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FLOW CYTOMETRIC ANALYSIS OF SPERMATOGENESIS IN THE DIATOM *THALASSIOSIRA WEISSFLOGII* (BACILLARIOPHYCEAE)[1]

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ABSTRACT

Flow cytometry was used to detect and quantify sexual differentiation in the centric diatom Thalassiosira weissflogii *(Grun.). Size (light scatter), chlorophyll, protein and DNA contents were measured for each cell throughout the process of differentiation. Male gametes were small round cells characterized by one complement of DNA and a lower protein and chlorophyll content than vegetative cells. Male gamete formation was induced by a long period of darkness (2 days) followed by a transfer to continuous light. Up to 30% of the initial cell population produced male gametes which appeared in the culture 14 h after release from darkness. Male gamete production was also detected in exponentially growing cultures in continuous light, but to a much smaller degree.*

Key index words: diatom; flow cytometry; sexual differentiation; spermatogenesis

In all eukaryotic phytoplankters, mitotic cell division is the predominant mechanism for cell reproduction. In diatoms the presence of a rigid silica frustule around the cell can result in a progressive size reduction with each round of cell division (MacDonald 1869, Crawford 1981). One way for cells to escape from this size reduction is through gametogenesis and sexual reproduction (von Stosch 1951, Lewis 1984). In most centric diatoms (Fig. 1), male gametes are formed by multiple mitoses occurring within the same frustule (spermatogonangium), producing small round, differentiated cells

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(spermatogonia), which undergo meiosis to yield the small haploid sperm cells (Drebes 1977). In contrast, female gametes (oogonia) are large elongated cells usually formed after two degenerative meiotic divisions (Drebes 1977). Fertilization results in an auxospore which produces a new cell of diameter 3 to 5 times larger than the average vegetative cell (von Stosch 1951, Costello and Chisholm 1981).

Differentiation of vegetative cells into sexual cells depends not only on their size, but also on environmental factors. Sexuality can be induced experimentally by shifts in light or temperature under nutrient replete conditions (Drebes 1966, Manton et al. 1969, Werner 1971) or by nutrient depletion (Davis et al. 1973, French and Hargraves 1985). No systematic study of these factors has been made, however, and the incidence of sexual reproduction under typical culture conditions is poorly documented. We report here a study of the production of male gametes in the marine diatom *Thalassiosira weissflogii*, and the analysis of the process using flow cytometry.

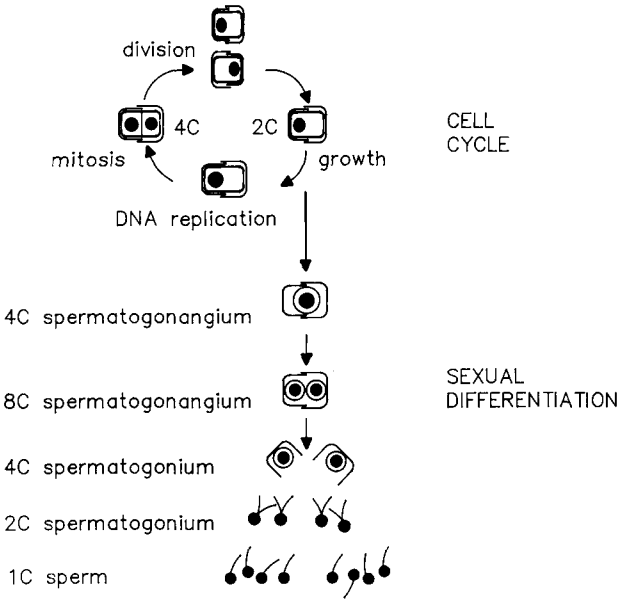

FIG. 1. Development of spermatogenesis in centric diatoms as described by Drebes (1977).

MATERIALS AND METHODS

Culture conditions. Thalassiosira weissflogii (Grun.) clone "Actin" was obtained from the culture collection of Dr. R. R. L. Guillard (Bigelow Laboratories) and was maintained in batch culture at 20° C, in f/2 enriched seawater (Guillard 1975), filter-sterilized through 0.22 μm pore size filter. Experiments were performed in batch cultures grown at 100 $\mu E \cdot m^{-2} \cdot s^{-1}$ in 100 to 500 mL Erlenmeyer flasks in f/2 enriched seawater with 0.2 mM N as $(NH_4)_2SO_4$ and 0.2 mM Si as $Na_2SiO_3 \cdot 9H_2O$.

Cell counting and fixation. After collection, samples were immediately transferred to a water bath maintained at −1° C to prevent any further population growth and cell metabolism. Cell concentrations were measured with a Model Z_f Coulter counter and an aliquot of each sample was fixed in methanol as described in Olson et al. (1983). Methanol served as an excellent fixative for cells enclosed in a frustule (vegetative cells and spermatogonia), but not for the more fragile naked male gametes; for this stage the recovery rate after fixation was only 5%. The analyses on fixed cells, therefore, yield only qualitative information for male gametes.

Cell staining. Cells were rinsed out of methanol, resuspended in Phosphate Buffer Saline and stained for DNA and protein using respectively propidium iodide (PI: 5 mg/L) and fluorescein isothyocyanate (FITC: 4.2 mg/L) in presence of RNase (40 mg/L). This procedure is a modification of the Crissman and Steinkamp (1982) method described in Vaulot et al. (1986).

Flow cytometric measurements. All analyses were performed with a Coulter Epics V flow cytometer/cell sorter (Coulter Electronics, Hialeah, Fla.) using the 488 nm laser line as the excitation light source. Details of the technical aspects of flow cytometry can be found in Shapiro (1985) and expanded descriptions of its application to phytoplankton appear in Trask et al. (1982), Olson et al. (1983), Yentsch et al. (1985) and Chisholm et al. (1986). Briefly, the instrument measures several parameters on individual cells at a very rapid rate (typically 10^3 cells per second); it also has the capability of physically sorting out cells of interest, according to a predetermined suite of characteristics, allowing one to verify signal interpretation through microscopic examination of the sorted cells.

In this study the parameters of interest to us were forward angle light scatter (FLS), red autofluorescence of chlorophyll (above 630 nm), green fluorescence (between 515 and 560 nm) of the protein-FITC complex and red fluorescence (above 630 nm) of the DNA-PI complex. The first two parameters were measured simultaneously on live cells taken directly from storage at −1° C and the last two were measured on cells fixed in methanol and stained with the appropriate fluorochromes. Between 10^4 to 2×10^5 cells were analyzed per sample.

Data analysis. The data obtained with flow cytometry are presented as two-dimensional contour plots of the measured parameters expressed in relative units (e.g. Fig. 2). The instrument could be calibrated to give absolute values, but for our purposes this was not necessary. Each contour encloses regions where cell density is equal to or larger than a given value. When distinct subpopulations were observed (e.g. Fig. 2), the *relative concentration* of each subpopulation was obtained by counting all the cells observed in the region ascribed to the subpopulation, and dividing this number by the number of cells observed in the total population. The *absolute concentration* of cells in the subpopulation was then obtained by multiplying its relative concentration by the absolute concentration of the total population as measured by the Coulter counter. This indirect procedure was necessary because the flow cytometer does not yield directly absolute concentration values.

RESULTS AND DISCUSSION

Production of male gametes by *T. weissflogii* was first observed during an experiment designed to study the light/dark control of the cell cycle of this species (Vaulot 1985, Vaulot et al. 1986). In this experiment, a population in exponential growth was placed in the dark for 34 h and then transferred back to constant light (t = 0). Samples of cells were taken hourly from the time of transfer to light, and analyzed using flow cytometry. Measurements of forward angle light scatter (FLS), which is related to cell size, and of red fluorescence, which is proportional to cellular chlorophyll revealed that after three hours of exposure to light (t = 3), the initially homogeneous population had begun to split into two subpopulations (Fig. 2). Both had the same average

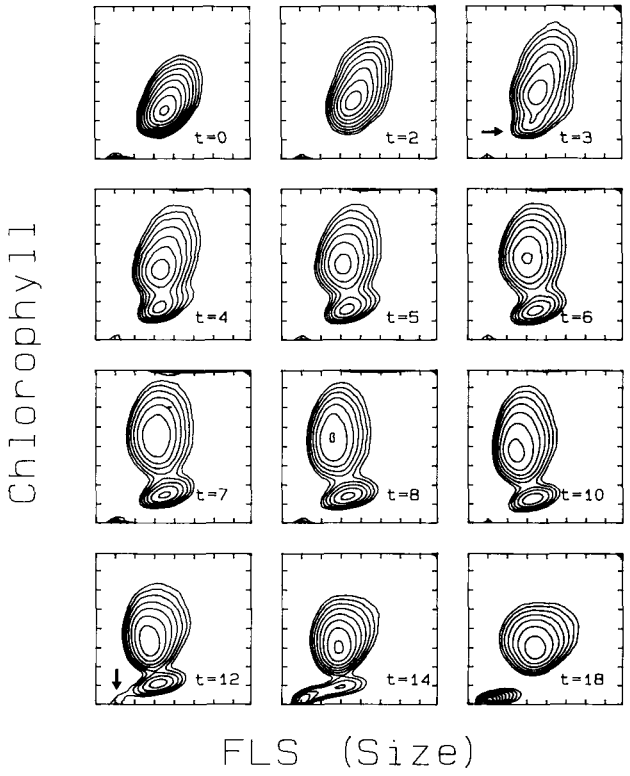

FIG. 2. A culture of *T. weissflogii* previously maintained in the dark for 34 h was released into continuous light at t = 0. Joint distributions of Forward angle Light Scatter (FLS), which is related to size, and of chlorophyll autofluorescence (above 630 nm) were measured on live cells by flow cytometry at the indicated times (hours). Both parameters are expressed in relative units on a linear scale and the contours connect equal cell densities. Note the formation of spermatogonangia at time t = 3, which are characterized by low chlorophyll fluorescence (arrow); note also the ensuing formation of male gametes characterized by very low FLS and chlorophyll, beginning at time t = 12 (arrow).

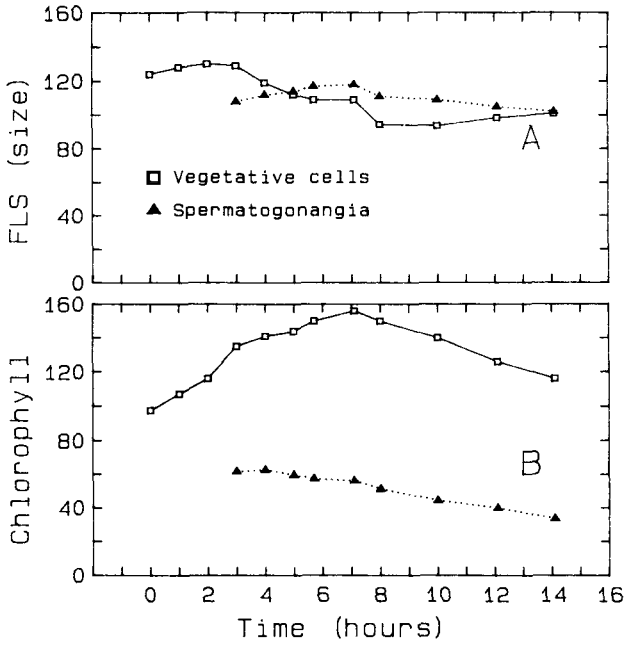

FIG. 3A, B. Time evolution of the average cell FLS (A) and chlorophyll content (B) (relative units), for vegetative cells and spermatogonangia from the experiment described in Figure 2. While both populations had similar FLS (size), the chlorophyll content of the spermatogonangia was reduced two to three times in comparison to vegetative cells.

size but they had different chlorophyll fluorescence (Fig. 3A, B). One of the subpopulations had a low chlorophyll fluorescence which decreased over time (Fig. 3B) and the other had a higher initial chlorophyll fluorescence which increased up to t = 8 (Fig. 3B). The low chlorophyll subpopulation disappeared very rapidly after t = 12 (Fig. 2) and was replaced by a third subpopulation of cells characterized by a three-fold reduction in size (FLS) and a ten to twenty-fold reduction in chlorophyll fluorescence (t = 14 and 18).

Microscopic examination of fresh samples taken at t = 10 revealed that a number of cells contained from two to eight small round differentiated bodies, fitting the description of spermatogonangia of other centric diatom species (Fig. 1, Drebes 1977). At t = 14 a number of frustules had opened up, liberating small round cells, about 3–6 μm in diameter and containing a few tiny chloroplasts, which correspond to the description of male gametes of centric diatoms (Fig. 1, Drebes 1977). In a separate set of experiments (E. Armbrust, pers. comm.), the subpopulations observed on the flow cytometric distributions (Fig. 2) were sorted using the cell sorting capability of the flow cytometer and it was confirmed by optical microscopy that the population of very small cells seen in the lower left corner of the dual-parameter distributions at t = 14 and t = 18 (Fig. 2) was composed of male gametes and that the low chlorophyll subpopulation from which it originated (t = 3 to t = 10; Fig. 2) was composed of spermatogonangia.

The time evolution of cellular DNA and protein distributions was also monitored by flow cytometry to follow more closely the time course of the mitotic and meiotic divisions associated with gamete formation (Fig. 4). When an asynchronous population of *T. weissflogii* is placed in the dark, cells arrest in either the G_1 stage (2 complements of DNA—abbreviated as 2C DNA; Fig. 1) or the G_2 stage (4C DNA) of the cell cycle (Vaulot 1985, Vaulot et al. 1986). This can be seen clearly in the joint protein and DNA distribution at t = 0 (Fig. 4). All the cells had either a 2C or a 4C DNA content: no cells had an intermediary DNA content characteristic of the DNA synthesis phase, S. After 2 h of light exposure (t = 2) a portion of the dark-arrested G_1 cells initiated DNA synthesis, underwent mitosis and continued to reproduce asexually (Vaulot 1985, Vaulot et al. 1986).

At t = 3 (Fig. 4), a subpopulation of G_1 cells with relatively low protein content (arrow at t = 3 in Fig. 4) initiated DNA synthesis. These cells completed the S phase within two hours (t = 5) but did not

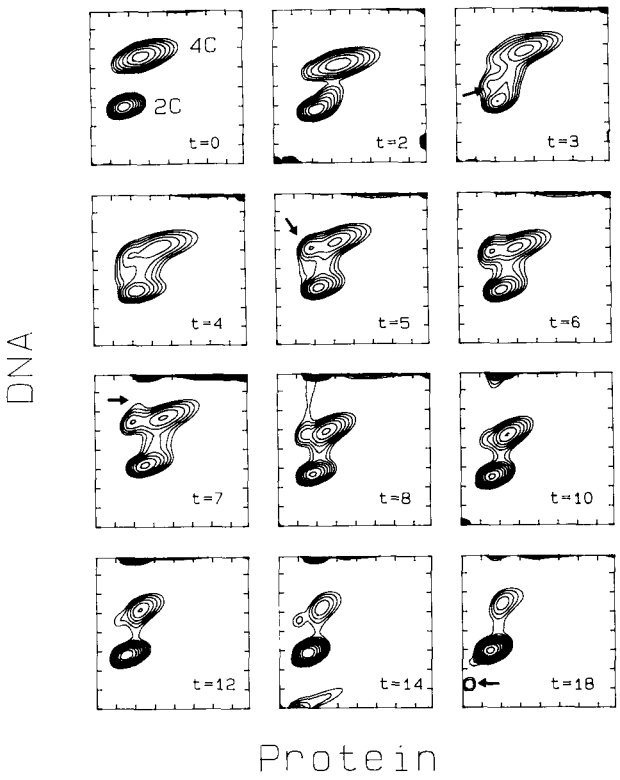

FIG. 4. Joint distributions of protein and DNA measured on fixed cells stained with fluorescein isothyocyanate (FITC) and propidium iodide (PI) from the experiment described in Figure 2. Note the small subpopulation of cells initiating DNA synthesis at t = 3 (2C spermatogonangia). At t = 5 this subpopulation reached the 4C DNA level (4C spermatogonangia), but remained at a lower protein level than vegetative G_2 cells. At t = 7 a new round of DNA synthesis occurred in these cells and they went off the DNA scale (8C spermatogonia). Cells with a 1C DNA appeared at t = 18 (1C gametes).

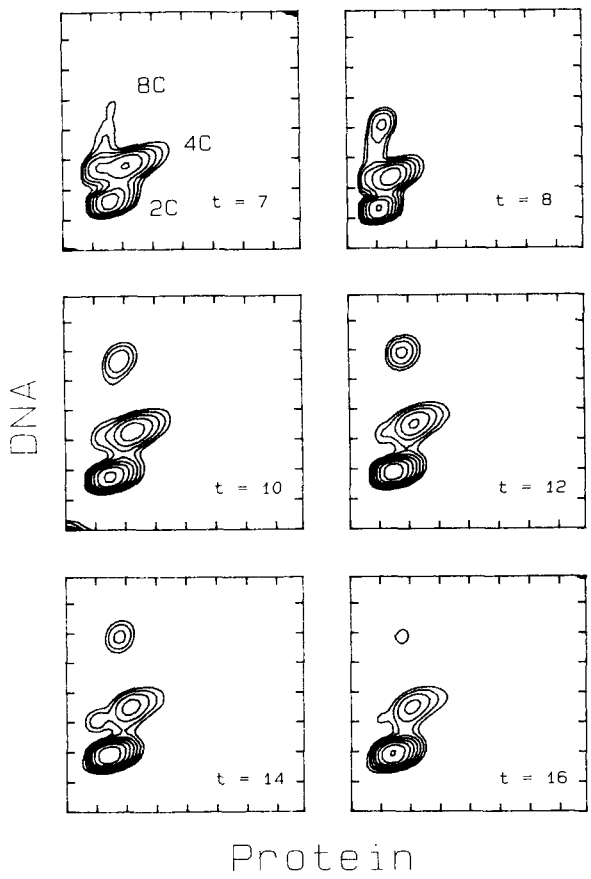

FIG. 5. Samples from Figure 4 measured on an expanded DNA scale so that the 8C spermatogonangia can be seen clearly at t = 10.

merge with the vegetative G_2 + M cells, maintaining a lower protein content (arrow, t = 5). Two hours later these "G_2" cells (4C spermatogonangia; Fig. 1) initiated a second round of DNA synthesis (arrow, t = 7), which was completed by t = 12 yielding cells with 8C DNA (8C spermatogonangia; Fig. 1). These cells are off scale on the DNA axis in Fig. 4 and can be seen on an expanded DNA scale (Fig. 5). Between t = 14 and t = 18, the 8C DNA population disappeared and was replaced by a 1C DNA population with very low protein (arrow, t = 18, Fig. 4).

We can estimate the abundance of each subpopulation (Fig. 6) by piecing together the information obtained on both live (Fig. 2) and fixed (Figs. 4 and 5) samples. Spermatogonangium concentrations estimated from FLS-chlorophyll distributions (not shown) agreed very well with those estimated from protein-DNA distributions (Fig. 6). About 30% of the initial dark-arrested population became involved in gametogenesis and produced spermatogonangia. On the average, six 1C gametes were produced per spermatogonangium (Fig. 6). Since the number of 1C gametes must be a power of 2 if there are no degenerative nuclear divisions (Fig. 1), this suggests that some cells released 4 gametes and some 8. Only one DNA replication took place inside the spermatogonangium in the former case and two in the latter case. Such variability in the number of premeiotic DNA replications has also been observed in the centric diatom *Lithodesmium undulatum* (Manton et al. 1969).

With the flow cytometric "signature" of both spermatogonangia and male gametes in mind, we examined previous data sets to see how common gametogenesis was in our experiments and to try to gain insights into the possible triggers. Evidence of spermatogenesis was found in the data from a total of four dark release experiments which were performed over a period of six months. The magnitude of the response was however different from experiment to experiment, and in one case only the spermatogonangium stage was evident. Spermatogonangia were also often observed in populations grown in continuous light (Fig. 7A, B), although always in very low proportions (less than 5%).

Female gametes of centric diatoms (oogonia) are usually larger than vegetative cells, contain an increased number of chloroplasts (Drebes 1977) and

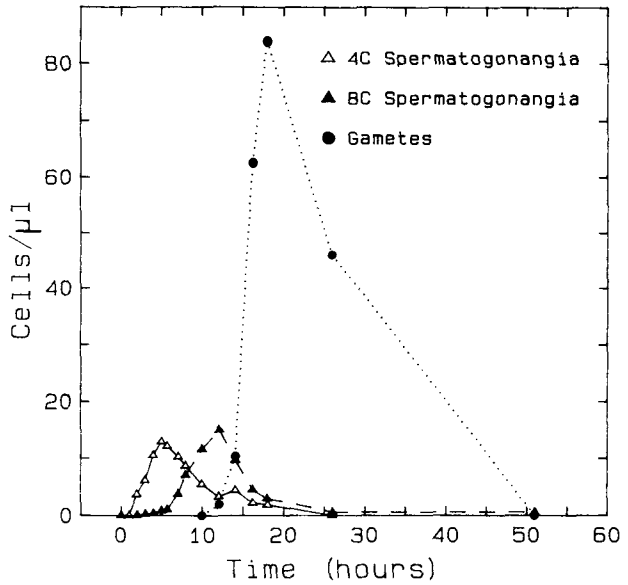

FIG. 6. Time evolution of the absolute concentrations of 4C and 8C spermatogonangia and 1C male gametes from the experiment described in Figure 2. Spermatogonangium concentrations were estimated from protein-DNA distributions, while male gamete concentrations were estimated from FLS-chlorophyll distributions.

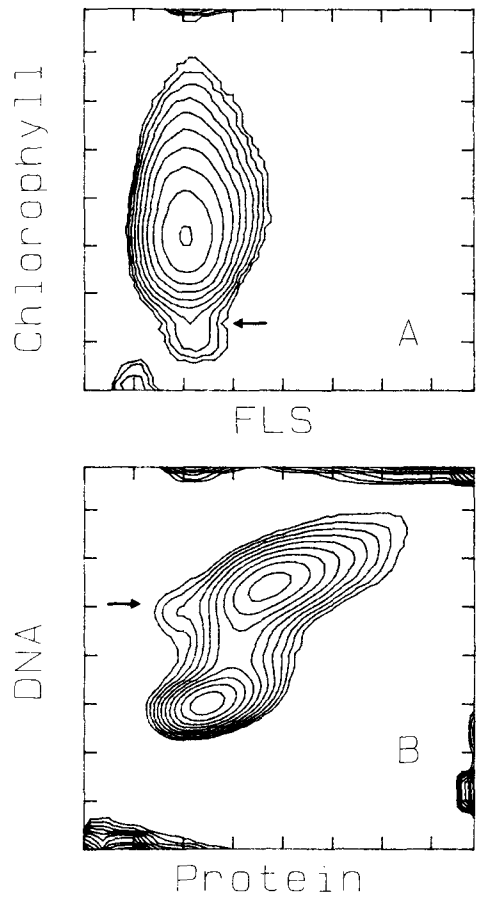

FIG. 7A, B. Joint distributions of FLS-cholorphyll (A) and protein-DNA (B) in a culture growing exponentially in continuous light. 2% of the total population was composed of spermatogonangia (arrow).

have a 1C DNA content. They should therefore appear below vegetative cells and to the right of the male gametes in the protein vs. DNA flow cytometric measurements. Such cells were not identified in the experiments described here (Figs. 4 and 7B), but auxospores have been previously recorded in the same strain under a different set of conditions (Costello and Chisholm 1981), thus we know that the strain is capable of oogenesis. The dependence of male and female induction on different environmental factors has also been observed in other diatom species (Holmes 1966, Migita 1967, Drebes 1977).

Conclusions. Gamete formation has important consequences for the study of diatom populations in the laboratory. In particular, successful sexual reproduction results in the appearance of a subpopulation of large cells, derived from auxospores, which can have very different growth characteristics from normal vegetative cells (Davis et al. 1973, Costello and Chisholm 1981). For strains which have been maintained for a long period of time in culture (such as the one used in these experiments), frequent sexual reproduction can also result in a genetic drift of their physiological characteristics, although this is rarely reported in the literature.

The occurrence of gametogenesis and sexual reproduction in phytoplankton cultures is usually only detected when it affects a large fraction of the population and is thus easily noticeable by classical methods such as microscopic observation (Gallagher 1983) or electronic (Coulter) counting (Davis et al. 1973, Costello and Chisholm 1981). Flow cytometry can be used to discriminate between the different sexual stages, permitting a precise quantification of their relative abundance, even when it is as low as a few percent (Fig. 7). In particular, this allows one to detect spermatogenesis not only under special sets of conditions such as light/dark shifts, but also during exponential growth in continuous light.

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KINETICS OF NUTRIENT UPTAKE AND GROWTH IN PHYTOPLANKTON[1]

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ABSTRACT

Microscopic algae can grow rapidly in natural waters that are extremely low in essential macro and micro nutrients. Yet, their nutrient uptake systems exhibit only mediocre nutrient affinities, the saturation constants being often 10–1000 times the (estimated) ambient concentrations. The large difference which exists between the saturation constants for growth (K_μ) and short term uptake (K_ρ) are due to the acclimation capabilities of the organisms. Over the acclimation range, K_μ to K_ρ, the algae can maintain maximum growth rate by modulating both their internal nutrient quotas (Q) and their maximum short term nutrient uptake rates (ρ_{max}) in response to variations in external nutrient concentrations. The commonly assumed hyperbolic relationships for steady growth and uptake (viz "chemostat theory") are coherent with a hyperbolic expression for short term uptake including a variable maximum (ρ_{max}). The ratio of the saturation constants for growth and uptake is then directly related to the extreme in quotas and maximum uptake rates:

$$K_\mu / K_\rho = Q_{min} / Q_{max} \cdot \rho^{lo}{}_{max} / \rho^{hi}{}_{max}.$$

This result is applicable even when the exact hyperbolic laws are not. Published data on Fe, Mn, P and N limitation in algae are generally in accord with the theory and demonstrate a wider acclimation range for trace than for major nutrients.

Key index words: acclimation; growth limitation; iron; manganese; nitrogen; nutrient uptake; phosphorus; saturation constants

As testified by the extraordinary abundance of literature on the subject, the relationship between limiting nutrient concentration and phytoplankton growth rate is seen as a key physiological issue determining the primary production in many aquatic ecosystems. Typically, batch and continuous cul-

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