Vol. 39: 85–95, 2005 | AQUATIC MICROBIAL ECOLOGY
Aquat Microb Ecol | Published April 28

Cell cycle dependent expression of toxicity by the ichthyotoxic prymnesiophyte *Chrysochromulina polylepis*

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ABSTRACT: The coupling of toxicity expression with cell-cycle phases was studied in the toxic marine prymnesiophyte *Chrysochromulina polylepis* Manton & Parke, Clone B1511. Cell synchronisation of cultures in exponential or early stationary growth phases under nutrient-replete conditions was achieved by manipulation of the photoperiod. Chlorophyll *a* (chl *a*) and cell number increased in a stepwise manner, but were asynchronous, with chl *a* increasing during the light period and cell number increasing during the dark period. In the course of the light period, nearly all cells clustered in the G1 (Gap 1) phase, which lasted for about 20 h. DNA synthesis (S phase) occurred mainly in the dark during a discrete period (about 4 h) and G2 (Gap 2) and mitosis (M) were always completed before the end of the dark period. Toxicity expression, measured by the erythrocyte lysis assay (ELA), exhibited a dramatic drop in LC_{50} values (increase in toxicity) during the light period, although this effect was less pronounced after the first 2 generations of cell division when the cultures had entered the stationary phase. Similarly, haemolytic activity per unit cell volume decreased by a factor of 3 to 4 during the dark period over the first 48 h, but became irregular towards the end of the experiment. In this study, the light-dependent effect on toxicity and relationship to discrete phases of the cell cycle are demonstrated for the first time in a prymnesiophyte.

KEY WORDS: Cell cycle · Ichthyotoxins · Phytoflagellates · Prymnesiophytes · *Chrysochromulina polylepis*

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INTRODUCTION

Phytoflagellates are particularly well represented among the numerous toxigenic microalgae responsible for harmful algal blooms in coastal marine and brackish waters throughout the world (Hallegraeff 1993). Most of these blooms are attributed to dinoflagellates, but other flagellate groups, including raphidophytes, pelagophytes and prymnesiophytes are often implicated, particularly in incidents of fish kills and other mass faunal mortalities. For example, in May 1988, the marine flagellate *Chrysochromulina polylepis* Manton et Parke (Prymnesiophyceae) caused a devastating toxic bloom, resulting in extensive fish kills in the Kattegat and Skagerrak, which connects the Baltic Sea with the North Sea (Dahl et al. 1989). The bloom covered an area of approximately 7.5×10^4 km^2 and reached concentrations of 10×10^7 cells l^{-1} during the peak of the bloom (Dahl et al. 1989, Granéli et al. 1993). In later stages, the bloom was essentially monospecific, with *C. polylepis* as the dominant microalgal species. The bloom exhibited strong toxicity to various marine organisms, leading to severe ecological damage to wild biota and to high economic losses at fish farms along the Norwegian and Swedish coasts (Rosenberg et al. 1988, Nielsen et al. 1990, Skjoldal & Dundas 1991). Subsequently, *Chrysochromulina* blooms (not only of *C. polylepis*) have been repeatedly observed, some of which have caused fish mortalities (Tangen & Brieby 1988, Tangen 1989, Edvardsen &

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Paasche 1998 [review], Johnsen et al. 1999). The definitive causes of these blooms remain unknown, but in addition to meteorological, hydrographical and chemical conditions that promote growth and toxin production in *Chrysochromulina* spp., adverse effects on planktonic grazers may also have played a role in bloom development (Nielsen et al. 1990, John et al. 2002).

The chemical characterisation of *Chrysochromulina polylepis* toxin(s) is still lacking. The mode of action of these toxins is apparently non-selective, causing interference mainly with membrane functions, and thus organisms ranging from protozoans to fish are known to be affected (Skjoldal & Dundas 1991, Gjøsæter et al. 2000). Yasumoto et al. (1990) described the toxins as glucolipids and/or fatty acids, but John et al. (2002) showed that a toxic and an apparently non-toxic clone of *C. polylepis* exhibited the same lipid and fatty acid composition.

Curiously, prior to the major ichthytoxic event in Scandinavia, *Chrysochromulina polylepis* had been considered to be non-toxic to fish (Manton & Parke 1962) and only slightly toxic to a bryozoan, *Electra pilosa* (Jebram 1980). Toxicity of *C. polylepis* was demonstrated to be highly variable within and among strains of this species (review by Edvardsen & Paasche 1998). Little is known of the factors triggering toxicity, but unbalanced nutrient conditions such as high levels of ambient nitrogen (N) and limiting amounts of phosphorus (P)—conditions that exist in many coastal regions due to human activity—seem to play an especially important role in the manifestation of *C. polylepis* toxicity (Edvardsen et al. 1990, 1996, Edvardsen 1993). Laboratory experiments have confirmed that toxicity of *C. polylepis* is strongly dependent on the pH of the culture medium (Schmidt & Hansen 2001) and the cellular N:P ratio (Johansson & Granéli 1999).

In order to define the mechanisms involved in the expression of toxicity in *Chrysochromulina polylepis*, it is necessary to identify the exogenous and endogenous factors that induce and regulate toxin production at the cellular level. This can best be achieved by cell-cycle analysis of synchronised or at least highly phased cultures. The entrainment of microalgal cells towards synchronous division is most often attempted by exogenous control via regulation of light and/or nutrient concentration and supply rate.

Circadian rhythms among the phytoflagellates are well known to be associated with both nutrient assimilation and light utilisation. Among dinoflagellates, diel vertical migration has been shown to be nutrient-mediated, although not obviously linked to toxin biosynthesis (MacIntyre et al. 1997). Light plays a critical role in regulating rhythmic physiological processes, e.g. cell division, nutrient assimilation, bioluminescence, toxin production, the onset of sexual reproduction, vertical migration, etc. (Taroncher-Oldenburg et al. 1997). The cell division cycles of most phytoflagellate species are therefore phased or synchronised to photocycles (Chisholm 1981, Chisholm et al. 1984, Gerath & Chisholm 1989). For experimental purposes, manipulation of the light regime (as opposed to nutrients, temperature or use of metabolic inhibitors) allows virtually instantaneous alteration in magnitude and frequency of the exogenous regulator.

The eukaryotic cell cycle is generally divided into 4 phases: M (mitosis), G1 (Gap 1), S (DNA synthesis), and G2 (Gap 2). Although early work on the unusual nuclei of certain phytoflagellates, particularly the 'mesokaryotic' dinoflagellates (summarised by Rizzo 1987), led to suspicions that this pattern may not be consistently maintained, Chisholm (1981) showed that this cell-cycle sequence is universal for all eukaryotic microalgae. The cell cycle is initiated with mitotic division (M phase), followed by G1 phase, where the cells are metabolically very active, and S phase when DNA synthesis occurs. The G2 phase, where the cell contains 2 copies of nuclear DNA, terminates at mitotic division, and ultimately cytokinesis occurs. Among prymnesiophytes, such as *Chrysochromulina polylepis*, this asexual mitotic cycle continues with successive cycles of cell division, and sexuality is rarely, if ever, observed. As in dinoflagellates (except *Noctiluca scintillans*), the vegetative, authentic cell type of *C. polylepis* (e.g. Clone B1511) is haploid (n), with the diploid stage restricted to cell-cycle events subsequent to the S phase. Vegetative, alternate cells of *C. polylepis* (e.g. Clone B11), however, may be either haploid or diploid (Edvardsen & Vaulot 1996).

The aim of the present work was to investigate whether or not the expression of toxicity in *Chrysochromulina polylepis* is coupled to a defined period within the cell cycle, as previously shown for dinoflagellates (Taroncher-Oldenburg et al. 1997, Pan et al. 1999).

MATERIALS AND METHODS

Synchronisation conditions and sampling procedure. Experiments were conducted with a toxic, haploid clone (B1511) of *Chrysochromulina polylepis*. Clone B11, which did not express toxicity according to various bioassays, was used as a control in the erythrocyte lysis assay (ELA). Both clones were isolated by Bente Edvardsen, University of Oslo, and originate from the Oslofjord, Norway (59° 00′ N, 10° 45′ E). Each clone represents 1 of the 2 morphologically different cell types of *C. polylepis*, previously described in detail (Paasche et al. 1990, Edvardsen & Paasche 1992,

Edvardsen & Vaulot 1996). *C. polylepis* was grown in enriched seawater medium IMR 1/2 (Eppley et al. 1967) supplemented with 10 nM selenite in batch cultures at 15°C under white fluorescent light with a photon flux density of 45 µmol s^{-1} m^{-2}, applied on a 14:10 h light:dark regime. For synchronisation, unialgal cultures in exponential growth phase were inoculated with 1×10^4 cells ml^{-1} in sequence into 0.05, 0.5, 1, 5 and finally into 10 l flasks to reach a final concentration of 1.5×10^5 cells ml^{-1} before each transfer. We gently bubbled 5 and 10 l cultures with sterile-filtered air to provide CO_2 and to achieve homogenous cell distribution. Sampling was done from 3 parallel cultures during exponential growth throughout a culture period of 72 h at 2 h intervals, starting at a cell concentration of about 4×10^4 cells ml^{-1}. Samples were collected from 10 l cultures via a silicone-rubber tube with an inner diameter of 3 mm by gently applying a vacuum created by drawing on a 50 ml syringe. Samples were immediately stored on ice and, after determination of the cell numbers, processed according to their respective analytical procedure. During the dark period, samples were collected in darkness under a red darkroom safety light to avoid disturbance of synchronous growth and algal metabolism. The degree of synchronisation of *C. polylepis* cultures was calculated by the algorithm of Engelberg (1961).

Determination of cell concentration and size. Samples (2 ml) were diluted in 18 ml of sterile seawater pre-cooled to 15°C to determine cell concentration. Cell numbers were counted with a Multisizer II particle counter (Coulter Electronics) equipped with a 100 µm aperture. Cells were counted within a size window of 5 to 12 µm, which excluded background particles present in the seawater. Average cell size with standard deviation (SD) was calculated with Coulter Multisizer Software. Calculation of specific growth rate (µ, d^{-1}) was performed using the formula:

$$\mu = [\ln(C_1) - \ln(C_0)]/1$$

where C_1 is the cell concentration at Time 1 and C_0 is the cell concentration at Time 0.

Growth rate (k, divisions d^{-1}) was calculated as:

$$k = \mu/\ln(2)$$

Chlorophyll determination. Samples for chlorophyll *a* (chl *a*) determination were prepared by filtering 5 ml of algal culture in triplicate onto glass microfibre filters GF/F (Whatman) followed by extraction with 10 ml 90% acetone at –20°C. Chl *a* concentrations were measured by fluorometry (Gamma Analysen Technik, Type 10AK) using an excitation wavelength of 435 nm and emission fluorescence at 670 nm. Fluorescence of other pigments was subtracted by measuring the samples again after acidification with 1 N HCl. Chl *a* concentration (ng ml^{-1}) was determined using the formula (Arar & Collins 1992) as follows:

$$\text{chl } a = F_m/(F_m - 1) \times (F_0 - F_a) \times K_x \times (\text{Vol}_{Ex}/\text{Vol}_S)$$

where F_m is the acidification coefficient of chl *a* standard (= 2.18), F_0 is the relative fluorescence before acidification, F_a is the relative fluorescence after acidification, K_x is the calibration factor (= 2.48), Vol_{Ex} is the extract volume (= 10 ml), and Vol_S is the sample volume (= 5 ml).

Cell-cycle analysis. Samples (20 ml) of *Chrysochromulina polylepis* culture were fixed with 0.25% glutaraldehyde, stained with 5 µM SytoxGreen (Molecular Probes) and subsequently analysed for relative DNA content using a FACS Vantage flow cytometer (Becton-Dickinson) equipped with an Innova Enterprise II 621 laser, as previously described (Eschbach et al. 2001a). In brief, at least 1×10^4 cells were analysed per sample at a pressure of 1 psi. Dot-plots and histograms were created with the WinMDI 2.8 software (Joseph Trotter, Scripps Research Institute, La Jolla, CA, USA). Cell-cycle analysis was achieved with the Multicycle software (Phoenix Flow Systems). The number of cells in a certain cell-cycle phase was expressed as the percentage of the total cell number in the sample. The duration of single cell-cycle phases was determined based on the algorithm developed by Beck (1978) for synchronised cell cultures. The duration of G1, S and G2+M phases was calculated for 2 consecutive complete cell cycles of *C. polylepis*.

Toxicity determination. The toxicity of *Chrysochromulina polylepis* was determined with an improved version of the ELA, as previously described (Eschbach et al. 2001b). This assay is based on the lysis of carp erythrocytes resulting from membrane perturbation activity of *C. polylepis* toxin(s), and the subsequent photometric measurement of released cellular contents at 414 nm. We performed 2 types of toxicity measurements: (1) 4.8×10^6 cells were sampled every 2 h throughout the 72 h sampling period to measure haemolytic activity; (2) 6.4×10^6 cells were sampled 8 h after the start of the light period (since preliminary experiments had indicated high toxicity at this point), every day throughout the sampling period (i.e. after 24, 48 and 72 h), to measure toxicity kinetics. Samples were centrifuged for 15 min at $3200 \times g$ at 4°C in 15 ml tubes to yield cell pellets of 0.4, 0.8 or 1.6×10^6 cells per tube, depending on the cell concentration in the culture at the time of sampling. Pellets were frozen immediately and stored at –20°C until analysis.

The kinetic analysis was done in order to determine the optimal incubation time (IT_{opt}) in the ELA, which varied in preceding experiments. Preliminary work showed that toxicity of synchronised *Chrysochromulina polylepis* cultures was high at 8 h after the start

of the light period; therefore, this sampling point was selected for toxicity kinetic measurements (E. Eschbach unpubl. data). Different levels of toxicity were simulated by exposing the erythrocytes to dilutions of algal extracts corresponding to 1, 2, 3, 4 and 5 × 10^5 cells ml^{-1} (= final concentration in the ELA). Lytic activity was determined at 4 h intervals for 24 h. The percentage erythrocyte lysis (haemolysis) was then plotted against incubation time to yield a series of curves showing the degree of haemolysis as a function of incubation time and concentration of algal extract (Fig. 1). The IT_{opt} were chosen where the differences in haemolytic activity among the algal extract dilutions showed the greatest differences, allowing for an optimal detection of difference in toxicity.

Haemolytic measurements with the ELA were performed in triplicate according to Eschbach et al. (2001b). Haemolytic activity was calculated as the percentage of lysis of carp erythrocytes, by comparing absorbance mediated by the lytic activity of algal extracts with absorbance obtained from completely lysed erythrocytes, defined as 100% lysis.

To determine the cell concentration that resulted in 50% erythrocyte lysis after 16 h exposure (LC_{50} 16 h values), logarithmically transformed algal cell concentrations were separately plotted against erythrocyte lyses for each sampling point. LC_{50} was subsequently calculated from the linear part of the resulting curves using linear regression analysis and plotted against time. Haemolytic activity was also calculated relative to the cell volume at each respective sampling point, to take into account the changing cell volume during cell growth. The cell volume was calculated from the cell-size measurements, assuming the algal cells to be spherical. For these calculations, the haemolytic activity of 5 × 10^5 algal cells ml^{-1} (final concentration) was divided by the cell volume ($4/3\pi r^3$, where r = cell radius).

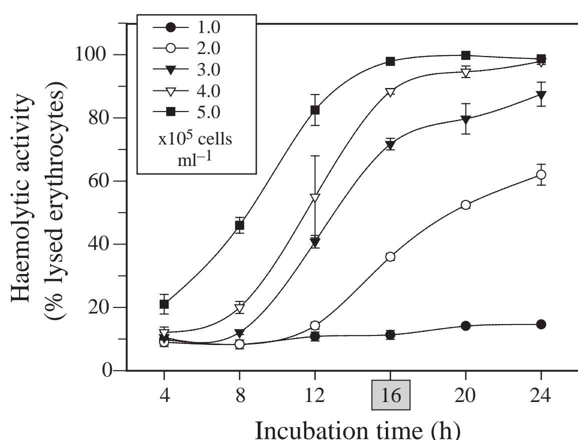

Fig. 1. *Chrysochromulina polylepis*. Toxicity kinetics for determination of optimal incubation time (IT_{opt}) of algal extracts with erythrocytes in erythrocyte lysis assay. Different toxicity of algal extracts was simulated in these measurements by exposing erythrocytes to dilutions of algal extracts corresponding to 1, 2, 3, 4 and 5 × 10^5 cells ml^{-1}. Haemolytic activity of the diluted extracts was measured at 4 h intervals for 24 h

RESULTS

Synchronous growth kinetics and chl *a* synthesis

All results described herein originate from 3 independently sampled 10 l batch cultures of *Chrysochromulina polylepis* Clone B1511 growing under identical synchronising conditions. Growth of *C. polylepis* Clone B1511 in batch culture under synchronising conditions is depicted in Fig. 2A. Sampling was initiated at a cell density of approximately 4 × 10^4 cells ml^{-1} and was continued for a 72 h period, indicated by the arrow in Fig. 3. Given an inoculum of 1 × 10^4 cells ml^{-1} on Day 1, the cells reached early exponential growth phase within the first day in the 10 l cultures. A maximum cell density of about 3.5 × 10^5 cells ml^{-1} was achieved within 10 d after the first inoculation.

Chrysochromulina polylepis cultures showed a stepwise increase in both cell number and cellular chl *a* concentrations during the 72 h sampling period (Fig. 2A). This is because of the experimental culture procedure, which produced 58% synchronisation of the algal population during the first 24 h of the sampling period. After this initial period, synchronisation increased in successive steps of approximately 5%, yielding 63 and 68% synchronisation after 48 and 72 h, respectively.

Increases in chl *a* and cell number were shifted in time, with chl *a* increasing during the light period and cell number increasing during the dark period. Increases in cellular chl *a* began after the switch from dark to light and continued throughout the entire light period. Conversely, a decrease in chl *a* content occurred after the light to dark switch and continued throughout the entire dark period due to reduction of cell content during cell division (Fig. 2B). During the first 24 h of the sampling period, an increase in cell number was initiated and then terminated within the same dark period, yet through the next two 24 h periods, this increase in cell number extended 2 h and finally 4 h into the light period (Fig. 2A).

The growth rate of *Chrysochromulina polylepis* decreased over time from 0.82 to 0.65–0.61 divisions (div.) d^{-1} during the 3 d experiment. Cell size (equivalent spherical diameter) varied periodically during the 72 h sampling period from a maximum of 8.3 µm to a minimum of 6.7 µm (Fig. 2D). Towards the end of the sampling period, cells became smaller, as indicated by a maximum cell size of only 7.5 µm on Day 3 of the sampling period.

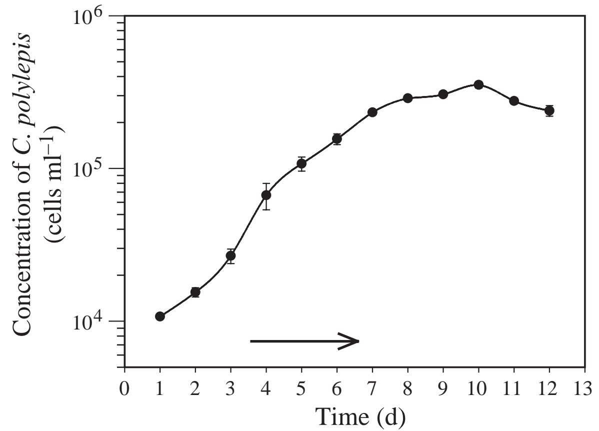

Fig. 3. *Chrysochromulina polylepis* Clone B1511. Mean (±SD; n = 3) concentration over a 2 wk period in synchronised batch cultures. Arrow indicates sampling period of 72 h

Cell-cycle analysis

Flow cytometry determination of the relative DNA content of *Chrysochromulina polylepis* revealed successive cell-cycle phases typical for eukaryotic cells (Fig. 2C). Single distinct peaks for the G1, S and G2+M phases were obtained for each cell cycle (G2 and M phases cannot be distinguished by flow cytometry because cells in these respective phases contain the same amount of DNA).

Our results (Fig. 2C) show that *Chrysochromulina polylepis* proceeds, as expected, through the typical phases of a eukaryotic cell cycle, consisting of mitosis (M phase) followed by the metabolically active G1 phase, the S phase of DNA synthesis and the S and M phases separating the G2 phase. As summarised in Table 1 and illustrated in Fig. 2C. The S phase in *C. polylepis* takes place during a discrete period of approximately 4 h following G1 phase. The G2 and M phases occur within 1 to 2 h after the S phase.

DNA synthesis (S phase) always began 2 h before and was completed by the end of the dark period (Fig. 2C). During the first 48 h of the sampling period, a peak of DNA synthesis appeared after 2 h in the

Fig. 2. *Chrysochromulina polylepis* Clone B1511. Data from 3 synchronised batch cultures during the 72 h sampling period. (A) Algal cell concentration and chlorophyll *a* (chl *a*) concentration as a function of time; (B) chl *a* content per cell (derived from data in A); (C) percentage of cells in G1, S and G2+M phases of cell cycle; (D) average cell size (equivalent spherical diameters); (E) toxicity of algal cells expressed as haemolytic activity relative to cell volume (HA/Vol) and as cell concentration required for 50% erythrocyte lysis (LC_{50}). Data points are mean (±SD) of 3 independent measurements; error bars omitted from graphs with more than 1 curve (A,C,E) for clarity. Shaded areas: 10 h dark periods; G1: Gap 1; S: DNA synthesis; G2+M: Gap 2+mitosis phases

Table 1. *Chrysochromulina polylepis* Clone B1511. Duration of cell-cycle phases in 2 consecutive cycles, calculated according to Beck (1978) using time intervals from 6 to 48 h and from 30 to 72 h, respectively. Results are expressed in absolute numbers (h) and as percent (%) of total duration (Total) of a complete cell cycle. Means ± SD are calculated from 3 independent 10 l batch cultures. G1: Gap 1; S: DNA synthesis; G2+M: Gap 2 and mitosis

Phase	1st complete cycle (6 to 48 h)		2nd complete cycle (30 to 72 h)	
	h	%	h	%
G1	19.6 ± 0.3	78.7 ± 1.1	22.0 ± 0.6	79.3 ± 2.2
S	4.3 ± 0.9	17.1 ± 3.5	4.1 ± 0.1	14.8 ± 0.5
G2+M	1.0 ± 0.7	4.1 ± 2.6	1.6 ± 0.8	5.8 ± 2.9
Total	24.8 ± 1.9	100	27.8 ± 1.6	100

dark. The third S phase peak was shifted by 4 h within the third dark period. Cell division (G2+M) started and was completed during the dark periods of the first 48 h of the sampling period. By the end of the sampling period, cell division extended into the light period and the delay between the S and G2+M peaks was reduced to 2 h. An increase in the number of cells in the S and G2+M phases was always synchronous with a decrease in the number of cells in the G1 phase. During the first 48 h of the sampling period, nearly all cells clustered in G1 by the end of the dark period.

The duration of the cell-cycle phases, determined according to Beck (1978), was calculated during the 6 to 48 h interval shown in Fig. 2C for the first cycle, and the 30 to 72 h interval for the second complete cycle. The G1 phase occupied most of the cell cycle of *Chrysochromulina polylepis*, with 19.6 ± 0.3 and 22.0 ± 0.6 h (n = 3) duration for the first and the second complete cycle, respectively (Table 1). The mean duration of the S phase was 4.3 ± 0.9 and 4.1 ± 0.1 h (n = 3), whereas the G2+M phase lasted 1.0 ± 0.7 and 1.6 ± 0.8 h (n = 3) in successive cycles. Total cell-cycle duration increased slightly from 24.8 ± 1.9 to 27.8 ± 1.6 h (n = 3) during the 72 h sampling period.

Toxin assays

Calculations of lytic activity from the ELA, based on percentage haemolysis at different time points, showed that 16 h was the optimal incubation time for sensitive high-resolution measurements with the ELA (Fig. 1). Extracts of the ichthyotoxic Strain B1511 and the non-toxic B11 clone of *Chrysochromulina polylepis* revealed different haemolytic capacities, but the differences were a matter of degree, not absolute (Fig. 4). Specifically, B1511 extracts yielded nearly 100% erythrocyte lysis after an incubation time of 16 h, yet B11 extracts also exhibited about 40% lysis for this period.

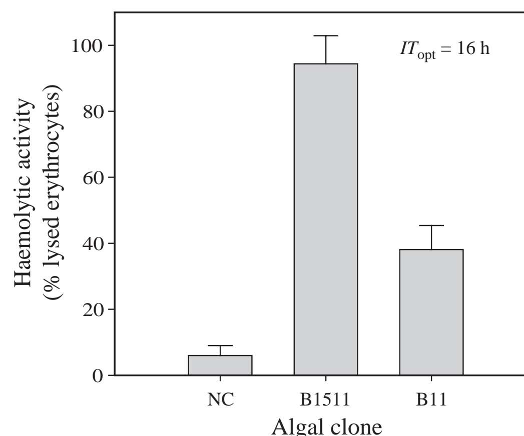

Fig. 4. *Chrysochromulina polylepis*. Comparison of haemolytic activity of extracts of ichthyotoxic Clone B1511 versus almost non-toxic Clone B11. Extracts of 5×10^5 cells ml^{-1} of each clone were incubated for 16 h (optimal incubation time, IT_{opt}). Histograms represent mean (+SD) of 6 independent measurements. NC: negative control

As depicted in Fig. 2E, the cellular lytic capacity of Strain B1511, expressed as LC_{50} and as haemolytic activity per unit algal cell volume, varied periodically throughout the sampling period. Haemolytic activity dropped during the initial dark periods, resulting in an increase of more than 3 orders of magnitude in the number of algal cells required for 50% erythrocyte lysis during the first 48 h. In the third dark period, the phenomenon was less pronounced.

The haemolytic activity of algal cells as expressed per unit algal cell volume varied over the sampling period in a similar fashion. During the first 48 h, lytic activity per unit cell volume decreased by a factor of 3 to 4 during the dark periods and became irregular towards the end of the experiment.

DISCUSSION

Synchronisation and growth

Analyses of synchronised cultures are useful to enhance the signal from induced toxin production and provide insights into regulation, because the induction of a given biosynthetic pathway is phased to the cell division cycle and essentially occurs at the same time point for all cells in a culture. Establishment of synchronous growth conditions was therefore essential for analysis of toxin production in relation to the cell cycle of *Chrysochromulina polylepis*. A high degree of synchronisation was necessary to allow clear identification of cell-cycle stages in following temporal changes in toxin synthesis throughout the 3 d sampling period.

Alternative methods have been employed to achieve synchronisation of microalgal cultures. The block-release approach involves use of metabolic inhibitors (van Dolah et al. 1998, Ng et al. 1999) or light and/or nutrient deprivation techniques (Olson & Chisholm 1986, Vaulot et al. 1986, Taroncher-Oldenburg et al. 1997, Pan et al. 1999). The block-release method is based on the fact that under unfavourable growth conditions cells may be arrested in the G1 phase of the cell cycle (then termed G0 phase). In theory, and often in practice, the initial block of the cell cycle yields cells completely arrested in G0; after release, cells will proceed through the rest of the cell-cycle stages as a homogenous population (Pardee et al. 1978).

For *Chrysochromulina polylepis* experiments, it was possible to entrain or phase the cell-division cycle to near synchronicity by means of a repeated photoperiod. Although not always possible to apply, this method has clear advantages over the block-release method for studying periodic responses since no sudden stress is necessary to induce synchronicity. At least in the case of metabolic inhibitors, there is also less potential for introducing artefacts into the cell cycle. Partial synchronisation induced by light:dark cycles has been obtained for the haptophytes *Phaeocystis* spp. (Vaulot et al. 1994, Jacobsen 2002), *Prymnesium parvum* (Chisholm 1981, Larsen & Edvardsen 1998) and *C. polylepis* (Edvardsen & Vaulot 1996), and has also been reported for many other microalgae including chlorophytes of the genera *Chlorella*, *Scenedesmus*, and *Chlamydomonas* and the euglenophyte *Euglena* (Wanka et al. 1972, Ober 1975, Carré & Edmunds 1993, Lemaire et al. 1999). In practice, the induction of synchronous (as opposed to merely phased) cell division by manipulation of the photoperiod is only possible when the length of the cell-division cycle closely approximates the length of the photoperiod (1 div. d^{-1}).

High levels of synchronisation in *Chrysochromulina polylepis* were obtained by the sequential inoculation of increasingly larger culture volumes of cells from early exponential growth phase entrained to a 14:10 h light:dark regime. A favourable factor in these experiments was that high growth rates may be achieved (although not necessarily sustained) by *C. polylepis* populations. Under routine culture conditions, growth rates of ca. 0.5 div. d^{-1} (data not shown) were obtained with this strain of *C. polylepis*, but under exceptional growth conditions growth rates as high as 1.3 div. d^{-1} have been achieved with this species (Edvardsen & Paasche 1992, Throndsen et al. 1995). In our toxicity expression experiments, *C. polylepis* reached maximum growth rates of 0.82 div. d^{-1} in early exponential growth phase—close to the 'ideal' rate of 1.0 div. d^{-1}. Attempts to enhance synchrony in *C. polylepis* by exposure to prolonged darkness (2 to 3 d = 2 to 3 cell division cycles) yielded cultures exhibiting long lag-phases, and sometimes the cultures never recovered at all (U. John et al. unpubl. data).

Synchronous growth was indicated by the stepwise increase in cell numbers and chl *a* content following the well-known scheme for photosynthetic flagellates of cell division during the dark period and chl *a* synthesis during the light period (Fig. 2A). This pattern has also been shown in the dinoflagellate studies of Taroncher-Oldenburg et al. (1997) and Pan et al. (1999). Growth rates showed a successive decrease from 0.82 (Day 3.5) to 0.65 (Day 4.5) and finally to 0.61 div. d^{-1} (Day 5.5) Fig. 3 indicates that the cultures entered the transition from late exponential to early stationary phase by this time.

Unbalanced growth is one effect of batch cultures, whereby high metabolic needs cannot be compensated by nutrient uptake over extended periods of time because of the exceeding high growth rates of the cells and progressively increasing biomass. The maximum growth rate (μ_{max}) for each strain/species is limited by intrinsic factors such as the maximal rate of synthesis of bioenergetic metabolites, DNA and proteins. Below this threshold, growth rates are limited by external factors, such as rate-limiting supply of nutrients, irradiance or diffusion of CO_2. Continuous aeration of our cultures mitigated the effects of carbon limitation and, to some degree, pH changes. It is unlikely that the nutrients in the medium used in our cultures were exhausted during our short experimental time, since IMR 1/2 is a highly nutrient-enriched medium. In this regard, Schmidt & Hansen (2001) calculated that their batch cultures of *Chrysochromulina polylepis*, grown on comparable *f*/2 medium used approximately 10 to 20% of available nutrients in their medium by the end of a 2 to 3 wk time course. In our experiments, the cultures were harvested much earlier (on Days 3 and 6), suggesting that macronutrient (including carbon) limitation did not play a role in decreasing growth rates.

In this study, the incident photon flux density was rather low (45 µmol photons m^{-2} s^{-1}), but this selection of light intensity was shown in preliminary experiments with this strain to sustain maximal growth rate. Nevertheless, it is possible that the cultures may have become light-limited as cell concentration increased. Increasing cell concentration in batch cultures can influence growth rate as well as synchronisation efficiency through a variety of mechanisms including a rise in 'self-shading' from the increasing biomass. For example Lemaire et al. (1999) observed that in *Chlamydomonas reinhardii* cultures, higher synchronisation efficiency in more dilute cultures could be attributed to better homogeneity of the culture, and hence a better light quality and quantity received by

each cell. Lower light quality and quantity and reduced cell size reduce the number of cells which can potentially complete the cell cycle and hence reduce synchronisation efficiency. However, in our experiment the synchronisation efficiency actually increased with increasing cell concentration.

Cell-cycle analysis

The cell cycle of *Chrysochromulina polylepis* has been previously analysed by flow cytometry (Edvardsen & Vaulot 1996). In our experiments, cell-cycle analysis was accomplished by quantifying the amount of DNA in fixed cells (Grey et al. 1990), but we used an improved method based on glutaraldehyde fixation (Eschbach et al. 2001a). At the beginning of our experiments, *C. polylepis* cultures were in quasi-steady state (roughly balanced growth) and the cells passed through S and G2+M phases during the dark period (Fig. 2C). Mitotic division in the dark is typical for many other microalgae, although there are exceptions to this rule, particularly for shade-adapted benthic species (Pan & Cembella 1998). During the light period, all *C. polylepis* cells accumulated in the G1 phase, during which growth and other metabolic functions, such as chlorophyll and toxin biosynthesis, were carried out.

After the second round of cell division, there appeared to be a slight temporal shift of the cell-cycle phases during the dark period. The second complete cell cycle lasted 3 h longer than the first cycle. Careful examination of the individual phases of the cell cycle indicated a progressive increase in the length of the G1 phase. Other reports support the finding that lengthening of the generation time accompanies decreasing growth rates and is attributable to expansion of a single cell-cycle phase, namely the G1 phase (Olson & Chisholm 1986, Micheli et al. 1996). As previously noted, for species grown phototrophically, such as the *Chrysochromulina polylepis* cultures in the current experiments, light is of primary importance for activating physiological processes and regulating progression through cell-cycle events. The observed elongation of the G1 phase is consistent with the observation of Olson & Chisholm (1986) (and references therein) that light and nutrient limitation primarily influence processes in the G1 phase, resulting in prolongation and a decrease in cell size. Light- and cell size-dependent transition points have also been documented in a variety of photoautrophic microalgae, including *Amphidinium carteri* (Olson & Chisholm 1986), *Hymenomonas carterii* and *Thalassiosira weisflogii* (Vaulot et al. 1986), and *Alexandrium fundyense* (Taroncher-Oldenburg et al. 1997). Cells that have passed the transition point will complete the cell cycle regardless of external conditions. Whether they pass this point depends on, for instance, light intensity and day length or cell size; if the cells do not pass the transition point they stay arrested in the G1 or G0 phases.

Toxin biosynthesis

In previous studies on the relationship of toxin synthesis to cell cycle phases in marine dinoflagellates, toxin production was found to be restricted to a defined period of the cell cycle. In the planktonic dinoflagellate *Alexandrium fundyense*, a causative organism responsible for paralytic shellfish poisoning, saxitoxin analogues were produced during a 10 h period within the G1 phase, with slight time shifts in the synthesis of different derivatives (Taroncher-Oldenburg et al. 1997). Nevertheless, there were some important differences in the phasing of toxin production among dinoflagellates: in *A. fundyense*, biosynthesis of the tetrahydropurine neurotoxins was restricted to the G1 phase, whereas this was not strictly true for the polyketide-derived toxins of *A. ostenfeldii* (spirolides) (John et al. 2001) and *Prorocentrum lima* (okadaic acid derivatives; dinophysistoxins, DTX) (Pan et al. 1999). For the shade-adapted benthic dinoflagellate *P. lima* (a cause of diarrhetic shellfish poisoning), the DTX derivatives produced during the beginning of the light period ('morning') in the G1 phase were different than those synthesised later in the photocycle ('afternoon') during the S and G2 phases (Pan et al. 1999). In all cases, toxin biosynthesis in these dinoflagellates is not continuous, but follows a strict time schedule coupled to their cell cycle, and is light-dependent.

In *Chrysochromulina polylepis*, under non-limiting nutrient conditions, toxicity is highest in the mid-exponential growth phase, whereas in phosphorus-limited cultures, cells remain highly toxic in the stationary phase (Edvardsen et al. 1996). However, the effects of nutrient limitation must be carefully interpreted because such limitation can lead to toxin accumulation in cells merely by reduced cell division. Hence, an increase in toxin cell quota does not necessarily result from an increase in the rate of toxin production (Taroncher-Oldenburg et al. 1997, Cembella 1998, John et al. 2001). Nevertheless, the fact that highest cell toxicity in *C. polylepis* was recorded during the exponential growth phase indicates that the rate of toxin synthesis is highest when the growth rate is maximal (Edvardsen et al. 1996).

Quantitative and qualitative analysis of the toxins produced by *Chrysochromulina polylepis* is hampered by the fact that these ichthyotoxic substances are chemically poorly described. Without certainty of the

chemical structures of these toxins, it is not possible to employ chemical analytical techniques, e.g. liquid chromatography with fluorescence or mass detection, in studies of toxin expression through the cell cycle. Nevertheless, because these toxins are known to cause lysis of cells, the mechanism that damages the gills of fishes (Skjoldal & Dundas 1991), the ELA can be employed to estimate potential toxic activity. The assay has recently been improved for more sensitivity and high sample throughput (Eschbach et al. 2001b) and can be used as a proxy for ichthyotoxicity estimates.

Simonsen & Moestrup (1997) showed haemolytic capacity for 8 different *Chrysochromulina* species, but only *C. polylepis* was toxic to *Artemia* sp. In our experiments, cell extracts of Clones B1511 and B11 of *C. polylepis* expressed different haemolytic effects (Fig. 4), and therefore the differences in haemolytic capacity of the 2 clones are assumed to be related to the toxicity.

In the work of Yasumoto et al. (1990), the haemolytic capacity and the ichthytoxicity of *Chrysochromulina polylepis* were mainly attributed to galactolipids, 1-acyl-3-digalacto-glycerol and polyunsaturated fatty acids (PUFAs). However, John et al. (2002) demonstrated that Clone B11, which showed no toxicity towards *Artemia* sp. larvae, and Clone B1511, which was highly toxic towards these crustaceans, exhibited no difference in lipid and PUFA composition. This led to the conclusion that the difference in toxicity may be explained by substances other than lipids or PUFAs.

As has been reported for dinoflagellates (Taroncher-Oldenburg et al. 1997, Pan et al. 1999), toxicity (as lytic activity) of *Chrysochromulina polylepis* also appears to be induced by light and is discontinuous over the cell cycle. The LC_{50} increases as the haemolytic activity drops during the middle of the dark period and decreases at the end of the dark period until the first few hours of the light period, leading to an intracellular accumulation of the toxin(s) (Fig. 2E). The dramatic decrease in toxicity per cell (expressed as increased LC_{50}) corresponds to cell-division events and the increase in toxicity with the start of light period and early G1 phase when the daughter cells produce the toxic/haemolytic compounds. However, this rhythmic behaviour for the lytic activity lasted only for the first 48 h of the sampling period; thereafter, lytic activity of the cells showed large variation, resulting in a less rhythmic course of the haemolytic activity curve (Fig. 2E). The induction of toxicity was not pronounced in the third light:dark cycle, although synchronisation efficiency was even higher than in the 2 earlier light:dark cycles, leading to the suggestion that light is one of the major factors regulating toxin production and confirming earlier findings that toxicity decreases in the early stationary phase. This observation is also consistent with the results of Pan et al. (1999), who found that toxin production in *Prorocentrum lima* was simultaneously initiated when the dark-arrested cells were released to the regular light:dark cycle. However, with our experiments we cannot distinguish if toxicity is light- or G1-dependent. This question should be answered in future experiments, which should take circadian regulation into account.

In conclusion, *Chrysochromulina polylepis* proceeds through a typical eukaryotic cell cycle with a distinct DNA synthesis phase. Toxin production in *C. polylepis* (and presumably other haptophytes growing photoautotrophically) can be studied with synchronised cultures in a manner similar to that for dinoflagellates (Taroncher-Oldenburg et al. 1997, Pan et al. 1999). Moreover, the analysis of toxin biosynthesis and its regulation at the gene and protein levels must be advanced in order to understand the mechanisms of toxin induction under different environmental conditions. Now that we have demonstrated that toxin biosynthesis (or at least expressed toxicity) in *C. polylepis* is switched on within a narrow time window at the beginning of the G1 phase of the cell cycle, it is possible to pursue directed molecular biological approaches to understand these phenomena.

Acknowledgements. This research was supported as part of the BMBF TEPS project 03F0244. We thank M. Henschke (Technical University, Aachen, Germany) for helping with the calculation of synchronisation efficiency and cell-cycle phase durations.

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Editorial responsibility: William Li, Dartmouth, Nova Scotia, Canada

Submitted: April 7, 2004; Accepted: January 7, 2005
Proofs received from author(s): April 12, 2005