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DETERMINING NUTRIENT- AND PREDATOR-LIMITATION OF SYNECHOCOCCUS POPULATION SIZE USING CELLULAR rRNA CONCENTRATIONS

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Paul Wesley Lepp

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DETERMINING NUTRIENT- AND PREDATOR-LIMITATION OF *SYNECHOCOCCUS* POPULATION SIZE USING CELLULAR rRNA CONCENTRATIONS

By

Paul Wesley Lepp

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ABSTRACT

DETERMINING NUTRIENT- AND PREDATOR-LIMITATION OF *SYNECHOCOCCUS* POPULATION SIZE USING CELLULAR rRNA CONCENTRATIONS

By

Paul Wesley Lepp

Cyanobacteria of the genus *Synechococcus* are important contributors to primary productivity, community composition and trophic interactions in marine environments. Despite their importance, the factors controlling the size of *Synechococcus* populations are not well understood. Substantial evidence exists to suggest that synechococci are often substrate limited. However, the evidence does not preclude the possibility of predator control of synechococci population size. The goal of this research was to distinguish nutrient-limitation from predator-limitation of the size of *Synechococcus* populations in both laboratory cultures and the environment. Nutrient- or predator-limitation was distinguished based on a plot of population size versus cellular 16S rRNA; which was indicative of specific growth rate.

Light-limited batch cultures of both *Synechococcus* spp. strain PCC 6301 and WH 8103 exhibited a positive correlation between specific growth rate and the cellular concentrations of both RNA and DNA. The cellular concentration of nucleic acids decreased during light periods and increased during dark periods in both *Synechococcus* sp. strain PCC 6301 and *Synechococcus* sp. strain WH 8103 entrained by diel cycles. The cyclical variation in cellular nucleic acids concentrations during diel cycles was a function of the timing of cell division.

Predator-prey models were applied to a chemostat culture of *Synechococcus* sp. strain PCC 6301 prey and a *Tetrahymena pyriformis* predator. In the absence of predation, *Synechococcus* responded to increases in nutrients by increasing equilibrium population size, without a concurrent increase in growth rate. Addition of the predator increased the

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specific growth rate of the *Synechococcus* population approximately 10-fold while decreasing the size of the *Synechococcus* population by one-seventh.

The cellular 16S rRNA concentrations and size of *Synechococcus* populations were examined at oligotrophic and eutrophic stations in the Gulf of Mexico. Prey-dependent models predicted that a nutrient-limited population would be larger and have slower specific growth rates than a population limited by predation. The distribution of population size versus specific growth conformed to the predictions of prey-dependent models better than those of ratio-dependent models. A significant portion of the synechococci examined appeared to be limited in population size by predation, in agreement with the reports of heavy grazing of pelagic *Synechococcus* populations.

To r
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To my wife, Cheryl, who endured years of separation in order to make this work possible. It would not have been possible to complete this work without her love, understanding and patience.

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Chapter 1

GENERAL INTRODUCTION

Introduction and Specific Aims

Cyanobacteria of the genus *Synechococcus* are reported to contribute from approximately 2% of total primary productivity in coastal waters up to 46% of total primary productivity in pelagic waters. Synechococci (30, 54) have proven to be both ubiquitous and abundant by epifluorescence microscopy (5, 9, 30, 54, 55), flow cytometry (7, 34) and nucleic acid sequence analysis (18, 47), since their discovery in marine environments. Despite their significant contribution to total primary production and marine community structure, little is known about the mechanisms controlling their population size. An understanding of the mechanisms which control *Synechococcus* populations is essential in determining the rate of energy transfer to other trophic levels (21), the role of synechococci in determining microzooplankton composition and the construction of models of trophic interactions.

The goal this research was to determine whether the size of *Synechococcus* populations in the environment was primarily nutrient-limited or predator-limited. Nutrient-limitation was distinguished from predator-limitation of population size by modeling predator-prey interactions in chemostats containing a *Synechococcus* sp. prey and a *Tetrahymena pyriformis* predator. Prey-dependent models of predator-prey interactions predicted that predator-limited populations would have higher specific growth rates than nutrient-limited populations. The relative differences in growth rates was measured using oligonucleotide probes complementary to 16S rRNA, the concentration of which was correlated with specific growth rates. Finally, this same approach was applied to synechococci populations in the Gulf of Mexico in order to distinguish nutrient-limitation from predator-limitation of population size *in situ*.

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Background and Significance

Synechococcus physiology and ecology. Picoplankton are defined as organisms from 0.2 μm to 2 μm in size (48). These organisms are responsible for a large portion of the photosynthetic activity in the open ocean (16). Picophytoplankton communities are often numerical dominated by members of the genus *Synechococcus* (5, 7, 55). Populations of *Synechococcus* range from $1x10^3$ cells ml^{-1} to $1x10^6$ cells ml^{-1} within the euphotic zone (5, 7, 9, 45, 54, 55). Beyond mere abundance, the ubiquitous nature of *Synechococcus* is evident from counts by epifluoresence microscopy of samples from the southern Pacific (55), the subartic Pacific (11), the tropical Atlantic (9, 54, 55) and the northern Atlantic (5, 21). Cloned 16S rRNA sequences have revealed *Synechococcus* spp. to be abundant in both the Pacific (47) and the Atlantic (19); results that are supported by flow cytometry (34).

Members of the genus *Synechococcus* are distinguished from other cyanobacteria by their unicellular, coccobacilli morphology, transverse binary fission in one plane and the absence of a polysaccharide sheath (46). Marine *Synechococcus* spp. can be divided into two distinct groups. The first group lacks phycoerythrin and does not have an elevated salt requirement. This halotolerant group has been found within the continental shelf margin but never in the open ocean. The second group contains phycoerythrin as its primary light harvesting pigment and possesses an elevated salt requirement. This group has been isolated on and off the continental shelf (55).

Synechococcus is capable of fixing 10 fg C $cell^{-1}h^{-1}$ (9, 40, 45), principally via the reductive pentose phosphate pathway (20). This reduction is tightly coupled to a diel light cycle (23, 44, 45) and probably occurs within carboxysomes (51). It is believed that up to half of the photosynthates produced are released, typically as glycolate, into the environment and capable of supporting multiple trophic levels (16). This is supported by

the fact that the growth rate is saturated at light intensities of approximately 50 $\mu Em^{-2}s^{-1}$ and photosynthesis at approximately 100 $\mu Em^{-2}s^{-1}$ (21, 40, 45). The photosynthetic capacity of *Synechococcus* is often saturated by oceanic surface irradiance that can exceed 1000 $\mu Em^{-2}s^{-1}$ on cloudless days.

Characteristic of cyanobacteria and chloroplasts, reducing equivalents are provided by photosystems I and II (20). Although *Synechococcus* contains chlorophyll *a*, its primary light harvesting pigments are phycocyanin, phycoerythrin, allophycocyanin and allophycocyanin B (20). These accessory pigments, sequestered in phycobilisomes arranged along the outside of parallel stacks of thylakoid membranes, results in a light harvesting complex capable of supporting a maximum growth rate μ_{max} at 50 $\mu Em^{-2}s^{-1}$ (40) compared with approximately 100 $\mu Em^{-2}s^{-1}$ for eukaryotic algae (20). The low light level at which maximal growth occurs is believed to contribute to the competitive edge that allows *Synechococcus* to dominate many marine phytoplankton communities (20).

In addition to light, *Synechococcus* exhibits a high affinity for nitrate K_s = 30 μM (15, 22), requires less than 0.1 nM manganese and less than 0.1 pM zinc (4). Synechococci also benefit from their small size approximately 1 μm x 0.6 μm (55), which results in a surface:volume ratio that aids substrate uptake (16). High substrate affinity and small size make *Synechococcus* extremely efficient at nutrient uptake and a key link in micronutrient recycling and trophic transfer in pelagic systems (29). Should the *Synechococcus* population become nitrogen limited, a number of diazotrophic *Synechococcus* have been reported (25, 28) which could quickly displace non-nitrogen fixing species.

Despite the advantages in oligotrophic environments afforded by small size and high affinity for scarce resources, there is substantial support in the literature for adopting

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the position that *Synechococcus* is often substrate limited. Reports indicate that *Synechococcus* may at times be limited for light (38), trace metals (11, 39), in particular Fe^{2+} (24, 36, 37), nitrogen (22, 41), and phosphorus (31, 32, 56).

However, the evidence for substrate limitation of *Synechococcus* is far from conclusive and does not preclude the possibility of predator control. One of the foremost predictions of ecological theory is that predation can be a major factor in controlling population size (26, 27, 33, 42). An inverse correlation has been found between phytoplankton biomass and grazer density in lakes (41) and rivers (57). One would expect to find similar forces at work upon the *Synechococcus* in marine environments where flagellates and protozoa are known predators (8, 14, 16, 31, 32, 43). The reported ***in situ*** grazing coefficient for flagellates and protozoan specific to *Synechococcus* range from 0 to 1.7 d^{-1} (6, 8). Grazing pressure can reportedly reduce the population of *Synechococcus* by 30 to 50% (6, 8). Another possible control mechanism is parasitism by *Synechococcus*-specific bacteriophage, which can be considered predation for the purposes of mathematical models. Viruses have been reported to reduce phytoplankton primary productivity by as much as 78% (29, 50). However, more recent work has demonstrated that natural populations of *Synechococcus* are largely phage resistant (53). The effects of predation and nutrient limitation on *Synechococcus* populations can be predicted by mathematical models of predator-prey interactions, such as a prey-dependent model.

Prey-dependent model of population control. In the absence of predators or free of predator control, bacterial populations tend to reach an equilibrium population density that is dependent on the concentration of the limiting substrate. Such a population is referred to as being controlled by bottom-up forces. This situation is analogous to a chemostat in which the specific growth rate is set by the flow of the limiting nutrient through the system (17).

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According to traditional predator-prey theory the composition of a food chain is dramatically altered by the addition of a predator species and can be mathematically described by the coupled differential equations, first described by Lotka (35) and Volterra (52):

$$dN/dt = \mu N - qN - gNP \qquad 1.1$$

$$dP/dt = egNP - mP \qquad 1.2$$

where N and P are the size of the prey and predator populations at time t. The term μ describes the specific growth rate of the prey species. The term q describes the loss of individual organisms, which may be due to inherent mortality or efflux of the prey from the system. The predator grazing coefficient is described by g. The trophic function eg defines the conversion of consumed prey into predator biomass and m the mortality rate of the predator.

The most significant result of this addition is the reduction of the equilibrium prey population, referred to as top-down control (26, 27, 33, 42). The end result is that with the addition of each trophic level the *bottom* trophic level is alternately limited by available substrate or predation, depending the number of links in the chain Figure 1.1.

By definition, the size of an equilibrium population is unchanging with time and therefore the specific growth rate must equal the rate at which individuals are removed from the system (26, 27, 33, 42). Loss of individuals within natural pelagic populations may be attributed to several causes: inherent mortality, emigration, sinking and predation. Of these causes, it is probable that only predation has a significant impact on marine *Synechococcus*. It is assumed for the purposes of this model that the rate of emigration from surface water is compensated for by the rate of immigration from the surrounding water mass. Likewise, the 2.5 cm d^{-1} sinking rate of *Synechococcus* makes loss by this

Equilibrium Population Size (N)

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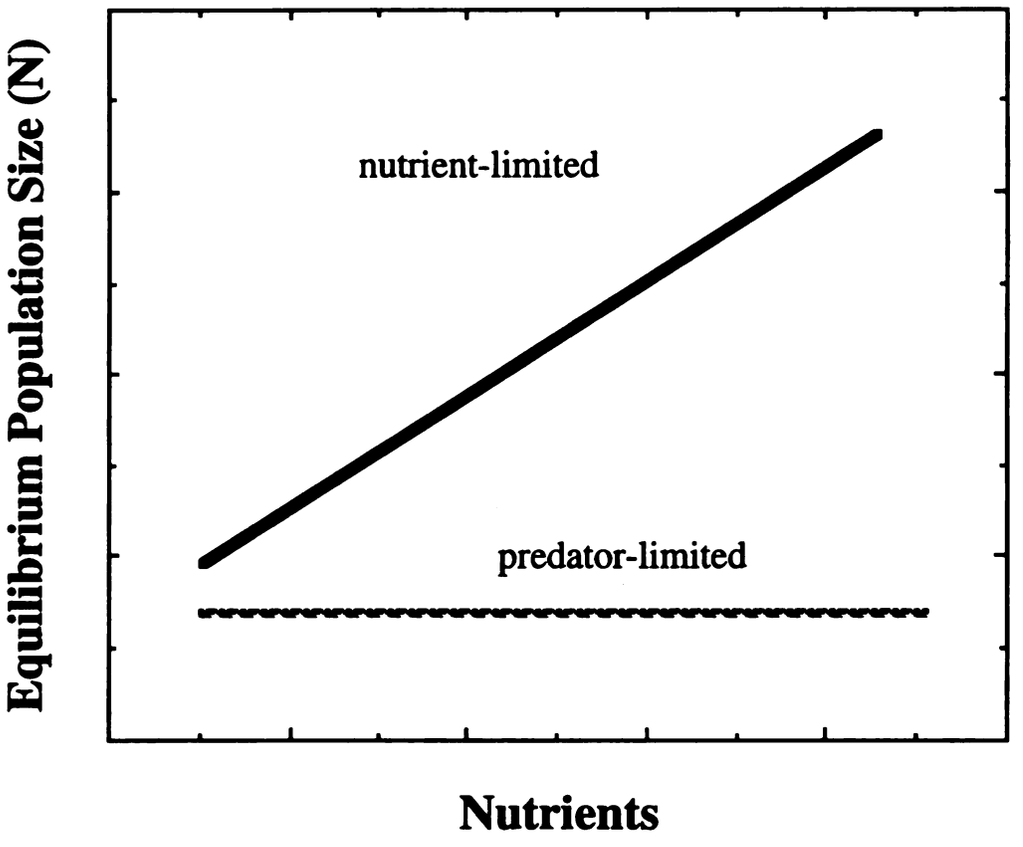

Figure 1.1 - Predicted relationship between *Synechococcus* equilibrium population size based on potential nutrients and trophic interactions (Adapted from 27).

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means negligible due to the high degree of mixing of surface water (16). *Synechococcus* may be lost by the sinking of "sea-snow" that originates from zooplankton fecal pellets (49). However, this loss may be viewed as the result of predation. Within an environment dominated by *Synechococcus* and absent a predator, the mortality rate within a single trophic level would be due to the small loss by sinking and inherent mortality. The specific growth rate of *Synechococcus* would be constant and equal to the rate of sinking loss and inherent mortality even while the equilibrium population density increases along a nutrient gradient. Thus, a plot of the equilibrium population size versus the specific growth rate μ would result in a vertical line Figure 1.2. The appearance of a predator would increase the mortality rate of the *Synechococcus* prey, at equilibrium, and would be paralleled by an increase in the specific growth rate. Therefore, the introduction of a second trophic level would result in a horizontal line if the equilibrium *Synechococcus* population density was plotted against its specific growth rate Figure 1.2. Adding a third trophic level, a predator specific to the *Synechococcus* predator, would release *Synechococcus* from predator control. The *Synechococcus* population again becomes substrate limited and results in a vertical plot of equilibrium *Synechococcus* population size versus specific growth rate. Thus, vertical and horizontal lines should be indicative of substrate limitation or predator limitation, respectively Figure 1.2. Although prey-dependent models are widely accepted, alternative models of predator-prey interactions exist. These alternative models differ significantly from prey-dependent models in the predicted outcome of trophic interactions.

Alternative models and predictions. An implicit assumption of the Lotka-Volterra model is that predator-prey interactions are random and analogous the principle of mass action in chemical systems (45). This analogy is one of the reasons that Lotka-Volterra type models have recently come into contention (45). It has been argued that natural systems are heterogeneous both spatially and in terms of the temporal scales of predator-prey interactions and reproduction. As a result, natural systems cannot be

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modeled on the homogenous systems characterized by mass action. Ratio-dependent models (1-3) of predator-prey interactions have been proposed to provide a mathematical

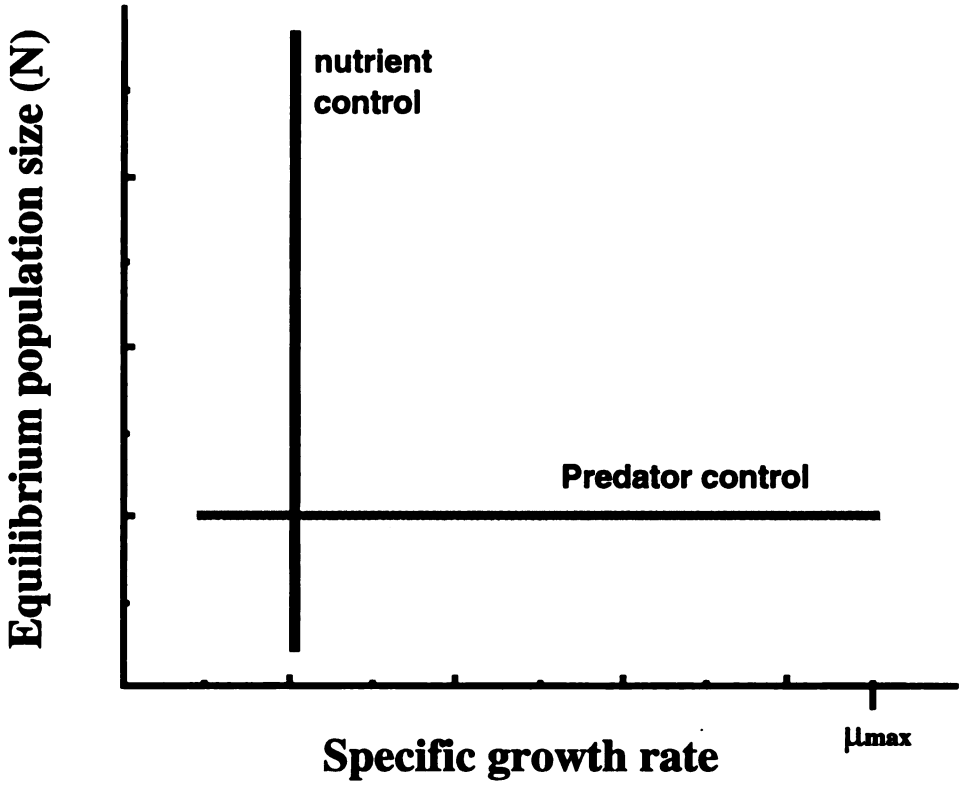

Figure 1.2 - Model of equilibrium population size and specific growth for predator-prey interactions.

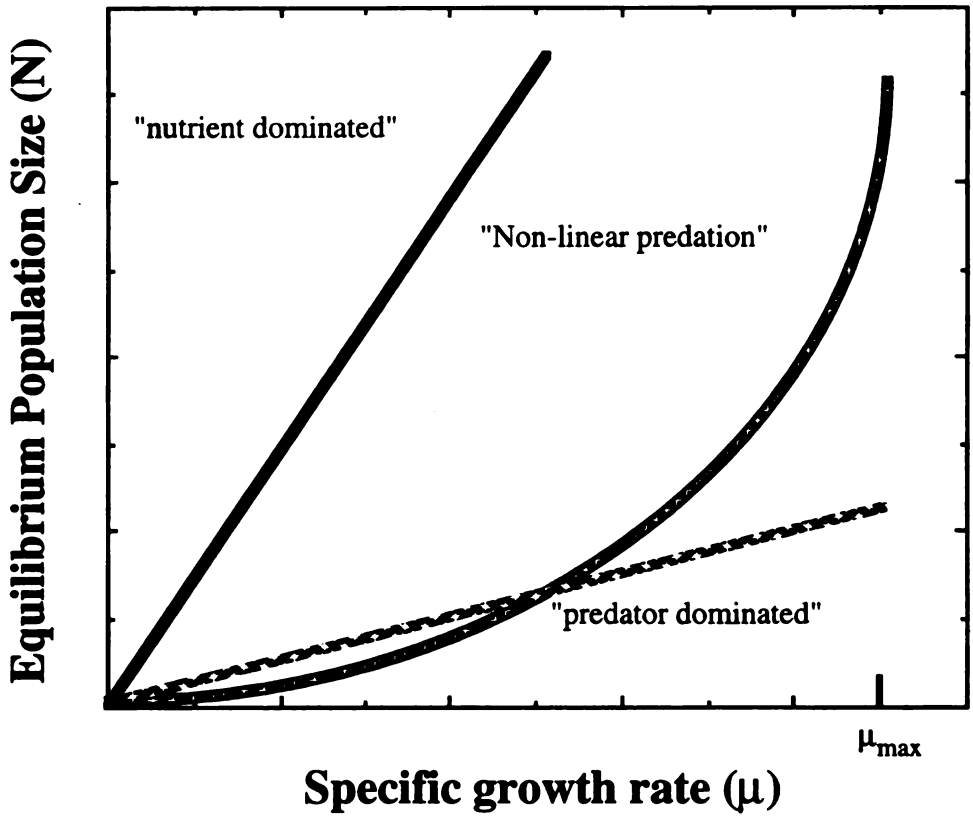

Figure 1.3 - Alternative relationships between *Synechococcus* equilibrium population size and specific growth rate (μ) for raio-dependent interactions.

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description of the heterogeneity in natural systems. In ratio-dependent models the prey density and mortality rate is dependent on the ratio of prey to predators (N/P):

$$dN/dt = \mu N - qN - gN/PP \qquad 1.3$$

$$dP/dt = egN/PP - mP \qquad 1.4$$

where N and P are the size of prey and predator populations at time t. The term μ describes the specific growth rate of the prey species. The term q describes the loss of individual organisms, which may be due to inherent mortality or efflux of the prey from the system. The predator grazing coefficient is described by g. The trophic function eg defines the conversion of consumed prey into predator and m the mortality rate of the predator.

The predictions of the interactions between trophic levels derived from equations 1.3 and 1.4 differ significantly from those predicted by equations 1.1 and 1.2 Figures 1.1 and 1.2. According to ratio-dependent models, an increase in primary productivity leads to an increase in the equilibrium population size at all trophic levels Figure 1.3. Furthermore, there is no lower productivity limit on the number of trophic levels that may be supported by a system. A plot of the equilibrium *Synechococcus* population versus the specific growth rate may result in one of several alternatives relationships predicted by ratio-dependent models. A linear correlation between the equilibrium population density and specific growth rate would suggest that both substrate concentration and predation contribute to the equilibrium population size and specific growth rate. A slope of greater than one >1 would indicate that nutrient input has a relatively greater effect upon the equilibrium population size than predation. Likewise, a slope of less than one <1 would indicate the opposite. A curvilinear increase in consumption rate would be the result of predator pressure giving way to substrate limitation 1 (Figure 1.3).

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The implicit assumption of the ratio-dependent model is that temporal or spatial heterogeneity exists within the system. It is unlikely that temporal heterogeneity could be produced by differences in generation times between *Synechococcus* and its predators because such differences are small. The estimated 1 to 2 divisions per day (13) of flagellates is comparable to the reported replication rate of *Synechococcus* (4, 10). This would exclude temporal heterogeneity as justification for application of a ratio-dependent model to this system. The other possible basis for the application of the ratio-dependent model is spatial heterogeneity. Spatial heterogeneity is principally the result of refugia for the prey population. One possible refugia for *Synechococcus* is the abundant marine microaggregates known as "sea-snow" (49). However, recent phylogenetic analysis failed to detect *Synechococcus* within these microaggregates (12). Thus, it is unlikely that the planktonic *Synechococcus* use microaggregates as refugia.

Synechococci populations both in the laboratory and the environment were analyzed in the context of the afore mentioned models. In chapters 2 and 3 the nucleic acids concentrations of a freshwater cyanobacterium and a marine cyanobacterium were studied in response to changes in specific growth rate and during a 12 h light: 12 h dark diel cycle. In chapter 4 oligonucleotide probes specific for cyanobacterial 16S rRNA were used to document the changes in specific growth rate of synechococci populations in response to nutrient-limitation or predator-limitation. In chapter 5 synechococci population size and specific growth within the Gulf of Mexico were examined, using oligonucleotide probes specific for cyanobacterial 16S rRNA, in light of the predictions of prey-dependent and ratio-dependent models. Finally, chapter 6 discusses how the results of the previous chapters apply in a broad context to predator-prey interactions within the microbial world and the future directions such techniques and models may take.

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Chapter 2

CONCENTRATIONS OF NUCLEIC ACIDS OF *SYNECHOCOCCUS* SP. STRAIN PCC 6301 DURING GROWTH IN CONTINUOUS LIGHT AND LIGHT:DARK CYCLES

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Abstract

Cellular nucleic acids concentrations were determined for *Synechococcus* sp. strain PCC 6301 grown in either light-limited batch cultures, phosphate-limited chemostat cultures or batch cultures entrained by 12 h light:12 h dark (diel) cycles. Both light-limited batch cultures and phosphate-limited chemostat cultures exhibited an exponential increase in cellular RNA concentrations, cellular 16S rRNA concentrations and cellular DNA concentrations that correlated with increases in growth rate. The ratio of RNA to DNA remained constant with increases in growth which suggests a lack of transcriptional regulation of ribosomal RNA operons. Cellular nucleic acids concentrations decreased during light periods and increased during dark periods in cultures entrained by a diel cycle; a result that was unexpected in light of experiments demonstrating a circadian increase in transcriptional activity during the subjective light periods and a decrease in transcriptional actitvity during subjective dark periods. The mean cellular RNA concentration during diel growth was comparable to the cellular RNA concentration at the same growth rate under constant illumination. Cellular 16S rRNA concentrations were closely correlated with cellular DNA concentrations during a diel cycle. The correlation between cellular DNA concentration and cellular 16S rRNA concentration, during a diel cycle, is consistent with the hypothesis that cellular 16S rRNA concentration is not transcriptionally regulated. Cellular 16S rRNA concentration also correlated with ribosomal RNA operon copy number which suggests that gene-dosage is the primary mechanism regulating cellular 16S rRNA concentrations in synechococci. The cyclical variation in cellular nucleic acids concentration during diel cycles appears to be a function of the timing of cell division.

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Introduction

Cellular nucleic acids concentration is proportional to growth rate in a number of heterotrophic bacteria (32, 33, 55). Nucleic acids concentration is also proportional to growth rate in both filamentous and unicellular cyanobacteria under constant illumination (43, 45). The nucleic acids content of bacteria is predominately composed of rRNA and one-third of ribosomal rRNA (w/w) is composed of 16S rRNA. The correlation between cellular 16S rRNA concentrations and growth rate presents the possibility of using probes complementary to 16S rRNA to determine *in situ* growth rates.

The concentration and synthesis of nucleic acids have typically been studied in cyanobacteria grown under constant illumination followed by a single dark incubation period (3, 17, 18, 22, 24, 46, 60, 63). Growth under constant illumination, however, may not accurately reflect the physiological state of synechococci populations growing under the diel cycles found in the environment (16). Cyanobacteria entrained by a diel cycle, for example, exhibit circadian rhythms in cell division (48, 62), amino acid uptake (10), nitrogenase activity (21, 27, 28), the expression of *psb*A (35-37) and the expression of *dna*K (10). Circadian rhythms are not found in continuously illuminated cultures of cyanobacteria.

In this study, we demonstrate that increases in cellular RNA concentrations and cellular DNA concentrations correlate with increases in growth rate in light-limited batch cultures and phosphate-limited chemostat cultures. Increases in cellular 16S rRNA concentrations also correlate with increases in growth rate. Cellular RNA, cellular 16S rRNA and cellular DNA concentrations, however, fluctuated in a regular pattern over the course of a diel cycle.

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Materials and Methods

Bacterial strains and culture conditions. *Synechococcus* sp. strain PCC 6301 (ATCC 27144) was maintained at room temperature (RT) on BG-11 (1) agar at 86$\mu Em^{-2}s^{-1}$. *Synechococcus* sp. strain PCC 6301 batch cultures were grown at 37°C. Growth rate was varied by changing the photon fluence rate from 15 $\mu Em^{-2}s^{-1}$ to 64.4 $\mu Em^{-2}s^{-1}$ in light-limited batch cultures. Diel cultures were entrained on a 12 h light: 12 h dark diel cycle for at least seven generations prior to the start of each diel experiment.

Chemostat cultures of *Synechococcus* sp. strain PCC 6301 were grown on modified Cg medium (29). Chemostats were illuminated at 162 $\mu Em^{-2}s^{-1}$ at 37°C and aerated at a rate of 40 $ml \cdot min^{-1}$ with 3% CO_2 (balance air). Phosphate-limited growth was measured at three different reservoir concentrations. Phosphate concentrations within the reservoirs were 5 µM, 10 µM and 15 µM K_2HPO_4. Growth rate was varied by changing the dilution rate from 20 $ml \cdot min^{-1}$ to 365 $ml \cdot min^{-1}$ in a 250 ml Bellco Spinner flask (Bellco Glass Inc., Vineland, NJ). The culture was considered to have reached a stable equilibrium after maintaining the same optical density at 750 nm and 600 nm for three volume changes of the culture vessel.

Cell density was determined by microscopic direct counts (34) in a Petrohoff-Hausser chamber (Hausser Scientific Partnership, Horsham, PA) or using a particle counter and channelyzer (Coulter Electronics Inc., Miami, FL). Growth rate was defined as the reciprocal of the generation time, given in hours.

Chemical determination of RNA and DNA. *Synechococcus* sp. strain PCC 6301 cells were harvested by centrifugation of 10 ml of log-phase culture at 16,000 x*g* for 25 min at 4°C. Cellular RNA concentration was measured by the orcinol assay (13), using adenosine monophosphate as a standard. Cellular DNA concentration was measured by the

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diphenylamine assay (13), using deoxyribose as a standard. Cellular RNA concentrations were estimated to be twice that of the AMP standard based on the approximately 55% G+C ratio of *Synechococcus* PCC 6301 16S rRNA and 23S rRNA (19, 41). Cellular DNA concentrations were calculated as 4.84 times the deoxyribose standard concentration based on the 52-53% G+C ratio of the *Synechococcus* PCC 6301 genome (12) and the difference between the average molecular weight of a nucleotide and the molecular weight of deoxyribose.

Nucleic acids isolation and hybridization. *Synechococcus* sp. strain PCC 6301 cells were harvested by centrifugation of 1.5 ml log-phase batch culture or stable chemostat culture at 16,000 x*g* for 25 min at 4°C. Cells were disrupted mechanically and nucleic acids isolated (61). The RNA extraction efficiency was 65.7±2.3 % based on the comparison between whole cell orcinol assays and orcinol assays of extracted nucleic acids. RNase contamination was reduced by using either virgin polypropylene or glassware treated with diethylpyrocarbonate (Sigma Chemical Co., St. Louis, MO) and baked at 240°C for 3 h.

Nucleic acids were hybridized with a cyanobacterial-specific oligonucleotide probe (7); this probe was complementary to the small subunit rRNA at positions 360-376 (*Escherichia coli* numbering). The cyanobacterial-specific probe was 5' end-labeled with γ-^{32}P-ATP (56) to a specific activity of ca. 0.5 μCi·pmol^{-1} probe. End-labeled probes were purified on TSK-DEAE columns (Supleco, Bellefonte, PA) equilibrated with 50 mM NH_4OAc (7).

Nucleic acids from batch cultures or chemostat cultures were denatured by the addition of phosphate-buffered gluteraldehyde [pH 7.0] to a final concentration of 1.5% and incubated at RT for 15 min. Purified *Synechococcus* sp. strain PCC 6301 16S rRNA (7) was denatured in the same manner and a standard curve constructed from 1 ng to 75 ng of 16S rRNA. *Escherichia coli* (*E. coli*) nucleic acids were denatured in the same manner

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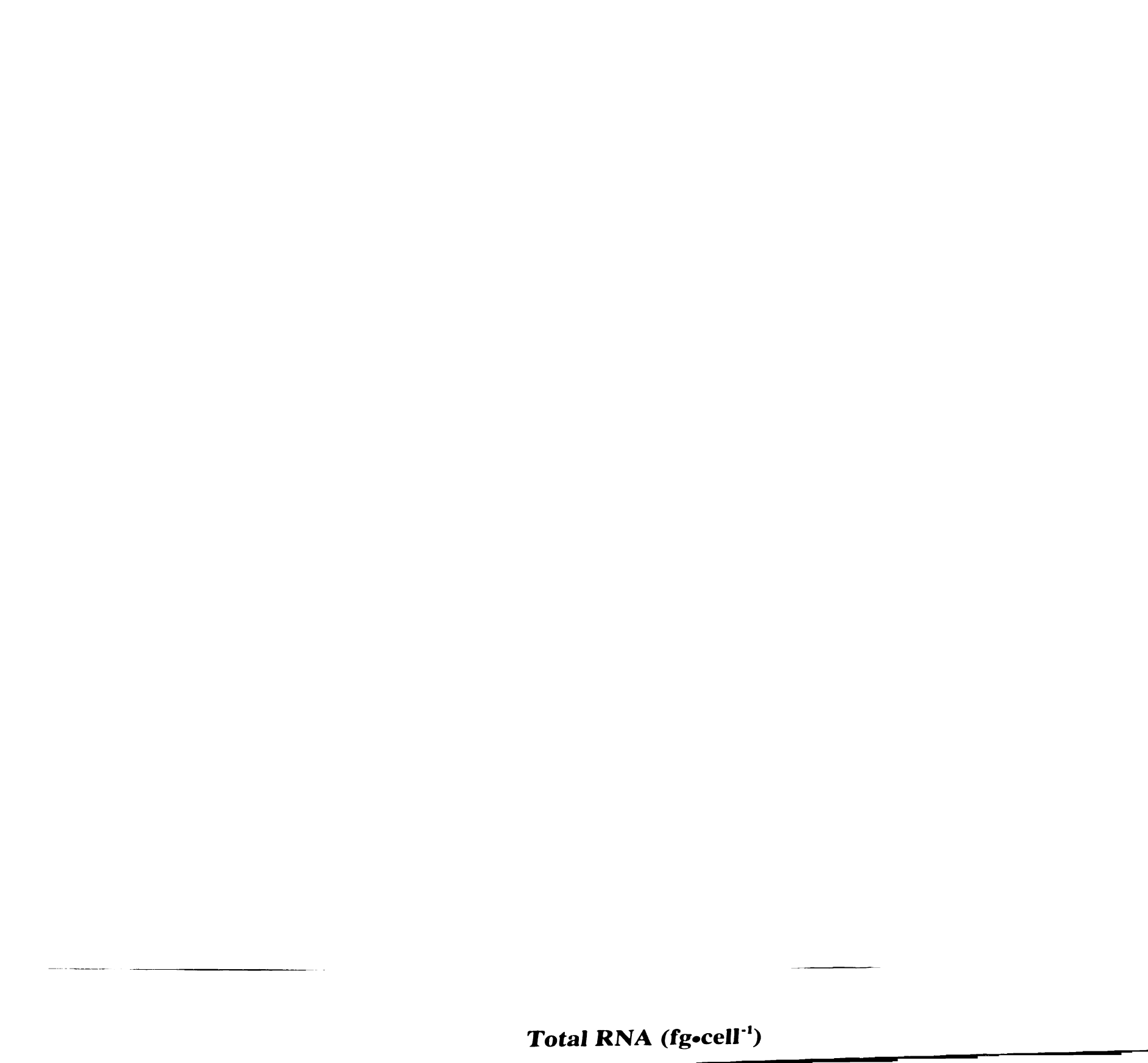
and used to determine non-specific binding of the probe. Nucleic acids from experimental samples, 16S rRNA standards and *E. coli* were applied to MagnaCharge nylon membranes (Micron Separation Inc., Westbourgh, MA) using a slot-blot apparatus (Millipore, Bedford, MA). Approximately 1 μg, 500 ng and 250 ng from each experimental sample and *E. coli* were applied to nylon membranes. Nucleic acids were UV-crosslinked (Stratalinker 1800, Stratagene Inc., La Jolla, CA) to nylon membranes according to the manufacturer's instructions. Membranes were prehybridized for 30 min at 37°C in 10 ml hybridization buffer [6X SET (56), 0.5% SDS, 1X Denhardt's solution (56), 100 $\mu g \cdot ml^{-1}$ poly(A)], followed by hybridization for 14-16 h at 37°C in 10 ml hybridization buffer containing $1x10^6$ $CPM \cdot ml^{-1}$ ^{32}P-radiolabeled probe. Membranes were washed twice at RT and once at 48°C for 30 min each in wash buffer [2X SET, 0.5% SDS]. Standards and samples were quantified using a radioanalytical image system (AMBIS Inc., San Diego, CA).

Results

Cellular RNA and DNA concentrations of light-limited batch cultures. Differential growth rates were established for light-limited batch cultures of *Synechococcus* sp. strain PCC 6301 by varying photon fluence rates. Continuous photon fluence rates of 15 $\mu Em^{-2}s^{-1}$ to 64.4 $\mu Em^{-2}s^{-1}$ resulted in growth rates of 0.008 h^{-1} to 0.051 h^{-1}, respectively. Cellular RNA and DNA concentrations were determined colorimetrically. Cellular RNA concentrations increased from 23.9±2.2 $fg \cdot cell^{-1}$ to 45.5±0.9 $fg \cdot cell^{-1}$ with increases in growth rate (Figure 2.1; Table 1). Cellular DNA concentrations


Total RNA (fg•cell-1)

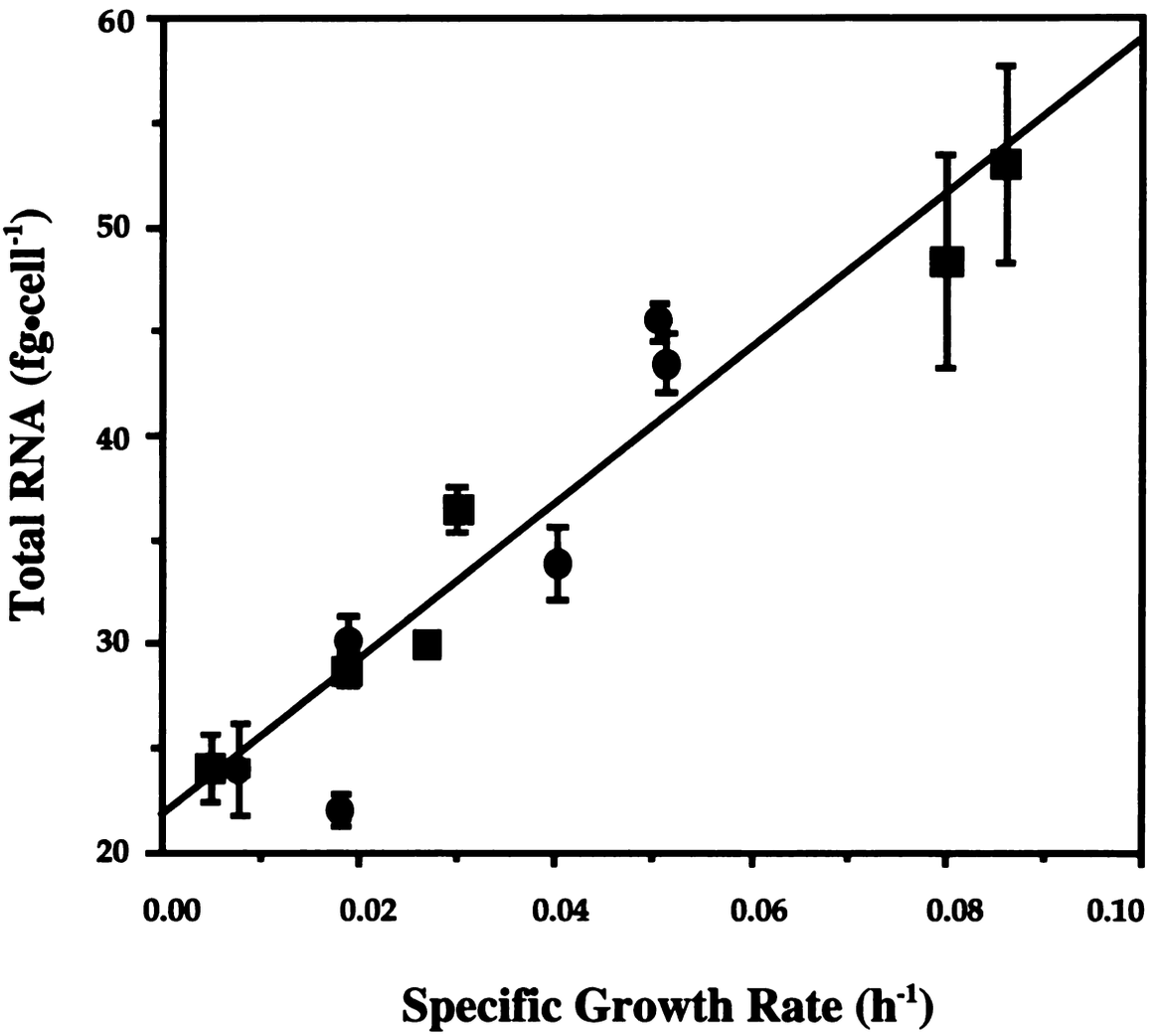


Figure 2.1 - Total cellular RNA, as determined by orcinol, from light-limited batch cultures (●) and phosphate-limited chemostat cultures (■), as a function of growth rate ($r^2 = 0.92$). Error bars represent standard errors (n = 3).

Table 2.1 - Physiological parameters of *Synechococcus* sp. strain PCC 6301 in light-limited batch culture.

Growth Rate (h^{-1})	Total RNA (fg • $cell^{-1}$)	Total DNA (fg • $cell^{-1}$)	RNA/DNA[a]	cell vol. ($mm^3 \cdot cell^{-1}$)	RNA ($fg \cdot mm^3$)
0.008	23.9 ± 2.2	11.4 ± 0.5	2.10	1.22	19.6
0.018	21.9 ± 0.7	7.6 ± 0.7	2.88	1.27	17.3
0.019	30.1 ± 1.2	12.7 ± 0.4	2.37	1.27	23.7
0.040	33.8 ± 1.8	17.3 ± 1.2	1.95	1.37	24.7
0.051	44.5 ± 1.2	18.1 ± 0.8	2.46	1.43	31.1

[a]PCC 6301 mean ± s.e. = 2.35 ± 0.18

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increased from 11.4±0.5 fg·cell^{-1} to 18.6±1.1 fg·cell^{-1} with increasing growth rate (Table 2.1). The ratio of RNA to DNA remained a constant 2.4±0.3 over a range of growth rates in *Synechococcus* sp. strain PCC 6301 (Table 2.1).

Cellular RNA and 16S rRNA concentrations of phosphate-limited chemostat cultures. Growth rates were varied from 0.005 h^{-1} to 0.09 h^{-1} in each of three chemostats fed by reservoirs containing 15 μM, 10 μM or 5 μM phosphate. Phosphate limitation does not preferentially restrict ribonucleic acid pools, but results in a proportional loss from all macromolecular pools with decreases in growth rate (20). Cellular RNA concentrations increased exponentially from 24.0±1.6 fg·cell^{-1} to 53.0±4.8 fg·cell^{-1} with increases in growth rate (Figure 2.1). Cellular 16S rRNA concentrations were determined using ^{32}P-radiolabeled, cyanobacterial-specific, oligonucleotide probes. Cellular 16S rRNA concentrations increased exponentially from 4.6±1.9 fg·cell^{-1} to 19.7±0.45 fg·cell^{-1} with increasing growth rate (Figure 2.2); regardless of the phosphate concentration within the reservoir.

Cellular RNA, 16S rRNA and DNA concentrations of diel batch cultures. Batch cultures of *Synechococcus* sp. strain PCC 6301 were entrained in a 12 h light: 12 h dark diel cycle at 64.4 μEm^{-2}s^{-1}. Entrained cultures had a growth rate of 0.03 h^{-1} (Figure 2.3) over the course of a single diel cycle (24 h). The growth rate was 0.06 h^{-1} during the light periods of a diel cycle because cell division was arrested in the absence of light. Cell division resumed upon reexposure to light. Entrained cultures did not exhibit a noticeable lag in growth upon being reexposed to light. Cellular RNA concentrations gradually decreased from approximately 40.8±4.0 fg·cell^{-1} to 32.7±1.1 fg·cell^{-1} during light periods and gradually returned to 38.0±1.4 fg·cell^{-1} fg·cell^{-1} during subsequent dark periods (Figure 2.4A). The trend observed in cellular RNA concentrations was observed in cellular 16S rRNA concentrations as well. Cellular 16S RNA concentrations gradually

Cellular 16S rRNA (fg·cell^{-1})

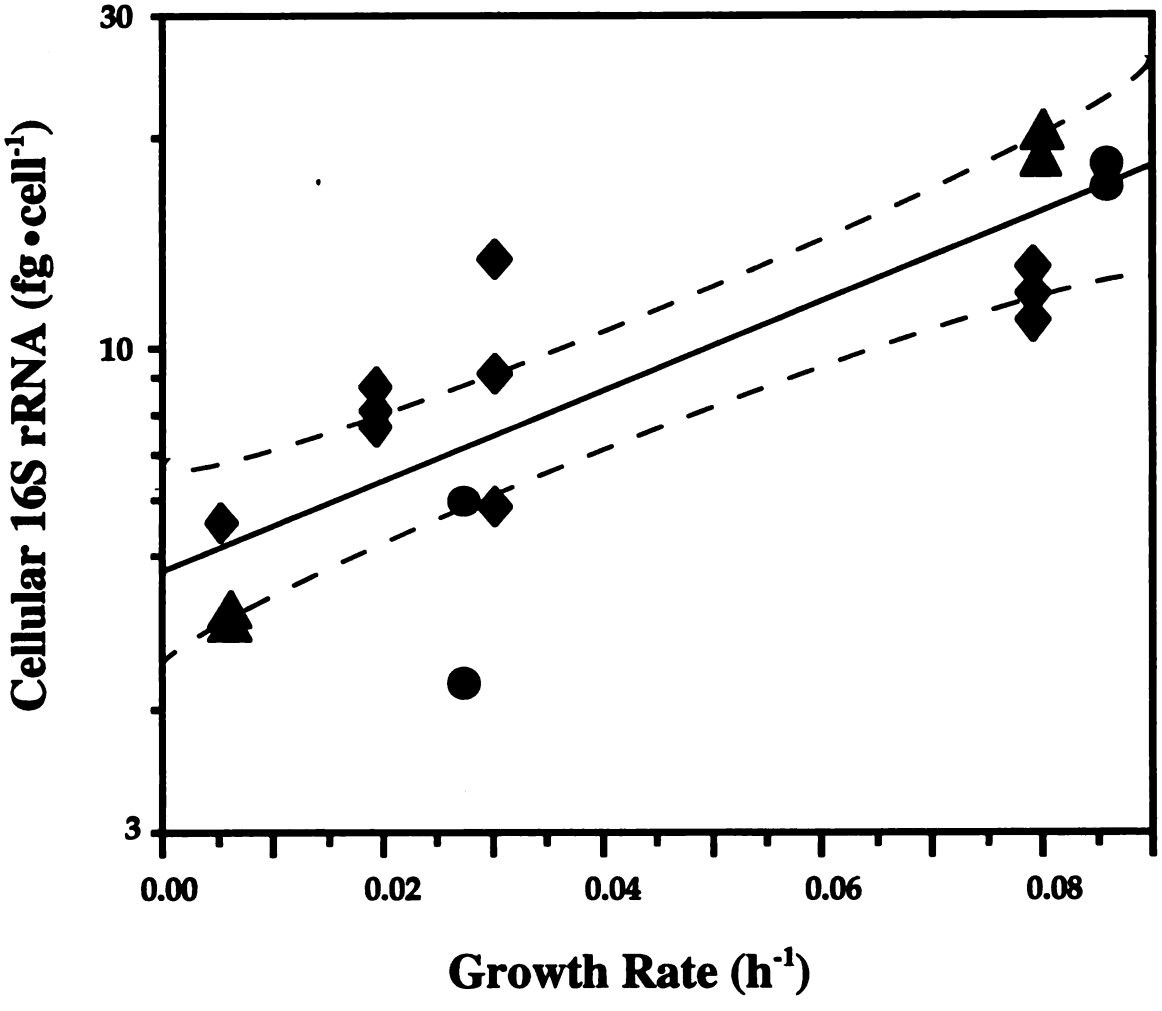

Figure 2.2 - Cellular 16S rRNA concentrations of *Synechococcus* PCC 6301 from phosphate-limited chemostats. Phosphate concentrations of nutrient reservoirs were 15 µM (♦), 10 µM (▲) and 5 µM (●). Dashed lines indicate 95% confidence intervals ($p < 0.001$; r2 = 0.67).

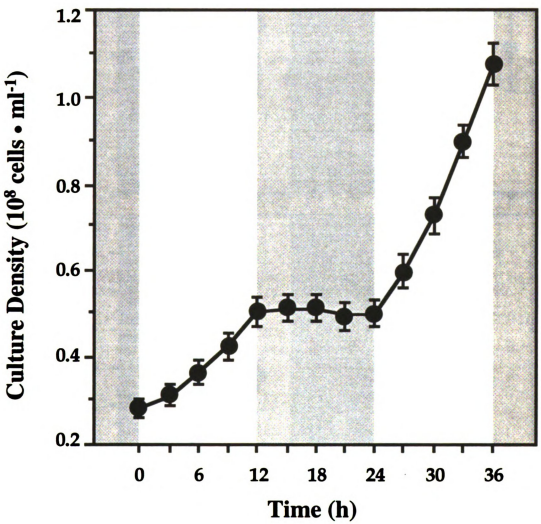

Figure 2.3 - Cell density of *Synechococcus* sp. strain PCC 6301 during diel growth. Each day consisted of 12 h light: 12 h dark (shaded area). Error bars represent standard errors (n=6).

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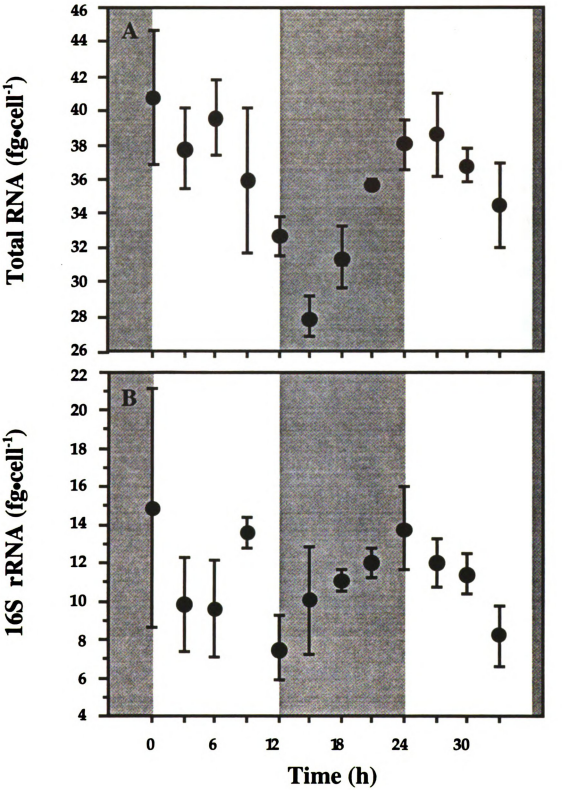
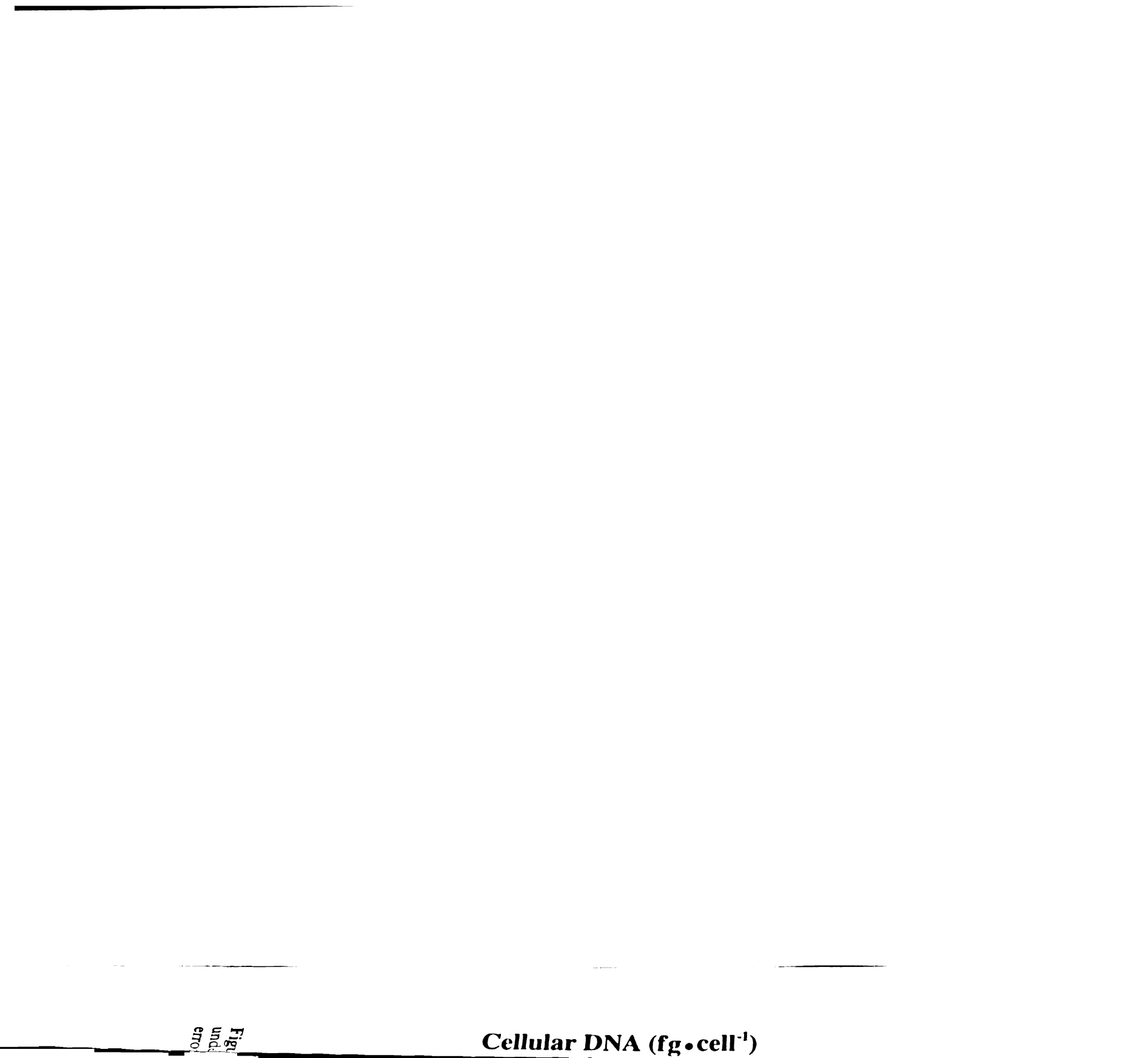


Figure 2.4 - Cellular RNA concentrations of *Synechococcus* sp. strain PCC 6301 grown under a 12 h light: 12 h dark (shaded area) diel cycle. (A) Cellular RNA concentrations. (B) Cellular 16S rRNA concentrations. Error bars represent standard error (n=6).



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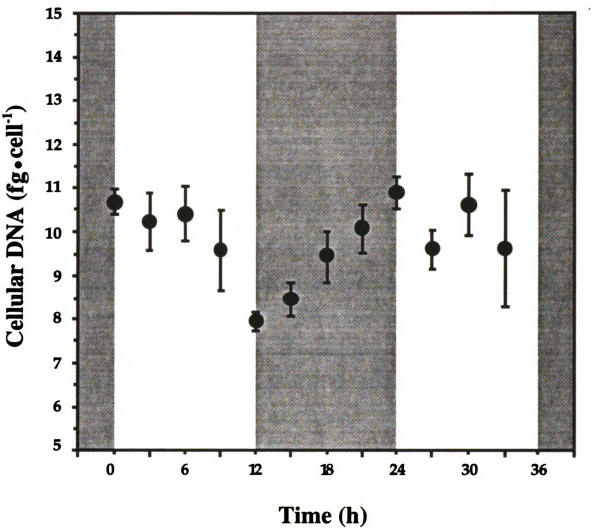

Figure 2.5 - Cellular DNA concentrations of *Synechococcus* sp. strain PCC 6301 grown under a 12 h light: 12 h dark (shaded area) diel cycle. Error bars represent standard error (n=6).

decreased from approximately 14.9±6.2 fg·cell^{-1} to 7.5±1.7 fg·cell^{-1} during light periods and gradually returned to 13.8±2.2 fg·cell^{-1} during subsequent dark periods (Figure 2.4B). The trend exhibited by cellular RNA concentrations and cellular 16S rRNA concentrations was also observed in cellular DNA concentrations (Figure 2.5). Cellular DNA concentrations gradually decreased from 10.7±0.3 fg·cell^{-1}, corresponding to approximately 3 genomes (23, 25), to a low of 7.9±0.2 fg·cell^{-1}, corresponding to approximately 2 genomes, during light periods before returning to 10.9±0.4 fg·cell^{-1} during subsequent dark periods.

Discussion

Light-limited, batch cultures of *Synechococcus* sp. strain PCC 6301 displayed an exponential increase in both cellular RNA concentrations and cellular DNA concentrations that correlated with increases in growth rate. The increase in cellular RNA concentrations is comparable to previous reports (45) of an approximately 28.7 fg·cell^{-1} to 44.9 fg·cell^{-1} increase in cellular RNA concentrations over the same range of growth rates in continuously illuminated, light-limited batch cultures. The exponential relationships between RNA, DNA and growth rate observed in *Synechococcus* sp. strain PCC 6301 have also been observed in *Anabaena variabilis* (43). The correlated increase in nucleic acids with increased growth rate appears to be a general physiological feature of cyanobacteria, as well as heterotrophic bacteria (32, 33, 55). The cellular DNA concentrations in this study are approximately 2.4 times the values (4.9 fg·cell^{-1} to 7.8 fg·cell^{-1}) reported previously for light-limited batch cultures (45). The apparent discrepancy between the two experiments may be attributed to previous studies reporting the deoxyribose concentration, calculated from the standard, as a DNA concentration when in

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fact there is a 2.4 fold difference in molecular weight between deoxyribose and nucleotides of DNA. The cellular DNA concentrations in this study agrees with the number of genomes per cell previously reported (6) for *Synechococcus* sp. strain PCC 6301.

In heterotrophic bacteria, such as *E. coli*, the rate of transcription from rRNA operons increases with increases in growth rate (51). The increase in transcription rates from rRNA operons is reflected in a linear increase in the RNA/DNA ratio with increasing growth rate in heterotrophic bacteria (14, 32, 49, 55). Unlike in heterotrophic bacteria, the ratio of cellular RNA concentration to cellular DNA concentration remained constant over a range of growth rates in *Synechococcus* sp. strain PCC 6301 (Table 1), as reported previously (45). The constant RNA/DNA ratio may indicate a lack of transcriptional regulation of the rRNA operons in *Synechococcus* sp. strain PCC 6301. A lack of transcriptional control of stable rRNAs is also indicated by the constant ratio of cellular rRNA concentrations to cellular tRNA concentrations in *Synechococcus* sp. strain PCC 6301 (44). The constant rRNA/tRNA ratio in *Synechococcus* sp. strain PCC 6301 contrasts with the situation in heterotrophic bacteria where the rRNA/tRNA ratio increases with increasing growth rate (33, 55). The rRNA/tRNA ratio increased from approximately 1.8 to 4.5 with increases in growth rate in *Salmonella typhimurium* (33). Similar increases in rRNA/tRNA ratios have been observed in *E. coli* (55) and *Bacillus subtilis* (15); although these increases were smaller than those reported for *S. typhimurium*.

It was originally argued (9, 44) that the constant ratio of rRNA to tRNA, the lack of induction or repression of key enzymes by their respective substrates, and the constant ratio of RNA to DNA are indicative of a general lack of transcriptional control in cyanobacteria. Transcriptional control has been demonstrated, however, for glycogen phosphorylase (58), glucose-6-phosphate dehydrogenase (58) and *psb*A (40) in cyanobacteria. Although not all gene expression lacks transcriptional control the constant RNA to DNA ratio observed in this study (Table 1) and by Mann and Carr (45) supports the conclusion that rRNA is not transcriptionally regulated in cyanobacteria.

Even though rRNA does not appear to be transcriptionally regulated, cellular RNA concentrations increased exponentially with increases in growth rate in phosphate-limited, chemostat cultures of *Synechococcus* sp. strain PCC 6301 (Figure 2.1). Cellular RNA concentrations have also been demonstrated to increase exponentially from approximately 20 fg·cell^{-1} to 40 fg·cell^{-1} over a range of dilution rates from 0.025 h^{-1} to 0.1 h^{-1} in light-limited chemostats (54). Cellular RNA concentrations of 50-60 fg·cell^{-1} at a dilution rate of 0.09 h^{-1} have been reported for *Synechococcus* sp. strain PCC 6301 in Ca^{2+}-limited chemostats at nonlimiting photon fluence rates (65). The cellular RNA concentration in Ca^{2+}-limited chemostats is comparable to the cellular RNA concentration observed at a growth rate of 0.09 h^{-1} in phosphate-limited chemostats (Figure 2.1). One exception to the correlation between cellular RNA concentration and growth rate, however, has been noted. Cellular RNA concentration was found to be independent of growth rate in *Synechococcus* sp. strain PCC 6301 grown in Mg^{2+}-limited chemostats (64). *Synechococcus* sp. strain PCC 6301 possessed a uniformly high concentration of RNA in Mg^{2+}-limited chemostats regardless of growth rate. Low Mg^{2+} concentrations were postulated to reduce ribosome efficiency which resulted in a reduction in growth rate. If ribosome efficiency had not been reduced by low Mg^{2+} concentrations the RNA concentration would have reflected growth rate. Cellular RNA concentrations, therefore, can be used to measure growth rate except in cases of Mg^{2+} limitation.

The correlation between cellular RNA concentration and growth rate was also expected to apply to cellular rRNA concentrations because rRNA comprises a constant 75% of total RNA in continuously illuminated cultures of cyanobacteria (44). This hypothesis was tested using cyanobacterial-specific oligonucleotide probes complementary to the small subunit rRNA. Cellular 16S rRNA concentrations increased exponentially with increases in growth rate in phosphate-limited, chemostat cultures of *Synechococcus* sp. strain PCC 6301 (Figure 2.2). The correlation between cellular 16S rRNA concentrations and growth

rate presents the possibility of using group-specific probes complementary to 16S rRNA to determine *in situ* growth rates.

Determining the *in situ* growth rate of cyanobacteria by using 16S rRNA probes requires an understanding of the diel variations of cellular RNA concentrations. The variation in cellular RNA concentrations was examined in batch cultures of *Synechococcus* sp. strain PCC 6301 entrained in a 12 h light: 12 h dark diel cycle and possessing a diel growth rate of 0.043 h^{-1}. Cellular RNA concentrations gradually decreased during the light periods of a diel cycle (Figure 2.4A). The decrease in cellular RNA concentrations does not appear to be due to a decrease in rates of RNA synthesis. High rates of incorporation of ^{3}H- and ^{14}C-uracil (18, 22, 38, 58) indicate that RNA synthesis rates do not decrease in the light. Continuous rates of RNA synthesis are also indicated by the accumulation of *rbcL* gene transcripts (encoding the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase) in *Synechococcus* sp. strain PCC 8801 (11) and transcripts of the *psb*A1 gene (encoding the D1 protein of PSII) in *Synechococcus* sp. strain PCC 7942 (36) in the light. The decrease in cellular RNA concentrations appears to be the result of cell division. Cell division occurred only upon exposure to light in *Synechococcus* sp. strain PCC 6301 (Figure 2.3). During these light periods the rate of cell division exceeded the cells ability to double its cellular RNA content and therefore RNA concentrations decreased on a per cell basis.

Cellular RNA concentrations decreased during the first 3 h of dark periods. This decrease in cellular RNA concentrations does not appear to be due to cell division because cell division is arrested during dark periods. The transient decrease in cellular RNA concentrations during the first 3 h of darkness coincides with decreases in the light-specific transcripts of the *psbA1* gene (36) and the *rbcL* gene (11) which offset the increase in 16S rRNA concentrations during the same period (Figure 2.4B). Cellular RNA concentrations increase approximately 33% over the remaining nine hours of darkness. The 33% increase in cellular RNA concentrations is similar to the 22% increase observed by Herdman *et al*

(24). The increase in cellular RNA concentrations coincides with increase in a dark-specific transcript of unknown function (63), transcripts of the nitrogen fixing gene *nifH* (11), transcripts encoding glycogen phosphorylase and glucose-6-phosphate dehydrogenase (58), and ribosomal RNAs (53, 58, 63). Incorporation of ^{3}H- and ^{14}C-uracil into both stable and unstable RNA species indicates that RNA continues to be synthesized throughout the dark period (17, 18, 22, 38, 53, 58). The majority of dark ^{14}C-uracil incorporation was into RNA, with little incorporation into nucleotide pools (22). The small amount of ^{14}C-uracil incorporation into nucleotide pools suggests a rapid turnover within nucleotide pools which is also indicative of continuous RNA synthesis. The small amount of ^{14}C-uracil incorporation into nucleotide pools in *Synechococcus* sp. strain PCC 6301 is unlike the incorporation in *E. coli* (47) where up to half of the ^{14}C-uracil accumulates in nucleotide pools. The mean cellular RNA concentration during diel growth was comparable to the cellular RNA concentration at the same growth rate under constant illumination.

The decrease in cellular RNA concentrations was accompanied by a gradual decrease in cellular 16S rRNA concentrations during the light period of a diel cycle (Figure 2.4B). Like cellular RNA concentrations, the drop in cellular 16S rRNA concentrations appears to be due to cell division. Cellular 16S rRNA concentrations doubled during the subsequent dark period. The steady increase in 16S rRNA concentrations has previously been observed in synchronized cultures of *Synechococcus* sp. strain PCC 6301 placed in the dark (17, 53, 58, 63). A rise in 16S rRNA concentrations during the night was also observed in *in situ* assemblages of synechococci (39). This assemblage of synechococci exhibited a marked decline in 16S rRNA during the first 4.5 h of darkness which was due to an increase in population density.

The accumulation of 16S rRNA in the dark implies *de novo* synthesis of ribosomal RNAs (17, 18, 58) without concurrent cell division. The net result of these two factors is an increase in 16S rRNA concentrations on a per cell basis. Cellular 16S rRNA

concentrations increased by approximately 6 fg·cell^{-1} during the course of a dark period. Cellular 16S rRNA comprises approximately one-third of ribosomal RNA. The approximately 6 fg·cell^{-1} increase in cellular 16S rRNA concentration would have resulted in a 18 fg·cell^{-1} increase in ribosomal RNA. The calculated 18 fg·cell^{-1} increase in cellular rRNA concentrations is in agreement with the observed increase of approximately 18 fg·cell^{-1} in cellular RNA concentrations and accounts for the majority of the 33% increase in cellular RNA concentrations.

Cellular DNA concentrations gradually decreased from approximately 3 genomes to 2 genomes, during light periods, before returning to 3 genomes during subsequent dark periods (Figure 2.5). Like cellular RNA concentrations, the decrease in cellular DNA concentrations was due to the increase in population size. The average of 3 genomes observed is consistent with the 3 genomes·cell^{-1} reported by Binder and Chisholm (6) for continuously illuminated *Synechococcus* sp. strain PCC 6301 with a growth rate (0.07 h^{-1}) similar to the growth rate during light periods in our diel experiments. Cell division can be synchronized in cyanobacteria by a period of dark incubation. Synchronized cultures of *Synechococcus* sp. strain PCC 6301 have shown continuous incorporation of radiolabeled thymine for up to 8 h after reentering the dark (60). The observed increase in cellular DNA concentrations seems to be dependent on the phase of the cell cycle of the culture upon reentering the dark (46). Synchronous cultures of *Synechococcus* sp. strain PCC 6301 have shown increases of 10-19% in cellular DNA concentrations within the first 4 h of dark incubation before decreasing to a plateau maintained over the next 12-23 h (46). The continuous, dark DNA synthesis observed in the entrained cultures of *Synechococcus* sp. strain PCC 6301 used in this study contrasts with the lack of dark DNA synthesis in asynchronous cultures of *Synechococcus* sp. strain WH 8101 (2) and *Synechococcus* sp. strain PCC 6301 synchronized by a single dark incubation period (22, 46). It would appear that entrained cultures of cyanobacteria possess greater resources for the synthesis

of DNA during the dark than asynchronously growing cultures or cultures synchronized by a single dark period.

One possible explanation for the increase in cellular DNA concentrations is the initiation of genome replication during the dark. Replication has been demonstrated by the continuous incorporation of radiolabeled thymine into synchronized cultures of *Synechococcus* sp. strain PCC 6301 for up to 8 h after reentering the dark (60). The initiation of DNA replication is presumed to be either inhibited (16, 24) or aborted (46) in the absence of light. The initiation of genome replication is, by inference, a light-dependent reaction. Cellular nucleic acids concentrations were measured during diel growth by two sampling regimes in our study. The first regime briefly exposed cultures to light (<10 min) during sampling in the dark portion of the diel cycle. This regime raised concerns that brief exposure to light may have allowed a portion of the population to initiate genome replication during the dark. This concern was addressed by a second sampling regime in which all culture manipulations were performed in the dark during the dark phase of the diel cycle. Both regimes produced similar cellular DNA concentrations and the same trend of increasing cellular DNA concentration during the dark. These results demonstrated that brief exposure to light does not significantly perturb cell cycle events.

A second possible explanation for the increase in cellular DNA concentrations is the initiation of genome replication shortly before entering the dark and continued DNA synthesis throughout the dark period of a diel cycle. Continuous DNA synthesis throughout the dark period would imply a genome replication time (C) in excess of 12 h. A replication time of 12 h is significantly longer than the 65 min C period estimated by Mann and Carr (45) from shift-up experiments with asynchronous cultures of *Synechococcus* sp. strain PCC 6301. A difference in C periods would indicate that genome replication times vary between light and dark periods or with growth rate. Variations in genome replication times are not without precedence in the literature (4, 5). Genome replication times have been reported to vary between 60 min and 4 h for cultures of *Synechococcus* sp. strain

PCC 6301 (24, 46, 60). DNA synthesis has been reported to "run-out" within 3 h of placing a synchronous culture of rapidly growing *Synechococcus* sp. strain PCC 6301 in the dark (6). Synchronous cultures of *Synechococcus* sp. strain PCC 6301 with generation times of 8 h were reported to have DNA synthesis periods of up to 8 h (3). The long replication time reported by Asato and Folsome would be consistent with our observations of long C period in slowly growing cultures of *Synechococcus* sp. strain PCC 6301. At present we cannot distinguish between a slow DNA synthesis rate and initiation of DNA synthesis during the dark.

It is unlikely that the increase in cellular DNA concentrations can be explained by an increase in plasmid copy number. *Synechococcus* sp. strain PCC 6301 contains two cryptic plasmids of approximately 48.9 kb and 7.9 kb (42, 57, 66). The approximately 2.8 fg·cell^{-1} increase in cellular DNA would require the cell to carry more than 53 copies of the largest plasmid, an event that seems improbable.

The pattern of decreasing cellular nucleic acids concentration during light periods and increasing cellular nucleic acids concentrations during dark periods is in part a consequence of the dynamics of the growth rate of the cyanobacteria population. Population density increased exponentially when exposed to light, but was arrested immediately upon entering the dark phase of a diel cycle (Figure 2.3). The immediate arrest of cell division has also been observed in *Synechococcus* sp. strain PCC 6301 (22, 46), *Synechococcus* sp. strain WH 8101 (2) and *Oscillatoria agardhii* (67) in the absence of light. The immediate arrest of cell division contrasts with the increase in population size observed in *Synechococcus* sp. strain WH 8101 and *Synechococcus* sp. strain WH 7803 during the first 3 h of darkness (5, 6, 68). This contrast may be explained by the longer generation times of *Synechococcus* sp. strain WH 8101 and *Synechococcus* sp. strain WH 7803 in these experiments. Variability in the period of cell division (D) has been documented (8, 26) and is expected to be longer in slowly growing cells. A protracted D period may have allowed cell division that was initiated before entering the dark period to continue to completion in

the dark in slowly growing *Synechococcus* WH 8101 and *Synechococcus* WH 7803. The extended period of cell division would have resulted in an increase in cell density during the first few hours of darkness. Cell counts demonstrated that *Synechococcus* sp. strain PCC 6301 does not undergo reductive cell division during the dark such as the reductive division exhibited by heterotrophs during starvation (52). Furthermore, based on the constant optical density at 600 nm and 750 nm (data not shown) biomass did not appear to decrease as might be expected of heterotrohic bacteria during a downshift in nutrients. Population density increased without the noticeable 30 min to 4 h lag time that has previously been observed in *Synechococcus* sp. strain PCC 6301 (6, 24) and in marine *Synechococcus* sp. strain PCC 6301 (2, 68) emerging into the light from the dark. The lag period, however, may have been shorter than the sampling period (3 h) used in this study.

The cellular nucleic acids concentration of cyanobacteria correlates with growth rate under conditions of continuous illumination. This correlation is true regardless of whether growth rate is limited by available nutrients or light.

Superficially the periodic absence of a cyanobacterium's primary energy source (light) may be considered analogous to carbon-starvation in heterotrophic bacteria. Heterotrophic bacteria, such as *E. coli*, quickly degrade ribosomal RNAs when faced with starvation conditions (30, 31). Cyanobacteria, however, continue to accumulate ribosomal RNA despite being confronted by starvation conditions. Degradation of ribosomes would be detrimental in the long run for a photoautotroph faced with periodic, short-term starvation. The ability to retain high concentrations of active ribosomes allows *Synechococcus* sp. strain PCC 6301 to quickly resume exponential growth upon being reexposed to light (Figure 2.3).

The cyclical rise and fall of cellular RNA concentrations appear to be a consequence of initiation of genome replication and rRNA synthesis. *Synechococcus* sp. strain PCC 6301 contains two ribosomal RNA operons per genomes (*rrn*) (19, 50) and therefore contains approximately 4 rRNA operons-cell^{-1} at the beginning of the dark period and 6

rRNA operons-cell^{-1} at the end of the dark period. Although the cellular 16S rRNA concentration doubles by the end of the dark period, 16S rRNA molecules per rRNA operon only increase from approximately 2100 16S rRNA molecules per operon to 2780 16S rRNA molecules per operon. The transcription rate of rRNA operons, therefore, is nearly invariant based on the ratio of 16S rRNA molecules to 16S rRNA operons within the cell. This result supports the hypothesis that rRNAs are not transcriptionally regulated and that the correlation between cellular RNA concentrations and growth rate is due to gene dosage effects. Gene dosage effects have also been postulated to account for the observed correlation between growth rate and cellular ribulose-1,5-bisphosphate carboxylase/oxygenase concentrations (59). The implication of this hypothesis is that the growth rate is regulated or restricted by the DNA content of the cell. Cellular rRNA concentrations appear to be influenced primarily by genome copy number and gene dosage effects (50). Genome copy number and gene dosage effects may be one mechanism responsible for the expression of circadian rhythms in prokaryotes.

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Chapter 3

Concentrations of Nucleic acids of *Synechococcus* sp. strain WH 8103 During Growth in Continuous Light and Light:Dark Cycles

Abstract

Light-limited batch cultures of *Synechococcus* sp. strain WH 8103 exhibited a positive correlation between specific growth rate and the cellular concentrations of both RNA and DNA. Like heterotrophic bacteria, the ratio of RNA to DNA increased with growth rate in *Synechococcus* sp. strain WH 8103. Cellular RNA and 16S rRNA concentrations generally decreased during light periods and increased during dark periods in cultures entrained by a diel cycle. There was a significant increase in total cellular RNA concentrations and cellular 16S rRNA concentrations during the last 3h of the dark period that may indicate an increase transcription rates. The trend in cellular DNA concentrations during a diel cycle differed from that observed in *Synechococcus* sp. strain PCC 6301. Cellular DNA concentrations of *Synechococcus* sp. strain WH 8103 increased during the first half of the dark period before decreasing to basal levels during the later half of the dark period. A similar trend was observed during the light period of a diel cycle. The variation in cellular nucleic acids concentration during diel cycles may be in part a function of the timing of cell division, in addition to transcriptional regulation.

Introduction

Since their discovery in marine environments, synechococci (19, 40) have proven to be both ubiquitous and abundant (15, 19, 26, 40, 41). It has also been recognized that marine synechococci are significant contributors to primary production within the world's oceans (16, 26, 41).

There has been little investigation, however, in marine synechococci into the changes in cellular nucleic acids concentrations during diel cycles or accompanying changes in specific growth rates. Cellular nucleic acids concentrations are proportional to growth

rate in a number of heterotrophic bacteria (20, 21, 33) and both filamentous and unicellular cyanobacteria under constant illumination (23, 25, 28). Kramer (23) has demonstrated a correlation between total cellular RNA concentrations and specific growth rates in the marine cyanobacterium *Synechococcus* sp. strain WH 7803. Radiolabeled uracil incorporation demonstrated that stable RNA concentrations increased with growth rate in *Synechococcus* sp. strain WH 7803. The correlation between cellular 16S rRNA concentrations and growth rate presents the possibility of using probes complementary to 16S rRNA to determine *in situ* growth rates of marine synechococci, as has been done with sulfate-reducing bacteria in a biofilm (32).

The concentration and synthesis of nucleic acids have typically been studied in cyanobacteria grown under constant illumination followed by a single dark incubation period (1, 13, 14, 17, 18, 29, 36, 38). The same is true of the incorporation studies of Kramer (23) in *Synechococcus* sp. strain WH 7803. Growth under constant illumination, however, may not accurately reflect the physiological state of synechococci populations growing under the diel cycles found in the environment (12).

Another significant reason to study *Synechococcus* sp. strain WH 8103 is recent evidence by Binder and Chisholm (2) that synechococci display two distinct replication phenotypes. To date all studies of cellular RNA concentrations have been done with marine and freshwater synechococci exhibiting the asynchronous replication phenotype. These organisms contain even or odd numbers of complete genomes (eg, 3 or 4 genomes•cell^{-1}). The study of the variations in cellular RNA concentrations in *Synechococcus* sp. strain WH 8103 represents the first study of the variations in cellular RNA concentrations in a *Synechococcus* sp. exhibiting a synchronous replication phenotype (2). Organisms exhibiting a synchronous replication phenotype conform to the Helmstetter-Cooper model of genome replication (5, 9) and possess an even number of genomes.

In this study, we demonstrate that increases in cellular RNA concentrations and cellular DNA concentrations corelate with increases in growth rate in light-limited batch cultures of *Synechococcus* sp. strain WH 8103. Unlike *Synechococcus* sp. strain PCC 6301, the RNA/DNA ratio increased with growth rate in *Synechococcus* sp. strain WH 8103. It is argued that this increasing ratio is indicative of transcriptional control, primarily of the rRNA operons. The cellular concentration of total RNA and 16S rRNA in *Synechococcus* sp. strain WH 8103 displayed trends similar to those of *Synechococcus* sp. strain PCC 6301 during a diel cycle, except for an increase in last hours of the dark period that may also be indicative of up-regulation of transcription rates from the rRNA operons. The trends in cellular DNA concentrations in *Synechococcus* sp. strain WH 8103 during a diel cycle are also discussed in context of the trends observed in *Synechococcus* sp. strain PCC 6301.

Materials and Methods

Bacterial strains and culture conditions. *Synechococcus* sp. strain WH 8103 (provided by John Waterbury, Woods Hole Oceanographic Institute, Woods Hole, MA) was maintained at RT on SN liquid media (41) at 86 $\mu Em^{-2}s^{-1}$.

Synechococcus sp. strain WH 8103 batch cultures were grown at 25°C. Growth rate was varied by changing the photon fluence rate from 15 $\mu Em^{-2}s^{-1}$ to 53 $\mu Em^{-2}s^{-1}$ in light-limited batch cultures. Diel cultures were entrained on a 12 h light: 12 h dark diel cycle for at least seven generations prior to the start of each diel experiment at 64.4 $\mu Em^{-2}s^{-1}$.

Cell density was determined using a particle counter and channelyzer (Coulter Electronics Inc., Miami, FL). Cell volume was determined using a particle counter and

channelyzer (Coulter Electronics Inc.). Specific growth rates (μ; h^{-1}) of *Synechococcus* sp. strain WH 8103 in batch cultures were calculated from eq. 2.1.

Chemical determination of RNA and DNA. *Synechococcus* sp. strain WH 8103 cells were harvested by centrifugation of approximately $6x10^8$ cells from log-phase cultures at 16,000 x*g* for 25 min at 4°C. Cellular RNA concentration was measured by the orcinol assay (10), using adenosine monophosphate as a standard. Cellular DNA concentration was measured by the diphenylamine assay (10), using deoxyribose as a standard. Cellular RNA concentrations were estimated to be twice that of the AMP. Cellular DNA concentrations were calculated as 4.84 times the deoxyribose standard concentration based the difference between the average molecular weight of a nucleotide and the molecular weight of deoxyribose.

Nucleic acids isolation and hybridization. *Synechococcus* sp. strain WH 8103 cells were harvested by centrifugation of approximately $9x10^7$ cells of log-phase batch culture at 16,000 x*g* for 25 min at 4°C. Cells were disrupted mechanically and nucleic acids isolated (37). RNase contamination was reduced by using either virgin polypropylene or glassware treated with diethylpyrocarbonate (Sigma Chemical Co., St. Louis, MO) and baked at 240°C for 3 h.

Nucleic acids were hybridized with a cyanobacterial-specific oligonucleotide probe (4); this probe was complementary to the small subunit rRNA at positions 360-376 (*Escherichia coli* numbering). The cyanobacterial-specific probe was 5' end-labeled with γ-^{32}P-ATP (34) to a specific activity of ca. 0.5 μCi·pmol^{-1} probe. End-labeled probes were purified on TSK-DEAE columns (Supleco, Bellefonte, PA) equilibrated with 50 mM NH_4OAc (4).

Nucleic acids from batch cultures or chemostat cultures were denatured by the addition of phosphate-buffered gluteraldehyde [pH 7.0] to a final concentration of 1.5%

and incubated at RT for 15 min. Purified *Synechococcus* sp. strain WH 8103 16S rRNA (4) was denatured in the same manner and a standard curve constructed from 1 ng to 75 ng of 16S rRNA. *Escherichia coli* (*E. coli*) nucleic acids were denatured in the same manner and used to determine non-specific binding of the probe. Nucleic acids from experimental samples, 16S rRNA standards and *E. coli* were applied to MagnaCharge nylon membranes (Micron Separation Inc., Westbourgh, MA) using a slot-blot apparatus (Millipore, Bedford, MA). Approximately 1 μg, 500 ng and 250 ng from each experimental sample and *E. coli* were applied to nylon membranes. Nucleic acids were UV-crosslinked (Stratalinker 1800, Stratagene Inc., La Jolla, CA) to nylon membranes according to the manufacturer's instructions. Membranes were prehybridized for 30 min at 37°C in 10 ml hybridization buffer [6X SET (34), 0.5% SDS, 1X Denhardt's solution (34), 100 $\mu g \cdot ml^{-1}$ poly(A)], followed by hybridization for 14-16 h at 37°C in 10 ml hybridization buffer containing $1x10^6$ $CPM \cdot ml^{-1}$ ^{32}P-radiolabeled probe. Membranes were washed twice at RT and once at 48°C for 30 min each in wash buffer [2X SET, 0.5% SDS]. Standards and samples were quantified using a radioanalytical image system (AMBIS Inc., San Diego, CA).

Results

Cellular RNA and DNA concentrations of light-limited batch cultures. Differential growth rates were established for light-limited batch cultures of *Synechococcus* sp. strain WH 8103 by varying photon fluence rates. Continuous photon fluence rates of 15 $\mu Em^{-2}s^{-1}$ to 53 $\mu Em^{-2}s^{-1}$ resulted in growth rates of 0.014 h^{-1} to 0.037 h^{-1}. Cellular RNA and DNA concentrations were determined colorimetrically. Cellular RNA concentrations increased from 15.6±1.2 $fg \cdot cell^{-1}$ to 29.2±1.2 $fg \cdot cell^{-1}$ with increases in growth rate

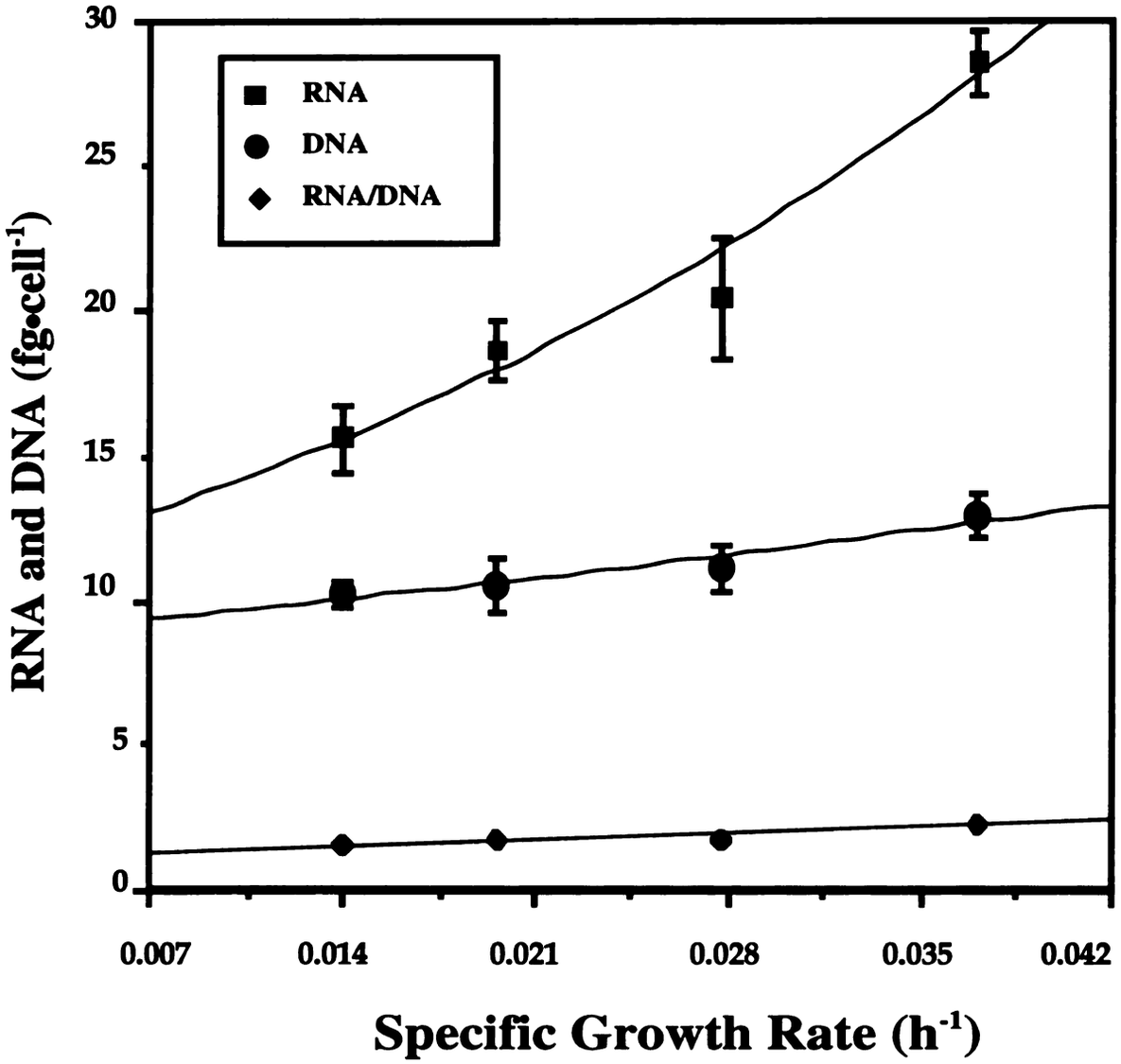

Figure 3.1 - Concentrations of nucleic acids of *Synechococcus* sp. strain WH 8103 from light-limited batch cultures. Error bars represent standard errors (n=3).

Table 3.1 - Physiological parameters of *Synechococcus* spp. strain WH 8103 in light-limited batch culture.

Growth Rate (h^{-1})	Total RNA ($fg \cdot cell^{-1}$)	Total DNA ($fg \cdot cell^{-1}$)	RNA/DNA[a]	cell vol. ($\mu m^3 \cdot cell^{-1}$)	RNA ($fg \cdot \mu m^3$)
0.014	15.6 ± 1.2	10.2 ± 0.5	1.53	0.330	47.3
0.019	18.6 ± 1.0	10.6 ± 0.9	1.75	0.360	51.7
0.028	20.4 ± 2.2	11.2 ± 0.8	1.82	0.364	56.0
0.037	29.2 ± 1.2	12.9 ± 0.8	2.26	0.390	74.9

(Figure 3.1). Cellular DNA concentrations increased from 10.2±0.5 fg·cell^{-1} to 12.91±0.8 fg·cell^{-1} with increasing growth rate (Figure 3.1). The ratio of RNA to DNA increased from 1.5 to 2.3 with increases in growth rate (Figure 3.1). The concentration of RNA increased on a per volume basis from 47.3 fg•μm^3 to 74.9 fg•μm^3 with increasing growth rate (Table 3.1).

Cellular RNA, 16S rRNA and DNA concentrations of diel batch cultures. Batch cultures of *Synechococcus* sp. strain WH 8103 were entrained in a 12 h light: 12 h dark diel cycle at 64.4 μEm^{-2}s^{-1}. Entrained cultures had a growth rate of 0.015 h^{-1} (Figure 3.2) over the course of a single diel cycle (24 h). Entrained cultures exhibited a marked decline in cell density 3 h after the beginning of the dark period. Cell density declined from 1.45x10^8 cells•ml^{-1} to 1.23x10^8 cells•ml^{-1} during this period; a decline of 15%. Entrained cultures did not exhibit a noticeable lag in growth upon being re-exposed to light and possessed a growth rate of 0.044 h^{-1} during the light periods of a diel cycle. There was a 30% drop in both cellular RNA and 16S rRNA concentrations during the first 3 h of the dark period (Figure 3.3). Both total cellular RNA and 16S rRNA concentrations gradually increased 18% over the following 6 h during the dark period. Transcription of RNA and 16S rRNA appeared to be up-regulated during the final 3h of the dark period; the concentration of both increasing 66%. Total cellular RNA concentrations increased to a high of 24.9±2.2 fg·cell^{-1} during the first few hours of the light period before gradually declining to 16.2±1.8 fg·cell^{-1} at the beginning of the next dark period (Figure 3.3A). Cellular 16S rRNA concentrations showed a similar increase during the first few hours of the light period, increasing to a high of 19.4±4.2 fg·cell^{-1}, but then dropped precipitously to 7.1±3.3 fg·cell^{-1}; a 60% decrease (figure 3.3B). Cellular 16S rRNA concentration appeared to increase slightly over the following 6 h to 9.1±2.4 fg·cell^{-1} at the beginning of the next dark period.

Cellular DNA concentrations exhibited a trend that was nearly the opposite of that exhibited by cellular RNA concentrations and cellular 16S rRNA concentrations. Cellular DNA concentrations gradually increased from 4.1±0.4 fg•cell^{-1} to 6.7±0.3 fg•cell^{-1} during the first 6h of the dark period (Figure 3.4). This increase corresponds to an increase in genome copy number from approximately 1 to 1.5 genomes•cell^{-1} (2). Cellular DNA concentrations gradually fell to 4.0±0.7 fg•cell^{-1} during the final 6h of the dark period. Unlike the trend observed in cellular RNA concentrations, cellular DNA concentrations increased gradually over the first 9h of the light period, to 5.3±0.1 fg•cell^{-1}, before decreasing to 4.2±0.1 fg•cell^{-1} at the beginning of the next light period.

Discussion

Light-limited, batch cultures of *Synechococcus* sp. strain WH 8103 exhibited a correlation in both cellular RNA and DNA concentrations with increases in growth rate. The correlation between RNA, DNA and growth rate observed in *Synechococcus* sp. strain WH 8103 have been observed in both heterotrophic bacteria (20, 21, 33) and cyanobacteria (23, 25, 28). Both cellular RNA and DNA concentrations were approximately half those of *Synechococcus* sp. strain PCC 6301 at comparable growth rates (Figure 2.1 and 3.1). Total cellular RNA concentrations for *Synechococcus* sp. strain WH 8103, however, were significantly higher than *Synechococcus* sp. strain PCC 6301 on a per volume basis (Table 2.1 and 3.1). The high per volume RNA concentrations in *Synechococcus* sp. strain WH 8103 were more comparable to the concentrations observed in *E. coli* than the concentrations observed in *Synechococcus* sp. strain PCC 6301.

Synechococcus sp. strain WH 8103 possessed one genome equivalent at slow growth rates (2) and were comparable to those of *Synechococcus* sp. strain PCC 6301 at low growth rates. However, cellular DNA concentrations did not increase as quickly with

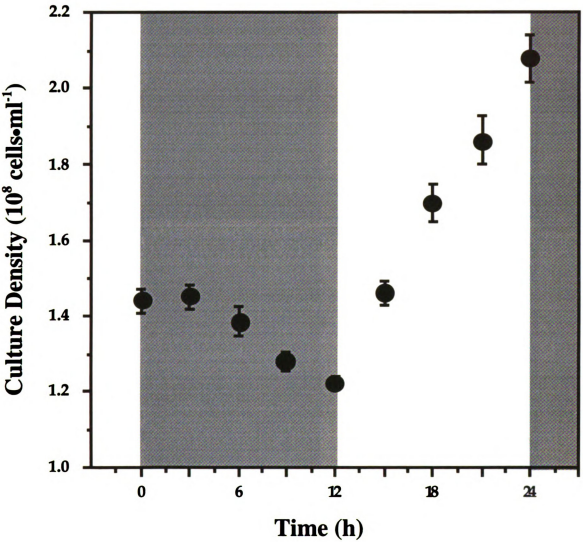

Figure 3.2 - Cell density of *Synechococcus* sp. strain WH 8103 during diel growth. Each day consisted of 12 h light: 12 h dark (shaded area). Error bars represent standard errors (n=6).

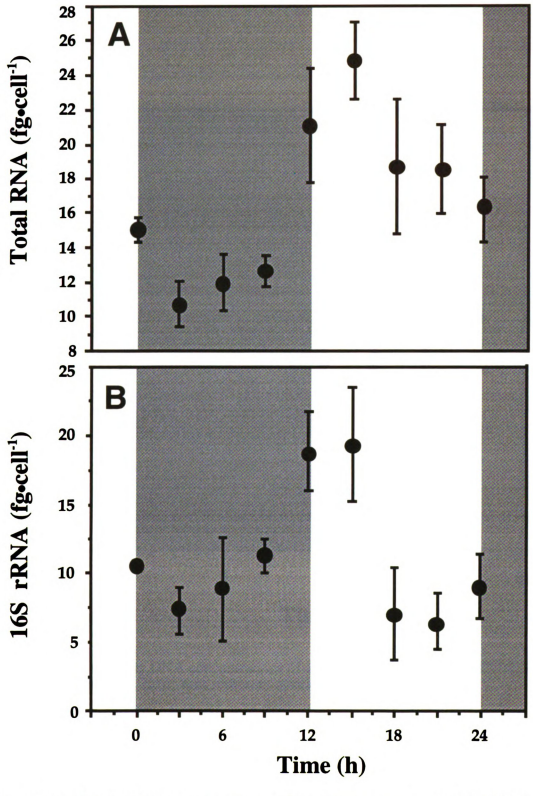

Figure 3.3 - Cellular RNA concentrations of *Synechococcus* sp. strain WH 8103 during diel growth. (A) Total cellular RNA concentrations.
(B) Cellular 16S rRNA concentrations. Error bars represent standard errors (n=4).

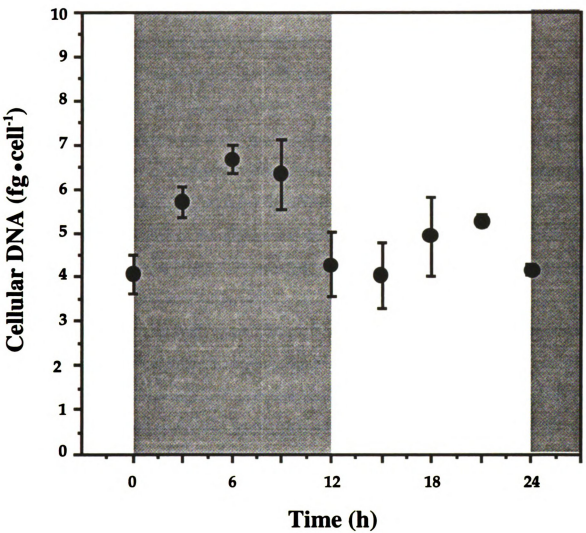

Figure 3.4 - Cellular DNA concentrations of *Synechococcus* sp. strain WH 8103 during diel growth. Error bars represent standard errors (n=6).

growth rate in *Synechococcus* sp. strain WH 8103 as in *Synechococcus* sp. strain PCC 6301. The ratio of RNA to DNA increased with growth rate in *Synechococcus* sp. strain WH 8103 as a result of the cellular RNA concentration increasing more rapidly than cellular DNA concentration (Figure 3.1). This increase in the RNA/DNA ratio is similar to increase in RNA/DNA ratio observed in heterotrophic bacteria (11, 20, 30, 33). The increasing RNA/DNA ratio in heterotrophic bacteria primarily reflects an increase in rRNA concentration resulting from increased transcription rates from the rRNA operons (31). The increase in RNA/DNA ratios in *Synechococcus* sp. strain WH 8103 would appear to indicate that *Synechococcus* sp. strain WH 8103 is capable of increasing transcription rates from the rRNA operons. The increasing RNA/DNA ratios observed in *Synechococcus* sp. strain WH 8103 are unlike those of *Synechococcus* sp. strain PCC 3601 which exhibited constant RNA/DNA ratios (Table 2.1) and is arguably incapable of regulating transcription rates from its rRNA operons (7, 27). .

As in *Synechococcus* sp. strain PCC 6301, understanding the diel variations in the concentrations of nucleic acids in *Synechococcus* sp. strain WH 8103 is a required first step in using rRNA concentrations to determine *in situ* growth rates. The variation in cellular nucleic acids concentrations were examined in batch cultures of *Synechococcus* sp. strain PCC 8103 entrained in a 12 h light: 12 h dark diel cycle and possessing a diel growth rate of 0.015 h^{-1}.

Total cellular RNA and 16S rRNA concentrations decreased by 30% during the first 3 h of dark periods (Figure 3.3). This decrease in cellular RNA concentrations does not appear to be due to cell division because cell division was arrested during dark periods. The transient decrease in cellular RNA concentrations during the first 3 h of darkness is similar to that observed in *Synechococcus* sp. strain PCC 6301 and probably originates from a decreases in light-specific transcripts (8, 22). Total cellular RNA and 16S rRNA concentrations increased approximately 18% over the following 6 hours of the dark period. This increase is similar to the 33% increase observed in *Synechococcus* sp. strain PCC

6301 over a 9 h period (Figure 2.4) and the 22% increase in *Synechococcus* sp. strain PCC 6301 in the dark observed by Herdman *et al* (18). It is reasonable to assume that this increase is due to dark specific transcripts, such as those encoding glycogen phophorylase and glucose-6-phosphate dehydrogenase, as has been documented in *Synechococcus* PCC 6301 (35). The concentration of both total cellular RNA and 16S rRNA increased by 66% during the final 3h of the dark period. This increase is likely a consequence of up-regulation of transcription rates from the rRNA operons. This up-regulation supports the hypothesis drawn from the increase in RNA/DNA ratios, that *Synechococcus* sp. WH 8103 is capable of transcriptional regulation of the rRNA operons. The gradual increase in 16S rRNA concentrations during dark periods followed by a dramatic increase in 16S rRNA concentrations is similar to the trend observed *in situ* for marine synechococci by Kramer and Singleton (24) using a 1.5-kb probe. The rapid increase in total cellular RNA and 16S rRNA shortly before light periods would be expected of an organism exhibiting a circadian rhythm. Both total cellular RNA and 16S rRNA concentrations increased slightly during the first few hours of the light period, but were followed a 60% drop in concentration over the succeeding 3h. This decrease may be partially explained by increases in cell density that exceed transcription rates, as was seen in *Synechococcus* sp. strain PCC 6301. Cellular concentrations of total RNA gradually decreased over the course of the light period and entered the next dark period at a concentrations comparable to that observed at the beginning of the first dark period. Cellular 16S rRNA concentrations remain moderately stable over the final 6h of the light period. The large drop in 16S rRNA followed by relatively stable 16S rRNA concentrations may represent a down-regulation in rRNA transcription rates supporting the interpretation that increasing RNA/DNA ratios are indicative of transcriptional regulation.

Cellular DNA concentrations exhibited a trend that was nearly the opposite of that exhibited by cellular RNA concentrations and cellular 16S rRNA concentrations. Genome copy number increased from approximately 1 to 1.5 genomes•cell^{-1} (2) during the first 6h

of the dark period (Figure 3.4). This trend was similar to that observed in *Synechococcus* sp. PCC 6301 (Figure 2.5) and like *Synechococcus* sp. strain PCC 6301 the mechanism driving the increase in DNA concentration may be the initiation of replication before entering the dark period and continued DNA synthesis during the dark period. Genome copy number in *Synechococcus* sp. strain WH 8103 was half that observed in *Synechococcus* sp. strain PCC 6301 and is consistent with the findings of Binder and Chisholm (2). Binder and Chisholm (2) found that *Synechococcus* sp. strain WH 8103 possessed 1 to 2 genomes•cell^{-1} and regulated replication in a manner consistent with the Helmstetter-Cooper model (5, 9). *Synechococcus* sp. strain PCC 6301 possessed between 2 and 6 complete genomes resulting from an asynchronous genome replication phenotype (3). Cellular DNA concentrations decreased during the final 6 h of the dark period. This decrease was unlike the decrease during light periods observed in *Synechococcus* sp. PCC 6301 where the decrease in cellular DNA concentrations appeared to be due cell division. The decrease in cellular DNA concentration in *Synechococcus* sp. WH 8103 occurred during the dark period, when cell division was arrested, and therefore the decrease could not originate from the same mechanism as in *Synechococcus* sp. PCC 6301. The decrease in cellular DNA concentrations in *Synechococcus* sp. strain WH 8103 did coincide with a noticeable decrease in cell density during the later half of the dark period and will be discussed later. Cellular DNA concentrations increased from a mean of 1 genome•cell $^{-1}$ to 1.25 genome•cell^{-1} during the first 9h of the light period. Again the trend observed *Synechococcus* sp. strain WH 8103 contrasted with the trend of decreasing cellular DNA concentrations observed in *Synechococcus* sp. strain PCC 6301 during the light periods of a diel cycle. It appears as if the rate of replication exceeded the growth rate of the population resulting in a temporary increase in cellular DNA concentrations. The increased DNA concentrations may precede the peak percentage of dividing, which occurs shortly before entering dark periods most *Synechococcus* spp. (2, 6). Cellular DNA concentrations then decrease to 1 genome•cell^{-1} before entering the next dark period.

As in *Synechococcus* sp. strain PCC 6301 the observed trends in nucleic acids concentrations in *Synechococcus* sp. strain WH 8103 may be in part a consequence the dynamics of population growth. Cell division appeared to be arrested shortly after entering the dark period (Figure 3.2). There was a significant decrease in cell density during the last 9 h of the dark period. This same decrease was noted by Binder and Chisholm (2) in *Synechococcus* sp. strain WH 8101, however, they offered no explanation for its origin. One possible explanation for the decrease in cell density is due to lysis by cyanophage. To date a large number of cyanophage from three families of DNA viruses (Myoviridae, Styloviridae and Podoviridae) have been isolated from marine *Synechococcus* spp. (39, 42). These cyanophage were isolated by enrichment from filtered seawater on various strains of marine synechococci and plaque purification. However, it is possible that temperate bacteriophage were resident within the synechococci population before the addition of filtered seawater and may be resident in many of the axenic marine *Synechococcus* spp. currently available. The replication of DNA viruses may account for some or all of the increase in cellular DNA observed during the first half of the dark period. Likewise, cell lysis by cyanophage may also account for the decrease in cellular DNA concentrations during the later half of the dark period. The technique used to measure cellular DNA concentrations required an initial wash and centrifugation of cell material in 1X SSC, a procedure that would have left the soluble DNA from lysed cells in the supernatant. Cell density increased linearly during the whole of the light period. A linear increase in cell density can arguably be observed in the results of Binder and Chisholm (2) and could be the result of cell lysis by cyanophage.

Comparison of the macromolecular composition and diel response of *Synechococcus* sp. strain WH 8103 with that *Synechococcus* sp. PCC 6301 reveals a number of fundamental differences between the two organisms. Most notably *Synechococcus* sp. WH 8103 exhibits an increase in RNA/DNA ratios with growth rate that is absent in *Synechococcus* sp. PCC 6301. *Synechococcus* sp. WH 8103 exhibits

trends in nucleic acids concentrations during diel cycles that are similar those observed in *Synechococcus* sp. PCC 6301 with the exception of what may be up-regulation of transcription rates shortly before entering the light period.

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Chapter 4

Use of 16S rRNA probes to determine nutrient-limitation or predator-limitation of the size of *Synechococcus* PCC 6301 populations

Abstract

Currently there is controversy whether *in situ* microbial population size is controlled primarily by available nutrients (bottom-up control) or by predation (top-down control). Prey-dependent models predict that as the availability of nutrients increase, prey populations not controlled by predation will respond by increasing the size of the population without a concurrent increase in the mean growth rate of the population. Likewise, populations controlled by predation are predicted to respond to an increase in available nutrients by an increase in growth rate without a concurrent increase in population size, as a result of an increase in the predator population size. Models of predator-prey interactions were applied to a two tier trophic system consisting of *Synechococcus* PCC 6301 prey and a *Tetrahymena pyriformis* predator in chemostat culture. *Synechococcus* PCC 6301 responded to increases in available nutrients by increasing equilibrium population size without a concurrent increase in growth rate in the absence of a *Tetrahymena pyriformis* predator. Addition of a *Tetrahymena pyriformis* predator increased the growth rate of the synechococci population 7- to 10-fold, as measured by cellular 16S rRNA concentration, while simultaneously decreasing the synechococci population size by one-seventh. Increases in nutrients to the two-tier trophic system were not consumed by the synechococci population because *Synechococcus* had already achieved maximal growth rates. Clearance rates of synechococci by *T. pyriformis* were comparable to previous reports. This work demonstrates that group-specific 16S rRNA probes can be used to distinguish between control of population size by nutrient-limitation or predation.

Introduction

The contribution of bacteria to oceanic production via the microbial loop (3, 16) has become well document in the last decade and is now recognized as an intregal part of

the oceanic food web (67). Picophytoplankton, photoautotrophic organisms 0.2 μm to 2 μm in diameter, are responsible for a significant percentage of the photosynthetic activity in the open ocean and are often numerically dominated by members of the genus *Synechococcus* (23, 26, 50, 83). This dominance may be due to high substrate affinity and small size that makes *Synechococcus* extremely efficient at nutrient uptake and a key link in micronutrient recycling and trophic transfer in pelagic systems (8, 39). The highly efficient nutrient uptake and the possibility of fixing nitrogen (38) would seem to indicate that *Synechococcus* is rarely nutrient limited in the natural environment.

Despite the advantages in oligotrophic environments afforded by small size and high affinity for scarce nutrients, there is substantial support in the literature for adopting the position that *Synechococcus* populations are often substrate limited. *Synechococcus* spp. may at times be limited for light (57), trace metals (11, 12, 59), in particular Fe^{2+} (4, 25, 30, 54, 55), nitrogen (27, 60), and phosphorus (43, 46, 80, 84).

However, the evidence for nutrient-limitation of *Synechococcus* is far from conclusive and does not preclude the possibility of predator control. One of the foremost tenants of ecological theory is that predation can be a major factor in controlling population size (32, 33, 49, 62). An inverse correlation has been found between phytoplankton biomass and grazer density in lakes (60) and rivers (85). One would expect to find similar forces at work upon the *Synechococcus* in marine environments where microflagellates and ciliates are known predators (10, 21, 43, 46). *In situ* grazing coefficients for flagellates and ciliates specific to *Synechococcus* range from 0 to 0.2 h^{-1} (8, 10, 21). Grazing pressure can reportedly reduce the population of *Synechococcus* by 30 to 100% (8, 10). Another possible control mechanism is parasitism by *Synechococcus* specific bacteriophage, which can be considered predation. Viruses have been reported to reduce phytoplankton primary productivity by as much as 78% (34, 40, 76). However, more

recent work has demonstrated that natural populations of *Synechococcus* are largely phage resistant (82).

Although attempts at determining whether population size and growth rate of heterotrophic bacteria as a whole are controlled by nutrient-limitation (bottom-up forces) or predation (top-down forces) have been made (5), there have been few attempts to determine whether a specific-group or genus of bacteria is controlled by nutrient-limitation or predation; primarily due to technical limits of delimiting a specific group (66). To date, most investigations of microbial predator-prey interactions have been done on entire communities of heterotrophic bacteria. The trophic interactions of *Synechococcus* spp. are essential to understanding pelagic food webs (8). This work was designed to address the predator-prey interactions within a restricted group, the synechococci, and to demonstrate that 16S rRNA probes can be used to distinguish top-down from bottom-up control of any group for which a specific probe can be designed.

Materials and Methods

Strains and culture conditions. *Synechococcus* sp. strain PCC 6301 (ATCC 27144) was maintained at room temperature (RT) on BG-11 (1) agar at 86 $\mu Em^{-2}s^{-1}$. Batch cultures of *Synechococcus* sp. strain PCC 6301 were grown at 64.4 $\mu Em^{-2}s^{-1}$ in modified Cg (36) containing 0.25 μM to 20 μM K_2HPO_4. Maximal growth rate and K_s were determined from a double reciprocal plot of the initial growth rate of each phosphate-limited culture.

Tetrahymena pyriformis (ATCC 30039) was maintained at RT on rich media containing 5.0 $g \cdot L^{-1}$ protease peptone (Difco, Detroit, MI), 5.0 $g \cdot L^{-1}$ tryptone peptone (Difco, Detroit, MI) and 11 μM K_2HPO_4.

Chemostat cultures of *Synechococcus* sp. strain PCC 6301 were grown on phosphate-limiting Cg medium (37). Chemostats were illuminated at 162 $\mu Em^{-2}s^{-1}$ at 37°C and aerated at a rate of 40 $ml \cdot min^{-1}$ with 3% CO_2 (balance air). Phosphate-limited growth was measured at three different reservoir concentrations. Phosphate concentrations within the reservoirs were 5 μM, 10 μM and 15 μM K_2HPO_4. Growth rate was varied by changing the dilution rate from 20 $\mu l \cdot min^{-1}$ to 365 $\mu l \cdot min^{-1}$ in a 250 ml Bellco Spinner flask (Bellco Glass Inc., Vineland, NJ). The culture was considered to have reached a stable equilibrium after maintaining the same optical density at 750 nm and 600 nm for three volume changes of the culture vessel.

Phosphate concentration within each chemostat containing only *Synechococcus* PCC 6301 was calculated using the formula:

$$S = (K_s * D)/(\mu_{max} - D) \qquad (4.1)$$

The Fiske and Subbarow (22, 29) assay for inorganic phosphate was used to determine phosphate concentrations within chemostats.

Cell density was determined by microscopic direct counts (45) in a Petrohoff-Hausser chamber (Hausser Scientific Partnership, Horsham, PA) or using a particle counter and channelyzer (Coulter Electronics Inc., Miami, FL). Specific growth rates (μ; h^{-1}) of *Synechococcus* sp. strain PCC 6301 in batch cultures were calculated from eq. 2.1.

Tetrahymena pyriformis (*T. pyriformis*) was enumerated by stereoscopic counts of

thirty 100 µl aliquots taken from three 1 ml chemostat subsamples at each time point. Cell volume of *T. pyriformis* was calculated as an ellipsoid (13) from the equation:

$$V = (4/3)\pi w^2 l \qquad (4.2)$$

where w is one-half the width of the organism and l is one-half of the length of the organism. A value of 0.11 pg C·µm^{-3} was used for conversion of ciliate biovolume to carbon equivalents (75, 79).

Nucleic acids isolation and hybridization. *Synechococcus* sp. strain PCC 6301 cells were harvested by centrifugation of approximately 9x10^7 cells of log-phase batch culture or stable chemostat culture at 16,000 x*g* for 25 min at 4°C. Cells were disrupted mechanically and nucleic acids isolated (74). The RNA extraction efficiency was 65.7±2.3 % based on the comparison between whole cell orcinol assays and orcinol assays of extracted nucleic acids. RNase contamination was reduced by using either virgin polypropylene or glassware treated with diethylpyrocarbonate (Sigma Chemical Co., St. Louis, MO) and baked at 240°C for 3 h.

Nucleic acids were hybridized with a cyanobacterial-specific oligonucleotide probe (7); this probe was complementary to the small subunit rRNA at positions 360-376 (*Escherichia coli* numbering). The cyanobacterial-specific probe was 5' end-labeled with γ-^{32}P-ATP (70) to a specific activity of ca. 0.5 µCi·pmol^{-1} probe. End-labeled probes were purified on TSK-DEAE columns (Supleco, Bellefonte, PA) equilibrated with 50 mM NH_4OAc (7).

Nucleic acids from batch cultures or chemostat cultures were denatured by the addition of phosphate-buffered gluteraldehyde [pH 7.0] to a final concentration of 1.5%

and incubated at RT for 15 min. Purified *Synechococcus* sp. strain PCC 6301 16S rRNA (7) was denatured in the same manner and a standard curve constructed from 1 ng to 75 ng of 16S rRNA. *Escherichia coli* (*E. coli*) nucleic acids were denatured in the same manner and used to determine non-specific binding of the probe. Nucleic acids from experimental samples, 16S rRNA standards and *E. coli* were applied to MagnaCharge nylon membranes (Micron Separation Inc., Westbourgh, MA) using a slot-blot apparatus (Millipore, Bedford, MA). Approximately 1 mg, 500 ng and 250 ng from each experimental sample and *E. coli* were applied to nylon membranes. Nucleic acids were UV-crosslinked (Stratalinker 1800, Stratagene Inc., La Jolla, CA) to nylon membranes according to the manufacturer's instructions. Membranes were prehybridized for 30 min at 37°C in 10 ml hybridization buffer [6X SET (70), 0.5% SDS, 1X Denhardt's solution (70), 100 $\mu g \cdot ml^{-1}$ poly(A)], followed by hybridization for 14-16 h at 37°C in 10 ml hybridization buffer containing $1x10^6$ $CPM \cdot ml^{-1}$ ^{32}P-radiolabeled probe. Membranes were washed twice at RT and once at 48°C for 30 min each in wash buffer [2X SET, 0.5% SDS]. Standards and samples were quantified using a radioanalytical image system (AMBIS Inc., San Diego, CA).

Results

Kinetics of phosphate-limited growth in *Synechococcus* sp. strain PCC 6301. Batch cultures of *Synechococcus* PCC 6301 were grown at 64.4 $\mu Em^{-2}s^{-1}$ in Cg media (36) containing 0.25 µM to 20 µM K_2HPO_4. The maximal specific growth rate approached 0.13 h^{-1} (Fig. 4.1A), in close agreement with maximum specific growth rate reported by Ihlenfeldt and Gibson (36). The K_s was calculated to be 0.55 µM K_2HPO_4.

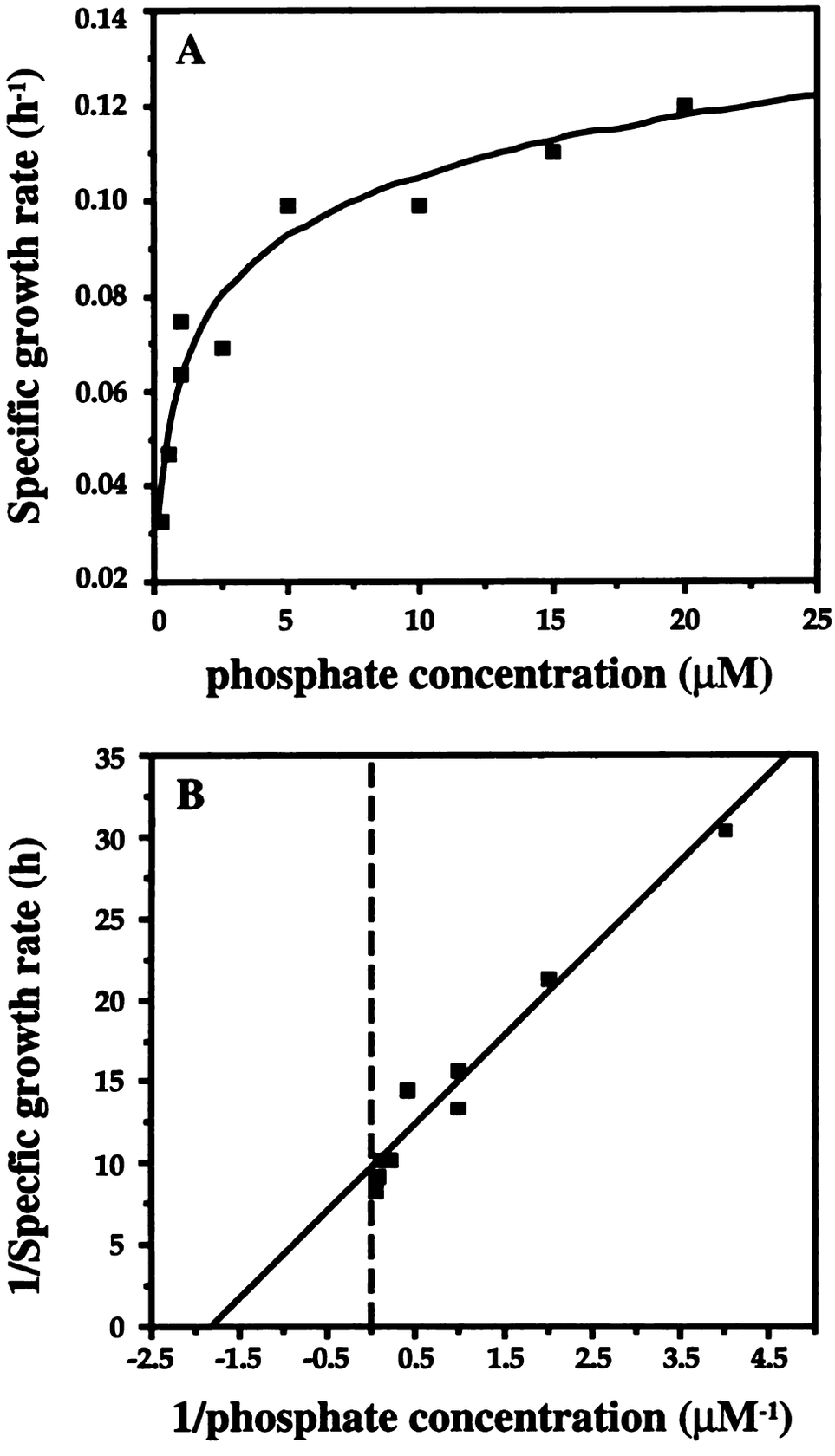

Figure 4.1 Kinetics of phosphate utilization by *Synechococcus* sp. strain PCC 6301.

based on a double-reciprocal plot of growth rate and phosphate concentration (Fig. 4.1B) and agrees with the 0.5 μM K_m reported by Grillo and Gibson (31).

***Synechococcus* sp. strain PCC 6301 population size in phosphate-limited chemostats.** Dilution rates were varied from 0.005 h^{-1} to 0.086 h^{-1} in each of three chemostats fed by reservoirs containing 15 μM, 10 μM or 5 μM phosphate. The concentration of phosphate within each chemostat increased from 0.02 μM to 0.8 μM with increasing dilution rate and was independent of the phosphate concentration supplied by each reservoir (Table 4.1). The internal phosphate concentration for each chemostat was below the K_s at all but the highest dilution rate, which indicates that the growth of each culture was phosphate-limited. Chemostats supplied by 5 μM, 10 μM and 15 μM phosphate reservoirs resulted in mean equilibrium population sizes of 4.1×10^7 cells·ml^{-1}, 7.6×10^7 cells·ml^{-1}, and 1.4×10^8 cells·ml^{-1}, respectively (Fig. 4.2; Table 4.1). The equilibrium population size did not vary significantly for each reservoir phosphate concentration regardless of the dilution rate. However, extensible properties such as cell volume and mass varied with dilution rate (Table 4.1). Cell yield was approximately 8.4 mg cells·mg^{-1} phosphate.

***Synechococcus* sp. strain PCC 6301 population size under predation pressure by *Tetrahymena pyriformis*.** In the absence of predation, *Synechococcus* sp. strain PCC 6301 reached an equilibrium population size of approximately 1.4×10^8 cells·ml^{-1} in chemostats supplied by a 15 μM phosphate reservoir (Fig. 4.3; Table 4.2). The equilibrium population size of *Synechococcus* sp. strain PCC 6301 decreased to 1.9×10^7 cells·ml^{-1}, upon the introduction of *T. pyriformis* (Fig. 4.3). *T. pyriformis* was inoculated into the chemostat at an initial concentration on 250 cells·ml^{-1}. After an initial

Table 4.1 - Physiological parameters of *Synechococcus* sp. strain PCC 6301 grown in phosphate-limited chemostats.

Reservoir phosphate concentration	Dilution rate (h^{-1})	Chemostat PO_4 conc. (μM)[a]	Cell density ($cell \cdot ml^{-1}$)	Dry cell mass ($pg \cdot cell^{-1}$)	Cell volume (μm^3)
5 μM	0.005	0.02	4.9×10^7	ND[b]	ND[b]
	0.027	0.14	4.2×10^7	0.638±0.003	1.30
	0.086	0.80	3.3×10^7	0.791±0.004	1.60
10 μM	0.006	0.02	8.7×10^7	0.341±0.002	1.23
	0.020	0.08	6.6×10^7	0.440±0.002	ND[b]
	0.080	0.67	6.9×10^7	0.903±0.007	1.56
15 μM	0.012	0.05	1.4×10^8	0.241±0.003	1.20
	0.020	0.08	1.5×10^8	0.381±0.035	1.25
	0.030	0.14	1.1×10^8	0.566±0.045	1.34
	0.080	0.67	1.5×10^8	1.127±0.024	1.56

[a]Calculated from eq. 4.1
[b]Not Done

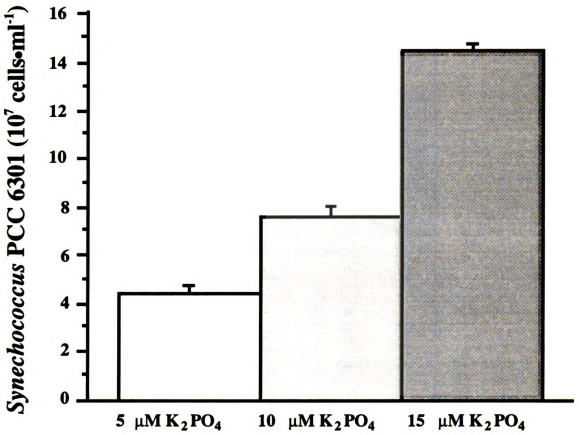

Figure 4.2 -*Synechococcus* sp. strain PCC 6301 mean population size in chemostats supplied by reserviors containing 5 μM, 10 μM and 15 μM K_2PO_4.

Table 4.2 - Physiological parameters of *Synechococcus* sp. strain PCC 6301 in the presence and absence of *T. pyriformis* predation.

	15 µM phosphate without *T. pyriformis*	15 µM phosphate with *T. pyriformis*	30 µM phosphate with *T. pyriformis*
Synechococcus **(cells•ml^{-1})**	14×10^7	1.9×10^7	2.0×10^7
T. pyriformis **(cells•ml^{-1})**	NA[a]	25.5 ± 3.3	25.1 ± 1.8
PO_4 (µM) calculated:	0.1	2.1	17.1
measured:	ND	8.6 ± 0.2	21.3 ± 2.1
Dilution rate (h^{-1})	0.012	0.012	0.012
Synechococcus **16S rRNA (fg•cell^{-1})**	6.5 ± 0.4	27.5 ± 4.6	23.0 ± 0.7
Calculated *Synechococcus* growth rate (h^{-1})[c]	0.016	0.157	0.127

[a]Not applicable
[c]Specific growth rate calculated from fig. 2.2.

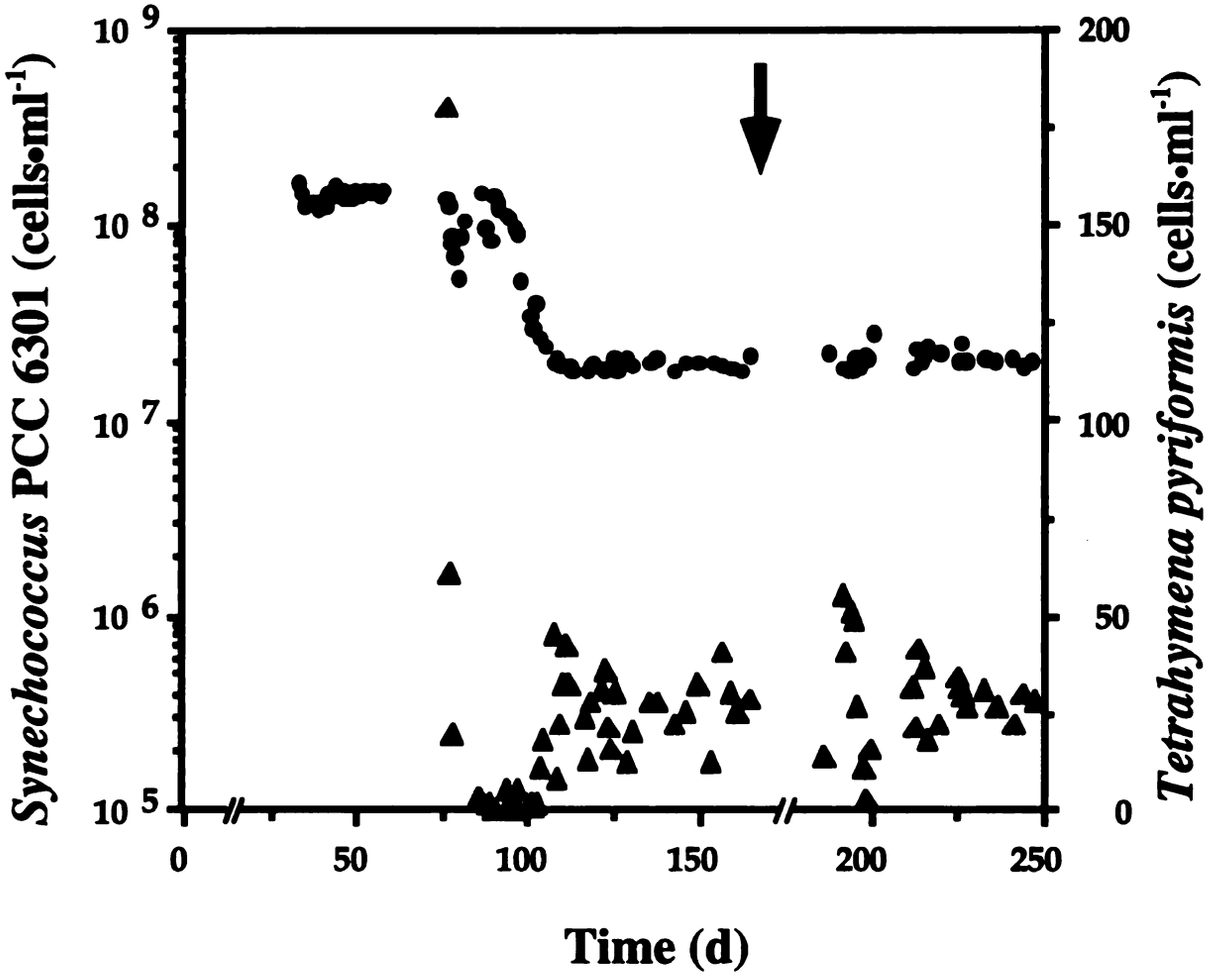

Figure 4.3. *Synechococcus* PCC 6301 population size with and without predation. *T. pyriformis* (▲) was inoculated at day 75. *Synechococcus* sp. strain PCC 6301(●) was established at a reservior phosphate concentration of 15 µM. Arrow indicates increase in reservoir phosphate concentration from 15 µM to 30 µM.

decrease, *T. pyriformis* reached an equilibrium population size of approximately 25 cells·ml^{-1} (Fig. 4.3; Table 4.2). An increase in the reservoir concentration to 30 µM phosphate did not result in an increase in the equilibrium size of either population (Table 4.2).

At equilibrium the grazing rate (g), of the *T. pyriformis* predator was calculated as described by Curds and Cockburn (13), from the difference between the potential cyanobacterial prey population size ($1.4x10^8$ cells·ml^{-1}) and the actual cyanobacterial prey population size ($1.9x10^7$ cells·ml^{-1}) multiplied by the dilution rate and divided by the *T. pyriformis* population size:

$$g = (D/P) \cdot (N_p/N_r) \qquad (4.3)$$

where D is the chemostat dilution rate, P is the *T. pyriformis* predator population size, N_p is the potential *Synechococcus* population size and N_r is the realized *Synechococcus* population size. The grazing rate of *T. pyriformis* was $9.6x10^4$ cyanobacteria·ciliate^{-1}· h^{-1}. A specific clearance rate of 5 µl·ciliate $^{-1}$·h^{-1} was calculated for each *T. pyriformis* by dividing the grazing rate of *T. pyriformis* by the standing crop of cyanobacerial prey.

T. pyriformis had an approximate body volume of $6.5x10^4$ µm^3 (Fig. 4.4) and cleared $1.05x10^5$ body volumes·h^{-1}. Based on biovolume the carbon content of *T. pyriformis* was calculated to be 2.53 ng C. *T. pyriformis* possessed a conversion efficiency of ca. 0.4%, assuming 0.21 pg C·*Synechococcus*$^{-1}$ (10, 83). The low conversion efficiency resulted in a *T. pyriformis* yield of $2.5x10^{-7}$ ciliate·cyanobacterium^{-1}.

Cellular 16S rRNA concentrations of *Synechococcus* sp. strain PCC 6301. *Synechococcus* sp. strain PCC 6301 had a cellular 16S rRNA concentration of 6.5±0.4 fg·cell^{-1} at a dilution rate of 0.012 h^{-1} (Table 4.2). There was a significant

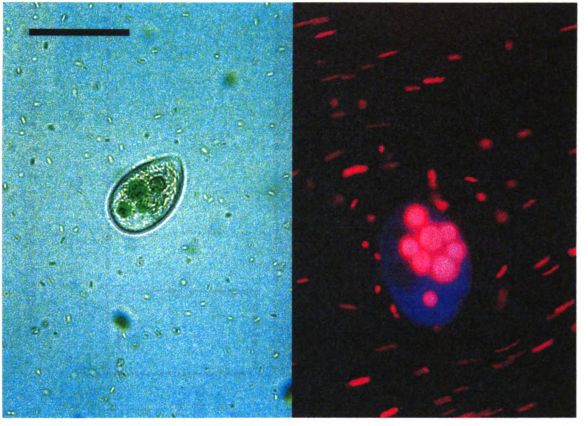

Figure 4.4 - *Tetrahymena pyriformis* grazing *Synechococcus* sp. strain PCC 6301. A) Phase contrast micrograph of wet mount. B) Epifluorescent micrograph of wet mount. Synechococci appear as red cells streaming past or within vacuoles of *T. pyriformis*, which appears blue. Bar represents 50 μm.

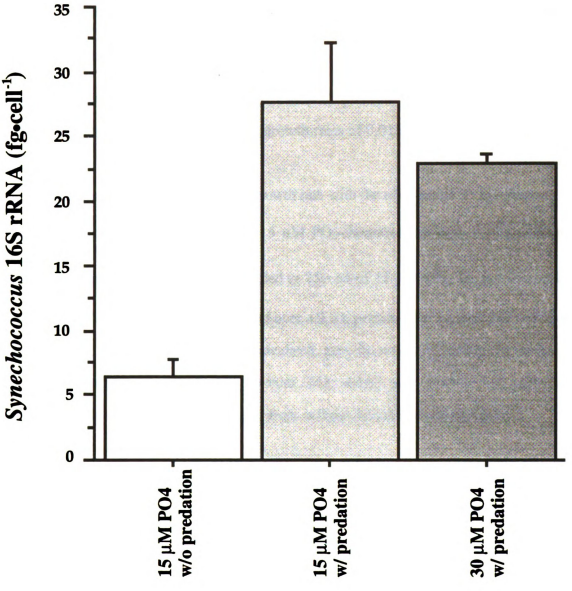

Figure 4.5 - Cellular 16S rRNA concentrations of *Synechococcus* sp. strain PCC 6301 in the presence and absence of predation by T. pyriformis. Chemostats were supplied by either 15 µM or 30 µM K_2PO_4 reserviors. Error bars represent standard deviations (n = 3).

($p<0.0001$) increase in *Synechococcus* sp. strain PCC 6301 cellular 16S rRNA concentration with the addition of a *T. pyriformis* predator; resulting in a cellular 16S rRNA concentration of 27.5±1.6 fg·cell^{-1} (Fig. 4.5; Table 4.2). Increasing the phosphate concentration within the reservoir to 30 μM did not result in a significant ($p>0.1$) increase in cellular 16S rRNA concentration compared to the chemostat supplied by a 15 μM phosphate reservoir (Fig. 4.5; Table 4.2). Based on previous results (Fig 2.2), these 16S rRNA concentrations corresponded to a growth rates of 0.016 h^{-1}, 0.157 h^{-1}, and 0.127 h^{-1}, respectively.

The increase in *Synechococcus* growth rate with the addition of *T. pyriformis* was tested by filtering an aliquot from the 15 μM PO_4-chemostat through a 10 μm filter to exclude the predator. The filtrate was added to 250 ml of 15 μM PO_4-Cg medium and the growth rate of *Synechococcus* determined over a 2.5 h period. The growth rate, calculated from eq. 2.1, was 0.12 h^{-1}. The calculated growth rate approaches the maximum *Synechococcus* growth rate on Cg medium (Fig. 4.1A) and confirms the increase in growth rates, due to predation, calculated from cellular 16S rRNA concentrations.

Chemostat phosphate concentrations with and without predation. In the absence of predation the chemostat phosphate concentration was calculated to be 0.1 mM phosphate. This concentration increased to 8.6±0.2 mM phosphate upon introduction of the *T. pyriformis* predator (Table 4.2). Increasing the phosphate concentration from 15 mM to 30 mM within the reservoir resulted in a corresponding increase in phosphate concentration to 21.3±2.1 mM within the chemostat, indicating the increased phosphate concentration was not utilized by the cyanobacteria for growth.

Discussion

Growth of a single trophic level.

Developments in ecological theory and models have lead many to the conclusion that naturally occurring populations are limited in size by either available nutrients or predation (32, 33, 49, 62). These two mechanisms can be mathematically modeled by coupled differential equations first proposed by Lotka and Volterra (51, 81):

$$dN/dt = \mu N - qN - gNP \qquad (4.4)$$

$$dP/dt = egNP - mP \qquad (4.5)$$

where N and P are the prey and predator populations at time t. The term μ describes the specific growth rate of the prey species. The term q describes the loss of individual organisms, which may be due to inherent mortality or efflux of the prey from the system. The predator grazing coefficient is described by g. The trophic function (eg) defines the conversion of consumed prey into predator and m the mortality rate of the predator.

Based on the traditional prey-dependent models (eqs. 4.4 and 4.5), there are two possible outcomes to increases in substrate concentration: 1) The prey population (N) increases in size, 2) The prey population (N) maintains the same density while the predator population (P) increases. Which of these outcomes prevails depends upon the number of trophic interactions within the system (24, 32, 33, 49, 62).

Under odd numbered trophic levels the controlling factor in prey population density is the availability of limiting substrate. In the simplest case of a single trophic level the community consists solely of the prey population (P = 0), reducing equations 4.4 and 4.5 to:

$$dN/dt = \mu N - qN \qquad (4.6)$$

Since the system is assumed to be in long term equilibrium dN/dt becomes zero (dN/dt = 0). The cellular growth rate of the individuals comprising the population is balanced by the loss of individuals from the system:

$$\mu N = qN \qquad (4.7)$$

Therefore, while an increase in available nutrients results in a increase in the equilibrium population size, the specific growth rate remains constant. In the absence of predation or free of predator control, bacterial populations tend to reach an equilibrium population dependent on the concentration of the limiting substrate. Such a population, typical of a food chain containing an odd number of links, is controlled by bottom-up forces. This situation is analogous to a chemostat in which the specific growth rate is set by the flow of the limiting nutrient through the system, but population size is set by the concentration of the limiting substrate (58, 61).

Before testing this hypothesis in chemostat cultures of *Synechococcus* sp. strain PCC 6301 it was necessary to determine the parameters governing phosphate-limited growth of *Synechococcus* sp. strain PCC 6301. *Synechococcus* sp. strain PCC 6301 was grown in batch culture supplemented with 0.25 μM to 20 μM K_2HPO_4 in order to establish the maximum growth rate and K_s of the organism on Cg media. The K_s was 0.55 μM (Fig 4.1B), in agreement with Grill and Gibson (31), and the maximum specific growth rate approached 0.13 h^{-1} (Fig. 4.1A). Using these parameters the phosphate concentration within phosphate-limited chemostats supporting the growth of *Synechococcus* sp. strain PCC 6301 was calculated.

The hypothesis that the concentration of available nutrients controlled population size was tested using nine single-stage chemostats fed by reservoirs of Cg media containing 5 μM, 10 μM, and 15 μM phosphate. Dilution rates of approximately 0.005 h^{-1}, 0.03 h^{-1}, and 0.08 h^{-1} were established in all three chemostats. The phosphate concentration within each chemostat was calculated (eq. 4.1) to be approximately 0.02 μM, 0.14 μM and 0.67 μM at each dilution rate, irrespective of the phosphate concentration supplied by the reservoir (Table 4.1).

As predicted for a microbial community consisting of a single or odd number of trophic levels, the community biomass increased with an increasing supply of limiting nutrient. The population size of *Synechococcus sp.* strain PCC 6301 increased by approximately $4x10^7$ cells·ml^{-1} for each 5 μM increase in phosphate supplied form the reservoir to each chemostat. The increase in population size was independent of the dilution rate of the chemostat (Figure 4.1). However, extensible properties such as cell volume and mass correlated with dilution rate (Table 4.1).

Similar increases in population size have been observed in nutrient addition experiments with natural assemblages of bacterioplankton and phytoplankton. Bottle enrichments from the Widdel and Scotia seas showed a sizable response by phytoplankton to iron additions, as measured by chlorophyll a concentrations (14). Kivi (43) observed a significant response of the phytoplankton population to NH_4+ addition to *in situ* enclosures in the Baltic Sea.

Nutrient limitation of phytoplankton population size has also been observed in a gradient of temperate lakes (56), the Mediterranean Ocean (78), and the Antarctic Ocean (15). Glover and colleagues (27) observed a bloom of synechococci concurrent with an increase in nitrate concentration, of unknown origin, in the Sargasso sea. Greene and colleagues (30) suggested that low intracellular iron concentrations limit photochemical

energy conversion efficiency, reducing both photosynthetic capability and growth rate. Such limitations were elegantly demonstrated in the IRONEX experiments in high-nitrate, low-chlorophyll (HNLC) waters off the coast of the Galapagos. A single *in situ* iron amendment simulated a 4-fold increase in primary production, a 3-fold increase in chlorophyll a concentrations, and a 2-fold increase in phytoplankton biomass, composed predominately of synechococci (54). A similar response was observed during sustained iron inputs during IRONEX II (4).

Control of population size by nutrient-limitation is not limited to photoautotrophic bacteria. Pace found that heterotrophic bacteria were nutrient-limited in two lakes in Michigan (63). Billen and colleagues (5) also observed a linear correlation between increased biomass and increased bacterial production and concluded that in general assemblages of heterotrophic bacteria were nutrient-limited. However, Billen *et al* (5) observed that biomass and population size were not proportional over the range of environments examined because different size classes of bacteria dominate different environments. They also concluded that these nutrient-limited populations had low growth rates (0.001-0.1), comparable to those used in this study.

Growth with two trophic levels.

According to traditional predator-prey theory, the composition of a food chain is dramatically altered by the addition of a predator species. The most significant result of this addition is the reduction of the equilibrium prey population referred to as top-down control (24, 32, 33, 49, 62). In an even numbered food chain such as this the predator species exhibits substrate-limited growth. Increases in available nutrients are transferred up the trophic chain to the predator population without changing the prey equilibrium population size.

This result can be seen chemostat cultures of *Synechococcus* sp. strain PCC 6301 to which a *T. pyriformis* predator had been added (Fig. 4.3). In the absence of predation *Synechococcus* sp. strain PCC 6301 reached an equilibrium population size of $1.4x10^8$ cells•ml^{-1} in chemostats supplied by a 15 μM phosphate reservoir (Table 4.2). The addition of *T. pyriformis* resulted in decrease of the *Synechococcus* sp. strain PCC 6301 prey population size to ca. $2x10^7$ cells•ml^{-1} (Fig 4.3). The predator population reached an equilibrium population size of 25 predators•ml^{-1} with a grazing rate (g) of $1.15x10^5$ synechococci• ciliate^{-1}•h^{-1} (eq. 4.3).

The population size of *T. pyriformis* is low compared to the greater than $1x10^3$ ciliates•ml^{-1} reported for ciliates raised on heterotrophic bacteria (10, 13, 79) and the 400 ciliates•ml^{-1} for the algivorous *Urotricha furcata* (73). Although ciliate concentrations in this study were low for laboratory cultures, they were typical of those of freshwater pelagic environments (6) and 10-fold higher than typical concentrations of ciliates observed in marine pelagic environments (21).

The low *T. pyriformis* population was probably due to the low nutrient value of *Synechococcus* PCC 6301 reflected by the low $2.5x10^{-7}$ ciliate•cyanobacterium^{-1} yield and a conversion efficiency of 0.4%. The yield and conversion efficiency of *T. pyriformis* raised on *Synechococcus* sp. strain PCC 6301 is low compared to the 2 to 54 % conversion efficiency reported for ciliates raised on heterotrophic bacteria (13, 69, 79). However, ciliates isolated from marine pelagic waters by Caron *et al*, exhibited cell yields that averaged 20% less when raised on synechococci compared to heterotrophic bacteria (10).

The grazing rate of *T.* pyriformis on synechococci is approximately 200-fold higher than the grazing rate (ca. 500 vibrio•ciliate•h^{-1}) reported for the smaller ciliate *Uronema marinum* (2). The grazing rate of *T. pyriformis* in this study is also significantly higher than the holotrichuous ciliate *Euplotes mutabilis*, with an approximate volume of $2x10^5$ μm^3, which has been reported to have grazing rates of 4.3 to $10.5x10^3$ bacteria•ciliate^{-1}•h^{-1}

on a bacterial population of $5x10^6$ to $30x10^6$ bacteria$\cdot ml^{-1}$ (79). The high grazing rates of *T. pyriformis* observed is most likely a product of the large size of *T. pyriformis* and the suspension feeding mode employed by the organism (19).

A specific clearance rate of 5 $\mu l \cdot ciliate^{-1} \cdot h^{-1}$ was calculated for each *T. pyriformis* by dividing the grazing rate of *T. pyriformis* by the standing crop of cyanobacerial prey. *T. pyriformis* had an approximate body volume of $6.5x10^4$ μm^3 (Fig. 4.4) and therefore cleared $1.05x10^5$ body volumes$\cdot h^{-1}$. This high clearance rate agrees with the high clearance rates observed in pelagic ciliates (71), and indicates that ciliates are capable of supporting population growth at the very low prey densities typical of oligotrophic systems ($1x10^6$ bacteria$\cdot ml^{-1}$) or on prey with low nutrient value, such as synechococci.

Although, microflagellates are considered the major predator of bacterioplankton (46), ciliates and cladocerans are also thought to contribute to bactivorous grazing, especially of cyanobacteria (10, 41, 71). Cyanobacteria with a mean cell volume of 0.5 μm^3 *in situ* are more likely to be effectively grazed by ciliates than smaller (0.07 μm^3) heterotrophic bacteria (19, 20, 71). Grazing coefficients of protozoan on synechococci have been estimated to range from 0 to 0.83 d^{-1} and estimated to consume 22-100% of synechococci production (8, 10).

Unfortunately, prey-dependent models of predator-prey interactions could not be distinguished from ratio-dependent models of predator-prey interactions in the *Synechococcus* - *T. pyriformis* system used in this study because the low conversion efficiency and high grazing rate did not allow either population to respond to increases in available nutrients. Likewise, nutrients could not be lowered without the predator population going extinct from washout.

Macromolecular response under single and multiple trophic levels.

The growth rate of the cyanobacteria prey population increased in order to maintain the population the under intense grazing pressure by the *T. pyriformis* predator population. The growth rate of the predator was governed by the dilution rate of the chemostat and ultimately by the flux of phosphate through both trophic levels.

In order to maintain a prey population at equilibrium, the specific growth rate (μ) of the prey population increased in order to balance the increase in grazing pressure resulting from a larger predator population. Assuming that cellular losses (q) are negligible compared to the grazing rate of the predator population and the prey population is at equilibrium ($dN/dt = 0$), then the cellular growth rate of the *Synechococcus* prey population can be described by the equation:

$$\mu = gP \qquad (4.8)$$

the terms of which were defined earlier.

One means of measuring changes in cellular growth rates is to monitor changes in macromolecular composition. Cellular nucleic acids concentration is proportional to growth rate in a number of heterotrophic bacteria (42, 44, 68). Nucleic acids concentration is also proportional to growth rate in both filamentous (47) and unicellular cyanobacteria (53) under constant illumination (Figure 2.1). The correlated increase in nucleic acids with increased growth rate appears to be a general physiological feature of cyanobacteria, as well as heterotrophic bacteria (42, 44, 68). The nucleic acids content of bacteria is predominately composed of rRNA and one-third of ribosomal rRNA (w/w) is composed of 16S rRNA. The correlation between cellular 16S rRNA concentrations and growth rate permitted the use of cyanobacterial-specific probes complementary to 16S rRNA to determine *in situ* growth rates (Figure 2.2).

The increased grazing pressure imposed by introduction of *T. pyriformis* on *Synechococcus* sp. strain PCC 6301 was accompanied be an increase in cellular 16S rRNA concentrations from 6.5 fg·cell^{-1} to 27.5 fg·cell^{-1} (Fig. 4.4). This 21 fg·cell^{-1} increase corresponded to an increase in growth rate from 0.016 h^{-1} to 0.15 h^{-1} (Figure 2.2), and represents a roughly 85 fg·cell^{-1} increase in total cellular RNA (52). The growth rate calculated from the cellular 16S rRNA concentration was near the maximal rate of 0.12-0.14 h^{-1} (Figure 4.1A) for *Synechococcus* sp. strain PCC 6301 growing on Cg medium (36).

The increase in specific growth rate of the *Synechococcus* population was also indicated by the increase in the phosphate concentration within the chemostat. The chemostat phosphate concentration increased from 0.1 μM to 8.6±0.2 μM upon the addition of the predator (Table 4.2). The 8.6±0.2 μM chemostat phosphate concentration was over 15-fold higher than the K_s of *Synechococcus* sp. strain PCC 6301 (Figure 4.1B). The high chemostat phosphate concentration supports the argument that the specific growth rate of the *Synechococcus* population was not limited by available nutrients and was near the maximum rate for Cg medium.

In order to confirm that the growth rate calculated from cellular 16S rRNA concentrations was near maximal, a subsample of the 15 μM chemostat was filtered through a 10 μm filter to remove the predator. The filtrate containing synechococci cells was used to inoculate Cg medium. The specific growth rate of the synechococci population was measured over a 2 h period, before any possible shift-up in growth rate could occur (53), and calculated to be 0.12 h^{-1} based on eq. 2.1 (Fig. 3.6; r^2=0.989).

The growth rate of autotrophic picoplankton was reported to increase from 0.08 h^{-1} and 0.2 h^{-1} due to protozoan grazing in the Mediterranean Sea (21); values comparable to those observed in this chemostat. However, the authors attributed increased growth rate to

utilization of protozoan excretions rather than grazing. Grazing coefficients (g) of 0 to 0.035 h^{-1} have been reported for protozoan subsisting on synechococci along the Atlantic coast (8) ; results that are comparable to those observed in these chemostat experiments.

Because the cyanobacterial population was growing near its maximum rate, any additional increase in available nutrients would not be expected to increase the population size of either the prey or the predator and consequently would not further increase the growth rate of the prey population. Increasing the available nutrient would result is an increase in the phosphate concentration within the chemostat that was unutilized by either population (77). The phosphate concentration was calculated to be 0.1 μM within chemostats containing only *Synechococcus* PCC 6301 and fed by a 15 μM phosphate reservoir. The chemostat phosphate concentration increased to 8.6±0.2 mM with the addition of the *T. pyriformis* predator. The chemostat phosphate concentration with *Synechococcus* sp. strain PCC 6301 growing near maximally can be calculated from a modification of an equation proposed by Thingstad (77), assuming no nutrient regeneration:

$$P_c = P_r - (\mu_{max} N / Y_N D) \qquad (4.9)$$

where P_c is the chemostat phosphate concentration, P_r is the phosphate concentration of the reservoir, μ_{max} is the maximum growth rate of the synechococci, N is the synechococci population size, Y_N is the synechococci yield and D is the dilution rate. The phosphate concentration within the chemostat was expected to be approximately 2.1 μM based on eq. 4.9. The 6.5 μM difference in phosphate concentrations may be attributable to phosphate regeneration by *T. pyriformis* . Increasing the reservoir phosphate concentration to 30 μM

resulted in an increase in the chemostat phosphate concentration to 21.3±2.1 μM, indicating that the additional phosphate was unutilized by either predator or prey population because the *Synechococcus* prey population was already growing at μ_{max}.

Nutrient regeneration

T. pyriformis is capable of excreting or regenerating phosphate resulting from nucleic acid degradation (48). The expected concentration of phosphate within the predator-limited chemostat was 2.1 μM based on eq. 4.9. The actual phosphate concentration within the chemostat was ca. 8.6 μM, indicating that *T. pyriformis* may have been responsible for regeneration of up to 6.5 mM phosphate. Nutrient regeneration by protozoa appears to be a key component of bacterioplankton and phytoplankton growth and maintenance in pelagic systems (9, 21, 28, 35, 65). Hutchins (35), for example, demonstrated that ^{59}Fe-labelled synechococci transferred iron to organisms >5 mm in size. The reciprocal experiment demonstrated that ^{59}Fe was returned to the <5 mm size fraction dominated by synechococci (35). Because not all of the available phosphate was utilized, additional organisms could have perhaps been supported in this system. The increase in available nutrients suggests that predation may led to an increase in diversity.

Multilevel trophic responses

Prey-dependent predator-prey models predict that the addition of a third trophic level results in the prey population (N_1) being released from predator control and the

population once again comes under substrate limited control. Under these conditions the predator population (P_2) comes under the control of its own predator (P_3) and the population density remains the same over a range of increasing potential productivity or nutrient increases. A crucial difference between one and three level trophic chains is that the predator continues to graze upon the prey population in a three tiered trophic system. According to this scenario an equilibrium prey population is maintained by the cellular growth rate which is proportional to the ratio of the third trophic level population to the prey population, the term m_jP/e_{ij} being constant.

$$\mu_1 = (P_3/N_1)(m_2P_2/e_{12}) \qquad (4.10)$$

Because P_3 is negligible compared to N_1, the cellular growth rate is nearly constant across a range of increases in potential primary productivity. Similar to the ecosystem composed of a single trophic level, the cellular growth rate remains nearly constant while the prey population increases in size. The result is a "stair step" effect, where cellular growth rate remains constant while the population increases alternating with plateaus of increasing cellular growth rate and constant population size (fig. 1.2).

The end result is that with the addition of each trophic level the bottom trophic level is alternately limited by available substrate or predator population, depending on whether there is an even or odd number of links in the chain (Fig. 4.6) (24, 32, 62). Examples of such a control mechanism were observed by Pace (63), who found that bacterioplankton population size was unaffected by manipulations of the top level predator, whereas the bacterioplankton predator was strongly affected by these manipulations.

Protozoan and microzooplankton appear to be a link to higher trophic levels by grazing heterotrophic bacteria, cyanobacteria and eucaryotic algae (64, 71). The rate of protozoan predation of bacteria is reportedly sufficient to balance net bacterial production

and limit bacterial population size (86). Protozoan grazing is believed to strongly influence bacterial community composition (18). However, the transfer of microbial biomass to higher trophic levels remains equivocal (17, 72).

This research demonstrates that group-specific probes complementary to 16S rRNA can be used to determine whether a particular population is controlled primarily by available resources (bottom-up control) or by predation (top-down control).

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Chapter 5

Evidence of predator-limited *Synechococcus* population size in the Gulf of Mexico based on indirect estimates of differences in growth rate in relation to population density

Abstract

Synechococcus is a significant contributor to global CO_2 fixation and often numerically dominates the phytoplankton component of a pelagic community. Predator-prey models predict that a nutrient-limited population will be larger and have slower specific growth rates than a population limited by predation. Since macromolecular composition is correlated with growth rate in synecococci it is possible to use specific oligonucleotide probes complementary to 16S rRNA to determine the relative differences in cellular growth rate between two populations. Synechococci population size and specific growth rates were examined at a number of oligotrophic and eutrophic stations in the Gulf of Mexico over a four year period. The distribution of population size versus specific growth conformed more closely to the predictions of simple predator-prey models than to those of ratio-dependent models. In addition, a significant portion of the synechococci examined appeared to be limited in population size by predation, in agreement with the reports of heavy grazing of pelagic synechococci populations.

Introduction

Cyanobacteria of the genus *Synechococcus* contribute from 2 to 46% of total primary productivity in coastal and oceanic waters (9, 23, 53, 42). *Synechococcus* population sizes range from $1x10^3$ cells ml^{-1} to $1x10^6$ cells ml^{-1} within the euphotic zone (6, 9, 5, 53, 63, 62). Beyond mere abundance, the ubiquitous nature of *Synechococcus* is evident from counts by epifluoresence microscopy of samples from the southern Pacific (62), the subartic Pacific (11), the tropical Atlantic (63, 9, 62) and the northern Atlantic (23, 6). Cloned 16S rRNA sequences have revealed *Synechococcus* spp. to be abundant in both the Pacific (56) and the Atlantic (19); results that are supported by flow cytometry

(42). Due to its high abundance and ubiquity, *Synechococcus* is undoubtedly an important contributor to the global carbon cycle.

The Louisiana-Texas shelf ecosystem is one region where anthropogenic inputs may have a profound effect on *Synechococcus* population size and dynamics. Nutrient loading from the Mississippi River into estuarine and coastal ocean environments have increased dramatically over the past half century. For example, nitrate concentrations in the lower Mississippi and carbon accumulation on the adjacent shelf have doubled since 1950 (47). A consequences of this nutrient-enhanced production is increased sedimentation of organic matter resulting in hypoxic zones in benthic environments (43).

Despite the significant contribution of *Synechococcus* to total primary production and marine community structure, little is known about the mechanisms controlling their population size. An understanding of the mechanisms which control *Synechococcus* populations is the first step in determining the rate of energy transfer to other trophic levels (23), micronutrient recycling, the role of synechococci in determining microzooplankton composition (7, 21) and the construction of future models of trophic interactions.

Synechococcus spp. may at times be limited for light (37), trace metals (11, 12, 38), in particular Fe^{2+} (4, 15, 18, 35, 36), nitrogen (17, 39), and phosphorus (23, 25, 48, 52). However, the evidence for nutrient-limitation of *Synechococcus* is far from conclusive and does not preclude the possibility of predator control. ***In situ*** grazing coefficients for flagellates and ciliates specific to *Synechococcus* range from 0 to 0.2 h^{-1} (9, 13, 23, 25) and can reduce *Synechococcus* production by 30 to 100% (7, 9). Predator-prey models are capable of distinguishing nutrient-limitation from predator-limitation by plotting equilibrium *Synechococcus* population density and the specific growth rate (Figure 1.2). One means of measuring changes in cellular growth rates is to monitor changes in macromolecular composition. Nucleic acids concentration is correlated to growth rate in both heterotrophic bacteria (22, 24, 44) and cyanobacteria (29, 34). The correlation between cellular 16S rRNA concentrations and growth rate permits the use of

cyanobacterial-specific probes complementary to 16S rRNA to determine *in situ* growth rates (Chapters 1 and 2).

To date, most investigations of microbial predator-prey interactions have been done on entire communities of heterotrophic bacteria. The trophic interactions of *Synechococcus* spp. is essential to understanding pelagic food webs (7). This work was designed to address the predator-prey interactions within a restricted group, the synechococci, and to demonstrate that 16S rRNA targetted probes can be used to distinguish top-down from bottom-up control for any microbial group for which a specific probe can be designed.

Materials and Methods

Sampling sites and protocol.

Synechococcus spp. populations were sampled at 12 stations in the Gulf of Mexico off the coast of Louisiana (Figure 5.1). The decrease in salinity is indicative of the influence of the nutrient rich waters of the Mississippi River (65).

Synechococci can be enumerated using epifluorescent counts following separation from other phycoerythrin containing organisms by size fractation of seawater samples. Seawater was initially collected, from a 1 m depth, through a 120 µm nylon mesh using a pneumatic pump (Wilden Pump & Engineering Co., Colton, CA) operating at less than 15 psi. The collected seawater was prefiltered through a 10 µm filter (Millipore Corp., Bedford, MA) before passing through a 1 µm filter (Micron Separations Inc., Westboro, MA). Aliquots (5 to 20 ml) of the final filtrate were concentrated on 0.22 µm black polycarbonate filters (Poretics Corp., Livermore, CA) for enumeration of the autofluorescent synechococci. Utilizing the autofluorescent properties of the

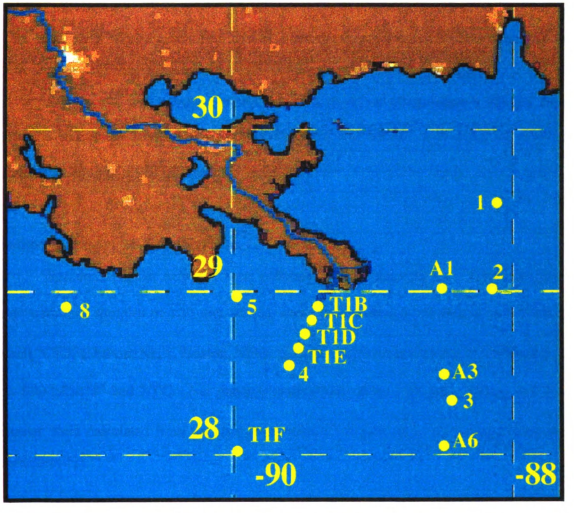

Figure 5.1 - Sampling sites within the Gulf of Mexico.

photosynthetic pigments, the *Synechococcus* population size was determined by direct epifluorescent counts of 300-400 cells distributed among 20 to 30 random fields using a Zeiss 48.77.09 filter set . Synechococci cells from 5 to 25 L of 1 μm filtrate were collected onto either 0.22 μm, 113 mm dia. polycarbonate membranes filter (Millipore Corp., Bedford, MA) at <15 psi or onto 0.22 μm Gelman filtration cartridges (Gelman Sciences, Ann Arbor, MI). Polycarbonate filters or filter cartridges were sealed in either zip-lock bags or with parafilm, immediately frozen and kept at -80°C until extracted.

Station T1f was sampled every 5 m to a depth of 80 m using a Niskin rosette sampler. Aliquots from station T1f enumeration and nucleic acid isolation were not prefiltered through a 120 μm mesh.

Estimated rates of CO_2 fixation.

Surface seawater samples were collected as described above. Triplicate 250 ml light and dark samples of 120 μm, 10 μm and 1 μm filtrates were spiked with 5-20 μCi $NaH[^{14}C]O_3$ (DuPont NEN, Boston, MA). Samples were incubated on board for 4-5 h at ca. 800 $\mu Em^{-2}s^{-1}$ and 20°C. Net primary productivity of the 120 μm, 10 μm and 1 μm filtrates were calculated from the change in cellular ^{14}C relative to ^{14}C incorporation from seawater (42).

Estimated specific growth rate and grazing coefficients by dilution culturing.

Seawater samples, from a 1 m depth, were passed through a 120 μm nylon mesh, as described above. Several liters of the 120 μm filtrate was filtered through a 0.2 μm filter (Micron Separations Inc., Westboro, MA) to remove microorganisms. Aliquots of the 120

μm filtrate were mixed with the 0.2 μm filtrate in ratios of 1:4, 1:10 or left undiluted. Each aliquot was divideded into triplicate 500 ml subsamples. Samples were incubated for 24 h on deck in ambient light and cooled by running seawater.

Synechococci cell density was determined by collection of 5 to 15 ml of each dilution subsample on black 0.22 μm polycarbonate filters and enumeration as described above. The rate of increase of synechococci population size for each dilution was calculated using the formula:

$$r = \mu\text{-}g = (\ln N_t - \ln N_0)/T \qquad (5.2)$$

where μ is specific growth rate, g is the mortality rate (due primarily to grazing), N_0 is the population size at time 0 and N_t is the population size at time T. Growth and grazing coefficients were determined by linear regression of the rate of increase in synechococci population size r at each dilution. Specific growth rate (k) was determined from the y-intercept and the grazing coefficient was equivalent to the slope of the regression.

Nucleic acid isolation and probing.

Nucleic acids were isolated from 113 μm Millipore filters by adding 5 ml lysis buffer (40 mM EDTA [pH 8.0], 400 mM NaCl, 0.75 M Sucrose, 50 mM Tris·HCl [pH 8.0]) and 1 mg·ml^{-1} lysozyme (Sigma Chemical Co., St. Louis, MO) and incubating for 20 min at 37°C. SDS (Sigma Chemical Co., St. Louis, MO) was added to 1% and 100 mg·ml^{-1} Proteinase K (Sigma Chemical Co., St. Louis, MO) followed by a second 20 min incubation at 37°C. Filters were then incubated at 55°C for 10 min and gently agitated. The lysate was removed and the filters washed with 2 ml TE (10 mM Tris·HCl [pH 8.0], 1 mM EDTA [pH 8.0]) and combined with the lysate. Lysates where extracted with an equal

volume of phenol (0.1 M Tris•HCl [pH 8.0] saturated), followed by a phenol:chloroform extraction and a chloroform extraction. Nucleic acids were precipitated by the addition of 0.1 volume 3 M sodium acetate (Sigma Chemical Co., St. Louis, MO), 3 volumes 100% EtOH and centrifugation at 16,000 xg for 2 h.

Nucleic acids were isolated from 0.22 µm Gelman filtration cartridges (Gelman Sciences, Ann Arbor, MI) by sealing the ends of the cartridge with size 000 rubber stoppers, Duraseal (DiversifieDBiotech, Boston, MA) and teflon clamps (Cole-Parmer, Vernon Hills, IL). A 60 ml syringe and gasket was used to force 10 ml 1X SSC (cold) through the outlet port. The 1X SSC filtrate was collected through the cartridge air vent. Another 10 ml 1X SSC (cold) containing 1% SDS was added through the cartridge air vent and the cartridge incubated at 90°C for 30 min in a hybridization oven (Hoefer Scientific Inst., SF, CA). The lysate was collected through the air vent and combined with the previous filtrate. Another 10 ml 1X SSC was forced through the outlet and combined with the previous lysates. Lysates where extracted with an equal volume of phenol (0.1 M Tris•HCl [pH 8.0] saturated), followed by a phenol:chloroform extraction and a chloroform extraction. Nucleic acids were precipitated by the addition of 0.1 volume 3 M sodium acetate (Sigma Chemical Co., St. Louis, MO), 3 volumes 100% EtOH and centrifugation at 16,000 xg for 2 h.

RNase contamination was reduced by using either virgin polypropylene or glassware treated with diethylpyrocarbonate (Sigma Chemical Co., St. Louis, MO) and baked at 240°C for 3 h.

Nucleic acids were hybridized with a cyanobacterial-specific oligonucleotide probe (5); this probe was complementary to the small subunit rRNA at positions 360-376 (*Escherichia coli* numbering). The cyanobacterial-specific probe was 5' end-labeled with g-^{32}P-ATP y to a specific activity of ca. 0.5 mCi•pmol^{-1} probe. End-labeled probes were

purified on TSK-DEAE columns (Supleco, Bellefonte, PA) equilibrated with 50 mM NH_4OAc (5).

Nucleic acids from batch cultures or chemostat cultures were denatured by the addition of phosphate-buffered gluteraldehyde [pH 7.0] to a final concentration of 1.5% and incubated at RT for 15 min. Purified *Synechococcus* sp. strain PCC 6301 16S rRNA (5) was denatured in the same manner and a standard curve constructed from 1 ng to 75 ng of 16S rRNA. *Escherichia coli* (*E. coli*) nucleic acids were denatured in the same manner and used to determine non-specific binding of the probe. Nucleic acids from experimental samples, 16S rRNA standards and *E. coli* were applied to MagnaCharge nylon membranes (Micron Separation Inc., Westbourgh, MA) using a slot-blot apparatus (Millipore, Bedford, MA). Approximately 1 mg, 500 ng and 250 ng from each experimental sample and *E. coli* were applied to nylon membranes. Nucleic acids were UV-crosslinked (Stratalinker 1800, Stratagene Inc., La Jolla, CA) to nylon membranes according to the manufacturer's instructions. Membranes were prehybridized for 30 min at 37°C in 10 ml hybridization buffer [6X SET (45), 0.5% SDS, 1X Denhardt's solution (45), 100 $mg \cdot ml^{-1}$ poly(A)], followed by hybridization for 14-16 h at 37°C in 10 ml hybridization buffer containing $1x10^6$ $CPM \cdot ml^{-1}$ ^{32}P-radiolabeled probe. Membranes were washed twice at RT and once at 48°C for 30 min each in wash buffer [2X SET, 0.5% SDS]. Standards and samples were quantified using a radioanalytical image system (AMBIS Inc., San Diego, CA).

Results and Discussion

Synechococci population size along nutrient gradients

Synechococci population density was determined by epifluorescent counts of surface water samples (<3m) taken along a nutrient gradient from the Gulf of Mexico (Figure 5.1). *Synechococcus* cell densities ranged from $2x10^2$ $cells \cdot ml^{-1}$ to $2x10^5$ $cells \cdot ml^{-1}$

(Tables 5.1-5.4). Synechococci cell densities in the surface waters of the Gulf of Mexico are comparable to those of previous reports (10, 16, 31, 32, 48-50). The 30 fold range in population densities in the Gulf of Mexico is comparable to both the seasonal range in cell densities observed in Woods Hole Harbor and along a 5400 mi north-south transect along the eastern coast of the Americas (50).

It has been proposed that *Synechococcus* spp. are at times variously limited for light (37), trace metals (11, 12, 38), in particular Fe^{2+} (4, 15, 18, 35, 36), nitrogen (17, 39), and phosphorus (23, 25, 48, 52). The decrease in light transmittance moving from oligotrophic stations, such as station 3, to the eutrophic stations heavily influenced by drainage from the Mississippi, such as station T1b, indicate that light is attenuated by silt and cell biomass from the Mississippi River. However, light attenuation does not appear to be a factor limiting synechococci population size since no correlation between percent transmittance and synechococci cell density was observed, results consistent with the findings of Lohrenz *et al* (33). Lorenz *et al* (33) were unable to find a correlation between the rate of carbon fixation in 10 μm seawater filterates and either salinity or *in situ* nitrate concentrations. Percent transmittance may also be used as an indicator of nutrient loading (6). The lack of correlation between percent transmittance and synechococci cell density is also an indication that synechococci population size is not nutrient-limited, a result consistent with the lack of correlation between primary production and nutrient loading noted by Lohrenz (33).

Rates of carbon fixation

It is commonly assumed that the specific growth rate of cyanobacteria is portional its rate of carbon fixation; and at least one method of determing specific growth rates of synechococci has been developed based on this assumption (50). However, recent evidence indicates that slow growing mats of cyanobacteria are capable of high rates of

Table 5.1 - Synechococci population density for October 27 to November 11, 1993.

Site	Percent Transmittance	Synechococci (10^4 cell·ml^{-1})	16S rRNA (fg·cell^{-1})	Carbon fixed (fg·cell^{-1}·h^{-1})
0.5	72	4.13	10.3	21.4
1	52	10.60	2.0	32.8
2	78	4.16	4.5	12.1
3	80	3.80	5.9	6.6
4	82	1.36	19.2	7.9
5	45	20.90	2.1	23.4

Table 5.2 - Synechococci population density for March 14 to March 20, 1994.

Site	Percent Transmittance	Synechococci (10^4 cell·ml^{-1})	16S rRNA (fg·cell^{-1})	Carbon fixed (fg·cell^{-1}·h^{-1})
0.5	39	4.60	6.8	137.6
1	61	1.94	1.8	21.6
2	49	3.00	14.1	48.4
3	71	4.89	0.6	0.9
4	54	8.78	4.9	136.2
5	39	2.90	14.0	121.6
A1	34	2.04	12.6	205.3
A3	75	1.87	3.4	148.8
A6	74	3.60	19.6	19.9

Table 5.3 - Synechococci population density for November 19, 1994 to November 22, 1994.

Site	Percent Transmittance	Synechococci (10^4 cell·ml^{-1})	16S rRNA (fg·cell^{-1})
3	92.6	0.36	3.9±0.2
5	43.6	1.39	11.4±0.3
8	55.0	1.23	19.5±2.9

Table 5.4 - Synechococci population density for February 16, 1996 to February 21, 1996.

Site	Percent Transmittance	Synechococci (10^4 cell·ml^{-1})	16S rRNA (fg·cell^{-1})
t1a	0.2	0.00	0.0
t1b	1.5	0.02	12.7±1.1
5 (2/16/96)	36.0	0.91	4.1±0.8
5 (2/22/96)	45.3	0.94	12.5±1.4
t1c	35.3	0.74	1.6±0.2
t1d	40.1	1.71	4.1±0.8
t1e	56.5	2.01	1.0±0.1
4	62.0	11.20	0.1±0.1
t1f	62.3	3.30	0.8±0.1

carbon fixation in the absence of high growth rates (40). This assumption was tested by measuring rates of carbon fixation attributable to synechococci and attempting to correlate these rates with cellular 16S rRNA concentrations, which has been correlated with growth rate (Chapters 1 and 2). The maximum photosynthetic capacity (P_{max}) of *Synechococcus* spp. was determined by shipboard incubation at 25°C and ca. 800 $mEm^{-2}s^{-1}$ in fall 1993, spring 1994, and fall 1994. Integrated sea-surface irradiance averaged 935 $mEm^{-2}s^{-1}$ from November 19 to November 20, 1993. Since mean surface irradiance was comparable to shipboard incubation irradiance, P_{max} measurement are believed to be an accurate estimate of *in situ* photosynthetic rates. P_{max} varied from 0.2 $fg{\cdot}cell^{-1}$ to 205.3 $fg{\cdot}cell^{-1}$ (Tables 5.1-5.4), rates comparable to *in situ* measurements from previous reports (16, 31, 50), further supporting the position that P_{max} estimates were an accurate reflection of *in situ* surface photosynthetic rates by synechococci. There was no discernible correlation between photosynthetic rates and nutrient loading of a particular sampling site, in agreement with the findings of Lohrenz (33).

Estimated growth rates by dilution culturing

In the fall of 1994 synechococci growth rates were determined at stations 3, 5 and 8, using the dilution method of Hasset and Landry (27, 28). *Synechococcus* spp. cellular 16S rRNA was also determined for each station. Of the three station examined only two, stations 5 and 8, exhibited a significant increase in growth rate by diluting out predation pressure. Instantaneous growth rates were 0.026, 0.48 and 3.4 d^{-1} at stations 3, 5 and 8, respectively (Figure 5.2). Grazing coefficients, presumably due to microflagellates (9) and ciliates (46), were 0.35 and 2.0 d^{-1} at stations 5 and 8. These grazing coefficients appeared to be capable of consuming approximately 75% of the daily net primary production of synechococci. Dilution culturing was not significantly correlated with growth rate at station 3, indicating that grazing was not a significant component of mortality within this population.

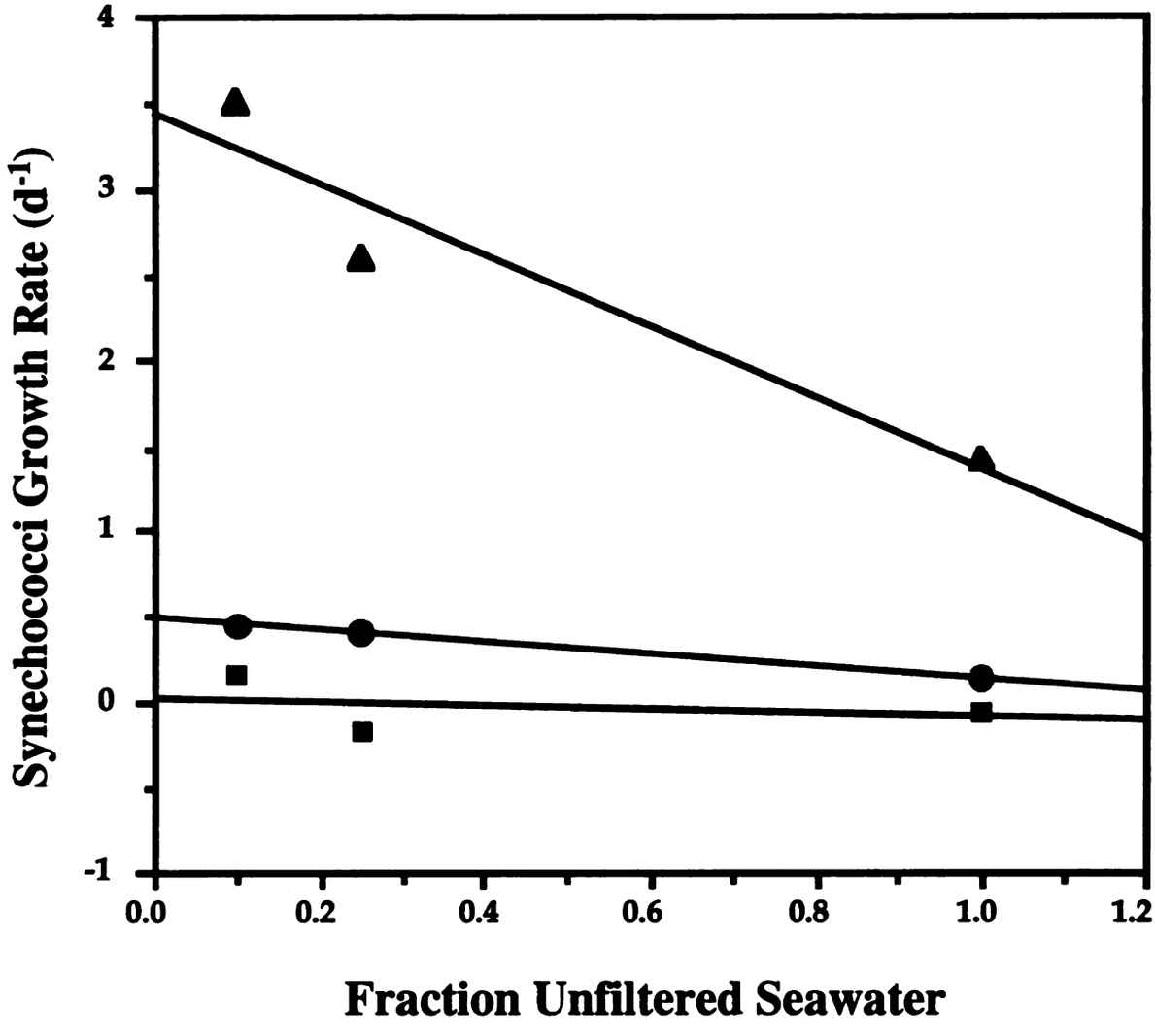

Figure 5.2 - Estimated growth rate of Synechococci determined by the dilution method of Landry and Hasset (25). See text for growth rate and grazing coeffecients. November 1994 sampling of stations (■) 3, (●) 5 and (▲) 8.

The growth rate at stations 3 and 5 are comparable to those reported Campbell and Carpenter (7) using the Landry and Hasset technique. the growth rate at station 8 was markedly higher than the other two stations and comparable to the growth rates reported by Waterbury (50) using a similar approach.

Synechococcus cellular 16S rRNA concentrations were determined all three stations (Table 5.3). Cellular 16S rRNA concentrations were significantly correlated with growth rates determined by the dilution culturing technique (r^2=0.75, $p<0.01$).

Cellular 16S rRNA concentrations and growth rates

According to simple models of predator-prey interactions, population size is variously controlled by either available nutrients (bottom-up forces) or predation (top-down forces) (19, 20, 30, 41). Nutrient-limited populations are expected to possess relatively slow cellular growth rates (Figure 1.2), whereas predator-limited population sizes are expected to be small compared to nutrient-limited populations and to possess relatively high growth rates in order to maintain the population under grazing pressure (Figure 1.2). Relative difference in growth can be distinguished using group-specific rRNA probes because cellular RNA concentrations, in particular rRNA concentrations, reflect growth rate (22, 24, 44) (Figure 2.2). *Synechococci* cellular 16S rRNA concentrations were determined at each station and plotted versus population size at each station (Figure 5.3). The greater than 10-fold variation in 16S rRNA concentration in the environmental samples exceeded the 2 fold variation in 16S rRNA concentrations attributable to diel variations (Figures 2.4 and 3.3). The distribution of large slow growing populations versus small rapidly growing populations conforms to the predictions of simple prey-dependent models (14, 41) of predator-prey interactions (Figure 1.2), suggesting both resource-limitation and predator-limitation are responsible for controlling these populations at different times or locales. The eutrophic stations 1 and 5, sampled in the fall of 1993, had populations of $1x10^5$ cells$\cdot$ml^{-1} or higher, and cellular 16S rRNA concentrations less

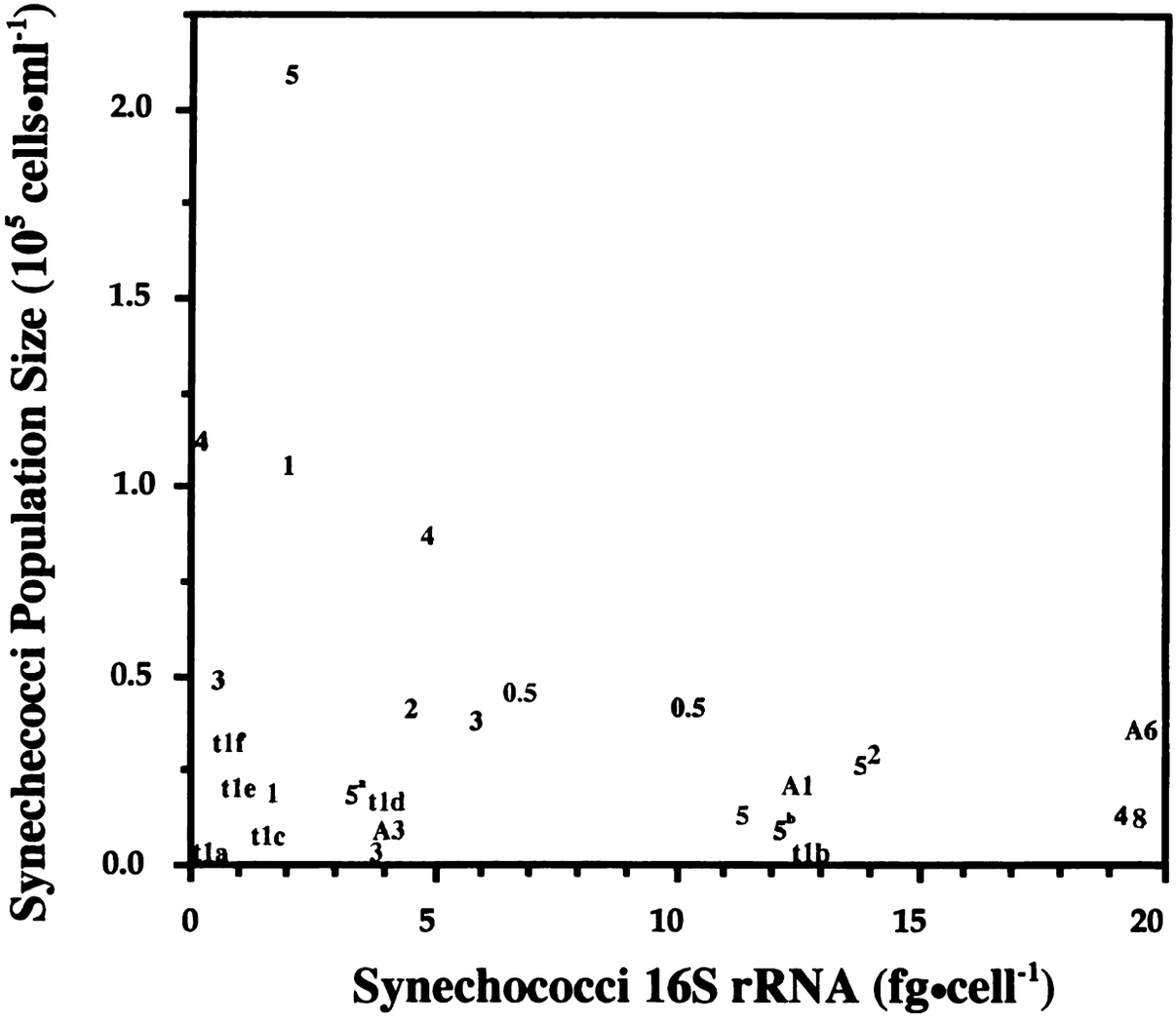

Figure 5.3 - Cellular 16S rRNA concentration of Synechococci populations in the Gulf of Mexico. Station colors correspond to the following sampling dates: (black) October-November 1993, (blue) March 1994, (red) November 1994 and (green) February 1996. Station numbers correspond to those in figure 5.1 and tables 5.1 through 5.4.

than 5 fg·cell^{-1}, indicating slow growth rates (Figure 5.3; Table 5.1). Stations A6 and 8, sampled in the spring of 1994 and the fall of 1994, respectively, both possessed small synechococci population densities and greater than 10 fg·cell^{-1} of 16S rRNA, indicating a rapidly growing population. A few stations, such as 4 and 5, exhibited considerable variation in both population size and 16S rRNA concentration. One explanation for this variation may be seasonal variation in nutrient inputs combined with seasonal variability in grazing intensity. A second explanation is the possiblity of "patchiness" in both nutrients and predator populations; although this explanation would require that the "patches" maintain their integrity over several days in order for the effects of added nutrients or predation to manifest themselves in the physiology of the relatively slow growing *Synechococcus* populations.

The distribution of larger, slower growing synechococci populations and smaller, faster growing, however, are inconsistent with the predictions of ratio-dependent models (1-3) of predator-prey interactions. If synechococci population size conformed to the predictions of ratio-dependent models the expected distribution would also include relative larger, faster growing populations (Figure 1.3). The absence of these larger, growing synechococci populations would suggest that the effects of predation are not dampened in a ratio-dependent manner.

The range of 16S rRNA concentrations observed in the Gulf of Mexico are similar to observed in pure cultures of *Synechococcus* PCC 6301 at specific growth rates of 0.007 to 0.08 h^{-1}. These growth rates are comparable to estimates of rates of grazing on synechococci determined dilution and inhibition techniques, reported by Campbell and Carpenter (7). The high growth rates are consistent with heavy grazing limiting population size and primary production, as postulated by Lohrenz (33).

There was no apparent correlation between nutrient loading and population size or growth rate. Large, slow growing populations of synechococci appeared in both eutrophic and oligotrophic marine sites. This pattern may indicate patchiness in nutrients

accompanied by a non-steady state population. This pattern could also indicate patchiness in predator population size or predator populations alternately controlled by their own bottom-up and top-down forces. For example, mesozooplankton grazing may limit protozoan population size and therefore grazing intensity, just as protozoan are capable of limiting bacterial population size. Stations with large slowly growing synechococci populations may consist of an odd number of trophic levels. In an odd numbered trophic system the *Synechococcus* spp. predator population would be limited by its own predator. This second level of predation would release the synechococci population from predator control and return the population to a nutrient-limited status (14, 41).

It appears that a significant number of synechococci populations in the shelf environment of the Gulf of Mexico, are controlled primarily by predation, regardless of whether the site is oligotrophic or eutrophic. These results are consistent with the work of Carpenter and Campbell (7), who found grazing rate of 0 to 0.08 h^{-1} of synechococci off the NY sound. It is also consistent with the predictions of Caron etal (9) that heterotrophic nanoflagellates (microflagellates) are the major predators of synechococci and capable of consuming 100% of the daily synechococci production. This contrasts with the results of recent experiments in the high-nitrogen:low-chlorophyll waters off the coast of the Galapagos islands (35), where it was demonstrated that iron was the element limiting synechococci population size/production.

Depth profile

A depth profile of synechococci population size was conducted at station T1f (28°01.03'N, 90°07.23'W) in February of 1996 (Figure 5.4). The synechococci population was approximately $2.5x10^4$ cells·ml^{-1} to a depth of 20 m. The population peaked at $1.1x10^5$ cells·ml^{-1} at 25 m. The population then declined with depths greater than 25 m and tended towards 0 at 80 m, the approximate bottom of the euphotic zone. The

distribution and cell density of synechococci is similar to the 20 m peak observed by Li (31) at a comparable latitude off the Moroccan coast and the 20 m peak observed at nertic front station P1 near Woods Hole Harbor, MA (16). In contrast the synechococci population peak at 25 m is deeper than the surface maximums typically observed by Waterbury *et al* (50) and much shallower than the approximately 100 m maximums observed at the oligotrophic ALOHA station near Hawaii (8). Chlorophyll *a* concentration displayed the same trend as synechococci cell density, peaking at 0.32 $mg \cdot L^{-1}$ at 25 m (Figure 5.5). This covariation between chlorophyll *a* and *Synechococcus* population size has been noted previously (8) in the vertical population of station ALOHA. The rapid decline in both cell density and chlorophyll *a* concentration after 25 m is typical of synechococci populations below the chlorophyll maximum (8). Synechococci populations typically peak at the chlorophyll maximum in oligotrophic waters (8). The synechococci population peak, the chlorophyll *a* peak and rapid decrease in both below 25 m are evidence that a chlorophyll maximum exists at this station that is much shallower than previously reported for the oligotrophic ALOHA station (8). Cellular 16S rRNA concentrations increased steadily for the first 20 m before peaking at 2.9 $fg \cdot cell^{-1}$ (Figure 5.4). The cellular 16S rRNA concentration fell to nearly zero at 25 m before increasing gradually to 1.7 $fg \cdot cell^{-1}$ and again decreased to low at 80 m. The high 16S rRNA concentrations near the surface indicate that surface populations possess a higher specific growth rate relative to populations at depth at this station. The high surface growth rates seen here are consistent with the high synechococci surface productivity reported by Waterbury *et al.* (51) in oligotrohic waters. Microzooplankton population size increased with depth to an approximate peak of 3000 $cells \cdot ml^{-1}$ at 35 m before decreasing to undetectable levels at 80 m (Figure 5.5). Low synechococci 16S rRNA concentrations coincided with the maximum microzooplankton population. If microzooplankton grazing had a significant impact upon synechococci population size there should be a correlated increase in the growth rate and the cellular 16S rRNA concentration of the synechococci

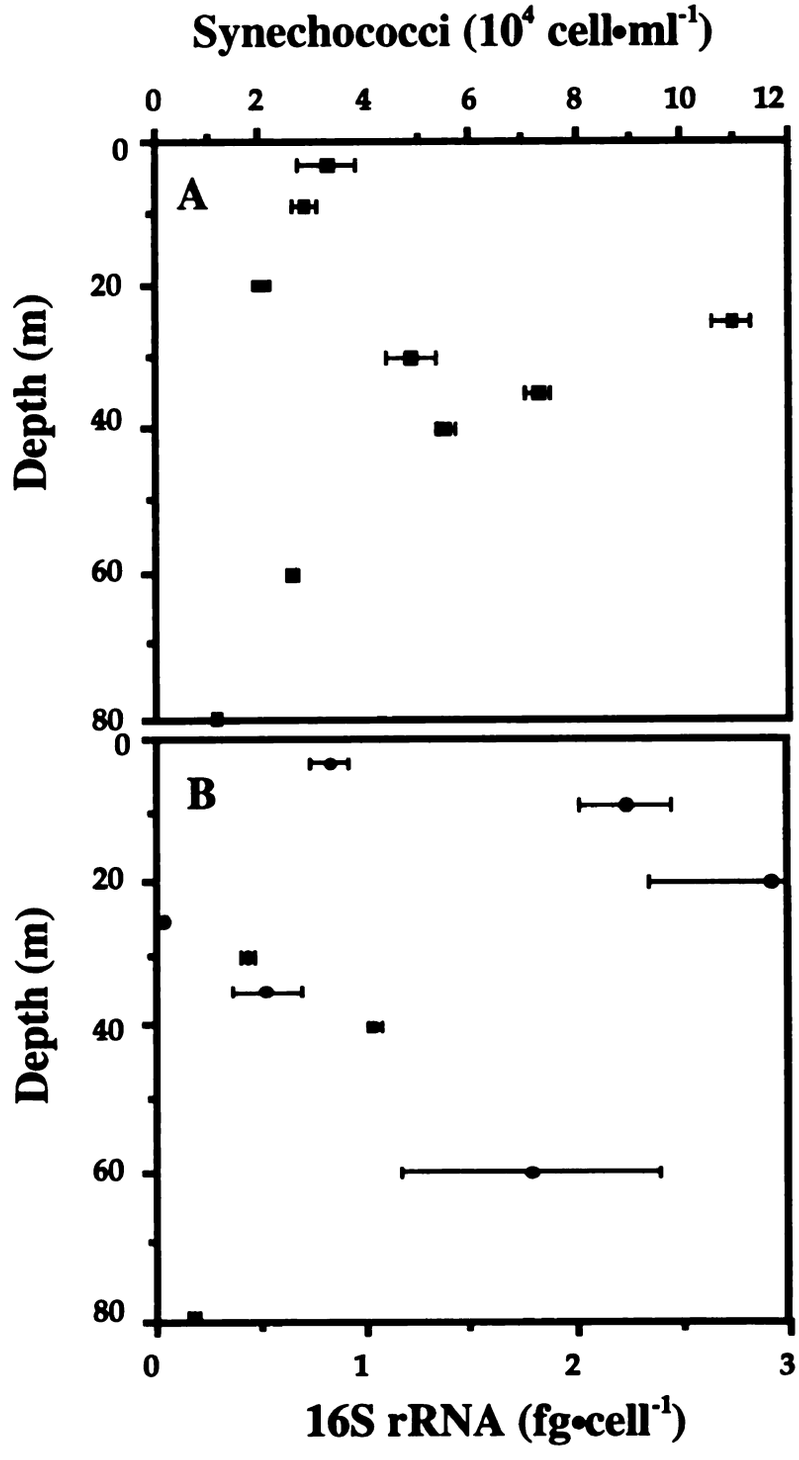

Figure 5.4 - Depth profile of *Synechococcus* populations. (A) *Synechococcus* population size. (B) *Synechococcus* cellular 16S rRNA concentrations.

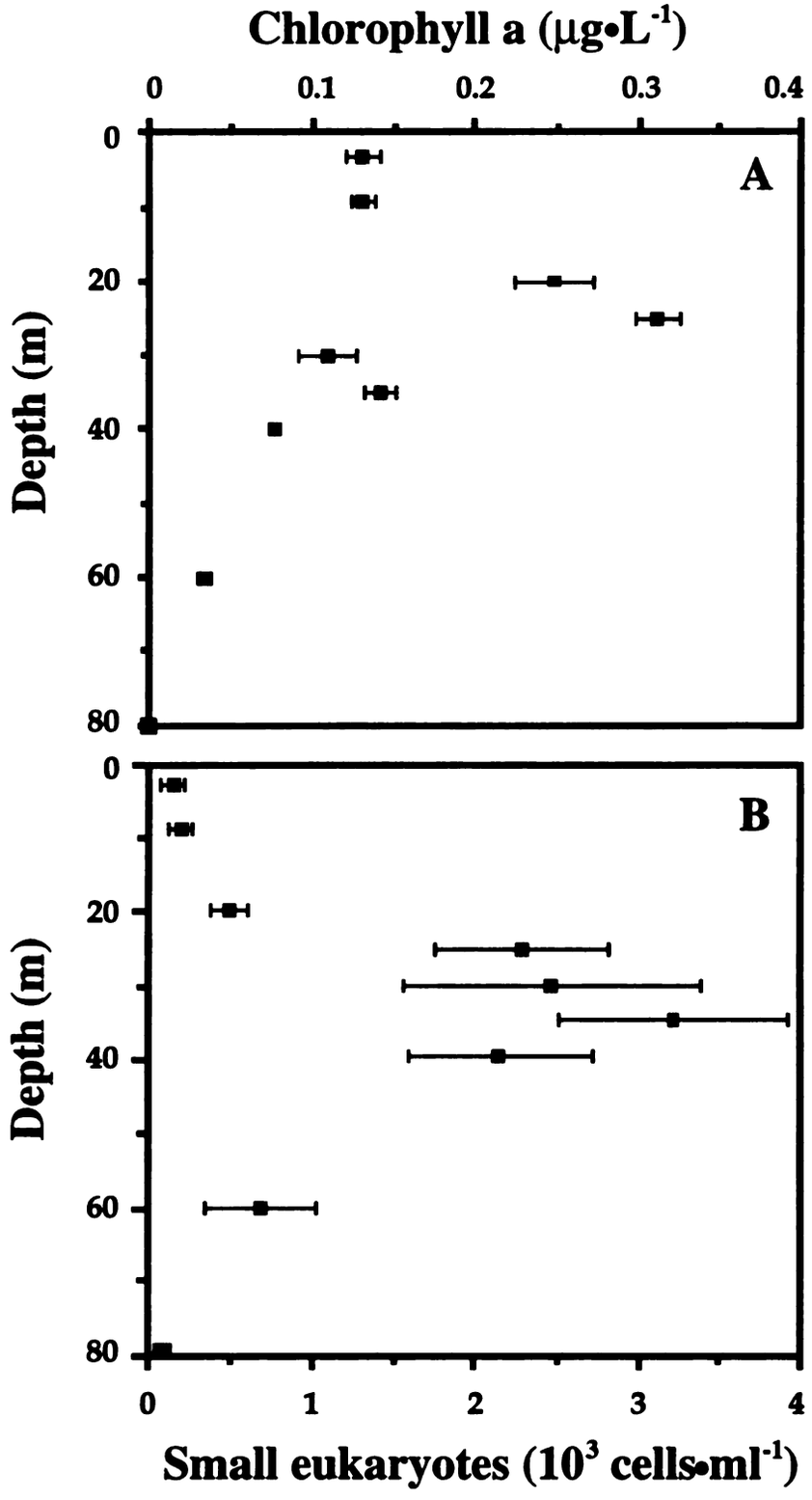

Figure 5.5 - Depth profile of station t1f. (A) Chlorophyll a concentrations. (B) Small eukaryote population size.

population (Figure 2.2). The low cellular 16S rRNA concentrations coincident with the high microzooplankton population indicates that microzooplankton do not have a significant impact on synechococci population size. It appears that the population growth rate at this station is controlled by available nutrients (bottom-up forces). The larger and faster growing synechococci population, at 25 m within the depth profile, bounded by a very slow growing population is similar to previous observations (26, 53), although the maximum synechococci population in this study appears to be bounded on a single side by a low growth population. Wood (53) has interpreted this characteristic depth profile of maximum population growth bounded by negative population growth as evidence for bottom-up forces controlling synechococci populations in oligotrophic waters, an interpretation consistent with the observations at this particular station.

Synechococcus cellular 16S rRNA concentrations were consistent with prey-dependent models of predation and previous reports of high grazing coeffecients. These results do, however, appear to be inconsistent with the predicted outcome of ratio-dependent predation. Cellular 16S rRNA concentrations and the prediction of predator-limitation also correlated with growth rate and grazing coeffecients estimated from dilution cultures. There was no apparent correlation between 16S rRNA concentrations and rates of carbon fixation. This work demonstrates that 16S rRNA probes can be used to distinguish top-down from bottom-up control of any group for which a specific probe can be designed and address general ecological questions.

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Chapter 6

Summary and Conclusions

Over thirty years ago it was demonstrated that cellular nucleic acids concentrations were proportional to growth rate in *Salmonella typhimurium* and *Escherichia coli* (6, 12). A recent synthesis of the literature on the relationship between cellular nucleic acids concentrations and growth rate has revealed that nucleic acids concentrations are proportional to growth rate in all heterotrophic bacteria examined to date (5). Nucleic acids concentrations are also proportional to growth rate in both filamentous and unicellular cyanobacteria under constant illumination (7, 9). The work presented in this series of studies demonstrates that cellular nucleic acids concentrations are proportional to growth rate in the freshwater cyanobacterium *Synechococcus* sp. strain PCC 6301 and the marine cyanobacterium *Synechococcus* sp. strain WH 8103. Furthermore, this proportionality is maintained over a range of growth rates which are ecologically relevant. The nucleic acids content of bacteria is predominately composed of rRNA (6, 8) and one-third of ribosomal rRNA (w/w) is composed of 16S rRNA. The correlation between cellular 16S rRNA concentrations and growth rate presents the possibility of using probes complementary to 16S rRNA to determine *in situ* growth rates. Probes complementary to 16S rRNA were used to demonstrate that 16S rRNA was proportional to growth rate in *Synechococcus* sp. strain PCC 6301 (Figure 2.2). This relationship was assumed to hold for *Synechococcus* sp. strain WH 8103, as well, a position supported by the fact that cellular 16S rRNA concentrations mirrored those of total cellular RNA concentrations during the course of a diel cycle (Figure 2.4).

The constant RNA/DNA ratios of *Synechococcus* sp. strain PCC 6301 contrasts with the increasing RNA/DNA ratios of *Synechococcus* sp. strain WH 8103. The constant RNA/DNA ratio observed in *Synechococcus* sp. strain PCC 6301 has been used to argue that *Synechococcus* sp. strain PCC 6301 is not capable of transcriptional regulating the majority of its genes. This position is further supported by the fact that both total RNA and 16S rRNA concentrations mirror those of DNA concentrations during the course of diel growth (Figure 1.3 and 1.4). The possible lack of transcriptional regulation hints that

many of the observed features of circadian rhythms may be a result of gene dosage effects (10).

By implication then the increasing RNA/DNA ratios observed in *Synechococcus* sp. strain WH 8103 would indicate that this organism possesses the ability to transcriptionally regulate the majority of its genes, including the ribosomal RNAs. This position is supported by examination of the nucleic acids concentrations of the organism during diel growth. Although the trends are similar to those of *Synechococcus* sp. strain PCC 6301 there is one notable exception. During the final 3h of the dark period there was a large increase in both total cellular RNA concentrations and cellular 16S rRNA concentrations (Figure 2.2). This dramatic increase is even more remarkable in view of the fact that cellular DNA concentrations had dropped to a low equivalent to one genome•cell^{-1} (Figure 2.3). The ability of *Synechococcus* sp. strain WH 8103 to dramatically increase cellular RNA concentrations from a single genome copy would indicate that the organism is capable of transcriptionally regulating its *rrn* operons. This evidence is also consistent with the interpretation that increasing RNA/DNA ratios indicate transcriptional control.

Once it was established that 16S rRNA probes were capable of distinguishing relative difference in ecologically relevant growth rates, it was a natural progression to use this technique to address ecological questions. One such question was whether cellular 16S rRNA concentrations could be used as a proxy for cellular growth rate to distinguish between nutrient limitation and predator limitation of a cyanobacterial population. This question was addressed in laboratory experiments using a model system consisting of a chemostat population of a *Synechococcus* sp. strain PCC 6301. In the absence of predation *Synechococcus* sp. strain PCC 6301 equilibrium population size was dependent upon the concentration of available limiting nutrients, in this case phosphate, within the reservoir supplying the chemostat culture vessel (Figure 3.1). The population size remained largely unaffected by the specific growth of the individual organisms which was determined by the dilution rate of the chemostat and ultimately the flux of the limiting

nutrient through the system. Ecological theories of predator-prey interactions predicted that the addition of a predator would decrease the equilibrium prey population size while simultaneously increasing the specific growth rate of the individuals within the prey population. The addition of the protozoan predator *T. pyriformis* to a chemostat culture of *Synechococcus* sp. strain PCC 6301 resulted in a 7-fold decrease in the equilibrium population size of *Synechococcus* sp. strain PCC 6301. The grazing pressure imposed by *T. pyriformis* resulted in *Synechococcus* sp. strain PCC 6301 reaching its maximal specific growth rate (Figure 3.1), which was reflected in the high cellular concentrations of cellular 16S rRNA compared to the low cellular 16S rRNA concentrations observed in the absence of predation (Table 3.1; Figure 3.3). Based on prey-dependent models of predator-prey interaction, increases in available nutrients supplied by the reservoir should result in an increase in the equilibrium population size of the *T. pyriformis* predator, while the equilibrium population size the *Synechococcus* sp. strain PCC 6301 prey remains unchanged. However, the larger *T. pyriformis* population would result in increased grazing pressure forcing a compensatory increase in growth rate and therefore cellular 16S rRNA concentrations in the *Synechococcus* sp. strain PCC 6301 prey. Unfortunately, because the specific growth rate of the *Synechococcus* sp. strain PCC 6301 population was already at maximal, the increase in available limiting nutrients went unutilized (Table 3.2). It was therefore not possible to distinguish between ratio-dependent models of predator-prey interactions and prey-dependent models of predator-prey interactions using the particular predator-prey system from these studies. Nor was it possible to distinguish between ratio-dependent and prey-dependent models by reducing the supply of limiting nutrient without the already low *T. pyriformis* predator population washing out of the system. Regardless of the short-comings of this system it was possible to demonstrate that 16S rRNA probes in combination with a knowledge of the equilibrium prey population size could be used to distinguish nutrient-limited prey populations from predator-limited populations.

Having demonstrated that predator-prey interactions conform to model predictions and that 16S rRNA probes could be used to distinguish nutrient-limited prey populations from predator-limited populations, these methods were applied to a nutrient gradient within the Gulf of Mexico. There is a well established nitrate and phosphate gradient where the oligotrophic water of the Gulf of Mexico are influenced by nutrient loading from the Mississippi River drainage system. If synechococci population size was limited by available nutrients it would be a reasonable expectation to find a decreasing synechococci population size moving from the near shore, eutrophic waters, heavily influenced by the Mississippi River, to the oligotrophic water of the open ocean within the Gulf of Mexico. Conversely, if synechococci population sizes were limited by predation then one would expect an increase in growth rates but not population size , along the gradient, as available nutrients were passed up the trophic chain to an increasing predator population.

Synechococci population sizes in the Gulf of Mexico were primarily small with a significant number of these populations containing high concentrations of 16S rRNA, indicative of rapid growth rates (Figure 4.1). It is significant that there were no populations in the upper right quadrant of figure 5.3, comprised of large quickly growing synechococci populations. Such populations would be predicted from ratio-dependent models of predator-prey interactions (1-3). Rather, the Gulf of Mexico environment appeared to be composed of a gradient between large, slow growing synechococci populations and small, fast growing synechococci populations. These results conform to the predictions of prey-dependent models of predator-prey interactions (4, 11) and appear to be inconsistent with predictions of ratio-dependent models of predator-prey interactions. Thus, it appears that some synechococci populations were nutrient-limited while others were predator-limited. There was no clear correlation between the trophic status of the local environment (oligotrophic vs. eutrophic) and whether a synechococci population was nutrient-limited or predator limited. This could be a result of increasing number of trophic

levels along the gradient, resulting in alternate switching between nutrient-limitation and predator-limitation (4, 11).

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