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Acquisition and use of bicarbonate by *Emiliania huxleyi*

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Summary

• Bicarbonate acquisition mechanisms and the kinetics of dissolved inorganic carbon (DIC) use in photosynthesis and calcification were investigated in *Emiliania huxleyi*.
• Photosynthesis was measured using O_2 evolution and ^{14}C incorporation and calcification was measured with ^{14}C. Noncalcifying (coccolith-free) cells were produced from calcifying (coccolith-bearing) cells of the same strain of *E. huxleyi*, so that photosynthesis could be monitored independently from calcification.
• Neither photosynthesis nor calcification was saturated at the ambient DIC concentration of seawater. In coccolith-bearing cells, both processes showed biphasic kinetics with DIC concentration, with a hiatus located at 1 mM. The same biphasic pattern and similar rates of photosynthesis were found in the coccolith-free cells. Inhibitor experiments showed that *E. huxleyi* acquires bicarbonate mainly by an anion exchange protein, but external carbonic anhydrase can be activated at low concentrations of DIC.
• We conclude that the biphasic kinetics of photosynthesis and calcification are caused by the presence of two bicarbonate acquisition mechanisms and also, since calcification does not enhance photosynthesis in this coccolithophore, we question the current view that the two processes are tightly coupled.

Key words: *Emiliania huxleyi*, dissolved inorganic carbon, kinetics, photosynthesis, calcification, anion exchange protein, carbonic anhydrase.

Introduction

Emiliania huxleyi is currently the most abundant coccolithophore (Winter & Siesser, 1994). It forms large blooms in coastal waters in mid-latitudes, and since the coccoliths backscatter light, these blooms can be seen clearly in satellite images.

E. huxleyi has a major role in the global biogeochemical cycling of elements, especially carbon, calcium and sulphur. The cells produce dimethyl sulphide, which assists in the formation of cloud condensation nuclei, and in shallow seas, the production of coccoliths accelerates carbon flux from the atmosphere to the ocean bed (Holligan *et al.*, 1993). Towards the ends of blooms, calcification also has an effect on the sea-atmosphere interchange of carbon by causing an increase in the partial pressure of CO_2 (pCO_2) in surface waters (Westbroek *et al.*, 1993). This is due to the uptake and assimilation of bicarbonate during the formation of calcite plates where two molecules of solute are transformed into a molecule each of a solid and a dissolved gas:

$$2HCO_3^- + Ca^{2+} CaCO_3 + CO_2 + H_2O \qquad \text{Eqn 1}$$

It is currently thought that photosynthesis and calcification are closely coupled and that the CO_2 produced when calcite plates are made is assimilated via the Calvin cycle, but at the ends of blooms the two processes can become uncoupled, hence the rise in pCO_2 (Robertson *et al.*, 1994).

Although the substrate for Rubisco (ribulose-1,5 bisphoshate carboxylase/oxygenase) is CO_2, the dominant form of dissolved inorganic carbon (DIC) in seawater is HCO_3^-. The equilibria between CO_2, HCO_3^- and CO_3^{2-} depend principally on pH and concentration, and to a lesser extent on temperature and salinity. At present, at the ambient seawater pH of 8.2, 90% of DIC is HCO_3^- and less than 1% is CO_2 but, with increasing concentrations of atmospheric CO_2, these proportions are likely to change (Stumm & Morgan, 1996). Estimates of the precise extent to which CO_2 concentrations will increase vary, but the uncertainties associated with biological consequences of this are far greater. Since the major calcifiers are also photosynthetic and consume DIC in both processes, the relationship

between calcification and photosynthesis needs to be examined more closely.

E. huxleyi was chosen for the present investigation because it is the Earth's major cool water calcifier. There are now many strains in culture, often with different calcifying capacities, including some non-calcifying strains (Paasche, 2001). Some authors have compared high and low calcifying strains in order to examine the influence of various factors on calcification. This approach is vulnerable to the criticism that strains that have different calcifying capacities also vary in other ways, including their DIC transporting abilities (Elzenga *et al.*, 2000; Paasche, 2001). Exploratory experiments in our laboratory showed that it was possible to produce genotypically identical, bicarbonate using, non-calcifying cells of the same high-calcifying strain by the simple expedient of omitting calcium from the growth medium. We therefore used coccolith-bearing and coccolith-free cells of the same strain of *E. huxleyi* to see if calcification had an effect on photosynthesis, and used coccolith-bearing (plated) cells to monitor calcification.

The extent to which *E. huxleyi* uses the bicarbonate ion has been the subject of some discussion (Paasche, 1964; Sikes *et al.*, 1980; Nimer & Merrett, 1992), but it now seems established that calcifying strains use bicarbonate for photosynthesis as well as for calcification (Buitenhuis *et al.*, 1999), even if the precise mechanisms of bicarbonate transport into cells remain unclear (Paasche, 2001). Extracellular carbonic anhydrase, which catalyses the dehydration of H_2CO_3 followed by the penetration of CO_2 into the cell, is active in seawater in a large number of algae (Aizawa & Miyachi, 1986; Sültemeyer *et al.*, 1993; Badger & Price, 1994; Nimer *et al.*, 1997), but not in growing cells of *E. huxleyi* (Sikes & Wheeler, 1982; Nimer *et al.*, 1994; Sekino & Shiraiwa, 1994). Although Nimer *et al.* (1996) found no external CA activity in *E. huxleyi*, they did record a halving of the DIC concentration of the medium in which cells of a high-calcifying strain were incubated. This dramatic uptake could be explained by the action of another DIC acquisition mechanism such as the anion exchange protein, AE1. However, the addition of 0.5 mM DIDS (4,4′-diisothiocyanato-stilbene-2,2′-disulfonic acid), a specific inhibitor of this bicarbonate transporter, did not decrease the photosynthetic rate and the rapid removal of DIC from the medium remained unexplained.

Initial experiments in our laboratory, which monitored photosynthesis over a wide range of DIC concentrations, showed that the kinetics did not follow a simple hyperbolic function but appeared to be biphasic. Paasche (1964) and Sekino *et al.* (1996) had also found some evidence of this. If the kinetics are indeed biphasic, this suggests that *E. huxleyi* possesses two or more mechanisms for transporting DIC into the cells. Hence, the present study used a wide range of DIC concentrations and specific inhibitors of DIC uptake in order to ascertain the kinetics and mechanisms of bicarbonate transport.

Methods

Organism

Axenic cultures of the coccolithophorid, *Emiliania huxleyi* (Lohmann) Hay & Mohler calcifying strain PCC.B11 were supplied by the Marine Biological Association Culture Collection, Plymouth, UK. Cells were grown in 250 ml conical flasks containing 100 ml of Harrison's artificial seawater medium (Harrison *et al.*, 1980), at pH 8.2. Medium and glassware were autoclaved for 20 min at 120°C. Cultures were grown at 15°C, on a rotating shaker at 100 rpm, under a continuous downwelling photon flux density (PFD) of 120 μmol m^{-2} s^{-1} provided by white fluorescent lights. Cells grown using this procedure possessed calcite plates. Coccolith-free, noncalcifying cells were produced from two sequential subcultures of coccolith-bearing cells inoculated into Harrison's medium from which $CaCl_2$ was omitted (Fig. 3). This technique does not starve the cells of calcium, but reduces the concentration below that needed to construct plates (Herfort *et al.*, in prep.). The ability of coccolith-free cells to calcify was not compromised by the treatment since coccoliths were readily produced again when these cells were reinoculated into Ca^{2+}-containing medium.

Cultures in mid-exponential growth phase (3–4 d after inoculation) were harvested following centrifugation for 10 mins at 150 × g. Immediately before measurements of photosynthesis or calcification were made, coccolith-bearing cells were resuspended in a simplified DIC-free medium (355.6 mM NaCl, 24.4 mM Na_2SO_4, 7.9 mM KCl, 46.2 mM $MgCl_2{\cdot}6H_2O$, 8.6 mM $CaCl_2{\cdot}2H_2O$) which was buffered with 25 mM bicine and the pH adjusted to 8.2. Coccolith-free cells were resuspended in the same medium without the $CaCl_2$.

Measurement of photosynthesis

O_2 production O_2 evolution was recorded using Clark-type oxygen electrodes (Rank Brothers, Cambridge, UK and Hansatech, Norfolk, UK). A 5-ml sample of a DIC-free suspension of 1.5–2 × 10^6 cells ml^{-1} was incubated in each chamber at 15°C and a PFD of 500 μmol m^{-2} s^{-1}. This PFD is saturating for photosynthesis and calcification (Nielsen, 1995). The sample was left to photosynthesise for about 5 min, in order to deplete any internal carbon pool. When the carbon compensation point was reached, additions of bicarbonate were made through the capillary tube in the chamber lid. The slope of the recorder trace of O_2 vs time gave the rate of O_2 evolution.

Radioactive carbon incorporation Photosynthetic ^{14}C incorporation was determined using the procedure described by Paasche (1963). DIC plus 0.22 MBq of $NaH^{14}CO_3$ were simultaneously pipetted into 20 ml of DIC-free suspension containing 5 × 10^5 cells ml^{-1}. The total radioactivity of each flask was assayed by immediately withdrawing 20 μl of the suspension and transferring it to a scintillation vial containing

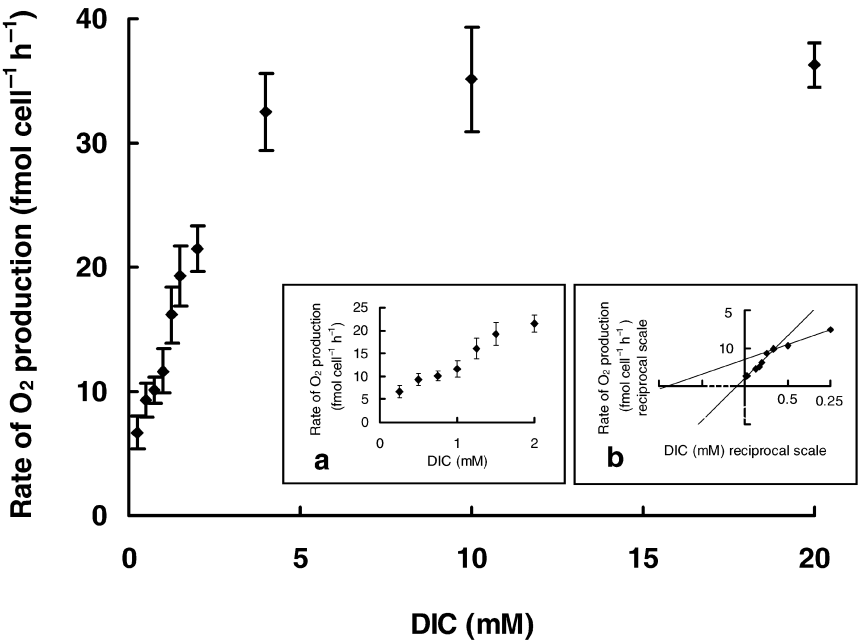

Fig. 1 The effect of different external DIC concentrations on the net O_2 production rate of coccolith-bearing cells of *Emiliania huxleyi*. Data points are means ± SE (n = 7–11). (a) Detail from the main graph, restricted to low dissolved inorganic carbon (DIC) concentrations, in order to highlight the biphasic kinetics of the photosynthetic rate, with a hiatus located at 1 mM DIC. (b) Double reciprocal plot of the data from the main graph. This shows that twin slopes are a good fit for these data and that the kinetic parameters differ below and above 1 mM DIC.

5 ml of scintillation fluid (Ecoscint, National Diagnostics, Hull, UK). Each flask was incubated on a shaker at 15°C and a PFD of 500 µmol (m^2 s^{-1}). After 3 h, six samples were taken from each treatment. Two millilitres of culture were filtered through cellulose nitrate filters (0.2 µm pore size, 25 mm diameter) and rinsed twice with 2 ml of seawater. Three of the six samples were put directly into scintillation vials containing 5 ml fluid, whilst the remaining three filters were fumed with concentrated HCl for 30 s to dissolve the calcium carbonate before rinsing and placing in scintillant. Radioactivity in the vials was measured using a Tri-carb 2200 liquid scintillation analyser. Photosynthetic rates were calculated from the acid-fumed filters and the calcification rate was obtained by subtracting the measured radioactivity in the fumed filters from that in the non-fumed filters.

Scanning electron microscopy (SEM)

Cultures of *Emiliania huxleyi* grown in Ca^{2+}-free medium or in medium containing 9.1 mM calcium were harvested during the mid-exponential growth phase. 2 ml of cells were added to a solution of 3% (w/v) of glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 8.2). After fixation for 2 h on a rotator at room temperature, the cells were filtered onto a 0.45-µm cellulose nitrate filter and washed with cacodylate buffer (pH 8.2), followed by rinsing with deionised water. The filters were dried overnight and then fixed onto the SEM stubs and coated with gold. Photographs were taken with a JEOL Scanning Microscope (JSM-35).

Other methods

pH was measured with a 420-A Orion pH meter calibrated at pH 7 and 9.2. Photon flux density was determined with a Hansatech quantum meter type QRT1. Cell counts were made using a Fuchs-Rosenthal haemacytometer with 0.2 mm and 1/16 mm^2 ruling. A minimum of 400 cells was counted on each occasion.

The membrane-impermeable carbonic anhydrase inhibitor, acetazolamide (N-[5-sulfamoyl-1,3,4-thiadiazol-2-yl] acetamide), was dissolved in distilled water and the pH raised to 11 to allow complete dissolution and then readjusted to 8.2 for experiments (Beer & Rehnberg, 1997). The AE1 inhibitor, SITS (4-acetamido-4′-isothiocyanato-stilbene-2,2′-disulfonic acid), was dissolved in distilled water and DIDS in dimethyl sulphoxide (DMSO). Care must be taken with both disulfonic stilbenes after they have been dissolved. The manufacturer warns that both SITS and DIDS are sensitive to light so, in this study, solutions were prepared daily and were protected with aluminium foil.

Results

Photosynthesis and calcification in coccolith-bearing cells of *Emiliania huxleyi*

The photosynthetic response of coccolith-bearing cells to a wide range of DIC concentrations shows that, in this microalga, the rate was not saturated at the ambient seawater concentration of about 2 mM. Fig. 1 shows that photosynthetic O_2 evolution at 20 mM DIC was 70% faster than at 2 mM DIC. (V = 36.3 fmol O_2 $cell^{-1}$ h^{-1} and V = 21.5 fmol O_2 $cell^{-1}$ h^{-1}, respectively). At first glance, the kinetics seem to follow a simple hyperbolic pattern, but closer examination of the photosynthetic response at low DIC concentrations shows that the relationship is biphasic (Fig. 1 inset a). A double reciprocal plot of the data (Fig. 1 inset b) shows this clearly.

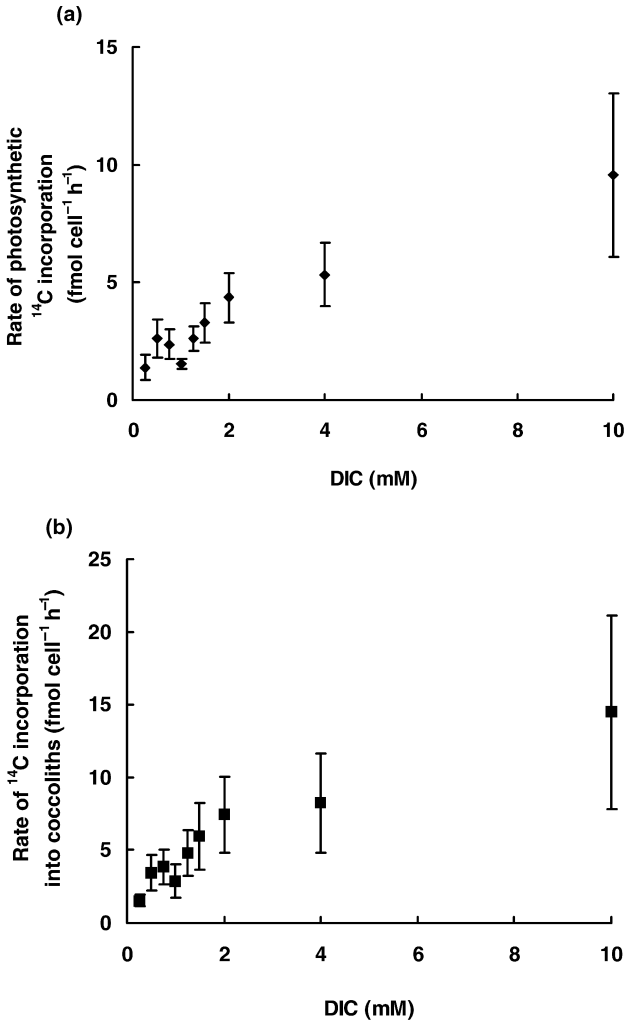

Fig. 2 The effect of different external DIC concentrations on ^{14}C incorporation into coccolith-bearing cells of *Emiliania huxleyi*. (a) Photosynthesis measured as the rate of ^{14}C incorporation into acid stable compounds. Data points are means ± SE (n = 4). (b) Calcification measured as the rate of ^{14}C incorporation into coccoliths. Data points are means ± SE (n = 4).

Below 1 mM DIC, the apparent $K_{0.5}$ was 0.3 mM and the V_{max} was 14.1 fmol O_2 cell^{-1} h^{-1} whilst, above 1 mM DIC, the $K_{0.5}$ was 2.6 mM and the V_{max} was 46.5 fmol O_2 cell^{-1} h^{-1}. Thus in *E. huxleyi*, photosynthetic O_2 evolution shows high affinity, low V_{max} kinetics below 1 mM DIC and low affinity, high V_{max} kinetics above 1 mM DIC.

The same biphasic pattern was evident when ^{14}C uptake was used to measure photosynthesis (Fig. 2a) in plated cells of this coccolithophore, and again when calcification rate was measured by $^{14}C_2$ incorporation into the coccoliths (Fig. 3b). It is possible that the low rates shown at 1 mM are outliers, but the consistency of the pattern in both types of cell, the fact that the results were obtained using different, independent methods of measurement, plus the high precision of the data at that point, all indicate that this is an accurate result.

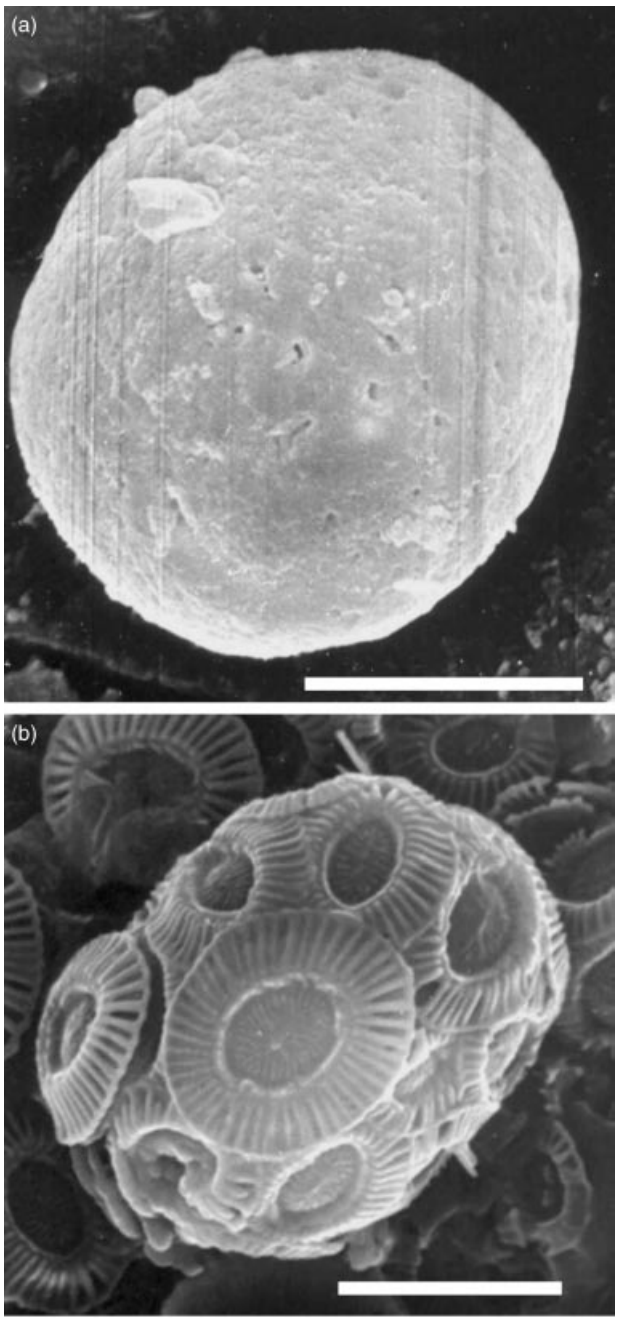

Fig. 3 Scanning electron micrographs of (a) coccolith-free and (b) coccolith-bearing cells of *Emiliania huxleyi* grown in calcium-free and normal media respectively. Bars, 5 μm.

Photosynthesis in coccolith-free cells of *Emiliania huxleyi*

The appearance of a coccolith-free cell of this alga is shown in Fig. 3(a). These cells were produced from the calcifying strain (PCC.B11) of *E. huxleyi* by sub-culturing coccolith-bearing cells into growth medium lacking $CaCl_2$. For comparison, a plated cell with well-developed coccoliths (grown in complete

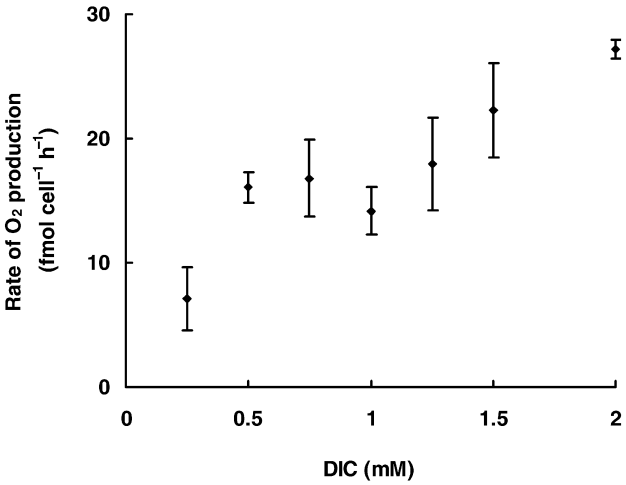

Fig. 4 The effect of different external dissolved inorganic carbon (DIC) concentrations on the net O_2 production rate of coccolith-free cells of *Emiliania huxleyi* grown and incubated in calcium-free medium. Data points are means ± SE (n = 3–5).

medium containing 9.1 mM $CaCl_2$) is shown in Fig. 3(b). The light-saturated rates of photosynthesis were the same in cells with and without coccoliths. In coccolith-free cells, photosynthesis at different DIC concentrations showed the same biphasic pattern as in the coccolith-bearing cells (Fig. 4). This is an interesting result since it shows clearly that the kinetics of photosynthesis in *E. huxleyi* are the same whether or not calcification is taking place.

Identification of an anion exchange protein and an external carbonic anhydrase

Bicarbonate transport in animal cells such as mammalian erythrocytes has been the subject of a great deal of research and one result of this has been the development of numerous specific inhibitors. Two of these, the membrane-impermeable compounds SITS and DIDS, were first employed with algae in an investigation of bicarbonate transport in the red seaweed *Chondrus crispus* (Smith & Bidwell, 1989). In this study we added SITS or DIDS to actively photosynthesising cultures of *E. huxleyi* in the oxygen electrodes and measured any change in rate.

Figure 5(a) is a double reciprocal plot of O_2 evolution at different DIC concentrations with and without 0.5 mM SITS. The inset graph shows the dose–response curve for SITS of a culture photosynthesising in 2 mM DIC. At 0.5 mM SITS, the inhibition was 53%, and at 1.5 mM SITS, oxygen evolution rates were reduced below the compensation point. The reciprocal plot indicates that the inhibition was competitive since the apparent $K_{0.5}$ was increased by SITS from 3.2 to 7.3 mM while the V_{max} remained similar with and without SITS, at 34.7 and 43.5 fmol O_2 cell^{-1} h^{-1}, respectively.

Fig. 5(b) shows the results of a similar experiment using 50 μM DIDS. At this concentration, DIDS clearly inhibits about 50% of photosynthesis in *E. huxleyi*, and the inset shows that 0.1 mM DIDS inhibits photosynthetic O_2 production by 70%. This inhibitor caused a five-fold decrease in the affinity of the cells for the substrate; the $K_{0.5}$ being 3.2 mM for the control and 14.8 mM in the presence of 50 μM DIDS. V_{max} values were similar at 43.4 and 52.6 fmol cell^{-1} h^{-1} for the control and inhibited cells, respectively. The effect of DMSO alone was tested at 2 and 10 mM DIC. Although this solvent did not decrease the photosynthetic rate at high DIC concentration, it had a small inhibitory effect (< 10%) at 2 mM DIC. The results are presented without correction for this inhibition at 2 mM DIC.

The effect of acetazolamide, a membrane-impermeable, specific inhibitor of carbonic anhydrase activity, was also tested using photosynthesising cultures grown at 2 mM DIC. No significant external CA activity was evident in these cultures.

Since it is well-known that carbonic anhydrase can be induced, or at least activated, under 'low DIC' conditions (Matsuda & Colman, 1995; Sasaki *et al.*, 1997; Kaplan *et al.*, 1998), the SITS and acetazolamide experiments were repeated with plated cells of *E. huxleyi* grown at 0.5 mM DIC rather than at 2 mM DIC. Figure 6 shows that SITS is clearly still active at 0.5 mM DIC and, as expected, a given concentration of this competitive inhibitor has a more potent effect at lower substrate concentration (Cabantchik & Greger, 1992). At 2 mM DIC for example, 0.25 mM SITS inhibited photosynthesis by 26%, whereas at 0.5 mM DIC, the same concentration of inhibitor caused 74% inhibition.

Figure 7 shows that in contrast to the absence of significant inhibition when 0.1 mM acetazolamide was added to *E. huxleyi* grown and incubated at 2 mM DIC, there was significant inhibition in cells grown and incubated at 0.5 mM DIC (t-test: p = 0.03). The inset shows the dose–response of acetazolamide for cells grown and incubated at 0.5 mM DIC.

Discussion

Dissolved inorganic carbon is not generally considered an important limiting factor for the growth and photosynthesis of marine phytoplankton (Redfield, 1958). It is present at a concentration two to three orders of magnitude higher than the commonly cited main limiting factors, nitrogen and phosphorus, and five to six orders of magnitude more than iron. *Emiliania huxleyi*, however, is regarded as an exception (Falkowski & Raven, 1997). Paasche (1964) was the first to demonstrate that photosynthesis in *E. huxleyi* was not saturated at 2 mM DIC; in fact the strain he isolated from Oslo Fjord required 15 times the DIC concentration of seawater to achieve saturation. In the present study, photosynthesis was not saturated at 2 mM DIC in either the coccolith-bearing or the coccolith-free form of strain PCC.B11. Also, by measuring photosynthetic rate at very small increments in the concentration of DIC, we have shown that the kinetics are biphasic.

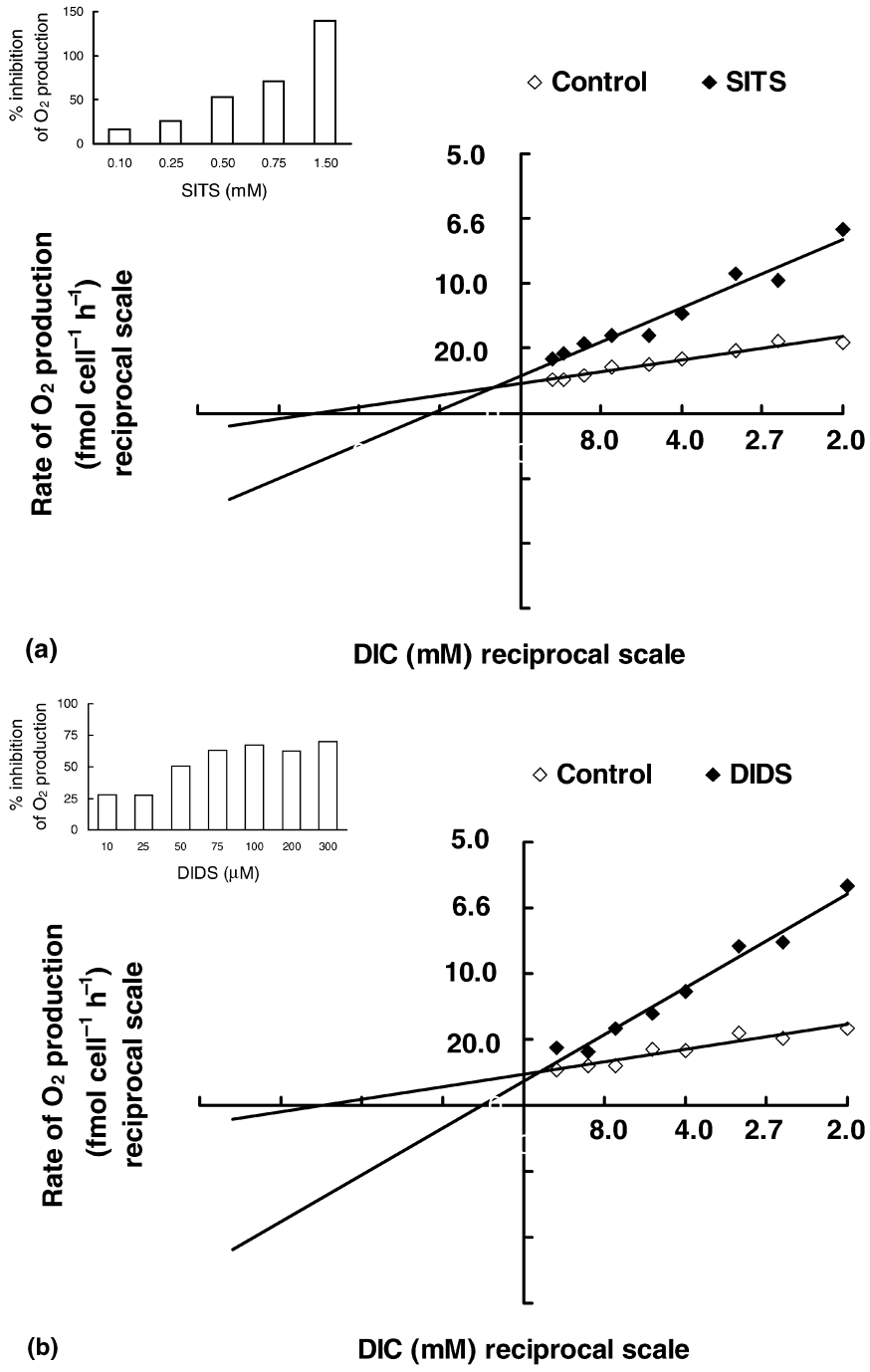

Fig. 5 The effect of different external dissolved inorganic carbon (DIC) concentrations on photosynthetic O_2 production by coccolith-bearing cells of *Emiliania huxleyi* (a) with or without the addition of 0.5 mM SITS (control, open diamonds; SITS, closed diamonds) and (b) with or without the addition of 50 μM DIDS (control, open diamonds; DIDS, closed diamonds). SITS and DIDS are specific inhibitors of the anion exchange protein, AE1. Data are presented as double reciprocal plots in order to highlight the difference in kinetic parameters. Data points are means of six measurements. Inserts: Dose-response for SITS or DIDS and photosynthetic O_2 production in coccolith-bearing cells of *E. huxleyi* grown and incubated at 2 mM DIC. Values greater than 100% indicate that cells were consuming O_2.

This is not an entirely novel discovery. Paasche suspected that the kinetics of photosynthesis with DIC in *E. huxleyi* may have been unorthodox, but regarded it as a function of his specific strain. Sekino *et al.* (1996) working with a South Pacific strain of *E. huxleyi* did not consider the kinetics to be biphasic until they used a double reciprocal transformation, when they noted that the data fitted a linear model only if twin slopes were used. In this investigation, the kinetics of photosynthesis in coccolith-bearing, actively calcifying cells were clearly biphasic without transformation of the data and when the rates were measured by two independent methods.

The biphasic kinetics suggested either that two processes or that two mechanisms of transport were involved. The idea that the hiatus between the two phases at 1 mM DIC might be caused by the onset of calcification at that concentration, and that the CO_2 produced during calcite formation was stimulating photosynthesis above 1 mM, was convincingly

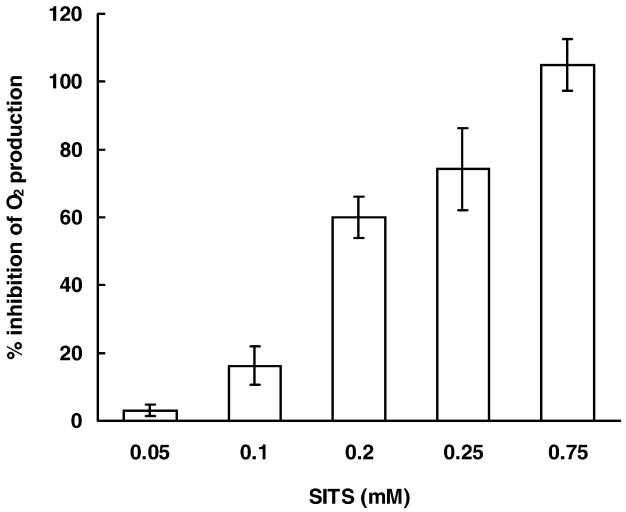

Fig. 6 The dose-response for SITS and photosynthetic oxygen production rate in coccolith-bearing cells of *Emiliania huxleyi* grown and incubated at 0.5 mM dissolved inorganic carbon (DIC). Data points are means ± SE (n = 9–11). Values greater than 100% indicate that cells were consuming O_2.

refuted by measurements of calcification over the same range of DIC concentrations. Not only was it clear that calcification occurred at lower DIC concentrations than 1 mM, but the kinetics of calcification were also biphasic with a hiatus located at 1 mM. In addition, the photosynthetic kinetics of coccolith-free, non-calcifying cells of the same strain of *E. huxleyi* showed the same pattern. It was clear therefore, that the biphasic pattern was not caused by the interaction of the twin processes of photosynthesis and calcification, but by two mechanisms affecting both processes.

Although it is well-established that bicarbonate is the major source of carbon for both photosynthesis (Paasche, 1964; Nimer & Merrett, 1992; Buitenhuis *et al.*, 1999) and calcification (Paasche, 1964; Sikes *et al.*, 1980; Buitenhuis *et al.*, 1999) in *E. huxleyi*, the precise mechanisms of uptake remained unidentified. There are many reports that this alga lacks external carbonic anhydrase activity (Sikes & Wheeler, 1982; Nimer *et al.*, 1994; Sekino & Shiraiwa, 1994), and the presence of a bicarbonate transporter has also been discounted (Nimer *et al.*, 1996). By contrast, in this study we provide strong evidence, using both DIDS and SITS, that bicarbonate transport is indeed mediated via an anion exchange protein in *E. huxleyi*. The discrepancy between our data and those of Nimer *et al.* (1996) may arise from methodological differences. We used short-term incubations in oxygen electrode chambers, whereas the pH drift experiments took 12 h. In our experiments, SITS and DIDS were exposed to light for only a short time (15–20 min), and after the initial clear depression of the photosynthetic rate caused by addition of the inhibitors, no recovery was evident. We have found that exposure to bright light for 5 h completely inactivates DIDS, so it is possible that 12 h of continuous light, even at a PFD of 50 µmol (m^{-2} s^{-1}), inactivated the inhibitor during the pH drift experiments of Nimer *et al.* (1996).

In the present study, inhibitors of AE1 showed that this bicarbonate transporter is active at all concentrations of DIC tested. AE1 is thus the main means by which this strain of *E. huxleyi* acquires DIC, but an additional mechanism is required to explain the high affinity, low V_{max} kinetics below 1 mM DIC. The reversible bicarbonate-dehydroxylating enzyme, carbonic anhydrase is induced or activated at low concentrations of DIC. External CA activity has been detected

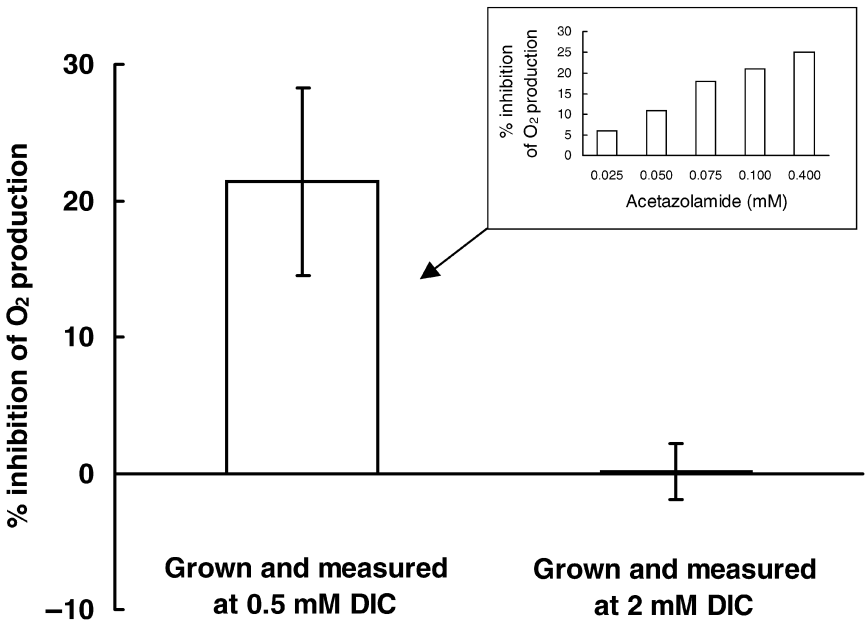

Fig. 7 The effect of 0.1 mM acetazolamide, a specific inhibitor of extracellular carbonic anhydrase, on the rate of photosynthetic O_2 production in coccolith-bearing cells of *Emiliania huxleyi* grown and measured at 0.5 mM dissolved inorganic carbon (DIC) or 2 mM DIC. Data points are means ± SE (n = 11). Insert: The dose-response of acetazolamide on photosynthetic O_2 production in coccolith-bearing cells of *E. huxleyi* grown and incubated at 0.5 mM DIC.

in *E. huxleyi* in cells in either the stationary phase of growth (Nimer *et al.*, 1994; Nimer & Merrett, 1996) in ambient seawater, or in the exponential growth phase at 1 mM DIC and pH 8.7, when the concentrations of both CO_2 and HCO_3^- were limited (Nimer *et al.*, 1997). In *E. huxleyi* PCC.B11, the rate of photosynthesis was significantly decreased by an inhibitor of external CA in cells grown and incubated at 0.5 mM DIC. Thus, we infer that the biphasic pattern is caused by the interaction of two bicarbonate acquisition mechanisms. At low DIC concentration, bicarbonate uptake is supported by both AE1 and external CA, but by AE1 alone at higher concentrations. This could explain the low rates at 1 mM DIC shown in Figs 2 and 4. At this concentration, we suggest that CA is switched off and AE1 alone cannot support the same rates as the two mechanisms acting together. AE1 acts more efficiently at higher DIC concentrations.

We do not yet know what switches off or represses CA around 1 mM DIC. AE1 is a chloride-bicarbonate exchanger and as more bicarbonate is imported at higher external concentrations of DIC, more chloride leaves the cells. CA has been shown to be inhibited by high concentrations of chloride ions (Stemler, 1986) so, if significantly higher Cl^- concentrations accumulate in the periplasmic space, this might be a mechanism for switching off CA. We realise that it would be impossible to increase the bulk phase chloride concentration by more than a few percent, but recent work with red blood cell CA and AE1, shows them to be closely associated in a carbon-transporting 'metabolon' (Sterling *et al.*, 2001). In this way, the molecular architecture of the two proteins could allow small changes in chloride concentration to have a disproportionate impact on CA.

The relationship between the processes of calcification and photosynthesis in organisms such as corals and coccolithophores continues to be controversial. The fact that calcification proceeds faster in the light is the central observation that has led to the view that photosynthesis somehow supports calcification. The reverse idea, that calcification functionally precedes photosynthesis via CO_2 production during calcite deposition, is of more recent origin (McConnaughey, 1994; McConnaughey & Whelan, 1997), and is a view that our results do not support. Somewhat surprisingly, or at least counter-intuitively, even at the lowest DIC concentration tested, 0.25 mM, the photosynthetic rate of calcifying cells was no faster than that in non-calcifying ones (6.7 and 7.1 fmol $cell^{-1}$ h^{-1}, respectively).

A similar conclusion was drawn by Gattuso *et al.* (2000) who used a standard (11.40 mmol kg^{-1}) and a low (2.85 mmol kg^{-1}) calcium medium with the zooxanthellate coral *Stylophora pistillata* in order to investigate the effect of calcification on photosynthesis. The low calcium medium caused a 2.0–2.4-fold decrease in calcification rate, but photosynthesis was not significantly different from that in the standard medium. In our study, we made two sequential subcultures of coccolith-bearing *E. huxleyi* cells into calcium-free medium, but enough calcium remained to support rapid photosynthesis. Moreover, the coccolith-free cells, produced by this treatment, were healthy looking and grew at 1.51 divisions d^{-1}, the same rate as the coccolith-bearing cells (1.57 divisions d^{-1}). This work is in direct conflict with Nimer *et al.* (1996) who claim that photosynthesis and calcification in *E. huxleyi* are equally dependent on the Ca^{2+} concentration in the medium. Our results demonstrate clearly that photosynthesis is far less dependent on Ca^{2+} than calcification and that although bicarbonate acquisition drives both processes, its uses in photosynthesis and in calcification may be quite independent.

The presence of a bicarbonate ion exchanger has been recorded for rather few algae, although it is widespread in higher animals. For example, AE1 constitutes 25% of the membrane proteins of mammalian erythrocytes (Smith, 1988), where its role is to transport bicarbonate out rather than in. It is possible that its importance among marine plants may be underestimated. At the current DIC concentration of seawater, *E. huxleyi* employs only AE1 for bicarbonate transport, although this mechanism is operating well below its maximum capacity and, in that sense, this alga would seem better adapted to an ocean with a high DIC concentration. Historically, the coccolithophores emerged during the Tertiary when the concentrations of both CO_2 and HCO_3^- were much higher than today (Lovelock & Whitfield, 1982; Dismukes *et al.*, 2001; Paasche, 2001). Perhaps AE1 is an atavistic uptake mechanism and external CA is a supplementary DIC scavenger analogous to the C_4 metabolism in terrestrial plants, which arose as atmospheric CO_2 concentrations were declining in the Late Tertiary. CA certainly seems the more common means by which modern marine plants acquire DIC (see Colman *et al.*, 2002 for review). It is true, though, that studies of DIC uptake deal almost exclusively with concentrations of 2 mM and below (e.g. Nimer *et al.*, 1996; Tortell *et al.*, 1997; Franklin & Badger, 2001). Also, if CA activity has been found, further mechanisms tend not to be sought, and only when CA is absent does screening for AE1 occur (e.g. Dong *et al.*, 1991). This was also the case when Nimer *et al.* (1997) screened a variety of marine phytoplanktonic algae and showed AE1 to be present in only three of the 18 species. AE1 is present too in *Gracilaria gaditana* (Andria *et al.*, 1999), *Enteromorpha intestinalis* (Larsson *et al.*, 1997) and *Ulva* sp. (Drechsler *et al.*, 1993; Axelsson *et al.*, 1995). This study is the first to report the existence in *E. huxleyi* of a bicarbonate transporter that is sensitive to SITS and DIDS. We suggest that AE1 will prove detectable in a far wider range of marine plants if these are screened at higher than ambient concentrations of DIC.

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