Low water column nitrogen fixation in the Mediterranean Sea: basin-wide experimental evidence

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ABSTRACT: The abundance and nitrogen fixation rates of *Trichodesmium* sp. integrated down to the deep chlorophyll maximum as well as nitrogen fixation rates in size-fractionated discrete surface water samples were measured across the Mediterranean Sea. The abundance of *Trichodesmium* sp. was generally low (<50 trichomes m^{-3}), except at one station in the Tyrrhenian Sea where the water temperature was $>20^{\circ}\text{C}$ and *Trichodesmium* sp. was present at a higher density (ca. 700 trichomes m^{-3}). Across the basin, size-fractionated nitrogen fixation rates were low (<250 $\text{nmol N}_2 \text{m}^{-3} \text{h}^{-1}$). The highest rate was measured at a station characterized by relatively high concentrations of total dissolved P and dissolved Fe. High $\delta^{15}\text{N}$ values ($8.04 \pm 2.03\text{‰}$) of the particulate organic nitrogen were in accordance with the observed low water column nitrogen fixation rates across the Mediterranean Sea.

KEY WORDS: Nitrogen fixation · *Trichodesmium* sp. · Mediterranean Sea

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INTRODUCTION

Water exchanges in the semi-enclosed Mediterranean Sea occur mainly through the Strait of Gibraltar. Across the Strait of Gibraltar, water circulation is intriguingly characterized by an unbalanced flow of N-depleted (organic and inorganic nitrogen) inflow of Atlantic surface water compared to the outflow of N-enriched deep Mediterranean waters (Béthoux & Copin-Montégut 1986). The deep Mediterranean waters have an anomalous nitrogen to phosphorus (N:P) ratio increasing from 22 in the western basin to 24 to 29 in the east (Krom et al. 1991, Béthoux et al. 2002). These values are well above the Redfield molar ratio of 16 that is typical for the ocean. These

high N:P ratios indicate that the Mediterranean Sea is enriched with N compared to P or, alternatively, that the system is highly deprived of P, with P-limitation increasing from the west to the east (Moutin & Raimbault 2001). Several sources of external combined N have been identified in the Mediterranean Sea in order to explain these high N:P ratios. These sources include terrestrial discharges (natural and anthropogenic, UNEP/WMO 1997, Béthoux & Copin-Montégut 1986), riverine discharge, atmospheric deposition (Kouvarakis et al. 2001, Sandroni et al. 2007, Markaki et al. 2008), and the biological fixation of atmospheric dinitrogen (Béthoux & Copin-Montégut 1986, Garcia et al. 2006, Sandroni et al. 2007). Terrestrial discharges in the Mediterranean Sea have N:P ratios of <8 . Whereas

the Mediterranean P budget can be balanced by heavy terrestrial loads (Béthoux & Copin-Montégut 1986), the N budget remains unbalanced when only terrestrial nutrient loads are taken into account. Atmospheric deposition of N and to a lesser extent P has recently been recognized as important sources of nutrients in the Mediterranean region (Markaki et al. 2008, Koçak et al. 2010). These atmospheric depositions possess high N:P ratios of up to 22, increasing from the western to the eastern basin (Markaki et al. 2003). Hence, atmospheric deposition represents an important source of nitrogen and provides a link to the high N:P ratio in the system (Markaki et al. 2008, Koçak et al. 2010). Atmospheric deposition of N can account up to 61 % of the total N budget in the Eastern Mediterranean Sea (Krom et al. 2004). Ridame et al. (2003) suggested that the high N:P ratio in the Mediterranean Sea may be the result of a combination of the atmospheric deposition of N and the biological fixation of atmospheric dinitrogen. Based on nitrogen isotope ratio analysis of phytoplankton, suspended particles and nitrate along the Mediterranean basin in different seasons, Pantoja et al. (2002) estimated that biological N_2 fixation may provide up to 20 and 90 % of new nitrogen in the western and eastern basins, respectively. Actual biological N_2 fixation rate measurements have only been published within the last 5 yr (Garcia et al. 2006, Rees et al. 2006, Sandroni et al. 2007, Ibello et al. 2010, Bonnet et al. 2011, Yoyev et al. 2011) with values ranging from very low ($<4 \mu\text{mol N m}^{-2} \text{ d}^{-1}$, Ibello et al. 2010) to extremely high rates measured in a Cyprus eddy in one study ($129 \mu\text{mol N m}^{-2} \text{ d}^{-1}$; Rees et al. 2006). Few of the studies (Ibello et al. 2010, Bonnet et al. 2011) have measured actual biological N_2 fixation rates on a basin-wide scale despite previous estimations of differences in the contribution of biological N_2 fixation to new nitrogen in the western and eastern basins (Pantoja et al. 2002). Therefore more extensive measurements are needed in order to assess the importance of this process in the Mediterranean Sea on a basin-wide scale. In addition, studies on the occurrence and abundance of the globally important diazotrophic cyanobacterium *Trichodesmium* has not been done on a basin-wide scale in the Mediterranean Sea except for some rare reports of its presence (Margalef 1969, Le Moal & Biogala 2009) and absence (Man-Aharonovich et al. 2007). Moreover, a limited number of studies dealt with the effect of physico-chemical factors on N_2 fixation in the Mediterranean Sea. Temperature (Langlois et al. 2005), combined N (Fu & Bell 2006), P (Sañudo-Wilhemly et al. 2001), trace metals such as Fe, Mo, V (Marino & Howarth 1990, Berman-Frank et al. 2001, Tovar-Sánchez & Sañudo-Wilhelmy 2011) and atmospheric dust as a source of Fe have all been attributed as factors that control N_2 fixation in the sea.

Here, we have measured N_2 fixation and the occurrence and abundance of the N_2 -fixing cyanobacterium *Trichodesmium* in the Mediterranean Sea and assessed their spatial variability. We have correlated these data with physico-chemical factors (temperature, inorganic nutrients and trace metal [Fe, V, Mo] availability). These investigations were carried out during 2 research cruises that took place during spring-summer of 2006 and 2007 (THRESHOLDS I and THRESHOLDS II, respectively) covering transects from the western to the eastern parts of the Mediterranean Sea.

MATERIALS AND METHODS

Study area and sampling. The study was carried out on board of the oceanographic vessel BIO García del Cid during 2 cruises to the Mediterranean Sea: (1) Cruise THRESHOLDS I, from June 5 to June 30, 2006, (35.6 to 41.9°N , 3.6 to 30.1°E ; Fig. 1), and (2) Cruise THRESHOLDS II, from May 4 to June 6, 2007 (31.5 to 39.35°N , 3.5 to 29.5°E ; Fig. 1). The THRESHOLDS (thresholds of environmental sustainability) cruises were originally planned to evaluate the critical values of pressures (e.g. contaminants, inorganic, and organic nutrient inputs) beyond which an indicator (e.g. biodiversity, water, and sediment quality) shifts to a different state. Each of the cruises consisted of 2 legs, starting from Spanish waters (off Barcelona) to the Black Sea, and back. For the purpose of this paper, data were collected from both legs of THRESHOLDS I and from one leg (east to west transect) of THRESHOLDS II.

At each station during THRESHOLDS I and II, temperature, salinity, and fluorescence were recorded down to 200 m depth using a SeaBird 911 Plus conductivity-temperature-depth (CTD) system, mounted on a General Oceanics rosette sampler, equipped with 12 l Niskin bottles. Chlorophyll *a* (chl *a*) concentrations were determined fluorometrically (Parsons et al. 1984) using a Turner Design fluorometer after pigment extraction overnight with 90 % acetone. Samples for the determination of the dissolved inorganic phosphate (DIP; PO_4^{3-}), total dissolved phosphorus (TP), and the nitrate plus nitrite ($\text{NO}_3^- + \text{NO}_2^-$) concentrations were kept frozen until analyzed in a Bran + Luebbe AA3 autoanalyzer following standard spectrophotometric methods (Hansen & Koroleff 1999). Ammonium was measured according to Kérouel & Aminot (1997).

Trace metal sampling and analysis. Surface seawater (1 m depth) samples were collected from a Zodiac deployed 2 km away from the research vessel in order to avoid contamination of the samples. Seawater was pumped through acid-cleaned Teflon tubing coupled to a C-flex tubing (for the Cole-Parmer

peristaltic pump head), filtered through an acid-cleaned polypropylene cartridge filter (0.22 μm ; MSI, Calyx®), and collected in a 0.5 l low-density polyethylene plastic bottle. Samples were acidified on board to pH < 2 with Ultrapure-grade HCl (Merck) in a class-100 HEPA laminar flow hood, and stored for at least one month before extraction. Metals (Fe, Mo, and V) were pre-concentrated by the APDC/DDDC organic extraction method of Bruland et al. (1979), and analyzed by ICP-AES (Perkin Elmer Optima 5300 DV). The accuracy of the pre-concentration method and analysis was established using Seawater Reference Material for Trace Elements (NASS5, NRC-CNRC) with recoveries for samples from THRESHOLDS I and II of Fe: 94.9 and 95.5%; Mo: 97.9 and 96.8%; V: 84.8 and 107.8%, respectively. Aerosol samples were collected using a high-volume collector (MCV: CAV-A/HF) with a plastic filter holder into an acid-washed cellulose filter (Whatman 41). A microwave acid digestion procedure (Pekney & Davidson 2005) followed by ICP-AES (Perkin Elmer, Optima 5300 DV) was used to measure total Fe levels in the aerosol filters.

***Trichodesmium* sp. counts, N_2 fixation rates, $\delta^{15}\text{N}$ and particulate organic nitrogen.** *Trichodesmium* abundance was determined during THRESHOLDS I and II. Samples were taken from vertical plankton hauls using a plankton net of 50 μm pore size and casted down to the depth of the deep chlorophyll maximum (DCM), which varied from 30 to 130 m (depending on the station). The vertical plankton hauls were done 1 to 3 times and recovered at a speed of 20 m min^{-1} . The sample volumes were pooled (amounting to ~200 ml of concentrated plankton cast samples from each station,

wherein 60 ml was preserved with 40 ml buffered formaldehyde [at 4 % final conc.]). The rest of the fresh unpreserved sample was used for determination of N_2 fixation rates as described below. The fixed samples were stored at room temperature until identification and counting of *Trichodesmium* sp. using the Utermöhl technique (Lund et al. 1958) and the recommendations contained in the UNESCO manual (Hasle 1978). The samples (total fixed volume of 100 ml) were allowed to settle for 24 h, and filaments were counted on the full settling chamber using an inverted microscope. When estimating the abundances of *Trichodesmium* sp., the total volume of water casted, the diameter of the plankton net, the depth of each cast, and the fraction of the pooled concentrated sample counted, were taken into account.

During both cruises, N_2 fixation rates were measured using the acetylene reduction assay (ARA) (Stal 1988, Capone 1993) in surface water samples (5 m depth) collected using 20 l Niskin bottles and in the plankton cast samples. During THRESHOLDS II, the surface water samples were also fractionated through 12 μm polycarbonate isopore filters in order to measure the N_2 fixation in this fraction. Six 2 l samples from surface waters (whole and fractionated to <12 μm) and six 20 ml subsamples of pooled concentrated samples from plankton net casts were filtered (pressure < 100 mm Hg to minimize disruption of cells) through Whatman GF/F filters and assayed for acetylene reduction. The filters were placed in 10 ml crimp top vials, humidified with ~0.5 ml filtered GF/F seawater, and sealed with a rubber stopper and an aluminum cap. After sealing, 2 ml of acetylene were injected using gas-tight Hamilton syringes. The vials

Fig. 1. Location of the stations during THRESHOLDS I carried out during June 2006 and THRESHOLDS II carried out during May 2007, where *Trichodesmium* sp. abundance and N_2 fixation rates were determined

were subsequently incubated (3 in the dark and 3 at ambient light attenuated to the light intensity at 5 m depth by using a neutral density screen) for 3 h (09:00 to 12:00 h) in on-deck aquaria with running circulating seawater to keep ambient temperature. After incubation, the headspace of the incubation vials was sampled using a gas-tight Hamilton syringe and transferred to and stored in evacuated Hungate tubes until analysis on land. The rubber stoppers of the Hungate tubes were further sealed with melted candle wax to minimize gas losses. Ethylene (C_2H_4) and acetylene (C_2H_2) were measured using a gas chromatograph (Agilent Technologies, model HP-5890) equipped with a flame ionization detector (FID), previously calibrated with standard C_2H_4 in helium and C_2H_2 at $4.157 \mu\text{mol } C_2H_4 \text{ l}^{-1}$ and $40.750 \text{ mmol l}^{-1}$ original concentrations (Carburus Metalicos). The column was a Varian wide-bore column (ref. CP7584) packed with CP-PoraPLOT U (27.5 m length, 0.53 mm inside diameter, 0.70 mm outside diameter, 20 μm film thickness). Helium was used as the carrier gas at a flow rate of 30 ml min^{-1} . Hydrogen and air flow rates were set at 60 and 400 ml min^{-1} , respectively. The split flow was used so that the carrier gas flow through the column was at 4 ml min^{-1} at a pressure of 5 psi. Oven, injection and detector temperatures were set at 52, 120, and 170°C , respectively. To determine the detection limit of the method, we considered the analyses of 8 blanks (4 sets done during THRESHOLDS I, and 4 sets done during THRESHOLDS II). The blanks were treated and calculated the same way as the samples. The detection limit was set as $3\times$ the standard deviation of the blanks. The results (production of C_2H_4 from C_2H_2) are considered significant when the production of C_2H_4 was $>3\times$ the standard deviation of the contaminating C_2H_4 in the C_2H_2 . The detection limits for C_2H_4 production for THRESHOLDS I and II cruises were 0.22 and $0.022 \text{ nmol } C_2H_4$, respectively. Taking into account the maximum volume of water filtered in each cruise and incubation times, the detection limits for THRESHOLDS I and II cruises translates to 1.4×10^{-3} and $1.5 \times 10^{-4} \text{ nmol } N_2 \text{ m}^{-3} \text{ h}^{-1}$, respectively.

$\delta^{15}\text{N}$ and total particulate organic nitrogen (PON) were analyzed in THRESHOLDS I from 1.5 to 5 l surface water (5 m depth) filtered on pre-combusted (6 h, 550°C) Whatman GF/F filters. The filters were washed three times with 5 ml Milli-Q in order to remove salts and were subsequently freeze-dried and stored at -20°C until analysis. PON was analyzed using a Carlo Erba Instruments FLASH 1112 elemental analyzer. Nitrogen isotope ratios were determined using a ThermoFinnigan Delta Plus isotope ratio mass spectrometer connected on-line to the Carlo Erba Instruments FLASH 1112 elemental analyzer. Data are expressed in the conventional delta notation:

$$\delta^{15}\text{N}_{\text{sample}} (\text{‰}) = [(R_{\text{sample}} - R_{\text{standard}})/R_{\text{standard}} - 1] \times 1000$$

where R is the ratio of ^{15}N to ^{14}N , and reported relative to air N_2 (standard). Average reproducibility based on duplicate measurements was $<0.2 \text{ ‰}$.

Data and statistical analyses. The acetylene reduction rates were calculated following the equations in Stal (1988) using acetylene as an internal standard, which circumvents inaccuracies due to gas losses during experimental handling, storage, and transport. Acetylene reduction was converted to N_2 fixation using the conversion factor of acetylene reduced: N_2 reduced of 4:1 (Jensen & Cox 1983). Normal distribution of data was checked with the Shapiro-Wilk test (Shapiro & Wilk 1965), and, if necessary, data were logarithmically transformed prior to analyses. Pearson correlation analyses were used to determine the correlations between N_2 fixation rates, $\delta^{15}\text{N}$ -PON, total PON and *Trichodesmium* sp. abundance and the various physico-chemical factors measured (temperature, aerosol Fe, dissolved Fe, V and Mo, PO_4^{3-} , TP, $\text{NO}_3^- + \text{NO}_2^-$). The statistical analyses were performed using the SPSS program.

RESULTS

Fig. 2 shows the distribution of sea surface temperature (SST, $^\circ\text{C}$) and salinity (psu) during THRESHOLDS I (June 2006) and THRESHOLDS II (May 2007). During THRESHOLDS I, SST were generally higher (18.5 to 24.8°C , Fig. 2A) than THRESHOLDS II (17.6 to 21.8°C , Fig. 2C). Salinity ranges were generally similar in both cruises (37.1 to 39.3 psu) except for lower salinity values in the Marmara Sea and adjacent inland Black Sea during THRESHOLDS I (Fig. 2B,D). During THRESHOLDS II, the eastern basin of the Mediterranean Sea revealed lower SST and salinity than the western part, while during THRESHOLDS I, SST and salinity did not differ much between the eastern and western basin (Fig. 2). During THRESHOLDS I, the concentrations of $\text{NO}_3^- + \text{NO}_2^-$ in the upper 200 m varied from below detection to $1.01 \mu\text{mol N l}^{-1}$ at 5 m, and increasing with depth to $8.34 \mu\text{mol N l}^{-1}$ at 200 m. During THRESHOLDS II, the concentrations of $\text{NO}_3^- + \text{NO}_2^-$ varied between 0.09 and $0.42 \mu\text{mol N l}^{-1}$ at 5 m and generally increased with depth (data not shown). Higher values of $\text{NO}_3^- + \text{NO}_2^-$ were found during THRESHOLDS I in the eastern stations, and during THRESHOLDS II in the western stations. During THRESHOLDS I, PO_4^{3-} concentrations ranged below detection to $26.37 \mu\text{mol P l}^{-1}$ (in the eastern part of the Mediterranean Sea) without a clear variation with depth. TP varied from 0.05 to $0.50 \mu\text{mol P l}^{-1}$, and increased with depth to $5.35 \mu\text{mol P l}^{-1}$ at 200 m (data not shown). During THRESHOLDS II, PO_4^{3-} concentrations also varied from below the detection limit to $0.13 \mu\text{mol}$

Fig. 2. Surface distributions of (A,C) temperature and (B,D) salinity along the west-east transects in the Mediterranean Sea during (A,B) THRESHOLDS I and (C,D) THRESHOLDS II

$P\ l^{-1}$, whereas TP varied from 0.15 to 0.53 $\mu\text{mol P l}^{-1}$ at 5 m without a clear variation with depth. During THRESHOLDS II, NH_4^+ concentrations at 5 m varied from 0.02 to 0.25 $\mu\text{mol N l}^{-1}$ without a clear variation with depth (data not shown). Chl *a* profiles during the THRESHOLDS cruises can be found in Regaudie-de-Gioux et al. (2009). During THRESHOLDS I, chl *a* concentration varied between 0.1 mg chl *a* m^{-3} at 5 m to 7.5 mg chl *a* m^{-3} in the DCM. Lower chlorophyll concentrations were

Fig. 3. Surface distributions of (A) iron (Fe) analyzed in aerosol samples (ng m^{-3}), (B) dissolved Fe (nM), (C) dissolved molybdenum (Mo) (nM), and (D) dissolved vanadium (V) (nM) along the west-east transects in the Mediterranean Sea during THRESHOLDS I

found during THRESHOLDS II, which varied from 0.02 mg chl *a* m^{-3} at 5 m to 1.8 mg chl *a* m^{-3} in the DCM.

Fe in the aerosols sampled during THRESHOLDS I ranged from 43 to 2607 ng m^{-3} air filtered. The highest amount was found at Stn 29 in the western basin (Figs. 1 & 3A). During THRESHOLDS I, dissolved Fe concentration at 5 m ranged from 1.7 to 8.2 nM, without a clear trend along the west-east transect (Fig. 3B). During THRESHOLDS II, dissolved Fe concentration at 5 m

Fig. 4. Surface distributions of (A) dissolved iron (Fe) (nM) and (B) dissolved molybdenum (Mo) (nM) along the west-east transects in the Mediterranean Sea during THRESHOLDS II

ranged from 1.87 to 6.82 nM (with only one high value of 16 nM) (Fig. 4A). During THRESHOLDS I, the concentrations of dissolved molybdenum varied from 41 to 147 nM, with the lowest concentrations occurring at the higher latitude stations (Fig. 3C). During THRESHOLDS II, molybdenum concentrations were more or less constant (128.6 ± 3.1 nM; Fig. 4B). Vanadium levels were also quite variable during THRESHOLDS I and ranged from 6.4 to 32 nM, with the lowest values occurring in the higher latitude stations (Fig. 3D).

The abundance of *Trichodesmium* sp. in stations during THRESHOLDS I ranged from 0.5 to 692 trichomes m^{-3} over the depth of the vertical hauls (average from surface down to DCM). The very high value was observed at Stn 32c (close to Stn 32) (Fig. 5C). During THRESHOLDS II, the abundance varied between 4 and 41 trichomes m^{-3} (Fig. 6D). During THRESHOLDS I, the abundance of *Trichodesmium* sp. was significantly correlated with N_2 fixation in the plankton cast samples (Pearson $r = 0.73$, $p < 0.05$, $n = 11$). During THRESHOLDS II, when N_2 fixation was also measured in the $<12 \mu m$ fraction in surface water samples, the rates were similar to those measured in the non-size fractionated whole surface water samples except at one station (Fig. 6A,B). N_2 fixation measured in the light and dark were significantly correlated with each other ($p < 0.05$) in each of the fractions in both cruises. N_2 fixation was highest in whole surface water samples measured in the dark during THRESHOLDS I at Stn 18 (Fig. 5A), and it

Fig. 5. N_2 fixation rates in (A) whole discrete surface samples, and (B) plankton cast samples integrated from surface to DCM, measured in the light and in the dark, and (C) *Trichodesmium* sp. abundance ($>50 \mu m$) during THRESHOLDS I. Error bars in (A) and (B) denote standard deviation of triplicate samples

coincides with relatively high TP and dissolved Fe concentrations (Fig. 7). $\delta^{15}N$ values ranged between +5.3 and +12.8‰, with no clear trends from west-east transect during THRESHOLDS I (Fig. 8A). During THRESHOLDS I, particulate organic nitrogen (PON) ranged from 0.0003 to $1.13 \mu mol N l^{-1}$, with the highest concentrations occurring in the eastern part of the transect (Fig. 8B).

Fig. 6. N_2 fixation rates in (A) whole discrete surface samples, (B) <12 μm discrete surface samples, and (C) plankton cast samples integrated from surface to DCM, measured in the light and in the dark, and (D) *Trichodesmium* sp. abundance (>50 μm) during THRESHOLDS II. Error bars in (A) and (B) denote standard deviation of triplicate samples

Fig. 7. Plot between N_2 fixation rates in whole discrete surface samples measured in the dark during THRESHOLDS I with (A) total dissolved phosphorus (TP) concentrations and in which the average is 27 ± 19 nmol N_2 m^{-3} h^{-1} at TP < 0.45 μM , and (B) with dissolved Fe concentrations and in which the average is 30 ± 20 nmol N_2 m^{-3} h^{-1} at dissolved Fe < 4 nM. Highest values were found at Stn 18 (Sea of Marmara, adjacent to the Black Sea)

PON correlated significantly with N_2 fixation in whole, unfractionated samples measured in the light and dark (Pearson $r = 0.72$ and $r = 0.80$, respectively; $p < 0.05$, $n = 9$).

DISCUSSION

Basin-wide occurrence of the key marine diazotrophic filamentous cyanobacterium *Trichodesmium* has not been reported previously in the Mediterranean Sea. Here we report on the presence of this important N_2 fixing organism throughout the Mediterranean Sea except at >35°N, 20°E (Aegean Sea and Black Sea) where *Trichodesmium* sp. was absent (Figs. 1 & 5C).

Fig. 8. (A) $\delta^{15}\text{N}$ values, and (B) PON concentrations in surface water samples during THRESHOLDS I

When present in the Mediterranean Sea, *Trichodesmium* sp. occurred in much lower abundances (<50 trichomes m^{-3}) than in other regions where basin-scale surveys of *Trichodesmium* sp. have been conducted (Tyrell et al. 2003, Davis & McGillicuddy 2006, Fernández et al. 2010). The question to be answered was whether the occurrence of *Trichodesmium* sp. (which occurred as single trichomes rather than in the typical colony forms of this organism: tufts or puffs) in the Mediterranean Sea is due to drift or to local growth of the organism. It has been suggested that the occurrence of *Trichodesmium* sp. at higher latitudes with water temperatures $<20^\circ\text{C}$ appears to be due to drift and not due to local net growth based on the direct physiological effects of temperature on diazotrophic growth of *Trichodesmium* sp. (Breitbart et al. 2007). During the THRESHOLDS cruises, only at one station in the Tyrrhenian Sea (between Sardinia and Sicily) where temperature was $>20^\circ\text{C}$ (Fig. 2), *Trichodesmium* sp. abundance was high (~ 700 trichomes m^{-3}) (Figs. 1 & 5C). At the other stations, *Trichodesmium* sp. abundance was low (Figs. 5C & 6D), and further investigations are needed to know whether these are drifted populations of *Trichodesmium* sp. or whether they proliferated locally.

Low numbers of *Trichodesmium* sp. in the Mediterranean basin, low rates of N_2 fixation in plankton cast

samples ($>50 \mu\text{m}$) and the data of size-fractionated N_2 fixation ($<12 \mu\text{m}$ size fractions) in discrete surface water samples indicate the presence of other N_2 fixers in the region. These may be unicellular cyanobacteria (UCYN₂-Fix lineage; Le Moal & Biegala 2009) and/or *Richelia intracellularis* (Venrick 1974), a heterocystous cyanobacterium occurring as an endosymbiont in the diatom *Rhizosolenia* whose diameter ranges from 2.5 to 170 μm (Wimpenny 1966). A previous study has indicated a high diversity of diazotrophs, with a dominance of the cyanobacterium *Richelia intracellularis* associated with the diatom *Hemiaulus hauckii* in the Eastern Mediterranean (Levantine, off the Israeli coast) identified through molecular identification and expression of *nifH*, one of the structural genes of nitrogenase (Man-Aharonovich et al. 2007). N_2 -fixing heterotrophs may also be present in the water column because considerable rates of N_2 -fixation have been detected in samples incubated in the dark. Alternatively, the dark N_2 -fixation can be carried out by the phototrophs that can satisfy the energy and reducing equivalents demands by respiratory means, which explains the significant correlation ($p < 0.05$) between N_2 fixation rates measured in the light and dark in each of the fractions in both THRESHOLDS cruises.

Our data on N_2 fixation rates based on the ARA for integrated samples of $>50 \mu\text{m}$ (accounting for *Trichodesmium* sp.) are similar in range compared to the majority of recent published N_2 fixation rates in the Mediterranean Sea for total community integrated samples based on ^{15}N tracer experiments (Ibello et al. 2010, Bonnet et al. 2011, Yoguev et al. 2011; Table 1). This suggests that total community integrated samples during the THRESHOLDS cruises (if taking into account the $<50 \mu\text{m}$ fraction—whole water samples were only measured in discrete surface [5 m] samples thus precluding comparisons with previous published estimates in the Mediterranean) could be higher than previous estimates in the Mediterranean Sea (Ibello et al. 2010, Bonnet et al. 2011, Yoguev et al. 2011; Table 1). This can be due to differences in the techniques used between the THRESHOLDS cruises and other studies (ARA measures gross N_2 fixation [Mulholland 2007] and the stable isotope $^{15}\text{N}_2$ method measures net actual incorporated N_2 into biomass [Montoya et al. 1996]) and/or temporal and spatial variability of the measurements. Also, different diazotroph groups might have been present at different times and they might have different physiologies. Nevertheless, N_2 fixation in whole fractions on discrete surface samples measured using ARA during the THRESHOLDS cruises along the Mediterranean basin were low (from non detectable to $<500 \text{ nmol N m}^{-3} \text{ h}^{-1}$) compared to rates reported in other oligotrophic systems in the Atlantic Ocean. In whole fractions of discrete surface samples,

up to $1000 \text{ nmol N m}^{-3} \text{ h}^{-1}$ or $12 \text{ } \mu\text{mol N m}^{-3} \text{ d}^{-1}$ (assuming 12 daylight h) were reported using the $^{15}\text{N}_2$ method (Fernández et al. 2010).

The low N_2 fixation rates across the Mediterranean basin found in this investigation agreed with the high natural $\delta^{15}\text{N}$ -PON values (mean $\pm$ SD: $8.04 \pm 2.03\text{‰}$) across the Mediterranean basin. The $^{15}\text{N}/^{14}\text{N}$ approach has been used as an indication of N_2 fixation. The utilization of nitrate is selective for the lighter isotope of nitrogen ^{14}N and results in N products (biomass), which are depleted in ^{15}N relative to the source nitrate. In closed systems, the remaining nitrate will progressively be enriched in ^{15}N , consequently producing biomass enriched in ^{15}N , and through mass-balance considerations, the $^{15}\text{N}/^{14}\text{N}$ ratio of biomass approaches that of the original nitrate source (Struck et al. 2001). N_2 fixation is not selective for isotopes, and therefore the $^{15}\text{N}/^{14}\text{N}$ ratio of the biomass produced from fixed dinitrogen differs little from the substrate (N_2) and produces biomass of low $\delta^{15}\text{N}$ (approx. -2 to 0‰) (Struck et al. 2001). The natural $\delta^{15}\text{N}$ -PON values displayed here were $>+5.3\text{‰}$ with no clear trend from the west-east transect across the Mediterranean Sea. These values are higher than the values reported in a previous study (-2.1 to $+4.11\text{‰}$), displaying a decreasing gradient from west to east (Ibello et al. 2010).

Studies aiming at elucidating the effect of physico-chemical factors on N_2 fixation in the Mediterranean Sea are few, and the factors controlling N_2 fixation in this basin are therefore not well understood. We made an attempt to correlate the N_2 fixation data with physico-chemical factors (temperature, inorganic nutrients and trace metal [Fe, V, Mo] availability). None of these factors tested resulted in significant correlations with the measured N_2 fixation rates here. However, sea surface temperature, which was significantly higher during THRESHOLDS I than in THRESHOLDS II ($p < 0.05$, independent t -test of means; Fig. 2), can be an important factor in explaining the significantly higher ($p < 0.05$, independent t -test of means) N_2 fixation rates of whole water surface samples during THRESHOLDS I than in THRESHOLDS II (Table 1). From previous studies in the ocean, phosphorus (Sohm et al. 2008), iron (Kustka et al. 2003) or a combination of both (Mills et al. 2004) are also known to limit N_2 fixation. The Mediterranean Sea has been described as a phosphate-depleted basin (Ribera d'Alcalà et al. 2003) and inorganic phosphate (PO_4^{3-}) concentrations, are often close to the detection limit. Hence, P could be an important limiting factor for Mediterranean biological N_2 fixation activities. Contradictory reports have been published with regard to the role of P limitation in controlling biological N_2 fixation in the Mediterranean. Microcosm experiments with DIP additions revealed that DIP was a key controlling factor in stations in the

eastern and western basins (but not in the central basin) (Ridame et al. 2011). However, in the previous basin-wide experiment in the Mediterranean Sea (Ibello et al. 2010), and in the present study, no co-variation was found between N_2 fixation rates and DIP nor dissolved organic P (DOP), which may be used as an alternative source of phosphorus by N_2 fixers (Rees et al. 2006). Although we did not obtain evidence for a correlation on a basin-wide scale for the role of P in controlling biological N_2 fixation in the Mediterranean Sea, we did observe high N_2 fixation rates coinciding with high TP in one station (Stn 18 in the Sea of Marmara, adjacent to the Black Sea; Fig. 7A). This station showed a 1 order of magnitude higher rate of N_2 fixation than the other stations which was more uniform ($27 \pm 19 \text{ nmol N}_2 \text{ m}^{-3} \text{ h}^{-1}$) at a TP of $< 0.45 \text{ } \mu\text{M}$ (Fig. 7A). PON was also highest at Stn 18 (Fig. 8B). Clearly more studies are needed to address the role of P in controlling biological N_2 fixation in the Mediterranean.

The role of Fe, the other possible controlling factor of N_2 fixation, has been subject of investigations in the Mediterranean Sea (Ridame et al. 2011). The range of the extensive basin-wide surface concentrations of dissolved Fe (1.7 to 8.2 nM and 1.87 to 6.82 nM [with one high value of 16.7 nM] during THRESHOLDS I and II, respectively) is similar to what has been reported in previous studies in the Mediterranean Sea (e.g. 5.8 to 11.8 nM in Saager et al. 1993, 0.13 to 4.8 nM in Sarthou & Jeandel 2001). The wide range of dissolved Fe concentration found in the present study suggests wide spatial variability of its distribution in the Mediterranean Sea. Previous studies have shown the impact of Saharan dust deposition on dissolved Fe in the Mediterranean Sea (Guerzoni et al. 1999, Guieu et al. 2002), and other studies have dealt with temporal variability of dust Fe dissolution in seawater in the Mediterranean Sea (Wagener et al. 2008). The source of the dissolved Fe and its spatial variability found during THRESHOLDS remains to be elucidated since no correlation was found between the Fe aerosol deposition and dissolved Fe ($p > 0.05$). Microcosm experiments conducted in the Mediterranean Sea showed no limitation by Fe (Ridame et al. 2011). However, we found that the highest N_2 fixation rates that we observed in Stn 18 (Fig. 7B) coincided with one of the highest dissolved Fe concentrations measured in this basin-wide survey ($\sim 5 \text{ nM}$; Fig. 3B). Nevertheless, it is clear that the role of Fe in controlling N_2 fixation activities in the Mediterranean remains to be elucidated.

Aside from Fe, there are other bioactive trace metals that may play an important role in regulating N_2 fixation, such as molybdenum (Mo) (Howarth & Cole 1985, Marino & Howarth 1990), and vanadium (V) both of which have not been investigated into much depth (Tovar-Sánchez & Sañudo-Wilhelmy 2011). Here, we

Table 1. Actual rate measurements of nitrogenase activity of phytoplankton reported in the Mediterranean Sea. DCM: deep chlorophyll maximum; PAR: photosynthetically active radiation

Location	Sample	Incubation conditions	N ₂ fixation rate in original units reported	N ₂ fixation rate (nmol N m ⁻³ d ⁻¹)	Method	Source
35.6–41.9° N, 3.6–30.1° E Mediterranean east-west transect, THRESHOLDS I cruise, 10 stations (spring 2006)	Whole surface (5 m) > 50 µm integrated cast (pooled sample, surface to DCM, 30–130 m)	Light 3 h Dark 3 h Light 3 h Dark 3 h	9–51 nmol N ₂ m ⁻³ h ⁻¹ 7–236 nmol N ₂ m ⁻³ h ⁻¹ 0.07–7.05 nmol N ₂ m ⁻³ h ⁻¹ 0.05–4.65 nmol N ₂ m ⁻³ h ⁻¹	216–1224 1.68–169.2	Acetylene reduction assay, calculated using the theoretical C ₂ H ₂ :N ₂ ratio of 4:1. Hourly rates converted to daily rates assuming 12 daylight hours. 17 % of the samples were below the detection limit of the method.	Present study
31.5–39.35° N, 3.5–29.5° E Mediterranean east-west transect, THRESHOLDS II cruise, 8 stations (spring 2007)	Whole surface (5 m) <12 µm surface (5 m) > 50 µm integrated cast (pooled sample, surface to DCM, 50–120 m)	Light 3 h Dark 3 h Light 3 h Dark 3 h Light 3 h Dark 3 h	nd–15 nmol N ₂ m ⁻³ h ⁻¹ nd–26.7 nmol N ₂ m ⁻³ h ⁻¹ nd–20.1 nmol N ₂ m ⁻³ h ⁻¹ nd–92.2 nmol N ₂ m ⁻³ h ⁻¹ nd–0.046 nmol N ₂ m ⁻³ h ⁻¹ nd–0.055 nmol N ₂ m ⁻³ h ⁻¹	nd–360 nd–482.4 nd–1.10	Acetylene reduction assay, calculated using the theoretical C ₂ H ₂ :N ₂ ratio of 4:1. Hourly rates converted to daily rates assuming 12 daylight hours. 46 % of the samples were below the detection limit of the method.	Present study
32.09° N, 34.34° E Offshore Israel station TB200, (monthly 2006–2007)	Whole integrated (down to 150 m), Stratified period (May–December)	24–30 h	0.03–9.7 µmol N m ⁻² d ⁻¹	0.2–64.6	N ₂ fixation rates using ¹⁵ N uptake method (in parallel with primary production, ¹³ C/ ¹⁵ N label technique) according to Mulholland et al. (2006).	Yogev et al. (2011)
32.10° N, 34.14° E Offshore Israel station TB1000, (monthly 2006–2007)	Whole integrated (down to 150 m), Stratified period (May–December)	24–30 h	0.7–4.7 µmol N m ⁻² d ⁻¹	4.6–31.3	Areal rates converted to volumetric rates taking into account the depth the values were integrated (i.e. 150 m). 36 % of the samples were below the detection limit of the method.	
33.14–34.08° N, 25–33.09° E Mediterranean Levantine east-west Israel–Crete transect, SESAME cruise, 5 stations (Sep 2007)	Whole (surface, 5–20 m; above DCM, 40–60 m; DCM, 90–120 m; below DCM, 150–180 m)	24–30 h	0–0.04 nmol N l ⁻¹ d ⁻¹	0–40		
43.12–42.83° N, 5.87–38.8° E Mediterranean east-west transect, BOUM cruise, 17 stations (summer 2008)	Whole integrated (subsurface down to 130 m), Stn A, eastern Mediterranean Whole integrated (subsurface down to 160 m), Stn B, eastern Mediterranean Whole integrated (subsurface down to 145 m), Stn C, western Mediterranean	24 h 24 h 24 h	10.2–14.8 µmol N m ⁻² d ⁻¹ 11.8–18.6 µmol N m ⁻² d ⁻¹ 0–0.4 µmol N m ⁻² d ⁻¹	78–114 74–116 0–2.7	N ₂ fixation rates using ¹⁵ N ₂ tracer method according to Montoya et al. (1996). Areal rates converted to volumetric rates taking into account the depth the values were integrated (i.e. 130–160 m).	Bonnet et al. (2011)

(continued on next page)

Table 1 (continued)

Location	Sample	Incubation conditions	N ₂ fixation rate in original units reported	N ₂ fixation rate (nmol N m ⁻³ d ⁻¹)	Method	Source
Mediterranean east-west transect, TRANSMED cruise, 6 stations (spring 2007)	Whole, integrated in the euphotic layer (down to 1 % surface PAR, 50 m) <10 µm, integrated in the euphotic layer (down to 1 % surface PAR, 50 m)	24 h 24 h	1–4 µmol N m ⁻² d ⁻¹ 0.5–1.7 µmol N m ⁻² d ⁻¹	20–80 10–34	N ₂ fixation rates using ¹⁵ N ₂ tracer method according to Montoya et al. (1996). Areal rates converted to volumetric rates taking into account the depth the values were integrated (i.e. 50 m). 31 % of the samples were below the detection limit of the method.	Ibello et al. (2010)
43.4° N, 7.9° E, central Ligurian Sea DYFAMED station (Monthly, Jan–Oct 2004)	Whole, integrated (down to 60 m)	Light 12 h	22–245 µmol N m ⁻² d ⁻¹	367–4083	N ₂ fixation rates using ¹⁵ N uptake method (in parallel with primary production, ¹³ C/ ¹⁵ N label technique) according to Diaz & Raimbault (2000). Areal rates converted to volumetric rates taking into account the depth the values were integrated (i.e. 60 m)	Garcia et al. (2006), Sandroni et al. (2007)
33.2–33.5° N, 32.1–32.4° E, Cyprus eddy control stations (spring 2002)	Whole, discrete subsurface at 16 m	24 h	129 ± 9 nmol N l ⁻¹ d ⁻¹	129 000 ± 9000	N ₂ fixation rates using ¹⁵ N ₂ tracer method according to Montoya et al. (1996).	Rees et al. (2006)

presented a basin-wide distribution of these micro-nutrients, but they did not show any notable spatial variability across the basin nor a correlation with N₂ fixation rates.

Extensive biological N₂ fixation in the Mediterranean Sea has been inferred from previous studies. Calculations imply a 7 to 41 % contribution of biological N₂ fixation to the Mediterranean Sea nitrogen budget (Béthoux & Copin-Montégut 1986) and isotopic data indicate that biological N₂ fixation contributes 46 to 70 % of the eastern basin new nitrogen (Sachs & Repeta 1999). Considering the recently increasing amount of data of actual rates of biological N₂ fixation (Garcia et al. 2006, Sandroni et al. 2007, Ibello et al. 2010, Bonnet et al. 2011, Yogeve et al. 2011, present study), the next step would be to re-estimate the contribution of biological N₂ fixation to new nitrogen on a basin-wide scale and to re-assess the importance of this process in the Mediterranean Sea. Moreover, not only water column biological N₂ fixation should be considered but also benthic N₂ fixation. Benthic N₂ fixation associated to *Posidonia oceanica* meadows can be an important source of new nitrogen. *P. oceanica* is an endemic angiosperm in the Mediterranean Sea, which occupies 50 000 km² (50 % occupation in the Mediterranean Sea floor) and globally covering 2 % of the sea area (Béthoux & Copin-Montégut 1986). To date, N₂ fixation rates associated to *P. oceanica* meadows in the Mediterranean Sea have not been reported, yet preliminary data reveal high N₂ fixation in the phyllosphere of *P. oceanica* (up to 1824 nmol N m⁻² h⁻¹; Agawin et al. unpubl. data).

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