

ORIGINAL ARTICLE

Temporal variation of *Synechococcus* clades at a coastal Pacific Ocean monitoring site

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Marine cyanobacteria from the genus *Synechococcus* are found throughout the world's oceans and are important contributors to global primary productivity and carbon cycling. Cultured isolates and environmental DNA clone libraries of *Synechococcus* have demonstrated the diversity of these microbes. However, the natural distribution of this diversity through space and time and the ecological significance of their distribution are still poorly understood. To understand the seasonal dynamics of *Synechococcus* diversity, we have developed a quantitative PCR strategy using the gene encoding as a subunit of DNA-dependent RNA polymerase (*rpoC1*) and applied it to a 3-year time series of surface samples from the Scripps Institution of Oceanography pier (La Jolla, CA, USA), a coastal site in the northeastern Pacific Ocean. *Synechococcus* from clades I and IV were dominant throughout the time series and correlated with total *Synechococcus* abundance. The relative abundance of these two dominant clades showed evidence of a seasonal cycle. *Synechococcus* from clade IV were typically more abundant, but those from clade I dominated during periods just before the annual spring bloom of *Synechococcus*. *Synechococcus* from clades II and III were absent during spring and early summer, but appeared at low abundances in late summer and winter possibly due to changes in circulation in the Southern California Bight. As the first long-term time series describing *Synechococcus* population diversity, these temporal dynamics were used to interpret the genetic/genomic diversity observed in the environment and the potential factors regulating their distribution. *The ISME Journal* (2009) 3, 903–915; doi:10.1038/ismej.2009.35; published online 9 April 2009

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Introduction

Marine cyanobacteria from the genus *Synechococcus* have a fundamental biogeochemical role in the world's oceans. Due to their photosynthetic lifestyle and sheer abundance, they are significant participants in oceanic primary production and the global carbon cycle (Johnson and Sieburth, 1979; Waterbury *et al.*, 1979; Glover, 1985; Li, 1994; Smith and Kemp, 2001; Richardson and Jackson, 2007). As a component of the picophytoplankton, this group accounts for 40% of global ocean primary productivity (Agawin *et al.*, 2000). In some coastal environments, *Synechococcus* accounts for 20% of the primary productivity (Li, 1994). But to properly understand their role in the environment, we need a better understanding of the ecological niches they occupy,

how they adapt to changing environments and how these adaptations are linked to their evolution and diversification.

There is a strong foundation of genetic information describing the diversity of marine *Synechococcus*. Most marine *Synechococcus* form a monophyletic group based on phylogenetic trees constructed with 16S rRNA, *rpoC1* and other conserved gene sequences and are closely related with the other major marine cyanobacterial genus, *Prochlorococcus* (Palenik, 1994; Toledo and Palenik, 1997; Rocap *et al.*, 2002; Fuller *et al.*, 2003; Zeidner *et al.*, 2003; Chen *et al.*, 2004; Gibson *et al.*, 2006; Jenkins *et al.*, 2006; Penno *et al.*, 2006).

Within the marine *Synechococcus* group, distinct clades have been recognized based on *rpoC1* gene phylogenies (Palenik, 1994; Ferris and Palenik, 1998). These clades are supported by other gene phylogenies and have been named numerically with approximately 10 clades currently identified (Rocap *et al.*, 2002; Fuller *et al.*, 2003; Ahlgren and Rocap, 2006; Penno *et al.*, 2006). However, as these clades have been delineated based on gene

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sequence phylogenies, phenotypic traits that define each *Synechococcus* clade have not been identified except for motility, which is found only in clade III strains (Toledo *et al.*, 1999). In addition, little is known about biogeographical patterns in *Synechococcus* clades and their causes.

The spatial biogeography of *Synechococcus* diversity has been broadly described. *Synechococcus* from clades I, II and IV are the most common throughout the world's oceans (Zwirgmaier *et al.*, 2008). Clades I and IV appear to be the dominant type in temperate, coastal environments (Brown and Fuhrman, 2005; Brown *et al.*, 2005; Zwirgmaier *et al.*, 2007; Zwirgmaier *et al.*, 2008) and clade II is distributed in subtropical/tropical environments (Fuller *et al.*, 2006; Zwirgmaier *et al.*, 2008) and in off-shore, oligotrophic environments (Ferris and Palenik, 1998; Toledo and Palenik, 2003; Ahlgren and Rocap, 2006).

Few studies, however, have examined the temporal changes in *Synechococcus* diversity and abundance. These few studies have all sampled the Gulf of Aqaba in the Red Sea where *Synechococcus* from clade II are dominant (Fuller *et al.*, 2005; Muhling *et al.*, 2005, 2006; Penno *et al.*, 2006). The genetic composition of *Synechococcus* from the Gulf of Aqaba has been shown to change over time, possibly due to changes in nutrient concentrations or viral infections (Muhling *et al.*, 2005) but these studies were of short duration, typically 1 year, and used Southern hybridization, clone library or DNA fingerprinting methods which provided relative quantification only.

In this study, we have developed a quantitative PCR (qPCR) method based on *rpoC1* phylogeny to assess the abundance of the three major *Synechococcus* clades, clades I, II and IV and a fourth clade, clade III. The *rpoC1* gene sequence has proven to be a robust genetic marker for describing *Synechococcus* diversity from several marine environments (Palenik, 1994; Toledo and Palenik, 1997; Ma *et al.*, 2004; Muhling *et al.*, 2006). We have applied this qPCR method to a 3-year time series located at the Scripps Institution of Oceanography (SIO) pier, a coastal Pacific site of the Southern California Bight, and the data were analyzed in relation to physical and chemical parameters from the same site. As the first long-term time series describing *Synechococcus* population diversity, these temporal dynamics provide essential information to understand the interplay between genetic/genomic diversity, physiology and the environment.

Materials and methods

Sample collection

Surface seawater samples were collected from the end of the SIO pier in La Jolla, CA (32°53'N, 117°15'W) weekly in the morning from January 2005 to January 2008. The sampling site is beyond

the surf zone and has a bottom depth of 7 m on average with a spring tidal range of 2 m. From the seawater samples, duplicates of 1 ml seawater were preserved with 0.25% glutaraldehyde (Sigma-Aldrich, St Louis, MO, USA), incubated at room temperature for 15 min, and stored at -80 °C until analyzed by flow cytometry. For DNA analysis, either 500 or 1000 ml of the sample was filtered through a 0.2 µm Supor filter disc (Pall Life Sciences, Ann Arbor, MI, USA) to collect microorganisms and the filter disc was stored at -80 °C.

Sample collection was timed to coincide with chlorophyll, temperature, salinity and nutrient (nitrate (NO₃⁻), ammonia (NH₄⁺), phosphate (PO₄³⁻), nitrite (NO₂⁻) and reactive silicate) measurements collected biweekly through the Southern California Coastal Ocean Observing System (SCCOOS) program.

Flow cytometry

Flow cytometry was used to assess the abundance of total *Synechococcus* cells. When using flow cytometry, *Synechococcus* cells are distinguished from other picoplankton by the fluorescence of their phycoerythrin pigments. The samples were analyzed at the highest flow rate using a FACSort flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA) as described by Collier and Palenik (2003) but without applying a compensation matrix. Duplicates were counted for samples also used for DNA extraction and qPCR analysis.

DNA extraction

DNA was extracted from microorganisms collected on 0.2 µm Supor filter discs (Pall) from monthly to bimonthly samples. The filter discs were first incubated in 12.5 mg/ml lysozyme (Roche Applied Science, Indianapolis, IN, USA), 20 mM EDTA (Sigma), and 50 mM Tris (Sigma), pH 8.0 for 30 min at 37 °C. SDS (Bio-Rad, Hercules, CA, USA) and proteinase K (Sigma) were added to final concentrations of 1% and 1 mg ml⁻¹, respectively, and incubated for 2.5 h at 55 °C. RNA was removed by adding 2 mg ml⁻¹ RNase (Sigma) for 5 min at room temperature. Proteins were removed by extracting twice with an equal volume of 25:24:1 phenol/chloroform/isoamyl alcohol (Invitrogen, Carlsbad, CA, USA) and once with 24:1 chloroform/isoamyl alcohol (Fisher Scientific, Pittsburg, PA, USA). Using phenol/chloroform dissolved the Supor filter and increased the DNA extraction efficiency. The DNA in the aqueous phase was purified using a silica gel column (DNeasy Blood and Tissue Kit; Qiagen, Valencia, CA, USA) following the manufacturer's protocol and eluted from the column using 400 µl of the manufacturer's buffer. DNA was quantified using PicoGreen (Quant-iT; Qiagen), a DNA-binding fluorescent dye, following the manufacturer's protocol.

Cultured representatives of *Synechococcus* clades (CC9311 for clade I, CC9605 for clade II, WH8102 for clade III and CC9902 for clade IV) were counted, filtered and their genomic DNA extracted following the same procedure as for environmental samples. On average, 10^{10} cells were filtered for DNA extraction in a final volume of 400 μ l. The DNA extractions from cultured strains were used to test the specificity of clade-specific qPCR primers and as concentration standards.

qPCR

For each clade, two oligonucleotide primers were designed to amplify 145–304 bp fragments of the *rpoC1* gene (Table 1). *rpoC1* is a single copy gene and thus the problems when using the 16S rRNA gene as the marker locus of normalizing cell number with multiple 16S rRNA gene copies were avoided.

The primer sequences are unique for a given clade based on an alignment of all available *Synechococcus rpoC1* gene sequences in the nonredundant (nr) GenBank database from both environmental and cultured sources. A partial alignment of primer regions is provided in Supplementary Table S1. Using DNA from cultured representatives (see above), the primers were tested for clade specificity using a range of annealing temperatures in PCR reactions. An appropriate annealing temperature was chosen (Table 1) so that DNA only from the targeted clade amplified.

qPCR reactions consisted of 1X Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA, USA), 30 nM Rox reference dye (Stratagene), 0.8 μ M of each primer (Integrated DNA Technologies, Coralville, IA, USA), 0.1 mg/ml BSA (New England BioLabs, Ipswich, MA, USA), and template DNA. Ten-fold serial dilutions of environmental DNA (generally ranging from 5 ng to 0.05 pg DNA) were used as templates in the qPCR reactions. The serial dilutions served as replicates and to dilute any PCR inhibitors occurring in the DNA extractions. DNAs from cultured strains of *Synechococcus* were serially diluted over 6 orders of magnitude for use as concentration standards. Using a Stratagene 3000P

machine to acquire SYBR Green fluorescence data, the reactions were incubated at 95 °C for 10 min followed by 40 cycles of 95 °C for 30 s, the clade-specific annealing temperature for 30 s and 72 °C for 30 s, and ended with 95 °C for 1 min, a ramp down to 55 °C, and a ramp up to 95 °C at 0.2 °C s⁻¹ to assess the dissociation curve of the amplified product.

Template DNAs for the standard curves were obtained from cultured cells that were counted before the DNA extraction, thus the amount of DNA added to each standard qPCR reaction represents the DNA from a known number of cells taking into account any inefficiencies in the DNA extraction. For environmental samples, the threshold cycle (C_t) determined from each qPCR was converted to the number of cells based on the standard curve. The number of cells in each qPCR was converted to cells per ml based on the volume of seawater filtered for each sample and the volume of the DNA extraction used in the qPCRs (no. of cells in the qPCR/(volume of seawater filtered)/(volume of DNA extracted \times volume of DNA in qPCR)).

rpoC1 clone library

Two cyanobacteria-specific primers (SAN-157F = 5' YTN AAR CCN GAR ATG GAY GG and SAC-1039R = 5' CYT GYT TNC CYT CDA TDA TRT C, the numbers in the primer names denote the first nucleotide where the primers are located along the *rpoC1* gene) were designed (by J Collier) to amplify an 841 bp fragment of the *rpoC1* gene. These primers generate a longer fragment of the *rpoC1* gene and are a better match for marine cyanobacteria than previously published primer pairs (Palenik and Haselkorn, 1992; Muhling *et al.*, 2006). Using the DNA extracted from a sample collected from the SIO pier on 12 May 2005, PCR was used to amplify the *rpoC1* gene fragment from the cyanobacterial population. Template DNA (20 ng) was combined with 1.33 μ M of each primer and 25 μ l of Platinum PCR SuperMix (Invitrogen). This mixture was incubated at 94 °C for 2 min, followed by 30 cycles at 94 °C for 30 s, 52 °C for 1 min and 72 °C for 1 min, and ending with an incubation at 72 °C for 10 min.

Table 1 *Synechococcus* clade-specific qPCR primers

Clade	Model strain	Primer	Primer sequence (5'–3')	Temp. (°C)	Size (bp)	Eff. (%)
I	CC9311	RPOC362F-I	TGA AAG GGA TYC CCA GTT ATG T	59	304	96.3
		RPOC665R-I	CCC TTA CTI CCA GCA ATC TC			
II	CC9605	RPOC378F-II	CTA CGT GGC CAT CCT GCT	60	170	93.6
		RPOC547R-II	TCG GAR TCT TCG GCG TAG ATC			
III	WH8102	RPOC578F-III	GTG CTG AGG CGC TCA AGC AA	65	144	94.4
		RPOC721R-III	TGG CCA CGA AGT TAT CGA TGA C			
IV	CC9902	RPOC306F-IV	CCG TCA TCG GAT GGG CTT	56	159	99.1
		RPOC464R-IV	TCA CCT GGI TCA AGA ACG AC			

The number in the primer name denotes the first nucleotide where the primers are located along the *rpoC1* gene.

Temp. is the annealing temperature used for clade-specific amplification. Size is the nucleotide length of the PCR product in base pairs (bp). Eff. is the amplification efficiency calculated from the slope of the standard curves (Figure 2).

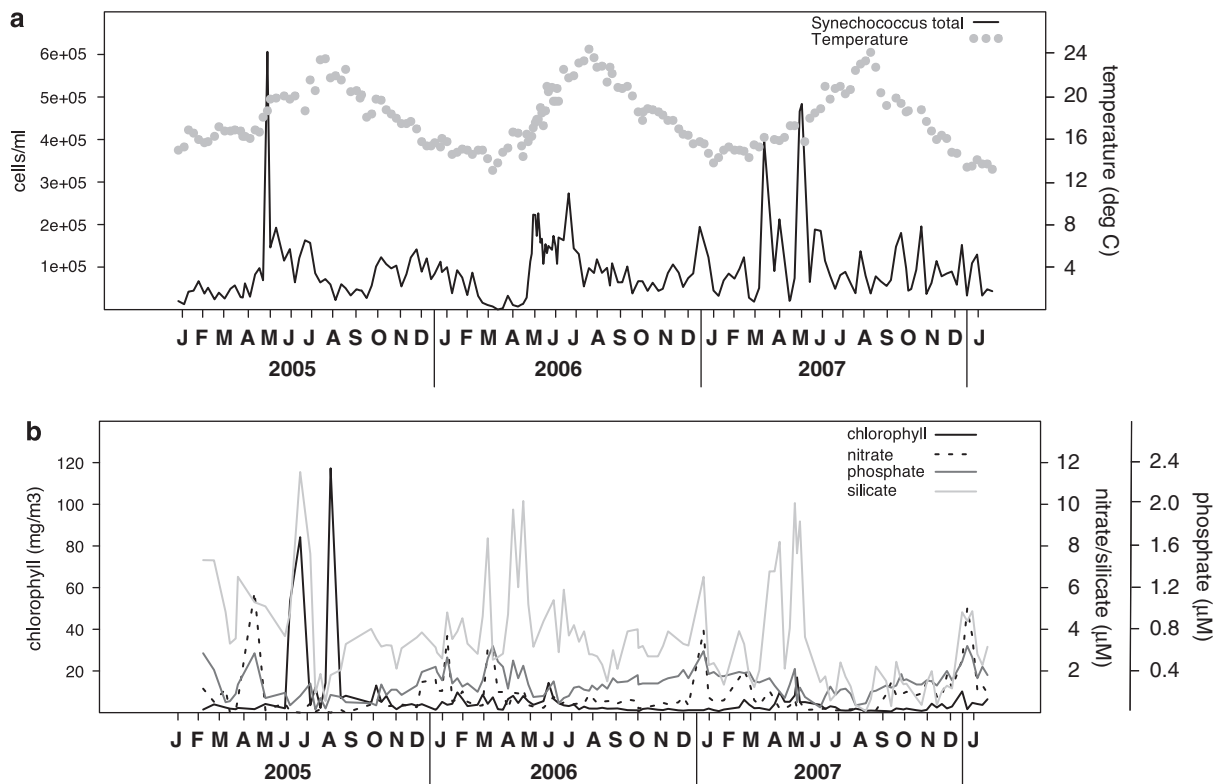


Figure 1 Three-year time series of weekly surface samples from the Scripps Institution of Oceanography (SIO) pier of (a) total *Synechococcus* abundances and temperature; (b) chlorophyll, nitrate, phosphate and silicate concentrations. Time is presented as the first letter of the month for each year.

The resulting PCR product was ligated into pCR4-TOPO plasmid vectors and cloned via transformation into TOP10 chemically competent *Escherichia coli* cells following the manufacturer's protocol (Invitrogen). Plasmid DNA was prepared from 21 clones using the QIAprep Spin Miniprep Kit (Qiagen) following the manufacturer's protocol. The *rpoC1* gene fragment insertion was sequenced (SeqXcel, San Diego, CA, USA) from both ends using the T3 and T7 sequencing primers provided by the manufacturer (Invitrogen).

The *rpoC1* gene fragment sequences were trimmed of plasmid and primer sequences and deposited in GenBank (accession numbers FJ790799–FJ790819). The sequences were aligned to known *rpoC1* gene sequences from cultured representatives of marine cyanobacteria, that is, *Synechococcus* and *Prochlorococcus*, available through GenBank. A phylogenetic tree was calculated using the neighbor-joining algorithm in PAUP (v.4.0b10) with distances calculated using the hky85 model. A 50% majority-rule consensus tree calculated from 1000 bootstrap replicates provided statistical support.

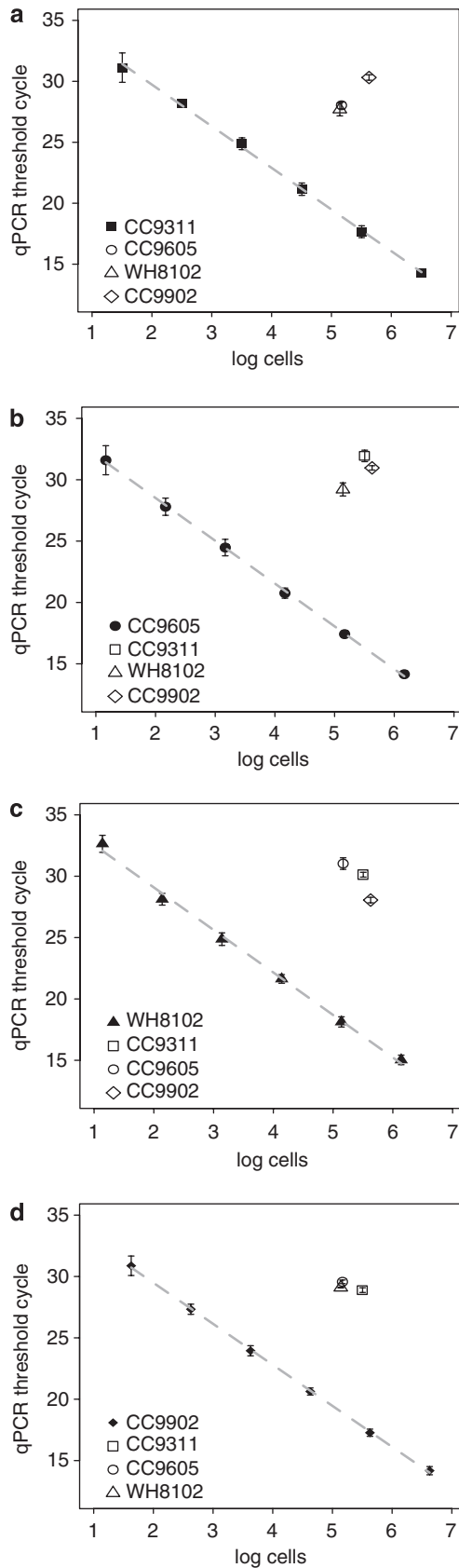
Correlation analyses

Weekly total *Synechococcus* abundances, temperature, chlorophyll, salinity and nutrient measurements were subjected to pairwise correlation

analyses. A separate dataset of bimonthly to monthly clade-specific abundances, total *Synechococcus* abundances, temperature, chlorophyll, salinity and nutrient concentrations was also analyzed. Except for temperature, the data were log transformed ($\log(x+1)$) before analysis to obtain an approximately normal distribution of data values. The Pearson's product-moment correlation coefficient (r) and the nonparametric Spearman's correlation coefficient (ρ) were calculated using JMP v. 5.0.

Results

From weekly samples over 3 years, total *Synechococcus* abundances were determined using flow cytometry. By examining the total abundances, a clear annual pattern emerged (Figure 1). Consistently in mid-May, *Synechococcus* abundance increased above basal fluctuations indicating that *Synechococcus* blooms on an annual basis. The timing of the blooms is very consistent, with the annual abundance peaks occurring on 12 May 2005 (6.06×10^5 cells ml^{-1}), 18 May 2006 (2.27×10^5 cells ml^{-1}) and 17 May 2007 (4.84×10^5 cells ml^{-1}). Second abundance peaks occurred in 2006 at the end of June (29 June 2006, 2.73×10^5 cells ml^{-1}) and in 2007 at the end of March (26 March 2007, 3.95×10^5 cells ml^{-1}), but, in general, *Synechococcus*



abundances remained below 2×10^5 cells ml^{-1} throughout the rest of the year. Abundance minimums occurred throughout the year; the lowest in the time-series was 319 cells ml^{-1} on 23 March 2006. Total *Synechococcus* abundances weakly correlated with temperature (Pearson's $r=0.30$, $P=0.0001$, Spearman's $\rho=0.28$, $P=0.0002$) and negatively with phosphate ($r=-0.31$, $P=0.005$, $\rho=0.22$, $P=0.016$), but did not correlate significantly with salinity, chlorophyll or any of the other measured nutrients. The lack of correlation with chlorophyll concentration is not surprising as larger phytoplankton, such as diatoms and dinoflagellates, can dominate more than 70% of the chlorophyll (Melissa Carter, personal communication).

Primers were developed for qPCR to assess the genetic composition of the *Synechococcus* population during this time series. The primers were tested for specificity against genomic DNA from cultured representatives. At the annealing temperature used, the primers were clade specific and did not amplify DNA from any nontarget strain (Figure 2; Supplementary Figure S1). All primers quantified DNA over 6 orders of magnitude equivalent to 10^2 to 10^7 cells. Based on the amount of seawater filtered for the DNA extraction, the detection limit for this method is approximately 10 cells ml^{-1} .

From bimonthly to monthly samples, qPCR was used to evaluate the diversity of the *Synechococcus* population from the SIO pier. *Synechococcus* from clades I and IV consistently dominated this coastal environment throughout the year (Figure 3a and b). Both clade I and clade IV abundances significantly correlated with total *Synechococcus* concentrations (Figure 4a and b; Supplementary Table S2), and the abundance maxima of these clades coincided with the annual blooms in total *Synechococcus* (Figure 3a and b). The clade I and clade IV abundances were also strongly correlated to one another (Figure 4c; Supplementary Table S2).

These two clades accounted for an average of $168 \pm 54\%$, of the total *Synechococcus* abundance (Figure 3c). The values ranged from 83% to 335% with the exception of 12 May 2005 where the two clades accounted for only 34% of the total abundance (see below). The qPCR assay may have overestimated the clade-specific abundances because dividing cells with replicated chromosomes

Figure 2 Range and specificity of quantitative PCR (qPCR) reactions using dilutions of genomic DNA from cultured *Synechococcus* strains: (a) clade I qPCR primers targeting CC9311; (b) clade II qPCR primers targeting CC9605; (c) clade III qPCR primers targeting WH8102; (d) clade IV qPCR primers targeting CC9902. The threshold cycle is the PCR cycle at which the fluorescence signal begins to increase. The number of cells in each qPCR reaction, rather than the amount of DNA, is plotted on the x axis. Closed symbols indicate qPCR reactions using DNA from *Synechococcus* strains targeted by the clade-specific qPCR primers. Open symbols indicate qPCR reactions where DNAs from nontarget strains were assayed.

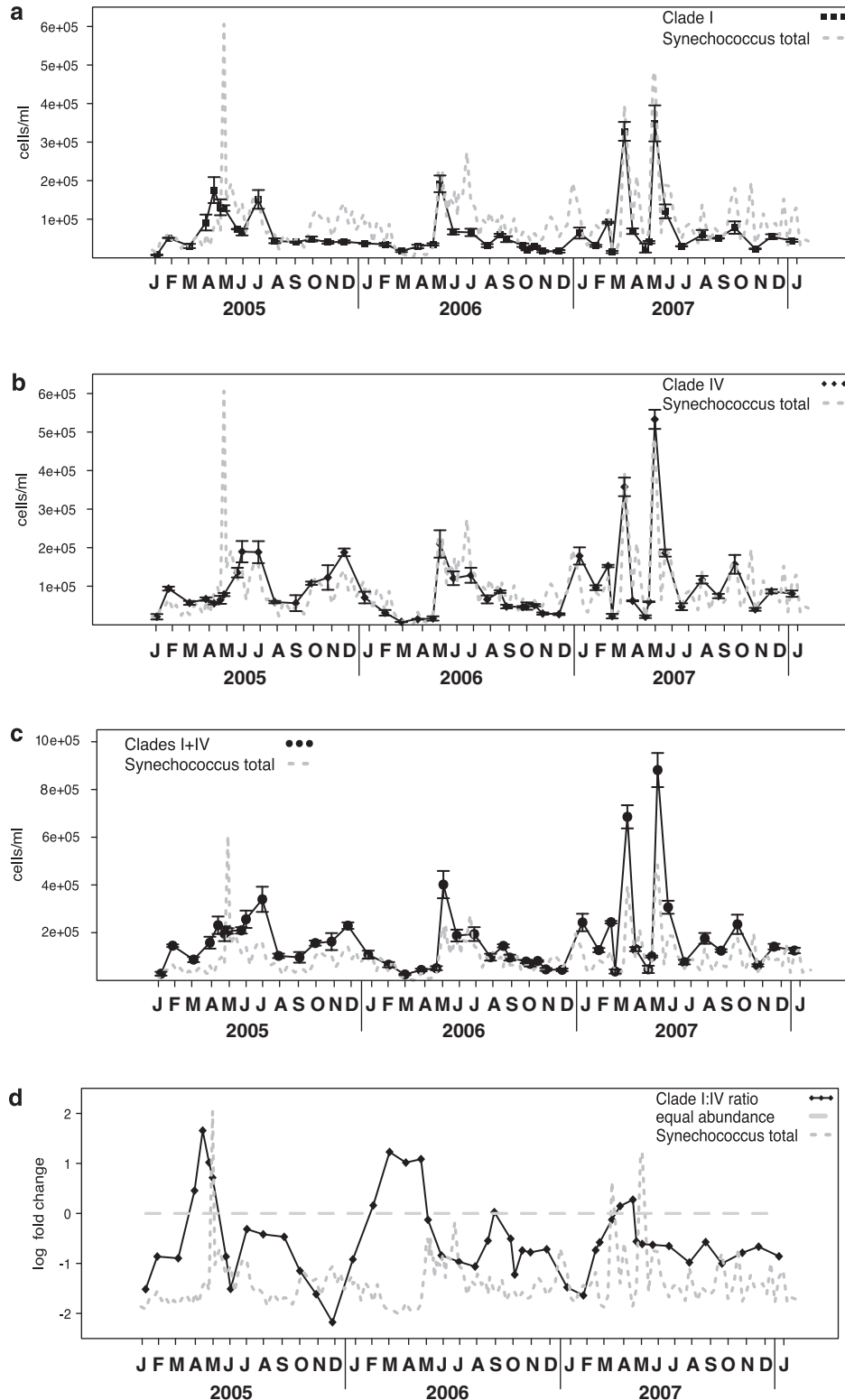
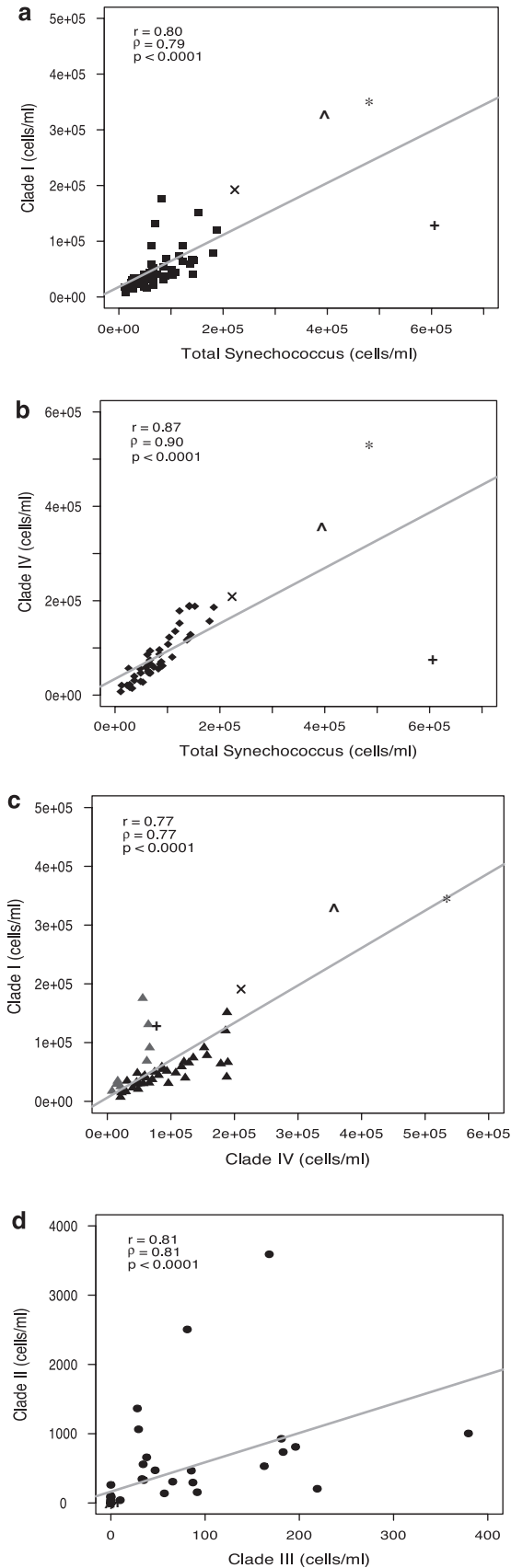


Figure 3 Abundances of the two dominant clades over 3 years from Scripps Institution of Oceanography (SIO) pier surface samples: (a) clade I abundances; (b) clade IV abundances; (c) clade I+clade IV abundances; (d) log₂ fold change of clade I to clade IV abundances. Positive values indicate a greater abundance of clade I in the environment. Negative values indicate a greater abundance of clade IV. Zero (0) indicates that each clade was in equal abundance (dashed gray line). In each panel, the dotted gray line indicates total *Synechococcus* abundances, measured weekly. Clade-specific abundances were measured monthly.



would be counted as single cells by the flow cytometer. This suggests that cells in the environment typically contained replicated chromosomes, but not the cultured cells used for qPCR standards.

The ratio of clade I to clade IV abundances was used to assess changes in the abundance of these two dominant clades relative to one another. For most of the year, clade IV *Synechococcus* were at least twice as abundant as clade I (\log_2 fold change < -1.0 ; Figure 3d). However, each year just before the annual peak in total *Synechococcus*, clade I increased to twice the abundance of clade IV (\log_2 fold change > 1.0 ; Figure 3d).

Synechococcus from clades II and III rarely occurred in this environment or occurred in low abundances (Figure 5). Clade II was typically more abundant than clade III, but both clades appeared in the winter when the water is colder and more oligotrophic, and also during the summer when the water is more likely to be stratified. The abundance of these clades was not correlated with total *Synechococcus* (Supplementary Table S2), but clade II abundances were significantly correlated with clade III abundances suggesting their dynamics may be influenced by similar mechanisms (Figure 4d).

An *rpoC1* clone library was generated to determine the composition of the *Synechococcus* population on 12 May 2005. On this date, a bloom of total *Synechococcus* was sampled, but qPCR failed to indicate which were the dominant clade types. This sample is clearly an outlier based on the plots of clade I and clade IV abundances versus total *Synechococcus* (Figures 4a and b). However, phylogenetic analyses of the sequences from the clone library indicated that the majority of the *Synechococcus* from this sample belonged to clades I and IV (Figure 6).

On examining the *rpoC1* sequences from the library, the clade I and clade IV qPCR primers do match the respective *rpoC1* sequences from this clone library, so it is not known why the qPCR assay failed to recognize the dominance of these two clades on this date. Chemical inhibitors coextracted with the environmental DNA may have inhibited the qPCR reaction. However, dilution of the environmental DNA should have avoided this problem.

Figure 4 Scatter plots of clade-specific and total *Synechococcus* abundances from samples assayed by quantitative PCR (qPCR) over the 3-year time series: (a) clade I versus total *Synechococcus* abundances; (b) clade IV versus total *Synechococcus* abundances; (c) clade I versus clade IV abundances; (d) clade II versus clade III abundances. In each panel, samples from peaks in *Synechococcus* abundance are represented by the symbols: + (12 May 2005), x (14 May 2006), ^ (26 March 2007) and * (17 May 2007). In (c), gray symbols represent samples just before the *Synechococcus* bloom where clade I abundances exceeds clade IV. Results from correlation analyses (Pearson's correlation coefficient (r) and Spearman's correlation coefficient (ρ) and its associated P value) are provided. The best linear fit is shown as a light gray line.

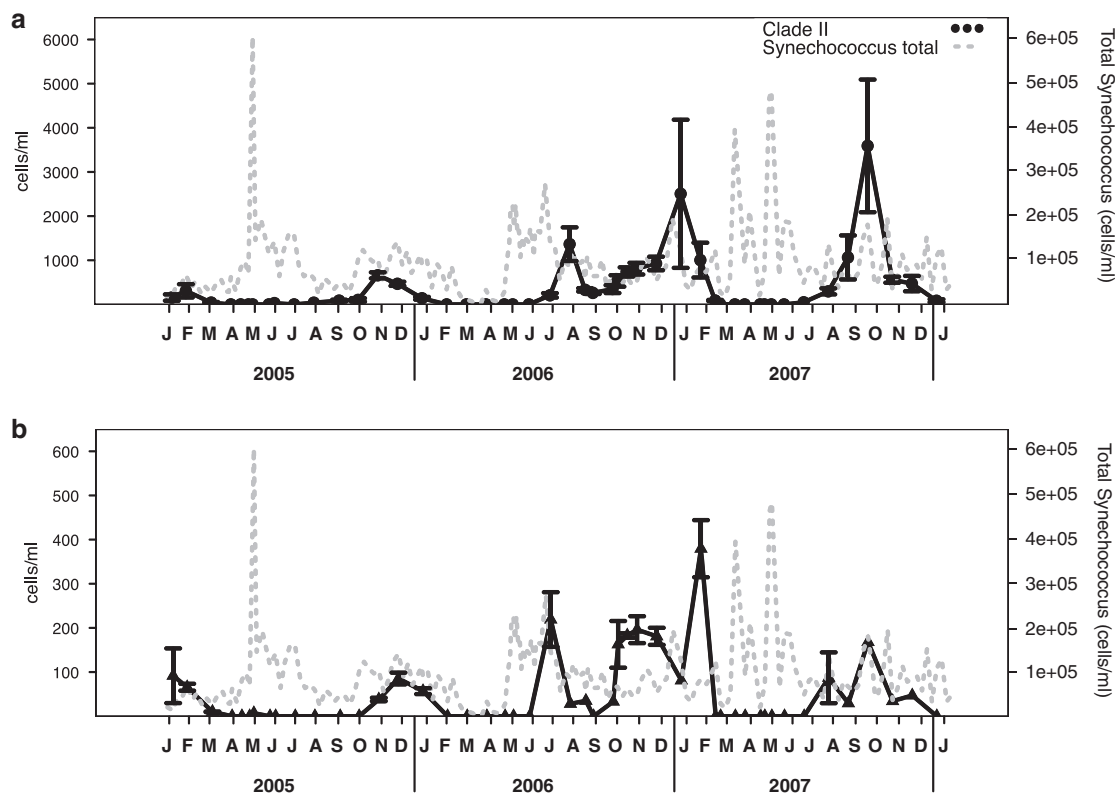


Figure 5 Abundances of the two minor clades over 3 years from Scripps Institution of Oceanography (SIO) pier surface samples: (a) clade II abundances; (b) clade III abundances. In each panel, the dotted gray line indicates total *Synechococcus* abundances, measured weekly. Clade-specific abundances were measured monthly.

Seventeen and three environmental sequences grouped with strong support to cultured clade I and clade IV strains, respectively. For clade I, the environmental sequences grouped as two separate, but strongly supported, clades distinct from cultured strains. This suggests that the genetic diversity of the *Synechococcus* population in the environment can differ significantly from that of isolated strains as also seen recently in a metagenomic study at our monitoring site (Palenik *et al.*, 2009).

Discussion

We have developed a qPCR strategy to quantify *Synechococcus* clades, as defined by *rpoC1*, 16S rRNA and 16S–23S internal transcribed spacer sequences (Ferris and Palenik, 1998; Rocap *et al.*, 2002; Fuller *et al.*, 2003). This approach enabled us to document the changes in *Synechococcus* diversity from a 3-year time series of a coastal Pacific Ocean site. From this time series, it is clear that the *Synechococcus* population was dominated by clades I and IV throughout the year (Figure 3a and b). Secondly, the abundances of these two dominant clades were correlated with the total *Synechococcus* abundance, which showed annual abundance peaks

in the month of May (Figures 4a and b). A third intriguing result was that the abundances of *Synechococcus* from clade I relative to those from clade IV did not remain static, but changed throughout the year with evidence of a seasonal cycle. In particular, although *Synechococcus* from clade IV were generally the dominant type, *Synechococcus* from clade I dominated over clade IV just before the annual abundance peaks of total *Synechococcus* (Figure 3d).

Seasonal pattern of total *Synechococcus* abundance

The annual pattern of total *Synechococcus* abundance from this coastal Pacific Ocean site differs from that of a temperate coastal Atlantic site (Woods Hole Harbor) and an oligotrophic site (Sargasso Sea). At the SIO pier, abundance maxima above 2×10^5 cells ml⁻¹ last approximately 1 week and generally occur as the temperature rises above 17 °C. In contrast, from Woods Hole Harbor, *Synechococcus* blooms when the water reaches 6 °C and the bloom period lasts months, from April until June, with high abundances of *Synechococcus* (10^5 per ml) observed until November (Waterbury *et al.*, 1986). In the Sargasso Sea, *Synechococcus* peaks in March/April with the bloom period lasting several months (Durand *et al.*, 2001).

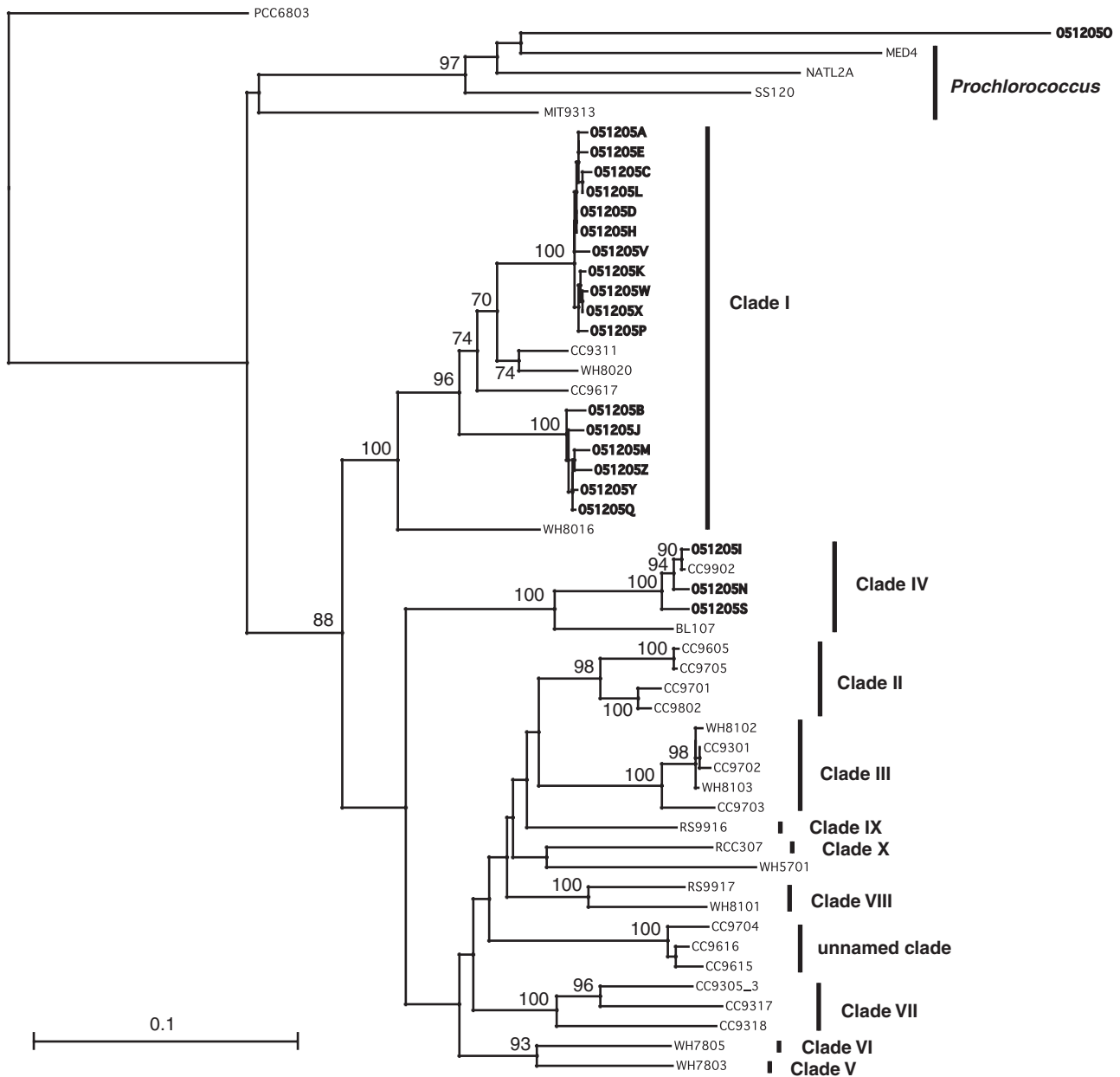


Figure 6 Neighbor-joining tree calculated from an alignment of *rpoC1* sequences from a Scripps Institution of Oceanography (SIO) pier sample on 12 May 2005 and cultured isolates. Sequences from the SIO pier sample are in bold. Values at nodes indicate percentage bootstrap support based on 1000 replicates. Values less than 70% are not shown. The scale bar indicates 10% divergence.

A rise beyond a threshold temperature appears to be a consistent factor that coincides with the timing of bloom initiation in both coastal Pacific and Atlantic oceans. Higher temperature likely causes an increased growth rate of *Synechococcus* resulting in greater abundances (Agawin *et al.*, 1998). The duration of the blooms, however, is probably more dependent on other environmental factors such as grazing pressure or nutrient availability. At the SIO pier, the relatively short bloom period suggests that mortality rates (for example, through grazing or viral lysis) also increased causing a rapid decrease in *Synechococcus* abundance.

Codominance of Synechococcus from clades I and IV
Using qPCR, we have shown that *Synechococcus* from both clades I and IV dominate the SIO pier coastal environment, yet they have different temporal dynamics. The codominance of the *Synechococcus* population by clades I and IV has been documented in other coastal environments of the Atlantic Ocean, Mediterranean Sea, eastern South Pacific Ocean, California coast and Arctic Ocean and their dominance is thought to be restricted to higher latitudes above 30°N and below 30°S (Brown and Fuhrman, 2005; Brown *et al.*, 2005; Zwirgmaier *et al.*, 2007, 2008). In the Eastern South Pacific and

the Atlantic, clade IV is more abundant than clade I, but in the Arctic they occur equally (Zwirgmaier *et al.*, 2008). However, these biogeographic surveys may have missed the temporal aspects of *Synechococcus* diversity.

From our 3-year time series, we show that the dominance of one clade over the other is dynamic over time and exhibits seasonal patterns. In particular, *Synechococcus* from clade I exceeds those from clade IV just before the annual abundance peak suggesting that the environment regularly favors clade I (or disfavors clade IV) to the disadvantage of clade IV. However, the relative importance of genetic compared to environmental factors driving these clade-specific changes in *Synechococcus* abundance is poorly understood, but it is likely that both play significant roles.

A matching temporal dataset from the SIO pier of temperature, salinity, and nitrate, phosphate, silicate and ammonia concentrations was analyzed to examine potential bottom-up environmental effects driving *Synechococcus* clade dynamics. Unfortunately none of these significantly correlated with clade-specific abundances (Supplementary Table S2). However, this does not mean that bottom-up effects are insignificant factors in dictating *Synechococcus* abundance or diversity. Other nutrients that have not been measured could have a significant influence on *Synechococcus* dynamics. *Synechococcus* have been shown to use urea as a nitrogen source (Collier *et al.*, 1999) and other organic nutrients are likely important to their survival (Moore *et al.*, 2002, 2005; Zubkov *et al.*, 2003; Bronk *et al.*, 2007). Trace metals may also play a significant role (Hutchins *et al.*, 1999; Leao *et al.*, 2007; Dupont *et al.*, 2008).

The top-down environmental effects on *Synechococcus* populations are also not well understood. A wide diversity of both protozoan grazers (Caron *et al.*, 1991; Strom, 1991; Christaki *et al.*, 1999, 2002; Guillou *et al.*, 2001; Jeong *et al.*, 2005) and viruses (Suttle and Chan, 1993; Waterbury and Valois, 1993; Wilson *et al.*, 1993) have been identified that prey on *Synechococcus*. These mortality agents can be the dominating factor regulating total *Synechococcus* abundances (see for example, Baudoux *et al.*, 2007), but their overall diversity, specificity to *Synechococcus* types and impact as selective agents remain largely unknown.

Genetic information in association with biogeographic data provides another basis for understanding the factors driving the dominance of clades I and IV in coastal environments and their coexistence. Genome sequences from cultured representatives of clades I and IV are available (Palenik *et al.*, 2006; Dufresne *et al.*, 2008) as are sequences from representatives of several other clades (Palenik *et al.*, 2003) providing a strong backbone of genetic information for this microbial taxon.

CC9311, a representative of clade I isolated from the California Current, has a genome with increased

sensory and response regulator genes, fewer phosphate scavenging genes, a greater capacity for metal transport and usage and more genes related to organic nutrient use than an open ocean strain (Palenik *et al.*, 2006). These genes may be key to their survival in a coastal location with higher nutrient availability and a more variable, dynamic environment compared to an oligotrophic one.

The genomes of two clade IV isolates, CC9902 and BL107, have also been sequenced. These strains have fewer sensory histidine kinase and response regulator genes than CC9311 and could represent a 'specialist' lifestyle (Dufresne *et al.*, 2008). The genomes of CC9902 and BL107 are approximately 0.38 and 0.33 Mb smaller than CC9311, respectively, and have fewer clade-specific genes. With a larger genetic repertoire, clade I types may be more versatile or 'opportunists' (Dufresne *et al.*, 2008). For example, CC9311 has genes capable of transporting iron (II) or halogenating compounds via a vanadium-dependent bromoperoxidase (Palenik *et al.*, 2006), but without more specific physiological investigations, the correlations between genetic composition and niche adaptation remain hypothetical.

The correlations between genetic composition and ecological niche are further complicated by the tremendous genetic diversity that exists within *Synechococcus* clades. By examining the diversity captured by our *rpoC1* clone library, *Synechococcus* that were dominant in the environment grouped separately from cultured representatives (Figure 6). In addition, as with other microbial taxa (Medini *et al.*, 2005; Tettelin *et al.*, 2005; Kettler *et al.*, 2007), *Synechococcus* genomes contain mostly conserved core genes, but many highly variable regions exist containing genes with a diversity of functions, likely the result of horizontal gene transfer events, that may be significant to their physiology and ecological niche (Palenik *et al.*, 2009).

Occasional occurrence of Synechococcus from clades II and III

For the two minor clades, clades II and III, their presence in this coastal environment may be more reflective of physical oceanographic patterns. Their occurrence in late summer coincides with periods of strong stratification (Palacios *et al.*, 2004). Stratification has the effect of reducing the input of colder, more saline and nutrient-rich waters from below, therefore surface waters become nutrient depleted. In the fall and winter, stratification is weaker, but there is a consistent northward flow, the Inshore Countercurrent, in the coastal regions of the Southern California Bight in contrast to the southward flow that dominates all other times of the year (Hickey, 1979; Lynn and Simpson, 1987; Di Lorenzo, 2003). The source of the northward flowing waters is the off-shore eastern boundary current of the North Pacific gyre, the California Current, that has turned toward the shore south of the US-Mexico border,

then divides and turns north into the Southern California Bight as the Inshore Countercurrent and south alongside Baja California (Hickey, 1979; Lynn and Simpson, 1987). This northward flow brings more oligotrophic, nutrient-poor waters to the coast and may advect *Synechococcus* from clades II and III that are relatively more abundant off-shore toward the coast (Ferris and Palenik, 1998; Toledo and Palenik, 2003). Although our sampling site is on the coastal shelf, the site is influenced by these major circulation trends, as well as by alongshore currents, cross-shelf tidal currents, and currents generated by local wind stress (Winant and Bratkovich, 1981).

Both stratification and the northward Inshore Countercurrent may have the effect of lowering nutrient concentrations of surface waters in the Southern California Bight and these conditions may explain the occurrence of *Synechococcus* from clades II and III at the SIO pier. However, we were unable to find a significant relationship between nutrient concentrations and the abundance of these clades.

An alternative explanation may be the advection of *Synechococcus* from clade II from the south. If clade II is predominantly distributed in subtropical/tropical coastal environments (Zwirgmaier *et al.*, 2008), then northward flow may bring these *Synechococcus* types to Southern California from the south. Observing a transition from clade I and clade IV dominance in temperate environments to clade II closer to the equator would support this hypothesis. A similar shift in *Synechococcus* clade dominance has been observed in the Atlantic Ocean (Zwirgmaier *et al.*, 2007).

The observation of *Synechococcus* from clades II and III in late summer and winter loosely coincides with the presence of *Prochlorococcus* at this site. *Prochlorococcus* is usually absent at the SIO pier during the spring months, although in addition to advection to and away from this location, their absence may also be due to increased grazing pressure (Worden *et al.*, 2004).

Conclusion

The biogeography of environmental microbes is a fundamental piece of information that is critical to understanding their evolution, adaptability and function in their respective ecosystems (Ramette and Tiedje, 2007). In addition, as *Synechococcus* are major contributors to primary production, their biogeography and the genetic and environmental factors influencing the composition of the population has important implications for understanding carbon cycling and other globally important biogeochemical processes. Through the use of qPCR, we have provided an important tool for studying *Synechococcus* biogeography, ecology and evolution. Although we have used qPCR to assess the

temporal dynamics of four globally distributed *Synechococcus* clades, this method can be applied to any scale of spatial and temporal sampling. With these tools in hand, the key is to discover the scales at which adaptation occurs and how genetic diversity influences and informs us of their ecological niches.

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References

- Agawin NSR, Duarte CM, Agusti S. (1998). Growth and abundance of *Synechococcus* sp. in a Mediterranean Bay: seasonality and relationship with temperature. *Mar Ecol Prog Ser* **170**: 45–53.
- Agawin NSR, Duarte CM, Agusti S. (2000). Nutrient and temperature control of the contribution of picoplankton to phytoplankton biomass and production. *Limnol Oceanogr* **45**: 591–600.
- Ahlgren NA, Rocap G. (2006). Culture isolation and culture-independent clone libraries reveal new marine *Synechococcus* ecotypes with distinctive light and N physiologies. *Appl Environ Microbiol* **72**: 7193–7204.
- Baudoux AC, Veldhuis MJW, Witte HJ, Brussaard CPD. (2007). Viruses as mortality agents of picophytoplankton in the deep chlorophyll maximum layer during IRONAGES III. *Limnol Oceanogr* **52**: 2519–2529.
- Bronk DA, See JH, Bradley P, Killberg L. (2007). DON as a source of bioavailable nitrogen for phytoplankton. *Biogeosciences* **4**: 283–296.
- Brown MV, Fuhrman JA. (2005). Marine bacterial microdiversity as revealed by internal transcribed spacer analysis. *Aquat Microb Ecol* **41**: 15–23.
- Brown MV, Schwalbach MS, Hewson I, Fuhrman JA. (2005). Coupling 16S-ITS rDNA clone libraries and automated ribosomal intergenic spacer analysis to show marine microbial diversity: development and application to a time series. *Environ Microbiol* **7**: 1466–1479.
- Caron DA, Lim EL, Miceli G, Waterbury JB, Valois FW. (1991). Grazing and utilization of chroococcoid cyanobacteria and heterotrophic bacteria by protozoa in laboratory cultures and a coastal plankton community. *Mar Ecol Prog Ser* **76**: 205–217.
- Chen F, Wang K, Kan JJ, Bachoon DS, Lu JR, Lau S *et al.* (2004). Phylogenetic diversity of *Synechococcus* in the Chesapeake Bay revealed by ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO) large subunit gene (rbcL) sequences. *Aquat Microb Ecol* **36**: 153–164.
- Christaki U, Courties C, Karayanni H, Giannakourou A, Maravelias C, Kormas KA *et al.* (2002). Dynamic

- characteristics of *Prochlorococcus* and *Synechococcus* consumption by bacterivorous nanoflagellates. *Microb Ecol* **43**: 341–352.
- Christaki U, Jacquet S, Dolan JR, Vaulot D, Rassoulzadegan F. (1999). Growth and grazing on *Prochlorococcus* and *Synechococcus* by two marine ciliates. *Limnol Oceanogr* **44**: 52–61.
- Collier JL, Brahmsha B, Palenik B. (1999). The marine cyanobacterium *Synechococcus* sp. WH7805 requires urease (urea amidohydrolase, EC 3.5.1.5) to utilize urea as a nitrogen source: molecular-genetic and biochemical analysis of the enzyme. *Microbiology* **145**: 447–459.
- Collier JL, Palenik B. (2003). Phycoerythrin-containing picoplankton in the Southern California Bight. *Deep Sea Res Part II* **50**: 2405–2422.
- Di Lorenzo E. (2003). Seasonal dynamics of the surface circulation in the Southern California Current System. *Deep Sea Res Part II* **50**: 2371–2388.
- Dufresne A, Ostrowski M, Scanlan DJ, Garczarek L, Mazard S, Palenik BP *et al.* (2008). Unraveling the genomic mosaic of a ubiquitous genus of marine cyanobacteria. *Genome Biol* **9**: R90.
- Dupont CL, Barbeau K, Palenik B. (2008). Ni uptake and limitation in marine *Synechococcus* strains. *Appl Environ Microbiol* **74**: 23–31.
- Durand MD, Olson RJ, Chisholm SW. (2001). Phytoplankton population dynamics at the Bermuda Atlantic Time-series station in the Sargasso Sea. *Deep Sea Res Part II* **48**: 1983–2003.
- Ferris MJ, Palenik B. (1998). Niche adaptation in ocean cyanobacteria. *Nature* **396**: 226–228.
- Fuller NJ, Marie D, Partensky F, Vaulot D, Post AF, Scanlan DJ. (2003). Clade-specific 16S ribosomal DNA oligonucleotides reveal the predominance of a single marine *Synechococcus* clade throughout a stratified water column in the Red Sea. *Appl Environ Microbiol* **69**: 2430–2443.
- Fuller NJ, Tarran GA, Yallop M, Orcutt KM, Scanlan DJ. (2006). Molecular analysis of picocyanobacterial community structure along an Arabian Sea transect reveals distinct spatial separation of lineages. *Limnol Oceanogr* **51**: 2515–2526.
- Fuller NJ, West NJ, Marie D, Yallop M, Rivlin T, Post AF *et al.* (2005). Dynamics of community structure and phosphate status of picocyanobacterial populations in the Gulf of Aqaba, Red Sea. *Limnol Oceanogr* **50**: 363–375.
- Gibson AH, Jenkins BD, Wilkerson FP, Short SM, Zehr JP. (2006). Characterization of cyanobacterial *glnA* gene diversity and gene expression in marine environments. *FEMS Microbiol Ecol* **55**: 391–402.
- Glover HE. (1985). The physiology and ecology of the marine cyanobacterial genus *Synechococcus*. *Adv Aquat Microbiol* **3**: 49–107.
- Guillou L, Jacquet S, Chretiennot-Dinet MJ, Vaulot D. (2001). Grazing impact of two small heterotrophic flagellates on *Prochlorococcus* and *Synechococcus*. *Aquat Microb Ecol* **26**: 201–207.
- Hickey BM. (1979). The California Current System—hypotheses and facts. *Prog Oceanogr* **8**: 191–279.
- Hutchins DA, Witter AE, Butler A, Luther GW. (1999). Competition among marine phytoplankton for different chelated iron species. *Nature* **400**: 858–861.
- Jenkins BD, Zehr JP, Gibson A, Campbell L. (2006). Cyanobacterial assimilatory nitrate reductase gene diversity in coastal and oligotrophic marine environments. *Environ Microbiol* **8**: 2083–2095.
- Jeong HJ, Park JY, Nho JH, Park MO, Ha JH, Seong KA *et al.* (2005). Feeding by red-tide dinoflagellates on the cyanobacterium *Synechococcus*. *Aquat Microb Ecol* **41**: 131–143.
- Johnson PW, Sieburth JM. (1979). Chroococcoid cyanobacteria in the sea: a ubiquitous and diverse phototropic biomass. *Limnol Oceanogr* **24**: 928–935.
- Kettler GC, Martiny AC, Huang K, Zucker J, Coleman ML, Rodrigue S *et al.* (2007). Patterns and implications of gene gain and loss in the evolution of *Prochlorococcus*. *PLoS Genet* **3**: 2515–2528.
- Leao PN, Vasconcelos MTSD, Vasconcelos VM. (2007). Role of marine cyanobacteria in trace metal bioavailability in seawater. *Microb Ecol* **53**: 104–109.
- Li KW. (1994). Primary production of prochlorophytes, cyanobacteria, and eukaryotic ultraphytoplankton: measurements from flow cytometric sorting. *Limnol Oceanogr* **39**: 169–175.
- Lynn RJ, Simpson JJ. (1987). The California Current System—the seasonal variability of its physical characteristics. *J Geophys Res* **92**: 12947–12966.
- Ma Y, Jiao NZ, Zeng YH. (2004). Natural community structure of cyanobacteria in the South China Sea as revealed by *rpoC1* gene sequence analysis. *Lett Appl Microbiol* **39**: 353–358.
- Medini D, Donati C, Tettelin H, Massignani V, Rappuoli R. (2005). The microbial pan-genome. *Curr Opin Genet Dev* **15**: 589–594.
- Moore LR, Ostrowski M, Scanlan DJ, Feren K, Sweetsir T. (2005). Ecotypic variation in phosphorus acquisition mechanisms within marine picocyanobacteria. *Aquat Microb Ecol* **39**: 257–269.
- Moore LR, Post AF, Rocap G, Chisholm SW. (2002). Utilization of different nitrogen sources by the marine cyanobacteria *Prochlorococcus* and *Synechococcus*. *Limnol Oceanogr* **47**: 989–996.
- Muhling M, Fuller NJ, Millard A, Somerfield PJ, Marie D, Wilson WH *et al.* (2005). Genetic diversity of marine *Synechococcus* and co-occurring cyanophage communities: evidence for viral control of phytoplankton. *Environ Microbiol* **7**: 499–508.
- Muhling M, Fuller NJ, Somerfield PJ, Post AF, Wilson WH, Scanlan DJ *et al.* (2006). High resolution genetic diversity studies of marine *Synechococcus* isolates using *rpoC1*-based restriction fragment length polymorphism. *Aquat Microb Ecol* **45**: 263–275.
- Palacios DM, Bograd SJ, Mendelssohn R, Schwing FB. (2004). Long-term and seasonal trends in stratification in the California Current, 1950–1993. *J Geophys Res* **109**: C10016.
- Palenik B. (1994). Cyanobacterial community structure as seen from RNA polymerase gene sequence analysis. *Appl Environ Microbiol* **60**: 3212–3219.
- Palenik B, Brahmsha B, Larimer FW, Land M, Hauser L, Chain P *et al.* (2003). The genome of a motile marine *Synechococcus*. *Nature* **424**: 1037–1042.
- Palenik B, Haselkorn R. (1992). Multiple evolutionary origins of prochlorophytes, the chlorophyll B-containing prokaryotes. *Nature* **355**: 265–267.
- Palenik B, Ren QH, Dupont CL, Myers GS, Heidelberg JF, Badger JH *et al.* (2006). Genome sequence of *Synechococcus* CC9311: insights into adaptation to a coastal environment. *Proc Natl Acad Sci USA* **103**: 13555–13559.
- Palenik B, Ren Q, Tai V, Paulsen IT. (2009). Coastal *Synechococcus* metagenome reveals major roles for

- horizontal gene transfer and plasmids in population diversity. *Environ Microbiol* **11**: 349–359.
- Penno S, Lindell D, Post AF. (2006). Diversity of *Synechococcus* and *Prochlorococcus* populations determined from DNA sequences of the N-regulatory gene *ntcA*. *Environ Microbiol* **8**: 1200–1211.
- Ramette A, Tiedje JM. (2007). Biogeography: an emerging cornerstone for understanding prokaryotic diversity, ecology, and evolution. *Microb Ecol* **53**: 197–207.
- Richardson TL, Jackson GA. (2007). Small phytoplankton and carbon export from the surface ocean. *Science* **315**: 838–840.
- Rocap G, Distel DL, Waterbury JB, Chisholm SW. (2002). Resolution of *Prochlorococcus* and *Synechococcus* ecotypes by using 16S-23S ribosomal DNA internal transcribed spacer sequences. *Appl Environ Microbiol* **68**: 1180–1191.
- Smith EM, Kemp WM. (2001). Size structure and the production/respiration balance in a coastal plankton community. *Limnol Oceanogr* **46**: 473–485.
- Strom SL. (1991). Growth and grazing rates of the herbivorous dinoflagellate *Gymnodinium* sp from the open subarctic Pacific Ocean. *Mar Ecol Prog Ser* **78**: 103–113.
- Suttle CA, Chan AM. (1993). Marine cyanophages infecting oceanic and coastal strains of *Synechococcus*—abundance, morphology, cross-infectivity and growth characteristics. *Mar Ecol Prog Ser* **92**: 99–109.
- Tettelin H, Masignani V, Cieslewicz MJ, Donati C, Medini D, Ward NL *et al.* (2005). Genome analysis of multiple pathogenic isolates of *Streptococcus agalactiae*: implications for the microbial ‘pan-genome’. *Proc Natl Acad Sci USA* **102**: 13950–13955.
- Toledo G, Palenik B. (1997). *Synechococcus* diversity in the California current as seen by RNA polymerase (*rpoC1*) gene sequences of isolated strains. *Appl Environ Microbiol* **63**: 4298–4303.
- Toledo G, Palenik B. (2003). A *Synechococcus* serotype is found preferentially in surface marine waters. *Limnol Oceanogr* **48**: 1744–1755.
- Toledo G, Palenik B, Brahmsha B. (1999). Swimming marine *Synechococcus* strains with widely different photosynthetic pigment ratios form a monophyletic group. *Appl Environ Microbiol* **65**: 5247–5251.
- Waterbury JB, Valois FW. (1993). Resistance to co-occurring phages enables marine *Synechococcus* communities to coexist with cyanophages abundant in seawater. *Appl Environ Microbiol* **59**: 3393–3399.
- Waterbury JB, Watson SW, Guillard RRL, Brand LE. (1979). Widespread occurrence of a unicellular, marine, planktonic, cyanobacterium. *Nature* **277**: 293–294.
- Waterbury JB, Watson SW, Valois FW, Franks DG. (1986). Biological and ecological characterization of the marine unicellular cyanobacterium *Synechococcus*. *Can Bull Fish Aquat Sci* **214**: 71–120.
- Wilson WH, Joint IR, Carr NG, Mann NH. (1993). Isolation and molecular characterization of five marine cyanophages propagated on *Synechococcus* sp. strain WH7803. *Appl Environ Microbiol* **59**: 3736–3743.
- Winant CD, Bratkovich AW. (1981). Temperature and currents on the southern California shelf—a description of the variability. *J Phys Oceanogr* **11**: 71–86.
- Worden AZ, Nolan JK, Palenik B. (2004). Assessing the dynamics and ecology of marine picophytoplankton: the importance of the eukaryotic component. *Limnol Oceanogr* **49**: 168–179.
- Zeidner G, Preston CM, Delong EF, Massana R, Post AF, Scanlan DJ *et al.* (2003). Molecular diversity among marine picophytoplankton as revealed by *psbA* analyses. *Environ Microbiol* **5**: 212–216.
- Zubkov MV, Fuchs BM, Tarran GA, Burkill PH, Amann R. (2003). High rate of uptake of organic nitrogen compounds by *Prochlorococcus* cyanobacteria as a key to their dominance in oligotrophic oceanic waters. *Appl Environ Microbiol* **69**: 1299–1304.
- Zwirgmaier K, Heywood JL, Chamberlain K, Woodward EMS, Zubkov MV, Scanlan DJ. (2007). Basin-scale distribution patterns lineages in the Atlantic Ocean. *Environ Microbiol* **9**: 1278–1290.
- Zwirgmaier K, Jardillier L, Ostrowski M, Mazard S, Garczarek L, Vault D *et al.* (2008). Global phylogeography of marine *Synechococcus* and *Prochlorococcus* reveals a distinct partitioning of lineages among oceanic biomes. *Environ Microbiol* **10**: 147–161.

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