AUTHOR COMMENTS – REPLY TO REVIEWERS

We thank the reviewers for their time and for the beneficial comments and suggestions. We have carefully gone over all the suggestions and comments and have clarified or changed the text and figures accordingly. We have also added all missing statistics as requested. Below is a point by point answers to the reviewer concerns (**in bold**).

Anonymous Referee #1

This manuscript describes field measurements of nitrogen fixation carried out in the Mediterranean Sea MS) during spring. The presented data is interesting and nicely complements existing data on the MS (mainly from summertime). The work is technically sound and the conclusions are well supported by the presented data. Further, the manuscript is very well written and easy to read (Thank you!).
Thus, I would support publication in Ocean Science after consideration of minor comments/technical corrections as outlined below:

pg 3, l 8: I think there is a "rates" twice in this sentence
Corrected.

pg 3, l 19: I think it would be more appropriate to write, e.g. ". . .as the gene encoding part of the nitrogenase complex. . ." as the gene itself is not mediating N2 fixation.
The text was changed according to the referee's suggestion.

pg 3, l 27 and l 29: up to 35% "to" new primary production and _ 0.5 – 2% "to" the new production rather than "of"
Corrected.

pg 5, l 9: I believe that Wilson et al. (2012) used less volume in their incubations, i.e. they added 50 ml of 15N2-enriched water to a 4.5 L bottle yielding 1.5 atom%. Please check the values.
Currently the only available atom % is from the Wilson et al., (2012) paper and thus we used it. We clarified this point in the methods i.e. *"... resulted in a final*

$^{15}N_2$ enrichment of 1.5 atom% after adding 50 ml of $^{15}N_2$-enriched water to a 4.5 L bottle (Wilson et al., 2012)...".

pg 6, l 10: the detection limit for silicic acid should be at least as high as the precision

Indeed the limit of quantification (0.07 µM) is similar to the precision (0.06 µM). However, the limit of detection may be smaller as reported. We noted in the text that the limits of quantification are similar to the precisions.
Clarification was added at the Methods section: *"note that for silicic acid the limit of quantification is similar to the precisions...".*

pg 7, l 14: "NO2-" and "NO3-" instead of NO2 and NO3.

Corrected.

pg 7, l 18: I think there is a "." missing after Rhodes Gyre and then continue with "Dissolved" Method section:

Corrected.

For completeness, I think you should mention which stations are defined as Eastern Mediterranean Sea (EMS) and which as Western MS (WMS) (also related to Figure 3)

Clarification was added in the Methods section: "*... Seawater samples collected east of the Sicily strait were defined as Eastern Mediterranean (EMS) stations, while west of it as Western Mediterranean (WMS)...*"

pg 8, l 19: "fold" instead of "folds"

Corrected.

pg 8, l 27 and pg 9, l 1: on Figure 1/map the easternmost station is 294; maybe the two stations (290 and 294) got mixed up here?

The station's number is correct and as seen in the map (i.e. figure 1). We simply started the track in the Aegean Sea and went south to the easternmost station off Lebanon. We sampled first the "Rhodes Gyre"-influence station (290) and then went east to the easternmost station (294).

pg 9, l 5-8: I think this should be the other way around (it is correct a bit further down in the manuscript): the eastern basin, values were below 1 (suggesting more heterotrophy) and in the western basin above 1 (suggesting more autotrophy)
Corrected.

pg 9, l 15: “WMS” instead of “WNS”
Corrected

pg 10, l 18: I think what you mean is “heterotrophic diazotrophs” or “heterotrophic bacteria” ; diazotrophic bacteria alone would also include cyanobacteria
Corrected to "heterotrophic bacteria".

pg 11, l 10: To me this seems more like “observations” or “differences” rather than “changes”
Corrected to "differences".

pg 11, l 14: delete “so” or replace by “thus” or “therefore”
Corrected to "therefore".

pg 12, l 17: see above, I think you meant heterotrophic diazotrophs rather than just bacterial diazotrophs
Corrected to "heterotrophic diazotrophs".

Table 2 and Figure 3: chose one of these consistently: nano- or picoeukaryotes
We now use only pico-eukaryotes throughout the text.

Figure 5 B: It might be worth putting some error bars on these ratios.
The figure was modified; error bars and statistics were added.

Anonymous Referee #2

The paper presents new data of N2 fixation rates measured over the Mediterranean Sea in spring with the new 15N2 enriched seawater method. Also, the authors

estimated the contribution of N2 fixation to primary production in both western and eastern basins. Major revisions need to be done to improve the manuscript.
First, statistical tests need to be performed before concluding (data on fig 5 a-b and 6).
Statistical tests were carried out and the figures were modified accordingly (see attached new figures).
Please see specific replies to queries and suggestions below.

A large part of the manuscript is dedicated to the contribution of heterotrophic diazotrophs versus phototrophs to N2 fixation without phylogenetic analyses. The authors attributed dark N2 fixation to heterotrophic diazotrophs. Nevertheless, it has been shown that unicellular diazotrophic cyanobacteria from group B and C performed N2 fix during the dark period. Consequently, N2 fix measured during the dark period does not reflect specifically the N2 fixing activity of heterotrophic diazotrophs. Based on the data presented in this paper, I suggest to remove parts of the manuscript concerning N2 fixation from heterotrophs versus phototrophs.

We are aware that unicellular cyanobacteria (group B and C) perform N_2 fixation during the dark period and have discussed this throughout the manuscript (see as an example page 11 lines 25-33). Yet, we have planned our experimental incubations so as to minimize their impact on total N_2 fixation by performing 48 hours incubations in the dark. The lack of energy (sunlight) for such a long time (i.e. 48 hours) would have diminished their impact as they require light energy to fix N_2. Furthermore, the issue of heterotrophic-dominated diazotrophy (mostly in the eastern basin) is also supported by the low chlorophyll concentrations (Fig.1), low primary productivity (Table 2) and the low contribution of N_2 fixation to the primary productivity (Fig. 5).

Pg3 l9 : six years
Corrected.

Pg3 l12 : please remove Rahav et al 2012 which is in prep
We updated the reference, i.e. Rahav et al. 2013.

Pg3 : please add that some studies reported high rates of N2 fix during summer stratification in MS: 7.5 nmol N L-1 d-1 (Sandroni et al., 2007) and up to 129 nmol N L-1 d-1 Rees et al., 2006

The Rees et al. (2006) measurement was not included as these extremely high rates (higher than almost any other oligotrophic system in the world) were not confirmed in any other study of N_2 fixation in the EMS. These high rates could have been a singular exception yet do not reflect the usually low rates of N_2 fixation in the EMS

The Sandroni et al., paper was added to the DYMAFED rates data.

Pg 3 : line 13 : The DYFAMED site, located in the central zone of the Ligurian Sea, is protected from coastal inputs by the presence of Ligurian Current (eg Marty et al., 2002…)

We have clarified this in a revised sentence *"... N_2 fixation rates at the central zone of the Ligurian Sea station in the NW Mediterranean (DYnamique des Flux de mAtiére en MEDiterranée- DYFAMED) are..."*

Pg3 l16: please add information about the diazo group which dominates the diazo community : bacteria ? unicellular cyano ? DDA ?

The diazotrophic community found in the MS is diverse- as stated in the paragraph, and no one group was found to dominate over the other. Thus we cannot address the referee suggestion. We have added more detail on the players found within the diazotrophic community see P. 3 lines 15-24.

Pg3 l21:precise if Tricho was observed in open or coastal waters of MS

While Trichodesmium has been seen in MS waters as single filaments or small aggregate colonies, there is only one report of a Trichodesmium bloom observed around the Lesvos island in the Aegean Sea close to Turkey (Spatharis et al., 2012). We do not have any other evidences from the literature of *Trichodesmium* sp. Blooms in either coastal or open waters of the MS,

Pg3 l25 : add “new” in the sentence “the contribution of N2 fixation to new primary production”

Corrected.

Pg4 l1:add results from Sandroni et al 2007 about the seasonal variability of the contribution of N2 fix to NP, in northwestern MS (Sandroni et al 2007 table 1).

Data was added.

Pg4 l14 : X to Y April 2011

Corrected.

Pg4 l21: how did you collect the seawater?

Sentence was changed: *"... Subsurface seawater (6-8 m depth) was collected using low pressure pump and placed...".*

When did you collect the sw (morning ?) ?

Seawater was collected upon our arrival to station mostly during the morning time, except for st. 294 where water was collected at ~ 1 pm. However all experiments started at the same time (~7 am).

We added the sentence: *"....* *Incubations began in early morning (~ 7 am) and were..."*

"4.6L PC bottles"…are you sure about the volume (4.3?) ?

Our bottles are 4.6 L.

did you acid washed the material (bottles….) ?

The bottles were acid washed and rinsed with sample water 3 times prior each experiment. Information was adde

Did you add 15N2 and 13C in the same incubated bottles (precise it) ?

We double labeled our bottles with both ^{13}C and $^{15}N_2$. Clarification was added to text: *"... After the enriched-seawater and ^{13}C were added (i.e. double labeling), the bottles were...".*

Did you make replicates of bottles for 15N2 and 13C fix (standard deviation on table 2, n=?)?

We made all experiments in triplicates, as mentioned in the experimental design, hence the standard deviations presented in table 2.

Pg4 l23 : precise the % of 13C in NaH13CO3 and the volume added in bottles

Data was added to text: *"...460 µl of 200 mmol L^{-1} $NaH^{13}CO_3$... ".*

Pg5 l13 : the incubation period for N2 fix is generally 24h. here you chose a very long incubation time of 48h. please justify this choice.

We performed long incubations because of the comparison between the "light" (representative of a full diel cycle) versus "dark" N_2 fixation rates. As 24 hours incubation can also account for diazotrophic autotrophs that fix N_2 in the dark (having gained energy during the light hours), we decided to make long incubations thus fully estimate their overall contribution to the total N_2 fixation (and estimate the relative contribution of heterotrophs...). We assume 48 hours of dark incubations reflected the activity of mainly heterotrophic diazotrophs that do not require light energy for dinitrogen fixation.

Did you make measurement of N2 fix with an incubation of 24h to check if rates were similar (if done, add these data)? If not, discuss about an eventual underestimation of the rates

We conducted 24 hours incubations at all stations, however only for the "light" samples (please see explanation above). We got no significant difference between the rates obtained (R^2= 0.91, n= 24 t-test, P< 0.05). As we compared these "light" rates to the "dark" rates, we only show in the text the 48 hours measurements. We added details of this in the text: *"... We also compared the obtained rates with 24 hours incubations (conducted in parallel) and got no significant statistical difference between the rates (R^2= 0.91 n= 24 p< 0.05, Fig. S1)..."* We also added a figure to the supplementary data which shows the correlation between the 24 hours and 48 hours incubations (i.e. Figure S1).

P5: add the incubation period for 13CO2 fix ?

The ^{13}C and N_2 fixation measurements were carried out in parallel. We have re-emphasized it in the text.

Pg5 l15-19: unicellular diazo cyanobacteria from group B and C perform N2 fix during the dark period. Consequently, N2 fix measured during the dark period does not reflect specifically the N2 fixing activity of heterotrophic diazo.
The comparison between rates obtained under "light" and "dark" periods suggest a complementary evidence for heterotrophic diazotrophy. We are aware that some autotrophs might also fixed N_2 in the dark bottles; however the long incubation period should resulted in dominancy of heterotrophs. We state in the text here and also in the discussion that the rates obtained in the "dark" bottles represents mainly heterotrophic diazotrophy and not all of it. We fill it is a good and appropriate innovating method that might complement our understanding of the role heterotrophic diazotrophs hold in the oceans.

Pg5,l22-24: give some details about reproducibility and precision. Add detection limit for particulate C and N. Precise if you measured the POC or total particulate carbon on the GFF filters
Data was added to the text.

Pg5 l25 : not clear. Do you mean that you used the measured POC/PON ratio to convert N2 fixation to primary production ?
We used the POC: PON ratio obtained from OUR measurements (n=3) and not according to the "conventional" ~6.6:1 Redfield ratio. Our previous work from the EMS showed these ratios are higher (~9:1, Yogev et al. 2011).
We added the averaged POC:PON measured and used here to Table 2.

P6 l6: add some details about the sampling of nutrients: depth? Filtration ? freezing ?
Data was added.

L12: volume of filtration for chla determination ?
Data was added.

Add a § on statistical analyses on data presented Fig 5A-B, Fig. 6
Statistical analyses were added to both figures.

Fig 1 and 2 : could be nice to have only one figure with chla data and localization of sampling stations

We compiled figures 1 and 2 as suggested. All figures numbers were changed accordingly throughout the text.

Table 1 : remove data of nutrients (NO3+NO2) which are below the detection limit

Corrected.

Si(OH)4 :add ’4’

Corrected.

No data for nanoeukaryote ?

The data is presented in Table 2.

Table 1 : add the surface mixed layer depth for every station

Data was added to Table 1.

Table 1 : 620 ngC L-1 is wrong for Proch at station 290; it is 62. Check all the data in table 2 please

Corrected. All data was rechecked.

Fig 3: please add which are the EMS and WMS stations (in particular stx 312 ?)

We have clarified this issue in the first paragraph of the methods section.

Fig 3: no figure for picoeukaryote ?

The term "nano"-eukaryotes was changed to "pico"-eukaryotes throughout the text to avoid confusion.

table 2+§results : the spatial variability of the POC/PON ratio could be interesting. Could you add the data in table 2 for the 8 stations?

The data was added to the table.

Fig 5a : please add the data for stations 338 and 339 (4°45W and 7°W)

Due to time restrains we did not perform "dark" N_2 fixation measurements at stations 338 and 339 and thus cannot present it.

Fig. 5b – Calculate the standard deviation of the ratio and add it on the figure

The standard deviation of the ratio was calculated and added to the figure.

Fig. 5b – add the ratios for stx 338 and 339

The data is unavailable- we did not perform dark incubations in these stations.

Fig. 5b – add the results of statistical test

Results were added.

Fig. 5b – which are the data from station 312 and 304 (same longitude) ?

The figure was changed so now the X axis is the station's numbers and not longitude. Moreover, we color-code the stations so the EMS and WMS stations look different. We hope it is now easier to distinguish between stations and satisfies the referee.

Fig 5B : give R2 and n

The data was added to the figure: R^2= 0.64, n=6.

Fig. 6 : add the standard deviation and results of stat test

Standard deviation and ANOVA analyses were added.

Pg7 l15 : remove “0.01 µM” of NO3+NO2 because below detection limit (0.075 µM)

We changed the sentence to*: "... NO_2^-+NO_3^- (DIN) increased from east to west from below detection in the Ionian Sea to..."*

L19: DIP : from 0.01 to 0.24 µM (stx 333)

Corrected.

Add a sentence on the spatial variability of silicic acid

Sentence was added: "*... Silicic acid ($Si(OH)_4$) concentration was lowest in the westernmost stations- at the entrance to the MS (0.44 µM), and increased toward the east with highest concentration observed at the easternmost station (1.10 µM) (Table 1)..."*

Pg8 l2: Replace' stx 290' by station in the Levantine basin (st. 290)

Corrected.

Pg8: Add a sentence on the spatial variability of nanoeukaryote (Fig. 3C)

We now refer, as suggested by referee #1, to all as pico-eukaryotes to avoid confusion. Our flow cytometer takes particles smaller than 5 µm thus the entire "pico" fraction is counted (< 2µm), while only a small portion of the "nano" (2-20 µm).

Pg8 l18-20: 'The springtime rates of N2 fixation at all stations were 3–7 folds higher than measurements published previously during summertime' . But the methods used to measure N2 fix (bubbling in summertime and 15N2 enriched sw in springtime) were different as well as the incubation period (24 and 48h). From Wilson et al., 2012 'a 2- to 6-fold increase in the rate of 15N2 assimilation was measured when 15N2-enriched seawater was added to the seawater sample compared to the addition of 15N2 as a gas bubble.'

We address this issue in details in the discussion (P.11 lines 6-20). We compared both methods in MS waters prior to the cruise and observed a 2-3 fold increase in rates using the enriched seawater method (n= 18). If we assume a 2-fold underestimate in the reported summer rates, we still observe significant seasonal differences between this study and summertime periods. This suggests that methodological differences alone cannot account for the seasonal changes we observed.

As for the incubation periods, in long incubations (i.e. > 24 hours) these differences might be lower because the gas bubble would be equilibrated within several hours of the incubation. Yet we observed seasonal differences after using a moderate 2 fold difference to compare the rates.(P11 line 16-20).

Pg8 l21 – pg9 l5-8: again you can not exclude the dark N2 fix by unicellular diazo cyanobacteria without phylogenetic analyses

We agree that some of the dark N_2 fixation might be also attributed to unicellular cyanobacteria; we simply suggest that the rates measured within these bottles were predominantly performed by heterotrophic N_2-fixers.

However, we emphasized this point in the discussion: ***"... 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, although we cannot exclude that some of the dark N_2 fixation was performed by unicellular cyanobacteria...".***

Pg9 l1-2 : give the standard deviation on 0.16 nmol N L-1 d-1 (stx 294) and perform a statistical test to compare the rates at stations 290 and 294

We added the stdev and performed a t-test, the two stations are significantly different (P= 0.02).

P9 l6: Perform a statistical test on the ratios and add the results on fig 5B- Mention that the ratios determined at stx 290 and 294 are really close to 1 (give the values); I am not sure that ratios at stx 312, 316, 304, 290, 294 will be statistically different.

The stdev of the ratios was added.

Pg9-§3-3 : this paragraph should be moved in 'discussion'

The paragraph mostly includes results (i.e. ratio >1 <1 etc.) with only a short explanation (2 lines) of the methodology behind it. We feel it is important to leave it in the results for clarity.

you used the measured C/N ratio to convert N2 fix in PP in order to estimate to contribution of N2 fix to PP. These C/N ratios are representative of the whole planktonic community and are not specific to diazo organisms. Please discuss about this (and please add the values of C/N in table 2)

We agree with the referee that some of the POC and PON measured are not related to diazotrophs at all but to other organisms. Yet our use of the ratio POC: PON instead of the ~6.6 Redfield ratio is more accurate in this system where this ratio is usually higher by ~30% (see Yogev et al. 2011).

We added the referee important comment to the methods: ***"... Although the measured POC and PON are representative of the whole planktonic community and are not specific to diazotrophs, our previous experience in the EMS suggests higher POC: PON ratio than the conventional 106:16 Redfield ratio (Yogev et al., 2011, Rahav et al., 2013) and thus were used to calculate the % contribution....".***

Pg10 l17 : a new figure with data of N2 fix and PP could be interesting to show this no correlation in EMS and probably over the whole MS (add R2).
We want to keep the paper as short as possible, thus would like to avoid adding more figures. We did add the R^2 of the correlation in the EMS.

The authors conclude that no correlation between N2 fix and PP 'suggests that N2 fix is attributed mainly to diazo bacteria'. others explanations should be given. For example we can hypothesize that diazotrophs and non diazotrophic phytoplankton are limited or co-limited by different nutrients.
The suggested explanation was added: *"... This suggests that N_2 fixation is attributed mainly to heterotrophic bacteria or that diazotrophs and non diazotrophic phytoplankton is limited or co-limited by different nutrients..."* (P. 10 lines 2-5).

L25 : remove from the manuscript, results and conclusions from Rahav et al., 2012 which is in preparation; Works cited in the manuscript should be accepted for publication or published already
The work has been accepted for publication in JGR. All citations and reference were corrected accordingly.

L28: ANOVA : Have you previously test the homogeneity of variances?
Yes we did test the homogeneity of variances.

L27 : Is there a correlation between PP and N2 fix in WMS (R2=?) ?
N_2 fixation and PP were correlated in the WMS stations. All statistical data was added to the text.

Pg11 L1-5 : do you have an idea of the order of magnitude of abundance of Richelia in EMS and WMS ? Richelia could also contribute to N2 fix in EMS (L5)
We did not count the *Richelia* along the cruise track. However, Bar-Zeev et al. (2008) reported the lack of large-scale diatom–diazotroph blooms and low rates of N_2 fixation by these diazotrophs in the EMS.

P12 l19-25 : you need the results of the stat test before concluding

The statistical test was carried out (please see new figure 4B).

Pg12 L29 : Is it possible to convert the nifH transcripts into abundances ?

For *Trichodesmium* and UCYN-B such a conversion is possible on 1:1 basis, assuming that there is one *nifH* gene copy per genome (Zehr et al., 2008) and one genome copy per cell (Luo et al., 2012). In the MS we have a highly diverse population, thus we fill it would be misleading to perform such a conversion.

Pg13 l21 'Higher contribution of N2 fixation to primary production (4–8 %) was measured in the western basin compared to the eastern basin (_2 %, Fig. 6)'. you need the results of the stat test before concluding

The statistical analyses were added to the figure and text.

Abstract and conclusions : 'These rates were 3–7 fold higher than N2 fixation rates measured previously in the Mediterranean Sea during summertime.' I suggest to remove '3-7 fold' because of large differences in methodology (incubation period, 15N2 addition) between rates measured in summer and spring

We removed "3-7 fold" from the conclusion and left it in the discussion where the methodological issues and incubation period differences is explained in details.

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

Rahav E.[1], Herut B.[2], Levi A.[1], Mulholland M.R.[3], and Berman-Frank I.[1]

[1] Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat Gan 52900, Israel.

[2] Israel Oceanographic and Limnological Research, National Institute of Oceanography, Haifa 31080, Israel.

[3] Department of Ocean, Earth and Atmospheric Sciences, Old Dominion University, 4600 Elkhorn Avenue, Norfolk, Virginia 23 529-0276, USA.

Submitted to Ocean Sciences as part of a special issue on the Meteor Cruise #M84/3 Spring 2011

REVISED – 1 March 2013

Correspondence to: Ilana Berman-Frank (Ilana.berman-frank@biu.ac.il)

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 nmol N L^{-1} d^{-1}, respectively). These rates were 3-7 fold higher than N_2 fixation rates measured previously in the Mediterranean Sea during summertime and we estimated that methodological differences alone did not account for the seasonal changes we observed. Higher contribution of N_2 fixation to primary production (4- 8 %) was measured in the western basin compared to the eastern basin (~2%). Our data indicates that these differences between basins may be attributed to changes in N_2-fixing planktonic communities and that heterotrophic diazotrophy may play 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 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).

Prokaryotic dinitrogen (N_2) fixation is now recognized as a globally important input of new oceanic N (reviewed in Gruber, 2008) that can be subsequently transferred to other planktonic groups (Mulholland et al., 2004; Mulholland and Capone, 2009). However, reported rates of N_2 fixation from the MS are limited to a few studies from the last six years and most are restricted to surface waters and the summer season. Typical rates of N_2 fixation during summer from both the eastern and western basins of the MS are generally low, ranging from undetectable to ~ 0.15 nmol N L^{-1} d^{-1} (Ibello et al., 2010; Ridame et al., 2011; Yogev et al., 2011; Rahav et al., 2013), However, N_2 fixation rates at the central zone of the Ligurian Sea station in the NW Mediterranean (DYnamique des Flux de mAtiére en MEDiterranée- DYFAMED) are higher ranging from 2-17 nmol N L^{-1} d^{-1} (Garcia et al., 2006; Sandroni et al., 2007).

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; Yogev et al., 2011). In the MS organisms expressing *nifH*, as the gene encoding part of the nitrogenase complex, include unicellular cyanobacteria, diatom-diazotroph assemblages, proteobacteria, methanogenic archaea, anaerobic bacteria, and purple sulfur bacteria. (Man-Aharonovich et al., 2007; Yogev et al., 2011). The filamentous cyanobacterium *Trichodesmium* has been sporadically observed in extremely low abundances (Yogev et al., 2011) and one bloom event of this genus was recorded from the Aegean Sea around the Lesvos island (Spatharis et al., 2012).

The contribution of N_2 fixation to new primary productivity in the MS was mostly tested during the stratified period in summer and appears to vary between the Eastern and Western basins. In the western basin, N_2 fixation was shown to contribute up to 35% to 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% to the new production (Yogev et al., 2011, Rahav et al., 2013). Yearly variability in the contribution of N_2 fixation to new primary productivity was also observed in the DYFAMED station ranging from 1% to 28% (Sandroni et al., 2007).

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 in spring and compare these with similar measurements made during 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 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) between the 4th and 28th of April 2011. Eight stations were sampled along an east to west transect across 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) (Figure 1 and Table 1). Seawater samples collected east of the Sicily strait were defined as Eastern Mediterranean (EMS) stations, whereas samples collected to the west of Sicily strait were defined as Western Mediterranean (WMS) stations.

2.2 Experimental design

Subsurface seawater (6-8 m depth) was collected using low pressure pump and placed in triplicate 4.6-liter polycarbonate Nalgene bottles. $NaH^{13}CO_3$ (Sigma) was added to obtain an enrichment of approximately 10 % of the ambient dissolved inorganic carbon (460 µl of 200 mmol L^{-1} $NaH^{13}CO_3$) (Mulholland and Bernhardt, 2005). $^{15}N_2$ uptake measurements were measured using a newly developed ^{15}N-enriched seawater protocol (Mohr et al., 2010). Enriched seawater was prepared by 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 hour. The degassed water was transferred into septum capped Nalgene bottles with no headspace, and 1 ml of $^{15}N_2$ gas (99%) was injected per 100 ml of seawater. The bottles were shaken vigorously until the bubble disappeared. Aliquots of this $^{15}N_2$- 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 $^{15}N_2$ enrichment of 1.5 atom% after adding 50 ml of $^{15}N_2$-enriched water to a 4.5 L bottle (Wilson et al., 2012).

After the enriched-seawater and ^{13}C were added (i.e. double labeling), the bottles were well shaken, and incubated on-deck at ambient surface seawater temperatures, maintained with running surface water pumped on board. Incubations began early in the morning (~ 7 am) and the incubators were covered with either neutral density screening to simulate ambient light, or under complete darkness for 48 hour incubations. We also compared the obtained rates with 24 hours incubations (conducted in parallel) and obtained no significant difference between the rates (R^2= 0.91 n= 24 P< 0.05, Figure S1). The incubations under ambient irradiance (representative of a full diel cycle) record the activities of both autotrophic and heterotrophic diazotrophs. Whereas, we assume that the 48 hours 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.

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 °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. For isotope ratio mass spectrometry, standard curves to determine N and C mass were done with each sample run. Samples were run only when standard curves had R_2 values >0.99. At masses >4.7 µg N, the precision for the atom percent ^{15}N measurement was _0.0001% based on daily calibrations made in association with sample runs and calibrations averaged over runs made over several years. For most of the results reported here, the masses were >4.7 µg N. However, samples with <4.7 µg N were only used if the precision was 0.0001% for that sample run. Standard masses ranged from 1.2 to 100 µg N and from 9.4 to 800 µg C. In addition to daily standard curves, reference standards and standards run as samples were run every six to eight samples.

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.

Although the measured POC and PON are representative of the whole planktonic community and are not specific to diazotrophs, our previous experience in the EMS suggests higher POC: PON ratio than the conventional 106:16 Redfield ratio (Yogev et al., 2011, Rahav et al., 2013) and thus were used to calculate the % contribution.

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).

2.4 Inorganic nutrients

Duplicate water samples were collected in 15-mL acid-washed plastic scintillation vials from surface (6-8 m) and immediately frozen at -20 °C. Nutrients were determined in the laboratory ~ 4 months after the cruise using a segmented flow Skalar SANplus System Instrument as detailed in Kress and Herut (2001). The precision of the nitrate+nitrite, ortho-phosphate and silicic acid measurements were 0.02, 0.003 and 0.06 µM, respectively. The limits of quantification were 0.075 µM, 0.008 µM and 0.07 µM for nitrate+ nitrite, ortho-phosphate and silicic acid, respectively (note that for silicic acid the limit of quantification is similar to the precisions).

2.5 Chlorophyll *a* extraction

Duplicate seawater samples (500 ml) taken twice a day across the MS (n= 94) were filtered onto glass fiber filters. The filters were stored at -20 ^{0}C in a dark box until analysis within 2-3 days. Samples were extracted in 5 ml 90% acetone overnight, at 4 ^{0}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.

2.6 Picophytoplankton abundance

The abundance of picophytoplankton 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 respectively). Samples of 1.8 ml were fixed immediately at room temperature with 23 µl of 25 %

gluteraldehyde (Sigma G-5882) retained at room temperature for 10 min, subsequently frozen in liquid nitrogen, and kept at -80 ^{0}C until analyzed. Samples were fast-thawed at 37 ^{0}C, and counted using a FACScan Becton Dickinson flow cytometer, fitted with an Argon laser (488 nm) for 10 to 15 min or until 30000 cells were counted (Vaulot et al., 1989). Pico/nano phytoplankton carbon (C) biomass was calculated from cell counts assuming 175 fg C cell^{-1} for *Synechococcus* cells 53 fg C cell^{-1} for *Prochlorococcus* cells, and 2100 fg C cell^{-1} for pico-eukaryotes (Campbell and Yentsch, 1989).

3 Results

3.1 East-west distribution of physical, chemical and phytoplankton parameters

The physical, chemical and biological parameters of the surface waters at each station are provided in Tables 1 and 2. Overall, surface temperatures and salinities increased from west to east from 14.7 to 18.1 °C and 36.3 to 39, respectively. NO_2^- +NO_3^- (DIN) increased from east to west from below detection in the Ionian Sea to 1.39 µM at the Gulf of Cadiz station (Table 1). In contrast, Station 290 (NW Levantine Basin) had high surface concentrations of DIN (0.86 µM), probably due to upwelling of deeper waters within the cyclonic Rhodes Gyre. Dissolved inorganic phosphorus (DIP) ranged from 0.01 to 0.24 µM in surface waters across the entire Mediterranean Sea (MS) (Table 1). Silicic acid ($Si(OH)_4$) concentration was lowest in the westernmost stations- at the entrance to the MS (0.44 µM), and increased toward the east with highest concentration observed at the easternmost station (1.10 µM) (Table 1).

Chlorophyll (Chl *a*) concentrations increased from east to west across the MS. Surface Chl *a* concentrations were ~0.03 µg L^{-1} at the eastern basin stations and up to 0.31 µg L^{-1} at the Strait of Gibraltar- the western-most station (Figure 1). *Synechococcus* dominated the picophytoplankton ranging from as low as 2.26 x 10^6 cells L^{-1} to 3.27 x 10^7 cells L^{-1} in the eastern and western basin, respectively (Figure 2, Table 2). Using a cell: carbon conversion ratio of 175 fg C cell^{-1} (see methods), this represents a range of 396 ng C L^{-1} to 5723 ng C L^{-1}. In the eastern basin, the picoeukaryote abundances (~2.1x10^4 to 7.5x10^4 cell L^{-1}) and biomass (44 to 158 ng C L^{-1}) were low except in the Levantine basin (Station. 290) where higher abundances

($4.36x10^5$ cell L^{-1}) and biomass (916 ng C L^{-1}) were measured (Figure 2, Table 2). *Prochlorococcus* abundances and biomass from the surface waters were generally low throughout the whole MS, especially at the Shikmona Eddy (Station 294) and the Ionian Sea (station 304) (Figure 2, Table 2).

3.2 Primary productivity and N_2 fixation rates

Photosynthetic carbon fixation rates ranged from 0.21 to 0.74 µg C L^{-1} d^{-1} in the eastern basin, and 0.76 to 1.39 µg C L^{-1} d^{-1} at the western Mediterranean stations. Much higher rates were measured at the Strait of Gibraltar (15.04± 1.6 µg C L^{-1} d^{-1}) and in the Gulf of Cadiz (8.22 µg C L^{-1} d^{-1}) (Table 2).

N_2 fixation rates obtained across the MS exhibited a strong zonal gradient from the eastern to western basins (Figure 3A and Table 2). The lowest N_2 fixation rates were measured in the eastern basin, ranging from 0.10± 0.02 nmol N L^{-1} d^{-1} in the Ionian Sea, to 0.15± 0.01 nmol N L^{-1} d^{-1} at Station 290 (affected by the Rhodes Gyre) (Figure 3A and Table 2). N_2 fixation rates increased gradually toward the west ranging from 0.22 ± 0.03 in the Tyhrranean Sea to 2.35± 1.12 nmol N L^{-1} d^{-1} at the westernmost station at the Strait of Gibraltar (Figure 3A and Table 2). The springtime rates of N_2 fixation at all stations were 3-7 fold higher than measurements published previously during summertime (Figure 3B).

In addition to total N_2 fixation (measured in light bottles under ambient diel irradiance), we examined N_2 fixation rates in bottles incubated for 48 hours in the dark. While some unicellular cyanobacteria fix N_2 during the dark hours, they require light energy to fuel the process. We assumed that after 48 hours in the dark, the contribution by these diazotrophs will be negligible and most N_2 fixation would be due to heterotrophic diazotrophs that do not require light for the N_2 fixing process (Postage, 1979). The N_2 fixation rates from 48 hour dark incubations showed a similar east-west trend as observed in light bottle incubations (Figure 4A); within the eastern basin, N_2 fixation in dark incubations were lowest at the easternmost Station 290 (0.11± 0.02 nmol N L^{-1} d^{-1}) and highest at Station 294 in the Shikmona Eddy (0.16± 0.01 nmol N L^{-1} d^{-1}) (Figure 4A). In the western basin N_2 fixation rates in dark incubation bottles rates ranged from 0.20± 0.05 to 0.40± 0.11 nmol N L^{-1} d^{-1} (Figure 4A).

We compared rates of light and dark N_2 fixation (Figure 4B) to estimate the relative contribution of autotrophic versus heterotrophic N_2 fixation. In the western basin, light:dark estimates of N_2 fixation were always > 1, suggesting the

predominance of autotrophic N_2 fixation. In the eastern basin light:dark N_2 fixation rates were < 1 suggesting a preponderance of heterotrophic diazotrophs (Figure 4).

3.3 The contribution of N_2 fixation to primary productivity

We calculated the percent contribution of N_2 fixation to total primary productivity during springtime based on rates of N_2 fixation measured in the light bottle incubations and the associated C fixation estimated using an the average particulate C:N ratio obtained at each station (Table 2, and see Yogev et al. 2011 and Rahav et al., 2013). New production due to N_2 fixation was ~2% of the total primary productivity at the EMS stations and increased by a factor of 2 to 4 in the western Mediterranean Sea (WMS), ranging from 3.5% in the Adriatic Sea to 8.5% in the Alboran Sea. The percent contribution of N_2 fixation to primary production in the Gulf of Cadiz, near the Strait of Gibraltar that connects the Mediterranean Sea with the Atlantic Ocean, was 2.3% (Figure 5).

4 Discussion

This study provides the first springtime measurements of N_2 fixation in surface waters along an east- west transect across the Mediterranean Sea (MS). We focused sampling at representative stations from different water provinces in the MS (Figure 1, Table 1). Our results yielded N_2 fixation rates in surface waters that are 3-7 fold higher (Figure 3A, Table 2) than published rates from two summertime basin-wide N_2 fixation studies (Ibello et al., 2010; Bonnet et al., 2011), routine stations off the Israeli coast (Yogev et al., 2011), and a Levantine Basin transect (Rahav et al., 2013). Moreover, the gradient of increasing N_2 fixation rates from east to west coincide with the east-west gradient in surface Chl.*a* (Figure 1) and primary productivity (Table 2).

Seasonal measurements of N_2 fixation rates in the MS have been made at two monitoring stations, one located west of the Israeli coastline (Levantine Basin) (Yogev et al., 2011) and the other off the coast of France, the DYFAMED station (Ligurian Sea) (Garcia et al., 2006; Sandroni et al., 2007). Rates of N_2 fixation in surface waters from the Levantine Basin were uniformly low (~ 0.01 nmol N L^{-1} d^{-1}) and did not show any seasonality (Yogev et al., 2011). In contrast, at the WMS time series station (DYFAMED), higher rates of N_2 fixation were measured during April and August (4-7.5 nmol N L^{-1} d^{-1}, 10 m) relative to other months (< 2 nmol N L^{-1} d^{-1}, 10 m), which were associated with higher primary productivity rates (Sandroni et al., 2007).

The Shikmona Eddy (Station 294) and the Ionian Sea (Station 304), representing ultraoligotrophic conditions had lower nutrient and Chl.*a* concentrations than the more productive cyclonic Rhodes Gyre station (Station 290). Yet similar N_2 fixation rates were measured at all three stations (Figure 3A, Table 2) and there was no correlation between N_2 fixation and primary production (R^2= 0.18, n=9, t-test, P> 0.05). This suggests that N_2 fixation is attributed mainly to heterotrophic bacteria or that diazotrophs and non diazotrophic phytoplankton is limited or co-limited by different nutrients. Heterotrophic bacteria are known to compete for N with autotrophs in the nutrient-depleted surface waters of the EMS (Thingstad et al., 2005; Tanaka et al 2007) and molecular fingerprinting suggests a highly diverse heterotrophic community of *nifH* phylotypes (Man-Aharonovich et al., 2007; Yogev et al., 2011). Heterotrophic diazotrophs may out-compete other bacteria in an N-impoverished system because they can acquire N from the abundant N_2 pool. Evidence for heterotrophic diazotrophy was found in both surface and aphotic depths in the EMS (Rahav et al., 2013).

Higher DIN (Table 1) and Chl *a* concentrations were measured in the more productive WMS compared to the EMS (Figure 1, Table 2). Concurrently, N_2 fixation rates in the WMS were also higher (ANOVA, $P < 0.05$) ranging from 0.22 to 0.86 nmol N L^{-1} d^{-1} (Figure 3A, Table 2) and correlated with PP (R^2= 0.82, n=12, t-test, P< 0.05), suggesting photoautotrophic associated N_2 fixation. Indeed, relatively high diatom abundances were detected in surface waters of the WMS (> 100 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 (Figure 3A, Table 2). Moreover, these springtime N_2 fixation rates were 7-fold higher than those measured previously during summer by Ibello et al., (2010) (2.35 nmol N L^{-1} d^{-1} versus 0.3 nmol N L^{-1} d^{-1}, respectively). These differences 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 therefore 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 ($^{15}N_2$) seawater addition method (Mohr et al., 2010) rather than the gas bubble $^{15}N_2$ addition method (Montoya et al. 1996). 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 waters demonstrated a 2-3 fold increase in rates using the enriched seawater method (n= 18). However, in long incubations (>24 hours), 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). Yet, research demonstrates that the abundant and widely distributed unicellular group A cyanobacteria are photoheterotrophs (Moisander et al., 2010). Further, many heterotrophic 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 stations, the ratio of light:dark bottle N_2 fixation was usually < 1 (Figure 4B)

suggesting that heterotrophic diazotrophs may be the dominant N_2 fixers, although we cannot exclude that some of the dark N_2 fixation was performed by unicellular cyanobacteria. In the western basin, this ratio was generally > 1 suggesting that autotrophic diazotrophs predominated (Figure 4B). 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 hours) 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 ~ 40% of the *nifH* transcripts attributed to heterotrophic bacteria (Man-Aharonovich et al., 2007; 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 N_2 fixation (Konno et al., 2010). In this study we used GF\F filters to measure planktonic N_2 fixation (nominal pore size of ~0.7 µm, see methods), as is a common practice. Thus, it is possible we could have missed N_2 fixation by very small bacteria diazotrophs and thereby underestimated total planktonic N_2 fixation.

Based on results from studies conducted during summer in the EMS, N_2 fixation accounted for only 0.7-2 % of primary productivity at stations in the Levantine basin (Yogev et al., 2011, Rahav et al.2013), but increased to ~ 6% in the more productive Rhodes Gyre and Cyprus Eddy (Rahav et al. 2013). Consistent with these results, during a summer transect across the Mediterranean (BOUM campaign), N_2 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 N_2 fixation rates (2- 4 fold) at both basins and a similar spatial trend. Higher contribution of N_2 fixation to primary production (4- 8 %) was measured in the western basin compared to the eastern basin (~2%, Figure 5). These differences between the two basins are probably attributed to changes in N_2-fixing planktonic communities and other environmental aspects. Summertime data from the EMS demonstrated a significant positive correlation between N_2 fixation

rates and bacterial production suggesting a higher involvement of heterotrophic diazotrophs in the ultraoligotrophic EMS (Rahav et al., 2013).

5 Conclusions

This study provides the first direct measurements of N_2 fixation rates in surface-waters across the MS during springtime. N_2 fixation rates were measured using the newly modified ^{15}N-uptake method (Mohr et al., 2010) during a spring transect and were 3- 7 fold higher than measurements made in surface waters during the stratified summer period. Methodological differences cannot fully account for the higher rates of N_2 fixation observed during this cruise and we suggest that the higher rates are due to seasonal variability in primary productivity and environmental factors. N_2 fixation was higher and contributed more to total primary production in the western basin than in the eastern basin. While our data suggests that N_2 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 N_2 fixation during spring. We suggest that future investigations should include N_2 fixation rate measurements and phylogenetic identity of diazotrophs at both photic and aphotic depths to better constrain the contribution of N_2 fixation to N budgets as well as the total and new production within the Mediterranean Sea.

Acknowledgments

Many thanks for the help provided by the captain and crew of the R/V *Meteor*. We would also like to thank Dr. Toste Tanhua, the Chief Scientist of this cruise, for allowing us to take part in this campaign. This research was supported by the Israel Science Foundation grant (996/08) to I. B-F and B.H. This study is in partial fulfillment of a Ph.D. thesis for Eyal Rahav from Bar Ilan University.

References

Bar Zeev, E., T. Yogev, D. Man-Aharonovich, N. Kress, B. Herut, O. Beja, and I. Berman-Frank: Seasonal dynamics of the endosymbiotic, nitrogen-fixing cyanobacterium *Richelia intracellularis* in the Eastern Mediterranean Sea, ISME J., 2, 911-92. 2008.

Berman, T., D. W. Townsend, S. Z. Elsayed, C. C. Trees, and Y. Azov: Optical transparency, chlorophyll and primary productivity in the Eastern Mediterranean near the Israeli coast, Oceanol. Acta., 7, 367-372, 1984.

Bonnet, S., Grosso, O., and Moutin, T.: Planktonic dinitrogen fixation along a longitudinal gradient across the Mediterranean Sea during the stratified period (BOUM cruise), Biogeosciences, 8, 2257–2267, doi:10.5194/bg-8-2257-2011, 2011.

Bosc, E., A. Bricaud, and D. Antoine: Seasonal and interannual variability in algal biomass and primary production in the Mediterranean Sea, as derived from 4 years of SeaWiFS observations, Global Biogeochem. Cy., 18, GB1005, doi:10.1029/2003GB002034, 2004.

Campbell, J. W., and C. M. Yentsch: Variance within homogeneous phytoplankton populations .1. Theoretical framework for interpreting histograms, Cytometry, 10, 587-595, 1989.

Capone, D. G., and J. P. Montoya: Nitrogen fixation and denitrification, Methods Microbiol., 30, 501-51, 2001.

Dong, J. D., Y. Y. Zhang, Y. S. Wang, S. Zhang, and H. K. Wang: Spatial and seasonal variations of cyanobacteria and their nitrogen fixation rates in Sanya Bay, South China Sea, Sci. Mar., 72, 239-251, 2008.

Falkowski, P. G., R. T. Barber, and V. Smetacek: Biogeochemical controls and feedbacks on ocean primary production, Science, 281(5374), 200-206, 1998.

Garcia, N., P. Raimbault, E. Gouze, and V. Sandroni: Nitrogen fixation and primary production in Western Mediterranean, Comptes. Rendus. Biologies., 329, 742-750, 2006.

Großkopf, T., W. Mohr, T. Baustian, H. Schunck, D. Gill, M. M. M. Kuypers, G. Lavik, R. A. Schmitz, D. W. R. Wallace, and J. LaRoche: Doubling of marine dinitrogen-fixation rates based on direct measurements, Nature, 488, 361-364, 2012.

Gruber, N. : The marine nitrogen cycle: Overview of distributions and processes, in: Nitrogen in the marine environment, edited by D. Capone, D. Bronk, M. Mulholland and E. Carpenter, Elsevier, Amsterdam, 1-50, 2008.

Halm, H., P. Lam, T. G. Ferdelman, G. Lavik, T. Dittmar, J. LaRoche, S. D'Hondt, and M. M. M. Kuypers:, Heterotrophic organisms dominate nitrogen fixation in the South Pacific Gyre, ISME J., 6, 1238-1249, 2012.

Holm-Hansen, O., C. J. Lorenzen, R. W. Homes, and J. D. H. Strickland: Fluorometric determination of chlorophyll., Journal du Conseil Permanent Internationale pour L'Exploration de la Mer., 30, 2-15, 1965.

Ibello, V., Cantoni, C., Cozzi, S., and Civitarese, G.: First basin-wide experimental results on N_2 fixation in the open Mediterranean Sea, Geophys. Res. Lett., 37, L03608, doi:10.1029/2009GL041635, 2010.

Ignatiades, L., O. Gotsis-Skretas, K. Pagou, and E. Krasakopoulou: Diversification of phytoplankton community structure and related parameters along a large-scale longitudinal east-west transect of the Mediterranean Sea, J. Plankton. Res., 31, 411-428, 2009.

Karl, D., A. Michaels, B. Bergman, D. Capone, E. Carpenter, R. Letelier, F. Lipschultz, H. Paerl, D. Sigman, and L. Stal:, Dinitrogen fixation in the world's oceans, Biogeochemistry, 57, 47-98, 2002.

Konno, U., U. Tsunogai, D. D. Komatsu, S. Daita, F. Nakagawa, A. Tsuda, T. Matsui, Y. J. Eum, and K. Suzuki: Determination of total N_2 fixation rates in the ocean taking into account both the particulate and filtrate fractions, Biogeosciences, 7, 2369-2377, 2010.

Kress, N., and B. Herut: Spatial and seasonal evolution of dissolved oxygen and nutrients in the Southern Levantine Basin (Eastern Mediterranean Sea):

chemical characterization of the water masses and inferences on the N : P ratios, Deep-Sea Res. Pt. I, 48, 2347-2372, 2001.

Krom, M. D., S. Brenner, N. Kress, and L. I. Gordon: Phosphorous limitation of primary productivity in the Eastern Mediterranean Sea, Limnol. Oceanogr., 36, 424-43, 1991.

Krom, M. D., K. C. Emeis, and P. Van Cappellen: Why is the Eastern Mediterranean phosphorus limited?, Prog. Oceanogr., 85, 236-244, 2010.

Le Moal, M., and I. C. Biegala: Diazotrophic unicellular cyanobacteria in the Northwestern Mediterranean Sea: A seasonal cycle, Limnol. Oceanogr., 54, 845-855, 2009.

Le Moal, M., H. Collin, and I. C. Biegala: Intriguing diversity among diazotrophic picoplankton along a Mediterranean transect: a dominance of *Rhizobia*, Biogeosciences, 8, 827-840, 2011.

Man-Aharonovich, D., N. Kress, E. Bar Zeev, I. Berman-Frank, and O. Beja: Molecular ecology of *nifH* genes and transcripts in the Eastern Mediterranean Sea, Environ. Microbiol., 9, 2354-236, 2007.

Marty, J. C., N. Garcia, and P. Rairnbault: Phytoplankton dynamics and primary production under late summer conditions in the NW Mediterranean Sea, Deep-Sea Res. Pt. I, 55, 1131-1149, 2008.

Moisander, P. H., R. A. Beinart, I. Hewson, A. E. White, K. S. Johnson, C. A. Carlson, J. P. Montoya, and J. P. Zehr: Unicellular cyanobacterial distributions broaden the oceanic N_2 fixation domain, Science, 327, 1512-1514, 2010.

Mohr, W., T. Grosskopf, D. W. R. Wallace, and J. LaRoche: Methodological underestimation of oceanic nitrogen fixation rates, PloS One, 5,e12583. doi:10.1371/journal.pone.0012583, 2010.

Mourino-Carballido, B., R. Grana, A. Fernandez, A. Bode, M. Varela, J. F. Dominguez, J. Escanez, D. de Armas, and E. Maranon: Importance of N_2 fixation vs. nitrate eddy diffusion along a latitudinal transect in the Atlantic Ocean, Limnol. Oceanogr., 56, 999-1007, 2011.

Moutin, T., and P. Raimbault: Primary production, carbon export and nutrients availability in western and eastern Mediterranean Sea in early summer 1996 (MINOS cruise), J. Marine. Syst., 33, 273-288, 2002.

Mulholland, M. R., G. Boneillo, and E. C. Minor: A comparison of N and C uptake during brown tide (*Aureococcus anophagefferens*) blooms from two coastal bays on the east coast of the USA, Harmful Algae, *3*, 361-376, 2004.

Mulholland, M. R., and P. W. Bernhardt: The effect of growth rate, phosphorus concentration, and temperature on N_2 fixation, carbon fixation, and nitrogen release in continuous cultures of *Trichodesmium* IMS101, Limnol. Oceanogr., 50, 839-849, 2005.

Mulholland, M. R. and Capone, D. G.: Dinitrogen fixation in the Indian Ocean, in: Indian Ocean Biogeochemical Processes and Ecological Variability, edited by: Wiggert, J. D., Hood, R. R., Naqvi, S. W. A., Brink, K. H., and Smith, S. L., American Geophysical Union, Washington, DC, 167–186, 2009.

Ohlendieck, U., K. Gundersen, M. Meyerhofer, P. Fritsche, K. Nachtigall, and B. Bergmann: The significance of nitrogen fixation to new production during early summer in the Baltic Sea, Biogeosciences, 4, 63-73, 2007.

Rahav, E., B. Herut, N. Stambler, E. Bar Zeev, M. R. Mulholland, and I. Berman-Frank: Uncoupling between dinitrogen fixation and primary productivity in the Eastern Mediterranean Sea, J. Geophys. Res-Biogeo., 118, 1–8, doi:10.1029/2012JG002055, 2013.

Ridame, C., M. Le Moal, C. Guieu, E. Ternon, I. C. Biegala, S. L'Helguen, and M. Pujo-Pay: Nutrient control of N_2 fixation in the oligotrophic Mediterranean Sea and the impact of Saharan dust events, Biogeosciences, 8, 2783-2783, 2011.

Riemann, L., H. Farnelid, and G. F. Steward: Nitrogenase genes in non-cyanobacterial plankton: prevalence, diversity and regulation in marine waters, Aquat. Microb. Ecol., 61, 225-237, 2010.

Sandroni, V., P. Raimbault, C. Migon, N. Garcia, and E. Gouze: Dry atmospheric deposition and diazotrophy as sources of new nitrogen to northwestern Mediterranean oligotrophic surface waters, Deep-Sea Res. Pt. I, 54, 1859-1870, 2007.

Siokou-Frangou, I., U. Christaki, M. G. Mazzocchi, M. Montresor, M. R. d'Alcala, D. Vaque, and A. Zingone: Plankton in the open Mediterranean Sea: a review, Biogeosciences, 7, 1543-1586, 2010.

Spatharis, S., N. Skliris, A. Meziti, and K. Kormas: First record of a *Trichodesmium erythraeum* bloom in the Mediterranean Sea: a result of climate change?, Can. J. Fish. Aquat. Sci., 69, 1444-1455, 2012.

Tanaka, T., T. Zohary, M. D. Krom, C. S. Lawe, P. Pitta, and S. Psarra: Microbial community structure and function in the Levantine Basin of the Eastern Mediterranean, Deep Sea Res. I, 54, 1721-1743, 2007.

Tanaka, T., et al.,: Lack of P-limitation of phytoplankton and heterotrophic prokaryotes in surface waters of three anticyclonic eddies in the stratified Mediterranean Sea, Biogeosciences, 8, 525-538, 2011.

Thingstad, F. T., U. L. Zweifel, and F. Rassoulzadegan: P limitation of heterotrophic bacteria and phytoplankton in the northwest Mediterranean, Limnol. Oceanogr., 43, 88-94, 1998.

Thingstad, T. F., M. D. Krom, R. F. Mantoura, G. A. Flaten, S. Groom, and B. Herut: Nature of phosphorus limitation in the ultraoligotrophic Eastern Mediterranean, Science, 309, 1068-1071, 2005.

Vaulot, D., C. Courties, and F. Partensky: A simple method to preserve oceanic phytoplankton for flow cytometric analyses, Cytometry, 10, 629-635, 1989.

Wilson, S. T., D. Bottjer, M. J. Church, and D. M. Karl: Comparative assessment of nitrogen fixation methodologies, conducted in the oligotrophic North Pacific Ocean, Appl. Environ. Microb., 78, 6516-6523, 2012.

Yogev, T., E. Rahav, E. Bar-Zeev, D. Man-Aharonovich, N. Stambler, N. Kress, O. Beja, M. R. Mulholland, B. Herut, and I. Berman-Frank: Is dinitrogen fixation significant in the Levantine Basin, East Mediterranean Sea?, Environ. Microbiol., 13, 854-87, 2011.

Zehr, J. P., and B. B. Ward: Nitrogen cycling in the ocean: New perspectives on processes and paradigms, Appl. Environ. Microb., 68,1015-1024, 2002.

Zehr, J. P., and R. M. Kudela: Nitrogen cycle of the open ocean: from genes to ecosystems, in *Annual Review of Marine Science, Vol 3*, edited by C. A. Carlson and S. J. Giovannoni, pp. 197-225, 2011.

Table 1 – Physical and chemical characteristics of the surface seawater (6-8 m) of the MS stations sampled during April 2011. BD- below detection limit; MLD- mixed layer depth.

Station number	290	294	304	312	316	333	338	339
Location	Levantine basin	Shikmona Eddy	Ionian Sea	Adriatic Sea	Tyrrhenian Sea	Alboran Sea	Strait of Gibraltar	Gulf of Cadiz
Position	34°20'N, 27°30'E	34°00'N, 34°25'E	35°36'N, 17°15'E	41°15'N, 18°00'E	38°36'N, 11°30'E	36°06'N, 2°48'E	35°57'N, 4°45'W	35°54'N, 7°00'W
Temperature (°C)	17.0	18.1	17.1	14.7	16.2	16.7	17.8	17.7
Salinity	39.0	39.0	38.3	38.5	37.2	36.3	36.3	36.4
MLD (m)	46	49	30	28	21	45	44	38
NO_2+NO_3 (µM)	0.86± 0.05	0.07± 0.01	BD	0.39± 0.09	0.54± 0.16	0.63± 0	0.56± 0.23	1.39± 0.84
PO_4 (µM)	0.05± 0.01	0.05	0.01	0.02± 0.02	0.02± 0.01	0.24± 0.18	0.07± 0.02	0.06± 0.03
$Si(OH)_4$ (µM)	1.10± 0.18	0.97± 0.06	0.79	0.95± 0.17	0.81± 0.32	0.61± 0.08	0.48± 0.13	0.44± 0.04

Table 2- Biological characteristics of the surface seawater (6-8 m) of the MS stations sampled during April 2011.

Station number	290	294	304	312	316	333	338	339
Chlorophyll ($\mu g\ L^{-1}$)	0.04± 0.01	0.03± 0	0.02± 0.01	0.11± 0.03	0.04± 0	0.18± 0.01	0.31± 0.01	0.07± 0.02
Synechococcus (cell L^{-1})	$1.33x10^7$	$2.26x10^6$	$3.86x10^6$	$1.78x10^7$	$1.16x10^7$	$2.68x10^7$	$3.27x10^7$	$4.94x10^6$
Prochlorococcus (cell L^{-1})	$1.17x10^6$	$8.32x10^4$	$3.17x10^5$	$1.14x10^6$	$1.24x10^6$	$2.60x10^6$	$1.60x10^6$	$3.57x10^6$
pico-eukaryotes (cell L^{-1})	$4.36x10^5$	$2.08x10^4$	$7.53x10^4$	$2.23x10^5$	$7.35x10^5$	$2.53x10^6$	$3.69x10^6$	$1.46x10^6$
Synechococcus (ng C L^{-1})	2328	396	676	3115	2030	4690	5723	865
Prochlorococcus (ng C L^{-1})	62	4	17	60	66	138	85	19
pico-eukaryotes (ng C L^{-1})	916	44	158	468	1544	5313	7749	3066
POC:PON	9.3± 2.5	9.2± 0.8	8.3± 0.7	7.6± 0.7	7.4± 0.5	8.2± 1.7	8.6± 1.6	6.4± 0.3
Primary productivity ($\mu g\ C\ L^{-1}\ d^{-1}$)	0.74± 0.01	0.53± 0.02	0.21± 0.01	1.39± 0.87	0.76± 0.13	0.78± 0.26	15.04± 1.61	8.01± 1.79
N_2 fixation (nmol $N\ L^{-1}\ d^{-1}$)	0.15± 0.01	0.12± 0.02	0.10± 0.02	0.29± 0.02	0.22± 0.03	0.86± 0.17	2.35± 1.12	0.39± 0.14

Figures Legends

Figure 1 – Map of the sampling locations (triangles): 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). Background (circle): Spatial distribution of chlorophyll *a* concentrations in surface waters (6-8 m) along the Meteor M84/3 cruise track (n= 94).

Figure 2 –Picophytoplankton distribution of *Synechococcus* (A), *Prochlorococcus* (B) and pico-eukaryotes (C) in the surface waters (6-8 m) of the Eastern (black circle) and Western (white circle) Mediterranean Sea. n= 21 and n=12 for the eastern and western basins respectively.

Figure 3 – 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., 2013, Yogev et al., 2011, Ibello et al., 2010 and Bonnet et al., 2011.

Figure 4 – 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). The asterisk above the columns represents statistically significant differences (one-way ANOVA, $P< 0.05$) for mean values of N_2 fixation rates in each station, and B) the resulting ratio between rates of N_2 fixation from ambient lighting and dark incubations. n= 3 for each incubation type at each station.

Figure 5 – The percent contribution of N_2 fixation to primary productivity (PP) of surface-waters sampled across the MS during the spring period. The letters above the columns represent statistically significant differences (one-way ANOVA and a Fisher LSD means comparison test, $P< 0.05$) for mean values of % contribution between stations. .