

Reply to the referees on behalf of all co-authors

General reply to the referees

We would like first to thank the reviewers for their relevant comments and suggestions which helped us to improve our manuscript.

One of the main questions raised by both reviewers concerns the community of diazotrophs in the Mediterranean Sea, and referee 2 advised us to introduce additional information on this topic. We agree and we propose a revised version of the manuscript including new results on the response of unicellular diazotrophic cyanobacteria (UCYN) to nutrient/Saharan dust additions, including microscopy cell counts of fluorescently labelled UCYN as well as molecular genetic assays, as suggested by referee 2. These new complementary results allow us to 1) improve our knowledge on the nutrient(s) controlling the abundance of these unicellular diazotrophic cyanobacteria and 2) better characterize the community of Mediterranean N₂ fixers.

Among the UCYN community, two cells types have been observed in our samples: a small one (0.8-1.5 µm) which has been previously identified as UCYN-A (Le Moal et al., 2011) and a large one (2.5-3.2 µm). The phylogenetic analysis demonstrates for the first time the affiliation of large UCYN to *Crocospaera watsonii* in the Mediterranean Sea. P and PFe additions stimulated slightly only the growth of the small UCYN at the eastern station (St. C) while Saharan dust additions led to a strong development of *Crocospaera* (up to 10-fold), at stations A and B.

These new results have been introduced in the manuscript in a concise way, as follow:

- **The title** has been changed into
“Nutrient control and Saharan dust impact on N₂ fixation and abundance of unicellular diazotrophic cyanobacteria in the oligotrophic Mediterranean Sea”
- **Introduction.** A paragraph has been added to introduce the problematic of UCYN in the Mediterranean Sea.
- **Materials & Methods, section 2.3 Sampling and Analysis:** two paragraphs concerning UCYN cell types enumeration and UCYN identification have been added.
- **Results, sections 3.1, 3.2, and 3.3:** results concerning the UCYN community have been introduced at the end of each section, after results on N₂ fixation rates.

- **Discussion, sections 4.1, 4.2, and 4.3.** Paragraphs have been added to discuss new results on the response of the UCYN community to nutrient and Saharan dust additions.
- **Figures:** Two figures (Fig. 3 and 4) have been added:

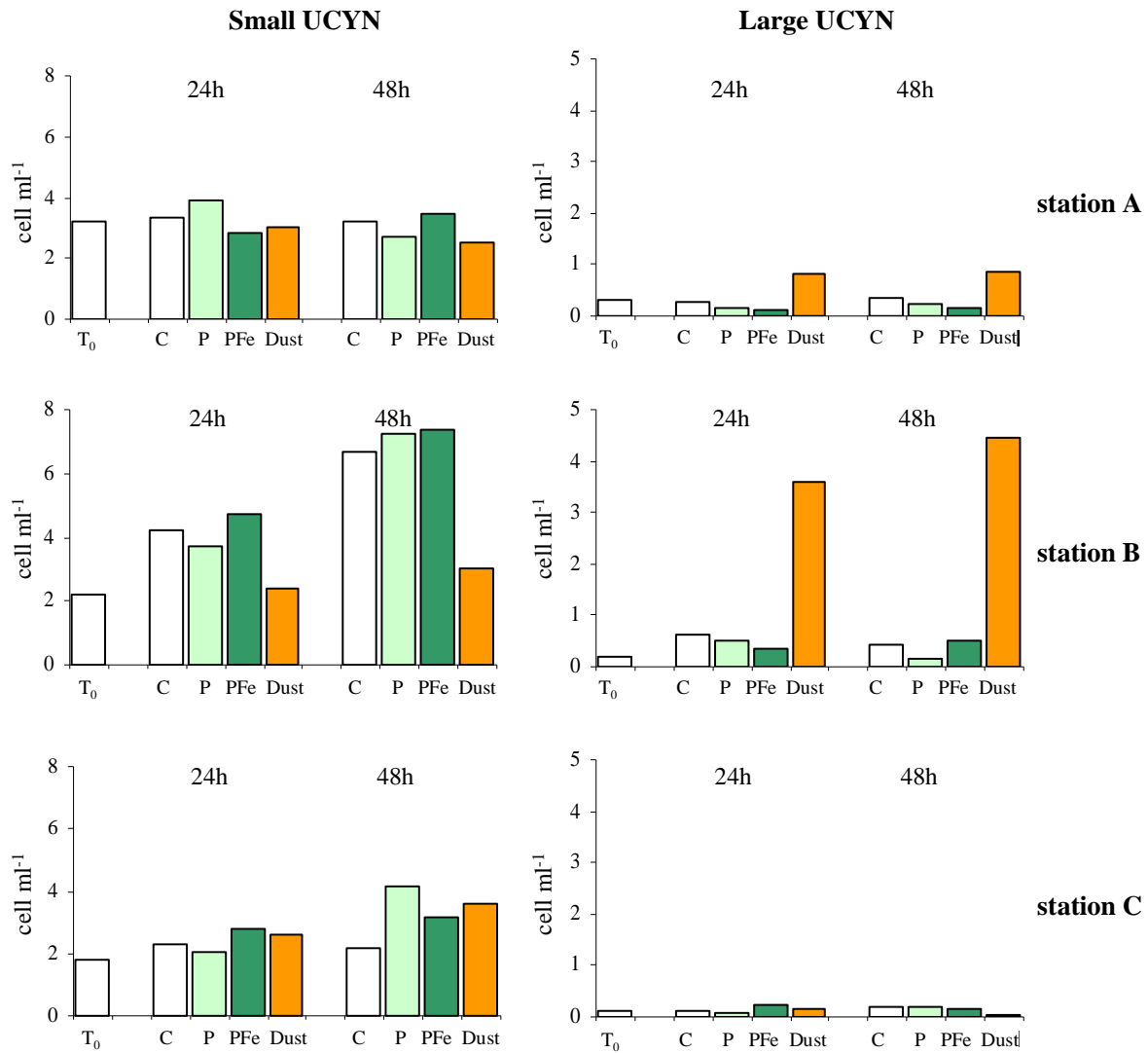


Figure 3: Concentrations of UCYN (cell ml⁻¹) in the control (C), P, PFe and dust treatments at t=24h and t=48h, at stations A, B and C. Two cell types were identified in the 0.2-10 μm size fraction, a small one (0.8-1.5 μm, left panel) and a large one (2.5-3.2 μm, right panel).

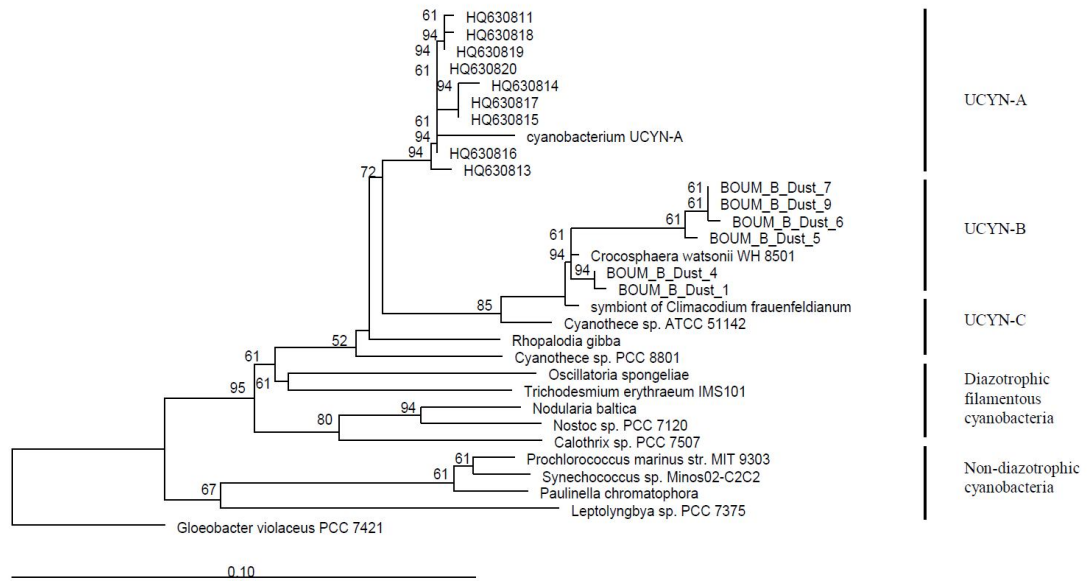


Figure 4: Phylogenetic tree of 16S rDNA sequences from Cyanobacteria. Sequences obtained in this study are referred to (i) the oceanographic transect BOUM, (ii) the station at which they were sampled (A, B, or C), and (iii) their clone number. Bootstrap values >50% are indicated at the nodes. Scale bar = 0.1 substitution per nucleotides.

Specific reply to referee 1

The changes in the text appear below in grey.

1) “Mo is generally considered conservative relative to salinity. Given the high salinities reported, it is a safe assumption that it can be excluded as a limiting factor.”

As compared to others trace metals concentrations in seawater, the concentration of dissolved Mo is high (mean of ~110 nM, Collier, 1985). Some authors have shown that in spite of this high concentration in surface seawater, Mo can nevertheless limit N₂ fixation due to the high concentration of sulfate in seawater which can inhibit molybdate assimilation by phytoplankton (Howarth and Cole 1985; Cole et al., 1993; Marino et al., 2003). For this reason and in spite of the conservative behavior of Mo, we think that bioavailable Mo could be a potential element controlling N₂ fixation in the Mediterranean Sea. We added in the text (section 4.3) the conservative behavior of Mo in seawater (in grey):

“The concentration of bioavailable molybdenum (Mo), nickel (Ni), zinc (Zn) and cobalt (Co) could all be potential candidates as limiting factors in N₂ fixation and growth of diazotrophs. As a cofactor of the nitrogenase enzyme, Mo is strongly suspected to play a central role in the control of N₂ fixation (Howarth and Cole 1985; Cole et al. 1993; Zerkle et al., 2006). As the mean concentration of dissolved Mo in seawater is high and invariant with salinity, Mo is considered as a conservative element in seawater. Despite the high Mo concentration in surface seawater, the high level of sulfate can competitively inhibit molybdate uptake (MoO₄²⁻) by N₂ fixers (Howarth and Cole 1985), but it is unclear how important this competition is in nature (Paerl et al., 1987; Paulsen et al., 1991; Tuit et al., 2004).”

2) “Consideration might be taken into account of the interactive properties of P and Fe given their propensity to precipitate when added as high concentrations. How could this have affected bioavailability within the microcosms?”

We added in “Materials and Methods, section 2.1” the following sentences (in grey):

‘Fe exists in seawater primarily as thermodynamically stable Fe(III) and more than 90% of the DFe is bound to organic ligands (Van den Berg, 1995). DIP as orthophosphate can react with Fe³⁺ to form a precipitate. As the ratio of Fe³⁺/Fe(III)’ (Fe(III)’: dissolved inorganic iron) is ~10⁻¹⁰ at pH 8.1 (Hudson et al., 1992), the chemical precipitation of orthophosphate and ferric iron can be considered as negligible in seawater. Moreover, iron hydroxides (Fe(OOH)) have a high affinity for orthophosphate (Stumm and Morgan, 1996). In seawater, the DIP removal through this scavenging process could be particularly significant in marine sediments

and hydrothermal environments (Krom and Berner, 1980; Feely et al., 1990; Bjerrum and Canfield, 2002). In the surface waters of the Mediterranean Sea, the concentrations of free organic complexing ligands occur in summer at concentrations (from 4 to 6 nM, Van den Berg, 1995; Wagener et al., 2008) well above those of dissolved iron ensuring full complexation of iron (Van den Berg, 1995; Wagener et al., 2008). In our microcosm experiments, 2 nM of DFe were added in the PFe treatment. Knowing that the initial DFe concentration ranged from 1.2 to 2.3 nM, we can strongly assume that the 2 nM of added DFe were kept in solution by this ligand excess. Consequently, the added DFe has probably not precipitated into iron hydroxides meaning that the DIP scavenging onto Fe(OOH) was negligible. So, it appears that the addition of DIP and DFe in the microcosms did not probably change the availability of both nutrients.

3) “Many of the "newly" discovered unicellular diazotrophs are found at maximum concentrations deeper in the water column (below the SML). Did you attempt to quantify concentration versus depth before choosing SML samples? “

We didn't quantify the concentration of unicellular diazotrophs versus depth before choosing SML samples because molecular techniques used to quantify or at least identify microorganisms such as unicellular diazotrophs are time consuming and/or not applicable at sea. Moreover, the BOUM cruise permitted the first study on distribution of diazotrophs across the entire Mediterranean Sea, and consequently no information on the depth distribution of these organisms was available before microcosm experiments were conducted. The choice of the SML sampling was motivated by the fact that the SML is strongly impacted by atmospheric deposition during the stratification period and that microorganisms living in the SML are the ones which benefit most from these atmospheric nutrient inputs. Since our bioassay experiments have been conducted, two studies on the vertical distribution of diazotrophs in the Mediterranean Sea have been published (Le Moal et al., 2011; Yogeve et al., 2011). Le Moal et al. (2011) reported by TSA-FISH technique the exact quantification of unicellular diazotrophic cyanobacteria (UCYN) at stations A, B, and C, from the BOUM transect, at two contrasted depths: at 12.5m which was nutrients depleted and at the upper deep chlorophyll maximum (DCM⁺) which was nutrients repleted. It appears that UCYN were present in low concentrations (1-6 cell ml⁻¹) at 12.5m but were on average 5.5 times more abundant than at DCM⁺. This result was consistent with the study of Yogeve et al., (2011), who detected UCYN from group A only in surface waters in the eastern Mediterranean Sea. Thus, the SML seems to be the most favourable layer for the development of UCYN, and we

think it is relevant to include results concerning their response to nutrient/dust additions to understand why they are present only in low concentrations.

I would be curious to see the results of the same experiments from deeper water samples, both as a function of biomass and the change in dust/nutrient availability farther from the atmospheric source.

We agree that it would be also very interesting to realize nutrient bioassay experiments deeper in the Mediterranean water column to study the response of the diazotrophic community which is probably different than that recovered in the SML. Indeed, Yogeve et al. (2011) demonstrated with clone libraries of *nifH* transcript that the community of active diazotrophs varied with depth. Nevertheless, further analyses using specific oligonucleotides will be necessary to quantify by TSA-FISH and/or quantitative PCR the Mediterranean diazotrophs other than cyanobacteria. Assuming that the community of diazotrophs varies with depth, we can strongly suspect that the nutrient controlling N₂ fixation and growth of diazotrophs varies with depth.

How would one quantify trace nutrient availability as dust particles settled through the water column?

The release of DIP and DFe from Saharan dust depends on different factors, the dust load in seawater being one of them. Ridame and Guieu (2002) for P and Bonnet and Guieu (2004) for Fe, showed that the percentage of dissolution decreases as the particle concentration (Pc) increases and reported a negative power relationship between both variables. This relationship may have critical consequences on the fertilizing potential of mineral dust which will ultimately depend on particle vertical distribution within the water column. The sedimentation rate of the Saharan particles in seawater can be computed as a function of particle size by use of Stokes' law (Ridame and Guieu, 2002). A size fraction $\leq 1 \mu\text{m}$ (diameter) that represents up to 90% of the total number of Saharan aerosols (Chester and Johnson, 1971) has very weak settling velocity in seawater ($<5 \text{ cm d}^{-1}$) if not self-aggregating to form larger particles or associated to larger biogenic aggregates. In the absence of parameterization of those processes, dust depositing at the surface of the ocean is often assumed to distribute homogeneously within the SML. Pulido-Villena et al., (2010) have deduced from observations in large mesocosms that the transient DIP increase in surface of the water right after an artificial seeding with Saharan dust was attributed to a nonhomogeneous vertical distribution of the mineral particles after dust addition. Artificial seeding experiments in large

mesocosms coupled with optical measurements indeed have shown that the particulate export following a dust event is a non-linear multi-step process and that this export is controlled in part by the formation of organic-mineral aggregates (Bressac et al., submitted). Vertical distribution of dust in the column water does thus certainly impact on the physical speciation of nutrients which is related to their potential to fertilize oceanic surface waters.

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