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# Culture of Chondracanthus teedei and Gracilariopsis longissima in a traditional Salina from Southern Spain.

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# Culture of *Chondracanthus teedei* and *Gracilariopsis longissima* in a traditional Salina from Southern Spain.

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## ABSTRACT

The cutivation 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,* being 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.

Key words: Earthen ponds; Net growth rate; Macroalgal cultivation; Nitrogen; Yield.

## 1. 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Geomorphological and archaeological evidence as well as historical documents suggest that salt exploitation and related activities (e.g. salted fish products) have been economically important in Cadiz Bay since Phoenician times (5th 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 are developed during summer and autumn. During winter and spring, the lock-gates remain opened and generally no exploitation activities are developed (Alonso-Villalobos et al., 2004; Alonso-Villalobos and Ménanteau, 2004).

Until the early 20th century the salt extraction was profitable and relevant for the local economy. However, after 1930, when industrial refrigeration become 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 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 communications 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, makes 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 optimize 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).

*Gp. 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 bioetanol and other compounds of interest for pharmaceutical and biotechnological applications (Stabili et al., 2012; Shukla et al., 2016). *Gp. 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).

*C. 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 carrageenean 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.

## 2. Material and method

### *2.1. Study site*

Field cultivation studies on *C. teedei* and *Gp. longissima* were conducted at "Salina de la Esperanza", located in southern Spain (36º30'39" N, 6º09'34-35" 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 is possible to observe an increase in the biomass of the red seaweeds thriving in these environments (our pers. obs.).

### *2.2. 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 meter), quality of the biomass (%N) and abundance of epiphytes. In order to accomplish with these objectives, the experimental trial was developed following a randomized complete block design.

#### *2.2.1. Raft cultivation method*

Two rectangular rafts (1.3 m x 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 minimize 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, minimizing the displacement and ensuring the parallel position of the seeded lines to the main water flow.

#### *2.2.2. 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 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 hours after seaweeds 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.

### *2.2.3. 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 meters (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.

### *2.2.4. Seasonality*

Three culture cycles of seven 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.

## 2.3. Culture monitoring

After seven 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 60ºC for at least 48 hours and grinded using a porcelain mortar. These grinded samples were stored in eppendorf tubes in a desiccator with silica gel until 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 g FW $week^{-1}$ $m^{-1}$.

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

Equation 1.- $DGR\ (\%\ day^{-1}) = 100\ \ln(FW_f\ FW_0^{-1})\ t^{-1}$

where DGR is the relative daily growth rate; $FW_f$ is the final fresh weight after t days of culture; $FW_0$ is the initial fresh weight; and t is the number of days of culture.

## 2.4. 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+ pH1 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 µm). Nutrients were determined by a Skalar SAN++ CFA autoanalyser. In the case of SS, five litres of seawater from the study site were transported to the lab. Just before filtration, the five litres of seawater were shaken to resuspend SS. Subsequently, using a vacuum pump between 2 and 0.5 litres 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 60ºC, for at least 24 hours before the filtration and for at least 48 hours 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.

### *2.5. 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 meter of rope, and per g of cultivated seaweed) did not accomplished with homoscedasticity assumption even after data transformation. For this reason, a PERMutational univariate Analisis Of Variance (PERMANOVA) was used instead. These PERMANOVA analyses were based on Euclidean distances.

## **3. Results**

### *3.1. 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 ºC (February), to 27.6 ºC (June). Regarding salinity, the value 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

### *3.2. 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 ± 1.19 g $m^{-1}$ $week^{-1}$; March-April, 6.76 ± 2.27 g $m^{-1}$ $week^{-1}$).

When growth was expressed as DGR, the 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 ± 1.60 % $day^{-1}$). Similar growth rates were reached during January-February (2.01 ± 0.37 % $day^{-1}$) and March-April (1.77 ± 0.51 % $day^{-1}$). 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 these losses remarkably high during May-June (53.27 ± 13.98%) and minimal during January-February (6.18±10.30%) and March-April (8.71±7.31%). No significant effects were observed for water motion, seedling density and the interactions between the studied factors (Table 2).

Regarding *Gp. longissima*, the 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. 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 ± 0.99 g $m^{-1}$ $week^{-1}$).

When growth was expressed as DGR, the ANOVA (Table 3; Fig. 4a and 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 ± 0.62 % $day^{-1}$) and the lowest during May-June (0.32 ± 1.55 % $day^{-1}$, 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 ± 19.72 %) than during the January-February (6.79 ± 10.84 %) and March-April (13.70 ± 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 ± 19.04 %, respectively).

### *3.3. Tissue N concentration*

The percentage of tissue N in *C. teedei* varied seasonally (three-way ANOVA, F $F_{2,36}$ = 24.86; p-value < 0.001), showing a significant interaction with water motion conditions (three-way ANOVA, F $F_{2,36}$ = 10.53; p-value < 0.001). The percentage of tissue N was the lowest during January-February (1.21 ± 0.15%) and maximal during May-June (1.68 ± 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 and b). The percentage of tissue N was lower in March-April (1.31 ± 0.25%) than in January-February (1.53 ± 0.26%) and May-June (1.54 ±

0.29%). Overall, tissue N was higher under high (1.53 ± 0.28) than under low water motion (1.39 ± 0.27%). In this species the C:N ratio was constant through the experiment (i.e. not 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.

### *3.4. Epiphytes*

The 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 per g *C. teedei*), and the lowest in January-February (0.04 ± 0.08 g epiphytes per g *C. teedei*), showing March-April intermediated values (0.10 ± 0.05 g epiphytes per g *C. teedei*). Overall, the highest relative abundance of epiphytes was found at low seedlings 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 seedlings densities.

It was also observed 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* (Table 3). The highest relative epiphyte abundances were reached during May-June (0.54 ± 0.68 g epiphytes per g *Gp. longissima*), and the lowest in January-February (0.23 ± 0.12 g epiphytes per g *Gp. longissima*), showing March-April intermediated values (0.34 ± 0.32 g epiphytes per g *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 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).

## **4. 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 growth faster under high water motion conditions (i.e. >100 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 farms 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 close tide 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 (aprox. 3.5%). The comparison of DGRs between these two studies is difficult since the culture periods and methods differed significantly (1 week vs. 5 weeks; cage vs. rope culture). However, growth patterns were similar. *Gp. longissima* living in tide creeks also evidenced a marked loss of biomass in June (-22 % $day^{-1}$). 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 tide 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 a optimum temperature for growth between 20 and 25ºC 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 a 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 30ºC 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 macrophytes development and productivity vary significantly between species (Peteiro and Freire, 2013; Sato et al., 2017). A rapid water motion can suppose a physical stress for the thallus, producing 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 suppose 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 is regulated by water motion conditions (Carrington et al., 2001). *Gp. longissima* and *C. teedei* contains agar and carrageenan respectively (Wakibia et al., 2001; Pereira and Mesquita, 2004), which confers some biomechanical characteristic that has been hypothesized 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 seaweeds 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 loses between high and low water motion conditions for *C. teedei;* and in the case of *Gp. longissima,* the differences in seedling loses 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 suppose a desirable trait for cultivation, since it preclude or limit 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 decreased 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, being easier the biomass processing (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 remarkable 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. *Gp. 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 et al., 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 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 competence for the 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 essayed 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 seedlings densities should be essayed in future studies in order to optimise this variable. In the case of *Gp. longissima*, the positive effect 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 that observed in field studies developed near fish farms (Hernandez 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 implies some theoretical advantages in comparison with open sea cultivation, such as: 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, specially if fish are also cultured in a global design based on integrated multitrophic aquaculture. Thus, to optimize 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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## References

Abreu MH, Pereira R, Yarish C, Buschmann AH, Sousa-pinto I (2011). IMTA with *Gracilaria vermiculophylla*: Productivity and nutrient removal performance of the seaweed in a land-based pilot scale system. Aquaculture 312:77–87.

Anderson M, Gorley R, Clarke KR (2008). PERMANOVA+ for PRIMER: Guide to Software and Statistical Methods. pp. 204.

Alonso-Villalobos C, Gracia Prieto FJ, Ménanteau L (2003). Las salinas de la Bahía de Cádiz durante la antigüedad: Visión geoarqueológica de un problema histórico. SPAL Rev Prehist y Arqueol la Univ Sevilla 12:317–332.

Alonso-Villalobos C, Ménanteau L (2004). Métodos y técnicas de explotación salinera. In: Salinas de Andalucía. Consejeria de Medio Ambiente, Junta de Andalucía, Sevilla, Spain, pp 47–51.

Alonso-Villalobos C, Ménanteau L, Rubio-García JC, Severo-Aguiló P (2004). Una visión histórica de las salinas andaluzas. In: Salinas de Andalucía. Consejería de Medio Ambiente, Junta de Andalucía, Sevilla, Spain, pp 25–46.

Avila M, Piel MI, Caceres JH, Alveal K (2011). Cultivation of the red alga *Chondracanthus chamissoi*: Sexual reproduction and seedling production in culture under controlled conditions. J Appl Phycol 23:529–536.

Britton RH, Johnson AR (1987). An ecological account of a Mediterranean salina: The Salin de Giraud, Camargue (S. France). Biol Conserv 42:185–230.

Bulboa C, Véliz K, Sáez F, Sepúlveda C, Vega L, Macchiavello J (2013). A new method for cultivation of the carragenophyte and edible red seaweed *Chondracanthus chamissoi* based on secondary attachment disc: Development in outdoor tanks. Aquaculture 410-411:86–94.

Burfeind DD, Udy JW (2009). The effects of light and nutrients on *Caulerpa taxifolia* and growth. Aquat Bot 90:105–109.

Carrington E, Grace SP, Chopin T (2001). Life history phases and the biomechanical properties of the red alga *Chondrus crispus* (Rhodophyta). J Phycol 37:699–704.

Choi HG, Kim YS, Kim JH, Lee SJ, Park EJ, Ryu J, Nam KW (2006). Effects of temperature and salinity on the growth of *Gracilaria verrucosa* and *G. chorda*, with the potential for mariculture in Korea. J Appl Phycol 18:269–277.

Chopin T, Buschmann AH, Halling C, Troell M, Kautsky N, Neori A, Kraemer GP, Zertuche-González JA, Yarish C, Neefus C (2001). Integrating seaweeds into marine aquaculture systems: a key toward sustainability. J. Phycol. 37:975–986.

Creed JC, Norton TA, Kain JM (1997). Intraspecific competition in *Fucus serratus* germlings: The interaction of light, nutrients and density. J Exp Mar Bio Ecol 212:211–223.

Duarte CM, Dennison WC, Orth RJW, Carruthers TJB (2008). The charisma of coastal ecosystems: Addressing the imbalance. Estuar Coast 31:233-238.

Engkvist R, Malm T, Nilsson J (2004). Interaction between isopod grazing and wave action: A structuring force in macroalgal communities in the southern Baltic Sea. Aquat Ecol 38:403–413.

Faucci A, Boero F (2000). Structure of an epiphytic hydroid community on *Cystoseira* at two sites of different wave exposure. Sci Mar 64:255–264.

Friedlander M, Kashman Y, Weinberger F, Dawes CJ (2001). *Gracilaria* and its epiphytes: 4. The response of two *Gracilaria* species to *Ulva lactuca* in a bacteria-limited environment. J Appl Phycol 13:501–507.

Ganesan M, Thiruppathi S, Jha B (2006). Mariculture of Hypnea musciformis (Wulfen) Lamouroux in South east coast of India. Aquaculture 256:201–211.

Gerard VA, Mann KH (1979). Growth and production of *Laminaria longicruris* (Phaeophyta) populations exposed to different Iintensities of water movement. J Phycol 15:33–41.

Guiry MD (1984). Structure, life history and hybridization of atlantic *Gigartina teedii* (Rhodophyta) in culture. Br Phycol J 19:37–55.

Guiry MD, Guiry GM (2017). AlgaeBase. World-wide electronic publication, National University of Ireland, Galway. http://www.algaebase.org

Hanisak MD (1983). The nitrogen relationship of marine macroalgae. In: Carpenter EJ, Capone DG (eds) Nitrogen in the Marine Environment. Academic Press, New York, pp 669-730.

Hernández I, Cara CL, Sánchez-García J, Macías M, Robyn L, Bermejo R (2016). Cultivos de macroalgas en el litoral gaditano: estado actual y perspectivas para su desarrollo. Algas 52:5-10.

Hernandez I, Fernandez-Engo MA, Perez-Llorens JL, Vergara JJ (2005). Integrated outdor culture of two estuarine macroalgae as biofilters for dissolved nutrients from *Sparus aurata* waste waters. J Appl Phycol 17:557-567.

Hernandez I, Peralta G, Perez-Llorens JL, Vergara JJ, Niell FX (1997). Biomass and growth dynamics of *Ulva* species in Palmones River estuary. J Phycol 33:764–772.

Hernández I, Pérez-Pastor A, Vergara JJ, Martínez-Aragón JF, Fernández-Engo MÁ, Pérez-Lloréns JL (2006). Studies on the biofiltration capacity of *Gracilariopsis longissima*: From microscale to macroscale. Aquaculture 252:43–53.

Hortas F, Muñoz-Pascual G, Pérez-Hurtado A (2004). Avifauna de las salinas atlánticas. In: Salinas de Andalucía. Consejeria de Medio Ambiente, Junta de Andalucía, Sevilla, Spain, pp 223–231.

Huo YZ, Xu SN, Wang YY, Zhang JH, Zhang YJ, Wu WN, Chen YQ, He PM (2011). Bioremediation efficiencies of *Gracilaria verrucosa* cultivated in an enclosed sea area of Hangzhou Bay, China. J Appl Phycol 23:173–182.

Hurd CL (2000). Water motion, marine macroalgal physiology, and production. J Phycol 36:453–472.

Hurtado AQ, Agbayani RF, Sanares R, De Castro-Mallare MTR (2001). The seasonality and economic feasibility of cultivating *Kappaphycus alvarezii* in Panagatan Cays, Caluya, Antique, Philippines. Aquaculture 199:295–310.

Israel A, Martínez-Goss M, Friedlander M (1999). Effect of salinity and pH on growth and agar yield of *Gracilaria tenuistipitata* var. *liui* in laboratory and outdoor cultivation. J Appl Phycol 11:543–549.

Kain JM, Destombe C (1995). A review of the life-history, reproduction and phenology of *Gracilaria*. J Appl Phycol 7:269–281.

Kloareg B, Quatrano RS (1988). Structure of the cell walls of marine algae and ecophysiological functions of the matrix polysaccharides. Oceanogr Mar Biol An Annu Rev 26:259–315.

Kumar M, Kumari P, Gupta V, Reddy CRK, Jha B (2010). Biochemical responses of red alga *Gracilaria corticata* (Gracilariales, Rhodophyta) to salinity induced oxidative stress. J Exp Mar Bio Ecol 391:27–34.

Leigh EGJ, Paine RT, Quinn JF, Suchanek TH, (1987). Wave energy and intertidal productivity. Proc Natl Acad Sci USA 84:1314–1318.

Lourenço SL, Barbarino E, Nascimento A, Freitas JNP, Diniz GS (2006). Tissue nitrogen and phosphorus in seaweeds in a tropical eutrophic environment: What a long-term study tells us. J Appl Phycol 18:389-398.

Martínez-Aragón JF, Hernández I, Pérez-Lloréns JL, Vázquez R, Vergara JJ (2002). Biofiltering efficiency in removal of dissolved nutrients by three species of estuarine macroalgae cultivated with sea bass (*Dicentrarchus labrax*) waste waters 1. Phosphate. J Appl Phycol 14:365–374.

Masero JA, Pérez-Hurtado A (2001). Importance of the Supratidal Habitats for Maintaining Overwintering Shorebird Populations : How Redshanks Use Tidal Mudflats and Adjacent Saltworks in Southern Europe. The Condor 103, 21–30.

Molina-Montenegro MA, Muñoz AA, Badano EI, Morales BW, Fuentes KM, Cavieres LA (2005). Positive associations between macroalgal species in a rocky intertidal zone and their effects on the physiological performance of *Ulva lactuca*. Mar Ecol Prog Ser 292:173–180.

Neori A, Chopin T, Troell M, Buschmann AH, Kraemer GP, Hallin C, Shpigel M, Yarsh C (2004). Integrated aquaculture: rationale, evolution and state of the art emphasizing seaweed biofiltration in modern mariculture. Aquaculture 231:361-391.

Padhi S, Swain PK, Behura SK, Baidya S, Behera SK, Panigrahy MR (2011). Cultivation of *Gracilaria verrucosa* (Huds) Papenfuss in Chilika Lake for livelihood generation in coastal areas of Orissa State. J Appl Phycol 23:151–155.

Parages ML, Figueroa FL, Conde-Álvarez RM, Jiménez C (2014). Phosphorylation of MAPK-like proteins in three intertidal macroalgae under stress conditions. Aquat Biol 22:213–226.

Pereira L (2012) A review of the nutrient composition of selected edible seaweeds. In: Vitor H. Pomin (ed) Seaweed: Ecology, Nutrient Composition and Medicinal Uses. Nova Science Publishers, Inc. pp 15-47.

Pereira L, Mesquita JF (2004). Population studies and carrageenan properties of *Chondracanthus teedei* var. *lusitanicus* (Gigartinaceae, Rhodophyta). J Appl Phycol 16:369–383.

Pérez-Lloréns JL, Brun FG, Andría J, Vergara JJ (2004). Seasonal and tidal variability of environmental carbon related physico-chemical variables and inorganic C acquisition in *Gracilariopsis longissima* and *Enteromorpha intestinalis* from Los Toruños salt marsh (Cádiz Bay, Spain). J Exp Mar Bio Ecol 304:183–201.

Pérez-Lloréns JL, Hernández I, Vergara JJ, Brun FG, León Á (2016). ¿Las algas se comen? Un periplo por la biología, la historia, las curiosidades y la gastronomía. Servicio de Publicaciones de la Universidad de Cádiz. pp 336.

Peteiro C, Freire Ó (2011). Effect of water motion on the cultivation of the commercial seaweed *Undaria pinnatifida* in a coastal bay of Galicia, Northwest Spain. Aquaculture 314:269–276.

Peteiro C, Freire Ó (2013). Biomass yield and morphological features of the seaweed *Saccharina latissima* cultivated at two different sites in a coastal bay in the Atlantic coast of Spain. J Appl Phycol 25:205–213.

R Development Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/.

Rothman MD, Anderson RJ, Boothroyd CJT, Kemp FA, Bolton JJ (2009). The gracilarioids in South Africa: Long-term monitoring of a declining resource. J Appl Phycol 21:47–53.

Ryder E, Nelson SG, McKeon C, Glenn EP, Fitzsimmons K, Napolean S (2004). Effect of water motion on the cultivation of the economic seaweed *Gracilaria parvispora* (Rhodophyta) on Molokai, Hawaii. Aquaculture 238:207–219.

Sadoul N, Walmsley J, Charpentier B (1998). Salinas and Nature Conservation. In: AJ Crivelli, Jalbert J (eds) Conservation of Mediterranean wetlands n°9. Station Biologique de la Tour du Valat, Arles (France). pp 99.

Sato Y, Yamaguchi M, Hirano T, Fukunishi N, Abe T, Kawano S (2017). Effect of water velocity on *Undaria pinnatifida* and *Saccharina japonica* growth in a novel tank system designed for macroalgae cultivation. J Appl Phycol 29:1429-1436

Shukla R, Kumar M, Chakraborty S, Gupta R, Kumar S, Sahoo D, Kuhad RC (2016). Process development for the production of bioethanol from waste algal biomass of *Gracilaria verrucosa*. Bioresour Technol 220:584–589.

Stabili L, Acquaviva MI, Biandolino F, Cavallo RA, de Pascali SA, Fanizzi FP, Narracci M, Petrocelli A, Cecere E (2012). The lipidic extract of the seaweed *Gracilariopsis longissima* (Rhodophyta, Gracilariales): A potential resource for biotechnological purposes? N Biotechnol 29:443–450.

Steentoft M, Irvine LM, Farnham WF (1995). Two terete species of *Gracilaria* and *Gracilariopsis* (Gracilariales, Rhodophyta) in Britain. Phycologia 34:113–127.

Stevens CL, Hurd CL (1997). Boundary-layers around bladed aquatic macrophytes. Hydrobiologia 346:119–128.

Torrejón J (1994). El área portuaria de la Bahía de Cádiz. Tres mil años de puerto. In: Puertos Españoles En La Historia. Ministerio de Obras Públicas, Transportes y Medio Ambiente. pp 117–145.

Torrejón J (1997). Las salinas de la bahía de Cádiz. In: Congreso Internacional de La Comisión de Historia de La Sal. Granada, pp 169–194.

Vásquez JA, Alonso-Vega JM (2001). *Chondracanthus chamissoi* (Rhodophyta, Gigartinales) in northern Chile: ecological aspects for management of wild populations. J Appl Phycol 13:267-277.

Villazán B, Salo T, Brun FG, Vergara JJ, Pedersen MF (2015). High ammonium availability amplifies the adverse effect of low salinity on eelgrass Zostera marina.

Mar Ecol Prog Ser 536:149–162.

Wheeler PA, Björnsäter BR (1992). Seasonal fluctuations in tissue nitrogen, phosphorus, and N:P for five macroalgal species common to the Pacific Northwest coast. J Phycol 28:1-6.

Wakibia JG, Anderson RJ, Keats DW (2001). Growth rates and agar properties of three gracilarioids in suspended open-water cultivation in St . Helena Bay , South Africa. J Appl Phycol 13:195–207.

Yang MY, Macaya EC, Kim MS (2015). Molecular evidence for verifying the distribution of *Chondracanthus chamissoi* and *C. teedei* (Gigartinaceae, Rhodophyta). Bot Mar 58:103–113.

Zhou W, Sui Z, Wang J, Chang L (2013). An orthogonal design for optimization of growth conditions for all life history stages of *Gracilariopsis lemaneiformis* (Rhodophyta). Aquaculture 392–395:98–105.

Zinoun M, Cosson J, Deslandes E (1993). Influence of Culture Conditions on Growth and Physicochemical Properties of Carrageenans in *Gigartina teedii* (Rhodophyceae — Gigartinales). Bot Mar 36:131–136.

**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.

**Fig. 2.-** Current velocity measured under high (black; Lock-gate) and low (grey; Inner) water motion conditions in a 24 hours periods in October 2015.

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

**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.

**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. 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.

**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.

Dear Editor Michael A. Borowitzka,

Please find our response (in grey) to the comments posed by the reviewer (in black) and yourself regarding of our manuscript entitled “Culture of *Chondracanthus teedei* and *Gracilariopsis longissima* in a traditional salina from Southern Spain” that was submitted for publication to the Journal of Applied Phycology (JAPH-D-17-00693). We appreciate your comments that undoubtedly have improved this new version of the manuscript. Dr. Liam Morrison, a native English speaker with a wide knowledge about applied phycology, also revised this final version of the manuscript in order to correct the grammar.

Journal of Applied Phycology
**Manuscript JAPH-D-17-00693**

Comments for the Author:

Reviewer #1: Authors have improved the manuscript accordingly but I still have some comments before the manuscript can be recommended for acceptance.

1) The use of abbreviated genus name in *Gracilariopsis longissima* is not consistent throughout the manuscript, i.e. G. longissima in Page 4 Line 28, Line 36 and Page 16 Line 18 but Gp. longissima in Page 7 Line 3 and elsewhere. Authors need to be very careful in formatting the species name as wrong input may led to taxomony confusion. Please check thoroughly.
It has been modified following referee recommendations.

2) The species name is commonly written out in full when it first appears in the abstract and then abbreviated in the rest of the abstract, and again written out in full when it first appears in the main text (Introduction) and is then abbreviated upon further use. Authors should check the manuscript thoroughly, i.e. *Chondracanthus teedei* (Martens ex Roth) and Gracilariopsis longissima (S.G. Gmelin) Steentoft M, L.M. Irvine & W.F. Farnham in Page 5 Line 21-22 should be abbreviated; *Chondracanthus teedei* in Page 13 Line 36, and *Gracilariopsis longissima* and *Gracilaria verrucosa* in Page 14 Line 29 should also be abbreviated.
The manuscript has been revised and modified accordingly.

3) The use of seasonality and calendar month need to be consistent between the figures and the main text. Figures 3, 5, 7 and 8 are illustrated in seasonality (Winter, Spring etc.) but their corresponding results are explained in calendar month in the main text (Page 10 Line 29-33, Page 12 Line 8-12 and Line 28-31, and Page 13 Line 3-7. This may cause confusion to the reader. Authors may use calendar month in all the figures in line with the main text and add the respective seasonality information in the Figure Legend.

Figures 3, 5, 7 and 8 have been modified following referee recommendations.

4) The use of "nitrogen" (e.g. Page 14 Line 5, Page 17 Line 24, etc.) and "N" (e.g. Page 12 Line 10 and Line 16, Page 17 Line 33, etc.) need to be consistent, i.e. when to use "nitrogen" and when to use "N"? Please check thoroughly.

Except in key words, where we used "Nitrogen". We replaced "nitrogen" by "N" in the entire manuscript.

5) When refer to the specific species in the Discussion, authors should mention the name of the species rather than their genus, i.e. Page 15 Line 27, are you referring to *Gracilariopsis* genus or *Gp. longissima*? Page 15 Line 28, *Chondracanthus* genus or *C. teedei*? Please check the same for *Gracilariopsis* in Page 17 Line 8, Line 15 and Line 18.

Now, the name of the species is specified.

6) Sentence structure and English grammar need to be enhanced to improve readability, e.g. Page 5 Line 13-14, Page 7 Line 17-21, Page 10 Line 29-33, and Page 15 Line 32-33.

These sentences have been rewritten and Dr. Liam Morrison, a native English speaker with a wide knowledge in phycology, revised the final version manuscript to correct grammar mistakes.

Page 5 Line 13-14

Previously: "This information will be key in case that cultures are developed in these traditional salinas."

Now: "This information will be critical for the further development of seaweed cultures in traditional salinas."

Page 7 Line 17-21

Previously: "These sites were selected as close as possible to ensure the existence of similar water conditions (temperature, salinity, dissolved oxygen and inorganic nutrient concentration, etc.) avoiding in this way the effect of confounding factors. Basic cultivation structures were placed perpendicular to the current so that ropes were parallel to the main flow."

Now: "These sites were selected as close as possible to ensure the existence of similar water conditions (temperature, salinity, dissolved oxygen and inorganic nutrient concentration, etc.)." The information about the parallel position of the ropes to the main flow was removed since this was previously specified and become repetitive.

Page 10 Line 29-33

Previously: "When growth was expressed as DGR, the ANOVA (Table 2) showed that seasonality, water motion and the interaction between both factors had a significant effect on the DGR of *C. teedei*. **The maximum percentages of DGR were reached during January-February (2.01 ± 0.37 % day$^{-1}$) and March-April (1.77 ± 0.51 % day$^{-1}$), being the net growth**

**minimum or negative during May-June (-0.32 ± 1.60 % day$^{-1}$), when negative DGR were observed especially in low water motion conditions (high water motion, 0.75 ± 1.30 % day$^{-1}$; low water motion, -1.41 ± 1.05 % day$^{-1}$).** Usually, *C. teedei* showed higher DGRs in high water motion (Fig. 3). However, significant differences in growth between water motion conditions were only observed during May-June."

Now: "When growth was expressed as DGR, the 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 ± 1.60 % day$^{-1}$). Similar growth rates were reached during January-February (2.01 ± 0.37 % day$^{-1}$) and March-April (1.77 ± 0.51 % 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."

Page 15 Line 32-33

Previously: "It is noteworthy that not distinction between entangled and attached epiphytes was done when the cultures were developed."

Now: "It is noteworthy that no distinction between entangled and attached epiphytes was done when the biomass of epiphytes was assessed."

7) Typo: No closing parenthesis after "… Steentoft M, L.M. Irvine & W.F. Farnham" in the abstract (Page 2 Line 5).

Done.

Editors Comments:

Please also correct the following:

The format of the references is not quite correct (punctuation of authors names & no full stops in journal title abbreviations – see attached Style Guidelines).

It has been modified according to JAPH Guidelines.

Fig 2. Correct the format of the unit on the Y-axis – should be cm s-1 [with the -1 in superscript).

Figures 2, 7 and 8, and the main text (e.g. equation 1) have been modified replacing "/" by "$^{-1}$".

There is also an incorrect use of the term %DGR – you measure the growth rate as a percentage i.e % day-1, whereas %DGR implies a percentage of the growth rate. You repeat this by saying “Percentage of Daily Growth Rate” in the figure legends. You should just use DGR and in Figs 3 & 4 the axis label should be ‘DGR (% day-1)’ [with the -1 in superscript]

It has been corrected accordingly.

In the figure and table legends also indicate what is n, the number of independent replicates used to calculate the standard deviation. You also need to indicate what the letters above the error bars indicate.

The number of independent replicates is now indicated in the legends of Figures 3, 4, 5, 6, 7 and 8, and also in table 1.

Now in legends of figures 3, 4, 5 and 6 is specified " Letters over the bars represent significant differences between treatments."

Now in legends of figures 7 and 8 is specified " Letters over the box-plots represent significant differences between treatments."

Figure 1

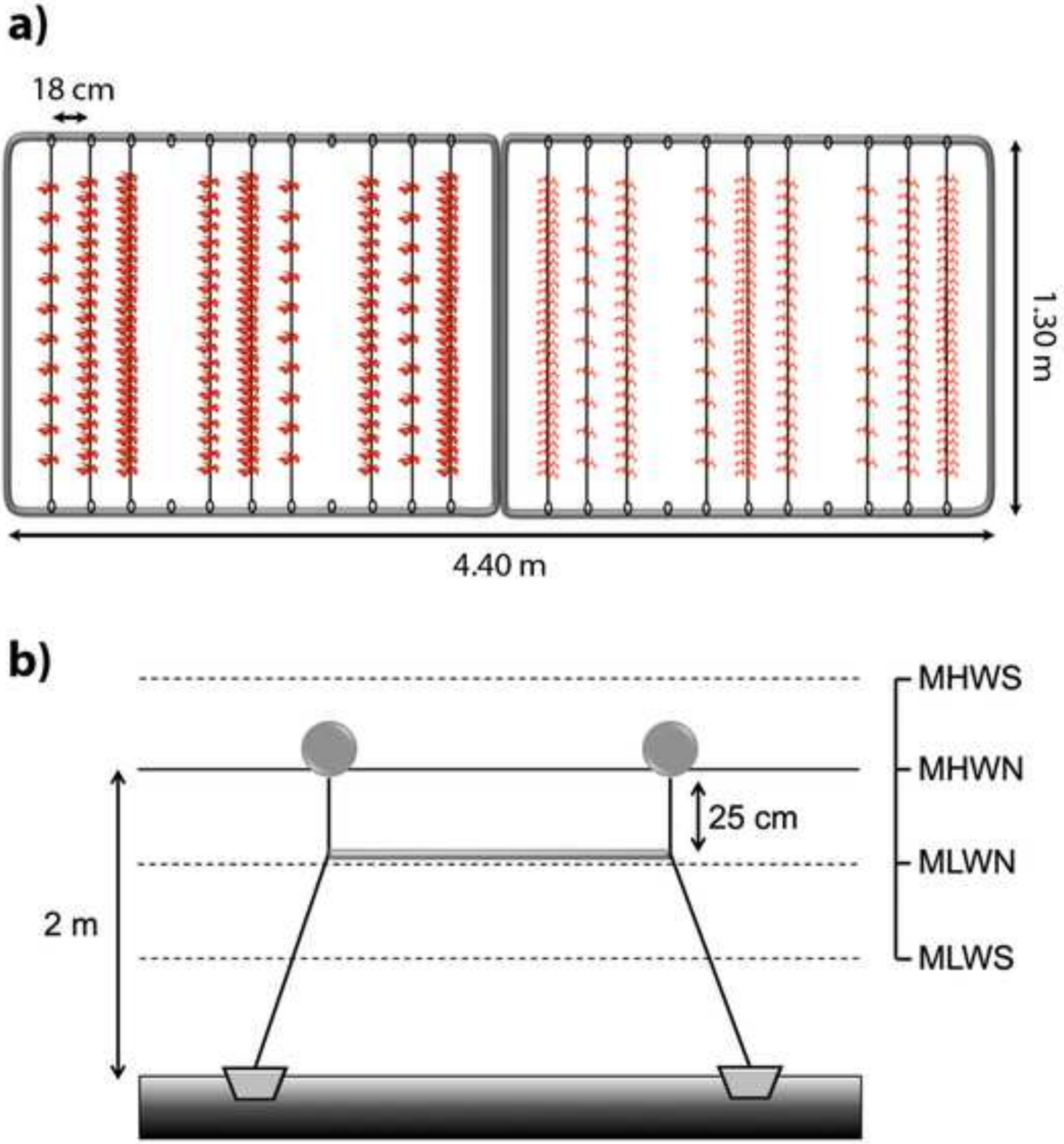
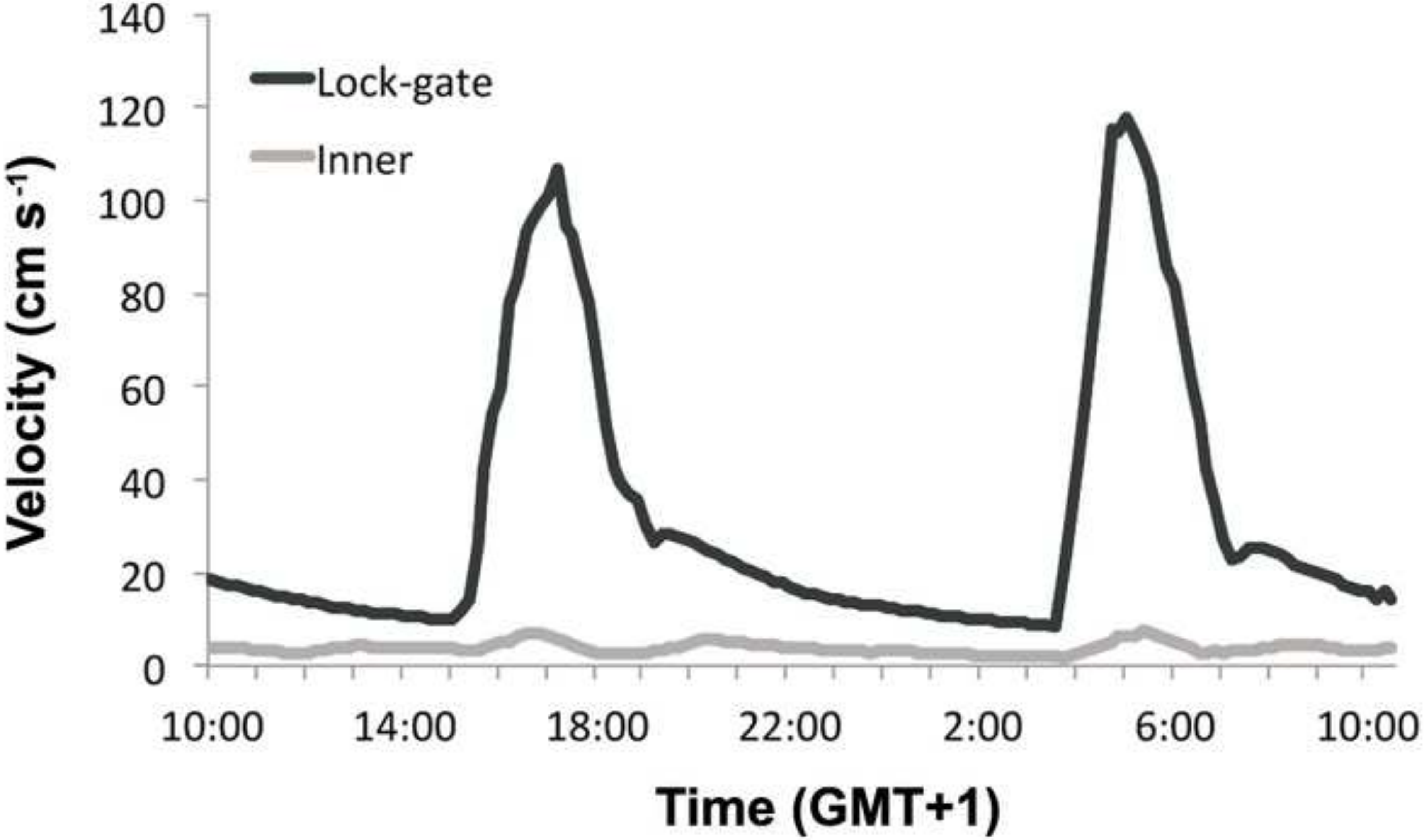


Figure 2


Lock-gate
Inner
Velocity (cm s-1)
140
120
100
80
60
40
20
0
10:00
14:00
18:00
22:00
2:00
6:00
10:00
Time (GMT+1)

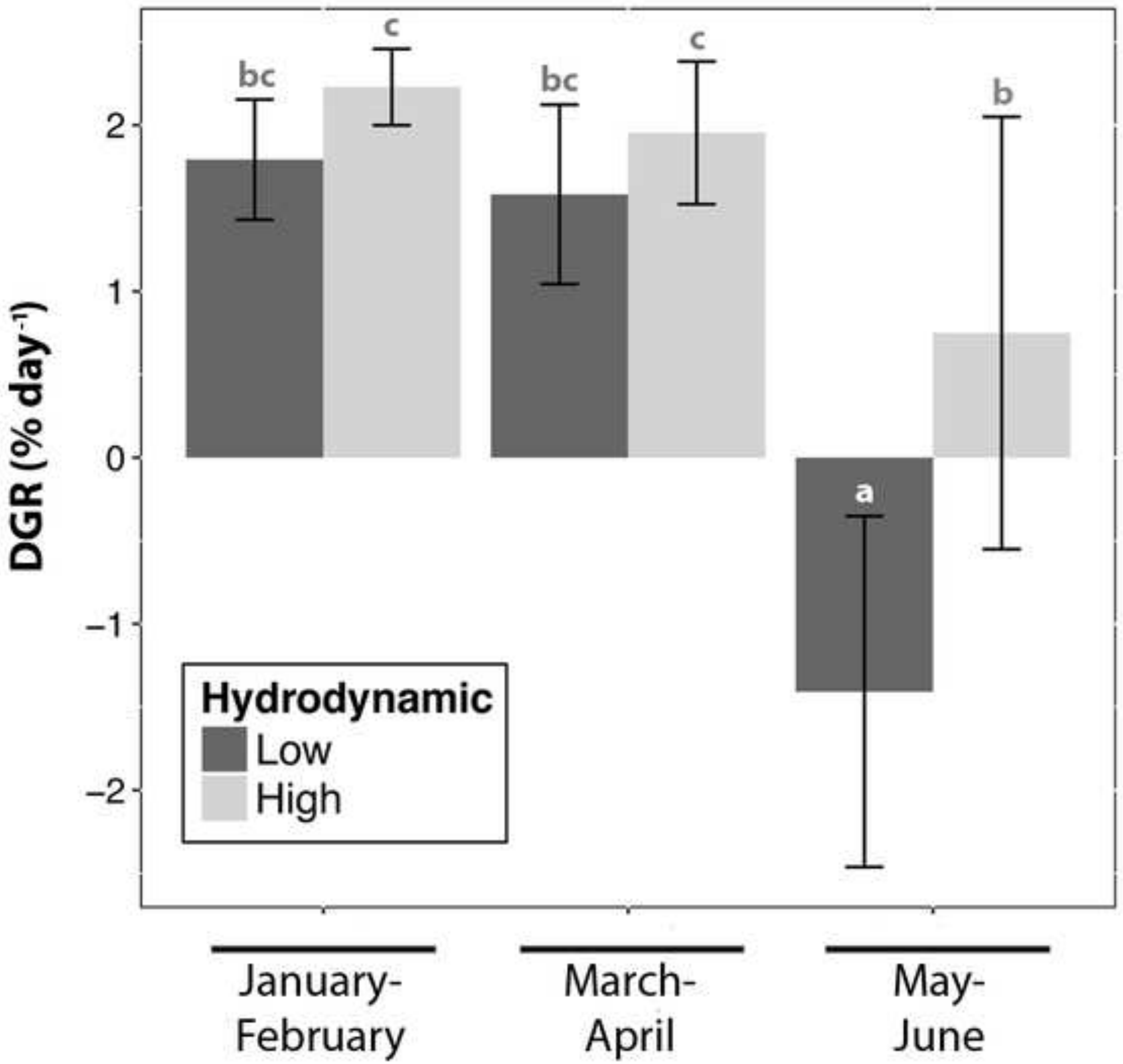

DGR (% day⁻¹)
2
1
0
−1
−2
bc
c
bc
c
b
a
Hydrodynamic
Low
High
January-
February
March-
April
May-
June

Figure 4

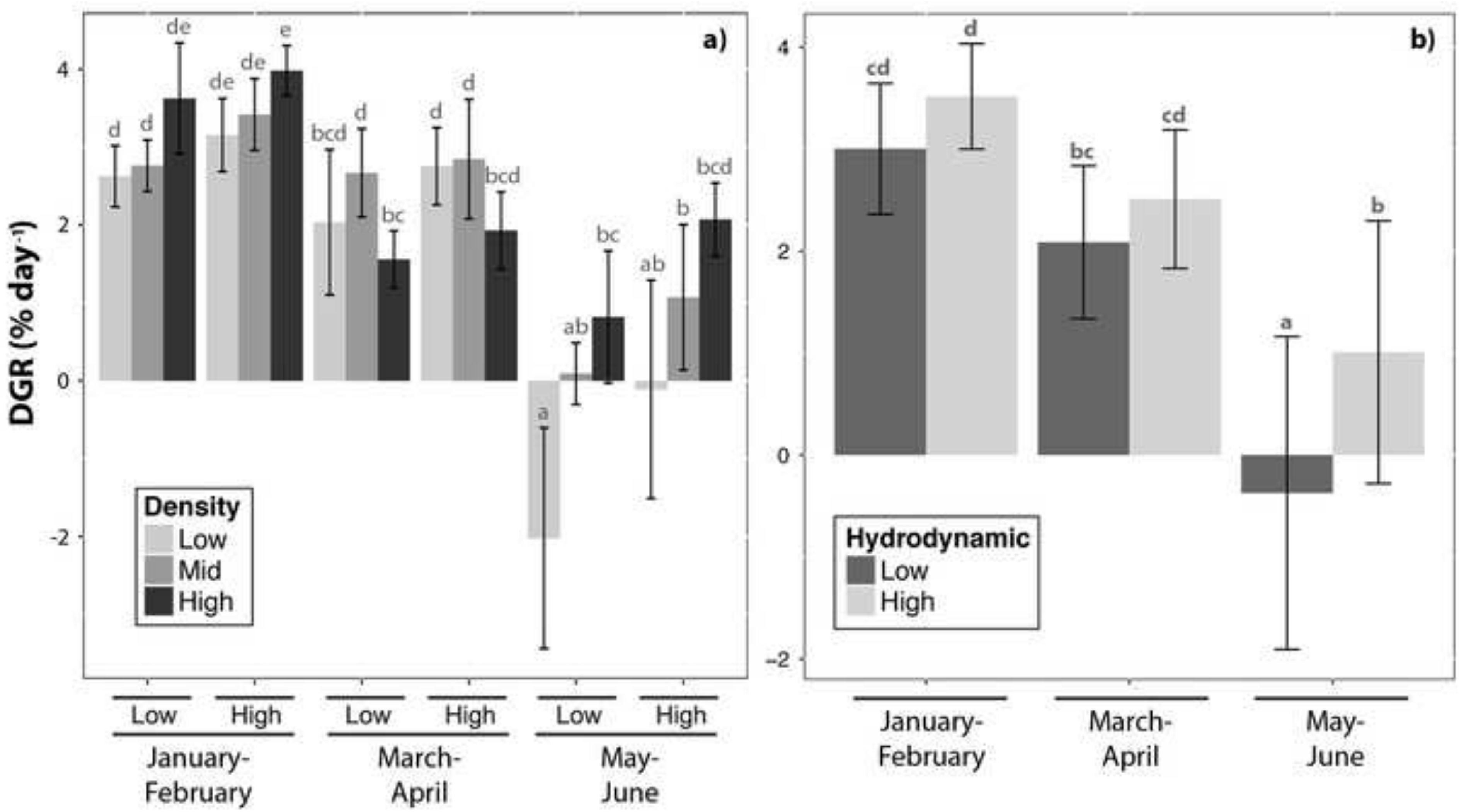

a)
DGR (% day⁻¹)
4
2
0
-2
de
e
de
de
d
d
bcd
d
bc
d
d
bcd
bc
ab
a
ab
b
bcd
Density
Low
Mid
High
Low
High
Low
High
Low
High
January-February
March-April
May-June
b)
4
2
0
-2
cd
d
bc
cd
a
b
Hydrodynamic
Low
High
January-February
March-April
May-June

Figure 5

Click here to download colour figure Fig5.tif

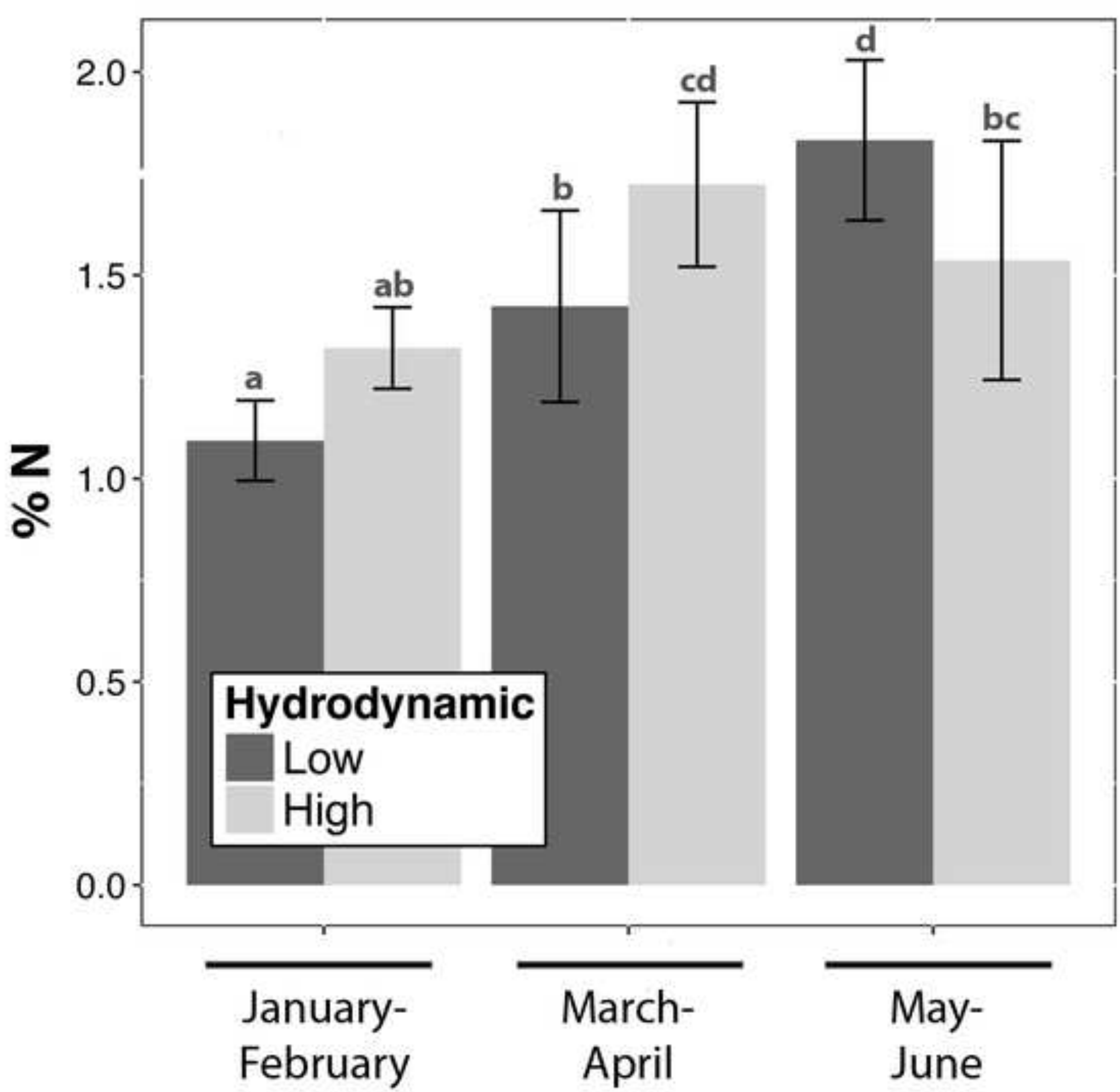


Figure 6

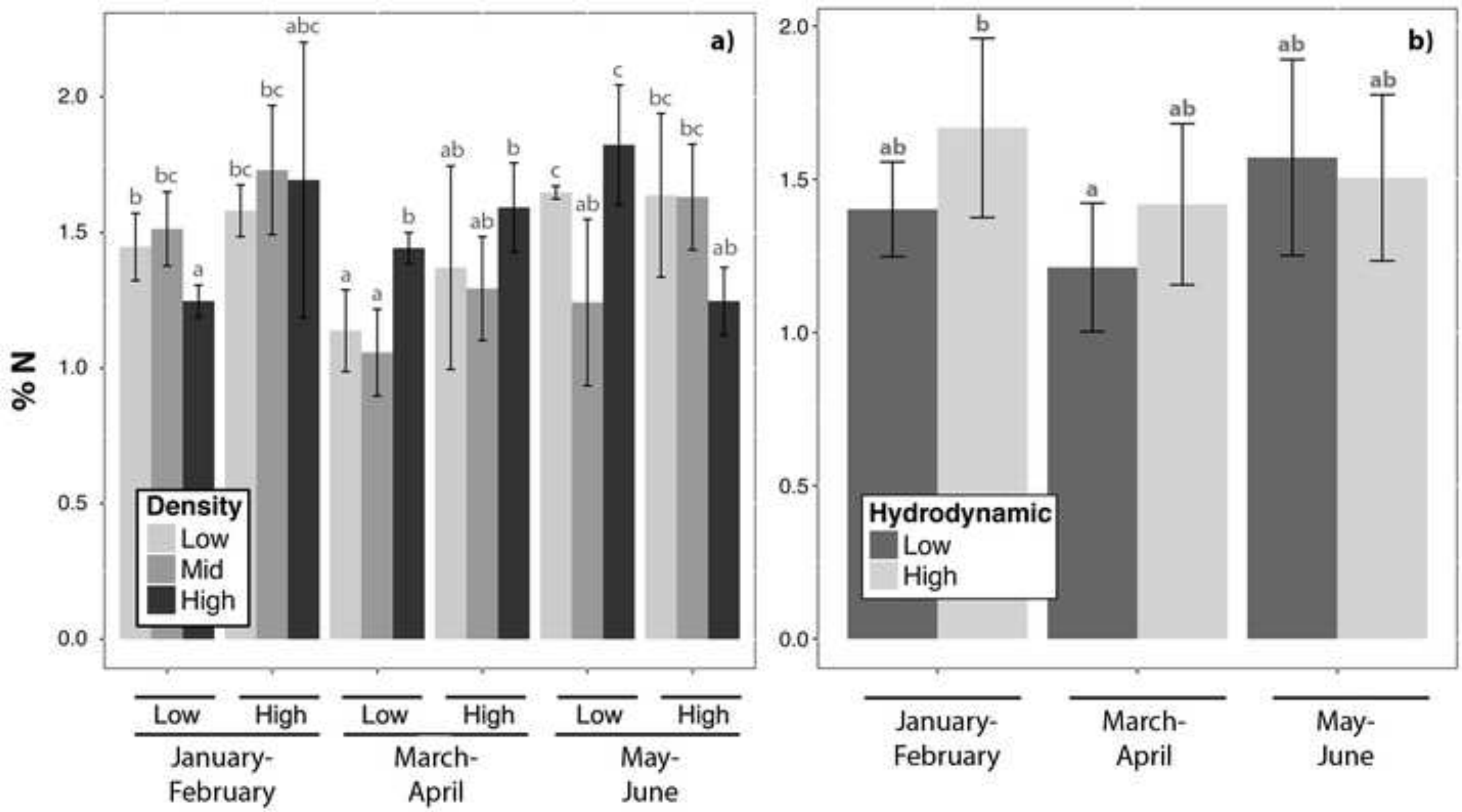
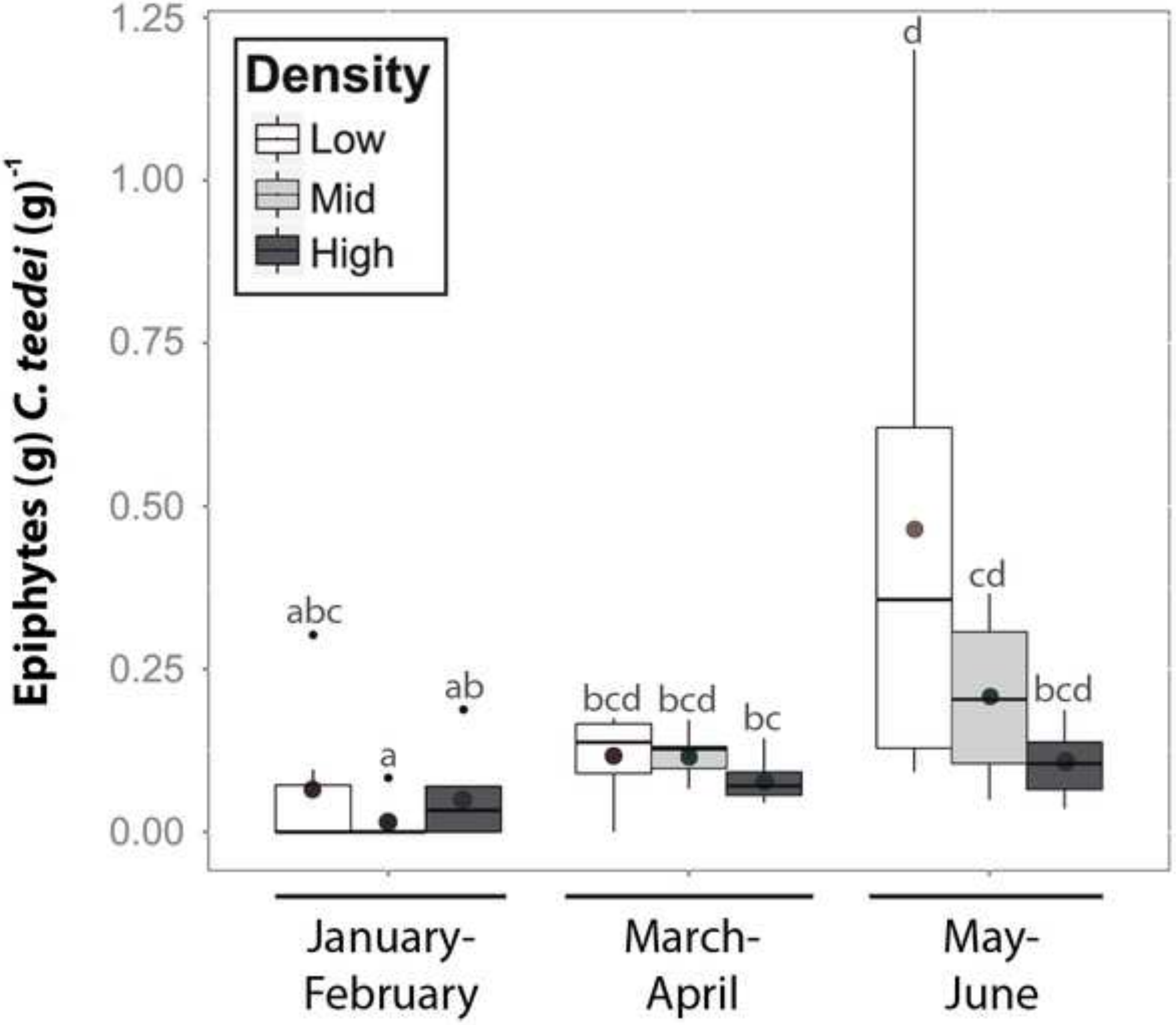
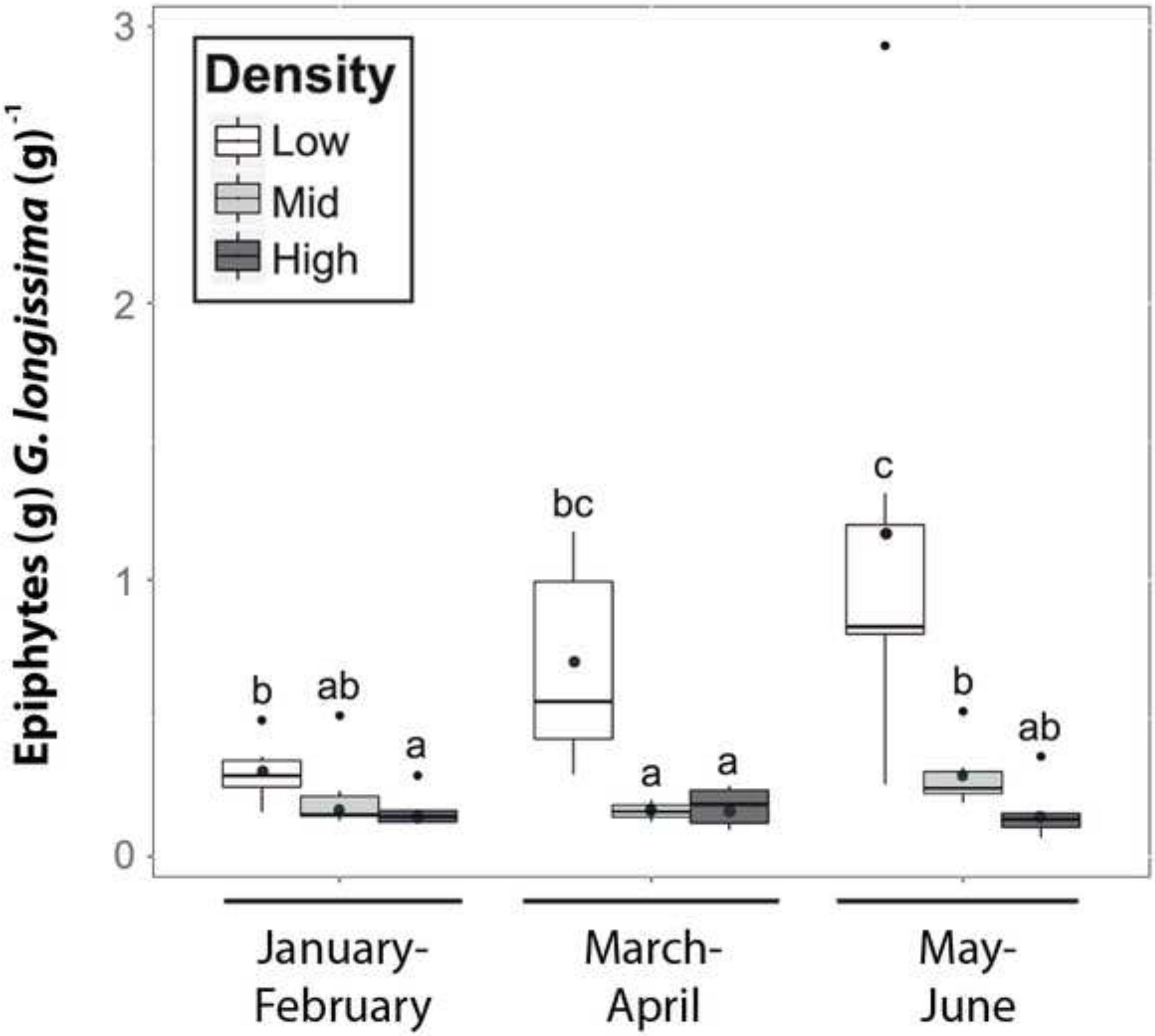

a)
% N
2.0
1.5
1.0
0.5
0.0
b
bc
a
bc
bc
abc
a
a
b
ab
ab
b
c
ab
c
bc
bc
ab
Density
Low
Mid
High
Low
High
Low
High
Low
High
January-February
March-April
May-June
b)
2.0
1.5
1.0
0.5
0.0
ab
b
a
ab
ab
ab
Hydrodynamic
Low
High
January-February
March-April
May-June

Figure 7


Density
Low
Mid
High
Epiphytes (g) *C. teedei* (g)$^{-1}$
1.25
1.00
0.75
0.50
0.25
0.00
abc
a
ab
bcd
bcd
bc
d
cd
bcd
January-February
March-April
May-June


Epiphytes (g) G. longissima (g)$^{-1}$
0
1
2
3
Density
Low
Mid
High
b
ab
a
bc
a
a
c
b
ab
January-February
March-April
May-June

| | Winter | | | Early Spring | | | Late Spring | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Mean ± sd** | **Max** | **Min** | **Mean ± sd** | **Max** | **Min** | **Mean ± sd** | **Max** | **Min** |
| Temp. (ºC) | 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 1 -** Mean, maximum and minimum values for the different environmental variables monitored during the different culture cycles (n= 7).

|  |  | DGR |  | Yield |  | Seedling |  | %N |  | Epiphytes |  |
|---|---|---|---|---|---|---|---|---|---|---|---|
|  | **Df** | **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*** | 12633 | 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 |  |

**Table 2 -** Results of three-way ANOVA analysis testing the effects of the factors "Seasonality" (S; 3), "Seeadling 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.

|  |  | DGR |  | Yield |  | Seedlings |  | %N |  | Epiphytes |  |
|---|---|---|---|---|---|---|---|---|---|---|---|
|  | **Df** | **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 |  |

**Table 3 -** Results of three-way ANOVA analysis testing the effects of the factors "Seasonality" (S; 3), "Seeadling 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.

| **Reference** | ***Gracilariopsis*** | | | **Reference** | ***Chondracanthus*** | | |
|---|---|---|---|---|---|---|---|
| | **DGR (% d$^{-1}$)** | **%N** | | | **DGR (% d$^{-1}$)** | **%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 |
| Hernández 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 | | | | |
| Zhou et al., 2011 | <0.5 - 16 | - | F | | | | |
| He et al., 2014 | 1.1 - 3 | 3.9 - 4.2 | P | | | | |

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