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Heterotrophic and autotrophic contribution to dinitrogen fixation in the Gulf of Aqaba

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ABSTRACT: We evaluated the seasonal contribution of heterotrophic and autotrophic diazotrophy to the total dinitrogen (N_2) fixation in the photic zone of a pelagic station in the northern Gulf of Aqaba, Red Sea. N_2 fixation rates were highest during a *Trichodesmium* bloom in winter (0.7 nmol N l^{-1} d^{-1}), decreased 7-fold 1 wk later throughout the upper 200 m (~0.1 nmol N l^{-1} d^{-1}), and were significantly coupled with both primary and bacterial productivity. N_2 fixation rates were generally higher in the upper 200 m (~0.4 nmol N l^{-1} d^{-1}) during the thermally stratified summer and were correlated solely with bacterial productivity. Experimental enrichment of seawater by phosphorus (P) enhanced bacterial productivity and N_2 fixation rates during both seasons by 3- to 5-fold. Moreover, during the stratified season, experimental amendments to seawater applying a combination of the photosynthetic inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea and a mixture of amino acids increased both bacterial productivity and N_2 fixation rates. Our findings from the northern Gulf of Aqaba indicate that in the photic zone, a shift occurs in the diazotrophic community from phototrophic and heterotrophic populations in winter, including the cyanobacteria *Trichodesmium,* to predominantly heterotrophic diazotrophs in summer. These heterotrophic diazotrophs may be both carbon and P limited as illustrated by their response to additions of P and amino acids.

KEY WORDS: Autotrophic diazotrophs · Heterotrophic diazotrophs · Primary productivity · Bacterial productivity · N_2 fixation · Gulf of Aqaba · P limitation

INTRODUCTION

The Gulf of Aqaba, located at the northern tip of the Red Sea, is surrounded by land on 3 sides and is characterized by a thermohaline circulation pattern caused by high evaporation rates (1 cm d^{-1}; Wolf-Vecht et al. 1992, Biton & Gildor 2011). The gulf hydrology is characterized by a strong seasonal variability mainly due to deep winter mixing (>300 m; Labiosa & Arrigo 2003) and strong summer stratification (Manasrah et al. 2007). Stratification enhances oligotrophy, with surface inorganic nutrient concentrations depleted during summer, and nitrogen (N) and phosphorus (P) levels usually close to their detection limits (Fuller et al. 2005, Mackey et al. 2009, Meeder et al. 2012). During winter, deep vertical mixing advects inorganic nutrients from depth to the surface, with P and N reaching ~0.1 and ~2 µM, respectively (Lindell & Post 1995, Meeder et al. 2012).

The picophytoplankton (<2 µm) predominate the phytoplankton populations in this system and are comprised mainly of *Synechococcus, Prochlorococcus*, and picoeukaryotes (Sommer 2000, Mackey et al. 2007, 2009, Iluz et al. 2009). The winter deep mixing as well as sporadic nutrient inputs during summer (e.g. Saharan dust events; Paytan et al. 2009)

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often induce blooms of larger phytoplankton such as diatoms (Lindell & Post 1995, Mackey et al. 2007) and the diazotrophic (dinitrogen-fixing) cyanobacteria *Trichodesmium* spp. (Post et al. 2002).

Pelagic dinitrogen (N_2) fixation by diazotrophs is an important source of new bioavailable N in oligotrophic marine systems, converting N from the otherwise unavailable pool of atmospheric N_2 to ammonia (Falkowski 1997). N_2 fixation occurs in nitrate-depleted surface photic layers of tropical oceans where diazotrophic phototrophs such as *Trichodesmium* spp. (Capone et al. 2005), unicellular cyanobacteria (Zehr & Kudela 2001, Montoya et al. 2004), and non-photosynthetic diazotrophic bacterioplankton (Halm et al. 2012) are predominantly responsible for this process.

In the Gulf of Aqaba, several groups of diazotrophs have been identified based on their nucleic acid sequences, including the heterotrophic *Alpha-* and *Gammaproteobacteria*, as well as the autotrophic *T. erythraeum* and the unicellular cyanobacterial group A (Foster et al. 2009). Yet, detailed fixation rates are unavailable and the contribution of diazotrophs to new production in the Gulf of Aqaba is still unknown. A study examining the $\delta^{15}N$ signatures of zooplankton in the Gulf of Aqaba concluded that diazotrophs play only a minor role as a source of recently fixed new N to zooplankton nutrition in both winter and summer (Aberle et al. 2010). However, this claim was based on indirect interpretation of isotopic signatures from a higher trophic level. N_2 fixation rates from the Gulf of Aqaba were measured directly in only 2 studies using the $^{15}N_2$ assimilation technique (Foster et al. 2009, Rahav et al. 2013a), while another study measured species-specific rates (based on acetylene reduction) for concentrated *Trichodesmium* colonies using mesh plankton nets (Post et al. 2002). During the stratified summer (September), Foster et al. (2009) measured N_2 fixation rates ranging from undetectable to a maximum of 1.2 nmol N l^{-1} d^{-1}. The highest rates measured by Foster et al. (2009) were during March (1.9 nmol N l^{-1} d^{-1}), when the water column was mixed.

Nutrient availability, especially P and iron, regulates the distribution and abundance of diazotrophs, with high requirements for both (e.g. Raven 1988, Berman-Frank et al. 2001, Sañudo-Wilhelmy et al. 2001, Kustka et al. 2003). In the Gulf of Aqaba, large diazotrophs such as *Trichodesmium* can be limited by P while small cells with larger surface area to volume ratios were not as impacted (Mackey et al. 2007). Competition for nutrients is not limited to autotrophic phytoplankton but also occurs between phytoplankton and bacteria, especially in oligotrophic systems, as demonstrated in the ultra-oligotrophic Levantine basin of the Mediterranean Sea (Thingstad et al. 2005). There, heterotrophic bacteria out-competed phytoplankton for P when additions were made to the ambient seawater and P cycling was shunted to the microbial loop (Thingstad et al. 2005). In a situation where N and P (or iron) are limiting, diazotrophs could have an advantage over phytoplankton that cannot access atmospheric N_2. Moreover, heterotrophic diazotrophs, typically small cells (<1 µm) with large surface area to volume ratios, may have a further competitive advantage over larger autotrophic diazotrophs such as *Crocosphaera* or the filamentous *Trichodesmium*. We therefore hypothesized that in the oligotrophic northern Gulf of Aqaba, heterotrophic diazotrophy may also have a competitive advantage over autotrophic diazotrophs and provide new bioavailable N for utilization by the marine food web. Our assumption was also based on the diversity of nitrogenase reductase (*nifH*) genes previously reported in the Gulf of Aqaba (Foster et al. 2009) which included heterotrophic diazotrophs and our recent study showing heterotrophs in both photic and aphotic water of the Gulf of Aqaba (Rahav et al. 2013a, O. Levitan et al. unpubl. data). Moreover, the abundance of cyanobacterial diazotrophs was also limited (Foster et al. 2009, O. Levitan et al. unpubl. data). The filamentous cyanobacterium *Trichodesmium* is found in very low numbers throughout the year and in 1996 increased in numbers averaging ~100 colonies m^3 from March to August (Post et al. 2002). Small *Trichodesmium* blooms have been identified only sporadically (e.g. fall 1997, Post et al. 2002), and in the last few years usually after winter advection and mixing. Thus, in this study, we evaluated the contribution of heterotrophic and autotrophic diazotrophy to the total N_2 fixation from a pelagic station in the northern Gulf of Aqaba in late winter while the water column was mixed and during summer stratification. We also examined the role of organic carbon (C) and P availability in regulating heterotrophic diazotrophy by experimental nutrient enrichments.

MATERIALS AND METHODS

Water samples were collected from the RV 'Rotenberg' at Stn A (29° 28′ N, 34° 55′ E) located at the northern tip of the Gulf of Aqaba during the mixed (March 2010) and during the stratified (September 2010, July 2012) periods. Previous observations of water profiles derived from 5 yr surveys (www.iui-

eilat.ac.il/Research/NMPMeteoData.aspx) show that Stn A accurately characterizes the northern Red Sea waters (see Fig. S1 in the Supplement, available at www.int-res.com/articles/suppl/m522p067_supp.pdf). During March, 2 samplings were carried out. The first sampling took place 3 d after a flood event (flooding occurred on 3 March 2010, and sampling took place on 6 March 2010), and the second sampling 12 d later (18 March, see below for more details).

Samples were collected using 12 l Niskin bottles mounted on a rosette equipped with a CTD (Seabird 19 Plus) and fluorometer (Turner designs, Cyclops7 for real-time chlorophyll *a* [chl *a*] fluorescence). Seawater for all analyses was dispensed into transparent 4.6 l Nalgene incubation bottles. The filled Nalgene bottles were placed in transparent outdoor incubators with continuously flowing surface seawater to maintain ambient surface-water temperatures and irradiance. The incubators were shaded with neutral density screening to simulate *in situ* irradiance conditions obtained using a photosynthetically active radiation detector (Licor LI-1400).

Inorganic nutrients

Water samples were collected in 15 ml acid-washed plastic scintillation vials and kept frozen until they were analyzed ~1 mo later. Nutrients were determined using a segmented flow Skalar SANplus System as described by Kress & Herut (2001). The precision of nitrite + nitrate (NO_2+NO_3), and phosphate (PO_4) measurements were 0.02 µM and 0.003 µM, respectively. The limit of detection (2 times the standard deviation of the blank) was 0.075 µM for NO_2+NO_3 and 0.008 µM for PO_4.

Chl *a* extraction

Duplicate seawater samples were filtered onto glass fiber filters (25 mm Whatman GF/F, ca. 0.7 µm in pore size). The filters were stored at −20°C in a dark box until analysis within 2 to 3 d. Samples were extracted in 5 ml of 90% acetone overnight at 4°C in the dark. Chl *a* concentrations were determined using a Turner Designs (TD-700) fluorometer with a 436 nm excitation filter and a 680 nm emission filter (Holm-Hansen et al. 1965). Blank filters were also stored in 90% acetone under the same conditions as those of the samples. Pure chl *a* (Sigma C6144- from *Anacystis nidulans*) was used to calibrate the measurements.

Trichodesmium abundance

Trichodesmium cell densities were determined after concentrating 10 l of surface seawater from our study site and counting 3 subsamples. *Trichodesmium* cell number and size were estimated using a Sedgewick-Rafter Cell (S50) and a light microscope (Nikon Eclipse 80i equipped with DXM 1200F Nikon camera) using 40× magnification.

N_2 fixation rates

N_2 fixation rates were measured on field samples using the $^{15}N_2$ assimilation technique described by Montoya et al. (1996) and Mulholland et al. (2006). Water was added to 4.6 l polycarbonate Nalgene bottles that were sealed with septum tops and spiked with 9 ml of $^{15}N_2$ (98%). Bottles were incubated for 24 h under ambient surface seawater temperatures and covered with neutral density screening as described above. To terminate the incubations, water was filtered onto pre-combusted 25 mm GF/F filters (450°C for 4 h), and filtered samples were analyzed on a Europa 20/20 mass spectrometer equipped with an automated N and C analyzer preparation module. N_2 fixation was calculated according to Mulholland et al. (2006) using N solubility factors described by Weiss (1970). Most of our samplings were performed during 2010, prior to the implementation of the newly developed enriched seawater method (Mohr et al. 2010). A comparison between the traditional $^{15}N_2$ injection (Montoya et al. 1996) and the modified enriched seawater method (Mohr et al. 2010) was conducted during the July 2012 sampling (Fig. S2 in the Supplement).

Primary productivity

Photosynthetic C fixation rates were estimated by determining the ^{13}C uptake rates (Mulholland & Bernhardt 2005) from the same 4.6 l polycarbonate Nalgene bottles in which ^{15}N uptake was measured. Bottles were amended with highly enriched (99%) $NaH^{13}CO_3$ (Sigma) to obtain 1% of the ambient dissolved inorganic C. Parallel dark bottles (n = 3) taken at each sampling depth were also incubated and subtracted from the light bottles of the same depth to correct for dark C fixation. Incubations were terminated by immediately filtering the entire contents of the incubation bottle onto pre-combusted 25 mm GF/F filters (450°C for 4 h). Filters were stored at −20°C

and then dried and pelleted in tin disks before their analysis using the Europa 20/20 mass spectrometer.

Bacterial productivity rates

Bacterial productivity was estimated using the [4,5-^{3}H]-leucine (Amersham, specific activity: 160 Ci mmol^{-1}) incorporation method and normalized to 24 h (Simon et al. 1992). Briefly, 3 aliquots (1.7 ml each) from each sample were incubated with 100 nmol l^{-1} of [4,5-^{3}H]-leucine for 4 h at ambient room temperature in the dark. Preliminary experiments indicated that this was a saturating level of ^{3}H-leucine and that incorporation was linear during this time (not shown). Triplicate seawater samples immediately amended with trichloroacetic acid (TCA) served as controls. The incubations were terminated with 100 µl of cold (4°C) TCA (100%) followed by the micro-centrifugation protocol (Smith & Azam 1992). After adding 1 ml of scintillation cocktail (Ultima-Gold) to each vial, the samples were counted using a TRI-CARB 2100 TR (Packard) liquid scintillation counter. We used a conversion factor of 3.1 kg C mol^{-1} with an isotope dilution factor of 2.0 to calculate bacterial production (BP; Simon & Azam 1989).

Addition of P

Amendments of orthophosphate (PO_4^{-3}) solution (Sigma) were added during early March (3 d after flood event) and September samplings into 4.6 l polycarbonate Nalgene bottles when $^{15}N_2$ was added, bringing the seawater to a final concentration of 0.5 µM PO_4^{-3}. The incubation times were identical to that of the un-amended controls, and incubations were terminated under the same conditions (see above).

Addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and amino acid mixture

The photosynthetic inhibitor DCMU, dissolved in dimethyl sulfoxide, was added to a final concentration of 50 µM, which is the effective minimal concentration required for photosynthetic inhibition (Clavier & Boucher 1992), in 4.6 l polycarbonate Nalgene bottles along with $^{15}N_2$. Furthermore, a mixture of 20 amino acids (Sigma A9906) was added, bringing the seawater to a final concentration of 500 nM of dissolved organic C (DOC). We hypothesized that the combination of DCMU and amino acids would promote heterotrophy over autotrophy by supplying DOC and dissolved inorganic N sources from the amino acids while suppressing photosynthesis, as DCMU blocks the linear electron flow from PSII to the plastoquinone.

RESULTS

Seasonal changes of productivity in the water column

The upper mixed layer of the study site differed between September 2010 and July 2012 (summer) and March 2010 (winter) (Fig. 1A). In March, the upper 200 m was mixed, whereas the water column

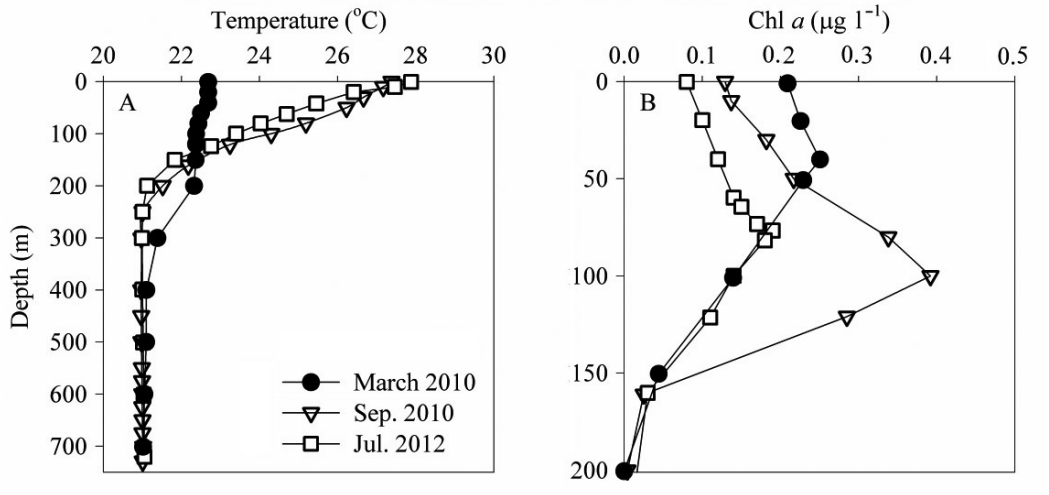

Fig. 1. Depth profiles of (A) temperature and (B) chlorophyll (chl *a*) during the mixed (March 2010) and the stratified (September 2010 and July 2012) periods. Note the different *y*-axis scales

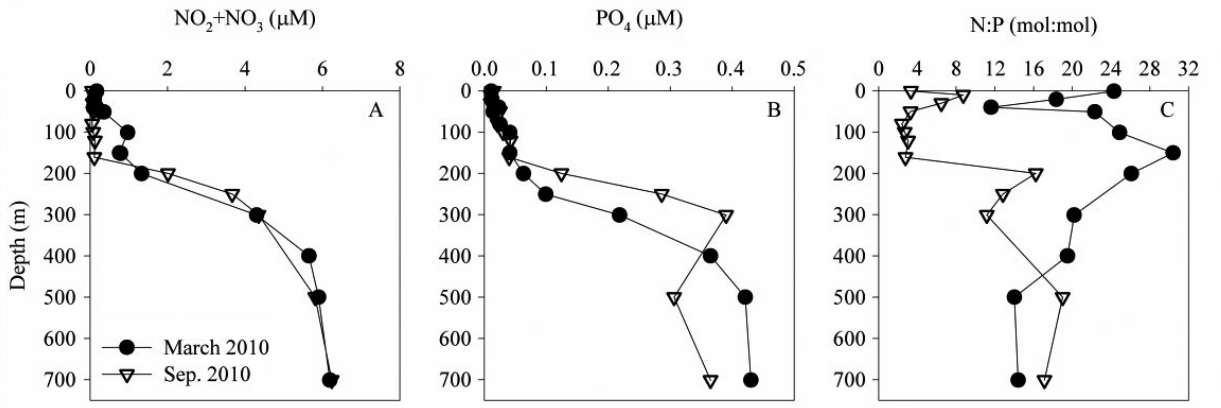

Fig. 2. Depth profiles of (A) nitrate + nitrite, (B) orthophosphate, and (C) N:P (mol:mol) ratio during the mixed (March 2010) and stratified (September 2010) periods. Data from July 2012 are unavailable

was much warmer during summer (July and September) and exhibited full stratification in July (Fig. 1A). The average sea surface temperature was 23°C in March and 27 to 28°C in July and September. At a depth of 400 m, no seasonal temperature effect was observed, and the seawater temperature remained at ~21°C (Fig. 1A).

During our samplings, the highest surface (5–20 m) chl *a* concentration was detected during the winter sampling, with 0.19 µg l^{-1} relative to a concentration of 0.14 µg l^{-1} during stratification (Fig. 1B). The deep chlorophyll maximum (DCM) differed between periods, with the shallowest DCM (~50 m) recorded during March, increasing to 80 m in mid-July, and reaching 100 m at the end of the summer (September). The chl *a* concentration of the DCM in March was 0.25 µg l^{-1}, while during summer it varied from 0.19 µg l^{-1} in June to 0.33 µg l^{-1} in September (Fig. 1B).

Inorganic nutrient concentrations in the upper 50 m were low in March, averaging ~0.15 µM and 0.01 µM for NO_2 + NO_3 and PO_4, respectively (Fig. 2A,B). During summer, NO_2 + NO_3 were slightly above the detection limit in the upper 160 m (0.1 µM), whereas PO_4 was were negligible in the upper 50 m (0.01 µM; Fig. 2A,B). The maximal nutrient concentrations were found below 200 m at all samplings (Fig. 2A,B). During March, the N:P ratio (mol:mol) was higher (~20:1) than the conventional 16:1 Redfield ratio (Redfield et al. 1963), except at 40 m (12:1), while during summer, the N:P was lower than 16:1 in the upper 200 m (~2 to 8:1; Fig. 2C).

BP rates were uniformly low throughout the upper 200 m in March (0.2–0.8 µg C l^{-1} d^{-1}), whereas higher BP rates were usually obtained during July and September (1.2–3.7 µg C l^{-1} d^{-1}), excluding the upper 10 m in July 2012 (Table 1). Surface primary productivity (PP) ranged from 2.4 to 3.1 µg C l^{-1} d^{-1} in March, whereas during the summer months PP was lower, ranging from 0.3 to 0.6 µg C l^{-1} d^{-1} (Table 1). PP declined with depth during both the mixed and stratified periods, with non-detectable rates at 100 m and 200 m, respectively, where <0.1% of surface irradiance was measured (Table 1).

The BP:PP ratio provides a measure of the metabolic status of the environment, i.e. whether the environment is predominantly heterotrophic or autotrophic (Lagaria et al. 2010, Rahav et al. 2013b). A ratio <1 indicates higher autotrophic fixation of C relative to heterotrophic C fixation, and, conversely, when this ratio is >1, higher heterotrophic to autotrophic production. In this study, the BP:PP ratio in March was generally <1, except for depths below the

Table 1. Bacterial and primary productivity rates (BP and PP, respectively) measured at different sampling periods. The values noted are the range, and the number of repetitions (n) is stated in parentheses. DCM: deep chlorophyll maximum

Sampling depth	March 2010 (Mixed period)	July 2012 (Stratified period)	September 2010 (Stratified period)
BP (µg C l^{-1} d^{-1})			
Surface to DCM	0.7–0.8 (6)	0.3 (3)	1.9–3.7 (12)
DCM	0.3–0.4 (6)	1.2–1.8 (3)	2.3–2.9 (3)
Below DCM	0.2–0.4 (6)	2.2–2.3 (3)	1.5–2.3 (6)
PP (µg C l^{-1} d^{-1})			
Surface to DCM	2.4–3.1 (6)	0.6 (3)	0.3–0.6 (6)
DCM	0.7–1.3 (3)	0.2 (3)	0.4 (3)
Below DCM	0–0.4 (9)	0 (3)	0–0.4 (6)

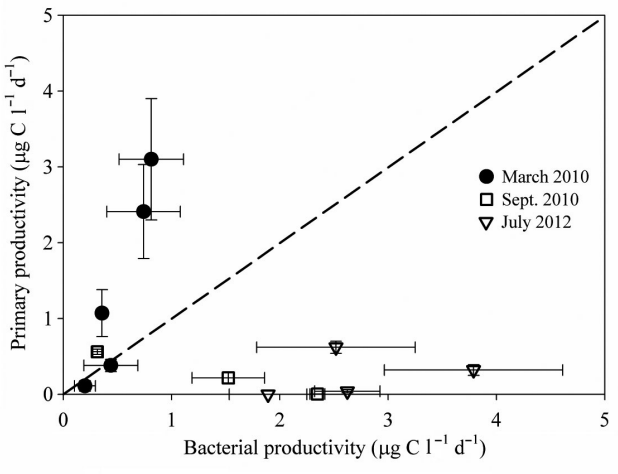

Fig. 3. Relationship between primary productivity versus bacterial productivity from all depths and samplings. The solid line represents a ratio of 1 between the variables, where C is fixed by bacterial and primary productivity equally

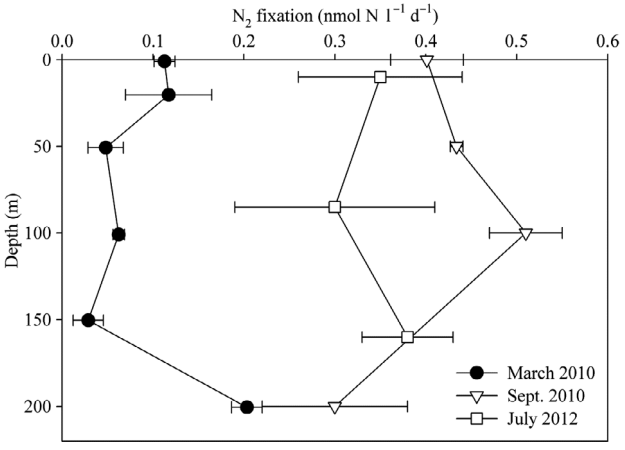

Fig. 4. Depth profiles of dinitrogen (N_2) fixation during the mixed (March 2010) and stratified periods (September 2010 and July 2012)

DCM, where it approximated 1 (Table 1). In both July 2012 and September 2010, the BP:PP ratio was always >1 (Table 1, Fig. 3).

N_2 fixation rates were uniformly low within the upper 150 m in March (~0.1 nmol N l^{-1} d^{-1}), while in the aphotic zone (>200 m) higher N_2 fixation rates were recorded (0.2 nmol N l^{-1} d^{-1}, Fig. 4). During summer stratification, surface N_2 fixation rates were ~0.4 nmol N l^{-1} d^{-1} in July, and increased to a maximum of 0.5 nmol N l^{-1} d^{-1} at 100 m depth in September (Fig. 4).

The measured rates of N_2 fixation were examined with the corresponding heterotrophic metabolism (BP) and phototrophic C fixation (PP) (Fig. 5). BP correlated significantly and positively with N_2 fixation for both the winter (R^2 = 0.98, p = 0.003, n = 15) and summer samplings (R^2 = 0.52, p = 0.01, n = 21; Fig. 5A). No correlation with PP was apparent for either the July or the September samplings (Fig. 5A). In March, PP and N_2 fixation were positively (R^2 = 0.84, n = 15), though not significantly, correlated (p = 0.07; Fig. 5B).

Addition of phosphorus

The response of productivity (bacterial and primary) and diazotrophy to amendment of PO_4 (P) was tested on surface (10 m) seawater in March while rel-

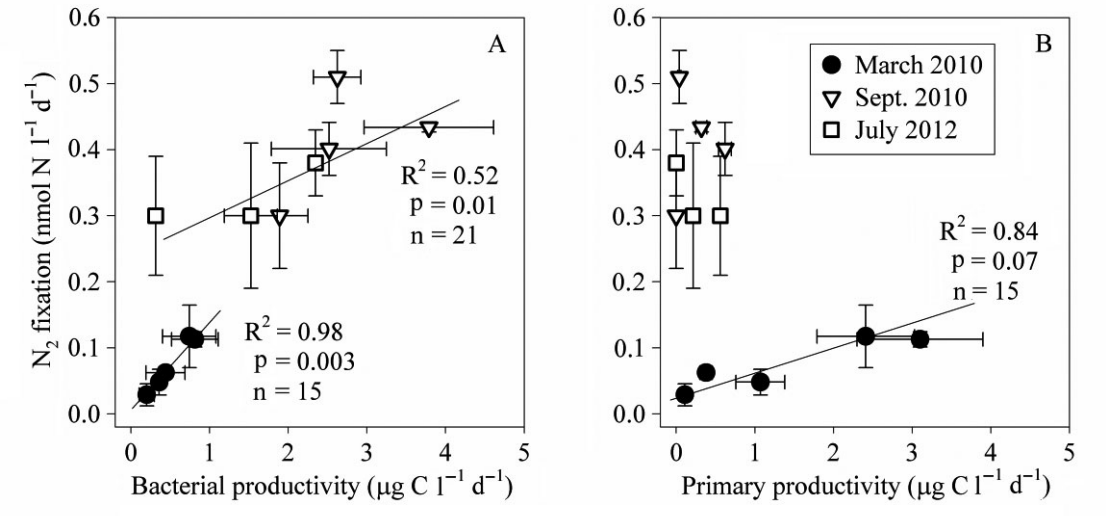

Fig. 5. Relationship between dinitrogen (N_2) fixation and (A) bacterial productivity and (B) primary productivity during the mixed (March 2010) and stratified periods (September 2010 and July 2012). The correlation coefficients are given in the graphs

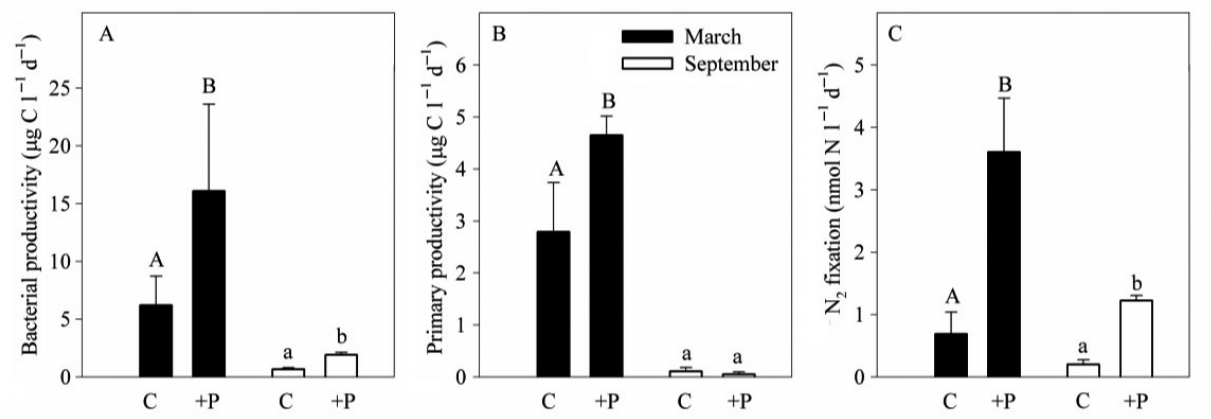

Fig. 6. Effect of phosphorus (P) addition on (A) bacterial productivity, (B) primary productivity, and (C) dinitrogen (N_2) fixation during the mixed (black bars) and stratified periods (white bars). Values are means + SD from 3 independent replicates performed for each control and treatment. The letters above the columns represent statistically significant differences (ANOVA, $p < 0.05$) between mean values for P addition (+P) versus unamended controls (C; no addition)

atively high *Trichodesmium* abundances were observed ($2.3 \pm 2.0 \times 10^3$ trichomes m^{-3}, ~1 wk prior to the detailed depth sampling described here) and again in September when *Trichodesmium* abundance was $<0.1\times 10^3$ trichomes m^{-3}. The initial BP rates were high in March compared with the September sampling (6.0 and 0.70 µg C l^{-1} d^{-1}, respectively; Fig. 6A). During both seasons, a positive significant increase was observed after the addition of P with ~3-fold higher rates in P-amended samples compared with the seawater controls (>16 and 1.9 µg C l^{-1} d^{-1} for March and September respectively, Fig. 6A). Moreover, in March, the PP rates increased significantly in response to the addition of P (from 2.8 to 4.7 µg C l^{-1} d^{-1}; Fig. 6B), while in September the initial PP rates were much lower (~0.1 µg C l^{-1} d^{-1}), and there was no significant change 24 h after P amendment (Fig. 6B). N_2 fixation rates were significantly enhanced by additions of P (ANOVA, $p < 0.05$), with 5-fold higher rates measured in March (3.6 nmol N l^{-1} d^{-1},) and 3-fold higher rates obtained in September (1.2 nmol N l^{-1} d^{-1}; Fig. 6C).

Photoautotrophic versus heterotrophic diazotrophy during July 2012

To examine the physiological contribution of photoautotrophic and heterotrophic diazotrophs during the summer stratification, while fixed N concentrations (NO_3+NO_2) are close to their analytical detection limits (Fig. 2A), we experimentally manipulated the system by the combined addition of the photosynthetic inhibitor DCMU and a mixture of 20 amino acids to water collected from 3 depths within the photic layer (10, 85, and 160 m; Table S1 in the Supplement). The additions resulted in 2- to 8-fold higher rates of BP at all depths sampled (Fig. 7A), whereas the PP rates were reduced by 70 to 80% compared with those of the control treatments (i.e. without any addition; Fig. 7B). The additions also stimulated N_2 fixation rates with a 2- to 4-fold increase in rates at all depths (Fig. 7C). The maximal enhancement of N_2 fixation rates was observed in water from the DCM (85 m), in which N_2 fixation rates increased from 0.3 to 1.2 nmol N l^{-1} d^{-1} (Fig. 7C).

DISCUSSION

Our results highlight the complexity and variability of N_2 fixation in the Gulf of Aqaba with seasonally differential contributions of heterotrophic and autotrophic diazotrophs. The additions of new N via diazotrophy could enhance production in the nutrient-depleted surface waters of the oligotrophic Gulf of Aqaba, especially during the long stratified period (April to November annually). This process may be especially important, as the Gulf of Aqaba lacks other significant external inputs of N via rivers or high precipitation. Diazotrophs also require P for the energetically demanding process of N_2 fixation, with P availability placing further controls on N_2 fixation rates in many marine environments (Sohm et al. 2011, Moore et al. 2013). The oligotrophic Gulf of Aqaba is considered P-limiting for PP during a significant part of the year (Chen et al. 2007). This limitation can be observed by the greater than Redfield (16:1) N:P ratios in the Gulf of Aqaba under 'typical' conditions (i.e. no flood or other inputs; Fig. 2C and Meeder et al. 2012), in the BP:PP ratio >1 during the

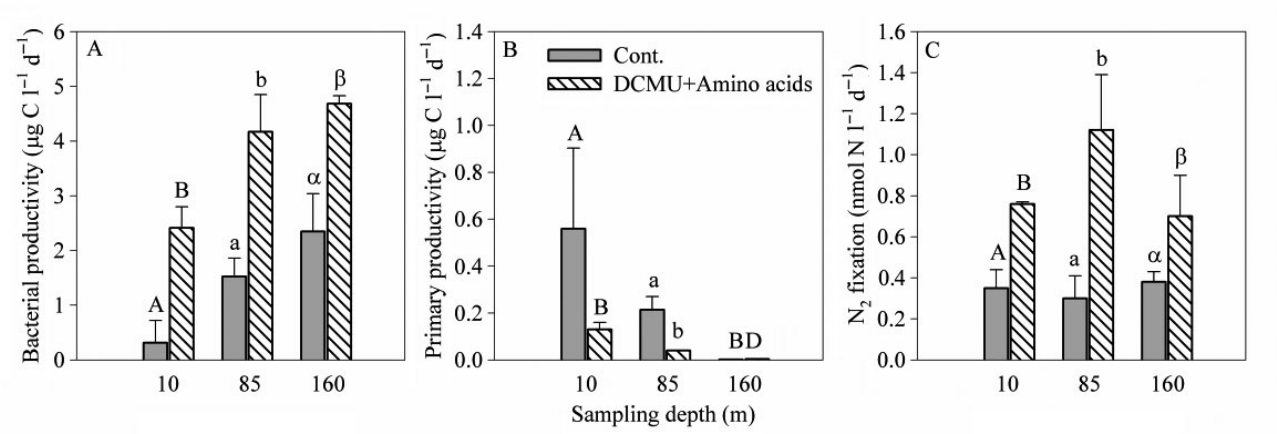

Fig. 7. Effect of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and a mixture of amino acids on (A) bacterial productivity, (B) primary productivity, and (C) dinitrogen (N_2) fixation during the stratified summer period (July 2012). Values are means + SD from 3 independent replicates performed for each control and treatment. The letters above the columns represent statistically significant differences (ANOVA, $p < 0.05$) between mean values for DCMU + amino acid additions and control treatments between depths. For more details, see 'Materials and methods'. BD: below detection

July and September samplings (Fig. 3 and Table 1) indicating a heterotrophic rather than autotrophic metabolic dominance, and in the increased alkaline phosphatase levels of some groups of phytoplankton induced by limited P availability (Mackey et al. 2007) under stratified conditions.

Yet, when seawater samples from different stations in the northern Gulf of Aqaba were amended with PO_4 (0.4 µM) in 2006, N_2 fixation rates were not stimulated (Foster et al. 2009). In contrast, our experimental PO_4 amendments (0.5 µM) significantly enhanced N_2 fixation rates both in early March, when large *Trichodesmium* colonies were counted (see below), and in September (Fig. 6). This response corresponds to findings showing that larger phytoplankton (including *Trichodesmium*) were P-limited in the Gulf of Aqaba, whereas smaller phytoplankton (picophytoplankton) were not (Mackey et al. 2007).

Natural alleviation of P limitation was recorded after the flood at the beginning of March (www.ims.gov.il). Flooding enriched NO_2 + NO_3 and PO_4 concentrations in surface waters and produced a subsequent increase in *Trichodesmium* abundance and corresponding high rates of PP (Fig. 6B) and N_2 fixation (Fig. 6C). With the decline in available P 1 wk after the flood (50% reduction in PO_4 concentrations), *Trichodesmium* numbers substantially declined (>90%), resulting in lower N_2 fixation and PP rates (Fig. 4, Table 1).

As in any natural environment, spatial and temporal variability exists in community composition and diversity as well as in the resulting metabolic processes. Thus, while the ambient P concentrations (<0.05 µM) were similar in both our study and that of Foster et al. (2009), the response of the community to enhanced P concentrations differed between the studies, illustrating the natural variability of community responses to P limitation (see also Mackey et al. 2007) and highlighting the necessity of spatial and temporal sampling approaches to determine both composition and metabolic function.

The differential responses to P availability and limitation may also result from the altered composition of diazotrophs and the ensuing structure of the diazotrophic community. Marine diazotrophy was traditionally linked to autotrophic metabolism and primary productivity attributed to a predominance of cyanobacterial diazotrophs in the photic surface layers (Capone et al. 1997, Gruber, 2008). Newer research has demonstrated increased diversity of diazotrophs (Zehr & Kudela 2011). Our findings show that in the Gulf of Aqaba, the close coupling between autotrophs and diazotrophs is only found during the mixed period and when cyanobacteria such as *Trichodesmium* are abundant in the gulf waters (Fig. 5). Thus, *Trichodesmium* responded to the flood-derived nutrient inputs in March, and N_2 fixation rates increased in parallel with higher BP and PP rates; these were all enhanced further upon addition of P (Table S1, Fig. 6A,B). Yet, we cannot rule out that the correlation of N_2 fixation with both BP and PP during March can also imply that only 1 of these variables (PP or BP) was indeed coupled with N_2 fixation while the other was indirectly related. For example, it is possible that in March only PP was coupled with N_2 fixation, while the increase in BP was related to the enhanced PP and not to diazotrophy per se.

During times of stratification, however, N_2 fixation rates were uncoupled from PP, and addition of external P enhanced only BP (Fig. 6A) and N_2 fixation

(Fig. 6C). We assume that the increase in BP and decline in PP in September was caused by heterotrophic bacteria bypassing and outcompeting primary producers for the inorganic P as was previously shown during the CYCLOPS campaign in the eastern Mediterranean Sea (Thingstad et al. 2005). Moreover, the diazotrophic heterotrophic fraction could utilize this resource and increase N_2 fixation rates, explaining the positive correlation between BP and N_2 fixation and the uncoupling between N_2 fixation and PP (Fig. 5).

Although BP is not diazotroph specific and represents the uptake of a large part of the bacterial community (Sebastián & Gasol 2013), the positive correlation obtained in all sampling periods between N_2 fixation and BP suggests that heterotrophic bacteria were also fixing N_2 (Fig. 5; Rahav et al. 2013a). Moreover, to further understand the contribution of heterotrophy to N_2 fixation during summer, we experimentally provided heterotrophs with a competitive advantage over autotrophs (not only diazotrophs). Our main objective was to inhibit photosynthesis (using DCMU and darkness; see 'Materials and methods') of autotrophic diazotrophs requiring photosynthetically derived energy for N_2 fixation (Postgate 1998). Simultaneously, we stimulated the heterotrophic community by addition of an amino acid mixture that supplies high concentrations of dissolved and particulate organic C and N. The additions resulted in a dramatic decline in PP rates by ~80% at all depths sampled (photic depth; 0–160 m), whereas both BP and N_2 fixation rates were substantially increased (Fig. 7, Table S1). While the elevated concentrations of N sources delivered by the addition of amino acids can theoretically depress N_2 fixation rates, Knapp (2012) showed that N_2 fixation can continue at substantial rates in the presence of as much as 30 µM NO_3 and/or 200 µM NH_4^+. Thus, the amino acid responses further highlight the important role that heterotrophic diazotrophs could play in the Gulf of Aqaba, at least during summer.

Whether contributed via autotrophic or heterotrophic diazotrophs, the total rates of N_2 fixation in the photic layer of Stn A correspond with previous measurements from the Gulf of Aqaba. The mean N_2 fixation rates measured throughout the entire water column during the summer months (July and September) were 2- to 3-fold higher than the rates obtained in March and were similar to reported rates from the Gulf of Aqaba (~0.01 to 0.8 nmol N l^{-1} d^{-1}, Foster et al. 2009, Rahav et al. 2013a) as well as other oligotrophic areas (Mague et al. 1974, Capone et al. 1997, Karl et al. 2002, White et al. 2007, Rahav et al. 2013b,c). The high variability in N_2 fixation rates during March reported by Foster et al. (2009) may imply that they also measured rates under both *Trichodesmium* 'bloom' and 'non-bloom' scenarios or they could reflect inter-annual changes.

The generally low rates of N_2 fixation measured in this system may underestimate actual rates. Our N_2 fixation measurements were carried out mostly during 2010, prior to the publication of the newly modified methodology for measuring N_2 fixation rates (Mohr et al. 2010) that involves the addition of enriched ($^{15}N_2$) seawater rather than adding $^{15}N_2$ as gas bubbles (i.e. 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, Rahav et al. 2013c), yet in long incubations (24 h), the underestimation of N_2 fixation using the bubble method is reduced, as the gas bubbles should have equilibrated within the first several hours of the incubation (Mohr et al. 2010, Mulholland et al. 2012). Moreover, comparison between the 2 methods in July 2012 (surface waters, n = 8) yielded an insignificant (1-way ANOVA, $p > 0.05$) 1.4- to 1.9-fold increase in rates using the enriched seawater method (Fig. S2). Currently, it is impossible to convert between the methods. Yet, even if we assume a 50% underestimation of N_2 fixation rates, we still observe temporal differences in N_2 fixation rates between the studied periods, suggesting that methodological differences alone cannot account for the observed seasonal changes.

Due to the lack of studies on N_2 fixation in the Gulf of Aqaba, it is currently impossible to discuss inter-annual differences and obtain any patterns for longer timescales. Studying this biologically important process in this system, including its aphotic layers (Bonnet et al. 2013, Rahav et al. 2013a), at finer spatial and temporal resolution may provide important data on biogeochemical cycling of N and C in the Gulf of Aqaba and thus might help clarify the interactions and contributions of diazotrophy to the marine food web. Recent data demonstrating expanded niches and roles of heterotrophic diazotrophy globally suggest that N_2 fixation may be severely underestimated in the global budget (Fernandez et al. 2011, Hamersley et al. 2011, Bonnet et al. 2013, Farnelid et al. 2013, Rahav et al. 2013a, Bentzon-Tilia et al. 2014). Subsequent efforts should be undertaken in this field to understand the scope of interactions between heterotrophic and autotrophic diazotrophs. Moreover, according to climate change predictions, the surface oceans in the future will be characterized by a more

stable thermal stratification and exacerbated limitation of inorganic nutrients for primary production. These conditions may provide heterotrophic diazotrophs with a competitive advantage over autotrophic diazotrophs, which could impact community composition and structure as well as the functioning of the biological pump.

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