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Growth limitation in marine red-tide dinoflagellates: effects of pH versus inorganic carbon availability

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ABSTRACT: The effects of dissolved inorganic carbon (DIC) on the growth of 3 red-tide dinoflagellates (*Ceratium lineatum*, *Heterocapsa triquetra* and *Prorocentrum minimum*) were studied at pH 8.0 and at higher pH levels, depending upon the pH tolerance of the individual species. The higher pH levels chosen for experiments were 8.55 for *C. lineatum* and 9.2 for the other 2 species. At pH 8.0, which approximates the pH found in the open sea, the maximum growth in all species was maintained until the total DIC concentration was reduced below ~0.4 and 0.2 mM for *C. lineatum* and the other 2 species, respectively. Growth compensation points (concentration of inorganic carbon needed for maintenance of cells) were reached at ~0.18 and 0.05 mM DIC for *C. lineatum* and the other 2 species, respectively. At higher pH levels, maximum growth rates were lower compared to growth at pH 8, even at very high DIC concentrations, indicating a direct pH effect on growth. Moreover, the concentration of bio-available inorganic carbon (CO_2 + HCO_3^-) required for maintenance as well as the half-saturation constants were increased considerably at high pH compared to pH 8.0. Experiments with pH-drift were carried out at initial concentrations of 2.4 and 1.2 mM DIC to test whether pH or DIC was the main limiting factor at a natural range of DIC. Independent of the initial DIC concentrations, growth rates were similar in both incubations until pH had increased considerably. The results of this study demonstrated that growth of the 3 species was mainly limited by pH, while inorganic carbon limitation played a minor role only at very high pH levels and low initial DIC concentrations.

KEY WORDS: pH · Growth limitation · Competition · Marine · Phytoplankton · Dissolved inorganic carbon · DIC

INTRODUCTION

During the past 15 to 20 yr, it has been heavily debated whether the growth and photosynthesis of phytoplankton can be limited by inorganic carbon in natural seawaters. One reason for much of this discussion has been the rising atmospheric CO_2 concentrations due to the combustion of fossil fuels. The oceans absorb CO_2 from the atmosphere, which not only causes CO_2 concentrations to rise, but it also makes the oceans more acidic. If CO_2 emissions continue to rise following current trends, the average pH of the oceans may decrease from pH 8.2 in 1950 to pH 7.8 in 2100 (e.g. Wolf-Gladrow et al. 1999, Raven et al. 2005). It has been speculated that such changes in carbonate chemistry may affect plankton primary production and shift the dominance of species (e.g. Tortell et al. 2002, Rost et al. 2003).

A much less debated but related topic has been whether algae may become limited by pH and/or inorganic carbon during periods of elevated pH in coastal waters due to algal blooms (Hansen 2002, Hinga 2002). The literature on natural variations in pH in coastal marine waters is still quite sparse. However, in coastal waters like the North Sea and the German Bight, the pH may increase to 8.7 during algal blooms in the early summer from May to June (Kempe & Pegler 1991, Brussard et al. 1996). In more enclosed coastal water

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bodies, the pH may become even higher. In Mariager Fjord (Denmark), a fjord with a sill (maximum depth of 30 m), the median pH was close to 9 during summer (May to August) over a 10 yr study period from 1990 to 1999 (Hansen 2002), with peak pH values of 9.75. Winter pH values here were between 7.5 and 8. In the coastal Santo André Lagoon (Portugal), Macedo et al. (2001) found that the pH was above 8.7 for most of the year, with peak values of 9.6. Thus, recent studies have suggested that periods of highly elevated pH occur in coastal waters, which may in fact be much more common phenomena than generally considered.

Dinoflagellates are important members of the phytoplankton community; they occasionally form conspicuous red-tides or blooms in coastal waters and embayments. These blooms can have enormous effects, both ecologically as well as economically, since some dinoflagellates produce potent toxins that can either directly or indirectly affect the entire food web. The common red-tide dinoflagellates *Heterocapsa triquetra* and *Prorocentrum minimum* are thus far reported to be the species associated with high pH events (e.g. Macedo et al. 2001, Hansen 2002). The tolerance to high pH is, however, species-specific, and the upper pH growth limits for dinoflagellates have been found to vary between 8.4 and 10.2 (see Hansen 2002). Among the most pH sensitive dinoflagellates are common red-tide species belonging to the genus *Ceratium*.

The reason for the sensitivity of some dinoflagellates to high pH is still unknown. Negative influence on growth and photosynthesis could be due to a direct pH effect, i.e. the effect of different proton ion concentrations on the algae. Furthermore, it could be due to an indirect pH effect, e.g. pH-mediated changes in the chemical speciation of the dissolved inorganic carbon (DIC) pool. In the marine environment, DIC concentrations may vary from ca. 2.4 mM in oceanic waters to 1.2 mM in brackish waters (e.g. Thomas & Schneider 1999, Key et al. 2004), but it is always in excess relative to other macronutrients such as nitrogen and phosphorus (Key et al. 2004). Despite the high concentrations of inorganic carbon in seawater, most of it (>90%) is present in the form of HCO_3^-, and only ca. 10 to 20 µM (~1%) is available as CO_2 at pH 8 to 8.2.

During periods of elevated pH in the marine environment, not only are DIC concentrations lowered, but also the chemical speciation of the DIC pool changes. Thus a greater proportion of the inorganic carbon pool is in the form CO_3^{2-}, which most likely is unavailable for algae. Very little is available as CO_2. At pH 9, in fact, only 0.1% of the DIC pool is in the form of CO_2. Low CO_2 availability may impose restrictions on algae, since their primary carboxylating enzyme, RubisCO, is limited to CO_2 for carbon fixation. Moreover, studies on *in vitro* abilities of RubisCO show that its affinity for CO_2 is very low, especially in the form II RubisCO found in dinoflagellates (Whitney & Andrews 1998). To avoid the risk of carbon limitation in seawater, most microalgae have thus developed so-called 'carbon concentrating mechanisms' (CCMs) that enhance the intracellular CO_2 concentration at the site of carboxylation (Badger et al. 1998).

Despite their ecological importance, relatively limited and often contradictory information is available on the inorganic carbon acquisition of dinoflagellates (see Giordano et al. 2005). Dinoflagellates have been shown to be able to accumulate inorganic carbon during photosynthesis between 5- and 70-fold relative to the ambient concentrations, depending on growth conditions and species investigated (e.g. Berman-Frank et al. 1998, Leggat et al. 1999). Such accumulation requires active uptake of either CO_2 or HCO_3^- or both. Studies by Colman et al. (2002) and Dason et al. (2004) on *Heterocapsa oceanica* and *Amphidinium carterae* found no evidence for HCO_3^- use, and they suggested that photosynthesis and growth were in fact CO_2 limited in marine environments. In a recent paper, carbon acquisition was investigated in 3 common marine red-tide dinoflagellates: *Ceratium lineatum*, *Heterocapsa triquetra* and *Prorocentrum minimum* (Rost et al. 2006a). For all 3 species, HCO_3^- uptake rates were high, contributing more than 80% relative to net photosynthesis at pH 8.0. When the dinoflagellates were acclimated at high pH, the affinity and maximum uptake rates of HCO_3^- even increased. These results suggest that photosynthesis in marine dinoflagellates is not limited by inorganic carbon even at high pH, which may occur during red tides in coastal waters.

Studies on inorganic carbon limitation of marine dinoflagellates have mainly assessed the short-term responses in photosynthesis. Data are largely lacking on the possible effects of DIC limitation on dinoflagellate growth rate, a highly relevant ecological parameter that does not necessarily reflect the response of photosynthesis. An exception is the study by Clark & Flynn (2000), who studied the growth rate of 2 dinoflagellate species as a function of DIC concentration at pH 8.3. They found that the growth rate of these dinoflagellates was >90% saturated at a DIC concentration of >1.6 mM relative to the growth rates obtained at a DIC concentration of 2.0 mM, which is typically found in natural seawater. However, to what extent the observed relationship between dinoflagellate growth and DIC may be affected by pH is unknown.

In the present study, we therefore aimed to determine (1) the effect of DIC concentration on the growth of 3 common red-tide dinoflagellates (*Ceratium lineatum*, *Heterocapsa triquetra* and *Prorocentrum minimum*), at 2 constant pH levels and (2) the growth of

these species in pH-drift experiments initiated at either 2.4 or 1.2 mM DIC, representing the range of concentrations found in natural marine habitats.

MATERIALS AND METHODS

Isolation and maintenance of phytoplankton cultures. Three marine dinoflagellates — *Ceratium lineatum*, *Heterocapsa triquetra* (strain K-0481) and *Prorocentrum minimum* (strain K-0295) — were selected for this study and provided by The Scandinavian Culture Collection of Algae and Protozoa, Department of Phycology and the Marine Biological Laboratory, University of Copenhagen. The dinoflagellates were grown as non-axenic cultures using the f/2 medium (Guillard & Ryther 1962) based on natural seawater (salinity 30 psu) at 15 ± 1°C following a light:dark cycle of 16:8 h. Illumination was provided by cool white fluorescent lamps, and cultures were kept at an irradiance of 25 µmol photons m^{-2} s^{-1}. Irradiance was measured using a LI-COR LI-1000 radiation sensor equipped with a spherical probe.

Experimental conditions and analysis. All experiments were carried out at an irradiance of 60 µmol photons m^{-2} s^{-1}. The cultures were acclimated to this irradiance for at least 14 d prior to each experiment. Cells from exponentially growing cultures were used for inoculation. In all treatments, bottles were filled to capacity and mounted on a plankton wheel (1 rpm) in order to keep cells in suspension. For enumeration of cells, subsamples were fixed in acidic Lugol's iodine (2.5% final concentration). Cells were counted in a Sedgewick-Rafter chamber or a multidish well (Nunclon®). Each count was based on at least 400 cells. Growth rates (µ) were measured as increase in cell densities and were calculated as:

$$\mu\ (d^{-1}) = \frac{(\ln N_1 - \ln N_0)}{t}$$

where N_0 and N_1 are concentrations of cells at time t_0 and t_1, respectively, and t is the difference in time between t_0 and t_1. A minimum of 3 sampling points was included in the calculation. All experiments were carried out in triplicate. The pH was measured using a Sentron® pH-meter (Argus X) equipped with Red-line probe, which is an ISFET® sensor (Semi-conductor Ion Field Effect Transistor) with a precision of 0.01 U. The pH sensor was 2 point calibrated using Sentron NBS (National Bureau of Standards) buffers of pH 7 and 10.

The concentration of DIC in the growth medium was measured by injection of samples (80 µl) into a bubbling chamber containing 0.5 ml 20 mM HNO_3. The liberated CO_2 was measured with an infrared gas analyser (ADC, MK3), using nitrogen gas as a carrier. Concentrations of CO_2 and HCO_3^- were calculated from DIC, pH, salinity and temperature of the medium using dissociation constants of Mehrbach et al. (1973).

Experimental setup. In the first set of experiments, growth rates of the dinoflagellates *Ceratium lineatum*, *Heterocapsa triquetra* and *Prorocentrum minimum* were measured at 2 separate pH levels as a function of the DIC concentration. In the case of *C. lineatum*, these pH levels were 8.0 and 8.55, and for both *H. triquetra* and *P. minimum*, they were pH 8.0 and 9.2. These pH levels were selected based on the evidence that all species grow at their maximum growth rates at pH 8.0, while at the higher pH levels (8.55 and 9.2), growth rates are about half the maximum (Hansen 2002).

Growth media (f/2) with different concentrations of DIC were obtained by mixing growth media with normal and very low DIC concentrations. The latter media (DIC < 1 µM) was achieved by acidifying the growth medium (to pH < 3), followed by heating to 110°C for 30 min and shaking or aerating the medium. The pH of the medium used in the experiments was then adjusted by the addition of 0.1 or 1.0 M NaOH or HCl.

The cultures were allowed to adjust to the experimental conditions for at least 7 d. Each experiment was initiated by the inoculation of between 20 and 100 cells ml^{-1} in 265 ml tissue culture flasks filled to capacity. Daily or 3 times per week, the pH of the culture media was measured, and subsamples (6 ml) were taken for enumeration of cells. After subsampling, the bottles were refilled to capacity with f/2 growth medium with the same pH and DIC content as the respective experimental bottle. If the pH differed by more than 0.03 U from the set point, it was adjusted by addition of small amounts of 0.1 M NaOH or HCl.

In the second set of experiments, the dinoflagellates were inoculated (initial concentration of 200 to 500 cells ml^{-1}) at a pH of 8.0 at initial DIC concentrations of ~1.2 and 2.4 mM and allowed to grow well into stationary growth phase (up to 22 d). These DIC concentrations were selected to cover the variation typically found in marine and brackish waters. The f/2 growth medium contains macronutrients, micronutrients and vitamins in excess, leaving out any kind of nutrient limitation of algal growth in the experimental bottles, even at very high pH (e.g. Schmidt & Hansen 2001, Hansen 2002, Lundholm et al. 2004).

The second set of experiments was carried out in 750 ml tissue culture flasks. Every 2 to 3 d, the pH of the culture medium was measured, and subsamples were withdrawn for enumeration of cell concentration (3 ml) and for measurements of the DIC concentration. The experimental bottles were not refilled after each sampling.

RESULTS

DIC limitation of marine dinoflagellate growth rates

In the semi-continuous experiments carried out at pH 8.0, all 3 dinoflagellates grew at their maximum rate at levels above ~0.4 and 0.2 mM DIC for *Ceratium lineatum* and the 2 other species, respectively (see Fig. 1). Below this DIC level, growth rates decreased, hence cell division was carbon limited. Maximum growth rates, apparent half-saturation constants ($K_{½}$) and DIC concentrations required for maintenance (compensation points) were estimated directly from the plots, since the curves could not be adequately described by a Michaelis-Menten fit (with or without introducing a compensation point). The data revealed that *Prorocentrum minimum* and *Heterocapsa triquetra* had low apparent $K_{½}$ as well as compensation points, with *P. minimum* being the most efficient species (see Fig. 1, Table 1). In comparison to the other species, *C. lineatum* had higher apparent $K_{½}$ and required higher DIC levels for maintenance (Table 1). At high pH, growth rates were lower in all 3 species even at the highest DIC levels, indicating that the pH itself affected algal growth and not DIC availability. Besides this, DIC became a limiting factor for growth at much higher DIC concentrations than at experiments

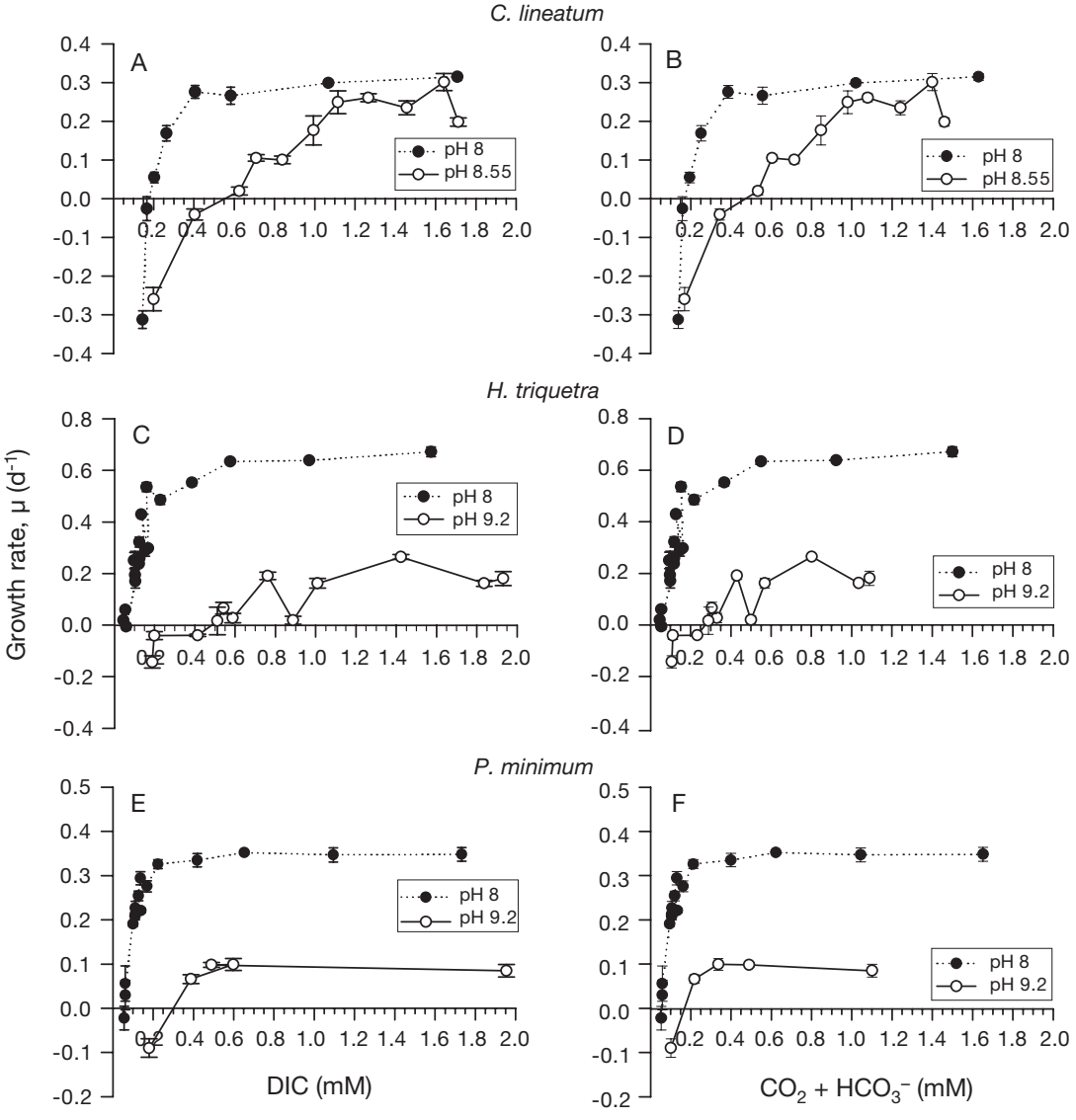

Fig. 1. *Ceratium lineatum, Heterocapsa triquetra, Prorocentrum minimum.* Growth rate of the 3 red-tide dinoflagellates as a function of (A,C,E) total dissolved inorganic carbon (DIC) and (B,D,F) available inorganic carbon ($CO_2 + HCO_3^-$). Data points with error bars represent treatment means ± 1 SE, n = 3

carried out at pH 8.0. This was evident in terms of both higher apparent $K_{1/2}$ and compensation point for growth (Fig. 1, Table 1).

To investigate the extent that pH and DIC can be limiting at the naturally occurring levels of pH and DIC, pH-drift experiments were carried out at 2 levels of initial DIC and an initial pH of ca. 7.8 to 8.0. In all cases, the growth at the 2 initial DIC levels was similar for the first 8 d, irrespective of the initial DIC concentration (Student's *t*-test, $p > 0.05$; Fig. 2, Table 2). The growth rates were also similar to the rates obtained from the experiments carried out at pH 8 and constant DIC concentrations (Student's *t*-test, $p > 0.05$; see Fig. 1A,C,E). Thus, both sets of data suggest that DIC is not limiting growth in these dinoflagellates at a pH of ca. 8 to 8.4 and natural levels of DIC concentrations. In the pH-drift experiments, pH reached a maximum of 8.7 with the pH-sensitive species *Ceratium lineatum*, irrespective of whether the experiments were initiated at a high DIC of 2.4 mM or a low DIC of 1.2 mM (Fig. 2). Final DIC concentrations in these experiments were 1.7 and 0.8 mM DIC, respectively. In the case of the pH-tolerant species (*Heterocapsa triquetra* and *Prorocentrum minimum*), pH reached 9.4 and 9.7, respectively, when grown at initially high DIC concentrations (Fig. 2). In the incubations initiated at a low DIC, however, pH only reached 9.2 and 9.55 for *H. triquetra* and *P. minimum*, which is significantly lower compared to the experiments carried out at an initially higher DIC concentration (Student's *t*-test, $p < 0.01$; Fig. 2). The final DIC concentrations in the experiments initiated at low DIC were around 0.7 and 0.6 mM for *H. triquetra* and *P. mini-*

Table 1. *Ceratium lineatum, Heterocapsa triquetra, Prorocentrum minimum.* Apparent growth compensation points and half saturation constants ($K_{1/2}$) for concentrations of total dissolved inorganic carbon (DIC) and bioavailable inorganic carbon ($CO_2 + HCO_3^-$) for the 3 dinoflagellates from experiments at a constant pH (see Fig. 1)

pH	Compensation point (mM)		$K_{1/2}$ (mM)	
	Total DIC	$CO_2 + HCO_3^-$	Total DIC	$CO_2 + HCO_3^-$
Ceratium lineatum				
8.0	0.18	0.18	0.28	0.28
8.55	0.60	0.50	0.90	0.80
Heterocapsa triquetra				
8.0	0.05	0.05	0.10	0.10
9.2	0.60	0.30	1.00	0.45
Prorocentrum minimum				
8.0	0.05	0.05	0.10	0.10
9.2	0.30	0.15	0.35	0.20

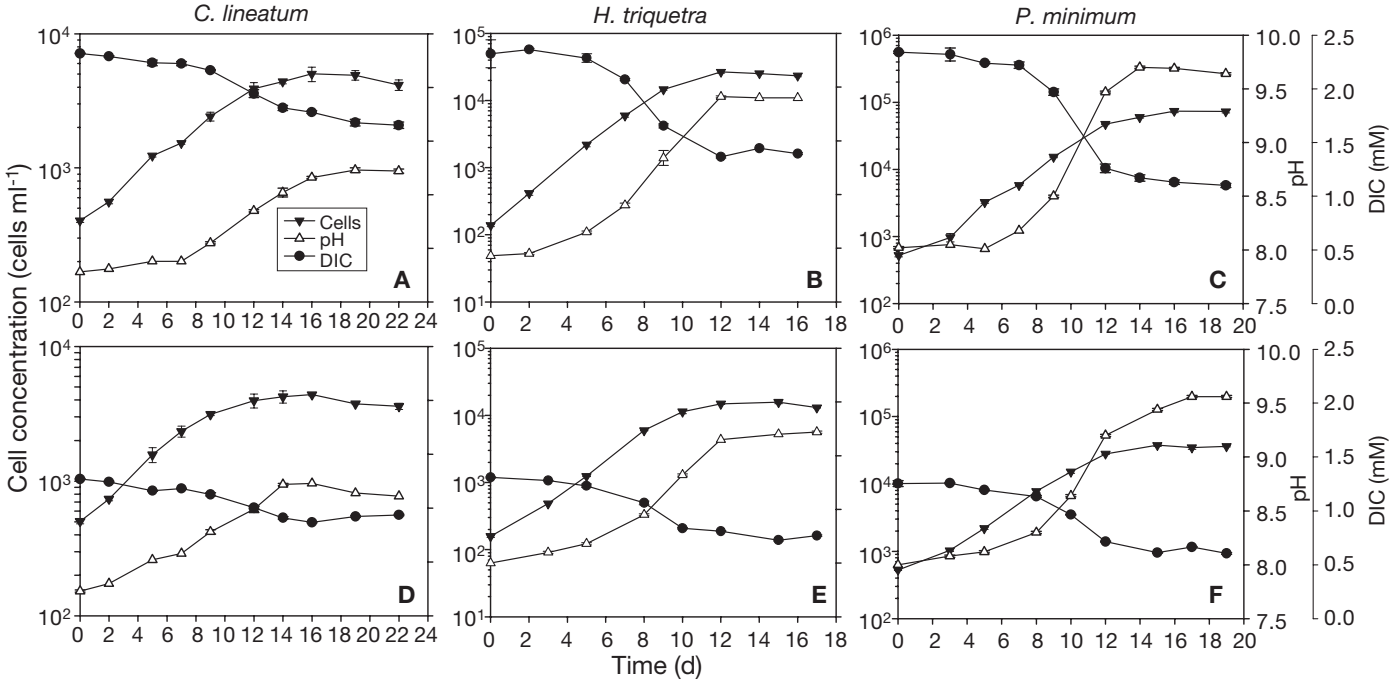

Fig. 2. *Ceratium lineatum, Heterocapsa triquetra, Prorocentrum minimum.* Cell concentration, pH and total dissolved inorganic carbon (DIC) as a function of time for pH-drift experiments at initial DIC concentrations for the 3 dinoflagellates. Initial DIC (A–C) 2.4 mM and (D–F) 1.2 mM. Data points with error bars represent treatment means ± 1 SE, n = 3

Table 2. *Ceratium lineatum, Heterocapsa triquetra, Prorocentrum minimum.* Exponential growth rates of the 3 dinoflagellates in pH-drift experiments at dissolved inorganic carbon (DIC) concentrations of 2.4 and 1.2 mM. No significant differences exist between growth rate and initial DIC concentration for any species (Student's *t*-test, $p > 0.01$)

Phytoplankton species	Growth rate (d^{-1}) ± SD DIC 2.4 mM	DIC 1.2 mM
Ceratium lineatum	0.20 ± 0.01	0.22 ± 0.03
Heterocapsa triquetra	0.54 ± 0.02	0.51 ± 0.03
Prorocentrum minimum	0.39 ± 0.01	0.41 ± 0.02

mum, respectively. In initially high DIC treatments, concentrations decreased to 1.4 and 1.2 mM for these 2 species, respectively (Fig. 2).

For *Heterocapsa triquetra*, the final pH of 9.2 in the pH-drift experiment, initiated at low DIC, is similar to the high pH selected for the semi-continuous experiments; and thus data can be directly compared between the 2 sets of experiments. Comparisons showed that the 2 different sets of experiments both give population maintenance ($\mu = 0$) at a DIC concentration of 0.7 mM at pH 9.2 (compare Figs. 1 & 2).

The amount of DIC removed from the media by the algae was much smaller for the cultures initiated with the low DIC concentration compared to those initiated at high DIC concentrations (Fig. 2). This reduced uptake of DIC in cultures initiated at low DIC concentration was mimicked in lower final cell densities. DIC consumed per cell produced did not show significant differences between the 2 treatments (Fig. 3, Student's *t*-test, $p > 0.01$).

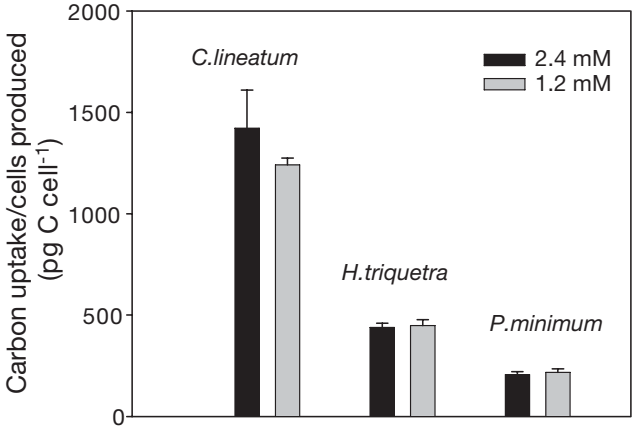

Fig. 3. *Ceratium lineatum, Heterocapsa triquetra, Prorocentrum minimum.* Dissolved inorganic carbon (DIC) taken up per number of cells produced during pH-drift experiments. Data represent treatment means ± 1 SE, n = 3

DISCUSSION

DIC or pH limitation of marine dinoflagellate growth rates

It has been speculated that changes in carbonate chemistry and corresponding changes in seawater pH may affect plankton primary production and shift the dominance of species (e.g. Wolf-Gladrow et al. 1999, Tortell et al. 2002, Rost et al. 2003, Raven et al. 2005). Unfortunately, very few data are available on the relationship between the concentration of DIC (at constant pH) and algal growth rate (e.g. Clark & Flynn 2000). Most studies on CO_2-related responses in phytoplankton have used responses in photosynthesis, either by monitoring C-fixation or O_2 evolution, to deduce the potential of carbon limitation. Moreover, many of these studies have used changes in pH or pCO_2 to manipulate the carbonate system. Under these conditions it is difficult to distinguish between the effect of carbon limitation and an effect of pH (see Hansen 2002).

Here, we studied the effect of DIC concentration on growth in 3 red-tide dinoflagellates, which are all predominantly HCO_3^- users, despite their different tolerances to high pH. In fact, >80% of the total inorganic carbon fixed in these species is derived from uptake of HCO_3^-, even at pH 8 (Rost et al. 2006a). Among other aspects of carbon acquisition, the HCO_3^- uptake was assessed using 2 different approaches in thei study: (1) the ^{14}C disequilibrium technique (Espie & Colman 1986) and (2) mass spectrometric carbon flux measurements (Badger et al. 1994), both yielding similarly high HCO_3^- preference in these species. In the present experiments at pH 8, all 3 dinoflagellates grew close to their maximum growth rate at DIC levels above ~0.4 mM, and the apparent $K_{½}$ for growth were quite low (Table 1, Fig. 1). The 3 red-tide dinoflagellates studied herein are thus not limited in their growth rates by inorganic carbon at pH 8, taking into account that natural levels of DIC range from 2.1 to 2.4 mM in the open ocean to ~1.2 mM in brackish waters (e.g. Thomas & Schneider 1999, Key et al. 2004). Clark & Flynn (2000) reached a similar conclusion in their study of 9 species of marine phytoplankton, including 2 dinoflagellates. Thus, recent claims (Colman et al. 2002, Dason et al. 2004) that marine dinoflagellates may be restricted to CO_2-rich environments cannot be supported by any of these studies. See Rost et al. (2006a) for a thorough discussion of the results obtained by Colman et al. (2002) and Dason et al. (2004).

Another issue addressed here is how high pH affects the ability of the dinoflagellates to grow at low DIC concentrations. Our results demonstrate that pH has a great influence on the ability of dinoflagellates to grow

at any DIC concentration, but particularly at the low DIC levels. *Ceratium lineatum* is a highly pH-sensitive species that stops growing at pH 8.7 at natural concentrations of DIC (Hansen 2002; Fig. 2). At pH 8.55, which is quite close to its pH limit for growth, the growth rate of *C. lineatum* decreases at DIC concentrations below 1.0 to 1.1 mM and stops at a concentration of 0.5 mM. However, in the pH-drift experiments with *C. lineatum*, pH has reached 8.7 under both high and low DIC treatments, suggesting that pH alone sets the limits for growth. Consequently, growth rates will probably not be limited by DIC in its natural environment. This is in contrast to *Heterocapsa triquetra* and *Prorocentrum minimum*, which can grow up to pH 9.4 and 9.75, respectively (Hansen 2002; Fig. 2). For these species, inorganic carbon limitation could be detected at very high pH and low DIC concentrations. Such conditions can potentially be found in productive fjords and estuaries in which the DIC pool is low. Under all other field conditions, our results showed that pH, and not DIC, will be the limiting factor for growth of these 2 dinoflagellate species.

Does photosynthesis respond to DIC limitation in the same way as growth?

The relationship obtained here between growth and inorganic carbon concentration is in many aspects similar to the relationship between photosynthesis and inorganic carbon concentration on the same species and strains in a previous publication (Rost et al. 2006a). Both data sets indicate the operation of a CCM based on use of HCO_3^- in all 3 species. Moreover, responses in photosynthesis as well as growth rates suggest that these species do not suffer from severe carbon limitation at natural concentrations of inorganic carbon. Nevertheless, important differences between the 2 data sets can be observed.

A direct comparison of data on growth rates and photosynthesis for 2 of the species, *Heterocapsa triquetra* and *Prorocentrum minimum*, reveal significant differences in the DIC-dependence of both processes. At pH 8.0, *H. triquetra* and *P. minimum* both have an apparent $K_{1/2}$ for growth of ~0.10 mM (CO_2 + HCO_3^-), which is lower than the $K_{1/2}$ values estimated for photosynthesis, around ~0.45 and 0.16 mM (CO_2 + HCO_3^-) for the 2 species, respectively. In experiments carried out at high pH (9.1 to 9.2), the $K_{1/2}$ constants for growth were 0.45 and 0.20 mM (CO_2 + HCO_3^-) for *H. triquetra* and *P. minimum*, respectively. $K_{1/2}$ values for photosynthesis, however, were estimated to be as low as 0.03 and 0.013 mM (CO_2 + HCO_3^-), respectively, for high pH acclimated cells. Thus, especially at high pH, the $K_{1/2}$ obtained for photosynthesis and growth were quite different. There may be several reasons for these discrepancies between the 2 different measures.

Assays on photosynthesis and carbon fluxes by membrane-inlet mass spectrometry (Badger et al. 1994) are performed to unravel underlying mechanisms and regulation of carbon acquisition. As a tradeoff for this detailed analysis, assay conditions often have to differ from the conditions during growth. In Rost et al. (2006a), cells were acclimated to different pH and a constant high DIC, while assays were performed at pH 8.0 and changing DIC concentration. A constant and rather low pH is a prerequisite for carbon flux measurements, since at high pH the proportion of CO_2 relative to the overall inorganic carbon diminishes, exacerbating the analysis and calculations of this technique. Consequently, results from such assays show the potential of differently acclimated cells under the respective assay condition and not necessarily the fluxes occurring in the acclimation.

Nevertheless, there may also be other reasons for differences observed between $K_{1/2}$ estimated for photosynthesis and growth rates with regard to inorganic carbon concentration. The lower $K_{1/2}$ for growth compared to the $K_{1/2}$ for photosynthesis may potentially be explained by treatment-dependent variations of cell carbon content. Such changes in the carbon quota between treatments have, however, not been observed in our study (Fig. 3). Another explanation for the observed differences in the responses of growth and photosynthesis to inorganic carbon concentration at high pH could be that the loss of inorganic carbon by cells via leakage increases at high pH or lower CO_2 concentrations, causing carbon acquisition to be less energy-efficient (Raven & Lucas 1985, Rost et al. 2006b). Last, but not least, Rost et al. (2006a) reported use of higher light intensities, and assays showed short-term responses, while growth rates reflected the integrated response over days (including the dark phase).

Cause of the direct pH effects on algal growth at high pH and high DIC concentrations

Effects of high pH on algae have commonly been attributed to the process of photosynthesis. However, these negative effects of elevated pH on algae may not necessarily be coupled to DIC uptake, fixation or leakage. It is known that the growth of at least some non-photosynthesizing heterotrophic dinoflagellates and ciliates are affected by a pH as low as 8.7 to 9.0 (Pedersen & Hansen 2003). This raises the question concerning the cause of these negative effects of high pH on the algae when DIC concentrations are high. By selecting the f/2 growth medium, we have ensured that

nutrients, including micronutrients, should not be limiting growth, because they are added in excess (see Schmidt & Hansen 2001, Hansen 2002, Lundholm et al. 2004). High pH may, however, affect the solubility of some macronutrients and micronutrients. Olsen et al. (2006) showed that precipitation of phosphorus occurs at high pH but is relevant only at extremely high values (pH 10). At high pH, trace metals such as Zn, Fe, Co or Cu tend to form hydroxides, which are less soluble in seawater. In f/2 medium, concentrations are very high, and the chelator EDTA is present. Since EDTA binds these metals, it will supply a pool of free trace metals. This pool may become smaller at high pH, but owing to the large EDTA-metal reservoir, it will not be exhausted.

A much more likely explanation for the observed effects of pH has to do with the fact that elevated extracellular pH may influence intracellular pH as has been found in some phytoplankton species (Nimer et al. 1994, Dason & Colman 2004). In the cell, intracellular pH regulates cellular processes, and since pH is known to influence enzymatic processes, changes in intracellular pH could affect cell growth (Smith & Raven 1979, Raven 1980, 1993). Experiments on *Skeletonema costatum* at increasing external pH showed changes in cellular amino acid content that were related to metabolic changes and leakage of organic material (Taraldsvik & Myklestad 2000). Similarly, studies on 2 dinoflagellate species showed that external pH changes from 8 to 7 were associated with a lowering of internal pH, which was used to explain an observed decrease in cell growth (Dason & Colman 2004). Hence changes in external pH could affect processes involved in growth that are not directly associated to photosynthesis. Generally, maintenance of a stable intracellular pH is important for phytoplankton cells. In spite of changes in external pH, maintenance of a relatively stable internal pH is associated with energy expenses related to, e.g., membrane transport processes (Smith & Raven 1979, Raven 1980, Puceat 1999, Gerloff-Elias et al. 2006). Such energy expenses may increase at elevated extracellular pH and hence affect cell growth. Nevertheless, how extracellular pH affects intracellular pH and how they together affect growth and photosynthesis definitely deserve more attention in the future.

CONCLUSIONS

In conclusion the present study has presented data on both pH-sensitive and pH-tolerant red-tide dinoflagellate species. The results suggest that these common marine red-tide dinoflagellates are not limited by inorganic carbon at pH 8.0, a pH level close to that in the open sea. However, in productive waters such as fjords and embayments, the pH may reach high values and thus affect pH-sensitive species such as the red-tide dinoflagellate *Ceratium lineatum*. Other species such as *Heterocapsa triquetra* and *Prorocentrum minimum* can tolerate these much higher pH levels. Our data suggest direct pH effects on the growth of *C. lineatum* in the natural environment, while inorganic carbon is not limiting at natural levels of DIC. Growth of the 2 other species was also mainly affected by pH itself, and inorganic carbon limitation probably only plays a role in productive DIC-poor, brackish waters. In some aspects our findings deviate considerably from results obtained previously on photosynthesis. Since responses in photosynthesis do not necessarily mimic the patterns in growth rates, interpretation based entirely on responses to photosynthesis, especially in terms of species succession, should be interpreted cautiously.

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