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Nitrate reductase is known to be the enzyme regulating the reduction of nitrate to nitrite in plants. This reduction is the first and rate-limiting step in the transformation of inorganic nitrate to a cellular nitrogen form. Since this reduction process is essential to phytoplankton growth when nitrate is the only available nitrogen source, the species with the highest capability to reduce nitrate, or the highest nitrate reductase activity, should be the species most favored to dominate a phytoplankton population under nitrate-limiting conditions. To study this hypothesis, the nitrate reductase activities of two species were studied; these species are co-occurring yet dominate under different conditions. *Thalassiosira nordenskioldii* is the dominant species in Auke Bay, Alaska in early spring when nitrate levels are decreasing from 15 μM to 1 μM, and the temperature is about 5 C. *Skeletonema costatum* is dominant in mid-summer when

nitrate levels are below 1 μM and the temperature is above 12 C. The results show that at 15 C, Skeletonema has a higher enzyme activity at all nitrate levels than does Thalassiosira, which is consistent with the hypothesis. In addition, Thalassiosira shows a higher enzyme activity at 10 C, nearer its optimal temperature for growth, than at 15 C, suggesting that temperature affects species succession through its influence on enzyme activity. The applicability of Michaelis-Menten kinetics to this reduction reaction, based on the enzyme activities measured for these two species, is doubtful but inconclusive. The results also have implications for such concepts as nitrate-limited growth and the Redfield model relating nutrient, O_2 and CO_2 changes in the ocean.

Nitrate Reductase Activity as a Factor Influencing the Seasonal Succession of Marine Phytoplankton

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NITRATE REDUCTASE ACTIVITY AS A FACTOR INFLUENCING THE SEASONAL SUCCESSION OF MARINE PHYTOPLANKTON

INTRODUCTION

The observed seasonal cycle of marine phytoplankton blooms has been explained, at least in part, as a consequence of the nutrient regime of the oceans. Winter regeneration of inorganic nutrients in the water allows a spring phytoplankton bloom and this bloom dies off as the nutrients are depleted and become the limiting factor in further production. Over the period of a complete seasonal cycle, there is often a succession of phytoplankton blooms and within each bloom a given species may be dominant. It is therefore possible to speak not only of the seasonal succession of phytoplankton blooms but also of a succession of species. The available nutrients may not only control the gross numbers of phytoplankton that can exist within a bloom at any given time in the cycle, but also may be a factor in determining which of the competing phytoplankton species will be dominant in that bloom.

The possibility of such an influence by the nutrient level on the seasonal succession of species led to studies of the varying abilities of different species to utilize limiting levels of nitrate, such ability being reflected in their nitrate uptake rates (Dugdale, 1967; Eppley and Coatsworth, 1968). The equation derived in these investigations

to describe the relation between nitrate concentration and uptake rate was found to be analogous to the Michaelis-Menten equation (Mahler and Cordes, 1966) describing the kinetics of enzyme reactions.

$$v = \frac{(V)(S)}{k + S} \tag{1}$$

Where one equates uptake rate with growth rate as Dugdale (1967) did, the terms of equation (1) denote as follows:

v - rate of nitrate uptake or specific growth rate

V - maximum uptake rate or maximum growth rate

S - concentration of nitrate in the medium

k - concentration of nitrate at which the uptake or growth rate is half its maximum ($v = 1/2\ V$)

In an alternate form,

$$\frac{1}{v} = \left(\frac{k}{V}\right)\left(\frac{1}{S}\right) + \frac{1}{V} \tag{2}$$

By plotting $1/v$ versus $1/S$, one obtains a straight line whose slope represents k/V and whose intercept is $1/V$.

The immediate value of equations (1) or (2) is to describe the kinetics of nutrient uptake. But even more important is the fact that it provides a means of correlating phytoplankton species succession with nutrient concentration. Because V and k are constants under given physical conditions of light, temperature, etc. (Dugdale, 1967), they may be considered as growth characteristics with unique values

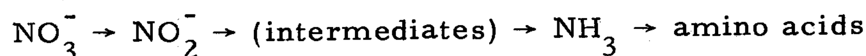
for each species of phytoplankton. The lower the k value, the greater would be the nitrate uptake rate or growth rate. Under conditions in which it is the nutrient level which is limiting growth, the equation predicts that the dominant species would be the species with the lowest value of k. Or, as Eppley (1968) explains concerning the cycle of blooms and dominant species, "Each species should show a lower k value than the preceding one if declining level of the nutrient in question is indeed significant in species succession."

To test the validity of such a correlation between nitrate level and species dominance and succession, Eppley, Rogers and McCarthy (1969) studied the k values for nitrate uptake of various oceanic and neritic phytoplankton species. In comparing these values, they found that the oceanic species were characterized by lower k values than all but one of the neritic species. This would tend to support the idea that the species dominant at low nitrate levels would be the one with a low k value, since the oceanic environment has a much lower concentration of nutrients than the neritic and the oceanic species were found to have correspondingly lower k values.

In their studies of uptake rates, Dugdale (1967) and others (Eppley, 1968 and 1969) equated the uptake rate with growth rate. But nutrient uptake (absorption into the cell) and nutrient assimilation (transformation of the absorbed inorganic nutrient into a form which can be utilized by the cell) are two distinct processes. In the

case of nitrate, assimilation is the process of reducing nitrate to ammonia and then synthesizing amino acids and proteins. It may be that growth should be correlated not with uptake but with assimilation. This would mean that in a bloom of competing species in which growth is nitrate-limited, the dominant species might be expected to exhibit the greatest ability to assimilate nitrate.

The assimilation of nitrate has been rather extensively studied in higher plants and several reviews have been written (Kessler, 1964; Beevers and Hageman, 1968). The pathway of the complete reduction process is not known because all the intermediates have not been isolated and identified. It is known, however, that the process involves at least the following reactions (Kessler, 1964):

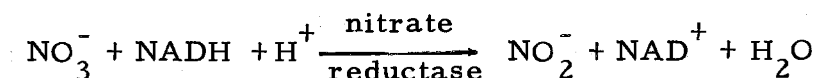


$$NO_3^- \rightarrow NO_2^- \rightarrow \text{(intermediates)} \rightarrow NH_3 \rightarrow \text{amino acids}$$

In considering the rate of this assimilation process, it is sufficient to consider the rate-limiting step of the overall reduction process. Indications are that the first step, the reduction of nitrate to nitrite involving the enzyme nitrate reductase, is the rate-limiting step. This is supported by several facts. First of all, nitrate has been found to accumulate within the phytoplankton cell whereas nitrite does not accumulate (Eppley, 1968). Second, since nitrite is toxic to the cell, it would be advantageous for control of the overall reduction process to come in this first step so that nitrite is never produced in excess.

Such control is provided by the observed repression of the enzyme nitrate reductase by ammonia and nitrite accumulated within the cell (Beevers, 1968). And finally, it has been found that nitrate reductase consistently exhibits a lower activity than nitrite reductase, meaning that the first reduction step is slower than the second (Schrader, Ritenour and Hageman, 1968). It is probably the first step then that controls the rate of the overall nitrate assimilation process, and the rate of this first step depends on the activity of the enzyme nitrate reductase.

The presence of nitrate reductase has already been demonstrated in both higher plants and phytoplankton (Beevers and Hageman, 1968). The reduction reaction which this enzyme regulates requires the presence of a reduced pyridine nucleotide cofactor, which is most often NADH, or reduced nicotinamide adenine dinucleotide (Beevers, Flesher and Hageman, 1964; Schrader, 1968). The stoichiometry of this reaction has been shown to be (Evans and Nason, 1953):



$$NO_3^- + NADH + H^+ \xrightarrow[\text{reductase}]{\text{nitrate}} NO_2^- + NAD^+ + H_2O$$

Since this is an enzymatic reaction, its kinetics should follow the Michaelis-Menten equation, which has the same form as the equation already presented for nutrient uptake. According to this equation, the rate of reduction of nitrate to nitrite depends on the nitrate concentration. But the rate is also controlled by the activity of nitrate

reductase itself, and the activity of this enzyme *in vivo* has been shown to be influenced not only by nitrate level but also by light, temperature, pH, plant age and other physical and physiological factors (Beevers, 1968).

This thesis was based on the hypothesis that, under conditions in which nitrate is the growth-limiting factor, the ability of given species of marine phytoplankton to reduce nitrate may be an important parameter controlling species succession. In order to test this hypothesis, the nitrate-reducing ability of different species was to be expressed quantitatively by measuring the enzyme activities of the species at given nitrate levels and under constant physical conditions. Then by comparing the observed activities of co-occurring species, it could be determined whether, as the hypothesis suggests, the species dominant when the nitrate level is relatively low is also the species exhibiting the higher enzyme activity.

The choice of the two phytoplankton species used in this investigation was based on information contained in a study of Auke Bay, Alaska (Bruce, 1968). In this estuarine environment, the nitrate concentration in the euphotic zone (0-8 m depth) varies seasonally from a maximum of 27 μM in the winter to a minimum of 0-0.05 μM from May through August (Figure 1). Superimposed on this nitrate cycle is a succession of four to five phytoplankton blooms (Figure 1) occurring between mid-April and mid-September of each year, with

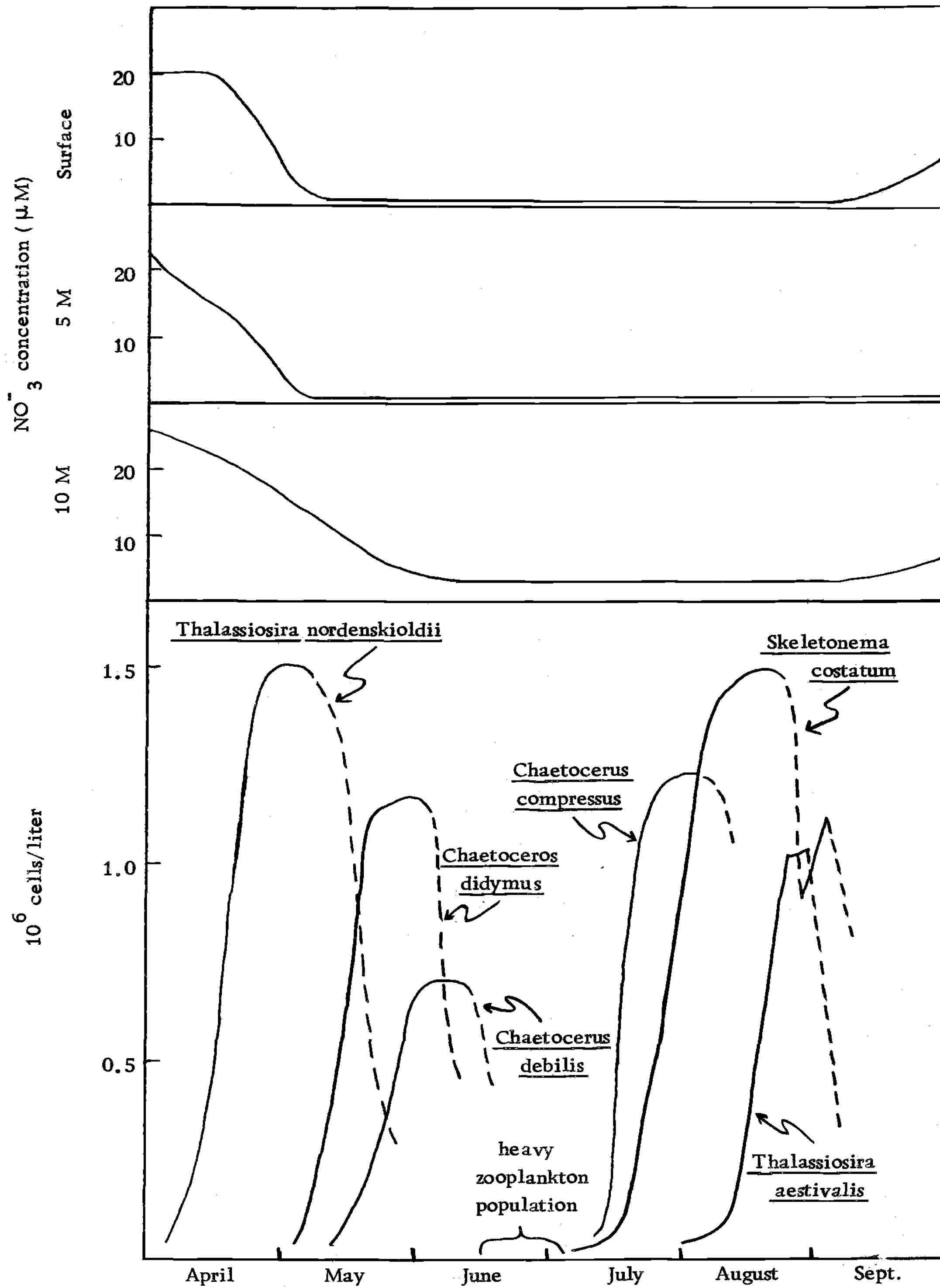


Figure 1. Seasonal cycle of nitrate and succession of phytoplankton blooms observed in Auke Bay, Alaska. (From Bruce, 1968.)

the dominant species in each bloom constituting 60-80% of the total population. The first of these blooms covers about three weeks, starting the third week of April and continuing through the first week of May. During this time, the nitrate level is slowly depleted from 15 μM to about 1 μM. The dominant species of this bloom is the diatom *Thalassiosira nordenskioldii*. The fourth bloom in the series covers about a month from the fourth week in July to the fourth week in August. The nitrate at this time is slowly increasing from 0.05μM to about 0.5 μM. In this case the dominant species is the diatom *Skeletonema costatum*. On the basis of this observed seasonal succession, *Thalassiosira nordenskioldii* and *Skeletonema costatum* were chosen as the experimental species; *Thalassiosira* representing a species more abundant at relatively high nitrate levels and *Skeletonema* at low nitrate levels.

METHODS

The experimental diatoms were grown in the laboratory under physical conditions similar to the normal environmental conditions found in Auke Bay. Thus the culture salinity was adjusted to 20‰ and the temperature in all experiments ranged between 10 C and 15 C, which approximates the observed range of summer surface temperatures (Bruce, 1968). The light intensity, which is between 200 and 300 langleys per day during the bloom cycle in Auke Bay (Bruce, 1968), was maintained in the laboratory at only 15 langleys per day in order to slow down the growth of the cells. In addition, the lights were continuously on in order to eliminate possible diurnal variations in enzyme activity which have been observed to result from varying light intensities (Hageman, Flesher, and Gitter, 1961). Culture media of filtered seawater, autoclaved and buffered with bicarbonate, contained phosphate, silicate, thiourea, vitamins and a mixture of metals in quantities based on a procedure proposed by Guillard and Ryther (1962). The nitrate in the media varied according to the intent of each experiment, but in all cases was the limiting nutrient.

Two sizes of cultures were used in this investigation. Two-liter cultures were used to grow cells at a given nitrate level and to determine the enzyme activity corresponding to this level. This level was maintained by determining the nitrate concentration in the water

each day and adding more nitrate as it was needed to regain the initial nitrate level. These cultures were continued until enough cells were present to allow measurement of their enzyme activity, at which time all cells were removed by continuous-flow centrifugation. The 15-liter cultures were used to grow cells at steadily decreasing nitrate concentrations and to follow the changes in their enzyme activity as the nitrate level decreased. No nitrate was added to the culture except the initial addition of about 140 μM. Beginning about the second day after inoculation of the culture, a certain volume of the 15-liter culture was harvested each day by continuous-flow centrifugation. The cells from this volume were collected for analysis of enzyme activity and the cell-free effluent from the centrifuge was returned to the 15-liter culture. Since no nitrate was added beyond the initial level and only a portion of the cells were removed each day, the remaining cells steadily depleted the nitrate in the culture medium. The centrifugation process was repeated daily until the cells had either completely depleted the nitrate or the cells themselves were no longer dividing, at which point all the cells were harvested. With both the two-liter and 15-liter cultures, samples of the effluent from the centrifuge were collected, forzen and saved for analysis of the nitrate concentration in the culture medium at the time of removal of the cells.

Because of the instability of nitrate reductase at even

moderately high temperatures (Evans, 1953), cells were harvested at 5 C and the in vitro preparation was thereafter kept cold. In warmer weather, an ice bath was necessary to maintain the temperature of the 15-liter cultures during the three- to four-hour harvesting process.

Once the cells had been harvested, they were suspended in 10 ml of a cold 0.2 M KH_2PO_4 - K_2HPO_4 buffer, pH 7.9, which was 1.0 mM in dithiothreitol. This suspension was sonicated to rupture the cell walls and centrifuged. The supernatant was saved as the crude extract for the nitrate reductase assay, and the centrifugate saved for the protein analysis.

The assay for nitrate reductase activity was adapted from a method used by Eppley, Coatsworth and Solòrzano (1969), in which NADH reacts with nitrate in the presence of nitrate reductase from the crude extract, reducing nitrate to nitrite. In the procedure followed here, 2 ml of the crude extract were added to each of four test tubes containing 0.5 ml of 36 mM $NaNO_3$ and 0.3 ml of 0.9 mM NADH (this solution was prepared fresh each day to prevent decomposition of the NADH in solution). The reaction in the two control tubes was stopped immediately by addition of 0.2 ml of 1 M zinc acetate and 5 ml of 95% ethanol. The other two tubes were incubated for one hour at 23-25 C before stopping the reduction reaction. After the reaction had been stopped, the resulting suspension was centrifuged

and the supernatant analyzed for nitrite concentration by the addition of 1 ml each of sulfanilamide and n-(1-napthyl)-ethylenediamine dihydrochloride (solutions prepared according to Strickland and Parsons, 1965, p. 75). The intensity of the color developed after 15 minutes was read at 540 mμ. The difference between the average absorbances of the reaction and control tubes was calculated and from this, using a standard curve of nitrite versus absorbance, the amount of nitrite produced per hour as a result of the enzyme activity was determined.

Nitrate reductase activity is expressed as μM NO_2^- produced/mg protein-hour. To determine the protein in the harvested cells, the centrifugate of the original suspension of cells in buffer was suspended in 10 ml of 5% trichloroacetic acid and allowed to stand for several hours. This suspension was centrifuged, the precipitate suspended in 10 ml of 1 N NaOH and allowed to stand in the refrigerator overnight. After centrifuging, 0.20 ml portions of the supernatant were analyzed for protein by the method of Lowry _et al_. (1951). The absorbances were read at 500 mμ after one hour, at which time color development was complete. The amount of protein was determined on the basis of the average absorbance of the 0.20 ml protein samples, using a protein calibration curve.

Nitrate concentration in the culture media was determined using a Technicon auto-analyzer. This method reduces the nitrate in the sample to nitrite by passing it through a cadmium reducing column.

The nitrite is then determined photometrically. Samples used for the nitrate determinations had been taken at the time of harvesting of the cells and were kept frozen until they were analyzed.

RESULTS

The activity of nitrate reductase for each group of cells harvested and the corresponding nitrate level in the culture medium at the time of harvesting are given in Table 1. These results are considered in groups of related experiments. One set of two-liter cultures (A) looked at the enzyme levels of the two species of diatom at 15 C and at the extreme nitrate level of greater than 1200 μM. Under these conditions, Skeletonema exhibited a much higher enzyme activity than did Thalassiosira. Another set of two-liter cultures (B) was designed so that, by daily monitoring of the nitrate level in the culture, this level was maintained between zero and 20 μM NO_3^- during the entire growth period. Although a greater quantity of Thalassiosira was harvested from these cultures in terms of protein, the enzyme level of Thalassiosira proved to be unmeasurable whereas that of Skeletonema was about 1.5 μM NO_2^-/mg protein-hour. With one exception (Thalassiosira 6/22 to 6/26), the 15-liter cultures (C) were all grown at 15 C under the same light intensity. The five resulting enzyme activities for Skeletonema ranged from 0.93 to 7.9 μM NO_2^-/mg protein-hour, while the three activities for Thalassiosira were all much lower, ranging from 0.06 to 1.1 μM NO_2^-/mg protein-hour. In the Skeletonema cultures, the nitrate reductase activity decreased with decreasing nitrate levels, whereas

Table 1. Nitrate reductase activities, temperatures, medium nitrate concentrations and cell numbers for all experiments.

Species	Sampling date	Culture temp. (° C)	10^6 cells/liter (±18%)	Activity $\frac{\mu M\ NO_2^-}{\text{mg protein-hr}}$ (±2.8%)	Medium NO_3^- conc. (μ M) (±3.7%)
(A) Two-liter cultures; > 1200 μM NO_3^-					
Skeletonema	3/19	15	39	18	> 1200[1]
	4/21	15	33	30 (fresh) 16 (frozen)	> 1200[1]
Thalassiosira	3/17	15	3.7	0.58	> 1200[1]
(B) Two-liter cultures; 0-20 μM NO_3^-					
Skeletonema	5/17	15	17	1.7	< 0.1
	5/21	15	17	1.5	0.1
Thalassiosira	5/28	10	4.4	-[3]	0.3
	6/4	10	4.4	-[3]	0.1
(C) 15-liter cultures; < 140 μ M NO_3^-					
Skeletonema	3/26	15	21	7.9	70-140[1]
	3/27	15	50	0.93	52
	3/28	15	42	-[3]	0
	3/30	15	8.4	-[3]	0
	4/24	15	3.9	6.3	130
	4/25	15	6.9	-[3]	120
	4/26	15	9.2	5.1	92
	4/27	15	8.4	-[3]	68
	4/28	15	9.6	-[3]	42
	4/29	15	3.6	2.0	27
	4/30	15	2.0	-[3]	19
Thalassiosira	3/26	15	4.9	0.53	70-140[1]
	3/27	15	3.5	1.1	10
	3/29	15	2.0	0.06	0
	6/22	10	1.2	-[3]	150
	6/23	10	3.1	0.08[2]	120
	6/24	10	3.1	3.3	88
	6/25	10	2.6	-	65
	6/26	10	1.7	3.7	51

[1] exact NO_3^- concentration not known

[2] doubtful value

[3] below level detectability or no reaction

[4] relative errors calculated for duplicate samples within each experiment.

no such trend was observed with Thalassiosira. The Thalassiosira culture of 6/22 to 6/26 was grown at 10 C because the summer heat had necessitated using a colder constant temperature room to keep the cells alive. The 15 C cold room could no longer be maintained at that temperature. The results of this culture are worth noting because two of the activities observed (6/24, 6/26) are much higher than those of the Thalassiosira cells grown at 15 C. The validity of the third activity (6/23) is doubtful because the nitrite reaction mixtures started losing their normal red color and becoming an abnormal green before the 15-minute period for color development was over.

A two-liter experiment (Skeletonema 4/21) (A) was run to see if freezing a crude enzyme extract would affect the activity. This was done by freezing 5 ml of a 10-ml extract, thawing it the next day and comparing its activity to that of the 5 ml of fresh extract. Since the activity was nearly halved by freezing, all assays were run on fresh extracts.

Table 2 presents the data correlating cell numbers with weight of protein. The weight of protein per cell for Thalassiosira is almost a factor of ten greater than for Skeletonema, as might be expected from the difference in cell sizes of the two species. One might also note that with both the 15-liter cultures Thalassiosira 3/26 to 3/29 and Skeletonema 4/24 to 4/30, the weight of protein per cell seems to increase steadily with the age of the culture. In addition,

Table 2. Total number of cells and protein content of all samples.

Species	Sampling date	Temperature °C	Volume filtered	10^6 cells in sample	Protein in sample (mg)
Skeletonema costatum					
2-liter	3/19	15	2	79	4.4
16-liter	3/26	15	8	165	4.5
	3/27	15	8	398	17
	3/28	15	8.25	346	13
	3/30	15	16	135	13
15-liter	4/9	15	7.5	190	8.4
	4/10	15	8.0	258	11
2-liter	4/21	15	2	66	4.3
15-liter	4/24	15	10	39	1.8
	4/25	15	9.9		
	4/26	15	12	110	6.9
	4/27	15	11		
	4/28	15	12.25		
	4/29	15	9	32	2.3
	4/30	15	0		
2-liter	5/17	15	2	34	1.2
2-liter	5/21	15	2	34	1.1
Thalassiosira nordenskioldii					
2-liter	3/17	15	2	7.4	5.8
15-liter	3/26	15	7.5	37	25
	3/27	15	6.75	24	17
	3/29	15	15	30	24
2-liter	5/28	10	2	8.8	1.9
2-liter	6/4	10	2	8.3	1.1
15-liter	6/22	10	0		
	6/23	10	7.5	23 *	4.1
	6/24	10	7.5	23	5.1
	6/25	10	7.5	19	4.0
	6/26	10	15	26 *	5.9

*cells lost in centrifugation - number cells in sample uncertain

Thalassiosira appears to have less protein per cell in the cultures grown at 10 C than in those grown at 15 C.

The growth and nitrate utilization data for a continuous culture of *Skeletonema* at 15 C (4/24 to 4/30) and one of *Thalassiosira* at 10 C (6/22 to 6/26) are given in Table 3. The cell division rate of *Skeletonema* was much higher than that of *Thalassiosira*. Moreover, *Thalassiosira* took up approximately four times as much nitrate per cell produced as did *Skeletonema*, so that *Skeletonema* could produce more cells given the same total amount of nitrate. In both cases, the nitrate required per cell division steadily increased as the culture got older.

Table 3. Cell production as a function of nitrate depletion in two 15-liter experiments.

Species	Sampling date	Divisions/ day	cells produced daily	cells produced cumulative	NO_3^- depletion µmoles daily	NO_3^- depletion µmoles cumulative	Daily NO_3^- depletion / cell produced (10^{-12} moles)
Skeletonema costatum - grown at 15 C							
	4/24						
	4/25	1.8	74	75	214	214	2.9
	4/26	2.0	103	178	424	639	4.1
	4/27	2.2	98	276	360	999	3.7
	4/28	2.1	111	387	391	1390	3.5
	4/29	1.0	28	415	216	1610	7.8
	4/30	0.50	8.6	423	123	1730	14
Thalassiosira nordenskioldii - grown at 10 C							
	6/22						
	6/23	1.4	28	28	450	450	16
	6/24	1.0	24	52	429	879	18
	6/25	0.69	14	66	351	1230	24
	6/26	0.43	6.8	73	210	1440	31

$$\frac{\text{cumulative } NO_3^- \text{ depletion}}{\text{cumulative cell production}} \rightarrow 4.1 \times 10^{-12} \text{ moles } NO_3^-/\textit{Skeletonema} \text{ cell}$$

20×10^{-12} moles NO_3^-/Thalassiosira cell

4.8 times as much NO_3^-/cell in Thalassiosira as in Skeletonema

DISCUSSION

These experiments indicate that, at comparable nitrate levels in the medium, the nitrate reductase activities observed for *Skeletonema costatum*, a diatom most abundant when nitrate levels are low in Auke Bay, are in all cases higher than the corresponding activities of *Thalassiosira nordenskioldii*, most abundant at higher nitrate levels. Of all the experiments, the two-liter cultures (B) in which the nitrate level was at all times between zero and 20 μM are the most comparable to the environment actually encountered in Auke Bay. In these cultures, both species were grown at nearly their respective optimal temperatures (10 C in the *Thalassiosira* cultures, 15 C for *Skeletonema*). The results show that the activity of *Thalassiosira* was unmeasurable while that of *Skeletonema* was about 1.5 μM NO_2^-/ mg protein-hour. Thus within the 10 C to 15 C range observed here, *Skeletonema* seems much better adapted to assimilate nitrate over the entire range of nitrate levels from zero to greater than 1200 μM than is *Thalassiosira*. As suggested in the introduction, this could be a very important factor in determining which of the two competing species is dominant, and under conditions in which nitrate is the growth-limiting factor, would make *Skeletonema* the much more favored species.

If *Skeletonema*'s higher enzyme activity makes it better adapted

to assimilate nitrate, an essential function in phytoplankton growth, the question arises as to why we find *Thalassiosira* and not *Skeletonema* dominant in the first spring bloom in Auke Bay. This phenomenon is not unique to Auke Bay. Conover (1956) observed that *Thalassiosira* is the closest competitor of *Skeletonema*, both occurring under the same physical conditions, and yet an early spring bloom of *Skeletonema* is rare. In the coastal waters south of Long Island, as in Auke Bay, *Skeletonema* grows best from July to September (Curl and McLeod, 1961). Obviously there are more factors influencing seasonal succession and species dominance here than just nitrate reductase activity, and a combination of such factors must make *Thalassiosira* the species best adapted to exist in the early spring conditions of Auke Bay and other neritic environments.

From mid-April to mid-May in Auke Bay, when *Thalassiosira* is dominant, the light intensity increases steadily from 200 to 300 langleys/day, the temperature at the surface increases from 4 C to 6 C, and the nitrate concentration decreases from slightly more than 20 μM to as little as 0.5 μM (Bruce, 1968). Laboratory experiments using *Skeletonema* and *Thalassiosira* indicate that *Thalassiosira* should be dominant under these conditions. Thus Curl (1961) found that at 5 C *Skeletonema* could attain only 5% of its maximal photosynthetic rate, regardless of the light intensity. Also the cell division rate was between 1.0 and 1.2 divisions per day at 15 C and above,

regardless of the nitrate level, whereas it was only 0.47 at 10 C and 0.21 at 5 C, even with excess nitrate available. Thus the low early spring temperatures in Auke Bay would seem to be very unfavorable to *Skeletonema* growth and could explain the fact that it is not dominant in the first spring bloom.

Jitts *et al.* (1964) also found light and temperature to be important factors controlling the competition between *Thalassiosira* and *Skeletonema*. He says, "Species succession is favored not only when the temperature rises above the optimum range for one species into the optimum range for another, but these optima themselves depend on the mean light level." He found that, although the optimal temperatures for both these species of diatom were above the 4 C to 6 C range of early spring temperatures in Auke Bay, both species could grow at these low temperatures. However, *Skeletonema* could grow and divide at low temperatures only if the light was above 576 langleys/day, which it is not in early spring in Auke Bay. *Thalassiosira*, on the other hand, thrived at low temperatures if the light level was low. Thus the combination of low light and low temperatures encountered in early spring in Auke Bay prevents *Skeletonema* from thriving and leads to the dominance of *Thalassiosira*.

The influence of temperature and light on species succession may not be independent of the influence of enzyme activity, since enzyme activity itself depends on both temperature and light. The

level of an enzyme present at any given time is determined by the difference in rate of synthesis and rate of degradation of the enzyme. Temperature conditions influence the rate of degradation; the higher the temperature, the faster the breakdown of the enzyme (Kannagara and Woolhouse, 1967). Light, on the other hand, influences the rate of synthesis. Enzyme synthesis seems to be directly related to protein synthesis, and both are enhanced by light. Thus Hageman and Flesher (1960) reported that the nitrate reductase level and the 5% TCA fraction (protein) in corn seedlings placed in the dark increased when the seedlings were transferred to the light. Kannagara (1967) found that nitrate reductase disappears completely after four to five hours in the dark. A second and indirect influence of light on enzyme activity results from the fact that both the energy for the reduction of nitrate and the production of the cofactor NADH are intimately tied up with photosynthesis and respiration, which are themselves affected by light.

Since enzyme activity depends on temperature and light conditions, it is possible that it is partly through their effect on nitrate reductase activity that these two physical factors influence species succession and dominance. The results of the two 15-liter cultures of Thalassiosira, the one at 15 C and the other at 10 C support this idea. The nitrate reductase activities for the cells grown at 10 C are above 3 μM NO_2^-/mg protein-hour, whereas the highest value

obtained for the 15 C culture is 1.1 μM NO_2^-/mg protein-hour. Since all conditions but temperature were the same for the two cultures, the differences in enzyme activity may well be a result of the temperature difference. This would mean that the cells have higher nitrate reductase activities the closer the temperature is to the optimal temperature for the species. Thus, in mid-April to mid-May, when the temperature in Auke Bay is around 5 C and much nearer optimal for Thalassiosira than for Skeletonema, Thalassiosira may actually have a higher nitrate reductase activity than does Skeletonema, resulting in its dominance. By the beginning of August, when the temperature is 13 C or higher, the enzyme activity of Thalassiosira should have decreased to nearly the levels found in the 15 C culture. Then the activity of Skeletonema would be higher and the latter would become the dominant species.

As already stated, the reduction reaction involving nitrate reductase is an enzyme reaction whose kinetics should follow the Michaelis-Menten equation (2) relating enzyme activity or reaction rate to nitrate concentration. If such a relationship exists, a plot of 1/v (1/activity) versus 1/S (1/nitrate concentration in the medium) should yield a straight line. Such a plot of the data for Skeletonema is presented in Figure 2. The scatter of the various points, especially the points 3/27, 5/17 and 5/21 (the latter two are off the graph), would seem to indicate that this enzyme reaction is not governed by

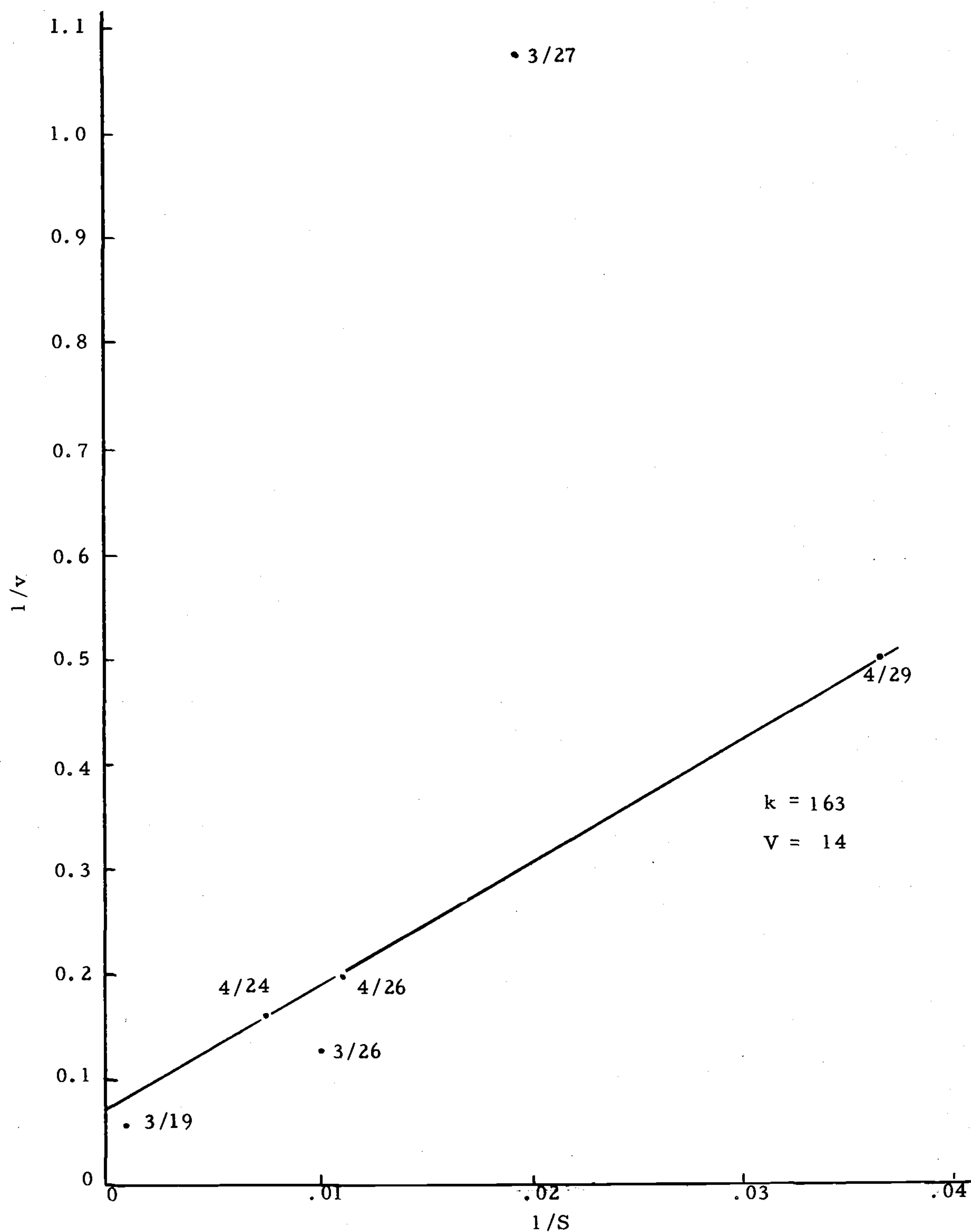


Figure 2. Michaelis-Menten plot of enzyme activity (1/v) and nitrate concentration (1/S) for experiments using Skeletonema (line drawn and extrapolated to obtain constants K and V for one 15-liter culture.

Michaelis-Menten kinetics. However, it is probable that the enzyme reaction rate (activity) is controlled not by the nitrate concentration in the culture medium but by the concentration in the cell itself. Caperon (1965) found that nitrate accumulates in an intracellular pool, demonstrating that absorption is faster than assimilation. This accumulation of nitrate within the cell is also supported by the work of Eppley (1968), who found that about 84% of the nitrate absorbed by _Ditylum brightwelli_ cells in the dark and 40% of that absorbed in the light was still present in the cells as nitrate. If cells store nitrate until it can be reduced, the nitrate concentration in the cells could be significantly different from that in the culture medium. Thus the nitrate reductase reaction itself may be governed by Michaelis-Menten kinetics, but activity as a function of the nitrate level in the medium is not necessarily so governed.

Figure 2 is not sufficient evidence, however, for the conclusion that Michaelis-Menten kinetics do not apply to this data. Of the points plotted here, the three points 4/24, 4/26 and 4/29 represent cells grown in the same 15-liter culture, and these points do fall on a straight line. It is possible that unintended variations in culture conditions caused the scatter among the points of Figure 2 and not the absence of Michaelis-Menten kinetics. Nevertheless, one should not necessarily expect the Michaelis-Menten equation to apply because of the differences between culture and intracellular nitrate

concentrations already discussed.

Few values for nitrate reductase activity of different phytoplankton species are available in the literature, but some comparisons can be made. Eppley (1968) reports a nitrate reductase activity in *Ditylum brightwelli* of up to 5 μmoles NO_2^-/mg protein-hour for cells grown in a medium of about 75 μM NO_3^-. This value would be comparable to those obtained in this study for *Skeletonema* and higher than those for *Thalassiosira.* Eppley also notes that the activity increases with an increase of nitrate level in the culture, as was found with *Skeletonema.* In a later paper, Eppley (1969a) reports a k value for *Ditylum brightwelli* of 110 μM NO_3^-. If we compute a k value for *Skeletonema* on the basis of the line determined by the three points 4/24, 4/26 and 4/29 in Figure 2, we obtain k equal to 163 μM NO_3^-. This again is comparable to the value for *Ditylum brightwelli.*

In a third paper by Eppley (1969b), the k values reported for nitrate uptake by *Ditylum brightwelli* and *Skeletonema* are 0.6 and 0.4-0.5 μM NO_3^- respectively. These are both extremely lower than the corresponding k values for nitrate assimilation of 110 and 163 μM NO_3^-. This is further proof that nitrate uptake and assimilation are distinct processes. Furthermore, oceanic nitrate levels are higher or comparable to the k values for nitrate uptake but never approach the k values for nitrate assimilation. Thus uptake would be expected to proceed much faster than assimilation, resulting in

the intracellular accumulation of nitrate already mentioned.

The data from the experiments indicates several interesting features of the growth patterns of these two species of phytoplankton. A plot of cell numbers per sample versus weight of protein for the same sample (Figure 3) indicates a linear relationship for Skeletonema. A similar plot for Thalassiosira (Figure 4) can be separated into points from the experiments run at 10 C and those run at 15 C. This produces two straight lines of quite different slope. Thus it seems that under constant physical conditions of light and temperature, the amount of protein per cell is relatively constant and independent of nitrate concentration. If these physical conditions are changed, as with the temperature of the Thalassiosira cultures, the cellular protein content changes too. In the case of Thalassiosira, protein content or cell size was much smaller at 10 C than at 15 C. This could mean that the nearer the physical conditions are to optimal, the faster the cell division rate. Without a corresponding increase in the rate of production of cellular organic compounds, a decrease in cell size would result. Jitts et al. (1964) hypothesizes just such a case, saying that if the cell division rate and the photosynthetic rate are affected differently by light and temperature, a change in physical environment may cause a change in cell size.

The data for the Skeletonema culture 4/24 to 4/30 indicate that changes in cell size, as indicated by the amount of nitrate needed to

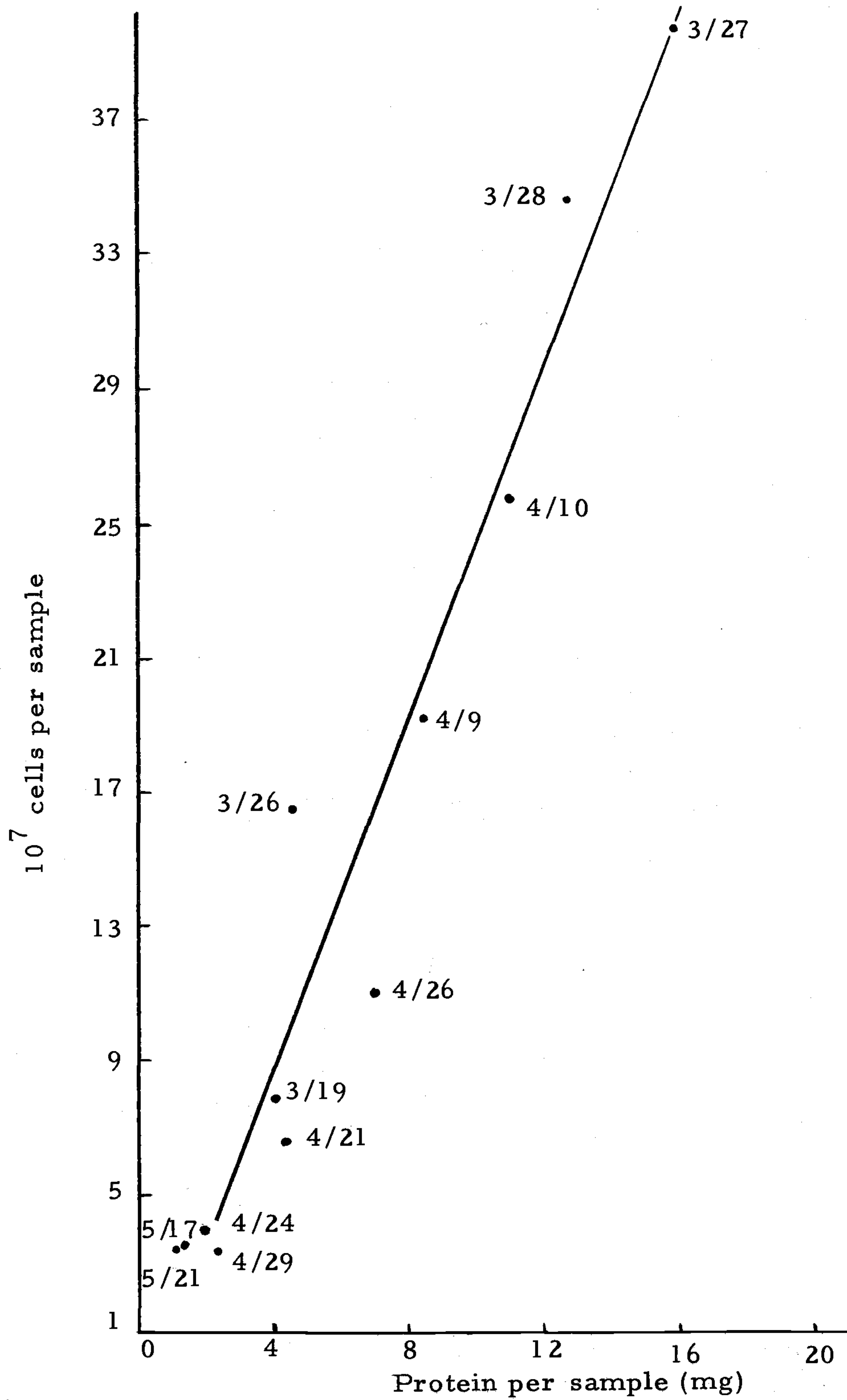


Figure 3. Protein as a function of cell numbers for samples of Skeletonema cultures.

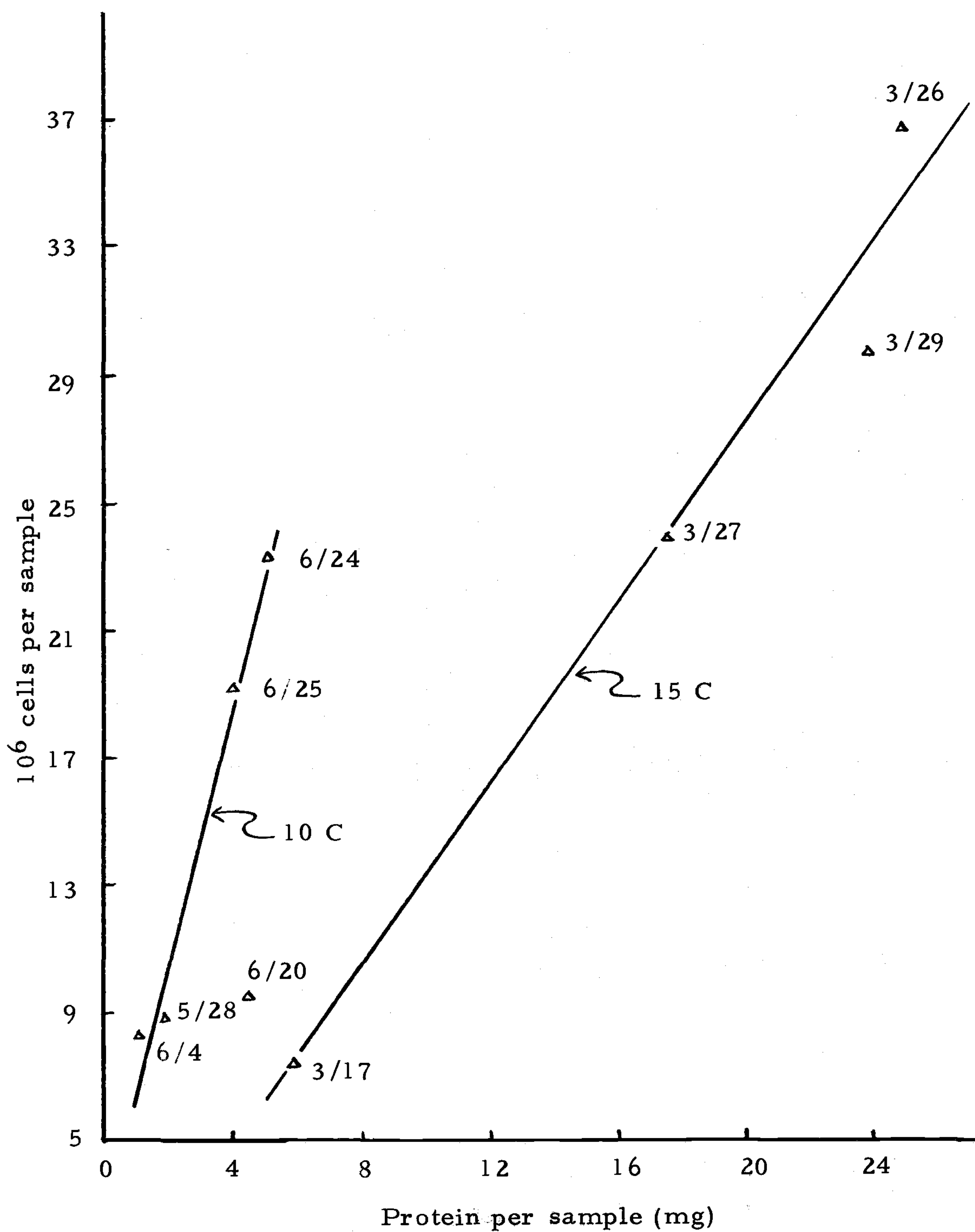


Figure 4. Protein as a function of cell numbers and temperature for samples of <u>Thalassiosira</u> cultures.

produce one cell, may be related to changes in the cell division rate. If we compare the doubling rates for this culture (Figure 5) with the nitrate uptake per cell produced (Figure 6), we see that while the division rate increased from 4/25 to 4/27, the nitrate uptake per cell decreased from 4/26 to 4/27; the division rate leveled off from 4/27 to 4/28 as did the nitrate uptake; then the division rate dropped drastically and the nitrate uptake soared up. Thus, if the division rate is constant, nitrate uptake per cell is constant. If the division rate decreases, the nitrate uptake per cell increases and vice versa. This trend is also apparent from the Thalassiosira data plotted in Figures 5 and 6; the division rate steadily decreased and the nitrate uptake per cell steadily increased. One may conclude that the division rate changes faster than the rate of synthesis of cellular material over the period of growth of the culture. This causes a change in cell size as indicated by the varying amount of nitrate needed to produce one cell. As a result, cells in log phase growth, when cell division rate is rapidly increasing, are smaller than cells found later in the culture when the division rate is rapidly decreasing.

Figure 7 shows the total cell production versus medium nitrate depletion for the two 15-liter cultures Skeletonema 4/24 to 4/30 and Thalassiosira 6/22 to 6/26. The Thalassiosira culture was inoculated from a small culture whose nitrate level was less than 20 μM, while the Skeletonema inoculum was taken from a culture whose nitrate

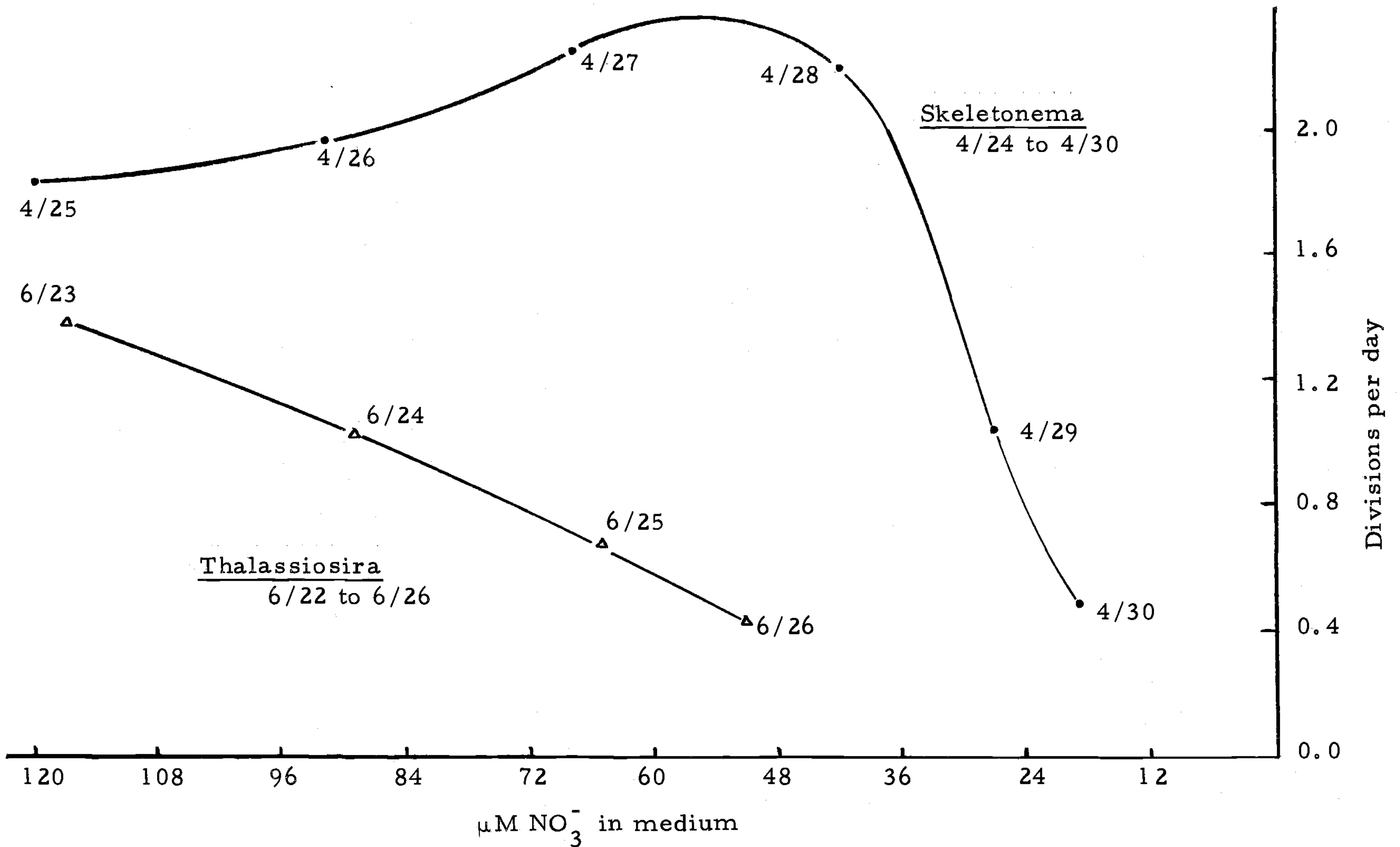


Figure 5. Cell division rates as a function of culture age and nitrate concentration for two 15-liter cultures.

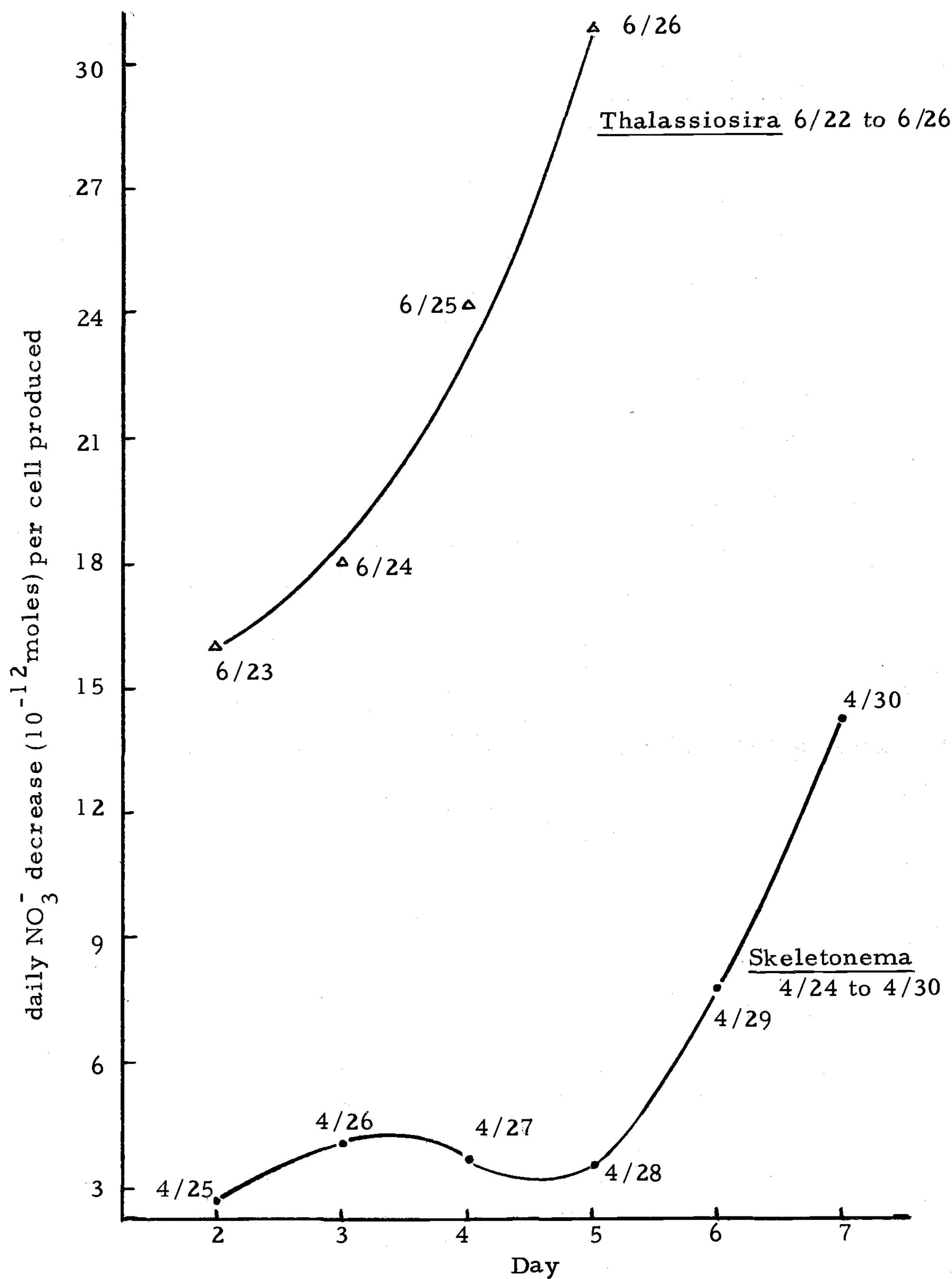


Figure 6. Daily nitrate decrease per cell produced as a function of culture age for two 15-liter cultures.

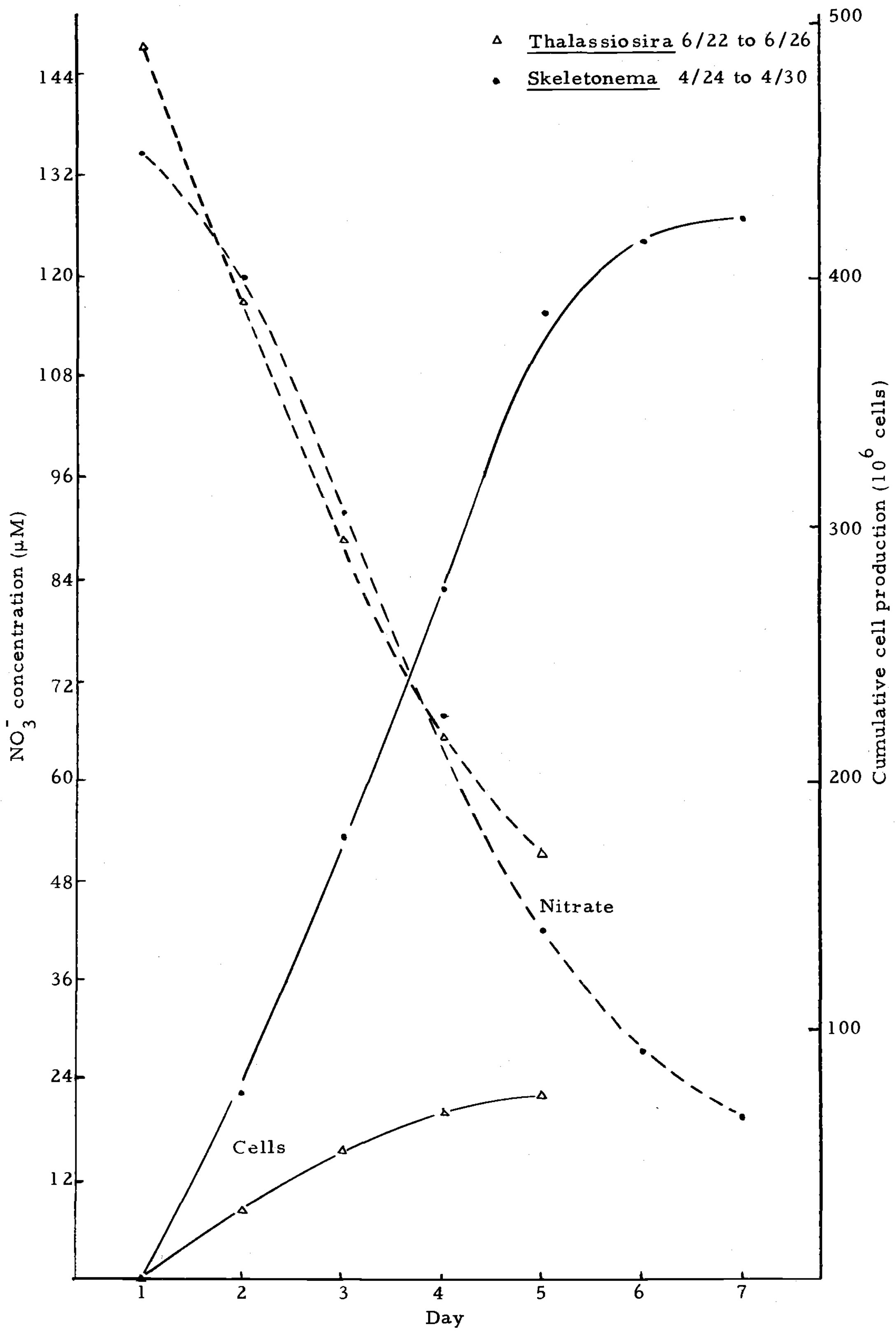


Figure 7. Nitrate concentration and cumulative cell production as a function of time for two 15-liter cultures.

level was greater than 1200 M. Thalassiosira immediately began to deplete the nitrate in the 15-liter culture whereas the Skeletonema culture shows a lag phase in nitrate depletion. This is probably due to the nitrate-starved condition of the cells of the Thalassiosira inoculum, as opposed to the Skeletonema inoculum of cells which had stored up intracellular nitrate from a nitrate-rich medium and could thus continue to grow for some time without absorbing nitrate from their new medium. Their initial nitrate-sufficient condition also explains the increase of nitrate uptake per cell from 4/25 to 4/26 in Figure 6, which would not normally be expected from cells whose division rate was increasing.

Figure 7 shows a much earlier leveling off of nitrate depletion and cell production for Thalassiosira than for Skeletonema, in spite of the fact that both had been at comparable nitrate levels. By the fifth day the doubling rate of Thalassiosira was only 0.43 divisions/day while that of Skeletonema was still 2.10 divisions/day. In both cases production leveled off before the nitrate was completely depleted and with other nutrients still available in excess, perhaps because of the initial unnaturally high nitrate level in the cultures.

Skeletonema proved to be a much more adaptable organism than Thalassiosira in several ways. Thalassiosira cells grown in an enriched medium initially containing 1750 μM NO_3^- soon became misshapen, being very elongated rather than their normal octagonal

shape. These elongated cells did not form chains and their growth rate was slower than normal. Thus high nitrate levels seem to be inhibiting to growth of *Thalassiosira*. Also, *Thalassiosira* cells transferred from one culture to a second one at a slightly different temperature failed to grow. This temperature variance intolerance was also demonstrated by the fact that the effluent from the centrifuge had to be equilibrated to the temperature of the remaining 15-liter culture before being poured back in or the remaining cells would not continue to grow. Neither of these problems was encountered while working with *Skeletonema*, which is viable over a much wider range of conditions.

CONCLUSION

Skeletonema costatum, a species which is dominant in Auke Bay, Alaska in late summer when nitrate levels are very low and the temperature is above 12 C, was found to have higher nitrate reductase activities at all nitrate levels at 15 C than *Thalassiosira nordenskioldii*, which is dominant in early spring when nitrate levels are higher but the temperature is around 5 C. In addition, the enzyme activity of *Thalassiosira* was found to be higher at 10 C, near its optimal temperature for growth, than at 15 C. These results are consistent with the hypothesis that the ability to assimilate nitrate, as measured by nitrate reductase activity, may be an important parameter influencing seasonal species succession under nitrate-limiting conditions. The species dominant at low nitrate levels should have and was found to have a higher enzyme activity. The data for *Thalassiosira* further suggest that it is partially through its effect on enzyme activity that temperature influences growth rate and species dominance. Finally, in looking at the relation of the nitrate reductase activity to the nitrate concentration in the medium, the normal Michaelis-Menten kinetics of enzyme reactions may not be applicable.

These results are significant in themselves. But they also have implications for relating chemical and biological processes in

the oceans. First of all, they indicate that the nitrate level in the oceans plays an important role in determining not only total numbers of phytoplankton in a bloom but also which species may be dominant in a given bloom. Second, the concept of growth-limiting nitrate levels in the oceans should connote not only that there is insufficient nitrate to allow production of large numbers of cells, but also that the cells have a much lower nitrate reductase activity at low nitrate levels and thus production is slower. Finally, the fact that different species have different nitrate reductase activities means that each phytoplankton species is unique in its efficiency of utilization of nitrate. It has already been shown that the ratio of C:N in phytoplankton can change as nitrate becomes limiting, and it may be that the degree to which this ratio changes depends on the enzyme activity of each species. In addition, if phytoplankton absorb nitrate faster than they reduce it, as indicated by pools of intracellular nitrate, then a change in nitrate in the water may not produce the change in O_2 and CO_2 which would be predicted by the Redfield model.

SUGGESTIONS FOR FURTHER INVESTIGATIONS

The ideas discussed in this thesis siggest several areas for further investigation. First, nitrate reductase levels should be correlated with intracellular nitrate levels to see if, indeed, a Michaelis-Menten relation of reaction rate to nitrate level is found. Second, the activities of cells grown in a chemostat where the nitrate level can be maintained below 20-30 μM should be measured, since these are the conditions encountered in the ocean. And third, the effect of temperature and light on enzyme levels should be studied, since it may be in this way that physical factors control biological growth and species dominance.

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