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High rates of nitrogen fixation during an in-situ phosphate release experiment in the Eastern Mediterranean Sea

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[1] Nitrogen fixation and $\delta^{15}N$ were measured within a warm-core eddy during an in-situ phosphate experiment (CYCLOPS) in the Eastern Mediterranean, with experimental procedures performed on unconcentrated, bulk water. Mean rates of 129 nmol-N $L^{-1}d^{-1}$ were measured at control stations in the absence of phosphate (<2 nmolL^{-1}), inferring the bioavailability of DOP to diazotrophs. In P-enriched waters, rates increased by 48% to 197 nmol-N $L^{-1}d^{-1}$ five days after addition. $\delta^{15}N$ of particulate material was homogenous throughout the upper mixed layer, but changed with time at both amended and control stations from +3.8‰ on day 1, to −0.6‰ on day 4–5 before returning to +3.1‰ after 9 days. This trend matched the observed response in other components of the biota and biogeochemistry in the P-enriched patch. In-vitro addition experiments indicated that diazotrophy was not limited by Fe availability. **Citation:** Rees, A. P., C. S. Law, and E. M. S. Woodward (2006), High rates of nitrogen fixation during an in-situ phosphate release experiment in the Eastern Mediterranean Sea, *Geophys. Res. Lett.*, *33*, L10607, doi:10.1029/2006GL025791.

1. Introduction

[2] Geochemical evidence [e.g., *Gruber and Sarmiento*, 1997] has indicated that the biological fixation of N_2 in the marine environment is far greater than was previously thought. Recent work has validated this hypothesis, and fixation rates have been reported at unprecedented levels [*Montoya et al.*, 2004], whilst the diversity and abundance of diazotrophs other than *Trichodesmium sp.* [*Capone et al.*, 1997] is becoming more apparent [*Zehr et al.*, 2001; *Karl et al.*, 2002].

[3] The Eastern Mediterranean Sea has great potential for nitrogen fixation, being an ultra-oligotrophic basin which does not exhibit Redfield stoichiometry. N:P ratios of ~28:1 occur in deep water [*Krom et al.*, 1991], and surface waters are characterised by elevated temperatures and very low inorganic nitrogen. *Gruber and Sarmiento* [1997] found high N* values in the Mediterranean indicative of nitrogen fixation which they suggested could support up to 30% of the export flux; this is supported by indirect estimates from stable-isotopic evidence [*Bethoux and Copin-Montegut*, 1986; *Pantoja et al.*, 2002] which indicate that up to 90% of new nitrogen could be supplied by this route. In contrast, *Krom et al.* [2004] present a revised nitrogen budget for the Eastern Mediterranean within which they find balance without diazotrophy, and attribute the low ^{15}N natural abundance ($\delta^{15}N$) signature to other processes.

[4] This study was undertaken during the CYCLOPS project in which phosphate was added to surface waters of the Cyprus Eddy in the Levantine Basin to investigate the nature of regional P limitation. This experiment was performed under a Lagrangian framework with the inert tracer SF_6 and phosphate added over 2 x 2 km^2 patch of seawater to a ~20m surface mixed layer [*Law et al.*, 2005]. The chemical and biological changes in the system were then tracked for 9 days until the patch had relaxed to a dilution in excess of 95%. The detailed results of the CYCLOPS P addition experiment are presented elsewhere [e.g., *Thingstad et al.*, 2005].

2. Methods

[5] Following deployment of the PO_4^{3-} on the 17 May 2002, seawater samples were collected daily using a profiling CTD system from stations identified by their SF_6 signature as either IN or OUT of amended waters (Figure 1).

[6] At all stations, samples were collected for the determination of nitrogen fixation rate from 16m at 1100 local time each day in acid cleaned cubitainers of 5L or 10L, with care taken to avoid light shock. Either ^{15}N-N_2 (99 atom%; Isotech Ltd) or local air was added to each cubitainer at 2ml gas L^{-1} of seawater. Samples were incubated on-deck in tanks maintained at sea-surface temperature and light screened using blue filters corresponding to ~55% surface irradiance. Incubations were terminated after 24h by filtering 4.8L onto 25mm GF/F filters, which were then stored at −20°C. Each day a single ^{15}N amended sample was filtered immediately following ^{15}N-N_2 addition to rule out initial enrichment of ^{15}N in the particulates. On day 9, a single experiment was performed to investigate the impact of iron availability upon diazotrophic activity by adding 6nmol L^{-1} $FeCl_3$ to 3 cubitainers, $^{15}N_2$ and incubating for 48 hours. Particulate nitrogen and ^{15}N atom% were measured using continuous-flow stable isotope mass-spectrometry (PDZ-Europa 20-20 and GSL) [*Owens and Rees*, 1989], with rates determined according to *Montoya et al.* [1996]. Instrument precision was better than 0.55% CV based on urea standards (PDZ-Europa Ltd) analysed throughout the run (mean ± 1 s.d. = 0.3663 atom% ± 0.0020, n = 12) in the range 0.5–1.5 μmol-N. The mean particulate N content of samples was 0.73 μmol (range 0.56–1.21 μmol-N). Samples incubated with ^{14}N (air) additions and run in parallel to ^{15}N amended samples showed no significant deviation from the expected atom% of 0.366.

[7] Samples were collected at the same time as experimental samples for the determination of $\delta^{15}N$ in particulate

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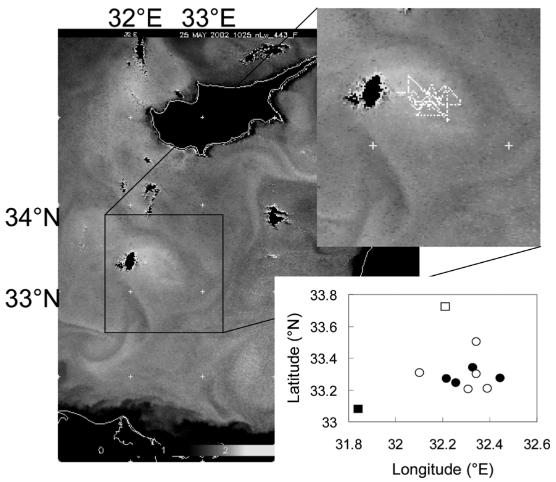

Figure 1. Location of the Cyprus Eddy (25 May 2002) within the Levantine Basin of the Eastern Mediterranean Sea, identified by SeaWiFS normalised water-leaving radiance at 443 nm. Upper inset shows the ships track (white dots) between days 7 and 9, the lower inset identifies the position of stations within the Cyprus Eddy, which were IN (solid circles) and OUT (open circles) of the SF_6/PO_4^{3-} labelled patch. Two stations were also occupied outside of the eddy on day 5 (solid square) and day 9 (open square). SeaWiFS image processed by the Plymouth Marine Laboratory, Remote Sensing Data Analysis Service. SeaWiFS data courtesy of NASA SeaWiFS Project and Orbimage.

material, and additionally at two stations outside of the eddy (Figure 1). Handling and filtration for enriched and natural abundance samples was done in different areas of the ship, with filter storage in separate freezers to avoid contamination. Water was collected at 5 depths between 16 and 120 m (12 – 300 m for the 2 out of eddy stations), with 4.8L filtered onto 25mm GF/F filters which were stored at −20°C. $\delta^{15}N$ was measured using continuous-flow stable isotope mass-spectrometry. Samples were referenced to 1 μmol-N urea standards (PDZ-Europa Ltd.), with a precision better than 0.7‰.

[8] Samples were collected for the analysis of NO_3^-, NO_2^-, NH_4^+ and PO_4^{3-} using nanomolar techniques and were analysed onboard ship within 2 hours of collection as described in *Krom et al.* [2005].

3. Results and Discussion

[9] Immediately after addition, the concentration of PO_4^{3-} rose from 1 – 2 to 110 nmol L^{-1} in surface waters, but then decreased rapidly to background levels after 7–8 days [*Law et al.*, 2005]. N_2 fixation rates determined over the 9 day period ranged between 115 nmol-N L^{-1} d^{-1} to 197 nmol-N L^{-1} d^{-1} (Figure 2). The OUT stations showed little variation in rate at 129 ± 9 (mean ± 1 s.d.) nmol-N L^{-1} d^{-1}, whilst higher rates (146 – 197 nmol-N L^{-1} d^{-1}) were determined in the PO_4^{3-} amended waters. Replicate incubations (n = 5 and n = 3 respectively) on days 6 and 7 gave rates of 162 ± 10 and 132 ± 20 nmol-N L^{-1} d^{-1}, that is 6% and 15% CV respectively. In addition a time series experiment on day 5 with rate measurements at 8, 16 and 24 hours showed a linear increase (r^2 = 0.97), consistent with the findings of *Montoya et al.* [2004]. Iron addition had no effect on nitrogen fixation, with a mean rate (± 1 s.d.) of 146 ± 16 nmol-N L^{-1} d^{-1} relative to the control rate of 145 nmol-N L^{-1} d^{-1}.

[10] The rates of nitrogen fixation presented here are the first reported for this area and are of particular significance due to their magnitude, which although large, are not unprecedented. Rates were uniform over 24 hours, and comparison of the mean OUT station rate with other reported hourly rates shows that they are only 8–27% of the high rates determined in the Arafura Sea by *Montoya et al.* [2004]; and 128%–240% of maximum rates determined in the N.E. Atlantic by P. Kaehler and A. Oschlies (personal communication, 2005) and *Voss et al.* [2004] respectively.

[11] The organisms responsible for the high rates of fixation during the present study remain unidentified. Neither *Trichodesmium sp.* nor *Richelia sp.* (diazotrophic heterocystous cyanobacteria associated with diatoms) were found, nor have they been recorded in the Eastern Mediterranean [*Krom et al.*, 2003] (S. Psarra, personal communication, 2005). The autotrophic community was dominated by the picoplankton [*Psarra et al.*, 2005] of which >40% was *Synechococcus sp. Synechococcus sp.* have been identified as potential diazotrophs [*Karl et al.*, 2002], although a recent survey of the tropical and sub-tropical Atlantic revealed no evidence of the *nifH* gene in *Synechococcus sp* [*Langlois et al.*, 2005]. A diverse range of diazotrophic organisms have been identified, including heterotrophic bacteria [*Zehr et al.*, 2001; *Falcón et al.*, 2004; *Montoya et al.*, 2004, *Langlois et al.*, 2005]. The in situ P addition to surface waters resulted in an initial decrease in phytoplankton biomass due to grazing [*Thingstad et al.*, 2005], so that the heterotroph/autotroph ratio increased from 1.05 to 2.8, and although bacterial biomass remained constant, bacterial production approximately doubled [*Pitta et al.*, 2005]. The increase in nitrogen fixation in the P-enriched waters suggests that either, the diazotrophic community avoided the increased grazing pressure, or that the observed fixation rates were net rates which may have been higher in the absence of grazing.

[12] The Eastern Mediterranean is considered an ultra oligotrophic environment although it receives significant input of Saharan dust [*Guerzoni et al.*, 1999] which is a

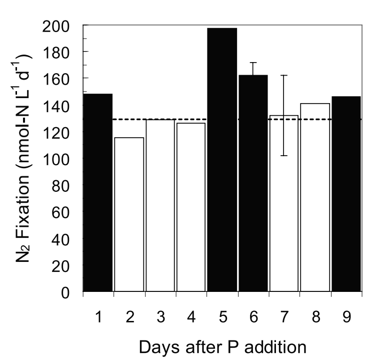

Figure 2. Nitrogen fixation rates within the Cyprus Eddy following PO_4^{3-} addition on day 0 (17 May 2002) at IN stations (solid bar) and OUT stations (open bar). The dotted line is equivalent to the mean rate at OUT stations.

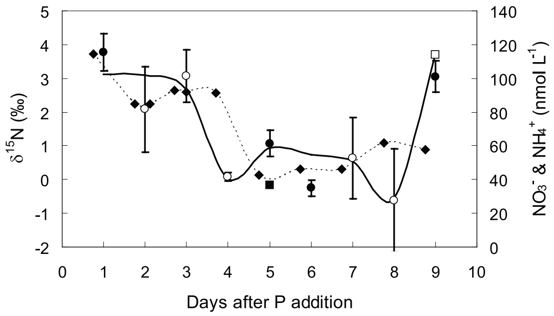

Figure 3. δ^{15}N of particulate material within the Cyprus Eddy following PO_4^{3-} addition on day 0 (17 May 2002). Mean δ^{15}N values ± 1 s.d. for 3 depths in the upper 30m are shown for stations IN (solid circles) and OUT (open circles) of the SF_6/PO_4^{3-} patch. The solid line represents the mean δ^{15}N for 5 depths in the upper 120m. δ^{15}N is also shown for two stations which were occupied outside of the Cyprus Eddy on day 5 (solid square) and day 9 (open square). DIN (solid diamonds) is presented for IN stations only.

source of N, P and Fe [*Herut et al.*, 1999]. High regional dust deposition results in surface waters being Fe-replete, with concentrations of 2 – 4 nmol L^{-1} [*Krom et al.*, 2003]. Consequently, although Fe is a fundamental requirement for nitrogenase it is unlikely to limit diazotrophy, as confirmed by the in-vitro Fe amendment incubations. We found no direct relationship between nitrogen fixation rate and PO_4^{3-} concentration or the N:P ratio of inorganic nutrients. The patch experienced rapid dilution with phosphate returning to background levels after 7–8 days, and so the biological response was limited and most apparent at days 3–5 [*Law et al.*, 2005; *Thingstad et al.*, 2005], consistent with the observed increase in N fixation (Figure 2). Although the maximum rates were recorded in the P-amended waters, the high and relatively stable OUT station rates in the absence of dissolved inorganic P indicate that the nitrogen fixing community were accessing the DOP pool. DOP was present in greater concentrations (50–60 nmol l^{-1}) [*Krom et al.*, 2005] than PO_4^{3-}, although little is known about the availability of DOP to diazotrophic organisms. *Dyhrman et al.* [2006] have identified an enzyme in *Trichodesmium* with the capacity for hydrolysis of phosphonates, which were previously considered a refractory component of the DOP. *Krom et al.* [2005] hypothesised that the major fraction of DOP in the E. Mediterranean is refractory, whilst *Thingstad and Mantoura* [2005] show that P release from the action of alkaline phosphatase on DOP maintained a supply of 2.2 nmol-P L^{-1} h^{-1}. Assuming Redfield stoichiometry this would supply six times the P requirement to support the observed nitrogen fixation rates.

[13] The δ^{15}N signature of particulate material is widely used as an indicator of the nitrogen source [e.g., *Karl et al.*, 2002]. For phytoplankton and bacterioplankton the range is in the order of −2‰ to +5‰, depending on whether the dominant nitrogen source is: regenerated N −1.5‰ to −2.1‰ [*Altabet*, 1988]; atmospheric N_2 0‰ to −1.0‰ [*Wada*, 1980; *Wada and Hattori*, 1991; *Carpenter et al.*, 1997]; or DON +1‰ to +3.9‰ [*Abell et al.*, 1999; *Knapp et al.*, 2005]. The δ^{15}N value for NO_3^- is generally accepted to be ≈ +5‰ although this is lower in the Mediterranean ranging from 3.4‰ in the west to 2.5‰ in the east [*Pantoja et al.*, 2002]. During this study the δ^{15}N of particulate nitrogen (Figure 3) showed homogeneity throughout the upper mixed layer (16 – 120m), but temporal heterogeneity, with a trend throughout the water column (mean δ^{15}N ± 1 s.d. for 5 depths 16 – 120 m) from +3.1 ± 1.0‰ on day 1 to 0.0 ± 0.9‰ on days 4–8, returning to 3.5 ± 1.2‰ on day 9. This temporal heterogeneity but vertical homogeneity was apparent in other environmental variables, with POC and PON showing high day-to-day variability but uniformity with depth (P. Wassman, personal communication, 2006), but could not be attributed to hydrodynamical or meteorological conditions. At the 2 stations outside of the eddy that were not influenced by P addition, the surface waters showed a similar pattern to contemporary IN stations on day 5 and day 9 suggesting that the trends observed in δ^{15}N were independent of the P addition.

[14] N fixation measurements were made over a DIN range of 42 – 101 nmol L^{-1} (Figure 3), and some consistency was apparent between DIN and δ^{15}N, with decreases in both around day 4–5 suggestive of increased N fixation at lower DIN. At present there is no consensus over the concentration of DIN or DON at which nitrogen fixation becomes viable; there is an acknowledgement that in general the synthesis of nitrogenase is suppressed by NH_4^+ and induced by the depletion of fixed N. A weak negative correlation (r^2 = 0.50, $p > 0.05$) was apparent between DIN concentration and N fixation, although is based on data from both IN and OUT station, and so interpretation is complicated by the phosphate addition. Execution of this study under a Lagrangian framework should result in observation of robust temporal trends in DIN, δ^{15}N and nitrogen fixation. The time-scales are however different for each process, and they should not be expected to be intimately linked. It should also be noted that interpretation of the origin of the light δ^{15}N is complicated by the potential utilisation of more than one nitrogen source (N_2, NH_4^+ and DON), the assimilation rates of which may be highly variable. The decrease in δ^{15}N from +3.8‰ to −0.1‰ between days 1 and 6 equates to the fixation of 1.27 μmol l^{-1} of N_2 which approximates to 212 nmol-N L^{-1} d^{-1} so that the mean fixation rate determined over this period can account for approximately 70% of the observed change in δ^{15}N. However the decrease in δ^{15}N was not temporally uniform, but resulted from an abrupt change (Δ 3‰) between days 3 and 4.

[15] Our data confirm previous observations that diazotrophic organisms are active within the Eastern Mediterranean Sea, and that nitrogen fixation contributes a significant component to the δ^{15}N-PON. This study was performed within a warm-core eddy, and so the observed rates cannot be extrapolated to the entire E. Mediterranean with confidence, due to potential differences in the microbiology in the isolated surface waters of these features. *Groom et al.* [2005] report that remotely sensed chlorophyll-a concentration was lower across the eddy than waters outside, and it is possible that factors such as the deep nutricline, that characterise warm-core eddies, may select for N fixation.

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