ORIGINAL ARTICLE

Diazotrophic bacterioplankton in a coral reef lagoon: phylogeny, diel nitrogenase expression and response to phosphate enrichment

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We investigated diazotrophic bacterioplankton assemblage composition in the Heron Reef lagoon (Great Barrier Reef, Australia) using culture-independent techniques targeting the *nifH* fragment of the nitrogenase gene. Seawater was collected at 3 h intervals over a period of 72 h (i.e. over diel as well as tidal cycles). An incubation experiment was also conducted to assess the impact of phosphate (PO_4^{3-}) availability on *nifH* expression patterns. DNA-based *nifH* libraries contained primarily sequences that were most similar to *nifH* from sediment, microbial mat and surface-associated microorganisms, with a few sequences that clustered with typical open ocean phylotypes. In contrast to genomic DNA sequences, libraries prepared from gene transcripts (mRNA amplified by reverse transcription-polymerase chain reaction) were entirely cyanobacterial and contained phylotypes similar to those observed in open ocean plankton. The abundance of *Trichodesmium* and two uncultured cyanobacterial phylotypes from previous studies (group A and group B) were studied by quantitative-polymerase chain reaction in the lagoon samples. These were detected as transcripts, but were not detected in genomic DNA. The gene transcript abundance of these phylotypes demonstrated variability over several diel cycles. The PO_4^{3-} enrichment experiment had a clearer pattern of gene expression over diel cycles than the lagoon sampling, however PO_4^{3-} additions did not result in enhanced transcript abundance relative to control incubations. The results suggest that a number of diazotrophs in bacterioplankton of the reef lagoon may originate from sediment, coral or beachrock surfaces, sloughing into plankton with the flooding tide. The presence of typical open ocean phylotype transcripts in lagoon bacterioplankton may indicate that they are an important component of the N cycle of the coral reef.

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Introduction

Coral reefs are important habitats as hotspots of biological diversity and productivity in the oligotrophic ocean (Capone, 1996). Tropical coral reefs are also among the most threatened marine ecosystems because of their susceptibility to elevated surface water temperatures (Hughes *et al.*, 2003; Pandolfi *et al.*, 2003). Most research in these habitats has focused upon the biology of conspicuous large metazoa and coral holobionts (Glynn, 1976, 1991; Koop *et al.*, 2001; Gardner *et al.*, 2003; Saxby *et al.*, 2003), whereas the microbial ecology of coral reef

ecosystems has received considerably less attention (Moriarty *et al.*, 1985; Moriarty and Hansen, 1990; Capone *et al.*, 1992; Capone, 1996; Wild *et al.*, 2004a, b, 2005). Several studies have demonstrated an enormous diversity of coral-associated bacteria and archaea (Cooney *et al.*, 2002; Rohwer *et al.*, 2002; Kellogg, 2004; Wegley *et al.*, 2004), and a recent study of reef flat sediment bacterial and diazotrophic assemblages demonstrated a large degree of patchiness, indicating that these systems have high diversity (Hewson and Fuhrman, 2006). Understanding the ecology of functional groups of bacterioplankton may help to further resolve nutrient cycling budgets in coral reef ecosystems (Charpy-Roubaud *et al.*, 1990; Charpy, 2001; van Duyl *et al.*, 2006).

Diazotrophic prokaryotes play a critical role in marine ecosystems as a source of N in marine food webs. Nitrogen fixation is carried out by a diverse suite of prokaryotes in marine plankton, including

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colonial and unicellular cyanobacteria and other eubacteria. Diazotrophic activity in oligotrophic waters supports phytoplankton N demand, and fixed N rapidly enters food webs (Carpenter and Romans, 1991). The ecology of diazotrophs has been investigated in both coastal and open ocean waters, however there have been no previous reports of diazotroph phylogeny in coral reef lagoons (RLs).

Gene-based approaches have been widely used to elucidate the ecology of microorganisms in natural systems. In the case of marine bacterioplankton, it is estimated that approximately 99% of taxa are as yet uncultured, including several dominant groups commonly observed in seawater using culture-independent techniques (Azam, 1998). Studies targeting functional genes have considerably advanced our understanding of the diversity and activity of biogeochemical processes in the ocean. Open ocean nitrogen fixation was traditionally ascribed to large colonial taxa of cyanobacteria (e.g. *Trichodesmium*), until studies of expression of the nitrogenase gene (*nifH*) in seawater revealed active nitrogenase expression by other cyanobacteria and bacterioplankton (Zehr *et al.*, 2001).

High bacterial growth rates (Moriarty *et al.*, 1985; Moriarty and Hansen, 1990) and low inorganic nutrient concentrations (Charpy-Roubaud *et al.*, 1990; van Duyl *et al.*, 2006) imply nutrient limitation of microorganisms in coral reef waters. Nitrogen limitation of microorganism growth is believed to promote nitrogen fixation by microorganisms in coral reef sediments (Capone *et al.*, 1992; Capone, 1996; Koop *et al.*, 2001) and surfaces (Charpy-Roubaud *et al.*, 2001; Charpy-Roubaud and Larkum, 2005). Nitrogen fixation rates have been documented in coral reef bacterioplankton associated with the $<10\mu\text{m}$ size fraction in two coral RLs (Indian Ocean and New Caledonia), equivalent to 20–40% of new production (Charpy, 2005). However, the supply of nitrogen to lagoons and surrounding waters is controlled by reef morphology (Dufour *et al.*, 2001), and the concentration of organic N and

inorganic N in coral lagoons is typically higher than in surrounding waters owing to turbulent mixing on sediment surfaces (Charpy-Roubaud *et al.*, 1990). Reef cavities, which are present throughout the coral reef framework, are a source for oxidized nitrogen species (however not for NH_4^+ , possibly due to nitrification) (van Duyl *et al.*, 2006). Tidal flushing through coral reef framework may thus cause enhanced nutrient (including nitrogen) concentration in lagoon waters, which may ultimately control planktonic biogeochemical processes.

This study aimed to determine whether hydrodynamic shifts associated with tidal influence, together with diel cycling, influence the observed patterns in diversity and expression of nitrogenase (*nifH*, nitrogen fixation) genes in coral RLs. Water column samples were collected at regular time intervals and over several tidal cycles, and the phylogeny, abundance and expression patterns of diazotrophs studied. A phosphate-enrichment experiment was conducted simultaneously to examine the relationship between phosphate availability and nitrogen cycling activities. Our results indicate that the composition and activity of diazotroph assemblages is affected by tidal cycles, and that some diazotrophs are responsive to phosphate availability.

Materials and methods

Sampling location

Samples were collected near the Heron Island Research Station, situated on Heron Reef, Great Barrier Reef, Australia, in January 2006. Samples for the diel coral RL study were taken approximately 5 m from the high tide mark on the reef flat (total water depth was approximately 50 cm at low tide, and 2.5 m at high tide) within the gutter zone of the reef (Figure 1). The samples for the phosphate-enrichment experiment were taken at 1545 from the Gutter Region in approximately 50 cm of water (total water depth), while the tide was receding.

Figure 1 Map of sampling locations in the Heron Reef lagoon. The dominant flow directions are indicated during flood and ebb tides.

Reef lagoon sampling

Reef lagoon sampling began on 1745 on 2 January 2006, and concluded at 0015 on 5 January 2006. Samples were collected from the sampling location every 4–6 h using an acid-washed and sample-rinsed bucket, which was submerged to collect water from the current. The water was then poured into duplicate 4 liter LDPE cubitainers (Nalgene I-Chem, Rockwood, TN, USA), which were transported immediately to the laboratory for processing. One cubitainer was filtered for DNA analysis, whereas the other was processed for RNA retrieval.

Phosphate-enrichment experiment

Samples were withdrawn from the sampling location by immersing sample-rinsed 1.2 l Whirl Pak bags (Nasco, Fort Atkinson, WI, USA) directly into seawater. A total of 52 samples were collected, and half of the bags were enriched with $1 \mu\text{M}$ K_2HPO_4 . The incubations were then placed in outdoor aquaria with 25% attenuated photosynthetically active radiation and with a flow-through seawater supply (to maintain temperature $\pm 1^\circ\text{C}$ ambient). Bags were harvested every 4–6 h after inoculation. DNA samples were harvested every 12 h, whereas RNA samples were retrieved every 6 h. The incubation experiment continued for a total of 72 h.

Filtration for nucleic acids

Samples for DNA analysis were filtered through $10 \mu\text{m}$ pore-size polyester, 25 mm diameter filters (Osmonics, Trevose, PA, USA) and onto $0.2 \mu\text{m}$ pore size, 25 mm diameter Supor filters (Pall Gelman, East Hills, NY, USA) filters (PO_4^{3-} enrichment experiment) or 47 mm diameter $0.2 \mu\text{m}$ Supor filters, both using a peristaltic pump filter manifold. After processing, the filters were placed in sterile Whirl Pak bags and frozen at -70°C until extraction. Samples for RNA analysis were filtered through 25 mm, $0.2 \mu\text{m}$ Supor filters, placed after processing in a Whirl Pak bag and frozen at -70°C until analysis.

Extraction of DNA and RNA

DNA was extracted from samples in the RL sampling using a phenol/chloroform protocol (Fuhrman *et al.*, 1988) with modifications for 47 mm diameter filters (Hewson and Fuhrman, 2004). DNA on 25 mm diameter filters (i.e. the PO_4^{3-} enrichment experiment) was processed following a xanthogenate-based extraction procedure (Tillett and Neilan, 2000). The reason that two extraction procedures were followed for DNA is that in our experience, the xanthogenate protocol provides the best recovery from smaller-sized filters with less sample volume and with some downstream polymerase chain reaction (PCR) inhibition, whereas the phenol/chloroform protocol provides the best DNA recovery

in larger sampling volumes or filter sizes with little PCR inhibition.

RNA samples (both 47 and 25 mm filters) were extracted using the RNEasy kit (Qiagen, Valencia, CA, USA) with the following modifications. After placing the filter in a microcentrifuge tube containing approximately $100 \mu\text{l}$, 0.1 mm diameter glass beads (BioSpec Inc., Bartlesville, OH, USA), the filters were amended with $350 \mu\text{l}$ buffer RLT, and the tubes bead-beaten in a BioSpec beadbeater for 6 min. After bead-beating, the samples were then centrifuged for 1 min at $3000g$ to remove large cell debris, after which the supernatant was transferred to a new eppendorf tube. After this step, the manufacturer's protocols were followed for RNA recovery. DNA was quantified after extraction using Pico Green Fluorescence (Molecular Probes Inc., Eugene, OR, USA), and RNA quantified using Ribo Green Fluorescence (Molecular Probes Inc.) in an Applied Biosystems International 7500 real-time PCR system (Foster City, CA, USA).

Clone library preparation

A clone library of *nifH* was prepared for each of five DNA and two RNA samples. The samples used for Heron Island represented RL DNA samples at midnight (20113, 20014) and midday (20125, 20126), RNA samples at those same times (20115 and 20127, respectively) and at the beginning (19410, 20053), middle (20107, 20108) and end (20148, 20149) of the PO_4^{3-} incubation experiment. A total of 283 clones were sequenced from all samples. The clone libraries were prepared as follows:

The PCR was used to amplify *nifH* from DNA and reversely transcribed RNA (see next section) using degenerate primers and a nested approach (Zehr and McReynolds, 1989; Zehr *et al.*, 1998; Zani *et al.*, 2000). Briefly, $2 \mu\text{l}$ of DNA or cDNA was added to PCR mixtures containing $1 \times$ PCR buffer, 4.0 mM MgCl_2 , 0.2 mM of each dNTP (Promega PCR Nucleotide Mix, Madison, WI, USA), 100 pmol each of primers *nifH3* (5'-ATRTTRTTNGCNGCRTA-3') and *nifH4* (5'-TTYTAYGGNAARGGNGG-3'), $40 \text{ ng } \mu\text{l}^{-1}$ bovine serum albumin (Sigma # 7030, St Louis, MO, USA), and 2.5 U of Taq Polymerase (Promega). The PCR were performed in a MJ Research Dyad (Hercules, CA, USA) thermal cycler with an initial heating step at 95°C for 3 min, followed by 25 cycles of denaturation at 95°C for 30 s each, annealing at 57°C for 30 s and extension at 72°C for 45 s. The cycling protocol ended with a final extension step at 72°C for 7 min. After thermal cycling, the products ($2 \mu\text{l}$) were subjected to a second round of PCR, identical to the first, but containing primers *nifH1* (5'-TGYGAYCCNAARGCNGA-3') and *nifH2* (5'-ANGCCATCATYTCNCC-3'), which were subjected to 30 PCR cycles. PCR products were run on a 25 cm long, 1.2% agarose gel, for 1.5 h at 85 V, which was poststained in $1 \times$ SYBR Gold for 30 min. Amplicons were visualized on a BioRad GelDoc

system (Hercules, CA, USA). Bands corresponding to *nifH* amplicons (i.e., at 324 bp) were excised using a sterile razor blade and placed immediately into sterile 2 ml microcentrifuge tubes. Amplified DNA was extracted from the gel slices using Gel Recovery kits (Zymo Research Inc., San Diego, CA, USA).

RNA amplification followed the same protocol as DNA, but with an initial reverse transcriptase step to convert RNA into cDNA. This comprised the Superscript III (Invitrogen, Carlsbad, CA, USA) protocol, where 7 μ l of extracted RNA (containing 0.25–0.5 ng RNA) was treated with 1 μ l of reverse transcriptase, 1 μ l of RNase OUT and 100 pmol each of primers *nifH*2 and 4, and incubated at 50 min at 45°C. cDNA (2 μ l) was added as template material in the PCR.

The purified DNA amplicons were cloned into the PGEM-T Easy Vector II system (Promega), which utilized blue/white discrimination of transformed JM109 competent *Escherichia coli* cells. Transformants were picked into 1 ml overnight Luria-Bertani broth liquid cultures containing 12% glycerol. After incubation at 300 r.p.m. in a shaking 37°C incubator, transformant cultures were frozen, and sent to the University of Florida Interdisciplinary Center for Biotechnology Research for sequencing using TempliPhi preparation of sequencing template material and an Amersham MegaBACE capillary sequencer.

Phylogenetic analysis

Sequence chromatograms were first consulted to resolve ambiguous bases, using the Chromas Lite chromatogram viewer (Technelysium Pty Ltd, Mt Gravatt, Australia). Sequences were transferred to the BioEdit (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) program (Hall, 1999). Sequences were searched against the nonredundant nucleotide GenBank database (NCBI, www.ncbi.nlm.nih.gov) using the online BLASTN function (Altschul *et al.*, 1997). Closest matches in GenBank for each sequence or cluster of sequences were assembled into a BioEdit project file. All sequences within the database were then aligned using HMMER (profile hidden Markov model software, www.sanger.ac.uk or hmmer.wustl.edu) (Eddy, 1998) and phylogenetic trees constructed using ClustalX (Thompson *et al.*, 1997) including bootstrap support of 1000 replicates. Phylogenetic trees were viewed using the Treeview program (Page, 1996). The *nifH* sequences in this study have been deposited in GenBank under accession numbers EF174667–EF174886.

Quantitative PCR of *nifH* phylotypes

Quantitative PCR (qPCR) and reverse transcription-polymerase chain reaction (qRT–PCR) was conducted on samples from the RL and PO_4^{3-} enrichment studies using primer and probe sets specific to group A and group B cyanobacteria, *Trichodesmium*, and a heterocystous endosymbiont in diatoms (Short *et al.*, 2004; Church *et al.*, 2005a,b).

Additionally, two new primer probe sets (Azosp_1 and HIProt_1), specific to phylotypes that had a high occurrence in clone libraries (i.e. >5 sequences in each cluster) and which were present in both RF and PO_4^{3-} enrichment studies were designed with the aid of the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Table 1). Quantitative PCR was conducted on DNA extracts and cDNA according to the protocol as follows. Briefly, 2 μ l of DNA extract was placed in each of duplicate 25 μ l qPCR reactions containing 1 $\times$ Master Mix (ABI Taqman Universal PCR Master Mix, Foster City, CA, USA), 400 pmol of each primer and 200 pmol of probe. The qPCR thermal cycling program consisted of a 10 min heating step at 60°C, followed by a hot start at 95°C for 10 min. After this initial treatment, the amplification cycling program consisted of 15 s at 95°C, followed by annealing at 60°C for 1 min (for group A, group B and *Trichodesmium* primer/probe sets) or 57°C for 1 min (for Azosp_1 and HIProt_1 primer/probe sets), after which fluorescence data were collected over 60 thermal cycles. Dilution series (tenfold dilutions over eight orders of magnitude) of plasmids containing *nifH* amplicons as inserts were used for standards. Six negative controls were also run in addition to the qPCR standards. The method for calculation of *nifH* quantity is outlined in Church *et al.* (2005a,b).

Oligonucleotide microarray analysis

The *nifH* 60-mer oligonucleotide microarray consisted of 768 *nifH* probes (Moisander *et al.*, 2006) with each probe duplicated within each array slide. The microarray was constructed using the same slide chemistry as an earlier version of the array constructed with 96 probes and described in Moisander *et al.* (2006). Additionally, six control probes were included on the array, repeated throughout the array and used as positive and negative controls as described previously (Moisander *et al.*, 2006). The microarray probes were 5'-modified with acrydite for signal amplification. Probes were included on the array that targeted sequences distributed in all the known clusters of the *nifH* phylogenetic tree (Zehr *et al.*, 2003). Based on prior tests of hybridization stringency in the

Table 1 Primer and probe sets used in quantitative PCR in this study for detection of noncyanobacterial diazotrophic phylotypes

Primer name	Primer direction	Primer/probe sequence (5'-3')
HIProt_1	F	TCCACGCGTTTAAATCCT
	R	CTCAACACAGCGCACACA
Azosp_1	Probe	ATGTATTAAAAGTCGGTTACGGC
	F	TGAGATGGGCTCGGTT
	R	GGACCACCGGATTCAA
	Probe	GAAGGATGTTCTGAAGATTGGTTAC

conditions used, crosshybridization at a >77% sequence identity was expected in microarray hybridizations. However, the specificity is generally higher than the low limit (Moisander *et al.*, submitted).

Amplifications of *nifH* for microarray hybridizations were carried out following protocols for the clone libraries with the exception that amplification for each sample was carried out in triplicate, to provide a more representative PCR sample. After the first round of PCR the replicates were combined, and this pooled product was used as a template in the second round PCR reaction. PCR products were gel purified, quantified and then biotinylated using the Brightstar Psoralen-Biotin kit (Ambion, Austin, TX, USA) according to the manufacturer's protocols. In each biotinylation reaction, 10 μ l of PCR product at 5 ng μ l⁻¹ was used in 20 μ l initial reaction volume (the rest was 5 μ l TE buffer and 5 μ l Positive Control Mix, which contained PCR product targets for human gene oligonucleotides printed on the microarray, as described in a previous study (Moisander *et al.*, 2006)). Before hybridization, the biotinylated products (target) were denatured at 96°C for 5 min, then 13.2 μ l target was added to the hybridization buffer containing 2 $\times$ SSPE, 1% sodium dodecyl sulfate and 30% formamide (final concentrations) with 25 μ l final volume. The target was added on microarray slides that were covered with microarray cover slips and then hybridized at 50°C for 18 h. After hybridization, the slides were washed and subjected to secondary staining with streptavidin Alexa-555 stain (Molecular Probes) as described previously (Moisander *et al.*, 2006). The slides were scanned using an Axon 4000B (Molecular Devices, Sunnyvale, CA, USA) microarray scanner with 10 μ m pixel size. Detection threshold was determined and the data were normalized using the positive controls as previously described (Moisander *et al.*, 2006).

Results

Different extraction protocols were used for DNA and RNA from collected bacterioplankton samples. This was because there exists no single protocol with equal extraction efficiency for both nucleic acid types. Some variation in the results between RNA and DNA may have resulted because different extraction protocols were used.

Phylogeny of diazotrophic assemblages

nifH was amplified from two size fractions (>10 and 10–0.2 μ m) of DNA from a total of five samples (283 clones total); two samples from the diel RL sampling, representing midnight and midday, and three from the PO₄³⁻ amendment experiment (controls from initial, 24 and 48 h). The *nifH* clone libraries contained a large number of distinct

phylotypes (collectively 120, and between 17 and 46 per library), with few repeat sequences.

The DNA library contained roughly even numbers of sequences from Cluster I (cyanobacteria and proteobacteria) and Cluster III (anaerobic bacteria and archaea) *nifH*. Two Cluster I sequences were present in four of the five libraries (Figure 2), the first most similar to *Vibrio* sp. and the other phylotype was most similar to the sequence from *Azospirillum* sp. Most sequences in the libraries were noncyanobacterial, with few representatives clustering with known unicellular diazotrophic cyanobacteria. Of cyanobacterial phylotypes, most recovered from RL waters were similar to *Chroococcidiopsis thermalis* originally cultured from gypsum rock (Boison *et al.*, 2004), although sequences similar to those recovered from marine mats, *Xenococcus* sp. and *Myxosarcina* sp. were also recovered. Most noncyanobacterial sequences were most similar to uncultured diazotrophic phylotypes originating in marine and estuarine sediments, or phylotypes from marine microbial mats. Cluster III sequences (Figure 3) detected in Heron Island were most similar to phylotypes recovered from the Chesapeake Bay (Steward *et al.*, 2004) and from marine mats in Guerrero Negro, Baja California (Omeregie *et al.*, 2004a,b). Despite the presence of *Trichodesmium* blooms in the region at the time of sampling only one *Trichodesmium*-like sequence was recovered from the samples.

In contrast to the DNA library, RNA-derived sequences were exclusively cyanobacterial phylotypes at both midnight and midday. At midnight, the sequences clustered with group B unicellular diazotrophic cyanobacteria (similar to *Crocospaera watsonii* WH8501), and with phylotypes of heterocystous cyanobacteria typically found as endosymbionts in higher organisms (e.g. *Richelia* sp.). The midday sequences clustered with the uncultivated group A unicellular diazotrophic cyanobacteria, and also the heterocystous phylotypes found in the midnight sample. Noncyanobacterial diazotroph sequences were not detected in the two RNA-based libraries.

Quantitative PCR of nifH

The abundances of group A, group B and *Trichodesmium* phylotypes were below the detection threshold of approximately 1 copy per milliliter of our qPCR assays. However, gene transcripts of group A, group B and *Trichodesmium nifH* were detected in RNA extracts from bacterioplankton in lagoon waters, and group A *nifH* transcripts were detected in the PO₄³⁻ incubations. The two primer sets designed around noncyanobacterial sequences that were present in clone libraries were not detected in either DNA or RNA, suggesting their abundance and expression was also below detection thresholds.

The expression patterns of different phylotypes echoed observations of diel cycles in previous

Figure 2 Phylogenetic representation of Cluster I *nifH* sequences derived from Heron Island bacterioplankton. The tree was constructed by first aligning sequences using HMMER (Eddy, 1998), then tree constructed using ClustalX using a neighbor joining algorithm and bootstrap support of 1000 iterations. The clusters of sequences for which qPCR primer/probe sets were developed are indicated by arrows. The group A and group B cyanobacterial diazotrophic sequences are indicated by parenthesis.

Figure 3 Phylogenetic representation of Cluster III *nifH* sequences derived from Heron Island bacterioplankton. The tree was constructed by first aligning sequences using HMMER (Eddy, 1998), then tree constructed using ClustalX using a neighbor-joining algorithm and bootstrap support of 1000 iterations.

studies (Zehr *et al.*, 2007). The group A cyanobacterial phylotype was only detected during daylight hours, however it was not detected in several day samples (Figure 4). The group B cyanobacterial phylotype was detected mostly at night and until dawn, however was also detected during the day in one sample. *Trichodesmium* was detected during both day and night. The greatest transcript abundance was detected for group B cyanobacteria at midnight. Expression of *nifH* was detected primarily during slack and ebb tides in the lagoon.

Comparison of microarray fingerprints

Microarray fingerprints from DNA and RNA were consistent with *nifH* sequence libraries obtained for the same samples. Hybridizations with the highest signal intensity varied between samples, and between size fractions. Most DNA fingerprints had greatest signal intensity from a probe designed for a sequence recovered from the North Pacific Ocean (AY191947, putatively similar to *nifH* from *Rhodobacter azotoformis* (Falcon *et al.*, 2004)). Targets in the $>10\ \mu\text{m}$ size fraction collected between 1000 and 1030 (20082 and 20113) were detected by probes for sequences originating from *Spartina*-associated microbial communities, *Trichodesmium thiebautii*, *Lyngbya majuscula* and noncyanobacterial sequences from the Chesapeake Bay plankton and estuarine sediments. The $0.2\text{--}10\ \mu\text{m}$ size fraction (20114) had similar hybridizations. The hybridizations from the $>10\ \mu\text{m}$ sample at midnight to 0100 (20113, 20126 and 20145) had detected noncyanobacterial and cyanobacterial microbial mat sequences, as well as plankton sequences from the Chesapeake Bay, which was largely the same as for the $0.2\text{--}10\ \mu\text{m}$ size fraction (20114, 20125 and 20146) (Figure 5).

In the RNA daylight sample (20127), probes for cyanobacterial diazotrophic phylotypes similar to group A cyanobacteria (clones HT19S19 and HT1902), as well as to the heterocystous cyanobacteria *Cylindrospermopsis raciborskii*, *Anabaena* sp. and *Tolypothrix* sp. detected target. The night sample (20115) was dominated by signal from hybridization to *C. watsonii* WH8501, *Anabaena* sp., *Nostoc* sp., *C. raciborskii*, as well as to other group B cyanobacterial phylotypes previously observed at Station ALOHA (clones HT265A107 and HT1900).

Phosphate enrichment experiment

No gene transcripts of group A, *Trichodesmium*, HIProt_1 and Azosp_1 phylotypes were detected in RNA extracts, and no phylotype was detected in DNA extracts from the phosphate enrichment experiment. Group B gene transcripts were detected in all except one incubation (a control incubation after 12 h) (Figure 6), and numbers of gene transcripts corresponded with gene copy abundances detected in lagoon diel samples. Unlike the RL sampling, gene expression tightly followed light availability, with greatest transcript abundance in the dark, and least during day. There was no significant difference between controls and PO_4^{3-} incubations in transcript abundance during the dark period at initial, after 24 and 48 h incubation, however after both 24 and 48 h, the addition of PO_4^{3-} significantly ($P < 0.05$, Student's *t*-test) decreased transcript abundance relative to controls.

Discussion

The results show that diazotroph assemblages are present in coral RL bacterioplankton, that they are comprised of noncyanobacterial as well as cyano-

Figure 4 Quantitative PCR results for the lagoon sampling, over three days. Symbols below the x-axis line indicate that the phylotype was not detected in the sample. The black bars on top of the graph indicate the length of the dark phase. Solid line indicates the tidal height. Error bars = s.e.

Figure 5 Results of microarray hybridization analysis of diazotroph assemblages based upon amplicons of the *nifH* gene. Shown are the 10 strongest hybridization signals in each sample as a percentage of total hybridization signal.

bacterial phylotypes and that they are likely to be actively fixing nitrogen in this environment. However, our results also suggest that diazotrophic assemblage composition and activity may be responsive to interactions between the water column, sediment, beachrock or benthic faunal surfaces, both directly in terms of the types of diazotrophs that may be sloughed into plankton, or indirectly by controls benthos place upon the availability of inorganic and organic materials. Moreover, our results suggest that although diazotrophs may follow diel cycles in dinitrogenase gene expression, observations of this cycling at a fixed location are affected by hydrodynamics, which may contribute to high variability in the diazotroph abundances over the diel cycle.

Phylogeny of diazotrophic assemblages

Sequence libraries from the RL and from controls in the PO_4^{3-} incubation experiment demonstrated

that diazotrophic assemblages contain both non-cyanobacterial and cyanobacterial components, including phylotypes that are well documented in other systems (e.g. unicellular diazotrophic cyanobacteria) (Zehr *et al.*, 2001; Falcon *et al.*, 2002; Mazard *et al.*, 2004; Bird *et al.*, 2005; Church *et al.*, 2005a,b; Langlois *et al.*, 2005). However, there were several unexpected features of the sequence libraries.

At the time of sampling, large and dense *Trichodesmium* surface aggregations were noted in waters offshore of Heron Reef, and it was anticipated that sequences of this colonial phylotype may dominate clone libraries, particularly those prepared from the $>10\mu\text{m}$ size fraction. However, only a single sequence was found which was similar to *Trichodesmium erythraeum*; and gene transcript abundances for *Trichodesmium* were low throughout the sampling period. The low representation in clone libraries was confirmed by microarray analysis. Furthermore, *Trichodesmium* could not be detected

Figure 6 Quantitative PCR results for the PO_4^{3-} -enrichment experiment for group B unicellular cyanobacterial diazotrophs. The black bars on top of the graph indicate the length of the dark phase. Error bars = s.e. Significant differences between control and PO_4^{3-} -enriched incubations ($p < 0.05$, Student's *t*-test) are given by *.

using qPCR of genomic DNA, suggesting that within our samples they comprised <1 cell per milliliter.

The fate of *Trichodesmium* in marine waters has been somewhat of a mystery, because grazing by planktonic copepods (O'Neil and Roman, 1994), disintegration by viruses (Ohki, 1999; Hewson *et al.*, 2004) and autocatalytic mortality (Berman-Frank *et al.*, 2004) account each for only a small proportion of total biomass under natural conditions in the open ocean. A previous study of *Trichodesmium* viral activity in a coral cay suggested that most filaments in lagoon waters visibly contained phage, whereas those outside the reef did not (Ohki, 1999). Hence, one possible reason that the *Trichodesmium* were not observed in our libraries is that they had disintegrated during residence in lagoonal waters. It is also possible that the filaments were removed by grazing of corals themselves as the blooms advected over the reef crest. Ciliates and other eukaryotes within the same size range as *Trichodesmium* colonies are removed rapidly from lagoon waters (50–70% of total production) (Ferrier-Pages and Gattuso, 1998) so it is reasonable to assume that *Trichodesmium* may be trapped and consumed by feeding corals.

The second unexpected result was the large number of Cluster III and microbial mat phylotype *nifH* sequences derived from plankton samples. Cluster III *nifH* sequences include those from anaerobic bacteria and archaea (Zehr *et al.*, 2003), and although a few have been observed in open ocean surface waters (Zehr *et al.*, 2001; Church *et al.*, 2005a,b), their diversity there is typically not as great as observed in the lagoon libraries (Zehr *et al.*, 2001; Falcon *et al.*, 2002; Church *et al.*, 2005a,b). The large number of sequences from multiple libraries clustering with a putative *nifH2* phylotype of *C. thermalis* was surprising, because this group is infrequently observed in marine plankton clone libraries (Zehr *et al.*, 1998, 2001). This group of

cyanobacteria includes relatives that are terrestrial dessication- and UV (ultraviolet)-resistant (Billi and Caiola, 1996; Billi *et al.*, 2000; Boison *et al.*, 2004); hence, occurrence of cyanobacteria related at the nitrogenase level in coral reef bacterioplankton suggested that they may have originated from rock surfaces, and become sloughed off from the tideline.

High diversity of Cluster III diazotrophs in the lagoon water suggests these organisms are resuspended from benthos to the overlying waters at certain tidal phases. As with most coral reefs, the Heron Reef lagoon is higher in elevation than the low-tide water level. During flood tides, water entering the RL advects over coral and sediment surfaces at the reef crest, and is also flushed through the reef framework (Haberstroh and Sansone, 1999). At low tide, corals produce mucus, which serves as osmoprotectant and UV 'sunscreen', which is heavily colonized by microorganisms, and is highly labile for bacterial uptake (Wild *et al.*, 2004a,b, 2005). Coral surfaces contain an extremely diverse suite of bacteria (Rohwer *et al.*, 2002) and archaea (Kellogg, 2004; Wegley *et al.*, 2004), which are coral-specific (Rohwer *et al.*, 2002). Additionally, coral sediments have been demonstrated to contain diverse and active diazotroph assemblages (Hewson and Fuhrman, 2006). Thus, some of the mat-forming bacteria on coral tissue surfaces may become planktonic when they are attached to the coral mucus. We speculate that the presence in plankton of diazotrophs similar to cyanobacteria, which are characteristically dessication-resistant and UV-tolerant, may also result from this resuspension. We also speculate that these cyanobacteria may be associated with exposed surfaces at low tide, possibly on the coral tissues itself.

Surprisingly, all nitrogenase sequences in our libraries expressed as transcripts were cyanobacterial. The phylotypes expressing their genes – group A, group B and a group most closely related to

Richelia – have been observed previously in open ocean bacterioplankton (Zehr *et al.*, 1998, 2001; Falcon *et al.*, 2002, 2004; Mazard *et al.*, 2004; Church *et al.*, 2005a,b; Langlois *et al.*, 2005).

The *nifH* microarray results were coherent with clone library observations. DNA-based microarrays contained a wide diversity of detectable hybridizations, and dominant signals came from phylotypes with greatest representation in our clone libraries. In most fingerprints, the signal was dominated by a hybridization to a phylotype from the vicinity of Station Aloha (Falcon *et al.*, 2002). However, RNA-based microarray results demonstrated that almost all signal came from cyanobacterial phylotypes observed in clone libraries. Interestingly, most of the phylotypes hybridized from the $>10\ \mu\text{m}$ size fraction were also present in the $0.2\text{--}10\ \mu\text{m}$ size fraction fingerprint. This may lend further support to the idea that bacterial diazotrophs were particle-associated (possibly on coral mucus), where some become trapped on the $10\ \mu\text{m}$ filter, whereas others disassociate from the particles and pass through, becoming trapped on the $0.2\ \mu\text{m}$ filter.

The microarray fingerprints also demonstrated heterogeneity in bacterioplankton assemblages over time within the lagoon, as well as between different size fractions. Samples collected on different tidal cycles at the same stage (high slack) shared some of the same components, however, the proportions of hybridization signal was partitioned differently between phylotypes. There was 60% similarity between all microarray fingerprints, in line with observations of benthic bacterial variability on Heron Reef (Hewson and Fuhrman, 2006). This suggests that different water masses may have been present within the lagoon, or that the dynamics of resuspension of benthic- or surface-associated organisms is variable in time or space.

Diel expression patterns of diazotrophs

Nitrogenase activity follows a diel cycle in most autotrophic organisms, potentially regulated by oxygen tension (Gallon, 1981), circadian rhythm (Grobelaar and Huang, 1992) and the C:N content of cells (Rabouille *et al.*, 2006). As nitrogenase is oxygen sensitive (Hill, 1992), it was initially believed that cyanobacteria were either limited to nitrogen fixation at night, or within specialized cells, which isolated O_2 produced during photosynthesis from the nitrogenase enzyme (Gallon, 1981). However, it is now known that nitrogen fixation occurs in nonheterocystous filamentous and unicellular diazotrophic microorganisms during daylight and when cells are actively photosynthesizing (Villareal, 1991; Prufert-Bebout *et al.*, 1993; Zehr *et al.*, 2001). Diel expression cycles of nitrogenase in distinct diazotrophic groups have been studied previously at a station in the open ocean (Zehr *et al.*, 2001; Montoya *et al.*, 2004; Church *et al.*, 2005a,b), and within bottle incubations

(Zehr *et al.*, 2007). Both of these studies concur that although the group B cyanobacterial diazotroph phylotype (which includes the cultivated *C. watsonii*) fixes N_2 at night, the group A cyanobacterial diazotroph phylotype (of which there are no cultivated representatives) appears to fix N_2 during the day, at the same time as *Trichodesmium* and *Richelia intracellularis*.

Observations by qPCR of the expression pattern of three groups of diazotrophs (group A and B unicellular cyanobacteria) and *Trichodesmium* indicated that although most of the pattern was coherent with previous studies (i.e. group A and *Trichodesmium* expressed during the day, group B at night), there were exceptions. At several times within each illumination phase, nitrogenase expression in phylotypes at their illumination optimum was not detected. For example, during the light phase, nitrogenase activity was only detected in group A cyanobacteria twice, both at slack or ebb tides. Similarly, *Trichodesmium* expression was only detected at slack high and ebb tides during the light phase. As group B transcripts were observed once at dusk on the third sampling day, for the other two dark periods, expression was only detected at slack high and ebb tides. We believe this could be a consequence of enhanced nutrient availability in lagoon waters during a part of the tidal cycle.

During flood tide, advection of waters through the reef framework and across sediment surfaces likely brings into lagoon waters large concentrations of inorganic and organic nutrients, including N (Haberstroh and Sansone, 1999). This effect has been observed previously in coral lagoons in Tahiti (Charpy-Roubaud *et al.*, 1990). Nitrogenase activity is inhibited in cultures by the addition of NH_4^+ (Klugkist and Haaker, 1984), an effect brought about at the transcription level by binding of a nitrogenase operon-encoded protein binding to the nitrogenase gene and blocking transcription (Merrick, 1992). Thus, if higher concentrations of inorganic nutrients are present in lagoon waters upon flooding, this may prevent expression of *nifH* until nitrogen starvation. Organic or inorganic nutrients brought into lagoonal waters are rapidly assimilated by bacterioplankton (their growth rates of 1.5–3/days place a heavy demand upon available resources) (Sorokin, 1994; Ferrier-Pages and Gattuso, 1998), and as a consequence nitrogen limitation may occur within hours of becoming available. Thus, at slack or high tide, the expression pattern we observed may be a consequence of nitrogen limitation. However, it is important to note that we cannot discount the possibility that some of the variation in expression pattern may be due to different abundances of diazotrophs advecting on and off the reef with different tides.

The RNA-based microarrays provided comparable results to the expression patterns observed via qPCR. During the dark phase, the hybridization

signal was dominated by the group B cyanobacterial phylotypes and heterocystous cyanobacteria, whereas during the day the signal was dominated by signal from group A cyanobacteria.

Interestingly, the two diazotrophic, noncyanobacterial phylotypes, which occurred in multiple libraries and repeated within libraries could neither be detected by qPCR in DNA nor in RNA. This suggests that overall diazotroph abundance was low in the samples analyzed, assuming that commonly occurring components of clone libraries are dominant, or at least subject to the same PCR biases in qPCR as in normal PCR.

Response of diazotroph phylotypes to phosphate enrichment

The results of the PO_4^{3-} enrichment experiment lent weight to the idea that tidal enrichment of lagoon waters may cause noise in the expression patterns of different diazotrophic phylotypes. Unlike the lagoon sampling, the expression pattern of group B diazotrophs in incubation bags demonstrated greater expression during the dark phase than during the light phase, with highest expression at midnight, and least at dawn and dusk. Group A, *Trichodesmium*, and a fourth phylotype, *Richelia* were not detected in incubations in either DNA or RNA. Furthermore, the group B phylotype was not detected in DNA from the incubations, suggesting their abundance fell below the detection threshold for this assay of one gene copy per milliliter.

PO_4^{3-} had no significant impact upon the expression rate of group B unicellular diazotrophs. PO_4^{3-} concentrations on coral reefs are typically low owing to adsorption of P onto carbonate surfaces (Charpy, 2001). The addition of PO_4^{3-} to microatolls has been shown to stimulate the activity of nitrogen fixation in sediments (Koop *et al.*, 2001). Previous bottle incubation experiments of oligotrophic surface waters indicated that PO_4^{3-} had no significant impact on the abundance or expression ratio of four groups of diazotrophs, however these experiments were only 48 h in duration (Zehr *et al.*, 2007). Our results suggest that responses to inorganic nutrient addition may occur over longer time scales, perhaps in line with growth rates of open ocean cyanobacteria.

Conclusions

Our results demonstrate that diazotrophs are present and are an active component of the microbial loop in coral reef ecosystems. These data suggest that coral reefs may be sources of bacterioplankton taxa to the lagoon, however active nitrogen fixation appears to be constrained to oligotrophic, open ocean cyanobacterial taxa advected into the coral reef from surrounding waters, which are impacted by reef nutrient cycling. Finally, this study raises interesting

questions about the fate of diazotrophic organisms as they are advected into surrounding, interreef waters.

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