**Biogeosciences
Discussions**

Is the distribution of *Prochlorococcus* and *Synechococcus* ecotypes in the Mediterranean Sea affected by global warming?

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Title Page

Abstract	Introduction
Conclusions	References
Tables	Figures

◄◄   ►►

◄   ►

Back   Close

Full Screen / Esc

Printer-friendly Version

Interactive Discussion

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Title Page

Abstract | Introduction

Conclusions | References

Tables | Figures

◄ | ►

◄ | ►

Back | Close

Full Screen / Esc

Printer-friendly Version

Interactive Discussion

Abstract

Biological communities populating the Mediterranean Sea, which is situated at the northern boundary of the subtropics, are often claimed to be particularly affected by global warming. This is indicated, for instance, by the introduction of (sub)tropical species of fish or invertebrates that can displace local species. This raises the question of whether microbial communities are similarly affected, especially in the Levantine basin where sea surface temperatures have risen in recent years. In this paper, the genetic diversity of the two most abundant members of the phytoplankton community, the picocyanobacteria *Prochlorococcus* and *Synechococcus*, was examined on a transect from the South coast of France to Cyprus in the summer of 2008 (BOUM cruise). Diversity was studied using dot blot hybridization with clade-specific 16S rRNA oligonucleotide probes and clone libraries of the 16S–23S ribosomal DNA Internal Transcribed Spacer (ITS) region. Data were compared with those obtained during the PROSOPE cruise held almost a decade earlier, with a focus on the abundance of clades that may constitute bioindicators of warm waters. During both cruises, the dominant *Prochlorococcus* clade in the upper mixed layer at all stations was HLI, a clade typical of temperate waters, whereas the HLII clade, the dominant group in (sub)tropical waters, was only present at very low concentrations. The *Synechococcus* community was dominated by clades I, III and IV in the northwestern waters of the Gulf of Lions and by clade III and groups genetically related to clades WPC1 and VI in the rest of the Mediterranean Sea. In contrast, only a few sequences of clade II, a group typical of warm waters, were observed. These data indicate that local cyanobacterial populations have not yet been displaced by their (sub)tropical counterparts. This is discussed in the context of the low phosphorus concentrations found in surface waters in the eastern Mediterranean basin, as this may constitute a barrier to the colonization of these waters by alien picocyanobacterial groups.

1 Introduction

Due to their ubiquity and abundance, marine cyanobacteria are widely recognized as being major contributors to global photosynthetic biomass and primary production (Campbell et al., 1994; Goericke and Welschmeyer, 1993; Li, 1994; Partensky et al., 1999). Two genera dominate the picophytoplankton size fraction (0.2–2 µm), *Prochlorococcus* and *Synechococcus*. Although these genera co-occur in tropical and temperate areas, their abundance patterns differ not only spatially but also seasonally (Johnson et al., 2006; Tai and Palenik, 2009; Zwirglmaier et al., 2008). Indeed, *Synechococcus* prefers well-lit, nutrient-rich waters and usually blooms in spring when the water column is mixed, whereas *Prochlorococcus* is most abundant in oligotrophic waters and reaches maximal concentrations in summer and fall when waters are stratified, with a deeper sub-surface maximum than *Synechococcus* (Campbell et al., 1994; DuRand et al., 2001; Lindell and Post, 1995). Both genera are culturable and this has considerably facilitated studies on their physiological and genetic diversity. Each comprises a number of clades or "ecotypes" (sensu Coleman and Chisholm, 2007) exhibiting distinct ecophysiologies and spatial distributions.

For *Prochlorococcus*, several different ecotypes coexist in the water column. The upper part of the euphotic zone is occupied by high light-adapted (HL) ecotypes. HLII (also called eMIT9312) dominates in permanently stratified waters from the equator to subtropics, and is progressively replaced at high latitude by HLI (also called eMED4; Johnson et al., 2006; Zwirglmaier et al., 2007, 2008). The distinct latitudinal distribution pattern of *Prochlorococcus* HLI and HLII ecotypes is well supported by the different temperature ranges for growth of representative isolates (Johnson et al., 2006; Zinser et al., 2007). These complementary distributions make variations of the relative concentrations of these two HL ecotypes a good potential bioindicator of community shifts as a result of temperature changes of oceanic waters. Two further HL clades (HNLC1 and HNLC2) have recently been shown to occur specifically in warm, iron-depleted areas of the ocean, such as the eastern Equatorial Pacific upwelling and the tropical Indian Ocean (Rusch et al., 2010; West et al., 2010).

Until recently, it was thought that the bottom of the euphotic layer was dominated by the low light-adapted LLIV ecotype (also called eMIT9313), but recent evidence using qPCR analyses with refined ecotype-specific primers suggests that it co-occurs with the LLII ecotype (also called eSS120), and the latter one could even outnumber LLIV (Malmstrom et al., 2010). A fifth ecotype (LLI, also called eNATL) was found to occupy an intermediate niche in the water column, in the vicinity of the thermocline (Johnson et al., 2006; Zinser et al., 2006, 2007). Clone library sequencing has also revealed the presence of several additional but still uncultured LL clades, including NC1, a potentially abundant group found at depth at several stations of the Atlantic and Pacific oceans (Martiny et al., 2009b), and two LL clades (LLV and LLVI) seemingly specific for low oxygen zones (Lavin et al., 2010). However, at high latitudes, the lower stability of the water column progressively simplifies these depth distribution patterns, since only HLI and LLI ecotypes appear able to survive in mixed, cool water conditions (Johnson et al., 2006).

For *Synechococcus*, some general distribution patterns can also be defined for several of the ten clades defined by Fuller and coworkers (Fuller et al., 2003), although the genetic and/or physiological bases behind these patterns are much less well understood than for *Prochlorococcus*. Clades I and IV generally co-occur at latitudes above 30° N/S and seem to be restricted to near coastal waters in the lowest part of their latitudinal distributions (Tai and Palenik, 2009; Zwirglmaier et al., 2007, 2008; Mazard et al., 2011). In contrast, clade II seems to be abundant in warm, coastal or shelf areas and is thought to be the (sub)tropical counterpart of clades I/IV, although some overlap may occur in the boundary zone, i.e. between 30° and 35°, in both the Southern and Northern Hemispheres (Zwirglmaier et al., 2008). The latitudinal distribution of clade III appears to be broader, but cells belonging to this ecotype seemingly prefer oligotrophic, offshore waters. All other clades are generally found at lower concentrations than clades I to IV and their distribution patterns are therefore less clearly defined (Zwirglmaier et al., 2008).

Title Page

Abstract | Introduction

Conclusions | References

Tables | Figures

◄ | ►

◄ | ►

Back | Close

Full Screen / Esc

Printer-friendly Version

Interactive Discussion

Diversity of picocyanobacteria in the Mediterranean Sea

D. Mella-Flores et al.

A number of recent studies have reported striking changes in the distribution and/or diversity of a variety of Mediterranean organisms, such as fish and marine invertebrates (for recent reviews, see Coll et al., 2010; Lejeusne et al., 2009). Indeed, it seems that more and more tropical or subtropical species are colonizing this marine area, often replacing local species (Bianchi, 2007). Whether the community structure of marine prokaryotes is also affected by global warming, and in particular picocyanobacteria that are key players in the productivity of the Mediterranean Sea, has been little studied so far. In the present paper, we use dot blot hybridization analysis and clone library sequencing to compare the vertical and horizontal distributions of *Prochlorococcus* and *Synechococcus* ecotypes/clades between two cruises (PROSOPE and BOUM) held some nine years apart in the Mediterranean Sea and which followed comparable transects through both western and eastern basins.

2   Methods

2.1   Sampling

Water samples were collected at several stations during two independent cruises in the Mediterranean Sea, the PROSOPE cruise (Productivity of Oceanic Pelagic Systems) that was held from 9 September to 1 October 1999 aboard the French research vessel R/V *La Thalassa* (for details on hydrology; see Garczarek et al., 2007; Marie et al., 2006; Crombet et al., 2011) and the BOUM cruise (Biogeochemistry from the Oligotrophic to the Ultra-oligotrophic Mediterranean) held from 22 June to 18 July 2008 aboard the R/V *L'Atalante* (Fig. 1). Additional samples were collected at the BOUS-SOLE site (located near the PROSOPE cruise DYF site; see Fig. 1) and during the BOUSSOLE 88 cruise held on 15 June 2009 aboard the R/V *Tethys II*. At each station, 4 to 8 l water samples were retrieved from 4 to 6 discrete depths using 12 l Niskin bottles, fitted on a Seabird CTDO rosette sampler (model SBE 911) equipped with in situ conductivity, temperature, depth, oxygen as well as chlorophyll fluorescence

sensors. Seawater was subsequently filtered through a 20 μm meshed silk (Whatman Co.) and 47 mm diameter, 3 μm pore size polycarbonate Nuclepore filters (Whatman, Maidstone, UK). Picoplanktonic cells were then collected by gentle filtration onto 47 mm diameter, 0.2 μm pore size Pall Supor-200 filters (Gelman Inc., Ann Arbor, MI). The fil-
5 ter was transferred into a cryovial containing 1.8 ml of DNA lysis buffer (20 mM EDTA, 400 mM NaCl, 0.75 mM sucrose, 50 mM Tris pH 9), quickly frozen in liquid nitrogen and stored at −80 °C until extraction. At all stations and depths, samples were taken and analyzed to determine nutrient concentrations, nitrate and phosphate, as previously described (see Raimbault et al., 1990), *Prochlorococcus* and *Synechococcus*
10 cell concentrations by flow cytometry (Marie et al., 1999; Obernosterer et al., 2008) and pigment content (Ras et al., 2008). All these data are available on-line on the BOUM cruise database (http://www.com.univ-mrs.fr/BOUM/).

2.2 DNA extraction

DNA was extracted from the filters largely following the method described by Massana
15 et al. (2004) but with slight modifications. Briefly, filters from selected stations and depths were first cut into small pieces using clean scissors and resuspended by strong vortexing in lysis buffer, used as storage buffer. Samples were then incubated at 37 °C for 45 min in the presence of lysozyme at a final concentration of 1 mg ml^{-1}, then 1 h at 50 °C with proteinase K and SDS (final concentrations of 0.2 mg ml^{-1} and 1 % *w/v*, re-
20 spectively). Nucleic acids were extracted consecutively with phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1) before being precipitated with 2.5 vol of 100 % (*v/v*) cold ethanol and 0.1 vol of 3 M sodium acetate (pH 5.2) at −20 °C. Nucleic acids were recovered by centrifugation, washed twice with 70 % ethanol (*v/v*), resuspended in sterile water and stored at −80 °C.
25 Strains representative of the different *Prochlorococcus* and *Synechococcus* clades, obtained from the Roscoff Culture Collection (RCC, http://www.sb-roscoff.fr/Phyto/RCC/) or held at Warwick University, were used as controls in dot blot hybridization experiments (Table 1). Their DNA was extracted as described by Eguchi et

al. (2001). Briefly, 50 ml of late logarithmic phase cells were pelleted and then re-suspended in 567 µl of TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA). Cells were lysed at 60 °C for 4 h after addition of SDS (0.5 % *w/v* final) and proteinase K (250 mg ml^{-1} final). NaCl (100 µl of 5 M) and CTAB (80 µl of 10 % *w/v* hexadecyltrimethyl ammonium bromide in 0.7 M NaCl; Sigma-Aldrich, St Louis, MO, USA) were added and mixed thoroughly and samples incubated at 65 °C for 10 min. Nucleic acids were purified from CTAB complexes by successive chloroform:isoamyl alcohol (24:1; 1 vol) and phe-nol:chloroform:isoamyl alcohol (25:24:1; 1 vol) extractions before being precipitated by addition of 0.6 vol isopropanol. Nucleic acids were then purified successively in 50 %, 70 % and 100 % (*v/v*) ethanol, resuspended in sterile water and kept at −20 °C for fur-ther use. The quality and quantity of environmental or strain DNA was monitored using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA).

2.3 PCR amplification for dot blot hybridization analysis

16S rDNA sequences were amplified from control strains and environmental DNA us-ing the primers OXY107F (GGACGGGTGAGTAACGCGTG) and OXY1313R (CTTCAY-GYAGGCGAGTTGCAGC; Fuller et al., 2003).

PCR reactions were carried out in a total reaction volume of 100 µl with 1.0–10 ng of template DNA per reaction. The reaction mix consisted of 200 nM forward and reverse primers, 200 µM dNTPs, 2.0 mM MgCl$_2$ and 2.5 U of Taq DNA polymerase (Qiagen, Valencia, CA). For amplification of environmental DNAs, BSA (bovine serum albumin, Sigma-Aldrich, St Louis, MO, USA) was added at 1.0 mg ml^{-1} and hot start PCR was routinely performed. The reaction cycle consisted of an initial denaturation step of 4 min at 96 °C and 1 min at 80 °C, then primers, dNTPs and Taq were added to the reaction mix, followed by 25–30 cycles of 94 °C for 45 s, 55 °C for 30 s 72 °C for 1 min, with a final extension at 72 °C for 6 min. PCR products were purified using the Qiaquick PCR purification kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The amplification reaction was repeated until sufficient DNA amount was available for each sample, then PCR products were pooled for dot blot analysis.

2.4 Dot blot hybridization

Hybridization conditions were as previously described (Fuller et al., 2003; West and Scanlan, 1999). Oligonucleotide probes (30 ng, dual HPLC grade purified) targeting each *Prochlorococcus* and *Synechococcus* clade (Table 1) were end-labeled with γ^{32}P-ATP using T4 polynucleotide kinase in 1X forward kinase buffer (Invitrogen, Carlsbad, CA) at 37 °C for 1 h. Unincorporated nucleotides were then removed by purification through a mini Quick Spin DNA Column (Roche, Basel, Switzerland). Purified 16S rDNA amplicons from environmental DNA and control strains were blotted onto Zeta-Probe nylon membranes using a Bio-Dot microfiltration apparatus (both from Bio-Rad, Laboratories Ltd., Hemel Hempstead, Herts, UK) and hybridized with labeled clade-specific oligonucleotide probes as described previously by Fuller et al. (2003; see Table 1). Hybridization signals were quantified using a Phosphorimager (Typhoon Trio) and ImageQuant TL software (both from GE Healthcare, Buckinghamshire, UK).

Relative hybridization of clade-specific probes to total oxygenic phototroph 16S rDNA sequences were calculated as described previously (Fuller et al., 2003) by normalizing the individual *Prochlorococcus* and *Synechococcus* probe signals to the signal for total amplified DNA, as determined with the EUB338 probe. The dot blot hybridization data as well as other environmental parameters were visualized using Ocean Data View software, designed by R. Schlitzer (http://odv.awi.de).

2.5 Clone libraries and phylogenetic analyses

16S–23S ribosomal DNA Internal Transcribed Spacer (hereafter ITS) regions were amplified using the 16S-1247f (CGTACTACAATGCTACGG) and 23S-241r (TTCGCTCGC-CRCTACT) primer set (Rocap et al., 2002). PCR reactions were performed in 25 µl volumes containing 1.25 U Taq polymerase (Promega, Madison, WI), 1X buffer provided with the polymerase, 1.5 mM MgCl$_2$, 1.25 mM of each dNTP, 0.5–1 µM of each primer and 0.5–1 µl of environmental DNA template. The reaction cycle consisted of an initial denaturation step of 5 min at 95 °C, followed by 25–30 cycles of 1 min at 94 °C, 1 min

at 52 °C and 2 min at 72 °C with a final extension at 72 °C for 10 min. To minimize PCR biases, the number of PCR cycles was adjusted between 25 to 30 cycles in order to obtain a very faint band on agarose gels. Ten parallel reactions were performed for each sample followed by a three-cycle reconditioning step (Thompson et al., 2002) by transferring 2.5 µl each of the initial reaction mixture into 22.5 µl of fresh PCR mixture. PCR products were then pooled, purified using the Qiaquick kit PCR purification kit (Qiagen, Valencia, CA) and cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). The presence of an insert was screened by PCR amplification of colonies using the vector specific M13 primers. The size of inserts, as measured by agarose gel electrophoresis, was also used to specifically enrich *Synechococcus* sequences since the ITS sequence is generally longer in *Synechococcus* than in *Prochlorococcus* (except LLIV strains; Rocap et al., 2002). Between 69–80 clones from each sample were sequenced bi-directionally on an ABI3130 automated sequencer using Big Dye terminator Cycle sequencing kits version 3.0 (both from Applied Biosystems, Foster City, CA), according to the manufacturer's instructions.

A total of 746 environmental ITS sequences were obtained and primer regions were excluded for the analysis. To avoid redundancy, only 149 sequences, including only one sequence per cd-hit cluster as defined with a percent identity cutoff of 99 % (Li and Godzik, 2006) and all BOUM sequences from the four new clades described in this study, were deposited in the Genbank nucleotide sequence database under the following accession numbers: XXXXXXX–YYYYYY. Their phylogenetic affiliation was determined using Pplacer v1.0, which places sequences into a reference tree (Matsen et al., 2010). First, a reference alignment of full-length ITS sequences was made with 97 selected sequences of isolates and environmental clones representative of the whole diversity of *Synechococcus* and *Prochlorococcus* clades/ecotypes known so far, including four novel groups that were revealed by preliminary phylogenetic analyses of our own sequences (see Results). The alignment was done using MAFFT v6.847 taking into account the RNA secondary structure (Q-INS-i option; Katoh and Toh, 2008), then manually refined using BioEdit v7.0.5.3 (Hall, 1999). The general

Diversity of picocyanobacteria in the Mediterranean Sea

D. Mella-Flores et al.

time-reversible (GTR+I+G) model of nucleotide substitution was selected according to JModeltest v0.1.1 (Posada, 2008) and the corrected Akaike information criterion (Posada, 2009) for the phylogenetic inference of each sequence by Maximum Likelihood (ML) using PhyML v3.0 (Guindon and Gascuel, 2003) with gamma and invariant sites distributions.

Environmental sequences were then aligned with a HMM built from the reference alignment using tools from the HMMER v3.0 suite (http://hmmer.janelia.org/), with default parameters. Using this alignment and the reference tree, the most probable location for each environmental sequence was determined using Pplacer and this was visualized by additional branches in the tree (see the Supplement). The same affiliation of environmental sequences was obtained with two independent methods (not shown): (i) a clustering strategy based on pairwise-distances between reference and environmental sequences (Needleman-Wunsch algorithm as implemented in Emboss; Needleman and Wunsch, 1970) and (ii) a strategy based on a ML analyses of a large multiple sequence alignment including both reference and environmental sequences using RaxML (Stamatakis, 2006).

While a full-length ITS sequence reference alignment was necessary for Pplacer, phylogenetic analyses were performed after removing a number of hypervariable regions and gaps, leaving a total of 1135 positions, including tRNAs. Bayesian inference was then conducted using Mr Bayes v3.1.2 (Huelsenbeck and Ronquist, 2001), starting with a random tree, run two million generations in four chains and with a burn-in of 2000 generations, using parameters similar to the ML method. Neighbor-Joining (NJ) based on the F84 distance model and Maximum Parsimony (MP) trees were inferred using Phylip v3.69 (Felsenstein, 1989). Robustness of inferred topology was supported by bootstrap resampling (1000) with ML, NJ and MP. Very similar tree topologies were obtained for the full-length and the cut versions of the reference alignment except for slight modifications of the relative position of a few groups, essentially within sub-cluster 5.1B (see Results).

3 Results

3.1 Hydrology

The Mediterranean Sea is a semi-enclosed sea undergoing extensive water mixing during winter, a seasonal phenomenon that injects nutrients from deep waters into surface waters (Marty et al., 2002). These nutrients are rapidly consumed by phytoplankton during late winter/early spring blooms, which occur in surface waters in the northern Mediterranean Sea (as seen by satellite imagery; D'Ortenzio and Ribera d'Alcala, 2009) and at depth further South (Crombet et al., 2011). By early summer, the upper mixed layer becomes oligotrophic and the chlorophyll biomass reaches annual minima over the whole Mediterranean Sea (D'Ortenzio and Ribera d'Alcala, 2009).

Figure 2a–c shows high resolution plots of the vertical distribution of temperature, salinity and fluorescence along the BOUM cruise transect held in late June–early July 2008. These graphs show the occurrence of a strong vertical thermal stratification along the whole transect (Fig. 2a) and a strong horizontal salinity gradient increasing from W to E (Fig. 2b), with a marked halocline located in the Ionian Sea. This gradient separates western Atlantic waters entering the Mediterranean Sea in the Straits of Gibraltar, from the high salinity waters of the east Mediterranean Sea. Also notable is the progressive eastwards deepening of the fluorescence maximum (Fig. 2c), a proxy for the deep chlorophyll maximum (DCM), associated with a comparable deepening of nutriclines (not shown, but see Crombet et al., 2011; Pujo-Pay et al., 2011). These summertime hydrological characteristics were globally similar to those previously reported for the PROSOPE cruise in late summer-early fall (see Garczarek et al., 2007; Marie et al., 2006), including sea surface temperatures (SST) above 24 °C over most of both transects (except at the three northernmost stations of BOUM and the westernmost station of PROSOPE), and comparable depths of the upper mixed layer (25–30 m) in the common parts of both cruises. The main hydrological difference between the two cruises was the position of the halocline, located more eastward during BOUM (in the Ionian Sea) than during PROSOPE (off the Algerian coasts).

Diversity of picocyanobacteria in the Mediterranean Sea

D. Mella-Flores et al.

Title Page

Abstract	Introduction
Conclusions	References
Tables	Figures

◀◀ ▶▶

◀ ▶

Back   Close

Full Screen / Esc

Printer-friendly Version

Interactive Discussion

SST data for the 1985–2010 period were retrieved from the National Oceanic and Atmospheric Administration (http://iridl.ldeo.columbia.edu/SOURCES/.NOAA/; Reynolds et al., 2002) in order to calculate the summer SST anomaly for the same period, i.e. the difference between the average SST for the three warmest months (July, August and September) of any given year and the mean summer SST averaged over the last 25 yr. Figure 3 shows both the monthly SST variations and the evolution of summer SST anomalies in the 1° latitude-longitude grid areas surrounding the three long-term stations (hereafter Sta.) of the BOUM cruise, located in the Algero-Provencal basin (Sta. A), the Ionian basin (Sta. B) and the Levantine basin (Sta. C). Over this 25-yr period, the SST range between the coldest (March) and warmest (August) months tended to enhance at Sta. A (about 1 °C) due to an increasing trend for August SST and almost constant March SST, whereas at Sta. B and C, SST increased for both March and August (Fig. 3a–c). The summer SST anomaly showed an increasing trend in the eastern Mediterranean basin (Fig. 3d–f), with the strongest rate of increase (+0.05 °C per year) observed at the easternmost Sta. C (Fig. 3f). It must be noted though that when considering the sole period between the two cruises (1999–2008), there were no significant SST increase at the latter station. A somewhat different pattern was observed at Sta. B, where despite the globally increasing trend (+0.028 °C per year), summer SST anomalies were much more variable from year to year. In the western basin at Sta. A, the summer SST anomaly trend was quite low (only +0.012 °C per year) over the last 25 yr, despite a few exceptionally strong anomalies (e.g. −1.16 °C in 1996 and +1.87 °C in 2003).

3.2 Vertical distributions of marine picocyanobacteria

Figure 2d–e shows the vertical distributions of *Prochlorococcus* and *Synechococcus* during the BOUM cruise. The highest *Synechococcus* abundances (range: 3.5–8.3 ×10^4 cells ml^{-1}; Table 2) were observed in subsurface, mesotrophic waters of the Gulf of Lions, i.e. at the northernmost stations of the Algero-Provencal basin (Sta. 25–27), and the depth of maximum abundance progressively increased southwards to

Title Page

Abstract | Introduction

Conclusions | References

Tables | Figures

|◀ | ▶|

◀ | ▶

Back | Close

Full Screen / Esc

Printer-friendly Version

Interactive Discussion

reach 75 m at Sta. A. From Sta. 22 to 18, fairly high *Synechococcus* abundances (range: 0.63–1.93 × 10^4 cells ml^{-1}) were found down to 50 m, whereas from the Sicily Strait (Sta. 17) to Sta. B cell densities dropped very rapidly with depth, with typically a 2- to 3-fold decrease between surface (ca. 5 m) and 25 m waters. Comparatively,
5 in the same area during the PROSOPE cruise (Sta. 5 and 6), surface abundances of *Synechococcus* were about two-fold higher than on the BOUM cruise and fairly high cell densities (>10^4 cells ml^{-1}) were observed down to 70 m (Garczarek et al., 2007). East of BOUM Sta. 3, the *Synechococcus* maximum systematically occurred around 75 m, i.e. about 25 m above the DCM (Table 2). Since the PROSOPE transect did not
10 extend as far east as the BOUM cruise, it is not possible to say whether this marked deep *Synechococcus* maximum, possibly related to the extreme oligotrophy of the upper layer in the eastern Mediterranean Sea waters, already existed in 1999 (but see Fig. 5d in Tanaka et al., 2007, for a profile made in May 2002). In terms of integrated *Synechococcus* concentrations, the highest value of the BOUM cruise occurred
15 at Sta. 25 (2.5 × 10^8 cells cm^{-2}) and the lowest at Sta. B (2.81 × 10^7 cells cm^{-2}; Table 2), consistent with the very different nutrient regimes of these two stations (Crombet et al., 2011).

For *Prochlorococcus*, the highest abundance during the whole BOUM cruise was observed at Sta. 1 at a depth of 71 m (1.84 × 10^5 cells ml^{-1}). This mid-depth abundance
20 maximum extended over most of the transect from Sta. A (Algero-Provencal basin) to Sta. 1 (Ionian Sea). At Sta. B, the most oligotrophic station of the transect (i.e., exhibiting the lowest integrated nitrate and phosphate concentrations over the top 150 m layer; see Moutin et al., 2002), the layer of high *Prochlorococcus* cell abundance extended very deeply, with average values as high as 4.7 × 10^4 cells ml^{-1} at 140 m. East
25 of Sta. 1, the *Prochlorococcus* abundance maximum progressively deepened down to 100 m with a concomitant decrease of cell densities (Table 2 and Fig. 2d). The maximum concentration of *Prochlorococcus* specific pigment divinyl-Chl-*a* followed a similar trend, though it was sometimes deeper in the Ionian basin due to strong photoacclimation in these very transparent, ultra-oligotrophic waters (Fig. 2f).

Diversity of picocyanobacteria in the Mediterranean Sea

D. Mella-Flores et al.

3.3 Distribution of *Prochlorococcus* and *Synechococcus* lineages in the Mediterranean Sea

3.4 *Prochlorococcus*

The distribution of the main *Prochlorococcus* ecotypes at selected stations of the BOUM cruise (Fig. 4) and at Station BOUSSOLE, independently sampled in June 2009 (see the Supplement), was analyzed by dot blot hybridization using 16S rRNA-targeted oligonucleotides. Results showed that over the whole transect, the dominant HL ecotype was HLI, whereas HLII was present at very low concentrations. Analysis of the depth distribution of HLI showed that its peak abundance generally occurred around 25 m in the western basin and 75 m in the eastern basin (except at Sta. 5) then rapidly decreased below these depths. Deep *Prochlorococcus* populations were dominated by LL ecotypes belonging to the LLI and/or LLIV clades; the oligonucleotide probe used here did not allow us to discriminate between these two populations, but previous studies have shown that LLI (eNATL) occurs at mid-depth (generally at the base of the thermocline) and LLIV (eMIT9313) at the bottom of the euphotic zone (Johnson et al., 2006; Zinser et al., 2006). At Sta. 13, the vertical distribution of LL ecotypes exhibited two clearly separated abundance maxima (with the upper one likely dominated by LLI and the bottom one by LLIV), a feature apparently related to an anomaly in the hydrological structure. Indeed, a low-salinity/high-nutrient layer was observed at 50 m, likely translating a recent event of downwelling of modified Atlantic Waters below Mediterranean waters (see Fig. 2b for salinity and Crombet et al., 2011, for nutrients). The *Prochlorococcus* LLII (eSS120) ecotype was only abundant in the northwestern part of the Mediterranean Sea, BOUM Sta. 25 and Sta. BOUSSOLE, where notable hybridization signals could be detected from the DCM down to the bottom of the photic layer (see Fig. 4 and the Supplement).

The distribution of the main *Prochlorococcus* clades was previously analyzed in September 1999 during the PROSOPE cruise using the same molecular approach (Garczarek et al., 2007) and gave comparable results. Indeed, the HLI population

dominated in the upper layer over the whole transect, whereas HLII was negligible, except at Sta. 5 (located near BOUM Sta. 16; Fig. 1), where the HLII hybridization signal reached 8 % at 25 m, one fifth of the HLI signal.

Synechococcus

The set of clade-specific oligonucleotide probes designed by Fuller et al. (2003), which targets the nine major marine *Synechococcus* clades of sub-cluster 5.1 (i.e. individual probes against clades I, II, III, IV, VIII and IX, and a single probe against clades V, VI and VII) as well as sub-cluster 5.3 (formerly sub-cluster 5.1 clade X; see Dufresne et al., 2008), was used to study the distribution of these clades in the Mediterranean Sea at selected stations of the BOUM (Fig. 5) and PROSOPE cruises (Fig. 6), as well as at Sta. BOUSSOLE (see the Supplement).

Clade I was most abundant in the vicinity of the DCM at mesotrophic stations of the Gulf of Lions (i.e. Sta. 25 during BOUM, Sta. DYF during PROSOPE and the nearby Sta. BOUSSOLE during BOUSSOLE 88), and was therefore found in fairly cold waters ($<17\,°$C). In the rest of the BOUM transect, clade I was sometimes found at low abundance at depth, generally within the deep *Synechococcus* maximum. Clade IV exhibited a similar distribution pattern as clade I, though its relative abundance was significantly higher than clade I in the northern part of the Algero-Provencal basin and lower at other stations of the BOUM cruise. Similarly during the PROSOPE cruise, clade IV was dominant over clade I at Sta. 1 (Straits of Gibraltar) and the reverse at Sta. 5, where a significant abundance maximum of clade I cells occurred at 60 m (Fig. 6).

Clade III was present in the upper mixed layer at all stations of the BOUM transect with its maximum signal observed in surface waters. It was the predominant *Synechococcus* clade at Sta. 25, 5 and 9 (Fig. 5) and the hybridization signal extended throughout the top 100 m of most of the Levantine basin. Similarly, during the PROSOPE cruise, clade III was also the dominant clade in surface waters and its relative abundance as well as vertical extension increased eastwards (Fig. 6).

Diversity of picocyanobacteria in the Mediterranean Sea

D. Mella-Flores et al.

The probe targeting clades V, VI and VII gave strong relative hybridization signals (up to 15.5 %) at all stations and all depths examined during both the PROSOPE and BOUM cruises. During the latter cruise, it was the dominant *Synechococcus* group at most depths from Sta. A to B, with a maximal relative abundance found at the level of the *Synechococcus* density maximum.

The hybridization signals for clade II were never significant (<1.0 %; Fig. 5) during the BOUM cruise. In contrast, during the PROSOPE cruise, low but significant clade II hybridization signals (up to 4.4 %) were observed in surface waters of the southwestern Mediterranean basin (Fig. 6), corresponding to a population that likely originated from the Atlantic Ocean. Clade VIII was virtually undetectable at all examined stations of both BOUM and PROSOPE cruises. Clade IX was detectable only at the mesotrophic Sta. 25 during BOUM at all sampled depths (Fig. 5, Table 2) and at Sta. BOUSSOLE (not shown) but not during PROSOPE. Finally, cells belonging to sub-cluster 5.3 were only detected in the Levantine basin during BOUM, with highest abundance in the DCM, i.e. below the *Synechococcus* density maximum.

3.5 ITS clone libraries

In order to explore in greater detail the genetic diversity of the Mediterranean Sea populations of picocyanobacteria, we sequenced clone libraries generated from the ITS region using general primers targeting both *Prochlorococcus* and *Synechococcus* (Rocap et al., 2002). Clone libraries were obtained from two selected depths at four stations of the BOUM cruise (A, B, 5 and C), as well as two depths of the BOUS-SOLE site, sampled in mid-June 2009. In order to maximize the number of *Synechococcus* sequences in the data set, the two depths were chosen in the upper mixed layer and above the *Prochlorococcus*-dominated DCM (hereafter called "surface" and "mid-depth" samples, respectively). We sequenced 69–80 clones from each library and performed phylogenetic analyses using four methods (ML, Bayesian, NJ and MP; Fig. 7). In only 2 out of 8 BOUM clone libraries (Sta. B at 12 m and Sta. C at 5 m) as well as at both depths at Sta. BOUSSOLE, the proportion of sequences attributable

Diversity of picocyanobacteria in the Mediterranean Sea

D. Mella-Flores et al.

to *Synechococcus* was higher than *Prochlorococcus*, and the large variability of this ratio (from 74:1 to 1:69) likely reflected the variable relative abundances of these two genera in the starting samples. Environmental sequences from each individual library were positioned using Pplacer v1.0 (see Methods) in a reference tree containing 97 ITS sequences from strains or environmental samples representative of the whole ITS diversity reported so far in the *Prochlorococcus* and *Synechococcus* groups, including four new groups described below (see Fig. 7 and the trees of the Supplement generated using Pplacer for each library).

Most sequences related to *Prochlorococcus* were assignable to the HLI clade, with the majority of sequences originating from "surface" samples at Sta. BOUSSOLE, A and 5 and from "mid-depth" samples at Sta. B and C. *Prochlorococcus* LLI were also present in abundance, but only in "mid-depth" samples, except at Sta. B where 4 sequences were obtained from the "surface" sample (Fig. 7). There were no sequence attributable to either LLII, LLIII or LLIV in the whole data set but, as mentioned earlier, this result was expected since these ecotypes are abundant only near the bottom of the euphotic zone.

For *Synechococcus*, clade III was the dominant group at most stations except in the "mid-depth" sample from BOUSSOLE and in surface waters of Sta. A (Fig. 7). In contrast, many clade I and IV sequences were retrieved from Sta. BOUSSOLE and a few clade I sequences from Sta. A, but none from the three stations of the eastern Mediterranean basin. The next most abundant group of sequences belonged to the WPC1 clade, a lineage recently described based on culture isolates from the east China Sea and East Sea (Choi and Noh, 2009). This group was systematically present in surface waters at all sites and sometimes extended to mid-depth. All other *Synechococcus* clades contributed for less than 12 sequences and can therefore be considered as minor (at least for the dataset considered here). These included 10 sequences attributable to clade II, originating from various stations and depths along the BOUM transect with half coming from surface waters of Sta. BOUSSOLE. Eight clade VI sequences were retrieved in surface at Sta. C, while single sequences of this clade and

Diversity of picocyanobacteria in the Mediterranean Sea

D. Mella-Flores et al.

the closely related clade CB3 (previously only observed in Chesapeake Bay; Chen et al., 2006) were retrieved from 10 m at BOUSSOLE.

Interestingly, sequences from four new, uncultured *Synechococcus* clades, so-called MS1 through MS4, were observed in clone libraries (Fig. 7). MS1 was inferred to be phylogenetically located at the base of the group formed by clades II (which includes groups XV and WPC2), III, IV and CRD2 (Ahlgren and Rocap, 2006). It is noteworthy however, that in trees made with full-length ITS sequences, MS1 in fact grouped with clade III, though with low bootstrap support (see the Supplement). MS2 clustered with the above mentioned WPC1 clade using all phylogenetic methods except MP. The final two clades, MS3 and MS4, were related to one another and together formed a new group within sub-cluster 5.3. Given their phylogenetic position, supported by strong bootstrap values, and the relatively large distance between these two new clades and members of the initial sub-cluster 5.3, we propose to split the latter sub-cluster into two groups, so-called 5.3A and 5.3B (see Fig. 7).

MS1 was found both in the western and eastern basins (Sta. A, B and C) and comprised only "surface" sequences, whereas the three other uncultured clades were only retrieved in the eastern part of the transect, with the latter two mainly found in the *Synechococcus* abundance maximum.

4 Discussion

4.1 Hydrology-driven variations in picocyanobacterial population structure

Despite its relatively modest size, representing about 0.7 % of the cumulative surface of marine areas, the Mediterranean Sea displays a large variety of hydrological conditions in summer, with a strong N-S gradient of SST (17–24 °C; Fig. 2a) and W-E gradient of salinity (37.4–39.6 PSU; Fig. 2b). Nutrient availability also varied tremendously along the BOUM transect, with a progressive N-S and W-E deepening of nutriclines, that was much steeper for the phosphocline than for the nitracline (Moutin et al., 2011; Pujo-Pay et al., 2011).

D. Mella-Flores et al.

The spatial heterogeneity of hydrological conditions observed along the BOUM transect translated into large variations in the vertical structure of both *Prochlorococcus* and *Synechococcus* populations, which seemed to respond differently to these physico-chemical changes. In the northern part of the Algero-Provencal basin, both genera formed subsurface abundance maxima, but the latter genus generally outnumbered the former one (e.g. at Sta. 25 at all depths). Although this may simply be due to the greater physiological fitness (potentially allowing faster growth) of *Synechococcus* with respect to *Prochlorococcus* in these particular conditions, we cannot exclude some antagonistic relationships between these groups or differential sensitivity to predation by local grazers and/or cyanophages. In the southern part of the Algero-Provencal basin, the Sicily Strait and the eastern Ionian Sea, *Prochlorococcus* was the dominant group at depth, while *Synechococcus* dominated in the upper mixed layer, with abundances usually above 10^4 cells ml^{-1}. This near surface *Synechococcus* maximum is somewhat unusual compared to other oligotrophic areas with surface chlorophyll concentrations <0.1 µg Chl l^{-1}, such as the Atlantic (Li et al., 1992; Olson et al., 1988, 1990; Zubkov et al., 2000) or Pacific gyres (Campbell et al., 1997; Grob et al., 2007), where *Synechococcus* are generally more homogeneously distributed in the upper 100–150 m layer and maximal cell densities are typically 2–5 times lower. This raises the question of the mechanism of maintenance of such high *Synechococcus* cell densities in surface waters of this nutrient-poor region of the Mediterranean Sea. Given the relatively close proximity of aerosol source emissions, it is possible that nutrient enrichment of surface waters by atmospheric deposition, may play a significant role in this phenomenon (Bonnet et al., 2005), though it has been reported that some, copper-rich Saharan dust types may in fact be toxic to *Synechococcus* (Paytan et al., 2009). It is also worth noting that most stations between sites A and B exhibited a N:P ratio between 20 and 40 (integrated over the top 150 m; see Crombet et al., 2011), i.e. above the Redfield ratio (N:P = 16) indicating P-depletion in these waters. However, both *Prochlorococcus* (e.g. MED4) and oceanic strains of *Synechococcus* (e.g. WH8103 and WH8012) display a cellular N:P ratio in the same range (21–43), and

therefore have a low P requirement, which can even be further reduced by 3- or 4-fold when grown under P depletion (Bertilsson et al., 2003; Heldal et al., 2003). Additionally, it was previously shown that *Synechococcus* populations from the Mediterranean Sea have a high affinity for orthophosphate and an elevated P uptake rate (Moutin et al., 2002). Thus, *Synechococcus* seems to be particularly well adapted to sustain the moderate P depletion occurring between Sta. A and B. Nevertheless, east of Sta. B, the intensity of P depletion increased dramatically (integrated N:P ratios were between 50 to more than 100 at most stations; see Crombet et al., 2011) which might explain in part the complete change in picocyanobacterial population structure observed between the Ionian sea and the Levantine basin. In the latter region, *Synechococcus* formed a marked deep abundance maximum around 75 m and the *Prochlorococcus* maximum deepened down to 100 m. Even though the fluorescence of *Prochlorococcus* cells was too faint for them to be counted in the upper mixed layer, several indices suggest that cell densities were low in surface waters of this region (see also Tanaka et al., 2007). These include undetectable levels of the *Prochlorococcus*-specific pigment divinyl-Chl-*a* (Fig. 2f) and the fact that only one sequence attributable to *Prochlorococcus* was retrieved in the ITS clone library obtained from the 5 m sample at Sta. C. For *Synechococcus*, even though there were still about 1–2×10^3 cells per ml in surface waters, an enrichment study performed on-board the BOUM cruise showed that these cells were co-limited by both N and P at Sta. C, but only N-limited at Sta. A and B (Tanaka et al., 2011).

The population structure of marine picocyanobacteria was globally comparable along common parts of the BOUM and PROSOPE cruises held about a decade apart, although it is worth noting that in surface waters south of the Algero-Provencal basin and the Sicily Strait *Synechococcus* abundances were two-fold lower during BOUM than during PROSOPE and the corresponding integrated cell concentrations were also 20–40 % lower (Table 2 and data not shown). This might be due to an increase in oligotrophy of this area, but we cannot exclude that this could merely be a result of seasonal differences and/or an aeolian dust transport event that enriched surface waters shortly

prior to the PROSOPE cruise (Bonnet et al., 2005).

4.2 Factors determining the distribution of cyanobacterial ecotypes

4.2.1 *Prochlorococcus*

A number of recent studies have shown that the phylogenetic composition of *Prochloro-coccus* populations is tightly driven by environmental factors, primarily by light and secondarily by temperature. When the water column is stratified, there is a clear vertical partitioning of ecotypes, with HLI occupying the upper layer, LLI an intermediate layer (generally the thermocline depth) and LLII-IV the base of the lit layer (Johnson et al., 2006; Zinser et al., 2006). Interestingly, this vertical partitioning has a genomic basis, since HL and LLI strains possess a number of adaptations to the deleterious effects of high irradiance and UV stress (including DNA repair genes, such as photolyases) that are absent in "true LL" ecotypes, i.e., LLII-IV (Kettler et al., 2007; Partensky and Garczarek, 2010; Rocap et al., 2003). In the present study, the LL-specific oligonucleotide probe used for dot blot hybridization analysis did not allow us to distinguish LLI (or eNATL) from LLIV (or eMIT9313) ecotypes, but the fact that ITS clone libraries from "mid-depth" (30 to 75 m; see Fig. 7) contained many LLI and no "true LL" sequences clearly indicated that a vertical partitioning of ecotypes comparable to that previously observed in the Atlantic and Pacific Ocean (Johnson et al., 2006; Malmstrom et al., 2010) is also occurring in the Mediterranean Sea. Surprisingly, the LLII- (or eSS120-) specific probe detected significant levels of this ecotype only at the northwestern Mediterranean Sea stations (Sta. DYF during PROSOPE, Sta. 25 during BOUM and Sta. BOUSSOLE during BOUSSOLE 88). This suggests that the distribution of LLII in the Mediterranean Sea is restricted to temperate, mesotrophic areas. However, this clade was found to be abundant at depth at both long-term stations BATS (Bermuda Atlantic Time Series) and HOT (Hawaii Ocean Time series; Malmstrom et al., 2010) and its occurrence in such tropical and subtropical, oligotrophic waters somewhat contradicts this assumption. So it is probable that our LLII-specific probe in fact

Diversity of picocyanobacteria in the Mediterranean Sea

D. Mella-Flores et al.

Title Page

Abstract	Introduction
Conclusions	References
Tables	Figures

◄ | ►

◄ | ►

Back | Close

Full Screen / Esc

Printer-friendly Version

Interactive Discussion

D. Mella-Flores et al.

did not target exactly the same population as did the qPCR primers used by Malmstrom and coauthors.

The second most important factor determining *Prochlorococcus* ecotype distribution is temperature. Indeed, a clear latitudinal partitioning of HLI and HLII ecotypes was observed during two consecutive AMT transects through the Atlantic ocean in late summer/early fall (Johnson et al., 2006; Zwirglmaier et al., 2007). Although both HL clades co-occurred at low latitudes, HLII was always the dominant clade from 28° S to 33° N, then the ratio was progressively reversed at higher latitude, so that HLI became dominant above 32° S or 38° N. If one extrapolates the HLI and HLII concentrations reported in the N Atlantic by Johnson et al. (2006) to the latitudinal range covered by the BOUM and PROSOPE transects in the Mediterranean Sea (33.6–43.2° N), one should have expected to find the HLI:HLII abundance ratio ranging roughly from 1:1 in the Ionian and Levantine basins to 2000:1 at the northernmost Sta. 27. However, the HLII hybridization signal was most often lower than 1 % of the EUB signal, except in subsurface waters at PROSOPE Sta. 5 (see Fig. 3 in Garczarek et al., 2007). This low abundance of the HLII ecotype in the Mediterranean Sea was independently confirmed by the occurrence of only one HLII sequence (at Sta. C) in our ITS clone libraries compared to 316 HLI sequences.

The scarcity of the HLII ecotype in the southern part of the Mediterranean Sea, in particular in the Levantine basin, is somewhat puzzling. Indeed, the growth temperature range of strains representative of this ecotype (MIT9312 and MIT9215) is 16–30 °C, with growth optima between 23 and 28 °C. In contrast, HLI strains (MED4 and MIT9515) can grow in the 11–28 °C range, with growth optima between 22 and 26 °C (Johnson et al., 2006; Zinser et al., 2007). In field populations, the curve of abundance as a function of temperature differs even more dramatically between the two HL ecotypes, since HLI abundance peaks at ca. 19 °C and declines drastically above 25 °C, whereas HLII abundance increases with temperature from less than 10 cells ml^{-1} at 14 °C up to 1–4 × 10^5 cells ml^{-1} at 25–28 °C (Zinser et al., 2007). The average SST over the 1999–2008 period for the coldest and warmest month (i.e. March and August,

respectively) were 14.5–25.8 °C, 16.6–27.6 °C and 17.3–28.0 °C for Sta. A, B and C, respectively (Fig. 2a–c). Although the wintertime SST at Sta. A was close to the lower limit of the HLII growth temperature range, suggesting that this ecotype might not be able to survive year-round in these waters, the SST range recorded at Sta. B and C were completely compatible with its growth temperature range. Furthermore, the Mediterranean Sea is connected to the Red Sea via the Suez Canal and it was shown that the dominant *Prochlorococcus* ecotype year-round in the Gulf of Aqaba is HLII (Fuller et al., 2005), constituting a possible reservoir of HLII cells for the southeastern Mediterranean Sea. Thus, temperature alone cannot explain why this ecotype was so scarce in the open waters of the eastern Mediterranean Sea in summer. It is noteworthy that Feingersch et al. (2010) analyzed a metagenomic database from samples collected in surface waters off Haifa and suggested that the dominant *Prochlorococcus* genotype belonged to HLII, so it is likely that coastal waters of the Levantine basin might display a higher HLII to HLI ecotype ratio than offshore waters.

The scarcity of HLII cells in open sea waters suggests that environmental factors other than temperature may limit the growth of this ecotype and the extreme phosphorus deficiency of this area is possibly involved in this phenomenon. Indeed, it was shown that the HLI strain MED4, isolated from the western Mediterranean Sea, displays a much larger set of P uptake and assimilation genes than strains (or natural populations) from P-replete areas (Coleman and Chisholm, 2010; Kathuria and Martiny, 2011; Martiny et al., 2006, 2009a). Accordingly, MED4 is capable of utilizing a broader range of organic P compounds than does the HLII strain MIT9312, which only uses ATP as organic P source and lacks significant alkaline phosphatase activity, an enzyme which hydrolyzes organic P to orthophosphate, even under conditions of P starvation (Moore et al., 2005). Thus, HLII cells entering the Mediterranean Sea via the Suez Canal may not be able to cope with the extreme P depletion they face in these open sea waters. It should be mentioned however, that summer picocyanobacterial populations in the Gulf of Aqaba (Red Sea) displayed elevated levels of PstS, a high-affinity P uptake protein, indicating at least some capacity of HLII cells to respond

to P starvation (Fuller et al., 2005). Furthermore, HLII is also the dominant HL ecotype at the Bermuda Atlantic Time Series (BATS) Sta., an area displaying low P levels, like the Mediterranean Sea (Malmstrom et al., 2010; Zinser et al., 2006, 2007). Hence, HLII cells can acquire the capacity to cope with severe P stress, though it is as yet

5 unknown whether they can gain it from HLI cells (i.e., by lateral transfer of P uptake genes) and at what frequency.

4.2.2 *Synechococcus*

Whilst for *Prochlorococcus* the same phylogenetic groups were found at compara-ble relative depths from one end of the Mediterranean Sea to the other (Fig. 4; see

10 also Garczarek et al., 2007), we observed large variations in the genetic diversity of *Synechococcus* populations both vertically and horizontally along the BOUM and PROSOPE cruise transects (Figs. 5 and 6). It must be noted that the two molecular approaches used here to assess genetic diversity within this genus provided slightly different pictures of the *Synechococcus* community structure. For instance, dot blot

15 hybridization seemingly indicated that the two most abundant *Synechococcus* groups in the Mediterranean Sea were clade III and the phylogenetic cluster formed by the closely related clades V/VI/VII. It is worth noting though, that the clade V/VI/VII probe also recognizes *Synechococcus* strain MITS9220 which is now phylogenetically placed in the CRD1 clade (Fig. 7). While the predominance of clade III was confirmed by clone

20 library sequencing, since the majority of *Synechococcus* ITS sequences belonged to this clade, there were fewer sequences attributable to clades V, VI, VII, CRD1 or to the closely related clade CB3 than would have been expected from hybridization results (Fig. 7). Moreover, there is a single mismatch between this probe and sequences of the recently described clade WPC1 (Choi and Noh, 2009), which appears to be widely

25 distributed in the Mediterranean Sea (Fig. 7). It is therefore possible that the clade V/VI/VII probe can also recognize this particular group. In any case, cells targeted by this probe were found everywhere in the Mediterranean Sea, with highest abun-dances (up to 15.5 % relative hybridization) in the nutrient-rich waters of the northern

Algero-Provencal basin (Figs. 5 and 6). This is consistent with previous literature on other regions of the ocean, since this group was observed throughout the AMT transect in the Atlantic Ocean, with the highest concentrations in temperate waters of the Southern and Northern Hemispheres (e.g., up to 28 % relative hybridization off Cape Town) and in upwelling waters of the northern Arabian Sea (up to 53 % relative hybridization; Zwirglmaier et al., 2007, 2008).

Clade III was most abundant in the surface layer at all stations of the western basin and the Ionian Sea (Fig. 5). This result was confirmed by the much larger number of clade III sequences obtained in clone libraries from "surface" than "mid-depth" (except at Sta. A, but only three clade III sequences were retrieved at this station). This is consistent with earlier studies which showed that clade III was preferentially found in the upper mixed layer (Toledo and Palenik, 2003; Toledo et al., 1999). However, in the Levantine basin, its depth distribution extended to the whole euphotic layer and this seems to be directly related to the deepening of the *Synechococcus* density maximum in this area (Fig. 2e). Zwirglmaier et al. (2008) previously suggested that clade III was an "oligotrophic" clade since they observed that members of this clade were confined to a fairly narrow window of nitrate and phosphate concentration, even though they displayed no obvious latitudinal preference. Genomic analysis of the clade III isolate *Synechococcus* sp. WH8102, indeed shows that it contains a large number of nutrient transporters but a reduced regulatory machinery (Dufresne et al., 2008; Palenik et al., 2003; Scanlan et al., 2009), strengthening the idea that members of this clade are well adapted to stable, low-nutrient environments. Moreover, mutation and gene expression studies have confirmed a well-developed cellular response to P-stress in this strain (Tetu et al., 2009; Ostrowski et al., 2010) that includes the expression of an array of high-affinity periplasmic P-binding proteins and at least three organic phosphatases that could provide a competitive advantage for scavenging P from alternative sources when inorganic P is scarce.

Clades I and IV were both most abundant in mesotrophic waters of the Mediterranean Sea (though with higher signals for clade IV at all depths), suggesting that both

clades are adapted to high-nutrient/low-temperature waters. Faint hybridization signals were also observed in the southern Mediterranean Sea with maximal values for both clades often occurring at the same depth. These maximal values generally coincided with the deep *Synechococcus* maximum, which was generally found below the
18 °C isoline (Fig. 2), confirming the preference of these clades for cool waters. The co-occurrence of *Synechococcus* clades I and IV has been well documented in the Atlantic and Pacific Oceans, where they constitute the dominant *Synechococcus* groups at latitudes above 30° N/S (Brown and Fuhrman, 2005; Zwirglmaier et al., 2007, 2008; Tai et al., 2011; Tai and Palenik, 2009). Nevertheless, the ratio of abundances of the
two clades may vary both spatially and temporally. Indeed, it was observed at a coastal site off South California that these clades displayed different seasonal cycles (Tai and Palenik, 2009). Whilst clade IV was the dominant type over most of the year, clade I outnumbered clade IV just before the annual abundance peak of *Synechococcus* cell density. Genome sequences of one clade I strain (CC9311) and two clade IV strains
(CC9902 and BL107) have been obtained so far (Dufresne et al., 2008; Palenik et al., 2006; Scanlan et al., 2009). The former has a slightly larger genome than the latter, and encodes a larger set of proteins involved in signal sensing (such as histidine kinases and response regulators) and metal binding (including multiple genome encoded copies of ferritin and Zn-binding metallothioneins). Thus, clade I might have been ex-
pected to be better adapted to the variability experienced by coastal/mesotrophic environments than its clade IV counterpart, which somewhat contradicts the higher relative abundance of clade IV over clade I in nutrient-rich areas (Tai and Palenik, 2009; this study). Thus, understanding the factors that allow the co-occurrence of clades I and IV, but also the dominance of one clade over the other will likely require extensive compar-
isons of genomes from many more strains of both clades and/or refined comparative physiological studies in culture (including co-cultures).

The presence of sub-cluster 5.3 at depth in the Levantine basin during BOUM, with the highest abundance observed at Sta. 5, is consistent with ITS sequencing, since seven sequences of this group were retrieved at this station from the 75 m sample.

Previously, Fuller et al. (2006) reported the presence of this group (ex-clade X) in subsurface samples in warm, coastal waters of the Arabian Sea, i.e., in a fairly different niche. However, we showed here that this sub-cluster is more diverse than previously thought and thus it is not unexpected that, like for sub-cluster 5.1, different clades within this group might be adapted to different habitats.

Both dot blot hybridization and clone library sequencing suggest that clades II, VIII and IX are only minor components of the *Synechococcus* community in the Mediterranean Sea. So far, all characterized clade VIII strains, including a number isolated from the Gulf of Aqaba (Fuller et al., 2003, 2006), are phycocyanin-containing halo-tolerant isolates, and this may explain why members of this clade have never been found in significant numbers in open ocean samples (Zwirglmaier et al., 2008). Clade IX has only been found at low levels in warm and coastal sub-surface waters of the northern Arabian Sea (Fuller et al., 2006) and was undetectable elsewhere, so its preferred niche remains unclear. The low abundance of clade II in the Mediterranean Sea, suggested by dot blot hybridization and confirmed by the occurrence of few ITS sequences in clone libraries, is somewhat more surprising. In the California Current (Toledo and Palenik, 2003), the Red Sea (Fuller et al., 2003), the northern part of the Arabian Sea (Fuller et al., 2006) and the region of the Morocco upwelling (Zwirglmaier et al., 2007), this clade constituted a large proportion of the *Synechococcus* population (up to 75 % relative hybridization) in surface waters in tropical and subtropical areas. Indeed, Zwirglmaier et al. (2008) showed that this clade was particularly well represented in warm waters, ranging from 22 °C to 28 °C, while other clades either possess a larger temperature tolerance (7–28 °C for clades III and V/VI/VII) or are restricted to cooler waters (7–18 °C for clades I and IV). Thus, one might have expected to find notable abundances of clade II in the Mediterranean Sea, at least at the easternmost Sta. C, where SST reaches 27 °C in summer (Fig. 2a). Thus, like for *Prochlorococcus* HLII, some environmental factors other than temperature appear to hinder the proliferation of this clade, at least during the warmest months of the year. Perhaps cells of this clade cannot cope with the lower temperatures found in winter (down to 17 °C),

have a lower capacity to deal with low dissolved inorganic phosphate concentrations and/or use alternative P sources than their clade III (and possibly WPC1?) counterparts. However, in easternmost Mediterranean Sea coastal waters, Feingersch et al. (2010) already showed that clade II (together with clade III) comprised the majority

5 of the *Synechococcus* community. As mentioned above, low winter water temperatures across the western Mediterranean Sea, may represent a strong geo-physical barrier to the migration of clade II into the eastern Mediterranean from the Atlantic. The occurrence of clade II in coastal waters of the eastern Mediterranean Sea is more likely explained by westward Lessepsian migration of this clade from the Red Sea, where it

10 dominates the *Synechococcus* community in Spring (Fuller et al., 2005), via the Suez Canal.

5   Conclusions

A number of recent studies suggest that a global increase in the temperature of the Mediterranean Sea has occurred in the last few decades (Saaroni et al., 2003; this

15 study) and that this oceanic region is particularly vulnerable to global change (see e.g., Belkin, 2009). However, the intensity of this rise may vary locally, as shown here by the variation in average summer SST anomalies over the 1985–2010 period, in the vicinity of the three long term stations of the BOUM cruise. Interestingly, the increasing trend was more marked in the eastern basin (up to 0.5 °C per decade at Sta. C) than in

20 the western basin (0.12 °C per decade at Sta. A). These trends are consistent with climate models, which forecast that by the end of the 21st century the Mediterranean Sea SST's will be, on average, 3 to 4 °C warmer than today (Coll et al., 2010; Hertig and Jacobeit, 2008; Somot et al., 2006). Even if these SST variations may seem relatively modest compared to seasonal SST variations (Fig. 3), this change could potentially

25 induce drastic changes in the population structure of marine picocyanobacteria, if the annual temperature range better matches the growth temperature optima of alien lineages than that of native populations.

Here, we studied the genetic diversity and distribution of *Prochlorococcus* and *Synechococcus* clades/ecotypes during the PROSOPE and BOUM cruises held almost a decade apart, using two different and complementary molecular approaches. For *Prochlorococcus*, there was a large phylogenetic diversity down the water column at any given site but the same ecotypes were found from one end of the Mediterranean Sea to the other. The dominant clade in the upper mixed layer was HLI during both cruises. Thus, even though the Mediterranean Sea is located in the boundary zone between the distribution areas of HLI and HLII ecotypes and we have evidence that HLII is present in this area, changes that occurred in the hydrology of the Mediterranean Sea during the period 1999-2008 were not sufficient to cause a shift in the composition of the HL population. Similarly, Malmstrom et al. (2010) followed the distribution of *Prochlorococcus* ecotypes at the long-term Sta. HOT (off Hawaii) and BATS (off Bermuda) for 5 yr and observed no significant change in the *Prochlorococcus* clade composition for any given month over the period considered, although strong seasonal variations did occur in the relative proportion of the different clades.

For *Synechococcus*, analyses of the distribution of the different clades reported here for the Mediterranean Sea not only strengthened the generalizations made by previous studies in other parts of the world ocean (Bouman et al., 2006; Johnson et al., 2006; Zwirglmaier et al., 2007, 2008), but also provided some new insights into the distribution of some of the rarer phylogenetic groups, such as clade IX. The large relative abundance of cells detected by the probe targeting clades V/VI/VII/CRD1 and the broad distribution of this group clearly highlights a need for the design of further probes, or the use of alternate approaches that can precisely map the distribution of individual clades or genotypes in the environment. Also, clone library sequencing revealed the importance in the Mediterranean Sea of new clades (MS1, MS2 and the novel sub-cluster 5.3B) for which specific probes would also need to be designed to more precisely assess their distribution in the world ocean.

In conclusion, even if the increased trend in SSTs has not yet caused any dramatic change in picocyanobacterial community structure, our study points out the need for

long term temporal studies in the Mediterranean Sea, and especially in the Levantine basin which is seemingly the most affected by global warming. Our results advocate that the abundance of phytoplankton clades (particularly *Prochlorococcus* HLII and *Synechococcus* clade II) can serve as powerful bioindicators to augment the classical physico-chemical parameters usually measured to monitor the impact of global warming in the Mediterranean Sea.

Supplementary material related to this article is available online at: http://www.biogeosciences-discuss.net/8/4281/2011/ bgd-8-4281-2011-supplement.pdf.

Title Page

Abstract | Introduction

Conclusions | References

Tables | Figures

◄ | ►|

◄ | ►

Back | Close

Full Screen / Esc

Printer-friendly Version

Interactive Discussion

References

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Title Page

Abstract | Introduction

Conclusions | References

Tables | Figures

◀◀ | ▶▶

◀ | ▶

Back | Close

Full Screen / Esc

Printer-friendly Version

Interactive Discussion

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Title Page

Abstract	Introduction
Conclusions	References
Tables	Figures

|◄ | ►|

◄ | ►

Back | Close

Full Screen / Esc

Printer-friendly Version

Interactive Discussion

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Title Page

Abstract | Introduction

Conclusions | References

Tables | Figures

◀ | ▶|

◀ | ▶

Back | Close

Full Screen / Esc

Printer-friendly Version

Interactive Discussion

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Title Page

Abstract | Introduction

Conclusions | References

Tables | Figures

◀◀ | ▶▶

◀ | ▶

Back | Close

Full Screen / Esc

Printer-friendly Version

Interactive Discussion

Table 1. Characteristics of 16S rDNA oligonucleotide probes used for dot blot hybridization. Abbreviations: *Syn.*, *Synechococcus*; *Proc.*, *Prochlorococcus*.

Probe names	Sequence (5′-3′)	Wash Temp. (°C)	Target organism	Reference sequence[1]	References
EUB338	GCTGCCTCCCGTAGGAGT	46	Eubacteria	all	Amann et al. (1990)
SYN1006	CTCTCAAGTTTCCAAGAG	44	*Syn.* clade I	CC9311	Fuller et al. (2003)
SYN1006RS	CTCTCCTGTTTCCAAGAG	42	*Syn.* clade II	RS9903	Idem
SYN262	GATGCCTTGGTAGGCCTT	53	*Syn.* clade III	WH8103	Idem
SYN635	AAGCCCCTCAGTTTCCAC	50	*Syn.* clade IV	5 × 15[2]	Idem
SYN1280	GAGCCACGGTTTATGAGA	51	*Syn.* clade V–VII	WH7803, WH8018, S9220	Idem
SYN620	CACTGCCACGATGGAGTT	51	*Syn.* clade VIII	RS9913	Idem
SYN1000	GGTTTCCCAGAAATTCGC	45	*Syn.* clade IX	RS9916	Idem
SYN1007	ACCCTCCGGTTTCCCAGA	48	*Syn.* clade X	RCC307	Idem
S2PRO640R	ATACTCAAGCCTTTCAGTTT	41	*Proc.* HLII	TAK9803-2	Zwirglmaier et al. (2007)
S1PRO634R	GCCGATCAGTTTCCACTG	50	*Proc.* HLI	PCC9511	West and Scanlan (1999)
DPRO634R	GCCAATCAGTTTCCACTG	46	*Proc.* LLI/LLIV	NATL1, MIT9303	Idem
SARG634R	GCCCTTCAGTTTCCACTG	44	*Proc.* LLII	SS120	Idem

[1] DNA from the same samples was used to draw hybridization curves for each probe (cf. materials and methods).
[2] Environmental clone.

Table 2. Maximal and integrated concentrations of *Prochlorococcus* and *Synechococcus* at each station of BOUM and PROSOPE cruises. Abbreviations: DCMD, Deep Chlorophyll Maximum Depth; MAC, Maximum Abundance Concentration; MAD, Maximum Abundance Depth; IC, Integrated Concentration; *Syn.*, *Synechococcus*; *Proc.*, *Prochlorococcus*; n.a., not available.

Cruise	Station	DCMD (m)	MAC *Proc.* (cells ml^{-1})	MAD *Proc.* (m)	IC *Proc.* (cells cm^{-2})	MAC Syn. (cells ml^{-1})	MAD Syn. (m)	IC Syn. (cells cm^{-2})
BOUM	27	30	5.19×10^4	19	1.6×10^8	3.54×10^4	10	1.23×10^8
	26	37	1.05×10^4	25	n.a.	8.35×10^4	25	2.38×10^8
	25	51	3.40×10^4	25	6.2×10^7	7.73×10^4	41	2.50×10^8
	24	71	3.85×10^4	51	2.1×10^8	3.23×10^4	51	2.07×10^8
	23	75	1.01×10^5	50	4.3×10^8	3.05×10^4	50	1.74×10^8
	A	88	1.38×10^5	75	$5.60 \times 10^8 \pm 1.13 \times 10^8$ ($n = 15$)	1.01×10^4	75	$4.0 \times 10^7 \pm 5.93E+06$ ($n = 21$)
	22	74	6.69×10^4	75	5.9×10^8	1.72×10^4	25	1.23×10^8
	21	87	5.64×10^4	51	2.8×10^8	1.18×10^4	5.9	5.80×10^7
	20	78	7.56×10^4	76	3.6×10^8	1.28×10^4	51	8.93×10^7
	19	67	7.43×10^4	76	3.0×10^8	1.93×10^4	51	8.80×10^7
	18	81	8.97×10^4	51	5.7×10^8	1.73×10^4	5.2	1.15×10^8
	17	77	4.74×10^4	65	3.6×10^8	8.82×10^3	6.0	6.71×10^7
	16	73	5.58×10^4	51	3.0×10^8	8.42×10^3	5.3	4.30×10^7
	15	98	1.06×10^5	76	8.1×10^8	1.35×10^4	5.1	6.47×10^7
	14	118	1.00×10^5	75	6.4×10^8	1.16×10^4	4.9	3.78×10^7
	13	140	1.17×10^5	81	6.8×10^8	1.23×10^4	5.1	5.90×10^7
	12	120	8.15×10^4	75	5.7×10^8	1.22×10^4	4.9	4.81×10^7
	B	141	8.42×10^4	75	$6.55 \times 10^8 \pm 1.19 \times 10^8$ ($n = 23$)	8.09×10^3	5.0	$2.81 \times 10^7 \pm 4.85E+06$ ($n = 22$)
	1	78	1.84×10^5	71	7.3×10^8	5.05×10^3	101	4.10×10^7
	2	n.a.	5.67×10^4	100	5.7×10^8	6.47×10^3	75	4.70×10^7
	3	104	4.02×10^4	125	2.4×10^8	7.38×10^3	50	5.90×10^7
	4	98	2.13×10^4	101	1.9×10^8	1.43×10^4	75	7.14×10^7
	5	114	6.94×10^4	100	2.6×10^8	1.55×10^4	75	5.43×10^7
	6	117	8.75×10^4	125	4.1×10^8	9.28×10^3	75	6.17×10^7
	7	104	9.38×10^4	101	2.6×10^8	2.42×10^4	76	1.41×10^8
	8	98	3.68×10^4	100	3.3×10^8	1.60×10^4	76	7.91×10^7
	9	128	4.83×10^4	121	2.7×10^8	9.55×10^3	76	6.47×10^7
	10	108	4.34×10^4	100	3.7×10^8	1.02×10^4	75	5.56×10^7
	11	109	6.35×10^4	100	4.0×10^8	1.31×10^4	75	6.34×10^7
	C	108	5.86×10^4	100	$2.73 \times 10^8 \pm 4.076 \times 10^7$ ($n = 23$)	1.81×10^4	75	$6.61 \times 10^7 \pm 1.11 \times 10^7$ ($n = 23$)
PROSOPE	1	49	4.54×10^4	29	2.5×10^8	9.89×10^4	49	5.64×10^8
	2	40	3.80×10^4	29	1.7×10^8	5.61×10^4	15	2.49×10^8
	3	5	2.23×10^5	36	3.3×10^8	2.44×10^4	5	9.07×10^7
	4	78	9.57×10^4	60	4.5×10^8	1.87×10^4	12	7.51×10^7
	5	55	6.95×10^4	55	4.1×10^8	1.93×10^4	6	6.77×10^7
	6	85	1.25×10^5	70	6.0×10^8	1.37×10^4	29	6.28×10^7
	MIO	90	1.84×10^5	70	$6.93 \times 10^8 \pm 2.11 \times 10^8$ ($n = 28$)	2.15×10^4	70	$1.04 \times 10^8 \pm 5.89 \times 10^7$ ($n = 28$)
	DYF	47	1.15×10^5	26	$3.30 \times 10^8 \pm 8.03 \times 10^7$ ($n = 23$)	6.26×10^4	47	$2.24 \times 10^8 \pm 1.12 \times 10^8$ ($n = 23$)

Title Page

Abstract | Introduction

Conclusions | References

Tables | Figures

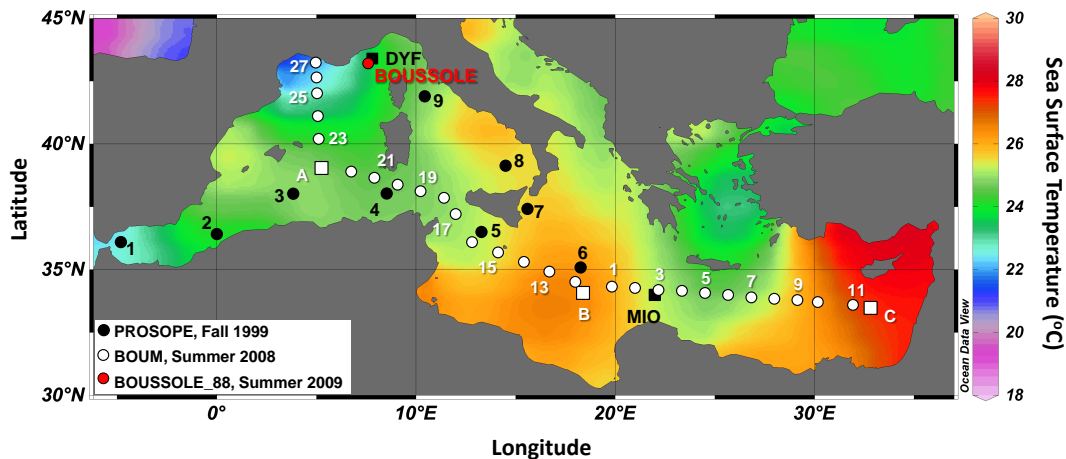|◀ | ▶|

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Back | Close

Full Screen / Esc

Printer-friendly Version

Interactive Discussion

Diversity of picocyanobacteria in the Mediterranean Sea

D. Mella-Flores et al.

Fig. 1. False-color composite satellite image of sea surface temperature of the Mediterranean Sea in July 2008 (data: NOAA). The sampling stations of the PROSOPE, BOUM and BOUSSOLE 88 cruises, numbered in order of occupation, are indicated in black, white and red, respectively. Short- and long-term stations are indicated by dots and squares, respectively.
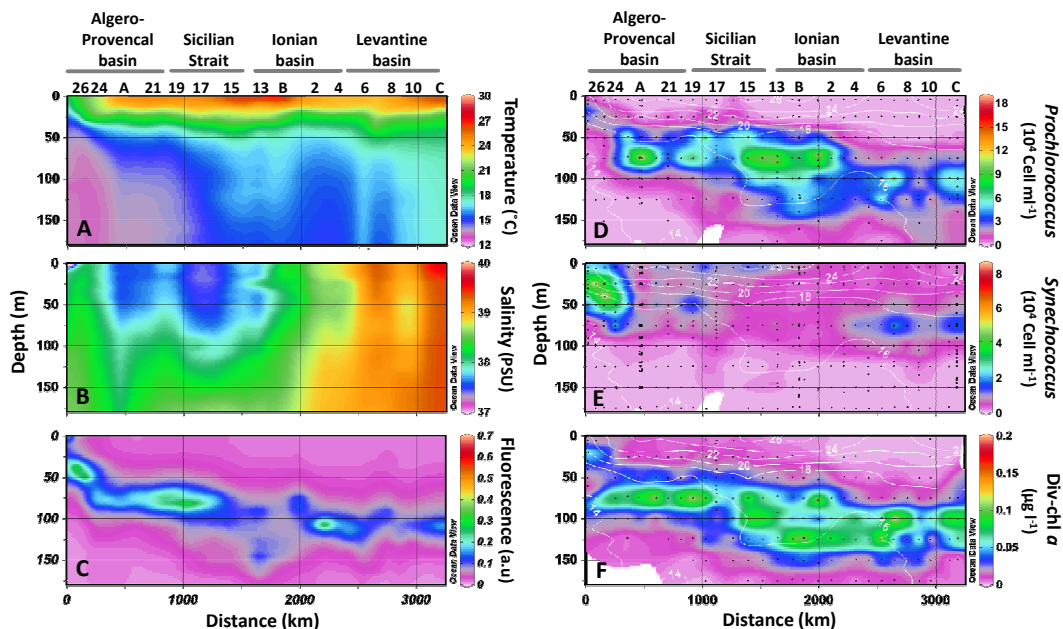
Fig. 2. Depth profiles of temperature **(A)**, salinity **(B)**, fluorescence **(C)**, *Prochlorococcus* abundance **(D)**, *Synechococcus* abundance **(E)** and concentration of the *Prochlorococcus*-specific pigment divinyl-Chl-*a* **(F)** along the BOUM cruise track. For each panel, the x-axis represents the cumulative distance in kilometers from a site near the French coast (43.21° N, 4.93° E) and the y-axis represents depth in meters. The positions of the main marine regions are indicated at the top of the graph as well as the number (or letter) of every two stations along the transect. For panels **(D)** to **(F)**, white contour lines represent temperature in °C and black dots correspond to sampled depths.
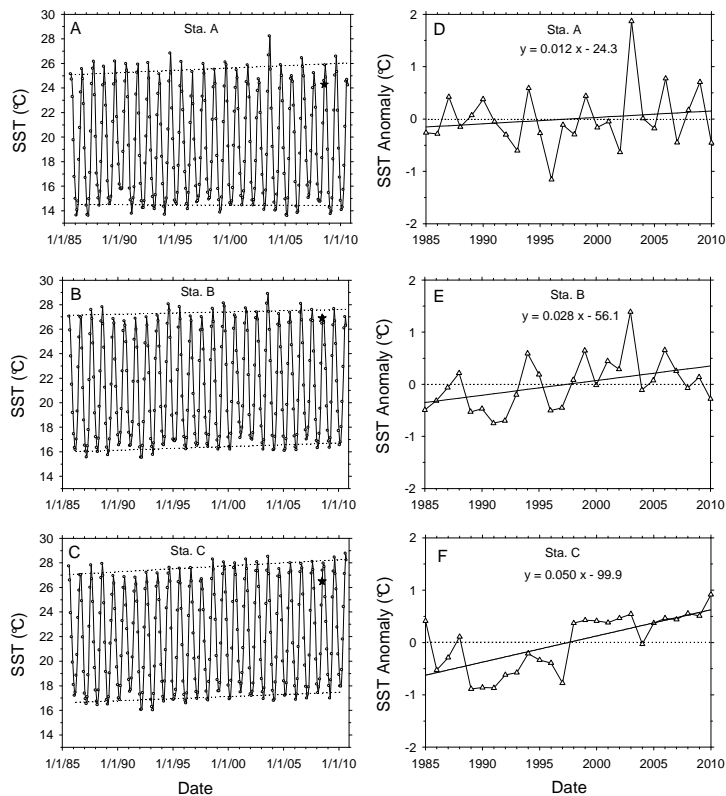
Fig. 3. Time plots over the 1985-2010 period of sea surface temperature (SST) as measured by satellite **(A, B, C)** and average SST anomaly for the three summer months **(D, E, F)** in the 1° latitude-longitude grid around the long term stations of the BOUM cruise (Sta. A, B and C; data: NOAA). The 25-yr trend for the coldest (March) and warmest (August) months (dotted lines) and the actual SST measured on board during the BOUM cruise (stars) are indicated on plots **(A–C)**. The trend curve (plain line) and equation parameters are shown on plots **(E–F)**.

Title Page

Abstract | Introduction

Conclusions | References
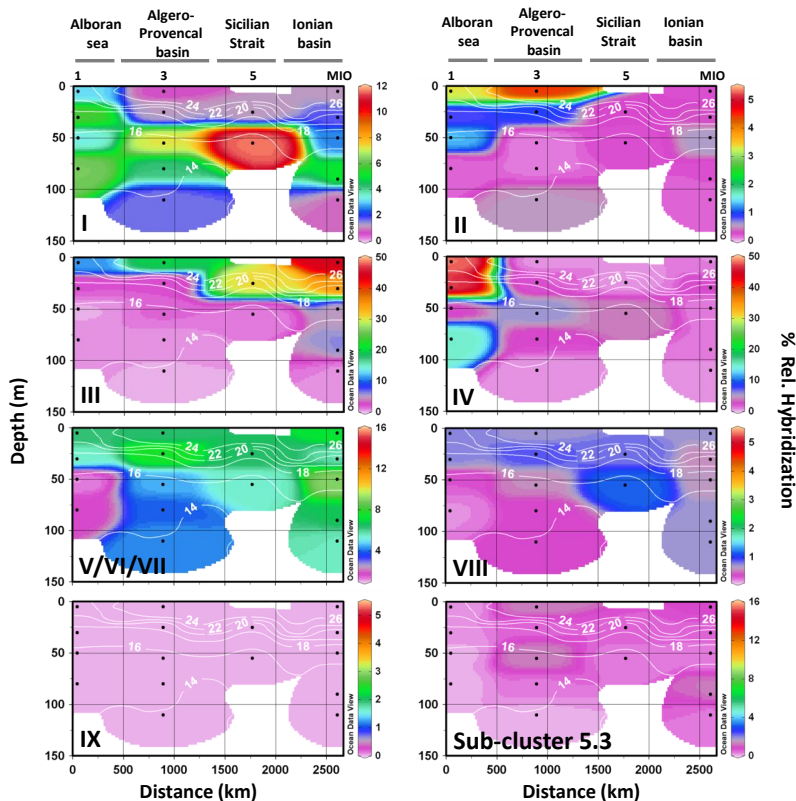
Tables | Figures

|◄ | ►|

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Back | Close

Full Screen / Esc

Printer-friendly Version

Interactive Discussion
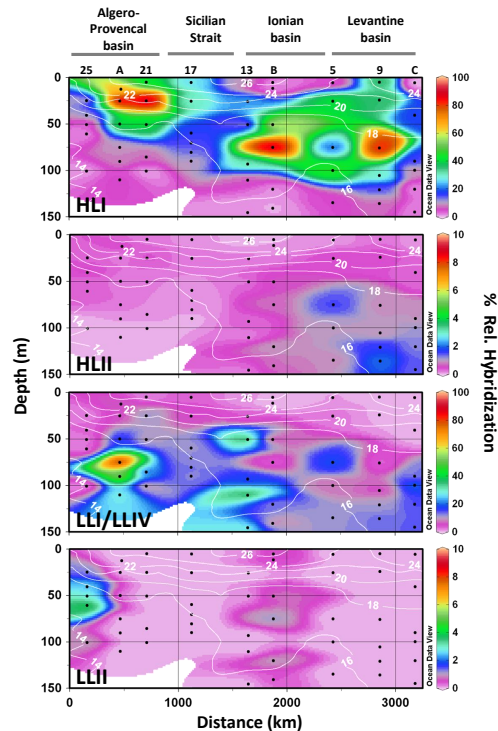
Fig. 4. Vertical distribution of *Prochlorococcus* ecotypes along the BOUM cruise track analyzed by dot blot hybridization using 16S rRNA gene specific oligonucleotide probes targeting HLI (high light ecotype I or eMED4), HLII (high light ecotype II or eMIT9312), LLI/LLIV (low light ecotypes I or eNATL and IV or eMIT9313) and LLII (low light ecotype II or eSS120). Contour plots indicate the % relative hybridization. The positions of the main marine regions are indicated at the top of the graph as well as the number (or letter) of the stations that have been sampled for dot blot hybridization. Details of panels are as described in Fig. 2. Note the different ranges of % relative hybridization values between panels.

Fig. 5. Vertical distribution of different phylogenetic groups of *Synechococcus* along the BOUM cruise track, as analyzed by dot blot hybridization using 16S rRNA gene specific oligonucleotide probes targeting sub-cluster 5.1 clades I to IX and sub-cluster 5.3. Details of panels are as described in Fig. 2. Note the different ranges of % relative hybridization values between panels.

Fig. 6. Vertical distribution of different phylogenetic groups of *Synechococcus* along the W-E part of PROSOPE cruise track, as analyzed by dot blot hybridization using 16S rRNA gene specific oligonucleotide probes targeting sub-cluster 5.1 clades I to IX and sub-cluster 5.3. For each panel, the x-axis represents the cumulative distance in kilometers from a site W of Sta. 1 (36.2° N–5.8° W) and the y-axis represents depth in meters. Details of panels are as described in Fig. 2. Note the different ranges of % relative hybridization values between panels.

Title Page

Abstract | Introduction

Conclusions | References

Tables | Figures

◄ | ►

◄ | ►

Back | Close

Full Screen / Esc

Printer-friendly Version

Interactive Discussion

Fig. 7. (Caption on next page.)

Title Page

Abstract | Introduction

Conclusions | References

Tables | Figures

◀ | ▶

◀ | ▶

Back | Close

Full Screen / Esc

Printer-friendly Version

Interactive Discussion

Fig. 7. Reference Maximum Likelihood (ML) tree of ITS sequences (based on a sequence alignment of 1135 positions, including tRNA) and table summarizing the number of clones of the different *Synechococcus* and *Prochlorococcus* clades/ecotypes for each clone library obtained from samples collected during the BOUM cruise. Novel environmental groups discovered during BOUM (MS1-4) were included in the reference tree and are shown in bold italics letters. The nomenclature used to name the BOUM samples is as follows: stX_Ym_cZ where X indicates the station number, Y, the sampling depth in meters and Z, the clone number. For sequence submission, the prefix "BOUM_" has been added in front of the sequence name. The clade/ecotype number or name as reported in the literature (Ahlgren and Rocap, 2006; Chen et al., 2006; Choi and Noh, 2009; Fuller et al., 2003; Dufresne et al., 2008) are indicated in colored letters for each group. Sequences belonging to the *Synechococcus* marine sub-cluster 5.3 were used as an outgroup. Numbers at nodes of the phylogenetic tree correspond to bootstrap values for ML analyses, posterior probability of Bayesian method (ranging between 0 and 1), and bootstrap values for Neighbor-Joining and Parsimony methods, respectively. Bootstraps, represented as a percentage, were obtained through 1000 repetitions and only values higher than 60 % are shown. The scale represents 0.07 substitution per nucleotide. Branches corresponding to the *Prochlorococcus* HL ecotypes and *Synechococcus* marine sub-cluster 5.3 have been cut for readability (their full length is 0.36 and 0.5 substitution per nucleotide, respectively). The table on the right indicates the number of sequences in clone libraries constructed for each station and depths; their vertical position in the table corresponds to their clade affiliation in the tree, as determined by Pplacer (see text). Numbers in the table are highlighted with increasing degree of shading.