Supplementary Information

High levels of heterogeneity in diazotroph diversity and activity within a putative hotspot for marine nitrogen fixation

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MATERIALS AND METHODS

Sample Collection

Vertical profiles of temperature, salinity, oxygen, photosynthetically active radiation (PAR), and fluorescence were measured at each site using a conductivity-temperature-depth (CTD) recorder (Seabird SBE 911; Supplementary Table 1). Samples were collected from the surface at all CTD stations and from the chlorophyll (indicated by fluorescence) maximum (cmax) when it was discernible. When the water column was well-mixed, for example exhibiting no stratification of temperature or salinity, and no peaks in fluorescence, samples were collected from the surface only.

Inorganic nutrient analysis

Dissolved nutrient analyses (NO_3^- + NO_2^-, PO_4, NH_3, SiO_2) were conducted on board the research vessel using fresh samples. Nitrate + nitrite, phosphate and silicate concentrations were determined via Flow Injection Analysis (Lachat; detection limit 0.02 µmol L^{-1}).

Ammonium samples were analysed using an AA3HR Segmented Flow nutrient analyser (Seal Analytical; detection limit 0.01 µmol L^{-1}).

Photosynthetic pigment analysis

Seawater samples (2.2 L) were filtered through 0.74 µm glass fibre filters (GF/F, Whatman, UK), snap-frozen in liquid nitrogen and stored at -80°C prior to extraction in 90% acetone. The photosynthetic and photoprotective pigment composition was measured using High Performance Liquid Chromatography (HPLC) via the gradient elution procedure of van Heukelem and Thomas (2001) on a Waters HPLC system with an Agilent 1200 series column.

^{15}N-N_2 Incubation Experiments

Briefly, acid-rinsed 4 L Nalgene polycarbonate incubation bottles were filled to overflowing via silicone tubing directly from Niskin bottles. Bottles were capped headspace free, followed by an injection of 3 ml ^{15}N-N_2 (98 atm%, Sigma Aldrich) using a gas tight syringe. Bottles were inverted 100 times and then maintained at *in situ* sea surface temperatures in deck board incubators continuously filled with flow-through surface seawater, and *in situ* irradiance was simulated by shading with shade cloth and appropriate layers of blue filter (061 Mist Blue, Lee Filters), according to PAR values obtained from vertical CTD casts. Samples for whole community (WC) and unicellular size fraction (USF) rates were incubated in triplicate for 24 h and terminated by filtration, either directly onto a pre-combusted GF/F filter (0.7 µm; Whatman, Kent, UK; WC) or through a 10 µm Isopore membrane filter (EMD Millipore, Billerica, MA, USA) and then onto a GF/F filter (USF). Filters were stored at -20 °C until

46 further analyses. At all stations, 2-4 L of seawater was filtered onto a pre-combusted GF/F
47 (Whatman) for ^{15}N natural abundance analyses using independent sampling equipment to
48 prevent any cross-contamination.

49 In the laboratory, GF/F filters were dried at 60 °C for 48 h and isotopic composition and total
50 particulate nitrogen and carbon were determined using an elemental analyser (Thermo
51 Finnigan MAT Conflo IV) coupled to an isotope ratio mass spectrometer (Thermo Finnigan
52 Delta XP) at the Research Corporation of the University of Hawaii. Assimilation rates were
53 calculated as previously described (Montoya et al. 1996).

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55 RNA extraction

56 Total RNA was extracted from filters using the *mir*Vana miRNA Isolation Kit (Ambion) as
57 previously described (Frias-Lopez et al., 2008; Stewart et al., 2010), with a slight
58 modification. Briefly, samples were thawed on ice then filters were transferred to RNase-free
59 5 ml centrifuge tubes prior to adding 750 µl Lysis/Binding buffer (Ambion), to enable
60 complete access to all cells on the filter. Then, total RNA was extracted following the
61 *mir*Vana protocol and genomic DNA was removed following the TURBO DNA-free
62 (Ambion) protocol. DNase treated RNA was concentrated and purified following the
63 manufacturer's protocol for the RNeasy MinElute Cleanup Kit (Qiagen). Total RNA was
64 eluted in 25 µl nuclease-free water, yield was quantified using the Qubit™ RNA Assay Kit
65 (Invitrogen) and RNA quality was checked on a Nanodrop™ spectrophotometer.

66 Superscript III™ Reverse Transcriptase was used to synthesise single stranded cDNA from
67 purified total RNA (40 ng) using 2 pmol of gene specific nifH3 reverse primer (5'-

ATRTTRTTNGCNGCRTA-3'; Zani et al. 2000). Resultant *nifH* cDNA was immediately used as template for *nifH* PCR amplification.

nifH PCR amplification

DNA or cDNA template (2 µl) was added to 25 µl of GoTaq Colourless Mastermix (Promega, USA), along with 2 µl of both forward and reverse primer (0.4 µM final concentration) and 19 µl of molecular grade nuclease-free water. The first stage of the PCR used nifH4 and nifH3 primers and the following reaction conditions: 95 °C initial denaturation (GoTaq activation), and 30 cycles of 95 °C denaturation (1 min), 48 °C annealing (1 min) and 72 °C extension (1 min), followed by a final extension at 72 °C (10 min). The second stage used the nifH1 and nifH2 primer pair and 1 µl of PCR product from the first stage as template, following the same reaction conditions as the first stage PCR.

References

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Supplementary Figure Legends

Supplementary Figure 1. Maximum likelihood phylogenetic tree of *nifH* nucleotide sequences recovered during this study (purple diamond) and cultured and environmental *nifH* sequences publically available. Scale bar represents 0.1 base pair substitutions, SCS= South China Sea, NPSG= North Pacific Subtropical Gyre, SPG= South Pacific Gyre. Clusters defined by Zehr et al. (2003) are indicated, as are sequences that have previously been reported as potential *nifH* PCR contaminants, and sequences attributed to be UCYN-A ecotypes (Thompson et al. 2014) and the γ-24774A11 group (Moisander et al. 2008, 2014).

Supplementary Figure 2. Relative abundance (% sequences) of *nifH* OTUs representing > 96 % of sequences, in the ATS and Coral Sea during the Austral spring (SS) and winter (WS) at the surface (A and C) and cmax (B and D).

Supplementary Table 1. Measured biotic and abiotic parameters at each site and depth during spring 2012 (ss2012_t07; SS) and winter 2013 (ss2013_t03; WS) voyages.

Supplementary Table 2. Marginal test results from distance based linear modelling (distLM) of diazotroph (*nifH* gene) composition in relation to available predictor variables. Variables in bold displayed significant ($P < 0.05$) explanatory power and were subsequently used in distance based redundancy analysis (dbRDA).