

# Effect of lowered pH on marine phytoplankton growth rates

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**ABSTRACT:** Continued anthropogenic carbon emissions are expected to result in an increase in atmospheric CO<sub>2</sub> concentration to 700 ppm by the end of this century. This will cause a corresponding drop in the global average surface water pH of the oceans by ~0.4 units to ~7.8 and an increase in the CO<sub>2</sub> concentration of seawater. Ocean acidification may potentially both stimulate and reduce primary production by marine phytoplankton. Data are scarce on the response of marine phytoplankton growth rates to lowered pH/increased CO<sub>2</sub>. Using the acid addition method to lower the seawater pH and manipulate the carbonate system, we determined in detail the lower pH limit for growth rates of 2 model species of common marine phytoplankton. We also tested whether growth and production rates of 6 other common species of phytoplankton were affected by ocean acidification (lowered to pH 7.0). The lower pH limits for growth of the dinoflagellate *Heterocapsa triquetra* and the cryptophyte *Teleaulax amphioxeia* were pH ~6.0 and 6.3, respectively. The growth rates of these 2 species were significantly reduced in the range of pH 6.4 to 6.5. Cell volume, growth, and production rates of the 6 other phytoplankton species were statistically similar in the pH range of ~7.0 to 8.5. Our results and literature reports on growth at lowered pH indicate that marine phytoplankton in general are resistant to climate change in terms of ocean acidification, and do not increase or decrease their growth rates according to ecological relevant ranges of pH and free CO<sub>2</sub>. We speculate about whether common natural pH fluctuations in time and space from 7.0 to 9.0 make phytoplankton capable of tolerating near-future ocean acidification. However, due to the less fluctuating pH environment of oceanic regions compared to coastal regions, truly oceanic species may be more sensitive to lowered pH than coastal species.

**KEY WORDS:** Marine phytoplankton · Lowered pH · Growth rates · Primary production · Ocean acidification

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## INTRODUCTION

By the end of the present century, burning of fossil fuels will have doubled atmospheric CO<sub>2</sub> concentration and increased the total inorganic carbon (TCO<sub>2</sub>) content of the surface ocean by ~12% compared to preindustrial levels (Houghton et al. 2001). The associated drop in the average surface water pH from ~8.2 to ~7.8 represents one of the most rapid ocean acidification events on earth over the past 300 Myr (Caldeira & Wickett 2003). Equilibrium pools of TCO<sub>2</sub> species (i.e. free CO<sub>2</sub>, HCO<sub>3</sub><sup>-</sup>, and CO<sub>3</sub><sup>2-</sup>) will change, and in-

creased free CO<sub>2</sub> could potentially enhance phytoplankton primary production (Riebesell et al. 2007). This is because free CO<sub>2</sub> concentrations in the oceans are presently at limiting levels for RUBISCO, the enzyme responsible for the first step in photosynthesis (Beardall & Raven 2004). However, most phytoplankton species possess CO<sub>2</sub> concentrating mechanisms (CCMs), which increase free CO<sub>2</sub> in the vicinity of RUBISCO and saturate photosynthesis in the present ocean (Giordano et al. 2005). On the other hand, a pH drop of ~0.4 units, as predicted, represents a ~150% increase in the H<sup>+</sup> concentrations, which may affect

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intracellular pH, membrane potential, energy partitioning, and enzyme activity (Beardall & Raven 2004, Riebesell 2004, Giordano et al. 2005). Thus, ocean acidification may reduce phytoplankton growth rates through direct pH effects.

Experimental data on the effects of lowered pH and increased free CO<sub>2</sub> on growth and productivity of phytoplankton are few and do not show a clear pattern (Iglesias-Rodriguez et al. 2008, Langer et al. 2009). Some studies dealing with natural plankton communities, using micro- and mesocosms, have shown altered species composition in response to lowered pH and increased free CO<sub>2</sub>, while other studies have shown very limited effects on species composition and community production (Tortell et al. 2002, Kim et al. 2006, Feng et al. 2009, Nielsen et al. in press). While micro- and mesocosm studies have the advantage of allowing the analyses of complete, natural phytoplankton communities, the conclusions from such studies are difficult to interpret, and experimental artifacts may be common. (1) Since plankton communities are not subjected to sedimentation in closed experimental bottles, as they are in nature, a few fast-growing species (i.e. non-motile diatoms) tend to dominate such communities within 1 wk of incubation, making a proper community description difficult. (2) Dilution (typically 50% d<sup>-1</sup>) is often applied to the incubation bottles to avoid nutrient limitation and increased pH during the experiment. This further contributes to the problem of using mesocosm incubations because slow-growing rare species are removed. (3) Observed apparent effects of lowered pH and increased free CO<sub>2</sub> on phytoplankton may be indirect rather than direct, if grazers and competitors are affected (i.e. heterotrophic protists; Pedersen & Hansen 2003). (4) Tests of the required acclimation period of natural communities are impossible, and a long acclimation period will change the system compared to the community that was originally sampled. Thus, due to the problems with mesocosm studies, there is a need for controlled laboratory experiments to determine the isolated effects of lowered pH and increased free CO<sub>2</sub> on common marine phytoplankton species and strains.

Despite the increasing scientific and public awareness of the potential effects of ocean acidification, relatively few laboratory studies have focused on effects of lowered pH and increased free CO<sub>2</sub> on marine phytoplankton monocultures. So far no or very limited effects of low pH on diatoms, prymnesiophytes, and dinoflagellates have been found (Burkhardt et al. 1999, 2001, Rost et al. 2003). However, some focus has been on calcifying phytoplankton organisms (mainly the ecologically important coccolithophorids), because these are expected to be particularly vulnerable (Riebesell 2004). This is due to the fact that the satura-

tion state of the oceans with respect to calcite and aragonite will decrease with decreasing concentrations of CO<sub>3</sub><sup>2-</sup> (Riebesell et al. 2000). However, for strains of the well-studied coccolithophorid *Emiliania huxleyi*, unaffected, decreased, or increased calcification rates and photosynthesis have been found in lowered pH and increased free CO<sub>2</sub> (Riebesell et al. 2000, Iglesias-Rodriguez et al. 2008, Langer et al. 2009). Overall, the few laboratory experiments on marine phytoplankton species—in general subjected to lowered pH and increased free CO<sub>2</sub>—have indicated that maximum growth rates can be maintained at pH 7.8 and even as far down as pH 7.0 (Swift & Taylor 1966, Chen & Durbin 1994, Taraldsvik & Mykkestad 2000).

Two main approaches have been used to manipulate pH and the carbonate system in studies involving phytoplankton and responses to lowered pH and increased free CO<sub>2</sub>. These are based on CO<sub>2</sub> bubbling or acid/base additions (Hurd et al. 2009). Heated discussions on which technique is most suitable have resulted in several recent papers considering different effects of the 2 methods (Hurd et al. 2009, Schulz et al. 2009, Shi et al. 2009). The main difference lies in their different effects on the carbonate speciation, the total pool of inorganic carbon (TCO<sub>2</sub>), and alkalinity of seawater medium. CO<sub>2</sub> bubbling leads to an increase in TCO<sub>2</sub> while alkalinity is kept constant and pH decreases. This reflects the changes related to ocean acidification due to increased atmospheric CO<sub>2</sub>. In the HCl addition method, TCO<sub>2</sub> is kept stable while total alkalinity and pH decrease. Nevertheless, there is emerging recognition that the use of the 2 techniques depends on the questions being asked (Hurd et al. 2009, Schulz et al. 2009, Shi et al. 2009). For example, when investigations focus on phytoplankton calcification, the calcite and aragonite saturation states depend on the alkalinity and the CO<sub>3</sub><sup>2-</sup> concentration. A problem with CO<sub>2</sub> bubbling is that growth of several species, especially dinoflagellates, is negatively affected by the stress introduced by the bubbles. On the other hand for non-calcifying species, saturation states are of no concern, while growth limitation by TCO<sub>2</sub> could be a potential problem. However, several studies have shown that most phytoplankton species possess CCMs and are only limited by TCO<sub>2</sub> at very low concentration (Hansen et al. 2007, Hurd et al. 2009). The difference in TCO<sub>2</sub> between the acid-addition and CO<sub>2</sub>-bubbling represents only a small difference in HCO<sub>3</sub><sup>-</sup> concentration (Brewer 1997), which should not affect photosynthesis and growth (Hurd et al. 2009). Measuring growth rates of 2 diatom species at lowered pH, Chen & Durbin (1994) tested both methods and reported no differences.

Here we focused on the effects of lowered pH (by HCl addition) on the growth rates of 8 common species of phytoplankton representing all major marine

classes. The study addressed 3 main questions: (1) Are common phytoplankton species sensitive to pH in the range that can be expected by the end of this century (~7.8)? (2) How low is the lower pH limit for growth of selected model coastal phytoplankton species? (3) Does the acclimation period in pH manipulation studies affect growth and production rates?

## MATERIALS AND METHODS

**Phytoplankton cultures.** The 8 phytoplankton species included in this study (Table 1) originated either from the Scandinavian Culture Collection of Algae & Protozoa (SCCAP, www.sccap.dk) or were established from water samples (60 ml) collected from land in coastal harbors in Denmark and Norway during 2007 and 2008 (5 strains). Preferably, newly isolated strains were used, due to potential artefacts by laboratory maintenance (Hurd et al. 2009, Lakeman et al. 2009). Our choice of species was based on an attempt to include the most common coastal marine phytoplankton groups (diatoms, dinoflagellates, prymnesiophytes, and cryptophytes), and to cover a large size range of organisms (Table 1).

Isolation of single cells was done with the use of a drawn-out Pasteur pipette, and the cells were washed by transferring them through 3 drops of fresh medium (L1) using an Olympus CKX-40 inverted microscope with a 20× objective. Depending on species, successful culture establishment was achieved from 10 to 70% of the isolated cells. The used strains were deposited in SCCAP (Table 1), if not otherwise stated. All strains were maintained in F/2 medium at a salinity of 30 and a temperature of 15°C, under a light intensity of 20 to 50  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  in a light:dark cycle of 14:10 h.

**Experiments and conditions.** In Expt 1, the goal was to determine at what level of lowered pH the growth rates of 2 common species of phytoplankton, viz. the dinoflagellate *Heterocapsa triquetra* and the cryptophyte *Teleaulax amphioxeia*, were affected compared to growth rates at natural levels (pH ~8.1 to 8.5). Moreover we wanted to determine the lower pH tolerance for positive growth rates. Two to 3 wk before setting up the experiment, the stock cultures were introduced and acclimated to a higher experimental light intensity of 150  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , than the light conditions in which they were maintained (see above). The acclimatization to light conditions was carried out by keeping the pH at ~8 by diluting with fresh medium of pH 7.9 every 3 to 4 d, and cell concentrations were kept at <5000 cells  $\text{ml}^{-1}$  to maintain balanced exponential growth. Experimental salinity was 30 and temperature was 15°C (similar to culture maintenance). Light was measured with a spherical sensor (Li185B, Li-Cor) and provided in a light:dark cycle of 14:10 h. All treatments were run in closed sterile polycarbonate bottles (270 ml) that were filled to capacity to avoid gas exchange with the air. All treatments were run in triplicate. To create a homogenous light field, the experimental bottles were illuminated from below and positions changed randomly during sampling every day.

During the last 1 to 4 d of acclimation to light intensity, pH was adjusted stepwise, by 0.5 pH units  $12 \text{ h}^{-1}$ , until the culture medium had reached the pH of the specific treatment. The pH in the fresh medium was adjusted by adding 0.1 to 1.0 molar HCl and NaOH. The pH was measured to the nearest 0.01 unit with a pH meter (Copenhagen pHM-83 Autocal). The pH sensor was calibrated on a daily basis using IUPAC buffers pH 7.0 and 10.0. To keep a constant pH during the incubation period (up to 10 d), low cell concentrations were used by diluting the cultures during sampling with fresh medium of desired pH. Following 4 d of exponential growth in the triplicate bottles, samples for enumeration of cell concentrations were taken every day for 5 to 6 d (see Fig. 1), and the sample volume was replaced with fresh medium with a pH of the corresponding pH treatment. pH was measured both before and after sampling and dilution. Cell concentrations were measured manually by counting at least 300 Lugol's acid (1% final concentration)-fixed cells in Sedgewick-Rafter chambers. The dilution rate corresponded approximately to the growth rate (see Fig. 1), and sample volumes ranged from 10 to 60% of the

Table 1. Species included in this study, their geographic and temporal origin, cell volume, and strain accession number at the Scandinavian Culture Collection of Algae & Protozoa (SCCAP: www.sccap.dk). na: information not available

Phytoplankton group Species	Origin	Year of isolation	Cell volume ( $\mu\text{m}^3$ )	Strain accession no.
<b>Dinoflagellates</b>				
<i>Prorocentrum minimum</i>	Skagerrak	2008	1000	K-1138
<i>Prorocentrum micans</i>	Skagerrak	2008	11000	K-1137
<i>Karlodinium veneficum</i>	North Sea	2007	600	K-1413
<i>Heterocapsa triquetra</i>	Baltic	2007	1960	K-1133
<b>Cryptophytes</b>				
<i>Rhodomonas marina</i>	Kattegat	1990	760	K-0435
<i>Teleaulax amphioxeia</i>	The Sound	2009	300	na
<b>Diatom</b>				
<i>Coscinodiscus granii</i>	USA	1994	310000	K-1048
<b>Prymnesiophyte</b>				
<i>Prymnesium parvum</i>	na	na	110	K-0623

total volume. From the obtained growth curves (cell concentration as a function of time), growth rates were estimated (see calculations).

The concentration of  $\text{TCO}_2$  was measured in triplicate in fresh medium and in the experimental bottles on the final day of the growth rate experiments (see Figs. 1 & 2), over the range of treatments from pH 6.0 to 8.6. Measurements were done using an infrared gas analyzer (IRGA) and a bicarbonate standard ( $2 \text{ mmol l}^{-1}$ ), according to Hansen et al. (2007). From pH, salinity, temperature, and  $\text{TCO}_2$ , we calculated the concentration of the carbon species in the medium (see below).

In Expt 2, we studied potential reduction of growth rates and cell size (cell volume;  $\mu\text{m}^3$ ) and calculated the production rates ( $\mu\text{m}^3 \text{ d}^{-1}$ ) of 6 different species of coastal phytoplankton at naturally occurring levels of pH 7.5, 8.0, and 8.5. We also included 1 treatment of, for marine conditions, unusually low pH ( $\sim 7.0$ ). The species selected were the dinoflagellates *Karlodinium veneficum*, *Prorocentrum micans*, and *P. minimum*; the diatom *Coscinodiscus granii*, the haptophyte *Prymnesium parvum*, and the cryptophyte *Rhodomonas marina*. All conditions were the same as in Expt 1, except that in Expt 2, at the lowest cell concentrations, including the large diatom *C. granii* ( $310\,000 \mu\text{m}^3$ ) and the large dinoflagellate *P. micans* ( $11\,000 \mu\text{m}^3$ ), all cells

in 20 ml of sample were settled in multidishes (24-well dishes) and counted, using an inverted microscope. In these treatments of lower cell concentrations, at least 100 cells were counted. The reason for using lower cell concentrations was due to the large size and therefore the higher influence of photosynthesis on the pH of the experimental medium. Moreover, in Expt 2, cell dimensions (length & width) were measured manually on the first 20 Lugol-fixed cells encountered on micrographs taken at  $250\times$  magnification using an inverted microscope, and the measure-tool in Adobe Photoshop CS3. The cells in each pH treatment were grouped across the experimental period ( $>5 \text{ d}$ ) by mixing equal cell numbers from each sampling occasion, before sedimentation for at least 12 h in sedimentation chambers. A high precision object-micrometer was used to convert pixels to  $\mu\text{m}$ .

**Calculations and statistics.** Exponential growth rates in  $\mu \text{ (d}^{-1}\text{)}$  were calculated as:

$$\mu = \ln(X_{t_2} - X_{t_1}) / t_2 - t_1 \quad (1)$$

where  $X_{t_2}$  and  $X_{t_1}$  is the cell concentration at the end ( $t_2$ ) and start ( $t_1$ ) of the sampled interval (24 h), respectively. Due to the daily dilutions during samplings, growth rates were averaged in each replicate bottle, over a period of 5 to 6 d of exponential growth (see

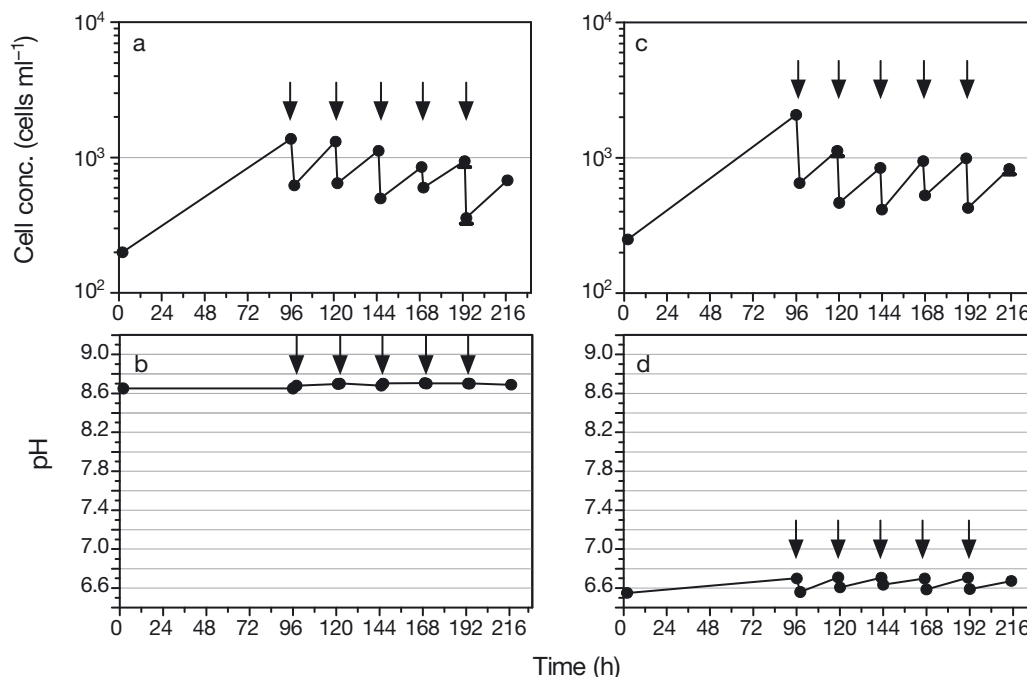


Fig. 1. *Heterocapsa triquetra*. Example of growth rate estimation from Expt 1 including a high and a low pH treatment. This same method was used in both Expt 1 and Expt 2. Cell concentration (a,c) and pH (b,d) are shown as a function of time. The first 4 d (96 h) represented the acclimation period, while the subsequent 5 d were included in the estimation of acclimated balanced growth rates. Arrows indicate time of dilutions and sampling. Total inorganic carbon ( $\text{TCO}_2$ ) was measured in the medium used for dilutions and at the final sampling point. The carbonate system at the different pH is presented in Fig. 2. Data points are means  $\pm$  SE ( $n = 3$ )

Fig. 1). Only linear parts of semi-log plots of concentration as a function of time were included (see Figs. 3 & 5). Concentrations of free  $\text{CO}_2$ ,  $\text{HCO}_3^-$ , and  $\text{CO}_3^{2-}$  were calculated according to Plath et al. (1980) from measured  $\text{TCO}_2$  (see above), pH, salinity, and temperature of the medium using the dissociation constants of Mehrbach et al. (1973). In Expt 2, cell size ( $\mu\text{m}^3$ ) was estimated by assuming simple geometric shapes, and was based on length and width measurements (see above). Under the assumption that potential effects of Lugol's fixation on cell size was similar within species at the ranges of pH used, net specific production rates ( $\mu\text{m}^3 \text{cell}^{-1} \text{d}^{-1}$ ) were calculated as the product of exponential growth rate ( $\text{d}^{-1}$ ) and specific cell size ( $\mu\text{m}^3$ ) and compared. All statistical analyses were done using the free statistical software R and 1-factor analysis of variance (ANOVA), and Tukey's multiple comparisons test was used to test for differences in growth rates, production rates, and cell size between the pH treatments. A significance level of 0.05 was chosen.

## RESULTS

### pH and the carbonate system

At pH levels  $>7.0$ , we were able to keep pH constant during the incubation period (Fig. 1a,b) with only small changes in pH between samplings (change of  $<0.05$  pH units). At pH  $<7.0$ , the pH varied at most 0.2 pH units (at pH 6.0) from the treatment pH between dilutions with fresh medium during sampling (Fig. 1c,d).

We measured the concentration of  $\text{TCO}_2$  and calculated the carbon speciation in our medium and in the experimental medium after incubations (Fig. 2). This was done at 4 different pH levels in fresh medium and in experimental medium at the end of the incubation period (7–8 d) at 9 pH levels from Expt 1. The pH range tested covered the range of pH and free  $\text{CO}_2$  used in all treatments in both experiments.  $\text{TCO}_2$  concentrations in the fresh medium varied from 0.7 to 1.9  $\text{mmol l}^{-1}$  over the range of pH treatments tested (6.0 to 8.6; Fig. 2a). Calculated free  $\text{CO}_2$  in the fresh medium decreased as a function of pH and varied from 4 to 331  $\mu\text{mol l}^{-1}$  (Fig. 2b) while  $\text{CO}_3^{2-}$  increased as a function of pH (data not shown). In the medium sampled at the end of the incubation period,  $\text{TCO}_2$  was in the range of  $\sim 0.8$  to 1.8  $\text{mmol l}^{-1}$  and free  $\text{CO}_2$  ranged from 4 to 441  $\mu\text{mol l}^{-1}$  over a range from pH 6.0 to 8.6 (Fig. 2). Thus, the concentration of  $\text{TCO}_2$  and free  $\text{CO}_2$  did not decrease noticeably in our experimental bottles during the course of the growth rate experiments at different pH values. From pH  $\sim 7.0$  to 8.5, which repre-

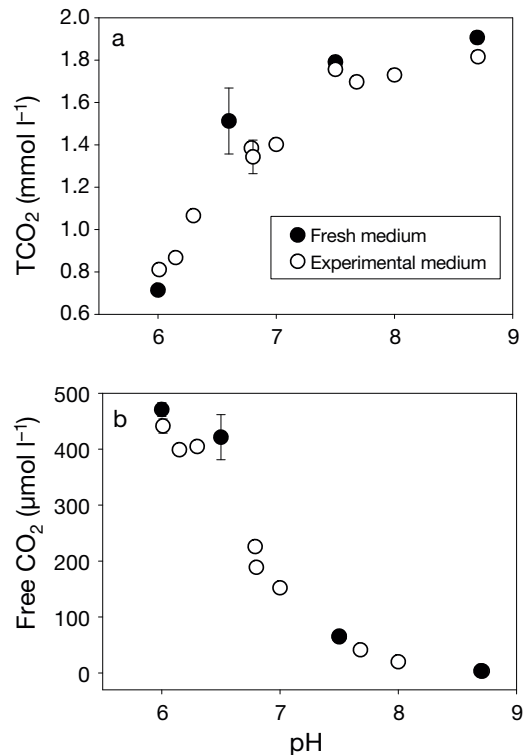


Fig. 2. Expt 1. Carbonate system manipulated with HCl additions. (a) Total inorganic carbon ( $\text{TCO}_2$ ) and (b) free  $\text{CO}_2$  as a function of pH in the medium and in the medium at termination of the experiment after 7 to 8 d. pH was measured using a 2-point calibration pH meter that was calibrated daily using IUPAC buffers of pH 7 and 10.  $\text{TCO}_2$  was measured using an infrared gas analyzer (IRGA) compared with a 2.0 mM bicarbonate standard solution. Estimations of  $\text{CO}_2$  were based on Plath et al. (1980) and Mehrbach et al. (1973). Data points are means  $\pm$  SE ( $n = 3$ )

sented the pH range used in Expt 2,  $\text{TCO}_2$  was kept relatively constant from  $\sim 1.5$  to 1.8  $\text{mmol l}^{-1}$ , and free  $\text{CO}_2$  varied from  $\sim 150$  to 5  $\mu\text{mol l}^{-1}$ .

### Growth rate as a function of lowered pH in two common species of phytoplankton

In Expt 1, the concentration of cells increased exponentially as a function of time, and no lag phase was observed in any of the pH treatments for both species (Fig. 3). At pH 5.8, the growth rate of the dinoflagellate *Heterocapsa triquetra* was negative (Fig. 4a), but increased as a function of pH from pH 6.0. *H. triquetra* maintained a maximum growth rate of  $\sim 0.9 \text{d}^{-1}$  in the range of pH 6.9 to 8.7 (Tukey's test,  $p > 0.05$ ; Fig. 4a). Below a pH of 6.9, the growth rate of *H. triquetra* was reduced and was  $0.7 \text{d}^{-1}$  at pH 6.7, significantly different from the maximum growth rates (Tukey's test,  $p < 0.05$ ; Fig. 4a).



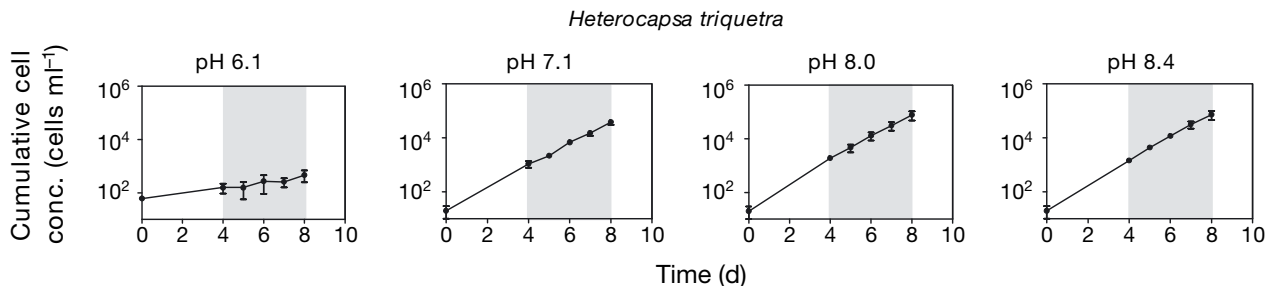


Fig. 3. *Heterocapsa triquetra*. Expt 1. Lower pH limit for growth. Cumulative cell concentrations as a function of time in 4 of the 11 treatments. Data points are means  $\pm$  SE ( $n = 3$ ). Shaded areas represent data points included in estimations of balanced acclimated growth rates (Fig. 4). Data for *Teleaulax amphioxeia* not shown. No significant differences were observed between the growth rates in the acclimation period (time 0 to 4) and the acclimated growth rates ( $p > 0.05$ )

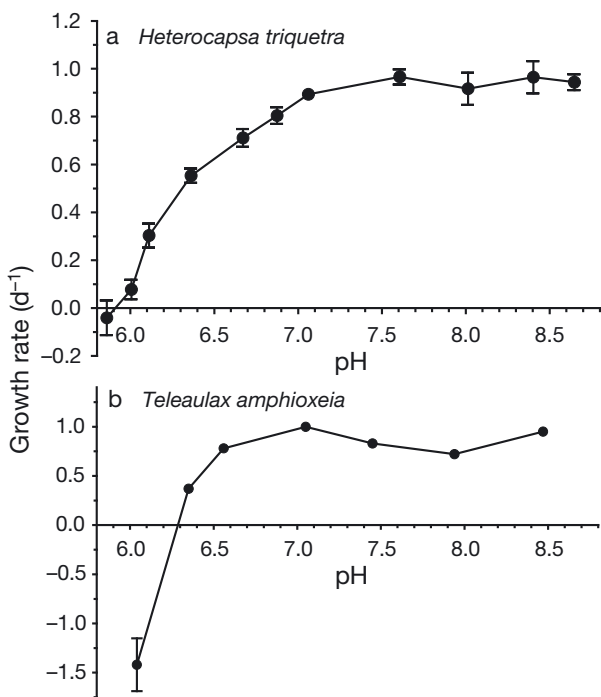


Fig. 4. *Heterocapsa triquetra* and *Teleaulax amphioxeia*. Expt 1. Lower pH limit for growth. Growth rate of (a) the dinoflagellate *H. triquetra* and (b) the cryptophyte *T. amphioxeia* as a function of lowered pH. The cultures were grown under nutrient and light replete conditions and acclimation to pH during a period of 4 d where pH was kept constant. Growth rates were averaged over a period of 5 d and represented linear parts on semi-log growth curves as shown in Fig. 3. Growth rate of *H. triquetra* was constant and unaffected by pH levels  $> 6.6$ , and half their maximum growth rate was achieved at pH  $\sim 6.3$ . Growth rates of *T. amphioxeia* were unaffected in the pH range of 6.4 to 8.5. Data points are means  $\pm$  SE ( $n = 3$ )

At pH 6.0, the growth rate of the cryptophyte *Teleaulax amphioxeia* was negative (Fig. 4b), but increased as a function of pH to a maximum level of  $\sim 0.8$   $d^{-1}$  in the pH range of 6.6 to 8.5 (Tukey's test,  $p < 0.05$ ). Below a pH of 6.6, the growth rate of *T. amphiox-*

*eia* was reduced and was  $0.4$   $d^{-1}$  at pH 6.4, which was significantly different from maximum growth rates (Tukey's test,  $p < 0.05$ ; Fig. 4b).

#### Growth rate, cell size, and production rate of six common phytoplankton species at lowered pH

In Expt 2, 6 species of phytoplankton were grown in 4 pH treatments ( $\sim 7.0$ ,  $\sim 7.5$ ,  $\sim 8.0$ , and  $\sim 8.5$ ). No apparent lag phase was observed in any treatments, and the experimental cultures continued balanced growth immediately after inoculation into the 4 experimental pH levels (Fig. 5).

Growth rates in all 6 species were similar in all 4 pH treatments (ANOVA,  $p > 0.05$ ; Fig. 6). Likewise, cell size and production rates were similar in all 4 pH treatments (ANOVA,  $p > 0.05$ ). Thus, no significant effects of lowered pH and increased free  $CO_2$  in the ranges studied here ( $\sim 7.0$  to 8.5 and 5 to 150  $\mu mol\ l^{-1}$ , respectively) were found in any of the tested strains on any of the measured parameters (Fig. 6).

## DISCUSSION

### Marine phytoplankton growth rates at lowered pH

Our results show that lowered pH and corresponding increased free  $CO_2$  in the range of average surface ocean levels proposed by the end of this century (pH 7.8 and free  $CO_2$   $\sim 30$   $\mu mol\ l^{-1}$ , Houghton et al. 2001) neither increased nor decreased growth and production rates of the 8 species of phytoplankton tested, representing diatoms, dinoflagellates, cryptophytes, and haptophytes. Not many laboratory studies have focused on lowered pH and marine phytoplankton growth rates. However, our findings agree well with previously published phytoplankton growth rates obtained in the laboratory at lowered pH of similar

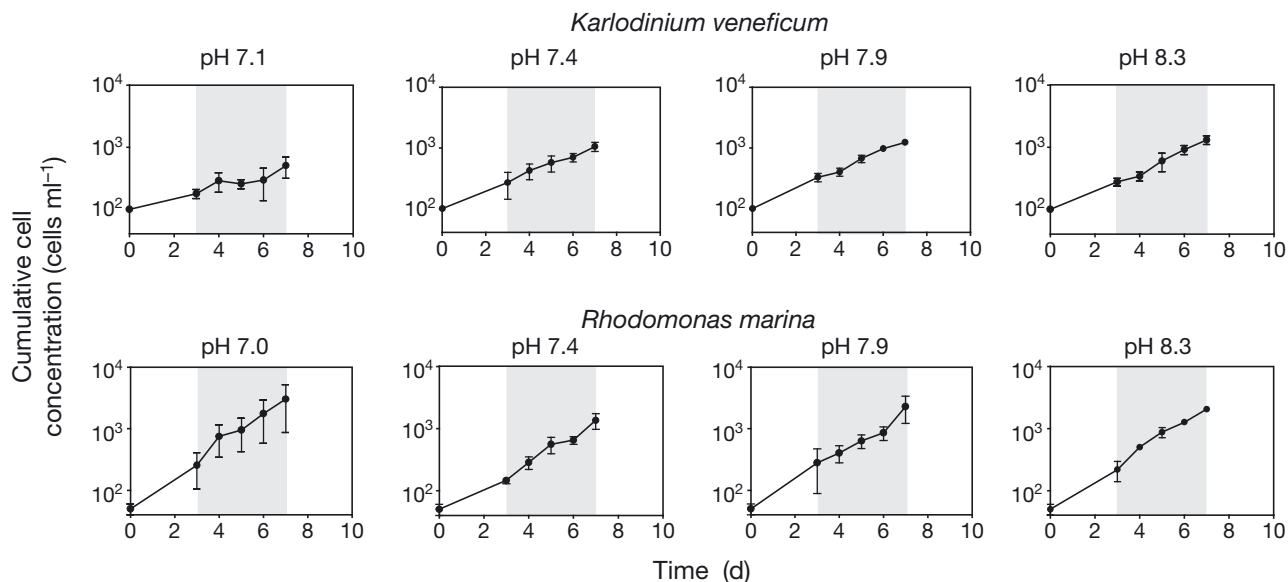


Fig. 5. *Karlodium veneficum* and *Rhodomonas marina*. Expt 2. Effects of lowered pH on growth rates of 6 common phytoplankton species (2 examples are given here). Cumulative cell concentration as a function of time in semi-continuous batch cultures of one slow-growing species (the dinoflagellate *K. veneficum*) and one fast-growing species (the cryptophyte *R. marina*), grown at 4 levels of pH. Data points are means  $\pm$  SE ( $n = 3$ ). Shaded areas represent points used in growth rate estimations, while the first 3 d represent the acclimation period to pH

range (~7.8–8.5; Table 2, Fig. 7). We collected a substantial part of the published literature on phytoplankton balanced growth rates at lowered pH and increased free  $\text{CO}_2$  and found a total of 33 species including 49 strains studied in the pH range of ocean acidification (pH ~7.8; Table 2). Across this range, reported effects on acclimatized balanced growth rates have only been reported in the calcifying oceanic coccolithophorid *Emiliana huxleyi*. Concerning the most studied species *E. huxleyi* (Table 2), there is no clear pattern with regard to effects on its growth rate at lowered pH/increased  $\text{CO}_2$ , in the range of average ocean acidification (pH ~7.8). The growth rates of most strains of this ecologically important coccolithophorid are unaffected at pH 7.8, while a few strains slightly reduce or increase their growth rates (Iglesias-Rodriguez et al. 2008, Langer et al. 2009, Ridgwell et al. 2009). On the other hand, all other species and strains tested (33 species and 49 strains in total) had similar growth rates at pH ~7.8, compared to present day levels (pH 8.1–8.2). This indicates that marine phytoplankton, in general, are adapted to tolerate the modeled global average surface water drop of pH due to ocean acidification by the year 2100 (pH ~7.8). This seems to apply to most major taxonomic groups of phytoplankton (Table 2).

A major concern in ecophysiological experiments on phytoplankton is acclimation, involving cellular regulation and resource allocation of cultures when introduced to a new environment (Brand 1982, Hurd et

al. 2009, Barcelos e Ramos et al. 2010). Expression of essential metabolic processes of eukaryotic microorganisms changes with the environment, and before realistic comparisons between treatments can be made, cultures need to stabilize into a new physiological state and achieve balanced growth under the experimental growth conditions. These often differ from the conditions under which the cultures are maintained. Therefore, phytoplankton ecologists often acclimatize their cultures for several generations (often 5 to 10 generations). Previous studies on the effects of lowered pH and increased free  $\text{CO}_2$  on phytoplankton growth rates differed considerably with respect to the duration of the acclimation period (Table 2). However, in *Emiliana huxleyi*, acclimation to increased free  $\text{CO}_2$  and lowered pH has recently been shown to occur within hours (Barcelos e Ramos et al. 2010). The generality of these findings is limited by only 1 other report, where no differences were found between photosynthetic response after 1 and 5 h of acclimation of 2 species of phytoplankton (Chen & Durbin 1994). In our study, after a sudden drop of pH by 1 unit  $\text{d}^{-1}$ , the cultures did not show any sign of a lag phase, but continued balanced growth immediately. This adds 8 species to the list of species able to respond and acclimatize quickly to pH and  $\text{CO}_2$  changes, which has large implications for literature comparisons across different acclimation periods and may simplify interpretations of studies with natural incubations (shipborne and mesocosm studies).

Spatial variation in the magnitude of ocean acidification will occur, affecting some areas more than others (McNeil & Matear 2008). Full strength oceanic seawater contains high  $\text{TCO}_2$  concentrations (~2.2 to 2.4 mM) which act to buffer pH changes. However, in coastal waters with a generally lower content of  $\text{TCO}_2$ , pH fluctuates considerably more in time and space (Hansen 2002, Middelboe & Hansen 2007, Wootton et al. 2008). In coastal surface waters, pH fluctuations

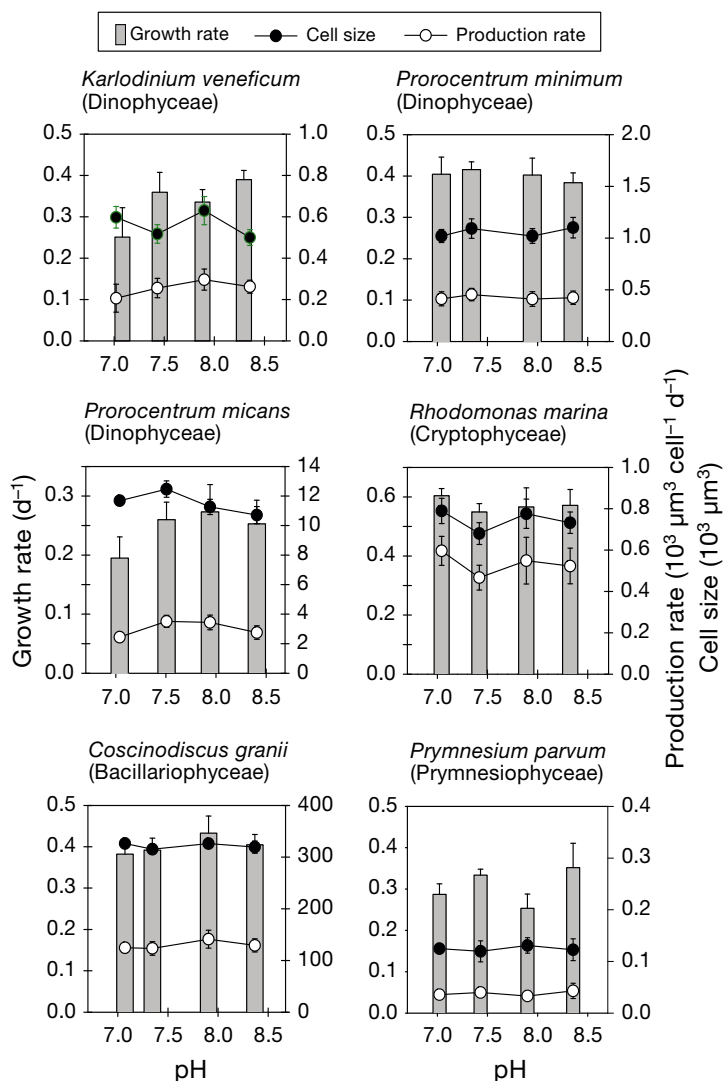


Fig. 6. Expt 2. Effects of lowered pH on common species of coastal marine phytoplankton. Growth rates (d<sup>-1</sup>), cell volume (μm<sup>3</sup>), and production rates (μm<sup>3</sup> cell<sup>-1</sup> d<sup>-1</sup>) of 6 species grown at 4 different pH levels. The cultures were grown under nutrient and light replete conditions for 1 wk and acclimation to pH during a period of 3 d where pH was kept constant. No significant differences between growth rates, cell volume, and production rates under the different pH treatments were observed in any of the tested species ( $p > 0.05$ ). Data points are means  $\pm$  SE ( $n = 3$ )

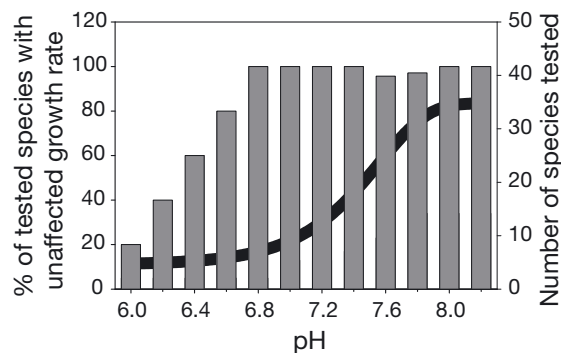


Fig. 7. Percentage of phytoplankton species tested as a function of pH, in which acclimated balanced growth rates are unaffected, compared to maximum growth rates (grey bars). Affected growth rates were defined as a >20% reduction of maximum growth rates. The black line represents the number of species tested as a function of pH. Fig. 7 is based on Table 2

from 7.5 to 9 at daily and seasonal time scales are common (Hinga 1992, 2002, Hansen 2002, Middelboe & Hansen 2007, Wootton et al. 2008). Therefore, it is necessary to consider even larger ranges of lowered pH and increased  $\text{CO}_2$ , when evaluating potential effects of ocean acidification on phytoplankton growth rates. If we widen the lowered pH range to include these natural fluctuations (pH 7.5 to 9.0), the number of literature reports on phytoplankton growth rates decreases markedly. Nevertheless, all of the 23 species tested thus far down to a pH of 7.5, including the 8 species tested here, maintained their maximum growth rates (Table 2, Fig. 7). Natural variations in pH occur at several scales in environments where phytoplankton photosynthesis takes place. In general, pH decreases as a function of water column depth, and bottom layer water with pH close to 7.0 has been reported from nature (Byrne et al. 2010). In a survey of pH in Monterey Bay, California (USA), pH generally ranged from 7.6 at 500 m depth to 8.2 at the surface (Byrne et al. 2010). On the continental shelf of western North America, Feely et al. (2008) reported upwelling of corrosive bottom water of pH 7.6 up to a depth of 50 m. In estuaries, pH varies at small spatial scales, often resulting in environmental gradients (Hinga 2002). Alkalinity and  $\text{TCO}_2$  concentration in river water vary considerably depending on the geology of the catchment areas. In estuaries with relatively low  $\text{TCO}_2$  levels, pH measurements below 7.5 are common, and even pH values as low as 6.5 have been observed in nature (Hinga 2002). On even smaller spatial and temporal scales, rainfall (which often has a pH of ~5), may also affect the pH environment of phytoplankton (Bates & Peters 2007).

Future ocean acidification may affect all of these natural pH extremes, indicating that even pH levels <7.5



Table 2. (continued overleaf) Marine phytoplankton groups, species, and strains studied in the laboratory for effects of lowered pH/increased CO<sub>2</sub> on balanced acclimatized growth rates. The pH range for maximum exponential growth rate is defined as the pH over which growth rates were at their maximum levels and varied < 20% ; lower pH at which exponential growth is affected is defined as the pH at which a >25% reduction of growth rate compared to maximum growth rate occurred; and the lower pH limit for growth was defined as the pH at which growth = 0.0 d<sup>-1</sup>. Only studies using replicated acclimatized growth rates obtained over at least 3 d are included. nd: not determined; na: information not available

Phytoplankton group Species Strain ID	pH range for max. exponential growth rate	Lower pH at which exponential growth is affected	Lower pH limit for growth	Duration of acclimation period to pH	Source
<b>Dinoflagellates</b>					
<i>Prorocentrum minimum</i>					
K-0295	7.3–8.9	nd, but <7.3	nd	2 d	Hansen (2002)
K-1138	7.0–8.4	nd, but <7.0	nd	3 d	Present study
<i>Prorocentrum micans</i>					
K-1137	7.0–8.4	nd, but <7.0	nd	3 d	Present study
PML 97	7.5–8.8	nd, but <7.5	nd	2 d	Kain & Fogg (1960)
<i>Heterocapsa triquetra</i>					
K-0481	7.6–9.1	nd, but <7.6	nd	2 d	Hansen (2002)
na	6.7–8.6	6.6	5.9	4 d	Present study
<i>Ceratium lineatum</i>					
na	7.4–8.5	nd, but <7.4	nd	2 d	Hansen (2002)
<i>Ceratium fusus</i>					
na	7.35–8.2	nd, but <7.35	nd	<2 d	Søderberg & Hansen (2007)
<i>Ceratium furca</i>					
na	7.6–8.8	nd, but <7.6	nd	<2 d	Søderberg & Hansen (2007)
<i>Ceratium tripos</i>					
na	7.5–8.6	nd, but <7.5	nd	<2 d	Søderberg & Hansen (2007)
<i>Karlodinium veneficum</i>					
K-1413	7.0–8.3	nd, but <7.0	nd	3 d	Present study
<i>Scrippsiella trocoideae</i>					
na	7.8–9.2	nd, but <7.8	nd	9 generations	Burkhardt et al. (1999)
<i>Alexandrium minutum</i>					
T1	5.5–8.5	nd, but <5.5	nd	5 d	Hwang & Lu (2000)
<b>Diatoms</b>					
<i>Thalassiosira oceanica</i>					
13.1	7.1–8.9	nd, but <7.1	nd	<1 d	Chen & Durbin (1994)
<i>Thalassiosira pseudonana</i>					
3H	7.1–8.9	nd, but <7.1	nd	<1 d	Chen & Durbin (1994)
<i>Thalassiosira weissflogii</i>					
CCMP 1336	7.7–8.6	nd, but <7.7	nd	7 generations	Shi et al. (2010)
<i>Thalassiosira oceanica</i>					
13.1	7.85–8.5	nd, but <7.85	nd	7 generations	Shi et al. (2010)
<i>Thalassiosira punctigera</i>					
na	7.8–9.1	nd, but <7.8	nd	9 generations	Burkhardt et al. (1999)
na	7.5–8.5	nd, but <7.5	nd	na	Riebesell et al. (1993)
<i>Ditulum brightwelli</i>					
na	7.7–8.5	nd, but <7.7	nd	na	Riebesell et al. (1993)
<i>Rizosolenia cf. alata</i>					
na	7.7–8.5	nd, but <7.5	nd	na	Riebesell et al. (1993)
<i>Phaeodactylum tricornutum</i>					
CCAP 1052/1A	7.8–9.1	nd, but <7.8	nd	9 generations	Burkhardt et al. (1999)
CCAP 1052/1A	7.3–8.9	nd, but <7.3	nd	na	Johnston (1996)
CCMP 630	7.7–8.5	nd, but <7.7	nd	7 generations	Shi et al. (2010)
<i>Skeletonema costatum</i>					
na	6.5–8.5	nd, but <6.5	nd	3 d	Taraldsvik & Mykkestad (2000)
<i>Coscinodiscus granii</i>					
K-1048	7.1–8.4	nd but <7.1	nd	3 d	Present study
<i>Coscinodiscus wailesii</i>					
na	7.8–8.6	nd, but <7.8	nd	9 generations	Burkhardt et al. (1999)

Table 2 (continued)

Phytoplankton group Species Strain ID	pH range for max. exponential growth rate	Lower pH at which exponential growth is affected	Lower pH limit for growth	Duration of acclimation period to pH	Source
<b>Diatoms (continued)</b>					
<i>Asterionella glacialis</i> na	7.8–8.9	nd, but <7.8	nd	9 generations	Burkhardt et al. (1999)
<i>Asterionella japonica</i> na	7.5–8.5	nd, but below 7.5	nd	2 d	Kain & Fogg (1958a)
<b>Coccolithophorids</b>					
<i>Emiliana huxleyi</i>					
PML B92/11	7.8–8.6	nd, but <7.8	nd	Unclear	Rost et al. (2002)
PML 92A	7.46–8.30	nd, but <7.46	nd	7 and 14 d	Leonardos & Geider (2005)
RCC1256	7.81–8.56	7.81	nd	12 generations	Langer et al. (2009)
RCC1238	7.71–8.56	nd, but <7.71	nd	12 generations	Langer et al. (2009)
RCC1216	7.71–8.56	nd, but <7.71	nd	12 generations	Langer et al. (2009)
RCC1212	7.71–8.56	nd, but <7.71	nd	12 generations	Langer et al. (2009)
CCMP371	7.9–8.1	nd, but <7.9	nd	7 generations	Feng et al. (2008)
NZEH(COWPO6)	7.85–8.1	7.77	nd	9 generations	Iglesias-Rodriguez et al. (2008)
CCMP 374	7.8–8.5	nd, but <7.85	nd	7 generations	Shi et al. (2010)
CCAP 920/2	7.0–8.3	nd, but <7.0	nd	na	Johnston (1996)
PML 92	8.0–8.5	7.8	nd	na	Johnston (1996)
<i>Coccolithus leptoporus</i>					
AC365	7.86–8.44	nd, but <7.86	nd	10 generations	Langer et al. (2006)
<i>Coccolithus pelagicus</i>					
AC400	7.81–8.56	nd, but <7.81	nd	10 generations	Langer et al. (2006)
<b>Prymnesiophytes</b>					
<i>Isochrysis galbana</i>					
na	7.5–8.6	nd, but <7.5	nd	2 d	Kain & Fogg (1958b)
<i>Prymnesium parvum</i>					
K-0623	7.1–8.4	nd, but <7.1	nd	3 d	Present study
<i>Cricosphaera elongata</i>					
Miliport strain 62	7.1–7.8	6.4	nd	<14 d	Swift & Taylor (1966)
<b>Cryptophytes</b>					
<i>Teleaulax amphioxeia</i>					
na	6.1–8.9	6.1	05.Sep	3 d	Present study
<i>Rhodomonas marina</i>					
K-0435	7.1–9.5	nd but <7.1	nd	3 d	Present study
<b>Cyanobacteria</b>					
<i>Prochlorococcus</i> sp.					
CCMP1986	7.8–8.2	nd, but <7.8	nd	14 d	Fu et al. (2007)
<i>Synechococcus</i> sp.					
CCMP1334	7.8–8.2	nd, but <7.8	nd	14 d	Fu et al. (2007)
<i>Trichodesmium</i> sp.					
IMS101	7.8–8.2	nd, but <7.8	nd	60 d	Barcelos e Ramos et al. (2007)

may be common in an acidified ocean. Apart from our study, only a few laboratory studies of marine phytoplankton growth rates have used seawater pH at such extreme levels (Swift & Taylor 1966, Chen & Durbin 1994, Johnston 1996, Hwang & Lu 2000, Taraldsvik & Mykkestad 2000, Hansen 2002, Søderberg & Hansen 2007). Overall, these previous studies all reported a very high tolerance to lowered pH (Table 2, Fig. 7). The growth rate of the brackish water calcifying coccolithophorid *Cricosphaera elongata* at pH 7.0 was not significantly reduced compared to maximum growth

rates in the pH range of 7.8 to 8.6 (Swift & Taylor 1966), and growth rates of the coastal diatom *Skeletonema costatum* remained constant (2.4 divisions d<sup>-1</sup>) from pH of 6.5 to 8.5 (Taraldsvik & Mykkestad 2000). Chen & Durbin (1994) investigated the growth rates of one oceanic and one coastal species of diatom (*viz. Thallasiosira oceanica* and *T. pseudonana*) under a pH range of 7.0 to 9.5 and found no reduction of growth rates of both species at the lowest pH tested (pH 7.0). In our study, growth rates of the dinoflagellate *Heterocapsa triquetra* were not significantly reduced until pH was

lowered to 6.5, while the growth rates of the cryptophyte *Teleaulax amphioxeia* were unaffected by lowered pH down to ~6.1. The dinoflagellates *Karlodinium veneficum*, *Prorocentrum minimum*, and *P. micans*, the cryptophyte *Rhodomonas marina*, the diatom *Coscinodiscus granii*, and the haptophyte *Prymnesium parvum* all maintained maximum growth and production rates down to a pH of ~7.0. In summary, the unaffected growth rates of 17 species tested so far at pH levels <7.5 indicate that phytoplankton growth rates in general are tolerant of the ecologically relevant changes in pH and free CO<sub>2</sub> (Table 2, Fig. 7). We speculate that adaptation of phytoplankton to the range of present and past pH fluctuations in the ocean (Pearson & Palmer 2000, Hansen 2002, Hinga 2002, Ridgwell & Schmidt 2010), can explain the wide tolerance of these organisms to lowered pH levels. However, most of the species tested at lowered pH (<7.5) represent coastal species, and truly oceanic species are likely to be adapted to a smaller range of pH, and are therefore likely to be more sensitive to pH changes. Only one oceanic species, the diatom *T. oceanica*, has been tested at pH <7.5, and this species was able to grow unaffected down to pH 7.0 (Chen & Durbin 1994). More data on the tolerance to lowered pH of growth rates of oceanic species are needed to evaluate a potential difference between oceanic and coastal phytoplankton.

#### Lower pH tolerance limit for marine phytoplankton growth

Here we report the absolute lower pH tolerance limits for positive growth rates of the first 2 tested marine phytoplankton species at pH levels of ~6.0. At the high range of pH, tolerance limits for growth of marine phytoplankton are well studied, and is in general around 9, but with large species-specific variation (Hansen 2002). At high levels of pH, a larger fraction of TCO<sub>2</sub> is present as CO<sub>3</sub><sup>2-</sup>, which is unavailable for phytoplankton photosynthesis, and it has been discussed whether growth at high pH is mainly reduced by TCO<sub>2</sub> limitation or by direct pH effects (Hansen et al. 2007). However, several studies on this controversy between TCO<sub>2</sub> and pH limitations have clearly indicated that limitation is due to direct pH effects, and very low levels of TCO<sub>2</sub> are required to limit growth rates (<0.5 mM; Hansen et al. 2007, Søderberg & Hansen 2007). At lowered pH, despite the fact that TCO<sub>2</sub> content in our medium was reduced compared to higher pH levels (~0.8 mM at pH 6 and ~1.4 mM at pH 7, due to water/air contact during preparation), most TCO<sub>2</sub> was present as available CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup>. Therefore, it is very likely that the reduced growth rates

observed (close to their lower limits for growth) for the 2 species *Heterocapsa triquetra* and *Teleaulax amphioxeia* were not due to TCO<sub>2</sub> limitation, but to direct pH effects. The most probable direct effect may be an inability to maintain a favorable intracellular pH. Our results indicate that marine phytoplankton are well adapted to do so, even at very low pH levels by marine standards. Insensitivity of phytoplankton growth rates to even lower pH has been reported. The marine dinoflagellate *Alexandrium minutum* is able to grow at maximum rates even down to pH 5.5 (Hwang & Lu 2000). Regulation of intracellular pH in phytoplankton has only been studied in freshwater species. Some of these are able to maintain a constant intracellular pH of ~7.0, across an extracellular pH range of 3.0 to 8.0 (Lane & Burris 1981). The freshwater cryptophyte *Cryptomonas* sp., which is closely related to *Rhodomonas marina* investigated in the present study, tolerated wide pH fluctuations from pH 4.4 to 9.65 and was able to regulate its cell volume over this range (Weisse & Stadler 2006). Some common freshwater species of the green algal genus *Chlamydomonas* show positive growth rates over more than 7 pH units and keep their intracellular pH constant over this range (Spijkerman 2005, Gerloff-Elias et al. 2006). In addition to affecting intracellular pH, low extracellular pH may directly affect membrane potential, energy partitioning, and enzyme activity (Beardall & Raven 2004, Riebesell 2004, Giordano et al. 2005). Considering the ongoing ocean acidification, the absolute lower limits for growth and intracellular pH regulation of marine phytoplankton need more attention.

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