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Nutrient control of N_2 fixation in the oligotrophic Mediterranean Sea and the impact of Saharan dust events

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Abstract. A better understanding of the factors controlling N_2 fixation is a pre-requisite for improving our knowledge on the contribution of N_2 fixation process in the nitrogen cycling. Trace-metal clean nutrient/dust addition bioassays (+P, +PFe, +dust) were performed at three stations located in the western, central and eastern Mediterranean Sea, in summer 2008 as part of the BOUM cruise. The main goals were (1) to investigate the nutrient factor(s) limiting N_2 fixation (uptake of $^{15}N_2$) and (2) to evaluate the potential impact of a Saharan dust event on this biological process during the stratification period. Initially, surface waters at the three stations were DIP-depleted (<10 nM) while the DFe concentrations were relatively high (from 1.2 to 2.3 nM) most likely due to atmospheric iron accumulation in the surface mixed layer. At all stations, Saharan dust input relieved the ambient nutrient limitation of the diazotrophic activity as demonstrated by the strong stimulation of N_2 fixation (from 130 % to 430 %). The highest dust stimulation of N_2 fixation was recorded at the station located in the eastern basin. The response of diazotrophic activity to nutrient additions was variable between the sampled stations suggesting a spatial variability of the factor controlling N_2 fixation over the whole basin. At all stations, N_2 fixation was not limited by Fe nor co-limited by P and Fe. At the western station, N_2 fixation was DIP limited while at the eastern one, N_2 fixation was first DIP limited, then was limited by one or several chemical element(s) released by dust. Our results demonstrated that a Saharan dust input was able to relieve these successive on going limitations. Very interestingly, at the station located in the central basin, N_2 fixation was not limited by the availability of P yet it was strongly stimulated by dust addition (x3.1). A chemical element or a combination of several, released by the added dust may have been responsible for the observed stimulations of N_2 fixation. These results indicated that Saharan dust pulses to the surface Mediterranean waters, in addition to P and Fe, could be a source of chemical(s) element(s) that are necessary for metabolic processes and therefore influence rates of N_2 fixation.

1 Introduction

The Mediterranean Sea is one of the most oligotrophic areas of the world's ocean (Krom et al., 2004; Bosc et al., 2004) characterized by a strong gradient of nutrients and primary production decreasing eastward (Béthoux et al., 1998; Moutin and Raimbault, 2002; Lagaria et al., 2011; Pujo-Pay et al., 2011). Based on the short dissolved inorganic

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phosphorus (DIP) turnover times, the Mediterranean Sea can be considered as a Low Phosphate Low Chlorophyll (LPLC) environment (Moutin et al., 2008). Another interesting feature is the unusually high dissolved inorganic N:P ratios of 22–23:1 and 28–30:1 for the western and eastern basins, respectively (Marty et al., 2002; Pujo-Pay et al., 2011) which may result from an intense N_2 fixation (Gruber and Sarmiento, 1997; Ribera d'Alcala et al., 2003). This hypothesis is still under debate as recent direct measurements of N_2 fixation rates showed very low values through the open Mediterranean Sea (Ibello et al., 2010; Bonnet et al., 2011) indicating that N_2 fixation may represent a low source of external nitrogen input on an annual scale (Krom et al., 2010). At the north-western DYFAMED site (DYnamique des Flux Atmosphériques en MEDiterranée, 43°25 N 07°52 E, 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). Apart from the extremely high N_2 fixation rate (129 nmol N L^{-1} d^{-1}) measured in spring in the Eastern basin (Cyprus eddy, Rees et al., 2006), N_2 fixation rates in the Mediterranean Sea are low when compared to other oligotrophic environments (see review in a companion paper, Bonnet et al., 2011).

Most of the Mediterranean studies focusing on diazotrophs reported low abundance of these organisms. While *Trichodesmium* sp. and *Richelia* sp. (symbionts hosted within diatoms) are known to form massive blooms in the Atlantic and the Pacific Oceans (Mague et al., 1974; Venrick, 1974; Capone et al., 1997; Carpenter et al., 1999), only low concentrations of these diazotrophic filamentous cyanobacteria have been mentioned in the Mediterranean Sea (Bar-Zeev et al., 2008; Le Moal and Biegala, 2009; Crombet et al., 2011; Yogev et al., 2011). The presence of smaller unicellular diazotrophs affiliated to Archeae, Proteobacteria and Cyanobacteria was recently reported both in the western and eastern basins (Man-Aharonovich et al., 2007; Le Moal et al., 2011; Yogev et al., 2011). Picoplanktonic ($<3\,\mu m$) 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 high concentrations during one summer at the coastal station (Le Moal and Biegala, 2009). A combination of environmental parameters was suspected to be responsible for such massive development of the UCYN community: the unusually warm sea surface temperature, detectable concentration of DIP and unusually high urban pollution event.

The recognition of the importance of N_2 fixation in the global cycling of carbon and nitrogen has recently encouraged intensive studies concerning the major factors that control N_2 fixation and the growth of diazotrophs, particularly for the filamentous diazotroph *Trichodesmium.* From field studies, it appears that in the world ocean N_2 fixation may be limited by either phosphorus (Sañudo-Wihlemy et al., 2001; Moutin et al., 2005; Sohm et al., 2008), iron (Wu et al., 2000; Kustka et al., 2003) or co-limited by both (Mills et al., 2004). Due to the high iron content of the nitrogenase enzyme complex (Raven, 1988; Kustka et al., 2003), N_2 fixation process can be controlled by iron supply. Diazotroph phosphorus limitation could also occur in oceanic areas strongly impacted by Fe-rich mineral dust input (Wu et al., 2000). Whether Fe or P is the chemical element ultimately limiting oceanic N_2 fixation remains an open question. In the Mediterranean Sea, the factors controlling N_2 fixation are still poorly known. As the Mediterranean Sea has been described as a phosphate-depleted basin, phosphorus can be logically suspected to be the limiting nutrient for diazotrophic activity.

The Mediterranean Sea is strongly impacted by episodic Saharan dust deposition (e.g. Guerzoni et al., 1999; Guieu et al., 2010a). After the seasonal phytoplanktonic bloom in spring, the surface mixed layer (SML) is isolated from deeper waters by a strong stratification, and becomes nutrient-depleted. During the whole stratification period, atmospheric inputs are therefore the main source of allochtonous nutrients to oligotrophic surface waters. By bringing new nutrients such as DIP (e.g. Ridame and Guieu, 2002; Pulido-Villena et al., 2010) and Fe (e.g. Bonnet and Guieu, 2006) to the Mediterranean surface waters, Saharan dust deposition is strongly suspected to play a key role in the control of N_2 fixation.

In order to understand why the N_2 fixation rates are most of the time low in the Mediterranean Sea, it is important to focus on the environmental controlling factors. As the Mediterranean surface waters are DIP-depleted in summer, the BOUM (Biogeochemistry from the Oligotrophic to the Ultra-Oligotrophic Mediterranean, see details in Moutin et al., 2011) cruise provides a good platform to investigate the nutrient factors (including Fe) controlling N_2 fixation. The main objectives of this study were: (1) to determine if the concentrations of P as DIP and Fe are limiting N_2 fixation along a trophic gradient in the Mediterranean Sea, and (2) to evaluate the potential impact of a Saharan dust event on this metabolic activity. A microcosm approach was chosen in this study as nutrient addition experiments have proven to be very useful in identifying potential chemical limitations or co-limitation of biological activity during cruises. A comparison of data collected from both shipboard iron enrichments and in situ enrichments suggested that shipboard experiments provide a robust indication on the limiting chemical factors of the phytoplankton community (Gall et al., 2001).

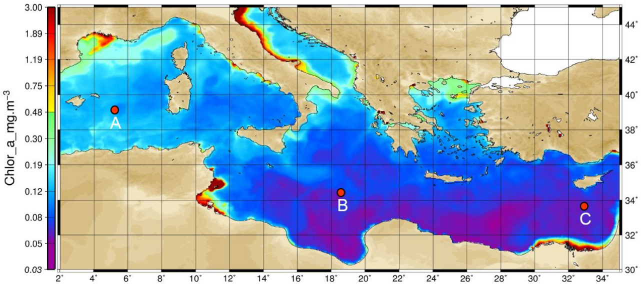

Fig. 1. Locations of the stations A, B, C where microcosm experiments were performed and satellite chlorophyll concentration (MODIS, NASA) during the BOUM cruise.

2 Materials and methods

2.1 Microcosm experiments

Experiments were carried out on board R/V *L'Atalante*, during the European BOUM cruise (Biogeochemistry from the Oligotrophic to the Ultra-Oligotrophic Mediterranean) in the Mediterranean Sea between the 16 June and the 20 July 2008 (http://www.com.univ-mrs.fr/BOUM/) (Moutin et al., 2011). The cruise track is shown in Fig. 1. The trace-metal clean nutrient/dust addition experiments were performed at three 5-days drifting stations (see locations on Fig. 1) representative of LNLC (Low Nutrient Low Chlorophyll) conditions: station A located in the western basin (39°11′ N, 5°35′ E), station B in the Ionian Sea (34°13′ N, 18°45′ E) and station C in the Levantine Sea (33°63 N, 32°65 E). These three stations were located at the center of warm-core anti-cyclonic 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 nutrient/dust additions on N_2 fixation.

Manipulations took place under a laminar flow hood inside a clean container. Materials were first acid-washed (HCl Suprapur). Unfiltered subsurface seawater (8 m depth) was collected within the surface mixed layer with a trace metal clean Teflon pump system. 4.5-liter polycarbonate bottles were filled with seawater and immediately enriched with DIP (dissolved inorganic phosphorus) (+P), DIP and iron (+PFe) and Saharan dust (+dust). Nutrients were added to final concentrations of 30 nM KH_2PO_4 and 2.0 nM $FeCl_3$. 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 Fe^{3+} to form a precipitate. As the ratio of Fe^{3+}/Fe(III)' (Fe(III)': dissolved inorganic iron) is $\sim 10^{-10}$ 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 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). 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 in the PFe treatment 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.

Pre-weighted amounts of the Saharan dust (see "Saharan dust characterization" below) were added to seawater to obtain a final particulate concentration of 1.1 mg L^{-1}. One unamended treatment served as control (C). Each treatment was performed in triplicate. The microcosms were then placed in an on-deck incubator with circulating surface seawater and covered by a screen to simulate the appropriated irradiance (~50 % ambient light level representing the light intensity encountered at 8 m). Over the 48 h-incubation, the bottles were regularly manually stirred. For each station, the incubation started in the morning. Before the nutrient/dust additions, seawater was sampled for initial determination of the N_2 fixation rate, chlorophyll-*a* and concentrations of nutrients including dissolved iron. N_2 fixation was determined in all treatments between 0 and 24 h and between 24 h and 48 h.

2.2 Saharan dust characterization

A fine fraction of a composite Saharan soil (<20 µm) was used for the dust additions. This dust was shown to be representative of the Saharan aerosol carried over the Mediterranean Sea (Guieu et al., 2002; Ridame and Guieu, 2002). The final concentration of dust in the bioassays was 1.1 mg L^{-1}. Assuming an homogeneous dust distribution within the surface layer (Blain et al., 2004), this dust concentration was representative of a realistic dust deposition of 8 g m^2 (Loÿe-Pilot and Martin, 1996; Ternon et al., 2010; Guieu et al., 2010b), vertically diluted within the 8 m deep surface layer. 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 (Guieu et al., 2002; Ridame and Guieu, 2002). It has to be noted that the dust used in our experiment was different from the one used by Ternon et al. (2011).

2.3 Sampling and analysis

N_2 fixation rate: 5 mL of $^{15}N_2$ gas (99 %, EURISOTOP) were added at two selected time points (0 and 24 h) to 4.5 L polycarbonate bottles equipped with septum caps using a gas-tight syringe for $^{15}N_2$ uptake determination between 0–24 h and 24–48 h. $^{15}N_2$ tracer was added to obtain a ∼10 % final enrichment. 24 h after on-deck incubation, 4.5 L were filtered onto pre-combusted 25 mm GF/F filters, and filters were stored at −20 °C. Sample filters were dried at 40 °C before analysis. N concentration in the particulate matter as well as ^{15}N-enrichment were quantified with a mass spectrometer (Delta plus, ThermoFisher Scientific, Bremen, Germany) coupled with a C/N analyzer (Flash EA, ThermoFisher Scientific) via a type III-interface. Standard deviations (SD) were 0.004 µmol L^{-1} for particulate nitrogen and 0.0001 atom % for ^{15}N enrichment. N_2 fixation rates were calculated by isotope mass balanced as described by Montoya et al. (1996). 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).

Chlorophyll-*a*: For initial determination, 2.3 L were filtered on 25 mm GF/F. After extraction in 90 % acetone, fluorescence was measured on a Hitaschi F-4500 spectrofluorometer, following Neveux and Lantoine (1993).

Nutrients: For initial determination, samples for nitrate (NO_3^-), nitrite (NO_2^-) and phosphate (PO_4^{3-}) were immediately analysed on board after collection according to classical methods using the automated colorimetric technique (Wood et al., 1967; Tréguer and Le Corre, 1975), on a segmented flow Bran Luebbe autoanalyser II. The precision of measurements was 20 nM, 5 nM and 5 nM for NO_3^-, NO_2^-, and PO_4^{3-}, respectively. The detection limits, defined as three times the standard deviation of the blank were 20 nM, 10 nM and 10 nM for NO_3^-, NO_2^-, and PO_4^{3-}, respectively.

Total dissolved iron: Initial dissolved iron (DFe) concentration was measured on filtered seawater (<0.2 µm, Sartobran cartridge filters) by flow injection with online preconcentration and chimiluminescence detection (FIA – CL) (see details in Ternon et al., 2011). The detection limit was 12 ± 2 pM and the reagent blank was 35 ± 8 pM.

2.4 Statistical analysis

For N_2 fixation rate, treatment means were compared using a one-way ANOVA and a Fisher LSD means comparison test (α=0.05). For each incubation (0–24 h and 24–48 h), means that were not significantly different ($p > 0.05$) were labeled with the same letter (A, B, C for data at 0–24 h and a, b, c for data at 24–48 h).

Table 1. Initial chemical and biological properties of the surface seawater used for the incubation experiments. [DIP] (Dissolved Inorganic Phosphorus) and [NO_x] (NO_3^-+NO_2^-) were under detection limit at all stations.

	Station A	Station B	Station C
Temperature (°C)	24.46	26.96	25.24
salinity	37.43	38.63	39.60
Chlorophyll-*a* (ng L^{-1})	45.3	22.0	13.0
N_2 fixation (nmol N L^{-1} d^{-1})	0.10	0.08	0.09
DFe (nM)	1.2	2.3	1.3

3 Results

3.1 Initial characteristics of the sampled surface waters

Stations A, B and C located at the core of anti-cyclonic eddies had contrasting chemical and biological characteristics (Table 1). The initial concentration of chlorophyll-*a* decreased substantially along a west to east gradient (from 45.3 ng L^{-1} at station A to 13 ng L^{-1} at station C) (Table 1; Fig. 1). At the three stations, DIP and NO_x (NO_3^- + NO_2^-) concentrations at the sampling depth (8 m depth) were below the detection limit (<10 nM and <20 nM, respectively) as in the entire SML (Pujo-Pay et al., 2011). Dissolved iron concentrations were the highest at station B (2.3 nM) and were in the same order of magnitude at stations A and C (1.2 nM and 1.3 nM, respectively). Initial N_2 fixation rates in surface waters measured in the control treatment (0–24 h) were very similar at the three study sites (Table 1) as demonstrated by the low standard deviation (mean: 0.10 ± 0.02 nmol N $L^{-1}d^{-1}$).

3.2 Response of N_2 fixation to nutrient additions

The biological response of diazotrophic activity after nutrient additions was variable between the studied stations. At stations A (western basin) and C (eastern basin), DIP additions alone or those associated with Fe, stimulated significantly N_2 fixation relative to the control ($p < 0.05$; Fig. 2, Table 2). At station A, N_2 fixation was 3.2-fold and 5-fold higher in the P and PFe treatments respectively, as compared to the unamended control at $t = 48$ h (Fig. 2). At station C, additions of P and PFe resulted in a final N_2 fixation rate 3-fold and 2.5-fold higher, respectively, than that in the control. At both stations, addition of DIP alone or in combination with Fe had a more pronounced effect on N_2 fixation rate at $t = 48$ h (t_{24-48h}) than at $t = 24$ h (t_{0-24h}) (Fig. 2). Statistically, the stimulations of N_2 fixation after P and PFe additions at stations A and C were not significantly different (Table 2). Surprisingly, at station B (central basin), N_2 fixation rate was not stimulated by addition of DIP alone or in combination with Fe (Fig. 2).

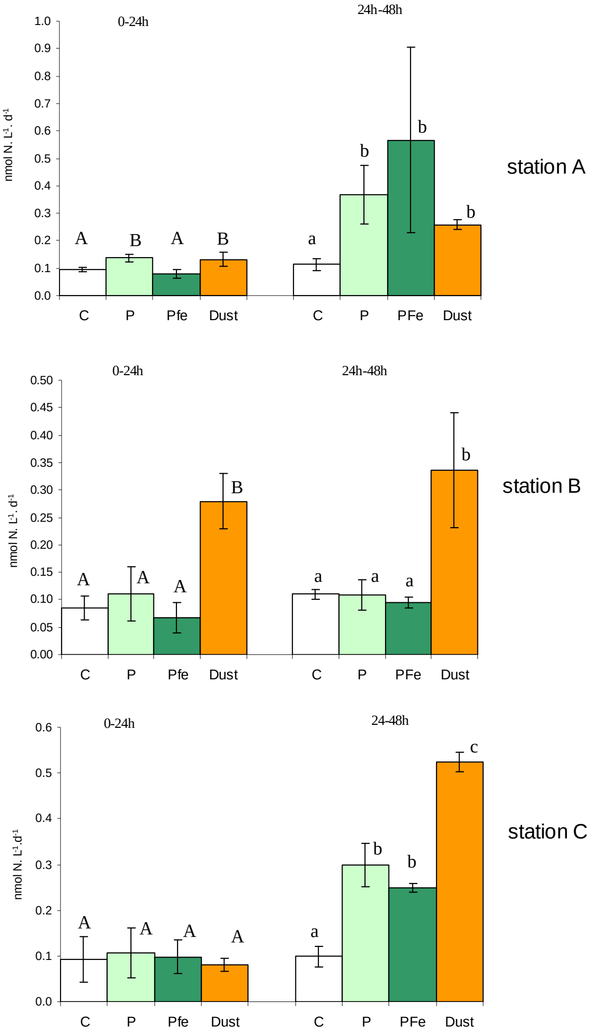

Fig. 2. N_2 fixation rate in control (C), P, PFe and dust treatments during incubation times (0–24 h and 24–48 h) at stations A, B and C. Treatment means were compared using a one-way ANOVA and a Fisher LSD means comparison test (α=0.05). For each incubation (0–24 h and 24–48 h), means that are not significantly different are labeled with the same letter (A, B, C for analysis at 0–24 h and a, b, c for analysis at 24–48 h).

3.3 Response of N_2 fixation to Saharan dust additions

Saharan dust additions stimulated significantly N_2 fixation at all the sampled stations, however, the intensity and the time required for an increase in measured rates varied (Fig. 2; Table 2). The highest stimulation of N_2 fixation was observed at station C (eastern basin) with a 5.3-fold increase relative to the control, at $t = 48$ h ($p < 0.05$). Dust addition led to a 2.3-fold and 3.1-fold increase in N_2 fixation at $t = 48$ h at stations A and B, respectively. This increase in N_2 fixation was not statistically different between stations A and B. The N_2 fixation stimulation after dust addition was higher at 48 h than at 24 h at stations A and C while it was similar at station B.

Table 2. Results of the statistical comparison (ANOVA, FISHER test) between the different treatments (control, +P, +PFe, +dust) at the end of the experiment for N_2 fixation ($t_{24-48\text{h}}$). NS: the difference between the two treatments is not statistically different; S: the difference between the two treatments is statistically different ($p < 0.05$).

	A	B	C
P vs. control	S	NS	S
PFe vs. control	S	NS	S
dust vs. control	S	S	S
P vs. PFe	NS	NS	NS
P vs. dust	NS	S	S
PFe vs. dust	NS	S	S

4 Discussion

4.1 Environmental conditions versus N_2 fixation

The Mediterranean waters remained strongly stratified at the studied stations representative of summer conditions (Moutin et al., 2011). At the three stations, the DIP and NOx concentrations in the SML were extremely low, typical of LNLC environments (Pujo-Pay et al., 2011). Initial N_2 fixation rates in the surface waters of the sampled stations were homogeneous and low (mean: 0.10 ± 0.02 nmol N L^{-1} d^{-1}). These values were consistent with measurements along the BOUM transect (Ternon et al., 2011; Bonnet et al., 2011) and with previous data over the entire basin (Ibello et al., 2010; Yogev et al., 2011). They were within the range of the lowest rates measured in Atlantic and Pacific Oceans (Raimbault and Garcia, 2008; Needoba et al., 2007; Bonnet et al., 2009). Associated with low N_2 fixation rates, concentrations of UCYN measured during the BOUM cruise, in surface waters at the studied stations were low (Le Moal et al., 2011). At the sampled stations, initial N_2 fixation activity was most likely due to microorganisms in the <10 µm size fraction as filamentous cyanobacteria such as *Trichodesmium* sp. and *Richelia intracellularis* were under detection limit (<0.004 cell ml^{-1}, Le Moal et al., 2011) at all stations. Moreover, Bonnet et al. (2011) reported that during the BOUM transect, 50 % to 100 % of the N_2 fixing activity were performed within the picoplanktonic fraction (<3 µm) in the surface layer. Based on DIP turnover times, Moutin et al. (2005) estimated that a critical concentration of 9 nM of DIP, corresponding to a DIP turnover time of 50 h, was necessary to sustain the growth of *Trichodesmium*. As DIP turnover times measured in the surface waters of stations A, B and C were lower than 10 h (Tanaka et al., 2011; Mauriac et al., 2011), the very low DIP availability could explain the absence of *Trichodesmium* at these stations.

The sea surface temperature at the studied stations ranged from 24.5 °C to 27 °C (Table 1) which is within the temperature range where unicellular diazotrophs were found most abundant (Church et al., 2008; Moisander et al., 2010). Therefore, we hypothesize that temperature was not a limiting factor of the diazotrophic community in summer in the Mediterranean Sea, as also mentioned by Yogev et al. (2011).

4.2 What is controlling Mediterranean N_2 fixation? Phosphorus versus iron

Diazotrophs, by their nature, should not be limited by the availability of combined nitrogen in the environment. Due to the necessity to synthesize the nitrogenase enzyme, N_2 fixation imposes additional iron demands for growth beyond those attributed to photosynthesis and respiration. Less is known about the role of phosphorus in controlling N_2 fixation and diazotrophs growth in the open ocean. Phosphorus is an important nutrient controlling the distribution and the activity of diazotrophs (Moutin et al., 2005; Sohm et al., 2008) but it is not clear if the availability of phosphorus through the high ATP requirement controls directly the N_2 fixation process or/and indirectly through the CO_2 fixation of phototrophic diazotrophs.

Iron: Our results showed that during the summer stratification, Fe did not limit or co-limit with DIP, N_2 fixation at any studied stations. The DFe concentrations at the three stations were relatively high (from 1.2 to 2.3 nM) probably due to atmospheric iron accumulation in the SML (Sarthou and Jeandel, 2001; Bonnet and Guieu, 2006). Our results suggested that a DFe concentration of the order of 1.2 nM and higher did not limit the Mediterranean N_2 fixation. These field observations confirmed those obtained from culture studies (Berman-Frank et al., 2007) in which similar DFe concentrations were not inhibiting the growth and N_2 fixation of unicellular diazotrophic cyanobacteria. Our results confirmed that N_2 fixation should not be limited by iron in oceanic areas strongly impacted by Fe-rich mineral dust deposition as suggested by Wu et al. (2000).

Phosphorus: In spite of DIP concentrations under detection limit ($<$10 nM) in the SML at the three stations, the response of N_2 fixation after DIP additions were variable between stations. The microcosm experiments revealed that phosphorus as DIP was a key controlling factor of N_2 fixation in both western (st. A) and eastern basins (st. C). Surprisingly, in the central Mediterranean Sea (st. B), DIP addition did not stimulate N_2 fixation in spite of undetectable DIP concentration at the sampled depth before addition. N_2 fixation could be limited by another chemical element or co-limited by DIP and a chemical element different from Fe. 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 DIP addition did not led to a significant stimulation of the rates. The different responses of N_2 fixation to DIP additions at the three sampled stations indicated a spatial variability of the factor controlling the N_2 fixing activity over the whole basin. While phosphorus availability seemed to be the proximate nutrient limiting N_2 fixation in the western (st. A) and eastern Mediterranean Sea (st. C), it was not the case in the central one (st. B).

4.3 Impact of Saharan dust input on N_2 fixation

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 Aquaba (Foster et al., 2009). As the Mediterranean Sea is strongly impacted by Saharan dust deposition, we tested the potential impact of such an event on N_2 fixation. Saharan dust additions induced a strong stimulation of N_2fixation at the three sampled stations with different intensities. The highest dust stimulation of N_2 fixation was recorded at station C located in the eastern basin (x5.3).

At the sampled stations A (western basin) and C (eastern basin), Saharan dust additions as well as DIP additions significantly stimulated N_2 fixation ($p < 0.05$). At station A, the stimulation of N_2 fixation was statistically similar in the dust and P treatments ($t_{24\mathrm{h}}$ and $t_{48\mathrm{h}}$) (Table 2) while at station C, Saharan dust led to an increase in N_2 fixation significantly higher than after DIP addition (Table 2): up to 3-fold (+P) versus up to 5.3-fold (+dust) after 48 h.

Our results demonstrate that Saharan dust was a source of bioavailable P and that such a dust input relieves the on-going DIP-limitation of N_2 fixation at western and eastern stations (A and C). Saharan dust is recognized to be a significant source of DIP in oligotrophic environments such as the Mediterranean Sea during the periods of stratification (Ridame and Guieu, 2002). 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). At the western station (st. A), N_2 fixation was DIP limited alone while at the eastern one (st. C), N_2 fixation was first DIP limited then progressed to limitation by one or several chemical element(s) released by dust. In this way, Saharan dust input was able to relieve the successive on-going N_2 fixation limitations.

One of the most conspicuous results of this study was that in the central Mediterranean Sea (station B) where no DIP limitation was evident, only Saharan dust was able to increase N_2 fixation rate (x3.1). At this station, N_2 fixation was either: (1) limited by a chemical element other than DIP; or (2) co-limited by DIP and a chemical element different from Fe. Chemical elements released by the dust (in addition to DIP) such as trace metals may be responsible for the observed stimulation. In addition to be a source of iron to the ocean surface waters (see e.g. Jickells et al., 2005), Saharan dust deposition represents a source of trace elements such as Co, Ni, Mo, Mn, Zn, and Cd, which are essential for phytoplanktonic activity (e.g. Morel and Hudson, 1985; Morel et al., 1991). The concentrations of these essential trace elements in seawater are so low as to limit their availability to phytoplankton (Morel et al., 1991).

Beyond iron, little is known about the role of trace metals in the N_2 fixation process and growth of diazotrophs. The concentration of bioavailable molybdenum (Mo), nickel (Ni), zinc (Zn) and cobalt (Co) could all be potential candidates as limiting factors in N_2 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 N_2 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 (MoO_4^{2-}) by N_2 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). Ni, a component of the hydrogenase enzyme, could be a limiting factor for N_2 fixation (Ho and Hu, 2010). The gene coding for Ni–superoxide dismutase was found in the genomes of most marine cyanobacteria, including unicellular N_2-fixing cyanobacteria *Crocosphaera* (Dupont et al., 2008). Also, the genome of UCYN-A (UCYN from group A) includes a hydrogenase for nickel-iron uptake (Tripp et al., 2010). The use of HCO_3^- requires carbonic anhydrase dehydration to CO_2 for fixation by Rubisco. Since most of the cellular Zn quota is present in carbonic anhydrase, low levels of dissolved Zn in the ocean (Ellwood and Van den Berg, 2000) suggest its potential to limit the growth of autotrophic N_2 fixers, as has been suggested for diatoms by Morel et al. (1994). Co has also a function in carbonic anhydrase and is a core constituent of vitamin B12 (Raux et al., 2000) involved in central metabolic processes (e.g. Banerjee and Ragsdale, 2003). A recent study showed that *Crocosphaera* is a major producer of vitamin B12 as compared to the non N_2-fixing cyanobacteria *Synechococcus* or heterotrophic bacteria suggesting that *Crocosphaera* has high Co requirement (Bonnet et al., 2010).

Saharan dust contains Mo, Ni, Zn and Co (Guieu and Thomas, 1996; Eglington et al., 2002) but to what extent the dust input changes their distribution in the water column is not well known in the Mediterranean Sea. Moreover, the bioavailability of those metals, which is probably not directly related to their total dissolved concentrations in the oceans (Ellwood and van den Berg, 2001; Wang et al., 2009; Dupont et al., 2010) is also poorly documented. While a very limited database does exist for some of these metals in the Mediterranean Sea (Boyle et al., 1985; Tankere et al., 1995; Vega and Van den Berg, 1997; Morley et al., 1997), there is no data on the bioavailable fraction of these metals for the Mediterranean waters. Identifying and quantifying those metals in atmospheric deposition and how they impact the distribution in the water column is essential for understanding the spatial and temporal distribution of diazotrophs and N_2 fixing activity in the Mediterranean Sea. Clearly many open questions do remain about the metal requirements for the different diazotrophs which are highly variable within the diazotrophic community as demonstrated for Fe and Mo in cultured *Crocosphaera watsonii* and *Trichodesmium erythraeum* (Berman-Franck et al., 2001; Tuit et al., 2004; Berman-Franck et al., 2007).

4.4 Who was responsible for the different N_2 fixation responses?

The spatial variability in the responses of N_2 fixation could be explained by a spatial variability of the diazotrophic communities. Species composition of unicellular diazotrophs has been well characterized at stations A, B, and C of the BOUM oceanographic cruise in a companion paper (Le Moal et al., 2011). A new marine group of α-proteobacteria affiliated to *Bradyrhizobium* was discovered in surface waters of the three stations (Le Moal et al., 2011). Closest terrestrial counterparts of these *Bradyrhizobium* are able to fix nitrogen as free living cells (Masson-Boivin et al., 2009). While UCYN-A were detected only at station A (surface waters), γ-proteobacteria and other *Rhizobium* than *Bradyrhizobium* were recovered at stations B and C, respectively (Le Moal et al., 2011). The well-known marine diazotrophic cyanobacteria *Trichodesmium* sp., *Richelia* sp., UCYN-B (UCYN from group B as *Crocosphaera* sp.) and UCYN-C (UCYN from group C as *Cyanothece* sp.) were not detected in surface waters of stations A, B and C (Le Moal et al., 2011). However, their contribution to N_2 fixation rate cannot be excluded as they could be present in surface waters at concentrations under detection limit. *Bradyrhizobium*, UCYN-A, *Rhizobium* and γ-proteobacteria could be the main contributors to the diazotrophic activity in the surface Mediterranean waters.

The factors that constrain the growth and N_2 fixing activity of each diazotrophic group are poorly known as few of them have been cultured. Each of these species has likely its own nutrient/metal requirements and strategies to acquire them, as previously demonstrated for P among diazotrophic cyanobacteria (Dyhrman and Haley, 2006; Dyhrman et al., 2006), suggesting that difference in species composition may induce different global diazotrophic community responses. Other factors may also contribute to the different responses of the diazotrophic activity such as the relative abundance of each species and the physiological status within a given group.

5 Conclusions

The factor limiting the diazotrophic activity during summer was shown to be variable in the Mediterranean Sea: phosphorus as DIP was shown to be the limiting nutrient of N_2 fixation in the western and eastern stations while at the central station, DIP could either be not limiting N_2 fixation or co-limiting, with a chemical element different from Fe. The spatial variability of the diazotrophic communities at the three stations could explain the spatial variability in limitation. At the three stations, Saharan dust alleviated the nutrients stress of diazotrophic activity. 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. One of the most conspicuous results is that a trace element different from Fe and P present in Saharan dust does impact N_2 fixation. 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 Oceans. 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. In addition, in the future, a warming atmosphere and surface waters could potentially increase the stratification of the surface waters in the Mediterranean Sea (Somot et al., 2008) and other oceanic areas such as subtropical gyres (Bopp et al., 2001). In response to this, the biogeochemical impact of the Saharan deposition events on the diazotrophic community could be more pronounced in particular during periods of stratification.

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