

## ORIGINAL ARTICLE

# A novel cohabitation between two diazotrophic cyanobacteria in the oligotrophic ocean

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The cyanobacterial genus *Trichodesmium* is biogeochemically significant because of its dual role in nitrogen and carbon fixation in the oligotrophic ocean. *Trichodesmium* species form colonies that can be easily enriched from the water column and used for shipboard rate measurements to estimate their contribution to oceanic carbon and nitrogen budgets. During a July 2010 cruise near the Hawaiian Islands in the oligotrophic North Pacific Subtropical Gyre, a specific morphology of *Trichodesmium* puff-form colonies were examined under epifluorescent microscopy and found to harbor a colonial endobiont, morphologically identified as the heterocystous diazotrophic cyanobacterium *Calothrix*. Using unialgal enrichments obtained from this cruise, we show that these *Calothrix*-like heterocystous cyanobionts (hetDA for ‘*Trichodesmium*-associated heterocystous diazotroph’) fix nitrogen on a diurnal cycle (maximally in the middle of the light cycle with a detectable minimum in the dark). Gene sequencing of *nifH* from the enrichments revealed that this genus was likely not quantified using currently described quantitative PCR (qPCR) primers. Guided by the sequence from the isolate, new hetDA-specific primers were designed and subsequent qPCR of environmental samples detected this diazotroph from surface water to a depth of 150 m, reaching densities up to  $\sim 9 \times 10^3 \text{ l}^{-1}$ . Based on phylogenetic relatedness of *nifH* and 16S rRNA gene sequences, it is predicted that the distribution of this cyanobiont is not limited to subtropical North Pacific but likely reaches to the South Pacific and Atlantic Oceans. Therefore, this previously unrecognized cohabitation, if it reaches beyond the oligotrophic North Pacific, could potentially influence *Trichodesmium*-derived nitrogen fixation budgets in the world ocean.

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## Introduction

Nitrogen ( $\text{N}_2$ ) fixation is a critical metabolism providing as much as half of the bioavailable nitrogen to ocean surface waters (Montoya *et al.*, 2004; Capone *et al.*, 2005). In the oligotrophic oceans, time-series measurements indicate that  $\text{N}_2$ -fixing bacteria (diazotrophic) can bring more fixed N into surface waters than upwelling of  $\text{NO}_3^-$  (Karl *et al.*, 1997; Dore *et al.*, 2002). As phytoplankton in the oligotrophic ocean are frequently nitrogen (N) limited, the fixed nitrogen provided by diazotrophs is a key controlling factor for marine primary production (Capone *et al.*, 1997; Falkowski *et al.*, 1998; Hutchins *et al.*, 2007; Sohm *et al.*, 2011) and long-term carbon sequestration (Sarmiento *et al.*, 2006; Gruber and Galloway, 2008).

Biologic  $\text{N}_2$  fixation is an exclusively prokaryotic process mediated by the nitrogenase enzyme complex, the site where dinitrogen gas is reduced to form two ammonium ions (Postgate, 1982). The nitrogenase enzyme has been described in both heterotrophic and autotrophic organisms spanning the bacterial and archaeal domains (Zehr *et al.*, 2003), but no eukaryotes have been identified that can fix nitrogen without the aid of a prokaryotic symbiont (Sohm *et al.*, 2011; Zehr, 2011). Although there are many genes involved in the nitrogen fixation pathway, the active enzymes are encoded by the functional genes *nifH*, *nifD* and *nifK* (Postgate, 1982). The *nifD* and *nifK* genes encode dinitrogenase, the Mo-Fe containing tetramer that possesses the active site for  $\text{N}_2$  reduction (Mortenson and Thorneley, 1979), whereas the *nifH* gene encodes dinitrogenase reductase, the iron-containing protein component in the nitrogenase enzyme complex. Amplification, cloning and sequencing of the *nifH* gene has provided a powerful molecular tool for assessing the diversity of marine organisms capable of bringing new N into the photic marine ecosystem (Zehr *et al.*, 1998; Zehr, 2011). Field data from many groups have shown that marine cyanobacterial diazotrophs can be delineated into five groups: UCYN-A (a unicellular symbiont to a

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eukaryotic alga), Group B (e.g. *Crocospaera* spp.), Group C (e.g. *Cyanothece* spp.), non-heterocystous filamentous forms (*Trichodesmium* spp.) and the heterocystous filamentous forms (e.g. *Richelia*, *Calothrix*) (Foster et al., 2006; Moisaner et al., 2010; Sohm et al., 2011; Zehr, 2011; Thompson et al., 2012).

The *Trichodesmium* genus is comprised of four distinct clades based on functional and ribosomal genetic analysis: (I) *Trichodesmium spiralis*, *T. thiebautii*, *T. hildebrandtii*; (II) novel/uncultured; (III) *T. erythraeum*; and (IV) *T. tenue*, *T. contortum* (Lundgren et al., 2005; Hynes et al., 2012). *T. spiralis* was previously named *Katagnymene spiralis* and under this description was found to span the subtropical North and South Pacific Oceans, at certain stations and depths constituting 100% of the *Trichodesmium*/*Katagnymene* morphologically characterized, biomass (Lundgren et al., 2001). *Trichodesmium* spp. have been known to form blooms throughout the nitrogen-limited Atlantic and Pacific Oceans (Letelier and Karl, 1996), as well as the Arabian and Red Seas (Marvin-DiPasquale and Capone, 1998; Post et al., 2002). In the North Pacific Subtropical Gyre, *Trichodesmium* spp. dominate a recurring annual phytoplankton bloom (Dore et al., 2008). Despite decades of study, oceanic molecular surveys of *Trichodesmium* spp. frequently identify signatures from unidentified *Trichodesmium* phylotypes in field samples, demonstrating that not all members of this group have been recognized or characterized (Janson et al., 1999; Lundgren et al., 2005; Hynes et al., 2009; Chappell et al., 2012). Furthermore, relatively little is known about the distribution, specific microbial associations, enumeration and contribution of each of these phylotypes to *Trichodesmium*-specific global oceanic N<sub>2</sub> fixation (Hmelo et al., 2012).

In addition to *Trichodesmium*, filamentous, heterocystous diazotrophs (including both free-living and symbiotic cell types) have been detected throughout the North Pacific Subtropical Gyre in abundances up to 10<sup>5</sup> cells l<sup>-1</sup> (Church et al., 2005a,b, 2008; Foster et al., 2006; Zehr et al., 2007). Three distinct groups of heterocystous diazotrophs have been detected in the North Pacific subtropical ocean: Het\_1, Het\_2 and Het\_3 (the latter aligning with other members of the heterocystous genus *Calothrix* in sequence analysis) (Church et al., 2005a, 2008; Karl et al., 2008). The previously described heterocystous diazotrophs *Richelia* spp. and *Calothrix* spp. have been detected throughout the North Pacific Subtropical Gyre (Church et al., 2008; Foster et al., 2010) and are almost exclusively found in association with the diatoms *Rhizosolenia* spp. and *Chaetoceros* spp. (Foster et al., 2006, 2011), respectively. These diatom–diazotroph associations are well documented and globally distributed through the oligotrophic ocean and are thought to have an important role in carbon sequestration (Venrick, 1974; Janson et al., 1999; Subramaniam et al., 2008; Karl et al., 2012; Villareal et al., 2012).

In this study, we report the characterization of a heterocystous diazotroph, not previously observed or enumerated, cohabitating with colonies of the abundant and cosmopolitan global nitrogen fixer *Trichodesmium*. The heterocystous diazotroph (hetDA for ‘*Trichodesmium*-associated heterocystous diazotroph’) will be referred to as a hetDA ‘cyanobiont’ hereafter because it is currently the most accurate description when one considers that the absolute nature of the association (i.e., parasitic/symbiotic/opportunistic) is still unknown. We quantified the *in situ* abundance of the new hetDA cyanobiont via *nifH* gene using quantitative PCR (qPCR), enumerated the ratio of *Trichodesmium* spp. colonies cohabitating with this cyanobiont and measured the N<sub>2</sub>-fixation rates from a hetDA enrichment in controlled laboratory conditions.

## Materials and methods

### Field sampling

Sampling in the subtropical North Pacific Ocean was conducted aboard the R/V *Kilo Moana* on a 10-day cruise (KM10-13) near Station ALOHA (22° 45'N, 158° 00'W) in July 2010. Samples for *in situ* environmental DNA extraction were collected from a Sea Bird CTD (conductivity temperature depth) rosette. Ten liters of seawater were gravity filtered onto 10-µm polycarbonate filters from Niskin bottles triggered at discrete depths (5, 25, 45, 75, 125, 150 and 175 m). Samples were frozen and stored in liquid nitrogen until DNA extraction using MoBio PowerPlant DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA), and amended with an initial liquid nitrogen freeze/thaw cycle to facilitate cell lysis. Environmental DNA was stored at –20 °C and later used for genomic DNA PCR, cloning and sequencing, as well as qPCR enumeration.

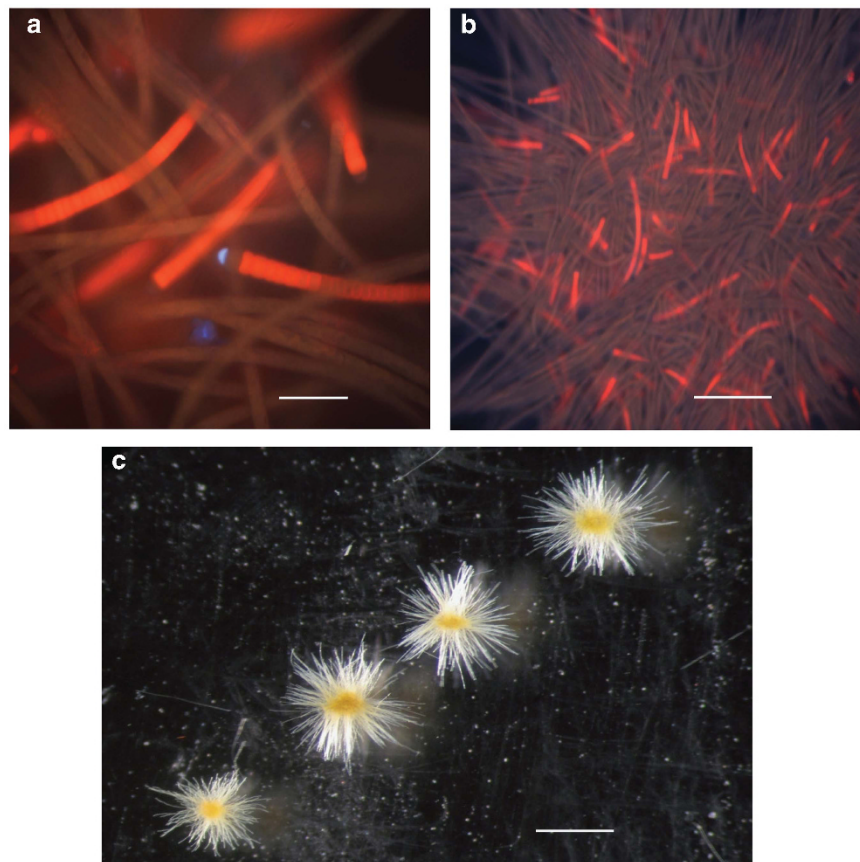
Colonies of *Trichodesmium* spp. were enriched from the top 10 m with a 130-µm mesh plankton net (Sea-Gear Corporation, Melbourne, FL, USA) that was hand towed at the surface for 20–30 min using a 30 m line. Immediately following the final haul to the surface, the contents of the tow were poured into 5 l polycarbonate beakers and taken into the laboratory. Individual *Trichodesmium* colonies were carefully handpicked using a polypropylene transfer bulb pipette and then gently washed several times with 0.2-µm-filtered local seawater, to remove organisms not closely associated with the colonies before observation on a Zeiss AxioStar epifluorescent microscope with the Zeiss Axiovision software/Axiocam and Zeiss dissecting microscope (Zeiss, Pleasanton, CA, USA).

All hand-picked *Trichodesmium* colonies were screened for hetDA cells using epifluorescent microscopy. Colonies with the cohabitation were collected and filtered onto a 5.0 µm polycarbonate filter and frozen in liquid nitrogen for further

laboratory analysis (Figure 1). After microscopic confirmation of cohabitation, select individual colonies were placed in 14 ml VWR Polystyrene Culture Tubes containing 10 ml of 0.2  $\mu\text{m}$  sterilized YBCII media and incubated on board ship at 24 °C on a 12:12 h light–dark cycle. Upon return, tubes were immediately placed into a Percival incubator (Percival Scientific Inc., Perry, IA, USA) at 24 °C and mimicking day–night conditions of 100  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  during the 12 h light cycle. After 1 month, visibly dark green filamentous growth was observed in tube KM1013-14. This enrichment culture was maintained in sterile YBC II media and transferred to fresh YBCII media every 14 days. To purify the filamentous, heterocystous cells, the culture was filtered through a 60  $\mu\text{m}$  cell strainer. Cell aggregates were retained and this enrichment was maintained as a semicontinuous batch culture throughout the study. Once the enrichment was stably maintained in the laboratory, the material was streaked on Sigma 1% tissue culture grade, Type VII agar, YBCII plates to obtain clonal, unialgal hetDA cultures.

#### DNA extraction and 16S and *nifH* cloning and sequencing

To phylogenetically identify the hetDA cells in the culture, 16S rRNA and *nifH* genes were amplified, cloned and sequenced from genomic DNA extracted from the enrichment using the same procedure as the environmental depth profiles (see above). Primers CYA 106F and 781a/b, which amplify a conserved 16S ribosomal DNA cyanobacteria-specific region, were used as described previously (Nubel *et al.*, 1997). The *nifH* partial gene sequence was amplified using the primers and nested PCR protocol as described by Zehr and Turner (2001). Primer sequences are found in Table 1. Nuclease-free water additions were used for negative controls and all reactions were performed on an Applied Biosystems 9700 thermocycler (Life Technologies, Carlsbad, CA, USA). Amplicons were separated on 1.2% agarose gels, DNA bands were visualized using SYBR-safe (Invitrogen, Carlsbad, CA, USA) and desired products purified with QIAquick Gel Purification Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The purified product was



**Figure 1** Epifluorescent images of *Trichodesmium* and heterocystous cyanobiont taken while on board the R/V *Kilo Moana* in July of 2010. (a) DAPI (4',6-diamidino-2-phenylindole) long-pass filter set micrograph of heterocystous cyanobacterium (bright red tapered filament, bulbous heterocyst, blue cap) and thinner, lighter *Trichodesmium* filaments. Scale bar is 10  $\mu\text{m}$ . (b) Same *Trichodesmium* colony as pictured in (a), at lesser magnification. Shorter, bright red filaments are the heterocystous cyanobacteria, surrounded by longer, thinner *Trichodesmium* filaments. Scale bar is 50  $\mu\text{m}$ . (c) Image taken under dissecting microscope. Colonies were handpicked from onboard net-tow and each was confirmed to contain cyanobionts using the epifluorescent microscope. Scale bar is 4 mm. Dark centers are concentrated with hetDA *Calothrix*-like cyanobionts.



cloned using the TOPO-TA 2.1 Kit (Invitrogen) and transformed clones were picked for plasmid miniprep using the PureLink Quick Plasmid Miniprep Kit (Invitrogen). Purified minipreps (500 ng) were amplified with T3 and T7 primers (5 pmol ml<sup>-1</sup>) following the manufacturer's instructions, and sent to Genewiz sequencing facility (San Diego, CA, USA) for Sanger sequencing using T3 and T7 forward and reverse primers, respectively.

Sequences were trimmed, assembled and aligned using the MUSCLE algorithm. Phylogenetic trees were created using Geneious Pro version 5.6.2 (Biomatters Ltd., San Francisco, CA, USA). PhyML consensus trees were constructed (Guindon and Gascuel, 2003) using the Jukes Cantor genetic distance model with 1000 bootstrap replicates. Trees were constructed using nucleotide sequences for each of the 16S rRNA partial gene sequence (681 bp), and the *nifH* partial gene sequence (321 bp) (Figures 2a and b), respectively. Sequences from heterocystous cyanobacteria groups traditionally found at Sta. ALOHA were included in Figure 2b to determine whether hetDA had been sequenced in previous

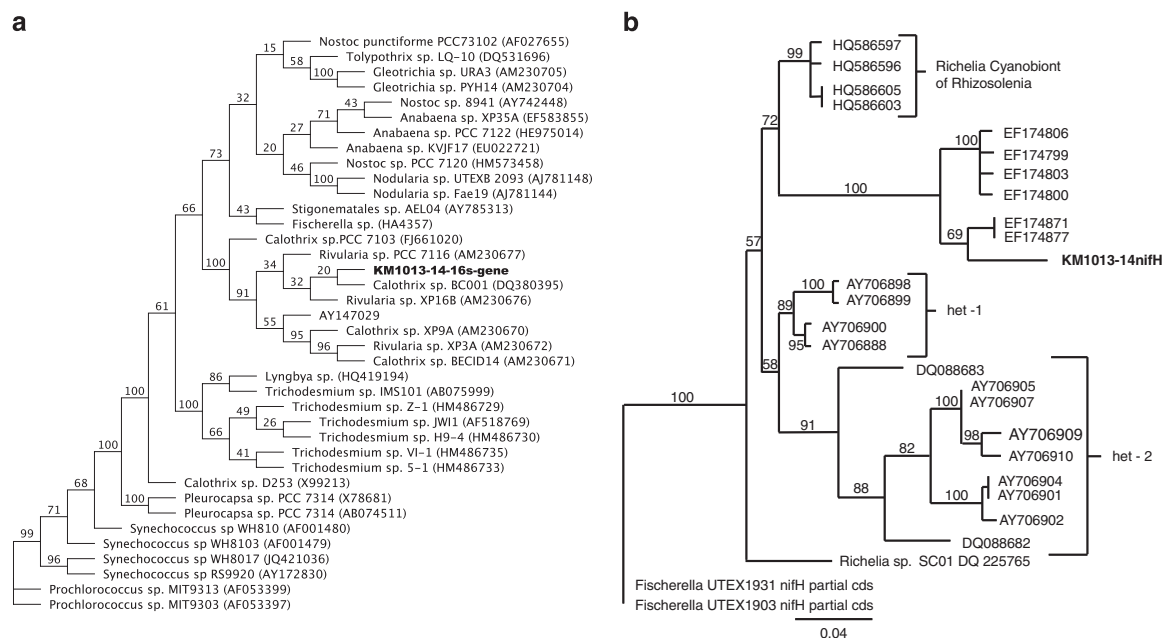
diazotroph surveys (Church *et al.*, 2008). The 16S rRNA and *nifH* sequences obtained in this study have been deposited into NCBI GenBank Short Read Archive under accession numbers KJ093757 and KJ721229, respectively.

### Quantitative PCR

Real-time qPCR (25 µl total volume, reaction conditions: 95 °C for 5 min, 40 cycles of 95 °C for 30 s, 48 °C for 45 s, 70 °C for 30 s standard melt curve at the end) was performed on environmental DNA extracts at depths from 5 to 175 m on an Applied Biosystems StepOnePlus Real Time PCR system and processed using StepOne Software Version 2.1 (Applied Biosystems, Foster City, CA, USA) as previously described (Reese *et al.*, 2014). DNA was amplified with the QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA, USA) using the KM1013-14F/R primers given in Table 1. Standards were made from linearized plasmids containing the 237 bp gene fragment of interest, which was cloned from amplified hetDA DNA extracted from

**Table 1** Primer nucleotide sequences used for PCR and qPCR analyses

Primer names	Forward primer 5' → 3'	Reverse primer 5' → 3'	Gene target/assay
<i>nifH</i> 3/ <i>nifH</i> 4	ATRTTTRTTNGCNGCRTA	TTYTAYGGNAARGGNGG	<i>nifH</i> /PCR
<i>nifH</i> 1/ <i>nifH</i> 2	TGYGAYCCNAARGCNGA	ANDGCCATCATYTCNCC	<i>nifH</i> /PCR
KM1013-14F/R	CCAAAGCACAAACAACCGTA	CCACCACATACAACGTCACC	<i>nifH</i> /qPCR
Het_3F/R or SC01F/R	CGGTTTCCGTGGCGTACGTT	AATACCACGACCAGCACAAC	<i>nifH</i> /qPCR
CYA106F/CYA781a/b	CGGACGGGTGAGTAACGCGTGA	GACTACTGGGGTATCTAATCCCAT	16S/PCR
		GACTACAGGGGTATCTAATCCCTTT	



**Figure 2** Maximum-likelihood consensus trees relating hetDA cloned 16S and *nifH* sequences to environmental clones and isolates. PhyML trees were constructed in Geneious Pro version 5.6.2 using the Jukes Cantor distance model and 1000 bootstrap replicates. (a) Community 16S rRNA relatedness based on 681 bp 16S rRNA nucleotide sequence. (b) Phylogenetic tree of *nifH* gene nucleotide sequence relating hetDA enrichment culture to known heterocystous cyanobacteria found in the oligotrophic North Pacific (groups het\_1, het\_2 and het\_3) as well as clones of similar sequence distributed globally.

laboratory enrichment culture. Standard plasmids were quantified using a Qubit fluorometer (Applied Biosystems) and diluted over six orders of magnitude ( $1.6 \times 10^1$ – $1.6 \times 10^6$ ) with three replicates at each dilution. Sensitivity was calculated with precision to  $10^1$ – $10^2$  target molecules per reaction. No-template negative controls were carried out and yielded no detectable signals. Primer concentration was optimized at 150 nM to eliminate primer dimer and non-target melt curves. Copy number was calculated assuming a molecular mass of 660 Da per base pair of DNA and using the following formula for calculating the PCR target copy number (Jin and Mattes, 2010):

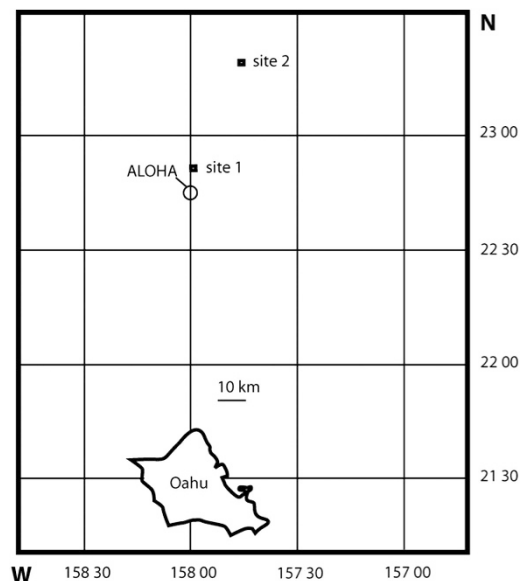
$$\frac{\text{gene copies}}{\text{lofwater}} = \frac{\text{concentration} \left( \frac{\text{ng}}{\mu\text{l}} \right) \times \frac{6.022 \times 10^{23}}{\text{mol}}}{\text{amplicon size} \left( \frac{\text{bp}}{\text{gene copy}} \right) \times \frac{10^9 \text{ ng}}{\text{g}} \times \frac{660 \text{ g}}{\text{mol}}}$$

#### Primer design

Primers were designed using Serial Primer 3.0 and verified using the NCBI Basic Local Alignment Search Tool (BLAST) database, including environmental clones, for specificity. Previously described SC01/Het\_3 primers (Foster and Zehr, 2006; Foster *et al.*, 2010), Het\_1 primers (Church *et al.*, 2005b) and Het\_2 primers (Church *et al.*, 2008) were tested on nucleic acids extracted from the enriched KM1013-14 culture to ensure that these primers did not amplify hetDA. No amplification was seen using the Het\_1, Het\_2 or SC01/Het\_3 primer sets (data not shown). Amplification of the KM1013-14 *nifH* gene was observed using the *nifH* qPCR primers designed specifically for this study, KM1013-14F and R (Table 1). To further test the specificity of the newly designed *nifH* primers, a clone library was generated from environmental DNA extracts from stations 1 and 2 (Figure 3) collected on the R/V *Kilo Moana*. Briefly, the clone library was generated as follows: whole environmental template DNA was amplified using our optimized qPCR conditions and primers (excluding the final melt curve), the resulting PCR products were cloned and sequenced as above.

#### Laboratory $N_2$ -fixation rate

The nitrogenase activity of the hetDA enrichment was evaluated via the acetylene reduction (AR) assay (Capone, 1993). All cultures were grown in autoclaved, sterilized YBCII media with no added nitrogen sources according to the protocol described in Chen *et al.*, 1996. Nitrogen fixation was measured on the hetDA batch culture over a 26-h incubation period (Capone, 1993; Fu *et al.*, 2008). Briefly, immediately before acclimation for the AR assay, whole water enrichments of the hetDA culture were filtered through 60  $\mu\text{m}$  cell tissue strainers and only aggregates of dark green filamentous hetDA cells were retained, to ensure there would be minimal



**Figure 3** Map of sample collection locations from 17–19 July 2010 on cruise KM10-13. Open circle indicates Station ALOHA ( $22^\circ 45' \text{N}$ ,  $158^\circ 00' \text{W}$ ), small solid squares represent locations where CTD rosette and Niskin bottles were deployed. Longitude and latitude (degrees and minutes) indicated on figure axes.

contamination from potential single-celled cyanobacterial diazotrophs. After filtration, aggregates were transferred back into fresh, sterile YBC II media and 80 ml of this semicontinuous enrichment culture was aliquoted into glass serum vials, placed back in the incubator and allowed to acclimate. Cultures were incubated at  $24^\circ \text{C}$  with a light–dark cycle of 12:12 h and an incident photon flux density of  $175 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ . Vials were then capped with butyl rubber stoppers and crimp sealed. Headspace of vials was sequentially spiked with 5 ml acetylene (time=0) and evolution of ethylene gas was measured by gas chromatography every hour for 3 h in biologic triplicate. Ethylene evolution was measured on a Shimadzu gas chromatograph model GC-8A using a flame ionization detector (Shimadzu Scientific Instruments, Columbia, MD, USA) and 200  $\mu\text{l}$  injection volume (Chappell and Webb, 2010).

Total chlorophyll was extracted and AR rates were normalized to fluorometrically measured chlorophyll *a* (Chl*a*). Following each AR assay, cultures were filtered onto combusted Whatman GF/F (Fisher Scientific, Pittsburg, PA, USA), placed in 90% acetone and incubated overnight in the dark at  $-20^\circ \text{C}$  to extract the Chl*a*. Total Chl*a* was quantified based on the fluorescence technique (Parsons *et al.*, 1984) using a Turner Designs TD-700 fluorometer (Turner Designs, Sunnyvale, CA, USA). Outliers in biologic triplicates were identified using R version 2.9.0 (<http://www.r-project.org/>).

## Results

#### Field and laboratory observations

While on board the RV *Kilo Moana*, a total of 441 *Trichodesmium* puff colonies were handpicked from

net tows and screened on the Zeiss epifluorescent microscope using a DAPI (4',6-diamidino-2-phenylindole) long-pass filter. Of these, 347, or ~75%, were found to contain the unidentified phycocyanin-rich, heterocystous cyanobiont (hetDA). The hetDA cells were not observed cohabitating with tuft colonies, thereby indicating potential morphologic specificity. *Trichodesmium* trichomes appeared orange because of the presence of the photopigment phycoerythrin, whereas the heterocystous cyanobionts were a distinct red, tapered, filament that had a well-defined heterocyst at one end, and a blue cap at the 'top' of the heterocyst (Figure 1a). It was also noted that when the net tow receptacle was left for a short time without agitation, all of the *Trichodesmium* puff colonies that sank to the bottom of the receptacle contained the hetDA cyanobiont. This sinking indicates reduced colony buoyancy when cyanobionts are present. There appeared to be no size discrimination in cohabitation by the cyanobiont: *Trichodesmium* colonies that contained the cyanobiont ranged from smallest observed to largest observed, 1.4–3.4 mm diameter (Figure 1c).

Attempts to reconstitute the cohabitation in laboratory cultures were performed with multiple successes. YBCII media (Chen *et al.*, 1996) were not conducive to reconstitution. However, SO media, consisting of 75% seawater and 25% MilliQ water, and devoid of a fixed nitrogen source (Webb *et al.*, 2001), exhibited repeated success. Attempts were made with two distinct *T. thiebautii* isolates VI-1 and H9-4 (Hynes *et al.*, 2012). Both *Trichodesmium* strains demonstrated a propensity to incorporate hetDA filaments into puff colonies within 48 h of culture combination. In the first 24 h, *Trichodesmium* spp. and hetDA cells remained physically separate (Figure 4a), but during days 2–4 the hetDA cells were incorporated into healthy *Trichodesmium* puff colonies (see Figure 4b). After this window, the *Trichodesmium* filaments tended to be outcompeted by hetDA in culture and gradually died off, until only dark green *Calothrix*-like filaments could be seen. Attempts to coculture members of the *Trichodesmium* clade III (specifically, type strain IMS101) with the hetDA cyanobiont were never successful, regardless of media and culture conditions.

#### Genotypic identification and analyses

Using total genomic DNA extracted from the laboratory hetDA enrichment, the *nifH* and 16S rRNA, and genes were PCR amplified, cloned and Sanger sequenced. BLAST comparisons were performed to identify the nucleotide and amino-acid sequences in NCBI, with the highest sequence identity to those amplified from the hetDA enrichment. Amino-acid level comparison of the hetDA *nifH* partial sequence (324 bp) indicated that heterocystous cyanobacterium culture C1C5 (accession number AF227947), obtained from enriching N<sub>2</sub>-fixing organisms from Bahamian stromatolites (Steppe *et al.*, 2001) was 99%

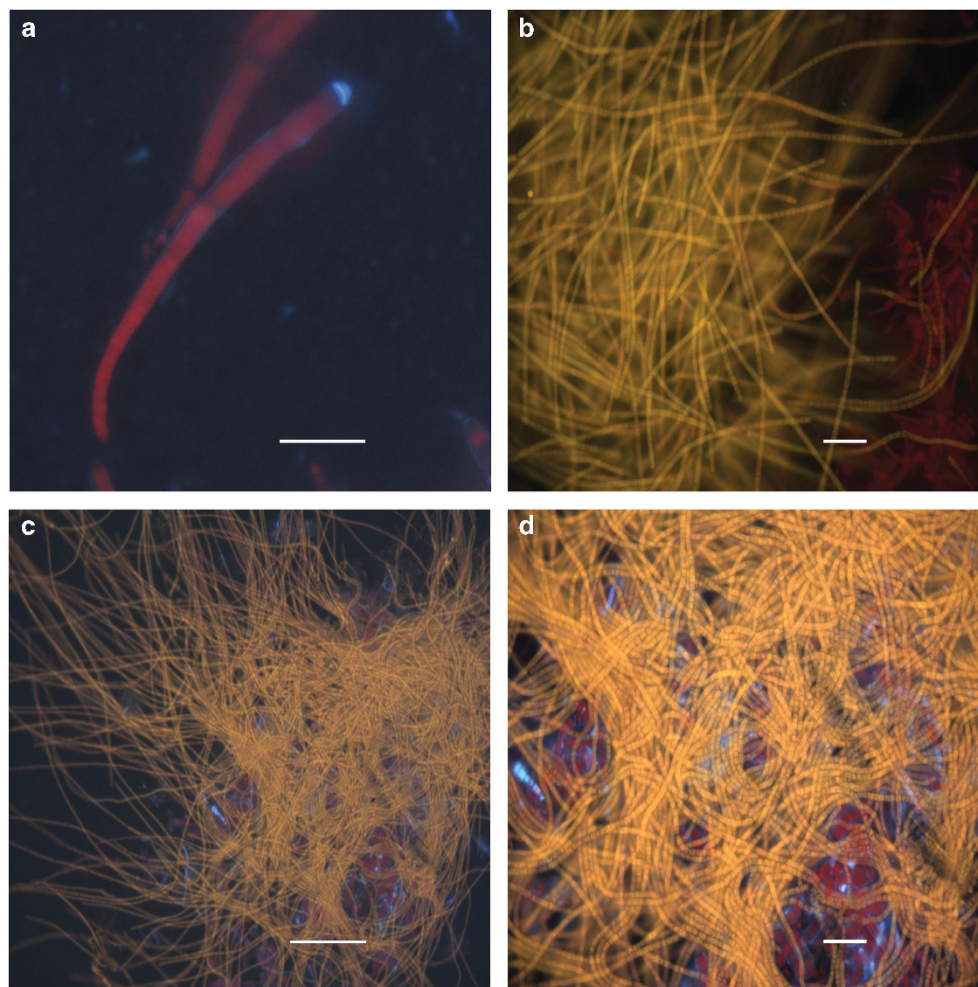
identical at the amino-acid level. Interestingly, the *nifH* hetDA nucleotide sequence did not identify most closely with Bahamian sequence (AF227947), but instead was more similar to putative heterocystous cyanobacterial *nifH* sequences (95% nucleotide sequence identity over 317 bp, accession numbers EF174877 and EF174871), obtained from Heron Island in the Great Barrier Reef area of the South Pacific (Hewson *et al.*, 2007). This is demonstrated in the resulting phylogenetic tree (Figure 2b) where KM1013-14 and environmental clone EF174877 fall into the same branch with 69% bootstrap support. Staying within the same branch of the Nostocales, the top cultured hits (>98% nucleotide sequence identity) for the 750 bp hetDA 16S rRNA gene sequence include either *Rivularia* or *Calothrix* spp. that had been enriched from marine carbonate rock surfaces in Puerto Rico (*Calothrix* sp. BC001), littoral zone of the Baltic Sea (*Rivularia* sp. XP16B) or from a small planktonic marine cyanobacterial colony in La Paz, Baja California (Rippka *et al.*, 1979; Chacón *et al.*, 2006; Sihvonen *et al.*, 2007) (Figure 2a). In all of the aforementioned studies, the apparent habitat for the closest hits to hetDA were surface associated or aggregated organisms, findings that are consistent with the hetDA–*Trichodesmium* aggregate observed at Sta. ALOHA.

*Trichodesmium* colonies known to contain the heterocystous cyanobiont were screened and collected during cruise KM10-13 (see Materials and methods). DNA was extracted from these colonies and the *nifH* partial gene sequence was amplified and cloned. Fourteen clones were trimmed and assembled using a CLUSTALW alignment with gap open cost of 15. The 14 clones generated had >99% nucleotide sequence identity. The consensus identity sequence was used to search the NCBI database for closest relatives of the environmental clones. Nucleotide sequence search (BLASTn), excluding environmental clones, of the consensus sequence returned *K. spiralis* (now *T. spiralis*), *T. erythraeum* and *T. thiebautii* as the closest relatives represented in culture (99%, 98% and 98% identical, respectively).

#### Quantitative PCR

Samples were analyzed in triplicate technical replicates at each depth and values were within a standard deviation of 5% (Supplementary Figure 1). Overall, an average of 10<sup>2</sup> copies *nifH* gene l<sup>-1</sup> was measured at site 1, and 10<sup>3</sup> copies *nifH* l<sup>-1</sup> at site 2 (Table 2). A peak in the number of copies was observed at 75 m at site 1. Below 75 m, the number of copies was below the limit of detection (10<sup>1</sup> gene copies per reaction). Site 2 showed a higher gene copy number for all depths and a peak cell density at 100 m (8.7 × 10<sup>3</sup> copies *nifH* l<sup>-1</sup>). Based on a two-way analysis of variance, experimental values for gene copy number were not significantly different between the two sites. Standard curves were run in triplicate with each environmental depth profile and yielded between





**Figure 4** Epifluorescent images of *Trichodesmium* H9-4 and hetDA maintained and reconstituted in laboratory culture. All images were taken through DAPI (4',6-diamidino-2-phenylindole) long-pass filter (a) hetDA heterocystous filaments by themselves, observed after 2 years in laboratory culture. Scale bar bottom left is 10  $\mu\text{m}$ . (b) *Trichodesmium* H9-4 and hetDA filaments after 24 h in the same liquid culture vessel. Filaments are not yet physically intertwined, but the majority of puff colonies were observed to have the heterocystous cyanobionts clinging to their fringes. Scale bar is 40  $\mu\text{m}$ . (c) *Trichodesmium* H9-4 and hetDA after 48 h in the same culture bottle. Colony was taken from the same liquid culture as the colony pictured in (b). Heterocystous filaments are clearly intertwined in the *Trichodesmium* H9-4 puff colony. Scale bar is 100  $\mu\text{m}$ . (d) Image (c) at greater ( $\times 400$ ) magnification.

95% and 105% efficiency and a linear regression through  $10^1$  copies per reaction (Supplementary Figure 1). Fourteen clones were generated from the qPCR product. They aligned to each other over 247 bp with <1% base pair mismatch, indicating that no unintended targets were amplified.

qPCR values were plotted as a function of depth against cruise metadata for temperature and relative fluorescence units (Supplementary Figure 2). No correlations were found between heterocyst-forming cyanobacteria abundance and temperature, depth or chlorophyll profiles (other than a general decrease in cell abundance below 150 m).

#### Culture $N_2$ -fixation rates

Diel AR was run over the 26 h period 0000–2400–0200 hours, using the same batch culture of hetDA (Figure 5). Each experiment resulted in peak fixation

rates of  $\sim 4.5\text{--}5.5 \mu\text{m C}_2\text{H}_4 \text{ mg Chla}^{-1} \text{ h}^{-1}$ , occurring principally between 1000 and 1400 hours. This value is in accord with previous nitrogen fixation calculations for related heterocystous diazotrophs (Foster *et al.*, 2010, 2011). The minimum ethylene production was between 0.0 and  $0.3 \mu\text{m C}_2\text{H}_4 \text{ mg Chla}^{-1} \text{ h}^{-1}$ . The baseline measured rate was slightly higher than this minimum signal during dark hours between 1900 and 2400 hours. This indicates basal background nitrogen fixation carried out by hetDA, which appears to be maintained at a lower, but measureable, rate well into the dark cycle.

## Discussion

To our knowledge, this is the first report of high numbers of heterocystous  $N_2$ -fixing cyanobacteria cohabitating with *Trichodesmium* colonies in the

open ocean. One documented strategy used to optimize energy yields in a nutrient-limited habitat, such as the oligotrophic open ocean, is symbiotic metabolite exchange among microbial consortia (Biebl and Pfennig, 1978; Orphan *et al.*, 2001; Foster *et al.*, 2011; Prokopenko *et al.*, 2013). However, because these two diazotrophic primary producers are presumably competing for the same nutrients and producing the same metabolic byproducts, the nature of this relationship remains unclear. One explanation is that very few grazers are thought to prey on *Trichodesmium* spp. (Hawser *et al.*, 1992; O'Neil and Roman, 1992, 1994); therefore, these large filamentous colonies offer protective habitat in the open ocean, and they are known to harbor a community of heterotrophic bacteria and other filamentous cyanobacteria (Paerl and Bebout, 1989; Siddiqui *et al.*, 1992; Sheridan *et al.*, 2002; Hewson *et al.*, 2009). Measurements of diurnal

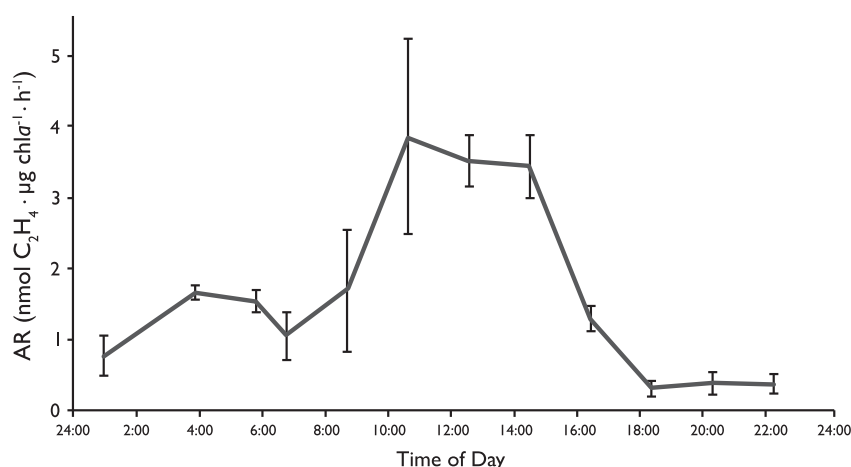
nitrogen fixation have shown that *Trichodesmium* nitrogen-fixation rates drop to below detection level at the start of the dark cycle (e.g., Capone *et al.*, 2005; Staal *et al.*, 2007). However, heterocystous diazotrophs are known to express *nifH* transcripts into the middle of the dark cycle (Zehr *et al.*, 2007; Poretsky *et al.*, 2009), a time when *Trichodesmium* are reaching N-storage depletion. Dark cycle, unintentional excretion of fixed bioavailable N by associated heterocystous cyanobacteria could be an advantage to the *Trichodesmium* colonies harboring the endosymbiont as *Trichodesmium* spp. are known to fix nitrogen in the middle of the light cycle but experience N starvation at night (Berman-Frank *et al.*, 2001). According to our AR assay results, the hetDA cyanobionts appear to have a basal rate of nitrogen fixation through the dark cycle. In associated colonies, this could alleviate the N stress experienced by *Trichodesmium* cells at night.

Others have calculated the abundance of various heterocystous diazotrophic groups using qPCR of the *nifH* gene at Sta. ALOHA (e.g., Church *et al.*, 2005a,b, 2008). However, because of specificity of the primers used, we believe that the heterocystous cyanobacterium described in this study was not detected in those previous studies and therefore has not been quantified at Sta. ALOHA or any other location. The relative abundance of the heterocystous cyanobacterial group described in this study is in the range of total heterocystous cyanobacteria cell densities measured previously,  $10^2$ – $10^3$  l<sup>-1</sup> (Church *et al.*, 2008), indicating that this new phylotype has a measurable and perhaps significant role in C and N cycling in the subtropical North Pacific Ocean. Previous surveys at Sta. ALOHA have quantified average *Trichodesmium* cell abundance at  $10^5$  l<sup>-1</sup> (Church *et al.*, 2008). Combining previous surveys and the qPCR results reported herein, the relative abundance of *Trichodesmium* cells to the newly described cyanobiont ranges from 10:1 to 100:1.

**Table 2** Summary of ship location and number of *nifH* gene copies l<sup>-1</sup> seawater as a function of depth at two sample sites

Site/date collected	Site 1 17 July 2010	Site 2 19 July 2010
CTD cast time	1630 hours	2130 hours
Latitude (N)	22.8	23.3
Longitude (W)	–157.9	–157.8
SST (°C)	25.9	25.2
Depth (m)	<i>nifH</i> gene copies per l	
5	$1.6 \times 10^2$	$1.2 \times 10^3$
25	$1.7 \times 10^2$	$2.6 \times 10^3$
45	$3.2 \times 10$	$5.2 \times 10^2$
75	$1.9 \times 10^2$	$1.4 \times 10^3$
100	BDL	$8.7 \times 10^3$
125	BDL	$1.5 \times 10^3$
150	BDL	$3.5 \times 10^3$
175	BDL	BDL

*nifH* values are means of triplicates, standard error <5%. Abbreviations: BDL, below detection limit; CTD, conductivity temperature depth; SST, sea surface temperature.



**Figure 5** Summary of hetDA enrichment acetylene reduction over the diel cycle. Data points are the means of biologic triplicates at each timepoint. Standard error bars are shown on graph. Sample vial headspace was saturated with acetylene every 2–3 h and 500 µl gas samples were removed from headspace for analysis every hour following acetylene addition.



The hetDA cyanobiont has yet to be quantified *in situ* at any location outside the stations surveyed in this study; thus, a wider environmental survey using the *nifH* qPCR primers designed in this study would elucidate questions on the global importance of these cells.

Based on phylogenetic comparison of the *nifH* gene nucleotide sequence, the hetDA endosymbiont is most closely related to a heterocystous cyanobacterium found in the South Pacific-Indian Oceans (uncultured environmental clones EF174877 and EF174871) (Hewson *et al.*, 2007). Comparisons made in the NCBI database for 16S rRNA nucleotide sequence revealed that closest relatives to hetDA have geographic ranges from the Baltic Sea (i.e. BECID14 accession no. AM230671) (Sihvonen *et al.*, 2007) to the subtropical South Pacific Ocean (i.e. BC001 accession no. 230670) (Hewson *et al.*, 2007). These clones were found in near-island, benthic environments. However, the closely related cells found in this study were collected in the oligotrophic open ocean. It was noted that *Trichodesmium* colonies that contained the heterocystous cyanobiont sank to the bottom of the collection vessel; however, colonies that did not contain the hetDA cells appeared to be more buoyant. This field result with freshly collected colonies suggests that the hetDA cells have negative buoyancy compared with *Trichodesmium*, a supposition that was consistent with laboratory observation of hetDA enrichment cultures. It is possible that the hetDA cells are taking advantage of the buoyancy and range of *Trichodesmium* to exploit a new habitat: the open ocean. Without association with *Trichodesmium* colonies, the cyanobiont would likely sink out of the photic zone. Furthermore, the dominant light-harvesting pigment of *Trichodesmium* cells is phycoerythrin, whereas it is phycocyanin in the cyanobiont. Assuming that the light levels were high enough, wavelength partitioning could ensure that the two cell types are not directly competing for available light.

Gliding motility was also observed in the hetDA culture. Field observations showed that cyanobiont cells concentrate in the center of *Trichodesmium* colonies, and cell motility would indicate that the hetDA cells preferentially inhabit this niche within the colony. It is well known that algae can release significant amounts of recently fixed carbon as molecules and polymers that fall into both the dissolved or particulate phases, and *Trichodesmium* is no exception (e.g., Nagata, 2000; Berman-Frank *et al.*, 2007; Claquin *et al.*, 2008). In *Trichodesmium*, this carbon (and presumably N) supports a wide array of heterotrophic organisms (Paerl and Bebout, 1989; Hmelo *et al.*, 2012) with activities so significant that the center of the colonies can go anoxic (Paerl and Bebout, 1988) and even allow the growth of active denitrifying bacteria (Wyman *et al.*, 2013). Oxygen diffusion across the *Calothrix*-like cyanobionts' heterocystous would be minimized in the

oxygen-deplete center of the *Trichodesmium* colony bundle, thereby lessening cellular damage and nitrogen fixation inhibition. Additionally, it is also known that *Trichodesmium* moves Fe-rich minerals to the center of the colony where they are subsequently dissolved (Rueter *et al.*, 1992; Rubin *et al.*, 2011). According to 16S rRNA gene sequence analysis, *Rivularia* strain PCC7116 has the highest sequence identity to strain hetDA, and it is known to be a facultative photoheterotroph (Rippka *et al.*, 1979); thus, it is possible that hetDA has photoheterotrophic capabilities as well. And, it is quite possible that the hetDA cells live an opportunistic, photoheterotrophic lifestyle, taking advantage of enriched carbon polymers, fixed nitrogen, dissolved Fe and low molecular oxygen in the center of *Trichodesmium* colonies.

Finally, N<sub>2</sub>-fixing organisms, similar to *Trichodesmium*, can contribute to long-term carbon sequestration (Sarmiento *et al.*, 2006; Gruber and Galloway, 2008). Here we suggest that the newly documented heterocystous cohabitation could have a role in the previously documented efficiency of N<sub>2</sub>-fixation-mediated downward particle flux (Dore *et al.*, 2002). This is corroborated by the 13-year (1992–2004) observation of a large and rapid particle export flux at Sta. ALOHA, dubbed the summer export pulse (Karl *et al.*, 2012). This period of high primary productivity include diatom–diazotroph associations and bloom conditions for various phytoplankton, and it is possible that the faster-sinking *Trichodesmium* colonies harboring the hetDA cyanobiont could contribute to this flux. This pulse occurs principally from 15 July–15 August, exactly overlapping with our observation in July 2010 of *Trichodesmium* harboring N<sub>2</sub>-fixing hetDA cyanobionts. Further work at Sta. ALOHA needs to be carried out to test any possible role of hetDA in this downward flux.

## Conflict of Interest

The authors declare no conflict of interest.

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