

Haemolytic activity and allelopathy of the haptophyte *Prymnesium parvum* in nutrient-limited and balanced growth conditions

Pauliina Uronen^{1,2,*}, Sirpa Lehtinen², Catherine Legrand³, Pirjo Kuuppo², Timo Tamminen²

¹University of Helsinki, Tvärminne Zoological Station, 10900 Hanko, Finland

²Research Programme for the Protection of the Baltic Sea, Finnish Environment Institute, PO Box 140, 00251 Helsinki, Finland

³Department of Biology and Environmental Science, University of Kalmar, 39182 Kalmar, Sweden

ABSTRACT: *Prymnesium parvum* (Haptophyceae) was grown in 3 different nutrient treatments in semi-continuous cultures, where the N:P molar ratios were modified to 80:1, 4:1 and 16:1. The nutrient concentrations in the media affected the cellular nutrient content of *P. parvum*. The phosphorus content showed larger flexibility in the cells and determined more the cellular nutrient ratios than nitrogen or carbon. Toxicity was measured as haemolytic activity 14 times during the culturing. All the cultures were toxic during the exponential growth, but toxicity increased in the nutrient limited cultures during semi-continuous dilution. The allelopathic effect of *P. parvum* was examined with low cell densities (final abundance 2 and 5×10^3 cells ml⁻¹) on cryptomonad *Rhodomonas salina*. With lower cell density, cell structures of *R. salina* were damaged in one-third of the cells and the cell density decreased slightly. With higher *P. parvum* cell density, less than half of the *R. salina* cells remained in the nutrient limited cultures after 23 h. *P. parvum* grown in nutrient balanced cultures negatively affected *R. salina* cells. The cellular N:P ratio, which was scaled to the Redfield ratio, could explain 67 and 75% of the variation in the haemolytic activity and the allelopathic effect, respectively. Our results confirm the hypothesis that nutrient deficiency increases toxicity of *P. parvum*. The haemolytic activity varied during semi-continuous culturing, but it was significantly dependent on intracellular N:P ratios. Due to the allelopathic effect, formation of a *P. parvum* bloom may accelerate after a critical cell density is reached when the competing species are eliminated.

KEY WORDS: *Prymnesium parvum* · Toxicity · Haemolytic activity · Allelopathy · C:N:P stoichiometry · Harmful algal bloom · Phytoplankton · Nutrients

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INTRODUCTION

Blooms of the haptophyte *Prymnesium parvum* have been reported around the world in brackish and marine waters (Edvardsen & Paasche 1998). Studying the characteristics of *P. parvum* has been motivated by the large economic losses it has caused, especially in fish farms (Edvardsen & Paasche 1998). *P. parvum* damages fish by destroying their gills (Yariv & Hestrin 1961, Shilo 1971) through toxins the cells excrete into the water (Shilo & Aschner 1953, Shilo & Rosenberger 1960). Taxonomical research suggests that haptophytes *P. parvum* and *P. patelliferum* represent differ-

ent generations rather than separate gene pools, both belonging to the species *P. parvum* (Larsen 1999).

Nutritional conditions influence the toxicity of *Prymnesium parvum*. Its toxicity may increase when nutrients are limited, which causes cellular stress and finally leads to excretion of toxins into the water (Johansson & Granéli 1999b). This phenomenon is not unique to *P. parvum*. It is found in another phytoplankton species *Chrysochromulina polylepis* (another haptophyte), which is also more toxic when nitrogen and phosphorus are limited (Johansson & Granéli 1999a). Furthermore, dinoflagellates like *Alexandrium minutum* may contain more toxins in phosphorus

*Email: pauliina.uronen@environment.fi

(Lippemeier et al. 2003) or nitrogen-limited conditions (Béchemin et al. 1999). Frangópulos et al. (2004) have studied the link between nutrient uptake affinity and toxicity in *Alexandrium* species. They found a correlation between cellular toxicity of *Alexandrium* species and a lower ability to take up inorganic phosphorus. They showed that those species, which are the poorest competitors in nutrient-limited conditions, were the most toxic ones (Frangópulos et al. 2004).

Bloom-forming species manage to outcompete co-occurring algae, and thereby reach a dominant status in the phytoplankton community. Species, which form blooms, must therefore have special characteristics that support this development. Smayda (1997) listed 4 adaptations that may have evolved to give advantage to some species in situations when other species are better competitors for nutrients: (1) nutrient-retrieval migrations, (2) mixotrophic nutritional tendency, (3) allelochemically enhanced interspecific competition, and (4) antipredatory defence mechanisms. The last 3 adaptations on Smayda's list are characteristics of *Prymnesium parvum*. They may help to explain how *P. parvum* achieves better growth and forms dense blooms. *P. parvum* is a mixotroph and it can feed phagotrophically on different-sized prey (Nygaard & Tobiesen 1993, Tillmann 1998, Legrand et al. 2001, Martin-Cereceda et al. 2003). It, however, cannot use nitrogen, which is bound to dissolved organic matter (Stolte et al. 2002). *P. parvum* is allelopathic to co-existing algae by excreting toxins into the water (Fistarol et al. 2003, Granéli & Johansson 2003b, Legrand et al. 2003), and it may immobilise its prey before utilising it (Skovgaard & Hansen 2003). In addition, it may use its toxins to damage or reduce the growth of its grazers, for example dinoflagellates, ciliates and copepods (Nejstgaard et al. 1995, Nejstgaard & Solberg 1996, Koski et al. 1999, Fistarol et al. 2003, Granéli & Johansson 2003a, Rosetta & McManus 2003, Tillmann 2003). Allelopathy is important in aquatic interactions (Legrand et al. 2003) as it can affect algal succession (Keating 1977, 1978). Thus, the ability of *P. parvum* to use both allelopathic and mixotrophic features provides it to outcompete other algae and harm its own grazers.

The maximum cell density reported in *Prymnesium parvum* blooms is 1600×10^3 cells ml⁻¹ (Edvardsen & Paasche 1998). Edvardsen & Paasche (1998) report that relatively dense blooms, i.e. cell abundance over $50\text{--}100 \times 10^3$ cells ml⁻¹, are usually needed to cause fish kills. On the other hand, Kaartvedt et al. (1991) have reported an extensive fish kill with cell abundance of less than 2×10^3 cells ml⁻¹, indicating that *P. parvum* may harm aquatic organisms at lower densities, as well. Copepods and microzooplankton

can also be afflicted by *P. parvum* at low cell densities (Nejstgaard et al. 1995, Nejstgaard & Solberg 1996, Koski et al. 1999, Granéli & Johansson 2003a, Rosetta & McManus 2003). The importance of allelopathy of *P. parvum* on the structure of plankton communities has been examined at high cell densities, for example $460 \times 10^3 \pm 27$ cells ml⁻¹, by Fistarol et al. (2003), but evidence that allelopathy plays a role at lower cell densities (less than or equal to 10×10^3 cells ml⁻¹) is scarce (Skovgaard & Hansen 2003, Skovgaard et al. 2003).

Therefore, we focused our experiments on relatively low cell densities of *Prymnesium parvum* (final cell densities 2 and 5×10^3 cells ml⁻¹) and observed the effects on the algae cryptomonad *Rhodomonas salina*. *R. salina* was chosen as a target species, because it is a common cryptomonad in the Baltic Sea, and it occurs in the plankton community at the same time as *P. parvum*. Additionally, *R. salina* and *P. parvum* are close in size and form, and therefore they may be competitors for resources and the same grazers may potentially graze on them.

Besides the effects of *Prymnesium parvum* on *Rhodomonas salina*, our other aim was to follow the toxicity of *P. parvum* as haemolytic activity over a long time period, as current information is based on measurements made of a single sampling during culture growth (Johansson & Granéli 1999b). Cellular organic nutrient contents were used to estimate nitrogen and phosphorus minimum demands in the cells, and to calculate cellular nutrient ratios. The aim was to evaluate the significance of nutrient limitation on both cellular toxicity and the allelopathic potential of *P. parvum*.

MATERIALS AND METHODS

To examine the combined effect of nutrient limitation and low cell densities, we used *Prymnesium parvum* cultures grown in three different nutrient treatments. The experiments were carried out with diluted cultures, and they contained both *P. parvum* cells and their growth media. Allelopathy is usually examined using cell-free filtrates excluding the bias of potential competition between the cells and potential phagotrophy. Since we worked with low cell densities, it was necessary to keep the *P. parvum* cells in the samples to ensure continuous toxin production during the experiments, because excreted toxins are quickly eliminated in light (Reich & Parnas 1962, Meldahl et al. 1995).

***Prymnesium parvum* and *Rhodomonas salina* cultures.** *P. parvum* strain KAC 39 (Kalmar Algal Collection, University of Kalmar) was grown in nine replicate 15 l, non-axenic batch cultures in modified *f*/20

medium (1/20 of modified *f* medium) (Guillard & Ryther 1962), in Nalgene polycarbonate vessels for 8 d. The growth media was prepared from filtered (0.2 µm Sartobran capsule) and autoclaved Baltic Sea water (salinity 6 psu). Nutrients, trace metals and vitamins were filter-sterilised before being added to the seawater. The final nutrient concentrations of the modified *f*/20 medium were 58 µM NO₃ and 3.6 µM PO₄, which gave the Redfield atomic N:P ratio of 16:1. Vitamins were added according to the MET 44 medium (Schöne & Schöne 1982), modified to a final concentration of 0.5, 0.5 and 0.1 µg l⁻¹ of vitamins B₁₂, biotin and thiamin, respectively. Trace metals were added in 1/10 of the L1 medium (Andersen et al. 1997).

On Day 8 the batches were turned into semi-continuous cultures (Johansson & Granéli 1999b): 20% of the culture was removed and the same amount of new media was added daily. The 9 batches were divided randomly into 3 groups (3 replicates of each nutrient treatment): in +NP the N:P ratio of the added media was 16:1 (58 µM NO₃ and 3.6 µM PO₄); in -N it was 4:1 (16 µM NO₃ and 4 µM PO₄) and in -P it was 80:1 (80 µM NO₃ and 1 µM PO₄). The cultures were aerated gently, and the temperature was kept at 16°C. Irradiance was 330 µmol photons m⁻² s⁻¹ outside the containers on the bright side and <100 µmol m⁻² s⁻¹ on the dark side (measured with Li-Cor LI-1000 DataLogger). The light: dark cycle was 14 h light: 10 h dark. After 7 d of semi-continuous culturing, samples were taken daily from the outgoing medium for nutrient, cell density and chemical composition (C, N, P) analyses.

Cryptomonad *Rhodomonas salina* (TV22/4, Tvärminne Zoological Station) was used in the experiments as a non-toxic target species. It was cultured in the modified *f*/20 medium as a non-axenic batch at 18°C, in 75 µmol photons m⁻² s⁻¹ (Li-Cor LI-1000 DataLogger) with a light: dark cycle of 14 h light: 10 h dark.

Allelopathic effect of *Prymnesium parvum* on *Rhodomonas salina*. On Days 16 and 18, subsamples of *P. parvum* grown in different nutrient conditions (-N, -P, +NP) were diluted with corresponding nutrient modified media to a final cell density of 10 000 cells ml⁻¹. In parallel, *R. salina* culture was diluted with *f*/20 medium to a cell density of 10 000 cells ml⁻¹. Five ml of *P. parvum* and 5 ml of *R. salina* (Expt I), and 2 ml of *P. parvum* and 8 ml of *R. salina* (Expt II) were mixed to a total volume of 10 ml in 20 ml glass vials. Thus, the final *P. parvum*:*R. salina* cell ratios were 1:1 (Expt I) and 1:4 (Expt II), i.e. 5000 + 5000 cells ml⁻¹ (Expt I) and 2000 + 8000 cell ml⁻¹ (Expt II), respectively. Control treatments were done by mixing 5 ml of corresponding growth media (*f*/20, *f*/20-N or *f*/20-P) with 5 ml of diluted *R. salina* culture in 20 ml glass vials. The mixtures were incubated in the same light and tempera-

ture conditions as described above for the *P. parvum* cultures. Triplicate samples were removed at different time intervals (0, 15, 60, 120, 180, 360 min and 23 h), and the incubations were stopped at each time point by fixing the samples with glutaraldehyde (final conc. 2% v/v).

The cell number of *Rhodomonas salina* was counted with a light microscope using the Utermöhl technique (Utermöhl 1958). At least 400 cells were counted in each sample. The counted total cell density included both intact cells as well as cells with a visible cell shape but a damaged cell structure. The allelopathic effect of *Prymnesium parvum* was expressed as the proportion of damaged *R. salina* cells relative to the total number of cells present at each time point. Feeding cells of *P. parvum* were also observed as Skovgaard et al. (2003), however, the abundance was low (<2 to 3 %) during the incubations in all samples.

Analytical procedures. Nutrients and cellular chemical composition: Free inorganic and particulate organic nutrients were measured daily after seven days of semi-continuous culturing. Inorganic nutrients (NO₃-N, PO₄-P) were measured spectrophotometrically using standard procedures (Grasshoff et al. 1983, Koroleff 1983). As the replicate cultures followed each other well according to cell density and free inorganic nutrient concentrations, the replicate culture samples were pooled before measuring the particulate organic nutrients. Samples (20 ml) for particulate organic C and N (POC, PON) were filtered onto acid-washed and precombusted Whatman GF/F filters and stored individually in petri dishes. Samples (10 ml) for particulate organic phosphorus (POP) were prepared similarly, but were placed into acid-washed, precombusted scintillation vials with an addition of MgSO₄, dried at +80°C and stored capped until being analyzed according to Solorzano & Sharp (1980). The POC and PON were analyzed from the filters with a mass spectrometer (Roboprep/Tracermass, Europa Scientific UK).

Cell densities: Cell density in the *Prymnesium parvum* cultures was counted from fresh samples using a particle counter ELZONE (Particle Data). Daily growth rates (µ) were calculated using the equation:

$$\mu = \ln N_1 - \ln (N_0 \times 0.8)$$

where N_0 and N_1 represented cell densities just before the 20% dilution on consecutive days.

Haemolytic activity: Haemolytic activity (HA) was determined from *Prymnesium parvum* cells and cell-free filtrate. Three replicate cultures were pooled before measuring the haemolytic activity. Cell-free filtrate was collected after filtering 10 ml of the culture onto GF/C glass microfibre filters. Haemolytic sub-

stances in the *P. parvum* cells were extracted with 2 ml of methanol from the filter (Igarashi et al. 1998, Johansson & Granéli 1999a). The HA of both cell methanol extract and the GF/C-filtrate on horse blood cells was measured in duplicate (Igarashi et al. 1998, Johansson & Granéli 1999a). The horse blood was washed and resuspended in isotonic phosphate buffer (Fistarol et al. 2003), after which 200 µl of horse blood cell suspension in the isotonic phosphate buffer and 50 µl of the cell methanol extract or the GF/C-filtrate was pipetted into microplate wells (Falcon, 96 wells) in different ratios (0 to 100%). A standard curve was made with saponin (Sn). After 1 d incubation at room temperature in the dark, the microplates were centrifuged (3000 rpm, 5 min, Beckman Allegra TM centrifuge), and the supernatant (110 µl) was transferred to new microplate wells (black Costar®, 96 wells). The absorbance at 540 nm was read on a Fluostar 403 microplate reader. The results were expressed as saponin equivalent (SnEq) µg ml⁻¹ in *P. parvum* cultures and calculated further to saponin equivalent (SnEq) pg cell⁻¹.

Statistical analyses. To analyse the results of the *Prymnesium parvum* cultures, we used the 1-way repeated measures analysis of variance (ANOVA). To analyse the effects on *Rhodomonas salina*, we used the 1-way analysis of variance and the *t*-test. If conditions for ANOVA (normality and equality of variances) were not met, the non-parametric Kruskal-Wallis test was used instead. The Student-Newman-Keuls method (SNK) was used for pairwise multiple comparisons if a significant difference between the groups was found. Analyses were performed using SigmaStat for Windows 3.0.1 (SPSS) software.

RESULTS

Prymnesium parvum cultures

Growth

Three replicate *Prymnesium parvum* cultures were grown for each nutrient treatment. During Days 15 to 26, the cultures which were diluted with nutrient balanced (+NP) media had the highest cell density, while in the -P and -N cultures the cell densities corresponded to 86 ± 5% and 41 ± 5% of the +NP cultures, respectively (Table 1, Fig. 1).

Cellular content

Nutrient concentrations used for daily dilutions during semi-continuous culturing set the limits for cell growth. With nutrient balanced media, the total particulate organic nitrogen and phosphorus concentrations were close to the inorganic nitrogen and phosphorus concentrations in the growth media (Table 1). In the -P cultures, the particulate organic nitrogen concentration was high and the particulate organic phosphorus concentration remained low (Table 1). In the -N cultures, the nitrogen concentration was slightly higher in the particulate organic form than in the inflowing media, and the concentration of particulate organic phosphorus was lower than in the inflowing media (Table 1).

The POP content per cell differed significantly between treatments (ANOVA, *p* < 0.001) (Table 1, Fig. 2). The highest content was in the -N treatment (SNK, *p* < 0.001) indicating that *Prymnesium parvum* cells are capable of storing significant amounts of phosphorus, whereas in the -P treatment, the POP content per cell was the lowest (SNK, *p* < 0.001). The PON content in the cells differed significantly in the treatments as well

Table 1. *Prymnesium parvum* cultures on Days 15 to 26 with 20% daily dilution. Inorganic nutrients (NO₃-N and PO₄-P) in the inflowing and outflowing media (n = 10); daily growth rate (n = 12); particulate organic C, N and P in the cultures and per cell; particulate organic C:N, C:P and N:P molar ratios (n = 11) and haemolytic activity (n = 11). Mean ± SD. Levels of significance: *** *p* < 0.001; ** *p* < 0.01; * *p* < 0.05; ns: not significant; ^{a,b,c} indicate groups of treatments with significant differences with at least *p* < 0.05. POC: particulate organic C; PON: particulate organic N; POP: particulate organic P

	+NP	-N	-P
Nutrients in the inflowing media			
NO ₃ -N (µM)	58	16	80
PO ₄ -P (µM)	3.6	4	1
N: P	16.1:1	4:1	80:1
Nutrients in the outflowing media			
NO ₃ -N (µM)	1.1 ± 0.2	0.5 ± 0.1	11.6 ± 1.7
PO ₄ -P (µM)	0.6 ± 0.2	2.2 ± 0.2	0.3 ± 0.2
Cell density (cells ml ⁻¹)***	3.6 ± 0.2 × 10 ^{5a}	1.5 ± 0.2 × 10 ^{5b}	3.1 ± 0.1 × 10 ^{5c}
Daily growth rate ^{ns}	0.21 ± 0.07	0.18 ± 0.06	0.22 ± 0.08
POC (µM)***	622.7 ± 56.9 ^a	261.6 ± 59.8 ^b	617.0 ± 79.1 ^a
PON (µM)***	59.0 ± 4.0 ^a	24.1 ± 4.0 ^b	60.5 ± 4.8 ^a
POP (µM)***	3.8 ± 0.4 ^a	2.1 ± 0.4 ^b	1.9 ± 0.3 ^c
POC (pg C cell ⁻¹)**	20.5 ± 1.9 ^a	22.2 ± 3.8 ^{a,b}	24.6 ± 2.8 ^b
PON (pg N cell ⁻¹)***	2.3 ± 0.1 ^a	2.4 ± 0.2 ^b	2.8 ± 0.2 ^c
POP (pg P cell ⁻¹)***	0.32 ± 0.03 ^a	0.46 ± 0.06 ^b	0.19 ± 0.03 ^c
POC: PON ^{ns}	10.5 ± 0.6	10.8 ± 1.1	10.2 ± 0.9
POC: POP***	166.4 ± 15.5 ^a	124.3 ± 11.7 ^b	339.6 ± 68.1 ^c
PON: POP***	15.8 ± 1.2 ^a	11.6 ± 0.9 ^b	33.1 ± 4.4 ^c
Haemolytic activity (SnEq pg cell ⁻¹)***	105.0 ± 17.9 ^a	135.9 ± 24.1 ^b	200.1 ± 34.5 ^c

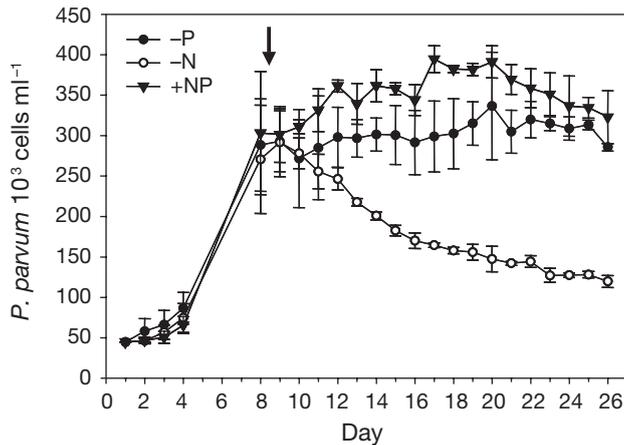


Fig. 1. *Prymnesium parvum*. Cell density (cells ml⁻¹) in the cultures with 3 nutrient treatments. The molar N:P ratio was 80:1, 4:1 and 16:1 in the dilution media used in the -P, -N and +NP treatments, respectively. The arrow shows the beginning of the 20% daily dilution. Values are mean ± SD (n = 3)

(ANOVA, $p < 0.001$): the PON content per cell was higher in the -P treatment than in the +NP or -N treatments (SNK, $p < 0.001$ for both), and furthermore, the PON content per cell was higher in the -N treatment than in the +NP treatment (SNK, $p < 0.05$). The POC content per cell was higher in the -P cultures than in the nutrient balanced cultures (ANOVA, $p < 0.01$; SNK, $p < 0.01$), but there was no difference between the other treatments (Table 1, Fig. 2).

The average N:P molar ratio in the particulate form ranged from 11.6 to 33.1, with a minimum value of 10.1 in the -N treatment and a maximum value of 40.4 in the -P treatment (Fig. 3). In the nutrient balanced treatment, the cultures were growing steadily with a PON:POP molar ratio close to the Redfield ratio (Table 1, Fig. 3), whereas in the -P cultures the cells were phosphorus limited with a high PON:POP ratio. On the contrary, in the -N cultures the PON:POP ratio was lower than the Redfield ratio of 16:1, which indicates nitrogen limitation in the cells. Thus, there was a significant difference in the PON:POP ratios between the treatments (ANOVA, $p < 0.001$) (Table 1, Fig. 3). In all cultures, the POC:PON ratio was higher (10.2–10.8) than the Redfield atomic ratio (6.6:1) (Table 1)

Haemolytic activity

All the cultures were haemolytic (Fig. 4). During Days 16 to 26, *Prymnesium parvum* cells had the highest haemolytic activity (HA) in the -P cultures and in the -N cultures, whereas HA was lowest in the +NP cultures (Table 1, Fig. 4). The haemolytic activity varied within the treatments during this period. However, all the treatments differed significantly from each

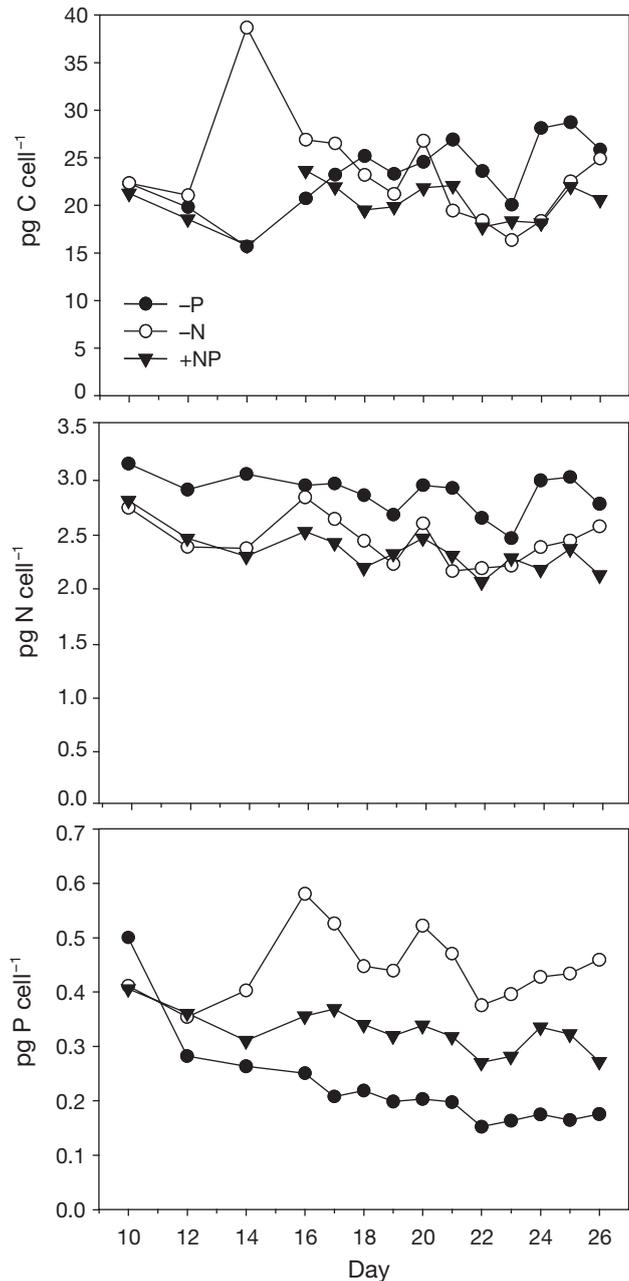


Fig. 2. *Prymnesium parvum*. Particulate organic carbon (POC pg cell⁻¹), nitrogen (PON pg cell⁻¹) and phosphorus (POP pg cell⁻¹) in the -P, -N and +NP treatments

other when compared over the whole time period (ANOVA, $p < 0.001$; SNK, $p < 0.001$ for all treatments). Analyses with cell-free filtrates showed no HA.

In order to study the relation of HA and nutrient limitation in more detail, the N:P molar ratios were scaled in proportion to the Redfield ratio (Fig. 5). Hence, for example a ratio of 32:1 was interpreted as an equally severe limitation as a ratio of 8:1, because both ratios represented a 2-fold departure from to the Redfield ratio of 16:1. As a result, this scaling

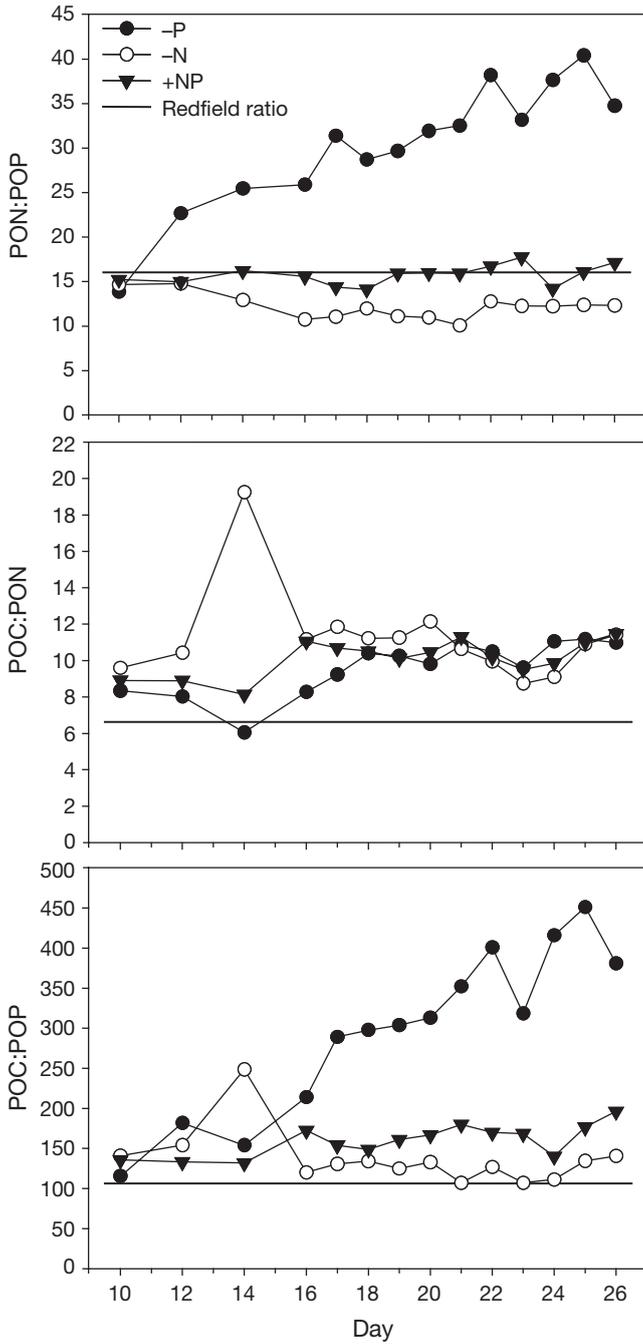


Fig. 3. *Prymnesium parvum*. Particulate organic nutrient ratios (atomic N:P, C:N and C:P) in the -P, -N and +NP treatments

procedure ensured that both nitrogen and phosphorus limitation were taken into account with the same magnitude. The relation of the cellular PON:POP ratios and HA had a high positive correlation (Pearson product moment correlation analysis 0.817, $p < 0.0001$), and the scaled N:P ratio could explain as much as 67% (linear regression, $r^2 = 0.667$; $p < 0.001$) of the variation in the HA (Fig. 5).

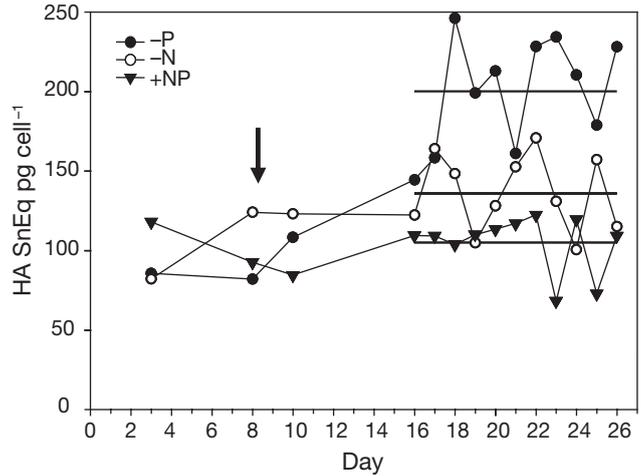


Fig. 4. *Prymnesium parvum*. Haemolytic activity (HA SnEq pg cell⁻¹) in the -P, -N and +NP treatments. The arrow shows the beginning of the semi-continuous dilution with nutrient modified media. The lines represent the mean haemolytic activity in each treatment during the apparent steady-state growth (16 to 26 d)

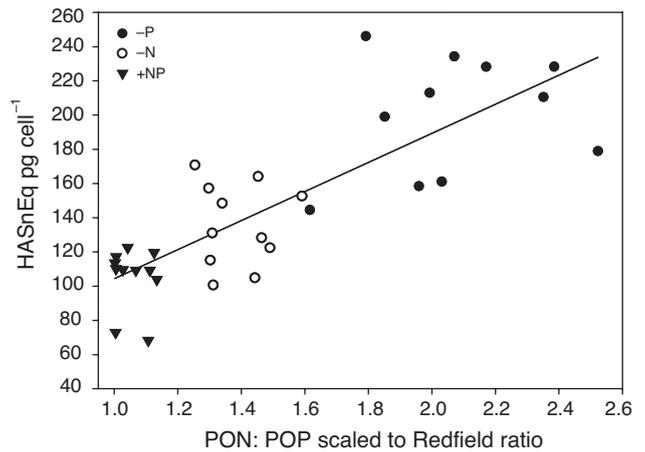


Fig. 5. *Prymnesium parvum*. Haemolytic activity (HA SnEq pg cell⁻¹) versus the PON:POP ratio on Days 16 to 26. The PON:POP ratios are scaled to the Redfield ratio and values under 16:1 are scaled inversely. Regression line $r^2 = 0.667$; $p < 0.001$ ($n = 33$)

Effect on *Rhodomonas salina*

Expts I and II

During Expt I (*Prymnesium parvum* cell density 5×10^3 cells ml⁻¹), the *Rhodomonas salina* total cell density decreased in all treatments (ANOVA, $p < 0.001$; compared all times points within each treatment) (Fig. 6A). Already during the first hour of incubation, the *R. salina* cell density decreased in the -N and -P treatments to $80 \pm 0\%$ and $77 \pm 3\%$, respectively (Kruskall-Wallis, $p < 0.05$; SNK, $p < 0.05$ for both treatments).

Thus, after 1 h, the cell density was significantly lower in the -N and -P treatments than in the +NP treatment (ANOVA, $p < 0.001$; SNK, $p < 0.05$ for both). *R. salina* cells were also destroyed in the +NP treatment, but at a slower rate than in the nutrient deficient treatments. Finally, at the end of the 23 h incubation, the *R. salina* cells were destroyed most efficiently in treatments -N and -P, and least in the +NP treatment (ANOVA, $p < 0.05$; SNK, $p < 0.05$ for both) (Fig. 6A). No significant difference could be found when comparing treatments -N and -P. In Expt I, the proportion of damaged *R. salina* cells, i.e. those cells that were present and with a visible cell form but had damage in the cell structure, rose to 11–18% within 15 min (ANOVA, $p < 0.001$; SNK, $p < 0.05$ in all treatments) (Fig. 6B). Within 3 h, the percentage of damaged cells increased to $37 \pm 4\%$ in treatment -N; to $42 \pm 4\%$ in treatment -P, and to $26 \pm 3\%$ in treatment +NP (SNK, $p < 0.05$ in all treatments) (Fig. 6B). After 23 h, the proportion of damaged

cells had decreased significantly in treatments -P and +NP (ANOVA, $p < 0.001$; SNK, $p < 0.05$), because the cells in worst condition had lysed and the whole cell density had decreased (Fig. 6B).

In Expt II, with a lower *Prymnesium parvum* cell density (2×10^3 cells ml^{-1}), the effect on *Rhodomonas salina* was weaker. Significant effects could only be measured in the -N treatment, where $80 \pm 6\%$ and $79 \pm 11\%$ of the cells were present after 1 and 6 h, respectively (ANOVA, $p < 0.05$; SNK, $p < 0.05$) (Fig. 7A). Although only minor effects of *P. parvum* on *R. salina* were seen in terms of the cell density, it was still possible to observe damage in the *R. salina* cell structures over 23 h (Fig. 7B), and the treatments differed significantly from each other (ANOVA, $p < 0.05$).

In control treatments, the *Rhodomonas salina* cell density increased slightly and the proportion of damaged cells ranged between 0 and 4% during the whole incubation time (Fig. 6A,B).

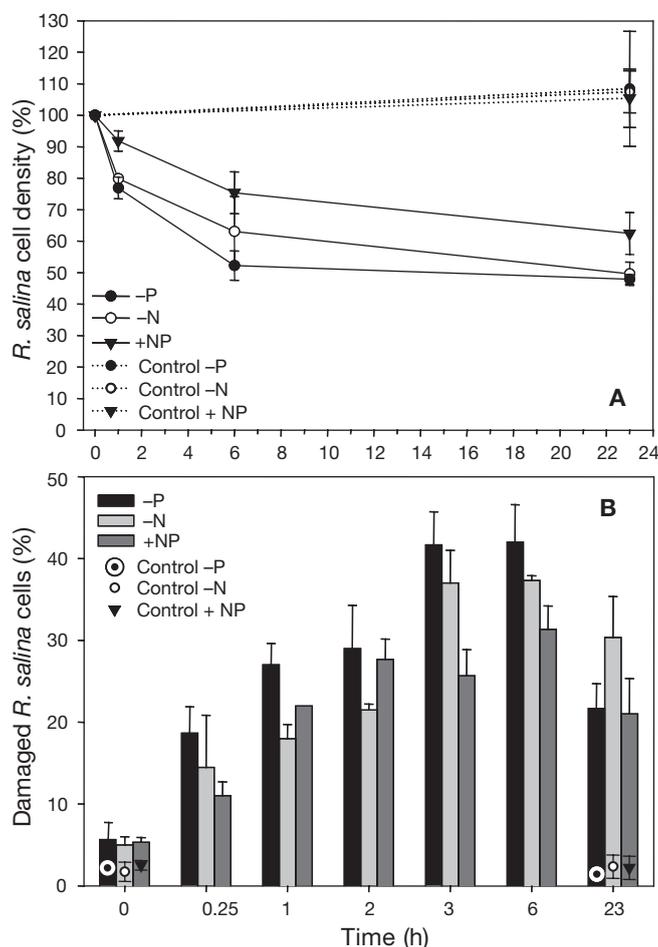


Fig. 6. *Rhodomonas salina*. Expt I. (A) Total cell density (% of the beginning) and (B) damaged cells (% of the total cell density). Mean + SD ($n = 3$) in the -P, -N and +NP treatments and in the -P, -N and +NP controls. Note the continuous scaling of x-axis in (A) and categorical scaling in (B)

Comparison of Expts I and II

After 23 h, the experiments with different *Prymnesium parvum* cell densities differed significantly from each other. In treatments -P and -N, the higher *P. parvum* cell density lysed *Rhodomonas salina* cells more than the lower *P. parvum* cell density (t -test, $p < 0.01$ for both treatments). In contrast, in the +NP treatment no statistical difference was found between the experiments. In the -P treatment, the experiments differed after 1 h, whereas there was no difference in the -N treatment at the same time.

In the experiment with the higher *Prymnesium parvum* cell density, the cellular PON:POP ratio, which was scaled to the Redfield ratio, could explain 75% of the variation in the allelopathic effect ($r^2 = 0.751$; $p < 0.001$) (Fig. 8A). However, no dependence could be found in the second experiment with the lower ratio of *P. parvum* and *Rhodomonas salina* ($r^2 = 0.000$) (Fig. 8B).

DISCUSSION

Nutrients

In the nutrient-balanced cultures, *Prymnesium parvum* grew steadily with a PON:POP ratio close to 16:1, which indicated that the cells were not limited by phosphorus or nitrogen. In contrast, nutrient limitation was obvious in the -N and the -P cultures, as the particulate organic nutrient ratios deviated from the Redfield ratio (Table 1, Fig. 3), and the residual inorganic concentrations confirmed the limitation patterns (Table 1). In the phosphorus-limited cultures the phos-

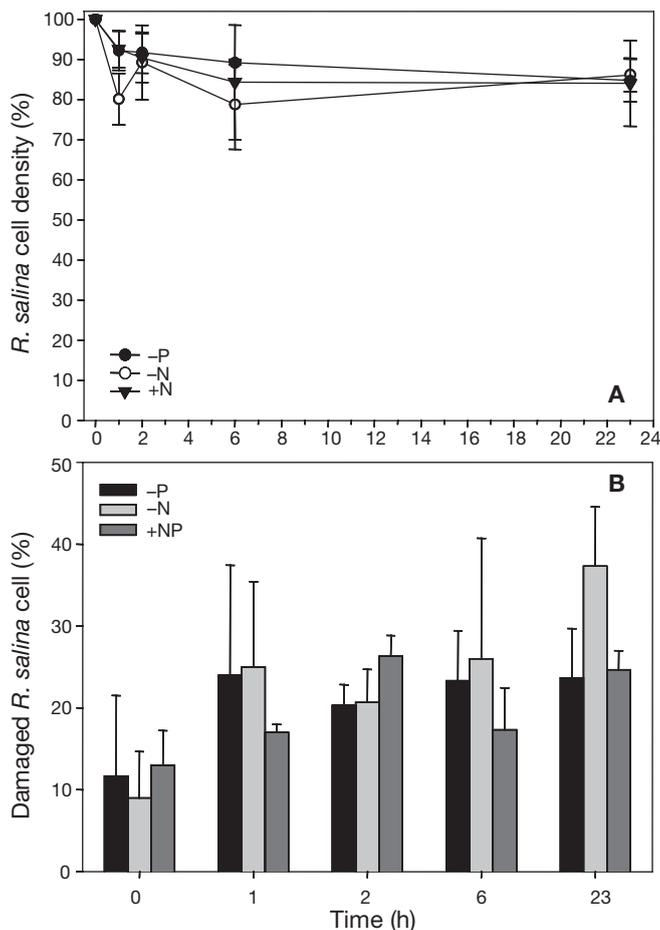


Fig. 7. *Rhodomonas salina*. Expt II. (A) Total cell density (% of the beginning) and (B) damaged cells (% of the total cell density). Mean + SD (n = 3) in the -P, -N and +NP treatments. Note the continuous scaling of x-axis in (A) and categorical scaling in (B)

phorus content per cell was on average $0.19 \text{ pg P cell}^{-1}$, which was lower than previously reported values ($0.3 \pm 0.1 \text{ pg P cell}^{-1}$ [Johansson & Granéli 1999b]). The average PON contents of 2.4 and $2.3 \text{ pg N cell}^{-1}$ in the nitrogen-limited and nutrient balanced cultures were also lower than reported previously (2.7 ± 0.3 and $3.7 \pm 0.7 \text{ pg N cell}^{-1}$, respectively [Johansson & Granéli 1999b]). Thus, the values from our cultures can be used as estimates of minimum nutrient demands in the *P. parvum* cells for maintaining the very basic cellular functions.

The mean phosphorus content in the cells was 1.7- and 2.4-fold higher in the +NP and -N cultures than in the -P cultures, respectively. In contrast, the nitrogen and carbon contents per cell were in the same range in all the treatments (Table 1). This means that flexibility of the cellular phosphorus content was higher than the flexibility of the carbon or nitrogen contents. The cellular functions have different demands for carbon, phos-

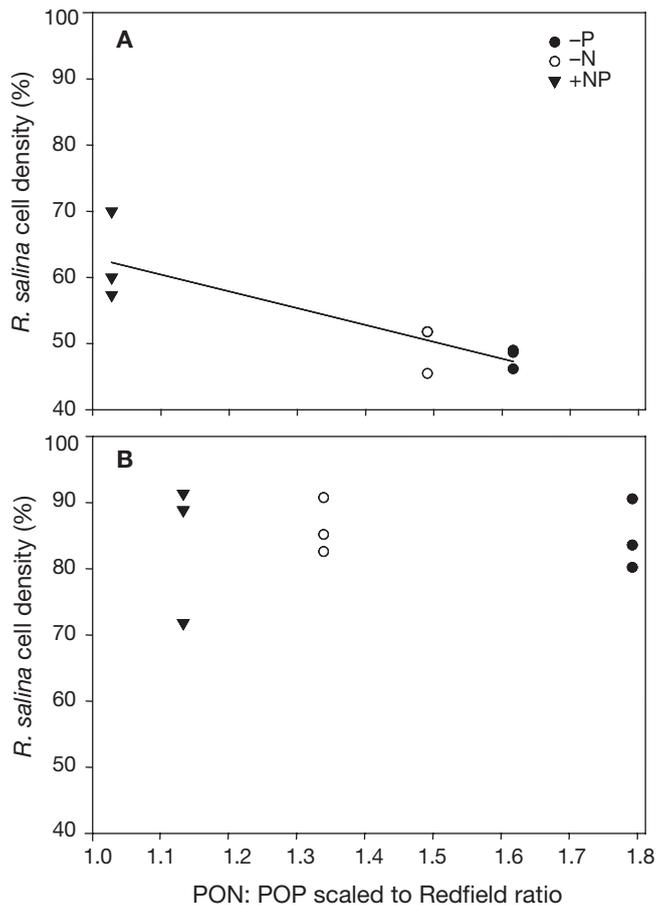


Fig. 8. *Pymnesium parvum*. Allelopathic effect versus the PON:POP ratio. *Rhodomonas salina* cell density after 23 h (% of the beginning) indicates the allelopathic effect. The PON:POP ratios are scaled to the Redfield ratio and values under 16:1 are scaled inversely. (A) Expt I regression line $r^2 = 0.751$; $p < 0.001$ and (B) Expt II not significant (n = 9)

phorus and nitrogen ratios (Sterner & Elser 2002). Because nitrogen and phosphorus are used for different purposes in the cell, the tolerance for enduring limitation of these nutrients differs as well. In the balanced and phosphorus-limited cultures, growth rates were determined by the dilution rate of semi-continuous culturing (Table 1). Thus, the cells could maintain the given growth rate with the nutrients provided, although they could not maintain the Redfield N:P ratio in the phosphorus-limited cells. Consequently, the N:P ratio increased continuously during culturing. In contrast, nitrogen limitation was more constricted: the nitrogen-limited cells had a N:P ratio closer to the Redfield ratio, and the nitrogen concentration used in the -N cultures could not support as high cell densities as the concentrations used in the -P and the +NP cultures. In our -P cultures, there could have been indications of accumulation of carbon in the cells (Skovgaard et al. 2003), as the carbon content in the cells was higher and the cell

density was lower than in the +NP cultures. However, the POC:PON ratio was only slightly higher than expected from the Redfield ratio, and there was no difference between the nutrient treatments.

The Redfield ratio is generally used in nutrient balanced treatments, because it is considered an estimate of nutrient requirement ratio for algae. However, the optimum N:P ratio may vary between algal species (Hecky & Kilham 1988), and the critical N:P ratio, which determines whether nitrogen or phosphorus is the limiting nutrient for the species, may range between species and regions (Geider & La Roche 2002). For example, the critical N:P ratio has been found to be as high as 40 to 50:1 in the haptophyte *Pavlova lutheri* with high growth rates (Terry et al. 1985). To our knowledge, there are no studies on the nutrient requirements of *Prymnesium parvum*, which could elucidate the actual critical N:P ratio in this species.

Haemolytic activity

Imbalance in the cellular nutrient composition may disturb the metabolism in the cells, which may lead to an increase in toxin production (Johansson & Granéli 1999b, Fig. 5). In our study, haemolytic activity of *Prymnesium parvum* was measured 14 times during the whole culturing period. Daily results revealed that HA varied largely within the treatments during Days 16 to 26. Despite the variation within the treatments, all the treatments differed significantly from each other. In the +NP treatment, cellular HA was the lowest while the PON:POP ratio was close to the Redfield ratio. In contrast, the phosphorus limited -P cultures were the most haemolytic ones (Fig. 4). Similar results, based on HA measurements at one single sampling occasion during culturing, were presented in a previous study (Johansson & Granéli 1999b). Our results confirm that the cellular nutrient ratio determines to a large extent the haemolytic activity of *Prymnesium parvum*, and validate the conclusion that nutrient stress increases toxicity in *P. parvum*.

HA could be measured in all cultures already during the exponential growth phase, i.e. before the beginning of semi-continuous dilution with the three nutrient treatments (Fig. 4). After commencing dilution with the nutrient depleted media, HA started to increase in the -P and in the -N cultures. In contrast, HA remained, on average, at the same level in the +NP treatment during the 26 days of culturing. These results indicate that *P. parvum* cells grown in balanced medium are haemolytic. One reason for this result could be the relatively high cell density in the cultures. The cell density in our -P and +NP cultures were 6 to 7 times higher than reported in previous studies (Johansson &

Granéli 1999b, Granéli & Johansson 2003b). Therefore, the species could be toxic in balanced conditions as well, if the cell concentration is high enough. The cultures were continuously aerated with gentle bubbling to prevent increase of the pH, which may influence the growth rate of phytoplankton species and strengthen the toxic effects of harmful algae (Schmidt & Hansen 2001, Hansen & Hjorth 2002).

HA was not detected in any of the *Prymnesium parvum* cell-free filtrates. However, other studies carried out with these cultures showed that the filtrates had toxic effects on planktonic organisms (Barreiro et al. 2005, A. Giannakourou et al. unpubl., S. Sopanen et al. unpubl., P. Uronen et al. unpubl.). These results indicate that the toxicity level in the filtrate might have been under the detection limit in the HA test, or that the substances toxic to other plankton might have been different from haemolysins.

Allelopathic effects on *Rhodomonas salina*

Both *Prymnesium parvum* cell densities, which were used in the allelopathic experiments, were relatively low compared to the densities that have usually been reported to cause severe damage to fish (Edwardsen & Paasche 1998). However, even with these densities it was possible to measure effects on the cryptomonad *Rhodomonas salina*.

When *Prymnesium parvum* was used at the cell density of 5×10^3 cells ml⁻¹, up to half of the *Rhodomonas salina* cells disappeared during the 23 h incubation, and over one third of the remaining cells were in bad condition: they were swollen or the cell membranes were damaged. The variation in the allelopathic effect could mainly be explained with the cellular nutrient ratio (Fig. 8A) and thus, the nutrient limited treatments caused significantly more damage to *R. salina* than the nutrient balanced cultures. Although the nutrient balanced cultures were toxic as well when the haemolytic activity was used as an indicator, the effect on *R. salina* remained weaker. In the control treatments, exposure to nutrient limiting growth media did not harm *R. salina* cells, which indicates that the nutrient limited media did not cause the harmful effect.

When *Prymnesium parvum* was used at the lower cell density (2×10^3 cells ml⁻¹), 80 to 85 % of the *Rhodomonas salina* cells remained in the mixtures after 23 h, and *P. parvum* grown in the nutrient balanced cultures destroyed the *R. salina* cells to the same degree as *P. parvum* grown in the -N and -P treatments. However, the cells had similar damage in their cell membranes than observed in the higher *P. parvum* cell density. Thus, the allelochemicals had some effects on *R. salina*, but the effects were weak. Our results are in accordance with

the suggestion that mortality caused by *P. parvum* could be affected not only by the density of *P. parvum* itself but also by the density of the target organisms (Tillmann 2003). The target algae may bind toxins from the water and hence reduce the free toxin molecules.

Bloom dynamics

A phytoplankton bloom starts with a low cell density, and several factors determine whether it may eventually develop. *Prymnesium parvum* has several properties which enable it to gain dominance in the plankton community and finally form blooms. Allelopathy is widely recognised as a means for algae to interact with each other, and inhibit the growth of their competitors (Inderjit & Dakshini 1994). Consequently, this may enable formation of toxin-producing blooms, when other species are eliminated (Legrand et al. 2003). The effect can be even stronger under conditions where competing species suffer from stress, for example nutrient limitation (Fistarol 2004).

In the long run only slight changes in survival and growth rate may have a strong impact on the species composition (Riegman 1998). Our experiments focused on the first steps in a potential bloom formation process. We observed that with a relatively low cell density, the allelopathic influence of *Prymnesium parvum* was very weak, but it could still harm the co-existing cryptomonads. When the higher cell density was used, the negative effects on the cryptomonad cell density were obvious. It is interesting to notice that when the *P. parvum* cell density was low, differences between the different nutrient treatments could not be found: all the nutrient treatments had a similar weak effect on *Rhodomonas salina*. However, the dilutions for the experiments were made with fresh media with corresponding nutrient modifications, which may have temporarily relieved the nutrient limitation of the *P. parvum* cells, and thereby may have weakened the effects between the treatments. On the contrary, when *P. parvum* was used at the higher cell density, the effect on the target species was stronger in the nutrient deplete cultures than in the nutrient balanced cultures.

Since the cultures used were non-axenic, there were bacteria available for *Prymnesium parvum*. It may feed on associated bacteria, and thereby improve the phosphorus concentration in the cells (Nygaard & Tobiesen 1993, Legrand et al. 2001). Also, bacteria in the different cultures may have been nutrient limited, which may have affected their growth and their ability to degrade the released toxins. However, to our knowledge there are no studies on how effective bacteria are in degrading the toxins of *P. parvum*. On the other hand, *P. parvum* may have a positive indirect effect on

bacteria, because damage in coexisting algae can lead to increased release of dissolved organic matter into the water, which in turn, increases the biomass of bacteria (P. Uronen et al. unpubl.).

The changes in the *Prymnesium parvum* abundance have further impacts on other algae: The increase in its own cell density and toxin production stimulates the feeding behaviour of *P. parvum* itself (Skovgaard & Hansen 2003, Skovgaard et al. 2003). In addition, the effects on protozoan and metazoan grazers depend on the cell concentration of *P. parvum* and on the availability of other algae: small amounts of *P. parvum* together with good food may even improve the fecundity of the grazers like copepods (Koski et al. 1999), and ciliates may grow well as long as a non-toxic alga is available (Rosetta & McManus 2003). Negative effects may occur when the cell density of *P. parvum* increases, the cells suffer from nutrient limitation, or *P. parvum* is the single food source. These reasons may lead to increased mortality of ciliates (Fistarol et al. 2003, Granéli & Johansson 2003a, Rosetta & McManus 2003) or the feeding and reproduction rates of rotifers and copepods may decrease (Nejstgaard & Solberg 1996, Barreiro et al. 2005, S. Söpanen et al. unpubl.). Hence, after a critical cell density is reached by the harmful species, the development of the bloom may accelerate dramatically due to the combined effect of eliminating competitors and relieving grazing losses.

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