Limnol. Oceanogr., 54(1), 2009, 219–233

Seasonality of N_2 fixation and *nifH* gene diversity in the Gulf of Aqaba (Red Sea)

R. A. Foster,[1] *A. Paytan, and J. P. Zehr*
Department of Ocean Sciences, University of California, 1156 High Street, Santa Cruz, California 95064

Abstract

Nitrogen (N_2) fixation rates were determined on bulk water collected in the Gulf of Aqaba, Red Sea, during fall (stratified, oligotrophic) and spring (deep mixing, mesotrophic) seasons. N_2 fixation rates were low yet consistently measurable in both seasons, and maximum rates of 1.0 ± 0.1 nmol N L^{-1} d^{-1} and 1.9 ± 0.2 nmol N L^{-1} d^{-1} occurred in the fall and spring, respectively. Amendment with inorganic phosphate did not always increase rates. Rates were uninfluenced by dissolved organic phosphorus treatments. The highest rate (2.1 nmol N L^{-1} d^{-1}) was measured in spring, when surface seawater was amended with an aerosol dust filter. The *nifH* deoxyribonucleic acid (DNA) gene diversity was analyzed in surface and depth profile samples. Most (58) *nifH* sequences were similar to the previously identified cluster I (containing sequences from proteobacteria and cyanobacteria) sequences. A small subset of sequences (11) was most similar (>96.5) to *nifH* nucleotide sequences of cyanobacteria, including *Trichodesmium* spp. and the unicellular cyanobacterial group A. Sequences similar to cluster III (which contains sequences from many anaerobes) lineages were only retrieved from fall libraries. Quantitative polymerase chain reaction (qPCR) assays were used to estimate the *nifH* abundance for select phylotypes. *Trichodesmium* and a γ proteobacteria had maximum abundances at 60-m depth in the water column in fall 2005 (1.4×10^5 and 2.3×10^2 *nifH* gene copies L^{-1}, respectively). Group A was undetected in all samples, and *Trichodesmium* and γ proteobacteria were undetected in the spring 2007. The *nifH* transcription as determined by quantitative reverse-transcription PCR (qRT-PCR) assays was at the detection limit (1–10 transcripts per reaction) for all phylotypes. This study is the first assessment of *nifH* diversity and rates of $^{15}N_2$ fixation in the Gulf of Aqaba.

There has recently been increased interest in the marine nitrogen cycle, in particular, the diversity, distribution, and activity of nitrogen (N_2)–fixing microorganisms. However, N_2 fixation rates have not been measured in many areas of the world's ocean. Studies of the diversity and distribution of marine diazotrophs have largely focused on the open-ocean subtropical and tropical gyres. Here, we report on N_2 fixation rates and the diversity of N_2-fixing microorganisms in the Gulf of Aqaba.

The Gulf of Aqaba is a semienclosed body of water located in the northeastern extension of the Red Sea. It is 165 km long and approximately 15 km wide and is separated from the northern Red Sea by a shallow sill (240 m) at the Straits of Tiran. Concentrations of inorganic nutrients in the surface waters of the Gulf are oligotrophic (phosphate and nitrate rarely exceed 0.1 and 1 μmol L^{-1}, respectively; Fuller et al. 2005), and a shallow but stable thermocline is present for most of the year, except during the winter, when winds drive convective mixing of deep, higher-nutrient water to the surface. Fluvial nutrient inputs are minimal since the gulf is bounded by desert. Chlorophyll *a* (Chl *a*) concentrations are lowest during the summer and fall stratification period, and phytoplankton populations during these seasons are composed mostly of *Synechococcus* and *Prochlorococcus*, respectively (Lindell and Post 1995). During the winter mixing period, the abundance of picoeukaryotes and larger phytoplankton (i.e., diatoms and the dinoflagellate *Ceratium* sp.) increase, and Chl *a* concentrations reach an annual maximum (Genin et al. 1995).

In other regions of the world's oceans where similar oligotrophic conditions persist, diazotrophs can be abundant, contribute significantly to primary production, and provide a source of new nitrogen to the euphotic zone (Capone et al. 1997; Karl et al. 2002). Several groups of diazotrophs have been identified in the Gulf of Aqaba, including the colonial nonheterocystous cyanobacterium *Trichodesmium* spp., the unicellular cyanobacterium *Synechocystis* spp. (*Cyanothece* spp.), and unicellular and heterocystous cyanobionts of dinoflagellates and diatoms, respectively (Kimor and Golansky 1977; Gordon et al. 1994; Post et al. 2002). Although sporadic occurrences of these diazotrophic groups have been observed by microscopy during summer stratification, and episodic blooms have been reported for *Trichodesmium* spp., to our knowledge there has been only one report of N_2 fixation rates in the Gulf of Aqaba, which was based on acetylene reduction rate measurements for concentrated *Trichodesmium* spp. colonies (Post et al. 2002).

[1] Corresponding author (rfoster@ucsc.edu).

Acknowledgments

We thank the captains and crews of the R/V *Malkat Shva* (2005, 2007) for their assistance in the field collections. We thank our colleagues at the Interuniversity Institute for Marine Science in Eilat, Israel, for assisting in data collection and providing laboratory space and equipment during the study. Specifically, we thank Asaph Rivlin for assistance in sampling, Tania Rivlin for processing nutrient analysis, and Anton F. Post for insight about phytoplankton ecology in the Gulf of Aqaba. We also thank K. R. M. Mackey for her help with the chlorophyll *a* analysis. R.A.F. also thanks Tom R. Cole for his support during this research. The authors also appreciate the comments and helpful suggestions of two anonymous reviewers.

This research was supported by grants from the National Aeronautics and Space Administration (NASA) New Investigator Program NAG5-12663, the North Atlantic Treaty Organization (NATO) Science for Peace Program SfP 982161 grant to A. Paytan, and a Gordon and Betty Moore Foundation Marine Microbial Initiative award to J. P. Zehr.

Recently, rates of N_2 fixation by small (<10 μm) diazotrophs have been reported in the open ocean. These rates can equal or, in some areas, exceed those reported for the larger colonies and trichomes of *Trichodesmium* spp. (Montoya et al. 2004; Zehr et al. 2007). Molecular genetic analyses have shown that there are several other groups of nitrogenase genes (*nifH*) in addition to those of *Trichodesmium* spp. (Zehr et al. 2001). Thus, we now know that there are several groups of *nifH*-containing organisms in marine open-ocean ecosystems, including several groups of unicellular cyanobacteria (groups A, B, and C), three groups of heterocystous cyanobionts (het-1, het-2, het-3), and also gamma (γ) proteobacteria (Zehr et al. 2001; Church et al. 2005*a*; Foster and Zehr 2006). Since oligotrophic conditions in the Gulf of Aqaba are similar to those environments where these diazotrophic phylotypes have been found, we expected to detect these organisms in the water column of the Gulf.

The primary aim of this study was to identify and determine the relative distribution of the various diazotrophic phylotypes and to measure N_2 fixation rates of natural assemblages of microorganisms in the Gulf of Aqaba. Our field investigations were conducted during peak stratification in two fall seasons, when dissolved iron (Fe) concentrations are typically high (Chase et al. 2006). Iron is important to phytoplankton since cofactors of photosynthesis require Fe (Kustka et al. 2002). Furthermore, Fe is critical for diazotrophs as the nitrogenase enzyme is composed of two proteins with Fe components, and thus the enzyme has a high iron requirement. In addition to Fe, phosphorus (P) is often also considered a limiting nutrient for phytoplankton growth; in particular, N_2 fixation can be significantly reduced under conditions of low Fe and P (Sañudo-Wilhelmy et al. 2001; Karl et al. 2002).

To investigate the degree of limitation with respect to P availability (Fe is plentiful in the gulf), we conducted nutrient addition experiments including additions of dissolved inorganic and organic P and aerosol dust filters. A third sampling to capture different environmental conditions was conducted in early spring of 2007 (March) prior to stratification and coincident with extreme vertical mixing throughout the water column. Surface samples were collected from several coastal stations and from depth profiles at offshore stations to determine the diversity of *nifH*-containing organisms in the Gulf of Aqaba. Quantitative polymerase chain reaction and reverse transcription–polymerase chain reaction assays (qPCR and qRT-PCR) were used to estimate the gene abundance and messenger ribonucleic acid (mRNA) gene expression for major *nifH* gene groups.

Methods

Sample collection for N_2 fixation experiments—Twelve experiments were conducted (1–12) to estimate N_2 fixation rates in the Gulf of Aqaba. Eight experiments were conducted during fall (September 2005 and October 2006) when the water column was stratified, and four experiments were conducted in the early spring (March 2006) when the water column was mixed. A summary of the source water and conditions for the 12 experiments is outlined in Table 1.

Seawater for experiments 1, 5–7, 9, and 12 was collected from the surface (3–5 m) at the respective stations (Table 1) into 20-liter acid-washed and sample water–rinsed carboys (Fig. 1). The seawater was kept in the dark and under ambient temperature conditions until it was dispensed into the 2.5-liter (fall 2005) or 4.25-liter (fall 2006 and spring 2007) incubation bottles. Seawater for experiments 2 and 10 was collected from 20-, 40-, and 80-m depths at Sta. A (Fig. 1) using a conductivity-temperature-depth (CTD) rosette sampler equipped with 10-liter Niskin bottles. The seawater was dispensed directly into 2.5-liter (fall 2005) or 4.25-liter (spring 2007) incubation bottles, which were placed in the shade until returned to the Interuniversity Institute (IUI) for Marine Sciences field station in Eilat (within a few hours). Seawater for experiments 3, 4, 8, and 11 was collected by hand from the pier of the IUI (Fig. 1).

The bottles were immediately placed in a continuously flowing seawater pool upon return to the laboratory at IUI in order to maintain ambient surface-water temperatures. The pool was shaded with neutral density screening to mimic irradiance conditions. The bottles collected from 20-, 40-, and 80-m depth were wrapped in additional screening to mimic 50%, 25%, and 10% incident surface irradiance, respectively.

Experiments were initiated (T_0) with the addition of 1.5 or 3 mL of $^{15}N_2$ gas (98% + $^{15}N_2$, D Chem), where 1.5 mL were added in the fall 2005 incubations and 3 mL were added in the fall 2006 and spring 2007 incubations. All experiments were performed with triplicate incubation bottles, such that three bottles were harvested at the end of the incubation point (incubation length varied for the different experiments) for $^{15}N_2$ uptake analyses (Table 1). The entire contents of the experimental bottle (2.5–4.5 liters) were filtered onto 25-mm-diameter precombusted GF/F filters (Whatman) using gentle filtration and a Gast vacuum pump. The filters were placed in separate petri dishes and desiccated in an oven at 60°C for 12 h. Particulate nitrogen and ^{15}N atom% for each filter were measured using an isotope ratio mass spectrometer (Europa Integra) at the University of California (UC)–Davis Stable Isotope Facility, and fixation rates were calculated according to Montoya et al. (1996) using the recommended solubility constants reported by Hamme and Emerson (2004).

In order to assess if dissolved inorganic phosphorus (DIP) or dissolved organic P (DOP) was potentially limiting nitrogen fixation, amendments of sodium phosphate monobasic (NaH_2PO_4, final concentration: 0.4 μmol L^{-1}) and glycerol phosphate (final concentration: 3.0 μmol L^{-1}), respectively, were added at the time of the $^{15}N_2$ spikes. Inorganic P concentrations were very low (<0.05 μmol L^{-1}) in the Gulf of Aqaba during the fall 2005 and 2007 experiments, and therefore our additions of DIP were a means to assess P limitation on N_2 fixation. In experiment 3, we added DOP, as there is some evidence from genome annotations and growth studies and gene expression analysis suggesting that some marine cyanobac-

Table 1. Summary of $^{15}N_2$ experiments using bulk water collected in the Gulf of Aqaba, Red Sea.

Treatment	Experiment number	Date	Source water	Incubation time (h)	Average rate (nmol N L^{-1} d^{-1})
Depth profiles	2	07 Sep 05	20 m	24	1.2±0.3*
Fall			40 m	24	1.0±0.4
			80 m	24	0.6±0.1*
	10	15 Mar 07	20 m	48	bd–0.8†
Spring			40 m	48	bd–0.8†
			80 m	48	0.4–0.9†
No addition	6	10 Oct 06	I-1	52	0.3±0.5
	7	11 Oct 06	I-3	48	0.8±0.2
	12	19 Mar 07	I-3	48	bd
	1	04 Sep 05	I-6	26	0.1±0.1
	3	08 Oct 06	I-6	48	bd
	9	13 Mar 07	I-6	49	1.9±0.2
	3	08 Sep 05	IUI pier	33.5	0.8±0.1
	6	10 Sep 05	IUI pier	25	1.0±0.1
	8	12 Oct 06	IUI pier	48	bd
	11	17 Mar 07	IUI pier	48	0.5±0.4‡
DOP amendment§	3	8 Sep 05	IUI pier	33.5	0.6±0.1‡
DIP amendment‖	6	10 Oct 06	I-1	52	0.2±0.2
	7	11 Oct 06	I-3	48	1.0±0.2
	5	8 Oct 06	I-6	48	bd
	9	13 Mar 07	I-6	49	0.9±1.6
	4	10 Sep 05	IUI pier	25	1.0±0.1
	8	12 Oct 06	IUI pier	48	bd
	11	17 Mar 07	IUI pier	48	1.4±0.3‡
Dust amendment¶	12	19 Mar 07	I-3	48	0.1±0.2
	9	13 Mar 07	I-6	49	2.1±1.2

* Indicates statistical significance from paired *t*-test ($p<0.08$) or one-way ANOVA (depth profile fall 2005 only).
† Values are given as a range, since only two replicate bottles were incubated.
‡ Indicates statistical significance from paired *t*-test controls ($p<0.05$).
§ Dissolved organic P (DOP) amendment, or 3.0 μmol L^{-1} (final concentration).
‖ Dissolved inorganic phosphorous (DIP) amendment, or 0.4 μmol L^{-1} (final concentration).
¶ Aerosol dust filters were added to incubations as a treatment; trajectory information is described in Methods section.

teria may metabolize DOP (e.g., phosphonate) compounds (Dyhrman and Haley 2005; Dyhrman et al. 2005). Experiments 9 and 11 in spring 2007 were amended with dust filters such that the treatments received approximately 3 mg aerosol dust collected at the IUI during the fall 2006.

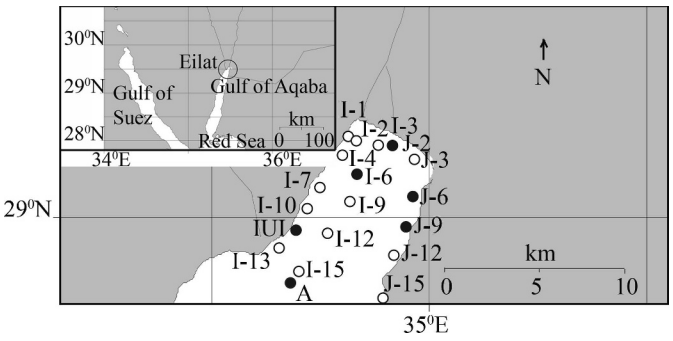

Fig. 1. Map of sampling locations in the Gulf of Aqaba, Red Sea. The locations within the Israeli and Jordanian waters are indicated with I-, and J-, respectively. Filled circles designate samples where *nifH* genes were amplified in fall 2005. The *nifH* genes were recovered from I-3 and Sta. A in these subsequent (more limited) collections (fall 2006, spring 2007). Note that Sta. B is not shown on the map, since it is located further south at 29°22.608′N, 34°53.894′E.

For each experiment, a T_0 (initial) and an end time (final) samples were filtered where the three replicate T_0 samples served as an estimate for natural abundance. These T_0 replicates were run for each experiment and in parallel with the nutrient addition (DIP, dust, DOP) experiments (3–0, 11–12); a no-addition "control" was also included. Since previous work has reported that the natural $\delta^{15}N$ of particulate matter in the Gulf of Aqaba does not vary much (e.g., between −2‰ and +2‰; A. Paytan unpubl.), incubations without the $^{15}N_2$ tracer were not considered necessary and were not included. In order to assess if the amendments (P or dust) were significantly different than the no-addition controls, a paired *t*-test was run on the three replicates for each treatment using SigmaStat 3.5 (Systat software). Similarly, to determine if the estimated fixation rates in the depth profile of experiment 2 were significantly different, one-way ANOVAS were run on the three replicate bottles per each depth using SigmaStat 3.5. In depth profile experiment 10, only duplicate bottles were used, and, as such, a mean cannot be determined and assessments of statistical significance are not possible. The rates from the latter experiment were reported as ranges, and they are provided as a comparison to the earlier measures in experiment 2.

The duration of incubation for the first set of experiments in fall 2005 was 24–33 h, and the volume was

2.5 liters. The estimated rates from these experiments were considered low (0.1–1.2 nmol N L^{-1} d^{-1}). Our initial conclusion was that at such low rates, it would be better to increase the incubation volume to 4.5 liters and the incubation time to 48 h, as others have used these conditions in similar field experiments (Montoya et al. 2004; Needoba et al. 2007; Zehr et al. 2007). In addition, the increase in the incubation time was necessary because others have shown that nutrient amendments, in particular, additions of P, can take 3–5 d to elicit a response (Rees et al. 2006). Indeed, the utilization of organic P (i.e., DOP) requires the synthesis of enzymes, and thus longer incubations are desirable. Since similar rates of fixation were found in all three seasons of experiments, we concluded that the potential bottle effects of a longer incubation period in experiments 5–12 were minimal.

Sample collection for nutrient and Chl a analyses—Samples for nutrient analysis were collected into acid-washed high density polyethylene (HDPE) bottles and filtered through 0.45–mm–pore size filters. Samples were collected from the selected depths at Stas. A and B using the CTD rosette sampler. Samples were analyzed for nitrite (NO_2), nitrate (NO_3), and soluble reactive phosphate (SRP) using colorimetric methods described by Grasshoff et al. (1999) and modified for a Flow Injection Autoanalyzer (FIA, Lachat Instruments Model QuickChem 8000). SRP was preconcentrated by a factor of 20 using the magnesium coprecipitation (MAGIC) method (Karl and Tien 1992), followed by measurement using the FIA. The FIA was fully automated, and peak areas were calibrated using standards prepared in low-nutrient filtered seawater over a range of 0–10 μmol L^{-1}. The precision of the methods in this study was 0.05 μmol L^{-1} for NO_2 and NO_3, and 0.01 μmol L^{-1} for SRP (Fuller et al. 2005).

Samples for Chl *a* measurements were collected from select depths at Stas. A and B using the CTD rosette sampler. Replicate water samples (250 mL) were filtered through Whatman GF/F filters. Filters were placed in 10-mL borosilicate glass vials, and Chl *a* was extracted with 90% acetone saturated with $MgCO_3$ (10 mL) per filter for 24 h in the dark at -20°C. Extract was analyzed fluorometrically before and after acidification with 3.7% HCl on a Turner fluorometer.

Sample collection for deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) extractions—Surface seawater from 11 stations along the coast of Israel (I) and six stations along the Jordan (J) coastline was collected into 20-liter carboys using a small boat (Fig. 1) in fall 2005. In addition, seawater was collected from six discrete depths at Stas. A (29°27.815′N, 34°55.830′E) and B (29°22.608′N, 34°53.894′E) using a CTD (Fig. 1). Temperature and salinity were continuously recorded during CTD casts. In fall 2006, sampling was limited to Stas. I-1, I-3, I-6, and the IUI pier, and in spring 2007, surface water was collected from Sta. I-6 and the IUI pier (Fig. 1). In addition, six depths at Sta. A were sampled in spring 2007.

Samples (2 liters) for DNA extractions were filtered through 25-mm-diameter 0.2–μm–pore size Supor filters (Pall Corporation) held in Swinnex filter holders (Millipore), using a peristaltic pump. The filters were folded in half, placed in sterile 2-mL bead-beater vials (Biospec Products) containing a 0.2-g mixture of sterile 0.1- and 0.5-mm glass beads (Biospec Products), frozen in liquid nitrogen, and stored at -80°C until extraction. In fall 2006 and spring 2007, surface seawater samples (2 liters) were also collected from the IUI pier over 24 h at 4–5-h increments for quantitative reverse transcription–polymerase chain reaction (qRT-PCR) assays. Samples were filtered and placed in bead-beater tubes as described previously, and 300 μL of RLT buffer (Qiagen) amended with beta-mercaptoethanol was added prior to storage in liquid nitrogen. Additionally, during fall 2005 $^{15}N_2$ incubation experiments, parallel bottles for mRNA expression were also incubated, and 2-liter samples were collected at 5–6-h increments over the 24–26-h incubation. These RNA samples were preserved as described already. In our previous experience with other oligotrophic areas, filtration of 2 liters for RNA (and 2 liters DNA) is usually within the potential detection limit (0–10 copies) of our qPCR assays, and thus we chose 2 liters as a minimum for collection.

DNA extraction—DNA was extracted from samples collected during fall 2005 using the xanthogenate method (Tillett and Neilan 2000) with 500 μL of 2× xanthogenate buffer (200 mmol L^{-1} tris(hydroxymethyl)aminomethane [TRIS], 40 mmol L^{-1} ethylenediaminetetraacetic acid [EDTA], 1.6 mol L^{-1} ammonium acetate, 2% sodium lauryl sulfate [SDS], 2% xanthogenate) added to the bead-beater tubes (Biospec Products) containing filter samples and the bead mixture. The tubes were agitated for 2 min using a bead beater (Biospec Products), incubated for 2 h at 70°C, and placed on ice for 30 min. The tubes were centrifuged briefly at 8000 × g, and the supernatant was transferred to a clean 1.5-mL centrifuge tube with an equal volume of 70% ethanol. DNA was precipitated for 30 min at 20,000 × g in a centrifuge. Ethanol supernatants were removed, and 500 μL of 90% ethanol were added, samples were centrifuged again at 20,000 × g for 10 min, and the ethanol supernatants were removed. The tubes were left to dry overnight, and re-eluted in 30 μL of DNAse-free 5-kD-filtered water (Ambion). This DNA extraction method resulted in inhibition of the qPCR assays; therefore, an alternate extraction method was used for fall 2006 and spring 2007 samples. In these samples, DNA was extracted using a commercially available kit by Qiagen (Plant Mini-kit). Modifications of the manufacturer's protocol have been previously described in detail (Foster et al. 2007). Modifications included a 2-min agitation step with a bead beater, and the final re-elution volume was 35 μL of Elution Buffer [EB] buffer (Qiagen).

Gene nifH PCR, cloning, and sequencing—A nested PCR method was used to amplify a 359-base-pair (bp) fragment of the *nifH* gene with two sets of degenerate primers (Zehr and Turner 2001). The PCR amplifications were performed with a Gene Amp 1800 thermal cycler (Applied Biosystems). Reaction mixtures were composed of 2 μL of

template DNA, 32.5 μL of nuclease-free water, 8.0 μL of 25 mmol L^{-1} $MgCl_2$, 5.0 μL of 10× Dynazyme reaction buffer (Qiagen), 2.0 μL of 10 mmol L^{-1} each of the dNTPs (deoxynucleosides) (dATP, dCTP, dGTP, dTTP), 0.5 μL each of 100 μmol L^{-1} nifH3 and nifH4 primers (Zehr and Turner 2001), and 0.5 μL (0.5 U) of *Taq* polymerase (Qiagen) (total final reaction volume of 50 μL). Reaction conditions consisted of an initial denaturing step at 94°C for 4 min, then 25 cycles of 30 s at 94°C, 57°C, and 72°C, followed by a final extension at 72°C for 7 min. In the second round of PCR, the same reaction mixture and conditions were used; however, nifH1 and nifH2 primers (Zehr and Turner 2001) were used and 1 μL (compared to 0.5 mL) of *Taq* polymerase was added. Nuclease-free water additions were used for negative controls in the PCR reactions. Amplification was not detected in the negative controls.

The products of the PCR reactions were analyzed on a 1.2% agarose gel. For samples that produced a 359-bp product, the band was excised and purified with the QIAquick Gel purification kit (Qiagen) according to the manufacturer's protocol, and bands were eluted in 35 μL of EB buffer (Qiagen). Three microliters of product were immediately cloned using the PGEM Vector system (Promega) following the manufacturer's protocol. Four to 24 white colonies per ligation were restreaked onto new plates and inoculated in 1 mL of 2× Luria Bertani media amended with a final concentration of 100 μg mL^{-1} carbinicillin in sterile 96-deep-well blocks. Cells were incubated overnight at 37°C and agitated. Plasmids were purified using the Montáge Plasmid $Miniprep_{96}$ Kit (Millipore) following the manufacturer's partial lysate protocol.

Two microliters of purified plasmid miniprep were sequenced using SP6 or T7 primers (5 pmol mL^{-1}) with the ABI Prism BigDye terminator sequencing ready-reaction kit (Perkin-Elmer) and at the Berkeley DNA Sequencing Facility (University of California) using an Applied Biosystems 48 capillary 3730 machine (Applied Biosystems). Vector and primer sequences were removed using Seqlab of the GCG Wisconsin Package Version 10.3 (San Diego). For each library, sequences were considered different if they differed by more than one base pair (bp). Seventy-three sequences were deposited in GenBank under accession numbers: DQ825711–DQ825753, DQ831007–DQ831008, and EU151773–EU151800.

The edited *nifH* DNA sequences were imported into an ARB database (Ludwig et al. 2004) containing all *nifH* sequences available in the National Center for Biotechnology Information (NCBI) database as of August 2007. Sequences were aligned with a *nifH* PFAM (protein family) using HMMR (software for making a Hidden Markov Model) (run within GCG software). Phylogenetic analysis of *nifH* nucleotide sequences was performed in ARB using a Jukes-Cantor correction factor (Jukes and Cantor 1969) and 1000 bootstrap replicates. The *nifH* amino-acid-sequence phylogenetic analysis was based on translated representative DNA sequences using a Kimura correction factor and 1000 bootstrap replicates.

RNA extraction and measurement of nitrogenase activity (cDNA synthesis)—Quantitative RT-PCR assays were used to analyze the diel pattern of *nifH* expression from bottles incubated in parallel with the $^{15}N_2$ uptake experiments or from water collected directly from the IUI pier. RNA was extracted using a commercially available kit (QIAGEN RNeasy) with minor changes to the recommended protocol. The bead-beater tubes containing RNA filter samples, RLT buffer, and beads were placed inside a bead-beater machine (Biospec Products) and agitated for 2 min. The tubes were centrifuged for 1 min at 8000 × g, the supernatant was applied to QIAGEN RNeasy minicolumns, and the RNA was purified following the QIAGEN RNeasy protocols. To ensure no DNA contamination, samples were treated for 1–1.5 h at room temperature with DNase I using QIAGEN on-column DNase I RNA extraction protocol. RNA was eluted in 35 μL of RNase-free water provided in the kit and stored frozen at −80°C.

For qRT-PCR assays, total RNA was reverse-transcribed using Super-Script III first-strand cDNA synthesis kit (Invitrogen) according to the manufacturer's recommendations. The reaction mixtures contained 2 μL of RNA template, 0.5 pmol L^{-1} of each reverse primer (nifH2 and nifH4) (Zehr and Turner 2001), 1 mmol L^{-1} dNTP mixture, 1× RT buffer, 5 mmol L^{-1} $MgCl_2$, 10 mmol L^{-1} dithiothreitol, 1 unit (U) RNaseOUT (Invitrogen), and 1U SuperScript III reverse transcriptase (RT). A second set of reactions was set up as described previously; however, these had no SuperScript III reverse transcriptase, and so they served as our negative controls (No RT). The reaction was performed at 55°C for 50 min, 85°C for 5 min, and then the tubes were placed on ice. One microliter of RNase H was added to each reaction mixture, and tubes were incubated at 30°C for 20 min to eliminate residual RNA. The cDNA was stored at −20°C until used in the qPCR assays.

For the qRT-PCR assays, we used the TaqMan® primers and probes described by Church et al. (2005*a*,*b*) to evaluate the expression of *nifH* for *Trichodesmium*, the unicellular group A phylotype, and for a γ proteobacterial group. These three lineages were chosen because these sequence types were found in the clone libraries from fall 2005. For the DNA templates extracted from the depth profiles of Sta. A (fall 2005, spring 2007) and Sta. B (fall 2005), an entire suite of oligonucleotides specific for the other common diazotrophs in oligotrophic environments was also run. These included primers and probes for the *nifH* gene of the unicellular groups B and C, and three heterocystous symbionts of diatoms (het-1, het-2, het-3) (Church et al. 2005*a*; Foster et al. 2007).

For all TaqMan® PCR, the 25-μL reactions contained 12.5 μL of 2× TaqMan® buffer (Applied Biosystems), 8.0 μL of 5-kD-filtered nuclease-free water (Ambion), 0.5 μmol L^{-1} each of the forward and reverse primers, 0.25 μmol L^{-1} of fluorogenic probe, and 2 μL of cDNA or DNA template. Reactions were replicated in quadruplicate, where the fourth replicate was used to estimate the reaction efficiency (*see* below). Two microliters of 5-kD-filtered nuclease-free water (Ambion) were added for the no-template controls (NTCs), and the No-RT controls were run the same as the RT samples.

PCR amplifications were conducted in an ABI 7500 Real Time PCR system (Applied Biosystems) with the following parameters: 50°C for 2 min, 95°C for 10 min, and 45 cycles

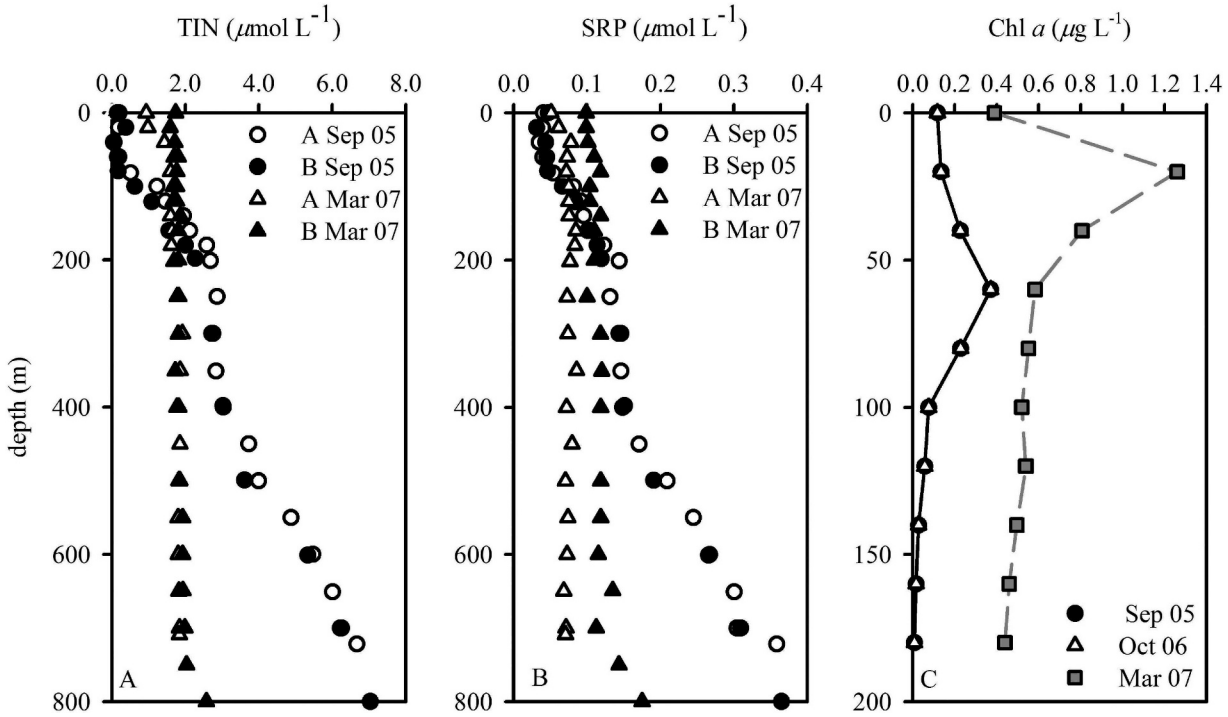

Fig. 2. Depth profiles from Stas. A and B of measured nutrient concentrations: (A) TIN: NO_3 + NO_2; (B) SRP: soluble reactive phosphorous; and (C) extracted Chl *a* during the September 2005, October 2006, and March 2007 field collections. Note that only the depth profile of Chl *a* from Sta. A is shown.

of 95°C for 15 s, followed by 60°C for 1 min. Gene copy abundances were calculated from the mean number of cycle (C_t) value of three replicates and the standard curve for the appropriate primer and probe set (*see* below). In some samples, only two of the three replicates produced an amplification signal; these were noted as detectable, but not quantifiable (dnq).

Standard curves—For each primer and probe set, duplicate standard curves were made from 10-fold dilution series ranging from 1 to 10^8 gene copies per reaction. The standard curves were made from linearized plasmids of the cloned target *nifH* gene. Regression analyses of the number of cycles (C_t) of the standard curves were calculated in Excel.

PCR efficiency—The PCR efficiency for each sample was determined as previously described by Short et al. (2004) using the following formula

$$X_n = X_0(1 + E_x)^n \quad (1)$$

where X_0 is the initial number of target molecules, n is the number of cycles (C_t), and E_x is determined by using the calculated X_n with the C_t value from the fourth replicate of each sample, which contained 2 μL of plasmid plus 2 μL of sample DNA. The E_x value was converted to a percentage, and samples that amplified with less than 95% efficiency were considered to be inhibited.

Results

Environmental conditions—In September 2005, surface temperature (26.3°C to 27.0°C) and salinity (40.1 to 40.4) were relatively uniform in the surface waters at all 18 sampling stations. Concentrations of combined total inorganic nitrogen (TIN: NO_2^- + NO_3^-) in the surface Jordanian waters were higher than in Israel, measuring >1 μmol L^{-1} in three of the six stations (J-2, J-3, J-15), and >3 μmol L^{-1} at two sites (J-2 and J-15). Sta. I1 had similar surface nutrient concentrations as the remaining three Jordanian stations (0.1–0.7 μmol N L^{-1}). All other surface samples from the Israel stations were close to the detection limit for nitrogen (0.02–0.07 μmol N L^{-1}). Concentrations of SRP were similarly low in the surface samples of Jordan and Israel, with concentrations never exceeding 0.07 μmol L^{-1}.

During both fall seasons (September 2005 and October 2006), the mixed-layer depth was ~20 m at Stas. A and B. Generally, the nutrient conditions during both fall seasons and at both stations (A and B) were consistent. For example, at Sta. A, TIN was <0.2 μmol L^{-1} in the upper 60 m, and SRP concentrations were low (<0.04 μmol L^{-1}) or below the detection limit (0.02 μmol L^{-1}) in the upper water column (Fig. 2A,B). Similar nutrient conditions were found at Sta. B; however, there were low concentrations of combined nitrogen and phosphate down to 100 m. A sharp maximum of NO_2^- was measured at 80 m at both Stas. A and B. The maxima in biomass as determined by extracted Chl *a* were at 60 and 80 m during the field seasons of September 2005 (0.371 μg L^{-1}) and October 2006 (0.228 μg L^{-1}), respectively (Fig. 2C).

In spring 2007, the water column was well mixed down to 750 m (depth of deepest sample), and TIN and SRP were significantly higher (>1.5 μmol L^{-1}) than in the fall

sampling seasons and varied little throughout the water column (Fig. 2A). The depth of the Chl *a* maximum was shallower (~25 m), and nitrate concentration was higher (>1.2 μg L^{-1}), than in either fall season (Fig. 2C). The surface concentrations of both TIN and SRP were slightly higher at Sta. B than Sta. A in the spring 2007 sampling (Fig. 2A,B). TIN in deep water (below 200 m) was similar between stations, and SRP remained slightly elevated at Sta. B (Fig. 2A,B).

Amplification of nifH, diversity, and phylogenetic analysis—In order to identify the potential diazotrophic populations in the Gulf of Aqaba, samples were taken for standard *nifH* PCR amplification and sequencing analyses during each of the field seasons.

In fall 2005, the *nifH* gene amplified from five of the surface stations, four of which were along the Israeli (I-3, IUI) and Jordanian (J-6, J-9) coastlines (Fig. 1, filled circles). The fifth surface sample was collected at Sta. I-6, approximately 2.5 km from the shoreline (Fig. 1). In the nested PCR reactions performed on the replicate depth profiles collected in fall 2005 from Stas. A and B, *nifH* did not amplify from any of the Sta. B samples, but it did amplify from samples from all depths at Sta. A, with the exception of the sample at 100 m. In addition, *nifH* amplified from the replicate samples collected from 40- and 60-m depth of Sta. A. Although, in fall 2006 and spring 2007, fewer samples were collected for *nifH* amplification due to time constraints, we were able to collect and amplify *nifH* from the surface samples, which previously showed positive amplification results. Specifically, *nifH* clone libraries were generated from surface samples of Stas. A, IUI, and I-3 during fall 2006, and similarly, from a surface sample of Sta. A in spring 2007. The *nifH* gene was also amplified from a filter that had been used to collect airborne particulates in October 2006. These particulates are assumed to represent aerosols containing Fe and P, but they may also contain microorganisms. The aerosol sampler was located on the rooftop of the IUI station, and collected particles from local desert dust (Chen et al. 2008).

Of the 73 unique *nifH* sequences recovered from 17 separate libraries, most of the sequences (58 sequences, 79% of total) were most similar (>80%) to cluster I (Chien and Zinder 1996; Zehr et al. 2003) *nifH* sequences (Fig. 3). Twelve (16%) sequences aligned with cluster III *nifH* sequences, which include anaerobic bacteria and sulfate reducers (Fig. 4). Three of the 12 sequences were shorter than 359 bp and were left out of further analyses. The remaining nine sequences were obtained from three separate libraries derived from surface samples during the fall 2006, and from the aerosol filter sample, which was used to collect dust in fall 2006. Six sequences were derived from the dust-filter clone library and are distantly related to a sequence obtained from an oil-contaminated marine soil sample (accession no: DQ077987). The three other cluster III sequences were derived from the surface I-3 station sample, two of which were similar and cluster with other planktonic *nifH* sequences from the tropical North Pacific near Heron Island, Australia, and the Mediterranean Sea. An additional sequence obtained in fall 2005 is distantly related to a clade made of cluster II *nifH* gene sequences (data not shown).

The 10 sequences most similar to the cyanobacterial (cluster IB) lineage were only obtained from the fall 2005 libraries and were amplified from surface and from subsurface samples (20, 40, and 60 m) from Sta. A and from one surface sample (Sta. J-6) (Fig. 3). Three of these 10 sequences were 97.2–99.4% identical to *Trichodesmium erythraeum* nucleotide sequences. Two other sequences were 97–100% similar in amino-acid sequence to sequences amplified from field samples from the eastern Mediterranean Sea (Man-Aharonovich et al. 2007) and the subtropical Pacific Ocean (PO), one (AF059637) of which has been designated as a unicellular group A phylotype (Zehr et al. 2001) (Fig. 3). One sequence was similar and clustered with a low bootstrap value (53%) with an environmental sequence from the Neuse River (AF035519). The remaining four sequences were distantly related to other environmental sequences from the Atlantic Ocean and unicellular-like sequences (i.e., *Cyanothece*) (Fig. 3).

Twenty-one sequences derived from the fall 2005 and 2006 samples were distantly related to the 1G, 1H, and 1L *nifH* lineages, which are composed of several proteobacterial sequences, including the *Vibrio diazotrophicus* sequence (Fig. 3). Two of the latter 21 sequences amplified from the I-3 sample of fall 2006 clustered with high bootstrap support (85%) to environmental sequences derived from Lake Michigan and Tomales Bay, which have been identified as *nifH* lineage 1H. Two sequences derived from fall 2006 collection at I-3 and from the aerosol filter were similar and distantly related to the 1H lineage. Two sequences amplified from samples collected at 40-m depth from Sta. A and one sequence from the surface of Sta. J-6 were similar and supported by a high bootstrap value (82%) with environmental sequences derived from the eastern Mediterranean Sea (MS) and the Atlantic (AO) and Pacific (PO) Oceans.

Seven other sequences from the fall 2005 and 2006 libraries were distantly related and supported by a high bootstrap value (89%) with two representatives of the cluster 1A *nifH* lineage (Fig. 3) and another sequence derived from the Mediterranean Sea. One sequence amplified from the 40-m depth sample collected at Sta. A in fall 2005 was strongly supported (97%) with *Azoarcus* sp. *nifH* sequence, which represents the cluster 1P *nifH* lineage (Fig. 3). Sixteen other sequences formed two separate bootstrap-supported clades (72 and 88, respectively) composed of sequences previously described as lineages 1J and 1K (Fig. 3). These sequences were obtained from surface samples (fall 2005, fall 2006, spring 2007) and from samples collected at deeper depths from Sta. A in fall 2005 and spring 2007 (40 m, 80 m). The latter sequences clustered with other environmental sequences derived from the Atlantic Ocean, Pacific Ocean, and Mediterranean Sea and marine stromatolites (Fig. 3).

Quantitative PCR—In order to estimate the depth dependent-distribution and abundance for some of the more commonly reported diazotrophic phylotypes, qPCR assays were applied to DNA extracts from samples

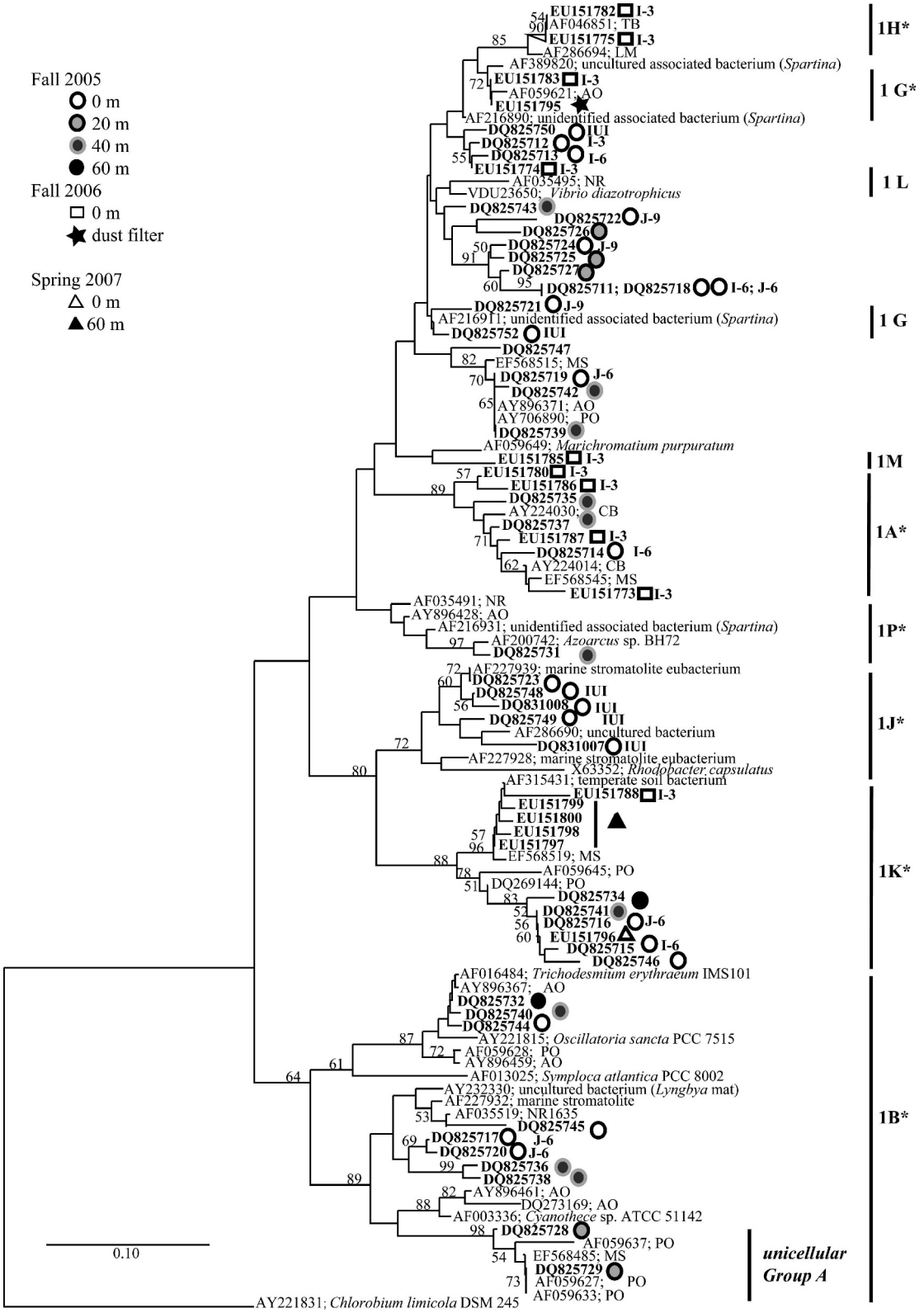

Fig. 3. Neighbor-joining phylogenetic tree of cluster I *nifH* amino-acid sequences. Sequences determined in this study are shown in bold and compared with the most closely related amino-acid sequences of *nifH* genes from cultured and uncultured microorganisms. One sequence (accession number: DQ825730) was not included in the analysis because the sequence was short. Distances were calculated using the Kimura correction in ARB, trees were bootstrapped 1000 times, and bootstrap values >50% are indicated at the nodes. *Chlorobium limicola* (accession number: AY221831) was used to root the tree. The key to the left on figure indicates the seasons and depths from which sequences were derived from Sta. A, and the other surface stations are indicated after the sequence accession number. The

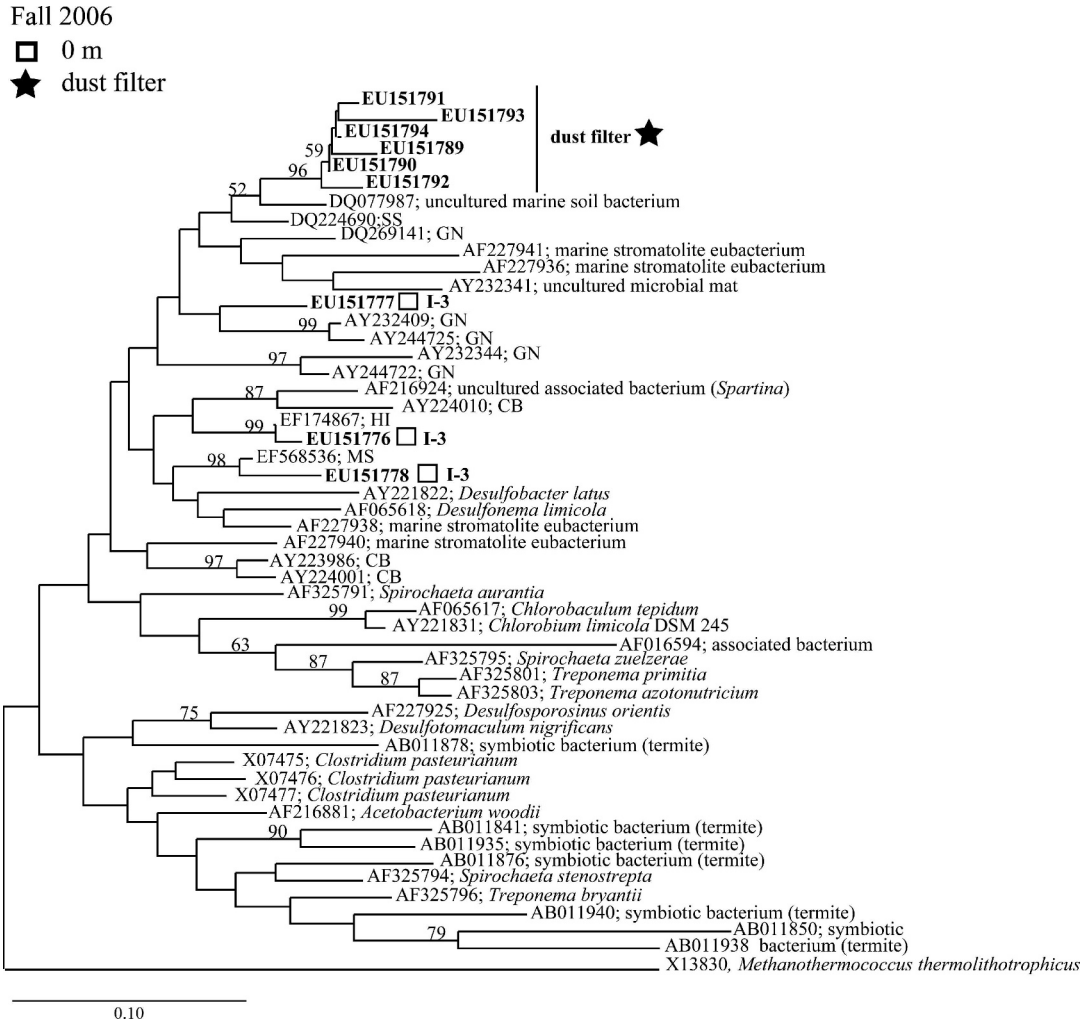

Fig. 4. Neighbor-joining phylogenetic tree of cluster III *nifH* amino-acid sequences obtained from upper ocean plankton and dust (aerosol) samples collected in the Gulf of Aqaba. Sequences determined in this study are shown in bold and compared to the most closely related *nifH* amino-acid sequences from cultured and uncultured microorganisms. Distances were calculated using the Kimura correction in ARB, trees were bootstrapped 1000 times, and bootstrap values >50% are indicated at the nodes. *Methanothermococcus thermolithotrophicus* (accession number: X13830) was used to root the tree. The environments where the *nifH* sequences were obtained are abbreviated as follows: SS, microbial mat from Bahamas, San Salvador; GN, microbial mat from Guerrero Negro, Mexico; CB, marine sediments of Chesapeake Bay; MS, planktonic sample from Mediterranean Sea.

collected from Stas. A and B during fall 2005 and Sta. A during spring 2007 collections. These depth-dependent distributions were estimated using a TaqMAN® qPCR approach with previously designed primer and probe sets for the following phylotypes: *Trichodesmium* spp., γ proteobacteria, three unicellular cyanobacterial groups (A, B, C), and three heterocystous symbiotic cyanobacterial groups (het-1, het-2, het-3) (Church et al. 2005*b*; Foster et al. 2007).

In fall 2005, only the *Trichodesmium* and γ proteobacteria phylotypes were detected in the depth profile samples of Stas. A and B (Fig. 5). Many of the qPCR reactions (10, 42%) had poor PCR amplification efficiencies (<95%). Both phylotypes were detected at higher concentrations at Sta. A than at Sta. B (Fig. 5). The *Trichodesmium* spp. sequence was detected at 80 m, and 1.1×10^4 and 1.4×10^5 gene copies L^{-1} were found in the 40-m and 60-m depth samples of Sta. A, respectively. At Sta. B, *Trichodesmium*

←

sequences derived from the aerosol filter library are designated with a star. The letter designations on right of figure indicate the previously identified subclades of the cluster I lineage (Zehr et al. 2003), and asterisks designate those supported with bootstrap >50%. The environments where *nifH* sequences were obtained are abbreviated as follows: AO, Atlantic Ocean; CR, Chophank River (Maryland); LM, Lake Michigan (Michigan); MS, Mediterranean Sea; NR, Neuse River (North Carolina); PO, Pacific Ocean (Sta. ALOHA); RS, Gulf of Aqaba; TFCLY, soil Cuevo del Indio National Park (Caracas, Venezuela).

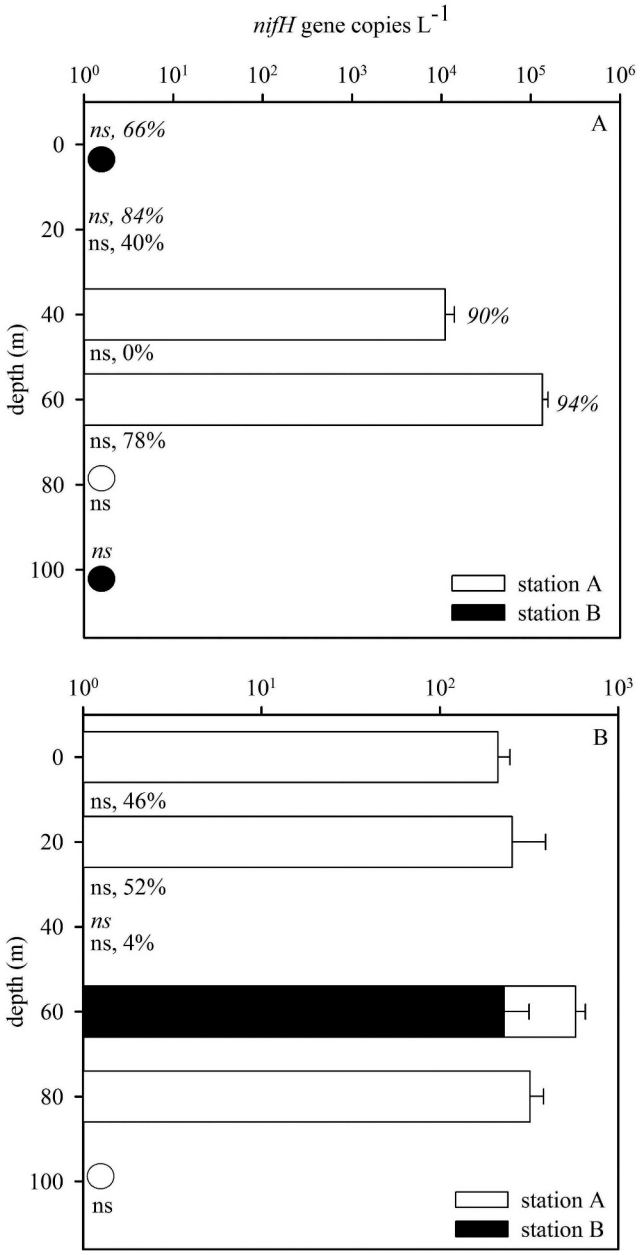

Fig. 5. Depth profiles of *nifH* gene copy abundance for (A) *Trichodesmium* and (B) γ proteobacteria phylotypes at Stas. A and B in fall 2005. Circles indicate samples that had a detectable signal but were not quantifiable, i.e., at least one of three replicate reactions resulted in detectable amplification; "ns" indicates where there was no detectable signal in any of the replicate reactions. The estimated reaction efficiencies <95% are indicated in italics for Sta. A and in regular print for Sta. B. Note that the *x*-axis is a logarithmic scale.

was detected in the surface (0 m) and deepest samples (100 m). At Sta. A, the γ proteobacteria *nifH* genes were less abundant than *Trichodesmium nifH* genes, but the γ proteobacteria phylotype was detected in more of the samples (five of the six depth samples; 0, 20, 60, 80, 100 m), and it exhibited a mid-depth maximum abundance (5.7×10^2 *nifH* gene copies L^{-1}) in the 60-m depth sample at Sta. A (Fig. 5). A similar depth maximum (2.3×10^2 gene copies L^{-1}) was recorded at Sta. B (Fig. 5). The qPCR reactions for the γ proteobacteria were more efficient (9 of 12 reactions had efficiencies >95%) than the reactions for *Trichodesmium*.

Two other phylotypes were detected in two of three replicates, which we report as detected but not quantifiable (dnq). The *Calothrix* symbiont of *Chaetoceros* diatoms was dnq from the 60-m and 0-m depth samples of Sta. B. Secondly, the group A phylotype was dnq at two depths (0 and 60 m) of Sta. A from fall 2005. During spring 2007, no phylotypes were detected in the qPCR reactions.

In order to identify the organisms that were actively expressing the *nifH* gene, and therefore likely to be the microorganisms responsible for N_2 fixation in the uptake experiments, RNA samples were collected approximately every 6–8 h in parallel incubation bottles to the $^{15}N_2$ uptake experiments of September 2005. In October 2006 and March 2007, sampling time and the equipment (bottles) was limited to allow collections from surface seawater samples from the IUI pier approximately every 5–6 h over a 24-h period. Although, nutrient concentrations, temperature, and salinity are fairly consistent throughout the surface of the Gulf of Aqaba, it should be noted that we consider the results from these diel *nifH* gene expression qRT-PCR assays to be only a preliminary survey of the Gulf of Aqaba. Given more materials, time, and budget, it would have been desirable to make more collections around the Gulf Aqaba. Within a given season, however, others have reported on similar conditions around the gulf (Chase et al. 2006; Mackey et al. 2007; Chen et al. 2008), and therefore our sampling at the pier was considered representative of a particular season.

A similar quantitative approach with the TaqMAN® oligonucleotides specific for *Trichodesmium*, unicellular group A, and the γ proteobacteria phylotypes was applied to the RNA extracts from September 2005 after a reverse-transcription (RT) step. None of the No RT controls amplified and only 3 of 14 RNA samples (two diel experiments) produced a detectable signal from one of the three replicates with the *Trichodesmium* primer and probe set. Since N_2 fixation rates were low, it is likely that the RNA transcription rates were at the detection limit (1–10 copies) of the qRT-PCR assay. Similarly, *Trichodesmium*, unicellular group A, and γ proteobacteria *nifH* transcription was not detected in the October 2006 and March 2007 diel RNA samples.

N_2 fixation rates—In September 2005, N_2 fixation rates were measurable, but low (0.1–1.2 nmol N L^{-1} d^{-1}) (Table 1). Although samples were taken at multiple time points during the September 2005 incubation (8–16 h) experiments, we used the final time point because shorter incubations did not yield enough particulates for analyses. In subsequent field seasons, October 2006 and March 2007, we doubled the volume (4.5 liters) and increased the time of incubation (48–52 h). Similarly, low rates were measured in all seasons, suggesting the low rates measured are not a result of short incubation time or of limited sample volume but rather represent the actual low fixation rates in this area (Table 1).

N_2 fixation rates measured in experiments 1–4 of September 2005 were variable and ranged from 0.1 to 1.2 nmol N L^{-1} d^{-1}, where the highest rate measurements were detected in seawater collected at 20-m depth at Sta. A (Table 1). Similar rates as those measured at 20-m depth were obtained in experiment 4 for the surface seawater sample from the IUI pier (Table 1). The lowest rates of N_2 fixation were measured in experiment 1, where rates were measured in bulk surface water from Sta. I-6 (Fig. 1; Table 1). The addition of DOP or DIP did not result in a significant increase in N_2 fixation; in fact, although not statistically significant, we note that in experiment 3, the bottle with a DOP amendment had a N_2 fixation rate that was 0.6 nmol N L^{-1} d^{-1} compared to 0.8 nmol N L^{-1} d^{-1} in the parallel control sample not receiving P addition (Table 1). At Sta. A, the bulk water collected from 20-m depth had a significantly (ANOVA, $p < 0.05$) higher N_2 fixation rate than the seawater assayed from 80 m (Table 1).

In two of the four experiments from the October 2006 field season, N_2 fixation rates were below detection (Table 1). In both these experiments, DIP was added to the bulk water collected from the IUI pier and Sta. I-6. Both these stations were selected because in the previous field season, N_2 fixation rates were measurable, and *nifH* genes were amplified from these samples. Low rates (0.2–0.3 nmol N L^{-1} d^{-1}) were determined in experiment 6 (Sta. I-1), and DIP amendments had no measurable effect on the N_2 fixation rates. Significantly (*t*-test, $p < 0.004$) higher fixation rates (0.8–1.0 nmol N L^{-1} d^{-1}) were measured in the incubation of surface seawater collected from I-3 (experiment 7), which were similar to the highest rates measured in the experiments from the previous field season at Sta. A and the IUI pier. Again, addition of DIP had no detectable effect on the N_2 fixation rate.

The highest rates of N_2 fixation were measured in experiment 9 during March 2007 (Table 1). In the bottles without addition and one with a dust filter added, N_2 fixation rates were 1.9 and 2.1 nmol N L^{-1} d^{-1}, respectively. These rates were higher than the rate in the seawater amended with DIP (0.9 nmol N L^{-1} d^{-1}) (Table 1); however, the differences in the mean values amongst the treatments and control were not significant (one-way ANOVA, $p = 0.844$; *t*-tests, $p = 0.844$, $p = 0.416$, $p = 0.440$ for control vs. +DIP, control vs. dust, and dust vs. +DIP, respectively). Thus, the lower N_2 fixation with the DIP addition and the slightly higher N_2 fixation rate with a dust addition cannot be attributed to their respective amendments. Consistent with the low effect of DIP amendment on fixation rates in our samples, in experiment 11, where increased N_2 fixation rate with DIP addition was observed, the increase was only 36% over the unamended control bottles.

The rate of N_2 fixation in seawater collected from three depths at Sta. A was low and/or at the detection limit. The addition of a dust filter to bulk seawater collected from the pier at IUI in experiment 12 had only a small effect—the N_2 fixation rate was low (0.1 ± 0.2 nmol N L^{-1} d^{-1}) but detectable, whereas N_2 fixation was not detected in the no-addition bottle (Table 1).

Discussion

During the fall of 2005 and 2006, the surface waters of the Gulf of Aqaba were stratified with low concentrations of inorganic nitrogen ($NO_3^- + NO_2^-$). An earlier study conducted at the same field sites in August 2003 and March 2004 as those presented here reported high dissolved Fe concentrations at the surface (Chase et al. 2006). In parallel to our study, Chen et al. (2007) collected and reported the atmospheric dry deposition and associated nutrient inputs for the north coast of the Gulf of Aqaba between August 2003 and September 2005. They found high atmospheric input of seawater soluble Fe (0.3 nmol m^{-3}). The very low N concentration in surface water and the lower than Redfield stoichiometric (16 : 1) N : P ratios observed in the fall of 2005 and fall of 2006 in the surface and down to 80-m depth (consistent with those previously reported for the Gulf of Aqaba surface water during strong summer stratification; Lindell and Post 1995) suggest potentially N-limiting conditions and ample available Fe, which should stimulate N_2 fixation (Zehr and Capone 2002).

Despite the favorable conditions for N_2 fixation in the gulf's surface water, the N_2 fixation rates reported here for the Gulf of Aqaba are low and fall within the range of previously reported rates for the smaller, presumably unicellular cyanobacteria. For example, previous $^{15}N_2$ uptake experiments for size-fractioned (<10 μm) bulk water assays have reported rates ranging from 0.01 to 22.2 nmol N L^{-1} h^{-1} (Dore et al. 2002; Montoya et al. 2004; Needoba et al. 2007). In most of these studies, a similar phytoplankton composition (based on *nifH*), with unicellular microbes, in particular, group A and related unicellular cyanobacterial phylotypes, was reported.

Since proteobacteria and *Trichodesmium* phylotypes, and to a lesser degree unicellular and heterocystous cyanobacteria, were detected by qPCR and in clone libraries, it seems plausible that the rates reported here represent predominantly the fixation by the bacterioplankton community together with only a small contribution by *Trichodesmium* spp. filaments. The highest concentrations of *Trichodesmium* were from deeper depths (40 and 60 m) than the depths where water was collected for assessing N_2 fixation (except experiments 2 and 10); thus, our estimates may be lower than actual depth-integrated values because they did not capture the depths of highest *Trichodesmium* density.

Most of the *nifH* sequences obtained from September 2005 and October 2006 clone libraries and all the sequences from March 2007 were similar to cluster I γ, α, and unidentified proteobacterial phylotypes. An increasing number of *nifH* sequences within the proteobacterial radiation of cluster I has been reported from diverse habitats, including most major ocean basins (Zehr et al. 2001; Church et al. 2005*a*; Langlois et al. 2005). In addition, there were representative sequences from cluster II and III lineages detected in the fall 2006 and spring 2007 libraries. Two of our sequences clustered with unicellular group A sequences, a phylotype which has been reported in high abundances from the subtropical and tropical Atlantic and Pacific Oceans (Zehr et al. 2001; Church et al. 2005*a*;

Foster et al. 2007). Thus, it seems that the bacterial populations of Aqaba were similar in composition to other oligotrophic regions of the world's oceans. Although, the relative abundance and depth distribution of the dominant phylotypes (Fig. 5) were quantitatively similar to those reported in other studies, there were, however, surprisingly low estimates for their transcript abundance, especially in the context of the fall seasons when nitrogen was definitely limiting.

Our estimates of the γ proteobacteria *nifH* phylotype densities were within the same order of magnitude (10^3 *nifH* gene copies L^{-1}) reported in the subtropical Pacific Ocean (Sta. ALOHA) (Church et al. 2005*a*,*b*; Zehr et al. 2007) and within the abundance range recently reported in a qPCR study in the tropical North Atlantic for other γ proteobacteria phylotypes (Langlois et al. 2008), yet the *nifH* transcripts (cDNA) were undetectable in the diel samplings. Similarly low transcript abundance was found for *Trichodesmium* and unicellular group A phylotypes. Considering the low rates of N_2 fixation measured in the $^{15}N_2$ uptake assays, we concluded that the transcript abundance was likely at the detection of the qRT-PCR assay (1–10 copies) (Novak et al. 2006). Increasing the volume of water collected for RNA could increase detection, as there would be a higher concentration of target; however, RNA is highly unstable, and the longer filtration time required to filter more sample volume could also compromise the integrity of the sample. Ideally, we want to compare our studies from all over the world with other published works, and thus it is an ongoing challenge to determine a consistent sampling and processing strategy from one location to the next, even when the same environmental conditions are observed and/or expected.

During late summer in the Gulf of Aqaba, Fe concentrations have been reported as high as 6 nmol L^{-1}, which is not considered to be limiting (Chase et al. 2006). Phosphorus (P), on the other hand, is often considered limiting during the Gulf of Aqaba's summer stratification, at least for the larger phytoplankton taxa (Stihl et al. 2001; Mackey et al. 2007), and at times even for the picoplankton populations (*Synechococcus* and *Prochlorococcus*) (Fuller et al. 2005). Indeed, P concentrations were at the detection limit during fall 2005 and 2006. Enhanced alkaline phosphatase activities, the enzyme responsible for P regeneration from dissolved organic matter (DOM), have been measured in the Gulf of Aqaba and show a seasonal maximum during late summer (Stihl et al. 2001; Mackey et al. 2007). Previous studies in the gulf have suggested that P limitation may explain the absence of *Trichodesmium* spp. in the gulf, particularly during seasons when oligotrophic conditions are most obvious (Mackey et al. 2007). We assumed that P was likely limiting, since it was nearly undetectable in the fall samplings, and therefore it was surprising that the addition of P (as DIP or DOP amendments) in our incubations did not result in significant and consistent increases in N_2 fixation rates.

Few of the amendment experiments that measured an increase or decrease in rates were considered statistically significant. There was only one experiment (experiment 11) of six DIP-amended incubations where a marked increase (t-test; $p < 0.005$) in activity was observed after DIP addition. This suggested that at least in our experiments, P was not likely a limiting factor for N_2 fixation. Similar results have been reported for the North Pacific Subtropical Gyre (Zehr et al. 2007). The DOP addition experiment (experiment 3) resulted in a significant (t-test, $p < 0.005$) decrease in N_2 fixation rate from 0.8 nmol N L^{-1} d^{-1} in the unamended controls to 0.6 nmol N L^{-1} d^{-1} in the DOP additions. We interpreted the decrease as an indication that our amendment (glycerol phosphate) may have contained an unidentified contaminant (s) or possibly an N source. We note that as evidenced from the *nifH* diversity analyses and the qPCR assays, the microbial composition in our experiments was dominated by the smaller N_2-fixing organisms, which presumably have lower N_2 fixation rates and potentially lower nutrient quotas (Van Mooy et al. 2006). In addition, *Trichodesmium* spp. colonies, which are considered to have high N_2 fixation rates, were absent or present at low abundances in our samples.

If the bacterioplankton community was primarily responsible for the N_2 fixation measured in our incubations, this could explain the relatively low rates and the lack of response to P enrichment. Smaller bacterial cells have higher surface area–to–volume ratios (Vogel 1988) and thus are more efficient in utilizing the little available P in surface waters and may have higher P uptake efficiency and a lower P requirement, as has been observed in other picocyanobacteria (Van Mooy et al. 2006). A second possibility is that the short duration of incubation was not long enough to elicit a response, particularly for utilizing DOP, which requires synthesis of enzymes for utilization. Other reports of the effect of P additions on N_2 fixation have indicated that it took on the order of 3–5 d before a biological response was apparent (Rees et al. 2006).

The addition of P could have caused an increase in competition amongst the planktonic community or a shift in the community structure within the incubation bottles. For example, in the eastern Mediterranean Sea, an area well characterized by ultraoligotrophic and P-starved conditions, Thingstad et al. (2005) and Tanaka et al. (2007) observed a decline in chlorophyll with the addition of phosphate to a surface eddy. They suggested that P amendments bypassed the phytoplankton community via rapid uptake by heterotrophic bacteria. Higher abundances of heterotrophic ciliates and close predator–prey relationships were observed in the upper 50 m and were mechanistic for rapid transfer of a limiting nutrient to higher trophic levels (Tanaka et al. 2007). A similar scenario could have occurred in experiments where alternate populations other than the diazotrophs were more favored by the P additions. Yet another possibility is that since we assayed bulk water without a prefiltration step, grazers, e.g., ciliates, could have consumed the active diazotrophic community.

In September 2005, several *nifH* sequences very similar to *Trichodesmium* sp. *nifH* sequences were recovered. In addition, a few filaments (trichomes) of *Trichodesmium* spp. were observed by microscopy (data not shown). *Trichodesmium* spp. have been previously observed in early and late summer in the Gulf of Aqaba, and sometimes

through the fall (Kimor and Golandsky 1977; Gordon et al. 1994; Post et al. 2002). Thus, it was not unexpected to recover several *nifH* sequences with high sequence identity (>96%) to the *nifH* sequences of *Trichodesmium* spp. The estimates of *Trichodesmium nifH* gene copy abundances (10^4–10^5 copies L^{-1}) by qPCR were similar to the cell abundances previously reported for *Trichodesmium* spp. in the gulf during early fall (10^2–10^6 colonies m^{-3}) (Post et al. 2002). The lack of detection of *Trichodesmium* by qPCR or clone library analyses in the subsequent fall (2006), when similar hydrographic conditions existed, was unexpected. Similar to other oligotrophic environments, populations of *Trichodesmium* within the Gulf of Aqaba appear to be patchy in distribution, and they are not observed every year (A. Post pers. comm.).

A subsurface maximum of *Trichodesmium* spp. *nifH* gene copy abundance was observed at 60-m depth at Sta. A. This was consistent with a previous observation from the Gulf of Aqaba, where puff-shaped colonies of *Trichodesmium* were more common in the bottom half of the photic zone (Post et al. 2002). *Trichodesmium* spp. colonies are capable of vertical migration through the water column (Villareal and Carpenter 1990; Letelier and Karl 1998). Interestingly, maximum abundances were observed at the base of the euphotic zone where P and combined nitrogen had highest concentrations, and this might be a possible alternative niche for *Trichodesmium* spp. when the euphotic zone is deep (100 m). Furthermore, it might also help explain why transcript abundances assayed by qRT-PCR were low or below detection, as we did not analyze samples from the depth of highest *Trichodesmium* densities.

An interesting and unexpected result from the diversity study was the recovery of *nifH* sequences from the aerosol dust filter. Two lineages (cluster I and III) were recovered, one of which was composed of a sequence most similar to sequences recovered from other planktonic samples, including a surface sample from Sta. I-3. The second lineage was most similar to an uncultured soil bacterium within cluster III. One of the highest N_2 fixation rates in spring 2007 was observed in the incubation bottle amended with an aerosol dust filter. These sequence diversity data in the context of the $^{15}N_2$ uptake experiments warrant future studies, since the addition of dust might introduce microbial communities as well as nutrients or toxins.

As a final note, the comparison of sequences from the clone libraries of fall 2005 with those generated from the fall 2006 and spring 2007 should be interpreted with caution. Since there was inhibition in the qPCR assays from the fall 2005, we changed the extraction method to improve reaction efficiency in the subsequent year's samplings. This, however, inevitably might bias amplification and cloning. We did, however, recover similar sequence types from all three seasons, which provides confidence that the extraction methods were fairly consistent.

The rates of N_2 fixation reported here are low, but they provide a good representation of background, nonbloom conditions within the Gulf of Aqaba. The transcript abundance is consistent with the low rates, as these were at the limit of detection (<10 copies), and it suggests that transcription was low. Several novel lineages of γ proteobacterial *nifH* sequences were recovered that provide the preliminary sequence data for other applications, i.e., qPCR, that will allow for better estimates of their abundances and activity (qRT-PCR) in the Gulf of Aqaba and beyond. Furthermore, the N_2 fixation rates measured here may be a good indication of the contribution of N_2 fixation by the bacterioplankton community in other oligotrophic settings. The results from our limited study of two fall seasons and one spring season warrant future investigations during other seasons (summer and winter) of the year to determine further the seasonal variation in N_2 fixation rates and to examine if the community of diazotrophs is changing in the gulf as well. Collectively, the 12 experiments reported here on N_2 fixation rates are the first of their kind in the Gulf of Aqaba, and although the community response to the P amendments was not consistent, rates of N_2 fixation were consistently measured in an environment without other significant external N sources. This latter result is important and relevant to other regions of the world's oceans where similar environmental conditions persist and consistently, albeit low, rates of N_2 fixation have been measured.

References

Capone, D. G., J. P. Zehr, H. W. Paerl, B. Bergman, and E. J. Carpenter. 1997. *Trichodesmium*: A globally significant marine cyanobacterium. Science **276:** 1221–1229.

Chase, Z., A. Paytan, K. S. Johnson, J. Street, and Y. Chen. 2006. Input and cycling of iron in the Gulf of Aqaba, Red Sea. Glob. Biogeochem. Cy. **20:** GB3017, doi:10.1029/2005GB002646.

Chen, Y., S. Mills, J. Street, D. Golan, A. Post, M. Jacobson, and A. Paytan. 2007. Estimates of atmospheric dry deposition and associated input of nutrients to the Gulf of Aqaba seawater. J. Geophys. Res. **112:** D04309, doi:10.1029/2006JD007858.

———, A. Paytan, Z. Chase, C. Measures, A. J. Beck, S. A. Sañudo-Wilhelmy, and A. F. Post. 2008. Sources and fluxes of atmospheric trace elements to the Gulf of Aqaba, Red Sea. J. Geophys. Res. **113:** D05306, doi:10.1029/2007JD009110.

Chien, Y. T., and S. H. Zinder. 1996. Cloning, functional organization, transcript studies, and phylogenetic analysis of the complete nitrogenase structural genes (nifHDKZ) and associated genes in the archaeon *Methanosarcina barkeri* 227. J. Bact. **178:** 143–148.

Church, M. J., B. D. Jenkins, D. M. Karl, and J. P. Zehr. 2005a. Vertical distributions of nitrogen-fixing phylotypes at Station ALOHA in the oligotrophic North Pacific Ocean. Aquat. Microb. Ecol. **38:** 3–14.

———, B. D. Jenkins, C. Short, D. M. Karl, and J. P. Zehr. 2005b. Temporal patterns of nitrogenase (*nifH*) gene expression in the oligotrophic North Pacific Ocean. Appl. Environ. Microbiol. **71:** 5362–5370.

Dore, J. E., J. R. Brum, L. M. Tupas, and D. M. Karl. 2002. Seasonal and interannual variability in sources of nitrogen supporting export in the oligotrophic subtropical North Pacific Ocean. Limnol. Oceanogr. **47:** 1595–1607.

Dyhrman, S. T., P. D. Chappell, S. T. Haley, J. W. Moffet, E. D. Orchard, J. Waterbury, and E. A. Webb. 2005. Phosphonate utilization by the globally significant marine diazotroph *Trichodesmium*. Nature **439:** 68–71.

———, and S. T. Haley. 2005. Phosphorus scavenging in the unicellular marine diazotroph *Crocosphaera watsonii*. Appl. Environ. Microbiol. **72:** 1452–1458.

Foster, R. A., A. Subramaniam, C. Mahaffey, E. J. Carpenter, D. G. Capone, and J. P. Zehr. 2007. Influence of the Amazon River plume on distributions of free-living and symbiotic cyanobacteria in the western tropical North Atlantic Ocean. Limnol. Oceanogr. **52:** 517–532.

———, and J. P. Zehr. 2006. Characterization of diatom–cyanobacteria symbioses on the basis of *nifH*, *hetR* and 16S rRNA sequences. Environ. Microbiol. **8:** 1913–1925.

Fuller, N. J., D. Marie, M. Yallop, T. Rivlin, N. J. West, A. F. Post, and D. J. Scanlan. 2005. Dynamics of community structure and phosphate status of picocyanobacterial populations in the Gulf of Aqaba, Red Sea. Limnol. Oceanogr. **50:** 363–375.

Genin, A., B. Lazar, and S. Brenner. 1995. Vertical mixing and coral death in the Red Sea following the eruption of mount Pinatubo. Nature **377:** 507–510.

Gordon, N., A. D. L. Angel, A. Neori, N. Kress, and B. Kimor. 1994. Heterotrophic dinoflagellates with symbiotic cyanobacteria and nitrogen limitation in the Gulf of Aqaba. Mar. Ecol. Prog. Ser. **107:** 83–88.

Grasshoff, K., M. Ehrhardt, K. Kremling, and L. G. Anderson. 1999. Methods of seawater analysis. Weinheim.

Hamme, R. C., and S. R. Emerson. 2004. The solubility of neon, nitrogen and argon in distilled water and seawater. Deep-Sea Res. I **51:** 1517–1528.

Jukes, T. H., and C. R. Cantor. 1969. Evolution of protein molecules, p. 21–132. *In*: H. N. Munro [ed.], Mammalian protein metabolism. Academic Press.

Karl, D. M., A. Michaels, B. Bergman, D. G. Capone, E. J. Carpenter, R. Letelier, F. Lipschultz, H. W. Paerl, D. Sigman, and L. Stal. 2002. Dinitrogen fixation in the world's oceans. Biogeochemistry **57/58:** 47–98.

———, and G. Tien. 1992. MAGIC: A sensitive and precise method for measuring dissolved phosphorous in aquatic environments. Limnol. Oceanogr. **37:** 105–116.

Kimor, B., and B. Golandsky. 1977. Microplankton of the Gulf of Eilat: Aspects of seasonal and bathymetric distributions. Mar. Biol. **42:** 55–67.

Kustka, A. E., E. J. Carpenter, and S. A. Sañudo-Wilhelmy. 2002. Iron and marine nitrogen fixation: Progress and future directions. Res. Microbiol. **153:** 255–262.

Langlois, R., D. Hümmer, and J. Laroche. 2008. Abundances and distributions of the dominant *nifH* phylotypes in the northern Atlantic Ocean. Appl. Environ. Microbiol. **74:** 1922–1931.

———, J. Laroche, and P. A. Raab. 2005. Diazotrophic diversity and distribution in the tropical and subtropical Atlantic Ocean. Appl. Environ. Microbiol. **71:** 7910–7919.

Letelier, R., and D. M. Karl. 1998. *Trichodesmium* spp. physiology and nutrient fluxes in the North Pacific Subtropical Gyre. Aquat. Microb. Ecol. **15:** 265–276.

Lindell, D., and A. F. Post. 1995. Ultraphytoplankton succession is triggered by deep winter mixing in the Gulf of Aqaba (Eilat), Red Sea. Limnol. Oceanogr. **40:** 1130–1141.

Ludwig, W., and others. 2004. ARB: A software environment for sequence data. Nucleic Acids Res. **32:** 1363–1371.

Mackey, K. R. M., R. G. Labiosa, M. Calhoun, J. H. Street, A. F. Post, and A. Paytan. 2007. Phosphorous availability controls phytoplankton community dynamics and taxon specific nutrient status in the Gulf of Aqaba, Red Sea. Limnol. Oceanogr. **52:** 873–885.

Man-Aharonovich, D., N. Kress, E. B. Zeev, I. Berman-Frank, and O. Beja. 2007. Molecular ecology of *nifH* genes and transcripts in the eastern Mediterranean Sea. Environ. Microbiol. **9:** 2354–2363.

Montoya, J. P., C. M. Holl, J. P. Zehr, A. Hansen, T. A. Villarea, and D. G. Capone. 2004. High rates of N_2 fixation by unicellular diazotrophs in the oligotrophic Pacific Ocean. Nature **430:** 1027–1032.

———, M. Voss, P. Kahler, and D. G. Capone. 1996. A simple, high-precision, high-sensitivity tracer assay for N_2 fixation. Appl. Environ. Microbiol. **62:** 986–993.

Needoba, J. A., R. A. Foster, C. Sakamoto, J. P. Zehr, and K. S. Johnson. 2007. Nitrogen fixation by unicellular diazotrophic cyanobacteria in the temperate oligotrophic North Pacific Ocean. Limnol. Oceangr. **52:** 1317–1327.

Novak, R. T., M. B. Glass, J. E. Gee, D. Gal, M. J. Mayo, B. J. Currie, and P. P. Wilkens. 2006. Development and evaluation of a real-time PCR assay targeting the type III secretion system of *Burkholderia pseudomallei*. J. Clin. Microbiol. **44:** 85–90.

Post, A. F., Z. Dedej, R. Gottlieb, H. Li, D. N. Thomas, M. El-Absawi, A. El-Naggar, M. El-Gharabawi, and U. Sommer. 2002. Spatial and temporal distribution of *Trichodesmium* spp. in the stratified Gulf of Aqaba, Red Sea. Mar. Ecol. Prog. Ser. **239:** 241–250.

Rees, A. P., C. S. Law, E. Malcom, and S. Woodward. 2006. High rates of nitrogen fixation during in situ phosphate release experiment in the Eastern Mediterranean Sea. Geophys. Res. Lett. **33:** L10607, doi:10.1029/2006GL025791.

Sañudo-Wilhelmy, S. A., A. Kustka, C. J. Gobler, D. A. Hutchins, D. G. Capone, J. A. Raven, and P. M. Digiacomo. 2001. Phosphorus limitation of nitrogen fixation by *Trichodesmium* in the central Atlantic Ocean. Nature **411:** 66–69.

Short, S. M., B. D. Jenkins, and J. P. Zehr. 2004. Spatial and temporal distribution of two diazotrophic bacteria in the Chesapeake Bay. Appl. Environ. Microbiol. **70:** 2186–2192.

Stihl, A., U. Sommer, and A. F. Post. 2001. Alkaline phosphatase activities among populations of the colony-forming diazotrophic cyanobacterium *Trichodesmium* spp. (cyanobacteria) in the Red Sea. J. Phycol. **37:** 310–317.

Tanaka, T., and others. 2007. Microbial community structure and function in the Levantine Basin of the eastern Mediterranean. Deep-Sea Res. I **54:** 1721–1743.

Thingstad, T. F., and others. 2005. Nature of phosphorus limitation in the ultraoligotrophic eastern Mediterranean. Science **309:** 1068–1071.

Tillett, D., and B. A. Neilan. 2000. Xanthogenate nucleic acid isolation from cultured and environmental cyanobacteria. J. Phycol. **36:** 251–258.

Van Mooy, B. A. S., G. Rocap, H. F. Fredricks, C. T. Evens, and A. H. Devol. 2006. Sulfolipids dramatically decrease phosphorus demand by picocyanobacteria in oligotrophic marine environments. Proc. Natl. Acad. Sci. USA **103:** 8607–8612.

Villareal, T. A., and E. J. Carpenter. 1990. Diel buoyancy regulation in the marine diazotrophic cyanobacterium *Trichodesmium thiebautii*. Limnol. Oceanogr. **35:** 1832–1837.

Vogel, S. 1988. Life's devices: The physical world of animals and plants. Princeton Univ. Press.

Zehr, J. P., and D. G. Capone. 2002. N_2 fixation in the marine environment, p. 2211–2221. *In* G. Bitton [ed.], Encyclopedia of environmental microbiology. John Wiley & Sons.

———, B. D. Jenkins, S. M. Short, and G. F. Steward. 2003. Nitrogenase gene diversity and microbial community structure: A cross-system comparison. Environ. Microbiol. **5:** 539–554.

———, J. P. Montoya, B. D. Jenkins, I. Hewson, E. Mondragon, C. M. Short, M. J. Church, A. Hansen, and D. M. Karl. 2007. Experiments linking nitrogenase gene expression to nitrogen fixation in the North Pacific Subtropical Gyre. Limnol. Oceanogr. **52:** 169–183.

———, AND P. J. TURNER. 2001. Nitrogen fixation: Nitrogenase genes and gene expression, p. 271–286. *In* J. H. Paul [ed.], Methods in marine microbiology. Academic Press.
———, J. B. WATERBURY, P. J. TURNER, J. P. MONTOYA, E. OMOREGIE, G. F. STEWARD, A. HANSEN, AND D. M. KARL. 2001. Unicellular cyanobacteria fix N_2 in the subtropical North Pacific Ocean. Nature **412:** 635–638.

Edited by: Wade H. Jeffrey

Received: 11 February 2008
Accepted: 12 September 2008
Amended: 18 September 2008