PICOCYANOBACTERIAL CELLULAR PHYSIOLOGY AND TROPHIC INTERACTION WITH A HETEROTROPHIC NANOFLAGELLATE

by

CHRISTOPHER D. BURBAGE

(Under the Direction of Brian J. Binder)

ABSTRACT

Prochlorococcus and *Synechococcus* are two closely related picocyanobacteria that together are responsible for a large proportion of the total primary production in open ocean environments. Relatively little is known about the growth rates and trophic interactions of these organisms in the field. This dissertation focuses on characterizing the growth physiology of representative strains of *Prochlorococcus* (MIT9312) and *Synechococcus* (WH8103), and exploring the potential role of these organisms as prey for a model heterotrophic nanoflagellate (*Paraphysomonas imperforata*). Two proposed approaches for assessing picocyanobacterial growth rates in the field would use either cellular RNA content or population DNA distributions as the basis for estimating growth rate. In this study I show that biomass-normalized RNA content is linearly related to growth rate in *Synechococcus* WH8103. Re-analysis of previously published data suggests that many (though not all) other *Synechococcus* strains behave similarly, and therefore that cellular RNA may represent a reasonable approach for estimating *in situ* growth rates in natural *Synechococcus* populations. However, the non-linear relationship observed here (and in previous studies) for *Prochlorococcus* indicates that application of the approach may be problematic in this case. The cell cycle study in this dissertation is the first to systematically characterize the relationship between growth rate and cell cycle behavior in

Prochlorococcus. I show that *Prochlorococcus* and *Synechococcus* display a notable degree of similarity with respect to this behavior. The combined duration of the replication and post-replication phases varied with growth rate in both species, suggesting that typical strategies for calculating growth rates from DNA data may need to be modified. Furthermore, I found that cell mass at the start of DNA replication decreased with increasing growth rate, indicating that the initiation of chromosome replication may not be a simple function of cell biomass, as previously suggested. Regarding picocyanobacteria as potential prey items for heterotrophic flagellates, I found that *P. imperforata* could graze and grow upon both these strains, but that *Synechococcus* was the preferred prey. This preference was flexible, however, and could be modulated by the ratio of prey types and/or the overall concentration of available prey.

INDEX WORDS: cell cycle, growth rate, *Paraphysomonas imperforata*, protozoan grazing, *Prochlorococcus*, RNA, *Synechococcus*

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DEDICATION

I would like to dedicate this dissertation to my grandmother who never got to see me complete this dissertation. Without your support and generosity during the early years of grad school I never would have made it this far. Additionally I would like to also dedicate this dissertation to my parents. I am truly thankful for your support and understanding over the years. I'm sure you wondered how long it was going to take but I finally finished. Lastly I would like to dedicate this dissertation to Laura, my wife, without your never ending support and help I would have never made it, for that I am truly thankful.

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CHAPTER 1

INTRODUCTION / LITERATURE REVIEW

The world's oligotrophic oceans contribute approximately 25% of the earth's net primary production (Field et al. 1998). Primary production in these environments is dominated by picophytoplankton, a group of very small (diameter ≤ 2 μm) photosynthetic organisms. Among the picophytoplankton are two closely related groups of unicellular cyanobacteria, *Prochlorococcus* and *Synechococcus* (termed picocyanobacteria), that together account for a large proportion of overall photosynthetic biomass and primary production in many oceanic environments (Waterbury et al. 1986, Goericke & Welschmeyer 1993, Li 1995, Liu et al. 1997, Veldhuis et al. 1997, Partensky et al. 1999). *Synechococcus* were first recognized as being abundant in marine waters in the late 1970s by Johnson and Sieburth (1979) and Waterbury et al. (1979). *Prochlorococcus* was not characterized until 10 years later (Chisholm et al. 1988), though it had apparently been observed originally by Johnson and Sieburth in their 1979 study where it was described as "Type II" *Synechococcus* cells. *Prochlorococcus* is unusual in possessing divinyl Chlorophyll-a and –b, and generally lacking typical cyanobacterial phycobiliproteins. Nevertheless, molecular sequencing of *Prochlorococcus* and *Synechococcus* 16S ribosomal RNA (rRNA)(Urbach et al. 1992, Urbach & Chisholm 1998) and the RNA polymerase gene *rpoC1* (Palenik & Haselkorn 1992) demonstrated that these two picocyanobacteria are closely related. Since their respective discoveries, the abundance of *Prochlorococcus* and *Synechococcus* has been observed in the North Atlantic (Chisholm et al. 1988, Olson et al. 1990, Li et al. 1992, Li et al. 1995, Buck et al. 1996, Partensky et al. 1996,

Worden & Binder 2003a, Zinser et al. 2006), the South Atlantic (Zubkov et al. 1998, Johnson et al. 2006), Caribbean Sea (McManus & Dawson 1994), central, eastern and equatorial Pacific Ocean (Campbell & Vaulot 1993, Campbell et al. 1994, Ishizaka et al. 1994, Binder et al. 1996, Worden & Binder 2003a), the Arabian Sea (Campbell et al. 1998), the Indian Ocean (Veldhuis et al. 1997), the Mediterranean Sea (Vaulot et al. 1990), and the Red Sea (Veldhuis & Kraay 1993, Lindell & Post 1995).

Based on the photophysiology of two *Prochlorococcus* co-isolates from the same water sample (with 97% rRNA similarity), Moore et al. (1998) identified two sub-groups or "ecotypes" of *Prochlorococcus*: a low-light adapted group with a relatively high Chlorophyll-b:a ratio (high-B/A), and a high-light adapted group with a low Chlorophyll-b:a ratio (low-B/A). Subsequent examination of the 16S-23S transcribed spacer sequence (ITS) regions from 32 different *Prochlorococcus* isolates reconfirmed this division of *Prochlorococcus* into high-B/A and low-B/A ecotypes, but also allowed the subdivision of the high-B/A ecotype into four genetically distinct clades (Rocap et al. 2002). Recently, Zinser et al. (2006) and Johnson et al. (2006) used a quantitative PCR-based approach to establish that these *Prochlorococcus* ecotypes have distinct distributions in the Atlantic Ocean with respect to depth and location.

Numerous physiological correlates among *Prochlorococcus* ecotypes have been described. As discussed above, observed photophysiological differences were used to first establish the existence of *Prochlorococcus* ecotypes (Moore et al. 1998). Moore et al. (2002) also observed differences in nitrogen use among different ecotypes. All of the *Prochlorococcus* isolates in their study were able to use NH_4 and urea, but surprisingly none could use NO_3, and only four (all of which were high-B/A ecotypes found near the nitracline) were able to use NO_2. Whole genome analysis of a low-B/A *Prochlorococcus* isolate (MED4) and a high-B/A isolate

(MIT9313) confirmed these observations: both organisms lack the genes required for NO_3 utilization, but MIT9313 possesses homologs of the genes required for NO_2 utilization. Consistent with these patterns of nitrogen utilization, Moore et al. (2002) found that high-B/A ecotypes of *Prochlorococcus* were restricted to the deep euphotic zone near the nitracline. While low-B/A ecotypes were dominant, though not restricted to the NO_3-depleted waters near the surface, and suggested that this distribution may reflect resource partitioning of N among closely related ecotypes.

Prochlorococcus isolates also display a high degree of variability in gene content with respect to phosphorus acquisition, although in this case not necessarily congruent with their rRNA phylogeny (Martiny et al. 2006). Rather, the differences in phosphorus scavenging genes may reflect differences in phosphorus availability in the oceans from which the respective strains were isolated. *Prochlorococcus* in general, and to a lesser extent *Synechococcus*, appear to have a reduced P requirement owing to the substitution of sulfo-lipids for phospho-lipids in their cell membrane (Van Mooy et al. 2006). This diminished demand for phosphorus may give *Prochlorococcus* a selective advantage in the North Pacific Subtropical Gyre and other strongly oligotrophic areas, accounting for this organism's dominance in these areas.

As is the case for *Prochlorococcus*, marine *Synechococcus* can also be divided into distinct phylogenetic clusters (Scanlan 2003). Marine cluster A (MC-A or Cluster 5.1) includes both oceanic and coastal strains. These strains contain phycoerythrin as their major light-harvesting pigment and have elevated growth requirements for Na^+, Cl^-, Mg^{2+}, and Ca^{2+}. Many MC-A strains are motile, and some have been shown to chromatically adapt to changing light conditions. Marine cluster B (MC-B or Cluster 5.2) contains only coastal *Synechococcus* isolates with phycocyanin as their major light harvesting pigment, phycoerythrin is absent. Most

MC-B strains are somewhat halotolerant. Marine cluster C (MC-C or Cluster 3) contains both coastal and brackish water *Synechococcus* isolates. Most strains in this cluster contain phycocyanin as their major light harvesting pigment, although there are some isolates that produce C-phycoerythrin and are capable of chromatic adaptation (Scanlan 2003).

Analysis of *Synechococcus* ITS regions permits *Synechococcus* cluster MC-A to be partitioned into six distinct clades, three of which are associated with a particular phenotype (motility, chromatic adaptation, and lack of phycourobilin) (Rocap et al. 2002). Ahlgren and Rocap (2006) further assessed the diversity of open ocean MC-A *Synechococcus* using a combination of culture isolates and ITS clone libraries. They distinguished seven total *Synechococcus* phylotypes, including two new ecotypes. Isolates of these ecotypes displayed chromatic adaptation and requirements for specific nitrogen sources. Ahlgren and Rocap (2006) concluded that, as in its close relative *Prochlorococcus*, light and nitrogen utilization are important factors in ecotype differentiation in the marine *Synechococcus* lineage.

Picocyanobacterial Growth Rates:

Although considerable understanding about the phylogeny and distribution of *Prochlorococcus* and *Synechococcus* in oceanic environments has been achieved over the past decade or so, our knowledge of the growth and mortality of these groups remains limited. The measurement of *Prochlorococcus* and *Synechococcus* growth rates in the field is difficult, owing to the dynamic nature of microbial systems. One early attempt at estimating *in situ* *Prochlorococcus* growth rates involved measurements of ^{14}C incorporation specifically into divinyl Chlorophyll-a (Goericke & Welschmeyer 1993): *Prochlorococcus* growth rates in the Sargasso Sea were estimated to be fairly low (0.1-0.3 day^{-1}). However, this methodology was later determined to be problematic due to issues of photoacclimation (Cailliau et al. 1996).

An additional method that has been utilized in measuring *Prochlorococcus* and *Synechococcus* growth rates is the selective inhibition technique. This method relies on an inhibitor's effect upon either the eukaryotic grazers or the prokaryotic prey. Liu et al. (1995) used the selective inhibitor technique to measure both the growth and grazing rates of *Prochlorococcus* and *Synechococcus*. They reported growth rates in the range of 0.1-0.5 and up to 1.0 day^{-1} for *Prochlorococcus* and *Synechococcus*, respectively.

A widely used method for estimating *Prochlorococcus* and *Synechococcus* growth rates in the field is the dilution assay. In this assay, dilution of cyanobacterial prey items with filtered seawater reduces their grazing pressure and leaves their growth rate unchanged, allowing growth and grazing to be separated (Landry & Hasset 1982, Landry et al. 1995). The dilution assay has been used to determine *Prochlorococcus* and/or *Synechococcus* population growth rates in the equatorial, subtropical, and subarctic Pacific, the tropical and subtropical North Atlantic, and the Arabian Sea (see Worden & Binder 2003a and references therein). Although considerable variability has been observed, in a very general way growth and grazing rates are approximately balanced, falling in the range of one doubling (or consumption of one day's growth) day^{-1} (Reckerman & Veldhuis 1997, Rivkin et al. 1999, Kuipers & Witte 2000, Worden & Binder 2003a). Despite its widespread use, the dilution assay relies on a number of assumptions (e.g. linearity of functional responses, insignificant grazer growth) that lead to questions as to the accuracy of this methodology (Evans & Paranjape 1992, Dolan et al. 2000, Agis et al. 2007).

The ^{14}C, inhibitor, and dilution approaches all involve incubations, and as such may be subject to various "bottle effects" that confound their interpretation. One approach that avoids incubations entirely is cell cycle analysis. This method tracks the progression of a phased population through its cell cycle over a 24h period, providing an estimate of the fraction of the

population that divides during that period, and thus allowing the calculation of the population growth rate (Carpenter & Chang 1988, Chang & Carpenter 1988, Chang & Carpenter 1991). In theory this method should not be influenced by grazing or other mortality terms, and thus is thought to provide an estimate of intrinsic growth rate of the population of interest. Because natural *Prochlorococcus* populations are very strongly phased in their cell division (Partensky et al. 1999, Jacquet et al. 2001, Binder & DuRand 2002), and because their cellular DNA content can be assessed with relative convenience using flow cytometry, this group represents an excellent target for the cell cycle approach. Vaulot et al. (1995) were the first to apply this approach to *Prochlorococcus* in the equatorial Pacific; a limited number of other studies have since applied it to *Prochlorococcus* populations in the subtropical North Pacific, the Sargasso Sea, and in the context of Fe-enrichment studies in the equatorial Pacific (Liu et al. 1999, Mann & Chisholm 2000, Worden & Binder 2003a). The cell cycle approach is not without its drawbacks: it is labor intensive, requiring high-frequency sampling of the same population over a 24 h period. Furthermore, its application to *Synechococcus* populations can be problematic due to staining difficulties, lack of strict phasing, and variation in cell cycle behavior (Binder & Chisholm 1995). Perhaps most importantly, our current knowledge of cell cycle behavior in *Prochlorococcus* is extremely limited, and critical analysis of the various components of this assay as applied to this group have yet to be performed.

The use of biochemical properties as a proxy for instantaneous growth rate measurements is another approach that has been explored for its potential in determining picocyanobacterial growth rates. This sort of approach would be independent of bottle incubations and in theory could provide an instantaneous measurement of growth rate for the organism of interest. Cellular ribosomal RNA (rRNA) is an attractive target for this biochemical index approach.

Ribosome content (and therefore rRNA) is well-correlated with growth rate in model prokaryotes (see Kerkhof & Ward 1993 and references therein), and has been used as a proxy for marine heterotrophic bacteria with some success (Dortch et al. 1983, Kemp et al. 1993, Kramer & Singleton 1993). Binder and Liu (1998) found that the relationship between growth rate and cellular rRNA was tri-phasic in marine *Synechococcus* strain WH8101. At low growth rates ($<0.7\ d^{-1}$), rRNA $cell^{-1}$ remained fairly constant; at growth rates between 0.7 and 1.6 d^{-1}, cellular rRNA content increased proportionally with increasing growth rate; and at the highest growth rates ($>1.6\ d^{-1}$) rRNA $cell^{-1}$dropped abruptly. Similar patterns were subsequently observed in other *Synechococcus* strains, and in a *Prochlorococcus* (Worden & Binder 2003b), but this pattern is apparently not universal (e.g. Lepp & Schmidt 1998). Clearly, further work is required before rRNA can be used reliably as an indicator of growth rate in natural *Synechococcus* and *Prochlorococcus* populations.

Consumption of Picocyanobacteria by Heterotrophic Protozoa:

Although *Prochlorococcus* and *Synechococcus* contribute significantly to the overall net primary production in open ocean ecosystems (see above), little is known about the fate of that production. Estimates of *in situ Prochlorococcus* and *Synechococcus* mortality rates have been limited. In general, dilution experiments suggest that growth is balanced by grazing, as is to be expected, in natural picocyanobacterial communities (Landry & Hasset 1982, Landry et al. 1995, Reckerman & Veldhuis 1997, Landry et al. 1998, Lessard & Murrell 1998, Kuipers & Witte 2000, Worden & Binder 2003a). However, the identity and grazing behavior of the organisms that consume *Prochlorococcus* and *Synechococcus* have not been widely studied.

Culture experiments have demonstrated that various ciliates and flagellates can have quite varied abilities to graze and to grow upon *Prochlorococcus* and *Synechococcus*. Christaki

et al. (1999) showed that both the herbivorous ciliate *Strombidium sulcatum* and the bactivorous ciliate *Uronema* sp. could graze on *Prochlorococcus* and *Synechococcus*, though the latter was preferred. *S. sulcatum* was able to grow on both prey items, whereas *Uronema* sp. grew poorly on *Synechococcus* and not at all on *Prochlorococcus*.

It is generally assumed that heterotrophic nanoflagellates are the major grazers on picocyanobacteria in open ocean systems (Reckerman & Veldhuis 1997, Caron et al. 1999, Christaki et al. 2001, Cuevas & Morales 2006). Guillou et al. (2001) isolated two such nanoflagellates (*Picophagus flagellatus* and *Symbiomonas scintillans*) from environments where *Prochlorococcus* and *Synechococcus* were abundant, and presented them with various combinations of these prey cell types. *P. flagellatus* ingested both *Prochlorococcus* and *Synechococcus*, but preferred the latter when both were offered together. *S. scintillans*, on the other hand, did not feed on either prey item. In another study the bactivorous flagellate *Pseudobodo* sp., as well as a mixed nanoflagellate community from the field, grazed upon both *Prochlorococcus* and *Synechococcus* cell types, but in neither case was flagellate growth observed (Christaki et al. 2002). Given the diversity of responses observed within only two studies (looking at a total of four different model flagellates) it is clear that much more work needs to be done before even a rudimentary understanding of the relationships between heterotrophic nanoflagellates and picocyanobacterial prey can be developed.

Objectives of the dissertation:

The overall goal of this dissertation is to examine the effects of growth rate upon the physiology and macromolecular composition of the two picocyanobacteria *Prochlorococcus* and *Synechococcus*, and to characterize the grazing response of a model heterotrophic nanoflagellate grazer to these organisms.

Specific Objectives include:

1. Quantify the cellular RNA and protein content of the *Prochlorococcus* (MIT9312) and *Synechococcus* (WH8103) as a function of growth rate.
2. Examine the relationship between cell cycle behavior and light-limited growth rate in *Prochlorococcus* and *Synechococcus*.
3. Characterize the grazing behavior of *Paraphysomonas imperforata* when feeding on varying concentrations and prey ratios of *Prochlorococcus* and *Synechococcus*.

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CHAPTER 2

CELLULAR RNA AND PROTEIN CONTENT OF *PROCHLOROCOCCUS* (MIT9312) AND *SYNECHOCOCCUS* (WH8103) (CYANOBACTERIA) AS A FUNCTION OF GROWTH RATE

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Abstract:

The unicellular cyanobacteria *Prochlorococcus* and *Synechococcus* are important primary producers in marine ecosystems. Our understanding of the dynamics of natural populations of these organisms is hampered by a lack of convenient methods for measuring their growth rates *in situ*. One approach for making such measurements uses cellular RNA content as a biochemical index reflecting growth rate. Here we examine the relationship between growth rate and cellular RNA and protein levels in oceanic isolates of *Prochlorococcus* (strain MIT9312) and *Synechococcus* (strain WH8103). In *Synechococcus* WH8103, cellular RNA content increased linearly with growth rate. In *Prochlorococcus* MIT9312, however, the relationship was decidedly non-linear, with RNA levels remaining approximately constant at low growth rates (< ~0.6 d^{-1}) and increasing at higher rates. Cellular protein content generally decreased as growth rate increased in both strains, although in *Prochlorococcus* it abruptly increased at the very highest growth rates achieved. Consistent with theoretical predictions, protein-normalized RNA was linearly related to growth rate in *Synechococcus* WH8103. In contrast, RNA/protein was related to growth rate in a non-linear, tri-phasic manner in *Prochlorococcus*. Reanalysis of published biomass-normalized RNA data suggests that this difference may reflect a general (though not universal) difference between *Synechococcus* and *Prochlorococcus*. Thus, appropriately normalized cellular RNA may represent a reasonable approach for assessing in situ growth rates in *Synechococcus*, but the tri-phasic relationship between growth rate and RNA/protein in *Prochlorococcus* would complicate such application to this group of cyanobacteria.

Key index words: cyanobacteria; growth rate; *Prochlorococcus*; RNA; *Synechococcus*

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Introduction:

The picocyanobacteria *Prochlorococcus* and *Synechococcus* together account for a large fraction of the primary production in open ocean systems (Waterbury et al. 1986, Goericke & Repeta 1993, Li 1995, Liu et al. 1997, Partensky et al. 1999, Maranon et al. 2003). Despite their ecological significance, our knowledge of the *in situ* growth rates of these populations and our understanding of the factors that regulate these rates remain rudimentary.

Measuring *in situ* growth rates among natural phytoplankton populations generally, and picophytoplankton in particular, remains problematic. Among the approaches for making such measurements, dilution incubations (Landry & Hasset 1982, Landry et al. 1995, Worden & Binder 2003, Selph et al. 2005) and cell cycle analysis (Carpenter & Chang 1988, Vaulot et al. 1995, Liu et al. 1997, Shalapyonok et al. 1998, Vaulot & Marie 1999, Mann & Chisholm 2000) are the most widely employed methods. Although both these approaches provide reasonable estimates of picocyanobacterial growth rates, they are not without drawbacks that limit their use. Dilution experiments are labor intensive and subject to bottle effects. Furthermore, they depend upon numerous assumptions, a number of which have been questioned (Evans & Paranjape 1992, Dolan et al. 2000, Agis et al. 2007). The cell-cycle analysis approach is also labor intensive, due to the high frequency sampling required for accurate growth rate estimates (Vaulot et al. 1995, Shalapyonok et al. 1998, Vaulot & Marie 1999, Mann & Chisholm 2000). In addition, natural *Synechococcus* populations may not be well suited for this type of growth rate

determination due to difficulties in DNA staining and variable diel cell-cycle patterns (Binder & Chisholm 1995, Vaulot et al. 1996).

Given the short-comings of these methods, it is worthwhile to consider alternative approaches for estimating growth rates among natural picocyanobacterial populations. One class of potential approaches involves the use of a biochemical property as a proxy for instantaneous growth rate measurements (Furnas 1990). This sort of approach would be independent of incubations and in theory could provide an instant snapshot of the growth status of the population of interest. Ribosomal RNA (rRNA) has been proposed as a particularly attractive proxy for growth rate owing to the relatively low variability of ribosomal protein synthesis efficiency, at least among model heterotrophic bacteria (see Kerkhof & Ward 1993 and references therein). Given this stability in ribosome efficiency, cellular rRNA content can be expected to reflect cellular protein synthesis rate. During balanced growth, protein synthesis rate should in turn be closely related to cellular growth rate, particularly when normalized to cellular protein concentration (Bremer & Dennis 1996). Given that rRNA comprises a large fraction of total cellular RNA in prokaryotes, it may also be expected to be related to growth rate in a relatively direct way (see Binder & Liu 1998). Several studies have used cellular RNA quotas of marine bacterial isolates or natural communities to make inferences about *in situ* growth rates among these organisms (Dortch et al. 1983, Kemp et al. 1993, Kramer & Singleton 1993). In the case of mixed microbial communities, bulk RNA measurements may be difficult to interpret due to varied growth rates among the mixed taxa. This problem can be ameliorated to some extent by measuring per-cell rRNA using 16S rRNA probes (Lee & Kemp 1994).

The relationship between growth rate and RNA content in cyanobacteria was first explored in the thermophilic freshwater *Synechococcus* strain PCC6301 (formerly *Anacystis*

nidulans)(e.g. Mann & Carr 1974, Parrott & Slater 1980, Lepp & Schmidt 1998). Cellular RNA content in this strain was observed to increase with growth rate, although the exact form of the relationship differed among studies (being reported as exponential, sigmoidal, or linear).

Binder and Liu (1998) examined the relationship between growth rate and cellular RNA content in a marine *Synechococcus*, WH8101. In this strain, cellular rRNA (and total RNA) levels were related to growth rate in a tri-phasic manner: at growth rates <~0.7 day^{-1}, rRNA $cell^{-1}$ remained approximately constant; at growth rates between ~0.7 and 1.6 day^{-1}, rRNA $cell^{-1}$ increased with increasing growth rate; and at growth rates >~1.6 day^{-1}, rRNA $cell^{-1}$ dropped abruptly. This same sort of tri-phasic relationship was observed subsequently in two other marine *Synechococcus* strains (WH7803 and WH8007) and in a *Prochlorococcus* strain (Med4) (Worden & Binder 2003), but not in *Synechococcus* WH8103 (Lepp & Schmidt 1998).

Given the apparently complex nature of the RNA versus growth rate relationship in marine *Synechococcus* and *Prochlorococcus*, and the relatively restricted set of strains that have been investigated in this context to date, it is clear that further characterization of the relationship is required before RNA-based growth rate estimates for natural picoplankton populations can be undertaken. Here we present results regarding cellular RNA content and growth rate in two strains representative of important picocyanobacterial groups in open-ocean environments: *Prochlorococcus* strain MIT9312 and *Synechococcus* strain WH8103. MIT9312 is a high-light adapted strain of *Prochlorococcus* with a relatively low Chl b/a_2 ratio (low-B/A ecotype from *Prochlorococcus* clade II), typical of upper mixed layer *Prochlorococcus* populations (Moore & Chisholm 1999, Zinser et al. 2006). Strain WH8103 is representative of open ocean *Synechococcus* (marine clade A, group III) in that it contains phycoerythrin as a major light harvesting pigment, has elevated salt requirements for growth, and is motile (Scanlan 2003).

Methods:

Culture growth. *Prochlorococcus* strain MIT 9312 was provided by S. W. Chisholm (Massachusetts Institute of Technology, Cambridge, MA, USA) and grown in seawater-based Pro99 Medium (see Moore et al. 2002). *Synechococcus* strain WH8103 was provided by J. B. Waterbury (Woods Hole Oceanographic Institution, Woods Hole, MA, USA) and grown in SN medium (Waterbury et al. 1986) prepared with artificial sea water (McLachlan 1964). It should be noted that neither the *Prochlorococcus* nor the *Synechococcus* isolates used in this study were axenic, though heterotrophic bacteria numbers were always <5% of the total cyanobacterial cell concentration. Semi-continuous 25 ml cultures were maintained at 25˚C under constant light in borosilicate tubes as described previously (Binder & Liu 1998). Light was provided by Cool-White fluorescent lamps. A range of photon flux densities between 2.3 and 143 µmol photons·m^{-2}·sec^{-1} was maintained by placing culture tubes in different locations within the incubator and shading with black nylon window screening. Light intensities were measured with a scalar PAR meter (Biospherical Instruments, Inc., San Diego, CA). Culture growth was monitored by *in vivo* fluorescence (Brand et al. 1981), using a Turner Designs model 10 fluorometer equipped with a chlorophyll analysis accessory kit (Turner Designs, Inc., Sunnyvale, CA). Specific growth rates (day^{-1}) were calculated as described previously (Binder & Liu 1998). Cultures were maintained at a given light level and growth rate for no less than 10 generations prior to sampling. Samples from at least three successive transfers of the same culture served as replicates for all analyses.

Cellular RNA Analysis. Cellular RNA content was assayed flow cytometrically using SYBR Green II as described in Binder and Liu (1998). Samples were preserved with 1% paraformaldehyde (final concentration) and frozen in liquid nitrogen for later analysis. Frozen

samples were thawed at approximately 30°C, resuspended in 90% methanol as described previously (Binder & Chisholm 1990), and extracted overnight at -20°C. Extracted samples were centrifuged (23,000 × g/10 min/10˚C) and resuspended in 100 µl phosphate buffered saline (PBS), spun again, resuspended in 500 µL PBS and incubated at 37°C with or without RNase I ($1U \cdot \mu l^{-1}$ final concentration; Ambion, Inc., Austin, TX) for 30 minutes. After the RNase treatment, samples were centrifuged once more and resuspended in 500 µL PBS plus potassium citrate (30 mM final concentration), stained with SYBR Green II (Molecular Probes, Inc., Eugene, OR) at a final concentration of 0.01% of the stock solution, and analyzed flow cytometrically. SYBR Green fluorescence of RNase-treated samples has been shown to reflect DNA content in *Synechococcus* (Binder & Liu 1998). The difference between this DNA-derived fluorescence and the fluorescence of stained samples not treated with RNase is taken to reflect RNA content.

Flow Cytometric Analysis. Stained samples were analyzed on a modified EPICS 753 flow cytometer (Beckman Coulter, Fullerton, CA) equipped with a 6-W Argon Ion laser tuned for blue excitation (488 nm, 1000 mW) and focused with a spherical lens assembly described previously (Binder et al. 1996). Green fluorescence from SYBR Green was collected through a 525 nm band-pass filter. Red fluorescence from chlorophyll was collected through a 680 nm band-pass filter and, together with forward angle light scatter (FALS) was used to identify *Prochlorococcus* and *Synechococcus* cells. Fluorescence and FALS signals were normalized to standard fluorescent polystyrene beads (0.474 µm diameter; Polysciences, Inc., Washington, PA) that were added to each sample. All data were collected as list modes and analyzed with WinList software (Verity Software House, Inc., Topsham, ME).

Protein Analysis. For bulk protein analysis, 23 ml (approximately 10^9 cells) of MIT9312 or WH8103 cultures were filtered onto previously ashed GF/F filters and stored at -85ºC until analysis. Blanks were prepared by filtering 23 ml of newly prepared medium through GF/F filters and storing as described above. Bulk protein concentrations were determined similar to Peterson (1977) using a commercial kit (Micro Lowry Total Protein Kit, Sigma-Aldrich Co. St. Louis, MO, USA); bovine serum albumin was used as the protein standard. All samples were corrected for measured blank protein values, which corresponded to less than 20 and 14 % of the total protein concentration measured per sample for MIT9312 and WH8103 medium, respectively.

Curve Fitting. A non-linear curve fitting algorithm (SigmaPlot Software, SPSS Inc., Chicago,IL) was used to fit the following modified beta function (Johnson et al. 1995) to the growth rate versus RNA relationships as described previously (Worden & Binder 2003):

$$RNA \cdot cell^{-1} = a + b(c \cdot \mu)^p (1 - c \cdot \mu)^q \qquad \text{(Equation 1)}$$

where μ is the specific growth rate and a, b, c, p, and q are fitted parameters. The choice of this equation is entirely empirical, and based upon its ability to describe the previously observed non-linear trends discussed above. Analysis of variance was used to test the significance of this non-linear fit, relative to a simple linear model.

Results and Discussion:

Culture Growth. The relationships between irradiance levels and growth rate for *Prochlorococcus* strain MIT9312 and *Synechococcus* strain WH8103 were similar to those reported previously by us for these strains, growing under the same conditions (Burbage & Binder 2007) (Fig. 2.1). Growth rates varied between 0.29-0.94 and 0.22-1.34 day^{-1} for MIT9312 and WH8103 respectively, and in both cases the μ versus I relationship was well

described by a hyperbolic tangent function (Jassby & Platt 1976) (MIT9312: r^2=0.97, $p<0.0005$; WH8103: r^2=0.96, $p<0.0005$). Neither MIT9312 nor WH8103 showed any signs of photoinhibition under the range of light intensities employed here.

The maximum MIT9312 growth rate observed here (and in Burbage & Binder 2007), 0.94 d^{-1}, was somewhat higher than previously reported for this strain (0.78 d^{-1})(Moore & Chisholm 1999) and for MED4, a closely related *Prochlorococcus* strain (0.63–0.74 d^{-1})(see Moore et al. 1995, Moore & Chisholm 1999, Worden & Binder 2003). This likely reflects the fact that in these other studies cells were growing under a 14:10 L:D cycle, whereas in the present case they were exposed to constant light. Likewise, the previously reported maximum growth rate for *Synechococcus* WH8103 growing under a L:D cycle (1.0 d^{-1})(Moore et al. 1995) is lower than that observed here (1.3 d^{-1}) under constant light.

Cellular growth rate versus RNA. The relationship between growth rate and total cellular RNA content for MIT9312 and WH8103 is shown in Fig. 2.2. In MIT9312 the relationship appeared to be comprised of two phases: at growth rates below approximately 0.6 d^{-1} cellular RNA levels remained relatively constant, whereas at growth rates above this point cellular RNA increased approximately linearly. In WH8103 cellular RNA levels appeared to increase linearly with increasing growth rate over the entire range of growth rates tested (r^2=0.92, $p<0.0005$).

The relationship between cellular RNA levels and growth rate we observed for MIT9312 is reminiscent of the non-linear relationship reported by Worden and Binder (2003) for *Prochlorococcus* strain MED4 (a closely related, high light-adapted strain). In that study, rRNA $cell^{-1}$ was relatively stable at low growth rates, and increased linearly at intermediate rates.

However at the highest growth rate (~0.8 d^{-1}), rRNA $cell^{-1}$ decreased dramatically. We observed no such decrease in RNA $cell^{-1}$ in MIT9312.

The linear relationship between cellular RNA and growth rate reported here for WH8103 is consistent with the observations of Lepp and Schmidt (1998) for the same *Synechococcus* strain grown under similar conditions (though over a narrower range of growth rates). As described in the Introduction, however, this linear relationship is by no means universal among *Synechococcus* strains, a number of which appear to show a non-linear relationship similar to that described for *Prochlorococcus* above (Binder & Liu 1998, Worden & Binder 2003)(however see Normalized Cellular RNA versus Growth Rate, below).

Cellular protein. Cellular RNA content alone is unlikely to serve as a robust proxy for growth rate among different *Prochlorococcus* or *Synechococcus* strains: all other things being equal, larger cells should be expected to have higher RNA levels than smaller cells at a given growth rate. Therefore numerous metrics have been used to normalize cellular RNA levels to cellular biomass, including cell volume (Binder & Liu 1998, Lepp & Schmidt 1998), light scatter (Worden & Binder 2003), and dry weight (Parrott & Slater 1980, Kramer & Morris 1990). In the present study, we normalize RNA to protein content. As explained previously, protein is a particularly appropriate normalizing factor because RNA/protein can be expected to be proportional to growth rate, assuming balanced growth and constant ribosome efficiency.

In both *Prochlorococcus* and *Synechococcus,* cellular protein content was greatest at the lowest growth rates (54 and 288 fg protein·$cell^{-1}$ in MIT9312 and WH8103 respectively) and generally decreased to minimum values (31 and 166 fg protein·$cell^{-1}$) as growth rate increased (Fig. 2.3). Overall, mean cellular protein content of each strain varied approximately 1.7-fold over the range of experimental growth rates employed in this study. The relationship between

growth rate and protein content for *Synechococcus* strain WH8103 was reasonably well described by a linear regression model (r^2=0.85, $p<0.005$). In the case of MIT9312 there also appeared to be a linear decrease in cellular protein content as growth rates progressed from low to moderate values; however protein content abruptly increased again at the highest experimental growth rates (0.92 and 0.94 day^{-1})(Fig. 2.3).

Generalities about the relationship between biomass and growth rate in picocyanobacteria are difficult to make. The present study presents the first examination of this relationship in a *Prochlorococcus* strain, but observations are available for a number of *Synechococcus* strains. The expectation from model heterotrophic prokaryotes is that mean cell size will increase with growth rate (Bremer & Dennis 1996). This pattern has indeed been observed in a few *Synechococcus* strains (e.g. Mann & Carr 1974, Lepp & Schmidt 1998). However, in other studies (of the same strains in some cases), cellular biomass has been found to decrease with increasing growth rate (e.g. Kana & Glibert 1987), or to exhibit a more complex relationship with growth rate (e.g. Parrott & Slater 1980, Kramer & Morris 1990, Binder & Liu 1998). Given the differences in biomass measures (e.g. volume, dry weight, C content) and growth conditions employed in these various studies, it is perhaps not surprising that a coherent picture of the biomass versus growth relationship in *Synechococcus* has not yet emerged. Our observations of generally decreasing protein biomass with increasing growth rate in both WH8103 and MIT9312 is consistent with the previously reported trends in carbon biomass in these same strains growing under the same conditions (Burbage & Binder 2007).

Normalized Cellular RNA Versus Growth Rate. When normalized to cellular protein, RNA levels in MIT9312 appeared to vary with growth rate in a non-linear , tri-phasic fashion, such that the increase in RNA·$protein^{-1}$ accelerated up until a growth rate of ~0.8 d^{-1}, beyond

which it decreased abruptly (Fig. 2.4). This trend was well-described by the modified beta function, which accounted for significantly more of the overall variation than did a linear function (Table 2.1). In WH8103, RNA·protein^{-1} increased approximately linearly with growth rate (Fig. 2.4); in this case the beta function did not explain significantly more than a simple linear model (Table 2.1).

The tri-phasic trend observed here in MIT9312 is remarkably similar to that reported by Worden and Binder (2003) for MED4, a closely related *Prochlorococcus* strain, despite differences in growth conditions (L:D vs. constant light), RNA assay (rRNA-targeted probes vs. SyberGreen II), and biomass normalization (FALS vs. protein) employed in these two studies (Fig. 2.5A, Table 2.1). The biological significance of this non-linearity, particularly the abrupt decrease in RNA/protein at the maximum growth rate, is unclear at present. Binder and Liu (1998) hypothesized that this phenomenon might be related to the transition from light-limited to light-saturated growth. Further data are required before this hypothesis can be evaluated in MED4 or MIT9312. In any case, the abrupt decrease in RNA/protein at growth rates close to the maximum reflects a corresponding increase in the apparent ribosome efficiency at the these growth rates (see below).

Previous studies have suggested that biomass-normalized RNA varies non-linearly with growth rate in marine *Synechococcus* as well (Binder & Liu 1998, Worden & Binder 2003). However among the few strains studied, the evidence for non-linearity in *Synechococcus* is in fact relatively weak: re-analysis of the original data from these studies indicates that the modified beta function provides a statistical improvement over the simple linear model in only one case (WH8101)(Table 2.1, Fig. 2.5B&C). The basis of this difference among *Synechococcus* strains is not clear: it does not appear to be related to phylogeny, growth conditions, or RNA assay

employed. The possibility that it reflects differences in the chosen biomass-normalization cannot be excluded at present: the single significantly non-linear relationship was observed in the only study in which RNA was normalized to cell volume (Binder & Liu 1998) as opposed to forward light scatter or protein (Worden & Binder 2003, present study)). We have found that the latter two parameters are well-correlated in *Prochlorococcus* and *Synechococcus* (not shown). Given the very limited data currently available, the extent to which the observed differences in biomass-normalized RNA vs. growth rate reflect true differences among closely related *Synechococcus* strains, or rather are the result of differences in the biomass metric employed, remains to be seen.

Apparent Ribosome Efficiency. Ribosome efficiency (defined here as protein synthesis rate per ribosome) underlies the relationship between rRNA content and growth rate. Given growth rate, cellular rRNA, and cellular protein content, and assuming balanced growth and invariant protein turnover rate, ribosome efficiency can be calculated (Schleif 1967, Bremer & Dennis 1996, Binder & Liu 1998). For the present case, if we further assume that total RNA:rRNA is relatively constant (Binder & Liu 1998), an apparent ribosome efficiency can be calculated as $\mu \cdot P/R$, where P and R are the cellular protein and RNA content, respectively. Because R in this study is a relative measure, the calculated apparent ribosome efficiency is in relative units as well.

Apparent ribosome efficiency varied relatively little (83 ± 7) for *Synechococcus* over the range of growth rates examined (Fig. 2.6). In *Prochlorococcus*, relative overall variation was higher (37 ± 11), owing largely to an abrupt increase in efficiency at the highest growth rates. This increase is clearly the mathematical result of the decrease in RNA/protein that occurred at these same growth rates (Fig. 2.4). In both *Synechococcus* and *Prochlorococcus*, there is a

suggestion that apparent ribosome efficiency decreases (albeit slightly) at the lowest growth rates examined (Fig. 2.6). Such decreases have been observed in slowly growing *E. coli* (Koch 1970, Ingraham et al. 1983) and in *Synechococcus* WH8101 (see below). In the case of *E. coli* this phenomenon has been attributed to the presence of a pool of inactive ribosomes, rather than a decrease in efficiency among all ribosomes; its significance in *Synechococcus* remains to be established.

The relatively constant apparent ribosome efficiency in *Synechococcus* WH8103 sharply contrasts the observations of Binder & Liu (1998) for WH8101, in which this efficiency varied linearly with growth rate, and changed by a factor of approximately 6 overall. This discrepancy may be explained in part by the fact that in the latter study cellular protein content was not directly measured, but rather was assumed to be proportional to cell volume. Nevertheless, it seems unlikely that variation in the protein:volume ratio would be sufficient to explain the variation in calculated ribosome efficiency, and therefore these results may reflect real differences between the way rRNA, protein, and growth rate are regulated in these two strains. Note that WH8101 seems exceptional among *Synechococcus* in its relationships between both cell size and RNA versus growth rate, as discussed above.

Apparent ribosome efficiency was considerably lower in *Prochlorococcus* than in *Synechococcus*, regardless of any growth rate-related variation (Fig. 2.6). This reflects the fact that RNA/Protein is considerably higher in the latter (Fig. 2.4). It is theoretically possible that these contrasts might be related to differences in the rRNA:total RNA ratio in these two strains, rather than differences in ribosome efficiency. However, a similar contrast between *Prochlorococcus* and *Synechococcus* biomass-normalized RNA was reported by Worden & Binder (2003), who measured rRNA directly. Therefore, it appears that *Prochlorococcus* and

Synechococcus may differ fundamentally in the protein synthesizing efficiency of their ribosomes.

Conclusions. The data and analysis presented here suggest that the relationship between cellular RNA content and growth rate may be different in *Prochlorococcus* and *Synechococcus*. Although previous studies have indicated that this relationship is distinctly non-linear in marine *Synechococcus* strains, re-analysis of that data combined with the new data presented here indicate that linearity in the relationship may be the rule rather than the exception in *Synechococcus*. It is not possible to exclude the tri-phasic model suggested previously (as represented here by the modified beta function), and in fact for one strain (WH8101) there is strong evidence favoring that model (Binder & Liu 1998). In the 3 other strains examined to date, however, a simple linear model explains the data just as well (statistically speaking) as the more complex model. This suggests that biomass-normalized RNA might be reasonably used as a proxy for growth rate in natural populations. However, owing to the variety of RNA and biomass metrics employed to date, we cannot assess at present whether a single normalized-RNA vs. growth calibration will work for the range of *Synechococcus* strains likely to comprise a natural community.

The story for *Prochlorococcus* is substantially different: in this case, only two strains have been examined, but in both cases biomass-normalized RNA was clearly related to growth rate in the tri-phasic manner described earlier. This non-linear relationship would likely hamper attempts to use RNA as an indicator of growth rate in natural *Prochlorococcus* populations. *Prochlorococcus* also appears to differ from *Synechococcus* with respect to ribosome efficiency: data from this study and from Worden & Binder (2003) indicate that biomass-normalized

RNA/protein is higher, and that ribosome efficiency is lower in *Prochlorococcus*, regardless of growth rate.

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Table 2.1. Comparison of linear and non-linear (modified Beta model, see text) regressions of biomass-normalized rRNA (or RNA) vs. growth rate in *Prochlorococcus* and *Synechococcus* in the present and previously published studies.

Group [1]	Isolate	Measured Parameter [2]	n	Beta Model r^2	Beta Model p-value	Linear Model r^2	Linear Model p-value	Model Comparison [3] p-value	Ref [4]
Synechococcus									
Marine Clade-A	WH8103	RNA prot^{-1}	8	0.99	0.004	0.99	<0.0001	0.46	*a*
	WH7803	rRNA FALS^{-1}	14	0.67	0.022	0.65	0.0005	0.79	*b*
Marine Clade-B	WH8101	rRNA, RNA vol^{-1}	16	0.84	0.0002	0.64	0.0002	0.008	*c*
	WH8007	rRNA FALS^{-1}	10	0.93	0.004	0.87	<0.0001	0.30	*b*
Prochlorococcus									
Clade II Low-B/A	MIT9312	RNA prot^{-1}	7	0.99	0.021	0.62	0.036	0.008	*a*
Clade I Low-B/A	MED4	rRNA FALS^{-1}	6	0.99	0.083	0.39	0.184	0.014	*b*

[1] Phylogenetic grouping per Rocap *et al.* 2002 and Scanlan *et al.*2003 for *Prochlorococcus* and *Synechococcus*, respectively.

[2] Prot: protein, FALS: Forward Angle Light Scatter, vol: cell volume.

[3] Significance of additional explanatory power of the modified Beta function (df = n - 5) as compared with a simple linear regression (df = n-2)(F-Test).

[4] References: (*a*) present study, (*b*) Worden & Binder 2003, (*c*) Binder & Liu 1998.

Figure Legends:

FIG. 2.1. Relationship between specific growth rate and light intensity for *Prochlorococcus* strain MIT9312 (closed symbols) and Synechococcus strain WH8103 (open symbols). Standard errors calculated from successive transfers of each culture, as described in Methods. Lines are best-fit hyperbolic tangent function for *Prochlorococcus* and *Synechococcus* cultures, respectively. Error bars not shown are contained within the symbols.

FIG. 2.2. Variation in total cellular RNA content with growth rate in *Prochlorococcus* strain MIT9312 (closed symbols) and *Synechococcus* strain WH8103 (open symbols). Means and error bars as in Fig. 2.1. Broken line shows linear regression for *Synechococcus* data. Note scale differences for the two cell types.

FIG. 2.3. Relationship between cellular protein content and growth rate in *Prochlorococcus* strain MIT9312 (closed symbols) and *Synechococcus* strain WH8103 (open symbols). Means and error bars as in Fig. 2.1. Line shows linear regression of *Synechococcus* data. Note different y-axis scales for *Prochlorococcus* and *Synechococcus*.

FIG. 2.4. Relationship between protein-normalized cellular RNA and growth rate in *Prochlorococcus* strain MIT9312 (closed symbols) and *Synechococcus* strain WH8103 (open symbols). Error bars as in Fig. 2.1. Lines show linear regression (broken) and modified beta function fit (solid) for *Synechococcus* and *Prochlorococcus*, respectively.

FIG. 2.5. Comparison of the relationships between biomass-normalized RNA (or rRNA) and growth rate in marine *Synechococcus* and *Prochlorococcus* in this and previous studies. Both X and Y values are scaled relative to the maximum reported values for each strain in each study. See Table 1 for references and details about measured parameters. Lines show linear regressions or (if they provide a better fit – see Table 2.1) modified beta-function fits. (A) *Prochlorococcus* Clade I and II low b/a strains MED4 and MIT9312, respectively; (B) *Synechococcus* Marine Clade A strains WH7803 and WH8103; (C) *Synechococcus* Marine Clade B strains WH8007 and WH8101.

FIG. 2.6. Apparent ribosome efficiency versus growth rate in *Prochlorococcus* strain MIT9312 (closed symbols) and *Synechococcus* strain WH8103 (open symbols). Ribosome efficiency calculated from the RNA and protein $cell^{-1}$ data, as described in text.

FIG. 2.1

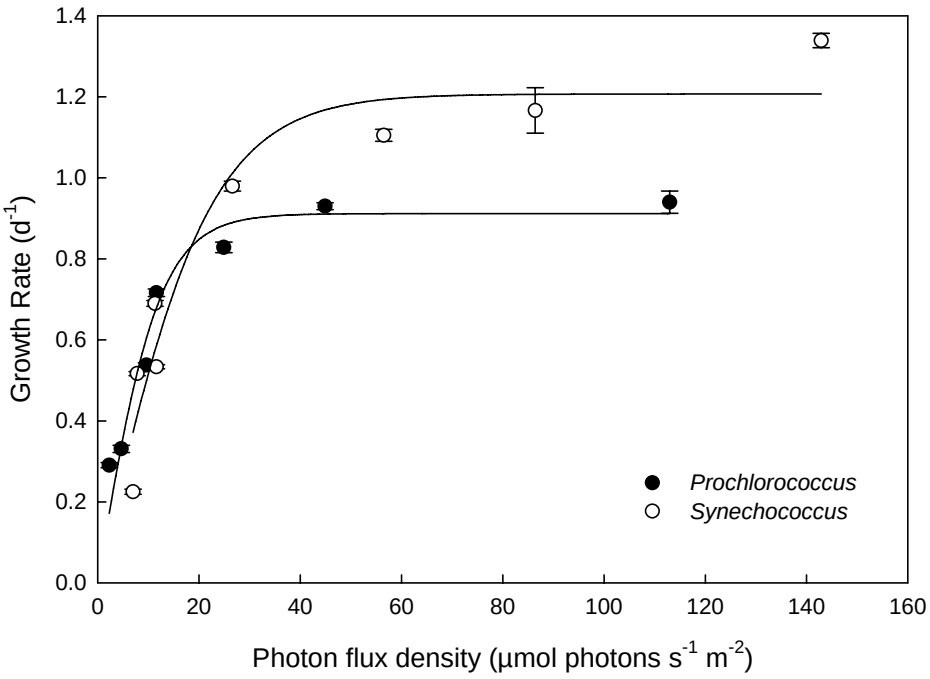

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Growth Rate (d-1)
0
20
40
60
80
100
120
140
160
Photon flux density (µmol photons s-1 m-2)
Prochlorococcus
Synechococcus

FIG. 2.2

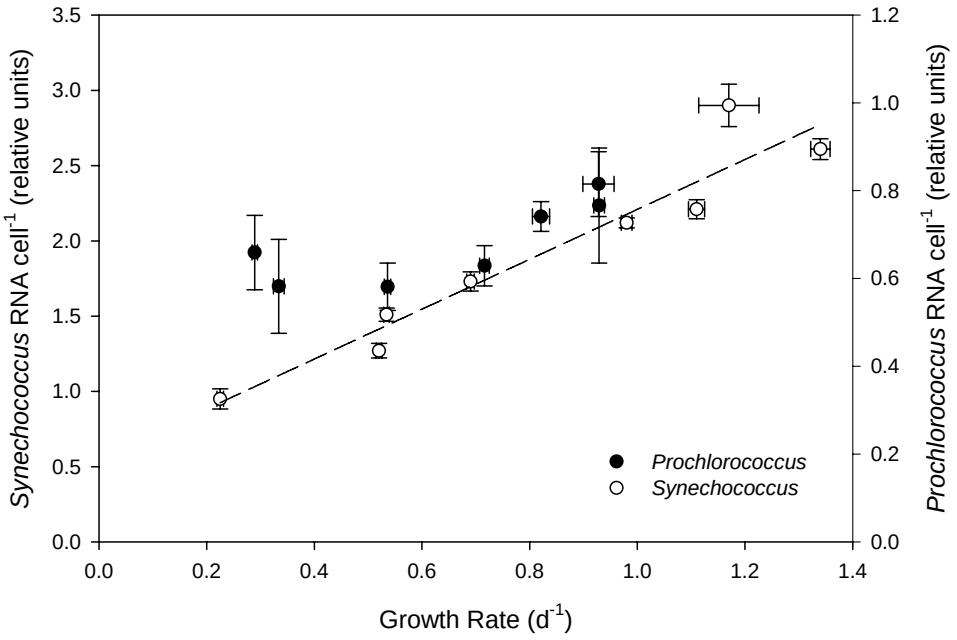

Synechococcus RNA cell-1 (relative units)
Prochlorococcus RNA cell-1 (relative units)
Growth Rate (d-1)
Prochlorococcus
Synechococcus
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
0.2
0.4
0.6
0.8
1.2
1.4

FIG. 2.3

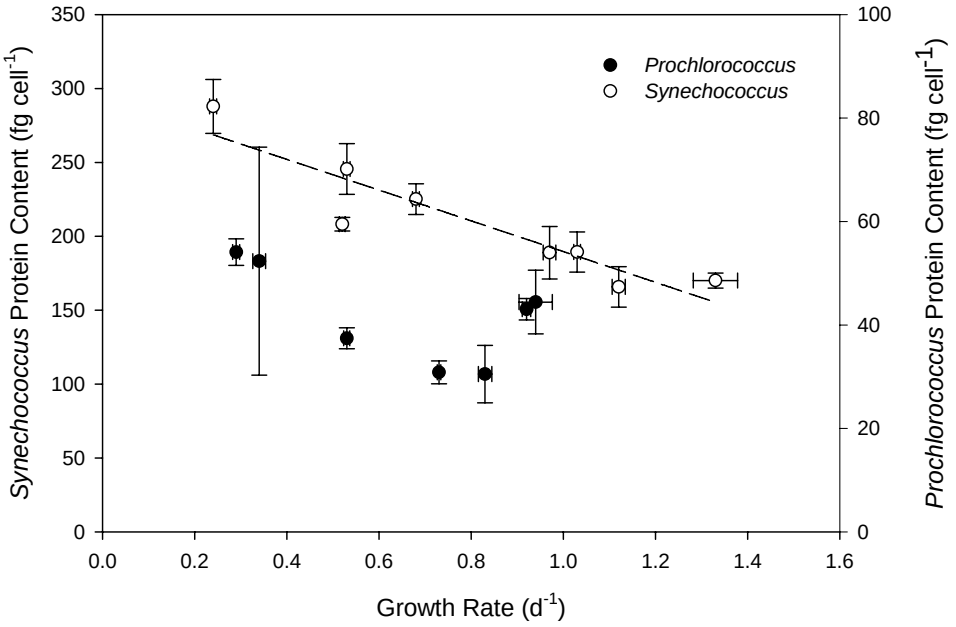

Prochlorococcus
Synechococcus
Synechococcus Protein Content (fg cell-1)
Prochlorococcus Protein Content (fg cell-1)
Growth Rate (d-1)
0
50
100
150
200
250
300
350
0
20
40
60
80
100
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6

FIG. 2.4

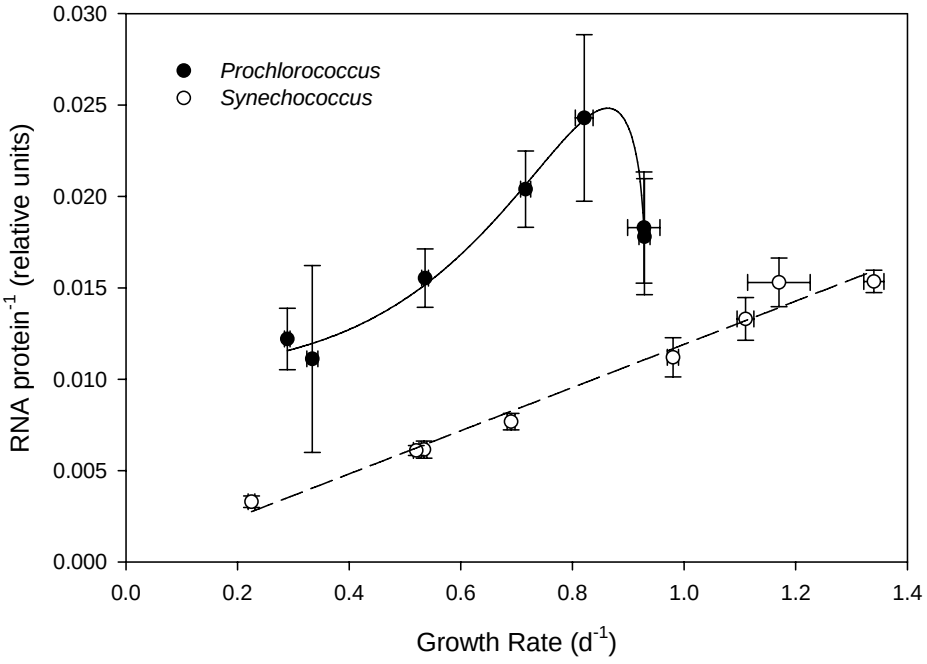

0.030
0.025
0.020
0.015
0.010
0.005
0.000
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Prochlorococcus
Synechococcus
RNA protein-1 (relative units)
Growth Rate (d-1)

FIG. 2.5

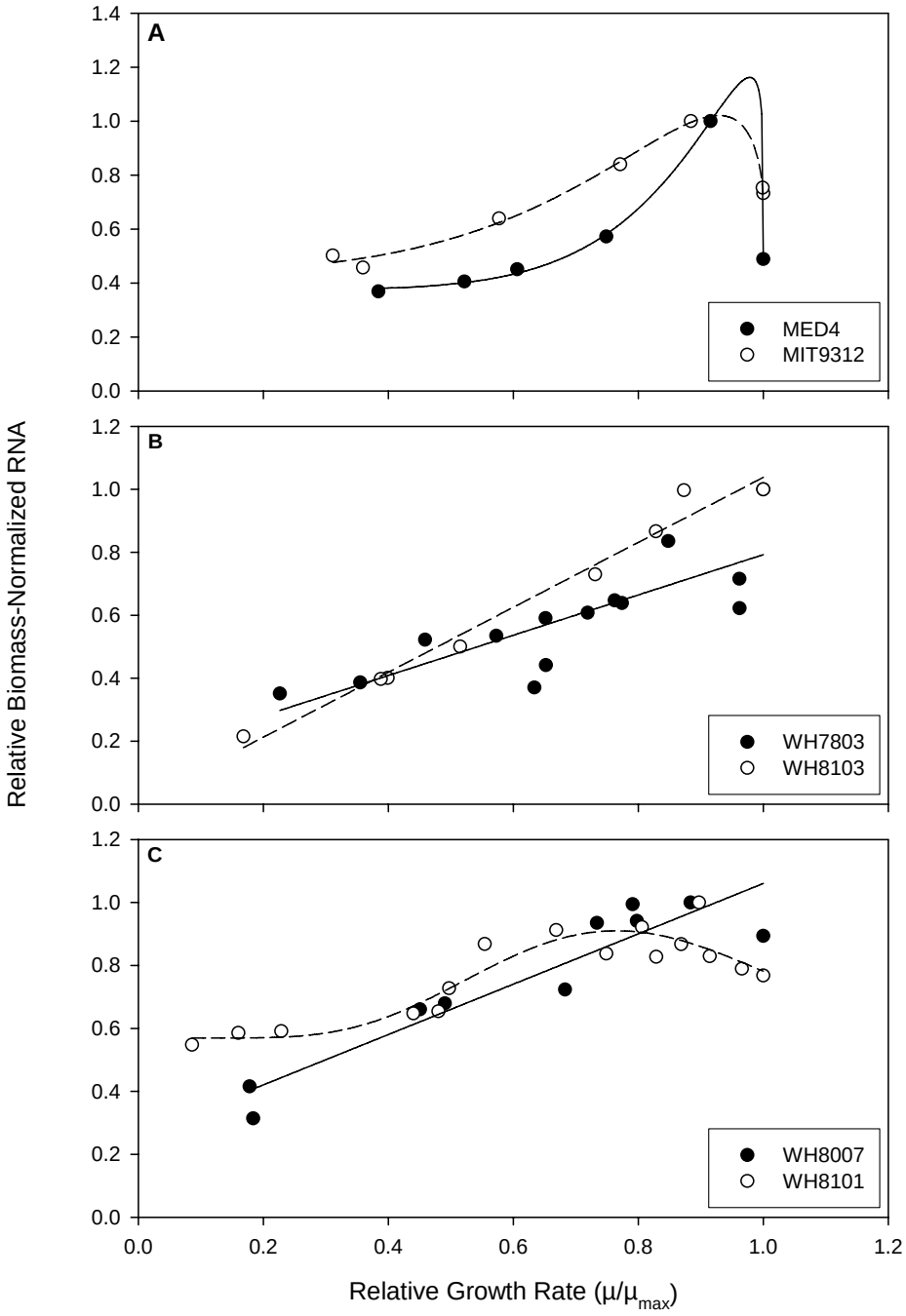
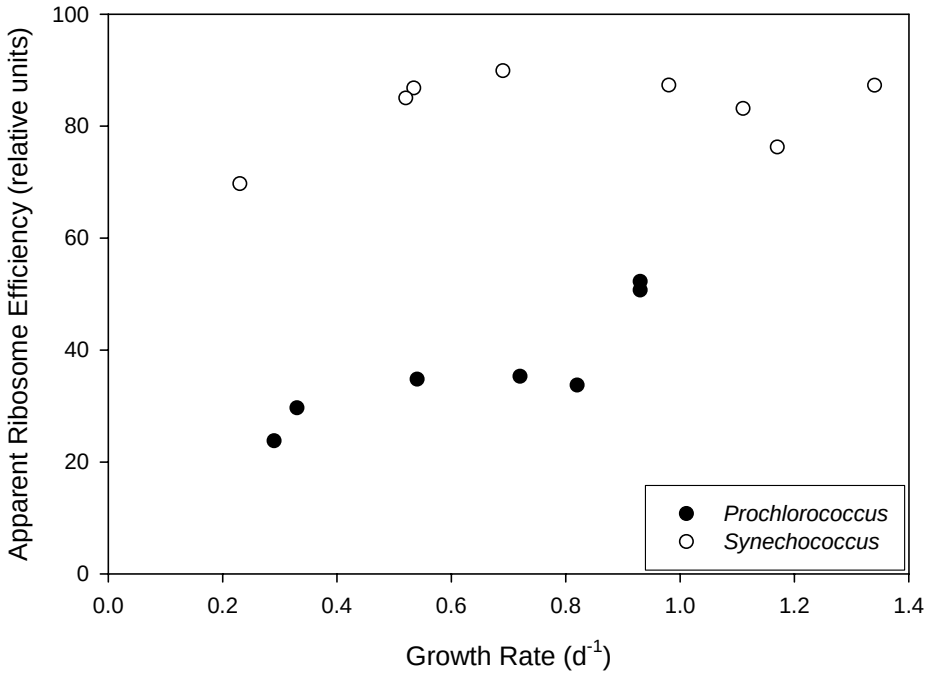

A
MED4
MIT9312
B
WH7803
WH8103
C
WH8007
WH8101
Relative Biomass-Normalized RNA
Relative Growth Rate (μ/μmax)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4

Fig. 2.6


Apparent Ribosome Efficiency (relative units)
100
80
60
40
20
0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Growth Rate (d-1)
Prochlorococcus
Synechococcus

CHAPTER 3

RELATIONSHIP BETWEEN CELL CYCLE AND LIGHT-LIMITED GROWTH RATE IN *PROCHLOROCOCCUS* (MIT9312) AND *SYNECHOCOCCUS* (WH8103) (CYANOBACTERIA)[1]

[1] Burbage, C.D., and Binder, B.J. 2007. *Journal of Phycology*, 43(2):266-274. Reprinted here with permission of publisher.

Abstract:

Prochlorococcus (strain MIT9312) and *Synechococcus* (strain WH8103) cell cycle behaviors were compared over a wide range of light-limited growth rates. These two species displayed a notable degree of similarity with respect to the various cell cycle parameters examined. The presence of bimodal DNA distributions across the entire range of growth rates examined indicates that overlapping rounds of chromosome replication do not occur in either of these species. Chromosome replication time, *C*, was constrained to a fairly narrow range of values (4.7±1.1 and 4.0±1.0 h for MIT9312 and WH8103, respectively), and did not appear to vary with growth rate. The post-DNA replication period, *D*, was maximal (10-20 hours) in both strains at the lowest growth rates, and decreased monotonically with increasing growth rate to minimum values of 2-3 h. The combined duration of the chromosome replication and post replication periods (*C* + *D*), a quantity often used in the estimation of *Prochlorococcus in situ* growth rates, varied approximately 2.4 fold over the range of growth rates examined. In both strains cell mass was highest at the lowest growth rates (75 and 260 fg C·cell^{-1} in MIT9312 and WH8103, respectively) and decreased 2-3 fold as growth rates increased. Cell mass was well correlated with forward angle light scatter (FALS). Cell mass at the start of replication appeared to decrease with increasing growth rate, indicating that the initiation of chromosome replication in *Prochlorococcus* and *Synechococcus* is not a simple function of cell biomass, as previously suggested.

Acknowledgements:

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Introduction:

Prochlorococcus spp. and *Synechococcus* spp. are two of the most abundant photosynthetic microorganisms in the world's oceans. Together these two closely related genera account for a considerable amount of the photosynthetic biomass (Waterbury et al. 1986, Partensky et al. 1999, Zubkov et al. 2000, DuRand et al. 2001) and primary production (Chavez 1989, Li et al. 1992, Maranon et al. 2003) in open ocean ecosystems. Natural populations of *Prochlorococcus* and *Synechococcus* have been shown to be composed of a variety of genetically and physiologically distinct ecotypes that occupy different ecological niches (Moore et al. 1998, Moore and Chisholm 1999, Scanlan 2003, Toledo and Palenik 2003, Johnson et al. 2006, Zinser et al. 2006).

Despite their ecological importance we still have only a rudimentary understanding of the factors that regulate *Prochlorococcus* and *Synechococcus* population growth in the field. It has been well established through laboratory and field experiments that these two groups exhibit strong diel patterns that are linked to their cell division cycle (Partensky et al. 1999, Jacquet et al. 2001a, Binder and DuRand 2002). In general *Synechococcus* and *Prochlorococcus* divide in the early to late afternoon and the late afternoon to early evening period, respectively (Jacquet et al. 2001a, Binder and DuRand 2002). This pattern of phased cell division, combined with the fact that cellular biomass increase is confined to the light period, results in a strong diel pattern in mean cell size in *Prochlorococcus* and *Synechococcus* populations (Olson et al. 1990, Vaulot et

al. 1995, DuRand and Olson 1996, Jacquet et al. 2001a, Binder and DuRand 2002). In turn, this pattern is likely to influence protozoan grazing on these populations, the rates of which have been shown to be sensitive to prey size (e.s. Gonzalez et al. 1990, Kinner et al. 1998). Thus, cell cycle dynamics in *Prochlorococcus* and *Synechococcus* can be expected to influence both population growth and mortality in natural systems.

To date there have been only a relatively limited number of studies addressing marine cyanobacterial cell cycle regulation (see review in Asato 2003). Among most marine *Synechococcus* strains examined thus far cell cycle behavior is consistent with the slow-growth case of the prokaryotic cycle model developed by Cooper and Helmstetter (1968), in which cells inherit exactly one chromosome copy, and rounds of chromosome replication are non-overlapping. This behavior is (at least superficially) indistinguishable from the typical eukaryotic cell cycle, and predicts that in an exponentially growing population the frequency distribution of DNA·cell^{-1} will be bimodal (Slater et al. 1977). A number of marine *Synechococcus* strains have been shown to display such bimodal DNA distributions (Binder and Chisholm 1995). In the well-studied coastal *Synechococcus* strain WH8101, bimodal distributions occur over a wide range of light- and N-limited growth rates (Armbrust et al. 1989, Binder 2000). Chromosome replication time (*C* in the Cooper-Helmstetter model) in this strain remains approximately constant in the face of varying growth rates, while the lengths of pre- and post-replication periods (*B* and *D*) both increase with decreasing growth rate. Binder (2000) suggested that the initiation of replication in WH8101 may be linked to the achievement of a specific cell size, as it is in *E. coli* (Donachie 1968, Donachie and Blakely 2003), and that this critical size remains approximately constant except at very slow growth rates.

In contrast, Binder and Chisholm (1995) showed that *Synechococcus* strain WH7803, an open ocean strain (marine cluster A, group V (Scanlan 2003)), contains a multimodal DNA distribution that indicates the presence of more than two genome copies per cell. This distribution is very similar to that observed in the freshwater *Synechococcus* strain PCC 6301 (Binder and Chisholm 1990) and in specific mutants of *E. coli* in which the initiation of replication among multiple chromosome copies within each cell is asynchronous (Skarstad and Boye 1988). It should be noted that Liu et al. (1999) observed no such multimodal DNA distributions in N-limited WH7803 populations.

DNA distributions of all strains of *Prochlorococcus* examined thus far have been bimodal, again suggesting cell cycle regulation that is consistent with the slow growth case of the Cooper-Helmstetter model, involving discrete chromosome replication and pre- and post-replication periods. To date there has been no systematic study of the influence of growth rate on the length of these cell cycle periods in *Prochlorococcus*.

In the present paper we examine the influence of light-limited growth rate on the cell cycle behavior of *Prochlorococcus* strain MIT9312 and *Synechococcus* strain WH8103 growing exponentially under constant light. MIT9312 is a high-light adapted strain of *Prochlorococcus* with a relatively low Chl b/a_2 ratio (low-B/A ecotype from *Prochlorococcus* clade II), typical of surface *Prochlorococcus* populations (Moore and Chisholm 1999, Zinser et al. 2006). Strain WH8103 is typical of open ocean *Synechococcus* (from marine cluster A, group III) in that it contains phycoerythrin as a major light harvesting pigment, has elevated salt requirements for growth, and is motile (Scanlan 2003).

Methods:

Culture growth. *Prochlorococcus* strain MIT 9312 was provided by S. W. Chisholm (Massachusetts Institute of Technology, Cambridge, MA, USA) and grown in seawater-based Pro99 Medium (per Moore et al. 2002). *Synechococcus* strain WH8103 was provided by J. B. Waterbury (Woods Hole Oceanographic Institution, Woods Hole, MA, USA) and grown in SN medium (per Waterbury et al. 1986) prepared with artificial seawater (see Goldman and McCarthy 1978). Semi-continuous 25 ml cultures were maintained at 25˚C under constant light in 25 mm borosilicate tubes as described previously (Binder and Liu 1998). Light was provided by Cool-White fluorescent lamps. A range of photon flux densities between 2.3 and 143 µmol photons·m^{-2}·sec^{-1} were maintained by placing culture tubes in different locations within the incubator and shading with black nylon window screening. Light intensities were measured with a scalar PAR meter (Biospherical Instruments, Inc., San Diego, CA). Culture growth was monitored by *in vivo* fluorescence (Brand et al. 1981), using a Turner Designs model 10 fluorometer equipped with a chlorophyll analysis accessory kit (Turner Designs, Inc., Sunnyvale, CA). Specific growth rates (day^{-1}) were calculated as described previously (Binder and Liu 1998).

Cellular DNA Analysis. For DNA analysis, cells were preserved with 1% paraformaldehyde (final concentration) and frozen in liquid nitrogen for later analysis via flow cytometry (Vaulot et al. 1989). Preserved cells were thawed at approximately 30˚C for 5 minutes and then stained with Hoechst 33342 (Molecular Probes, Eugene, OR) at a final concentration of 0.5 µg·ml^{-1} and analyzed flow cytometrically. A modified EPICS-753 flow cytometer (Beckman Coulter, Fullerton, CA) equipped with a 6-W Argon Ion laser tuned for UV output (280 mW) and focused with a confocal lens assembly was used for this analysis. Hoechst

fluorescence was measured between 408 and 475 nm; forward angle light scatter (FALS) was measured with a custom collection lens and photo multiplier tube (PMT) set-up similar to that described by Binder et al. (1996). Single-parameter histograms of Hoechst fluorescence for *Prochlorococcus* and *Synechococcus* cells represent the DNA frequency distributions for these populations and were used for all further cell cycle analysis. Calibration beads (1 and 2 μm Flow Check high intensity green alignment grade particles, Polysciences, Inc., Washington, PA) were added to all cell cycle analysis samples as internal standards; cellular FALS and Hoechst fluorescence were normalized to these beads.

Cell Cycle Analysis. ModFit software (Verity Software House, Topsham, ME) was used to deconvolute single parameter DNA frequency distributions into *g1*, *s*, and *g2* subpopulations as in Binder (2000). Briefly, a nonlinear curve fitting algorithm was applied to fit two gaussian curves (with equal coefficients of variation) and a broadened trapezoid to the observed DNA distribution. The sizes of these constituent subpopulations were then used to estimate the prokaryotic cell cycle parameters *B* (time between cell birth and the start of chromosome replication), *C* (chromosome replication time), and *D* (time between the end of chromosome replication and cell division) (Cooper and Helmstetter 1968, Helmstetter 1996) based on the equations of Slater et al. (1977).

CHN Analysis. For CHN analysis 23 ml (approximately 10^9 cells) of MIT9312 or WH8103 cultures were filtered onto previously ashed GF/F filters and then stored at -85˚C until analysis. Blanks were prepared by filtering 23 ml of newly prepared culture medium through ashed GF/F filters, and stored as described above. Particulate C and N were measured on a Carlo Erba CHN NA1500 Analyzer (Carlo Erba Instruments Milan, Italy) using a poplar leaf standard (48.16 %C and 2.59 %N) as the reference material. All samples were corrected for measured

blank C and N values, which correspond to approximately 9 μg C and 4 μg N·filter^{-1} for MIT9312 medium, and 14 μg C and 1 μg N·filter^{-1} for WH8103 medium.

Initiation Biomass Calculation. Cell size at the time of the initiation of chromosome replication was estimated from the mean biomass of the asynchronous populations as described in Binder (2000). Briefly, the ratio of the initiation mass to the average cell mass in an exponentially growing culture was calculated as:

$$M_i / M_{avg} = \frac{2^{B/T_d}}{2\ln(2)} \qquad \text{(Equation 1)}$$

where T_d is the generation time and B is as defined above (Wold et al. 1994). The initiation mass, M_i was then calculated as the product of this ratio and the measured average cell mass (fg C·cell^{-1}). In this paper we refer to this carbon-based estimate of M_i as the $M_{i(C)}$.

A second approach for estimating M_i, based on the FALS of *s*-phase cells, and independent of calculated cell cycle parameters and mean biomass measures, was also employed. Within the *s*-phase subpopulation, log FALS was regressed against DNA content, and the biomass at initiation taken to correspond to the extrapolated FALS value at a DNA content of 1 genome equivalent (represented by $FALS_i$)(see Boye et al. 1996, Binder 2000). These FALS values were converted to biomass using the observed relationship between mean FALS and measured cell mass among the experimental cultures. We refer to this M_i estimate as $M_{i(FALS)}$. In order to avoid interference from *g1*- and *g2*-phase cells, the FALS versus DNA regression was restricted to that portion of the DNA distribution in which *s*-phase cells comprised ≥ 90% of the total cells, as estimated from the *g1*, *s*, and *g2* deconvolution. We excluded from this analysis samples in which the above criteria was not met anywhere in the DNA distribution. Two MIT9312 cultures and one WH8103 culture were so-excluded.

Results:

Prochlorococcus strain MIT9312 and *Synechococcus* strain WH8103 growth rates in this study varied between 0.28-0.95 and 0.22-1.33 day^{-1}, respectively; their dependence on light intensity was well-described by hyperbolic tangent functions (MIT9312: r^2=0.95, p<0.0005; WH8103: r^2=0.92, p<0.0005)(Jassby and Platt 1976). Estimated μ_{max} values for MIT9312 and WH8103 were 0.95 and 1.33 day^{-1}, respectively; estimated initial slopes (α) were 0.074 and 0.055 (Figure 3.1). Neither strain showed any obvious sign of photoinhibition over the range of light intensities employed here.

DNA frequency distributions for both MIT9312 and WH8103 were bimodal at all growth rates examined (Figure 3.2). Deconvolution of these distributions into *g1*, *s*, and *g2* sub-populations allowed us to calculate the length of each of these phases (*B*, *C*, and *D* respectively) for each of the growth rates tested (Figure 3.3). Overall there was a notable degree of similarity between *Prochlorococcus* MIT9312 and *Synechococcus* WH8103 with respect to each of these cell cycle parameters. Chromosome replication times (*C*) in MIT9312 and WH8103 were relatively invariant over the range of growth rates examined, averaging 4.7±1.1 (mean±SD) and 4.0±1.0 hours, respectively (Figure 3.3A). The significance of the apparent spike in WH8103 growing at 1.05 day^{-1} is not known at present; it was well-replicated among serial cultures at that growth rate, but was not reflected in cultures growing at slightly higher or lower rates. The length of the post-replication period (*D*) was maximal (10-20 hours) in both strains growing at their lowest growth rates (Figure 3.3B). As growth rate increased *D* declined rapidly, reaching minimum values of approximately 2.9 and 2.2 in MIT9312 and WH8103, respectively. In MIT9312 *D* appeared to increase abruptly at the two highest (light-saturated) growth rates. The length of the pre-replication period (*B*) was maximal at the slowest growth rates (29-33 hours),

and decreased gradually as growth rate increased, reaching minimum values of approximately 6.1 and 4.3 in MIT9312 and WH8103, respectively (Figure 3.3C). The combined length of time required for DNA replication and cell division (*C+D*) is an important factor in the calculation of *in situ* growth rates based on diel changes in population DNA frequency distributions (see Discussion). This factor varied over a range of approximately 2.4 and 5-fold in *Prochlorococcus* and *Synechococcus*, respectively (Figure 3.3D).

The relationship between mean cell mass and growth rate in MIT9312 and WH8103 is shown in Figure 3.4A. Cell mass was greatest (75 and 260 fg C·cell^{-1}, respectively) at the lowest growth rates in both organisms, and decreased gradually to minimum values (22 and 129 fg C·cell^{-1}) at the highest growth rates. This represents a 2-3 fold decrease in mean cell mass over the range of experimental growth rates employed here. Average cellular carbon was related to FALS according to the power function FALS = a (C·cell^{-1}) ^{b}, with b = 1.74 (Figure 3.4B). This relationship was not significantly different for *Prochlorococcus* or *Synechococcus* considered individually (p=0.40; analysis of covariance).

Using these mean cell mass data coupled with the cell cycle phase data, we calculated the cell mass at the point of initiation of chromosome replication ($M_{i(C)}$), as described in the Methods section. For MIT9312 and WH8103 $M_{i(C)}$ ranged from approximately 23-108 and from 127-285 fg C·cell^{-1}, respectively (Figure 3.5A). In both strains, $M_{i(C)}$ was negatively related to growth rate (*Prochlorococcus*: r^2=0.59, p=0.043; *Synechococcus*: r^2=0.84, p=0.0013); the slopes of these relationships were not significantly different (p=0.24).

We used a second, FALS-based method to independently estimate initiation cell mass ($M_{i(FALS)}$), as described in the methods section (Figure 3.5B). $M_{i(FALS)}$ showed much the same relationship with growth rate as $M_{i(C)}$ did in WH8103, however in MIT9312 $M_{i(FALS)}$ did not vary

systematically with growth rate (r^2=0.96, p=0.0008; r^2=0.03, p=0.8 for WH8103 and MIT9312 respectively). Thus, our two estimates of M_i ($M_{i(FALS)}$ and $M_{i(C)}$) were well correlated for WH8103 (r=0.94, p=0.002), but not for MIT9312 (r=0.62, p=0.27).

Discussion:

The bimodal DNA distributions observed for both *Prochlorococcus* strain MIT9312 and *Synechococcus* strain WH8103 at all experimental growth rates indicate that cell cycle regulation in both of these organisms conforms to the slow-growth case of the Cooper-Helmstetter model (Cooper and Helmstetter 1968). Thus neither culture exhibits multiple chromosome copies nor the "asynchronous initiation" of chromosome replication described previously for *Synechococcus* strains WH7803 and PCC 6301 (Binder and Chisholm 1990, Binder and Chisholm 1995). In this regard the results presented here for WH8103 are very similar to those obtained by Armbrust et al. (1989) and Binder (2000) for the coastal *Synechococcus* strain WH8101, which displayed a clear bimodal DNA distribution even at growth rates as high as 1.8 day^{-1}. Likewise, the bimodal MIT9312 DNA distributions we observed are similar to previously reported *Prochlorococcus* DNA distributions in laboratory cultures and in the field (e.g. Vaulot et al. 1995, Parpais et al. 1996, Marie et al. 1997, Shalapyonok et al. 1998, Liu et al. 1999, Mann and Chisholm 2000). Shalapyonok et al. (1998) documented ultradian growth (generation time < 1 day) for *Prochlorococcus* populations both *in situ* and in culture. Consistent with our results for rapidly growing MIT9312 there was no evidence of overlapping rounds of chromosome replication even under these conditions.

The chromosome replication times (C) we observed in MIT9312 and WH8101 were very similar (means = 4.7 and 4.0 h, respectively), and in neither strain did C appear to vary systematically with growth rate (Figure 3.3A). C values of similar magnitude (approximately 2-

5 h) have been reported previously for *Synechococcus* strains WH7805, WH8101, and WH8103 (Armbrust et al. 1989, Binder and Chisholm 1995, Binder 2000). Liu et al. (1999) likewise reported similar *C* values (2.3 – 4.1 h) for N-limited *Synechococcus* WH7803 growing at relatively high rates (≥ 0.6 d^{-1}), but in contrast to our observations for WH8103, they found *C* to increase as growth rate decreased, reaching values as high as 26 h at growth rates of 0.1 d^{-1}. The explanation for this difference is not immediately clear. It could reflect true differences in cell cycle regulation among different *Synechococcus* strains. Alternatively, this difference might arise from changes in cell cycle behavior under light-limited versus N-limited conditions. Binder (2000) found that in *Synechococcus* WH8101, *C* increased slightly at slow growth rates under both N- and light-limitation, but in neither case was it ever greater than ~7.5 h, even at growth rates comparable to the slowest examined by Liu et al. (1999).

Binder (2000) also observed *C* to increase slightly as light-limited growth approached 1.5 d^{-1}, and then to shift downward at the highest growth rates. He suggested that this abrupt change might reflect a physiological shift from light-limited to light-saturated growth (see also Binder and Liu 1998). It is tempting to suggest that the spike in *C* observed here in WH8103 at 1.05 d^{-1}, and the subsequent decrease at growth rates beyond this point, reflects this same sort of shift. Without more data for growth rates in the vicinity of this transition point, however, this interpretation must remain tentative.

The *C* values reported here for *Prochlorococcus* MIT9312 are consistent with previous estimates of 4–6 h made for *Prochlorococcus* strains SS120, PCC9511, and CCMP 1378 (Parpais et al. 1996, Jacquet et al. 2001b). In contrast, Parpais et al. (1996) report *C* values of approximately 9–25 h in five other *Prochlorococcus* strains, all of which however were growing at very low rates (~0.2 d^{-1}). Although this suggests that *C* might increase as growth rate

decreases, we observed no such effect in MIT9312, at least for growth rates down to 0.28 d^{-1}. Further investigation of intra-specific growth rate-mediated variation in *Prochlorococcus* cell cycle behavior is required before these different observations can be adequately explained.

The asymptotic decrease in *D* (the time between replication and cell division) observed here in WH8103 and MIT9312 (Figure 3.3B) is consistent with the previously reported behavior of *Synechococcus* WH8101 and WH7803 (Armbrust et al. 1989, Liu et al. 1999, Binder 2000). Maximum observed *D* values in these strains ranged between approximately 15 and 23 h. Asymptotic values (reached at high growth rates) were ~2.3 h in WH8103 and MIT9312 (present study), ~2.8 h in WH8101 (Binder 2000), and 4–5 h in WH7803 (Liu et al. 1999). Reported *D* values in other *Prochlorococcus* strains (representing a range of arbitrary growth rates) range from ~1.5–25 h (Parpais et al. 1996, Jacquet et al. 2001b). In the present study, *D* in both strains appeared to increase abruptly as growth rates increased to their highest values. Again, this shift may reflect physiological changes associated with the transition from light-limited to light-saturated growth.

The time between cell birth and the initiation of chromosome replication (*B*) showed a consistent decrease with increasing growth rate in both MIT9312 and WH8103. Similar trends have been observed in *Synechococcus* WH8101 and WH7803 (Armbrust et al. 1989, Liu et al. 1999, Binder 2000). In the present study, *B* showed transient drops at growth rates of 0.86 and 1.05 day^{-1} (in MIT9312 and WH8103, respectively), reflecting the transient increases in *C* and/or *D* values observed at these growth rates, as discussed above.

The combined duration of the replication and post-replication periods (*C*+*D*) can be used in conjunction with diel cell cycle phase dynamics to estimate the growth rates of natural *Prochlorococcus* populations (e.g. Vaulot et al. 1995, Mann and Chisholm 2000). Carpenter and

Chang (1988) developed an approach for directly estimating this duration, based on the difference in timing between the observed peaks in *s* and *g2* frequency over the course of the day. However, this estimate can be problematic, given typical sampling intervals (≥ 2 h) and the difficulty of precisely locating the *s* and *g2* peaks. This has led some researchers to assume a constant *C+D* over some subset of their data, for the purposes of calculating *in situ* *Prochlorococcus* growth rate (Vaulot et al. 1995, Liu et al. 1997, Shalapyonok et al. 1998, Mann and Chisholm 2000). Our results show that within a single *Prochlorococcus* strain, *C+D* can vary by a factor of approximately 2.4 (Figure 3.3D), suggesting that any such assumptions of invariant *C+D* should be applied with caution.

The cellular biomass (fg C·cell^{-1}) measured here for MIT9312 and WH8103 are consistent with the values reported by Bertilsson et al. (2003) for the same or closely related *Prochlorococcus* and *Synechococcus* strains. In that study, the biomass of *Prochlorococcus* strain Med 4 (a low-B/A clade I ecotype closely related to MIT9312; Rocap et al. 2002) growing at ~0.39 day^{-1} was found to be ~46-61 fg C·cell^{-1}, values that are bracketed by our measurements for MIT9312 growing at slightly lower and higher rates (74.5 and 35.4 fg C·cell^{-1}, respectively). Bertilsson et al. (2003) reported that the biomass for WH8103 growing at 0.35-0.41 day^{-1} was 213-244 fg C·cell^{-1}, as compared with our measurements of 184-271 fg C·cell^{-1} in the same strain at comparable growth rates. In the present study, both MIT9312 and WH8103 exhibited decreased biomass with increasing growth rate (Figure 3.4A). This is the first report examining the relationship between biomass and growth rate in a *Prochlorococcus* strain, but a similar trend has been observed in *Synechococcus* WH7803 over a range of nitrogen-limited growth rates (Liu et al. 1999). In contrast, Binder and Liu (1998) reported that in *Synechococcus* WH8101, cell biovolume increased at relatively high light-limited growth rates (though it decreased again at

higher, light-saturated rates). This disparity could be the result of changes in cellular carbon:biovolume ratios associated with changes in growth rate; this ratio is known to be sensitive to growth conditions in heterotrophic bacteria (e.g. Vrede et al. 2002). Alternatively, it may reflect true differences between the growth physiology of WH8101 (a coastal MC-B isolate) and WH8103 and WH7803 (open ocean MC-A strains). Over the range of growth rates examined here, there was a 2-4 fold difference in cellular carbon content in MIT9312 and WH8103 cells. This relatively large range of cellular biomass within single species brings into question the accuracy of *Prochlorococcus* and *Synechococcus* standing stock calculations that assume a single cellular carbon value, and argues strongly for using more direct estimates of cellular biomass for such calculations.

The relationship we observed between FALS and mean cellular carbon content was similar to those derived by Binder et al. (1996) and DuRand et al. (2001), based on diel FALS changes in natural *Prochlorococcus* populations, and on volume measurements in cultured *Synechococcus* strains, respectively. If we assume that carbon content is proportional to cell volume among this restricted group of cells, our estimated coefficient for the power function relating cell carbon to FALS translates to a cell diameter-based coefficient of ~5.2. This agrees remarkably well with the estimates of 5.5 and 5.4 made in these previous studies, and is in reasonable accord with the theoretical value of 6 based on Mie theory for cells in this size range (Morel and Bricaud 1986).

Bertilsson et al. (2003) reported FALS to be a linear function of cell carbon among a number of *Prochlorococcus* and *Synechococcus* strains, but their data are equally well-described by a power function with a diameter-based coefficient of 3.5 (data from their Fig. 3, $r^2 = 0.998$), somewhat lower than the coefficients discussed above. Because the relationship between cell

size and light scatter is sensitive to the angle over which that scatter is measured, it is plausible that this difference in coefficients is related to the geometry of light scatter detection in the different flow cytometers employed in these studies: the instruments used in Binder et al. (1996), DuRand et al. (2001), and the present study all had similar optical geometries (all were versions of Coulter EPICS V/753 models), whereas the geometry of the instrument used by Bertilsson et al. (2003) (a Becton Dickinson FACSscan) differs from these in numerous ways. These observations underscore the importance of using instrument-specific calibrations if cell mass is to be estimated from flow cytometric light scatter measurements.

In *E. coli*, chromosome replication is initiated when cell biomass, normalized to replication origin copy number, reaches a critical value or "initiation mass" (M_i)(Donachie and Blakely 2003). If this model is extended to *Prochlorococcus* and *Synechococcus*, in which overlapping rounds of replication generally do not occur (and replication origin copy number therefore = 1), this M_i translates simply to cell mass at the start of replication. Binder (2000) found that in *Synechococcus* WH8101 cell volume at the start of replication is approximately constant, suggesting that the M_i model is applicable to *Synechococcus*, and that M_i is relatively invariant across growth rates (as is the case of *E. coli*). In contrast, in the present study we found that in both *Synechococcus* strain WH8103 and *Prochlorococcus* strain MIT9312, $M_{i(C)}$ decreased significantly with increasing growth rate (Figure 3.5A). This same trend was apparent in $M_{i(FALS)}$ for WH8103, but not MIT9312 (Figure 3.5B). It should be noted however that in the latter case, because $M_{i(FALS)}$ couldn't be calculated for the slowest-growing cultures (see Methods), the range of growth rates examined in this context was relatively narrow. Thus we consider $M_{i(C)}$ to be a better estimator of true M_i in this particular case. Although the observation of decreasing M_i with increasing growth rate does not mean that cell mass is uninvolved in the

initiation of chromosome replication in these organisms, it does argue that the regulation of initiation is more complex than previously suggested, involving at the very least M_i values that themselves are modulated by changes in growth rate. Again, the discrepancy between the results of Binder (2000) and our present results may reflect the use of biovolume in one and cellular carbon content in the other, as discussed above. However in both studies, the trends in *Synechococcus* $M_{i(FALS)}$ versus growth rate were consistent with the trends in biovolume- or carbon-based M_i, respectively. This suggests that the observed differences between *Synechococcus* WH8101 and WH8103 may reflect real differences in cell cycle physiology between these organisms.

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Figure Legends:

FIG. 3.1. Relationship between specific growth rate and light intensity for *Prochlorococcus* strain MIT9312 (closed symbols) and *Synechococcus* strain WH8103 (open symbols). Shown are the means and standard errors for the specific cultures analyzed for DNA distributions. Lines are the best-fit hyperbolic tangent function for *Prochlorococcus* and *Synechococcus* cultures respectively. Error bars not shown are contained within the symbols.

FIG. 3.2. Examples of DNA frequency distributions for *Prochlorococcus* strain MIT9312 (A and B) and *Synechococcus* strain WH8103 (C and D) growing at low and high growth rates (indicated in each panel). Deconvoluted *g1*, *s*, and *g2* subpopulations shown as stippled and hatched areas, respectively. Solid line indicates the sum of these three sub populations, and broken line shows the actual data. X-axis: cellular DNA content (as genome equivalents); Y-axis: relative cell number (scale varies from panel to panel). In all cases, the distributions are normalized such that the *g1* peaks correspond to 1.0 genome equivalents.

FIG. 3.3. Magnitude of cell cycle parameters, expressed as absolute time, versus growth rate in MIT9312 (closed symbols) and WH8103 (open symbols). Values for *C*, *D*, *B*, and (*C*+*D*) are shown in panels A, B, C, and D respectively. Error bars represent SE of growth rates and cell cycle parameter values from replicate cultures at each experimental light intensity. Error bars not shown are contained within symbol.

FIG. 3.4. (A) Variation of mean cellular carbon content with growth rate in *Prochlorococcus* strain MIT9312 (closed symbols) and *Synechococcus* strain WH8103 (open symbols). Error bars

represent SE of growth rates and cellular carbon content values from replicate cultures at each experimental light intensity. Error bars not shown are contained within symbol, except when only one datum is available (indicated by inverted triangles ▼). (B) Relationship between log FALS and log cellular carbon content for *Prochlorococcus* and *Synechococcus*. Line represents the least squares regression for all cultures, taking log FALS as the independent variable.

FIG. 3.5. Variation in *Prochlorococcus* strain MIT9312 and *Synechococcus* strain WH8103 chromosome initiation cell mass with respect to growth rate. (A) Chromosome initiation mass, $M_{i(C)}$ determined using cell cycle parameters and measured mean C·cell^{-1}. (B) Chromosome initiation mass, $M_{i(FALS)}$ determined using *s*-phase FALS estimates (see Methods for details). Lines represent least squares linear regressions.

FIG. 3.1

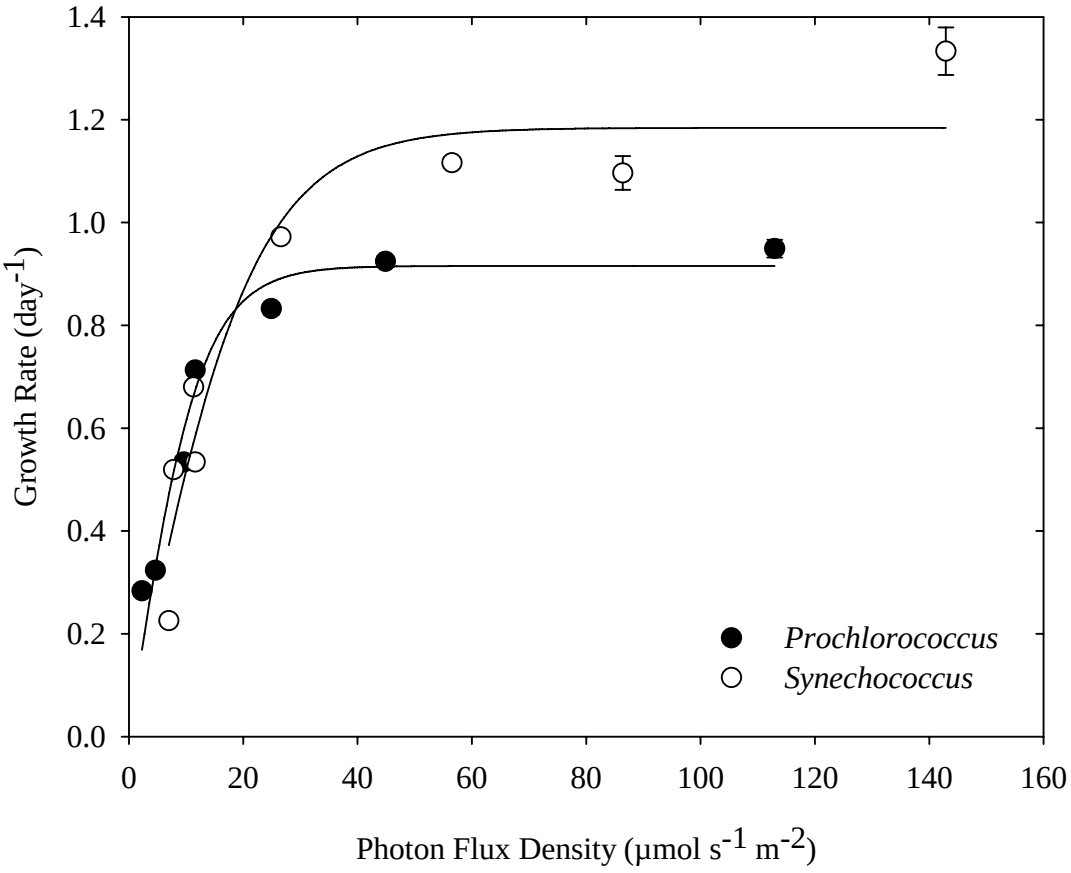

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Growth Rate (day-1)
0
20
40
60
80
100
120
140
160
Photon Flux Density (µmol s-1 m-2)
Prochlorococcus
Synechococcus

FIG 3.2

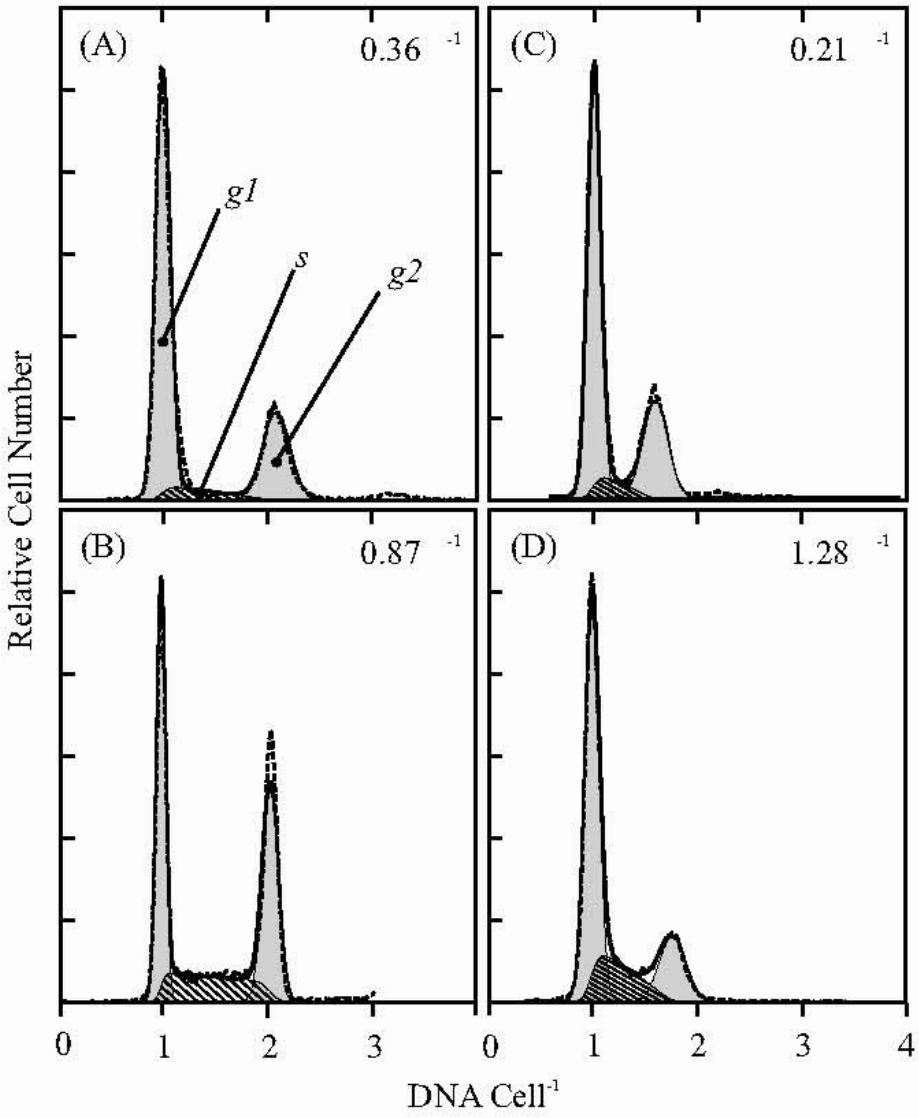

(A)
0.36 -1
g1
s
g2
(C)
0.21 -1
(B)
0.87 -1
(D)
1.28 -1
Relative Cell Number
0
1
2
3
0
1
2
3
4
DNA Cell-1

FIG 3.3

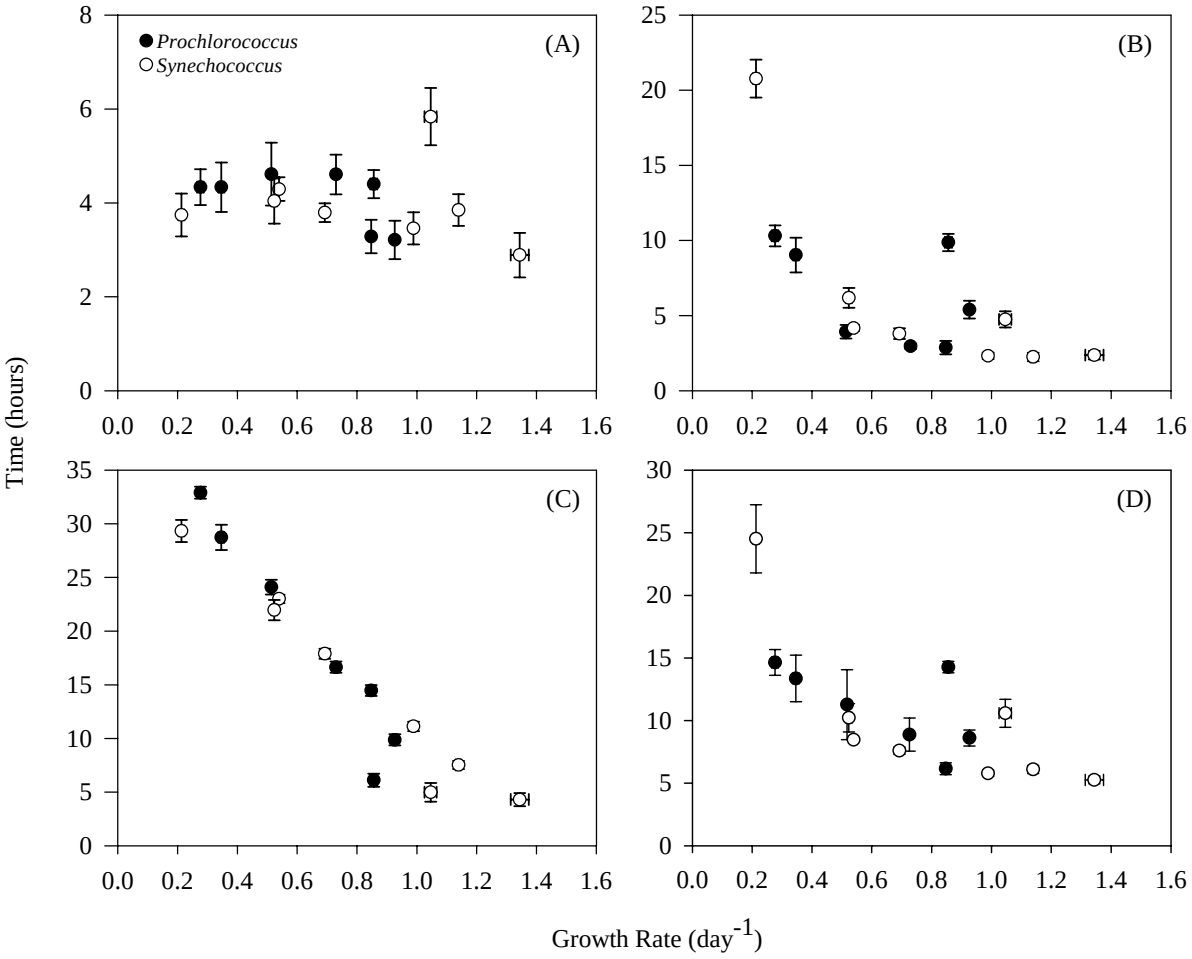

Prochlorococcus
Synechococcus
(A)
(B)
(C)
(D)
Time (hours)
Growth Rate (day-1)

FIG 3.4

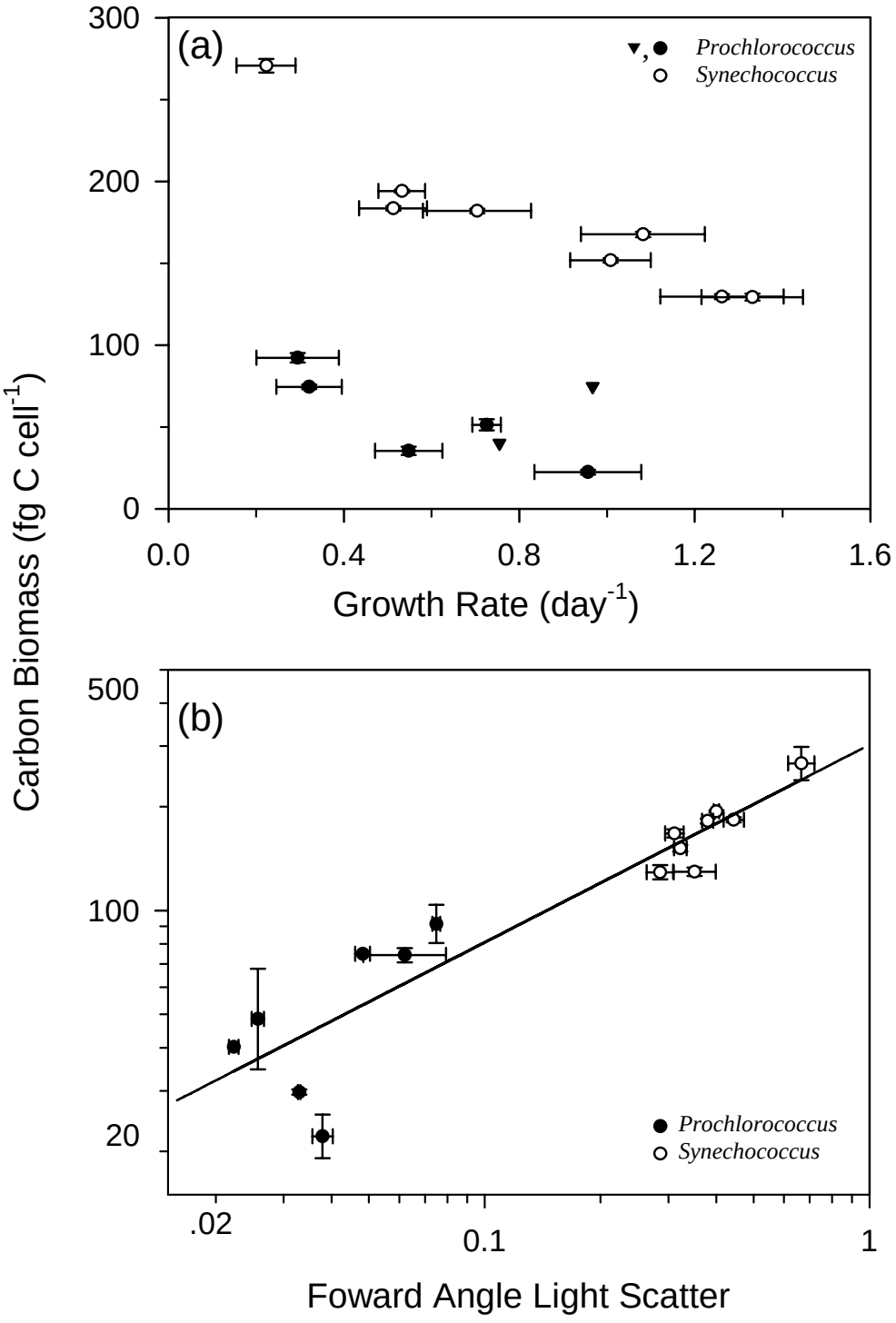
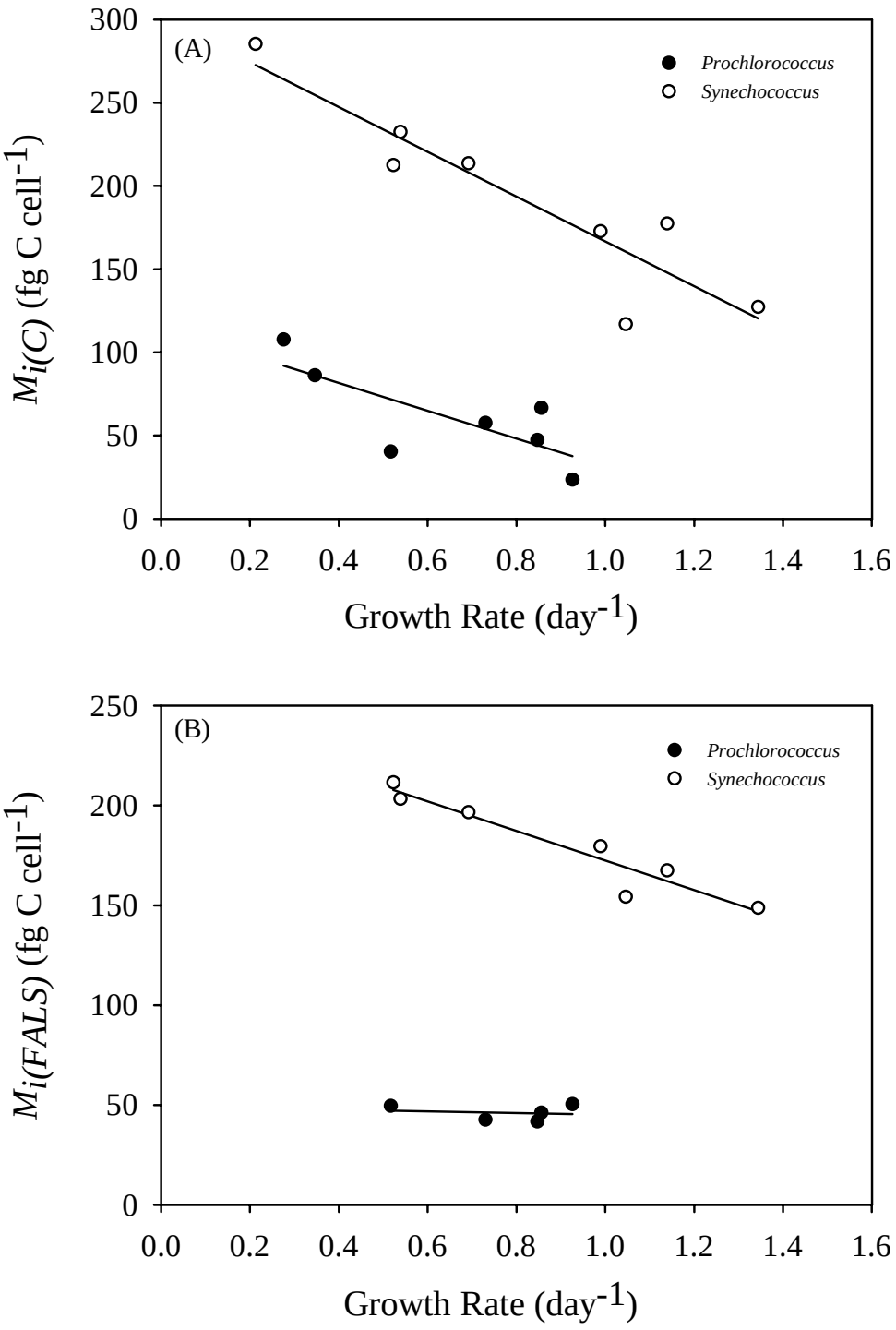

(a)
Prochlorococcus
Synechococcus
300
200
100
0
0.0
0.4
0.8
1.2
1.6
Growth Rate (day-1)
Carbon Biomass (fg C cell-1)
(b)
500
100
20
.02
0.1
1
Prochlorococcus
Synechococcus
Foward Angle Light Scatter

FIG 3.5


(A)
Prochlorococcus
Synechococcus
$M_{i(C)}$ (fg C cell^{-1})
Growth Rate (day^{-1})
(B)
Prochlorococcus
Synechococcus
$M_{i(FALS)}$ (fg C cell^{-1})
Growth Rate (day^{-1})

CHAPTER 4

EFFECTS OF PREY CONCENTRATION AND RATIO ON THE GRAZING ACTIVITY OF A HETEROTROPHIC NANOFLAGELLATE FEEDING ON *PROCHLOROCOCCUS* AND *SYNECHOCOCCUS*

Burbage, C.D., and Binder, B.J. To be submitted to *Journal of Experimental Marine Biology and Ecology*.

Abstract:

Prochlorococcus and *Synechococcus* are important primary producers in open ocean ecosystems, yet little is known about the fate of their production in these systems. Heterotrophic nanoflagellates are generally assumed to be the primary consumers of *Prochlorococcus* and *Synechococcus* in the field. Here we examine the grazing behavior of the heterotrophic nanoflagellate *Paraphysomonas imperforata* with respect to *Prochlorococcus* (strain MIT9312) and *Synechococcus* (strain WH8103) prey. This flagellate actively ingested both picocyanobacteria, with clearance rates ranging up to 7 and 24 nL grazer^{-1} hr^{-1} for MIT9312 and WH8103, respectively, and was capable of growing on a diet of either organism. Rates of ingestion upon *Prochlorococcus* prey items increased monotonically with prey concentration over approximately 4 orders of magnitude (~1×10^4 - 4×10^8 cells mL^{-1}), while *Synechococcus* ingestion rates plateaued and ultimately declined at the highest prey concentrations tested (~5×10^5 cells mL^{-1}). When offered an approximate 1:1 *Prochlorococcus* to *Synechococcus* prey ratio, *P. imperforata* showed a strong preference for *Synechococcus* regardless of prior prey history. However, this preference was not constant: under conditions of gradually increasing *Prochlorococcus*: *Synechococcus* ratio, *Synechococcus*-raised *P. imperforata* prey preference switched from *Synechococcus* to *Prochlorococcus*. *Prochlorococcus*-raised *P. imperforata* displayed no such prey preference switching, preferring *Synechococcus* at prey ratios as high as ~1500.

Introduction

In oligotrophic regions of the world's oceans *Prochlorococcus* and *Synechococcus* often dominate the photosynthetic community in terms of abundance and contribution to primary productivity (Glover et al. 1986, Waterbury et al. 1986, Chisholm et al. 1988, Mackey et al. 2002, DiTullio et al. 2003, Maranon et al. 2003, Moran et al. 2004). *Prochlorococcus* cell concentrations are typically in the range of 10^4 to $>10^5$ cells mL^{-1} in the euphotic zone of the tropical and sub-tropical oceans (Chisholm et al. 1988, Partensky et al. 1999b). *Synechococcus* has a more cosmopolitan distribution, occurring in significant concentrations (10^2 to 10^5 cells mL^{-1}) in coastal and open ocean waters in tropical through temperate latitudes (Waterbury et al. 1986, Partensky et al. 1996, Grob et al. 2007). *Prochlorococcus* and *Synechococcus* can account for a large percentage (32-89%) of the total primary production in open ocean regions (see Goericke & Welschmeyer 1993, Li 1995, Liu et al. 1997, Veldhuis et al. 1997). Yet surprisingly little is known about the trophic connections between these picocyanobacteria and the rest of the marine microbial food web. Potential fates of picocyanobacterial production in the open ocean include consumption by protozoa (e.g. Caron et al. 1999, Sanders et al. 2000, Christaki et al. 2001), release as DOM via phage-mediated lysis (see Suttle & Chan 1994, Sullivan et al. 2003, Muhling et al. 2005), and possibly export out of the euphotic zone (see Waite et al. 2000). The tight coupling generally observed between picocyanobacterial growth rate and grazing mortality in field data suggests that protozoan grazing may be the dominant fate for *Prochlorococcus* and *Synechococcus* production (Caron et al. 1999, Sanders et al. 2000, Christaki et al. 2001).

Heterotrophic nanoflagellates are thought to be the major consumers of both *Prochlorococcus* and *Synechococcus* in natural systems (Reckerman & Veldhuis 1997, Caron et al. 1999, Christaki et al. 2001, Cuevas & Morales 2006). A limited number of studies have

addressed the ability of nanoflagellates to consume and/or grow on *Prochlorococcus* and *Synechococcus*. Guillou et al. (2001) found that one recently isolated heterotrophic nanoflagellate, *Symbiomonas scintillans*, consumed neither *Prochlorococcus* nor *Synechococcus*. *Picophagus flagellatus* actively grazed both and grew exceptionally well on a diet of *Prochlorococcus*, while only growing slowly on *Synechococcus* prey items. In contrast, Christaki et al. (2002) found that for both the lab-raised flagellate *Pseudobodo* sp., and a mixed flagellate community, *Prochlorococcus* and *Synechococcus* were actively grazed, but neither supported flagellate population growth. *Strombidium sulcatum* and *Uronema sp.*, two species of ciliates, have also been shown to be able to graze *Prochlorococcus* and *Synechococcus*. *Strombidium sulcatum* was shown to grow on both prey types, while *Uronema sp.* was shown to only grow on a diet of *Synechococcus* prey items (Christaki et al. 1999).

These studies established the palatability of *Prochlorococcus* and *Synechococcus* as prey items that support growth in some protozoan grazers, however there is still very little known about the behavior of flagellates presented with a choice between these two picocyanobacterial prey items. Bactiverous protozoa have been shown to be quite selective in the prey items that they choose to consume (Verity 1991, Jürgens & DeMott 1995, Monger et al. 1999, Boenigk et al. 2002, Matz et al. 2002), however, to date there is only one published report explicitly quantifying the grazing preferences of a heterotrophic nanoflagellate fed a mixed diet of *Prochlorococcus* and *Synechococcus* prey items (Guillou et al. 2001). In that study, *P. flagellatus* grazed both picocyanobacteria with comparable efficiency when each was presented singly, but displayed a strong preference for *Synechococcus* when the two were offered together (Guillou et al. 2001).

Given the presumed importance of nanoflagellate grazers as consumers of *Prochlorococcus* and *Synechococcus* production in the open-ocean, it is important that we gain a clearer understanding of the grazing behavior of nanoflagellates upon these two co-occurring picocyanobacteria both singly and together. Here we examine the functional response, grazing preference and influence of prey history of a heterotrophic nanoflagellate (*Paraphysomonas imperforata*) growing on *Synechococcus* (WH8103) or *Prochlorococcus* (MIT9312) prey. *P. imperforata* is a cosmopolitan, heterotrophic Chrysophyte that has been studied extensively as a model bacteriovore (Caron et al. 1990, Sin et al. 1998, Fu et al. 2003); WH8103 and MIT9312 are representative of *Synechococcus* and *Prochlorococcus* groups that dominate the picocyanobacteria in open-ocean surface waters (Moore & Chisholm 1999, Scanlan 2003, Zinser et al. 2006).

Materials and Methods:

Culture Conditions. *Prochlorococcus* strain MIT9312, was obtained from S. W. Chisholm (Massachusetts Institute of Technology, Cambridge, MA, USA) and grown in Pro99 medium (see Moore et al. 2002). *Synechococcus* strain WH8103 was obtained from J. B. Waterbury (Woods Hole Oceanographic Institute, Woods Hole, MA, USA) and grown in SN medium (see Waterbury et al. 1986). Both *Prochlorococcus* and *Synechococcus* were grown under constant light conditions at a growth rate of approximately 0.69 day^{-1}. Neither the *Prochlorococcus* nor the *Synechococcus* cultures were axenic, but heterotrophic bacterial cell concentrations were <5% of picocyanobacterial concentration (as measured prior to all grazing experiments). The heterotrophic nanoflagellate *Paraphysomonas imperforata* (*P. imperforata*) was obtained from D. A. Caron (University of Southern California, Los Angeles, CA, USA) and grown in autoclaved seawater with an amendment of either *Prochlorococcus* (MIT9312) or

Synechococcus (WH8103) as prey. All *P. imperforata* cultures (including experimental cultures) were maintained at 16°C in the dark. Individual cultures of *P. imperforata* were maintained on either *Prochlorococcus* or *Synechococcus* for approximately one year prior to grazing experiments.

Experimental Design. Prey consumption by *P. imperforata* was examined in three different sets of grazing experiments. The first was designed to measure the functional grazing response of *P. imperforata* to *Prochlorococcus* and *Synechococcus* prey items individually. Protozoa cultures that were raised historically on either *Prochlorococcus* or *Synechococcus* (50 mL; ~2 $\times 10^3$ *P. imperforata* mL^{-1}) were amended with exponentially growing *Prochlorococcus* or *Synechococcus*, respectively, to yield six different prey concentrations. Prior to the start of each individual experiment *P. imperforata* cultures were examined via phase contrast microscopy to ensure that the flagellates were still actively grazing. Control treatments consisted of prey or flagellates by themselves. All flasks were incubated at 16°C in the dark (so as to prevent picocyanobacterial growth) for <24 hours. The short length of these experiments was important so as to limit the effect of protozoan grazer growth upon ingestion rate calculations. Flasks were sampled approximately every 2 hours and the decrease in prey concentration used to calculate grazing rates (see Calculations below).

The second set of grazing experiments was designed to measure prey preferences for flagellates raised on *Prochlorococcus* or *Synechococcus*, respectively. *Prochlorococcus*- and *Synechococcus*-raised *P. imperforata* were incubated with three different combinations of prey: *Prochlorococcus* alone, *Synechococcus* alone, or a mixture of *Prochlorococcus* and *Synechococcus* (Table 1). Controls and incubation conditions were as described for the first experiment. Prey preference was determined by comparison of clearance rates (see Calculations

below) for specific prey types (*Prochlorococcus* or *Synechococcus*) in flasks containing both prey together per Jürgens & DeMott (1995). *P. imperforata* prey preference (*PP*) was calculated as:

$$PP = \frac{CR_{Pro} - CR_{Syn}}{CR_{Pro} + CR_{Syn}} \qquad \text{(Equation 1)}$$

where CR_{Pro} and CR_{Syn} are the clearance rates for *Prochlorococcus* and *Synechococcus* in the mixed treatment, respectively. *PP* values can fall within a range of +1 to -1, with +1 representing uptake of only *Prochlorococcus*, -1 representing uptake of only *Synechococcus*, and 0 indicating non-selective feeding.

The third set of experiments was designed to examine the effect of prey ratio upon the prey preference of *P. imperforata*. In this set of experiments *Prochlorococcus*- and *Synechococcus*-raised *P. imperforata* were presented with gradually increasing *Prochlorococcus*: *Synechococcus* ratios as follows: To initiate the experiment, individual *P. imperforata* cultures were allowed to graze their respective prey down to minimal levels (~10^3 cells mL^{-1}) at which point a large aliquot of *Synechococcus* prey was added (raising the ambient *Synechococcus* prey concentration to ~7×10^6 cells mL^{-1}). Triplicate sub-samples (~50 mL each) were removed periodically (~ every 3 hours) thereafter and amended with small aliquots of *Prochlorococcus* prey (bringing their ambient concentration to ~1×10^7 cells mL^{-1}). These "sub-experiments" were then incubated for 1-2 hours. Samples for flagellate and prey enumeration were taken at the start and the end of each sub-experiment, and used to calculate ingestion and clearance rates and prey selectivity values for each *P. imperforata* culture at each time point.

Calculations. Grazing rates (g , mg carbon hr^{-1}) were calculated as the slope of the linear regression of corrected prey concentration (cells or mg C mL^{-1}) over time. Prey

concentrations were corrected by the fractional change in the no-flagellate controls over corresponding time periods.

Protozoan ingestion rates (IR, mg carbon protozoa^{-1} hour^{-1}) were determined as:

$$IR = \frac{g}{Flagellates \cdot mL^{-1}} \qquad \text{(Equation 2)}$$

where g represents the grazing rate (defined above), and $Flagellates \cdot mL^{-1}$ corresponds to the *P. imperforata* cell concentration at the start of the experiment.

Clearance rates (CR, nL protozoa^{-1} hour^{-1}) were determined as:

$$CR = \frac{IR}{\overline{N}} \qquad \text{(Equation 3)}$$

where IR represents ingestion rate (see above), and $\overline{N}$ represents the mean concentration of the prey of interest over the course of the experimental time period.

Carbon Analysis. Approximately 10^9 cells (23 mL) of exponentially growing MIT9312 or WH8103 cultures were filtered onto previously-ashed GF/F filters and stored at -85°C until analysis. GF/F filters have been shown to efficiently retain both *Prochlorococcus* and *Synechococcus* cells (see Bertilsson et al. 2003). Blanks were prepared by filtering 23 mL of freshly prepared culture medium through ashed GF/F filters, and stored as described above. Particulate C was measured on a Carlo Erba CHN NA1500 Analyzer (Carlo Erba Instruments, Milan, Italy) using a poplar leaf standard (48.16 %C) as the reference material. All samples were corrected for measured blank C values, which corresponded to approximately 19 μg C filter^{-1} for both MIT9312 and WH8103 medium (<10% of average total carbon value measured). These per-cell carbon values were used to calculate prey C concentration from measured cell concentrations.

Cell Counts. Samples for *Prochlorococcus* and *Synechococcus* enumeration were preserved with 1% paraformaldehyde (final concentration) and frozen in liquid nitrogen for later analysis. Cell counts were performed using a modified Coulter EPICS 753 flow cytometer (Binder et al. 1996) as described previously (Burbage & Binder, submitted).

P. imperforata counts were performed in a manner similar to that described by Sherr et al. (1993) and Sherr and Sherr (1993). Briefly, 2 mL samples were fixed with 10 μL alkaline Lugol's solution (10 g iodine + 20 g potassium iodide + 10 g sodium acetate, dissolved in 140 mL of distilled water), 50 μL borate-buffered formalin, and 40 μL sodium thiosulfate (3 g $Na_2S_2O_3$ dissolved in 100 mL distilled water). Fixed samples were stored at 4°C in the dark prior to staining and mounting (no longer than 6 hours). Cells were stained with DAPI (50 μg/mL) and primulin (250 μg/mL) and filtered onto 0.8 μm 25 mm black polycarbonate filters that were then mounted on microscope slides. Slides were stored at 0°C in the dark until they were counted on an epifluorescence microscope.

Results:

Functional Response Experiments. *P. imperforata* actively ingested and grew on both *Prochlorococcus* and *Synechococcus*. Ingestion rates on *Prochlorococcus* increased monotonically over ~4 orders of magnitude of prey concentration (Fig. 4.1A). This increase was well described by a power function ($r^2 = 0.95$, $p < 0.001$) with a slope of 0.87, which is slightly, but significantly, less than 1 ($p = 0.02$). In practical terms, this means that *Prochlorococcus* ingestion rate increased somewhat more slowly than *Prochlorococcus* concentration, as reflected in the decreasing trend in clearance rate for this prey (Fig. 4.1B). *Synechococcus* ingestion increased in a manner similar to *Prochlorococcus* over low to moderate *Synechococcus* concentrations, but beyond ~0.1 mg C mL^{-1}, ingestion appeared to plateau. At the highest

concentration tested (~9 mg C mL^{-1}), calculated ingestion and clearance rates were negative (reflecting net growth of *Synechococcus* over the course of the grazing trial)(Fig. 4.1). *Synechococcus* clearance rates appeared to increase with concentration over the lower half of concentration treatments, but abruptly decreased at concentrations above ~0.1 mg C ml^{-1} (Fig. 4.1B). At prey concentrations below this level, clearance (and ingestion) rates for *Synechococcus* were consistently higher than those for *Prochlorococcus* (clearance rates = 16-24 and 1-7 nL $grazer^{-1}$ h^{-1}, for *Synechococcus* and *Prochlorococcus* respectively). Cell- based rates (versus the carbon-based rates quoted above) revealed qualitatively similar patterns.

Short Term Prey Preference Experiments. *P. imperforata* cells ingested both individually offered *Prochlorococcus* and *Synechococcus* prey with reasonable efficiency, regardless of their long-term grazing history: at the approximately equal prey concentrations employed in this experiment (~4×10^6 cells mL^{-1}), flagellates raised on either *Prochlorococcus* or *Synechococcus* cleared both these prey at rates of 1.3 – 2.5 nL $grazer^{-1}$ h^{-1} (Table 1). However, the ratio of *Prochlorococcus* to *Synechococcus* clearance rates for a given flagellate culture appeared to be influenced by prey exposure history, being significantly higher in *Prochlorococcus*-raised versus *Synechococcus*-raised *P. imperforata* (*Pro*:*Syn* clearance rate ratios = 1.3 and 0.5, respectively; p=0.01). In contrast, when offered both prey types together, *P. imperforata* strongly preferred *Synechococcus*, regardless of its prey exposure history. Under this circumstance, clearance rate on *Prochlorococcus* dropped to very low levels for both *Prochlorococcus*- and *Synechococcus*-raised flagellates, while *Synechococcus* clearance rate remained unchanged or increased. The corresponding selectivity indexes reflect strong preference for *Synechococcus* in both cases.

Protozoan Selectivity Dynamics. To what extent is the preference for *Synechococcus* over *Prochlorococcus* dependent on the availability of each prey species? To answer this question, we presented *P. imperforata* with an ever-decreasing *Synechococcus* concentration, holding *Prochlorococcus* concentration constant. The results indicate that at least for *Synechococcus*-raised flagellates, prey preference is plastic: in the face of decreasing concentrations of *Synechococcus* prey these cells shifted their preference to the alternate prey type, as indicated by changes in clearance rate and calculated selectivity index (Figs. 4.2D & 4.3). Note that unlike ingestion rate, these two parameters take into account changes in prey concentration, and thus reflect true changes in grazer behavior. For *Prochlorococcus*-raised cells, flexibility in prey preference is not apparent: although clearance rate of *Prochlorococcus* appeared to increase as the *Prochlorococcus*:*Synechococcus* ratio increased, clearance of *Synechococcus* remained high, and the selectivity index thus remained negative (Figs. 4.2C & 4.3).

When viewed in relation to *Synechococcus* concentration alone (rather than *Prochlorococcus*:*Synechococcus* ratio), *Synechococcus* clearance rates appeared to vary similarly for both *Prochlorococcus*- and *Synechococcus*-raised *P. imperforata* (Fig. 4.4A). Interestingly, *Synechococcus* clearance rate was highest at prey concentrations on the order of $\sim 3 \times 10^5$ cells mL^{-1}, and dropped off above and below this point. This behavior is similar to our functional response observations, presented above. Owing to the experimental design, *Prochlorococcus* concentrations varied little over the course of these experiments, although they were different for the *Synechococcus*-raised and *Prochlorococcus*-raised treatments, respectively (Fig 4.4B). Thus the variation in *Prochlorococcus* clearance rates in these experiments was not directly related to *Prochlorococcus* concentration.

Total prey concentration may have influenced the selectivity we observed in this experiment. Because of the higher *Prochlorococcus* concentration employed for the *Prochlorococcus*-raised grazer treatment, total available C was generally higher in this treatment than in the *Synechococcus*–raised treatment (Fig. 4.5). Within each treatment, however, total C varied with *Synechococcus* concentration (and therefore with *Prochlorococcus*: *Synechococcus* ratio). The net result is that selectivity, as measured in this experiment, appears to be strongly related to total prey carbon, as well as prey ratio.

Discussion:

Prochlorococcus and *Synechococcus* abundance in open-ocean surface water typically ranges between 10^2 and 10^5 cells mL^{-1} (Waterbury et al. 1986, Li 1995, Partensky et al. 1999a, Partensky et al. 1999b, Johnson et al. 2006). For the cultures used in the present study, this corresponds to 10^{-6}-10^{-3} and 10^{-5}-10^{-2} mg C mL^{-1} for *Prochlorococcus* and *Synechococcus*, respectively. Thus, the range of experimental prey concentrations employed here covers and extends beyond the higher range of natural concentrations.

In our functional response experiment, *P. imperforata* ingested *Prochlorococcus* at rates that increased with prey concentration over the entire range tested. Clearance rates for these prey ranged between 1 and 7 nL $grazer^{-1}$ hr^{-1}, and appeared to decrease with increasing prey concentration. Ingestion of *Synechococcus*, on the other hand, reached a plateau at prey concentrations higher than ~0.1 mg C mL^{-1} (~4.7×10^5 cells mL^{-1}); grazing appeared to stop at the very highest *Synechococcus* concentrations tested (9 mg C mL^{-1}). Calculated *Synechococcus* clearance rate initially increased with concentration, but then decreased at high concentrations, suggestive of a sigmoidal, Type III functional response (see Holling 1959). Although not designed to address this issue, the prey-ratio experiment revealed a similar pattern in

Synechococcus clearance rate versus concentration (Fig. 4.4A). Maximum observed *Synechococcus* concentrations in the field generally fall in the range of ~5×10^5 cells mL^{-1}, approximately the prey concentration at which *Synechococcus*-raised ***P. imperforata*** reached its maximum ingestion rate in this experiment. Thus the decreased ingestion and clearance rates observed here at higher *Synechococcus* concentrations may not be directly relevant to the natural system.

These results are generally consistent with those of Christaki et al. (2002), who examined grazing on *Prochlorococcus* and *Synechococcus* by the bactiverous nanoflagellate *Pseudobodo* sp. and by a mixed natural flagellate population. In that study, as in ours, ingestion of *Prochlorococcus* increased approximately linearly with prey concentration over the entire range of *Prochlorococcus* concentrations tested. Ingestion of *Synechococcus*, on the other hand, increased only up until a specific prey concentration was reached; beyond which ingestion rates appeared to drop. This is consistent with our observations of ***P. imperforata*** grazing on *Synechococcus*. Remarkably, the *Synechococcus* concentration at which ingestion behavior appeared to change was approximately the same in both studies: ~5×10^5 cells mL^{-1} in Christaki et al. (2002), and ~4.7×10^5 cells mL^{-1} (corresponding to 6.6×10^{-2} µg C mL; Fig 4.1A) in the present study. The clearance rates observed by Christaki et al. (2002) and by Christaki et al. (2005) were of the same order as those reported here. Though the perceived relationships between clearance rate and prey concentration in the Christaki et al. (2002) were not identical to the relationships shown in our study, the variability in their data could well accommodate the trends we report. It is interesting to note that in marked contrast to our observations of ***P. imperforata***, neither *Pseudobodo* sp. nor the mixed natural flagellate population showed significant growth on *Prochlorococcus* or *Synechococcus* (Christaki et al. 2002). This could

reflect differences in the history of the flagellate cultures used in the two studies: in ours, *P. imperforata* was raised for many generations on *Prochlorococcus* or *Synechococcus*, while in Christaki et al. (2002) the flagellates were raised on heterotrophic bacteria prior to the grazing experiments. Alternatively, this difference may reflect true differences between the flagellates used in each study.

Prey Preference. *Prochlorococcus* and *Synechococcus* co-occur over vast areas of the world's oceans (Partensky et al. 1999a, Partensky et al. 1999b, Johnson et al. 2006). Although numerous physiological differences are thought to influence the relative abundance of each of these groups (Moore et al. 1995, Moore et al. 2002, Toledo & Palenik 2003, Johnson et al. 2006, Zinser et al. 2007), top-down control by (nanoflagellate) grazers is likely to be important as well. Although *Prochlorococcus* tends to extend deeper into the water column than does *Synechococcus*, in surface waters grazers will be presented with a choice of picocyanobacterial prey. Interactions between flagellate grazers and *Prochlorococcus* and *Synechococcus* may therefore have a profound influence on the abundance and activity of these groups.

The behavior of *P. imperforata* with respect to picocyanobacterial prey was weakly influenced by prey exposure history, and strongly influenced by the presence of alternate prey. *Prochlorococcus*- and *Synechococcus*-raised *P. imperforata* achieved somewhat higher clearance rates for their 'native' prey than for their alternate prey, when these prey were presented individually. However, when challenged with a (~1:1) mixture of *Prochlorococcus* and *Synechococcus*, *P. imperforata* displayed a very strong preference for *Synechococcus*, regardless of its previous prey experience (Table 1).

These results are consistent with those of Guillou et al. (2001), the only other study to date examining picocyanobacterial prey preference by a heterotrophic nanoflagellate. In that

study, the small heterotrophic flagellate *Picophagus flagellatus* was shown to clear *Prochlorococcus* and *Synechococcus* at comparable rates when presented with these prey alone, but to strongly prefer *Synechococcus* when both prey types were available (selectivity index calculated from their data = -0.4), much as we observed here. In a related study, Christaki et al. (1999) examined picocyanobacterial prey preference in a ciliate (*Strombidium sulcatum*). This ciliate grazed *Synechococcus* much more effectively than it did *Prochlorococcus*, whether these prey were presented individually or together; the clearance rates for these two prey types remained unchanged in the individual versus mixed treatments.

Taken together, these results suggest that for some flagellates at least, grazing impact on *Prochlorococcus* in natural systems may be reduced in the presence of *Synechococcus* or other alternate prey. The factors responsible for the apparent preference of grazers for *Synechococcus* are not known at present. One obvious candidate is cell size, which is known to exert a strong influence on prey preference in many protozoa (see review by Jürgens and Matz (2002)). This preference need not reflect grazer behavior per se; simple encounter rate models predict significant size-related effects on clearance rate. In the present case, however, our calculations indicate that differences in neither prey size nor motility would result in sufficiently different encounter rates to explain the degree of prey preferences we observed (see Appendix). Other potential factors that could influence the preference for *Synechococcus* versus *Prochlorococcus* include differences in C:N:P ratios (Bertilsson et al. 2003), cell membrane lipid composition (Van Mooy et al. 2006), and cell surface hydrophobicity (Monger et al. 1999).

Influence of prey ratios on grazer behavior. The experiment discussed above established that *P. imperforata* displays a strong apparent preference for *Synechococcus* when presented in conjunction with *Prochlorococcus* prey at approximately equal abundances.

However in many open ocean environments, *Prochlorococcus* is often present at considerably higher concentration than *Synechococcus*. To what extent might this differential availability influence the prey preference behavior of grazers? Data from the third experiment suggest that flagellates may display considerable flexibility with regard to this behavior. In particular, *Synechococcus*-raised flagellates clearly relaxed their discrimination against *Prochlorococcus* prey as *Synechococcus* grew scarcer, and in fact grazed on *Prochlorococcus* selectively when the ratio was greater than ~100 (Fig. 4.3). In contrast, *Prochlorococcus*-raised *P. imperforata* displayed no such prey switching, even at ratios above1000.

Prey ratio-based selection among heterotrophic nanoflagellates has been shown previously by Jürgens and DeMott (1995), who experimented with the nanoflagellates *Bodo saltans* (Kinetoplastidae) and *Spumella sp.* (Chrysomonadida) grazing on mixtures of inert latex beads and live bacteria. In these experiments both flagellates exhibited shifts in prey selectivity with changes in prey ratios. Flagellates that were raised under food-limiting conditions showed a slight preference for beads when both particles were offered simultaneously. When satiating concentrations of live bacteria were added, however, these same flagellates developed a strong discrimination against the inert beads. As bacterial concentrations were depleted over the following 24 h, discrimination against the inert beads began to decrease gradually, and ultimately the grazing preference for beads returned. Jürgens and DeMott (1995) conclude that this type of behavior is consistent with predictions of optimal diet models, which should favor behaviors that maximize net nutritional gains (Stephens & Krebs 1986). Optimal diet models examine how behaviorally flexible predators might adjust their prey preferences when feeding on mixtures of low and high quality prey items. These models predict that discrimination against low quality prey items should be high when preferred prey particles are abundant and weak when preferred

prey particles are scarce. In our study the *Synechococcus*-raised *P. imperforata* fed in a manner consistent with these optimal diet models in that it displayed prey ratio-based selectivity. The lack of such flexibility in *Prochlorococcus*-raised *P. imperforata* can also be explained in the context of this theory: because overall prey concentration was consistently higher in this experiment, the flagellate may have found itself satiated at all prey ratios. Under this scenario, favoring the more abundant but presumably lower quality alternate prey may not be advantageous.

On the other hand, invariant prey preference as observed here in *Prochlorococcus*-raised *P. imperforata* has also been documented for some marine protozoan grazers. Ilse et al. (2004) reported that four ciliate grazers all displayed constant prey preferences when offered two potential prey items in prey selection experiments. Neither total prey density nor feeding history had any effect on prey preferences in this study. It is possible, therefore, that differences in prey selectivity observed in the present study reflect real differences in the grazing behavior between *Synechococcus*- versus *Prochlorococcus*-raised *P. imperforata*. These two cultures were maintained exclusively on their respective prey for over a year, during which time they could conceivably have undergone selection for different grazing behavior. Further study is clearly needed if we are to fully understand the influence of preconditioning on prey preference behavior among flagellates grazing on picocyanobacteria.

Appendix:

We used two different models to calculate prey-normalized encounter rate (E, ml grazer^{-1} h^{-1}) between *P. imperforata* and its picocyanobacterial prey in our experiments. Prey-normalized encounter rate is analogous to clearance rate; thus absolute encounter rate (prey grazer^{-1} h^{-1}) = prey-normalized encounter rate × prey concentration.

The following parameters are used in these calculations:

v_{graz} = swimming speed of *P. imperforata* = 200 μm s^{-1} (Fenchel 1987)

v_{prey} = swimming speed of prey cells (see below for specific values)

v_{Syn} = swimming speed of *Synechococcus* = 25 μm s^{-1} (Brahamsha 1999)

v_{Pro} = swimming speed of *Prochlorococcus* = 0 μm s^{-1}

r_{graz} = radius of *P. imperforata* cells= 3 μm

r_{prey} = radius of prey cells (see below for specific values)

r_{Syn} = radius of *Synechococcus* = 0.45 μm (Morel et al. 1993)

r_{Pro} = radius of *Prochlorococcus* = 0.30 μm (Morel et al. 1993)

Model I, Gerritsen & Strickler 1977. This model uses a relatively simple geometric argument to calculate the encounter rate between motile predators and motile prey. Prey size is not considered.

$$E = \frac{\pi}{3} r_{graz}^2 \left(\frac{3v_{graz}^2 + v_{prey}^2}{v_{graz}} \right)$$

Plugging in the values given above,

E_{Syn} = 5.68 ×10^3 μm^3 $grazer^{-1}$ s^{-1}

E_{Pro} = 5.65 ×10^3 μm^3 $grazer^{-1}$ s^{-1}

These normalized encounter rates yield a Prey Preference value of -0.003. Clearly, differences in encounter rates related to differences in prey swimming speed cannot account for the Prey Preference values we observed in our experiments.

Model II, Shimeta 1993. This model combines a direct interception encounter model with a Brownian diffusion model. It takes into account prey size, but doesn't consider prey

motility. Note that for the grazer and prey sizes relevant here, this model yields results very similar to the force-balance model of Monger & Landry (1990).

$E = E_R + E_D$

E_R and E_D are the direct-encounter and diffusion-driven encounter rates, respectively.

$$E_R = \frac{3\pi}{2} v_{graz} \cdot r_{prey}^2$$

$$E_D = 7.98 D^{2/3} \cdot v_{graz}^{1/3} \cdot r_{graz}^{4/3}$$

where D = Brownian Diffusivity = $KT/(6\pi\mu\, r_{prey})$

K = Boltzmann's Constant = 1.38×10^{-16} g cm^2 s^{-2} °K^{-1}

T = absolute temperature (°K)

μ = Dynamic Viscosity = 1×10^{-3} kg m^{-1} s^{-1}

Plugging in the values given above,

$E_{Syn} = 3.14 \times 10^2$ grazer^{-1} μm^3 s^{-1}

$E_{Pro} = 2.46 \times 10^2$ grazer^{-1} μm^3 s^{-1}

These normalized encounter rates yield a Prey Preference value of -0.12. Thus according to this encounter rate model, although differences in prey size could result in a small degree of apparent prey preference in favor of the larger prey (*Synechococcus*) in our experiments, these size differences are insufficient to explain the large Prey Preference values we observed (see Fig. 4.3).

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Table 4.1. Prey preference experiments[†]. Values are Means ±SE.

Treatment	Initial Concentration 10^6 Cells mL^{-1} (μg C mL^{-1})		Ingestion Rate Cells $flagellate^{-1}$ h^{-1} (ng C $flagellate^{-1}$ h^{-1})		Clearance Rate nL $flagellate^{-1}$ h^{-1}		Selectivity Index
	Pro	*Syn*	*Pro*	*Syn*	*Pro*	*Syn*	
Pro-raised *P. imperforata* ($2.2 \pm 0.9 \times 10^4$ cells mL^{-1})							
Pro	4.6 ±0.3 (93 ±6)	0	7.6 ±0.5 (0.15 ±0.01)	–	1.6 ±0.1	–	
Syn	0	3.8 ±0.2 (540 ±30)	–	5.0 ±0.7 (0.10 ±0.01)	–	1.3 ±0.2	
Pro + *Syn*	4.1 ±0.2 (83 ±5)	3.7 ±0.1 (510 ±20)	1.0 ±0.8 (0.03 ±0.02)	5.1 ±0.7 (0.10 ±0.01)	0.2 ±0.2	1.4 ±0.2	-0.6
Syn-raised *P. imperforata* ($4.8 \pm 0.8 \times 10^3$ cells mL^{-1})							
Pro	4.2 ±0.1 (85 ±1.7)	0	5.3 ± 2.4 (0.11 ±0.05)	–	1. 3 ±0.6	–	
Syn	0	4.0 ±0.1 (560 ±10)	–	10.0 ±1.1 (0.20 ±0.02)	–	2.5 ±0.3	
Pro + *Syn*	4.3 ±0.1 (85 ±2)	3.8 ±0.1 (540 ±20)	-1.2 ±0.3[‡] (-0.02 ±0.01)	13.5 ±2.0 (0.27 ±0.04)	-0.3 ±0.1[‡]	3.5 ±0.5	-1.0[‡]

[†] *Pro* = *Prochlorococcus*, *Syn* = *Synechococcus*

[‡] Negative grazing rates reflect net prey growth during grazing trial, and are set = 0 for Selectivity Index calculation.

Figure Legends:

FIG. 4.1. Functional response of *P. imperforata* grazing on *Prochlorococcus* strain MIT9312 (closed symbols) and *Synechococcus* strain WH8103 (open symbols). (A) Ingestion rate vs. prey concentration (note log scales). Points show mean ± SE for each treatment; solid line is linear regression for *Prochlorococcus*, broken line is a 3rd order polynomial fit. Inverted triangles on the baseline indicate individual trials in which calculated ingestion rates were negative (i.e. net growth of prey was >0). (B) Clearance rate vs. prey concentration. Lines show the clearance rates calculated from the corresponding fitted ingestion rate relationships in (A). Negative clearance rate indicates net growth (above control).

FIG. 4.2. Relationship between ingestion rate (A and B) and clearance rate (C and D) versus *Prochlorococcus*: *Synechococcus* prey ratio (in terms of cells mL^{-1}) for *Prochlorococcus*- (A, C) and *Synechococcus*- (B, D) raised *P. imperforata*. Closed and open symbols show ingestion or clearance of *Prochlorococcus* and *Synechococcus*, respectively. Lines indicate time course of the experiments, starting at the lowest prey ratios in each case.

FIG. 4.3. Relationship between selectivity value (*PP*) and prey ratio in *Prochlorococcus*- and *Synechococcus*-raised *P. imperforata* (closed and open symbols, respectively). *PP* values of -1 indicate exclusive grazing on *Synechococcus*, values of +1 indicate exclusive grazing on *Prochlorococcus*.

FIG. 4.4. Clearance rate for *Prochlorococcus* (A) and *Synechococcus* (B) as a function of their respective concentration in the prey ratio experiments with *Prochlorococcus*- and *Synechococcus*-raised *P. imperforata* (closed and open symbols, respectively).

FIG. 4.5. Relationship between selectivity value (*PP*) and total prey carbon (*Prochlorococcus* biomass + *Synechococcus* biomass, mg C mL^{-1}) in the prey ratio experiments with *Prochlorococcus*- and *Synechococcus*-raised *P. imperforata* (filled and open symbols, respectively).

FIG. 4.1

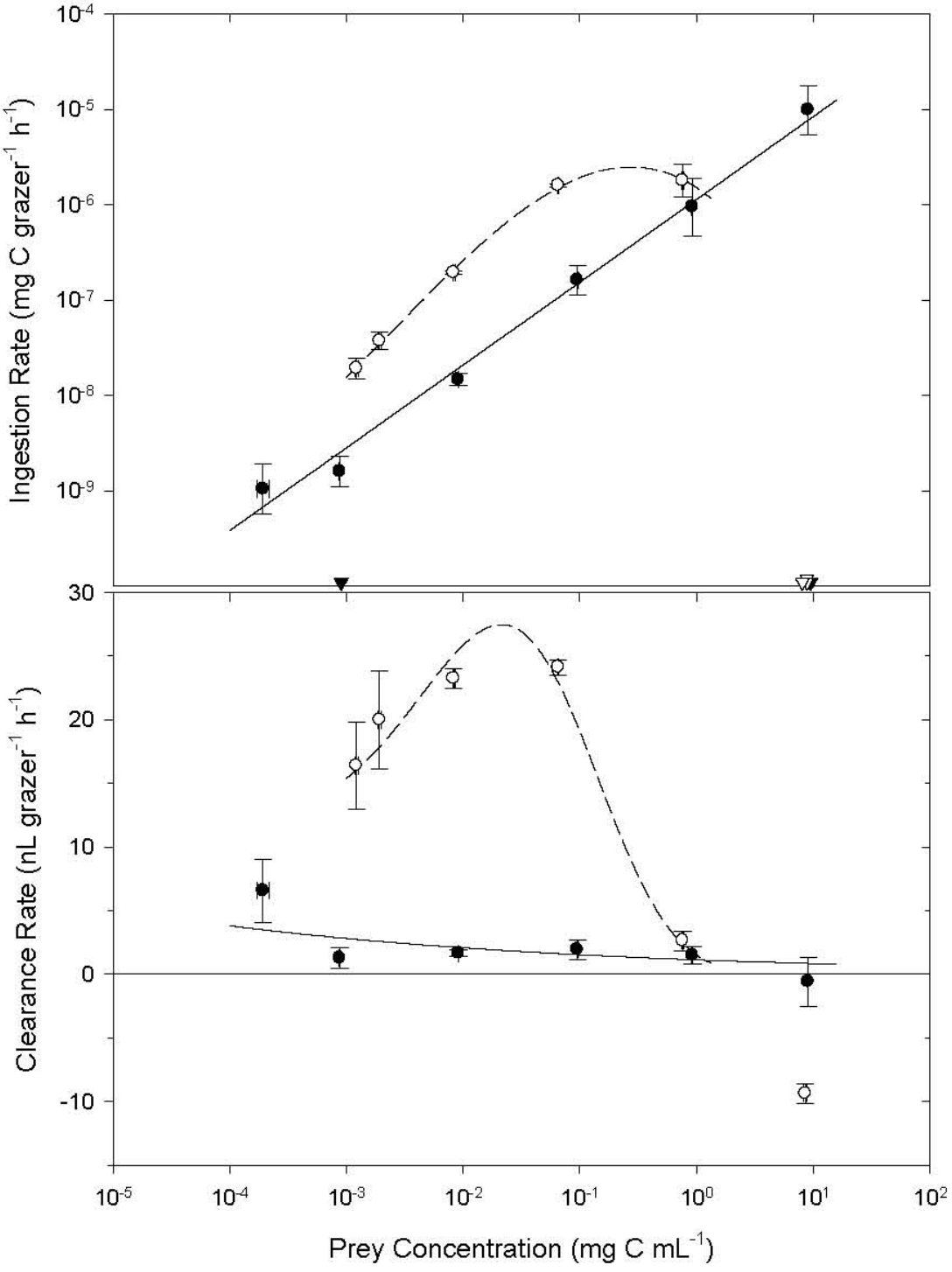

Ingestion Rate (mg C grazer^{-1} h^{-1})
10^{-4}
10^{-5}
10^{-6}
10^{-7}
10^{-8}
10^{-9}
30
Clearance Rate (nL grazer^{-1} h^{-1})
20
10
0
-10
10^{-5}
10^{-4}
10^{-3}
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
Prey Concentration (mg C mL^{-1})

FIG 4.2

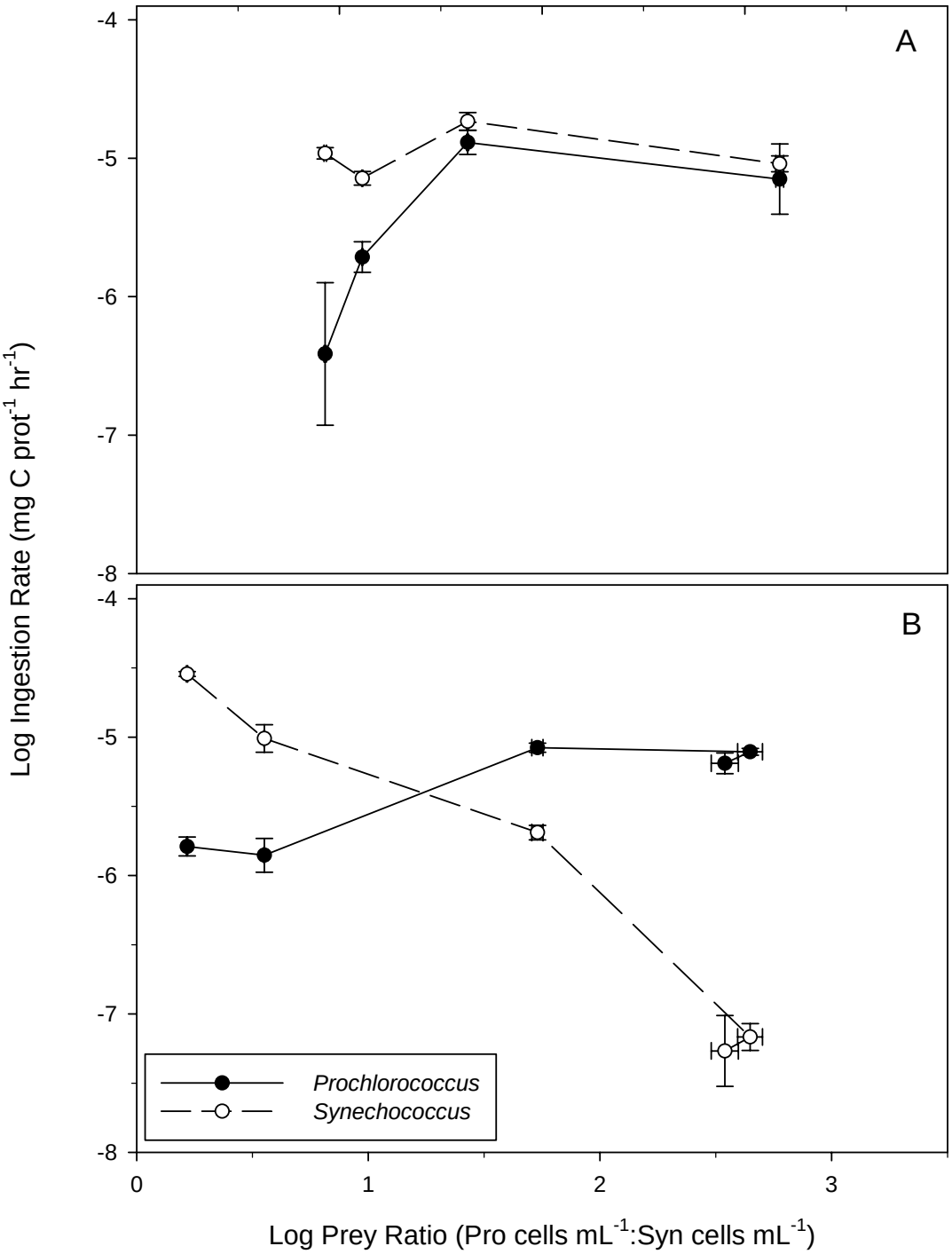

A
B
-4
-5
-6
-7
-8
0
1
2
3
Log Ingestion Rate (mg C prot-1 hr-1)
Log Prey Ratio (Pro cells mL-1:Syn cells mL-1)
Prochlorococcus
Synechococcus

FIG 4.2 (cont.)

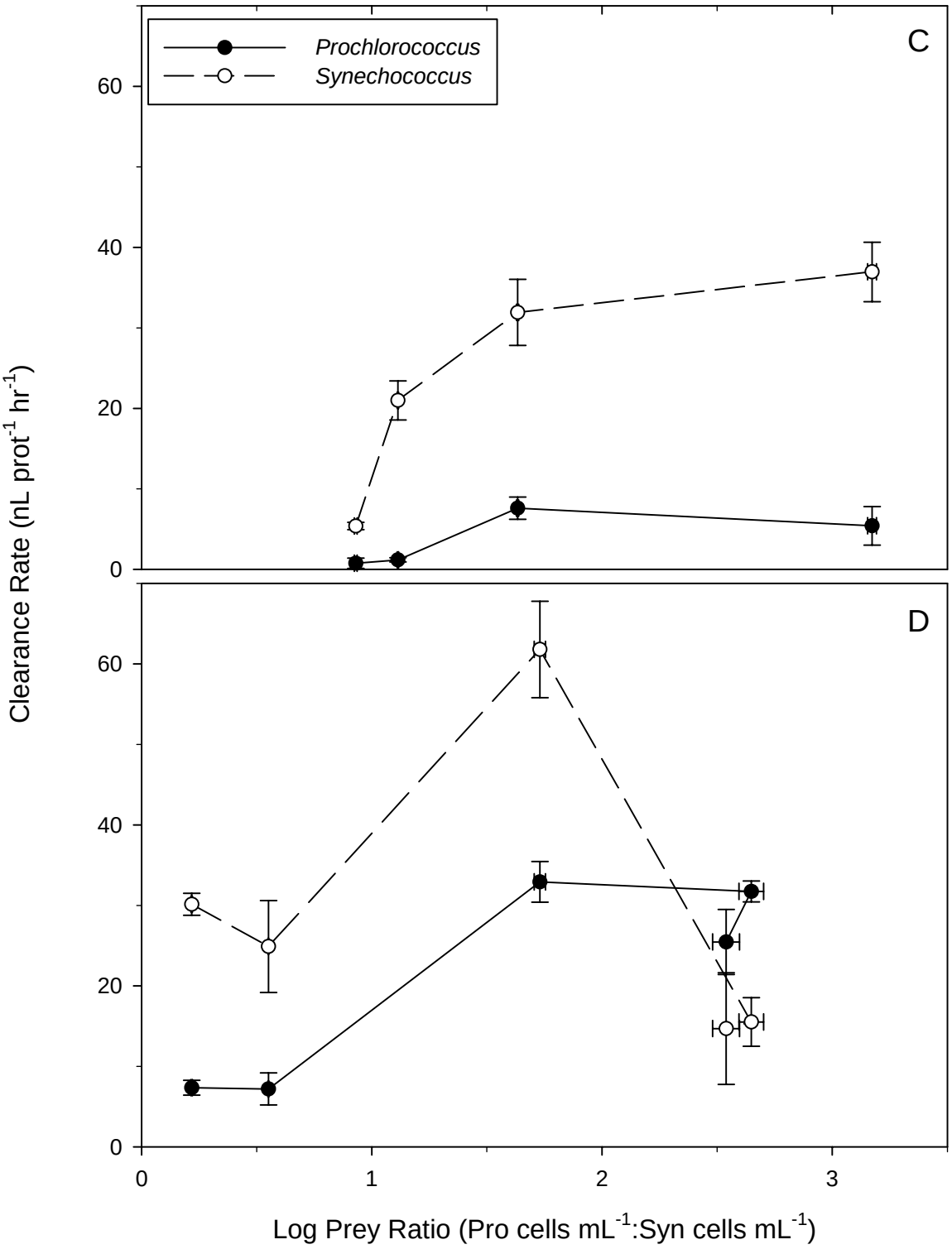

Prochlorococcus
Synechococcus
C
D
Clearance Rate (nL prot-1 hr-1)
Log Prey Ratio (Pro cells mL-1:Syn cells mL-1)
0
20
40
60
0
1
2
3

FIG 4.3

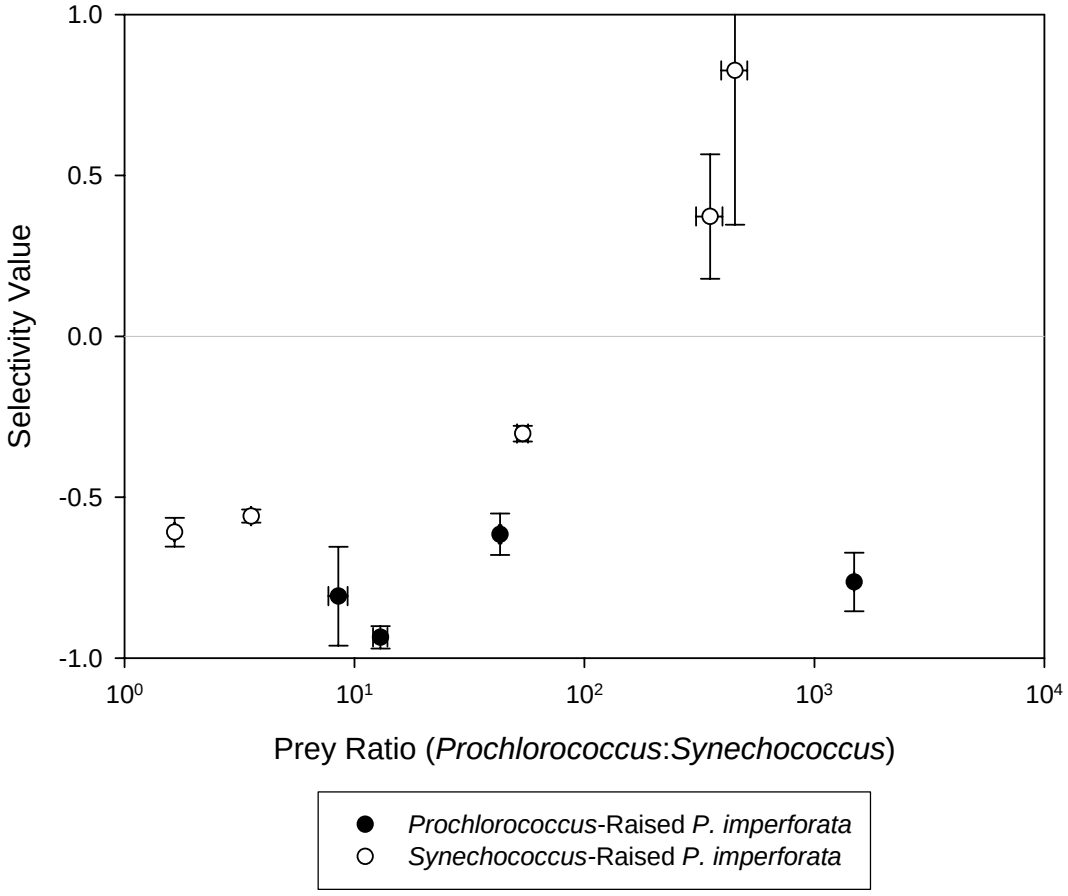

1.0
0.5
0.0
-0.5
-1.0
Selectivity Value
10⁰
10¹
10²
10³
10⁴
Prey Ratio (*Prochlorococcus*:*Synechococcus*)
Prochlorococcus-Raised *P. imperforata*
Synechococcus-Raised *P. imperforata*

FIG 4.4

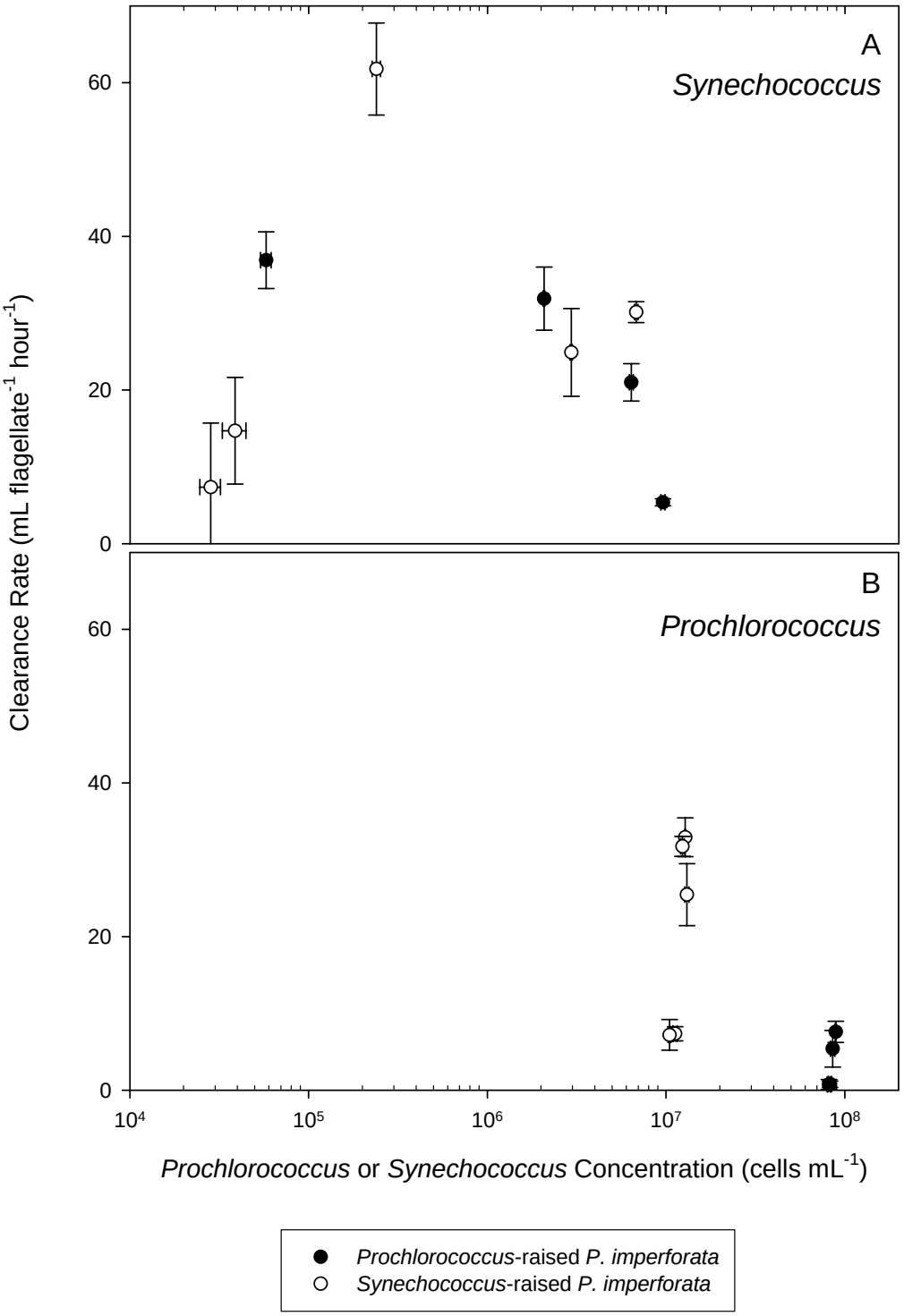
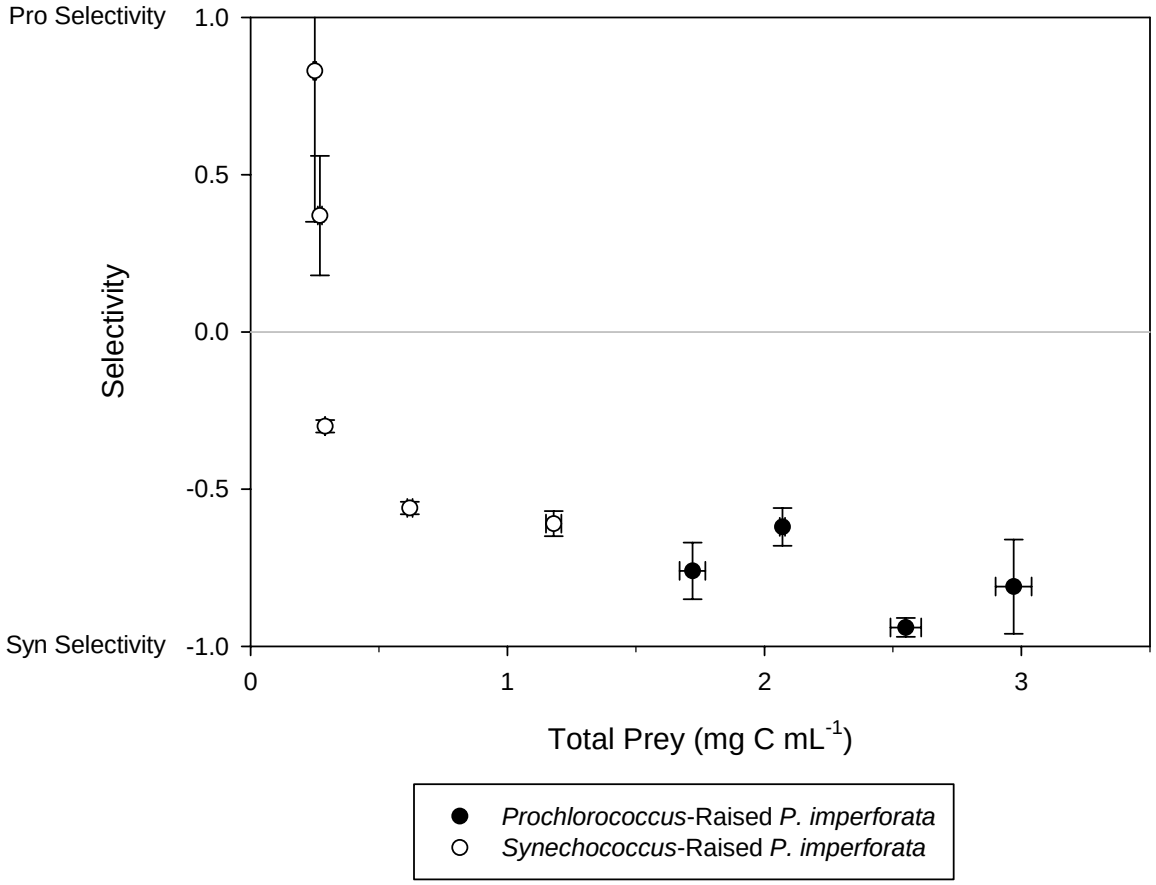

A
Synechococcus
B
Prochlorococcus
Clearance Rate (mL flagellate-1 hour-1)
0
20
40
60
10⁴
10⁵
10⁶
10⁷
10⁸
Prochlorococcus or Synechococcus Concentration (cells mL-1)
Prochlorococcus-raised P. imperforata
Synechococcus-raised P. imperforata

FIG 4.5


Pro Selectivity
Syn Selectivity
Selectivity
1.0
0.5
0.0
-0.5
-1.0
0
1
2
3
Total Prey (mg C mL-1)
Prochlorococcus-Raised P. imperforata
Synechococcus-Raised P. imperforata

CHAPTER 5

SUMMARY / CONCLUSION

Summary:

To gain a better understanding of *Prochlorococcus* and *Synechococcus* growth physiology and trophic interaction with heterotrophic nanoflagellate grazers, a series of controlled experiments were designed and implemented in the laboratory. The specific goals of this study were to:

1. Quantify the cellular RNA and protein content of *Prochlorococcus* (MIT9312) and *Synechococcus* (WH8103) as a function of growth rate.

   Major findings from this aspect of the study were:

   a. Biomass-normalized cellular RNA content increased in a linear fashion with growth rate in *Synechococcus*, while in *Prochlorococcus* it displayed a tri-phasic pattern (remaining fairly constant at low growth rates, increasing in a linear manner at intermediate growth rates, and decreasing abruptly at the highest growth rates).

   b. Cellular protein content (the cellular biomass estimate utilized in this study) generally decreased with increasing growth rate in both *Prochlorococcus* and *Synechococcus*. However *Prochlorococcus* showed an abrupt increase in cellular protein content at the highest experimental growth rates.

   c. Reanalysis of published biomass-normalized RNA data suggests that the use of cellular RNA content as a means of estimating *in situ* growth rates is a reasonable

strategy for *Synechococcus*, but may be problematic for *Prochlorococcus* (owing to the observed nonlinearities in the RNA vs. growth relationship).

2. Examine the relationship between cell cycle behavior and light-limited growth rate in *Prochlorococcus* and *Synechococcus*.

   Major findings from this part of the study were:

   a. *Prochlorococcus* MIT9312 and *Synechococcus* WH8103 DNA distributions were bimodal across the entire range of experimental growth rates employed in this study, indicating that rounds of chromosome replication are initiated synchronously and do not overlap in these organisms.

   b. Chromosome replication time, *C*, was restricted to a fairly narrow range of values (4.7±1.1 and 4.0±1.0 hours for *Prochlorococcus* and *Synechococcus*, respectively) and did not vary with growth rate. The post-DNA replication phase, *D*, reached its highest value (10-20 hours) at the lowest experimental growth rates for both strains of picocyanobacteria and decreased monotonically with increasing growth rate to minimum values of 2-3 hours.

   c. The combined duration of chromosome replication and post-DNA replication phases in *Prochlorococcus* varied approximately 2.4-fold over the range of growth rates examined (variation in *Synechococcus* was even higher) . This suggests that the assumption of invariant *C+D* commonly used in cell cycle-based growth rate calculations may be inappropriate.

   d. Both *Prochlorococcus* and *Synechococcus* cell biomass estimates were at maximum values (75 and 260 fg C $cell^{-1}$ in MIT9312 and WH8103, respectively)

at the lowest experimental growth rate levels and decreased 2- to 3-fold as growth rates increased.

e. Cell mass at the start of chromosome replication appeared to decrease with increasing growth rate indicating that the initiation of chromosome replication in *Prochlorococcus* and *Synechococcus* is not determined solely by cell biomass, as has been previously suggested.

3. Characterize the grazing behavior of *Paraphysomonas imperforata* when feeding on varying concentrations and prey ratios of *Prochlorococcus* and *Synechococcus*.

Major findings from this aspect of the study were:

a. The heterotrophic nanoflagellate grazer used in this study actively ingested both *Prochlorococcus* and *Synechococcus* prey items with clearance rates reaching maximum values of 7 and 24 nL grazer^{-1} hr^{-1} (for MIT9312 and WH8103, respectively). *Prochlorococcus* ingestion rates increased monotonically with prey concentrations over 5 orders of magnitude, while *Synechococcus* ingestion rates plateaued and eventually declined in value with increasing growth rates.

b. When challenged with *Prochlorococcus* and *Synechococcus* prey in equal proportions, both *Prochlorococcus*- and *Synechococcus*-raised *Paraphysomonas imperforata* strongly preferred *Synechococcus* prey. Under conditions of gradually increasing *Prochlorococcus*: *Synechococcus* ratio however, *Synechococcus*-raised *P. imperforata* switched its prey preference to *Prochlorococcus* when *Synechococcus* prey items became scarce. *Prochlorococcus*-raised *P. imperforata* displayed no such plasticity in prey

selection in this experiment, preferring *Synechococcus* prey items even when *Prochlorococcus*: *Synechococcus* ratios were >1000.

c. Overall prey ration may modulate the prey switching response: the shift to a preference for *Prochlorococcus* occurred only at low total prey concentrations.

Conclusions:

The objective of this dissertation was to examine the physiology and ecology of the two picocyanobacteria *Prochlorococcus* and *Synechococcus*. More specifically it was my intention to explore the effect of growth rate upon the physiology and macromolecular composition of these two important marine cyanobacterial. Additionally I aimed to characterize the grazing response of a model heterotrophic nanoflagellate when presented with a choice of both of these cyanobacteria.

Prochlorococcus and *Synechococcus*, as stated in earlier chapters, are among the dominant photosynthetic organisms in the world's marine oligotrophic gyres. The in situ growth rates of these organisms must be assessed if we are to fully understand their role in the marine microbial food web, the fate of the carbon they fix, and that factors that influence their activity and distribution. Methodologies for determining the growth rates of *Prochlorococcus* and *Synechococcus* cell populations, as reviewed previously, can be problematic. In this dissertation I examined both the potential use of macromolecular components for estimating growth rate, and the biological relationships between growth rate and the cell cycle parameters used in calculating growth rates via the cell cycle assay.

The use of macromolecular components as a proxy for growth rate has long been considered a potentially valuable approach for microbial growth rate estimates, owing to its independence from bottle incubations and their associated artifacts (see Chapter 2). For reasons

discussed previously, cellular RNA content is a particularly attractive target for this sort of methodology. Though previous studies have added greatly to our understanding of the relationship between growth rate and RNA content in *Prochlorococcus* and *Synechococcus*, the small number of strains examined to date makes it difficult to fully understand cellular RNA dynamics with respect to growth rate. The additional information provided by the examination of *Prochlorococcus* strain MIT9312 and *Synechococcus* strain WH8103 allowed us to make a more thorough evaluation of this growth rate methodology. In my study I examined the relationship between cellular RNA content and growth rate as determined via cellular RNA staining. My measurements of RNA in *Synechococcus* strain WH8103, along with the re-analysis of previous studies indicate that there is a reasonably robust relationship between growth rate and RNA content in this group. Application of this approach for estimating the growth rate in *Prochlorococcus* strain MIT9312 reconfirmed that this method might be problematic for *Prochlorococcus* strains, given the tri-phasic nature of their RNA vs. growth rate relationship. For future study it may prove useful to examine a broader scope of *Prochlorococcus* ecotypes to determine if this tri-phasic relationship is cosmopolitan among the *Prochlorococcus* genus or specific for the high-light ecotypes that have been examined to date. Additionally, cell permeability issues still need to be addressed. For unknown reasons, staining of *Prochlorococcus* and *Synechococcus* with SYBR Green RNA stain proved to be extremely problematic without a permeability treatment. These permeabilization steps unfortunately lead to the loss of naturally occurring photosynthetic pigments important in distinguishing between various phytoplankton groups.

The effects of growth rate upon variables important in the cell cycle assay were also examined in this dissertation. As stated previously (Chapters 1 and 3), the cell cycle assay is

another methodology that has been utilized in the estimation of field picocyanobacterial growth rates. The cell cycle assay uses time estimates of the various cell cycle phases to determine species specific *in situ* growth rates. Unlike the previously discussed RNA methodology, the cell cycle assay requires intensive sampling so as to obtain accurate estimates of the lengths of the various cell cycle phases. My study represents the first systematic characterization of the effects of growth rate upon *Prochlorococcus* cell cycle behavior. My results showed a surprising degree of similarity between *Prochlorococcus* and *Synechococcus* in regard to the magnitude of the different cell cycle parameters. In addition the growth rate-dependent variation in the lengths of the cell cycle phases were very comparable between groups. Of particular interest was the relatively high degree of variability in the combined duration of DNA replication (C) and post replication times (D). Due to the difficulty in determining the lengths of both the *s*- and *g2+m*-phases, a constant value is often applied when calculating growth rates via the cell cycle assay. The 2.4 fold variation in C+D values reported in Chapter 3 could translate to over- or under-estimates of *Prochlorococcus* growth rates on an equivalent order. In combination with the 2-3 fold variation I observed in *Prochlorococcus* and *Synechococcus* cell biomass, this could lead to a greater than 5 fold variation in production estimates for these groups. Ultimately these results suggest that assumptions of set/constant C+D (and cellular biomass) values must be applied with caution in the future when utilizing the cell cycle assay to determine cell growth rates.

If we are to gain a clearer understanding of *Prochlorococcus* and *Synechococcus* population dynamics, in addition to understanding their growth we must also consider factors affecting their mortality. Though *Prochlorococcus* and *Synechococcus* can both be found in the euphotic zone of the world's oligotrophic gyres, their respective dominance has been shown to vary spatially and temporally. In the Sargasso Sea, for example, *Prochlorococcus* is generally

the dominant picocyanobacteria during well-stratified summer conditions while *Synechococcus* increases in abundance (to levels equal to or greater than that of *Prochlorococcus*) during well-mixed winter conditions. Causes for this apparent shift in picocyanobacterial dominance are thought to center around the availability of inorganic nitrogen, though the impact of protozoan grazing control cannot be disregarded. Presently we have a very poor understanding of top-down control factors for the oligotrophic gyres of the world's oceans. Given that *Prochlorococcus* and *Synechococcus* generally co-occur, I thought it important to examine the effects of varying prey ratios upon the grazing characteristics of a heterotrophic nanoflagellate grazer. Prey selectivity has long been known to exist among protozoan grazers, and there were already indications in the literature that *Synechococcus* may be generally preferred over *Prochlorococcus*. This was however the first study to address the effect of prey availability on heterotrophic nanoflagellate protozoan prey preference. As discussed in Chapter 4, *P. imperforata* seemed to show a general preference for *Synechococcus* prey items when prey concentrations were high. However when prey concentrations became low, *Prochlorococcus* became an apparently more sought after prey particle. It should be stated that the apparent preference for *Synechococcus* in these experiments may be due in part (but not wholly - see Chapter 4 Appendix) to an increased potential encounter rate between *P. imperforata* and *Synechococcus* prey items (as compared to *Prochlorococcus* prey items) owing to the larger size and motility of *Synechococcus* in this study. Extrapolating these results to the field should be done with caution. In these experiments prey concentrations were significantly higher than those found in the field. Additionally, the behavior of one heterotrophic nanoflagellate can hardly be expected to correspond to the general behavior of all heterotrophic nanoflagellate grazers in the field. We presently have very little understanding of the diversity of the heterotrophic protozoan

grazers, however one could imagine that they are equally as diverse as the prey items upon which they graze. Nevertheless, these results do point to the importance of considering the abundance of alternate prey when assessing the grazing mortality of picocyanobacteria in the field.