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Characterization of agar from *Gracilaria tikvahiae* cultivated for nutrient bioextraction in open water farms

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ABSTRACT

Gracilaria tikvahiae, an endemic western North Atlantic red alga, was cultivated for nutrient bioextraction in urbanized estuarine waters in Long Island Sound and the Bronx River Estuary, USA. This study assesses the feasibility of an integrated approach of using *G. tikvahiae* produced in this bioextraction system as sustainable biomass source for agar production. Agars were extracted after alkaline pre-treatment and characterized in terms of gelling strength, chemical composition, chemical structure and gel structure. Results indicated that this seaweed performed similar to other cultivated *Gracilaria* in terms of extraction yield and gelling strength of the agar. Differences between sites were not significant in terms of agar gel strength, though yield was higher at Long Island Sound. The extracted agars were sulfated, methylated and with no detectable pyruvate substituents. It is possible to use an integrated strategy of nutrient bioextraction in urbanized estuarine waters and agar exploitation with *G. tikvahiae*.

1. Introduction

With increasing ecological concerns and with the world aiming to build a sustainable future, the use of biodegradable and biocompatible materials is becoming a true necessity of modern times. In this respect, seaweed polysaccharides have long been explored in the manufacture of biomaterials covering a broad spectrum of areas as diverse as food, biomedical, pharmaceutical and biotechnological sciences (Matsuhashi, 1990; Pereira & Yarish, 2008; Rinaudo, 2008). Agars extracted from some red seaweeds are made up of two main fractions: agarose and agaropectin. Agarose is a neutral polysaccharide responsible for the gelling ability of agar and its basic repeating unit is the alternating 1,3-linked β-D-galactopyranose and 1,4-linked 3,6-anhydro-α-L-galactopyranose. Agaropectin results from the presence of several substituent groups in the basic repeating unit such as sulfates, methyl ethers and pyruvates at different positions along the polysaccharide chain and constitutes the non-gelling polymer fraction (Lahaye & Rochas, 1991; Rees, 1969). Depending on the molecular weight and on the type, pattern and degree of substitution, different functional properties are achieved and a wide range of applications can be considered (Villanueva, Sousa, Goncalves, Nilsson, & Hilliou, 2010).

The ability to produce commercial grade seaweed polysaccharides in sufficient amounts that enable a broader exploitation of these biomaterials is a crucial step towards its sustainability. Commercial grade agars are mainly extracted from red seaweeds, including species of *Gelidium* and/or *Gracilaria/Gracilariopsis*. Bacteriological or pharmaceutical agars and agarose are traditionally produced from wild harvested *Gelidium*. *Gracilaria/Gracilariopsis* species usually lead to weaker agar gels, suitable for food applications, but these taxa are easily cultivated. *Gracilaria/Gracilariopsis* have been mostly cultivated in two Asian countries, China and Indonesia. These two countries produce approximately 98% of global production (FAO, 2016; Kim, Yarish, Hwang, Park, & Kim, 2017). In the Americas, Chile produces nearly 13 tons of dried extract powder per year with an annual value of US $29 million (FAO, 2016). Agars from *Gelidium* present typically a low degree of substitution and, thus low sulfate content, resulting in agars with high gel strength. On the other hand, agars from *Gracilaria/Gracilariopsis* have usually higher sulfate contents resulting in lower gelling capability. However, geographic factors, seasonal variations, growth stages, nutrient availability and environmental conditions can influence

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the synthesis, yield and chemistry of agar leading to a high heterogeneity of agars (Lahaye & Rochas, 1991). The extraction conditions (e.g. time, temperature, solvent to seaweed ratio) can also be refined to produce agars with an enhanced gelling ability (Arvizu-Higuera, Rodriguez-Montesinos, Murillo-Alvarez, Munoz-Ochoa, & Hernandez-Carmona, 2008; Sousa, Alves, Morais, Delerue-Matos, & Goncalves, 2010). Chemical treatments are also used for improving the properties of extracted agars. Alkaline hydrolysis of agars from *Gracilaria* prior to extraction allows the conversion of L-galactose 6-sulfate units to the 3,6-anhydro-L-galactose residues responsible for the ability of the polymer to form a gel.

Agar's unique gelling properties make it particularly suitable for food applications. Its gelling strength is high, even at low concentrations, gelation is reversible, though it only melts above 80 °C avoiding the need for refrigeration (an advantage over gelatin), and it can retain its gelling ability even at high temperature, allowing proper sterilization. Furthermore, its high temperatures resistance widens its usability, allowing, e.g., its use as thickening or stabilizing agent in the baking industry. In adition, it is tasteless and does not need the presence of extra reagents to induce gelation, being preferred over a wide range of other phycocolloids or gums. In fact, ca. 90% of the produced agar is used for food applications (McHugh, 2003), and its price is generally higher than for other food grade phycocolloids (Porse & Rudolph, 2017). Nevertheless, other emerging food applications include its use in low fat food as a fat replacer, in prebiotics, and as an edible film-forming or coating-forming agent.

No short term shortage of *Gracilaria* is predicted, due to the growth of Chinese and Indonesian production and processing capacities, and its price has decreased significantly in this last decade (Porse & Rudolph, 2017). However, this decrease in the price of raw materials did not have relevant impact in the final price of food grade agar. Due to the alarming decrease in natural populations of *Gelidium*, high grade agar is becoming scarce (Porse & Rudolph, 2017; Santos & Melo, 2018).

Seaweeds can be used as biofilters, removing nutrients, heavy metals and other organic and inorganic matters from the ecosystem. This process is now called nutrient bioextraction (Kim, Kraemer, & Yarish, 2014, 2015a; Rose et al., 2015). Some species of *Gracilaria* have proven to be good candidates for nutrient bioextraction removing inorganic nutrients from urbanized coastal waters (Abreu et al., 2009; Abreu, Pereira, Yarish, Buschmann, & Sousa-Pinto, 2011; Kim et al., 2014; Rose et al., 2015). The high volume of biomass generated in these nutrient bioextraction systems can lead to a sustainable source of these hydrocolloids.

Gracilaria tikvahiae is native to western Atlantic Ocean, extending from Nova Scotian, cold temperate regions, to Florida and into the Caribbean, warm subtropical regions (Ganesan, 1989; Littler & Littler, 2000; Mathieson & Hehre, 1986; McLachlan, 1979; Schneider, Suyemoto, & Yarish, 1979). This alga is known to be a highly opportunistic species occurring in eutrophic estuaries and bays (Peckol & Rivers, 1995). *G. tikvahiae* may grow up to 40 cm long and its thallus color can be highly variable, ranging from dark green to red and brown (Littler, Littler, Bucher, & Norris, 1989). Green mutants have been reported (Kim, Mao, Kraemer, & Yarish, 2015b). Its branches spread irregularly and can be either somewhat flattened or cylindrical (Littler et al., 1989). Interestingly, particular morphtypes can persist even when cultured under uniform conditions, suggesting the morphological differences are genetically controlled (Patwary & Meer, 1984). *Gracilaria tikvahiae* is a euryhaline species (Bird, Chen, & McLachlan, 1979; McLachlan & Bird, 1984). For example, *G. tikvahiae* collected from Tampa Bay, FL survived salinities between 8 and 60 psμ and was reported to grow well between 15 and 35 psμ (Bird & McLachlan, 1986). Furthermore, *G. tikvahiae* grows well at high temperature up to 29 °C but was found to not grow at prolonged sub-optimal temperatures (e.g. < 15 °C; Lapointe, Rice, & Lawrence, 1984; Gorman, Kraemer, Yarish, Boo, & Kim, 2017).

There have been attempts to cultivate *Gracilaria tikvahiae* to use the biomass as a source of agar in North America (Bird, Hanisak, & Ryther, 1981; Cheney, Mar, Saga, & Meer, 1986; Craigie & Jurgens, 1989; Craigie & Wen, 1984). These authors have not only cultivated local populations of *G. tikvahiae* but also developed selected mutants. Some mutants (e.g. MP-40 and MP-44) showed superior characteristics in terms of their agar quality in comparison to the wild type clones (Patwary & Vandermeer, 1983; 1984). Although the extraction of agar from *Gracilaria tikvahiae* was a very active pursuit at laboratory scale in the 1980s, we are not aware of any studies on agar extraction from this species during recent years. Furthermore, no studies were found dealing with the integrated exploitation of agars from bioextraction systems, nor with the agar extraction from *G. tikvahiae* cultivated in open waters.

This study follows a field-scale evaluation of *Gracilaria tikvahiae* aquaculture as a nutrient bioextraction strategy in Long Island Sound and the Bronx River Estuary, USA. Therefore, the objective of this study was to assess the feasibility of integrating the exploitation of agars in bioextraction systems using *Gracilaria tikvahiae*. Seaweeds were cultivated at two different open water farms, Long Island Sound (LIS) and at the mouth of the Bronx River estuary (BRE), and the agar quality from the cultivated *Gracilaria* biomass was monitored in terms of yield, gel strength (GS), sulfate and 3,6-anhydro-L-galactose content contents (3,6-AG), gel structure and NMR chemical and structural profile.

2. Materials and methods

Commercial agar (ref. A-7002 with an ash content of 2–4.5%) was obtained from Sigma-Aldrich Co. (St. Louis, MO). All other chemicals were of analytical grade.

2.1. Cultivation and sampling

Gracilaria tikvahiae (G-RI-ST$_1$) was cultivated by means of two, 50 m long-line culture systems at two, near-shore sites in Long Island Sound (LIS; Fairfield, CT; 41º06.882′ N/73º15.277’ W) and at the mouth of the Bronx River Estuary (BRE; Bronx, NY; 40° 48.047′N/73° 52.164′W). The long-lines were deployed at two depths (0.5 m and 1.0 m) in July 28, 2011 in LIS and in September 20, 2011 at the BRE site. Twenty gram bundles of *G. tikvahiae* thalli were inserted into nylon rope (5/8” high liner sink line) for every condition tested. The samples were randomly collected from each seaweed culture unit and then transferred to the laboratory in a cooler within 2 h. After the material was washed with autoclaved seawater, the samples were dried in an oven at 55 °C until they were completely dried.

Salinity at the LIS site during the growing season ranged from 26 to 30 psμ. The salinity at the BRE site was slightly lower and ranged from 20 to 25 psμ. Light penetration did not differ between sites during the growing season. At the LIS site, the light penetration was 81.2% (SD ± 9.2%) at 0.5 m and 53.2% (SD ± 14.4%) at 1.0 m deep, during midday on cloudless days. At the BRE site, it was 80.5% (± 10.0%) at 0.5 m and 48.2% (± 4.9%) at 1.0 m deep during midday on cloudless days. The water temperature was measured at both culture depths during sampling using a YSI 556 MPS meter. The temperature was similar at both sites and at both culture depths. The water temperature from July to September was 22–24 °C and started to drop below 20 °C in October and reached below 13 °C in early November. Water samples (n = 3) were also collected adjacent to the longlines at 1.0 m and then transferred to the laboratory in a cooler within 2 h. The samples were filtered through 0.45 μm glass microfiber filters (Whatman, Buckinghamshire, UK) and kept at −20 °C until measurements were made. Inorganic nutrients were analyzed using a SmartChem Discrete Analyzer (Unity Scientific, LLC, Brookfield, CT, USA). Nitrogen and phosphorus concentrations in the water column at the LIS site during the month of July 2011 ranged for 2.7–3.4 μmol L^{-1} and 0.9–1.2 μmol L^{-1}, respectively. The nitrogen and phosphorus concentrations at this site started to increase from late August and were as high as 8.4 and 4.7 μmol L^{-1}, respectively. The nutrient concentrations

at the BRE site were significantly higher than those at the LIS site (37–55 μmol L^{-1} of nitrogen and 14–19 μmol L^{-1} of phosphorus, respectively, during the months of August through October 2011).

2.2. Agar extraction and purification

Agar was hot-water extracted from the dried biomass using previously optimized conditions (Villanueva et al., 2010). The dried seaweeds (4 g) were pre-treated with 200 mL of NaOH 6% (w/w) at 85 °C for 3.5 h. The mixture was washed several times with tap water and neutralized with 200 mL of acetic acid 0.5% (w/w) for 1 h at room temperature. The extraction was then performed with 200 mL distilled water at 85 °C for 2 h and the mixture was filtered whilst still hot with a filter cloth (100% cotton). Agar was recovered through a freeze-thawing process, washed, dehydrated with ethanol (96%) and dried at 60 °C overnight. The obtained products were milled in a coffee grinder. Finally, agar samples were purified by heat solubilization at 0.2% (w/w) followed by centrifugation in a Beckman Coulter Allegra 25R centrifuge (40 °C, 21000 × *g* for 1 h) and dried at 60 °C.

2.3. Gel strength

Agar (1.5% w/w) was solubilized with distilled water at boiling temperature until complete dissolution. 15 g of the hot solution were poured into a cylindrical container with 30 mm diameter covered and allowed to rest at room temperature for ca. 20 h. A texture analyzer (TA-XT2 from Stable Micro Systems, Surrey, England) equipped with a cylindrical probe with 10 mm diameter was used for the gel strength determination. The rate of penetration was set at 0.2 mm/s and the experiment was performed at least in triplicate for each agar sample. Gel strength was considered to be the stress required for breaking the gel surface, with the test parameters used.

2.4. Gelling and melting temperatures

Agar (1.5% w/w) was solubilized with distilled water at boiling temperature until complete dissolution. Gelling and melting temperatures were measured in a controlled stress rheometer AR-G2 (TA Instruments, New Castle, USA) fitted with a parallel cross hatched plate geometry (40 mm diameter, gap 1000 μm). The solution was de-gassed and poured onto the pre-heated plate of the rheometer. Liquid paraffin oil was used to prevent water loss. A temperature ramp from 80 to 20 °C at the rate of 2 °C/min was applied. The sample was equilibrated for 30 min at 20 °C and heated from 20 to 95 °C with the same rate of 2 °C/min. Storage and viscous moduli were recorded at the end of the equilibration time at 20 °C. All experiments were performed with a frequency of 6.28 rad/s and 1.0% strain. Preliminary strain sweep tests were performed to ensure that the used strain was within the linear visco-elastic region.

2.5. Chemical analyses

Sulfate content was estimated by turbidimetry using the method with $BaCl_2$ after hydrolysis, as described by Jackson and McCandless (1978). Agar samples (approx. 20 mg) were pre-hydrolyzed in 10 mL HCl of 1 mol/L concentration and subsequently diluted to a final volume of 50 mL. The precipitating reagent was then added to the samples and the optical density read at a wavelength of 500 nm using a UV/Vis spectrometer (Jasco, V-630 Bio). Sulfate standards were prepared with sodium sulfate at final concentrations ranging from 0.002 to 0.09% (w/w) and treated in the same manner as the hydrolyzed samples. The 3,6-anhydro-L-galactose content (3,6-AG) was determined through the resorcinol–acetal colorimetric method (Yaphe & Arsenault, 1965). D-fructose was used as standard. All chemical determinations were made in triplicate.

2.6. Structure

The samples with highest and lowest gelling ability were further characterized through Nuclear Magnetic Resonance spectroscopy (NMR), attenuated total reflectance Fourier-transform infrared spectroscopy (ATR-FTIR) and intrinsic viscosity measurements. The microstructure of their gels was imaged by Scanning Electron Microscopy (SEM).

The NMR experiments were carried out at CEMUP (Centro de Materiais da Universidade do Porto, Porto, Portugal) following a procedure described elsewhere (Sousa et al., 2013). All spectra (1H, 13C and 1H−13C correlations recorded through a phase-sensitive HSQC (heteronuclear single quantum coherence)) were acquired non-spinning at 80 °C in a 400 MHz Bruker Avance III spectrometer and using a 5 mm QNP probe equipped with a z gradient coil. The purified agar powders were dissolved in D2O to a final concentration of 15 mg/mL and using TSP as internal reference (δH = −0.017 ppm; δC = −0.18 ppm). For details on the acquisition parameters as well as the shorthand notation used for the chemical nomenclature of agar units (i.e. 3,6-anhydro-α-L-galactose, LA; 6-O-methyl-β-D-galactose, G6M; 2-O-methyl-3,6-anhydro-α-L-galactose, LA2M; α-L-galactose 6-sulfate, L6S) previous papers can be consulted (Sousa et al., 2010, 2013).

The FTIR spectra of extracted agars were recorded using an ALPHA FTIR Spectrometer (Bruker, USA), by acquiring 60 scans with 4 cm^{-1} resolution.

A Cannon-Fenske viscometer for transparent liquids (according to ASTM D-2515) was used to measure intrinsic viscosities ([η]; mL/g) following the procedure described by Sousa et al., 2012. Diluted agar solutions were prepared using 0.75 mol/L NaSCN to inhibit agar aggregation. Concentrations were set to obtain relative viscosities from 1.2 to 2.0 (approximately), to allow linear regression according to Huggins and Kraemer relations, and extrapolation to zero. Viscosity average molecular weight (Mv) for each agar sample was calculated using the Mark-Houwink relationship (Rochas & Lahaye, 1989):

$$[\eta] = 0.07 M_v^{0.72} \quad (1)$$

The microstructure of the agar gels was confirmed by Cryogenic Scanning Electron Microscopy (CryoSEM) at CEMUP. For each sample, a small volume of the gel (approx. 1–3 mm^3) was frozen in slushy nitrogen (−210 °C), transferred to an ALTO 2500 cryo-preparation chamber and placed onto a cool stage (−150 °C), where it was fractured exposing the internal surface. The ice formed on the exposed fractured surface was removed by sublimation at −90 °C for 1.5 min. The sample was then sputter-coated with an Au−Pd thin film at −150 °C for 40 s, from a sputter head using ultra-pure argon gas. The analysis was performed at −150 °C in a JEOL JSM 6301F scanning electron microscope equipped with a Gatan ALTO 2500 cryo-preparation chamber using an accelerating voltage of 15 kV and working distances (WD) of 15 mm.

2.7. Thermal behavior

The thermal properties of agar samples were assessed through differential Scanning Calorimetry using a PerkinElmer DSC 6000. Samples were cooled to −20 °C, heated up to 160 °C, cooled back to −20 °C and re-heated up to 200 °C, at a heating or cooling rate of 10 °C/min. At least two replicates were made for each sample.

Thermal gravimetric analyses were performed in a PerkinElmer TGA 4000 (PerkinElmer, Massachusetts, EUA). Samples were heated from 25 °C to 600 °C at a heating rate of 10 °C/min under a nitrogen atmosphere. At least two replicates were made for each sample.

2.8. The properties of agar-based films

Film forming solutions were prepared by heating 1.5% agar

solutions up to 95 °C and the temperature was kept for 30 min, until complete dissolution. Samples were left to cool to 70 °C, and glycerol was added as plasticizer to a final content of 0.15 $g/g_{dry\ agar}$. 40 g of each film-forming solution were poured into polyoxyethylene methylene (POM) petri dishes with a diameter of 12 cm. Films were dried at 40 °C for 18 h and equilibrated for 48 h at 53% RH, in a desiccator at 20 °C with a saturated salt solution of $Mg(NO_3)_2$, before further analyses.

Film thickness was measured with a digital micrometer (No. 293–561, Mitutoyo, Japan) at 6 random positions for each film.

Tensile strength (TS) and elongation at break (EB) were determined in a TA. HDplus texture analyzer (Stable MicroSystems) equipped with tensile test attachments, following the guidelines of ASTM D882-91 standard method, as described by Costa et al., 2018. The tests were replicated eight times.

Water vapor permeability (WVP) was measured gravimetrically according to ASTM E96-92 standard test. The equilibrated films were sealed tightly to a permeation cell containing distilled water (100% relative humidity (RH)) and placed inside a desiccator with silica gel (0% RH). Weight loss was monitored every 2 h for 10 h. Testing was performed in triplicate. WVP was calculated by linear regression, using Eq. (2), as described by Silva, Mauro, Goncalves, & Rocha, 2016:

$$WVP = \frac{\Delta m \times x}{\Delta P \times A \times t} \quad (2)$$

Δm represents the weight loss (g), x is the average film thickness (m), A is the permeation area, t is duration (s) and ΔP is the difference of the water vapor partial pressure at 20 °C (2337 Pa) between the two sides of film. Three replicates were made.

2.9. Statistical analyses

All statistical analyses were made using the data analysis software Statistica version 8.0 (StatSoft, Inc, Tulsa, OK, USA). The influence of time (random) and depth (fixed, two levels) on yield, gel strength, sulfate and 3,6-anhydro-α-L-galactose contents were analyzed by factorial analysis of variance (ANOVA). A separate analysis was made for location (fixed, two levels), including also time (only samples from October and November) and depth (fixed, two levels), as data were more limited for the BRE site. For significant differences from ANOVA, variances were tested for homogeneity and statistically significant differences were analyzed *a posteriori* with Scheffé's multiple comparisons test. The significance level was defined as $p \leq 0.05$, for all tests.

3. Results and discussion

Results obtained for agar extracted from *Gracilaria tikvahiae* are presented in Tables 1–5 and Figs. 1–8. *Gracilaria* was collected in the summer and fall of 2011. The extraction conditions were based on early optimization studies concerning *G. vermiculophylla* from the Portuguese coast (Villanueva et al., 2010).

3.1. Yield, gel strength and physico-chemical properties

Yields for LIS samples after the purification step (Fig. 1) ranged from 13 to 17% (15–19% prior to purification). A wide range of extraction yields can be found in the literature, due to different seaweeds and extraction protocols applied, but yield values of near 20% are frequently reported for pre-treated agar from different *Gracilaria* spp. without re-extraction steps (e.g. Arvizu-Higuera et al., 2008; Lewis & Hanisak, 1996; Skriptsova & Nabivailo, 2009; Villanueva et al., 2010). Nevertheless, extraction yields below 10% were reported for *G. tikvahiae* (Lopezbautista & Kapraun, 1995), making the yields achieved in this work significant. However, the low yield reported by Lopezbautista & Kapraun was obtained in July, with very high water temperatures (ca. 30 °C). Yields for the BRE samples were significantly lower than those from LIS samples in October and November and ranged from 11 to 12% (Figs. 1 and 2). No significant differences were observed in agar yields between *Gracilaria* cultivated at different depths at each site. Overall extraction yields unrelated with light intensity were also reported for *Gelidium pulchellum* (Sousa-Pinto, Murano, Coelho, Felga, & Pereira, 1999), though higher amounts of agar with a lower melting point were extracted with increased irradiance.

At the LIS site, seasonal variations were significant (Table 1), the yield being higher during August which generally decreased until November (Fig. 1). From the literature, these results with higher yields in summer and steep decreases in autumn and winter were expected for sites within a temperate climate (e.g. Marinho-Soriano and Bourret (2003), for *Gracilaria bursa-pastoris* and Martin et al. (2013), for *G. gracilis*). Slight seasonal differences in yield and strong seasonal differences in gel strengths were also reported for agars from *G. tikvahiae* (Bird & Hinson, 1992). The yield of agar has often been positively correlated with temperature and salinity and negatively with nitrogen content, as reported for *G. bursa-pastoris* (Marinho-Soriano & Bourret, 2003) or *G. gracilis* (Martin et al., 2013). With the decrease of nitrogen, for instance, protein synthesis decreases in favor of polysaccharide synthesis. Water temperature was higher at the beginning of August, and decreased slightly until the beginning of October and declined sharply after that. The tissue nitrogen content started to increase also from late August at the LIS site. Differences in nitrogen content and salinity may also justify the differences in agar yield between both sites: BRE had considerable higher nitrogen content and slightly lower salinity, leading to concomitant lower yields.

Seasonal differences in the gel strength were also statistically significant (Fig. 3; Table 1). With the exception of some samples from late October and November, all samples presented acceptable GS (from 580 to 893 g/cm^2). This gel strength was in fact higher than that in another study using the same species (Lopezbautista & Kapraun, 1995). Another statistical analysis was made excluding the out-lying October and November samples, when temperature started decreasing sharply. In this new statistical analysis, differences in GS at different depths became significant (Table 1), particularly for samples in September and early October. This result may be related with faster temperature variations (drops) near the surface (at 0.5 m).

Gel strength was highest in September and lowest in late October and early November (Fig. 3). For *Gracilaria* at 1.0 m, values in November (610 g/cm^2) were similar to values at mid-August (580 g/cm^2). The growth rate of another *Gracilaria* (*Gracilaria multipartita*) has been negatively correlated with agar gel strength (Givernaud, El Gourji, Mouradi-Givernaud, Lemoine, & Chiadmi, 1999), with the GS decreasing from February to July; increasing from August to October, decreasing in November–December and increasing again in January and February, on the Atlantic coast of Morocco. The results obtained at LIS and BRE are similar, with the autumn decrease in growth rate starting slightly earlier, due to the colder climate at these sites in comparison to Morocco.

Light has been inversely correlated in the literature with increasing gel strength (Villanueva, Hilliou, & Sousa-Pinto, 2009). However, the present study showed that the *Gracilaria* grown at 0.5 m had a higher GS than the same seaweed grown at 1.0 m during summer (Fig. 3). In autumn, however, no defined trend was observed: the samples from LIS in late October and November had higher a GS at 1.0 m (lower light intensity), and the samples from BRE and LIS in the beginning of October had a higher GS when grown at 0.5 m. Nevertheless, as the seaweeds were cultivated in open-water farms, weather may be governing the differences, being the seaweeds cultivated nearer the surface more vulnerable to weather changes.

Temperature decreased slowly from August to early October. From late October, a steep decrease was observed at both cultivation sites (Kim et al., 2014). This together with high variations in diffuse attenuation coefficients (with different trends at each site) may have been responsible for the inconsistent results in the values of GS during this

Table 1
Results from the statistical analyses (ANOVA and Scheffé's test (main differences).

	Yield	GS	Sulfate	3,6-AG
From Aug to Nov				
Depth	Not significant	Not significant	Significant	Significant
Seasonality	Significant	Significant	Significant	Significant
Scheffé's test	Different in August and late autumn	Different in late August, September and November	Different in August and late October	Different at 0.5 m in late August
From Aug to early Oct				
Depth	Not significant	Significant	Significant	Significant
Seasonality	Significant	Significant	Significant	Significant
Scheffé's test	Different in August and October	Different also in September, at different depths	Different at 0.5 m in September and October	Different at 0.5 m in late August
From Oct to Nov				
Location	Significant	Not significant	Significant	Significant
Depth	Not significant	Not significant	Significant	Not significant
Seasonality	Significant	Significant	Significant	Significant
Scheffé's test	Only different for site location	Different in early October	Different in late October (0.5 and 1 m) and November (1 m)	Different at 1 m in November

last tested period.

Though depth of cultivation seemed to have an effect opposite to the expected in gel strength, this did not happen with 3,6-AG and sulfate contents (Fig. 4). In fact, at least until early October, the sulfate content was globally higher when biomass was grown at the shallower depths (and higher light intensities) and the 3,6-AG content seemed to be globally lower from material cultivated at the shallower depths. It has been often reported that darkness has a negative effect on sulfate content and a positive effect on 3,6-AG content. For instance, it was reported that a 10 days dark post-harvest treatment of *Chondrus* improved the gelling properties of the extracted carrageenan through the decrease in sulfate and increase in 3,6-AG content, in a similar way to the alkali extraction pre-treatment (Villanueva et al., 2009).

This probably means that, in spite of the effect of darkness on 3,6-AG and sulfate contents, other factors were also conditioning agar performance, such as sulfate position, molecular weight, other substituent groups (such as methyl) and/or the presence of other storage polysaccharides (e.g. floridean starch). Furthermore, despite the alkali pre-treatment, the sulfate content was still moderate and no relationship was found between differences in the contents of sulfate and 3,6-AG. Therefore the sulfate groups were probably not mainly in the form

Table 3
Chemical shifts assignments by ^{1}H NMR for alkali-treated agar.

	Ref. Shifts (ppm) (Villanueva et al., 2010)	LIS 9–15; 1M	LIS 9–15; 0.5M	LIS 11-04; 1M	BRE 11-02; 1M
G1	4.55	4.54	4.54	4.55	4.54
G2	3.63	3.62	3.62	3.63	3.62
G3	3.76	3.76	3.76	3.75	3.76
G4	4.11	4.11	4.11	4.12	4.10
G5	3.69	3.66	3.67	3.67	3.67
G6	3.73(1)/3.79(2)	3.75/3.82	3.73/3.81	3.68/3.83	3.68/3.82
G6M	3.41	3.40	3.41	3.41	3.41
LA1	5.13	5.12	5.13	5.13	5.13
LA2	4.11	4.11	4.11	4.12	4.10
LA3	4.55	4.54	4.54	4.55	4.54
LA4	4.65	4.65	4.65	4.65	4.65
LA5	4.55	4.54	4.54	4.55	4.54
LA6	4.02(3)/4.24(4)	4.02/4,29	4.02/4,28	4.03/4.24	4.04/4.24
LA2M	3.52	3.51	3.51	3.52	3.51
L6S	5.28	n.d.	n.d.	5.21	5.21

n.d. not discernible.

Table 2
Rheological data obtained through dynamic rheological measurements in a stress-controlled rheometer for agar from two sites and two different depths from August to November (storage modulus, G'; viscous modulus, G''; gelling temperature, Tg; melting temperature, Tm; mean ± SD).

	Depth (m)	Date	G' (Pa)	G'' (Pa)	Tg (°C)	Tm (°C)
LIS	1.0	8-16-11	9617 ± 5	129 ± 6	47.9 ± 0.3	89.7 ± 0.5
LIS	0.5	8-16-11	12860 ± 113	252 ± 20	48.7 ± 0.1	90.2 ± 0.7
LIS	0.5	8-26-11	12375 ± 1379	226 ± 29	47.2 ± 0.3	89.5 ± 0.6
LIS	1.0	8-26-11	11690 ± 622	162 ± 1	47.1 ± 0.1	90.4 ± 0.1
LIS	0.5	9-15-11	16780 ± 240	315 ± 2	45.8 ± 0.1	89.0 ± 0.1
LIS	1.0	9-15-11	13070 ± 1174	268 ± 10	45.6 ± 0.3	90.9 ± 0.9
LIS	0.5	10-7-11	14043 ± 2397	283 ± 33	46.7 ± 0.4	89.7 ± 1.3
LIS	1.0	10-7-11	10860 ± 240	164 ± 4	48.6 ± 0.4	89.3 ± 0.5
LIS	0.5	10-24-11	14624 ± 2214	315 ± 51	44.9 ± 0.3	90.4 ± 1.5
LIS	1.0	10-24-11	11564 ± 984	276 ± 20	45.8 ± 0.6	90.7 ± 0.5
LIS	1.0	11-4-11	13500 ± 1004	261 ± 24	46.7 ± 0.4	88.1 ± 0.3
LIS	0.5	11-4-11	13710 ± 42	246 ± 2	45.2 ± 0.1	83.3 ± 0.4
BRE	0.5	10-5-11	14890 ± 2263	281 ± 16	46.4 ± 0.1	87.8 ± 1.6
BRE	1.0	10-5-11	12405 ± 148	235 ± 45	47.1 ± 0.4	85.3 ± 0.4
BRE	1.0	10-19-11	12530 ± 679	250 ± 22	46.5 ± 0.6	87.8 ± 0.5
BRE	0.5	10-19-11	16430 ± 1372	311 ± 11	45.3 ± 0.8	87.4 ± 3.1
BRE	1.0	11-2-11	11440 ± 184	187 ± 6	47.0 ± 0.1	83.0 ± 0.1
BRE	0.5	11-2-11	15850 ± 764	301 ± 9	45.6 ± 0.3	86.0 ± 1.5

Table 4
Thermal properties and molecular weight.

	LIS 9–15; 1M	LIS 9–15; 0.5M	LIS 11-04; 1M	BRE 11-02; 1M
Mv (kDa)	338	302	180	131
T_1 (°C)	n.d.	98.5 ± 0.1	93.7 ± 0.9	97.7
T_2 (°C)	n.d.	131 ± 1	128 ± 1	126
T_3 (°C)	136 ± 7	138 ± 3	139 ± 7	138 ± 1
ΔH_3 (J/g)	246 ± 37	172 ± 10	191 ± 11	232 ± 63
T_{glass} (°C)	22.8 ± 0.1	22.7 ± 0.3	22.6 ± 0.1	22.7 ± 0.1
c_p (J/(g°C)	0.0345 ± 0.008	0.0485 ± 0.005	0.0410 ± 0.002	0.0395 ± 0.006
Water content (%) (TGA)	15.1 ± 0.2	15.7 ± 1.4	16.1 ± 0.5	16.7 ± 0.4
Tsd	197 ± 8	197 ± 3	211 ± 9	206 ± 1
Td	277 ± 2	283 ± 2	289 ± 1	239 ± 1

n.d. not discernible; Tsd – temperature at which the degradation ramp starts; Td – temperature at which degradation rate is maximum (measure as the negative peak in the first derivative curve).

of L6S (Givernaud et al., 1999).

Rheological parameters for various agar gels are presented in Table 2. The storage or elastic modulus (G′) is a measure of the solid nature of a sample and is expected to be positively correlated with the gel strength. In fact, G' at 20 °C followed a similar trend to the gel strength, being highest in September. The storage modulus was significantly different at different depths being consistently higher at 0.5 m, confirming the general trend of GS (with the exceptions in GS referred above). Site differences were not visible. Melting temperatures of around 90 °C (Table 2) are typical of agar samples suitable as agar standards (Arvizu-Higuera et al., 2008) according to the United States Pharmacopeia (USP) criteria (Tm > 85 °C). Differences related to depth and seasonality were only significant in November. Agars from the LIS site presented slightly higher melting temperature than agars from BRE (with higher nitrogen content). Gelling temperatures were slightly higher than the typical gelling temperatures of commercial agars, which are generally below 40 °C, but common in agar from *Gracilaria sp*., particularly if highly methylated. Despite the good gelling ability, the gel temperatures above 32–36 °C made these agars unsuitable for direct use in bacteriological grade applications (Skriptsova & Nabivailo, 2009).

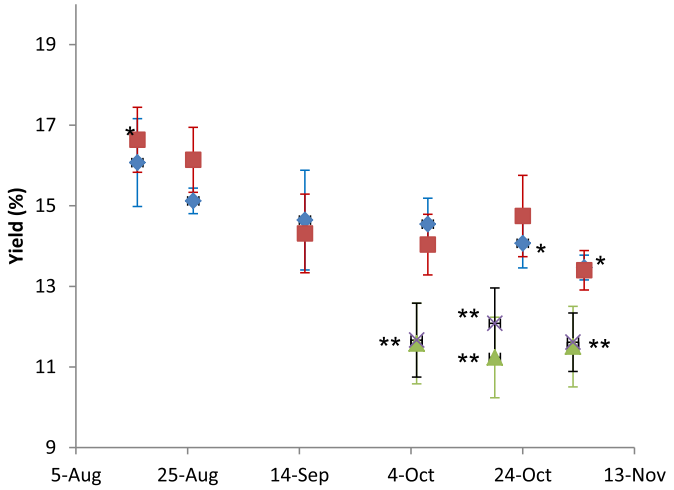

Fig. 1. Effective yield ($g_{purified\ extract}/g_{seaweed}$): ♦ Long Island Sound 0.5 m; ■ Long Island Sound 1.0 m; ▲ Bronx River Estuary 0.5 m; × Bronx River Estuary 1.0 m (* significant differences in seasonality; ** significant differences in site location), mean ± SD.

3.2. NMR, FTIR and molecular weight

The ^{13}C and ^{1}H chemical assignments of alkali-treated agars were obtained through NMR spectroscopy. The chemical shift assignments by ^{1}H NMR are presented in Table 3. The 12 characteristic ^{13}C signals of agarose and the presence of substituent groups such as sulfate, O-methyl, pyruvic acid ketals, and other monosaccharides in the agar backbone were inspected at typical signals by comparison with previous studies on agar NMR characterization (Rodriguez, Matulewicz, Noseda, Ducatti, & Leonardi, 2009; Villanueva et al., 2010).

The backbone substitutions, induced by physiological (i.e. during the life cycle of the alga) and environmental aspects (i.e. growth conditions, season of collection, cultivation site) as well as the extraction conditions, are crucial factors since they define the final properties of the polysaccharide. For instance, the alkaline treatment of agar causes the conversion of the α-L-galactose 6-sulfate (L6S) in 4-linked 3,6-anhydro-α-L-galactose (LA) units, leading to a significant increase in gel strength. L6S residues were detected through a minor peak ~5.17 ppm in the ^{1}H spectra and a minor cross-peak in the HSQC at (~5.17; 102.1) ppm attributed to H1 and C1 of L6S, respectively. This peak was undetected in LIS September samples that showed the highest gel strength. The peak was still present (though very small) in BRE and LIS November samples, with the lowest gel strength. The persistence of this signal, after alkali treatment, indicated that these samples had originally more L6S groups (i.e. before the alkaline pre-treatment, in accordance with previous studies reporting higher sulfate content in the cold season (e.g. Romero et al., 2007).

Furthermore, samples isolated from biomass grown at both LIS and BRE sites in November presented a minor peak at 62.8 (^{13}C spectra) that may correspond to amylose from contaminating floridean starch. This peak was absent from the September samples. Therefore, under the more adverse environmental conditions for seaweed growth, storage polysaccharide accumulation was starting to be relevant, as could be expected. Furthermore, the presence of the floridean starch may have been responsible for the differences the behavior between the rheological parameters (namely, storage and viscous moduli) and gel strength for the BRE samples and those from LIS in November. In fact, despite

Table 5
LIS agar's films properties.

Thickness (mm)	Mechanical properties		WVP (g/(msPa))
	Tensile strength (MPa)	Elongation at break (%)	
0.049 ± 0.005	16.8 ± 1.2	11.2 ± 1.7	$2.6 \times 10^{-11} \pm 0.1 \times 10^{-11}$

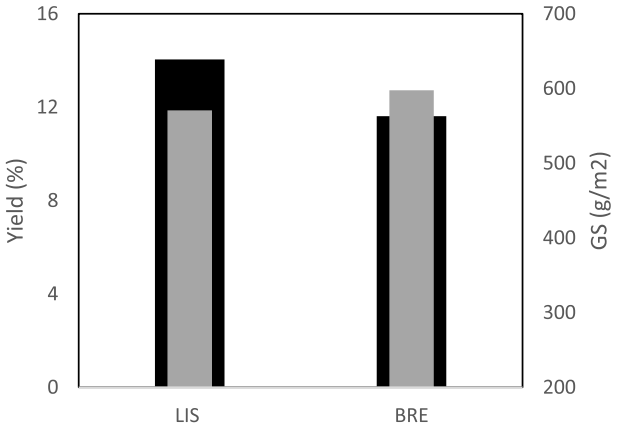

Fig. 2. Overall comparison of yield and GS between the two sites - Long Island Sound (LIS) and Bronx River Estuary (BRE) (only data from October and November was considered) - ■ Yield; ■ GS, mean ± SD.

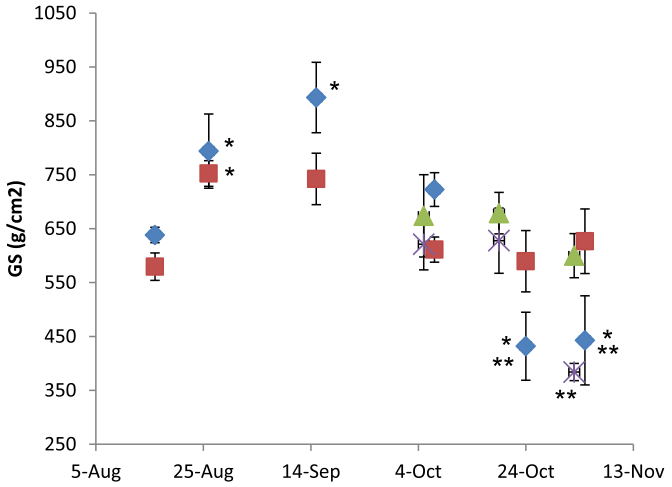

Fig. 3. Gel strength at two different sites and two different depths: ♦ Long Island Sound 0.5 m; ■ Long Island Sound 1.0 m; ▲ Bronx River Estuary 0.5 m; × Bronx River Estuary 1.0 m (* significant differences in seasonality; ** significant differences in site location), mean ± SD.

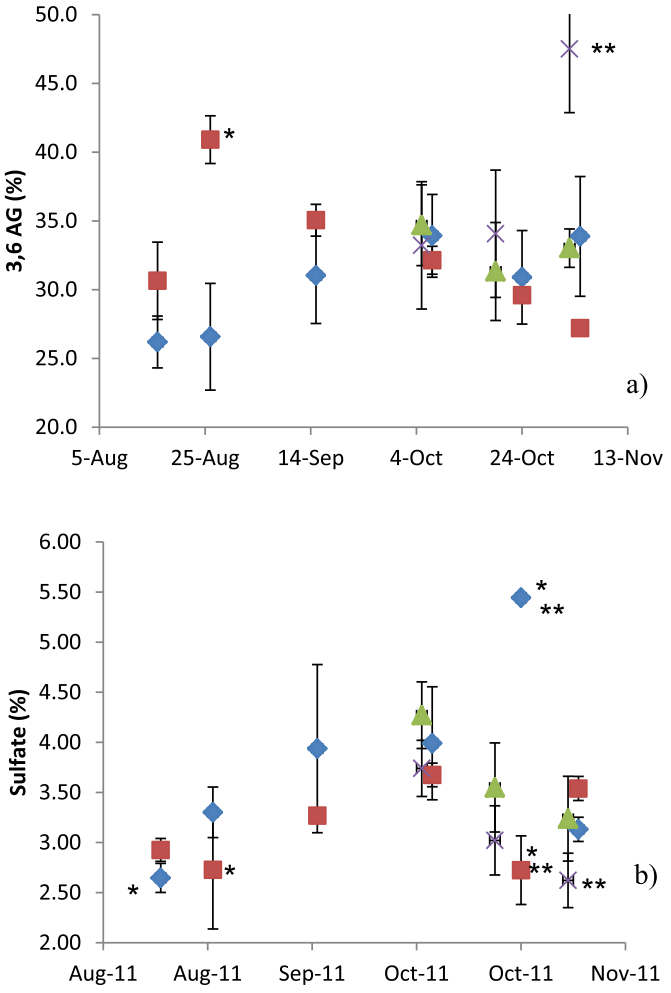

Fig. 4. Chemical properties of the extracted agar: a) 3,6-AG content; b) sulfate content; ♦ Long Island Sound 0.5 m; ■ Long Island Sound 1.0 m; ▲ Bronx River Estuary 0.5 m; × Bronx River Estuary 1.0 m (* significant differences in seasonality; ** significant differences in site location), mean ± SD.

the general decrease in the GS after September, storage and viscous moduli were higher. Floridean starch generally has a negative effect on the GS of agar (Rodriguez et al., 2009). An unidentified minor peak in the LIS November samples was present at 64.3 (^{13}C).

Nevertheless, overall the HSQC pattern was typical of agar samples in all cases (Fig. 5), with all characteristic signals from agarose being easily identified, confirming that this was the main extracted component. Overall samples presented significant degrees of methylation substitution at C6 of the G units, which was detected with the presence of a peak at about 60 ppm in the ^{13}C spectra, due to the downshift in the nearest carbon resonance (Usov, 1984), and a sharp singlet at 3.41 ppm. The presence of methylated substituents was probably responsible for the high gelling temperature (above 45 °C), as they are known to raise the gel setting temperature. For instance, Falshaw, Furneaux, and Stevenson (1998) reported an increase in the melting temperature from 33 to 37 °C for non-methylated agar samples to 42–49 °C for naturally methylated material. The presence of 4-O-methyl-L-galactose has also been associated with the age of *G. tikvahiae* (Craigie & Jurgens, 1989). In fact, these authors concluded that young tissues had only traces of 4-O-methyl-L-galactose groups and that they were markedly present in the agar extracted from mature seaweeds. Therefore, the presence of significant methylation could be related with the age of the seaweeds, as *G. tikvahiae* thalli were harvested after some growth period.

Methylation on the C2 of LA units, detected by the presence of small peaks in the region 3.51–3.52, was also observed but at a much lower extent. The LIS sample from November seemed to have a different pattern related to the methylated groups, namely the LA2M groups, with a more intense peak in the 1D ^{1}H NMR spectra (Fig. 5; 3.51 ppm), when compared to the September LIS sample. This observation may be responsible for the differences in the Tm. In fact, the Tm values for the LIS samples in November were significantly lower than that from the LIS samples taken in other months. Pyruvate residues were not found in any of the samples.

FTIR spectra are presented in Fig. 6 and generally confirmed the NMR results. Spectra of all samples were very similar and the typical bands for agar samples were easily identified: 1370 and 1250 cm^{-1} are referred to be related with total sulfate (ester sulfates and S=O stretching vibration, respectively), 930 cm^{-1} is related with the C–O vibration of LA units, (Sousa et al., 2012). The broad absorption region between 1100 and 1000 cm^{-1} as well as the band at 1150 cm^{-1} are common to all polysaccharides and could be assigned C–O–H bending and to C–O and C–C stretching (Warren, Perston, Royall, Butterworth, & Ellis, 2013). The characteristic peak at 1640 cm^{-1} is assigned to C=O stretching (Selvalakshmi, Vijaya, Selvasekarapandian, & Premalatha, 2017). The band at 890 cm^{-1} is also typical of agar samples and could be related with the C–H bending at the anomeric carbon in G residues. The absence of peaks at 805 and 830 cm^{-1} indicated no sulfates at C2 in LA and G moieties, respectively, and the small shoulder at 850 cm^{-1}

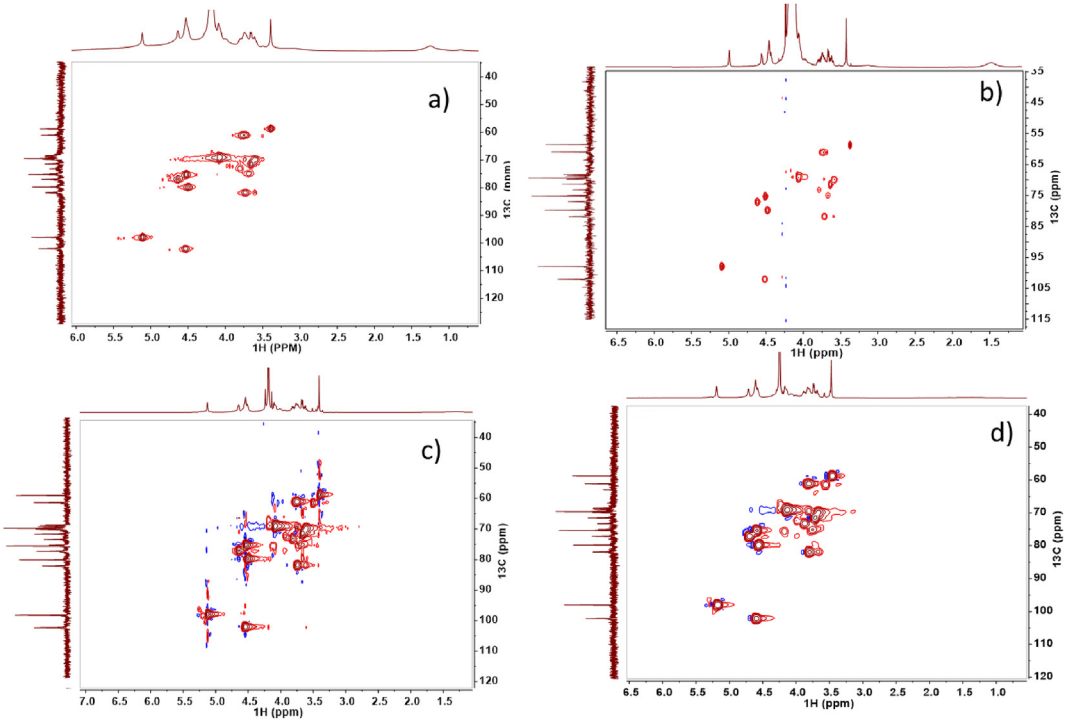

Fig. 5. HSQC spectra with the corresponding 1D NMR spectra of each nucleus for the alkali-treated agar from: a) Long Island Sound 0.5 m in 15/09; b) Long Island Sound 1.0 m in 15/09; c) Bronx River Estuary 1.0 m in 02/11; d) Long Island Sound 1.0 m in 04/11.

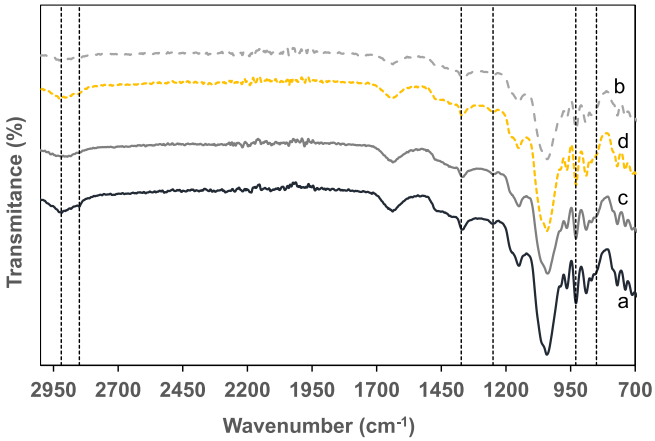

Fig. 6. FTIR spectra for the alkali-treated agar from: a) Long Island Sound 0.5 m in 15/09; b) Long Island Sound 1.0 m in 15/09; c) Bronx River Estuary 1.0 m in 02/11; d) Long Island Sound 1.0 m in 04/11.

indicated low amount of sulfates at C4 of G moieties (Romero, Villanueva, & Montano, 2008). Furthermore, the absence of a band at 820 cm^{-1} indicated no or very low sulfates at C6 of G moieties (Rochas, Lahaye, & Yaphe, 1986), as expected due to the alkali pre-treatment. Nevertheless, a small shoulder could be seen in the BRE sample, confirming the results from NMR. Bands at 740 and 770 cm^{-1} are related with skeletal bending of the galactose ring (Rhein-Knudsen, Ale, Ajalloueian, Yu, & Meyer, 2017). Finally, the band at 2920 cm^{-1} is related with the C–H, being a good measure of total sugar content (Rochas et al., 1986). The small shoulder on this band at 2850 cm^{-1} is typical of highly methylated agars which is once more in accordance with NMR results.

In terms of molecular weight measurements, samples from September had a much higher viscosity average molecular weight than samples from November, as expected from the GS values. Furthermore samples from the BRE site had the lowest gelling strength. Depth of

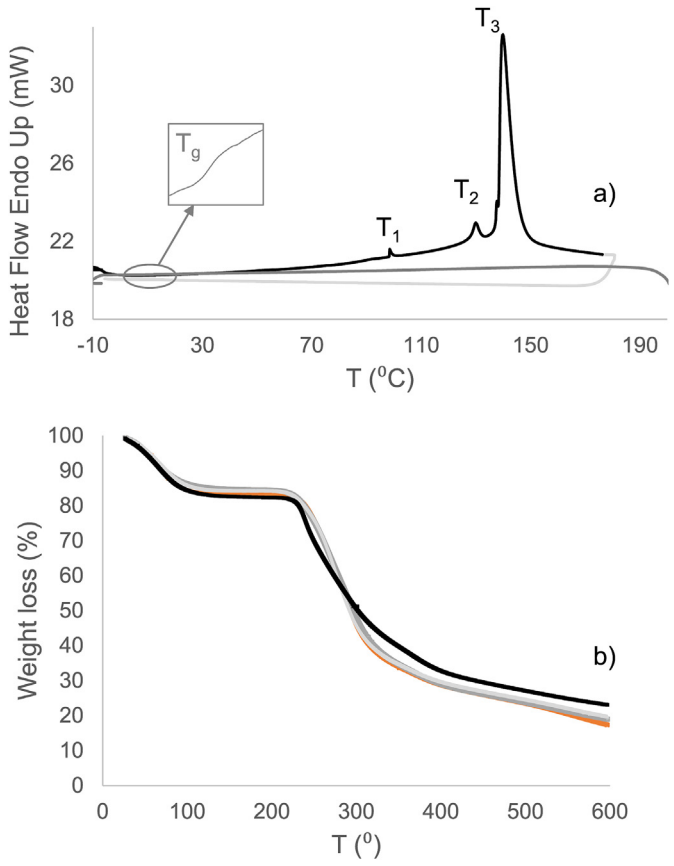

Fig. 7. Thermal behavior for the alkali-treated agar: a) Typical DSC thermogram (alkali-treated agar from Long Island Sound 1.0 m in 15/09): - First heating cycle; -- Cooling cycle; -- Second heating cycle; b) TGA: -- Long Island Sound 1.0 m 15/09; -- Long Island Sound 0.5 m 15/09; -- Long Island Sound 1.0 m 4/11; – Bronx River Estuary 1.0 m 2/11.

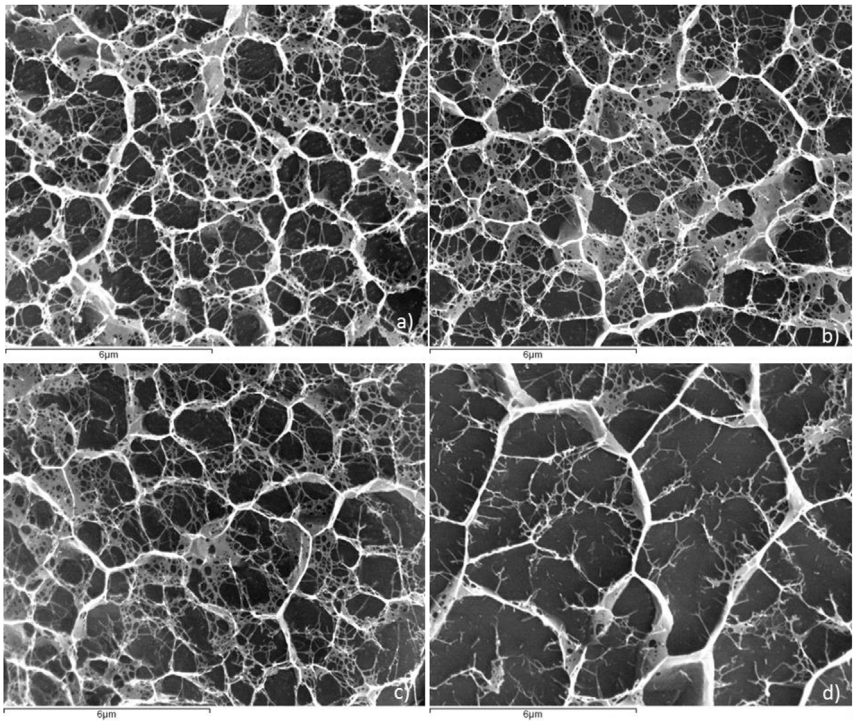

Fig. 8. CryoSEM pictures of agar gels: a) Long Island Sound 1.0 m 15/09; b) Long Island Sound 0.5 m 15/09; c) Long Island Sound 1.0 m 4/11; d) Bronx River Estuary 1.0 m 2/11; error bar corresponds to 6 µm in all pictures.

cultivation did not seem to have a significant effect, though the values for samples cultivated at 1 m were slightly higher, unlike the GS.

3.3. DSC and TGA

A typical thermogram achieved for DSC is presented in Fig. 7a. Three main endotermic peaks were detected in the first heating cycle: one slightly below 100 °C, one around 130 °C and the biggest one near 140 °C. The first peak is usually assigned to the residual water present in the sample (Ouyang et al., 2018). Peaks in the range of 126–144 °C correspond to agar transitioning into the disordered state, from double helix to single chain. A corresponding transition was also seen in the rheological tests with high water content (gel melting T_m), but occurred at lower temperatures. This process was not reversible for low moisture samples (i.e. the sample does not resume its double helical state), but the second heating cycle showed a shift around 22.7 °C (Table 4) for all analyzed agar samples, normally assigned to a glass transition. A commercial agar was used for comparison purposes and also showed a glass transition temperature at 23.4 ± 0.4 °C. In fact, glass transition temperatures referred to in literature for low moisture agars but their final value is highly dependent on the type of agar, the moisture content and the drying history (Cooke, Gidley, & Hedges, 1996; Mitsuiki, Yamamoto, Mizuno, & Motoki, 1998).

Thermogravimetric analyses indicate two different regions of weight loss (Fig. 7b): a first one, up to 130 °C, assigned to the removal of adsorbed and bond water and a second one starting from ca. 200 °C, depending on the sample, assigned to thermal degradation. All samples presented similar weight loss patterns, though slight differences could be seen for the BRE sample in the thermal degradation region. In particular, the value of the temperature that corresponds to the minimum of the first derivative curve was significantly lower than the samples from the LIS site, corresponding to a maximum degradation rate at a lower temperature.

3.4. Gelling and film forming ability

3.4.1. The gel structure

As referred to in the introduction, the main food applications of agar are related with its gelling ability. Samples from late August and September presented quite high gel strengths matching the values of high quality commercial agars, confirming the commercial viability of the LIS agars. According to the criteria defined by the Japanese Specifications for Processed Agar (JSPA), all samples would be first grade food agar, with a GS higher than 350 g/cm^2 (Skriptsova & Nabivailo, 2009). However, though late August to early October alkali-treated agars could be superior grade agars using these criteria (GS higher than 600 g/cm^2), their higher gelling temperatures hamper bacteriological and pharmaceutical applications.

Micrographs obtained from the cryo-SEM analyses of gels made from the extracted agar were in accordance with expectations resulting from the analyses of their chemical and physical properties (Fig. 8).

Data derived from agar gels of samples grown at different depths did not present any significant differences. The LIS gel from November presented a slightly more open network, which agreed with the slightly lower gelling strength, probably due to the residual presence of L6S groups. The BRE gels from November samples grown at 1.0 m produced the coarser network, due to the lowest gel strength, in spite of the high 3,6 AG value and low sulfate content. As the presence of L6S was similar to the LIS November sample, molecular weight was probably governing the gel structure. In fact, the molecular weight from the BRE sample was significantly lower than from the LIS sample (Table 4).

3.4.2. Films

Though the application as a food additive is the typical use in the food industry, much research work has been made assessing the viability of using agar-based materials for food packaging and edible coatings with interesting results. Therefore, agar samples collected at the LIS site from August till early October were mixed in a single batch and their edible-film forming ability was also tested. Mechanical properties, thickness, thermal properties (TGA) and water permeability

of the films were assessed for benchmarking with agar-based films described in the literature.

Transparent and flexible films were obtained, with an average thickness of ca. 50 μm (Table 5). Values found in literature for films produced by casting using formulations with agar and glycerol ranged from 37 to 60 μm (Arham, Mulyati, Metusalach, & Salengke, 2016; Kanmani & Rhim, 2014; Malagurski et al., 2017; Phan, Debeaufort, Luu, & Voilley, 2005; Shankar & Rhim, 2016).

As for mechanical properties, the dispersion of values found in the literature is also high. Film forming solutions have agar concentrations ranging from 1 to 3% and glycerol (plasticizer) contents of 0–0.5 $g/g_{dry\ agar}$ (Arham et al., 2016; Atef, Rezaei, & Behrooz, 2014; Freile-Pelegrín et al., 2007; Kanmani & Rhim, 2014; Malagurski et al., 2017; Mohajer, Rezaei, & Hosseini, 2017; Phan et al., 2005; Rhim, 2011; Shankar & Rhim, 2016; Sousa & Goncalves, 2015; Wu, Geng, Chang, Yu, & Ma, 2009). Though typical films conditioning for mechanical properties assessment is made at 50 or 53% relative humidity (RH), this is not frequently the case. As water has a strong plasticizer effect, mechanical properties will strongly differ upon RH variations. The achieved tensile strength (ca. 17 MPa) and elongation at break (ca. 11%) are within the expected from the results reported with similar conditioning at 50–53% RH. Freile-Pelegrín et al. (2007) achieved a TS of 3.2 MPa and EB of 6.5% with 1.5% agar solutions without glycerol, but the agar used had a lower Mv (100 kDa) than the samples from LIS. Mohajer et al. (2017) reported a TS of 18.7 MPa and an EB of 30%, using 0.3 $g/g_{dry\ agar}$ of glycerol, Arham et al. (2016) reported a TS of 20 MPa and a EB of 15% using 0.15 $g/g_{dry\ agar}$ of glycerol and Atef et al. (2014) reported a TS of 18 MPa and a EB of 19 using 0.3 $g/g_{dry\ agar}$ of glycerol.

In terms of barrier to water, another important property for packaging and in particular, for food packaging, the dispersion in reported results is even higher. Phan et al. (2005), analyzed WVP conditioning samples at different RH and using different humidity gradients. For 3% agar solutions with 0.15 $g/g_{dry\ agar}$ of glycerol, they reported a WVP of 6×10^{-11} g/(msPa), achieved conditioning films at 57% (slightly higher than the used in this work). Nevertheless, the results are in the same order of magnitude as this work, but slightly higher. Sousa & Gonçalves (2015) also reported values of 4.6×10^{-11} g/(msPa) for alkaline treated agars from *Gracilaria vermicullophyla* with no glycerol and Atef et al. (2014) reported 1.6×10^{-10} g/(msPa) but using 0.33 $g/g_{dry\ agar}$ of glycerol and a commercial food grade agar. These are promising results, with water vapor permeability matching the values of cellophane as reported by Phan et al. (2005).

The properties of agar films are highly variable and strongly depend on several factors, including: the film forming methodology; plasticizer type and content; the presence of other additives; the source of agar and agar's properties; RH at which films are conditioning before analyses. Nevertheless, it can be concluded that films formulated with LIS agar samples matched the properties of other agar films and may be an interesting alternative application, both as edible films (as external packaging) or edible coatings (in direct contact with food). However, and as happens for other hydrocolloids, agar's price is still not competitive for packaging applications. Furthermore, biopolymers' performance continues to be a drawback for bioplastics applications. Therefore, most published articles involve strategies to improve mechanical and barrier properties including the inclusion of a reinforcing agent, such as nanoparticles, or the formulation in composites, taking advantage of synergies or interesting properties from other biopolymers (e.g. Malagurski et al., 2017; Shankar & Rhim, 2016).

4. Conclusions

Previous studies showed that *Gracilaria tikvahiae* cultivated in open farms could be effectively used for nutrient bioextraction from urbanized estuarine waters, namely in Long Island Sound and the Bronx River Estuary, USA. Though *Gracilaria/Gracilariopsis* spp. are commonly used for agar production, information on agar from *G. tikvahiae* was scarce, probably because *Gracilaria/Gracilariopsis* have been mostly cultivated in China and Indonesia, and this particular species is not grown there. Furthermore, most reported yields for *G. tikvahiae* were low, not encouraging further industrial exploitation. Nevertheless, this study showed that it is possible to use *G. tikvahiae* biomass, cultivated in open waters, for agar production, which matches the performance of other *Gracilaria* agars used for food applications. Furthermore, this study also proved that an integrated strategy of nutrient bioextraction and agar exploitation is feasible, using the biomass from the first process as a sustainable source of seaweeds for the extraction of the hydrocolloid.

Acknowledgments

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