

Growth limitation due to high pH and low inorganic carbon concentrations in temperate species of the dinoflagellate genus *Ceratium*

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ABSTRACT: The effects of high pH and low DIC (dissolved inorganic carbon) concentrations on growth rates of the dinoflagellate *Ceratium* were studied using 2 different approaches: (1) pH drift experiments, in which both pH and DIC change during the experiment, and (2) constant pH and DIC experiments at a range of fixed pH and DIC levels. The upper pH limits for growth were between 8.7 and 9.1 for the 3 species tested (*C. furca*, *C. tripos* and *C. fusus*) and thus within an environmentally relevant pH range. Hence, for *Ceratium* spp. and other dinoflagellates with upper pH tolerances for growth below or around 9, high pH will most likely periodically be a limiting factor in coastal areas. Different upper pH limits for growth were found using either the pH drift or the constant pH methods for the larger *Ceratium* species, but, for the smaller species, the 2 methods provided similar results. The results therefore suggest a combined pH/DIC limitation for large species such as *C. tripos* and *C. furca* in natural waters.

KEY WORDS: pH · Inorganic carbon · DIC · *Ceratium* · Phytoplankton

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INTRODUCTION

Seawater possesses a high buffer capacity (compared to most freshwater habitats) due to large amounts of dissolved inorganic carbon (DIC). This has previously led to the general belief that the pH of seawater is stable around pH 8.2. Recent research, however, has shown that the pH may rise to values >9.5 in nutrient-enriched fjords and lagoons due to photosynthetic CO₂ uptake by phytoplankton (Macedo et al. 2001, Hansen 2002). During algal blooms, when algal CO₂ uptake from the water is larger than CO₂ replacement from the atmosphere, the pH increases and the carbon equilibrium moves towards CO₃²⁻: CO₂ ↔ HCO₃⁻ ↔ CO₃²⁻ (Chen & Durbin 1994). In oceanic waters, the amount of DIC is usually around 2 mM, and, at atmospheric equilibrium (pH 8.2), the majority of this is in the form of HCO₃⁻ (approximately 90%), whereas CO₂ comprises <1% and CO₃²⁻ ca. 9% (Riebesell 2004).

Effects of high pH on algal growth can be either direct or indirect. High pH could, for example, directly affect the efficiency of proton pumps in the

cell wall. Indirect effects of high pH could, for example, occur in the form of inorganic carbon limitation. Inorganic carbon limitation can occur if the algae are unable to utilise or have a low affinity for HCO₃⁻. Inside the cell it is necessary to convert HCO₃⁻ to CO₂, since the sole substrate for carbon fixation by the primary carboxylating enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is CO₂. The interconversion of CO₂ and HCO₃⁻ is catalysed by internal carbon anhydrase (CA). External CA can also be employed in the conversion of HCO₃⁻ to CO₂ outside the cell and subsequent CO₂ uptake (Badger 2003). Whether all marine phytoplankton are able to utilise both HCO₃⁻ and CO₂ and whether uptake of bicarbonate is a more energy-demanding process than CO₂ uptake are topical issues (Burkhardt et al. 2001, Bartual & Gálvez 2002, Riebesell 2004). If marine phytoplankton is CO₂ limited, it implies that an increase in atmospheric CO₂ would promote phytoplankton productivity (Hein & Sand-Jensen 1997, Schippers et al. 2004) and perhaps increase the frequency or magnitude of blooms.

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Dinoflagellates belonging to the genus *Ceratium* are common in temperate waters, especially in the late summer and fall (Smetacek 1981, Jansen et al. 2006). In Scandinavian coastal waters, *Ceratium* spp. can comprise up to 90% of the protist biomass, and these species are thus of great ecological importance (Hansen & Larsen 1992). Recent experiments have shown that some *Ceratium* species like *C. furca* and *C. tripos* are very sensitive to high pH (Schmidt & Hansen 2001), with upper pH limits for growth as low as 8.3 to 8.4. Thus, we were puzzled as to how algae with such low pH limits for growth, periodically can be dominating species in coastal waters (Lindahl & Hernroth 1983). Furthermore, extensive pH and DIC data already exist for the species *C. lineatum* (Hansen 2002, Rost et al. 2006, Hansen et al. 2007), which facilitate comparisons of the species within the genus and provide additional information regarding their carbon acquisition. Rost et al. (2006) have demonstrated that *C. lineatum* is able to use HCO_3^- and that direct HCO_3^- uptake accounts for 85% of the carbon uptake even at pH 8.

The aim of the present study was to examine the effects of high pH on growth rates of 3 species of *Ceratium* (*C. furca*, *C. fusus* and *C. tripos*) and to establish upper pH limits for growth and survival. This has been done by (1) using different constant pH levels and (2) allowing the algae themselves to generate a drifting pH due to their uptake of inorganic carbon. The results of the 2 methods were compared, since both methods have previously been used to describe pH effects on phytoplankton (e.g. Hinga 2002). Three species of the same genus covering a wide range of cell volumes have been chosen to elucidate a possible relationship between size and pH tolerance.

Since the effects of high pH could be indirect due to low (photosynthetically available) DIC concentrations, the DIC limits for growth were also examined for *Ceratium furca*. The results were used to separate the effects of high pH and low DIC. Finally, the effects of light were investigated to examine whether an increased pH tolerance would occur when more energy was provided in the form of light (Bartual & Gálvez 2002). An increased pH tolerance could occur if growth at high pH is more energy demanding, due to

an extra energy cost when using HCO_3^- as carbon substrate or due to an extra energy demand in order to maintain a stable internal pH.

MATERIALS AND METHODS

Culture and general experimental conditions.

Three species of the dinoflagellate genus *Ceratium* were used in this study. The origin and isolation dates of these cultures are given in Table 1. The cultures (non-axenic) were kept in 250 ml Nunclon flasks at $15 \pm 1^\circ\text{C}$, and mounted on a plankton wheel with 1 rpm at a photon flux density of $100 \mu\text{m photons m}^{-2} \text{s}^{-1}$ in a 16:8 h light:dark cycle. Illumination was provided by cool white fluorescent lamps and irradiance was measured using a Li-1000, Li-Cor sensor, equipped with a spherical probe. The growth medium consisted of autoclaved seawater with a salinity of 30 ± 1 psu enriched with *f/2* nutrients added after autoclaving (Guillard 1983).

Measurements of cell density were done according to Utermöhl (1958), and algal growth rates were calculated assuming exponential growth: $\mu \text{ (d}^{-1}\text{)} = (\ln N_1 - \ln N_0)/t$, where N_0 and N_1 are the cell concentrations per millilitre at time t_0 and t_1 , respectively, and t is the difference in time (d) between t_0 and t_1 samples. Dilutions were taken into account by subtracting the removed amount of cells after each sampling occasion. A minimum of 400 cells was counted at all sampling occasions, except in the case of negative growth at high pH. At these occasions it was not possible to remove such large quantities of medium as to provide 400 cells, but instead a volume of 10 ml was counted. This rather large volume ensured that the standard error (SE) was kept within reasonable limits (maximum SE was 0.06). In all experiments, a 2 d lag phase was excluded from the growth rate calculations to ensure that exponential growth was obtained.

The pH of the culture medium was measured with Sentron Isfet pH meters with Red line or Argus X probes, which were calibrated using pH buffers 7 and 10. Total concentration of DIC was measured using an infrared gas analyser, IRGA (ADC, MK3). Water sam-

Table 1. *Ceratium* spp. Maximum growth rates and upper pH limits for growth (\pm SD)

	<i>C. fusus</i>	<i>C. furca</i>	<i>C. tripos</i>
Max. growth rate (d^{-1})	0.32	0.26	0.14
Max. pH for growth (constant pH)	8.73	9.08	8.92
Max. pH for growth (pH drift)	8.76 (± 0.02)	8.82 (± 0.01)	8.85 (± 0.03)
Sampling location, year	The Sound, 2003	The Sound, 2003	The Sound, 1995
Donor	L. M. Söderberg	L. M. Söderberg	P. J. Hansen

ples (80 µl) were injected into a small chamber containing HNO₃ (20 mM), and the liberated CO₂ gas was transferred to the IRGA using nitrogen gas as a carrier.

Constant pH experiments with 3 species of *Ceratium*. In the constant pH experiments both the pH (and the total DIC concentration) was largely held constant during incubation because of the low cell concentrations (initial concentration 15 cells ml⁻¹). The initial total DIC concentration was between 1.6 and 1.7 mM. The pH was manipulated at the start of the experiment by the addition of 1 M NaOH. At the highest pH levels of 8.8, 9.0 and 9.2, the pH was gradually elevated for 1 to 2 d prior to the experiment. Experiments were carried out in triplicate. Every second day, 10 ml samples were removed and fixed with Lugol's iodine for enumeration. Growth rates are based on 3 or 4 consecutive cell counts. The flasks were refilled to capacity, and pH was adjusted with small amounts of 1 M NaOH or HCl in order to keep the pH constant (pH was adjusted to ±0.02 pH units every second day). In between adjustments the pH varied by a maximum of ±0.1 in the individual flasks, except at pH 7.5 when the pH increased during the experiments from 7.45 to 7.96 for *C. furca* and to 7.67 for *C. tripos*.

pH drift experiments with 3 species of *Ceratium*. In the pH drift experiments the algae decrease the total DIC pool as the population proliferates and pH increases. The initial total DIC concentration was between 1.6 and 1.7 mM. Experiments were performed using 3 or 4 replicates. The initial pH was around 8 (7.95 to 8.15), and the experiments were terminated when the algae went into stationary growth phase and no further increase in pH was observed. The initial concentration was 15 cells ml⁻¹ for *C. fusus*, 25 cells ml⁻¹ for *C. tripos* and 50 cells ml⁻¹ for the experiment with *C. furca*. Every second or third day (around noon), the pH was measured and 5 to 10 ml samples were removed and fixed for enumeration. The flasks were refilled with medium of the same pH (±0.05 pH units).

At termination of the experiment with *Ceratium furca*, the medium was filtered (GF/C glass microfiber filters) and frozen for later analysis of inorganic nutrients (N and P) to ensure that no nutrient limitation had occurred during the experiments. NO₂⁻, NO₃⁻, NH₄⁺ and PO₄³⁻ were analysed according to Valderrama (1995). In order to follow the decrease in the DIC pool, the total DIC was measured every second or third day for the pH drift experiment with *C. furca*.

An extra set of pH drift experiments was carried out to ensure that the growth rates during the pH drift experiments were not affected by lack of vitamins (B₁₂, thiamine, or biotin). In the original recipe by Guillard (1983), it is recommended that both the stock solution and the working solution of vitamins are autoclaved.

This procedure could possibly affect the quality of the vitamins, and for this reason a working stock solution based on non-autoclaved vitamins was tested. The non-autoclaved vitamin solution was prepared with autoclaved distilled water and stored in the freezer in small Eppendorf tubes until use. The effects of the 2 methods were studied in drift pH experiments with *Ceratium furca*. Both vitamin solutions were added to the media after autoclaving.

Growth rate of *Ceratium furca* at different DIC concentrations. *C. furca* was selected for this experiment. Both DIC and pH were kept constant during the experiment, which was performed at pH 8.0 and 9.0. The initial cell concentration was 15 cells ml⁻¹. The experiment was carried out in triplicate. Different DIC concentrations were used ranging from 0.2 to 3.4 mM DIC. In order to achieve the low DIC concentrations (below 1.6 mM), the pH was lowered to pH 3 by addition of 1 M HCl, and aerated until the CO₂ had gassed off. The DIC-enhanced medium was obtained by adding 0.5 M HCO₃⁻. Every second day 10 ml samples were removed for enumeration, and additional 2 ml samples were removed for DIC analysis. The flasks were refilled to capacity, and pH was adjusted to maintain the desired level. The pH was adjusted to ±0.05 every second day and varied by a maximum of ±0.16 between adjustments. The DIC concentrations were converted to photosynthetically available inorganic carbon (CO₂ + HCO₃⁻) for a better comparison of pH 8.0 and 9.0, since the speciation of inorganic carbon is highly pH dependent. The amount of photosynthetically available inorganic carbon was calculated according to Plath et al. (1980) and Mackereth et al. (1978).

Effects of irradiance on the pH tolerance for growth. This experiment was also carried out using *Ceratium furca* as a test organism. The DIC concentration was largely held constant, due to the low cell concentrations (initial concentration 15 to 30 cells ml⁻¹). The initial total DIC concentration was 1.4 to 1.7 mM in this experiment, and this did not change in the individual bottles during incubation. The pH was gradually elevated (during 2 d) prior to the start of the experiment by addition of 1 M NaOH. Experiments were carried out in triplicate. Every second day 10 ml samples were removed for enumeration. The flasks were refilled to capacity, and pH was adjusted with small amounts of 1 M NaOH or HCl in order to keep pH constant. The tested light regimes were 25 and 200 µm photons m⁻² s⁻¹ to ensure both subsaturating and saturating photon flux densities, on a 16:8 h light:dark basis. At both light intensities a pH range of 8.15 to 9.15 was tested. The algae were adapted to the appropriate photon flux density for at least 5 d prior to the experiment.

RESULTS

Constant pH experiments

The upper pH limits for growth using the constant pH approach were 8.73, 8.92 and 9.08 for *Ceratium fusus*, *C. tripos* and *C. furca*, respectively (see Table 1 for further results). A sharp decline in growth rates was observed within 0.1 pH unit of the upper pH limit (Fig. 1). The lower pH limits for growth of the *Ceratium* species were not determined, but no significant differences were observed in the growth rates at pH ~7.5 compared to the growth rates at pH ~8.0 for any of the 3 species ($p > 0.05$, Students' *t*-test).

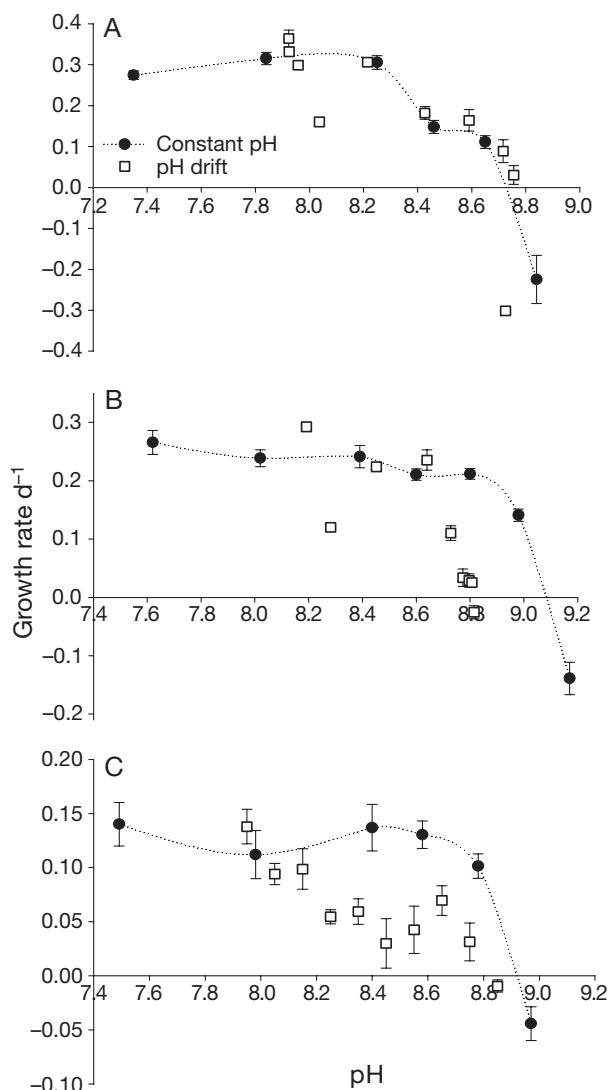


Fig. 1. *Ceratium* spp. pH drift and pH constant experiments for (A) *C. fusus*, (B) *C. furca* and (C) *C. tripos*. For *C. tripos*, data points represent data pooled within 0.1 pH units. Error bars = SE

pH drift experiments

The upper pH limit for growth obtained in the pH drift experiments was approximately 8.8 for all 3 species (Fig. 1). For the 2 largest species, *Ceratium tripos* and *C. furca*, these upper pH limits for growth were lower than those obtained in the pH constant experiments, while the pH limit for the smallest species, *C. fusus*, was the same irrespective of the approach used (Fig. 1, Table 1). Consult Table 2 for cell sizes.

In case of the largest species, *Ceratium tripos*, the growth rate started to decline at pH 8.25 in the pH drift experiment, while a reduction in growth rate in the constant pH experiment was not observed before pH had exceeded pH 8.8 (Fig. 1C). The growth of *C. furca* was unaffected up to pH 8.65 in the pH drift experiment, but the growth rate declined dramatically between pH 8.7 and 8.8 (Fig. 1B). Thus, for *C. furca*, the growth rates observed in the pH drift and constant pH experiments only differed at high pH. As opposed to *C. tripos* and *C. furca*, the pH drift and constant pH experiments for *C. fusus* yielded almost identical results with regard to the pH-dependent growth rate (Fig. 1A).

To ensure that pH was the only limiting factor in the pH drift experiment, inorganic macronutrients (N and P) and DIC were analysed at the end of the pH drift experiment (*Ceratium furca*). Table 3 shows that N and P remained in excess during the experiment. The final total DIC concentration in the pH drift experiment was 1.2 mM, which at pH 8.82 corresponds to 0.91 mM photosynthetically available DIC (= CO₂ + HCO₃⁻) (Table 4).

Furthermore, the quality of the vitamin solutions was examined by comparing the 2 different methods of preparing vitamin solutions (with/without heat treatment). The 2 methods gave very similar results (Fig. 2), and neither growth rates nor pH were significantly different at the termination of the experiment ($p > 0.05$, Students' *t*-test). Thus, the quality of the autoclaved vitamins was not affected to an extent that it became limiting for the growth of *Ceratium furca*.

Growth rate of *Ceratium furca* at different DIC concentrations

To test if the final DIC concentrations in the pH drift experiment were limiting for the growth of the larger *Ceratium* species, an experiment was carried out with *C. furca* in which the growth dependency of DIC at pH 8.0 and 9.0 was studied. Carbon concentrations remained constant during the experiments (Fig. 3). The results clearly show that the growth of *C. furca* is pH limited at pH 9.0, even at very high photosynthetic

Table 2. *Ceratium* spp. Measured sizes (\pm SD). Volume and surface areas are calculated using simple geometric formulae according to Hillebrand et al. (1999), with minor modifications (for *C. fusus* both the hypothecal and the apical horn have been calculated as cylinders). The calculations are based on microscopic measurements of 10 to 20 live cells from each species sampled in the exponential phase

	<i>C. fusus</i>	<i>C. furca</i>	<i>C. tripos</i>
Volume (μm^3)	31 000 (\pm 6330)	68 000 (\pm 14 432)	111 000 (\pm 11 656)
Surface area (μm^2)	26 000	37 000	51 000
Surface:volume ratio	0.83	0.55	0.46

Table 3. *Ceratium furca*. Macronutrient concentrations (N and P) at the beginning and at the termination of the *C. furca* pH drift experiment (\pm SD, n = 3). n.a.: SD is not available, since n = 1, due to sampling error

	$\text{NO}_3 + \text{NO}_2$ (μM)	NH_4 (μM)	PO_4 (μM)
Start concentration	1010.47 (\pm 0.73)	0.73 (n.a.)	45.74 (\pm 0.10)
Final concentration	943.74 (\pm 9.26)	0.90 (\pm 0.17)	35.31 (\pm 1.44)

Table 4. *Ceratium furca*. Carbon data for the pH drift experiment with *C. furca* (\pm SD). DIC: dissolved inorganic carbon

	CO_2 (mM)	HCO_3^- (mM)	$\text{CO}_2 + \text{HCO}_3^-$ (mM)	Total DIC (mM)
Start carbon conc. (pH 8.13)	0.013	1.46	1.48	1.57 (\pm 0.01)
Final carbon conc. (pH 8.82)	0.002	0.90	0.91	1.20 (\pm 0.05)

cally available inorganic carbon concentrations. However, the data also demonstrate that the ability of *C. furca* to grow at low photosynthetically available inorganic carbon concentrations depends on pH.

The growth rate at pH 8 is constant at a photosynthetically available inorganic carbon concentration above 0.5 mM. At a concentration of 0.2 mM, the cells

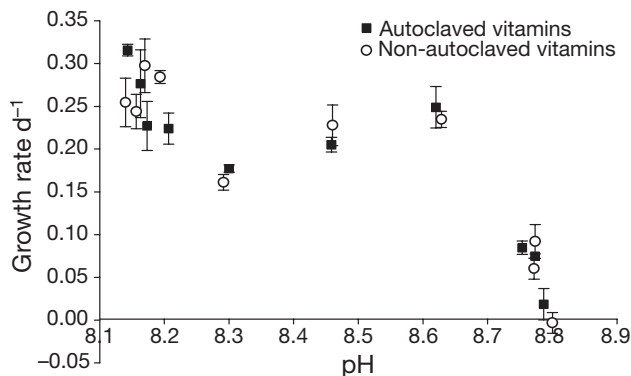


Fig. 2. *Ceratium furca*. Autoclaved and non-autoclaved vitamins in pH drift experiments with *C. furca*. Error bars = SE

are still able to grow at approximately half the maximum growth rate; thus, DIC limitation of the growth rate at pH 8 does not take place unless the DIC concentration is very low. At pH 9.0, on the other hand, *Ceratium furca* is not able to grow until the photosynthetically available inorganic carbon concentration exceeds approximately 0.8 mM, corresponding to a total DIC concentration of 1.2 mM. Thus, the final photosynthetically available DIC concentration of the pH drift experiment (0.91 mM, see Table 4) approaches the DIC limit for growth at pH 9.0.

Effects of irradiance on the pH tolerance for growth

Since the DIC limit for growth was higher at pH 9 than at pH 8, it could be due to an extra energy cost when converting HCO_3^- to CO_2 inside the cell or that direct HCO_3^- uptake is a more energy-demanding process than CO_2 uptake. For this reason, the effects of light on the growth rate were studied.

The growth rate of *Ceratium furca* at 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ was 0.24 d^{-1}

(Fig. 4), which is the same growth rate as was observed at 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ in the constant pH experiment (both at pH 8.0). Thus, the growth rate of *C. furca* was saturated at $\leq 100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. The corresponding growth rate at 25 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ was 0.14 d^{-1} (at pH 8.0). The growth rates remained constant up to pH

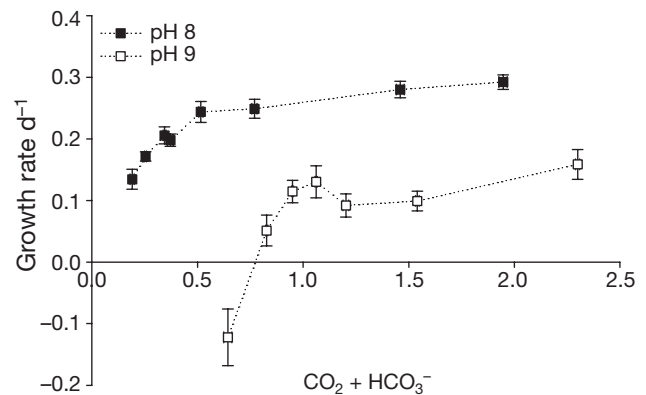


Fig. 3. *Ceratium furca*. Effects of photosynthetically available dissolved inorganic carbon on the growth rate of *C. furca*. Vertical error bars = SE (n = 12); horizontal error bars (SE) are covered by the data points

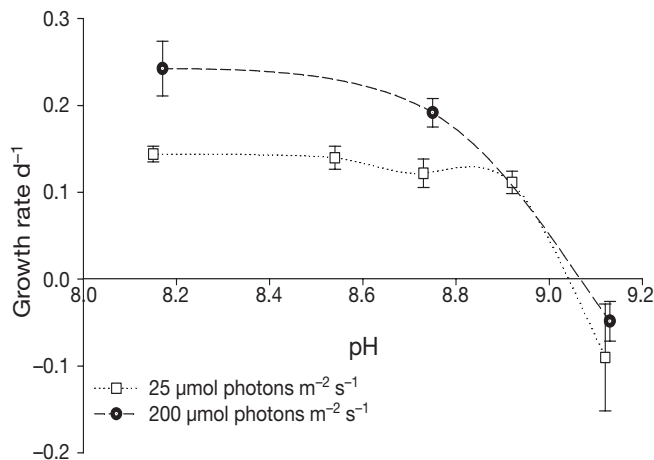


Fig. 4. *Ceratium furca*. Effects of light on the growth rate of *C. furca*. Error bars = SE (n = 9)

8.7–8.9 at both 25 and 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, and at both irradiances the growth rates approached zero around pH 9.05 to 9.10. Even though the data points at 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ are scarce, the observed change in light intensity did not alter the pH tolerance of *C. furca* and the growth rates for the 2 different light regimes did not differ significantly at pH 9.15 (Mann-Whitney *U*-test, >0.05).

DISCUSSION

Upper pH limits for *Ceratium* spp.

The upper pH limit for growth in the pH drift experiments was approximately 8.8 for all the *Ceratium* species tested. These pH limits for growth are significantly higher than the previously reported upper pH limits for growth of 8.3 and 8.4 for *C. tripos* and *C. furca*, respectively (Schmidt & Hansen 2001). The experimental conditions in Schmidt and Hansen's study are comparable to the present study, but the low growth rates of *C. tripos* and *C. furca* combined with frequent sampling and subsequent dilutions in their study could have prevented a true stationary growth phase and a maximum obtainable pH. In the present study, the ability to measure even very low growth rates has been increased due to longer periods of time between the samplings. Of course it is not possible to completely rule out clone variations, but the *C. tripos* strain used in both studies is the same.

In contrast, the upper pH limits for growth obtained in the constant pH experiment (Table 1) are comparable to the results obtained by Pedersen & Hansen (2003a) using natural plankton communities grown in natural seawater at different constant pH levels.

Two different methods for determining pH tolerance for growth

In the present study, 2 different methods (constant pH and drift pH) were used to test the pH limits for growth, and the results demonstrate that the 2 methods do not always produce comparable results. But why do the 2 methods give a different outcome in some cases?

An important difference between the 2 types of experiments is the cell density. The cell density is low throughout the constant pH experiment, due to low initial cell concentrations and the short duration of the experiment. For the pH drift experiments, on the other hand, the final cell concentrations were ~ 700 , 950 and 1300 cells ml^{-1} for *Ceratium tripos*, *C. furca* and *C. fusus*, respectively. The pH in high-density cultures is susceptible to daily variations, and an experiment with the dinoflagellate *Heterocapsa triquetra* reveals that pH in laboratory cultures can vary as much as 0.2 pH units during the day (Havskum & Hansen 2006). The pH was measured at noon in the present pH drift experiments, and the pH could thus have increased a further 0.1 to 0.15 pH units during the afternoon and evening. This daily pH variation would not have occurred in the constant pH experiments due to the lower cell densities. Thus, some of the observed differences between the pH drift and constant pH experiments might be explained by diurnal pH variations. This might explain the results for *C. furca*, but it also raises the question as to why *C. fusus* then do not show a similar response.

The high cell concentrations could theoretically result in depletion of nutrients, vitamins, light, or inorganic carbon. Regarding nutrients, both macro- and micronutrients were added in excess and at the end of the pH drift experiment with *Ceratium furca*, inorganic N and P were still available in excess (Table 3). Most dinoflagellates are in need of vitamins B₁₂, thiamine, or biotin (Gaines & Elbrächter 1987), but the different treatments of the vitamins did not result in different growth rates, and therefore it can be concluded that vitamins were not the limiting factor in our pH drift experiment.

The experimental irradiance (100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) was based on unpublished data by P. J. Hansen & T. G. Nielsen on the growth rate of *Ceratium furca*, *C. tripos* and *C. fusus* under different light regimes to ensure saturating light conditions with regard to growth rates. Since the growth rate of *C. furca* in the present experiments was the same at 100 and 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, irradiance was not a limiting factor in the pH drift experiments. Moreover, the experiments carried out at 2 different irradiances resulted in the same pH limit for growth for *C. furca*. Thus, light limitation cannot have been the reason for the lower pH tolerances observed in the pH drift experiment.

Finally, the DIC concentration was measured during the pH drift experiment with *Ceratium furca*, and the final DIC concentration was compared to the DIC limits for growth at pH 8 and 9 (Table 4, Fig. 3). The latter experiment demonstrates that the final DIC concentration in the pH drift experiment for *C. furca* does, in fact, approach the DIC limit at pH 9 and hence a co-limitation of pH and DIC in the pH drift experiment is a possible explanation for the different results in the 2 types of experiments. Under the assumption that *C. furca*, *C. tripos* and *C. fusus*, like *C. lineatum*, are able to use HCO_3^- as a carbon source, a combined limitation of high pH and low DIC applies for both *C. furca* and *C. tripos* at the end of the pH drift experiment. This is in contrast to *C. fusus*, which in our experiments is limited exclusively by pH, since the pH drift and the constant pH experiment yielded similar results.

Effects of irradiance on the upper pH limits for growth

The combined effects of light, DIC and pH on growth rates have, to our knowledge, not been studied on *Ceratium* spp. or any other dinoflagellate, but Bartual & Gálvez (2002) have examined the effects of light, DIC and pH on the diatom *Phaeodactylum tricornutum*. They found that the growth rate was significantly reduced at high pH and low DIC at subsaturating light intensities. Similarly, the growth rate remained constant at both high pH and low DIC at high light intensities. The authors suggest that this can be explained by an energy-demanding uptake of HCO_3^- and that a competition between N metabolism and C metabolism is observed under subsaturating light. These results are in contrast with the present results in which different light regimes did not result in different pH limits for growth. For *C. furca*, it does not seem that growth at high pH is more energy demanding than growth at pH 8. Whether this is due to fixed HCO_3^- and CO_2 proportions of the cell's total carbon uptake at changing pH levels or whether the HCO_3^- uptake does not require more energy than CO_2 uptake is speculative. But it can be concluded that the carbon uptake at high pH is not limited by lack of energy.

A possible effect of high pH could instead be less efficient proton pumps which, in turn, could result in lack of regulation of the internal pH and lack of control of pH-sensitive enzymatic processes or lead to cell leakage (Raven 1980, Taraldsvik & Mykkestad 2000).

Natural pH and natural DIC concentrations

Are our conclusions still relevant when extrapolating from laboratory to nature? The pH of marine waters has historically not received much attention, but recent

research demonstrates that the pH of fjords and lagoons can rise to as high as 9.5 during summer (Macedo et al. 2001, Hansen 2002). But to what extent are these pH increases just single incidents during extraordinary blooms? It is important to distinguish between oceanic and coastal areas, since recent data prove that high pH (≥ 9) is a common phenomenon in coastal waters during the summer months: In Mariager Fjord, Denmark, the median pH is close to 9 during the summer (May to August) every year (Hansen 2002). In the German Bight, pH values of 8.7 were measured in May and June 1986 (Kempe & Pegler 1991), and, in the Southern Bight of the North Sea, the pH was reported to rise from 7.9 to 8.7 during an intense *Phaeocystis* bloom (Brussaard et al. 1996). In contrast, the open oceans are in general oligotrophic and thus significant pH fluctuations are not expected to occur (Clark & Flynn 2000). Besides the seasonal pH variations in coastal waters, diurnal variations are also reported to take place. Diurnal pH variations are not restricted to dense laboratory cultures; in closed or small bodies of water the pH can vary from 8.2 to 8.9 during the day due to photosynthesis and respiration (Millero 1996). In conclusion, *Ceratium* spp. and other phytoplankton species with pH limits for growth ≤ 9 will experience pH limitations in coastal waters, and pH is thus an important factor in the regulation of coastal phytoplankton species composition. In the waters surrounding Denmark, *Ceratium* spp. are often the dominant species in the late summer phytoplankton. However, it is missing in many eutrophic Danish fjords and bays, even though chemical and physical parameters suggest that they ought to be present (Jensen 1994). High pH is thus a likely contributing factor for the distribution of *Ceratium*, and should as such be taken into consideration.

Naturally, for the pH to increase, the DIC must be depleted faster than CO_2 equilibration with the atmosphere. Therefore, the pH drift experiment was designed to mimic the coexisting pH rise and CO_2 depletion in nature during phytoplankton blooms. The total DIC concentrations in marine waters correlate in general with salinity; the higher the salinity, the higher the DIC (Thomas & Schneider 1999). The typical DIC concentration in oceanic waters is approximately 2.2 mM. In brackish waters like, for example, the Baltic Sea (salinity ~4 to 31 psu), the DIC concentration varies from approximately 1.2 to 2.2 mM (Thomas & Schneider 1999). Thus, the initial DIC concentrations of 1.6 to 1.7 mM in the pH drift experiment are representative of many estuarine areas. The above-mentioned natural DIC concentrations represent situations in which the CO_2 is close to air-sea equilibrium, and thus much lower DIC concentrations can occur during blooms (Codispoti et al. 1982), especially in nutrient-enriched

sheltered waters, such as bays, lagoons and fjords. However, additional data on DIC concentrations and pH in coastal waters during blooms are much needed.

Based on these inorganic carbon concentrations found in nature and the DIC requirements of *Ceratium furca*, it is unlikely that *C. furca* will experience DIC limitation in nature at pH 8.0 to 8.2. In contrast, at pH 9.0, the total DIC limit for *C. furca* is as high as 1.2 mM and a combined pH/DIC limitation at high pH is therefore possible in nature.

Thus, it seems likely that small *Ceratium* species in nature will often experience growth limitations by high pH and that larger *Ceratium* species most likely will experience a combined pH/DIC limitation at high pH.

Upper pH limits for dinoflagellates — Does size matter?

For *Ceratium furca* and *C. tripos* it is worth noticing that not only are the final pH in the pH drift experiments different from the maximum tolerable pH found in the constant pH experiments, but the growth rates measured during the pH drift experiment are affected at an earlier stage; this especially applies to *C. tripos* (Fig. 1C). These results indicate a size dependency in the ability to maintain a constant growth rate during drifting pH and concomitant DIC depletion. This apparent DIC limitation at high pH could be due to different abilities in inorganic carbon uptake or in the ability to minimize carbon leakage.

A possible size dependency of pH tolerances could be explained by the difference in the surface:volume ratio, in the same way that smaller cells have a more efficient nutrient uptake due to their larger surface:volume ratio (Raven 2003). Data on the smaller *Ceratium lineatum* (approximately 8000 μm^3) support this size-dependency hypothesis, since pH drift and pH constant experiments give the same pH tolerances for growth (Schmidt & Hansen 2001, Hansen 2002). Furthermore, pH drift experiments with *C. lineatum* using varying initial DIC concentrations resulted in similar pH values in the stationary phase and hence indicate that pH was the main limiting factor (Hansen et al. 2007).

Growth limitation by high pH is thus a relevant issue for *Ceratium* spp., but does this apply to dinoflagellates in general? For dinoflagellates as a group the currently published pH limits for growth vary from 8.7 to 10.1 (Table 5) and thus cover almost the whole range of pH tolerances at present known to apply to phytoplankton (see Hansen 2002). The pH limits for growth of *C. tripos*, *C. furca* and *C. fusus* are accordingly among the lowest measured dinoflagellate pH tolerances for growth. Nevertheless, pH tolerances for growth ≤ 9 are not uncommon for dinoflagellates.

For diatoms, it seems that small species have a higher pH tolerance than larger species, probably due to a better regulation of their internal pH as they have a larger surface:volume ratio (Lundholm et al. 2004). For dinoflagellates, the results indicate that a similar semi-logarithmic relationship exists between pH tolerance and size (Fig. 5). The species that are able to tol-

Table 5. List of pH tolerances for growth for dinoflagellates based on the literature. Data are exclusively from studies in which growth was not considered as being nutrient limited. Asterisks refer to heterotrophic dinoflagellates. 1: Schmidt & Hansen (2001); 2: Humphrey (1975); 3: Lee et al. (2003); 4: Hansen (2002); 5: Lundholm et al. (2005); 6: Møgelhøj et al. (2006); 7: Barker (1935); 8: Pedersen & Hansen (2003b)

Species	pH limit	Type of experiment	Source	Cell vol. (μm^3)
<i>Alexandrium ostenfeldii</i>	8.9	pH drift	1	17 000
<i>Alexandrium tamarense</i>	8.9	pH drift	1	17 160
<i>Amphidinium carterae</i>	>10.1	Non-constant pH	2	650 ^a
<i>Amphidinium</i> sp.	>9.5	Constant pH	3	350 ^a
<i>Ceratium lineatum</i>	8.8, 8.8	pH drift, constant pH	1, 4	8100
<i>Gymnodinium mikimotoi</i>	9.0	pH drift	1	5200
<i>Gymnodinium splendens</i>	>8.9	Non-constant pH	2	50 000 ^a
<i>Gyrodinium dominans</i> *	9.35	Constant pH	8	10 400 ^b
<i>Heterocapsa triquetra</i>	9.4, 9.4, 9.5, 9.8	2 × pH drift, constant pH, pH drift	1, 5, 4, 6	2050
<i>Oxyrrhis marina</i> *	>9.9	Constant pH	8	4600 ^c
<i>Peridinium</i> sp.	~9.5	Non-constant pH	7	3000 ^a
<i>Prorocentrum gracile</i>	~9.5	Non-constant pH	7	1900 ^a
<i>Prorocentrum micans</i>	9.9, 9.9, ~9.5	2 × pH drift, non-constant pH	1, 6, 7	10 000
<i>Prorocentrum minimum</i>	9.6, 9.6, 9.9	Constant pH, 2 × pH drift	4, 1, 6	1040

^aCell volume was calculated according to Hillebrand et al. (1999); cell dimensions were taken from the original papers or, if not listed there, from Steidinger & Tangen (1997)
^bCell volume from Hansen (1992)
^cCell volume from P. J. Hansen (unpubl. data)

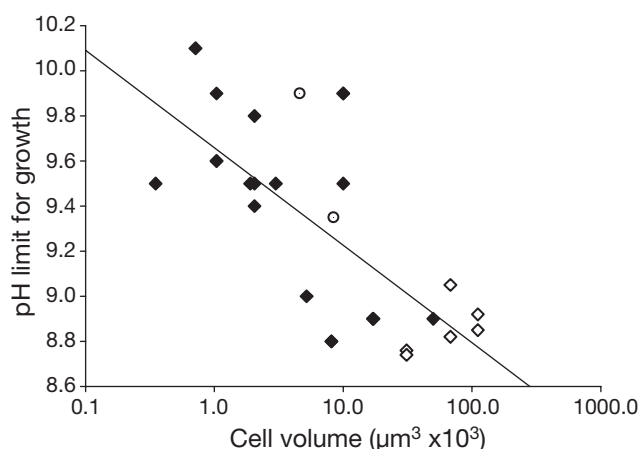


Fig. 5. Relationship between cell volume and pH limits for growth for dinoflagellates. Open data points represent results from present study; filled data points refer to Table 5 (dotted circles refer to heterotrophic dinoflagellates). Both pH drift and constant pH experiments have been included. Linear regression for all data points, $r^2 = 0.51$

erate high pH are all small. To confirm this trend more studies on the upper pH tolerance of large dinoflagellates are needed, however.

All the dinoflagellates in Table 5 are neritic and/or oceanic species, but since none of the species are exclusively neritic in their distribution, no conclusions can be made on the pH tolerance of neritic versus oceanic species. Even though the upper pH limits for growth for dinoflagellates do not differ from pH limits for phytoplankton in general, a mesocosm experiment indicates that dinoflagellates tend to dominate when pH is high (Hinga 1992). It has been suggested that mixotrophic dinoflagellates may have an advantage during high pH, due to phagotrophic carbon acquisition, since uptake of organic carbon has little impact on the charge balance of the cell (Hinga 1992). The pH limits for growth for dinoflagellates are measured for photoautotrophic carbon uptake and do not take the possible advantage of mixotrophy at high pH into consideration. Further studies are required to test this hypothesis.

CONCLUSIONS

The pH drift experiment was designed to mimic the coexisting pH rise and inorganic carbon depletion in nature during phytoplankton blooms, and thus the effects of high pH and low photosynthetically available DIC cannot be separated in these experiments. Our results illustrate that pH drift experiments in some cases give lower pH limits for growth than constant pH experiments; a likely explanation for the lower pH limit for growth is the combined pH/DIC limitation at high pH in the present pH drift experiments. Under

natural conditions, high pH can be a limiting factor for the growth of *Ceratium* and many other dinoflagellate species with pH tolerances below or around 9, especially in coastal waters during summer. Low DIC, on the other hand, appears only to limit very large species such as *C. furca* and *C. tripos* at high pH.

Acknowledgements. We thank C. Esplund and E. Granéli, University of Kalmar, for analysis of nutrients. This research was funded by the Danish Natural Research Council Project No. 21-03-0449 for P.J.H.

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Editorial responsibility: Howard Browman (Associate Editor-in-Chief), Storebø, Norway

Submitted: December 19, 2006; Accepted: July 7, 2007
Proofs received from author(s): November 6, 2007