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Acclimation Responses of *Gracilaria* sp. (Rhodophyta) and *Enteromorpha intestinalis* (Chlorophyta) to Changes in the External Inorganic Carbon Concentration

J. R. Andría*, F. G. Brun, J. L. Pérez-Lloréns and J. J. Vergara

Departamento de Ecología, Facultad de Ciencias del Mar, Universidad de Cádiz, 11510 Puerto Real, Cádiz, Spain

* Corresponding author

The acclimation responses of two intertidal macroalgae, *Gracilaria* sp. and *Enteromorpha intestinalis*, to different dissolved inorganic carbon (DIC) levels were investigated under laboratory conditions. The effect of DIC availability on growth rate, biochemical composition (C, N, pigments and Rubisco) and on the degree of inhibition of external and total carbonic anhydrase activities (by acetazolamide and 6-ethoxyzolamide, respectively), and of a putative HCO_3^- exchanger protein (sensitive to the inhibitor 4,4′-diisothiocyanatostilbene-2,2′-disulfonate, DIDS) was species-specific. Pigment and Rubisco contents co-varied negatively with DIC availability in *Gracilaria* sp. However, no such pattern was observed in *Enteromorpha intestinalis*. The mechanisms of DIC uptake were also modulated by the external DIC concentration. Under limiting DIC conditions, the induction of mechanisms for CO_2 acquisition above the diffusive rate was observed in *Gracilaria* sp., while a repression of the DIDS-sensitive mechanism was obtained for *Enteromorpha intestinalis*. The results revealed the plasticity of these intertidal macroalgae to acclimate to different ambient DIC levels, and indicate the important role of DIC as a factor controlling biochemical and physiological processes.

Introduction

Macroalgae thriving in intertidal shallow waters in temperate latitudes are daily exposed to high temperatures and irradiance levels, which often results in increased photosynthesis and growth rates, particularly when the nutrient inputs (N and P) are high. Under such conditions, low dissolved inorganic carbon (DIC), especially CO_2, concentrations could be an important factor controlling photosynthesis and growth, since high pH values adjacent to the photosynthesizing thalli are achieved (Beer and Israel 1990). It is likely that most of the species occurring in such fluctuating habitats have developed adaptive strategies to overcome these potentially limiting DIC conditions.

Several studies have been focused on the physiological responses of marine macroalgae to changing ambient DIC availability (Axelsson *et al.* 1989 a, b, García-Sánchez *et al.* 1994, Rivers and Peckol 1995). Although biochemical responses and adaptation mechanisms for carbon acquisition were regarded mainly as unrelated topics, from the above studies it can be concluded that both aspects should be considered when studying the effect of changing DIC availability on the algal physiology.

Gracilaria sp. and *Enteromorpha intestinalis* are the most abundant macroalgae in a temperate eutrophic salt-marsh of Cádiz Bay (Southern Spain), being daily subjected to abrupt changes of temperature, pH, irradiance, DIC and nutrient levels, especially during low tide (Andría *et al.*, unpublished data). In such a fluctuating system, the ability to take up nutrients at low levels as well as a longer-term storage capacity could give competitive advantages to *Gracilaria* sp. compared with fast-growing opportunistic species such as *Enteromorpha intestinalis*, especially when transient nutrient shortages occur. Evidence on such competitive advantages has been widely reported for N uptake (Rosenberg and Ramus 1982, Fujita 1985, Anderson *et al.* 1996), whereas, as far as we are aware, no study has been carried out on the selective ability of these two species to take up DIC. The aim of this work was to gain some insight into what specific physiological responses (tested under laboratory conditions) can determine competitive advantages in *Gracilaria* sp. and *Enteromorpha intestinalis* when growing under different DIC conditions. It was carried out by checking the effect of DIC availability on: 1) growth, 2) biochemical composition and 3) carbon acquisition mechanisms in these two co-occurring macroalgae with different resource use and growth strategies.

Materials and Methods

Plant material

Gracilaria sp. and *Enteromorpha intestinalis* (L.) Nees were collected in the tidal shallow creeks of Los Toruños salt marsh (Cádiz Bay, Southern Spain) during spring and summer. Individuals of *Gracilaria* sp. har-

vested from these populations were initially identified as *Gracilaria gaditana* nom. prov. (M. Steentoft, personal communication), although ongoing hybridization experiments have revealed them taxonomically closer to *Gracilaria gracilis* (Stackhouse) Steentoft, Irving *et* Farnham (C. Destombe, personal communication). Healthy thalli were cleaned of epiphytes, cut in fragments and maintained for 24 h in the laboratory in aerated 20 L aquaria with filtered natural sea water (NSW, 35 psu) at room temperature and under daylight prior to the experiments.

Acclimation to different DIC levels

Algae were cultured for 4 days in clear cylindrical Plexiglass chambers (8 cm diameter, 24 cm high) filled with 1 L of buffered filtered NSW at different dissolved inorganic carbon (DIC) levels: low (0.15 mM), normal (2.13 mM) and high (4.11 mM). A biological buffer (Tris, 25 mM) was used to maintain a stable pH value (8.2) and a constant proportion of the different inorganic carbon (C_i) species during photosynthesis. This raised the question of a possible reduction in the DIC concentration of the culture media due to formation of carbamates, which was considered unlikely under our experimental conditions (homogeneous media without precipitation of carbonates; cf. March 1992). Low DIC culture medium was prepared by bubbling NSW with air previously passed through a freshly prepared 6 N KOH solution for 12 h. High DIC medium was obtained by enrichment with $NaHCO_3$ up to a final concentration approximately twice the DIC level of NSW. The media were enriched with 40 μM NO_3^- and 2 μM $H_2PO_4^-$ and renewed every 48 h to prevent nutrient limitation. Prior to its use (0 and 48 h), the DIC concentration of each freshly prepared culture medium was measured using a Shimadzu TOC 5050 analyzer by the high-temperature catalytic oxidation method (Chen and Wangersky 1996), since the use of a pH buffer did not allow estimation of DIC by titration.

The cylinders were placed into a transparent aquaria filled with deionized water and connected to a water reservoir provided with a cooler and a heater system to achieve a nearly constant temperature (19 ± 1 °C). The initial biomass density was about 1.2 g fresh weight (FW) per litre in each cylinder. Plants were grown at a constant photon flux density (PFD) of 230 μmol photons $m^{-2}\,s^{-1}$ (LiCor LI-193SA) supplied by cool fluorescent tubes (Philips TL20W/54 RS) and the photoperiod was set at a 12:12, light:dark cycle.

During the experiments, the culture media were agitated by gently bubbling CO_2-free air from the bottom to avoid changes in DIC level due to CO_2 diffusion from air to culture medium. The air had previously been silica-gel dried and then passed through a flask containing NaOH pellets.

Measurements of O_2 evolution rates

Measurements of net photosynthesis rates (NPS) as a function of DIC concentration (photosynthesis-DIC curves) were carried out by a Clark-type O_2 electrode using DIC-free artificial sea water (as in Andría *et al.* 1999 a). The range of DIC concentrations (0 to 3.5 mM) was achieved by injecting different volumes of a 20 mM $NaHCO_3$ solution (freshly prepared, also dissolving NaCl in distilled water up to a final salinity of 35 psu to avoid osmotic changes) into the incubation chamber once a zero net O_2 exchange rate was achieved. Triplicate measurements were carried out at a saturating PFD of 396 μmol photons $m^{-2}\,s^{-1}$ (cosine collector, LiCor LI-192SA) under control conditions, as well as in the presence of inhibitors of DIC uptake mechanisms. Data were fitted to the Michaelis-Menten curve by an iterative procedure and to the Hanes-Woolf equation, and apparent kinetic parameters such as maximum photosynthesis rate (NPS_{max}) and half-saturation constant ($K_{1/2}$) were calculated. In addition, photosynthetic conductance based on DIC concentration (g_p) was calculated from the initial slope of the curves using the equation:

$$g_p\ (m\ s^{-1}) = (NPS \cdot FW{:}S) / (PQ \cdot [DIC]), \qquad (1)$$

where NPS is the net photosynthesis rate expressed as μmol $O_2\ g^{-1}$ FW s^{-1}, FW:S is the ratio between fresh weight and surface area (g FW m^{-2}), PQ is the photosynthetic quotient and [DIC] is the concentration of DIC (μmol m^{-3}).

Inhibitors and carbonic anhydrase assays

Inhibitors of the carbonic anhydrase (CA) activity with varying capacity to penetrate into the cell, acetazolamide (AZ) and 6-ethoxyzolamide (EZ), as well as the inhibitor of the direct HCO_3^-uptake via an anion exchanger, 4,4′-diisothiocyanatostilbene-2,2′-disulfonate (DIDS), were applied (Sigma-Aldrich Química). It is generally assumed that AZ does not penetrate the cell wall and therefore only acts on surface-accessible CA, while EZ penetrates into the cell inhibiting external and internal CA. Stock solutions of AZ and EZ were prepared in 0.05 N NaOH at a concentration of 50 mM, the final concentration applied in the assays being 100 μM. Stock solution of DIDS (50 mM) was prepared in Milli-Q deionized water and the concentration used for the assays was 300 μM.

To assess whether the concentration of the different inhibitors was enough to saturate the inhibition of photosynthesis in *Gracilaria* sp. and *Enteromorpha intestinalis*, triplicate measurements of the percentage of inhibition as a function of the inhibitor concentration (10−5000 μM AZ, 50−3600 μM DIDS) were carried out at a saturating PFD of 396 μmol photons $m^{-2}\ s^{-1}$. The rates of inhibition did not show significant differences with the inhibitor concentration for either macroalgae, being sufficient to saturate the inhibition at ten and six times lower con-

centration than those used in our experiments for AZ and DIDS, respectively (data not shown).

The external CA activity was measured by the potentiometric method based on the time required for a drop of 0.4 units in the pH range from 8.1 to 7.1 at 0−5 °C for non-enzymatic (buffer) and enzymatic (thallus fragments) reactions (as in Haglund and Pedersen 1992). The external CA activity values were expressed as relative activity units on a fresh weight basis (REA g^{-1} FW).

Estimation of inorganic carbon requirement and maximum diffusive CO_2 entry

Net mean requirement of C_i for *Gracilaria* sp. and *Enteromorpha intestinalis* subjected to different DIC treatments, was estimated by using the equation:

$$[\mu\text{mol } C_i\ m^{-2}\ s^{-1}] = [(C_{int}) \cdot (DW{:}FW) \cdot (FW{:}S) \cdot \mu] / [AW_C \cdot P], \quad (2)$$

where C_{int} is the internal C content expressed as mass of C per unit dry weight (DW) basis (μg C g^{-1} DW), DW:FW is the ratio between dry and fresh weight, FW:S is the ratio between fresh weight and surface area (g FW m^{-2}), μ is the relative growth rate expressed as s^{-1}, AW_C is the atomic weight of C, and P is the photoperiod (12 h of light per day; 0.5).

The maximum diffusive CO_2 entry was calculated using the equation

$$J = D/\delta \cdot (CO_{2\ bulk} - CO_{2\ in}), \quad (3)$$

where J is the diffusive CO_2 flux (μmol CO_2 m^{-2} s^{-1}), D is the CO_2 diffusion coefficient ($1.5 \cdot 10^{-9}$ m^2 s^{-1}, Raven 1991), δ is the unstirred boundary layer thickness (μm), and $CO_{2\ bulk}$ and $CO_{2\ in}$ are the CO_2 concentrations (μM) in the bulk phase and the inner side of the plasmalemma, respectively. The external CO_2 concentration in sea water ($CO_{2\ bulk}$) for a given salinity, temperature and pH was calculated according to Mehrbach *et al.* (1973), taking into consideration the apparent dissociation constants for carbonic acid in sea water (Dickson and Millero 1987). To maximize the theoretical CO_2 diffusive gradient around the cell, a value of $CO_{2\ in} = 0$ was assumed. An unstirred-layer of 100 μm thickness, close to the value given by Israel and Beer (1992) for *Gracilaria conferta* (Schousboë *ex* Montagne) Montagne under stirred conditions, was used for *Gracilaria* sp. For *Enteromorpha intestinalis*, a value of 150 μm was assumed according to Koch (1993). Theoretical O_2 production was calculated using a photosynthetic quotient of 1.07 for *Gracilaria* sp. (Andría *et al.* 1999 a) and of 1.17 for *Enteromorpha intestinalis* (Axelsson 1988).

Growth estimation

Biomass was measured during the experiments to estimate growth. The mean relative growth rate (μ), expressed as % d^{-1}, was calculated according to the exponential model

$$\mu = [\ln(w_2/w_1)/(t_2 - t_1)] \cdot 100, \quad (4)$$

where w_2 and w_1 are FW at the times t_2 and t_1.

Analytical methods

The analytical measurements were performed on thallus fragments harvested at the end of the experiments. Quadruplicate samples (0.1 g FW) per treatment were frozen in liquid nitrogen and maintained at −80 °C until analyses. *Gracilaria* sp. samples were ground in phosphate buffer 0.1 M pH 6.5 at 4 °C, extracted overnight and centrifuged at 19000 g for 25 min (SIGMA Laborzentrifugen GmbH 2K15, Germany). The content of the phycobiliproteins (PBP), r-phycoerythrin (RPE) and r-phycocyanin (RPC), were determined spectrophotometrically (Unicam UV/Vis Spectrometer UV2) from the supernatant fraction using the chromatic equations of Beer and Eshel (1985). The pellet fraction was resuspended in 90 % (v:v) acetone, extracted overnight at 4 °C and spun down at 19000 g for 25 min. Chlorophyll *a* (Chl *a*) concentration was determined spectrophotometrically according to Talling and Driver (1963).

Thalli of *Enteromorpha intestinalis* were also sampled to determine the lipo-soluble pigment content by grinding in 90 % (v:v) acetone, extracting overnight at 4 °C and filtering. The Chl *a* concentration was determined according to Jeffrey and Humphrey (1975).

Parallel samples from both macroalgae were oven-dried at 60 °C for 48 h to determine total C and N content (Perkin-Elmer 240-C elemental autoanalyzer).

For Rubisco determination, quadruplicate samples were powdered in liquid nitrogen and sonicated for two cycles of 10−20 s (MICROSON ultrasonic cell disruptor) in 1 mL of ice-cold TCA-acetone (10 % w:v). Proteins were precipitated for 1 h at −20 °C, and centrifuged at 20,000 g for 5 min. Pellets were washed in 0.5 mL of ice-cold 100 % acetone and air dried. This procedure was repeated twice to extract proteins completely. Protein concentration was determined using the bicinchoninic acid protein assay (Smith *et al.* 1985) with bovine serum albumin (BSA) as standard (Pierce, Rockford, IL, U. S. A.). The assay was run on a Bio-Tek EL304 i microplate reader using DeltaSOFT3 software for Macintosh. Samples were prepared for electrophoresis following Greene *et al.* (1991), subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 14 % resolving gel, 6 % stacking gel) according to Laemmli (1970), and loaded on an equal protein basis (as in Andría *et al.* 1999 b). Different dilutions of partially purified Rubisco from spinach (R-8000; Sigma) were used as standards during the electrophoretic measurements. Gels were scanned (AGFA studio star desktop colour scanner), and the area and mean intensity of the bands,

identified as Rubisco large subunits (by size), were quantified by an image analysis program (NIH Image 1.55). Relative units of Rubisco were calculated as a percentage of the values obtained for samples subjected to normal DIC conditions.

Statistics

The results are expressed as the mean value ± SE. Statistical analyses (one and two-way ANOVAs, and post-hoc least significant difference (LSD) test) were applied to check the significance of the results ($p < 0.05$).

Results

Characterization of mechanisms of DIC acquisition in *Enteromorpha intestinalis*

As the mechanisms of DIC acquisition in *Gracilaria* sp. have been recently described in an earlier work (as *Gracilaria gaditana* nom. prov. in Andría *et al.* 1999 a), our research was initially focused on the characterization of the pathways for C_i utilization in *Enteromorpha intestinalis*.

As a first approach, photosynthesis-DIC curves in *E. intestinalis* were measured. Photosynthesis followed a saturation kinetic with a maximal capacity (NPS_{max}) of 107.4 ± 3.5 μmol O_2 g^{-1} FW h^{-1}, and a half-saturation constant ($K_{1/2}$) of 329.6 ± 42.9 μM DIC (Fig. 1, control). This monophasic pattern was further confirmed by the Hanes-Woolf plot (data not shown). The effect of inhibitors (AZ and DIDS) on photosynthesis-DIC curves was also checked (Fig. 1). A saturation curve with lower NPS_{max} and $K_{1/2}$ than the control was recorded (64.8 ± 2.2 μmol O_2 g^{-1} FW h^{-1} and 118.1 ± 23.1 μM DIC, respectively) following AZ addition. In contrast, DIDS reduced the photosynthetic affinity for DIC ($K_{1/2}$ increased up to 1182.4 ± 328.6 μM DIC), but no significant changes in NPS_{max} (125.1 ± 14.9 μmol O_2 g^{-1} FW h^{-1}) were recorded. Inhibition caused by DIDS was higher at DIC concentrations below 1 mM, decreasing down to a nearly zero value at higher DIC levels (Fig. 1, inset). The addition of AZ resulted in higher inhibition rates than DIDS at high DIC concentrations, but a stimulation of the photosynthesis, instead of inhibition, was obtained when AZ was applied at low DIC levels (Fig. 1, inset).

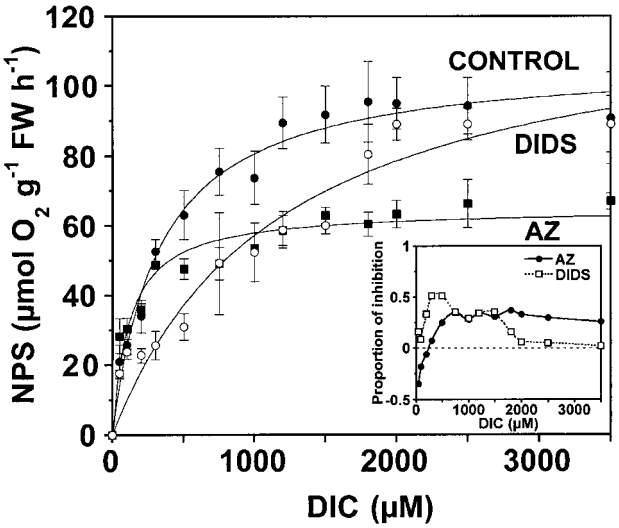

Fig. 1. Net photosynthetic O_2 evolution (NPS) as a function of dissolved inorganic carbon (DIC) concentration in *Enteromorpha intestinalis*. The photosynthetic response was tested either in the presence of the anion exchanger protein inhibitor (DIDS, 300 μM) or of acetazolamide (AZ, 100 μM), an inhibitor of external CA. The figure inset shows the proportion of inhibition in the presence of AZ and DIDS, expressed as $[(NPS_{control} - NPS_{inhib})/NPS_{control}]$, as a function of DIC concentration. Data are presented as mean ± SE (n = 3).

The presence of extracellular CA activity in *E. intestinalis* was further investigated by using the potentiometric method, which provided a value of 2.68 ± 0.21 REA g^{-1} FW.

Biochemical responses to different DIC treatments

Relative growth rates, net mean C_i requirements and biochemical composition were affected after culturing *Gracilaria* sp. and *Enteromorpha intestinalis* at different DIC levels. Relative growth rate was significantly affected by DIC level in *Gracilaria* sp., with values increasing as DIC level rose (Table I). In contrast, growth rate was not significantly affected by DIC treatments in *Enteromorpha intestinalis* (Table II). Overall, mean growth rates were higher in *Gracilaria* sp. than in *Enteromorpha intestinalis*, although a higher variance was achieved for the latter.

No significant differences in tissue nutrient contents (C and N) were obtained for *Gracilaria* sp. (Table I). In contrast, lower C and higher N contents were recorded when *Enteromorpha intestinalis* was cultured under low DIC conditions (Table II).

The net mean C_i requirement (calculated from growth rate and internal C content data) for both macroalgae decreased when grown at low DIC levels. The C_i requirement for *Gracilaria* sp. increased with external DIC concentration, but no differences were observed in *Enteromorpha intestinalis* when grown at normal or high DIC levels (Tables I and II).

Pigment content was also affected by DIC level. In *Gracilaria* sp., RPE content decreased significantly under low and high DIC conditions (this decline being more pronounced at high DIC level), while no significant differences in RPC among treatments were observed (Table I). A significant increase in Chl *a* was recorded at low DIC level for both species. In addition, the high DIC treatment also promoted a significant decrease in Chl *a* in *Gracilaria* sp. (Tables I and II).

Different levels of DIC did not affect significantly the total protein content for either macroalgae (Tables I and II). Relative units of Rubisco (scaled to

Table I. Relative growth rates (μ), net mean inorganic carbon requirement and biochemical composition of *Gracilaria* sp. cultured at different dissolved inorganic carbon (DIC) levels for 4 days.

Variable	Low DIC	Normal DIC	High DIC	F-values
Relative growth rate: μ (% d^{-1})	1.48 ± 0.56[a]	5.19 ± 0.38[b]	6.20 ± 0.04[c]	41.25**
Internal C content (% DW)	27.11 ± 0.55	28.15 ± 0.62	28.27 ± 0.37	ns
Net mean C_i requirement (μmol C_i m^{-2} s^{-1})	0.35	1.28	1.53	–
Internal N content (% DW)	3.29 ± 0.12	3.09 ± 0.04	2.89 ± 0.12	ns
C:N atomic ratio	9.67 ± 0.47[a]	10.63 ± 0.29[a]	11.48 ± 0.46[b]	4.79*
R-phycoerytrin (mg g^{-1} DW)	6.52 ± 0.38[a]	8.10 ± 0.32[b]	5.10 ± 0.57[c]	11.74**
R-phycocyanin (mg g^{-1} DW)	1.16 ± 0.11	1.22 ± 0.01	0.89 ± 0.10	ns
Chlorophyll *a* (mg g^{-1} DW)	0.99 ± 0.07[a]	0.76 ± 0.03[b]	0.54 ± 0.02[c]	20.81***
Total protein (mg g^{-1} DW)	46.11 ± 3.01	48.21 ± 2.96	51.21 ± 4.25	ns
Relative Units of Rubisco per protein	149.19 ± 13.17[a]	99.86 ± 1.68[b]	63.97 ± 3.30[c]	29.35***
Relative Units of Rubisco per g DW	143.26 ± 17.85[a]	100.00 ± 6.85[b]	68.52 ± 8.33[b]	9.71**

Data are presented as mean ± SE. F-values of one-way ANOVA with replication of growth rates (n = 2) and biochemical variables (n = 4) are presented in the last column. Significance levels are *** = $p < 0.001$; ** = $p < 0.01$; * = $p < 0.05$; ns = no significant difference ($p > 0.05$). Different superscript letters for significant differences at 5% confidence level (LSD test).

Table II. Relative growth rates (μ), net mean inorganic carbon requirement and biochemical composition of *Enteromorpha intestinalis* subjected to different dissolved inorganic carbon (DIC) levels for 4 days.

Variable	Low DIC	Normal DIC	High DIC	F-values
Relative growth rate: μ (% d^{-1})	1.50 ± 1.50	4.80 ± 0.12	4.72 ± 1.24	ns
Internal C content (% DW)	26.60 ± 0.31[a]	30.04 ± 0.24[b]	30.39 ± 0.36[b]	45.91***
Net mean C_i requirement (μmol C_i m^{-2} s^{-1})	0.04	0.15	0.15	–
Internal N content (% DW)	3.13 ± 0.05[a]	2.58 ± 0.05[b]	2.45 ± 0.05[b]	51.75***
C:N atomic ratio	9.93 ± 0.18[a]	13.62 ± 0.37[b]	14.48 ± 0.37[b]	35.54***
Chlorophyll *a* (mg g^{-1} DW)	5.44 ± 0.25[a]	2.97 ± 0.23[b]	2.60 ± 0.14[b]	54.15***
Total protein (mg g^{-1} DW)	26.85 ± 2.43	34.71 ± 2.60	33.59 ± 0.74	ns
Relative Units of Rubisco per protein	105.00 ± 25.24[a]	100.45 ± 3.06[a]	22.12 ± 1.99[b]	10.01**
Relative Units of Rubisco per g DW	86.27 ± 28.73[a]	100.00 ± 4.86[a]	21.33 ± 1.51[b]	6.22*

Data are presented as mean ± SE. F-values of one-way ANOVA with replication of growth rates (n = 2) and biochemical variables (n = 4) are presented in the last column. Significance levels are *** = $p < 0.001$; ** = $p < 0.01$; * = $p < 0.05$; ns = no significant difference ($p > 0.05$). Different superscript letters for significant differences at 5% confidence level (LSD test).

total protein and DW) were also determined. For both species, relative units of Rubisco per protein significantly decreased at high DIC levels, especially in *Enteromorpha intestinalis* (Tables I and II). Rubisco content significantly increased at low DIC concentration when compared with normal DIC in *Gracilaria* sp., but not in *Enteromorpha intestinalis*.

Kinetic parameters for carbon acquisition in different DIC treatments

Changes in the apparent kinetic parameters for carbon acquisition after culturing the algae at different DIC levels were obtained (Fig. 2, Table III). Dual kinetics were recorded for *Gracilaria* sp. (Fig. 2 a), with a high affinity phase at low DIC concentrations (below 0.75 mM DIC) and a low affinity phase at higher DIC levels (as in Andría *et al.* 1999 a,b). The DIC treatments did not affect significantly NPS_{max} values for the high affinity phase, but a substantial increase in $K_{1/2}$ was recorded for the high DIC-grown algae. In the low affinity phase, a significant decrease in NPS_{max} and $K_{1/2}$ was recorded for algae previously grown at low DIC level. However, under high DIC conditions, NPS_{max} was higher than that at normal DIC level, whereas $K_{1/2}$ displayed a similar value (Fig. 2 a, Table III).

In *Enteromorpha intestinalis*, $K_{1/2}$ increased significantly after culturing at a high DIC level. Significant differences were obtained for NPS_{max} and $K_{1/2}$ values when comparing low and high DIC treatments (Fig. 2 b, Table III).

Different levels of DIC did not affect photosynthetic conductance (g_p) in *Gracilaria* sp., whilst a decrease was recorded for *Enteromorpha intestinalis* cultured at a high DIC level.

Response to inhibitors of carbon uptake

The effect of the inhibitors on DIC acquisition was checked by photosynthesis (NPS) measurements at

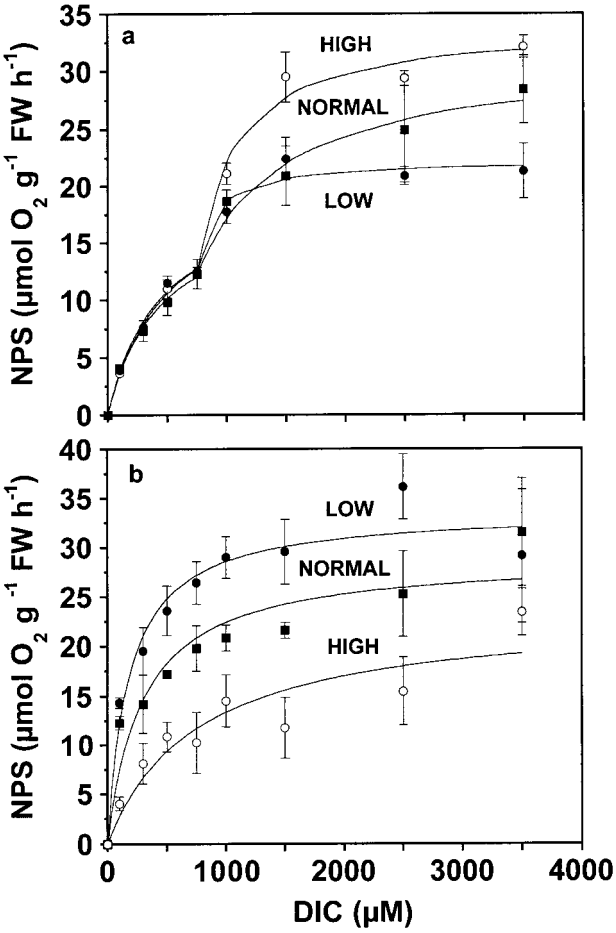

Fig. 2. Net photosynthetic O_2 evolution (NPS) as a function of dissolved inorganic carbon (DIC) concentration for *Gracilaria* sp. (a) and *Enteromorpha intestinalis* (b) subjected to low (0.15 mM), normal (2.13 mM) and high (4.11 mM) DIC levels for 4 days. Data are presented as mean ± SE (n = 3).

saturating DIC (2.2 mM DIC) after culturing algae at different DIC levels (Figs 3 and 4).

A significant decrease in the NPS of the controls (no inhibitors added), measured at 2.2 mM DIC, was recorded after growing *Gracilaria* sp. at a low DIC level (Fig. 3, inset table). High DIC concentration increased significantly the inhibition caused by AZ and EZ (Fig. 3). Exposure to EZ caused a higher inhibition than AZ in algae grown at normal and high DIC, whereas no differences were observed at low DIC levels. The remaining NPS measured following EZ addition was higher than that solely supported by diffusive CO_2 entry regardless of the DIC treatment, especially for low DIC grown algae. No significant differences following DIDS addition were obtained, but a constitutive percentage of inhibition of about 35% was measured regardless of the DIC treatment.

In contrast to *Gracilaria* sp., a significant increase in NPS of the controls was obtained at 2.2 mM DIC after growing *Enteromorpha intestinalis* at low DIC level (Fig. 4, inset table). No significant differences among DIC treatments were recorded when AZ was applied, although a slightly higher inhibition was obtained for algae previously grown at low DIC concentrations. The inhibition caused by EZ and DIDS significantly increased when algae were cultured under DIC-enriched conditions (normal and high DIC treatments). The remaining NPS measured following EZ addition were higher than that solely supported by diffusive CO_2 entry for algae cultured at low DIC concentration. However, a reduction of photosynthesis below this theoretical level was obtained for algae subjected to normal and high DIC treatments.

Discussion

These results, together with others recently obtained (Andría *et al.* 1999 a,b), suggest that *Gracilaria* sp. and *Enteromorpha intestinalis* have developed specific physiological strategies in response to DIC availability. The first evidence arose from the characterization of the mechanisms of DIC acquisition. The development of mechanisms for use of ionic DIC as an alternative source for photosynthesis in addition to diffusive CO_2 entry has been described as a major response to changes in the ambient DIC levels both in red (Cook *et al.* 1986, Smith and Bidwell 1987, 1989, Mercado *et al.* 1997) and green macroalgae (Drechsler and Beer 1991, Björk *et al.* 1992, 1993, Axelsson *et al.* 1995). Our results indicate the presence of two HCO_3^--utilizing mechanisms in *E. intestinalis* from Cádiz Bay: an extracellular CA-mediated mechanism with a high maximal capacity but a low DIC affinity, and a direct HCO_3^- transport (via a DIDS-sensitive mechanism) with comparatively lower maximal capacity and higher DIC affinity. Similar findings were obtained by Larsson *et al.* (1997) for the same species collected from a Swedish rockpool. Despite the apparent existence of the same two HCO_3^- utilization mechanisms in *E. intestinalis* and *Gracilaria* sp. (cf. Andría *et al.* 1999 a), the shape of the photosynthesis-DIC curves was different. While a simple saturation curve was obtained for *Enteromorpha intestinalis*, a biphasic kinetic pattern was recorded for *Gracilaria* sp. (Andría *et al.* 1999 a), denoting different features for DIC acquisition between these two algae.

The cultivation of *Gracilaria* sp. and *Enteromorpha intestinalis* at different DIC levels (under saturating light conditions) also caused changes in biochemical composition. The acclimation of *Gracilaria* sp. to limited and enriched DIC conditions resulted in changes of pigments and Rubisco levels which were fairly coupled and coordinated. It suggests a regulation of the photosynthesis process at two levels: light absorption and carbon fixation. External DIC concentration also modulated the growth rates, inorganic carbon requirements and apparent kinetic parameters for carbon acquisition. Thus, a substantial reduction of growth and of maximal photosynthetic capacity was obtained under DIC limitation. This has been previously reported for other *Gracilaria* species (in *G.*

Table III. Kinetic parameters from photosynthesis-dissolved inorganic carbon (DIC) curves for *Gracilaria* sp. and *Enteromorpha intestinalis* subjected to different DIC levels for 4 days.

Kinetic parameters	*Gracilaria* sp.			
	High affinity phase			
	Low DIC	Normal DIC	High DIC	F-values
NPS_{max} (μmol O_2 g^{-1} FW h^{-1})	20.0 ± 2.5	18.9 ± 2.0	20.8 ± 1.6	ns
$K_{1/2}$ (μM DIC)	427.8 ± 23.2[a]	432.2 ± 19.7[a]	483.3 ± 14.5[b]	7.54*
r^2	0.992	0.994	0.998	–
g_p (10^{-3} m s^{-1})	4.6	4.9	5.3	–
	Low affinity phase			
NPS_{max} (μmol O_2 g^{-1} FW h^{-1})	22.8 ± 1.2[a]	29.2 ± 1.5[b]	35.3 ± 1.8[c]	50.74***
$K_{1/2}$ (μM DIC)	869.5 ± 38.2[a]	1176.8 ± 50.7[b]	1208.9 ± 47.4[b]	49.00***
r^2	0.878	0.960	0.970	–
g_p (10^{-3} m s^{-1})	2.7	2.3	2.8	–
	Enteromorpha intestinalis			
	Low DIC	Normal DIC	High DIC	F-values
NPS_{max} (μmol O_2 g^{-1} FW h^{-1})	33.7 ± 1.8[a]	29.0 ± 2.6[ab]	23.4 ± 3.9[b]	9.49*
$K_{1/2}$ (μM DIC)	179.4 ± 26.9[a]	292.3 ± 54.5[a]	748.2 ± 135.4[b]	37.05***
r^2	0.954	0.996	0.868	–
g_p (10^{-3} m s^{-1})	2.4	1.2	0.5	–

Data were calculated by fitting the Michaelis-Menten equation applying an iterative procedure and taking into account the two phases of the dual kinetic pattern of the photosynthesis-DIC curves in *Gracilaria* sp. (high- and low affinity phases). F-values of one-way ANOVA with replication (n = 3) are presented in the last column. Significance levels are *** = $p < 0.001$; ** = $p < 0.01$; * = $p < 0.05$; ns = no significant difference ($p > 0.05$). Different superscript letters for significant differences at 5% confidence level (LSD test).

secundata Harv. f. *pseudoflagellifera* May by Lignell and Pedersen 1989, and in *G. tenuistipitata* Zhang *et* Xia by García-Sánchez *et al*. 1994) and was attributed to the lack of carbon in the medium, since an increase in such variables was obtained under replete DIC conditions. However, in *Enteromorpha intestinalis*, the changes in the pigment and Rubisco levels were not coupled. Thus, Chl *a* content increased under DIC limitation (compared to normal DIC conditions) and the Rubisco levels were unaffected. It could reflect a more pronounced effect of DIC limitation on light absorption processes than on carbon fixation pathways. The opposite pattern was obtained when comparing high DIC with normal DIC conditions. The growth rate, internal C and N contents and Chl *a* concentration were unaffected by an enriched DIC-growth regime, contrasting with the sharp decrease in Rubisco content and in the apparent DIC affinity. These results agreed with those obtained by Björk *et al*. (1993), where the growth rate of *Ulva* sp. was unaffected by an increase in CO_2 levels of the medium (up to 5% CO_2) compared to air-CO_2 grown algae (normal DIC conditions), although a substantial decrease in DIC affinity was obtained. In this context, the reduction of the photosynthetic DIC affinity under high DIC levels has been widely described (Johnston and Raven 1990, Mercado *et al*. 1997), being often related to changes in the external and internal CA activities (Haglund and Pedersen 1992, Björk *et al*. 1993, García-Sánchez *et al*. 1994).

The results from the inhibitor assays also show a certain effect of the DIC availability on the regulation of the C_i acquisition mechanisms in *Gracilaria* sp. and *Enteromorpha intestinalis*. Data from *Gracilaria* sp. grown under limiting DIC conditions showed a CO_2 use at a higher rate than its diffusive entry. Following EZ addition (external and internal CA activities inhibited) the remaining NPS was much higher than that solely supported by CO_2 diffusion. Since the potential contribution of the direct HCO_3^-transport (DIDS-sensitive mechanism) to photosynthesis would require the transformation of HCO_3^- to CO_2 by intracellular CA activity prior to carbon fixation by Rubisco, it can be considered that this NPS was supported by an enhanced CO_2 entry. Two possibilities could be considered: direct CO_2 transport (described for microalgae such as *Chlamydomonas reinhardtii* Dangeard by Sültemeyer *et al*. 1989) and facilitated CO_2 entry in acidic regions of the cell membrane (Lucas 1983, Price and Badger 1985, Björk *et al*. 1992, Axelsson *et al*. 2000), both

mechanisms may or may not be associated with the external CA activity. In this context, a mechanism

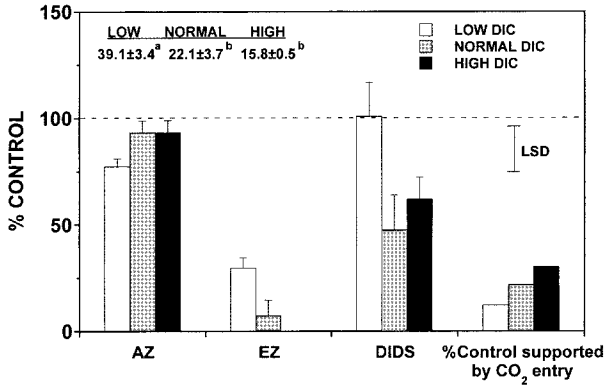

Fig. 3. Net photosynthetic O_2 evolution (NPS) of the controls (expressed as μmol O_2 g^{-1} FW h^{-1}; inset table) and rates (expressed as percentage of the controls) remaining after addition of inhibitors of CA (acetazolamide, AZ, and 6-ethoxyzolamide, EZ, 100 μM), and of anion-exchange protein (DIDS, 300 μM) in *Gracilaria* sp. cultured at different dissolved inorganic carbon (DIC) levels for 4 days. Percentages of NPS of the controls solely supported by CO_2 entry were calculated assuming a diffusive model (diffusion coefficient of $1.5 \cdot 10^{-9}$ m^2 s^{-1}, Raven 1991), using an unstirred boundary layer thickness of 100 μm (Israel and Beer 1992) and a photosynthetic quotient of 1.07 (Andría *et al.* 1999 a). Data are presented as mean ± SE (n = 3). Vertical bars indicate least significant differences (LSD). Different superscript letters for significant differences at 5% confidence level (LSD test).

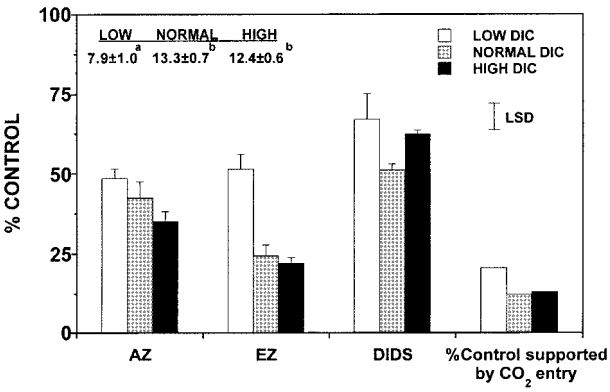

Fig. 4. Net photosynthetic O_2 evolution (NPS) of the controls (expressed as μmol O_2 g^{-1} FW h^{-1}; inset table) and rates (expressed as percentage of the controls) remaining after addition of inhibitors of CA (acetazolamide, AZ, and 6-ethoxyzolamide, EZ, 100 μM), and of anion-exchange protein (DIDS, 300 μM) in *Enteromorpha intestinalis* cultured at different dissolved inorganic carbon (DIC) levels for 4 days. Percentages of NPS of the controls solely supported by CO_2 entry were calculated assuming a diffusive model (diffusion coefficient of $1.5 \cdot 10^{-9}$ m^2 s^{-1}, Raven 1991), using an unstirred boundary layer thickness of 150 μm (Koch 1993) and a photosynthetic quotient of 1.17 (Axelsson 1988). Data are presented as mean ± SE (n = 3). Vertical bars indicate least significant differences (LSD). Different superscript letters for significant differences at 5% confidence level (LSD test).

based on H^+ excretion improving the overall capacity of the external CA-dependent HCO_3^- utilization has been recently proposed for *Laminaria saccharina* (L.) Lamouroux (Axelsson *et al.* 2000), with such a mechanism being inhibited by proton buffers (competing with HCO_3^- for H^+). If such H^+ extrusion operates in *Gracilaria* sp. facilitating the CO_2 entry, the presence of a proton buffer (Tris) in the culture medium would affect carbon acquisition and photosynthetic performance, especially when growing under DIC limitation. Thus, we cannot rule out that the enhanced CO_2 entry displayed by *Gracilaria* sp. under DIC limitation is due to the operation of a mechanism with similar features to that proposed for *Laminaria saccharina*. At this point, current studies are in progress in our group to discern between the two possibilities mentioned above, as well as to study the effect of pH buffers on DIC acquisition in *Gracilaria* sp. Algae grown at high DIC level displayed a higher inhibition by EZ, with photosynthetic O_2 production decreasing to a level close to that supported by diffusive CO_2 entry. It is suggested that the induction of the mechanisms described above was mainly found in those algae previously maintained at limiting DIC conditions.

Limitation of DIC reduced the effect of DIDS on the photosynthesis rate of *Enteromorpha intestinalis* when measured at normal DIC conditions. According to Axelsson *et al.* (1991) and Larsson (1998), the contribution of the direct HCO_3^- transport to photosynthesis of green macroalgae is pH-dependent, displaying a minimum at pH values of the seawater around 8.2−8.4. It would imply a lower contribution of this mechanism to photosynthetic DIC acquisition by *E. intestinalis* under our experimental conditions (pH 8.2), especially for low DIC grown algae. In fact, the inhibitor assays showed a slight increase in the external CA activity (a more efficient carbon uptake mechanism at pH 8.2) for these algae. Addition of EZ resulted in a sharp reduction of photosynthesis in plants grown under replete DIC conditions, whereas under DIC limitation, the NPS values remained slightly higher than those theoretically achieved by CO_2 diffusion (probably also indicating an active CO_2 use).

Our results suggest a species-specific response to changes in DIC availability which could be related to the differential distribution of *Gracilaria* sp. and *Enteromorpha intestinalis* in their habitat. While *Gracilaria* sp. mainly occurs subtidally in shallow pools, *Enteromorpha intestinalis* occurs in mixed populations with *Gracilaria* sp., but is restricted to the uppermost locations, being able to withstand long periods exposed to air. Under submerged conditions (only these were tested in this work), *Gracilaria* sp. appears to acclimate more efficiently to strong DIC limitation (0.15 mM) than *Enteromorpha intestinalis*, which was indicated by a coordinated regulation of the photosynthesis process at the levels of light ab-

sorption and carbon fixation, as well as by the induction of mechanisms for an enhanced CO_2 uptake. However, *E. intestinalis* could have certain competitive advantages for the light utilization and carbon acquisition (directly taking up HCO_3^- formed from atmospheric CO_2 by CA-catalyzed hydration in the surrounding water layer) under emerged conditions, which would help to explain the differential location of these two co-occurring macroalgae in the tidal creeks.

In conclusion, a species-specific response to variation in DIC availability was observed in *Gracilaria* sp. and *Enteromorpha intestinalis*, pointing out the relevance of ambient DIC level as a factor controlling proccesses at biochemical and physiological levels, and probably playing an important role in the location of these species in their habitat. The results also reflected the capacity of these intertidal algae to rapidly respond to different DIC conditions.

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