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Mechanisms of inorganic carbon acquisition in *Gracilaria gaditana* nom. prov. (Rhodophyta)

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Abstract. The mechanisms for acquisition of dissolved inorganic carbon (DIC) in the red macroalga *Gracilaria gaditana* nom. prov. have been investigated. The capacity for HCO_3^- use by an extracellular carbonic anhydrase (CA; EC 4.2.1.1), and by an anion exchanger with similar properties to that of red blood cells (AE1), has been quantified. It was illustrated by comparing O_2 evolution rates with those theoretically supported by CO_2, as well as by photosynthesis-pH curves. Both external and internal CA, and a direct uptake were involved in HCO_3^- use, since photosynthesis and pH evolution were affected by acetazolamide, 6-ethoxyzolamide (inhibitors of external and total CA, respectively) and 4,4′-diisothiocyanatostilbene-2,2′-disulfonate, (DIDS; an inhibitor of HCO_3^- exchanger protein). The activity of the external CA was detected by a potentiometric method and by an alternative method based on the study of O_2 evolution after addition of CO_2 and acetazolamide. The latter method showed a residual photosynthetic rate due to direct HCO_3^- use. Inhibitors caused a reduction in the pH compensation points in pH-drift experiments. The CO_2 compensation points for photosynthesis increased when the inhibitors were applied, indicating a suppression of the pathways involved in the carbon-concentrating mechanism. The net photosynthesis rates as a function of DIC concentration displayed a biphasic pattern that could be supported by the occurrence of the two mechanisms of HCO_3^- use. The potential contribution to HCO_3^- acquisition by the DIDS-sensitive mechanism was higher after culturing at a high pH. Our results suggest that the HCO_3^- use by *Gracilaria gaditana* is carried out by the two DIC uptake mechanisms. These operate simultaneously with different affinities for DIC, the indirect HCO_3^- use by an external CA activity being the main pathway. The presence of a carbon-concentrating mechanism confers eco-physiological advantages in a fluctuating ecosystem subjected daily to high pHs and low DIC concentrations.

Key words: Anion exchanger – Carbonic anhydrase – *Gracilaria* – Inorganic carbon uptake – pH-drift – Photosynthesis

Introduction

Carbon is a fundamental resource for photosynthesis. The total amount of dissolved inorganic carbon (DIC) in water includes CO_2, HCO_3^- and CO_3^{2-}. It is stated that CO_2 can freely cross artificial membranes with similar properties to the plasmalemma or chloroplast envelope by diffusion (Gutknecht et al. 1977), although Sültemeyer and Rinast (1996) found a higher CO_2 resistance by using biological membranes. In natural sea water, at normal pH (8.0–8.2) and 20 °C, the concentration of HCO_3^- is about 2 mM while that of CO_2 is only about 10 μM. Since the half-saturation constant (K_s) of ribulose-1, 5-bisphosphate carboxylase-oxygenase for CO_2 ranges from 30 μM to 60 μM in marine macroalgae (Kerby and Raven 1985; Cook and Colman 1987), it seems likely that those macrophytes able to use HCO_3^- would possess advantages compared to those relying solely on diffusive CO_2 entry. In a theoretical analysis based on organism size, Raven (1991) pointed out that larger macrophytes hardly achieved the observed growth rates by diffusive CO_2 entry. In fact, most of the marine macroalgae tested have been proposed to be "HCO_3^- users" (Beer and Eshel 1983; Sand-Jensen and Gordon 1984; Bidwell and McLachlan 1985; Giordano and Maberly 1989; Maberly 1990; Johnston 1991).

Abbreviations and symbols: α = photosynthetic efficiency; AZ = acetazolamide; CA = carbonic anhydrase; DIC = dissolved inorganic carbon; DIDS = 4,4′-diisothiocyanatostilbene-2,2′-disulfonate; EZ = 6-ethoxyzolamide; K_s = half-saturation constant; NPS = net photosynthesis rates; NPS_{max} = light-saturated photosynthetic rate

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Several pathways for inorganic carbon acquisition (termed carbon-concentrating mechanisms; Johnston and Raven 1987) have been proposed in addition to diffusive CO_2 entry: H^+/HCO_3^- cotransport or OH^-/HCO_3^- antiport systems (Lucas 1983), HCO_3^- dehydration in acidic regions in the cell wall created by H^+ extrusion (Lucas 1983), ATPase-dependent HCO_3^- transport (Raven and Lucas 1985), surface-bound carbonic anhydrase (CA; EC 4.2.1.1) that mediates HCO_3^- dehydration to CO_2 (Smith and Bidwell 1989), and active CO_2 transport (Sültemeyer et al. 1989). The main pathway of HCO_3^- use in red macroalgae is the extracellular dehydration of HCO_3^- by CA to form CO_2, which is taken up into the cells (Smith and Bidwell 1987, 1989; Gómez-Pinchetti et al. 1992; Mercado et al. 1997b). This type of HCO_3^- use has been reported in several species of *Gracilaria* such as *G. sordida* (as *G. secundata*; Lignell and Pedersen 1989), *G. tenuistipitata* (Haglund and Pedersen 1992; Haglund et al. 1992; García-Sánchez et al. 1994) and *G. conferta* (Israel et al. 1991; Israel and Beer 1992). A HCO_3^- transport system (probably coupled to transmembrane H^+/OH^- fluxes) has been also suggested in *G. conferta* (Israel and Beer 1992), as well as a direct HCO_3^- uptake in 15 species of red macroalgae (Cook et al. 1986, 1988).

The direct uptake of HCO_3^- has been demonstrated, so far, in *Ulva lactuca* (Drechsler and Beer 1991; Axelsson et al. 1995) and *Enteromorpha intestinalis* (Larsson et al. 1997). In these green macroalgae, HCO_3^- uptake could be facilitated by a mechanism with similar properties to the anion-exchange protein of red blood cells (AE1; Sharkia et al. 1994), such as being sensitive to the inhibitor 4,4′-diisothiocyanatostilbene-2,2′-disulfonate (DIDS; Drechsler et al. 1993, 1994). A direct uptake of HCO_3^- via a DIDS-sensitive mechanism can be induced in *U. lactuca* when grown at high pH, while under normal pH conditions the CA-mediated mechanism sensitive to the CA inhibitor acetazolamide (AZ), is the main method of HCO_3^- utilization (Axelsson et al. 1995). The presence of two HCO_3^--utilizing mechanisms has been also established in *E. intestinalis* (Larsson et al. 1997). These authors reported a DIDS-sensitive mechanism with a low maximal capacity and a high affinity for DIC, and a CA mechanism with a high maximal capacity and a low affinity for DIC.

As far as we are aware, direct HCO_3^- uptake by a DIDS-sensitive mechanism has only been shown in green marine macroalgae. Our results showed that photosynthetic O_2 evolution rates as a function of DIC concentration (photosynthesis vs. DIC curves) displayed a biphasic kinetic pattern in *Gracilaria gaditana*. This could be evidence of the existence of either a double mechanism of DIC acquisition or a mechanism that operates with a variable affinity at different DIC concentrations. Based on this, the aim of this work was to describe the mechanisms of DIC utilization in *G. gaditana* by the use of inhibitors of DIC acquisition. Our results suggest that, in addition to possessing an external CA activity, *G. gaditana* is able to use HCO_3^- by a DIDS-sensitive mechanism.

Materials and methods

Plant material. *Gracilaria gaditana* nom. prov. was collected in the tidal channels of the Los Toruños salt marsh (El Puerto de Santa María, Southern Spain) during autumn 1997. Plants were cleaned of epiphytes and maintained for several months in the laboratory in aerated 20-L aquaria with filtered (GF/C; Whatman, Maidstone, Kent, U.K) natural sea water (NSW, 35‰), enriched with 40 μM $NaNO_3$ and 2 μM $NaH_2PO_4 \cdot 2H_2O$ (final concentrations). The temperature was maintained at 19 ± 1 °C, and the photon fluence rate (PFR) at 85 μmol photons m^{-2} s^{-1} (GRO-LUX, F-36 W/GRO-T8; Sylvania Erlangen, Germany) in a 12-h light/12-h dark cycle. The PFR was quantified with a quantum spherical PAR sensor (Li-193SA; LiCor Lincoln, Neb., USA) connected to a data logger (LiCor Li-1000).

Oxygen exchange rates. Net photosynthesis rates (NPS) were measured, at 19 ± 1 °C, with a polarographic O_2 electrode (Hansatech, Norfolk, UK) lit by a high-intensity light source (LS2; Hansatech) and connected to a chart recorder (L6512; linseis, Selb, Germany). In triplicate experiments, photosynthesis-PFR relationships were studied at different pHs in buffered filtered NSW (25 mM) and at 10 PFRs from 0 to 2180 μmol photons m^{-2} s^{-1} (cosine collector, LiCor LI-192SB). Biological buffers Mes, for pH 6.5, and Tris-(hydroxymethyl) aminomethane (Tris), for pH 8.2 and 9.2, were used. The light-saturated photosynthetic rate (NPS_{max}) and photosynthetic efficiency (α) were estimated according to Jassby and Platt (1976). Photosynthesis-pH relationships were also studied in triplicate experiments at a saturating PFR. Thalli and sea water were replaced after every measurement. Other experimental conditions were set as described above.

Photosynthesis-DIC relationships were studied in DIC-free artificial sea water [modified "Marine Culture Medium" (Woelkerling et al. 1983) by omitting sodium bicarbonate and boric acid] buffered at pH 8.2 with 25 mM Tris. The sea water was further bubbled with air which was previously passed through a freshly prepared 6 N KOH solution. The range of DIC concentrations (0–3500 μM) was achieved by injecting different volumes of a 20-mM $NaHCO_3$ solution into the incubation chamber once a zero net O_2 exchange rate was achieved. Triplicate measurements were carried out at a saturating PFR. The data were fitted to the Michaelis-Menten curve by an iterative procedure and to the Hanes-Woolf equation.

Inhibitors and CA assays. The inhibitors 6-ethoxyzolamide (EZ) and AZ (Sigma-Aldrich Química) were used. It is generally assumed that AZ cannot penetrate the cell and therefore only acts on surface-accessible CA (Moroney et al. 1985) although this assumption has been questioned (Williams and Turpin 1987). The EZ penetrates into the cell, inhibiting external and internal CA. The presence of an alternative HCO_3^- utilization mechanism (a direct uptake by an anion exchanger), was checked by using DIDS, (Sigma-Aldrich Quimica). Both AZ and EZ were dissolved in 0.05 N NaOH at a concentration of 50 mM (100 μM final concentration), while DIDS was dissolved in Milli-Q distilled deionized water at a concentration of 50 mM (300 μM final concentration).

The CA activity was measured by two different methods, a potentiometric method based on the time required for a drop of 0.4 units in the pH range from 8.4 to 7.4 at 0–2 °C for nonenzymatic (buffer) and enzymatic (thalli fragments) reactions (as in Haglund and Pedersen 1992), and a method based on the enhancement of CO_2 diffusive entry when the external CA activity is inhibited by AZ (Mercado et al. 1997a). The CA activity values were expressed as relative enzyme activity units calculated on a fresh weight basis [REA $(g\ FW)^{-1}$], and as external CA percentage, respectively.

pH-drift experiments and photosynthetic quotient determination. *Gracilaria gaditana* thalli were placed in a water-jacketed glass reactor containing 150 mL of filtered NSW. The initial pH of sea water was quickly adjusted to 7.9. The pH changes of the medium

caused by photosynthesis were measured with a pH electrode (Crison micropH; Crison Instruments, Barcelona, Spain) connected to a pH meter (micropH 2002). The pH values were recorded at 5-min intervals (LiCor Li-1000 data logger). The PFR was saturating and temperature was set at 19 ± 1 °C. The experiments lasted 6–15 h to reach a final pH value, the pH compensation point, which was achieved when the pH changed less than 0.01 units in 1 h (Maberly 1990). These experiments were also performed with inhibitors of CA activity and the HCO_3^- exchanger (AZ, EZ and DIDS), to characterize the mechanisms of DIC use in *G. gaditana*. The photosynthetic quotient was determined, in NSW, according to Axelsson (1988) by fitting an oxygen probe (Crison Oxi-92) and a pH electrode to the incubation chamber.

Induction experiments. The potential contribution of the two HCO_3^- acquisition pathways were checked after culturing the algae at different pH values. Thalli were maintained in 1 L of NSW at pHs of 6.5, 8.2 and 9.2 for 48 h. Measurements of photosynthetic O_2 evolution at low (450 μM) and high (1500 μM) DIC concentrations were performed in the Hansatech O_2 electrode chamber, at pH 8.2, without inhibitors (control) and with AZ and DIDS, as described above.

Spontaneous dehydration of HCO_3^- and other calculations. The rate of uncatalyzed formation of CO_2 from HCO_3^- in sea water, for a given salinity, temperature and pH, was calculated according to Johnson (1982), taking into consideration the apparent dissociation constants of carbonic acid in sea water, according to Dickson and Millero (1987). It was assumed that under alkaline pH conditions the CO_2 in sea water (CO_{2equil}), as well as the CO_2 formed by spontaneous dehydration (CO_{2dehyd}), were consumed almost instantaneously by photosynthesis at a rate causing the CO_2 concentration to approach zero (Haglund et al. 1992). Theoretical O_2 production was calculated using a photosynthetic quotient value of 1.067 according to our measurements.

The DIC concentration in its different forms was calculated from pH, salinity and temperature data, assuming a constant alkalinity. Photosynthetic rates, estimated as ΔpH (g FW)$^{-1}$ min^{-1}, and carbon uptake (μmol C m^{-2} s^{-1}) were calculated from the time-dependent pH variation, the incubation volume, and thallus weight:surface ratio. To calculate the theoretical CO_2 uptake, both for CO_{2equil} and CO_{2dehyd}, a diffusive model was applied, using a diffusion coefficient of $1.5 \cdot 10^{-9}$ m^2 s^{-1} (Raven 1991), and an unstirred-layer thickness of 100 μm, close to the value given by Israel and Beer (1992) for *Gracilaria conferta* under stirred conditions.

Statistical analysis. The results were expressed as the mean value ± SD. The effect of inhibitor treatments on photosynthetic parameters was analysed by a two-way analysis of variance (ANOVA). The minimum significant differences were calculated for the variation of these parameters. In all cases the significance level was set at a 5% probability.

Results

As a first approach, the mechanisms of DIC acquisition were studied by means of photosynthesis-DIC curves (Fig. 1). The photosynthetic response of *G. gaditana* to different DIC concentrations did not follow a simple saturation curve, but a biphasic pattern. Two lines were derived from a Hanes-Woolf plot, suggesting the existence of two kinetically different processes (Fig. 1, inset). Two alternative explanations can be given: the existence of one mechanism of DIC acquisition operating with different affinities, or the presence of two uptake mechanisms operating simultaneously. To analyze whether *G. gaditana* is using HCO_3^- in addition to CO_2, O_2 evolution rates as a function of plant biomass were calculated (Fig. 2). The photosynthetic quotient (1.067) was determined to calculate theoretical O_2 evolution rates supported by spontaneously formed CO_2 from HCO_3^-. The results showed that O_2 production was a saturating function of plant biomass, with the NPS higher than the maximum theoretical rate supported by CO_2 formed by uncatalyzed HCO_3^- dehydration (Fig. 2), at the highest biomass tested.

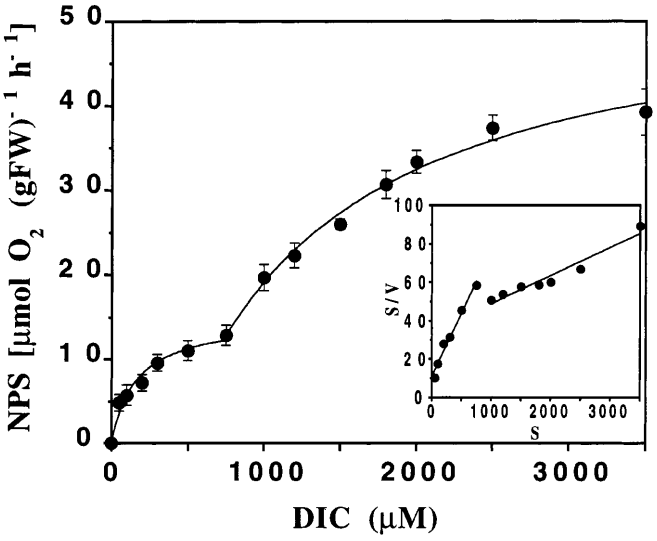

Fig. 1. Rates of net photosynthetic O_2 evolution (NPS) as a function of DIC concentration for *Gracilaria gaditana*. Data are presented as means ± SD (n = 3). A Hanes-Woolf plot is shown in the inset graph

Additional evidence of HCO_3^- use came from the photosynthesis-pH curve, and the use of inhibitors of DIC acquisition mechanisms. The photosynthesis-pH

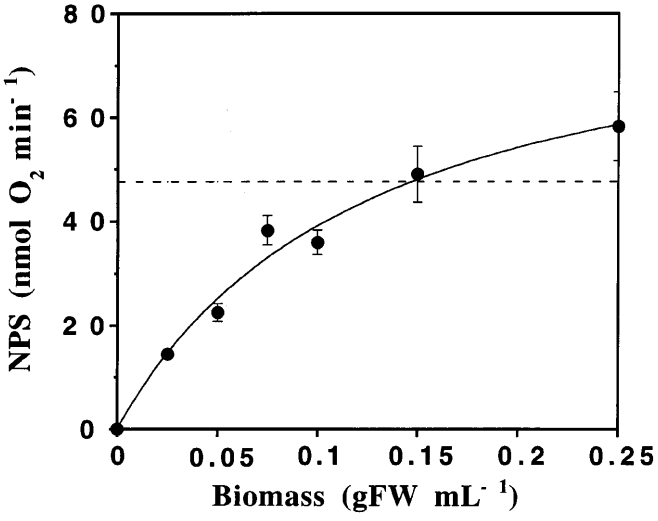

Fig. 2. Rates of net photosynthetic O_2 evolution (NPS) at different plant biomass for *Gracilaria gaditana* in a total volume of 2 mL. The measurements were performed in buffered natural seawater at pH 8.2 (25 mM Tris). The photon fluence rate was saturating. The *dashed line* shows the maximum NPS solely supported by uncatalyzed HCO_3^- dehydration. Data are presented as means ± SD (n = 3)

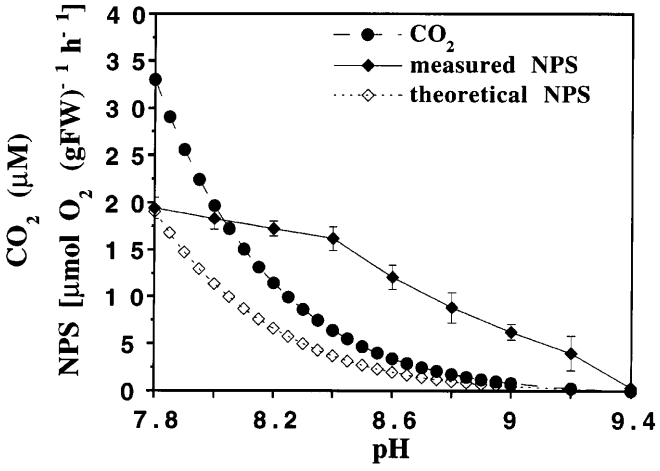

Fig. 3. Measured and theoretical net photosynthetic O_2 rates (NPS), and CO_2 concentration as a function of pH for *Gracilaria gaditana*. The theoretical O_2 evolution was calculated assuming that CO_2 was consumed instantaneously by photosynthesis. Theoretical NPS was calculated using a photosynthetic quotient of 1.067, the biomass and time of incubation. Data are presented as means ± SD (n = 3)

curve (Fig. 3) showed that the measured O_2 evolution rates were higher than those theoretically supported by CO_2, in the pH range from 7.8 to 9.4. The effect of inhibitors on the photosynthetic parameters was tested by photosynthesis-photon fluence rate curves at different pH values (Fig. 4). The NPS_{max} and α decreased with pH. Both CA inhibitors produced a significant decrease in NPS_{max} and α only at pH 8.2, but EZ did not cause a further inhibition compared to AZ. At pH 9.2, the drastic reduction of photosynthesis caused by pH masked the inhibitor effects. The photosynthetic O_2 evolution rates were measured in buffered artificial sea water (pH 8.2) under a non-saturating DIC concentration of 750 μM, without inhibitors (control) and with AZ, EZ or DIDS (Fig. 5). The NPS was reduced by AZ more than by DIDS (70.2% and 48.2%, respectively). The combination of AZ + DIDS, which should inhibit both DIC acquisition mechanisms, further reduced O_2 evolution rate by 77.2%, while EZ and EZ + DIDS inhibited about 79.8 and 93.1%, respectively. These results suggested that *G. gaditana* can use HCO_3^- by mechanisms involving both CA activity and a DIDS-sensitive pathway.

The DIC utilization by an extracellular CA was further investigated by two methods. The potentiometric method rendered a value of external CA activity of 5.69 ± 0.12 REA $(g\ FW)^{-1}$. Figure 6 shows the O_2 evolution after CO_2 addition and the subsequent effect of AZ. After NPS became zero (Fig. 6, slope c) the addition of 50 μL of ice-cold CO_2-saturated distilled water caused an immediate increase in the rate of O_2 evolution (Fig. 6, slope a), followed by a gradual decrease, to approach a constant level (Fig. 6, slope b). Following the addition of AZ, a substantial reduction of O_2 evolution was recorded (Fig. 6, slope c′). However, some O_2 evolution was still detected, which indicated the use of HCO_3^- by a mechanism other than external CA.

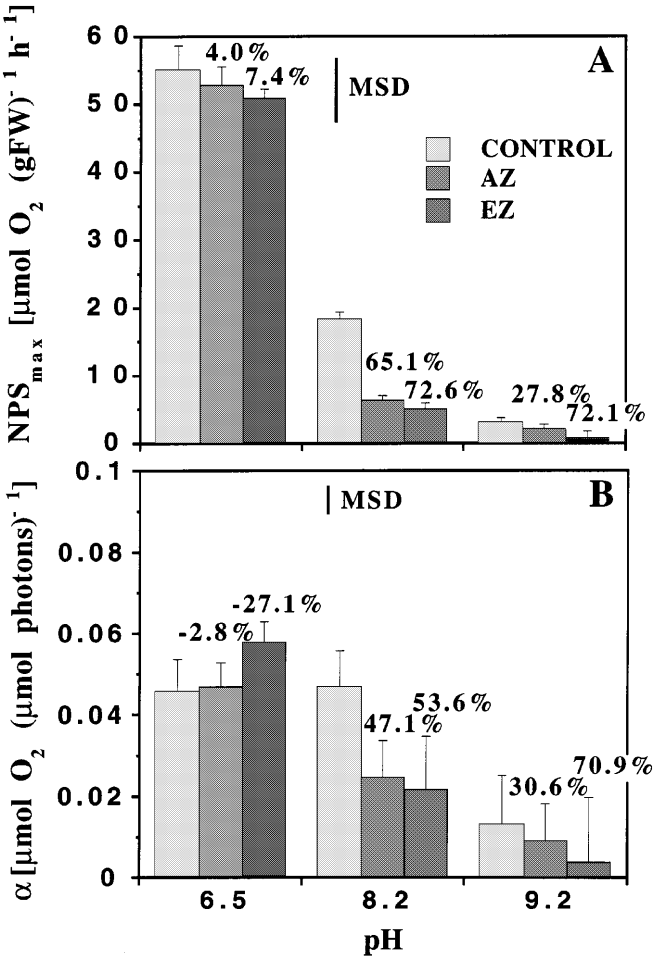

Fig. 4A,B. Light-saturated rate of photosynthesis (NPS_{max}) (**A**) and photosynthetic efficiency (α) (**B**) at different pH values for *Gracilaria gaditana*. The effect of AZ and EZ (100 μM) on both parameters was determined. *Numbers above the columns* indicate the percentage inhibition compared with the control. Data are presented as means ± SD (n = 3). The *vertical bar* indicates the minimum significant differences (*MSD*)

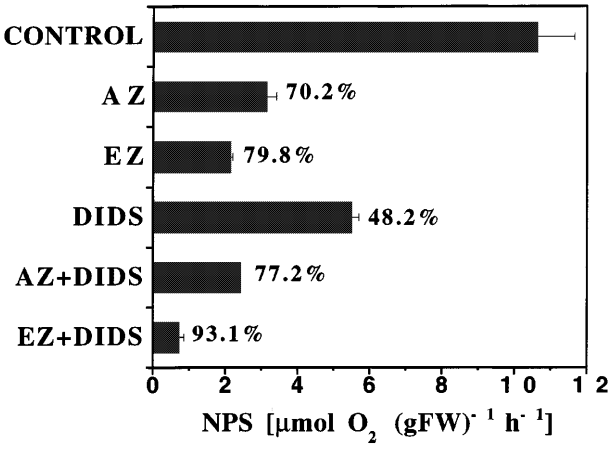

Fig. 5. Net photosynthesis rates (NPS) for *Gracilaria gaditana* subjected to CA (AZ and EZ, 100 μM), and to anion-exchange protein (DIDS, 300 μM) inhibitors. *Number associated with the columns* indicate the percentage inhibition compared with the control. Data are presented as means ± SD (n = 3)

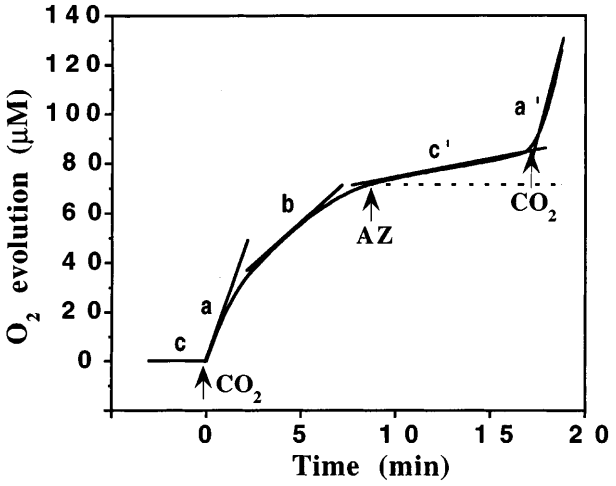

Fig. 6. Oxygen-electrode tracing obtained for *Gracilaria gaditana* after applying CO_2 and an external CA inhibitor, AZ (100 μM). The experiment was performed in the O_2 electrode chamber containing artificial sea water buffered with Tris (pH 8.7). The figure shows one of three experiments performed with similar results

The further addition of CO_2 promoted a new increase in the rate of O_2 evolution (Fig. 6, slope a′) which was greater than the initial rate. This method gave a value of external CA activity of 0.30 ± 0.02% (Mercado et al. 1997a). These results indicate the presence of an extracellular CA activity, as well as a remaining photosynthetic activity based on HCO_3^- transport.

pH-drift experiments were also performed in natural sea water (Fig. 7). The pH of the medium of control thalli increased up to 9.40 in 9 h. The pH compensation points decreased when inhibitors were added. The AZ-treated algae reached a higher pH compensation point than the DIDS-treated ones (9.24 and 9.07, respectively). However, AZ reduced the initial slope in contrast to DIDS. The AZ + DIDS treatment caused a further reduction in the pH compensation point. The EZ- and EZ + DIDS-treated algae increased the pH up to 8.2.

The NPS derived from the pH-drift data were plotted versus CO_2 and HCO_3^- concentrations (Fig. 8). The NPS as a function of CO_2 concentration showed a double component: a diffusive component at high CO_2 concentrations (linear phase), and an enzymatically mediated component at low CO_2 levels (Fig. 8A). The diffusive CO_2 entry was substantially lowered by the addition of the inhibitors and the enzymatically mediated component was also affected (Table 1). The CO_2 compensation points increased as a result of the addition of AZ, DIDS, AZ + DIDS, EZ, and EZ + DIDS. In EZ and EZ + DIDS treatments, the enzymatically mediated component disappeared. The NPS as a function of HCO_3^- concentration showed that inhibition caused by AZ was higher than that promoted by DIDS at high

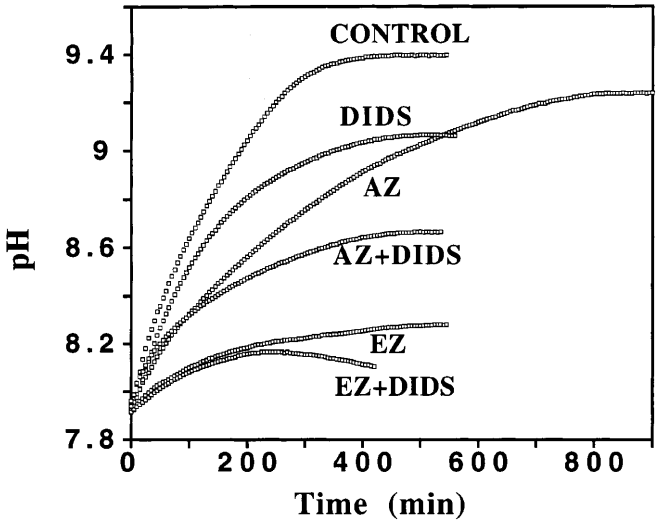

Fig. 7. pH-drift experiments for *Gracilaria gaditana* in natural sea water. The effects of CA (AZ and EZ, 100 μM) and of anion-exchange protein (DIDS, 300 μM) inhibitors were tested

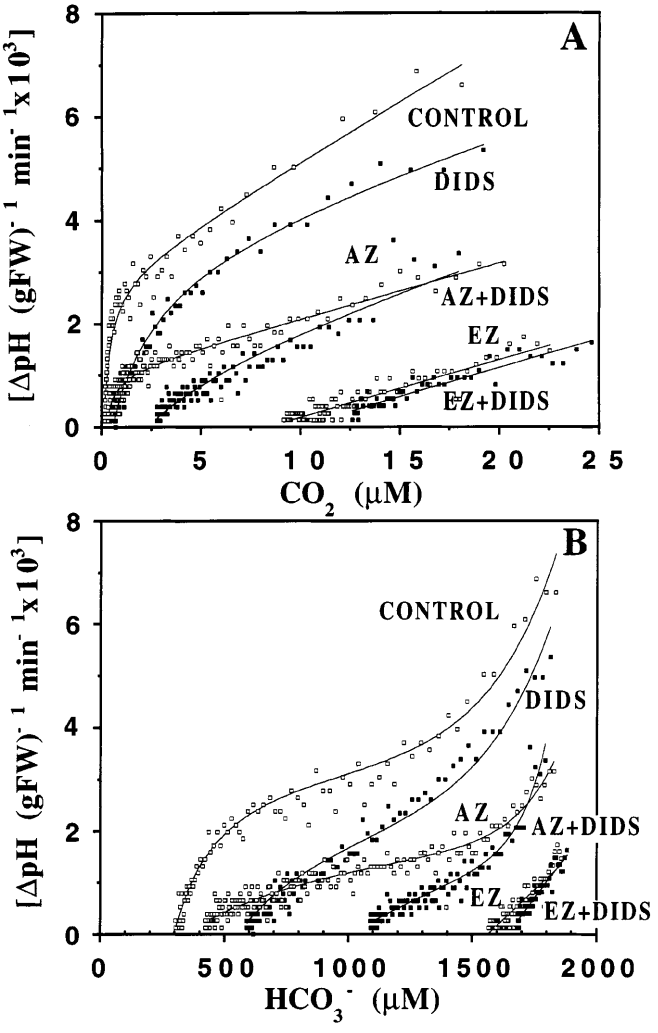

Fig. 8A,B. Photosynthesis rates, expressed as ΔpH (g FW)$^{-1}$ min^{-1}, as a function of external CO_2 (**A**) and HCO_3^- (**B**) concentrations for *Gracilaria gaditana*. Data were derived from pH-drift experiments. **A** Data were fitted to a linear plus a Michaelis-Menten equation by an iterative procedure. **B** Data were fitted to an exponential curve plus an Edwards and Walker equation

Table 1. Effect of inhibitors on the rate of diffusive CO_2 entry (slope of the diffusive component, b) and on the kinetic parameters of the enzymatic component (V_{max} and K_s) in *Gracilaria gaditana*. Data were calculated by fitting a Michaelis-Menten plus a linear equation using an iterative procedure (*r*, regression coefficient)

	$b \times 10^{-4}$ [(Δ pH (g FW)$^{-1}$ min^{-1}) (μM CO_2)$^{-1}$]	*r*	V_{max} [(Δ pH (g FW)$^{-1}$ min^{-1}]	K_s [μM CO_2]	*r*
Control	2.29 ± 0.09	0.98	4.33 ± 0.07	0.22 ± 0.01	0.98
DIDS	1.22 ± 0.05	0.99	4.62 ± 0.07	1.87 ± 0.07	0.99
AZ	1.07 ± 0.04	0.96	2.14 ± 0.03	0.21 ± 0.02	0.96
AZ + DIDS	1.22 ± 0.06	0.95	2.26 ± 0.12	3.49 ± 0.49	0.95
EZ	1.11 ± 0.04	0.93	–	–	–
EZ + DIDS	1.12 ± 0.06	0.94	–	–	–

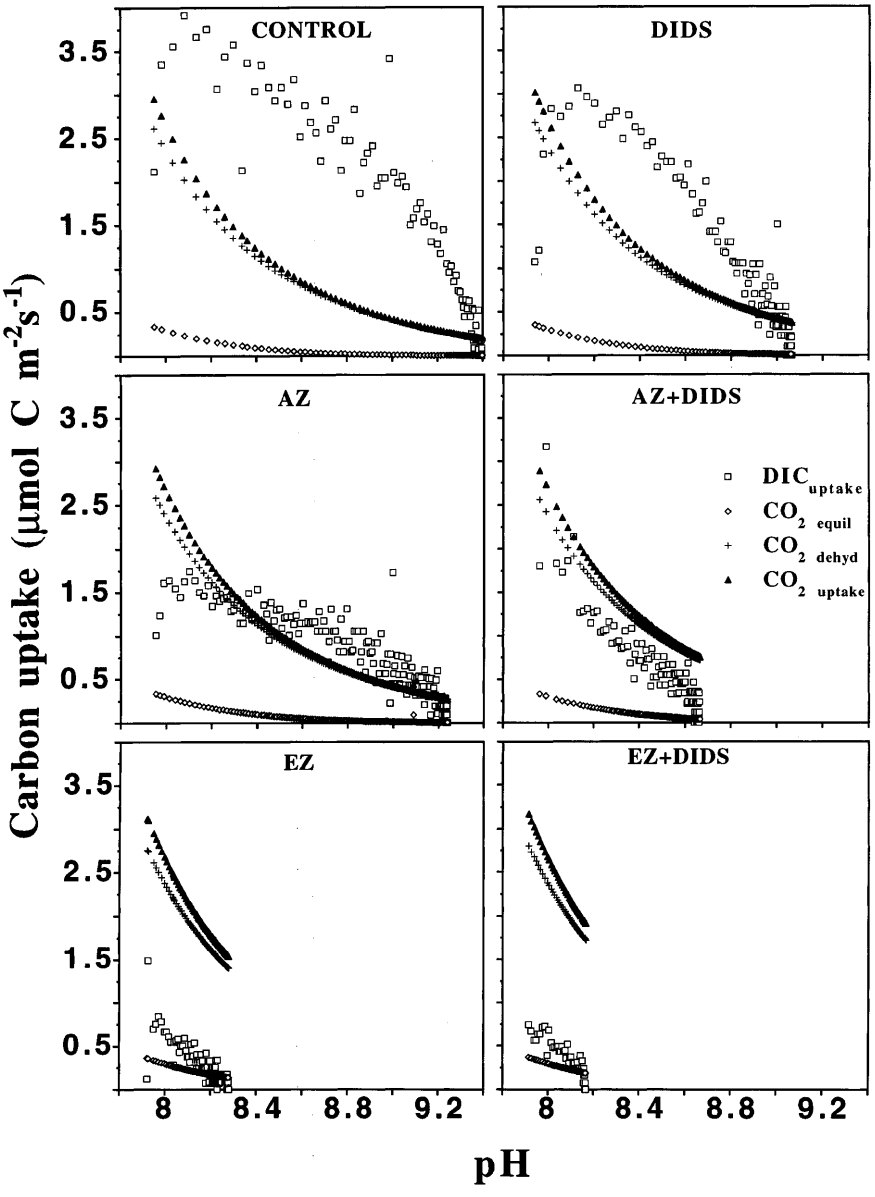

Fig. 9. Carbon uptake, expressed as μmol C m^{-2} s^{-1}, as a function of pH for *Gracilaria gaditana*. The DIC uptake data (DIC_{uptake}) were obtained from pH-drift experiments. The theoretical CO_2 uptake data ($CO_{2uptake}$) represent the incorporation of CO_2 achieved from uncatalyzed HCO_3^- dehydration (CO_{2dehyd}) plus the CO_2 in equilibrium (CO_{2equil})

HCO_3^- concentrations, while the opposite response occurred at low HCO_3^-, the HCO_3^- compensation point being lower in AZ-treated algae (Fig. 8B). The two curves crossed at a HCO_3^- concentration of ca. 700–800 μM.

The DIC uptake, derived from pH-drift experiments, and the theoretical CO_2 entrance by diffusion, as a function of pH, are shown in Fig. 9. The DIC uptake was higher than that theoretically supported by diffusive CO_2 entry, suggesting the use of HCO_3^- (Fig. 9, control).

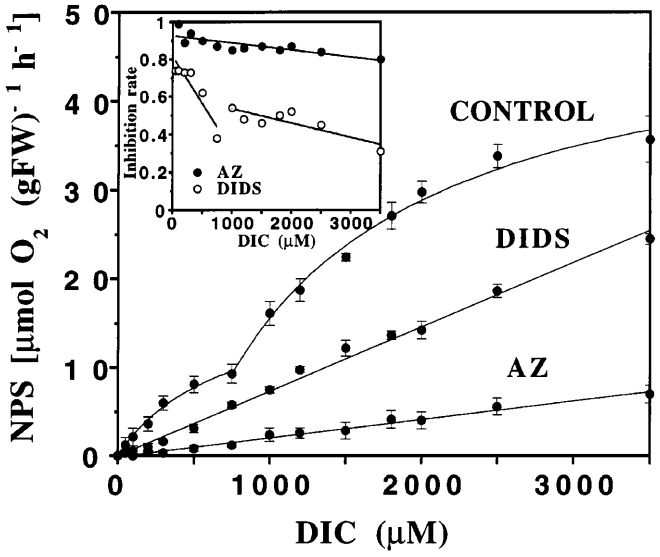

Fig. 10. Net photosynthetic O_2 evolution (NPS) as a function of DIC concentration for *Gracilaria gaditana*. The photosynthetic response was tested either in the presence of the anion exchanger protein inhibitor DIDS (300 μM) or of AZ (100 μM), an inhibitor of external CA. *Inset* shows the inhibition rates in the presence of AZ or DIDS, expressed as $[(NPS_{control} - NPS_{inhib})/NPS_{control}]$, as a function of DIC concentration

In DIDS-treated algae, DIC acquisition was lower than the theoretically calculated diffusive CO_2 entry at high pH (about 9.0), while AZ affected DIC acquisition more drastically, being lower than the theoretically calculated diffusive CO_2 entry even at low pH (8.0–8.4). The DIC uptake was lower than CO_2 acquisition by diffusion in AZ + DIDS-treated algae. It was further inhibited when EZ and EZ + DIDS were used.

As was shown above, photosynthesis-DIC curves displayed a biphasic pattern in *G. gaditana* (Figs. 1, 10): a first phase at low DIC (below 750 μM DIC) with a V_{max} of 14.7 ± 1.3 μmol O_2 (g FW)$^{-1}$ h^{-1} and a K_s of 158 ± 40 μM, and a second phase at higher DIC, with V_{max} of 41.4 ± 3.5 μmol O_2 (g FW)$^{-1}$ h^{-1} and a K_s of 1390 ± 235 μM. This dual pattern disappeared when AZ or DIDS were applied, following a linear fitting, being not saturated in the range of tested DIC concentrations. The inset figure shows the inhibition rates produced by AZ and DIDS at different DIC concentrations. Inhibition caused by DIDS was higher at a DIC concentration below 750 μM, while that caused by AZ was much less affected by DIC concentration.

Induction experiments were performed by culturing *G. gaditana* at different pHs for 48 h. Table 2 shows the rate of photosynthesis under a low (450 μM) and a high (1500 μM) DIC concentration, for algae cultured under different pHs. External CA was the main mechanism of HCO_3^- use regardless of the pH treatment and the DIC level. However, the contribution of a DIDS-sensitive mechanism was variable at low DIC levels, the inhibition being higher as the pH rose, while no variation was denoted at a high DIC level. The NPS was sharply reduced when AZ + DIDS were added at low DIC for algae cultured at a pH of 9.2.

Discussion

The ability to use HCO_3^- in macroalgae can be demonstrated by comparing the DIC uptake rates as a function of the dry weight:volume ratio (Maberly 1990). Use of HCO_3^- occurs when the DIC uptake rates are higher than those supported by CO_2 spontaneously formed from HCO_3^-. A similar comparison has been performed, indicating that *G. gaditana* exceeded the theoretical maximum rate at a biomass above 0.15 g FW mL^{-1}. The photosynthesis-pH curves also suggest that *G. gaditana* is able to use HCO_3^- for photosynthesis, since NPS values were above those supported by CO_2, contrasting with pure "CO_2 users" (Sand-Jensen and Gordon 1984). The pH compensation point obtained in pH-drift experiments also supports the occurrence of HCO_3^- use for photosynthesis, since those species relying solely on CO_2 use do not raise the pH above 9.0 (Maberly 1990).

The HCO_3^- use by an external CA-mediated mechanism has been previously demonstrated in red marine macroalgae (Smith and Bidwell 1987, 1989; Lignell and Pedersen 1989; Gómez-Pinchetti et al. 1992; Haglund and Pedersen 1992; García-Sánchez et al. 1994; Mercado et al. 1997b). The occurrence of an indirect HCO_3^-

Table 2. Net photosynthetic O_2 evolution rates (NPS) for *Gracilaria gaditana* precultured in buffered natural sea water at pH values of 6.5, 8.2 and 9.2. Measurements were performed in buffered DIC-free artificial sea water, at pH 8.2 and at two DIC levels (450 and 1500 μM), as well as in the presence of specific inhibitors of two HCO_3^- utilization mechanisms (AZ, 100 μM; DIDS, 300 μM). Data are mean values (± SD) for $n = 3$

pH	Control [μmol O_2 (g FW)$^{-1}$ h^{-1}]	AZ [% of control]	DIDS [% of control]	AZ + DIDS [% of control]
450 μM DIC				
6.5	11.2 ± 0.4	42.3 ± 3.1	83.5 ± 4.5	34.8 ± 5.0
8.2	12.9 ± 0.2	37.1 ± 1.0	77.2 ± 9.4	45.4 ± 2.9
9.2	13.1 ± 0.6	45.3 ± 1.5	67.4 ± 9.5	18.8 ± 3.2
1500 μM DIC				
6.5	23.3 ± 2.1	54.9 ± 5.6	81.1 ± 6.2	45.4 ± 1.6
8.2	22.8 ± 1.2	45.9 ± 4.0	94.0 ± 8.0	44.6 ± 3.6
9.2	15.5 ± 1.3	38.9 ± 1.0	82.8 ± 1.6	48.2 ± 2.3

use by this mechanism was evidenced by the potentiometric method which showed a moderate external CA activity for *G. gaditana* in comparison to other red algae (Giordano and Maberly 1989; Mercado et al. 1997a). An alternative method based on the O_2 evolution study and AZ use (Mercado et al. 1997a), further reinforced the existence of an external CA activity. Additionally, this method also suggested the existence of a HCO_3^- use by a mechanism other than external CA, since the photosynthetic O_2 evolution was not completely abolished by AZ. Mercado et al. (1997a) tested this method in 14 species of macroalgae. Three types of response occurred after addition of AZ: (i) macroalgae unable to use HCO_3^- did not displayed only slope *a*, with similar shape after AZ application, reflecting a CO_2-dependent rate of O_2 evolution; (ii) macroalgae with external CA displayed slope *b* distinct from zero and O_2 evolution was zero after addition of AZ (external CA inhibited), and (iii) macroalgae without external CA but displaying a direct HCO_3^- use showed slope *b* but this did not decrease after application of AZ. In the present experiments, a new type of response in between situations (ii) and (iii) has been found. Another way of investigating the presence of an external CA activity is based on the effect of specific inhibitors, such as AZ or dextran-bound sulfonamide, on photosynthetic parameters (Palmqvist et al. 1990; Haglund et al. 1992; Björk et al. 1993; Axelsson et al. 1995). The reduction of NPS_{max} and α, when AZ was applied, also suggested indirect utilization of HCO_3^- by an external CA-mediated mechanism.

The capacity to take up HCO_3^- directly in a variety of red macroalga species was suggested by Cook et al. (1986, 1988), but no evidence was provided about the mechanisms involved. It has been found that HCO_3^- uptake can be facilitated by a mechanism with similar properties to the anion exchanger of red blood cells (AE1; Drechsler et al. 1993,1994; Sharkia et al. 1994), such as being inhibited by DIDS. Its addition reduces photosynthetic rates when HCO_3^- is taken up directly (Drechsler and Beer 1991) although DIDS, like most of the disulfonic stilbene derivatives, not only inhibits HCO_3^- uptake but also other anion channels and anion transporters. A DIDS-sensitive mechanism has been previously reported in green macroalgae (Drechsler and Beer 1991; Drechsler et al. 1993,1994; Larsson et al. 1997). To our knowledge, this is the first report that provides evidence of the presence of a direct HCO_3^- transport via a DIDS-sensitive mechanism in a red marine macroalga. Despite the observation that AZ decreased NPS more than DIDS, showing the importance of an external CA-mediated mechanism, the NPS declined about 50% when DIDS was applied.

Based on the pH-drift technique, several authors have reported the ability of marine macroalgae to use HCO_3^- (Axelsson and Uusitalo 1988; Surif and Raven 1989; Maberly 1990). It is assumed that the changes in the composition of the enclosed sea water during the pH-drift experiments would be almost the same as those occurring in the boundary layer during periods of low hydrodynamic activity or high irradiation levels (Axelsson and Uusitalo 1988). In our study, the pH-drift technique established that the two pathways of DIC utilization were operating simultaneously in *G. gaditana*. The pH compensation points decreased when AZ, DIDS and AZ + DIDS were added, indicating the existence of both HCO_3^- uptake mechanisms. These experiments provided further information about the photosynthetic responses to CO_2 and HCO_3^- by *G. gaditana*. An indication of the existence of a carbon-concentrating mechanism is a low CO_2 compensation point (Johnston and Raven 1987; Reiskind et al. 1988; Johnston 1991). A suppression of the carbon-concentrating mechanism by specific inhibitors (AZ and DIDS) would imply an increase of the CO_2 compensation point, as shown in our results. The combined addition of AZ + DIDS further increased the CO_2 compensation point, the maximum effect being caused by EZ and EZ + DIDS. Under the latter conditions, *G. gaditana* was not able to photosynthesize at the CO_2 concentration of the natural sea water (ca. 10 μM).

Carbon uptake rates were estimated from pH-drift experiments. The detection limit of this method is high, since pH differences of 0.01 units correspond to a difference of about 0.2% of the DIC, which is close to the detection limits of the best methods for direct determination of DIC in sea water (Axelsson and Uusitalo 1988). These data were calculated assuming a constant alkalinity in the pH range from 7.9 to 9.4. Uusitalo (1996) reported that photosynthetic CO_2 and HCO_3^- uptake do not generally change the alkalinity, excepting for some species such as *Ascophyllum nodosum* and *Polysiphonia nigrescens*, at pHs above 9.5. In fact, Maberly (1990) pointed out that at pHs higher than 9.7 the calculated DIC values were higher than those measured directly. Our results suggest that a DIDS-sensitive mechanism operates at low DIC levels and at high pHs, while the external CA-mediated mechanism acts at higher DIC concentrations and at a wider pH range. This is further supported by the HCO_3^- compensation points. By applying a selective inhibitor for one of the HCO_3^- utilization mechanisms at a time, it was possible to assess the capacity of the other (Larsson et al. 1997). In the presence of AZ, photosynthetic HCO_3^- utilization should be based on HCO_3^- uptake via a DIDS-sensitive mechanism and vice versa. The lower HCO_3^- compensation point obtained when AZ was applied compared to DIDS-treated algae, indicated that the direct HCO_3^- transport was more important at lower DIC concentrations than the external CA-mediated mechanism.

It can be proposed that *G. gaditana* is able to use HCO_3^- by two complementary mechanims which are operating simultaneously. This could explain the dual kinetic pattern of the photosynthesis-DIC curves. This proposal is further supported by the induction experiments at different pHs. The potential contribution of the DIDS-sensitive mechanism increased when the algae were precultured under high-pH conditions and the NPS were measured under low DIC concentrations. However, the external CA-mediated mechanism was not suppressed, indicating that the two mechanism are complementary. These results are different from those

reported for the green macroalgae *Ulva lactuca* (Axelsson et al. 1995) and *Enteromorpha intestinalis* (Larsson et al. 1997), where the two mechanisms do not occur simultaneously, the DIDS-sensitive mechanism being inducible under high pH. Populations of *G. gaditana* occur in tidal shallow creeks in the Los Toruños salt marsh (Cádiz Bay). In this environment, temperature, irradiance and nutrient levels are high, specially at low tide. This promotes high photosynthesis and growth rates, causing high pH values and reducing the DIC levels in the surrounding sea water. Under such conditions, high photorespiration rates would be expected (Johnston and Raven 1987). The occurrence of a carbon-concentrating mechanism could be a strategy to improve the efficiency of DIC utilization and to avoid carbon leakage by photorespiration.

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