Vol. 31: 259–265, 2003 | AQUATIC MICROBIAL ECOLOGY
Aquat Microb Ecol | Published April 3

Effects of nutrient limitation on food uptake in the toxic haptophyte *Prymnesium parvum*

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ABSTRACT: The haptophyte *Prymnesium parvum* Carter is toxic and frequently responsible for harmful algal blooms in coastal waters. It is a mixotrophic species having the capability to feed on various planktonic microorganisms. It is frequently suggested that mixotrophic algae may obtain inorganic nutrients through phagotrophy and that nutrient depletion should then lead to increased food uptake. To study this, we investigated the feeding activity of *P. parvum* in semi-continuous, nutrient-limited cultures, using the cryptophyte *Rhodomonas baltica* as prey. *P. parvum* showed to be an active predator under all conditions investigated. After 2 h of incubation with prey, 40% of *P. parvum* cells were either feeding or contained recently formed food vacuoles. However, under the conditions used, no difference in feeding activity was found between treatments. On the contrary, the feeding activity was similar in *P. parvum* cultures that had been grown under N-limiting, P-limiting, N- and P-limiting, as well as under nutrient-replete conditions. It cannot be excluded that *P. parvum* under limiting nutrient conditions may acquire nutrients to be used in photosynthetic growth through phagotrophy. It is evident, however, that the species also feeds when inorganic nutrients are present in concentrations sufficient to support maximum phototrophic growth.

KEY WORDS: Food uptake · Phagotrophy · Mixotrophy · Toxic algae · Prymnesiophyceae · Nutrient limitation · Semi-continuous cultures · Harmful algal blooms

INTRODUCTION

Blooms of the toxic haptophyte *Prymnesium parvum* Carter have recurrently been recorded in various parts of the world for decades (Moestrup 1994, Edvardsen & Paasche 1998). These blooms are often associated with fish kills, and have caused large damage to the carp industry in Israel (Reich & Aschner 1947) and salmon and trout aquaculture in the Norwegian fjords (Kaartvedt et al. 1991). Consequently, *P. parvum* has been suggested to be one of the most harmful microalgal species worldwide (Igarashi et al. 1996). The toxin of *P. parvum* is harmful not only to fish; it also causes cell lysis in other microorganisms (Shilo 1971). The majority of *P. parvum* blooms have occurred at low salinities (1 to 5 psu; Edvardsen & Paasche 1998), but laboratory studies have shown that the species is able to thrive at salinities up to at least 30 psu (Larsen & Bryant 1998). However, even though the autecology and toxicity of *P. parvum* has been extensively studied (e.g. Rahat & Jahn 1965, Shilo 1971, Larsen & Bryant 1998), the potential importance of uptake of particulate food in the species has been widely ignored. The original description of *P. parvum* (Carter 1937) included an illustration of a cell containing what resembled a food vacuole. The phagotrophic capabilities of *P. parvum* were subsequently confirmed, and we now know that the species (including *P. parvum* f. *patelliferum* [Green, Hibberd et Pienaar] A. Larsen) is omnivorous and able to prey on bacteria (Nygaard & Tobiesen 1993, Legrand et al. 2001) as well as on protists of a wide size range (Tillmann 1998).

Mixotrophic flagellates, i.e. flagellates that are both photosynthetic and able to take up particulate food (e.g. Stoecker 1998), are known to possess a variety of feeding behaviours reflecting different mixotrophic

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life strategies (Jones 1997, Stoecker 1998). Some mixotrophic flagellates are able to supplement photosynthesis with heterotrophic carbon assimilation (Andersson et al. 1989, Sanders et al. 1990, Skovgaard 1996). In other cases, food uptake by phytoflagellates is rather a means to acquire essential growth factors (Kimura & Ishida 1989, Skovgaard 2000). It has also been speculated that some mixotrophic algae use phagotrophy as a mechanism to acquire macronutrients for use in photosynthetic growth (Caron et al. 1993, Arenovski et al. 1995, Legrand et al. 1998, Stoecker 1998). Feeding may thus supply the organism with nitrate and phosphate when concentrations of dissolved inorganic nutrients in the surrounding water are low. Mixotrophy should then be particularly advantageous during conditions of nutrient limitation and unfavourable nutrient conditions should stimulate food uptake (Jones et al. 1993, Stoecker 1998, Granéli et al. 1999a). In some cases, field data support this theory (Li et al. 2000, Smalley & Coats 2002), but attempts to provide experimental evidence are rare (Veen 1991, Stoecker et al. 1997, Li et al. 2000, Legrand et al. 2001).

In addition to having phagotrophic capabilities, *Prymnesium parvum* has been shown to be able to grow photosynthetically using amino acids as its sole nitrogen source (Rahat & Hochberg 1971). It is, therefore, natural to assume that the species is able to satisfy its need for macronutrients through phagotrophy as well. Here, we have investigated this relationship between nutrient limitation and feeding in *P. parvum*. As a measure of feeding activity of *P. parvum*, we determined the frequency of cells that contained food vacuoles or were in the process of engulfing a prey item. The aims of this study were to determine: (1) if feeding activity of *P. parvum* grown in semi-continuous, nutrient-limited cultures differs from when the cells are grown in nutrient-replete cultures; and (2) if such a potential stimulation of feeding activity by nutrient limitation is dependent on which of the macronutrients (N or P) limit growth.

MATERIALS AND METHODS

Growth of algae and preparation of medium. *Prymnesium parvum* (Kalmar University Algal Collection, KAC 39) was cultivated non-axenically in f/10-medium (0.1 strength f-medium; Guillard & Ryther 1962) prepared from GF/C-filtered and pasteurised, coastal seawater (Baltic Sea: 0.10 µM NO_3^-, 0.02 µM PO_4^{3-}, NH_4^+ was below the detection limit). The original salinity of the seawater, 7 psu, was adjusted to 10 psu by adding NaCl. Initially, 9 batch cultures were set up in 3 l Pyrex bottles each containing 2 l f/10-medium. The culture bottles were gently aerated and kept in a thermostat room at 15°C under a light:dark cycle of 16:8 h. Irradiance, measured inside the culture bottles with a QSL-100 Quantum Scalar Irradiance Meter (Biospherical Instruments), was 90 to 100 µmol photons m^{-2} s^{-1}. Cell densities were monitored at least every second day by counting >400 cells in Lugol's fixed samples in a Sedgewick Rafter counting cell (Graticules).

Semi-continuous cultures. The 9 *Prymnesium parvum* batch cultures were turned into semi-continuous cultures through daily dilutions, based on the set-up described in Johansson & Granéli (1999). The cultures were diluted at a daily rate of 15%. This corresponds to a daily algal growth rate, µ, of 0.14 d^{-1}, when $\mu = (\ln N_1 - \ln N_0)/t$, and N_0 and N_1 represent cell density at the start and the end of the growth period, t, respectively. We started diluting the cultures after they had been growing exponentially for 13 d. Culture volumes of 300 ml were replaced by modified f/10-medium with different N and P concentrations: 3 bottles received ingoing medium in which the NO_3^- concentration was assumed to be limiting for growth at an N:P ratio of 3.2:1 (–N, 23.0 µM NO_3^- and 7.2 µM PO_4^{3-}); 3 bottles received medium where both NO_3^- and PO_4^{3-} were assumed to be limiting although at a balanced Redfield atomic N:P ratio of 16:1 (–N & P, 58.0 µM NO_3^- and 3.6 µM PO_4^{3-}); and another 3 bottles were refilled with medium in which PO_4^{3-} was assumed to be limiting for growth at an N:P ratio of 80:1 (–P, 58.0 µM NO_3^- and 0.73 µM PO_4^{3-}). Trace metals and vitamins were added to all treatments in amounts corresponding to full strength f/10-medium. After 16 d of growth with daily dilutions, it became clear that the treatments would reach steady state at different cell densities. The –N & P cultures stabilised at 6×10^5 cells ml^{-1}, whereas the –P cultures seemed to stabilise at 4×10^5 cells ml^{-1} and the –N cultures at 2×10^5 cells ml^{-1} (Fig. 1). To study and compare feeding activities in unmanipulated sub-samples of the 3 treatments, similar cell densities were needed. We chose a cell density of 7×10^4 cells ml^{-1}. To obtain this cell density for all treatments, nutrient concentrations in the ingoing media were changed: in the –N medium both N and P concentrations were reduced by a factor of 2, in the –N & P medium by a factor of 6 and in the –P medium by a factor of 4. All cultures were then diluted to a cell density of 1.5×10^5 cells ml^{-1} in order to reach the desired cell density of 7×10^4 cells ml^{-1} more rapidly. These nutrient concentrations in the ingoing media (Table 1) were kept for the rest of the 49 d experiment. In addition to the nutrient-limited, semi-continuous cultures, 3 bottles containing nutrient-replete cultures, grown in f/10-medium, were also used in the experiment. When cell densities reached 7×10^4 cells ml^{-1}, these cultures were diluted daily with 30% d^{-1} and the removed culture volumes were

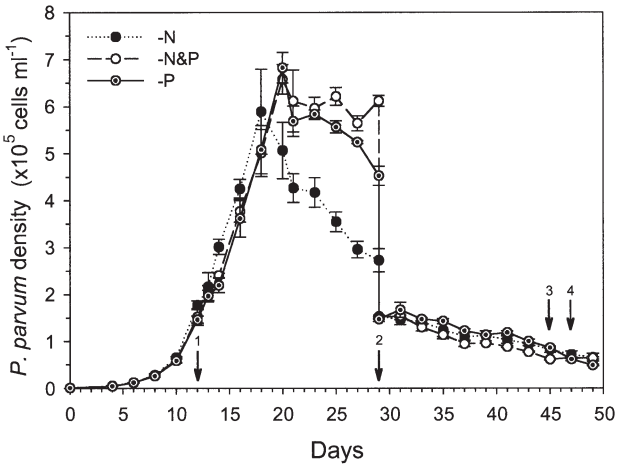

Fig. 1. *Prymnesium parvum*. Development in cell densities of the 3 semi-continuous treatments. Arrows denote: 1, onset of daily dilutions of 15% d^{-1}; 2, adjustments of nutrient concentrations and dilution of cultures; 3, feeding experiments; 4, feeding experiments and measurements of cellular compositions. Means ± SE, n = 3

replaced with f/10-medium. This dilution rate corresponded to the maximum growth rate of *P. parvum* and the cells were thus kept in exponential growth at their maximum growth rate, reaching the same cell density every day just before dilution.

Feeding experiments. Feeding activity of *Prymnesium parvum* cells having grown under different nutrient regimes was studied by mixing 10 ml *P. parvum* culture with 2 ml dense *Rhodomonas baltica* culture (KAC 30) in 20 ml glass vials. Final cell densities were approximately 6.5×10^4 prey cells ml^{-1} and the predator:prey ratio approximately 1:1. All feeding experiments were started 3 to 4 h after the onset of the light period, and the vials were incubated without agitation at temperature and light conditions similar to those of the semi-continuous cultures. To determine a suitable incubation time for the feeding experiments, we measured the percentage of feeding cells after 0, 10, 30, 60, 120, 180 and 240 min in the –N treatment. After incubation, the samples were fixed with glutaraldehyde (final concentration: 2%) and percentages of feeding cells were counted microscopically (≥400 cells counted per sample). To estimate the toxicity of *P. parvum*, the percentages of lysed prey cells were counted, i.e. prey cells that had a visibly disintegrated cell membrane. A 2 h incubation time was selected for subsequent feeding experiments. Whether agitation had any influence on feeding was tested by performing a feeding experiment (–N treatment) in which the vials were incubated on a plankton wheel rotating 1 round min^{-1}. This proved not to be the case (data not shown).

Feeding experiments were then performed for each of the 4 treatments between Days 45 and 47. For the –N, –P and –N & P treatments, feeding experiments were also made using prey cells that had been rinsed gently with nutrient-depleted seawater. This rinsing was done in order to rule out any possible effect of nutrients added along with the prey. Prey concentration for all above feeding experiments was kept at approximately 6.5×10^4 prey cells ml^{-1}. To ensure that no grazing-induced prey limitation occurred due to the relatively high predator:prey ratio used, a series of grazing experiments was also performed in which the predator:prey ratios were lowered (for the –N treatment only) by reducing the predator density (range: 0.2 to 7.2×10^4 cells ml^{-1}). Prey density was kept at 6.5×10^4 cells ml^{-1}.

Nutrient measurements. Concentrations of NO_3^-, NH_4^+ and PO_4^{3-} were measured in GF/C-filtered seawater and in GF/C filtrate of the outgoing culture media of the different treatments (Valderama 1995). In addition, particulate organic carbon (POC), nitrogen (PON) and phosphorus (POP) contents of *Prymnesium parvum* cells were measured. From each treatment, 3 × 150 ml culture volumes were retained on precombusted GF/C filters. The filters were then dried at 65°C for 48 h and analysed for POC and PON using a CHN elemental analyser (model NA 1500, Fisons Instruments). POP was measured as described in Solórzano & Sharp (1980).

RESULTS

Growth

On Days 0 to 12, i.e. while still kept as batch cultures, the specific growth rate of *Prymnesium parvum* in nutrient-replete, full-strength f/10-medium was 0.39 to 0.40 d^{-1} in the 3 different treatments (Fig. 1). Following the onset of daily dilution with the nutrient-limited growth media (–N, –N & P and –P) at Day 13, several

Table 1. Concentrations of inorganic N and P in the daily added (ingoing) and in the outgoing media of semi-continuous *Prymnesium parvum* cultures. The f/10-medium was assumed to be nutrient-replete and, therefore, not included in nutrient analyses. bd: below detection limit

Treatment N:P ratio	–N (3.2:1)	–N & P (16:1)	–P (80:1)
Added daily (µM)			
PO_4^{3-}	3.60	0.60	0.18
NO_3^-	11.50	9.70	14.50
Outgoing (µM)			
PO_4^{3-}	3.75	0.03	bd
NO_3^-	0.26	0.29	0.99

Table 2. Cell densities and growth rates of *Prymnesium parvum* semi-continuous cultures, and cellular contents and molar ratios of organic C, N and P. Particulate organic carbon, POC; particulate organic nitrogen, PON; particulate organic phosphorus, POP. Growth rates are means of the last 2 wk of experiments. SE in parentheses, n = 3. *Means for parameter varied significantly among treatments (ANOVA, $p < 0.05$); [a,b,c]within the same parameter, having different letters indicates that means are significantly different at $p < 0.05$ (Tukey's), [I] and [II] are significantly different at $p < 0.10$ (Tukey's)

Treatment	–N	–N & P	–P	f/10
Cell densities				
($\times 10^4$ cells ml^{-1})	7.2 (0.4)	6.4 (0.2)	6.2 (0.4)	8.7 (0.8)
Growth rates				
(d^{-1})*	0.11 (0.01)^a	0.11 (0.01)^a	0.10 (0.01)^a	0.40 (0.01)^b
Cellular contents				
(pg C/N/P cell^{-1})				
POC*	30.0 (1.2)^a	27.8 (1.8)^a	40.8 (2.4)^b	25.4 (1.6)^a
PON*	1.9 (0.1)^I	2.0 (0.1)	2.6 (0.3)	2.6 (0.1)^{II}
POP*	0.47 (0.04)^a	0.48 (0.02)^a	0.23 (0.02)^b	0.58 (0.02)^a
Molar ratios				
N:P*	8.9 (0.6)^a	9.3 (0.4)^a	25.0 (1.0)^b	9.8 (0.6)^a
C:P*	166.1 (8.9)^a	151.0 (10.8)^a	466.7 (12.1)^b	114.1 (9.4)^c
C:N*	18.6 (0.3)^a	16.2 (0.6)^a	18.7 (0.9)^a	11.6 (0.3)^b

days were required for nutrient concentrations in the cultures to attain the new, lower level. Therefore, some increase in cell densities continued until Day 20 (Fig. 1), after which cell densities began to decrease. At Day 29, nutrient concentrations in the added media were changed and the cultures were diluted to 1.5×10^5 cells ml^{-1}. Because nutrient concentrations in the added media were lowered, cell densities decreased even further (Fig. 1) and the cultures reached steady state at 6 to 7×10^4 cells ml^{-1} between Days 37 and 49. At this point, average daily growth rate was dictated by the dilution rate and was thus between 0.10 and 0.11 d^{-1} (Table 2). The limiting component of the daily added nutrients was, thus, exploited prior to each new nutrient addition. This was the time period when feeding experiments were performed (Fig. 1). The nutrient-replete f/10 cultures were diluted by 30% daily, reaching a cell density of 6 to 7×10^4 cells ml^{-1} after the daily dilution. Due to the higher growth rate, these cultures increased to $8–10 \times 10^4$ cells ml^{-1} at the end of every 24 h period, corresponding to an average growth rate of 0.40 d^{-1} (Table 2).

Nutrients, POC, PON and POP

Concentrations of the limiting nutrient were consistently low in outgoing culture media (Table 1), i.e. low NO_3^- concentrations in the –N and –N & P treatments and low PO_4^{3-} concentrations in the –P and –N & P treatments. Cells in all nutrient-limited treatments had a tendency of higher C-content than the nutrient-replete cells growing in f/10-medium. However, only for the cells of the –P treatments was this difference statistically significant (Tukey's $p < 0.05$) (Table 2). Cellular N-content, on the other hand, was significantly lower in the –N treatment (Tukey's $p < 0.10$) and had a tendency of also being lower in the –N & P treatment. Similarly, cellular P-content was significantly lower in the –P cultures (Tukey's $p < 0.05$). These cellular C-, N- and P-contents are reflected in the molar ratios: due to the lower P-content, cells from –P cultures had a considerably higher N:P ratio than in the other treatments (Table 2), and cellular C:P and C:N ratios in all nutrient-limited cultures were significantly higher than in the f/10 cultures (Tukey's $p < 0.05$) (Table 2).

Feeding

Feeding activity in *Prymnesium parvum*, determined as percentage of cells feeding or containing food vacuoles, increased linearly with incubation time for 2 h until a feeding activity of ~40% was reached (Fig. 2). Since maximum percentage of feeding cells was reached after 2 h of incubation, this period was chosen for all subsequent feeding experiments. Lysis of the prey, *Rhodomonas baltica*, was almost instantaneous (Fig. 2). After 10 min of incubation of the mixture of prey and the *P. parvum* cells grown under N-limitation, 96% of the prey cells had already been lysed, and this

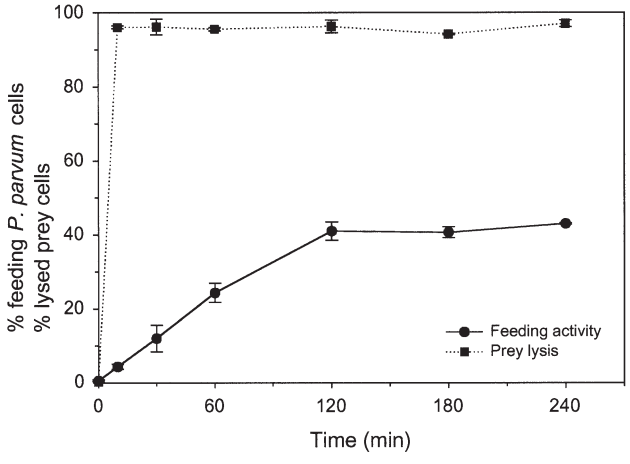

Fig. 2. Food uptake by *Prymnesium parvum* and lysis of prey, *Rhodomonas baltica*, as functions of incubation time (–N treatment). Means ± SE, n = 3

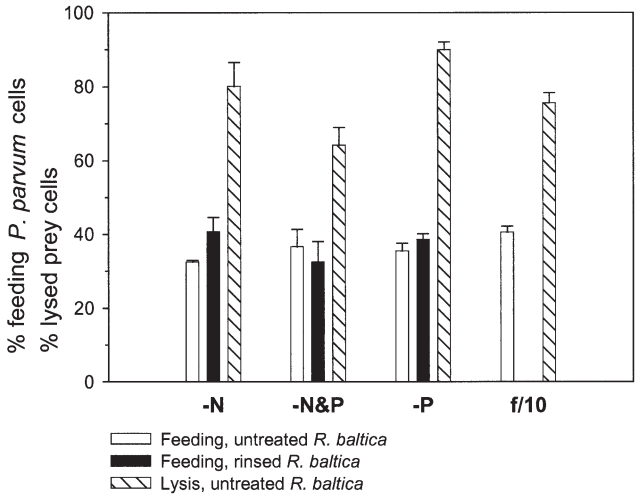

Fig. 3. Feeding activity of *Prymnesium parvum* and lysis of prey, *Rhodomonas baltica*, in the 3 different treatments, –N, –N & P, –P and in f/10. For the –N, –N & P, and –P treatments, feeding was also determined using prey cells that were rinsed in nutrient-depleted medium prior to feeding experiments. Bars represent means, error bars are SE, n = 3

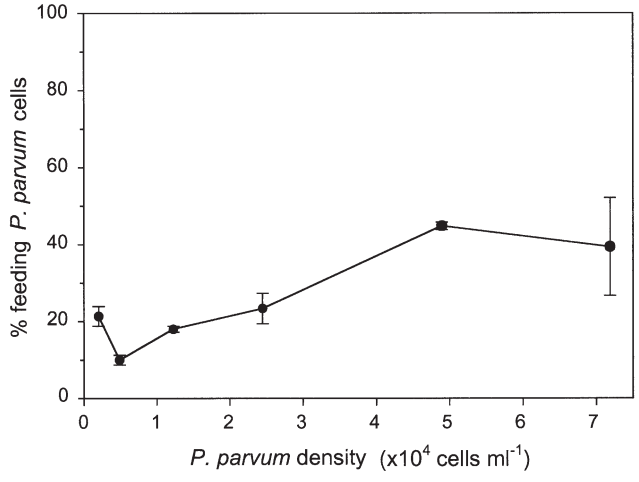

Fig. 4. Feeding by *Prymnesium parvum* at different predator densities (–N treatment). Prey, *Rhodomonas baltica*, densities were similar in all experiments. Means ± SE, n = 3

percentage of lysed prey cells remained consistently high for the longer incubation times.

The frequency of feeding *Prymnesium parvum* cells was between 33 and 41% (Fig. 3). There was no difference between percentages of feeding cells in the 4 treatments, including the f/10 treatment where *P. parvum* was growing under nutrient-replete conditions (ANOVA, $p > 0.01$). Rinsing of prey cells in nutrient-depleted seawater led to minor variations in feeding percentages (Fig. 3), but also when using rinsed prey cells, there was no significant difference between percentages of feeding *P. parvum* cells in the nutrient-limited treatments (ANOVA, $p > 0.01$). Percentages of *P. parvum* cells feeding on rinsed prey cells were within the same range as when feeding on prey cells that had not been rinsed. This showed that nutrient enrichment due to the addition of prey had no short-term effect on feeding. Amounts of lysed prey cells in the different treatments were between 64 and 90% with the –P cultures having the tendency of inducing most cell lysis (Fig. 3). However, the method of counting frequency of lysed prey cells did result in somewhat variable results. In the –N time series, for example, the percentages of lysed prey cells (Fig. 2) reached a level that was even higher than in the –P treatment (Fig. 3).

The above-described feeding experiments were performed using a predator:prey ratio close to 1:1. To ensure that no food limitation occurred due to this relatively high predator density, a series of feeding experiments were performed with lower predator densities (0.2 to 7.2 × 10^4 *Prymnesium parvum* ml^{-1}) (Fig. 4). These experiments proved that there was no food limitation induced by predator grazing. On the contrary, feeding activity increased with increasing predator density, suggesting that a higher predator density stimulated feeding: at 0.2 to 2.5 × 10^4 *P. parvum* ml^{-1}, 10 to 23% of the cells were feeding, whereas above ~5 × 10^4 *P. parvum* ml^{-1}, 40 to 45% were feeding or contained food vacuoles after 2 h of incubation with prey (Fig. 4).

DISCUSSION

Nutrient limitation

That the assumed nutrient-limited *Prymnesium parvum* cultures used here were in fact nutrient-limited, is supported by the measured low amounts of the limiting nutrient in the outgoing media (Table 1). In addition, the cellular chemical compositions revealed nutrient limitation. Nutrient limitation in phytoplankton cells usually leads to increased cellular C-content (Cembella et al. 1984, Johansson & Granéli 1999), which was most pronounced for the –P treatment of the present study. For the –N and –N & P treatments, there were tendencies of increased cellular C-content (Table 2). Nutrient-limited cells also generally contain lowered levels of the limiting nutrient (Sakshaug & Olsen 1986), and this was seen at various levels for all nutrient-limited treatments of this study. The –P treatment had elevated N:P and C:P ratios, indicating P-limitation (Healey & Hendzel 1980, Cembella et al. 1984). Likewise, the elevated C:N ratios of the –N and

–N & P treatments were indicators of N-limitation (Sakshaug & Holm-Hansen 1977, Granéli et al. 1999b). The –P treatment also had a high C:N ratio, which could indicate N-limitation. However, it is more likely that this high C:N ratio merely reflects the very high C-content of these P-limited cells. The fact that cell growth was significantly suppressed in all 3 nutrient-limited treatments as compared with the f/10 treatment also showed that nutrient limitation was present in these 3 treatments.

Feeding

It has been suggested that, in cultures, *Prymnesium parvum* f. *patelliferum* primarily feeds when cultures are senescent (Tillmann 1998). This was not supported by our experiments, which showed that *P. parvum* is capable of ingesting prey during exponential, non-limited growth. Other studies have shown that phagotrophy in mixotrophic algae may be related to nutrient limitation (Nygaard & Tobiesen 1993, Stoecker 1998, Legrand et al. 2001), but no such direct relationship was found for *P. parvum* in this study. On the contrary, when prey was present, nutrient conditions did not influence feeding in *P. parvum*. It has previously been reported that bacterivory in *P. parvum* f. *patelliferum* in batch cultures is suppressed by excess dissolved phosphate (Legrand et al. 2001). This may seem contradictory to our results. However, caution must be taken when comparing these results as Legrand et al. (2001) used batch cultures for the experiment and bacteria as prey. Nevertheless, our experiments indicated that feeding in *P. parvum* is not a direct response to nutrient limitation. This may imply that the species does not utilise phagotrophy as a means to obtain inorganic nutrients for use in phototrophic growth.

A heterotrophic mode of nutrition is more energetically favourable than photosynthetic growth (Raven 1997), and heterotrophs generally have higher growth rates than phototrophs of similar size (Banse 1982, Raven 1995). Since *Prymnesium parvum* is able to grow heterotrophically in the dark with glycerol as the energy source (Rahat & Jahn 1965), it is conceivable that it is also able to grow partially heterotrophically on particulate food as well. In that case, it is an energetic advantage for *P. parvum* to feed even when conditions are optimal for phototrophic growth and no relationship between feeding and nutrient conditions should then be expected. Such a mixotrophic strategy is different from what has been observed in the mixotrophic dinoflagellates *Prorocentrum minimum* (Stoecker et al. 1997) and *Karlodinium micrum* (Li et al. 2000, as *Gyrodinium galatheanum*), in which adverse nutrient conditions were found to stimulate feeding. Interestingly, the latter 2 species have not been shown to be able to use ingested carbon for strictly heterotrophic growth.

Feeding activity of *Prymnesium parvum* appeared to become stimulated by high densities of the predator itself (Fig. 4), even though prey densities were similar in all feeding experiments. A possible explanation for this is that excretion of toxin by *P. parvum* stimulates feeding, e.g. by inducing lysis in prey cells, thereby making them more susceptible for predation. This issue has been studied in detail in Skovgaard & Hansen (2003). In the present study, we estimated *P. parvum* toxicity towards *Rhodomonas baltica* by counting frequencies of lysed prey cells, and we found no clear difference between toxicity in the 4 treatments. In other studies, however, P limitation has been observed to increase toxicity of *P. parvum* (Shilo 1971, Dafni et al. 1972, Johansson & Granéli 1999). Therefore, one cannot exclude that nutrient limitation may cause *P. parvum* to become more toxic under certain growth conditions, indirectly inducing higher feeding activity in the species.

We conclude that what makes *Prymnesium parvum* successful in producing blooms (Edvardsen & Paasche 1998) is not its potential capacity to obtain inorganic nutrients through food uptake. It is rather the combination of mixotrophic and allelopathic capabilities that makes *P. parvum* a strong competitor, thereby facilitating build-up of blooms by this species.

Acknowledgements. Financial support was provided by the European Commission (EC) (Research Directorate General-Environment Programme-Marine Ecosystems) to the University of Copenhagen and Kalmar University (co-ordinators P.J.H. and E.G., respectively) through the BIOHAB project 'Biological control of harmful algal blooms in European coastal waters: role of eutrophication' (Contract EVK3-CT99-00015). The BIOHAB project is part of the EC EUROHAB cluster. Additional funding was provided by the Swedish Research Council for Environment, Agricultural Sciences and Spatial planning (Formas) to E.G.

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Editorial responsibility: David Caron, Los Angeles, California, USA

Submitted: August 28, 2002; Accepted: January 19, 2003
Proofs received from author(s): March 6, 2003