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Dynamics of community structure and phosphate status of picocyanobacterial populations in the Gulf of Aqaba, Red Sea

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Abstract

We monitored the genetic diversity and phosphate (P) status of *Prochlorococcus* and *Synechococcus* populations during an annual cycle (1999–2000) in the Gulf of Aqaba, Red Sea, using DNA probes and antibodies against the protein PstS, a molecular marker of P stress. The usual spring *Synechococcus* bloom was not observed in this year, and the picophytoplankton was dominated by *Prochlorococcus* from April to December. A single high light (HL)-adapted ecotype of *Prochlorococcus*, HLII, dominated, and this dominance extended even to the base of the euphotic zone for much of the year. Low light (LL)-adapted ecotypes only appeared between July and October and were confined to waters deeper than 50 m. The *Synechococcus* population was also dominated by a single clade (clade II), with the exception of a peak of clade III genotypes in June. Highest PstS expression from the collective *Synechococcus* and *Prochlorococcus* populations, indicative of P stress, was observed during the acutely oligotrophic summer, whereas low expression occurred during the winter and spring. *Prochlorococcus* abundance appeared unaffected by P stress and only decreased with the beginning of winter mixing, whereas P stress might have caused the decline of the *Synechococcus* population in May 1999.

Unicellular cyanobacteria of the genera *Synechococcus* and *Prochlorococcus* are known to be widespread and abundant throughout the world's oceans (see Partensky et al. 1999a,b; Scanlan 2003 for reviews), contributing significantly to marine and global primary production. The wide oceanic distribution of these organisms is mirrored by high physiological and genetic diversity of cultured isolates and natural populations (Moore et al. 1995; Scanlan and West

2002; Scanlan 2003). Marine *Synechococcus* comprises at least 10 lineages within marine cluster A (MC-A; Fuller et al. 2003), and although these might correspond to different ecotypes, the relationship between diversity and physiology is not yet well defined. However for *Prochlorococcus*, specific high light (HL)- and low light (LL)-adapted ecotypes defined by photosynthetic pigment characteristics and photophysiology (Moore et al. 1998; Moore and Chisholm 1999; Garcarek et al. 2000) are indeed phylogenetically distinct (Moore et al. 1998; Urbach et al. 1998). Furthermore, this phylogenetic separation is of ecological relevance because in situ studies that used the gene markers 16S rRNA (West and Scanlan 1999; West et al. 2001), *rpoCI* (Ferris and Palenik 1998), *cpeB* (Steglich et al. 2003), and *rbcL* (Wawrik et al. 2003) showed that the HL and LL ecotypes exhibited depth-dependent distributions. In addition, HL-adapted *Prochlorococcus* can be subdivided into two further clades, HLI and HLII (Moore et al. 1998; Urbach et al. 1998; West and Scanlan 1999), that show different oceanic distributions (West et al. 2001).

Although the light gradient is clearly a major factor dictating the vertical distribution of *Synechococcus* and *Pro-*

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chlorococcus, little is known of how nutrient and temperature gradients, or water column stability, influence community dynamics. The few in situ studies that have been performed indicate that warmer temperatures and water column stability are associated with higher *Prochlorococcus* abundance (Lindell and Post 1995; Cavender-Bares et al. 2001), whereas *Synechococcus* are more tolerant of mixed water conditions (Lindell and Post 1995) and lower temperatures, and their concentration has been linked with higher nitrate concentrations (Blanchot et al. 1992).

In terms of nutrient acquisition, *Synechococcus* are versatile, being able to use nitrate, nitrite, ammonium, urea, and some amino acids (Moore et al. 2002). In contrast, *Prochlorococcus* cannot use nitrate but can grow on ammonium and urea, and some LL-adapted strains can metabolize nitrite (Moore et al. 2002). In addition, *Prochlorococcus* can supplement their nitrogen (N) requirement by the uptake of amino acids (Zubkov et al. 2003). *Synechococcus* and *Prochlorococcus* show similar phosphorus (P) acquisition capacity though, utilizing phosphate and numerous organic P sources (Scanlan et al. 1997; Rippka et al. 2000). The complete genome sequences for three *Prochlorococcus* strains (one HL-adapted and two LL-adapted) and one *Synechococcus* strain fully support these observations but also indicate that the success of these marine cyanobacteria in nutrient-deplete waters might be partly a result of their utilization of novel organic sources of N and P, such as cyanates and phosphonates (Dufresne et al. 2003; Palenik et al. 2003; Rocoap et al. 2003).

The relationship between *Prochlorococcus* and *Synechococcus* community dynamics and nutrients is clearly complex and will necessitate further in situ studies that take into account the multiple sources of N and P available to them. However, a more direct approach to studying the response of organisms to their environment, or their activity, is through the use of molecular markers in which the expression of a gene or protein is used to indicate a given stress or a metabolic activity. In *Prochlorococcus* and *Synechococcus*, a high-affinity P uptake protein, PstS, is expressed in response to P starvation (Scanlan et al. 1993). An antibody raised against PstS allowed the detection of P stress in natural *Synechococcus* populations with the use of single-cell immunofluorescence (Scanlan et al. 1997). These authors also found that this antibody recognizes PstS in *Prochlorococcus* cells but not marine heterotrophic bacteria. Similarly, assays for alkaline phosphatase activity can be used to indicate P stress in the bulk planktonic community (Li et al. 1998) or in a given species with the use of a specific antibody (Dyhrman and Palenik 2001). Nitrogen stress has also been followed in natural *Synechococcus* populations by quantifying expression of the *ntcA* gene, the global nitrogen regulator (Lindell and Post 2001).

To understand marine cyanobacterial community dynamics better, there is a need for more studies combining genetic and physiological data with environmental parameters. To address this, we have carried out a detailed genetic analysis of picocyanobacterial community structure in relation to flow cytometry and physical and chemical properties of the water column, at Sta. A in the Gulf of Aqaba, at the northern end of the Red Sea, over an annual cycle during February

1999–January 2000. This site was chosen because, despite its proximity to land, it is typical of an open ocean gyre in its biogeochemistry; in addition, it is subject to strong seasonal variation in water column structure (see Wolf-Vecht et al. 1992; Lindell and Post 1995). The oligotrophic nature of these Gulf waters results from the influx of relatively nutrient-deplete surface waters from the northern Red Sea, from which it is separated by a shallow sill (240 m) at the Straits of Tiran. Furthermore, because the Gulf is bounded by deserts, fluvial nutrient input is negligible. To analyze picocyanobacterial community structure both spatially and temporally at this site, we used 16S rDNA oligonucleotides specific for individual *Synechococcus* clades and *Prochlorococcus* ecotypes, which we had developed in previous studies (West and Scanlan 1999; Fuller et al. 2003). We also used antibodies raised against a component, PstS, of the high-affinity P uptake system of these organisms (Scanlan et al. 1997) to evaluate whether changes in nutrient status were coupled with any obvious changes in subsequent population structure. We used PstS expression here, rather than the more widely used alkaline phosphatase activity measurements, because PstS antibodies specifically target the collective *Synechococcus* and *Prochlorococcus* populations for P stress, separate from the rest of the picoplanktonic community. Furthermore PstS probing and alkaline phosphatase activities report on different aspects of the P stress response, PstS responding to ambient P levels (i.e., both organic and inorganic phosphate) and alkaline phosphatase activities reporting on ambient inorganic phosphate levels (the enzyme being switched on when inorganic phosphate supply is low).

Materials and methods

Sampling—Monthly water samples were collected from the northern tip of the Gulf of Aqaba at Sta. A (29°28'N, 34°55'E) in the surface 200 m between February 1999 and January 2000, and then again in September 2000, aboard the Research Vessel (RV) *Suella* (Feb 99) and the RV *Sea Surveyor* (Mar 99–Jan 00, Sep 00). Samples were obtained from discrete depths with 30-liter Niskin bottles on a hydrographic cable (Feb 99–Sep 99) or with a Rosette sampler of 12-liter Go-Flo bottles (Oct 99–Jan 00, Sep 00). Conductivity, temperature, and barometric pressure were measured simultaneously with a CTD (model STD-plus, Applied Microsystems). For ammonium measurements, 20 ml of water was taken by syringe directly from the collection bottle before any water had been removed. This was then filtered through two 25-mm-diameter GF/F filters, which had been combusted at 450°C with the use of an in-line filtration system and stored in an air-tight polycarbonate Nalgene bottle at 6°C until analysis. For DNA extraction, seawater (5 or 10 liters) was filtered and stored, and the DNA was extracted as previously described (Fuller et al. 2003). The 1% isolume (the depth to which 1% of incident surface light penetrates and thus the base of the euphotic zone) was calculated as three times the Secchi depth (Brown et al. 1992).

Oxidized nitrogen measurement—Duplicate 10-ml samples of seawater were filtered through acid-washed 25-mm-

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diameter 0.2- μm -pore-size cellulose acetate filters into acid-washed 10-ml screw-capped tubes and stored at -20°C until analysis. Total oxidized nitrogen (TON) and nitrite were measured with a Quick Chem 8000 flow injection nutrient analyzer (Lachat Instruments) with a detection limit of 20 nmol L^{-1} as described previously (Lindell and Post 2001). Nitrate measurements were calculated by the subtraction of nitrite from TON.

Phosphate measurement—Soluble reactive phosphate (SRP) was concentrated from duplicate samples by the MAGIC method, described previously (Karl and Tien 1992), from 500 ml of seawater, with 250-ml polycarbonate centrifuge pots, collecting the precipitate by centrifugation with a GS 3 rotor with a Sorvall RC-5B centrifuge. The two pellets were resuspended in a total of ~ 10 ml of 1 mol L^{-1} HCl in acid-washed 10-ml screw-capped tubes and stored at -20°C until analysis. SRP was measured by the molybdenum blue method (Murphy and Riley 1962) modified for analysis with a Quick Chem 8000 flow injection nutrient analyzer (Lachat Instruments) and with a detection limit of 10 nmol L^{-1} .

Ammonium measurement—The method is based largely on protocol A described by Holmes et al. (1999), with a detection limit of 20 nmol L^{-1} , except that smaller volumes were used. Triplicate 4-ml samples were placed in screw-cap tubes, and 1 ml of the working reagent (WR) was added to two of them. Samples with WR were mixed and incubated for 3–8 h in the dark. Fluorescence readings were taken with a Hoefer DyNA Quant 200 fluorometer, with a 365 ± 7 nm excitation lamp and a 460 ± 15 nm emission filter. The sample's fluorescence reading with no WR was subtracted from those with WR, with water as a blank. To avoid matrix effects, a standard curve was made by adding NH_4Cl to seawater that had been collected from surface waters of Sta. A during the nutrient-deplete summer, filtered through two GF/F filters, and stored at 6°C .

Flow cytometric analysis—Between February 1999 and January 2000, duplicate 1-ml samples were preserved with 1% (w/v) paraformaldehyde and 0.05% (v/v) glutaraldehyde and picophytoplankton enumerated as described previously (Marie et al. 1999; Fuller et al. 2003). During September 2000, duplicate 2-ml subsamples from each depth were preserved with 0.2% (w/v) paraformaldehyde, after which they were quickly frozen and stored in liquid nitrogen. Picophytoplankton abundance in these samples was determined on a modified FacScan flow cytometer (Becton Dickinson) suited for analysis of the dimly fluorescent surface populations of *Prochlorococcus* (Dusenberry and Frankel 1994). A 10-ml suspension of 0.474- μm fluoresbrite microspheres (Polysciences) was used as an internal standard for both cell-specific scatter and fluorescence.

Chlorophyll *a* measurement—Chlorophyll *a* (Chl *a*) was extracted and measured as previously described (Fuller et al. 2003).

PCR amplification—Initially 16S rDNA sequences both from environmental DNA and from laboratory strains were

amplified with primers OXY107F and OXY1313R (West et al. 2001), which specifically amplify oxygenic phototrophs and are based on primers described previously (Urbach et al. 1992). Amplification conditions were the same as reported previously (Fuller et al. 2003). Later amplification of *Synechococcus* 16S rDNA from environmental DNA was performed with the new primer SYN172F (5'-AATA-CCCATATGCCGMGAG-3') in conjunction with OXY1313R. This primer pair amplifies all known sequences from *Synechococcus* MC-A (clades I–X; Fuller et al. 2003), some MC-B strains, no freshwater *Synechococcus* strains, all LL *Prochlorococcus* strains, some HLI *Prochlorococcus* strains, but no HLII *Prochlorococcus* strains. SYN172F was designed by the probe design function in the ARB program (Ludwig et al. 2004). Amplification conditions were the same as for OXY107F and OXY1313R, except that the concentration of SYN172F was 50 nmol L^{-1} . Environmental DNA was amplified in duplicate, and the products were pooled for further use.

PstS measurement—Phytoplankton were concentrated from 100-liter seawater volumes, prefiltered through 20- μm mesh by tangential flow filtration, and centrifuged as previously described (Scanlan et al. 1997). A subsample of the concentrate was fixed and analyzed by flow cytometry as described above to enumerate *Prochlorococcus* and *Synechococcus*. Relative expression of PstS in the picocyanobacteria was assessed by western blot analysis as described previously (Scanlan et al. 1993, 1997) with the use of a polyclonal PstS antiserum that cross-reacts with PstS from both *Synechococcus* and *Prochlorococcus*. Protein gels for the annual cycle were loaded on an equal cell number basis (total 1.5×10^7 cells = [*Synechococcus* + *Prochlorococcus*]) with the use of flow cytometry data from the concentrates. The protein gels for samples taken in September 2000 were loaded on an equal protein concentration basis because cell numbers were not available for these samples. All western blots were repeated at least once. To allow semiquantification of the PstS expression level and interblot comparisons, a standard curve was prepared with a *Synechococcus* WH 7803 purified cell wall extract that was enriched in PstS (see fig. 2B in Scanlan et al. 1993). Cross-reacting bands were revealed by the ECL Plus western blotting detection system (Amersham Pharmacia Biotech UK) according to the manufacturer's instructions and quantified against a standard curve of PstS by densitometric analysis with Total Lab software (Phoretix).

Dot blot hybridization—Blots of 16S rDNA PCR products were constructed, prehybridized, and hybridized as previously reported (West and Scanlan 1999). Briefly, environmental products (30 ng) pooled from duplicate reactions were blotted in triplicate, as were membrane blanks for subtraction from final readings. Blots were also constructed for calibration curves with control DNAs. ^{32}P -labeled oligonucleotides were hybridized simultaneously to environmental and control blots in the same hybridization vessel, and all blots from the same probe were subsequently exposed in the same phosphorimager cassette. Oligonucleotide probes and their stringency wash temperatures were used as previously

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Table 1. 16S rDNA oligonucleotide probes used in this study.

Probe	Sequence (5'–3')	Target organism(s)	Reference
SYN1006	CTC TCA AGT TTC CAA GAG	<i>Synechococcus</i> clade I	Fuller et al. 2003
SYN1006RS	CTC TCC TGT TTC CAA GAG	<i>Synechococcus</i> clade II	Fuller et al. 2003
SYN262	GAT GCC TTG GTA GGC CTT	<i>Synechococcus</i> clade III	Fuller et al. 2003
SYN635	AAG CCC CTC AGT TTC CAC	<i>Synechococcus</i> clade IV	Fuller et al. 2003
SYN1280	GAG CCA CGG TTT ATG AGA	<i>Synechococcus</i> clades V, VI and VII	Fuller et al. 2003
SYN620	CAC TGC CAC GAT GGA GTT	<i>Synechococcus</i> clade VIII	Fuller et al. 2003
SYN1000	GGT TTC CCA GAA ATT CGC	<i>Synechococcus</i> clade IX	Fuller et al. 2003
SYN1007	ACC CTC CGG TTT CCC AGA	<i>Synechococcus</i> clade X	Fuller et al. 2003
SYN1258	TTG TCC TCG CGA ACT TGC	<i>Synechococcus</i> clades I–VII and X, <i>Prochlorococcus</i> sp. MIT9303	Fuller et al. 2003
S1PRO634R	GCC GAT CAG TTT CCA CTG	HLI <i>Prochlorococcus</i>	West and Scanlan 1999
S2PRO640R	ATA CTC AAG CCT TTC AGT TT	HLII <i>Prochlorococcus</i>	This study
DPRO634R	GCC AAT CAG TTT CCA CTG	LL <i>Prochlorococcus</i> (except SS120)	West and Scanlan 1999
SARG634R	GCC CTT CAG TTT CCA CTG	<i>Prochlorococcus</i> sp. SS120	West and Scanlan 1999
MIT1023R	TGC GTT CCC AAA GGC ACT	<i>Prochlorococcus</i> sp. MIT9303	West and Scanlan 1999
EUB338	GCT GCC TCC CGT AGG AGT	Eubacteria	Amann et al. 1995

reported, having been optimized against control DNAs from a range of cyanobacterial strains for *Synechococcus* probes (Fuller et al. 2003) and for *Prochlorococcus* and eubacterial probes (West and Scanlan 1999; see Table 1). However, the *Prochlorococcus* HLII-specific probe S2PRO640R is a modification of the published probe S2PRO634R to improve specificity (data not shown), for which the stringency wash temperature was determined as 41°C. Hybridization of *Synechococcus* probes to SYN172F-OXY1313R products was quantified with a Fujifilm FLA-5000 phosphorimager and Total Lab software (Phoretix). Hybridization of *Synechococcus* and *Prochlorococcus* probes to OXY107F-OXY1313R products was quantified with a Molecular Dynamics phosphorimager and Image Quant software (Molecular Dynamics). Minimum detection limits were ~0.1 ng of DNA. Levels of relative hybridization of the probes were calculated as previously reported, normalizing the individual cyanobacterial probe signals against the signal for total amplified DNA with the EUB338 probe (West and Scanlan 1999). Hence, relative hybridization represents the probe signal as a proportion of total DNA amplified with the specific primer set used.

Statistical analyses—Following tests for normality, Pearson's correlation coefficients were determined for selected genotype and cell abundance data. Multivariate statistical analysis of the physical, chemical, and biotic data from the annual cycle was performed with the dot blot hybridization data set OXY107F-OXY1313R and the computer program CANOCO (version 4.0). A preliminary detrended correspondence analysis (DCA) was run and showed short gradient lengths (<2.0 SD), which indicated that linear methods were more appropriate. A partial redundancy analysis (RDA; ter Braak 1994) ordination was carried out with variations in species abundance of *Synechococcus* and *Prochlorococcus* "partialled out" by setting them as covariables, allowing us to relate the residual variation to the impact variables (John Birks pers. comm.). Nutrient data were log transformed prior to analysis. Environmental variables were selected by a Forward Selection option with Monte Carlo re-

strictions. Only those variables with $p < 0.05$ have been included.

Results

Seasonal cycle: Physical and chemical profiles—Depth profiles of nutrients and temperature throughout the year in the north of the Gulf of Aqaba (Figs. 1, 2A) reveal the strong seasonal changes in the water column and its oligotrophic nature throughout most of the year. The upper part of the water column was nearly homogeneously mixed down to depths of >300 m during the winter months, with very little variation in temperature or nutrients. This winter mixing increased the concentration of phosphate and nitrate in surface waters, although these still barely exceeded 0.1 and 1 $\mu\text{mol L}^{-1}$, respectively (Fig. 1A,B). Winter mixing at the beginning of 1999 reached a depth of 500 m by early March (data not shown). By the end of April, a slight thermocline provided a stable surface mixed layer (SML), which coincided with a sharp decline in nitrate and phosphate concentrations in surface waters. Surface waters remained stratified throughout the summer until November, when the thermocline had descended to below the euphotic zone. Despite winter mixing, nitrate and phosphate concentrations remained low through to January 2000. Ammonium concentrations (Fig. 1C) were consistently low (~70 nmol L^{-1}) throughout the period measured (Jul 99–Jan 00). Thermocline and nutrient conditions in September 2000 were similar to those in September 1999 (Fig. 3A,B).

Picophytoplankton abundance—Picocyanobacterial numbers were low during February–March 1999 (Fig. 2A) but increased greatly with the onset of stratification in mid-April, a feature coinciding with falls in nitrate and phosphate concentrations (Fig. 1). Maximum *Synechococcus* abundance was observed at the end of April ($3.9 \times 10^4 \pm 430$ cells ml^{-1} at 60 m depth), followed by a decline through the summer before another increase from November to January, with a maximum of $3.0 \times 10^4 \pm 530$ cells ml^{-1} in January 2000. In contrast, *Prochlorococcus* abundance continued to in-

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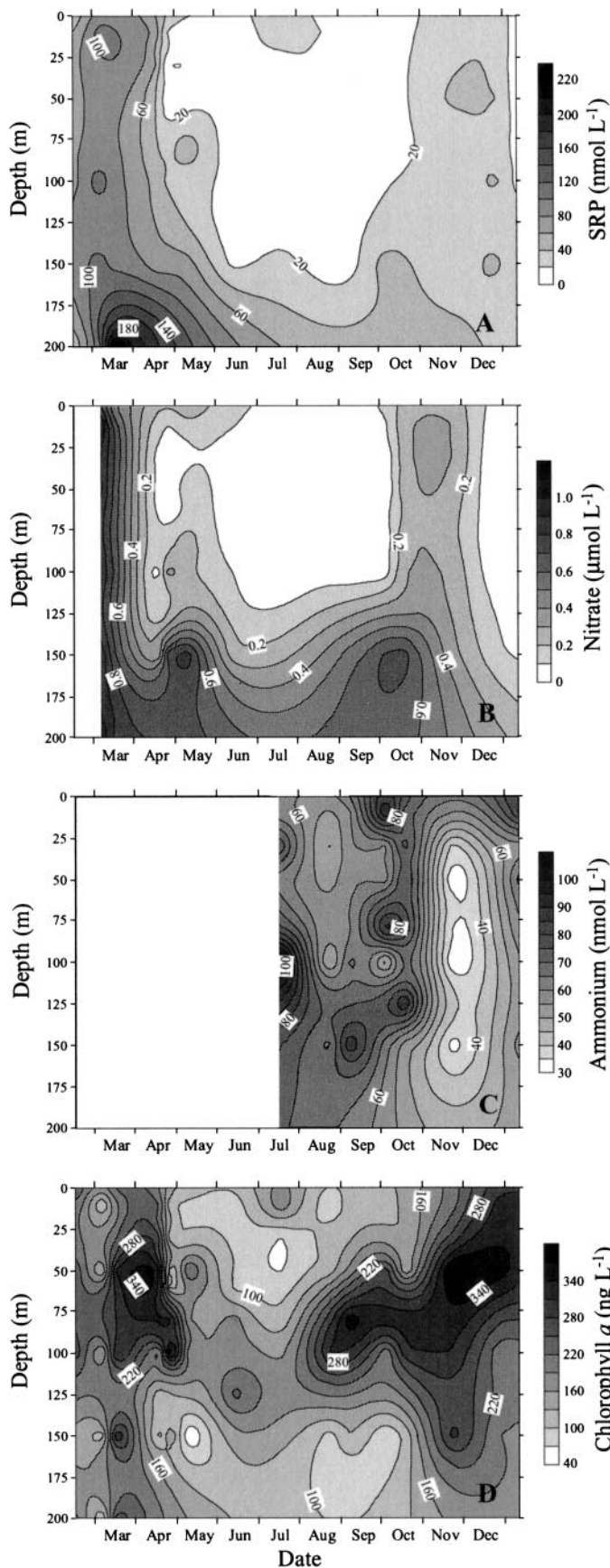


Fig. 1. Contour plots over the 1999 annual cycle of (A) soluble reactive phosphate, (B) nitrate, (C) ammonium, and (D) Chl *a*.

crease throughout the summer, reaching a maximum in June ($2.3 \times 10^5 \pm 900$ cells ml^{-1} at 80 m depth) and remaining high through to November. Although *Prochlorococcus* abundance decreased during the winter, cell numbers were still relatively high ($4.1 \times 10^4 \pm 870$ cells ml^{-1}) in January 2000. Photosynthetic eukaryotes remained at relatively low concentrations ($<6 \times 10^3$ cells ml^{-1}) throughout the year. With the onset of stratification in April 1999 through to the following January, *Prochlorococcus* numerically dominated the picophytoplankton in the euphotic zone. Cell counts in September 2000 were similar to those in September 1999 (Fig. 3B).

Prochlorococcus community diversity and dynamics—Oxygenic phototroph 16S rDNA from depth profiles was hybridized with oligonucleotides specific to the different *Prochlorococcus* ecotypes (see Table 1), revealing their relative distribution over the 1999 calendar year and in September 2000 (Fig. 2B, 3D). The HLI ecotype and the LL-adapted strains SS120 and MIT9303 were not detected in any samples from Sta. A. In addition, no *Prochlorococcus* ecotypes could be detected before April because cell numbers were too low to allow detection. HLII ecotypes were first detected in mid-April, corresponding well with an increase in *Prochlorococcus* cell numbers (Fig. 2A). These HLII ecotypes then dominated the *Prochlorococcus* population for the rest of the year, extending even below the base of the euphotic zone (defined by the 1% isolume; Fig 2B). LL ecotypes (other than those represented by strains SS120 and MIT9303) first appeared in summer with the relative hybridization signal for this group increasing dramatically ($0.5\% \pm 0.06$ to $42\% \pm 3.1$) between July and August. The LL ecotypes were always found preferentially below the SML, with the strongest signal below 100 m. However, these ecotypes only dominated over HLII ecotypes at the base of the euphotic zone or deeper, and only between August and September 1999. During 4–18 October, the LL ecotype signal decreased significantly, and no signal was detectable after mid-October. This marked decline in LL ecotype hybridization signal coincided with a deepening of the SML as surface waters cooled. The profile from September 2000 (Fig. 3D) shows that, although the relative hybridization values are lower, the trend is similar to that from September 1999: HLII ecotypes were present throughout the water column and LL ecotypes were detected only in the deeper waters, dominating at the base of the euphotic zone. This differential distribution of *Prochlorococcus* ecotypes correlates extremely well with flow cytometric observations that were available only from the September 2000 water column, in which distinct HL (“dim *Prochlorococcus*”) and LL (“bright *Prochlorococcus*”) populations could be discriminated and enumerated by the intensity of their red fluorescence (Fig. 3C).

Pearson’s correlation coefficients determined for selected genotypes (according to relative hybridization data) and cell abundance data over the period from February 1999 to January 2000 showed a significant positive correlation between *Prochlorococcus* cell counts and the HLII ecotype signal ($p < 0.001$), as well as a strong negative correlation between the LL ecotype signal and *Synechococcus* cell counts ($p < 0.001$).

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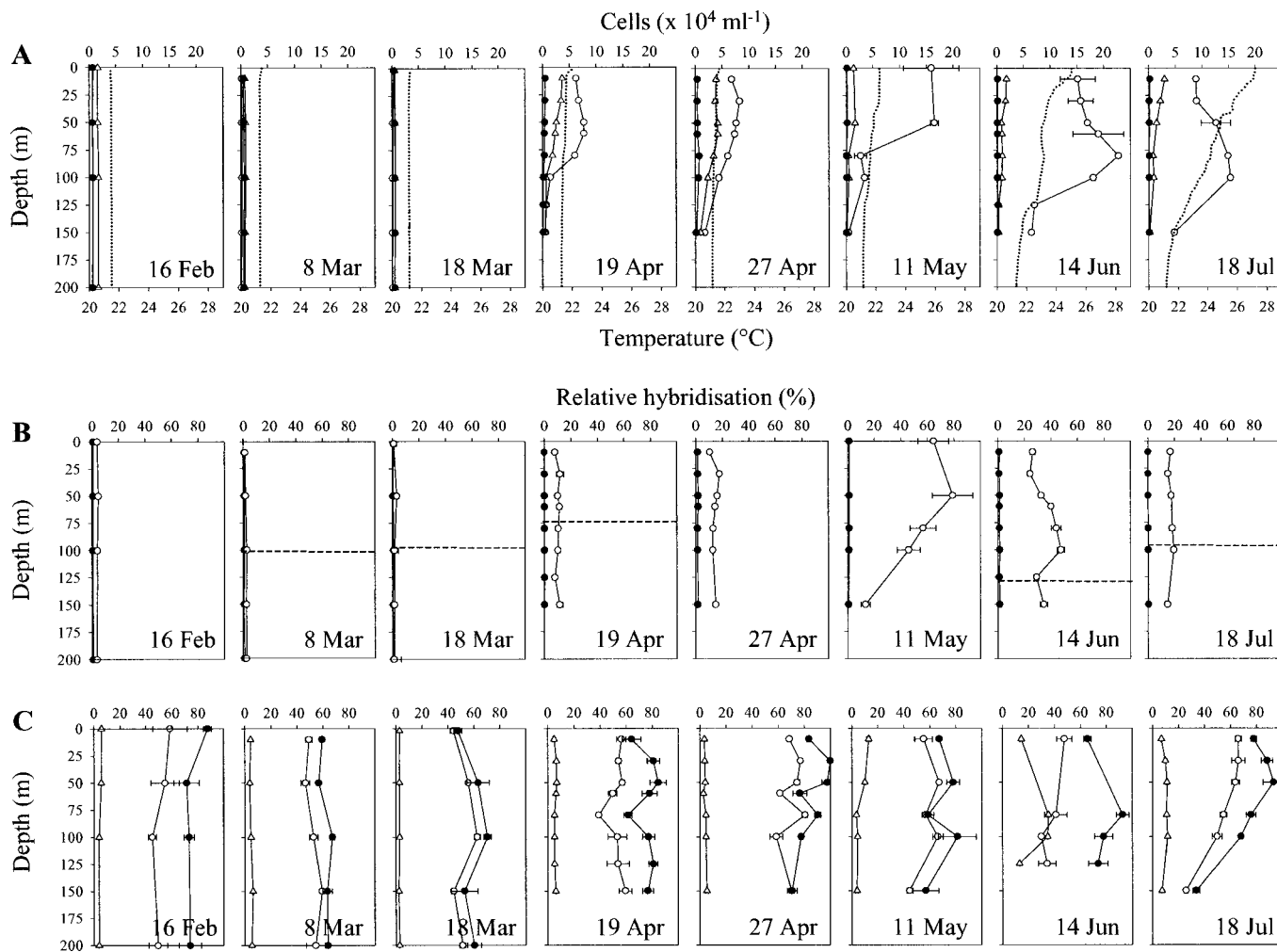


Fig. 2. Vertical profiles over the 1999 annual cycle of (A) temperature and *Synechococcus*, *Prochlorococcus*, and picoeukaryote abundance (mean \pm SD) as determined by flow cytometry; (B) relative hybridization values (mean \pm SD) of HLII and LL *Prochlorococcus* ecotypes (as a proportion of all amplified by primers OXY107F and OXY1313R); and (C) relative hybridization values (mean \pm SD) of *Synechococcus* clade II and III genotypes and “general MC-A *Synechococcus*” (clades I–VII, X; as a proportion of all amplified by primers SYN172F and OXY1313R) derived from dot blot data. The horizontal dashed line in panel B represents the depth of the 1% isolume.

Synechococcus community diversity and dynamics—An initial assessment of *Synechococcus* community diversity at Sta. A was made by hybridizing oxygenic phototroph 16S rDNA with *Synechococcus* clade-specific oligonucleotide probes (see Table 1). This assessment (data not shown) revealed the predominance of a single *Synechococcus* clade, clade II, throughout the water column for the entire year. From April onward, however, relative hybridization signals were low, even for the dominant clade II genotypes, presumably as the result of low total *Synechococcus* compared with *Prochlorococcus* cell numbers in the water column. To improve the resolution of *Synechococcus* clade dynamics, a new forward primer, SYN172F, was designed that amplifies all known MC-A *Synechococcus* and LL *Prochlorococcus* strains but no HLII *Prochlorococcus* strains. This primer was used to amplify environmental DNA in conjunction with OXY1313R prior to hybridization with the *Synechococcus* clade-specific oligonucleotides. These dot blot data showed

much more clearly the dominance of a single marine *Synechococcus* clade, clade II, throughout the sampling period, often accounting for >50% of the relative hybridization signal (Fig. 2C). For certain samples, some discrepancy between the clade II and “general *Synechococcus*” (SYN1258) hybridization signal could not be accounted for by the summation of the hybridization values for the remaining clades. Of these minor clades, clade III was the most abundant, yet accounted for <10% of the signal for most of the year. Relative hybridization values for clade III were proportionately higher through the summer months, compared with winter, peaking in June when values exceeded clade II at 100 m. Relative hybridization values for each of the minor clades (including clades VIII and IX, which are not detected by the general MC-A *Synechococcus*, SYN1258, probe) remained <1% throughout the euphotic zone during most of the sampling period and never exceeded 2% (data not shown). The *Synechococcus* community structure profile from September

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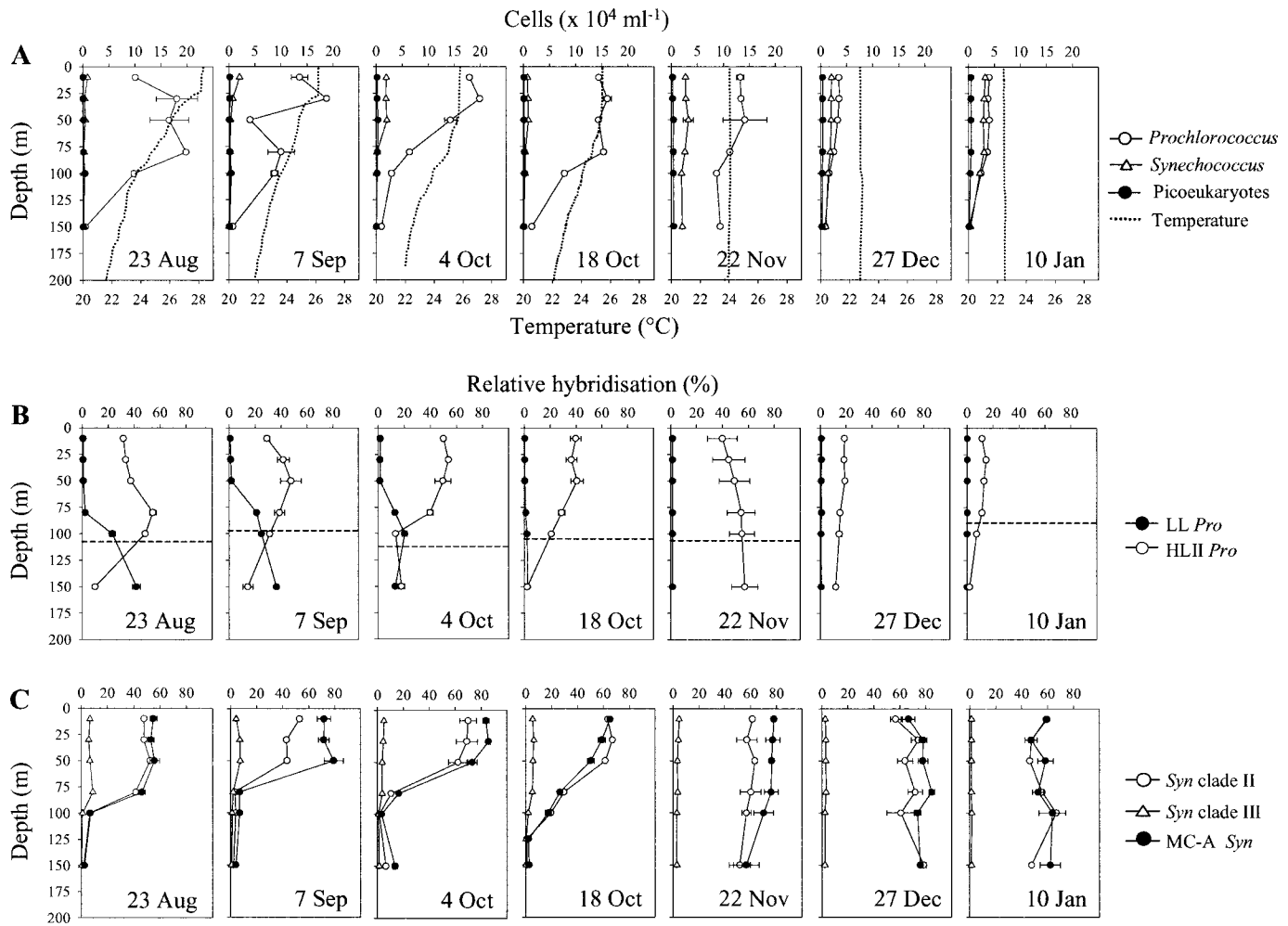


Fig. 2. Continued.

2000 (Fig. 3E) was very similar to that from September 1999, but clade II accounted for an even greater proportion ($85\% \pm 3.8$) of the total MC-A *Synechococcus* signal.

Picocyanobacterial P status—Corporate expression of the PstS protein by *Synechococcus* and *Prochlorococcus* was measured over the annual cycle at 10 and 100 m (Fig. 4). Two general trends were observed: (1) PstS expression was low in the spring and then increased in the summer before decreasing in the winter, and (2) SRP concentration was high in the early spring and then decreased in the summer before increasing in the winter. No absolute correlation between depth and level of PstS expression was observed over the annual cycle; however, PstS expression was usually greater at the surface than at depth on any given date. In contrast to the data for September 1999, the depth profile of PstS expression from September 2000 showed a marked decline between 5 and 25 m, with no detectable expression below 50 m (Fig. 5). Expression correlated negatively with SRP concentration, which was $<4 \pm 3 \text{ nmol L}^{-1}$ in the upper 50 m and $>25 \pm 3 \text{ nmol L}^{-1}$ below 50 m.

As expected, these trends (1 and 2 above) show an inverse relationship and indicate that PstS is useful for showing a P

stress response in *Prochlorococcus* and *Synechococcus*. However, low PstS expression was sometimes observed when SRP concentration was also low (e.g., 7 Sep), suggesting that other bioavailable P forms were present at that time.

Multivariate analysis—To define the environmental parameters that allow proliferation of a specific picocyanobacterial population, a partial redundancy analysis (pRDA) was performed on a suite of environmental variables and the OXY107F-OXY1313R dot blot hybridization data (Fig. 6). The first two pRDA axes explained 19.6% of the variance in the species data and 76.9% of the variance in the correlations and the class means of species (genotypes) with respect to the environmental variables. An examination of the canonical coefficients and the intraset correlations of environmental variables with the first two axes of the pRDA indicated that the first axis was mainly a phosphate gradient, whereas the second axis described nitrate concentration and biomass (Chl *a*) as the important gradients.

The *Synechococcus* clades III and IX correlated positively with SRP concentration, whereas both HL and LL *Prochlorococcus* ecotypes correlated negatively with SRP. Addition-

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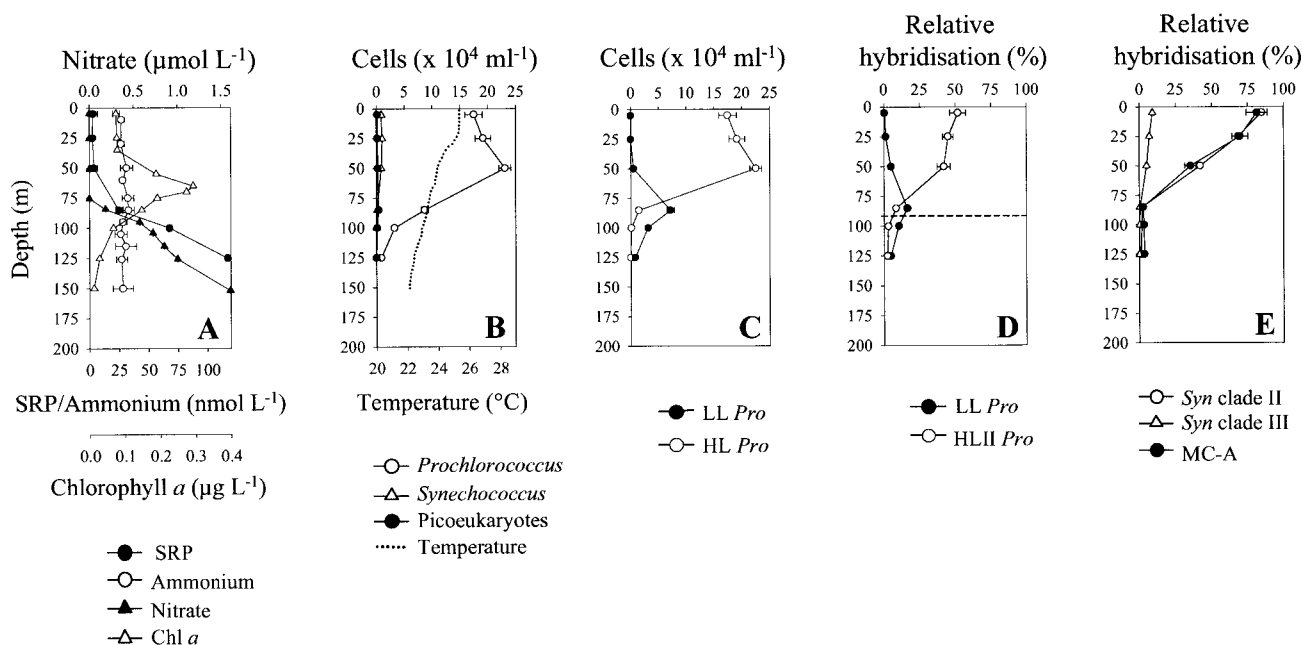


Fig. 3. Vertical profiles from September 2000 of (A) Chl *a*, nitrate (mean \pm SD), soluble reactive phosphate (mean \pm SD), and ammonium (mean \pm SD); (B) temperature and *Synechococcus*, *Prochlorococcus*, and picoeukaryote abundance (mean \pm SD) as enumerated by flow cytometry; (C) abundance of HL and LL *Prochlorococcus* ecotypes (mean \pm SD) as enumerated by flow cytometry; (D) relative hybridization values (mean \pm SD) of HLII and LL *Prochlorococcus* ecotypes (as a proportion of all amplified by primers OXY107F and OXY1313R); and (E) relative hybridization values (mean \pm SD) of *Synechococcus* clade II and III genotypes and “general MC-A *Synechococcus*” (clades I–VII, X; as a proportion of all amplified by primers SYN172F and OXY1313R) as determined by dot blot analysis. The horizontal dashed line in panel D represents the depth of the 1% isolume.

ally, *Synechococcus* clades II and X showed a positive correlation with the abundance of photosynthetic picoeukaryotes, whereas clades V, VI, and VII showed a positive correlation with algal biomass (Chl *a*). For nitrate concentration, *Synechococcus* clades II and V–VII were negatively correlated, whereas *Synechococcus* clade III showed no correlation. HLII *Prochlorococcus* showed a weak negative correlation and LL *Prochlorococcus* ecotypes were uncorrelated with nitrate concentration.

Discussion

Temporal studies of *Synechococcus* and *Prochlorococcus* populations in the North Atlantic Ocean, the Pacific Ocean, and the Red Sea (Lindell and Post 1995; Campbell et al. 1997; DuRand et al. 2001) have provided invaluable data on picocyanobacterial community structure. However, we now know that these populations are composed of several ecotypes or diverse clades; therefore, the dynamics of these populations are better resolved by molecular approaches. Here we present for the first time an annual study of *Synechococcus* and *Prochlorococcus* community structure dynamics combining genetic analyses with flow cytometric data, physical and chemical data, and an assessment of the P status of these populations.

Prochlorococcus numerically dominated the picophytoplankton from April 1999 to January 2000, whereas *Synechococcus* cell concentrations remained low throughout the year. This contrasts markedly with a previous study carried out between September 1992 and September 1993 (Lindell

and Post 1995) that showed the succession of blooms of *Synechococcus* and *Prochlorococcus* in the spring and summer, respectively. These striking differences in the picophytoplankton dynamics between the two studies could be explained by the different depths of winter mixing and their subsequent effects on nutrient concentrations. During the 1992–1993 winter, the water column was mixed to a depth of 600 m at Sta. A and exceeded depths of 1,000 m at southerly sites in the Gulf of Aqaba (Genin et al. 1995; Lindell and Post 1995), leading to nitrate concentrations as high as $2.8 \mu\text{mol L}^{-1}$ by mid-March (Lindell and Post 1995). In contrast, water column mixing during the 1998–1999 winter descended to 300–500 m (see also Sommer et al. 2002), and nitrate concentrations were only $0.7 \mu\text{mol L}^{-1}$ in surface waters by mid-March (Fig. 1). *Synechococcus* have a high N requirement (Moore et al. 2002; Heldal et al. 2003) because of their N-rich phycobilisomes; therefore, the lower nitrate concentrations might have been a factor in preventing a spring bloom. Because *Prochlorococcus* cannot utilize nitrate, but rely instead on more reduced N forms for growth (Moore et al. 2002; Zubkov et al. 2003), they might be less sensitive to lower concentrations of this nutrient.

Molecular diversity analyses showed that *Prochlorococcus* and *Synechococcus* populations were dominated by a single ecotype or clade. The HL-adapted *Prochlorococcus* ecotype, HLII, was the most abundant ecotype throughout 1999 and in September of the following year. These results are supported by sequencing of *ntcA* and *cpeB* environmental clones from the same site sampled in 1997 and September 2000, respectively (Lindell 2000; Steglich et al. 2003), and

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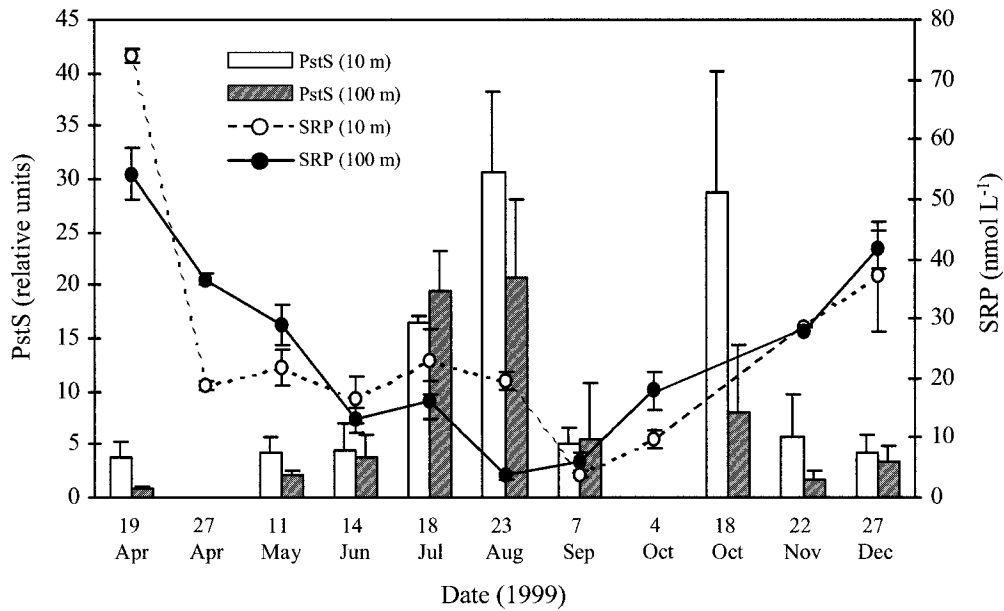


Fig. 4. Corporate expression of the phosphate-binding protein, PstS, by marine *Synechococcus* and *Prochlorococcus* during the 1999 annual cycle (mean \pm SD) at 10 and 100 m. Respective SRP concentrations (mean \pm SD) are plotted with the data from Fig. 1.

suggests a dominance of the HLII ecotype in this waterbody over longer time scales. Furthermore, 16S rDNA sequences from clone libraries from two sites in the Arabian Sea have also shown the presence of HLII, rather than HLI, ecotypes

(Zubkov et al. 2003), suggesting that HLII is the dominant *Prochlorococcus* HL ecotype throughout this oceanic region. In contrast to the HLI ecotype found in the eastern North Atlantic Ocean (West and Scanlan 1999) and the Mediterranean Sea (West et al. unpubl.), the HLII ecotype extended to the base of the euphotic zone well below the 1% isolume throughout most of the year (see Fig. 2B). This extension of HLII ecotypes to the base of the euphotic zone agrees well with our previous fluorescent in situ hybridization data from the same site (West et al. 2001). This distribution might be explained by a lack of competition from other *Prochlorococcus* ecotypes because the LL form only appeared in late summer. The late emergence of LL ecotypes from July to October 1999 and their reappearance in September 2000 (Figs. 2B, 3C,D) suggests a seasonal occurrence. The delay in colonization of LL ecotypes at Sta. A could be explained by differences in growth rates between ecotypes (from the small “seed” population remaining after winter mixing) or by invasion or repopulation of *Prochlorococcus* originating from niches and refuges within the Gulf of Aqaba or from the main body of the Red Sea, south of the Straits of Tiran. Should seeding be required from the main body of the Red Sea, a mechanism could then be envisaged whereby HLII ecotypes are transported via rapidly moving surface waters into the northern tip of the Gulf. LL ecotypes, however, would probably be photoinhibited by the high light intensities in such surface waters (Moore and Chisholm 1999); hence, their seeding populations would be confined to the slower moving, deeper waters. The sudden disappearance of LL ecotypes after mid-October might have been a result of the cooling and deepening of the SML at this time; however, a specific loss of LL ecotypes through selective grazing or viral lysis cannot be discounted.

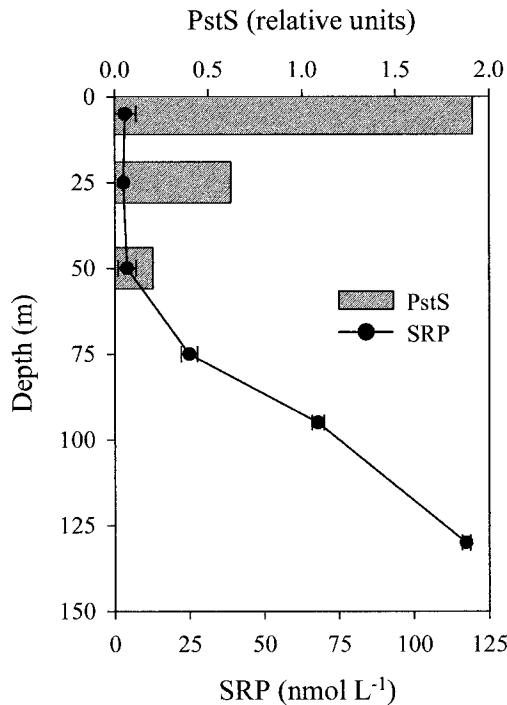


Fig. 5. Corporate expression of the phosphate-binding protein, PstS, by marine *Synechococcus* and *Prochlorococcus* down a vertical profile in September 2000. SRP concentrations (mean \pm SD) are also plotted. N.B., the relative units of PstS expression between Figs. 4 and 5 are not directly comparable.

The *Synechococcus* population in the Gulf of Aqaba was

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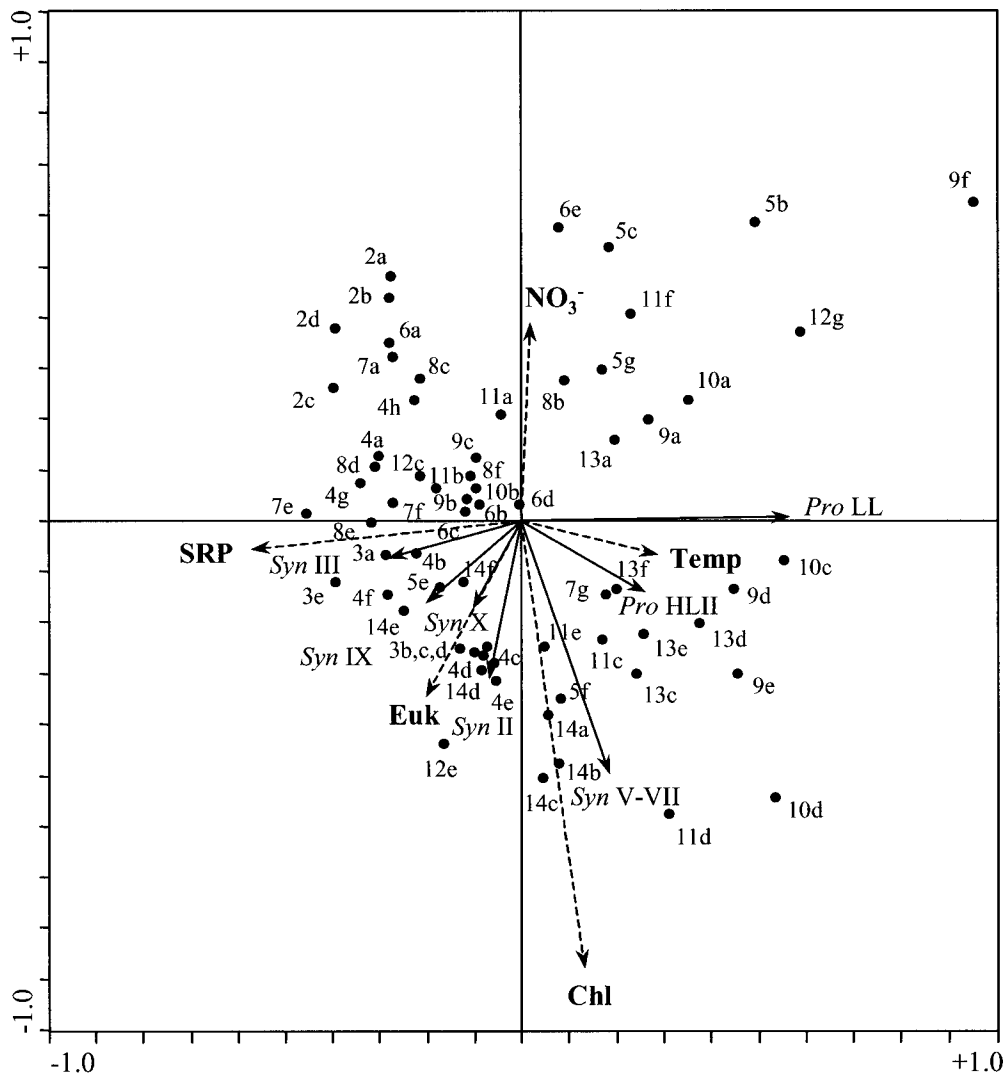


Fig. 6. Partial RDA ordination diagram of *Synechococcus* and *Prochlorococcus* dot blot hybridization data obtained from OXY107F-OXY1313R PCR amplicons (solid lines) with environmental variables (dotted lines). Data points are labeled with the number according to the date of sampling from 8 March 1999 (2) to 27 December 1999 (14). The associated letters represent depths in the profile; (a) the shallowest sampling station. NO_3^- , nitrate concentration; SRP, soluble reactive phosphate concentration; Temp, temperature; Euk, picoeukaryote abundance; Chl, Chl *a* concentration. Arrows pointing in roughly the same direction indicate a high positive correlation, arrows crossing at right angles indicate a near-zero correlation, and arrows pointing in the opposite direction have a high negative correlation.

also dominated by a single genotype, clade II, throughout 1999 and in September 2000 (Figs. 2C, 3E). Indeed, our own phylogenetic analysis of 27, 16S rDNA sequences derived from flow-sorted *Synechococcus* cells from the Arabian Sea (Zubkov et al. 2003) revealed that 26 of the sequences corresponded to clade II and 1 to clade III. This outcome suggests that members of clade II are dominant throughout this oceanic region, as well as over the ~3-yr time scale of these different studies. Furthermore, it is possible that members of *Synechococcus* clade II have a more global distribution because isolates phylogenetically affiliated with clade II have been obtained from both the Gulf of Mexico and the Sargasso Sea (Waterbury et al. 1986; Fuller et al. 2003), where-

as clone libraries and immunofluorescent studies have shown their presence in oligotrophic waters offshore from California (Ferris and Palenik 1998; Toledo and Palenik 2003). The recent immunofluorescent work showed that the serotype of *Synechococcus* CC9605 (a member of clade II) was more abundant in the SML compared with deeper waters (Toledo and Palenik 2003). However, our data suggest that members of this clade can dominate the *Synechococcus* population even at the base of the euphotic zone.

Because *Synechococcus* accounted for only a small proportion of the total picocyanobacterial abundance, low relative hybridization values were obtained when 16S rDNA was amplified with the OXY107F-OXY1313R primers. A

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Synechococcus-biased forward primer (SYN172F) used with OXY1313R allowed a much clearer resolution of the *Synechococcus* clade dynamics over the course of the year, demonstrating the importance of primer pair choice. However, the general MC-A *Synechococcus* probe (SYN1258) gave hybridization values often <100%, even for the more specific SYN172F-OXY1313R primer pair (Fig. 2C). The apparent decrease in relative hybridization values of *Synechococcus* genotypes below the SML from July to October (Fig. 2C) can be explained by the appearance of *Prochlorococcus* LL ecotypes in these samples, which numerically outnumber *Synechococcus* cells (Fig. 2A,B) and which are amplified by the SYN172F-OXY1313R primer set. Where LL ecotypes are absent, the low SYN1258 values suggest the presence of other noncharacterized MC-A *Synechococcus* 16S rDNA clades in this water column. Such a proposal is supported by *rpoC1* restriction fragment length polymorphism data from clone libraries obtained from these annual cycle samples that indicate the presence of novel genotypes (Mühling et al. unpubl.). These novel genotypes might correspond to *Synechococcus* strains CC9616 and CC9317, whose *rpoC1* sequences do not correlate with strains whose 16S rDNA sequence is known (Ferris and Palenik 1998).

To try to identify the response of *Prochlorococcus* and *Synechococcus* to their environment, we chose to use a specific molecular marker of P stress (PstS) in *Prochlorococcus* and *Synechococcus* (Scanlan et al. 1997) because previous studies had already shown evidence of P stress in cyanobacteria in the Red Sea (Li et al. 1998; Stihl et al. 2001). The combined *Prochlorococcus* and *Synechococcus* community showed marked seasonal changes of PstS expression over an annual cycle, whereas the single depth profile for September 2000 showed highest PstS expression, indicative of P stress, in the upper 50 m where SRP concentrations were extremely low. Similarly, high alkaline phosphatase (AP) activities, also indicative of P stress, which were correlated with *Synechococcus*, were observed in surface waters at the same site in the Gulf of Aqaba in 1996 (Li et al. 1998). Indeed, AP added exogenously to seawater samples showed that AP could release inorganic phosphate during November–December and April–May, but not during June–August, suggesting low concentrations of organic P hydrolyzable by this enzyme in the same region of the Gulf during the summer of 1998 (Stihl et al. 2001).

Because *Prochlorococcus* cells greatly dominated the picocyanobacterial population during the summer months (Fig. 2A), we would suggest then that most of the PstS expression observed during this period can be attributed to this genus. Although the *Prochlorococcus* population might therefore have exhibited P stress, it remained abundant throughout the summer months and showed a negative correlation with SRP. Other studies have shown a lack of correlation of *Prochlorococcus* abundance with nutrients and instead indicated that temperature was more important in *Prochlorococcus* dynamics (Cavender-Bares et al. 2001). Indeed, our study also suggests a positive correlation between *Prochlorococcus* ecotypes and temperature (Fig. 6). In contrast, *Synechococcus* abundance was low in summer, which corresponded with greater picocyanobacterial PstS expression and lower SRP concentrations. Indeed, the decline in *Synechococcus* abun-

dance in May 1999, coinciding with the onset of PstS expression, suggests that P stress might have been a causal factor in this decline. Certainly, *Synechococcus* have higher cell P quotas compared with *Prochlorococcus* (Bertilsson et al. 2003; Heldal et al. 2003); therefore, their proliferation might be restricted under low P concentrations.

SRP measurements alone are, however, insufficient for assessing the P status of these picocyanobacteria. This is evidenced by low PstS expression co-occurring with low SRP concentration in some instances (Fig. 4). We would suggest that the explanation for this low PstS expression, at a time when SRP measurements alone might indicate stress, is the bioavailability of other, likely organic, P sources at that time. This hypothesis would be consistent with earlier AP data from the Gulf of Aqaba (Stihl et al. 2001). It is already known that PstS expression in *Synechococcus* sp. strain WH 7803 is repressed by the addition of organic P sources such as dCTP, *p*-nitrophenyl phosphate, glucose-6-phosphate, or glycerol phosphate (Scanlan et al. 1997). Hence, it is quite possible that utilization of organic P sources such as P esters and phosphonates, which are now known to be major components of marine dissolved organic phosphorus (Kolowitz et al. 2001), would not be measured as SRP but would repress PstS expression. Certainly, the complete genome sequences of *Synechococcus* and *Prochlorococcus* indicate the presence of genes encoding alkaline phosphatases as well as those for phosphonate uptake, showing their potential to use organic P substrates (Palenik et al. 2003; Rocap et al. 2003).

Multivariate statistical analysis of the molecular community structure (dot blot hybridization) and environmental parameter datasets indicates that specific *Synechococcus*–*Prochlorococcus* clades respond somewhat differently to the dominant environmental gradients of phosphate and nitrate. For example, different *Synechococcus* clades showed varying responses to nitrate concentrations at Sta. A, albeit the “signal” is weak. Further use of large datasets, coupled with high-resolution sampling to fully capture the dynamics of P and N cycling and their influence on community structure, is now required to demonstrate the generality of the correlations found here, particularly with respect to specific clades and their potential adaptation to different niches. Even so, given the potential for specific picocyanobacterial clades to respond differently to environmental variables, this needs to be borne in mind, particularly when considering correlations that have previously been drawn between picocyanobacterial flow cytometric (i.e., cell abundance) data and such parameters.

This study shows the dominance of the *Prochlorococcus* HLII ecotype and *Synechococcus* clade II in the northern tip of the Red Sea with punctual appearances of the *Prochlorococcus* LL ecotype and the *Synechococcus* clade III genotype in the summer, demonstrating the utility of an annual study. *Synechococcus* and *Prochlorococcus* population dynamics were different in this study compared with those in 1992–1993, particularly with respect to the absence of a significant spring *Synechococcus* bloom, which emphasizes the need for long-term molecular studies. Furthermore, the use of PstS antibodies, which allowed for the specific interrogation of the *Synechococcus* and *Prochlorococcus* populations for their P status and which showed these organisms

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to be P stressed during the summer months, demonstrates the utility of markers that can target particular components of the bacterioplankton, rather than bulk enzyme measurements. The availability of whole genome sequences will greatly aid the development of new molecular markers of physiological state which, combined with DNA and antibody probes, will help unlock the complex processes influencing *Prochlorococcus* and *Synechococcus* population dynamics.

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