

Food uptake in the harmful alga *Prymnesium parvum* mediated by excreted toxins

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Abstract

Toxin production is widespread among aquatic microalgae, suggesting a functional advantage for organisms producing toxic compounds. However, the biological role of algal toxin production is only vaguely understood. Here, we show that excretion of a toxic substance in the phagotrophic phytoflagellate *Prymnesium parvum* (Prymnesiophyceae) constitutes a mechanism to immobilize and seize motile prey. Feeding frequency of *P. parvum* in dilute batch cultures was low when fed the motile prey *Heterocapsa rotundata* (dinoflagellate). However, dense cultures caused immobilization of *H. rotundata* cells, thereby allowing *P. parvum* to feed on them. In contrast, when fed a nonmotile prey—the diatom *Thalassiosira pseudonana*—feeding frequency was high, even in dilute *P. parvum* cultures. We could demonstrate that feeding frequency of *P. parvum* on *H. rotundata* was positively correlated with the measure of the toxic effect causing immobilization and lysis of prey cells. The fact that the toxic effect on *H. rotundata* was found in cell-free filtrate of *P. parvum* cultures suggests that immobilization and lysis of prey cells were caused by the excretion of toxins.

Blooms of planktonic algae are common in aquatic environments, and harmful algal blooms cause substantial commercial problems for the exploitation of marine and freshwater resources, as well as for recreational purposes (e.g., Hallegraeff 1993). The harmful effects of algal blooms are typically from toxins produced by the algae. The question of why algae produce toxins has, therefore, been a point of much speculation, but few facts exist on possible biological or ecophysiological roles of toxin production. Toxins might function as grazing deterrents (Verity and Stoecker 1982). Another possibility is that toxin-producing algae inhibit and thereby outcompete other phytoplankton competitors through the release of allelopathic substances (Maestrini and Bonin 1981; Schmidt and Hansen 2001). It has also been suggested that some toxic substances produced by algae primarily function as hormones or regulate ion channels (Turner et al. 1998).

In this report, we present data on the role of toxin excretion by a toxic alga, the prymnesiophyte flagellate *Prymnesium parvum* in feeding. The species is mixotrophic (i.e., it is photosynthetic), but it also feeds phagotrophically on other microorganisms, such as bacteria (Nygaard and Tobiesen 1993) and other protists (Tillmann 1998, *P. parvum* f. *patelliferum* [Green, Hibberd et Pienaar] A. Larsen). Blooms of *P. parvum* occur in brackish and coastal waters where they have caused much damage to the aquaculture industry (Reich and Aschner 1947; Moestrup 1994). Most *P. parvum* blooms have been reported from Europe, but the species is ubiquitous and has caused toxic blooms worldwide (Moes-

trup 1994; Edvardsen and Paasche 1998). *P. parvum* has ichthyotoxic capabilities (Otterstrøm and Steemann Nielsen 1940), and blooms of *Prymnesium* spp. are often associated with fish kills (Moestrup 1994; Edvardsen and Paasche 1998). Not only is *P. parvum* toxic to fish, it produces hemolytic substances (Yariv and Hestrin 1961) that cause lysis of both prokaryotic and eukaryotic cells (Shilo and Rosenberger 1960; Tillmann 1998). The range of toxic effects caused by *P. parvum* suggests that perhaps more than one toxic compound is produced by the organism (Shilo and Rosenberger 1960), and considerable effort has been put into purifying and describing the structure of these toxins (summarized by Shilo 1971). Recently, Igarashi et al. (1999) described the gross structures of two *Prymnesium* toxins—prymnesin-1 and prymnesin-2—but still, no straightforward method exists to quantify them because standards for use in, for example, chromatographic techniques are not commercially available. It is possible, however, to determine relative concentrations of *Prymnesium* toxin by measuring the toxin's hemolytic potential (Simonsen and Moestrup 1997).

Tillmann (1998) speculated that *Prymnesium* could use toxin to kill potential prey organisms prior to ingestion. This would be advantageous because it does not have any morphological structure for capturing swimming prey cells as do many other phagotrophic flagellates (Fenchel 1982; Gaines and Elbrächter 1987). In this study, we have investigated the possible relation between feeding and toxin excretion in *P. parvum* by quantifying feeding frequency and, simultaneously, determining the toxic effect exerted by *P. parvum* culture filtrate on its prey.

Methods

Algal cultures were obtained from the Scandinavian Culture Center for Algae and Protozoa, Denmark (*P. parvum*: K-0081 and *Heterocapsa rotundata*: K-0483), from the Culture Collection of Algae and Protozoa, U.K. (*Thalassiosira pseudonana*: CCAP1085/12), and from the culture collection of the Marine Biological Laboratory, Denmark (*Rhodomonas marina*). The organisms were grown nonaxenically in

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batch cultures in f/10 medium (Guillard and Ryther 1962) prepared from pasteurized seawater (salinity 30‰; temperature 15°C; irradiance 90 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, measured with a QSL-100 Quantum Scalar Irradiance Meter, Biospherical Instruments). Triplicate 500-ml batch cultures of *P. parvum* were set up in erlenmeyer flasks and the development in cell densities was monitored for 22 d. Every second day, samples were withdrawn and counted with a microscope and a Sedgewick–Rafter counting cell (Graticules), and pH was measured using a Sentron pH meter (model 2001, Sentron). At each sampling occasion, toxic effect on the prey and feeding frequency of *P. parvum* were measured using *H. rotundata* as the prey/target cell as described below.

The toxic effect of GF/C-filtered *P. parvum* cultures was quantified as the filtrate's capacity to induce cell lysis or immobilization in *H. rotundata* cells by modification of the method described by Schmidt and Hansen (2001). *H. rotundata* cells (500) corresponding to 5–7 μl of culture volume were added to 0.3-ml cell-free, GF/C-filtered *P. parvum* culture contained in 0.330-ml multidish wells (Nunc). After 2 h, number of immobile or disintegrated *H. rotundata* cells was counted with an inverted microscope, and total cell numbers were then counted following fixation in Lugol's solution. For control experiments, *P. parvum* culture filtrate was replaced by 0.3-ml GF/C-filtered f/10 medium.

Feeding frequency was quantified by determining the percentage of *P. parvum* cells that were phagotrophically active after 2 h of incubation with food. Prey concentration was kept constant in all feeding experiments in order to avoid any effect of food concentration on measured feeding frequencies. Triplicate incubations were set up in 20-ml glass vials containing 10 ml *P. parvum* culture. Small volumes (0.5–1.0 ml) of dense cultures of either *H. rotundata* or *T. pseudonana* were then added, corresponding to final prey concentrations of $5 \times 10^4 \text{ cells ml}^{-1}$ (actual range: $4.7\text{--}5.2 \times 10^4 \text{ cells ml}^{-1}$). After 2 h, the mixture was fixed in glutaraldehyde (final concentration 2%), and with an Olympus CK40 inverted microscope at $\times 400$ magnification, total numbers of *P. parvum* cells were counted, as well as the numbers of *P. parvum* cells that either contained food vacuoles or were fixed in the process of engulfing a prey cell.

Two series of feeding experiments were performed. In the first series, the toxic effect exerted by *P. parvum* culture filtrate on *H. rotundata* and the feeding frequency of *P. parvum* offered *H. rotundata* prey were determined every 2–3 d in triplicate batch cultures, in which *P. parvum* cell densities increased exponentially over time (Fig. 1). In the second series, feeding frequency was again measured as function of *P. parvum* cell densities, but this time different cell densities were obtained by diluting a dense *P. parvum* culture ($3 \times 10^5 \text{ cells ml}^{-1}$) with f/10 medium. To test whether the effect of toxic filtrate on feeding depended on whether the prey was a motile or a nonmotile organism, feeding frequencies were then measured using both *H. rotundata* (motile) and *T. pseudonana* (nonmotile) as prey.

An additional feeding experiment was carried out to examine whether filtrate from a dense *P. parvum* culture was able to induce feeding in a culture with low feeding frequency. A culture of low cell density ($2 \times 10^4 \text{ P. parvum cells ml}^{-1}$) was diluted 50% with GF/C filtrate from a dense

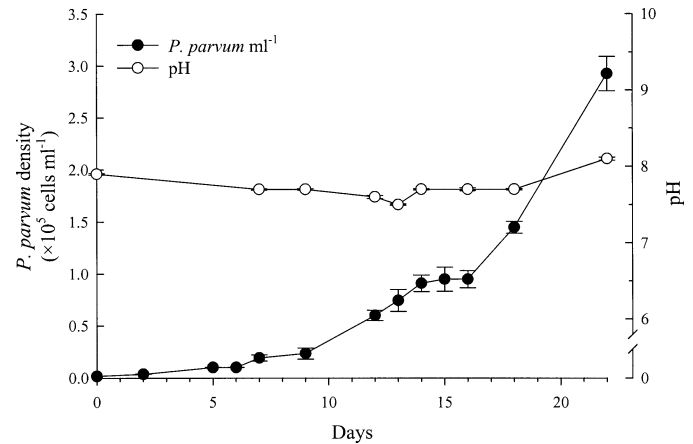


Fig. 1. Development of cell density and pH in the three parallel *P. parvum* batch cultures. Symbols represent means \pm SE.

culture ($2 \times 10^5 \text{ cells ml}^{-1}$) immediately before a feeding experiment was performed. As controls, feeding frequencies were determined following 50% dilution with either f/10 medium or GF/C filtrate of a dense *Rhodomonas salina* culture ($2 \times 10^5 \text{ cells ml}^{-1}$). Prey was *H. rotundata* and the concentration was, as throughout this study, $5 \times 10^4 \text{ cells ml}^{-1}$.

P. parvum and *H. rotundata* swimming speeds were measured using a standard video system mounted on an inverted microscope. Cell suspensions were kept in tissue culture flasks (Greiner), and average swimming speeds were determined based on 12 recorded swimming tracks for each species.

Results

Cell densities of *P. parvum* in the three parallel batch cultures increased exponentially from 1.6×10^3 to $2.9 \times 10^5 \text{ cells ml}^{-1}$ during the 22-d period (Fig. 1), corresponding to an average growth rate of 0.34 divisions d^{-1} . These cell densities did not alter pH in the culture medium substantially (Fig. 1). As the *P. parvum* cultures grew denser, toxic effect exerted by the culture filtrate on *H. rotundata* increased (Fig. 2). Toxic effect on *H. rotundata* cells appeared at $0.1 \times 10^5 \text{ P. parvum cells ml}^{-1}$, at which point a minor fraction of the *H. rotundata* cells became immobile by exposure to the filtrate. When *P. parvum* cell densities had exceeded $1.0 \times 10^5 \text{ cells ml}^{-1}$, most *H. rotundata* cells (60–80%) were affected within 2 h after exposure to the filtrate (Fig. 2). At *P. parvum* densities $> 3 \times 10^5 \text{ cells ml}^{-1}$, exposure to the filtrate led to total disintegration of *H. rotundata* cells (data not shown).

As the batch cultures grew denser, feeding frequency, expressed as the percentage of *P. parvum* cells exhibiting phagotrophic activity, also increased. At $1.6 \times 10^3 \text{ P. parvum cells ml}^{-1}$, 0.1% of the cells were feeding 2 h after the addition of prey, but when the predator density had grown to $2.9 \times 10^5 \text{ P. parvum cells ml}^{-1}$, feeding frequency increased to 20.1% (Fig. 3), even though the prey concentration was constant in all feeding experiments performed ($5 \times 10^4 \text{ H. rotundata cells ml}^{-1}$). Feeding frequency of the predator, *P. parvum*, was thus positively correlated with its cell density,

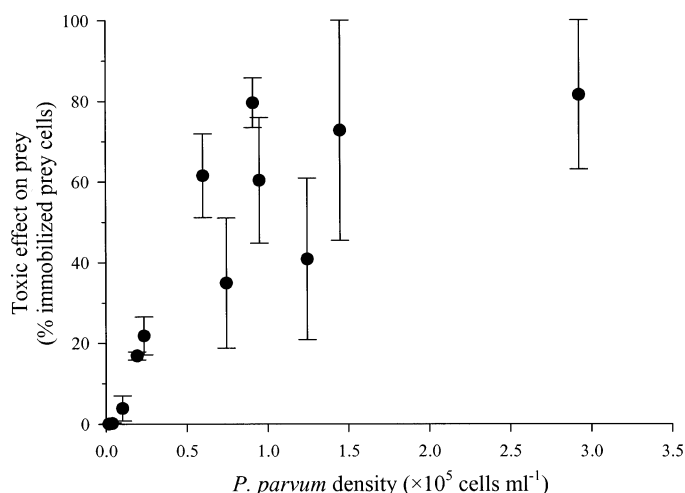


Fig. 2. Toxic effect of *P. parvum* filtrate on *H. rotundata* as a function of *P. parvum* cell density in the three batch cultures of Fig. 1. *P. parvum* cell density varied with age of batch cultures. The toxic effect was determined as the efficiency of *P. parvum* culture filtrate to immobilize prey, *H. rotundata*. Symbols represent means of three replicates \pm SE.

and as a result, there was a positive correlation between toxic effect of the culture medium and feeding frequency in *P. parvum* (Fig. 4).

Modifying *P. parvum* cell density in cultures by means of dilution with f/10 medium yielded feeding frequencies comparable to the above when the prey was *H. rotundata* (Fig. 5). At constant food concentration (5×10^4 *H. rotundata* cells ml $^{-1}$), *P. parvum* feeding frequency decreased as a response to dilution of the culture and consequently lower predator density (Fig. 5). Hence, at 2.3×10^5 *P. parvum*

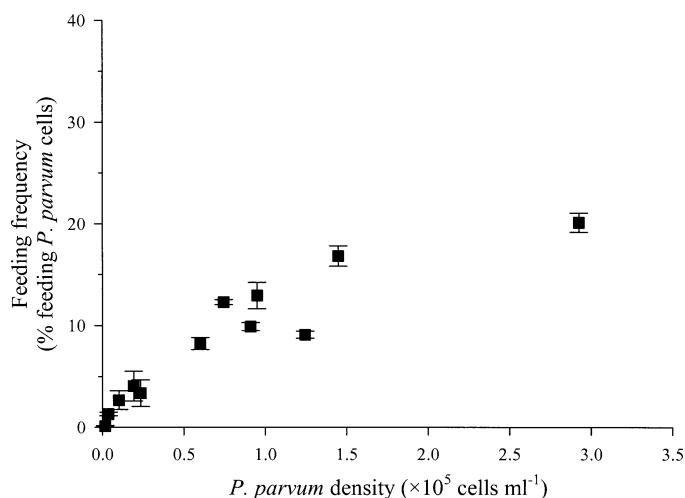


Fig. 3. Feeding frequency of *P. parvum* on *H. rotundata* as a function of *P. parvum* cell density in the three batch cultures of Fig. 1. *P. parvum* cell density varied with age of batch cultures. Feeding frequency was defined as the percentage of *P. parvum* cells feeding or containing food vacuoles after 2 h of incubation with prey. Prey concentrations were similar (5×10^4 prey cells ml $^{-1}$) at all predator cell densities. Symbols represent means of three replicates \pm SE.

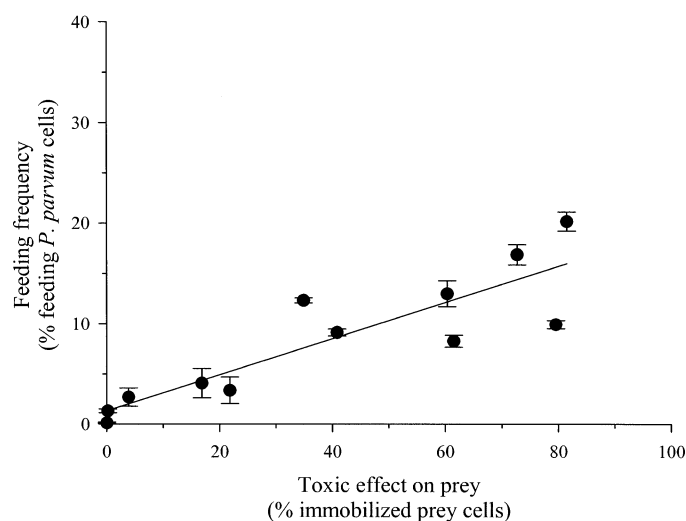


Fig. 4. Relationship between *P. parvum* feeding frequency and toxic effect on prey in the three batch cultures of Fig. 1. Toxic effect on the prey and feeding frequency were determined using *H. rotundata* as target/prey organism. Prey concentration was constant throughout the experiment (5×10^4 prey cells ml $^{-1}$); toxic effect on the prey increased as result of increasing *P. parvum* cell densities. Symbols represent means of three replicates \pm SE. Line represents linear regression ($r^2 = 0.77$).

cells ml $^{-1}$, feeding frequency was 21%, whereas feeding frequency had decreased to 5% after dilution of the predator to 1×10^4 cells ml $^{-1}$. When *T. pseudonana* was offered as food, however, feeding frequency was not correlated with predator density, but was consistently between 22 and 34%, even at predator densities as low as 1×10^4 *P. parvum* cells ml $^{-1}$ (Fig. 5).

In a *P. parvum* culture of low cell density (2×10^4 cells ml $^{-1}$), it was possible to induce a higher feeding frequency when preying on the motile prey *H. rotundata* by adding

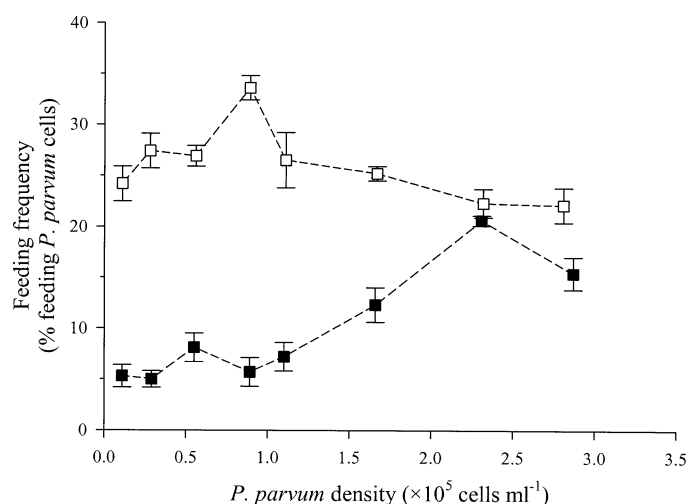


Fig. 5. Feeding frequency of *P. parvum* on *H. rotundata* and on *T. pseudonana* as functions of *P. parvum* cell density. Predator density was altered by diluting with f/10 medium. Prey concentration was similar at all predator cell densities (5×10^4 prey cells ml $^{-1}$). Symbols represent means of three replicates \pm SE.

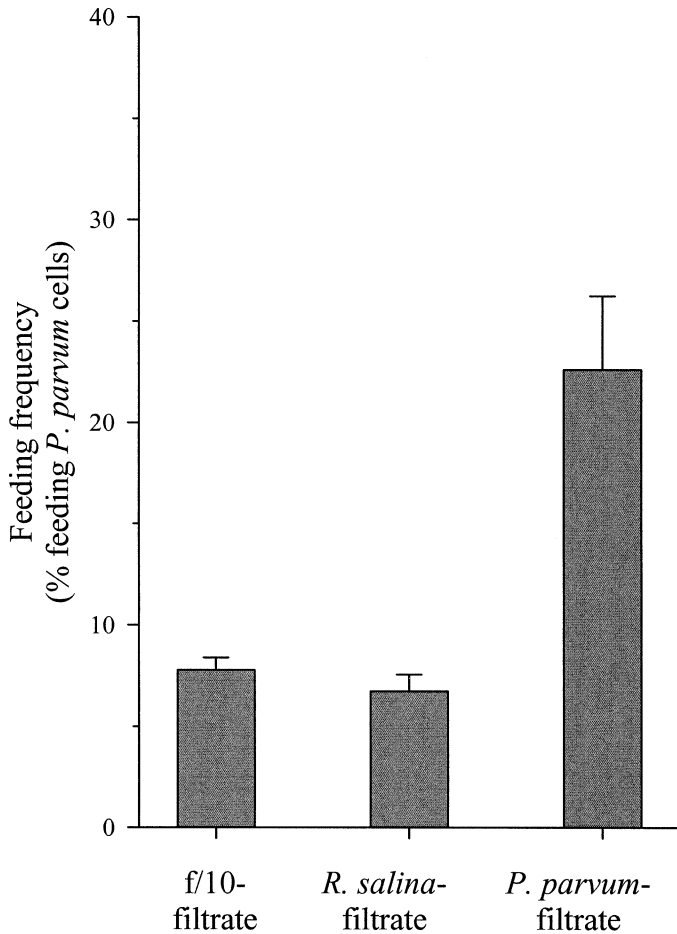


Fig. 6. Feeding frequencies of *P. parvum*. Prey was the motile dinoflagellate *H. rotundata*. Prior to the addition of prey, a 50% volume of either f/10 medium, a filtrate of a dense culture of the nontoxic cryptophyte *R. salina* (2×10^5 cells ml^{-1}), or a filtrate of a dense culture of the toxic *P. parvum* (2×10^5 cells ml^{-1}) were added to *P. parvum* in cultures of low cell densities (2×10^4 cells ml^{-1}). Bars represent means of six replicates \pm SE.

50% volume of a dense *P. parvum* culture (2×10^5 cells ml^{-1}). Feeding frequency on *H. rotundata* increased to 23% after the addition of this toxic filtrate (Fig. 6), whereas feeding frequency in the control experiment, in which 50% volume f/10 medium was added, was only 8% (Fig. 6). Adding 50% volume of filtrate from a dense culture of the nontoxic cryptophyte *R. salina* (2×10^5 cells ml^{-1}) did not induce a feeding frequency higher than in the control.

In microscopical observations, *P. parvum* seemed to move slowly in comparison with *H. rotundata*. This was confirmed through measurements of swimming speeds (Table 1; Fig. 7). Mean swimming speed of *P. parvum* was $17.5 \mu\text{m s}^{-1}$, whereas *H. rotundata* had a mean swimming speed of $73.4 \mu\text{m s}^{-1}$. In addition, *H. rotundata* exhibited a characteristic swimming pattern in which it made frequent jumps (i.e., short-lasting, rapid movements with a mean swimming speed of $398.2 \mu\text{m s}^{-1}$). These jumps were spontaneous elements of the swimming pattern but also occurred as provoked escape jumps (e.g., when a cell was disturbed by

Table 1. Motility characteristics for the prymnesiophyte *Prymnesium parvum* and the dinoflagellate *Heterocapsa rotundata*. Numbers are means of 12 measurements.

	Swimming speed ($\mu\text{m s}^{-1} \pm \text{SE}$)	Jump speed ($\mu\text{m s}^{-1} \pm \text{SE}$)	Jump length ($\mu\text{m} \pm \text{SE}$)
<i>P. parvum</i>	17.5(1.5)	—	—
<i>H. rotundata</i>	73.4(4.3)	398.2(25.0)	231(31)

bumping into another cell) (Fig. 7). Mean length of jumps was $231 \mu\text{m}$ (Table 1).

Discussion

P. parvum feeding frequency was independent of its density when feeding on the nonmotile diatom *T. pseudonana* (Fig. 5). However, when fed the motile dinoflagellate prey *H. rotundata*, feeding frequency increased from $<5\%$ of the population being in the process of feeding at low *P. parvum* cell densities to 20% feeding at high *P. parvum* densities (Figs. 3, 5). The latter was comparable to the feeding frequency observed when the nonmotile *T. pseudonana* was offered as prey (Fig. 5). The reason for the increased feeding frequency in dense *P. parvum* cultures was that cells of *H. rotundata* were immobilized as *P. parvum* increased in cell density. Not only was there a direct relationship between the percentage of *H. rotundata* cells immobilized and *P. parvum* feeding frequency (Fig. 4), but the addition of a filtrate from a dense *P. parvum* culture to a dilute *P. parvum* culture with an initial low feeding frequency also resulted in an increase of feeding frequency (Fig. 6) because the addition of this filtrate led to immobilization of the prey.



Fig. 7. Selected typical swimming tracks of (A) *P. parvum* (0.48 s between symbols) and (B) *H. rotundata* (0.16 s between symbols). Arrowhead indicates the point of contact with another cell, provoking the tracked *H. rotundata* cell to perform a jump. Arrows denote direction of motion. Scale bar = $50 \mu\text{m}$.

Evidence thus indicates that *P. parvum* is able to feed only on immobile prey. This is in agreement with the observations of Tillmann (1998), reporting that food items always became immobile and rounded prior to ingestion. The short haptonema ($\sim 5 \mu\text{m}$) of *P. parvum* is not involved in food uptake (Tillmann 1998), and the species has no other morphological structure for capturing swimming prey cells. The motile prey in the present study, *H. rotundata*, is a faster swimmer than *P. parvum*; in addition, it is able to perform jumps whereby it can escape predators (Fig. 7; Table 1). Hence, *H. rotundata* must be immobilized in order for *P. parvum* to seize and ingest it, which in the present study started to occur when *P. parvum* densities exceeded $\sim 0.1 \times 10^5$ cells ml^{-1} . In contrast to *P. parvum*, some prymnesiophytes have a longer haptonema (30–100 μm) that is involved in the feeding process. In *Chrysochromulina* spp. the haptonema is involved in the capture and transport of prey to the cytostome positioned in the posterior end of the cell. This enables *Chrysochromulina* spp. to feed on motile prey (Kawachi et al. 1991; Jones et al. 1993).

Recent studies on interactions between algae have shown that both high pH and the excretion of toxins can lead to immobilization of other algae in mixed cultures (Schmidt and Hansen 2001). Because pH in our experiments never exceeded 8.2, pH as a factor causing immobility of *H. rotundata* cells can be ruled out, suggesting that excreted toxin was the cause of immobilization. This agrees with the fact that *P. parvum* is known to produce toxins that damage cell membranes and cause cell lysis (Yariv and Hestrin 1961). These cell-lysing properties of *Prymnesium* toxin are found in the cellular fraction of *Prymnesium* cultures (Shilo and Rosenberger 1960), as well as in cell-free culture filtrate (Yariv and Hestrin 1961).

Phagotrophy is common in toxic microalgae with allelopathic capabilities (Gaines and Elbrächter 1987), and one could speculate that toxicity also plays a role in food uptake in other species. More studies are needed, however, before concluding how widespread this mechanism might be among phagotrophic, toxic algae. To some extent, the toxin-mediated feeding in *P. parvum* can be compared with the “ambush-predator” feeding behavior suggested for the dinoflagellate *Pfiesteria* spp. (Burkholder and Glasgow 1995). *Pfiesteria* spp. are able to feed on finfish allegedly killed by toxin excreted by the algae (Burkholder and Glasgow 1995), but in contrast to *P. parvum*, *Pfiesteria* spp. possess a specialized feeding apparatus, a peduncle (Steidinger et al. 1996). This peduncle enables *Pfiesteria* spp. to feed on live, motile microorganisms, and the prey does not need to be immobilized or killed prior to ingestion (Burkholder and Glasgow 1995).

Blooms of *P. parvum* can become very dense, up to 10^6 cells ml^{-1} (reviewed by Edvardsen and Paasche 1998), and in some cases, the plankton communities of such blooms consist of virtually monospecific *P. parvum* populations (Otterstrøm and Steemann Nielsen 1940; Reich and Aschner 1947; Comín and Ferrer 1978). According to our study, such cell concentrations of *P. parvum* are beyond what must be assumed to be toxic for other plankton organisms. It is likely, therefore, that the monocultural nature of these blooms are facilitated by the disintegration of other planktonic micro-

organisms through cell lysis and, perhaps, by *P. parvum* predation on other organisms. The possible role of phagotrophy in the build-up of *P. parvum* blooms is unknown, but its feeding strategy might explain why this species is so successful in producing blooms: by excreting toxin, *P. parvum* is able to affect severely and even kill photosynthetic competitors through the excretion of lytic substances (e.g., Yariv and Hestrin 1961), and it might also stimulate its own growth by feeding on the affected organisms. Which potential benefits *P. parvum* obtains from the ingestion of particulate food still, however, needs to be investigated.

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