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Cell-cycle response to nutrient starvation in two phytoplankton species, *Thalassiosira weissflogii* and *Hymenomonas carterae**

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Abstract

The influence of nutrient deprivation on cell-cycle progression was examined in two phytoplankton species, the diatom *Thalassiosira weissflogii* (actin) and the coccolithophorid *Hymenomonas carterae* (cocco II). The diatom was starved for nitrogen, silicon or both, whereas only nitrogen limitation was examined in *H. carterae*. In both species, nitrogen-starved cells were arrested in the early part of the cell cycle (G_1 phase). In the diatom, silicon-starvation arrested cells in late G_1 phase and also in the last part of the cell cycle ($G_2 + M$). In all cases, cell-cycle arrest could be reversed by addition of fresh medium, but cell-cycling times during the first generation were increased in comparison to those in nutrient replete, steady-state growth conditions. These results supply evidence for simultaneous dual-nutrient limitation of population growth and provide a mechanistic interpretation for the division patterns observed in cultures where nutrients are supplied periodically.

Introduction

Essential nutrients such as nitrogen, phosphorus or silicon often limit phytoplankton growth in natural waters. In the absence of a renewable supply of these nutrients, as in batch cultures, nutrient depletion results in a retardation and eventual cessation of population growth. When a nutrient is periodically supplied, population-division patterns can be entrained (Olson and Chisholm, 1983) in the same way they can be entrained by a periodic supply of light, as is the case with the daily photocycle (Chisholm, 1981). In fluctuating environments with period lengths shorter than phytoplankton generation times, there is a large variability in the conditions which an individual cell encounters during its "life" or cell cycle. Population behavior thus cannot be reduced to that of an "average" cell: one must understand the details of the control exerted by external conditions on the cell cycle in order to be able to interpret patterns of population growth (Chisholm *et al.*, 1984).

The cell cycle is classically divided into four phases, G_1, S, G_2 and M (Mitchison, 1971). The nuclear genome is replicated during the S phase, M corresponds to the period of mitosis and cell division, and G_1 and G_2 refer to "gaps" in the cycle during which most of the cell growth takes place. It has been proposed by Spudich and Sager (1980) that progression through a restricted part of the cell cycle of the phytoplankton species *Chlamydomonas reinhardtii* is dependent on light. This segment is bounded by a transition point beyond which cells can complete their cycle and divide in the dark. This hypothesis has received strong theoretical and experimental support for several species (Chisholm *et al.*, 1984; Donnan *et al.*, 1985; Heath and Spencer, 1985; McAteer *et al.*, 1985; Vaulot *et al.*, 1986), and has been shown to be useful for interpreting cell-division patterns in light/dark cycles (Vaulot and Chisholm, 1987).

Requirements for certain growth factors are also restricted to limited segments of the cell cycle of animal and plant cells (Pardee *et al.*, 1978; Johnston *et al.*, 1980); the location of the segment can vary, depending on the factor (Gould *et al.*, 1981; Olashaw *et al.*, 1983). It is likely that such a mechanism also exists for nutrient limitation of phytoplankton growth. Indeed, Olson *et al.* (1986) and Olson and Chisholm (1986) have shown that when generation times are lengthened due to nitrogen limitation, only the G_1 phase of the cell cycle expands, suggesting that progression through the rest of the cycle does not depend upon the acquisition of nitrogen.

In order to test this hypothesis, the population-growth dynamics of two marine phytoplankton species (a diatom

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and a coccolithophorid) were followed in detail during nutrient starvation. The cell-cycle position of individual cells was monitored, using flow cytometry, as growth was arrested due to nitrogen or silicon depletion and then reinitiated following the addition of fresh medium. The results suggest that the availability of these nutrients controls only certain segments of the cell cycle of the two species under study and that the location of these segments is both species- and nutrient-dependent.

Materials and methods

Culture conditions

Thalassiosira weissflogii (actin) and *Hymenomonas carterae* (cocco II) were obtained from the culture collection of Dr. R. R. L. Guillard (Bigelow Laboratory, Boothbay Harbor, Maine, USA). Both were maintained at 20 °C in batch cultures, under a light intensity of 100 μE m^{-2} s^{-1}; the culture medium was *f*/2-enriched seawater which had been filter-sterilized through a 0.22 μm pore-size filter (Guillard, 1975). For the experiments, batch cultures were grown in *f*/2-enriched seawater with 0.2 m*M* nitrogen as $(NH_4)_2SO_4$. Three concentrations of silicon, as $Na_2SiO_3 \cdot 9\ H_2O$, were used in experiments with *T. weissflogii:* 0.4 m*M* Si (N:Si = 0.5), 0.2 m*M* Si (N:Si = 1) or 0.1 m*M* Si (N:Si = 2).

Cell counting, fixation, and staining

Cells were counted using a Model Z_f Coulter Counter (Coulter Electronics, Hialeah, Florida, USA) and were fixed in methanol for flow-cytometric analysis as described by Vaulot *et al.* (1986). In preparation for staining, cells were centrifuged and rinsed twice out of methanol with phosphate saline buffer (PBS: 10.9 g l^{-1} $Na_2HPO_4 \cdot 7\ H_2O$; 3.9 g l^{-1} KH_2PO_4; 5.0 g l^{-1} NaCl). DNA was stained with either Hoechst 33342 (1 μg ml^{-1}; Sigma Co, St. Louis, Missouri, USA), or propidium iodide (5 μg ml^{-1}; Sigma Co), in the presence of RNase (40 μg ml^{-1}; RASE, Worthington Division) to eliminate RNA-prodidium-iodide fluorescence (Crissman and Steinkamp, 1982).

Flow cytometric measurements

Cells stained with Hoechst 33342 were analyzed on a flow cytometer built around an Olympus BH-2 epifluorescence microscope (Olson *et al.*, 1983). Cells stained with propidium iodide were analyzed using a Coulter Epics V flow cytometer/cell sorter (Coulter Electronics, Hialeah, Florida) as described by Vaulot *et al.* (1986). Side-by-side comparisons showed that the two methods and instruments yielded essentially identical results.

DNA distribution analysis

DNA distributions (Fig. 1) were analyzed to determine the proportion of cells in the different phases G_1, S and $G_2 + M$. The G_1 and $G_2 + M$ fluorescence peaks were modelled as Gaussian distributions and the S phase as a sum of 3 to 5 broadened rectangles, the number of which was determined to cover optimally the S phase. This model is especially suitable for synchronized populations where the proportion of cells in S is large. The optimal parameter set was obtained by a non-linear Marquardt algorithm (P. Dean, personal communication). In *Thalassiosira weissflogii,* $G_2 + M$ included a post-cytokinesis doublet stage during which sister cells remained attached even though wall formation was completed. The duration of this stage was about 2 h (Vaulot *et al.*, 1986). In *Hymenomonas carterae,* the G_1 peak was skewed to the right, in such a way

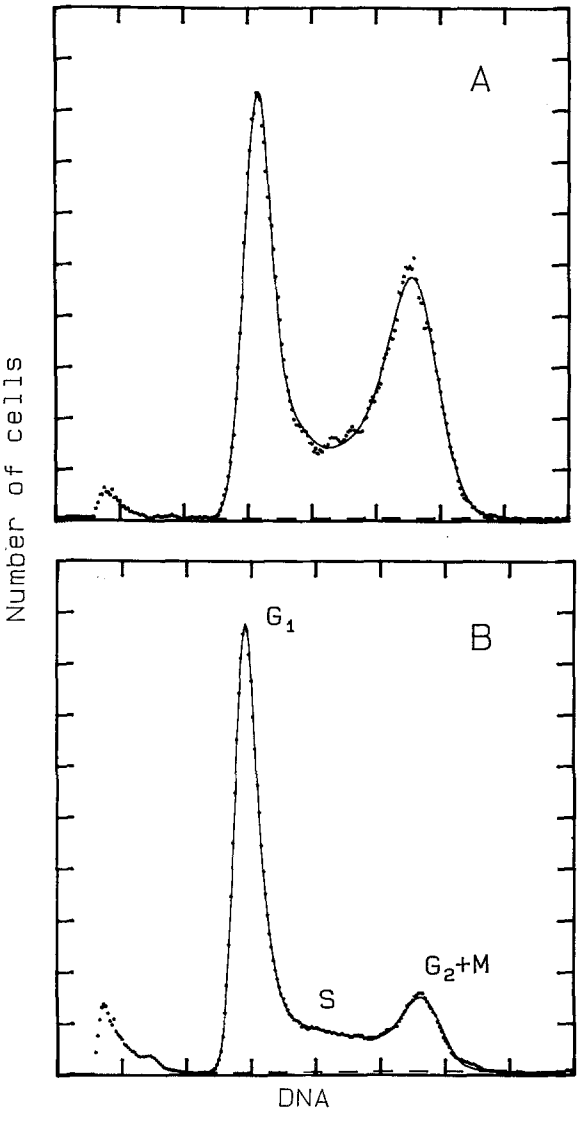

Fig. 1. *Thalassiosira weissflogii* (A) and *Hymenomonas carterae* (B). DNA distributions measured by flow cytometry in exponentially growing populations. DNA was stained with propidium iodide (PI) in the presence of RNase. S corresponds to the replication of nuclear genome, M to mitosis and cell division and G_1 and G_2 to "gaps" in cell cycle. Points represent raw data and line represents distribution reconstructed from results of the analysis (see "Materials and methods – DNA distribution analysis")

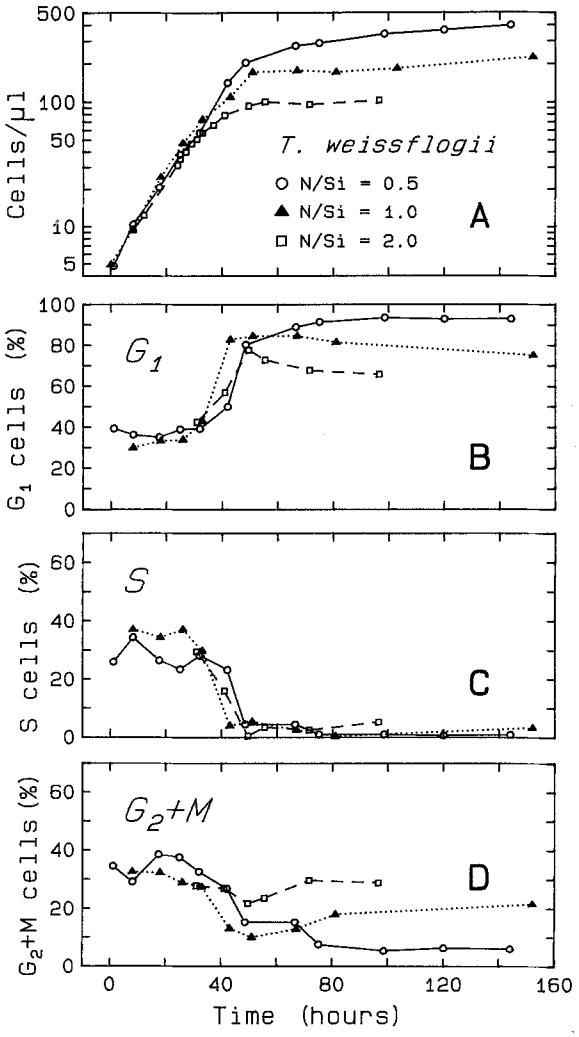

Fig. 2. *Thalassiosira weissflogii.* Growth in batch cultures in three media with increasing N:Si ratios limiting growth for nitrogen alone (N:Si=0.5), nitrogen and silicon (N:Si=1), and silicon alone (N:Si=2). (A) Cell concentration; (B, C, D) proportion of cells in the different cell-cycle phases

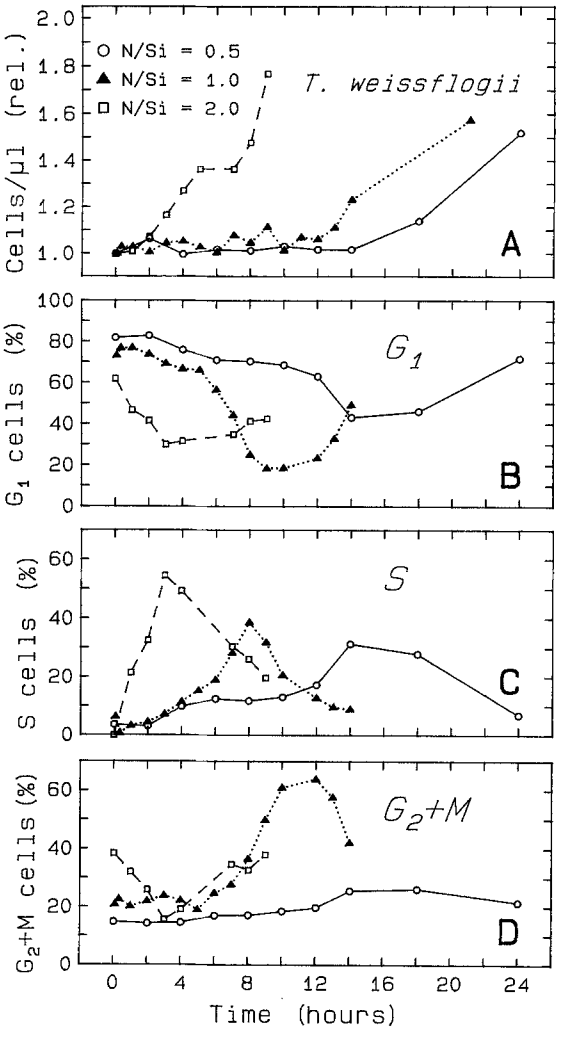

Fig. 3. *Thalassiosira weissflogii.* Stationary-phase cultures diluted with fresh medium at $t=0$ h. (A) Relative cell concentration assuming a cell concentration of 1 at $t=0$ h. (B, C, D) proportion of cells in the different cell-cycle phases

that the analysis method always yielded a relatively large proportion of cells in the early part of the S phase. For reasons described in Vaulot *et al.* (1986), this is believed to be an analysis artifact; these cells were thus assigned to G_1 rather than S.

Results

Thalassiosira weissflogii

The growth of *Thalassiosira weissflogii* was studied in three different media with equal amounts of nitrogen and decreasing amounts of silicon resulting in N:Si ratios of 0.5, 1 and 2. These ratios yielded populations decreasingly starved for nitrogen and increasingly starved for silicon. When the N:Si ratio was equal to 1, simultaneous additions of nitrogen and silicon were necessary to induce growth resumption after the cultures had reached stationary phase (Vaulot, 1985). In contrast to most animal and phytoplankton cells, *T. weissflogii* cells spend only a small proportion of their cell cycle in G_1 during exponential growth (Table 1). In all three media, nutrient limitation began to retard the population division rate and to affect cellular DNA distributions around $t=30$ h, i.e., from one (for N:Si=2) to three (for N:Si=0.5) generations before cell division actually stopped (Fig. 2). The amount of silicon in the medium had, however, no influence on the DNA distributions until the late exponential phase ($t=50$ h, Fig. 2B, C, D). Cells were apparently submitted to two successive depletions: (1) before $t=50$ h, nitrogen-depletion was prevalent and caused a marked increase in the fraction of the cells in G_1 (Fig. 2B); (2) after $t=50$ h, cells began also to experience silicon-depletion, the extent of which was proportional to the N:Si ratio in the medium. The final number of cells arrested in G_2+M, which ranged from less than 10% for N:Si=0.5 to more than 35% for N:Si=2, reflected increasing degrees of silicon-starvation.

Following dilution with fresh medium (Fig. 3), division resumed after a lag. The length of this lag increased with the degree of nitrogen-starvation (Fig. 3A; Table 1). In the

Table 1. *Thalassiosira weissflogii* and *Hymenomonas carterae.* Average duration (h) of cell-cycle phases during exponential growth. Phase lengths were computed from mean generation time and percentage of cells in the corresponding phase, assuming exponential age distribution (Slater *et al.*, 1977; Olson *et al.*, 1983)

Species	Generation time	Length of cell-cycle phases		
		G_1	S	G_2+M
T. weissflogii				
Average ($n=5$)	8.1	2.1	2.7	3.4
H. carterae[a]				
Average ($n=4$)	12.9	7.2	3.1	2.7

[a] In *H. carterae,* the first peak of S obtained by the mathematical analysis of the DNA histograms was included into G_1 (see Vaulot *et al.*, 1986)

Table 2. *Thalassiosira weissflogii* and *Hymenomonas carterae.* Average duration (h) of S phase, lag phase and median time to division after nutrient limitation recovery. Duration of S phase was estimated from delay between rise of cells in S and rise of cells in G_2+M (Figs. 3C, D, 5B). Lag phase was defined as delay between addition of fresh medium and beginning of cell division (Figs. 3A; 5A). Median division time was defined as delay between addition of fresh medium and time at which 50% of the cells had divided (Figs. 3A; 5A); it is to be compared to the mean generation time in exponential growth (Table 1)

Treatment	N:Si ratio	S phase	Lag phase	Median division time
T. weissflogii				
Si-starved	2.0	3	1	9
N/Si-starved	1.0	3	12	18
N-starved	0.5	9	14	24
H. carterea				
N-starved	–	3	12	17

case of silicon-starvation (N:Si = 2), this lag was completely eliminated, since cells arrested in G_2+M began to divide immediately (Fig. 3A). In all cases, DNA synthesis was initiated right after the addition of fresh medium, but the kinetics of population entry into the S phase depended on medium composition (Fig. 3B). In the case of the nitrogen-starved population (N:Si = 0.5), entry into the S phase occurred very gradually and at a constant rate during the lag phase (Fig. 3B), indicating that cells were arrested at different positions throughout G_1. In the case of silicon-starvation (N:Si = 2), cell entry in S was very synchronous (Fig. 3B), indicating that most cells in G_1 were arrested close to the G_1/S boundary. Finally, in the case N:Si = 1, entry in S appeared to be intermediate between the two other cases. Both the transit time through S and the median time between fresh medium addition and division increased with the degree of nitrogen-starvation (Table 2), implying that nitrogen-starvation had less reversible effects on the cell cycle of *Thalassiosira weissflogii* than silicon-starvation.

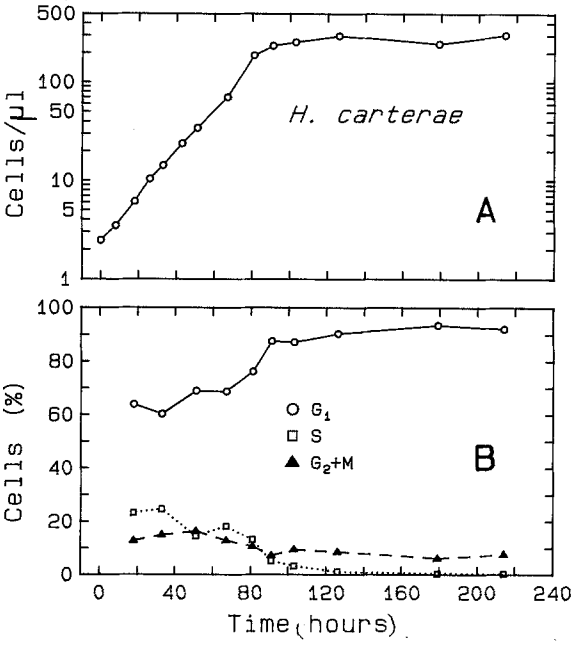

Fig. 4. *Hymenomonas carterae.* Growth in batch culture in nitrogen-limiting medium (0.2 m*M* nitrogen). (A) Cell concentration; (B) proportion of cells in the different cell-cycle phases

Hymenomonas carterae

When *Hymenomonas carterae* was grown in batch culture with 0.2 m*M* nitrogen, the initial phase of exponential growth (Fig. 4A), was followed by entry into stationary phase at 2×10^5 cells ml^{-1}. At this point, resumption of division could be achieved by the addition of nitrogen, proving that growth had stopped as a consequence of nitrogen-starvation (Vaulot, 1985). During exponential growth, cells spent most of their time in the G_1 phase of the cell cycle, as determined by the analysis of the DNA distributions (Fig. 4B; Table 2). The effect of nitrogen-starvation on the cell cycle was noticeable before division stopped: by $t=70$ h, the proportion of cells in G_1 had begun to increase and that of cells in S to decrease. In the stationary phase, most of the cells were arrested in G_1, with only 8% of the cells left in G_2+M and none in S (Fig. 4B).

In order to follow the recovery of nitrogen-starved cells, a culture in early stationary phase was diluted in half with fresh medium ($t=0$ h, Fig. 5). Six hours after medium addition G_1 cells began to synthesize DNA, as indicated by the increase in the number of cells in S at this time (Fig. 5B). This implies that these cells were initially arrested far from the G_1/S boundary at the beginning of G_1, or that the cells "regressed" in G_1 during the starvation period. The S phase of these cells lasted about 3 h, which is close to the transit time realized during exponential population growth (Table 1). The first cells began to divide 12 h after the addition of fresh medium (Fig. 5A), and half of the population had divided by $t=17$ h. Median cell generation-time, therefore, increased by 30% over the unperturbed conditions (Table 1).

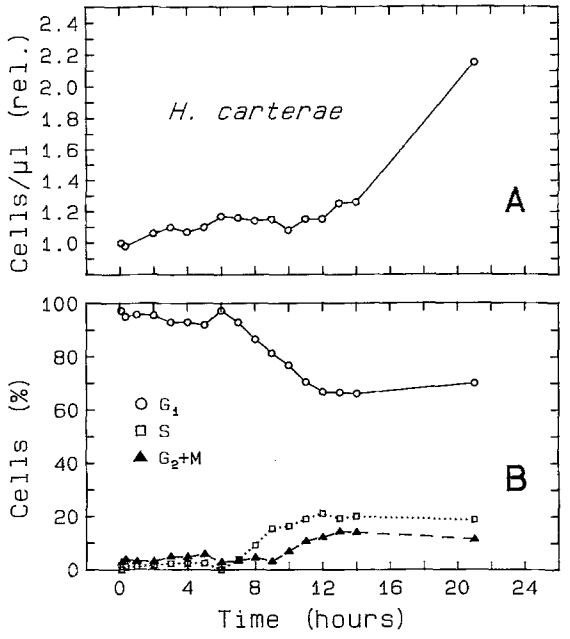

Fig. 5. *Hymenomonas carterae.* Nitrogen-limited stationary-phase culture diluted with fresh medium at $t=0$ h. (A) Relative cell concentration assuming a cell concentration of 1 at $t=0$ h; (B) proportion of cells in the different cell-cycle phases

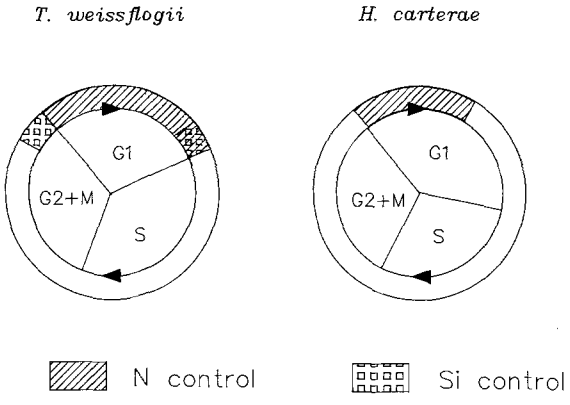

Fig. 6. *Thalassiosira weissflogii* and *Hymenomonas carterae.* Localization of the cell-cycle segments which are dependent upon availability of nitrogen and silicon

Discussion

The cell cycle is composed of two parallel cycles (Mitchison, 1971): the DNA cycle which encompasses genome replication and nuclear division, and the growth cycle which includes the macromolecular synthesis necessary to ensure the doubling of all cell components between two divisions. Since cell populations maintain a limited range of size and composition, it is necessary for these two cycle which includes the macromolecular syntheses necessumed that the growth cycle controls the DNA cycle, although the reverse can also be true (Singer and Johnston, 1985). This control has been hypothesized to be mediated through the attainment of a level of protein (Alberghina and Sturani, 1981) or RNA (Darzynkiewicz *et al.*, 1982) necessary for DNA replication to begin.

In the present study, nitrogen-starvation only perturbed progression through the initial part of the cell cycle G_1 in both *Thalassiosira weissflogii* and *Hymenomonas carterae* (Fig. 6). This confirmed the observations of Olson *et al.* (1986) that changes in steady-state nitrogen-limited growth rate in these species only affected the duration of G_1. It appears, therefore, that nitrogen primarily controls cell growth before DNA synthesis, as might be expected since it is a major component of proteins. The DNA cycle is indirectly affected by nitrogen limitation, since failure to complete G_1 prevents the initiation of DNA synthesis. In *Hymenomonas carterae,* nitrogen-starved cells were blocked far from the G_1/S boundary suggesting that, like some animal cells (Pardee *et al.*, 1986), the coupling between the growth and DNA cycle might be regulated well before the actual beginning of DNA synthesis.

Silicon appears to exert a more direct control on the DNA cycle (Fig. 6). In *Thalassiosira weissflogii,* part of the cell population was arrested at the G_1/S boundary by silicon starvation, reflecting a silicon requirement for the initiation of DNA synthesis. This is most likely related to the established silicon requirement for DNA polymerase and TMP-kinase activity found in the diatom *Cylindrotheca fusiformis* (Darley and Volcani, 1969; Sullivan and Volcani, 1973). The rest of the population was arrested in G_2+M as a result of the post-cytokinesis silicon requirement for frustule formation (Sullivan and Volcani, 1981). The precise localization of silicon control in the cell cycle established here explains why silicon deprivation is such an efficient means of synchronizing diatom cell populations (Darley and Volcani, 1971).

In the intermediate situation, where the N:Si ratio in the medium (1.0) is similar to the average stationary-phase N:Si intracellular ratio, which is different from the exponential-phase one (0.5 according to Brzezinski, 1985), the two controls can occur in sequence during late exponential phase, nitrogen control followed by silicon control, resulting in a split of the initial population into two subpopulations, each arrested by a different deficiency.

The concept of cell cycle is useful to interpret growth behavior of phytoplankton batch-cultures and especially the lag phase observed after dilution with fresh medium. Two kinds of hypothesis have been formulated to explain such lag phase. It has been proposed that cells "condition" the new medium by releasing some organic materials such as chelating agents which make the medium more favorable for growth (Johnston, 1964). Alternatively, it has been hypothesized that cells adjust to their new medium during the lag phase (Spencer, 1954). Cell arrest in specific cell-cycle segments upon nutrient deprivation provides an other interpretation. When stationary-phase cells are supplied with fresh medium, they resume cycling from their arrest location, such that if the cells are arrested at the end of their cycle there is no lag (e.g. silicon-starvation in the diatom), while if they are arrested at the beginning of their cycle there is a long lag (e.g. nitrogen-starvation).

The results of this study and a parallel work on the light control of the cell cycle (Vaulot *et al.*, 1986) provide a

mechanistic explanation for population division patterns observed in fluctuating environments. The behaviors of *Thalassiosira weissflogii* and *Hymenomonas carterae* are qualitatively similar under nitrogen control (this study) and different under light/dark control (Olson *et al.*, 1986; Vaulot *et al.*, 1986): *T. weissflogii* has two light-dependent control points, one in G_1 and the other in G_2+M, while *H. carterae* has only one in G_1. If nitrogen and light are both supplied periodically, the control which acts earlier in the cell cycle will synchronize the population division pattern, since cells have always to wait for the earlier requirement to be met, before being able to proceed in their cycle and meet the second requirement. This would explain why periodic nutrient pulses override the photoperiod in determining cell-division phasing in *T. weissflogii* but not in *H. carterae* (Olson and Chisholm, 1983), since nutrient control of the cell cycle acts earlier than the light control in the former, but not in the latter species.

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