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Influence of different nutrient conditions on cell density, chemical composition and toxicity of *Prymnesium parvum* (Haptophyta) in semi-continuous cultures

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Abstract

The influence of various nitrogen (N) and phosphorus (P) levels on the cell density, chemical composition and toxicity of the marine haptophyte *Prymnesium parvum* were studied. A non-axenic strain of *P. parvum* was grown in semi-continuous cultures under either N- or P-limited conditions or nutrient-sufficient conditions (N:P = 1:1, 160:1 and 16:1, respectively). Cell toxicity was measured on two occasions at steady state using a haemolytic test. Haemolytic activity was determined as saponin nano-equivalents (SnE) and HE_{50} (50% haemolysis). Haemolytic activity was demonstrated in all treatments. However, haemolytic activity was significantly higher in *P. parvum* cells grown under N- or P-limited conditions (287.7 ± 14.0 and 256.8 ± 38.1 SnE cell^{-1}, respectively) compared to cells grown under non-limiting conditions (42.4 ± 3.3 SnE cell^{-1}). Our results document, for the first time, enhanced haemolytic activity in *P. Parvum* cells irrespective of which nutrient (N or P) was limiting growth. Our results suggest that the toxicity of *P. parvum* is related to cellular physiological stress, due to nutrient limitation rather than to the direct involvement of either N or P in toxin synthesis.

Keywords: Cell density; Chemical composition; Haemolytic activity; Nutrient limitation; *Prymnesium parvum*; Toxicity

1. Introduction

Since a first report in the 1920s (Liebert and Deerns, 1920), toxic blooms of

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Prymnesium parvum (Haptophyta), have frequently been reported from coastal marine and brackish waters around the world (Otterstrøm and Steeman Nielsen, 1940; Shilo, 1971; Bjergskov et al., 1990; Holmquist and Willén, 1993). Blooms of this microalgae have often been associated with massive fish mortalities (Shilo, 1964; Holdway et al., 1978; Kaartvedt et al., 1991; Lindholm and Virtanen, 1992; Holmquist and Willén, 1993), resulting in negative impacts on the marine ecosystem and large economic consequences for commercial aquaculture. These events have resulted in intensive studies of *P. parvum*, yet, knowledge about ecophysiology and factors that influence its production of toxins is poor.

The species is euryhaline (Shilo, 1971), but dense blooms resulting in fish kills have so far been reported only from brackish waters (Parnas and Abbott, 1965; Moestrup, 1994). In contrast to many other toxin-producing algae, *P. parvum* excretes its toxins into the surrounding water (Shilo and Ashner, 1953; Skulberg et al., 1993). The biological activity of *P. parvum* toxins is not due to a single compound, but to a set of toxins with ichthyotoxic, cytotoxic and haemolytic activity (Shilo, 1981), indicating a complex nature of the toxins. All three effects of the toxins appear to depend upon a single mechanism, a change in permeability of cell membranes, which causes the membranes to become cation permselective (Shilo, 1971, Collins, 1978). The most striking effect of increased membrane permeability is the lethal effect on aquatic gill breathing animals, such as fish and molluscs, due to a loss of the selective cell permeability in the gills (Shilo, 1967; Meldahl et al., 1994; Hallegraeff, 1993).

A large obstacle in understanding the regulation of toxicity in this species has been that the toxicity varies substantially both in nature and in culture, and seems to be expressed only under certain environmental conditions (Ulizur and Shilo, 1964; Dafni et al., 1972; Larsen et al., 1993). Several studies have demonstrated that toxin production in phytoplankton is not a fixed component of algal metabolism. Instead, both the extent to which algae accumulate toxins and the number and quantity of individual toxins of algae are strongly influenced by environmental growth conditions (Plumley, 1997). Blooms of *P. parvum* often occur in eutrophic areas (Collins, 1978) where N and P availability can vary substantially. As both the availability and composition of nutrients have a significant impact on the algal community structure and biomass, and also on phytoplankton biochemistry, changes in nutrient supply may ultimately affect the production of toxins. Shilo (1967) first reported a connection between low levels of P in the medium and high toxicity of *P. parvum*. This relationship has later been confirmed both in *P. parvum* (Dafni et al., 1972) and the closely related *Chrysochromulina polylepis* (Edvardsen et al., 1990; Edvardsen, 1993; Simonsen and Moestrup, 1997). These observations suggest a possible connection between nutrient limitation and high toxicity. However, most studies of *P. parvum* toxicity have involved only investigations of the influence of P-limitation. Information about how nutrient limitation, including both N and P, influence *P. parvum* toxicity is rare. As N-limitation is more common in marine environment its possible influence on phytoplankton toxicity is of great ecological significance.

The aim of this study was to examine whether toxicity in *P. Parvum* was stimulated under N- and/or P-limitation. Toxicity was quantified by measuring the haemolytic activity of *P. Parvum* cells grown in semi-continuous cultures under different N:P supply ratios.

2. Methods

2.1. Algal culture conditions

A non-axenic strain of *P. parvum* (LAC 12 CCMP 708) was grown as batch cultures in 12 Pyrex bottles containing 2 l of f/20 medium (Guillard and Ryther, 1962). The cultures were grown at a temperature of 16°C and with PAR (Photosynthetic Active Radiation) of 200 μmol m^{-2} s^{-1} (cool-white fluorescent tubes) under a 16:8-h light/dark cycle for 23 days. The medium was prepared with filtered (Gelman A/E, 25 mm, nominal pore size 0.45 μm) and autoclaved aged natural sea water (initial nutrient concentrations; 1.2 μM NO_3^-, 0.1 μM PO_4^{3-}, 0.3 μM NH_4^+) adjusted to a salinity of 9‰ with milli-Q water. After the batch cultures reached high cell densities (about 1 week after inoculation, 90×10^3 cells ml^{-1}) 40% of the culture volume (0.8 l) was sampled at a set time each day (10:00 am) and replaced with an equal volume of new, enriched media to attain steady-state conditions (Table 1). Three N:P supply ratios were used (four replicates of each); N:P = 1:1, 16:1 or 160:1. The daily additions of inorganic nutrients were as follows: N-limited (-N; 2.0 μM NO_3^-, 2.0 μM PO_4^{3-}), non-limited (NP; 32 μM NO_3^-, 2.0 μM PO_4^{3-}) and P-limited conditions (-P; 32 μM NO_3^-, 0.2 μM PO_4^{3-}). Trace metals, iron and EDTA were added to all cultures at levels corresponding to medium f/20 (Guillard and Ryther, 1962). Vitamins ($B_{12,}$ biotin and thiamin) were added following the method of Schöne and Schöne (1982).

The outgoing medium (0.8 l) was used for the following measurements: inorganic nutrients (NO_3^-, NH_4^+ and PO_4^{3-}); cell density; particulate nitrogen (PON), phosphorus (POC) and carbon (POC); and toxicity (haemolytic activity).

2.2. Cell densities and chemical analyses

Phytoplankton samples (50 ml) were preserved with acid Lugol's solution and cells were counted using a particle counter (HIAC/ROYCO 9064) calibrated by manual

Table 1
Inorganic nutrient concentrations (μM) in the outgoing media and in the daily added new media in semi-continuous cultures of *P. parvum*[a]

	N:P ratio		
	1:1	16:1	160:1
Cell density (10^3 cells ml^{-1})	12.8±0.4	42.0±4.9	38.8±4.3
Daily added concentrations			
PO_4^{3-} (μM)	2.0	2.0	0.2
NO_3^- (μM)	2.0	32.0	32.0
NO_4^+	0.0	0.0	0.0
Concentrations in outgoing media			
PO_4^{3-} (μM)	2.1±0.6	1.2±0.6	n.d
NO_3^- (μM)	0.2±0.0	22.9±4.8	26.2±5.2
NH_4^+	0.1±0.0	0.2±0.1	0.2±0.0

[a] n.d., not detected. Samples taken on day 22; $n = 4$.

counting (of at least 400 cells) using an inverted microscope (Nikon Diaphot). Nitrate, ammonium and phosphate in the water were analysed on five occasions (day 13, 16, 18, 20 and 22) using standard methods for seawater analyses (Valderama, 1995). The cellular contents of C, N and P were analysed on day 18 and 22, in cells retained on 25 mm precombusted (450°C, 2 h) Gelman A/E filters after filtering 300 ml of cultures. The filters were dried at 65°C for 24 h, and analysed for POC and PON with a CHN elemental analyser (Fisons Instruments model NA 1500), while POP was analysed following the method of Solórzano and Sharp (1980).

2.3. Extraction of haemolytic substances

Since the daily sampled culture volumes (0.8 l) were not sufficient to carry out all of the analyses at the same time, analyses of the haemolytic activity were made one day after particulate nutrient analyses were performed. Samples for test of haemolytic activity were taken on day 19 and 23. Aliquots (400 ml) from each culture were filtered onto 47-mm precombusted (450°C, 2 h) glass-fiber filters (Gelman A/E, mesh size 1 μm). Cells retained on the filters were extracted in a chloroform/methanol/water (13:7:5, v/v) phase system. Fractions were separated using a separation funnel, and the toxic substances were recovered in the lower chloroform phase. The chloroform fraction was thereafter evaporated to dryness in a vacuum centrifuge (21°C, Savant, Speed Vac SC 200) and the residues were redissolved in 70% methanol. The final extracts were stored at -20°C for a maximum of 2 weeks prior to analysis.

2.4. Haemolytic procedure

The haemolytic test was performed according to the method developed by Simonsen and Moestrup (1997), with the following modifications. One ml of algal extract was added to 4 ml of 2.5% horse blood (Swedish National Veterinarian Institute) in isotonic phosphate buffer. After a 30-min incubation at 37°C, the mixture was centrifuged for 5 min at $530 \times g$ and haemolytic activity was determined spectrophotometrically (Beckman DU 650) by measuring the absorbance at 540 nm (10 mm cuvette). The tests were made in triplicate, and methanol (70%) was used as an optical blank. A standard haemolytic curve based on concentrations of saponin (Sigma S-2149) in an isotonic phosphate buffer was used as reference, and the haemolytic activity of the cells was determined as saponin equivalents. The results are expressed as saponin nano-equivalents per cell (SnE cell^{-1}) and as HE_{50} (amount of cells causing 50% haemolysis). The haemolytic activity was also related to the cellular carbon content in order to compensate for differences in carbon content among treatments. The HE_{50} of the phytoplankton was calculated according to Simonsen and Moestrup (1997) as:

$$HE_{50} = C \cdot 0.227 \cdot S^{-1}$$

Where C is the concentration of cells (cells ml^{-1}), S is saponin equivalents (mg ml^{-1}), and 0.227 is the concentration of saponin (mg ml^{-1}) causing 50% haemolysis of the horse blood cells.

2.5. Statistical analyses

Statistical analyses were performed with SYSTAT using analysis of variance (ANOVA) and Tukey's test to examine differences in algal toxicity and chemical composition between different nutrient conditions. ANOVA were used to test if toxicity of *P. parvum* was related to the cellular content of N and P.

3. Results

3.1. Inorganic nutrient concentration

In cultures growing under P-limited conditions (N:P = 160:1), inorganic P was below the analytical detection limit (0.05 μM) in the outgoing medium, while excess amounts of inorganic N (26.2±5.2 μM) were measured throughout the entire steady-state period (Table 1). In N-limited cultures (N:P = 1:1), inorganic N (0.2±0.0 μM) was close to the detection limit (0.1 μM) in the outgoing medium, while high inorganic P concentrations (2.1±0.6 μM) were always found. Non-limited cultures (N:P = 16:1) contained excess amounts of both N (22.9±4.8 μM) and P (1.2±0.3 μM) during the entire steady-state period (Table 1). The concentrations of NH_4^+ were low in all treatments during the entire experimental period (Table 1).

3.2. Cell densities

Steady-state conditions were attained in all cultures within 11 days after daily dilution started (Fig. 1). After the daily dilution started there was a rapid drop in cell density in all treatments. In semi-continuous cultures the cell density at steady-state is a function of the concentration of the limiting nutrient. In the present study, the daily nutrient additions were not enough to support the cell density of 90×10^3 cells ml^{-1} that were reached in the non-limited batch cultures (Fig. 1). As a result, the cultures experienced a rapid drop in cell density until steady-state conditions were attained. The cell densities differed significantly among the different treatments (ANOVA, $p < 0.0001$, Fig. 1). Nitrogen-limited cultures reached an average cell density of 12.8×10^3 cells ml^{-1} ($n = 4$) at steady state, which was approximately one third of the cell density found in P-limited cultures (38.8×10^3 cells ml^{-1}, $n = 4$), and the non-limited cultures (42.0×10^3 cells ml^{-1}, $n = 4$). Pairwise comparisons showed that the cell densities of cultures growing at low levels of N were significantly lower than the cell densities measured under P-limited (Tukey's, $p < 0.001$) and non-limited conditions (Tukey's, $p < 0.001$). Phosphorus limitation had lesser influence on cell density than N limitation and no statistical difference (Tukey's, $p > 0.05$) could be measured in cell density between P-limited and non-limited cultures.

3.3. Cellular chemical composition

The chemical composition of the cells is given in Table 2. There was a significant

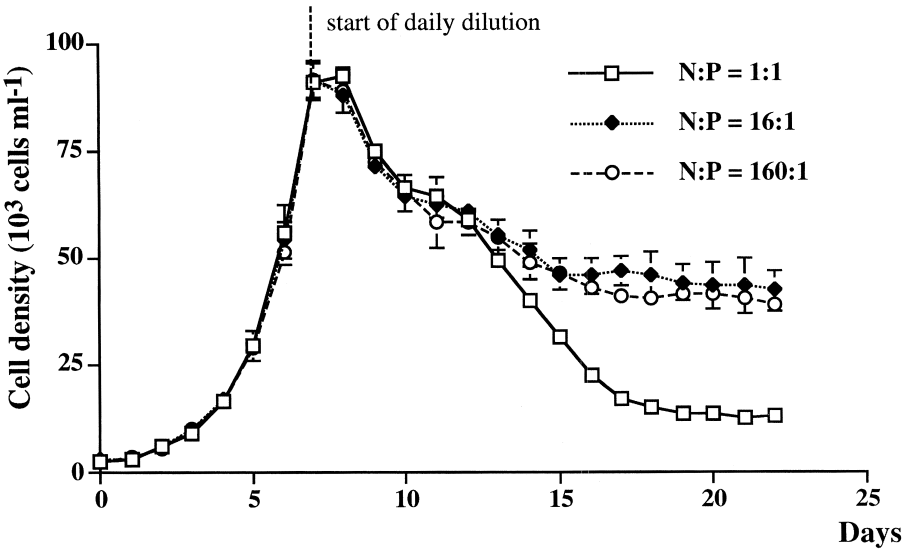

Fig. 1. Cell densities±S.D. of *P. parvum* grown in semi-continuous cultures under nitrogen limitation, non limitation and phosphorus limitation; $n=4$.

(ANOVA, $p<0.001$) difference in the cellular carbon content among treatments. Cells limited by N showed a significantly higher cellular carbon content (43.7±2.4 pg C cell^{-1}) than P-limited cells (29.4±5.1 pg C cell^{-1}, Tukey's, $p<0.01$) and non-limited cells (32.8±3.7 pg C cell^{-1}, Tukey's, $p<0.001$). No significant differences in cellular carbon content were found between P-limited and non-limited cells (Tukey's, $p>0.05$). There was a small, but significant (ANOVA, $p<0.05$), difference in the cellular content of N among treatments (Table 2). However, pairwise comparisons showed no significant differences. Cells grown under low P conditions contained significantly lower (ANOVA,

Table 2
Cell density, cellular C, N and P content and cellular N:P, C:P and C:N ratios in semi-continuous cultures of *P. parvum* at steady state[a]

	N:P ratio		
	1:1	16:1	160:1
Cell density(10^3 cells ml^{-1})	12.8±0.4	42.0±4.9	38.8±4.3
POC (pg C cell^{-1})	43.7±2.4**	32.8±3.7	29.4±5.1
PON (pg N cell^{-1})	2.7±0.3	3.7±0.7	3.9±0.8
POP (pg P cell^{-1})	1.1±0.3	0.6±0.1	0.3±0.1*
N:P	6.0±2.2*	14.3±3.1	24.3±1.9*
C:P	112.9±36.8	146.3±18.5	216.3±31.9*
C:N	19.1±0.9**	10.4±1.1	8.9±0.9

[a] Samples were taken on day 22; $n=4$.
[*] Levels of significance.

$p < 0.01$) contents of P (0.3±0.1 pg P cell^{-1}) than both N-limited cells (1.1±0.3 pg P cell^{-1}, Tukey's, $p < 0.01$) and non-limited cells (0.6±0.1 pg P cell^{-1}, Tukey's, $p < 0.01$).

Cellular N:P ratios (Table 2) varied significantly among treatments (ANOVA, $p < 0.001$). The average N:P value of 6.0±2.2 obtained for *P. parvum* cells growing under N-limitation was significantly lower than the corresponding N:P values of 14.3±3.1 and 24.3±1.9, obtained, respectively, for non-limited (Tukey's, $p < 0.01$) and P-limited cells (Tukey's, $p < 0.01$). The N:P value in P-limited cells was significantly higher than the N:P value of non-limited cells (Tukey's, $p < 0.01$). There was a significant (ANOVA, $p < 0.0001$) difference in particulate C:N among treatments. Nitrogen-limited cultures showed a significantly higher C:N value of 19.1±0.9, compared to the corresponding values of 8.9±0.9 for P-limited (Tukey's, $p < 0.001$) and 10.4±1.1 for non-limited (Tukey's, $p < 0.001$) cultures. The C:N value for P-limited cells was not significantly different from that of non-limited cells (Tukey's, $p > 0.05$). Also, C:P values differed significantly among treatments (ANOVA, $p < 0.01$). Cultures grown in media low in P showed a significantly higher C:P value (216.3±31.9) than N-limited (112.9±36.8, Tukey's, $p < 0.01$) and non-limited cells (145.2±18.5, Tukey's, $p < 0.05$). Nitrogen-limited and non-limited cells did not differ significantly with respect to cellular C:P ratios (Tukey's, $p > 0.05$).

3.4. Haemolytic activity

Haemolytic activity was demonstrated in all treatments (Fig. 2). The haemolytic activity varied significantly among different treatments (ANOVA, $p < 0.0001$). In both N- and P-limited cultures, a significantly higher haemolytic activity (287.7±14.0 and 256.8±38.1 SnE cell^{-1}, respectively) was detected compared to cultures grown under non-limiting conditions (42.4±3.3 SnE cell^{-1}, Tukey's, $p < 0.01$ for both cases). The average haemolytic activity per cell was higher under N-limiting conditions than under P-limiting conditions, but this difference was not significant (Tukey's, $p > 0.05$). The HE_{50} values (Fig. 2) of N- (0.52±0.08 10^6 cells ml^{-1}) and P-limited (0.58±0.09 10^6 cells ml^{-1}) cultures were significantly lower than that of non-limited (3.43±0.26 10^6 cells ml^{-1}) cultures (ANOVA, $p < 0.01$). The decrease in HE_{50} values was more pronounced under N-limitation than under P-limitation but were not significantly different from each other (ANOVA, $p > 0.05$). To compensate for differences in cellular carbon among treatments the haemolytic activity was related to cellular carbon content (Fig. 3). A significant difference were found among treatments (ANOVA, $p < 0.0001$), with significantly higher values for both N- and P-limited cultures (6.6±0.2 10^3 and 9.0±2.2 10^3 SE (g C)$^{-1}$, respectively) compared to cultures grown under non-limiting conditions (1.3±0.2 10^3 SE (g C)$^{-1}$, Tukey's, $p < 0.01$ for both cases). In contrast to what we found when haemolytic activity were related to cell density the haemolytic activity per carbon was higher under P-limiting conditions than under N-limiting conditions. However, no significant difference was found between N- and P-limited cultures (Tukey's, $p > 0.05$). Haemolytic activity per cell was inversely proportional to cellular N and P content (ANOVA, $p < 0.05$ for both cases).

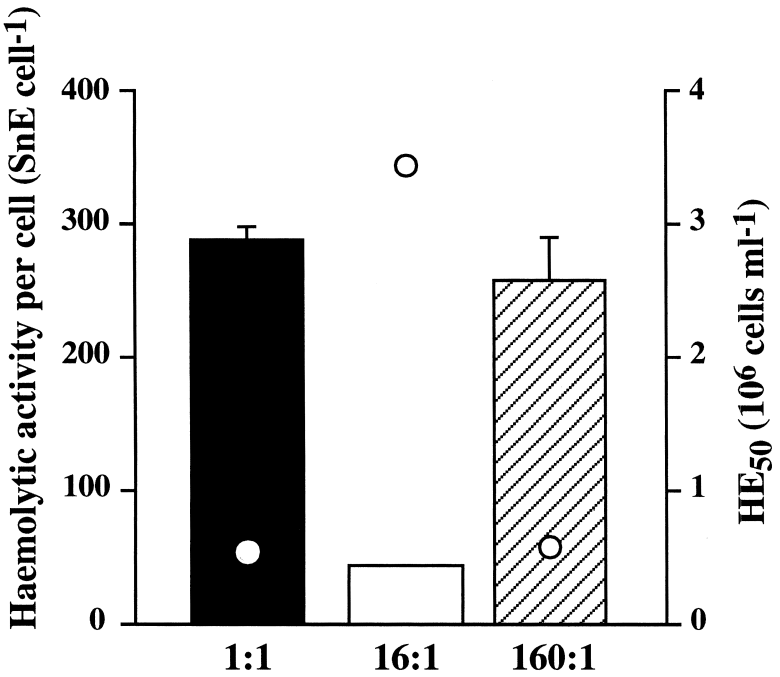

Fig. 2. Haemolytic activity±S.D. (bars) and HE_{50} values (circles) of *P. parvum* grown in semi-continuous cultures under nitrogen limitation (N:P=1:1), non limitation (N:P=16:1) and phosphorus limitation (N:P=160:1). Samples taken on day 23; $n=4$.

4. Discussion

This study documents a relationship between nutrient limitation and haemolytic activity in *Prymnesium parvum* cells. Haemolytic activity of *P. parvum* cells was demonstrated when the cells were grown under N-limited, P-limited as well as non-limited conditions. However, it was significantly higher under nutrient limiting conditions compared to non-limiting conditions. Furthermore, our results showed an enhanced haemolytic activity in *P. parvum* cells irrespective of which nutrient (N or P) was limiting, suggesting that toxin production is related to cellular physiological stress, in this case due to N- or P-limitation.

4.1. Nutrient limitation of P. parvum cells

In this study we have used the cellular content of C, N and P as indicators of N- and P-limitation. It is known that the environmental conditions under which phytoplankton grow influences their cellular chemical composition (e.g. Harrison et al., 1990). A general effect of nutrient limitation is a reduction in the intracellular levels of the limiting nutrient (Cembella et al., 1984; Sakshaug and Olsen, 1986; Darley, 1988). In the present study, cells grown in both the low N and the low P media showed a

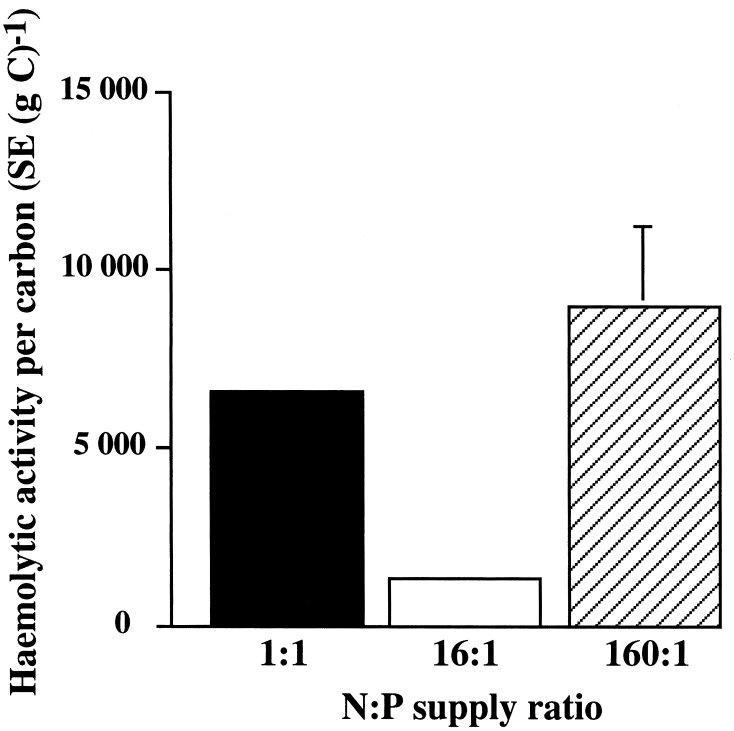

Fig. 3. Haemolytic activity related to cellular carbon content in *P. parvum* grown in semi-continuous cultures under three different N:P supply ratios.

significantly lower cellular content of N and P, respectively, compared to the non-limited cells. These results are consistent with limitation by N and P. Nutrient limitation is usually associated with increasing cellular contents of carbon (Cembella et al., 1984; Latasa and Berdalet, 1994), as a result of the accumulation of carbon when cell division ceases. In our study, the cellular carbon content in P-limited cells did not differ significantly from that of non-limited cells, indicating that P-limitation had only a moderate effect on intracellular carbon content.

Cellular nutrient compositions are also used as indicators of N- and P-limitation. Phytoplanktons growing near nutrient-saturated growth rates typically have C:N:P ratios close to 106:16:1 (Redfield, 1958). Under P-limited growth, C:P and N:P ratios increase, but under N-limited growth C:N increases and N:P decreases (Healey and Hendzel, 1980; Sakshaug and Holm-Hansen, 1977; Goldman et al., 1979). In our study, N-limited cultures showed a low N:P ratio (6.0 ± 2.2) compared to the corresponding value (14.3 ± 3.1) for non-limited cultures, indicating a strong N-limitation. Phosphorus-limited cells showed a N:P ratio of 24.3 ± 1.9, which was significantly higher than the N:P ratio of 14.3 ± 3.1 found in non-limited cultures, which imply a shortage of P. The C:P ratios of P-limited cells were significantly higher (216.3 ± 31.9) than those of non-limited cells (146.3 ± 18.5). Furthermore, the C:N ratios of cells limited by N were significantly

higher (19.1±0.9) than the C:N value in non-limited cells (10.4±1.1). All these results reflect a limitation of either N or P in the cells.

Although, the analysis of chemical composition indicated P-limitation of the cells grown at the lowest P supply, the cell densities of P-limited cultures did not differ from non-limited cultures in a distinguishable way. As the growth rate was identical (e.g. dilution rate) for all treatments, the cell density at steady-state is merely a function of the limiting nutrient. This implies that cells grown in the low P medium were only moderately limited. The concentration of inorganic P was below the detection limit in the culture medium removed daily, indicating that all inorganic P (0.2 μM PO_4) added daily was removed by the *P. Parvum* cells. However, a low concentration in the surrounding media does not necessarily imply nutrient limitation. Nutrients control algal growth only if nutrient demand exceeds availability over the time-scale of cellular growth and reproduction (Harris, 1986). Regular measurements of nutrient concentration in the water give no information on this.

Several phytoplankton species are able to store excess P in intracellular pools for use during time of limitation (Darley, 1988). This can not be the case in the present study as the cellular content of P was low in these cultures. *Prymnesium parvum* is able to use different organic P sources (McLaughlin, 1958) such as bacteria (Nygaard and Tobiesen, 1993). The above experiments were carried out with non-axenic cultures, and we can not eliminate the possible role of bacteria as a source of P in the growth of *P. Parvum* (e.g. phagotrophy). Nygaard and Tobiesen (1993) demonstrated a close connection between P-limitation and increased phagotrophy in *P. parvum*. It is likely that bacteria could have supplied the cells with enough P to sustain cell division but not enough for storage, which could explain the lower intracellular content of P in cells grown under P-limited conditions.

4.2. Toxicity vs. nutrient limitation

To our knowledge this is the first time that N-limitation has been reported to promote toxicity of *P. parvum* cells. In contrast, Ulizur (1970, quoted in Shilo, 1971) reported that nitrate limitation does not have an effect on the toxicity. However, evidence to support this conclusion was not published. The enhancement of toxicity under P-limitation, found in this study, is in agreement with earlier studies showing that a low inorganic P content in the water can increase the toxicity of *P. parvum* cells up to 10–20 times (Shilo, 1967; Dafni et al., 1972). Similar results were shown in more recent studies of *P. parvum* (Meldahl et al., 1994, 1995) and the related *P. patelliferum* (Larsen et al., 1993; Meldahl and Fonnum, 1993). Recently, enhanced toxicity due to either N- or P-limitation has also been reported for the closely related *Chrysochromulina polylepis* (Edvardsen et al., 1996). Thus, limiting conditions of any of the two most crucial nutrients during a bloom might lead to enhanced toxicity of these flagellates.

The results reported by Edvardsen et al. (1996) were based on a test where, toxicity of *C. polylepis* cells was tested against the brine shrimp *Artemia salina*. In the present study, *P. parvum* was not tested against *Artemia*. However, in agreement with our results, the strain used here has been shown to increase its toxicity against *Artemia* under both N- and P-limited conditions (results to be published). This is of interest since

haemolytic activity of phytoplankton cells is not always accompanied by toxicity to aquatic organisms (Simonsen and Moestrup, 1997).

Previous studies have shown that it is not necessarily a simple relationship between cell density and toxicity of *P. parvum* populations. High toxicity has been observed with very low algal numbers, whereas in other cases mass development of *P. parvum* did not result in any observable toxic effects in nature (Shilo, 1967). These observations indicate that growth and toxicity is regulated by different factors. A comparison of the capacity of *P. parvum* to produce toxins under different environmental conditions has shown that growth and toxin synthesis have different optimal requirements (Shilo, 1971), and it has been argued that toxicity of *P. parvum* is fully expressed only when growth is limited (Dafni et al., 1972). This is supported by our results, as the cells in the non-limited cultures, in contrast to our findings for the N- and P-limited cells, did not exhibit enhanced haemolytic activity. This suggests a connection between enhanced toxicity and nutrient limitation. However, it appears to reflect more a physiological stress than direct linkage between limitation of N or P and toxicity, as neither N nor P is a major constituent of the toxin complex (Igarashi et al., 1996) or apparently involved in its synthesis. This conclusion is also supported by the fact that several other environmental stress factors such as light intensity and salinity have been shown to have substantial influence on the toxicity of *P. parvum* (Shilo, 1967; Larsen et al., 1993)

When haemolytic activity was related to cellular carbon content, the highest activity was found under P-limited conditions. This is contradictory to what we found when haemolytic activity was related to cell density. The reason for this difference seems to be found in dilution effects rather than in factual differences in haemolytic activity among the two treatments, as no significant differences were found between P-limited and N-limited cultures in any of the tests. Nitrogen-limited cultures had lower cell density and higher haemolytic activity per cell, whereas P-limited cultures had lower carbon content and consequently a higher haemolytic activity per carbon.

The influence of nutrient availability on phytoplankton toxicity has been shown for microalgae other than haptophytes. Boyer et al. (1985, 1987) reported that the saxitoxin producing dinoflagellate *Alexandrium tamarense* increases its toxin production under P-limitation. Anderson et al. (1990) later confirmed these findings. A similar response has also been reported for the dinoflagellate *Gymnodinium catenatum* (Reguera and Oshima, 1990) and the diatom *Pseudo-nitzschia multiseries* (Bates et al., 1991, 1992, 1996; Pan et al., 1996a). Experiments on the okadaic acid producing dinoflagellates *Prorocentrum lima* and *Dinophysis acuminata* showed that toxin content is enhanced under both N- and P-limitation (McLachlan et al., 1994; Sohet et al., 1995; Johansson and Granéli, 1996). However, phytoplankton can produce an array of toxins that are of distinct chemical nature. The effect of different environmental conditions on the production of these substances can vary substantially and are likely to be species specific. Therefore, it is difficult to discern any general trends or to make a conclusive picture about factors regulating phycotoxin synthesis.

Despite extensive research during the last years the knowledge about the ecological role of algal toxins is still scarce. One explanation that has been discussed is that toxin production is a mechanism used to control the growth of competing algae and potential grazers when growth is limited. Several studies have shown that algal toxins have a

negative influence on both herbivorous zooplankton (Ives, 1985; Huntley et al., 1986; Targett and Ward, 1991) and other algae (Keating, 1977; Myklestad et al., 1995; Windust et al., 1996). *Prymnesium* toxins have mainly been reported as a problem to aquatic gill breathing animals as they disrupt the selective cell permeability of the gill tissue (Shilo, 1967). However, toxic effects on copepods (Nejsgaard and Solberg, 1996) and other algae (Arlstad, 1991) have also been reported. A negative influence by a toxin on competing organisms and grazers would certainly be of value for any phytoplankton species. A species ability to dominate in a specific natural environment depends on its ability to compete successfully for the growth-limiting resource. Thus, from an ecological point of view, an increased toxin production when nutrients are limiting would be a great advantage, giving the algae an opportunity to proliferate where they would otherwise be incapable of competing with competitively superior species of algae.

Phytoplankton toxins are regarded as secondary metabolites (Bates et al., 1996; Pan et al., 1996b; Windust et al., 1996). Recently, considerable improvements have been made in understanding how environmental conditions affect the production of secondary metabolites (Vining, 1990; Hashimoto and Yamada, 1994; Jensen and Fenical, 1994). A general trend seems to be that cells frequently produce low amounts of secondary metabolites when grown under optimal conditions but exhibit enhanced synthesis under specific conditions that limit growth (Plumley, 1997). Our study shows that toxicity of *P. parvum* meets these criteria.

5. Conclusions

In the present study we show that the haemolytic activity of *P. parvum* is influenced by different N:P supply ratios. *Prymnesium parvum* cells grown under nutrient limiting conditions showed a significantly higher haemolytic activity than cells grown under non limiting conditions (N:P = 16:1). This occurred independently of the growth-limiting nutrient (N or P), suggesting a relationship between high toxicity and limitation of nutrients. However, a direct linkage between limitation of N or P and toxicity seems unlikely, as neither N nor P is a major constituent of the toxin complex (Igarashi et al., 1996) or apparently involved in its synthesis. It is more likely that the increase in toxicity is a result of physiological stress, in this due to N- and P-limitation, and the related limitation of growth. It is generally accepted that the availability of inorganic nutrients (N and P) is an important factor for the regulation of growth and toxicity in potentially toxic phytoplankton species. In many coastal areas, where the majority of toxic blooms occur, N:P ratios have changed considerably due to high erratic inputs of antropogenic N and P, leading to an unbalanced N:P ratio (compared to the Redfield ratio). Potentially toxic phytoplankton may thus become toxic when exposed to a given nutrient regime resulting from eutrophication. The connection between nutrient limitation and enhanced haemolytic activity observed in this study is in agreement with this idea.

The mechanism of enhanced toxicity under N- or P-limitation might improve the competitive ability of *P. parvum* under conditions of severe nutrient limitation. However,

we need more information about toxin metabolism and the ecological role of these toxins in order to increase our understanding of the growth and succession of *P. parvum*.

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