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Key Points:
- Volumetric N_2 fixation rates in aphotic waters were as high as previous euphotic rates
- N_2 fixation was higher in more ventilated and oxygenated waters containing fresher organic matter
- Deep N_2 fixation contributed largely to depth-integrated rates and poorly to total nitrogen inputs

Supporting Information:
- Table S1

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Basin-wide N_2 fixation in the deep waters of the Mediterranean Sea

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Abstract Recent findings indicate that N_2 fixation is significant in aphotic waters, presumably due to heterotrophic diazotrophs depending on organic matter for their nutrition. However, the relationship between organic matter and heterotrophic N_2 fixation remains unknown. Here we explore N_2 fixation in the deep chlorophyll maximum and underneath deep waters across the whole Mediterranean Sea and relate it to organic matter composition, characterized by optical and molecular methods. Our N_2 fixation rates were in the range of those previously reported for the euphotic zone of the Mediterranean Sea (up to 0.43 nmol N L^{-1} d^{-1}) and were significantly correlated to the presence of relatively labile organic matter with fluorescence and molecular formula properties representative for peptides and unsaturated aliphatics and associated with the presence of more oxygenated ventilated water masses. Finally, and despite that the aphotic N_2 fixation contributes largely to total water column diazotrophic activity (>50%), its contribution to overall nitrogen inputs to the basin is negligible (<0.5%).

1. Introduction

The Mediterranean Sea is characterized by high nitrogen-to-phosphorus (N:P) ratios in deep water masses, which increase from 20 to 22:1 in the western subbasin to 24–28:1 in the eastern subbasin [*Krom and Herut*, 2004; *Pujo-Pay et al*., 2011]. These ratios are well above the canonical Redfield ratio (16:1) and have been claimed to be caused either by external nutrient inputs with high N:P values [*Krom and Herut*, 2004; *Ludwig et al*., 2009; *Markaki et al*., 2010; *Pujo-Pay et al*., 2011; *Rodellas et al*., 2015] or alternatively by dinitrogen (N_2) fixation (the reduction of N_2 to ammonia by prokaryotic microbes named diazotrophs) [*Sachs*, 1999]. While early geochemical balance and isotope fractionation studies suggested a high contribution of N_2 fixation to new production in the Mediterranean Sea [*Bethoux and Copin-Montegut*, 1986; *Sachs*, 1999; *Pantoja et al*., 2002], direct studies that measured biological N_2 fixation rates through $^{15}N_2$ tracer experiments in sunlit waters found that diazotrophic activity in this basin is very low (typically <0.5 nmol N L^{-1} d^{-1}) [*Ibello et al*., 2010; *Bonnet et al*., 2011; *Yogev et al*., 2011; *Rahav et al*., 2013a, 2013b]. These low rates could partly be due to methodological underestimations and to the fact that the majority of studies were performed during the summer, which is the most oligotrophic season (as discussed in *Rahav et al*. [2013a]), but still the contribution of N_2 fixation to new production is thought to be low [*Bonnet et al*., 2011; *Rahav et al*., 2013a].

Diazotrophs include cyanobacteria, other bacteria, and archaea [*Zehr et al*., 2003]. In the sunlit waters of the Mediterranean Sea, unicellular cyanobacteria (UCYN) and filamentous cyanobacteria in symbiosis with diatoms (diatom-diazotroph associations, DDAs) have been reported as the most abundant diazotrophs [*Bar-Zeev et al*., 2008; *Le Moal et al*., 2011]. Recent investigations have, however, shown that heterotrophic non-cyanobacterial diazotrophs, which are thought to outnumber autotrophic cyanobacterial diazotrophs in the world's oceans [*Farnelid et al*., 2011], are also present in the eastern Mediterranean Sea [*Man-Aharonovich et al*., 2007; *Yogev et al*., 2011; *Rahav et al*., 2013c]. Some heterotrophic diazotrophs are not constrained by the availability of light and are therefore able to live below the euphotic layer. *Rahav et al*. [2013c] measured significant N_2 fixation rates in the aphotic waters of the Levantine Basin at depths extending from the base of the euphotic zone down to 720 m (up to 0.38 nmol N L^{-1} d^{-1}) and related this

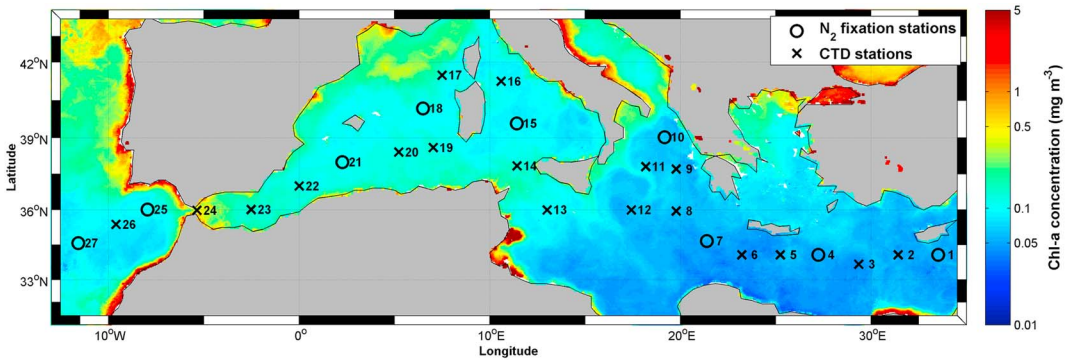

Figure 1. Stations sampled during the HOTMIX cruise superimposed to a satellite image of chlorophyll *a* (Chl *a*) concentrations (mg m^{-3}) obtained from the National Aeronautics and Space Administration (NASA) Goddard Earth Sciences Data and Information Services Center Giovanni (NASA GES DISC) online database for the month of May 2014. Stations sampled for nutrients and DOM are depicted by crosses, while stations where N_2 fixation rates were measured are depicted by open circles.

fixation to sinking organic material such as transparent exopolymeric particles (TEPs) or other aggregates providing carbon resources for heterotrophic diazotrophs. These authors also reported an enhancement of N_2 fixation rates upon the addition of TEP and of organic substrates such as amino acids. Similar bioassays have been conducted in the aphotic waters off coastal Peru [*Bonnet et al*., 2013] and in sunlit waters in coastal Denmark [*Severin et al*., 2015] and suggest that heterotrophic N_2 fixation is regulated by organic matter availability, but the relationship between the diversity and activity of heterotrophic diazotrophs with the quantity and composition of organic matter has not been studied in detail [i.e., *Riemann et al*., 2010]. Moreover, the relative contribution of aphotic N_2 fixation to whole water column diazotrophic activity at the scale of the Mediterranean Sea basin has never been quantified. In the light of the high N:P ratios observed in deep waters of the Mediterranean Sea, we explore here the magnitude of aphotic N_2 fixation rates and its contribution to total nitrogen inputs to the basin. Furthermore, we investigate the relationship between N_2 fixation at the DCM and in deep waters to the quality and quantity of the in situ organic matter pools.

2. Methods

2.1. Sampling Strategy and Hydrographic Data

The HOTMIX cruise took place between 27 April and 29 May 2014 aboard the R/V *Sarmiento de Gamboa* across the Mediterranean Sea (Figure 1). Seawater was collected with a General Oceanics rosette, equipped with 24 L Niskin bottles. Hydrographic data (temperature, conductivity, depth (CTD)) were recorded with a SBE911+ CTD equipped with fluorescence and oxygen sensors.

Seawater samples were collected from four levels: the deep chlorophyll maximum (DCM, 1% photosynthetically active radiation, ranging between 60 and 130 m along the cruise), the Levantine Intermediate Water (LIW, located at 200–300 m in the eastern basin and at 400–500 m in the western basin), the oxygen minimum (located at 900–1000 m), and the Eastern and Western Mediterranean Deep Water (EMDW and WMDW, located at >1500 m). Two more stations were sampled at the exit of the Strait of Gibraltar (25 and 27; Figure 1), where we included sampling of the Mediterranean Water (MW), which is a mixture of LIW and North Atlantic Central Water (NACW).

Oxygen solubility was calculated using the equations outlined in *United Nations Educational, Scientific and Cultural Organization* [1986], and apparent oxygen utilization (AOU, μmol O_2 kg^{-1}) was computed by subtracting dissolved oxygen concentrations from oxygen solubility at saturation with respect to the atmosphere.

2.2. N_2 Fixation Rates

Samples for N_2 fixation measurements were collected at stations 1, 4, 7, 10, 15, 18, 21, 25, and 27 (Figure 1). The samples were taken in duplicate 4.3 L polycarbonate bottles (Nalgene) fitted with teflon-lined septa and darkened with black fabric to reproduce aphotic conditions. To assay N_2 fixation, 5 mL of high-purity $^{15}N_2$ gas

(Cambridge Isotopes, 98.9% atom ^{15}N) were injected to each bottle. The bottles were transferred to a temperature-controlled room set at 13°C. Samples taken from the DCM were incubated in on-deck incubators connected to the underway system seawater and darkened with layers of mesh gauze to simulate in situ light levels. The temperature of the on-deck incubators ranged between ~14 and 19°C along the cruise. Incubations lasted 24 h.

The samples were filtered through precombusted (450°C, 6 h) GF/F filters (Whatman) and stored at −20°C until being analyzed with an Integra2 Analyzer, calibrated every 10 samples using reference material (IAEA-N1). N_2 fixation rates were calculated with the equations of *Montoya et al*. [1996]. At every station and sampling depth an extra 4.3 L bottle was collected and immediately filtered to determine the background atom % ^{15}N in the particulate nitrogen (PN). Considering the PN linearity limit of the mass spectrometer (2.32 μg N), 3 times the standard deviation of our background (time zero atom % ^{15}N) values, our usual filtration volume, and incubation time, our volumetric N_2 fixation rate detection limit was 0.031 nmol N L^{-1} d^{-1}.

Our rates could be somewhat underestimated due to incomplete dissolution of $^{15}N_2$ gas [*Großkopf et al*., 2012]. The alternative dissolved $^{15}N_2$ method is prone to trace metal contaminations [*Klawonn et al*., 2015] and thus was not used here. Moreover, the potential underestimation of the $^{15}N_2$ bubble method is minimized in long (24 h) incubations [*Mulholland et al*., 2012], and the contamination of $^{15}N_2$ gas stocks has been shown to be minimal for the brand used in this study [*Dabundo et al*., 2014].

2.3. Heterotrophic Prokaryotic Abundance and Production

Prokaryotic abundance was determined by flow cytometry using a FACSCalibur instrument (BD Biosciences) [*Gasol and del Giorgio*, 2000]. Prokaryotes were stained with SYBR Green I (Molecular Probes, Invitrogen) and detected in side scatter against green fluorescence plots. Yellow green Polysciences 1 μm beads were used as a reference.

Prokaryotic heterotrophic production was assayed by ^{3}H-Leucine (specific activity = 112 Ci $mmol^{-1}$) assimilation into proteins [*Kirchman et al*., 1985], using the microcentrifugation procedure [*Smith and Azam*, 1992]. Leucine was added at a 20 nM concentration. Four replicates and two trichloroacetic acid fixed controls were done per sample.

2.4. Microbial Electron Transport System Respiratory Activity

Electron transport system (ETS) determinations were performed on 3–8 L samples filtered onto 47 mm GF/F filters as described in *Arístegui and Montero* [1995].

2.5. Inorganic Nutrients

Samples for the analysis of nitrate and phosphate concentrations were collected in 50 mL polyethylene tubes and determined immediately on board using a Skalar autoanalyzer SAN++ following the colorimetric methods of *Grasshoff et al*. [1999].

2.6. Particulate Organic Matter

For the quantification of particulate organic carbon (POC) and nitrogen (PON) concentrations 2–4 L seawater samples were filtered onto precombusted GF/F filters and analyzed on a Perkin Elmer 2400 CHN. TEP concentrations were measured using the Alcian blue method [*Passow and Alldredge*, 1995] on triplicate samples (0.4–2 L) filtered through 0.4 μm polycarbonate filters (Poretics).

2.7. Dissolved Organic Matter

Dissolved organic matter (DOM) samples were collected in acid-clean 5 L polycarbonate carboys and filtered through precombusted GF/F filters under positive pressure with high-purity N_2 in an all-glass filtration system. The filtrates were collected in acid-clean Teflon bottles for solid phase extraction prior to molecular-level analysis. Approximately 10 mL of the filtrate were collected in precombusted glass ampoules for dissolved organic carbon (DOC) analysis with a Shimadzu TOC-V analyzer. The precision of the instrument was tested on a daily basis with DOC reference materials (provided by D.A. Hansell, University of Miami, USA).

The absorption spectra of the colored DOM (CDOM) were determined between 250 and 750 nm at 1 nm intervals in 10 cm path length quartz cuvettes using a Perkin Elmer lambda 850 spectrophotometer. The absorbance was converted to Neperian absorption coefficient (per meter) according to *Green and Blough*

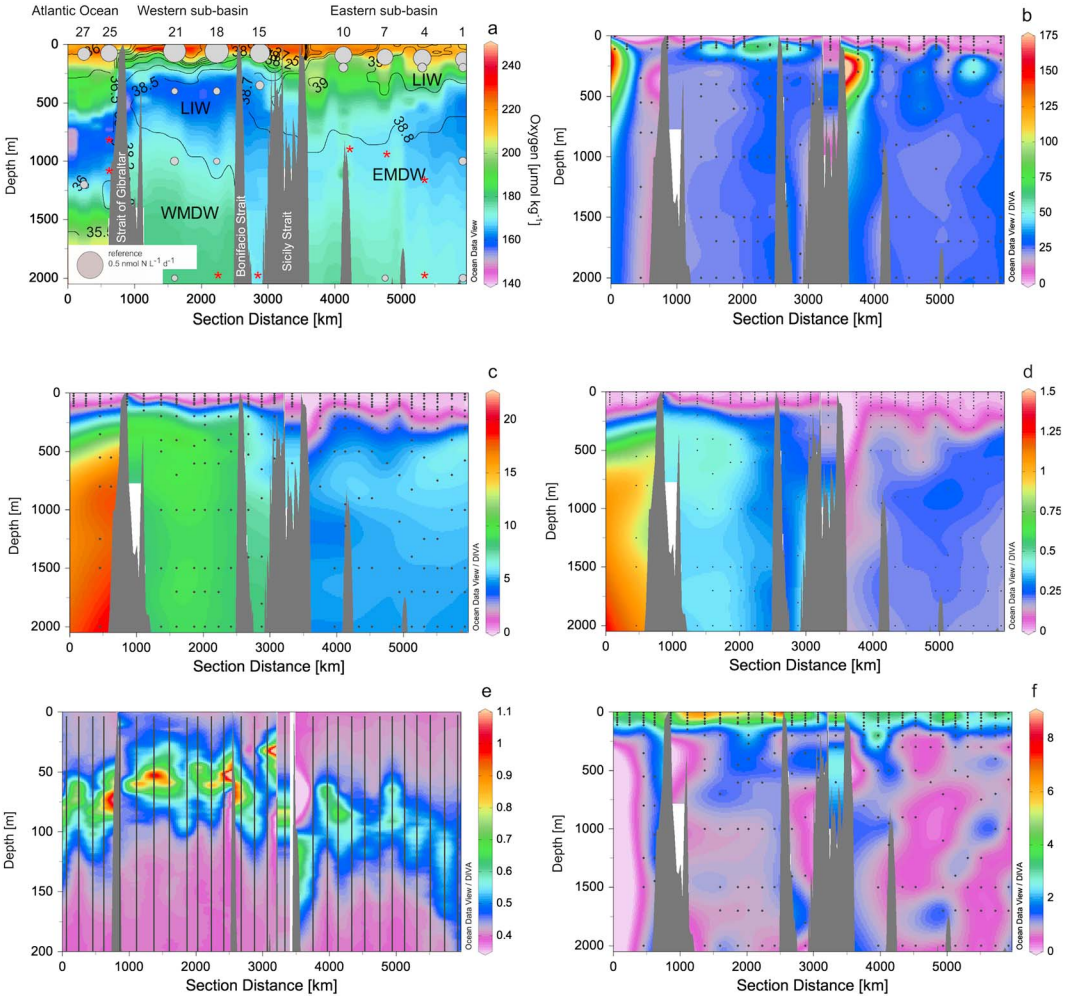

Figure 2. (a) N_2 fixation rates (sized grey circles, a size reference corresponding to a rate of 0.5 nmol N L^{-1} d^{-1} is displayed at the lower left corner), superimposed on dissolved oxygen (µmol kg^{-1}; color scale) and salinity (isolines) data. Red asterisks indicate sites where N_2 fixation activity was below the detection limit (<0.031 nmol N L^{-1} d^{-1}), numbers over the panel indicate the number of the station as depicted in Figure 1, (b) nitrate to phosphate (N:P) ratio, (c) nitrate concentrations (µ*M*), (d) phosphate concentrations (µ*M*), (e) Chl *a* concentrations (mg m^{-3}), and (f) POC concentrations (µ*M*).

[1994]. The carbon-specific absorption coefficient at 254 nm (a_{CDOM}(254)/DOC) was used for Spearman correlations. The fluorescence of CDOM (FDOM) was recorded with a Perkin Elmer LS55 luminescence spectrofluorometer. Excitation and emission slit widths were 10 nm. Single-point measurements were performed at the classical peaks defined by *Coble* [1996] representative of humic- (peak C: 340/440 nm) and protein-like (peak T: 280/350 nm) DOM. Milli-Q water was used as a reference blank, and the analyses were carried out at room temperature in a 1 cm quartz fluorescence cell. Carbon-specific FDOM of peaks C and T were used for Spearman correlations, along with the ratio peak C/peak T.

Samples for ultrahigh-resolution mass spectrometry analyses were extracted (solid phase) with Agilent PPL cartridges as described in *Dittmar et al*. [2008]. After extraction, the cartridges were rinsed with acidified ultrapure water and frozen at −20°C. Subsequently, they were dried by flushing with high-purity N_2 and eluted with 6 mL of methanol. The efficiency of the extraction was 47.3 ± 3.9% on a carbon basis. Methanol extracts were molecularly characterized with a 15 Tesla Fourier transform ion cyclotron resonance mass spectrometer (Solarix FT-ICR-MS, Bruker Daltonik GmbH) using an electrospray ionization source in negative mode (Bruker Apollo II). Molecular formulae were ascribed to the detected masses as outlined in *Seidel et al*. [2014]. The aromaticity and unsaturation degree of each compound were evaluated according to its molecular formula and were presented as the modified aromaticity index (AI-mod) and double bond equivalents (DBE), respectively

Table 1. Depth-Integrated Aphotic N_2 Fixation Rates and Their Contribution to Total Water Column Activity and to Total Nitrogen Inputs to Each Subbasin in the Mediterranean Sea

Subbasin	Aphotic Integrated N_2 Fixation (μmol N m^{-2} d^{-1})[a]	Photic (Surface to 0.1% PAR) Integrated N_2 Fixation (μmol N m^{-2} d^{-1})[b]	Total Water Column Integrated N_2 Fixation (μmol N m^{-2} d^{-1})	Percent Contribution of Aphotic N_2 Fixation to Total Integrated N_2 Fixation	Volume Below the DCM (km^3)	N Inputs From Aphotic N_2 Fixation ($\times 10^9$ mol yr^{-1})[a]	Total N Inputs per Subbasin ($\times 10^9$ mol yr^{-1})[b]	Percent Contribution of Aphotic N_2 Fixation to Total N Inputs
Eastern	17.83–91.06 ($n = 16$)	1.0–4.0 ($n = 18$)[b] 0–0.4 ($n = 81$)[c]	17.83–95.06	96–100	1.98×10^6	0.35–0.70	181[e]/293[f]	0.39[d]/0.24[e]
Western	37.95–68.96 ($n = 15$)	2.5–3.7 ($n = 24$)[b] 10–76 ($n = 72$)[c]	40.45–144.96	48–94	1.31×10^6	0.23–0.38	78–94[e]/126–142[f]	0.40–0.48[d]/0.28–0.31[e]

[a]This study.
[b]*Ibello et al.* [2010].
[c]*Bonnet et al.* [2011].
[d]Considering 6 months of activity per year.
[e]Considering a conservative submarine groundwater discharge (SGD) (only freshwater inputs) [*Rodellas et al.*, 2015].
[f]Considering the median SGD for the whole Mediterranean Sea [*Rodellas et al.*, 2015].

[*Koch and Dittmar*, 2006]. In addition, we ascribed the identified molecular formulae identified to compound groups according to established molar ratios, AI-mod, DBE, and heteroatom contents [*Seidel et al.*, 2014] (see Table S1 in the supporting information). A detailed discussion of molecular DOM quality will be given elsewhere (Martínez-Pérez et al., manuscript in preparation).

3. Results and Discussion

3.1. Does Aphotic N_2 Fixation Contribute Significantly to Total Nitrogen Inputs in the Mediterranean Sea?

Deep N_2 fixation rates during this study ranged from 0.13 to 0.43 nmol N L^{-1} d^{-1} at the DCM and from undetectable (<0.031 nmol N L^{-1} d^{-1}) to 0.07 nmol N L^{-1} d^{-1}, in the mesopelagic and bathypelagic waters below (Figure 2a). The highest rates were observed thus at the DCM, and these were higher in the western as compared to the eastern basin (0.31–0.43 and 0.17–0.24 nmol N L^{-1} d^{-1}, respectively), which is in agreement with previous west-east decreasing gradients observed for euphotic N_2 fixation in the Mediterranean Sea [*Bonnet et al.*, 2011; *Rahav et al.*, 2013b]. Conversely, slightly higher rates were observed at the LIW level in the eastern subbasin (~0.06 nmol N L^{-1} d^{-1}) as compared to the western subbasin (0.03–0.05 nmol N L^{-1} d^{-1}). At the oxygen minimum level (900–1250 m), N_2 fixation was undetectable at stations 4, 7, and 10 (eastern subbasin) and station 15 (western subbasin). At stations where N_2 fixation at the oxygen minimum was detectable, the rates were similar (0.03–0.05 nmol N L^{-1} d^{-1}) to those detected at the level of the MDW at stations 1, 7 (eastern subbasin), and 21 (western subbasin). In the northeast Atlantic N_2 fixation was only detected at the DCM and at the oxygen minimum level at station 27 (Figure 2a). The eastward decrease in N_2 fixation activity coincided with an increase in the N:P ratios of surface waters (Figure 2b), a general decrease in nitrate and phosphate concentrations (Figures 2c and 2d), and the deepening of the DCM (Figure 2e), with the surface concentrations of POC being higher in the western basin.

Our aphotic N_2 fixation rates are in the same range as those reported previously from the surface Mediterranean Sea [*Ibello et al.*, 2010; *Bonnet et al.*, 2011; *Yogev et al.*, 2011; *Rahav et al.*, 2013b], and they are also comparable with previous results from the mesopelagic to bathypelagic waters of the Southern California Bight (0.07 nmol N L^{-1} d^{-1}) [*Hamersley et al.*, 2011], the Levantine Basin and the Gulf of Aqaba (0.01–0.38 nmol N L^{-1} d^{-1}) [*Rahav et al.*, 2013c], and the eastern tropical South Pacific (<1 nmol N L^{-1} d^{-1}) [*Bonnet et al.*, 2013]. This fact highlights the relevance of aphotic N_2 fixation, which has been largely disregarded in past studies. The resulting contribution of aphotic N_2 fixation to total water column activity (using previously published data as a reference) is 48–94% and 96–100% in the western and eastern subbasins, respectively (Table 1). This high contribution of the aphotic layer to total water column N_2 fixation is comparable to the values reported

from the South Pacific (80–90%) [*Bonnet et al*., 2013; *Benavides et al*., 2015] and the eastern Mediterranean Sea (up to 75%) [*Rahav et al*., 2013c] and could potentially alleviate nitrogen limitation of aphotic bacterial communities [*Rahav et al*., 2013c]. It is currently not known whether aphotic N_2 fixation occurs homogenously throughout the water column or if it takes place in hot spots of microbial activity such as particles or ecotones [*Riemann et al*., 2010], which gives whole water column integrated rates some uncertainty. Our sampling strategy does not allow us to solve this question.

Earlier geochemical-oriented studies suggested that N_2 fixation provided a significant input of nitrogen to the Mediterranean Sea, accounting for the high N:P ratios observed in deep waters [*Sachs*, 1999; *Pantoja et al*., 2002]. Considering the depth-integrated N_2 fixation rates measured in our study (17.83–95.06 and 40.45–144.96 μmol N m^{-2} d^{-1} for the eastern and western subbasins, respectively; Table 1), a volume of 1.39×10^6 and 2.15×10^6 km^3 for the western and eastern subbasins, respectively [*Manca et al*., 2004], and an average DCM depth of 100 m [*Macías et al*., 2014], we estimate yearly total nitrogen inputs associated with aphotic N_2 fixation of 0.35-0.70 and 0.23–0.38 $\times 10^9$ mol N yr^{-1} for the eastern and western subbasins, respectively (considering a conservative diazotrophic activity during 6 months/yr; Table 1). Taking into account all other inputs of nitrogen to the Mediterranean Sea (atmospheric, riverine, and submarine groundwater discharge (SGW)), aphotic N_2 fixation would contribute for $<$0.5% of total nitrogen inputs in the basin (Table 1). The atmospheric, riverine, and SGW sources of nitrogen feature high N:P ratios (65–102, 36–47, and 80–430, respectively) [*Huertas et al*., 2012; *Rodellas et al*., 2015], well above those of the deep Mediterranean Sea waters (28.84 and 25.73 for the east and west subbasins, respectively—as calculated using nutrient data from our cruise). Hence, these high N:P ratios are the main factors responsible for the recurrently measured phosphorus limitation [*Krom and Herut*, 2004], and thus, we infer that the contribution of aphotic N_2 fixation to the high N:P ratios of the deep waters of the Mediterranean Sea is negligible.

3.2. Nature of Aphotic N_2 Fixation in the Mediterranean Sea

Previous molecular analyses identified UCYN and DDAs as recurrent diazotrophs in the sunlit waters of the Mediterranean Sea [*Bar-Zeev et al*., 2008; *Le Moal et al*., 2011]. At the global scale non-cyanobacterial *nifH* genes outnumber cyanobacterial ones [*Farnelid et al*., 2011], and recent studies suggest that non-cyanobacterial diazotrophs may also dominate N_2 fixation activity at the surface of the Mediterranean Sea [*Man-Aharonovich et al*., 2007; *Yogev et al*., 2011; *Rahav et al*., 2013a, 2015]. Non-cyanobacterial diazotrophs inhabiting aphotic waters are presumably heterotrophic and rely on organic matter for their nutrition [*Riemann et al*., 2010]. Absolute organic matter inputs to the aphotic zone accounting for deep prokaryotic activity include the transport of DOM from the surface to deep waters via water mass sinking processes, the settling of organic particles, the release of DOM by migrating zooplankton, and the in situ production of DOM by chemolithoautotrophic bacteria [*Arístegui et al*., 2009]. In the Mediterranean Sea DOM inputs to aphotic waters are quantitatively important given the high frequency and various sites of deep water formation [*Santinelli et al*., 2010]. Together with the high temperatures observed in aphotic waters ($>$12.5°C), these peculiar conditions are thought to promote prokaryotic activity at depth [*Santinelli et al*., 2010; *Boutrif et al*., 2011; *Luna et al*., 2012].

Detailed descriptions of the variability of nutrients, DOM, prokaryotic heterotrophic production, and diversity parameters during this cruise can be found elsewhere (Martínez-Pérez et al.; Gasol et al., manuscript in preparation). Because the DCM level is not strictly aphotic, we considered the separate and combined contributions of only aphotic and aphotic and DCM N_2 fixation rates for correlations with dissolved inorganic nutrients, bacterial abundance and activity, and DOM data (Table 2). Despite the lower number of variables that were significantly correlated with N_2 fixation once the DCM was removed from the correlations (Table 2), the recurrent observations of heterotrophic diazotrophs in the DCM waters of the Mediterranean Sea [*Man-Aharonovich et al*., 2007; *Yogev et al*., 2011] suggest that DOM exerts a nonnegligible control of diazotrophic activity in this level. In our study N_2 fixation rates showed a significant negative correlation with N:P ratios, which agrees with the low N:P requirements of diazotrophic activity [*Capone and Knapp*, 2007]. Significant positive correlations were observed with prokaryotic abundance, prokaryotic heterotrophic production, and ETS, suggesting a relationship between aphotic N_2 fixation and bacterial activity, as has been previously observed before in the Levantine Basin [*Rahav et al*., 2013c]. When examining the relationship between N_2 fixation and DOM compositional parameters (FDOM, CDOM, and FT-ICR-MS), we observed

Table 2. Bonferroni-Corrected Significant Spearman Correlations Between N_2 Fixation Rates, Organic Matter, and Bacterial Activity Variables

Variable	Correlation Coefficient	Significance (Two Tailed)
N:P	−0.924	<0.0005
POC	0.730	0.001
PON	0.785	<0.0005
TEP	0.781	<0.0005
Prokaryotic abundance[a]	0.845	<0.0005
Prokaryotic heterotrophic production	0.850	<0.0005
ETS	0.648	0.004
AOU[a]	−0.829	<0.0005
DOC[a]	0.810	<0.0005
Peak C/DOC[a]	−0.635	0.005
Peak T/DOC	0.627	0.002
Peak C/Peak T[a]	−0.833	<0.0005
*a*CDOM(254)/DOC	0.655	0.003
Double bond equivalent (DBE[b])	−0.697	0.001
% peptides[ca]	0.525	0.025
% highly unsaturated compounds[ca]	−0.519	0.027
% unsaturated aliphatics[ca]	0.664	0.003
% oxygen-rich unsaturated aliphatics[ca]	0.558	0.016

[a]Correlations that remain significant after removing DCM N_2 fixation rates from the analysis.
[b]*Koch and Dittmar* [2006].
[c]Refers to DOM molecular formula information, categorization according to *Seidel et al*. [2014].

significant negative correlations with DBE as a measure for the unsaturation of the DOM, some optical indicators of the refractory nature of CDOM such as the C-specific humic-like substances (peak C/DOC) and the ratio of fluorescence of refractory humic-like to fresh protein-like substances (peak C/peak T), the percentage of highly unsaturated compounds, and AOU (Table 2). This suggests that N_2 fixation rates were higher in oxygenated waters containing presumably fresher organic materials, which is also supported by the positive correlations found with TEP, the carbon-specific fluorescence of protein-like substances (calculated as the ratio of peak T/DOC), the carbon-specific absorption coefficient at 254 nm of *Weishaar et al*. [2003] that indicated the presence of carbon double bonds in DOM, and the percentage of peptides and unsaturated aliphatics (as determined by FT-ICR-MS; Martínez-Pérez et al., manuscript in preparation) (Table 2).

The relationship of heterotrophic N_2 fixation with the quantity and/or quality of organic matter has been seldomly studied. *Rahav et al*. [2013c] found a positive correlation between N_2 fixation and TEP in the mesopelagic zone of the Levantine Basin. These authors suggested that TEP are utilized as organic carbon rich and low oxygen microniches for N_2 fixation in the water column. Other studies have also observed an enhancement of aphotic N_2 fixation rates upon the addition of amino acids or carbohydrates [*Bonnet et al*., 2013; *Rahav et al*., 2013c; *Loescher et al*., 2014], suggesting that aphotic diazotrophic activity depends on labile organic matter inputs to deep water layers. Most studies on the phylogenetic *nifH* gene composition of aphotic waters have reported a high diversity (usually encompassing all four *nifH* clusters) [*Hewson et al*., 2007; *Hamersley et al*., 2011; *Bonnet et al*., 2013; *Rahav et al*., 2013c; *Benavides et al*., 2015]. Although we did not characterize the diazotrophic community in this study, we assume that a diverse diazotroph community can also be expected too in the aphotic waters across the Mediterranean Basin, probably dominated by heterotrophic bacteria as reported in the sole study that has explored aphotic *nifH* diversity in Mediterranean Sea waters [*Rahav et al*., 2013c]. Although *nifH* sequences from aphotic diazotrophs are becoming increasingly available, the metabolic properties of these phylotypes are largely unknown [*Riemann et al*., 2010]. On the one hand, non-cyanobacterial diazotrophs may present a versatile metabolism, upregulated/downregulated according to changes in the composition and/or lability of DOM. Alternatively, their abundance and activity may fluctuate according to DOM dynamics. A pioneering genomics study targeting diazotrophic Alphaproteobacteria and Gammaproteobacteria isolated from coastal Denmark waters showed that these organisms devoted an important part of their genome to aromatic hydrocarbon metabolism, although diverse systems related to carbohydrate and fatty acid metabolism were also found [*Bentzon-Tilia et al*., 2015], suggesting a flexible metabolism. Conversely, *Severin et al*. [2015] found that the taxonomic composition of non-cyanobacterial

diazotrophs in laboratory cultures changed dramatically upon exposure to a labile organic carbon mix and low oxygen conditions (10–16% saturation), indicative of a more specialized metabolism.

In the Mediterranean Sea, the high renewal rate of deep waters and their higher temperature as compared to other oceanic basins likely selects for diazotrophs different to those in low oxygen pelagic zones such as the oxycline of the Baltic Sea or the oxygen minimum zone off Peru [*Dekaezemacker et al*., 2013; *Farnelid et al*., 2013]. Modeling studies have found that the loss of fixed nitrogen via anammox and denitrification in oxygen minimum zones creates a deficit of nitrogen related to phosphorus which is replenished by N_2 fixation inputs [*Deutsch et al*., 2007], which is supported by the significant N_2 fixation rates reported from these areas [*Fernández et al*., 2011; *Dekaezemacker et al*., 2013; *Loescher et al*., 2014]. Because oxygen irreversibly destroys the nitrogenase enzyme system, autotrophic cyanobacterial diazotrophs use temporal and/or spatial segregation strategies to avoid oxygen deactivation [*Berman-Frank et al*., 2003; *Zehr*, 2011], while heterotrophic diazotrophs are thought to either thrive in oxygen minimum zones or inhabit other oxygen-poor habitats or microhabitats such as suspended particles [*Paerl and Prufert*, 1987; *Riemann et al*., 2010]. For example, aphotic N_2 fixation activity has been previously related to moderate or low dissolved oxygen levels [*Hamersley et al*., 2011; *Bonnet et al*., 2013; *Loescher et al*., 2014]. Nevertheless, significant N_2 fixation rates have also been reported from fully oxygenated mesopelagic waters ($>200\ \mu mol\ L^{-1}$) [*Rahav et al*., 2013c]. In this study we found significant negative correlations between AOU and N_2 fixation rates (Table 2), and N_2 fixation rates were undetectable in most oxygen minimum levels sampled along the transect (Figure 2). This suggests that aphotic diazotrophs in the Mediterranean Sea prefer newer more oxygenated waters presumably containing less refractory organic matter, which is characteristic of newly formed deep water masses [*Santinelli et al*., 2006], as discussed above. Alternatively, and given the positive correlations observed between N_2 fixation and POC and PON concentrations (Table 2), we can also infer that aphotic diazotrophs use particles as oxygen-poor microzones in these oxygenated waters. However, trap-measured organic particle fluxes in the Mediterranean Sea are low given the ultraoligotrophic character of its overlying sunlit waters [*Meador et al*., 2010], potentially rendering few microniches for aphotic diazotrophs relying on particles to avoid nitrogenase deactivation by oxygen.

4. Conclusions

Aphotic N_2 fixation represented >50% of total water column integrated diazotrophic activity across the Mediterranean Sea basin. While deep diazotrophic activity may contribute importantly to the fixed nitrogen stock in mesopelagic to bathypelagic remote open ocean areas where terrigenous and atmospheric inputs are minimal, its contribution to the semienclosed basin of the Mediterranean Sea, which presents a high drainage to surface area ratio, is negligible. We thus infer that the high N:P ratios recurrently observed in deep waters of the Mediterranean Sea are not greatly impacted by diazotrophic activity. Nevertheless, the N_2 fixation rates measured in the aphotic zone during this study are in the same range as those reported before for the euphotic zone in the Mediterranean Sea and aphotic N_2 fixation rates reported from the South and the North Pacific Oceans. N_2 fixation rates were higher in oxygenated waters with lower AOU and correlated positively to indicators of the labile nature of the available organic matter, suggesting that diazotrophs take advantage of the presence of the fresher organic material present in these newer, presumably more recently subducted water masses. Although evidence on active N_2 fixation in aphotic depths is accumulating, we currently ignore the metabolic requirements of these diazotrophs. Future investigations coupling genomic and proteomic studies combined with organic matter chemical analyses will further our understanding of the requirements and functioning of deep how deep diazotrophic communities.

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