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Biogeosciences Discussions

Interactive comment on "Is the distribution of *Prochlorococcus* and *Synechococcus* ecotypes in the Mediterranean Sea affected by global warming?" *by* D. Mella-Flores et al.

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Reviewer: This discussion paper by Mella-Flores et al. addresses an interesting and timely question as to whether the composition of the picocyanobacteria assemblages in the Mediterranean Sea have been impacted by rising temperatures over the past decade. To do this the researchers assessed the total population size of both Synechococcus and Prochlorococcus using flow cytometry as well as ecotype composition using dot blot hybridization with clade-specific probes on cruises 9 years apart. The first was in September 1999 and the second in July 2008. The methodology used is generally appropriate and was well implemented. The findings are interesting and per-

haps even a little surprising, that despite an increase in water temperatures between the two sampling periods: that the ecotype composition is generally similar between the two cruises and ecotypes known/thought to be more adapted to higher temperatures have not significantly increased in their relative abundance. The authors therefore conclude that, at least so far, increased temperatures have not led to changes in picocyanobacterial composition in the Mediterranean Sea. The manuscript is generally well written and the major conclusions valid. I have mainly minor comments. However, one aspect that complicates interpretation of the findings, mainly for the differences that were found for Synechococcus assemblages, is the different seasons in which the cruises took place. This coupled with the lack of seasonal analysis to assess whether seasonality could explain some of these differences, and lack of multi-year analysis to ascertain the reproducibility of the findings year after year, renders some of the discussion overstated. Furthermore, some of the Discussion is not sufficiently backed up by data or focused enough, and occasionally also contradicts itself (see Specific Comments for details). Therefore, besides some minor changes to the manuscript, the Discussion needs to be shortened and focused and some of the arguments revisited.

Response: We have already addressed the main concern raised in these general comments, i.e. the different seasons in which the cruises took place, in our response to Referee #1 point (1) above. Other concerns are addressed in the specific comments part below.

Specific Comments

Methods:

Reviewer: It should be noted here that surface populations of Prochlorococcus can not be accurately enumerated by flow cytometry. Alternatively, this could be mentioned in the Results when Prochlorococcus abundances are first mentioned, on page 4294. Right now this is stated in passing in the Discussion only on page 4301 line 13.

Response: To address this comment, we added the following sentence in the Methods

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section: " Prochlorococcus and Synechococcus cell concentrations were measured by flow cytometry, as detailed previously (Marie et al., 1999; Obernosterer et al., 2008). Due to the dim fluorescence of Prochlorococcus cells in the upper mixed layer, populations were not completely resolved from the background noise at most stations, so that for integrating cell numbers up to the surface, we assumed that the concentration measured at the bottom of the mixed layer was the same throughout the top layer. "

Reviewer: P4289, line 14-20: The normalization procedure needs to be clarified. The authors state that relative hybridization of the clades is to total oxygenic phototrophs yet a general eubacterial probe was used for this. It seems to be explained in a clearer manner in the figure legend of Figure S1 and perhaps an explanation similar to this should be included in the Methods.

Response: We clarified this point by adding the following detail (underlined) in the methods section: 16S rDNA sequences were amplified from control strains and environmental DNA using the primers OXY107F (GGACGGGTGAGTAACGCGTG) and OXY1313R (CTTCAYGYAGGCGAGTTGCAGC), which specifically target sequences from oxygenic phototrophs (Fuller et al., 2003).

Indeed, this explains why we could then use "a general eubacterial probe" after blotting the amplified material and nevertheless detect only total oxygenic phototrophs. A full description of the method can be found in Fuller et al. (2003), so we don't think a more detailed description is necessary here.

Results:

Reviewer: P4293, line24. It should be clarified in the text that the analyses being discussed are total genus abundances as determined by flow cytometry (my assumption) or combined hybridization blots (if this is actually the case).

Response: Change made as requested. The initial sentence was amended as follows (underlined text): " Figure 2D-E shows the vertical distributions of Prochlorococcus

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and Synechococcus during the BOUM cruise, as determined by flow cytometric cell counting."

Reviewer: P4294, lines 18-29. It would be nice if maximum and minimum integrated numbers for Prochlorococcus would be presented in a similar way in which these were provided for Synechococcus.

Response: Change made as requested. The following sentence was added: "In terms of integrated Prochlorococcus concentrations, the highest value of the BOUM cruise occurred at Sta. 15 (8.08 x 108 cells cm-2) and the lowest at Sta. 25 (6.23 x 107 cells cm-2; Table 2)."

Reviewer: P4294, line 28. State how it was determined that the increase in dvChl a was due to strong photoacclimation, with “as determined from...” Was chl a per cell assessed by flow cytometry and found to increase with depth? If not, then please qualify statement with “probably due to...”

Response: As this was not done systematically, we followed the referee’s recommendation and added "likely due to..."

Reviewer: P4296, lines 12-21. Isn’t it true that clade I was more abundant than clade IV at Sta 5 on both cruises? Please change wording to clarify this.

Response: Yes it is. We slightly changed the wording to make this clearer (text underlined), as follows: "Clade IV generally exhibited a similar distribution pattern to clade I. However, its relative abundance was significantly higher than clade I in the northern part of the Algero-Provencal basin (Sta. 25 and BOUSSOLE) and lower at all other stations of the BOUM cruise."

Reviewer: P4296, lines 26-28. According to Fig. 6, clade IV was considerably more abundant than clade III in the Alboran Sea (assuming that the scale for clade IV for this figure is accurate – see comment below).

Response: True. The text was changed as follows (underlined text was added): "

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Similarly, during the PROSOPE cruise, clade III was also the dominant clade in surface waters (except in the Alboran Sea) and its relative abundance as well as vertical extension increased eastwards (Fig. 6)

Reviewer: P4298, line 20. No Synechococcus or no clade I Synechococcus? Please clarify.

Response: To make this clearer the text was changed as follows (modifications underlined): "In contrast, many clade I and IV sequences were retrieved from Sta. BOUSSOLE and a few clade I sequences from Sta. A, but no sequence from either clade from the three stations of the eastern Mediterranean basin."

Tables and Figures

Reviewer: Table 1: It is not so clear what the reference sequence refers to despite the footnote. That used to design the probe? In fact the meaning of the footnote is not clear and I could not find what the authors are referring to in the Methods on page 4289. It would be more useful for the reader if, rather than the actual reference sequences used to design the probes, strain names representative of the clades that have the most meaning to the reader be used. I propose that these be the fully sequenced strains for Synechococcus that are published in Dufresne et al. 2008 and Scanlan et al. 2009. For example this would include CC9605 and WH8109 instead of RS9903, WH8102 instead of WH8103, CC9902 and BL107 for clade IV and so on. For Prochlorococcus these should correspond to the representative ecotypes discussed in the introduction and presented in the legend of figure 4. For example, use MIT9312, MED4 and MIT9313. If the reference sequence itself is important to retain in the Table, then this information should be included as an additional column of the table. It seems to be explained in a clearer manner in the figure legend of Figure S1 and perhaps an explanation similar to this should be included in the Methods. of WH8103, CC9902 and BL107 for clade IV and so on. For Prochlorococcus these should correspond to the representative ecotypes discussed in the introduction and presented in the legend

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of figure 4. For example, use MIT9312, MED4 and MIT9313. If the reference sequence itself is important to retain in the Table, then this information should be included as an additional column of the table.

Response: We followed the referee's suggestion to provide in an additional column the names of Synechococcus and Prochlorococcus strains that have been sequenced and that are the typical reference for each clade (except clade VII, for which there is no sequenced representative so far), as we explain in footnote 1.

However this information cannot replace the one mentioning the strains or environmental samples that we actually used as controls for dot blots. So we left this column (now renamed "Control sample") in the revised version. The corresponding footnote was indeed incomplete, so to address the referee's concern, we modified it as follows (new text is underlined): "2Strains (or environmental sample in the case of clade IV) representative of the target group and used here as controls for dot blot hybridizations. DNA from the same samples was also used to draw hybridization curves for each probe (cf. materials and methods)"

Reviewer: There appears to be a typo (a number: 5 x 15) instead of a reference sequence strain for the SYN635 probe.

Response: No this was not a typo, but the name of an environmental sample (also mentioned in Fuller et al., 2003). Indeed for clade IV, there were no strain available in culture by the time we did the first series of dot blot hybridizations (i.e. for PROSOPE samples). We then used the same control sample for the BOUM cruise. So we have left the text as is.

Reviewer: Figure 2: Please clarify in the 4th line of the legend that the x-axis represents the cumulative distance towards to the east from the mentioned site.

Response: We respectfully disagree with the referee and preferred not to change the initial text. Indeed the transect went first South, then East (see Fig. 1) so this modifica-

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tion would make the legend incorrect.

Reviewer: Figure 5: It would be very helpful to the reader if a clade strain representative of each clade be presented in the legend as is done for Prochlorococcus in the Figure 4 legend. I suggest these strains be those that have had their genomes fully sequenced (see comment regarding Table 1).

Response: Doing so would considerably lengthen the legend and we preferred to simply refer the reader to Table 1 which, in its revised version (see above), now shows the correspondence between clades and (sequenced) reference strains. So we added the following sentence in the legend of Figures 5 and 6: "See Table 1 for correspondence to sequenced strains."

Reviewer: Fig. 5 and 6: Is the scale for clade IV in figure 5 and figure 6 supposed to be different

Response: Yes. Indeed, the relative abundances of clade IV cells were too different between BOUM and PROSOPE for us to use the same scale in both graphs.

Reviewer: Figure 7: The type is too small to see in a printed version of the figure. It needs to be increased significantly or else the entire figure needs to be larger to make the print readable. Also on a screen it is necessary to increase the figure to a very large size to read the print easily, which prevents visualizing the entire tree all at once.

Response: We are aware that the type is rather small but this is the largest we could possibly use for this figure. We think the problem is in part due to the unusual landscape format used for Biogeosciences Discussion (BGD) manuscripts, but this should be solved in the final format which will be a classical A4 format. In the latter format, the resolution is quite sufficient to read correctly the text after printing it on an A4 page. Furthermore, the most important information, i.e. the clade numbers, is readable even under the BGD format. Note that the revised version contains accession numbers for all the new sequences added.

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Reviewer: Please provide a key for the level of shading on the right side of the figure.

Response: The shading is automatically generated by Excel and is conditioned by the highest value of the dataset, so we cannot simply answer this point. However, this is likely not critical.

Reviewer: Legend should read 0.07 substitutions rather than substitution.

Response: Change made as suggested.

Reviewer: Figure S2: These trees are also impossible to read with the font size used. Placing fewer trees on the page and increasing their size will help.

Response: The important point we want to make with this Figure is where are the clone library sequences showing up in the reference tree at each station. For this, the resolution of this figure and the use of colors are sufficient to show the essential information (i.e. the assignment of environmental sequences to a given clade and their relative abundance). This figure printed in A4 format is sufficient to quickly compare the different stations, but we added a recommendation in the revised legend, as follows: "It is recommended to print this Figure in A3 format to see details". Indeed, splitting the plots between different pages, as suggested by the referee, confuses the comparative power of the phylogenies and goes against the intended aim of this figure.

Discussion:

Reviewer: P4300, lines 10-13: Are the abundances of Synechococcus really higher than Prochlorococcus in the surface waters in the southern part of the Algero-Provencal basin, Sicily Strait and Ionian Sea when one considers that flow cytometry cannot detect surface populations of Prochlorococcus?

Response: No. See reply to referee #1, point (2-a).

Reviewer: Are these findings also borne out from the dot blot hybridizations? This does not seem to be the case for these waters when comparing figure 4 and 5, assuming

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that both Synechococcus and Prochlorococcus are normalized to the same parameter in the same way. This should also be double checked for the northern Algero-Provencal basin (discussed in lines 5-8), although it seems to be possible from the dot blots, but it is hard to ascertain because of differences in scale in Figure 4 and 5. and the need to combine a number of Synechococcus clades.

Response: As mentioned in the response to referee #1, we pointed out in the revised version of the Fig. 2 legend that these distribution data were determined by flow cytometric cell counting. Indeed, dot blot hybridization values obtained for Prochlorococcus and Synechococcus are only semi-quantitative and cannot really be used to compare absolute abundances of specific lineages of these two genera so we prefer not to present such an analysis.

Reviewer: P4300, lines 13-18. Near surface maxima for Synechococcus occurs during blooms soon after stratification of the water column (see Lindell and Post 1995, DuRand et al. 2001). Could this be the situation here? Is much known about the water column conditions at these stations in the month or so prior to sampling?

Response: No. We have no specific information on the water column conditions at these stations in the period prior to sampling. However, we know from the literature that stratification generally occur several months earlier, during late winter/early spring, as we mention at the beginning of the results section.

Reviewer: P4300, lines 19-21: I don't think sufficient information is available to state that there is maintenance of high Synechococcus cell densities in surface waters. Is this the case year round? And from year to year? This is not so clear from the results regarding the Prosope cruise.

Response: The referee is true that we cannot ascertain that there is "maintenance" of high Synechococcus in surface waters. So the revised text was changed as follows (modifications underlined): "This raises the question of why do such high Synechococcus cell densities occur in surface waters of this nutrient-poor region of the Mediter-

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ranean Sea."

Reviewer: The ensuing Discussion from P4300 line 20 to P4301 line needs to be revisited in light of the responses to the above questions and written in a more focused and concise manner.

Response: The ensuing discussion has been changed in particular by adding a sentence and references which mentions the responsiveness of Synechococcus to nutrient additions, as follows: " Indeed, aerosols are enriched in inorganic nutrients (Fe, N and P, but with a N:P ratio usually much higher than the Redfield ratio of 16:1; Paytan et al., 2009) and natural, nutrient-starved Synechococcus populations can respond quickly to such enrichment by rapid increases in cell concentrations (Hutchins et al., 2003; Mackey et al., 2009; Tanaka et al., 2011, Ternon et al., 2011). "

Response: We also reorganized the rest of the paragraph as suggested, though it is not any shorter because we have had to answer a request of the other referee who asked us to discuss about copper.

Reviewer: P4302, line 7: Do the authors mean HL (i.e. either HLI or HLII)?

Response: Yes. Change made as suggested.

Reviewer: P4302, line 12, 17: I wonder if the term “true LL” ecotype is appropriate. The LL nature of the ecotypes is related to their adaptation to LL levels, not to their ecological position in the water column. As such these could be “true LL” adapted strains that are found higher up in the water column. According to Malmstrom et al. (2010) it is not that the LLI (eNATL) ecotype is less adapted to LL than the other LL ecotypes, rather that the LLI ecotype can better withstand light shock and thus may be capable of residing higher in the water column at depths subjected to mixing events.

Response: To take this comment into account, we changed "true LL" for "strictly LL" in the revised text.

Reviewer: P4304 line 15 to P4305 line 6: The P depletion arguments seems very

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unlikely to explain the lack of HLII ecotype in these waters for the very reasons stated by the authors. In addition to the arguments mentioned by the authors, Coleman & Chisholm (2010) found that there are more HLII types than HLI types (Table S2) at both HOT and BATS (with BATS being considered more P-deplete) despite the larger set of P uptake genes in the HLI MED4 strain, making the presence of such genes unlikely to be the reason for more HLI types than HLII types in the Mediterranean Sea. Therefore there does not seem to be a valid reason to invoke P depletion as a reason for the lack of HLII types in the Mediterranean Sea.

Response: The paragraph at the end of the section 4.2.1 has been completely rewritten and shortened. We removed the focus on P and we now develop a different argument, as follows: " Thus, to explain the scarcity of HLII cells in open Levantine surface waters in summer, despite seemingly optimal temperatures for its growth, we hypothesize that, in this area, the upper mixed layer exhibits a combination of features that make these waters unfavorable to growth of Prochlorococcus HL populations in general, as attested by undetectable divinyl-Chl a concentrations (see Fig. 2). These features likely include extremely low macronutrient concentrations, triggering N and P co-limitation of growth, and/or high and potentially toxic levels of copper (Mann et al., 2002; Paytan et al., 2009), though some other yet-to-be identified factors may also be involved. We further assume that local HLI populations are able to survive at low cell concentrations under these stress conditions, whereas alien HLII populations entering the Mediterranean Sea via the Suez Canal cannot and are rapidly outcompeted and eliminated."

Reviewer: P4309, lines 1-3. Once again, giving the reason of low P levels for the lack of clade II Synechococcus strains does not sit well with their relative high abundance in oceanic regions with low P levels (Fuller et al. 2005, Zwirglmaier et al. 2008). Overall section 4.2.1 and 4.2.2 should be written more concisely.

Response: All of the final part of section 4.2.2 has been suppressed in the revised text for the sake of conciseness, since the arguments are the same as those developed before for Prochlorococcus (section 4.2.1).

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Reviewer: P4310, line 17: It would be helpful to briefly restate the major generalizations the authors are referring to here.

Response: Change made as suggested. The revised text now reads (changes underlined): "For Synechococcus, analyses of the distribution of the different clades reported here for the Mediterranean Sea strengthened generalizations made by previous studies in other parts of the world ocean, including the fact that clades I, III and IV are locally the most abundant groups, with clades I and IV co-occurring and being restricted to cool waters, while clade III is ubiquitously found in oligotrophic areas (Bouman et al., 2006; Johnson et al., 2006; Zwirglmaier et al., 2007; Zwirglmaier et al., 2008)"

Reviewer: P4310, line 23-25. Such methodologies, such as quantitative PCR, have already been used to differentiate between distinct Prochlorococcus and Synechococcus ecotypes (see publications from the Chisholm and Palenik labs (such as Ahlgren et al. 2006 EM, Tai Palenik 2009 etc). This should be mentioned here.

Response: Change made as suggested. The revised text now reads (changes underlined): " These data clearly point to a need for the design of further probes, or the use of alternate approaches, such as quantitative PCR (Tai and Palenik, 2009), hybridization to liquid bead-arrays (Tai et al., 2011), or high throughput sequencing using a recently developed functional gene marker (petB) with high taxonomic resolution (Mazard et al., in press), which allow a precise mapping of the distribution of individual clades or genotypes in the environment."

Reviewer: P4311, line 1-2: This statement is not based and should be clarified. In what way does this study point out the need for long term temporal studies for the Mediterranean Sea in relation to global warming, if so far no significant effect has been seen? Because there might be change once greater temperature differences occur? So that there is a better baseline from which to measure potential differences in the future? Or simply because it is important to understand the temporal difference occurring in the Mediterranean Sea irrespective of the impacts of global warming?

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Response: We have better argued why time series would be useful in the revised conclusion, as follows (changes underlined): "Our study advocates the interest of bioindicators (such as Prochlorococcus HLII or the Synechococcus clade II) to complement the classical physico-chemical parameters usually measured to monitor the impact of global changes on marine ecosystems. It also points out the need for time series analyses of the microbial diversity in the Mediterranean Sea, especially in the Levantine basin, which is seemingly the most affected by global warming and which exhibits a number of atypical hydrological traits for an oligotrophic area. Such studies would not only show whether the different taxonomic groups display different annual patterns of abundance (Malmstrom et al., 2010; Tai and Palenik, 2009), but also help unveil what factors, besides temperature, control the composition of these microbial communities"

Reviewer: P4311, lines 2-6: The statement that following HLII Prochlorococcus and clade II Synechococcus is important for monitoring global warming seems to contradict one of the major conclusions arrived at in the Discussion: that the low levels of these two clades are probably explained by factors other than temperature in the Mediterranean Sea.

Response: Prochlorococcus HLII and Synechococcus clade II definitely constitute good potential bioindicators of an eventual colonization of temperate waters by subtropical populations of cyanobacteria on a global scale. The situation of the eastern Mediterranean Sea is somewhat more complex than the rest of the ocean because this is a semi-closed sea and it shows a number of atypical hydrological traits (e.g. low P, strong salinity), in addition to being subjected to global warming like the rest of the world ocean, so multiple factors likely need to be followed.

Technical Comments

Reviewer: P4290, line 17. “from the analysis” instead of “for the analysis”

Response: We modified the sentence so it now reads: A total of 746 environmental ITS sequences were obtained. In further analysis of these sequences primer regions

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were excluded.

Reviewer: P4290, line 21. The accession numbers have not been provided.

Response: They have been added in the revised version.

Reviewer: P4291, line 10. see the Supplementary Information (?)

Response: Here we followed the instructions provided by the editor so let as is.

Reviewer: P4295, line 20. “translating” seems to be strange terminology in this context. Do the authors mean “probably due to: : :” ?

Response: Change made as suggested.

Reviewer: P4296, line 1. Use of the word “whereas” makes the statement a little ambiguous as it is not clear if this whereas relates to differences between HLI and HLII or between the two cruises. Assuming that relatively high numbers of HLII types were found at Sta 5 in both cruises, then “and” would be clearer.

Response: Change made as suggested.

Reviewer: P4301, line 5. “sustain” seems inappropriately used here. Do the authors mean: maintain numbers under sustained P depletion?

Response: We rather chose to replace "sustain" by "withstand" as it better corresponds to our original idea.

Reviewer: P4306, line 10. “where” instead of “but” seems more appropriate if I understand the meaning here.

Response: Change made as suggested.

Interactive comment on Biogeosciences Discuss., 8, 4281, 2011.