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Nutrients, but not genetic diversity, affect *Gracilaria chilensis* (Rhodophyta) farming productivity and physiological responses[1]

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ABSTRACT

In terrestrial plants, it is well known that genetic diversity can affect responses to abiotic and biotic stress and have important consequences on farming. However, very little is known about the interactive effects of genetic and environmental factors on seaweed crops. We conducted a field experiment on *Gracilaria chilensis* to determine the effect of heterozygosity and nutrient addition on two southern Chilean farms: Ancud and Chaica. In addition to growth rate and productivity, we measured photosynthetic responses, photosynthetic pigment concentration (chlorophyll *a* and phycobiliproteins), C:N ratio (C:N), and epiphytic load. Nutrient addition affected the growth rate, productivity, phycobilin and C:N content, but not the epiphytic load. These results were independent of the heterozygosity of the strains used in the experiments. Interestingly, depending on the sampled sites, distinct photosynthetic responses (i.e., maximal quantum yield, F_v/F_m and maximal electron transport rate, ETR_{max}) to nutrient addition were observed. We propose that thallus selection over the past few decades may have led to ecological differentiation between *Gracilaria chilensis* from Chaica and Ancud. The lack of effect of heterozygosity on growth and physiological responses could be related to the species domestication history in which there is a limited range of genetic

variation in farms. We suggest that the existing levels of heterozygosity among our thalli is not sufficient to detect any significant effect of genetic diversity on growth or productivity in Metri bay, our experimental site located close to the city of Puerto Montt, during summer under nitrogen limiting conditions.

Key index words: epiphyte load, growth, heterozygosity, photosynthesis, pigment content, seaweed.

Abbreviations: C:N, carbon:nitrogen ratio; RLC, rapid light curves; ETR, electron transport rate; α_{ETR}, electron transport efficiency; $\Delta F/F_m'$, effective quantum yield; *Ek*, saturation irradiance; ETR_{max}, maximal electron transport rate; F_v/F_m, maximal quantum yield of fluorescence; NPQ, non-photochemical quenching; NPQ_{max}, maximal non-photochemical quenching.

INTRODUCTION

Various green, brown, and red seaweeds have been recognized as organisms of high economic interest, and thus the acquisition of raw material is gradually shifting from natural population harvesting to aquaculture and intensive farming (Hafting et al. 2015, Buschmann et al. 2017, Valero et al. 2017). Even if seaweed-based ecosystems are potentially very productive, the long-term success of the macroalgal aquaculture production will depend, at least, on the following issues: 1) frequency and intensity of the abiotic and biotic disturbances affecting the farms, 2) quality of the genomes being cultivated (e.g., selection of highly productive or resistant thalli) and genetic diversity preserved in both farms and natural populations, and 3) efficiency of farms' management strategies (Santelices 1999). In farmed

seaweeds, several studies have focused on establishing how abiotic and biotic factors affect productivity (e.g., Kuschel and Buschmann 1991, Pizarro and Santelices 1993, Yang et al. 2015), and only few studies have been carried out focusing on understanding the interaction among genetic diversity and abiotic and biotic conditions for determining seaweed aquaculture production (Valero et al. 2017, Gallegos-Sánchez et al. 2018). However, in terrestrial plants and seagrasses, it is well known that differences in genetic diversity can affect the responses to stress both in wild populations and major crops (Zhu et al. 2000, Reusch et al. 2005).

In *Gracilaria chilensis*, studies carried out during the early 1990s showed that populations from different localities responded differently under laboratory conditions (Santelices and Ugarte 1990). Likewise, differences of growth responses between two *G. chilensis* populations were also detected in the field (Buschmann et al. 1992). These results suggest the existence of genetic variability associated with geographic variation at the species level. The existence of environment-dependent differences in fitness between genotypes may be highly relevant when developing strategies for managing responses to abiotic stress and reduce potential crop failure in the long term (Jump et al. 2008). Furthermore, recent studies based on the use of neutral genetic markers were able to determine that the intense cultivation of *G. chilensis* during the last three decades had generated clear modification of the life-history traits of farmed populations (Guillemin et al. 2008). Moreover, serious concerns were raised about crop vulnerability because of reduced genetic variation upon which *G. chilensis* production was initiated (Guillemin et al. 2008, Guillemin et al. 2014). In *G. chilensis*, aquaculture is sustained mainly by clonal reproduction focused particularly on the diploid phase of the haplo-diplontic life cycle. *Gracilaria chilensis* diploids are characterized by a higher growth rate than haploid individuals (Guillemin et al. 2013, Gallegos-Sánchez et al. 2018) and selection for "rapidly growing thalli" could take place in farms. Besides the

predominance of the diploid phase in farms, a strong reduction in genotypic diversity has been reported (Guillemin et al. 2008).

It is known that high density seaweed farms maintained under monoculture conditions are more susceptible to pests in general, and to epiphytes in particular (Friedlander 1992, Buschmann et al. 1995). Epiphytic load is indeed one of the major biological problems reported in *Gracilaria* farms (Buschmann et al. 2001). In *G. chilensis,* the existence of widespread clones dominating some farms (Guillemin et al. 2008) may have serious impacts leading to a reduction of productivity. In fact, several studies have shown that genetic diversity leads to positive effects in marine systems: higher resilience in seagrass and eelgrass (Hughes and Stachowicz 2004, Reusch et al. 2005) and an increase in productivity in the congeneric *G. vermiculophylla* under stressful conditions (Gerstenmaier et al. 2016). On the other hand, conflicting results regarding the effect of genetic diversity on fitness in slowly growing clonal species have been obtained. For example, in the seagrass *Posidonia oceanica*, experimental results indicated that genetic diversity could enhance survival of transplants (Procaccini and Piazzi 2001). Nonetheless, low mortality rates have been reported in meadows subjected to aquaculture impacts when the genotypic and genetic diversity were lower (Diaz-Almela et al. 2007). In spite of the discrepancies, it is generally assumed that genetic impoverishment negatively affects the future capacity of adaptation and response to environmental changes of populations, thereby potentially threatening their mid-term survival (Arnaud-Haond et al. 2010), and, ultimately, affecting the efficiency of crops management plans (Massa et al. 2013).

Another relevant factor that can influence the productivity of a seaweed crop is the availability, uptake, and assimilation of nutrients (Rees 2003, Reef et al. 2012). It is well known that nutrient limitation has been associated with several negative impacts on seaweed ecophysiology (i.e., an increased vulnerability to photoinhibition by different radiations and

slower growth; Dean and Jacobsen 1986, Döhler et al. 1995, Figueroa et al. 2010). Moreover, stress due to nitrogen limitation is known to depress photosynthetic responses beyond the simple reduction of photosynthetic pigments levels in red seaweeds (Dawes et al. 1984). High nutrient concentrations do not necessarily induce higher growth since they can promote epiphytic and planktonic algal blooms, reduce light availability for photosynthesis, and ultimately cause significant loss of productivity and biomass in macroalgae (Buschmann and Gómez 1993, Mateo et al. 2006). Epiphytic load can significantly affect growth through reduction of light and nutrient availability in *G. chilensis* (Buschmann and Gómez 1993). Interestingly, in this species it has been established that a continuous supply of nutrients could induce an increase in epiphyte growth, whereas this was not observed when a more periodically spaced pulse of nutrient occurs (Pickering et al. 1993). It seems then, that not only nutrient concentration, but also periodicity of nutrient availability in the seawater can affect *G. chilensis* farms productivity.

In this study, we investigated the potential effects of genetic diversity (heterozygosity) and nutritional supply on growth rate, productivity and physiological responses of farmed *G. chilensis* thalli from two different localities. In order to better understand the effect of distinct physiological parameters on *G. chilensis* growth responses, we also quantified photosynthetic pigment concentration (chlorophyll *a* and phycobiliproteins), photosynthetic activity, C:N content, and epiphyte load in our experimental plots.

MATERIALS AND METHODS

Experimental site and algal material sampling sites

The experiment was conducted in southern Chile, at an intertidal sandy beach located at the marine station CEACIMA of Universidad de Los Lagos (Metri Bay: 41°36´ S, 72°43´ W, 30 km east of the city of Puerto Montt; Fig. 1A). The tidal amplitude in the area varies between 5 to 7 m (Buschmann et al. 1995), and the experimental site was at 0.5 m of the lowest tidal level. The algal material was sampled from two farmed localities (Ancud: 41°52´ S, 73°48´ W and Chaica: 41°38´ S, 72°39´ W; Fig. 1A) during spring season (September, southern hemisphere). In total, 260 healthy thalli of *Gracilaria chilensis* were sampled (130 in Ancud and 130 in Chaica). In each farm, the collected thalli were separated at least by 10 m to avoid, as much as possible, sampling fragments of the same clone (following Guillemin et al. 2008). Each thallus was individually bagged before being transported to Metri within an insulated container. Thalli were cleaned with fresh water and all epiphytic macroalgae were removed by hand. Thalli were individually tagged with color nylon threads and all marked thalli were kept in 1000 L tanks (i.e., one tank per locality). Thalli were maintained at 10°C under constant aeration and operated under flow-through water circulation system pumping water directly from Metri Bay. A 3 cm fragment of apical tissue was excised from each thallus, and immediately placed into silica gel for rapid dehydration. The dry thallus material was stored for subsequent DNA extraction and genetic analyses.

Phase determination and detection of putative clonal genotypes

Only tetrasporophytes were selected for the experiment to prevent differences in growth rate between life cycle phases (demonstrated for *Gracilaria chilensis*, see Guillemin et al. 2013 and Gallegos-Sánchez et al. 2018) that could confound effects of the experimental

manipulations. Direct observation under stereoscope microscope (Stemi DV4, Zeiss, Jane, Germany) was used for phase determination of all individuals presenting development of reproductive structures. When the individuals were not mature, sex markers published for *G. chilensis* (Guillemin et al. 2012) were used to determine ploidy. DNA extraction and PCR amplification reactions were carried out following protocols described by Cohen et al. (2004) and Guillemin et al. (2012), respectively, and the amplification products were visualized in 1.5 % agarose gel (w/v) after adding 2 µl of GelRed™ (Biotium, Fremont, CA, USA).

To detect possible presence of clones, all tetrasporophytes were genotyped using the six microsatellite loci available for *G. chilensis* following Guillemin et al. (2005). As above, total genomic DNA was extracted following Cohen et al. (2004). PCR reactions were performed and PCR products were analyzed on an ABI fragment analyzer (ABI 3100 Sequencer, Applied Biosystems, Foster City, CA, USA; Guillemin et al. 2005). Raw allele sizes were scored with GENEMARKER v.2.6.3 (SoftGenetics, State College, PA, USA) and multilocus genotypes (MLGs) were determined using Genclone v.2.0 (Arnaud-Haond and Belkhir 2007). The number of heterozygous loci was calculated for each MLG. For repeated MLGs, only one thallus was kept (the thallus with the highest biomass) as algal material for experimental plots set-up (see below for more details).

Experimental design

The field experiment was performed during late summer (25 February to 27 March 2017), a period characterized by low concentrations of nitrogen in Metri Bay that limit the growth of *Gracilaria chilensis* (Troell et al. 1997). The experiment consisted of 12 PVC frames of 1 m^2 size (plot), installed 2 m from each other, in rows parallel to the coastline (Fig. 1B).

Plots were fixed to the seabed at a depth of 0.5 m below the lowest tide level. Each plot was composed by four subplots (experimental units) of 0.25 m^2 and each experimental unit was composed by four parallel ropes attached to the plastic frame (Fig. 1, B and C). In each experimental unit, 16 thallus fragments (5 g of fresh weight each), originating from the same MLG, were entwined in ropes. This technique is classically used in *G. chilensis* aquaculture in Chile to secure growing thalli in farms (Halling et al. 2005). Each rope held 4 thallus fragments separated by approximately 12 cm. For each farm, the same MLG was subjected to two nutrient treatments (i.e., with or without nutrient addition, see below for more details). In total 12 distinct MLGs from Ancud and 12 distinct MLGs from Chaica were entwined in ropes (noted 1 to 12 in Fig. 1B). MLGs of Ancud and Chaica were chosen randomly from thalli, maintained in 1000 L tanks at CEACIMA, which had a fresh weight greater than 160 g (i.e., 12 thallus fragments x 2 conditions x 5 g). Experimental units were randomly sorted at the beginning of the experiment, to constitute the plots and they were randomly swapped each week between the fixation anchors established in the Metri Bay (Fig. 1C). Five temperature data loggers (Tidbit®, Hobo, Onset Computer Corporation, Bourne, MA, USA) were attached to the plots to record temperature every 30 min, during the course of the experiment. Concentrations of dissolved inorganic N (NO_3^-) and P ($PO^3_4^-$) in Metri Bay seawater were analyzed weekly during the study period by methods described in Strickland and Parson (1972).

Once a week, plots were removed from the field and soaked in 1000 L tanks, located at the marine station CEACIMA, for 5 h following Lapointe (1987). Plots submitted to the treatment "without nutrient addition" were soaked in coastal seawater pumped directly from the Metri Bay. Plots submitted to the treatment "with nutrient addition" were soaked in seawater pumped from the Metri Bay, within which commercial NPK fertilizer (CNA, Santiago, Chile) were added to obtain a final concentration of 0.1 $g \cdot L^{-1}$. The 30N:1P ratio of

the seawater obtained after additions of nutrients have been showed to allow for maximal growth of seaweed (Atkinson and Smith 1983). To facilitate nutrient uptake during soaking, thalli in both 1000 L tanks were aerated. The experiment was ended after 30 d.

Growth rate and productivity estimates

Fresh weight of each thallus fragment was measured at the beginning and after 30 days of the experiment with an analytical balance (accuracy 0.0001 mg, Radom, Radwag, Poland). Specific growth rate (SGR) was calculated as the percentage of fresh weight gain per day according to the formula: $SGR= [\ln (Wf \cdot Wi^{-1})/ (tf-ti)] \times 100$; where Wi= initial fresh weight, Wf= final fresh weight, and tf-ti= number of days between measurements. For each experimental unit, the biomass productivity was determined by measuring the fresh weight of all 16 thallus fragments at the beginning and at the end of the experiment. The biomass increment was calculated per area (m^2) and per time (d).

Pigment content

Pigment content was examined in three thallus fragments per subplot, once, at the end of the experiment. For each thallus fragment, 0.1 to 0.7 g of fresh weight was sampled just after plot removal and tissue was wrapped with aluminum foil before deep-freezing in liquid nitrogen. Tissues were transported to the laboratory in liquid nitrogen and stored in a -80°C freezer until pigment extraction.

Chlorophyll *a* (chl *a*) was extracted in N,N-dimethyl-formamide for 24 h at 4°C in darkness according to Marquardt et al. (2010). The chl *a* concentration was measured using a microplate reader (Infinite M200 PRO, TECAN, Männedorf, Switzerland) and expressed as

µg · mL^{-1} using the dichromatic equations of Porra et al. (1989). Chl *a* content was expressed as mg · g^{-1} dry weight (DW). Phycobilins were solubilized in water-soluble extract using phosphate buffer (pH 6.8) following the protocol by Gómez et al. (2005). Water-soluble extracts were centrifuged for 10 to 15 min at ca. 5000*g*. and supernatants were used for spectrophotometric determination of the phycoerythrin and phycocyanin concentrations using the equations described by Beer and Eshel (1985):

Phycoerythrin: [(A564 - A592) - (A455-A592) · 0.20] · 0.12 (1)

Phycocyanin: [A618 - A645) - (A592-A645) · 0.15] · 0.15 (2)

Fluorescence measurements

Algal thallus fragments were collected at the end of the experimental period (three thalli per subplot) and were incubated for 20 min in dark before measurement of maximal quantum yield of fluorescence (F_v/F_m, an indicator of the maximal quantum efficiency and photoinhibition; Schreiber et al. 1995) with a Junior PAM (Walz GmbH, Effeltrich, Germany). The electron transport rate (ETR, µmol electrons · m^{-2} · s^{-1}), as rapid light curves (RLC), was determined after 20 s exposure in 12 increasing irradiances of blue light provided by the Junior-PAM. ETR was calculated according to Schreiber et al. (1995) as follows:

$ETR = \Delta F/F_m \,.\, E \,.\, A \,.\, F_{II}$ (µmol electrons · m^{-2} · s^{-1}) (3)

where $\Delta F/F_m'$ is the effective quantum yield, E is the incident PAR irradiance expressed in µmol photons · m^{-2} · s^{-1}, A is the thallus absorptance and F_{II} is the fraction of chlorophyll related to PSII (400-700 m), using 0.15 in red macroalgae according to Grzymski et al. (1997) and Figueroa et al. (2003). The absorptance (A) was calculated with a LI-250A radiometer (LI-COR Biosciences, Lincoln, NE, USA) according to the formula: A=1-Et/Eo,

where Eo is the incident irradiance of PAR (photosynthetically active radiation) and Et is the transmitted irradiance with the algae being located on the light sensor (Figueroa et al. 2009).

As an estimator of photosynthetic efficiency, the initial slope of ETR (α_{ETR}) and maximum ETR (ETR_{max}) were obtained from the tangential function reported by Eilers and Peeters (1988) and the saturated irradiance (Ek_{ETR}) was calculated by dividing ETR_{max} per α_{ETR}. Non-photochemical quenching (NPQ, used as an estimator of photoprotective response to high light stress) was calculated according to Schreiber et al. (1995) as: NPQ = (F_m – $F`_m$)/$F`_m$. Maximal non-photochemical quenching (NPQ_{max}) was obtained from the tangential function of NPQ versus irradiance function (αNPQ) according to Eilers and Peeters (1988), following Celis-Plá et al. (2015).

Carbon and nitrogen content

Three thallus fragments, of 1 to 2 g of fresh weight, were sampled per subplot at the end of the experiment. Fragments were dried in a heating oven (series FD, Binder, Tuttlingen, Germany) until reaching a constant weight. Total intracellular carbon and nitrogen contents were determined using three replicates per fragment with a CNHS LECO-932 elemental analyzer (LECO, St. Joseph, Michigan, USA).

Epiphytic load

For epiphyte load, three thallus fragments per subplot were randomly selected at the end of the experiment, individually bagged and transported to the CEACIMA marine station in insulated coolers. All visible epiphytes were removed by hand. Simple taxonomic classification of epiphytes was carried out using direct observation under stereoscopic

microscope (Leica, Wetzlar, Germany) and following the descriptions provided by Hoffmann and Santelices (1997). Epiphytes were classified under four taxonomic groups: *Polysiphonia* spp., *Ulva* spp., *Rhizoclonium* spp. and *Ectocarpus* spp. Both epiphytes and *Gracilaria chilensis* thallus fragments were gently blotted on paper towel and fresh weight was measured using an analytical balance (Radom, Radwag, Poland). Epiphyte load was estimated as the biomass of epiphyte divided by the biomass of *G. chilensis* (g epiphytes $\cdot$ g^{-1} *Gracilaria chilensis*). Total epiphyte load and epiphyte load of *Ulva* spp. and *Polysiphonia* spp. were estimated.

Statistical analysis

All analyses were performed in R (3.2.4 version; Cayuela 2011). Data were tested for homogeneity of variances and normal distribution using Levene's and Shapiro-Wilk tests, respectively. When existence of non-normal residuals and/or heteroscedasticity was detected, data were logarithmically transformed (for C:N ratio, ETR_{max} and *Ek*) or Box-Cox transformed (for NPQ_{max} and phycocyanin concentration) prior analyses. For pigments concentration, carbon and nitrogen content, fluorescence measurements and epiphytic load, statistical significance of means were tested with two-way analyses of variance (2-way ANOVA, Treatment x Locality of origin, Treatment: with or without nutrient addition, Locality of origin: Ancud or Chaica) followed by Tukey's honest significant difference (HSD) tests. Effects were considered significant if $p < 0.05$. The number of biological replicates was 12 (i.e., number of distinct subplot or MLGs) for all response variables. Measurements made on the three thallus fragments per MLGs for pigments concentration, carbon and nitrogen content, fluorescence measurements and epiphytic load were considered as technical replicates and averaged before analyses.

Effect of treatment and level of heterozygosity were tested on growth rate and productivity using 3-way ANOVA (Treatment x Locality of origin x Heterozygosity level). Three levels of heterozygosity were defined after genotyping thalli from Ancud and Chaica (see results below for more details) and used as a fixed factor. As no differences were observed between thalli localities of origin for growth rate ($F_{2,34}$=0.12, P=0.89) and productivity ($F_{2,33}$=0.57, P=0.57), data from Ancud and Chaica were pooled.

RESULTS

Monitoring of Metri Bay environmental parameters

During the time-course of the experiment, seawater temperature and phosphate concentration were relatively constant with average value of 15.8 °C ± 1.0 and 0.06 µM ± 0.01, respectively. Nitrate concentrations were always lower than 4 µM, varying between 0.7 µM ± 0.01 and 3.9 µM ± 0.01.

Heterozygosity of Ancud and Chaica thalli

MLGs showing between 0 and 5 heterozygous loci were observed in our algal material and genotypes were classified in three level of heterozygosity: 1) low level (0 to 1 heterozygous loci, 6 and 5 MLGs for Ancud and Chaica, respectively), 2) intermediate level (2 to 3 heterozygous loci, 4 and 3 MLGs for Ancud and Chaica, respectively) and 3) high level (4 to 5 heterozygous loci, 2 and 4 MLGs for Ancud and Chaica, respectively).

Growth rate and productivity

Both, relative growth rate and productivity at 30 d, were significantly affected by the nutrient addition treatments (Fig. 2, A and B). Thalli with nutrient addition presented a significantly higher growth rate ($F_{1,34}$=38.02, P<0.0001; Fig. 2A) and productivity ($F_{1,33}$= 27.19, P<0.0001) than the ones without nutrient addition. No significant differences were observed between levels of heterozygosity for growth rate ($F_{2,34}$= 1.24, P= 0.30) or productivity ($F_{2,33}$= 0.05, P=0.95). However, both growth rate and productivity at 30 d seemed to increase slightly between MLGs with low, intermediate, and high level of heterozygosity for nutrient added thalli (Fig. 2A).

Physiological responses

Chlorophyll *a* concentration was roughly similar in all groups of thallus fragments (no significant effect of nutrient addition treatment, locality of origin, or their interaction), however, both phycocyanin and phycoerythrin concentrations were significantly affected by the nutrient addition treatments ($F_{1,43}$= 8.87, P=0.004 and $F_{1,44}$= 9.89, P=0.003 for phycocyanin and phycoerythrin content, respectively; see Table 1). The phycocyanin and phycoerythrin concentrations of thalli grown without nutrient addition represented between 70 to 80 % of the one measured in thalli grown with nutrient addition (Table 2).

There was a significant interaction of treatment and locality of origin with the maximal quantum yield of fluorescence (F_v/F_m) and the maximal electron transport rate (ETR_{max}) measured in *G. chilensis* (Tables 1 and 2). F_v/F_m and ETR_{max} were significantly higher in thalli from Chaica grown without nutrient addition, while no significant differences between treatments were detected in thalli from Ancud (Table 1). No significant effect of treatment, locality of origin, or their interaction were observed on the electron transport efficiency (α_{ETR}), the saturation irradiance (*Ek*) and the maximal non-photochemical quenching (NPQ_{max}).

The C:N ratio significantly increased under the no nutrient addition treatment, regardless of the locality of origin of the algal material (Fig. 3). No significant interaction was observed between nutrient addition treatment and locality of origin on C:N ratio ($F_{1,140}$= 0.522, P = 0.471). However, when treated as independent factors, a significant effect of nutrient addition treatment and locality of origin on the C:N ratio ($F_{1,140}$= 107.454, P <0.0001 and $F_{1,140}$= 14.369, P= 0.0002, respectively) was observed (see also Fig. 3).

Epiphytic load

After 30 d, only four epiphytic genera were observed on the *Gracilaria chilensis* thalli growing in our experimental plots. However, the epiphytic communities were slightly distinct depending on the thallus group under study: *Ectocarpus* spp. was observed on nutrient-added thalli from Chaica (maximum of 0.02 g epiphyte · g^{-1} *G. chilensis*) and on no nutrient added thalli from Ancud (maximum of 0.04 g epiphyte · g^{-1} *G. chilensis*), while *Rhizoclonium* spp. was observed on thalli sampled from both localities but only when growing with nutrient addition (maximum of 0.13 and 0.02 g epiphyte · g^{-1} *G. chilensis* on thalli from Ancud and Chaica, respectively). *Polysiphonia* spp. and *Ulva* spp. were observed on all thallus groups. Epiphytic load was generally low (total load <0.25 g epiphytes · g^{-1} *G. chilensis*; Table 2) and roughly similar for all groups of thallus fragments (no significant effect of nutrient addition, locality of origin or their interaction; Table 1).

DISCUSSION

Our results confirm that nutrient addition has significant positive effects on growth rate and productivity of farmed *Gracilaria chilensis* during summer at Metri Bay. These results

support previous studies that demonstrated that *Gracilaria* crops cultivated near salmon farms show increased tissue nitrogen levels and higher growth rates (Troell et al. 1997, Abreu et al. 2009). Nutrient addition had a significant impact on *G. chilensis* ecophysiology with higher phycobilin concentrations and lower C:N ratio measured in thalli that underwent nutrient addition. Photosynthetic parameters, as F_v/F_m and ETR_{max}, showed significant interaction between treatment and locality of origin. On the other hand, no significant effect of heterozygosity level on *G. chilensis* growth rate was observed during our experiment.

Our study shows that *G. chilensis* crops growth and productivity could be highly influenced by nutrient availability. This result is concordant with experimental evidence obtained in various species of the *Gracilaria* genus. Indeed, growth and photosynthetic capacity of *G. tikvahiae* in the field was enhanced by phosphate and ammonia addition (Lapointe 1987), while pulses of nutrients leaded to significant increase in biomass in *G. cornea* (Navarro-Ángulo and Robledo 1999) and growth rate in *G. chilensis* (Pickering et al. 1993).

Understanding how seaweeds use various sources of inorganic and organic nitrogen is key for successful development of aquaculture (Hanisak 1990). The capacity of the genus *Gracilaria* to uptake and store nitrogen in excess of immediate requirements, and use it to sustain growth during subsequent periods of nutrient deficiency, has been utilized in cultures to minimize the growth of epiphytes (Lapointe 1985). However, the nutrient requirements for optimal seaweed crop growth can vary seasonally and spatially as it may depend on other environmental factors as light intensity and/or seawater temperature. Moreover, a fine-tuned balance between higher growth rate and epiphyte control should be considered as critical when establishing farm management strategies for nutrient supplement (Hanisak 1990). One of the major problems in seaweed mariculture, such as *Gracilaria* farms, is the high epiphyte load, which often occur in nitrogen rich conditions (Pickering et al. 1993). Indeed, a negative

effect of epiphyte abundance on farm production has been observed in *Gracilaria* (Buschmann and Gómez 1993). However, at the end of our monthly experiment, we did not detect any significant effect of nutrient addition on the epiphyte load and a maximum of 0.25 g epiphytes · g *G. chilensis*$^{-1}$ thalli was observed in our plots, regardless of the nutrient treatment applied. The methodology used to supplement the nutrient, using 5 h N pulses once per week, could explain our results. Pickering et al. (1993) demonstrated that nitrogen addition administered by pulses at interval of 7-10 d increased *Gracilaria* thalli growth rate without increasing epiphytic load. The authors proposed that this result could be due to starvation of the fast-growing epiphyte species during periods of low nitrogen access (i.e., in-between nutrient pulses). Since *Gracilaria* growth seems to be improved by nutrient addition, co-cultivation with fish had been proposed in order to simultaneously reduce the risk of eutrophication and diversify source of economic income (Troell et al. 1997). However, evidence exists that a continuous nutrient supply can increase epiphyte loads in *Gracilaria* culture, negatively affecting farm productivity (Fletcher 1995). Indeed, high levels of epiphytes were observed on *Gracilaria* cultivated near salmon farms under elevated and constant nutrient availability (Halling et al. 2005).

The C:N ratio increase in our *G. chilensis* no-nutrient added-thalli suggest that the growing environment in the oligotrophic water of Metri Bay in the summer was not highly favorable to production of some nitrogen-rich organic metabolites, such as soluble proteins and phycobiliproteins (Collén et al. 2004, Figueroa et al. 2010), and that nitrogen uptake was not enough to induce optimal growth rates (Duarte 1992). Collén et al. (2004) observed that under nutrient limitation, *Gracilaria* cellular metabolism decreases. This situation rapidly affects phycobilin content, but not photosynthetic efficiency. Indeed, the decrease in F_v/F_m in *G. tenuistipitata* was shown to occur only at the very end of the experiment, a period potentially characterized by an acute lack of nitrogen (Collén et al. 2004). Our study

showed a significant interaction between treatment and locality of origin of the thalli on two photosynthetic parameters: F_v/F_m and ETR_{max}. The effect of nutrient addition was only detectable on the Chaica thalli and led to lower values of F_v/F_m and ETR_{max}. The maximum quantum yield is used regularly as an early warning of physiological stress in seaweeds (Figueroa et al. 2006). Therefore, it is possible that strains from Chaica were under stress due to low nutrient availability at the end of our experiment (Parkhill et al. 2001), a state that could have ultimately affected their maximal photosynthetic capacity.

The effects of nutrient treatment on these different photosynthetic responses between Ancud and Chaica thalli could be due to differences in cultivation techniques and thallus selection could have leaded to ecological differences between *G. chilensis* from these two farms. The two farms are established in distinct habitats: Ancud is located at the mouth of an estuary with muddy substrate, while Chaica is located in a sandy bay with very strong tidal influence. Evidence suggests that seaweeds from distinct origins, but cultivated under the same conditions in the field can show differences in morphology or chemical constituents (Hanisak et al. 1990, Buschmann et al. 1992). For red seaweeds, such as *Kappaphycus* or *Gracilaria*, potential adaptation of local landraces or cultivars to their growing environment has been proposed and could be particularly important to sustain cultures when growing conditions are not optimum (Valero et al. 2017).

Contrary to our expectations, no significant effect of heterozygosity on growth rate was observed during our experiment. A recent study published by Gallegos-Sánchez et al. (2018) explored the relationship between heterozygosity and growth rate in *G. chilensis* cultured under different salinity conditions. However, only two levels of heterozygosity (two and three heterozygous loci) were found among their experimental thalli using five microsatellite markers. At the end of the experiment, only thalli characterized by two heterozygous loci showed a decrease in SGR at the lower salinity. The authors concluded

that diploids with a higher number of heterozygous loci could display lower growth rate plasticity than those exhibiting less heterozygosity. In our experiment, thalli with zero to five heterozygous loci were observed using six microsatellite markers, but we did not find sufficient differences in heterozygosity among our groups to detect any significant effect of genetic diversity on growth or productivity in the conditions encountered in the Metri Bay during summer.

Gerstemeier et al. (2016) demonstrated that increases in genotypic diversity could increase productivity of *Gracilaria vermiculophylla*, and suggested that the positive effects of genetic diversity may only be observed in this species when grown in a highly stressful environment (mid intertidal during the summer). However, they concluded that the impacts that the genetic diversity can have on *Gracilaria* productivity vary temporally and spatially for reasons that remain unclear. It should also be noted that they explored these patterns in a non-native population of this seaweed, though they focused on diploid thalli. In our specific case, we consider the summer conditions during the experimental time as stressful environment (due to lower nitrate concentrations found in the Metri Bay), but in contrast to Gerstenmaier et al. (2016), the genetic diversity did not affect *Gracilaria chilensis* growth or productivity under stressful conditions.

CONCLUSIONS

In conclusion, these results highlight that the abiotic factors, more than the genetic response, affect growth and physiological responses of two *Gracilaria chilensis* farmed populations. This result could be related to the species domestication history in which there is a limited range of genetic variation in farms. However, even if we did not detect a significant impact of genetic diversity on *G. chilensis* productivity, its impact on farms productivity when subjected to changes of environmental factors, other than nutrient

limitation, remains to be tested in more detail. For *G. chilensis*, farming in Chile has unconsciously lead to a reduction of genetic diversity over the past few decades and this may negatively affect the growth of populations undergoing challenging conditions in the future. In this context, more studies considering interactions between genetic and abiotic factors are needed, in order to plan for better sustainability of seaweed farms in response to global change. Our study shows that nutrient limitation is a relevant factor for *G. chilensis* farming and the pulse nitrogen addition had no visible effect on epiphyte load. However, *G. chilensis* farms are generally located at the mouth of large estuaries, a habitat where trends of increasing eutrophication have been reported worldwide. It will be of interest, in the future, to test the response of distinct genotypes to nitrogen concentrations higher than the one reported as best for optimal growth.

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Table 1. Results of ANOVA analyses on A) pigment contents, B) photosynthetic parameters and C) epiphytic load for *Gracilaria chilensis* thalli sampled from two localities (L) and submitted to two contrasting fertilization treatments (F.T). Localities: Ancud and Chaica, fertilization treatments: with nutrient addition or without nutrient addition. Significant values are noted in bold; *: p <0.05, **: p<0.01.

Response variables	Fertilization Treatment (F.T)				Locality (L)				Interaction (L x F.T)			
	Sum Sq	df	F	p	Sum Sq	df	F	p	Sum Sq	df	F	p
A)												
Chlorophyll *a*	0.214	1	0.042	0.8377	12.741	1	2.531	0.119	15.125	1	3.004	0.090
Phycocyanin	0.639	1	8.874	**0.0047****	0.077	1	1.066	0.308	0.014	1	0.191	0.664
Phycoerytrin	2.132	1	9.890	**0.0030****	0.100	1	0.466	0.499	0.1440	1	0.668	0.418
B)												
F_v/F_m	0.002	1	0.867	0.3599	0.005	1	2.203	0.149	0.014	1	5.799	**0.023***
ETR_{max}	0.027	1	0.058	0.8120	0.852	1	1.804	0.190	2.515	1	5.322	**0.029***
α_{ETR}	<0.001	1	1.487	0.2328	<0.001	1	0.823	0.372	<0.001	1	2.960	0.096
Ek	0.225	1	0.423	0.5209	1.156	1	2.173	0.152	1.205	1	2.265	0.143
NPQ_{max}	0.003	1	0.039	0.8439	0.003	1	0.382	0.541	0.002	1	0.021	0.884
C)												
Ulva spp.	2.165	1	0.746	0.398	4.319	1	1.487	0.236	0.243	1	0.084	0.775
Polysiphonia spp.	4.102	1	0.720	0.409	2.328	1	0.409	0.532	3.631	1	0.637	0.437
Total epiphyte load	<0.001	1	0.048	0.833	0.004	1	1.875	0.213	0.0001	1	0.050	0.829

Table 2. Ecophysiological measures in *Gracilaria chilensis* thalli from Ancud and Chaica submitted to two contrasting fertilization treatments: with nutrient addition or without nutrient addition. Pigment content (chlorophyll *a*, Phycocyanin, Phycoerytrin, mg · g^{-1} DW) (A), photosynthetic parameters (B), and epiphyte load (g epiphytes · g^{-1} *G. chilensis*) (C). Data are means ± SD (for each measurement n=12 per plot). Distinct uppercase letters denote significant differences after Tukey test.

	Ancud		Chaica	
	With nutrient addition	Without nutrient addition	With nutrient addition	Without nutrient addition
A)				
Chlorophyll *a*	7.68 ± 2.28[a]	6.70 ± 2.73[a]	7.59 ± 1.99[a]	8.85 ± 1.88[a]
Phycocyanin	0.86 ± 0.20[a]	0.70 ± 0.37[a]	0.89 ± 0.32[a]	0.73 ± 0.20[b]
Phycoerytrin	1.79 ± 0.45[a]	1.26 ± 0.41[b]	1.77 ± 0.56[a]	1.46 ± 0.42[a]
B)				
F_v/F_m	0.63 ± 0.03[a]	0.60 ± 0.06[a]	0.61 ± 0.07[a]	0.67 ± 0.02[b]
ETR_{max}	21.66 ± 15.55[a]	12.42 ± 9.85[a]	9.15 ± 6.56[b]	14.49 ± 9.39[a]
α_{ETR}	0.03 ± 0.00[a]	0.03 ± 0.01[a]	0.02 ± 0.01[a]	0.03 ± 0.01[a]
Ek	801.87 ± 536.59[a]	457.98 ± 300.28[a]	469.97 ± 547.71[a]	491.81 ± 354.75[a]
NPQ_{max}	1.50 ± 0.82[a]	1.76 ± 1.54[a]	1.34 ± 0.64[a]	1.58 ± 1.24[a]
C)				
Ulva spp.	0.03 ± 0.04[a]	0.07 ± 0.10[a]	0.01 ± 0.03[a]	0.03 ± 0.03[a]
Polysiphonia spp.	0.09 ± 0.19[a]	0.14 ± 0.01[a]	0.12 ± 0.05[a]	0.05 ± 0.05[a]
Total epiphyte load	0.25 ± 0.23[a]	0.25 ± 0.12[a]	0.16 ± 0.10[a]	0.07 ± 0.10[a]

Figure 1. A) Map of the Chilean coast showing the sampling locations of the two farms in the X Region, Ancud and Chaica and the experimental site in southern Chile, Metri Bay. B) Schematic representation of the experimental set up in the field. In grey: plots with the treatment of nutrient addition (N+) and in white: plots without the nutrient addition treatment (N-). The numbers inside each subplot represent one of the twelve genotypes used per locality. C) Underwater plots installation. All the treatments were randomly interdispersed.

Figure 2. Comparison of the growth rate after 30 d (A) and productivity (B) of *Gracilaria chilensis* thalli categorized under three level of heterozygosity (LOW, low level of heterozygosity, MLGs with 0 to 1 heterozygous loci; INT, intermediate level of heterozygosity, MLGs with 2 to 3 heterozygous loci and HIGH, high level of heterozygosity, MLGs with 4 to 5 heterozygous loci) under two contrasting fertilization treatments: with nutrient addition (box-plot filled in grey) or without nutrient addition (box-plot filled in white). Box plot whiskers show the 1%–99% range values; the horizontal line in each box plot shows the median, and the colored segment shows the quartile range (25%–75%). Values outside of the whisker range are plotted as dots. As no differences were observed between populations of origin, data from Ancud and Chaica were pooled.

Figure 3. Comparison of internal C:N ratio in *Gracilaria chilensis* thalli, sampled from two localities (Ancud and Chaica), after 30 d of grow under two contrasting fertilization treatments: with nutrient addition (grey bars) or without nutrient addition (white bars). Distinct letters denote significant differences between treatments ($P < 0.05$).