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Effects of the toxic haptophyte *Prymnesium parvum* on the survival and feeding of a ciliate: the influence of different nutrient conditions

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ABSTRACT: We studied the growth and feeding response of the ciliate *Euplotes affinis* when exposed to algal cultures of *Prymnesium parvum* and *Rhodomonas* cf. *baltica* as monocultures or as mixtures. Cultures of *P. parvum* grown under nutrient-limited (N or P) or nutrient-sufficient conditions were tested for toxicity against *E. affinis*. Ciliates grew well when fed *R.* cf. *baltica*, but avoided grazing on monocultures of *P. parvum*, regardless of algal concentration. Increasing abundances of *P. parvum* decreased survival of the ciliate, even if supplied as a mixture together with high concentrations of *R.* cf. *baltica* as an alternative prey. This implies that *P. parvum* produces substances that were fatal to the ciliate when released to the medium. The lethal effect of *P. parvum* was dependent on the physiological status of the cells, with the highest toxicity in nutrient-stressed cultures. Our results suggest that toxin production in *P. parvum* may be a chemical defense to repel predators.

KEY WORDS: *Prymnesium parvum* · Toxic algae · Grazing avoidance · Nutrient limitation · Grazing · Growth · Ciliate

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INTRODUCTION

Toxic incidents of the haptophyte *Prymnesium parvum* have been known since the end of the last century (Strodtmann 1898). Since then, toxic blooms have been reported from brackish water localities in Europe, the Middle East, Ukraine, China and the USA (Moestrup 1994, Edvardsen & Paasche 1998). These blooms have strongly affected coastal marine ecosystems and caused economic problems for commercial aquaculture. Therefore, it is important to understand the selective forces leading to bloom formation of this species. The ability of a specific phytoplankton species to become dominant and form blooms in natural environments is, apart from its competitive ability, also dependent on mortality losses. Grazing by herbivorous zooplankton is considered a major loss factor for the development of phytoplankton blooms (Watras et al. 1985, Uye 1986). Adaptations of algae to escape grazing would therefore directly favour the ecological success of that particular species. Several studies have shown that a number of phytoplankton species have the ability to produce toxic substances that stun, kill or repel potential grazers (Ives 1985, 1987, Sykes & Huntley 1987, Hansen 1989, 1995, De Mott & Moxter 1991, De Mott et al. 1991, Carlsson et al. 1995, Kamiyama & Arima 1997). Thus, one of the reasons for toxin production in phytoplankton may be to escape grazing.

Among haptophytes, several species produce toxic substances with negative effects on other marine organisms. The toxins have mainly been considered a problem for gill-breathing animals as they destroy the selective permeability of the gill tissue (Yariv & Hestrin 1961). However, there is also evidence for toxic effects on potential grazers. For instance, moderate abundances of *Prymnesium patelliferum* have a strong negative effect on copepod feeding and reproduction (Nejstgaard et al. 1995, Nejstgaard & Solberg 1996). Nielsen et al. (1990) reported a high mortality of microzooplankton and copepods during a bloom of *Chrysochromulina polylepis* in the Kattegatt in 1988. In addition, Carlsson et al. (1990) showed that the *C. polylepis*

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toxin was lethal to the ciliate *Favella ehrenbergi*. From these results, it is evident that several haptophyte species have the capacity to become toxic or unpalatable to both ciliates and copepods. However, although several physiological and ecological studies of *P. parvum* have been performed, there have, to our knowledge, been no previous studies on interactions between *P. parvum* and zooplankton. *Prymnesium* toxins have a generalised membrane action (e.g. to destroy the permeability of cell membranes) and thus may affect organisms ranging from protozoa to fish (Igarashi et al. 1998, Sasaki et al. 2001). A relevant question to ask, therefore, is whether the potential for toxin production provides *P. parvum* with a selective advantage as a chemical defence against grazing.

Previous studies have shown that limiting conditions of either nitrogen or phosphorus enhance the toxic effect of *Prymnesium parvum* (Shilo 1971, Meldahl et al. 1994, Johansson & Granéli 1999), suggesting that toxin production is a defence mechanism used to improve the competitive ability of *P. parvum* under conditions of severe nutrient competition. In the present study we investigated the survival and feeding of the ciliate *Euplotes affinis*, when exposed to mixed cultures of *P. parvum* and *Rhodomonas* cf. *baltica*, which all coexist in natural phytoplankton communities (Thomsen 1992, Johansson 2002). Cultures of *P. parvum* were grown under nitrogen (N) or phosphorus (P) deficient or nutrient-sufficient conditions in order to compare differences in the toxic response of the ciliates related to nutrient conditions of the algae.

MATERIALS AND METHODS

Experimental organisms. A toxic strain of the haptophyte *Prymnesium parvum* (CCMP 708, equivalent spherical diameter [ESD] 7 to 9 µm) and a strain of the non-toxic chryptophyte *Rhodomonas* cf. *baltica* (Kalmar Algal Collection [KAC] 30, 6 to 8 µm ESD) were obtained from KAC. *R.* cf. *baltica* was selected as the control species since it is similar in size (thus the grazers will have an alternative food source of the same size range which is not toxic to them) to *P. parvum*. The ciliate *Euplotes affinis* (40 to 70 µM) was isolated from a surface water sample from Kalmar Bay (Baltic Sea), Sweden, in July 1999. Stock cultures of *P. parvum* and *R.* cf. *baltica* were grown in autoclaved aged coastal seawater (7‰) with f/10 enrichment (Guillard & Ryther 1962). Vitamins (B12, biotin and thiamine) were added following the method of Schöne & Schöne (1982). Cultures of *E. affinis* were grown in autoclaved seawater (7‰) enriched with EDTA, in polystyrene culture bottles (50 ml, Nunclon), to which *R.* cf. *baltica* was added every second day (at concentrations of 10^3 to 10^4 cells ml^{-1}). The ciliates were transferred to new media every week. All cultures were maintained at 20°C on a 16 h light:8 h dark cycle under an irradiance of 100 µmol m^{-2} s^{-1} provided by 36 W cool-white fluorescent lamps.

Ingestion rates of *Euplotes affinis*. The ingestion rates of the ciliate *E. affinis* were determined at 8 concentrations (0.5 to 12.0 × 10^3 cells ml^{-1}) of either *Rhodomonas* cf. *baltica* or *Prymnesium parvum* in monocultures, or in mixtures of the 2 species (Table 1). To obtain different experimental concentrations of the 2 algae, the cultures were diluted with autoclaved seawater (nutrient concentrations: 0.1 µM PO_4^{3-} and 1.2 µM NO_3^-). The experiments were performed in multiwells (Falcon multiwell, 24-well). Using a micropipette, 4 ciliates (together with 10 to 20 µl of medium) were added to each well, which were then filled with 2 ml of algal suspension (3 replicates for each algal concentration). After 4 h of incubation, the ingestion rates were calculated from the disappearance of algal cells in the suspension following the method of Frost (1972). The algal numbers were, after preservation with Lugol's solution, counted initially and at the end of incubations using a flow cytometer (FACS Calibur, Becton Dickinson). Grazing experiments were carried out at low light intensity (15 µmol m^{-2} s^{-1}) to ensure minimal growth of the prey during the experiments.

Growth of *Euplotes affinis*. The response of *E. affinis* to different mixtures of *Prymnesium parvum* and *Rhodomonas* cf. *baltica* was studied in multiwells (Falcon multiwell, 24-wells). *E. affinis* were incubated together with: (1) *R.* cf. *baltica* (10^3 cells ml^{-1}, monocultures), (2) *R.* cf. *baltica* (10^3 cells ml^{-1}) and various concentrations of *P. parvum* (2 to 32 × 10^3 cells ml^{-1}) in mixed cultures, and (3) with only autoclaved algal medium (f/10) that served as a starvation control. In order to test differences in indirect growth response of *E. affinis* related to nutrient conditions of the water,

Table 1. Ingestion rate (±SD) for the ciliate *Euplotes affinis* feeding on cells of *Rhodomonas* cf. *baltica* (R) in the presence of *Prymnesium parvum* (P). The negative values of the ingestion rate are an artefact due to better growth of *R.* cf. *baltica* in the presence of *E. affinis* than in the controls where *R.* cf. *baltica* was growing without ciliates (time = 4 h, n = 3)

Algal suspension (10^3 cells ml^{-1})		Ingestion rate (cells ciliate h^{-1})
R	**P**	
10	0	35 ± 7
10	2	29 ± 6
10	4	27 ± 6
10	8	14 ± 2
10	16	−18 ± 2
10	32	−24 ± 6

P. parvum was, prior to the experiment, grown as batch cultures under 3 different nutrient concentrations; 14.5 µM NO_3^-:3.6 µM PO_4^{3-} (nitrogen-deficient, N:P = 4:1), 58 µM NO_3^-:3.6 µM PO_4^{3-} (nutrient-sufficient, N:P = 16:1) and 58 µM NO_3^-:0.9 µM PO_4^{3-} (phosphorus-deficient, N:P = 64:1). *R.* cf. *baltica* was grown as batch cultures under a nutrient concentration of 58 µM NO_3^-: 3.6 µM PO_4^{3-} (nutrient-sufficient, N:P = 16:1). Cells of *P. parvum* were harvested in mid-exponential phase (nutrient-sufficient cultures) or late exponential/stationary growth phase (N- or P-limited cultures), while *R.* cf. *baltica* were harvested at mid-exponential phase. The concentrations of NO_3^-, PO_4^{3-} and NH_4^+ in the cultures were analysed according to Valderama (1995). To attain different experimental concentrations of the 2 algae, the cultures were diluted with autoclaved seawater (nutrient concentrations: 0.1 µM PO_4^{3-} and 1.2 µM NO_3^-). Each algal concentration consisted of 12 replicate wells initially containing 4 ciliates and 2 ml of algal suspension. This means that initially we had a total of 48 ciliates (the sum of the 12 replicate wells) for each of the treatments. The growth response of the ciliates were measured after 12, 24, 36 and 48 h by sampling 3 out of 12 replicate wells at each time which were fixed with Lugol's solution and counted under a microscope. The underlying assumption was that the measured ciliate density of the wells taken at each point in time was representative for all 12 replicate wells. Thus, our results in the figures are based on the multiplication of the the ciliate/algal cell numbers in each of the 3 sampled wells, by 12. The same experimental procedure was used for *P. parvum* cultures grown at different nutrient conditions. Experiments were carried out at constant low light intensity (15 µmol m^{-2} s^{-1}) to ensure minimal growth of the prey during the experiments.

Toxicity test: survival of *Artemia salina* nauplii. Prior to the growth experiment, the toxicity of *Prymnesium parvum* cultures grown under different nutrient conditions was tested using an *Artemia*-assay. The reason to use this test is because, although some of the toxins are identified (see Igarashi et al. 1998, Sasaki et al. 2001), a chemical method will only be possible after all the toxins have been identified. Eggs of *A. salina* (Mackay Marine Brine Shrimp) were hatched according to Vanhaecke et al. (1981) using filtered (90 mm membrane filter [Gelman Science] mesh size 0.45 µm) autoclaved seawater with a salinity of 7‰ and pH 8.0. The eggs were kept under constant aeration at a temperature of 25°C with PAR of 100 µmol m^{-2} s^{-1} under continuous light. After 18 to 24 h, hatched *Artemia* nauplii (Developmental Stage 1) were transferred to fresh seawater and kept for an additional 24 h. The transfer was made in order to be sure that all the nauplii used for the test had reached the same developmental stages. The nauplii were kept under the same conditions as the eggs. The tolerance of *A. salina* to the different cultures of *P. parvum* was examined on nauplii 48 h after egg hatching began (Developmental Stages 2 and 3).

The different cultures of *Prymnesium parvum* were diluted with filtered, autoclaved seawater (7‰) to give a dilution series of 100, 50, 30, 10, 5, and 2.5%. Filtered autoclaved seawater was used as a control. Ten ml of each dilution was added to a 10 ml well (Falcon multiwell, 6-wells), 10 nauplii were transferred to each well, and 3 replicates were used for each algal concentration. After 24 h in darkness at a temperature of 25°C, the mortality of nauplii at each concentration was examined. Dead nauplii were observed under a microscope and the nauplii were considered dead if no movement of the appendages was observed within 10 s (Vanhaecke et al. 1981). The mortality was transformed into probit units according to Hewlett & Plackett (1979) and plotted against log-transformed cell concentration. From the regression line, the 50% mortality (24 h; LC_{50}) was calculated.

RESULTS

Ingestion rate

Maximum ingestion rate of *Euplotes affinis* was reached at a prey concentration of approximately 6 × 10^3 *Rhodomonas* cf. *baltica* cells ml^{-1} (Fig. 1). When *Prymnesium parvum* was offered as the only food source, grazing was not observed at any concentration, resulting in negative ingestion rates. Experiments in which *E. affinis* was offered a mixed food source con-

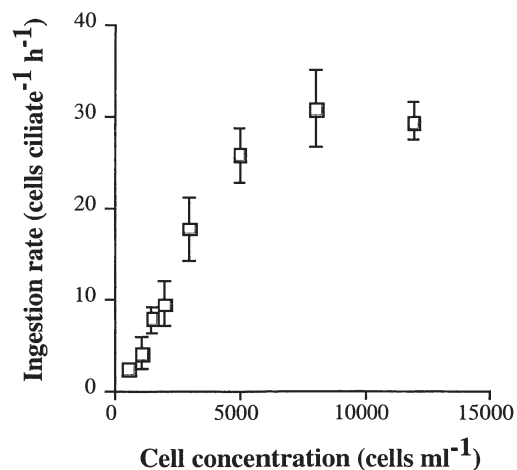

Fig. 1. Ingestion rates for *Euplotes affinis* fed increasing concentrations of *Rhodomonas* cf. *baltica* (time = 4 h, n = 3)

sisting of a fixed cell concentration of *R.* cf. *baltica* (10 × 10^3 cells ml^{-1}) and various concentrations of *P. parvum* showed that the feeding on *Rhodomonas* cells decreased as the concentration of *P. parvum* increased (Table 1, *t*-test). Thus, *P. parvum* were not only rejected as a food source, but their presence also inhibited feeding on normally edible phytoplankton cells (Table 1).

Growth of *Euplotes affinis*

When *Euplotes affinis* was exposed to a fixed concentration of *Rhodomonas* cf. *baltica* and various concentrations of *Prymnesium parvum* cells grown under nutrient sufficient conditions, the growth of *E. affinis* was unaffected at *P. parvum* concentrations below 8 × 10^3 cells ml^{-1} (Fig. 2). At a concentration of 8 × 10^3 *P. parvum* cells ml^{-1}, the cell numbers of *E. affinis* increased during the first 36 h and then declined. At *P. sparvum* concentrations above 8 × 10^3 cells ml^{-1} the ciliates were unable to sustain growth and the number of ciliates decreased (Fig. 2).

When the ciliates were exposed to a fixed concentration of *Rhodomonas* cf. *baltica* and various concentrations of *Prymnesium parvum* cells grown under N- or P-deficient conditions, the ciliates showed a different growth response. The ciliates were growing well at 2 × 10^3 *P. parvum* cells ml^{-1} (Fig. 2), but at concentrations exceeding 2 × 10^3 cells ml^{-1} *Euplotes affinis* was not able to grow and survival decreased as the concentration of *P. parvum* increased (Fig. 2). *E. affinis* exposed to *P. parvum* cells grown under nutrient-deficient conditions (N or P) showed a higher mortality than cells exposed to nutrient-sufficient *P. parvum* cells (Fig. 3), suggesting that nutrient limitation stimulated the toxin production in *P. parvum*.

Survival of *Artemia salina* nauplii

The tolerance of *Artemia salina* nauplii to different *Prymnesium parvum* cultures was dependent on the physiological status of the *P. parvum* cells. The LC_{50} value of cultures grown under nutrient-sufficient conditions was calculated to be 26.5 × 10^3 cells ml^{-1} (Fig. 4). *Artemia salina* was less tolerant to *P. parvum* cultures grown under N- or P-deficient conditions, resulting in LC_{50} values of 3.0 × 10^3 and 2.0 × 10^3 cells ml^{-1}, respectively (Fig. 4).

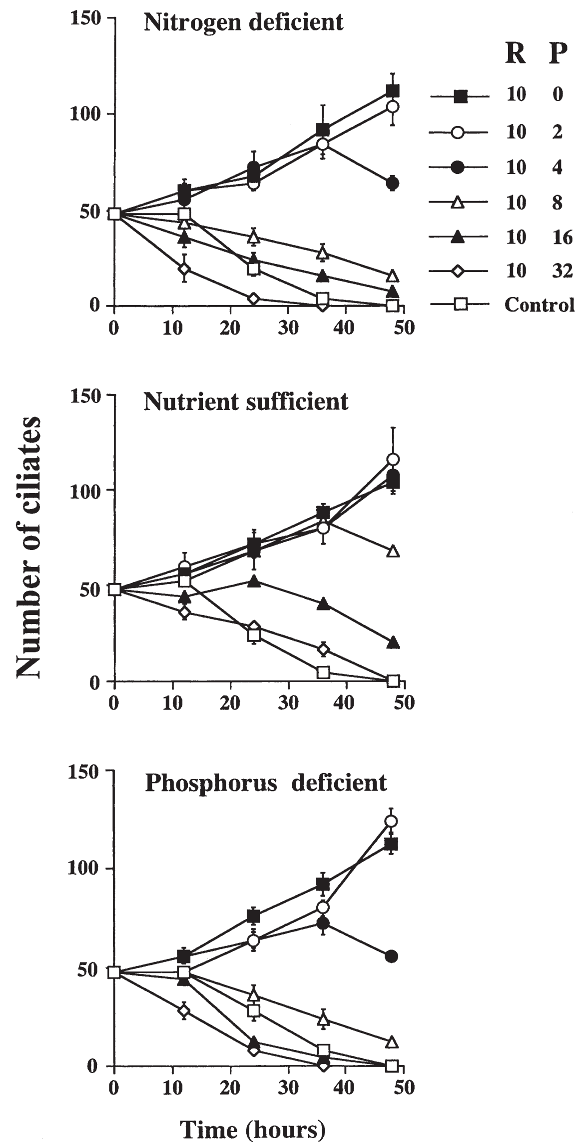

Fig. 2. Growth respones of *Euplotes affinis* when fed monocultures of *Rhodomonas* cf. *baltica* (R) or mixtures of *R.* cf. *baltica* and various concentrations of *Prymnesium parvum* (P) cells grown under nitrogen-deficient, nutrient-sufficient or phosphorus-deficient conditions. Pure algal medium was used as starvation control

DISCUSSION

In the present study, we demonstrate an inhibitory feeding response of the haptophyte algae *Prymnesium parvum* on the ciliate *Euplotes affinis*. The ciliate was growing well when fed monocultures of the *Rhodomonas* cf. *baltica*, whereas it avoided grazing on monocultures of *P. parvum*, irrespective of the algal concentration. Furthermore, we found that increasing abundances of *P. parvum* increased the mortality of the ciliate, even if supplied as a mixture together with high concentrations of *R.* cf. *baltica* as an alternative prey. This suggests that toxic substances excreted into the medium were responsible for the observed mortality.

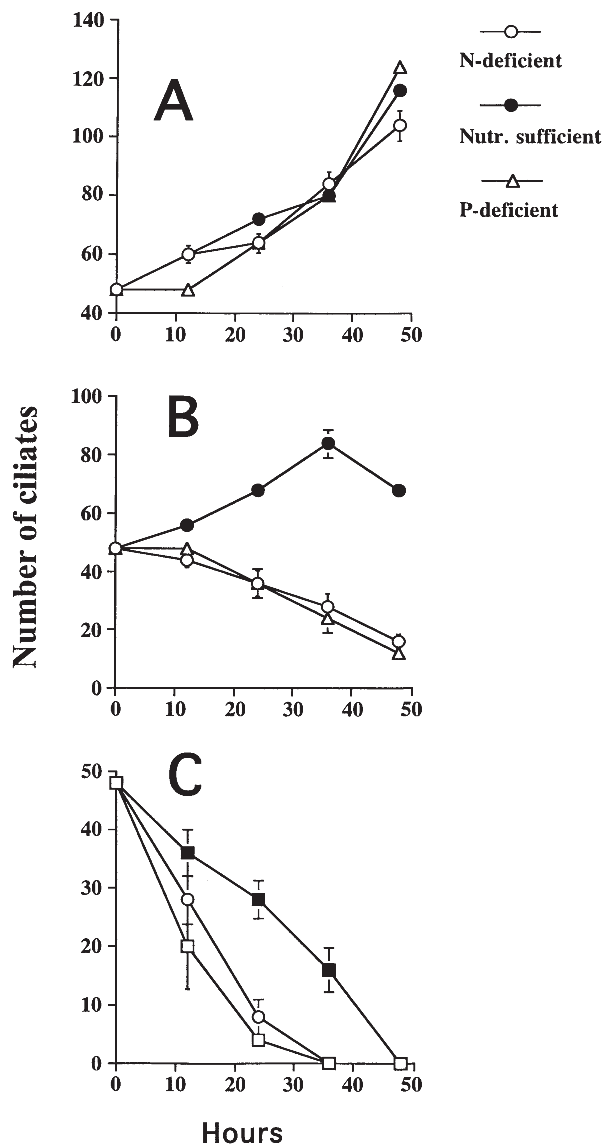

Fig. 3. *Euplotes affinis* related to differences in nutrient conditions. Growth responses of *E. affinis* exposed to mixtures of *Rhodomonas* cf. *baltica*. and *Prymnesium parvum* as follows: (A) 10 000:2000, (B) 10 000:8000, and (C) 10 000:32 000. *P. parvum* cells were grown under N- and P-deficient and under nutrient-sufficient conditions. *R.* cf. *baltica* cells were grown solely under nutrient-sufficient conditions

Granéli & Johansson (2003) have shown that addition of *P. parvum* filtrates from cultures which had grown under N- or P-deficient conditions contained toxic substances able to kill several phytoplankton species. There was a clear difference in the degree of growth inhibition of the ciliate, whether nutrient-limited or nutrient-sufficient *P. parvum* cultures were offered as prey, suggesting that the survival of the ciliate in the presence of toxic *P. parvum* cells was strongly influenced by the physiological status of the *P. parvum* cells. It is well known that *P. parvum* under certain conditions produces highly potent toxins that are released to the water (Shilo 1967). These toxins have a broad spectrum of different biological effects, including ichthyotoxic, neurotoxic, cytotoxic, hepatotoxic and hemolytic activity towards a range of marine organisms (Valkanov 1964, Shilo 1971). Several studies have shown that the toxic effect of *P. parvum* is enhanced under nutrient stress (Shilo 1971, Johansson & Granéli 1999), and toxic substances released to the medium have been reported to suppress growth of other algae (Granéli & Johansson in press) and cause fish mortality under nutrient-limiting conditions (Aure & Rey 1992). Although the toxicity of cell-free filtrates of *P. parvum* was not tested in the present study, it seems likely that a similar mechanism was responsible for the negative effect of *P. parvum* cells on the *E. affinis* cells.

Several phytoplankton species have been reported to release toxic substances with negative effects on both ciliates and copepods. Huntley et al. (1986), for instance, reported that toxic substances released by the dinoflagellate *Protoceratium reticulatum* induced mortality of the copepod *Calanus pacificus*. Similarly, certain dinoflagellate species have been found to secrete substances with a negative influence on the ciliate *Favella ehrenbergii* (Hansen 1989, 1995, Hansen et al. 1992). Among haptophytes, Carlsson et al. (1990) showed that *Chrysochromulina polylepis* releases toxic substances that are lethal to the ciliate *F. ehrenbergi*. In laboratory experiments, both *Prymnesium patelliferum* and *C. polylepis* strongly affect copepod feeding and reproduction (Nielsen et al. 1990, Nejstgaard et al. 1995, Nejstgaard & Solberg 1996). These results support the conclusion that toxin production in phytoplankton can be a mechanism to escape grazing. In spite of several studies of toxic interactions between phytoplankton and potential grazers, the mechanism has rarely been related to the nutrient condition of the ambient water. Carlsson et al. (1990) demonstrated that cells of the haptophyte *C. polylepis* were more lethal to the ciliate *F. ehrenbergii* when grown under P-limited conditions compared to cells grown under nutrient-sufficient conditions. Further, Hansen (1989) and Hansen et al. (1992) found that the toxic dinoflagellates *Alexandrium tamarense* and *A. ostenfeldii* produce substances that are lethal to the ciliate *F. ehrenbergii* when released to the medium. The toxicity of the medium was dependent on the growth phase, with the highest toxicity during the stationary phase. This suggests a close relationship between growth limitation and excretion of toxic substances by certain phytoplankton.

Negative effects on predators due to production of toxic substances would be valuable for any phyto-

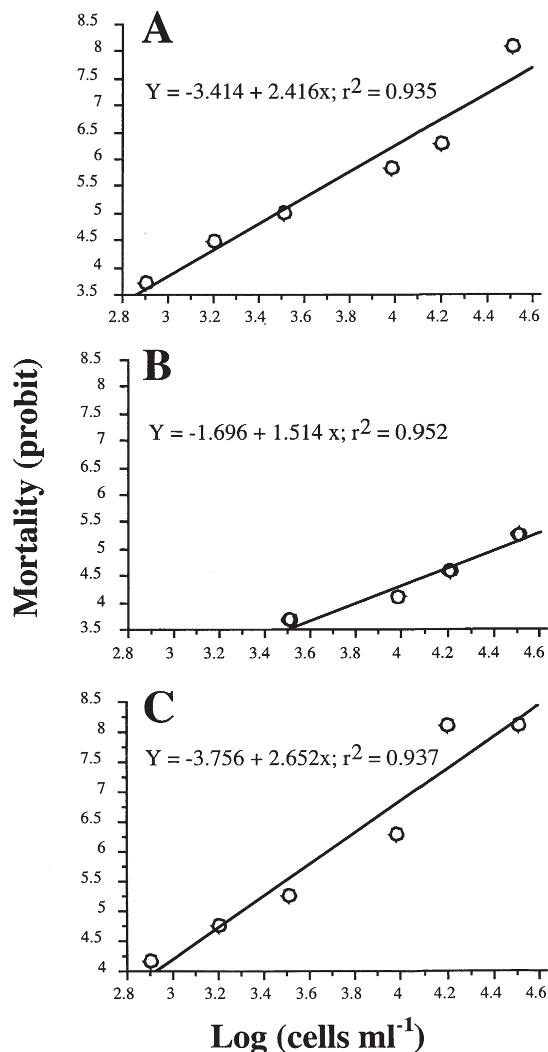

Fig. 4. Dose-response relationship of cultures of *Prymnesium parvum* on nauplii of *Artemia salina*. Cultures of *P. parvum* were grown under (A) nitrogen-deficient, (B) nutrient-sufficient or (C) phosphorus-deficient conditions

plankton species. The underlying assumption is that blooms of these algal species can develop without regulation due to zooplankton grazing, which have been considered an important loss factor during bloom formation of phytoplankton (Watras et al. 1985). Several studies have reported that blooms of toxic phytoplankton species may initiate and develop in natural waters as a result of reduction or absence of grazers due to toxic interactions. For instance, the large bloom of *Chrysochromulina polylepis* in 1988 in Scandinavian waters has been suggested to develop partly due to reduced grazing (Dahl et al. 1989, Maestrini & Granéli 1991).

Field observations have shown that *Prymnesium parvum* are capable of forming more or less monospecific blooms, which may persist in the water for several weeks (Lindholm et al. 1999). This suggests a mechanism to inhibit or avoid zooplankton grazers. However, to our knowledge, there is no information on the importance of zooplankton grazing for the development of *P. parvum* blooms in nature. Nevertheless, the present study implies that zooplanktonic grazers, at least in the case of *Euplotes affinis*, avoid grazing on monocultures of *P. parvum*. This is supported by Valkanov (1964) and Lindholm et al. (1999), which reported a substantial reduction in the abundance of zooplankton during natural blooms that were heavily dominated by *P. parvum*. At the beginning of a bloom, *P. parvum* will occur together with other phytoplankton species. The present study showed that *E. affinis* grew well at low concentrations of *P. parvum* (2 to 4 × 10^3 cells ml^{-1}) if an alternative prey was present at high concentrations. Similar relationships have been reported between ciliates and other toxic phytoplankton species (Verity & Stoecker 1982, Hansen 1995). That is, ciliates can thrive among algae producing potent exotoxins, as long as the density of these algae is low and the algae do not dominate the phytoplankton assemblage.

The mechanism responsible for the lethal effect on *Euplotes affinis* cells is not clear. On several occasions in the present study we observed that in some of the wells ciliates were completely absent, suggesting cell lysis. Hansen (1989) reported that the dinoflagellate *Alexandrium tamarense* excretes toxic substances to the medium that acts on the cell membrane of ciliates, making them swell and subsequently lyse. The toxins produced by *Prymnesium* cells are also known to act on cell membranes and disrupt the ability of selective permeability (Shilo 1967), which Meldahl et al. (1996) thought was probably due to an influx of Ca^2. However, recent studies by Igarashi et al. (1998), have shown that the haemolytic activity of prymnesins are not affected by Ca^2 but by the origen of the blood cell being tested. Several studies have shown that *Prymnesium* toxins damage cell membranes and exert lytic effects on various cell types including blood cells, human liver cells and amnion cells (Shilo & Rosenberger 1960, Meldahl & Fonnum 1993, Johansson & Granéli 1999, Granéli & Johansson 2003). Although we did not see the ciliate cells lyse, it is possible that *P. parvum* toxins have a similar mode of action on ciliates.

In conclusion, our results show that *Prymnesium parvum* were not grazed by the ciliate *Euplotes affinis*. *P. parvum* had a negative effect on the survival of the ciliate, even if supplied as a mixture together with a non-toxic prey. This implies that *P. parvum* produced substances that are lethal to the ciliate when released to the medium. The toxic effect of *P. parvum* was

dependent on the physiological status of the algal cells, with the highest toxicity in nutrient-stressed cultures. This was clearly demonstrated by a higher mortality when *E. affinis* were incubated with *P. parvum* cells grown under nutrient-limited (N or P) conditions, compared to when incubated with nutrient-sufficient cells. From these results we suggest that toxin production in *P. parvum* may be a chemical defence against grazers, and that toxic interactions between *P. parvum* and potential grazers may be an important mechanism in the development and maintenance of blooms of this species in nature.

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