Reply to the referees on behalf of all co-authors

General reply to the reviewers

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 worldwide unicellular diazotrophic cyanobacteria and 2) better characterize the community of Mediterranean N_2 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 *Crocosphaera 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 *Crocosphaera* (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 N_2 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 N_2 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:

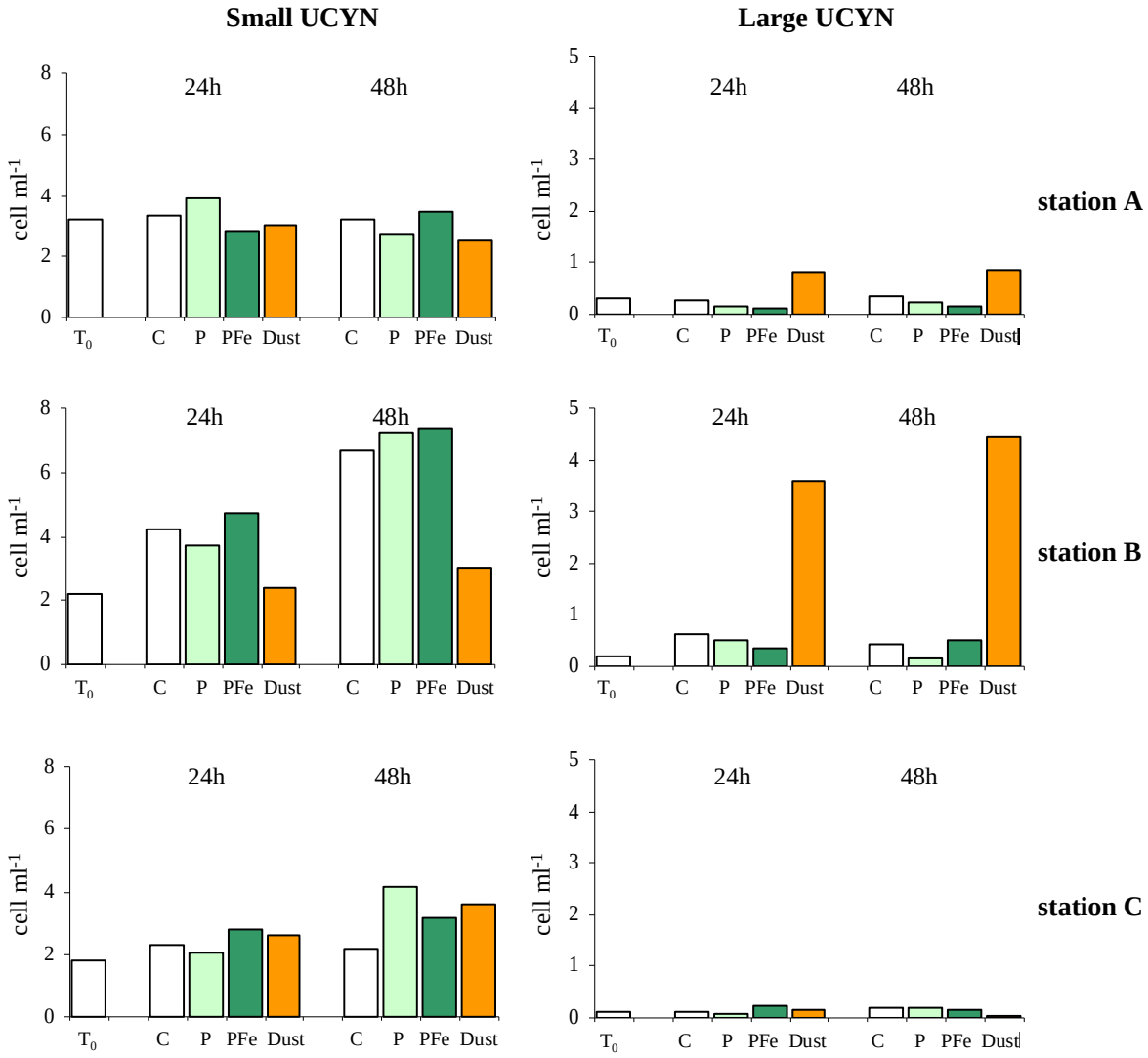

Figure 3: Concentrations of UCYN (cell ml^{-1}) in the control (C), P, PFe and dust treatments at t=24h and t=48h, at stations A, B and C. Two cell types were identified in the 0.2-10 µm size fraction, a small one (0.8-1.5 µm, left panel) and a large one (2.5-3.2 µm, right panel).

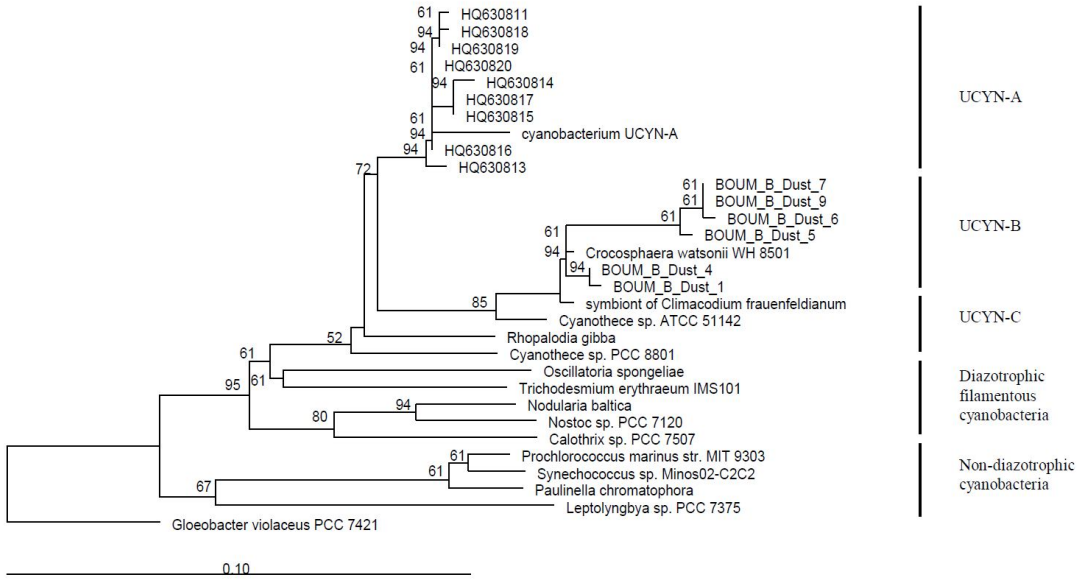

Figure 4: Phylogenetic tree of 16S rDNA sequences from Cyanobacteria. Sequences obtained in this study are referred to (i) the oceanographic transect BOUM, (ii) the station at which they were sampled (A, B, or C), and (iii) their clone number. Bootstrap values >50% are indicated at the nodes. Scale bar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

Specific reply to referee 2

Is the chemical characterization of the dust amendment known?

Yes, the total content of P, Fe and N in the Saharan dust was given in section 2.2, §Saharan dust characterization: "The total contents of phosphorus, iron and nitrogen in the dust were on average 0.14 % ± 0.01 %, 4.97 % ± 0.12 % and 0.11 % ± 0.01 % in weight, respectively". The estimated concentration of DIP released by dust in the dust treatments was given in section 4.3: "We used a relationship between the percentage of DIP released from Saharan dust in seawater as a function of dust concentration (Ridame and Guieu, 2002), to estimate that about 10 nM of DIP were released by dust in the bottles with dust added which was lower than the DIP addition in the bottles enriched by DIP (30 nM)." As Fe does not seem to be a controlling factor of N_2 fixation in the Mediterranean Sea during summer, we did not focus on the release of DFe from dust.

If dust deposition is in fact a major influence on N2 fixing activities and community structure it would be valuable to identify if the rates increase or decrease over the seasons by these amendments?

N_2 fixation rates measured over a year in the north-western Mediterranean Sea exhibit a marked seasonal variability with the highest values recorded in surface during spring and summer (Sandroni et al., 2007). In this study, no relationship was found between the concentration of DIP or DFe and the N_2 fixation rates. To date, the parameters controlling this temporal variability in the Mediterranean Sea are unknown.

In the open Mediterranean Sea, during the summer stratification period (from June to September), the surface waters are isolated from intermediate and deep waters. Consequently, the surface mixed layer is nutrient depleted. During this season, the atmosphere becomes the primary pathway for inputs of new nutrients to the surface waters, and, during this same period, the frequency and the magnitude of Saharan pulses are greatest over the Mediterranean Sea (Bergametti et al., 1989; Loye-Pilot and Martin, 1996). Therefore, the summer season appears to be the period when N_2 fixation could benefit most from Saharan dust inputs, in the Mediterranean Sea.

And finally, it would have been interesting to include or report on 13C/14C label(s) experiments (assuming 13C uptake was also measured) as carbon fixation is an important component of N2 fixation.

Our study is focused on the chemical factors controlling N_2 fixation and the abundance of UCYN in the Mediterranean Sea and the potential impact of a Saharan dust event. A companion paper from Bonnet et al., (2011) is focusing on the biogeochemical significance of N_2 fixation in the Mediterranean Sea, in particular through the estimations of N supply by N_2 fixation and contribution of N_2 fixation to the new primary production during the stratification period. They show that the contribution of N_2 fixation to the new primary production is negligible at the station C (0-0.3%) and increases to reach 9% at stations A and B.

Indeed, we did measure primary production (^{13}C uptake) in our samples. Assuming a molar C:N ratio in UCYN of 7 (data for *Crocosphaera* in Fu et al., 2008), we roughly estimated that the contribution of N_2 fixation to primary production was negligible (<5%) in all treatments (P, PFe, dust) at all stations. Nevertheless, we assumed through this calculation (i) that the small UCYN, previously demonstrated to be UCYN-A (Le Moal et al., 2011), are photosynthetic, which is arguable as UCYN-A were recently found to lack the oxygen-producing photosystem II complex of the photosynthetic apparatus (Zehr et al., 2008), (ii) that these small UCYN were also characterized by a C/N ratio of 7 as *Crocosphaera* and (ii) that N_2 fixation was exclusively realized by UCYN which is not realistic as diazotrophs other than cyanobacteria have been discovered in the surface waters at stations A, B, and C (Le Moal et al., 2011). Knowing these uncertainties, we did not include these data and calculations in the manuscript.

Page 2633, line 4. Which diazotrophic cyanobacterial picoplankton are the authors referring to? Are these the unicellular groups (i.e. A, B, C)?

Yes, we are referring to the UCYN from group A, B and C. To clarify the sentence and introduce the new results on the response of UCYN to nutrient/dust additions, we changed in the 'introduction', the sentence and added information on these UCYN-A, B, and C as follow:

"Picoplanktonic (<3 μm) unicellular diazotrophic cyanobacteria (UCYN) as free living cells largely dominated communities of diazotrophic cyanobacteria across the entire open Mediterranean Sea and throughout the year at a coastal station in the north-western basin (Le Moal and Biegala, 2009; Le Moal et al., 2011). Despite their low concentrations throughout the year and across the sea, these organisms were able to reach one of the highest concentrations ever recorded for UCYN during one summer at the coastal station (Le Moal and Biegala, 2009). A combination of environmental parameters was suspected to be responsible from such massive development of the UCYN community, the unusually warm sea surface temperature, detectable concentration of DIP and unusually high urban pollution

event. This community of UCYN was dominated by small cells (0.7-1.5 µm) whose affiliation to the uncultivated group A (UCYN-A) was demonstrated in the open western basin (Le Moal et al., 2011). Slightly larger cells (2.5-3.2 µm) were also observed at both coastal and open Mediterranean Sea and hypothesized to be affiliated to *Crocosphaera watsonii* (UCYN-B) or *Cyanothece* sp., (UCYN-C) (Le Moal and Biegala, 2009; Le Moal et al., 2011). "

For the non-expert on time series stations and research programs in the Mediterranean Sea, DYFAMED (pg 2632, line 15) and BOUM (pg 2634, line 3) will not be recognized.

In the "introduction" section, we added some information (in grey) concerning the DYFAMED station and the BOUM program :

"At the north-western DYFAMED site (DYnamique des Flux Atmosphériques en MEDiterranée, 43°25N 07°52E, http://www.obs-vlfr.fr/sodyf/), N_2 fixation rates measured over a year ranged from 0.5 to 7.5 nmol N L^{-1} $12h^{-1}$ with the highest values recorded in surface during spring and summer (Garcia et al., 2006; Sandroni et al., 2007; Marty et al., 2008)."

"As the Mediterranean surface waters are DIP-depleted in summer, the BOUM cruise (Biogeochemistry from the Oligotrophic to the Ultra-Oligotrophic Mediterranean, see details in Moutin et al., 2011) provides a good frame to investigate the nutrient factors (including Fe) controlling N_2 fixation."

Page 2632, line 22. After which phytoplankton bloom? Is this in reference to a seasonal bloom or are a potential scenario?

We assumed that this comment refers to page 2633, line 22. Total chlorophyll-a and primary production are maximal each year in surface layers during spring (March and April) revealing a seasonal phytoplanktonic bloom (Marty et al., 2002). The sentence in the introduction has been modified (in grey): "After the seasonal phytoplanktonic bloom in spring, the surface mixed layer (SML) isolated from deeper waters by a strong stratification becomes nutrient-depleted."

In the results and in the methods section, references to anti-cyclonic eddies are made; maybe the authors should provide a brief description on these features and their potential importance/influence/relevance to their study on N2 fixation?

The following paragraph has been added in "Materials and Methods, section 2.1": "These three stations were located at the center of warm-core anticyclonic eddies (see details in

Moutin et al., 2011). The sampling of eddies was motivated by the fact that these systems are quasi-stable, and the horizontal advection is negligible. The eddies of about 100 km in diameter (Moutin et al., 2011) were easily identified by a local deepening of isotherms leading to a strong depletion in nutrients (Pujo-Pay et al., 2011), a decrease in chlorophyll-a and N_2 fixation rate (Bonnet et al., 2011). For these reasons, they represented good sites to study the impact of nutrients/dust additions on N_2 fixation.

Page 2636, line 6. In section 2.1, N2 fixation rates were described for 0-24 and 24-48 hr incubations, here it is described at 0 and 24 hr?

The sentence has been modified in the text (section 2.1) for more clarity: "5 mL of $^{15}N_2$ gas (99%, EURISOTOP) were added at two selected time points (0 and 24h) to 4.5 L polycarbonate bottles equipped with septum caps using a gas-tight syringe for $^{15}N_2$ uptake determination between 0-24h and 24-48h."

Page 2637. Is the initial N2 fixation rate, the 0-24 hr incubation or time 0? If it is in reference to the bottles sacrificed at the start of the experiment, or time 0, is this really possible to measure given the new evidence of underestimation due to dissolution of N2 bubble? Mohr W, Grosskopf T, Wallace DRW, LaRoche J. (2010). Methodological underestimation of oceanic nitrogen fixation rates. PLoS One 9: 1–7.

The initial N_2 fixation rates correspond to the 24h-rates measured in the control treatments (between 0 and 24h). We changed the sentence for more clarity (section 3.1): "Initial N_2 fixation rates in surface waters measured in the control treatments (0-24h) were very similar at the three study sites (Table 1) as demonstrated by the low standard deviation (mean: 0.10 ± 0.02 nmol N $L^{-1}d^{-1}$)".

Moreover, we added the following sentence in section 2.3: "It has to be noted that the N_2 fixation rates measured by the $^{15}N_2$-tracer addition method may have been underestimated due to incomplete $^{15}N_2$ gas bubble equilibration, as recently shown by Mohr et al., (2010).

Page 2639, line 14. Note the reference to Needoba et al. 2007 study and maximum abundance of small diazotrophs within 22-24°C temperature is to a study off the coast of N. California with SST and temperatures within the mixed layer which were below 20°C.

We did a mistake. In section 4.1, the reference to Needoba et al. (2007) has been removed.

Sections 4.2-4.3. These sections might be combined into one as it appears repetitive.

As we added new results on the response of the UCYN abundances to nutrient/dust additions, we chose to keep these 2 sections separated: one about the response of the UCYN community to P and Fe additions and the other one about the response to a dust event. We made efforts to be concise and not repetitive.

An additional reference that might be useful for comparison of rates and responses to P and dust amendments is an earlier study in the Red Sea: Foster et al. 2009. Seasonality of N2 fixation and nifH gene diversity in the Gulf of Aqaba (Red Sea) Limnol. Oceangr. 219-233.

Foster et al. (2009) presented results obtained after nutrient/aerosols addition to coastal/pier waters of the Gulf of Aquaba. They showed that DIP and dust filter additions had no significant effect or a low effect on N_2 fixation rates (+36%).

We added in:

section 4.2 'Phosphorus': "Similar response of N_2 fixation was observed in the North Pacific Ocean (Needoba et al., 2007; Zehr et al., 2007) and Red Sea (Foster et al., 2009) where addition of DIP did not led to a significant stimulation of the rates."

Section 4.3: "Results from previous bioassay experiments have shown that a Saharan dust addition was able to significantly increase N_2 fixation in the tropical Atlantic waters (Mills et al., 2004; Maranon et al., 2010) while no significant stimulation was recorded in the Gulf of Aqaba (Foster et al., 2009)."

Sections 4.3. Could the dust also contain some contaminants, trace metals, chemicals, which could be toxic to cells if these were in high concentrations in the dust? Was there any evidence, i.e. decrease in biomass, which could suggest a negative effect to dust amendments?

As well as a source of N and P, atmospheric aerosols deposition is also an important source of trace metals to the open ocean. Paytan et al., (2009) have recently observed a toxic effect on phytoplankton growth (chla) after addition of African aerosols in surface seawater of the Red Sea. They suggested that this effect could be due to the high copper concentration in aerosols. Aerosols contain also organic molecules which could impact the phytoplankton and bacterial communities but very little is known about the characterization and solubility of the organic matter associated with the Saharan dust. Nevertheless, in our study we did not observe a decrease of chla (data not shown), N_2 fixation or UCYN abundance after the dust amendments

excepted at station B where the abundance of the small cells (UCYN-A) decreased (-50%) as compared to the control. As the small cell concentration did not decrease at stations A and C after dust additions, we hypothesize that dust did not inhibit the development of the small cells (UCYN-A).

Section 4.4. It is not clear if the various phylotypes referenced as present were from this study or a parallel study. A bit more introduction to where these results come from. For example, (i) the percent similarity of the phylotypes (Bradyrhizobium, UCYN-A), abundance by qPCR or qRT-PCR? (ii) It is not clear why some groups would be excluded over others by volume filtered? (iii) How is it concluded that 50-100% of the activity is attributed to the smaller size fraction? This section could be improved by including more of information or connectivity between the present study and the phylogenetic diversity studies which are referenced.

This section 4.4 has been deleted; the discussion on diazotrophic organisms has been clarified, improved with new information on UCYN, and introduced in sections 4.2 and 4.3

More specifically:

(i) the percent similarity of the phylotypes *Bradyrhizobium* and UCYN-A with their closest relative is 94% and 98%, respectively. We didn't add these information in the discussion as it refers to the result of the companion paper of Le Moal et al. (2011, Table 2). To date, only UCYN have been quantify by TSA-FISH technique in the Mediterranean Sea, and no information on the quantification of non-cyanobacterial diazotrophs is available.

(ii) Our sentence was not clear enough to be understood: the topic was not to exclude some groups over others by volume filtered, but to discuss the fact that the activity of *Trichodesmium sp., Richelia intracullaris*, and UCYN-B cannot be excluded, although they were under detection limit at stations A, B, and C (Le Moal et al., 2011). Indeed, filtered volumes used to measure N_2 fixation rates were higher than the ones used to detect diazotrophs, and we cannot exclude that these diazotrophic cyanobacteria would have been detected in bigger filtered volume. Nevertheless, this sentence has been clarified and now concerns only filamentous cyanobacteria, as UCYN-B have been detected in Saharan dust enrichment experiments.

(iii) By measuring N_2 fixation rates both in the total and in the picoplanktonic size fraction (< 3µm), Bonnet et al. (2011) have shown that 50-100% of the total N2 fixing activity can be attributed to the < 3µm size fraction.

Suggestion. Is it possible to take the rates and information reported here in a more global context? For example, how do the rates reported here compare with other rates?

In section 5 'conclusions', the following sentence has been added : "In spite of strong increases after dust additions, the rates remained low (maximum of 0.52 nmol N L^{-1} d^{-1} at station C) as compared to those measured in tropical Atlantic and Pacific Oceans".

Also, in section 5, we added: "This underlines the importance of Saharan dust deposition on the N_2 fixing activity in the Mediterranean Sea and potentially in all oligotrophic areas impacted by dust deposition such as the tropical Atlantic and Pacific. Predictions of future oceanic dust deposition are model dependent and will be controlled by land use changes, as well as climate effects (Tegen et al., 2004; Mahowald et al., 2006). Modeling studies predict for the next 100 years a strong increase (+200%) to a high decrease (-60%) of the global dust flux with different regional deposition patterns (Woodward et al., 2005; Mahowald et al., 2006) suggesting large uncertainties in these predictions."

Or, given the measurements of N2 fixation rates, it should be possible to estimate the amount of new nitrogen added to the euphotic zone by N2 fixation, i.e. what percent of new production is from N2 fixation measured during the given study?

From the data obtained during the BOUM transect, Bonnet et al., (2011) presented the estimations of N supply to the euphotic zone by N_2 fixation and the contribution of N_2 fixation to the new primary production during the stratification period in the Mediterranean Sea. The authors have shown that while the biogeochemical impact of N2 fixation in the eastern basin seems negligible, N2 fixation is able to sustain up to 35% of new primary production during the stratified period and accounts for up to 25% of the external "new" N supply to the western basin during that period.

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