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RESEARCH ARTICLE

Coupling of Saxitoxin Biosynthesis to the G_1 Phase of the Cell Cycle in the Dinoflagellate *Alexandrin fundyense*: Temperature and Nutrient Effects

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ABSTRACT The correlation between changes in length of the different cell cycle stages and the toxicity of *Alexandrium fundyense* Balech was studied in semi-continuous cultures. Growth rates ranging from 0.031 d^{-1} to 0.36 d^{-1} were established at different temperatures or levels of phosphate limitation. In all treatments, G_1 was the phase with the longest duration. Decrease in growth rate was associated with an increase in duration of the different cell cycle stages. Toxin content was always directly correlated to the duration of the G_1 phase. In both the temperature treatments and the phosphate limitation experiments, toxin production rates remained constant for the respective range of conditions, implying that the variations in toxin content observed were a result of increasing periods of biosynthetic activity. Toxin accumulation was directly correlated to protein biosynthesis in all temperature treatments. In contrast, toxin content showed little correlation with protein content as phosphate limitation increased. Significant differences in toxin composition were observed between the temperature and phosphate treatments. Total concentrations of GTX II and III and C I and II were significantly higher in the phosphate-limited cultures, while the levels of STX, NEO and gonyautoxins I and IV remained virtually unchanged. We conclude that toxin biosynthesis in *A. fundyense* is coupled to the G_1 phase of the cell cycle, that toxin synthesis is not down-regulated by phosphate deprivation and that interconversions among saxitoxin derivatives are influenced by the availability of phosphate. Copyright © 1999 John Wiley & Sons, Ltd.

Key words: *Alexandrium fundyense*; cell cycle; dinoflagellate; physiology; saxitoxin

INTRODUCTION

Dinoflagellates in the genus *Alexandrium* are responsible for outbreaks of paralytic shellfish poisoning (PSP) in temperate coastal areas around the world (Anderson, 1989). PSP is caused by saxitoxin (STX) and its over 20 known derivatives, a group of highly nitrogenous alkaloids with a marked affinity and blocking capacity for sodium channels (Shimizu, 1993). STX is biosynthesized by *Alexandrium* sp. and subsequently accumulates in bivalves filter-feeding on the dinoflagellate; ingestion of these toxic shellfish causes PSP symptoms at higher levels of the trophic chain, including humans (Sommer *et al.*, 1937). Knowledge of the mechanism of toxin biosynthesis and its regulation in dinoflagellates is crucial for a complete understanding of this phenomenon.

Considerable work has been done on the influence of growth-limiting physico-chemical factors such as nutrient concentrations, light and temperature on the ecophysiology of this genus and its capacity to produce toxins (Proctor *et al.*, 1975; White, 1978; Hall, 1982; Boyer *et al.*, 1987; Ogata *et al.*, 1987; Anderson *et al.*, 1990b; Flynn *et al.*, 1994; Matsuda *et al.*, 1996). Under nitrogen starvation, light limitation or non-optimal salinity conditions, toxin content (=toxin cell quota) is reduced concomitantly with a decrease in the availability of the limiting factor. Only in the case of phosphate starvation and temperature limitation has the contrary been observed – an increase in toxin content with increasing

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stress. Phosphorus is incorporated into four main organic compound fractions in the cells: RNA, DNA and phosphorus included in lipids and in esters that have a direct bearing on cell metabolism. Furthermore, phosphates play an important metabolic role as substrates for the fine regulation of biochemical activity in the cells via protein kinases (Pardee *et al.*, 1978). Temperature is a general modulator of growth and biosynthetic rates in phytoplankton and can therefore influence the accumulation of specific metabolites (Jitts *et al.*, 1964).

In addition to changes in the toxin content, toxin composition (i.e. the absolute or relative amounts of the different STX derivatives present in the organism) has also been shown to vary under different limiting conditions (Boczar *et al.*, 1988; Anderson *et al.*, 1990a; MacIntyre *et al.*, 1997). These variations are a result of physiological changes that regulate the sequence of transformations leading to the different derivatives. These pathways have not yet been elucidated. Only recently have several enzymes involved in specific interconversions among STX derivatives been characterized, but the information is still fragmented with respect to the sequence of interconversions (Oshima, 1995; Yoshida *et al.*, 1996). Observation of accumulation patterns for the different STX derivatives in synchronized cultures of *A. fundyense* suggests a sequence and directionality to these interconversions beginning with C I and II (Taroncher-Oldenburg *et al.*, 1997), but no specific pathway has yet been outlined, as the direct precursor or parent compound for the series of the STXs has not been determined. The sequence of interconversions cannot be rooted at a specific origin or parent compound with our present knowledge (Shimizu, 1993).

In parallel to the influence of the above-mentioned environmental factors on toxin production, a more immediate effect of limiting conditions has been observed on the growth rate of toxic dinoflagellates (Proctor *et al.*, 1975; White, 1978; Hall, 1982; Watras *et al.*, 1982; Anderson *et al.*, 1990b; Matsuda *et al.*, 1996). The general trend in this case is a reduction in growth rate with an increase in the limitation or stress. It has further been documented for several groups of phytoplankton that such changes in growth rate are tightly correlated to changes in the duration of the different cell cycle stages (Vaulot, 1985; Olson and Chisholm, 1986; Cetta and Anderson, 1990). Depending on the organism, changes in the doubling time can be reflected in changes in the duration of specific phases of the cell cycle (Guiget *et al.*, 1984; Olson *et al.*, 1986; Yamaguchi and Honjo, 1989).

The eukaryotic cell cycle is divided into four main stages. Following the mitotic division of the mother cell, the two daughter cells undergo a first period called G_1, during which they synthesize essential cell constituents and grow in size. Once appropriate internal and external conditions are met the cell commits to a new division. This process follows three steps. First, the cells replicate their DNA, thereby doubling their genetic information, during the S phase. A second metabolically active period follows, called G_2, in preparation for the final phase, mitosis, M, during which cells undergo cytokinesis and divide their genetic information among their offspring. Progression through the cell cycle is highly influenced by environmental conditions, as these determine the metabolic and energetic status of the cell (Yamaguchi and Honjo, 1989). Cells will grow more slowly if their metabolism is not optimal and a successful division process is compromised (Pardee *et al.*, 1978). Conversely, metabolic processes regulated by cell cycle events will also be affected.

In algae, biochemical pathways such as DNA synthesis and a variety of specific enzyme activities are modulated in a periodic, cell cycle-dependent fashion (Kates and Jones, 1967; Vassef *et al.*, 1973; Forde and John, 1974). These biosynthetic pathways are influenced by conditions affecting normal progression through the cell cycle (Mitchison, 1969; Mitchison *et al.*, 1991). The lengthening of a specific cell cycle stage will thus result in longer metabolic activities and hence higher product accumulations. In the case where the pathway of interest is only influenced by the cell cycle dynamics and no other factors, this would translate into a constant ratio between the concentration of the metabolite and the length of the cell cycle phase of interest. This is equivalent to the existence of a constant cell cycle-specific production rate of the compound.

Previous studies have shown an inverse correlation between growth rate and toxin content in *A. tamarense* (Proctor *et al.*, 1975; Ogata *et al.*, 1987) and a direct correlation between specific growth rate and specific toxin production rate in the closely related organism *A. fundyense* (Anderson *et al.*, 1990b). We have also shown that toxin production is discontinuous and restricted to a portion of the G_1 phase of the cell cycle in synchronously growing *A. fundyense* (Taroncher-Oldenburg *et al.*, 1997). These results lead us to the conclusion that there should be a correlation between the rates of toxin production or toxin accumulation and length of G_1. The aim of this study was to determine if there is any direct correlation between variations in toxin production, measured as accumulation of saxitoxin in a cell, and changes in duration of the different stages of the cell cycle under different conditions that affect growth rate and thus cell cycle progression. Such a correlation would confirm that STX biosynthesis is discontinuous and restricted to the G_1 cell cycle stage.

MATERIALS AND METHODS

The Organism

The dinoflagellate *Alexandrium fundyense* (strain

Table 1. Growth rates of *Alexandrium fundyense* grown in semi-continuous culture under phosphate limitation or at different temperatures

Temperature[a] (°C)	PO_3^- limited cultures[b] dil. rate (day^{-1})	Growth rate[c] μ (day^{-1})
20		0.277
17		0.24
15		0.194
13		0.186
11		0.18
10		0.15
8		0.121
20	0.35	0.36
20	0.17	0.17
20	0.069	0.064
20	0.052	0.045
20	0.035	0.031

[a] The daily dilution rate was equivalent to the growth rate of exponentially growing cultures at the same temperature.
[b] Cultures were diluted once a day with low phosphate medium.
[c] In all treatments the standard deviation obtained from the four replicates was less than 8% of the mean value.

GtCA29) was used throughout this study. A culture was established in January 1985 from a cyst isolated from Gulf of Maine sediments, 20 miles east of Portsmouth, New Hampshire (USA). That culture was used to isolate a single cell to establish a clonal culture. Prior to the experiments, cultures were maintained at 15 °C in f/2 medium (Guillard and Ryther, 1962) modified by addition of H_2SeO_3 (10^{-8}M) and by reducing the concentration of $CuSO_4$ to 10^{-8}M. Vineyard Sound seawater (0.2 µm filtered, 31‰ salinity) was used as the medium base. Throughout the experiments, irradiance of *c.* 250 $\mu Em^{-2}s^{-1}$ was provided by cool white fluorescent bulbs on a 14:10 h light:dark cycle.

Temperature Experiment with Semi-continuous Cultures

Effects of temperature on toxin content, toxin composition, protein content and cell cycle phase lengths were studied using a temperature-gradient bar (Watras *et al.*, 1982), in which a range of seven different temperatures (8 °C, 10 °C, 11 °C, 13 °C, 15 °C, 17 °C and 20 °C) was established (Table 1). Twenty-eight 25 × 150 mm culture tubes containing 25 ml of sterile filtered f/2 medium were inoculated with exponentially growing cells and acclimated, four per treatment, to each temperature over two transfer cycles. Growth was monitored throughout the experiment using *in vivo* fluorescence measurements obtained on a Turner Designs model 10 fluorometer (Turner Designs, Sunnyvale, CA). Semi-continuous cultures were established after specific growth rates were determined for the different temperatures. Following the second transfer, these rates were used as the respective daily dilution rates of fresh medium for each treatment in order to maintain a constant cell density in the cultures over a period of 12 days. The cell densities ranged from 6000 cells ml^{-1} at 8 °C to about 8000 cells ml^{-1} at 20 °C. At the end of the experiment, the four tubes per temperature were combined, two by two. The resulting two samples per treatment were then harvested and aliquots prepared for toxin, DNA and protein analysis.

Low Phosphate Experiment with Semi-continuous Cultures

To study the effect of phosphate limitation on toxin content, toxin composition, protein content and cell cycle, semi-continuous cultures of *A. fundyense* were established at 20 °C and in f/2 medium in which the concentration of PO_4^{3-} was lowered from 36 µM to 1.8 µM (f/40 [PO_4^{3-}]). Five different dilution rates were applied to the different cultures, four per treatment, in order to provide five different phosphate limited growth rates (0.5, 0.25, 0.1, 0.075 and 0.05 divisions per day) (Table 1). Growth was monitored throughout the experiment using *in vivo* fluorescence measurements obtained on a Turner Designs model 10 fluorometer. After two transfers the cultures were kept at a cell density of approximately 3000 cells ml^{-1} for a 12-day period, following which the four tubes per dilution rate were combined, two by two. The resulting two samples per treatment were then harvested and aliquots prepared for toxin, DNA and protein analysis.

Flow Cytometric Analysis of DNA

Ten milliliter aliquots of culture were preserved with 5% formalin (v/v) and stored at 4 °C. The cells were centrifuged (1700 × *g*, 23 °C, 3 min) and the pellet resuspended in 10 ml of ice cold methanol and stored at 4 °C to extract chlorophyll. The samples were then further concentrated by centrifugation. A subsample of the methanol-preserved material was transferred into a 1.5 ml microcentrifuge tube and pelleted at 2000 rev min^{-1}. The supernatant was removed and the cells washed with 1 ml PBS (40mM Na_2HPO_4, 22mM KH_2PO_4, 85mM NaCl). After centrifugation, the pellet was resuspended in 0.5 ml propidium iodide (PI) staining solution (4 µg ml^{-1} PI, 1250 units RNaseA ml^{-1}) and allowed to react in the dark for at least 2 h prior to analysis on an Epics V flow cytometer (Coulter Electronics, Hialeah, FL) (Olson *et al.*, 1986). The proportions of cells in G_1, S and G_2 + M were determined as described elsewhere (Cetta and Anderson, 1990). The duration of the cell cycle stages was calculated using the equations of Slater *et al.* (1977):

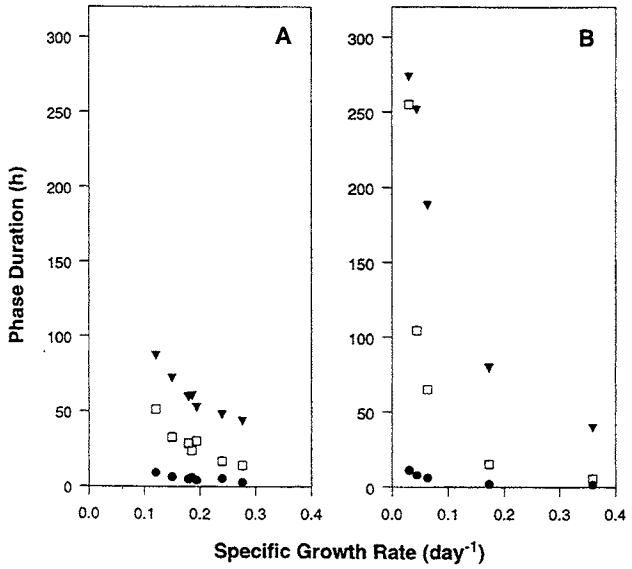

Figure 1. Cell cycle phase durations in *Alexandrium fundyense* at different growth rates in semi-continuous culture, under nutrient replete or phosphate-limited conditions. (G_1 ▼, $G_2 + M$ □, S ●). (A) Cells grown in f/2 medium at seven different temperatures ranging from 8 to 20 °C (see Table 1 for correspondence of μwith temperature of treatment). (B) Cells grown at different levels of phosphate starvation (see Table 1 for correspondence of μ with phosphate depletion regimes)

$$T(G_1) = -(t_D/\ln 2)\ln(1 - [P(G_1)/2])$$

$$T(S) = (t_D/\ln 2)\ln(1 + P(S)/[1 + P(G_2)])$$

$$T(G_2) = (t_D/\ln 2)\ln(1 + P(G_2))$$

where $T(x)$ is the duration of each cell cycle stage, t_D represents the doubling time of the population under balanced growth conditions and $P(x)$ is the proportion of cells in each specific cell cycle stage.

Toxin Analysis

Between 1.5 and 2.5×10^5 cells were collected by centrifugation ($3000 \times g$, 23 °C). The resulting pellet was rinsed into a centrifuge tube and resuspended in 0.5 M acetic acid for extraction by sonification. The extracts were stored at −20 °C prior to analysis. The extracts were analyzed by HPLC (Oshima *et al.*, 1989) incorporating the modifications previously described by Anderson *et al.* (1994).

Soluble Protein

Approximately 30 000 cells were collected by centrifugation ($3000 \times g$, 23 °C) for protein quantification. After aspiration of the supernatant, the pellet was stored frozen at −20 °C. For analysis, 1 ml of 0.1 N NaOH was added to the pellet and the suspension sonified for 15 s to disrupt the cells. Samples were then digested for 20 min at 80 °C, centrifuged, and the supernatant removed and neutralized with 0.1 volumes of 1.0 N HCl (Binder and Anderson, 1990). A 50 μl fraction of the neutralized solution was mixed with 1 ml of bicinchoninic acid (BCA) working solution (Pierce, Rockford, IL), and the absorbance of duplicate samples determined at 562 nm.

The following abbreviations are used throughout this text: STX = saxitoxin; NEO = neosaxitoxin; GTX 1,2,3, 4,5 = gonyautoxins I, II, III, IV and V; C2 = toxin C2; G_0, G_1 and G_2 = Gap 0, Gap 1 and Gap 2 phases of the cell cycle; S = synthesis phase; M = mitosis.

RESULTS

Growth and Cell Cycle Dynamics

The growth rate of *A. fundyense* decreased as temperatures decreased (Table 1). The slope obtained from a linear regression of growth rate against temperature ($a = -1.65$; $R^2 = 0.93$) matched the slope of a theoretical curve ($a = -1.67$) representing a doubling of growth rate with a 10 °C increase in temperature (i.e. a Q_{10} of 2; figure not shown). Decrease in growth rate was associated with an increase in duration of the different cell cycle stages, G_1 and $G_2 + M$ showing the most variation relative to the respective increase in generation time and S the stage showing the least (Figure 1A).

In the phosphate-limited cultures, growth rates decreased as phosphorus supply rates decreased due to reductions in the dilution rate of the cultures (Table 1). Discrepancies between the dilution rates and actual growth rates are a result of variations associated with pipetting the small dilution volumes. Only the empirically determined growth rates were subsequently used for calculations. Similar to the temperature experiment, decreases in growth rate also resulted in a lengthening mostly of G_1 and $G_2 + M$ relative to the increase in generation time (Figure 1B).

In all temperature and phosphate limitation treatments, G_1 was always the phase with the longest duration. The length of G_1 was comparatively longer in the phosphate depletion experiment than in the temperature treatments (Figure 1A and B). As an example, at 11 °C and with a specific growth rate of 0.18, G_1 was 59, $G_2 + M$ 28 h and S 4.7 h long, while at a growth rate of 0.17 under phosphate limitation, G_1 was 79 h, $G_2 + M$ 15 h and S 1.8 h long (Figure 1).

Variations in Toxin Content

Toxin content was higher at lower growth rates for both experiments and was higher under phosphate limitation compared to the replete medium conditions (Figure 2A).

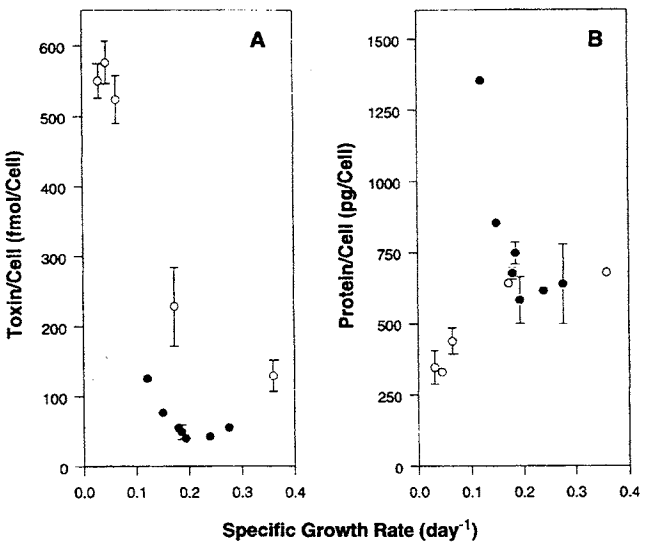

Figure 2. Changes in (A) total toxin levels and (B) protein concentrations in *Alexandrium fundyense* grown in semi-continuous culture under temperature-limited conditions in f/2 medium (●) or under phosphate-limited conditions (○). Error bars represent SE

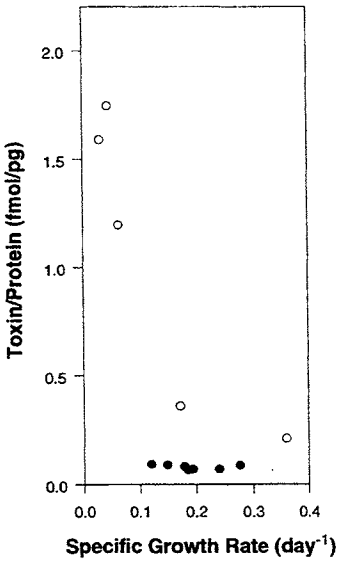

Figure 3. Ratio of toxin/protein contents in *Alexandrium fundyense* at different growth rates in semi-continuous culture and under temperature-limited conditions in f/2 medium (●) or under phosphate-limited conditions (○)

In the temperature experiment, and over the range of temperatures studied (20 °C → 8 °C), the toxin cell quota increased by a factor of 2.1. Over the range of growth rates studied under phosphate-limiting conditions (0.36 → 0.031), the toxin cell quota increased by a factor of 4.8. Toxin content under phosphate limitation was approximately four times as high as in some of the treatments in the temperature experiment at the same growth rate. These toxin levels and differences in toxicity between nutrient-replete and phosphate-limited treatments coincide with observations by other authors (Boyer *et al.*, 1987; Anderson *et al.*, 1990b).

Variations in Protein Concentrations

Figure 2B shows the variability in protein concentrations for cells grown at different temperatures. Similar to what was observed for toxin content (Figure 2A), the levels of protein increased as growth rate decreased. A different pattern was revealed in the phosphate-limited cultures (Figure 2B). In this case the protein levels decreased at lower growth rates. At rates between 0.2 and 0.4 day^{-1} the levels of protein, 649 ± 87 pg cell^{-1}, were similar between the two experimental treatments and coincided with values previously reported in the literature (Boyer *et al.*, 1987; Anderson *et al.*, 1990b).

Correlation between Toxin and Protein Contents

The ratios of toxin content to protein per cell were determined for each treatment. In the temperature experiment this ratio was constant at a value of 0.079 ± 0.006 fmol of toxin per pg of protein (Figure 3). The slope of the linear regression through the data, a = 0.013, was not statistically different from zero ($p > 0.95$, C.I. 93%). This ratio was not constant in the phosphate-limited cultures but increased as growth rate decreased ($p < 0.005$) (Figure 3).

Correlation between Toxin Contents and Cell Cycle Phase Durations

Cell cycle phase durations and toxin content were normalized in both experiments against the lowest values of the respective data series. These normalized values showed a clear overlap in the relative changes of G_1 duration and toxin content (Figure 4). A linear regression through the normalized data resulted in a slope of a = 0.97 ($R^2 = 0.98$). In both experiments the relative increases in duration of G_2 and S are different from the concurring relative changes in toxin content. In this case the slopes were a = 0.46 ($R^2 = 0.97$) and a = 0.1 ($R^2 = 0.88$) respectively.

Toxin Production Rates

Four different net toxin production rates (fmol cell^{-1} h^{-1}) were calculated for each of the experimental treatments. (Note that production rate is used as an equivalent term for toxin accumulation since we do not know the extent of toxin leakage or catabolism.) First, the overall net toxin production rates were determined (Anderson *et al.*, 1990b; Taroncher-Oldenburg *et al.*, 1997). Under steady-state conditions the specific rate of

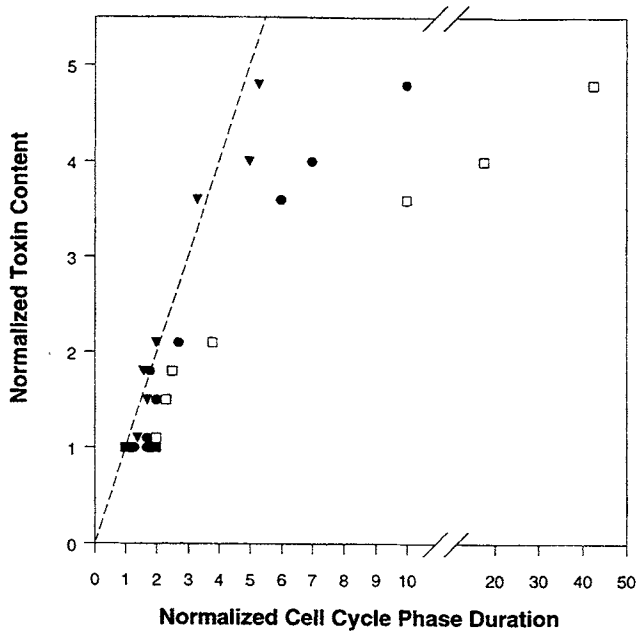

Figure 4. Relative changes in cell cycle stage durations of G_1 ▼, $G_2 + M$ □, S ● against toxin content, for *Alexandrium fundyense* in semi-continuous culture (dashed line represents 1:1 correlation). Data from experiments under temperature-limited conditions in f/2 medium and under phosphate-limited conditions are pooled. Data were normalized against the lowest value of the corresponding series (Figure 2)

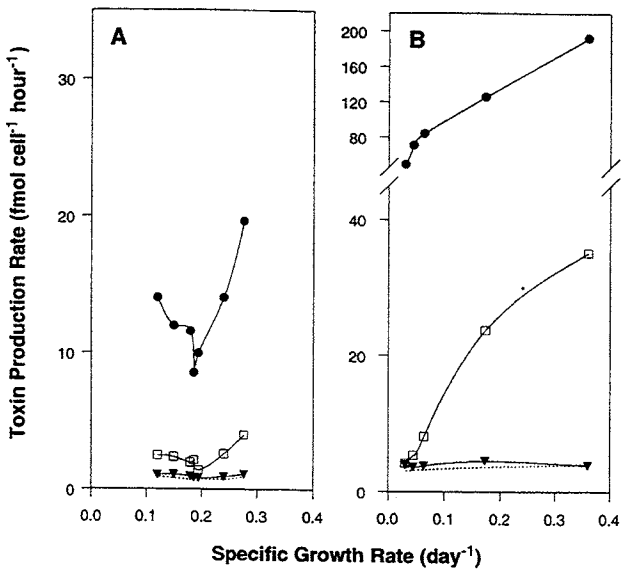

Figure 5. Overall net toxin production rate, R_{Tox} (.....), and cell cycle stage specific toxin production rates, $R_{Tox}(G_1)$ (–▼–), $R_{Tox}(G_2 + M)$ (–□–) and $R_{Tox}(S)$ (–●–), for *Alexandrium fundyense* in semi-continuous culture under (A) temperature-limited conditions in f/2 medium or (B) phosphate-limited conditions. Note scale changes on ordinates

toxin production must equal the specific growth rate of the organism, resulting in a constant toxin concentration per cell. Hence, the net toxin production rate per cell becomes a function of the doubling rate of the organism and the steady state toxin concentration. This can be expressed with the following equations:

$$\mu_{Tox} = \mu$$

where μ_{Tox} (h^{-1}) is the specific toxin production rate and μ the specific growth rate (h^{-1});

$$R_{Tox} = k[Tox] = (\mu/\ln 2)[Tox] = (\mu_{Tox}/\ln 2)[Tox]$$

where R_{Tox} (fmol cell^{-1} h^{-1}) is the net toxin production rate, k (h^{-1}) is the doubling rate and [Tox] (fmol cell^{-1}) represents the toxin cell quota.

We further determined the three cell cycle stage-specific net toxin production rates, i.e. the rates at which toxin would be synthesized if all production occurred exclusively during one of the three phases of the cell cycle (G_1, S or $G_2 + M$). These rates are described by the following equation:

$$R_{Tox}(x) = [Tox]/T(x)$$

where $R_{Tox}(x)$ is the net toxin production rate for each cell cycle stage ($x = G_1$, S or $G_2 + M$) and $T(x)$ is the duration of each cell cycle stage.

The values of R_{Tox} (fmol cell^{-1} h^{-1} ± SD) remained constant at a mean value of 0.77 ± 0.12 for all of the experimental temperatures (Figure 5A). The slope of the linear regression through the data, a = −0.01, was not statistically different from zero ($p > 0.94$, C.I. 96 %). A similar pattern was observed for the five phosphate depletion regimes that resulted in a mean value of 3.34 ± 0.20 (Figure 4B). A linear regression through the data also resulted in a slope, a = 0.06, that was not significantly different from zero ($p > 0.89$, C.I. 92%). The results for $R_{Tox}(x)$ (fmol cell^{-1} h^{-1} ± SD) for both experiments are plotted in Figure 5A and B. $R_{Tox}(G_1)$ was constant with a mean value of 0.94±0.21 in the temperature experiment (Figure 5A) and 3.56 ± 0.43 in the phosphate depletion treatments (Figure 5B). $R_{Tox}(S)$ and $R_{Tox}(G_2 + M)$ showed variations within and between experiments. Linear regressions through these data showed that the slopes of $R_{Tox}(S)$ and $R_{Tox}(G_2 + M)$ were significantly different from zero in both experiments ($p < 0.005$ for all four curves). The slopes of $R_{Tox}(G_1)$ were a = −0.01 for the temperature experiment and a = 0.05 for the range of phosphate limitations. Both slopes were statistically not different from zero ($p > 0.97$, C.I. 95% and $p > 0.9$, C.I. 93 % respectively).

Variations in Toxin Composition

The changes in concentrations (fmoles per cell) and

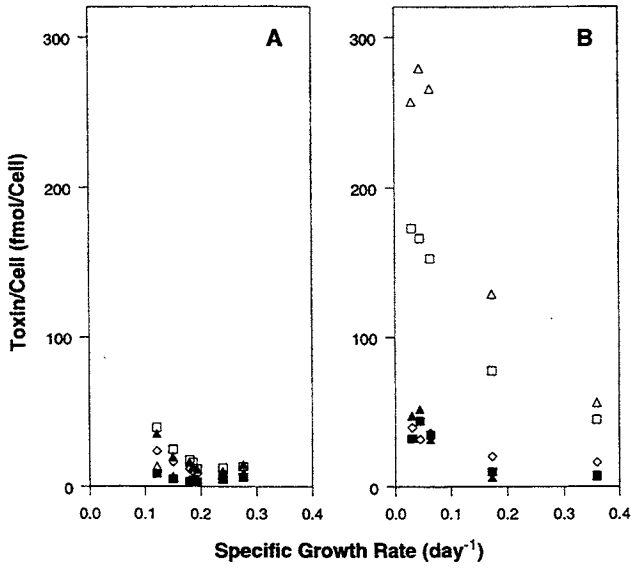

Figure 6. Toxin composition expressed in fmoles per cell of the different toxin derivatives present in *Alexandrium fundyense* cells in semi-continuous culture and under temperature limited conditions in f/2 medium (A) or under phosphate limited conditions (B). (□ C I,II, ■ GTX I,IV, △ GTX II,III, ▲ NEO, ◇ STX).

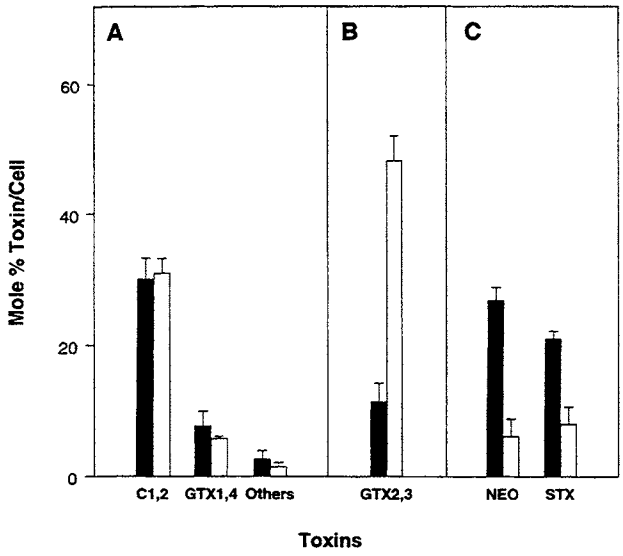

Figure 7. Toxin composition expressed in mole per cent of total toxin present in *Alexandrium fundyense* cells in semi-continuous culture and under temperature-limited conditions in f/2 medium (■) or under phosphate-limited conditions (□). The relative proportions of the different toxins fall within three distinct groups by either (A) remaining constant, (B) increasing or (C) decreasing under phosphate limitation. Error bars represent SD; $n = 7$ for the temperature experiment, $n = 5$ for the phosphate treatments

relative proportions (mole per cent of total toxin) of the eight main saxitoxin derivatives (STX, NEO, GTX I,IV, GTX II,III, C I,II) were determined for the different growth rates in both experiments. Variations in concentration of STX derivatives within each experiment were proportional to the changes in total toxin concentration, resulting in constant relative proportions of the toxins for each of the two experiments (Figure 5). Differences were only observed in the absolute concentrations and mole per cent of the different derivatives between treatments. The concentrations of C I,II and GTX II,III were higher by at least an order of magnitude in cells grown under phosphate starvation compared to cells at similar growth rates in replete medium (Figure 6). At similar growth rates, no differences in concentration of the other toxins were observed between both experiments (Figure 6). Analysis of the relative proportions provided a different picture. The percentages of C I,II, GTX I,IV and the traces of other derivatives (Others, Figure 7) did not vary significantly. The relative proportions of GTX II,III increased significantly under phosphate stress ($12\% \pm 3 \rightarrow 48\% \pm 4$) while NEO and STX decreased from $27\% \pm 2$ and $21\% \pm 1$ in the temperature experiment to $6\% \pm 3$ and $8\% \pm 2$, respectively under phosphate limitation (Figure 7).

DISCUSSION

In the past, toxin production in *Alexandrium fundyense* has been correlated to several environmental factors including nutrient availability, salinity and temperature (Proctor *et al.*, 1975; White, 1978; Hall, 1982; Boyer *et al.*, 1987; Ogata *et al.*, 1987; Anderson *et al.*, 1990b; Matsuda *et al.*, 1996). Only recently has the correlation of toxin production with a specific physiological process, the cell cycle, been described (Taroncher-Oldenburg *et al.*, 1997). Those studies showed that in synchronized batch cultures STX biosynthesis is turned on only during the G_1 phase of the cell cycle. In this article we describe a series of independent experiments showing that toxin content is directly correlated to the length of the cell cycle and, more precisely, to the duration of the G_1 phase of the cell cycle. Semi-continuous cultures of *Alexandrium fundyense* were utilized as they allowed the effects of growth limitation on toxin production dynamics to be analyzed while the extracellular conditions to which the cells had adapted remained stable. In continuous cultures, as well as semi-continuous cultures, the growth rate of an organism is determined by the rate of supply of the limiting nutrient (Monod, 1950; Fogg, 1965).

In one case, we examined the extent to which temperature-induced changes in growth affected toxin production and its linkage to cell cycle events under nutrient-replete conditions. In contrast, phosphate-limited treatments were established to study the effects of nutrient-limited growth on cell proliferation, cell cycle dynamics and toxin production at constant temperature. Phosphate limitation is the only known instance, besides

low temperature, in which saxitoxin biosynthesis is enhanced compared to non-limiting conditions (Hall, 1982; Boyer *et al.*, 1987; Anderson, 1990).

The influence of phosphate availability and temperature on toxin and protein production together with cell cycle dynamics and their relationships are discussed below.

The Cell Cycle

Over the range of growth rates studied, we observed differing effects of temperature limitation and phosphate starvation on the lengths of the G_1 and G_2 phases of the cell cycle. As was observed for the dinoflagellate *Amphidinium carterii* over a range of decreasing temperatures (Olson *et al.*, 1986), the length of both G_1 and G_2 increased with decreasing growth rate in *A. fundyense* (Figure 1A). A similar pattern was observed under phosphate limitation. In this case, the length of G_1 was greater and the G_2 phase was shorter at all growth rates compared to temperature-limited growth (Figure 1A and B). Similar patterns of proportional or general expansion of all cell cycle phases under growth limiting conditions have been observed in mammalian systems and yeast (Rivin and Fangman, 1980; Guiget *et al.*, 1984). The mechanistic explanation is that both energy and nutrient limitation act on processes that control cell cycle progression (e.g. DNA replication and protein biosynthesis). Thus, temperature limitation causes a general decrease in enzyme activity, resulting in lower growth rates and, in the case of *A. fundyense*, a non-proportional expansion of the different cell cycle stages.

Under phosphate limitation we observed not only a longer duration of the G_1 phase, but also a disproportional expansion of the different phases. The most plausible explanation for these patterns is that phosphate is an essential building block of DNA and hence is a limiting factor for cell cycle progression when present at low concentrations. This suggests that the mechanism controlling cell cycle length under phosphate limiting conditions is a delay of the transition from G_1 to the S phase as the cell needs additional time to accumulate enough phosphate to ensure a successful DNA replication process (Pardee *et al.*, 1978). At lower growth rates induced by more severe phosphate limitation, a second mechanism might become operative. Phosphate is not only an essential element for DNA synthesis, but is also a necessary component of energy transfer, via ATP and related compounds, and is critical to the regulation of protein activity by means of enzyme phosphorylation. At extremely low phosphate concentrations, protein metabolism in preparation for mitosis would be restricted, lengthening G_2. Low protein levels at slow growth rates corroborate this interpretation (Figure 2B). Temperature limitation and phosphate limitation thus affect the cell cycle dynamics in different ways by modulating separate regulatory machineries.

Toxin Production and Nitrogen Pools

Saxitoxin biosynthesis is dependent on the availability of the amino acid arginine (Arg), its main building block (Shimizu, 1993). Arg is one of the principal constituents of proteins and its metabolism is regulated by temperature and nutrient levels, among other factors, especially nitrogen and phosphate (Meister, 1965). Similarly, STX biosynthesis is dependent on factors such as the nitrogen status of the cells and phosphate availability in the medium (Hall, 1982; Boyer *et al.*, 1987; Anderson *et al.*, 1990b; Matsuda *et al.*, 1996). It thus became of interest to look at the correlation between toxin levels and protein accumulation under the different experimental treatments in this study. If toxin and protein production were directly correlated, the ratio of the two components would remain constant under all physiological conditions. We saw such a pattern in the temperature experiment, but not with phosphate limitation, where toxin concentrations became progressively uncoupled from protein concentrations in the cell as the limitation increased (Figure 3). This ratio distribution suggests selective maintenance of enzymes of toxin biosynthesis at low phosphate concentrations. It can thus be hypothesized, that toxin production is advantageous to *Alexandrium* as it becomes progressively phosphate-limited.

Previous work has shown that during exponential growth of *Alexandrium*, pools of Arg do not show any significant differences between phosphate-limited and nutrient-replete cultures (Anderson *et al.*, 1990b). One can surmise that protein and toxin production could be inversely related as a result of protein catabolism and subsequent channeling of Arg through the general Arg pool into STX anabolism. Such a scenario would be supported by the fact that cellular levels of Arg as well as those of other amino acids are under tight control in order to preclude high accumulations detrimental to the cell's physiology (Meister, 1965). However, analysis of the variations in toxin, protein and Arg content at low temperatures reveals a direct correlation between these factors (Anderson *et al.*, 1990b) (Figure 3), suggesting that the regulatory pathway of STX biosynthesis does not represent a straight recycling of protein-derived Arg into an alternative storage compound, the toxin. Accumulations of protein and STX at low temperatures in this study show the same direct proportionality (Figures 2A and B). We conclude that toxin production is not a direct response to a cell's need to balance its nitrogen pools, but the result of a specific pathway integrated in the general nitrogen metabolism of the cell.

Toxin Production and the Cell Cycle

Our previous work with synchronized cultures in nutrient-replete medium has shown that STX biosynthesis is turned on during the G_1 phase of the cell cycle (Taroncher-Oldenburg *et al.*, 1997). The main hypothesis we wanted to test with the experiments reported here was whether toxin production and the levels of toxin accumulation were related to one specific cell cycle stage or another under growth limiting conditions. We first set out to determine if levels of STX accumulation, duration of the different cell cycle stages and the deduced toxin accumulation rates for each stage were correlated. First we compared the relative increases in toxin content and cell cycle phase duration for both experiments (Figure 4A and B). A clear overlap was observed between the trends and values of relative durations of G_1 and the normalized toxin concentrations. These results clearly indicated that toxin production was related to the length of the G_1 cell cycle phase. We then went on to determine the cell cycle stage-specific, as well as overall net, toxin production rates. A comparison of these parameters showed similar patterns and values for the net and the G_1-specific production rates (Figure 5A and B). The other two cell cycle-specific rates ($G_2 + M$ and S) showed not only a high degree of uncoupling from the overall rates, but their trends also contradicted basic concepts in metabolic regulation. Typically, the end product of a biosynthetic pathway accumulates as a function of enzymatic rates and the length of the time interval during which synthesis occurs. The two extreme cases result in two different scenarios. In the first case, high product concentrations are derived from increases in production rates as a result of biochemical induction of the responsible enzymes. Alternatively, enzymatic rates can remain constant and accumulation of the end product can be the consequence of longer periods of biosynthetic activity. This appears to be the case with STX production, as shown by our results (Figure 5). In both the temperature treatments and the phosphate limitation experiments, toxin production rates remained constant for a range of conditions, implying that the variations in toxin content observed (Figure 2A) were a result of increasing periods of biosynthetic activity. This increase in the length of the toxin production was directly proportional to the changes in duration of the G_1 phase of the cell cycle.

The G_2 and S specific toxin production rates fall into a third category in which an increase in metabolite concentration would be related to an actual decrease in production rates. Such dynamics are to be expected only in the case of a storage product where regulation of biosynthesis is not coupled to metabolite accumulation. One of the possible roles postulated in the past for saxitoxin is as a storage compound for nitrogen (Loeblich, 1984; Boyer *et al.*, 1987; Anderson *et al.*, 1990b). Our data show a coincidence of decreasing protein concentrations with an increase in toxin levels under phosphate limitation (Figure 2), which would support such a hypothesis, considering that nitrogen was not a limiting factor in those cultures and protein anabolism was probably compromised by the lack of phosphate. However, work by other authors showing no evidence for reallocation of toxin-nitrogen into protein-nitrogen (Hall, 1982; Boyer *et al.*, 1985; Usup, 1995; Flynn *et al.*, 1996), together with our observation of a parallel increase of toxin content and protein levels under the different temperature limitation regimes (Figure 2A and B), contradicts such a theory.

Toxin Production and Phosphate Availability

The role of phosphate in a cell's metabolism is a complex one. The physiological changes we observe under different phosphate starvation regimes are the result of the cumulative effects of phosphate limitation on the entire range of physiological processes in the cell. We observed that both the net and the G_1-specific toxin production rates increased by a factor of approximately four in all phosphate-limited regimes compared to the nutrient replete ones over the same range of growth rates (Figure 5A and B). This confirmed previous toxin data comparisons for phosphate-limited cultures of *Alexandrium* where toxin levels were consistently higher, also by a factor of four (Boyer *et al.*, 1987; Anderson *et al.*, 1990b). This enhancement of toxin production suggests that phosphate starvation is not acting directly on the biosynthetic pathway but rather increases the rate of substrate supply via down-regulation of other cellular processes (Anderson *et al.*, 1990b). Moreover, the rate of toxin production remains at a constant level throughout the range of phosphate limitation regimes (Figure 5B). This suggests that the rate of approximately 3.3 fmol $cell^{-1}\ h^{-1}$ is the maximum rate of toxin production under phosphate stress for *Alexandrium fundyense*.

It is of interest that phosphate deprivation does not limit toxin production, i.e. no reduction in the toxin production rate is observed (Figure 5A and B), which implies that phosphate is not an essential cofactor or substrate in saxitoxin biosynthesis. This is further corroborated by the observation that toxin production is uncoupled from protein accumulation under phosphate starvation (Figure 3). Protein metabolism is dependent on phosphate availability. If STX biosynthesis were phosphate dependent we should observe a similar pattern of variation as the one recorded for protein metabolism. Instead, opposite trends were followed (Figure 2B).

We conclude that STX biosynthesis is not directly regulated by phosphate, but is indirectly modulated by

the effects of phosphate starvation on other cellular processes, such as protein and amino acid metabolism.

Toxin Production Rate and Growth Rate

The results reported here show that net toxin production rate is independent of growth rate under the conditions studied. Net toxin production rates had been previously determined under phosphate limitation in *A. fundyense* (Anderson, 1990). The average toxin production rate was of approximately 75 fmol cell^{-1} day^{-1} (=3.12 fmol cell^{-1} h^{-1}). This value coincides with our value of 3.34 fmol cell^{-1} h^{-1}. In that instance the toxin production rates were suggested to be correlated to growth rate based on their increase at faster growth rates. The discrepancy between those data and the results reported here may lie in the high variability seen in the first case. This is further substantiated by the fact that we observed constant toxin production rates in nutrient-replete cultures and under varying temperatures. Toxin production rates have also been shown to be constant over a range of nitrogen limiting conditions (Anderson *et al.*, 1990b). These data suggest that once a specific limitation threshold is reached, toxin production rates are not affected by further limitation. This has important implications regarding the production of STX as it suggests possible regulatory mechanisms of the biosynthetic pathway. A more precise biochemical interpretation of the data is not possible at this point because none of the enzymes involved in the pathway has been isolated yet. A concerted effort in determining the exact sequence of intermediates and the nature of the enzymes responsible for their processing is necessary in order to overcome this last hurdle of understanding the regulatory mechanisms involved in the biosynthesis of STX.

Toxin Composition

Toxin composition in *Alexandrium* had long been considered a stable feature of the different species or strains throughout a broad range of physico-chemical conditions (Hall, 1982; Boyer *et al.*, 1987; Ogata *et al.*, 1987) and therefore used as a chemotaxonomic character (Cembella *et al.*, 1987). Only more recently have changes in toxin composition over the life cycle or under varying physiological conditions been documented (Boczar *et al.*, 1988; Anderson, 1990; MacIntyre *et al.*, 1997). Our results add one more example in which a significant change in toxin composition was observed when *Alexandrium* was subjected to phosphate-limited conditions and grown in semi-continuous culture in nutrient replete medium (Figure 6). The most significant changes occurred at the level of absolute accumulation of the carbamate derivative GTX II,III and the sulfocarbamoylated pair C I,II (Figure 6). No significant differences were observed for the total concentrations of the two other carbamate derivatives, GTX I,IV, NEO or STX (Figure 6). Transformations among STX derivatives, in particular 11-α-β-*O*-sulfatation of STX and 16-*N*-sulfatation of the resulting pair GTX II,III, are enhanced by phosphate starvation, as previously shown by Anderson *et al.* (1990a). In a similar scenario to the one discussed in the context of enhancement of total toxin production under phosphate limiting conditions, the apparent increase in sulfatation activity is most probably the result of an increase in substrate availability for these enzymes, rather than an induction of enzyme activity. The characterization of the two enzymes responsible for these reactions in *Gymnodinium catenatum* showed that their activities were not dependent on the presence of phosphate (Yoshida *et al.*, 1996). We can further explain the lack of a parallel and significant change in the total concentration of the pair GTX I,IV, via the equivalent transformation of the 1-*N*-hydroxy STX derivative series, by the fact that an 11-β-β-*O*-sulfatation activity of NEO has not yet been isolated, and that the two sulfatases of *G. catenatum* do not recognize NEO or the pair GTX I,IV as substrates (Yoshida *et al.*, 1996). A plausible explanation for the observed patterns would therefore be the presence of specific 1-*N*-hydroxylases or oxidases of the kind observed before in *Alexandrium tamarense* (Oshima, 1995) that would in turn transform STX and GTX II,III into NEO and GTX I,IV respectively. Such enzymes are always ATP dependent and hence inhibited in the absence of phosphate.

The resulting scheme of transformations is outlined in Figure 8. It is necessary to point out that the arrows follow opposite directions from those we had postulated in previous experiments with synchronous cultures (Taroncher-Oldenburg *et al.*, 1997), but a comparison of both sets of observations after reinterpretation of the toxin accumulation patterns in those synchronous cultures results in convergence. In that instance, we observed first an accumulation of C I,II followed by GTX II,III and STX and finally GTX I,IV and NEO with a slight delay. We interpreted this to be the sequence in which these compounds were formed (i.e. that C I,II were the 'parent' compounds). These data can now be reinterpreted in light of our current results as a process during which C I,II accumulates as a result of the rapid transformation of its direct precursor, GTX II,III, until its equilibrium concentration is reached. This then results in the subsequent accumulation of GTX II,III until its concentration reaches a maximum and STX, the precursor of this latter transformation starts building up. The hydroxylases/oxidases involved in the transformation of STX and GTX II,III into NEO and GTX I,IV only start showing higher activities once the sulfatation reactions that are competing for their substrates stabilize, suggesting that the K_m of the sulfatases is lower than that of the hydroxylases/oxidases.

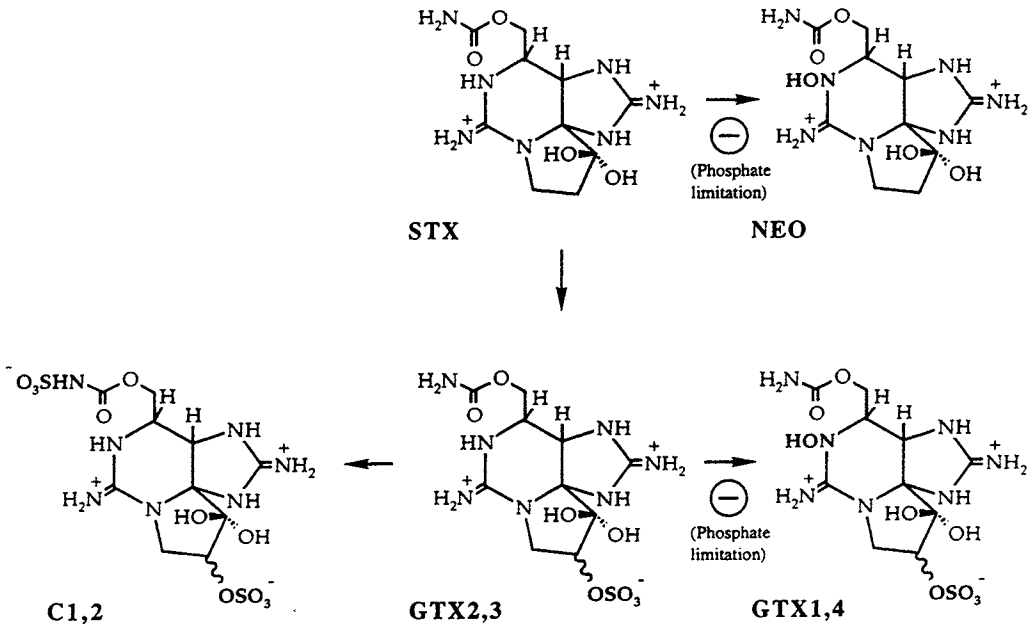

Figure 8. Suggested scheme of interconversions among the main STX derivatives synthesized by *Alexandrium fundyense*. The arrows show the most plausible direction for the transformations; ⊖ denotes possible inhibition of specific enzyme activity

One last comment addresses the divergent results obtained when toxin composition is analyzed as absolute values of toxin derivative cell quota or as derivative mole per cent of total toxin in intraspecific comparisons between sets of toxin data where the total toxin content has varied significantly (Figures 6 and 7). In such cases the relative amounts of the different derivatives have to be contrasted with their actual concentrations in order not to distort the real changes in composition occurring in the cells. This is illustrated by our results. In the temperature experiment, all derivatives are loosely grouped at similar levels of concentration, C I,II consistently showing the highest concentration (Figure 6A). In contrast, under phosphate-limiting conditions, GTX II,III are very clearly present at the highest concentration, with C I,II coming second and the rest of the derivatives all clustering at similar concentrations to those observed in the temperature treatments (Figure 6B). These results indicate a pattern of enhanced accumulation of GTX II,III and C I,II under phosphate limitation. Analysis of these same data by normalizing the concentration of each derivative to total toxin provides a different picture (Figure 7). A clear change can still be seen for GTX II,III but the proportion of C I,II remains constant. In addition, NEO and STX also show a significant decrease. These differences between analytical approaches reflect the loss of information associated with representing toxin concentrations as relative amounts of the total toxin. While not invalidating mole per cent representations as a means for comparing different organisms or determining qualitative, and to a certain extent, quantitative variations for one species, our results imply that changes in the physiology of toxin interconversions can only be inferred from the absolute values of the toxin cell quota for each derivative.

Summarizing the toxin composition portion of this study, the patterns of change we observed, together with the fact that within treatments the variation in toxin composition among the different growth rates was minimal, suggest that phosphate starvation affects toxin speciation at the level of hydroxylase/oxidase activities. In an integrated scheme of the STX biosynthetic pathway, interconversions among the toxins would be determined in part by the presence or absence of the appropriate enzymes, but net total toxin production would be the result of the accumulation of only one precursor or parent compound. This precursor could be one of the toxins or another unknown compound (Shimizu, 1993). Further efforts to identify and isolate intermediate compounds are necessary in order to determine the exact sequence of the biosynthetic pathway.

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