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Cell density, chemical composition and toxicity of *Chrysochromulina polylepis* (Haptophyta) in relation to different N:P supply ratios

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Abstract The influence of different N:P supply ratios on cell accumulation, chemical composition and toxicity of the marine haptophyte *Chrysochromulina polylepis* was examined in semi-continuous cultures. A non-axenic strain of *C. polylepis* was exposed to five different N:P supply ratios (N:P = 1:1, 4:1, 16:1, 80:1 and 160:1, by atoms), in order to create a range of N- and P-limited conditions. The toxicity per cell in *C. polylepis* was determined on four occasions at steady state cell density using the haemolytic activity of the cells expressed as saponin nanoequivalents. Haemolytic activity was demonstrated in all treatments, and increased in the algae when cell growth was nutrient limited (N:P = 1:1, 4:1, 80:1 and 160:1), compared to cells grown under non-limiting conditions (N:P = 16:1). This occurred regardless of the growth-limiting nutrient (N or P) and became more pronounced as nutrient limitation increased. In P-limited cultures the haemolytic activity per cell increased linearly with the cellular N:P ratio, whereas the N-limited cultures showed an opposite trend. The haemolytic activity per cell showed an inverse relationship with both cellular N and cellular P content. Cells limited by P showed a higher haemolytic activity than cells limited by N. The results suggest that toxicity in *C. polylepis* is strongly influenced by the physiological state of the algae. This may partially explain the large variability previously observed in the toxicity of *C. polylepis* blooms. The potential ecological significance of our findings is also discussed.

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Introduction

The occurrence of toxic algal blooms and their possible relation to antropogenic activities has received increasing attention for many years. The discharge of nutrients (N and P) to coastal waters has increased dramatically around the world over the past several decades due to human activities (Smayda 1990). This has resulted in a shift in the nutrient composition of the water in many coastal areas. Such changes are believed to influence not only algal species composition, in favour of harmful algal species, but also to promote toxicity in species traditionally not considered toxic (Smayda 1990; Hallegraeff 1993). The marine haptophyte *Chrysochromulina polylepis* is widely distributed in coastal waters in northern Europe (Edvardsen and Paasche 1998) and has received considerable attention over the past decade due to the broad environmental impact attributed to an intensive bloom along the Scandinavian coastline in 1988. The bloom had a negative impact on a wide range of naturally occurring marine organisms, as well as farmed fish (Rosenberg et al. 1988; Dahl et al. 1989; Underdahl et al. 1989; Nielsen et al. 1990; Kaas et al. 1991; Robertson 1991). Prior to this bloom there were no records of mass occurrences of this species, and earlier studies have not found it very toxic (Manton and Park 1962; Jebram 1980). During the 1990s, bloom events of this species have been reported on several occasions in Scandinavian waters (Tangen 1989; Edvardsen and Paasche 1998 and references therein). However, no harmful effects on marine biota were reported in relation to these blooms. These observations imply that toxicity of this species is highly variable and is expressed only under certain environmental conditions.

Chrysochromulina polylepis produces a non-selective toxin, which shows the same type of toxic activity, mainly on cell membranes, as that produced by the closely related and well-known fish killer *Prymnesium parvum*, which have been thoroughly studied. Shilo (1967) first showed a connection between P-limitation

and enhanced toxicity in *P. parvum*. This was also later confirmed in *C. polylepis* (Edvardsen et al. 1990, 1996; Edvardsen 1993; Meldahl et al. 1994). It is also possible that low levels of N promote toxicity in these species. Results are, however, contradictory (Shilo 1967 and references therein; Dafni et al. 1972; Edvardsen et al. 1996; Johansson and Granéli 1999). Nutrient conditions just before the bloom in 1988 were characterised by low concentrations of phosphorus and elevated concentrations of nitrate, probably due to a strong input from the southern North Sea, resulting in a N:P ratio well above the Redfield ratio of 16:1. It has been suggested that this shift in nutrient composition of the water contributed to the exceptional high toxicity of the *C. polylepis* cells during the bloom (Dahl et al. 1989; Maestrini and Granéli 1991; Skjoldahl and Dundas 1991). All these observations suggest a possible relationship between enhanced toxicity and limitation of N or P in the medium. However, as the toxin(s) produced by *C. polylepis* are mainly composed of carbon and contain very little N or P (Yasumoto et al. 1990), a direct linkage between N- or P-limitation and toxin production seems unlikely. This gives us reason to ask whether toxicity in *C. polylepis* is a response to physiological stress, due to N- or P-limitation, rather than the involvement of N or P in toxin synthesis.

The objective of the present study was to investigate the influence of N- and P-stress on the toxicity of the marine haptophyte *Chrysochromulina polylepis* grown in semi-continuous cultures under different N:P supply ratios.

Materials and methods

Culture and growth conditions

A non-axenic strain of *Chrysochromulina polylepis* (LAC 14) was first grown as 15 batch cultures (3.0 litres) in *f*/20 medium (Guillard and Ryther 1962). The cultures were grown at a temperature of 16 ± 1 °C, and with PAR (photosynthetic active radiation) of 200 μmol m^{-2} s^{-1} (cool white fluorescent tubes) under a 16 h light: 8 h dark cycle for 26 d. The culture medium was prepared with filtered (Gelman A/E glass fibre filter, 900 mm, mesh size 0.45 μm) and autoclaved, aged natural seawater (initial nutrient concentration: 1.4 μ*M* NO_3^-, 0.1 μ*M* PO_4^{3-}, 0.2 μ*M* NH_4^+) with a salinity of 21‰. Once the batch cultures reached high cell densities (75 to 80×10^3 cells ml^{-1}) the experiment was run as semi-continuous cultures. Daily, 30% of the culture volumes (0.9 litres) were removed at a set time and replaced with an equal volume of new, enriched media in order to attain steady state conditions (Table 1). Five different N:P supply ratios were used (three replicates of each): 1:1, 4:1, 16:1, 80:1 and 160:1. Inorganic nutrients were supplemented in order to give the algae a daily addition corresponding to the following concentrations: 2.0 μ*M* NO_3^-, 2.0 μ*M* PO_4^{3-} (1:1); 8.0 μ*M* NO_3^-, 2.0 μ*M* PO_4^{3-} (4:1); 32.0 μ*M* NO_3^-, 2.0 μ*M* PO_4^{3-} (16:1); 32.0 μ*M* NO_3^-, 0.4 μ*M* PO_4^{3-} (80:1); and 32.0 μ*M* NO_3^-, 0.2 μ*M* PO_4^{3-} (160:1). Trace metals, iron and EDTA were added to all cultures at levels corresponding to *f*/20 medium (Guillard and Ryther 1962). Vitamins (B_{12}, biothin and thiamine) were added following the method of Schöne and Schöne (1982).

Several variables were measured in the outgoing media, including inorganic nutrients (NO_3^-, PO_4^{3-}, NH_4^+), cell density, particulate nitrogen (PON), phosphorus (POP) and carbon (POC) and haemolytic activity.

Cell density and chemical analyses

Phytoplankton samples (50 ml) were preserved with acid Lugol's solution, and cell numbers were counted using a particle counter (HIAC/ROYCO 9064), calibrated by manual counting (of at least 400 cells) using an inverted microscope (Nikon Diaphot). The concentrations of NO_3^-, PO_4^{3-} and NH_4^+ in the media were analysed on five occasions according to IOC standard protocols (Valderama 1995). Cellular contents of C, N and P were analysed on Days 15, 19, 22 and 25 in cells retained on 25 mm precombusted (450 °C, 2 h) Gelman A/E glass fibre filters after filtering of 300 ml of the cultures. The filters were dried in an oven at 65 °C for 24 h, and analyses of POC and PON were performed with a CHN elemental analyser (Fisons Instruments Model NA 1500), while POP was analysed according to the method of Solorzano and Sharp (1980). For blanks, samples of milli-Q water was filtered and treated as above for POC, PON and POP. Elemental molar ratios were calculated from cellular C, N and P values.

Extraction of haemolytic substances

Since the daily sampled culture volumes (0.9 litres) were not sufficient to carry out all the analyses, measurements of haemolytic activity were performed 1 d after cellular nutrient analyses (i.e. on Days 16, 20, 23 and 26). From each culture flask 400 ml was filtered onto precombusted (450 °C, 2 h) glass fibre filters (Gelman A/E, 47 mm, nominal pore size 1.0 μm). Cells retained on the filters were extracted in a chloroform-methanol-water (13:7:5) phase system. Fractions were separated using a separation funnel, and the haemolytic substances were recovered in the lower chloroform phase. The chloroform fraction was then evaporated to dryness under nitrogen flow and residues were redissolved in 70% methanol. The final extracts were stored at −20 °C for a maximum of 2 weeks before analysis.

Haemolytic procedure

The haemolytic test was carried out using the method developed by Simonsen and Moestrup (1997), with minor alterations. One millilitre of algal extract was added to 4 ml of 2.5% horse blood (Swedish National Veterinarian Institute) in an isotonic phosphate buffer. After 30 min of incubation at 37 °C, the mixture was centrifuged (Jouan MR 1822) for 15 min at 530 ×*g*, and haemolytic activity was determined spectrophotometrically (Beckman DU 650) by measuring the supernatant at 540 nm. The test was done in triplicates, 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 a reference, and the haemolytic activity of the cells was determined as saponin equivalents. The results are expressed as saponin nano-equivalents per cell (SnE $cell^{-1}$).

Statistical analyses

Statistical analyses were performed with SYSTAT (SPSS, Chicago) using ANOVA and Tukey's test to examine differences in haemolytic activity and chemical composition between algae grown under different nutrient supply ratios. Linear regression analysis was used to test if haemolytic activity of *Chrysochromulina polylepis* was related to the cellular content of N or P or to the cellular N:P ratio.

Results

Cell densities

Steady state conditions were attained in all cultures within 2 weeks of the first daily dilution (Fig. 1).

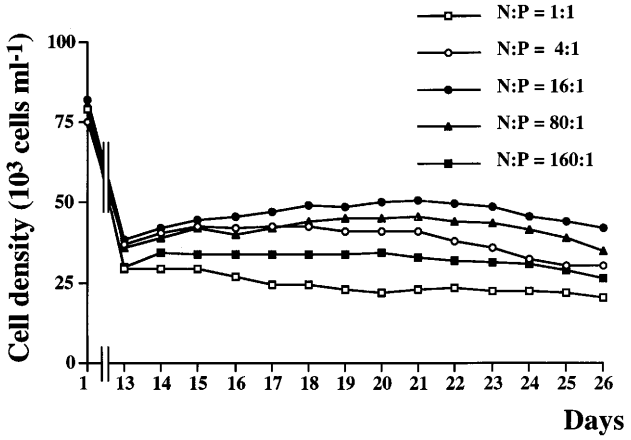

Fig. 1 *Chrysochromulina polylepis*. Cell density of cultures grown semi-continuously under five different N:P supply ratios

Non-limited cultures (N:P = 16:1) contained excess amounts of both NO_3^- and PO_4^{3-} during the entire steady state period, while either NO_3^- or PO_4^{3-} were limiting at the other supply ratios (Table 1). Changes in the N:P supply ratio influenced the final cell yield at steady state substantially, resulting in significant differences among treatments (ANOVA, $p < 0.0001$). The cell density ranged from 21.8×10^3 cells ml^{-1} in cultures with the lowest N:P supply ratio (N:P = 1:1) to 49.7×10^3 cells ml^{-1} in non-limited cultures (N:P = 16:1). One interesting feature was that cultures limited by N generally exhibited lower cell density than the corresponding cultures limited by P (Fig. 1). Pairwise comparisons showed that severely N-limited cultures (N:P = 1:1) had significantly lower cell concentration at steady state than the cultures grown at N:P supply ratios of 4:1, 16:1 and 80:1 (Tukey, $p < 0.05$, 0.001 and 0.01, respectively). A significant difference in cell density was also measured between severely P-limited cultures and non-limited cultures (Tukey, $p < 0.05$).

Cellular chemical composition

No significant difference (ANOVA, $p > 0.05$) could be measured in cellular C content among treatments, although a trend towards increasing concentrations of C per cell could be discerned under both N- and P-limitation (Table 2). In contrast to the relative constancy of the C content, cellular P concentrations varied substantially among treatments (ANOVA, $p < 0.0001$). As expected, an increase in the N:P supply ratio caused a general decrease in the cellular content of P. This led to a decreasing trend in the cellular P content from severely N-limited cultures to severely P-limited cultures, with mean values ranging from 0.69 ± 0.06 to 0.28 ± 0.04 pg P cell^{-1} (Table 2). Pairwise comparisons revealed significant differences between cultures grown at high N:P supply ratios (80:1 and 160:1) and cultures growing at N:P supply ratios of 16:1 (Tukey, $p < 0.01$ and 0.001, respectively), indicating limitation of P in both treatments. A decrease in the N:P supply ratio resulted in less N on a per cell basis as shown in Table 2. There was a small but significant (ANOVA, $p < 0.05$) difference in the cellular N content between different treatments, and pairwise comparisons proved it to be significantly lower in cultures exposed to the lowest N supply (N:P = 1:1) than in non-limited cultures (N:P = 16:1, Tukey, $p < 0.05$), whereas no significant

Table 1 Inorganic nutrient concentrations (μM) in the outgoing media and in the daily additions of new media in semi-continuous cultures of *Chrysochromulina polylepis* ($n = 3$)

	N:P supply ratios				
	1:1	4:1	16:1	80:1	160:1
Cell density (10^3 cells ml^{-1})	21.8 ± 0.4	40.5 ± 6.1	49.7 ± 3.3	44.6 ± 10.1	34.1 ± 0.9
Daily added conc.					
PO_4^{3-}	2.0	2.0	2.0	0.4	0.2
NO_3^-	2.0	8.0	32.0	32.0	32.0
Conc. in outgoing media					
PO_4^{3-}	7.0 ± 2.3	7.0 ± 1.9	7.5 ± 1.2	0.1 ± 0.0	0.0 ± 0.0
NO_3^-	0.1 ± 0.0	0.5 ± 0.2	122.5 ± 10.3	127.9 ± 12.7	130.0 ± 9.8
NH_4	0.1 ± 0.0	0.2 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	0.5 ± 0.0

Table 2 *Chrysochromulina polylepis*. Effect of N:P supply ratios on cell density, cellular C, N and P content and cellular N:P, C:P and C:N ratios in semi-continuous cultures at steady state ($n = 3$)

Cell parameter	N:P supply ratios				
	1:1	4:1	16:1	80:1	160:1
Cell density (10^3 cells ml^{-1})	21.8 ± 0.4	40.5 ± 6.1	49.7 ± 3.3	44.7 ± 10.1	34.1 ± 0.9
Carbon (pg cell^{-1})	29.7 ± 2.9	27.8 ± 2.8	25.6 ± 3.5	26.7 ± 2.3	26.5 ± 2.6
Nitrogen (pg cell^{-1})	2.7 ± 0.4	3.2 ± 0.3	3.9 ± 0.3	3.6 ± 0.3	3.6 ± 0.1
Phosphorus (pg cell^{-1})	0.7 ± 0.1	0.6 ± 0.0	0.5 ± 0.1	0.4 ± 0.0	0.3 ± 0.0
C:N ratio	12.9 ± 0.7	10.3 ± 1.7	7.7 ± 1.4	8.8 ± 1.1	8.5 ± 0.9
N:P ratio	8.7 ± 1.9	11.6 ± 1.5	16.0 ± 0.6	20.5 ± 0.6	29.5 ± 5.0
C:P ratio	111.6 ± 19.1	117.5 ± 4.8	122.2 ± 21.6	180.7 ± 26.5	249.3 ± 30.2

differences were found between the other treatments. Fig. 2 summarises the relationship between cellular N and P content for cells grown under different N:P supply ratios.

The cellular C:N ratios were generally high in this study, with mean values above the Redfield ratio of 6.6 (by atoms, Redfield 1958) in all treatments (Table 2). A decrease of the N:P supply ratio led to higher cellular C:N ratios, with significant differences between treatments ($p < 0.05$). Pairwise comparisons, however, could only establish a significant difference between non-limited (N:P = 16:1) and severely N-limited (N:P = 1:1) cultures (Tukey, $p < 0.05$). The cellular N:P ratios did not mirror the supply ratio, but showed a tendency to change in the direction of the composition of the medium, resulting in a significant difference among treatments (ANOVA, $p < 0.0001$, Table 2). The cellular N:P ratios increased gradually from severe N-limitation (N:P = 1:1) to severe P-limitation (N:P = 160:1), with mean values ranging from 8.7 ± 1.9 to 29.5 ± 5.0. Pairwise comparisons demonstrated a significant difference between severely N- and P-limited cultures and non-limited cultures (Tukey, $p < 0.05$ and 0.001, respectively), which is indicative of strong N- and P-limitation, respectively. This difference was not seen between moderately N- or P-limited cultures and non-limited cultures (Tukey, $p > 0.05$ in both cases), suggesting a lesser degree of limitation in these treatments. Cellular C:P ratios increased with increasing N:P supply ratios, with significant differences among treatments (ANOVA, $p < 0.0001$). Both the cultures grown at N:P supply ratios of 80:1 and the cultures grown at 160:1 exhibited significantly higher C:P values than the non-limited cultures (Tukey, $p < 0.05$ and 0.001, respectively), indicating that P was in shortage in these cultures. The C:P values were significantly higher in cells grown at N:P supply ratios of 160:1 than in cells grown at N:P supply ratios of 80:1 (Tukey, $p < 0.05$).

Haemolytic activity

Haemolytic activity was demonstrated in all treatments, but the changes in N:P supply ratios influenced the haemolytic activity significantly (ANOVA, $p < 0.0001$, Fig. 3). The haemolytic activity was higher in cultures exposed to nutrient-limiting conditions (both N and P) than in cultures grown under non-limiting conditions. Moreover, the haemolytic activity increased as the nutrient limitation increased, suggesting a close relationship between haemolytic activity and the availability of N and P in the media. For both N- and P-limited cultures the haemolytic activity increased with increasing nutrient limitation in a similar manner. This indicates that the production of haemolytic substances increased as a function of nutrient stress. To evaluate this further, haemolytic activity per cell was plotted as a function of cellular N:P ratios (Fig. 4) and cellular N and P content

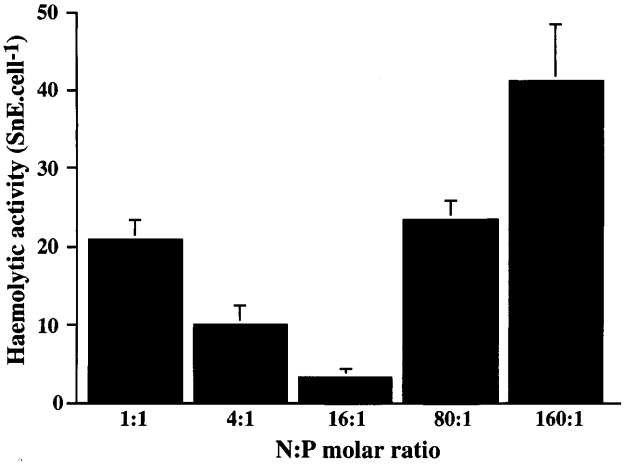

Fig. 3 *Chrysochromulina polylepis*. Haemolytic activity per cell at steady state in semi-continuous cultures grown under five different N:P supply ratios

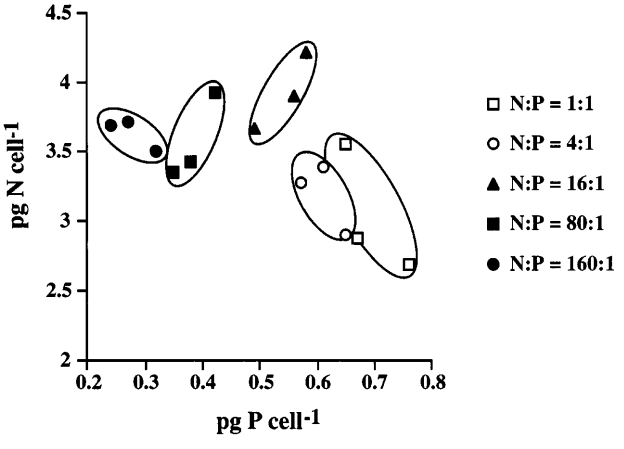

Fig. 2 *Chrysochromulina polylepis*. Relationship between cellular N and P content of cultures grown semi-continuously under five different N:P supply ratios

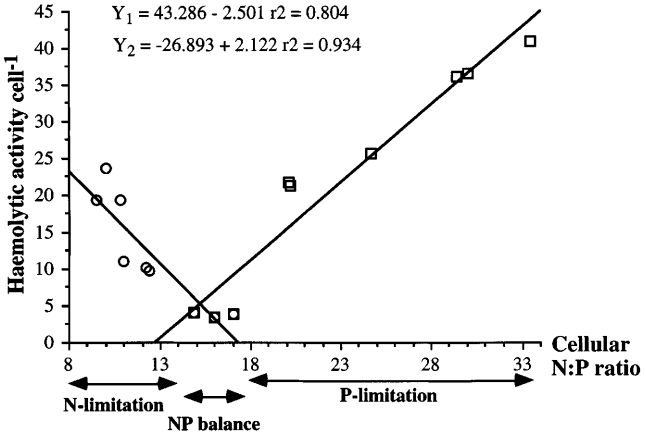

Fig. 4 *Chrysochromulina polylepis*. Relationship between haemolytic activity per cell and cellular N:P ratio in semi-continuous cultures growing under five different N:P supply ratios. Regression for N-limited cultures: Y_1; for P-limited cultures: Y_2

(Fig. 5a, b). In P-limited cultures the haemolytic activity per cell increased linearly (r^2 = 0.93) with the cellular N:P ratio, whereas the N-limited cultures showed an opposite trend (r^2 = 0.80). The haemolytic activity per cell showed an inverse relationship with both cellular N and cellular P content (r^2 = 0.86 and 0.91, respectively). These results demonstrate a strong relationship between cellular nutrient stress and haemolytic activity of *Chrysochromulina polylepis* cells. The highest haemolytic activity was measured in cells exposed to the highest N:P supply ratio (41.0 ± 3.3, Fig. 3). Those cells showed more than 12 times higher activity than the non-limited cells. For both N- and P-limited cultures the haemolytic activity per cell was higher in severely nutrient-limited cultures than in moderately limited cultures (Tukey, $p < 0.01$ and 0.001, respectively). Cultures limited by P showed higher haemolytic activity than corresponding cultures limited by N.

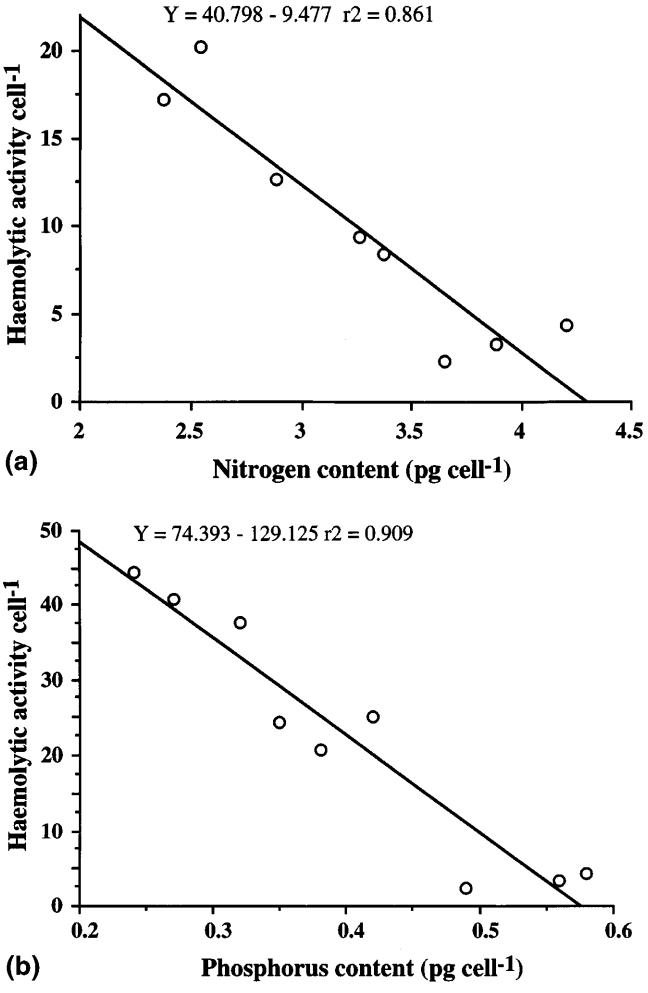

Fig. 5 *Chrysochromulina polylepis*. Relationship between haemolytic activity per cell and **a** cellular N content in semi-continuous cultures of growing under N-limited and non-limited conditions and **b** cellular P content in semi-continuous cultures growing under P-limited and non-limited conditions

Discussion

Two important aspects emerge from our experiments. First, there are marked differences in haemolytic activity between *Chrysochromulina polylepis* cells grown under different N:P supply ratios. Second, the difference in haemolytic activity can be related to differences in the availability of nutrients, that is, to differences in nutrient stress.

Nutrient limitation of *Chrysochromulina polylepis*

Phytoplankton is regularly subjected to environmental conditions that are less than optimal for them and respond to changes in the availability of nutrients by adapting their chemical composition to maintain growth (Laws and Bannister 1980). Analyses of chemical composition can therefore be a valuable tool for measuring the degree of cell limitation. A common feature of nutrient limitation is a decrease in the cellular concentration of the limiting nutrient (Cembella et al. 1984; Sakshaug and Olsen 1986). In our study, cells grown at high N:P supply ratios had significantly lower P content per cell than the cells grown under balanced conditions. This is a clear indication of P-limitation under both 80:1 and 160:1 supply ratios. A similar decrease in the cellular content of N was observed also in cultures grown under low N:P supply ratios, but the N-limitation seemed to be less severe than the P-limitation since the N content per cell in cultures grown at a N:P supply ratio of 4:1 was not statistically different from the N content in non-limited cultures. In comparison, Dahl et al. (1989) reported values of 3.2 to 4.2 pg N cell^{-1} in culture experiments on natural populations of *C. polylepis*, which is in the range of those found by us, whereas the P concentrations reported here (0.3 to 0.7 pg P cell^{-1}) were lower than the values (0.7 to 1.0 pg P cell^{-1}) reported by those authors. This strengthens the conclusion that P-limitation was more severe than N-limitation in the present study. In the literature, nutrient limitation has often been related to an increase in cellular C (Sakshaug et al. 1984; Latasa and Berdalet 1994). In the present study, C content per cell was relatively constant and did not reveal strong indications of either N- or P-limitation.

Cellular nutrient ratios are also used as indicators of N- and P-limitation. Phytoplankton growing close to maximum rates typically has a C:N:P ratio near 106:16:1 (Redfield 1958). Under P-limited growth, C:P and N:P increase, and under N-limited growth C:N increases and N:P decreases (Healey 1975; Sakshaug and Holm-Hansen 1977; Goldman et al. 1979). Although, the optimal ratios may vary substantially between different species there are, in the literature, certain intervals in cellular nutrient ratios that have to be differed from in order for the algae to be considered nutrient limited. The cellular N:P ratio of 8.7 found in the cultures grown under the lowest N:P supply ratio, in the present study, would be

in the range of strong N-limitation (Paasche and Erga 1988), whereas the mean value of 11.6 found for the moderately N-limited cultures is indicative of little or no N-limitation according to Healey (1975). In P-limited cultures the cellular N:P ratios were above 20.0 under both 80:1 and 160:1 N:P supply ratios, which is indicative of severe P-limitation (Healey 1975; Paasche and Erga 1988). The cellular C:N ratios in the 1:1 and 4:1 treatments increased in response to the low N:P supply ratio of the medium and are indicative of severe and moderate N-limitation, respectively. The C:P ratios indicated the presence of P-limitation in the cultures exposed to the two highest N:P supply ratios, becoming more severe as the N:P supply ratio rose.

On the basis of both cellular nutrient concentrations and nutrient ratios, the cultures grown under N:P supply ratios of 1:1 and 160:1 showed clear signs of N- or P-limitation, respectively. Indications of N- or P-limitation were also seen in the cultures grown under N:P supply ratios of 4:1 and 80:1, but the limitation was less severe in those treatments.

Haemolytic activity versus nutrient stress/limitation

The production of haemolytic substances in *Chrysochromulina polylepis* was clearly influenced by the N:P ratio of the medium. Both high and low N:P supply ratios resulted in higher haemolytic activity on a per cell basis compared to cells grown under N:P balanced conditions. The increase in haemolytic activity under P-limited growth is consistent with earlier experiments on batch cultures of this species, which resulted in an increase of haemolytic activity under P-limited conditions, similar to the results reported here (Edvardsen et al. 1990; Meldahl et al. 1994). Shilo (1967, 1971) also reported a similar relationship, for the closely related *Prymnesium parvum*. These results stress the importance of P availability as a regulation factor for toxin production of those haptophytes. In the present study we have also demonstrated enhanced haemolytic activity in *C. polylepis* cultures during N-limitation, showing that the haemolytic activity is enhanced under nutrient limitation irrespective of which nutrient (N or P) is limiting. These results suggest a relationship between nutrient limitation (N or P) and enhanced toxin production. However, a direct connection between N- or P-limitation and toxin production seems unlikely, as neither N nor P is a major component of the toxin complex (Yasumoto et al. 1990) or apparently involved in the toxin synthesis. Previous studies of *C. polylepis* toxicity in relation to nutrient limitation have provided evidence for a marked variability of the toxicity in batch cultures as the algal population changes from an exponential to a stationary phase (Edvardsen et al. 1990; Edvardsen 1993; Meldahl et al. 1994). In the referenced works, the changeover was reflected by an increase in toxicity per cell at arrested cell growth, suggesting that growth and toxin production have different optimal requirements and that toxicity is fully expressed only when growth is limited. This is supported by our results, as the cells in the non-limited cultures, in contrast to the findings for the N- and P-limited cells, did not exhibit enhanced haemolytic activity at steady state (Fig. 3). Based on this differing response, we conclude that enhancement of the haemolytic activity of *C. polylepis* is related to cellular physiological stress. This is suggested by the enhanced haemolytic activity as physiological stress increases with increasing nutrient limitation, demonstrated by the inverse relationship observed between haemolytic activity and cellular N and P contents (Fig. 5a, b). When haemolytic activity was related to the cellular N:P ratio we found that in P-limited cultures the haemolytic activity per cell increased linearly with the cellular N:P ratio, whereas the N-limited cultures showed an opposite trend, which further supports our conclusion. Recently, enhanced toxicity due to either N- or P-limitation has also been reported for the closely related *P. parvum* (Johansson and Granéli 1999). An increase in toxicity in relation to suppressed cell growth, in *C. polylepis*, independent of the growth limiting nutrient, has also been reported by Edvardsen et al. (1996). Thus, one of the metabolic strategies under nutrient-limited growth conditions, in haptophytes, seems to be based on production of toxic substances. The results reported by Edvardsen et al. (1996) were based on a test in which toxicity of *C. polylepis* cells was tested against the brine shrimp *Artemia* sp. In the present study, *C. polylepis* was not tested against *Artemia* sp. However, the strain used here has, in agreement with our results, been shown to increase its toxicity against *Artemia* sp. under both N- and P-limited conditions (results to be published). This is of interest since haemolytic activity of phytoplankton cells is not necessarily accompanied by toxicity to aquatic organisms (Simonsen and Moestrup 1997).

Interestingly, in 1988, when the exceptionally toxic bloom of *Chrysochromulina polylepis* occurred along the Scandinavian coast, the N:P ratio of the water was high (N:P = 24:1, Dahl et al. 1989), consistent with the present study. The concentrations of inorganic P in the bloom water were at the detection level while there was still some unused nitrate (Dahl et al. 1989), which may have stressed the bloom population and, in turn, enhanced toxin production as demonstrated in the present study.

Previous studies have demonstrated that the toxicity of *Chrysochromulina polylepis* and the related *Prymnesium parvum* is not regulated by nutrient stress alone. Several environmental factors that influence growth, such as light (Rahat and Jahn 1965; Dafni et al. 1972) and salinity (Shilo and Rosenberger 1960; Padilla 1970; Larsen et al. 1993; Moestrup and Arlstad 1993) have also been shown to influence toxicity. A common feature of those studies is that toxicity increases as the growth decreases, indicating that toxin production is associated with cellular physiological stress. These studies have provided evidence that a number of variables, besides nutrient limitation, may be responsible for the

expression of toxicity of those flagellates, suggesting that many different environmental stresses tie-up into a common response. In this context the link between toxin production and growth metabolism assumes an interesting ecological aspect. The production of toxins may provide *C. polylepis* with a selective advantage in the form of a chemical defence, used at times when the cells are not growing optimally. Toxic effects of *C. polylepis* on other phytoplankton species (Arlstad 1991; Myklestad et al. 1995) and grazers (Carlsson et al. 1990) have been demonstrated in laboratory culture studies. In situ and experimental observations by Nielsen et al. (1990) during the bloom of 1988 demonstrated deleterious effects on ciliates, bacteria and copepods. Thus, from an ecological point of view, increased toxin production when nutrients are limiting would be a great advantage, providing the algae with an opportunity to proliferate where they would not be capable of competing with otherwise competitively (i.e. for nutrients) superior species of algae.

A comparison of the haemolytic activity reported by Johansson and Granéli (1999) for *Prymnesium parvum* cells and the haemolytic activity measured in *Chrysochromulina polylepis* in this study reveals some interesting aspects. First, in *P. parvum* the highest haemolytic activity was measured under N-limiting conditions, while in *C. polylepis* the highest haemolytic activity was observed under P-limiting conditions. Second, *P. parvum* showed 6 to 14 times higher haemolytic activity per cell than *C. polylepis* cells when grown under N:P supply ratios of 160:1 and 1:1, respectively. The reason for these marked differences may reflect genetic or metabolic differences in the ability to produce haemolytic substances.

Recently, experiments on the okadaic acid–producing dinoflagellates *Prorocentrum lima* and *Dinophysis acuminata* showed that toxin production increases under both N- and P-limitation (McLachlan et al. 1994; Sohet et al. 1995; Johansson et al. 1996). Thus, the availability of N and P seems to play a key role in toxin regulation in several phytoplankton species. One of the greatest deficits in our knowledge about toxin production in phytoplankton, however, is the lack of explanations for the differences in response to nutrient stress among species. Phytoplankton toxins include an array of compounds, chemically distinct and belonging to remarkably diverse families of products. How changes in nutrient availability and composition affect the production of these substances may vary substantially and are likely to be species specific. Therefore, it is difficult to see any general trends or to draw a conclusive picture of nutrient availability as a regulating factor for toxin synthesis in marine phytoplankton.

In summary, the results obtained in this study demonstrate that the haemolytic activity of *Chrysochromulina polylepis* is influenced by the N:P ratio of the medium. *C. polylepis* cells grown under unbalanced N:P supply ratios showed a significantly higher haemolytic activity than cells grown under balanced N:P supply ratios (N:P = 16:1). This occurred independently of the growth-limiting nutrient (N or P) and became more pronounced as nutrient limitation became more severe. These results strongly suggest that the increase in haemolytic activity of *C. polylepis* is a response to cellular physiological stress; in this case due to N- and P-limitation. It is generally accepted that in several potentially toxic phytoplankton species the availability of inorganic nutrients (N and P) is an important factor for the regulation of growth and toxicity. In many coastal areas where toxic blooms occur, N:P ratios have changed considerably due to high, erratic inputs of N and P from human activities, leading to an unbalanced N:P ratio (compared to the Redfield ratio). Potentially toxic phytoplankton may thus become toxic when exposed to an unbalanced nutrient regime caused by eutrophication. The direct relationship observed between nutrient limitation and the production of haemolytic substances in this study is consistent with this idea. The mechanisms behind this behaviour, and exactly how N- and P-limitation acts to stimulate the toxicity in *C. polylepis* is not yet fully understood and needs further exploration.

The importance of environmental factors for the regulation of toxins demonstrated in this study may help to explain the large variability frequently observed in the toxicity of marine phytoplankton both in cultures and in the natural environment. An increase in toxicity as a response to limiting nutrients appears to be a general trend in diverse taxonomic groups, including haptophytes. However, due to the species-specific responses of phytoplankton under nutrient-limited conditions (Sakshaug et al. 1983), more work should be done in comparing different phytoplankton species and groups in order to establish the ecological significance of toxin production in phytoplankton.

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References

Arlstad S (1991) Den allelopatiske Effekt af *Prymnesium parvum*, *Prymnesium patelliferum*, *Chrysochromulina polylepis*. MSc thesis, University of Copenhagen, Copenhagen

Carlsson P, Granéli E, Olsson P (1990) Grazer elimination through poisoning: one of the mechanisms behind *Chrysochromulina polylepis* blooms. In: Granéli E, Sundström B, Edler L, Anderson DM (eds) Toxic marine phytoplankton. Elsevier, New York, pp 116–122

Cembella AD, Antia NJ, Harrison PJ (1984) The utilisation of inorganic and organic phosphorus compounds as nutrients by eukaryotic microalgae: a multidisciplinary perspective. Part 2. CRC Critical Rev Microbiol 10: 13–79

Dafni Z, Ulitzur S, Shilo M (1972) Influence of light and phosphate on toxin production and growth of *Prymnesium parvum*. J gen Microbiol 70: 199–207

Dahl E, Lindahl O, Paasche E, Throndsen J (1989) The *Chrysochromulina polylepis* bloom in Scandinavian waters during spring 1988. In: Cosper EM, Bricelj VM, Carpenter EJ (eds) Coastal and estuarine studies. Vol. 35. Novel phytoplankton blooms. Springer-Verlag, Berlin, pp 383–405

Edvardsen B (1993) Toxicity of *Chrysochromulina* species (Prymnesiophycae) to the brine shrimp, *Artemia salina*. In: Smayda TJ, Shimizu Y (eds) Toxic phytoplankton blooms in the sea. Elsevier, New York, pp 681–686

Edvardsen B, Larsen A, Kristiansen S, Paasche E (1996) The effect of growth stage, salinity, temperature and nutrient limitation on the toxicity of *Chrysochromulina polylepis* (Prymnesiophyte). In: Toxicity, autecology and life history of *Chrysochromulina polylepis* (Haptophyta). Dr. scient. thesis, University of Oslo, Oslo, Norway

Edvardsen B, Moy F, Paasche E (1990) Hemolytic activity in extracts of *Chrysochromulina polylepis* grown at different levels of selenite and phosphate. In: Granéli E, Sundström B, Edler L, Anderson DM (eds) Toxic marine phytoplankton. Elsevier, New York, pp 284–289

Edvardsen B, Paasche E (1998) Bloom dynamics and physiology of *Prymnesium* and *Chrysochromulina*. In: Anderson DM, Cembella AD, Hallegraeff GM (eds) The physiological ecology of harmful algal blooms. Springer-Verlag, Heidelberg, pp 193–208

Goldman J, Graham SJ, Peavey DG (1979) Growth rate influence on the chemical composition of phytoplankton in oceanic waters. Nature 229: 210–215

Guillard RR, Ryther JH (1962) Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt, and *Detonula confervacea* (Cleve) Gran. Can J Microbiol 8: 229–239

Hallegraeff GM (1993) A review of harmful algal blooms and their apparent global increase. Phycologica 27: 25–42

Healey FP (1975) Physiological indicators of nutrient deficiency in algae. Tech Rep Fish mar Serv, Can 585: 1–30

Jebram D (1980) Prospection for a sufficient nutrition for the cosmopolitic marine bryozoan *Electra pilosa* (Linnaeus). Zool Jb (Abt Syst Ökol Geogr Tiere) 107: 368–390

Johansson N, Granéli E (1999) The influence of different N:P supply ratios on cell density, chemical composition and toxicity of *Prymnesium parvum* (Haptophyta) in semi-continuous cultures. J exp mar Biol Ecol 239: 243–258

Johansson N, Granéli E, Yasumoto T, Carlsson P, Legrand C (1996) Toxin production by *Dinophysis acuminata* and *D. acuta* cells grown under nutrient sufficient and deficient conditions. In: Yasumoto T, Oshima Y, Fukuyo Y (eds) Harmful and toxic algal blooms. Intergovernmental Oceanographic Commission of UNESCO, Paris, pp 277–280

Kaas H, Larsen J, Møhlenberg FJ, Richardson K (1991) The *Chrysochromulina polylepis* bloom in the Kattegatt (Scandinavia), May–June 1988. Distribution, primary production and nutrient dynamics in the late stage of the bloom. Mar Ecol Prog Ser 79: 151–161

Larsen A, Eikrem W, Paasche E (1993) Growth and toxicity in *Prymnesium patelliferum* (Prymnesiophycae) isolated from Norwegian waters. Can J Bot 71: 1357–1362

Latasa M, Berdalet E (1994) Effect of nitrogen and phosphorus starvation on the pigment composition of cultured *Heterocapsa* sp. J Plankton Res 16: 83–94

Laws EA, Bannister TT (1980) Nutrient- and light-limited growth of *Thalassiosira fluviatilis* in continuous culture, with implications for phytoplankton growth in the ocean. Limnol Oceanogr 25: 457–473

Maestrini SY, Granéli E (1991) Environmental conditions and ecophysiological mechanisms which led to the 1988 *Chrysochromulina polylepis* bloom: an hypothesis. Oceanol Acta 14: 397–413

Manton I, Park M (1962) Preliminary observations on scales and their mode of origin in *Chrysochromulina polylepis* sp. nov. J mar biol Ass UK 42: 565–578

McLachlan JL, Marr JC, Conlon-Kelly A, Adamson A (1994) Effects of nitrogen concentration and cold temperature on DSP-toxin concentration in the dinoflagellate *Prorocentrum lima* (Proro-centrales, Dinophycae). Nat Toxins 2: 263–270

Meldahl AS, Edvardsen B, Fonnum F (1994) Toxicity of four potentially ichtyotoxic marine phytoflagellates determined by four different test methods. J Toxic envirl Hlth 42: 289–301

Moestrup Ø, Arlstad S (1993) The toxicity of *Chrysochromulina* and *Prymnesium* in relation to salinity and growth phase. In: Proceedings 6th International Conference on Toxic Marine Phytoplankton. October 18–22, 1993, Nantes, France. IFREMER, Nautes, p 141 (abstract)

Myklestad SM, Ramlo B, Hestman S (1995) Demonstration of strong interaction between the flagellate *Chrysochromulina polylepis* (Prymnesiophycae) and a marine diatom. In: Lassus P, Arzul G, Erard-le Denn E, Gentien P, Marcaiillou-Le Baut C (eds) Harmful marine algal blooms. Lavoisier Intercept Ltd, New York, pp 633–638

Nielsen TG, Kiørboe T, Bjørnsen PK (1990) Effects of a *Chrysochromulina polylepis* subsurface bloom on the planktonic community. Mar Ecol Prog Ser 62: 21–35

Paasche E, Erga SR (1988) Phosphorus and nitrogen limitation of phytoplankton in the inner Oslofjord (Norway). Sarsia 73: 229–243

Padilla GM (1970) Growth and toxigenesis of the chrysomonad *Prymnesium parvum* as a function of salinity. J Protozool 17: 456–462

Rahat M, Jahn LT (1965) The growth of *Prymnesium parvum* in the dark and a note on its ichtyotoxin formation. J Protozool 12: 246–250

Redfield AC (1958) The biological control of chemical factors in the environment. Am Scient 46: 205–222

Robertsson A (1991) Effects of a toxic bloom of *Chrysochromulina polylepis* on the common dog-whelk, *Nucella lapillus*, on the Swedish west coast. J mar biol Ass UK 71: 569–578

Rosenberg R, Lindahl O, Blanck H, (1988) Silent spring in the sea. Ambio 17: 289–290

Sakshaug E, Andresen K, Myklestad S, Olsen Y (1983) Nutrient status of phytoplankton communities in Norwegian waters (marine, brackish, and fresh) as revealed by their chemical composition. J Plankton Res 5: 175–196

Sakshaug E, Granéli E, Elbrächter M, Kayser H (1984) Chemical composition and alkaline phosphatase activity of nutrient-saturated and P-deficient cells of four marine dinoflagellates. J exp mar Biol Ecol 77: 241–254

Sakshaug E, Holm-Hansen O (1977) Chemical composition of *Skeletonema costatum* (Grev.) Cleve and *Pavlova* (Monochrysis) *lutheri* (Droop) Green as a function of nitrate-, phosphate- and iron-limited growth. J exp mar Biol Ecol 29: 1–34

Sakshaug E, Olsen Y (1986) Nutrient status of phytoplankton blooms in Norwegian waters and algal strategies for nutrient competition. Can J Fish aquat Sciences 43: 389–396

Schöne HK, Schöne A (1982) A weakly enriched sea-water medium for ecological studies on marine plankton algae, some examples of its application. Botanica mar 25: 117–122

Shilo M (1967) Formation and mode of action of algal toxins. Bact Rev 31: 180–193

Shilo M (1971) Toxins of Chrysophycae. In: Kadis S, Ciegler A, Ajl SJ (eds) Microbial toxins. Algal and fungal toxins. Vol. 7. Academic Press, New York, pp 67–103

Shilo M, Rosenberger RF (1960) Studies on the toxic principles formed by the chrysomonad *Prymnesium parvum* Carter. Ann NY Acad Sci 90: 866–876

Simonsen S, Moestrup Ø (1997) Toxicity tests in eight species of *Chrysochromulina* (Haptophyta). Can J Bot 75: 129–136

Skjoldahl HR, Dundas I (1991) The *Chrysochromulina* bloom in the Skagernak and the Kattegat in May–June 1988: environmental conditions, possible causes and effects. No. 175, ICES Cooperative Research Report, Copenhagen

Smayda TJ (1990) Novel and nuisance phytoplankton blooms in the sea: evidence for a global epidemic. In: Granéli E, Sundström B, Edler L, Anderson DM (eds) Toxic marine phytoplankton. Elsevier, New York, pp 29–40

Sohet K, Perira A, Braeckman JC, Houvenaghel G (1995) Growth and toxicity of *Prorocentrum lima* (Ehrenberg) dodge in different culture media: effects of humic acids and organic phosphorus. In: Lassus P, Arzul G, Erard-le Denn E, Gentien P, Marcaiillou-Le Baut C (eds) Harmful marine algal blooms. Lavoisier Intercept Ltd, New York, pp 669–674

Solorzano L, Sharp JH (1980) Determination of total dissolved phosphorus and particulate phosphorus in natural waters. Limnol Oceanogr 25: 754–758

Tangen K (1989) Algal blooms in Norway in 1989. Red Tide Newsl 2: 2–3

Underdahl B, Skulberg OM, Dahl E, Aune T (1989) Disastrous bloom of *Chrysochromulina polylepis* (Prymnesiophycea) in Norwegian coastal waters 1988: mortality in marine biota. Ambio 5: 265–270

Valderama JC (1995) Methods of nutrient analysis. In: Hallegraeff GM, Anderson DM, Cembella AD (eds) Manual of harmful marine microalgae. No. 33, IOC and Guides, UNESCO, Paris

Yasumoto T, Underdahl B, Aune T, Hormazabal V, Skulberg OM, Oshima Y (1990) Screening for hemolytic and ichtyotoxic components of *Chrysochromulina polylepis* and *Gyrodinium aureolum* from Norwegian coastal waters. In: Granéli E, Sundström B, Edler L, Anderson DM (eds) Toxic marine phytoplankton. Elsevier, New York, pp 436–440