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Effects of increased CO_2 levels on growth, photosynthesis, ammonium uptake and cell composition in the macroalga *Hypnea spinella* (Gigartinales, Rhodophyta)

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Abstract The red seaweed *Hypnea spinella* (Gigartinales, Rhodophyta), was cultured at laboratory scale under three different CO_2 conditions, non-enriched air (360 ppm CO_2) and CO_2-enriched air at two final concentrations (750 and 1,600 ppm CO_2), in order to evaluate the influence of increased CO_2 concentrations on growth, photosynthetic capacity, nitrogen removal efficiency, and chemical cellular composition. Average specific growth rates of *H. spinella* treated with 750 and 1,600 ppm CO_2-enriched air increased by 85.6% and 63.2% compared with non-enriched air cultures. CO_2 reduction percentages close to 12% were measured at 750 ppm CO_2 with respect to 5% and 7% for cultures treated with air and 1,600 ppm CO_2, respectively. Maximum photosynthetic rates were enhanced significantly for high CO_2 treatments, showing P_{max} values 1.5-fold higher than that for air-treated cultures. $N–NH_4^+$ consumption rates were also faster for algae growing at 750 and 1,600 ppm CO_2 than that for non-enriched air cultures. As a consequence of these experimental conditions, soluble carbohydrates increased and soluble protein contents decreased in algae treated with CO_2-enriched air. However, internal C and N contents remained constant at the different CO_2 concentrations. No significant differences in data obtained with both elevated CO_2 treatments, under the assayed conditions, indicate that *H. spinella* is saturated at dissolved inorganic carbon concentrations close by twice the actual atmospheric levels. The results show that increased CO_2 concentrations might be considered a key factor in order to improve intensively cultured *H. spinella* production yields and carbon and nitrogen bioremediation efficiencies.

Keywords Ammonium uptake · Increased CO_2 concentration · Culture · *Hypnea spinella* · Photosynthesis · Rhodophyta

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Introduction

The photosynthetic acclimation and response of aquatic photoautotrophs to a predictable CO_2-enriched environment has been a subject of interest in recent years (Bowes 1993; Gao and McKinley 1994; Raven and Falkowski 1999; Collins and Bell 2004; Wu et al. 2008). Such environmental predictions suggest that a doubled atmospheric CO_2 concentration by the mid-end of this twenty-first century will bring a proportional increase of dissolved CO_2 (together with a slight decrease of pH and marginal increases of HCO_3^-) at the ocean surface (Stumm and Morgan 1981; Raven and Falkowski 1999), affecting the photosynthetic control of CO_2 levels (Bowes 1993; Gao et al. 1993). This fact, closely related with the increase of coastal activities which produce important amounts of wastes, including inorganic nutrients (Troell et al. 2003), makes micro- and macroalgae interesting organisms to predict possible impacts (Beardall et al. 1998; Raven and Falkowski 1999), responses (Johnston and Raven 1990; Beer and Koch 1996; Kübler et al. 1999; Andría et al. 2001; Collins and Bell 2004), and remediation processes by considering biomass production through cultivation techniques (Laws and Berning 1991; Gao et al. 1991, 1993; Gao and McKinley 1994; Keffer and Kleinheinz 2002; Doucha et al. 2005; Israel et al. 2005).

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Macroalgae, in particular, have been the object of additional interest for CO_2 remediation (see review by Gao and McKinley 1994) because of their solar energy conversion capacity, high productivity values (higher than most productive terrestrial crops), and the possibility of being, in many cases, intensively cultivated. However, examples exploring the capacity to reduce excessive ambient CO_2 through land-based cultivation of macroalgae are scarce in comparison to data obtained with microalgae cultures (Ho et al. 2011). In a recent attempt, flue gases from a power plant containing 12–15% CO_2 were applied to a long-term tank culture of *Gracilaria cornea*, with additions of N and P, resulting in a significant enhancement of algal growth that prove the ability of CO_2 bioremediation of seaweed cultures (Israel et al. 2005).

In addition, macroalgal cultures are being used as efficient biofilters for nutrient recycling and treatment of fishpond effluents (Neori et al. 2004). The effect of these waste effluents (rich in C, N, and P) has been reported to enhance seaweed growth, and biofilters can thus reduce the overall environmental impact. In these cultivation systems operating under high algal densities, especially when nitrogen and phosphorus are available, the inorganic carbon sources could become limited (Bidwell et al. 1985; Friedlander and Levy 1995); therefore, such highly productive units could be considered potential sinks for CO_2. The longer-term response of photosynthesis and growth to CO_2 enrichment will depend on the availability of mineral nutrients and the way they are used by the plant because the higher growth rates will lead to an increased demand for nutrients, particularly inorganic nitrogen and phosphorus (Stitt and Krapp 1999; Xu et al. 2010). Algae might then use nutrients in a more efficient way, e.g., increasing uptake and assimilation rates, and improving the efficiency of bioremediation of these biofiltration units.

In recent years, interest to produce seaweed species in poly-aquaculture systems together with the possibility to obtain year-round biomass is high. From the considered possibilities, the genus *Hypnea* is an economically interesting group of red macroalgae, with some species, i.e., *Hypnea musciformis*, being reported as cultivable under different conditions (Guist et al. 1982; Ganesan et al. 2006). However, intensive culture of *Hypnea* has not been as successful if compared with other genera like *Gracilaria*, *Chondrus*, or *Ulva*. In our facilities, the species *Hypnea spinella* has been successfully cultivated in tanks using fish pond effluents as nutrients source during the last years, resulting in average annual yields close to 30 g dry weight m^{-2} day^{-1} (109 t DW ha^{-1} $year^{-1}$) and mean ammonium biofiltration efficiencies close to 70% (Gómez-Pinchetti et al. 2002). Biomass produced in these biofilters has shown a high dietary value for the abalone *Haliotis tuberculata coccinea*, a species of potential importance for mariculture in the Canary Islands (Viera et al. 2005).

The effects of CO_2 enrichment on seaweeds are still largely unknown and results on different basic aspects seem to be contradictory, i.e., improved (Gao et al. 1991, 1993; Zou 2005) or decreased (García-Sánchez et al. 1994; Israel and Hophy 2002) growth and photosynthetic rates; enhanced nutrient assimilation (Gao et al. 1993; Gordillo et al. 2001; Zou 2005); or decreased capacity to use HCO_3^- (Johnston and Raven 1990; García-Sánchez et al. 1994; Mercado et al. 1999), all of them affecting cellular components. These different responses, which could reflect species-specific capabilities, might be altered or maintained depending on the habitat (Murru and Sandgren 2004), culture conditions, or duration of acclimation to elevated CO_2 concentrations (Beardall et al. 1998; Andría et al. 2001; Zou 2005; Zou and Gao 2009). Moreover, the affinity of algae for CO_2 is mainly related with the development of carbon concentrating mechanisms (CCMs; see review by Giordano et al. 2005). Those algal species that are believed to be saturated for growth via these CCMs are expected to be insensitive to increased CO_2 concentrations (Beardall et al. 1998; Kübler et al. 1999), and species that exhibit carbon-limited photosynthesis and growth might have a positive response to CO_2 enrichments (Raven 1997; Gordillo et al. 2001).

Considering the controversy regarding the effects of elevated CO_2 on the growth of different seaweed species, the aim of the present study was to evaluate, at a laboratory scale, how the combined effects of increased CO_2 and N–NH_4^+ availability affect growth, photosynthetic capacity, nutrient removal efficiency, and adaptation responses of intensively cultured *H. spinella*. The results provide basic information to be considered in the development of new algal-based cultures and bioremediation of increased CO_2 and inorganic waste nutrients.

Materials and methods

Hypnea spinella (C. Agardh) Kützing (Gigartinales, Rhodophyta) was collected at Hoya del Pozo (27°59′ N, 15°22′ W), east coast of Gran Canaria (Canary Islands, Spain). After collection, algae have been intensively cultivated in tanks at the greenhouse facilities at the Center of Marine Biotechnology (CBM, ULPGC). Individuals were random selected, and 2-cm apical fragments, free from epiphytes, were carefully cut and used for indoor experiments.

Algal fragments, 8 g fresh weight (FW), were cultivated in 1-L flasks, sealed with rubber caps, and fitted with independents inlet and outlet. Air was continuously bubbled into the culture medium, at the bottom of the flask, through a thin glass tube. One liter filtered (0.22 μm) seawater enriched with 140 μM NH_4Cl and 14 μM KH_2PO_4 was used as the culture medium. Nutrient addition was carried out every day, at the start of the light period,

throughout the whole experimental period in order to restore nitrogen and phosphorus sources.

For 16 days, cultures were supplied with three different CO_2 concentration treatments by bubbling with air (360 ppm CO_2), CO_2-enriched air at 750 ppm, and CO_2-enriched air at 1,600 ppm. Controlled air flow rate was 550 mL min^{-1}. Four replicates were set for each treatment.

Light was provided by fluorescent white lamps (Sylvania Daylight F36W, Germany) in a LD 16:8 photoperiod (darkness from 0000 to 0800 A.M.). Irradiance, measured with a quantum spherical PAR sensor (SPQA 2770) connected to a radiometer LI-1000 datalogger (LI-COR Inc.), was 100 μmoL photons m^{-2} s^{-1}. Temperature in the culture chamber was maintained at 23±1°C.

During the first 7 days, cultures were adapted to the described conditions, then harvested and re-stocked to the initial density (8 g L^{-1}). Growth rates, CO_2 reduction percentages, in vivo photosynthesis and chlorophyll fluorescence parameters, and ammonium uptake were measured during the next 9 days.

Daily pH variations from the cultures were continuously measured with an ICP-100 (Gantner Electronic, Germany) data acquisition system.

Biomass increase was measured in each culture to estimate growth. The specific growth rate (SGR) was calculated as the percentage of increase in FW biomass per day according to the exponential equation by D'Elia and DeBoer (1978)

$$\mathrm{SGR}\left(\%\ \mathrm{day}^{-1}\right) = 100\ \ln(W_t/W_0)/t$$

where W_0 is the initial biomass and W_t the biomass weighted at t days. FW was determined after blotting water of the thalli with tissue paper. The dry weight/fresh weight ratio (DW/FW, 0.11 for *H. spinella*) was obtained after drying 10 g samples at 80°C to constant weight. Algae density variations during the experimental period due to growth were considered to normalize the data.

CO_2 levels at the air outlet of each culture flask were daily recorded during a 2-h period (from 1000 to 1200 A.M.) with a non-dispersive infrared gas analyzer, IRGA (EGM-4, PP Systems, UK) that measures CO_2 concentrations with a precision of 1 μatm. A desiccation column filled with anhydrous calcium sulfate (Drierite) was placed before the gas entry into the analyzer to remove humidity from the air and avoid interferences in the measurements. An algal-free flask for each treatment was used as a control to monitor the established CO_2 equilibrium. The CO_2 level at the outflow of the algal-free flask was measured to verify the CO_2 concentration after diffusion into the medium, and it was considered as the CO_2 concentration at the inflow. For the calculation of CO_2 reduction percentages, differences between CO_2 concentrations at the inflow and outflow were used.

Photosynthesis rates and respiration were measured as oxygen evolution and consumption by *H. spinella* thalli (0.15–0.20 g FW). Measurements were carried out, every 2 days during the morning, in an Illuminova Light Dispenser System ('Light Pipette', Illuminova, Sweden) equipped with a light source, an incubation chamber with a micro-oxygen electrode (MI-730, Microelectrodes Inc., USA) inserted, a quantum sensor, and a computer.

Thalli were introduced inside the chamber, and the cuvette was filled with culture medium, directly taken from each corresponding control (algal-free) treatment flask, magnetically stirred, and temperature-controlled at 23±1°C by using a circulator water bath. Five pulses of 2 min were programmed at increasing irradiances of 25, 50, 150, 300, and 600 μmol photons m^{-2} s^{-1} with a 2-min dark period after each light pulse. O_2 saturation (%) was measured and recorded each 2 s. The maximum photosynthetic rate under light-saturated condition (P_{max}), the photosynthetic efficiency calculated as the initial slope under light-limited conditions (α), and the dark respiration rate at the initial 2-min darkness period (R_d) were derived from photosynthesis–irradiance (P–E) curves using the hyperbolic tangent formulation (Jassby and Platt 1976; Henley 1993):

$$P = [P_{max} \times \tanh(\alpha \times E/P_{max})] + R_d$$

Compensation (E_c) and light saturation (E_k) irradiances were also estimated from the data obtained. Photosynthetic rate values were expressed as oxygen evolution per biomass and time units (μmol O_2 g^{-1} DW min^{-1}).

In order to analyze possible stress situations caused by continuously increased CO_2 conditions, in vivo chlorophyll fluorescence of photosystem II (PSII) was daily measured with a plant efficiency analyzer (Hansatech Ltd., UK) operated at 100% of the excitation light (3,500 μmol photons m^{-2} s^{-1}) during a 5-s saturating pulse. Measurements were recorded after algae were dark-adapted for a 10-min period. Basal fluorescence (F_0) and maximum fluorescence (F_m) values were used to calculate the variable fluorescence (F_v=F_m−F_0). The optimal quantum yield of the PSII was calculated as F_v/F_m.

Ammonium concentration measurements were made, immediately after sampling, according to the colorimetric method described by Parsons et al. (1984). The decrease in $N\text{–}NH_4^+$ from the culture medium was determined each 30 min since the medium was renovated (140 μM NH_4Cl) until all the ammonium was depleted. Ammonium uptake rates were calculated for each time interval, and corrections were made for dry biomass and volume changes in the culture medium due to sampling. The equation used was:

$$V = [(S_t - S_{t+\Delta t}) \times \mathrm{vol}]/(\Delta t \times \mathrm{DW})$$

where V is the ammonium uptake rate (μmol N–NH_4^+ g^{-1} DW h^{-1}), t is time (h), Δt is the time interval (h), S_t the initial concentration of NH_4^+ at time t (μM), $S_{t+\Delta t}$ is the NH_4^+ concentration at time $t+\Delta t$ (μM), vol is volume (L), and DW is the dry weight.

Ammonium uptake rates were plotted against the corresponding average substrate concentration, and the kinetics uptake parameters, V_{max} (maximum uptake rate) and K_s (semi-saturation constant), were estimated from the Michaelis–Menten model using a nonlinear least-squares regression. Uptake affinity, V_{max}/K_s, was also calculated. To test significant differences between CO_2 concentration treatments on NH_4^+ uptake, the Michaelis–Menten equation was transformed to the linear equation, $S/V = (1/V_{max})S + (K_m/V_{max})$ for the Hanes–Woolf plot (Dowd and Riggs 1965).

Analytical measurements of freeze-dried biomass were carried out at the end of the experimental period. Protein content was determined by the Kjeldahl method ($N\times6.25$). Total soluble carbohydrates were quantified according to the phenol–sulphuric acid method (Dubois et al. 1956) after samples were digested in 10% (*w*/*v*) trichloroacetic acid for 60 min at 90°C. Total carbon and nitrogen contents and C/N ratios in a molar basis were estimated using a CNH elemental analyzer (2400 Elemental Analyzer, Perkin Elmer).

Data are expressed as mean values with standard deviations ($n\geq3$). Significance of variations on measured parameters was determined by one-way analysis of variance after testing for data normality and homogeneity. Tukey's test was conducted to evaluate statistical differences between treatments. Significance level was 5%. Analyses were performed using Sigma Plot and SPSS software (SPSS Inc., USA).

Results

The SGR of *H. spinella* during the experimental period increased significantly ($p<0.05$) with the increased CO_2 levels (Fig. 1). The growth rates of cultures treated with air (2.8±0.7% per day) were lower than those treated with CO_2-enriched air at 750 and 1,500 ppm (5.2±0.9 and 4.5±0.5% per day). Compared with air-treated cultures, relative enhancements of growth were 85.6% and 63.2% for cultures treated with 750 and 1,600 ppm CO_2, respectively.

Daily pH fluctuations of the medium for the CO_2 treatments assayed are presented in Fig. 2. During darkness periods, increased CO_2 concentrations (750 and 1,600 ppm CO_2) resulted in a higher decrease of pH values in the medium with respect to the air-treated cultures. pH values below 8.0 were measured for cultures treated with CO_2-enriched air at 1,600 ppm. During light periods, photosynthetic activity in all the treatments assayed resulted in pH increases to reach stable constant levels, between 8.2 and 8.3, after an 8-h period. This dynamic was daily observed during the whole experimental period.

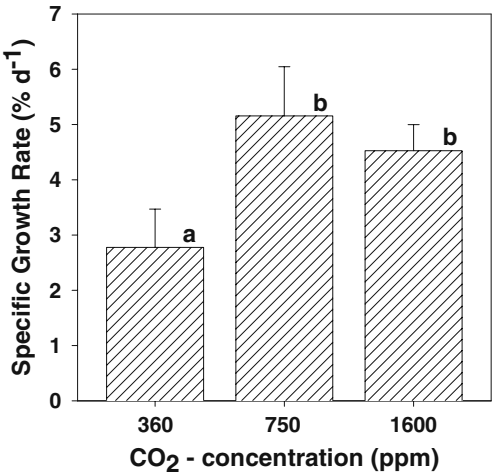

Fig. 1 SGR of *H. spinella* cultures treated with air (360 ppm CO_2) and CO_2-enriched air (750 and 1,600 ppm CO_2). Letters *a* and *b* denote significant differences ($p<0.05$). Data are the mean±SD ($n=4$)

CO_2 reduction percentages and consumption rates by *H. spinella* were affected by increased CO_2 concentration treatments (Table 1). The highest percentage of CO_2 reduction (12%) was measured at the 750-ppm CO_2-enriched cultures compared with those treated with 360 and 1,600 ppm CO_2 that showed reduction percentages of 5% and 7%, respectively.

Photosynthetic rates vs. irradiance curves (*P–E*) for *H. spinella* treated with air (360 ppm CO_2) and CO_2-enriched air (750 and 1,600 ppm CO_2) are shown in Fig. 3. Maximum photosynthetic rates (P_{max}) were significantly higher ($p<0.01$) for both CO_2-enriched treatments with respect to air, reaching

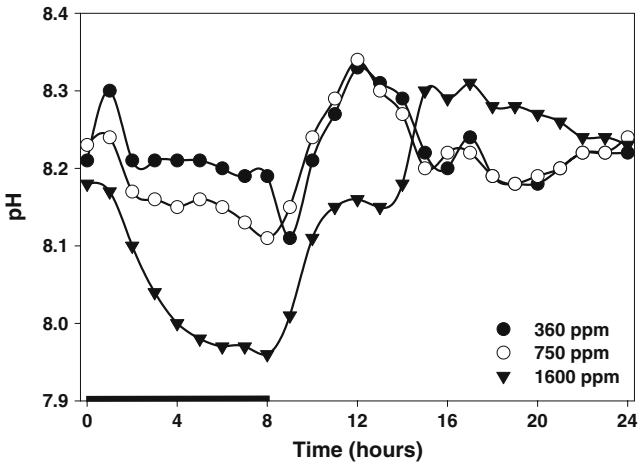

Fig. 2 Daily fluctuations dynamic of pH in the culture medium of *H. spinella* treated with air (360 ppm CO_2) and CO_2-enriched air (750 and 1,600 ppm CO_2). The *black line* indicates the dark period

Table 1 CO_2 concentration and reduction percentages of *H. spinella* cultures treated with air (360 ppm CO_2) and CO_2-enriched air (750 and 1,600 ppm CO_2)

	CO_2 (ppm)		
	360	750	1,600
CO_2 input (ppm)	360±5	750±28	1,643±21
CO_2 output (ppm)	341±2	657±21	1,537±18
Reduction (%)	5	12	7

Measurements were daily recorded during a 2-h period (from 1000 to 1200 A.M.). Data are the mean±SD ($n \geq 30$)

mean P_{max} values of 27.3 and 29.1 μmol O_2 g^{-1} DW min^{-1} compared with 19.1 μmol O_2 g^{-1} DW min^{-1} for air-treated cultures (Table 2). However, no significant differences for respiration (R_d) and photosynthetic efficiency (α) were found for algae grown under the different CO_2 treatments (Table 2). Significant increases ($p<0.05$) for E_c and E_k were observed for algae treated with 1,600 ppm CO_2-enriched air.

Values for daily measured maximum quantum yield (F_v/F_m), which varied from 0.53 for 1,600 ppm CO_2 to 0.56 for 750 and 360 ppm CO_2, were stable during the experimental period and showed no significant differences between treatments (Table 2), indicating the healthy physiological status of algae under the different assayed conditions.

Nonlinear ammonium depletion curves showed similar patterns for the different CO_2 treatments assayed (Fig. 4). A faster N–NH_4^+ consumption from the medium was observed for *H. spinella* treated with 750 ppm CO_2, 1.0 and 0.5 h faster than algae treated with air (360 ppm) and CO_2-enriched air at 1,600 ppm, respectively. Removal of the whole NH_4^+ concentration in air treatments (360 ppm CO_2) lasted for 4.5 h.

Table 2 Photosynthetic parameters estimated from *P–E* curves and chlorophyll fluorescence optimal quantum yield (F_v/F_m) for *H. spinella* treated with air (360 ppm CO_2) and CO_2-enriched air (750 and 1,600 ppm CO_2)

	CO_2 (ppm)		
	360	750	1,600
P_{max}	19.27±2.73a	27.27±1.72 b	29.00±3.82b
R_d	−7.18±2.82a	−6.91±1.09a	−6.55±0.64a
α	0.23±0.09a	0.23±0.09a	0.23±0.09a
I_c	27.7±2.1a	26.0±1.9a	33.0±3.7b
I_k	78.5±13.8a	103.1±2.7a	145.7±19.4b
F_v/F_m	0.56±0.04a	0.56±0.03a	0.53±0.05a

Values followed by different letters denote significant differences ($p<0.05$) within each row. Data are the mean±SD ($n \geq 4$)

P_{max}=light-saturated photosynthetic rate (in μmol O_2 g^{-1} DW min^{-1}); R_d=rate of respiration in darkness (in μmol O_2 g^{-1} DW min^{-1}); α= initial slope at limiting irradiance levels (in μmol O_2 g^{-1} DW min^{-1} (μmol photons m^{-2})$^{-1}$); $E_c=R_d/\alpha$=compensation irradiance (in μmol photons m^{-2} s^{-1}); $E_k=P_{max}/\alpha$=light saturation parameter (in μmol photons m^{-2} s^{-1}); F_v/F_m=optimal quantum yield after 10-min dark adaptation

Data for the NH_4^+ uptake rates as a function of NH_4^+ concentration presented a biphasic pattern with a typical Michaelis–Menten hyperbolic phase at low concentrations (<80 μM, Fig. 5a) and an increasing linear response at concentrations higher than 80 μM (data not shown). Parameters calculated for ammonium uptake rate kinetics are presented in Table 3. Maximum ammonium uptake rates, V_{max}, were enhanced for *H. spinella* treated with CO_2-enriched air (mean values of 60.6 and 58.5 μmol NH_4^+ g^{-1} DW h^{-1} for 750 and 1,600 ppm CO_2) with respect to cultures treated with air (48.8 μmol NH_4^+ g^{-1} DW h^{-1} for

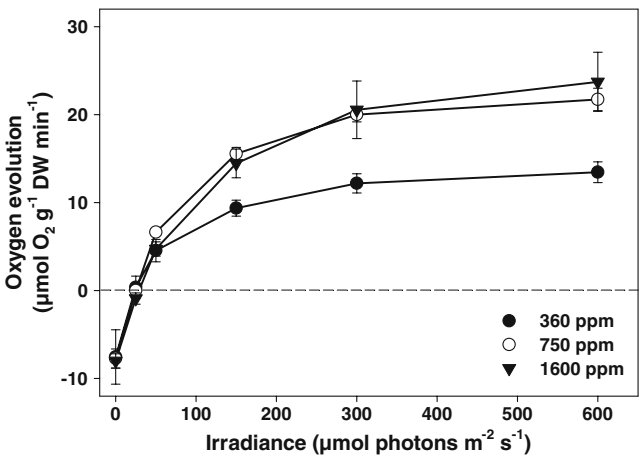

Fig. 3 Photosynthetic oxygen evolution vs. irradiance (*P–E*) curves obtained for *H. spinella* treated with normal air (360 ppm CO_2) and CO_2-enriched air (750 and 1,600 ppm CO_2). Data are the mean±SD ($n \geq 4$)

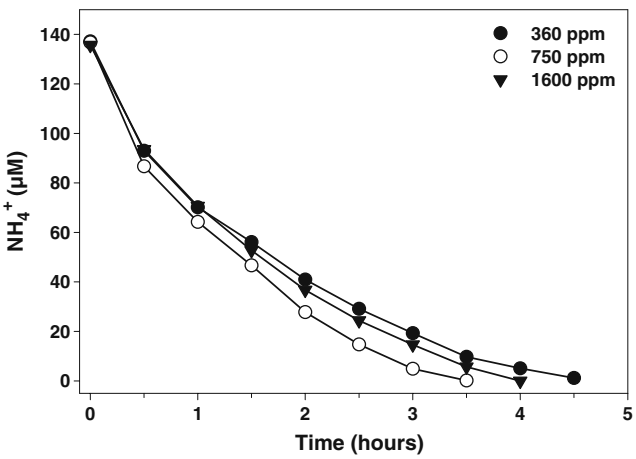

Fig. 4 Time course of ammonium depletion for cultures of *H. spinella* treated with normal air (360 ppm CO_2) and CO_2-enriched air (750 and 1,600 ppm CO_2). Data are the mean±SD ($n=4$). The coefficient of variance was <10%

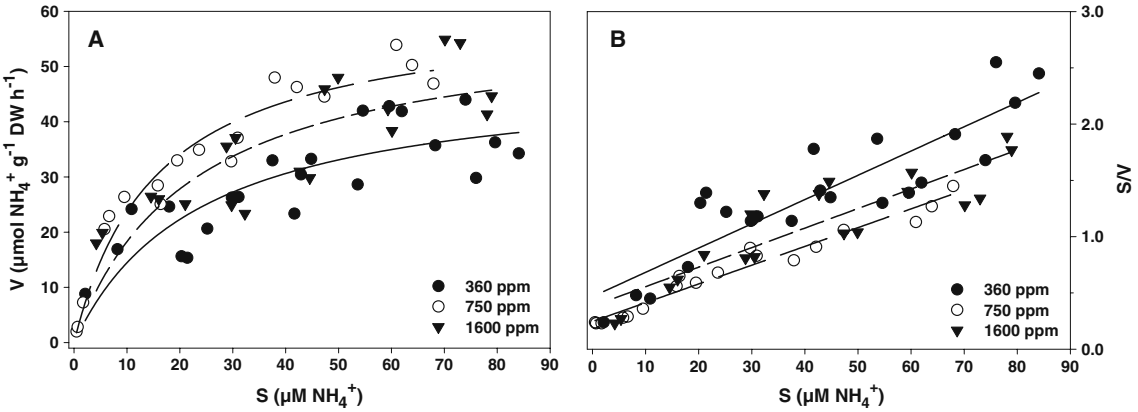

Fig. 5 Ammonium uptake rates for *H. spinella* treated with normal air (360 ppm CO_2) and CO_2-enriched air (750 and 1,600 ppm CO_2). **a** Best fits for the nonlinear Michaelis-Menten curves. **b** Hanes–Woolf linear plots of the same data: 360 ppm CO_2 (*solid dash*), 750 ppm CO_2 (*long dash*), 1,600 ppm CO_2 (*short dash*)

360 ppm CO_2). K_s values were significantly reduced in algae treated with CO_2-enriched air at 750 ppm (15.6 μM) with respect to air (23.7 μM for 360 ppm CO_2) and CO_2-enriched air with 1,600 ppm CO_2 (22.0 μM, Table 3). Hence, the NH_4^+ uptake affinity (V_{max}/K_s) was higher (3.90) for algae treated with CO_2-enriched air at 750 ppm. The linear Hanes–Woolf transformation of the data (Fig. 5b) indicated significant linear correlations of S/V and S for each plot. The results obtained showed significant differences for NH_4^+ uptake between cultures treated with air and cultures treated with CO_2-enriched air ($p < 0.001$). No significant differences were found between both CO_2-enriched air treatments.

The chemical cell composition of *H. spinella* was clearly affected by CO_2 treatments (Table 4). Total soluble carbohydrates were increased significantly in algae treated with CO_2-enriched air at 750 and 1,600 ppm, although no differences were found in the total carbon content. The protein contents decreased slightly in algae grown in CO_2-enriched air, but N content only decreased in 750 ppm CO_2-enriched air. As a result, a higher C/N ratio was measured for *H. spinella* treated with 750 ppm CO_2 (9.5) with respect to air (8.1 for 360 ppm CO_2) and CO_2-enriched air (8.8 for 1,600 ppm CO_2, Table 4). Algae with C/N values lower than 10 were considered nitrogen non-limited.

Discussion

Increased CO_2 concentration levels under culture conditions directly affected responses by *H. spinella* on the different aspects studied including growth, CO_2 removal, photosynthesis, ammonium uptake, and chemical cell composition. Continuous addition of CO_2-enriched air (750 and 1,600 ppm) to the culture medium affected the pH values since CO_2 supplied is dissolved, hydrated forming carbonic acid, and afterwards dissociated to bicarbonate, releasing H^+ ions and causing a decrease of pH (Stumm and Morgan 1981). Consequently, during dark periods, the decrease of pH was directly related to the

Table 3 Parameters of the Michaelis–Menten model (V_{max}, K_s) and uptake affinity (V_{max}/K_s) for the NH_4^+ uptake kinetics of *H. spinella* treated with air (360 ppm CO_2) and CO_2-enriched air (750 and 1,600 ppm CO_2)

	CO_2 (ppm)		
	360	750	1,600
V_{max} (μmol NH_4^+ g^{-1} DW h^{-1})	48.8±6.1a	60.6±3.9b	58.5±8.3b
K_s (μM)	23.7±8.4a	15.6±2.9b	22.0±9.1a
R	0.82	0.98	0.79
V_{max}/K_s	2.06	3.90	2.66

Correlation coefficient values, r, are also presented. Values followed by different letters denote significant differences ($p<0.05$) within each row. Data are the mean±SD ($n=4$)

Table 4 Chemical cell composition of *H. spinella* treated with air (360 ppm CO_2) and CO_2-enriched air (750 and 1,600 ppm CO_2)

	CO_2 (ppm)		
	360	750	1,600
Proteins (%)	20.8±0.8a	17.0±0.3b	19.1±0.8c
Carbohydrates (%)	25.9±1.5a	30.3±0.8b	30.9±1.9b
C/N ratio	8.1±0.3a	9.5±0.3b	8.8±0.4a,b
C (mg g^{-1} DW)	227.9±15.5a	238.9±2.3a	245.3±1.3a
N (mg g^{-1} DW)	32.8±2.0a	29.2±1.0a	32.4±1.5a

Values followed by different letters denote significant differences ($p<0.05$) within each row. Data are the mean±SD ($n=4$)

increase of dissolved CO_2, as it has been also shown for the red macroalga *Gracilaria* (Gao et al. 1993). In cultures aerated with air (360 ppm CO_2), pH variations measured in darkness were basically the result of respiration. However, the most pronounced decrease of pH in cultures supplied with CO_2-enriched air was caused not only by respiration but also by the continuous influx and dissolution of CO_2 into the medium. Therefore, pH increases during light periods, despite this continuous influx of CO_2, probably occurred as a consequence of CO_2 photosynthetic use and demonstrate how CO_2 consumption rates are higher than the dissolution rates into the medium. After an approximate period close to 8 h in light, the pH values became stable and constant for the treatments assayed. Since constant levels of pH indicate a balance between CO_2 dissolution and photosynthetic removal of Ci, *H. spinella* appears to be an effective user of CO_2, like it has been shown to be the general form of inorganic carbon transported across the plasma membrane in other red macroalgae (Smith and Bidwell 1989; Gómez-Pinchetti et al. 1992; Haglund et al. 1992; Israel and Beer 1992). The effects of nutrient uptake ($N–NH_4^+$ and $P–PO_4^{3-}$) on pH changes might be considered negligible compared with those related to Ci utilization (Axelsson 1988; Gao et al. 1991, 1993).

Photosynthesis was also significantly affected by CO_2 enrichment. Maximum photosynthetic rates were found to be higher for *H. spinella* grown with CO_2-enriched air compared with algae treated with air, indicating that photosynthesis in this species is not saturated by Ci at normal atmospheric CO_2 levels. Although P_{max} was increased 1.4-fold in cultures treated with 750 ppm CO_2 with respect to air treatment, no differences were found for cultures grown with 1,600 ppm CO_2 compared with 750 ppm CO_2. This result indicates that under CO_2 levels close by twice the actual atmospheric CO_2 concentration, the photosynthesis of *H. spinella* might be saturated by Ci. In a similar way, growth was markedly higher for *H. spinella* treated with CO_2-enriched air compared with algae grown with air. Hence, the enhanced growth rates obtained with algae cultured with high CO_2 concentrations were due to an increase in the photosynthetic activity associated to a higher Ci availability.

Previous results related to the effect of pH on the photosynthetic capacity by *H. spinella* showed a decrease of P_{max} close to 19% for thalli at pH 8 compared with values obtained at pH 6 (Costas et al. 2004). A decrease in the photosynthetic rate with an increase of pH can be attributed to changes in the availability of the preferred Ci source for photosynthesis (Haglund et al. 1992), indicating that CO_2 is the main Ci source for *H. spinella*, bicarbonate being an alternative source, as has been also described for other Gigartinales (Gómez-Pinchetti et al. 1992). A limited ability to use bicarbonate might be the reason why *H. spinella* exhibits carbon-limited growth and photosynthesis at current CO_2 ambient levels (360 ppm). In addition, seaweeds with efficient bicarbonate uptake systems increase the pH in the surrounding medium up to 9 (Maberly 1990), which is in contrast with maximal pH values, not higher than 8.4, measured for *H. spinella* treated with air (360 ppm CO_2). Although the photosynthesis rates were increased at saturating light with the increase in CO_2 concentrations, photosynthetic efficiencies (α) at low light intensity were not affected by CO_2, as was also observed for constant values of photochemical efficiencies (F_v/F_m) during the whole experimental period, indicating that algae were not physiologically affected by continuous addition of CO_2-enriched air at the concentrations assayed. It has been suggested that low light might limit photosynthesis and then photosynthetic rates would not be enhanced by increases in Ci availability (Maberly 1990; Levavasseur and Edwards 1991; Raven 1997; Mercado and Niell 2000; Zou and Gao 2009). Thus, growth and photosynthetic responses to CO_2 increases would be the result of interactions with other environmental factors such as light, temperature, or nutrient availability, which would be important factors to consider when scaling up *H. spinella*, and similar seaweed species, to intensive culture conditions.

Ammonium uptake rates were increased by high CO_2, indicating that elevated CO_2 enhances nitrogen assimilation, hence improving the growth of *H. spinella* as the result of a combined effect of increased CO_2 photosynthetic assimilation and nutrient uptake. An increase in N uptake is a common response found in macroalgae cultured at high CO_2 levels. Under such growing conditions, *Gracilaria* sp., *Gracilaria chilensis*, *Porphyra leucosticta*, *Ulva rigida*, *Hizia fusiforme*, and *Gracilaria lemaneiformis* showed enhancement of NO_3^- uptake and higher nitrate reductase activity (Gao et al. 1993; Mercado et al. 1999; Gordillo et al. 2001, 2003; Zou 2005; Xu et al. 2010), and this uptake enhancement was suggested to play a major role as more nitrogen is required to support higher growth rates, as it appears to be the case also for *Hypnea*.

From a practical point of view, these results suggest that intensive culture of *H. spinella* operated in biofilters might be enhanced by CO_2 supply to generate higher biomass productivities and better nitrogen biofiltration efficiencies. The use of flue gases for this purpose would also improve the ability of bioremediation of these biofilters, as has already been tested for *Gracilaria cornea* (Israel et al. 2005).

The increase of CO_2 levels in cultures clearly affected the chemical cell composition of *H. spinella* under the assayed conditions. Increases of Ci in high CO_2 treatments resulted in higher soluble carbohydrates, while soluble protein content showed a decrease, particularly in algae grown under 750 ppm CO_2. The total internal pools of C and N were not affected by CO_2. This fact has also been described for other

seaweeds grown at elevated CO_2 levels (García-Sánchez et al. 1994; Kübler et al. 1999; Mercado et al. 1999) which showed a decrease in phycobiliprotein, soluble protein, or Rubisco contents under CO_2-enriched conditions, although internal N contents were not significantly affected by the Ci levels.

The increase of soluble carbohydrates has been previously reported in seaweeds grown under high CO_2 levels. Gordillo et al. (2001) indicated that the increase of soluble carbohydrates in *U. rigida* occurred only under a high CO_2 level and N limitation, and concluded that it was related to N availability. However, an increase of soluble carbohydrates in *Gracilaria* sp. grown under high CO_2 was not clearly related with N availability, suggesting that the exposure and acclimation to high CO_2 would involve resource reallocation (Andria et al. 1999).

The decrease in soluble protein occurred at high CO_2 levels in spite of constant N cellular content, which might be explained if carbon assimilation through photosynthesis was coupled also to a higher demand for nitrogen, producing changes in the distribution of N between proteins and other nitrogen-containing compounds (Stitt and Krapp 1999). The slight decrease of soluble protein suggests that the demand for N exceeds its availability. In this study, ammonium was supplied daily in order to avoid N limitation, but its rapid consumption from the medium, while Ci is continuously available, would cause a greater demand for N. The effects of increased N availability should be assayed for a better understanding of the changes in biomass composition, for example by the cultivation of *H. spinella* in biofilters units where nitrogen is provided continuously through a regulated open flow.

Apart from this fact, identical cellular C and N contents in cultures grown with and without CO_2-enriched air show interactive effects between C and N metabolism. The results indicate that under no C and N limitation, pathways related to C fixation and N assimilation are highly coordinated and coupled (Huppe and Turpin 1994), resulting in no significant variations in basic cellular composition.

It can be concluded that when cultured at a laboratory scale under the combined effects of increased CO_2 concentrations (two and four times ambient CO_2) and ammonium availability, *H. spinella* shows positive responses as an effective user of CO_2, increasing growth, photosynthesis, and ammonium uptake rates. However, no significant differences were found for data obtained with both elevated CO_2 treatments, clearly indicating that *H. spinella* is CO_2-saturated at concentrations close by twice the actual atmospheric levels. From a practical point of view, the results can be used for intensive cultivation practices where environmental factors, such as light intensity or nutrient supply, could be controlled. Under such conditions, increased CO_2 concentrations, i.e., provided by flue gases, might be considered a key factor in order to improve production yields and C and N bioremediation efficiencies.

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