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Springtime contribution of dinitrogen fixation to primary production across the Mediterranean Sea

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Abstract

Dinitrogen (N_2) fixation rates were measured during early spring across the different provinces of Mediterranean Sea surface waters. N_2 fixation rates, measured using $^{15}N_2$ enriched seawater, were lowest in the eastern basin and increased westward with a maximum at the Strait of Gibraltar (0.10 to $2.35 \text{ nmol NL}^{-1} \text{ d}^{-1}$, respectively). These rates were 3–7 fold higher than N_2 fixation rates measured previously in the Mediterranean Sea during summertime. Moreover, comparisons between N_2 fixation rates measured during dark versus natural light incubations (48 h) show higher rates during dark incubations at the eastern Mediterranean stations but lower rates at the western stations. This suggests that heterotrophic diazotrophy has a significant role in the Eastern Mediterranean while autotrophic diazotrophy has a more dominant role in the Western basin.

1 Introduction

The Mediterranean Sea (MS) is frequently described as a “blue desert” with low phytoplankton biomass and primary production (Berman et al., 1984; Bosc et al., 2004; Ignatiades et al., 2009; Siokou-Frangou et al., 2010). The low primary production is due to the low concentration and supply of dissolved nutrients in surface waters during most of the year and this is exacerbated during spring through late fall when the water column is thermally stratified. Compounding the problem, there is export of underlying, nutrient-rich intermediate-depth water to the North Atlantic Ocean through the Strait of Gibraltar (Moutin and Raimbault, 2002; Krom et al., 2010).

Dissolved inorganic nitrogen (NO_3^- , NO_2^- , NH_4^+) is considered the proximate limiting nutrient for primary productivity in many oceanic regions (Falkowski, 1998), especially in low nutrient, low chlorophyll (LNLC) environments. While traditionally the MS has been considered phosphorus (P) limited (Krom et al., 1991; Thingstad et al., 1998), more recent publications demonstrate nitrogen (N) limitation or N and P co-limitation

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across the two sub-basins within the MS (Thingstad et al., 2005; Tanaka et al., 2011). Diazotrophs (i.e. N_2 fixers) are likely to have an advantage in N-limited environments because they are able to utilize the abundant dissolved N_2 , unavailable to most organisms, as an N source for growth (Capone and Montoya, 2001; Zehr and Ward, 2002).

5 Prokaryotic dinitrogen (N_2) fixation is now recognized as a globally important pathway of new oceanic N inputs (reviewed in Gruber, 2008) and this N can be subsequently transferred to other planktonic groups (Mulholland et al., 2004; Mulholland and Capone, 2009). However, reported rates of N_2 fixation rates from the MS are limited to a few studies from the last ~ 6 yr and most are restricted to surface waters and
10 the summer season. Reported rates of N_2 fixation during summer from both the eastern and western basins of the MS are generally low, ranging from undetectable to $\sim 0.15 \text{ nmolNL}^{-1} \text{ d}^{-1}$ (Ibello et al., 2010; Ridame et al., 2011; Yogeve et al., 2011; Rahav et al., 2012). However, N_2 fixation rates at a coastal-influenced station in the NW Mediterranean (Dynamique des Flux de matière en Méditerranée – DYFAMED) are
15 higher ranging from 2–17 $\text{nmolNL}^{-1} \text{ d}^{-1}$ (Garcia et al., 2006).

Diazotrophs contributing to N_2 fixation in the MS have been partially characterized (Man-Aharonovich et al., 2007; Bar Zeev et al., 2008; Le Moal and Biegala, 2009; Le Moal et al., 2011; Yogeve et al., 2011). In the MS organisms expressing *nifH*, as the gene mediating N_2 fixation, include unicellular cyanobacteria, diatom-diazotroph
20 assemblages, proteobacteria, methanogenic archaea, anaerobic bacteria, and purple sulfur bacteria. (Man-Aharonovich et al., 2007; Yogeve et al., 2011). The filamentous cyanobacterium *Trichodesmium* has been sporadically observed in extremely low abundances (Yogeve et al., 2011) and one bloom event of this genus was recorded from the Aegean Sea (Spatharis et al., 2012).

25 The contribution of N_2 fixation to primary productivity in the MS appears to vary between the Eastern and Western basins. In the western basin, N_2 fixation was shown to contribute up to 35% of new primary production during the stratified period (Bonnet et al., 2011), while in the Levantine basin and the Eastern Mediterranean Sea (EMS), N_2 fixation contributed only ~ 0.5 –2% of the new production (Yogeve et al.,

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2011). However, all of these estimates are based on measurements made during the stratified period in summer and so seasonal variability has not been assessed (Bonnet et al., 2011; Yogeve et al., 2011).

5 Here we present N_2 fixation and carbon uptake rate measurements from surface waters collected from a transect across the Mediterranean Sea during spring (before summer stratification). We calculate the contribution of diazotrophy to primary production during spring and compare these with similar measurements made from the stratified summer period to provide a more comprehensive seasonal assessment of N_2 fixation in the Mediterranean Sea. Additionally, we assessed the relative contribution of
10 heterotrophic versus autotrophic diazotrophy during springtime across the MS.

2 Material and methods

2.1 Sampling locations

This research was carried out aboard the R/V *Meteor* (cruise M84/3) during 24 days in April 2011. Eight stations were sampled along an east to west transect across
15 the Mediterranean Sea, each representing a different water mass with associated mesoscale characteristics. Stations included: the NW Levantine basin (St. 290), the anti-cyclonic Shikmona eddy (St. 294), the Ionian Sea (St. 304), the Adriatic Sea (St. 312), the Tyrrhenian Sea (St. 316), the Alboran Sea (St. 333), Strait of Gibraltar (St. 338), and the Gulf of Cadiz (St. 339) (Fig. 1 and Table 1).

2.2 Experimental design

20 Subsurface seawater (6–8 m depth) was collected and placed in triplicate 4.6-liter polycarbonate Nalgene bottles. $\text{NaH}^{13}\text{CO}_3$ (Sigma) was added to obtain an enrichment of approximately 10% of the ambient dissolved inorganic carbon (Mulholland and Bernhardt, 2005). $^{15}\text{N}_2$ uptake measurements were measured using a newly developed ^{15}N -enriched seawater protocol (Mohr et al., 2010). Enriched seawater was prepared by
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first degassing filtered (0.2 μm) natural seawater collected at the same site and depth using a vacuum (250 mbar) applied to continuously stirred seawater for ~ 1 h. The degassed water was transferred into septum capped Nalgene bottles with no headspace, and 1 mL of $^{15}\text{N}_2$ gas (99%) was injected per 100 mL of seawater. The bottles were

5 shaken vigorously until the bubble disappeared. Aliquots of this $^{15}\text{N}_2$ -sea enriched water were then added to the incubation bottles, with the enriched water constituting 5% of the total sample volume (i.e. 230 mL). Similar enriched seawater additions from the oligotrophic North Pacific Subtropical Gyre (NPSG) resulted in a final $^{15}\text{N}_2$ enrichment of 1.5 atom% (Wilson et al., 2012).

10 After the enriched-seawater was added, the bottles were well shaken, and incubated on-deck at ambient surface seawater temperatures, maintained with running surface water pumped on board. Incubators were covered with either neutral density screening to simulate ambient lighting, or under complete darkness for 48 h incubations. The incubations under ambient irradiance (representative of a full diel cycle) record the activities of both autotrophic and heterotrophic diazotrophs. Whereas, we assume that

15 the 48 h dark incubations reflected the activity of mainly heterotrophic diazotrophs who do not require light energy for dinitrogen fixation. We estimated heterotrophic contribution to N_2 fixation by comparing the dark incubations versus the bottles incubated under ambient diel irradiance.

20 Incubations were terminated by filtering water onto pre-combusted 25 mm GF/F filters (nominal pore size of 0.7 μm). Filters were then dried in an oven at 60 $^\circ\text{C}$ and stored in a dessicator until analysis. In the laboratory, samples for ^{15}N and ^{13}C analyses were pelletized in tin disks and then analyzed on a Europa 20/20 mass spectrometer equipped with an automated nitrogen and carbon analyzer.

25 The percent contribution of N_2 fixation to primary productivity was calculated based on the measured particulate carbon (POC) and nitrogen (PON) in each sample. Our previous experience in the EMS, suggests higher POC : PON ratio than the conventional 106 : 16 Redfield ratio (Yogev et al., 2011).

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2.3 Physical measurements

Measurements of temperature and salinity were taken at each station along the cruise track using an in situ conductivity, temperature and depth (CTD) sensor (Seabird 19 Plus).

5 2.4 Inorganic nutrients

Duplicate water samples were collected in 15-mL acid-washed plastic scintillation vials. Nutrients were determined using a segmented flow Skalar SANplus System Instrument as detailed in Kress and Herut (2001). The precision of the nitrate + nitrite, orthophosphate and silicic acid measurements were 0.02, 0.003 and 0.06 μM , respectively.

10 The limits of detection were 0.075 μM , 0.008 μM and 0.03 μM for nitrate + nitrite, orthophosphate and silicic acid, respectively.

2.5 Chlorophyll *a* extraction

Duplicate seawater samples taken twice a day across the MS ($n = 94$) were filtered onto glass fiber filters. The filters were stored at -20°C in a dark box until analysis

15 within 2–3 days. Samples were extracted in 5 mL 90% acetone overnight, at 4 $^\circ\text{C}$ in dark. Chlorophyll *a* (Chl *a*) concentrations were determined with a Turner Designs (TD-700) fluorometer, using a 436 nm excitation filter and a 680 nm emission filter (Holm-Hansen, 1965). A blank filter was also stored in 90% acetone under the same conditions as the samples.

20 2.6 Picophytoplankton abundance

The abundance of picophytoplankton and nanoeukaryotes was determined by flow cytometry. Taxonomic discrimination was based on the following parameters: cell side-scatter – a proxy of cell volume; forward scatter – a proxy of cell size; and orange and red fluorescence of phycoerythrin and of chlorophyll *a* (585 nm and 630 nm,

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diatom abundances were detected in surface waters of the WMS ($> 100 \text{ cells L}^{-1}$) associated with a small spring bloom (Oviedo et al., personal communication). *Richelia intracellularis*, a symbiotic N_2 fixing cyanobacterium, has been found associated with diatoms in the EMS previously (Bar-Zeev et al., 2008) and may have contributed to N_2 fixation in the WMS.

The highest N_2 fixation rates during this spring transect were observed at the westernmost station in the Strait of Gibraltar (Fig. 4a, Table 2). Moreover, these springtime N_2 fixation rates were 7-fold higher than those measured previously during summer by Ibbello et al. (2010) ($2.35 \text{ nmol NL}^{-1} \text{ d}^{-1}$ versus $0.3 \text{ nmol NL}^{-1} \text{ d}^{-1}$, respectively). These changes suggest seasonality of N_2 fixation and/or the abundance or activity of diazotrophic populations, or seasonal exchange of water and resident planktonic populations between the Eastern Atlantic Ocean and the MS through the Strait of Gibraltar.

During this study N_2 fixation rates were only measured in surface waters (upper 6–8 m) and so depth-integrated N_2 fixation rates could not be calculated. It is therefore conceivable that many autotrophic and heterotrophic diazotrophic groups populating other depths, such as the deep Chl *a* maximum (DCM), were not accounted for in our rate measurements. In addition, seasonal changes in the vertical distribution of diazotrophic microbes were not considered here. For example, a recent study from the eastern basin found no statistical difference in N_2 fixation rates measured in water collected from below the pycnocline at the DCM compared to surface waters during the stratified period, while during the winter mixing period, when the water column was mixed up to 150 m, the N_2 fixation rates were 2–3 fold higher at the DCM than in surface waters (Yogev et al., 2011).

Another methodological contribution to the higher N_2 fixation rates during spring throughout the MS was our use of the newly enriched ($^{15}\text{N}_2$) seawater addition method (Mohr et al., 2010) rather than the gas bubble $^{15}\text{N}_2$ addition method (Montoya et al., 1996). It has been shown that the gas bubble enrichment method may underestimate N_2 fixation rates by a factor of 2 or more in some circumstances (Großkopf et al., 2012; Wilson et al., 2012). Our preliminary comparison of both methods in MS

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waters demonstrated a 2–3 fold increase in rates using the enriched seawater method ($n = 18$). However, in long incubations ($> 24 \text{ h}$), the underestimate of N_2 fixation using the bubble method was reduced because the gas bubble should have equilibrated within the first several hours of the incubation (Mohr et al., 2010; Mulholland et al., 2012).

While it is impossible to convert from one method to another using a constant conversion factor, if we assume a 2-fold underestimate of previously reported summer N_2 fixation rates, we still observe significant seasonal differences in N_2 fixation rates between the early spring and fully stratified summer periods. This suggests that methodological differences alone cannot account for the seasonal changes we observed.

We examined the relative contribution of autotrophic and heterotrophic diazotrophs to the measured N_2 fixation rates using parallel natural light and dark bottle incubations. It has generally been assumed that diazotrophy in surface-waters is dominated by photoautotrophic cyanobacteria that use light energy to satisfy the energetic demands of N_2 fixation and acquire carbon (Karl et al., 2002). Recently, it was discovered that the abundant and widely distributed unicellular group A cyanobacteria are photoheterotrophs (Moisander et al., 2010). Further, many bacterial diazotrophs are present in surface waters (Riemann et al., 2010; Zehr and Kudela, 2011; Mulholland et al., 2012). Our results show that in the eastern basin, the ratio of light : dark bottle N_2 fixation was always < 1 suggesting that heterotrophic diazotrophs may be the dominant N_2 fixers. In the western basin, this ratio was generally > 1 suggesting that autotrophic diazotrophs predominated (Fig. 5). We acknowledge that some phototrophic diazotrophs fix N_2 during the dark, to avoid the inhibitory effects of oxygen, but we assume that our long incubation time in the dark (48 h) would have diminished their impact as they require light energy to fix N_2 .

Phylogenetic characterizations of diazotrophs in surface waters across this Mediterranean transect are currently unavailable. However, a diverse group of auto- and heterotrophic diazotrophs have been reported from the eastern basin with $\sim 40\%$ of the *nifH* transcripts attributed to heterotrophic bacteria (Man-Aharonovich et al., 2007;

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Bar-Zeev et al., 2008; Yogev et al., 2011). In the WMS, unicellular cyanobacteria (including UCYN-A) are present in low abundances year round and short blooms of 2000–5000 cells mL⁻¹ have been reported from a coastal station off Marseille during summer (June and July, Le Moal and Biegala, 2009). Another recent study suggested that cells < 0.7 μm in size, usually ignored during routine sampling, can contribute 50 % of the N₂ fixation (Konno et al., 2010). In this study we used GF/F filters to measure planktonic N₂ fixation (nominal pore size of ~0.7 μm, see methods), as is a common practice. Thus, it is possible we could have missed N₂ fixation by very small bacteria diazotrophs and thereby underestimated total planktonic N₂ fixation.

Based on results from studies conducted during summer in the EMS, N₂ fixation accounted for only 0.7–2 % of primary productivity at stations in the Levantine basin (Yogev et al., 2011; Rahav et al., 2012), but increased to ~6 % in the more productive Rhodes Gyre and Cyprus Eddy (Rahav et al., 2012). Consistent with these results, during a summer transect across the Mediterranean (BOUM campaign), N₂ fixation accounted for 6 to 35 % of new production at stations in the more productive western basin but only 0 to 0.3 % at the more oligotrophic eastern basin (Bonnet et al., 2011). Our springtime results show higher N₂ fixation rates (2–4 fold) at both basins and a similar spatial trend. Higher contribution of N₂ fixation to primary production (4–8 %) was measured in the western basin compared to the eastern basin (~2 %, Fig. 6). These differences between the two basins are probably attributed to changes in N₂-fixing planktonic communities and other environmental aspects. Summertime data from the EMS demonstrated a significant positive correlation between N₂ fixation rates and bacterial production suggesting a higher involvement of heterotrophic diazotrophs in the ultraoligotrophic EMS (Rahav et al., 2012).

5 Conclusions

This study provides the first direct measurements of N₂ fixation rates in surface-waters across the MS during springtime. N₂ fixation rates were measured using the newly

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modified ¹⁵N-uptake method (Mohr et al., 2010) during a spring transect and were 3–7 folds higher than measurements made in surface waters during the stratified summer period. Methodological differences cannot fully account for the higher rates of N₂ fixation observed during this cruise and we suggest that the higher rates are due to seasonal variability in primary productivity and environmental factors. N₂ fixation was higher and contributed more to total primary production in the western basin than in the eastern basin. While our data suggests that N₂ fixation rates across the MS are higher during spring than in the summer stratified period, our measurements were constrained to surface waters and thus we cannot provide depth integrated estimates of N₂ fixation during spring. We suggest that future investigations should include N₂ fixation rate measurements and phylogenetic identity of diazotrophs at both photic and aphotic depths to better constrain the contribution of N₂ fixation to N budgets as well as the total and new production within the Mediterranean Sea.

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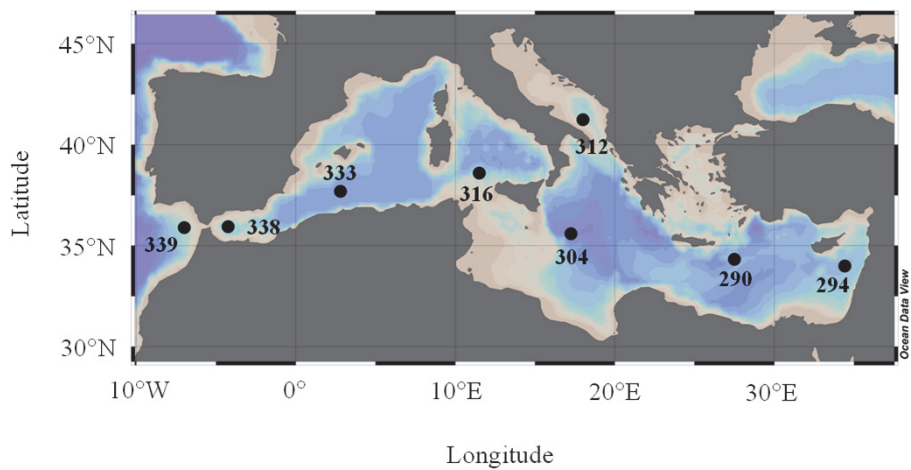


Fig. 1. Map of the sampling locations: NW Levantine Basin (St. 290), anticyclonic Shikmona Eddy (St. 294), Ionian Sea (St. 304), Adriatic Sea (St. 312), Tyrannian Sea (St. 316), Alboran Sea (St. 333), Strait of Gibraltar (St. 338) and Gulf of Cadiz (St. 339).

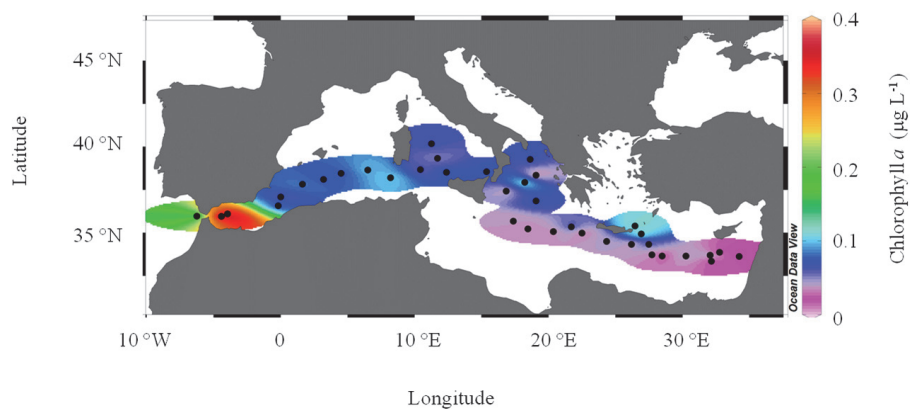


Fig. 2. Spatial distribution of chlorophyll *a* concentrations in surface waters (6 m) along the Meteor M84/3 cruise track during April 2011. $n = 94$.

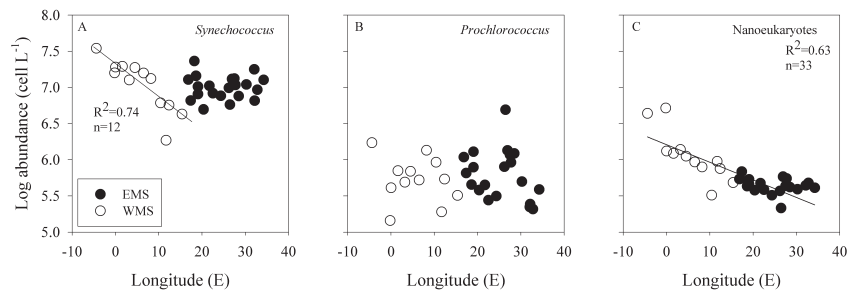


Fig. 3. Picophytoplankton distribution of *Synechococcus* (A), *Prochlorococcus* (B) and nanoeukaryotes (C) in the surface waters (6 m) of the eastern (black circle) and western (white circle) Mediterranean Sea. $n = 21$ and $n = 12$ for the eastern and western basins, respectively.

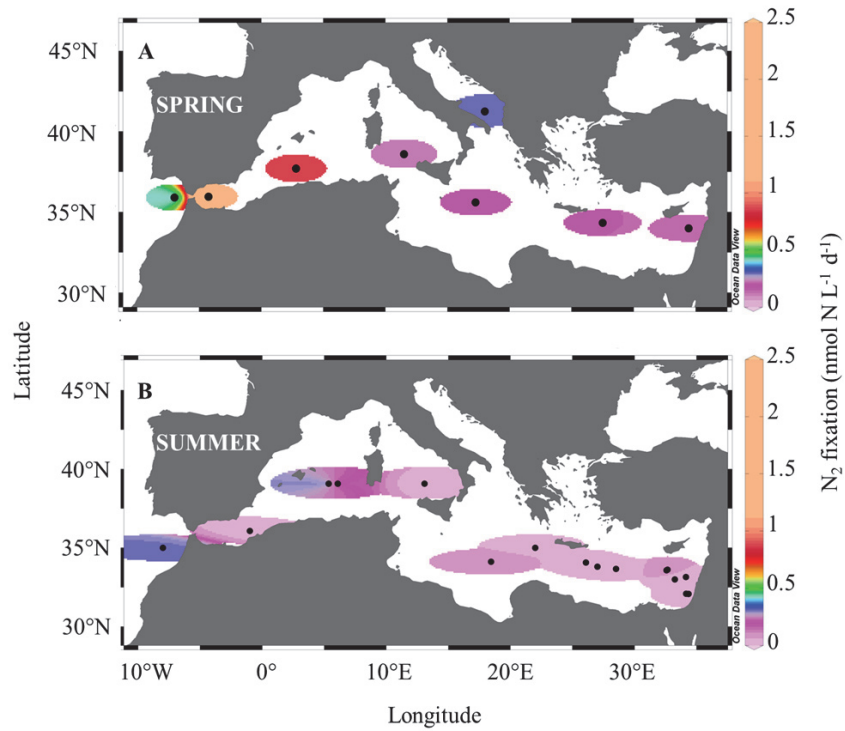


Fig. 4. Seasonal variations of N_2 fixation in the surface waters of the Mediterranean Sea. (A) Springtime rates measured in this study, (B) Summer data compiled from Rahav et al. (2012), Yogeve et al. (2011), Ibello et al. (2010) and Bonnet et al. (2011).

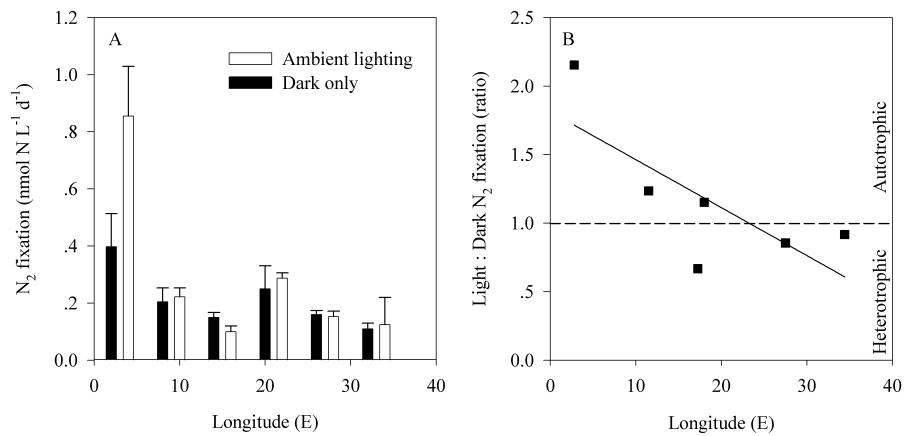


Fig. 5. (A) N_2 fixation rates of surface-waters from stations across the Mediterranean Sea for bottles incubated under ambient lighting (white bars) and in complete darkness (dark bars), and **(B)** the resulting ratio between rates of N_2 fixation from dark incubation and ambient lighting. $n = 3$ for each incubation type at each station.