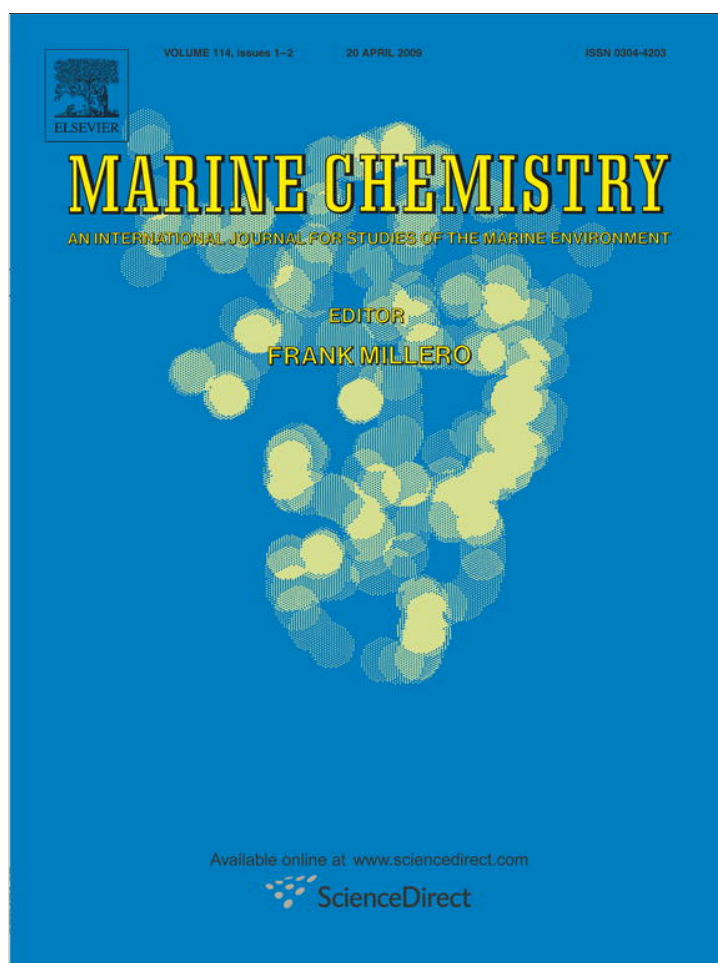


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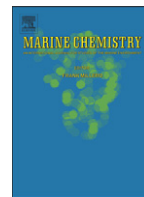


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Response of cell surface pH to $p\text{CO}_2$ and iron limitation in the marine diatom *Thalassiosira weissflogii*

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ARTICLE INFO

Article history:

Received 29 September 2008

Received in revised form 11 March 2009

Accepted 11 March 2009

Available online 17 March 2009

Keywords:

Diatom

Thalassiosira weissflogii

Boundary layer

Surface chemistry

pH sensor

SNARF

Iron

Barium

ABSTRACT

The physicochemical conditions immediately adjacent to the phytoplankton cell membrane, within the diffusion boundary layer, determine the speciation and reaction rates of biologically important nutrients such as iron and carbon dioxide. Over the past decade, several modeling efforts have attempted to calculate the supply of nutrients to phytoplankton surfaces using diffusion-reaction models. In these modeling efforts the pH within the cellular boundary layer is an important unknown and a direct measure of the cell surface pH is needed. We prepared and calibrated a new cell-bound, pH sensitive dye (a lectin–SNARF conjugate) that can be used to determine surface pH in living diatoms and applied the method to cultures grown under iron and carbon stress; conditions that are likely to elicit a pH response. We found that *Thalassiosira weissflogii* responded to iron limitation by reducing growth rates and increasing both the activity of phosphoenolpyruvate carboxylase (PEPCase) and the efflux of the organic acid malate, consistent with the iron-limited response of vascular plants termed Strategy I. Despite our predictions there is no proton efflux from iron or carbon stressed *T. weissflogii* and that the cell surface pH can be higher than the bulk seawater pH by as much as 0.4 units. High surface pH and increased PEPCase activity in low- $p\text{CO}_2$, high-iron grown cells is consistent with an efficient C4 type CO_2 concentrating mechanism. An increase in surface pH may impact cell surface precipitated iron and associated trace metals such as barium and may be an important factor to consider in paleo-export flux proxies when carbon dioxide is depleted.

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1. Introduction

Nutrient transport and chemical reactions occurring at phytoplankton cell surfaces are dependent on the physicochemical conditions immediately adjacent to the cell membrane within the diffusion boundary layer. Over the past decade, several modeling efforts have attempted to calculate the supply of nutrients to phytoplankton surfaces using diffusion-reaction models (Wischmeyer et al., 2003; Wolf-Gladrow and Riebesell, 1997; Zeebe et al., 1999, 2001, 2003). However, an important unknown in these models is the pH within the boundary layer. For example, Wolf-Gladrow and Riebesell (1997) calculate the supply of CO_2 to the surface of a phytoplankton cell using both diffusion and the conversion of bicarbonate to CO_2 within the cell boundary layer assuming a range of possible surface pHs. Wischmeyer et al. (2003) utilized a diffusion-reaction model to calculate the silicon speciation at the cell surface of a diatom within a presumably reasonable range of cell surface pH, and they concluded that transported Si is of the form $\text{Si}(\text{OH})_4$. Many of these modeling efforts could benefit from a direct measure of the cell surface pH.

Deviation of cell surface pH from the surrounding seawater is expected in a number of scenarios. Cell surface pH may be either higher or lower than bulk seawater due to inorganic carbon transport. A high surface pH is expected when phytoplankton take up CO_2 from seawater and a low pH might be expected if phytoplankton effluxed protons in order to facilitate conversion of bicarbonate to CO_2 within the boundary layer (Wolf-Gladrow and Riebesell, 1997). Efflux of protons and acidification of the boundary layer is also possible if phytoplankton respond to iron limitation in a manner similar to vascular plants. Vascular plants respond to iron limitation using two general strategies. The first, termed Strategy I, involves the induction of a ferric chelate reductase, the efflux of protons, an increase in organic acid synthesis via phosphoenolpyruvate carboxylase (PEPCase) and efflux of organic acids, mainly citrate, oxalate and malate. The second, termed Strategy II, involves the production of iron chelators (phytochelatins) and induction of a ferric chelate reductase (see Schmidt, 1999 for review). Eukaryotic phytoplankton are not known to produce phytochelatins, but do express a ferric chelate reductase like Strategy I plants and could potentially efflux protons although this has not been directly examined to date (Jones et al., 1987; Kustka et al., 2007; Maldonado and Price, 2000). Acidification can increase the availability of iron through dissolution of iron oxides and prolong the half life of Fe^{2+} (Shaked et al., 2005). In the sea, Fe^{2+}

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reoxidation rate will determine the bioavailability of iron reduced via plasmalemma reductases or through photochemical reduction (Kustka et al., 2005).

To examine the potential changes in cell surface pH given a range of $p\text{CO}_2$ and Fe availabilities, we developed a method utilizing the dual wavelength emission pH indicator seminaphthorhodafluor (SNARF) dye conjugated to a cell surface binding lectin. Cell surface pH can be measured in a variety of ways. The two most common methods are microelectrodes and pH sensitive fluorescent dyes. Microelectrodes suffer from significant voltage gradients at the cell surface and require a reference electrode situated close to the ion-sensitive electrode to avoid spurious changes in ion activity (Voipio et al., 1994). These difficulties can be overcome by using double-barreled microelectrodes which contain both a reference and pH electrode in the same tip but construction of such electrodes requires significant skill (Boyd and Gradmann, 1999a; Voipio et al., 1994). Fluorescent dyes have the advantage of being much simpler to use but can be difficult to calibrate and suffer from photobleaching during measurement. Here we overcome these problems by using the pH sensitive dye seminaphthorhodafluor (SNARF). While SNARF has not been used to measure cell surface pH to date, it has been used to measure cytoplasmic pH in algae and compares well with microelectrode measures of pH (Gibbon and Kropf, 1994; Kropf et al., 1995). The SNARF dye is excited at 550 nm and its emission at 583 nm and 618 nm is dependent on pH. This emission range is ideal due to the poor absorption by diatom photosynthetic pigments in this range of the visible spectrum. During exposure to excitation light the dye is photobleached resulting in an intensity decrease over time but the emission ratio remains stable. We calibrated this new method and applied it to diatoms grown under a range of iron and CO_2 availability, conditions under which we were likely to see a change in surface pH. We also report the response of growth rates, PEPCase activities and organic acid (malate) efflux as a function of culture conditions. We find that proton efflux by diatoms is not likely because cell surface pH can be higher than the bulk seawater pH by as much as 0.4 units but is never lower. The activity of PEPCase increases either under low CO_2 , consistent with its role in the diatom C4 carbon dioxide concentrating mechanism (Reinfelder et al., 2000, 2004) or under low-iron availability and may be responsible for the increase in accumulated dissolved-phase malate observed.

2. Materials and methods

2.1. Culture conditions

An axenic culture of the marine diatom *Thalassiosira weissflogii* (clone Actin) was obtained from the Provasoli-Giullard Center for the Culture of Marine Protozoa (CCMP). Axenicity was verified using acridine orange staining and epifluorescence microscopy. Continuous saturating irradiance was provided at a photon flux density of $160 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ using fluorescent bulbs below the cultures. Temperature was controlled at 20 °C. Duplicate cultures were maintained using Aquil artificial seawater medium with a salinity of 34 and a total alkalinity of $2494 \mu\text{mol kg}^{-1}$ (Morel et al., 1979; Price et al., 1988/89). Macronutrients were added at $100 \mu\text{mol L}^{-1} \text{NO}_3^-$, $100 \mu\text{mol L}^{-1} \text{Si(OH)}_4$ and $10 \mu\text{mol L}^{-1} \text{PO}_4^-$. Medium and culture flasks were microwave sterilized (Keller et al., 1988) in polycarbonate (PC) bottles. All medium preparation and sample handling was carried out in a class 100 flow hood. All plasticware was acid-cleaned by soaking in 10% reagent grade HCl for 2–5 d and rinsed five times with 18.2 M Ω cm deionized water (DDW). Iron was added to medium at a $p\text{Fe}$ ($-\log_{10}$ of unchelated ferric iron) of 19 (Fe replete) and 20.75 (Fe limited) corresponding to a total Fe concentration of 1.4 μM and 25 nM respectively. Unchelated metal concentrations $[M']$ were calculated using the thermodynamic equilibrium program Mineql (Westall et al., 1976).

High ($750 \pm 4 \mu\text{atm}$) and low ($100 \pm 4 \mu\text{atm}$) partial pressures of CO_2 were controlled by bubbling cultures with acid-washed plastic air-diffusers, using premixed gasses (Scott). To ensure that partial pressures remained constant during growth, pH was monitored daily.

2.2. Growth and biomass measurements

All measurements were made on cultures in mid-exponential phase of growth ($2\text{--}4 \times 10^4 \text{ cells mL}^{-1}$). Duplicate samples for cell counts and cell volume from each replicate flask were obtained using a Coulter (Model TAIL) particle counter fitted with a 70 μm aperture. Average specific growth rates (d^{-1}) were determined from linear regressions of natural log transformed cell counts versus time during the exponential phase of growth. Particulate organic carbon (POC) and nitrogen were obtained by filtering duplicate 20–40 mL aliquots of culture from each replicate flask onto pre-combusted (460 °C, 4 h) 25 mm A/E (Gelman) glass fiber filters and analyzed with a Carlo Erba NCS analyzer NA 1500. Carbon and nitrogen were calculated against a standard curve generated from sulfanilamide standards. Carbon incorporation rate was calculated from the product of carbon cell $^{-1}$ and the specific growth rate ($\mu^{-\text{d}}$).

2.3. PEPCase activity

Phosphoenolpyruvate carboxylase activities were measured (6 replicates from each replicate flask) in cells homogenized by sonication in 50 mmol L^{-1} bicine buffer (pH 8.3) containing $10 \text{ mmol L}^{-1} \text{NaHCO}_3$, 1.5 M glycerol, 1 mmol L^{-1} EDTA, $10 \text{ mmol L}^{-1} \text{MgCl}_2$, 5 mmol L^{-1} dithiothreitol (DTT) and 5 mg mL^{-1} bovine serum albumin (BSA) (Reinfelder et al., 2000). PEP-dependent ^{14}C -fixation was measured at 20 °C by addition of PEP (final concentration 5 mmol L^{-1}) and 0.2 $\mu\text{Ci NaH}^{14}\text{CO}_3$. Following a 15 min. incubation the reaction was stopped by addition of HCl (final concentration 1 mol L^{-1}). Samples were allowed to degas overnight and final fixed ^{14}C was determined by liquid scintillation counting.

2.4. Accumulated malate

L-malate concentration was determined (6 replicates from each replicate flask) in 0.2 μm PC filtered culture medium using malic enzyme dependent reduction of NADP^+ in a buffer containing 25 mmol L^{-1} Bicine (pH 8.3), 0.1 mmol L^{-1} EDTA, $2 \text{ mmol L}^{-1} \text{MgCl}_2$, $0.5 \text{ mmol L}^{-1} \text{NADP}^+$ and 0.2 units malic enzyme (Mollering, 1985). Reduction rate was measured as an increase in absorbance at 340 nm of NADPH as a function of time in a 15 min incubation. Absorbance was measured in 1 cm stirred cuvettes using a temperature controlled (20 °C) Cary 1E spectrophotometer (Varian). Rates were calculated using linear regression and calibrated against standard concentrations of malate in artificial seawater.

2.5. Boundary layer pH determination

Diatom surface pH was determined (3 replicates from each replicate flask) with SNARF conjugated to a lectin (wheat gluten, SIGMA-Aldrich). Lectins are proteins that selectively bind sugars and sugar moieties of glycoproteins and are ideal for cell surface labeling (Zhelev et al., 2005). The carboxylic acids of the lectin were coupled to the amine groups of carboxy SNARF-1 (Invitrogen, Cat# C-1270, $pK_a = 7.5$) in aqueous solution using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) and *N*-hydroxysulfosuccinimide according to manufacturers instructions (Pierce, Rockford, IL, also described in Grabarek and Gergely, 1990 and Staros et al., 1986). Following conjugation unreacted reagents were removed by overnight dialysis (10,000 MW cutoff).

To label cells, 30 mL of culture (about 1×10^6 cells) was filtered onto 3.0 μm PC membrane and rinsed with an isotonic solution of sorbitol (400 mmol L^{-1}) in deionized water. This step was necessary to avoid precipitation of the SNARF-lectin due to the high salt of Aquil medium. Cells were resuspended in the filter tower with 1 mL of sorbitol solution

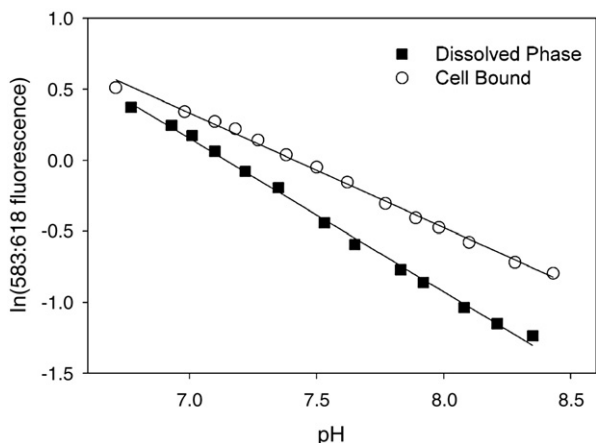


Fig. 1. Fluorescence ratio yield for SNARF–lectin conjugate both in dissolved phase and bound to azide killed cells as a function of pH (total scale). Excitation = 550 nm. For cell-bound dye, the response is described by the equation $y = (-0.93 \pm 0.04)X + (7.76 \pm 0.02)$ (parameter $\pm 95\%$ CI), $r^2 = 0.996$.

and incubated with SNARF–lectin (50 μ L) at room temperature (22 °C) for 5 min. Cells were then filtered, rinsed 3 times with 10 mL sorbitol solution and resuspended in 3 mL of Aquil medium. Preliminary experiments determined that cells labeled in this manner were not lost (fluorescence before and after treatment were equivalent) and continued to grow at exponential rates without a lag period (data not shown). Indeed use of sorbitol and its stereoisomer mannitol as osmoticants have no deleterious effects in either short term exposures (min–h) or long-term growth given low concentrations of sodium to support ion-dependent nutrient transport systems (Larson and Rees, 1994, 1996; Rees, 1995). Cells poisoned with azide (40 mM for 20 min) were labeled similarly and were used to calibrate the cell-bound SNARF–lectin fluorescence ratio.

The SNARF–lectin conjugate was calibrated both in solution phase and bound to azide killed cells. The pH was determined using a glass combination pH electrode (Orion) calibrated with NBS buffers (pH 4, 7, 10). Because the $p\text{CO}_2$, temperature and total DIC concentration are known in our culture system we corrected the electrode pH measures to the total pH scale using the CO2SYS software package (Lewis and Wallace, 1998) using the constants from refit data of Mehrbach et al. (1973) (Dickson and Millero, 1987). All pH values are reported on the total scale as defined by Lewis and Wallace (1998). For each calibration run culture medium (buffered with 50 mmol L⁻¹ HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) was set to each pH with NaOH or HCl and either SNARF–lectin or azide killed cells added. It was assumed that cell surface pH of azide killed cells was equivalent to the bulk medium because active ion pumping was abolished. The fluorescence ratio was measured in a temperature controlled and stirred 3 mL quartz cuvette using a custom spectrofluorometer with continuous xenon light source (Photon Technology International, Inc., Birmingham, NJ) with excitation set at 550 nm (20 nm bandwidth). Each emission intensity (583 nm and 618 nm, 5 nm bandwidth) was measured every 5 s for a period of 2 min.

Both CO₂ and iron treatment cultures were sampled as above but each sample was resuspended in Aquil medium (No HEPES, and the appropriate Fe concentration) that was equilibrated to the CO₂ treatment.

2.6. Statistics

For each measured variable the within bottle replicates were averaged and tested in a 2 replicate \times 4 treatment ANOVA using MS EXCEL 2003 (ver. 11.6355.6360) ANOVA: Two-factor with replication.

3. Results

The emission response of SNARF–lectin conjugate was pH dependent but the natural log linear behavior differed between dissolved phase and

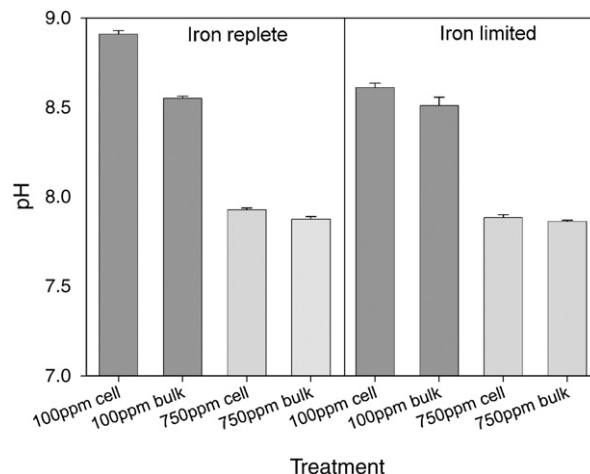


Fig. 2. Cell surface and bulk medium pH (total scale) as a function of $p\text{CO}_2$ and iron treatments. Cell surface pH was measured with SNARF–lectin conjugate. Bulk pH was calculated using CO2SYS (Lewis and Wallace, 1998). Error bars represent standard deviation ($n = 3$).

cell-bound dye (Fig. 1). A change in the calibration curve is an expected response to changing the electrostatic and solvent environment upon binding to the cell surface. Given the errors in the estimate of the linear regression parameters the precision of this method is ± 0.04 pH units.

Cell surface pH of *T. weissflogii* was dependent on both iron and carbon availability (Fig. 2). Bubbling with 100 μ atm CO₂ resulted in an equilibrium pH in the medium of about 8.55 and a significant rise in cell surface pH by 0.2 and 0.5 units for iron-limited and iron-replete cells respectively (Table 1). In high $p\text{CO}_2$ treatments, medium pH declined to 7.88 and there was not a detectable ($\Delta\text{pH} \leq 0.05$) difference between cell surface and medium pH.

Both growth rate and total dissolved malate were significantly affected by iron availability (Table 1). Growth rate was approximately halved in low-iron medium while the amount of malate effluxed during culture growth increased (Fig. 3). The total malate effluxed was independent of $p\text{CO}_2$ treatment but increased about 30% under Fe limitation concomitant with an increase in PEPCase activity (Fig. 3). Iron limitation resulted in an increase in PEPCase activity by roughly 2 fold. While a decrease in $p\text{CO}_2$ resulted in an increase in PEPCase activity by roughly 60%. Surprisingly, growth rate at low iron and high $p\text{CO}_2$ was slightly decreased relative to low $p\text{CO}_2$ (Fig. 3, Table 1).

4. Discussion

4.1. Carbon acquisition drives pH high at low CO₂

Cell surface pH was not observed to drop below the bulk medium pH in any of our treatments and we conclude that there is no significant proton efflux from *T. weissflogii* under our growth conditions. Only under low $p\text{CO}_2$ is there significant deviation of the cell surface pH from the bulk. This rise in pH is either due to a draw down of CO₂, conversion of HCO₃⁻ to

Table 1

Treatment effects tested using two-way ANOVA for measured parameters in a two replicate, two treatment design.

Treatment	Cell surface pH	ΔpH Surface pH – bulk pH	Growth	Malate	PEPCase activity
Fe	0.037	0.004	<0.001	0.029	0.003
CO ₂	<0.001	<0.001	0.032	0.346	<0.001
Fe \times CO ₂ interaction	0.097	0.022	0.676	0.813	0.097

Significant effects (p -values < 0.05) are in bold, $df = 1,1,1,4$ (Fe, CO₂, Fe \times CO₂, within).

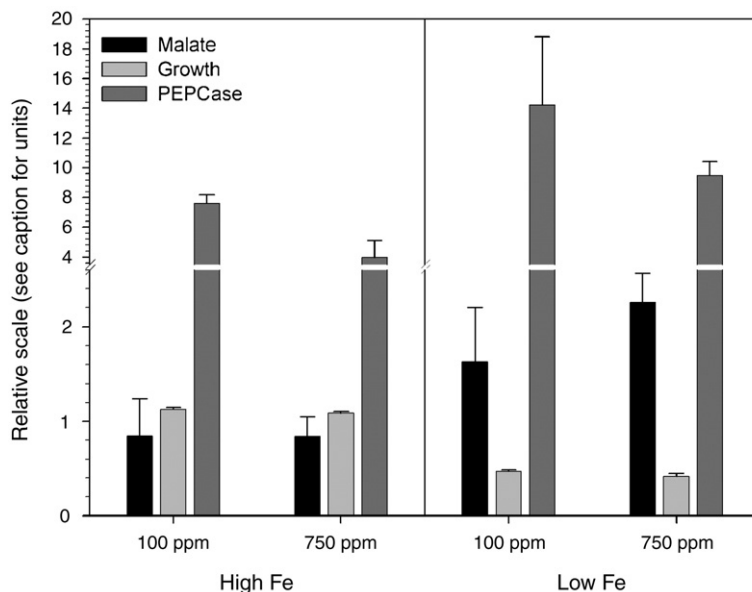


Fig. 3. Response of *T. weissflogii* to pCO₂ and iron treatments. Malate is the accumulated total in dimensionless units of dissolved-phase malate carbon normalized to total particulate carbon. Growth is in units of d⁻¹. PEPCase is in dimensionless units of carbon fixed by PEPCase cell⁻¹ d⁻¹ normalized to total carbon assimilated cell⁻¹ d⁻¹. Note log scale.

CO₂ (a reaction which consumes protons) at the cell surface or transport of bicarbonate and efflux of base from the cell. It is not clear which form of inorganic carbon (CO₂ or HCO₃⁻) traverses the membrane under our conditions. Assuming that the seawater carbon system is in equilibrium we calculate (using CO2SYS) that the cell surface pCO₂ is maximally 27 μatm under the high-iron, low-pCO₂ condition (Lewis and Wallace, 1998). This assumption is likely to be satisfied under our growth conditions because *T. weissflogii* expresses a cell surface carbonic anhydrase (CA) that maintains the CO₂–HCO₃⁻ equilibrium (Morel et al., 2002; Park et al., 2008). Calculating the steady-state pCO₂ concentration is not possible given our data (the activity of CA is not known) but if equilibrium cannot be assumed or if there is significant depletion of HCO₃⁻ at the cell surface a lower pCO₂ is required to satisfy the observed cell surface pH.

The depletion of CO₂ at the cell surface to roughly one third of ambient suggests a strong gradient of CO₂ from the bulk to the cytoplasm of *T. weissflogii* and an effective CO₂ concentrating mechanism. Photosynthetic organisms must concentrate CO₂ in their chloroplasts in order to saturate the primary enzyme of the photosynthetic carbon reduction cycle, ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO), and minimize energy wasting photorespiration (Andrews et al., 1971; Bowes et al., 1971) and many prokaryotic and eukaryotic phytoplankton have evolved a CO₂ concentrating mechanism (CCM) (Giordano et al., 2005). Recent work has shown that the marine diatom *T. weissflogii* uses a C4 pathway to concentrate CO₂ and the activity of PEPCase is critical to the functioning of the C4-CCM (Reinfelder et al., 2000, 2004). The C4-CCM utilizes two carbonic anhydrases, one at the cell surface and one in the cytoplasm to maintain HCO₃⁻ and CO₂ in thermodynamic equilibrium. Because PEPCase acts as a sink for bicarbonate, rapidly converting it to a C4 compound, we expect that intracellular CO₂ is at concentrations even

lower than 27 μatm thus facilitating a strong gradient for diffusive flux of CO₂. Assuming a zero CO₂ concentration in the cytoplasm the diffusive flux of CO₂ from a 27 μatm external concentration is able to satisfy at best 30% of the carbon necessary to account for a specific growth rate of 1.2 d⁻¹ (Subczynski et al., 1992). For this calculation we use a CO₂ assimilation rate of 2 × 10⁻¹⁶ mol C cell⁻¹ s⁻¹ (from the product of growth (μ) and carbon quota) and a cell radius of 5.5 μm and assume no CO₂ efflux, a CO₂ diffusivity in water of 1.95 × 10⁻⁵ cm² s⁻¹, a membrane permeability of 8 × 10⁻³ (Sültemeyer and Rinast, 1996), and a membrane thickness of 3.2 × 10⁻⁷ cm (see Subczynski et al., 1992). Given these assumptions we expect HCO₃⁻ to be an important source (>70%) of inorganic carbon to *T. weissflogii* at low pCO₂.

Martin and Tortell (2008) estimate that most (95% for *T. weissflogii*) of the DIC transported by diatoms is in the form of HCO₃⁻. The transport of two bicarbonate ions, conversion to CO₂ and CO₃²⁻ in the cytoplasm, and efflux of the CO₃²⁻ could result in a pH rise at the cell surface. This scenario is not expected because there are no known CO₃²⁻ transporters and when a diatom C4-CCM is engaged CO₃²⁻ is not a byproduct. In the β-carboxylation of PEP, PEPCase uses HCO₃⁻ as a substrate and produces oxaloacetate with a carboxylic acid in the C4 position, which deprotonates at physiological pH, and inorganic phosphate (Fig. 4). Hence, no carbonate is produced when using a C4-CCM. Importantly, the accumulation of base as a by product of transporting HCO₃⁻ is unavoidable and may be effluxed from the cell as possibly OH⁻. The co-transport of H⁺ with HCO₃⁻, a mechanism found in the freshwater alga *Chara corallina* (Lucas et al., 1983) could also cause a cell surface pH rise but is not likely because the proton gradient is not energetically favorable in marine systems (Boyd and Gradmann, 1999b). Symports in marine diatoms are Na⁺ dependent (Bhattacharyya and Volcani, 1980; Boyd and Gradmann, 1999b; Rees et al., 1980).

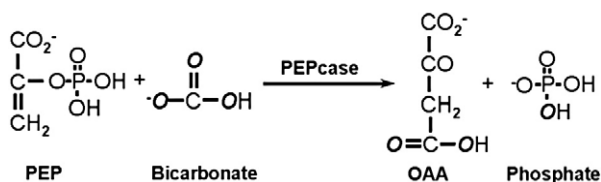


Fig. 4. The reaction catalyzed by PEPCase. The β-carboxylation of phosphoenolpyruvate (PEP) by bicarbonate is catalyzed by PEP carboxylase to form oxaloacetate (OAA) and inorganic phosphate. At physiological pH both carboxylic acids of OAA will be deprotonated.

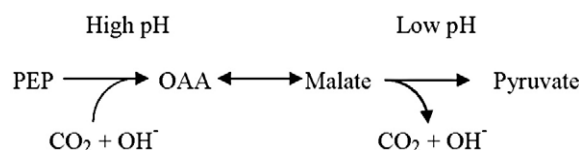


Fig. 5. The pH-stat according to Davies (1973). At high pH phosphoenol pyruvate (PEP) is carboxylated via PEP carboxylase (PEPCase) to form the four carbon compound oxaloacetic acid (OAA) which is converted to the more stable malate.

4.2. pH homeostasis

We expected an efflux of organic acids along with protons in our low-iron condition. In vascular plants, the efflux of protons was thought to result in alkalinization of the cytoplasm and increase in the activity of phosphoenolpyruvate carboxylase (PEPCase). This PEPCase dependent pH-stat was first suggested by Davies (1973) and is the dominant theory of cytoplasmic pH regulation in plants. Briefly, excess OH^- increases the activity of PEPCase to form oxaloacetate (OAA) which is rapidly converted to malate (Fig. 5). When H^+ concentration increases PEPCase activity decreases inhibiting malate production and malic enzyme activity increases to yield pyruvate. While this scheme is attractive, it is now known to be biochemically incorrect. The substrate for PEPCase is bicarbonate and the products of decarboxylation of malate are pyruvate, CO_2 , and NADH, hence there is no net change in pH (Fig. 5). Current pH-stat theory envisions ion pumping as the primary system and secondary involvement of PEPCase activity in a more complex system than the scheme proposed by Davies (1973) (Sakano, 1998). When PEP is consumed through activity of PEPCase it is replenished via glycolysis, which is proton producing. Consistent with its indirect involvement in pH regulation PEPCase activity has been shown to respond to treatments that cause cytoplasmic pH shifts (Gerendas and Ratcliffe, 2000; Gout et al., 1992). Activity of PEPCase has also been shown to respond to iron limitation and proton efflux, increasing as much as six-fold in iron-limited cucumber (*Cucumis sativus*) (Rabotti et al., 1995).

4.3. Malate efflux

Although there is no evidence for proton extrusion in *T. weissflogii* there was a significant increase in total dissolved malate in low-iron medium (Fig. 3, Table 1). Maximally, malate reached concentrations of $1 \mu\text{M}$ in the low-iron, high- CO_2 condition. Malate is an iron ligand but its stability constant is so low ($\log K = 8.4$) that it is unable to compete with OH^- at seawater pH. Given a pH of 8.1 and a malate concentration of $2 \mu\text{M}$ all of the Fe (100 pM) in typical seawater would be in the form of hydroxide species (Visual Minteq ver. 2.53). Malate cannot appreciably influence the speciation of iron in seawater and it remains a question of why malate would be effluxed from iron-limited diatoms. A possible explanation is that efflux is simply a function of the flux of carbon through the anapleurotic β -carboxylation pathway replenishing the carbon substrates lost during the synthesis of amino acids. This is consistent with the increased activity of PEPCase in our low-iron treatments and agrees well with work by Rueter and Ades (1987) that found the green alga *Scenedesmus quadricauda* preferentially incorporated nitrogen into protein when iron-limited.

4.4. Oceanographic implications

A high pH at the surface of a diatom has several consequences. The precipitation of iron oxyhydroxides and the incidental incorporation of phosphate and other trace metals is likely enhanced by the high local pH (Anderson and Morel, 1982; Hudson and Morel, 1989). This cell surface bound fraction has been shown to be an important component (30% to 90%) of Fe and P associated with field collected phytoplankton (Hutchins et al., 2002; Sanudo-Wilhelmy et al., 2004; Tovar-Sanchez et al., 2003). Laboratory studies have shown that Ba is incorporated in surface associated Fe-oxyhydroxides at levels that far exceed its internal cellular concentration (Sternberg et al., 2005). Since the Ba/Fe ratio in surface precipitates is pH dependent, increasing roughly six-fold across the pH range 7.5 to 8.7, we expect that, $p\text{CO}_2$, along with iron supply and growth rate should influence the barium flux to the sediments and its use as a paleoproxy in the oceans. Indeed high barite accumulation rates are negatively correlated with $p\text{CO}_2$ (Paytan and Griffith, 2007). It is thus possible that the ongoing increase in the $p\text{CO}_2$ of surface seawater and the

attending decrease in pH in the bulk solution and at the surface of phytoplankton will affect the scavenging and biological export flux of elements such as Fe, P and Ba.

Acknowledgements

We thank Satish Myneni for use of his spectrofluorometer. Funding was provided by the Center for Bioinorganic Chemistry NSF/CEBIC (NSF# CHE 0221978), a NSF Biocomplexity grant (DEB-0083566) to A. J. M. and F. M. M. M.

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