PHYSIOLOGICAL ECOLOGY OF *PROCHLOROCOCCUS*: A COMPARISON OF ISOLATES FROM DIVERSE OCEANOGRAPHIC REGIMES

by

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Submitted to the Department of Civil and Environmental Engineering in Partial Fulfillment of the Requirements for the Degree of Environmental Engineering and Aquatic Sciences

DOCTOR OF PHILOSOPHY
AT THE
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

September 1997

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ABSTRACT

Prochlorococcus, the newest member of the marine cyanobacterial picophytoplankton community, is uniquely different from the closely related marine A *Synechococcus*. *Prochlorococcus* are smaller and contain divinyl chlorophylls *a* and *b* (chl a_2 and chl b_2) as their primary photosynthetic pigments. They are generally more abundant and extend deeper in the water column than *Synechococcus* in oligotrophic oceans. To gain insight into the ecology of *Prochlorococcus*, this thesis examines the light-dependent physiology of this group in relation to *Synechococcus* and among different *Prochlorococcus* isolates.

An examination of the light-dependent growth response revealed that *Prochlorococcus* can grow faster than *Synechococcus* at lower irradiances. In addition, the small size and unique pigmentation of *Prochlorococcus* results in higher relative absorption efficiency in the blue wavelengths that penetrate into the deep euphotic zone of oligotrophic ocean waters. Differences in the light-dependent physiology also were apparent between two isolates of *Prochlorococcus*, SS120 and MED4. SS120 has higher chl b_2/chl a_2 ratios than MED4 and is adapted to growth at low irradiances but is completely photoinhibited in growth at the higher irradiances at which MED4 is growing maximally.

Coexisting populations of *Prochlorococcus* were observed by flow cytometry in single water samples in the north Atlantic, however, the physiological relationship of these coexisting populations was unknown. Isolates obtained from these populations were found to be physiologically and phylogenetically distinct when cultured under identical conditions in the laboratory, and their molecular phylogenetic relationship correlated with their physiology. The physiological differences between these coexisting isolates are similar to the differences observed between SS120 and MED4, leading to the hypothesis that isolates of the genus *Prochlorococcus* can be distinguished by their pigmentation, light utilization capabilities and phylogenetic relationship. The physiological response of ten different isolates from diverse oceanographic regimes was studied to examine this hypothesis. We found that the isolates

adapted for growth at low irradiances have higher chl b/a_2 ratios, chlorophyll *a*-specific absorption coefficients and photosynthetic efficiencies, and appear to have a different photoacclimative strategy relative to those adapted to high light levels. Low and high-light adaptation may be a general distinguishing characteristic within the genus *Prochlorococcus*.

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ACKNOWLEDGMENTS

As with any major endeavor in life, completing a Ph.D. dissertation takes dedicated perseverance to handle the intellectual and emotional challenges that come over the years. I would like to thank the members of the Chisholm lab, past and present, for all their help, discussions and, most importantly, their wonderful friendship. Special thanks go to Jeff Dusenberry for teaching me more than I ever wanted to know about flow cytometry, to Gabrielle Rocap for helping me be strong, and to Sheila Frankel for her support and perspective on science and life. I also want to acknowledge my colleague, Heidi Sosik, who has helped me tackle many scientific issues and particularly helped in the crucial last "push". I was extremely fortunate to have Penny Chisholm as my advisor. She provided encouragement and trust, and forced me to think and write independently. During my most trying moments, she was there as a friend, to which I am eternally grateful. Thanks also to Daniel, my husband and friend, for his caring support and encouragement over the years. And finally, my son, Evan, now almost two years old, has made my world brighter, richer and more complex.

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CHAPTER ONE

Introduction

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Phytoplankton community structure in oligotrophic oceans is dominated by prokaryotic and eukaryotic picophytoplankton less than 2 μm in diameter. About 15 years ago, a new member of the marine prokaryotic picoplankton community, *Prochlorococcus*, was discovered (Chisholm et al., 1988), pushing the lower limit for size and upper limit for abundance of marine phytoplankton. Because of its abundance, wide distribution, unique pigment composition and small size, the biology and ecological role of *Prochlorococcus* is being extensively studied.

The first recorded documentation of *Prochlorococcus* was by Johnson and Sieburth (Johnson and Sieburth, 1979), who observed them by transmission electron microscopy (TEM) in North Atlantic field samples. They included the unknown cells as members of *Synechococcus* because of their gross anatomical similarity to this genus. Sometime thereafter, Gieskes and Kraay (1983) discovered that a large fraction (>85%) of the measured chlorophyll in the North Atlantic was actually a derivative of chlorophyll, which they called "red-shifted" chlorophyll *a* because its Soret peak is shifted towards the red relative to "normal" chl *a* absorption. The "red-shifted" chl *a* was almost exclusively associated with the <1 μm fraction of filter-fractionated seawater, along with zeaxanthin which was the dominant carotenoid in this fraction. By 1988 flow cytometric signatures of cells exhibiting low chlorophyll fluorescence per cell (related to pigment content) and low right angle light scattering (related to cell size) relative to *Synechococcus* cells were observed in the Sargasso Sea (Chisholm et al., 1988; Li and Wood, 1988; Neveux et al., 1989). This characteristic flow cytometric signature coupled with HPLC pigment analysis and TEM was connected with the

"type-II" *Synechococcus* and "red-shifted" pigment data, allowing these unusual cells to be identified as a type of free-living, marine prochlorophyte (chlorophyll *a* and *b* containing photosynthetic prokaryotes, *sensu* Lewin, 1981) (Chisholm et al., 1988).

Prochlorococcus are distinguished from other picophytoplankton based on four major morphological and physiological characteristics: a) they are small in size (0.6 - 0.8 μm in diameter compared to 1.0 μm average diameter of *Synechococcus* and 2-3 μm average diameter of picoeukaryotic algae); b) they have chlorophyll *a* and chlorophyll *b*, in the divinyl form (chl a_2 and chl b_2, respectively), as their major photosynthetic pigments; c) they lack phycobiliproteins characteristic of other cyanobacteria (but see below); and d) they have low chlorophyll fluorescence and light scattering characteristics as measured by flow cytometry. These planktonic marine prochlorophytes were given the genus and species name *Prochlorococcus marinus* (Chisholm et al., 1992).

Since its identification and over the course of this dissertation, the spatial and temporal distribution of *Prochlorococcus* and its contribution to biomass and primary production have been explored throughout the major oceanographic provinces, particularly in relation to the co-occuring prokaryotic picoplankton, *Synechococcus*, and eukaryotic picophytoplankton. In addition to field studies, isolates of *Prochlorococcus* have been obtained from several regions of the world's oceans, and work on cultured cells includes phylogenetic analysis, pigment characterization and physiological studies. This introduction will review what is known about

the ecology and physiology of this important member of the picophytoplankton community in the open ocean.

ECOLOGY

Global, seasonal and vertical distributions

Prochlorococcus are easily identified based on their flow cytometric signature and/or on the presence of divinyl chlorophyll *a* (chl a_2), which, so far, is unique to these phytoplankton. Using these methods, *Prochlorococcus* have proven to be ubiquitous throughout the euphotic zone in the north Atlantic Ocean, the north, south and equatorial Pacific Oceans, the Mediterranean Sea, the northwestern Indian Ocean and Arabian Sea, and the Red Sea (Fig. 1,Table 1). *Prochlorococcus* are generally not found in coastal regions, with a few exceptions: the near coastal, eutrophic waters of the tropical northeastern Atlantic Ocean (Partensky et al., 1996), the surface waters of the Gulf of Policastro off southwestern Italy (Li et al. 1992), Suruga Bay off the coast of Japan (Shimada et al., 1995) and the low salinity, coastal waters of the Mediterranean Sea. In the latter two locations the presence of *Prochlorococcus* could be due to mixing dynamics (Vaulot et al. 1990). Generally, *Prochlorococcus* co-occur with *Synechococcus* and picoeukaryote populations (see e.g. Veldhuis and Kraay, 1993; Campbell and Vaulot, 1993; Olson et al., 1990).

Prochlorococcus abundance varies seasonally and vertically in the water column and are correlated with water column stability in the north Pacific (Campbell et al., 1997), north Atlantic (Olson et al., 1990), Mediterranean Sea (Li et al., 1993; Vaulot et al., 1990),

TABLE 1 - Observations of *Prochlorococcus* throughout the world's oceans.

LOCATION	REFERENCE
North Atlantic Ocean.....	Chisholm et al. 1988[1] Neveux et al. 1989[1] Olson et al. 1990[2] Veldhuis and Kraay, 1990[1] Li et al. 1992[1]; 1995[1] Goericke and Repeta, 1993[3] Veldhuis et al. 1993[2] McManus and Dawson, 1994[1] Graziano et al. 1996[1] Partensky et al. 1996[1]
Carribbean, Panama Basin, Gulf of Mexico, and Southern California Bight.....	Chisholm et al. 1988[1]
central & eastern North Pacific Ocean.....	Campbell and Vaulot, 1993[2] DiTullio et al. 1993[2] Ishizaka et al. 1994[4] Blanchot and Rodier, 1996[2]
equatorial Pacific Ocean.....	Everitt et al. 1990[3] Chavez et al. 1991[2] Vaulot et al. 1994[2]
western Pacific, Japan, China Sea, Banda Sea.....	Gieskes et al. 1988[3] Shimada et al. 1993[1], 1995[1], 1996[1] N. Jiao, personal communication[1]
Mediterranean Sea.....	Vaulot et al. 1990[2] Li et al. 1992[2]; 1994[2] Bustillos-Guzman et al.; 1995[3]
Northwestern Indian Ocean and Red Sea.....	Veldhuis and Kraay, 1993[1] Lindell and Post, 1995[1] Veldhuis et al. 1997[1]

Notes: presence indicated by [1]the combined use of flow cytometry and detection of chl a_2; [2]flow cytometry alone; [3]detection of chl a_2 alone; [4]epifluorescence microscopy.

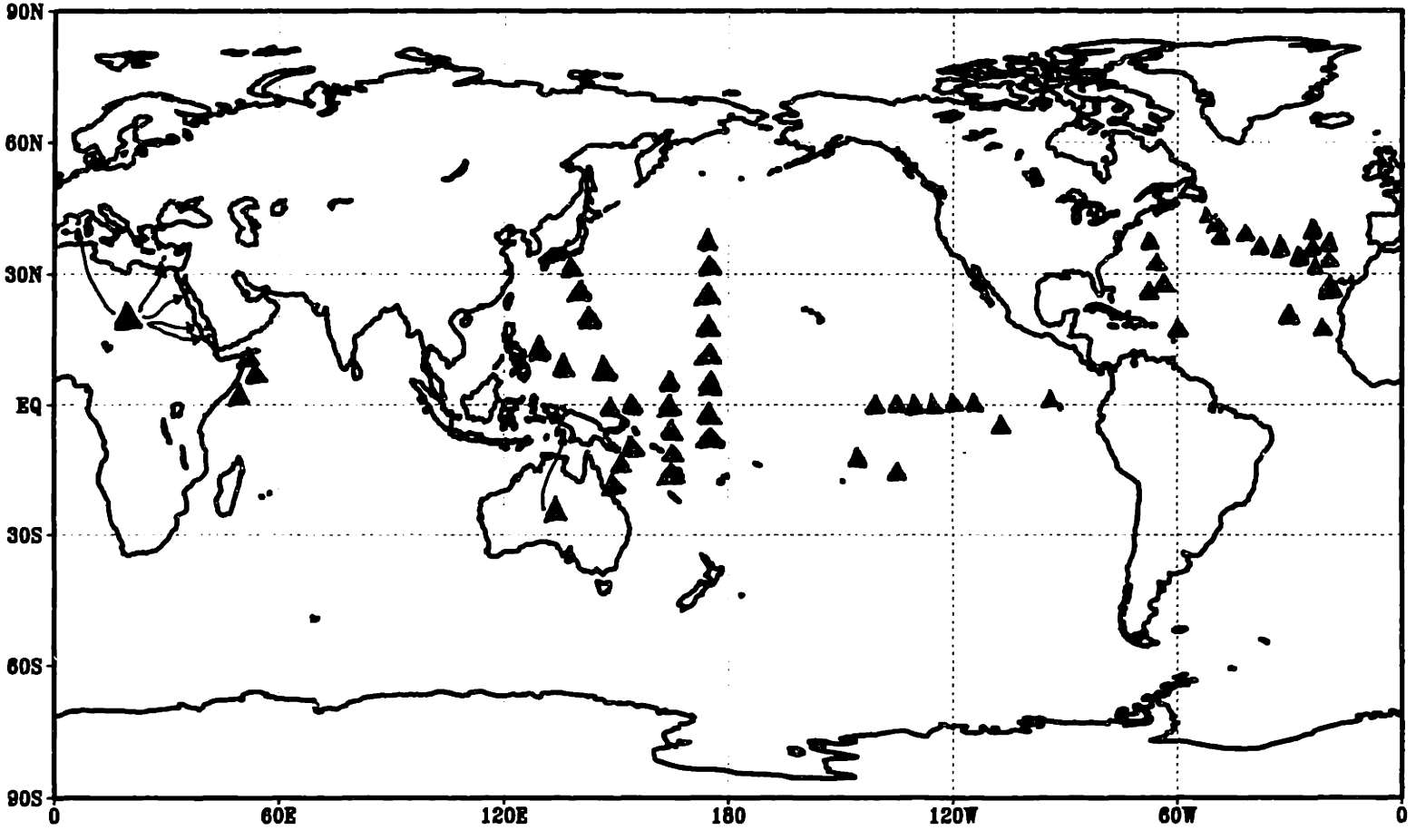

Figure 1 - Observations of *Prochlorococcus* in the world's oceans.

northwestern Indian Ocean (Veldhuis et al., 1997) and the Red Sea (Lindell and Post, 1995). Abundance maxima occur in the summer/fall months when the water column stabilizes, the mixed layer depth shoals, and the nitracline deepens. The pattern of vertical distribution varies seasonally and between oceanographic provinces and also differs from that of the *Synechococcus* and picoeukaryotic populations. In general, *Prochlorococcus* populations are present throughout the euphotic zone and extend to greater depths (in some places down to depths of 0.1% full surface sunlight, I_o) than *Synechococcus* (Campbell et al., 1997; Partensky et al., 1996; Shimada et al., 1996; Ishizaka et al., 1994; Veldhuis et al., 1993; Olson et al., 1990). The eukaryotic component of the picophytoplankton community also can extend to depths that *Prochlorococcus* can but in significantly fewer numbers (Campbell et al., 1997; Partensky et al., 1996; Shimada et al., 1996; Olson et al., 1990).

In the central and eastern North Atlantic when summer stratification sets in and the nitracline deepens (due to depletion within the surface of the euphotic zone), *Prochlorococcus* establishes a subsurface maximum usually associated with the deep chlorophyll maximum and almost always located slightly above the nitracline (Partensky et al., 1996; Li, 1995; Veldhuis et al., 1993; Olson et al., 1990). The median depth of the eukaryotic phytoplankton is similar to that of the *Prochlorococcus* population, whereas the *Synechococcus* population remains uniformly abundant in the surface waters and then declines with depth (Partensky et al., 1996; Li, 1995; Veldhuis et al., 1993; Olson et al., 1990). During the summer period, the integrated abundance of *Prochlorococcus* exceeds that of *Synechococcus* populations (Li, 1995; Veldhuis et al., 1993; Olson et al., 1990). In the winter as the mixed layer deepens and the

nitracline shoals, the depth-integrated abundance of *Prochlorococcus* and *Synechococcus* are roughly equal, and these picophytoplankton populations are distributed uniformly throughout the euphotic zone (Olson et al., 1990). Whereas *Prochlorococcus* and *Synechococcus* bloom in the summer and winter months, respectively, the picoeukaryotes appear to bloom in the spring (Olson et al., 1990).

Like the vertical profiles of *Prochlorococcus* in the North Atlantic, *Prochlorococcus* populations in the western and central North Pacific exhibit a subsurface maximum, usually associated with the subsurface chlorophyll maximum (Shimada et al., 1996; Ishizaka et al., 1994). In the subtropical North Pacific at station ALOHA (22_ 45' N, 158_ W), however, the characteristic vertical distribution of *Prochlorococcus* reveals a fairly uniform abundance in the upper 100 m, with a slight maximum below the surface mixed layer (about 75 m) and decreasing abundance down to about 200 m (Campbell et al., 1997). The picoeukaryotes display a subsurface maximum positively correlated with the deep chlorophyll maximum, whereas *Synechococcus* are generally restricted to the upper 100 m. (Campbell et al., 1997; Campbell and Vaulot, 1993). In contrast to the situation in the North Atlantic, *Prochlorococcus* at station ALOHA always exceed *Synechococcus* in abundance and undergo significant but relatively small range of variation with season (Campbell et al., 1997). The phasing in seasonal cycles for the picophytoplankton community at Station ALOHA, and possibly throughout the north Pacific, is similar to that observed in the Atlantic: peak abundance occurs for *Prochlorococcus* in summer/fall, *Synechococcus* in winter, and picoeukaryotes in spring (Campbell et al., 1997). Previously, the seasonal pattern in the

picophytoplankton community structure of the north Pacific was thought to differ from that observed in the Atlantic (Campbell and Vaulot, 1993) until more detailed time-series data was obtained for the Pacific (Campbell et al., 1997).

The seasonal succession of picophytoplankton in the Red Sea was similar to that seen in the subtropical Pacific and Atlantic Oceans, with water column stability being a major factor determining *Prochlorococcus* bloom conditions (Lindell and Post, 1995). In these oceanographic provinces, *Prochlorococcus* establish a subsurface maximum during the nutrient-depleted, summer stratification period, reaching the highest integrated water-column abundance (Veldhuis et al., 1997; Lindell and Post, 1995; Veldhuis and Kraay, 1993). The seasonal change in the *Prochlorococcus* population in the Red Sea is more dramatic as the population declines to undetectable levels during the nutrient-replete, deep winter mixing period (Lindell and Post, 1995). The eukaryotic algae bloom in the winter and the *Synechococcus* bloom during the spring period and again in the fall (Lindell and Post, 1995). During both SW and NE monsoon periods in the northwestern Indian Ocean when the water column is unstable, *Prochlorococcus* become a minor component of the phytoplankton community and *Synechococcus* dominates (Veldhuis et al., 1997).

Vertical variation in *Prochlorococcus* populations is also apparent in the Mediterranean Sea. Maximum cell concentrations ($1 - 4 \times 10^4$ cells ml^{-1}) are lower in the Mediterranean Sea than in the Atlantic and Pacific Oceans and do not differ considerably with season (Li et al., 1993; Vaulot et al., 1990). During the winter in stratified, offshore waters of

the northwestern Mediterranean Sea, *Prochlorococcus* exhibit a surface maxima and then decline in abundance with depth (Vaulot et al., 1990). In the eastern Mediterranean Sea (time of year is not given), a subsurface maximum was the typical feature, except at two stations where the water column was well-mixed and the vertical distribution of *Prochlorococcus* abundance was uniform (Li et al., 1993).

Abundance and contribution to biomass and primary production

Prochlorococcus are typically the most abundant phytoplankton in the oligotrophic oceans, up to 4 x 10^5 cells ml^{-1}, resulting in significant contributions of *Prochlorococcus* to phytoplankton and total microbial biomass and primary production. In the north Atlantic the contribution of *Prochlorococcus* to the total phytoplankton biomass, indicated by the ratio of chl a_2 to total chl *a*, ranges from 25 - 58% (Partensky et al., 1996; Li, 1995; McManus and Dawson, 1994; Goericke and Repeta, 1993; Goericke and Welschmeyer, 1993). In terms of carbon biomass, *Prochlorococcus* contributed only 15% of the depth-integrated total microbial carbon biomass and 11-32% of the total phytoplankton carbon biomass, based on cell counts and an assumed cellular carbon content of 59 fg C $cell^{-1}$ or carbon/light scatter considerations, despite the fact that *Prochlorococcus* abundance was an order of magnitude greater than both the *Synechococcus* and picoeukaryote abundance (Li, 1995; Li et al., 1992).

In the north Atlantic, Goericke and Welschmeyer (1993) estimated that the average contribution of *Prochlorococcus* to primary production was 28-38% of the total in the winter, characterized by no systematic variation with depth. In the summer, the *Prochlorococcus*

contribution decreased to 10-20% of the total in the surface layer and increased to 80% of the total at the 1.6% light level (Goericke and Welschmeyer, 1993). Another study in the North Atlantic compared the relative contributions of the ultraphytoplankton (< 3 μm in diameter) components to primary production in the north Atlantic by flow cytometric sorting of ^{14}C-labeled cells (Li, 1995; Li, 1994). The *Prochlorococcus* contribution to the seasonal and depth integrated primary production (11 - 57 %) is between that due to eukaryotic ultraphytoplankton (32 - 79%) and greater than that due to cyanobacteria (2 - 20%).

In the north Pacific, *Prochlorococcus* abundance is generally 1-2 orders of magnitude above that of *Synechococcus* and picoeukaryotes and its contribution to microbial biomass is higher than that estimated for the Atlantic. At station ALOHA, *Prochlorococcus* was the greatest contributor (39%) to the total chlorophyll biomass in the deep chlorophyll maximum layer (Letelier et al., 1993) and contributed 35% to depth-integrated microbial carbon biomass and more than 50% to depth-integrated photosynthetic carbon biomass, assuming 53 fg C cell^{-1} (Campbell et al., 1994). Using the same cellular carbon content for *Prochlorococcus*, depth-integrated carbon biomass for *Prochlorococcus* in the central equatorial Pacific ranged from 27 - 41% of the total photosynthetic biomass (Binder et al., 1996) and in the western tropical Pacific, the *Prochlorococcus* contribution was even higher (50 - 59 %), which is slightly greater than the picoeukaryotic contribution (Blanchot and Rodier, 1996).

Prochlorococcus contribution to primary production in the Pacific Ocean (Liu et al., 1997; Liu et al., 1995; Vaulot et al., 1995) was estimated from cell abundance and growth

rates, assuming a cellular carbon content for *Prochlorococcus* of 53 fg $cell^{-1}$ (Campbell et al., 1994). The contribution of *Prochlorococcus* to the total gross primary production increases as the euphotic zone of the water column becomes more oligotrophic: 5-39 % in the equatorial Pacific (Liu et al., 1997; Vaulot et al., 1995) and up to 82% in the subtropical North Pacific Ocean at Station ALOHA (Liu et al., 1997). At station ALOHA in the North Pacific, the integrated carbon production due to *Prochlorococcus* (382.2 mg C m^{-2} d^{-1}) was 25-fold higher than that produced by *Synechococcus* (14.6 mg C m^{-2} d^{-1}) (Liu et al., 1995). In the equatorial Pacific, *Prochlorococcus* daily integrated production rates varied between 174 and 488 mg C m^{-2} d^{-1}, comparable to rates measured in the north Pacific (Vaulot et al., 1995).

In the northwestern Indian Ocean, the *Prochlorococcus* and *Synechococcus* contributions to phytoplankton community structure differs from what is seen in the Atlantic and Pacific Oceans. *Prochlorococcus* populations are a factor of 2-5 less abundant (Veldhuis et al., 1997) and *Synechococcus* are 2-5 times more abundant (Veldhuis et al., 1997; Burkill et al., 1993) in the northwestern Indian Ocean compared to the Atlantic and Pacific Oceans. In the Red Sea, *Prochlorococcus* and *Synechococcus* maximum concentrations are comparable, whereas the eukaryotic algae concentration is always much lower. *Prochlorococcus* contributed as much as 57% to the total chlorophyll biomass in the southern Red Sea and in the Indian Ocean off the Somalian coast during upwelling conditions (Veldhuis and Kraay, 1993). Picophytoplankton in the Indian Ocean and Red Sea contribute substantially to primary production, though relative contributions by *Prochlorococcus* and *Synechococcus* were not presented (Veldhuis et al., 1997; Jochem et al., 1993; Owens et al., 1993).

In the Mediterranean Sea, *Prochlorococcus* range in population abundance from about 10^3 - 10^4 cells ml^{-1} and are estimated to contribute 5 - 31% of the total photosynthetic biomass in the surface waters and up to 75% below the deep chlorophyll maximum (Li et al., 1993; Vaulot et al., 1990).

Environmental factors regulating distributions and abundances

The now well-documented differences in spatial and seasonal distribution of the picophytoplankton community in the open ocean points to the fact that *Prochlorococcus* populations are regulated differently from the *Synechococcus* and picoeukaryote populations. Physical properties of the water, such as light, temperature and water column stability, are all important environmental determinants for *Prochlorococcus* distributions. It is clear that the latitudinal distribution of *Prochlorococcus* is limited, in part, by temperatures below 14 - 17°C. It is not found north of 43°N in the Atlantic and 39°N in the Pacific, whereas *Synechococcus* can be found in waters with temperatures below 12°C, extending its distribution much farther north (Ishizaka et al., 1994; Veldhuis et al., 1993; Veldhuis and Kraay, 1990; Olson et al., 1990; Waterbury et al., 1986). Only during winter months in the Mediterranean Sea has *Prochlorococcus* been detected at temperatures as low as 12.5°C (Vaulot et al., 1990).

The quantity and spectral quality of the subsurface light field is also a major determinant of *Prochlorococcus* distributions. *Prochlorococcus* are most abundant in waters

where light of blue wavelengths penetrates the deepest, and its population can extend to depths corresponding to ~0.01% I_o (see *vertical distributions* above). Several aspects of *Prochlorococcus* physiology enable this picoplankter to outcompete other phytoplankton in low blue light regimes (see Physiology Section).

Nutrient and trace metal limitation may play an important role in determining the distribution of *Prochlorococcus* populations. *Prochlorococcus* dominate the phytoplankton community structure (abundance maximum is typically above 10^5 cells ml^{-1}) and are growing at or near their maximum rates (as compared with studies on isolates, see below) in oligotrophic oceanic regions where macronutrients (primarily nitrate) are limiting and in the high-nutrient, low-chlorophyll regions of the equatorial Pacific where iron is the limiting nutrient (Liu et al., 1997; Campbell et al., 1997; Binder et al., 1996; Blanchot and Rodier, 1996; Partensky et al., 1996; Vaulot et al., 1995; Campbell et al., 1994; Veldhuis et al., 1993; Olson et al., 1990). *Prochlorococcus* chlorophyll fluorescence and light scattering properties were affected by iron addition during open ocean iron fertilization experiments (IronEx II) (Cavender-Bares et al. 1997). Other trace metals may also be important in determining depth distributions of *Prochlorococcus*. High copper concentrations in the surface waters of the Sargasso Sea in the summer may be partially responsible, in addition to photoinhibition, for the decrease in *Prochlorococcus* populations in the surface waters (J. Moffet, unpublished observation). On-deck incubations of natural picoplankton populations and laboratory experiments with isolates show that *Prochlorococcus* are more sensitive to copper than *Synechococcus* (E. Mann, unpublished results). In eutrophic and mesotrophic areas where

macronutrient and trace metal concentrations are higher, *Prochlorococcus* is absent or in low concentrations, and *Synechococcus* and picoeukaryotes are present in higher concentrations than in oligotrophic waters (Partensky et al., 1996; Waterbury et al., 1986).

The relationship of *Prochlorococcus* abundance maximum with nitrogen concentration appears to be complex. In the north Atlantic (McManus and Dawson, 1994; Olson et al., 1990) and some locations of the tropical and subtropical Pacific Oceans (Ishizaka et al., 1994; Shimada et al., 1993), *Prochlorococcus* abundance maximum is positively correlated with the nitracline. However, in other locations of the north Pacific, the peak abundance of *Prochlorococcus* is associated with the nitrate-depleted layer of the water column (Blanchot and Rodier, 1996; Campbell and Vaulot, 1993). Differences between oceanographic provinces and with depth may be related to different subpopulations of *Prochlorococcus* (see below) (Campbell and Vaulot, 1993).

Grazing pressure and viral infections are other likely controls of *Prochlorococcus* abundance. Small heterotrophic nanoflagellates and larger protozoa appear to be the primary grazers of picoplankton, with the grazers showing no apparent preference for a specific group of picoplankton (Reckermann and Veldhuis, 1997; Liu et al., 1995). Mortality rates due to grazing are roughly equal to *Prochlorococcus* growth rates (Reckermann and Veldhuis, 1997; Liu et al., 1997; Liu et al., 1995; Landry et al., 1995; Landry et al., 1995). Viruses are responsible for a sizeable fraction of cyanobacterial and heterotrophic bacterial mortality

(Proctor and Fuhrman, 1990) and are important to *Synechococcus* population dynamics (Waterbury and Valois, 1993).

In situ *growth and mortality rates*

In situ growth rates of *Prochlorococcus* can be estimated from the incorporation of ^{14}C into chl a_2 (Goericke and Welschmeyer, 1993), incubations and dilution experiments (Landry et al., 1995; Veldhuis et al., 1993) or a selective metabolic inhibitor method (Liu et al., 1995). All of these methods depend on sample incubations (usually 24 h), which is often a major source of error due to "bottle effects". Two other methods to estimate *in situ* growth rates eliminate the need for incubations and the associated errors: one method takes advantage of diel patterns in DNA distributions of different cell cycle stages (diel DNA distribution method; see Vaulot, 1992 for detailed description); the other exploits diel patterns in forward angle light scatter (i.e. cell size) as measured by flow cytometry (Binder et al., 1996; DuRand, 1995). The diel DNA distribution method works well for *Prochlorococcus* since DNA distributions of *Prochlorococcus* populations are tightly synchronized with the daily light cycle ([Shalapyonok, 1997 #889]; Liu et al., 1997; Binder et al., 1996; Partensky et al., 1996; Vaulot et al., 1995). For example, a diel pattern in the depth-integrated *Prochlorococcus* cell concentrations reveals a maximum at midnight and a minimum at 16:00-18:00 hours in the equatorial and subtropical north Pacific Ocean. Unfortunately, because of multiple DNA peaks in some strains of marine *Synechococcus* (Binder and Chisholm, 1995), the diel DNA distribution method is not always possible for measuring growth rates of natural populations of *Synechococcus*, but the diel changes in light scattering and incubation-based

methods have been used with some success (Liu et al., 1997; Landry et al., 1995; Liu et al., 1995; DuRand, 1995).

In the north Atlantic Ocean, growth rates for *Prochlorococcus* vary with depth ranging from 0.3 – 0.75 day^{-1} at the surface (DuRand, 1995; Goericke and Welschmeyer, 1993; Veldhuis et al., 1993) and generally decrease with depth (Partensky et al., 1996; Goericke and Welschmeyer, 1993; Veldhuis et al., 1993). In the Sargasso Sea during July 1993, however, *Prochlorococcus*-specific growth rates as high as 0.6 day^{-1} were measured at 90 m (DuRand, 1995).

Growth rates for the *Prochlorococcus* population in the Pacific Ocean are also variable. At station ALOHA in the North Pacific, *Prochlorococcus* populations grew at rates of 0.4 - 0.6 day^{-1}, whereas *Synechococcus* populations grew faster, reaching growth rates of 1.0 day^{-1} within the surface mixed layer (Liu et al., 1997; Liu et al., 1995). At the base of the euphotic zone below which *Synechococcus* populations were detected (as deep as 175 m in October 1993), *Prochlorococcus* populations grew at about 0.1 day^{-1} (Liu et al., 1995). In the equatorial Pacific, *Prochlorococcus* growth rates range from 0.1 - 0.78 day^{-1}, with the maximum rates occuring at 70 m depth along the western stretch and occurring at 15 - 45 m in the central and eastern portion of the equatorial Pacific (Liu et al., 1997; Binder et al., 1996; DuRand, 1995; Vaulot et al., 1995). Growth rates in the surface mixed layer reached as high as 0.55 – 0.7 day^{-1} (Liu et al., 1997; Binder et al., 1996; DuRand, 1995; Vaulot et al., 1995). However, growth rate estimates based on the dilution technique for *Prochlorococcus*

in surface waters (10 m) of the central equatorial Pacific were considerably lower (0.26 ± 0.26 day^{-1}) compared to the rates obtained from incubation-free methods, possibly due to photoinhibition at the high light levels over the course of the 24 hr incubation (Landry et al., 1995; Landry et al., 1995). The range and depth profiles of growth rate estimates for eukaryotic phytoplankton in the equatorial Pacific were similar to those of *Prochlorococcus*, whereas the *Synechococcus* rates were lower (DuRand, 1995).

The diel DNA distribution method for natural populations of *Prochlorococcus* in the northwestern Arabian Sea yielded high growth rates of 0.92 - 1.06 day^{-1} ([Shalapyonok, 1997 #889]). Based on cell counts over the course of 24 h incubation periods, net growth rates in the Indian Ocean and Red Sea were quite variable with depth, and in the southern Red Sea, very high values (1.8 day^{-1}) were measured in the surface waters (Veldhuis et al., 1997). Rates this high have never been reported for *Prochlorococcus* cultures and may be due to some methodological error. In the deeper euphotic zone at the low light levels, negative values of *Prochlorococcus* population growth were often measured, indicative of high grazing pressure (Reckermann and Veldhuis, 1997; Veldhuis et al., 1997).

Estimates of the grazing impact on picoplankton can be made in conjunction with growth rates estimates from dilution and selective inhibitor experiments. So far, *Prochlorococcus* mortality rates due to grazing have only been reported for the equatorial and north Pacific Ocean (Liu et al., 1997; Liu et al., 1995; Landry et al., 1995; Landry et al., 1995) and the Indian Ocean (Reckermann and Veldhuis, 1997). In both oceanographic

provinces grazing mortality varied, but overall they roughly balanced *Prochlorococcus* growth rates (Reckermann and Veldhuis, 1997; Liu et al., 1997; Liu et al., 1995; Landry et al., 1995; Landry et al., 1995), as is the case for *Synechococcus* (Landry et al., 1995; Burkill et al., 1993).

Pigment and chlorophyll fluorescence distributions

In stratified waters, the pigment content and mean cellular fluorescence of *Prochlorococcus* populations increases with depth, reflecting photoacclimation of pigments to decreasing surface light intensities (Goericke and Repeta, 1993; Veldhuis and Kraay, 1990; Neveux et al., 1989; Chisholm et al., 1988). In addition, significant increases with depth in the cellular ratio of the accessory pigment chl *b* to divinyl chl a_2 have been observed in all locations under stratified conditions (chl *b* can be attributed primarily to *Prochlorococcus* in oligotrophic, open ocean waters when evidence for other chl *b*-containing phytoplankton is minimal or absent; see Goericke and Repeta, 1993 and Letelier et al., 1993). Ratios of chl *b*/chl a_2 increase up to 20-fold from surface waters to the deep euphotic zone: 0.3 - 3.0 in the western tropical north Atlantic (McManus and Dawson, 1994) and Sargasso Sea ((Goericke and Repeta, 1993), < 0.1 - 1.5 in the northeastern Atlantic (Partensky et al., 1996), and 0.12 - 2.4 at station BM in the Red Sea (Veldhuis and Kraay, 1993).

The vertical distribution of flow-cytometrically measured cellular chlorophyll fluorescence for *Prochlorococcus* displays significant increases when the water column is stratified, which is attributed to photoacclimation of cellular pigment content but also may be

a result of changes in *Prochlorococcus* population composition (see below). In the north Atlantic Ocean, increases in chlorophyll fluorescence of approximately 20-fold were observed with depth for *Prochlorococcus* during springtime (Olson et al., 1990). In the western equatorial Pacific (Blanchot and Rodier, 1996) and at station ALOHA in the subtropical north Pacific (Campbell and Vaulot, 1993), increases in chlorophyll fluorescence with depth are more extreme, about 50-fold, inFor *Prochlorococcus* in the Red Sea, the mean chlorophyll fluorescence signal increases by a factor of 25, from the surface down to the 0.01% I_o depth (Veldhuis and Kraay, 1993).

Multiple Prochlorococcus populations

Bimodal distributions of *Prochlorococcus* chlorophyll fluorescence as measured by flow cytometry have been observed in the North Atlantic (chapter III of this thesis; Partensky et al., 1996; McManus and Dawson, 1994; Olson et al., 1991), the equatorial and North Pacific (Binder et al., 1996; Blanchot and Rodier, 1996; Campbell and Vaulot, 1993) and the Red Sea (Veldhuis and Kraay, 1993). The co-occurence of at least two populations of *Prochlorococcus* at Station ALOHA in the North Pacific was hypothesized by Campbell and Vaulot (1993) to explain these bimodal distributions: one population with high mean chlorophyll fluorescence and acclimated to low light and high nutrients (influx at nitracline), and the other population with lower mean chlorophyll fluorescence, acclimated to low nutrients and extending to the surface waters. Size-fractionated pigment analysis at this station revealed that *Prochlorococcus* of different size classes (1.2-0.65 μm and 0.65-0.22 μm) contained different chl *b*/chl a_2 ratios, supporting the idea that two physiologically

distinct subpopulations of *Prochlorococcus* coexisted (Letelier et al., 1993). In the North Atlantic, Goericke and Repeta (1993) also hypothesized the co-occurence of two types of *Prochlorococcus*, based on the broad range in chl *b*/chl a_2 ratio with depth that has not been observed for individual *Prochlorococcus* isolates in culture (Moore et al., 1995). The coexistence of two physiologically and genetically distinct *Prochlorococcus* has recently been demonstrated for two locations in the North Atlantic, since flow cytometrically sorted cells of each type maintained their relative fluorescence characteristics and pigment contents once established in culture (see chapter III).

PHYSIOLOGY AND PHYLOGENY

Isolates

Prochlorococcus was first isolated by Brian Palenik in 1988 from 120 m in the Sargasso Sea (the primary cultures were originally called LG and SARG). Since then many more isolates have been obtained from the Mediterranean Sea, the North Atlantic, and the South, Equatorial and North Pacific Ocean (Table 2). Five clonal decendants from the first isolate (SS2, SS35, SS51, SS52, and SS120) and two clonal isolates from a Mediterranean Sea isolate (MED1 and MED4) were obtained by serial dilution and are deposited at the Provassili-Guillard Center for Cultured Marine Phytoplankton (Chisholm et al., 1992). Cloned isolate SS120 has been designated the type culture, CCMP1375 (Chisholm et al., 1992). Only one of the isolates, the non-clonal primary culture, SARG, has been successfully made axenic through serial dilution (R. Rippka, personal communication).

TABLE 2 - *Prochlorococcus* isolates in culture. All cultures are primary cultures obtained by filter fractionation, except where noted.

ISOLATE NAME	COORDINATES	DEPTH	ISOLATOR	DATE ISOLATED
Atlantic isolates				
Sargasso Sea				
SARG (=LG)	28° 59'N; 64° 21'W	120 m	B. Palenik	30-May-88
SS120[a] (=CCMP1375[b])	"	"	"	"
SS35[a] (=CCMP1428)	"	"	"	"
SS51[a] (=CCMP1376)	"	"	"	"
SS52[a] (=CCMP1377)	"	"	"	"
SS2[a] (=CCMP1427)	"	"	"	"
MIT9301	34° 10.1'N; 66° 18.2'W	90 m	L. R. Moore	10-Jul-93
MIT9302[c,f]	34° 45.5'N; 66° 11.1'W	100 m	L. R. Moore	15-Jul-93
MIT9303[c,f]	"	"	"	"
MIT9401	station 14	surface	L. Aref	CH0694
Gulf Stream				
MIT9311[d,f]	37°30.8'N; 68°14.4'W	135 m	L. R. Moore	17-Jul-93
MIT9312[d,f]	"	"	"	"
MIT9313[d,f]	"	"	"	"
MIT9314	"	180 m	"	"
North Atlantic				
NATL2A	38° 59'N; 49° 33'W	30 m?	F. Partensky	Apr-90
NATL1	37° 39'N; 40° 1'W	30 m	"	"
Tropical Atlantic				
TATL1	21° 02'N; 31° 08'W	20 m	F. Partensky	Oct-91
TATL2	20° 25'N; 31° 08'W	30 M	"	"
Pacific isolates				
South Pacific				
MIT9107[e]	14° 60'S; 134° 60'W	25 m	J. Dusenberry	8-Aug-91
MIT9116[e]	"	"	"	"
MIT9123[e]	"	"	"	"
MIT9201	11° 60'S; 145° 25'W	surface	B. Binder	26-Sep-92
MIT9202	"	79 m	"	"
Equatorial Pacific				
MIT9211	0°; 140°W	1% Io	R. Olson	10-Apr-92
MIT9215	"	surface	B. Binder	3-Oct-92
MIT9321	1°N; 92°W	50 m	P. Chisholm	12-Nov-93
MIT9322	0° 16'N; 93°W	surface	P. Chisholm	16-Nov-93
MIT9515	5° 44.9'S; 107°5.25W	15 m	E. Mann	4-Jun-95
West Pacific				
GP2	8° 32.5'N; 136° 31.8'E	150 m	A. Shimada	10-Sep-92
SB	35° 00.9'N;138° 35.8'E	40 m	"	21-Oct-92
tropical Pacific				
PAC1	22° 45'N; 158° 00'W	100 m	L. Campbell	Apr-92

TABLE 2 - continued

Mediterranean isolates				
MED (=DV)	43° 12'N; 6° 52'E	5 m	D. Vaulot/F. Partensky	Jan-89
MED4[a] (=CCMP1378)	"	"	"	"
MED1[a] (=CCMP1426)	"	"	"	"

Notes for Table 2 - *a*: Clonal cultures, obtained by serial dilution of the isolate; *b*: culture number in Center for the Culture of Marine Phytoplankton (CCMP), Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, MA; *c, d, e*: These are separate subcultures from the same field sample; *f*: isolate obtained by flow cytometrically sorting away from other phytoplankton.

Analysis of light-dependent physiology of SARG (and SS120) and MED (and MED4) *Prochlorococcus* isolates has greatly aided the ecological interpretation of *Prochlorococcus* distribution in the field. This introduction will just touch on aspects of *Prochlorococcus* physiology since the rest of the thesis focuses on this. Nutrient physiology has yet to be explored for *Prochlorococcus* isolates, but will most likely provide valuable information for interpreting its ecology.

Ultrastructure

Prochlorococcus are the smallest known phytoplankton. The majority of cells pass through a 0.8 µm filter; and the average forward light scatter signal (related to cell size) is a third that of *Synechococcus* (Chisholm et al., 1988). Based on transmission electron micrographs (TEM), natural populations and isolates of *Prochlorococcus* are small, coccoid-shaped cells (ranging 0.45 - 0.8 µm in length and 0.35 - 0.6 µm in diameter) (Lichtle et al., 1995; Chisholm et al., 1992). The average size of SARG is slightly larger than that of MED

based on TEM measurements and Coulter Counter estimates (Lichtle et al., 1995; Morel et al., 1993; Chisholm et al., 1992).

Prochlorococcus is distinctly different from *Synechococcus* in its ultruastructure. TEM of cultured and natural populations of *Prochlorococcus* reveals that the thylakoid membranes are peripherally located and tightly appressed, with no evidence of phycobilisomes between the thylakoid membranes (Chisholm et al., 1988; Chisholm et al., 1992). Inter-isolate comparison reveals that the thylakoid membranes are concentrically arranged in SARG (Lichtle et al., 1995; Chisholm et al., 1992; Chisholm et al., 1988) and horseshoe-shaped in MED, observed in all section planes (Lichtle et al., 1995). In addition, both isolates contain carboxysome-like structures, reflecting the presence of autotrophic enzymes and glycogen storage granules, but gas vacuoles have not been observed (Lichtle et al., 1995; Chisholm et al., 1988).

Pigments

Prochlorococcus have a distinct suite of pigments not seen in any other prokaryotic or eukaryotic phototroph (Table 3). It does not contain "normal" monovinyl chlorophyll *a* (chl a_1) as its dominant photoreactive pigment but instead contains divinyl chlorophyll *a* (chl a_2) (Goericke and Repeta, 1992). Chl a_2 has a blue absorption peak that is shifted about 10 nm to the red relative to chl a_1 (Bazzaz, 1981), i.e. the "red-shifted" chlorophyll of Gieskes and Kraay (1983). This picophytoplanter represents the first known wild-type oxygenic phototroph which does not use "normal" chl *a* in its photosystem.

TABLE 3 - Comparative pigment content of *Prochlorococcus* and *Synechococcus*.

pigment type	*Prochlorococcus*[1]	marine *Synechococcus*[2]
chl a_1	-	+
chl a_2	+	-
chl b_1	+/-[3]	-
chl b_2	+	-
zeaxanthin	+	+
α-carotene	+	-
β-carotene	-	+
phycoerythrin	+/-[4]	+
phycocyanin	-	+
Mg 3, 8 divinyl-pheoporphyrin a_5	+	-
cryptoxanthin?	+/-	-

+ indicates presence, - indicates absence, +/- indicates presence in some but not all isolates.
[1]All pigment data for *Prochlorococcus* from Goericke and Repeta, 1992, except where indicated otherwise; [2]from Waterbury et al. 1979, Goericke and Repeta, 1992; [3]from Partensky et al. 1993, Moore et al. 1995; [4]from Hess et al. 1996.

Prochlorococcus cells also contain an unusual complement of accessory pigments: divinyl chlorophyll *b* (chl b_2), zeaxanthin, α-carotene (not found in other prokaryotic phototrophs), chl *c*-like pigment (possibly Mg 3,8 divinyl-pheoporphyrin a5), and an unknown carotenoid (Goericke and Repeta, 1992; Chisholm et al., 1988). Four isolates of *Prochlorococcus* also contain chl b_1 at high irradiances (chapter IV; Moore et al., 1995; Partensky et al., 1993). Pigment content is an important factor in determining the spatial distribution of algae and phytoplankton, and this holds true for *Prochlorococcus* as well. The divinyl chlorophylls *a* and *b* optimize absorption efficiency in the blue region of the visible spectrum (445-480nm), allowing *Prochlorococcus* populations to extend to the deep euphotic zone of oligotrophic waters where the light is primarily blue.

Recently, the presence of phycoerythrin has been reported in *Prochlorococcus* clone SS120 but was absent in MED4 and four other Atlantic isolates (Hess et al., 1996). The functional role of this phycobiliprotein in this isolate of *Prochlorococcus* awaits further investigation; some physiological role is suggested based on the presence of flow cytometrically-generated low orange fluorescence from the *Prochlorococcus* population in the deep euphotic zone of the tropical south Pacific (Hess et al., 1996).

Isolates from the Atlantic [SARG (and SS120) and NATL2 (from the North Atlantic)] and the Mediterranean Sea [MED and (MED4)] photoacclimate their pigments by increasing their cellular chlorophyll content as growth irradiance decreases, however, they differ significantly in their ratios of total chl *b* to chl a_2 (Partensky et al., 1993; Moore et al., 1995; Morel et al., 1993). The chl *b*/chl a_2 ratio of SARG and SS120 is 10-fold higher than that of MED and MED4 (Partensky et al., 1993; Moore et al., 1995), and that of NATL1 is intermediate (Partensky et al., 1993). The significant differences in chlorophyll content between the Sargasso Sea and Mediterranean Sea isolates result in higher mean chlorophyll fluorescence (as measured by flow cytometry) for the former (Moore et al., 1995), distinctly different absorption properties (Partensky et al., 1993; Moore et al., 1995; Morel et al., 1993) and different growth capabilities (see below) (Moore et al., 1995).

Absorption and scatter properties

The *in vivo* absorption spectrum of *Prochlorococcus* differs between isolates with different chl b/a_2 ratios. SS120, with its high ratio of chl b/a_2, has a prominent chl b_2

absorption peak in the blue in addition to the chl a_2 Soret peak, whereas MED4 does not (Partensky et al., 1993; Moore et al., 1995; Morel et al., 1993). Absorption efficiency of *Prochlorococcus* is comparable to and sometimes exceeds that of *Synechococcus*, primarily due to the smaller size of *Prochlorococcus* (Moore et al., 1995; Morel et al., 1993).

Prochlorococcus cells have the lowest forward light scattering signals relative to other phytoplankton, reflecting their small cell size (Chisholm et al., 1988). In addition, *Prochlorococcus* have a higher backscattering-to-scattering ratio relative to other algae, but the backscattering capacity remains relatively low (Morel et al., 1993). These scattering properties help explain some of the optical characteristics typical to oligotrophic Case 1 waters. In this water type, vertical profiles of beam attenuation are almost featureless and do not correlate with the vertical structure of fluorescence (or chlorophyll), in part because *Prochlorococcus*, the dominant phytoplankton generally associated with the deep chlorophyll maximum, has such low scattering that its impact upon the attenuation coefficient is almost undetectable (Morel et al., 1993).

Growth rate

The light saturated growth rates for cultured *Prochlorococcus* isolates range from 0.54 - 0.83 day^{-1} (chapter III & IV; Moore et al., 1995). Recently, rates as high as 0.99 day^{-1} have been measured by cell cycle analysis (diel DNA distribution method) for two isolates of *Prochlorococcus* ([Shalapyonok, 1997 #889]), indicating that the maximum achievable growth rate for *Prochlorococcus* has yet to be determined. Differences in growth capabilities

are apparent between *Prochlorococcus* isolates: SS120 exhibits growth at lower irradiances and is more easily photoinhibited than MED4 (Partensky et al., 1993; Moore et al., 1995). This difference in growth along with the distinctly difference pigment contents and flow cytometric chlorophyll fluorescence signals between these two isolates led to the hypothesis that SS120 is low light adapted and most likely predominates in the deep euphotic zone, whereas MED4 is high light adapted and predominates in surface waters (Moore et al., 1995). This hypothesis is consistent with the observation of co-occuring *Prochlorococcus* populations in the oceans (see above). Furthermore, physiological measurements on several other isolates (see chapters III & IV) support the low-light adapted and high-light adapted ecotype distinction.

Photosynthesis and photosynthetic apparatus

High photosynthetic efficiency (α) and quantum yields (ϕ^{CO2}) were reported for several *Prochlorococcus* isolates relative to other phytoplankton (chapter III & IV ; Shimada et al., 1996; Partensky et al., 1993). Unusually high light harvesting efficiency and near maximal quantum yields were measured for a natural population of *Prochlorococcus* dominating a deep secondary chlorophyll peak in the central Arabian Sea (Johnson et al., 1997). Consistent with its high absorption efficiency in the blue, *Prochlorococcus* was found to be more efficient at energy transfer in blue-violet light (400-460nm) than *Synechococcus*, based on photosynthetic action spectrum measurements (Shimada et al., 1996).

Photoacclimation of photosynthesis examined for three *Prochlorococcus* isolates,

(SARG, MED, and NATL1) revealed some atypical patterns in photoacclimation of P_{max} and α (Partensky et al., 1993). Photosynthesis-irradiance measurements on eight additional isolates reveal that inter-isolate differences and photoacclimation patterns in the photosynthetic parameters (P_{max}, α, ϕ_m, I_{max}, and I_b) relate to the cellular chlorophyll content (chapter III & IV) and are distinctly different between low-light adapted and high-light adapted *Prochlorococcus* isolates (chapter III & IV).

Major structural differences exist between the antenna complexes of SS120 and MED4 (Partensky et al., 1997). SS120 has a higher concentration of antenna complexes and lower chl a/b ratio in the antenna complexes relative to MED4. In addition, SS120 can differentially regulate its various apoproteins within the antenna complex in response to changes in growth irradiance, whereas MED4 maintains relatively constant concentrations of its apoproteins compared to the total thylakoid proteins as growth irradiance changes.

Characterization of the *psbA* gene encoding for the D1 protein component of the PSII reaction center in SS120 reveals that this low-light adapted isolate contains only a single copy of this gene (Hess et al., 1995). This single *psbA* gene encodes for the lower-light *iso*-form of the D1 protein, consistent with the low-light growth capability of this isolate (Hess et al., 1995).

Regulation of carbon fixation occurs in part by transcriptional control of ribulose bisphosphate carboxylase (RubisCO) gene expression (enzyme synthesis) in a

Prochlorococcus isolate from the Pacific Ocean (Pichard et al., 1996). In addition, the pattern of expression displays a diel rhythmicity implying circadian control of transcription of this gene when the culture was maintained under continuous illumination (Pichard et al., 1996).

Phylogeny

Based on sequence analysis of a variety of genes, *Prochlorococcus*, once considered the putative ancestor to chloroplasts, has been shown to be more closely related to cyanobacteria than to chloroplasts (Hess et al., 1995; Urbach et al., 1992; Palenik and Haselkorn, 1992). The lack of relationship to chloroplasts is corroborated by analysis of the chl *a/b*-binding genes of *Prochlorococcus*. The chl *a/b*-binding light harvesting protein is encoded by a constitutively expressed gene that is more closely related to the cyanobacterial *isiA* gene, which encodes a chl *a*-binding protein (CP43') in iron-starved cyanobacteria, than to the eukaryotic genes encoding for the chl *a/b*-binding proteins in chloroplasts (La Roche et al., 1997; La Roche et al., 1996). The dissimilarity of the light harvesting complex proteins of *Prochlorococcus* and chloroplasts was also seen immunologically (Lichtle et al., 1995).

Not only are *Prochlorococcus* isolates closely related to cyanobacteria, 16S rRNA gene sequence analysis indicates that they form a single marine picoplankton clade with marine A *Synechococcus* (Urbach et al., 1997). The physiological relationship of many of the isolates is reflected in the phylogenetic relationship. Several high-light adapted *Prochlorococcus* isolates cluster in a shallow branch of the marine picoplankton clade referred

to as a "high-light adapted" clade (Urbach et al., 1997; chapter III), whereas low-light adapted isolates are on separate, deeper branches (chapter III; G. Rocap, unpublished results).

GOAL OF THIS THESIS

The overall goal of this dissertation has been to characterize the light-dependent physiological ecology of the marine picoplankter, *Prochlorococcus*. At the beginning of this research very little was known about this organism. There were only a few publications related to *Prochlorococcus* in the field (Olson et al., 1990; Chisholm et al., 1988) and no publications about isolates of *Prochlorococcus*. Thus, my first goal was to compare the photoacclimative response of two *Prochlorococcus* isolates (SS120 and MED4) to that of an open-ocean strain of *Synechococcus*, WH8103 (see chapter 2, Moore et al., 1995). This work paved the way for the rest of the dissertation research to: (1) determine whether the physiological differences seen between SS120 and MED4 are due to the different depth or geographical location of isolation, (2) determine whether more than one physiological type of *Prochlorococcus* could coexist in a single location, (3) characterize the range of physiological response to light of various *Prochlorococcus* isolates from several geographical regions, (4) identify any physiological patterns which could be used to categorize *Prochlorococcus* isolates into groups, and (5) use the physiological information from laboratory studies on isolates to further our understanding of the ecology of *Prochlorococcus*.

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CHAPTER TWO

Comparative physiology of *Synechococcus* and *Prochlorococcus*: influence of light and temperature on growth, pigments, fluorescence and absorptive properties.

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Vol. 116: 259–275, 1995 | MARINE ECOLOGY PROGRESS SERIES Mar. Ecol. Prog. Ser. | Published January 12

Comparative physiology of *Synechococcus* and *Prochlorococcus*: influence of light and temperature on growth, pigments, fluorescence and absorptive properties

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ABSTRACT: *Prochlorococcus marinus* is abundant and widespread throughout the world's oceans and always co-occurs geographically with the marine cyanobacterium *Synechococcus*. In the Atlantic Ocean, these 2 picoplankters exhibit different spatial and seasonal distributions. In order to better understand the ecology of these species, we measured growth and photoacclimation responses including fluorescence excitation [$F^*_{ph}(\lambda)$] and *in vivo* absorption [$a^*_{ph}(\lambda)$] spectra over a range of growth irradiances for *P. marinus* (clone SS120) and *Synechococcus* WH8103, both isolated from the Sargasso Sea. To explore the physiological diversity of *P. marinus*, we measured the physiological responses of another *P. marinus* clone, MED4, isolated from the Mediterranean Sea. Growth rate as a function of temperature was also examined for all 3 clones. *P. marinus* SS120 and *Synechococcus* WH8103 have different temperature optima for growth, but these do not explain the different latitudinal distributions in the North Atlantic. *P. marinus* SS120 is adapted for growth at low light intensities relative to *Synechococcus* WH8103, which is consistent with the relative depth distribution of *P. marinus* and *Synechococcus* in the field. The light-dependent growth response of *P. marinus* MED4 is more similar to *Synechococcus* WH8103 than to *P. marinus* SS120. The unique pigment content of *P. marinus* (which contain divinyl chlorophylls *a* and *b*) results in maximal absorbance in the blue wavelengths. The high total chl *b*/chl *a* ratio of *P. marinus* SS120 enables it to absorb more light, grow faster than *Synechococcus* WH8103 (and *P. marinus* MED4) at low light intensities, and presumably to outcompete *Synechococcus* in the deep euphotic zone. At high growth irradiances, *P. marinus* SS120 contains measureable amounts of normal (monovinyl) chl *b*, whereas this pigment was not found in *P. marinus* MED4 at any growth irradiance. Photoacclimative changes in pigment ratios, and not package effect, account for most of the changes in $a^*_{ph}(\lambda)$ and $F^*_{ph}(\lambda)$ with light intensity for all 3 picoplankters. At high light intensities, zeaxanthin contributes substantially to $a^*_{ph}(\lambda)$ in the blue, but appears to transfer little or no excitation energy to the reaction centers, based on $F^*_{ph}(\lambda)$ measurements. For *P. marinus*, high absorption in the blue due to divinyl chl *a* and *b* relative to normal chl *a* and *b*, absorption due to zeaxanthin, and small cell size result in unusually high a^*_{ph} (blue) relative to a^*_{ph} (red).

KEY WORDS: *Prochlorococcus marinus* · *Synechococcus* · Light · Absorption · Pigments · Divinyl chlorophyll *a*

INTRODUCTION

Prochlorococcus marinus (Chisholm et al. 1992) is ubiquitous throughout the euphotic zone in tropical and subtropical oceans and contributes substantially to photosynthetic biomass and primary production (Chisholm et al. 1988, Campbell & Vaulot 1993, Goericke & Welschmeyer 1993). It has a unique suite of pigments, which includes divinyl chlorophyll *a* (chl a_2) as the principal light-harvesting pigment, and divinyl chl *b* (chl b_2), zeaxanthin, α-carotene and a chl *c*-like pigment as the main accessory pigments (Goericke &

Repeta 1992). Based on flow cytometric signatures and/or the presence of chl a_2, *P. marinus* has been found in the Atlantic Ocean (Neveux et al. 1989, Olson et al. 1990, Veldhuis & Kraay 1990), the tropical and subtropical Pacific (Chavez et al. 1991, DiTullio et al. 1992, Campbell & Vaulot 1993), the Mediterranean Sea (Vaulot et al. 1990, Vaulot & Partensky 1992), and the Red Sea (Veldhuis & Kraay 1993). *Synechococcus* has always been found in regions where *P. marinus* is present, although *P. marinus* often extends to lower depths (Olson et al. 1990, Campbell & Vaulot 1993, Veldhuis & Kraay 1993). *P. marinus* is usually found in abundances reaching 10^4 to 10^5 cells ml^{-1} (Olson et al. 1990, Vaulot et al. 1990, Chavez et al. 1991, Campbell & Vaulot 1993, Veldhuis & Kraay 1993) and can contribute up to 65% of total chl *a*, i.e. the sum of chl a_1 and chl a_2 (Veldhuis & Kraay 1990, Goericke & Welschmeyer 1993). Goericke & Welschmeyer (1993) measured the growth rate of *P. marinus* by measuring the incorporation of ^{14}C into chl a_2. Surface layer growth rates of *P. marinus* ranged from 0.1 to 0.5 d^{-1} and did not vary systematically over the seasons; growth rates at the subsurface chlorophyll maximum (SCM) ranged from 0.04 to 0.16 d^{-1}. These authors also calculated that the seasonally averaged contribution of *P. marinus* to the total primary productivity in the Sargasso Sea was 25%.

Prochlorococcus marinus has been observed in the North Atlantic only when surface water temperatures were above 15°C, suggesting that low temperature can influence the *P. marinus* distribution (Olson et al. 1990, Veldhuis et al. 1993). In the North Atlantic, *P. marinus* blooms after *Synechococcus* does (after the onset of spring stratification) and establishes a subsurface abundance maximum usually associated with the deep chlorophyll maximum (Olson et al. 1990). *P. marinus* is generally present throughout the euphotic zone during the winter, fall and spring, but during the early summer the majority of cells are largely restricted to the SCM (Olson et al. 1990). In the North Pacific (station ALOHA), *P. marinus* is uniformly abundant in the surface of the euphotic zone and declines in numbers with depth during all seasons (Campbell & Vaulot 1993). *Synechococcus* populations in both the North Atlantic and North Pacific are uniformly abundant in the surface and have no subsurface maximum yearround (Olson et al. 1990, Campbell & Vaulot 1993). *P. marinus* populations are more abundant and extend deeper in the water column than *Synechococcus* populations throughout most of the year in the oligotrophic North Atlantic (Olson et al. 1990, Veldhuis & Kraay 1993) and Pacific (Campbell & Vaulot 1993).

Prochlorococcus marinus seems to be particularly well adapted to growth in low light, as determined by laboratory studies (Partensky et al. 1993) and distributions observed in the field. At the 1.6% light level in the Sargasso Sea, *P. marinus* was found to grow twice as fast as other phytoplankton, based on ^{14}C incorporation into chl a_2 and chl a_1 (Goericke & Welschmeyer 1993). Morel et al. (1993) suggested that the ability of *P. marinus* to dominate the algal population in the deeper euphotic zone may be due, in part, to the optical properties of *P. marinus*. Because of its small size, it has a higher absorption efficiency than *Synechococcus*, even though both are well suited for absorbing the blue light available in oligotrophic waters (Morel et al. 1993).

In stratified waters, photoacclimation (used here to describe reversible light-induced alterations in the physiological or morphological characteristics of a population) of *Prochlorococcus marinus* populations is reflected by a change in cellular concentrations of chl a_2 and in mean cellular fluorescence (Olson et al. 1990, Veldhuis & Kraay 1990, 1993, Campbell & Vaulot 1993). Goericke & Repeta (1993) reported dramatic changes of the ratio of chl b_2/chl a_2 with depth at a station in the southern Sargasso Sea, ranging from 0.1 in the surface layer to 3.0 below the SCM.

These field observations led us to examine how light and temperature affect the growth rates of *Prochlorococcus marinus* and *Synechococcus* and to analyze the effects of light on the pigment composition of the cells. While differences in nutrient utilization are undoubtedly a critical environmental determinant, this factor could not be studied as axenic cultures have not yet been established for these species. In addition, we examined the extent of physiological differences between 2 *P. marinus* clones isolated from different parts of the world: the Sargasso Sea and the Mediterranean Sea.

METHODS

Culture conditions and growth measurements. Clonal cultures of WH8103, a high phycourobilin (PUB) *Synechococcus* strain isolated from the Sargasso Sea (obtained from John Waterbury, Woods Hole Oceanographic Institution, MA, USA), and *Prochlorococcus marinus* isolated at 120 m from the Sargasso Sea (*P. marinus* SS120; designated CCMP-1375 at the Center for the Culture of Marine Phytoplankton, Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, ME, USA) and from the surface of the Mediterranean Sea (*P. marinus* MED4; CCMP-1378) were maintained in a modified K/10 medium (Chisholm et al. 1992) supplemented with 50 µM urea and 10 nM $NiCl_2$. For measuring growth rate as a function of temperature, cultures were grown in glass test tubes in an aluminum temperature-gradient bar on a 14 h

light:10 h dark cycle at 90 μE m^{-2} s^{-1}, incident from below. For light experiments, cultures were grown in 250 ml polycarbonate bottles on a 14 h light:10 h dark cycle at 24 ± 1 °C. Different light levels and quality were generated using cool white fluorescent lamps in combination with neutral density (Rosco #3402, 3403, 3404) and blue (Rosco #69) filters. The blue filter, which transmits maximally at 440 nm with half-maximum transmission width of 130 nm, provides a light environment similar to that found in the deep euphotic zone of the Sargasso Sea (Jerlov 1976). The growth irradiance (μE m^{-2} s^{-1}) for the white and blue light regimes was matched using a recently calibrated quantum scalar irradiance meter (Biospherical QSL-100, San Diego, CA, USA). Of the white light irradiance, 47% is comprised of wavelengths between 380 and 540 nm, corresponding approximately to the wavelengths of light provided by the blue filters. Thus, the cells grown in white light had approximately half the energy in the blue wavelengths as the cells grown only in blue light of comparable total irradiance.

Cultures were acclimated to each irradiance level for several generations (until the mean fluorescence per cell was constant) before data was collected. Replicates for all growth experiments represent serial transfers from the same 'parent' culture. Cells were harvested in exponential growth phase for the pigment analyses, *in vivo* absorption spectra, and fluorescence excitation and emission spectra.

Growth rate was determined by sampling each culture at the same time of day over several generations using a Becton-Dickinson FACScan (San Jose, CA) flow cytometer to enumerate the cells. This instrument employs an argon-ion laser with 488 nm line as the excitation source. Fluorescence emission was obtained using a long-pass filter (>650 nm) for chlorophyll (red fluorescence) and a narrow band-pass filter (585 nm, half-maximum transmission width = 42 nm) for phycoerythrin (orange fluorescence). Mean chlorophyll fluorescence per cell is presented relative to standard fluorescent beads (0.57 μm diameter; Polysciences, Inc., Warrington, PA, USA). Data were analyzed using CYTOPC software (Vaulot 1989).

Pigment measurements. *Prochlorococcus marinus* and *Synechococcus* WH8103 cultures (25 to 75 ml) were collected on 25 mm Whatman GF/F filters using vacuum pressure of <75 mm Hg. Filters were stored in liquid nitrogen for 1 to 6 mo. For pigment analysis by reverse-phase high-pressure liquid chromotography (RP-HPLC), filters were extracted as described in Goericke & Welschmeyer (1993). All samples were analyzed on a C-18 column-based RP-HPLC system using a Rainin Dynamax 10 cm 3 μm C-18 column; the following solvents: A (MeOH:aqueous ammonium acetate, 85:15), B (MeOH), and C (acetone); and a linear ternary gradient (time; % solvent A, % solvent B, % solvent C): (0 min; 100, 0, 0), (5 min; 6, 92, 2), (12 min; 0, 100, 0), (15 min; 0, 90, 10), (18 min; 0, 40, 60), (20 min; 0, 20, 80), (24 min; 0, 20, 80), with a flow rate of 1.5 ml min^{-1}. The coefficient of variation for replicate analyses on this system was concentration dependent; for the major pigments discussed here it was in the range 1 to 5%.

Selected samples were also analyzed on a C-8 column-based RP-HPLC system (Goericke & Repeta 1993). On this system, chl a_1 is well separated from chl a_2 and its stereoisomer chl a_2'. Chl b_1 and chl b_2 are partially separated. The chromatographic systems were calibrated with zeaxanthin, α-carotene, chl b_2, and chl a_2 isolated from low-light cultures of *Prochlorococcus marinus* (clone MED4), and with chl a_1 and chl b_1 isolated from spinach. Pigments were quantified (Waters 990 photodiode array detector) using integrated absorbance at 440 nm and the extinction coefficients given by Goericke & Repeta (1993). The chl b_1/chl b_2 concentration ratio was determined for all samples from the ratio of the integrated absorbances at 468 and 478 nm (Goericke & Repeta 1993).

All pigments were identified based on retention time and on-line Vis-spectra. Mass spectra of chl a_2, chl b_1, and total chl b, i.e. the sum of chl b_1 and chl b_2, were recorded using liquid secondary ion mass spectrometry (LSIMS) with a VG dynamic LSIMS probe, acetone as a solvent and nitrobenzylalcohol as a matrix with polyethyleneglycol 600 and 1000 as an internal standard. Samples were ionized with a cesium ion gun operated at 35 kV. Pigments for this analysis were isolated from a higher plant and from pooled extracts of high-light cultures of *Prochlorococcus marinus* SS120.

Absorption spectra. An *in vivo* absorption spectrum for cells concentrated on a filter was run for each sample using a Beckman DU-7 (Irvine, CA) single-beam spectrophotometer following methods outlined by Mitchell & Kiefer (1988). Absorption spectra of cells collected on filters, rather than spectra of whole cell suspensions, were used in order to compare lab results to field results. Scans were run with a 2 nm bandpass at 600 nm min^{-1}. All samples were filtered using Whatman GF/F filters at low vacuum pressure (<75 mm Hg), placed on a glass slide, and run within 2 to 3 min of filtration to avoid potential artifacts (Stramski 1990). A GF/F filter saturated with culture medium was used as the blank, and the optical density of the filter sample (OD_f) at 750 nm was subtracted from all spectra to obtain $OD_f(\lambda)$.

In order to correct the absorption spectra of filter samples for the effects of pathlength amplification, a β-correction algorithm was empirically derived for our spectrophotometer using *Synechococcus* WH8103 and

Prochlorococcus marinus, following published methods (Mitchell & Kiefer 1988, Mitchell 1990). Each species was concentrated by centrifugation (11 000 rpm, 19 000 × *g*, 30 min, 10°C), and serial dilutions were made to cover a range of optical densities. Absorption spectra for these cell suspensions were obtained with an opal diffuser (Shibata 1958). For the same culture, there were no differences in shape between the absorption spectrum obtained using an opal diffuser and that obtained using an integrating sphere (H. Sosik & L. R. Moore unpubl. data). For each absorption spectrum of a cell suspension, a corresponding spectrum was determined using an equivalent pathlength (clearance area of filter multiplied by the length of the spectrophotometer cuvette) of cells collected on a filter.

The relationship between the $OD_f(\lambda)$ and the optical density of cells in suspension, $OD_s(\lambda)$, fits a second-order polynomial:

$$OD_s(\lambda) = a\, OD_f(\lambda) + b\, [OD_f(\lambda)]^2 \quad (1)$$

where a and b are the coefficients (inset, Fig. 1). The *in vivo* absorption spectrum for each phytoplankton culture was calculated as follows:

$$a^*_{ph}(\lambda) = 2.3\, A_f\, [OD_s(\lambda)] / V_f\, C_i \quad (2)$$

where A_f is the area of the cells on the filter (m^2), V_f is the volume of cells filtered (ml), $OD_s(\lambda)$ is the optical density of the suspension obtained by applying the correction algorithm (Eq. 1) to the measured values of $OD_f(\lambda)$, C_i is the amount of chl a_1 or chl a_2 in the sample (mg ml^{-1}), and the constant 2.3 converts units from log of base 10 to natural log (Mitchell & Kiefer 1988).

The relationship between OD_f and OD_s obtained for *Synechococcus* WH8103 differed (up to 30% at OD_f = 0.4) from that found previously for other phytoplankton species, including an unidentified strain of *Synechococcus* (Mitchell 1990) and *Synechococcus* WH7803 (Cleveland & Weidemann 1993). The relationship found for *Prochlorococcus marinus* differed even more, up to 50% lower OD_s is obtained when the optical density of cells filtered onto a GF/F filter reaches 0.4. To verify that the different β correction found for *Synechococcus* WH8103 and *P. marinus* in this study was not due to any systematic or procedural error, absorption of *Thalassiosira weissflogii* cells was also measured on our system and found to be consistent with that found by other researchers for other phytoplankton (Fig. 1) (Mitchell 1990, Cleveland & Weidemann 1993). A greater correction for *Synechococcus* WH8103 cells, and the even smaller *P. marinus* cells, may result from their small size. It is likely that the cells become embedded within the filter matrix rather than creating a layer on top of the filter, and the interaction of the filter scattering and the cell scattering (as small as it is; Morel et al. 1993) might increase the pathlength of amplification, similar to a thicker filter pad (cf. Mitchell 1990). Culture conditions such as nutrients and light which affect cell size might influence the extent of the β correction; however, this effect deserves more study.

The unique β correction of *Prochlorococcus marinus* and *Synechococcus* WH8103 becomes particularly important when estimating photosynthetic quantum yield. Depending on the optical density of the sample, if the β-correction algorithm for other phytoplankton is used

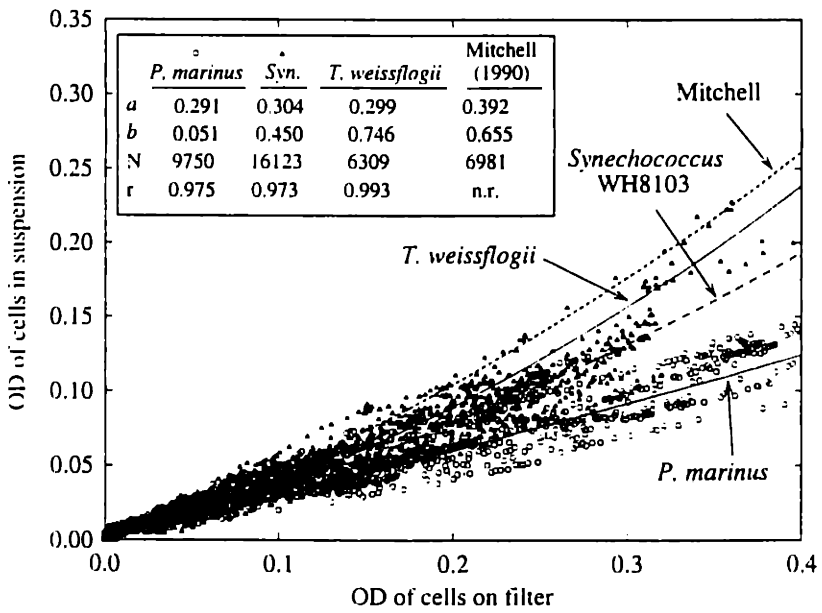


Fig. 1. Relationship between the optical density (*OD*) of cells measured in suspension and on a filter for *Synechococcus* WH8103 (▲) and *Prochlorococcus marinus* (○) from this study compared to the relationship obtained for *Thalassiosira weissflogii* in this study (individual data points not shown) and obtained by Mitchell (1990) for a collection of phytoplankton species, including *Synechococcus*. Inset: coefficients for Eq. (1) obtained by fitting N data points to a second-order polynomial. r: correlation coefficient; n.r.: not reported

for *P. marinus* (e.g. Partensky et al. 1993), the absorption spectrum could be overestimated by as much as a factor of 2 in the blue, resulting in a higher spectrally weighted chl *a*-specific absorption and, consequently, a lower photosynthetic quantum yield. Thus, the relatively high maximum quantum yields calculated by Partensky et al. (1993) would actually be *higher* if samples with $OD_f > 0.1$ were used and our *P. marinus*-specific β correction were used.

Spectral reconstruction. Whole-cell absorption spectra [$a_{ph}(\lambda)$; m^{-1}] were reconstructed and compared to *in vivo* absorption spectra as follows (Mann & Myers 1968, Bidigare et al. 1987, 1989a, b):

$$a_{ph}(\lambda) = \Sigma [a_i(\lambda)] c_i \quad (3)$$

where $a_i(\lambda)$ is the spectral specific absorption coefficient for each pigment, i, and c_i is the volume-based concentration (mg m^{-3}) of pigment i. The individual pigment-specific absorption coefficients [$a_i(\lambda)$] (Fig. 2) were determined by normalizing the HPLC-generated absorption spectra to the weight-specific absorption coefficient (Goericke & Repeta 1993), multiplying by 2.3 to convert from log of base 10 to natural log units, and shifting the wavelength of the pigment spectra as follows: (1) chl a_2 $a_i(\lambda)$ was shifted to the red by 6 nm for wavelengths greater than 480 nm; (2) zeaxanthin $a_i(\lambda)$ was shifted to the red by 10 nm; (3) the $a_i(\lambda)$ for α-carotene and the unknown carotenoid were shifted by 6 nm to the red; and (4) no shift was made for chl b_2 and the chl *c*-like $a_i(\lambda)$. The wavelength shifts were the best matches for the *in vivo* absorption maxima (see Fig. 11).

Fluorescence spectra. Because a spectrofluorometer was not readily available, samples for fluorescence excitation and emission spectra were fixed with 0.125% glutaraldehyde (Tousimis, Rockville, MD, USA) and frozen in liquid nitrogen for later analysis (Vaulot et al. 1989). A SLM-Aminco (Champagne-Urbana, IL, USA) SPF-500 spectrofluorometer with a Xenon arc lamp as the excitation source was used for the fluorescence excitation and emission spectra. Excitation spectra were determined by measuring fluorescence emission at 680 nm (the wavelength of maximum emission for both *Prochlorococcus marinus* and *Synechococcus* WH8103) as a function of excitation wavelength. Measurements were obtained in 1 nm increments (2 nm excitation band width) from 400 to 660 nm in 'ratio mode' which corrects for spectral variation due to the lamp. A media blank was subtracted from each spectrum. Quantum corrected excitation spectra were obtained by measuring the absorption and fluorescence excitation spectra of pure chl a_1, chl b_1 and phycoerythrin (Sigma, St. Louis, MO, USA), calculating the ratio of fluorescence excitation to absorption as a function of wavelength for these standards, and correcting the spectrum of each sample by dividing by this ratio. The rhodamine-B method of Melhuish (1962) was not appropriate for the spectrofluorometer used in this study.

To evaluate the influence of preservation on the analysis, spectra run on fresh aliquots and thawed aliquots which had been fixed and frozen were compared. For *Prochlorococcus marinus* SS120, the amplitude of the fluorescence excitation peak at 450 nm (due to chl a_2) did not change, whereas the peak at 480 nm (due predominantly to chl b_2) was reduced by 18% in the fixed and frozen sample relative to the fresh sample. In *P. marinus* MED4, the chl a_2 and chl b_2 fluorescence excitation peaks were reduced 22 and 12%, respectively, by the preservation process. Although these differences were significant, they did not obscure the systematic intraspecies changes in fluorescence spectra which accompanied changes in light intensity. In contrast, the changes in fluorescence excitation due to preservation for *Synechococcus* WH8103 (40% decrease in both phycoerythrin peaks with a concomitant increase of 27% in the chl a_1 fluorescence excitation peak) were too large to allow meaningful interpretation of the trends.

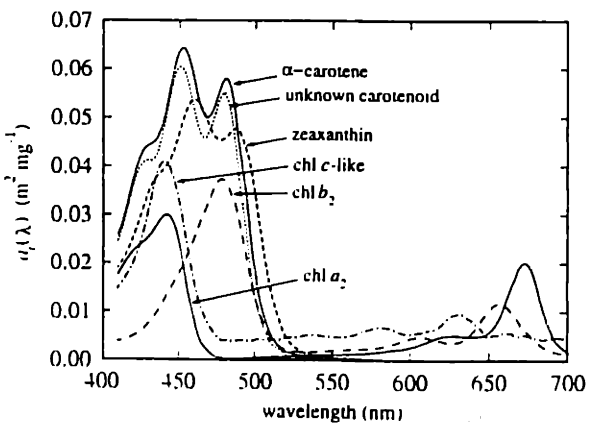


Fig. 2. Pigment-specific absorption coefficients [$a_i(\lambda)$] for the different pigments in *Prochlorococcus marinus*, wavelength shifted to match the *in vivo* absorption peaks. Each pigment is designated by a different line type

RESULTS AND DISCUSSION

Temperature optima

The 2 *Prochlorococcus marinus* clones had the same optimal growth temperature of 24 °C, but neither would grow at 28 °C, the optimum for *Synechococcus* WH8103 (Fig. 3). Between 15 and 22 °C, however, the growth rates were quite similar for all 3 strains. *Synechococcus* WH8103 would not grow below 15 °C under the conditions used in this study, differing from results previously

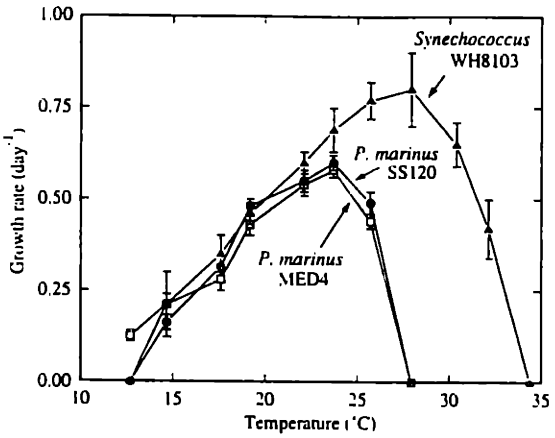


Fig. 3. Growth rate as a function of temperature for 3 oceanic picoplankters: *Prochlorococcus marinus* SS120 (●), *P. marinus* MED4 (□) and *Synechococcus* WH8103 (▲). Cultures were maintained at 93 μE m^{-2} s^{-1} under a 14 h light:10 h dark regime. Error bars are 1 SD from the mean based on 2 or 3 cultures

reported for several other marine *Synechococcus* clones (Waterbury et al. 1986, Castenholtz & Waterbury 1989). The relatively low upper-limit growth temperature for the 2 *P. marinus* clones most likely is strain-specific, since *P. marinus* has been detected at water temperatures as high as 28 °C in the equatorial and south Pacific (R. Olson, E. Zettler, J. Dusenberry & B. Binder unpubl. data), the north Pacific (Campbell & Vaulot 1993) and the Red Sea (Veldhuis & Kraay 1993).

Of the 3 clones examined in this study, only *Prochlorococcus marinus* MED4 grew at a temperature of 12.5 °C, comparable to that of the Mediterranean Sea in wintertime (Vaulot et al. 1990). The lower temperature limit for growth of *P. marinus* SS120 is consistent with the observation that *P. marinus* has only been detected in the North Atlantic when surface temperatures are greater than 15 °C (Olson et al. 1990, Veldhuis et al. 1993). However, the differences in optimal growth temperature cannot account for the difference in latitudinal and seasonal distributions of *P. marinus* and *Synechococcus* seen in the North Atlantic (Olson et al. 1990, Goericke & Welschmeyer 1993, Veldhuis et al. 1993). The depth-integrated abundance of *P. marinus* is the same as that of *Synechococcus* during the winter in the North Atlantic but is greater than that of *Synechococcus* at other times of the year when the surface temperature ranges from 22 to 27 °C (Olson et al. 1990).

Light-dependent growth rates

The light-saturated, maximum growth rates (μ_{max}) for the 2 *Prochlorococcus marinus* clones were similar: 0.53 ± 0.06 d^{-1} for *P. marinus* SS120 (Fig. 4A) and

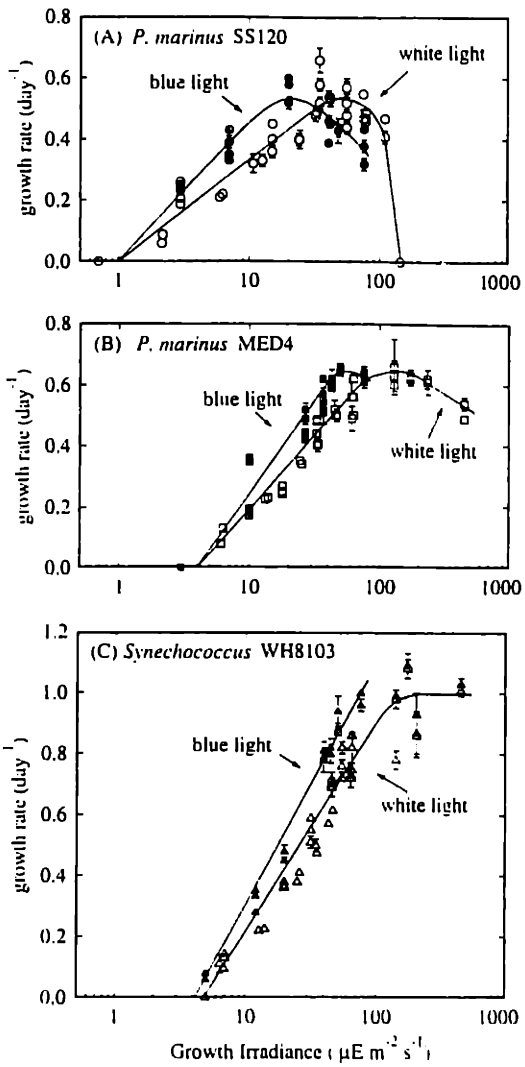


Fig. 4. Growth rate (± SD) as a function of both white and blue light irradiance. Cultures were maintained at 24.5 °C under a 14 h light:10 h dark regime. Cultures were adapted to the growth irradiance for at least 10 generations. Open symbols: white-light-grown cells; closed symbols: blue-light-grown cells. (A) *Prochlorococcus marinus* SS120, (B) *P. marinus* MED4, (C) *Synechococcus* WH8103

0.63 ± 0.06 d^{-1} for *P. marinus* MED4 (Fig. 4B). The μ_{max} for *Synechococcus* WH8103 was higher (1.0 ± 0.1 d^{-1}) (Fig. 4C). *P. marinus* SS120 (which was isolated from 120 m) had a significantly lower compensation light intensity (I_{comp}) for growth (the 95% confidence interval about I_{comp} did not overlap with those of *P. marinus* MED4 and *Synechococcus* WH8103) and grew faster at lower light levels than both *P. marinus* MED4 (which was isolated from the surface; cf. Partensky et al. 1993) and *Synechococcus* WH8103 (Table 1). *P. marinus* SS120 had positive growth rates at light intensities about 5 times lower than *Synechocccus*

Table 1. Light-dependent growth parameters (μE m^{-2} s^{-1}) for *Prochlorococcus marinus* and *Synechococcus* cultures grown in white and blue light. I_{comp}: compensation light level at which no growth occurs (= x-intercept for a linear fit to the light-limited region of the μ vs log(I) growth curve). Values in parentheses are the 95% confidence interval about I_{comp}. I_{max}: light level at which growth rate reaches a maximum; I_{inhib}: irradiance at which growth rate is first inhibited due to excess light

		I_{comp}	I_{max}	I_{inhib}
P. marinus	White	1 (0.4–2.4)	37 ± 8	110
SS120	Blue	1 (0.4–1.4)	17 ± 3	50
P. marinus	White	4 (2.6–6.7)	90 ± 13	450
MED4	Blue	4 (2.0–5.9)	49 ± 8	80
Synechococcus	White	5 (3.5–8.4)	142 ± 21	>450
WH8103	Blue	4 (3.5–6.1)	>80	>80

WH8103 (Fig. 4A, C). At the I_{comp} for *Synechococcus* WH8103, for example, *P. marinus* SS120 grew at about 0.3 d^{-1}. At 10 μE m^{-2} s^{-1} of blue light (typical of the wavelengths which penetrate deep in oligotrophic waters), the growth rate of *P. marinus* SS120 was about twice that of the other 2 picoplankters. These results are consistent with field observations which have shown that *P. marinus* extends deeper into the euphotic zone than *Synechococcus* (Chisholm et al. 1988, Olson et al. 1990, Campbell & Vaulot 1993, Veldhuis et al. 1993) and that *P. marinus* grows faster than other phytoplankton by a factor of 2 at the 1.6% light level in the Sargasso Sea (Goericke & Welschmeyer 1993).

Growth of *Prochlorococcus marinus* SS120 was inhibited at light intensities greater than 37 μE m^{-2} s^{-1}, and it did not grow at 140 μE m^{-2} s^{-1}, even after a several day adjustment period employing small incremental increases in irradiance (as suggested by Kana & Glibert 1987). Photoinhibition of this strain at relatively low growth irradiances is not consistent with the distribution of *P. marinus* in the Sargasso Sea, where they are present (Olson et al. 1990) and growing (Goericke & Welschmeyer 1993) in the surface waters when light intensities are in excess of 200 μE m^{-2} s^{-1}. In the surface layer during the winter, growth of *P. marinus* is comparable to the growth rates of the other phytoplankton, and during the summer, *P. marinus* grow at a rate corresponding to 60% of the growth rate of the other phytoplankton (Goericke & Welschmeyer 1993). The difference between our laboratory data and the field data could be explained by physical mixing (which could modulate the light regime experienced by these cells in the field), or by the existence of 2 (or more) different strains of *P. marinus* in the Sargasso Sea — one adapted for growth at high light, and one (e.g. *P. marinus* SS120) adapted for growth deep in the euphotic zone.

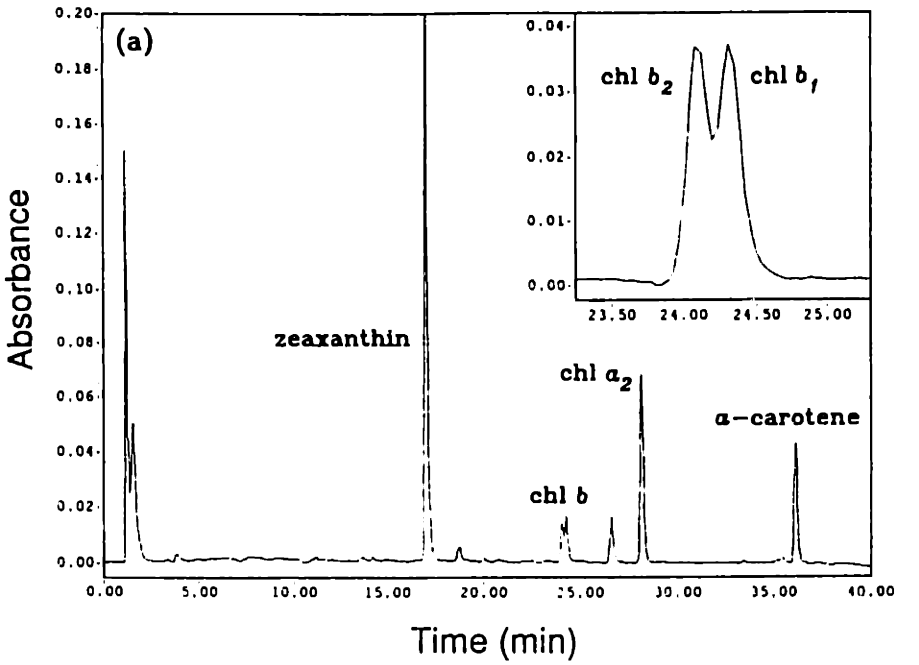
The light-dependent growth response of *Prochlorococcus marinus* MED4 has characteristics distinct from *P. marinus* SS120 and is consistent with characteristics expected for a high-light-adapted strain. The I_{max} for *P. marinus* MED4, for example, was more than twice that for *P. marinus* SS120 and more similar to the I_{max} for *Synechococcus* WH8103 (Table 1). *P. marinus* MED4 was photoinhibited only at the highest growth irradiance tested (450 μE m^{-2} s^{-1}). Thus, the differences between *P. marinus* MED4 and *P. marinus* SS120 could reflect differences between populations collected from different *depths* rather than different geographical locations.

For all 3 picoplankters, light-limited growth rates in blue light were higher than at the same irradiance of white light (Fig. 4). These differences can be explained if we consider the portion of photon flux density in the white light corresponding to the band of transmittance of the blue filters (47% of the white light irradiance is between 380 and 540 nm). This band of wavelengths includes the wavelengths of absorption by the major photosynthetic pigments: chl a_1, chl a_2, chl b_2, and PUB. The reduced growth rates of cells grown in white light relative to those in blue light can be explained solely on the basis of reduced levels of photosynthetically usable radiation: the graphs 'collapse' onto one another when the white light irradiance is expressed in terms of blue photon flux density (data not shown).

Cellular pigment content

Concentrations of chlorophylls and carotenoids were determined in light-limited and light-saturated cultures of all 3 picoplankton clones. The major pigments present in *Synechococcus* WH8103 were chl a_1, zeaxanthin and β-carotene, and the major pigments of low-light cultures of *Prochlorococcus marinus* SS120 and *P. marinus* MED4 were chl a_2, chl b_2, a chl *c*-like pigment, zeaxanthin, α-carotene, and an unknown carotenoid, consistent with previous reports (Guillard et al. 1985, Goericke & Repeta 1992). Analysis of the pigments on the C-18 column-based RP-HPLC system also revealed traces of β-carotene in the 2 *P. marinus* clones.

The analysis of high-light cultures of *Prochlorococcus marinus* SS120 on the C-8 column-based HPLC system revealed 2 partially separated chl *b* peaks (Fig. 5a) with on-line absorbance spectra typical of chl b_1 and chl b_2 (cf. Goericke & Repeta 1993). Chl a_1 was undetectable in these cultures, which would have eluted at 28.9 min, well separated from chl a_2 and its stereoisomer chl a_2' (Fig. 5a). We isolated chl a_2 and total chl *b* from high-light cultures of the *P. marinus* clone SS120 and recorded their mass spectra to prove



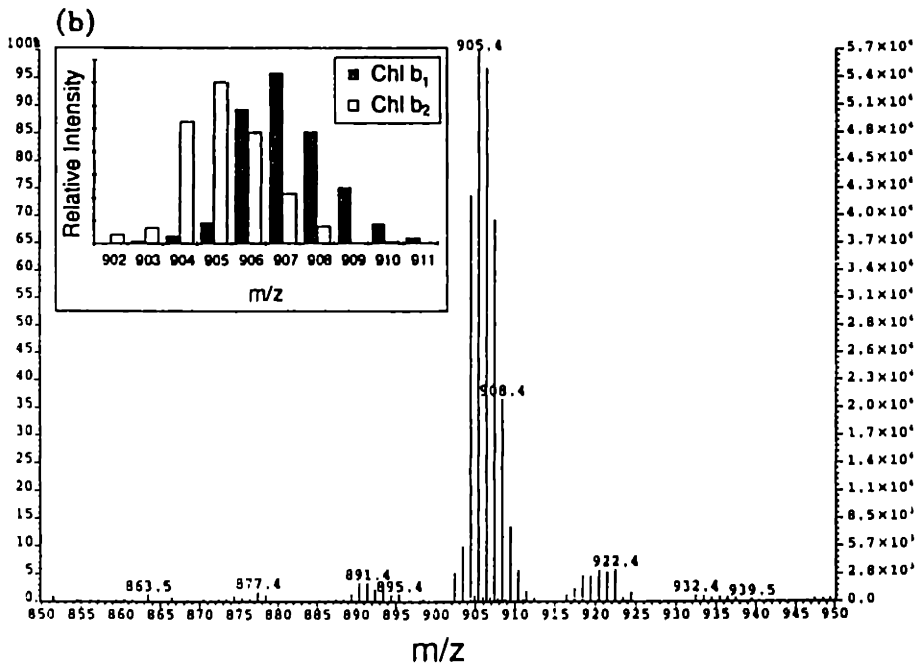


Fig. 5. (a) System II chromatogram (absorbance at 440 nm) of the pigments of the *Prochlorococcus marinus* SS120 grown at 75 μE m^{-2} s^{-1} under white light. Inset: partial separation of chl b_1 and chl b_2 when absorbance is measured at 473 nm. (b) Mass spectrum of total chl *b* isolated from high-light cultures of *P. marinus* clone SS120. The cluster at *m/z* 905 to 907 is due to chl b_1 and chl b_2 as demonstrated from the deconvoluted spectra (inset) which are identical to the spectra of chl b_1 (*m/z* 907) and chl b_2 (*m/z* 905)

the presence of these pigments and the absence of chl b_2 allomers. The major ion in the mass spectrum of chl a_2 was *m/z* 891, consistent with its molecular ion. The dominant peak in the mass spectrum of total chl *b* was a cluster around *m/z* 903 to 909 (Fig. 5b); notably absent were major signals at *m/z* 921, 934 and 937, which would have corresponded to the allomers of chl b_2 (cf. Otsuki et al. 1987). Using least-squares analysis, the cluster at *m/z* 903 to 909 was deconvoluted into 2 chl *b* spectra with molecular ions at *m/z* 905 and 907 (Fig. 5b, inset), which correspond to the molecular ions of chl b_2 and chl b_1, respectively.

The relative concentrations of chl b_1 and chl b_2 were determined in all cultures by recording the ratio of the integrated total chl *b* absorbances at 468 and 478 nm (A_{468}/A_{478}). The ratios of the integrated absorbances at 468 and 478 nm were 1.229 ± 0.004 (standard deviation) for pure chl b_1 isolated from higher plants and

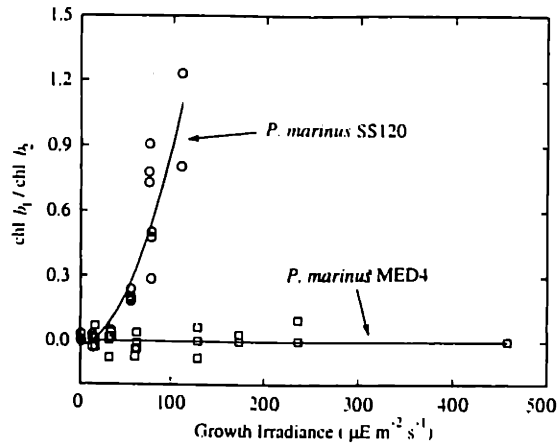


Fig. 6. Ratio of chl b_1 to chl b_2 in *Prochlorococcus marinus* SS120 (○) and *P. marinus* MED4 (□) grown under white light

0.837 ± 0.004 for pure chl b_2 isolated from the corn mutant ON8147 (Bazzaz 1981). The chl *b* from low-light cultures of *Prochlorococcus marinus* SS120 (< 20 μE m^{-2} s^{-1}) had an average A_{468}/A_{478} ratio of 0.84 ± 0.008 (N = 13), a value not significantly different from the value of pure chl b_2 ($0.10 > p > 0.05$). The ratio increased with increasing growth irradiances, indicating that *P. marinus* SS120 has measurable amounts of normal chl b_1 at high light (Fig. 6). Chl b_1 was not detected in previous analyses of pigment content in the *P. marinus* isolate from the Sargasso Sea because the light level used in those studies (Goericke & Repeta 1992) was below that at which chl b_1 begins to be synthesized. In contrast, *P. marinus* MED4 does not have any detectable levels of chl b_1 at any growth irradiances (Fig. 6), as indicated by an average A_{468}/A_{478} ratio of 0.839 ± 0.016 (N = 42), a value not significantly different ($p > 0.25$) than the value of 0.837 for pure chl b_2. The presence of chl b_1 in *P. marinus* SS120 and its absence in *P. marinus* MED4 has been suggested by other researchers (Partensky et al. 1993). For the rest of this paper, references to chl *b* in the *P. marinus* data refer to the sum of chl b_1 and chl b_2 (i.e. total chl *b*) for *P. marinus* SS120 and to chl b_2 only for *P. marinus* MED4.

Cellular chl *a* increased with decreasing light intensity in all 3 picoplankton (Fig. 7A). The chl a_2 per cell of *Prochlorococcus marinus* was 2 to 6 times lower than the chl a_1 of *Synechococcus* WH8103 at all light intensities. *P. marinus* MED4 had about 2.5 times the chl a_2 per cell, but 3 to 10 times less chl *b* per cell (Fig. 7B), than *P. marinus* SS120, such that the sum of the chl a_2 and total chl *b* was similar for both clones (data not shown). The total chl *b*/chl a_2 ratio for *P. marinus* SS120 was 10 times higher than the chl b_2/chl a_2 ratio for *P. marinus* MED4 at all growth irradiances, but

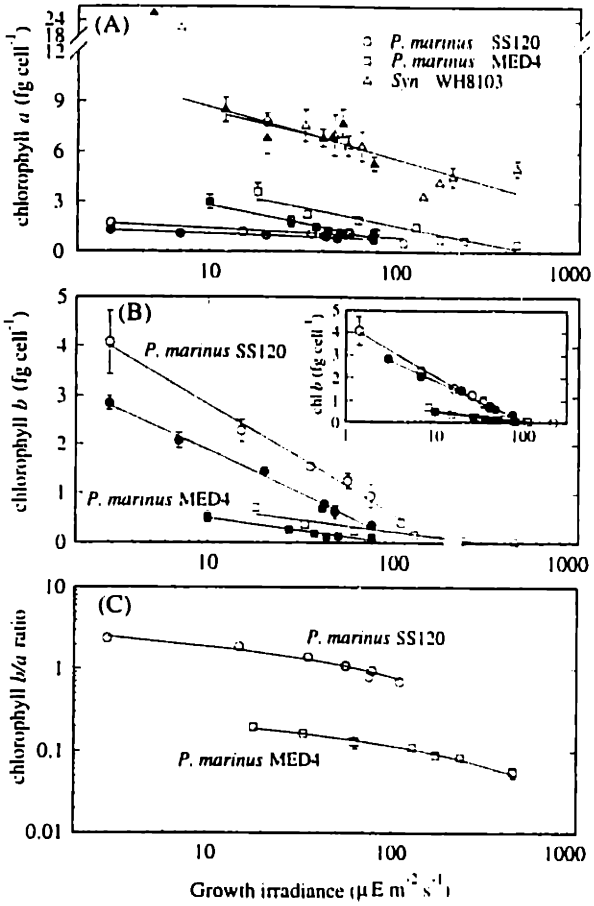


Fig. 7. (A) Cellular chl a_1 for *Synechococcus* and chl a_2 for *Prochlorococcus marinus* SS120 and *P. marinus* MED4. (B) Cellular chl *b* content of both *P. marinus* clones. The inset shows that the total chl *b* in the 2 *P. marinus* clones as a function of blue light and 'corrected' white light (see text) is the same. (C) Ratio of total chl *b* to chl a_2 as a function of white growth irradiance for the 2 strains. Symbols for all 3 panels are the same: *P. marinus* SS120 (○, ●), *P. marinus* MED4 (□, ■) and *Synechococcus* WH8103 (△, ▲); open symbols: white light; closed symbols: blue light. Error bars: 1 SD of the mean of 2 or 3 cultures

the percent change in the chl *b*/chl a_2 ratio over the growth irradiance range was the same (Fig. 7C).

Depth profiles of chl b_2/chl a_2 ratios in the field (Goericke & Repeta 1993) span a range which is greater than the ranges measured here for either of the 2 clones of *Prochlorococcus marinus*. The range of chl b_2/chl a_2 ratios measured in the deep euphotic zone of the Sargasso Sea is similar to the range of total chl *b*/chl a_2 ratio of *P. marinus* SS120 measured under blue and white light (0.4 to 2.4), whereas the range found in the surface waters of the Sargasso Sea is more similar to the range measured for *P. marinus* MED4 grown under blue and white light (0.05 to 0.2) (Goericke & Repeta 1993). As suggested by Goericke

& Repeta (1993), the sharp transition of chl b_2/chl a_2 ratios at depth may be due, in part, to changing populations of differently photoadapted (as opposed to photoacclimated) *P. marinus* populations: in the surface, the *P. marinus* population may consist of low chl b_2-type cells capable of growth at higher light, similar to *P. marinus* MED4; in the deeper euphotic zone, the population may shift to high chl b_2-type cells, similar to *P. marinus* SS120.

The chlorophyll content for all 3 picoplankters grown in blue light was lower than that of cultures grown in white light (Fig. 7A, B). When the white light irradiance was corrected for the amount of blue light wavelengths, however, the differences disappeared for the chlorophylls and other pigments (shown for total chl *b* only; Fig. 7B, inset). Thus, these *Prochlorococcus marinus* clones do not chromatically adapt to blue light. Rather, they respond to the amount of photosynthetically usable light energy available in both the blue and white light treatments.

Cellular concentrations of zeaxanthin varied by about a factor of 2 in the 2 *Prochlorococcus marinus* clones and *Synechococcus* WH8103 (Fig. 8A). The trends range from slightly increasing with irradiance in *P. marinus* SS120 (regression coefficient = 0.005 ± 0.001; $p < 0.001$) to no obvious variations with growth irradiance in *P. marinus* MED4 and *Synechococcus* WH8103, with the exception that low-light cultures of *Synechococcus* WH8103 have relatively high concentrations of zeaxanthin. These data confirm the results of Kana et al. (1988), who showed that cellular concentrations of zeaxanthin do not vary appreciably in nutrient-replete batch cultures of *Synechococcus* WH7803 grown under a wide range of white light intensities. In contrast to the results of Bidigare et al. (1989b) for *Synechococcus* WH7803, we found no difference in the average cellular zeaxanthin content for *P. marinus* and *Synechococcus* WH8103 cells grown in white light and those grown in blue light (blue light data not shown).

The content of zeaxanthin in *Synechococcus* WH8103 (3.3 ± 0.6 fg zeaxanthin cell^{-1}) was 1.7 times greater than that reported for *Synechococcus* WH7803 (Kana et al. 1988) and 3 times the amount in the *P. marinus* clones (Fig. 8A). However, when zeaxanthin content is normalized to estimated cell volume, there is no significant difference between *Synechococcus* WH8103 and *Prochlorococcus marinus*. [The volume of *Synechococcus* WH8103 (0.42 ± 0.03 μm^3 cell^{-1}) is estimated to be 3.2 times greater than that of *P. marinus* (0.13 ± 0.01 μm^3 cell^{-1}); Morel et al. 1993]. In addition, a significant, positive correlation ($p < 0.01$) was found between cellular zeaxanthin and the forward light scatter signal on the flow cytometer for all 3 organisms (data not shown). When zeaxanthin is normalized to forward light scatter and plotted against growth irradiance (Goericke pers. comm.), outliers disappear, in particular the low-light-grown *Synechococcus* WH8103 data points. These results point to the possibility of using zeaxanthin as a marker for prokaryotic biovolume in the field.

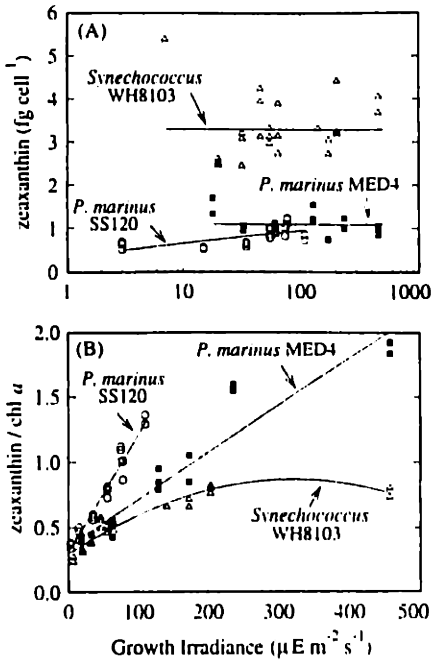


Fig. 8. Change in zeaxanthin and carotenes with change in white light intensity for *Synechococcus* WH8103 (▲) and the 2 *Prochlorococcus marinus* clones, SS120 (○) and MED4 (■). (A) Zeaxanthin per cell, (B) ratio of zeaxanthin to chl *a*

For all 3 picoplankton clones, the zeaxanthin to chl *a* ratio increased (driven by photoacclimative changes in chl a) with increasing growth irradiance (Fig. 8B). At high light intensities, the zeaxanthin/chl *a* ratio was highest for *Prochlorococcus marinus* MED4 and began to level off for *Synechococcus* WH8103. *P. marinus* SS120 has its highest ratio of zeaxanthin/chl *a* (about 1.5) at light intensities at which its growth rate was photoinhibited (Fig. 4A).

The α-carotene in the *Prochlorococcus marinus* cultures and the β-carotene in the *Synechococcus* cultures covaried with chl *a* (data not shown) such that the ratio of carotene to chl *a* was constant over the entire irradiance range for all 3 organisms (0.26 ± 0.02 for *P. marinus* SS120, 0.13 ± 0.02 for *P. marinus* MED4, and 0.09 ± 0.01 for *Synechococcus* WH8103). It is likely that these carotenes are associated with the photosynthetic apparatus, as suggested for β-carotene in *Synechococcus* WH7803 (Kana et al. 1988). In addition, both the chl *c*-like pigment and an unknown carotenoid in the *P. marinus* cultures increased with decreasing light (data not shown), also suggesting that these 2 pigments are involved in photosynthesis.

Flow-cytometrically induced red fluorescence

As expected, the mean red fluorescence per cell increased with decreasing growth irradiance in all 3 organisms (Fig. 9A, B). *Prochlorococcus marinus* SS120 had a greater mean cellular red fluorescence at a given irradiance than *P. marinus* MED4 (Fig. 9A) and the change in fluorescence per unit chl *a* was larger in *P. marinus* SS120 than *P. marinus* MED4 (Fig. 10A). This difference is reduced when fluorescence was normalized to the sum of chl a_2 and total chl *b* (Fig. 10B), as expected since the sum of total chlorophylls is the same for the 2 strains. However, the difference is not totally eliminated by this normalization. Even if the red fluorescence is plotted against the sum of all the pigments except zeaxanthin, the difference in slopes still exists ($p < 0.001$). *P. marinus* SS120 has a greater change in red fluorescence per pigment content because it absorbs more light than *P. marinus* MED4 at 488 nm (see below), which is the wavelength of the laser line used by the flow cytometer and which

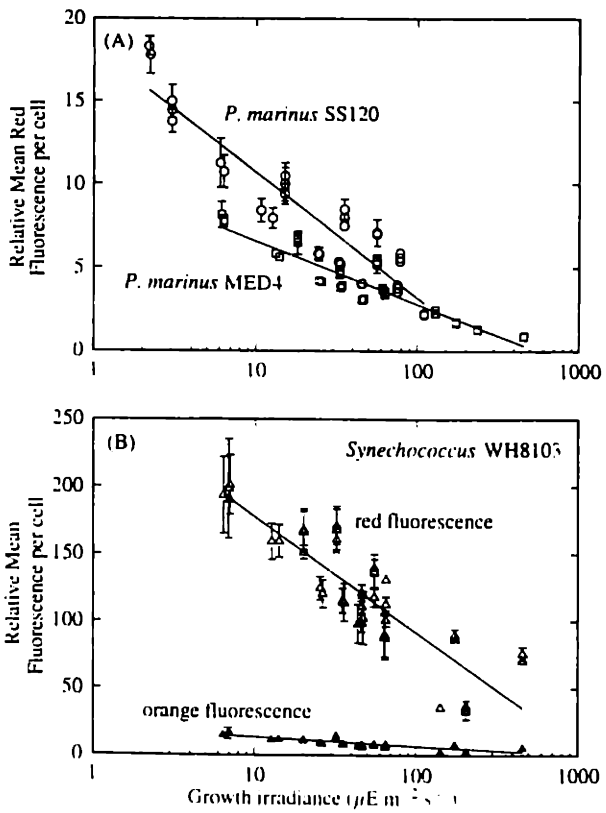


Fig. 9. Flow-cytometrically induced fluorescence per cell as a function of white growth irradiance. Symbols: the mean fluorescence of the population relative to standard beads; error bars: 1 SD of mean fluorescence. (A) *Prochlorococcus marinus* SS120 (○) and *P. marinus* MED4 (□). (B) *Synechococcus* WH8103. (Δ) mean red fluorescence per cell; (▲) mean orange (phycoerythrin) fluorescence per cell

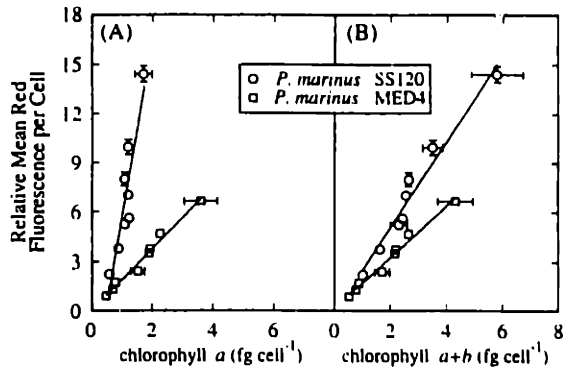


Fig. 10. Relative mean red fluorescence per cell as a function of chlorophyll for both *Prochlorococcus marinus* clones, SS120 (○) and MED4 (□) grown under white light intensity. (A) Cellular chl a_2, (B) sum of chl a_2 and total chl *b* per cell

is close to the *in vivo* absorption maxima of chl a_2. The linear relationship between the fluorescence and chl a_2, and the sum of chl a_2 and total chl *b* (Fig. 10), is indicative of a negligible pigment package effect in these small cells (Sosik et al. 1989, Veldhuis & Kraay 1990, Morel et al. 1993).

In the North Atlantic, the mean red fluorescence of *Prochlorococcus marinus* has been shown to increase sharply (about 5-fold) between the 2 and 3% light levels (Veldhuis & Kraay 1990). A similar sharp increase in fluorescence with depth was observed in the North Pacific between the 50 and the 120 m depths (Campbell & Vaulot 1993). These changes are greater than expected from photoacclimative increases in red fluorescence per cell for either *P. marinus* SS120 or *P. marinus* MED4 alone (Fig. 9A), but might be explained partially by a shift in population from a low chl b_2-type *P. marinus* population with relatively low mean red fluorescence in the surface waters to a high chl b_2-type *P. marinus* population with higher mean red fluorescence in the deep euphotic zone. This interpretation of the fluorescence data from field measurements is consistent with observations by other authors (Campbell & Vaulot 1993, Goericke & Repeta 1993, Veldhuis & Kraay 1993) and the possible existence of 2 (or more) strains of *P. marinus* which coexist in the same water column.

Chlorophyll-specific absorption

For all 3 picoplankters, the main blue absorption peak in the *in vivo* chl *a*-specific absorption (a^*_{ph}) spectrum decreased with decreasing growth irradiance, except for low-light cultures of *Prochlorococcus marinus* SS120 (Fig. 11A to C). As irradiance levels decreased, absorption due to total chl *b* (at 480 and 657 nm) increased relative to the chl a_2 absorption (at 449 and 673 nm) in

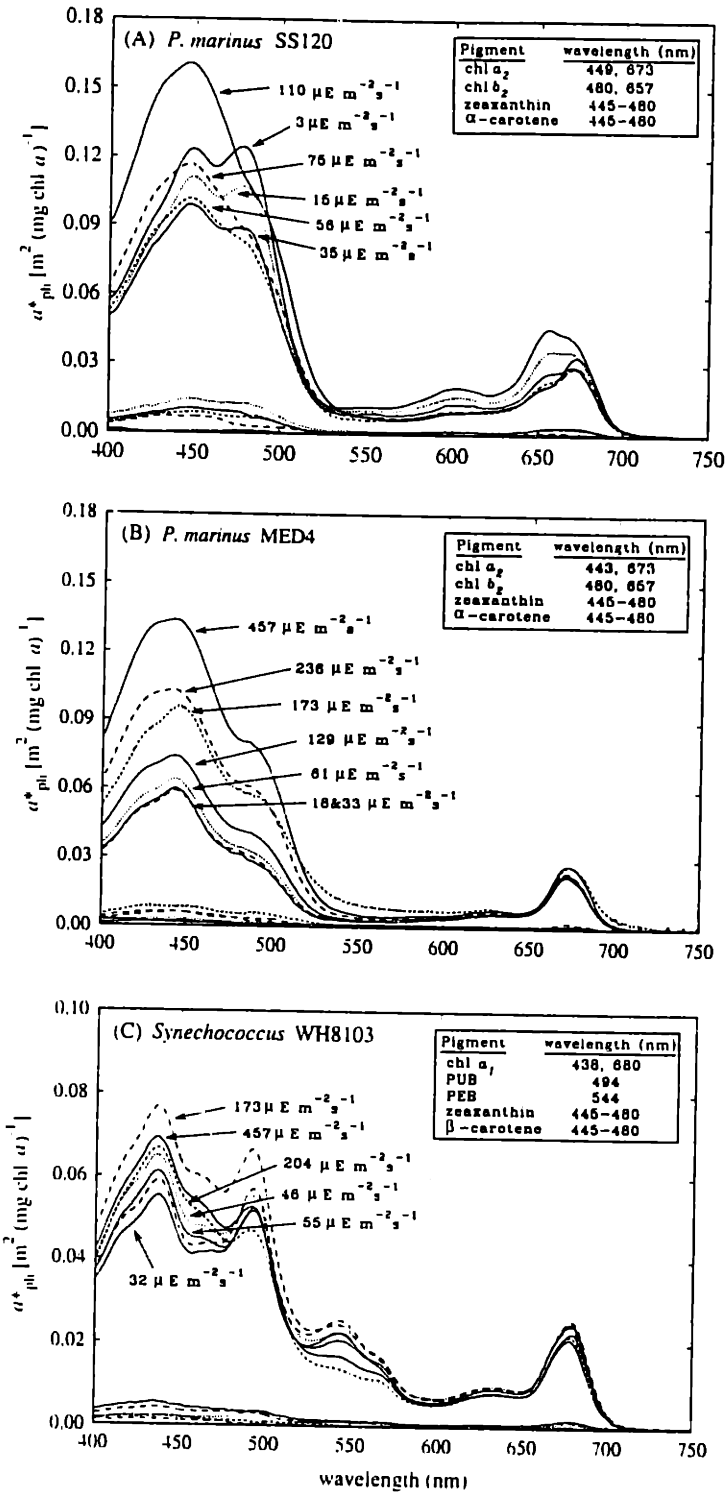


Fig. 11. *In vivo* chl *a*-specific absorption spectra [a^*_{ph} [m^2 (mg chl *a*)$^{-1}$]] for all 3 picophytoplankters. Wavelengths of maximum absorption for the various pigment peaks are listed in the insets (PEB: phycoerythrobilin; PUB: phycourobilin). Each line corresponds to an average spectrum of 2 or 3 cultures grown at different white-light irradiances. Lines at the bottom correspond to the standard deviation of each average spectrum. (A) *Prochlorococcus marinus* SS120, (B) *P. marinus* MED4, (C) *Synechococcus* WH8103

P. marinus SS120, resulting in an apparent increase in the height of the chl a_2 absorption peak in the blue as well as in the red (Fig. 11A). For *P. marinus* MED4, there was no change in the relative peak heights at 480 and 443 nm with changing growth irradiance (Fig. 11B) because the ratio of chl b_2 to chl a_2 is relatively small (Fig. 7C). The blue absorption of chl a_1 in *Synechococcus* WH8103 also decreased slightly as growth irradiance decreased (Fig. 11C), although not to the same extent and not as systematically as for *P. marinus* MED4. Overall, *P. marinus* SS120 had a higher a^*_{ph} in the blue wavelengths than *Synechococcus* WH8103.

To quantify the contribution of various pigments to the absorption spectra of *Prochlorococcus marinus*, absorption spectra were reconstructed from knowledge of the concentration of the individual pigments and their respective extinction coefficients. (Absorption spectra for *Synechococcus* WH8103 were not reconstructed because the phycobiliproteins were not measured.) Reconstructed absorption spectra for both *P. marinus* clones grown at high and low light levels closely matched the measured *in vivo* absorption spectra [$a_{ph}(\lambda)$, m^{-1}; not normalized to chl a_2] (Fig. 12A to D). The contribution by the individual pigments in each reconstructed spectrum reveals that the non-photosynthetic pigment, zeaxanthin, is a major contributor to absorption at both high and low irradiances in *P. marinus*. At the high growth irradiances, zeaxanthin absorption contributed 40 and 50% to the total absorption at 449 and 443 nm in *P. marinus* SS120 and *P. marinus* MED4, respectively (Fig. 12A, C). High contributions of non-photosynthetic pigment absorption to total absorption could have important implications for estimates of primary productivity and photosynthetic quantum yield (Bidigare et al. 1987, 1989b).

At the lowest growth irradiances, the zeaxanthin contribution to the total absorption at 443 nm decreased to 30% and chl a_2 increased to 51% of the total in *Prochlorococcus marinus* MED4; chl b_2 contributed ≤5% of the total absorption at 448 nm over all irradiance levels (Fig. 12C, D). The shoulder at 480 nm for *P. marinus* MED4 is due primarily to the second absorption peak of zeaxanthin, not chl b_2 (Fig. 12C, D). In low-light *P. marinus* SS120, the absorption peak at 449 nm is due to 42% absorption by chl b_2,

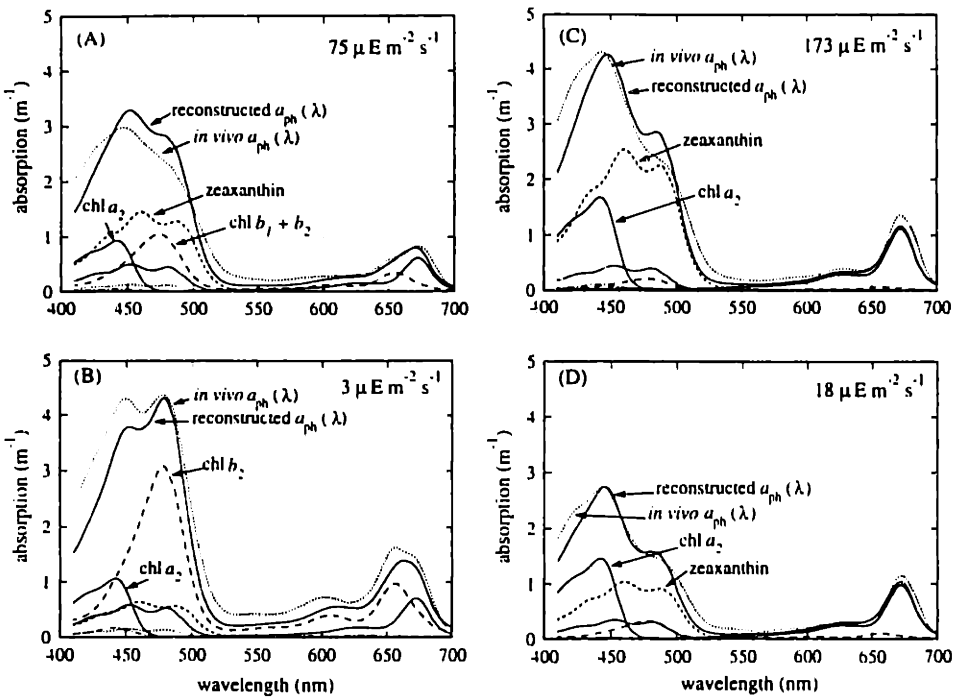


Fig. 12. Comparison of the *in vivo* absorption spectra, $a_{ph}(\lambda)$, m^{-1}, and respective reconstructed absorption spectra for high- and low-light-grown *Prochlorococcus marinus* cultures. Different pigments are designated by the same line types as used in Fig. 2. Only the pigments with the greatest contribution to the total absorption are indicated. (A) *P. marinus* SS120 grown at 75 μE m^{-2} s^{-1}, (B) *P. marinus* SS120 grown at 3 μE m^{-2} s^{-1}, (C) *P. marinus* MED4 grown at 173 μE m^{-2} s^{-1}, (D) *P. marinus* MED4 grown at 18 μE m^{-2} s^{-1}

20% absorption by chl a_2, and only 15% absorption by zeaxanthin (Fig. 12B). Thus, the large amount of absorption by chl b_2 at low growth irradiances keeps the absorption in the blue at relatively high values (Fig. 11A). For both *P. marinus* clones, absorption by α-carotene at the chl a_2 blue peak wavelength was 10 to 15% and that due to an unknown carotenoid and the chl *c*-like pigment was less than 5% over the range of growth irradiances (Fig. 12A to D).

The slight differences between the *in vivo* and the reconstructed absorption spectra could be due to several factors: (1) the wavelength shifts assumed for calculating $a_1(\lambda)$ may not be precisely correct; (2) we used the 662 and 653 nm extinction coefficients for chl a_2 and chl b_2, respectively (Goericke & Repeta 1993), which differ slightly from those measured by Shedbalkar & Rebeiz (1992); (3) imprecision in the correction of the filter absorption spectra may result in differences in the magnitude of absorption. Still, the close match between the reconstructed absorption spectra and the *in vivo* absorption spectra, in particular the lack of 'flattening' of absorption in the blue, is consistent with the findings of Morel et al. (1993) that the package effect in *Prochlorococcus marinus* is minimal. Bidigare et al. (1989b) concluded the same for *Synechococcus* WH7803 using similar methods.

The photoacclimative changes in chl *a* absorption peak height, quantified above using reconstructed absorption spectra, can also be illustrated by plotting the light-dependent ratio of chl *a* absorption in the blue relative to that in the red (B/R ratio; Mitchell & Kiefer 1988) (Fig. 13). For all 3 phytoplankters, the B/R ratio increased with increasing growth irradiance reflecting the trend in zeaxanthin to chl a ratio (Fig. 8B). *Prochlorococcus marinus* had a B/R ratio that increased to 5 as growth irradiance increased (see also Partensky et al. 1993), whereas 'typical' eukaryotic phytoplankton have B/R ratios of less than 2.5 (SooHoo et al. 1986, Maske & Haardt 1987, Mitchell & Kiefer 1988).

Several factors contribute to high B/R ratios in *Prochlorococcus marinus*. First, the ratios of accessory pigments (particularly chl b_2 and zeaxanthin) to chl a_2 are

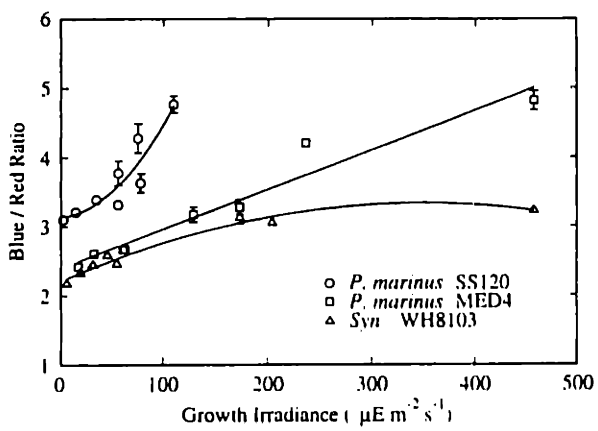


Fig. 13. B/R ratio (chl *a* absorption peak in the blue relative to that in the red) as a function of white growth irradiance. *Prochlorococcus marinus* SS120 (○), *P. marinus* MED4 (□), and *Synechococcus* WH8103 (△)

high. Secondly, the B/R ratio of pure chl a_2 and chl b_2 in solvent (diethyl ether) is about 15 and 50% higher than that for chl a_1 and chl b_1, respectively (see also Bazzaz 1981). Higher specific absorption in the blue wavelengths for chl a_2 and chl b_2 would provide an added advantage for *P. marinus* to absorb blue light relative to other phytoplankton. Thirdly, the small size of *P. marinus* results in a smaller package effect relative to most other phytoplankton and, hence, less flattening of the absorption spectrum in the blue region (Morel et al. 1993, Partensky et al. 1993).

High B/R ratios (>3) from *in vivo* a^*_{ph} measurements (corrected for detrital absorption) have been observed in the Sargasso Sea (Bricaud & Stramski 1990) and off the coast of southern California (H. Sosik & B. G. Mitchell unpubl. data) and are likely due to the presense of *Prochlorococcus marinus*, as hypothesized by Bricaud & Stramski (1990). In fact, B/R ratio could possibly be used to detect the presence of *P. marinus* in the field when HPLC or flow cytometry is not available.

Chlorophyll *a*-specific fluorescence excitation

The fluorescence emission spectra for both *Prochlorococcus marinus* clones is a single peak at 680 nm, the same wavelength as for *Synechococcus* WH8103 (data not shown). The chl *a*-normalized fluorescence [$F^*_{ph}(\lambda)$] of *P. marinus* SS120 increased as growth irradiance decreased (Fig. 14A), opposite to the trend in $a^*_{ph}(\lambda)$. This increase was due in large part to the increase in chl b_2 relative to chl a_2 (Fig. 7C), such that below irradiances of 56 µE m^{-2} s^{-1} the contribution of chl b_2 to fluorescence excitation exceeded that of chl a_2 (Fig. 14A). In fact, the ratio of the total chl *b* F^*_{pn} peak at 480 nm to that of the chl a_2 F^*_{pn} peak at 443 nm (*F*-ratio; Mitchell & Kiefer 1988) is positively correlated ($r = 0.87$, $p < 0.01$) with the total chl *b*/chl a_2 ratio for *P. marinus* SS120 (data not shown). For *P. marinus* MED4, the differences between high- and low-light chl a_2 $F^*_{pn}(\lambda)$ peaks are small compared to differences of the chl a_2 a^*_{ph} peak. However, fluorescence emission due to chl b_2 absorption F^*_{pn} (480 nm) was apparent when *P. marinus* MED4 was grown at low irradiances (Fig. 14B).

The opposite trend in the magnitude of the chl a_2 peak in fluorescence excitation spectra relative to the

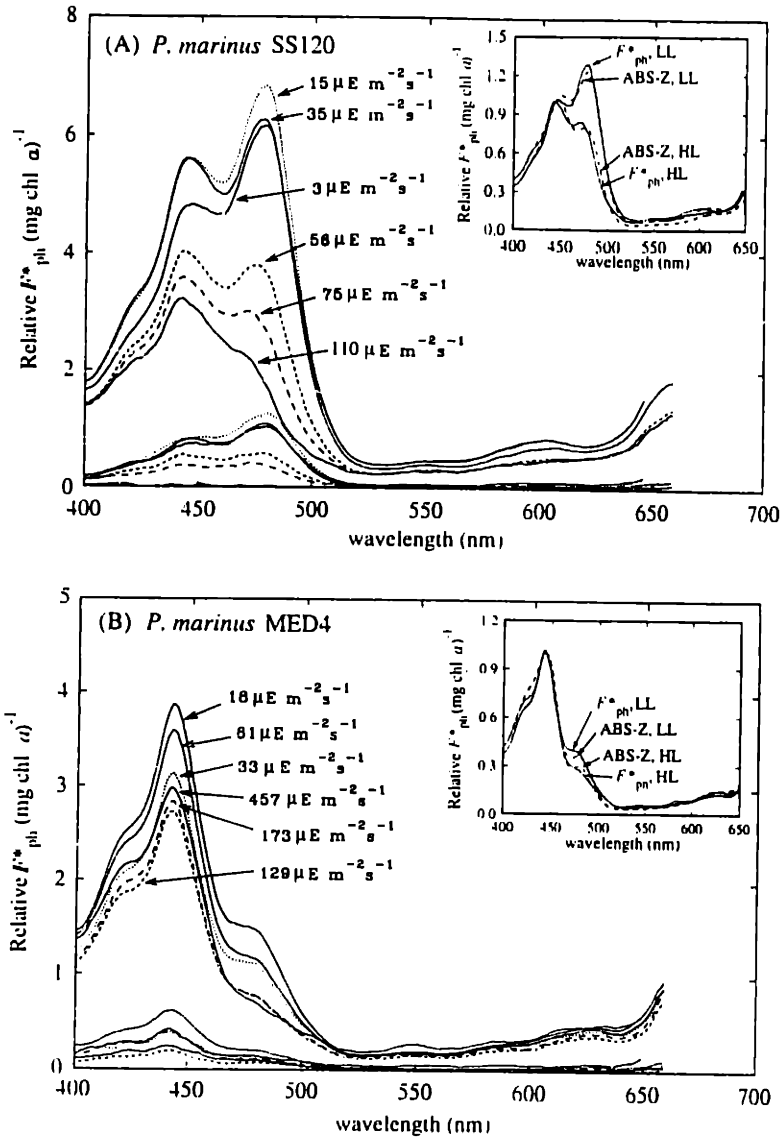


Fig. 14. Chl *a*-specific fluorescence excitation spectra at different growth irradiances. Fluorescence emission was measured at 680 nm. Each line corresponds to an average spectrum of 2 or 3 cultures grown at different white-light irradiances. Lines at the bottom (below a relative fluorescence of 1) correspond to the standard deviation of the average spectrum. (A) *Prochlorococcus marinus* SS120, (B) *P. marinus* MED4. Inset: comparison of $F^*_{ph}(\lambda)$ and chl *a*-specific reconstructed spectra, ABS-Z, generated by subtracting the zeaxanthin absorption from the reconstructed absorption and dividing by chl a_2 content at high (HL) and low (LL) light intensities. $F^*_{pn}(\lambda)$ was normalized to the chl a_2 peak of ABS-Z for easy comparison of spectral shapes. LL = 3 µE m^{-2} s^{-1} for *P. marinus* SS120 and 18 µE m^{-2} s^{-1} for *P. marinus* MED4; HL = 75 µE m^{-2} s^{-1} for *P. marinus* SS120 and 173 µE m^{-2} s^{-1} for *P. marinus* MED4

absorption spectra can be explained more fully by considering the non-photosynthetic role of zeaxanthin (Kana et al. 1988, Bidigare et al. 1989b). The fluorescence excitation spectra should be spectrally similar to the absorption spectra only if the light absorbed by the accessory pigments (e.g. chl b_2) is transferred with high (and equal) efficiency to chl a_2. Pigments not connected with the photosynthetic apparatus will not be observed in the excitation spectrum. To verify that zeaxanthin does not contribute to chl a_2 fluorescence, we subtracted zeaxanthin absorption from the chl a-normalized reconstructed absorption spectra (ABS-Z) and compared this to the corresponding measured $F^*_{ph}(\lambda)$ at both high and low growth irradiances. The comparison revealed that ABS-Z spectra for both *Prochlorococcus marinus* clones was spectrally similar to the measured $F^*_{ph}(\lambda)$, even when considering spectral distortion in $F^*_{ph}(\lambda)$ due to the preservation process (see 'Methods'). Furthermore, the trend of decreasing absorption in the blue with decreasing irradiance (Fig. 11A, B) was eliminated for *P. marinus* MED4 and reversed for *P. marinus* SS120, as was seen for $F^*_{ph}(\lambda)$. This indicates that zeaxanthin does not contribute to fluorescence emission, consistent with the non-photosynthetic role of zeaxanthin suggested by other authors (Kana et al. 1988, Bidigare et al. 1989b).

CONCLUSIONS

Prochlorococcus marinus SS120 is adapted to growth at low light, relative to *Synechococcus* WH8103 (Fig. 4, Table 1). This result supports observations that *P. marinus* is a very abundant (Olson et al. 1990) and fast-growing (Goericke & Welschmeyer 1993) picoplankter in the deep euphotic zone of the Sargasso Sea. A high abundance of *P. marinus* has been observed in the deep euphotic zone in other oceanic regions as well: the eastern North Atlantic (Veldhuis et al. 1993), the Red Sea (Veldhuis & Kraay 1993), the North Pacific (Campbell & Vaulot 1993) and the equatorial Pacific (R. Olson, E. Zettler, J. Dusenberry & B. Binder unpubl. obs.). Thus, it is likely that strains similar to *P. marinus* SS120 are present in these locations as well.

Differences in seasonal and latitudinal distributions of *Prochlorococcus marinus* and *Synechococcus* in the Sargasso Sea cannot be explained by the temperature-dependent growth responses observed in this study (Fig. 3). Other factors, such as nutrient availability, are likely to be important environmental determinants. In the North Atlantic, *P. marinus* appear to be limited to regions with temperatures above 15 to 18°C (Olson et al. 1990, Veldhuis et al. 1993). The presence of *P. marinus* strains other than those used in this study also may account for the different latitudinal and seasonal distributions observed in the North Atlantic.

The 2 clones of *Prochlorococcus marinus* used in this study differ significantly in their physiological responses to light. *P. marinus* MED4 is high-light adapted, exhibiting a light-dependent growth response similar to *Synechococcus* WH8103 (isolated from the surface of the Sargasso Sea), whereas *P. marinus* SS120 is low-light adapted. *P. marinus* MED4 has significantly reduced levels of chl b_2/chl a_2 relative to *P. marinus* SS120, which results in different absorption and fluorescence properties. Gene sequence analysis of the *rpoC1* RNA polymerase gene indicates that these 2 *P. marinus* clones are more divergent than 2 heterocyst-forming cyanobacteria from different morphological groups (Palenik & Haselkorn 1992), supporting the idea that these 2 *P. marinus* isolates are genetically distinct. On the other hand, these isolates are only 1.2% divergent in the slower-evolving 16S ribosomal RNA gene (E. Urbach unpubl. data), which suggests that these 2 clones are monophyletic. Analysis of 16S ribosomal RNA, *psbB* and combined *petB* and *petD* gene sequences has revealed that these 2 clones and other *P. marinus* isolates form a phylogenetic cluster (E. Urbach unpubl. data), suggesting that they should be considered a single genus, and possibly a single species. Further studies of the phylogenetic diversity of these organisms are needed to explore this issue.

It is possible that the differences in the physiological response to light between *Prochlorococcus marinus* SS120 and *P. marinus* MED4 are not due solely to the fact that they were isolated from 2 geographically distinct regions, but reflect the depth from which each was isolated. Several lines of evidence suggest that at least 2 strains of *P. marinus* may coexist at a given locality, and that these strains may be photoadapted for high and low light in ways that are similar to *P. marinus* MED4 and *P. marinus* SS120, respectively. (1) *P. marinus* are able to grow in the surface mixed layer of the Sargasso Sea (Goericke & Welschmeyer 1993), even though the Sargasso Sea *P. marinus* isolate is photoinhibited at high light. (2) Depth profiles of chl b_2/chl a_2 in the Sargasso Sea are suggestive of a high chl b_2-containing *P. marinus* at depth and a low chl b_2-containing *P. marinus* in the surface (Goericke & Repeta 1993). (3) At least 2 coexisting populations of *P. marinus* in the deep euphotic zone have been observed based on flow cytometric measurements in the North and South Pacific (Campbell & Vaulot 1993, B. Binder, R. Olson, J. Dusenberry & E. Zettler unpubl. obs.), the Red Sea (Veldhuis & Kraay 1993) and the Sargasso Sea (authors' unpubl. obs.).

The observed variability in chl *a*-specific absorption and fluorescence excitation spectra as a function of light is due to photoacclimative changes in pigment ratios

and not to pigment package effect, as evidenced by reconstructed absorption spectra. Our results differ from results for eukaryotic phytoplankton, where the package effect accounts for most of the changes in the blue region (chl *a* peak) of a^*_{ph} (Mitchell & Kiefer 1988, Berner et al. 1989). Reconstructed absorption spectra for *Prochlorococcus marinus* indicated that zeaxanthin is a major contributor to the magnitude and changes in the blue region of a^*_{ph} but does not contribute to chlorophyll emission, consistent with a non-photosynthetic role in these organisms (Kana et al. 1988, Bidigare et al. 1989b). Estimates of primary productivity and photosynthetic quantum yields could be incorrect by as much as 50 % if the absorption of light due to zeaxanthin is included. The use of chl *a*-specific photosynthetically active absorption (a^*_{ps}) quantified from $F^*_{ph}(\lambda)$ to eliminate variability in a^*_{ph} due to non-photosynthetically active pigments has been suggested (Sosik & Mitchell unpubl. data). An additional error in the estimates of primary productivity and quantum yields may occur if an incorrect β-correction algorithm is used to correct absorption of cells on filters from waters dominated by picoplankton, in particular *P. marinus*.

In vivo a^*_{ph} in the blue for *Prochlorococcus marinus* is higher relative to that measured for eukaryotes (Maske & Haardt 1987, Mitchell & Kiefer 1988, Berner et al. 1989), resulting in a high B/R ratio, which could be used as an indicator of *P. marinus* when more reliable methods of detection, such as flow cytometry or HPLC, are unavailable. The high a^*_{ph} in the blue is due not only to high pigment-specific absorption by accessory pigments but also to the small cell size of *P. marinus* (Morel et al. 1993). The small cell size and chl b_2 content of *P. marinus*, particularly *P. marinus* SS120, are key features which give *P. marinus* a competitive advantage over *Synechococcus* and other phytoplankton in the deeper euphotic zone where nutrients are relatively available.

Acknowledgements. This work was supported in part by NSF BSR 9020254, OCE 9000043, OCE 9012117, OCE 9022285, DIR 9101361 and MIT Sloan and Volvo Funds to S.W.C. and NSF grant OCE 91-01384 to D. Repeta and R.G. We thank Carl Johnson for his help with the mass-spec analysis, Neil Blough for the generous use of his spectrofluorometer, and Heidi Sosik and B. Greg Mitchell for helpful discussions about β corrections and absorption spectra. We also thank Brian Binder, Neil Blough, Rob Olson and B. Greg Mitchell for critical review of the manuscript.

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This article was submitted to the editor

Manuscript first received: December 2, 1993
Revised version accepted: September 12, 1994

CHAPTER THREE

Physiology and Molecular Phylogeny of Distinct Ecotypes of *Prochlorococcus* Coexisting in the Oceanic Euphotic Zone

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ABSTRACT

***Prochlorococcus*, a major component of the marine cyanobacterial picoplankton community, can contribute substantially to primary production in oligotrophic oceans[1;2;3]. *Prochlorococcus* are unique from the closely related marine A *Synechococcus* in that they contain divinyl chlorophylls *a* and *b* as their primary photosynthetic pigments4. Among isolates of *Prochlorococcus*, distinct differences in physiology have been observed[5;6;7;8;9], despite high 16S rRNA sequence similarity (97.0 - 99.9%)[10]. Recent evidence has revealed that at least two different populations of *Prochlorococcus* coexist in the Atlantic Ocean[11;12;13], the Pacific Ocean[14;15] and the Red Sea[16]. It has been hypothesized that these populations are physiologically distinct and may occupy different ecological niches[8;14;3]. We demonstrate that two such paired populations from the north Atlantic Ocean are physiologically and genetically distinct and can be distinguished as low or high-light adapted. Additionally, the physiology of these isolates is reflected in their phylogenetic relationship. This is the first study that directly connects physiological diversity with phylogenetic microdiversity in a single marine water sample.**

INTRODUCTION

Prochlorococcus , the most numerically abundant component of the open ocean cyanobacterial picoplankton community, is widely distributed throughout the oligotrophic oceans and is responsible for up to 82% of the primary production in some regions[1;2;3]. *Prochlorococcus* are small (0.6 - 0.8 μm in diameter), contain chlorophylls *a* and *b* in their

divinyl forms (chl a_2 and b_2), and can easily be identified by their characteristic flow cytometric signatures[4; 17].

Different physiological types of *Prochlorococcus* have been hypothesized to coexist in the north Atlantic Ocean[11; 12; 13], the equatorial and north Pacific Ocean[14; 15], and the Red Sea[16] based on flow cytometry and pigment measurements. Multiple *Prochlorococcus* populations have been identified in single water samples by their different relative cellular chlorophyll fluorescence and light scattering signals[11; 12; 14; 16]. In the north Pacific, these populations were observed to have different DNA histogram signatures as detected by flow cytometry[14], suggesting that they were physiologically distinct. Additional evidence for the coexistence of multiple types of *Prochlorococcus* comes from size-fractionated water samples (5 - 1.2 μm and 0.65 - 0.22 μm) which have been shown to contain different chl b/a_2 ratios[15]. Also, changes in chl b/a_2 ratios with depth in the Sargasso Sea exceed those observed for individual *Prochlorococcus* isolates[13].

METHODS

Flow cytometry and culturing

Field populations were identified as *Prochlorococcus* based on the characteristic low cellular chlorophyll fluorescence and low forward angle light scatter (FALS) of the flow cytometric signature[4]. Populations were designated as coexisting when two population densities with different fluorescence and forward angle light scatter were

distinguishable. Coexisting *Prochlorococcus* populations were sorted from each other into sterile test tubes of modified K/10 -Cu media with 1.17 μM EDTA[8] using an EPICS 753 flow cytometer (Coulter Corp.). Cells were grown at 21°C and 17 μmol Q m^{-2} s^{-1} blue light (14:10 LD) immediately after isolation on the ship, and at 24°C and 9 μmol Q m^{-2} s^{-1} white light (14:10 LD) back in the laboratory. Growth rates were calculated from cell counts obtained using FACScan flow cytometer (Becton-Dickinson, San Jose, CA). Flow cytometric data was analyzed using CYTOPC software[36].

Photosynthesis measurements

Photosynthesis-irradiance measurements were carried out following standard methods[37]. Briefly, exponentially growing cells were spiked with $NaH^{14}CO_3$ (0.1 μCi/ml culture; specific activity between 100,000-200,000 DPM). The spiked culture was dispensed into glass scintillation vials (1ml each) and incubated at 24 °C for 45 min over a range of irradiances obtained using very high output/daylight spectrum fluorescence bulbs attenuated with neutral density filters and measured with Biospherical QSL-100 scalar light meter (Biospherical Inc., San Diego, CA). Photosynthesis was terminated by acidifying with 2N HCl (100 μl) and shaking for 2 hours to allow for degassing of unincorporated ^{14}C. Liquid scintillation cocktail (9 ml, ScintiSafe Plus 50%, Fisher Scientific) was added and ^{14}C incorportation was counted on Beckman counter. Chl a_2-and cell-normalized data were fitted to the equation of Platt et al. (1980) using the curve-fitting program in SigmaPlot (Jandel Scientific): $P = P_s(1 - e^{-\alpha I/Ps})(e^{-\beta I/Ps})$, where P is the carbon fixation rate [fg C (fg chl a_2)$^{-1}$ h^{-1} or fg C $cell^{-1}$ h^{-1}], I is the irradiance (μmol Q m^{-2}

s^{-1}), P_s is the maximum, potential, light-saturated photosynthetic rate, and α is the initial slope [fg C (fg chl a_2)$^{-1}$ h^{-1} (μmol Q m^{-2} s^{-1})$^{-1}$ or fg C $cell^{-1}$ h^{-1} (μmol Q m^{-2} s^{-1})$^{-1}$]. The maximum rate of photosynthesis is obtained by the following equation: $P_{max} = P_s[\alpha/(\alpha+\beta)][\beta/(\alpha+\beta)]^{\beta/\alpha}$. Quantum yield, ϕ, is simply calculated at the ratio of α^{chl} to $\bar{a}^*_{ph}$ [mol C (mol Q)$^{-1}$]. Chl a_2 and chl b_2 (fg $cell^{-1}$) were measured spectrophotometrically[39]. Cell counts were obtained using a FACScan flow cytometer (Becton-Dickinson). *In vivo* chlorophyll a_2-specific absorption, $a^*_{ph}(\lambda)$ [m^2 (mg chl a_2)$^{-1}$], was obtained using an opal diffuser on a Beckman DU-640 spectrophotometer (Fullerton, CA), and the weighted average ($\bar{a}^*_{ph}$) was calculated over the photosynthetically available radiation range (400 - 700 nm).

Molecular phylogenetic analysis

Genomic DNA was isolated from cultures essentially as described[40]. 16S rDNA was amplified using the general eubacterial primers P8-27 (AGAGTTTGATCCTGGCTCAG) and P1504-1486R (CTTGTTACGACTTCACCCC). PCR reactions were in quintuplicate, each in a 25 µl volume using a final concentration of 0.5 µM each primer, 250 µM dNTP's and 1X cloned *Pfu* buffer (Stratagene, La Jolla, CA) and 500 ng genomic DNA. Reactions were overlaid with 50 µl sterile mineral oil, denatured at 94° for 5 minutes and 1µl (5U) *Pfu* DNA polymerase was added. Reactions were cycled at 94° for 1 minute, 50°for 1 minute, 72° for 2 minutes for 30 cycles with a final extension at 72° for 10 minutes. Quintuplicate reactions were pooled and purified using QIA quick kit (QIAGEN, Chatsworth, CA) and cloned using the pCR-Script kit

(Stratagene), both according to manufacturer's instructions. Colonies with inserts of appropriate size were sequenced on an automated sequencer (LI-COR, Lincoln, NE) using M13 Reverse dye labeled primers and Sequitherm Long Read cycle sequencing kits (Epicentre Technologies, Madison, WI). Partially sequenced clones were screened using the SIMRANK function of the Ribosomal Database Project (RDP)[41], and those determined to be cyanobacterial in origin were fully sequenced using the M13 Forward primer. A minimum of eight cyanobacterial clones were sequenced for each culture and all clones from a single culture were identical. These clones were then pooled for second strand sequencing using primers internal to the 16S rDNA. Sequences were aligned manually with other marine cyanobacterial sequences available in the RDP using the Genetic Data Environment[42]. Phylogenetic analyses employed the computer packages PHYLIP[43], Paup* (test version kindly provided by Dave Swofford) and FastDNAML[44; 45]. The sequence we obtained for MIT9303 is identical to that obtained by Urbach et al. (1997). Sequences have not been deposited yet in Genbank.

RESULTS AND DISCUSSION

In two locations in the North Atlantic we observed multiple *Prochlorococcus* populations in a single water sample located at the junction of two subsurface chlorophyll fluorescence maximum layers (Fig. 1). These populations could result from the mixing together of genetically identical *Prochlorococcus* cells acclimated to different light and/or nutrient regimes. Without knowledge of the mixing dynamics of the water column, flow cytometric signatures are not sufficient evidence that multiple *Prochlorococcus*

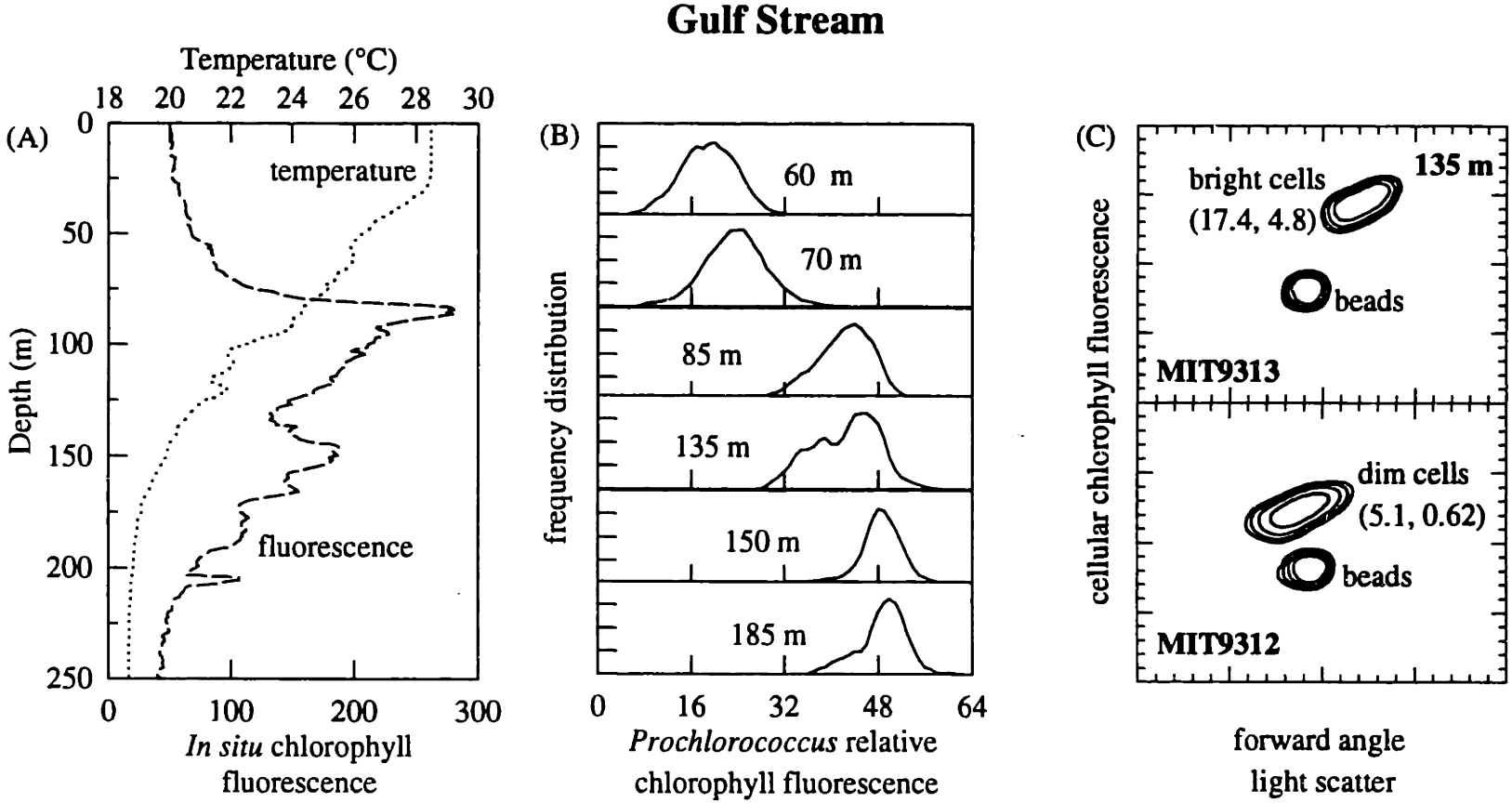

Figure 1 - Coexisting populations of *Prochlorococcus* observed in two locations: (A-C) the Gulf Stream, 135 m, 37° 30.8'N, 68° 14.4'W and (D-F) the northern Sargasso Sea, 100 m, 34° 45.5'N, 66° 11.1'W. (A,D) The physical features of both water columns were roughly similar. Depth profiles of temperature reveals a stratified water column with the mixed layer extending to 30 m in the Gulf Stream and 10 m in the Sargasso Sea. Two subsurface chlorophyll maxima were present at both locations: 90 m and 150 m in the Gulf Stream, 80 m and 125 m in the Sargasso Sea. (B,E) Chlorophyll fluorescence distribution of *Prochlorococcus* populations at various depths indicates that coexisting populations occurred at depths which coincide with

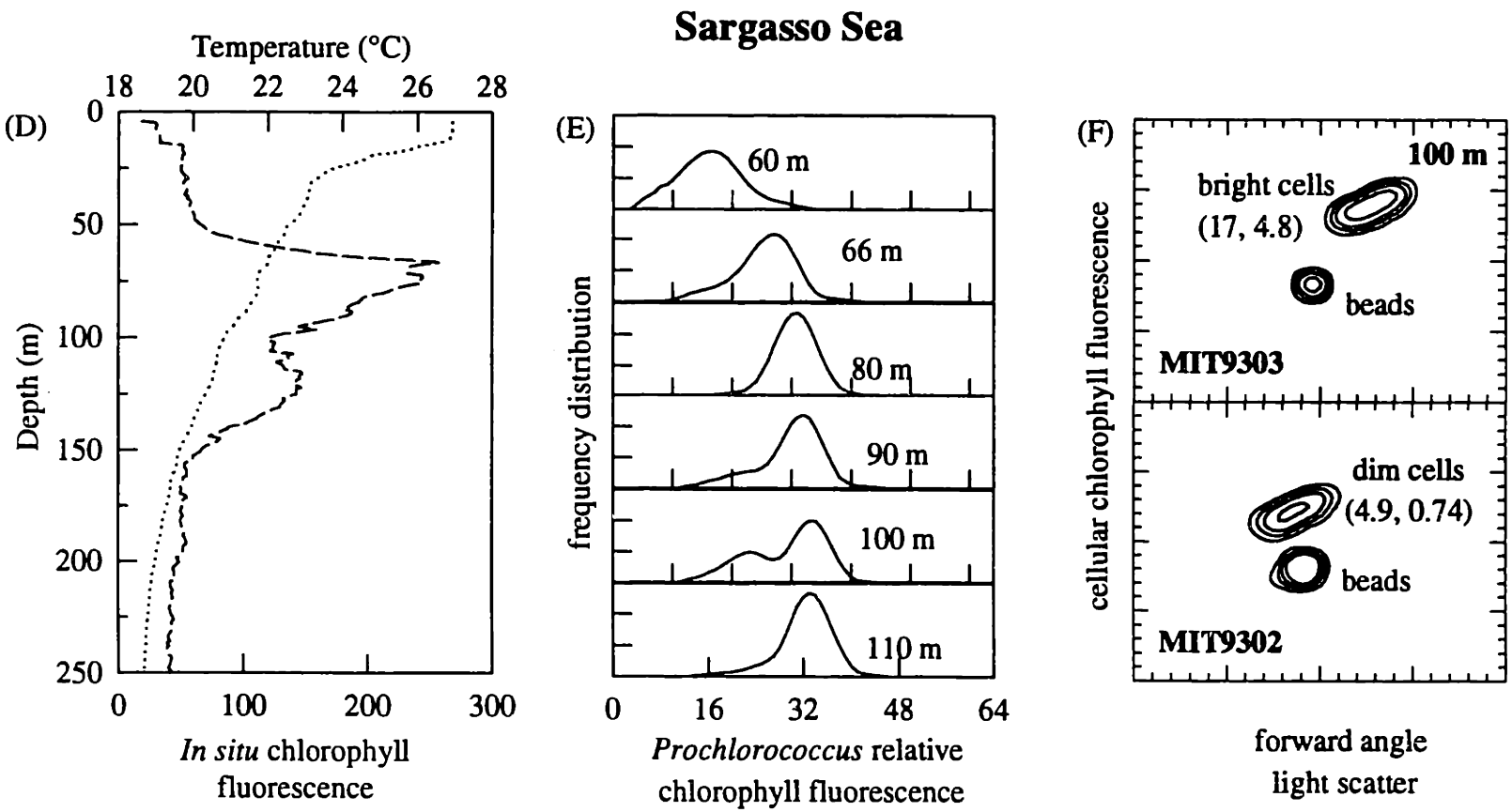

Figure 1 (cont'd) - the chlorophyll fluorescence minima between the two chlorophyll fluorescence maxima peaks. The height of the distributions were normalized to the highest peak for comparative purposes. (C,F) Numbers in parentheses refer to the flow cytometric chlorophyll fluorescence per cell and forward angle light scatter signal relative to 0.474 µm yellow-green fluorescent beads (Polysciences, Inc., Warrington, PA).

populations in a single water sample are physiologically and genetically distinct. In order to determine whether these coexisting populations were physiologically and genetically distinct, we flow-cytometrically sorted apart the high and low chlorophyll fluorescence cells from the coexisting populations in each of these locations and cultured them. After several years in culture under identical conditions, the cells sorted from the Gulf Stream water sample (isolates MIT9312 and MIT9313) and the Sargasso Sea water sample (isolates MIT9302 and MIT9303) have maintained their differences in relative red fluorescence and forward angle light scatter per cell (Fig. 1C,F), confirming that these coexisting *Prochlorococcus* populations are distinct phenotypes.

We hypothesized that the high fluorescence and low fluorescence cells are low and high-light adapted, respectively, because their different flow cytometric parameters reflected differences seen between the low-light adapted *Prochlorococcus* SS120 (= CCMP1375), isolated from 120 m in the Sargasso Sea, and the high-light adapted MED4 (= CCMP1378), isolated from the surface of the Mediterranean Sea[8]. To test this hypothesis, we compared these four isolates with respect to their light-dependent growth response. The pattern of growth rate as a function of irradiance differs between the two pairs, MIT9312 and MIT9313 (Fig. 2A) and MIT9302 and MIT9303 (Fig. 2B). The two isolates with high relative chlorophyll fluorescence per cell, MIT9303 and MIT9313 reached their light saturated maximum growth rate at lower irradiances (0.51 day^{-1} at 24 $\mu mol\ Q\ m^{-2}\ s^{-1}$ and 0.54 day^{-1} at 36 $\mu mol\ Q\ m^{-2}\ s^{-1}$, respectively) and showed complete photoinhibition of growth at irradiances where the isolates with low relative chlorophyll

fluorescence per cell, MIT9302 and MIT9312, were growing at or close to maximal rates (Fig. 2A-B).

To further characterize these isolates, we compared the pigmentation, absorption and photosynthetic properties for each isolate grown under low irradiance (9 μmol Q m^{-2} s^{-1}). The high relative cellular chlorophyll fluorescence isolates, MIT9303 and MIT9313, contain higher concentrations of chl a_2 and chl b_2 relative to the other isolates, MIT9302 and MIT9312 (Table 1). They also contain higher ratios of chl b_2/chl a_2, resulting in higher spectrally-weighted average chlorophyll a_2-specific absorption coefficient, $\bar{a}^{*}_{chl}$ (Table 1). Furthermore, the higher chl b_2 content of MIT9303 and MIT9313 drives a higher chl a_2-specific and cell-specific maximum photosynthetic capacity (P^{chla}_{max} and P^{cell}_{max}), is responsible for a greater photosynthetic efficiency (α_{chl} and α_{cell}), and results in the high maximum quantum yields (ϕ_{max}) (Table 1; Fig. 2C-F). Interestingly, on a per cell basis, MIT9303 and MIT9313, have the highest light harvesting efficiency and photosynthetic capacity measured for *Prochlorococcus* isolates[6; 18; 19]. The relative differences in the light dependent physiological parameters between the high fluorescence and low fluorescence isolates in this study is similar to the differences observed between SS120 and MED4, respectively[6; 8]. These data support the hypothesis that the high fluorescence isolates, MIT9303 and MIT9313, are adapted to growth at lower irradiances and that the low fluorescence isolates, MIT9302 and MIT9312, are high-light adapted.

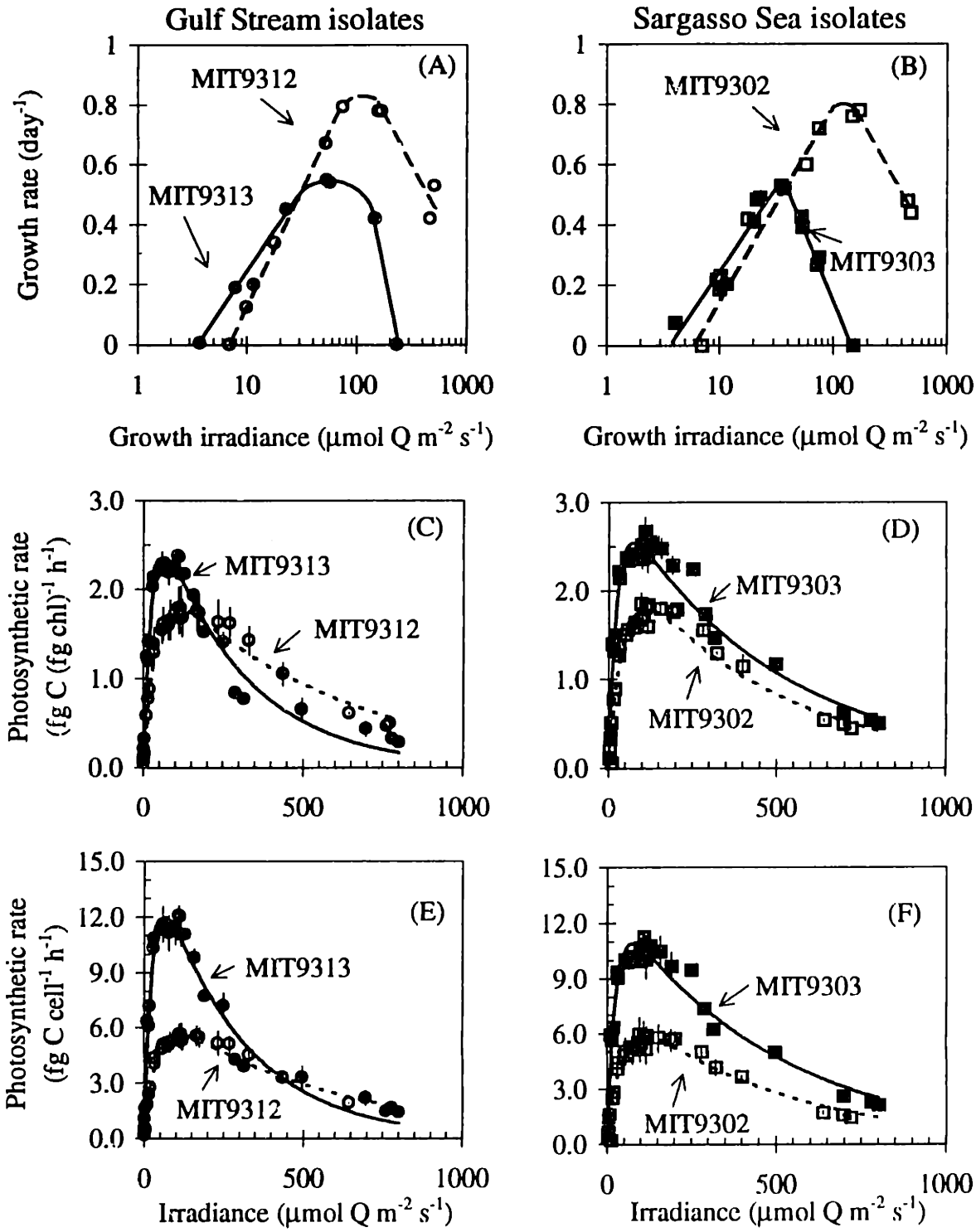

FIGURE 2 - Photosynthesis and growth as a function of irradiance for all four *Prochlorococcus* isolates. (A, B) Growth rate vs. growth irradiance. Photosynthesis-irradiance curves, chl a_2-based (C, D) and cell-based (E, F), are average curve-fits[38] of two replicate cultures for each isolate.

Parameter	Gulf Stream MIT9312	Gulf Stream MIT9313	Sargasso Sea MIT9302	Sargasso Sea MIT9303
pigmentation				
chl a_2	3.15 (± 0.09)	5.1 (± 0.1)	3.2 (± 0.3)	4.2 (± 0.2)
chl *b*/chl a_2	0.661 (± 0.004)	1.175 (± 0.007)	0.65 (± 0.01)	1.19 (± 0.01)
absorption				
$\bar{a}^*_{ph}$	0.0152 (± 0.0002)	0.0213 (± 0.0005)	0.0147 (± 0.0004)	0.0232 (± 0.0001)
photosynthetic				
α_{chla}	0.057 (± 0.004)	0.118 (± 0.005)	0.055 (± 0.002)	0.125 (± 0.006)
P^{chla}_{max}	1.8 (± 0.2)	2.4 (± 0.1)	1.8 (± 0.1)	2.54 (± 0.04)
α_{cell}	0.18 (± 0.01)	0.61 (± 0.04)	0.18 (± 0.02)	0.525 (± 0.007)
P^{cell}_{max}	6 (± 1)	12.0 (± 0.7)	5.9 (± 0.7)	10.9 (± 0.2)
ϕ_{max}	0.086 (± 0.006)	0.128 (± 0.008)	0.086 (± 0.006)	0.125 (± 0.005)

TABLE 1 - Physiological characteristics of *Prochlorococcus* isolates grown at 9 µmol Q m^{-2} s^{-1}. Chl a_2 is in units of fg $cell^{-1}$; chl *b*/chl a_2 are on a weight/weight basis. $\bar{a}^*_{ph}$ presented units of m^2 (mg chl a_2)$^{-1}$. Photosynthetic parameter units are as follows: α_{chl} and α_{cell} = fg C (fg chl a_2)$^{-1}$ h^{-1} (µmol Q m^{-2} s^{-1})$^{-1}$ and fg C $cell^{-1}$ h^{-1} (µmol Q m^{-2} s^{-1})$^{-1}$, respectively; P^{chla}_{max} and P^{cell}_{max} = fg C (fg chl a_2)$^{-1}$ h^{-1} and fg C $cell^{-1}$ h^{-1}, respectively; and ϕ_{max} = mol C (mol Q)$^{-1}$. Numbers in parentheses are ±1 SE of duplicate cultures.

We next explored whether the physiology of these four *Prochlorococcus* correlated with their phylogeny, as defined by 16S rRNA sequence analysis. The relationship between the two low-light adapted isolates, MIT9303 and MIT9313 (99.0% similarity), and between the high-light adapted isolates, MIT9302 and MIT9312 (99.5%), is closer than the relationship between their respective co-isolates: MIT9312 and MIT9313 are only 97.5% similar while MIT9302 and MIT9303 are 96.8% similar (Table 2). Further, a phylogenetic tree constructed from these and other cyanobacterial sequences shows that high-light adapted MIT9312 and MIT9302 cluster in the shallow branch of the tree referred to by Urbach et al. (1997) as the "high-light adapted clade". Additional members of this clade include the high-light adapted MED4 and two *Prochlorococcus* isolates from the Pacific, SB and GP2, which have low ratios of chl *b*/chl a_2[18]. The low-light adapted MIT9303 and MIT9313 are on separate, deeper branches, more closely related to the low-light adapted SS120 and to marine A *Synechococcus* isolates (Fig. 3). Thus, the phylogenetic relationship reflects the phenotypic relationship. It appears that at least two distinct ecotypes can be identified for isolates of the genus *Prochlorococcus*, distinguished by their different pigmentation and light utilization capabilities and supported by their phylogenetic relationship.

These two *Prochlorococcus* ecotypes with their different ratios of chl *b*/chl a_2 are analogous to two types of marine *Synechococcus* with different ratios of phycourobilin to phycoerythrobilin (PUB/PEB)[20, 21], the oceanic distribution of which is related to their relative abilities to efficiently utilize the green or blue light typical of coastal or

	1	2	3	4	5	6	7	8	9
1 *Prochlorococcus* MIT9302	-								
2 *Prochlorococcus* MIT9303	0.974	-							
3 *Prochlorococcus* MIT9312	0.997	0.974	-						
4 *Prochlorococcus* MIT9313	0.977	0.992	0.978	-					
5 *Prochlorococcus* SS120	0.984	0.976	0.984	0.98	-				
6 *Prochlorococcus* MED4	0.989	0.97	0.989	0.976	0.983	-			
7 *Synechococcus* WH7805	0.957	0.974	0.959	0.968	0.968	0.96	-		
8 *Synechococcus* WH8101	0.966	0.976	0.967	0.973	0.968	0.963	0.967	-	
9 *Synechococcus* WH8103	0.965	0.98	0.966	0.971	0.969	0.966	0.975	0.979	-

Table 2 - Similarity matrix in 16S rRNA gene sequence between isolates of *Prochlorococcus* and *Synechococcus.*

node	Distance	Parsimony
a	72	63
b	84	82
c	99	71
d	91	93
e	99	97
f	67	79
g	100	93
h	95	66

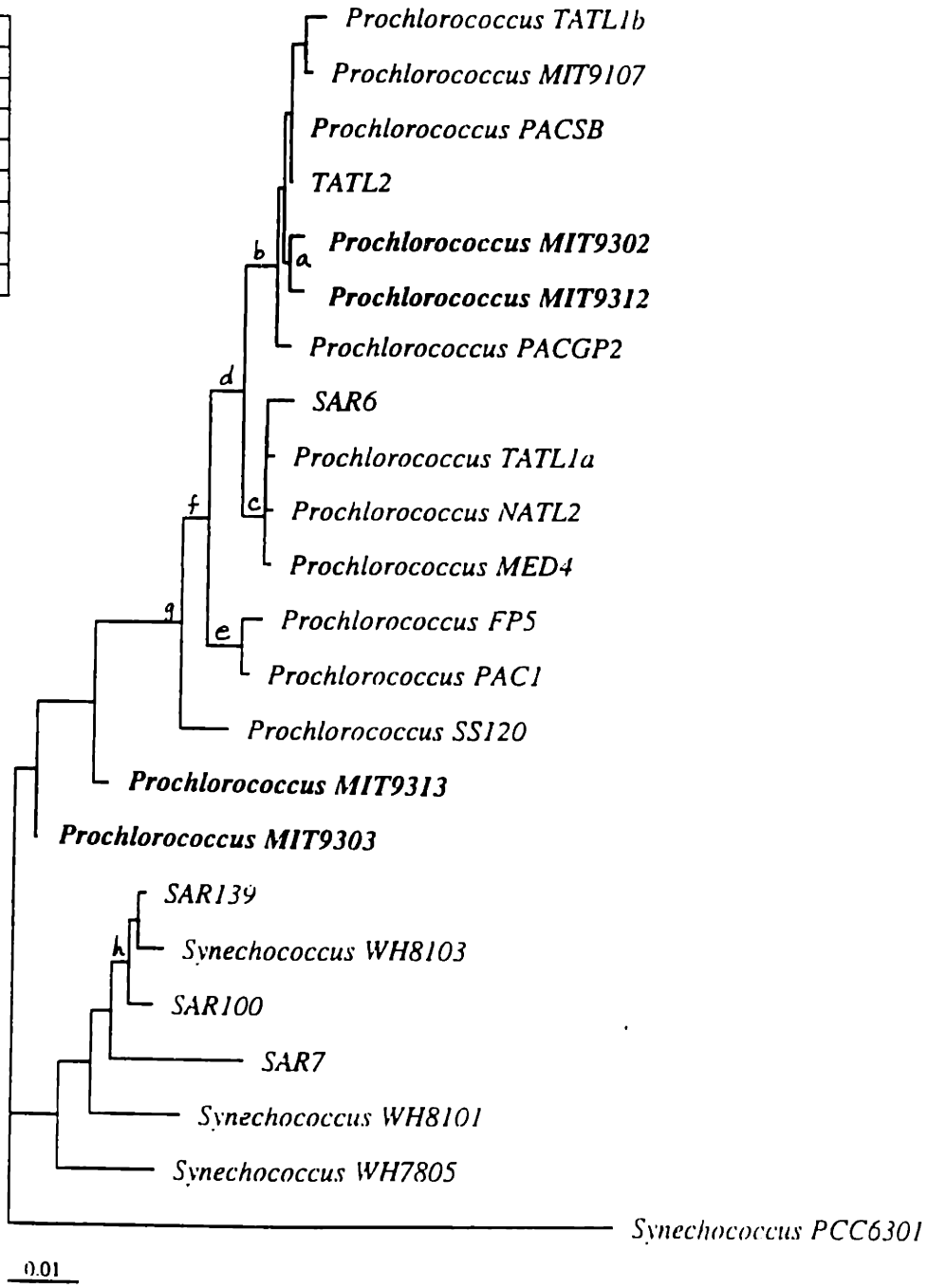

Figure 3 - Phylogenetic relationship of *Prochlorococcus* and *Synechococcus* isolates and environmental sequences from the Sargasso Sea, with the freshwater cyanobacterium *Synechococcus* PCC6301 used to root the tree. The tree was constructed using the maximum likelihood method (presented is the best tree of 10 random addition runs using global rearrangements as implemented by the program DNAML) and 1094 bases of the 16S rRNA. Bootstrap values from distance and parsimony analyses are presented out of 100 resampled datasets; values below 50 are not shown. (For distance analyses genetic distances were calculated using the Kimura 2 parameter model and trees were evaluated by the Fitch-Margoliash least squares criterion.) The scale bar for the horizontal branch lengths represents a 1% sequence difference.

oligotrophic ocean waters[22; 23]. A similar selective pressure may determine the vertical distributions of high and low-light adapted *Prochlorococcus*. It has been suggested[8; 14; 3] that low-light adapted, high chl *b*/chl a_2 *Prochlorococcus* predominate in the deeper portion of the euphotic zone and that high-light adapted, low chl *b*/chl a_2 *Prochlorococcus* predominate in the surface, with coexistence of the two ecotypes reflecting overlap of their different ecological niches. One could test the hypothesis of depth partitioning by low- and high-light adapted *Prochlorococcus*, as well as survey the seasonal and spatial distribution of the two ecotypes, by designing molecular probes which take advantage of the correlation of genotype with phenotype demonstrated above for the isolates in this study.

Partitioning of the water column by different ecotypes of *Prochlorococcus* may have important implications for estimates of oceanic primary production. For example, at an irradiance of 10 μmol Q m^{-2} s^{-1} (0.5% of full sunlight, I_o = 2000 μmol Q m^{-2} s^{-1}, equivalent to a depth of 117 m assuming a reasonable attenuation coefficient for oligotrophic waters of 0.045 m^{-1}), the contribution of the low-light adapted MIT9313 to primary production would be 3.4 times higher (5.01 vs. 1.48 mg C m^{-3}) than that of its high-light adapted co-isolate MIT9312 (using their respective α_{chl} values and chlorophyll contents, and assuming 10^4 cells ml^{-1} exposed to 14 h light). In open ocean provinces where *Prochlorococcus* dominates the phytoplankton assemblage, variability in pigmentation, absorption and photosynthetic parameters of the *Prochlorococcus*

populations may need to be considered in bio-optical models used for estimating oceanic biomass and primary production from remote sensing.

In addition to demonstrating the differences in physiology and molecular phylogeny between coexisting populations of *Prochlorococcus*, this study is the first to directly couple physiological diversity to molecular phylogenetic diversity within a single water sample. Closely related gene sequences belonging to the marine cyanobacterial picoplankton have been observed in single water samples of the open ocean[24; 25; 26; 27; 28]. Within the cyanobacterial marine picoplankton as a whole small differences in 16S rRNA sequences (between 0.1 - 4.1%) encompass significantly different physiologies, from the phycobiliprotein-containing *Synechococcus* to the chl b_2-containing, high and low-light adapted *Prochlorococcus*, with *Prochlorococcus* sequences accounting for up to 3.2% differences. Clusters of closely related gene sequences, or high phylogenetic microdiversity, is a common observation in many ecosystems[29; 24; 30]. The extent to which this microdiversity is due to physiological variability between microorganisms or simply results from multiple rRNA operons within a single strain[31] or artifacts such as formation of gene chimeras[32] has been unclear, especially since there are few matches of the environmental sequences to those from culture collections recorded in public databases. Recent studies indicate that the microdiversity within gene clusters of uncultivated heterotrophic bacteria represents closely related microbial populations, and that differential distributions of environmental RNA sequences indicates physiological and ecological variability[29; 33]. This study goes one step further and clearly demonstrates that

the high phylogenetic microdiversity observed for coexisting cyanobacterial picoplankton is due to physiological differences within the *Prochlorococcus* community, in addition to the well known co-occurrence of *Synechococcus* and *Prochlorococcus* populations[14; 34; 35]. High microbial diversity observed in other microbial communities determined by comparative gene sequence analysis independent of culturing may also result primarily from physiologically different microbial populations. Thus, a more complete understanding of the ecological significance of high microdiversity within microbial communities requires that the microbial physiology be determined in conjunction with the phylogenetic relationships.

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CHAPTER FOUR

Ecotypic Differentiation Among Isolates of the Marine Cyanobacterium *Prochlorococcus* in Response to Light Availability

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Introduction

The marine prokaryotic picophytoplankton, *Prochlorococcus*, are ubiquitous and abundant from temperate to tropical open ocean waters. *Prochlorococcus* is unique from other cyanobacteria in that it contains divinyl chlorophylls *a* and *b* (chl a_2 and chl b_2) as the primary photosynthetic pigments (Chisholm et al., 1988). Because *Prochlorococcus* populations usually surpass most other members of the phytoplankton community in abundance and depth in the water column, they contribute significantly to the total phytoplankton biomass and integrated primary production in the north Pacific, the north Atlantic, and the Red Sea (Liu et al., 1997; Partensky et al., 1996; Letelier et al., 1993; Veldhuis and Kraay, 1993; Goericke and Welschmeyer, 1993; Chisholm et al., 1988). Seasonal and spatial variations in abundance, pigment content and cellular chlorophyll fluorescence have been documented for *Prochlorococcus* populations in all locations (Campbell et al., 1997; Shimada et al., 1995; Partensky et al., 1996; Olson et al., 1990; Vaulot and Partensky, 1992; Veldhuis et al., 1997; Lindell and Post, 1995). Their populations are found throughout the euphotic zone, from the surface to depths of 200 m where the light level is as low as 0.1% of surface irradiance. In the Atlantic and Pacific Oceans and the Red Sea, *Prochlorococcus* establish subsurface abundance maxima in the summer months after the water column stabilizes and the nitracline deepens (Campbell et al., 1997; Partensky et al., 1996; Lindell and Post, 1995; Shimada et al., 1995; Veldhuis and Kraay, 1993; Olson et al., 1990). *Prochlorococcus* populations also exhibit large changes in cellular chlorophyll fluorescence and photosynthetic pigments (chl *b*/chl a_2 ratio) with depth (Partensky et al., 1996; Campbell et al., 1997; Shimada et al., 1996; Goericke and Repeta,

1993; Olson et al., 1990). These changes in cellular chlorophyll fluorescence and chl *b*/chl a_2 ratio with depth are due in part to photoacclimation but also are attributed to the coexistence of at least two different populations of *Prochlorococcus* (Moore et al., 1997; Partensky et al., 1996; Goericke and Repeta, 1993; Campbell and Vaulot, 1993).

Bimodal frequency distributions of *Prochlorococcus* chlorophyll fluorescence per cell as measured by flow cytometry have been observed in the equatorial and North Pacific (Binder et al., 1996; Blanchot and Rodier, 1996; Campbell and Vaulot, 1993), the North Atlantic (Moore et al., 1997; Partensky et al., 1996; McManus and Dawson, 1994; Olson et al., 1991) and the Red Sea (Veldhuis and Kraay, 1993). Campbell and Vaulot (1993) first hypothesized that these bimodal distributions result from the co-occurence of at least two populations of *Prochlorococcus*: one population with high mean chlorophyll fluorescence and acclimated to the low light and high nutrient conditions of the deep euphotic zone in a stratified water column; and the other population with lower mean chlorophyll fluorescence, acclimated to low nutrient conditions at the surface. Evidence from laboratory studies supports this hypothesis: physiological differences measured for two *Prochlorococcus* isolates, SS120 (=CCMP1375), isolated from 120 m in the Sargasso Sea, and MED4 (=CCMP1378), isolated from the surface of the Mediterranean Sea (Moore et al., 1995; Partensky et al., 1993), are consistent with the physiological attributes of *Prochlorococcus* populations co-occuring in the oceans, but the laboratory isolates were obtained from different oceanographic provinces. Thus, the question still remained as to whether these two ecotypes coexist in the ocean.

Moore et al. (1997) have answered this question by establishing that in two locations of the North Atlantic, bimodal distributions of *Prochlorococcus* chlorophyll fluorescence are a direct result of two physiologically and genetically distinct *Prochlorococcus* populations. Cells isolated from these coexisting populations exhibited different pigment content and growth and photosynthesis capabilities under low light intensities: the observed differences reflected those found between *Prochlorococcus* isolates SS120 and MED4. Phylogenetic analyses of 16S rRNA gene sequence for several *Prochlorococcus* isolates also indicate that isolates with low chl b/a_2 ratio form a distinct cluster, "high-light adapted" clade, and other isolates with high chl b/a_2 ratio branch separately from this cluster (Moore et al., 1997; Urbach et al., 1997). The physiological and phylogenetic evidence led us to hypothesize that isolates of the genus *Prochlorococcus* can be distinguished by their pigmentation, light utilization capabilities and phylogenetic relationship (Moore et al., 1997). Specifically, we proposed that *Prochlorococcus* isolates adapted[1] to low light grow well at low irradiances and show marked inhibition of growth at relatively low light levels, have high chl b/a_2 ratio, high chlorophyll *a*-specific absorption, and high photosynthetic efficiency relative to a high-light adapted ecotype.

In this paper, we examine whether the physiology of other *Prochlorococcus* isolates is consistent with this hypothesis. We extend the pigment, fluorescence and absorption dataset for the coexisting isolates from the North Atlantic (Moore et al., 1997) and present data from

[1] In this paper, the term "adapted" is used to refer to the phenotypically different traits between cells grown under identical conditions which make them genotypically different; whereas "acclimated" refers to the reversible physiological response of an isolate in response to some environmental stimulus.

four new *Prochlorococcus* isolates from the Pacific ocean. In addition, the photosynthesis-irradiance response for *Prochlorococcus* grown under two growth irradiances is examined to see whether the low and high-light adapted *Prochlorococcus* ecotypes have characteristic differences in their photosynthetic response and parameters. These studies show that all *Prochlorococcus* isolates examined to date can be distinguished as either low-light or high-light adapted, and that this may be a general distinguishing category for the genus *Prochlorococcus.*

Methods

Isolation and culture conditions

Ten isolates of *Prochlorococcus* (Table 1) were examined for their physiological response to light availability. *Prochlorococcus* isolates MIT9302, MIT9303, MIT9312 and MIT9313 were isolated by sorting on a flow cytometer as described in Moore et al. (1997) and the four isolates of *Prochlorococcus* from the Pacific ocean, MIT9201, MIT9202, MIT9211 and MIT9215, were isolated by filter fractionation as described in Chisholm et al. (1992). None of these eight isolates are clonal, however, they appear to be unialgal in that they maintain a coherent flow cytometric signature under each growth irradiance. In addition, 16S rRNA sequence analysis revealed single sequences for cultures of MIT9302, MIT9303, MIT9312, MIT9313 and MIT9211 (G. Rocap, personal communication); the 16S rRNA gene of MIT9201, MIT9202 and MIT9215 have not been sequenced yet. For the experiments in this study, cultures were grown under 14:10 light:dark cycle at 24 (±1) °C in the same media used for isolating (Table 2). The only exception to these conditions is for the

TABLE 1 - Isolation information for *Prochlorococcus* isolates used in this study. *Isolates obtained by filter fractionation; **isolates obtained by sorting on flow cytometer.

ISOLATE NAME	COORDINATES	DEPTH OF ISOLATION	DATE ISOLATED	ISOLATOR
Sargasso Sea				
SS120* (=CCMP1375)	28° 59'N; 64° 21'W	120 m	30-May-88	B. Palenik
MIT9302**	34° 45.5'N; 66° 11.1'W	100 m	15-Jul-93	L. R. Moore
MIT9303**	" "	"	"	"
Gulf Stream				
MIT9312**	37° 30.8'N; 68° 14.4'W	135 m	17-Jul-93	L. R. Moore
MIT9313**	" "	"	"	"
Mediterranean Sea				
MED4* (=CCMP1378)	43° 12'N; 6° 52'E	5 m	Jan-89	D. Vaulot
South Pacific				
MIT9201*	11° 60'S; 145° 25'W	surface	26-Sep-92	B. Binder
MIT9202*	" "	79 m	"	"
Equatorial Pacific				
MIT9211*	0°; 140°W	1% I_o (~83 m)	10-Apr-92	R. Olson
MIT9215*	"	surface	3-Oct-92	B. Binder

growth, pigment and fluorescence data for SS120 and MED4 taken from Moore et al. (1995), where the cultures were grown in a media that contained 10-fold higher concentration of EDTA (Table 2). After gradually increasing the growth irradiance, cultures were acclimated to each irradiance level for at least 10 generations before measurements were made. Different light levels were obtained using cool white fluorescent lamps in combination with layers of plastic neutral density lighting filters (Rosco 1-4 stop). For pigment analysis at each light level, cells were harvested from exponential growth phase (see below).

TABLE 2 - Final concentration of macronutrients and trace metals in media made with Sargasso Sea water. This media recipe was used for isolating all *Prochlorococcus* isolates and for obtaining the experimental data presented here. The only exception is that an EDTA concentration of 11.7 µM was used for isolating *Prochlorococcus* SS120 and MED4 and obtaining the data reported in Moore et al., 1995.

nutrient	final concentration
NaH_2PO_4	10 µM
NH_4Cl	50 µM
$(NH_2)_2CO$ (urea)	100 µM
trace metal mix	
Zn	8 nM
Co	5 nM
Mn	90 nM
Mo	3 nM
Se	10 nM
Ni	10 nM
Fe	1.17 µM
EDTA	1.17 µM

Growth rate and flow cytometric measurements

Cell enumeration, relative forward angle light scattering (FALS) and relative mean chlorophyll fluorescence per cell were determined using a FACScan flow cytometer with 488 nm excitation (15 mW Ar-ion laser) and >650 nm longpass emission detection (Becton-Dickinson, San Jose, CA) (as detailed in Moore et al., 1995). FALS and fluorescence per cell are reported relative to 0.57 µm diameter yellow-green, polystyrene microspheres (Polysciences, Inc., Warrington, PA). As a means of comparing the light-dependent growth response between isolates, two parameters were calculated: the compensation light level (I_{comp}), defined as the irradiance level at which growth is completely limited by low light, and the inhibition light level (I_{inhib}), defined as the irradiance level at which growth decreases due

to high light exposure. I_{comp} was determined as the x-intercept of the linear regression of the light-limited region of the μ vs log(I) curve ($\mu = 0$); I_{inhib} was calculated from the intersection of μ_{max} with the linear fit to the high light region of the μ vs log(I) curve.

Pigment measurements

Cultures were harvested for pigments by filtering (10-25 ml) onto 25 mm Whatman GF/F filters under low vacuum. Filters were stored in liquid nitrogen until extraction and analysis. Pigments were extracted as described in Goericke and Welschmeyer (1993) and analyzed on a C-8 column-based reverse-phase high-pressure liquid chromatograph (Beckman) (as described in Goericke and Repeta, 1993). All pigments were identified based on their relative retention times (Goericke and Repeta, 1993) and quantified using integrated absorbance at 440nm (chl a_2, zeaxanthin and α-carotene) or 478nm (chl b_1 and chl b_2). The chromatographic systems were calibrated with chl a_2, chl b_1, chl b_2, zeaxanthin and α-carotene obtained from cultures of *Prochlorococcus* by collecting each pigment as it eluted off the HPLC, drying under N_2(g), resuspending in 90% acetone, quantifying spectrophotometrically using known extinction coefficients (Goericke and Repeta, 1993), and then injecting a dilution series for each pigment on the HPLC system.

Absorption measurements

An *in vivo* absorption spectrum was obtained for each culture used in the photosynthesis-irradiance experiments (methods described below). A Beckman DU-640 spectrophotometer was used with an opal diffuser placed between the suspended cells and the

detector opening to minimize particle scattering effects (when necessary, cultures were concentrated by centrifugation, as described in Moore et al. (1995)). The spectrally-weighted average chl a_2-specific absorption coefficient, $\bar{a}^*_{chl}$ [m^2 (mg chl a_2)$^{-1}$], was calculated over the photosynthetically available radiation range (λ = 400 - 700 nm) as follows:

$$\bar{a}^*_{chl} = \frac{\int a^*_{chl}(\lambda) \cdot E(\lambda) d\lambda}{\int E(\lambda) d\lambda},$$

where $a^*_{chl}(\lambda)$ is the chl a_2-specific absorption and $E(\lambda)$ is the energy spectrum (W nm^{-1}) of the cool white fluorescent lamps used for growing the cultures.

Photosynthesis measurements

For photosynthesis-irradiance experiments, eight *Prochlorococcus* isolates were grown at 24 °C under 14:10 light:dark cycle at two growth irradiances (~9 µmol Q m^{-2} s^{-1} and ~70 µmol Q m^{-2} s^{-1}, except that the high photon flux density for MIT9303 was 52 µmol Q m^{-2} s^{-1}) using cool white fluorescent lamps. Photosynthesis, cell counts, and absorption measurements were made and cells harvested for pigment analysis during exponential growth phase. Measurements were performed at the same time of day (4-6 hr into light cycle) for each culture to avoid diurnal effects. Chl a_2 and total chl *b* were extracted as described above and quantified spectrophotometrically using the trichromatic equations of Jeffrey and Humphrey (1975). Photosynthesis was measured by incubating exponentially growing cells with $NaH^{14}CO_3$ (0.1 µCi/ml; specific activity between 100,000 - 200,000 DPM) at 23 ± 1 °C for 45 min and terminated by acidification, as described in Moore et al. 1997. Irradiances were obtained using very high output/daylight spectrum fluorescence bulbs attenuated with

Rosco neutral density light filters. Chl a_2- and cell-normalized data were fitted to the equation of Platt et al., 1980 using the curve-fitting program in SigmaPlot (Jandel Scientific) to obtain photosynthesis parameters: maximum rate of photosynthesis (P_{max} [fg C (fg chl a_2)$^{-1}$ h^{-1} or fg C cell^{-1} h^{-1}]), the initial slope of photosynthesis-irradiance curve (or light utilization efficiency; α [fg C (fg chl a_2)$^{-1}$ h^{-1} (μmol Q m^{-2} s^{-1})$^{-1}$ or fg C cell^{-1} h^{-1} (μmol Q m^{-2} s^{-1})$^{-1}$]), the irradiance at which photosynthesis is maximum (I_m [μmol Q m^{-2} s^{-1}]), the index of photoinhibition (I_b [μmol Q m^{-2} s^{-1}]). The maximum quantum yield of photosynthesis, ϕ_m, [mol C (mol quanta)$^{-1}$] was calculated from the ratio of α_{chla} to $\bar{a}^*_{chl}$. The rate values that are presented here are gross photosynthesis, as we make no adjustment for possible respiratory loss, which are assumed to be small due to the short incubation times used (45 min). All values reported are the mean (±1 SE) of duplicate cultures.

Statistical Analysis

Principal component analysis was performed using MultiVariate Statistics Package (Kovach Computing Services, Anglesey, Wales, UK). The raw data matrices were arranged with the isolates in columns and the physiological parameters in rows. The analysis was carried out on the correlation matrix of centered, standardized data (as suggested by Pielou, 1984) so that values from all the different physiological parameters could be used.

Results and Discussion

Growth rate v. irradiance

One of the defining characteristics distinguishing *Prochlorococcus* isolates is the light-

dependent growth response: the low-light adapted SS120, MIT9303 and MIT9313 grow well at very low irradiances (low I_{comp}) but are unable to grow at irradiances above 170 μmol Q m^{-2} s^{-1}, and the high-light adapted MED4, MIT9302 and MIT9312 are capable of growth up to irradiances as high as 450 μmol Q m^{-2} s^{-1} (Moore et al., 1997; Moore et al., 1995). To determine whether the four *Prochlorococcus* isolates from the Pacific follow this trend, we examined their growth as a function of irradiance (I_g). Three of these isolates, MIT9215, MIT9201, and MIT9202, have growth responses consistent with that described for the high-light adapted ecotype (Fig. 1A). They have I_{comp} values that fall within the range of mean I_{comp} values for the other three *Prochlorococcus* isolates adapted to high light (4.6 - 8.3 μmol Q m^{-2} s^{-1}). At higher I_g, they exhibit high growth rates (μ_{max} = 0.77 ± 0.05 for MIT9215, 0.79 ± 0.04 for MIT9201, 0.83 ± 0.05 for MIT9202) and do not begin to show inhibition in growth until above 200 μmol Q m^{-2} s^{-1} (Fig. 1A). Thus, based solely on the light-dependent growth response, we conclude that Pacific isolates MIT9215, MIT9201, and MIT9202 are high-light adapted.

The other isolate from the Pacific, MIT9211, can grow well at low light levels and has an I_{comp} within the range obtained for the three low-light adapted isolates (Fig. 1B). At higher irradiance levels, MIT9211 becomes inhibited in growth at an irradiance of 124 μmol Q m^{-2} s^{-1} (Fig. 1B inset), which is below the I_{inhib} range observed for the other Pacific isolates but only slightly lower than the I_{inhib} of the high-light adapted isolates (Fig. 1A). Whether MIT9211 can grow at irradiances above 230 μmol Q m^{-2} s^{-1} or whether it becomes completely photoinhibited should be determined. From the growth response data at hand, MIT9211

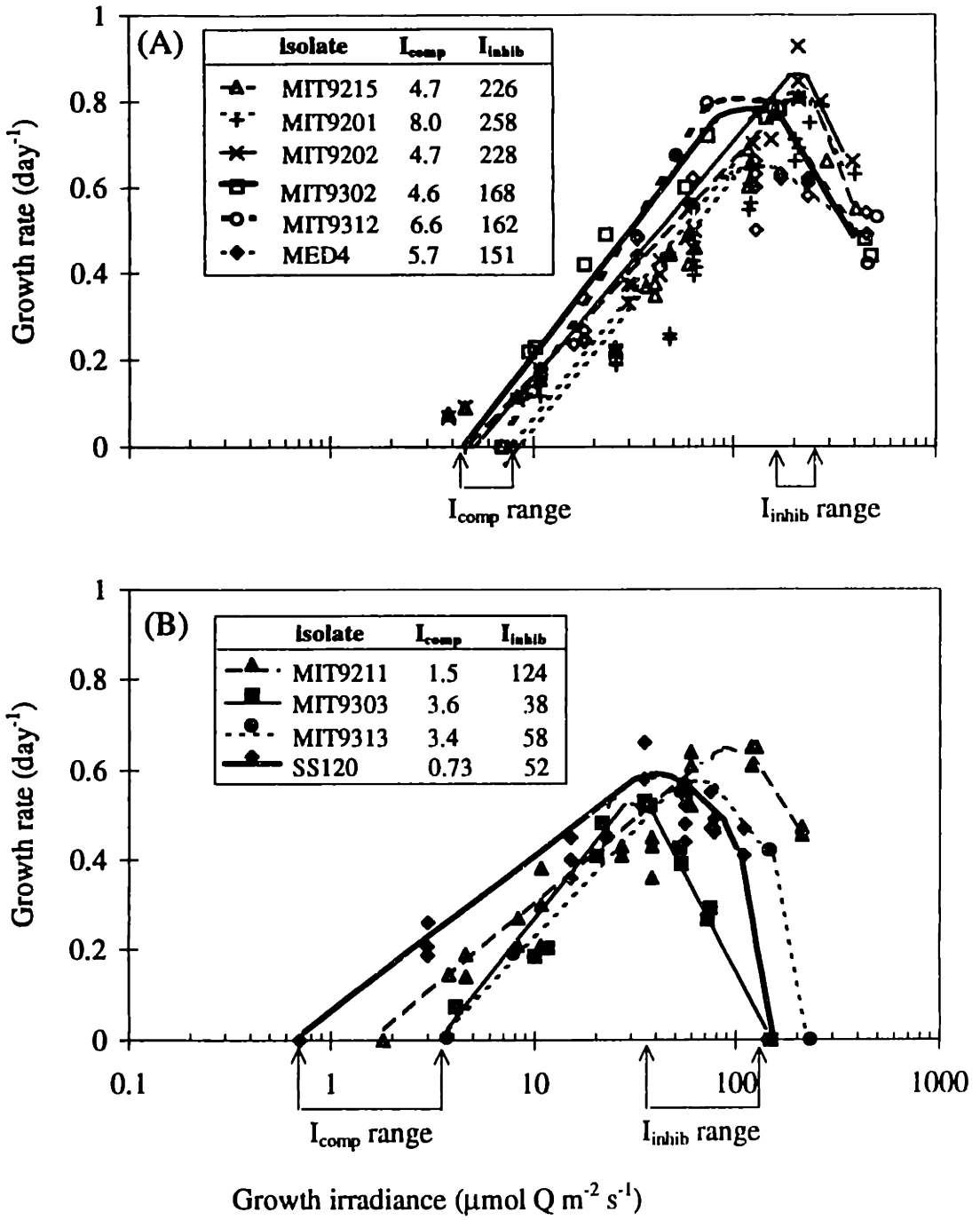


Figure 1 - Growth rate as a function of irradiance. (A) Low-light adapted isolates. (B) High-light adapted isolates. The range for mean values of I_{comp} and I_{inhib} are indicated on the abscissa. Data for SS120 and MED4 from Moore et al. 1995, and for MIT9302, MIT9303, MIT9312, and MIT9313 from Moore et al. 1997.

appears to be intermediate between the high-light adapted and low-light adapted *Prochlorococcus* ecotypes.

Differences in the light-dependent growth response for these isolates implies that the high-light adapted ecotypes occupy a different light-related ecological niche than the low-light adapted ecotypes. Under natural conditions, *Prochlorococcus* populations exhibit positive net growth rates over a wide light gradient, from surface irradiances to light levels as low as 0.1% I_o in the deep euphotic zone (Veldhuis et al., 1997; Liu et al., 1997; Partensky et al., 1996; Binder et al., 1996; Vaulot et al., 1995; Landry et al., 1995; Goericke and Welschmeyer, 1993). Estimates of growth rates for surface *Prochlorococcus* populations reach rates of 0.75 day^{-1}, similar to the μ_{max} of the high-light adapted isolates, indicating that *Prochlorococcus* populations in surface waters may not be severely photoinhibited at high irradiances (Veldhuis et al., 1997; Liu et al., 1997; Vaulot et al., 1995; Landry et al., 1995; Goericke and Welschmeyer, 1993).

Although the maximum light level at which the high-light adapted *Prochlorococcus* can grow was not reached in this study, it is likely that *Prochlorococcus* populations present in the surface mixed layer of the oceans are composed of the high-light adapted ecotype. The complete photoinhibition of the low-light adapted *Prochlorococcus* raises questions as to whether they could even survive exposure to irradiance levels in a surface mixed layer. For example, in an upper mixed layer extending to 70 m, PAR can range from 250-2000 μmol Q m^{-2} s^{-1}; these light levels far exceed the I_{inhib} of MIT211 and the low-light adapted SS120,

MIT9303 and MIT9313. Complete photoinhibition of growth due to high light exposure is not a common observation for most phytoplankton, but has been found for oceanic species of dinoflagellates at ~800 μmol Q m^{-2} s^{-1} ([Brand, 1981 #867]). Marine *Synechococcus*, closely related to *Prochlorococcus* based on molecular phylogeny (Palenik and Haselkorn, 1992; Urbach et al., 1992), show little or no photoinhibition of growth when measured at irradiance levels from 450 to 2500 μmol Q m^{-2} s^{-1} (Moore et al., 1995; Kana and Glibert, 1987).

Within and below the subsurface chlorophyll maximum, estimates of *Prochlorococcus* growth rates are diminished but still positive: between 0.1-0.2 day^{-1} at 150 m in the equatorial and subtropical North Pacific Ocean (Liu et al., 1997; Binder et al., 1996), 0.04-0.16 day^{-1} at the 1.6% light level in the Sargasso Sea (Goericke and Welschmeyer, 1993) and 0.39 day^{-1} between 90-120 m in the subtropical, northeastern Atlantic Ocean (Partensky et al., 1996). For comparison, at I_g of 10 μmol Q m^{-2} s^{-1} (corresponding to a depth of 118 m assuming a reasonable attenuation coefficient for oligotrophic waters, k = 0.045 m^{-1}, and I_o = 2000 μmol Q m^{-2} s^{-1}), the low-light adapted *Prochlorococcus* have a range of growth rates, 0.18 - 0.41 day^{-1}, extending above the range measured for the high-light adapted ecotype, 0.04 - 0.25 day^{-1}. Thus, as irradiance decreases deeper in the water column, *Prochlorococcus* adapted to lower light levels will outcompete those adapted to higher light (assuming all else is equal). Furthermore, low-light adapted *Prochlorococcus*, with their low I_{comp} values comparable to 0.04% - 0.18% of full sunlight, I_o (assuming I_o = 2000 μmol Q m^{-2} s^{-1}), would extend the euphotic zone to about the 0.1% I_o depth (153 m, assuming k = 0.045 m^{-1} and I_o = 2000 μmol

Q m^{-2} s^{-1}), 50% deeper than the traditional base of the euphotic zone (1% I_o depth, 102 m assuming the same k and I_o).

Pigment content

Another defining characteristic distinguishing *Prochlorococcus* isolates is the chl *b*/chl a_2 ratio. Under low growth irradiances, the low-light adapted isolates SS120, MIT9303 and MIT9313 have higher ratios of chl *b*/chl a_2 than the high-light adapted isolates MED4, MIT9302 and MIT9312 (Moore et al., 1997). This difference in chl *b*/chl a_2 ratio between SS120 and MED4 is maintained irrespective of growth irradiance (Moore et al., 1995; Partensky et al., 1993). Examination of the response of the chl b/a_2 ratio for the paired co-isolates MIT9303, MIT9313, MIT9302 and MIT9312 at relatively high growth irradiances reveals that the low-light adapted MIT9303 and MIT9313 consistently maintain fairly high ratios of chl *b*/chl a_2 (0.47 - 1.3), whereas their high-light adapted co-isolates, MIT9302 and MIT9312, maintain approximately 2-fold lower ratios at any given growth irradiance (Fig. 2A). Pigment trends for the *Prochlorococcus* isolates from the Pacific shows that ratios of chl *b*/chl a_2 of MIT9211 are 4 - 8 times higher than those of the three other Pacific isolates, MIT9201, MIT9202 and MIT9215 (Fig. 2B), reminiscent of the differences seen between chl *b*/chl a_2 ratios of SS120 and MED4.

By combining the chl *b*/chl a_2 ratio data for all the isolates in this study, as well as previously published data for the clonal *Prochlorococcus* isolates SS120 and MED4, two main clusters of isolates can be identified. (Fig. 2C). The four low-light adapted isolates,

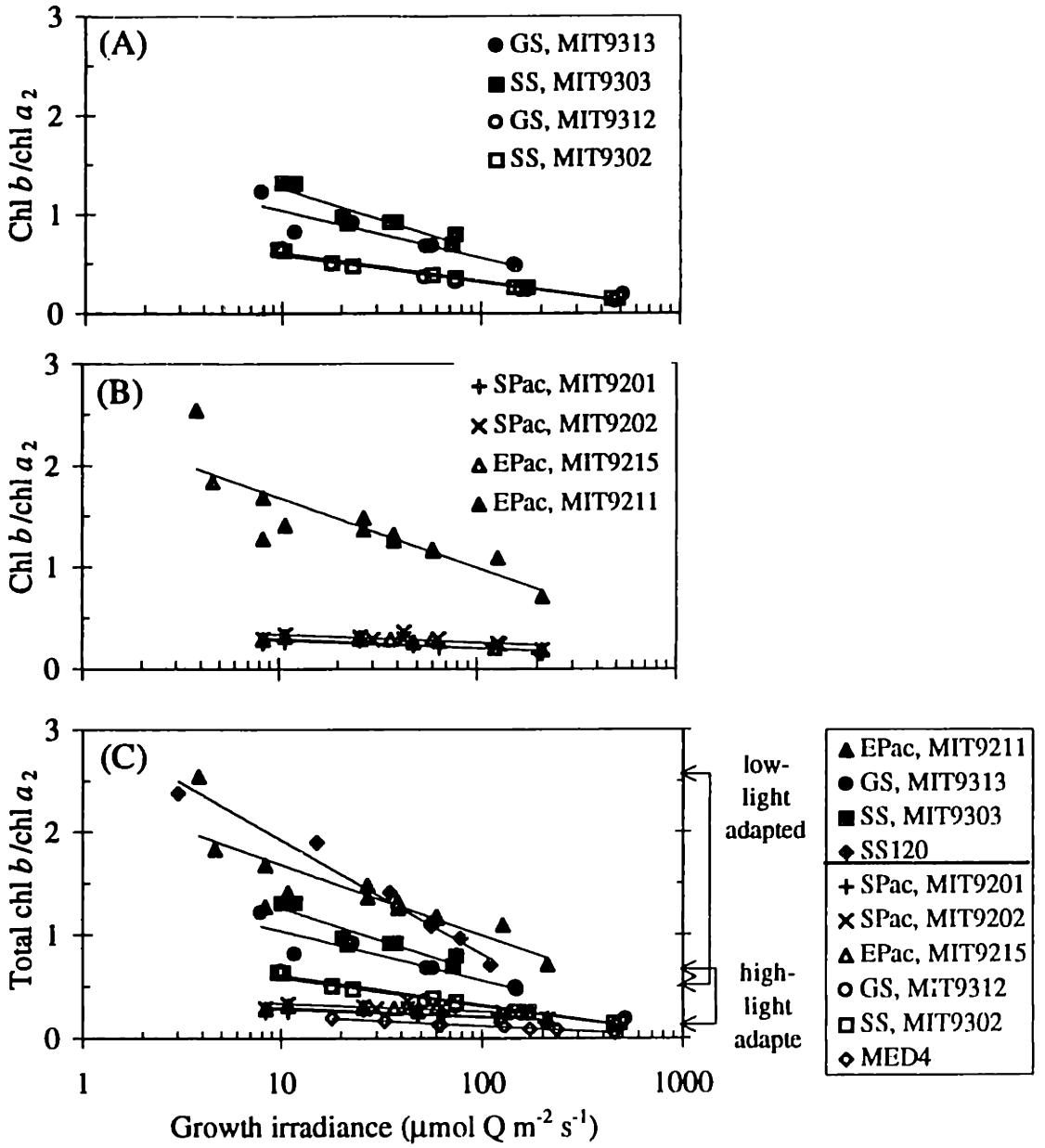


Figure 2 - Ratio of total chl *b*/chl a_2 (fg/fg) as a function of growth irradiance. (A) Sargasso Sea co-isolates, MIT9302 and MIT9303, and Gulf Stream co-isolates, MIT9312 and MIT9313. (B) Equatorial Pacific isolates, MIT9215 and MIT9211, and southeastern Pacific isolates, MIT9201 and MIT9202. (C) Compilation of total chl *b*/chl a_2 ratio for all isolates, including SS120 and MED4 from Moore et al. 1995. Symbols for each isolate in each panel are the same as used in Fig. 1.

SS120, MIT9303, MIT9313 and MIT9211, have relatively high ratios of total chl *b*/chl a_2 ranging from 0.47 - 2.6. The six isolates that exhibited a high-light adapted growth response cluster together with a ratio of chl b/a_2 (0.05 - 0.65) which is 2 - 10 fold lower across all growth irradiances compared to the other four isolates (Fig. 2C). Although the growth response of MIT9211 was not definitively that of a low-light adapted *Prochlorococcus*, its ratio of chl *b*/chl a_2 is similar to that of SS120 at all light levels, which clearly distinguishes it from the high-light adapted *Prochlorococcus*. [Because of its low I_{comp} and high ratio of chl *b*/chl a_2, we will refer to MIT9211 as low-light adapted for the rest of the paper, which later will be shown to be a reasonable characterization of this isolate.] The wide range of chl *b*/chl a_2 ratios, with no overlap of chl *b*/chl a_2 values between the two groups, strengthens the interpretation that large shifts in the chl *b*/chl a_2 ratios with depth observed in the Atlantic and Pacific Oceans result from both photoacclimation of cellular pigments and shifts in *Prochlorococcus* populations (Partensky et al., 1996; Goericke and Repeta, 1993).

Based on these chl b/a_2 ratios, we can speculate on the growth capability of other *Prochlorococcus* isolates, described by others, which have not been studied extensively with respect to growth as a function of light intensity. Chl b/a_2 ratios reported for three other isolates of *Prochlorococcus* indicate that a north Atlantic isolate, NATL1, falls close to MIT9313 in the low-light adapted group (chl b/a_2 range = 0.95 - 0.40 from 7.5 - 133 μmol Q m^{-2} s^{-1} white light, from Partensky et al., 1993), and two clonal isolates from the northwestern Pacific, GP2 and SB, fall into the high-light adapted group (chl b/a_2 = 0.17 and 0.13, respectively, when grown at 6 μmol Q m^{-2} s^{-1} blue-green light, from Shimada et al., 1996).

The association of the northwestern Pacific isolates, GP2 and SB, with the other low chl b/a_2 ratio, high-light adapted isolates is consistent with the 16S rRNA gene phylogeny which places these two isolates in a "high light-adapted clade" (Urbach et al., 1997).

Despite the correlation of chl b/a_2 ratios with low and high-light adapted growth capabilities of *Prochlorococcus* isolates, no correlation is observed for the cellular chlorophyll concentration. The paired co-isolates from the Atlantic have high values of chl a_2 per cell compared to other *Prochlorococcus* isolates (Fig. 3A). Within these pairs, the two low-light adapted isolates, MIT9303 and MIT9313, have similar cellular contents of chl a_2, roughly 1.25 - 2.3 times the amount of chl a_2 compared to their high-light adapted coisolates, MIT9302 and MIT9312. Chl a_2 content of MED4 is similar to that of MIT9302 and MIT9312. All four of the Pacific isolates have lower cellular concentrations of chl a_2, ranging from 2.3 fg cell^{-1} at the lowest I_g to 0.4 fg cell^{-1} at the highest I_g, similar to the levels in SS120 (Fig 3A).

A different inter-isolate pattern was observed for total cellular chl *b* content (chl b_1 + chl b_2 for some isolates, see below). The two low-light adapted isolates, MIT9303 and MIT9313, have the highest concentrations of chl *b*, whereas the high-light adapted *Prochlorococcus* isolates from the Pacific (MIT9201, MIT9202, MIT9215) along with MED4 have the lowest cellular concentration of total chl *b* (Fig. 3B). Intermediate between these two extremes are the cellular chl *b* contents of the low-light adapted isolates SS120 and MIT9211, and the high-light adapted isolates, MIT9302 and MIT9312 (Fig. 3B).

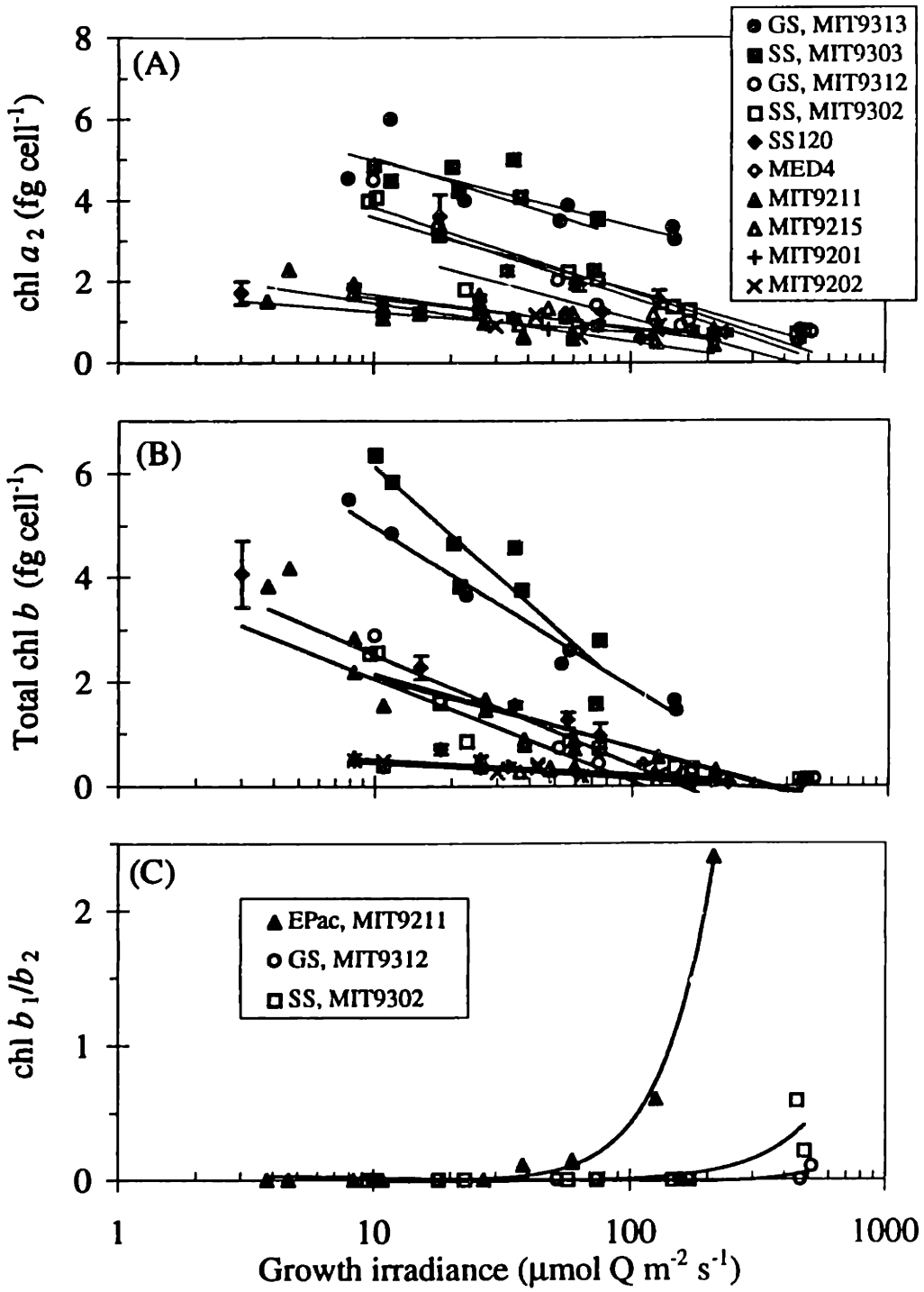


Figure 3 - Photoacclimative changes in chlorophyllous pigment content. (A) Chl a_2 per cell. (B) Total chl b per cell. (C) Ratio of chl b_1 to chl b_2 for isolates that contain measurable amounts of chl b_1: MIT9211, MIT9302 and MIT9312. Symbols same as for Fig. 1.

Several of the isolates (MIT9211, MIT9302 and MIT9312) contain the more typical, monovinyl chl b_1 in addition to divinyl chl b_2 as their major photosynthetic accessory pigment. Curiously, this trait appears to be unrelated to the light-dependent growth response of the isolate. The Pacific isolate MIT9211 contains significant amounts of chl b_1, which is first measurable in cultures grown at 38 µmol Q m^{-2} s^{-1} and increases in concentration as growth irradiance increases until chl b_1/chl b_2 reaches 2.4 at the highest growth irradiance (Fig. 3C). This is similar in trend and magnitude to that seen for SS120 (Moore et al., 1995; Partensky et al., 1993). Chl b_1 was also observed in small amounts (< 30% of total chl *b*) at the highest growth irradiances (> 400 µmol Q m^{-2} s^{-1}) in the high-light adapted isolates from the Sargasso Sea, MIT9302, and the Gulf Stream, MIT9312 (Fig. 3C). No evidence of chl b_1 was found in any of the other *Prochlorococcus* isolates examined.

Flow cytometric fluorescence per cell

Flow-cytometrically measured relative chlorophyll fluorescence per cell is not always be a reliable indicator of low or high-light adapted *Prochlorococcus*, especially at higher irradiances. The three low-light adapted isolates, SS120, MIT9303 and MIT9313, exhibit the highest levels of relative cellular chlorophyll fluorescence over the entire range of I_g, with values for MIT9303 and MIT9313 exceeding the values measured for all the other *Prochlorococcus* isolates and values for SS120 approaching those of the MIT9211, MED4, MIT9302 and MIT9312 (Fig. 4A). The high-light adapted Pacific isolates exhibit low relative chlorophyll fluorescence per cell across all irradiances. Intermediate between the extremes are relative cellular chlorophyll fluorescence exhibited by MIT9211, similar to those of three high-

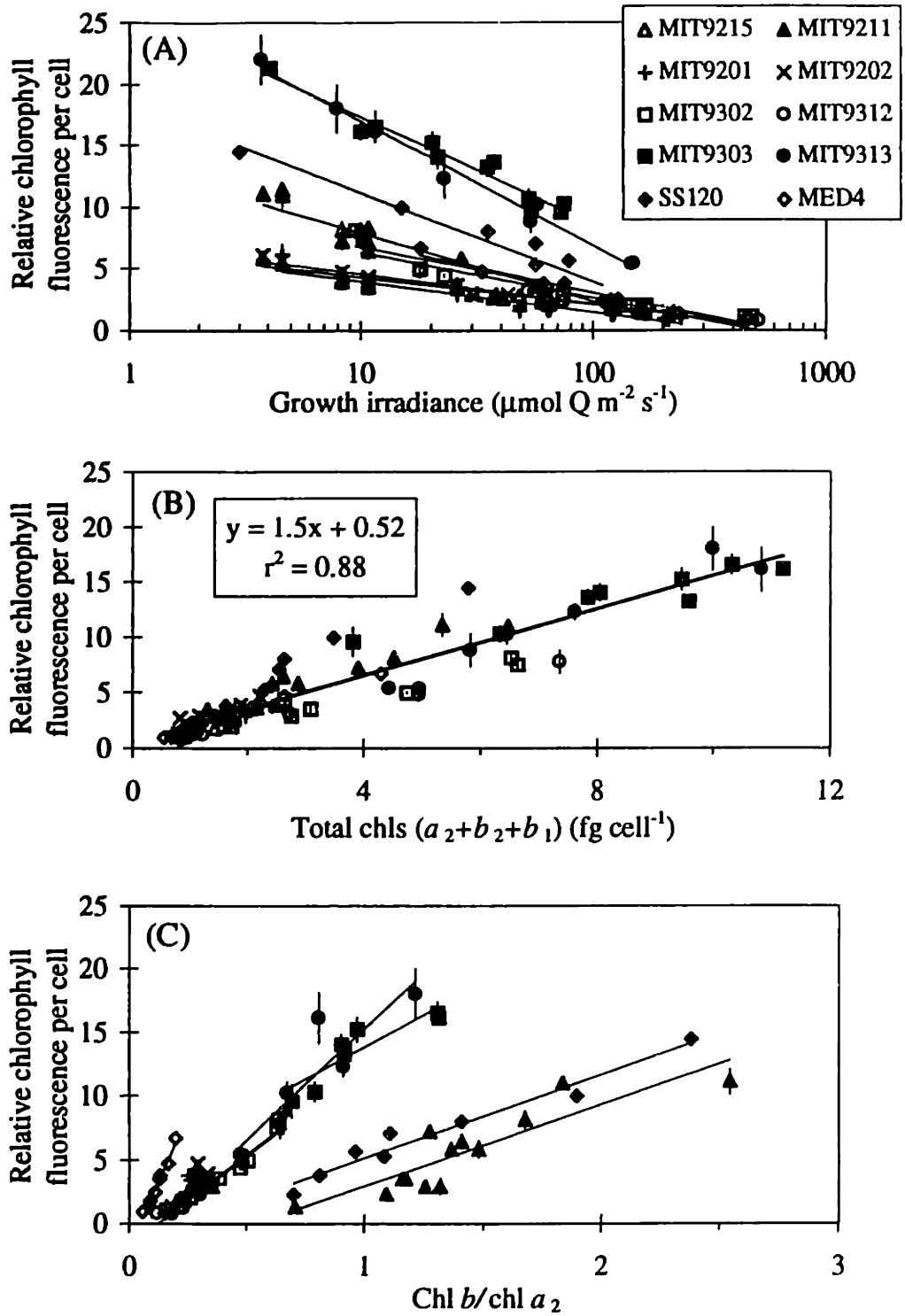


Figure 4 - Relative mean red (chlorophyll) fluorescence per cell. (A) As function of growth light intensity. (B) As function of total chlorophyll pigment content. (C) As function of chl b/a_2 ratio.

light adapted isolates MED4, MIT9302 and MIT9312, over most of its growth irradiances, except the lowest I_g (Fig. 4A). The variability in cellular chlorophyll fluorescence between isolates can be explained by the fact that both chl a_2 and chl *b* absorb light which can contribute to the fluorescence signal. Mean chlorophyll fluorescence per cell correlates well with total cellular chlorophyll content for all the isolates (Fig. 4B) and with the chl b/a_2 ratio for individual isolates (Fig. 4C). The similar levels of cellular chlorophyll fluorescence for MIT9211, MED4, MIT9302 and MIT9312 result from the fact that they have similar total cellular chlorophyll content over the range of light levels measured (0.73 - 6.5 fg cell^{-1}, 0.78 - 4.3, 0.8 - 6.6 fg cell^{-1} and 0.86 - 7.4 fg cell^{-1}, respectively).

Another interesting observation is that the low-light adapted isolates, SS120, MIT9211, MIT9303 and MIT9313, all exhibit small but significant amounts of flow-cytometrically induced orange (585 nm) fluorescence that increase with decreasing growth irradiance (data not shown). Low levels of orange fluorescence have been reported for a natural population of *Prochlorococcus* in the bottom euphotic layer of the tropical Pacific and attributed to the presence of small amounts of phycoerythrin (Hess et al., 1996). In addition, these authors detected phycoerythrin based on spectrofluorometric analysis of a soluble water extract from SS120, although the functional significance of this pigment awaits further investigation. It is possible that the three other low-light adapted isolates may also possess phycoerythrin. There is, however, another possible explanation. The orange fluorescence signal is collected using a 585 nm bandpass filter, which could transmit the tail of the chlorophyll fluorescence emission if the emission is large enough. This is particularly possible

with the high chl b_2-containing isolates, because of the coincidence of the laser excitation line and the absorption maximum of chl b_2. One could test for the presence of phycoerythrin in natural populations by measuring orange emission resulting from excitation at 515 nm (as decribed by Olson et al., 1988), and artifactual orange emission could be tested for by using a narrow bandpass filter which would not transmit emission wavelengths associated with chlorophyll *a*. Thus, caution should be exercised in interpreting the orange fluorescence signal of natural populations of *Prochlorococcus*.

Absorption properties

In vivo chl a_2-specific absorption spectra [$a^*_{ph}(\lambda)$] reflect changes in the ratio of chl *b* to chl a_2 in *Prochlorococcus* (Fig. 5 and 6). The low-light adapted isolates with high chl *b*/chl a_2 ratios, MIT9211, MIT9303 and MIT9313, exhibit a distinct peak associated with chl *b* (λ = 480 nm) when grown under low irradiance (Fig. 5). At high I_g, the main peak in the blue (λ = 449 nm) and the ratio of peak absorption in the blue relative to that in the red (a_{449}/a_{680}; referred to as B/R ratio in Moore et al., 1995) increase in magnitude (Fig. 5, 6A). Photoacclimative changes in the relative concentrations of chl *b* to chl a_2 for cells grown in high and low light were apparent in the increased ratio of the chl b_2 peak in the blue (λ = 480 nm) relative to the chl a_2 blue peak (a_{480}/a_{449}) (Fig. 5, 6B). And, as expected, the spectrally-weighted average chl a_2-specific absorption coefficients ($\bar{a}^*_{chl}$) are higher for isolates with high chl b/a_2 ratios (Fig. 6C). Bio-optical models which use $\bar{a}^*_{chl}$ for estimating oceanic primary production from remote sensing may need to account for shifts in *Prochlorococcus*

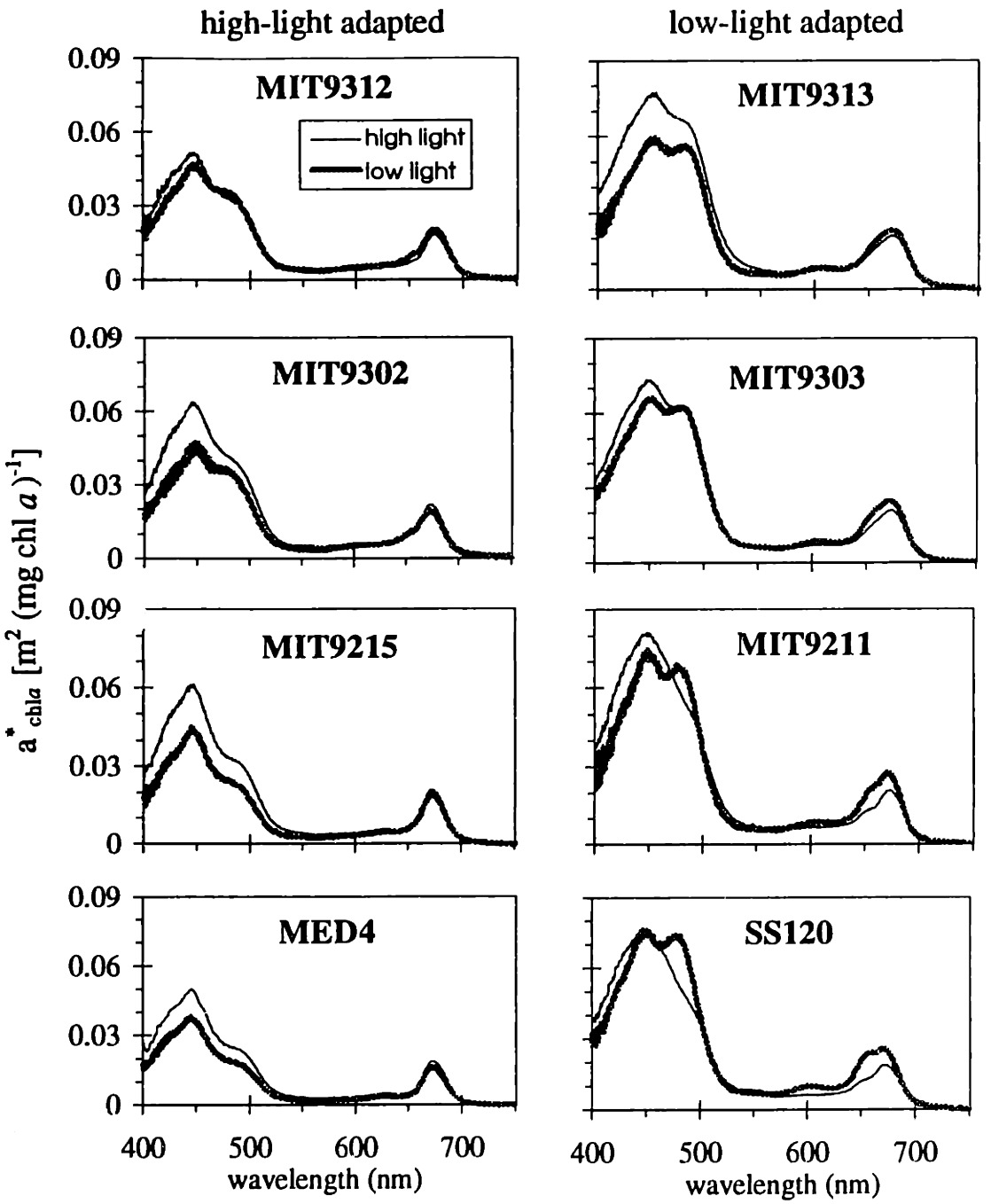


Figure 5 - Chl a_2-specific absorption spectra [m^2 (mg chl a_2)$^{-1}$] for each isolate grown at two light intensities: low light = 9 µmol Q m^{-2} s^{-1}; high light = 70 µmol Q m^{-2} s^{-1} (52 µmol Q m^{-2} s^{-1} for MIT9303).

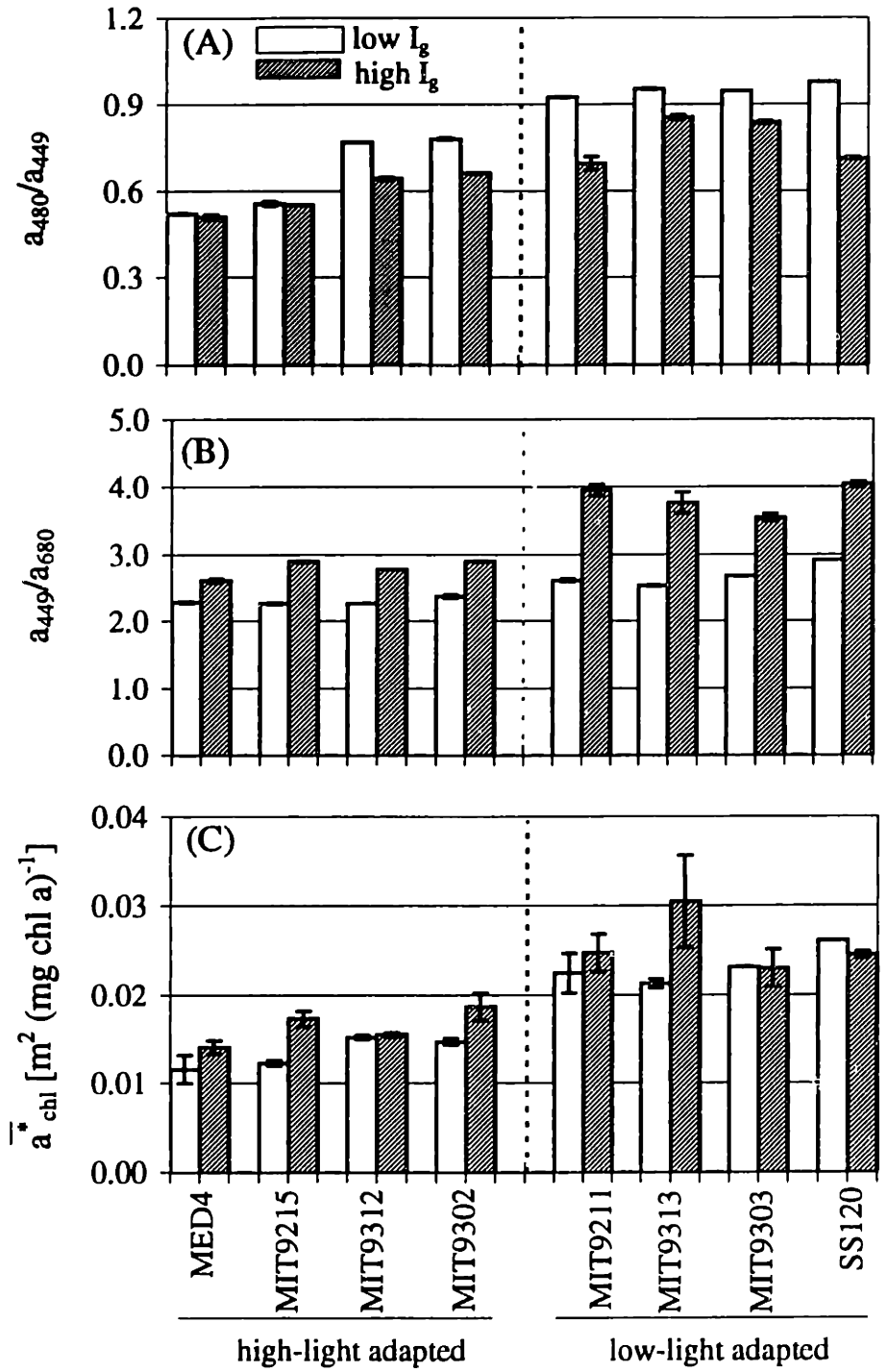


Figure 6 - Absorption properties of low and high-light adapted isolates grown at two growth irradiances: low light = 9 µmol Q m^{-2} s^{-1}; high light = 70 µmol Q m^{-2} s^{-1} (52 µmol Q m^{-2} s^{-1} for MIT9303). Error bars are ±1 SE of duplicate cultures. (A) a_{480}/a_{449}. (B) a_{449}/a_{680}. (C) $\bar{a}^*_{ph}$.

populations and their respective absorption coefficients and pigmentation in open ocean regimes where this group dominates the phytoplankton assemblage.

Photosynthesis characteristics and photoacclimative changes

Differences in photosynthesis-irradiance (P-I) response and associated photosynthetic parameters between the low-light adapted *Prochlorococcus* isolates, MIT9303 and MIT9313, and their coexisting, high-light adapted isolates, MIT9302 and MIT9312 were consistent with differences in their pigments and growth rates (Moore et al., 1997). To further explore this relationship, we examined the P-I response for these isolates grown at a higher irradiance and additionally looked at the P-I response for the high-light adapted isolates MIT9215 and MED4, and the low-light adapted isolates SS120 and MIT9211. [The P-I response for the two low-light adapted isolates MIT9201 and MIT9202 were not examined since their growth and pigment physiology is so similar to MIT9215.] At low growth irradiance, low-light adapted isolates SS120, MIT9303, MIT9313 and MIT9211 are, on average, about twice as effective at utilizing available light-energy on a per chl a_2 basis as the four high-light adapted isolates MED4, MIT9302, MIT9312 and MIT9215 (average $\alpha_{chla} = 0.11$ vs. 0.06) (Fig. 7A, 8A). When acclimated to high I_g, all the low-light adapted isolates exhibit approximately 2-fold lower α_{chla} such that the light harvesting efficiency does not differ significantly from that for the four high-light adapted isolates examined (Fig. 7A, 8A). The latter undergo fairly small photoacclimative changes in α_{chla} with no consistent trend with growth irradiance (Fig. 8A). The range of α_{chla} values [0.03 - 0.125 fg C fg chl a^{-1} h^{-1} (μmol Q m^{-2} s^{-1})$^{-1}$] is consistent with values previously reported for *Prochlorococcus* (Shimada et al., 1996; Partensky et al.,

1993) and are similar to what has been observed for *Synechococcus* (Shimada et al., 1996; Bidigare et al., 1989; Kana and Glibert, 1987). For natural phytoplankton populations in the oligotrophic north Atlantic where *Prochlorococcus* is typically abundant, the α_{chla} values measured are often lower than those reported here (Babin et al., 1996; Cleveland et al., 1989; Lewis et al., 1985). However, a very high value for α_{chla} [0.26 fg C fg chl a^{-1} h^{-1} (μmol Q m^{-2} s^{-1})$^{-1}$] was found for a population occupying a deep secondary chlorophyll maximum in the Arabian Sea (Johnson et al., 1997).

The differences in magnitude and photoacclimation of photosynthetic efficiency between isolates are correlated with pigment content for the low-light adapted isolates. Higher photosynthetic efficiency for cells grown under low irradiances can be attributed to an increase in energy transfer from the accessory pigments to chl a_2; this is supported by a strong, positive correlation between α_{chla} and the chl b/a_2 ratio for low-light adapted *Prochlorococcus* ($r^2 = 0.799$; Fig. 9A). A major role for accessory pigments in regulating photoacclimative changes in light harvesting efficiency has been observed for cyanobacteria (Kana and Glibert, 1987; Raps et al., 1983) and red algae (Levy and Gantt, 1988) undergoing photoacclimation of phycobiliprotein to chl *a* ratios. The regulation of α_{chla} for the high-light adapted isolates (MED4, MIT9215, MIT9302, MIT9312) is difficult to assess based on P-I measurements because the changes are small and appear unrelated to changes in pigmentation (Fig. 9A), possibly due to the small overall ratio of chl b/a_2 in these isolates.

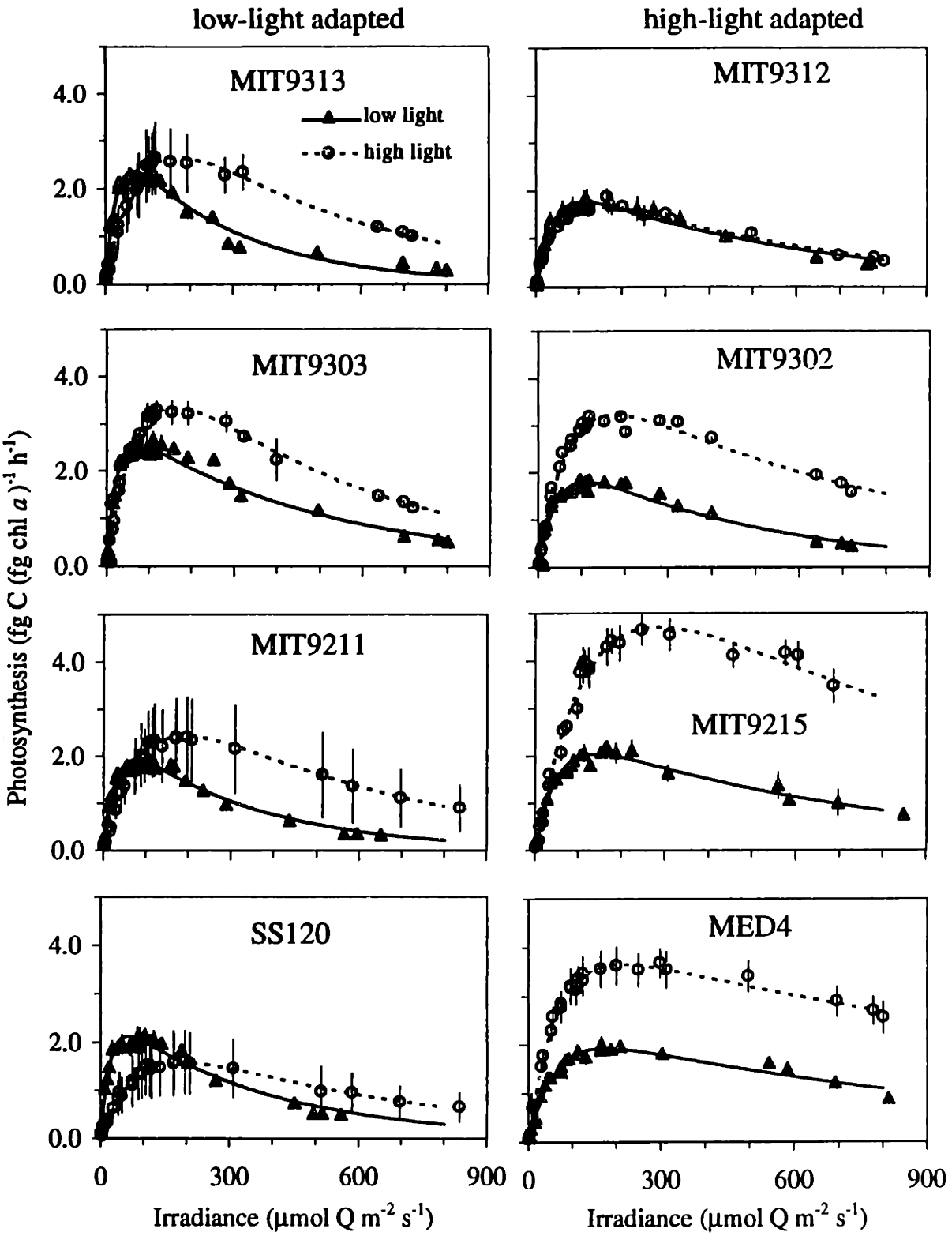


Figure 7 - P-I curves for each isolate grown at two light intensities: low light (triangles, solid line) = 9 µmol Q m^{-2} s^{-1}; high light (circles, dashed line) = 70 µmol Q m^{-2} s^{-1} (52 µmol Q m^{-2} s^{-1} for MIT9303). (A) Chl a_2-normalized photosynthesis.

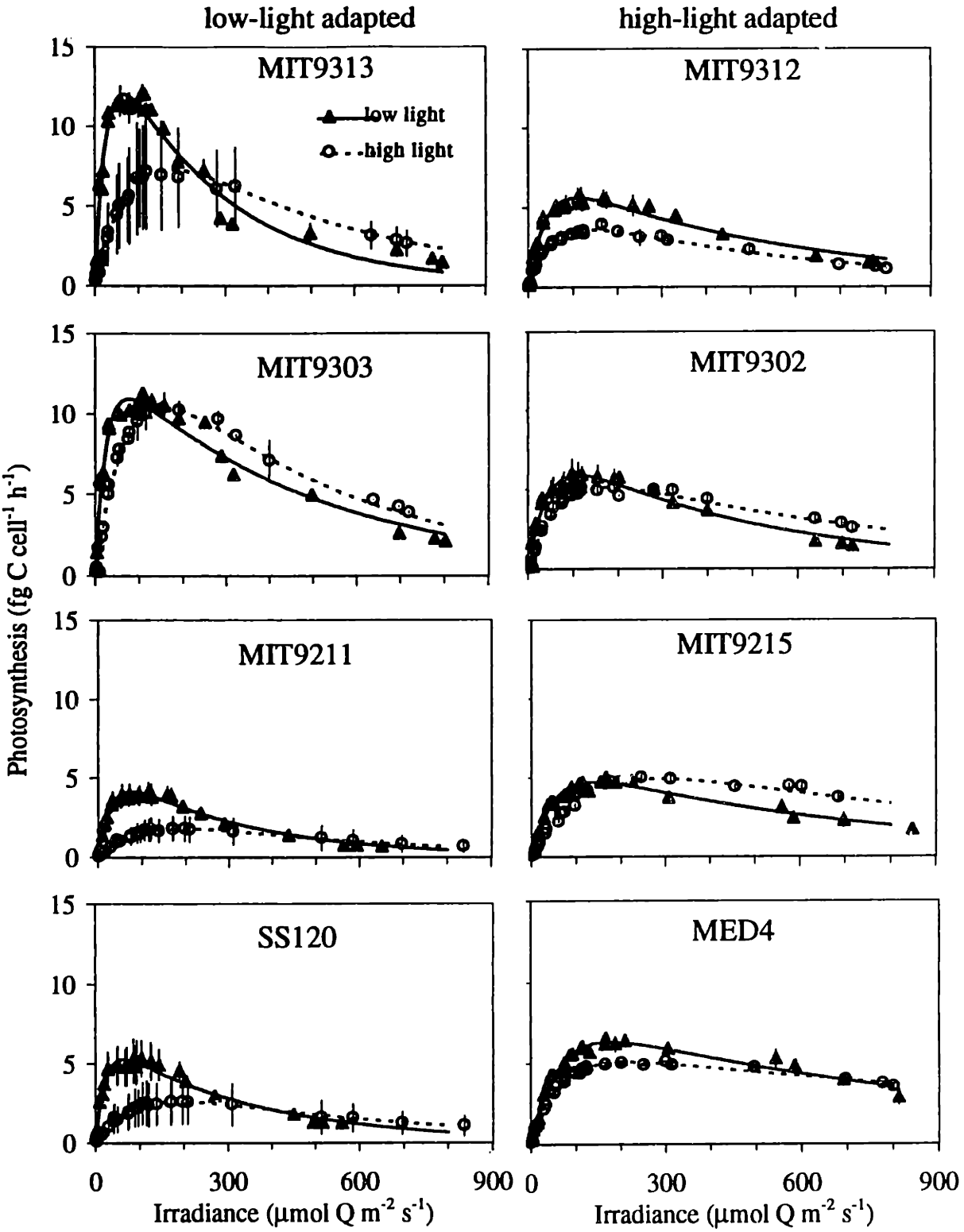


Figure 7 (cont'd) - (B) Cell-normalized photosynthesis. Error bars are ±1 SE of duplicate cultures. Curves presented for MIT9302, MIT9303, MIT9312 and MIT9313 grown under low light were previously reported in Moore et al. (1997).

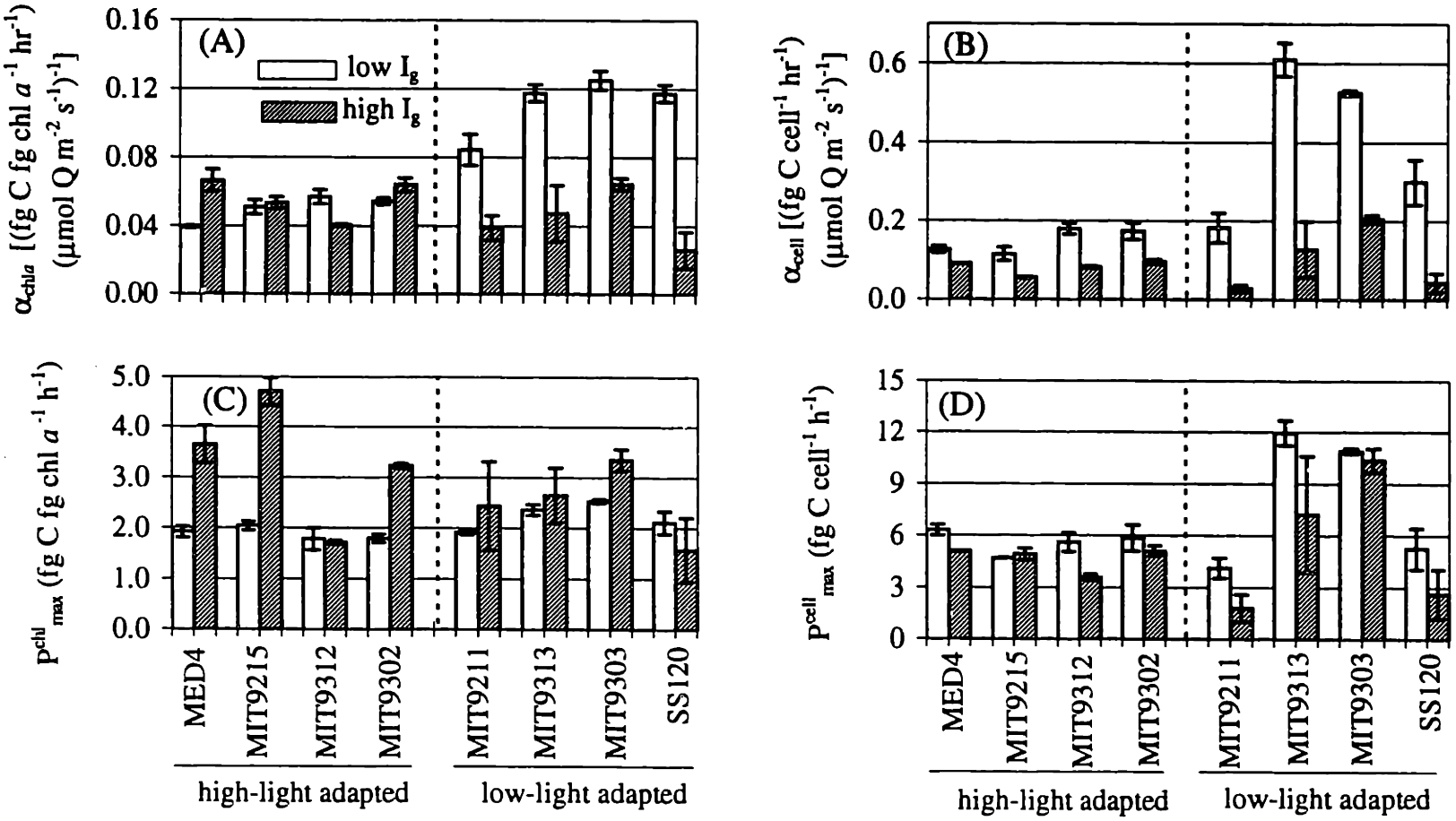


Figure 8 - P-I curve parameter values derived from the experiments described in Fig. 7. Error bars are ±1 SE of duplicate cultures. (A) α_{chla}; (B) α_{cell}; (C) P^{chl}_{max}; and (D) P^{cell}_{max}.

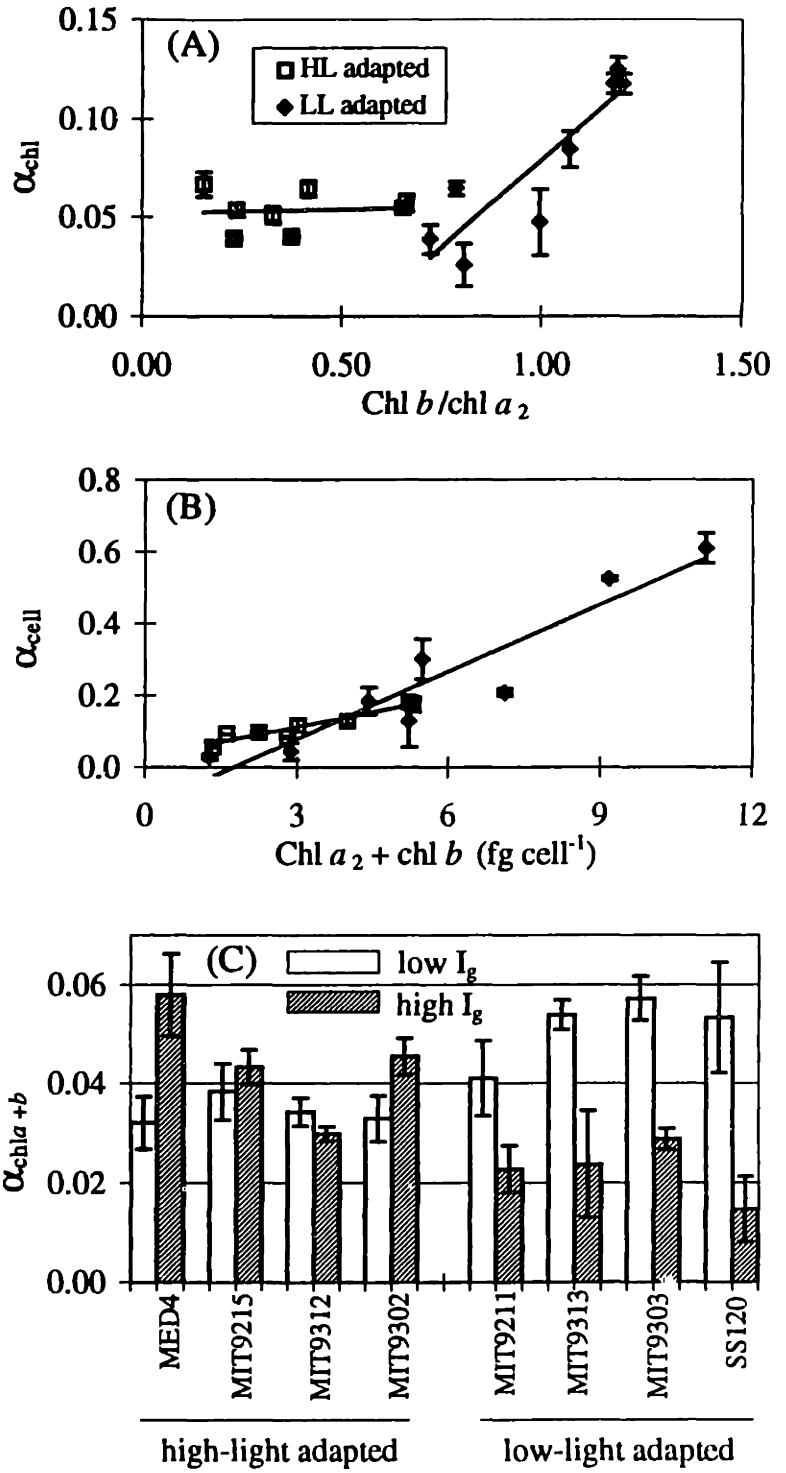


Figure 9 - (A) α_{chla} as a function of chl b/a_2 ratio. (B) α_{cell} as a function of total cellular chlorophyll. Low-light adapted (closed diamonds) and high-light adapted isolates (open squares); (C) α_{chla+b} for each isolate grown at two irradiances: low light = 9 µmol Q m^{-2} s^{-1}; high light = 70 µmol Q m^{-2} s^{-1} (52 µmol Q m^{-2} s^{-1} for MIT9303).

Measurements of photoacclimative changes in chl *a*/*b*-protein complexes of *Prochlorococcus* SS120 indicate that this isolate increases its concentration of antenna complexes but not the concentration of photosystem (PS) I and II cores as a result of decreasing growth irradiance (Partensky et al., 1997). Because the photoacclimative strategy of the other low-light adapted isolates, MIT9211, MIT9303, and MIT9313, are similar to that of SS120, e.g. increasing total chlorophyll concentration, chl b/a_2 ratio, and α_{chl} in response to lower I_g, it is likely that these isolates also increase the concentration of their antenna complexes. The high-light adapted MED4 was found to increase its antenna complexes while the PSI and PSII cores remained relatively constant with decreasing growth irradiance, though to a much lesser extent than SS120 (Partensky et al., 1997); it is possible that the other high-light adapted isolates, MIT9302, MIT9312, and MIT9215, exhibit this same response. Furthermore, because of the similar photosynthetic responses for isolates within each ecotype, we suspect that the light harvesting complexes of the two *Prochlorococcus* ecotypes will differ in ways similar to those described for SS120 and MED4 (Partensky et al., 1997).

All the *Prochlorococcus* isolates have higher cell-specific light harvesting efficiency (α_{cell}) when grown under low light compared to when grown under high light (Fig. 7B, 8B). This is in contrast to the findings of Partensky et al. (1993) where differences between *Prochlorococcus* isolates MED, SARG and NATL1 grown at a variety of I_g levels were not as significant, nor were any photoacclimative trends distinguishable (Partensky et al., 1993). At low I_g, the high-light adapted isolates MED4, MIT9215, MIT9312 and MIT9302 have similar values of α_{cell} ($\pm$ 20%), and at high I_g their α_{cell} was 27 - 53% lower (Fig. 7B, 8B). Values of

α_{cell} differed significantly between the low-light adapted isolates. SS120 and MIT9211 grown under high light have the lowest α_{cell} values, whereas MIT9313 and MIT9303 grown under low light exhibited the highest values of α_{cell} ever reported for *Prochlorococcus* (Shimada et al., 1996; Partensky et al., 1993) or *Synechococcus* (Kana and Glibert, 1987; Glibert et al., 1986). The variability in cell-specific light harvesting efficiency between all the isolates is well correlated with the total cellular chlorophyll content, though the relationship differs for the low and high-light adapted isolates (Fig. 9B), indicating different photoacclimative strategies between these two ecotypes. This difference is clearly demonstrated when the initial slope of the P-I curve is normalized to the total cellular chlorophyll content, α_{chla+b}. The four isolates adapted to low light increase their α_{chla+b} in response to decreasing I_g, indicative of increased light harvesting efficiency of the cellular pigments at lower I_g. Of the four high-light adapted isolates, MED4 and MIT9302 exhibit decreases in α_{chla+b}, and MIT9215 and MIT9312 show no change in α_{chla+b} to decreasing I_g (Fig. 9C).

Maximum photosynthetic rates on a per chl a_2-basis, P^{chla}_{max}, varied by 2.8 fold (1.7 - 4.7 fg C fg chl a^{-1} h^{-1}) with no consistent trend among the different isolates (Fig. 7A, 8C). Significant variability in P^{chla}_{max} also was observed among several isolates (Shimada et al., 1996; Moore et al., 1995; Partensky et al., 1993) and for cultures grown under different light levels (Partensky et al., 1993). The isolates in this study exhibit a wide range of cell-specific, maximum photosynthetic rates, P^{cell}_{max}, (1.8 - 12 fg C $cell^{-1}$ h^{-1}) (Fig. 7B, 8D). In contrast to the photoacclimative changes observed for P^{chl}_{max}, however, P^{cell}_{max} showed higher values (1.2 to 2.2-fold higher) or remained constant for cultures grown under low growth irradiances as

compared to cultures grown under high growth irradiances. Unexpectedly, the change in P^{cell}_{max} with I_g for SS120 and MED4 reported in this study are opposite that reported for their nonclonal, primary cultures, SARG and MED (Partensky et al., 1993). The reasons for this are unknown; though, perhaps the clonal derivatives differ slightly from the nonclonal, primary cultures, or cell cycle effects on P_{max} may have obscured trends between these two studies. As observed for α_{cell}, the two low-light adapted *Prochlorococcus* isolates MIT9303 and MIT9313 exhibited the highest light-saturated maximum photosynthetic rates under both growth irradiances examined (Fig. 8D), approaching (though not reaching) values reported for *Synechococcus* (Shimada et al., 1996; Kana and Glibert, 1987; Glibert et al., 1986; Waterbury et al., 1986). Increases in P^{cell}_{max} are correlated to increases in total cellular chlorophyll content for the low-light adapted isolates, and for two of the high-light adapted isolates, MED4 and MIT9312 ($R^2 = 0.912$; data not shown); this may indicate increased pigment-specific energy transfer.

Photoinhibition of photosynthesis at high light intensities is a consistent feature for all the isolates regardless of growth irradiance. All but two isolates (MIT9312 and MIT9303) show greater photoinhibition (index of photoinhibition, I_b, is lower) when grown under low photon flux density (Table 3). In general, the irradiance at which photoinhibition is first apparent is lower for the low-light adapted isolates than for the high-light adapted ones (Table 3); this is consistent with the relative differences in I_{inhib} of growth response between these two ecotypes (Fig. 1). Also, the irradiance at which $P^{chl\,a}_{max}$ is reached (I_m) is higher for all cultures grown in the higher irradiance level (Table 3).

TABLE 3 - Additional photosynthetic parameters obtained from the P-I curves presented in Fig. 7. Values for each strain are mean ± S.E. of duplicate cultures.

A). PHOTOINHIBITION INDEX, I_b

low light adapted

irradiance	MIT9211	MIT9313	MIT9303	SS120
low	315 (±1)	269 (±16)	465 (±24)	372 (±69)
high	528 (±112)	483 (±89)	494 (±23)	579 (±50)

high-light adapted

irradiance	MED4	MIT9215	MIT9312	MIT9302
low	1003 (±99)	690 (±71)	548 (±40)	450 (±42)
high	1694 (±66)	1032 (±14)	562 (±5)	745 (±59)

B). MAXIMUM QUANTUM YiELD OF PHOTOSYNTHESIS

low light adapted

irradiance	MIT9211	MIT9313	MIT9303	SS120	average
low	0.09 (±0.02)	0.127 (±0.008)	0.125 (±0.005)	0.106 (±0.005)	0.11 (±0.02)
high	0.04 (±0.01)	0.04 (±0.02)	0.068 (±0.003)	0.03 (±0.01)	0.04 (±0.02)

high-light adapted

irradiance	MED4	MIT9215	MIT9312	MIT9302	average
low	0.080 (±0.009)	0.096 (±0.009)	0.085 (±0.006)	0.085 (±0.005)	0.087 (±0.007)
high	0.114 (±0.005)	0.08 (±0.01)	0.062 (±0.001)	0.079 (±0.006)	0.08 (±0.02)

The maximum quantum yield of photosynthesis (ϕ_m) is close to its theoretical maximum value for all isolates grown under low light levels. High values of ϕ_m (up to 0.10 mol C mol quanta^{-1}) have been reported previously for *Prochlorococcus* in culture (Partensky et al., 1993) and under natural conditions (Johnson et al., 1997), and for *Synechococcus* (Bidigare et al., 1989; Iturriaga and Mitchell, 1986). Photoacclimative trends in ϕ_m are apparent between the two *Prochlorococcus* ecotypes. The four high-light adapted isolates, MED4, MIT9215, MIT9302, and MIT9312, showed only slight changes in ϕ_m when grown under high light. In contrast, the four low-light adapted isolates, SS120, MIT9211, MIT9303 and MIT9313, exhibited significant decreases in ϕ_m (1.9 to 4.2-fold) when grown under these conditions (Table 3). Lower ϕ_m values for phytoplankton acclimated to high light have been observed previously for *Prochlorococcus* (Partensky et al., 1993) and for *Dunaliella tertiolecta* (Sukenik et al., 1990). However, changes as large as observed here for SS120, MIT9211, MIT9303 and MIT9313 have been observed only for natural phytoplankton assemblages and attributed to nitrogen availability and/or high light absorption by nonphotosynthetic pigments (Babin et al., 1996; Sosik 1996; Prezelin et al. 1991; Kolber et al. 1990; Cleveland et al., 1989). Zeaxanthin, the major nonphotosynthetic pigment in *Prochlorococcus*, may be responsible for some of the photoacclimative changes in ϕ_m observed for the low-light adapted *Prochlorococcus*. Unfortunately, we did not measure zeaxanthin in the cultures used for P-I experiments, so we cannot quantify the importance of this pigment to photoacclimative changes in $\bar{a}^*_{chl}$ and ϕ_m.

One way to evaluate the ecological significance of ecotypic differentiation of

Prochlorococcus is to compare the contribution of each ecotype to the productivity at a given depth in the water column (0.5% I_o depth = 117 m, again assuming k = 0.045 m^{-1} and I_o = 2000 μmol Q m^{-2} s^{-1}). Given a population composed entirely of a low-light adapted *Prochlorococcus* ecotype ($\alpha_{chl\,a}$ range of 0.085 - 0.125 fg C fg chl^{-1} h^{-1} (μmol Q m^{-2} s^{-1}) $^{-1}$, chl *a* range of 2.2 - 5.1 fg $cell^{-1}$), the contribution of *Prochlorococcus* to primary production for this depth ranges between 1.6-5.0 mg C m^{-3}, assuming a mean population abundance of 10^4 cells ml^{-1} and 14 hr exposure to a mean incident irradiance of 10 μmol Q m^{-2} s^{-1}. If, instead, the population is composed of a high-light adapted ecotype ($\alpha_{chl\,a}$ range of 0.04 - 0.057 fg C fg chl^{-1} h^{-1} (μmol Q m^{-2} s^{-1}) $^{-1}$), then the primary production at this depth and light level would be substantially lower (1.6 - 3.4 times) at 1.0 - 1.48 mg C m^{-3}. The above analysis makes it clear that shifts in *Prochlorococcus* populations can influence the primary production, particularly in the deeper euphotic zone where differences between low-light and high-light adapted *Prochlorococcus* are most pronounced. Thus, as mentioned above for $\bar{a}^{*}_{chl}$, bio-optical models used for estimating primary production also may need to account for variability in *Prochlorococcus* photosynthetic parameters, particularly with depth.

Principle component analysis

Two principal component analyses of the physiological data confirmed the grouping of *Prochlorococcus* isolates as low and high-light adapted ecotypes. The first PCA utilized all ten isolates and is based on 4 growth rate parameters (I_{inhib}, I_{comp}, I_{max}, μ_{max}), the cellular chlorophyll fluorescence, chl *b* content, and chl b/a_2 ratio for cells grown under both low and high light. The first principal component, axis 1, accounted for 81.25% of the variance in the

data, with I_{inhib} and pigmentation-related parameters contributing most to the variance (Table 4). Thus, axis 1 separates the isolates into those with high I_{inhib}, and low fluorescence and pigmentation, corresponding to the 6 high-light adapted isolates, and those with low I_{inhib}, and high fluorescence and pigmentation, corresponding to the low-light adapted isolates (Fig. 10A).

A second PCA was carried cut on the 8 isolates on which P-I measurements were made; additional parameters included in this analysis were the P-I parameters and their low to high light ratios. The results yielded a grouping pattern consistent with that found from PCA1. The first and second principal components (axis 1 and 2) accounted for 60.3% and 23.1% of the variance, respectively. The primary separation of the isolates along the first axis is based on the chl b/a_2 ratio and the low light harvesting efficiency, α_{chla} (Table 4). Thus, isolates that have high ratios of chl b/a_2 and can efficiently harvest low light, i.e. the low-light adapted isolates, group separately from those that have lower chl b/a_2 and are less efficient at harvesting low light, i.e. the high-light adapted isolates (Fig. 10B). The four low-light adapted isolates are further separated by axis 2 into those that have low cell-specific photosynthetic capacity and efficiency (SS120 and MIT9211) and those with high P^{cell}_{max} and α_{cell} (MIT9303 and MIT9313) when grown under high light (Table 4, Fig. 10B). These two PCAs reveal that the 10 isolates in this study can be distinguished as low or high-light adapted by their pigmentation and growth and photosynthetic capabilities. The low-light adapted isolates are further distinguished at high growth irradiances by their different cell-specific photosynthetic parameters.

TABLE 4 - Eigenvector component loadings for the principal parameters (components) contributing to each axis.

PCA1		PCA2			
parameters	axis 1	parameters	axis 1	parameters	axis 2
I_{inhib}	+0.341	chl b/a_2, HL	-0.216	P^{cell}_{max}, HL	-0.338
chl fluorescence $cell^{-1}$	-0.339	chl b/a_2, LL	-0.215	α_{cell}, HL	-0.324
chl *b*, LL	-0.335	α_{chla}, LL	-0.216		
chl b/a_2, HL	-0.33				
chl b/a_2, LL	-0.328				

CONCLUSION

The hypothesis that *Prochlorococcus* isolates (and possibly all members of the *Prochlorococcus* genus) can be distinguished as low-light adapted or high-light adapted based on their different chl b/a_2 ratios and their light utilization capabilities is supported by characterization of the light-dependent physiological response of *Prochlorococcus* isolates from a variety of geographical provinces. Principal component analysis of the data further supports the ecotypic differentiation of *Prochlorococcus* and points to specific physiological parameters responsible for the groupings. Many of the light-related parameters also differ significantly between the two ecotypes (Table 5). Differences in the photoacclimative strategy of photosynthesis, related to their pigment content, was also apparent between the two ecotypes. However, since we considered P-I responses for cultures grown under only two light irradiance levels, a more complete study is necessary to establish detailed "ecotype-specific" patterns of photoacclimation, particularly for the high-light adapted isolates.

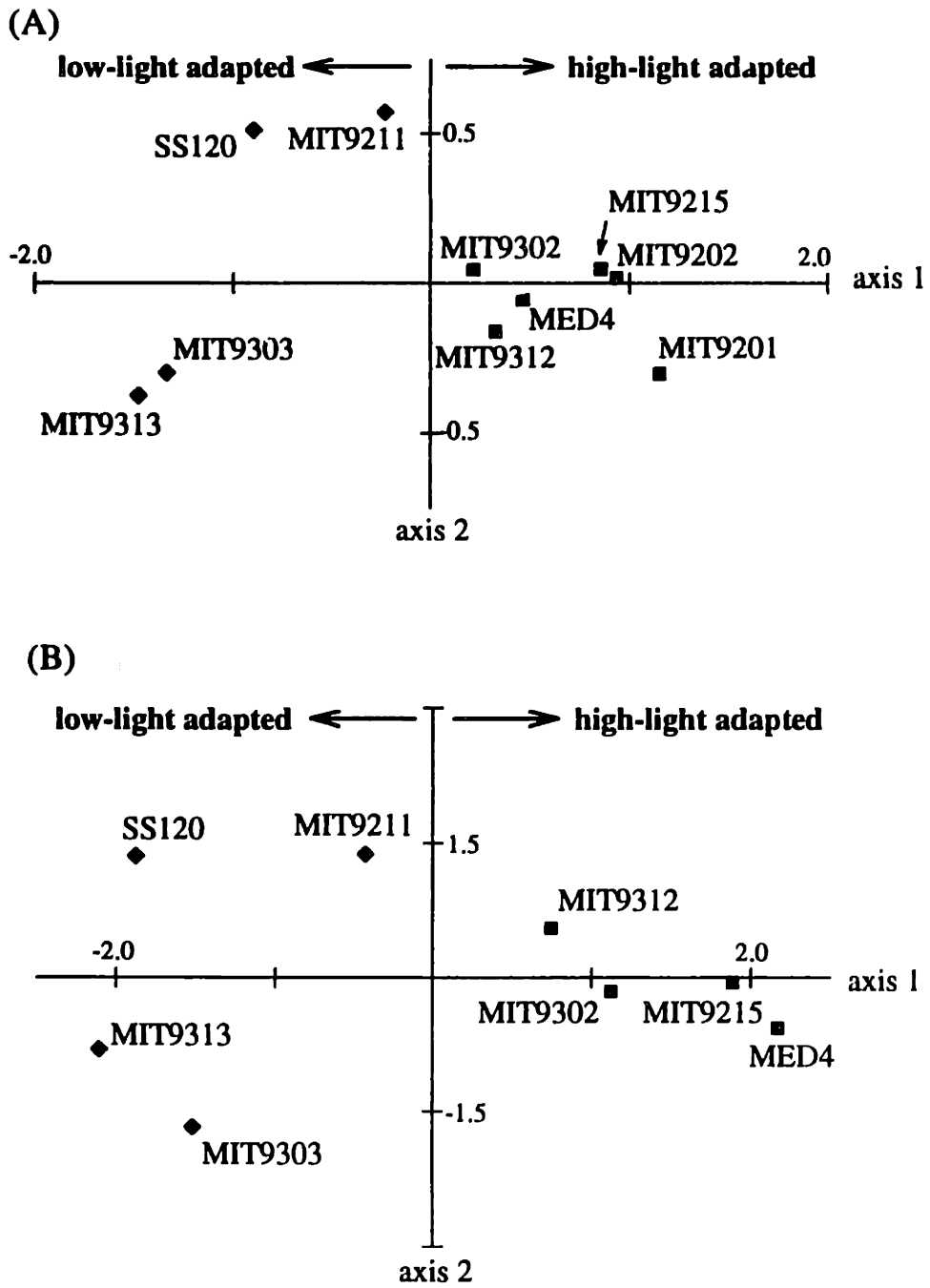


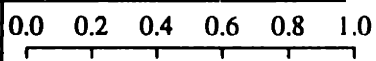
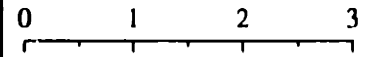
Figure 10 - Groupings of *Prochlorococcus* isolates resulting from principal component analyses of the physiological data. (A) PCA1 of all 10 isolates; (B) PCA2 of 8 isolates.

Phylogenetic analysis of *Prochlorococcus* isolates also correlates with the ecotypic differentiation associated with differences in chl b/a_2 ratio (Moore et al., 1997; Urbach et al., 1997; Scanlan et al., 1996). The high-light adapted isolates form a physiologically coherent group which is reflected in the 16S rRNA phylogenetic relationship of MED4, MIT9302 and MIT9312, all of which belong to the "high-light adapted" clade of the marine cyanobacteria picophytoplankton (Moore et al., 1997; Urbach et al., 1997). [16S rRNA genes have not been sequenced yet for MIT9201, MIT9202 and MIT9215]. The 16S rRNA gene sequences of SS120, MIT9303, MIT9313 (Moore et al., 1997; Urbach et al., 1997) and MIT9211 (G. Rocap, preliminary results) branch separately from the "high-light adapted" clade but do notform a distinct cluster (Moore et al., 1997; Urbach et al., 1997). In fact, MIT9303 and MIT9313 branch more deeply and closer to the marine A *Synechococcus* than any of the other *Prochlorococcus* isolates (Moore et al., 1997; Urbach et al., 1997), reflecting the separation of these two isolates from SS120 and MIT9211 seen in the PCA2 (Fig. 10B) and in several of the physiological parameters (total chl $a_2 + b$, relative chlorophyll fluorescence per cell, α_{cell}, and P^{cell}_{max}). It is possible that the high and low-light adapted ecotypes (and MIT9303 and MIT9313) are distinct species. For now, however, we distinguish them simply as ecotypes of the genus *Prochlorococcus*. Additional criteria, such as %G+C content (Priest and Austin, 1993), can be applied to delineate species.

These two physiologically distinct ecotypes of *Prochlorococcus* may occupy different ecological niches, particularly with respect to depth. The "depth" hypothesis that low-light adapted *Prochlorococcus* predominate in the deeper portion of the euphotic zone and high-

light adapted *Prochlorococcus* predominate in the surface, is supported by the distinct physiological responses to light availability of these two ecotypes. It may be possible to test the hypothesis of depth partitioning and to survey the seasonal and spatial distributions of the two *Prochlorococcus* ecotypes with the use of molecular probes designed to take advantage of the correlation between physiology and molecular phylogeny. Measuring the relative contribution of either *Prochlorococcus* ecotype to the total *Prochlorococcus* population is crucial to estimating the significance of ecotypic differentiation on estimates of primary production. Exploring other aspects of their physiology, such as response to nutrient and trace metal limitation, cell cycle patterns and controls, photosynthetic apparatus and genes, and viral susceptibility, as well as how these factors might correlate with light adaptation will be important for furthering the understanding of the ecological role of *Prochlorococcus*.

TABLE 5 - Comparative parameters for the low and high-light adapted *Prochlorococcus* ecotypes. Units: $\bar{a}^{*}_{chl}$ = m^2 mg chl a^{-1}, I_{comp}, I_{inhib}, I_m and I_b = µmol Q m^{-2} s^{-1}; α_x = fg C fg x^{-1} h^{-1} (µmol Q m^{-2} s^{-1})$^{-1}$ [x = chl a_2 or cell]; P^x_{max} = fg C fg x^{-1} h^{-1} [x = chl a_2 or cell]; ϕ_m = mol C mol quanta^{-1}. Statistical analysis for growth parameters are based on unpaired Student's t-test, and those for the pigment, absorption and P-I parameters are based on one-way, Model I ANOVA: ***$p < 0.01$, **$p < 0.05$, n.s. = not significantly different. The last column displays the relative ranges of the parameters for the low-light adapted (thin lines) and the high-light adapted (thick lines) *Prochlorococcus* ecotypes. For ease of comparison, the range limits are normalized to the high end value of the low-light adapted group. Note the scale is 0 - 1 for pigment and absorption parameters and 0 - 3 for growth and P-I parameters.

Physiological Characteristics	low-light adapted	high-light adapted	relative parameter ranges
pigment, & absorption parameters:			
total chl *b*/chl a_2 at all irradiances***	0.47-2.6	0.05-0.65	
total chl *b* at low I_g***	2.3-6.0	0.74-2.1	
chl fluor cell^{-1} at low I_g**	7.2-18	3.9-8.1	
chl fluor cell^{-1} at high $I_g$ns	3-10	2.3-3.8	
$\bar{a}^*_{ph}$, at low & high I_g***	0.022-0.029	0.012-0.018	
a_{480}/a_{450}, at low & high I_g***	0.70-0.98	0.51-0.78	
a_{450}/a_{680}, at low & high I_g***	2.5-4.1	2.3-2.9	
growth parameters:			
I_{comp}***	0.7-3.6	4.6-8	
I_{inhib}**	38-124	151-258	
P-I parameters:			
low growth irradiance			
α_{chl}***	0.085-0.125	0.04-0.055	
α_{cell}***	0.18-0.61	0.12-0.18	
P^{chl}_{max}***	1.93-2.54	1.8-2.05	
P^{cell}_{max}n.s	4.1-12	4.7-6.3	
ϕ_m***	0.09-0.127	0.080-0.096	
I_m***	65-77	110-177	
I_b***	269-465	450-1003	
high growth irradiance			
α_{chl}n.s.	0.03-0.065	0.041-0.067	
α_{cell}n.s.	0.03-0.10	0.057-0.097	
P^{chl}_{max}n.s	1.6-3.4	1.72-4.7	
P^{cell}_{max}n.s	3.0-10.4	3.6-5.1	
ϕ_m***	0.03-0.068	0.062-0.11	
I_mn.s.	162-190	142-286	
I_b**	483-579	562-1694	

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APPENDIX I

The β relationship for *Prochlorococcus*: method and instrument comparison

by

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Introduction

Quantitative determination of particulate absorption is difficult in much of the ocean due to low concentrations of particles. A common method for obtaining *in situ* absorption coefficients is to measure absorption for particles collected on a filter. One of the drawbacks to using this method is that the particulate absorption coefficient needs to be corrected for amplification of the optical pathlength due to multiple scattering by the glass-fiber filter (β effect) (Butler, 1962). Empirical algorithms (β relationship) have been derived to correct for this scattering artifact. Averaging of data from different sized phytoplankton (1 - 26 μm diameter size range) has yielded similar general β relationships (Cleveland and Weidemann, 1993; Bricaud and Stramski, 1990; Mitchell, 1990).

Recently, the β relationship obtained for three picophytoplankton, *Synechococcus* WH8103, *Prochlorococcus* SS120 and *Prochlorococcus* MED4, was found to differ from the relationship obtained for larger phytoplankton (Moore et al., 1995). The difference was more pronounced for *Prochlorococcus*, with an average cell diameter of 0.6-0.8 μm, than for *Synechococcus*, with an average cell diameter of 0.8-1.2 μm. The reasons for these differences simply could be the instrument used (single-beam spectrophotometers, Beckman models DU-7 and DU-640) or the experimental procedure, specifically, the suspension absorption was measured using an opal glass diffuser (Shibata, 1958) rather than an integrating sphere. Another possible reason may be related to the small cell size of the picoplankton, particularly *Prochlorococcus*. To test whether the method was responsible for the difference in the β relationship between the picoplankton and larger

phytoplankton, a comparison was made of the optical density (OD) of cells in suspension measured with an opal diffuser to that measured with an integrating sphere. To determine if instrument-specific difference could account for the different β relationships, absorption measurements of *Prochlorococcus* cultures were made with a dual beam spectrophotometer equipped with an integrating sphere and compared to published β relationships of *Prochlorococcus* and other phytoplankton.

Materials and Methods

Cultures

Prochlorococcus isolates SS120, MED4, MIT9312, MIT9313 and *Synechococcus* isolates WH7805 and WH8103 were grown in a modified K/10 media at 24°C, ~25 μmol Q m^{-2} s^{-1}, 14:10 LD, up to concentrations of 10^7-10^8 cells ml^{-1}. *Dunaliella tertiolecta* and *Thalassiasira weissflogii* were grown in F/2 media. Measurements were made on cultures in exponential growth phase.

Absorption measurements

A Perkin-Elmer Lambda 18 dual-beam spectrophotometer was used to measure absorption from 300-800 nm at 1 nm intervals, with slit width set to 4 nm and scan speed at 240 nm min^{-1}. For all cultures, suspension absorption was measured first using the standard optics of the instrument with the sample cuvette positioned in front of a piece of opal diffusing glass directly in front of the detector opening. An integrating sphere was then inserted into the instrument and suspension absorption was measured on the same

cultures. For filter absorption measurements, cells were filtered onto 25 mm glass-fiber filters (3.46 ml, filtered under low vacuum, <5 in. Hg), and the filters were placed on quartz discs located directly in front of the detector opening of the integrating sphere. Filtered seawater was used for blank references.

Results and Discussion

Suspension spectra for several phytoplankton species show little difference in spectral shape when measured using an opal diffuser compared to when measured using an integrating sphere (Fig. 1). Differences in magnitude between the two methods are also small, except for the two *Synechococcus* cultures which differ by as much as 35% at some wavelengths. Suspension absorption measured using an opal diffuser were noisier and nonlinear at wavelengths > 700 nm, making the null point correction more difficult. In fact, a baseline correction at $\lambda = 720$ nm works best. Measurements on three other isolates of *Prochlorococcus* (SS120, MIT9312, MIT9313; data not shown) yielded similar results as *Prochlorococcus* MED4. The regression of OD_{opal} vs $OD_{int.sph.}$ for all cultures results in a slope not significantly different from one (Fig. 2), illustrating that suspension absorption measured by either method will yield essentially the same spectra.

The β relationship (suspension OD v. filter OD) for four *Prochlorococcus* isolates (SS120, MED4, MIT9312 and MIT9313) measured on a dual beam spectrophotometer equipped with an integrating sphere results in a polynomial fit that lies essentially on top of the published curve fit for *Prochlorococcus* measured on a single beam spectrophotometer

with an opal diffuser (Fig. 3; Moore et al. 1995). Interestingly, a line fits the *Prochlorococcus* data as well as a polynomial ($r^2 = 0.967$ for a linear fit, r2 = 0.968 for a polynomial fit). Furthermore, the β relationship for *Prochlorococcus* is distinctly different from the β relationship obtained for larger phytoplankton [for comparison the curve fit published by Mitchell (1990) is plotted] (Fig. 3). Thus, the difference in the β relationship between *Prochlorococcus* and larger phytoplankton is not instrument or method dependent but appears to be related to some property of the cells. Other researchers have also found relatively low and linear β relationships, similar to those observed for *Prochlorococcus*, for small phytoplankton in the laboratory (Nelson et al., 1997; Helmut Maske, personal communication) and in the field where picoplankton numerically dominate (Bricaud and Stramski, 1990).

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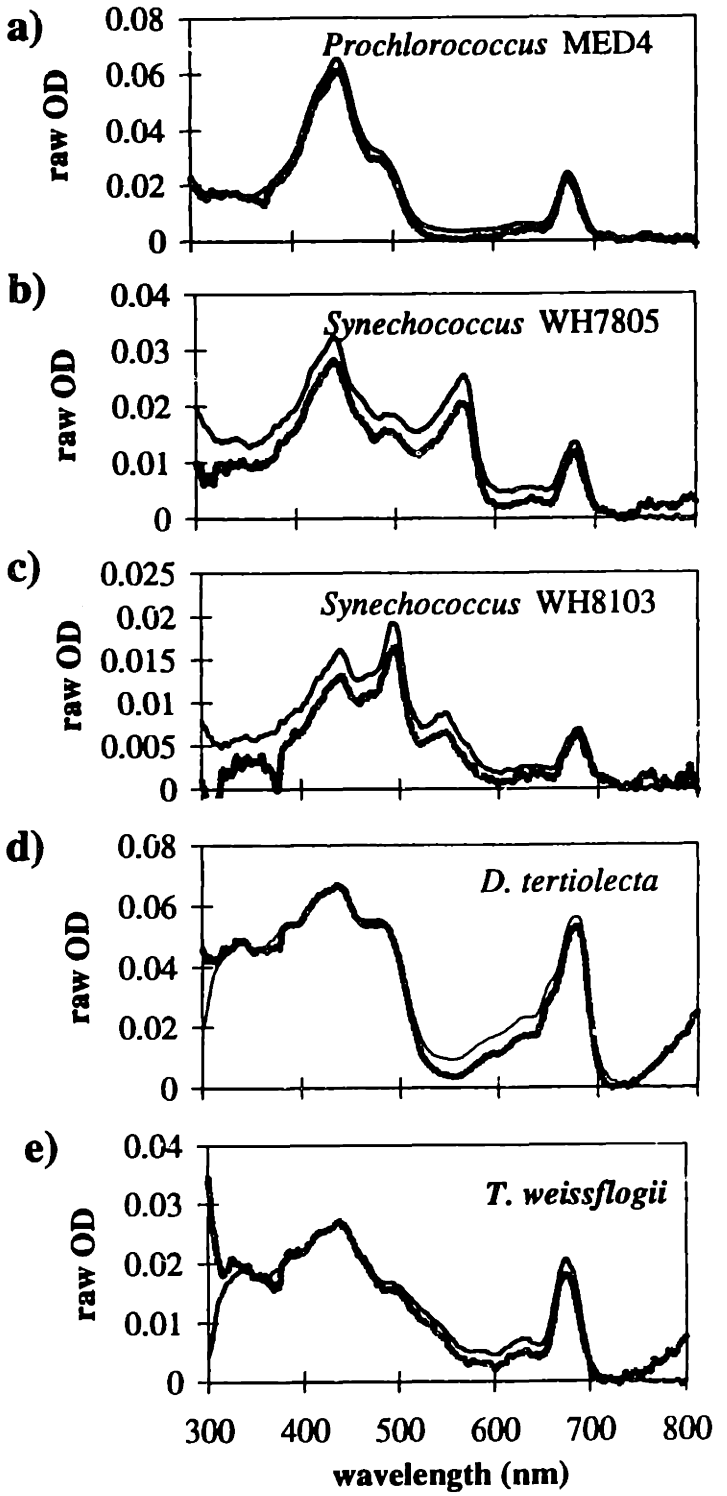

Figure 1 - Optical density (OD) spectra of phytoplankton cells in suspension measured with an integrating sphere (thin line) and with an opal glass diffuser (thick line). a) *Prochlorococcus* MED4; b) *Synechococcus* WH7805; c) *Synechococcus* WH8103; d) *D. tertiolecta*; e) *T. weissflogii*.

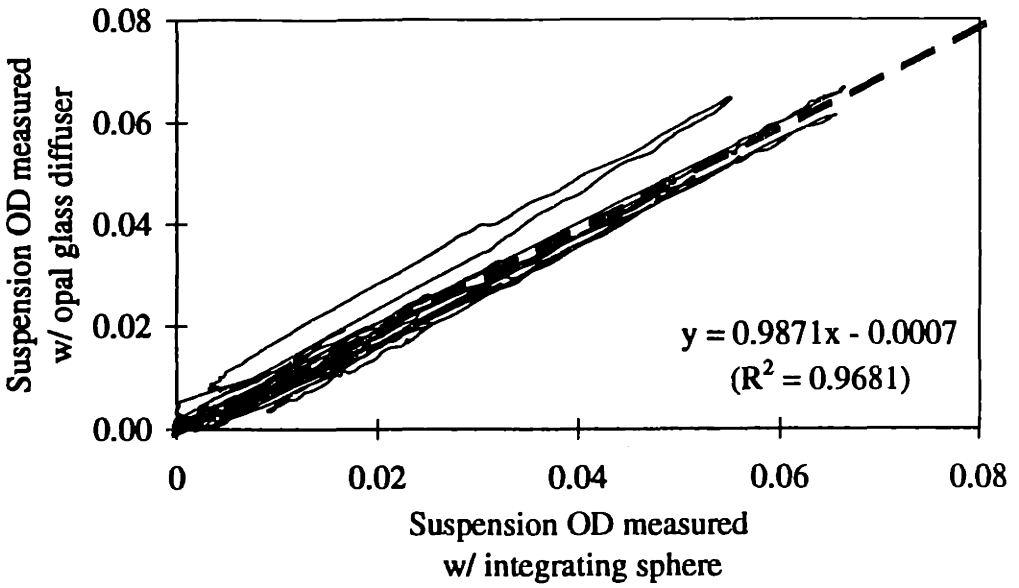

Figure 2 - Suspension OD measured with opal glass diffuser (OD_{opal}) vs suspension OD measured with an integrating sphere ($OD_{int\_sph.}$) for all 8 cultures used in this study. The linear regression (dashed line) yields a slope close to unity.

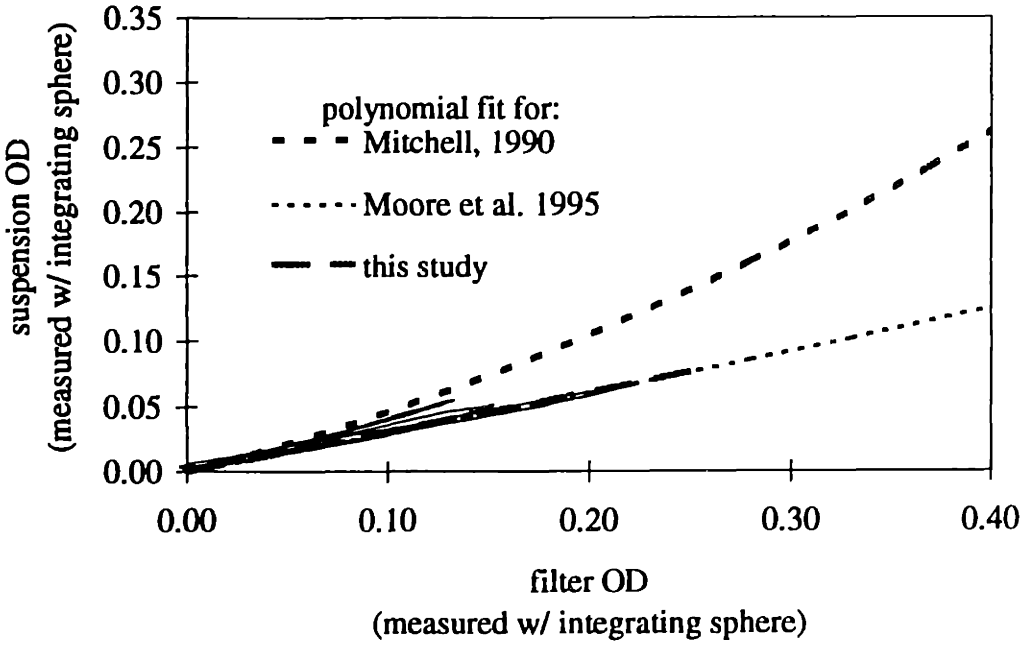

Figure 3 - Suspension OD (measured with an integrating sphere) vs filter OD for 4 strains of *Prochlorococcus* (thin solid line). The polynomial fit of this data set (thick long dashes) lies on top of the *Prochlorococcus* -specific β relationship published previously (short dashes; Moore et al., 1995). The β relationship for phytoplankton ranging in size from 2 - 20 μm (Mitchell, 1990) is shown for comparison.

APPENDIX II

The MIT *Prochlorococcus* collection:

media, maintenance, miscellaneous experiments and anecdotal observations

Introduction

Successful culturing of *Prochlorococcus* in the laboratory eluded researchers for years after they were first discovered (Johnson and Sieburth, 1979). In 1988, Brian Palenik obtained the first *Prochlorococcus* isolate, SARG from the Sargasso Sea. At first keeping that isolate alive and successfully transferring was "hit or miss", and required much concerted and dedicated effort, which included modifying the media composition, finding the best culture ware and cleaning protocols, and determining the proper light and temperature regimes. This appendix documents some of the more important procedures and observations that came out of the process of establishing the SARG and subsequent isolates. In addition, this appendix includes results which deserve mention but, unfortunately, did not fit into any of the main chapters of this dissertation. My hope is that these methods, observations and experiments serve as a starting point for further refinements and experimentation into the culturing and biology of *Prochlorococcus*.

Growth media

Several different recipes are used for successfully growing *Prochlorococcus* (Table 1). The original isolate SARG, the Mediterranean sea isolate MED, and two north Atlantic isolates NATL1 and NATL2 were obtained using the "CPTC-based" media (Table 1). This recipe was modified for the physiological studies carried out on the clonal isolates SS120 and MED4 ("PRO" media in Table 1; Moore et al., 1995), and additionally modified ("PRO2" medium; Table 1) for subsequent isolations and experiments.

Prochlorococcus have been isolated using a variety of methods, the easiest being filter

Table 1 - Composition of different media used for isolating and culturing *Prochlorococcus*

nutrient	"CPTC-based"[1]	"K/10 (-Cu)"[2]	"PRO"[3]	"PRO2"[4]
NaH_2PO_4		10 μM	10 μM	10 μM
Glycerophosphate	10 μM			
NH4Cl		50 μM	50 μM	50 μM
$(NH_2)_2CO$ (urea)	20 μM		50 μM	100 μM
trace metal mix				
EDTA[5]		11.7 μM	11.7 μM	1.17 μM
CPTC	100 μM			
$ZnCl_2$		8 nM	8 nM	8 nM
$CoCl_2$		5 nM	5 nM	5 nM
$MnCl_2$	10 nM	90 nM	90 nM	90 nM
Na_2MoO_4	10 nM	3 nM	3 nM	3 nM
Na_2SeO_3		10 nM	10 nM	10 nM
$NiCl_2$			10 nM	10 nM
$FeCl_3$	0.1 μM ($FeSO_4$)	1.17 μM	1.17 μM	1.17 μM

[1]used for original isolation of SARG, MED, NATL1 and NATL2; published in Chisholm et al. 1992
[2]used for maintaining *Prochlorococcus* until ~1990; published in Chisholm et al. 1992
[3]used for experiments published in Moore et al. 1995
[4]used for experiments presented in chapters III and IV, and for many isolations
[5]The brand of EDTA may be significant to culturing success. $Na_2EDTA\ 2H_20$ from Sigma Chemical Co (product #ED2SS) works well. Do not use Mallinckrodt brand.

fractionation of *Prochlorococcus* away from other phytoplankton through double-stacked 0.6 μm polycarbonate filters, as outlined in Chisholm et al., 1992. Since 1991, the MIT *Prochlorococcus* collection, 13 additional isolates have been obtained by using this isolation protocol as well as by flow-cytometrically sorting *Prochlorococcus* away from *Synechococcus* and other larger phytoplankton (see chapter III). Once back in the laboratory, screening and selection of the isolates until reliably survivable isolates are obtained can take months.

The PRO medium used for experiments on *Prochlorococcus* clones SS120 and MED4 was made with 11.7 μM EDTA from Sigma Chemical Co. After these experiments (~1991), a new trace metal mix (TMM) was made that mistakenly had a 10-fold lower EDTA

concentration (also made using Sigma brand EDTA); media made with this TMM is called PRO2. Before the mistake was realized, PRO2 media was used successfully for maintenance of *Prochlorococcus* stocks, obtaining new isolates and experiments on the Pacific isolates (MIT9201, MIT9202, MIT9211, MIT9215). At this point, I decided to go back to using PRO media; so the TMM for PRO media was remade, however, this time using EDTA from Mallinckrodt Chemical Co. Unfortunately, it took several weeks to months before I determined that many cultures were not growing well with PRO media made from this TMM. In fact, MIT9211 and MIT9303 were dying out in this PRO media. So, I switched back to PRO2 media (TMM made with Sigma brand EDTA at 1.17 μM), so I could obtain data for MIT9303 and compare to data obtained for MIT9302, MIT9312 and MIT9313.

Some differences in growth rate (Fig. 1) and pigment content (Fig. 2 and 3, are apparent for MIT9302, MIT9312 and MIT9313 grown in PRO media with 11.7 μM EDTA (Mallinckrodt brand) compared to PRO2 media with 1.17 μM EDTA (Sigma brand). Interestingly, differences in growth rate between these two media also has a light-dependent component. Growth in PRO media occurs at a higher rate at the lowest I_g, but the opposite is true at the higher irradiances (Fig. 1). In fact, there was no growth of MIT9313 at the higher irradiance level when grown in PRO media (Fig. 1B). Differences are also evident in the individual chlorophyll and zeaxanthin as well as the ratios of these pigments to chl a_2 (Fig. 2). For each isolate, growth in PRO2 media resulted in higher cellular concentration of chl a_2 and chl b_2 and a higher chl b_2/ a_2 ratio than when grown in PRO media (Fig. 2). Zeaxanthin and zeaxanthin/chl a_2 ratio are lower for cells grown in PRO2 media (Fig. 3A,B); however, no significant differences were observed for the cellular concentrations of α-carotene under either media (Fig. 3C,D).

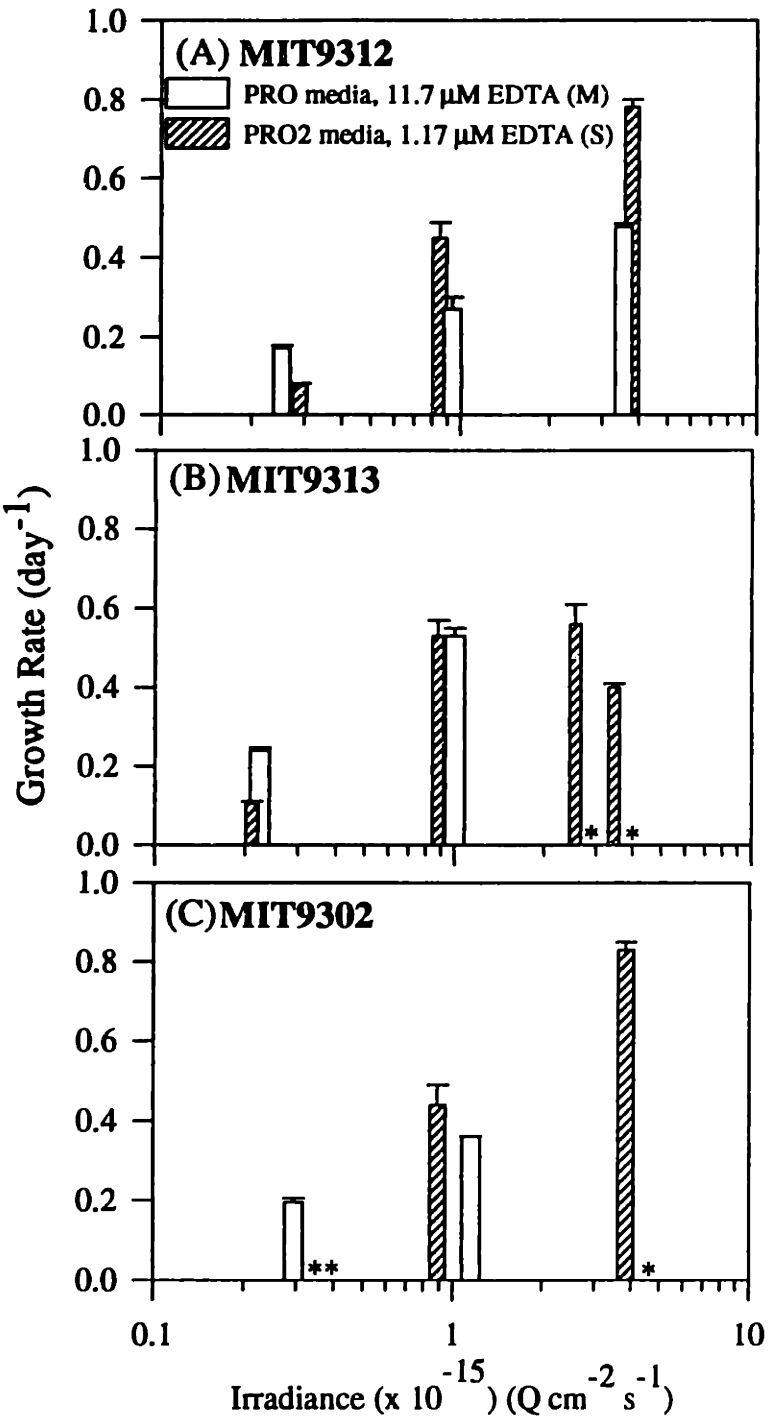

Figure 1 - Growth rate comparisons for *Prochlorococcus* isolates grown under a range of irradiance levels in two different media: PRO medium made with 11.7 µM EDTA (Mallinckrodt brand) and PRO2 medium made with 1.17 µM EDTA (Sigma brand). (A) MIT9312, (B) MIT9313, (C) MIT9302. *no growth at these light levels in PRO media; **no growth at this light level in PRO2 media.

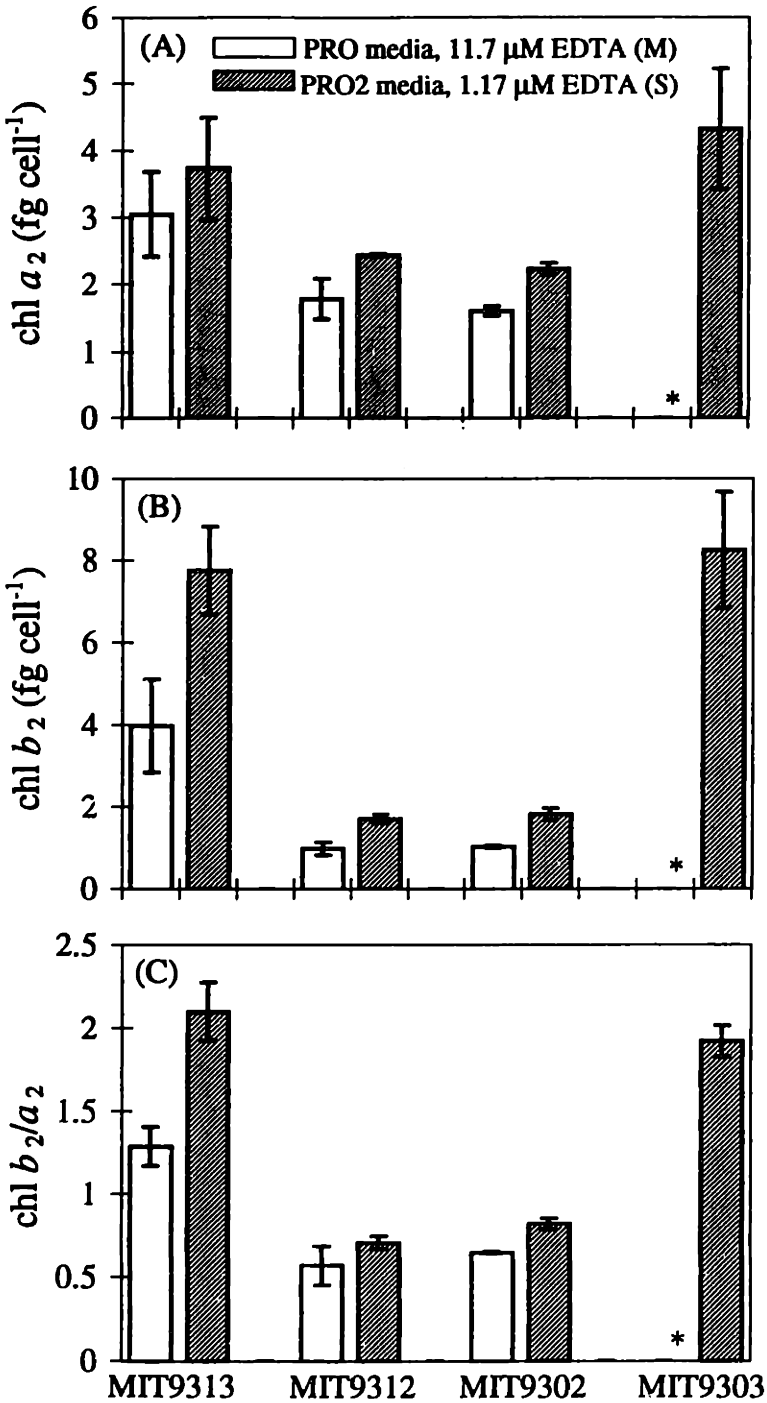

Figure 2 - Comparison of chlorophyll content for *Prochlorococcus* isolates, MIT9312, MIT9302, MIT9303 and MIT9313, grown in PRO and PRO2 media. Pigments were extracted and analyzed by HPLC as described in chapter IV. (A) chl a_2 per cell, (B) chl b_2 per cell, (C) ratio of chl b_2/ a_2. *no pigment data for MIT9303 because it could not grow in PRO media.

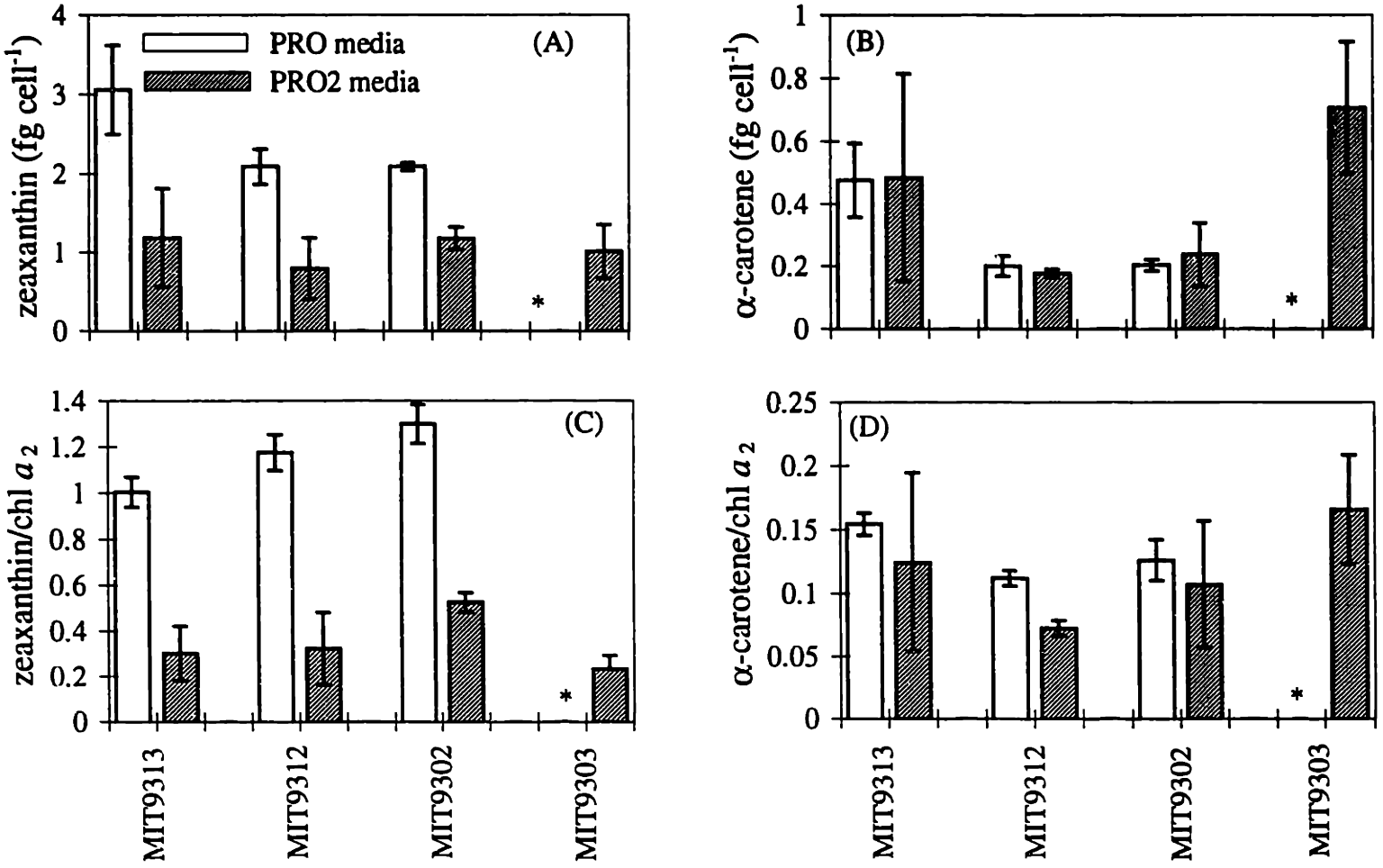

Figure 3 - Comparison of carotenoid pigment content for *Prochlorococcus* isolates, MIT9312, MIT9302, MIT9303 and MIT9313, grown in PRO and PRO2 media. Pigments were extracted and analyzed by HPLC as described in chapter IV. (A) zeaxanthin per cell, (B) α-carotene per cell, (C) ratio of zeaxanthin/chl a_2, (D) ratio of α-carotene /chl a_2. *no pigment data for MIT9303 because it could not grow in PRO media.

There are two variables in the above comparison, both of which could account for the observed differences. 1). The lower EDTA concentration in PRO2 media results in a higher concentration of free iron (Fe), as determined by trace ion composition analysis using MINEQL (Westall et al., 1976). Iron addition to a patch of water within the high nutrient/low chlorophyll regions of the equatorial Pacific, where iron is limiting, as been shown to result in increased cellular chlorophyll fluorescence of the natural *Prochlorococcus* populations (Cavender-Bares et al., 1997). The lower cellular concentration of chl a_2 in the cells grown in PRO media with lower Fe concentration implies that Fe may be limiting in this media. 2). The different *brands* of EDTA may also affect *Prochlorococcus*, possibly due to different trace contaminants. The TMM made with Sigma brand EDTA was distinctly lighter in color regardless of concentration of EDTA compared with the TMM made with the Mallinckrodt brand EDTA. When the TMMs and comparisons were originally made, the brand of EDTA was not considered a factor. Thus, additional experiments need to be done to determine which factor, concentration, brand of EDTA, or both is responsible for the observed differences in growth rate and pigment content.

A comparison of growth rates for the *Prochlorococcus* isolate SARG using different nitrogen sources indicates that it can grow on any nitrogen source, except more variability in growth rate was observed in the media that contained nitrate as the sole nitrogen source (Fig. 4). The results for media made with urea as the sole nitrogen source are questionable since urea degrades to NH_4 during autoclaving (J. Waterbury, personal communication). Growth on media made with urea as the sole nitrogen source is currently being revisited by L. Mann.

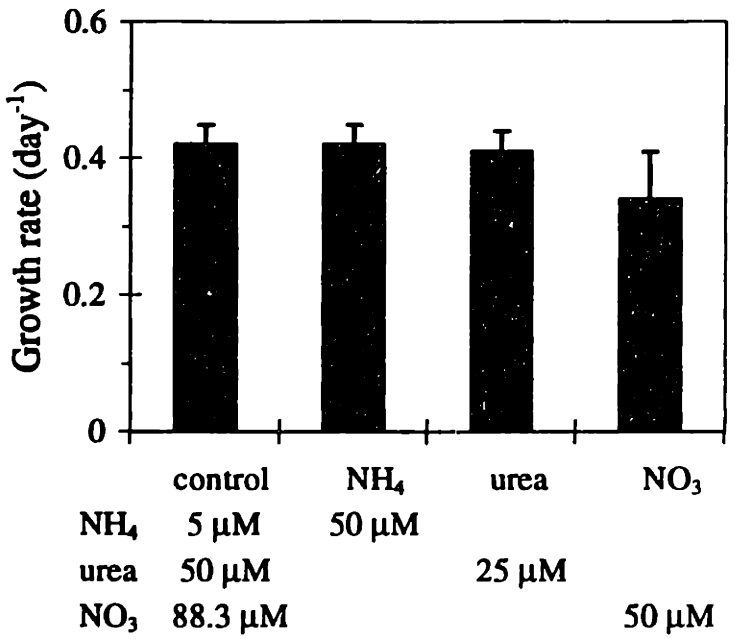

Figure 4 - Growth rates for *Prochlorococcus* isolate SARG grown in four different medias with different nitrogen compositions. The control media composition is similar to "K/10-Cu" except that it contains 1 nM Cu, uses 10 µM glycerophosphate instead of inorganic PO_4, and contains the following vitamin mixture: 0.21 nM biotin, 0.037 nM vitamin B12 and 30 nM thiamin. The control and "urea" media also had 0.2 µM Ni added to facilitate the utilization of urea by urease. The nitrogen composition and concentration (µM) in each media is indicated on the figure. Values correspond to the mean ± 1 S.E. of 8-10 replicates.

Prochlorococcus appear not to require the addition of any vitamins to the media in order to grow, though it is possible that some amount of vitamins remains in the seawater or that the bacterial contaminations provide a source of vitamins for the *Prochlorococcus* to utilize. Vitamins (0.21 nM biotin, 0.037 nM vitamin B_{12} and 30 nM thiamin) were successfully removed from the media with no difference in growth rate (data not shown) for *Prochlorococcus* isolates MED and SARG and clone SS120.

Nutrients and trace metal mix are autoclaved and stored in separate, acid-cleaned (see below) teflon bottles at 4 °C. They are added asceptically to seawater, and the sterile media is despensed asceptically to sterile cultureware and allowed to sit overnight before innoculation with cells.

Sargasso Sea water (SSW) is typically used for experimental work with *Prochlorococcus*, and coastal water off Woods Hole, MA (WHOI) is used for maintaining the stocks. Small decreases in growth rate were found for two *Prochlorococcus* isolates when grown in "PRO2" media made in WHOI water compared to the same media made in SSW (Table 2). Isolate MIT9515 also can grow in oligotrophic Pacific seawater (L. Mann, personal communication). Growth in lower salinity media is currently being attempted. To date, a few isolates (MIT9211, MIT9313, MIT9315) can grow in PRO2 media made with 90% seawater, and SS120 can grow in 75% seawater (J. Hahn & L. Mann).

Table 2 - Growth rates for SS120 and MIT9211 grown in PRO2 media made in Sargasso Sea water (SSW) or Woods Hole water (WHOI). Values are averages from 6 cultures (2 replicate cultures serially transferred 3 times) ± 1 SE of the mean. Data provided by L. Schaeder.

	SS120	MIT9211
SSW	0.411 ± 0.004	0.38 ± 0.02
WHOI	0.375 ± 0.008	0.35 ± 0.03

Maintenance of *Prochlorococcus* isolates

Twenty-eight separate isolates of *Prochlorococcus* obtained from different oceanographic regimes are maintained in the MIT culture collection (Table 3). Isolates are grown in "PRO2" media in glass test tube at 22 - 24 °C under 1 - 2 μmol Q m^{-2} s^{-1}, 14:10 L:D cycle.

Cultureware in a variety of shapes and materials have been used for successfully culturing *Prochlorococcus*: round and square bottles, flasks and tubes made out of glass, teflon, polycarbonate, or polystyrene. The glass, teflon and polycarbonate cultureware undergoes a 4-step cleaning and sterilization protocol when cultures are used for experiments: 1) overnight soak in 10% Micro detergent, tap water rinse (6X), distilled water rinse (6X), 2) overnight soak in 1N HCl, Milli-Q H_20 rinse (6X), 3) overnight soak in Milli-Q H_20, and 4) autoclave with Milli-Q H_20 in container. Steps 2 and 3 are eliminated for cultureware used for maintaining stocks, since no significant difference was seen in growth rates (data not shown). The polystyrene cultureware is disposable and comes pre-sterilized and does not undergo additional cleaning. It has often been observed that transfer from one type of container to another is accompanied by a lag in growth, especially when going into polysytrene cultureware.

Storage in liquid nitrogen

Prochlorococcus can be stored in liquid nitrogen (LN2) for future revival and growth (J. Hahn, personal communication), using a protocol similar to that used for marine *Synechococcus* (J. Waterbury, personal communication). To freeze, mix 0.5 ml of a dense

Table 3 - Isolation history of isolates in the MIT *Prochlorococcus* collection.

ISOLATE NAME	COORDINATES	DEPTH	ISOLATOR	DATE ISOLATED	ISOLATION METHOD	OTHER NAMES
Sargasso Sea						
SARG[a]	28° 59'N; 64° 21'W	120 m	B. Palenik	30-May-88	filtered	LG
SSW5[a]	"	"	"	"	"	
SS120[b]	"	"	"	"	"	CCMP-1375
SS35[b]	"	"	"	"	"	CCMP-1428
SS51[b]	"	"	"	"	"	CCMP-1376
SS52[b]	"	"	"	"	"	CCMP-1377
SS2[b]	"	"	"	"	"	CCMP-1427
MIT9301	34° 10.1'N; 66° 18.2'W	90 m	L. R. Moore	10-Jul-93	filtered	
MIT9302[c]	34° 45.5'N; 66° 11.1'W	100 m	L. R. Moore	15-Jul-93	sorted on fcm	
MIT9303[c]	"	"	"	"	"	
MIT9401	station 14	surface	L. Aref	CH0694	filtered	14S2c1
Gulf Stream						
MIT9311[d]	37° 30.8'N; 68° 14,4'W	135 m	L. R. Moore	17-Jul-93	sorted on fcm	
MIT9312[d]	"	"	"	"	"	
MIT9313[d]	"	"	"	"	"	
MIT9314	"	180 m	"	"	filtered	
North Atlantic						
NATL2A	38° 59'N; 49° 33'W	10 m	F. Partensky	Apr-90	filtered	FP5
NATL1	37° 39'N; 40° 1'W	30 m	"	"	filtered	FP12
Mediterranean Sea						
MED[a]	43° 12'N; 6° 52'E	5 m	D. Vaulot & F. Partensky	Jan-89	filtered	DV
MED4[b]	"	"	"	"	"	CCMP-1378
MED1[b]	"	"	"	"	"	CCMP-1426
South Pacific						
MIT9107[e]	14° 60'S; 134° 60'W	25 m	J. Dusenberry	8-Aug-91	filtered	SPac7
MIT9116[e]	"	"	"	"	"	SPac16
MIT9123[e]	"	"	"	"	"	SPac23
MIT9201	11° 60'S; 145° 25'W	surface	B. Binder	26-Sep-92	filtered	A16
MIT9202	"	79 m	"	"	filtered	B10
Equatorial Pacific						
MIT9211	0°; 140°W	1% Io (~83 m)	R. Olson	10-Apr-92	filtered	TT8-11
MIT9215	"	surface	B. Binder	3-Oct-92	filtered	C15
MIT9321	1°N; 92°W	50 m	P. Chisholm	12-Nov-93	filtered	
MIT9322	0° 16'N; 93°W	surface	P. Chisholm	16-Nov-93	filtered	
MIT9515	5° 44.9'S; 107°5.25W	15 m	E. Mann	4-Jun-95	filtered	

*a*Different names for the same isolate in the literature; *b*Clonal cultures, obtained by serial dilution of the isolate; *c, d, e*These are separate subcultures from the same field sample.

culture and 0.5 ml of 20% (v/v) dimethyl sulfoxide (DMSO, made in sterile media), dispense into cryovials and immediately put into LN2. To revive, thaw cryovial rapidly in 37 °C water bath just until melted, transfer to sterile eppendorf tube and centrifuge (10,000 rpm, 8 min, 4 °C). Pour off supernatent to remove DMSO, resuspend pellet in 1 ml fresh, sterile media, then transfer to sterile culture tube containing 3 ml fresh, sterile media. Leave tubes at low light for 24 hr before increasing light level. Culture can take about 1 month to grow to a measurable density. Storage in LN2 for a period of weeks has only been tested on a few isolates. Longer storage and revivability of all isolates need to be examined.

Cloning

Clonal *Prochlorococcus* cultures were obtained by serial dilution (with the help of S. Frankel). Exponentially growing cells were diluted down to a concentration of ≤ 0.1 cell per tube and dispensed into > 100 test tubes, so that the probability of being clonal (i.e. growth from a single cell) was > 95%, based on the Poisson probability distribution. After 4-6 weeks, the culture tubes were screened for cells using a FACScan flow cytometer. The *Prochlorococcus* isolate SARG yielded four clones, SS35, SS51, SS52 and SS120 (95.3% probability of being clonal) and the Mediterranean Sea isolate yielded two clones, MED1 and MED4 (97.3% probability). An additional clone, SS2, was obtained from recloning SS120 and has a 96.7% probability of being clonal. None of the isolates are axenic (i.e. all are contaminated with bacteria, presumably heterotrophs), despite the fact that the ratio of *Prochlorococcus* cells to other bacteria (as determined from the difference between microscopic counts of total DAPI-stained cells and flow cytometer counts of

Prochlorococcus) was 1.6 for MED and 1.9 for SS120 before the serial dilution. The ratio of *Prochlorococcus* cells to other bacteria was not determined for SARG before the serial dilution was carried out. The seven clonal isolates (indicated by CCMP# in Table 3) have been deposited at the Center for the Culture of Marine Phytoplankton, Bigelow Laboratory for Ocean Sciences (West Boothbay Harbor, MA).

Flow cytometric signatures

In the field, the presence of *Prochlorococcus* populations is usually determined by flow cytometric (FCM) observation of the characteristic *Prochlorococcus* signature, i.e. no orange fluorescence and low red fluorescence and scatter signals compared to *Synechococcus* (Chisholm et al., 1988). However, there is variability in FCM signatures between *Prochlorococcus* isolates when grown under nearly identical conditions, due to phenotypic differences between the isolates, not light or nutrient-dependent changes.

Seventeen of the twenty-eight *Prochlorococcus* isolates in the MIT *Prochlorococcus* collection have the characteristic FCM signature described above (Table 4, Fig. 5A). The other eleven isolates exhibit measureable orange fluorescence (OFL) and vary in the other FCM parameters (Table 4, Fig. 5B, C). This orange fluorescence may be indicative of the presence of phycoerythrin (as measured in SS120, see Hess et al., 1996) and/or the result of high red fluorescence passing through the orange fluorescence filters to the detectors on the FACScan (this is also discussed in chapter IV). Isolates MIT9303 and MIT9313 have the highest values of FCM parameters reported for *Prochlorococcus* (need to compare to *Synechococcus* & picoeuks) (chapter III and IV; Fig. 5C). Cells like these could possibly be

Table 4 - Flow cytometric signature characteristics of isolates in the MIT *Prochlorococcus* collection.

no orange fluorescence	low orange fluorescence	
low RFL, low FALS and SSC	medium/high RFL, low FALS and SSC	high RFL, high FALS and SSC
MED	SARG	MIT9303
MED1	SS120	MIT9313
MED4	SS35	
MIT9107	SS51	
MIT9116	SS52	
MIT9123[a]	SS2	
MIT9201	MIT9211	
MIT9202	NATL2A[b]	
MIT9215	NATL1[b]	
MIT9301		
MIT9302		
MIT9311		
MIT9312		
MIT9314		
MIT9321		
MIT9322		
MIT9401		

*a*this isolate has apparent low orange fluorescence characteristics due to clumping of cells. *b*these two isolates have FCM parameters intermediate between the two low orange fluorescence groups, however, no other physiology has been measured for these.

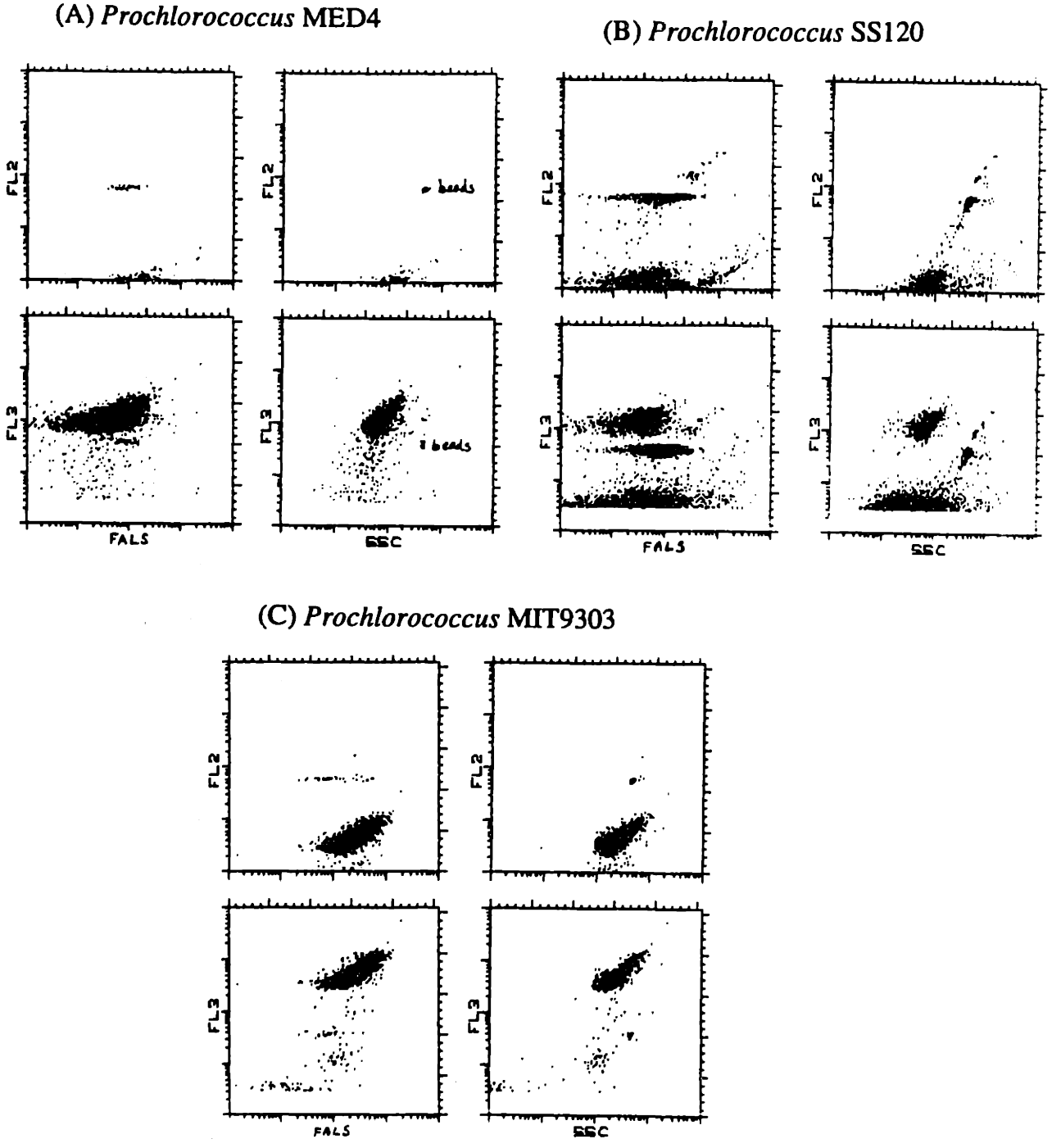

Figure 5 - Flow cytometric signature of three *Prochlorococcus* isolates. (A) MED4, typical for isolates that have no orange fluorescence (OFL); (B) SS120, typical for isolates with low OFL; (C) MIT9303, typical for this isolate and MIT9313 with low OFL, separate from the baseline, and high RFL and FALS. Panels for each culture are from left to right, top to bottom: OFL vs. forward angle light scatter (FALS); OFL vs. side scatter (SSC); red fluorescence (RFL) vs. FALS; RFL vs. SSC. Beads are 0.474 μm yellow-green, fluorescent bead standards (Polysciences, Inc., Warrington, PA). The cloud of signals at the bottom RFL panels for isolate SS120 are mostly cells that have lost measureable fluorescence and are most likely dying.

mistaken for a low phycoerythrin *Synechococcus* in a natural population. One isolate, MIT9123, has a FCM signature that extends over two orders of magnitude in RFL, SSC and FALS and exhibits some orange fluorescence (data not shown). However, this FCM signature is actually a result of cells that clump together based on two observations: 1) a layer and/or pellet of cells at the bottom of the test tube after a day or two in culture, and 2) the FCM signature becomes more like the "no orange fluorescence" signature after the cells are sonicated to break up the clumps (data not shown).

Forward angle light scatter

The forward angle light scattering (FALS) of 10 isolates was examined as a function of growth irradiance in conjunction with the light-dependent growth and pigment measurements presented in chapters II-IV. All of the isolates exhibit fairly constant FALS over the range of growth irradiances measured, and all but MIT9313 exhibited slight increases at the highest growth irradiances (Fig. 6). Two low-light adapted isolates, MIT9303 and MIT9313, exhibited significantly higher FALS, averaging around 1.2, than the other 8 isolates, with FALS varying between 0.1 - 0.6. Such a high FALS implies that these two isolates are larger than the other *Prochlorococcus* isolates. Size determinations by another method, such as measurements from transmission electron micrographs, will verify any differences in size between isolates.

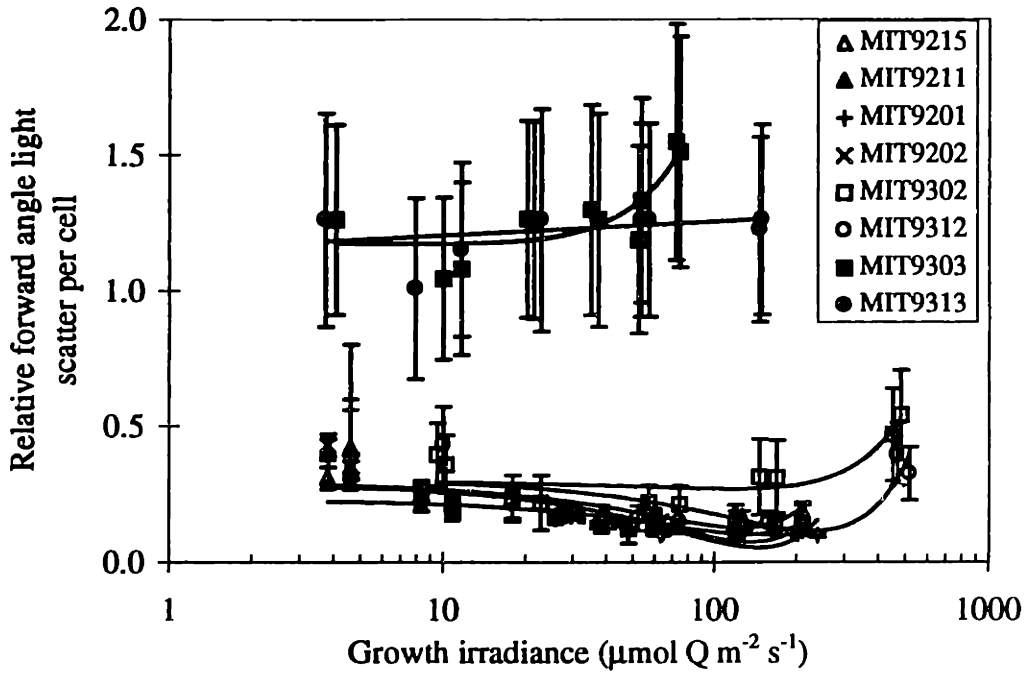

Figure 6 - Forward angle light scatter per cell for 10 *Prochlorococcus* isolates, relative to 0.474 µm standard bead scatter.

Population cycling in culture

Single cultures of SARG have been observed to undergo "cycling" of the population, i.e. the culture reaches some maximum cell density, decreases in abundance below detectable levels, and then repeats this cycle. Cycling was also detected by the culture alternating between a green color (when the cell concentration is maximal) and no color (when cells are undetectable). Two cultures of SARG growing in two different media recipes were followed through at least 3 cycles over the course of 7 months (Fig. 7). Cycling has also been observed (though not documented) in other isolates within the MIT *Prochlorococcus* collection. The reasons behind cycling of the *Prochlorococcus* population in a single culture have not been explored, though a couple possible explanations come to mind. One obvious possibility (and the most likely) is that the culture reaches its carrying capacity in terms of *Prochlorococcus* abundance, causing the majority of the cells to die; then the contaminating heterotrophic bacteria recycle the nutrients, allowing the remaining *Prochlorococcus* cells to grow. Another possibility is that viruses are playing some role.

Temperature optima and range

Natural populations of *Prochlorococcus* are present in surface waters of the equatorial and western Pacific where temperatures can be as high as 29 °C (R. Olson, B. Binder, J. Dusenberry, M. DuRand and E. Zettler, unpublished observations). The clonal *Prochlorococcus* isolates SS120 and MED4, however, could not grow at temperatures at or greater than 28 °C. The temperature range for growth for two *Prochlorococcus* isolates from the equatorial Pacific, MIT9211 isolated from deep euphotic zone (~83 m) and MIT9215

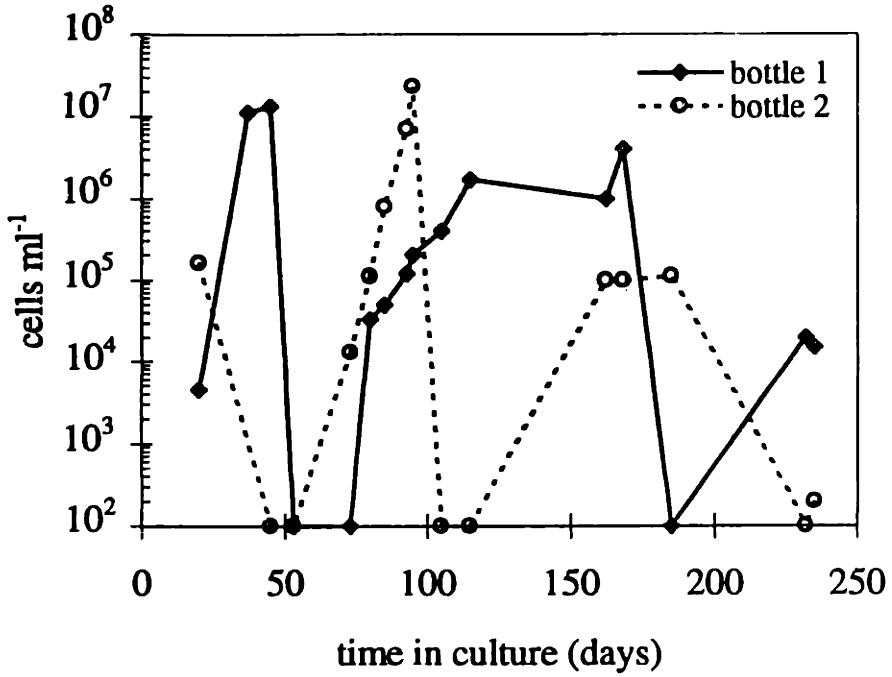

Figure 7 - Cell concentration changes in two cultures of *Prochlorococcus* isolate SARG followed over the course of 7 months. These two cultures were maintained in polycarbonate bottles (500 ml) at 21.5 C under 10:14 LD cycle at ~15 μmol Q m^{-2} s^{-1}. Bottle 1 is a culture grown in media with the same TMM as PRO, 10 μM glycerophosphate, 20 μM urea and 10 nM of Ni; bottle 2 is a culture grown in media with the control media described in figure 1.

isolated from the surface, was measured to determine whether the temperature maxima found for SS120 and MED4 applies to these isolates (this was work performed by an MIT undergraduate, J. Goodman, under my supervision). In addition, the temperature dependent growth was examined for a *Synechococcus* isolate, C20SYN, cultured from the same water sample from which *Prochlorococcus* isolate MIT9215 originated. The temperature maximum (T_{max}) for all three cyanobacteria is 25.5 °C (Fig. 8), only 1 °C higher than that observed for *Prochlorococcus* SS120 and MED4 (Moore et al., 1995). The temperature range differed for each of the cyanobacteria: 16.5 - 29.5 °C for *Prochlorococcus* MIT9211, 14 - 31.5 °C for *Prochlorococcus* MIT9215, and 12 - 29.5 °C for *Synechococcus* C20SYN. These results show that the T_{max} at which *Prochlorococcus* isolates can grow is variable, and that the highest T_{max} is associated with the isolate obtained from a location that reaches high *in situ* temperatures.

Pigment content and photoacclimative changes

The chlorophyll pigment content and light-dependent changes for *Prochlorococcus* isolates are reported in chapters II - IV of this dissertation. [The method used for extracting the pigments (adapted from Goericke and Repeta, 1993) is outlined in table 5.] However, only the cellular zeaxanthin content and zeaxanthin/chl a_2 ratio for SS120 and MED4 have been reported (Moore et al., 1995). Here, I present and compare the zeaxanthin and α-carotene pigment changes for the other *Prochlorococcus* isolates whose physiology was examined for this dissertation.

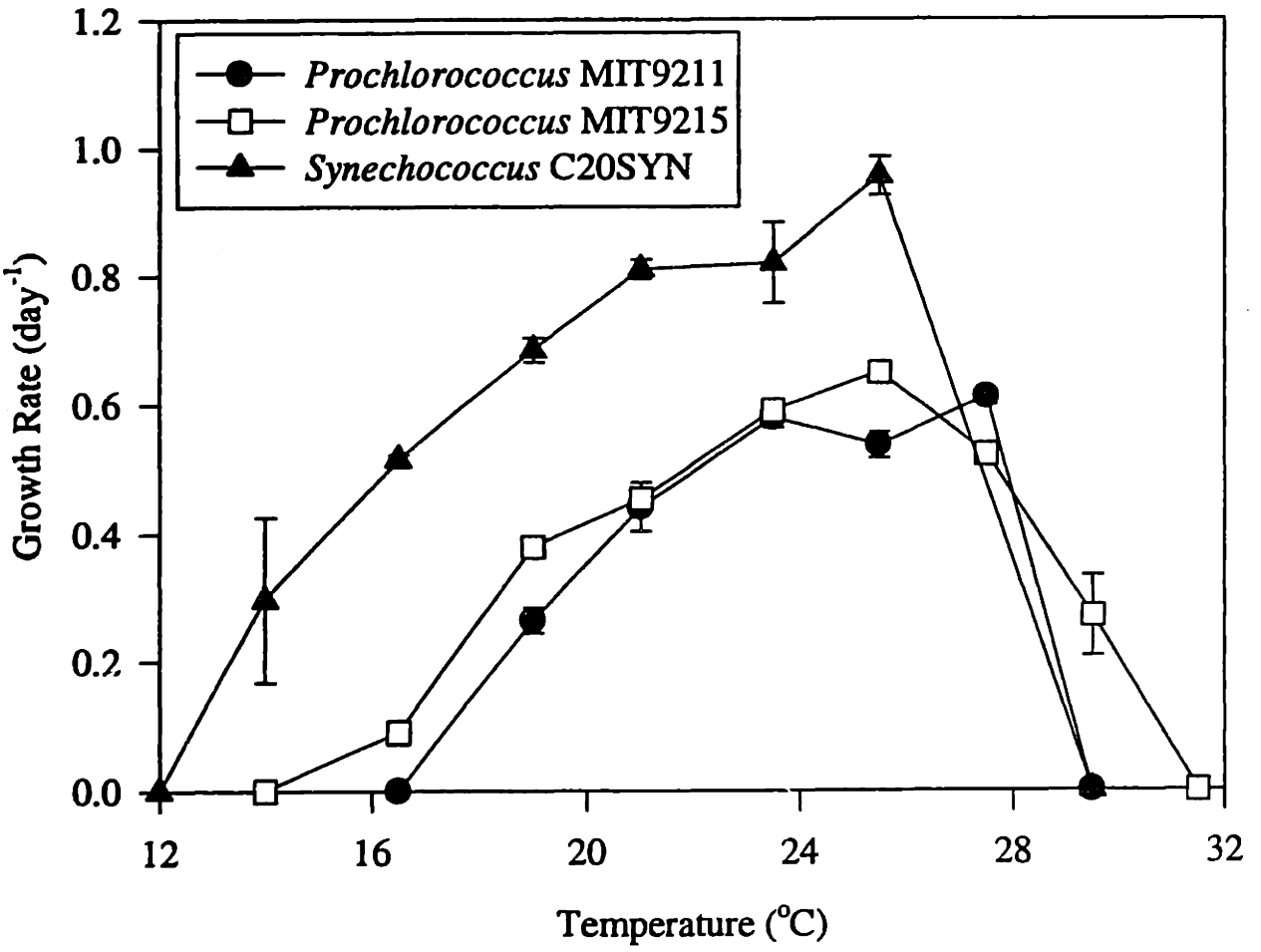

Figure 8 - Growth rate as a function of temperature for three marine picophytoplankton, *Prochlorococcus* MIT9211 (solid circle), *Prochlorococcus* MIT9215 (open square) and *Synechococcus* C20SYN (solid triangle). These cultures were grown at 83 μmol Q m^{-2} s^{-1} under a 14:10 light:dark cycle. Error bars are 1 S.E. from the mean of 2 - 4 cultures. Figure taken from Goodman, 1995.

Table 5 - Pigment extraction method for *Prochlorococcus*

Method Outline

30min extract

- filter cells under low vacuum (<5 in Hg) onto GF/F
- immediately put filter into 90% acetone (3ml) [or cryovial and freeze] in centrifuge tube
- let extract in dark in refrigerator for 30min
- close blinds and dim lights

grinding

- transfer acetone and filter to grinding tube, push filter to bottom
- keep on ice except during grinding
- grind at speed of 45-50, insert pestle (serrated, teflon) to bottom of tube 3 times
- filter should be ground into tiny pieces
- rinse pestle with 90% acetone (from squirt bottle) directly into grinding tube, remove any filter fragments caught in pestle serrations using forceps
- transfer ground filter and extract back to centrifuge tube
- rinse out grinding tube with more 90% acetone (from squirt bottle) and add to rest of extract
- keep <u>total</u> volume of extract as close to 10ml as possible and write down volume

30min extract

- allow to extract in dark in refrigerator for another 30min
- centrifuge to pellet filter fragments (Beckman table-top, speed=10, 3min)
- transfer supernatent to another centrifuge tube and put on ice until measuring its absorption
- extract rest of pigment from pellet: add 90% acetone (4ml total), resuspend pellet, let extract for 30min in dark in refrigerator, centrifuge, transfer supernatent

- measure absorption at 647nm, 664nm and 750nm, using 90% acetone as blank for both the main extract and extract from the pellet (about 10% of total)

<u>notes:</u>

- keep extracts cool and dark (on ice or in fridge, and covered with foil)
- keep lights dim and close curtains (or blinds)
- minimize exposure to acetone, both breathing and skin contact
- wear gloves, lab coat and safety glasses
- keep acetone in hood as much as possible
- cannot use polystyrene, acetone will dissolve
- grinding should take about 1min to avoid heating up extract
- grind filter completely

Cellular concentrations of zeaxanthin vary approximately 2-fold in most of the isolates. The four Pacific isolates had comparable concentrations, ranging from 0.8 - 1.8 fg cell^{-1} (Fig. 9A), similar to the concentration range measured for MED4 (Moore et al., 1995). The concentration of zeaxanthin in the high light adapted Atlantic isolates, MIT9302 and MIT9312, ranged from 3 - 7 fg cell^{-1}, with the lowest values reached when cells were at or near their μ_{max}. This concentration of zeaxanthin is about 3.8 times higher than that found in the Pacific isolates (Fig. 9A) and higher than the average cellular concentrations reported for *Synechococcus* WH8103 (Moore et al., 1995) and WH7803 (Kana and Glibert, 1987).

The low-light adapted isolates from the Atlantic, MIT9303 and MIT9313, showed more unusual patterns cellular zeaxanthin photoacclimation. Both of these isolates increased their zeaxanthin per cell to very high levels, up to 15 fg cell^{-1} for MIT9313 at the highest I_g (Fig. 9A). Additionally, these two isolates contain an unidentified pigment as indicated by a peak that elutes from the HPLC system 0.71 min. after the zeaxanthin peak (Fig. 10). Preliminary results from mass spectrometry analysis indicates that this pigment may be a 5,8 (or 5',8') epoxide of isocryptoxanthin (R. Goericke, personal communication), though more detailed work is underway to verify this. The peak height from this unknown carotenoid is inversely proportional to the zeaxanthin peak heights. Only MIT9313 showed a clear pattern of photoacclimation for this unknown pigment, with the peak for the unknown pigment surpassing that of zeaxanthin at the lowest I_g (Fig. 10). A pattern for this pigment in MIT9303 was difficult to determine, but appears to be opposite to that of zeaxanthin; none was detected in MIT9303 at high I_g (75 μmol Q m^{-2} s^{-1}). This unusual pigment has been observed in deep euphotic zone of the Sargasso Sea and Arabian Sea associated with the

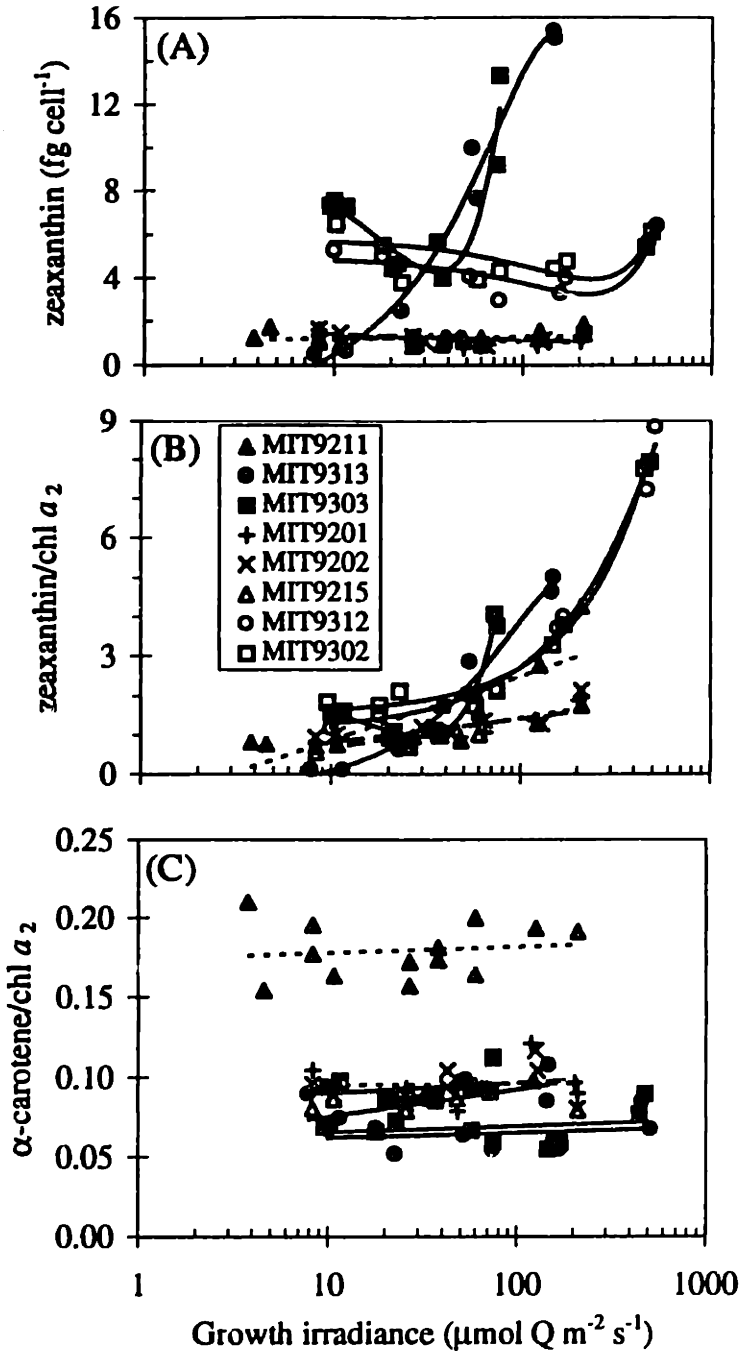

Figure 9 - Carotenoid pigments as a function of growth irradiance for *Prochlorococcus* isolates from the Pacific Ocean, MIT9201, MIT9202, MIT9215, MIT9211, and the coexisting isolates from the Atlantic Ocean, MIT9302, MIT9312, MIT9303, MIT9313. These isolates were grown and pigments analyzed by HPLC as decribed in chapter IV. (A) zeaxanthin per cell, (B) ratio of zeaxanthin/chl a_2, (C) ratio of α-carotene /chl a_2.

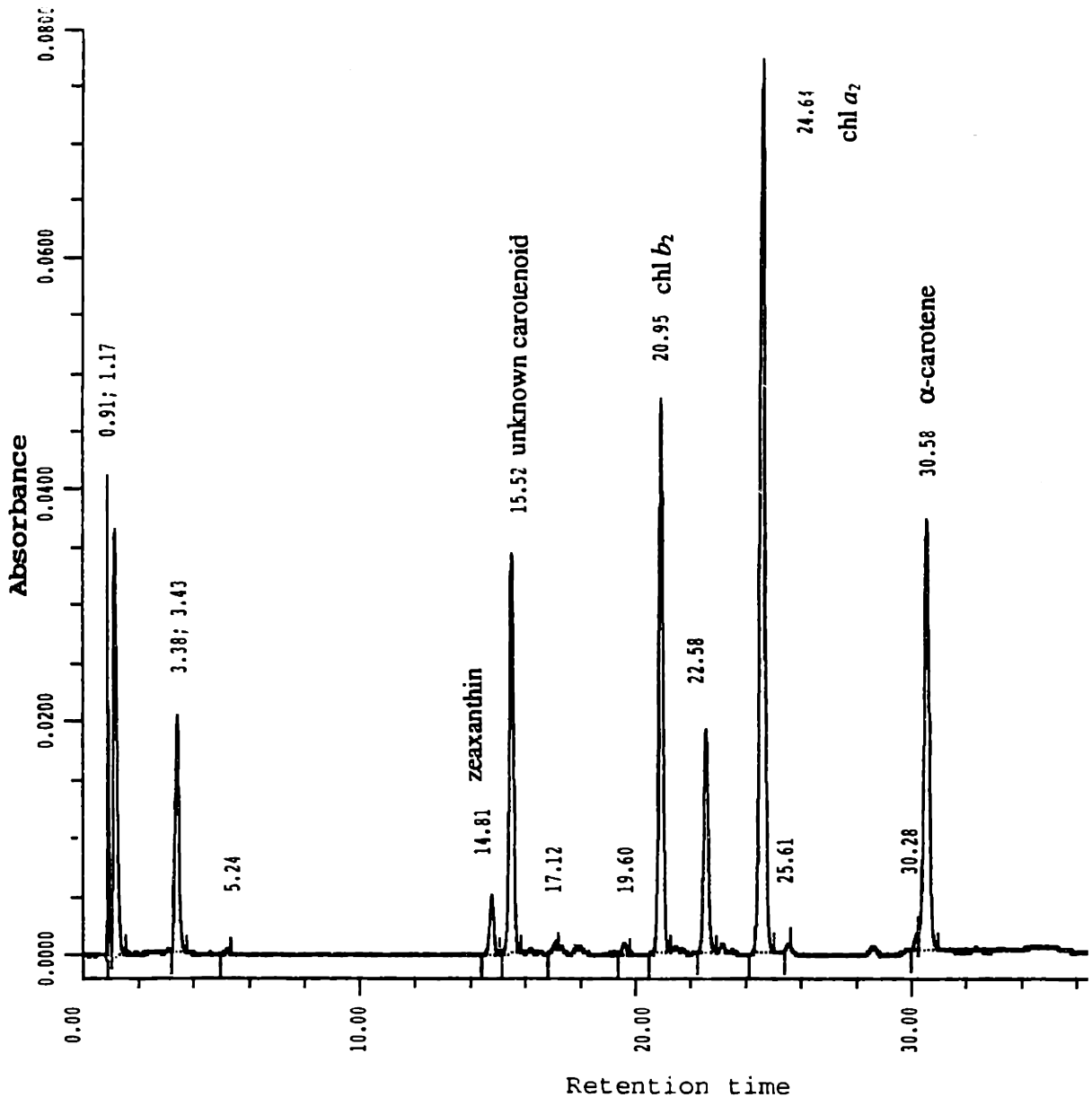

Figure 10 - HPLC chromatogram of a pigment extract from the low-light adapted *Prochlorococcus* isolate MIT9313 grown at 7.5 µmol Q m^{-2} s^{-1} (see chapter IV for details of the HPLC method). Absorbance is detected at 440 nm. The peaks corresponding to major pigments are indicated. Note that at this light level, the peak corresponding to an unknown pigment (t_r = 15.52) is much larger than the peak corresponding to zeaxanthin (t_r = 14.81).

Prochlorococcus population (R. Goericke, unpublished observations). The physiological significance of this novel pigment awaits identification and further study.

The ratio of zeaxanthin to chl a_2 increases with increasing I_g for all *Prochlorococcus* isolates (Fig. 9B). At high irradiances, the ratio for the Atlantic isolates far exceeds those of the other isolates due both to low chl a_2 concentration and high zeaxanthin concentrations. The high-light adapted Pacific isolates photoacclimate their zeaxanthin/chl a_2 ratio to a similar extent as MED4 (Moore et al., 1995).

The cellular concentration of α-carotene covaried with chl a_2 in all the isolates resulting in a constant ratio of α-carotene to chl a_2 over the entire range of photon flux densities (Fig. 9C). [The HPLC method used for this study does not separate α-carotene from β-carotene, however, we assumed only α-carotene is present in these isolates, consistent with findings for SS120 (Goericke and Repeta, 1992)]. No pattern in α-carotene per cell between low and high-light adapted ecotypes is evident. The α-carotene to chl a_2 ratio for MIT9211 (0.18 ± 0.02) fell halfway between the values reported SS120 and MED4 (Moore et al., 1995). Isolates, MIT9201, MIT9202, MIT9215, MIT9303 and MIT9313 have similar ratios (ranging from 0.087 to 0.100) and MIT9302 and MIT9312 have slightly lower ratios (0.06 to 0.07 (Fig. 9C).

Photosynthesis

To assure linearity over the incubation period used for the photosynthesis-irradiance (P-I) experiments presented in chapter 4, the experimental incubation time was determined by comparing the photosynthetic rate over several incubation times for six *Prochlorococcus*

isolates MIT9302, MIT9312, MIT9303, MIT9313, MIT9211, and MIT9215. The vials for each culture were incubated under a particular light level, not necessarily the same between isolates. All six of these isolates exhibit a constant rate of photosynthesis between 15 - 120 min (Fig. 11). The method outlined in table 6 was followed for the P-I measurements presented in chapter 4.

Carbon content

A rough estimate of cellular carbon content (C $cell^{-1}$) of *Prochlorococcus* isolates can be made from P-I measurements used in chapter IV. Multiplying the photosynthetic rate corresponding to the growth irradiance, P^{cell}_i by 14 hrs of light and dividing by the growth rate obtained for each culture (since respiration is not corrected for, the calculated C $cell^{-1}$ may be an overestimate, the extent of which is unknown) we found C $cell^{-1}$ values for most of the isolates similar to C $cell^{-1}$ estimates of natural *Prochlorococcus* populations (53 - 96 fg C $cell^{-1}$, see Veldhuis et al., 1997; Campbell et al., 1994; Li, 1994). Two Pacific isolates, MIT9211 and MIT9215 and one Atlantic isolate, MIT9312, averaged 61 ± 7 fg C $cell^{-1}$; the Mediterranean Sea isolate, MED4, and two Atlantic isolates, SS120 and MIT9302, averaged 94 ± 17 fg C $cell^{-1}$. However, two of the low-light adapted isolates, MIT9303 and MIT9313, had a surprisingly high cellular C content of 250 ± 56 fg C $cell^{-1}$, 2.7 - 4 times higher than the other isolates, and similar to C $cell^{-1}$ estimates of *Synechococcus* (Li, 1994). The higher C $cell^{-1}$for these two isolates is possibly a result of size, since C $cell^{-1}$ correlates strongly with flow cytometric FALS ($r^2 = 0.989$), considered a proxy for cell size (Fig. 12). These results point to the possibility that depth-integrated primary production due to *Prochlorococcus*,

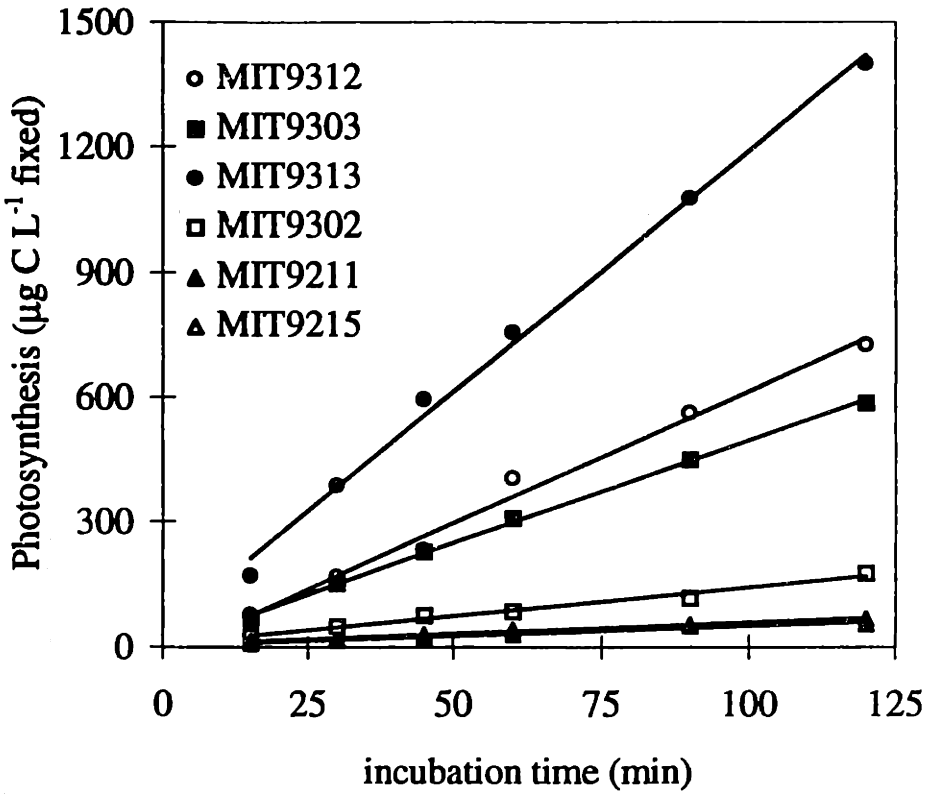

Figure 11 - Photosynthesis as a function of time for six *Prochlorococcus* isolates. The lines correspond to linear regressions of the data.

Table 6 - Experimental outline for photosynthesis-irradiance (P-I) measurements on *Prochlorococcus* using ^{14}C-bicarbonate. T_0 = initials @ t = 0 min; T_A = total specific activity; T_d dark controls.

Starting P-I experiment:

- put 19 sample vials in temperature-controlled, aluminum incubating bar (lights off)
- put 5 T_0 vials, 5 T_A vials, 5 T_d vials (covered w/ foil) in scint. vial box
- spike culture with ^{14}C bicarbonate ($NaH^{14}CO_4$, 10 µCi per ml; add 350µl per 35ml culture), mix by pipetting
- dispense 1ml into sample vials, T_0 vials and T_d vials using a repeat pipettor
- dispense 300ml into T_A vials
- cover T_0 vials, T_A vials, and T_d vials with foil
- turn on lights in incubator and start timer for 45 min
- immediately go to radioactive chemical hood with box of T_0 vials, T_A vials, and T_d vials
- add 100µl 2N HCl to T_0 vials and swirl to mix
- add 8.7ml cocktail (ScintiSafe 50 Plus from VWR) to T_A vials, cap & shake vigorously, cover with foil

During P-I experiment:

- count cells per ml of culture using FACScan
- preserve 1.2 ml of culture with 25% glutaraldehyde (0.1% final concentration) and store in LN2
- measure *in vivo* absorption spectrum (3ml) using opal diffuser and special cuvette holder
- measure *in vivo* fluorescence excitation and emission?
- filter 20 ml through GF/F filter, put into cryovial & freeze for HPLC analysis
- filter 20 ml through GF/F filter, put into 5 ml 90% acetone, cover with foil and extract in fridge overnight.

Ending P-IE experiment:

- turn off lights in incubator
- transfer vials from incubator to scint. vial box, cover with foil
- go to radioactive chemical hood with box
- add 100µl 2N HCl to sample vials, T_0 vials, and T_d vials
- put all vials on shaker table, cover with foil, set to low speed
- let shake 2 hr
- add 8ml cocktail to sample vials, T_0 vials, and T_d vials, cap & shake vigorously
- can count on liquid scintillation counter immediately

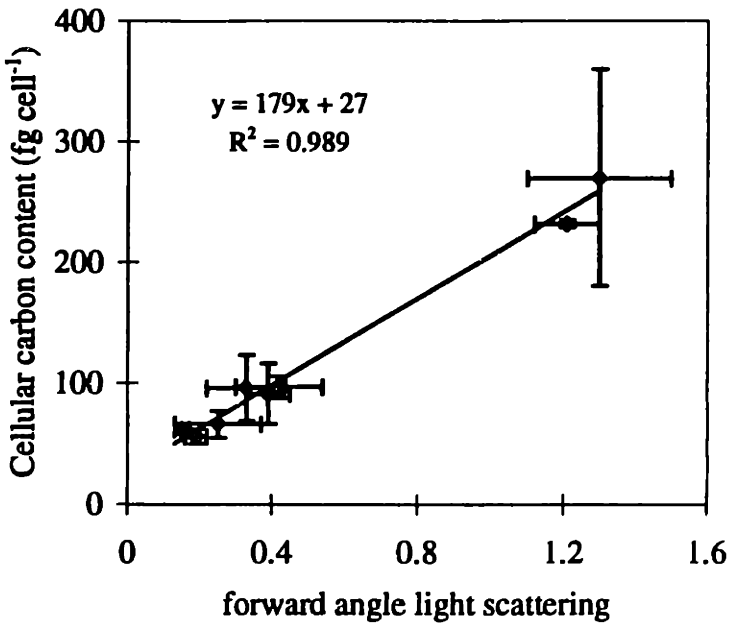

Figure 12 - The relationship between cellular carbon content and forward angle light scatter (FALS) for 8 *Prochlorococcus* isolates. Values for FALS represent the mean +/- 1 S.E. for cultures grown over a range of irradiance levels, and values for C $cell^{-1}$ represent mean +/- S.E. estimated from replicate cultures from photosynthesis-irradiance experiments grown at two photon flux densities (see chapter IV).

based on measurements of population abundance, growth rates and assumed C cell^{-1}, may be underestimated if a single C cell^{-1} value is applied to the entire water column.

Light deprivation

During the course of the light-dependent growth response experiments, I observed that some *Prochlorococcus* isolates (SS120, MIT9313 and MIT9303), could survive for months, as detected by flow cytometry, without exhibiting a net increase in cell numbers (growth rate ≤ 0), when placed in extremely low light levels ($I_g < \sim 3$ µmol Q m^{-2} s^{-1}). This led us to hypothesize that *Prochlorococcus* may be able to survive relatively long periods of exposure to sub-compensation light levels or darkness. In order to test this hypothesis, the effects of light deprivation on growth and cellular chlorophyll fluorescence was examined on two physiologically distinct isolates of *Prochlorococcus*, the high-light adapted MIT9312 and its low-light adapted co-isolate MIT9313 (this was work performed by an MIT undergraduate, C. Marx, with the help of me and another graduate student, J. Hahn; analysis and interpretation of the data was carried out under my supervision). Each isolate was exposed to four periods (1, 2, 4, and 8 weeks) of darkness (foil-covered test tubes) and four periods of subcompensation light level (1.7 µmol Q m^{-2} s^{-1}, 14:10 light:dark cycle at 21 °C in PRO2 media), which is below the I_{comp} for both isolates (6.6 µmol Q m^{-2} s^{-1} for MIT9312 and 3.4 µmol Q m^{-2} s^{-1} for MIT9313; chapter IV). After each period, the cultures were placed into 10 µmol Q m^{-2} s^{-1} and their concentration was followed. Cells were enumerated using Becton Dickenson FACScan flow cytometer and analyzed using CYTOPC (Vaulot, 1989).

During the periods of darkness, cell numbers of both isolates remained constant irrespective of the length of dark exposure (Fig. 13). After being returned to light, cell numbers of both isolates initially decreased but then began to increase, except for MIT9312 when exposed to 8 weeks of darkness. On average, the low-light adapted isolate MIT9313 began increasing in abundance before its high-light adapted isolate MIT9312 and exhibited faster growth rates upon recovery (data not shown). Results were similar when these isolates were exposed to sub-compensation light levels (Fig. 14), except that the time for recovery of growth was shorter and the growth rates were slightly higher. As with exposure to darkness, the low-light adapted isolate MIT9313 responded sooner and grew faster than the high-light adapted isolate MIT9312.

Changes in relative cellular red fluorescence (RFL) was variable between treatments and between isolates (Fig. 15). For both MIT9312 and MIT9313, RFL decreased during exposure to darkness and continued to decrease after being put back into the light for some period of time before increasing again. The exception to the RFL response just described is that MIT9312 did not increase its RFL after 4 and 8 weeks of darkness, consistent with the fact that it did not recover in cell abundance after these periods. During exposure to sub-compensation light levels, RFL showed a net increase for the low-light adapted isolate MIT9313 which continued for some time after the cultures were returned to 10 μmol Q m^{-2} s^{-1}, and then decreased (Fig. 16). The RFL of MIT9312 either remained constant or decreased during the sub-compensation light treatment and then increased after the cultures were put back into 10 μmol Q m^{-2} s^{-1} (Fig. 16).

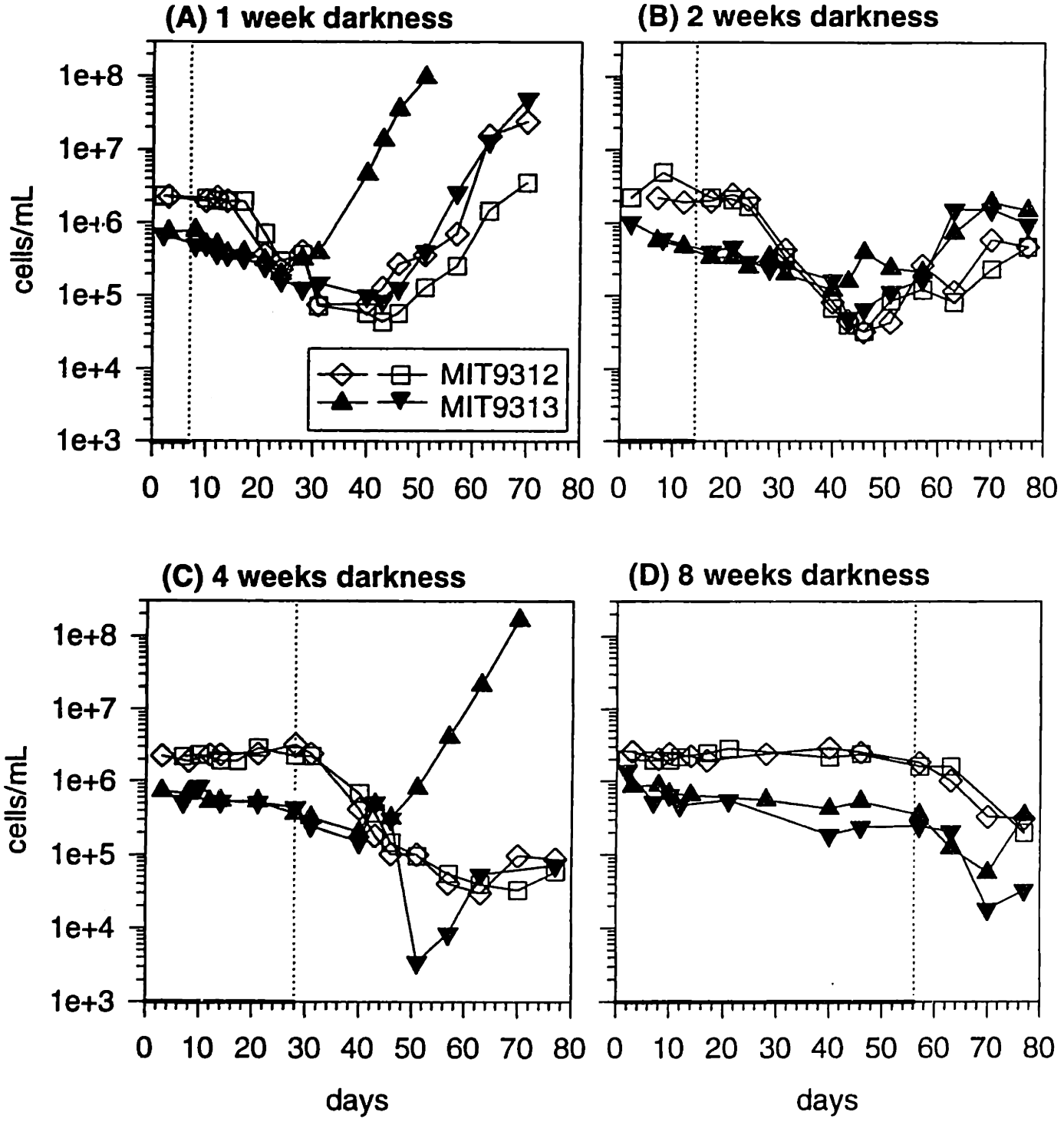

Figure 13 - Changes in cell concentration in MIT9312 (open symbols) and MIT9313 (closed symbols) during and after 1 week (A), 2 weeks (B), 3 weeks (C) and 4 weeks (D) of exposure to complete darkness. The dark bar and vertical dotted line indicate the period of darkness. Figure taken from Marx, 1997.

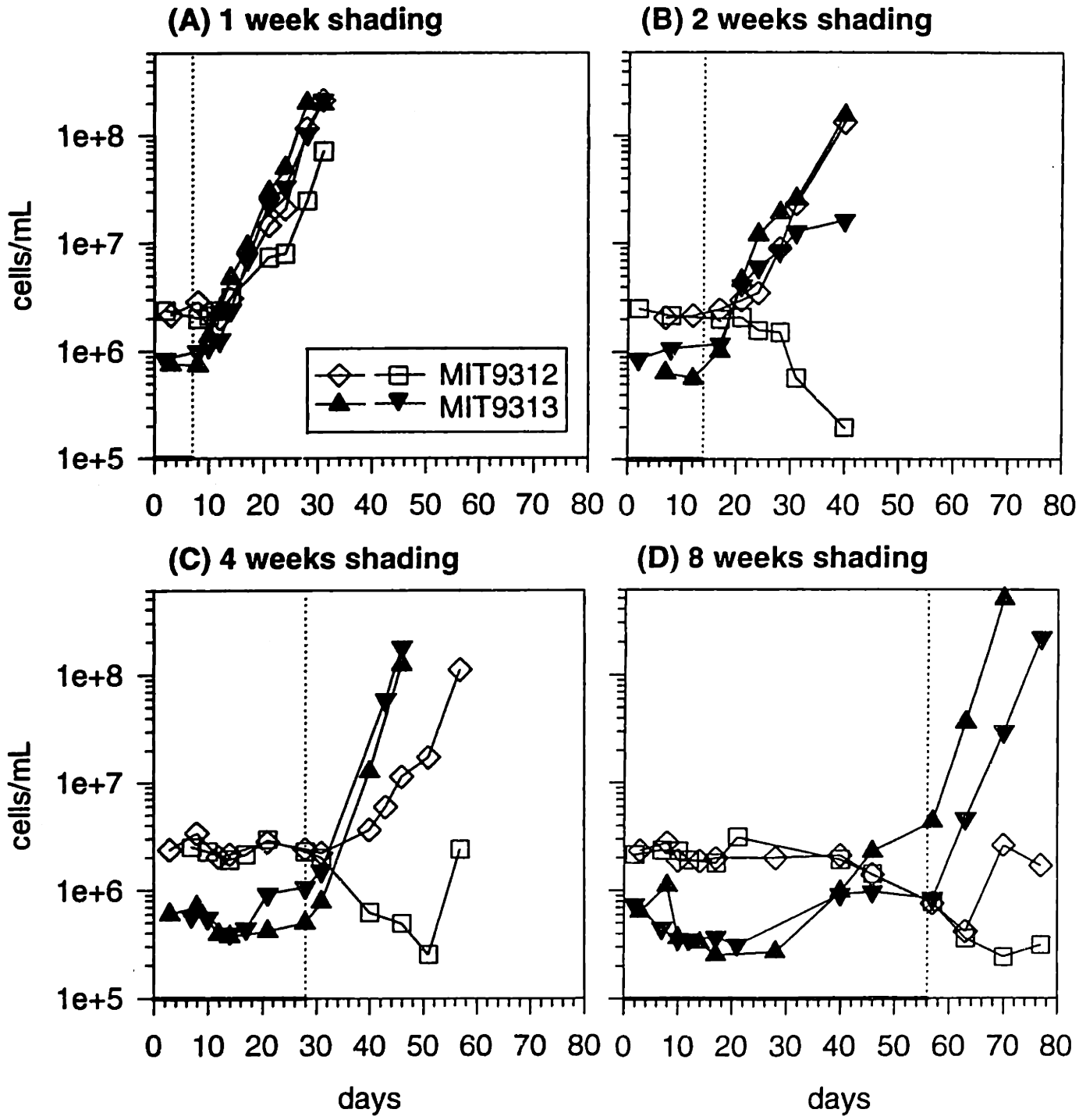

Figure 14 - Same as Fig. 13, except exposure is to a sub-compensation light level of 1.7 μmol Q m^{-2} s^{-1}.

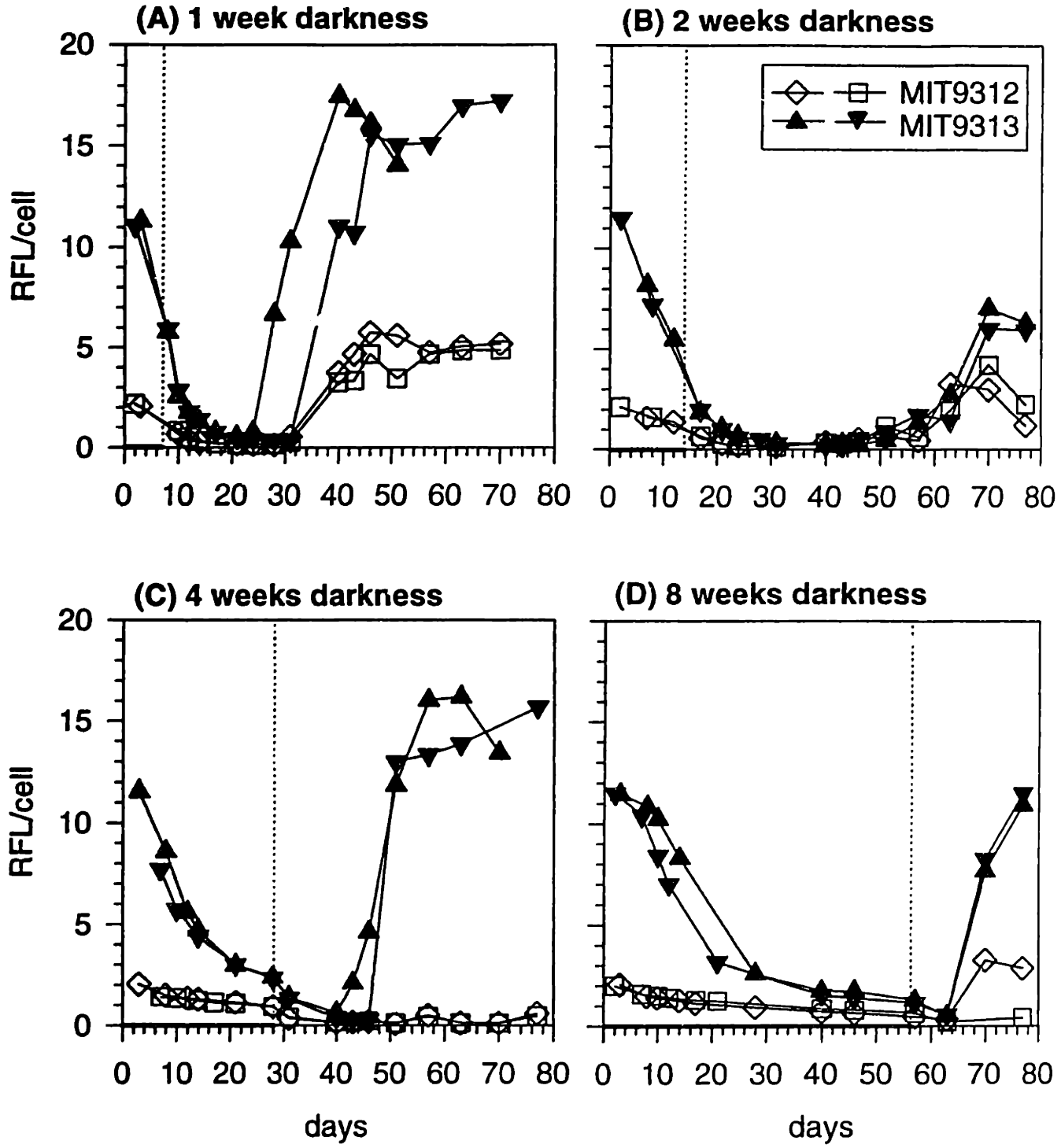

Figure 15 - Changes in relative red cellular fluorescence in MIT9312 (open symbols) and MIT9313 (closed symbols) during and after 1 week, 2 weeks, 4 weeks and 8 weeks of exposure to complete darkness. The dark bar and vertical dotted line indicate the period of darkness. Figure taken from Marx, 1997.

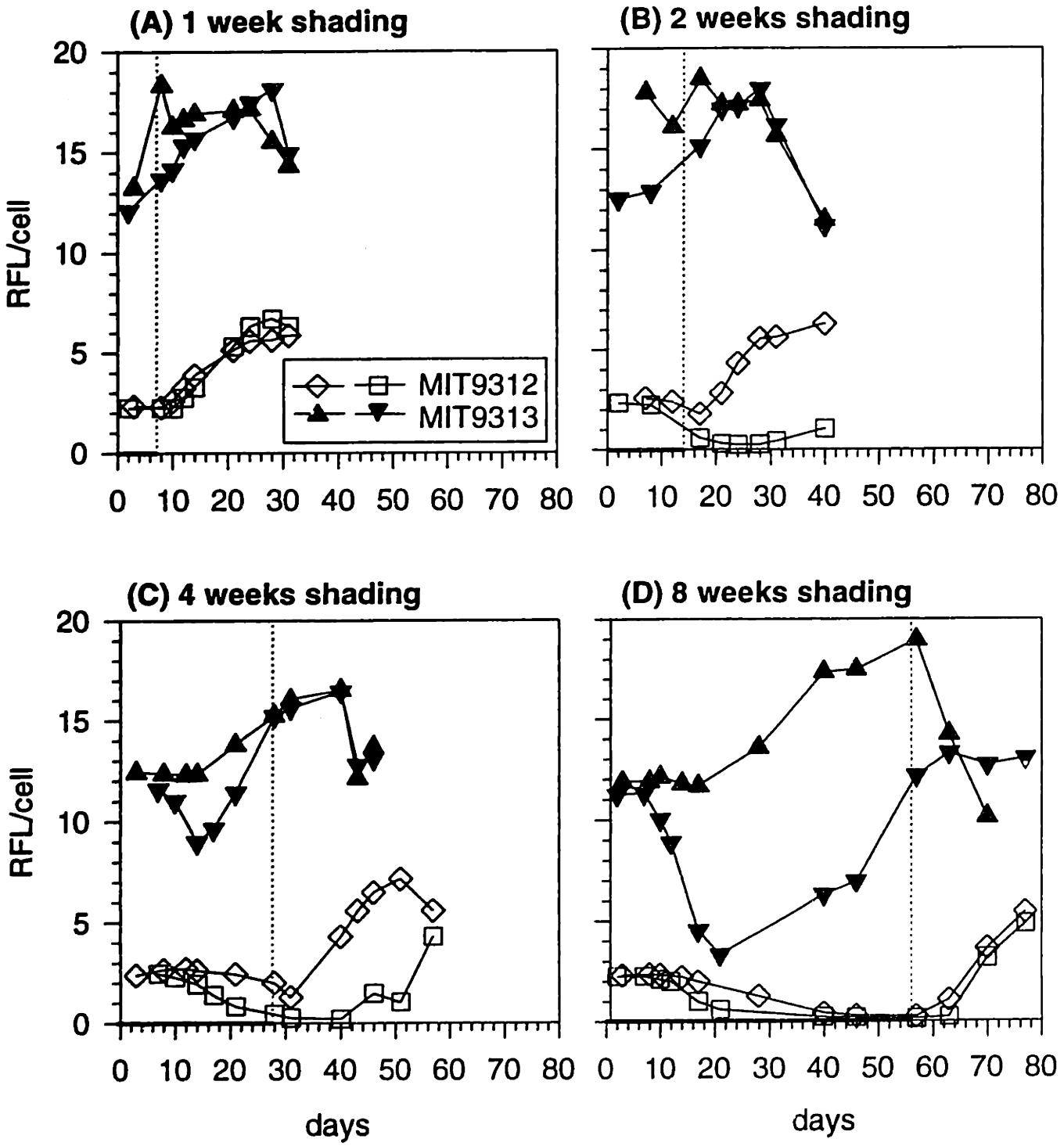

Figure 16 - Same as Fig. 15, except exposure is to a sub-compensation light level of 1.7 µmol Q m^{-2} s^{-1}.

These results show that *Prochlorococcus* can survive extended periods of sub-compensation light or darkness. The faster recovery times observed for the low-light adapted isolate MIT9313 indicates that it is better adapted to light deprivation than the high-light adapted isolate MIT9312. Additional studies need to be carried out to further understand the physiological and pigment changes responsible for the RFL response observed.

Future Work

Throughout the course of establishing and maintaining the isolates in the MIT *Prochlorococcus* collection, two basic culturing issues continue to draw attention but have not been pursued in a systematic manner. One of these issues concerns the media used to grow *Prochlorococcus*. Differences in EDTA concentrations and brand resulted in different growth and pigmentation response with an apparent light-dependent factor (see **Growth media** section). This needs to be explored further, especially to determine if the relatively low I_{inhib} for low-light adapted *Prochlorococcus* (chapter IV) is related, in part, to media composition. Additionally, entirely different media recipes are used by other researchers to grow *Prochlorococcus* (e.g. Shimada et al., 1995 and R. Rippka, personal communication). A comparison of media would be useful.

Obtaining axenic cultures is the other culturing issue that continues to prove difficult. Serially diluting out contaminating bacteria from the *Prochlorococcus* isolate SARG was successful, however it was necessary to dilute to below 10^{-2} cells (R. Rippka, personal communication), a very labor intensive approach. Many attempts have been made to grow *Prochlorococcus* on solid media with limited success: green colonies, presumably

Prochlorococcus, were formed on agar but subsequent transfer into liquid media was unsuccessful (J. Waterbury, personal communication). Another approach to obtaining axenic cultures is to flow cytometrically sort the *Prochlorococcus* cells away from the bacteria. *Prochlorococcus* isolates have been obtained from natural samples using this method (chapter III). This relatively straightforward technique has not been used for eliminating bacteria from *Prochlorococcus*, but it may prove to be the easiest method and deserves serious effort.

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