Carbon dioxide availability, intracellular pH and growth rate of the coccolithophore *Emiliania huxleyi*

N. A. Nimer¹, C. Brownlee², M. J. Merrett¹

¹School of Biological Sciences, University College of Swansea, Singleton Park, Swansea SA2 8PP, United Kingdom
²Marine Biological Association, The Laboratory, Plymouth PL1 2PB, United Kingdom

ABSTRACT: Growth of a high-calcifying strain of *Emiliania huxleyi* (Lohmann) Hay & Mohler was investigated in cultures aerated with varying concentrations of CO₂ in air and compared with growth in 0.03\% (v/v) CO₂. Cultures aerated with 0.1\% (v/v) CO₂/air under identical conditions resulted in approximately 40\% reduction in cell number and final cell yield. A concentration of 0.5\% (v/v) CO₂ completely inhibited growth. In the virtual absence of CO₂, cells could grow to the same levels as in those cultures aerated with air-equilibrated levels of CO₂, i.e. 0.03\% (v/v) CO₂. Measurement of internal pH (pHᵢ) gave comparable results using either the 5,5-dimethyl-2-[¹⁴C]oxazalidine-2,4-dione (DMO) method or a fluorescent probe technique (BCECF-AM). At external pH 8.3, intracellular pH of cells aerated with air was 6.9 whilst pHᵢ of cells aerated with 0.1\% (v/v) CO₂ was 6.4. Lowering external pH decreased growth rate for cultures aerated with 0.03\% (v/v) CO₂. A 30\% reduction in cell number and final cell yield occurred at pH 7.8 increasing to almost 60\% at pH 7.0; pHᵢ decreased at more acidic external pH down to 6.38 at pH 7.0. Carbon dioxide concentration and external pH appear to be equally important in the growth of high-calcifying cells. The significance of these results is considered in relation to the development of mesoscale blooms of *E. huxleyi*.

KEY WORDS: *Emiliania huxleyi* · Growth · Inorganic carbon · Intracellular pH

INTRODUCTION

Metabolic processes in microalgal cells are compartmentalized at the intracellular level, the proton concentration being maintained within narrow limits in the various compartments. The homeostasis of pH at all levels of cellular organisation is achieved by the balance between the rates of proton-producing and proton-consuming reactions and fluxes of H⁺ or OH⁻ through various transporters in conjunction with passive diffusion pathways (Haussinger et al. 1988, Tester 1990).

The internal pH (pHᵢ) in *Emiliania huxleyi* (Lohmann) Hay & Mohler cells has to be precisely regulated because the high- and low-calcifying strains show a negative membrane potential (Sikes & Wilbur 1982), as with all cells growing in seawater (Nimer et al. 1992). This will lead to the passive diffusion of protons into the cytosol, thus altering the pHᵢ. But this passive diffusion depends on the H⁺ permeabilities of the plasmalemma and the proton electrochemical gradient (ΔµH⁺), where ΔµH⁺ = Eₘ + 58(pHₑ - pHᵢ) = (-60) + 58(8 - 7.3) = -60 + 41 = -19 mV [where pHₑ is external pH, and Eₘ = RT/3F where R is gas constant, T is temperature (K) and F is Faraday constant] so the inward driving force for H⁺ is very small. Both H⁺ consuming reactions (including NO₃⁻, SO₄²⁻, reduction, DMSP production) and H⁺ producing reactions (Raven & Smith 1974, 1976) occur in microalgae and, in the case of *E. huxleyi*, this includes biologically driven intracellular calcification (Brownlee et al. 1994b), so a pH regulating mechanism is needed to counteract acidification of the cytosol.

In microalgae, CO₂ is the substrate for ribulose biphosphate carboxylase (Rubisco), but in high-calcifying cells of *Emiliania huxleyi*, HCO₃⁻ provides the inorganic carbon substrate for calcification (Sikes et al. 1980). In microalgae capable of the direct utiliza-
tion of HCO$_3^-$ as the exogenous inorganic carbon source for photosynthesis (Rees 1984, Patel & Merrett 1986, Dixon et al. 1987, Nimer & Merrett 1992). pH is expected to be an important factor in maintaining the CO$_2$/HCO$_3^-$ equilibrium within the cytosol. In high-calcifying cells of _Emiliania huxleyi_, H$^+$ fluxes at the Golgi/coccolith vesicle membrane will be important in relation to HCO$_3^-$ availability (Brownlee et al. 1994b). Since there is a close relationship between calcification and photosynthesis (Nimer & Merrett 1994b) cytosolic pH may be important in relation to the availability of inorganic carbon to Rubisco in the chloroplast.

Many species of marine phytoplankton will grow well when aerated with air enriched with CO$_2$ (Shiraiwa et al. 1988, Umino et al. 1991) but high-calcifying cells of _Emiliania huxleyi_ failed to grow when aerated with air enriched with 0.5% (v/v) CO$_2$. If the cells are unable to utilize this extra CO$_2$, the high ambient CO$_2$ concentrations will result in the passive diffusion of CO$_2$ across the plasma membrane and acidification of the cytosol.

The pH$_i$ in cyanobacteria (Falkner et al. 1976, Coleman & Colman 1981) and green algae (Lane & Burris 1981, Tromballa 1983, Gimmler et al. 1988, Goyal & Gimmler 1989), has been measured using 5,5-dimethyl-[14C]oxalidine-2,4-dione (DMO), while the fluorescent probe 2',7'-bis-(2-carboxyethyl)-5 (and -6) carboxyfluorescein acetoxyethyl ester (BCECF-AM) has been used in conjunction with dual wavelength fluorescence microscopy to measure the pH$_i$ in low-calcifying cells of _Emiliania huxleyi_ (Dixon et al. 1989). In the present study the 2 methods are compared and used to investigate possible potential interactions between inorganic carbon availability, pH, and growth.

**METHODS**

**Growth of cells.** Axenic cultures of a high-calcifying strain of _Emiliania huxleyi_ (Bigelow Laboratories No. 88E) were grown on the same medium as described previously (Nimer & Merrett 1992) using an initial inoculum of 10$^5$ cells ml$^{-1}$. When required, the pH of the medium was maintained using N-[tris(hydroxymethyl)-methyl] glycine (Tricine) for pH at 8.3 and 2-hydroxy-1-piperazine ethanesulfonic acid (HEPES) for pH 7.8 and 7.0. Cultures were gently aerated with air (0.03% v/v CO$_2$) or CO$_2$-enriched air (0.1% and 0.5% v/v CO$_2$) using a gas blender 852 (Signal Instrument Company Limited, Surrey, UK) at a flow rate of approximately 1 l h$^{-1}$ and grown at 15°C at a photon flux density of 50 μmol m$^{-2}$ s$^{-1}$ at the surface provided by cool white fluorescent lamps. At this flux density the growth rate was 0.5 divisions d$^{-1}$ (see Fig. 2) compared with 1.5 divisions d$^{-1}$ at higher photon flux densities (Brand 1982).

**Carbon starved cells.** Harvested cells were resuspended in fresh medium lacking NaHCO$_3$ and the suspension incubated at 20°C in a Clark-type oxygen electrode (Hansatech, Ltd, Kings Lynn, Norfolk, UK) at a photon flux of 500 μmol m$^{-2}$ s$^{-1}$ (Ellipsoid Halogen reflector bulb, Philips, Eindhoven, The Netherlands). The chamber was closed and the cells were allowed to deplete all endogenous carbon sources as measured by the cessation of oxygen evolution (compensation point). Over the relatively short period (ca 30 to 45 min) required to take cells to the compensation point, photoinhibition of inorganic-carbon-dependent photosynthetic oxygen evolution was not observed.

**Measurement of internal pH (pH$_i$).** Measurement of pH$_i$ using 5,5-dimethyl-2-[14C]oxalidine-2,4-dione (DMO): This method requires the measurement of the distribution of this radio-labelled weak acid between the intracellular space and an external medium of known pH. This was carried out as described previously (Goyal & Gimmler 1989) using the silicone oil centrifugation technique to separate cells from incubation medium. Harvested cells were resuspended in media of required pH at a density of 1 × 10$^6$ cells ml$^{-1}$. Cells were incubated with 0.95 μCi 14C-DMO in a total aliquot volume of 2.4 ml in the light (50 μmol m$^{-2}$ s$^{-1}$) at room temperature (18°C). A total of 50 μl of cell suspension was withdrawn and placed in a 400 μl microtube containing from bottom to top 50 μl 0.75% (w/v) sodium lauryl sulphate (SDS) in 1 M glycine, 60 μl Versilube F 50 silicone oil (specific gravity 1.050 and 70 centistokes viscosity) and centrifuged in a Microfuge B centrifuge (Beckman). The bottom portion of the tubes containing the cells was immediately frozen in liquid nitrogen, removed by razor blade, resuspended in 500 μl distilled water. Radioactivity was measured using 10 ml scintillation fluid in a Beckman LS 6800 liquid scintillation counter. The intracellular space and free water space taken down with the cells through the silicone oil was estimated using 5 μM 14C-inulin and 3H$_2$O. Separate incubations with each were for 20 s. 14C and 3H activities in the pellet were determined by liquid scintillation counting. The intracellular space was determined as the total water volume minus the inulin permeable space. pH$_i$ was calculated using the Henderson-Hasselbalch equation as described by Tromballa (1983).

**Measurement of pH$_i$ using 2',7'-bis(2-carboxyethyl)-5 (and -6) carboxyfluorescein acetoxyethyl ester (BCECF-AM):** This was as described previously (Dixon et al. 1989). The final intracellular concentration of BCECF-AM was 5.0 μM. Cells were settled on a cover slip pretreated with poly-L-lysine (slide adhesive solution) and incubated for 2 h in the light (50 μmol m$^{-2}$ s$^{-1}$). A perfusion system was used to study the effect of
external media on pH, on the same batch of cells under study.

Measurement of cell number. Cell number was determined using an improved Neubauer haemocytometer.

RESULTS

Time course of 14C-DMO uptake by Emiliania huxleyi

The relatively high surface area to volume ratio of Emiliania huxleyi cells allowed a rapid net influx of DMO, causing the cells to equilibrate with external DMO in less than 20 min at pH 8.3, 7.8 and 7.0 (Fig. 1). The final intracellular concentration of DMO was 7.5 μM.

pH, in carbon-starved and HCO3− replete cells

Comparison of the pH, using the DMO and the fluorescent probe technique for high-calcifying cells of Emiliania huxleyi (88E) at an external pH of 8.3 in the presence or absence of HCO3− gave similar values (Table 1). In the presence of 2 mM HCO3− the pH, of cells was 7.03 using the fluorescent probe and 6.77 by the DMO method. With carbon-starved cells the pH, fell to 6.33, measured by the fluorescent probe technique and to 6.15 by the DMO method. The pH, measured by the fluorescent probe technique was marginally higher than that measured by the DMO method (Table 1).

pH, in relation to CO2 availability

Cells were grown at different air-equilibrated levels of CO2 (0.03, 0.1 and 0.5% v/v CO2/air) maintaining the pH of the medium constant using organic buffers. Growth was not observed with 0.5% (v/v) CO2/air while 0.1% (v/v) CO2/air reduced the growth rate from 0.3 to 0.23 divisions d−1 producing approximately 65% of the final cell biomass of cultures grown on air levels of CO2 (0.03% v/v CO2/air) (Fig. 2). In contrast to elevated CO2 concentrations, decreasing the CO2 concentration to minimal levels in the culture medium did not affect the growth of the high-calcifying cells of Emiliania huxleyi (Fig. 2).

Cells were grown at pH 8.3 at 2 different CO2 concentrations to investigate the effect of CO2-enriched air on pH,. The pH, (measured by 14C-DMO) dropped by approximately 0.4 pH units from 6.77 when grown at air levels of CO2 (0.03% v/v CO2/air) to 6.38 when cells were grown at 0.1% (v/v) CO2/air (Table 2).

Table 1. Emiliania huxleyi. Effect of the presence of 2 mM HCO3− on pH, in high-calcifying cells using the fluorescent probe technique and the DMO method (growth media as described in ‘Material and methods’)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pH, measured by:</th>
<th>Fluorescent probe technique</th>
<th>DMO method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth media with 2 mM HCO3−</td>
<td>7.03 ± 0.14</td>
<td>6.77 ± 0.31</td>
<td></td>
</tr>
<tr>
<td>Growth media lacking HCO3−</td>
<td>6.33 ± 0.12</td>
<td>6.15 ± 0.29</td>
<td></td>
</tr>
</tbody>
</table>

*Intracellular pH monitored as the average (± SE) 480/450 excitation ratio of several (10) E. huxleyi cells loaded with the ratiometric pH indicator BCECF (Dixon et al. 1989). Cells were immobilized on 0.01% poly-L-lysine coated coverslips.

bPoints represent the average (± SE) of 3 replicate readings.
Fig. 2. *Emiliania huxleyi*. Growth of high-calcifying cells in relation to CO₂ concentration. Cell number in cultures grown at air levels of CO₂ (0.03% v/v CO₂/air) (○), 0.1% (v/v) CO₂/air (□), 0.5% (v/v) CO₂/air (∆) and CO₂-free air (■). All cultures grown at 15°C, 2 mM dissolved inorganic carbon, 20 μM NO₃⁻, pH 8.3, 50 μmol m⁻² s⁻¹ photon flux density. Points represent the average of 3 replicate readings.

stationary phase. When cells were grown at pH 8.3 the pHₐ at mid growth phase was 6.77, which was maintained almost unchanged during late growth phase and stationary phase (Table 2).

**DISCUSSION**

Possible limitations of the DMO technique have been reviewed (Boron & Roos 1976, De Michelis et al. 1979, Gimmler & Hartung 1988, Goyal & Gimmler 1989). In the present study the DMO method and the fluorescent probe technique gave comparable results and provided reliable methods for pHₐ measurements in high-calcifying cells of *Emiliania huxleyi*. The fluorescent probe technique has the advantage that it allows measurement of the spatial and temporal variation in pH of a single cell and the rapid change in pHₐ in response to external conditions.

Cytoplasmic pH in the high-calcifying cells of *Emiliania huxleyi* (Table 1) is more acidic than that reported for other plant cells (Smith 1979, Raven 1980) and other non-calcifying marine microalgae (Raven & Smith 1980, Burns & Beardall 1987) but is similar to values found for the low-calcifying cells (Dixon et al. 1989) with a pHₐ near neutrality. This suggests that the optimum pHₐ for cellular metabolic pathways in *E. huxleyi* may be different from some other algae. The dependence of various enzyme activities on pH relies mainly on enzyme protonation, a critical process for their biological function, which is controlled by pHₐ (Haussinger et al. 1988).

pHₐ was strongly dependent on the availability of HCO₃⁻ in the medium. With both pH measurement techniques the addition of HCO₃⁻ to carbon-starved cells resulted in an increase in pHₐ, while its removal resulted in the cytosolic acidification, suggesting a role of HCO₃⁻ in buffering cytosolic pH. HCO₃⁻ influx (Sikes & Wilbur 1982, Nimer & Merrett 1992) was shown previously to be very rapid (Nimer & Merrett 1992). The rise in pHₐ in the presence of HCO₃⁻ might be due to an increase in OH⁻ in the cytosol as a result of the production of CO₂ from HCO₃⁻ occurring in the chloroplast or the cytosol (Nimer & Merrett 1993, Brownlee et al. 1994b). However, if HCO₃⁻ transport across the plasmalemma was coupled to the export of OH⁻ or influx of H⁺, cytosolic alkalinization during HCO₃⁻ uptake would not be expected. Calcification in high-calcifying cultures of *Emiliania huxleyi* in a simulated seawater medium results in rapid utilization of dissolved inorganic carbon (DIC), largely as HCO₃⁻.

Table 2. *Emiliania huxleyi*. Effect of growth media pH and different CO₂ concentrations at pH 8.3 on pHₐ of high-calcifying cells using the DMO method at different stages of culture growth. Values represent the average (± SE) of 3 replicate readings.

<table>
<thead>
<tr>
<th>External pH</th>
<th>Mid growth phase</th>
<th>Late growth phase</th>
<th>Stationary phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.0</td>
<td>6.19 ± 0.15</td>
<td>6.07 ± 0.10</td>
<td>6.08 ± 0.17</td>
</tr>
<tr>
<td>0.03% (v/v) CO₂/air</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 7.8</td>
<td>6.52 ± 0.23</td>
<td>6.41 ± 0.24</td>
<td>6.32 ± 0.19</td>
</tr>
<tr>
<td>0.03% (v/v) CO₂/air</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 8.3</td>
<td>6.77 ± 0.13</td>
<td>6.71 ± 0.20</td>
<td>6.75 ± 0.30</td>
</tr>
<tr>
<td>0.03% (v/v) CO₂/air</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 8.3</td>
<td>6.38 ± 0.16</td>
<td>6.35 ± 0.24</td>
<td>6.37 ± 0.18</td>
</tr>
<tr>
<td>0.1% (v/v) CO₂/air</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3. *Emiliania huxleyi*. Growth of high-calcifying cells in response to different external pH. Cell number in cultures grown at air levels of CO₂ (0.03% v/v CO₂/air) at pH 8.3 (●), pH 7.8 (○) and pH 7.0 (△). All cultures grown at 15°C, 2 mM dissolved organic carbon, 20 μM NO₃⁻, 50 μmol m⁻² s⁻¹ photon flux density. Points represent the average of 3 replicate readings.
but external pH remains unaltered (Dong et al. 1993, Merrett et al. 1993) while alkalinity decreases. This suggests the absence of a HCO$_3^-$/OH$^-$ antiport or HCO$_3^-$/$\text{H}^+$ symport at the plasmalemma.

CO$_2$ exerts a dual effect on pH. First, it will lower pH$_1$. Since it can diffuse passively across the plasmalemma, if it is present externally at higher concentrations, a state of equilibrium has to be reached. Second, CO$_2$ fixation by Rubisco will have the primary effect of increasing pH$_1$ by removing CO$_2$ but, as a result of CO$_2$ fixation by Rubisco, a larger metabolic effect can be observed, owing to the accumulation of acidic products. This was observed when cells were grown on media of different external pH, so that, at more acidic pH, pH$_1$ was lower and this was reflected in a decrease in growth rates. High-calculifying cells of Emiliania huxleyi show lower photosynthetic oxygen evolution at more acidic pH (Nimer & Merrett 1992). When external pH was buffered at pH 8.3 and CO$_2$ levels increased from 0.03 to 0.1% (v/v) CO$_2$/air, pH$_1$ and growth rate decreased while at 0.5% (v/v) CO$_2$/air high-calculifying cells failed to grow, in part because of the decrease in pH$_1$. Many species of marine phytoplankton restrict intracellular pH to between 7.0 and 7.4 by ion transport mechanisms and a high buffering capacity of the cytosol and these show enhanced growth at elevated CO$_2$ concentrations (Beardall & Raven 1981, Gehl & Colman 1985, Sultemeyer et al. 1989). The inhibition of growth of the high-calculifying cells of E. huxleyi at elevated CO$_2$ may be related to the inability to generate adequate OH$^-$ to neutralize the protons produced by calcification (Nimer & Merrett 1993) and to counteract the acidification of the cytosol resulting from the passive diffusion of CO$_2$ at elevated ambient concentrations.

The response of Emiliania huxleyi to CO$_2$ and HCO$_3^-$ (Dong et al. 1993) may be one of several parameters that interact in the development of E. huxleyi blooms. Field studies show coccolithophore blooms succeed spring diatom blooms (Holligan et al. 1983) when these become limited by nutrient depletion of the upper mixed layer. The depletion in CO$_2$ following the spring diatom bloom (Codispoti et al. 1982) would favour E. huxleyi since it is able to obtain the carbon for growth and calcification from HCO$_3^-$ (Dong et al. 1993) and has a very short generation time compared to other calcifying phototrophs. The generation of CO$_2$ from HCO$_3^-$ in calcification may also confer advantages in terms of photon and nutrient costs of carbon fixation in calcifying cells (Brownlee et al. 1994a) compared to phytoplankton with CO$_2$-concentrating mechanisms (Raven & Johnston 1991). However, conditions at the beginning of a spring diatom bloom might also be expected to favour growth of E. huxleyi since recent studies suggest the diffusive CO$_2$ flux to the surface of a diatom cell may be rate limiting for growth (Riebesell et al. 1993). It is possible that diatoms also have the potential to use bicarbonate (Patel & Merrett 1986, Dixon & Merrett 1988) or alternatively that the CO$_2$ concentration may be important at the beginning of a spring bloom. The growth of calcifying cells of E. huxleyi is inhibited at external CO$_2$ concentrations that increase the growth rate of diatoms and this differential effect may occur at the onset of a spring bloom giving a faster growth rate for diatoms than for E. huxleyi.

Acknowledgement. This work was supported by the EEC funding to the MAS2-CT92-0038 project.

LITERATURE CITED


nudans caused by light-dependent proton flux into the thylakoidal space. Plant Physiol. 58: 717–718

This article was submitted to the editor

Manuscript first received: December 6, 1993
Revised version accepted: April 25, 1994