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Contribution of dinitrogen fixation to bacterial and primary productivity in the Gulf of Aqaba (Red Sea)

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Abstract

We evaluated the seasonal contribution of heterotrophic and autotrophic diazotrophy to the total dinitrogen (N_2) fixation in a representative pelagic station in the northern Gulf of Aqaba in early spring when the water column was mixed and during summer under full thermal stratification. N_2 fixation rates were low during the mixed period ($\sim 0.1\,\mathrm{nmol\,N\,L^{-1}\,d^{-1}}$) and were significantly coupled with both primary and bacterial productivity. During the stratified period N_2 fixation rates were four-fold higher ($\sim 0.4\,\mathrm{nmol\,N\,L^{-1}\,d^{-1}}$) and were significantly correlated solely with bacterial productivity. Furthermore, while experimental enrichment of seawater by phosphorus (P) enhanced bacterial productivity and N_2 fixation rates during both seasons primary productivity was stimulated by P only in the early spring. Metatranscriptomic analyses from the stratified period identified the major diazotrophic contributors as related to heterotrophic prokaryotes from the Euryarchaeota and Desulfobacterales (Deltaproteobacteria) or Chlorobiales (Chlorobia). Moreover, during this season, experimental amendments to seawater applying a combination of the photosynthetic inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and a mixture of amino acids increased both bacterial productivity and N_2 fixation rates. Our findings from the northern Gulf of Aqaba indicate a shift in the diazotrophic community from phototrophic and heterotrophic populations, including small blooms of the cyanobacterium *Trichodesmium*, in winter/early spring, to predominantly heterotrophic diazotrophs in summer that may be both P and carbon limited as the additions of P and amino acids illustrated.

1 Introduction

The Gulf of Aqaba, located at the tip of the Red Sea, is surrounded by land on three sides and characterized by a thermohaline circulation pattern caused by high evaporation rates ($1\,\mathrm{cm\,d^{-1}}$, Wolf-Vecht et al., 1992; Biton and Gildor, 2011). The Gulf hydrology is characterized by a strong seasonal variability mainly due to deep winter mixing

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(> 300 m, Labiosa and Arrigo, 2003) and strong summer stratification (Manasrah et al., 2007). Stratification enhances oligotrophy with surface inorganic nutrients concentrations depleted during summer, with 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 inorganic P and N reaching ~ 0.1 µM and ~ 2 µM, respectively (Lindell and Post, 1995).

The picophytoplankton fraction (< 2 µm) predominates the phytoplankton populations in this system and is 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 events of nutrient inputs during summer (i.e., Saharan dust events, Paytan et al., 2009) often induce blooms of larger phytoplankton such as diatoms (Lindell and Post, 1995; Mackey et al., 2007) and *Trichodesmium* spp. (Post et al., 2002).

Pelagic dinitrogen (N_2) fixation by diazotrophs is an important source of new N in oligotrophic marine systems, converting nitrogen 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 et al., 2001; Montoya et al., 2004), and, also non-photosynthetic diazotrophic bacterioplankton (reviewed in Riemann et al., 2010) are predominantly responsible for this process.

In the Gulf of Aqaba, several groups of diazotrophs have been identified based on their amino acid sequences, including α and γ proteobacteria, as well as *T. erythraeum* and the unicellular cyanobacterial group A (Foster et al., 2009). To our knowledge, only three studies were published on N_2 fixation from the Gulf of Aqaba: one showing actual rates using the $^{15}N_2$ assimilation technique (Foster et al., 2009); another based on indirect measurements of acetylene reduction rates for concentrated *Trichodesmium* spp. colonies (Post et al., 2002); and the third based on isotopic $\delta^{15}N$ estimations (Aberle et al., 2010). During the stratified summer (September), Foster et al. (2009) measured

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N_2 fixation rates ranging from undetectable to 1.9 nmol N L^{-1} d^{-1}. Although estimations based on isotopic analyses suggested that N_2 fixation plays a minor role during winter mixing (Aberle et al., 2010), the highest rates measured (1.9 nmol N L^{-1} d^{-1}) were during March, when the water column was mixed (Foster et al., 2009).

In this study, we evaluated the seasonal contribution of heterotrophic and autotrophic diazotrophy to the total dinitrogen fixation in a representative pelagic station in the northern Gulf of Aqaba in early spring the water column was mixed and during summer under full thermal stratification. We also experimentally examined the role of heterotrophic diazotrophy and the potential of P-limitation on diazotrophs in this system.

2 Materials and methods

Water samples were collected from the R.V. *Rotenberg* at sampling Station A (29°28′ N, 34°55′E) located at the northern tip of the Gulf of Aqaba (Fig. 1) during the mixed winter (March 2010) and the stratified summer periods (September 2010 and July 2012). 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 [Chl *a*] fluorescence). For metatranscriptome analyses samples were taken from surface and 3 depths within the photic layer during September 2010: 60 m, the DCM (approximately 100 m), and 130 m. Fifty L (60 m and DCM samples) or 200 L (130 m sample) of seawater were pre-filtered through 2 µm mesh and 10 L aliquots were vacuum filtered on Supor-400 0.45 µm filters (Pall. Corp.). Filters were immersed into 2 mL PGTX buffer (4.2 M phenol amended with 6.9 % v/v glycerol, 5 mM 8-hydroxyquinoline, 15.6 mM Na_2EDTA, 0.1 M sodium acetate, 0.8 M guanidine thiocyanate and 0.48 M guanidine hydrochloride (Pinto et al., 2009) and immediately frozen in liquid nitrogen. For all other analyses seawater was dispensed into 4.6 L Nalgene incubation bottles. The filled Nalgene bottles were placed in transparent outdoor incubators with continuously flowing seawater to maintain ambient surface-water tem-

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peratures. The incubators were shaded with neutral density screening to mimic in situ irradiance conditions.

2.1 Inorganic nutrients

Duplicate water samples were collected in 15 mL acid-washed plastic scintillation vials and kept frozen. Nutrients were determined using a segmented flow Technicon AutoAnalyser II (AA-II) system as described by Kress and Herut (2001). The precision of nitrite + nitrate ($NO_2 + NO_3$), phosphate (PO_4), and silicic acid ($Si(OH)_4$) measurements were 0.02, 0.003, and 0.06 µM, respectively. The limit of detection (2 times the standard deviation of the blank) was 0.075 µM for $NO_2 + NO_3$, 0.008 µM for PO_4, and 0.03 µM for $Si(OH)_4$.

2.2 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 2–3 days. 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 et al., 1965). A blank filter was 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.

2.3 Dinitrogen (N_2) fixation rates

N_2 fixation rates were measured on field samples using the $^{15}N_2$ assimilation technique described by Montoya (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 $^{15}N_2$ (99 %). Bottles were incubated for 24 h under ambient surface seawater temperatures and covered with neutral density screening as described above. To terminate the

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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 Carbon (C) analyzer preparation module. N_2 fixation was calculated according to Mulholland et al. (2006) using N solubility factors described by Weiss (1970).

2.4 Primary productivity

Photosynthetic C fixation rates were estimated by determining ^{13}C uptake (Mulholland and Bernhardt, 2005). Water samples were placed in clear 4.6 L polycarbonate Nalgene bottles and amended with highly enriched (99 %) $NaH^{13}CO_3$ (Sigma) to obtain 1 % of the ambient dissolved inorganic C and incubated under the same conditions as for ^{15}N incubations described above. Parallel dark bottles (n = 3–5) were also incubated and subtracted from the light bottles to correct for dark C fixation. Incubations were terminated by immediately filtering the entire contents of incubation bottles 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.

2.5 Bacterial productivity rates

Bacterial production was estimated using the ^3H-leucine (Amersham, specific activity: 160 Ci mmol^{-1}) incorporation method (Simon et al., 1992). Triplicate 1.7 mL samples were incubated for 4–8 h at in situ temperatures in the dark. Killed samples of triplicate trichloroacetic acid (Sigma) served as controls. One milliliter of high ^3H affinity (Ultima Gold) scintillation cocktail was added to samples that were subsequently counted using a TRI-CARB 2100 TR, PACKARD scintillation counter.

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2.6 Addition of phosphorus

Amendments of orthophosphate (PO_4) solution (Sigma) were added 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. The incubation times were identical to that of the unamended controls, and incubations were terminated under the same conditions (see above Sect. 2.3).

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

The photosynthetic inhibitor, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), dissolved in dimethyl sulfoxide, was added to a final concentration of 50 µM in 4.6 L polycarbonate Nalgene bottles along with $^{15}N_2$. Clavier and Boucher (1992) reported this as the most effective for photosynthetic inhibition by using a minimal concentration of DCMU. 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 (not necessarily diazotrophs) by supplying DOC and dissolved inorganic nitrogen (DON) sources from the amino acids while suppressing photosynthesis as DCMU blocks the linear electron flow from PSII to the plastoquinone.

2.8 RNA extraction and cDNA library preparation for sequencing

Total cellular RNA was extracted from cells on frozen filters following the hot phenol method (Steglich et al., 2006) and yielded 5–13 µg total RNA for each sample. For cDNA library preparation DNA was removed from total RNA by TURBO™ DNase (Ambion, USA) treatment and resulting RNA was depleted in ribosomal RNA using MICROBExpressTM (Ambion, USA). cDNA was prepared following the manufacturers' recommended protocol. The obtained cDNA libraries of the three depths (60, DCM

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and 130 m – see above) were mixed and analysed on an Illumina HiSeq2000 resulting in 2.3 million paired-ends reads of 100 nt length for each run.

2.9 Bioinformatic analyses

All 6309 protein sequences with a gene name matching "nif" or with the annotation "nitrogenase" were extracted from RefSeq database (www.ncbi.nlm.nih.gov/refseq; query: "(nif[Gene Name]) OR nitrogenase"; date: 28 February 2013). Using TBlastN, sequencing reads matching one of these with an E value $\leq 1 \times 10^{-9}$ were searched and extracted. This set of reads was compared against the NCBI nt database using BlastN at an E-value cut-off of 1×10^{-5} and by BlastX at an E value cut-off of 1×10^{-8}. The phylogenetic distribution was analysed with MEGAN 4.70.4 (Huson et al., 2011).

3 Results

3.1 Seasonal changes of productivity in the water column

The upper mixed layer of the Gulf of Aqaba showed a clear seasonal pattern between the stratified (September 2010 and July 2012) and mixed (March 2010) periods (Fig. 2a). During the latter period, the upper 200 m was relatively mixed, and the water column during summer was well stratified (Fig. 2a). The average sea surface temperature (SST) was 23 °C in March and 28 °C in July. At a depth of 350 m, no seasonal temperature effect was observed, and the seawater temperature remained at ~ 21 °C.

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. 2b). The deep chlorophyll maximum (DCM) value differed between periods, with the shallowest DCM (~ 50 m) recorded during March 2010 increasing to 80 m in mid-July 2012 and reaching 100 m at the end of the stratified period (September 2010). The Chl *a* concentration in the winter DCM was 0.25 µg L^{-1}, while during summer, the Chl *a* concentration in the DCM reached 0.33 µg L^{-1} (Fig. 2b).

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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. 3a, b). During summer (stratified water column), $NO_2 + NO_3$ was close to its detection limit in the upper 160 m (< 0.1 µM), whereas PO_4 was scarce in the upper 50 m (~ 0.01 µM; Fig. 3a, b). The maximal nutrient concentrations were found below 200 m for all samplings (not shown). 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 (N : P of 12 : 1; Fig. 2c), while during summer, the N : P was lower than 16 : 1 in the upper 200 m (~ 2 to 8 : 1; Fig. 2c).

Bacterial productivity (BP) rates were uniformly low throughout the upper 200 m during the mixed period (0.2–0.8 µg C L^{-1} d^{-1}), whereas higher BP rates were usually obtained during the summer samplings (1.2–3.7 µg C L^{-1} d^{-1}), except for the upper 10 m during July 2012 (Table 1). Primary productivity (PP) of the surface waters ranged from 2.4 to 3.1 µg C L^{-1} d^{-1} in March, whereas during the stratified period, 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 ratio between bacterial productivity (BP) and primary productivity (BP : PP) provides a measure of the metabolic status of the environment, i.e., whether the environment is predominantly heterotrophic or autotrophic (Lagaria et al., 2011; Rahav et al., 2013a). A ratio < 1 indicates higher autotrophic fixation of C relative to heterotrophic C fixation, and, conversely, when this ratio is > 1, there is higher heterotrophic production. In this study the BP : PP ratio was usually < 1 during March, except for depths below the DCM, where it was ~ 1 (Table 1). In the stratified periods, the BP : PP ratio was always > 1 (Table 1, Fig. 4).

N_2 fixation rates were uniformly low at the upper 150 m during March 2010 (~ 0.1 nmol N L^{-1} d^{-1}); however, in the aphotic zone (> 200 m), slightly higher N_2 fixation rates were recorded (0.2 nmol N L^{-1} d^{-1}, Fig. 5). During both samplings of the stratified period, N_2 fixation rates for the surface waters were ~ 0.4 nmol N L^{-1} d^{-1} in

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July, and increased to a maximum of 0.5 nmol N L^{-1} d^{-1} at 100 m depth in September 2010 (Fig. 5).

The relationship between N_2 fixation rates from March 2010 (mixed), July 2012 and September 2010 (stratified) was examined with the corresponding heterotrophic metabolism (BP) and phototrophic C fixation (PP) (Fig. 6). BP correlated significantly and positively with N_2 fixation during both the mixed (R^2 = 0.98, P = 0.003, n = 21) and stratified periods (R^2 = 0.52, P = 0.01, n = 21) (Fig. 6a). No correlation with PP was apparent for the stratified periods (Fig. 6a). In March, PP and N_2 fixation were positively (R^2 = 0.84, n = 15), although not significantly (P = 0.07), correlated (Fig. 6b).

To identify active diazotrophs, metatranscriptome analyses were carried out during the fully stratified water column in September 2010. Samples were collected from surface waters, 60 m, the DCM, and 130 m. The latter three, encompassing the depths in which maximum fixation rates were measured, were pooled and subjected to analytical metatranscriptomics. We found mRNA reads from two different groups of prokaryotes related to N_2 fixation, none of which were of cyanobacterial origin (Table S1 in the Supplement). One set of reads was closely related to sequences from the Euryarchaeota, more specifically the Methanosarcinales, which matched the *nifH*, *nifD* and *nifB* sequences of organisms such as *Methanosarcina mazei* strain Gö1 (Fig. 7). The second set of reads was of bacterial origin, indicating the Desulfobacterales (Deltaproteobacteria) or Chlorobiales (Chlorobia). For several of these reads, the top hit in a BlastN search came from the Desulfobacterales but the second hit from the Chlorobiales, or vice versa. For example, the sequence lcl|HWI-ST1172:64:D1FDDACXX:8:1206:19713:16690_1:N:0:ATCGCG (Table S1 in the Supplement) has in a BlastN search as top match a stretch in the genome sequence of a marine sulfate reducer, *Desulfobacterium autotrophicum* HRM2 (Strittmatter et al., 2009) with an *e* value of $4e^{-14}$ (76/95 identical residues, 80 % ID), whereas the hit ranked second is in the genome sequence of *Chlorobium phaeobacteroides* BS1, with an *e* value of $6e^{-12}$ (76/98 identical residues, 78 % ID). Sequences (SAR406) pointing to the green sulfur bacterial phylum, which includes the genus Chlorobium, were

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reported previously from two oceans, based on 16S rRNA libraries prepared from stratified waters and depths of 80 m and 120 m (Gordon and Giovannoni, 1996). The lack of sequences matching 100 % at DNA level and the fact that an identical number of matching nucleotides exists for the two different groups of bacteria found as the top hits in Genbank (in both cases 76 of 101 nt read length), make the algorithmic identification of the exact phylogenetic group impossible. Thus, these bacteria appear to be lacking cultured representatives. Nevertheless, our available evidence points to the Desulfobacterales or Chlorobiales as phylogenetic groups. At the protein level, the identified reads of bacterial origin encode different N_2 fixation proteins, such as the nitrogenase molybdenum-iron protein alpha and beta chains *NifA* and *NifB*, the nitrogenase MoFe cofactor biosynthesis protein *NifE*, or the nitrogenase iron protein *NifH*.

3.2 Addition of phosphorus

The response of productivity (bacterial and primary) and diazotrophy to the addition of PO_4 (P) was tested on surface (10 m) seawater during a natural *Trichodesmium* bloom from early March 2010 ($2.3 \pm 2.0 \times 10^3$ trichomes m^{-3}, 1 week prior to the detailed depth sampling described here and again in September 2010 (Table 2). The initial BP rates were high in March (accompanied by a *Trichodesmium* bloom) compared with the September sampling (6 and 0.7 $\mu g\,C\,L^{-1}\,d^{-1}$, respectively, Fig. 8a). During both seasons, a positive significant increase was observed after the addition of P. In March and September, P enrichment resulted in ~ three-fold increase over the non-amended controls (> 16 $\mu g\,C\,L^{-1}\,d^{-1}$ and 1.9 $\mu g\,C\,L^{-1}\,d^{-1}$ respectively, Fig. 8a). Moreover, during March, the PP rates increased significantly in response to the addition of P (2.8–4.7 $\mu g\,C\,L^{-1}\,d^{-1}$; Fig. 8b), while during September the initial PP rates were much lower (~ 0.1 $\mu g\,C\,L^{-1}\,d^{-1}$) and decreased 24 h after P was amended (although not significantly, Fig. 8b). N_2 fixation during the *Trichodesmium* bloom (March) was ~ 0.7 $nmol\,N\,L^{-1}\,d^{-1}$ at the surface whereas lower rates (0.4 $nmol\,N\,L^{-1}\,d^{-1}$) were observed at the surface during the stratified period (September; Fig. 8c). Enrich-

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ment of the surface (10 m) water with P elevated N_2 fixation rates significantly (ANOVA, $P < 0.05$) on both sampling dates, with five-fold higher rates measured during March (3.6 nmol N L^{-1} d^{-1},) and three-fold higher rates obtained in September (1.2 nmol N L^{-1} d^{-1}, Fig. 8c).

3.3 Photoautotrophic versus heterotrophic diazotrophy during July 2012

To examine the contribution of photoautotrophic and heterotrophic diazotrophs during the summer stratification we experimentally manipulated the system by the combined addition of the photosynthetic inhibitor DCMU and a mixture of 20 amino acids to water from 3 depths within the photic layer (10 m, 85 m, and 160 m) during July 2012 (stratified period; Table 2). The additions resulted in two to eight fold higher rates of BP at all depths sampled (Fig. 9a), whereas the PP rates were reduced by 70–80 % compared with those of the control treatments (i.e., without any addition; Fig. 9b). The additions also stimulated N_2 fixation rates with a 2 to 4 fold increase in rates at all depths (Fig. 9c). The maximal enhancement of N_2 fixation rates was observed in water from the DCM (85 m; Fig. 2c), in which N_2 fixation rates increased from 0.3 nmol N L^{-1} d^{-1} to 1.2 nmol N L^{-1} d^{-1} (Fig. 9c).

4 Discussion

4.1 Autotrophic vs. heterotrophic diazotrophy in the Gulf of Aqaba

Our results from the photic zone of the Gulf of Aqaba demonstrate spatial and temporal variability in the presence and activity of both autotrophic and heterotrophic diazotrophy. The depth distribution of N_2 fixation during March 2010, when the water column was mixed, was coupled with both PP and BP (Fig. 6), while during September 2010 and July 2012; N_2 fixation rates were coupled only with BP and were not correlated with PP (Fig. 6). This suggests that in March, both autotrophic and heterotrophic di-

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azotrophs contributed to N_2 fixation, whereas mostly heterotrophic diazotrophs were responsible for the rates obtained in September 2010 and July 2012. Moreover, these temporal changes were also reflected in the BP : PP ratio throughout the upper 200 m. During March, this ratio was < 1 from the surface to the DCM, indicating more C was fixed through the autotrophic pathway, and > 1 below the DCM suggesting heterotrophy was dominant over autotrophy (Fig. 4 and Table 1). In contrast, during the stratified periods the BP : PP ratio was always > 1 (Fig. 4 and Table 1). Therefore, we assume that, during the stratified period, heterotrophic diazotrophs were primarily responsible for the N_2 fixation rates measured in our incubations.

Earlier molecular analyses (*nifH* based) from Gulf of Aqaba waters clearly showed diverse autotrophic and heterotrophic populations (Foster et al., 2009) including representative sequences of diazotrophs from clusters I, II, and III (Zehr et al., 1998; Zani et al., 2000). Most of the cluster I sequences were similar to *T. erythraeum* nucleotide sequences and were derived from surface and subsurface depths (up to 60 m) from the same sampling station as that in our study (Station A, Foster et al., 2009). Moreover, representative sequences of heterotrophic diazotrophs were found from all locations and depths (up to 100 m) (Foster et al., 2009). Some of the identified bacterial–diazotrophic sequences (Foster et al., 2009) were similar to sequences amplified from field samples from the Eastern Mediterranean Sea (Man-Aharonovich et al., 2007), a system highly dominated by heterotrophic metabolic conditions (Rahav et al., 2013a,b). In the Levantine basin (Eastern Mediterranean Sea), Yogev et al. (2011) reported that ~ 30 % of the N_2 fixation rates obtained were attributed to a fraction of < 1 µm (i.e., bacterioplankton) and that ~ 40 % of the *nifH* transcripts found were from heterotrophic bacteria (Yogev et al., 2011). The presence of heterotrophic diazotrophs is known from photic zones in other oceanic environments (Fuhrman and Capone, 2001; Zehr et al., 2001; Short et al., 2004). Oligotrophic environments and/or surface oceanic layers may likely be dominated by small bacterial diazotrophs. This assumption is supported by the significantly positive coupling we found between N_2 fixation and BP rates during the ultraoligotrophic stratified period (Fig. 6). In this study the se-

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quences matching diazotrophs suggested the presence of mainly Methanosarcinales and Desulfobacterales (Deltaproteobacteria) or Chlorobiales Chlorobia (Table S1 in the Supplement).

Although frequently used in molecular ecology, the identification of the capability of an organism or of a population for N_2 fixation based on the presence of sequences matching the *nifH* gene alone may not suffice (Dos Santos et al., 2012). Therefore, we used all 6309 protein sequences from Genbank with the "nif" annotation as part of the gene name or with the "nitrogenase" annotation using TblastN for the initial filtering for possible N_2 fixation-related reads. Indeed, we found reads matching several different N_2 fixation-related genes, such as *nifH*, *nifD*, *nifB* and *nifE*. The reads found mean not only the presence of diazotrophs in our study site but also that these genes were actually expressed. An active diazotrophic group identified from our sequences was the Methanoarchaea, which is consistent with the reported high incidence of Euryarchaea in these waters (Ionescu et al., 2009). The second group could not be clearly identified but the available evidence points to unidentified bacteria, possibly related to the Chlorobiales or Desulfobacterales. Quantitatively, the read numbers were similar to the number of hits found for the *amoA* gene, another gene involved in the cycling of nitrogen through the microbial loop but unrelated to N_2 fixation.

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 dark, see Sect. 2.7) of autotrophic diazotrophs requiring photosynthetically derived energy for N_2 fixation (Postgate, 1998). Simultaneously, we stimulated the heterotrophic community using an amino acid mixture amendment, which supplies high dissolved and particulate organic carbon concentrations (DOC and POC respectively). The additions resulted in a dramatic decline in PP rates by ~ 80 % in all depths sampled (photic depth 0–160 m), whereas both BP and N_2 fixation rates were substantially increased (Fig. 9 and Table 2). These responses further highlight the important role heterotrophic diazotrophs play in the Gulf of Aqaba, at least during summer. Subse-

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quent efforts should be undertaken in this field to understand the scope of interactions between heterotrophic and autotrophic diazotrophs.

4.2 N_2 fixation during the mixed and stratified periods

The *Trichodesmium* bloom observed in the surface waters of the Gulf of Aqaba at the beginning of March ($2.3 \pm 2.0 \times 10^3$ trichomes m^{-3}) was most likely triggered by flood derived nutrient inputs that occurred several days earlier. This event enriched the upper layer with $NO_2 + NO_3$ and PO_4 (0.29 µM and 0.05 µM, respectively). We thus assume that prior to this event, *Trichodesmium*, was co-limited by N and P, as previously reported (Fennel et al., 2002; Hood et al., 2004). During this sporadic bloom, relatively high rates of N_2 fixation were measured (Fig. 9c), which were accompanied by high PP rates (Fig. 8b). This coupling between PP and N_2 fixation suggests a tight connection to autotrophic metabolism at the time of the bloom. Previous studies conducted in the Gulf of Aqaba reported that the majority of *Trichodesmium* species were found between 20–90 m (Post et al., 2002; Foster et al., 2009). While we did not sample the full water column we assume that the conditions inducing the surface bloom of *Trichodesmium* (i.e., flood-derived nutrients) would enhance N_2 fixation throughout the photic layer and integrated rates would therefore be high during this mixing event.

After the *Trichodesmium* bloom crashed ($0.1 \pm 0.1 \times 10^3$ trichomes m^{-3}, one week later), the surface N_2 fixation rates decreased by 85 % from 0.7 nmol N L^{-1} d^{-1} (Fig. 8c) to 0.11 nmol N L^{-1} d^{-1} (Fig. 5), and the PP rates decreased by 33 % from 3.1 µg C L^{-1} d^{-1} (Fig. 8b) to 2.8 µg C L^{-1} d^{-1} (Table 1). These measured background levels (i.e., no *Trichodesmium* bloom scenario) were in agreement with previous measurements from the Gulf of Aqaba during March. Foster et al. (2009) measured N_2 fixation ranging from ~ 0.01 to 0.8 nmol N L^{-1} d^{-1} during March 2007, and Iluz (personal communication, 2011) measured PP of ~ 2 µg C L^{-1} d^{-1} during the same period. Furthermore, after the *Trichodesmium* bloom crashed, the N : P ratios measured throughout the entire water column were higher (~ 20 : 1 than the standard Redfield

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stoichiometric ratios, Fig. 3c), which reflected the contribution of *Trichodesmium* to N_2 fixation during the previous week and utilization of P sources. The high N : P ratios may inhibit N_2 fixation therefore the rates we measured were uniformly low for all depths of the water column (Fig. 5). Alternatively, the high N : P signatures may also reflect the deposition of atmospheric nutrients with elevated N : P ratios (Herut et al., 1999; Moutin et al., 2008). Yet, the occurrence of a *Trichodesmium* bloom a week earlier provides a plausible scenario for recent N_2 fixation driving the high N : P signatures.

During September 2010 (stratified summer), the < 16 : 1 N : P Redfield stoichiometric ratio observed in the surface waters and at 200 m (Fig. 3c), combined with the low total inorganic nitrogen concentrations (Fig. 3a), suggested potentially N-limiting conditions that would provide a competitive advantage to diazotrophs (Gruber, 2008). The mean N_2 fixation rates measured throughout the entire water column were 2–3 fold higher than N_2 fixation rates obtained during March (Fig. 5) and were similar to previously reported rates from the Gulf of Aqaba (0.1–1.2 nmol N L^{-1} d^{-1}, Foster et al., 2009) and other oligotrophic areas characterized by seasonal thermal stratification (Mague et al., 1974; Karl et al., 2002; White et al., 2007). In such areas where DIN availability is limited in the upper surface waters, biological N_2 fixation can contribute considerably to the system (Sohm et al., 2011; Moore et al., 2013).

Our N_2 fixation measurements were carried out mostly during 2010, prior to the publication of the newly modified methodology measuring N_2 fixation rates (i.e., rates by Mohr et al., 2010) that involves the addition of enriched ($^{15}N_2$) seawater rather than adding $^{15}N_2$ as gas bubble (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., 2013b), yet in long incubations (24 h), the underestimation of N_2 fixation using the bubble method is reduced because the gas bubble should have equilibrated within the first several hours of the incubation (Mohr et al., 2010; Mulholland et al., 2012). Moreover, comparison between the methods during July 2012 (surface waters, $n = 8$) yielded an insignificant (one-way ANOVA, $P > 0.05$) increase of 1.4–1.9 fold in rates using the enriched sea-

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water method. 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 stratified and mixed periods. This suggests that methodological differences alone cannot account for the changes we observed.

4.3 Is N_2 fixation in the Gulf of Aqaba limited by PO_4 availability?

Phosphorus availability places further controls on N_2 fixation rates (Sohm et al., 2011). The oligotrophic Gulf of Aqaba is considered P-limited for primary production during most months (Chen et al., 2007), yet amending seawater with PO_4 (0.4 µM) during earlier seasonal samplings in 2006 showed no measurable effects on N_2 fixation rates at any of the sampling dates (Foster et al., 2009).

In contrast with the earlier findings (Foster et al., 2009); our experimental PO_4 amendments (0.5 µM) in 2010 and 2012 significantly enhanced N_2 fixation rates both during the *Trichodesmium* bloom in early March and under the fully stratified conditions in September (Fig. 8). However, while during the *Trichodesmium* bloom the increase in N_2 fixation rates was reflected both in enhanced BP and PP rates (Table 2, Fig. 7a, b), in September the added P did not enhance PP but only BP (Fig. 8).

We assume that the increase in BP and decline in PP was caused by heterotrophic bacteria bypassing and outcompeting primary producers for the additional 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. Thus the positive correlation between BP and N_2 fixation and the uncoupling between N_2 fixation and PP (Fig. 6, Table 2).

As in any natural dynamic environment spatial and temporal modifications exist in community composition and diversity as well as in resulting metabolic processes. Thus, while the ambient P concentrations (< 0.05 µM) were similar in both our study and that of Foster (2009) – the response of the community to enhanced P concentrations differed between the studies thus illustrating the natural variability of community re-

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sponses to P limitation (see also Mackey et al., 2007) and highlighting the necessity of spatial and temporal sampling approaches.

5 Conclusions

Our multiple analyses provide evidence that significant rates of N_2 fixation occur in the Gulf of Aqaba during both the mixed and stratified periods. During early spring (March), PP, BP, and N_2 fixation were coupled and limited by P, while during the stratified period (July and September), N_2 fixation was uncoupled from PP and tightly coupled to BP. This finding suggests that during winter and early spring the diazotrophic community comprises both autotrophic and heterotrophic diazotrophs including small blooms of *Trichodesmium* that can exploit nutrient inputs from winter storms and deep mixing, while during summer heterotrophic diazotrophs are the major contributors to this process and that they may be both P and carbon limited as the additions of P and amino acids illustrated.

Supplementary material related to this article is available online at: http://www.biogeosciences-discuss.net/10/10327/2013/bgd-10-10327-2013-supplement.pdf.

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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.

	Sampling depth	Mar 2010 (mixed period)	Jul 2012 (stratified period)	Sep 2010 (stratified period)
BP ($\mu g\,C\,L^{-1}\,d^{-1}$)	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 ($\mu g\,C\,L^{-1}\,d^{-1}$)	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)

DCM = deep chlorophyll maximum.

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Table 2. Results of the statistical comparison (ANOVA and Fischer's test) between the different experimental simulations (control, +P, +DCMU, and amino acids) at the end of the experiment for bacterial and primary production rates and dinitrogen fixation (BP, PP, and N_2 fixation). NS: the difference between the two treatments is not statistically significant; S: the difference between two treatments is statistically significant ($P < 0.05$).

	Sampling date	Thermal stability	Sampling depth	BP	PP	N_2 fixation
P vs. control	Mar 2010	mixed	10	S	S	S
	Sep 2010	stratified	10	S	NS	S
DCMU + amino-acids vs. control	July 2012	stratified	10	S	S	S
			85	S	S	S
			160	S	NS	S

P = phosphorus; DCMU =3-(3,4-dichlorophenyl)-1,1-dimethylurea

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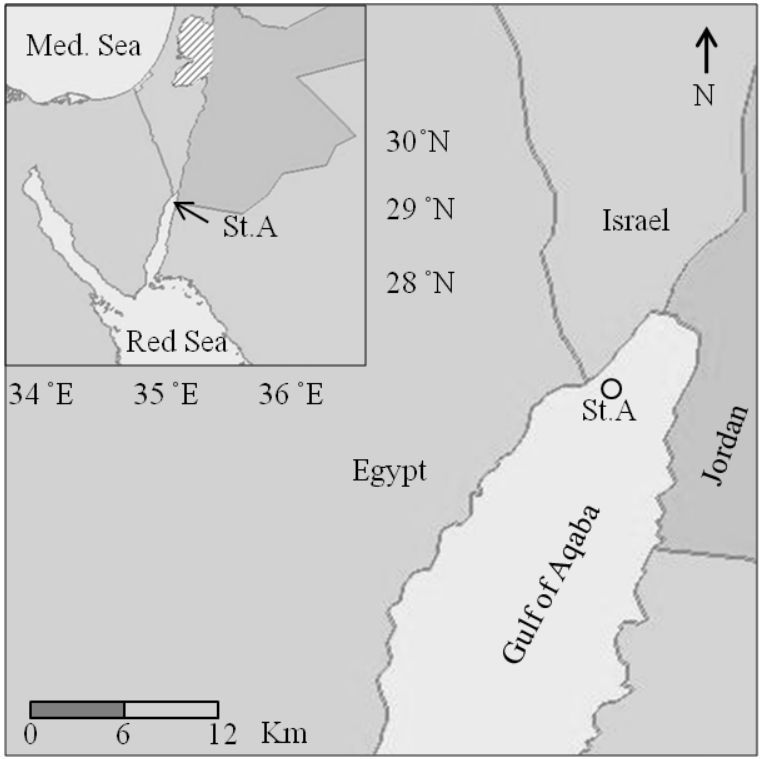

Fig. 1. Map of the Gulf of Aqaba and the sampling location.

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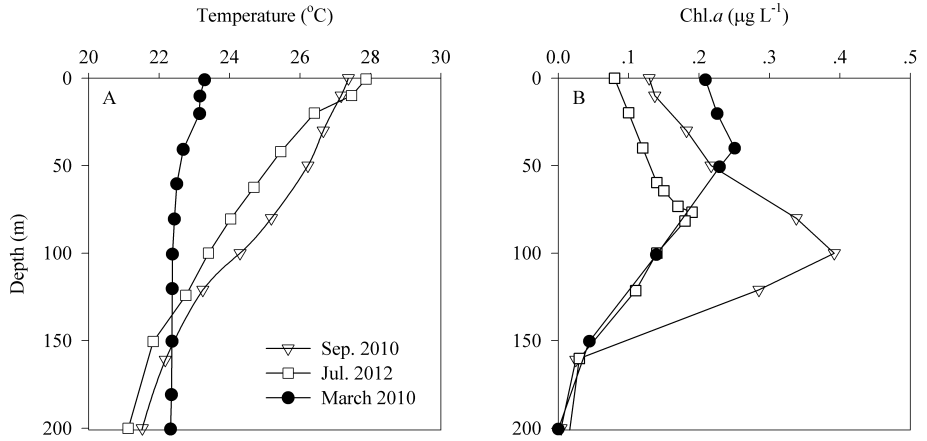

Fig. 2. Depth profiles of temperature **(A)** and Chl *a* **(B)** during the mixed (March 2010, black circles) and the stratified (September 2010 and July 2012, white triangles and white squares, respectively) periods.

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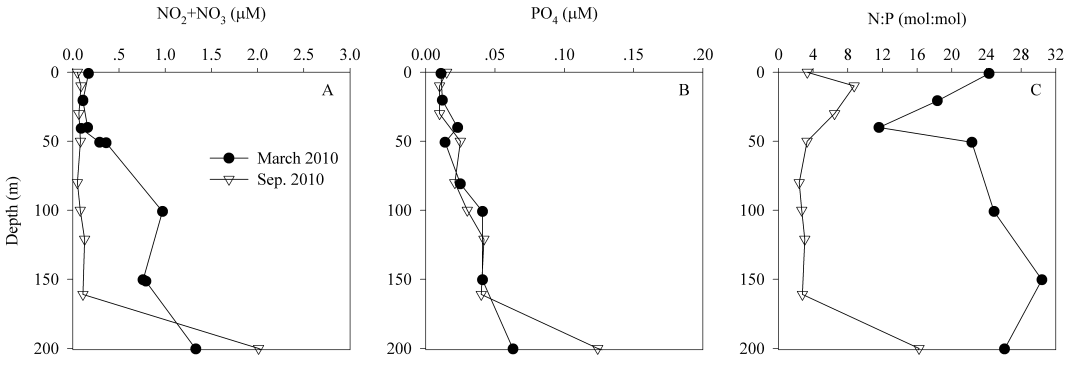

Fig. 3. Depth profiles of nitrate + nitrite **(A)**, orthophosphate **(B)**, and N : P (mol : mol) ratio during the mixed (March 2010, black circles) and stratified (September 2010, white triangles) periods. Data from July 2012 are unavailable.

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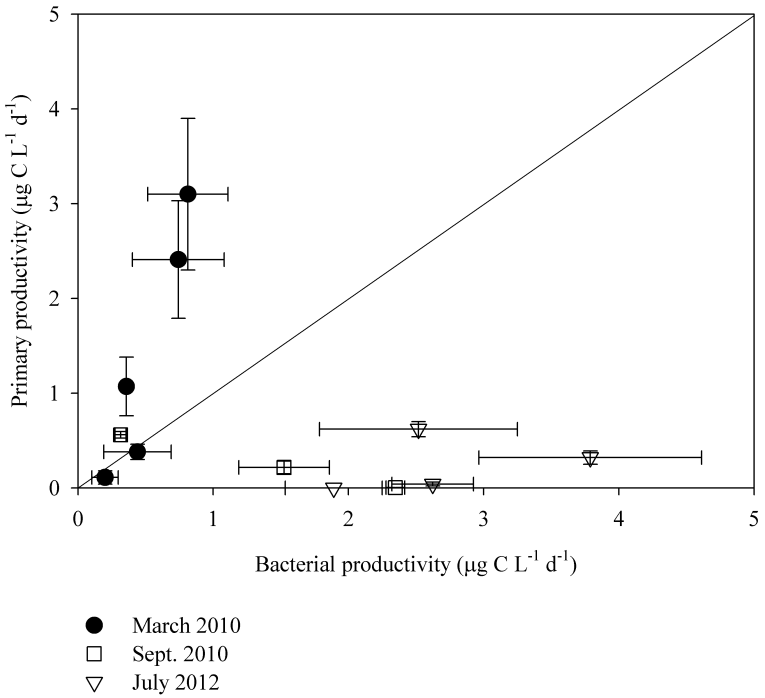

Fig. 4. Relationship between primary productivity versus bacterial productivity from all depths and samplings. The solid line represents a ratio of 1 between the variables which means that C is fixed by bacteria and primary productivity equally.

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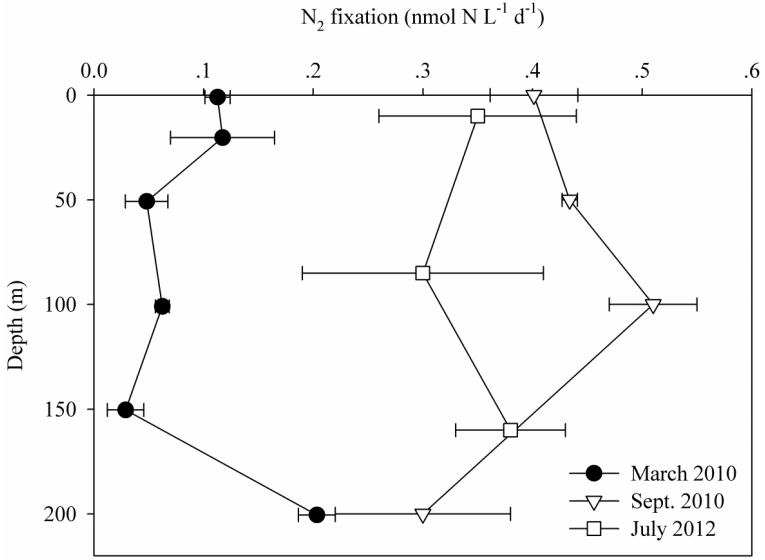

Fig. 5. Depth profiles of dinitrogen (N_2) fixation during the mixed (March 2010, black circles) and stratified periods (September 2010 and July 2012, white triangles and white squares, respectively).

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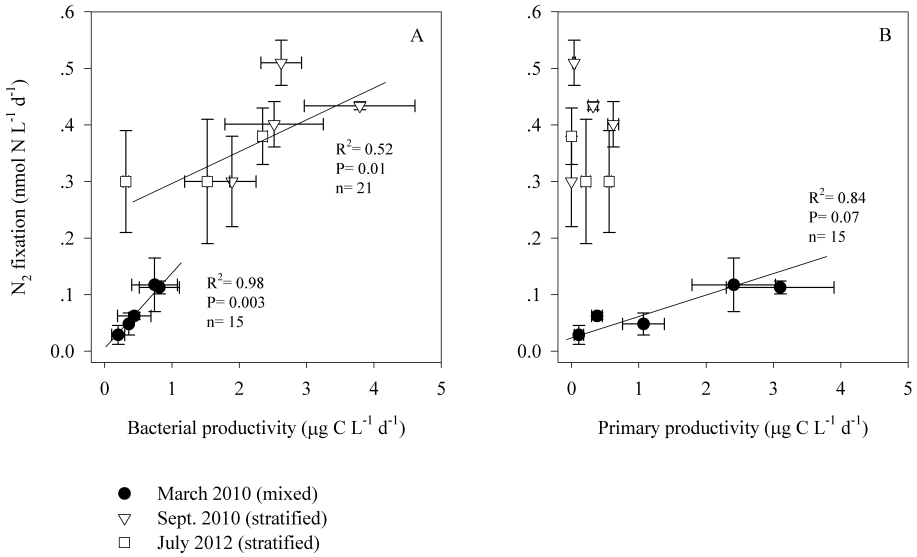

Fig. 6. Relationship between dinitrogen (N_2) fixation; bacterial productivity **(A)**; and primary productivity **(B)** during the mixed (March 2010, black circles) and stratified periods (September 2010 and July 2012, white triangle and white squares, respectively). The correlation coefficient is stated in the graph.

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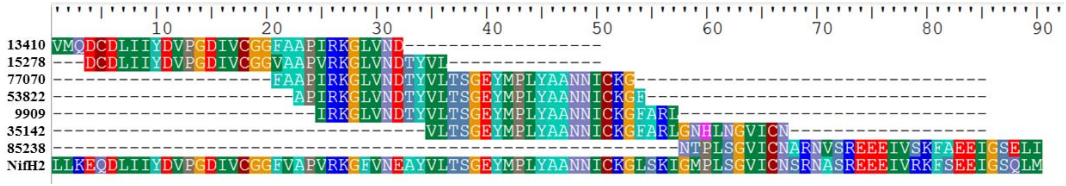

Fig. 7. Multiple alignments of selected transcriptome sequences translated to protein and compared to positions 115–204 of the *nifH2* sequence of *Methanosarcina mazei* strain Gö1. The read IDs are given to the left (compare also Table S1 in the Supplement).

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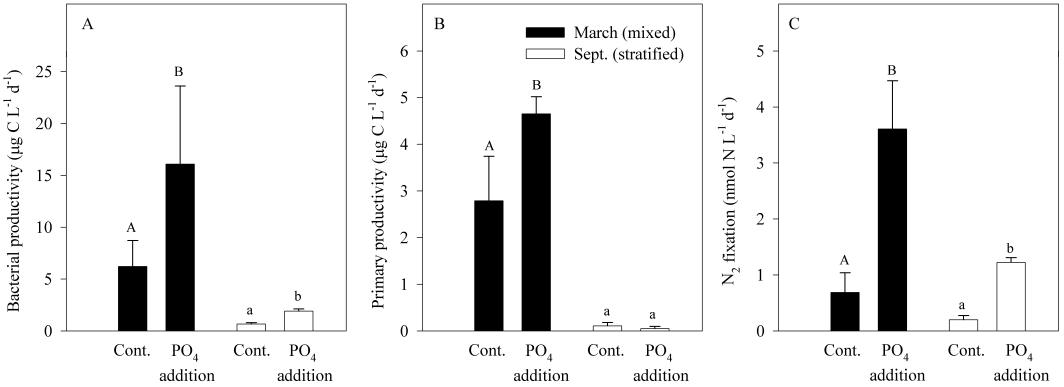

Fig. 8. The effect of phosphorus (P) addition on bacterial productivity **(A)**, primary productivity **(B)**, and N_2 fixation **(C)** during the mixed (black bars) and stratified periods (white bars). Values are averages and error bars are standard deviations from 3 independent replicates performed for each control and treatment. The letters above the columns represent statistically significant differences (ANOVA, $P < 0.05$) for mean values of P additions. Cont. = control (no addition).

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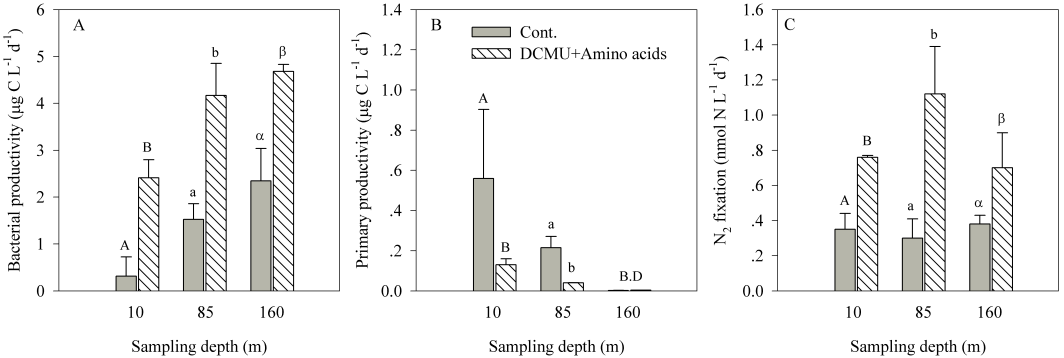

Fig. 9. The effect of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and a mixture of amino acids on bacterial productivity **(A)**, primary productivity **(B)**, and N_2 fixation **(C)** during the stratified summer period (July 2012). Gray bars represent the control, non-amended treatments and striped bars represent the additions. Values are averages and error bars ar standard deviations from 3 independent replicates performed for each control and treatment. The letters above the columns represent statistically significant differences (ANOVA, $P < 0.05$) for mean values of DCMU and amino acid additions between depths. For more details, see the Material and Methods section. Cont., control (no addition), B.D., below detection.

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