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Abstract approved: _____
H. C. Curl, Jr.

This research addresses the question: what is the relationship among nitrogen uptake, assimilation and phytoplankton growth during changing or transient nitrogen environments. A new culture system, diffusion culture, was developed to provide realistic low nitrogen environments. Physiological parameters for two species of diatom, Skeletonema costatum and Thalassiosira aestivalis were determined for a variety of nitrogen regimes, progressing from non-nitrogen limited, to low-nitrogen adapted, and to nitrogen starved cultures. Additions of nitrate, or nitrate and ammonium, were made to each regime; uptake rate, assimilation rate, and changes in cell physiological parameters were measured. Internal pools of nitrate, ammonium, and total free amino acids were measured and related to both conditioning regime and nitrogen perturbation. Two mechanisms for nitrate reduction were found, one that is coupled to nitrate uptake and another independent of uptake. The rate of nitrate reduction was not solely a function of internal nitrate concentration. Ammonium inhibited nitrate uptake, but the inhibition could

not be explained by changes in intracellular ammonium or free amino acids. A conceptual model of nitrate uptake for transient environments was developed.

Variations in Phytoplankton Physiological Parameters During Transient Nitrogen Environments

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Typed by Deanna L. Cramer for John Michael DeManche

This dissertation is dedicated to my daughter, Lori.

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VARIATIONS IN PHYTOPLANKTON PHYSIOLOGICAL PARAMETERS DURING TRANSIENT NITROGEN ENVIRONMENTS

INTRODUCTION

Marine phytoplankton are the major source of organic material for most of the world ocean. For this reason, understanding of the factors controlling the abundance of phytoplankton, their growth rates and the species composition of populations, is of primary importance both from an ecological and economic view. The abundance of phytoplankton and the transfer of phytoplankton growth through the marine food web depend on many factors; turbulence, sinking, horizontal and vertical advection, and grazing by herbivores. But the growth rate of phytoplankton populations, the rate of conversion of light and inorganic nutrients into organic material, is of overriding importance. The growth rate of marine phytoplankton is a complex function controlled by environmental factors including light, temperature and nutrient availability. One of the major questions in marine phytoplankton research is: what is the functional relationship among nutrient concentration, nutrient supply rates, phytoplankton nutrient uptake rate, and the assimilation of nutrients leading to phytoplankton growth? Of the major nutrients, nitrogen is the one which most often limits phytoplankton growth in marine environments (Thomas, 1966, 1969; Ryther and Dunstan, 1971). Light and temperature modify the rate at which nitrogen is used. Other nutrients such as phosphorus and silicon, and micronutrients such as vitamins and trace metals, may be temporarily limiting, or limiting in certain locations. However, on a world wide basis nitrogen

availability and the rate of supply of nitrogen to the lighted surface determine the extent of phytoplankton production.

Understanding the role of nitrogen in determining phytoplankton standing stocks, production rates, and species succession has been one of the major research goals in phytoplankton ecology for more than a decade. The problem is compounded by many factors including: 1) the non-constant influx of nitrogen into the upper, well-lighted portion of the water column; 2) the multiplicity of forms of nitrogen available for phytoplankton growth including nitrate, nitrite, ammonium, urea and amino acids; and 3) the variety of nitrogen utilization mechanisms and capabilities of various species of marine phytoplankton. An important question which has received little attention is: what is the relationship among uptake, assimilation and phytoplankton growth during transient or changing nitrogen environmental regimes with their correspondingly variable nitrogen influx rates and concentration changes? This dissertation represents an initial inquiry into aspects of this question.

Historical Perspectives

Uptake and Growth

One of the earliest statements concerning nutrient limitation was that of Liebig's Law of the Limit (Liebig, 1840) for terrestrial plants, stating that the total plant yield would ultimately be limited by that nutrient in least abundance relative to the needs of the plant. This concept was extended to marine environments by Redfield (1934) who made extensive analyses of the abundance of nutrients in deep sea water relative to the composition of plankton, finding that nutrients were utilized in proportion to the chemical composition of the

organisms. This concept was reflected in early models of phytoplankton production, with production assumed to be directly proportional to nutrient replenishment (Riley, 1946; Riley et al., 1949) and uptake either linearly proportional to nutrient concentration or constant when nutrient concentration exceeded some saturating level (Steele and Menzel, 1962). These models made gross estimates of phytoplankton standing stock based on measurable chemical parameters and assumptions relating standing crop to concentration of limiting nutrient, which was assumed to be phosphorus. That these models worked at all was partly fortuitous, partly a result of the choice of relationship between the measured parameters and the limiting nutrient, and partly the result of only looking at very coarse time scales on the order of weeks to months. What was gained in generality was lost in realism and precision. Subsequent efforts (Riley, 1956) indicated that nitrogen, not phosphorus, was the limiting nutrient. These early models were simplistic and empirical; they did not establish a cause-effect or functional relationship between nutrient availability and phytoplankton growth rates.

A new paradigm was introduced when Dugdale (1967) suggested that phytoplankton nutrient uptake followed a hyperbolic relationship with nutrient concentration, similar to that proposed by Monod (1950) for carbon uptake by bacteria. This functional relationship is analagous to the enzymatic kinetic function of Michaelis and Menten (1913).

Although several formulations have been used, the one of Dugdale (1967) is shown here and will be used in this research:

$$V = \frac{V_{max} \cdot (S)}{K_s + (S)} \qquad (1)$$

where V is the nitrogen specific uptake rate (nitrogen taken up per nitrogen biomass per unit time), V_{max} is the saturated or non-nitrogen limited uptake rate, (S) is the ambient substrate concentration and K_s is the half-saturation constant, i.e. the concentration at which uptake is equal to one-half the maximum rate. This equation describes a hyperbola with a zero intercept (uptake ceases as nutrient concentration approaches zero). Uptake, V, asymptotically approaches V_{max} as substrate concentration becomes infinite. Michaelis-Menten kinetics have certain inherent assumptions: 1) that the reaction proceeds by the reversible binding of substrate to an active site and subsequent irreversible transfer of substrate into the cell; 2) that the reaction is at steady state; 3) that only initial rates are measured, or that product inhibition or other internal feedback processes are insignificant; and 4) that the substrate concentration is not decreased or exhausted during the period that rates are measured. If Michaelis-Mention kinetics are to be extended to general models of phytoplankton uptake, additional assumptions are required: 1) that K_s is an inherent property of a phytoplankton population and not subject to short term environmental variations; and 2) that V_{max} is constant over short time periods. These assumptions were assumed valid, and much effort was expended trying to characterize these constants for a variety of environmental conditions and for different species of phytoplankton (Eppley et al., 1969; Eppley and Thomas, 1969; MacIsaac and Dugdale, 1969). Differences in half-saturation constants were proposed as one

factor determining relative competitive ability among species in nutrient limited environments (Eppley et al., 1969).

Difficulties in using this approach soon became apparent. It had long been noted that under laboratory conditions cell growth could continue for significant periods of time after nitrogen exhaustion from the medium (Ketchum, 1939). Furthermore, blooms of phytoplankton were observed under conditions of low environmental nitrogen without a preceding increase in ambient nitrogen (Bruce, 1969). The coupling function between nitrogen uptake and cell growth was unknown. When measurements of V_{max} and K_s were made, instead of the parameters being constant as assumed, great variability was found, even between clones of a single species. Light was found to be a controlling factor in determining maximum nitrate uptake rate V_{max} (MacIsaac and Dugdale, 1969). The measured intraspecific differences in K_s were thought to be due to selective pressures favoring low K_s in nutrient limited environments (Carpenter and Guillard, 1971). Laboratory methodological problems arose through failure to consider that uptake and growth (assimilation) were only loosely coupled processes and subject to both short-term and long-term adaptation. Early attempts at measuring V_{max} and K_s violated several of the assumptions required for Michaelis-Menten kinetics to be valid. Cultures were usually starved of nitrogen, then uptake rates were calculated by measuring the disappearance of nitrogen when various concentrations were added (Eppley and Coatsworth, 1968; Eppley, et al. 1969). This method suffered from the defect that initial rates were not measured, only an integrated rate over some long time period, often one hour or longer.

As investigators began using steady-state chemostats to produce phytoplankton populations with various degrees of nutrient limitation, major deviations from the uptake parameters of batch culture were observed. Chemostats are self regulating continuous cultures where the dilution rate (flow rate/vessel volume) determines the phytoplankton growth rate, for dilution rates less than the maximum growth rate of the phytoplankton. Phytoplankton growth rate, dN/dt, is given by the equation:

$$dN/dt = N \cdot \mu - N\ D\ , \tag{2}$$

where N is the phytoplankton biomass in terms of limiting nutrient, μ is the nutrient specific growth rate, and D is dilution rate. At steady state, $dN/dt = 0$, $\mu = D$, and nutrient specific uptake rate, V, equals the nutrient specific growth rate ($V = \mu$). Caperon and Meyer (1972a) could not detect a relationship between ambient nutrient concentration in steady state chemostats and nutrient uptake rate. Caperon and Meyer (1972a), working with nitrogen as limiting nutrient, and Droop (1973), working with vitamin B_{12}, suggested that the apparent discrepancy between uptake in chemostats and batch culture could be explained if nutrient per cell, or cell quota (Q), rather than ambient nutrient concentration, controlled cell growth rate. They found that the limiting nutrient per cell was a hyperbolic function of steady state dilution rate; i.e., a plot of $1/\mu$ vs. $1/Q$ was a straight line. Thus growth rate could be expressed as a function of limiting nutrient per cell:

$$\mu = \frac{\mu_{max} \cdot (Q-Q_o)}{(Q-Q_o) + K_g} \tag{3}$$

where μ_{max} is the maximum growth rate, Q_o is the cell quota when the growth rate is zero, and K_g is the rate constant for growth (Caperon and Meyer, 1972a). This equation is analogous to the Michaelis-Menten equation for uptake, with $(Q-Q_o)$ replacing (S). Since at steady state, uptake equals growth, Michaelis-Menten equations relating uptake rate to nutrient concentration can be algebraically combined to the growth equation to yield a relationship between ambient nutrient concentration and growth rate (Dugdale, 1976, 1977; Goldman, 1977). Mathematically, these models are equivalent. The one of Dugdale (1977) is representative:

$$\mu = \frac{V'_m S}{K_s + S} \tag{4}$$

where V'_m is no longer a constant but a variable such that the maximum specific uptake rate is a function of the maximum absolute uptake (per cell) divided by the amount of limiting nutrient per cell (Q). The rate constant for growth, K_g, is related to the constant for uptake as follows (Dugdale, 1976):

$$K_g \approx 2 \cdot Q_o \cdot K_s \frac{\mu}{V_{max}} \quad . \tag{5}$$

The net result is a rate constant for growth approximately an order of magnitude lower than that for uptake. This equation predicts ambient nutrient concentration at steady state to be much lower than expected had Q not been variable. The concentration predicted is near or below limits of detection for most nutrients, explaining the apparent lack of relationship between growth rate and ambient concentration in chemostats (Caperon and Meyer, 1972a).

The primary defect of such equations is that they were so derived as to be valid only at steady state. None of the assumptions used in the derivation need hold, a priori, for transient conditions (Goldman, 1977). Numerous alternative models can fit the experimental data for steady-state chemostats equally well, but have widely divergent predictions for uptake during transient conditions (F. M. Williams, personal communication). To apply a single steady-state equation for both uptake and growth in transient environments, several assumptions in addition to those already stated for Michaelis-Menten kinetics are required: that intracellular inorganic pools either do not represent a significant fraction, or represent a constant fraction, of total intracellular nutrient during transient environments; and that nutrient uptake and assimilation are coupled processes under transient as well as steady state environments.

Measurements of K_s in batch culture at very low phytoplankton concentrations confirmed the chemostat results for uptake and growth at low nutrient concentrations (Paasche, 1975; Steeman-Nielson, 1978). However, these low biomass batch cultures suffer from the same assumptions as steady-state chemostats. In essence, only the behavior of cells given several days adaptation to constant low nutrient levels is examined. They do not address the question: what are the functional relationships among uptake, growth, and nutrient concentration for cells exposed to transient nutrient environments which are changing faster than the cell's ability to adapt through alterations in cell quota?

A different approach was taken by Caperon and Meyer (1972b) and others (Conway and Harrison, 1977; Conway *et al.*, 1976) as a means of assessing the capacity for phytoplankton populations, with known degrees of nutrient limitation, to utilize nutrients. Chemostats were used to establish a known deficiency level in a culture, after which the culture was perturbed by a large addition of the limiting nutrient. This perturbation technique differed from previous analytical methods in that the time course of nutrient disappearance was monitored by sampling repeatedly at short time intervals. From this time series an integrated hyperbolic function could be fit directly to the data (Caperon and Meyer, 1972b) to yield estimates of V_{max} and K_s:

$$V_{max} \cdot t = S_o - S + [K_s \cdot \ln(S_o/S)] \quad , \tag{6}$$

where S_o is the initial nutrient concentration at time $t = 0$. A plot of $t/\ln(S_o/S)$ against $(S_o - S)/\ln(S_o/S)$ is a straight line with slope $1/V_{max}$ and intercept K_s/V_{max} on the $t/\ln(S_o/S)$ axis. Alternatively a non-linear least squares regression could be fit directly to the time series using Marquardt's algorithm (Conway, 1970). This perturbation approach avoided some of the theoretical problems of earlier chemical methods and most of the methodological ones. Several problems remained. Internal inorganic pools were still assumed to be insignificant, and only populations conditioned to the constant low-nutrient regimes of steady-state chemostats were studied. Furthermore, the estimation of V_{max} and K_s directly from exponential functions suffers from a statistical difficulty known as ill-conditioning (Cornish-Bowden, 1976), where a wide range of constants may fit the data equally well. This problem

is compounded when one may be fitting the wrong equation to the data set. In general it is very difficult to choose among nonlinear multiparameter models due to limited data and data variance at critical regions of the function. Over the long time periods necessary for measurements of these types, the theoretical justification for assuming the insignificance of product inhibition or other feedback processes is lacking (Cornish-Bowden, 1976). In fact, the results of time series measurements such as those by Caperon and Meyer (1972b) and others (Davis et al., 1978; Conway et al., 1976; Conway and Harrison, 1977) indicate that uptake following a perturbation is not a simple hyperbolic function of concentration, but may have several modes. These include a highly variable short term "surge uptake", V_s, immediately after the perturbation; a long term, internally controlled uptake rate not limited by nutrient concentration, V_i; and a concentration dependent uptake rate V_e. For a summary of some of the latest thinking on non-Michaelis-Menten uptake kinetics one is referred to Dugdale (1977). The issue is still controversial (McCarthy and Goldman, 1978; Davis et al., 1978; Droop, 1978; DeManche et al., 1979), and no clearly accepted paradigm for nutrient uptake has yet emerged.

Internal Pools

It has been long recognized that inorganic nitrogen could accumulate in marine phytoplankton cells. Ketchum (1939) postulated internal nutrient reservoirs to explain the continuation of growth observed after nutrient depletion in batch cultures. Eppley and Coatsworth (1968) found evidence for storage of intracellular nitrate that had been taken up in the dark. However, studies of the variations in internal nitrogen content as a function of nitrogen limitation or as a

function of fluctuations in the availability of inorganic nitrogen are scanty. Conover (1975) measured both inorganic and organic pools in the diatom Thalassiosira fluviatilis at non-limiting and starvation levels of nitrogen, and found nitrate pools as large as 420 m moles per liter of cell volume (mM) during growth on nitrate. The levels dropped to 20 mM or less during starvation. Ammonium pools were variable but in the range of 20 to 80 mM. Collos and Slawyk (1976) measured internal nitrate relative to total nitrogen in shipboard cultures of natural phytoplankton over a 56 h period, and found diurnal variations ranging from 3.1 to 20.6 ng-atom NO_3-N/ug-atom particulate-N (0.3 to 2.1%). Eppley and Rogers (1970) found internal ammonium and nitrate concentrations of 10 and 40 mmoles/liter of cell volume in the diatom Ditylum brightwellii after re-addition of nitrate to a culture which had a brief history of nitrate starvation. Bhovichitra and Swift (1977) measured internal concentrations of both ammonium and nitrate in large dinoflagellates following additions of nitrate. The presence of ammonium internally with nitrate as the only nitrogen source is not surprising since ammonium is an intermediate in the reduction of nitrate, and a key metabolite in both amino acid synthesis and chlorophyll production (Syrett, 1962).

A simplified schematic representation of nitrogen uptake and assimilation in phytoplankton is shown in Figure 1. Although nitrite is also an intermediate in nitrate reduction, it is usually not considered to be a significant fraction relative to nitrate or ammonium (Eppley and Rogers, 1968; Lundy, 1972).

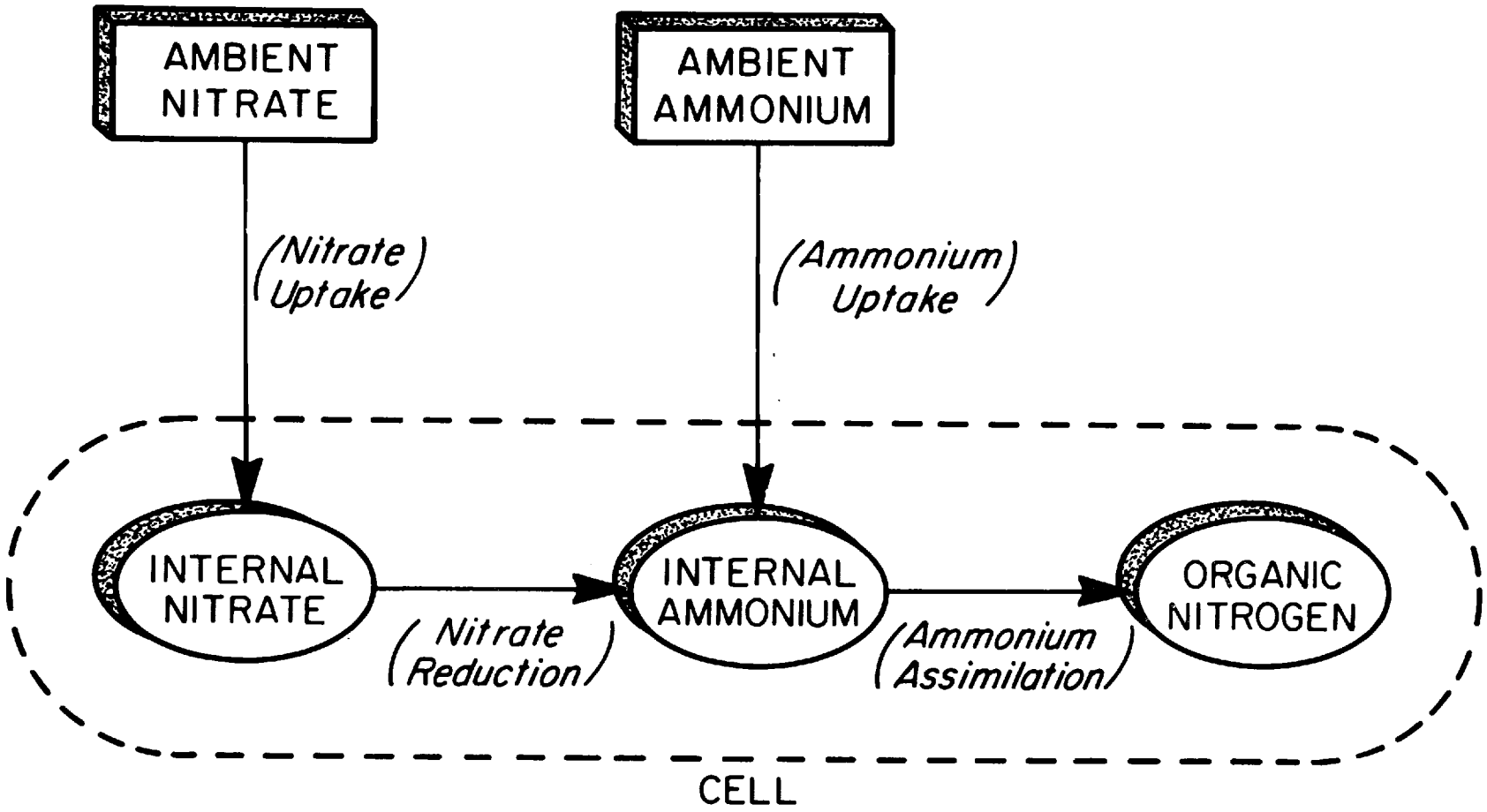

Figure 1. Schematic of nitrogen uptake and assimilation.

Models

Although many models of phytoplankton nutrient utilization have been developed (Droop, 1973; Caperon and Meyer, 1972a; Goldman, 1977), these models are based on the concept of a cell as a homogeneous unit which can be totally defined by integrated properties. Internal pools are included as total nutrient per cell, irrespective of form. Short term variations in internal parameters are ignored, as are changes in the relative importance of various fractions of internal nutrients.

Several attempts at explicitly incorporating internal cell nitrogen in its various fractions have been proposed or attempted. Williams (1970) compartmentalized cells into two fractions, structural and synthetic. Grenney et al. (1974) expanded this type of model into more practical fractions, namely inorganic, organic and structural nitrogen. Their model exhibited a good fit to Caperon's data set of Isochrysis cell number vs. dilution rate (Caperon, 1969), but required the assumption of an unreasonably large nitrogen excretion factor.

Further developments have been slow in coming, due to the increased complexity of the models. Dugdale (1977) has pointed out that further understanding of phytoplankton nutrient utilization, except under steady-state conditions, will require such internal control models. Recently, first generation internal control models have been developed for silica (Davis et al., 1978) and nitrogen (DeManche et al., 1979), but further developments await more complete experimental data.

Multiple Nitrogen Forms

Some of the complicating factors in modeling nitrogen utilization are the multiple forms of nitrogen available for phytoplankton, the transformation of one form into another within cells, and interaction or competition among forms. All marine phytoplankton can use ammonium, most can use nitrate and nitrite (Syrett, 1962), and many can use organic nitrogen such as urea (McCarthy, 1972) and amino acids (Bruce, 1969; Schell, 1974).

The sources of the three principal nitrogen forms, nitrate, ammonium, and urea, are different (Dugdale, 1967). Nitrate is supplied primarily by mixing and upwelling, while ammonium and urea primarily result from regeneration and anthropogenic input. The relationship between nitrogen form and utilization by phytoplankton is further complicated by observations that under some conditions ammonium is preferred over nitrate (Eppley and Rogers, 1970), while under other conditions uptake appears to be competitive (Bates, 1976; Bienfang, 1975), and under still other conditions nitrate is preferred to ammonium (Lundy, 1972).

Objectives

The research presented in this dissertation represents an effort to gain insight into the patterns of uptake and assimilation of various forms of nitrogen by marine phytoplankton; an effort to make inferences about the mechanisms involved; and an effort to evaluate the suitability of physiological parameters as indicators of nitrogen assimilatory capabilities. Several hypotheses relevant to current paradigms of nutrient utilization are tested, including: 1) that uptake and

assimilation of nitrogen are closely coupled processes; 2) that nitrate reduction is a function solely of internal nitrate concentration; 3) that internal pools of inorganic nitrogen and free amino nitrogen are small and constant, and need not be considered in models of phytoplankton nutrient utilization; and 4) that internal concentrations of ammonium or total free amino acids regulate and inhibit nitrate uptake during ammonium assimilation. A new conceptual framework was established to replace those hypotheses which were found to be inadequate for transient nitrogen environments, and to further investigate and model development of nitrogen utilization during transient nitrogen environments.

METHODS

Culture Methods

Two species of marine diatoms were used in this research, Skeletonema costatum (Greville) Cleve and Thalassiosira aestivalis (Gran). Both species are chain-forming centric diatoms and are abundant in temperate and subarctic coastal waters. They seldom bloom at the same time, but often one species will preceed the other in abundance. Both species were isolated from Auke Bay, Alaska, and were maintained in unialgal culture using a modified F/2 medium (Guillard and Ryther, 1962; Table 1). Cultures were not axenic, but bacteria were never abundant in exponentially growing cultures. Only innocula from log-phase cultures were used in these experiments.

Lighting for all experiments was provided by a bank of eight cool-white fluorescent bulbs on a continuous basis at 200 to 250 μ Einsteins $m^{-2}sec^{-1}$. The light was filtered through 6 mm thick, clear plexiglass, effectively removing all UV radiation.

Temperature control was maintained using an environmental room supplemented with a circulating water bath to within ±0.2°C.

Analytical Methods

Nutrients were measured using a Technicon Autoanalyzer®. Nitrate and nitrite were not determined separately but combined as total nitrate plus nitrite by the method of Wood et al. (1967). Ammonium was determined by the phenol hypochlorite method of Solorzano (1969). Amino acids (total) were measured using an automated

Table 1. Modified F/2 Culture Medium.

Major Ions, Concentration per liter of seawater:		
$NaH_2PO_4 \cdot H_2O$	5 mg	36.2 µg-atom P
Fe sequestrene (Iron EDTA)	0.5 mg	1.16 µg-atom Fe
$Na_2SiO_3 \cdot 9H_2O$	15 mg	53.5 µg-atom Si
$NaHCO_3$	0.1 g	
Thiourea	0.5 mg	
$NaNO_3$	Various amounts added in each experiment. Stock cultures maintained on 50 or 100 µg atom N liter.	
Vitamins, Weight per liter of seawater:		
Thiamin HCl	0.1 mg	
Biotin	0.5 µg	
B_{12} (Cyanocobalamin)	0.5 µg	
Trace Metals, Concentration per liter of seawater:		
$CuSO_4 \cdot 5H_2O$	0.098 mg	0.39 µg-atom Cu
$ZnSO_4 \cdot 7H_2O$	0.022 mg	0.076 µg-atom Zn
$CoCl_2 \cdot 6H_2O$	0.010 mg	0.042 µg-atom Co
$MnCl_2 \cdot 4H_2O$	0.18 µg	0.91 ng-atom Mn
$Na_2MoO_4 \cdot 2H_2O$	0.015 µg	0.062 ng-atom Mo

fluorometric method similar to the method of Benson and Hare (1975). Reagents were prepared according to their recommendations for a manual method. Equal volumes of reagent and sample were mixed, using a Technicon Pump II®, at a rate of 0.6 ml min^{-1} each, with a delay of approximately one-half minute between mixing and detection. Fluorescence was measured using an Aminco® fluorometer with a micro-flow-cell. Glutamic acid was used as a standard. Although the sensitivities for all amino acids are not equal (arginine is underestimated), the sensitivities for most are similar (Benson and Hare, 1975). The method has the advantage of being simple, sensitive, and uses only a small sample volume. Relative values for amino acid mixtures should be accurate unless major shifts in relative amino acid proportions occur for amino acids with large sensitivity differences. The method is not insensitive to ammonium; however, ammonium interference was minimized by reducing the time of reagent-sample mixing prior to detection. Full fluorescence development requires approximately one minute for ammonium but only one-half minute for amino acids. Interferences from ammonium were minimal except when ammonium was used as a nutrient. In such cases ammonium was determined separately and an equivalent amino-N correction(22.5% of the ammonium concentration) subtracted from the amino acid data. The method was relatively insensitive to both polypetide and protein nitrogen. Tests with polyglutamic acid and casein showed interferences of approximately 1%, and no corrections for protein nitrogen were applied to the amino acid data.

Cellular <u>in vivo</u> fluorescence was measured using an Aminco® fluorometer with a coproporphyrin standard as recommended by Turner

Designs®. All fluorescence values are reported in relative units with one unit equivalent to 50 µg coproporphyrin liter^{-1}.

Cell volume was determined with a model ZBI Coulter Counter® and a P64 Channelizer® using a 200 µ aperture. The Coulter Counter was calibrated against spherical latex particles (Particle Data Inc.®) and checked with an optical micrometer. The data from the Channelizer® were processed by an on line DEC PDP8® computer and were summed over the particle size spectrum to yield the volume of particles in units of 10^9 µm^3 liter^{-1}.

Particulate (cellular) carbon and nitrogen data were obtained with a Carlo Erba Elemental Analyzer®. Culture medium containing phytoplankton cells was gently filtered (less than 0.1 atm vacuum differential) through 13 mm Whatman GFA® glass fiber filters. Volume filtered ranged between 20 and 25 ml and required approximately two minutes to filter. The filters were not prewashed or precombusted. Blanks for carbon and nitrogen were low relative to sample size. Average blanks were subtracted from the calculations. After filtration the filters were desiccated at 60°C for one to two days, then stored in tightly capped glass vials until analysis. Acetanalide was used as a standard for the combustion. The precision of the analysis was approximately 5% for carbon and 10% for nitrogen for the size sample usually taken. The precision of the C/N ratio was slightly better than that for carbon, indicating that one of the largest sources of variability in the C and N analyses was the lack of precision of the actual volume filtered.

Diffusion Culture

Culture conditions ranging from non-nitrogen limited to various degrees of nitrogen limitation were created through the use of diffusion cultures. The diffusion culture is a modification of the dialysis culture used for bacterial work (Schultz and Gerhardt, 1969). As originally designed for bacteria, dialysis cultures provided a slow continuous flux of nutrients and were used in this form by Jensen et al. (1972) for marine phytoplankton. However, the low flux of dialysis cultures was not suitable for the type of physiological studies presented here. The flux across a membrane (usually dialysis tubing or membrane filter for dialysis cultures) is primarily a function of the concentration gradient across the membrane and follows Fickian diffusion laws where:

$$\text{Diffusion Rate} = \text{Diffusion Coefficient} \times \text{Concentration Gradient} \times \text{Area}.$$

As phytoplankton growth lowers the concentration on one side relative to the other, molecular diffusion increases, tending to reduce the gradient. The flux rate will continue to increase as the concentration gradient increases up to a maximum rate when the concentration on one side approaches zero. However, phytoplankton growth, which up to this time had been exponential, can continue in a linear mode with the rate of growth limited by the maximum diffusion rate. The specific growth rate necessarily decreases as the biomass increases and absolute uptake rate remains constant. Growth continues in this linear mode until the biomass becomes so large that: a) some nutrient other than nitrogen

becomes limiting; b) the culture is light limited; c) self-inhibition occurs; or d) the flux is inadequate to meet cell maintenance requirements. At this point cell scenescence and death increase rapidly. This progression of culture conditions, a consequence of unrestrained cell growth and limited nutrient flux, make the dialysis culture poorly suited to studying intermediate states of nitrogen limitation. I modified the typical dialysis culture by substituting nylon or polyester screens for the membrane filter, and by adding a high speed magnetic stir bar to the abiotic side, increasing the rate of diffusion several orders of magnitude (Table 2). Throughout the experiments reported here, a screen made of polyester (PECAP®, Tetko Inc.®) with a stated pore size of 10 μm was used. Actual pore size measured by passage of spherical latex particles was 16 μm. The stirring speed varied somewhat but was approximately 1000 rpm. The ratio of culture volume to screen surface varied depending upon the configuration used for the individual experiments but was approximately 10 cm throughout. In addition, an outflow was provided for the biotic chamber, whereby cells could be pumped out. The loss of culture through this outflow was continually replaced by maintaining constant volume in the biotic chamber through use of a level control. A typical diffusion culture system is shown in Figure 2. The outflow rate was minimal compared to the pumping rate through the lower chamber and to the diffusion rate. By setting the outflow rate equal to the anticipated growth rate, the biomass in the upper chamber could be held approximately constant. Moderate-biomass cultures (10 to 15 μg-atom particulate-N $liter^{-1}$) have been maintained for several days at ambient nitrate concentrations

Table 2. Diffusion Rates for Various Screens.*

Screen Type	Diffusion Rate (ml min^{-1})
80 µm mesh Nitex®	253
15 µm mesh Nitex®	91
10 µm mesh Nitex®	51
10 µm mesh PECAP®	42
1 µm mesh PECAP®	27
5 µm pore size Millipore®	7
0.45 µm pore size Millipore®	0.5

*For 4.5 cm diameter screens or filters used in diffusion culture apparatus (Figure 2) at maximum stirring speed permitted by magnetic stirrer.

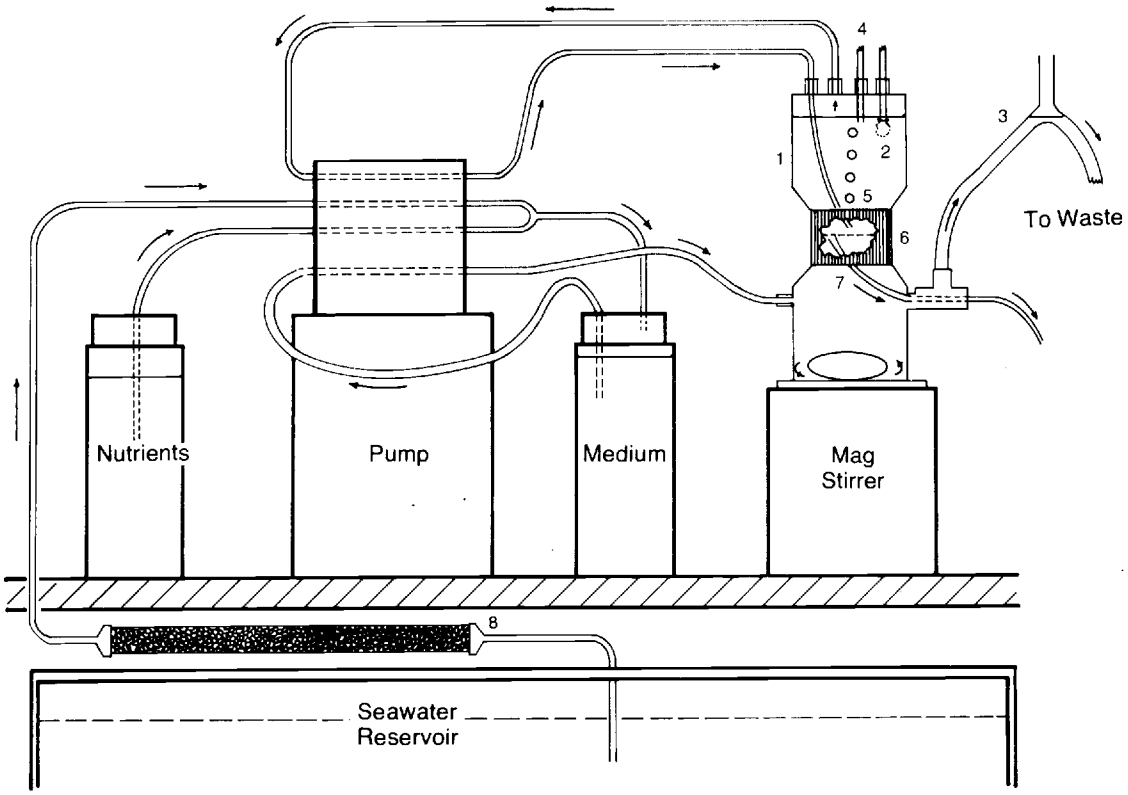

Figure 2. Schematic of diffusion culture apparatus: (1) upper chamber with phytoplankton; (2) 10 µm PECAP screen for cell free sample removal; (3) level control; (4) sample port for cell and medium removal; (5) bubbler; (6) 10 µm PECAP screen separating upper and lower chambers; (7) debubbler; (8) activated charcoal.

between 0.5 and 1.5 µg-atom NO_3-N liter^{-1} (Figure 3). High biomass cultures, up to 100 µg-atom particulate-N liter^{-1} could be supplied with a high flux of nutrient relative to demand, although this flux rate could not be held constant in the same sense as a chemostat. The nutrient regime produced by diffusion culture is probably more similar to that in nature than that of the chemostat (Jannasch, 1974).

Pulse Additions, Pool Measurements, and Time Series

The response of phytoplankton to the addition of a single pulse of limiting nutrient, nitrogen, was measured as follows. After a conditioning period of controlled or measured nitrogen deficiency (see METHODS - Diffusion Culture) or a period of nitrogen starvation in batch culture, the culture was harvested into batch culture (flask) and split into two fractions. All culture conditions (light, temperature) remained as before. One batch fraction was left untreated as a control. To the other fraction was added a small volume addition (less than 2 ml) of nitrogen, either $NaNO_3$ or NH_4NO_3, in low-N seawater. The addition was calculated to elevate the batch culture by approximately 12 µg-atoms N liter^{-1} for each form of nitrogen added. Immediately after the addition, two 15 ml samples were removed and sonicated. The sonication was accomplished with a Heat Systems Sonicator® model W350 using a microtip. Sonication time was 30 seconds at the maximum power rating for the microtip. This power-time combination was found to give almost total cell disruption (>95%) for diatoms as measured both visually and electronically with a Coulter Counter®. After sonication the samples were filtered through 13 mm Whatman GFA® glass fibre filters and

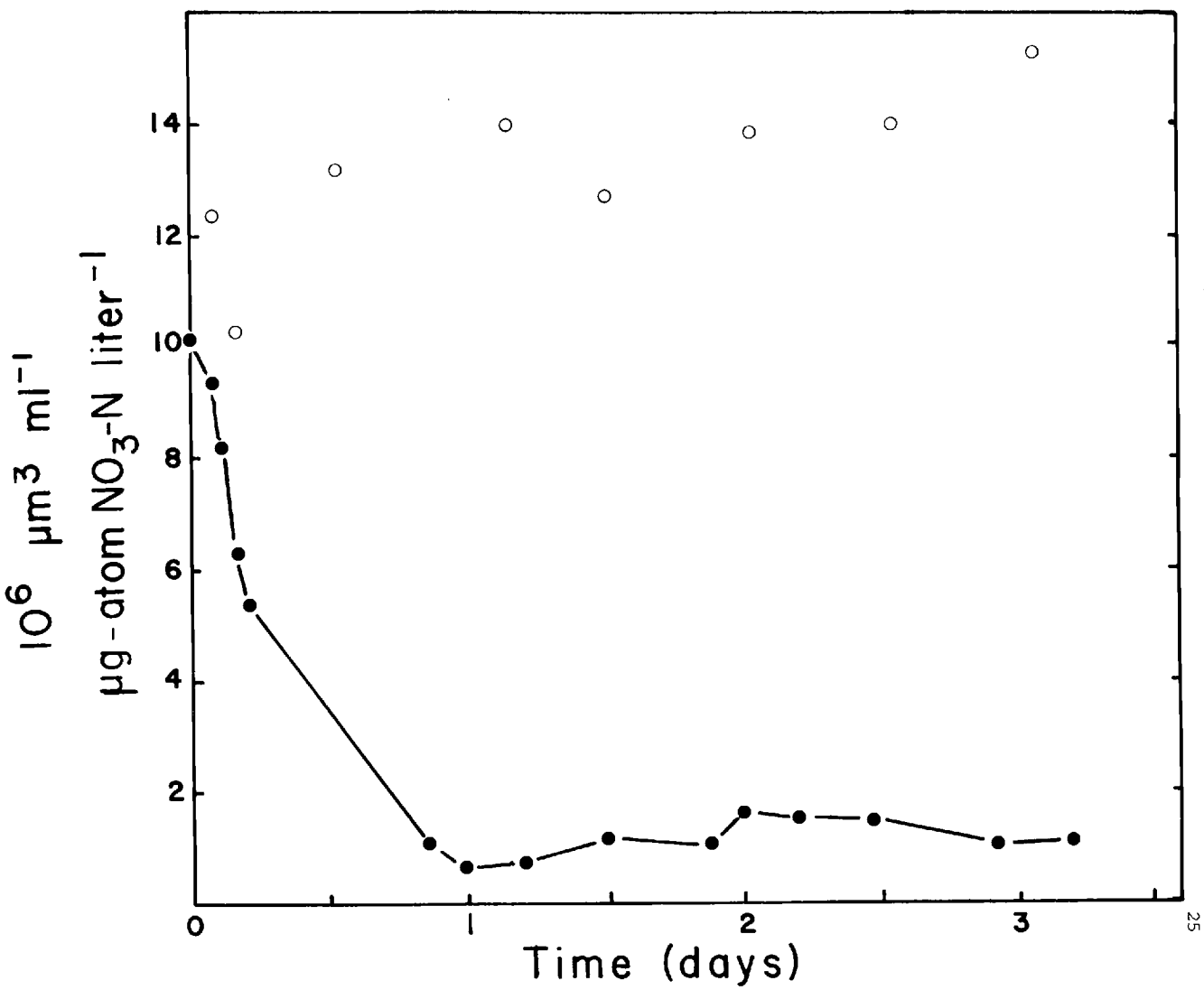

Figure 3. Ambient nitrate vs. time in a diffusion culture of Skeletonema costatum; ambient nitrate; ● ; phytoplankton biomass, O .

either quick frozen in polyethylene bottles or analyzed immediately. At approximately 15 min intervals, 30 ml samples were removed from the enriched culture. These samples were divided, 15 ml being sonicated as previously described to measure total nutrients (ambient plus cellular released by sonication). The other 15 ml were gently filtered through 13 mm Whatman GFA® filters, simultaneous with the sonicate sample, to measure ambient nutrients. Vacuum pressure differential was kept at or less than 0.1 atm. Higher pressures were found to increase cell breakage and increase variability between replicate samples. The filtrates were either quick frozen or analyzed immediately for nutrients.

The filtration of both ambient and sonicated nutrient samples was essential for reproducible results. Filtration of the sonicated samples removed fine particulates which interfere with the nutrient analyses, especially ammonium. Filtration was also found to reduce the variability among replicate frozen samples. Whatman GFA® filters have low but measurable filter blanks for both ammonium and amino-N, but not for nitrate. For 15 ml samples ammonium was elevated 0.77 μg-atom N $liter^{-1}$ and amino acids were elevated 0.56 μg-atom N $liter^{-1}$. The amino-N blank was barely significant ($p < .95$) and was not subtracted from the data values, since values less than zero would have been obtained in many instances. Ammonium blanks were significant ($p > .95$) and were subtracted from the data. Since both ambient and sonicated samples received similar filtrations, pool measurements (the difference between sonicated and ambient concentrations) were relatively unaffected by filter blanks.

The time series of data were tested for significance of slope or non-linearity by linear regressions for zero, first, or second order polynomials using an F test (Draper and Smith, 1966). The lines drawn through data points are for visual clarity only and may not be statistically significant.

Medium Preparation

Due to the large quantity of culture medium required for operating diffusion cultures (up to 20 liters per day for a one liter diffusion culture), and also because of the requirement that the nitrogen flux be easily alterable without affecting either total flow or other nutrients, an ozonization technique was developed for sterilization of seawater. Natural seawater (approximately 29 $^{o}/_{oo}$ salinity) was collected on high tides from the Marine Science Center, Oregon State University, Newport, Oregon. Following a preliminary filtration process, nutrients except nitrogen were added to make up modified F/20 medium (Table 1). The medium was innoculated with either Skeletonema costatum or Thalassiosira aestivalis, depending on which organism was to be used experimentally. The phytoplankton were allowed to grow until ambient nitrogen was decreased to a low level and the cells had settled to the bottom of the culture carboy. The seawater was then decanted, filtered, and ozonized for 30 min using an Alrond® model 45 ozonizer producing approximately 0.35 g O_3 hr^{-1} with a dry O_2 input. This ozonization procedure converted most of the ammonium and much of the organic nitrogen to nitrate. In combination with the biological stripping procedure, the resultant seawater had approximately one µg-atom nitrate-N $liter^{-1}$

and no detectable ammonium or amino acids. Furthermore the seawater was sterilized and remained toxic to phytoplankton or bacterial growth for several weeks. The ozonized seawater was pumped through a sterile column of Darco G-60 ® activated charcoal (20-40 mesh) just prior to use to remove the toxicity (DeManche et al., 1975). Following the detoxification, the seawater was mixed with concentrated sterile nutrients to make up modified F/4 medium (Table 1).

RESULTS

The following experiments cover a wide range of nitrogen regimes both in the preconditioning periods as well as in the type of pulsed nitrogen addition. The experiments presented here represent an initial inquiry into the dimensions of the transient nitrogen environment problem, not a definitive treatment of a single nutrient regime. In gaining a broad entry into the matrix of possible regimes, some replication was sacrificed. In return, many regimes were examined to determine areas fruitful for more detailed study. Patterns of uptake not predicted by current theories were observed, leading to a restructuring of some hypotheses, and support for alternative models.

Each experiment consisted of the perturbation of a culture by addition of nitrogen either as nitrate or a combination of nitrate and ammonium. For all experiments, samples were taken at various intervals for the following parameters: ambient nitrate, total nitrate (ambient plus intracellular), ambient amino acids, total amino acids (ambient plus intracellular), cell volume, particulate cellular carbon and nitrogen, and *in vivo* fluorescence. In all instances where ammonium was added, ambient and total ammonium (ambient plus intracellular), were also measured. From these data additional parameters were calculated: internal pool concentrations of nitrate, amino acids and ammonium; C/N ratios; and nitrogen specific uptake rates. The values of the internal parameters and their rates of change were used as indicators of the physiological state of the phytoplankton and as indicators of the ability of the phytoplankton to respond to new nitrogen environments.

The experimental results are organized according to organism and nitrogen conditioning regime. Experimental treatment of Skeletonema was much more extensive than that for Thalassiosira. Although each experiment is unique in that it is the product of a unique conditioning regime established by highly variable and not completely controllable culture conditions, I feel that a variety of regimes representative of the most common natural nitrogen environments have been included. The treatments can be classified into broad regime groups, representing a progression from non-nitrogen limited to extreme nitrogen deficiency. These groupings are:

1. Non-deficient: nitrogen not limiting at any time during the previous two days before perturbation.
2. Transitional: nitrogen concentration limited with variable flux less than one day prior to perturbation. Nitrogen flux continuous, i.e., no starvation.
3. Low-nitrogen adapted: ambient nitrogen concentration below that required for maximum growth rate for at least one day prior to perturbation. Nitrogen flux continuous.
4. Nitrogen starved: variable lengths of time following nitrogen exhaustion with no nitrogen flux.

The relationships among organism, nutrient history, and initial conditions are summarized in Table 3. Data for each experiment are contained in Appendix Tables II through XV. Nitrogen uptake and assimilation rates are calculated from the slopes of regressions of ambient or total nitrogen nutrient (nitrate or ammonia) against time for those data which are linear with time. Nitrogen-specific rates are calculated by

Table 3. Initial Conditions for Each Experiment. (1) mM/liter cell volume; (2) µg-atom N/liter; (3) atom/atom; (4) 10^9 μm^3/liter; and (5) relative fluorescence/10^9 μm^3. ± indicates one standard deviation; ---- indicates data not taken.

Experiment Name	History	Type of Pulse	NO_3 Pool(1)	Amino Acid Pool(1)	NH_4 Pool(1)	Cellular N(2)	C/N Ratio (3)	Cell Volume (4)	Fluor./ Cell Volume(5)
Skeletonema costatum									
S1	Non-limited	NO_3	82.9±17.1	192.8±17.3	---	43.2± 8.2	9.73±0.48	41.9	0.173
S2	Early starvation (< 2 hr)	NO_3	77.4± 2.1	165.0± 0.2	---	52.4± 0.5	9.81±0.76	39.5	0.131
S3	Starvation (1.6 days)	NO_3	5.1± 8.9	133.8± 7.7	---	36.8	14.0	26.0	0.044
S4a	Starvation (1.8 days)	NO_3	10.0± 6.8	79.1±19.6	53.6±8.3	39.8	15.4	42.2	0.046
S4b	Starvation (1.95 days)	NO_3+NH_4	12.2± 7.0	46.5; 30.0	10.8	45.3	14.3	44.3	0.033
S4c	Starvation (2.1 days)	NO_3	3.2; 2.0	29.2; 48.3	---	42.7	14.9	43.5	0.034
S5	Transition	NO_3	124.0±21.8	200.6±13.5	---	61.6± 4.3	9.01±0.08	53.7	0.094
S6a	Low N	NO_3	8.5± 5.8	57.3±18.7	12.4±1.2	73.4± 4.2	18.22±0.48	52.5	0.189
S6b		NO_3+NH_4							
S7	Low N	NO_3	6.5± 0.2	11.0± 4.1	---	18.8; 19.1	19.2; 19.7	36.2	0.062
Thalassiosira aestivalis									
T1	Low N	NO_3	95.0± 5.6	99.6± 8.9	---	149.0± 1.7	6.45±0.18	102.3	0.076
T2	Non limited and Early Starvation	NO_3	99.1; 116.4	77.8; 87.0	---	36.0	7.94	32.4	0.083

dividing the absolute uptake or assimilation rate by the value for cellular nitrogen.

Skeletonema costatum

S1 (Non-deficient)

This experiment consisted of a nitrate addition to a non-nitrogen limited diffusion culture of Skeletonema costatum. Pre-conditioning was at 18°C with continuous light at 250 μ Einsteins m^{-2} sec^{-1}. Nitrate concentration remained above 20 μg-atom NO_3-N $liter^{-1}$ for several days prior to the experiment. A few hours before the culture was harvested, the inflow to the lower chamber was switched to medium without nitrate, causing a dilution of the nitrate in the upper chamber from 22 μg-atom NO_3-N $liter^{-1}$ to 15 μg-atom NO_3-N $liter^{-1}$ within a few hours. At this time, the contents of the upper chamber were transferred to batch (flask) culture at the same light and temperature conditions. Nitrate was added to a portion of the culture increasing the ambient nitrate concentration to 27.8 μg-atom NO_3-N $liter^{-1}$. No significant differences between uptake and assimilation rates were observed, the mean for both being 1.69 day^{-1} as determined by linear regression (Figure 4). There were no apparent lags in either uptake or assimilation of nitrate. Following nitrate addition there were no significant ($p < .95$) changes in either internal nitrate or amino acid pools (Figure 5). This data set forms the basic non-limited data for Skeletonema to which other experiments were compared.

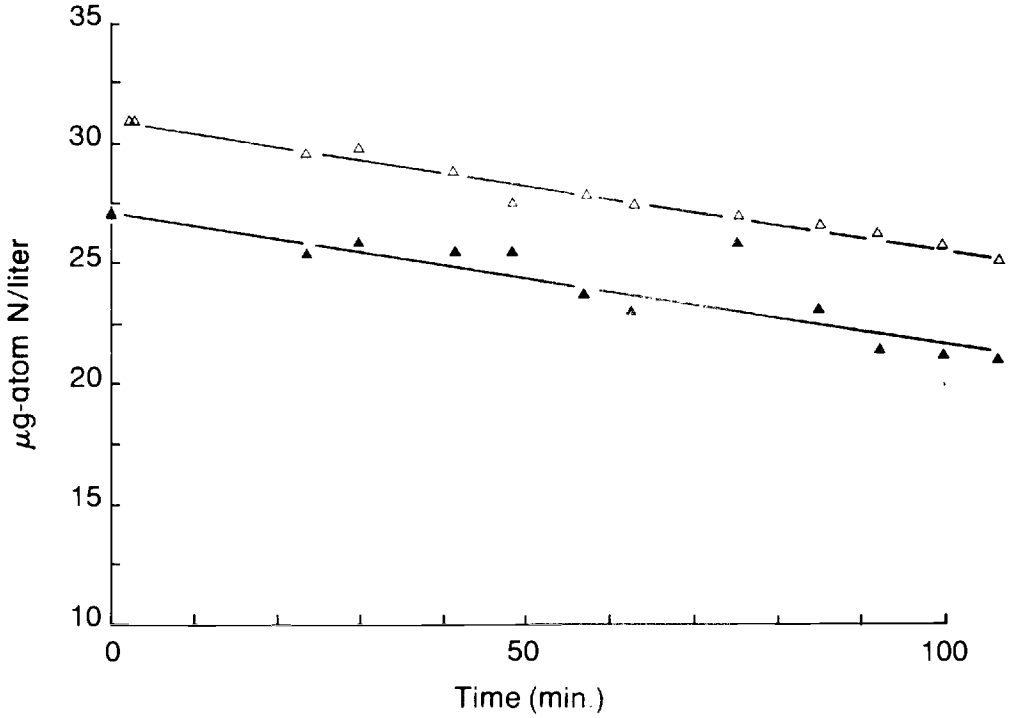

Figure 4. Experiment S1 (Skeletonema, non-deficient). Ambient nitrate (▲), and total nitrate (ambient + internal) (△), vs. time after nitrate addition.

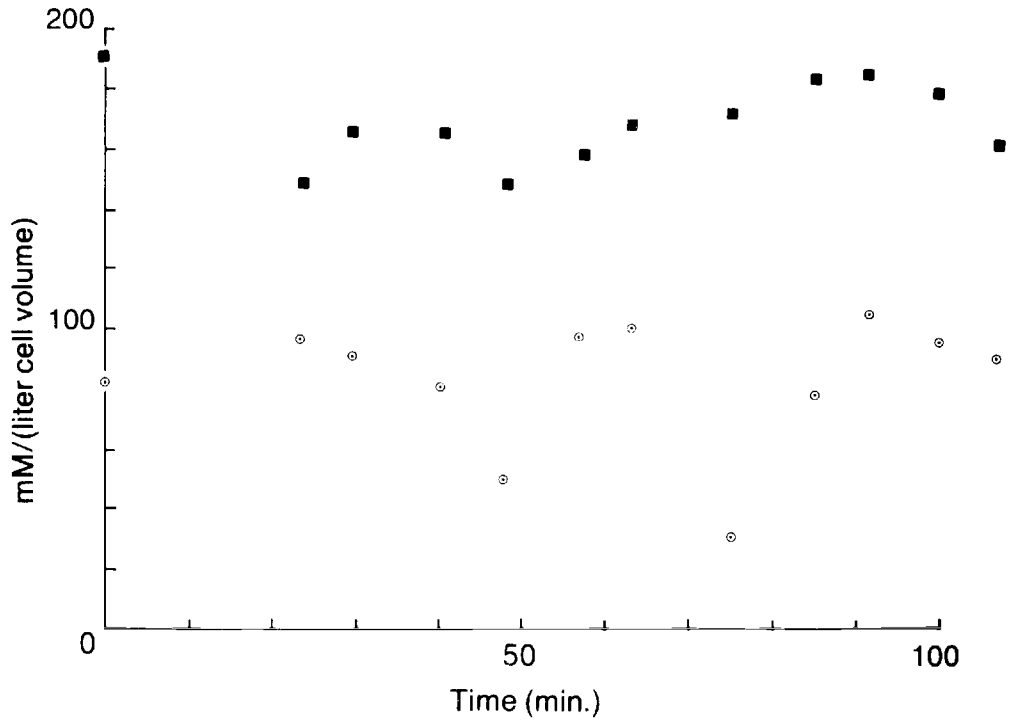

Figure 5. Experiment S1 (non-deficient). Intracellular nitrogen pools: nitrate (⊙), and amino acids (■), vs. time after nitrate addition.

S2 (Early Starvation)

This experiment was designed to test the response of Skeletonema costatum to the addition of nitrate following a brief period of nitrogen starvation. A non-nitrogen limited diffusion culture of S. costatum was cultured at 18°C and 250 μ Einsteins m^{-2} sec^{-1} continuous illumination, as in S1. At the time of harvest, ambient nitrate concentration was 12 μg-atom NO_3-N liter. The upper chamber contents were transferred to a flask maintained at the same light and temperature. The disappearance of nitrate was measured hourly until nitrate was exhausted from the culture. After approximately one hour of nitrate exhaustion, a new addition of nitrate was made and followed as previously described (Figure 6). The nitrogen-specific uptake rate prior to exhaustion was similar to that observed for S1 (1.51 day^{-1}). Initial post-addition nitrate uptake rate of 1.98 day^{-1} was significantly elevated ($p > .95$) from the previous non-limited uptake rate. The sustained difference between uptake and reduction led to the accumulation of significant quantities of nitrate internally, reaching a maximum pool size of 158 mM (Figure 7). Internal amino acid concentration showed a slight upward trend that was not statistically significant due to the large variance in amino acid data (Figure 7). No non-linearities in nitrate uptake rate were observed for the perturbed culture. Nitrate reduction rate for the culture receiving the nitrate addition was greater than for the control to which no addition was made (Appendix IX). In the nitrate addition culture, nitrate was reduced at an average absolute rate of 3.2 μg-atom N $liter^{-1}$ hr^{-1} during the two hour experiment, whereas the reduction rate in the control culture was only

0.82 µg-atom N liter^{-1} hr^{-1}. The reduction rate with nitrate addition was significantly non-linear ($p > .95$) showing a decrease in reduction rate with time. The initially elevated reduction rate decreased rapidly, returning to the non-limited rate within approximately one-half hour.

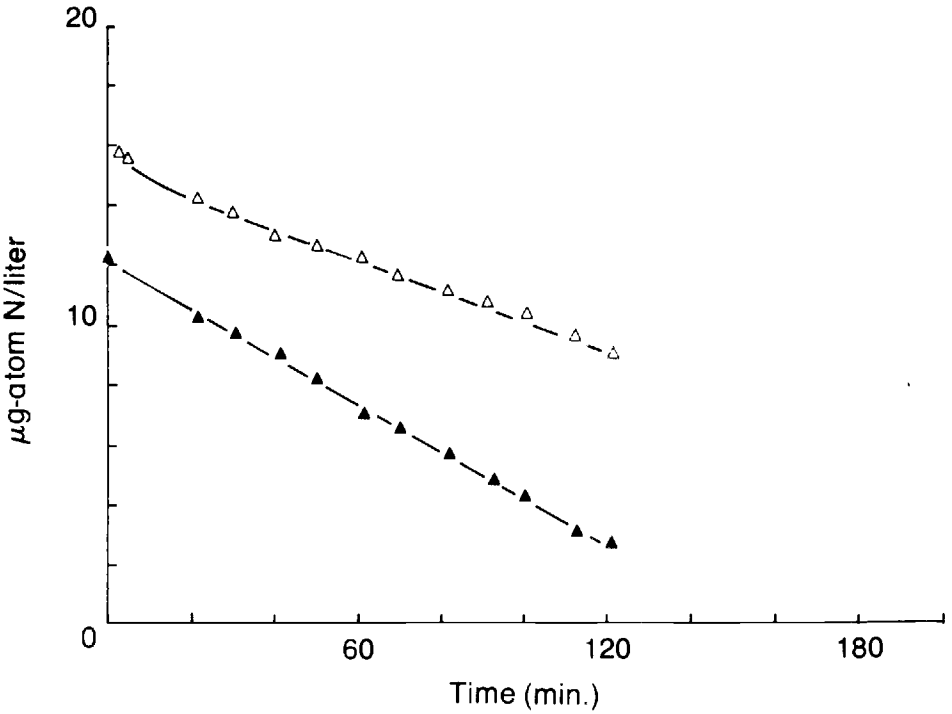

Figure 6. Experiment S2 (Skeletonema, early starvation). Ambient and total nitrate vs. time after nitrate addition. Symbols as Figure 4.

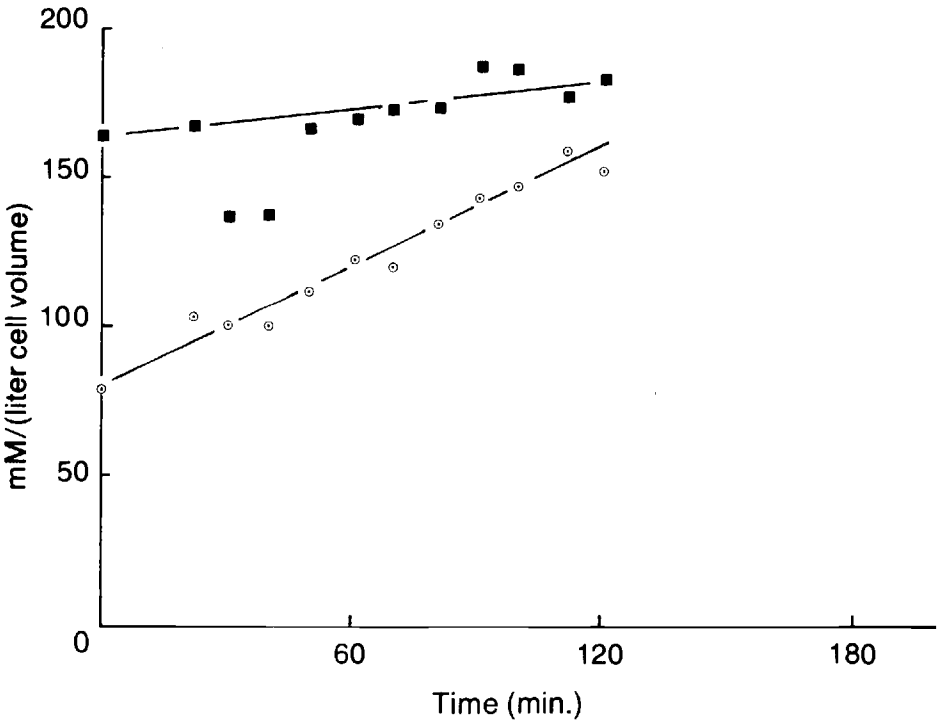

Figure 7. Experiment S2 (Skeletonema, early starvation). Intracellular nitrogen pools vs. time after nitrate addition. Symbols as Figure 5.

S3 and S4 (Starvation)

This series of four different additions of nitrogen to batch cultures of Skeletonema costatum was designed to test the response capabilities of S. costatum to new inputs of nitrate, or nitrate and ammonium, following a long period of nitrogen starvation. The series also demonstrated the degree of reproducibility between addition experiments with similar histories. All cultures were grown in batch cultures at 18°C under continuous illumination at 250 μ Einsteins m^{-2} sec^{-1}. Experiments S3 and S4 were both started from the same expontially growing parent culture, approximately two days apart. Disappearance of ambient nitrate from the culture was monitored to determine the time of nitrate exhaustion.

In experiment S3 an addition of nitrate was made to one culture 1.6 days after nitrate exhaustion, increasing the ambient nitrate concentration to 12.2 μg-atom NO_3-N $liter^{-1}$. Prior to nitrate exhaustion, the population had been growing exponentially with a growth rate (μ) of 1.3 day^{-1}. Internal nitrate increased linearly with time following the addition, at a rate of 0.26 mM min^{-1}. During this time there were no significant changes in the internal amino acid concentration (Figure 9). Nitrate uptake and reduction rates were significantly non-linear ($p > .95$) (Figure 8), with an initial lag period during which little or no nitrate was taken up. By the end of the three hour experiment, uptake and reduction rates had increased to 1.7 and 1.3 day^{-1} respectively (136 and 95% of the nonlimited rate before nitrate exhaustion).

The other culture was split into three identical flasks at 1.8 days after nitrate exhaustion to initiate experiment S4. To one flask

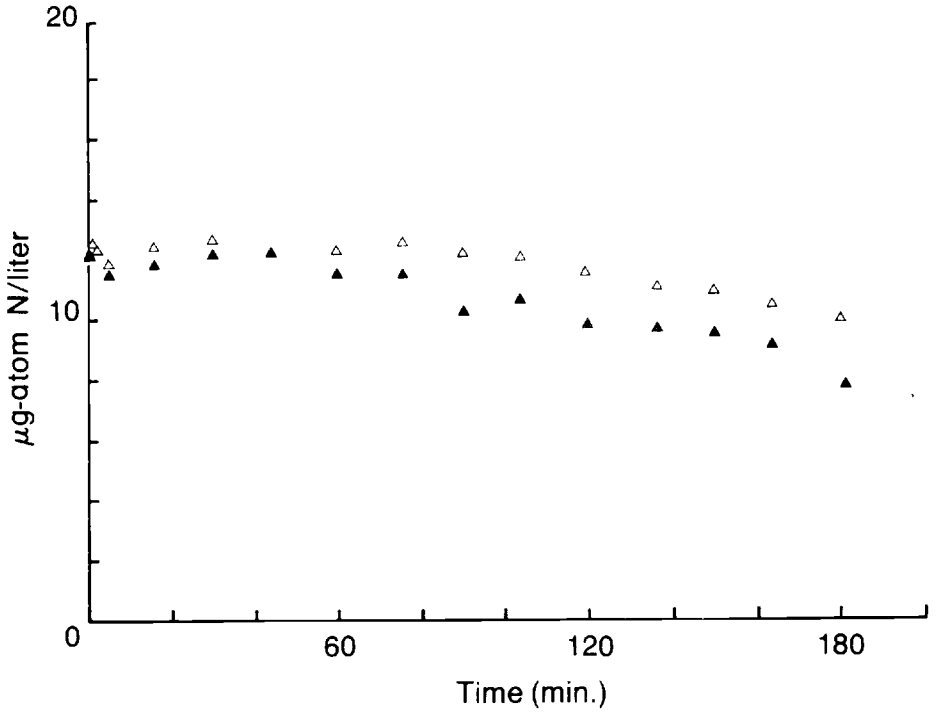

Figure 8. Experiment S3 (Skeletonema, 1.6 days starvation). Ambient and total nitrate vs. time after nitrate addition. Symbols as Figure 4.

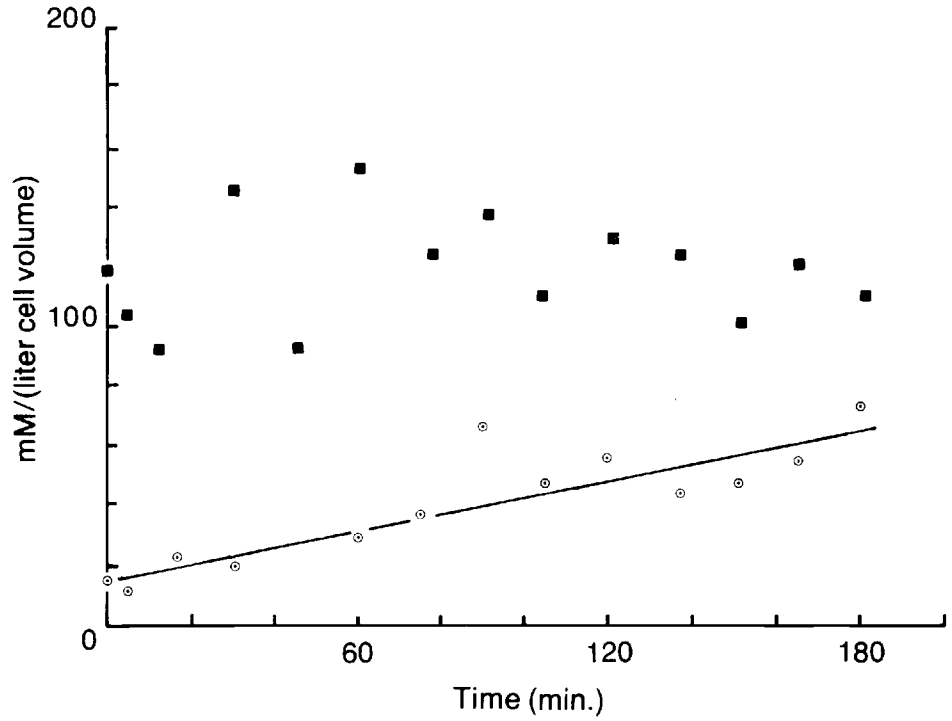

Figure 9. Experiment S3 (Skeletonema, 1.6 days starvation). Intracellular nitrogen pools vs. time after nitrate addition. Symbols as Figure 5.

(S4a) a pulse of nitrate was added, bringing the ambient concentration to 12 μg-atom NO_3-N liter^{-1}. At 1.95 days after nitrate exhaustion, ammonium nitrate was added to a second flask (S4b), increasing the ambient concentrations of both nitrate and ammonium to approximately 12 μg-atom N liter^{-1}. At 2.1 days after nitrate exhaustion, a pulse of nitrate only was added to the third flask (S4c), identically to S4a. The nitrate utilization pattern of S4a and S4c (Figures 10 and 14) were very similar to each other and to S3 (Figure 8). Both nitrate uptake and reduction rates were non-linear with time due to an initial lag period. Final uptake rates were 1.2 day^{-1} and 1.3 day^{-1}, respectively, for S4a and S4c. Nitrate pool-filling rates were also similar at 0.26 and 0.35 mM min^{-1}, respectively. No trends in amino acid pool levels were observed (Figures 11 and 15).

The uptake response of S4b (nitrate + ammonium) was distinctly different from the others. No significant nitrate uptake was observed. Ammonium uptake proceeded at a constant rate of 1.42 day^{-1}, or 109% of the non-limited growth rate (Figure 12). Internal ammonium pools increased rapidly immediately after ammonium addition, but quickly returned to near pre-addition levels (Figure 13). Amino acid pools increased for the culture receiving ammonium (S4b).

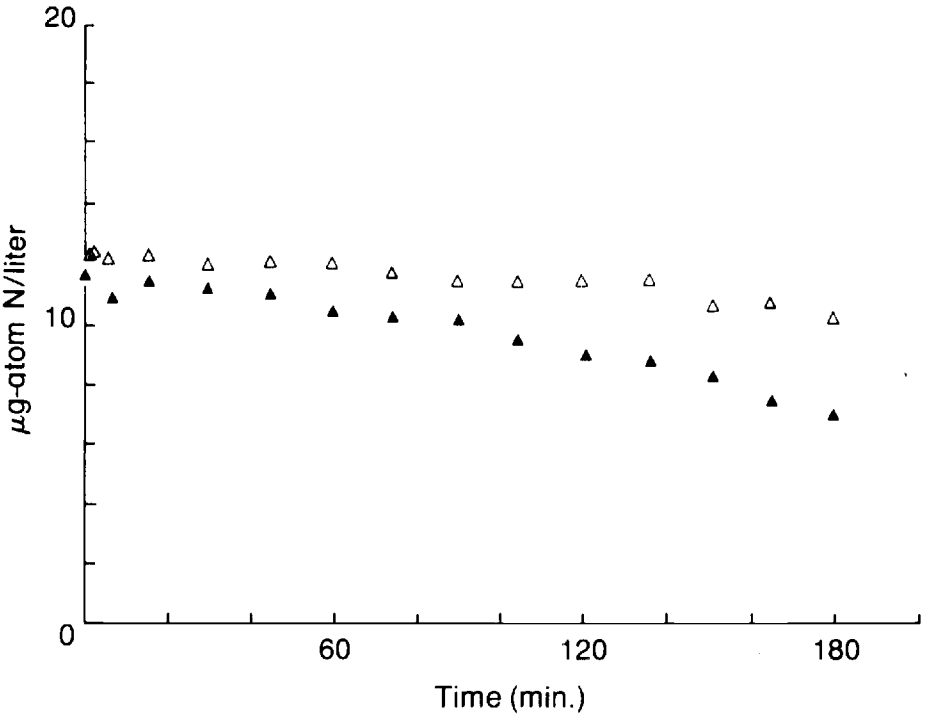

Figure 10. Experiment S4a (Skeletonema, 1.8 days starvation). Ambient and total nitrate vs. time after nitrate addition. Symbols as Figure 4.

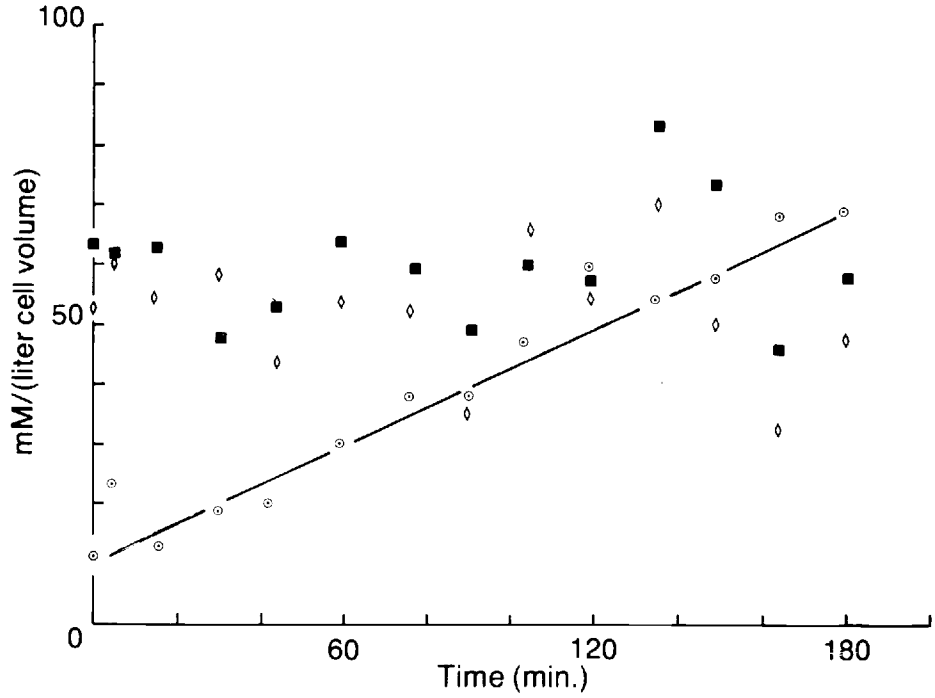

Figure 11. Experiment S4a (Skeletonema, 1.8 days starvation). Intracellular nitrogen pools: nitrate (⊙), amino acids (■), and ammonium (◊) vs. time after nitrate addition.

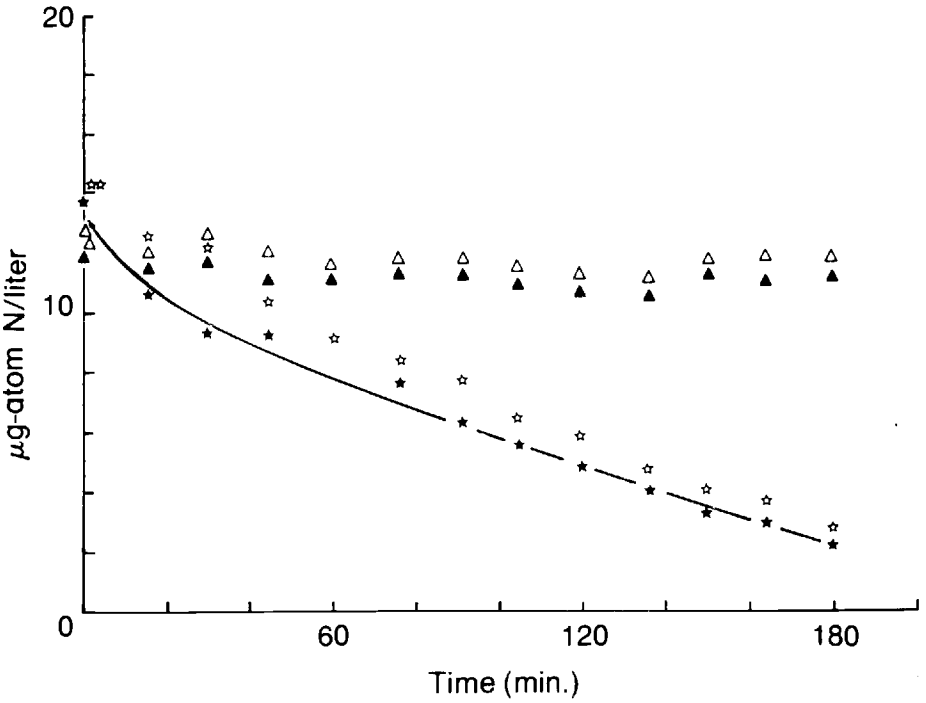

Figure 12. Experiment S4b (Skeletonema, 1.95 days starvation). Ambient nitrate (▲), total nitrate (△), ambient ammonium (★), and total ammonium (☆) vs. time after nitrate + ammonium addition.

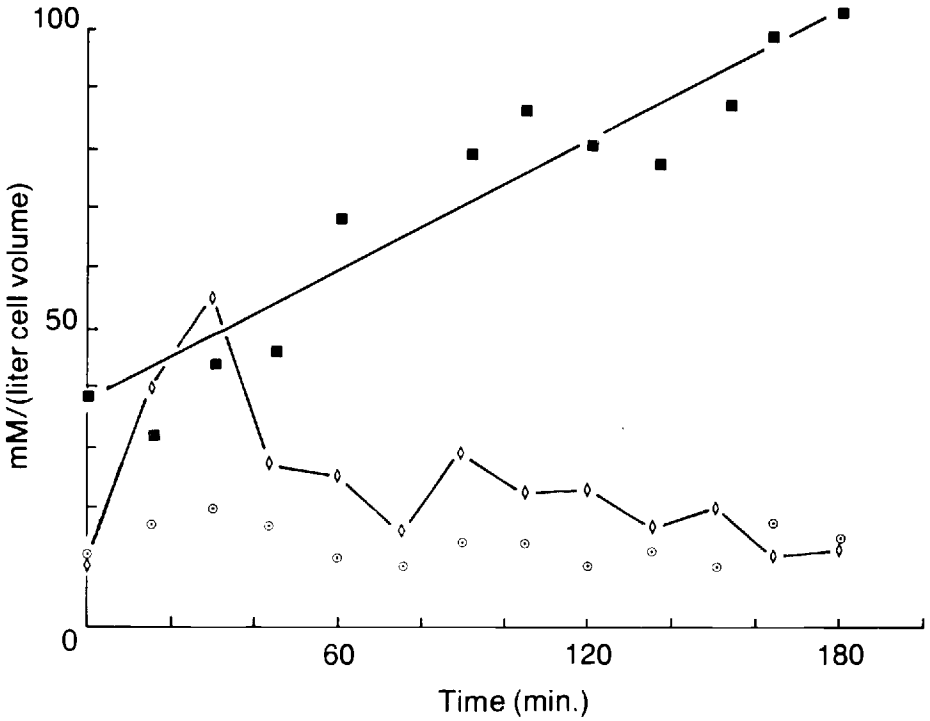

Figure 13. Experiment S4b (Skeletonema, 1.95 days starvation). Internal nitrogen pools vs. time after nitrate + ammonium addition, nitrate (⊙), ammonium (◊) and amino acids (■).

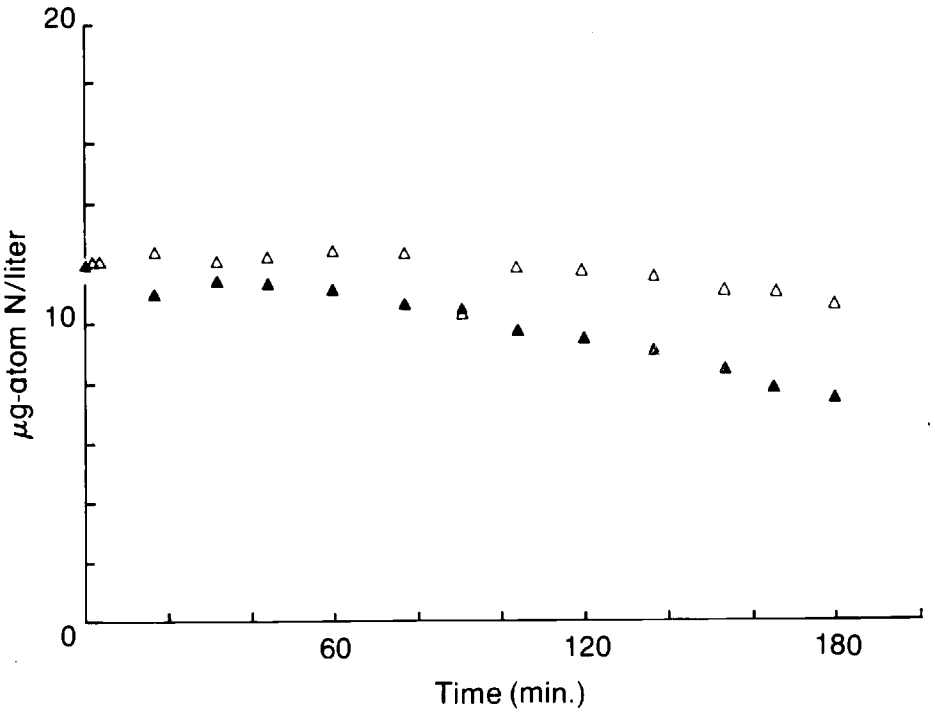

Figure 14. Experiment S4c (Skeletonema, 2.1 days starvation. Ambient and total nitrate vs. time after nitrate addition. Symbols as Figure 4.

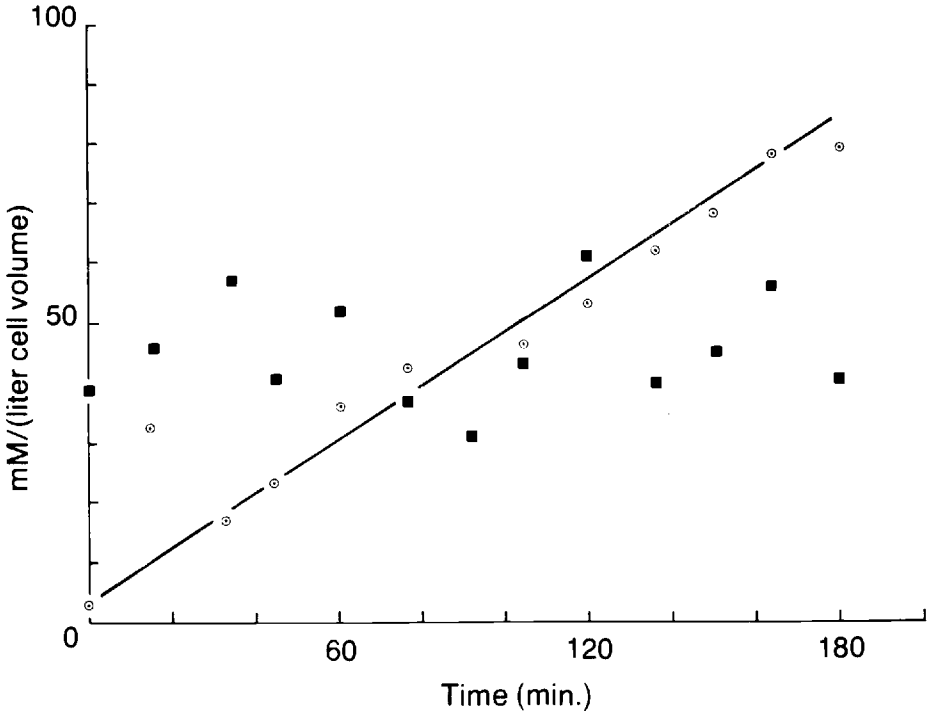

Figure 15. Experiment S4c (Skeletonema, 2.1 days starvation). Intracellular nitrogen pools vs. time after nitrate addition. Symbols as Figure 5.

S5 (Transition)

This experiment followed a nitrate addition to a transitional-state diffusion culture of Skeletonema costatum. Light and temperature conditions were identical to those for S1, although initial biomass was approximately 60% higher. The exact nitrogen preconditioning could not be determined; however, the culture had experienced several episodes of low nitrate during a five hour period prior to harvest. Approximately one hour before harvest, ambient nitrate was measured at 1.7 µg-atom NO_3-N liter^{-1}, and just prior to harvest, nitrate was at 0.7 µg-atom NO_3-N liter^{-1}. Due to an erratic diffusion rate, the culture experienced several periods of increased input prior to harvest. At the time of harvest, the culture had apparently just received one of these inputs since the initial nitrate concentration had risen to between 3 and 4 µg-atom NO_3-N liter^{-1}.

The culture was separated into two flasks, a control flask, and a flask to which an addition of nitrate was made. The nitrate concentration after the addition was approximately 16 µg-atom NO_3-N liter^{-1}. Both uptake and reduction rates for nitrate were elevated compared to S1. The nitrate uptake rate was significantly ($p > .95$) non-linear with an initial rate of 4.47 day^{-1} or an increase to 243% of the rate for S1 (Figure 16). Initial reduction rate was only slightly enhanced at 2.29 day^{-1}, or 135% of the rate for S1. The nitrate pool increased at a rate of 1.4 mM min^{-1}, to a maximum value of 199 mM (Figure 17). Amino acid pool levels may have decreased slightly (Figure 17). The high initial amino acid pool data are suspect because of analytical irregularities which occurred with those samples. The nitrate

reduction rate was significantly non-linear ($p > .99$), decreasing as the experiment progressed (see DISCUSSION - Nitrate Uptake and Reduction).

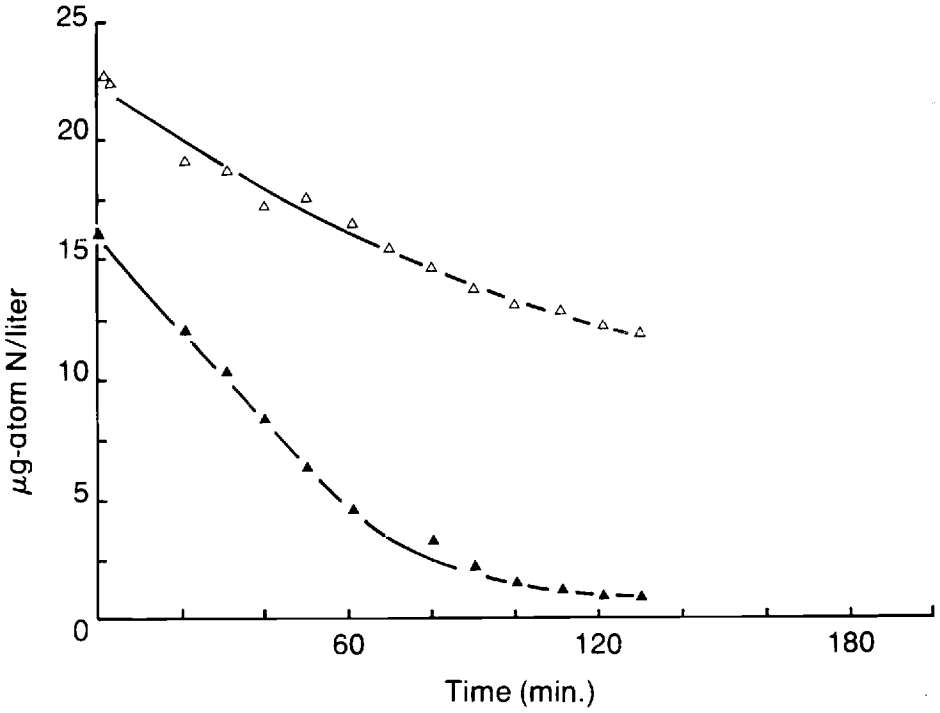

Figure 16. Experiment S5 (Skeletonema, transition). Ambient and total nitrate vs. time after nitrate addition. Symbols as Figure 4.

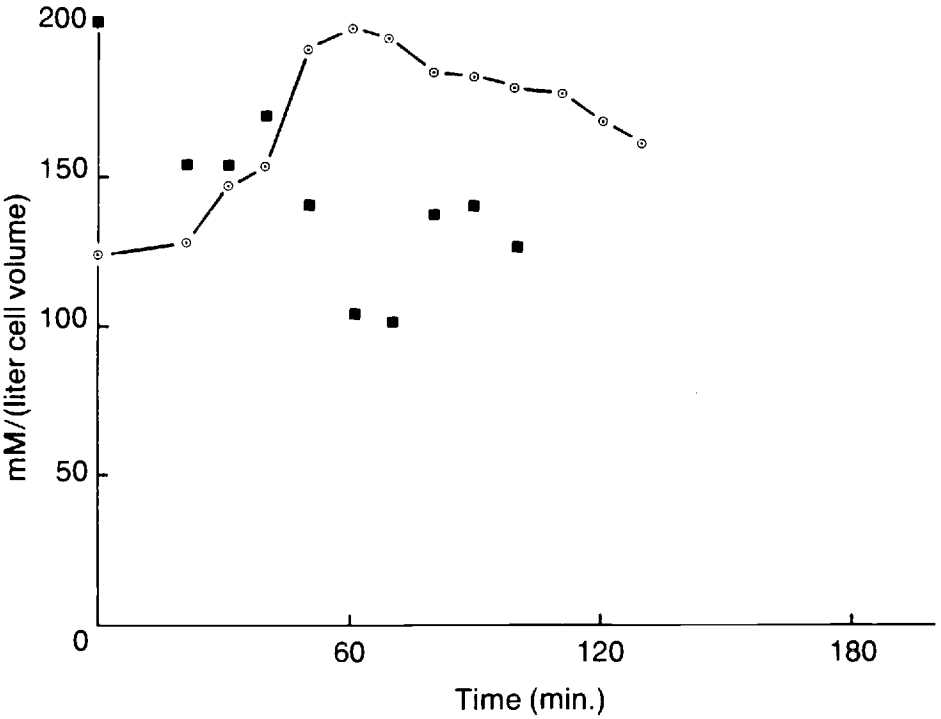

Figure 17. Experiment S5 (Skeletonema, transition). Intracellular nitrogen pools. Symbols as Figure 5.

S6 (Low Nitrogen Adapted)

In this experiment, designed to study the effects on Skeletonema costatum of adaptation to low ambient nitrate concentration, while still maintaining a high flux of nitrate, a culture was given two days adaptation to low nitrate in a diffusion culture. During this adaptation period upper chamber nitrate did not exceed 1 µg-atom NO_3-N liter^{-1}. The high biomass maintained a concentration gradient of approximately 20 µg-atom NO_3-N liter^{-1} between the upper and lower chambers. This large gradient resulted in a high flux rate of nitrate to the upper chamber. At the time of harvest, the phytoplankton population had small nitrate pools (8.5 mM) and only moderate amino acid pools (57 mM). Ammonium pools were found to be slightly higher than those for nitrate (12.4 mM vs. 8.5 mM)(Table 3). These ammonium pools were presumably the result of nitrate reduction, as no ambient ammonium was detected in the lower chamber medium.

The culture was split into three batch cultures. Immediate additions were made separately to two of the cultures, one of nitrate (15 µg-atom NO_3-N liter^{-1}) (S6a), and another of nitrate plus ammonium (16 and 10 µg-atom N liter^{-1}, respectively) (S6b). A third culture was left as a control. Light and temperature conditions remained constant.

In experiment 6A average nitrate uptake and reduction rates were 1.37 day^{-1} and 1.15 day^{-1}, respectively, for the three hour experiment (Figure 18). The uptake rate was constant, but a significant ($p > .95$) non-linear reduction rate was observed with an initially slower rate for the first half hour. The difference between uptake and reduction rates led to a rapid initial increase in the nitrate pool, followed by

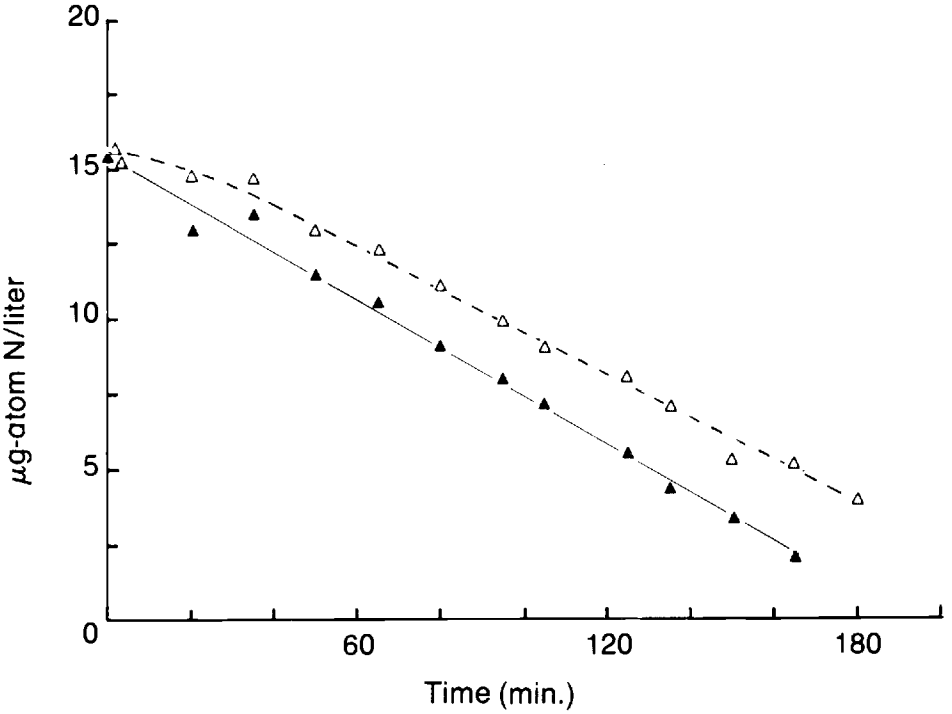

Figure 18. Experiment S6a (Skeletonema, low N). Ambient and total nitrate vs. time after nitrate addition. Symbols as Figure 4.

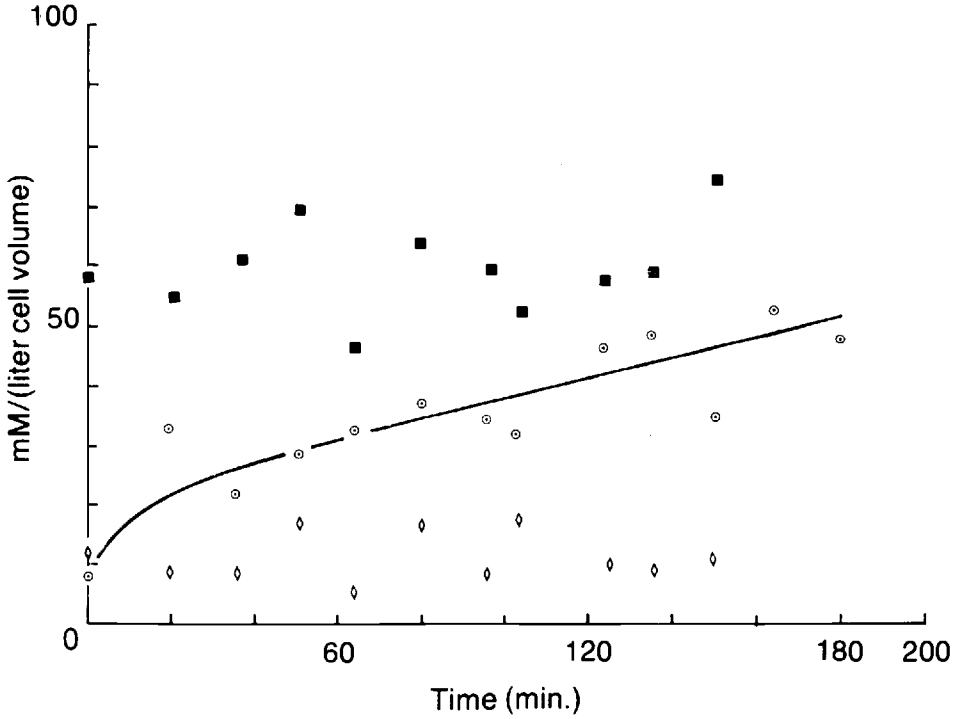

Figure 19. Experiment S6a. (Skeletonema, low N). Intracellular nitrogen pools vs. time after nitrate addition. Symbols as Figure 11.

a more gradual increase (Figure 19). Average nitrate pool concentration increased at a rate of 0.14 mM min^{-1} (Figure 19). Although this rate is much slower than either S2 or S5, the increase is significant ($p > .95$) over the three hour experiment. Both ammonium and amino acid pools remained constant (Figure 19).

In experiment S6b, both nitrate and ammonium were taken up simultaneously, although the average nitrate uptake rate in the presence of ammonium was only 36% of the rate when nitrate alone was added. Nitrate uptake and reduction rates were constant following the addition except for a small amount of nitrate which may have been taken up in the first fifteen minutes at the uninhibited rate (Figure 20). Nitrate reduction, with ammonium present, was 33% of the rate when only nitrate was added. Nitrate pool filling was also slowed to only 0.11 mM min^{-1}, or 62% of the rate without ammonium (Figure 21). Ammonium uptake was faster than the rate of nitrate uptake when nitrate only was added (2.51 day^{-1}) for a 183% enhancement (Figure 20). Ammonium pools remained statistically constant, but a possible trend similar to that for S4b was observed. There was an apparent rapid increase in internal ammonium immediately following the addition (Figure 21). After this "surge uptake" (Dugdale, 1977), the internal level slowly decreased back toward the initial values. The combined uptake of nitrate and ammonium nitrogen was equivalent to a specific uptake rate of 3.0 day^{-1}. Of the total inorganic nitrogen disappearing from the culture during the first 90 min, only 18% reappeared as internal amino acids. The reminder presumably was incorporated into more complex organic nitrogen compounds such as polypeptides and protein.

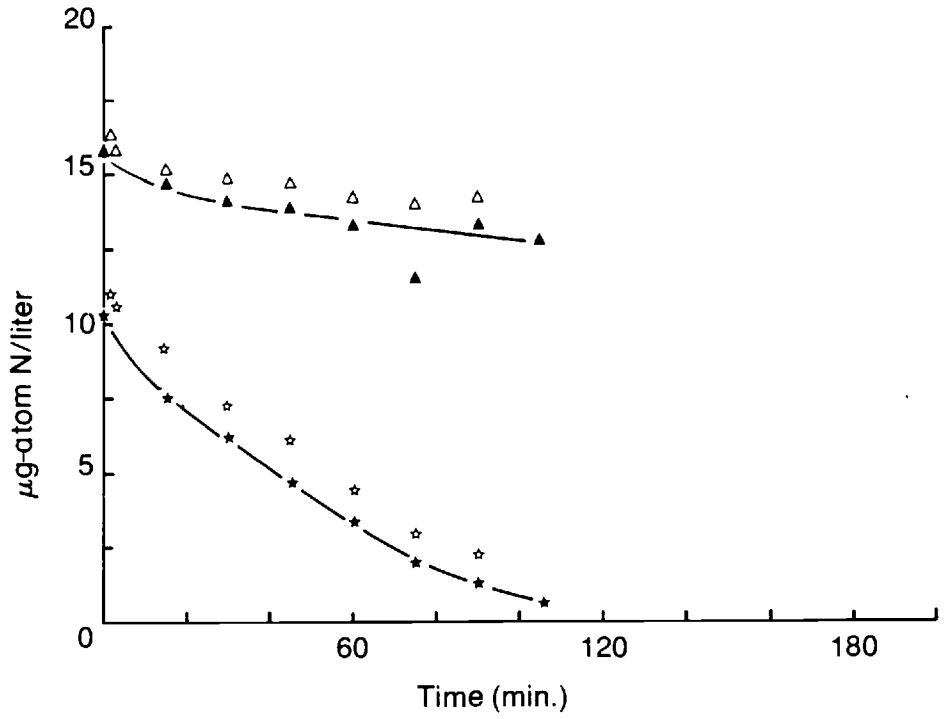

Figure 20. Experiment S6b (Skeletonema, low N). Ambient and total nitrate, and ammonium vs. time after nitrate and ammonium addition. Symbols as Figure 12.

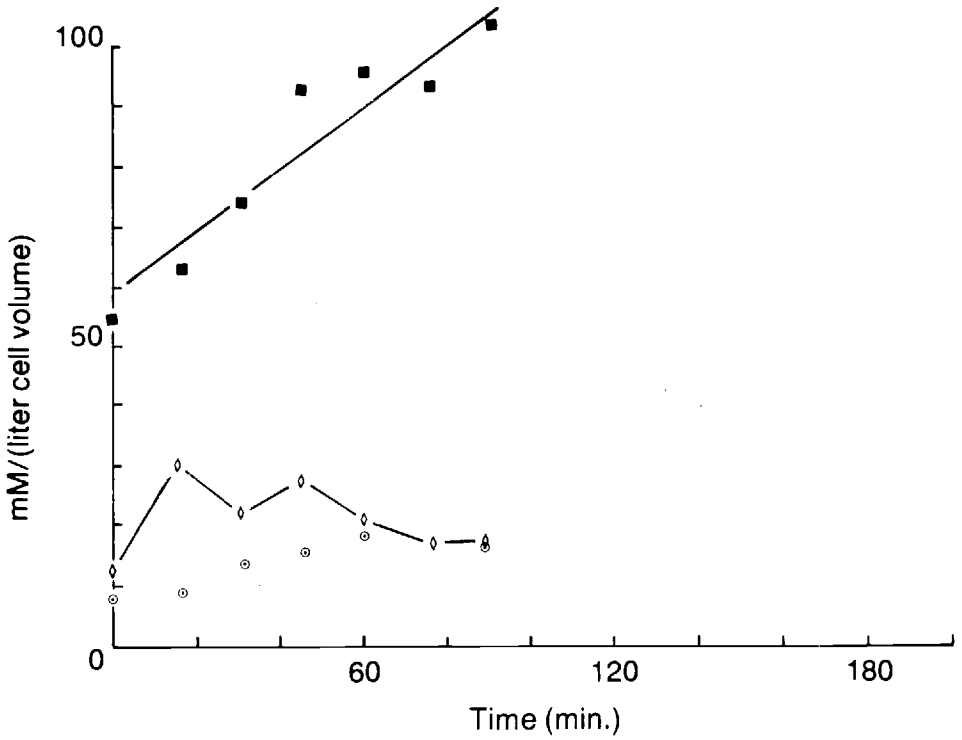

Figure 21. Experiment S6b (Skeletonema, low N). Intracellular nitrogen pools vs. time after nitrate and ammonium addition: nitrate, ammonium and amino acids. Symbols as Figure 13.

S7 (Low Nitrogen Adapted)

This experiment was similar to S6 in that the culture of Skeletonema costatum was adapted for several days to low ambient nitrate concentrations in a diffusion culture. However, an erratic diffusion rate, coupled with brief periods of nitrate starvation, produced a population with a nutrient history that could not be well defined. Initial parameters (Table 3) indicate a culture that was severely nitrogen limited, with low internal nitrate and amino acid pools.

The culture was harvested and, after receiving a pulse of nitrate, monitored as previously described. Both average uptake and reduction rates were enhanced relative to S1 (Figure 22). The initial nitrate uptake was the most rapid observed for S. costatum in these experiments (7.8 day^{-1}). This extremely rapid uptake rate, in spite of the enhanced reduction rate (2.6 day^{-1}), caused an extremely rapid increase in the internal nitrate pool (Figure 23). No significant decrease in nitrate reduction rate (as indicated by the linearity of the regression of total nitrate vs. time) was observed after nitrate exhaustion.

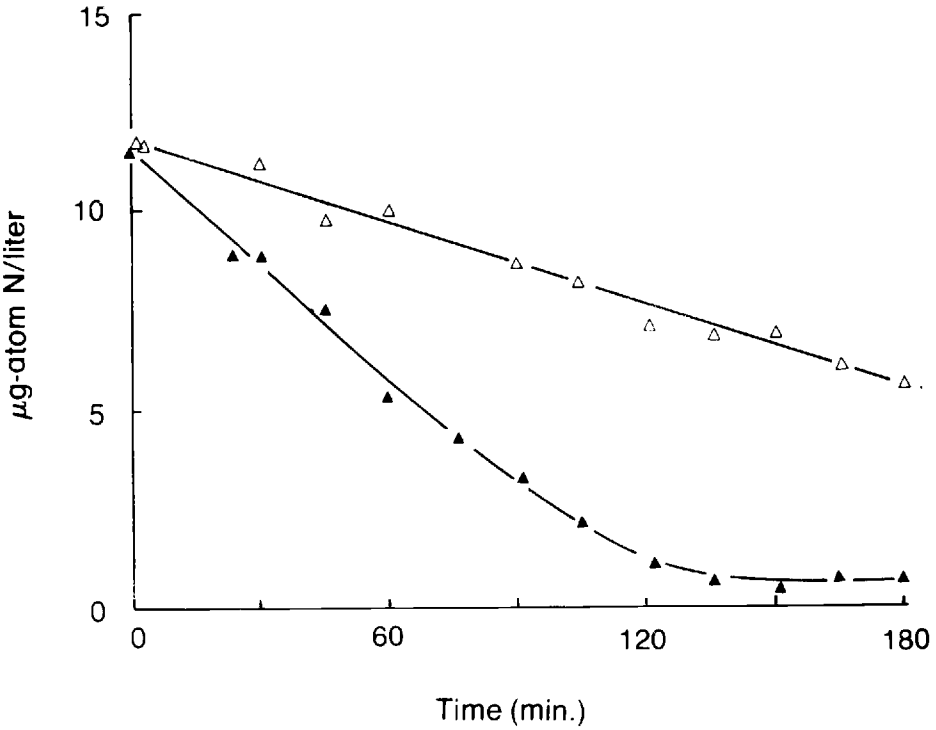

Figure 22. Experiment S7 (Skeletonema, low N). Ambient and total nitrate vs. time after nitrate addition. Symbols as Figure 4.

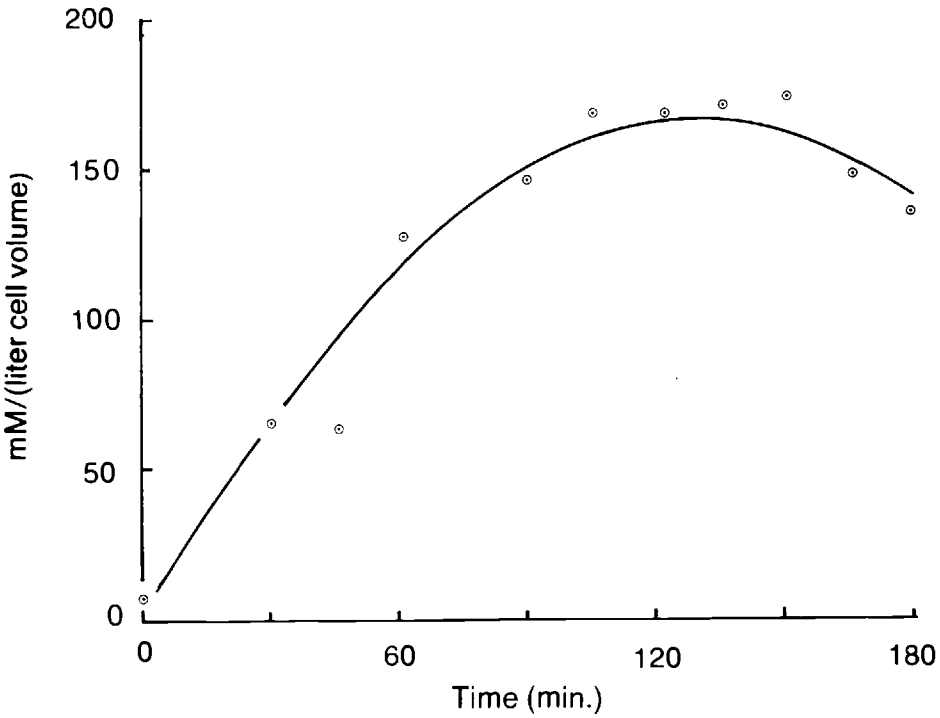

Figure 23. Experiment S7 (Skeletonema, low N). Intracellular nitrate pool vs. time after nitrate addition.

Thalassiosira aestivalis

The cultures using Thalassiosira aestivalis were not intended to range over a complete set of environmental nitrogen regimes. Instead, they were selected to give a comparison against the response of Skeletonema costatum for selected regimes. The preconditioning regimes were prepared so as to closely approximate the regimes for S. costatum. However, due to the limited reproducibility of diffusion cultures and differences in light-temperature optima for the two species, a rigorous comparison was not possible.

T1 (Low Nitrogen Adapted)

A culture of Thalassiosira aestivalis was maintained for two days in a low nitrate diffusion culture at 16°C and 200 μ Einsteins m^{-2} sec^{-1} continuous illumination. During this adaptation period the culture was provided with a high flux of nitrate, although ambient nitrate concentrations were near or below one μg-atom NO_3-N $liter^{-1}$. After this adaptation period, the culture was harvested and transferred to batch culture. Nitrate was added to a portion of the culture, raising the ambient nitrate concentration to 13 μg-atom NO_3-N $liter^{-1}$. The disappearance of nitrate was monitored as previously described. After the addition, nitrate was taken up rapidly, corresponding initially to a nitrogen specific uptake rate of 2.6 day^{-1}, or 3.1 times the non-nitrogen limited maximum growth rate for T. aestivalis under these light-temperature conditions. Initial reduction rate was also enhanced to 1.63 day^{-1}, or 1.9 times the non-limited rate (Figure 24). These rapid initial rates decreased quickly. A second order polynomial

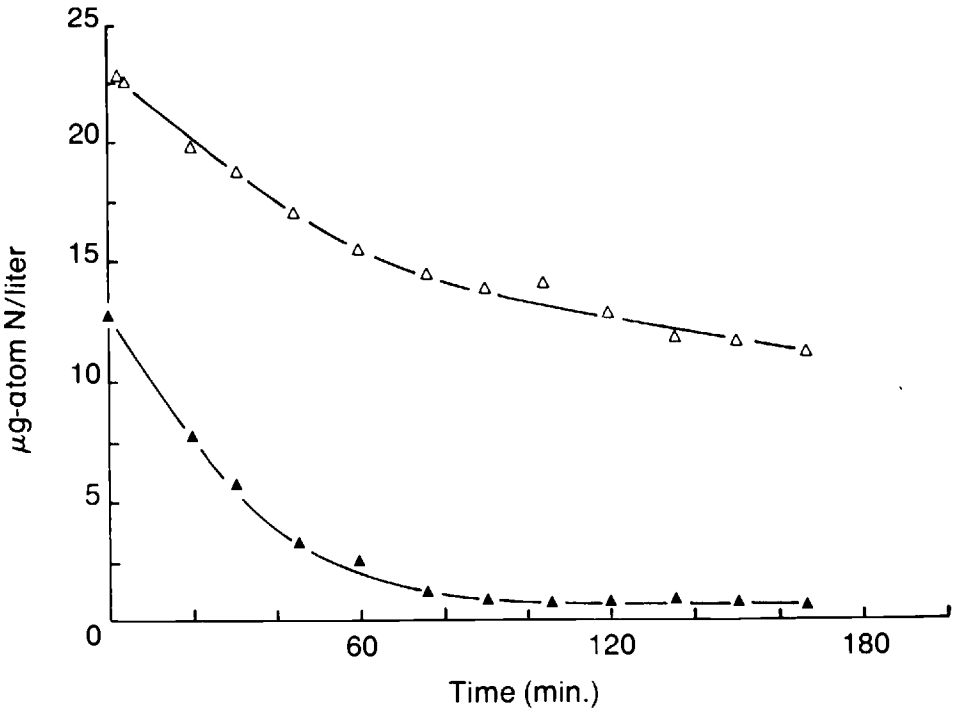

Figure 24. Experiment T1 (Thalassiosira, low N). Ambient and total nitrate vs. time after nitrate addition. Symbols as Figure 4.

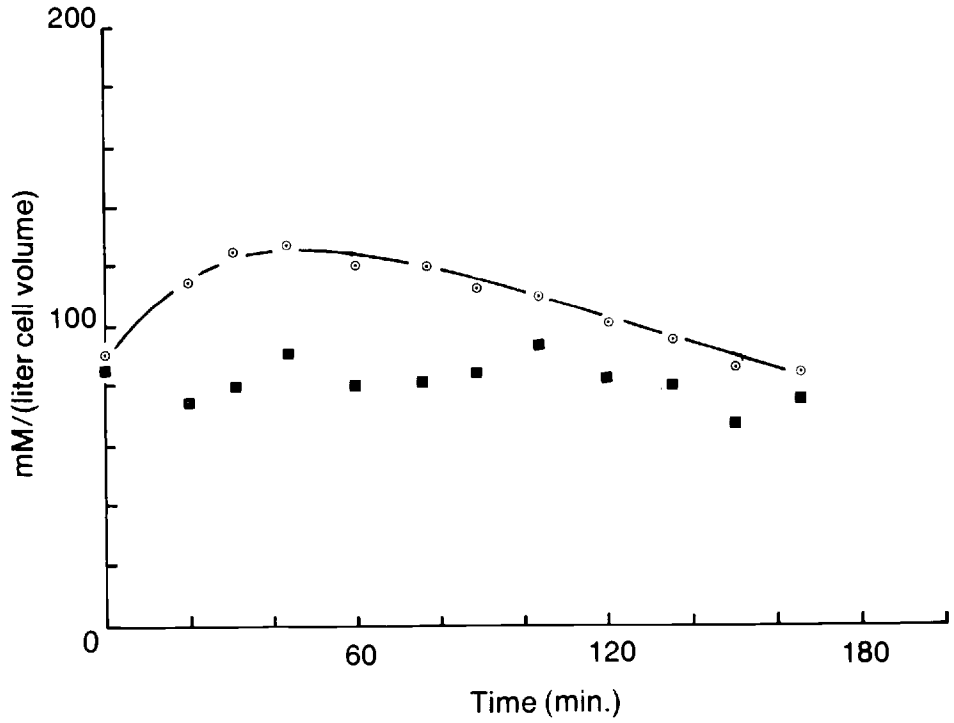

Figure 25. Experiment T1 (Thalassiosira, low N). Intracellular nitrogen pools vs. time after nitrate addition. Symbols as Figure 5.

regression gave the best significant fit to the uptake data. If the apparent half-saturation constant were calculated from this regression, a value of 4.9 µg-atom NO_3-N $liter^{-1}$ would be obtained. This contrasts to values of 0.5 to 1 µg-atom NO_3-N for non-limited batch cultures. The reduction rate of nitrate was significantly non-linear ($p > .99$), decreasing with time. This decrease in reduction rate was coincident with the decrease and cessation of nitrate uptake, but had no relation to the internal levels of nitrate (Figure 25). Nitrate reduction rates were, in fact, slowest at the time of maximum pool concentrations and fastest, initially, when nitrate pools were small. Amino acid pool concentrations showed no significant changes (Figure 25).

T2 (Early starvation)

A diffusion culture of Thalassiosira aestivalis was grown with the same environmental conditions as T1 (16°C and continuous light at 200 μ Einsteins m^{-2} sec^{-1}), except that nitrate was maintained at non-limiting concentrations. At the time of harvest ambient nitrate concentration was approximately four μg-atom NO_3-N $liter^{-1}$, and it had been at or above that level for at least the preceeding 24 hours.

The culture was transferred into a batch culture and allowed to take up the remaining nitrate. One-and-one-half hours after harvest, ambient nitrate was only 1.3 μg-atom NO_3-N $liter^{-1}$. At this time an addition of nitrate was made to the culture, increasing the ambient concentration to nine μg-atom NO_3-N $liter^{-1}$. Nitrate uptake was monitored as previously described. Approximately 1.5 hours after all the nitrate from the first pulse was taken up (243 min after the first pulse), a second pulse of nitrate was added.

After pulse 1, uptake and reduction were tightly coupled (Figure 26). There were no significant changes observed in nitrate pools (Figure 27). The uptake rate was rapid compared to previously measured non-nitrogen limited growth rates for T. aestivalis under similar light and temperature conditions. The factors contributing to this discrepancy are unknown, but probably have something to do with variations in the stock culture. Since no nitrate limitation had occurred at the time of the first pulse, this uptake rate for the first pulse was used as the basic non-limited rate to which the other cultures were compared. As in T1, a decrease in nitrate reduction (as indicated by change in slope) was observed coincident with the cessation of nitrate uptake

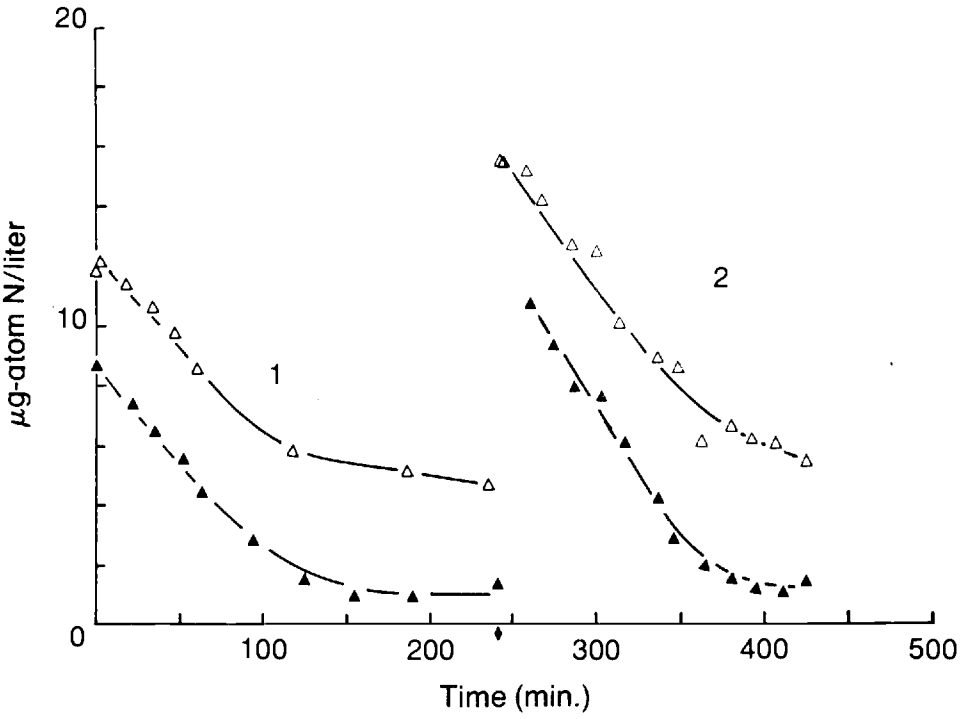

Figure 26. Experiment T2 (Thalassiosira, non-deficient). Ambient and total nitrate vs. time after nitrate addition. Symbols as Figure 4. Second nitrate addition 243 min after first addition.

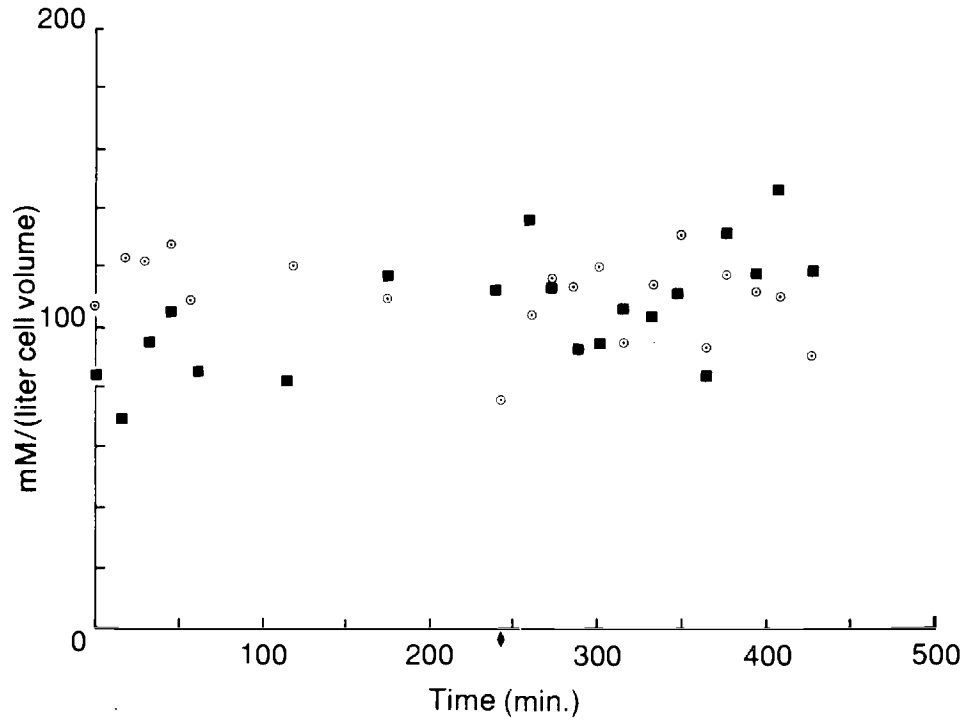

Figure 27. Experiment T2 (Thalassiosira, non-deficient). Intracellular nitrogen pools vs. time after nitrate additions. Symbols as Figure 5.

(Figure 26).

After pulse 2, the uptake and reduction of nitrate were essentially the same except that a brief lag in reduction rate may have occurred initially. The decrease in rate of nitrate reduction coincident with the cessation of nitrate uptake was noted again (Figure 26). Neither nitrate nor amino acid pools showed any apparent trends with either pulse (Figure 27).

DISCUSSION

Nitrate Uptake and Reduction

The difficulty in measuring nitrogen uptake and assimilation, especially in the field, prompted many investigators to search for simple enzymatic assays which, by themselves or in conjunction with measurements of ambient concentrations of inorganic nitrogen, could yield estimates of nitrogen transport. One enzyme proposed for such assays was nitrate reductase (NR), the enzyme thought responsible for the reduction of nitrate to nitrite (Eppley et al., 1969; Packard et al., 1971; Packard, 1973; Collos and Lewin, 1974; Packard and Blasco, 1974). Two assumptions were required: a) that the activity of free cellular nitrate reductase was a measure of the total potential for nitrate assimilation, and b) that accumulation of internal nitrate was insignificant, though no direct coupling was assumed between uptake and reduction. Recently, a new theory of nitrate reduction, independent of free cellular NR, has been proposed for higher plants (Butz and Jackson, 1977). If valid for marine phytoplankton, the mechanism proposed would invalidate the assumptions concerning NR and require a re-evaluation of research efforts along that line.

Butz and Jackson (1977) proposed that uptake and reduction of nitrate are coupled processes, mediated by a membrane bound nitrate reductase tetramer (Figure 28). One monomer, extending outside the cell, acts as a site for nitrate binding. Three monomers inside act as reduction sites, each site associated with a bound ATPase. The postulated sequence of events is as follows. Nitrate molecules bind

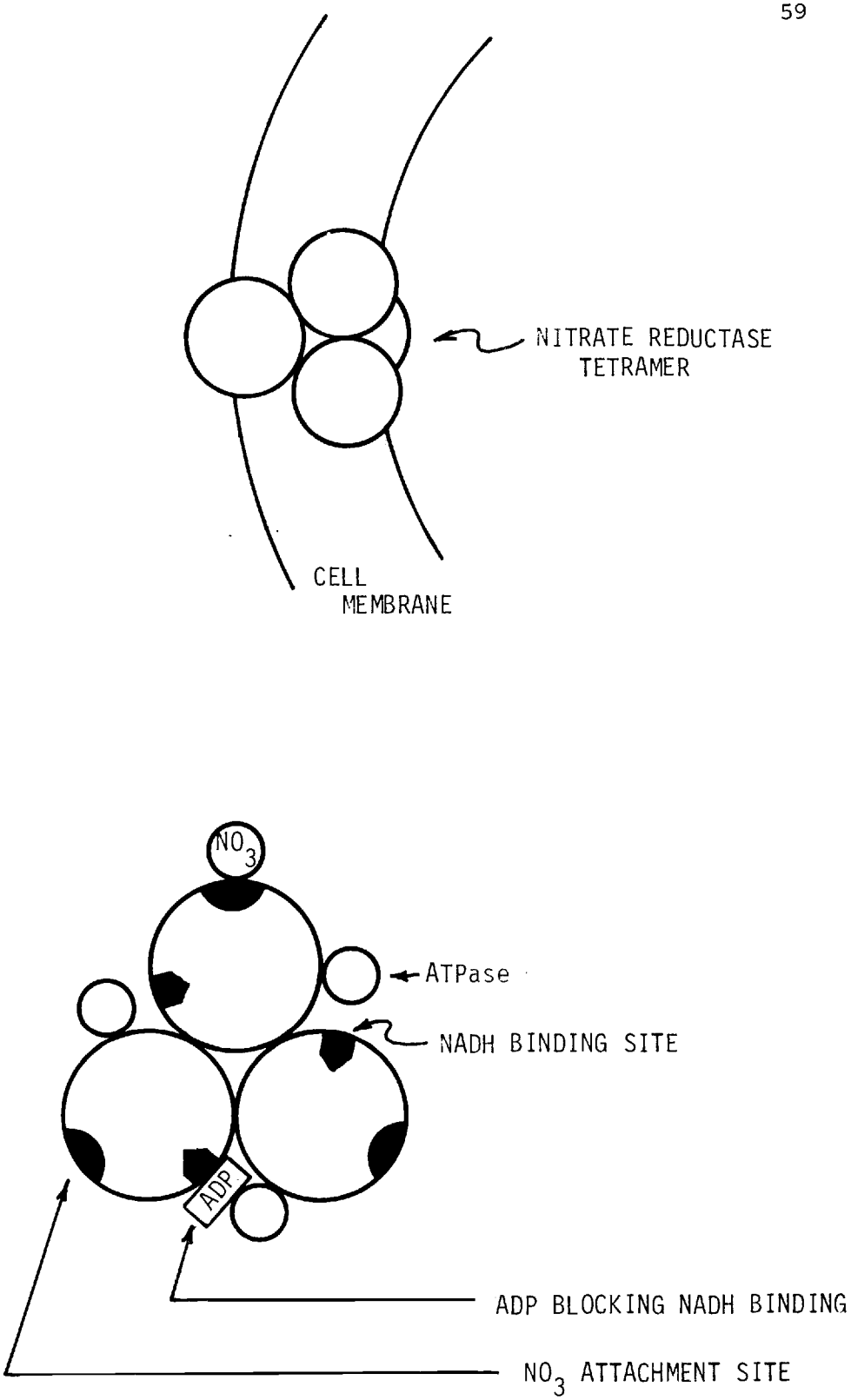

Figure 28. Coupled uptake-reduction mechanism for nitrate uptake and reduction. Redrawn from Butz and Jackson (1977).

one at a time to the outer monomer. These molecules are then transported sequentially to each of the three inner monomers, accompanied by the hydrolysis of three molecules of ATP to ADP. After the first and second transfers, the ADP molecules remain bound to the complex, blocking reduction of nitrate by NAD(P)H. A conformational change following the transfer of the third nitrate molecule frees the last ADP, allowing the reduction of a single nitrate to nitrite by NAD(P)H. The discharge of the nitrite molecule and NAD(P)H releases the other two nitrate molecules and their associated ADP's, returning the teramer to its original configuration. The net result is the uptake of three molecules of nitrate, the reduction of one to nitrite, an expenditure of energy and reducing power in the form of ATP and NAD(P)H, and an increase in the level of internal nitrate.

Butz and Jackson postulated that the ratio of uptake to reduction is variable, ranging from 1:1 to 3:1, and controlled by at least three factors: 1) modulation of the inhibition of reduction by internal levels of ADP, NAD(P), and thiol; 2) supplemental internal reduction via free NR (FNR); or 3) transport and reduction across the chloroplast membrane at a 1:1 ratio.

Coupled uptake-reduction (CUR) has implications for observations of nitrate uptake and utilization by marine phytoplankton. Regardless of the uptake:reduction ratio (U:R), reduction should stop when uptake stops if coupled reduction were the only means of nitrate reduction (Figure 29a). If free nitrate reductase (FNR) were the only means of reduction, there should be no immediate cessation of reduction, but only a gradual decrease following cessation of nitrate uptake,

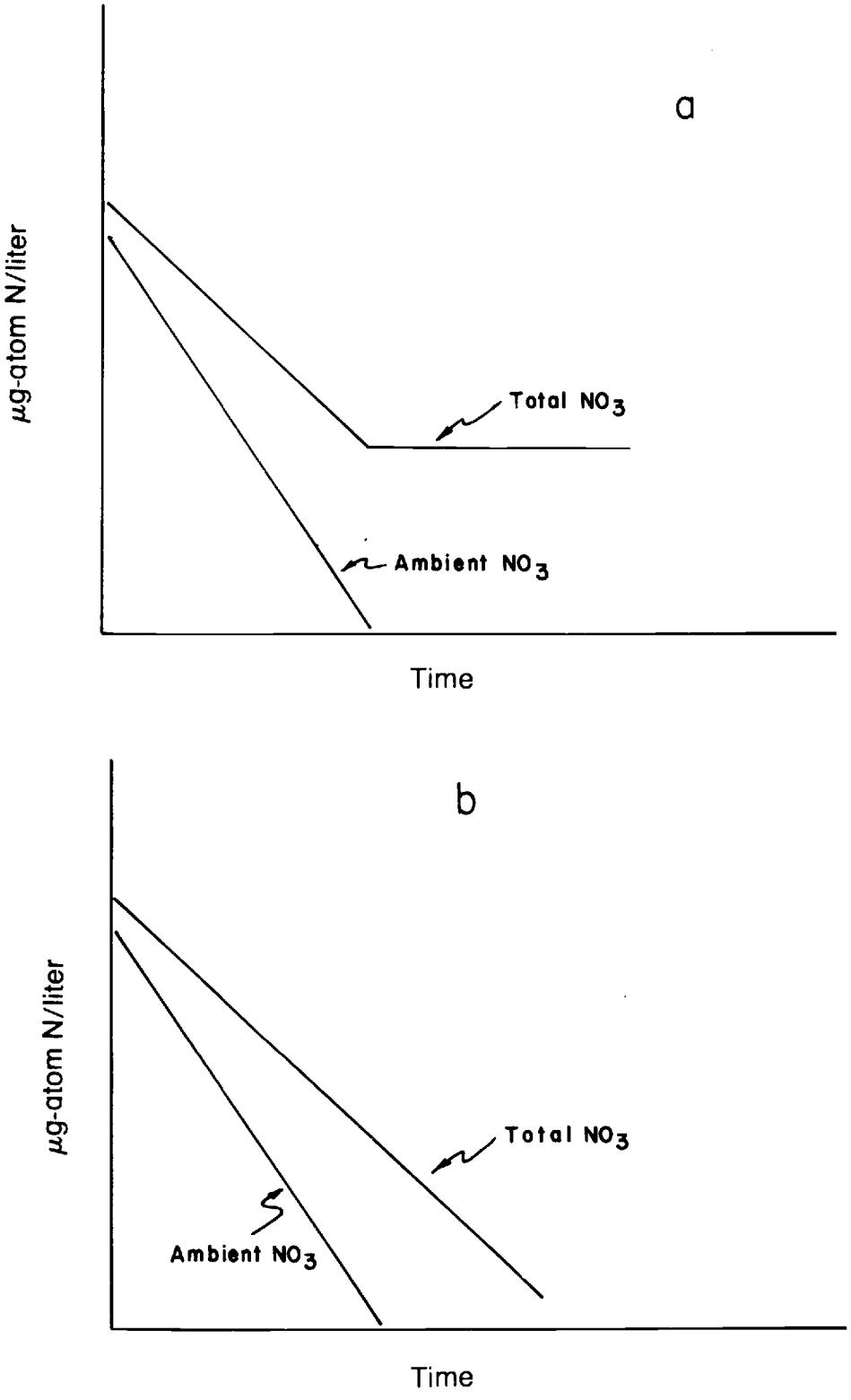

Figure 29. Predicted nitrate utilization patterns for (a) coupled uptake-reduction only; (b) uncoupled reduction only.

corresponding to a decrease functionally related to the internal nitrate concentration (Figure 29b). My experimental data indicate that both mechanisms may be important. Both experiments with Thalassiosira aestivalis, T1 and T2, support CUR as the primary mechanism for reduction. The slow down in nitrate reduction, as evidenced by the inflection in the plot of total nitrate vs. time (T1, Figure 24; T2, Figure 26), coincides with the cessation of uptake. That reduction does not completely cease is also apparent. The inflection supports the CUR theory, while the continued reduction at a much slower rate demonstrates the existence of an additional nitrate reductase uncoupled from uptake. This additional uncoupled nitrate reductase will be called FNR, but may not be identical to the free enzyme nitrate reductase measured by others (Eppley, et al., 1969). The non-linearities of nitrate disappearance (T1) make exact calculations difficult, but the ratio of initial reduction rate to final reduction rate [(CUR + FNR)/ FNR] is approximately 2.9, with FNR contributing only 35% to total reduction. Subtracting this fraction from the total initial reduction rate yields an uptake:reduction ratio based on CUR alone of 2.5 compared to the theoretical maximum of 3.0. The data from T2 (first pulse) are less exact, being derived from fewer data points over shorter time intervals. But given these limitations, FNR accounted for only 13.6% of the total nitrate reduction, with an uptake:reduction ratio of 1.3. These are the only data sets available which are sufficient to calculate coupled uptake:reduction ratios. However, a similar slowdown in reduction rate seems to occur with Skeletonema costatum (S5). The reduction rate after nitrate uptake ceased was

approximately 37% of the rate during uptake.

The relative importance of FNR vs. CUR appears to be quite variable. The post-uptake nitrate reduction rate for S7 (Skeletonema) was approximately 75% of the reduction rate during uptake. S7 is anomalous in other ways, suggesting that either an additional non-CUR uptake mechanism is present, or that the details of the uptake reduction coupling are more complicated than suggested by Butz and Jackson (1977). The ratio of total initial uptake to total intial reduction for S7 is 3.0, the maximum ratio predicted by Butz and Jackson. However, if the reduction which occurs after the cessation of uptake (presumably from free NR) is subtracted from the initial reduction, an unrealistic CUR uptake:reduction ratio of 12 is obtained. Further elucidation of the relative contribution of coupled uptake and reduction to marine phytoplankton must await further experimentation.

Other data can be found to support the hypothesis that both CUR and free NR mechanisms may be significant in marine phytoplankton. Collos and Slawyk (1976) found that for natural phytoplankton populations off the coast of Africa, free NR could account for only 12% of the total reduction during periods of nitrate uptake. After nitrate uptake ceased due to nitrate exhaustion, all reduction could be accounted for by free NR. In light of these results, it appears that the conventional NR assay (Eppley and Coatsworth, 1968) only measures that fraction of reduction mediated by free NR and misses that fraction mediated by CUR. Eppley and Coatsworth (1968) reached a similar conclusion for the diatom Ditylum brightwellii; that is, measured nitrate reductase could not account for all the reduction observed.

Furthermore, if their data are reinterpreted in light of CUR theory, an additional nitrate uptake mechanism is indicated, as I suggested for Skeletonema (S7). They observed uptake of nitrate in the dark, at one-fourth of the rate in the light, with essentially no reduction. Others have also noticed the dependence of nitrate uptake and light (Bates, 1974; MacIsaac and Dugdale, 1972). Falkowski (1975) reported evidence for a membrane bound ATPase responsible for uptake of nitrate by Skeletonema, with cyclic photophosphorylation as the primary energy source (Falkowski and Stone, 1975). One explanation for all these data could be that the membrane bound tetramer may serve a dual nitrate transport role, mediating both coupled uptake-reduction and uptake that is independent of reduction. In the dark, the supply of NAD(P)H and thiol decreases (Butz and Jackson, 1977), perhaps blocking reduction. If reduction is not mandatory for the release of the nitrate molecules bound to the inner monomers, but merely accelerates the release, one mechanism is sufficient to explain both light and dark uptake of nitrate. Furthermore, under conditions of high energy availability (such as for S7 with large carbon reserves) the rate of release from the membrane without reduction might be significant.

Internal Pools

The existence of internal nitrogen pools in marine phytoplankton has long been known. The significance of these pools, especially the role of inorganic nitrogen pools in cellular biochemical processes, and their role in physiological and ecological modeling, have been given little attention. Recently internal pools of nitrate have been pro-

proposed as indicators of nitrate utilization by natural phytoplankton populations (Collos and Slawyk, 1976). Models incorporating internal pools as explicit variables have been proposed for silica (Davis et al., 1978) and for nitrogen (DeManche et al., 1979). To be useful as indicators of physiological state, the levels of internal pools must be shown to relate to the nutrient history or assimilatory capacity of phytoplankton. For pools to be useful in models, a functional relationship coupling nitrogen uptake, internal pools, and cellular growth must be established. The data presented here suggest ways in which this coupling may be accomplished, but also point out some of the difficulties of a simplistic approach.

Skeletonema costatum displays a high degree of variability in the size of nitrate pools. If nitrogen history is divided into four broad regimes: non-limited, transient, low nitrogen adapted, and nitrogen starved (see RESULTS and Table 3), then ranges of nitrogen pool levels can be matched to each regime. Pool levels were divided into three ranges, low (undetectable to 30 mM/liter cell volume), intermediate (30 to 80 mM), and high (greater than 80 mM). For cultures with non-limited or transient histories, nitrate and amino acid pools were high. With starved or low nitrogen adapted cultures, nitrate pools were low and amino acid pools were intermediate to low.

Both non-limited and transient history cultures of Skeletonema showed significant decreases in nitrate pool levels following nitrate exhaustion from the medium (see Control Data, Appendix II and Appendix V). These observations support the conclusion that nitrate uptake and reduction by Skeletonema are only loosely or partially coupled.

Although decreases in amino acid pools were not apparent in the experimental treatments, a slow decrease in amino acid pools was noted for controls following nitrate exhaustion. After 1.6 days of nitrogen starvation, amino acid pools were still approximately 150 mM, decreasing to low levels over the next half day.

In contrast, the nitrate and amino acid pools of Thalassiosira aestivalis showed little variability with decreasing nitrate supply, reflecting the greater relative importance of coupled uptake and reduction for Thalassiosira versus Skeletonema. Cultures adapted for two days to low nitrate concentrations had nitrate and amino acid pools similar to those for non-limited cultures (Table 3).

The internal nitrate concentration (pool size) for both Skeletonema and Thalassiosira (for all regimes other than non-nitrogen limited) increased following an addition of nitrate. The rate of increase was highly variable, being most rapid for transient-history cultures (S5) and for one of the low-nitrogen adapted cultures (S7). The rate of increase was slowest or showed lags for the nitrogen starved cultures (S3 and S4) and for the other low nitrogen adapted Skeletonema culture (S6). In each case where an increase occurred, the rate of increase was constant over the two- to three-hour uptake experiments, unless interrupted by nitrate exhaustion from the medium. After an input of nitrate, nitrate pools of transition cultures or early starvation cultures (S5 and S2) reached levels greater than those for non-nitrogen-limited cultures. Internal nitrate pools appear to be the result of both adaptation to the long term nitrogen regime and response to short term nitrogen perturbations. The multiple factors

contributing to pool levels complicate the use of pools as indicators of physiological state unless the history of recent perturbations is known. However, the combined information from both nitrate and amino acid pools, along with the phytoplankton response to a perturbation by additional nitrate, may be sufficient to define the nitrogen history of the population and the capacity for nitrate assimilation. Since nitrate pool increases are minimal at the extremes of non-nitrogen limitation and starvation, the rate of increase should be maximal during transition. That is, the difference between uptake and reduction rates should be maximal during those periods when nitrogen supply rate is insufficient to meet the adapted uptake demands of the population, but before starvation has decreased the capacity for both uptake and reduction. With no recent perturbations, nitrate pools are an inverse function of nitrate deprivation.

In all experiments with nitrate as the sole nitrogen addition, no significant increases were observed in the amino acid pools after the nitrate addition. Small changes may have been obscured by "noise" in the amino acid analysis, or may have gone undetected if lags occurred before an increase began. The lack of increase also may indicate that nitrate reduction is the rate-limiting step in nitrate assimilation in marine phytoplankton as it is in higher plants (Beevers and Hageman, 1969). However, in both experiments where ammonium as well as nitrate was added (S4b, Figure 13; S6b, Figure 21), large increases in amino acid levels were observed. No comparable increases in ammonium pools were observed; in fact the trend for the starved culture was downward (Figure 13), probably reflecting a decrease in nitrogen catabolism.

The only evidence for "surge uptake" of ammonium (Dugdale, 1977) was observed for the low-nitrogen-adapted culture (S6b, Figures 20 and 21). Initial ammonium pools were only 12.4 mM (S.D. 1.2 mM), rose to 30 mM within 15 minutes after the addition, then slowly declined. This apparent surge in ammonium uptake was barely detectable, much less than that reported by other investigators (Conway et al., 1977; McCarthy and Goldman, 1979). The lack of significant surge uptake may have been the result of differences in degrees of nitrogen limitation for chemostats, diffusion culture, and nitrogen starved batch culture. McCarthy and Goldman (1979) found that for Thalassiosira psuedonana, surge uptake increased as dilution rate in a steady state chemostat decreased, i.e., as the nitrogen limitation increased. However, other physiological parameters (C/N ratio, nitrogen per cell volume, fluorescence per cell volume) indicate that the diffusion culture environment may not be comparable to any steady state environment. Another explanation for the apparent absence of ammonium surge uptake could be the prior conditioning on nitrate rather than ammonium. All reported instances of ammonium surge uptake were for cultures conditioned to ammonium. The failure to observe ammonium surge uptake, or large initial increases in the ammonium pool, following additions of both nitrate and ammonium to a nitrate deficient batch culture of Skeletonema (Lundy, 1974; DeManche et al., 1979), supports this explanation.

When both ammonium and nitrate were added to low-nitrogen (S6b, Figure 21) or nitrogen-starved (S4b, Figure 13) cultures of Skeletonema, a rapid increase in the amino acid pool was observed. For the low

nitrogen culture (S6b), the phytoplankton assimilated 2.6 times as much nitrogen during the first hour when both nitrate and ammonium were supplied, than when nitrate alone was given. Nitrate assimilation during this first hour was 25% of total nitrogen assimilation. Of the additional nitrogen assimilated (relative to the nitrate only culture), only 34% appeared as increased amino acids. The other 66% could have been assimilated into polypeptides or protein which are not detected by the amino acid analysis. Or the end products of nitrate and ammonium assimilation may be different. Lui and Roels (1972) found significant increases in amide nitrogen for the diatom Biddulphia aurita when grown on ammonium, but no increases for growth with nitrate.

Pools: Regulation and Control

Changes in nitrate pool levels reflect imbalances between uptake and reduction. If the coupled uptake-reduction (CUR) mechanism of Butz and Jackson (1977) discussed previously was the primary mechanism for both uptake and reduction, increases in the nitrate pool during nitrate uptake would be expected under most conditions. Only if inhibition of nitrate reduction were totally reversed would reduction equal uptake according to this mechanism. Otherwise, the ratio of uptake to reduction could be as large as 3:1, and pool increases would be a function of total uptake rate and the degree of imbalance between uptake and reduction. However, I have shown data (Figures 17, 23, 25) which indicate that, though CUR may be significant, additional internal nitrate reduction must be operating independently of uptake. The rela-

relative importance of CUR to free NR appears to be variable, a function of species and physiological state. Thus, nitrate pool increases are a function of the uptake:reduction ratio of CUR modified by the amount of additional internal free reduction. Also the existence of an additional uptake mechanism independent of CUR uptake cannot be ruled out at this time. From both physiological and ecological points of view, questions other than those concerning the mechanism of pool changes are equally interesting. These include: what mechanism stabilizes nitrate pools at a given level? what factors tend to destabilize this equilibrium level? and what is the time scale for response and adjustment?

During nitrate saturating conditions the internal nitrate pool for Skeletonema was approximately 80 mM, and was unaffected by additional nitrate input (S1, Figures 4 and 5). To maintain a constant nitrate pool requires some form of negative feedback control regulated by internal nitrate concentration. Otherwise even a small enhancement in uptake over reduction would lead to continually increasing pools. A simple form of control can be exerted by variations in the extent of internal nitrate reduction of the surplus nitrate uptake from the CUR uptake mechanism. If the non-limited internal nitrate concentration were less than the saturation concentration for internal free NR, then a fast response feedback loop is possible. Increases in internal nitrate concentration would lead to an increased reduction rate following Michaelis-Menten kinetics for free nitrate reductase. Thus the internal pool could remain stable at a level where reduction equals uptake. However, Eppley and Rogers (1970) found the in vitro half-

saturation constant for nitrate reductase in Ditylum to be only 0.11 mM, and Packard and Blasco (1974) reported a half-saturation constant for Skeletonema of only 0.033 mM. No data are available for in vivo apparent half-saturation constants for Skeletonema; however, no increase in reduction rate was observed in my experiments concurrent with increasing nitrate pools except for pools less than approximately 30 mM. The low-nitrogen-adapted culture (S6) and nitrate-starved cultures (S3 and S4), both with initially small nitrate pools, showed a lag in reduction rate until internal nitrate exceeded 30 mM (Figures 8, 9, 10, 11, 14, 15, 18 and 19). Though simple concentration dependent kinetics may be functioning at these low nitrate pool levels, for those cultures with high nitrate pools, another feedback mechanism must be controlling nitrate pool level.

At these saturating nitrate pool levels, the rate of nitrate reduction could be controlled by the total amount of free nitrate reductase. The data indicate that free NR is a variable fraction of total reduction, ranging from 14% to 75% of the total in these experiments. This mechanism would explain the constancy of reduction rate in spite of increasing internal nitrate concentration. The response time for induction of increased NR should be much longer than that required for simple enzymatic kinetic control. Eppley and Coatsworth (1968) found that several hours were required for induction. If the rate of production of internal NR were a function of internal nitrate concentration, similar lags could be expected before changes in internal nitrate could lead to changes in NR.

The uptake of nitrate also appears to be under internal control. Shifts in apparent half-saturation constants were observed for both Skeletonema and Thalassiosira during some of the perturbations. The most often observed response was constant nitrate uptake at the initial maximum rate until ambient nitrate levels were approximately one μg-atom NO_3-N liter^{-1} or less (Figures 18 and 26). However, Skeletonema (Figure 16) and Thalassiosira (Figure 24) both showed significantly non-constant nitrate uptake on occasion, with deviations from maximum uptake near five μg-atom NO_3-N liter^{-1}. This phenomenon may be a manifestation of the "shift up" reported by Dugdale (1977). However, both these cultures may have been in transitional state and both showed moderate initial nitrate uptake enhancement relative to non-deficient cultures. An alternative explanation may be that the cellular energy available for the enhanced nitrate uptake was insufficient to maintain the initial rate. In this case, the change in uptake rate would have no functional relationship to ambient nitrate concentration.

That ammonium can inhibit nitrate uptake has been well established (Eppley et al., 1969; Packard and Blasco, 1974). However, the extent of that inhibition as a function of either physiological state or environmental conditions is still uncertain. The variations in extent of inhibition have been mentioned previously (Bates, 1976; Bienfang, 1975; Lundy, 1974). McCarthy et al. (1977) devised a relative preference index for field studies to indicate the relative inhibition of nitrate uptake by ammonium. This index, RPI, is the ratio of the fraction of total nitrogen used as a single form of nitrogen to the fraction of total nitrogen nutrient concentration as the same form. For

nitrate:

$$RPI = \frac{NO_3 \text{ uptake/total nitrogen uptake}}{NO_3 \text{ concentration/total nitrogen}}$$

McCarthy et al. (1977) and others (Eppley et al., 1979) have found this RPI to be generally less than one for ammonium concentrations above 0.5 to 1.0 µg-atom NH_4-N liter^{-1}; however, much of the variability in RPI cannot be explained as a simple function of nutrient concentrations.

Conway (1977) proposed that the mechanism for ammonium inhibition of nitrate uptake operated through elevated amino acid levels associated with ammonium utilization. DeManche et al. (1979) proposed a similar mechanism with additional inhibition by higher ammonium pools proposed for ammonium assimilation compared to nitrate assimilation. Neither of these explanations are supported by the data presented here. Although amino acids did increase when ammonium was supplied, the increase was neither rapid enough nor large enough to account for the inhibition of nitrate utilization observed. Near the end of the experiment, when ammonium inhibition of nitrate utilization was nearly total, amino acid pools were smaller than those amino acid pools measured for non-limited cultures with nitrate as the sole nitrogen source (S6a, Figure 21). Ammonium pools also showed no change that could be associated with nitrate inhibition. Perhaps the assumption that phytoplankton cells are homogenious (Grenney et al., 1975; DeManche et al., 1979) is a gross oversimplification. In higher plants, nitrite reduction is concentrated in the chloroplasts (Hewitt, 1975) while ammonium assimilation may be concentrated in the mitochondria (Ritenour et al., 1967). If similar compartmentalization occurs in diatoms, as

proposed by Eppley and Rogers (1970), then only ammonium derived from ammonium uptake should be found near the cell membrane. Thus, nitrate uptake could be inhibited by an intercellular ammonium gradient resulting from ammonium uptake, not total internal ammonium concentration.

Transient Environments, Culture Conditions and Models

The data presented here demonstrate that transient nitrate environments can lead to highly variable uptake and reduction, and variations in inorganic nitrogen pools. Addition of ammonium significantly modifies the utilization of nitrate as well as causing changes in internal amino acid pools. Although these responses are intrinsically interesting from the view of a physiologist, their significance to biological oceanography derives from their effect on phytoplankton growth, species competition and succession, and the transfer of energy and materials to higher trophic levels. To be ecologically significant, two conditions must be met: 1) transient environments, as opposed to steady-state environments, must be significant components of marine nitrogen regimes; and 2) the magnitude of the effects of transients on phytoplankton must be sufficient to affect phytoplankton production, species succession or herbivore grazing.

Considering the time scale of phytoplankton response to changing nitrogen regimes observed here (hours to days), and the observation that establishment of steady-state adaptations by phytoplankton in chemostats requires several days (Caperon, 1969), the constancy of nitrogen flux required for establishment of a true equilibrium is seldom obtained in real marine systems (Jannasch, 1974). Variations in

nitrate flux can arise from breaking of internal waves, diurnal and semidurnal tidal mixing and episodic wind mixing. Even the pelagic ocean, often suggested as a steady-state environment, exhibits diurnal and other short-term variations in nitrogen concentration (Menzel and Spaeth, 1962). This lack of constancy has been proposed as one of the major factors contributing to the diversity of phytoplankton species (Hutchinson, 1961). Unfortunately, the information most critically lacking is data concerning the variation of nitrogen flux, the micro-scale of nitrogen abundance in time and space scales of relevance to marine phytoplankton, and the effect of complex variations in flux on phytoplankton.

Given that variable nitrogen environments are significant components of the marine environment, the significance of such environments for physiological studies of marine phytoplankton and implications for models of phytoplankton production and zooplankton interactions can be examined. Culture conditions can be classified according to the extent to which nutrient flux is sufficient to meet the growth requirements of the phytoplankton. Thus, both batch culture and chemostats, in one sense, are equivalent. In non-nitrogen limited batch culture, supply exceeds phytoplankton demand. In chemostats, at steady state, the physiological state of the phytoplankton has been self-modified so that growth rate and nutrient supply rate are in balance. This concept is shown diagramatically in Figure 30. If the time of adaptation required by cells to adjust their physiological state to match the nutrient flux were rapid compared to the rate of change of flux, then all nutrient regimes could be represented by line A.

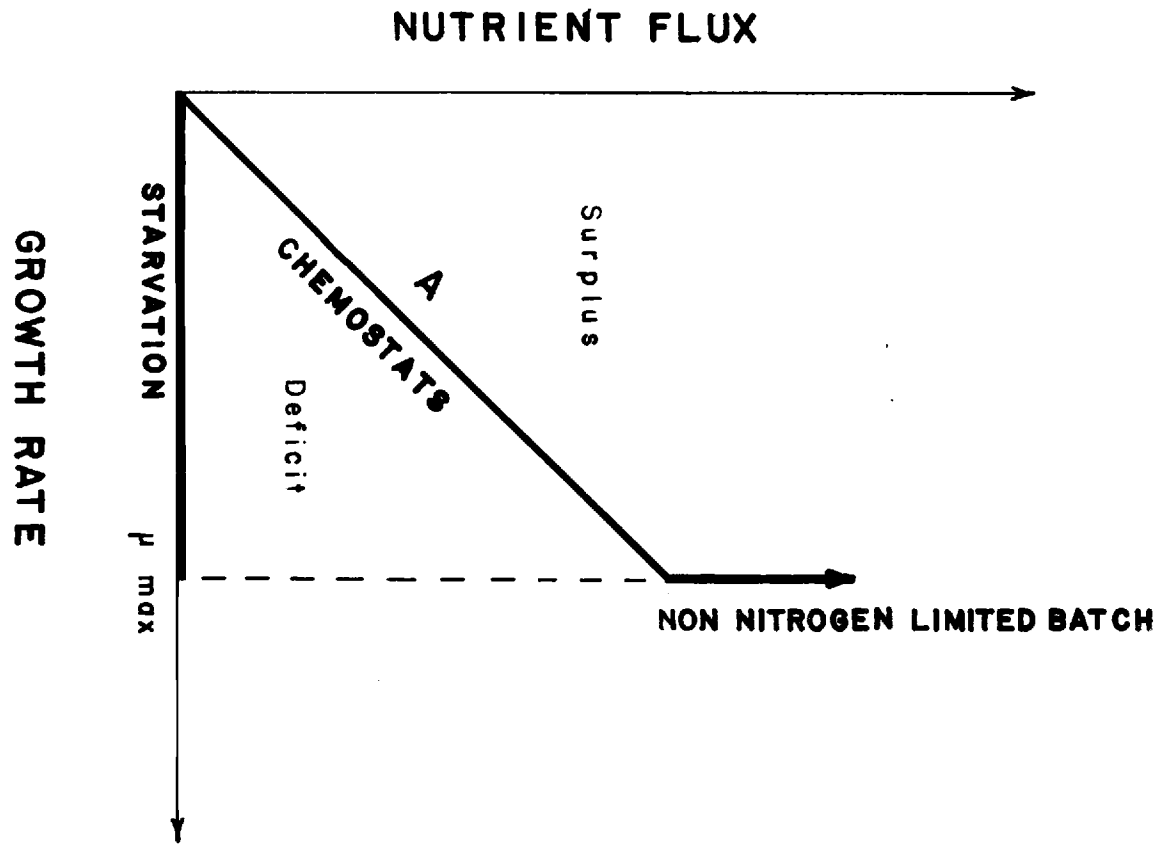

Figure 30. Nutrient supply vs. nutrient needs for phytoplankton growth.

However, changes in nutrient supply can occur much faster than adjustments in cellular physiology or changes in cell quota. The time scale of adapted responses to environmental perturbations is a critical factor in determining "optimum" strategy for survival (Slobodkin and Rapoport, 1974).

The fluctuating diffusion rates of the diffusion cultures used in this study provide one example of such rapidly changing nutrient flux. Nutrient exhaustion in batch culture provides another. Pulsed input of nutrients provides a third, though in the direction of surplus rather than deficiency. The most critical factor appears to be the degree to which nutrient supply is insufficient to meet adapted cell growth requirements. When cells are given sufficient time to adapt to a constant degree of nutrient limitation (steady-state chemostat), a functional relationship appears to develop between maximum uptake rate (as determined when nutrient is resupplied in excess) and steady-state growth rate. Although the exact form of the functional relationship is still uncertain (Dugdale, 1977; McCarthy and Goldman, 1979), in general V_{max}, the saturation uptake rate, increases as the steady-state dilution rate (growth rate) decreases. This effect is primarily attributed to a decrease in the amount of limiting nutrient per cell with decreasing dilution rates. McCarthy and Goldman (1979) have extended this concept to effects of nitrogen starvation relative to steady-state growth rate on maximum uptake rates. For one clone of Thalassiosira psuedonana (clone 3H), they found that additional enhanced uptake following a period of starvation could be attributed to a corresponding change in nitrogen per cell (cell quota) during the starvation period.

However, with a different clone of the same species (T. pseudonana, clone 13-1), they found an additional large enhancement of maximum uptake rate following a short starvation period (two hours), which could not be explained by changes in nitrogen per cell. Furthermore, the degree of enhancement depended upon the previous steady-state growth rate, increasing with decreasing growth rate. These starvation experiments lasted only two hours, but enhancement at two hours was greater than at 20 min. The effect of longer periods of deprivation were not reported.

Similar effects appear to be operating with Skeletonema costatum, with enhanced uptake rates observed after only short periods of nutrient insufficiency (S5) or starvation (S2). Longer periods of starvation (1.6 to 2.1 days) have an opposite effect, leading to lags in uptake and assimilatory capacity (S3 and S4). Short periods of starvation appear to have little effect on Thalassiosira aestivalis (T2), comparable to the effects noted by McCarthy and Goldman (1979) for T. pseudonana (clone 3H).

Obviously an additional variable is required for modeling phytoplankton nutrient response capabilities in real world environments with varying nutrient flux and phytoplankton growth adjustments. I suggest that an enhancement factor (E) be applied to the maximum uptake term for nutrient uptake, [V_m' in equation (4), p. 7] such that:

$$V_E = V_m' \cdot E \ ,$$

and

$$V_m' = V_m Q_m / Q \ ,$$

where V_E is the enhanced maximum uptake rate, V_m is the maximum saturated uptake rate (equivalent to the maximum attainable growth rate in steady-state chemostat or non-limited batch culture), Q is the cell quota (nutrient per cell), and Q_m is the maximum cell quota for cells growing at maximum growth rate. Based on my data (S2 and S5) and that of McCarthy and Goldman (1979), this factor, E, can be significant for short periods of deprivation (E equals 2.6 for S5, and approximately 2.2 for two hours starvation at 25% maximum growth rate for T. pseudonana (13-1)). For long periods of starvation, E is near zero (S3 and S4). This variable enhancement factor precludes prediction of uptake response from phytoplankton with an unknown environmental history without some additional information, hence the wide differences in response observed for two low nitrogen diffusion cultures of Skeletonema (S6 and S7). Not only must the growth potential be known, but the extent of deprivation and the functional relationship between deprivation and uptake enhancement must also be known. Such data are currently lacking and should be a major area for future research.

Controlled experiments can be carried out in the laboratory where uptake response capacity can be measured as a function of both potential growth rate and time of deprivation or starvation. From such data information can be provided for the creation of predictive models. However, information about both potential growth rate and the extent of deprivation are not so easily determined in field situations. A different approach is to model uptake capacity as a function of cellular nutrient quota, as described by Droop and others (Droop, 1975; Goldman, 1977; McCarthy and Goldman, 1979), but with modifications such

that the enhancement factor is related to nutrient deprivation through other cell properties. Numerous parameters have been suggested as indicators of physiological state, among them: C/N ratio (Eppley and Renger, 1974; Caperon and Meyer, 1972a; Donaghay et al., 1978), assimilation ratio (Curl and Small, 1965), and fluorescence number (Kiefer, 1973). None has yet proved to be a universally reliable indicator of cell physiological state. Much of the problem may relate to intrinsic differences among species. Both Skeletonema costatum and Thalassiosira aestivalis have quite different C/N ratios and fluorescence-to-cell-volume ratios under similar growth conditions (Table 4). Furthermore, Skeletonema showed marked changes in these ratios as culture conditions varied. However, the limited number of experiments with T. aestivalis, coupled with the unknown nutrient conditioning, make generalizations difficult at this time. The lack of understanding of the environmental factors contributing to changes in some cellular properties is demonstrated by comparison between S6 and S7. Both cultures were low-nitrate diffusion cultures. These cultures had, essentially, opposite responses to nitrate readdition; extreme enhancement of uptake for S7, normal or slightly slowed uptake for S6. Based upon nitrate pools and C/N ratios, both were extremely nitrate-limited. Yet the nitrogen per cell volume for S6 was much higher than that for S7 (1.4 vs. 0.52 μg-atom N/10^9 μm^3), and fluorescence/cell volume for S6 was three times that of S7. The ratio of fluorescence/cell volume appears to be the best indicator of the onset of nitrogen starvation (Table 4), dropping from 0.173 to 0.131 in approximately one hour, to 0.088 by the end of four hours, and to 0.03

Table 4. Initial Nitrate Uptake Rates, Enhancement, and Cell Parameters. (1) nitrate uptake relative to cellular nitrogen (day^{-1}); (2) initial nitrate uptake rate relative to non-limited nitrate uptake rate; (3) carbon/nitrogen ratio (atomic); (4) relative fluorescence/($10^9 \mu^3$); (5) cellular nitrogen per cell volume (μg-atom $N/10^9 \mu^3$); and (6) uptake during first hour significantly nonlinear. Value in parentheses is initial 20 min uptake rate.

Experiment	Initial uptake rate(1)	Relative enhancement(2)	C/N ratio(3)	Fluorescence/ cell volume(4)	N/cell volume(5)
Skeletonema costatum					
S1	1.84	1.00	9.7	0.17	1.03
S2	2.46	1.33	9.8	0.13	1.33
S3	0.45	0.24	14.0	0.04	1.41
S4a	0.66	0.36	15.4	0.05	0.94
S4b*	0.52	0.28	14.3	0.03	1.02
S4c	0.35	0.19	14.9	0.03	0.98
S5	4.47	2.43	9.0	0.09	1.15
S6a	1.60	0.87	18.2	0.19	1.40
S6b*	0.67	0.36	18.2	0.19	1.40
S7	7.76	4.22	19.4	0.06	0.52

Table 4 (continued)

Experiment	Initial uptake rate (1)	Relative enhancement (2)	C/N ratio (3)	Fluorescence/ cell volume (4)	N/cell volume (5)
Thalassiosira aestivalis					
T1	1.72 (2.60) (6)	0.66 (1.00) (6)	6.4	0.07	1.46
T2 (first pulse)	2.61	1.00	7.9	0.08	1.11
T2 (second pulse)	2.43	0.93	8.5	0.06	1.22

*with ammonium.

after two days of starvation. Except for cases of extreme starvation, the amount of enhanced uptake for Skeletonema is an increasing function of decreasing fluorescence/cell volume (Figure 31). Similar curves have been observed for steady-state cultures using nitrogen per cell as an indicator (McCarthy and Goldman, 1979). Nitrogen per cell was not measured in these experiments though both N/cell volume and C/N ratios were. No correspondence between either C/N ratio or N/cell volume and enhanced uptake were observed. Although a relationship between C/N ratio and growth rate has been observed for phytoplankton in chemostats (Donaghay et al., 1978), others report no change in C/N ratio with growth rate (McCarthy and Goldman, 1979). Both N/cell volume (or N/cell) and C/N ratios are slow-responding indicators and may not be indicative of short term physiological adaptations to short term variations in nutrient supply rate. These slow response parameters, though, may be useful in separating short-term from long-term adaptations. Lundy (1974) observed continued carbon fixation for at least 24 hours after nitrogen exhaustion. Contrary to the observations of Falkowski and Stone (1975), no cessation of carbon fixation was observed in these experiments concurrent with enhanced nitrate uptake. For my culture with greatest uptake enhancement (S7), the carbon specific growth rate during the period of enhanced nitrate uptake was 2.27 day^{-1} with a standard deviation of 0.54 day^{-1}, i.e., it was not significantly different from the non-nitrogen-limited rate. Carbon growth rates for the starved cultures (S3 and S4) were not significantly different from zero. Therefore, carbon fixation rates for short periods of time, even removed from nitrate flux, may be good

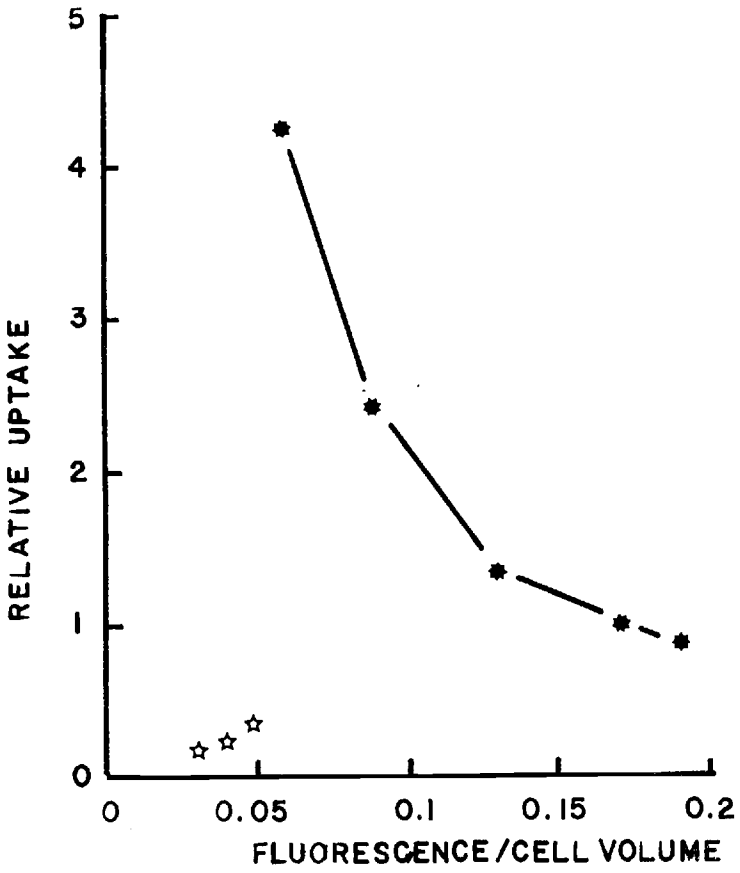

Figure 31. Relative uptake enhancement vs. fluorescence per cell volume for Skeletonema costatum. Starved cultures S3 and S4, ☆ . Data from Table 4.

indicators of long term adapted growth rate.

Finally, the significance of variations in internal pools and enhanced uptake cannot be overlooked in modeling interactions with zooplankton. McCarthy and Goldman (1979) have suggested that microscale pulses of ammonium excreted by zooplankton, coupled with enhanced uptake capabilities of phytoplankton under nitrogen stress, may be sufficient to meet phytoplankton nitrogen requirements without detectable changes in ambient nitrogen. Furthermore, the internal nitrogen pools should be considered for their effect upon zooplankton grazers. Internal nitrate, though not a large fraction of total nitrogen, represents a significant fraction (10 to 12% of total nitrogen) that is unavailable to grazers upon ingestion. This fraction becomes reavailable to phytoplankton due to "leakage" during grazing. Elevated amino acid pools, observed following a pulse of ammonium, could provide extra nitrogen to grazers. Combined with the excretion model of McCarthy and Goldman, the cycle of zooplankton excretion of ammonium, ammonium uptake by phytoplankton, and increased amino acid pools could lead to enhanced grazing by herbivores.

SUMMARY AND CONCLUSIONS

This research was an initial effort to break free from the assumptions imposed by steady-state nutrient kinetics on studies of marine phytoplankton. Once transient environments are permitted, the multiplicity of permutations seems endless. And yet it is just such a diversity of environments that must be understood first in the laboratory if we are to understand the role that highly variable, multiple-form nutrient flux has on determining both phytoplankton production and species succession. As knowledge of nutrient influences is coupled with understanding of physical mixing and turbulence, the interactions of light and non-limiting nutrients, and the loss due to sinking and zooplankton grazing, a more detailed and realistic picture of primary production will ensue. Finally one may hope to make predictions of the transfer of energy and materials from plants to higher trophic levels with some degree of confidence.

Much remains to be accomplished. This effort addressed only a few of the possible nitrogen regimes for only two species of phytoplankton at one light-temperature level. Hopefully, more complete patterns will become apparent as this research direction continues and expands. Based on this limited data set, some generalizations appear valid:

1. Two distinct mechanisms for nitrate reduction have been observed in marine phytoplankton. One is coupled to uptake and operates in a manner similar to the coupled uptake reduction mechanism proposed by Butz and Jackson (1977). The other is independent of uptake. Both mechanisms may exist simultaneously. When the coupled mode is dominant, reduction slows drastically when uptake stops from

nitrogen exhaustion.

2. The measured rate of total nitrate reduction is not a direct function of internal nitrate concentration. The fraction of nitrate reduction derived from the coupled uptake-reduction mechanism shows no relationship to internal nitrate. Only that fraction of nitrate reduction due to free nitrate reductase appears to be controlled by internal nitrate concentrations, and that control is limited to populations exposed to low nitrate environments. A mechanism of indirect control, with internal nitrate stimulating increased free nitrate reductase activity is proposed.

3. Internal pools of nitrate show great variability in response to varying nitrogen regimes. Under conditions of nitrogen stress, the levels become much smaller than normal. Populations which are in a state of nitrogen deprivation exhibit increases in the level of internal nitrate after a nitrate addition. This increase is caused by an enhanced uptake rate rather than a decreased reduction rate. Cultures that have been nitrogen starved for long periods may have delays before nitrate utilization is restored to near normal rates.

4. The response to ammonium additions is different than that for nitrate. Although surge uptake for ammonium may temporarily lead to a slight increase in internal ammonium, nitrogen stressed cultures show no sustained increase in internal ammonium during ammonium uptake. An increased concentration of free amino acids does occur following ammonium addition, but not nitrate additions. No delays in ammonium utilization were found in nitrogen starved cultures. Ammonium inhibits nitrate uptake, but the extent of that inhibition is variable. Initial

uptake and reduction of nitrate is not inhibited, inhibition requiring about 15 minutes to become effective.

5. The mechanism of ammonium inhibition of nitrate uptake and reduction is still unknown. However, control does not appear to be exercised through increased internal total amino acids nor by increased internal ammonium concentrations. A mechanism for inhibition based on ammonium uptake flux and cellular inhomogeneity is proposed.

BIBLIOGRAPHY

Bates, S. S. 1974. Effects of light and ammonium on nitrate uptake by two species of estuarine phytoplankton. M.S. Thesis. City College of New York.

_____. 1976. Effects of light and ammonium on nitrate uptake by two species of estuarine phytoplankton. Limnol. Oceanogr. 21: 212-218.

Benson, J. R., and P. E. Hare. 1975. o-Phthalaldehyde: fluorogenic detection of primary amines in the picomole range. Comparison with fluorescamine and ninhydrin. Proc. Nat. Acad. Sci. USA 72:619-622.

Bhovichitra, M. and E. Swift. 1977. Light and dark uptake of nitrate and ammonium by large oceanic dinoflagellates: Pyrocystis noctiluca, Pyrocystis fusiformis, and Dissodinium lunula. Limnol. Oceanogr. 22:73-83.

Bienfang, P. K. 1975. Steady state analysis of nitrate-ammonium assimilation by phytoplankton. Limnol. Oceanogr. 20:402-411.

Bruce, H. 1969. The role of dissolved amino acids as a nitrogen source for marine phytoplankton in an estuarine environment in Southeastern Alaska. Ph.D. Thesis, School of Oceanography. Oregon State University.

Butz, R. G. and W. A. Jackson. 1977. A mechanism for nitrate transport and reduction. Phytochemistry 16:409-417.

Caperon, J. 1969. Time lag in population growth response of Isochrysis galbana to a variable nitrate environment. Ecology 50:188-192.

Caperon, J. and J. Meyer. 1972a. Nitrogen limited growth of marine phytoplankton. I. Changes in population characteristics with steady state growth rate. Deep-Sea Res. 19:601-618.

_____. 1972b. Nitrogen limited growth of marine phytoplankton. II. Uptake kinetics and their role in nutrient limited growth of phytoplankton. Deep-Sea Res. 19:619-632.

Carpenter, E. J. and R. R. L. Guillard. 1971. Intraspecific differences in nitrate half-saturation constants for three species of marine phytoplankton. Ecology 52:183-185.

Collos, Y. and J. Lewin. 1974. Blooms of surf-zone diatoms along the coast of the Olympic Peninsula, Washington. IV. Nitrate reductase activity in natural populations and laboratory cultures of Chaetocerous armatum and Asterionella socialis. Mar. Biol. 25:213-221.

Collos, Y. and G. Slawyk. 1976. Significance of cellular nitrate content in natural populations of marine phytoplankton growing in shipboard cultures. Mar. Biol. 34:27-32.

Conover, S. A. M. 1975. Partitioning of nitrogen and carbon in cultures of the marine diatom Thalassiosira fluviatilis supplied with nitrate, ammonium or urea. Mar. Biol. 32:231-246.

Conway, G. R., N. R. Glass and J. C. Wilcox. 1970. Fitting nonlinear models to biological data by Marquardt's algorithm. Ecology 51: 503-507.

Conway, H. L. 1977. Interactions of inorganic nitrogen in the uptake and assimilation by marine phytoplankton. Mar. Biol. 39:221-232.

Conway, H. L. and P. J. Harrison. 1977. Marine diatoms grown in chemostats under silicate or ammonium limitation. IV. Transient response of Chaetoceros debilis, Skeletonema costatum, and Thalassiosira gravida to a single addition of the limiting nutrient. Mar. Biol. 43:33-43.

Conway, H. L., P. J. Harrison, and C. O. Davis. 1976. Marine diatoms grown in chemostats under silicate or ammonium limitation. II. Transient response of Skeletonema costatum to a single addition of the limiting nutrient. Mar. Biol. 35:187-199.

Cornish-Bowden, A. 1976. Principles of Enzyme Kinetics. Butterworths. London.

Curl, H. C., Jr. and L. F. Small. 1965. Variations in photosynthetic assimilation ratios in natural marine phytoplankton communities. Limnol. Oceanogr. 10:R67-R73.

Davis, C. O., N. F. Breitner, and P. J. Harrison. 1978. Continuous culture of marine diatoms under silicon limitation. 3. A model of Si-limited diatom growth. Limnol. Oceanogr. 23:41-51.

DeManche, J. M., P. L. Donaghay, W. P. Breese and L. F. Small. 1975. Residual toxicity of ozonized seawater to oyster larvae. Sea Grant Technical Bulletin #ORESU-T-75-003. Oregon State University. Corvallis.

DeManche, J. M., H. C. Curl, Jr., D. W. Lundy and P. L. Donaghay. 1979. The rapid response of the marine diatom Skeletonema costatum to changes in external and internal nutrient concentration. Mar. Biol. 53:323-333.

Draper, N. and H. Smith. 1966. Applied Regression Analysis. Wiley.

Droop, M. R. 1973. Some thoughts on nutrient limitation in algae. J. Phycol. 9:264-272.

Droop, M. R. 1975. The nutrient status of algal cells in batch culture. J. Mar. Biol. Assoc. U. K. 55:541-555.

_____. 1978. Comments on the Davis/Breitner/Harrison model for silicon uptake and utilization by diatoms. Limnol. Oceanogr. 23: 383-385.

Donaghay, P. L., J. M. DeManche and L. F. Small. 1978. On predicting phytoplankton growth rates from carbon:nitrogen ratios. Limnol. Oceanogr. 23:359-362.

Dugdale, R. C. 1967. Nutrient limitation in the sea: Dynamics, identification and significance. Limnol. Oceanogr. 12:685-695.

_____. 1976. Nutrient cycles. In Ecology of the Sea. D. Cushing and J. Walsh, eds. Blackwell, London. pp. 141-172.

_____. 1977. Modeling. In The Sea, vol. 6. Marine Modeling. E. D. Goldberg, I. N. McCave, J. J. O'Brien, J. H. Steele, eds. John Waley and Sons. pp. 789-806.

Eppley, R. W. and J. L. Coatsworth. 1968. Uptake of nitrate and nitrite by Ditylum brightwellii: Kinetics and mechanisms. J. Phycol. 4:151-156.

Eppley, R. W., J. L. Coatsworth, and L. Solorzano. 1969. Studies of nitrate reductase in marine phytoplankton. Limnol. Oceanogr. 14: 194-205.

Eppley, R. W. and E. H. Renger. 1974. Nitrogen assimilation of an oceanic diatom in nitrogen-limited continuous culture. J. Phycol. 10:15-23.

Eppley, R. W. and J. N. Rogers. 1970. Inorganic nitrogen assimilation of Ditylum brightwellii, a marine plankton diatom. J. Phycol. 6:344-351.

Eppley, R. W. and W. H. Thomas. 1969. Comparison of half-saturation constants for growth and nitrate uptake of marine phytoplankton. J. Phycol. 5:375-379.

Falkowski, P. G. 1975. Nitrate uptake in marine phytoplankton: (nitrate-chloride) - activated adenosine triphosphatase from Skeletonema costatum (Bacillariophyceae). J. Phycol. 11:323-326.

Falkowski, P. G. and D. P. Stone. 1975. Nitrate uptake in marine phytoplankton: energy sources and the interaction with carbon fixation. Mar. Biol. 32:77-84.

Goldman, J. C. 1977. Steady state growth of phytoplankton in continuous culture: Comparison of internal and external nutrient equations. J. Phycol. 13:251-258.

Grenney, W. J., D. A. Bella and H. C. Curl, Jr. 1974. Effects of intracellular nutrient pools on growth dynamics of phytoplankton. J. Wat. Pol. Cont. Fed. 46:1751-1760.

Guillard, R. R. L. and J. H. Ryther. 1962. Studies of marine planktonic diatoms. I. Cyclotella nana Hustedt and Detonula confervacea (Cleve.). Gran. Can. J. Microbiol. 8:229-239.

Hutchinson, G. E. 1961. The paradox of the plankton. Amer. Natur. 95:137-145.

Jannasch, H. W. 1974. Steady state and the chemostat in ecology. Limnol. Oceanogr. 19:716-720.

Jensen, A., B. Rystad and L. Skoglund. 1972. The use of dialysis culture in phytoplankton studies. J. Exp. Mar. Ecol. 8:241-248.

Ketchum, B. H. 1939. The absorption of phosphate and nitrate by illuminated cultures of Nitzschia closterium. Am. J. Bot. 26:339-407.

Kiefer, D. A. 1973. Chlorophyll a fluorescence in marine centric diatoms: Responses of chloroplasts to light and nutrient stress. Mar. Biol. 23:39-46.

Liebig, J. 1840. Chemistry in Its Application to Agriculture and Physiology. Taylor and Walton, London. 401 p.

Lundy, D. W. 1974. Rapid equilibrium response of a marine diatom to external and internal nutrient concentrations. M. S. Thesis. Oregon State University, Corvallis.

MacIsaac, J. J. and R. C. Dugdale. 1969. The kinetics of nitrate and ammonia uptake by natural populations of marine phytoplankton. Deep-Sea Res. 16:45-57.

McCarthy, J. J. 1972. The uptake of urea by marine phytoplankton. J. Phycol. 8:216-222.

McCarthy, J. J. and J. C. Goldman. 1979. Nitrogenous nutrition of marine phytoplankton in nutrient-depleted water. Science 203:607-672.

Menzel, D. W. and J. P. Spaeth. 1962. Occurrence of ammonia in Sargasso Sea waters and in rain at Bermuda. Limnol. Oceanogr. 7:159-162.

Paasche, E. 1975. Growth of the plankton diatom Thalassiosira nordenskioldii Cleve. at low silicate concentrations. J. Exp. Mar. Biol. Ecol. 18:173-183.

Michaelis, L. and M. L. Menten. 1913. Kinetics of invertase action. Biochem. Z. 49:333-369.

Monod, J. 1942. Recherches sur la Croissance des Cultures Bacteriennes. 2nd ed. Hermann and Cie, Paris, 210 pp.

Packard, T. T. 1973. The light dependence of nitrate reductase in marine phytoplankton. Limnol. Oceanogr. 18:466-469.

Packard, T. T. and D. Blasco. 1974. Nitrate reductase activity in upwelling regions. 2. Ammonia and light dependence. Tethys 6: 269-280.

Packard, T. T., D. Blasco, J. J. MacIsaac, R. C. Dugdale. 1971. Variations in nitrate reductase in marine phytoplankton. Inves. Pesq. 35:209-219.

Redfield, A. C. 1934. On the proportions of organic derivatives in sea water and their relation to the composition of plankton. James Johnstone Memorial Volume, 176-92. Liverpool: Lancashire Sea Fisheries. 348 pp.

Riley, G. A. 1946. Factors controlling phytoplankton populations on Georges Bank. J. Mar. Res. 6:54-73.

_____. 1956. Oceanography of Long Island Sound, 1952-54. IX. Production and utilization of organic matter. Bull. Bingham Oceanogr. Coll. 15:324-344.

Riley, G. A., H. Stommel and D. F. Bumpus. 1949. Quantitative ecology of the plankton of the Western North Atlantic. Bull. Bingham Oceanogr. Coll. 12:1-169.

Ritenour, G. L., K. W. Joy, J. Bunning and R. H. Hageman. 1967. Intracellular localization of nitrate reductase, nitrite reductase, and glutamic acid dehydrogenase in green leaf tissue. Plant Physiol. 42:244-237.

Ryther, J. H. and W. M. Dunstan. 1971. Nitrogen, phosphorus, and eutrophication in the coastal marine environment. Science 171: 1008-1013.

Schell, D. M. 1974. Uptake and regeneration of free amino acids in marine waters of Southeast Alaska. Limnol. Oceanogr. 19:260-270.

Schultz, J. S. and P. Gerhardt. 1969. Dialysis culture of microorganisms: Design, theory and results. Bact. Rev. 33:1-47.

Solorzano, L. 1969. Determination of ammonia in natural waters by the phenolhypochlorite method. Limnol. Oceanogr. 14:799-801.

Steele, J. H. and D. W. Menzel. 1962. Conditions for maximum primary production in the mixed layer. Deep-Sea Res. 9:39-49.

Syrett, P. J. 1962. Nitrogen assimilation. In Physiology and Biochemistry of Algae. R. A. Lewin, ed. Academic Press, New York, pp. 171-188.

Thomas, W. H. 1966. Surface nitrogenous nutrients and phytoplankton in the Northeastern Tropical Pacific Ocean. Limnol. Oceanogr. 11: 393-400.

_____. 1969. Phytoplankton nutrient enrichment experiments off Baja California and in the eastern equatorial Pacific Ocean. J. Fish. Res. Bd. Can. 26:1133-1145.

Wood, E. D., F. A. J. Armstrong, F. A. Richards. 1967. Determination of nitrate in seawater by cadmium-copper reduction to nitrite. J. Mar. Biol. Assoc. U. K. 47:23-31.

APPENDICES

Appendix I. Definitions.

1. Nitrogen exhaustion: the point in time when ambient inorganic nitrogen concentration becomes undetectable in batch culture, or when uptake ceases. Nitrogen starvation begins at this time.

2. Growth rate (μ): where $\mu = \ln(N_2/N_1)/(t_2-t_1)$, with N_1 and N_2 the biomass of phytoplankton nitrogen at time t_1 and t_2 respectively. In a steady-state chemostat, growth rate equals dilution rate equals uptake rate.

3. Internal pool: that quantity of free nitrogen within a cell in the form of nitrate, ammonium or amino acids per total cell volume. Values are expressed as a concentration in units of milli-moles nitrogen per liter of total cell volume (indicated as mM or mM/(liter cell volume). This concentration must be considered a minimum estimate of the actual internal concentration in that it assumes that the nitrogen in the pool is distributed uniformly throughout the cell volume. The values are calculated as the difference between ambient and sonicated (total) nitrogen divided by total cell volume as determined by Coulter Counter ^{R} measurements.

4. Specific uptake rate: the rate at which a particular nutrient (nitrogen) is taken up by phytoplankton, i.e., removed from the ambient medium, divided by the amount of that nutrient present in the phytoplankton biomass. Units are those of time^{-1} and are dimensionally equivalent to other specific growth rate measurements such as those for carbon, nitrogen, cell volume, or fluorescence.

5. Assimilation: the conversion within cells of inorganic nitrogen (nitrate or ammonium) into organic nitrogen. Measured as the

disappearance of total (sonicated) nitrate or ammonium from the medium.

6. Apparent half-saturation constant: the nutrient concentration at which uptake is half the maximum rate measured. The term is analgous to the Michaelis-Menten half-saturation constant. However, the term "apparent" is included to emphasize that the slowdown in uptake observed may in fact be functionally unrelated to the ambient concentration, i.e., uptake may be under some form of internal control or product inhibition and merely coincident with a particular nutrient concentration.

7. Coupled nitrate uptake-reduction (CUR): a mechanism whereby nitrate is taken up by cells and reduced at the point of transport into the cell. Nitrate reduction by this mechanism ceases when uptake ceases.

8. Free nitrate reduction (FNR): nitrate reduction that is independent of nitrate uptake. Possibly, but not necessarily, equivalent to nitrate reduction by unbound nitrate reductase.

APPENDICES II-XV*

*All nutrients (ambient NO_3, total NO_3, ambient NH_4, total NH_4, ambient amino acids, and total amino acids are in units of µg-atom N $liter^{-1}$. Cell volume units are 10^9 μm^3 $liter^{-1}$. Cell nitrogen units are µg-atom N $liter^{-1}$. Cell carbon units are µg-atom C $liter^{-1}$. C/N ratio is carbon/nitrogen on an atom/atom basis. Fluorescence is relative to 50 µg coproporphyrin $liter^{-1}$. Internal pools are expressed as mg atom-N/liter cell volume. Times are minutes after nitrogen additions.

	TIME (MIN)	AMBIENT NO_3	TOTAL NO_3	AMBIENT AMINO ACIDS	TOTAL AMINO ACIDS	CELL VOLUME	CELL NITROGEN	CELL CARBON	C/N RATIO	FLUORESCENCE	INTERNAL POOLS NO_3	INTERNAL POOLS AMINO ACIDS
NO_3 Addition	2.0	***	30.92	***	***	***	***	***	***	***	***	***
	2.5	***	30.92	***	***	***	***	***	***	***	***	***
	10.0	***	***	***	***	42.32	***	***	***	7.33	***	***
	24.0	25.26	29.44	2.64	8.98	***	46.30	443.1	9.57	***	97.5	147.9
	30.0	25.81	29.73	2.55	9.64	***	46.91	435.4	9.28	***	90.9	164.4
	41.0	25.37	28.87	2.69	9.87	***	45.94	433.5	9.44	***	80.3	164.8
	48.0	25.38	27.51	1.58	8.11	***	47.51	462.1	9.73	***	48.6	148.9
	56.5	23.60	27.87	1.79	8.66	***	41.58	419.2	10.08	***	96.6	155.4
	63.5	23.15	27.5	1.31	8.77	***	35.41	333.4	9.42	***	97.8	167.7
	75.0	25.68	26.95	0.70	8.29	***	43.52	414.4	9.52	***	28.2	168.8
	85.0	23.15	26.58	0.46	8.66	***	45.70	462.1	10.11	***	75.6	180.7
	92.0	21.54	26.35	0.61	8.95	***	52.96	500.3	9.45	***	105.3	182.5
	100.0	21.4	25.69	0.66	8.76	***	49.57	476.4	9.61	***	93.2	176.0
	106.5	21.16	25.24	0.82	8.19	42.25	49.33	490.8	9.95	6.93	88.1	159.1
Control	0.0	15.47	18.12	1.13	8.43	***	48.36	452.6	9.36	***	62.6	172.5
	7.0	14.23	18.16	0.74	9.47	***	33.83	347.7	10.28	***	92.9	206.3
	10.0	***	***	***	***	42.32	***	***	***	7.33	***	***
	15.0	14.35	18.19	0.67	8.88	***	47.51	454.5	9.57	***	90.7	194.0

	TIME (MIN)	AMBIENT NO_3	TOTAL NO_3	AMBIENT AMINO ACIDS	TOTAL AMINO ACIDS	CELL VOLUME	CELL NITROGEN	CELL CARBON	C/N RATIO	FLUORESCENCE	INTERNAL POOLS NO_3	INTERNAL POOLS AMINO ACIDS
NO_3 Addition	2.0	***	15.73	***	6.57	***	***	***	***	***	***	***
	3.0	***	15.54	***	6.79	***	***	***	***	***	***	***
	22.0	10.13	14.25	1.00	7.65	***	51.75	502.2	9.71	***	103.3	166.8
	30.5	9.82	13.83	0.49	5.97	***	56.60	511.7	9.04	***	100.2	136.9
	40.5	9.02	13.08	0.55	6.10	***	55.40	514.6	9.29	***	101.0	138.0
	50.0	8.11	12.65	0.31	6.39	***	55.40	509.8	9.20	***	112.4	165.9
	61.0	7.12	12.09	0.21	6.66	***	57.80	538.4	9.32	***	122.5	169.3
	70.0	6.45	11.32	0.34	6.68	***	56.60	521.3	9.21	***	119.5	172.3
	81.0	5.70	11.20	0.25	6.81	***	61.20	548.0	8.96	***	134.3	172.4
	91.0	4.86	10.74	0.25	7.41	***	56.60	509.8	9.01	***	143.0	186.3
	100.0	4.31	10.37	0.18	7.48	***	65.1	574.7	8.83	***	146.8	185.5
	111.5	3.05	9.62	0.11	7.22	***	60.20	528.9	8.79	***	158.3	176.7
	120.5	2.67	9.00	0.13	7.45	***	62.60	567.0	9.06	***	151.9	182.0
	140.0	***	***	***	***	43.73	***	***	***	5.42	***	***

	TIME (MIN)	AMBIENT NO_3	TOTAL NO_3	AMBIENT AMINO ACIDS	TOTAL AMINO ACIDS	CELL VOLUME	CELL NITROGEN	CELL CARBON	C/N RATIO	FLUORESCENCE	INTERNAL POOLS NO_3	INTERNAL POOLS AMINO ACIDS
Control	-520	***	***	***	***	32.3	***	***	***	4.24	***	***
	-240	11.82	***	***	***	***	***	***	***	***	***	***
	-180	8.27	***	***	***	***	***	***	***	***	***	***
	-110	3.86	***	***	***	***	***	***	***	***	***	***
	-60	1.03	***	***	***	***	***	***	***	***	***	***
	0.0	0.50	3.74	0.62	7.31	***	52.96	509.8	9.63	***	79.8	164.8
	8.5	0.46	3.56	0.21	6.93	40.57	51.99	476.4	9.16	3.55	76.1	165.0
	14.5	0.41	3.52	0.25	7.00	***	52.35	557.5	10.65	***	76.1	165.2
	127.0	0.38	1.91	0.28	5.57	42.50	52.35	586.1	11.20	5.31	35.6	136.3
	132.0	0.54	2.06	0.29	5.79	***	52.96	567.0	10.71	***	35.3	141.3
	139.0	0.43	1.74	0.12	5.18	***	54.17	612.8	11.31	***	30.36	122.8

	TIME (MIN)	AMBIENT NO_3	TOTAL NO_3	AMBIENT AMINO ACIDS	TOTAL AMINO ACIDS	CELL VOLUME	CELL NITROGEN	CELL CARBON	C/N RATIO	FLUORESCENCE	INTERNAL POOLS NO_3	INTERNAL POOLS AMINO ACIDS
NO_3 Addition	1.0	***	12.51	***	4.27	***	***	***	***	***	***	***
	1.5	***	12.28	***	4.16	***	***	***	***	***	***	***
	5.0	11.49	11.79	0.61	3.39	***	30.0	409.7	13.66	***	11.5	106.4
	15.0	11.86	12.50	1.36	3.78	***	40.2	490.8	12.21	***	24.2	91.6
	30.0	12.11	12.67	0.76	4.68	***	40.2	445.0	11.07	***	20.8	145.9
	45.0	12.09	***	0.71	3.21	***	39.8	443.1	11.13	***	***	91.5
	60.0	11.47	12.29	0.68	4.91	***	42.7	454.5	10.65	***	29.5	152.3
	75.0	11.51	12.56	0.66	4.21	***	36.6	438.3	11.98	***	37.2	125.7
	90.0	10.32	12.21	0.58	4.43	***	38.7	464.0	11.99	***	65.4	134.0
	105.0	10.63	12.04	0.78	3.95	***	37.5	447.8	11.94	***	48.3	108.5
	120.0	9.85	11.49	0.66	4.50	***	36.6	469.8	12.84	***	55.2	129.3
	135.0	9.77	11.12	0.65	4.44	***	34.8	447.8	12.87	***	44.7	125.5
	150.0	9.46	10.92	0.79	3.89	***	31.2	452.6	14.51	***	47.5	101.0
	165.0	8.95	10.64	0.78	4.56	***	35.6	455.5	12.80	***	54.1	121.1
	180.0	7.58	9.90	0.87	4.36	***	33.6	428.8	12.76	***	73.1	109.9
	195.0	***	***	***	***	32.29	***	***	***	1.74	***	***
Control	-85.0	0.96	0.83	0.76	4.03	***	45.7	422.1	9.24	***	0.0	138.6
	-82.0	***	0.82	0.56	4.15	***	39.8	405.9	10.20	***	***	150.8
	-80.0	0.49	0.71	0.51	3.87	***	40.8	407.3	9.98	***	9.2	141.2
	-75.0	0.45	0.76	0.69	4.39	***	42.1	433.5	10.20	***	13.0	154.8
	-40.0	***	***	***	***	24.85	***	***	***	1.09	***	***
	182.0	0.48	0.85	0.69	2.99	***	28.1	390.6	13.90	***	11.6	72.3
	187.0	0.38	0.82	0.47	2.76	***	31.2	438.3	14.05	***	13.8	71.6

	TIME (MIN)	AMBIENT NO_3	TOTAL NO_3	AMBIENT AMINO ACIDS	TOTAL AMINO ACIDS	AMBIENT NH_4	TOTAL NH_4	CELL VOLUME	CELL NITROGEN	CELL CARBON	C/N RATIO	FLUOR.	INTERNAL POOLS NO_3	INTERNAL POOLS AMINO ACIDS	INTERNAL POOLS NH_4
NO_3 Addition	1.0	***	12.10	***	2.75	***	***	***	***	***	***	***	***	***	***
	1.5	***	12.34	***	1.75	***	***	***	***	***	***	***	***	***	***
	5.0	11.04	12.05	0.23	2.85	1.04	3.63	***	34.6	581.4	16.80	***	23.9	62.0	61.4
	10.0	***	***	***	***	***	***	43.52	***	***	***	1.47	***	***	***
	15.0	11.59	12.15	0.65	3.31	0.30	2.58	***	43.3	628.1	14.51	***	13.3	62.8	54.0
	30.0	11.44	12.27	0.76	2.81	0.40	2.85	***	41.5	624.3	15.04	***	19.7	48.7	58.1
	45.0	11.08	11.86	0.70	3.00	0.67	2.48	***	42.1	619.5	14.71	***	19.9	54.5	42.9
	60.0	10.60	11.86	0.50	3.17	0.33	2.62	***	41.8	616.6	14.75	***	29.9	63.4	54.3
	70.0	***	***	***	***	***	***	42.53	***	***	***	1.64	***	***	***
	75.0	10.20	11.71	0.53	3.60	0.40	2.54	***	45.0	629.0	13.98	***	35.8	72.8	50.7
	90.0	10.09	11.64	1.33	3.84	0.67	2.15	***	42.3	607.1	14.35	***	36.7	59.6	35.1
	105.0	9.58	11.53	1.86	4.37	1.02	3.92	***	42.7	621.4	14.55	***	46.2	59.6	68.7
	120.0	8.87	11.43	1.78	4.13	0.76	3.09	43.31	43.3	614.7	14.20	1.52	60.7	55.6	55.9
	135.0	8.72	11.26	1.82	5.39	0.86	3.73	***	43.3	605.2	13.98	***	60.2	84.6	68.0
	150.0	8.25	10.59	1.71	4.89	0.89	2.95	***	45.1	619.5	13.74	***	55.5	75.5	48.8
	165.0	7.64	10.57	0.58	2.48	1.05	2.38	***	46.5	610.0	13.12	***	69.4	45.1	31.5
	180.0	7.16	10.23	0.51	2.81	1.22	3.10	43.45	49.3	640.5	12.99	1.46	72.8	54.5	44.6
Control	-36.0	0.27	0.56	0.91	3.40	0.32	2.32	***	***	***	***	***	6.9	59.0	47.4
	-34.0	0.22	0.35	0.72	4.19	0.00	2.08	***	***	***	***	***	3.1	82.2	48.3
	-31.0	0.22	1.02	0.74	5.16	0.06	2.72	***	***	***	***	***	19.0	104.7	63.0
	-29.0	0.59	1.06	0.49	3.46	0.39	2.64	***	***	***	***	***	11.1	70.4	53.3
	225.0	0.42	0.96	0.29	2.35	0.23	0.79	***	***	***	***	***	12.8	48.8	13.3
	228.0	0.38	0.69	0.36	1.66	0.17	0.57	***	***	***	***	***	7.3	30.8	9.5

	TIME (MIN)	AMBIENT NO_3	TOTAL NO_3	AMBIENT AMINO ACIDS	TOTAL AMINO ACIDS	AMBIENT NH_4	TOTAL NH_4	CELL VOLUME	CELL NITROGEN	CELL CARBON	C/N RATIO	FLUOR.	INTERNAL POOLS NO_3	INTERNAL POOLS AMINO ACIDS	INTERNAL POOLS NH_4
NO_3 + NH_4 Addition	1.0	***	12.98	*** *** ***	***	***	14.08	***	***	***	***	***	***	***	***
	1.5	***	12.35	***	0.04	***	14.12	***	***	***	***	***	***	***	***
	10.0	***	***	***	***	***	***	44.08	***	***	***	1.44	***	***	***
	15.0	11.27	12.02	0.93	0.45	10.59	12.37	***	44.5	643.4	14.46	***	16.9	0.0	40.2
	30.0	11.59	12.48	0.69	1.23	9.39	11.81	***	44.5	635.7	14.29	***	20.1	43.4	54.7
	45.0	11.19	11.95	0.37	1.67	9.17	10.35	***	46.9	667.2	14.23	***	17.2	45.9	26.8
	60.0	11.08	11.61	0.52	2.48	8.09	9.22	***	45.1	624.3	13.84	***	12.0	67.9	25.5
	75.0	11.32	11.78	0.20	***	7.62	8.35	***	49.3	652.9	13.24	***	10.4	***	16.5
	90.0	11.22	11.83	0.37	3.10	6.23	7.51	***	47.5	673.9	14.19	***	13.8	78.3	28.9
	95.0	***	***	***	***	***	***	45.38	***	***	***	1.62	***	***	***
	105.0	10.90	11.52	0.39	3.40	5.48	6.50	***	50.5	639.5	12.66	***	14.0	85.5	23.0
	120.0	10.84	11.31	0.23	3.32	4.72	5.75	***	50.0	657.7	13.15	***	10.6	80.0	23.3
	135.0	10.56	11.15	0.07	3.32	3.96	4.72	***	50.5	648.1	12.83	***	13.3	76.6	17.2
	150.0	11.30	11.71	0.04	3.78	3.31	4.22	***	50.7	662.4	13.07	***	9.3	86.4	20.6
	165.0	10.99	11.77	0.03	4.31	2.89	3.42	***	51.8	652.9	12.60	***	17.6	98.1	12.0
	170.0	***	***	***	***	***	***	43.35	***	***	***	1.51	***	***	***
	180.0	11.07	11.76	0.07	4.55	2.06	2.73	***	53.0	653.8	12.34	***	15.6	101.3	15.1
Control	0.0	0.42	00.96	0.29	2.35	0.23	0.79	***	***	***	***	***	12.2	46.5	12.6
	3.0	0.38	0.69	0.33	1.66	0.17	0.57	***	***	***	***	***	7.0	30.0	3.0
	220.0	0.42	0.56	0.32	1.59	***	***	***	***	***	***	***	3.2	28.7	***
	223.0	0.35	0.44	0.27	2.37	***	***	***	***	***	***	***	2.0	42.4	***

	TIME (MIN)	AMBIENT NO_3	TOTAL NO_3	AMBIENT AMINO ACIDS	TOTAL AMINO ACIDS	CELL VOLUME	CELL NITROGEN	CELL CARBON	C/N RATIO	FLUORESCENCE	INTERNAL POOLS NO_3	INTERNAL POOLS AMINO ACIDS
NO_3 Addition	1.0	***	12.01	***	2.17	***	***	***	***	***	***	***
	1.5	***	11.97	***	2.19	***	***	***	***	***	***	***
	10.0	***	***	***	***	43.16	***	***	***	1.48	***	***
	15.0	11.00	12.40	0.49	2.42	***	43.3	667.2	15.41	***	32.2	44.4
	32.0	11.25	12.00	0.46	2.88	***	42.1	610.0	14.49	***	17.3	55.7
	45.0	11.20	12.20	1.13	2.86	***	45.7	***	***	***	23.0	39.8
	60.0	10.96	12.52	1.18	3.42	***	44.5	641.4	14.41	***	36.1	51.5
	75.0	10.47	12.32	1.05	2.61	***	***	***	***	***	42.6	35.9
	90.0	10.51	***	0.38	1.72	***	***	***	***	***	***	30.8
	105.0	9.83	11.83	0.29	2.17	***	***	***	***	***	46.0	43.2
	120.0	9.33	11.65	0.37	2.99	***	***	***	***	***	53.4	60.3
	135.0	8.80	11.47	0.33	2.0[illegible]	***	***	***	***	***	61.4	39.8
	150.0	8.22	11.15	0.30	2.26	***	***	***	***	***	67.4	45.1
	165.0	7.79	11.15	0.30	2.73	***	***	***	***	***	77.3	55.9
	180.0	7.31	10.70	0.31	2.07	***	***	***	***	***	78.0	40.5
	190.0	***	***	***	***	45.47	***	***	***	1.26	***	***
Control	0.0	0.42	0.56	0.32	1.59	***	***	***	***	***	3.2	29.2
	3.0	0.35	0.44	0.27	2.37	***	***	***	***	***	2.1	48.3
	185.0	0.37	0.89	0.24	1.86	***	***	***	***	***	12.0	37.3
	189.0	0.36	0.83	0.31	1.84	***	***	***	***	***	10.8	35.2

	TIME (MIN)	AMBIENT NO_3	TOTAL NO_3	AMBIENT AMINO ACIDS	TOTAL AMINO ACIDS	CELL VOLUME	CELL NITROGEN	CELL CARBON	C/N RATIO	FLUORESCENCE	INTERNAL POOLS NO_3	INTERNAL POOLS AMINO ACIDS
NO_3 Addition	2.0	***	22.52	***	11.34	***	***	***	***	***	***	***
	3.0	***	22.30	***	11.54	***	***	***	***	***	***	***
	10.0	***	***	***	***	54.72	***	***	***	8.22	***	***
	21.0	12.02	19.12	0.64	9.18	***	64.46	557.5	8.65	***	127.3	153.1
	31.0	10.32	18.65	0.36	9.02	***	69.91	661.5	9.46	***	146.6	152.4
	40.0	8.37	17.22	0.26	10.08	***	71.12	642.4	9.03	***	153.2	170.0
	50.0	6.28	17.57	0.64	8.89	***	69.91	614.7	8.79	***	192.0	140.3
	61.0	4.54	16.48	1.24	7.49	***	74.75	624.3	8.35	***	199.0	104.2
	70.0	3.51	15.47	1.27	7.45	***	71.12	607.1	8.54	***	196.1	101.3
	80.0	3.26	14.70	0.09	8.60	***	72.81	719.7	9.89	***	184.2	137.0
	90.0	2.18	13.76	0.08	8.80	***	74.99	636.7	8.49	***	183.1	140.4
	100.0	1.48	13.02	0.16	8.32	***	74.75	647.2	8.66	***	179.2	126.7
	111.0	1.22	12.84	0.46	15.39	***	77.17	652.9	8.46	***	176.8	227.2
	121.0	1.02	12.24	0.53	14.84	***	75.96	664.3	8.75	***	167.7	213.8
	130.0	0.96	11.88	0.43	15.45	***	72.93	700.6	9.61	***	160.5	220.8
	135.0	***	***	***	***	68.32	***	***	***	6.39	***	***
Control	0.0	5.11	11.00	1.41	12.03	***	64.46	576.6	8.95	***	107.6	194.1
	5.0	2.13	10.14	0.80	10.93	***	56.59	514.6	9.09	***	146.4	185.1
	9.0	3.77	9.85	0.65	12.21	***	63.61	571.8	8.99	***	111.1	211.3
	140.0	0.58	6.08	2.00	12.84	***	72.33	714.9	9.88	***	76.9	151.6
	147.0	0.94	5.92	***	11.75	***	69.91	710.1	10.16	***	69.7	***
	155.0	0.85	5.53	1.90	11.41	71.49	69.91	702.5	10.05	6.40	65.5	133.0

	TIME (MIN)	AMBIENT NO_3	TOTAL NO_3	AMBIENT AMINO ACIDS	TOTAL AMINO ACIDS	AMBIENT NH_4	TOTAL NH_4	CELL VOLUME	CELL NITROGEN	CELL CARBON	C/N RATIO	FLUOR.	INTERNAL POOLS NO_3	INTERNAL POOLS AMINO ACIDS	INTERNAL POOLS NH_4
NO_3 Addition	2.0	***	15.63	***	3.44	***	0.66	***	***	***	***	***	***	***	***
	3.0	***	15.19	***	2.81	***	2.53	***	***	***	***	***	***	***	***
	20.0	13.01	14.81	0.02	2.99	0.16	0.63	***	85.4	1404.	16.44	***	33.88	55.86	8.85
	35.0	13.46	14.64	0.54	3.84	0.42	0.90	***	***	***	***	***	22.0	61.4	8.9
	50.0	11.44	12.98	0.54	4.29	0.33	1.26	***	90.2	1420.	15.74	***	28.4	69.4	17.2
	65.0	10.50	12.26	2.59	4.59	0.61	0.94	***	***	***	***	***	32.2	36.5	6.0
	80.0	9.04	11.09	0.68	4.17	0.41	1.31	***	94.2	1500.	15.92	***	37.2	63.2	16.3
	95.0	7.94	9.91	1.09	4.36	0.36	0.80	***	90.5	1416.	15.65	***	35.4	58.8	7.9
	105.0	7.13	8.97	1.51	4.45	0.55	1.54	***	97.8	1521.	15.55	***	32.8	52.5	17.7
	125.0	5.45	8.04	1.25	4.49	0.41	0.99	***	95.1	1349.	14.19	***	45.7	57.1	10.2
	135.0	4.26	7.04	1.14	4.43	0.44	0.95	***	96.5	1492.	15.46	***	48.7	57.8	8.9
	150.0	3.26	5.27	0.67	4.93	0.52	1.13	***	109.5	1502	13.72	***	34.9	74.0	11.0
	165.0	2.03	5.15	***	***	***	***	***	99.00	1506.	15.21	***	53.7	***	***
	170.0	***	***	***	***	***	***	58.31	***	***	***	7.71	***	***	***
	180.0	1.16	3.96	***	***	***	***	***	102.6	1549.	15.10	***	47.7	***	***
	1475.	0.52	0.46	***	***	***	***	***	94.1	1626.	17.28	***	***	***	***
Control	0.0	0.68	1.48	0.42	3.89	0.08	0.80	***	68.6	1225.	17.86	***	15.2	65.7	13.6
	7.0	0.73	1.00	0.51	4.18	0.08	0.73	***	74.9	1351.	18.04	***	5.1	69.5	12.3
	9.0	1.04	1.31	0.80	2.68	0.06	0.65	***	76.6	1437.	18.76	***	5.1	35.6	11.2
	10.0	***	***	***	***	***	***	52.80	***	***	***	10.0	***	***	***
	155.0	***	***	***	***	***	***	60.32	***	***	***	6.63	***	***	***
	1460	1.02	0.82	0.58	3.17	***	***	***	83.2	1797.	21.60	***	***	***	***

	TIME (MIN)	AMBIENT NO_3	TOTAL NO_3	AMBIENT AMINO ACIDS	TOTAL AMINO ACIDS	AMBIENT NH_4	TOTAL NH_4	CELL VOLUME	CELL NITROGEN	CELL CARBON	C/N RATIO	FLUOR.	INTERNAL POOLS NO_3	INTERNAL POOLS AMINO ACIDS	INTERNAL POOLS NH_4
NO_3 + NH_4 Addition	2.0	***	16.33	***	5.83	***	10.88	***	***	***	***	***	***	***	***
	3.0	***	15.78	***	6.09	***	10.64	***	***	***	***	***	***	***	***
	15.0	14.63	15.11	0.61	3.96	7.57	9.16	***	88.1	1368.	15.53	***	9.06	63.2	30.0
	30.0	14.07	14.80	0.85	4.83	6.14	7.28	***	89.3	1494.	16.73	***	13.7	74.3	21.3
	45.0	13.84	14.67	0.42	5.35	4.66	6.11	***	95.2	1607.	16.88	***	15.4	91.4	26.9
	60.0	13.25	14.22	0.51	5.73	3.32	4.46	***	95.3	1588.	16.66	***	17.8	95.9	20.9
	75.0	11.46	13.96	0.38	5.52	2.01	2.92	***	92.9	1492.	16.06	***	45.5	93.6	16.6
	90.0	13.30	14.24	0.81	6.51	1.35	2.32	***	***	1568.	***	***	16.9	102.7	17.5
	105.0	12.76	***	0.74	***	0.64	***	***	111.1	1588.	14.29	***	***	***	***
Control	0.0	0.68	1.48	0.42	3.89	0.08	0.80	***	68.6	1225.	17.86	***	15.2	65.7	13.6
	7.0	0.73	1.00	0.51	4.18	0.08	0.73	***	74.9	1351.	18.04	***	5.1	69.5	12.3
	9.0	1.04	1.31	0.80	2.68	0.06	0.65	***	76.6	1437.	18.76	***	5.1	35.6	11.2
	10.0	***	***	***	***	***	***	52.80	***	***	***	10.0	***	***	***
	155.0	***	***	***	***	***	***	60.32	***	***	***	6.63	***	***	***
	1460	1.02	0.82	0.58	3.17	***	***	***	83.2	1797.	21.60	***	***	***	***

	TIME (MIN)	AMBIENT NO_3	TOTAL NO_3	AMBIENT AMINO ACIDS	TOTAL AMINO ACIDS	CELL VOLUME	CELL NITROGEN	CELL CARBON	C/N RATIO	FLUORESCENCE	INTERNAL POOLS NO_3	INTERNAL POOLS AMINO ACIDS N
NO_3 Addition	1.0	***	11.66	***	0.49	***	***	***	***	***	***	***
	2.0	***	11.67	***	0.49	***	***	***	***	***	***	***
	23.5	9.04	***	0.36	0.59	***	23.9	394.4	16.50	***	***	***
	28.0	***	***	***	***	36.6	***	***	***	2.27	***	***
	30.5	8.85	11.13	0.19	0.71	***	20.9	388.2	18.58	***	63.0	14.4
	45.0	7.45	9.72	0.28	0.89	***	26.3	424.0	16.87	***	62.8	16.9
	60.0	5.29	9.90	0.23	1.56	***	29.4	416.8	14.18	***	127.5	36.8
	75.5	4.24	***	0.22	1.04	***	24.2	488.8	20.20	***	44.2	22.6
	90.0	3.30	8.66	0.46	2.57	***	28.8	441.2	15.32	***	148.2	58.3
	104.5	2.08	8.15	0.59	2.74	***	31.2	412.5	13.22	***	167.8	59.4
	112.0	***	***	***	***	34.0	***	***	***	2.41	***	***
	121.0	1.04	7.03	0.77	3.32	***	30.4	481.2	15.83	***	165.6	70.5
	135.0	0.61	6.78	0.78	2.81	***	32.4	526.0	16.24	***	170.6	56.1
	150.0	0.59	6.87	0.49	3.15	***	32.4	471.7	14.56	***	173.6	73.5
	165.0	0.77	6.08	0.51	2.88	***	31.2	516.5	16.56	***	146.8	65.5
	180.0	0.77	5.64	0.58	2.96	***	31.2	484.1	15.52	***	134.7	65.8
	185.0	***	***	***	***	37.9	***	***	***	2.65	***	***
Control	0.0	0.44	0.67	0.62	0.87	***	***	***	***	***	7.0	7.6
	9.0	0.41	0.65	0.58	1.13	32.7	18.8	360.6	19.18	3.57	7.1	16.8
	13.5	0.39	0.63	0.48	0.87	***	19.1	376.3	19.70	***	7.5	12.1
	96.0	***	***	***	***	40.1	***	***	***	3.07	***	***
	173.0	***	***	***	***	36.8	***	***	***	2.37	***	***
	183.5	0.30	1.13	0.84	2.34	***	27.5	435.4	15.84	***	22.4	40.4
	188.5	0.38	0.70	0.68	1.97	***	23.9	414.4	17.34	***	8.6	34.8
	192.5	0.34	0.76	1.81	1.98	***	25.1	473.6	18.87	***	11.3	4.6

	TIME (MIN)	AMBIENT NO_3	TOTAL NO_3	AMBIENT AMINO ACIDS	TOTAL AMINO ACIDS	CELL VOLUME	CELL NITROGEN	CELL CARBON	C/N RATIO	FLUORESCENCE	INTERNAL POOLS NO_3	INTERNAL POOLS AMINO ACIDS
NO_3 Addition	2.0	***	22.69	***	9.87	***	***	***	***	***	***	***
	3.0	***	22.54	***	10.02	***	***	***	***	***	***	***
	19.0	7.71	19.74	0.37	8.09	***	160.0	1069.	6.68	***	115.1	73.8
	30.0	5.61	18.81	0.67	9.11	***	165.5	1057.	6.39	***	124.7	79.8
	45.0	3.31	17.02	0.40	9.47	***	142.5	986.	6.93	***	127.4	91.7
	60.0	2.32	15.47	0.05	8.91	***	148.3	1063.	6.72	***	120.1	80.9
	75.0	1.02	14.35	0.17	8.89	***	164.3	1101.	6.70	***	119.7	81.4
	90.0	0.76	13.59	0.47	9.18	***	163.1	1101.	6.75	***	113.3	85.2
	98.0	***	***	***	***	118.0	***	***	***	6.75	***	***
	105.0	0.80	13.73	0.51	10.15	***	165.5	1111.	6.71	***	112.3	92.5
	120.0	0.78	12.59	0.54	9.21	***	170.4	1197.	7.03	***	100.8	83.2
	135.0	0.90	11.57	0.37	9.09	***	165.5	1139.	6.88	***	89.5	79.3
	150.0	0.82	11.46	0.50	7.70	***	146.2	1066.	7.29	***	87.8	67.6
	166.0	0.63	10.94	0.45	8.78	***	161.9	1164.	7.19	***	83.5	74.8
	171.0	***	***	***	***	122.0	***	***	***	7.14	***	***
Control	1.0	0.71	10.12	0.83	10.35	***	147.9	939.	6.35	***	91.9	93.0
	8.0	0.61	9.99	0.69	10.52	***	148.1	939.	6.34	***	91.6	96.0
	12.0	0.54	10.92	0.82	12.05	102.4	151.0	1004.	6.65	7.83	101.4	109.7
	1445	***	***	***	***	151.7	***	***	***	6.62	***	***
	1840	***	***	***	***	152.5	***	***	***	6.01	***	***
	2880	***	***	***	***	146.9	***	***	***	6.91	***	***

	TIME (MIN)	AMBIENT NO_3	TOTAL NO_3	AMBIENT AMINO ACIDS	TOTAL AMINO ACIDS	CELL VOLUME	CELL NITROGEN	CELL CARBON	C/N RATIO	FLUORESCENCE	INTERNAL POOLS NO_3	INTERNAL POOLS AMINO ACIDS
NO_3 Addition	1.0	***	11.85	***	3.26	***	***	***	***	***	***	***
	1.5	***	12.05	***	3.47	***	***	***	***	***	***	***
	15.0	7.16	11.25	0.88	3.15	***	45.7	339.6	7.43	***	124.9	69.3
	24.0	***	***	***	***	32.97	***	***	***	2.73	***	***
	30.0	6.37	10.45	0.62	3.74	***	43.3	308.6	7.13	***	123.2	94.2
	45.0	5.40	9.72	0.27	3.79	***	42.8	316.7	7.40	***	129.0	105.1
	60.0	4.68	8.43	0.19	3.02	***	47.5	344.4	7.25	***	110.7	83.5
	90.0	3.13	***	0.98	***	***	47.8	351.0	7.34	***	***	***
	120.0	1.55	5.83	0.67	3.56	***	49.9	359.6	7.21	***	120.7	81.5
	150.0	0.84	***	0.47	***	***	51.8	396.3	7.65	***	***	***
	180.0	1.00	5.02	2.42	6.71	***	51.8	395.4	7.63	***	108.3	115.6
	206.0	***	***	***	***	37.87	***	***	***	2.39	***	***
	243.0	1.58	4.52	0.78	5.14	***	47.2	432.6	9.17	***	75.7	112.3
Control	0.0	1.46	4.67	0.60	3.12	***	36.0	285.7	7.94	***	108.0	84.8
	3.0	1.24	5.01	0.68	3.50	***	***	***	***	***	126.8	94.9
	11.0	***	***	***	***	29.73	***	***	***	3.13	***	***

	TIME (MIN)	AMBIENT NO_3	TOTAL NO_3	AMBIENT AMINO ACIDS	TOTAL AMINO ACIDS	CELL VOLUME	CELL NITROGEN	CELL CARBON	C/N RATIO	FLUORESCENCE	INTERNAL POOLS NO_3	INTERNAL POOLS AMINO ACIDS
NO_3 Addition (243 min after first pulse)	1.0	***	15.20	***	4.86	***	***	***	***	***	***	***
	1.5	***	15.19	***	5.17	***	***	***	***	***	***	***
	20.0	10.49	14.32	0.17	5.36	***	48.1	410.2	8.53	***	96.8	139.7
	30.0	9.17	13.91	0.32	4.31	***	50.5	414.4	8.21	***	118.6	115.9
	45.0	7.84	12.49	0.18	4.29	***	50.5	454.5	9.00	***	114.7	101.4
	60.0	7.39	12.36	0.57	4.76	***	52.8	434.5	8.23	***	120.9	101.9
	75.0	5.83	9.80	0.19	4.31	***	54.8	462.1	8.43	***	95.2	107.9
	90.0	3.79	88.65	0.05	4.49	***	54.8	495.5	9.04	***	114.9	105.0
	105.0	2.62	8.25	0.09	4.70	***	54.8	462.1	8.43	***	131.3	111.7
	120.0	1.68	5.73	0.24	3.37	***	55.4	481.2	8.69	***	93.1	83.0
	135.0	1.31	6.49	0.22	5.55	***	53.0	452.6	8.54	***	117.4	130.8
	150.0	0.94	5.93	0.14	5.26	***	54.8	483.1	8.82	***	111.5	114.2
	165.0	0.83	5.73	0.13	7.76	***	56.0	500.3	8.93	***	107.9	168.1
	174.0	***	***	***	***	45.62	***	***	***	3.57	***	***
	180.0	0.95	5.06	***	7.97	***	54.2	500.3	9.23	***	89.2	***
Control	0.0	1.58	4.52	0.78	5.14	***	47.2	432.6	9.17	***	75.7	112.3