

Kill and eat your predator: a winning strategy of the planktonic flagellate *Prymnesium parvum*

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ABSTRACT: Interactions between the toxic and mixotrophic haptophyte *Prymnesium parvum* and the heterotrophic dinoflagellate *Oxyrrhis marina* were investigated in P-limited semi-continuous cultures and nutrient-replete batch culture experiments. When exposed to 100×10^3 cells ml^{-1} of P-limited *P. parvum*, starved *O. marina* initially ingested the algae, but ingestion was low (0.07 cells grazer $^{-1}$ h $^{-1}$) compared to the *O. marina* ingestion rate when the Cryptophyte *Rhodomonas* sp. was offered as food (2.75 cells grazer $^{-1}$ h $^{-1}$). Microscopic observations showed that low ingestion is due to toxic effects of *P. parvum* on the dinoflagellate. Cells of *O. marina* lost their normal cell shape and became rounded, hyaline and finally lysed. Rounded and partly lysed *O. marina* cells were rapidly attacked by several *P. parvum* cells, which formed larger aggregates around the remains of the *O. marina* cells. This was accompanied by phagotrophic ingestion of particulate material originating from disintegrating *O. marina*. Cell-free culture medium lysed *O. marina* cells, although to a lower degree compared to the effect of algal suspensions. *O. marina* mortality was not only reduced by diluting *P. parvum*, but also by increasing the dinoflagellate concentration. This clearly indicates that the toxin is removed from the system by its action, presumably by binding to the membrane. Under conditions where toxic effects were not apparent (nutrient-replete batch cultures, low cell concentrations, dim light), *P. parvum*, after an initial lag period, was rapidly ingested and sustained growth of *O. marina* at rates comparable to those estimated for *Rhodomonas* sp. Toxicity of *P. parvum* is thus a key factor in determining the interaction with protozoan grazers. If toxicity is low, *P. parvum* is a suitable prey for *O. marina*. At high toxicity levels, however, *O. marina* is rapidly killed and ingested by *P. parvum*, thus reversing the normal direction of grazing interactions between protozoa and algae.

KEY WORDS: *Prymnesium parvum* · *Oxyrrhis marina* · Toxic algae · Interaction · Grazing · Mixotrophy · Chemical defence

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INTRODUCTION

The haptophycean species *Prymnesium parvum* and *P. patelliferum* are well known to form dense blooms in brackish and coastal marine waters around the world (reviewed by Moestrup 1994, Edvardsen & Paasche 1998). There is increasing genetic evidence that *P. parvum* and *P. patelliferum*, despite their small differences in the fine-structural details of body scales (Green et al. 1982), should be considered as 1 species (Larsen & Medlin 1997, Larsen 1999), and I do so in the following discussion. Blooms of *Prymnesium parvum* have been associated with massive fish mortalities (e.g. Reich & Aschner 1947, Holdway et al. 1978,

Kaartvedt et al. 1991) and may cause severe damage to the whole ecosystem (Valkanov 1964, Petrova 1966). *P. parvum* produces a set of highly potent toxins commonly called prymnesin (Shilo 1981, Igarashi et al. 1996) which may be excreted into the surrounding waters (Shilo & Aschner 1953). The toxins have a broad spectrum of different biological effects. In addition to ichthyotoxic (Ulitzur & Shilo 1966) and pharmacological activities (Meldahl & Fonnum 1993, 1995) *Prymnesium* toxins exert lytic effects on various cell types, such as erythrocytes (Yariv & Hestrin 1961), bacteria (Ulitzur & Shilo 1970), and a number of nucleated cells (Shilo & Rosenberg 1960, Dafni & Gibberman 1972). Purified *Prymnesium* toxin preparation (prymnesin)

has been shown to be one of the most active lysins described (Ulitzur & Shilo 1970, Igarashi et al. 1998).

Acute toxicity towards 'standard toxicity test' systems, including blood cells, *Artemia* spp., cytological material or fish has been investigated in most studies. However, there is only limited information on interactions of *Prymnesium* spp. and co-occurring zooplankton grazers, although reduced or inhibited grazing is generally believed to be an important factor in harmful bloom dynamics (Fiedler 1982, Smayda 1997). Valkanov (1964) demonstrated qualitatively that copepod species showed no sign of mortality or exhaustion upon exposure to *P. parvum* and this obviously low sensitivity of copepods towards *Prymnesium* has been validated (Nejstgaard et al. 1995, Nejstgaard & Solberg 1996). A wide range of protozoan species, however, are severely affected upon exposure to *P. parvum* (Valkanov 1964). Likewise, laboratory experiments of Johannsson (2000) showed that increasing abundances of *P. parvum* increased mortality of the ciliate *Euplotes affinis*.

Toxin production is not the only distinctive characteristic of *Prymnesium*. Despite the intense research on *Prymnesium* in the last half century, it was only recently that phagotrophy of this genus was convincingly described (Nygaard & Tobiesen 1993, Tillmann 1998, Legrand et al. 2001). It was shown that *Prymnesium* is able to incorporate different sized particles (sometimes even larger than itself) including heterotrophic protozoans like amoeba or the heterotrophic dinoflagellate *Oxyrrhis marina* (Tillmann 1998). Based on qualitative observations, Tillmann (1998) speculated that *Prymnesium* toxin may be used to kill potential prey organisms prior to ingestion. However, quantitative data on that topic are still missing.

The aim of the present paper was to quantitatively analyse the interactions between *Prymnesium parvum* and a protozoan grazer. The heterotrophic dinoflagellate *Oxyrrhis marina*, a species that co-occurs with *Prymnesium* in a brackish pond in northern Germany (own observation), was chosen as the grazer.

MATERIALS AND METHODS

The heterotrophic dinoflagellate *Oxyrrhis marina* (Göttingen culture collection, Strain B21.89) was gradually adapted to a salinity of 10‰ 2 mo before the experiment started. Stock cultures held in 100 ml flasks were regularly fed *Dunaliella* sp. or *Rhodomonas* sp. pre-cultured with 10‰ f/10 medium (0.1 strength f-medium, Guillard & Ryther 1962). Cultures of *O. marina* used in the experiments were grown at 15°C to high densities until they became almost deprived of food. In experiments where it was desirable to keep the volume added to *Prymnesium parvum*

cultures as low as possible, *O. marina* cultures were gently concentrated by gravity filtration (Nucleopore filters, pore size 1 µm).

Semi-continuous culture experiments (P-limited).

Algal culture conditions: *Prymnesium parvum* (Kalmars University Culture Collection, strain KAC39) was cultivated non-axenically in f/10-medium prepared from GF/C-filtered and pasteurised coastal seawater (Baltic Sea). The original salinity of the seawater, 7‰, was adjusted to 10‰ by adding NaCl.

Triplicate P-limited semi-continuous cultures were established from batch cultures as described in detail by Skovgaard et al. (2003). The culture bottles (3 l Pyrex bottles) were gently aerated and kept in a controlled environment 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 µE m⁻² s⁻¹. Cell concentrations were monitored at least every second day by counting >400 cells in Lugol's fixed samples in a Sedgewick Rafter counting cell (Graticules). The cultures were diluted 15% daily by removing 300 ml of culture volume and replenishing it with modified f/10-medium where PO₄³⁻ was the limiting nutrient at an N:P ratio of 80:1 (14.5 µM NO₃⁻ and 0.18 µM PO₄³⁻). Trace metals and vitamins were always added in amounts corresponding to full strength f/10-medium. Daily dilution was performed at 10.00 h (3 h after onset of the light period). The culture volume withdrawn was then used for subsequent experiments. These experiments described below were carried out in a time period of 10 d.

Grazing of *Oxyrrhis marina*: An experiment was conducted to estimate grazing of *Oxyrrhis marina* on P-limited (-P) *Prymnesium parvum*. Five ml of a mixture of each *P. parvum* -P culture (final concentration: 100 × 10³ ml⁻¹) or *Rhodomonas* sp. (final concentration: 95 × 10³ ml⁻¹) and *O. marina* culture (final concentration: 400 ml⁻¹) were prepared in 20 ml glass vials and incubated at 15°C and 30 µE m⁻² s⁻¹. *O. marina* used in that experiment were feed with *Rhodomonas* sp. and then starved for 6 d to ensure that only low numbers of algae in food vacuoles were present. A fixed incubation time of 1 h was chosen to compare initial food uptake of the 2 different algal species. At the beginning and after 1 h incubation, 1 ml subsamples were pipetted into 2 ml Utermöhl chambers and fixed with 1% glutaraldehyde. After settlement, cells were inspected under an inverted microscope using fluorescence light (Zeiss filter set 14). Counts of the number of ingested algal cells, which were easily recognisable by their red (*P. parvum*) or orange (*Rhodomonas* sp.) fluorescence, of at least 200 individuals of *O. marina* allowed for the determination of food uptake rate (algae grazer⁻¹ h⁻¹).

Short-term negative effects: To analyse short-term negative effects of *Prymnesium parvum* -P on *Oxyrrhis marina*, 10 ml of a mixture of each *P. parvum* -P culture (final concentration: $100 \times 10^3 \text{ ml}^{-1}$) and *O. marina* culture (final concentration: 700 ml^{-1}) were prepared in 20 ml glass vials and incubated at 15°C and $30 \mu\text{E m}^{-2} \text{ s}^{-1}$. Filtered seawater instead of algal suspension served as a control. After 0, 0.5, 1, 2, 4, 6 and 10 h, 1 ml subsamples were pipetted into 2 ml Utermöhl chambers and fixed with Lugol's iodine. Preliminary microscopic examinations had shown that after exposure to *P. parvum* cultures, *O. marina* lost its motility, became rounded, hyaline and finally lysed. Rounded and partly lysed *O. marina* cells were rapidly attacked by several *P. parvum* cells forming larger 'aggregates' around the remains of the *O. marina* cells. To quantify the lytic effects of *P. parvum*, *O. marina* was counted in the whole chamber, and a cell was scored only if the normal cell shape was still visible. Results are expressed as percentage of 'intact cells' compared to the seawater control. In addition, one experiment was performed to compare the short-term effects on immobilisation and lysis of *O. marina*. Aliquots of 0.5 ml of a concentrated *O. marina* culture (final concentration: 1200 ml^{-1}) were mixed with 10 ml of *P. parvum* -P (final concentration: $95 \times 10^3 \text{ ml}^{-1}$) or with 10 ml of seawater as control. After a defined exposure time (0, 0.5, 1, 2, 4, 6 h) at ambient light and room temperature, subsamples were taken to estimate immobilisation and lysis. Immobilisation was estimated by counting the number of moving *O. marina* cells using a slightly modified droplet live-counting procedure as described by Tillmann & John (2002). Briefly, 200 μl of cell suspension were separated into 50 to 60 small droplets in a Petri dish and the number of moving *O. marina* cells was counted under a stereo microscope. Cell lysis was estimated by counting numbers of

intact *O. marina* cells in 0.5 ml fixed with Lugol's iodine. To follow the time course of the formation of 'round cells' and aggregates (see Figs. 1 & 2), the counting protocol used in this experiment included the enumeration of round cells and of aggregates with triplicate samples at 0, 0.5, 1 and 2 h and single samples at 4 and 6 h.

Dilution series: A concentration/response curve was recorded in order to estimate the cell concentration causing 50% effect (EC_{50}). In triplicate, 5 ml of a mixture of *Oxyrrhis marina* (final concentration: 800 ml^{-1}) and different amounts of *Prymnesium parvum* -P culture were prepared in 20 ml glass vials. Six different final *P. parvum* cell concentrations ($75, 56, 38, 19, 8$ and $0 \times 10^3 \text{ ml}^{-1}$) were prepared by appropriate dilution with fresh medium. Just after mixing and after 6 h of incubation at 15°C and $30 \mu\text{E m}^{-2} \text{ s}^{-1}$, 1 ml subsamples were fixed with Lugol's iodine and counted as described above. Mortality, defined as 100 minus the percentage of intact cells compared to the seawater control, was transformed to probits (Hewlett & Plackett 1979). EC_{50} values, defined as the number of *P. parvum* needed to induce 50% mortality after 6 h of incubation, were calculated by linear regression analysis of probits against log-transformed *P. parvum* concentrations.

Effect of filtrate: In one experiment, the effect of cell-free culture filtrate was tested. In triplicate, 5 ml of a mixture of *Oxyrrhis marina* (final concentration: 800 ml^{-1}) and either *Prymnesium parvum* -P culture (final concentration: $97 \times 10^3 \text{ ml}^{-1}$) or cell-free filtrate (prepared by gentle syringe filtration through a Whatman GFF filter and added at the same amount as the algal suspension), were prepared in 20 ml glass vials. Filtered seawater served as a control. Vials were incubated at 15°C and $30 \mu\text{E m}^{-2} \text{ s}^{-1}$. At time 0, 6 and 24 h subsamples were taken to estimate immobilisation and lysis as described previously.

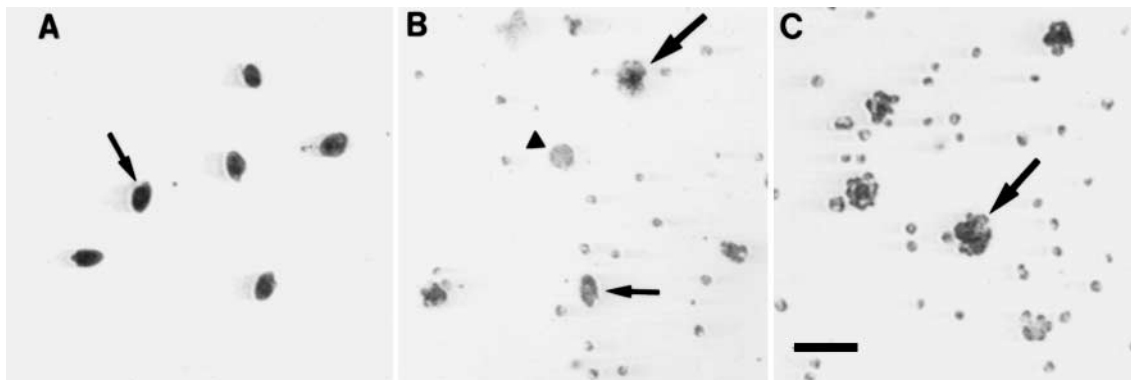


Fig. 1. Video prints of Lugol's fixed samples from the short-term experiments. (A) In control samples incubated with fresh medium, all cells of *Oxyrrhis marina* (thin arrow) exhibited the normal elongated cell shape. (B, C) In samples incubated with *Prymnesium parvum* -P, 'intact cells' of *O. marina* (thin arrow) as well as 'round cells' (arrowhead) and 'aggregates' (bold arrows) consisting of *P. parvum* around the remains of *O. marina* can be seen. Scale bar = $40 \mu\text{m}$

Addition of different *Oxyrrhis marina* concentrations: One experiment was carried out to study the effects of adding different amounts of *Oxyrrhis marina*. A series of equivalent *Prymnesium parvum* -P inoculum (final concentration: $20 \times 10^3 \text{ ml}^{-1}$) or seawater as a control was exposed to 8 different *O. marina* densities ranging from 220 to 81 000 cells ml^{-1} . Different *O. marina* concentrations were established by appropriate dilution of a concentrated *O. marina* culture.

Three replicates and 1 control were set up for each dinoflagellate concentration. After 6 h of incubation at 15°C and $30 \mu\text{E m}^{-2} \text{ s}^{-1}$, varying amounts were fixed with Lugol's iodine in order to obtain about 700 *O. marina* cells to count in the entire sample volume of the control sample. As described before, *O. marina* cells with a normal cell shape were counted. Percentage mortality was calculated by comparing cell numbers of samples with the seawater control.

Successive addition of *Oxyrrhis marina*: Six identical mixtures of 5 ml of *Prymnesium parvum* -P or seawater as a control and 50 μl of a concentrated *Oxyrrhis marina* were prepared in 20 ml glass vials, resulting in a final concentrations of $46 \times 10^3 \text{ ml}^{-1}$ and 900 ml^{-1} for *P. parvum* and *O. marina*, respectively. After 1 h incubation at 15°C and $30 \mu\text{E m}^{-2} \text{ s}^{-1}$, 1 ml subsamples were taken. To the remaining $\sim 4 \text{ ml}$ sample, 40 μl of a concentrated *O. marina* suspension were added (equivalent to $900 \text{ O. marina ml}^{-1}$). The whole procedure (1 h incubation, 1 ml subsamples, addition of *O. marina* with ca. 900 ml^{-1}) was repeated 5 times. All subsamples were fixed with Lugol's iodine and analysed as described above.

Batch culture experiment. To test whether 'low toxic' *Prymnesium parvum* supports growth of *Oxyrrhis marina*, the following experiment was carried out. A batch culture of *P. parvum* grown in filtered North Sea seawater diluted with distilled water to a salinity of 10‰ and enriched with f/10 nutrient solution was incubated at 15°C and $30 \mu\text{E m}^{-2} \text{ s}^{-1}$. Cell growth was monitored taking daily samples for cell counts. When the culture reached a density of about $50 \times 10^3 \text{ ml}^{-1}$, a series of equivalent *O. marina* inoculum (final concentration: 500 ml^{-1}) was exposed to 6 different *P. parvum* densities ranging from 0 to $42 \times 10^3 \text{ cells ml}^{-1}$. Different *P. parvum* concentrations were established by appropriate dilution of the *P. parvum* batch culture. A total volume of 15 ml of each mixture was prepared in 20 ml glass-vials. Vials containing the same concentrations of phytoplankton only served as control. At time 0, initial samples were taken for determination of cell concentrations. Subsequently, the volume of each vial was split into 3 wells of a multiwell plate (Nunc) and incubated at 15°C under dim light ($2 \mu\text{E m}^{-2}$

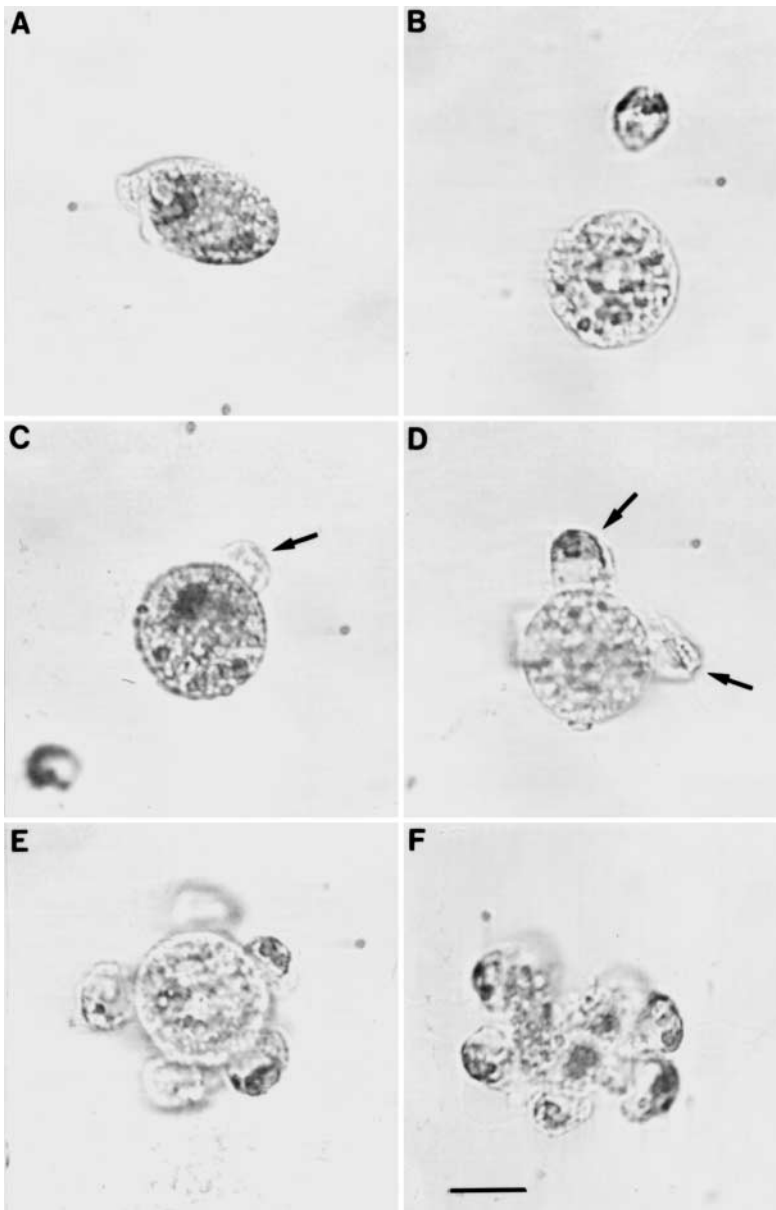


Fig. 2. Video prints of Lugol's fixed samples from the short-term experiments. (A) *Oxyrrhis marina* with its normal shell shape. (B) *O. marina* completely rounded off. (C, D, E) A varying number of *Prymnesium parvum* cells (arrows) have settled on the surface of *O. marina* round cells. (F) 'Feeding aggregate' with *O. marina* almost disintegrated. Note that the sequence of prints is not a true time course. Scale bar = 10 μm

s⁻¹). The same experiment was set up synchronously with *Rhodomonas* sp. as a non-toxic control species. Although *Rhodomonas* sp. and *P. parvum* differed slightly in size (microscopical size measurements using Lugol fixed samples; length × width (n = 50): 11.9 ± 0.6 × 7.0 ± 0.5 μm; 9.2 ± 0.9 × 5.5 ± 0.7 μm, respectively), the same algal concentrations were used as outlined above. After 2, 6 and 11 d incubation, a 1 ml subsample was taken from each well and fixed with Lugol's iodine. Cell concentrations of *O. marina* and *P. parvum* (or *Rhodomonas* sp.) were determined with an inverted microscope. Growth and ingestion for the periods 0 to 2 and 0 to 6 d were calculated from the initial cell concentrations and the cell concentrations of the accordant sampling day using the equations of Frost (1972) and Heinbokel (1978).

RESULTS

Semi-continuous culture experiments

Prymnesium parvum cell concentrations

Before the experiments started, cell numbers of *Prymnesium parvum* in the semi-continuous cultures slightly decreased from 140 to 120 × 10³ ml⁻¹. After the first 5 d of experiments, where concentrations were around 100 × 10³ ml⁻¹, cell concentrations steadily decreased down to 45 × 10³ ml⁻¹ by the end of the experiment.

Grazing of *Oxyrrhis marina* on *Prymnesium parvum*

Starved *Oxyrrhis marina* initially ingested *Prymnesium parvum* -P, but only at a very low rate (0.07 ± 0.01 algal cells grazer⁻¹ h⁻¹). When fed with the non-toxic control species *Rhodomonas* sp., the ingestion rate of *O. marina* was as high as 2.75 ± 0.02 algal cells grazer⁻¹ h⁻¹.

Short-term effects of *Prymnesium parvum* towards *Oxyrrhis marina*

Prymnesium parvum caused harmful, microscopically visible effects on the heterotrophic dinoflagellate *Oxyrrhis marina*. Cells of the dinoflagellate lost their normal cell shape and became rounded, hyaline and finally lysed (Figs. 1 & 2). Rounded and partly lysed *O. marina* cells were rapidly attacked by several *P. parvum* cells forming larger aggregates around the remains of the *O. marina* cells (Figs. 1 & 2). Live microscopy clearly showed the formation of posteriorly

located pseudopodia and subsequent ingestion of particulate material originating from disintegrating *O. marina* by *Prymnesium parvum* as described in detail by Tillmann (1998). As can be seen from Figs. 1 & 2, a clear differentiation between *O. marina* cells with their typical cell shape and rounded or partly lysed individuals in Lugol's fixed samples was possible. Lytic effects were analysed in a time course experiment. The number of intact *O. marina* cells, calculated as a percentage of initial values, remained constant with time for the seawater control. Cell lysis was evident, however, for the algal treatments. The number of intact cells sharply decreased during the first 2 h and then slowly reached a constant level of about 10% of the initial values (results not shown). In a similar experiment subsequently performed, the immobilising and lytic effect of toxins was simultaneously analysed (Fig. 3). Immobilisation of *O. marina* and cell lysis showed almost the same time course, indicating a close coupling between these 2 processes. The detailed enumeration of intact cells, round cells and of 'feeding aggregates' (consisting of *P. parvum* cells phagocytosing partly lysed *O. marina* remains) is shown in Fig. 4. In the control, numbers of intact *O. marina* cells stayed quite constant over time. When incubated with *P. parvum*, number of intact *O. marina* cells decreased with time. The number of round cells peaked after 30 min of incubation and subsequently declined again. In contrast, the number of feeding aggregates steadily increased during the first 2 h of incubation. The sum of intact cells, round cells and feeding aggregates slightly decreased with time indicating some total disintegration of *O. marina*. However, the data show that killed

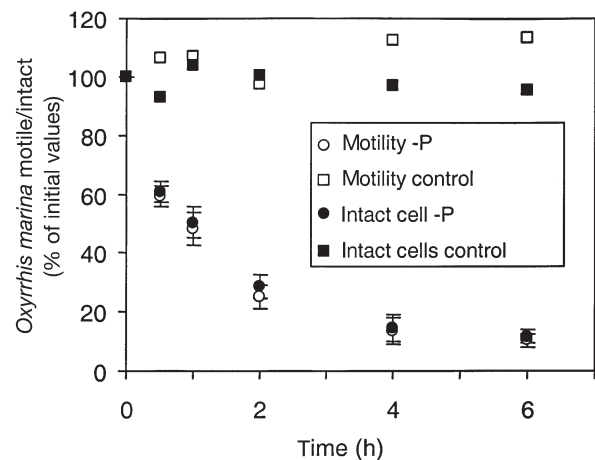


Fig. 3. Motile or intact *Oxyrrhis marina* (% of initial values) versus time when incubated with *Prymnesium parvum* -P (final concentration: 95 × 10³ ml⁻¹). Data points for experimental flasks represent mean values (n = 3) with error bars (±1 SD); control (n = 1)

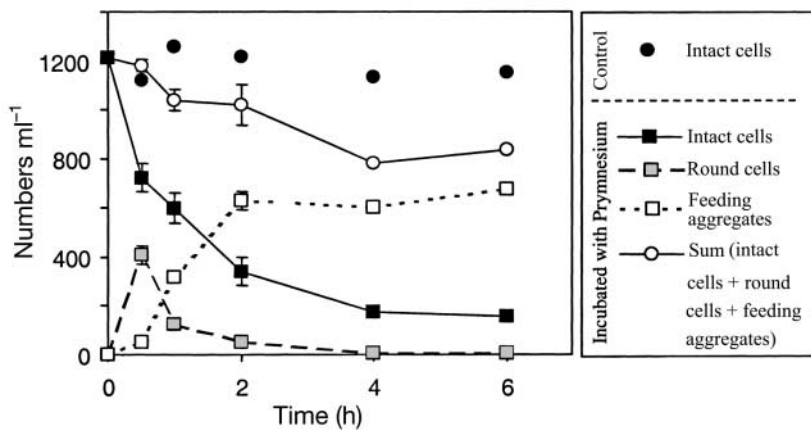


Fig. 4. Concentration of *Oxyrrhis marina* 'intact cells', 'round cells' and 'aggregates' and the sum of all 3 versus time, when incubated with *Prymnesium parvum* -P (final concentration: $95 \times 10^3 \text{ ml}^{-1}$). For a definition of the various cell types see Figs. 1 & 2. Data points for time 0, 0.5, 1 and 2 h represent mean values ($n = 3$) with error bars ($\pm 1 \text{ SD}$); 4 and 6 h ($n = 1$)

O. marina cells predominantly ended as feeding aggregates.

The cell concentrations causing 50% cell lysis were determined by recording a dilution/response curve (Fig. 5) which showed a sigmoid relation between percent of *Oxyrrhis marina* mortality and *Prymnesium parvum* cell concentration. The EC_{50} value, defined as the amount of algae needed to induce 50% mortality after 6 h of incubation, was $16 \times 10^3 \text{ cells ml}^{-1}$.

Effects of culture filtrate

A negative effect on *Oxyrrhis marina* also was obvious when testing *Prymnesium parvum* cell-free culture filtrate (Fig. 6). The immobilisation effect of filtrate, however, was lower compared to the effect of algal

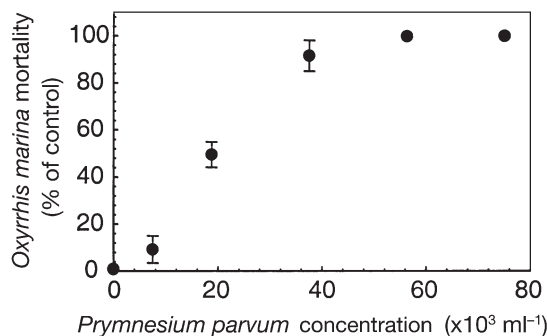


Fig. 5. Dilution/response curve for *Oxyrrhis marina* incubated with *Prymnesium parvum* -P. *O. marina* mortality (% of control) as a function of *P. parvum* cell concentration. Data points represent mean values ($n = 3$) with error bars ($\pm 1 \text{ SD}$)

suspensions (same cell concentration as used for the filtrate). The difference between cell free filtrate and algal suspension was most obvious for the lytic effect; after 6 h of incubation, the number of intact cells was not significantly different from the seawater control. After 24 h, the number of intact cells was reduced in only 2 of 3 parallel preparations causing the large SD in Fig. 6B.

Changing *Oxyrrhis marina* concentration

The percentage of *Oxyrrhis marina* mortality is not only reduced by diluting the *Prymnesium parvum* culture (see Fig. 5), but also by increasing the dinoflagellate concentration (Fig. 7). When exposed to $20 \times 10^3 \text{ P. parvum ml}^{-1}$, mortality was almost

100% for *O. marina* concentrations $< 1000 \text{ ml}^{-1}$. With increasing *O. marina* concentration, mortality declined to about 25% at a concentration of $8100 \text{ O. marina ml}^{-1}$. The effect of a reduced percentage mortality upon elevated target concentration is also apparent when *O.*

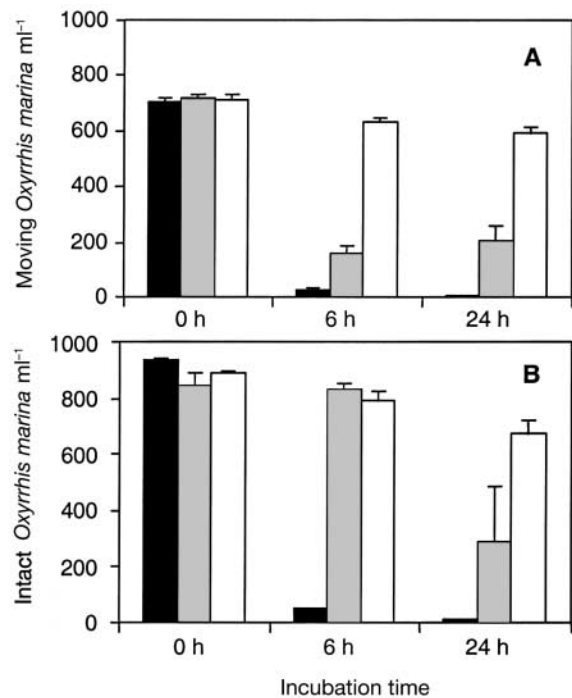


Fig. 6. Number per ml of (A) motile or (B) intact *Oxyrrhis marina* after 0, 6 and 24 h exposure to whole cells (black bars) or to culture filtrate (grey bars) of *Prymnesium parvum* -P (final concentration: $97 \times 10^3 \text{ ml}^{-1}$) or to fresh medium (control, white bars). Results are expressed as triplicate mean $\pm 1 \text{ SD}$

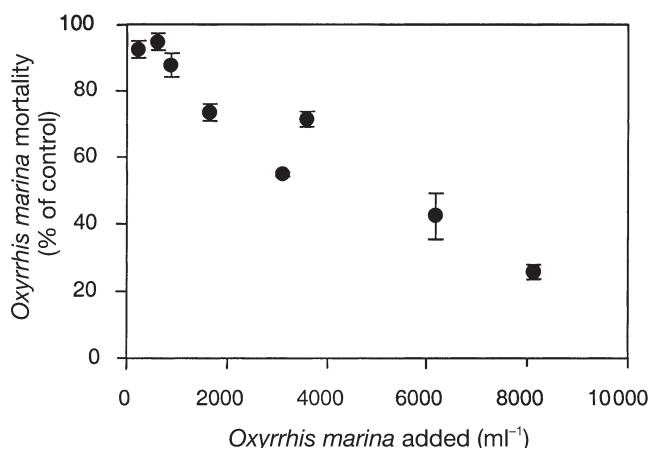


Fig. 7. *Oxyrrhis marina* mortality (% of control) as a function of initial *O. marina* concentration. Different *O. marina* concentrations were exposed to *Prymnesium parvum* -P (final concentration: $20 \times 10^3 \text{ ml}^{-1}$) for 6 h. Data points represent mean values ($n = 3$) with error bars ($\pm 1 \text{ SD}$)

marina mortality is recorded upon successive additions to the same algal suspension (Fig. 8). For the first 3 additions of *O. marina*, a mortality close to 100% was recorded. Upon further additions of *O. marina*, however, mortality declined, reaching 0 after the 5th addition.

Batch culture experiment

Under the environmental conditions and cell concentrations tested, *Prymnesium parvum* caused toxic effects on *Oxyrrhis marina*. The following experiment tested whether *O. marina* is able to graze and grow on *P. parvum* under conditions of 'low toxicity' of the algae. To achieve these conditions, *P. parvum* grown under nutrient-replete conditions was offered as food in a range of low cell concentrations at dim light (assuming that photosynthesis is a prerequisite of toxin production). Ingestion and growth of *O. marina* fed with *P. parvum* was compared to results when *Rhodomonas* sp. was offered as food. When estimated after 2 d incubation, ingestion of *Rhodomonas* sp. was high and almost linearly dependant on the initial algal concentration, reaching 14 cells grazer⁻¹ d⁻¹ at the highest *Rhodomonas* sp. concentration. In contrast, initial ingestion of *P. parvum* calculated for the 0 to 2 d period was comparably high only at the 2 lowest algal concentrations and dropped to almost 0 at the highest *P. parvum* concentration (Fig. 9A). Correspondingly, growth rate of *Oxyrrhis marina* increased with increasing *Rhodomonas* sp. concentrations but remained low with *Prymnesium parvum* as food. Furthermore, a negative growth rate, i.e. cell death, was observed at the highest *P. parvum* concentration (Fig. 9B). However,

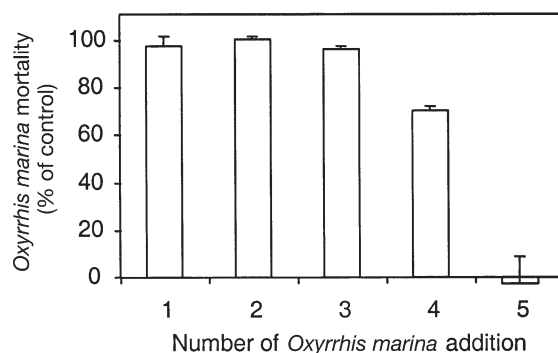


Fig. 8. Effect of successive addition of *Oxyrrhis marina* to *Prymnesium parvum* -P (final concentration: $46 \times 10^3 \text{ ml}^{-1}$). *P. parvum* (or fresh medium: control) was spiked with *O. marina*, incubated for 1 h and a subsample was withdrawn for the determination of numbers of intact *O. marina* (Addition 1). Subsequently, the same sample was spiked again with new *O. marina* and processed as described before (Addition 2). This procedure was repeated 5 times. The figure shows mortality of *O. marina* (% of control) after each addition. Data points represent mean values ($n = 6$) with error bars ($\pm 1 \text{ SD}$)

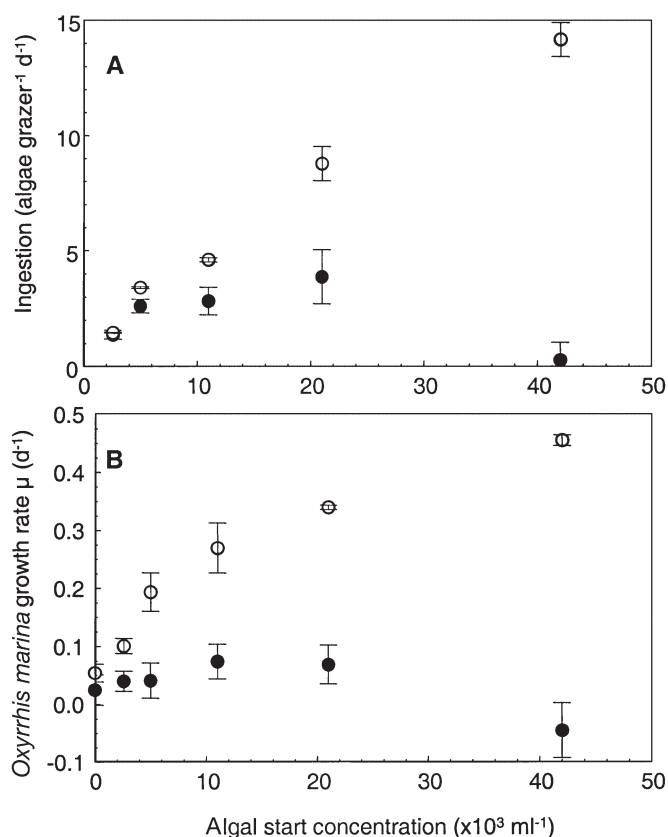


Fig. 9. *Oxyrrhis marina*. (A) Ingestion and (B) growth rate as a function of initial prey concentration calculated for the 0 to 2 d period when incubated with *Prymnesium parvum* (●) or *Rhodomonas* sp. (○). Data points represent mean values ($n = 3$) with error bars ($\pm 1 \text{ SD}$)

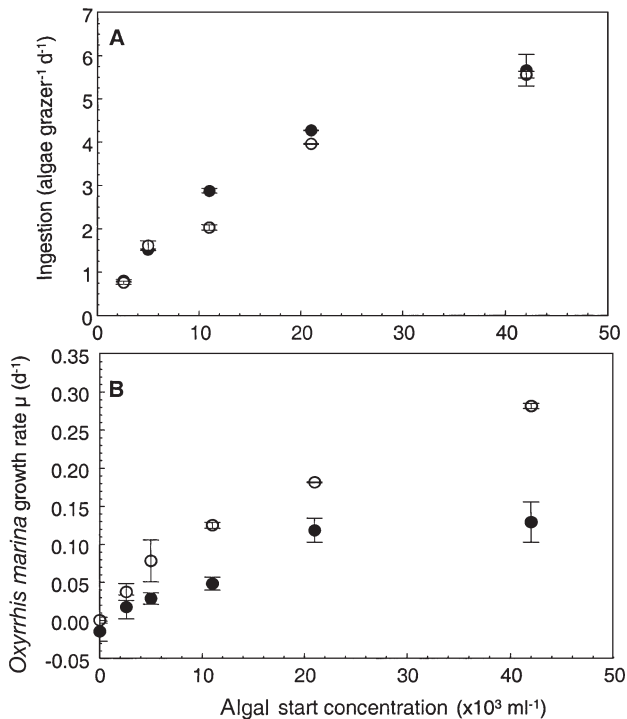


Fig. 10. *Oxyrrhis marina*. (A) Ingestion and (B) growth rate as a function of initial prey concentration calculated for the 0 to 6 d period when incubated with *Prymnesium parvum* (●) or *Rhodomonas* sp. (○). Data points represent mean values (n = 3) with error bars (±1 SD)

when calculated for the 0 to 6 d incubation period, it is evident that *O. marina* ingestion and growth had 'recovered' after the initial lag period; ingestion and growth of *P. parvum* was almost the same as for *Rhodomonas* sp. over the whole range of initial algal concentrations (Fig. 10A). Positive growth rates increasing with food concentration were observed for all *P. parvum* concentrations tested, although they were lower than growth rate recorded for the corresponding *Rhodomonas* sp. concentrations (Fig. 10B). At the final sampling after 11 d, *P. parvum*, like *Rhodomonas* sp., was drastically reduced in all experimental flasks, whereas cell numbers had slightly increased in the phytoplankton control cultures (Fig. 11A,B). For both algal prey, final concentrations of *O. marina* were linearly dependant on the initial algal concentration (Fig. 11C).

DISCUSSION

In the present study, toxic effects of the haptophyte *Prymnesium parvum* on the heterotrophic dinoflagellate *Oxyrrhis marina* were demonstrated. Toxicity of *P. parvum* is defined here by its lytic effects towards

O. marina. In agreement with these results, toxicity of *P. parvum* cultures used in the described semi-continuous culture experiment also has been proved by standard hemolytic tests and by its negative effects on a range of autotrophic algae (C. Legrand pers. comm.). It is well known that *P. parvum* under certain conditions produces highly potent toxins with a broad spectrum of different biological effects that are released to the water (Shilo 1967). *Prymnesium*'s toxins are ichthyotoxic (Ulitzur & Shilo 1966), pharmacologically active (Mel-dahl & Fonnum 1993, 1995) and exert lytic effects on various cell types such as erythrocytes (Yariv & Hestrin 1961), bacteria (Ulitzur & Shilo 1970) and a number of nucleated cells (Shilo & Rosenberg 1960, Dafni & Giberman 1972). In addition, negative effects towards a range of marine organisms have been reported.

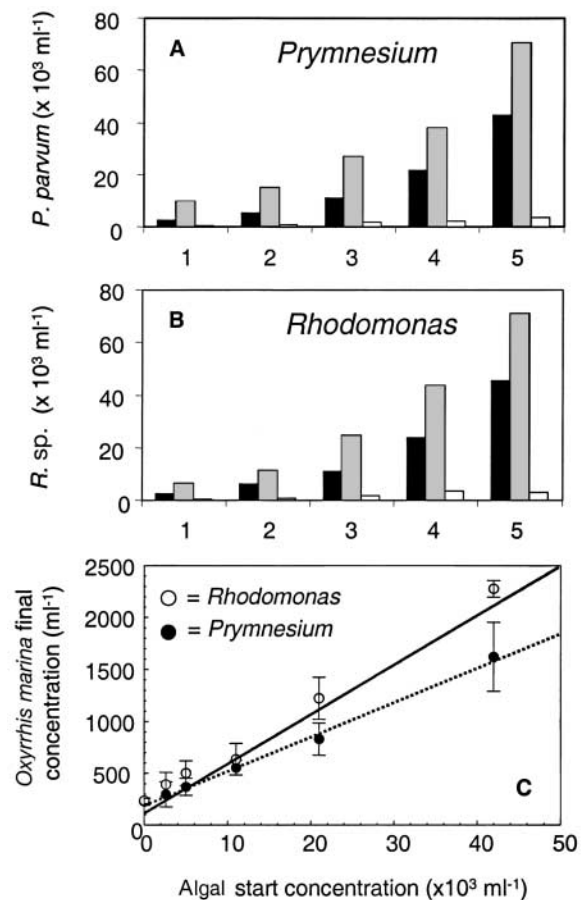


Fig. 11. Cell numbers of (A) *Prymnesium parvum* and (B) *Rhodomonas* sp. Initial concentrations (black bars) and final concentrations after 11 d incubation of control (grey bars) and experimental (white bars) flasks are given for the 5 different algal start concentrations (1 to 5 on the x-axis). (C) Final concentration of *Oxyrrhis marina* after 11 d as a function of initial prey concentration when fed with *P. parvum* (●) or *Rhodomonas* sp. (○). Data points represent mean values (n = 3) with error bars (±1 SD)

Valkanov (1964) briefly described the reactions of a broad range of different organisms upon exposure to *P. parvum*. Interestingly, he also tested *O. marina* and reported that all individuals died within 10 to 60 min after exposure to *P. parvum*.

Toxic effects of *Prymnesium* are known to be due to extracellular toxins (Shilo 1967). Accordingly, here, I showed that toxic effects towards *O. marina* were apparent when using cell free culture filtrate. However, lytic effects of cell-free filtrate occurred to a lesser degree compared to the effect of algal suspensions. This is most likely because of the known amphiphatic properties of the toxin molecules and their ability to form aggregates or micelles in solution (Ulitzur 1973). The formation of aggregates certainly affects the filterability of dissolved extracellular toxins. Using a set of filters of different pore size, Ulitzur (1973) convincingly showed that toxin micelles are typically larger than 0.6 μm and about 50% of hemolysis aggregates are larger than 1 μm , which is roughly the pore size of the glass fibre filter used in the present study. Furthermore, it is likely that toxin aggregates, rather than the individual molecules, are responsible for the haemolytic activity of the *Prymnesium* toxin (Ulitzur 1973, Moran & Hani 1974), as was also shown for the detergent sodium dodecyl sulfate (Armstrong 1957). However, neither the haemolysin nor the ichthyotoxin is solely extracellular, because both can be extracted from cells in large amounts (Shilo & Rosenberg 1960). There are, however, no indications of any ecological impact of intracellular toxins of *P. parvum*. In fact, negative effects of *P. parvum in situ* are always the consequence of a direct exposure of target organisms to extracellular toxins. Thus, if or to what extent the initially observed (but admittedly low) ingestion of *P. parvum* by *O. marina* and, hence, the incorporation of intracellular toxins is involved in negative effects towards grazers remains unknown.

Interestingly, percentage mortality of *Oxyrrhis marina* was not only reduced by diluting *Prymnesium parvum*, but also by increasing the dinoflagellate concentration. Moreover, upon repeated additions of *O. marina*, mortality declined, reaching 0 after the fifth addition. Supporting observations can be extracted from older literature. Padilla (1970) showed that the time course and extent of haemolysis were dependant on the applied concentrations of rat erythrocytes. Reich & Rotberg (1958), applying standard ichthyotoxicity tests with *P. parvum*, replaced dead fish with live ones and found profound differences in the time required to kill the fish. After the third addition, all fish remained alive and the authors suggested that the toxin is absorbed from the solution by the fish or by their heavy excretion of mucus (Reich & Rotberg 1958). Normally, percentage mortality recorded in toxicity

tests is assumed to be the result of a log-normal distribution in the sensitivity of individual target organisms to a given toxin concentration. In the case of *P. parvum* toxin, however, percentage mortality also seems to be determined by the apparent amount of toxin available per target organism, which itself is not constant but depends on the target concentration. This result underlines the need to standardise toxicity tests applied to *P. parvum* with respect to target concentration, in order to obtain reproducible and comparable results. More interesting, however, these results also clearly indicate that the toxin is removed from the system. By increasing the *O. marina* cell concentration, increasing amount of dissolved organic matter (DOM) were also added, and it is possible that DOM could alter toxicity. More probable, however, is that the majority of the toxin is removed from the system by its action, that is by binding to the membrane of the target organisms.

There are indications in the literature that the lysine molecules produced by *Prymnesium parvum* may become inert upon contact with cell surface. By using tritium-labelled toxin, Martin & Padilla (1971) found that after only 30 s exposure of erythrocytes to prymnesin, 40% of the toxin was removed from the supernatant fluid, and the majority of it was firmly bound. During the whole prolytic period (100 s) the toxin was bound to the erythrocytes in linear time course and was completely removed from the system during this interval (Rauckman & Padilla, cited in Martin & Padilla 1971). Moreover, rates of erythrocyte haemolysis induced by *P. parvum* toxin decrease upon addition of certain lipids (cephalin, cholesterol, phosphatidylcholin) (Martin & Padilla 1971, Igarashi et al. 1998). This effects was ascribed to a reduction in the effective concentration of prymnesin owing to the formation of a toxin-inhibitor complex (Martin & Padilla 1971). This suggests that prymnesin may react primarily with membrane sterols. Indeed, using liposomes as a model membrane system, Imai & Inoue (1974) showed that prymnesin caused severe damage to liposomes containing cholesterol but did not affect those without cholesterol. However, additional specific binding sites may exist, as Ulitzur & Shilo (1970) showed that prymnesin lysed spheroplasts or protoplasts of some bacteria whose membranes contain no cholesterol.

To conclude, toxicity of *Prymnesium parvum* in natural systems may be highly influenced by total biomass of co-occurring species and perhaps by concentrations of non-living particulate organic matter (POM) and DOM.

Nothing is known about long-term stability of such toxin/binding-sites complexes. In the experiment with successive addition of *Oxyrrhis marina*, lysis induced mortality reached 0 after 5 h of incubation, indicating

that toxin/binding-site complexes are stable at least in the magnitude of hours. If the *Prymnesium parvum* toxins are permanently removed from the system, addition of highly specific artificial binding sites to *P. parvum* blooms may represent a promising tool to detoxify bloom waters.

In the –P continuous culture experiment, P was the limiting nutrient as indicated by high particulate C/P ratios measured at the end of the experiment by Skovgaard et al. (2003). Under these conditions tested in the semi-continuous culture experiment, short-term toxic effects (cell lysis) prevent significant grazing of *Oxyrrhis marina* on *Prymnesium parvum*. This finding, however, does not necessarily lead to the conclusion that toxins are used to eliminate a potential predator, as *P. parvum* even lacking toxicity might be a poor prey for *O. marina* for reasons other than toxicity, e.g. size, handling problems or nutritional composition. It therefore was shown that, under certain conditions, *O. marina* is indeed a potential predator able to graze and grow on *P. parvum* with rates comparable to the non-toxic control species *Rhodomonas* sp. As nutrient replete semi-continuous cultures of *P. parvum* (100×10^3 cells ml⁻¹) grown under the same conditions as used for the P-limited cultures also caused cell lysis of *O. marina* (own unpubl. results), a different experimental set-up was chosen in order to minimise toxin production/accumulation. After batch culture growth of *P. parvum* in nutrient-replete medium without bubbling at relatively low light intensities, the incubation condition in particular constitutes of lower cell concentration ($<50 \times 10^3$ ml⁻¹) and dim light. A nutrient replete medium was chosen as there are several reports that toxicity of both *P. parvum* and *P. patelliferum* is enhanced by nutrient limitation (Shilo 1971, Dafni et al. 1972, Larsen et al. 1993, Meldahl et al. 1994, Johansson & Granéli 1999, Johansson 2000, Legrand et al. 2001). Bubbling was omitted because aeration of cultures was shown to be important in promoting intracellular toxin production (Igarashi et al. 1995). The importance of light conditions is more difficult to evaluate since light obviously has 2 different roles working in opposite directions. Light has been claimed to be essential for toxin production (Padan et al. 1967, Shilo 1967) with higher light conditions augmenting toxin production (Shilo & Aschner 1953). On the other hand, the extracellular toxins are known to be rapidly inactivated by exposure to visible and ultraviolet light (Parnas et al. 1962, Reich & Parnas 1962). This set of conditions is not meant to be exclusive; there may be other conditions that could also reduce toxic effects and allow *O. marina* to ingest *P. parvum* and grow in its presence (e.g. high concentrations of POM and DOM; see above). In any case, under the chosen conditions, negative effects (low ingestion, negative growth rate) were only apparent at the

beginning of the experiment at higher *P. parvum* concentrations. The obvious disappearance of these negative effects with time, i.e. high ingestion and growth when calculated for the 0 to 6 d period, might be due to acclimatisation of *O. marina* towards low amounts of toxin. More likely, however, is the possibility that a certain, albeit low, amount of extracellular toxin was present at the beginning, being introduced by inoculating *P. parvum* from the light grown culture. This initial stock of extracellular toxin then disappeared with time due to adsorption and/or degradation. Little is known about negative effects of *Prymnesium* toxins at lower, sublethal concentrations. For the closely related genus *Chrysochromulina*, Estep & MacIntyre (1989) proposed the concept of dasmotrophy, a theory of auxotrophy following induced osmosis. They speculated that extracellular toxins produced by *Chrysochromulina* simply punch holes in the cell membrane of other organisms, producing a transient nutrient leakage benefiting *Chrysochromulina*. John et al. (2002) recently showed that *Oxyrrhis marina* was not killed by the presence or by the ingestion of toxic *Chrysochromulina polylepis*, but that profound differences in ingestion and growth of *O. marina* when fed a toxic or a non-toxic clone of *C. polylepis* were apparent, indicating that the toxin deters grazers. The same may hold true for *P. parvum* toxins for a certain range of sublethal concentrations, as indicated by the initially low ingestion and growth of *O. marina* in the batch culture experiment.

In conclusion, *Prymnesium parvum* is a suitable prey for *Oxyrrhis marina* sustaining growth if toxicity is low. This indicates that protozoan grazing might be an important factor controlling *P. parvum* population dynamics prior to bloom build-up when environmental conditions prevent larger amounts of extracellular toxin to be produced and/or to accumulate in the water. However, when sufficient amounts of toxins are produced and released to the medium, they may act as a chemical defence against grazers. This may especially be the case for nutrient-limited bloom conditions, as nutrient stress is supposed to enhance toxin production (Shilo 1971, Johansson & Granéli 1999). *P. parvum* cell concentrations causing cell lysis of *O. marina* are well below reported bloom concentrations of *P. parvum* of up to 10^6 cells ml⁻¹ (reviewed by Edvardsen & Paasche 1998). Hence, elimination of grazers may be an important mechanism in the development and maintenance of dense blooms of this species in nature.

Prymnesium not only benefits from toxin production by not being grazed. The results clearly showed that there is an almost quantitative relationship between immobilisation/lysis of *Oxyrrhis marina* and the appearance of aggregates and phagotrophy. In addition to the known uptake of bacteria by *Prymnesium* (Nygaard & Tobiesen 1993, Legrand et al. 2001), the impressive

phagotrophic capacity of *Prymnesium* ingesting prey of different size, sometimes even larger than itself, was recently reported (Tillmann 1998). Microscopic observation supports the notion that *Prymnesium* intrinsically is a rather poor hunter: it is a poor swimmer and the stiff haptonema, which is poorly adapted to catching particles (in contrast to *Chrysochromulina*, see Kawachi et al. 1991), is not involved in the feeding process (Tillmann 1998). Food uptake by means of pseudopodia formed at the posterior part of the cell is rather slow and takes minutes (Tillmann 1998). Thus, *Prymnesium* seems to be unable to catch and ingest motile prey. Recently, it was convincingly shown that *Prymnesium* exotoxins are indeed used to immobilise and kill potential prey organisms prior to ingestion (Skovgaard & Hansen 2003). However, it still remains unknown whether or to what extent phagotrophy may lead to an increased growth rate of *Prymnesium*.

To conclude, toxicity of *Prymnesium parvum*, although its controlling factors are still insufficiently understood, is a key factor in determining the interaction with protozoan grazers. If toxicity is low, *P. parvum* is a suitable prey for *Oxyrrhis marina*. At high toxicity levels, however, *O. marina* is rapidly killed and ingested by *P. parvum*; thus, reversing the normal direction of grazing interactions between protozoa and algae.

Acknowledgements. Special thanks are due to Alf Skovgaard and Per-Juel Hansen for setting up the semi-continuous cultures and for the opportunity to work at the Marine Laboratory, Helsingør. I also thank Cathrine Legrand, Per-Juel Hansen and Alf Skovgaard for valuable discussions, Victor Smetacek and 3 anonymous reviewers for comments and suggestions on the manuscript, and Steve Pueppke for correcting the English. This work was supported by the European Commission (Research Directorate General-Environment Programme-Marine Ecosystems) 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.

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Editorial responsibility: John Dolan,
Villefranche-sur-Mer, France

Submitted: November 25, 2002; Accepted: February 3, 2003
Proofs received from author(s): May 6, 2003