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Effects of the pH/pCO_2 control method in the growth medium of phytoplankton

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Abstract

To study the effects of ocean acidification on the physiology of phytoplankton requires that the key chemical parameters of the growth medium, pCO_2, pH and Ω (the saturation state of calcium carbonate) be carefully controlled. This is made difficult by the interdependence of these parameters. Moreover, in growing batch cultures of phytoplankton, the fixation of CO_2, the uptake of nutrients and, for coccolithophores, the precipitation of calcite all change the inorganic carbon and acid-base chemistry of the medium. For example, absent pH-buffering or CO_2 bubbling, a sizeable decrease in pCO_2 occurs at a biomass concentration as low as 50 μM C in non-calcifying cultures. Even in cultures where pCO_2 or pH is maintained constant, other chemical parameters change substantially at high cell densities. The quantification of these changes is facilitated by the use of buffer capacities. Experimentally we observe that all methods of adjustment of pCO_2/pH can be used, the choice of one or the other depending on the specifics of the experiments. The mechanical effect of bubbling of cultures seems to induce more variable results than other methods of pCO_2/pH control. While highly convenient, the addition of pH buffers to the medium 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 pCO_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 total 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 pCO_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).

To study the response of phytoplankton to increasing pCO_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), the control of pCO_2/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. The most commonly used methods have been to bubble prepared air mixtures containing a given fraction of CO_2, 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 CO_2 as it is depleted by the growing culture. A similar result can be achieved in the absence of a buffer by using a 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 pCO_2 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/pCO_2 control in cultures of model phytoplankton species and examined their effects on the growth and calcification of the organisms under various conditions.

2 Materials and methods

2.1 Cultures

The marine diatom *Thalassiosira weissflogii* (clone Actin) was obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP). The strains CCMP 374 and PLY M219 (NZEH) 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 acid-cleaned polycarbonate bottles using 0.2-μm filtered Gulf Stream seawater. The culture media were enriched with 150 μM NO_3^-, 10 μM PO_4^{3-}, 100 μM SiO_2 and vitamins for *T. weissflogii*, and 100 μM NO_3^-, 6 μM PO_4^{3-} 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 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. Cell numbers 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 ln(cell number) vs. time.

2.2 pCO_2/pH manipulation

Targeted pCO_2 or pH values in culture medium were achieved by bubbling of commercially prepared air-CO_2 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 CO_2 bubbling

Prior to inoculations, seawater medium was gently bubbled with CO_2-enriched air (pCO_2 380 ppm or 750 ppm) until a desirable pH was achieved and remained stable. Bubbling was stopped for 24 h following inoculation; it was then resumed and continued till 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., 1988/1989) and pH adjusted with ultra pure HCl/NaOH. It was then introduced into culture medium at concentrations of 5 mM and 8 mM for *T. weissflogii* and *E. huxleyi*, respectively, to attain targeted pH.

2.2.3 Acid/base adjustment

pCO_2/pH of medium was adjusted by adding ultra pure HCl/NaOH to give desired pH. In experiments conducted at 750 ppm pCO_2, we also used a treatment in which an equimolar concentration of ultra pure $NaHCO_3$ was added simultaneously with the acid to maintain alkalinity constant.

2.3 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 ^{14}C incorporation. For short-term ^{14}C incorporation of bubbled cultures, $NaH^{14}CO_3$ was added into a subsample of the cultures and incubated for 2–4 h under the same conditions, but no CO_2 bubbling. For long-term ^{14}C incorporation, $NaH^{14}CO_3$ 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 μm 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% H_3PO_4 was added into the vials. Each vial was closed immediately with a cap containing a GF/D filter with 60 μl 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 ^{14}C with a LS6500 Multi-purpose Scintillation Counter (Beckman).

2.4 Calculation methods

Model calculations for a seawater medium containing 10 μM phosphate and 100 μM silicate were made according to DOE (1994) – except that the acidity constants for CO_2 in seawater are from Lueker et al. (2000) – using values of DIC=2030 μM and Alk=2310 μM, absent acid/base addition or CO_2 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 the known DIC or Alk of the medium, depending on the conditions.

The buffer factors γ_{DIC}, γ_{Alk}, β_{DIC}, β_{Alk}, ω_{DIC} and ω_{Alk} were calculated using the formulae in Egleston et al. (2009) except that we added a term to take into account the

relatively large phosphate concentration (10 μM) in the culture medium: $\frac{[H^+][HPO_4^{2-}]}{K_{hp}+[H^+]}$ was added to the equivalent borate term where it appears in the equations.

3 Results and discussion

3.1 Theoretical considerations

As mentioned above, the different methods used to control the pCO_2/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, resulting in changes in the chemistry of the medium, particularly pCO_2, pH, and Ω (the saturation state of calcium carbonate). These changes can be precisely calculated as illustrated in Fig. 1 for typical (not acidified) seawater conditions. Note that $\Omega=[Ca^{2+}]\,[CO_3^{2-}])/K_s$, such that $d\Omega/\Omega=d[CO_3^{2-}]/[CO_3^{2-}]$ in the presence of a large excess of Ca^{2+} when the solubility product of $CaCO_3$(s), K_s is constant. Here we used $K_s=4.3\times10^{-7}$ M^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 pCO_2, the changes in the medium chemistry are brought about by the fixation of CO_2, which decreases DIC, and to a lesser extent by the uptake of NO_3^-, which increases Alk by $\Delta Alk=-\Delta[NO_3^-]$. For a typical N:C ratio in the biomass of 0.16, $\Delta Alk=-0.16$ ΔDIC. 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 pCO_2 by approximately 24% when $-\Delta DIC>50$ μM, 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 20% for the same decrease in DIC. When the same cultures are bubbled at constant pCO_2, 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 pCO_2 decreases proportionally with DIC, i.e., only about 2.5% for $-\Delta DIC=50\,\mu M$. 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 μM C; this can be achieved within 0.5 pH units of the pKa 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 $CaCO_3$(s), in addition to the effects of CO_2 fixation and NO_3^- uptake. For cultures that are not bubbled at constant pCO_2, the net result is smaller changes in pCO_2, 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 (POC/PIC=1). Because Alk decreases along with DIC, Ω changes very little as the cells grow.

If a calcifying culture is bubbled at constant pCO_2, the decrease in DIC is a sizeable fraction of the decrease in Alk, about 75 μM ($\Delta DIC/DIC\sim-4\%$) for a PIC production of 50 μM ($\Delta Alk=-92\,\mu M$) (Fig. 1c). The pH decrease is small but the corresponding decrease in Ω, from 4.5 to 4.2, might be significant.

When the pH is maintained constant in a calcifying culture, there is a small decrease in pCO_2 as the organism fix CO_2 and calcify (Fig. 1d). The changes in DIC and Ω are similar to those in a bubbled culture.

The changes in chemistry illustrated in Fig. 1 depend on the initial composition of the medium so that new tedious calculations must be made for any new experiment in which pCO_2/pH is adjusted. In cultures where the object is to maintain sufficiently constant chemistry, the changes in DIC and Alk resulting from the growth of the phytoplankton must be kept relatively small and a reasonably accurate and simple method is provided by the use of buffers capacities (Egleston et al., 2009). Six parameters, γ_{DIC}, γ_{Alk}, β_{DIC}, β_{Alk}, ω_{DIC} and ω_{Alk} quantify the differential changes in pCO_2, pH and Ω upon changes in DIC and Alk, each defined as the inverse of a partial derivative; e.g., $\gamma_{DIC}=(\partial \ln pCO_2/\partial DIC)^{-1}$ (see Egleston et al., 2009). The values of the buffer capacities for each medium used in this study are given in Table 1. Small changes in pCO_2,

pH and Ω brought about by the growth of the phytoplankton and by the precipitation of calcite in the case coccolithophores can then be readily estimated:

$$\Delta pCO_2/pCO_2 = \Delta DIC/\gamma_{DIC} + \Delta Alk/\gamma_{Alk} \quad (1)$$

$$\Delta pH = -(\Delta DIC/\beta_{DIC} + \Delta Alk/\beta_{Alk})/2.3 \quad (2)$$

$$\Delta\Omega/\Omega = \Delta DIC/\omega_{DIC} + \Delta Alk/\omega_{Alk} \quad (3)$$

In the case of a non-calcifying organisms with a N:C ratio of 0.16 growing in a medium where pCO_2 is not fixed by bubbling, we can take $\Delta Alk = -0.16\Delta DIC = 0.16\Delta POC$. For a calcifying organism with N:C=0.16 and POC/PIC=1, $\Delta Alk = -(2\Delta PIC - 0.16\Delta POC) = -1.84\ \Delta PIC$.

If, pCO_2 is maintained constant, $\Delta[CO_2]=0$, such that

$$\Delta DIC = -\gamma_{DIC}/\gamma_{Alk} \times \Delta Alk \quad (4)$$

where ΔAlk is given as above by the nitrate uptake and the PIC formation.

If, pH is maintained constant, Δ[pH]=0, such that

$$\Delta Alk = -\beta_{Alk}/\beta_{DIC} \times \Delta DIC \quad (5)$$

where ΔDIC is given by the total POC and PIC in the medium.

For a given pH, the values of the buffer factors in Table 1 are all very similar to each other, signifying that the relative changes in pCO_2, $[H^+]$ and Ω upon changes in DIC and Alk all have similar magnitudes. (We note that a change in pH by only 0.1 units, represents a change of more than 20% in $[H^+]$). The buffer factors decrease with decreasing pH, reaching a minimum around pH=7.5 (Egleston et al., 2009). For non-bubbled cultures of a non-calcifying organism, where the dominant process is simply the decrease in DIC due to CO_2 fixation, the relative decrease in pCO_2 and the increase in pH during growth are thus more severe when the medium is acidified. For non-bubbled cultures of calcifying organisms where the decrease in DIC and Alk partially offset each other, the result depends on the PIC/POC ratio and the exact values

of the buffer factors. According to the values in Table 1, if PIC/POC=1, the relative decrease in pCO_2 is slightly smaller in acidified cultures, while the increase in pH is slightly larger. For calcifying organisms the decrease in DIC brought about by the decrease in Alk in bubbled cultures and the relative decrease in pCO_2 in constant pH cultures are higher at higher pCO_2/lower pH.

3.2 Maximum growth rates

Under nutrient-replete conditions at normal seawater pH, we observed identical maximum growth rates (μ=1.38±0.06 d^{-1}) in cultures of the diatom *T. weissflogii* whether the pH/pCO_2 of the medium was not controlled, pCO_2 fixed by bubbling, or pH buffered by addition of EPPS (Fig. 2a). In other experiments, we have obtained identical results with cultures maintained at a constant pH with a pH-stat (data not shown). Under similar conditions, we also observed identical maximum growth rates (μ=1.34±0.01 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 *E. huxleyi* strain PLY M219, an abundant calcifyer, also showed no difference in growth rate whether or not bubbled at ambient pCO_2 level (380 ppm) and in the presence or absence of EPPS (μ=1.36±0.05 d^{-1}) (Fig. 2c). Increasing the pCO_2 of these cultures by acidification in the presence or absence of buffer slightly increased their growth rate (μ=1.50±0.04 d^{-1}) but not when pCO_2 was fixed by bubbling (Fig. 2d; see below).

3.3 Changes in medium chemistry during growth

Based on a cellular C quota of 10 pmol/cell and an N:C ratio of 0.13 in *T. weissflogii* in our culture medium, the changes in medium composition over time in the absence of bubbling or pH control in the experiment of Fig. 2a can be calculated using the buffer capacities in Table 1 (Fig. 3a). After approximately 4 days of cultures, when the cells reach a concentration of ~5000 cells/mL, the pCO_2 of the medium decreases by about

25% and the pH increases by 0.09 units. This calculated change in pH agrees well with our actual measurement of 0.1 pH units.

Similar calculations for the experiments of Fig. 2c and d are shown in Figs. 3b and 3c, based on the measured composition of *E. huxleyi* strain PLY M219: POC=0.6 pmol/cell and PIC=0.5 pmol/cell at pH=8.1; POC=0.9 pmol/cell and PIC=0.65 pmol/cell at pH=7.8 (see below); and C:N=8 (To allow comparison among treatments we used an initial cell concentration of 350 cells/mL and a growth rate of 1.35 d^{-1} for Figs. 3b–e, instead of the actual data, and continued the calculations beyond the duration of the experiments). As seen in the figures, a sizeable (10%) decrease in pCO_2 and increases in pH occurs for cell concentrations around 60 000 cells/mL in the cultures at seawater pH without buffer or CO_2 bubbling (Fig. 3b). As expected, a similar relative decrease in pCO_2 occurs at a lower cell concentration, ca 30 000 cells/mL, in cultures at higher pCO_2/lower pH which are less well buffered (Fig. 3c).

To achieve a reasonable control of pCO_2 and/or pH in culture media with no buffer or CO_2 bubbling thus necessitates stopping the culture 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. The buffer capacities given in Table 1 provide a convenient means of calculating the appropriate additions necessary to maintain pCO_2 and pH within desired limits. For example, adding 46 μM HCl and 46 μM $NaHCO_3$ on day 4 in the cultures of Fig. 2a, would have adjusted the DIC to 2030 μM and the pH to 8.1, allowing for another doubling of the cell number before the pCO_2 decreases by 27% and the pH increases by 0.1 units.

To maintain constant conditions in a bubbled culture of a calcifying organism also requires periodic addition of either base or bicarbonate to make up for the loss of alkalinity and the accompanying decrease in DIC. This is illustrated in Fig. 3d which corresponds to the cultures bubbled at pCO_2=750 ppm in Fig. 2d. When cell densities reach about 80 000 cells/mL, the DIC, Ω and to a lesser extent pH begin decreasing along with Alk.

In a culture with constant low pH corresponding to the experiment in Fig. 2d, significant changes in DIC, pCO_2 and Ω also begin at about 80 000 cells/mL (Fig. 3e). A decrease by 10% in pCO_2 occurs at about 150 000 cells/mL.

3.4 Effects of bubbling.

The data of Figs. 2a–c show no difference in growth rates among cultures where pCO_2/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 (Fig. 2c). But the cultures bubbled with air at pCO_2=750 ppm, grew slightly slower than those in which pCO_2 was increased by acidification. We believe this small decrease in growth rate results from the mechanical effect of bubbling to which *E. huxleyi* appears particularly sensitive. We have seen adverse effects of bubbling on the growth of several phytoplankton species, and our experiments with bubbled cultures have yielded more variable results than those in which we used other methods to adjust pH/pCO_2. 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, or *E. huxleyi* at 200 cell/mL, bubbled cultures had difficulty getting started. To obtain reproducible results, we used a protocol in which bubbling was stopped for 24 h after inoculation, before we began monitoring growth. The pCO_2/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 CO_2 through bubbling to keep up with the rate of CO_2 fixation by the cells. As a result, when POC reaches values above 100 μmol C L^{-1} the pCO_2 of the cultures is often markedly lower than the nominal pCO_2 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 pCO_2, 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 pCO_2, and a negligible to moderate decrease in calcification, depending on the species (Feng et al., 2008; Riebesell et al., 2000).

A recent article reported increased calcification rate per cell and decreased growth rates with increasing pCO_2 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 (e.g. Riebesell et al. 2000) to the different methods used to adjust the pH/pCO_2 of the cultures, namely acid addition vs. CO_2 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 pCO_2/decreased pH by acid addition, with or without buffer, and remained unchanged upon bubbling of air at pCO_2=750 ppm (Fig. 2d and Table 2). In acidified cultures, we also measured significant increases in PIC and POC/cell. The resulting significant increases in rates of photosynthesis and calcification per cell at high pCO_2/low pH compared to low pCO_2/high pH are qualitatively consistent with the published data on the same *E. huxleyi* strain (Iglesias-Rodriguez et al., 2008b). The experiments with CO_2 bubbling did not allow the precise and convenient measurements of PIC and POC given by long-term incorporation of ^{14}C. Nonetheless, short-term ^{14}C incorporation experiments gave systematic data for the PIC:POC ratio that were virtually identical to those of the acidified cultures: PIC:POC=0.81±0.07 and 0.76±0.15 (n=5) vs. 0.81±0.03 (n=4) and 0.75±0.04 (n=6) in Table 2, at high and low pH, respectively. Thus, as observed in practically all previous

experiments with coccolithophores, the PIC/POC ratio of PLY M219 decreased slightly with increasing pCO_2, making it likely that a change in calcification in the nutrient-limited surface ocean will provide a slight negative rather than positive feedback to the ongoing increase in atmospheric CO_2. We note also that the concomitant increases in growth rate, POC and PIC observed at high pCO_2 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 pCO_2/pH, aside from the slightly lower growth rates of bubbled cultures.

3.6 Effects of buffer on Fe limitation

As shown in Fig.1, 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 essential trace metals (Mash et al., 2003) and may augment or inhibit their availability under some conditions. We thus conducted growth experiments with Fe-limited *T. weissflogii* at two different pH/pCO_2 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 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, Cu′ (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 Cu′ 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/pCO_2 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 pCO_2/pH of the medium was modified by bubbling of high pCO_2 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 pCO_2, 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, typically below a biomass of 50 μmol/L POC. Even when the pCO_2 or pH of such cultures is not controlled, the changes in DIC and Alk brought about by the growth of the organisms are too small to change significantly the chosen initial conditions. The decrease in Alk that results from precipitation of $CaCO_3$ partly compensates for the effects of decreasing DIC, and, as a result, pCO_2 and pH are less variable in calcifying than in non-calcifying cultures.

A convenient and widely used method to maintain the pCO_2 constant is to bubble air with a given fraction of CO_2 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 results in bubbled cultures than in cultures with other methods of pCO_2/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 pCO_2, particularly in non-calcifying cultures. But high concentrations of calcifying cells promote significant changes in pCO_2 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, pCO_2 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 CO_2 fixation, nutrient utilization and calcification may be allowable or even desirable if they are kept at reasonable levels.

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Table 1. Calculated buffer factors (γ_{DIC}, β_{DIC}, γ_{Alk}, β_{Alk}, ω_{DIC} and ω_{Alk}) for Aquil medium at different pCO_2/pH manipulated by CO_2 bubbling or addition of acid. See Materials and Methods for calculation methods.

Fig.	Treatment	pCO_2 (ppm)	pH	DIC (μM)	Alk (μM)	γ_{DIC} (mM)	$\beta_{DIC}=-\gamma_{Alk}$ (mM)	β_{Alk} (mM)	ω_{DIC} (mM)	ω_{Alk} (mM)
2a, 3a	acid	354	8.09	2030	2330	0.21	0.25	−0.28	−0.23	0.23
2c, 3b	acid	340	8.10	2030	2340	0.21	0.26	−0.29	−0.24	0.23
2d, 3c, 3e	acid	730	7.80	2030	2180	0.15	0.17	−0.18	−0.14	0.14
2d, 3d	bubbling	750	7.80	2150	2310	0.16	0.18	−0.19	−0.15	0.15

Table 2. Growth rate, POC, PIC, PIC/POC ratio, POC and PIC production rate of the coccolithophore *Emiliania huxleyi* PLY M219 at pH 8.10 and 7.80 adjusted by addition of acid or EPPS.

pH	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})
8.10	acid (n=2)	1.32±0.01	0.62±0.00	0.49±0.00	0.79±0.00	0.81±0.01	0.64±0.00
	EPPS (n=2)	1.41±0.04	0.57±0.04	0.47±0.04	0.83±0.02	0.80±0.03	0.66±0.04
	avg.±sd	1.36±0.06	0.59±0.04	0.48±0.02	0.81±0.03	0.81±0.02	0.65±0.02
7.80	acid (n=4)	1.48±0.02	0.93±0.11	0.68±0.07	0.73±0.03	1.37±0.15	1.00±0.10
	EPPS (n=2)	1.53±0.04	0.72±0.03	0.58±0.00	0.80±0.03	1.10±0.02	0.88±0.02
	avg.±sd	1.49±0.04	0.86±0.14	0.64±0.08	0.75±0.04	1.28±0.18	0.96±0.10

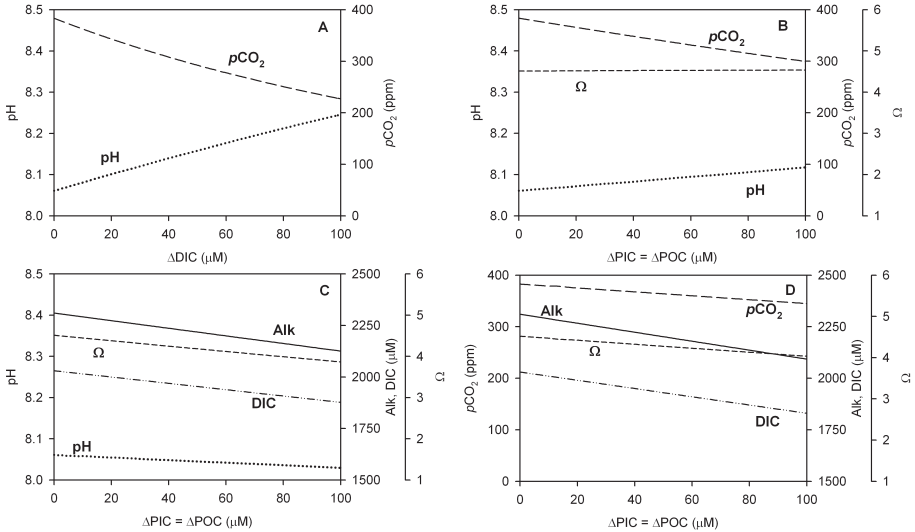

Fig. 1. Calculated chemical parameters of the Aquil seawater medium (normal pH) as a function of ΔDIC or ΔPIC. **(A)** Cultures of non-calcifying phytoplankton without pH/pCO_2 control; **(B)** cultures of calcifying phytoplankton without pH/pCO_2 control; **(C)** cultures of calcifying phytoplankton bubbled at constant pCO_2; and **(D)** cultures of calcifying phytoplankton maintained at constant pH. See Materials and Methods for calculation methods.

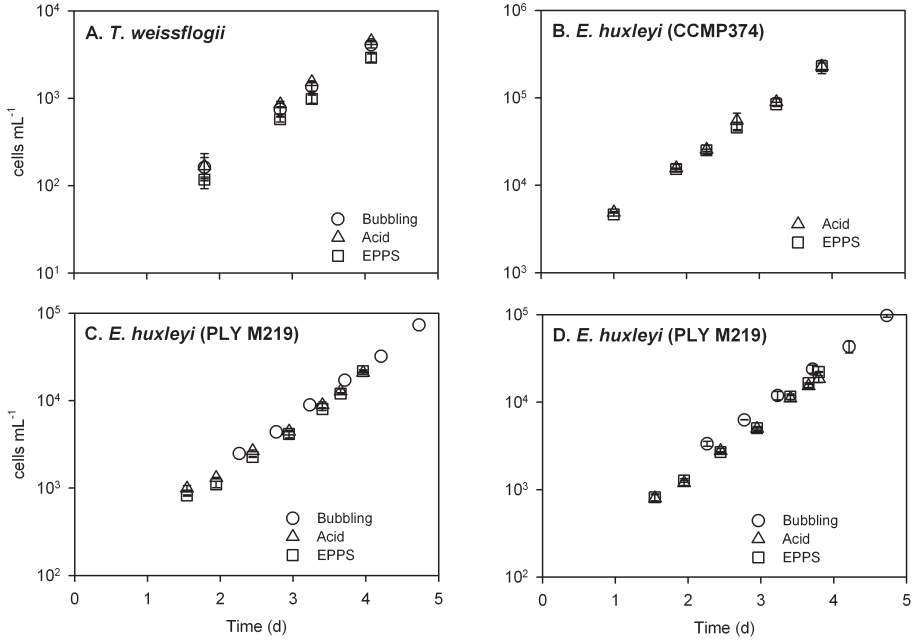

Fig. 2. Growth curves of nutrient-replete diatom *Thalassiosira weissflogii* and coccolithophore *Emiliania huxleyi* at different pH/pCO_2 manipulated by CO_2 bubbling, addition of acid, or EPPS. **(A)** *T. weissflogii* at pH=8.09; **(B)** *E. huxleyi* CCMP374 at pH=8.19; *E. huxleyi* PLY M219 (NZEH) at **(C)** pH=8.10 and **(D)** pH=7.80. Error bars represent standard deviation or the range of n=2–3.

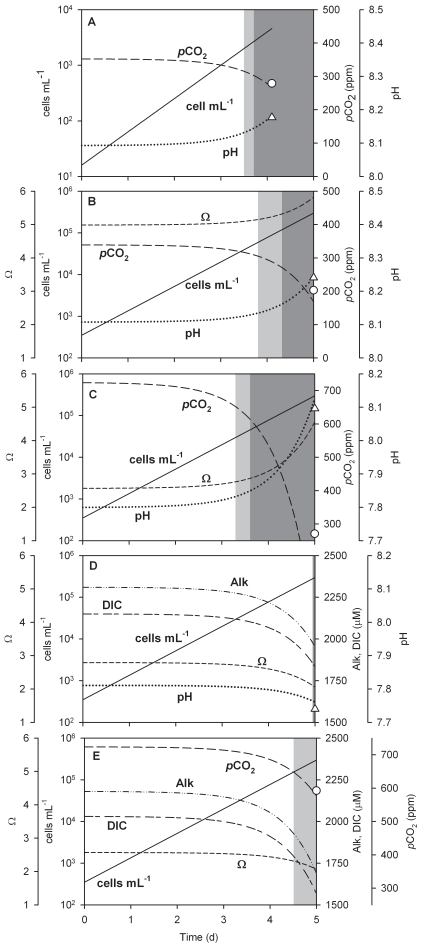

Fig. 3.

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Fig. 3. Cell density and chemical parameters calculated using the buffer factors in Table 1 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/pCO_2 control; **(B)** cultures of *E. huxleyi* PLY M219 at pH=8.10 without pH/pCO_2 control; **(C)** cultures of *E. huxleyi* PLY M219 and at pH=7.80 without pH/pCO_2 control; **(D)** cultures of *E. huxleyi* PLY M219 bubbled with air at 750 ppm pCO_2; and **(E)** cultures of *E. huxleyi* PLY M219 maintained at pH=7.80. The light grey areas in the figures indicate a change in pCO_2 larger than 10% of the initial value, and the dark grey areas indicate a change in pH larger than 0.05 units. The triangles and circles show the pH and pCO_2 at the end of the growth experiments obtained via full calculations, rather than using buffer factors. See Materials and methods for calculation methods.

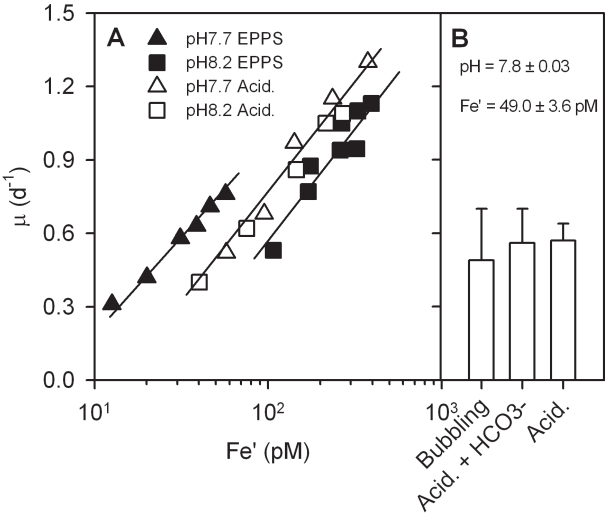

Fig. 4. Specific growth rate of Fe-limited diatom *T. weissflogii* **(A)** at pH 7.7 and 8.2 manipulated by addition of acid or EPPS, and **(B)** at pH 7.8±0.03 controlled by addition of acid, addition of equimolar of acid and bicarbonate, or CO_2 bubbling. Error bars in (B) represent standard deviation of n=8–9.