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Impact of cultivation of *Mastocarpus stellatus* in IMTA on the seaweeds chemistry and hybrid carrageenan properties

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ABSTRACT

The biomass yield potential of *Mastocarpus stellatus*, a commercially attractive carrageenophyte for foods and pharmaceutics, was investigated by cultivating the seaweeds in the nutrient-rich outflow of a commercial fish farm. Results from two consecutive 4 weeks experiments indicate that the cultivation of this seaweed produces a mean biomass of 21 to 40.6 g DW m^{-2} day^{-1} depending on the time of the experiment. DRIFT and CP–MAS NMR analyses of seaweeds indicate that cultivation during May affected quantitatively the seaweeds chemistry, and thus the chemical and gelling properties of native extracts of kappa/iota-hybrid carrageenan (KI). Overall, algal growth leads to the production of more sulphated KI, the percentage increase varying between 27% and 44% for the two experiments. However, alkali treatment of seaweeds before extraction reduces the variations in gelling properties of KI induced by the algal growth. This study demonstrates the capacity of growing *M. stellatus* in an integrated multi-trophic aquaculture system for the sustainable production of high value polysaccharides.

1. Introduction

Mastocarpus stellatus is a red marine macroalgae, and is one of the most predominant species at the intertidal zone of the Portuguese rocky coast (Araújo et al., 2009; Pereira & van de Velde, 2011). In Portugal, the value of the carrageenans extracted from wild collected *M. stellatus* has long been acknowledged (Santos & Duarte, 1991). An extensive study of this seaweed was more recently launched in order to optimize the alkali-extraction of the specific kappa/iota-hybrid carrageenan (KI) (Hilliou et al., 2006a; Azevedo et al., 2013), to screen for the season variability of the extracted KI (Hilliou et al., 2012) and to better rationalize the gelling properties of the KI by its chemical structure (Hilliou, Larotonda, Sereno, & Gonçalves, 2006b) or by the gel structure (Hilliou, Wilhelm, Yamanoi, & Gonçalves, 2009). The motivation for such a research effort lies in several facts: the industrial need for carrageenan is steadily increasing and alternatives to presently farmed carrageenophytes are to be found (Bixler & Porse, 2011); *M. stellatus* is in the GRAS list of seaweeds that can be used to extract food grade carrageenans (McHugh, 2003); *M. stellatus* is a model seaweed in the sense that only gametophytes can be harvested and thus only KI can be extracted instead of more complex hybrid carrageenans; KI are specific carrageenans for niche application in dairy industry (Bixler & Porse, 2011). KI are copolymers made of blocks of kappa-carrageenan (sequences of alternating 3-linked β-D-galactopyranose with a sulphate group on the fourth carbon of the sugar ring (**G4S**) and 4-linked α-D-anhydrogalactopyranose (**DA**)) and blocks of iota-carrageenan (sequences of alternating 3-linked β-D-galactopyranose with a sulphate group on the fourth carbon of the sugar ring (**G4S**) and 4-linked α-D-anhydrogalactopyranose bearing a sulphate group on the second carbon of the anhydro ring (**DA2S**)). Hybrid carrageenans differ from KI by the fact that blocks of **G4S–DA** sequences are separated from blocks of **G4S–DA2S** sequences by more sulphated carrageenan disaccharide units such as mu-carrageenan (**G4S–D6S**) and nu-carrageenan (**G4S–D2S6S**).

The integrated multi-trophic aquaculture (IMTA) systems are a way to diversify the aquaculture crops (Buschmann et al., 2008; Chopin et al., 2008), to increase the profitability (Chopin et al., 2008; Shi, Zheng, Zhang, Zhu, & Ding, 2013), to reduce the inorganic nutrient load from aquaculture effluents (Neori et al., 2004; Troell et al., 2003) and consequently to improve the social acceptability towards aquaculture (Barrington, Chopin, & Robinson, 2009). Several

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studies demonstrated that it is possible to cultivate economically valuable seaweeds in situ (Abreu et al., 2009; Marinho-Soriano, Panucci, Carneiro, & Pereira, 2009; Zhou et al., 2006), or using wastewaters from intensive or semi-intensive land-based systems (Buschmann, Troell, Kautsky, & Kautsky, 1996; Matos, Costa, Rodrigues, Pereira, & Sousa Pinto, 2006; Abreu, Pereira, Yarish, Buschmann, & Sousa-Pinto, 2011). The latter referenced studies focused on the production in an IMTA of agarophytes and carrageenophytes such as the Irish moss (*Chondrus crispus*) which is harvested for carrageenan production in several countries. *C. crispus* is morphologically similar to *M. stellatus* and presents the same photosynthetic capacity (Lohrmann, Logan, & Johnson, 2004). However, a comprehensive study assessing the potential of *M. stellatus* biomass yields and the effect of such cultivation on the seaweeds chemical properties and on the chemical–physical properties of the extracted high valued polysaccharides is missing in the literature.

The objectives of this study are (a) to evaluate the potential of biomass productivity of *M. stellatus* in an IMTA system, (b) to screen for possible changes in the chemical structure of *M. stellatus* after cultivation in an IMTA and (c) to check for the possible modification of the chemical–physical properties of KI extracted from grown seaweeds when compared with the polysaccharides isolated from the wild seaweeds.

2. Experimental

2.1. Seaweed tank system

This work was conducted at the intensive aquaculture farm A. Coelho e Castro (ACC) located in Póvoa de Varzim, where around 40 t of turbot (*Scophthalmus rhombus* Linnaeus, 1758), 5 t of sea bass (*Dicentrarchus labrax* Linnaeus, 1758) and 500,000 Senegalese sole juveniles (*Solea senegalensis* Kaup, 1858) are cultivated (Domingues, 2010). The water supply for the seaweed tanks was a mixture from water pumped from the sea (salinity of 34‰) and water from the recirculation system of the intensive fish farm, The experiment ran in two periods of 4 weeks: 29th of April 2010 to 27th of May 2010 (labelled as May experiment) and from the 2nd to 30th of June (labelled as June experiment). Before starting the May and June experiments, the tanks were stocked with new *M. stellatus* biomass collected from the same location in northern Portugal (Praia de Mindelo—41°19′N, 8°44′W). The seaweeds were cultivated in circular 1120 L polyethylene tanks (1.5 m^2, 80 cm deep, diameter 138 cm). Each tank contained an air diffuser placed at the bottom centre, so that seaweeds were grown suspended in the water column. The water flow was kept the same in every tank (200 L h^{-1}), meaning a water turnover rate of 0.18 vol h^{-1}. After flowing through the seaweed tanks, water was discharged into the sea. The mean water temperature recorded was 19.3 ± 0.1 °C in the first period and 20.0 ± 0.1 °C in the second one.

2.2. Productivity assessment

In the first and second periods, nine tanks were randomly "seeded" with 1, 2 or 3 kg m^{-2}, in order to evaluate the effect of the stocking density on the biomass yield. The seaweed biomass was harvested weekly, drained to remove the excess superficial water and then weighted (±10 g). After the weighting process, the seaweed biomass was re-stocked with biomass from the previous week of cultivation to the initial density in each tank.

Productivity P expressed in g (dry weight, DW) m^{-2} day^{-1} was determined by the formula:

$$P = \frac{DW}{SW}\frac{FW - IW}{Ad} \quad (1)$$

where FW is the final fresh weight, IW is the initial fresh weight, A is the area in m^2 of the tank, d is the number of days between sampling, DW is the seaweed dry weight and SW is the seaweed fresh weight. Three samples of cultivated *M. stellatus* were taken per stocking density, weighted and dried in an oven at 60 °C to calculate the dry weight (DW)/fresh weight (SW) ratios. The average DW/SW ratio was 0.29.

2.3. Seaweeds postharvest storage

Wild seaweeds and seaweeds cultivated during 5 weeks (May and June experiments) in all tanks were harvested for later chemical characterization and KI extraction. Right after harvesting, seaweeds were dried (24 h at 60 °C in a air convection oven) and stored in opaque plastic bags during a maximum duration of 6 months prior to KI extraction and chemical analysis, to allow for the preservation of seaweeds and KI chemical structures, as demonstrated elsewhere (Romero, Villanueva, & Montaño, 2008; Hilliou et al., 2012).

2.4. Spectroscopic analyses

The diffuse reflectance infrared Fourier transform (DRIFT) method used for the qualitative and semi-quantitative characterization of the native seaweed and the extracted KI is described in length elsewhere (Azevedo et al., 2013). Briefly, fronds or thalli of dried seaweeds were simply scratched onto the abrasive pad of the DRIFT accessory of the FTIR Spectrometer (Spectrum 100, PerkinElmer Ltd., United Kingdom). KI powder samples were directly deposited on the DRIFT accessory. 16 scans acquired at 4 cm^{-1} resolution were averaged to build a DRIFT spectrum. The semi-quantitative analysis of DRIFT spectra obtained with the DRIFT accessory was performed by computing the band intensity ratios A_{1240}/A_{2920}, A_{930}/A_{2920} and A_{805}/A_{845}, which correspond to the relative contents in sulphate, 3,6-anhydrogalactose (**DA**) with some contributions from the sulphated galactose (**G4S**), and iota-carrageenan, respectively, (see Villanueva, Hilliou, & Sousa-Pinto, 2009 for details). Preliminary tests showed no significant quantitative difference between the band intensity ratios computed from DRIFT spectra obtained with the fronds and from DRIFT spectra obtained with the thalli of collected dried seaweeds. In addition, band intensity ratios A_{1240}/A_{2920} and A_{930}/A_{2920} were not sensitive to variations in the stocking density of cultivation of the seaweeds. Thus, only seaweeds cultivated with the highest stocking density were used for chemical analysis with solid state NMR and for extraction of KI.

High resolution CP–MAS–HPD ^{13}C-NMR Nuclear Magnetic Resonance spectra of the powdered seaweeds samples were recorded at 75 MHz in a Bruker AVANCE III spectrometer, with a MAS of 10 kHz and an accumulation of 1000 scans.

2.5. Kappa/iota-hybrid carrageenan extraction

Three types of KI were obtained, namely KI isolated from native seaweeds (native KI), KI in the Na^+ form (KI Na^+) isolated from seaweeds treated with 1 wt% NaOH, and KI in the K^+ form (KI K^+) isolated from seaweeds treated with 5 wt% KOH. The KI extraction was performed using the set of optimized parameters obtained in an earlier study (Azevedo et al., 2013). Briefly, dried seaweeds were powdered and soaked at 80 °C during 5 h in NaOH solution (1 w/v%) or in KOH solution (5 w/v%). 5 M HCl solution was then added to the suspension to lower the pH down to 8–9, and suspensions were hearted at 90 °C for 1 h. This extraction was followed by an amylase treatment performed at 50–55 °C (1 mg of amyloglucosidase from *Aspergillus niger*, Fluka Biochemika, for 1 g of algal material), and by centrifugation of the suspension at 80 °C and 8000 rpm. Finally, the

Table 1
Productivity from the two sets of 4 weeks experiments conducted with *M. stellatus* under the specified culture conditions. Tabulated values of FW and (FW-IW) refer to individual 7 day periods of growth, and are not cumulative over the 4-week period of each trial.

Time	Culture conditions		IW (kg)	FW (kg)	(FW–IW) (kg)	Productivity		
	SD ($kg\,m^{-2}$)	Temp.(°C)				TAN input ($\mu mol\,TAN\,L^{-1}$)	Productivity ($g\,DW\,m^{-2}\,day^{-1}$)	Mean and s.e. ($g\,DW\,m^{-2}\,day^{-1}$)
29th April–27th May	1	19.3 ± 0.1	1.50	1.85	0.35	154.1 ± 12.1	10.7 ± 0.6	
	2	19.3 ± 0.1	3.00	3.85	0.85	151.2 ± 6.2	22.5 ± 2.0	
	3	19.3 ± 0.1	4.50	5.63	1.13	153.0 ± 6.1	29.6 ± 2.9	
2nd–30th June	1	20.0 ± 0.1	1.50	2.80	1.30	170.5 ± 12.1	40.0 ± 3.1	
	2	20.0 ± 0.1	3.00	4.61	1.61	179.0 ± 11.9	42.8 ± 3.1	40.6 ± 2.4
	3	20.0 ± 0.1	4.50	5.98	1.48	175.0 ± 7.5	38.9 ± 1.4	

supernatant was precipitated in 2.5 vol of ethanol (96%) and the precipitate was filtered through cotton cloth, washed twice with ethanol and oven dried at 60 °C for 24 h. In particular, the extraction of KI K^+ involves the KOH neutralization by addition of HCl and is followed by precipitation in ethanol, thus in the presence of large amount of KCl. It has been shown (Rochas, Rinaudo, & Landry, 1989) that this process removes irregular galactans from extracts of *Eucheuma cottonii*, which is not the case with ethanol precipitation in the presence of NaCl. As such, the chemical structures of KI Na^+ and KI K^+ are different (see Fig. 5) and no comparison between the two extracts will be carried out here.

2.6. Gels preparation and viscoelastic characterization

The experimental protocol used to prepare hot KI solutions (concentration 1 wt% in 1 mol L^{-1} NaCl for the KI Na^+, and in 0.1 mol L^{-1} KCl for both the native KI and the KI K^+), to produce and characterize the corresponding equilibrated gels in a stress-controlled rheometer (MCR 300, PaarPhysica) is described in Hilliou, Larotonda, Sereno and Gonçalves (2006b). The appropriate amount of KI powder was poured into the corresponding NaCl or KCl solution. These suspensions were then stirred during 1 h at 80 °C to ensure complete dissolution of KI. Hot KI solutions were gelled (cooling rate $-1\,°C\,min^{-1}$ from 80 °C down to 20 °C) in the concentric cylinders accessory (CC27) of the rheometer and dodecane was used to cover the shearing geometry to prevent any water loss. Small amplitude oscillatory shear (SAOS) measurements of storage modulus (G') and loss modulus (G'') for equilibrated gels (2 h at 20 °C) were made over the frequency-range 100–0.01 Hz, using a strain amplitude of 0.5%. The intrinsic viscosity of each KI was determined as described elsewhere (Azevedo et al., 2013). Briefly, KI solutions were prepared as for the characterization of gels, but using much smaller KI concentrations (from 0.01 w/v% to 0.06 w/v%) and 0.1 M NaCl as solvent. KI solutions were poured in a capillary viscosimeter (501–10 Type I, SCHOTT, Germany) and flowing times for each concentration were measured in triplicate. Intrinsic viscosities of each KI were extrapolated from the construction of the Huggins' plots.

2.7. Statistical analysis

A multi-factorial analysis of variance (MANOVA) was used to analyse the influence of time (fixed, 2 levels) and stocking density (fixed, 3 levels) on the productivity of *M. stellatus*. Cochran's heterogeneity of variances test was applied to the data. Significant differences between treatments were analysed a posteriori with a Student–Newman–Keuls (SNK) test ($\alpha = 0.05$). The productivity statistical analyses were performed using the software GMAV (EICC, The University of Sydney, Australia). A one-way analysis of variance (ANOVA) was used to determine the effects of cultivation time (2 levels, 6 replicates) on the seaweeds chemical properties assessed by DRIFT, and on the yield, gel and chemical properties of extracted KI. Tukey's studentized range (HSD) test was carried out as post hoc comparison procedure. Microcal Origin software (Microcal Software, Inc., Northampton, MA) version 8.0 was used to carry out the KI physical–chemical properties statistical analysis.

3. Results and discussion

3.1. Productivity of M. stellatus

Results from the two experimental periods are displayed in Table 1. The different stocking densities did not significantly affect the biomass yields of *M. stellatus* in the June experiment (ratio of means $F = 0.088$, and corresponding computed value of $p = 0.916$), whereas in May it was observed a linear increase in the productivity at higher stocking densities ($F = 6.03$; $p = 0.006$). Therefore, the mean and standard error of the nine replicates was applied to test the productivity in the June experiment. The time of experiment (May or June experiment) did influence significantly the productivity ($F = 34.02$, $p = 0.0001$) but no interaction between time and stocking density was found ($F = 2.96$, $p = 0.09$). The SNK test showed that the yield was significantly larger in time 2 ($40.57 \pm 2.37\,g\,DW\,m^{-2}\,day^{-1}$) than in time 1 ($20.95 \pm 3.29\,g\,DW\,m^{-2}\,day^{-1}$) (see Table 1). This result is explained by the higher water temperature measured (20.0 ± 0.1 °C) and by the larger mean total ammonium nitrate (TAN) input ($174.85 \pm 3.33\,\mu mol\,TAN\,L^{-1}$ vs. $152.80 \pm 2.52\,\mu mol\,TAN\,L^{-1}$) in the system found in June compared to May. There were no significant differences in TAN input between stocking densities ($F = 0.53$, $p = 0.95$). Regarding calculations of new biomass produced during the cultivation, for example in the tanks stocked with a density of $3\,kg\,m^{-2}$ the new biomass produced (FW-IW) constitutes 25 to 33% of the IW per week. As the tanks are restocked with the same algae, after 5 weeks (1 acclimation week and 4 weeks of experiments), most of the biomass is therefore new. In the first growing period, yields obtained in the $1\,kg\,m^{-2}$ density group are similar to those reported for *C. crispus* (ca. $8.4 \pm 2.60\,g\,DW\,m^{-2}\,day^{-1}$) during the spring by Matos et al. (2006). However, the productivity of *M. stellatus* at time 1 for the densities 2 and $3\,kg\,m^{-2}$ were clearly larger compared to *C. crispus* yet comparable to the ones obtained by Abreu et al. (2011) for *Gracilaria vermiculophylla* ($23.3 \pm 1.67\,g\,DW\,m^{-2}\,day^{-1}$). Overall, the productivity results in the June experiment are comparable to the results reported by Matos et al. (2006) for *G. vermiculophylla* (identified then as *Gracilaria bursa pastoris*) ($31.2 \pm 7.80\,g\,DW\,m^{-2}\,day^{-1}$), *C. crispus* ($36.6 \pm 11.10\,g\,DW\,m^{-2}\,day^{-1}$) and *Palmaria palmata* ($40.2 \pm 12.8\,g\,DW\,m^{-2}\,day^{-1}$). The present results position *M. stellatus* as a competitive seaweed for integrated aquaculture. In the June experiment, seaweeds stocked at the higher densities may have been deprived of light, which explains the absence of differences in productivity in this period. This is in agreement with results obtained for *G. bursa pastoris* (Matos et al., 2006) and for *G. vermiculophylla* (Abreu et al., 2011). The higher supply of TAN in the system found in June probably supported the better productivity found in this experiment. Still, the absence of differences in the productivity in June for the higher densities tested can also be

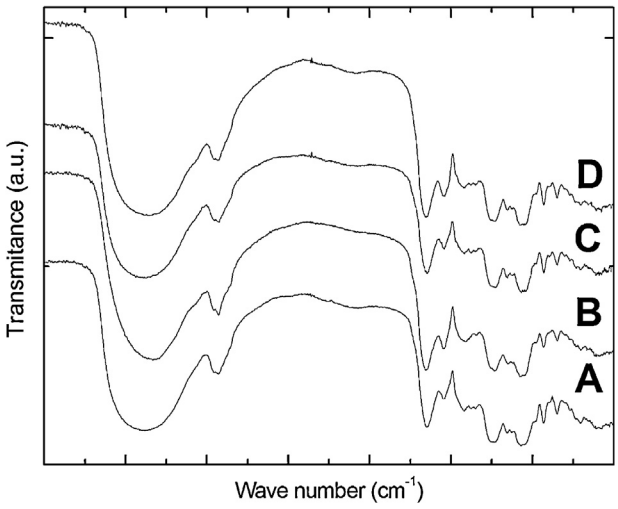

Fig. 1. DRIFT spectra of native *M. stellatus* harvested in April (A) and in June (C), and of *M. stellatus* collected after one month cultivation in IMTA during the period April–May (B) and June (D).

related to a limitation in the nutrients supply and may represent an important constraint on cultivation of this seaweed in larger densities.

M. stellatus grew consistently well in the circular 1120 L tanks throughout the 8 weeks of cultivation in all the stocking densities tested. Moreover, this seaweed was able to tolerate the changing weather conditions and nutrients in the water, highlighting the potential of this seaweed to be reared in an IMTA system. Still, a year round study should be carried out, in order to confirm the feasibility of the cultivation of *M. stellatus* during winter, when lower nutrient inputs are expected.

3.2. Variation in the chemical structure of M. stellatus

DRIFT spectra obtained with the fronds of representative seaweeds are presented in Fig. 1. They are very similar to the spectra presented elsewhere for wild *M. stellatus* and corresponding KI extracts (Azevedo et al., 2013). All the characteristic bands of carrageenan but the one assigned to the 2-sulphate of the α-D-anhydrogalactopyranose (**DA2S**, at 805 cm^{-1}), which is specific to iota-carrageenan, can be found in the DRIFT spectra of all *M. stellatus* seaweeds analysed in the present study. This is in contrast with the DRIFT spectra of extracted KI where the band at 805 cm^{-1} is resolved, along with the two other diagnostic bands for KI, namely the bands at 930 cm^{-1} and 845 cm^{-1} which are assigned to the 3,6-anhydrogalactose (**DA**) and the 4-sulphate of the β-D-galactopyranose (**G4S**), respectively (see below). Overall, DRIFT spectra of seaweeds grown during one month in the IMTA system are qualitatively similar to the DRIFT spectra of the wild seaweeds introduced in the tanks. The statistical analysis of the DRIFT band intensity ratios presented in Table 2 for the wild and cultivated seaweeds studied in May and June suggests that both total sulphate content (ratio A_{1240}/A_{2920}) and **DA** content (ratio A_{930}/A_{2920}) significantly increase during the cultivation of seaweeds in April. The simultaneous increase in sulphate content and **DA** content can be explained by the contribution of **G4S** to the band at 930 cm^{-1}. Such contribution was established in a study where the band intensity ratio A_{930}/A_{2920} computed from Infrared spectra of mixtures of carrageenan and agarose and desulphated carrageenan, linearly correlated with the total sulphate content of the mixtures obtained from turbidimetric method, ^{1}H-NMR and ionic capacity (Rochas, Lahaye, & Yaphe, 1986). Thus, the increase in sulphate content as determined from ratio A_{1240}/A_{2920} positively contributes to the increase in ratio A_{930}/A_{2920}. No significant variation in DRIFT data could be found with seaweeds cultivated in June (see corresponding *F* ratios and *p* values in Table 2).

Table 2
One-way ANOVA (*F* ratios and corresponding probabilities *p*) on the effect of cultivation on the DRIFT band intensity ratios A_{930}/A_{2920} and A_{1240}/A_{2920}, computed from DRIFT spectra of wild and cultivated *M. stellatus* seaweeds during the May and June experiments, and carrageenan composition of wild and cultivated *M. stellatus* seaweeds computed from CP–MAS–HPD ^{13}C-NMR peak intensities of carbons of galactopyranose and anhydrogalactopyranose residues. For DRIFT data the degree of freedom (*df*) for $N = 6$ and $n = 3$ was 2.

DRIFT	May		June	
	F	*p*	*F*	*p*
A_{930}/A_{2920}	65.3	0.001[a]	3.8	0.12
A_{1240}/A_{2920}	51	0.002[a]	0.99	0.37
CP–MAS ^{13}C-NMR	**Wild**	**Cultivated**	**Wild**	**Cultivated**
Kappa-carrageenan (mol%)	34.3	14	28.9	32.8
Iota-carrageenan (mol%)	27.1	42.5	27.1	27.0
Mu-carrageenan (mol%)	35.7	43.5	44.0	40.2
Nu-carrageenan (mol%)	2.9	0.0	0.0	0.0

[a] Significant ($p < 0.05$).

The CP–MAS–HPD ^{13}C-NMR spectra of seaweeds collected during cultivation in the IMTA are displayed in Fig. 2. There are no qualitative differences between the spectra which suggest that the chemical composition of *M. stellatus* is not qualitatively altered during the cultivation. Essentially, these spectra are qualitatively similar to spectra measured by Rochas and Lahaye (1989) with kappa-carrageenan and iota-carrageenan commercial powders as well as with *Hypnea musciformis* seaweeds. To the best of our knowledge, this is the only report where CP–MAS ^{13}C-NMR spectra of carrageenophytes and agarophytes are documented. Following Rochas and Lahaye (1989), the two broad peaks centred at 175 ppm and between 50 and 20 ppm are assigned to the carboxyl and methyl groups of pyruvates, respectively. Fig. 2 points towards a quantitative difference in the chemical composition of *M. stellatus* collected after one month cultivation in IMTA during the period April–May: the peak at 175 ppm is largely higher indicating more pyruvated seaweeds. These two signals are present in all dried samples, along with the 9 signals from 40 ppm to 120 ppm. We note here that no signal was measured between 50 and 20 ppm in ^{13}C spectra of KI extracted from wild *M. stellatus* (Azevedo et al., 2013). We thus suspect that the broad signal between 50 and 20 ppm also show contributions from lipids and pigments contained in the seaweed (Rochas & Lahaye, 1989) and removed from the KI during the extraction process. As such, the

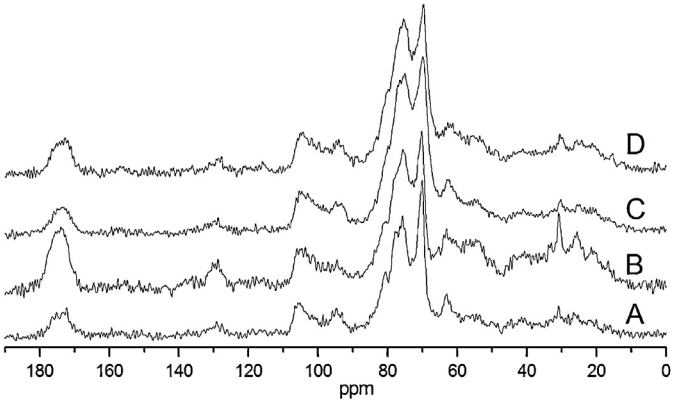

Fig. 2. CP–MAS–HPD ^{13}C-NMR spectra of native *M. stellatus* harvested in April (A) and in June (C), and of *M. stellatus* collected after one month cultivation in IMTA during the period April–May (B) and June (D).

Table 3
Assignment of computed Lorentzian lines to the carbons of the four carrageenan types.

carrageenan structural units									
Iota		Kappa		Mu		Nu		Chemical shift range (ppm)	Line number
G4S	**DA2S**	**G4S**	**DA**	**G4S**	**D6S**	**G4S**	**D2S,6S**		
C1		C1		C1		C1		107–104	1
					C1		C1	100	2
	C1		C1					97–94	3
		C3	C3		C4	C3	C4	82–81	4
C3	C5, C3, C4		C5, C4	C3			C2	80–79	5
C5	C2	C4, C5		C4, C5		C5		77	6
C4						C4		74	7
C2	C6	C2	C2, C6	C2	C3, C2, C5	C2	C3, C5, C6	72–70	8
C6		C6		C6		C6		63	9

important proportion of such contaminants evidenced in Fig. 2 is a limiting factor to a direct comparison with the chemical structure of KI reported in the literature and which are nearly free of pyruvate, lipids and pigments. Thus the present comparison should be taken with caution. For the quantitative analysis of NMR data, 9 lines were assigned a specific number of carbons, out from the 48 carbons of the four types of carrageenans, namely iota-, kappa-, mu- and nu-carrageenan, according to the respective chemical shifts as established in the work of van de Velde, Pereira and Rollema (2004). Fig. 3 details the quantitative analysis for the spectrum of the seaweed collected in April and introduced in the IMTA during the May experiment. The same procedure was carried out for the remaining samples. The spectrum between 40 ppm and 120 ppm is fitted with 9 Lorentzians of similar width. The assignment of the chemical shifts associated with each line is given in Table 3. The only parameters adjusted to the experimental spectrum are the 4 weighting factors corresponding to the composition of seaweeds in the four types of carrageenans and the common width of the Lorentzians. A residual line showing up at 55 ppm for all samples, and not assigned to any carrageenan, was also added in the fitting as to improve the quality of the adjustment to the line at 63 ppm (sixth carbon of the **G4S** unit). The outputs of this analysis are gathered in Table 2. Changes in seaweeds chemistry are identified for the May experiment, which is consistent with the DRIFT analysis. Cultivation and thus growth of seaweeds leads to a decrease in kappa-carrageenan which is compensated by an increase in both iota-carrageenan and the kappa-carrageenan precursor (mu-carrageenan). Results are different for the June experiment as no chemical change occurs during the growth of seaweeds, in agreement with the DRIFT analysis. Thus, the two sets of experiments lead to contradictory results and do not allow a clear understanding of the effect of algal growth on the seaweed chemistry. We suspect that seasonal variation between the two experiments brings additional complexity to the analysis. Indeed, the increase in relative content of sulphated carrageenans is in harmony with the seasonal variation assessed in an earlier study where *M. stellatus* collected in winter produced significantly more sulphated native KI (Hilliou et al., 2012). The percentage increase of sulphated KI contained in the seaweeds during cultivation can be estimated from the band intensity ratio A_{1240}/A_{2920} computed for the May experiment and corresponding to the total sulphate content of the seaweeds. This ratio increases by $44 \pm 10\%$ when compared to the ratio computed from wild seaweeds used to seed the tanks. This value is in harmony with the increase in iota-carrageenan (36%) and in mu-carrageenan (18%) (the latter correlates with a 36% increase in sulphate as this carrageenan shows and additional sulphate group when compared to iota-carrageenan) computed from the corresponding CP–MAS–HPD ^{13}C-NMR analysis (see Table 2). Nevertheless, results validate the use of CP–MAS–HPD ^{13}C-NMR for quantitatively assessing the chemical structure of seaweeds without relying on tedious extraction and chemical analysis of polysaccharides.

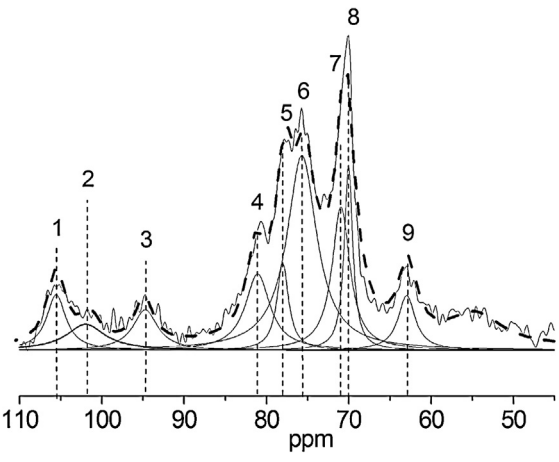

Fig. 3. CP–MAS–HPD ^{13}C-NMR spectrum of native *M. stellatus* harvested in April and computed spectrum (dashed line) using the nine Lorentzian lines (see text for details and Table 3 for line assignment).

3.3. Variations in chemical properties of extracted KI

The chemical compositions determined by DRIFT and ^{1}H-NMR analyses are displayed in Fig. 4 for all extracted hybrid carrageenans, along with their corresponding gel elasticity G_0, intrinsic viscosity $[\eta]$ and extraction yield. Examples of ^{1}H-NMR spectra of extracted hybrid carrageenans are given in Fig. 5. KI extracted from seaweeds studied during the May experiment do not present any significant change in their chemical structure assessed with the DRIFT analysis. This is hard to reconcile with the chemical variations detected for the corresponding seaweeds using both DRIFT and CP–MAS–HPD ^{13}C-NMR. In contrast to this, seaweeds cultivated in June with no impact on their DRIFT and NMR patterns, produce more sulphated native KI (a percentage increase of 27% in total sulphate content is computed from the comparison between A_{1240}/A_{2920} values of extracts from wild and cultivated seaweeds) with larger **DA** content but less iota-carrageenan units (see A_{1240}/A_{2920}, A_{930}/A_{2920} and A_{805}/A_{845}, respectively, in Table 4 and Fig. 4). This suggests the recovery of carrageenan with more biological precursors units (such as mu- and nu-carrageenan), together with more kappa-carrageenan units, as confirmed by the corresponding significant increase in the 5.11 ppm peak intensity calculated from ^{1}H-NMR spectra. The large viscosity of native KI solutions has a negative impact on ^{1}H-NMR spectral resolution and thus impedes the separation of signals assigned to mu- and iota-carrageenan at 5.25 and 5.31 ppm, respectively. Similar

Table 4
One-way ANOVA (*F* ratios) on the effect of cultivation on the chemical and gel properties of native KI, KI Na^+ and KI K^+ obtained from wild and cultivated *M. stellatus* seaweeds. In this case the degree of freedom (*df*) for $N = 6$ and $n = 3$ was 1 (wild and 4 weeks cultivation).

Carrageenan property	Native KI		KI Na^+		KI K^+	
	May	June	May	June	May	June
A_{805}/A_{845}	0.9	9.6[a]	<0.1[b]	54.5[a]	1.4	46.7[a]
A_{930}/A_{2920}	4.6	512[a]	0.7	216[a]	3.7	<0.1[b]
A_{1240}/A_{2920}	2.9	59.6[a]	<0.1[b]	229[a]	0.4	0.4
Kappa-carrageenan (mol%)	1.2	59[a]	0.5	12.1[a]	10.5[a]	<0.1[b]
Iota-carrageenan (mol%)	30.3[a,c]	10.2[a,c]	0.5	12.7[a]	8.4[a]	<0.1[b]
Nu-carrageenan (mol%)	91.5[a]	83.7[a]	n.a.	n.a.	n.a.	n.a.
Carrageenan yield	828[a]	18[a]	16.7[a]	0.4	14.2[a]	56.2[a]
G_0	37.6[a]	1.7	70.2[a]	0.9	1.4	9[a]
$[\eta]$	15,867[a]	426[a]	29.9[a]	13,321[a]	5222[a]	902[a]

[a] Significant ($p < 0.05$).
[b] Variation below the sensitivity of DRIFT and ^{1}H-NMR.
[c] Iota-plus mu-carrageenan content, as both ^{1}H-NMR peak intensities cannot be integrated separately.

experimental limitations were experienced in earlier studies (see for instance Fig. 2 in Hilliou et al., 2012). However, quantitative information can still be extracted by integrating both signal intensities. In this way a relative content in both iota- and mu-carrageenan is computed and corresponding values are labelled with an asterisk in Fig. 4. An overall decrease in this relative content is noted for the experiment conducted in June (see Fig. 4) which confirms the decrease in iota-content measured with DRIFT, in contrast to the overall increase noted in May. Overall, both DRIFT and ^{1}H-NMR analyses of native KI for the two periods indicate that 1 month IMTA of *M. stellatus* leads to an increase in sulphated carrageenan. Since the ^{1}H-NMR peak intensity assigned to nu-carrageenan drops significantly after cultivation in all cases, we assign the increase in sulphate content to mu-carrageenan, which is consistent with the corresponding increase measured with CP–MAS–HPD ^{13}C-NMR for seaweeds cultivated in May. Indeed, a percentage increase of 21% is computed from the comparison between the ^{1}H-NMR peak intensities assigned to both iota- and mu-carrageenan for native KI extracted from wild and cultivated seaweeds studied in May, which is in harmony with the percentage increase in mu-carrageenan (18%) computed from the CP–MAS–HPD ^{13}C-NMR analysis of corresponding seaweeds and with the increase of total sulphate content computed from DRIFT data ($44 \pm 10\%$).

^{1}H-NMR and DRIFT are not sensitive enough to detect any variation in the chemical structure of KI Na^+ extracted from seaweeds studied throughout May. This relates to the too small growth of seaweeds during May, which present a quantitative change in their chemical pattern but with no detectable impact on the chemistry of extracted KI Na+. The experiment conducted in June shows however that the growth of *M. stellatus* in the IMTA allows the recovery of more sulphated KI Na^+. Indeed, the band intensity ratio A_{1240}/A_{2920} shows a large (see corresponding large *F* ratio in Table 4) relative increase, which is concomitant with the large decrease in **DA**, and to a less extent with the decrease in A_{805}/A_{845}.

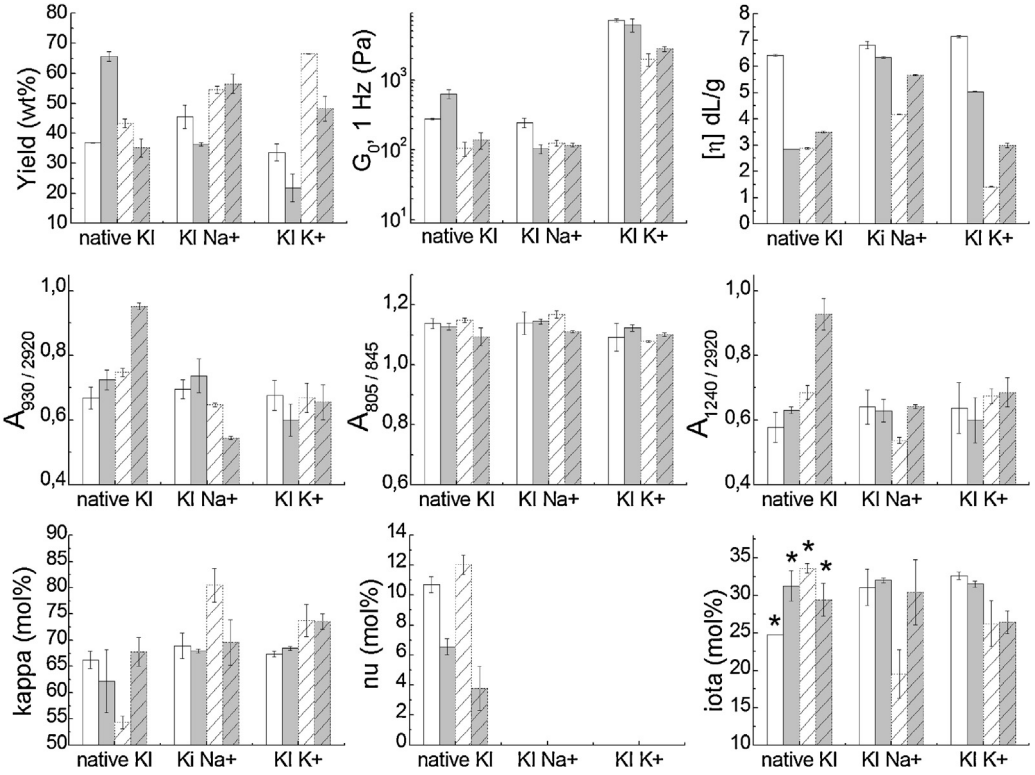

Fig. 4. Yield, gel elasticity G_0, intrinsic viscosity [η], DRIFT band ratio intensities and carrageenan composition (as determined by ^{1}H-NMR) of KI extracted from wild (white bars) and cultivated (grey bars) seaweeds during April (empty bars) and June (patterned bars). Asterisk in the lower right chart indicate that mu-carrageenan is also included in the calculation of the iota-carrageenan content (see text for detail).

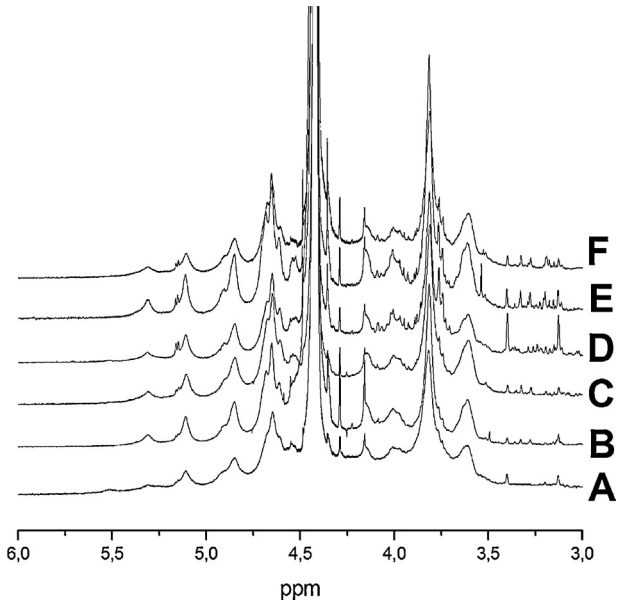

Fig. 5. ^{1}H-NMR spectra of KI extracted from wild *M. stellatus* harvested in April ((A) KI; (B)KI K^+; (C) KI Na^+) and from cultivated *M. stellatus* during the period April–May ((D) KI; (E) KI K^+; (F) KI Na^+).

In contrast to the DRIFT analysis of seaweeds, the band intensity ratio A_{930}/A_{2920} in spectra of KI is not affected by contributions from **G4S** as all carrageenan in KI show this sulphate group. Thus in this case, a decrease in A_{930}/A_{2920} relates indeed to a decrease in **DA** content. Combined with the increase in A_{1240}/A_{2920} and the decrease in A_{805}/A_{845}, we conclude that the increase in sulphated KI Na^+ relates to sulphate groups **D2S** or **D6S**. This trend is confirmed by the relative decrease in the intensity of ^{1}H-NMR signals assigned to kappa-carrageenan. No mu-carrageenan signal could be resolved in the ^{1}H-NMR spectra of KI Na^+. This comes as no surprise since the alkaline treatment use to extract this KI has been optimized to convert such biological precursors (Azevedo et al., 2013). Therefore, a significant increase in iota-carrageenan can be inferred from the spectra, which can be positively correlated with the increased content in sulphate detected in DRIFT, since this carrageenan possesses one additional sulphate (**DA2S**), when compared to kappa-carrageenan. However, this result only matches the decrease in A_{805}/A_{845} if this ratio is affected by the increasing contributions from biological precursors of iota-carrageenan (nu-carrageenan with **D2S** and **D6S**) suggested above, but the amount of nu-carrageenan is below the detection limit of ^{1}H-NMR as no signal shows up at 5.56 ppm (see Fig. 5). Overall the chemical analysis of KI Na^+ confirms that the growth of *M. stellatus* in the IMTA allows the production of more sulphated KI, which in this case will bear more iota-carrageenan units.

KI K^+ containing significantly more kappa-carrageenan but less iota-carrageenan was extracted from seaweeds cultivated in May when compared to KI K^+ extracted from wild seaweeds. In June, only a significant increase in A_{805}/A_{845} is found, which again suggests an evident discrepancy with the results achieved in May. If we consider however the *F* values listed in Table 4 for KI K^+, we note that variations in the chemical structure of KI K^+ extracted in May are less significant than the variation in the DRIFT obtained during June. Taking this into account, we may draw the following big picture: the cultivation of *M. stellatus* results in the production of KI K^+ with larger amounts of kappa-carrageenan and likely iota-carrageenan. As such, this result is consistent with the recovery of more sulphated native KI after cultivation, since the alkali conversion of more sulphated carrageenan using KOH should yield KI K^+ with more kappa- or iota-carrageenan units. *F* values computed for the native KI are overall larger than those computed for KI K^+. We thus suspect that the efficient alkali conversion of biological carrageenan precursors contained in seaweeds will smear out any significant variation in the chemical composition of the extracted KI K^+.

To summarize this part and making the connection with the chemical analysis of seaweeds, results suggest that the growth of *M. stellatus* in IMTA leads to the production of seaweeds containing more sulphated carrageenan. The alkali treatment of these seaweeds with K^+ however mitigates the chemical changes associated with the algal growth. Most of sulphated carrageenans are successfully converted into iota- or kappa-carrageenan and a KI K^+ with virtually constant chemical structure can be recovered. Algal growth during cultivation is at the origin of the biosynthesis of more biological carrageenan precursors with more sulphate groups.

3.4. Variations in the physical and gel properties of extracted KI

Carrageenan yields range from 20 wt% to 65 wt% for all extracts obtained, and are in harmony with earlier studies where similar extraction parameters were used (Azevedo et al., 2013). The statistical analysis of carrageenan yields reported in Table 4 indicates that seaweeds cultivation generates significantly less carrageenan, except for the experiment conducted in May where more native KI was obtained after cultivation. Differences in seaweeds growth during the cultivation study can be called here to explain the different results for the two experiments: growing seaweeds usually contain less carrageenan, and thus much larger KI yields are obtained from seaweeds collected in seasons corresponding to smaller growths (Hilliou et al., 2012).

The aquaculture affects significantly the intrinsic viscosity $[\eta]$ of all extracted KI as inferred from the statistical analysis presented in Table 4. The range of intrinsic viscosities reported in Table 4 is in agreement with values reported elsewhere for KI extracted from wild *M. stellatus* and for commercial kappa-carrageenan (Azevedo, Bernardo, & Hilliou, 2014). However a clear picture of the interplay between cultivation time and the molecular mass of recovered KI (which relates to $[\eta]$) cannot emerge from the data displayed in Fig. 3 since results obtained in May are at odd with results obtained in June. Hurtado, Critchley, Trespoey and Bleicher-Lhonneur (2008) also could not report any conclusive trend for the carrageenan molecular masses obtained from different cultivation tests. However these authors found that larger carrageenan yields correspond to polysaccharides with smaller molecular masses, which is in harmony with the negative correlation between the yields and $[\eta]$ ($R = -0.651$, $p < 0.001$).

Since KI is industrially attractive for its gel ability, we screened for possible improvement in the gels properties of all KI extracts, resulting from the IMTA of *M. stellatus*. Qualitatively, gels formed in NaCl with KI Na^+ are very similar to gels obtained from various wild *M. stellatus* seaweeds and using different alkali treatments (Souza, Hilliou, Bastos, & Gonçalves, 2011; Azevedo et al., 2013). In addition to the values of the gels elastic moduli G′ which are of the order of 100 Pa and to the minimum evidenced in the frequency dependence of the loss moduli G″ reported in Fig. 6, similarities include the two-step gelation mechanism measured during the cooling of the KI Na^+ hot solutions and which is illustrated in the inset of Fig. 6A. Gels with large enough strength and elasticity to compete with iota-carrageenan industrial grades, were even obtained with native KI, thus confirming a recent report (Azevedo et al., 2013). Nearly no effect of cultivation on the elasticity G_0 of KI K^+ gels is seen (a slightly significant increase is calculated with extracts from June, with *p*-value of 0.04), and there is no significant variation in the elasticity of gels prepared with native KI and KI Na^+ extracted from seaweeds studied in June. This set of data thus suggests that changes in the chemistry of seaweeds and in

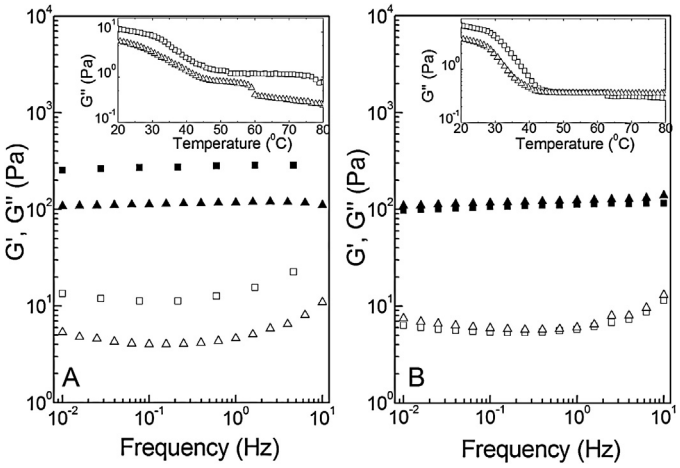

Fig. 6. Gel mechanical spectra (solid symbols G', empty symbols G'') of KI Na^+ gels in 1 mol L^{-1} NaCl measured at 20 °C: (A) KI Na^+ isolated from wild *M. stellatus* harvested in April (squares) and from cultivated *M. stellatus* collected after 1 month (May) in IMTA (triangles); (B) KI Na^+ isolated from wild *M. stellatus* harvested in June (squares) and from cultivated *M. stellatus* collected after 1 month (June) in IMTA (triangles). Insets: temperature dependence of the loss moduli G'' recorded during the cooling of hot 1 wt% KI Na^+ solutions in 1 mol L^{-1} NaCl prepared with KI Na^+ extracted from wild *M. stellatus* (empty squares) and cultivated *M. stellatus* (empty triangles).

the chemical–physical properties of extracted KI, which are related with the growth of *M. stellatus* in the IMTA, have no impact on the gel properties of the recovered carrageenans. A study where three different *Kappaphycus* species were grown in the effluents of a fish farm also reported that kappa-carrageenans recovered after seaweeds treatment with K^+ do not show any significant variation in the gelling properties when compared to kappa-carrageenan extracts from wild seaweeds (Rodrigueza & Montaño, 2007). In contrast to this, native KI and KI Na^+ extracted in May show a significant increase and decrease in the gel elasticity, respectively, after seaweed cultivation. Differences in salt and thus gelling conditions for the two products impede any comparison between the gel properties. In addition, and as underlined elsewhere (Hilliou et al., 2006b), G_0 depends on many interdependent parameters, some of them being very difficult to quantify (for instance the length of kappa-carrageenan blocks in the KI). Thus simple correlations between such parameters and gels properties are difficult to assess, and "model" KI are needed to establish gel-chemistry relationships (Souza et al., 2011).

4. Conclusions

The cultivation of *M. stellatus* integrated with fish aquaculture produces a significant amount of biomass. One month growth of *M. stellatus* in IMTA generates seaweeds with no qualitative chemical change, but possessing more pyruvates and biological carrageenan precursors. The kappa/iota-hybrid carrageenans extracted from these seaweeds show gel properties similar to the ones isolated from wild *M. stellatus*. This is explained by the alkali treatment of seaweeds prior to extraction which successfully converts the more sulphated biological precursors which develop during algal growth into kappa/iota-hybrid carrageenans. The quantitative chemical analysis of native kappa/iota-hybrid carrageenans extracted from wild and cultivated seaweeds studied in May suggest that algal growth in the IMTA corresponds to a percentage increase in sulphated kappa/iota-hybrid carrageenans ranging between 36% and 44%. The commercial value of this seaweed as a source of kappa/iota-hybrid carrageenans makes it a feasible choice for integrated aquaculture.

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