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Distribution and drivers of symbiotic and free-living diazotrophic cyanobacteria in the Western Tropical South Pacific

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Abstract

The abundance and distribution of cyanobacterial diazotrophs were quantified in two regions (Melanesian archipelago, MA and subtropical gyre, SG) of the Western Tropical South Pacific using *nifH* qPCR assays. UCYN-A1 and A2 host populations were quantified using 18S rRNA qPCR assays including one newly developed assay. All phylotypes were detected in the upper photic zone (0-50 m), with higher abundances in the MA region. *Trichodesmium* and UCYN-B dominated, composing 81-100% of *nifH* copies detected. Het-1 was the next most abundant, and co-occurred with het-2 and het-3. The two UCYN-A lineages were least abundant (<1.0-1.5 % of total *nifH* copies) and poorly detected (>47%). Abundance of the UCYN-A hosts mirrored their respective symbionts; UCYN-A1 and A2 however were detected while their respective hosts were below detection, suggesting a lower partner fidelity or free-living life history. Pairwise comparisons of abundance and environmental parameters supported two groups: deep (45 m) comprised of UCYN-A and surface (0-15m) comprised of *Trichodesmium*, het-1 and het-2, while UCYN-B overlapped. Temperature, salinity, and PAR, were positively correlated with the latter abundances except UCYN-A. Similar results were identified in a meta-analysis of 11 external datasets. Combined, our results indicate that conditions favoring the UCYN-A symbiosis differ from those of diatom diazotroph associations and free-living diazotrophs.

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1 Introduction

Biological di-nitrogen (N_2) fixation is considered a major source of new nitrogen (N) to oceanic ecosystems (Karl et al., 1997). N_2 fixation is an energetically expensive process, where N_2 gas is reduced to bioavailable ammonia (Howard and Rees, 1996) and is performed by a small but diverse group of bacteria and archaea. The nitrogenase enzyme, which is encoded by a suite of *nif*-genes, mediates N_2 fixation (Jacobson et al., 1989; Young, 2005). Nitrogenase has a high iron (Fe) requirement (Howard and Rees, 1996), and often N_2 fixers, or diazotrophs, are Fe- limited (Kustka et al., 2003; Raven, 1988). Nitrogenase is also sensitive to oxygen (O_2), which has been shown to negatively influence N_2 fixation efficiency (Meyerhof and Burk, 1928; Stewart, 1969). Thus, autotrophic diazotrophs (e.g. cyanobacteria) have evolved strategies, such as temporal and spatial separation of the fixation process, to protect their nitrogenase from O_2 evolution during photosynthesis (Berman-Frank et al., 2001; Haselkorn, 1978; Mitsui et al., 1986). N_2 fixation is widespread and occurs in marine, limnic and terrestrial habitats. In marine ecosystems it mainly occurs in the photic zone, closest to the surface, however, more recently, evidence has shown activity in deeper depths below the photic zone, including oxygen minimum zones (Benavides et al., 2016; Bonnet et al., 2013; Fernandez et al., 2011; Halm et al., 2009; Löscher et al., 2015).

N_2 fixation in the photic zone is often attributed to a diverse group of cyanobacteria. Traditionally, marine, photic dwelling diazotrophs are divided into two groups based on cell diameter, e.g. > 10 μm and < 10 μm size fractions. Diatom diazotroph associations (DDAs), symbioses between heterocystous cyanobacteria and a variety of diatom genera and large filamentous non-heterocystous *Trichodesmium* spp., compose the larger size fraction (>10 μm). *Trichodesmium* spp. occurs as free filaments or often in two morphologies of colonies: tufts/rafts and puffs. There are three defined lineages of the symbionts of DDAs based on their *nifH* phylogeny: het-1 and het-2 refers to the two the *Richelia intracellularis* lineages which

associate with diatom genera, *Rhizosolenia* and *Hemiaulus*, respectively, while the third lineage, het-3, is a symbiosis between the heterocystous *Calothrix rhizosoleniae* and *Chaetoceros compressus* diatoms (Foster et al., 2010; Foster and Zehr, 2006).

The unicellular diazotrophic cyanobacterial groups are divided into: UCYN-A, UCYN-B, and UCYN-C groups and are representatives of the <10 μm size fraction. The UCYN-A (*Candidatus Atelocyanobacterium thalassa*) group can be further delineated into 4 sub-clades (lineages), two (UCYN-A1, UCYN-A2) are identified as symbiotic with small prymnesiophyte microalgae (reviewed by Farnelid et al., 2016, see references within). The UCYN-B group has its closest cultured relative as *Crocosphaera watsonii* and lives freely, colonially, and also in symbiosis with the diatom *Climacodium frauenfeldianum* (Bench et al., 2013; Carpenter and Janson, 2000; Webb et al., 2009; Zehr et al., 2001). Often overlooked, is the observation that UCYN-B, when colonial or symbiotic could also be associated with the > 10μm size fraction. Less is known about the UCYN-C, and given that its *nifH* nucleotide sequence is 90% similar (Foster et al., 2007) to *Cyanothece* spp. ATCC51142, it is assumed to be analogous, and thus co-occur with the other < 10 μm size fraction. A diverse group of free-living heterotrophic bacteria (e.g. gamma proteobacteria) (Berthelot et al., 2015; Bombar et al., 2016; Halm et al., 2012; Langlois et al., 2005) and archaea (Zehr et al., 2005) are also within the < 10 μm size fraction.

The Tropical South Pacific Ocean (TSP) is considered one of the most oligotrophic regions in the World's oceans (Claustre and Maritorena, 2003) with a widespread N deficiency (Deutsch et al., 2007; Raimbault et al., 2007) and in the central SP gyre, some of the lowest concentrations of dissolved Fe in the world have been reported (Blain et al., 2008). One exception is the Western Tropical South Pacific (WTSP), harboring many islands with Fe rich sediments adding to an island mass effect (Shiozaki et al., 2014) and being influenced by multiple ocean currents, both surface and subsurface, that drive the distribution of dissolved

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nutrients, micronutrients, and the biota (Fitzsimmons et al., 2014; Gourdeau et al., 2008; Marchesiello and Estrade, 2010; Wells et al., 1999). The structure of these currents also promotes shearing instabilities and strong eddies (Qiu et al., 2009). Moreover, Van Den Broeck et al. (2004) suggested that the WTSP is phosphate limited, while Law et al. (2011) hypothesized that primary production and N_2 fixation in the WTSP follows the seasonality of cyclones, which in their wake, enrich surface waters with phosphate, and fuel primary and new production. An earlier investigation along a transect in the western equatorial Pacific estimated that 74% of the total N_2 fixation could be attributed to the <10 µm size fraction as abundances of unicellular cyanobacteria were high (17 cells mL^{-1}) (Bonnet et al., 2009). However, diazotroph quantification is lacking further South in tropical waters, despite being recently recognized as a hot spot of N_2 fixation, with average rates of ~570 µmol N m^{-2} d^{-1} (Bonnet et al., this issue), i.e. in the upper range (100-1000 µmol N m^{-2} d^{-1}) of rates gathered in the global N_2 fixation MAREDAT database (Luo et al., 2012).

The distribution and activity of diazotrophs in open ocean ecosystems are governed by different ambient environmental factors, including macronutrient availability (Moutin et al., 2008; Sañudo-Wilhelmy et al., 2001) and temperature (Messer et al., 2016; Moisander et al., 2010). There are also simultanous influences by several factors (i.e. co-limitation of nutrients, Mills et al., 2004). Moreover, most oceanic models of N_2 fixation assume that all diazotrophs are equally controlled by the same environmental parameters (Deutsch et al., 2007; Hood et al., 2004; Landolfi et al., 2015), despite well recognidzed differences in genetic repertoires for assimilating dissolved nutrient pools (e.g. dissolved organic phosphate, Dyhrman et al., 2006; Dyhrman and Ruttenberg, 2006), life histories (free, symbiotic, colonial), and cell sizes (µm to mm).

The primary aim of this study was to quantify diazotroph abundance and distribution in the WTSP with an emphasis on symbiotic N_2-fixing populations; both by ´at sea´ and lab

based quantitative approaches. For a more comprehensive investigation of the symbiotic diazotrophs we developed a new primer and probe set for quantification of the UCYN-A1 host. We also identified key environmental parameters, both biotic and abiotic, which influenced the distribution of diazotrophs in the WTSP and tested the congruency of these parameters in an additional 11 publicly available datasets. We hypothesized that the distribution and the underlying factors of the diazotrophic symbioses should differ due to the major differences in host taxonomy (e.g. diatom vs. prymnesiophyte), size (1-2 μm to 100's μm), and life history (free vs. symbiotic; chain forming). For comparison and for similarly divergent characteristics (symbiotic vs. free; colonial vs. single), several free-living (UCYN-B, *Trichodesmium* spp.) cyanobacterial diazotrophs were also included.

2 Materials and Methods

2.1 Sampling

Sampling was conducted on a transect in the WTSP during austral summer (19 Feb-5 Apr, 2015), on board the R/V *L'Atalante* (Fig. 1a). Nucleic acid samples were taken from 18 stations: three long duration (LD A, B and C) stations (approximately eight days duration) and 15 short duration (SD 1-15) stations (approximately eight hours duration). The cruise transect was divided into two geographic regions (Fig. 1a). The first region (Melanesian archipelago, MA) included SD 1-12, LD A and LD B stations (160º E-178º E and 170º-175º W). The second region (subtropical gyre, SG) included SD 13-15 and LD C stations (160º W-169º W). LD stations were chosen based on hydrographic conditions, satellite imagery, microscopic analyses of >10 μm cyanobacterial diazotrophs and the results of 'at sea' qPCR analyses of four unicellular diazotrophic targets (UCYN-A1, UCYN-A2, UCYN-B and UCYN-C) (see below and Moutin et al., this issue). Seawater (2.5 L) was collected into clean (10% bleach

rinsed) 2.75 L polycarbonate bottles from 6-7 discrete depths based on surface incident light intensity (100, 75, 54, 36, 10, 1, and 0.1%) once per station at both SD and LD stations using Niskin bottles (12 L) arranged on a Conductivity Temperature Depth (CTD) rosette.

After collection from the CTD rosette, seawater was immediately filtered onto a 0.2 µm pore size Supor filter (Pall Corporation, Pall Norden AB, Lund, Sweden) held within a 25 mm diameter swinnex filter holder (Merck Millipore, Solna, Sweden) using a peristaltic pump (Cole-Parmer, Masterflex, Easy-load II, USA). The filters were placed in pre-sterilized bead beater tubes (Biospec Bartlesville, OK, USA) containing 30 µL of 0.1 mm and 0.5 mm glass bead mixture, flash frozen in liquid nitrogen and archived at -80 ºC. Four additional DNA samples were collected from 4 discrete depths, (75, 50, 36, 10 % light), at 11 of the 18 stations, for the 'at sea' qPCR (see below) and filtered as described above.

2.2 Nutrient analyses

Seawater for nutrient analyses was collected from each station using the CTD rosette at the same depths as those collected for the nucleic acids. Seawater for inorganic nutrient analysis were collected in 20 mL high-density polyethylene HCL-rinsed bottles and poisoned with $HgCl_2$ to a final concentration of 20 µg L^{-1} and stored at 4°C until analysis. Dissolved nitrate and nitrite (NO_3^-+NO_2^-, DIN), phosphate (PO_4^{3-}, DIP) and silicate ($Si(OH)_4$, DiSi) concentrations were determined by standard colorimetric techniques using a segmented flow analyzer according to Aminot and Kérouel (2007) on a SEAL Analytical AA3 HR system (SEAL Analytica, Serblabo Technologies, Entraigues Sur La Sorgue, France). Quantification limits for nitrate, phosphate and silicate were all 0.05 µmol L^{-1}.

Cell abundances and microscopy observations. At the LD stations, 5 L of seawater was collected at the same depths and parallel with the nucleic acid samples from the CTD-rosette. Two sets of samples, one set each day, were taken on two different days (day 1 and 3 at each

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LD station) and immediately filtered onto a 47 mm diameter Poretics (millipore) membrane filter with a pore size of 5 µm using a peristaltic pump.

At the SD stations, the same collection was implemented, however a 25 mm diameter Poretics membrane filter was used. The high densities of cells on the latter made it impossible to properly enumerate the various cyanobacterial diazotrophs and as such these samples were used only for qualtitative observations (see below). Immediately after filtration, samples were fixed in 1 % paraformaldehyde (v/v) for 30 min prior to storing at -20 ºC. For enumeration, the filter was mounted on a glass slide and examined at under an Olympus BX60 microscope equipped with a filter for blue (460-490 nm) and green (545-580 nm) excitation wavelengths. Three areas (area = 0.94 mm^2) per filter were counted separately and values were averaged. When abundances were low, the entire filter (area = 1734 mm^2) was observed and cells enumerated. Due to weak fluorescence, only *Trichodesmium* colonies and free-filaments could be accurately estimated by microscopy and in addition, the larger cell diameter *Trichodesmium*, herafter referred to as *Katagynemene pelagicum*, was enumerated separately as these were often present albeit at lower cell densities. Other cyanobacterial diazotrophs, e.g *C. watsonii*-like, *C. rhizosoleniae* (het-3) and *R. intracellularis* (het-1, het-2) were also present on the larger 47 mm diameter samples, however fluorescence was weak and therefore difficult to enumerate. Pico-eukaryote populations, identified as round 1-3 µm diameter cells, with red excitation under the blue filter set, were also observed. For the latter populations, qualitative observations of presence and some details on cell integrity (e.g. fluorescence, frustule, free-living or symbiotic form) are included.

2.3 DNA extraction

The DNA from the 120 archived samples was extracted as described in Moisander et al. (2008), with a 30 second reduction in the agitation step in a Fast Prep cell disrupter (Thermo, Model FP120; Qbiogene, Inc. Cedex, France) and an elution volume of 70 µL. The nucleic

acid samples collected for the 'at sea' qPCR were extracted immediately after filtration using a modified version of the DNAeasy plant kit (Qiagen) total DNA extraction protocol. The modifications were an initial 2-minute agitation step using a bead beater (Biospec MiniBeadBeater-16, Model 607EUR; Biospec) and final elution volume was 25 µl.

2.4 Oligonucleotide design

A new primer and probe set was designed to amplify the UCYN-A1 host and was based on published 18S rRNA sequence (accession number JX291893) reported from N. Pacific gyre (station ALOHA) (Thompson et al., 2012). The design utilized the same 96 bp target region of the 18S rRNA used to amplify UCYN-A2 hosts described in Thompson et al. 2014 (Suppl. Table 1). The primers and probe for the UCYN-A1 host 18S rRNA gene assay are as follows: Forward, 5' AGGTTTGCCGGTCTGCCGAT-3'; Reverse, 5' GAGCGGGTGTCGGAGACGGAT-3'; Probe, 5'-FAM-CTGGTAGAACTGTCCT-TAMRA-3'. The forward, reverse and probe contain 2-4, 1, and 5 mismatches, respectively, to UCYN-A2 host sequences (accession number KF771248-KF771254) and the following closely related sequences (98-100%): uncultured eukaryote clones (station ALHOA: EU50069; Cariaco Basin: GU824119) *Chrysochromulina parkeae*: AM490994), *Braarudospaera bigelowii* TP056a: AB250784 *B. bigelowii* Furue-15: AB478413; *B. bigelowii* Funahama T3: AB478413; *B. bigelowii* Yastushiro-1 AB478414. The UCYN-A1 oligonucleotides specificity was tested *de nova* against the following closely related sequences derived from uncultured eukaryotic clonal sequences (accession numbers: EU500067-68; FJ537341; EU500138-39; EF695227; EU500141; EU499958; EF695229; EF695220). Only one mismatch was found in the forward probe for one sequence (EU500138). Finally, a cross reactivity test between the newly designed UCYN-A1 host oligonculeotides and a dilution series of the UCYN-A2 host template was run (see below).

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2.5 Quantitative PCR

Abundances of selected diazotrophs *nifH* gene copies (UCYN-A1, UCYN-A2, UCYN-B, UCYN-C, het-1, het-2, het-3 and *Trichodesmium* spp.) and the 18S rRNA of UCYN-A1 and A2 hosts were performed using previously published oligonucleotides and TaqMAN assays (Church et al., 2005; Foster et al., 2007; Moisander et al., 2010; Thompson et al., 2014) and the newly designed UCYN-A1 host oligonculeotides (Suppl. Table 1). The qPCRs were conducted in a StepOnePlus system (Applied Biosystems, Life Technologies, Stockholm Sweden) in fast (>40 min) mode with the following parameters: 95 ºC for 20 s, followed by 45 cycles of 95 ºC for 1 s and 60 ºC for 20 s.

Cross reactivity tests were run on two of the heterocystous symbiont (het-1 and het-2) oligonucleotides, the UCYN-A1 and UCYNA-2 oligonucleotides, and the newly designed UCYN-A1 host oligonucleotides and UCYN-A2 host primer and probe set. The standard curve for a particular target was run in reactions with the other primers and probe sets. For example, the UCYN-A1 TaqMAN host primers and probes were run in reactions with UCYN-A2 template DNA. The cross reactivity for the het-1 and het-2 primer and probe sets has been previously reported (Foster et al. 2007), however only when the assay is run in standard mode. Standard mode runs the holding, denaturation and annealing stages at the following longer intervals than in Fast mode: 11 min and 40 s, 14 s, and 40 s, respectively. Hence, we tested the cross-reactivity for the het primers and probes when run in fast mode, as the fast mode was used in our study. Similarly, the cross-reactivity between UCYN-A1 and UCYN-A2 were tested in fast mode at two annealing temperatures 60 ºC and 64 ºC; 64 ºC is the recommended annealing temperature for the UCYN-A2 assay (Thompson et al. 2014).

Reaction volume was 20 µL in all qPCRs and consisted of 10 µL of 2X TaqMan fast buffer (Applied Biosystems, 5.5 µL of nuclease free water (Sigma Aldrich Sweden AB, Stockholm Sweden), 1 µL each of the forward and reverse primers (10 µM), 0.5 µL of fluorogenic probe

(10 µM) and 2 µL of DNA extract. For standard mode runs, the latter master mix was identical with the exception of replacing the fast 2X buffer with the standard 2X buffer. For reactions quantifying *Trichodesmium* spp. *nifH* copies, SD 9 was excluded and 1 µL of DNA template was used for the remaining stations due to low template volume, and total reaction volume was adjusted by addition of 1 µL of nuclease free water. Reactions were performed in duplicates for the 'at sea' qPCR and in triplicates for the archived samples and lab based qPCR. For the 'at sea' qPCR, only four targets (UCYN-A1, UCYN-A2, UCYN-B, and UCYN-C) were quantified and only at the SD stations. No assays were processed at SD 5-6, 10-12, and 14 for the 'at sea' qPCR. Two µL of nuclease free water was used as template in no template controls (NTCs); no *nifH* copies were detected in the NTCs.

Gene copy abundance was calculated from the mean Ct value of the 3 replicates and the standard curve for the appropriate oligonucleotides in the lab based qPCRs. For the 'at sea' qPCR, a mean Ct value of 2 replicates was used to maximize the number of samples run on one amplification plate (96 well). In samples where 1 or 2 out of 3 replicates produced an amplification, signals were noted as detectable, but not quantifiable (dnq) and no amplification was noted as below detection (bd).

2.6 Standard curves and PCR efficiency

Standard curves were plotted and analyzed in Excel for each target based on the qPCR cycle threshold (Ct) values from known dilutions of synthesized target gene fragments (gBlocks®; Integrated DNA Technologies, Leuven Belgium) (359 bp *nifH* and 733 bp 18S rRNA for UCYN-A hosts). Tenfold dilutions were made starting with 10^8 to 10^1 gene copies L^{-1}. The PCR efficiency was determined as previously described (Short et al., 2004) for 12 samples run on the het-1, het-2, and het-3 primers and probe tests. The qPCR efficiency ranged from 90-99 % with an average of 94 % efficiency for the diazotroph targets het-1, het-2 and het-3.

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2.7 Statistics and data analysis

Skewness and normal distribution tests by descriptive statistics was performed in IBM SPSS (ver. 23) on the following parameters recorded during sample collection in the WTSP from the CTD package: depth (m), oxygen (ml L^{-1}), temperature (ºC), chlorophyll fluorescence (µg L^{-1}), photosynthetically active radiation (PAR; µmol photons m^{-2} s^{-1}), salinity (PSU), and gene copy abundances determined by qPCR. Significant skew was noted when skewness, divided by its standard deviation, exceeded 1.95. All but three targets (het-1, UCYN-B and *Trichodesmium* spp.) and three environmental parameters (temperature, salinity and oxygen) were significantly skewed (not normally distributed) even after LOG10 transformation. Therefore a non-parametric Spearman's rank correlation was conducted to test possible correlations between the targets and environmental parameters, where we assume that the het groups and UCYN-A clade is symbiotic, while UCYN-B is free living. The resulting correlation matrices were visualized in the form of a heat map of hierarchical clustering in R (ver. 3.2.2) using packages 'hmisc' and 'gplots'. Multivariate statistics by redundancy analysis (RDA) was conducted using the R package 'vegan'. T-tests, in IBM SPSS (ver. 23) were performed to characterize the different regions along the cruise transect based on environmental parameters, including nutrients, measured between stations and was reported as mean concentrations. For meta-analysis on the external dataset from 11 publically available datasets, sampled in the Atlantic, Pacific and South China Sea, data was acquired from the PANGAEA database and previous publications (Benavides et al., 2016; Bombar et al., 2011; Church et al., 2005, 2008, Foster et al., 2007, 2009; Goebel et al., 2010; Kong et al., 2011; Langlois et al., 2008; Moisander et al., 2008, 2010). We included only datasets with a minimum of 10 datapoints on the previously mentioned diazotrophic targets. Note that in all datasets the two UCYN-A phylotypes (A1 and A2) were not distinguished, and het-3 was excluded since it was rarely quantified. The meta-analysis was conducted using the software

OpenMEE (based on R package 'metafor'), where correlation coefficients from Spearman's rank were z-transformed (Fisher's) and tested using weighted random effect models. Graphical visualization of the mean abundances of the most numerous diazotrophs across the cruise transect was also performed in IBM SPSS (ver. 23).

3 Results

3.1 Hydrographic conditions

Near surface (0-5m) DIN concentrations were below the quantification limit (bq) in both the MA and SG regions, while the mean surface DIP and DiSi concentrations were below the quantification limit or low across all stations in the MA (bq-0.08 μM and 0.54-0.56 μM, respectively) and significantly ($p<0.001$; t-test) higher (0.18 ± 0.07 μM and 0.79 ± 0.04 μM, respectively) at the stations in the SG (Table 1). The upper 25-30 m of depth had stable temperatures of 29-30 ºC. The depth of the deep chlorophyll maximum (DCM) was between 70-165 m, except for LD B (DCM at 35 m), which was sampled during a degrading surface phytoplankton bloom, and a 30-day composite of the surface chlorophyll *a* (Chl *a*) confirmed the decreasing level of surface fluorescence measured by the CTD package at LD B (data not shown).

3.2 Comparison of 'at sea' and lab-based qPCR

In order expedite the sample processing for the 'at sea' qPCR, a shortened and modified DNA extraction protocol was performed, 4 depths were sampled, and 4 targets run (UCYN groups). In total, 44 samples can be compared with results from the parallel archive samples and we considered only when there was at least one order of magnitude difference in detection. A summary of the comparison, including the difference in *nifH* copy abundance is provided in Suppl. Table 2.

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In general, the 'at sea' and lab based qPCR were similar in quantifying the targets. Discrepancies were noted in 7, 8 and 11 samples, which had higher detection in the 'at sea' analyses for UCYN-A1, UCYN-A2 and UCYN-B, respectively. There were fewer instances (3, 4, and 5, respectively) of samples processed in the lab with the full extraction that had higher abundances for the UCYN-A1, UCYN-A2 and UCYN-B, respectively.

Horizontal and vertical distributions. *Trichodesmium* and UCYN-B were the most abundant diazotrophs and abundances ranged 10^4-10^6 *nifH* copies L^{-1} at multiple depths (4-6 depths) in the upper water column (0-35 m) (Fig. 1-2; Suppl. Tables 3). *Trichodesmium* represented 80-99% of total *nifH* genes detected at 9 out of 17 stations with highest detection in the MA and low to undetected in the SG. Microscopy observations and abundances of *Trichodesmium* spp. confirmed a high abundance of free filaments of *Trichodesmium* and *C. watsonii*-like cells at LD B, while colonies were in general rarely observed (Suppl. Table 5).

At stations where *Trichodesmium* was not the most abundant diazotroph (e.g. SD 2, 6, 7, 14, 15, and LD C), UCYN-B had the highest depth integrated *nifH* copy abundance. UCYN-B was also the most consistently detected diazotroph, and was quantifiable from all stations sampled accounting for for 81-100% of the total detected *nifH* gene copies in the SG. There was also a depth dependency for maximum abundance such that the average depth maximas of *Trichodesmium* and UCYN-B at the stations in the MA were 10 and 25 m, respectively. In the SG, the average depth maximum was the same for UCYN-B (25 m), while the average depth of the *Trichodesmium* maximum deepened to 31m.

Of the three heterocystous cyanobacterial symbiont lineages (het-1, het-2, het-3), het-1 was the most dominant (60% detection in total samples, 72 of 120 samples), and similar to *Trichodesmium*, had higher detection in the stations of the MA region. For example, at stations SD 2, 4 and 9, het-1 represented 10-15% of the total *nifH* genes quantified in the depth profiles, but in the total *nifH* genes quantified across the entire transect, het-1 only

represented 1.5 %. Abundances for het-1 ranged between 10^3-10^5 *nifH* copies L^{-1} (15 of the 18 stations) at multiple depths (0-90 m) and the average depth maximum at MA stations was closer to the surface (15 m) compared to the SG stations (60 m) (Fig. 1; Suppl. Table 3). Het-2 and het-3 co-occurred with het-1, however at lower abundances (10^2-10^4 *nifH* copies L^{-1}) and unlike het-1, were bd at all depths sampled in 1 and 3 stations, respectively, located in the SG. The averge depth of maximum abundance (17 m) for het-2 was similar to het-1 (15 m), while het-3 was deeper at 33 m (considering only the MA stations). Microscopy observations confirmed the presence of *R. intracellularis* at 5 SD stations of the MA and LD B and absence at the SD stations and LD C of the SG. Noticeable was the co-occurence of free filaments of *R. intracellularis* and degrading diatom cells (mainly belonging to the genus *Rhizosolenia*), especially at the SD 5, 6 and 7.

The unicellular symbiotic groups, UCYN-A1 and A2 (and their respective hosts), were the least detected targets. For example, UCYN-A1 was bd in 53% (63 of 120 samples) and UCYN-A2 was bd in 66% (79 of 120 samples) of samples. UCYN-A1 and A2 represented < 0.4 % of total *nifH* genes detected and UCYN-A symbionts were bd in the SG, except at LD C. When detected, average *nifH* abundance for UCYN-A1 and A2 were 8.60 x 10^4 and 4.60 x 10^4 *nifH* copies L^{-1}, respectively, and usually accounted for <1.0-1.5 % of the total *nifH* copies enumerated per station. One exception was at LD C in the SG, when UCYN-A1 and A2 accounted for 4 and 11%, respectively, of the total *nifH* gene copies, and were the second most abundant diazotroph (3.19 x 10^4 and 8.53 x 10^4 *nifH* copies L^{-1}). The average depth of maximum *nifH* abundance for the UCYN-A1 and A2 symbionts was consistently recorded at deeper depths (e.g. 55 and 58 m, respectively; 10 % light level).

The detection of the UCYN-A1 and A2 hosts mirrored the detection of their respective symbionts. However, in 22 and 15 samples, respectively, the UCYN-A1 and A2 symbionts were quantified while their hosts were bd. The UCYN-A hosts were never detected in samples

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where their respective symbionts were bd or dnq. When both UCYN-A host and symbiont were present, the abundances of the hosts were always one order of magnitude less than their respective symbionts, with the exception of two samples for UCYN-A1 symbionts where their respective host abundances were half, or nearly equal in abundance. UCYN-C was the least abundant unicellular diazotroph and was only quantified in the 'at-sea' qPCR where detection was poor and limited to the MA region (3 of 11 stations: 1-3 of 4 depths sampled) and abundances never exceeded 10^2 *nifH* copies L^{-1} (Suppl. Table 3).

3.3 Diazotroph and UCYN-A host covariation

Several significant correlations between the target diazotrophs and hosts were identified (Fig. 3; Suppl. Table 4a). The *nifH* gene copy abundances of *Trichodesmium* and UCYN-B were significantly positively correlated with each other ($p<0.01$). In addition, UCYN-B *nifH* gene copy abundance was significantly positively correlated with those of both UCYN-A symbionts (A1 and A2; $p< 0.01$) and UCYN-A2 host abundance ($p<0.05$). Abundances of UCYN-A1 and A2 were significantly positively correlated with each other, and in addition, with their respective host abundances ($p<0.01$). Lastly, the *nifH* copy abundances for het-1, het-2 and het-3 were significantly positively correlated with one another, and with the *nifH* copy abundances of *Trichodesmium* and UCYN-B ($p<0.01$). The only correlations that were not significant were between the UCYN-A (including their hosts) and *Trichodesmium* and the het-groups (with the exception of het-3, which correlated with the UCYN-A2 host ($p<0.05$)).

3.4 Influence of environmental conditions on diazotroph and UCYN-A host abundances in the WTSP

The abundances of UCYN-A1 and A2 were significantly positively correlated with salinity and depth ($p<0.02$ and $p<0.03$, respectively) (Fig. 3; Suppl. Table 4b). However, all other diazotrophs were significantly negatively correlated with salinity and depth ($p<0.01$). Moreover, *Trichodesmium*, UCYN-B, and the het-group (except het-3) were significantly

positively correlated ($p<0.01$) with PAR and temperature while UCYN-A1 and A2 were significantly negatively correlated ($p<0.02$) with the latter parameters. All diazotrophic targets, except UCYN-A1, UCYN-A2, and het-3, were significantly negatively correlated ($p<0.02$) with DIN concentration. Similarly, all diazotrophs, except UCYN-A2, were significantly negatively correlated ($p<0.02$) with DIP concentration, and all diazotrophs except UCYN-A1, A2 and het-3 were significantly negatively correlated ($p<0.001$) with DiSi concentration. The abundances of UCYN-A hosts, UCYN-A1 and UCYN-A2, and UCYN-B were significantly correlated ($p<0.001$ and <0.05) with dissolved oxygen. In general, the correlations between abundances and several hydrographic parameters divided the diazotrophs into two groups: the UCYN-A symbionts (and respective hosts) and all other diazotrophs.

Hierarchical clustering based on the Spearman's rank analyses resulted in the two major groups: (1) a shallow and (2) deeper euphotic zone, inferred from the negative and positive correlations, respectively, with depth (Fig. 3). For example, *Trichodesmium* and the symbiotic het-1 and het-2 lineages characterize an upper water column group 1 with significant clustering and positive correlations with temperature ($p<0.001$) and PAR ($p<0.003$), while only UCYN-A1 and A2 symbionts and their respective hosts represent group 2. UCYN-B was unique in an overlapping distribution, and resulted in positive significant correlations with both the shallow (group 1) and deep (group 2) euphotic zone diazotrophs (e.g. *Trichodesmium*, $p<0.001$ and UCYN-A1, $p<0.004$, respectively). The deeper dwelling group 2 significantly clustered and correlated positively with oxygen, depth, salinity and fluorescence ($p<0.03$, except for UCYN-A2 and fluorescence, $p<0.053$). Despite clustering with group 1, het-3 was less robust in a negative correlation with salinity ($p<0.01$).

The results from the Spearman's rank correlations were further confirmed and visualized in the RDA biplot (Fig. 4a), which explains parameter importance (Fig. 4b). Correlations with nutrients and PAR were omitted due to the limited number of data points.

Fluorescence, depth and salinity correlated positively with each other and negatively with temperature, while oxygen was uncorrelated with all other environmental parameters. The response variables UCYN-A1 and A2 and their respective hosts clustered with the explanatory variables: fluorescence, salinity and depth, with a dependency towards oxygen. On the other hand, the shallower euphotic group 1 (response variables *Trichodesmium*, het-1 and het-2) clustered closer to explanatory varaible temperature. In addition, most of the observed variance is explained by the two axes RDA1 (72 %) and RDA2 (22 %), indicative of depth and temperature, respectively, as the most important environmental parameters for diazotroph abundance in our study. Together they form a depth-temperature gradient (RDA1) where *Trichodesmium* occupies the warmest and shallowest waters, and UCYN-A occupies the coldest and deepest waters, among the investigated cyanobacterial diazotrophs.

3.5 Global drivers of diazotrophic abundance

We found consistency between our results in the WTSP and the correlations identified in the 11 external datasets by the non-parametric correlation analyses and meta-analyses (Suppl. Table 6). For example, in three of the external datasets, abundances of *Trichodesmium* spp., UCYN-B, and het-1, were significantly positively correlated with temperature and negatively correlated with the same three parameters as in our study in the WTSP: salinity, DIP, and DIN. The latter correlations were identified in two regions of the WTSP (tropical and subtropical) and in the northern South China Sea (NSCS). In contrast to a significant positive correlation between UCYN-A abundance and depth reported here in the WTSP, UCYN-A abundance was negatively correlated with depth in 4 of the 11 external datasets (two regions of the WTSP, Tropical Atlantic (TA), and NSCS). Moreover, and consistent with several of the other diazotrophs (*Trichodesmium*, UCYN-B, het-1), UCYN-A abundance was negatively correlated with DIP and DIN concentrations (5 and 3 additional external datasets, respectively) (Suppl. Table 6).

Meta-analysis revealed similar groupings (e.g. shallow and deep) as observed in the WTSP, however, the significance was less robust (Suppl. Table 6). For example abundances of *Trichodesmium* and het-1 and het-2 were significantly positively correlated with temperature and negatively correlated with salinity ($p<0.05$). No significance was found for UCYN-A abundance for the latter parameters, and UCYN-B abundance was un-correlated with salinity and significantly positively correlated with temperature ($p<0.05$). In addition, UCYN-A was the only diazotroph that was uncorrelated with het-2, while all other diazotrophs had a significant positive correlation with het-2 ($p<0.05$). Similar to our findings reported for the WTSP, all diazotrophs, except UCYN-A, correlated significantly negatively with depth, DIP and DIN concentrations ($p<0.05$) (except het-2 with DIP which was not significant). Finally, UCYN-B and het-1 abundances were significantly negatively correlated with chl *a* ($p<0.05$), while *Trichodesmium*, UCYN-A and het-2 were uncorrelated.

3.6 Cross reactivity tests

No amplification was detected for the newly designed UCYN-A1 host oligonucleotides run with the UCYN-A2 as template DNA and vice versa (Suppl. Fig. 1a).

Running the het assay in fast mode showed a lower cross-reactivity between the het-1 assay and the het-2 template than vice versa (the het-2 assay and het-1 template) (Suppl. Fig. 1b). In fact, no amplification was detected in the last two template additions and the Ct differences were > 9 when het-1 assay was run with het-2 templates. The UCYNA-2 assay detected the UCYN-A1 template in all but the last template addition and with Ct differences >3 (1 order of magnitude) while there was a 18-20 difference in Ct value (less gene copies) when UCYN-A1 assay was run in fast mode with UCYN-A2 templates at either annealing temperature (60° C or 64° C) and only the first three template additions (10^8-10^6 *nifH* copies μL^{-1}) were detected (Suppl. Fig. 1c-d).

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4 Discussion

4.1 Environmental conditions in the WTSP

The SP is one of the most oligotrophic regions of the world's oceans with chronically low dissolved nutrient concentrations, especially DIN, and thus, is considered an area primed for N_2 fixation. Likewise, we encountered surface hydrographic conditions in the WTSP that were consistently low in dissolved nutrient concentrations and similar to earlier reports for the equatorial Pacific (Bonnet et al., 2009; Dufour et al., 1999; Moutin et al., 2008; Van Den Broeck et al., 2004). The conservative tracers of temperature and salinity remained constant in the surface between the MA and SG regions, hence the elevated nutrient concentrations in the SG is likely not related to an eddy intrusion. The deviation away from a 16:1 relationship (Redfield ratio) (data not shown) in the upper 125 m in both regions (MA and SG) was indicative of DIN limitation. The low DIP concentrations in MA waters suggest utilization of DIP by diazotrophs in the absence of DIN, and likely other sources of nitrogen were available, e.g. dissolved organic nitrogen or N_2 fixation (Karl et al., 2001).

4.2 Detection of diazotrophs and application of 'at sea' qPCR

Trichodesmium, UCYN-B, and the het-groups are easily identifiable by standard epi-fluorescence microscopy, and so these populations can readily be observed 'at sea'. However, the UCYN-A1 and UCYN-A2, and their respective hosts, require a lengthy fluorescent *in situ* hybridization (FISH) protocol that is difficult to implement in the field. On the other hand, nowadays oceanographers have a suite of other molecular genetic tools, some of which are also 'sea-going' and autonomous (e.g. Robidart et al. 2014; Ottesen et al. 2013; Preston et al. 2011), thereby making quantification of microscopically unidentified microorganisms tangible by quantifying their genes, simultaneous with collection of hydrographic data. Here, we showed a rather efficient, steadfast (within 3 hrs of sample collection), and 'sea-going' nucleic acid extraction and qPCR to quantify diazotrophs by their *nifH* gene, which was used

in real time during the OUTPACE cruise to help locate the LD stations for the purpose of the project (see Moutin et al., this issue). The comparisons of the 'at sea' assays to the lab-based full extraction protocol and qPCR on archived samples indicated that the assays were consistent, and surprisingly the shortened DNA extraction performed 'at sea' had higher abundances for all three targets (UCYN-A1, UCYN-A2 and UCYN-B) in 16-25 % of the samples processed, depending on the target diazotroph. The 'at sea' (and lab-based) qPCRs could be appended with a multi-plexing approach to both increase and broaden the number of metabolic pathways (e.g. *narB, rbcL, nirS*) and/or phylotypes quantified simultaneously.

4.3 Abundance and vertical distribution of diazotrophs in the WTSP

Earlier work based on N isotope ratios (delta ^{15}N) of suspended particulate matter and dissolved organic N (DON) in the WTSP suggested that new production is likely fueled by N_2 fixation in this region (Hansell and Feely, 2000; Yoshikawa et al., 2005). The SP is also an area where high abundances of the unicellular diazotrophs, in particular UCYN-A and UCYN-B, have been previously reported (Biegala and Raimbault, 2008; Bonnet et al., 2009, 2015; Moisander et al., 2010) and account for a significant (74%) portion of the areal N_2 fixation (Bonnet et al., 2009). Hence, it was likely to encounter the presence of diazotrophic populations.

Recently UCYN-A and its various lineages have been highlighted as one of the most widespread and abundant diazotrophs (Farnelid et al., 2016 and references therein), which has led to the dramatic shift in the canonical paradigm of *Trichodesmium* as the only significant diazotroph. Surprisingly, here, we report abundances of the UCYN-A1 and UCYN-A2 lineages that are comparatively lower than earlier reports. In fact, UCYN-A1 and A2 were the least detected diazotrophs. Both UCYN-A phylotypes were largely restricted to the MA, with the exception of high densities (3.2×10^4 and 8.5×10^4 *nifH* copies L^{-1}, respectively) found at one depth (60 m) of LD C, which borders the MA region. Consistent with higher UCYN-A

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biomass at depth at LDC were microscopy observations of high abundances of picoeukaryotes similar in size and shape previously reported for the UCYN-A hosts (Krupke et al. 2013). The vertical distribution of UCYN-A1 (and A2) was similar to Moisander's et al. (2010) and others, including earlier studies in the North Pacific Ocean (NP) and NA, where maximum abundances of UCYN-A are common to deeper depths in the euphotic zone (below 45 m) (e.g. Bonnet et al., 2015; Foster et al., 2007; Goebel et al., 2010; Needoba et al., 2007). Likewise, we also observed as others (Cabello et al., 2016) that the UCYN-A based symbioses co-occur and typically have decreased abundance towards the DCM, and maximum abundances slightly above the nitracline.

Unlike UCYN-A phylotypes, UCYN-B and *Trichodesmium* were the most abundant diazotrophs in the WTSP, and UCYN-B in particular was the most detected phylotype (99% detection; dnq or higher in 119 of 120 samples). High abundances of *Trichodesmium* in the upper 10 m, including presence of surface slicks and free filaments, was widespread in the MA region and consistent with earlier observations of high surface densities further north in the SP (Moisander et al., 2010; Shiozaki et al., 2014). Surface slicks have also been reported elsewhere, e.g. the North Atlantic (NA) (Goebel et al., 2010; Langlois et al., 2005). The depth of maximum abundance for *Trichodesmium* deepened from the MA (10 m) region to the open gyre (SG, 31 m), which was similar to earlier reports in the equatorial Pacific (Bonnet et al., 2009). A niche partitioning has been suggested for *Trichodesmium* and unicellular diazotrophs in the SP (Bonnet et al., 2015; Moisander et al., 2010) and elsewhere (Goebel et al., 2010; Langlois et al., 2005; Messer et al., 2015). However, here in the WTSP, *Trichodesmium* abundance was correlated with UCYN-B, which is consistent with previous studies in other ocean basins, e.g. Atlantic Ocean (Foster et al., 2007, 2009; Langlois et al., 2008), and the South China Sea (Moisander et al., 2008). UCYN-B co-occurred with *Trichodesmium* in the surface samples, although at lesser *nifH* copy abundances, and more often UCYN-B had

subsurface maxima (35-70 m) in both regions (MA and SG) of the transect. The latter is also consistent with Moisander et al. (2010) who observed maximum abundances of UCYN-B north of the Fijian islands at 37m.

All 3 heterocystous symbiont phylotypes co-occurred and were widespread in the MA, with het-1 as the most abundant and most highly detected het group (70% detection or 84 of 120 samples). The early work of Moisander et al. (2010) detected het-1 in all but one of 26 stations sampled (56% detected, or 56 of 100 samples), and highest *nifH* copy densities were reported north east of our cruise transect. Moreover, Bonnet et al. (2015) detected het-1 and het-2 at the surface of one out of 10 stations west (approximately 10 degrees W) of our cruise transect. Het-2 and het-3 were not quantified by Moisander et al. (2010) and het-3 was not quantified by Bonnet et al. (2015). Therefore our study is among the first to report on the abundances and distributions for all 3 heterocystous diazotrophs in a large expanse of the SP. The 3 het phylotypes were however recently reported from a mesocosm (enclosed design) experiment in the Noumea lagoon, a low nutrient low chlorophyll (LNLC) region located along the New Caledonian coast (Turk-Kubo et al., 2015). In fact, het-1 and het-2 were among the most abundant diazotrophs in the first half of the experiment (Turk-Kubo et al., 2015). Two additional earlier studies have also reported microscopic observations of free-living *Richelia* in the same lagoon (Biegala and Raimbault, 2008; Garcia et al., 2007).

Highest densities (10^4-10^6 *nifH* copies L^{-1}) of the *Richelia* phylotypes were restricted to the western region of the MA, and in the upper 12 m, which is shallower than the subsurface maximum (25 m) commonly reported for het-1 (and het-2) in the Western Tropical North Atlantic (WTNA) and NP (Church et al., 2005; Foster et al., 2007; Goebel et al., 2010). Our microscopy observations from SD 5-7 and LD A indicated that near surface *Rhizosolenia* populations were in a moribund state since frustules were broken and free filaments of *Richelia* were observed. Our observations also coincide with a region of high backscattering

measurements in the upper water column (5-30 m) (Dupouy et al., this issue). Het-1 *nifH* copies were 4 orders of magnitude higher in abundance in the moored sediment traps of LD A (325 m: 2.0 x 10^7 *nifH* copies L^{-1}) and LD B (325 and 500m: 5.8 x 10^6 and 1.10 x 10^7 *nifH* copies L^{-1}, respectively) (Caffin et al., this issue) than the *nifH* copies detected in the overlying waters (3.11 x 10^3 *nifH* copies L^{-1} and 4.1 x 10^2 *nifH* copies L^{-1}, respectively). Combined, the latter observations suggest that a higher density of the het-1 population was likely present prior to our sampling and perhaps derived from a 'seed' population originating in the coastal regions of New Caledonia.

The UCYN-C phylotype was poorly detected in the 'at sea' assays (61% samples were bd and maximum abundance was 5.0x10^2 *nifH* copies L^{-1}), and as such was not enumerated in the archived samples. The low detection of UCYN-C is consistent with Taniuchi et al. (2012), who estimated that UCYN-C only represented a small portion of diazotrophs detected in the NW Pacific (Kuroshio Current). However, a recent study reported relatively high UCYN-C abundances in the open waters of the Solomon Sea (north of the MA) (Berthelot et al., submitted). UCYN-C has also been observed in the New Caledonian lagoon (Turk-Kubo et al., 2015), where it was the most dominant diazotroph in the first part of the aforementioned mesocom experiment (Turk-Kubo et al., 2015). Like most plankton, abundances can be patchy as was observed with UCYN-C in our study.

4.4 UCYN-A and host (co)-occurrence

Earlier and recent work has suggested a high host dependency (e.g. smaller and streamlined genomes), and selectivity in the UCYN-A based symbioses (Cabello et al., 2016; Cornejo-Castillo et al., 2016; Farnelid et al., 2016; Krupke et al., 2013, 2014; Thompson et al., 2012; Tripp et al., 2010). Moreover, the UCYN-A partnerships are also considered mutualistic, where the host and symbiont both benefit by exchange of metabolites (e.g. reduced C and N, respectively) (Krupke et al., 2014; Thompson et al., 2012); hence one would expect parallel

distributions for both partners. Some have argued that the partnership is also obligatory since few observations of free-living hosts have been reported and abundances of free symbionts assumed to be derived from disruption during sample preparation are always correlated with their hosts (Cabello et al., 2016; Krupke et al., 2014; Thompson et al., 2012). Thus, by use of our newly designed oligonucleotides for the UCYN-A1 host and previously designed oligonucleotides for the UCYN-A2 host (Thompson et al., 2014), we unexpectedly found that both UCYN-A1 and A2 were often (89% and 59%, respectively; not considering dnq) detected in the absence (or bd) of their respective hosts, while the hosts, when detected, always coincided with increased UCYN-A abundance. Our observations could result if the UCYN-A lineages can live freely, or in either a loose association, or perhaps with a wider range of hosts than previously thought and detected by the UCYN-A host assays. Presence of UCYN-A in the absence of their respective hosts could also indicate that the growth of symbiont and host is asynchronous, a pattern reported once in the het-1 or *Rhizosolenia-Richelia* symbioses (Villareal 1989).

The number of cells per partner lineage is considered specific as well, such that 1-2 UCYN-A1 cell is associated with a prymnesiophyte partner (UCYN-A1 host) and the larger *B. bigelowii* (UCYN-A2 host) host associates with multiple and variable numbers of UCYN-A2 cells to compensate for its higher N requirement (Cornejo-Castillo et al., 2016). On the contrary, we found evidence that there are multiple UCYN-A1 and A2 symbionts in both host types, which is somewhat surprising given that the host target gene (18S rRNA) is a multiple copy gene, meaning that we would expect higher gene copy numbers for each host. Nonetheless, we consistently observed higher abundances for the UCYN-A1 and A2 symbionts than their respective hosts. UCYN-A1 and A2 were 2-10 and 6-34 times, respectively, more abundant than their hosts. A symbiosome-like compartment has also been described attached to the UCYN-A2 host or residing free (Cornejo-Castillo et al., 2016).

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Thus, one plausible explanation for the higher abundances of the UCYN-A2, in particular, in the absence of their respective host, could result if our assays quantified UCYN-A2 residing in a dislodged free-floating symbiosome, or an overestimate of the UCYN-A2 due to cross-reactivity with UCYN-A3 lineage as expected by *in silico* tests (Farnelid et al. 2016). It is less likely that the UCYN-A2 was overquantified due to cross-reaction with UCYN-A1 templates since our cross-reactivity tests showed a weak cross reaction (see below).

4.5 Environmental influence on diazotroph abundances and distributions

The annual N inputs through biological N_2 fixation in the oceans is considered high, ranging 100-200 Tg N (Eugster and Gruber, 2012; Luo et al., 2012), yet large uncertainties remain in what factor(s) influence the abundance, distribution, and activity of marine diazotrophs. Initially, we hypothesized that conditions favoring a particular cyanobacterial diazotroph would differ given the contrasting life histories (free-living, colonial, and symbiotic). Moreover, we also suspected that the conditions promoting DDAs would differ from those influencing the UCYN-A based symbioses given the vast differences in the symbionts and hosts (e.g. genome content of symbiont, cell size of symbiont and hosts in the two systems; expected number of symbionts/host; host phylogeny: diatom vs. prymnesiophyte). Thus, determining the condition or sets of conditions that drive cyanobacterial diazotroph distribution, abundance, and activity is of great interest.

Hydrographic conditions and dissolved nutrient concentrations measured at the time of sampling were used to correlate diazotrophic abundance with various environmental parameters. Consistently, in two independent statistical tests, two groups emerged in the WTSP: 1) UCYN-A1 and A2 and their respective hosts 2) het-1, het-2 and het-3, UCYN-B and *Trichodesmium*. Thus, agreeing with our initial hypothesis that conditions favoring the UCYN-A based symbioses does differ from the conditions for DDAs, and in addition for the free-living cyanobacterial diazotrophs.

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Temperature is often cited as the most important driver of diazotroph abundance and distribution (Messer et al., 2016; Moisander et al., 2010). As shown earlier in the WTSP, both *Trichodesmium* spp. and UCYN-B were most abundant in warmer surface waters (> 27 ºC) in the north, while UCYN-A dominated in the cooler (24-26 ºC) southern waters of WTSP (Bonnet et al., 2015; Moisander et al., 2010). Likewise, we found similar abundances and temperature optima for the latter three diazotrophs and significant correlations between the various diazotrophs and temperature. In fact, all diazotrophs, except the UCYN-A lineages were significantly positively correlated with temperature in the WTSP. In addition to temperature, environmental parameters PAR, salinity and depth were also significantly influencing abundance and distribution. Moreover, the latter two variables drove the abundances of UCYN-A symbioses (A1 and A2) apart from the rest of the diazotrophs in the WTSP, including both free-living phylotypes and the symbiotic heterocystous lineages.

The maximum abundances at depth for UCYN-A1 and UCYN-A2 were slightly above or at the nitracline and coincided with higher measures of fluorescence from the CTD. The latter is consistent with observations of high UCYN-A abundances in coastal habitats (Bombar et al., 2014), estuaries (Messer et al., 2015), or in waters that are recently entrained with new nutrients (Moisander et al., 2010). Increased *nifH* copies and/or *nifH* gene expression for UCYN-A have also been reported from bioassay experiments amended with nutrients, including DIN, phosphate and iron (Krupke et al., 2015; Langlois et al., 2012; Moisander et al., 2012). The latter is in contrast with the data reported here in the WTSP (including the meta-analysis) and several of the external datasets (e.g. WTSP, TA, NA, NSCS), which finds a negative correlation between DIN and DIP concentrations and abundance of most of the diazotrophs, including UCYN-A. In the WTNA, waters with high DiSi concentration and low N:P ratios, driven by a disproportionate utilization of N relative to P, results in consistent and widespread blooms of the *Hemiaulus-Richelia* symbioses (het-2)

(Foster et al., 2007; Subramaniam et al., 2008). Across the cruise transect, DIP and DiSi concentrations were considered not limiting (Thierry Moutin, this issue), while DIN was below detection, hence conditions favoring symbiotic diatoms, and as reported here, the higher abundances of het-1 *nifH* gene copies and observations of *Rhizosolenia* hosts in the MA.

All the diazotrophs described here are either photoautotrophic or associated with photoautotrophic partners (UCYN-A, het-group). Therefore, light irradiance (e.g PAR) and availability will impact the abundance and distribution of the diazotrophic populations. Moreover, and related to light availability is the influence of day length or changes in the photoperiod which can influence diazotroph distribution, in particular the symbiotic diatoms (Karl et al., 2012). Results from CARD-FISH observations of the UYCN-A1 and A2 symbioses have reported a strong dependency on light intensity, which results in higher abundances nearer to the surface (Cabello et al., 2016). Presence in shallower waters is also thought to be strategic for avoiding competition (Cabello et al. 2016). However, in the WTSP, in 11 of the 14 stations where UCYN-A1 and A2 were detected at sub-surface depth maxima, the same lineages (and corresponding hosts) were undetected at the surface and a negative correlation was found with PAR. Microscopy observations also confirmed higher numbers of pico-eukaryotes at depth. Hence, it would appear that low light was a pre-requisite for high abundances of UCYN-A; while the other free-living diazotrophs and symbiotic het-1 and het-2 were positively correlated with PAR, and had maxima closer to the surface with higher PAR. Interestingly and unexpected was the lack of correlation between PAR and the UCYN-A host lineages, especially since it is the host partners that require light for photosynthesis.

In an attempt to identify the consistency in the correlation patterns identified in the WTSP with other regions of the world's ocean, the same statistical analyses were performed on 11 publically available datasets and subsequently run through a meta-analysis. Our

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statistical analyses provided coefficients and p-values for easy evaluation and comparisons between data sets for the influence of environmental parameter(s) and diazotrophs abundance. It confirmed that UCYN-A indeed stands out from the other diazotrophs in terms of environmental parameter influence, mainly by being uncorrelated with temperature, which for all other diazotrophs was a positive correlation. For most other environmental variables the pattern for UCYN-A does not hold true in the meta-analysis. However, for the other diazotrophs depth and salinity follow the same pattern as observed in the WTSP (except for UCYN-B being uncorrelated with salinity). Furthermore, what did unify all diazotrophs in the meta-analysis were their consistent negative correlations between abundance and concentrations of DIP and DIN, which was also observed in the WTSP and again UCYN-A was the exception.

In summary, the correlations observed in the WTSP were not always consistent with the meta-analysis of the external datasets. We attribute the inconsistencies in part to seasonal differences in sample collections, and the impact of an individual environmental parameter or sets of parameters on a local and regional scale that make it difficult to unambiguously explain the abundance and distribution patterns. Unlike our initial hypotheses, determining the condition or sets of conditions favoring one diazotroph or life history strategy (free-living vs. symbiotic) is complex and likely not all diazotrophs are influenced by the same condition in time and space.

4.6 Estimation of diazotrophs by nifH qPCR

When interpreting abundance estimates by qPCR there are a few assumptions to keep in mind. A caveat of qPCR assays assumes that there is one gene copy per cell. However, recent evidence in filamentous and heterocystous cyanobacteria reports evidence of polyploidy dependent on cell cycle (Griese et al., 2011; Sargent et al., 2016; Sukenik et al., 2012). Moreover, *Trichodesmium* may contain up to 100 genome copies per cell (Sargent et al.,

2016), thus a potential for overestimation. On the other hand, underestimation by qPCR is also plausible if one considers that DNA extraction efficiency is not 100% and can vary between species and DNA extraction kits (Mumy and Findlay, 2004), and if high probe specificity favors exclusion of closely related phylotypes for a particular target or lineage.

A final consideration with qPCR as shown here, is the degree of cross-reactivity in assays targeting closely related lineages (e.g. UCYN-A and het). Oligonucleotide specificity as a source of underestimation of the UCYN-A lineages was recently reviewed by a *de nova* analyses (Farnelid et al., 2016) showing the potential to underestimate UCYN-A sublineages since the widely used oligonucleotides for UCYN-A1 contains several mismatches to the other UCYN-A sublineages. The latter becomes important when the sublineages co-occur. Here, however, we highlight the potential to overestimate. For example, UCYN-A2 oligonucleotides amplified the UCYN-A1 templates, indicating a tendency to overquantify UCYN-A2 in the presence of A1. Moreover, when the annealing temperature was set to 64 ^{o}C, to distinguish between UCYN-A1 and A2 as recommended by Thompson et al. (2014), the assay still failed to separate the two sub-lineages when run in fast mode. Thus, the fast mode feature has a shortcoming that could influence a wider range of targets than the ones presented here. We observed the same cross-reactivity reported earlier (Foster et al., 2007) for het-1 and het-2 when run in fast mode and highlights the potential to overestimate het-2 if het-1 co-occurs at densities approximately 10^6 *nifH* copies L^{-1}. The latter observation has never been reported.

Conclusions

Consistent with earlier observations in the WTSP, we found diazotrophic cyanobacteria to be abundant. The most abundant cyanobacterial diazotrophs were UCYN-B, *Trichodesmium* and the symbiotic *Richelia* lineage het-1. Although the cell integrity and detection of het-1 in water column samples and those from depth (e.g. sediment traps)

indicated that the populations were in a senescent state, our work represents one of the first documentation of the three DDA populations in a wide expanse of the WTSP. In contrast to earlier work in the SP and other recent reports from global ocean surveys (Farnelid et al., 2016; Martínez-Pérez et al., 2016), we observed low abundances and poor detection of both UCYN-A (A1 and A2) lineages. According to our qPCR results, UCYN-A was also enumerated when their respective hosts were below detection, which contrasts to the assumed high fidelity and dependency in the partnerships; however, we cannot discount that the disparity in host-symbiont detection was not a result from qPCR oligonucleotide assay bias and/or overestimations indicated by our cross-reactivity tests.

Our initial hypothesis was that the condition or sets of conditions, which promote the distribution of one diazotroph, would differ. Moreover, the parameters for symbiotic diazotrophs should also differ from that of free-living phylotypes, and given the vast difference in hosts (diatoms and prymnesiophyte, respectively) and genome content for the het and UCYN-A symbionts, we further hypothesized divergent conditions favoring one symbiosis over another. In the WTSP, the same conditions favored abundances of both the free-living phylotypes and the diatom (het groups) symbioses. However, the same conditions impacted the abundance of UCYN-A based symbiosis negatively, hence, somewhat supporting our intial hypothesis that conditions for one symbiosis type would differ. In the external datasets, however, we observed differences in environmental conditions favoring abundances of the investigated diazotrophs compared to the WTSP, which underscores that diazotrophs are not similarly influenced by the same condition in time and space.

Multivariate approaches on numerous parameters and with high spatial resolution are required to understand the complex and often indirect effects that govern species distribution. Finally, this study highlights reliable quantification of *nifH* genes for various N_2 fixing cyanobacteria ‘at sea’ in the tropical open ocean and how environmental parameters influence

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distribution and abundance of diazotrophs differently both regionally and across ocean basins. However, it is of great interest to know, if the same parameters influence gene expressions (e.g. *nifH*), and ultimately N_2 fixation rates, in the same manner, thus, understanding the weight of environmental parameters influencing diazotrophic abundance and distribution. Given the global significance of N_2 fixation as a major new source of N to the oceans, the metanalysis presented here could be directly applicable to improving parameter constraints on model-based approaches for predicting areas prone to diaztrophy.

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Competing interests

The authors declare that they have no conflict of interest.

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1 Table 01. Summary of environmental conditions in the surface along the cruise transect.

Region	Stations	surface* DIN‡ µM	surface* DIP µM	surface* DiSi µM	surface* salinity (PSU)	surface* temp. ˚C
Melanesian archipelago (MA) 160˚ E-178˚ E 170 ˚W - 175 ˚W	SD1-12 LDA LDB	0.02 ± 0.01	0.03 ± 0.02	0.55 ± 0.10	35.13 ± 0.27	29.33 ± 0.45
Subtropical gyre (SG) 160 ˚W- 169˚W	SD13-15 LDC	0.01 ± 0.01	0.18 ± 0.07	0.79 ± 0.04	35.12 ± 0.10	29.34 ± 0.18

2 *5m depth, ‡NO_2+NO

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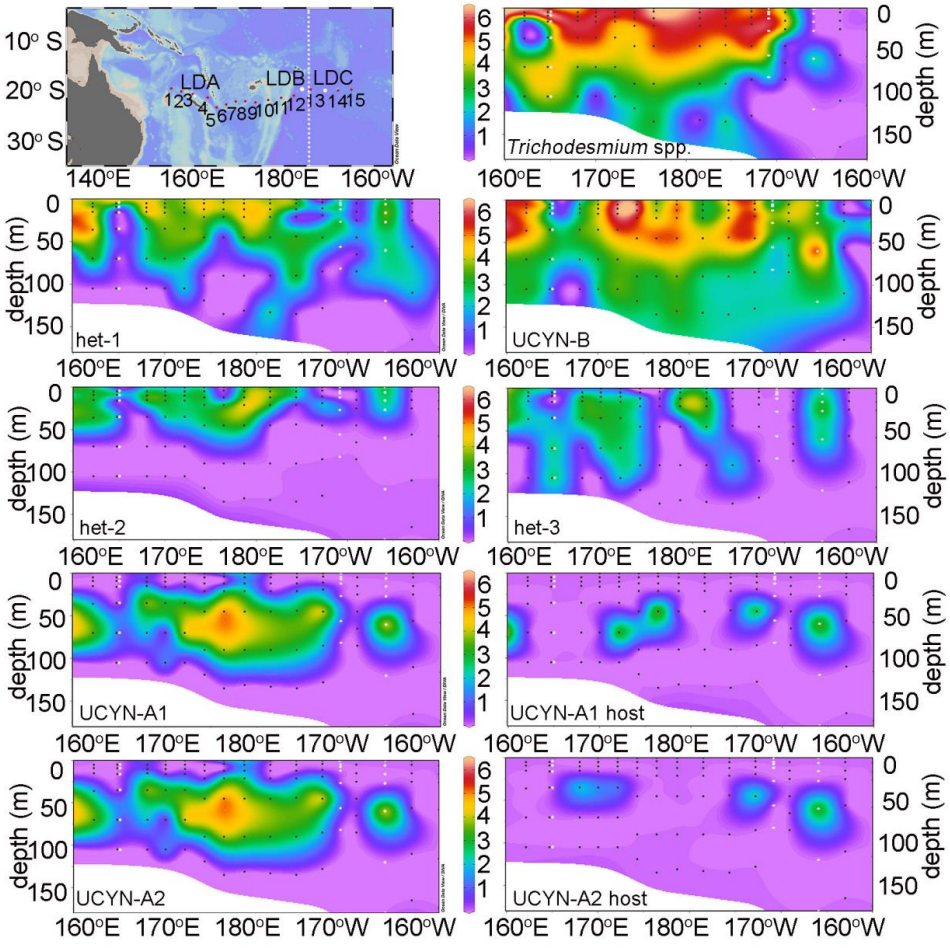

Figure 1. Sampling locations and the horizontal and vertical distributions of diazotrophs and the UCYN-A1 and UCYNA-2 hosts in the study area. Sampling depths are indicated as black dots (white for LD stations) and the abundances are the log *nifH* gene copy L^{-1} for the diazotrophs and 18S rRNA gene copies L^{-1} for the UCYN-A host lineages.

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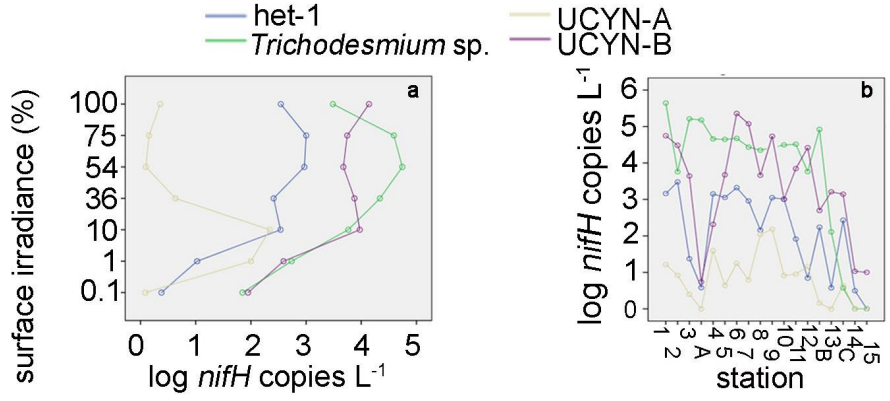

Figure 2 a-b. LOG10 transformed mean abundances for the 4 most abundant diazotrophs across the transect: het-1 (blue), *Trichodesmium* (green), UCYN-A (yellow) and UCYN-B (red). The mean *nifH* abundance values (log n*ifH* copies L^{-1}) shown as a function of (a) percent (%) surface irradiance and (b) at each station

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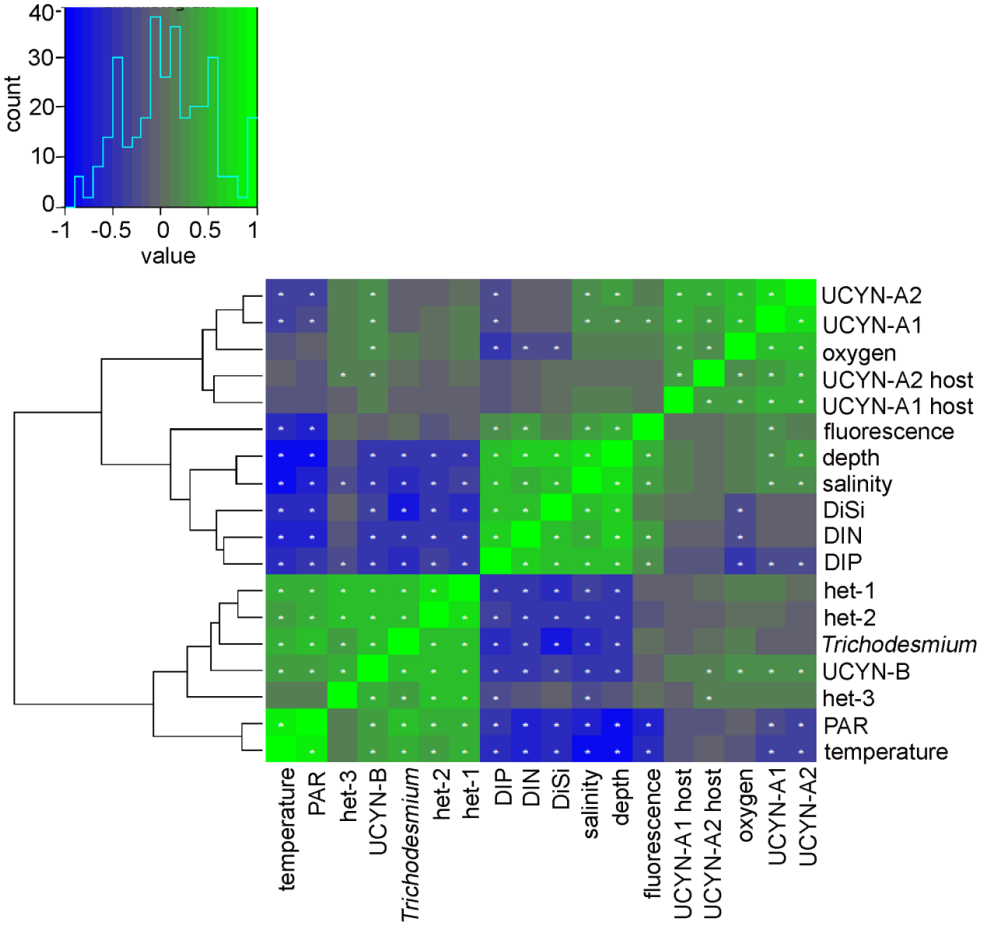

Figure 3. Hierarchical clustering heat map of Spearman's Rho results. The histogram shows negative (blue) and positive (green) values of correlation strength between parameters. Stars within cells mark significant correlations ($p<0.05$).

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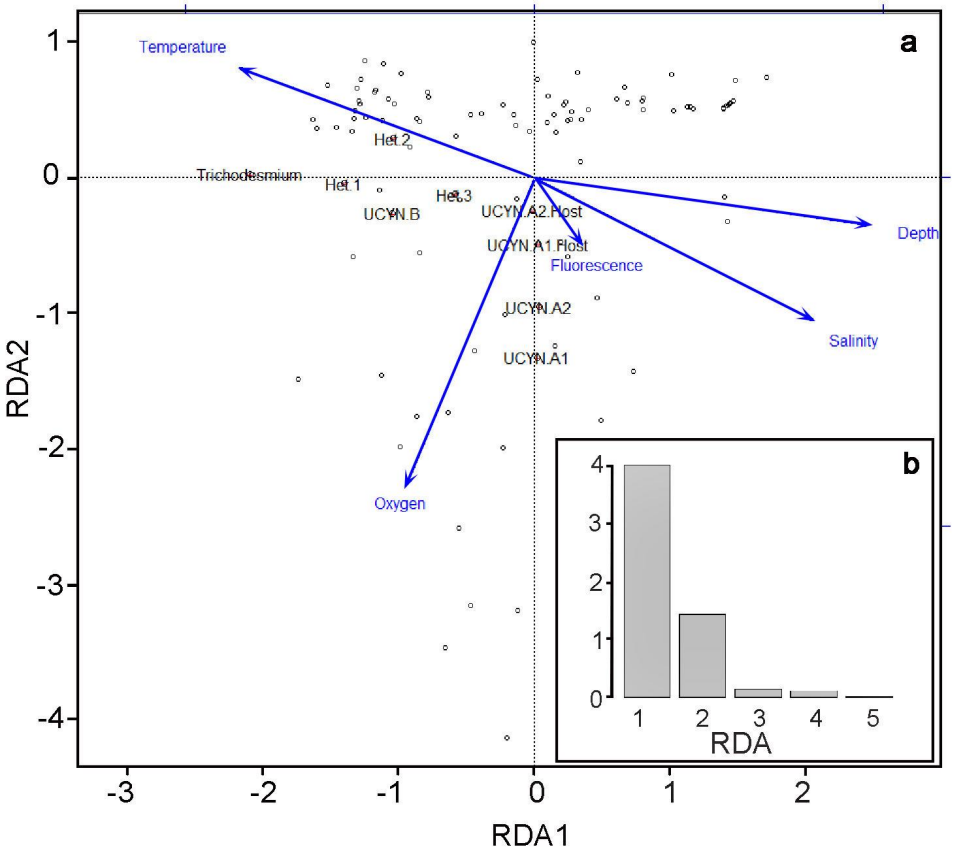

Figure 4 a-b. Multivariate RDA biplot (a), which also depicts variance of included parameters (b). As can be seen, a majority of the variance in the dataset is explained by the RDA1 and RDA2 axes meaning that most of the variance observed is explained by the included environmental parameters. The arrows are the constrained explanatory vectors with the dots representing the superimposed unconstrained response variables. PAR and nutrients (DIP and DIN) were omitted due to limited data.