Effects of high pH on the growth and survival of six marine heterotrophic protists

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ABSTRACT: The pH tolerance of the ciliates *Balanion comatum*, *Favella ehrenbergii*, *Rimostrombidium caudatum* and *R. veniliae* and the dinoflagellates *Gyrodinium dominans* and *Oxyrrhis marina* was studied using laboratory cultures at specific pH levels and prey concentrations. The results of these experiments divided the tested species into 2 groups: pH-tolerant species and pH-non-tolerant species. The tolerant group consisted of *B. comatum*, which experienced a reduction in growth when pH exceeded 9.5, and *O. marina*, which maintained its maximum growth within the pH limit of the experiment (pH 9.9). The pH-non-tolerant group consisted of 3 ciliates and 1 dinoflagellate. The most pH-sensitive species were *F. ehrenbergii*, *R. caudatum* and *R. veniliae.* Their growth rate became affected at pH 8.8 to 8.9 and they did not grow when pH exceeded 9.0. The more tolerant species of this group, *G. dominans*, experienced a reduction in its growth when pH exceeded 9.2, and negative growth when pH exceeded 9.4. In a different set of experiments with the same species, the algae were allowed to grow and thereby raise the pH. In these experiments, the pH-sensitive species *F. ehrenbergii*, *R. caudatum* and *R. veniliae* all died within 24 h when pH exceeded 9.3, whereas some cells of the more tolerant dinoflagellate *G. dominans* were able to survive at pH values around 10 for up to 5 d. Thus, heterotrophic protists differ in their pH limits for growth and in their survival response when exposed to pH exceeding their limits for growth. In nature, algal blooms may lead to elevated pH (>9). Our results suggest that such pH levels will kill many, but not all, heterotrophic protists. This may, at least temporarily, lead to a reduction in grazing control of such algal blooms, thereby further allowing their growth and persistence.

KEY WORDS: pH tolerance · Ciliates · Dinoflagellates · *Balanion comatum* · *Rimostrombidium caudatum* · *Rimostrombidium veniliae* · *Gyrodinium dominans* · *Favella ehrenbergii* · *Oxyrrhis marina*

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INTRODUCTION

Phytoplankton blooms in marine coastal eutrophic areas like bays, lagoons and fjords, are frequently reported during the summer period (e.g. Lindholm & Nummelin 1999, Macedo et al. 2001). The duration of these blooms may vary from days to months, depending on the weather conditions. Some of these often mono-specific blooms, caused by either dinoflagellates or diatoms, have been reported to elevate the pH of the surrounding water, the highest pH values reported being around pH 10 (Macedo et al. 2001, Hansen 2002). During such periods of strongly elevated pH, the biomass of grazers seems to be very low.

In the past decade, the ecological role of heterotrophic planktonic protists has been well studied. Numerous experiments dealing with the growth responses of these protists to different environmental parameters such as temperature, light and nutrients, and also biological parameters like prey quality and quantity, have been carried out (Aelion & Chisholm 1985, Nakamura et al. 1992, Jakobsen & Hansen 1997). However, we found only 1 study on the effects of high pH on the growth of heterotrophic dinoflagellates (Droop 1959a).

In closed culture vessels, pH values are found to increase significantly in a few days, even at relatively low phytoplankton concentrations (Schmidt & Hansen 2001). In literature regarding laboratory culturing of heterotrophic protists, it is often recommended that the

cells are grown on a relatively low prey concentration (Gifford 1985, 1993, Lessard 1993), which might be to avoid a potential increase in the pH value. An experiment with natural seawater incubated at different pH values related the reduction in species to high pH, but the specific cause of this reduction was not clearly determined (Pedersen & Hansen 2003, this issue).

The aim of this paper was to determine a possible effect of high pH on the growth rate and survival of heterotrophic protists under controlled conditions in the laboratory.

MATERIALS AND METHODS

Isolation and culture maintenance. Four ciliates, *Balanion comatum*, *Favella ehrenbergii*, *Rimostrombidium caudatum* and *R. veniliae*, and 2 heterotrophic dinoflagellates, *Gyrodinium dominans* and *Oxyrrhis marina*, were used in the present study. The phototrophic dinoflagellate *Heterocapsa triquetra* or the cryptophyte *Rhodomonas salina* was used as prey (Table 1). These algae were provided by the culture collection of the Marine Biological Laboratory, Helsingør, Denmark and chosen because of their known tolerance to high pH (Schmidt & Hansen 2001).

The algae were grown as non-axenic cultures in 20 psu autoclaved f/2 medium (Guillard 1972). *Rhodomonas salina* were grown in an aerated 500 ml Pyrex® glass flask at an irradiance of 70 µmol photons m^{-2} s^{-1} at 15 ± 1°C on a 16:8 h light:dark cycle, whereas *Heterocapsa triquetra* was grown in 270 ml transparent culture bottles on a plankton wheel (1 rpm) at 15 ± 1°C on a 16:8 h light:dark cycle at 50 µmol photons m^{-2} s^{-1}. Algal cultures were re-inoculated weekly in order to maintain exponentially growing cultures (Table 1).

The predators were grown in 20 psu, 0.20 µm filtered, autoclaved medium in 270 ml transparent culture bottles on a plankton wheel (1 rpm) at 15 ± 1°C on a 16:8 h light:dark cycle at 50 µmol photons m^{-2} s^{-1} (Table 1). All cultures were kept in exponential growth by re-inoculation of cells at weekly intervals. Irradi-

ance was measured using a LI-Cor®, LI-1000 radiation sensor equipped with a spherical probe.

Experimental conditions. The effect of high pH on the growth of ciliates and heterotrophic dinoflagellates was studied in 2 types of experiments. Prior to each experiment, all organisms were kept in exponential growth. All experiments were conducted in 270 ml transparent culture bottles filled with 20 psu either f/2 or f/20 medium, based on 0.20 µm filtered autoclaved seawater (Tables 1 & 2). The bottles were mounted on a plankton wheel (1 rpm) at 15 ± 1°C on a 16:8 h light:dark cycle at 50 µE m^{-2} s^{-1}. Sampling was conducted every 24 h, when samples of 2 to 3 ml for prey enumeration and 1 to 10 ml for predator enumeration were fixed in Lugol's iodine (final concentration 1%). All experiments were carried out in triplicates. Prey samples were counted in Sedgewick-Rafter® chambers at 100× magnification, whereas predators were counted in multidishes (Nunclon®) using an inverted microscope at 40 to 100× magnification.

pH was measured using a Sentron® 2001 pH meter with a Red Line electrode and a 2 point calibration. After sampling, fresh medium was added and the bottles were remounted on the plankton wheel.

Growth rates were calculated as an increase in cell number, assuming exponential growth, using the following equation: µ (d^{-1}) = $\ln(N_1 - N_0)/t$, where N_0 and N_1 are the number of cells at t_1 and t_0, respectively, and t is the time between sampling in days.

Constant pH. One prey species was mixed with 1 predator species, and the effect of high pH in the range of 8 to 9.9 was studied at distinct levels (Table 2). The pH limit was set by the pH tolerance of the prey species, *Heterocapsa triquetra* and *Rhodomonas salina*, which enter stationary growth phase at pH 9.43 and 9.93, respectively (Schmidt & Hansen 2001). The prey concentration and pH were kept constant during the experiment.

The pH was elevated with 0.1 and 1 M NaOH and adjusted within pH 0.01, with 0.1 M NaOH and HCl to the given pH. All bottles were preincubated under the standard experimental conditions of pH and prey con-

Table 1. Protists used in the experiments, their date of isolation, place of isolation, prey and growth medium

Predator species	Date of isolation	Place of isolation	Prey species	Growth medium
Ciliates				
Balanion comatum (Prostomatida)	Feb 2001	Helsingør	*Rhodomonas salina*	f/20
Favella ehrenbergii (Tintinnida)	Jun 2001	Limfjorden	*Heterocapsa triquetra*	f/2
Rimostrombidium caudatum (Strobilidae)	Feb 2001	Helsingør	*Rhodomonas salina*	f/20
Rimostrombidium veniliae (Strobilidae)	Jun 2001	Limfjorden	*Heterocapsa triquetra*	f/20
Dinoflagellates				
Gyrodinum dominans	May 1998	Helsingør	*Rhodomonas salina*	f/2
Oxyrrhis marina	1996	Helsingør	*Rhodomonas salina*	f/2

Table 2. Initial predator and prey concentrations in the constant pH and drifting pH experiments

| Species | | Constant pH | | Drifting pH | |
Predator	Prey	Predator (cells ml^{-1})	Prey (10^3 cells ml^{-1})	Predator (cells ml^{-1})	Prey (10^3 cells ml^{-1})
Rimostrombidium veniliae	*Heterocapsa triquetra*	2–7	2	2	2
Rimostrombidium caudatum	*Rhodomonas salina*	7	20	7	10
Favella ehrenbergii	*Heterocapsa triquetra*	3.5–5	2	6	1.2
Balanion comatum	*Rhodomonas salina*	5	50	5	50
Gyrodinum dominans	*Rhodomonas salina*	100–125	20	17	20
Oxyrrhis marina	*Rhodomonas salina*	100	25	100	25

centration, for at least 24 h and maximum 48 h before sampling. To avoid a chock effect at the high pH values (≥8.9), both predator and prey populations were grown at pH levels between 8.5 and 8.8 for 2 d, prior to the preincubation period. During sampling, the pH was measured and adjusted to the given pH, as a slight rise in pH usually occurred due to the photosynthetic activity of the algae. The pH values in these experiments are therefore the average values between the given pH and the pH values measured during sampling. At each sampling occasion, the prey cells were counted immediately and the bottles were diluted with 0.2 µm filtered, pH-adjusted media to keep prey concentrations relatively stable. The dilution rates were between 10 and 50%, depending on the growth of the prey. The experiments lasted for 3 to 4 d or until the predators had died.

Drifting pH. In the second type of experiments, 1 predator was mixed with a high initial prey concentration and allowed to grow. In this way, the predators were exposed to a pH increase mediated by the photosynthetic activity of the algae (Table 2). Sampling and pH measurements were conducted every 24 h and the experiments lasted either until the predators had died or depleted their prey.

RESULTS

The growth rates of all the tested species in both the constant pH experiment and the drifting pH experiment were not significantly influenced by pH below 8.8 (Figs. 1, 2 & 3). When exposed to higher pH, species could be divided into 2 groups: (1) non-tolerant species, which died out; and (2) tolerant species, which maintained positive growth within the limits of the experiments. Four of the 6 tested species belonged to the non-tolerant group; these species were the ciliates *Favella ehrenbergii*, *Rimostrombidium caudatum* and *R. veniliae* and the dinoflagellate *Gyrodinium dominans* (Figs. 1A–D & 2). The species

of the non-tolerant group all reached a pH level at which they could not maintain positive growth. Only the 2 tolerant species, the dinoflagellate *Oxyrrhis marina* and the ciliate *Balanion comatum*, were able to maintain growth throughout both types of experiments (Figs. 1E,F & 3).

The non-tolerant group of species could be divided into 2 subgroups based on the sensitivity to high pH. The first subgroup comprised the ciliate species *Rimostrombidium caudatum*, *R. veniliae* and *Favella ehrenbergii*. These species experienced a significant reduction (>20%) in their growth rate between pH 8.8 and 9, compared to the growth rate found at pH 8 (Table 3). A further increase by only 0.1 to 0.2 pH units resulted in negative growth, but not an immediate extinction of the population (Fig. 1A,B). However, when the pH level exceeded 9.2, all cells in the populations of both *R. caudatum* and *R. veniliae* died within 24 h; the same happened for *F. ehrenbergii*, but at pH 9.3 (results not shown). In the drifting pH experiment, *R. caudatum* and *F. ehrenbergii* grew until pH reached 9.0 and 9.3, respectively. During the following 24 h, the pH increased further and the ciliate populations were killed. *Rimostrombidium veniliae* grew until Day 3, at which time the pH was 8.9. During the following 24 h, pH increased further to 9.2 and the cell number decreased. After an additional 24 h and a further increase in pH, the entire population had died (Fig. 2A–C).

The second subgroup comprised the dinoflagellate *Gyrodinium dominans*, which showed a more gradual response to high pH (Fig. 1D). This species experienced a >20% reduction in its maximum growth rate at pH 9.2 (Table 3). A further increase in pH resulted in lower growth rates and when pH exceeded 9.4, the growth became negative. In the drifting pH experiment, *G. dominans* grew until pH 9.2 was reached. As the pH increased further, a reduction in cell number occurred, but in contrast to the members of the other subgroup, *G. dominans* survived for as long as 5 d, even though pH increased
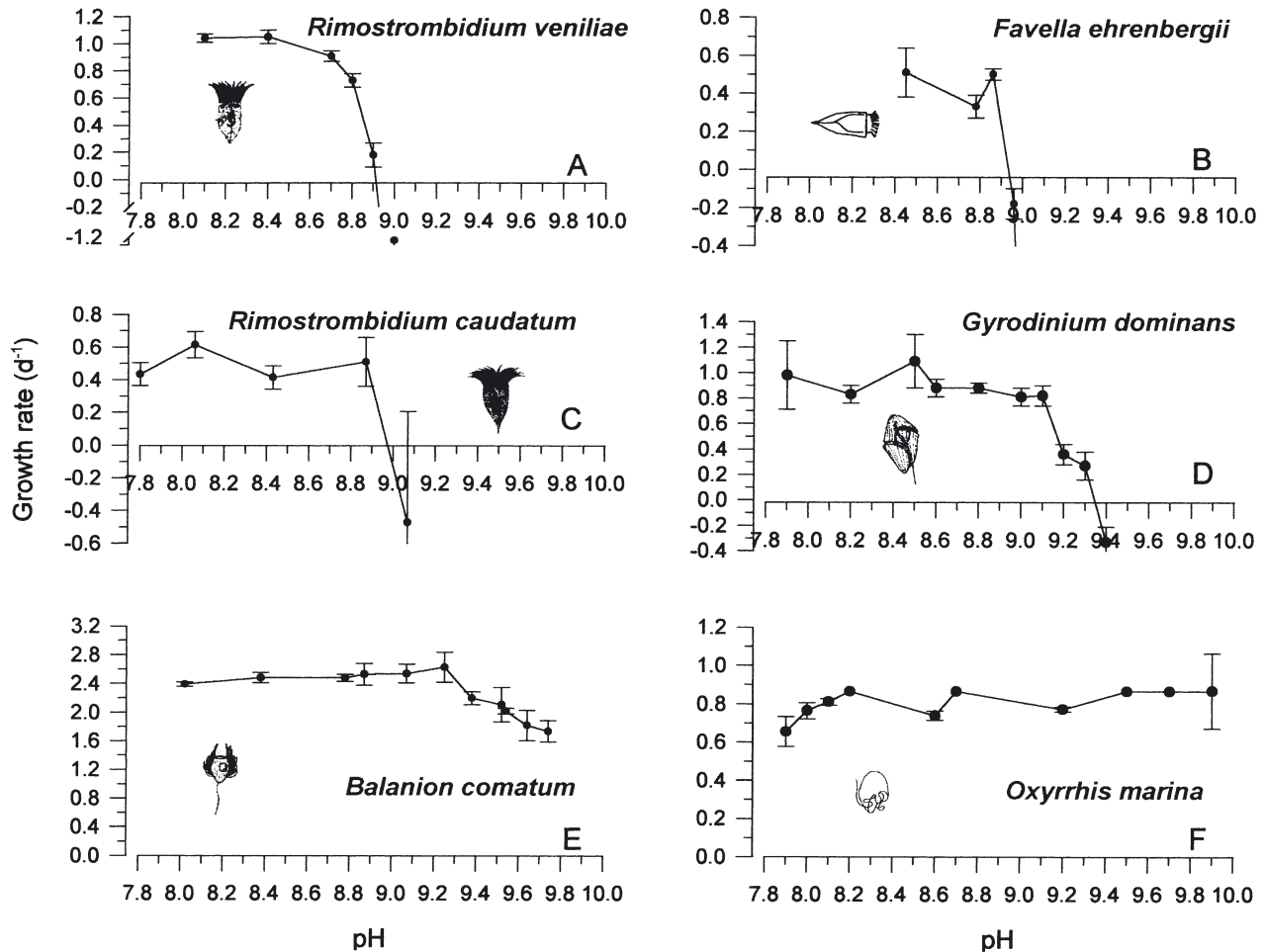
Fig. 1. Growth rate as a function of pH for 6 heterotrophic planktonic protist species (means ± SE). (A) *Rimostrombidium veniliae*, (B) *Favella ehrenbergii*, (C) *Rimostrombidium caudatum*, (D) *Gyrodinium dominans*, (E) *Balanion comatum*, (F) *Oxyrrhis marina*

further maintaining a value between pH 9.7 and 10.1 (Fig. 2D).

The tolerant group of species included the ciliate *Balanion comatum* and the dinoflagellate *Oxyrrhis marina*, which maintained a positive growth rate throughout both types of experiments. In the constant pH experiments, *B. comatum* grew at its maximum growth rate of 2.4 (d^{-1}) until pH 9.5, when a 20% reduction was observed (Fig. 1E and Table 3). Above this pH value, the growth rate decreased to reach 1.7 (d^{-1}) at pH 9.8. *O. marina* was found to be even more pH-tolerant (Fig. 1F); it grew at its maximum growth rate of 0.8 (d^{-1}) within the pH range studied. In the drifting pH experiment, *B. comatum* and *O. marina* grew throughout the experiment even at the pH values around 10, which made them able to eliminate *Rhodomonas salina* at the end of the experiments (Fig. 3).

DISCUSSION

Effect of high pH on heterotrophic protists

The literature on the effects of high pH on planktonic heterotrophic protists is very limited, with the exception of a study on the heterotrophic dinoflagellate *Oxyrrhis marina* and another on the effect of high pH on a natural planktonic community (Droop 1959a, Pedersen & Hansen 2003, this issue). While it was shown that *O. marina* was unaffected by even very high pH, the study on natural assemblages did suggest that high pH indeed affects heterotrophic protists in the natural plankton community.

In the present paper, we used 2 different methods to study the effect of pH on heterotrophic protists and they gave, in general, similar results. In some cases, however, the pH limits for growth were very difficult to
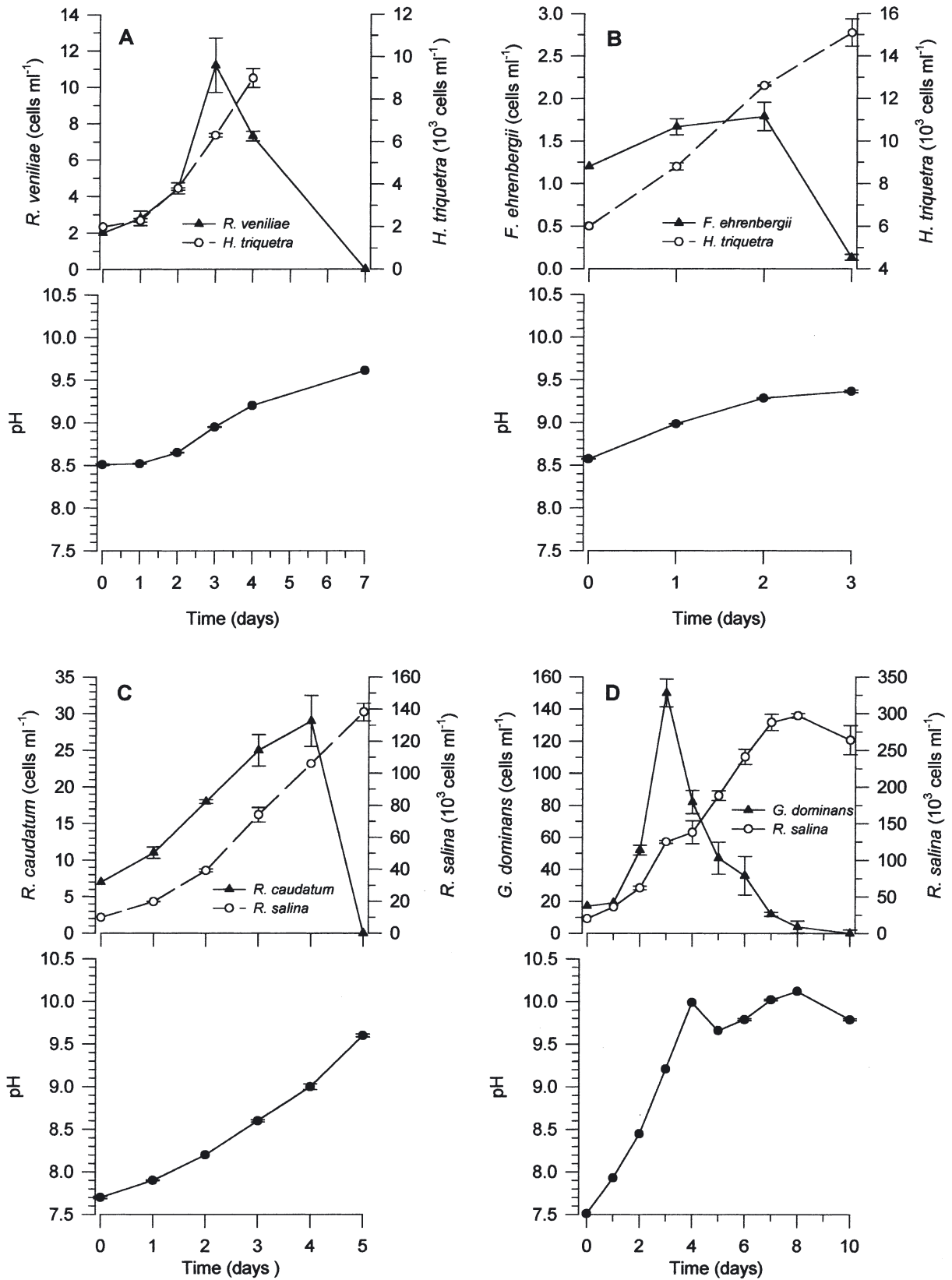
Fig. 2. Cell concentration (cells ml^{-1}) of the tested heterotrophic species in response to increasing prey concentration and pH (means ± SE). (A) *Rimostrombidium veniliae*, (B) *Favella ehrenbergii*, (C) *Rimostrombidium caudatum*, (D) *Gyrodinium dominans*
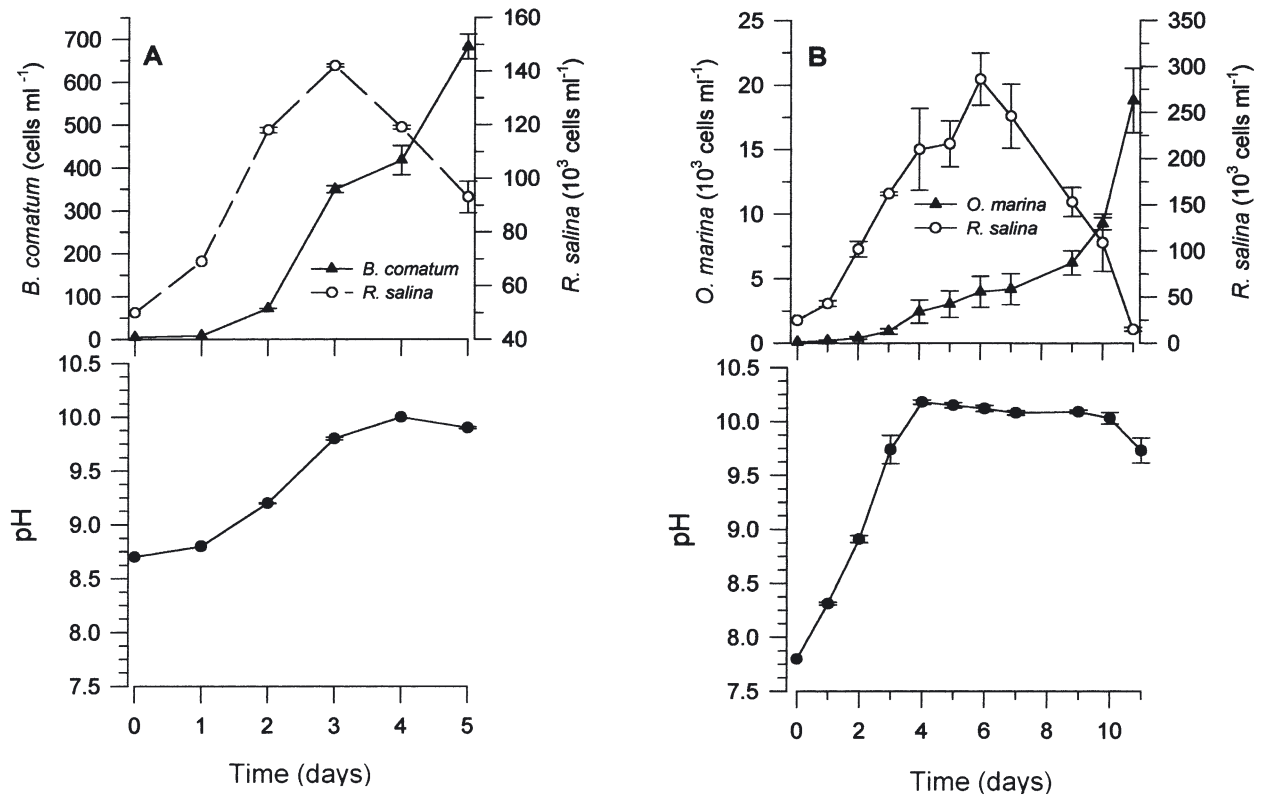
Fig. 3. Cell concentration (cells ml^{-1}) of the tested heterotrophic species in response to increasing prey concentration and pH (means ± SE). (A) *Balanion comatum*, (B) *Oxyrrhis marina*

identify using the drifting pH method, because pH could change significantly from day to day (by up to 0.8 pH units). Thus, the results from the constant pH experiments are best suited in regard to the growth limits of the tested species, because they represent close to steady-state growth rates at specific pH levels.

The most sensitive species were affected when the pH value exceeded 9.0; at this value, 3 out of the 6 tested species were not able to maintain positive growth (Table 3). These sensitive species were found to be the ciliates *Favella ehrenbergii, Rimostrombid-*

ium caudatum and *R. veniliae.* A less sensitive species, the dinoflagellate *Gyrodinium dominans*, was not able to grow when the pH exceeded 9.4 and it did not die quickly within the pH limits at which it was tested (pH 10.1). However, nothing can be concluded on the tendency of ciliates being more sensitive to elevated pH than heterotrophic dinoflagellates, because the number of tested species, especially the number of heterotrophic dinoflagellates, is low.

The pH-tolerant species in this experiment were the ciliate *Balanion comatum*, in which the growth rate

Table 3. The effect of pH on 6 heterotrophic protists. Maximum growth rate, the pH level at which a 20% reduction in growth was observed, the pH level where no growth was observed and the pH level causing quick death

Species	Max. growth rate (d^{-1})	Reduced-growth pH	No-growth pH	Quick-death pH
Ciliates				
Balanion comatum	2.4	9.5	Not found	Not found
Favella ehrenbergii	0.5	8.9	9.0	9.3
Rimostrombidium caudatum	0.5	8.9	9.0	9.2
Rimostrombidium veniliae	1.0	8.8	8.9	9.2
Dinoflagellates				
Gyrodinium dominans	0.9	9.2	9.4	Not found
Oxyrrhis marina	0.8	No reduction	Not found	Not found

was only slightly affected at pH above 9.5, and the dinoflagellate *Oxyrrhis marina*, which was not affected at all even when exposed to pH 10.2 (Figs. 1 & 3). Thus, our data on *O. marina* is in accordance with Droop (1959a), who also did not find any pH effects on its growth rate even at very high pH.

The obvious indirect effects of high pH encountered in nature, like starvation due to food limitation and malnourishment due to lack of suitable prey, were eliminated in this study. The prey items that we used are both known to be of a good food quality for the tested species, and they are both tolerant to high pH (Schmidt & Hansen 2001). The effect of high pH found on the sensitive species is therefore believed to be a direct effect. However, since no studies have been performed on cellular effects of high pH on heterotrophic protists, references are limited to the sparsely studied effects on phototrophic protists. Here, pH changes in the surrounding environment are proposed to affect the membrane transport processes and metabolic functions involved in cellular pH regulation, thereby causing changes in the biochemical composition, the rate of the metabolic processes and the extracellular production (Raven 1980, Nimer et al. 1994, Taraldsvik & Myklestad 2000). As these effects are equilibrium-controlled ion fluctuations, it would be harder to maintain a stable internal pH with increasing external pH. If these effects could be directly related to heterotrophic protists, this could explain the small intermediate pH range found between reduced and negative growth. Apart from direct effects of pH on the cells, elevated pH will influence the water chemistry. For example, elevated pH will reduce the availability of calcium ions in the water and possibly change the chelating activity of metals, thereby potentially making these metals toxic to the cells (e.g. Peterson et al. 1984). However, we have not been able to find any studies dealing with the possible metal toxicity to marine protists in the pH range studied here.

The reduction in growth found for *Balanion comatum* when pH exceeds 9.5 is believed to be due to either the direct cellular effects described above or a reduction in the food quality. Increasing pH is found to cause a decrease in the cellular content of C and N and also reduce the number of different amino acids found in the cell (Taraldsvik & Myklestad 2000). This might cause a reduced nutritious value of the algae cells; however, it is not possible to clearly determine which of these effects caused the decreasing growth rate. *Oxyrrhis marina* would probably not be affected by a potential reduction in the food quality, because it is an omnivore species and is able to consume clusters of bacteria (Lessard 1993).

The tolerance to high pH found among a few species is probably an adaptation to the extreme environment

in which these species are found to thrive. *Oxyrrhis marina* is in nature often found in tidal pools and nutrient-rich areas, and in the laboratory, it is a well-known pollutant of intensive phytoplankton cultures (Droop 1959b, Goldman et al. 1989, Fenchel et al. 1995), whereas *Balanion comatum* is found in both marine and freshwater areas and has been associated with estuarine dinoflagellate blooms (Stoecker et al. 1983, Jakobsen & Montagnes 1999).

This experiment has demonstrated that 3 out of 6 of the tested heterotrophic species experienced a reduction in their growth rate at pH 8.9 and showed negative growth rates at pH >9.0. Experiments conducted with phototrophic protists have shown a similar response. Here, half of the tested phototrophic species experienced a reduction in their growth at pH 9.0 and entered a stationary growth phase at pH >9.2 (Hansen 2002). The almost identical pH limits for growth found for both heterotrophic and phototrophic organisms might indicate the same type of cellular effects. This suggests that carbon limitation is not necessarily the inhibitor of growth found among phototrophs at high pH. It could equally well be that high pH causes direct cellular effects, like metabolic changes and alterations in the membrane confirmation as proposed by Taraldsvik & Myklestad (2000). This of course assumes that the affected phototrophs are able to utilise bicarbonate.

Effects of high pH on the interaction between phototrophic and heterotrophic protists

The effects of elevated pH in marine coastal waters will depend on both the duration of the pH elevation and the pH level itself. Here, 2 examples are considered.

An increase in pH to ~9.0 will probably cause a slight reduction in the grazing rates of the heterotrophic protists, as their growth rate will be reduced and some species will even die. However, the growth rate of many phototrophs will also be affected and a few species will enter the stationary growth phase or die (Schmidt & Hansen 2001, Pedersen & Hansen 2003). Overall this will result in a small reduction in the number of different species found among both heterotrophs and phototrophs. However, when normal pH is regained, e.g. by a mixing of the water column, the system will quickly regain its original status.

Longer periods of elevated pH (>9.0) seem to be self-perpetuating. The high pH will mediate species succession and cause a reduction in the species richness, due to reduced growth or death among about 50% of the heterotrophs and phototrophs (Hansen

2002, Pedersen & Hansen 2003). The reduction in growth, diversity and cell number of the heterotrophs will also result in a reduced and more species-specific grazing impact on the phototrophs. This decrease in the grazing will cause an increase in the net growth rate of the remaining pH-tolerant phototrophs. This in turn increases photosynthesis, which will result in a further increase in the pH. In extreme situations, this will lead to mono-specific phytoplankton blooms, with no efficient grazers present. In nature, examples of this are found in nutrient-rich shallow areas where *Prorocentrum minimum* often are found in mono-specific blooms during high pH, suggesting a lack of grazing (Macedo et al. 2001, Olesen 2001, Hansen 2002). However, the lack of grazing on *P. minimum* might not only be due to pH effects, because it is known to be of poor food quality for predators (Stoecker et al. 1981) and for being able to produce toxins when growth becomes limited (Trick et al. 1981, Grzebyk et al. 1997).

The inhibition of grazers due to high pH, as described above, is also found in freshwaters, where high pH is suggested to affect the structure and dynamics in the pelagial. Here, high pH is found to inhibit both fish and zooplankton and to increase the release of phosphate and ammonium from the sediment, which leads to a further increase in the phytoplankton growth, and thereby also leads to higher pH (O'Brien & deNoyelles 1972, Jeppesen et al. 1990, 1998, Hansen et al. 1991).

CONCLUSION

This study suggests that high pH (>9) will affect the growth of heterotrophic protists. The pH tolerance seems, however, to be species-specific, as pH-sensitive and pH-tolerant species can be found among both ciliates and heterotrophic dinoflagellates. Our knowledge of pH effects on heterotrophic protists is still quite limited, and the mechanisms of action remain unknown. Nevertheless, our data suggest that the sensitivity of heterotrophic protists to elevated pH may have important implications for the dynamics of algal blooms in coastal eutrophic areas which experience prolonged periods of elevated pH.

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