

Effects of the pH/ $p\text{CO}_2$ control method on medium chemistry and phytoplankton growth

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Abstract. The control of key chemical parameters in phytoplankton cultures, such as $p\text{CO}_2$, pH and Ω (the saturation state of calcium carbonate), is made difficult by the interdependence of these parameters and by the changes resulting from the growth of the organisms, such as CO_2 fixation, nutrient uptake and, for coccolithophores, calcite precipitation. Even in cultures where $p\text{CO}_2$ or pH is maintained constant, other chemical parameters change substantially at high cell densities. Experimentally we observed that various methods of adjustment of $p\text{CO}_2$ /pH – acid or base addition, use of buffers or pH-stats, or bubbling of CO_2 -enriched air – can be used, the choice of one or the other depending on the goals of the experiments. At seawater pH, we measured the same growth rates in cultures of the diatom *Thalassiosira weissflogii* where the $p\text{CO}_2$ /pH was controlled by these different methods. The pH/ $p\text{CO}_2$ control method also did not affect the rates of growth or calcification of the coccolithophore *Emiliania huxleyi* at seawater pH. At lower pH/higher $p\text{CO}_2$, in the *E. huxleyi* strain PLY M219, we observed increases in rates of carbon fixation and calcification per cell, along with a slight increase in growth rate, except in bubbled cultures. In our hands, the bubbling of cultures seemed to induce more variable results than other methods of $p\text{CO}_2$ /pH control. While highly convenient, the addition of pH buffers to the medium apparently induces changes in trace metal availability and cannot be used under trace metal-limiting conditions.

1 Introduction

There is a growing consensus that the ongoing increase in atmospheric carbon dioxide, CO_2 , as a result of anthropogenic activities will lead to a variety of physical, chemical and physiological effects on marine phytoplankton (Feely et al., 2004; Doney, 2006). Upon dissolution in the surface ocean, the additional CO_2 causes re-equilibration of the seawater carbonate system, increasing the concentrations of aqueous CO_2 (usually quantified by its partial pressure $p\text{CO}_2$) and bicarbonate ion, HCO_3^- , while decreasing that of the carbonate ion, CO_3^{2-} . These changes in the distribution of the various species of the dissolved inorganic carbon, DIC, which is the main acid-base buffer of seawater, result in an increase in the hydrogen ion, H^+ , concentration – i.e., a decrease in pH – and these interrelated chemical changes are commonly referred to collectively as ocean acidification. Of all these effects, the elevated $p\text{CO}_2$ and the lowered carbonate ion concentration have received the most attention. The former may facilitate inorganic carbon fixation in the dark reaction of photosynthesis and thus increase primary production in the ocean (Riebesell et al., 2007; Tortell et al., 2008); the latter could reduce precipitation of calcium carbonate by calcifying organisms such as coccolithophores (Riebesell et al., 2000; Feng et al., 2008). Changes in pH may affect a number of physiological processes, particularly the activity of important extracellular enzymes (Xu et al., 2006).

To study the response of phytoplankton to increasing $p\text{CO}_2$ /decreasing pH necessitates an experimental method to manipulate and control these parameters in laboratory cultures or field incubations. Unless one uses continuous cultures (which present their own difficulties, such as requiring large volume of medium which is particularly problematic in



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studies requiring trace metal clean conditions), the control of pCO₂/pH in a growing batch culture of phytoplankton is challenging as the growth of the organisms continuously changes the concentration of the inorganic carbon species (Rost et al., 2008). The most commonly used methods have been to bubble prepared air mixtures containing a given fraction of CO₂, or to add prescribed quantities of strong acids or base. Another possible technique, which has been widely used by microbiologists, but not recently by those who study the effect of acidification on marine phytoplankton, is to introduce a biologically benign buffer such as EPPS (4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid), in the growth medium to control the pH. In principle this method, which also leaves DIC constant, has the advantage of continuously readjusting the concentration of dissolved CO₂ to a nearly constant value as it is depleted by the growing culture. It should be noted that when cultures reach high biomass a sizable decrease in pCO₂ may be caused by the drawdown of DIC. A similar result can be achieved in the absence of a buffer by using a so called “pH-stat” which automatically delivers strong acid or base as required to maintain the pH of the medium constant.

These various methods have different effects on the carbonate system of seawater and thus, potentially, different biological consequences. For example bubbling of a gas at a given pCO₂ changes DIC and maintains alkalinity, Alk, constant, while the other methods leave DIC unadjusted but vary alkalinity. One can, of course, adjust Alk by adding bicarbonate simultaneously with strong acid. Other issues concern the possible mechanical effect of bubbling, the biological effects of an organic buffer in the growth medium, or the presence of an electrode which is prone to fouling and to introduce trace impurities if a pH-stat is used.

In this study we compare different methods of pH/pCO₂ control in cultures of model phytoplankton species and examine their effects on the growth and, when appropriate, calcification of the organisms under various conditions.

2 Materials and methods

2.1 Cultures

The marine diatom *Thalassiosira weissflogii* (CCMP 1336) was obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP). The strains CCMP 374 and PLY M219 (NZE) of the coccolithophore *Emiliania huxleyi* were obtained from CCMP and the Plymouth Culture Collection of Marine Algae in the UK, respectively. All the experiments were conducted in batch cultures in acid-cleaned polycarbonate bottles using a single batch of 0.2-μm filtered Gulf Stream seawater (DIC=1980±50 μmol kg⁻¹, Alk=2260±10 μmol kg⁻¹ and pH=8.08±0.02). The culture media were enriched with 150 μM NO₃⁻, 10 μM PO₄³⁻, 100 μM SiO₂ and vi-

tamins for *T. weissflogii*, and 100 μM NO₃⁻, 6 μM PO₄³⁻ and vitamins for *E. huxleyi*. Trace metal additions followed the Aquil recipe (Sunda et al., 2005). All cultures were maintained at 20°C under continuous light (~150 μmol quanta m⁻² s⁻¹). Experiments with *T. weissflogii* started with 20–100 cells ml⁻¹ and those with *E. huxleyi* started with 1000 cells ml⁻¹ for CCMP 374 and 150–500 cells ml⁻¹ for PLY M219. There was no pre-acclimation to the experimental conditions since we observed the same growth with and without pre-acclimation in our preliminary experiments. Cell number and volume, which can be converted to biomass, were determined using a Z2 Coulter® Particle Count and Size Analyzer (Beckman), and the specific growth rates were computed during exponential growth with a linear regression of natural logarithm (cell number) vs. time.

2.2 pCO₂/pH manipulation

Targeted pCO₂ or pH values in culture medium were achieved by bubbling of commercially prepared air-CO₂ mixtures, addition of buffer, or acid/base adjustment. Medium pH was monitored daily throughout the experiment. In some experiment we also used a pH-stat which delivered small amounts of strong acid (i.e., 10 mM HCl) whenever the pH increased by more than 0.02 units.

2.2.1 CO₂ bubbling

Bubbling experiments were performed in 250 ml polycarbonate bottles in the cap of which two small holes were made, one for the tubing supplying the gas and the other one to let the gas out. Prior to inoculations, seawater medium was gently bubbled with humidified CO₂-enriched air (pCO₂ 380 μatm or 750 μatm) until a desirable pH was achieved and remained stable. We found it useful to stop bubbling for the first day following inoculation to allow the new cultures to start growing. The bubbling of the cultures began on day two and continued until the end of the experiment.

2.2.2 Buffer

The buffer 4-(2-Hydroxyethyl)-1-piperazinepropanesulfonic acid (EPPS, Sigma Ultra) was chelexed (Price et al., 1988/89) and pH adjusted with ultra pure HCl/NaOH. It was then introduced into culture medium at concentrations of 8 mM and 5 mM for *T. weissflogii* and *E. huxleyi*, respectively, to attain targeted pH.

2.2.3 Acid/base adjustment

pCO₂/pH of medium was adjusted by adding ultra pure HCl/NaOH to give desired pH. In experiments conducted at 750 μatm pCO₂, we also used a treatment in which an equimolar concentration of ultra pure NaHCO₃ was added simultaneously with the acid to maintain alkalinity constant.

These experiments were performed in capped 250 ml polycarbonate bottles filled to the bottom of the neck, with a headspace of ~10 ml.

2.2.4 Measurements of DIC, total alkalinity and pH

The DIC concentration, total alkalinity and pH (on the total scale) of the seawater and culture media were analyzed by Membrane Inlet Mass Spectrometry (MIMS), Gran titration and potentiometry (Beckman ϕ 34 pH meter, which was calibrated against thymol blue measurement, Zhang and Byrne, 1996), respectively.

2.3 C/N ratio measurement

T. weissflogii cells were harvested at steady state and filtered onto a precombusted glass fiber filter (GF/F, 450°C, 4 h) and stored at -80°C. For analysis, each filter was dried overnight at 60°C, exposed to fuming HCl for 6 h, and dried overnight again at 60°C. Samples were packed in tin cups (Costech Analytical Technologies) and submitted for analysis on an elemental analyzer (Eurovector) connected to a continuous flow isotope ratio mass spectrometer (GVI Isoprime) at Rutgers Institute of Marine and Coastal Sciences.

2.4 PIC and POC production rate measurements

PIC and POC production rates in *E. huxleyi* PLY M219 were measured by either short-term or long-term ¹⁴C incorporation. For short-term ¹⁴C incorporation of bubbled cultures, 340 nM NaH¹⁴CO₃ was added into a subsample of the cultures and incubated for 2–4 h under the same conditions, but no CO₂ bubbling. For long-term ¹⁴C incorporation, 84 nM NaH¹⁴CO₃ was added into the culture medium before adding the inoculum or shortly after.

PIC and POC separation was as described previously (Paasche and Brubak, 1994) with slight modification. Briefly, cells were filtered onto 1 μ m polycarbonate filter under gentle vacuum, and rinsed with 5 ml seawater five times. The filters were then placed in 20 ml scintillation vials and 1 ml of 1% H₃PO₄ was added into the vials. Each vial was closed immediately with a cap containing a GF/D filter with 60 μ l phenethylamine absorbed in the filter. The vials were incubated at room temperature for 24 h with occasional shaking. Then vials and caps were separated and scintillation fluid was added before counting the radioactivity of ¹⁴C with a LS6500 Multi-purpose Scintillation Counter (Beckman).

2.5 Calculation methods

Model calculations for a seawater medium containing 10 μ M phosphate and 100 μ M silicate were made according to Dickson and Goyet (DOE, 1994) – except that the acidity constants for CO₂ in seawater are from Lueker et al. (2000) – absent acid/base addition or CO₂ bubbling. For the calculations of the acid-base chemistry of algal cultures over time,

the initial conditions were constrained by the measured initial pH and DIC or Alk of the medium, depending on the conditions.

3 Results and discussion

3.1 Theoretical considerations

As mentioned above, the different methods used to control the pCO₂/pH of a culture medium have different consequences for its carbonate chemistry, the DIC and Alk being modified in different ways by the various methods. These parameters are further affected by the growth of the phytoplankton in batch cultures, resulting in changes in the chemistry of the medium, particularly pCO₂, pH, and Ω . These changes can be precisely calculated for closed batch cultures as illustrated in Fig. 1 for typical (not acidified) seawater conditions. To calculate $\Omega = [\text{Ca}^{2+}] [\text{CO}_3^{2-}] / K_s$, we used $K_s = 4.3 \times 10^{-7} \text{ mol}^2 \text{ kg}^{-2}$, a value applicable to calcite at 20°C, 1 atmosphere of pressure and a salinity of 35.

In cultures of non-calcifying phytoplankton that are not bubbled with air at constant pCO₂, the changes in the medium chemistry are brought about by the fixation of CO₂, which decreases DIC, and to a lesser extent by the uptake of NO₃⁻, which increases Alk by $\Delta \text{Alk} = -\Delta [\text{NO}_3^-]$. For a typical N:C ratio in the biomass of 0.16, $\Delta \text{Alk} = -0.16 \Delta \text{DIC}$. In most cases, one can neglect the effects of the uptake of phosphate on the acid-base chemistry of the system as well as those due to the uptake of silicate in the case of diatoms. The net result is a decrease in pCO₂ by approximately 23% when $-\Delta \text{DIC} = 50 \mu\text{mol kg}^{-1}$, with a corresponding increase in pH by about 0.1 units (Fig. 1a). Although this is presumably not relevant to non-calcifying organisms, Ω increases by about 17% for the same decrease in DIC. When the same cultures are bubbled at constant pCO₂, the increases in DIC and pH brought about by the increase in Alk are negligible (calculations not shown). If the pH is maintained constant, the pCO₂ decreases proportionally with DIC, i.e., only about 2.4% for $-\Delta \text{DIC} = 50 \mu\text{mol kg}^{-1}$. We note that to maintain the pH constant within 0.05 units in a non-calcifying culture requires a pH buffer capacity of 2 mM when the biomass attains 100 $\mu\text{M C}$; this can be achieved within 0.5 pH units of the pK_a of a buffer added at a concentration of 4 mM (Morel and Hering, 1993).

In cultures of calcifying organisms, there is a sizeable decrease in Alk resulting from the precipitation of CaCO₃, in addition to the effects of CO₂ fixation and NO₃⁻ uptake. For cultures that are not bubbled at constant pCO₂, the net result is smaller changes in pCO₂, and pH than in non-calcifying cultures. This is illustrated in Fig. 1b for the case where calcification and carbon fixation occur at the same rate ($\Delta \text{POC} / \Delta \text{PIC} = 1$). Because Alk decreases along with DIC, Ω and pH change little as the cells grow, the extent of these changes becoming larger as the $\Delta \text{PIC} / \Delta \text{POC}$ ratio decreases.

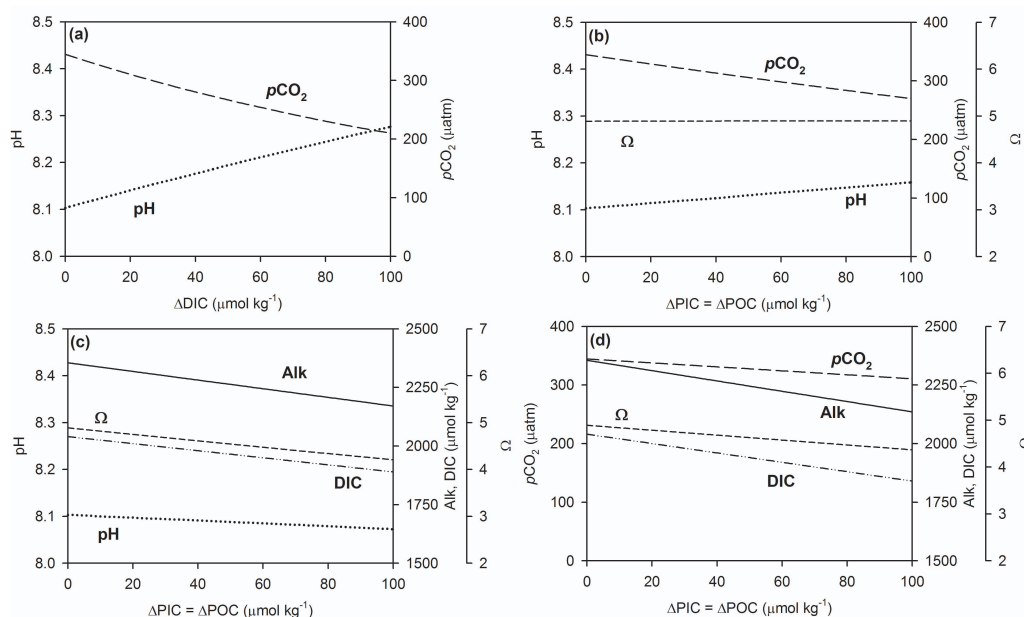


Fig. 1. Calculated chemical parameters of the Aquil seawater medium at normal pH containing 10 μM phosphate and 100 μM silicate as a function of ΔDIC or ΔPIC . (a) Cultures of non-calcifying phytoplankton without pH/pCO₂ control; (b) cultures of calcifying phytoplankton without pH/pCO₂ control; (c) cultures of calcifying phytoplankton bubbled at constant pCO₂; and (d) cultures of calcifying phytoplankton maintained at constant pH. Calculations were made according to DOE (1994) and Lueker et al. (2000).

If pCO₂ in a calcifying culture ($\Delta\text{PIC}/\Delta\text{POC}=1$) is maintained constant by bubbling, the decrease in DIC is a sizeable fraction of the decrease in Alk, about 75 $\mu\text{mol kg}^{-1}$ ($\Delta\text{DIC}/\text{DIC}\sim-4\%$) for a PIC production of 50 $\mu\text{mol kg}^{-1}$ ($\Delta\text{Alk}=-92 \mu\text{mol kg}^{-1}$) (Fig. 1c). The pH decrease is small but the corresponding decrease in Ω , from 4.8 to 4.5, might be significant.

When the pH is maintained constant in a calcifying culture (by pH-stat or buffer), there is a small decrease in pCO₂ as the cells fix CO₂ and calcify (Fig. 1d). The changes in DIC and Ω are similar to those in a bubbled culture (compare Fig. 1c to d).

3.2 Growth rates

Under nutrient-replete conditions at seawater pH, we observed identical maximum growth rates ($\mu=1.38\pm0.06 \text{ d}^{-1}$) in batch cultures of the diatom *T. weissflogii* regardless of whether the pH/pCO₂ of the medium was not controlled, pCO₂ manipulated by bubbling, or pH buffered by addition of EPPS (Fig. 2a). We also measured the same growth rate ($\mu=0.79\pm0.02 \text{ d}^{-1}$) in Fe-limited cultures of *T. weissflogii* where the pH/pCO₂ was controlled by bubbling or using a pH-stat (Fig. 2a). Under nutrient-replete conditions, we also observed identical maximum growth rates ($\mu=1.34\pm0.01 \text{ d}^{-1}$) for strain CCMP374 of the coccolithophore *E. huxleyi* in the presence or absence of EPPS (Fig. 2b). Strain CCMP374 does not calcify measurably in our cultures. Experiments with PLY M219, a highly calcified

strain of *E. huxleyi*, also showed no difference in growth rate whether or not bubbled at ambient pCO₂ level (380 μatm) and in the presence or absence of EPPS (Fig. 2c, Table 1). Increasing the pCO₂ of these cultures by acidification in the presence or absence of buffer significantly increased their growth rate ($p<0.05$, t-test) but not when pCO₂ was fixed by bubbling (Fig. 2d, Table 1; see below).

3.3 Changes in medium chemistry during growth

3.3.1 *T. weissflogii*

Based on a measured cellular C quota of 10.8 pmol cell⁻¹ and an N:C ratio of 0.14 in *T. weissflogii* in our culture medium, the changes in medium composition over time in the absence of bubbling or pH control in the nutrient-replete experiment of Fig. 2a can be calculated (Fig. 3a). After approximately 4 days of cultures, when the cells reach a concentration of $\sim 5000 \text{ cells ml}^{-1}$, the pCO₂ of the medium decreases by about 23% and the pH increases by 0.09 units. This calculated change in pH agrees well with our actual measurement of 0.1 pH units.

To achieve a reasonable control of pCO₂ and/or pH in culture media with no buffer or CO₂ bubbling thus necessitates ending the experiment at low cell density. If a high biomass is desired, then adding acid, or acid+bicarbonate, periodically in the medium can provide a suitable alternative to bubbling the cultures, adding a buffer, or using a pH-stat, each of which has its own drawbacks. For example,

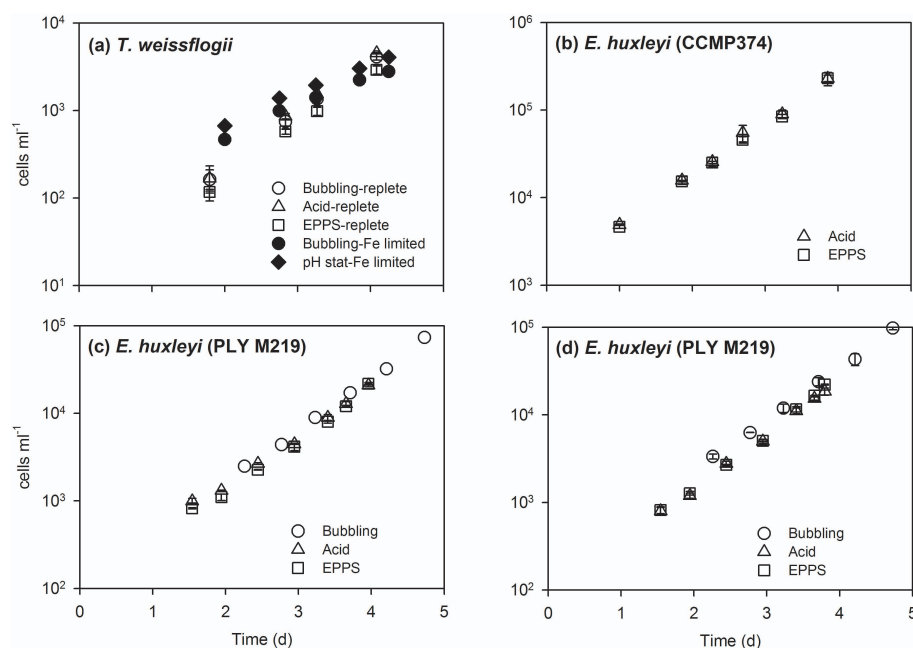


Fig. 2. Growth curves of the diatom *Thalassiosira weissflogii* and the coccolithophore *Emiliania huxleyi* at different pH/pCO₂ manipulated by CO₂ bubbling, addition of acid, use of a pH-stat, or EPPS addition. **(a)** *T. weissflogii* at pH=8.09 (nutrient-replete growth rate: Bubbling=1.34±0.18 d⁻¹, Acid=1.44±0.09 d⁻¹, EPPS=1.36±0.04 d⁻¹, mean±sd=1.38±0.06 d⁻¹, $p>0.05$, one-way ANOVA; Fe-limited growth rate: Bubbling=0.79±0.02 d⁻¹, pH-stat=0.79±0.03 d⁻¹, mean±sd=0.79±0.02 d⁻¹, $p>0.05$, one-way ANOVA); **(b)** nutrient-replete *E. huxleyi* CCMP374 at pH=8.19 (growth rate: Acid=1.34±0.06 d⁻¹, EPPS=1.34±0.02 d⁻¹, mean±sd=1.34±0.01 d⁻¹, $p>0.05$, one-way ANOVA); nutrient-replete *E. huxleyi* PLY M219 (NZEH) at **(c)** pH=8.10 (growth rate: Bubbling=1.35 d⁻¹, Acid=1.32±0.01 d⁻¹, EPPS=1.41±0.04 d⁻¹); and **(d)** pH=7.80 (growth rate: Bubbling=1.36±0.01 d⁻¹, Acid=1.48±0.02 d⁻¹, EPPS=1.53±0.04 d⁻¹). Error bars represent standard deviation or the range of $n=2-4$. All data are mean±sd. Data of bubbling treatments in (c) and (d) are from a single experiment; data from all replicate experiments are presented in Table 1.

adding 50 μmol kg⁻¹ HCl and 50 μmol kg⁻¹ NaHCO₃ on day 4 in the cultures of Fig. 2a, would have adjusted the DIC to 2040 μmol kg⁻¹ and the pH to 8.1, allowing for another doubling of the cell number before the pCO₂ decreases by 28% and the pH increases by 0.1 units.

3.3.2 *E. huxleyi*

Calculations for the experiments of Fig. 2c and d are shown in Fig. 3b and c, based on the measured composition of *E. huxleyi* strain PLY M219: POC+0.6 pmol cell⁻¹ and PIC=0.5 pmol cell⁻¹ at pH=8.1; POC=0.9 pmol cell⁻¹ and PIC=0.65 pmol cell⁻¹ at pH=7.8 (see below); and C:N=8.6 (Ho et al., 2003) (to allow comparison among treatments we used an initial cell concentration of 350 cells ml⁻¹ and a growth rate of 1.35 d⁻¹ for Fig. 3b–e, instead of the actual data, and continued the calculations beyond the duration of the experiments). As seen in the figures, a sizeable decrease in pCO₂ (10%) and increases in pH occurs for cell concentrations around 7×10⁴ cells ml⁻¹ in the cultures at seawater pH without buffer or CO₂ bubbling (Fig. 3b). As expected, a similar relative decrease in pCO₂ occurs at a lower cell concentration, ca. 3.5×10⁴ cells ml⁻¹, in cultures at higher pCO₂/lower pH which are less well buffered (Fig. 3c).

In cultures bubbled at pCO₂=750 μatm (Fig. 2d), when cell densities reach about 8×10⁴ cells ml⁻¹, the DIC, Ω and to a lesser extent pH begin decreasing along with Alk (Fig. 3d).

A quantitatively similar variation in Alk, DIC and Ω occurs in a culture with constant low pH corresponding to the experiment in Fig. 2d (Fig. 3e). In this case, a decrease by 10% in pCO₂ occurs at about 1.4×10⁵ cells ml⁻¹. To maintain constant conditions in a bubbled culture of a calcifying organism thus also requires periodic addition of either base or bicarbonate to make up for the loss of alkalinity and the accompanying decrease in DIC.

3.4 Effects of bubbling

The data of Fig. 2a–c show no difference in growth rates among cultures where pCO₂/pH are controlled by different methods. The cultures of *E. huxleyi* strain PLY M219 showed a small but systematic increase in growth rate at pH=7.8 compared to pH=8.1 in acidified cultures, with or without buffer, but the cultures bubbled with air at pCO₂=750 μatm, grew slightly slower than to those in which pCO₂ was increased by acidification (Fig. 2c and d, Table 1). We have seen adverse effects of bubbling on the growth of several phyto-

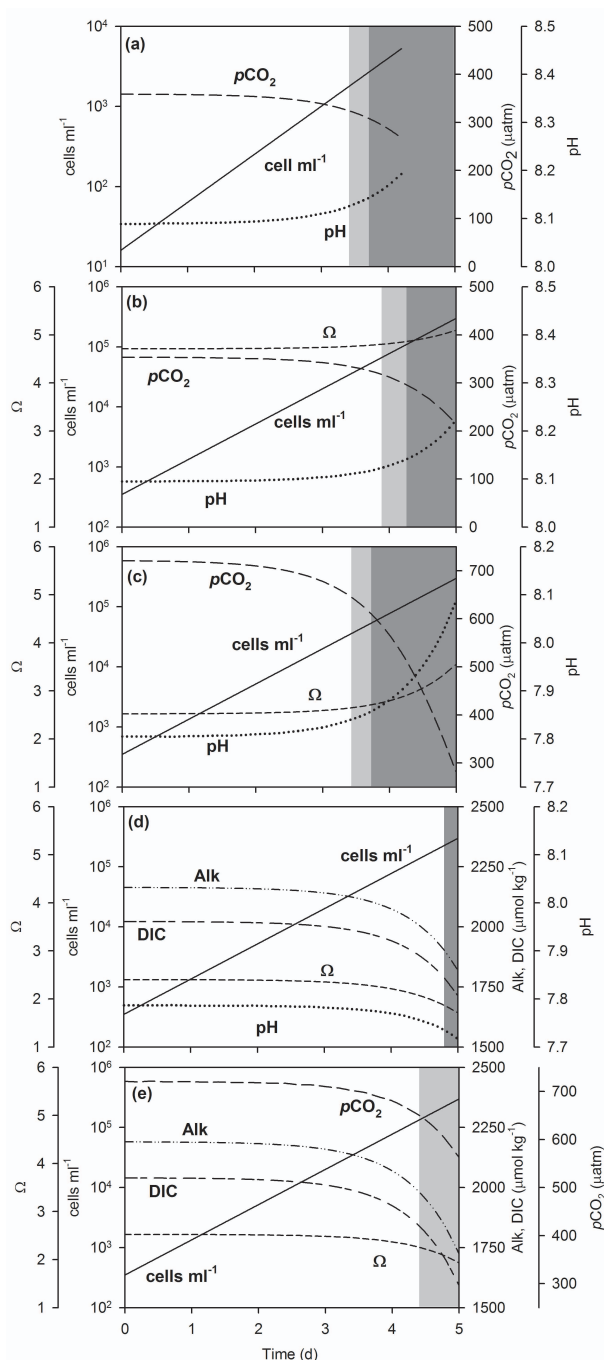


Fig. 3. Cell density and chemical parameters calculated as a function of time in cultures of *T. weissflogii* and *E. huxleyi* PLY M219 shown in Fig. 2. (a) Cultures of *T. weissflogii* at pH=8.09 without pH/pCO₂ control; (b) cultures of *E. huxleyi* PLY M219 at pH=8.10 without pH/pCO₂ control; (c) cultures of *E. huxleyi* PLY M219 at pH=7.80 without pH/pCO₂ control; (d) cultures of *E. huxleyi* PLY M219 bubbled with air at 750 μatm pCO₂; and (e) cultures of *E. huxleyi* PLY M219 maintained at pH=7.80. The light grey areas in the figures indicate a change in pCO₂ larger than 10% of the initial value, and the dark grey areas indicate a change in pH larger than 0.05 units. Calculations were made according to DOE (1994) and Lueker et al. (2000).

plankton species (Xu et al., unpublished Data), and our experiments with bubbled cultures have yielded more variable results than those in which we used other methods to adjust pH/pCO₂. We surmise this effect may result from the mechanical effect of bubbling (to which *E. huxleyi* appears particularly sensitive), since all chemical parameters were identical in the bubbled cultures and others for the first several days of the experiment. Others have also obtained results with a high degree of variability in bubbled cultures of strain PLY M219 (Iglesias-Rodriguez et al., 2008b). Bubbling seems particularly problematic at low cell densities, and, in our hands, upon inoculation of *T. weissflogii* at 20 cell ml⁻¹, or *E. huxleyi* at 200 cell ml⁻¹, bubbled cultures had difficulty getting started regardless of whether the cells had been pre-acclimated to bubbling condition. To obtain reproducible results, we used a protocol in which bubbling was stopped for 24 h after inoculation, before we began monitoring growth. The pCO₂/pH of closed culture vessels changes negligibly during this time. It should be also noted that when cultures (of any organism) reach high cell concentrations, it becomes difficult to supply enough CO₂ through bubbling to keep up with the rate of CO₂ fixation by the cells. As a result, when POC reaches values above 100 μmol C kg⁻¹ the pCO₂ of the cultures is often markedly lower than the nominal pCO₂ of the bubbled gas.

3.5 Calcification in coccolithophores

Because a decrease in calcium carbonate saturation might affect biological calcification, and hence the response of ocean chemistry to increasing pCO₂, many medium acidification experiments have focused on the question of calcification by coccolithophores. These experiments have generally shown a slight increase in growth rate with increasing pCO₂, and a negligible to relatively large decrease in calcification, depending on the species (Riebesell et al., 2000; Sciandra et al., 2003; Langer et al., 2006; Feng et al., 2008).

A recent article reported increased calcification rate per cell and decreased growth rates with increasing pCO₂ in the strain PLY M219 of the coccolithophore *E. huxleyi* (Iglesias-Rodriguez et al., 2008b). The authors have attributed the difference between their result and those of previous researchers to the different methods used to adjust the pH/pCO₂ of the cultures, namely acid addition vs. CO₂ bubbling (Iglesias-Rodriguez et al., 2008b; Iglesias-Rodriguez et al., 2008a). Measurements of photosynthetic and calcification rates in the experiments of Fig. 2d provide a test of this explanation.

As discussed above, the growth rates of PLY M219 cultures actually increased slightly rather than decreased when we increased pCO₂/decreased pH by acid addition, with or without buffer, and remained unchanged upon bubbling of air at pCO₂=750 μatm (Fig. 2d and Table 1). In acidified cultures, we also measured significant increases in PIC/cell and POC/cell ($p < 0.05$, t-test). The resulting significant increases in rates of photosynthesis and calcification per cell

Table 1. Growth rate, POC per cell, PIC per cell, PIC/POC ratio, POC and PIC production rates of the coccolithophore *Emiliania huxleyi* PLY M219 (data are mean±sd) at pH 8.10 and 7.80 adjusted by addition of acid or EPPS and by CO₂ bubbling.

Treatment	Growth rate* (d ⁻¹)	POC (pmol cell ⁻¹)	PIC (pmol cell ⁻¹)	PIC/POC	POC production (pmol cell ⁻¹ d ⁻¹)	PIC production (pmol cell ⁻¹ d ⁻¹)
pH 8.10	Acid (n=2)	1.32±0.01 ^a	0.62±0.00	0.49±0.00	0.79±0.00	0.64±0.00
	EPPS (n=2)	1.41±0.04 ^a	0.57±0.04	0.47±0.04	0.83±0.02	0.66±0.04
	mean±sd	1.36±0.06	0.59±0.04	0.48±0.02	0.81±0.03	0.65±0.02
	Bubbling (n=6)	1.32±0.07 ^a	n.d.	n.d.	0.83±0.09	1.04±0.24
pH 7.80	Acid (n=4)	1.48±0.02 ^a	0.93±0.11	0.68±0.07	0.73±0.03	1.37±0.15
	EPPS (n=2)	1.53±0.04 ^a	0.72±0.03	0.58±0.00	0.80±0.03	1.10±0.02
	mean±sd	1.49±0.04	0.86±0.14	0.64±0.08	0.75±0.04	1.28±0.18
	Bubbling (n=7)	1.29±0.07 ^b	n.d.	n.d.	0.79±0.19	1.15±0.35

* for treatments at each pH level, values with significantly different means ($p < 0.05$) are labeled with different letters (one-way ANOVA with post-hoc tests).

n.d.: not determined.

at high $p\text{CO}_2$ /low pH compared to low $p\text{CO}_2$ /high pH are qualitatively consistent with the published data on the same *E. huxleyi* strain (Iglesias-Rodriguez et al., 2008b). As in the previous study, we also observed a slight decrease in PIC/POC ratios though it may not be significant ($p = 0.06$, t-test). The experiments with CO₂ bubbling did not allow the precise and convenient measurements of PIC and POC given by long-term incorporation of ¹⁴C; nonetheless, short-term ¹⁴C incorporation experiments gave systematic data for the $\Delta\text{PIC}/\Delta\text{POC}$ ratio that were very similar to those of the acidified cultures (Table 1). The concomitant increases in growth rate, POC/cell and PIC/cell observed at high $p\text{CO}_2$ /low pH in PLY M219 probably indicate a control of calcification by cellular physiology rather than by the saturation state of calcite. From a methodological point of view we observed no significant differences in growth or photosynthetic rates, or in PIC/POC ratios between the different methods used to control $p\text{CO}_2$ /pH, aside from the slightly lower growth rates of bubbled cultures at higher $p\text{CO}_2$ /lower pH.

3.6 Effects of buffer on Fe limitation

As shown in Fig. 2, the presence of EPPS in the medium has no significant effect on the growth of nutrient-replete phytoplankton, and thus, presumably, no direct physiological effects on the organisms. But like all weak acids, EPPS can form weak complexes with metals (Mash et al., 2003). Complexes with Ca²⁺ or Mg²⁺ are expected to be too weak to affect the speciation of these metals in seawater, and indeed we saw no significant effect of EPPS on calcification by *E. huxleyi*. But EPPS complexes with essential trace metals may augment or inhibit their availability under some conditions. We thus conducted growth experiments with Fe-limited *T. weissflogii* at two different pH/pCO₂ in a medium buffered with EDTA, in the presence or absence of 8 mM EPPS. As expected, in the absence of EPPS the growth rate

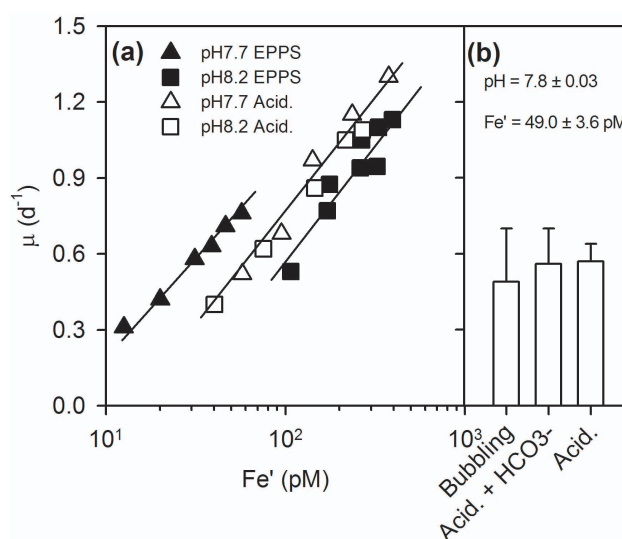


Fig. 4. Specific growth rate of Fe-limited diatom *T. weissflogii* (a) at pH 7.7 and 8.2 manipulated by addition of acid or EPPS, and (b) at pH 7.8 ± 0.03 controlled by addition of acid, addition of equimolar of acid and bicarbonate, or CO₂ bubbling. Error bars in (b) represent standard deviation of $n = 8-9$.

varied systematically with the calculated Fe', the concentration of unchelated Fe which depends on both the total Fe concentration and the pH (Sunda et al., 2005). Strikingly, the growth rate increased by about 50% at pH 7.7, but decreased by about 18% at pH 8.2 for a given calculated Fe' in the presence of EPPS compared to the no-buffer cultures (Fig. 4a). We surmise that these effects may be caused by the formation of Fe-EPPS complexes that change the availability of Fe in the culture medium in a pH-dependent manner, through the extent of Fe complexation and/or the reducibility of the complex. Previous studies have demonstrated that the

complexation of copper by TRIS (trishydroxymethylamino methane) reduces copper availability to algae by reducing the concentration of the free cupric ion, Cu²⁺ (Sunda and Guillard, 1976; Anderson and Morel, 1978). Another commonly used buffer, HEPES, has been shown to promote the production of exudates that cause a marked decrease in Cu²⁺ in *E. huxleyi* cultures (Vasconcelos and Leal, 2002).

Elucidating the underlying mechanism responsible for these effects of EPPS on Fe-limited cultures is beyond the scope of this study. Regardless of mechanisms, the substantial changes in growth rates caused by EPPS addition at low Fe and variable pH in *T. weissflogii* cultures would mask any possible effect of pH/pCO₂ under Fe-limited conditions. So the very convenient use of EPPS, or likely other pH buffers, for studying the effects of medium acidification on marine phytoplankton must be forgone in experiments involving metal limitation. We note that Fe-limited cultures in which the pCO₂/pH of the medium was modified by bubbling of high pCO₂ air, or initial acidification, with or without bicarbonate addition, all gave the same growth rates, albeit with some variability, particularly in the bubbled cultures (Fig. 4b).

4 Conclusions

Studying the effect of medium acidification on phytoplankton physiology poses unusual experimental difficulties, not because the experiments are technically challenging, but because all the key chemical parameters are interdependent and all are affected by the growth of the organisms. As a result, there is no easy method to study the physiological effects of a single parameter, such as pCO₂, pH or Ω, while maintaining all other parameters constant in a batch culture. The simplest method is to limit the experiments to sufficiently low cell concentrations. For example, keeping the biomass of diatom cultures below 20 μmol C kg⁻¹ (~250 μg C l⁻¹) will keep pH within 0.05 units and the pCO₂ within 10% of their initial values, even without any control method. In cultures of calcifying organisms the decrease in Alk that results from precipitation of CaCO₃ partly compensates for the effects of decreasing DIC, the extent of which depends on the ΔPIC/ΔPOC ratio. As a result, a higher biomass (e.g., ΔPOC=ΔPIC=40 μmol C kg⁻¹) can be allowed to maintain pH and pCO₂ within similar ranges.

A convenient and widely used method to maintain the pCO₂ constant is to bubble air with a given fraction of CO₂ in the growth medium. This technique maintains good control of the DIC and pH in cultures of non-calcifying phytoplankton. In calcifying cultures, the DIC decreases along with Alk, leading to potentially significant changes in pH and Ω at high cell concentrations. Presumably as a result of the mechanical effect of bubbling, we have found it more difficult to obtain reproducible growth rates in bubbled cultures than in cultures with other methods of pCO₂/pH control.

Controlling the pH of cultures by addition of a buffer or the use of a pH stat is a useful alternative to bubbling and gives good control of pCO₂, particularly in non-calcifying cultures. But high concentrations of calcifying cells promote significant changes in pCO₂ and Ω. We have unfortunately observed that the presence of buffers apparently affects the availability of trace metals, precluding their use in metal-limited experiments.

The extent to which one may want to control the acid-base chemistry of phytoplankton cultures depends on the goal of the experiments being conducted and the type of organism being studied. Different methods are then more appropriate, depending, for example, on whether pCO₂ or pH must be controlled, whether it matters or not that DIC or Ω vary, whether the organism calcifies or not, and if a high cell biomass is desired. If the goal is to mimic what may happen in the surface ocean, the changes brought about in culture media by CO₂ fixation, nutrient utilization and calcification may be allowable or even desirable if they are kept at reasonable levels.

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