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Acquisition of inorganic carbon by *Endarachne binghamiae* (Scytosiphonales, Phaeophyceae)

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Photosynthetic acquisition of inorganic carbon was studied in the brown seaweed *Endarachne binghamiae* J. Agardh. Photosynthesis was saturated at 245 μmol photons $m^{-2} s^{-1}$ and photoinhibition did not occur at an irradiance as high as 750 μmol photons $m^{-2} s^{-1}$. The dependence of O_2 evolution on inorganic carbon (Ci) concentration demonstrated that the normal Ci composition in natural seawater was not saturating for irradiance-saturated photosynthesis. Three lines of evidence demonstrated that *E. binghamiae* was able to acquire HCO_3^- as a source of Ci for photosynthesis: (i) the high value of photosynthetic conductance for CO_2 (220.6 μm s^{-1}); (ii) the high pH compensation point of 9.7; and (iii) the measured photosynthetic rates being in excess of the theoretical maximum rates supported solely by the CO_2 supply from the spontaneous dehydration of HCO_3^- in the bulk seawater. In order to establish the mechanism of Ci acquisition, specific inhibitors and a proton buffer were applied to examine their inhibitory effects on photosynthesis. No inhibitory effects were found for the proton buffer tris(hydroxymethyl)aminomethane and the anion exchanger inhibitor, 4,4′-diisothiocyano-stilbene-2,2′-disulphonate. By contrast, photosynthetic O_2 evolution in natural seawater was significantly depressed by the extracellular carbonic anhydrase (CA) activity inhibitor, acetazolamide, and the plasma membrane P-type H^+-ATPase inhibitor, vanadate. These results suggested that carbon acquisition from the natural seawater was mostly through the external CA-mediated HCO_3^- dehydration mechanism, and that P-type H^+-ATPase (proton pump) in the plasma membrane simultaneously functioned in photosynthesis of *E. binghamiae*. Additional experiments on the O_2 exchange versus pH value relationship indicated that, in contrast to photosynthesis, dark respiration of *E. binghamiae* was insensitive to the change of pH in the seawater, which resulted in a decreasing instantaneous balance between net carbon gain and respiratory carbon loss at high pH values in seawater.

Key words: *Endarachne binghamiae*, inorganic carbon, photosynthesis, seaweeds, respiration, carbon balance

Introduction

Seaweeds acquire exogenous inorganic carbon (Ci) for photosynthesis and growth. They are exposed to three forms of dissolved Ci in seawater: dissolved CO_2, bicarbonate and carbonate ions. In air-equilibrated seawater (20°C), because of the high alkalinity, the dominant form of Ci is bicarbonate; its concentration is about 2 mM, while that of dissolved CO_2 is only about 12 μM (Stumm & Morgan, 1996). Some seaweeds use only dissolved CO_2 (e.g. Surif & Raven, 1989; Maberly, 1990), whereas others can acquire both CO_2 and HCO_3^- as the exogenous carbon source to drive photosynthesis (Maberly, 1990; Raven, 1997). Considering the rather low concentrations and low diffusive rate of dissolved CO_2 in seawater, and the high K_m value (40–90 μM) of ribulose-1,5, bisphosphate carboxylase-oxygenase (RuBisCO) for CO_2 measured in seaweeds (Johnston, 1991), it seems likely that seaweeds able to acquire HCO_3^- would possess advantages compared to those depending only on diffusive entry of CO_2 from the seawater. Efficient HCO_3^- acquisition can allow an increase in the CO_2 concentration around RuBisCO and a decrease in the photorespiration rates (Beer, 1994; Raven, 1997) and thereby function as a carbon-concentrating mechanism (CCM) like that occurring in terrestrial C_4 plants.

While CO_2 can easily pass through biological membranes and is directly available for carbon fixation, the ionic species of Ci, HCO_3^- and CO_3^{2-} cannot pass unless transported by some mechanism to facilitate their acquisition (Axelsson *et al.*, 1995; Raven, 1997). The primary mechanism by which seaweeds acquire HCO_3^- is mediated by surface-bound (external) carbonic anhydrase (CA;

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EC 4.2.1.1) that catalyses the interconversion of HCO_3^- and CO_2 extracellularly (Björk *et al*., 1992, 1993; Haglund *et al*., 1992*a*, *b*; Johnson *et al*., 1992; Beer, 1994; Axelsson *et al*., 1995). Thus the HCO_3^- in seawater can be utilized indirectly as a CO_2 reservoir. Some seaweed species possess a mechanism for direct uptake of HCO_3^- through the plasma membrane, assumed to be facilitated by an anion exchange protein (Drechsler *et al*., 1993, 1994; Axelsson *et al*., 1995, 1999; Larsson *et al*., 1997). The mechanism of HCO_3^- utilization is closely related to the habitat and confers an advantage in natural selection to the algae (Maberly, 1990; Larsson *et al*., 1997; Mercado *et al*., 1998; Snoeijs *et al*., 2002).

The brown seaweeds demonstrate a large range in their capability and approach of acquiring the HCO_3^- pool in seawater. Most of the brown algae tested could acquire HCO_3^-, based on the results from the pH-drift experiments, comparison of the observed rate of photosynthetic oxygen evolution with the rate which could be supported solely by CO_2 arising from the uncatalysed dehydration of HCO_3^-, and the photosynthetic inhibition in the presence of special inhibitors of HCO_3^- acquisition (Cook *et al*., 1986; Johnston & Raven, 1986; Axelsson & Uusitalo, 1988; Surif & Raven, 1989; Maberly, 1990; Haglund *et al*., 1992*b*). Some brown algae have very high capacity for HCO_3^- use. For example, members of the littoral Fucaceae were able to extract almost all the dissolved Ci in seawater (Axelsson & Uusitalo, 1988; Ryberg *et al*., 1990). In contrast, a low capacity for HCO_3^- utilization was observed in *Laminaria hyperborea* (Maberly, 1990), *Durvillaea potatorum* (Raven *et al*., 1989) and *Phyllariopsis purpurascens* (Flores-Moya & Fernândez, 1998). It appears that HCO_3^- utilization is more efficient in littoral species than sublittoral species (Surif & Raven, 1989; Maberly, 1990; Mercado *et al*. 1998). Axelsson *et al*. (1989*a*, *b*) reported that species of the Fucaceae within the Fucales (*Pelvetia*, *Fucus* and *Ascophyllum*) had a 'photosynthetic buffering system', allowing the algae to carry out oxygen evolution without a concomitant uptake of Ci. Direct uptake of HCO_3^- was indicated for a filamentous brown alga *Ectocarpus siliculosus* (Schmid, 1998). Axelsson *et al*. (2000) suggested a mechanism involving a CO_2-concentrating capability located at the cell membrane, based on the fact that tris(hydroxymethyl)aminomethane (TRIS) and acetazolamide (AZ) alone were both capable of inhibiting HCO_3^- utilization almost completely in *Saccharina latissima* (as *Laminaria saccharina*). A proton-gradient-driven CO_2 pump in the cell membrane might be integrated with HCO_3^- dehydration via external CA activity. On the other hand, a few species of brown algae, such as *Desmarestia munda* (Jolliffe & Tregunna, 1970), *Carpophyllum* sp. (Dromgoole, 1978) and *Desmarestia aculeata* (Axelsson & Uusitalo, 1988) seemed to lack specific mechanisms for HCO_3^- utilization.

This study focused on the photosynthesis of the brown seaweed *Endarachne binghamiae* (Scytosiphonaceae, Phaeophyceae). This alga is an edible species with high economic value, being distributed in the warmer waters, such as in the northern and southern Pacific Ocean as well as in the Indian Ocean (Nizamuddin & Farooqi, 1968; Parente *et al*., 2003). Studies on the chemical constituents, morphology, life history and culture of this monotypic alga have been previously reported (Nizamuddin & Farooqui, 1968; Bano *et al*., 1987; Brophy & Murray, 1989; Gwo & Chen, 1999; Neto, 2000; Parente *et al*., 2003). However, to our knowledge, information on the photosynthetic characteristics of *E. binghamiae* is not available. The objective of the present work was to establish the relationship of photosynthesis vs irradiance and Ci concentration, with special reference to the mechanism of Ci acquisition.

Materials and methods

Algal materials and laboratory maintenance

Samples of *Endarachne binghamiae* J. Agardh were collected from rocks in the lower intertidal along the coast of Nanao Island, Shantou, China in April 2007. This alga is commonly found on rocks in the tidal region along Nanao Island. The ambient surface seawater temperature at the site of collection over the sampling period was around 25°C (this temperature level was used for all subsequent experimental work). Only healthy and non-damaged plants were selected, and any accumulated sediments and macroscopic epiphytes were gently removed. Samples were placed into a plastic barrel partly filled with natural seawater, kept cool and dark, and were transported to the laboratory within 3 h. The algae were then maintained in filtered natural seawater (salinity ca. 33) in a 30-l plexiglass aquarium at 25°C under 200 μmol photons $m^{-2} s^{-1}$ photosynthetically active radiation illuminated by a bank of cool-white fluorescent tubes under a 12-h : 12-h light–dark photoperiod. The seawater was renewed every day and was continuously aerated by a filter-pump in order to keep air equilibrium of the dissolved Ci. The algal samples were used for experiments within a period of 3d laboratory maintenance. After this period, the algal remains were discarded and fresh samples were collected again.

Photosynthetic O_2 evolution measurement

Photosynthetic rates were measured as O_2 evolution by using a Clark-type Oxygen Electrode (YSI Model 5300, USA) kept at 25°C with circulating water from a constant temperature bath (Cole Parmer, USA). The

illumination was provided by a halogen lamp. All of the subsequent photosynthetic measurements were made below 50% of air-equilibration oxygen concentrations to avoid the possible inhibitory influences of high oxygen tensions. Respiration measurements were carried out at 100% air-equilibrium oxygen concentrations in seawater. The fronds of *Endarachne binghamiae* were cut into small segments with a sterilized sharp razor and incubated for at least 3 h in the natural seawater under the same light-temperature condition as the laboratory maintenance of the algal materials described above. This pre-incubation aimed to minimize the possible effect of cutting damage of fronds cells (wound respiration) on the photosynthetic determination.

Photosynthesis vs irradiance and inorganic carbon characteristics

To obtain the net photosynthetic O_2 evolution rate (NPR) versus irradiance relationship (P–I curve), about 80 mg of fresh weight (FW) algal segments was transferred to the O_2 electrode chamber containing 8-ml sterilized natural seawater, which was magnetically stirred. The algal samples were allowed to equilibrate in the darkness (which was made by covering the photosynthetic chamber with an opaque cloth and switching off the light source) until the rate of oxygen consumption was constant, usually for approximately 4–6 min, and the respiratory rate (R_d) was monitored. The samples were then exposed to a series of increasing irradiance from 18.6 to 750 μmol photons $m^{-2} s^{-1}$, until there was no further increase in the rate of oxygen evolution. Irradiance was adjusted by altering the distance between the light source and the photosynthetic chamber. The levels of irradiance were quantified by a quantum sensor (SKP 200, ELE International). Change of the pH value in the chamber during the measurement was less than 0.1 unit.

To obtain the NPR at light saturation versus Ci concentration relationship (P–C curve), a sample of about 50 mg FW was incubated in the electrode chamber containing 8 ml Ci-free seawater. The Ci-free seawater was prepared prior to the measurements according to our previous studies (Zou *et al*., 2003, 2004). Ci was removed from the sterilized natural seawater by reducing the pH to less than pH 4.0 with the addition of 0.5 M HCl, followed by sparging with high purity N_2 gas for at least 5 h. Finally the pH in the seawater was adjusted to pH 8.1 with freshly prepared 0.5 M NaOH solution. The algae were left to photosynthesize to consume all the Ci present in the medium and in the algal cells until no further O_2 evolved, which took about 20 min. Different aliquots of $NaHCO_3$ stock solution were then injected into the electrode chamber to obtain the different Ci concentrations desired. Generally O_2 evolution was observed within 3–5 min after each addition of $NaHCO_3$.

The effects of buffers and inhibitors

Buffers are often used to maintain constant pH values for the reaction medium when photosynthetic measurements are made. However, it has been demonstrated that the buffers *per se* could inhibit the photosynthetic carbon acquisition in the marine brown seaweed, *Saccharina latissima* (Axelsson *et al*., 2000; Mercado *et al*., 2006) and in the seagrass *Zostera marina* (Hellblom *et al*., 2001). The inhibition was assumed to occur as the proton buffers interfered with the formation of acid zones involved in the external HCO_3^- dehydration on the thallus surface (Axelsson *et al*., 2000; Hellblom *et al*., 2001; Beer *et al*., 2002). Therefore, we investigated the possible inhibitory effect of the buffer on the photosynthesis of *E. binghamiae*. The buffer used here was TRIS (biological buffers, Sigma). NPR was determined at pH 8.0 and 9.0 with and without the addition of TRIS buffer, respectively. Different amounts of a 2 M TRIS stock solution (adjusted to generate the desired pH upon addition to seawater) were injected into the electrode chamber to achieve various final TRIS concentrations.

NPR in natural seawater (ca. pH 8.1 and Ci 2.2 mM) was determined in the presence of the inhibitors: AZ (Sigma), 4,4′-diisothiocyano-stilbene-2,2′-disulphonate (DIDS; Sigma), and orthovanadate (VAN; Sigma). It is generally thought that AZ cannot penetrate into the algal cells and inhibits only the extracellular CA activity (Haglund *et al*., 1992*a*, *b*); DIDS inhibits the direct uptake of HCO_3^- by the photosynthetic cells by means of action on the plasmalemma-located anion exchange protein (Drechsler *et al*., 1993, 1994; Axelsson *et al*., 1995); while VAN is an inhibitor of P-type H^+-ATPase in the plasma membrane and thus inhibits cellular H^+ extrusion (Beffagna & Romani, 1988; Marre *et al*., 1988; Karlsson *et al*., 1994; Snoeijs *et al*., 2002). An AZ stock solution (50 mM) was prepared with 0.5 M NaOH, while DIDS and VAN were directly dissolved in the seawater to give the final concentrations of 400 μM and 200 μM respectively, used in the experiments. When a steady O_2 evolution rate was achieved in the control seawater, or in the seawater with the presence of DIDS or VAN, AZ was added into the reaction chamber to a final concentration of 200 μM, then O_2 evolution was monitored. For all the inhibitor experiments, the pH changes during measurement were no more than 0.05. Such a small pH change should have a limited effect on the photosynthetic measurement, making any interpretation of the pH effect unnecessary.

The effects of pH values

Oxygen exchanges in darkness and at saturating irradiance were measured in natural seawater of various pH, at a constant dissolved Ci concentration (ca. 2.1 mM). Values of pH ranging from 7.0 to 10.0 were obtained by adding appropriate quantities of freshly prepared HCl or NaOH. The vessels containing the seawater samples of the desired pH were quickly stoppered to avoid CO_2 exchanges with the atmosphere. Before any measurements, the algal samples were acclimatized in seawater with the actual pH for 15 min. After this, the medium was replaced and the samples allowed to acclimatize for another 4–6 min, before the respiratory or photosynthetic measurements were performed. This latter

incubation did not cause any significant change in the pH of the reaction medium (less than 0.05 units).

Assays of CA activity and pH compensation point

The CA activity was assayed by the potentiometric method as described by Giordano & Maberly (1989). To obtain the pH compensation point, pH-drift experiments were conducted in sealed glass vials containing 0.5 g fresh algae and 20 ml natural seawater at 25°C and 450 µmol photons $m^{-2} s^{-1}$. The final pH values were obtained when there were no further increases (after 6–8 h).

Calculations and statistics

Parameters describing the photosynthesis vs irradiance and Ci relationships were estimated. For the photosynthetic response to irradiance, the light-saturated photosynthetic rate (P_{max}) was calculated from the mean values in the asymptote region of the P–I curve. Apparent photosynthetic efficiency (α) was estimated as the ascending slope at limiting irradiance levels. The light saturation (I_k) and compensation points (I_c) were calculated according to Henley (1993). For the Ci-dependent O_2 evolution rate, the Ci-saturated maximum rate of O_2 evolution (V_{max}) and the concentration of Ci ($K_{0.5}$) supporting half of V_{max} were estimated from double reciprocal plots of the rates of O_2 evolution and the Ci concentrations. The apparent photosynthetic conductance (g_p), i.e. the initial slope of P–C curve (Johnston *et al*., 1992; Mercado *et al*., 2000), was calculated based on the concentration of CO_2. The ratio between CO_2 and total dissolved Ci used to calculate g_p was 1.02×10^{-3}.

The theoretical rate of CO_2 supply derived from spontaneous hydration of HCO_3^- in seawater was calculated according to Miller & Colman (1980) and Matsuda *et al*. (2001). The assumption was made that the entire volume of the bathing medium was available for this uncatalysed conversion from HCO_3^- to CO_2 and that the alga consumed CO_2 at a rate causing the CO_2 concentration to approach zero. This gave a theoretical maximal rate of CO_2 formation from the uncatalysed conversion of HCO_3^- within the bulk seawater. A rate of observed O_2 evolution greater than the theoretical rate of CO_2 supply was considered as evidence for the ability of *E. binghamiae* to acquire external HCO_3^- as an exogenous source of Ci for photosynthesis.

The data were expressed as the mean values ± standard deviation (SD). The significance of the data was tested with statistical analysis using SPSS for Window version 10, including the analysis of variance (ANOVA) and Student's *t*-test. The significance level was set at $p < 0.05$.

Results

Figure 1 shows the dependence of *Endarachne binghamiae* NPR on the incident photon irradiance in natural seawater (i.e. at the normal pH 8.1 and Ci concentration ca. 2.2 mM), and Table 1 illustrates the parameters of the photosynthesis vs irradiance relationship. No photoinhibition was observed over the irradiance range tested (0–750 µmol photons $m^{-2} s^{-1}$). *E. binghamiae* thalli had a saturating irradiance of 245 µmol photons $m^{-2} s^{-1}$, and the maximum light-saturated NPR was 188.6 µmol $O_2 g^{-1} FW h^{-1}$ in natural seawater. The respiratory carbon loss was ca. 5% of the gross photosynthetic carbon gain in natural seawater at saturating irradiance, assuming a similar photosynthetic and respiratory quotient.

The dependence of irradiance-saturated NPR at natural seawater (pH 8.1) on Ci concentrations is shown in Fig. 2, and the properties of NPR vs. Ci

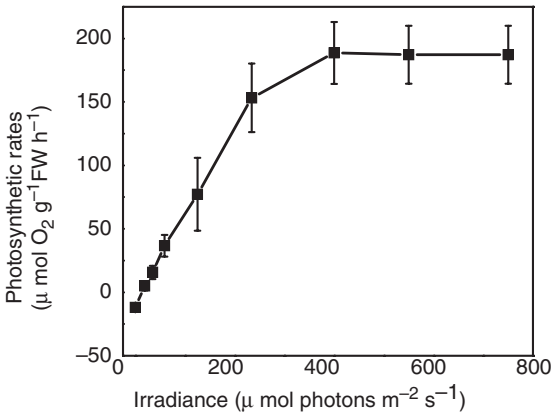

Fig. 1. Net photosynthetic oxygen evolution rate (mean ± SD; $n = 4$) of *E. binghamiae* as a function of photon irradiance. Measurements taken in natural seawater (pH 8.1, ca. 2.2 mM Ci) at 25°C. Abbreviations: Ci: inorganic carbon; FW: fresh weight.

Table 1. Photosynthetic parameters for the relationship between apparent photosynthetic O_2 evolution rates versus irradiance (P–I curve) and inorganic carbon (P–C curve) in *E. binghamiae*.

Parameters	Values
P–I curve	
P_{max} (µmol O_2 g^{-1} FW h^{-1})	188.6 ± 24.4
R_d (µmol O_2 g^{-1} FW h^{-1})	−11.9 ± 0.8
α [(µmol O_2 g^{-1} FW h^{-1})/ (µmol photons $m^{-2} s^{-1}$)]	0.82 ± 0.13
I_c (µmol photons $m^{-2} s^{-1}$)	14.9 ± 3.3
I_k (µmol photons $m^{-2} s^{-1}$)	244.5 ± 12.1
P–C curve	
V_{max} (µmol O_2 g^{-1} FW h^{-1})	292.7 ± 75.4
g_p (µm CO_2 s^{-1})	220.6 ± 51.5
$K_{0.5}$(Ci) (mM)	2.37 ± 0.43

Notes: Values are derived from Fig. 1 and Fig. 2. Values are means ± SD ($n = 4$).
Key: α: apparent photosynthetic efficiency; Ci: inorganic carbon; FW: fresh weight; g_p: photosynthetic conductance; I_c: light compensation point; I_k: light saturation point; $K_{0.5}$: concentration of Ci supporting half of V_{max}; P_{max}: light-saturated photosynthetic rate; R_d: dark respiratory rate; V_{max}: Ci-saturated maximum rate of O_2 evolution.

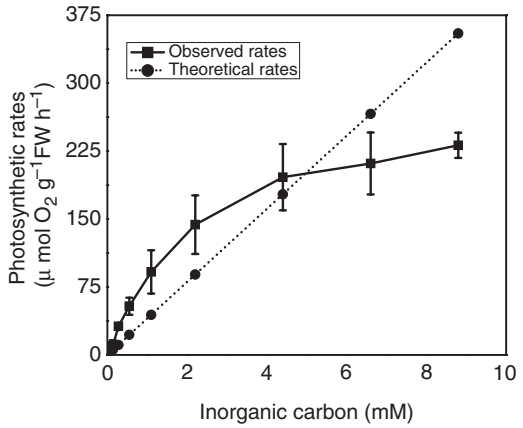

Fig. 2. Net photosynthetic oxygen evolution rate (mean ± SD; $n = 4$) of *E. binghamiae* as a function of inorganic carbon concentration at saturating irradiance (450 μmol photons $m^{-2} s^{-1}$). Measurements taken in natural seawater (pH 8.1, ca. 2.2 mM Ci) at 25°C. The dotted line shows the maximum photosynthetic rate supported solely by CO_2 deriving from the spontaneous HCO_3^- dehydration, which was calculated assuming that CO_2 was consumed instantaneously by photosynthesis. Abbreviations: Ci: inorganic carbon; FW: fresh weight.

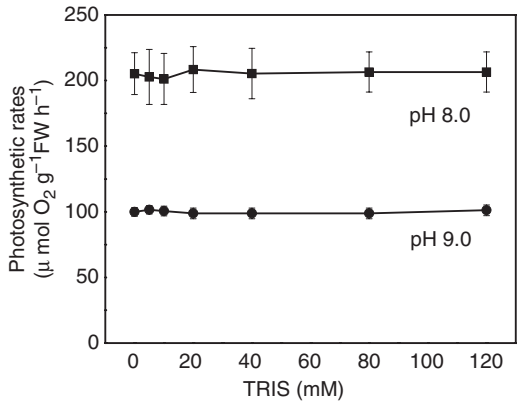

Fig. 3. Net photosynthetic oxygen evolution rate (mean ± SD; $n = 4$) of *E. binghamiae* as a function of TRIS (tris(hydroxymethyl)aminomethane) buffer concentration at saturating irradiance (450 μmol photons $m^{-2} s^{-1}$). Measurements taken in natural seawater (ca. 2.2 mM Ci) adjusted to two pH values: 8.0 and 9.0, at 25°C. Abbreviations: Ci: inorganic carbon; FW: fresh weight.

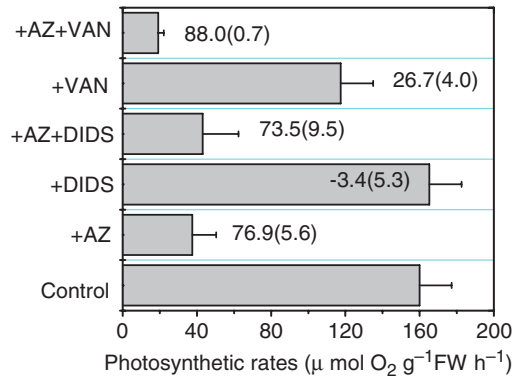

Fig. 4. Net photosynthetic oxygen evolution rates (mean ± SD; $n = 4$) of *E. binghamiae* after addition of external carbonic anhydrase inhibitor, acetazolamide (AZ, 200 μM of final concentration), an anion-exchanger inhibitor, 4,4′-diisothiocyano-stilbene-2,2′-disulfonate (DIDS, 400 μM of final concentration) and an inhibitor of P-type H^+-ATPase, vanadate (VAN, 200 μM of final concentration). The control was the photosynthetic rates obtained before adding the inhibitors. Measurements taken in natural seawater (pH 8.1, ca. 2.2 mM Ci) under saturating irradiance (450 μmol photons $m^{-2} s^{-1}$) at 25°C. Numbers associated with the columns indicate the percentage of inhibition compared with the control, standard deviations are given in parentheses. Abbreviations: Ci: inorganic carbon; FW: fresh weight.

relationship are presented in Table 1. Irradiance-saturated NPR was far from saturated with the normal seawater Ci concentration. NPR at the normal Ci levels was only about half of the Ci-saturated maximum photosynthesis. This is in concordance with the high value of $K_{0.5(Ci)}$ (2.37 mM; Table 1). Figure 2 also shows that only at concentrations of Ci above 5.0 mM, could the maximum theoretical rate of CO_2 formed via spontaneous HCO_3^- dehydration account for the measured NPR. Additionally, the photosynthetic conductance for CO_2 (g_p), estimated from the initial slope of P–C curve assuming that photosynthesis was supported solely by CO_2 diffusion, was 220.6 ± 51.5 μm s^{-1} for *E. binghamiae* in natural seawater (pH 8.1).

NPR of *E. binghamiae* thalli remained constant ($p > 0.1$) with varying concentrations of TRIS buffer, regardless of whether pH 8.0 or 9.0 was used for the photosynthetic measurement (Fig. 3). It is obvious that TRIS buffer had no inhibitory effects on NPR.

NPR at saturating irradiance was measured in natural seawater (pH 8.1, ca. 2.2 mM Ci) without inhibitors (control) and with AZ, DIDS, or VAN, respectively (Fig. 4). Both the external CA inhibitor, AZ and the P-type H^+-ATPase inhibitor, VAN, produced significant ($p < 0.01$) inhibition on NPR, with AZ having much greater inhibitory effect than VAN (inhibition percentage of 76.9% and 26.7%, respectively). The combination of AZ plus VAN, which was expected to depress both AZ-sensitive and VAN-sensitive mechanisms of Ci acquisition, reduced NPR by as much as 88.0%. No inhibitory effect ($p > 0.1$) was found for the anion exchanger inhibitor, DIDS.

The pH-drift experiments demonstrated that the final pH value of *E. binghamiae* thalli that could be achieved in a closed bathing medium was 9.7 (Table 2). Moreover, the presence of both external and internal carbonic anhydrase (CA) activities

Table 2. The pH compensation point and the external and total carbonic anhydrase (CA) activities in *E. binghamiae*.

pH compensation point	CA activity (REA g^{-1} FW)	
	External	Total
9.73 ± 0.08	15.8 ± 5.1	135.4 ± 19.4

Notes: Data are means ± SD ($n = 5$).
Abbreviations: FW: fresh weight; REA, relative enzyme activity.

was demonstrated by means of the potentiometric method, with the activity of external CA accounting for ca. 10% of the total CA activity (external plus internal; Table 2).

Rates of oxygen exchange under both darkness and saturating irradiance were measured in natural seawater adjusted to different pH values with constant Ci concentration (ca. 2.2 mM) (Fig. 5). The species of Ci available for support of the photosynthesis (HCO_3^- and/or CO_2) were modified by the changes in the pH of the seawater medium. The ratio of CO_2 to total Ci in the seawater was reduced from 0.5 to less than 0.0001 as a consequence of increasing the pH from 7.0 to 10.0. NPR remained unaltered in the pH region 7.0–8.0 (Fig. 5*a*). However, NPR decreased drastically as the pH increased from 8.0 to 10.0. The rates of measured photosynthesis were higher than those theoretically supported solely by CO_2 supply in seawater over the pH range from 7.5 to 10.0. The ratio of measured to the theoretical rates was increased by one order of magnitude with an elevation of one pH unit.

Dark respiration (R_d) was constant ($p > 0.1$) over the tested pH range of 7.0–10.0 (Fig. 5*b*). The ratio of R_d to gross photosynthesis (A_{gross}, i.e. NPR plus R_d) was maintained steady (ca. 5%) at pH 7.0, 7.5 and 8.0. However, the R_d/A_{gross} ratio increased slightly at pH 8.5 and 9.0 and was substantially increased at pH 9.5 and 10.0 (Fig. 5*c*).

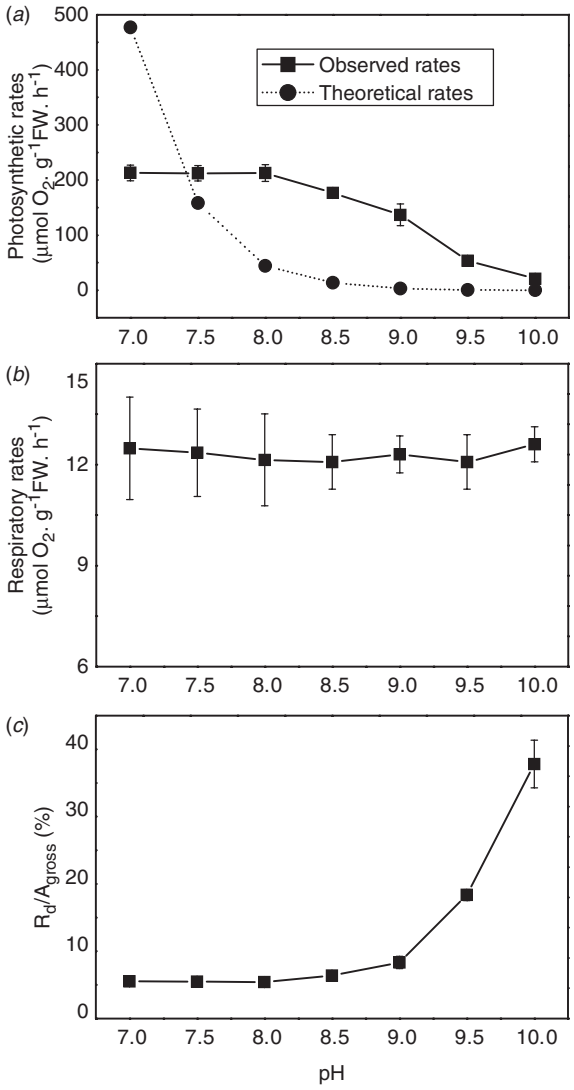

Fig. 5. Net photosynthetic oxygen evolution rate (NPR; *a*), dark respiratory rate (R_d; *b*) and the ratio of R_d to A_{gross} (*c*) in *E. binghamiae* as a function of pH values in seawater with constant inorganic carbon concentration. R_d was measured in the darkness, and A_{gross} was the gross photosynthetic oxygen evolution rate (i.e. NPR plus R_d). The experimental medium was natural seawater containing ca. 2.2 mM Ci, and the pH value was adjusted by HCl and NaOH. The dotted line in (*a*) shows the maximum photosynthetic rate supported solely by CO_2 supply deriving from the spontaneous HCO_3^- dehydration, which was calculated assuming that CO_2 was consumed instantaneously by photosynthesis. Vertical bars represent means ± SD ($n = 4$). When the bars are absent, the SD is smaller than the symbol size.

Discussion

The maximum light-saturating photosynthetic rate and dark respiratory rate (about 190 and 12 μmol O_2 g^{-1} FW h^{-1}, respectively) measured in natural seawater and ambient temperature in *Endarachne binghamiae* were much higher than those in *Hizikia fusiformis* (Zou & Gao, 2005), another brown seaweed species commonly found at the same sites and with the same growth period as *E. binghamiae* along the coast of Nanao Island, Shantou. This might mainly be due to the difference in morphology. *E. binghamiae* was characterized by flattened blades, whereas the thalli of *H. fusiformis* were coarsely branched. The value of saturating irradiance (245 μmol photons m^{-2} s^{-1}) of *E. binghamiae* estimated from the photosynthesis versus irradiance curve was lower than the general light levels (around 500 μmol photons m^{-2} s^{-1}), which can saturate photosynthesis in eulittoral seaweed species, as reviewed by Lüning (1981, 1990). However, the saturating irradiance of *E. binghamiae* was higher than the general level (60–150 μmol photons m^{-2} s^{-1}) of that observed in sublittoral

species. Photoinhibition did not occur at the highest irradiance level (750 μmol photons $m^{-2} s^{-1}$) we used during the measurement of the photosynthesis versus irradiance curves. However, in the present work, the NPR was measured after a relatively short exposure duration (i.e. minutes) to various irradiances, thus further work is needed to establish whether such a high irradiance as 750 μmol photons $m^{-2} s^{-1}$ is inhibitory over a longer exposure (for example, several hours).

Seaweeds acquire their exogenous carbon for support of photosynthesis from the dissolved Ci system of the seawater. This study presents three lines of evidence supporting the hypothesis that *E. binghamiae* thalli are capable of acquiring HCO_3^- for photosynthesis. Firstly, the theoretical photosynthetic rate which could be supported solely by CO_2 supply derived from the spontaneous dehydration of HCO_3^- in the bulk medium with 2.2 mM Ci was not high enough to account for the measured photosynthetic rate at pH 7.5 and above (Fig. 2, Fig. 5*a*). Secondly, the pH-drift experiment demonstrated that the final pH value that *E. binghamiae* could achieve in a closed medium was as high as pH 9.7. The final pH (i.e. pH compensation point) over pH 9.0 (equivalent to 0.6 μM CO_2 in seawater) has been considered as an indicator of HCO_3^- utilization in seaweeds (Axelsson & Uusitalo, 1988; Surif & Raven, 1989; Maberly, 1990; Johnston *et al.*, 1992), because the seaweed species which were restricted to acquiring CO_2 for photosynthesis could not raise the pH above 9.0. Thirdly, the initial slope of photosynthesis vs Ci curves (i.e. photosynthetic conductance) has been proposed as a useful parameter for determining the ability of a particular alga to acquire external HCO_3^- from the seawater (Johnston *et al.*, 1992; Mercado *et al.*, 1998, 2000). The value of photosynthetic conductance for CO_2 obtained in *E. binghamiae* was of the same order of magnitude as that found for seaweeds that can acquire HCO_3^- from seawater (Flores-Moya & Fernandez, 1998; Mercado *et al.*, 1998).

From the results obtained in this study, HCO_3^- acquisition by means of a direct uptake mechanism was unlikely to be present in *E. binghamiae* in natural seawater, because the photosynthetic rate was insensitive to the anion-exchange protein inhibitor, 4,4′-diisothiocyano-stilbene-2,2′-disulphonate (DIDS). In addition, a mechanism of direct HCO_3^- uptake would imply a photosynthetic capacity of low sensitivity to higher pH (Larsson *et al.*, 1997; Mercado *et al.*, 1998; Axelsson *et al.*, 1999; Mercado & Niell, 1999; Zou *et al.*, 2004). However, the photosynthetic rate of *E. binghamiae* at >pH 8.0 was sharply decreased in parallel with the pH increase (Fig. 5). This gave a further indication for a lack of direct HCO_3^- uptake in *E. binghamiae*.

Our results suggest that *E. binghamiae* uses the HCO_3^- dehydration mechanism to acquire HCO_3^- for photosynthesis. *E. binghamiae* thalli possessed extracellular CA activity, which accounted for more than 10% of the total CA. The extracellular CA catalyzes the conversion of HCO_3^- to CO_2, which is then taken up through the plasma membrane and finally fixed in photosynthesis. The role that the external CA played in photosynthesis was clearly recognized from the strong depression of photosynthetic rate by AZ (Fig. 5). Owing to the rather high catalytic efficiency of CA enzyme (a turnover rate of 600,000 s^{-1}; Falkowski & Raven, 1997), the HCO_3^- dehydration mechanism mediated by external CA conferred *E. binghamiae* with photosynthetic rates in normal seawater (pH ca. 8.0) similar to the rates in seawater of lower pH value (i.e. pH 7.0, 7.5) with higher equilibrium CO_2 concentrations (Fig. 5*a*). However, CA *per se* cannot bring about a disequilibrium between CO_2 and HCO_3^-, meaning that it cannot bring about a concentration of CO_2 higher than the equilibrium concentration at a certain pH (Falkowski & Raven, 1997). Therefore, the efficiency of the HCO_3^- dehydration mechanism mediated by external CA decreased sharply with increasing pH of the seawater. This gave the physiological explanation for the fact that the photosynthetic rate of *E. binghamiae* was sharply reduced at higher pH values.

It is worth noting another mode of HCO_3^- acquisition in seaweed species such as the brown algae *Laminaria* sp. (Axelsson *et al.*, 2000; Klenell *et al.*, 2002, 2004; Mercado *et al.*, 2006) and other macrophytes (Price & Badger, 1985; Hellblom *et al.*, 2001; Hellblom & Axelsson, 2003; Uku *et al.*, 2005). That is, a proton-gradient-driven CO_2 pump in the cell membrane based on the proton extrusion forming low pH (acid zones) on the thalli surface is integrated with HCO_3^- dehydration via external CA activity. Therefore the proton buffer TRIS and the external CA inhibitor, AZ, were both capable of inhibiting HCO_3^- acquisition almost completely. However, in the present study, there was no evidence that HCO_3^- acquisition by *E. binghamiae* involved acid zones on the thalli surface, because photosynthesis of this alga was insensitive to the proton buffer TRIS, in spite of the TRIS concentrations, or the pH values in seawater.

Vanadate (VAN) is the most well known and extensively adopted inhibitor of plasma membrane P-type H^+-ATPase in higher plants (Michelet & Boutry, 1995; Beer *et al.*, 2002) and algae (Karlsson *et al.*, 1994). It has been demonstrated to inhibit the HCO_3^- utilizing mechanism in macroalgae like the buffer sensitive brown alga

Laminaria sp. (Klenell *et al*., 2002, 2004), the red alga *Coccotylus truncatus* (Snoeijs *et al*., 2002) and the green alga *Cladophora glomerata* (Choo *et al*., 2002), most likely via inhibition of proton pumps. From the inhibitory effect of VAN on the photosynthetic rates, one might easily anticipate that a P-type H^+-ATPase (proton pump) was involved in carbon acquisition of *E. binghamiae* in natural seawater. Protons were extruded by the P-type H^+-ATPase pump in the plasma membrane. The protons would circulate across the cell membrane, creating a proton motive force and entering the cell again accompanied with HCO_3^- by means of a secondary symport transport, as suggested by Choo *et al*. (2002) in the green seaweed *Cladophora glomerata*. If so, the results of the present study might suggest that two pathways of carbon acquisition by *E. binghamiae* from natural seawater were operating simultaneously, i.e. external CA-mediated HCO_3^- dehydration and carbon uptake by the involvement of a VAN-sensitive P-type H^+-ATPase (proton pump).

Since AZ inhibited NPR much more that VAN (inhibition percentage was 76.9% and 26.7%, respectively), we proposed that external CA-mediated HCO_3^- dehydration mechanism was much more important than the VAN-sensitive mechanism in *E. binghamiae* during the period of photosynthesis. However, considering the fact that some part of photosynthesis must be supported by free CO_2 (partly obtained from non-catalysed HCO_3^--dehydration), it is obvious that there was an overlap between the inhibition by AZ (76.9%) and that by VAN (26.7%). In addition, the VAN-sensitive carbon uptake (i.e. the mechanism of the carbon uptake based on the plasma membrane P-type H^+-ATPase activity) would definitely be inhibited also by TRIS buffer (Klenell *et al*., 2004), but no effect of TRIS buffer was found in the present study with *E. binghamiae*. Therefore, it is possible that VAN may inhibit photosynthesis simply because functional proton pumps are vital for cell function and thereby VAN could interfere with net photosynthesis more directly and not by means of any HCO_3^- acquisition mechanism.

The capacity to acquire HCO_3^- from the bulk medium can usually be an important part of the CO_2 concentrating mechanism based on active Ci flux across the cell membranes. Photosynthesis of seaweeds is often saturated by the normal seawater Ci composition, largely because of their efficient utilization of HCO_3^- (Beer, 1994; Beer & Koch, 1996). However, *E. binghamiae* thalli were not saturated by the Ci concentrations in normal seawater, with high $K_{1/2(Ci)}$ values, although this alga was capable of acquiring HCO_3^-. It is suggested that the capacity to acquire HCO_3^- by the mechanism of external CA-catalysed HCO_3^- dehydration as well as any mechanism driven by a VAN-sensitive P-type H^+-ATPase in *E. binghamiae* in natural seawater operated in a way not sufficient to accumulate Ci intracellulary around RuBisCO and thereby to saturate the carboxylating potential.

It is of interest to note that, in contrast to photosynthesis, dark respiration showed no sensitivity to the changes of pH values (Fig. 5*b*). The pH of the seawater produced a tremendous influence on the equilibrium concentrations of CO_2. The CO_2 concentration in seawater with total Ci of 2.2 mM decreased from 0.8 mM to almost zero, when the pH increased from 7.0 to 10.0. Contrary to photosynthesis that uptakes CO_2 and releases O_2, respiration uptakes O_2 and produces CO_2. Therefore, one may expect that an instantaneous increase of the ambient CO_2 concentration would have an inhibitory effect on the respiration. In terrestrial higher plants, many reports have showed a decreased respiration in response to instantaneous increase in ambient CO_2 concentration (e.g. Amthor *et al*., 1992; Griffin *et al*., 1996; Amthor, 2000), which was usually attributed to an inhibition of cytochrome C oxidase activity (Gonzàlez-Meler *et al*., 2004). However, in the present study, we found no evidence that ambient CO_2 concentration exerted an effect on respiration in the seaweed *E. binghamiae*. This is consistent with the observations reported on some terrestrial plant species (e.g. Roberntz & Stockfors, 1998; Tjoelker *et al*., 1999). We suggest that the respiratory process(es), or the respiratory enzyme activities, in *E. binghamiae* remained relatively stable with the instantaneous changes of ambient CO_2 concentrations or pH values *per se*.

Our results demonstrate how pH values in seawater affected the carbon balance of *E. binghamiae*, owing to the differences between photosynthesis and respiration in response to the pH value. Assuming a photosynthetic quotient value of 1.0 in *E. binghamiae*, the percentage of carbon respired relative to gross carbon gained at saturating-irradiance increased appreciably with the increasing pH, especially at pH 9.5 and 10.0. This suggested an instantaneous decreasing balance between net carbon gain and respiratory carbon loss with increasing pH value in seawater.

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