Reply to the reviewers, on the behalf of all co-authors

General reply to the reviewers

We would like to greatly thank the referees for their relevant comment on the recovery of non-specific plastid sequences, and for their helpful spelling and grammar corrections.

The general comments of both reviewers concern the English language and the recovery of plastid sequences. We agree with all comments and adressed them as advised. Concerning the English language, all spelling and grammar errors noticed by reviewers #1 and #2 were adressed and some sections were re-written to improve the reading of the manuscript. Concerning the recovery of plastid sequences, the Referee #2 suggested that it was the result of non specific amplification, and that "the only way to know the obtained 16S rRNA sequences have an identical sequence in the Nitro821 primer region is to find a sequence identical to theirs in Genbank but obtained with a different primer pair that is further toward the end of the 16S rRNA gene than the Nitro821 primer".

Genbank closest relative sequences of each plastid groups have been looked for and 4 to 7 mismatches with the Nitro821 oligonucleotide were detected (see Table a). Biegala and Raimbault (2008) have demonstrated that the non-UCYN strains PCC73106 and PCC6803, which have 3 and 4 mismatches with Nitro821 respectively (see Table a), are not hybridized by the Nitro821 probe. Moreover, Mazard et al. (2004) showed that sequences having more than 1 mismatche with Nitro821 are not PCR amplified during Nitro821/Cya359 PCRs. So far, only the freshwater cyanobacteria *Planktothrix* has 1 mismatche with the Nitro821 oligonucleotide, however this cyanobacteria is unlikely to be detected in the marine environment, (Mazard et al., 2004).

We thus agree with Referee #2 that the plastid sequences are the result of non-specific amplifications made possible by the use of nested PCR on 16S rDNA gene. Consequently, the discussion on plastids is not correct and has removed entirely.

Table a. Number of mismatches between the Nitro821 oligonucleotide and 16S rDNA target sequences from plastids and two cyanobacteria.

Phyloge-netic group	Nb of clo-nes	Iden-tity (%)	Probe, target, or closest relative	Sequence*
			Nitro821	3'- CTT TGA TCC ACA CCG AAC - 5'
			Target	5'- GAA ACT AGG TGT GGC TTG - 3'
		99	Unc. bacterium clone S25_1306 (EF574962)	. . T . . . . T . . G . A
Group 2	5	91	*Ochromonas distigma* RCC21 (AY702136)	. . T . . . . . A . . . T . T . GT
Group 3	1	98	Unc. cyanobacterium SHAB462 (GQ348575)	. . T . . . . . A . . . T . T . GT
Group 4	1	91	Unidentified eukaryote OM270 (U70723)	. . T . . . . . A . . . T . . G . A
Group 5	1	96	Unidentified eukaryote OM270 (U70723)	. . T . . . . . A . . . T . . G . A
Group 6	3	87	*Crustomastix stigmatica* (FN563093)	. . . . G T . G GGA
Group 7	3	88	*Crustomastix stigmatica* (FN563093)	. . . . G T . G GGA
Group 8	2	94	*Dolichomastix tenuilepis* (FN563094)	A . . . . T . G GCT
Group 9	1	99	Unc. bacterium (AB307974)	. . T . . . . . . A . TTA A . T
			Gloeocapsa sp. strain PCC 73106	. . T . . . . . C . . A . . . . .
			Synechocystis sp. strain PCC 6803	. . T . . . . T . T C . .

** dots indicate bases identical to those of the target sequence.*

The paper has been modified as follow:

- **Material & Methods, section 2.4. PCR and cloning**: the nested protocol for 16S rDNA amplification has been removed.
- **Results, section 3.3. Diazotrophs species richness**: results concerning 16S rDNA phylogenetic analyses have been modified as follow: "UCYN specific 16S rDNA amplification was obtained only from station A and all the sequences were affiliated to UCYN-A". Moreover, according to the suggestion of Referee #2, we highlighted the discovery of the new marine group of *Bradyrhizobium* by adding a figure illustrating the percentage of each group of diazotrophs at station A, B, and C (Figure 7, see below).

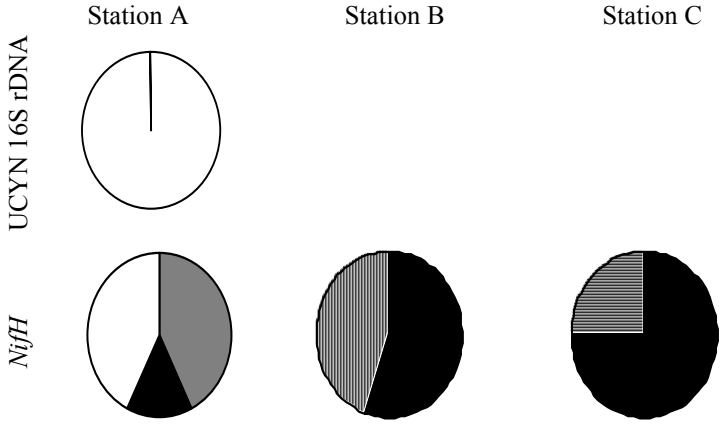

Fig. 7

Fig. 7. *Percentage of sequences from UCYN specific 16S rDNA and NifH clone libraries from station A, B, and C. Colors indicate phylogenetic groups from figure 5 and 6 in which sequences were recovered: UCYN-A group 1' (white); Bradyrhizobium group 2' (black); Bradyrhizobium group 3' (grey); rhizobia group 4' (horizontal lines); γ-proteobacteria group 5' (vertical lines).*

- **Discusssion: section 4.2. UCYN species richness recovery on 16S rDNA**: The discussion on plastids recovery has been removed and replaced by a discussion on the recovery of UCYN-A at station A and on the absence of positive 16S rDNA PCR amplification at station B and C, due to the too low cells concentration.
- **Fig 5:** all plastid sequences have been removed
- **Table 2:** all plastid sequences have been removed

Specific comments:

Title: Change to 'Intriguing diversity among diazotrophic picoplankton along a Mediterranean transect: a dominance of rhizobia'. (At the very least remove all mention of plastids). This modification has been done.

Throughout the manuscript italicise gene names (*nifH* etc). Also, use *Bradyrhizobium* throughout which is the genus name. Gene names have been italicized and Bradyrhizobia replace by *Bradyrhizobium* throughout the manuscript.

Abstract

Line 2: delete been. This has been corrected.

Line 17: delete the sentence starting 'Surprisingly, the….This sentence has been removed.

Lines 19-22: re-write these sentences: *Bradyrhizobium* sequences dominated *nifH* clone libraries from picoplanktonic size fractions. A few sequences of γ-proteobacteria were also detected in the central Mediterranean Sea. These sentences have been modified following the request of the referee.

Line 25: add comma after particles. The coma has been added.

Line 26: and using photosynthetic activity…. The word "using" has been added.

Line 26-27: Delete sentence beginning: 'Among UCYN further work… The sentence has been deleted.

Introduction:

p8781

Line 5: compared to Redfield (not Redfield's one). This has been corrected.

Line 11: punctual peaks. This has been corrected.

Line 13: replace 'massive' with a more appropriate description (or delete massive). The word "massive" has been deleted.

Line 15: re-write as follows: However, the importance of diazotrophy has been argued to be inconsistent with the known phosphate-starved conditions found in the Mediterranean Sea…… The sentence has been modified.

Line 17: controlled by phosphate… This has been corrected.

Line 25: larger ones (not large one). This has been corrected.

p8782

Line 11 replace semi-colon with comma (after Foster et al., 2007). This has been corrected.

Line 13-14: In addition to being free-living, UCYN have also been suggested to produce mucilage,….. The sentence has been modified.

Line 17: role in global…. This has been corrected.

Line 20: delete comma after Archaea. This has been corrected.

Line 21: be specific which subunit of nitrogenase the *nifH* gene encodes. The sentence has been modified as follow: "Organisms affiliated to UCYN-A, Proteobacteria, and Archaea were recovered in the south-eastern basin and were expressing their *nifH* gene, which encodes dinitrogenase reductase enzyme involved in nitrogen fixation process"

p8783

Line 3: the help of the quantitative…. This has been corrected.

Line 3-4: technique using the *nifH* gene, ….This has been corrected by "with the help of the quantitative polymerase chain reaction (qPCR) of *nifH* copies", as proposed by Referee #1.

Line 9: especially in the phylum Cyanobacteria …. This has been corrected.

Line 10: …which quantifies *nifH* gene copies per millilitre…. This has been corrected.

Lines 14-15: '….to investigate for the hypothetic picoplanktonic UCYN' needs re-phrasing since it is poor English. This sentence has been removed, since **t**he paragraph dedicated to molecular tools used to study the concentration and the species richness of diazotroph has been re-written, following the request of Referee #1.

Line 19-20: …as low concentration targets may be diluted too much during the different extraction steps. The sentence has been modified.

Line 21-24: DNA, a nested approach using the *nifH* gene was introduced….(Line 21 it is unclear what partially unsuccessful means here explain why) The sentence has been modified as follow: "To increase the amount of target within extracted DNA, a nested approach using the *nifH* gene was introduced (Zani et al., 2000). However, several studies still reported the inability to detect low concentrated diazotrophs at the DNA level, thus illustrating the need to develop an adapted PCR protocol for scarce microorganism in this field of research (e.g. Zani et al., 2000; Man-Aharonovich et al., 2007)".

Line 25: Planktonic diazotrophic activity has long been attributed…. This has been modified.

Line 26: …..expensive process via photosynthesis (i.e. delete owning to) This has been modified.

Line 27-28: ….acquire an autonomous carbon ….. This has been corrected.

p8784

Line 1: function has also recently been proven to be missing within the UCYN-A group (Zehr et al., 2008)….. The sentence has been modified.

Line 3: expression patterns… This has been corrected.

Line 6: the entire diazotrophic community…. This has been corrected.

Lines 8-10: delete these lines and replace with: To begin to address this we used here a combination of approaches: i) to assess the distribution….. Modifications have been done.

Line 20: diazotroph distributions and …. This has been changed.

p8786

Line 9: were counterstained with… This has been corrected.

Line 17: delete distribution (so sentence reads '….Nitro821-hybridized cells on filters …). The word "distribution" has been deleted.

Line 18: '….on triplicate counts… This has been corrected.

Line 20: This homogeneity allowed us to …. This has been corrected.

p8787

Line 1: '….emission wavelengths of… This has been corrected.

Line 11: '….using DNA extraction (Mazard et al., 2004).' This has been corrected.

Line 13: '……was discarded using a vacuum….. This has been corrected.

Line 18: '…..following the DNA extraction….. This has been corrected.

Line 21: give a reference for the general 27F/1515R primers. We have removed the nested protocol from the Material and Method section (Cf. General comments) and reference for the general 27F/1518R primers is not needed anymore.

p8788

Line 1: '….in 50 µl volumes. The first PCR used fixed cells on filters as... This has been corrected.

Line 10: '….according to the manufacturer's instructions. This has been corrected.

p8789

Line 5: How is the number of 12 *Crocosphaera* cells per PCR reaction obtained? We have had a sentence to explain this in the Material and Methods, section 2. 3. Flow cytometry: "1 cell ml^{-1} of *C. watsonii* corresponds to 12 cells per PCR reaction, according to UCYN counts realised on 0.2 µm pore-size filter portions from natural samples after TSA-FISH experiments."

Line 9: '…..primarily quantified using the TSA-FISH technique. The nested PCR protocol has been removed, and consequently this sentence also.

Line 17: '……using the TSA-FISH technique (Fig. 3). This has been corrected.

Line 26: '…..concentrated around the dinoflagellate nucleus….. This has been corrected.

p8790

Line 1: delete community. This has been corrected.

Line 4: DCM depth. This has been corrected.

Line 11-12: 16S rDNA clone libraries. This has been corrected.

Lines13-19: Delete the sentences beginning 'One sequence from station A….' to the end of the paragraph since this is non-specific PCR amplification. This paragraph on the plastid sequence recovery has been deleted.

p8791

Line 12: '….plankton net haul data…. This has been corrected.

Line 27: delete semi colon after (Goeble et al., 2008) and replace with a comma. This has been corrected.

p8792

Line 1: 'This latter morphological ….' This has been corrected.

Line 4: 'phylogenetic analysis belonged to group A… This has been corrected.

Line 7 and following lines: DELETE ALL OF SECTION 4.2 since it is incorrect. The discussion on plastids recovery has been removed and replaced by a discussion on the absence of positive 16S rDNA PCR amplification at station B and C, as well as on the identity of small and large Nitro821-targeted cells (cf. General coments).

p8794

Line 23: replace dominating with dominant. This has been done.

Line 24: delete 'belonging to a group of'. This has been done.

Line 24: *Bradyrhizobium.* This has been corrected.

Line 25-26: In addition to *Bradyrhizobium*,….. This has been corrected.

p8795

Line 9: freshwater (one word). This has been corrected.

Line 12: rhizobia. This spelling mistake has been corrected.

Lines 18-19: '…to contribute to two-thirds of…' This has been corrected.

Line 21-22: I think remarkable is a little overstating the finding – interesting would be better. "remarkable" has been changed in "interesting".

Line 23: whose closest relative is the freshwater strain…. These mistakes have been corrected.

Line 28-29: Despite being diazotrophic, phototrophic free-living bacteria….. The sentence has been modified.

p8796

Line 1: (Madigan, 1995; Riemann et al., 2010). We hypothesise though that similar to their closest relatives, the…. The sentence has been modified according to the request of the referee.

Line 3: '….acquire an independent source….' This has been corrected.

Line 6: '….proposes hypotheses for the…' This has been corrected.

Line 11: '….cyanobacterial development temperature,…..' This has been corrected.

Line 15: '….are considered to be at an ecological advantage compared to….' This has been corrected.

Line 18: '….than deeper and nitrate-enriched ones…..' This has been corrected.

Line 22-27: Delete these sentences since this is standard biochemistry. This has been done.

p8797

Line 2: inorganic phosphate, whose concentrations were…. This has been corrected.

Line 5: '…which is considered as the…' The sentence has been corrected.

Line 6: replace 'marine ocean' with 'marine waters'. This has been done.

Line 8: '...been shown to grow <u>in</u> deprived….' This has been corrected.

Line 11: 'Moreover, <u>the</u> phosphate enrichment…. This sentence has been removed because the citation of Ridame et al. is not appropriate anymore due to modification in this paper of the BOUM special issue.

Line 13: The requirements of these different cell types towards phosphate are not reflected …. The sentence has been modified.

Lines 15-17: re-write as follows: '….UCYN-B possess a broad spectrum of genes encoding i) a high affinity transport system to acquire inorganic phosphate and ii) for the scavenging of phosphomonoesters, the…… The sentence has been modified.

Lines 18-20: re-write as follows: 'In addition, we provide in this study (Table 3) an analysis of the UCYN-A genome with respect to P which, despite its reduced size, has a similar P genetic toolbox to that of other picoplanktonic….. The sentence has been modified.

Line 22: '….only marine cyanobacteri<u>um</u> that <u>has</u> the potential….. This has been corrected.

P8798

Lines 1-2: re-write as follows: 'While iron and phosphate seem to control the development of *Trichodesmium* and *Richelia*, temperature has recently been suspected to limit UCYN…. The sentence has been modified.

Line 7: delete 'the one'. This has been done.

Line 9: <u>seem</u> slightly relaxed. This has been corrected.

Line 10-11: We <u>suspect</u> carbon …. This has been corrected.

Line 13: '….making it probably dependent on an unknown….' This has been corrected.

Line 20-24: delete from 'Although it has not….' to the end of the paragraph. These sentences on plastids have been removed.

Line 27: '…show a significant degree… This has been corrected.

Line 29: 'limiting factors' not 'the limiting factors'. This has been corrected.

p8799

Line 3-4: (i) similar to their terrestrial…. This has been corrected.

Line 4: symbioses. This has been corrected.

Line 5-6: (ii) *Bradyrhizobium*, the most widely distributed diazotroph in this…. This has been corrected.

Line 7: delete the phrase 'which by many ways resemble plastids with their genome size and metabolic pathways'. This sentence has been deleted.

Lines 9-13: delete from 'This close relationship' to the end of the paragraph. This has been done.

Line 14: re-write as follows: 'To answer these hypotheses, further work will be ….. These mistakes have been corrected.

Line 16: environments. This has been done.

Lines 16-17: delete the sentence Among UCYN,…. This has been done.

Table 2: Delete all the plastid 16S sequences This has been done.

Fig 2 Legend : ……*C. watsonii*….time on different types of DNA template collected….. These mistakes have been corrected.

Fig. 5 Just show the cyanobacterial sequences and delete the rest of this phylogenetic tree. Plastid sequences have been removed.