



Culture of *Chondracanthus teedei* and *Gracilariopsis longissima* in a traditional salina from southern Spain

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Abstract

The cultivation of two red macroalgal species, *Chondracanthus teedei* (Martens ex Roth) and *Gracilariopsis longissima* (S.G. Gmelin) Steentoft M, L.M. Irvine & W.F. Farnham, was assessed in a traditional salina, a system of earthen ponds used for marine salt extraction taking advantages of solar evaporation and tidal cycle. Vegetative thalli of both species were cultivated in rafts holding polypropylene ropes, from January to June 2015, when lock-gates were opened during the period of no salt production. The effects of three factors in the net growth rate were analysed: seedling density, water motion and seasonality. Water motion and seasonality showed a significant effect in the growth of both species. Seedling density only showed a significant effect in the growth of *Gp. longissima*, where the growth rates improved at high seedling densities. Values of tissue N were generally lower than critical quotas, suggesting that maximum growth was limited by the concentrations of dissolved nutrients. In addition, the high salinity and temperatures in late spring seemed to condition the values of net growth rate. The study suggested that macroalgal cultivation of these two valuable species could be a promising complementary activity in the integrated management of the salina during winter and early spring, when salinity is lower than 40 PSU, if nutrients in the water are increased with the semi-intensive fish cultivation and the hydrodynamic conditions along the rafts are enhanced.

Keywords Rhodophyta · Earthen ponds · Net growth rate · Macroalgal cultivation · Nitrogen · Yield

Introduction

Geomorphological and archaeological evidence as well as historical documents suggest that salt exploitation and related activities (e.g. salted fish products) have been economically important in

Cádiz Bay since Phoenician times (fifth century BC) (Torrejón 1994; Alonso-Villalobos et al. 2003). This activity has generated a considerable natural and cultural legacy, modifying the natural saltmarsh landscape and geomorphology and determining the life style of the human population that inhabited this area for centuries (Britton and Johnson 1987; Alonso-Villalobos et al. 2004). Traditional salinas in the Southern Iberian Peninsula consist of a system of earthen ponds, creeks and lock-gates created in floodable saltmarshes, which, taking advantage of tides differences, light slopes and the solar evaporation, results in the salt extraction from seawater. This activity is usually combined with extensive fish aquaculture, and both activities occur during summer and autumn. During winter and spring, the lock-gates remain opened and generally no exploitation activities occur (Alonso-Villalobos et al. 2004; Alonso-Villalobos and Ménanteau 2004).

Until the early twentieth century, the salt extraction was profitable and relevant for the local economy. However, after 1930, when industrial refrigeration became feasible and technical advances in underground industrial salt exploitation reduced exploitation costs, traditional salinas were less competitive and suffered a strong decline (Torrejón 1997; Alonso-Villalobos et al. 2004). Between 1930 and 1999, more than 97% of these salinas

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ceased their activities in the area (Alonso-Villalobos et al. 2004). Since then, these lands have been naturally or anthropogenically filled and urbanised in many cases, resulting in the loss of their natural and cultural capital (Britton and Johnson 1987; Sadoul et al. 1998; Alonso-Villalobos et al. 2004; Duarte et al. 2008).

Currently, European aquaculture is stagnating, in contrast to the increasing rates of aquaculture production globally. In order to dampen this trend, the European Commission published different communication strategies for the development of the European aquaculture industry, which failed to increase the European production. This led to the publication in 2013 of a third Commission communication (COM (2013) 0229), aimed at achieving the sustainable development of European aquaculture. This document highlighted the necessity of securing sustainable development and growth of aquaculture through coordinated spatial planning. This document also recognised the role of extensive pond aquaculture supporting biodiversity and offering environmental services and business opportunities beside food production. In this sense, it is remarkable that the role of traditional salinas supporting a rich diversity of birdlife and a characteristic flora, which has been recognised elsewhere (Britton and Johnson 1987; Sadoul et al. 1998; Masero and Pérez-Hurtado 2001). This biodiversity and the geographical position of Cadiz Bay close to the Strait of Gibraltar make this area a pivotal point for bird migrations between Europe and Africa (Hortas et al. 2004). The importance of this has been recognised by international environmental conventions and laws (e.g. RAMSAR Convention, Europeans Birds Directive 79/409/EEC and Habitat Directive, 92/43/EEC). The development of seaweed mariculture in salinas in Cadiz Bay may be an excellent opportunity to promote the compatibility of a profitable commercial activity with the conservation of biodiversity and cultural heritage. The promotion of innovative practices of sustainable aquaculture has been proposed as the best solution to optimise its efficiency and maintain the health of coastal waters (Chopin et al. 2001).

Two native macroalgal species of marketable value relatively abundant in Cadiz Bay are ideal candidates for seaweed mariculture: *Chondracanthus teedei* (Martens ex Roth) and *Gracilariopsis longissima* (S.G. Gmelin) Steentoft M, L.M. Irvine & W.F. Farnham. These species were also selected because they potentially grow at high rates under elevated nutrient concentrations in the surrounding environment (Zinoun et al. 1993; Hernández et al. 2006; Huo et al. 2011).

Gracilariopsis longissima is an agarophyte belonging to the order of Gracilariales, which has a worldwide distribution (Guiry and Guiry 2017). This species and *Gracilaria gracilis* (Stackhouse) Steentoft M, L.M. Irvine & W.F. Farnham were considered similar in the past due to their morphological resemblance and were known by the name of *Gracilaria verrucosa* (Hudson) Papenfuss, a current synonym of *Gp. longissima* (Steentoft et al. 1995). These species have been used equally as a source of agar in South Africa (Wakibia et al. 2001;

Rothman et al. 2009), and probably in India and China, where the synonym *G. verrucosa* is still used (Huo et al. 2011; Padhi et al. 2011). The species have also been proposed as a potential source of bioethanol and other compounds of interest for pharmaceutical and biotechnological applications (Stabili et al. 2012; Shukla et al. 2016). *Gracilariopsis longissima* shows a typical Polysiphonia-type life history with three phases consisting of morphologically identical tetrasporophyte (2n) and gametophyte (n) phases and an additional carposporophyte (2n) phase (Kain and Destombe 1995). In southern Spain, *Gp. longissima* has been recently used for human consumption and commercialised under the name of Ogonori (Pérez-Lloréns et al. 2016).

Chondracanthus teedei is a carragenophyte belonging to the order Girgartinales. It is present in the North-East Atlantic Ocean, the Mediterranean Sea and the Black Sea (Yang et al. 2015). As for *Gp. longissima*, *C. teedei* shows a typical Polysiphonia-type life history (Guiry 1984). This species can be locally abundant, reaching high biomass densities, and show a high carrageenan content, making it a potentially important source of kappa:iota hybrid carrageenan (Pereira and Mesquita 2004; Pereira 2012). This species has also been traditionally used in some parts of Sicily for human consumption (Pérez-Lloréns et al. 2016). Furthermore, close species as the morphologically similar *Chondracanthus chamissoi* (Yang et al. 2015) is being commercialised in Chile and Japan, here under the name of Shinkin-nori, being also highly valuable (Vásquez and Alonso-Vega 2001; Bulboa et al. 2013).

The aim of this work was to study the technical feasibility of seaweed extensive mariculture in traditional salinas from southern Spain during winter and spring, when no exploitation activities are developed. The effects on culture performance of seaweed density, hydrodynamic conditions and seasonality, which have shown a relevant influence in previous seaweed cultivation studies (Wakibia et al. 2001; Ryder et al. 2004; Ganesan et al. 2006; Peteiro and Freire 2011), were assessed. This information will be critical for the further development of seaweed cultures in traditional salinas.

Material and method

Study site

Field cultivation studies on *C. teedei* and *Gp. longissima* were conducted at “Salina de la Esperanza”, located in southern Spain (36°30'39" N, 6°09'34–35" W), from January to June 2015. This salina is currently used for salt exploitation and extensive fish farming (e.g. grey mullets, European eel, seabream and seabass) in a traditional way. Two marked periods can be distinguished in this exploitation. The first period comprises from late spring to late autumn, when the lock-gates remain closed most of the time to avoid the escape of the fish and to obtain salt by evaporation in the shallowest parts of the earthen ponds. The closure of the lock-

gates and the intensive evaporation during summer produce a significant increase in salinity and temperature, and the water movement is dramatically reduced. This induces an important reduction in the biomass of red seaweeds that thrive in the salinas during the summer. The second period stretches from early winter to late spring, when the lock-gates are opened to obtain fish juveniles, and water conditions are similar to the surrounding Cadiz Bay, since there is a free exchange of water between the pond systems and the Bay due to tidal currents. During this period, it is possible to observe an increase in the biomass of the red seaweeds thriving in these environments (our pers. obs.).

Experimental design

Overall, the cultivation trial of the two species aimed to assess the feasibility of seaweed aquaculture in traditional salinas and the effects of the following factors: initial seaweed density (three levels: low, mid and high), hydrodynamic conditions (two levels: high and low) and seasonality (three levels: January–February, March–April and May–June) on culture performance (i.e. relative growth rate and weekly yield per metre), quality of the biomass (%N) and abundance of epiphytes. In order to accomplish with these objectives, the experimental trial was developed following a randomised complete block design.

Raft cultivation method

Two rectangular rafts (1.3 m × 2.2 m) made of PVC and braced with cable ties were used to provide the basic cultivation structure (Fig. 1a). Each raft held 9 parallel polypropylene ropes (3 mm diameter) spaced 18 cm and attached by two plastic shackles to the frame of the raft. The rafts were suspended at 30 cm of depth and disposed in such a way that braided polypropylene ropes were parallel to the main flow, with no physical interaction between them as a consequence of flow direction, separation between ropes and seaweed sizes. Ropes were placed in clusters of three to host the density treatment (see below). The three clusters of ropes were separated 36 cm (Fig. 1a) and the positions of the clusters were randomly swapped within the raft every week to minimise experimental errors. Each rope was then considered as an independent replicate (e.g. Wakibia et al. 2001; Peteiro and Freire 2013). Buoys were attached to the corners to promote buoyancy, and four concrete blocks of 8 kg each were set to fix the position (Fig. 1b). Due to the effect of tides, the anchor lines, connecting the concrete blocks and the raft, were long enough to allow the rafts being at a depth of 30 cm, minimising the displacement and ensuring the parallel position of the seeded lines to the main water flow.

Seeding lines at different densities

All the seaweeds were collected in a salina close to the culture site. Specimens of *C. teedei* and *Gp. longissima* were placed

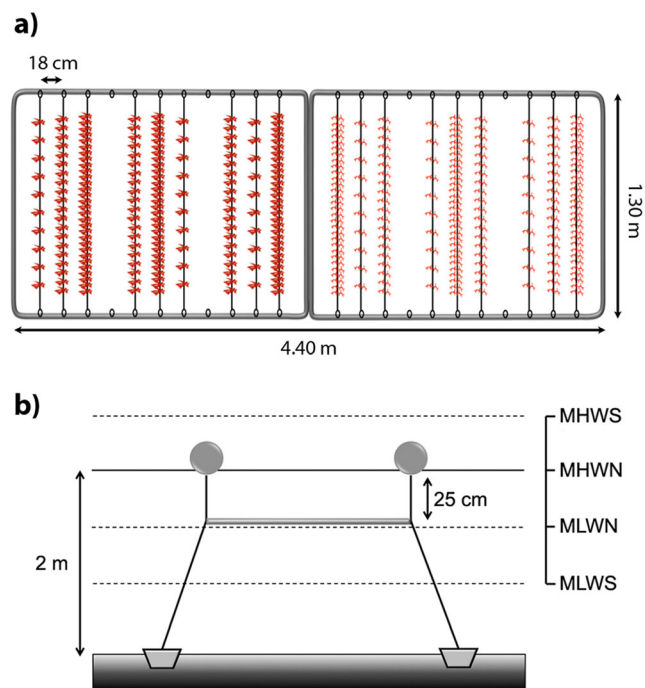


Fig. 1 Diagram of the basic cultivation structure (a) and schematic view of its position within the earthen pond (b). MHWS: mean high water springs, MHWN: mean high water neaps, MLWN: mean low water neaps, MLWS: mean low water springs

in a cooler box with gel packs and transported to the laboratory in dark and wet. Once in the lab, the specimens were rinsed with seawater and all epiphytes removed using a wet cloth. Thalli keeping the apical part of 10 ± 1 cm of *C. teedei* (0.5 ± 0.2 g) and 18 ± 2 cm of *Gp. longissima* (0.2 ± 0.1 g) were inserted between the twist of the braided ropes. Three different densities were tested: low, mid and high, placing the seaweed fragments at 10, 5 or 2.5 cm of distance, which corresponded with 10, 20 and 40 seaweed fragments per metre of rope, respectively. Three replicates were used per treatment and species, which made a total of 108 ropes. All the seeded ropes were placed in the rafts within 24 h after seaweed collection. Seeded ropes were transported to the experimental site in a cooler box with gel packs and wrapped individually in a wet cloth to avoid desiccation and seaweed breaking.

Raft deployment at different water motion conditions

To assess the effects of the water motion in the culture, two sites with contrasting hydrodynamic conditions were chosen, one next to the lock gate and other 40 m away from the former. These sites were selected as close as possible to ensure the existence of similar water conditions (temperature, salinity, dissolved oxygen, inorganic nutrient concentration, etc.). Due to spatial constraints, it was not possible to place more than one basic structure per site. To confirm water motion differences between sites, current velocity was recorded simultaneously during an entire tidal cycle on October 2015 (tide coefficient

0.75 approximately) using two vector D Doppler current metres (Nortek). The obtained results confirmed the existence of significant differences in water motion. In the conditions defined as low water motion, the current velocity was always lower than at high water motion conditions (Fig. 2), and the maximum current velocity did not reach values higher than 15 cm s^{-1} (maximum average inflowing current 12.5 cm s^{-1} ; maximum average outflowing current 5.48 cm s^{-1}). In contrast, maximum current velocities higher than 100 cm s^{-1} (maximum average inflowing current 141.6 cm s^{-1} ; maximum average outflowing current 25.9 cm s^{-1}) were observed at high water motion conditions.

Seasonality

Three culture cycles of 7 weeks each were conducted to assess the effect of seasonality in seaweed growth during the period in which lock-gates were opened. The first cycle (“Winter” or “January–February”) started on 15th of January 2015 and finished on 4th of March. The second cycle (“Early spring” or “March–April”) stretched from 6th of March to 22nd of April. Finally, the third cycle (“Late spring” or “May–June”) ranged from 23rd of April to 12th of June.

Culture monitoring

After 7 weeks of field culture, the ropes were collected, wrapped in wet paper and transported to the laboratory in a cooler box with gel packs to estimate different variables related to the culture performance (i.e. epiphyte biomass, tissue C and N content and growth). Once in the lab, seaweeds were counted, detached from the rope and rinsed with clean seawater to remove the epiphytes. Subsequently, the cultured seaweed and their respective epiphytes were weighted after removing water excess with filter paper until wet dots disappear. A small fragment (aprox. 2 g fresh weight) of the cultured seaweed was dried in the oven at $60 \text{ }^\circ\text{C}$ for at least 48 h and grinded using a porcelain mortar. These grinded samples were stored in Eppendorf tubes in a desiccator with silica gel until

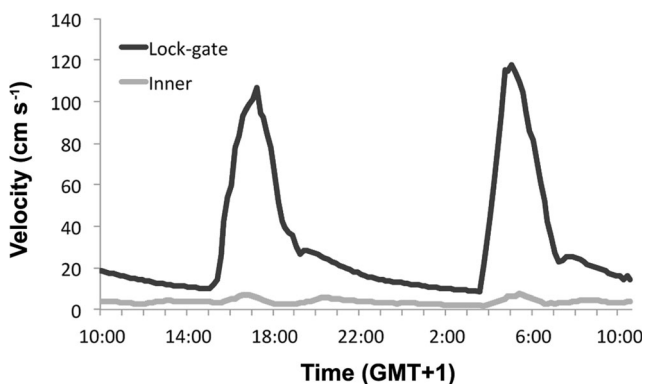


Fig. 2 Current velocity measured under high (black; lock-gate) and low (grey; inner) water motion conditions in a 24 h period in October 2015

sent to “Servizos de Apoio á Investigación” at the University of La Coruña (Spain), where tissue C and N contents were determined using a Flash combustion EA1108 elemental analyser (Carlo Erba Instruments).

Yield was calculated as the difference between the final and the initial fresh weight (FW) divided by the number of weeks of culture and the length of the rope and was expressed as $\text{g FW week}^{-1} \text{ m}^{-1}$.

To calculate the relative daily growth rate, an exponential growth was assumed (eq. 1):

$$\text{DGR } (\% \text{day}^{-1}) = 100 \times \ln(\text{FW}_f/\text{FW}_0)/t \quad (1)$$

where *DGR* is the relative daily growth rate; *FW_f* is the final fresh weight after *t* days of culture; *FW₀* is the initial fresh weight; and *t* is the number of days of culture.

Physico-chemical monitoring

During the cultivation period, physico-chemical variables were monitored weekly. Water temperature, pH, and dissolved oxygen were measured at 20 cm of depth using a portable multiparametric sonde (sensION+ pH 1 and DO6; HACH, respectively). Salinity was determined by a hand refractometer (ATAGO S-20E). Water samples were taken to measure suspended solids (SS) and dissolved inorganic nutrients (nitrate, nitrite, ammonium and phosphate) per triplicate. Each nutrient replicate consisted of 10 mL of filtered seawater. This water was filtered in situ using a syringe, a filter holder and a GF/F Whatman filter (effective pore size $0.7 \text{ } \mu\text{m}$). Nutrients were determined by a Skalar SAN++ CFA autoanalyser. In the case of SS, 5 L of seawater from the study site was transported to the lab. Just before filtration, the 5 L of seawater was shaken to resuspend SS. Subsequently, using a vacuum pump, between 2 and 0.5 L of water (depending of the amount of SS) were filtered through a glass fibre filter (Whatman GF/F). The filter was dried in an oven at $60 \text{ }^\circ\text{C}$, for at least 24 h before the filtration and for at least 48 h after the filtration. The concentration of SS was calculated as the difference in weight of the dried filter after and before filtration divided by the filtered volume.

Data analysis

Statistical analyses were performed using the software R version 3.2.1 (R Development Core Team 2017) and PERMANOVA+add-on PRIMER 6 (Plymouth Routines in Multivariate Ecological Research) software (Anderson et al. 2008). In all statistical analyses, significance was set at *p* value < 0.05 probability, and when it was necessary, they were based on 9999 permutations.

A three factorial ANOVA was performed to assess the effects of seeding density, hydrodynamic conditions and seasonality in

the yield, DGR, internal nutrient content, relative epiphyte abundance and % of seedlings lost. A post hoc Tukey test was applied to compare levels of treatment factors when main factors had a significant effect. In case of significant interactions, Tukey test was used to compare the levels of each factor within each level of the other factor and vice versa. Shapiro–Wilk normality test and Levene test were used to assess normality and homoscedasticity, respectively. Epiphyte abundance data (per metre of rope and per g of cultivated seaweed) did not accomplish with homoscedasticity assumption even after data transformation. For this reason, a PERMutational univariate analysis of variance (PERMANOVA) was used instead. These PERMANOVA analyses were based on Euclidean distances.

Results

Environmental conditions

The values of the different environmental variables monitored are shown in Table 1. Water temperature, salinity and SS tended to increase from the beginning to the end of the experiment. Water temperature varied from 9.7 °C (February) to 27.6 °C (June and salinity ranged from 33 to 48 PSU). In the case of SS, it was noticeable that during early spring, turbidity conditions were especially variable. In contrast, dissolved oxygen and pH decreased from winter to late spring suggesting an increase in the respiration rate in the water column throughout the experiment. Although no significant differences in inorganic N concentrations were observed between seasons, these tended to increase throughout the experiment. The main source of inorganic N was ammonium, which ranged between 2.89 and 9.17 μM, being generally between two- and five-fold higher than nitrate. Regarding dissolved phosphate, the highest concentrations were observed in late spring. Overall, phosphate concentrations remained below

0.5 μM, close to the detection limit. The dissolved N/P ratio was generally much higher than the Redfield value of 16:1.

Yield, DGR and seedling losses

The ANOVA (Table 2) indicated that the three factors assessed and the interaction between seedling density and seasonality had a significant effect on the weekly yield of *C. teedei*. During January–February and March–April, the yield was higher at high seedling densities (i.e. 2.5 cm of distance between seedlings) than at low (i.e. 10 cm) and medium (i.e. 5 cm) ones. The maximum yields were reached during these seasons at high seedling density (January–February, $6.11 \pm 1.19 \text{ g m}^{-1} \text{ week}^{-1}$; March–April, $6.76 \pm 2.27 \text{ g m}^{-1} \text{ week}^{-1}$).

When growth was expressed as DGR, ANOVA (Table 2) showed that seasonality, water motion and the interaction between both factors had a significant effect on the DGR of *C. teedei*. Regarding seasonality, the minimum DGR was obtained during May–June ($-0.32 \pm 1.60\% \text{ day}^{-1}$). Similar growth rates were reached during January–February ($2.01 \pm 0.37\% \text{ day}^{-1}$) and March–April ($1.77 \pm 0.51\% \text{ day}^{-1}$). In the case of hydrodynamic conditions, *C. teedei* usually showed higher DGRs in high than in low water motion (Fig. 3). However, significant differences in growth between water motion conditions were only observed during May–June.

The percentage of seedling losses in *C. teedei* varied significantly between seasons ($F_{2,36} = 27.79$; p value < 0.001), being remarkably high during May–June ($53.27 \pm 13.98\%$) and minimal during January–February ($6.18 \pm 10.30\%$) and March–April ($8.71 \pm 7.31\%$). No significant effects were observed for water motion, seedling density and the interactions between the studied factors (Table 2).

Regarding *Gp. longissima*, ANOVA (Table 3) revealed that the three factors assessed and the interactions between seedling density and seasonality, and seedling density and water motion had a significant effect on the weekly yield.

Table 1 Mean, maximum and minimum values for the different environmental variables monitored during the different culture cycles ($n = 7$)

	Winter			Early spring			Late spring		
	Mean ± sd	Max	Min	Mean ± sd	Max	Min	Mean ± sd	Max	Min
Temp. (°C)	14.23 ± 2.84	17.90	9.70	18.22 ± 3.58	22.30	12.60	24.58 ± 2.03	27.60	22.80
pH	8.33 ± 0.34	8.87	7.89	8.14 ± 0.2	8.40	7.85	8.12 ± 0.085	8.19	8.01
Salinity	37.57 ± 3.46	41.00	33.00	39 ± 2.97	43.00	34.00	44 ± 2.83	48.00	40.00
O2 (%)	100.63 ± 15.88	120.00	70.70	86.13 ± 12.98	111.50	75.20	70.47 ± 16.48	82.20	46.50
O2 (mg/L)	8.17 ± 1.23	9.27	5.40	6.42 ± 0.93	7.97	5.20	5.23 ± 1.34	6.29	3.33
SS (mg/L)	30.18 ± 7.14	46.00	22.88	53.06 ± 27.03	87.50	23.60	58.57 ± 11.47	79.00	51.67
NH ₄ ⁺	5.03 ± 1.57	7.84	3.38	4.85 ± 1.43	7.27	2.89	6.22 ± 1.96	9.17	3.96
NO ₂ ⁻	0.21 ± 0.11	0.36	0.07	0.22 ± 0.17	0.57	0.07	0.26 ± 0.19	0.63	0.09
NO ₃ ⁻	1.08 ± 0.99	2.56	0.10	1.63 ± 2.45	6.96	0.18	2.92 ± 3.87	10.31	0.28
PO ₄ ³⁻	0.06 ± 0.03	0.1	0.02	0.1 ± 0.03	0.14	0.06	0.18 ± 0.1	0.32	0.07

Table 2 Results of three-way ANOVA analysis testing the effects of the factors “Seasonality” (S; 3), “Seedling density” (S), and “Water Motion” (WM) on daily growth rate (DGR), yield (yield), seedling loose

(seedling), percentage of internal N (%N) and epiphyte abundance (epiphyte) in *Chondracanthus teedei* cultures. * p value < 0.05; ** p value < 0.01; *** p value < 0.001

	Df	DGR		Yield		Seedling		%N		Epiphytes	
		MS	F-value	MS	F-value	MS	F-value	MS	F-value	MS	Ps-F
S	2	29.78	52.08***	93.40	16.13***	12,633	122.5***	1.123	24.86***	0.216	9.05***
D	2	0.07	0.13	28.11	4.85*	182.9	1.77	0.047	1.035	0.089	3.71*
WM	1	13.16	23.02***	76.35	13.19***	44.3	0.43	0.081	1.787	0.086	3.58
SxD	4	0.68	1.19	26.32	4.54**	208.0	2.02	0.038	0.843	0.060	2.51
SxWM	2	4.61	8.07**	18.59	3.21	39.5	0.38	0.475	10.53***	0.068	2.84
DxWM	2	0.89	1.57	9.58	1.65	258.0	2.50	0.001	0.005	0.013	0.56
SxDxWM	4	0.60	1.06	2.42	0.42	122.1	1.18	0.015	0.335	0.013	0.54
Residuals	36	0.57		5.79		103.1		0.045		0.024	

In general terms, high seedling densities produced higher yield than low and medium densities. The differences between seedling densities were enhanced under high water motion, being clearer during January–February than in March–April and May–June. The maximum yield was reached during January–February at high seedling density ($5.22 \pm 0.99 \text{ g m}^{-1} \text{ week}^{-1}$).

When growth was expressed as DGR, ANOVA (Table 3; Fig. 4a, b) indicated that seasonality, water motion, seedling density and the interaction between seasonality and seedling density showed a significant effect on the DGR of *Gp. longissima*. The highest DGR was reached during January–February ($3.26 \pm 0.62\% \text{ day}^{-1}$) and the lowest during May–June ($0.32 \pm 1.55\% \text{ day}^{-1}$, ten-fold lower). In general terms, *Gp. longissima* showed higher DGR under high water motion than under low water motion conditions (Fig. 4b). However,

significant differences between high and low water motion conditions were only observed during May–June (Fig. 4b). On the other hand, during January–February and May–June, a positive effect of seedling density on growth was observed, showing the high seedling densities the highest DGRs (Fig. 4a). In contrast to these seasons, during March–April, the highest seedling densities yielded the lowest DGRs, while mid densities showed the highest.

Significant differences were observed in the percentage of seedling losses in *G. longissima* between the different seasons and for the interaction between seasonality and hydrodynamic conditions (Table 3). The losses were higher during May–June ($37.23 \pm 19.72\%$) than during the January–February ($6.79 \pm 10.84\%$) and March–April ($13.70 \pm 10.58\%$). During January–February and March–April, no differences in seedling losses between water motion conditions were observed, but in May–June, losses under high water motion almost doubled those of low water motion (47.78 ± 14.60 and $26.67 \pm 19.04\%$, respectively).

Tissue N concentration

The percentage of tissue N in *C. teedei* varied seasonally (three-way ANOVA, $F_{2,36} = 24.86$; p value < 0.001), showing a significant interaction with water motion conditions (three-way ANOVA, $F_{2,36} = 10.53$; p value < 0.001). The percentage of tissue N was the lowest during January–February ($1.21 \pm 0.15\%$) and maximal during May–June ($1.68 \pm 0.28\%$). In January–February and March–April, the highest tissue N occurred at high water motion conditions, but in May–June, the opposite trend was observed (Fig. 5). The C/N ratio followed the opposite trend, being maximal in January–February (25.65 ± 3.39) and minimal during May–June (17.58 ± 1.93).

The percentage of tissue N in *Gp. longissima* varied significantly between seasons and water motion conditions. Furthermore, a significant and complex third interaction was observed (Table 3; Fig. 6a, b). The percentage of tissue N was

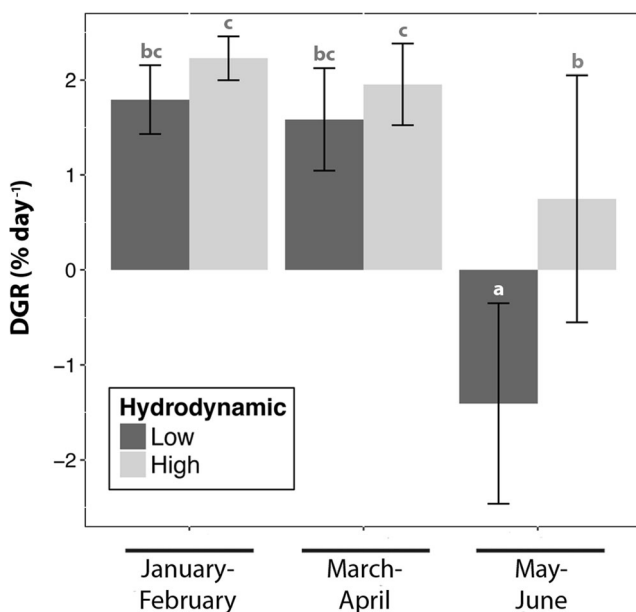


Fig. 3 DGR of *C. teedei* according to water motion conditions and season. Mean \pm standard deviation; $n = 9$. Letters over the bars represent significant differences between treatments

Table 3 Results of three-way ANOVA analysis testing the effects of the factors “Seasonality” (S; 3), “Seedling density” (S) and “Water Motion” (WM) on daily growth rate (DGR), yield (yield), seedling loose

(seedling), percentage of internal N (%N) and epiphyte abundance (epiphyte) in *Gracilariopsis longissima* cultures. **p* value < 0.05; ***p* value < 0.01; ****p* value < 0.001

	Df	DGR		Yield		Seedlings		%N		Epiphytes	
		MS	F-value	MS	F-value	MS	F-value	MS	F-value	MS	Ps-F
S	2	40.52	75.53***	17.76	28.32***	4582.3	27.79***	0.293	5.833**	0.43	4.37*
D	2	4.30	8.02**	39.68	63.28***	381.2	2.31	0.042	0.845	1.64	16.47***
WM	1	8.06	15.02***	9.40	15.00***	607.0	3.68	0.246	4.898*	0.50	5.04*
SxD	4	4.22	7.86***	2.31	3.68*	284.7	1.73	0.125	2.490	0.34	3.47*
SxWM	2	1.26	2.34	0.76	1.21	832.5	5.05*	0.141	2.798	0.16	1.63
DxWM	2	0.27	0.50	2.09	3.34*	84.2	0.51	0.085	1.696	0.20	2.02
SxDxWM	4	0.11	0.21	0.73	1.70	59.4	0.36	0.155	3.085*	0.13	1.36
Residuals	36	0.54		0.63		164.9		0.050		0.01	

lower in March–April ($1.31 \pm 0.25\%$) than in January–February ($1.53 \pm 0.26\%$) and May–June ($1.54 \pm 0.29\%$). Overall, tissue N was higher under high ($1.53 \pm 0.28\%$) than under low water motion ($1.39 \pm 0.27\%$). In this species, the C/N ratio was constant through the experiment (i.e. no effect of seasonality), showing specimens under low water motion conditions (22.71 ± 5.21) significant higher ratios than specimens in high (19.43 ± 3.16) water motion.

Epiphytes

PERMANOVA (Table 2) indicated a significant effect of seedling density, seasonality and their interaction in the relative epiphyte abundance growing on *C. teedei*. The highest relative epiphyte abundances were reached during May–June (0.26 ± 0.29 g epiphytes g^{-1} *C. teedei*), and the lowest in

January–February (0.04 ± 0.08 g epiphytes g^{-1} *C. teedei*), showing March–April intermediate values (0.10 ± 0.05 g epiphytes g^{-1} *C. teedei*). Overall, the highest relative abundance of epiphytes was found at low seedling densities and the lowest at high (Fig. 7). This trend was clearer in May–June than in March–April and was not observed during the January–February. When absolute values of epiphyte biomass were considered instead of relative abundances, the opposite effect of seedling density was observed (data not shown). In this case, the highest epiphyte biomass was observed at high seedling densities.

A significant effect of seedling density, seasonality, hydrodynamic conditions and the interaction between seasonality and seedling density in the relative abundance of epiphytes thriving on *Gp. longissima* was also observed (Table 3). The highest relative epiphyte abundances were reached during

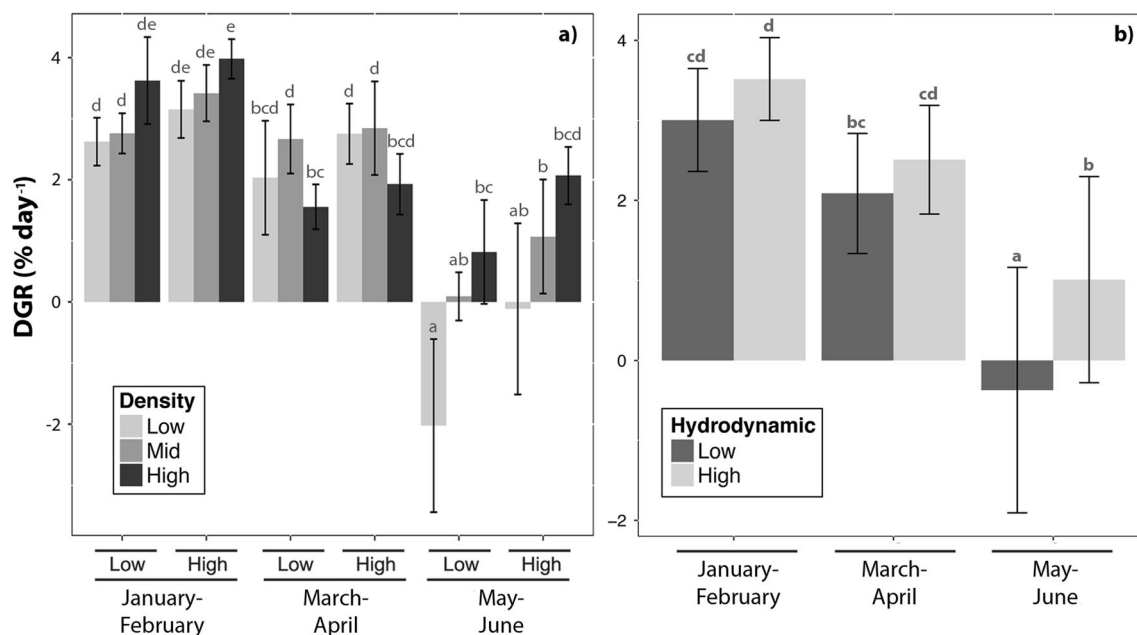


Fig. 4 DGR of *Gp. longissima* cultures according to initial seedling density, water motion and season (a; *n* = 3) and just considering water motion and seasonality (b; *n* = 9). Mean ± standard deviation. Letters over the bars represent significant differences between treatments

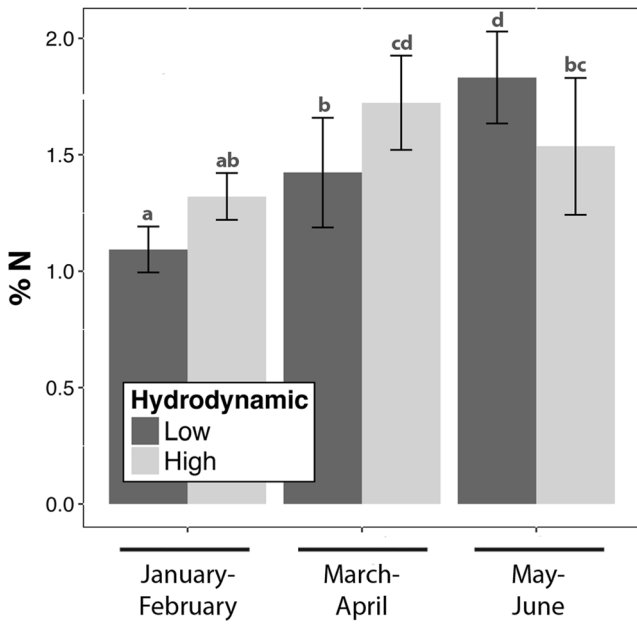


Fig. 5 Percentage of tissue N content in *C. teedei* according to water motion conditions and season. Mean ± standard deviation; $n = 9$. Letters over the bars represent significant differences between treatments

May–June (0.54 ± 0.68 g epiphytes g^{-1} *Gp. longissima*) and the lowest in January–February (0.23 ± 0.12 g epiphytes g^{-1} *Gp. longissima*), showing March–April intermediated values (0.34 ± 0.32 g epiphytes g^{-1} *Gp. longissima*). Overall, the highest relative abundance of epiphytes was found at low seedling densities and the lowest at high ones (Fig. 8). These differences in relative epiphyte abundances depended on the season, being minimal during January–February and

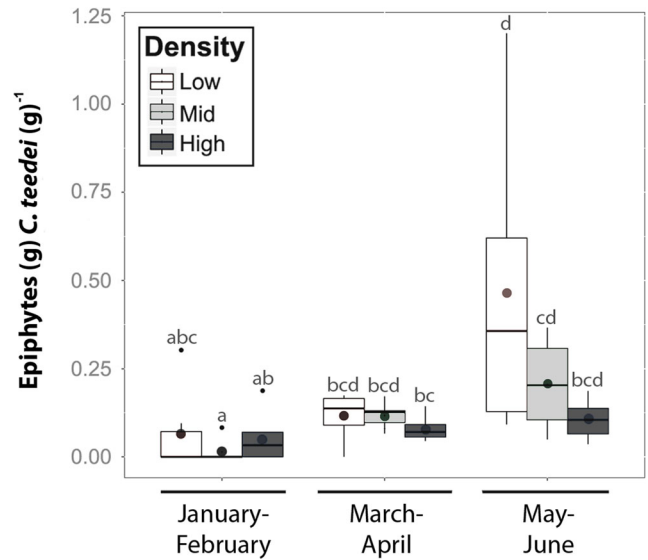


Fig. 7 Relative epiphyte abundance in *C. teedei* cultures according to initial seedling density and season ($n = 9$). Box plots indicate the mean (bigger dark dots), the median (bold line inside the box), the first and third quartile (upper and lower lines defining the box), the extreme values whose distance from the box is at most 1.5 times the inter quartile range (whiskers) and remaining outliers (dark dots). Letters over the box-plots represent significant differences between treatments

maximal in May–June (Fig. 8). Regarding water motion, slightly higher epiphyte abundances were found under low water motion. As in the case of *C. teedei*, when absolute values of epiphyte biomass were considered instead of relative abundances, the opposite effect of seedling density was observed (data not shown).

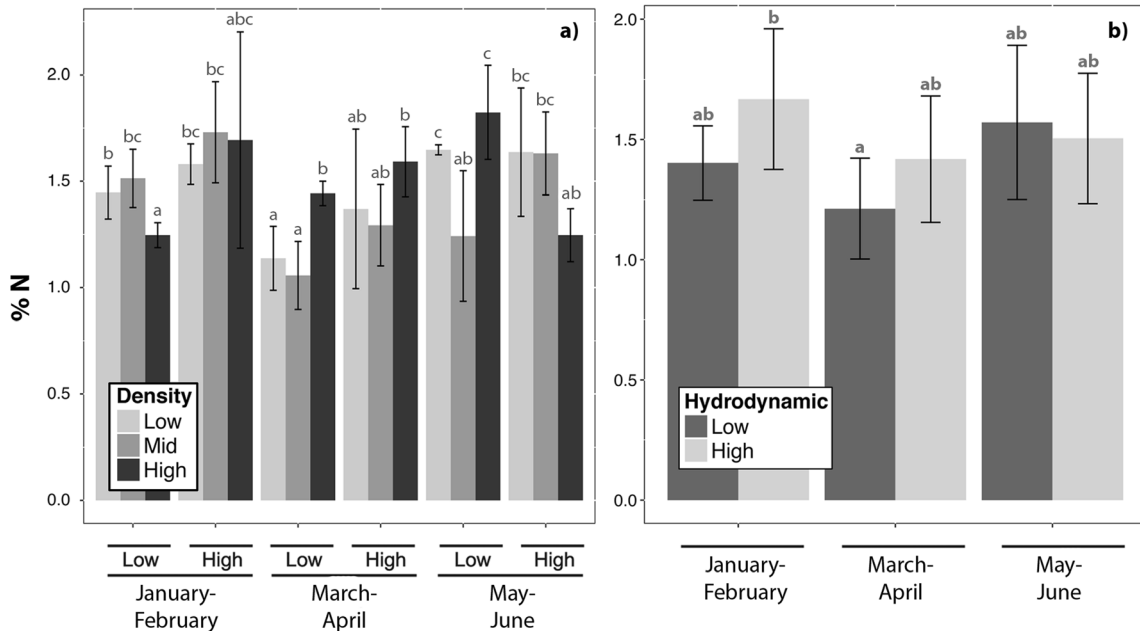


Fig. 6 Percentage of tissue N content in *Gp. longissima* cultures according to initial seedling density, water motion and season (**a**; $n = 3$), and just considering water motion conditions and season (**b**; $n = 9$). Mean

± standard deviation. Letters over the bars represent significant differences between treatments

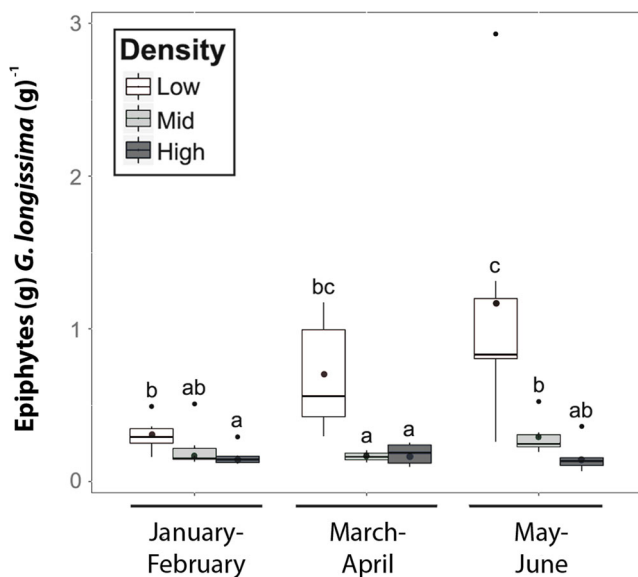


Fig. 8 Relative epiphyte abundance in *Gp. longissima* cultures according to initial seedling density and season ($n=9$). Box plots displayed as in Fig. 7. Letters over the box-plots represent significant differences between treatments

Discussion

This study has shown the feasibility of culture of two edible macroalgae in southern Spain as a complement of the traditional saline industry and the influence of three factors which clearly influenced the yield and quality of the algal biomass in terms of tissue N. The two species, *Gp. longissima* and *C. teedei*, reached the highest DGRs and showed the lowest relative abundance of epiphytes during January–February or March–April, suggesting that these periods are probably better for seaweed cultivation than May–June. Overall, the two species tended to grow faster under high water motion conditions (i.e. $> 100 \text{ cm s}^{-1}$). The positive effects of the enhanced flux were clearer during May–June, when both species showed the minimum DGRs. It is remarkable that during these months, null or negative DGRs were observed under low water motion, but still positive DGR values were observed at high water motion. On the other hand, the initial seedling density did not show any significant effect in the DGR or tissue N content in *C. teedei*. In the case of *Gp. longissima*, higher initial seedling densities showed positive effects for growth during January–February and May–June. Furthermore, the relative abundance of epiphytes tended to be lower under high seedling densities for both seaweeds. It was also remarkable that tissue N was usually lower than values found in *Gp. longissima* thriving near fish farm effluents (Pérez-Lloréns et al. 2004; Hernandez et al. 2005) or *C. teedei* living in polluted waters (Lourenço et al. 2006), and lower than the critical quota for growth estimated for *Gracilaria* and other macroalgae (ca. 2%, Hanisak 1983; Wheeler and Björnsäter 1992), which suggests that growth was constrained by nutrients.

The results of this study partially agreed with previous findings in natural populations of *Gp. longissima* thriving in a nearby tidal creek from Cadiz Bay (Pérez-Lloréns et al. 2004). This previous study identified the maximum peaks of tissue N in *Gp. longissima* during autumn and winter (approx. 3.5%). The comparison of DGRs between these two studies is difficult since the culture periods and methods differed significantly (1 vs. 5 weeks; cage vs. rope culture). However, growth patterns were similar. *Gp. longissima* living in tidal creeks also evidenced a marked loss of biomass in June ($-22\% \text{ day}^{-1}$). Salinity was more variable in our salina than in the saltmarsh (Pérez-Lloréns et al. 2004), while temperature and nutrients followed a similar pattern, but being phosphate concentrations slightly lower than in the tidal creeks. The high variability of salinity and also pH can produce an extra stress in the seaweeds thriving in the salina, which can partially explain the reduction in growth rates (Israel et al. 1999).

It is difficult to identify the factors or the interactions affecting negatively the DGR of the two species cultured in this study. In the case of *C. teedei*, Zinoun et al. (1993) observed a positive effect in growth of longer day lengths and identified an optimum temperature for growth between 20 and 25 °C under laboratory conditions with no nutrient limitation. Thus, the temperature and the longer day length observed during March–April and May–June should theoretically improve the DGR. Furthermore, nutrient concentrations in the water column remain constant or tended to increase from the start to the end of the experiment. However, in our study, the DGR in May–June was similar or even lower than in January–February. In this sense, the higher salinities and low dissolved oxygen observed in May–June could partially explain the low DGR reached. Otherwise, the combination of higher day lengths and higher temperatures in an unbalanced dissolved N/P environment can stress *C. teedei*, and thus reducing DGR (e.g. Creed et al. 1997; Burfeind and Udy 2009). In the case of *Gp. longissima* (= *G. verrucosa*), laboratory experiments revealed a maximum growth at 25‰ of salinity and 30 °C of temperature (Choi et al. 2006) with a slightly decrease in DGRs from 25 to 35‰ (maximum salinity assayed). Thus, these previous studies in combination with the high salinities observed suggest that the low DGR measured in traditional salinas could be consequence of high salinities reached during late spring, rather than temperature.

Further laboratory experiments assessing the role of salinity in the ecophysiology of both seaweeds species are currently being carried out and will be helpful to clarify if salinity is the main factor limiting growth or produce negligible effects during March–April and May–June. Preliminary results have shown that growth in *Gp. longissima* and *C. teedei* declines significantly under salinities higher than 35. Identify the factors limiting or restraining the growth will be key to propose new improvements and modifications in the structure and management of traditional salinas, to make seaweed aquaculture more feasible in these ponds.

The effect of water motion on marine macrophyte development and productivity varies significantly between species (Peteiro and Freire 2013; Sato et al. 2017). A rapid water motion can impose a physical stress for the thallus, leading to its detachment or breakage (e.g. Gerard and Mann 1979; Leigh et al. 1987; Hurd 2000). Correlations have been found between species zonation, ecological distribution and cell wall composition suggesting that matrix polysaccharides such as agar or carrageenan may impose an ecological advantage under determined ecological conditions through mechanical regulations (Kloareg and Quatrano 1988). The presence of these phycocolloids plays a structural role in algal tissues determining biomechanical properties, albeit it is not clear that carrageenan concentration and composition in the seaweed are regulated by water motion conditions (Carrington et al. 2001). *Gracilariopsis longissima* and *C. teedei* contain agar and carrageenan, respectively (Wakibia et al. 2001; Pereira and Mesquita 2004), which confers some biomechanical characteristic that has been hypothesised to be advantageous under high or moderate water motion conditions (Kloareg and Quatrano 1988). In fact, in the studied area (i.e. the Bay of Cadiz), these species dominate seaweed assemblages in rocky substrates where water velocity is enhanced, such as the lock gates of salinas, narrowing channels or bridge spans (our pers. obs.). In this case, there were no significant differences in seedling losses between high and low water motion conditions for *C. teedei*, and in the case of *Gp. longissima*, the differences in seedling losses between different water motion conditions depended on the season. However, epiphytes were significantly affected by water motion in the case of *Gp. longissima*, and a marginal significance was obtained in the case of *C. teedei* (p value = 0.066), being relative epiphyte abundances higher at low water motion. In this sense, the ability of these two species to thrive under high water motion conditions could be a desirable trait for cultivation, since it precludes or limits the settlement and development of epiphytes (Faucci and Boero 2000; Engkvist et al. 2004). It is noteworthy that no distinction between entangled and attached epiphytes was done when epiphyte abundances were assessed. This is important for culture performance because although both can cause deleterious effects in cultures, such as decrease in DGR as consequence of shading, nutrient competition or allelopathic compounds (Friedlander et al. 2001; Hernández et al. 2006), the removal of entangled epiphytes is easier. Interestingly, most of the epiphytes in high water motion conditions were hooked or entangled rather than attached, making biomass processing easier (pers. obs.).

On the other hand, water motion has a positive effect on seaweed development reducing the diffusion boundary layer along the algal surface, which favours the uptake of nutrients and carbon (Stevens and Hurd 1997; Hurd 2000). Overall, the cultures under high water motion conditions showed a higher DGR and percentages of internal N content

than seaweeds cultured under low water motion conditions, which can be attributed to the improvement in the uptake of nutrients and carbon (Figs. 5 and 6). However, it is worth mentioning that the highest mean percentage of internal N content for *C. teedei* was reached during May–June in low water motion conditions (Fig. 5). The lower internal %N observed in other conditions, which yielded significantly higher DGR, may be explained by a biomass dilution effect (Hernández et al. 2006). It means that although the internal %N is lower in January–February and March–April, the net N biomass increased, but in a lower rate than internal C, suggesting that growth was partially limited by N, as also indicated by the C/N ratios (data not shown).

As stated previously, the most important differences between seaweeds cultivated at high and low water motion conditions were found during May–June. *Gracilariopsis longissima* and *C. teedei* might be under different superimposed stresses related to high salinities and irradiances during this season. Thus, the auxiliary energy provided by enhanced water motion conditions can favour nutrient uptake through a reduction of the diffusion layer (Hurd 2000). This higher nutrient availability can improve the growth, photosynthesis and physiological responses to stress. Previous studies have shown non-linear responses of marine macrophytes to superimposed stresses under contrasting nutrient conditions (e.g. Villazán et al. 2015). Environmental stresses could lead to elevated energetic expenditures related to the synthesis of biocompounds (e.g. soluble proteins, phycobiliproteins or different enzymes) mediating physiological adaptations or reparation processes (e.g. Kumar et al. 2010; Parages et al. 2014; Villazán et al. 2015). In this sense, it is expected that macrophytes under low water motion conditions ($< 15 \text{ cm s}^{-1}$), which are more constrained by nutrient availability, exhibit less resistance to stressful conditions due to a lower fitness. This could partially explain why DGR values were negative at low water motion conditions, but positive at high water motion conditions during May–June.

The conspecific interactions of aggregation on marine macrophytes can be positive through facilitation or amelioration of environmental conditions, or negative through interspecific competition for space and other resources (Hernandez et al. 1997; Martínez-Aragón et al. 2002; Abreu et al. 2011). In seaweed aquaculture, it is important to find the optimal biomass that will maximise the biomass yield and limit the settlement of other undesired species on the cultivation ropes (e.g. Hurtado et al. 2001; Ganesan et al. 2006). In this study, the densities studied had no effect in the DGR of *C. teedei*, or the DGR was even enhanced at high densities in *Gp. longissima* during January–February and May–June. Furthermore, lower relative epiphyte abundances were found at the highest densities. Therefore, higher seedling densities should be examined in future studies in order to optimise this variable. In the case of *Gp. longissima*, the positive effect

Table 4 Values of DGR (% per day) and internal nitrogen percentage (%N) for this and previous studies in similar or related species

Reference	<i>Gracilariopsis</i>			Reference	<i>Chondracanthus</i>		
	DGR (% day ⁻¹)	%N			DGR (% day ⁻¹)	%N	
This study	0.3–3.2	1.3–1.5	F	This study	–0.3 – 2	1.2–1.7	F
Wakibia et al. 2001	4–11	–	F	Zinoun et al. 1993	1–8	1.8–4	L
Pérez-Lloréns et al. 2004	–24–8	1.8–3.5	F	Bulboa et al. 2005	0.1–3	–	F
Hernandez et al. 2005	6.5	4–5.2	P				
Hernández et al. 2006	5–6	3.9–5	P				
Choi et al. 2006	–0.7–3.1	–	F				
Padhi et al. 2011	4–9	–	F				
Huo et al. 2011	<0.5–16	–	F				
He et al. 2014	1.1–3	3.9–4.2	P				

observed in the DGR could be related to the shading produced by other specimens from the same rope. *Gp. longissima* and *C. teedei* thrive in the studied area under low irradiances, reaching high densities and dominating the intertidal assemblage during spring and early summer in determined environmental conditions (i.e. low light and high water velocity). In this sense, the relatively high irradiances in the culture environment combined with limited growth (higher salinities, %N < 2.0%; Fig. 6a) could produce a marked stress for *Gp. longissima*, especially during May–June, which could be ameliorated by a reduction in light exposure by auto-shading (e.g. Creed et al. 1997; Molina-Montenegro et al. 2005). Another possible explanation can be related to the reduction in the relative abundance of epiphytes, which can preclude or reduce the growth of *Gp. longissima* through allelopathic compounds (Friedlander et al. 2001; Hernández et al. 2006).

The present results of the biomass obtained in the cultures are promising, especially when these species are currently used in the regional food industry (Pérez-Lloréns et al. 2016). However, the obtained DGR for both species were generally lower in comparison with previous culture assays developed in different parts of the world with similar species (Table 4). The percentages of tissue N observed during the period of culture suggested values lower than critical quotas (i.e. growth below maximum rate). This is explained in part by the nutrient concentration in the field (Table 1), far from those of laboratory cultures or those observed in field studies developed near fish farms (Hernández et al. 2006) or other water polluted by dissolved nutrients (Lourenço et al. 2006). In this sense, it would be of great advantage to complement the salt production with an integrated fish cultivation (Neori et al. 2004). This nutrient limitation could also be explained by physiological factors affecting macroalgal nutrient uptake in the salina, especially temperature and salinity (Choi et al. 2006) or water motion (Stevens and Hurd 1997; Hurd 2000). The higher net N biomass in high water motion conditions for both species reveals an important role of this factor

determining nutrient availability. Water motion in the salina can be controlled and ropes can be placed within the structure of the lock-gates (Hernández et al. 2016), which will favour rate of nutrient diffusion. The present study has shown that macroalgal cultivation in the salinas has some limitations and that further research will be necessary to optimise culture performance with a limited investment; however, seaweed cultivation in traditional salinas has some theoretical advantages in comparison with open sea cultivation, such as the following: (i) since these salinas are located in land, the logistic of cultivation and monitoring is simplified; (ii) the effects of rough weather in the macroalgal culture can be more easily controlled; (iii) environmental conditions as water motion, light or nutrient concentration may be easier to modify or control, changing the physical structure of the earthen ponds (e.g. lock-gate systems, pond volume, channel width, deep and section) and nutrients can be managed, especially if fish are also cultured in a global design based on integrated multitrophic aquaculture. Thus, to optimise the efficiency and increase the possibilities of upscaling macroalgal cultures in this particular environment, further cultures should take advantages of improvements in the water flow, higher seedling density and the adoption of polytrophic practices based in integrated aquaculture. Furthermore, considering previous studies in *C. chamissoii* and *Gp. lemaneiformis* (Avila et al. 2011; Zhou et al. 2013) that found differences in optimal conditions for the development of different phases of the life cycle of these species (i.e. gametophytes vs. sporophytes), future studies should explore if these differences exist in the seaweed studied here and aim to identify the most suitable biological phase for their cultivation in salinas.

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