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

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ABSTRACT: Growth of a high-calcifying strain of *Emiliania huxleyi* (Lohmann) Hay & Mohler was investigated in cultures aerated with varying concentrations of $CO_2$ in air and compared with growth in 0.03% (v/v) $CO_2$. Cultures aerated with 0.1% (v/v) $CO_2$/air under identical conditions resulted in approximately 40% reduction in cell number and final cell yield. A concentration of 0.5% (v/v) $CO_2$ completely inhibited growth. In the virtual absence of $CO_2$, cells could grow to the same levels as in those cultures aerated with air-equilibrated levels of $CO_2$, i.e. 0.03% (v/v) $CO_2$. Measurement of internal pH ($pH_i$) gave comparable results using either the 5,5-dimethyl-2[$^{14}$C]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 $pH_i$ of cells aerated with 0.1% (v/v) $CO_2$ was 6.4. Lowering external pH decreased growth rate for cultures aerated with 0.03% (v/v) $CO_2$. A 30% reduction in cell number and final cell yield occurred at pH 7.8 increasing to almost 60% at pH 7.0; $pH_i$ 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 ($pH_i$) 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 $pH_i$. But this passive diffusion depends on the $H^+$ permeabilities of the plasmalemma and the proton electrochemical gradient ($\Delta\mu H^+$), where $\Delta\mu H^+ = E_m + 58(pH_e - pH_i) = (-60) + 58(8 - 7.3) = -60 + 41 = -19$ mV [where $pH_e$ is external pH, and $E_m = RT/3F$ 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 $NO_3^-$, $SO_4^{2-}$ 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, $CO_2$ is the substrate for ribulose biphosphate carboxylase (Rubisco), but in high-calcifying cells of *Emiliania huxleyi*, $HCO_3^-$ 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), $pH_i$ is expected to be an important factor in maintaining the $CO_2/HCO_3^-$ equilibrium within the cytosol. In high-calcifying cells of *E. 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 the passive diffusion of $CO_2$ across the plasmalemma 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-2[$^{14}$C]oxalidine-2,4-dione (DMO), while the fluorescent probe 2',7'-bis-(2 carboxyethyl)-5 (and -6) carboxyfluorescein acetoxymethyl 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, $pH_i$ 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 using 5,5-dimethyl-2[$^{14}$C]oxalidine-2,4-dione (DMO):*** This method requires the measurement of the distribution of this radiolabelled 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 $1 \times 10^6$ cells $ml^{-1}$. Cells were incubated with 0.95 µC$_i$ $^{14}$C-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 microfuge tube 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 $^{14}$C-inulin and $^3H_2O$. Separate incubations with each were for 20 s. $^{14}$C and $^3$H 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 acetomethylester (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 $pH_i$ 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 $^{14}C$-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.

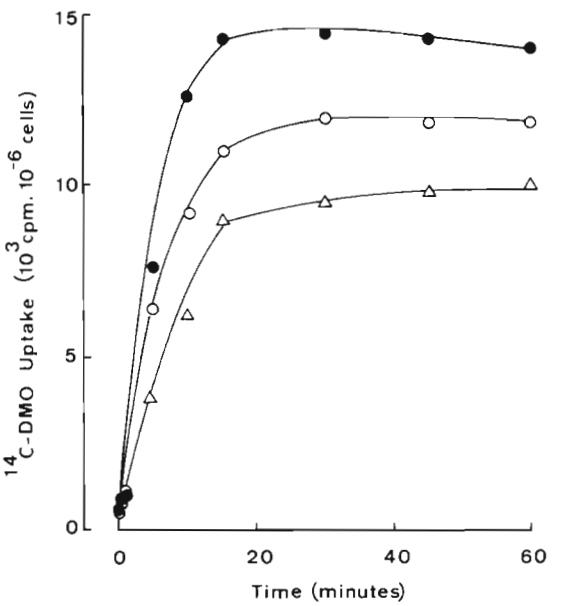


Fig. 1. *Emiliania huxleyi*. Time course of $^{14}C$-DMO uptake by high-calcifying cells at pH 8.3 (●), 7.8 (○) and 7.0 (△)

### $pH_i$ in carbon-starved and $HCO_3^-$ replete cells

Comparison of the $pH_i$ 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 $HCO_3^-$ gave similar values (Table 1). In the presence of 2 mM $HCO_3^-$ the $pH_i$ of cells was 7.03 using the fluorescent probe and 6.77 by the DMO method. With carbon-starved cells the $pH_i$ fell to 6.33, measured by the fluorescent probe technique and to 6.15 by the DMO method. The $pH_i$ measured by the fluorescent probe technique was marginally higher than that measured by the DMO method (Table 1).

### $pH_i$ in relation to $CO_2$ availability

Cells were grown at different air-equilibrated levels of $CO_2$ (0.03, 0.1 and 0.5% v/v $CO_2$/air) maintaining the pH of the medium constant using organic buffers. Growth was not observed with 0.5% (v/v) $CO_2$/air while 0.1% (v/v) $CO_2$/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 $CO_2$ (0.03% v/v $CO_2$/air) (Fig 2). In contrast to elevated $CO_2$ concentrations, decreasing the $CO_2$ 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 $CO_2$ concentrations to investigate the effect of $CO_2$-enriched air on $pH_i$. The $pH_i$ (measured by $^{14}C$-DMO) dropped by approximately 0.4 pH units from 6.77 when grown at air levels of $CO_2$ (0.03% v/v $CO_2$/air) to 6.38 when cells were grown at 0.1% (v/v) $CO_2$/air (Table 2).

### External pH, $pH_i$ and growth

The growth rate was reduced to 0.25 divisions $d^{-1}$ in cultures at external pH 7.8 and to 0.16 divisions $d^{-1}$ at external pH 7.0 (Fig. 3). In cells growing at external pH 7.0 the $pH_i$ was 6.19 in mid growth phase, declined to 6.07 by late growth phase and was maintained at this value throughout the stationary phase. At external pH 7.8, $pH_i$ was 6.52 at mid growth phase, declined to 6.41 by late growth phase and to 6.32 by the end of

Table 1. *Emiliania huxleyi*. Effect of the presence of 2 mM $HCO_3^-$ on $pH_i$ in high-calcifying cells using the fluorescent probe technique and the DMO method (growth media as described in 'Material and methods')

| Treatment | $pH_i$ measured by: | |
|---|---|---|
| | Fluorescent probe technique[a] | DMO method[b] |
| Growth media with 2 mM $HCO_3^-$ | 7.03 ± 0.14 | 6.77 ± 0.31 |
| Growth media lacking $HCO_3^-$ | 6.33 ± 0.12 | 6.15 ± 0.29 |

[a] 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

[b] Points represent the average (± SE) of 3 replicate readings

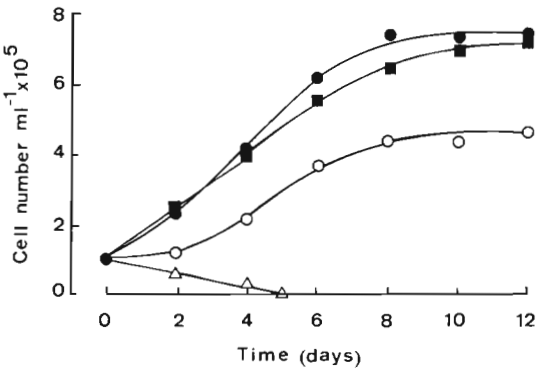


Fig. 2. *Emiliania huxleyi*. Growth of high-calcifying cells in relation to $CO_2$ concentration. Cell number in cultures grown at air levels of $CO_2$ (0.03% v/v $CO_2$/air) (●), 0.1% (v/v) $CO_2$/air (○), 0.5% (v/v) $CO_2$/air (△) and $CO_2$-free air (■). All cultures grown at 15°C, 2 mM dissolved inorganic carbon, 20 µM $NO_3^-$, pH 8.3, 50 µmol m$^{-2}$ s$^{-1}$ photon flux density. Points represent the average of 3 replicate readings

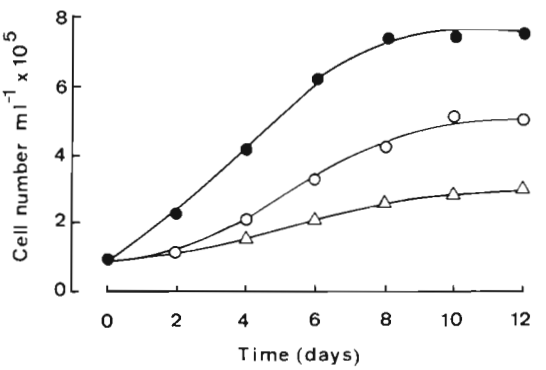


Fig. 3. *Emiliania huxleyi*. Growth of high-calcifying cells in response to different external pH. Cell number in cultures grown at air levels of $CO_2$ (0.03% v/v $CO_2$/air) at pH 8.3 (●), pH 7.8 (○) and pH 7.0 (△). All cultures grown at 15°C, 2 mM dissolved organic carbon, 20 µM $NO_3^-$, 50 µmol 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 $pH_i$ 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 has 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 $pH_i$ 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 $pH_i$ in response to external conditions.

Table 2. *Emiliania huxleyi*. Effect of growth media pH and different $CO_2$ concentrations at pH 8.3 on $pH_i$ of high-calcifying cells using the DMO method at different stages of culture growth. Values represent the average (± SE) of 3 replicate readings

| External pH | Mid growth phase | Late growth phase | Stationary phase |
|---|---|---|---|
| pH 7.0 0.03% (v/v) $CO_2$/air | 6.19 ± 0.15 | 6.07 ± 0.10 | 6.08 ± 0.17 |
| pH 7.8 0.03% (v/v) $CO_2$/air | 6.52 ± 0.23 | 6.41 ± 0.24 | 6.32 ± 0.19 |
| pH 8.3 0.03% (v/v) $CO_2$/air | 6.77 ± 0.13 | 6.71 ± 0.20 | 6.75 ± 0.30 |
| pH 8.3 0.1% (v/v) $CO_2$/air | 6.38 ± 0.16 | 6.35 ± 0.24 | 6.37 ± 0.18 |

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 $pH_i$ near neutrality. This suggests that the optimum $pH_i$ 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 pH (Haussinger et al. 1988).

$pH_i$ was strongly dependent on the availability of $HCO_3^-$ in the medium. With both pH measurement techniques the addition of $HCO_3^-$ to carbon-starved cells resulted in an increase in $pH_i$ while its removal resulted in the cytosolic acidification, suggesting a role of $HCO_3^-$ in buffering cytosolic pH. $HCO_3^-$ influx (Sikes & Wilbur 1982, Nimer & Merrett 1992) was shown previously to be very rapid (Nimer & Merrett 1992). The rise in $pH_i$ in the presence of $HCO_3^-$ might be due to an increase in $OH^-$ in the cytosol as a result of the production of $CO_2$ from $HCO_3^-$ occurring in the chloroplast or the cytosol (Nimer & Merrett 1993, Brownlee et al. 1994b). However, if $HCO_3^-$ transport across the plasmalemma was coupled to the export of $OH^-$ or influx of $H^+$, cytosolic alkalinization during $HCO_3^-$ 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 $HCO_3^-$,

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 $HCO_3^-/H^+$ symport at the plasmalemma.

$CO_2$ exerts a dual effect on $pH_i$. First, it will lower $pH_i$. 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 $pH_i$ 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, $pH_i$ was lower and this was reflected in a decrease in growth rates. High-calcifying 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, $pH_i$ and growth rate decreased while at 0.5% (v/v) $CO_2$/air high-calcifying cells failed to grow, in part because of the decrease in $pH_i$. 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-calcifying 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*.

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