



Physiological stress modulates epiphyte (*Rhizoclonium* sp.)-basiphyte (*Agarophyton chilense*) interaction in co-culture under different light regimes

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Abstract

Epiphytism is a common phenomenon in macroalgal aquaculture worldwide. This problem can affect the productivity and quality of the farmed species which may have socioeconomic consequences for the human communities that depend on this activity. In southern Chile, the aquaculture of the red macroalga *Agarophyton chilense* in the area of Maullín River has suffered losses in local production (> 90%) due to the proliferation of the filamentous green epiphyte *Rhizoclonium* sp. This epiphyte becomes entangled with *A. chilense* fronds, diminishing its quality and preventing its sale as raw material. Thus, the aim of this study was to investigate the effects of light intensity (90 and 180 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) on the competitive interaction between the epiphyte *Rhizoclonium* sp. and the basiphyte *A. chilense* co-cultured at different initial biomass proportions in a replace series experiment. At the end of the experiment, the presence of *Rhizoclonium* sp. did not affect negatively the growth of *A. chilense*, but both species showed stress responses (i.e. reduced F_v/F_m and C:N ratio, suggesting nutrient limitation) in co-culture compared with monocultures. The epiphyte-basiphyte interaction was not modulated by light availability at any initial co-culture proportion and could have been related to nutrient availability during experimentation. Using the replacement series approach, an interspecific competition was observed at all initial co-culture proportion under 180 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ while competition was found at the initial co-culture 1:1 under 90 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Our results suggest that the interaction in co-culture between both *A. chilense* and *Rhizoclonium* sp. seems to be regulated by nutrient availability as well as it demands utilization rather than light availability.

Keywords Aquaculture · Competition · Epiphytism · Nutrients · PAR · Replacement series · Rhodophyta · Chlorophyta

Introduction

Marine macroalgae experience interactions with biotic and abiotic factors. Within biotic interactions, inter and intraspecific competition for resources can occur with other macroalgae (Hurd et al. 2014). Epiphytism is a common interaction between macroalgae, where an epiphyte

grows on the surface of a basiphyte (i.e. substratum species), having often negative effects on the growth of the latest (Wahl 2008). The epiphyte usually has negative effects on the basiphyte by reducing light availability (Kuschel and Buschmann 1991; Buschmann and Gómez 1993), impeding gas and nutrient exchange from and to the water column (Svirski et al. 1993; Fletcher 1995) and increasing the dragging effect which causes rupture of the basiphyte thallus (Kuschel and Buschmann 1991; Buschmann and Gómez 1993; González et al. 1993; Fletcher 1995). In addition, epiphytes can damage the basiphyte thallus due to the penetration of rhizoids (González et al. 1993; Leonardi et al. 2006) and to the production and release of harmful allelochemicals (e.g. chemical compounds that may inhibit the growth of other species) (Svirski et al. 1993; Friedlander et al. 1996). The epiphyte-basiphyte interactions are complex and still need investigation for understanding its consequences at individual and community levels.

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Epiphytism is a typical phenomenon in macroalgal aquaculture worldwide (Svirski et al. 1993), especially in the cultivation of the agarophytic red macroalgae *Gracilaria* spp. (Buschmann et al. 1995, 1997; Fletcher 1995; Martín et al. 2013a; Santelices 2014). Cultivation of *Gracilaria* spp. has commonly been affected by the rapid development of undesirable epiphytes such as the green macroalgae *Ulva* spp., *Cladophora* spp. and *Rhizoclonium* spp. (Friedlander et al. 1987; Friedlander 1992; Buschmann and Gómez 1993; Pickering et al. 1993; Fletcher 1995). This problem can affect the productivity and quality of the farmed species which may have socioeconomic consequences for the human communities that depend on this activity. For example, in Chile, the red macroalgae *Agarophyton chilense* (C.J.Bird, J. McLachlan et E.C.Oliveira) C.F.D. Gurgel, J.N.Norris et S. Fredericq (basionym: *Gracilaria chilensis* C.J.Bird, J. McLachlan & E.C.Oliveira according to Gurgel et al. (2018), and Guiry and Guiry (2016)) is intensively farmed for agar extraction (Buschmann et al. 2017; Camus et al. 2019). During the year 2002, it reached a production 126,184 t (= US\$ 35,244,300), being the macroalgal species that most contributed to the country's income (Buschmann et al. 2005). In the area of Maullín River (southern Chile), during the summer of 2014, the local macroalgal aquaculture industry suffered large losses in the production of *A. chilense* (up to 93%) (Subpesca 2013) due to the proliferation of the filamentous green macroalga *Rhizoclonium* sp. (Subpesca 2015). This epiphyte becomes entangled with *A. chilense* fronds, diminishing its quality and preventing its sale as raw material. Consequently, in 2015, authorities declared the area of Maullín River as a “plague zone” as a result of the massive presence of *Rhizoclonium* sp. (Subpesca 2015).

The replacement series experiment is a methodology used for studying competitive interactions between two species (De Wit 1960). This methodology was designed to quantitatively assess the relative impact of inter- and/or intraspecific competition between two species at an overall density while the culture proportions between them are changed (De Wit 1960). The output obtained from this experimental design is useful to identify the magnitude of competition or the proportional combination between the two species where the growth is maximized (Rodríguez 1997; Jolliffe 2000; Williams and McCarthy 2001). The replacement series approach has been employed to study the interactions between terrestrial plants (Pantone 1995; Walck et al. 1999; Aminpanah et al. 2012; Vivian et al. 2013), nematodes (Stetina et al. 1997), bacteria (Wilson and Lindow 1994a, 1994b), fungi (Adee et al. 1990; Zitko and Timmer 1994) and macroalgae (Karez and Chapman 1998; Karez 2003).

Owing to the lack of studies on the mechanisms of the epiphyte-basiphyte interaction in the *A. chilense* aquaculture in southern Chile, we aimed to investigate the effects of non-saturating ($90 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) and saturating

($180 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) light intensities on the competitive interaction between the epiphyte *Rhizoclonium* sp. and the basiphyte *A. chilense* in a short-term replace series experiment (i.e. co-cultivated at different initial biomass proportions). We hypothesised that the non-saturating light intensity and the presence of the epiphyte will reduce the growth of the basiphyte in co-cultures compared with monocultures, indicating a competitive interaction between both species. This response will be magnified in co-cultures where the biomass of *Rhizoclonium* sp. is greater than that of *A. chilense*.

Materials and methods

Sample collection

Sixteen samples of both *Agarophyton chilense* and *Rhizoclonium* sp. were collected from Maullín River, southern Chile (Fig. 1), on June 2018 (autumn). Samples of each species were separately packed and transported in a cool box to the laboratory within 1 h of collection. Samples were lightly brushed and cleaned of visible epibionts using filtered seawater ($0.2 \mu\text{m}$, Whatman Polycap TC filter capsule, GE Healthcare Life Sciences, UK), and kept under acclimation conditions (15°C , $100 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$, 34 ‰) for 3 days.

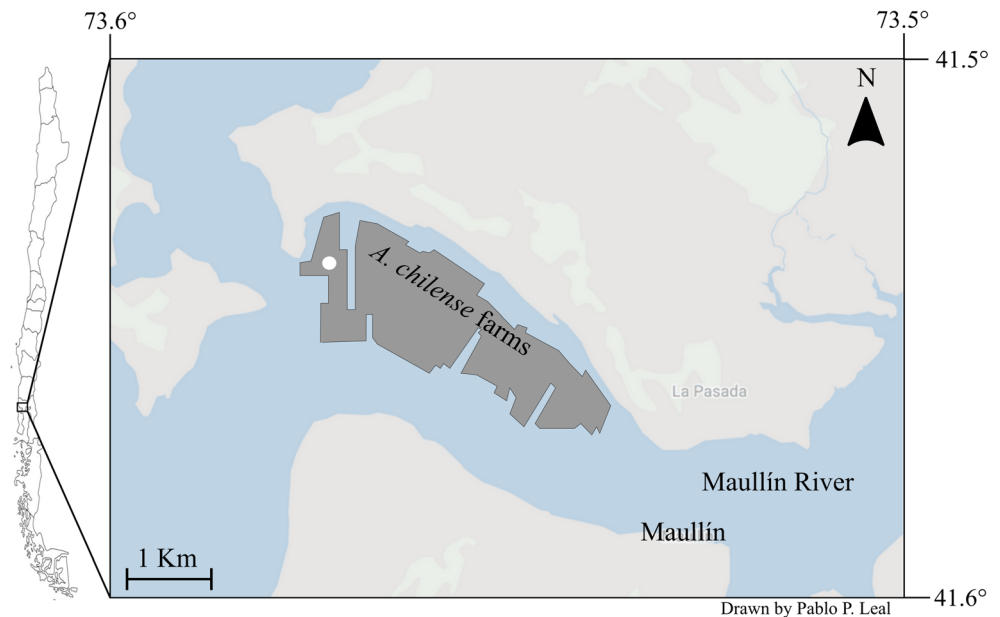
Co-culture experiment

After acclimation, samples of both species were co-culture ($n = 4$) at five different initial biomass proportions as *A. chilense*:*Rhizoclonium* sp. = 1:0, 2:1, 1:1, 1:2 and 0:1 (Fig. 2) and the actual biomass per proportion treatment is summarized in Table 1. Co-cultured macroalgae were exposed to two PAR intensities (saturating, $180 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ and under-saturating, $90 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) for 19 days (Fig. 2) inside a temperature-controlled growth room at 15°C . Seawater was enriched with $200 \mu\text{M NO}_3^-$ and $20 \mu\text{M PO}_4^{3-}$, aerated constantly using an air pump (air 550 R plus, SERA, Germany) and was renewed every 3 days. Light was provided by LED tubes (T8 integrated light, 18 W, white colour, TEJIE Ltd. San Antonio, Chile) and measured with a quantum sensor (LP471 PAR, Delta OHM S.r.l., Italy) connected to a light meter (photo-radiometer HD 2302.0, Delta OHM S.r.l., Italy). After acclimation, water content was also determined in both species by incubating tissue samples ($n = 4$) at 60°C for 72 h.

Growth rate

Fresh weight (FW) of both species at the different co-culture proportions under the two PAR intensities was determined at days 1 and 19. Macroalgal tissue was gently blotted dry with

Fig. 1 Diagram showing the study area ($\sim 3 \text{ Km}^2$) covered by the aquaculture farms of *A. chilense* in Maullín River, southern Chile. The sampling site (41.58° S ; 73.65° W) is indicated with a white circle. Modified from the map viewer by Subpesca (2013)



tissue before weighing, to remove excess water. Growth rate ($\% \text{ day}^{-1}$) was calculated on day 19 according to Yong et al. (2013):

$$\text{Growth rate } (\% \text{ day}^{-1}) = \left[(W_t/W_0)^{1/t} - 1 \right] \times 100$$

where W_0 is the initial size, W_t is the final size and t is the days of culture.

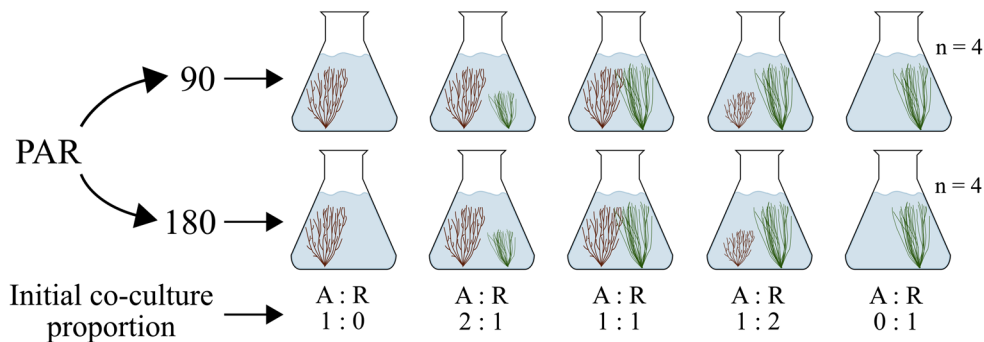
Maximal quantum yield (F_v/F_m)

Chlorophyll *a* fluorescence of photosystem II was measured using a pulse amplitude modulation fluorometer (diving-PAM, Walz, Germany). Macroalgae from the different treatment combinations were dark-adapted for 15 min before exposure to the PAM's photosynthetic active radiation (PAR, $0\text{--}422 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$). Then, F_v/F_m , which is a good indicator of maximal algal photosynthetic efficiency

(Figuroa et al. 2013), was calculated after 10–15 min of dark adaptation according to Schreiber et al. (1995).

Chlorophyll *a* content

The content of chlorophyll *a* was measured following different procedures (Seely et al. 1972; Speziale et al. 1984; MacIntyre and Cullen 2005; Su et al. 2010; Zhu et al. 2017). Frozen tissue samples ($0.1\text{--}0.15 \text{ g FW}$) from each species were placed in 15-mL test tubes. First, 4 mL of dimethyl sulfoxide (DMSO) was added and left to extract at 20° C (room temperature) for 20 min. Then, 4 mL of 90% acetone (v/v) was added to the tissue and left to fully extract pigments at 20° C for 30 min (MacIntyre and Cullen 2005). During this time, the test tubes were occasionally agitated. Visual observation showed no remaining pigments in the macroalgal tissue. After extraction, the



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Fig. 2 Diagram of the experimental design showing the different initial co-culture biomass proportions of *A. chilense* (A) and *Rhizoclonium* sp. (R) under two PAR intensities (90 and $180 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$) for

19 days. Four replicates of each co-culture proportion were prepared. Actual initial biomass (g FW) used in each co-culture proportion of both species is shown in Table 1

Table 1 Initial and final co-cultured biomass (g FW) of *A. chilense* and *Rhizoclonium* sp. for each co-culture proportion under the respective PAR treatment (PAR value between square brackets indicates mean $\pm$ SD, $n = 5$). Total initial nominal biomass for each co-culture proportion was 1 g of tissue in 250 mL of seawater. Data correspond to mean $\pm$ SD ($n = 4$)

PAR intensities ($\mu\text{mol photons m}^{-2} \text{ s}^{-1}$)	Co-culture proportions (<i>A. chilense</i> : <i>Rhizoclonium</i> sp.)	Actual co-cultured biomass (g FW)					
		Initial			Final		
		<i>A. chilense</i>	<i>Rhizoclonium</i> sp.	Total biomass in 250 mL	<i>A. chilense</i>	<i>Rhizoclonium</i> sp.	Total biomass in 250 mL
180 [181.71 $\pm$ 2.36]	1:0	1.11 $\pm$ 0.040	—	1.11	1.67 $\pm$ 0.076	—	1.67
	2:1	0.68 $\pm$ 0.020	0.34 $\pm$ 0.002	1.02	0.98 $\pm$ 0.117	1.21 $\pm$ 0.180	2.19
	1:1	0.53 $\pm$ 0.038	0.52 $\pm$ 0.012	1.05	0.80 $\pm$ 0.088	1.95 $\pm$ 0.376	2.75
	1:2	0.34 $\pm$ 0.011	0.67 $\pm$ 0.002	1.02	0.49 $\pm$ 0.042	1.88 $\pm$ 0.359	2.38
	0:1	—	1.03 $\pm$ 0.039	1.03	—	3.27 $\pm$ 517	3.27
90 [90.55 $\pm$ 1.11]	1:0	1.05 $\pm$ 0.027	—	1.05	1.66 $\pm$ 0.207	—	1.66
	2:1	0.68 $\pm$ 0.008	0.33 $\pm$ 0.004	1.02	1.00 $\pm$ 0.034	1.26 $\pm$ 0.57	2.26
	1:1	0.52 $\pm$ 0.019	0.52 $\pm$ 0.007	1.03	0.83 $\pm$ 0.038	2.55 $\pm$ 0.207	3.38
	1:2	0.34 $\pm$ 0.007	0.68 $\pm$ 0.003	1.02	0.52 $\pm$ 0.020	2.01 $\pm$ 0.190	2.53
	0:1	—	0.98 $\pm$ 0.024	0.98	—	3.15 $\pm$ 0.608	3.15

DMSO and acetone extracts were separately poured off and absorption of each extract was measured using a UV-visible spectrophotometer (Spectronic Genesys 5, Milton Roy Company, USA) at 663 and 645 nm. Concentrations of pigments were calculated using the equations given by Ritchie (2008).

C:N ratio

C:N ratio with values > 20 indicates stress by nutrient limitation (Wheeler and North 1981; Hurd et al. 2014; Gorman et al. 2017). Thus, after measuring growth, carbon (C) and (N) content were measured to determine potential nutrient limitation during the experiment. Tissue samples of 0.2 g FW from each species ($n = 4$) were dried in a drying oven (ca. 36 h at 60 °C). Dried tissue samples were powdered using a porcelain mortar and pestle and stored for C and N content measurements (TruSpec CHN analyser, LECO Corporation, USA).

Relative macroalgal yield

Relative yield for *A. chilense* (RY_A) and *Rhizoclonium* sp. (RY_R) were calculated from the weight of each sample at the different treatment combinations at day 19, according to the following equations (De Wit 1960; Bi and Turvey 1994):

$$\text{RY}_A = \frac{W_{AR}}{W_A} \quad \text{or} \quad \text{RY}_R = \frac{W_{RA}}{W_R}$$

where W_{AR} is the final FW (g) of *A. chilense* co-cultured with *Rhizoclonium* sp., W_A is the FW growth of *A. chilense* in

monocultures while W_{RA} is the final FW of *Rhizoclonium* sp. co-cultured with *A. chilense* and W_R is the final FW of *Rhizoclonium* sp. in monoculture.

Relative yield total (RY_T) was calculated according to the equation (De Wit 1960; Bi and Turvey 1994):

$$\text{RY}_T = \text{RY}_A + \text{RY}_R$$

Values of RY_T of 1.0 imply competition between both species for the same environmental resource, $\text{RY}_T > 1.0$ indicates a synergistic interaction and $\text{RY}_T < 1.0$ indicates a mutual antagonism (Bi and Turvey 1994).

Statistical analyses

Macroalgal growth rate ($\%\cdot\text{day}^{-1}$) data were logit transformed (Warton and Hui 2011), Fv/Fm (*Rhizoclonium* sp.), chlorophyll *a* content (*A. chilense*), C content (*Rhizoclonium* sp.) and N content (*A. chilense*), and relative macroalgal yield data were rank transformed (Potvin and Roff 1993) to satisfy ANOVA assumptions of normality (Kolgomorov-Smirnow test) and homogeneity of variances (Levene's test). The statistical significance of differences in growth rate, Fv/Fm, chlorophyll *a*, C and N content, C:N ratio and between initial co-culture proportions, PAR intensities and their interaction were tested using the two-way ANOVA ($P < 0.05$). The relative macroalgal yield between species, initial co-culture proportions, PAR intensities and their interaction was tested using the three-way ANOVA ($P < 0.05$). The post hoc Tukey test ($P < 0.05$) was applied when significant differences were obtained. The software

SigmaPlot version 12.0 (Systat Software, Inc., USA) was used to run all the statistical analyses.

Results

Species-specific growth rate

After 19 days, the growth rate ranged from 1.88 to 2.53%·day⁻¹ and 5.48 to 8.76%·day⁻¹ for *A. chilense* and *Rhizoclonium* sp. (Fig. 3), respectively. *Agarophyton chilense* growth rate was not significantly different between co-culture proportions nor PAR treatment (Table 2). *Rhizoclonium* sp. growth rate was not statistically different between co-culture proportions under 180 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (Fig. 3a) but was significantly faster at proportions

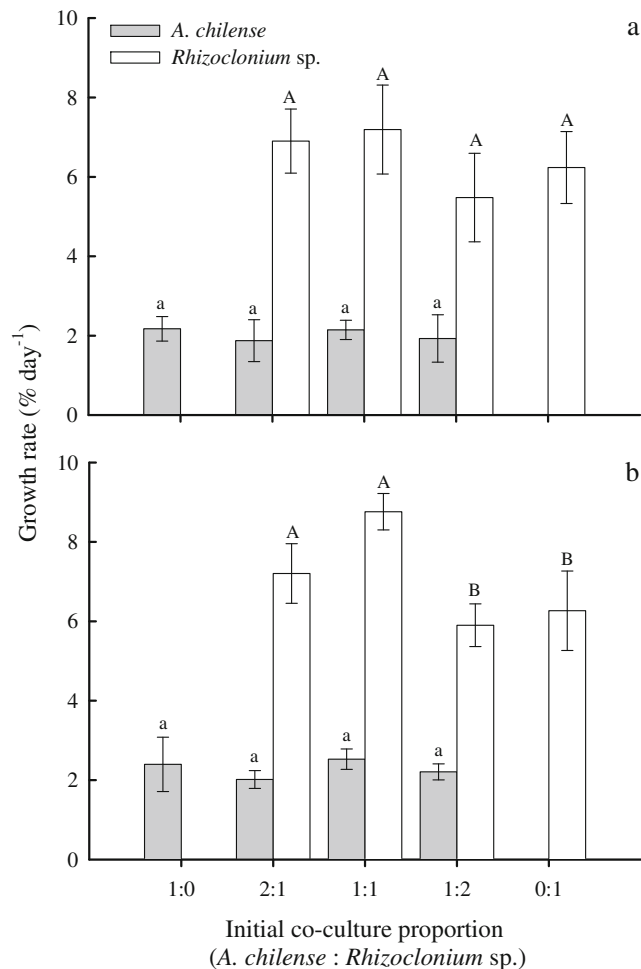


Fig. 3 Growth rate of *A. chilense* and *Rhizoclonium* sp. after 19 days of co-cultivation at different initial proportions under two PAR intensities of **a** 180 and **b** 90 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$. Bars represent mean $\pm$ SD ($n = 4$). Significant subgroups are defined by capital letters as $A > B$ for *Rhizoclonium* sp. (Tukey, $P < 0.05$). Statistical analyses for interspecific differences were not performed (see the “Materials and methods” section for details)

2:1 and 1:1 compared with 1:2 and 0:1 under 90 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (Fig. 3b; Table 2). Two-way interactions between independent variables did not significantly affect species-specific growth rate (Table 2). Water content of study species was $82.73 \pm 0.91\%$ and $76.33 \pm 3.94\%$ for *A. chilense* and *Rhizoclonium* sp., respectively.

Maximal quantum yield (F_v/F_m)

After 19 days F_v/F_m ranged from 0.29 to 0.44 and 0.36 to 0.69 for *A. chilense* and *Rhizoclonium* sp. (Fig. 4), respectively. *Agarophyton chilense* F_v/F_m was significantly higher at proportions 1:0 and 2:1 compared with 1:1 and 1:2 but was not affected by PAR treatments (Fig. 4; Table 3). *Rhizoclonium* sp. F_v/F_m was significantly higher at proportions 0:1 and 1:2 compared with 2:1 and 1:1 but was not affected by PAR treatments (Fig. 4; Table 3). Two-way interactions between independent variables did not significantly affect species-specific F_v/F_m (Table 3).

Chlorophyll *a* content

After 19 days, chlorophyll *a* content ranged from 0.11 to 0.16 $\text{mg g}^{-1} \text{ FW}$ and 0.12 to 0.37 $\text{mg g}^{-1} \text{ FW}$ for *A. chilense* and *Rhizoclonium* sp. (Fig. 5), respectively. *Agarophyton chilense* chlorophyll *a* content was not significantly different between co-culture proportions nor PAR treatment (Table 4). *Rhizoclonium* sp. chlorophyll *a* content was significantly higher at proportion 1:2 compared with 2:1, 1:1 and 0:1 under 180 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (Fig. 5a) but was significantly higher at proportions 1:2 and 0:1 compared with 2:1 and 1:1 (Fig. 5b; Table 4). Two-way interactions between independent variables did significantly affect chlorophyll *a* content of *Rhizoclonium* sp. (Table 4).

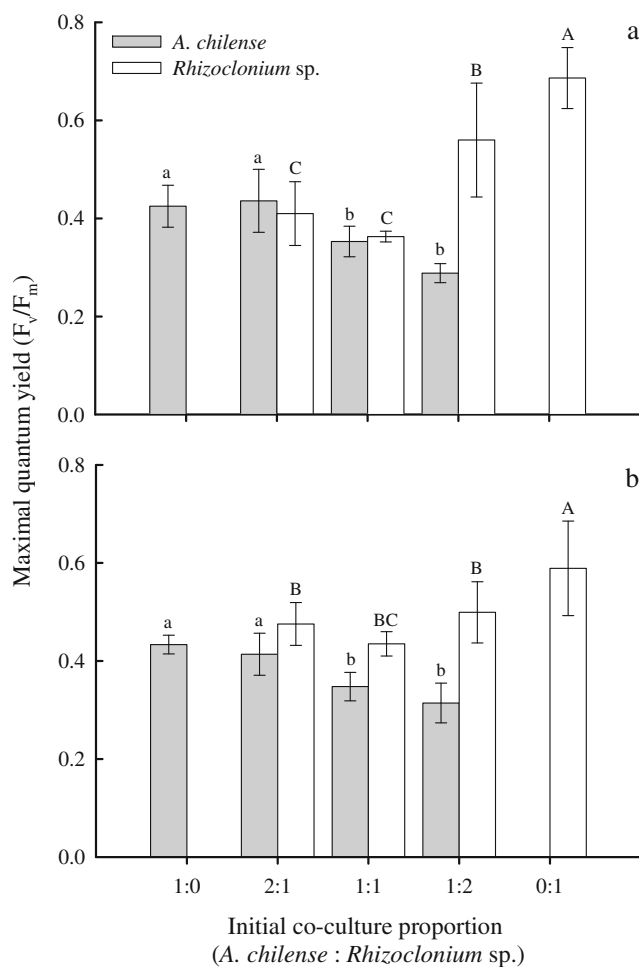
C and N tissue content, and C:N ratio

After 19 days, C tissue content ranged from 29.1 to 48.1% DW and 33.4 to 36.4% DW for *A. chilense* and *Rhizoclonium* sp. (Fig. 6), respectively. C tissue content in both *A. chilense* and *Rhizoclonium* sp. was not significantly different between co-culture proportions nor PAR treatment (Table 5). N tissue content ranged from 1.2 to 2.2% DW and 1.1 to 1.4% DW for *A. chilense* and *Rhizoclonium* sp. (Fig. 7), respectively. N tissue content in both *A. chilense* and *Rhizoclonium* sp. was not significantly different between co-culture proportions nor PAR treatment (Table 5).

C:N ratio ranged from 21.21 to 28.25 and 24.43 to 31.26 for *A. chilense* and *Rhizoclonium* sp. (Fig. 8), respectively. C:N ratio was higher under 90 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ than 180 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ for both species (Fig. 8; Table 5). *Agarophyton chilense* C:N ratio was not

Table 2 Two-way ANOVA and significance values for effects of co-culture proportions on growth rate of *A. chilense* and *Rhizoclonium* sp. under high (180 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) and low (90 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) PAR intensities

Variable	Source of variation	Degree of freedom	Sum of squares	Mean square	F	P
Growth rate of <i>A. chilense</i>	PAR	1	0.123	0.123	2.246	0.144
	Co-culture proportion	4	58.561	14.64	268.232	<0.001
	PAR $\times$ co-culture proportion	4	0.0756	0.0189	0.346	0.845
	Residual	30	1.637	0.0546		
	Total	39	60.396	1.549		
Growth rate of <i>Rhizoclonium</i> sp.	PAR	1	0.0139	0.0139	2.870	0.101
	Co-culture proportion	4	91.341	22.835	4720.912	<0.001
	PAR $\times$ co-culture proportion	4	0.0166	0.00415	0.859	0.500
	Residual	30	0.145	0.00484		
	Total	39	91.516	2.347		

**Fig. 4** Maximal quantum yield (F_v/F_m) of *A. chilense* and *Rhizoclonium* sp. after 19 days of co-cultivation at different initial proportions under two PAR intensities of **a** 180 and **b** 90 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Bars represent mean $\pm$ SD ($n=4$). Significant subgroups (Tukey, $P<0.05$) are defined by lowercase letters for *A. chilense* ($a>b$) and uppercase letters for *Rhizoclonium* sp. ($A>B>C$). Statistical analyses for interspecific differences were not performed (see the “Materials and methods” section for details)

statistically different between co-culture proportions under 180 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Fig. 8a) but was significantly higher at proportions 1:1 and 1:2 compared with 1:0 and 2:1 under 90 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ (Fig. 8b; Table 5). *Rhizoclonium* sp. C:N ratio was significantly higher at proportions 1:1, 1:2 and 0:1 compared with 2:1 under 180 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Fig. 8a), whereas it was significantly higher at proportion 1:1 compared with 2:1, 1:2 and 0:1 under 90 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Fig. 8b; Table 5). Two-way interactions between independent variables did not significantly affect species-specific C:N ratio (Table 5). Ratios of C:N were >20 in both species under all treatment combinations, suggesting nutrient limitation conditions after 19 days of experimentation.

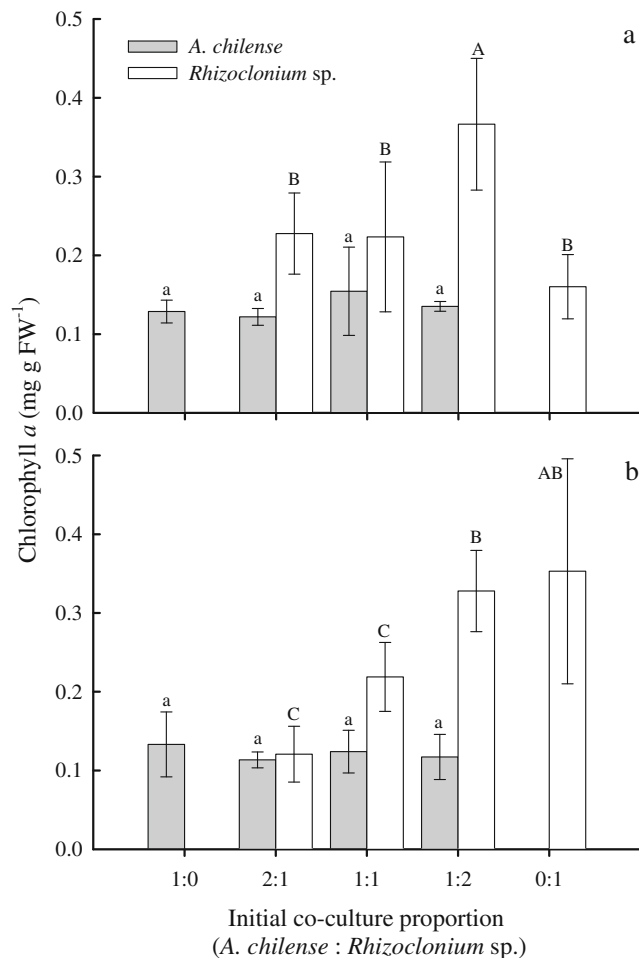
Relative macroalgal yield

After 19 days the relative macroalgal yield was not significantly different between PAR treatments for both species (Fig. 9; Table 6). RY_T was statistically higher than RY_A and RY_R but there were no significant differences between RY_A and RY_R under both PAR treatments (Fig. 9; Table 6). Two-way interactions (relative yield $\times$ co-culture proportion) did significantly affect the relative macroalgal yield (Table 6).

Under 180 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and after 19 days of cultivation, RY_T was close to 1 and not statistically different between initial co-culture proportions (Fig. 9a), indicating a competitive interaction between both species. Under 90 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, a statistically significant difference between RY_R and RY_A was observed at the initial co-culture proportion 1:1, resulting in a $RY_T = 1.34 \pm 0.14$ (Fig. 9b) that indicates a synergistic interaction between *A. chilense* and *Rhizoclonium* sp.

Table 3 Two-way ANOVA and significance values for effects of co-culture proportions on photosynthetic efficiency (F_v/F_m) of *A. chilense* and *Rhizoclonium* sp. under high ($180 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) and low ($90 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) PAR intensities

Variable	Source of variation	Degree of freedom	Sum of squares	Mean square	<i>F</i>	<i>P</i>
F_v/F_m of <i>A. chilense</i>	PAR	1	0.0000182	0.0000182	0.0152	0.903
	Co-culture proportion	4	0.999	0.250	208.052	<0.001
	PAR $\times$ co-culture proportion	4	0.00250	0.000624	0.520	0.721
	Residual	30	0.0360	0.00120		
	Total	39	1.037	0.0266		
F_v/F_m of <i>Rhizoclonium</i> sp.	PAR	1	9.025	9.025	0.346	0.561
	Co-culture proportion	4	4220.188	1055.047	40.462	<0.001
	PAR $\times$ co-culture proportion	4	274.537	68.634	2.632	0.054
	Residual	30	782.250	26.075		
	Total	39	5286.000	135.538		

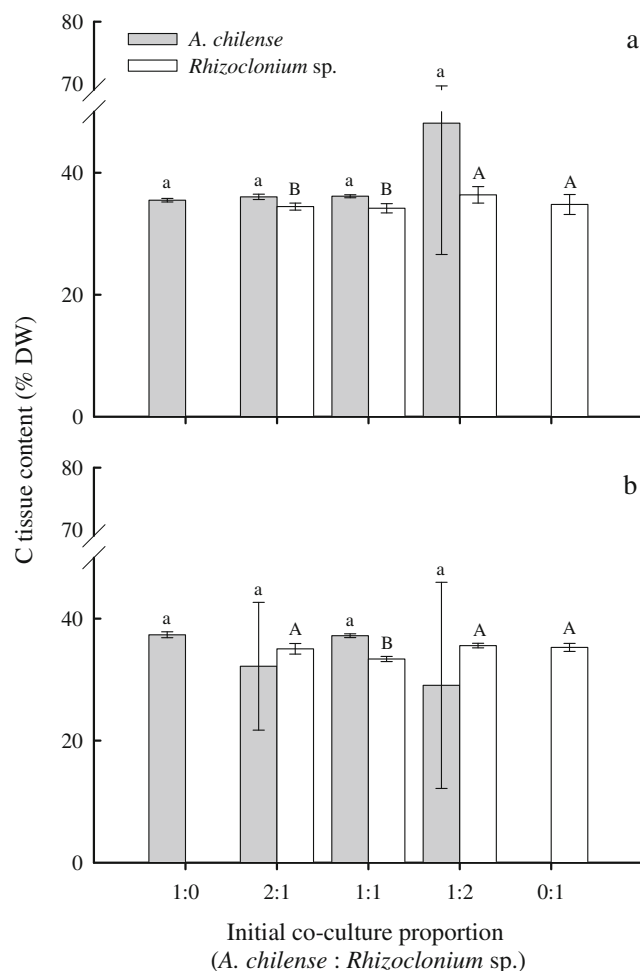
**Fig. 5** Content of chlorophyll *a* of *A. chilense* and *Rhizoclonium* sp. after 19 days of co-cultivation at different initial proportions under two PAR intensities of **a** 180 and **b** 90 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at different co-culture proportions. Bars represent mean $\pm$ SD ($n=4$). Significant subgroups (Tukey, $P<0.05$) are defined by uppercase letters for *Rhizoclonium* sp. ($A>B>C$). Statistical analyses for interspecific differences were not performed (see the “Materials and methods” section for details)

Discussion

Contrary to expectations, the presence of *Rhizoclonium* sp. did not have negative effects on the growth rate of *A. chilense* in co-cultures. In general, the growth rate of *Rhizoclonium* sp. was 3–4 times faster than that of *A. chilense* across experimental treatments, which is consistent with previous findings that show that green macroalgae have greater growth rate than red macroalgae (Friedlander 1992; Buschmann and Gómez 1993; Anderson et al. 1996; Chen et al. 2015). The fast growth of green macroalgae has been attributed to their higher nutrient uptake capacity and photosynthetic rates but lower capacity to store nitrogen compared with red macroalgae (Wallentinus 1984; Anderson et al. 1996; Pedersen and Borum 1997; Tyler and McGlathery 2006; Ale et al. 2011). Thus, it is possible to think that the co-cultured macroalgae used nitrogen in different ways during the experiment. For instance, C:N ratio was mainly modulated by the N content rather than C content, because N content trended to drop in co-cultures, especially in *Rhizoclonium* sp. at the 1:1 co-culture treatment. N varied between 1.2 and 2.2% DW in *A. chilense* and between 1.1 and 1.4% DW in *Rhizoclonium* sp., values that are close to 1% N DW, considered critical to limit growth in macroalgae (Wheeler and North 1981; Gerard 1982). N content in tissue of *A. chilense* and *Gracilaria* spp., has been reported to be $>2\%$ in N-sufficient environments (Abreu et al. 2009; Gorman et al. 2017). Our results may indicate that *A. chilense* might have utilized nitrogen to sustain a stable growth rate and chlorophyll *a* content, whereas *Rhizoclonium* sp. might have used nutrients to support a rapid growth, especially at initial co-culture proportions 2:1 and 1:1, independent of PAR intensities. However, studies on nutrient physiology and growth of both species

Table 4 Two-way ANOVA and significance values for effects of co-culture proportions on chlorophyll *a* of *A. chilense* and *Rhizoclonium* sp. under high (180 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) and low (90 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) PAR intensities

Variable	Source of variation	Degree of freedom	Sum of squares	Mean square	<i>F</i>	<i>P</i>
Chlorophyll <i>a</i> in <i>A. chilense</i>	PAR	1	160.000	160.000	2.054	0.162
	Co-culture proportion	4	2702.250	675.563	8.672	<0.001
	PAR $\times$ co-culture proportion	4	88.750	22.188	0.285	0.885
	Residual	30	2337.000	77.900		
	Total	39	5288.000	135.590		
Chlorophyll <i>a</i> in <i>Rhizoclonium</i> sp.	PAR	1	0.000722	0.000722	0.155	0.697
	Co-culture proportion	4	0.528	0.132	28.318	<0.001
	PAR $\times$ co-culture proportion	4	0.0994	0.0249	5.334	0.002
	Residual	30	0.140	0.00466		
	Total	39	0.768	0.0197		

**Fig. 6** C content in tissue of *A. chilense* and *Rhizoclonium* sp. after 19 days of co-cultivation at different initial proportions under two PAR intensities of **a** 180 and **b** 90 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at different co-culture proportions. Bars represent mean $\pm$ SD ($n=4$). Significant subgroups (Tukey, $P<0.05$) are defined by lowercase letters for *A. chilense* (a > b) and uppercase letters for *Rhizoclonium* sp. (A > B). Statistical analyses for interspecific differences were not performed (see the “Materials and methods” section for details)

(especially on *Rhizoclonium* sp.) still needed to better understand these differential responses in field and laboratory co-cultures.

Physiological parameters suggest that both *A. chilense* and *Rhizoclonium* sp. were stressed in co-culture conditions. In the case of photosynthesis, we observed a reduction of F_v/F_m in both species in co-cultures. The reduction in F_v/F_m indicates photosynthesis stress and photoinhibition in macroalgae (Hanelt 1996). It has been reported that nutrient limitation conditions can also alter the PSII functioning, which is evidenced by a reduction in F_v/F_m values in macro and microalgae (Berges et al. 1996; Gordillo et al. 2003; Fernández et al. 2020), and reduce the content of pigments (Lapointe and Duke 1984; Turpin 1991; Andria et al. 1999). In the present experiment, *Rhizoclonium* sp. under 90 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ showed reduced F_v/F_m and chlorophyll *a* content in co-cultures compared with monocultures, indicating nutrient limitation in co-culture conditions. Another variable that implies stress by nutrient limitation is C:N ratio with values >20 (Wheeler and North 1981; Hurd et al. 2014; Gorman et al. 2017) which occurred in both species across all treatment combinations at the end of our experiment. A possible reason for these results is that the culture medium (200 $\mu\text{M NO}_3^-$ and 20 $\mu\text{M PO}_4^{3-}$ enriched seawater) and/or renewal was not performed regularly enough, likely creating nutrient limitation conditions in co-cultures. However, these hypotheses need to be confirmed by evaluating the effects of sufficient and limiting nutrient availability in the interaction between *A. chilense* and *Rhizoclonium* sp. in co-culture conditions.

Despite the potential constraints of using the de Wit replacement series such as that the outcome depends on the total biomass density (Jolliffe 2000; Williams and McCarthy 2001), it has been proved to be a powerful method for studying the epiphyte-basiphyte interaction (Karez and Chapman 1998; Karez 2003). This method also allows us to obtain more

Table 5 Two-way ANOVA and significance values for effects of co-culture proportions on C and N tissue content, and C:N ratio of *A. chilense* and *Rhizoclonium* sp. under high (180 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) and low (90 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) PAR intensities

Variable	Source of variation	Degree of freedom	Sum of squares	Mean square	<i>F</i>	<i>P</i>
C content in <i>A. chilense</i>	PAR	1	160.240	160.2	1.866	0.182
	Co-culture proportion	4	8585.474	2146.369	24.999	< 0.001
	PAR $\times$ co-culture proportion	4	605.403	151.351	1.763	0.162
	Residual	30	2575.761	85.859		
	Total	39	11,926.878	305.817		
C content in <i>Rhizoclonium</i> sp.	PAR	1	0.225	0.225	0.00502	0.944
	Co-culture proportion	4	3757.188	939.297	20.970	< 0.001
	PAR $\times$ co-culture proportion	4	180.338	45.084	1.007	0.420
	Residual	30	1343.750	44.792		
	Total	39	5281.500	135.423		
N content in <i>A. chilense</i>	PAR	1	260.100	260.100	4.107	0.052
	Co-culture proportion	4	2980.500	745.125	11.765	< 0.001
	PAR $\times$ co-culture proportion	4	147.400	36.850	0.582	0.678
	Residual	30	1900.000	63.333		
	Total	39	5288.000	135.590		
N content in <i>Rhizoclonium</i> sp.	PAR	1	0.0373	0.0373	2.648	0.114
	Co-culture proportion	4	10.660	2.665	189.032	< 0.001
	PAR $\times$ co-culture proportion	4	0.0411	0.0103	0.729	0.579
	Residual	30	0.423	0.0141		
	Total	39	11.161	0.286		
C:N ratio of <i>A. chilense</i>	PAR	1	35.680	35.680	6.725	0.015
	Co-culture proportion	4	2645.021	661.255	124.632	< 0.001
	PAR $\times$ co-culture proportion	4	15.295	3.824	0.721	0.585
	Residual	30	159.170	5.306		
	Total	39	2855.166	73.209		
C:N ratio of <i>Rhizoclonium</i> sp.	PAR	1	11.240	11.240	4.473	0.043
	Co-culture proportion	4	3677.182	919.295	365.817	< 0.001
	PAR $\times$ co-culture proportion	4	9.597	2.399	0.955	0.446
	Residual	30	75.390	2.513		
	Total	39	3773.409	96.754		

information for a better interpretation of experimental results (Stetina et al. 1997). In our research, a $\text{RY}_T \sim 1$ for all initial co-culture proportions indicates an interspecific competition between both species under 180 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. This suggests that both species have similar resource demands, but different resource utilization (De Wit 1960; Vivian et al. 2013). For example, under saturating light, *Rhizoclonium* sp. may have utilized nutrients for supporting a fast growth rate while *A. chilense* might mainly have stored nutrients for a constant growth rate and photosynthesis. While, a $\text{RY}_T > 1$ was observed at the initial co-culture 1:1 under 90 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, meaning the existence of interspecific competition for different resources (De Wit 1960; Vivian et al. 2013). This result was mainly given by a faster growth rate of *Rhizoclonium* sp. in co-culture compared with monoculture. Probably, under non-saturating light, the stress condition

generated by the 1:1 co-culture treatment could have stimulated the growth in the epiphyte but not in the basiphyte, but this idea needs further research to be clarified. In addition, using the replacement series approach confirms that, under the actual experimental conditions, the presence of *Rhizoclonium* sp. did not affect the growth of *A. chilense* under this experiment conditions.

Species of genus *Gracilaria* and *Agarophyton* are the main source of agar production worldwide (Usov 2011; Hayashi et al. 2013; Alemañ et al. 2019). This cell wall polysaccharide is widely used in food, cosmetic and pharmaceutical industries (Buschmann et al. 2001; Shannon and Abu-Ghannam 2019) as well as in biological and medical research (Pangestuti and Kim 2015; Øverland et al. 2019). The properties of agar depend not only on abiotic factors such as season and environmental parameters (Chirapart and Ohno 1993; Marinho-

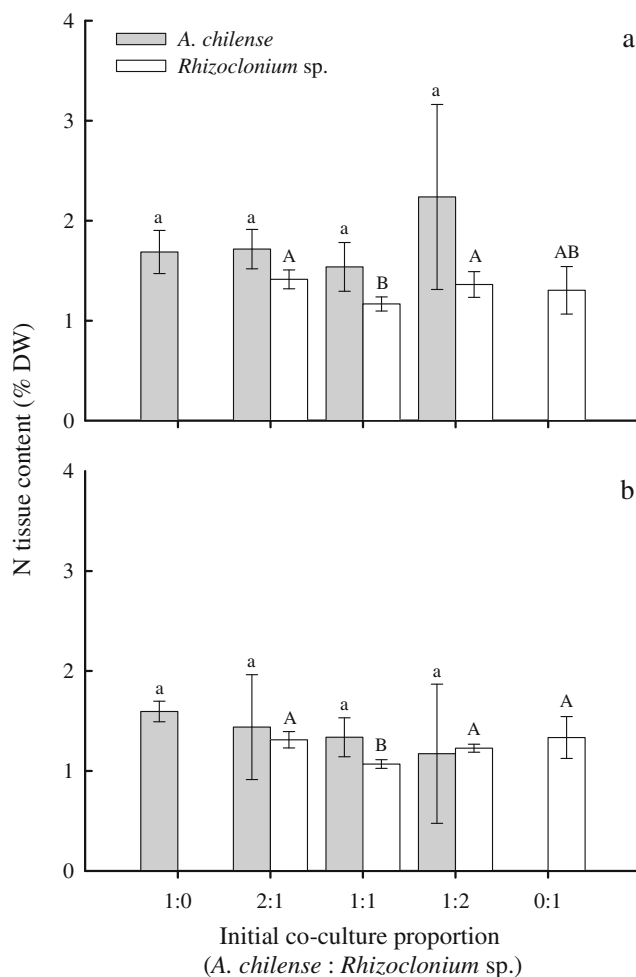


Fig. 7 N content in tissue of *A. chilense* and *Rhizoclonium* sp. after 19 days of co-cultivation at different initial proportions under two PAR intensities of **a** 180 and **b** 90 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at different co-culture proportions. Bars represent mean $\pm$ SD ($n=4$). Significant subgroups (Tukey, $P<0.05$) are defined by lowercase letters for *A. chilense* (a) and uppercase letters for *Rhizoclonium* sp. (A>B). Statistical analyses for interspecific differences were not performed (see the “Materials and methods” section for details)

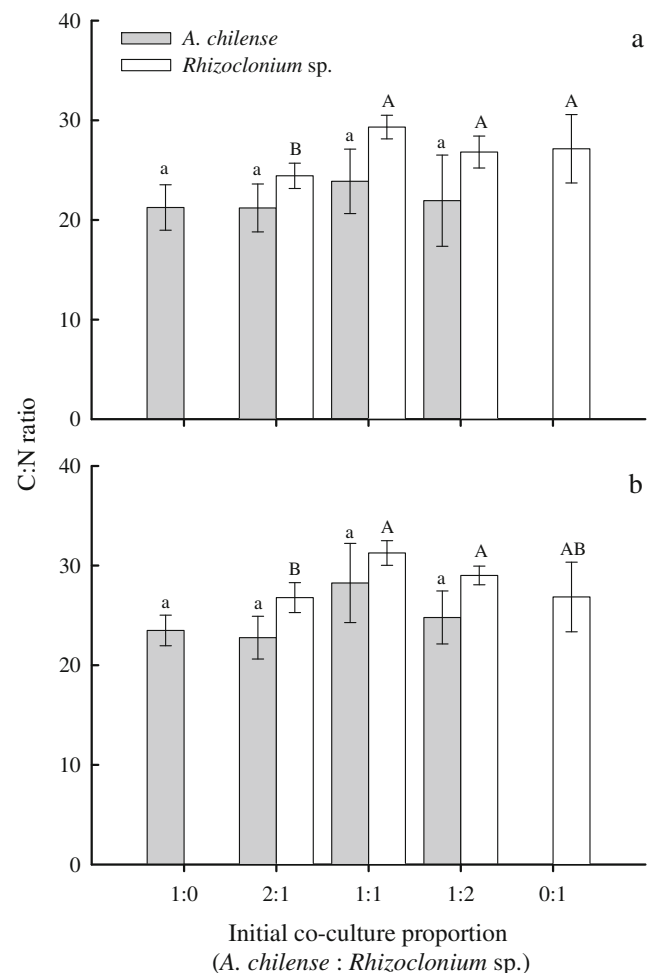


Fig. 8 C:N ratio of *A. chilense* and *Rhizoclonium* sp. after 19 days of co-cultivation at different initial proportions under two PAR intensities of **a** 180 and **b** 90 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at different co-culture proportions. Bars represent mean $\pm$ SD ($n=4$). Significant subgroups (Tukey, $P<0.05$) are defined by lowercase letters for *A. chilense* (a) and uppercase letters for *Rhizoclonium* sp. (A>B). Statistical analyses for interspecific differences were not performed (see the “Materials and methods” section for details)

Soriano et al. 2001; Vergara-Rodarte et al. 2010; Martín et al. 2013b), but also on specie-specific characteristics such as reproductive cycle, tissue age, growth and nitrogen content (Muñoz and Fotedar 2011; Ursi et al. 2013). For example, the yield of agar has been observed to rise with higher light intensity and nutrient availability in *A. chilense* (as *G. sordida* W.A.Nelson) and *Gracilariopsis longissima* (S.G.Gmelin) Steentoft, L.M.Irvine & Farnham (as *G. verrucosa* (Hudson) Papenfuss, nom. rejic.) (Ekman and Pedersén 1990). Thus, the content and yield of agar might be more affected than growth in highly epiphytized *A. chilense* farms although we did not observed effects of the presence of *Rhizoclonium* sp. on the growth of *A. chilense*. Similarly, an experimental decrease of 50% in light availability did not affect the final growth of *A. chilense* (as *G. chilensis*) compared with the epiphytic

effects of *Ulva* sp. (Buschmann and Gómez 1993). Additional experimental studies seem necessary to evaluate changes in agar properties of epiphytized red macroalgae.

Epiphytism has been a major problem for macroalgal aquaculture in southern Chile (Buschmann et al. 2001; Leonardi et al. 2006; Subpesca 2015). In farms of *A. chilense* from Maullín River, southern Chile, the load of *Rhizoclonium* sp. varied from 10 to 85% during spring-summer between 2014 and 2017 (Vidal et al. 2017). Although we used a similar epiphytic load (co-culture treatment 2:1, Table 1) to that observed in farms, no effects of *Rhizoclonium* sp. on the growth of *A. chilense* was observed in co-cultures under any PAR treatment. In the environment, *Rhizoclonium* sp. forms a skein of filaments that floats with the currents. The filamentous skein straightens out when attaches to an *A. chilense* thallus,

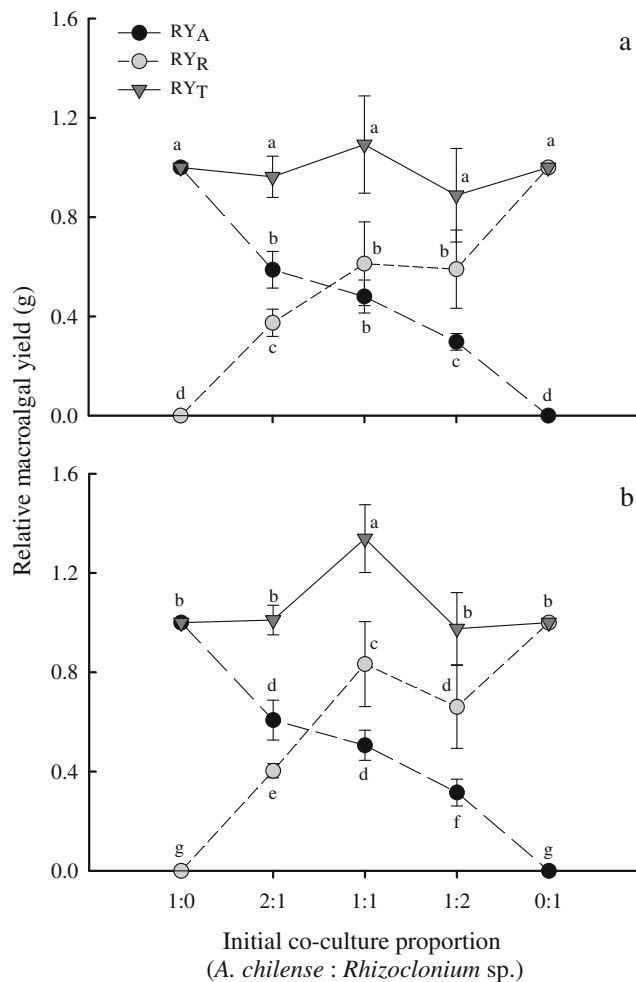


Fig. 9 Relative macroalgal yield of *A. chilense* and *Rhizoclonium* sp. after 19 days of co-cultivation at different initial proportions under two PAR intensities of **a** 180 and **b** 90 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at different co-culture proportions. RY_A , relative yield of *A. chilense*, RY_R , relative yield of *Rhizoclonium* sp. and RY_T , total relative yield. Circles and triangles represent mean $\pm$ SD ($n = 4$). Significant subgroups are grouped by lowercase letters as $a > b > c > d > e > f > g$ (Tukey, $P < 0.05$)

which better its exchange and acquisition of nutrients with the surrounding seawater. The physical contact between *Rhizoclonium* sp. and *A. chilense* may weaken the basiphyte tissue by limiting the availability of light and/or nutrients, making *A. chilense* thallus too weak to withstand strong water movement (Kuschel and Buschmann 1991; Buschmann and Gómez 1993). For example, the biomass of green and brown epiphytes, interacting with wave action at low tide, enhanced the dislodgement and removal of *A. chilense* from sandy substratum in the intertidal zone (Kuschel and Buschmann 1991; Buschmann and Gómez 1993). In our experiment, we did not observe this phenomenon because the water movement produced by bubbling was not strong enough to produce dislodgement of *A. chilense* co-cultured with *Rhizoclonium* sp. Nevertheless, the interaction between epiphytic weight and water movement is a factor to consider for ensuring a positive productivity in the intertidal farms of southern Chile (Buschmann and Gómez 1993).

In conclusion, *Rhizoclonium* sp. did not affect negatively the growth of *A. chilense* and this interaction was not modulated by light availability at any initial co-culture proportion. However, both species showed stress responses (i.e. reduced F_v/F_m and C:N ratio) in co-culture conditions. This result suggests that the epiphyte-basiphyte interaction in co-culture conditions may be regulated by other abiotic factors such as nutrient availability. Therefore, future studies should investigate responses of the epiphytic *Rhizoclonium* sp. to environmental factors for a better understanding of its physiology. Also, a study on the interaction between *A. chilense* and *Rhizoclonium* sp. under different levels of nutrients and other abiotic factors (e.g. temperature) in a replacement series experiment could enlighten the interspecific competition between both species in farms. Finally, it is also important to scale up these investigations (e.g. mesocosm experiment) and carry out some field experiments to determine the factors that may modulate the epiphyte-basiphyte interaction in aquaculture farms.

Table 6 Tree-way ANOVA and significance values for effects of co-culture proportions on the relative macroalgal yield a of *A. chilense* and *Rhizoclonium* sp. under high (180 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) and low (90 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) PAR intensities

Variable	Source of variation	Degree of freedom	Sum of squares	Mean square	F	P
Relative macroalgal yield	PAR treatments	1	448.533	448.533	3.861	0.052
	Relative yield	2	56,275.800	28,137.900	242.243	< 0.001
	Co-culture proportion	4	6467.583	1616.896	13.920	< 0.001
	PAR $\times$ relative yield	2	192.067	96.033	0.827	0.441
	PAR $\times$ co-culture proportion	4	824.717	206.179	1.775	0.141
	Relative yield $\times$ co-culture proportion	8	65,937.117	8242.140	70.958	< 0.001
	PAR $\times$ relative yield $\times$ co-culture proportion	8	322.183	40.273	0.347	0.945
	Residual	90	10,454.000	116.156		
	Total	119	140,922.000	1184.218		

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