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 $\text{mmol photons m}^{-2}\text{s}^{-1}$ and photoinhibition did not occur at an irradiance as high as 750 $\text{mmol photons m}^{-2}\text{s}^{-1}$. 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$_2$ ($220.6\text{ ms}^{-1}\text{Ci}^{-1}$); (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 $\mu$M (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 $\mu$M) 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;
EC 4.2.1.1) that catalyses the interconversion of HCO$_3^-$ and CO$_2$ extracellularly (Björk et al., 1992, 1993; Haglund et al., 1992a, 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; Snoeijjs 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; Larsson et al., 1997). 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 Phyllospadix purpurascens (Flores-Moya & Fernández, 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. (1989a, 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 (Schmidt, 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 (Sctosiphonaceae, 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 & Farooqi, 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 (Rd) 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$_3^-$ dehydoratation 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’-disothiocyano-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., 1992a, 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; Snoeijis et al., 2002). An AZ stock solution (50 μM) 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 efficiency (α) was estimated as the ascending slope at limiting irradiance levels. The light saturation (Iₘ) and compensation points (Iₜ) were calculated according to Henley (1993). For the Ci-dependent O₂ evolution rate, the Ci-saturated maximum rate of O₂ evolution (Vₘₐₓ) and the concentration of Ci (Kₐ₅) supporting half of Vₘₐₓ were estimated from double reciprocal plots of the rates of O₂ evolution and the Ci concentrations. The apparent photosynthetic conductance (gₚ), i.e. the initial slope of P-C curve (Johnston et al., 1992; Mercado et al., 2000), was calculated based on the concentration of CO₂. The ratio between CO₂ and total dissolved Ci used to calculate gₚ was 1.02 x 10⁻³.

The theoretical rate of CO₂ supply derived from spontaneous hydration of HCO₃⁻ 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 HCO₃⁻ to CO₂ and that the alga consumed CO₂ at a rate causing the CO₂ concentration to approach zero. This gave a theoretical maximal rate of CO₂ formation from the uncatalysed conversion of HCO₃⁻ within the bulk seawater. A rate of observed O₂ evolution greater than the theoretical rate of CO₂ supply was considered as evidence for the ability of E. binghamiae to acquire external HCO₃⁻ 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 O₂ 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 quotients.

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.](image)

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<th>Parameters</th>
<th>Values</th>
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<tr>
<td>P-I curve</td>
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<tr>
<td>Pₘₐₓ (μmol O₂ g⁻¹ FW h⁻¹)</td>
<td>188.6 ± 24.4</td>
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<tr>
<td>Rₙ (μmol O₂ g⁻¹ FW h⁻¹)</td>
<td>-11.9 ± 0.8</td>
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<tr>
<td>α [(μmol O₂ g⁻¹ FW h⁻¹)/ (μmol photons m⁻² s⁻¹)]</td>
<td>0.82 ± 0.13</td>
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<tr>
<td>Iₘ (μmol photons m⁻² s⁻¹)</td>
<td>14.9 ± 3.3</td>
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<tr>
<td>Iₜ (μmol photons m⁻² s⁻¹)</td>
<td>244.5 ± 12.1</td>
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<tr>
<td>P-C curve</td>
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<tr>
<td>Vₘₐₓ (μmol O₂ g⁻¹ FW h⁻¹)</td>
<td>292.7 ± 75.4</td>
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<tr>
<td>gₚ (μmol CO₂ s⁻¹)</td>
<td>220.6 ± 51.5</td>
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<td>Kₐ₅ (Ci) (mM)</td>
<td>2.37 ± 0.43</td>
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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; gₚ: photosynthetic conductance; Iₘ: light compensation point; Iₜ: light saturation point; Kₐ₅: concentration of Ci supporting half of Vₘₐₓ; Pₘₐₓ: light-saturated photosynthetic rate; Rₙ: dark respiratory rate; Vₘₐₓ: Ci-saturated maximum rate of O₂ evolution.
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...
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. 5a). 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. 5b). 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. 5c).

**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

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<th>pH compensation point</th>
<th>CA activity (REA g$^{-1}$ FW)</th>
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<tr>
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<td>External</td>
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<td>9.73 ± 0.08</td>
<td>15.8 ± 5.1</td>
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Notes: Data are means ± SD (n = 5).
Abbreviations: FW: fresh weight; REA, relative enzyme activity.

![Fig. 5.](image-url)
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. 5a). 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'-diisothiocyanato-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. 5a). 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 would be 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 than VAN (inhibition percentage was 76.9% and 26.7%, respectively), we proposed that external CA-mediated HCO\(_3\)\(^-\) dehydration and carbon uptake by the involvement of a VAN-sensitive P-type H\(^+\)-ATPase (proton pump).

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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