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Allelopathy in the prymnesiophyte *Chrysochromulina polylepis*: effect of cell concentration, growth phase and pH

Lisa Ejbye Schmidt, Per Juel Hansen*

Marine Biological Laboratory, University of Copenhagen, Strandpromenaden 5, 3000 Helsingør, Denmark

ABSTRACT: Seven species (8 isolates) of dinoflagellates were exposed to a dense *Chrysochromulina polylepis* suspension. All species (with 1 exception) lost their motility, indicating that *C. polylepis* produces toxins, allelochemicals which affect other algae. The role of cell concentration, growth phase, and pH in the ability of *C. polylepis* to immobilize the dinoflagellate *Heterocapsa triquetra* was studied in batch cultures. Loss of motility of *H. triquetra* cells could be detected at cell concentrations of *C. polylepis* above 3×10^4 cells ml^{-1}. Senescent cultures of *C. polylepis* did not immobilize *H. triquetra* cells. The ability of *C. polylepis* to immobilize *H. triquetra* cells was dependent on the pH of the growth medium. More non-motile *H. triquetra* cells were obtained in alkaline growth medium than in neutral or acidic media. Growth interactions between *C. polylepis* and 15 species (16 isolates) were also studied in mixed batch cultures using a nutrient replete growth medium. The algae selected for these experiments included diatoms, dinoflagellates, silicoflagellates, raphidophytes, euglenophytes, cryptophytes, and prasinophytes. *C. polylepis* had a harmful effect on all the tested algae, except the dinoflagellate *Prorocentrum minimum*. The harmful effect of *C. polylepis* was observed as an initial decrease in growth rate of the tested algae, followed by a decline in their population numbers. The harmful effect of *C. polylepis* on the tested algae could in a few cases be ascribed to the high pH in the culture medium. In most cases, however, the harmful effect was observed at a pH which did not affect the growth of these species when they were grown in monoculture. This indicates that toxins released by *C. polylepis* had a harmful effect on most of the tested algae in the mixed cultures.

KEY WORDS: Allelopathy · Toxicity · Growth phase · pH · *Chrysochromulina polylepis*

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INTRODUCTION

Species of *Chrysochromulina* (Prymnesiophyceae) are ubiquitous in the world's ocean, where they usually are found in concentrations of 1 to 100 cells ml^{-1} (e.g. Thomsen et al. 1994). A bloom of *Chrysochromulina polylepis* in Scandinavian waters in 1988, causing extensive fish kills, was the first report of a toxic bloom of a *Chrysochromulina* species. At the peak of the bloom, cell concentrations of *C. polylepis* reached 5 to 10×10^4 cells ml^{-1} (e.g. Edvardsen & Paasche 1998). Since then, a number of blooms of different *Chrysochromulina* species have been reported, some of which have caused fish mortality (reviewed by Edvardsen & Paasche 1998). The bloom of *C. polylepis* in 1988 was almost monospecific at its peak occurrence and the abundance of zooplankton (copepods, heterotrophic protists) was very low (Nielsen et al. 1990). Field and laboratory experiments demonstrated that *C. polylepis* inhibited the activity of planktonic bacteria, heterotrophic protists, and copepods (Nielsen et al. 1990, Tobisen 1991).

Experiments were not carried out on the interaction between *Chrysochromulina polylepis* and co-occurring planktonic algae during the bloom. However, *Cer-*

*Corresponding author. E-mail: pjhansen@zi.ku.dk

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atium species found at the peak of the bloom were obviously moribund, since their cytoplasm had shrunk and pigmentation was lost (Dahl et al. 1989, Nielsen et al. 1990). These observations led to the hypothesis that *C. polylepis* may produce toxic substances that affect other algae (Dahl et al. 1989, Nielsen et al 1990, Maestrini & Granéli 1991, Granéli et al. 1993). Myklestad et al. (1995) provided the first indications of allelopathy in *C. polylepis*. Addition of dense suspensions of *C. polylepis* to cultures of the diatom *Skeletonema costatum* resulted in growth inhibition of this alga. A similar effect was achieved using filtrates from dense *C. polylepis* cultures, indicating that toxic substances exuded from the algae to the medium caused the toxic effect. Some toxins, which interfere with cell membrane function, were extracted from *C. polylepis* cells during the bloom in 1988. The toxins were described as fatty acids (1-acyl-3-digalactosylglycerol and octadecapentenoic acid), but their chemical structures have not been fully elucidated (Yasumoto et al. 1990).

This paper investigates (1) the importance of cell concentration, growth phase and pH to the harmful effects of *Chrysochromulina polylepis* on the dinoflagellate *Heterocapsa triquetra* and (2) the possible harmful effect of *C. polylepis* on 16 isolates (15 species) of algae belonging to different phytoplankton taxa.

MATERIALS AND METHODS

Isolation and culture of algae. Information on isolation date, isolation place and clone designation of all the algae used in the present study is listed in Table 1. The Scandinavian Culture Collection of Algae and Protozoa, Botanical Institute, and the Marine Biological Laboratory, University of Copenhagen, Denmark, provided the algae. All algae were grown as non-axenic cultures in f/2 medium (Guillard 1983) based on autoclaved 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. The dimensions of all organisms were measured on live cells in the microscope (n = 20 to 30 cells) and cell volumes estimated using simple volumetric formulae (Table 1).

Table 1. List of algae used in the experiments, their clone designation, isolation place and time, and the cell volume. DK = Denmark, N = Norway, Au = Australia

Species	Clone	Isolation place and time	Cell volume (µm^3)
Bacillariophyceae			
Skeletonema costatum (Grev.) Cleve	K-0310	Kattegat, DK 1989	200
Cryptophyceae			
Rhodomonas marina (PA Dang.) Lemmerm.	K-0435	Kattegat, DK 1990	570
Dictyochophyceae			
Dictyocha speculum Ehrenb.	K-0301	Kattegat, DK 1989	1 800
Dinophyceae			
Alexandrium ostenfeldii (Paulsen) Balech et Tangen	K-0287,K-0324	Limfjorden, DK 1988	17 000
Alexandrium tamarense (M. Lebour) Balech	K-0055	Faroe Islands, DK	17 160
Ceratium furca (Ehrenberg) Claparede et Lachmann[a]		Øresund, DK 1995	35 000
Ceratium lineatum (Ehrenberg) Cleve[a]		Øresund, DK 1995	8 100
Ceratium tripos (OF Muller) Nitsch[a]		Øresund, DK 1995	110 000
Gymnodinium mikimotoi Adachi et Fukuyo	K-0260	Oslofjorden, N 1977	5 200
Heterocapsa triquetra Ehrenb.	K-0481	Øresund, DK 1988	2 050
Prorocentrum micans Ehrenb.	K-0335	Kattegat, DK 1989	10 000
Prorocentrum minimum (Pavill.) J. Schiller	K-0295	Kattegat, DK 1989	1 040
Prymnesiophyceae			
Chrysochromulina polylepis Manton & Parke	K-0259	Øresund, DK 1988	140
Chrysochromulina simplex Estep et al.	K-0272	Victoria, Au 1988	34
Euglenophyceae			
Eutreptiella gymnastica Throndsen	K-0333	Kattegat, DK 1988	850
Prasinophyceae			
Pyramimonas propulsa Moestrup et DRA Hill	K-0005	Port Phillip Bay, Au?	1 800
Raphidophyceae			
Heterosigma akashiwo (Hada) Hada	K-0246	Unknown locality	1 900

[a]Algae supplied by the Marine Biological Laboratory, Helsingør. Other algae originated from the Scandinavian Culture Collection of Algae and Protozoa, Botanical Institute, University of Copenhagen

Experimental conditions. All experiments were carried out at an irradiance of 60 µmol photons m^{-2} s^{-1}. The algae were adapted to this irradiance for at least 14 d prior to each experiment. Only cells from exponentially growing cultures were used for inoculation. For enumeration of cells, subsamples (2 ml) 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 were measured as increase in cell numbers and were calculated assuming exponential growth:

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

where N_0 and N_1 are number of cells at time t_0 and t_1, and t is the difference in time between samples. A minimum of 3 sampling points was included in the calculation. All experiments were carried out in triplicates. pH was measured using a Sentron® pH-meter (model 2001).

Effect of *Chrysochromulina polylepis* on the motility of *Heterocapsa triquetra*. Initial experiments had shown that *H. triquetra* which had been exposed to a dense culture of *C. polylepis* became non-motile. In order to quantitatively study the change in swimming behaviour, the following experiment was carried out: 0.25 ml *H. triquetra* culture (ca 500 cells) was mixed with 2 ml toxic culture of *C. polylepis* (1.5×10^5 cells ml^{-1}) in a multidish well. The number of cells lying non-motile at the bottom was enumerated after 10, 20, 30, 40, 50, 90, 120, and 150 min using an inverted microscope. A control experiment was carried out in a similar way using f/2 medium instead of the *C. polylepis* culture. The experiments were carried out in triplicates.

Based on the results from these experiments, the following experimental design was used in all subsequent experiments dealing with the effect of *Chrysochromulina polylepis* on the motility of *Heterocapsa triquetra* (unless otherwise stated): a *C. polylepis* culture (2 ml) was mixed with a *H. triquetra* culture (0.25 ml ca 500 cells) in a multidish well. The cultures of *H. triquetra* used in these experiments were kept in exponential growth at a concentration of 1×10^4 cells ml^{-1}. Prior to experiments, a part of the *H. triquetra* culture was diluted to attain a concentration of ca 2000 cells ml^{-1}. After 2 h of exposure to *C. polylepis*, the number of non-motile *H. triquetra* cells lying at the bottom of the well was enumerated. Subsequently, Lugol's (50 µl) was added to each well and the total number of *H. triquetra* cells in the well was enumerated after the cells had settled. This made it possible to calculate the percentage of cells which were affected by *C. polylepis* during the incubation. A control experiment was carried out using f/2 medium instead of the *C. polylepis* culture.

Effect of *Chrysochromulina polylepis* on the motility of 8 isolates (7 species) of dinoflagellates. The dinoflagellates were grown in Nunclon® tissue culture flasks (60 ml), which were mounted on a plankton wheel in order to keep the algae in suspension. A culture of *C. polylepis* was inoculated in a 1 l Pyrex® bottle containing 400 ml and allowed to grow to a cell density of 1.9×10^5 cells ml^{-1}. At this concentration, 2 ml of the *C. polylepis* culture were transferred to a multidish and mixed with 0.25 ml of the dinoflagellate culture. The number of non-motile dinoflagellates was enumerated after 2 h of exposure to the *C. polylepis* culture. Otherwise the experiment was carried out using the experimental design described in the section 'Effect of *Chrysochromulina polylepis* on the motility of *Heterocapsa triquetra*'.

Ability of *Chrysochromulina polylepis* culture to immobilize *Heterocapsa triquetra* cells: dependence of cell concentration and growth phase. *C. polylepis* was inoculated in a 1 l Pyrex® bottle containing 400 ml growth medium and allowed to grow. Daily pH and cell concentrations were measured and the culture was tested for its ability to immobilize *H. triquetra* cells. A control experiment was carried out with *Chrysochromulina simplex*, instead of *C. polylepis*, to study the effect of a non-toxic *Chrysochromulina* species on the motility of *H. triquetra*.

Immobilization of *Heterocapsa triquetra* cells by *Chrysochromulina polylepis*: the effect of pH. *Chrysochromulina polylepis* and *H. triquetra* were grown separately in 1 l Pyrex® bottles containing 400 ml growth medium. Three cultures of *C. polylepis* were inoculated and allowed to grow to different cell concentrations (1.8×10^4, 4.3×10^4, and 1.5×10^5 cells ml^{-1}). From each culture, 8 times 40 ml of the *C. polylepis* culture were transferred to small 60 ml tissue culture flasks and pH was altered by addition of 0.2 M HCl or NaOH, except for 1 flask in which the original pH was maintained. *H. triquetra* cells were added to each flask (0.9 ml containing 1×10^4 cells ml^{-1}) and pH was measured. Each mixture (2.25 ml) was added to a multidish well, and the number of non-motile *H. triquetra* cells was counted after 2 h. A similar experiment was carried out using f/2 growth medium, instead of the *C. polylepis* culture, in order to study the effect of pH itself on the motility of *H. triquetra*.

Interactions between *Chrysochromulina polylepis* and *Heterocapsa triquetra* in mixed batch cultures. All these experiments were carried out in Nunclon® tissue culture flasks (260 ml), which were mounted on a plankton wheel (1 rpm) in order to keep the algae in suspension.

In the first set of experiments, the growth responses of *Chrysochromulina polylepis* and *Heterocapsa triquetra* in monocultures and in mixed cultures were

studied. The cultures were inoculated and allowed to grow. Daily pH and cell concentrations were measured and the cultures containing *C. polylepis* were tested for their ability to immobilize *H. triquetra* cells. In the case of the mixed culture, the number of *H. triquetra* cells (both motile and non-motile) already in the culture was also measured. Control experiments (on both growth and motility) were carried out with *Chrysochromulina simplex*, instead of *C. polylepis*, to study the effect of a non-toxic *Chrysochromulina* species on *H. triquetra*.

In the second set of experiments, the importance of the initial *Chrysochromulina polylepis* concentration for the growth interaction between *C. polylepis* and *Heterocapsa triquetra* in mixed cultures was studied. Three different initial concentrations of *C. polylepis* were used (2×10^3, 5×10^3 and 1×10^4 cells ml^{-1}), while the initial concentration of *H. triquetra* was the same in all experiments (10 cells ml^{-1}). The cultures were inoculated and allowed to grow. pH and cell concentrations were measured daily.

Growth interactions between *Chrysochromulina polylepis* and algae belonging to different taxa. Growth interactions between *C. polylepis* and 15 isolates of algae (14 species) were carried out in 2 sets of growth experiments. In the first set of experiments, *C. polylepis* was grown in mixed cultures with 1 representative of the following algal classes: cryptophytes, diatoms, dictyophytes, dinoflagellates, euglenophytes, prasinophytes, and raphidophytes. In the second set of experiments, *C. polylepis* was grown in mixtures with 9 isolates (8 species) of dinoflagellates. *H. triquetra* was used in both sets of experiments to test the reproducibility of the experimental setup. Growth experiments were carried out in 260 ml tissue culture flasks, which were mounted on a plankton wheel (1 rpm) in order to keep the algae in suspension. Initial concentrations of the tested algae in these experiments were from 20 to 100 cells ml^{-1}, while the initial concentration of *C. polylepis* was 2000 cells ml^{-1}. The growth of the selected algae in monocultures was studied for comparison.

RESULTS

Effect of *Chrysochromulina polylepis* on the motility of *Heterocapsa triquetra* and other dinoflagellates

The dinoflagellate *Heterocapsa triquetra* lost its ability to swim when added to a suspension of *Chrysochromulina polylepis* containing 1.5×10^5 cells ml^{-1} (Fig. 1). After *H. triquetra* had been exposed to the suspension for 10 min, ca 25% of the *H. triquetra* cells were found non-motile at the bottom of a multidish well. After an exposure time of 50 min, all *H. triquetra* cells could be found non-motile at the bottom. Non-motile cells were not found in the control experiment using f/2 medium instead of the *C. polylepis* culture (Fig. 1).

Eight isolates of dinoflagellates were exposed to a *Chrysochromulina polylepis* suspension containing 1.9×10^5 cells ml^{-1} (Fig. 2). The percentage of cells which became non-motile after 2 h of exposure to the dense *C. polylepis* culture varied considerably among species (Fig. 2A,B). The motility of all species, except *Gymnodinium mikimotoi*, was affected by *C. polylepis*. *Heterocapsa triquetra* was the most affected species. After 2 h of exposure to the *C. polylepis* culture, 94% of the *H. triquetra* population had become non-motile. No difference in behavioural response was observed between the 2 different isolates of *Alexandrium ostenfeldii*, when they were exposed to the *C. polylepis* culture (Fig. 2A).

A significant negative relationship was found between the percentage of the dinoflagellates that became non-motile after 2 h of exposure to *Chrysochromulina polylepis* and their cell volume ($p = 0.0064$, $R^2 = 0.74$), if *Gymnodinium mikimotoi* (which was not affected) was left out (Fig. 2C, Table 2).

Effect of *Chrysochromulina polylepis* on the motility of *Heterocapsa triquetra*: dependence of cell concentration and growth phase

A batch culture of *Chrysochromulina polylepis* culture was initiated and allowed to grow. Daily, subsamples of the culture were tested for their ability to immobilize *Heterocapsa triquetra* cells. No effect on the motility of *H. triquetra* cells was observed when they were exposed to *C. polylepis* concentrations $<2 \times 10^4$ cells ml^{-1} (Fig. 3A–D). However, when the *C. polylepis* batch culture reached a cell concentration of

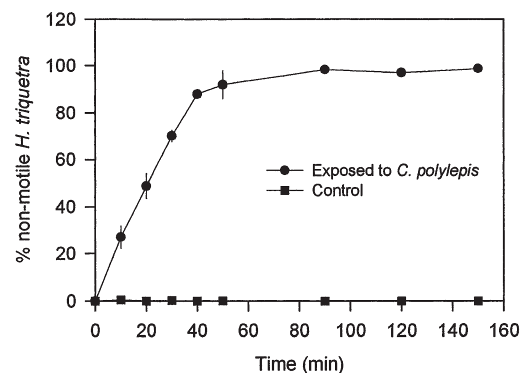

Fig. 1. Percentage of non-motile *Heterocapsa triquetra* cells found at the bottom of a multidish well as a function of time. (●) *H. triquetra* cells added to a *Chrysochromulina polylepis* culture (1.5×10^5 cells ml^{-1} at pH 8.36); (■) *H. triquetra* cells added to f/2-medium (Control). Data points refer to treatment means ± 1 SE (n = 3)

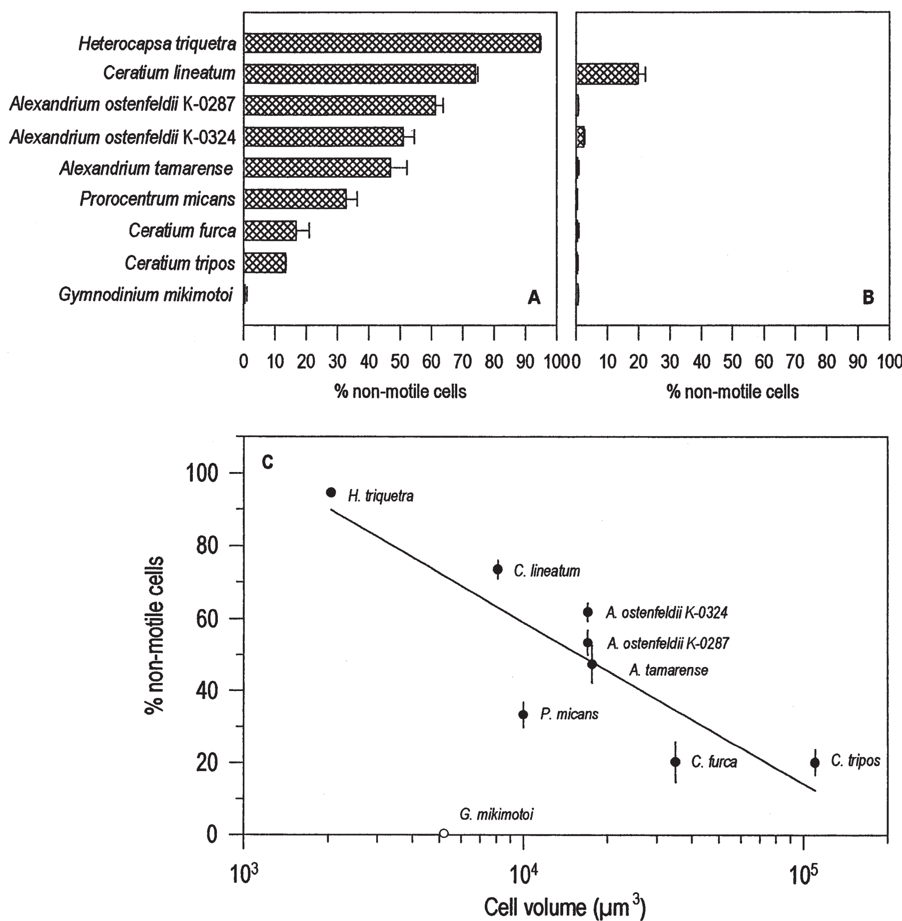

Fig. 2. (A) Percentage of non-motile dinoflagellates after 2 h of exposure to a *Chrysochromulina polylepis* suspension containing 1.9×10^5 cells ml^{-1}, after correction for non-motile cells found in the control experiment, pH = 8.8. (B) Percentage non-motile cells in the control experiment, pH = 8.8. (C) Relationship between the cell volume of the tested algae and percentage of non-motile cells observed when exposed to a dense suspension of *C. polylepis*. Data points represent treatment means ± 1 SE (n = 3). The solid line indicates a regression line. Only data points for those species affected were included in the regression (black dots). For details on the regression see Table 2

4×10^4 cells ml^{-1}, ca 50% of the *H. triquetra* population was immobilized (Fig. 3D). At higher cell concentrations of *C. polylepis*, the percentage of non-motile *H. triquetra* cells increased. Close to 100% of the *H. triquetra* population was immobilized at very high *C. polylepis* cell concentrations. Senescent cultures of *C. polylepis* did not immobilize *H. triquetra* cells (Fig. 3A,C).

pH was fairly constant until the *Chrysochromulina polylepis* culture reached a concentration of about 3.5×10^4 cells ml^{-1} (Fig. 3B). Above this cell concentration, the pH increased to about 8.8, as the culture reached stationary growth phase. The pH decreased to 7.9 in senescent cultures.

In a similar experiment, using the non-toxic *Chrysochromulina simplex* instead of *C. polylepis*, no immobilization of *Heterocapsa triquetra* cells was detected even at very high *C. simplex* concentrations (Fig. 3E,G,H). In this experiment, pH increased from 8.2 at the start of the experiment to about 9.2 when the culture had reached the stationary growth phase (Fig. 3F).

Immobilization of *Heterocapsa triquetra* cells by *Chrysochromulina polylepis*: the effect of pH

Chrysochromulina polylepis cultures were grown to 3 different cell densities, after which the culture medium was adjusted to pH ranging from 6.6 to 9.9 (Fig. 4). The pH in these cultures was between 8.1 and 8.2 before adjustment. In all the cases, the pH had a dramatic effect on the percentage of *Heterocapsa triquetra* cells that had become non-motile after 2 h of exposure to

Table 2. Linear regression analysis (p and R^2) for the relationship between size and toxicity of *Chrysochromulina polylepis* to other plankton algae in the motility experiment and in the mixed cultures. Significant relationships are marked ($p^* < 0.05$, $p^{**} < 0.01$)

	p	R^2	Number of species and clones (n)
Motility experiment			
All dinoflagellates except *Prorocentrum minimum* and *Gymnodinium mikimotoi*	0.0064**	0.74	8
Growth experiments with mixed culture			
All species and clones which were affected	0.22	0.11	15
All species where a direct effect of pH could be ruled out	0.042*	0.35	11
All dinoflagellates where a direct effect of pH could be ruled out	0.18	0.24	6

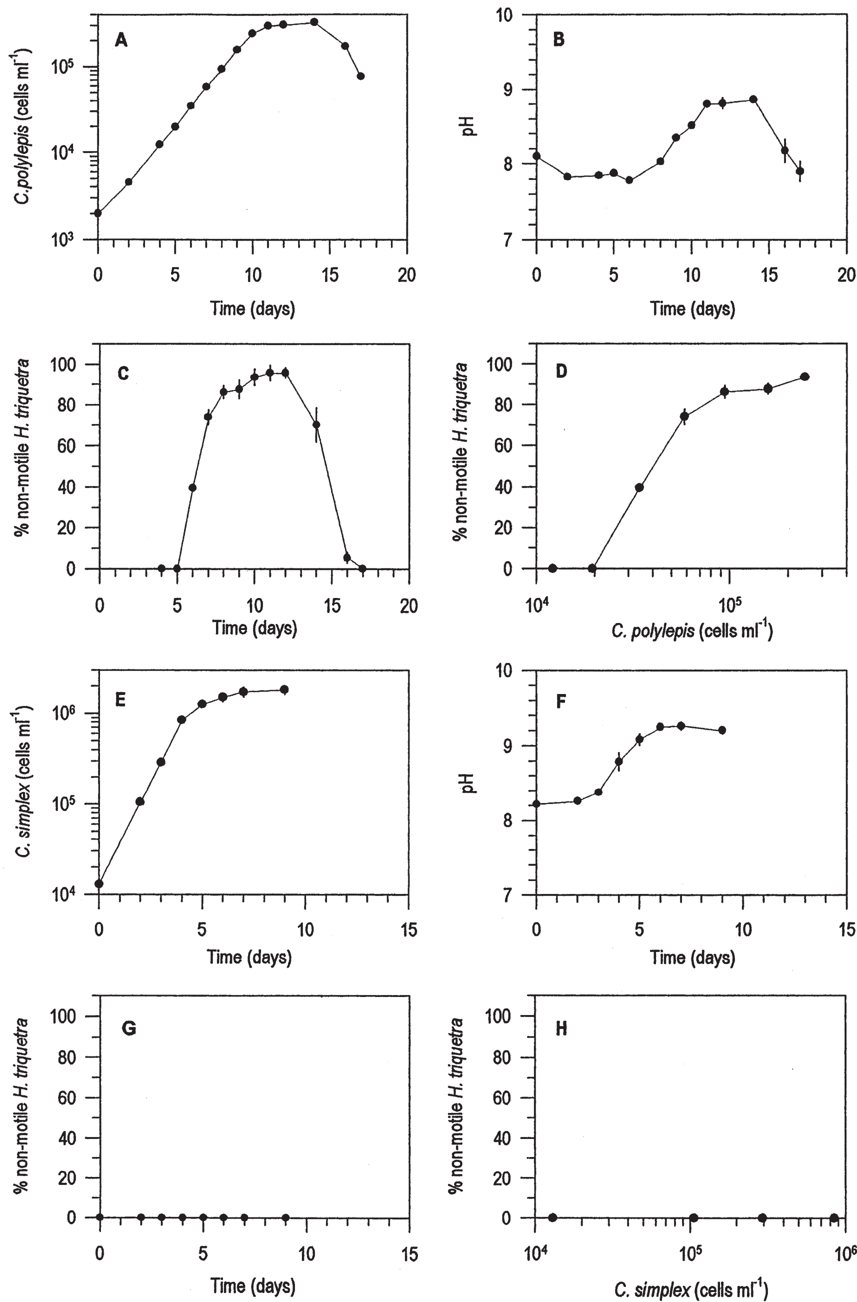

Fig. 3. Effect of *Chrysochromulina polylepis* on the motility of *Heterocapsa triquetra* and the control experiment with *C. simplex*. (A) Cell concentration of *C. polylepis* as a function of time in a batch culture. Exponential growth rate of *C. polylepis* (μ) = 0.48 ± 0.002 (mean ± 1 SE, n = 3). (B) pH in the *C. polylepis* culture as a function of time. (C) Percentage of non-motile *H. triquetra* cells as a function of time. (D) Percentage of non-motile *H. triquetra* cells as a function of cell concentration of *C. polylepis* in exponential growth phase. (E) Cell concentration of *Chrysochromulina simplex* as a function of time in batch culture. Exponential growth rate (μ) = 1.03 d^{-1} ± 0.069 (mean ± 1 SE, n = 3). (F) pH in a *C. simplex* culture as a function of time. (G) Percentage of non-motile *H. triquetra* cells as a function of time. (H) Percentage of non-motile *H. triquetra* cells as a function of cell concentration of *C. simplex* in exponential growth phase. All data points refer to treatment means ±1 SE (n = 3)

the *C. polylepis* culture. At the lowest *C. polylepis* concentration, the greatest percentage of non-motile *H. triquetra* cells was observed at a pH ranging from 8.9 to 9.6, while no effect on the motility of *H. triquetra* cells could be detected in a similar culture at pH < 7.7. At the highest *C. polylepis* concentration, the greatest percentage of non-motile *H. triquetra* cells was observed at pH > 7.25. Below this pH, the percentage of non-motile *H. triquetra* cells decreased with decreasing pH, and at pH 6.8 only motile cells were observed. An effect of pH itself on the swimming behaviour of *H. triquetra* could be detected at pH > 9.5 (Fig. 4).

Interaction between *Chrysochromulina polylepis* and *Heterocapsa triquetra* in a mixed batch culture

The interaction between of *Chrysochromulina polylepis* and *Heterocapsa triquetra* was studied in a mixed batch culture, which was initiated at low cell concentrations (Fig. 5). Non-motile *Heterocapsa triquetra* cells were first detected at a low percentage (8%) in the mixed culture at Day 6, when the concentration of *C. polylepis* had reached ca 3×10^4 cells ml^{-1} (Fig. 5E,F). The following day, the percentage of non-motile *H. triquetra* cells had increased to 72%, and the concentra-

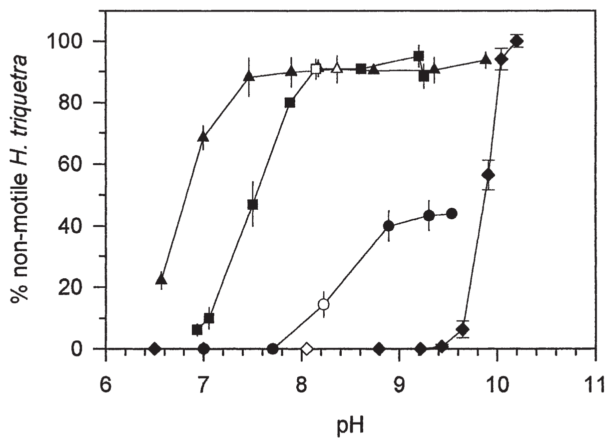

Fig. 4. Percentage of non-motile *Heterocapsa triquetra* cells as a function of pH in the growth medium of *Chrysochromulina polylepis*, using 3 different cell concentrations of *C. polylepis* (●: 1.8×10^4, ■: 4.3×10^4, ▲: 1.5×10^5 cells ml^{-1}) and a control (◆) using only growth medium. Open symbols refer to the initial values of pH in the individual experiments. Data points refer to treatment means ± 1 SE (n = 3)

tion of *C. polylepis* had reached 4.7×10^4 cells ml^{-1}. From Day 10 to 16, all *H. triquetra* cells in the mixed culture were non-motile. Between Days 16 and 18, all *H. triquetra* cells in the mixed culture became motile and only motile cells were observed in the mixed culture for the remaining study period (Fig. 5F).

Growth of *Heterocapsa triquetra* in the mixed culture was unaffected by the presence of *Chrysochromulina polylepis* until Day 6 (see Fig. 5A). The growth of the *H. triquetra* population stopped between Days 6 and 7 at a *H. triquetra* concentration of 200 cells ml^{-1}, and a dramatic decrease in the *H. triquetra* population was observed from Day 7 to Day 16. From Day 16, the *H. triquetra* population increased from ca 1.4 cells ml^{-1} to 550 cells ml^{-1} at the end of the experiment. When *H. triquetra* was grown in monoculture, it reached a cell concentration of ca 4×10^4 cells ml^{-1} (Fig. 5C).

The growth response of *Chrysochromulina polylepis* in the mixed culture with *Heterocapsa triquetra* was quite similar to that obtained when *C. polylepis* was grown in monoculture, at least for the first 16 d.

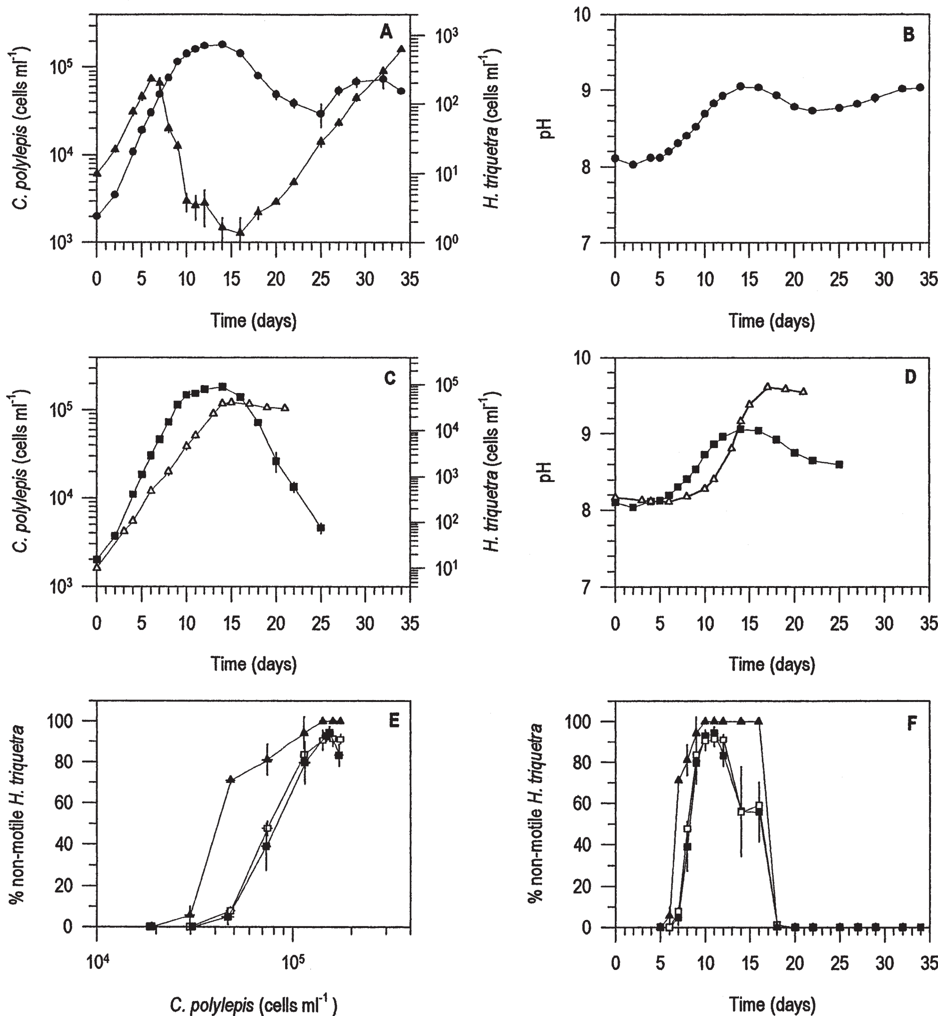

Fig. 5. Effect of *Chrysochromulina polylepis* on *Heterocapsa triquetra* in mixed batch cultures. (A) Cell concentrations of (●) *C. polylepis* and (▲) *H. triquetra*. Exponential growth rate of *C. polylepis* (μ) = 0.47 d^{-1} ± 0.013 (mean ± 1 SE, n = 3) using data from Days 2 to 6. (B) pH in the mixed culture. (C) Cell concentrations of (■) *C. polylepis* and (△) *H. triquetra* in monocultures. Exponential growth rate of *C. polylepis* (μ) = 0.48 d^{-1} ± 0.08 (mean ± 1 SE, n = 3) using data from Days 2 to 6. (D) pH in the monoculture of (■) *C. polylepis* and (△) *H. triquetra*. (E) Percentage of non-motile *H. triquetra* cells in the mixed culture as a function of *C. polylepis* concentration (only data from the first 12 d). (▲) *H. triquetra* cells already in the mixed cultures, (■) *H. triquetra* cells added to the mixed cultures and (□) *H. triquetra* cells added to monocultures of *C. polylepis*. (F) Percentage of non-motile *H. triquetra* cells as a function of incubation time (symbols the same as in E). Data points refer to treatment means ± 1 SE (n = 3)

However, if the culture was kept for a longer time, *C. polylepis* survived better in the mixed culture than in monoculture and to some extent even resumed growth (Fig. 5A,C). Thus, *H. triquetra* did not negatively affect *C. polylepis* in the mixed culture.

An increase in pH, from about 8 at the beginning of the experiment to 9.05 at the early stationary growth phase of *Chrysochromulina polylepis* culture, was observed in both the monoculture and in the mixture culture with *Heterocapsa triquetra*. As the *C. polylepis* population became senescent, pH decreased to 8.6–8.7. A slight increase in pH to ca 9 was found in the mixed cultures at the end of the experiment (Fig. 5B).

It is noteworthy that *Chrysochromulina polylepis* which had been grown in monoculture had the same ability to immobilize *Heterocapsa triquetra* cells as *C. polylepis* grown in the mixed culture (Fig. 5E). It is also noteworthy that the percentage of *H. triquetra* cells which became non-motile after 2 h of exposure to the *C. polylepis* culture was always low compared to that observed for *H. triquetra* cells that had been growing together with *C. polylepis* in the mixed culture (Fig. 5E). This indicates that the effect of *C. polylepis* on the motility of *H. triquetra* is not always fully developed within the exposure time used (2 h).

A similar experiment as that described above was carried out using the *Chrysochromulina simplex* instead of *C. polylepis* (Fig. 6). *C. simplex* did affect the swimming behaviour of *Heterocapsa triquetra* (Fig. 6E,F). When the mixed culture went into stationary growth phase, pH increased to 9.2 (Fig. 6B).

Effects of the initial concentration of *Chrysochromulina polylepis* on the growth response of *Heterocapsa triquetra* cells

The effect of the initial concentration of *Chrysochromulina polylepis* on the growth response of *Heterocapsa triquetra* in mixed cultures was also studied

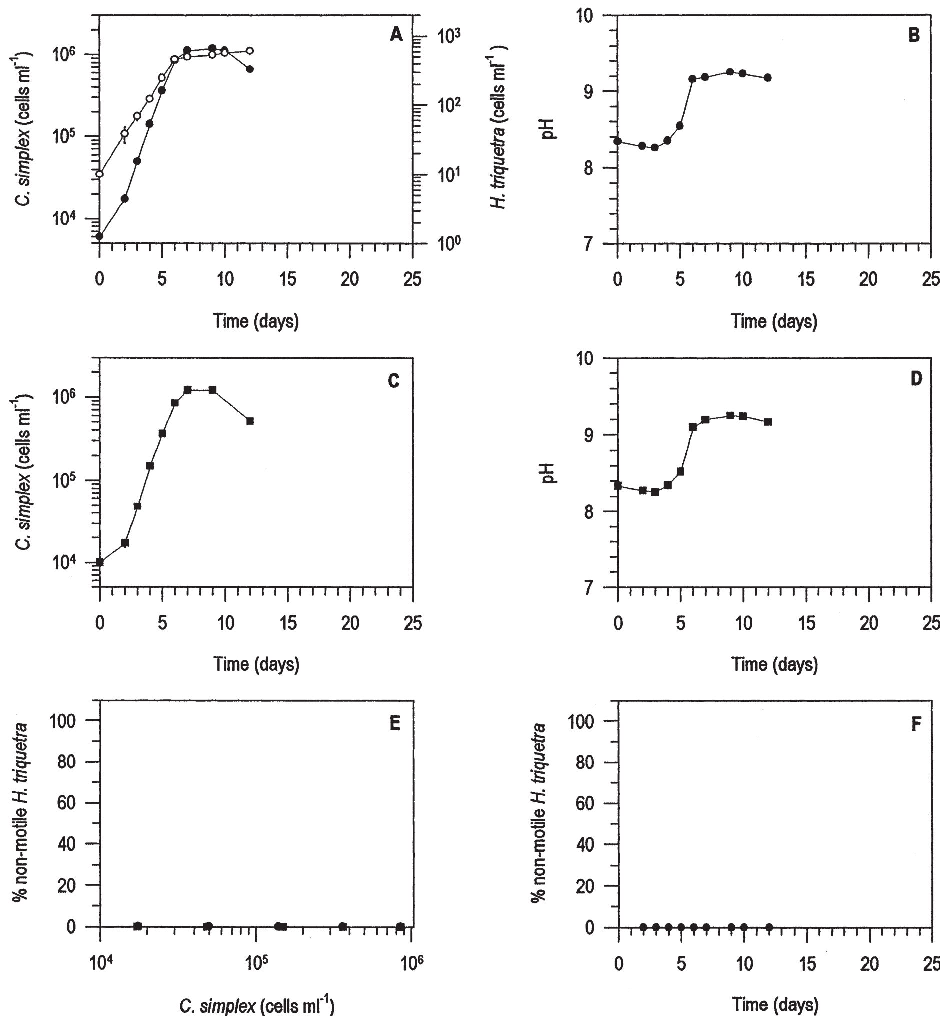

Fig. 6. Control experiments: the effect of *Chrysochromulina simplex* on *Heterocapsa triquetra* in a mixed batch culture. (A) Concentrations of (●) *C. simplex* and (○) *H. triquetra* in the mixed culture as a function of time. (B) pH in the mixed culture. (C) Concentration of *C. simplex* in the monoculture. (D) pH in the monoculture of *C. simplex*. (E) Percentage of non-motile *H. triquetra* cells in the mixed culture. (▲) *H. triquetra* cells already in the mixed culture, (■) *H. triquetra* cells added to the mixed cultures, and (□) *H. triquetra* cells added to monocultures of *C. simplex*. (F) Percentage of non-motile *H. triquetra* cells (symbols the same as in E). Data points refer to treatment means ± 1 SE (n = 3)

(Fig. 7). The initial growth rate of *H. triquetra* in the 3 mixtures was the same as that obtained when *H. triquetra* was grown in monocultures (compare Fig. 5C with Fig. 7A–C). A decrease in the *H. triquetra* population was observed on Day 4 in the culture with the highest initial *C. polylepis* concentration (Fig. 7A). In the 2 cultures which were initiated at lower *C. polylepis* concentrations, the decrease in *H. triquetra* numbers appeared some days later (Days 6 and 7). Nevertheless, and irrespective of the initial concentration of *C. polylepis*, the decline in *H. triquetra* numbers always began when the *C. polylepis* concentration reached ca 3×10^4 cells ml^{-1}. In all cases, pH increased from about 8 at the beginning of the experiment to 8.25, when the harmful effect was observed.

Growth interactions between *Chrysochromulina polylepis* and algae belonging to different taxa

Growth of 15 isolates (14 species) of algae in monoculture and in mixture with *Chrysochromulina polylepis* was also studied (Figs. 8 & 9). All algae were in an exponential growth phase at the beginning of the experiment. After some time in the mixed cultures with *C. polylepis*, the growth rate of all algae, except *Prorocentrum minimum*, was reduced. While the growth of some algae had already been affected between Days 2 and 3, the growth of other algae was not affected until between Days 6 and 7. Growth reduction of the tested algae was soon followed by a decline in their population densities. The growth response of *C. polylepis* in monoculture and in all mixed cultures was identical, indicating that *C. polylepis* was not positively or negatively affected by the other algae within the study period (Figs. 8 & 9).

The negative effect of *Chrysochromulina polylepis* on the growth of the other algae was in most cases observed at a pH which did not affect these species when they were grown in monocultures. However, it cannot be excluded that the negative effect of *C. polylepis* on the growth of *Ceratium furca*, *Ceratium tripos*, *Dictyocha speculum*, and *Gymnodinium mikimotoi* was due to a harmful effect caused by the high pH, rather than toxic substances released from *C. polylepis*.

For the algae in which a harmful effect of the high pH alone can be excluded, a reduction in growth rates of the algae was obtained at *Chrysochromulina polylepis* concentrations ranging from 1.1 to 5.19×10^4 cells ml^{-1} (Figs. 8 & 9, Tables 3 & 4). When the *C. polylepis* concentration required to induce a growth reduction was plotted against the cell size of all tested algae, no significant size relationship was found (Fig. 10, Table 2). However, omitting the 4 species in which a harmful effect of pH itself cannot be excluded, a significant relationship is found ($p < 0.05$), although the correlation coefficient is not very high ($R^2 = 0.35$). No significant size relationship was found when only dinoflagellates were taken into consideration (Table 4).

DISCUSSION

In the present study, *Chrysochromulina polylepis* had a harmful effect on almost all the algae tested. Three stages of harmful response could be identified: an early response which concerned itself with flagellar beating (documented for the dinoflagellates), a secondary response which saw a reduction in population growth rate and a third response which included population death. The fact that the tested algae were immobilized after a short-time exposure to a dense *C.*

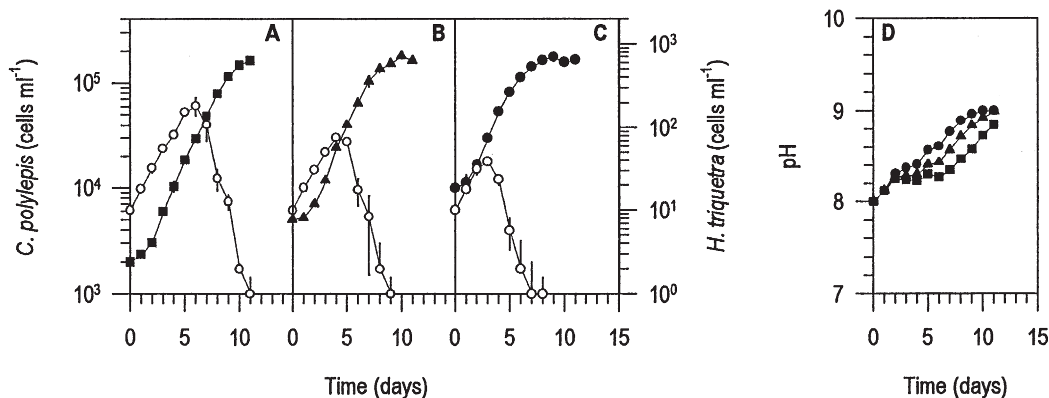

Fig. 7. Effect of the initial concentration of *Chrysochromulina polylepis* on the growth response of *Heterocapsa triquetra* in mixed batch cultures. (A–C) Concentrations of *C. polylepis* and *H. triquetra* with different initial concentrations of *C. polylepis* as a function of time. (○) Cell concentrations of *H. triquetra*, while filled symbols refer to the cell concentrations of *C. polylepis*. Initial concentrations of *C. polylepis* were (■) 2000, (▲) 5000, (●) and 10 000 cells ml^{-1}. (D) pH in the mixed cultures. Data points refer to treatment means ± 1 SE (n = 3)

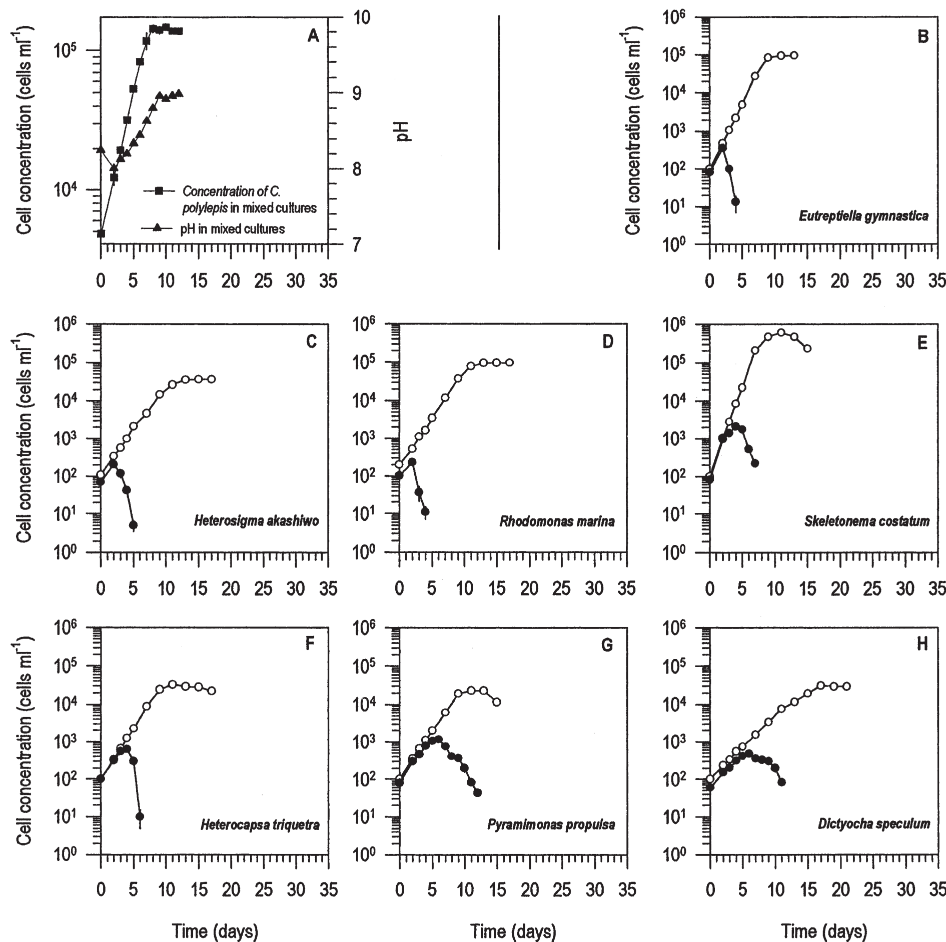

Fig. 8. Effect of *Chrysochromulina polylepis* on growth and survival of 7 algal species belonging to different taxa. (A) Average cell concentrations of *C. polylepis* in all the mixture experiments and pH. Exponential growth rate of *C. polylepis* (μ) = 0.47 d^{-1} ± 0.03 (mean ± 1 SE, n = 21) using data from Days 2 to 6. (B–H) Average cell concentrations of individual algal species (○) in monocultures and (●) in mixed cultures with *C. polylepis*. Data points represent treatment means ± 1 SE (n = 3)

polylepis culture suggests that the harmful response could be due to toxins released from *C. polylepis*. However, in the mixed growth experiments potentially harmful effects due to high pH itself must be taken into account, because high pH can affect growth and survival of marine phytoplankton, when it exceeds 9 to 9.5 (Goldman et al. 1982a,b, Chen & Durbin 1994), although some species can tolerate pH >9.5 (Humphrey 1975, Goldman et al. 1981).

A harmful effect caused by high pH could in most cases be excluded in the mixed growth experiments. This is because the reduced growth rates of the tested algae were observed at a pH which did not affect the growth of these species when they were grown in monoculture (Tables 3 & 4). However, in the cases of *Ceratium furca*, *Ceratium tripos*, *Dictyocha speculum*, and *Gymnodinium mikimotoi* it was impossible to differentiate between the harmful effect caused by a high pH and that caused by toxins released from *C. polylepis*. The fact that *C. furca* and *C. tripos* were negatively affected in the motility tests suggests that they must have been at least partly affected by toxins released by *C. polylepis*. Interestingly, *Gymnodinium mikimotoi* was not affected in the motility test, suggesting that it was mainly affected by the high pH in the mixture experiment and not by toxins released by *C. polylepis*.

Toxic effect of *Chrysochromulina polylepis*: dependence of cell concentration and growth phase

The experiments on the ability of *Chrysochromulina polylepis* to immobilize the dinoflagellate *Heterocapsa triquetra* clearly demonstrated how both the concentration and the growth phase of the *C. polylepis* culture affected its toxicity to another alga. *C. polylepis* cultures were not toxic to *H. triquetra* at *C. polylepis* concentrations below 2 to 3×10^4 cells ml^{-1} (Figs. 3D & 5E). Above this cell concentration, the toxic effect of *C. polylepis* cultures increased with cell density. However, senescent cultures of *C. polylepis* were non-toxic (Figs. 3D & 5E).

Several studies dealing with the effects of nutrient limitation on toxin content (as measured by a haemolytic test) of *Chrysochromulina polylepis* and another

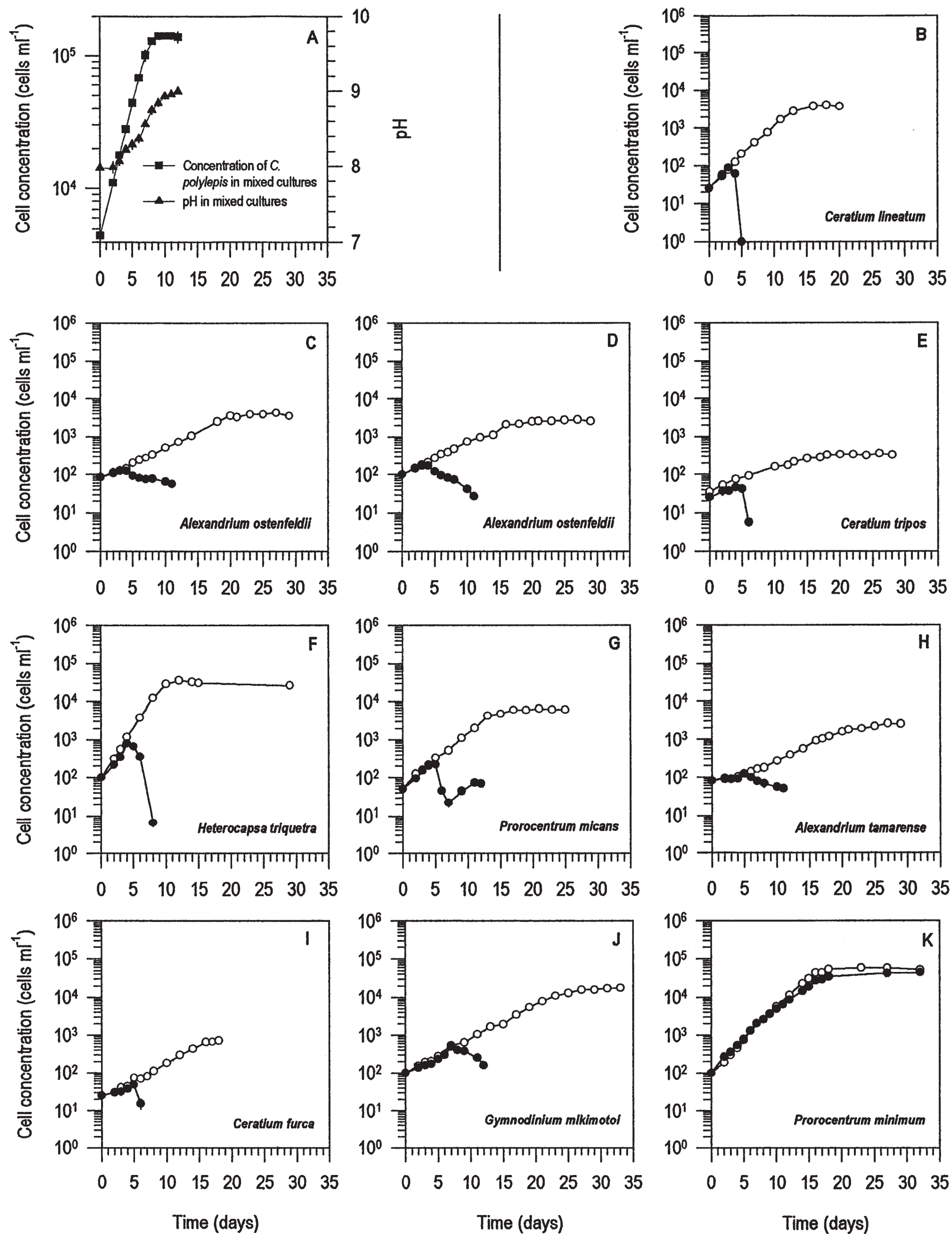

Fig. 9. Effect of *Chrysochromulina polylepis* on the growth and survival of 10 species of dinoflagellates. (A) Average cell concentration of *C. polylepis* in all the mixture experiments and pH. Exponential growth rate of *C. polylepis* (μ) = 0.45 d^{-1} ± 0.05 (mean ±1 SE, n = 30) using data from Days 2 to 6. (B–I) Average cell concentrations of individual algal species (○) in monocultures and (●) in mixed cultures with *C. polylepis*. Data points represent treatment means ±1 SE (n = 3)

prymnesiophyte *Prymnesium parvum* indicate that P limitation increases the cellular toxin content (Shilo 1967, 1971, Edvardsen & Paasche 1992, Meldahl et al. 1994, Johansson & Granéli 1999a,b). Recently, Johansson & Granéli (1999b) demonstrated that N limitation also leads to an increase in cellular toxin content in *C. polylepis*. This raises a question about the nutrient status of the senescent *C. polylepis* cultures in our experiments.

We used the f/2-medium algal growth medium (Guillard 1983) in which nitrogen and phosphorus are added in very high concentrations to the medium (Table 5). A simple calculation using the maximum cell concentration of *Chrysochromulina polylepis* and its cellular carbon content resulted in an estimated uptake of nitrogen and phosphorus by the cells of only about 11 and 16% of the nitrogen and phosphorus available, respectively (Table 5). Considering that the negative effect of *C. polylepis* was achieved at a cell concentration of about 10 times less than the maximum cell concentration, only about 1.1 to 1.6% of the available N and P in the medium was incorporated into cells at the time when the toxic effect first occurred in the mixed cultures. Thus, *C. polylepis* was neither N nor P limited in the present study.

So what actually did limit the growth of the *Chrysochromulina polylepis* cultures in the stationary growth phase in our study and why did this lead to non-toxic

Table 3. List of algae used in the experiments on growth interactions between *Chrysochromulina polylepis* and algae belonging to different taxa, including data on cell concentration of *C. polylepis* when the harmful effect occurred, and the pH in the culture medium under different conditions. See text for further details

Algal taxa	Concentration (×10^3 cells ml^{-1}) of *C. polylepis* when harmful effect occurred (mean ± SD)	pH in mixed culture when harmful effect occurred	pH in monoculture when exponential growth was affected*	pH in monoculture in stationary growth phase*
Bacillariophyceae				
Skeletonema costatum (Grev.) Cleve	12.9 ± 2.06	8.00	8.49	9.21
Cryptophyceae				
Rhodomonas marina (PA Dang.) Lemmerm.	13.1 ± 1.45	8.00	8.74	9.93
Dictyochophyceae				
Dictyocha speculum Ehrenb.	55.0 ± 2.01	8.34	8.30	8.81
Dinophyceae				
Heterocapsa triquetra Ehrenb.	19.3 ± 1.38	8.19	8.80	9.43
Euglenophyceae				
Eutreptiella gymnastica Throndsen	10.9 ± 0.55	8.00	9.00	9.22
Prasinophyceae				
Pyramimonas propulsa Moestrup et DRA Hill	30.4 ± 2.76	8.22	8.55	8.80
Raphidophyceae				
Heterosigma akashiwo (Hada) Hada	11.6 ± 1.40	8.00	8.52	9.15

*Criterion: growth is affected when the growth rate is reduced by >20%. The standard error of the mean (SEM) on pH is less than 0.3%

Table 4. List of dinoflagellates used in the mixed growth experiments with *Chrysochromulina polylepis*, including data on the cell concentration of *C. polylepis* when the harmful effect occurred, and pH in the culture medium under different conditions. See text for further details

	Concentration (×10^3 cells ml^{-1}) of *C. polylepis* when harmful effect occurred (mean ± SD)	pH in mixed cultures when harmful effect occurred*	pH in monoculture when exponential growth is affected*	pH in monoculture in stationary growth phase*
Alexandrium ostenfeldii (Paulsen) Balech et Tangen	30.5 ± 3.55	8.25	8.66	8.90
Alexandrium ostenfeldii (Paulsen) Balech et Tangen	27.4 ± 0.57	8.24	8.66	8.90
Alexandrium tamarense (M Lebour) Balech	29.5 ± 1.76	8.21	8.66	8.85
Ceratium furca (Ehrenberg) Claparede et Lachmann	43.9 ± 3.84	8.26	8.29	8.40
Ceratium lineatum (Ehrenberg) Cleve	18.0 ± 0.64	8.00	8.30	8.79
Ceratium tripos (OF Muller) Nitsch	41.4 ± 2.38	8.29	8.24	8.30
Gymnodinium mikimotoi Adachi et Fukuyo	150 ± 9.99	8.72	8.72	9.00
Heterocapsa triquetra Ehrenb.	25.7 ± 2.24	8.22	8.90	9.43
Prorocentrum micans Ehrenb.	51.9 ± 2.38	8.40	8.75	9.92
Prorocentrum minimum (Pavill.) J. Schiller	No effect of *C. polylepis*	–	9.20	9.62

*Criterion: growth is affected when growth rate is reduced by >20%. Standard error of the mean (SEM) on pH is less than 0.3%

cultures? One possible explanation could be that pH limited the growth of *C. polylepis* in stationary cultures and that this lead to non-toxic cultures. Another possible explanation could be inorganic carbon limitation. The toxins described for *C. polylepis* contain high amounts of carbon. Thus, a possible explanation for the loss of toxicity during the stationary growth phase in our experiments could be carbon limitation. The content of dissolved inorganic carbon (DIC) in the f/2 growth medium was on average 1.7 mM (P.J.H. unpubl. results). Again, a simple calculation using the maximum cell concentration of *C. polylepis* and its cellular carbon content results in an estimated uptake of carbon by the cells of only about 35% of the available amount of DIC (Table 5). However, when pH increased from 8 to 9, the concentration of inorganic carbon available as CO_2 decreased. Thus, if *C. polylepis* does not utilize bicarbonate very well at high pH, cultures may possibly become CO_2 limited. Future studies are required to test whether this is true or not.

It was interesting to observe that a *Chrysochromulina polylepis* culture, which had lost its toxicity in the stationary growth phase, did not regain its toxicity when positive growth was resumed, even though the cell concentration was considerably above a concentration which is usually toxic (Fig. 5A). This indicates

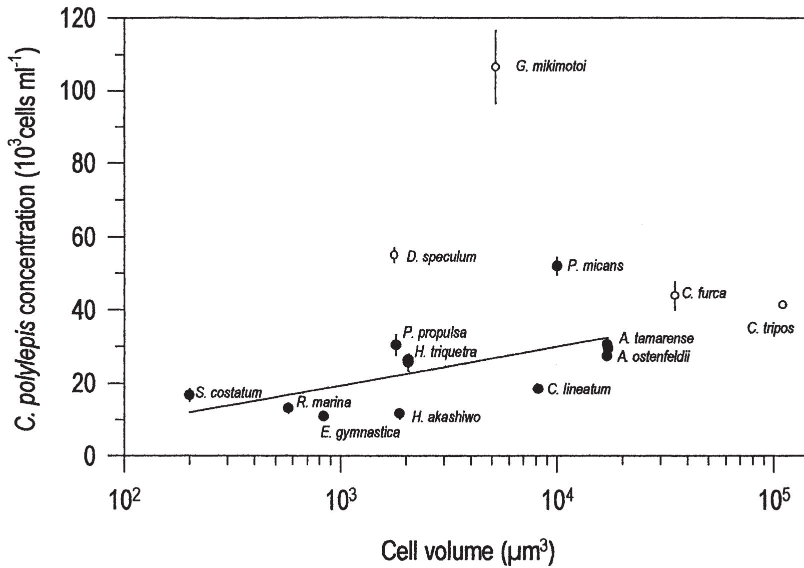

Fig. 10. Relationship between the cell volume of tested algae and the concentration of *Chrysochromulina polylepis* required to suppress their growth rate by >20%. (o) Those cases in which a direct effect of pH cannot be excluded. Solid line indicates regression line based on data where a direct effect of pH can be excluded. See text for further explanation

that a *C. polylepis* culture which has lost its toxicity requires a certain period under optimal growth conditions in order to become toxic again. This also explains some of our initial batch culture experiments with *C. polylepis*: cultures that had been inoculated with *C. polylepis* cells deriving from a stationary phase culture never became toxic (unpubl. data). For this reason, only inoculates from cultures of *C. polylepis* which had been growing in the exponential growth phase for a minimum of 2 wk were used. This is therefore an important aspect when testing algae for their ability to produce toxins.

It is also noteworthy that no evidence of enhancement or reduction of toxic effect was found in *Chrysochromulina polylepis* cultures, which had been grown together with *Heterocapsa triquetra*, as opposed to *C. polylepis* grown as unialgal cultures (Fig. 5E,F).

Table 5. Estimated total uptake of N and P in *Chrysochromulina polylepis* cultures that reached maximum cell concentration in f/2 growth medium. Addition of N and P to the seawater in the f/2 medium was 883 and 36 µM, respectively. The f/2 medium contained 1700 µM dissolved inorganic carbon (DIC). Estimates based on a red field ratio of 106 C:16 N:1 P (by mol). Cellular carbon content of *C. polylepis* was calculated using the carbon to cell volume equation published by Strathmann (1967)

Maximum cell concentration of *Chrysochromulina polylepis*	C uptake (µM)	N uptake (µM)	P uptake (µM)
3×10^5 cells ml^{-1}	626	94.4	5.90

Effect of pH on the toxicity of *Chrysochromulina polylepis*

The role pH played in the toxicity of *Chrysochromulina polylepis* to *Heterocapsa triquetra* cells was studied at 3 different *C. polylepis* concentrations using the motility test. In all cases, the highest toxicity of the *C. polylepis* cultures was observed in alkaline medium with a pH between 8 and 9 (Fig. 4).

Studies on the interaction between potentially toxic algae and other organisms rarely take the role pH plays in the toxicity into account. Exceptions are the studies by Ulitzur & Shilo (1964, 1966), who observed a similar effect of pH on the toxicity of the prymnesiophyte *Prymnesium parvum* to fish. They found that a change from pH 8 to 9 increased the toxicity by a factor of 4. At a pH of 7, no toxic effect to fish could be detected. Likewise, Proctor (1957) demonstrated that the toxic effect of the chlorophyte *Chlamydomonas reinhardi* to another chlorophyte *Haematococcus pluvialis* was highest at high pH, and the allelopathic effect disappeared when changing the pH in a cell-free medium of *C. reinhardi* from 10 to 7.5. Proctor suggested that the toxic effect was due to fatty acids and could demonstrate that the toxicity of palmin and oil acid to *H. pluvialis* was highest at alkaline pH.

In conclusion, pH may alter the toxicity of toxins produced by prymnesiophytes and other planktonic algae, and this should be considered when investigating the effect of toxic algae on other organisms. To what extent pH affected the toxicity of *Chrysochromulina polylepis* during the bloom in 1988 is uncertain, because data on pH from that period are completely lacking.

How pH affects the toxicity of algal toxins to other organisms is not well documented. Transformation into other derivatives of the toxin complex with a different degree of toxicity has been documented for the PSP-toxin complex (e.g. Boyer et al. 1986). Another possibility could be that release or breakdown of toxins in the medium is pH dependent (Simonsen & Moestrup 1997). This topic needs to be addressed further.

Is the toxic effect of *Chrysochromulina polylepis* on other algae dependent on their cell size?

Among the algae that were sensitive to the toxins of *Chrysochromulina polylepis*, a positive relationship was found between the cell concentrations of *C. polylepis* needed for a reduction in growth rate of the

exposed algae and their size (see Table 4 and Fig. 10). This could indicate that small cells are more sensitive than larger cells. However, the result should be interpreted with great caution. The reason is that these data were derived from the mixed culture experiments in which *C. polylepis* grew with a doubling time of ca 1.4 d. Thus, a comparison of data in such an experiment is only valid if the 'response time' to the toxic substance is similar among the tested species. This was probably not the case, because a significant negative relationship was found between the size of the exposed dinoflagellates and the percentage of cells found non-motile after 2 h of exposure to *C. polylepis* (Fig. 2, Table 2). This motility experiment was carried out at a very high concentration of *C. polylepis* (1.9×10^5 cells ml^{-1}), which in the mixture experiments was lethal to all the algae that were affected (Figs. 8 & 9). Thus, it may well be that what we first interpreted as a higher sensitivity of small algae to *C. polylepis*, may in fact be a size-dependent response time to the toxins instead.

Why do toxins released from *Chrysochromulina polylepis* not affect some algal species?

Yasumoto et al. (1990) demonstrated hemolytic effects of cell-free extracts from *Chrysochromulina polylepis* and *Gymnodinium mikimotoi* (= *Gyrodinium aureolum*). Chromatographic and mass spectrometric data indicate that these 2 algae produce toxic fatty acids (1-cyl-3-digalactogycerol and oktadecapentaen acid). Thus, the fact that *C. polylepis* and *G. mikimotoi* may produce the same toxins could be a likely reason why the toxins released by *C. polylepis* did not affect *G. mikimotoi*. *Prorocentrum minimum* has been connected to several toxic incidents in the past (Nakazima 1968, Tangen 1980). However, the toxins identified so far from *P. minimum* are structurally different from those of *C. polylepis* and display other symptoms (Trick et al. 1981, Grzebyk et al 1997). It is interesting that a study by Windust et al. (1996) on the effects of the DSP toxins okadaic acid and dinophysistoxin-1 on a variety of algae, found that these toxins, even at high doses, did not affect *P. minimum*. This may indicate that *P. minimum* may be more resistant to a variety of algal toxins; however, this needs to be studied in further detail.

Conclusion

Our results demonstrate that *Chrysochromulina polylepis* is toxic to a large variety of other planktonic algae at cell concentrations similar to those found during blooms in nature (e.g. Kaas et al. 1991, Edvardsen & Paasche 1998). Thus, the release of toxins by *C. polylepis* to the surrounding water may indeed explain why a bloom like the one in 1988 in Scandinavian waters became essentially monospecific at high cell concentrations. However, our results also demonstrate that factors such as pH and perhaps CO_2 limitation affect the toxicity of *C. polylepis* to other planktonic algae.

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