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ORIGINAL ARTICLE

Analogous nutrient limitations in unicellular diazotrophs and *Prochlorococcus* in the South Pacific Ocean

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Growth limitation of phytoplankton and unicellular nitrogen (N_2) fixers (diazotrophs) were investigated in the oligotrophic Western South Pacific Ocean. Based on change in abundances of *nifH* or 23S rRNA gene copies during nutrient-enrichment experiments, the factors limiting net growth of the unicellular diazotrophs UCYN-A (Group A), *Crocosphaera watsonii*, γ-Proteobacterium 24774A11, and the non-diazotrophic picocyanobacterium *Prochlorococcus*, varied within the region. At the westernmost stations, numbers were enhanced by organic carbon added as simple sugars, a combination of iron and an organic chelator, or iron added with phosphate. At stations nearest the equator, the nutrient-limiting growth was not apparent. Maximum net growth rates for UCYN-A, *C. watsonii* and γ-24774A11 were 0.19, 0.61 and 0.52 d^{-1}, respectively, which are the first known empirical growth rates reported for the uncultivated UCYN-A and the γ-24774A11. The addition of N enhanced total phytoplankton biomass up to 5-fold, and the non-N_2-fixing *Synechococcus* was among the groups that responded favorably to N addition. Nitrogen was the major nutrient-limiting phytoplankton biomass in the Western South Pacific Ocean, while availability of organic carbon or iron and organic chelator appear to limit abundances of unicellular diazotrophs. Lack of phytoplankton response to nutrient additions in the Pacific warm pool waters suggests diazotroph growth in this area is controlled by different factors than in the higher latitudes, which may partially explain previously observed variability in community composition in the region.

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Introduction

Availability of the macronutrients nitrogen and phosphorus (P) has an important role in controlling the magnitude of primary production and phytoplankton growth in oceans (Ryther and Dunstan, 1971; Falkowski, 1997). New nitrogen (N) from diazotrophic activity (fixation of N_2 by certain microorganisms, diazotrophs) supports carbon fixation in open ocean ecosystems (Karl *et al.*, 1997; Dore *et al.*, 2002), thus factors that control abundances of diazotrophs are relevant for regulation of ocean production.

Nitrogen is thought to be the most significant limiting nutrient in oligotrophic oceans with low nutrient and low chlorophyll, suggested by results from nutrient bioassays conducted in the North Atlantic (Graziano *et al.*, 1996; Mills *et al.*, 2004; Davey *et al.*, 2008) and Pacific (Van Wambeke *et al.*, 2008) Oceans. The presence and activity of microorganisms fixing atmospheric N_2 in oligotrophic oceans indicates scarcity of bioavailable N and relative sufficiency of other essential nutrients that allows growth of these microorganisms. While the filamentous cyanobacterium *Trichodesmium* is the most well described of the oceanic diazotrophs (Capone *et al.*, 1997), other important diazotrophs include filamentous cyanobacterial symbionts (Carpenter *et al.*, 1999) and unicellular diazotrophic autotrophs and heterotrophs, including the cyanobacteria *C. watsonii* and the uncultivated UCYN-A (Zehr and Turner, 2001; Montoya *et al.*, 2004; Langlois *et al.*, 2005; Church *et al.*, 2005a).

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Abundances of diazotrophs in open ocean are not as high as would be predicted from distribution of the widespread low nutrient and low chlorophyll areas, however, and their growth may be limited or controlled by other nutrients and growth factors, such as P (Sanudo-Wilhelmy *et al.*, 2001; Hynes *et al.*, 2009), iron (Fe) (Rueter, 1988; Sanudo-Wilhelmy *et al.*, 2001), or potential top–down effects by grazing or viral lysis. The low nutrient and low chlorophyll North Atlantic Ocean is thought to be one of the most iron-rich oceans owing to eolian inputs (Jickells *et al.*, 2005) and has diverse communities of diazotrophs (Orcutt *et al.*, 2001; Langlois *et al.*, 2008). Actively N_2-fixing *Trichodesmium* requires more Fe relative to non-diazotrophic phytoplankton (Berman-Frank *et al.*, 2001), however in the Atlantic its growth was suggested to be either P (Sanudo-Wilhelmy *et al.*, 2001; Hynes *et al.*, 2009) or Fe (Lenes *et al.*, 2001) limited. Co-limitation of bulk N_2 fixation by P and Fe was reported (Mills *et al.*, 2004), suggesting both nutrients may become limiting for diazotrophs in this ocean basin, supported by recent *in situ* gene expression data (Webb *et al.*, 2007). Differences in phosphorus utilization strategies among diazotrophs potentially contribute to their relative competitive success (Dyhrman *et al.*, 2007, 2009).

The South Pacific Ocean is a large ocean basin with a range of physicochemical characteristics, yet limited information is available on nutrient limitation of phytoplankton and diazotroph growth in this area. An upwelling area in the eastern equatorial South Pacific is characterized by abundant nitrate and low chlorophyll *a* (Chl *a*) in the surface waters (hence termed HNLC), and here iron has been found to limit phytoplankton photosynthetic efficiency and growth (Behrenfeld *et al.*, 1996; Coale *et al.*, 1996), while measurements across the oligotrophic South Pacific gyre reported low abundances of diazotrophs and variability in factors limiting phytoplankton, diazotrophs and bacterioplankton (Bonnet *et al.*, 2008; Moutin *et al.*, 2008; Van Wambeke *et al.*, 2008). In the western equatorial and southern South Pacific open ocean, both unicellular and filamentous diazotrophs have been recently detected at high abundances, and linked with high N_2 fixation rates in the size fraction corresponding with the unicellular groups (Campbell *et al.*, 2005; Bonnet *et al.*, 2009; Hewson *et al.*, 2009; Moisander *et al.*, 2010). Unicellular diazotrophs were also reported at high abundances from coastal lagoons in Australia and New Caledonia (Hewson *et al.*, 2007; Biegala and Raimbault, 2008). An uncultivated γ-Proteobacterium γ-24774A11, named after a *nifH* clone recovered from the South China Sea (Moisander *et al.*, 2008), is widespread in tropical oceans (Church *et al.*, 2005a; Hamersley *et al.*, 2011; Turk *et al.*, 2011). γ-24774A11 expressed *nifH* in previous studies (Bird *et al.*, 2005; Church *et al.*, 2005b) and in our study area (Moisander *et al.*, unpublished data) suggesting it is contributing to oceanic N_2 fixation, however its ecophysiology is poorly characterized to date.

In this study, we report nutrient-enrichment bioassay results from a 34-day transect across the oligotrophic South Pacific from Australia to Fiji, between latitudes 15S and 25S. The main goal was to compare nutrients limiting growth of the different unicellular diazotrophs, non-diazotrophic picocyanobacteria and heterotrophic bacteria, and the non-N_2-fixing plankton community. *In situ* growth rates of previously cultivated and uncultivated unicellular diazotrophs in field populations have not been previously published, thus the results are useful in estimates of diazotroph growth responses to environmental fluctuations in the open ocean.

Materials and methods

Six nutrient-enrichment experiments were carried out along the cruise track of R/V *Kilo Moana* (KM0703) in March–April 2007. Nutrient addition bioassays were carried out at stations 2, 5, 8, 13, 17 and 21 (Figure 1, Table 1). The incubation bottles were washed with 10% hydrochloric acid, rinsed three times with Milli-Q water or 0.2-μm filtered seawater (24 h old or less), and finally three times with water from the sampling site before filling them. Water was sampled between 14:00 and 18:30 h from the 5-m depth into 4.5-l polycarbonate bottles, using a high-pressure pump. The following nutrient

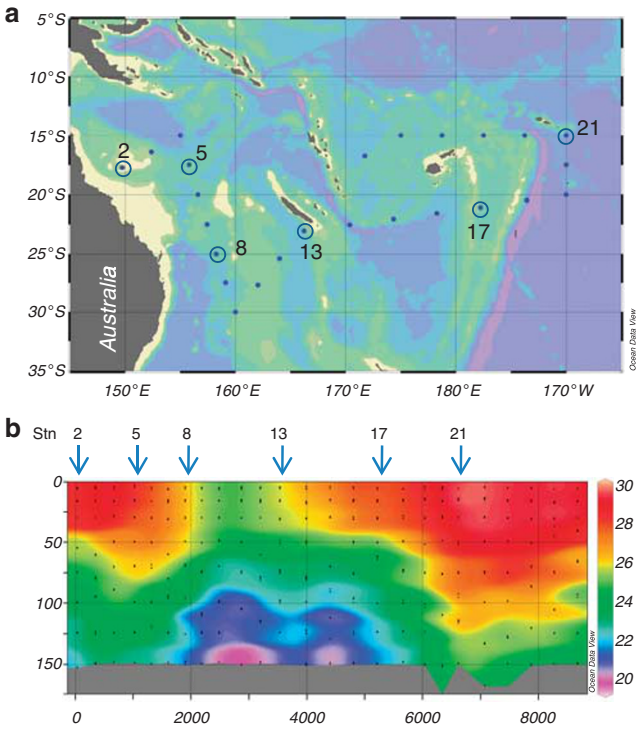

Figure 1 (**a**) Cruise transect and location of stations where bioassay experiments were initiated. (**b**) Temperature (°C) in the 150-m surface layers along the cruise transect (km) (Schlitzer, 2011).

Table 1 Physicochemical conditions at the start of the bioassays

Station	*NO_x (nM)*	*SRP (nM)*	*DFe (nM)*	*TDFe (nM)*	*DOC (μM)*	*TN (μM)*	*TDP (nM)*	*Chl a (μg l^{-1})*	*Fluor (fu)*	*Temperature (°C)*	*Salinity (psu)*	*O_2 (% sat)*	*Sigma-T (kg m^3)*
2	7	195	0.28	0.25	69.5	6.4	ND	0.10	0.06	28.22	35.09	95.35	22.39
5	3	115	ND	ND	66.3	5.0	ND	0.06	0.25	28.21	34.75	95.08	22.14
8	5	93	0.20	0.22	62.1	4.7	ND	0.07	0.06	25.81	35.39	95.55	23.39
13	6	79	ND	ND	ND	ND	419	0.13	0.08	25.52	35.50	94.45	23.56
17	9	26	0.38	0.92	74.7	5.0	312	0.12	0.05	27.37	35.08	93.65	22.66
21	2	97	0.34	0.83	77.8	5.9	1765	0.10	0.06	29.98	35.01	93.61	21.74

Abbreviations: Chl *a*, chlorophyll a concentration; DFe, dissolved iron; DOC, dissolved organic carbon; Fluor, *in situ* fluorescence; ND, not determined; NO_x, nitrate+nitrite; O_2, oxygen saturation; Sigma-T, potential density; SRP, soluble reactive phosphorus; TDFe, total dissolvable iron; TDP, total dissolved phosphorus.
All other parameters were detected at the 2–5 m depths except iron values are from the 5–15 m surface layer.

treatments were included in triplicate (all concentrations are final): (1) no additions, (2) 10 μM $NaNO_3 + NH_4Cl$ each (N), (3) 2 μM K_2HPO_4 (P), (4) 0.4 μM $FeCl_3$ and ethylenediaminetetraacetic acid (EDTA) each (Fe), (5) 0.4 μM glucose and mannitol each (GM), (6) N + P, (7) N + Fe, (8) P + Fe and (9) N + P + Fe + GM. The bottles were incubated for 70–78 h in an on-deck incubator with seawater flow-through, covered with two layers of neutral density screening to alleviate photoinhibition. The screening reduced the photosynthetically active irradiance to 35% of that in the air.

Responses to nutrient additions in phytoplankton growth were detected by measurements of Chl *a* concentration (Welschmeyer, 1994). A volume of 1.5 l sample (or less if the sample was very dense) was filtered through GF/F glass fiber filters (Whatman, Piscataway, NJ, USA) that were frozen at −20 °C and extracted and measured onboard. A volume of 5 ml of 90% aquaeous acetone was added to filters in 15-ml tubes that were vigorously vortexed for 20 s, then incubated at −20 °C for 24 h. Tubes were vortexed again for 20 s, filtrate cleared through a GF/F, then read with a Turner 10-AU fluorometer (Turner Designs, Sunnyvale, CA, USA) (Welschmeyer, 1994). Instrument stability was checked daily using solid standards.

Samples for DNA analyses were collected from two experimental containers per treatment by filtering 0.2–1.5 l of sample first through 10-μm polyester filters (Osmonics, Trevose, PA, USA), then through 0.2-μm Supor membrane filters (Pall Corp., Ann Arbor, MI, USA) using peristaltic pumps. Filters were placed in sterile tubes with glass beads and frozen in liquid nitrogen. For flow cytometric counts (FCM) of picocyanobacteria, 1.8-ml samples were preserved in ultrapure glutaraldehyde in PBS buffer (Tousimis, Rockville, MD, USA) at 1% final concentration, then frozen in liquid nitrogen for transport and stored at −80 °C until analysis. Slides for bacterial counts were prepared with the SYBR Green I method (Noble and Fuhrman, 1998; Patel *et al.*, 2007). A volume of 3 ml of sample preserved with formalin (2% final concentration), was filtered on 0.02-μm Anodiscs (Whatman), stained for 15 min and mounted on slides with low-density immersion oil. Slides were stored at −20 °C and counted under epifluorescence using a Zeiss Axioplan (Oberkochen, Germany) microscope. Two experimental replicates were analyzed for each treatment and a minimum of 200 bacterial cells and 20 fields were counted. Small, morphologically distinct eukaryotic phytoplankton could be observed and counted on these slides as well.

DNA was extracted using a modified Qiagen Plant Minikit (Valencia, CA, USA) protocol (Moisander *et al.*, 2008). Abundances of three groups of diazotrophs (UCYN-A, *C. watsonii* and γ-24774A11) were determined on the 0.2–10 μm size fraction filters by quantitative PCR using a 5′ nuclease assay with primer–probe sets listed in Supplementary Table S1.

Duplicate experimental treatments were analyzed with analytical duplicates each. The quantitative PCR (qPCR) methods followed previously published protocols (Moisander *et al.*, 2010). DNA extracts were diluted 1:10 (vol:vol) to amplification reactions. Inhibition was tested in separate reactions by running a plasmid standard (10^3–10^5 gene copies ml^{-1}) with 2 μl of the sample. Inhibition tests were done for each of the duplicate samples from each treatment, for each of the three diazotroph qPCR probe sets. Amplification efficiency was >95.6%.

Dominant *Prochlorococcus* and *Synechococcus* strains were also quantified with qPCR, using primers and probes designed in this study (Supplementary Table S1, Supplementary Methods). To generate plasmid standards, we designed PCR primers to amplify a 296-bp region of the cyanobacterial 23S rRNA gene. Primers were flanking the 5–300 region of the 23S rRNA gene in the MIT9312 genome. The PCR reaction mix consisted of the following components (all concentrations final): 5 μl 10× buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM forward and reverse primers each (Supplementary Table S1) (Eurofins MGM Operon, Huntsville, AL, USA), 0.3 μl Platinum Taq polymerase (Invitrogen, Carlsbad, CA, USA) and 2 μl DNA template. Reactions were filled to 50 μl reaction volume with nuclease-free water purified through a 5-kDa spin column. PCR conditions were as follows: initial 94 °C for 5 min, then for 30 cycles at 94 °C for 30 s, 57 °C 30 s, and 72 °C 30 s, and final 72 °C for 7 min. The products were electrophoresed on a 1.2% TAE gel, excised, purified and cloned into a pGEM-T vector (Promega, Madison, WI, USA). Plasmid purification was done using the Millipore (Billerica, MA, USA) Montage 96-well miniprep system. Sequencing was done at the University of California, Berkeley. The PCR resulted 23S rRNA gene fragments from both *Prochlorococcus* MIT9312/AS9601 lineage and *Synechococcus* CC9605, determined to be the dominant groups based on transcriptome libraries (Supplementary Methods). Linearized plasmids with these inserts were used as standards for the respective qPCR assays developed for HL *Prochlorococcus* and *Synechococcus* CC9605. The plasmids were linearized and amplified in a dilution series of 10–10^7 gene copies, included in parallel with all quantification runs. Genome of CC9605 has two identical copies of the 23S rRNA gene and we divided the 23S rRNA gene copies ml^{-1} by 2 to estimate cells ml^{-1} (assuming one genome per cell).

Physicochemical variables in the study area were measured as described in Moisander *et al.*, 2010. Samples for iron (Fe) measurements were collected by a MITESS ATE/VANE sampler (Bell *et al.*, 2002). Fe data reported here were collected from the 5–15 m depth within the mixed layer, where we assume Fe concentrations are uniform in the shallow mixed layer. Iron measurements are reported for dissolved iron (Fe, nM) and total dissolvable iron (TDFe, nM) (Zhang *et al.*, unpublished data). Background dissolved iron in the incubation bottles was tested at the end of one of the bioassays.

Comparisons of means among treatments for Chl *a* and qPCR data were carried out using 1-way analysis of variance with LSD *post-hoc* tests. qPCR data were pooled by geographic region (stations 8 and 13 formed one set, and stations 17 and 21 formed another set) (Supplementary Figure S1 and S2). Regression analysis was used for comparison of *Synechococcus* numbers by flow cytometry and qPCR. Some of the data were transformed to improve normality and homogeneity of variances, and tests were carried out in spite of small deviations from these assumptions in some cases (Underwood, 1997). Statistical tests were carried out using SPSS Statistics version 17 or 19 (SPSS, Armonk, NY, USA).

Results

Chl *a* concentrations were low at around 0.1 μg l^{-1}, and lowest at stations 5 and 8 (Table 1). Total nitrate + nitrite concentrations were in the nM concentration range, and very similar at all stations. Soluble reactive phosphorus concentrations were elevated at the most coastal station 2 and lowest at station 17. Highest dissolved and total dissolvable iron concentrations were measured at stations 17 and 21. Dissolved organic carbon (DOC) and temperature were the lowest at the westernmost stations, where total N also decreased. Temperatures at stations 8 and 13 were ~2–4 °C lower than at the other stations, thus surface water temperatures decreased toward higher latitudes. Parallel to this trend, density surfaces gradually shoaled along the North–South transect from stations 4 to 10 (Figure 1b, Moisander *et al.*, 2010).

In four of the six experiments (stations 2, 5, 8 and 13), total phytoplankton biomass was significantly higher in bottles with added N than in controls (Figure 2). At station 21, when iron was added with N there was a significant increase in Chl *a,* but not when N was added alone. In all experiments, Chl *a* increased the most if all nutrients were added. In some cases, 'All' treatment was not significantly different from the N addition (station 8), or NFe addition (stations 8 and 21). Phosphorus, Fe or organic carbon additions, when added alone, did not enhance Chl *a* over control in any of the experiments.

The responses to nutrient additions in *Synechococcus* were investigated by FCM and quantitative PCR (Figures 3–5) at four of the stations. Numbers from the two methods had a significant and strong positive relationship ($R^2 = 0.760$, $n = 33$, $P = 0.000$, Pearson correlation) and cell abundances estimated by qPCR were generally about 0.5–1 orders of magnitude greater than those from FCM. Addition of N resulted in an increase of *Synechococcus* abundances at stations 8, 13, 17 and 21.

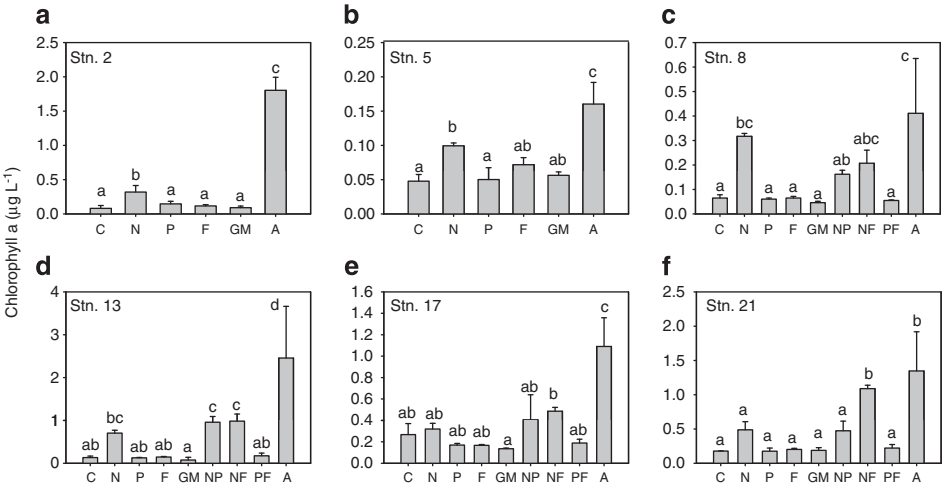

Figure 2 Chl *a* (μg l^{-1}) concentration in response to nutrient additions at stations 2 (**a**), 5 (**b**), 8 (**c**), 13 (**d**), 17 (**e**), and 21 (**f**). Significant differences between treatments are indicated with different letters: a < b < c < d, $P < 0.05$ ($n = 3$). C, control; N, nitrogen; P, phosphorus; F, iron; GM, glucose + mannitol; NP, nitrogen + phosphorus; NF, nitrogen + iron; PF, phosphorus + iron; A, nitrogen-phosphorus + iron + glucose + mannitol.

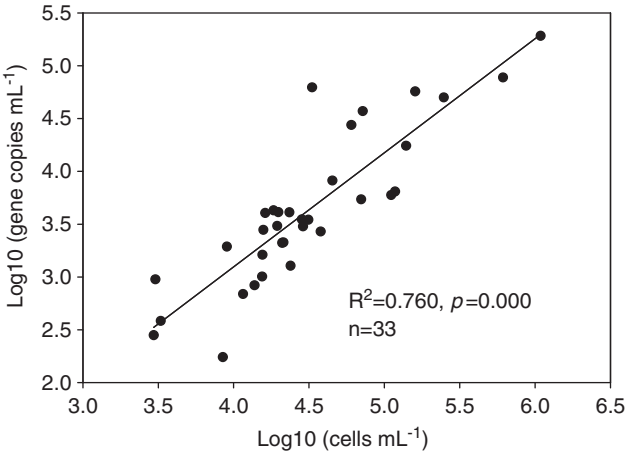

Figure 3 Relationship between abundances of *Synechococcus* in response to nutrients at stations 8, 13, 17 and 21 from FCM, and counts of cell equivalents from qPCR (23S copies l^{-1} were divided by 2 because the *Synechococcus* CC9605 genome has two identical copies of the 23S rRNA gene).

Synechococcus abundances were elevated in NP and NFe treatments significantly over the control (Supplementary Figure S1 and S2). Addition of P, Fe or carbon alone, or P and Fe in combination did not increase *Synechococcus* abundances above the control. *Synechococcus* had a particularly strong positive response to addition of all nutrients at station 21, observed in FCM, qPCR and microscopic counts (not shown).

The responses of *Prochlorococcus* were different from those of *Synechococcus* (Figures 4 and 5). In the westernmost stations, *Prochlorococcus* increased in abundance under Fe, PFe or organic carbon additions (Supplementary Figure S1). In N, P, NP, NFe and 'All' treatments, *Prochlorococcus* abundances remained at the level of the control or were lower (Supplementary Figure S1 and S2). Overall, the responses in *Prochlorococcus* populations were remarkably similar to those in unicellular diazotrophs at stations 8 and 13.

UCYN-A abundance was very low in station 17 and 21 waters (Moisander *et al.*, 2010) and, based on spot tests using the bioassays, was undetectable in most or all treatments. At the westernmost stations 8 and 13, UCYN-A abundances increased when either iron or organic carbon was added (Figure 4). Addition of P alone produced a negligible impact to UCYN-A abundances, but appeared to have an additive impact when added with iron at station 13. In N treatments, UCYN-A abundance was not different from control.

The responses of *C. watsonii* populations to nutrients were more variable. Iron and organic carbon appeared to stimulate abundances at station 8, while at stations 13 and 17, PFe addition resulted in elevated abundances. The analysis from stations 8 and 13 suggested elevated abundances of *C. watsonii* in iron and PFe treatments over those in N or NFe treatments. At station 21, abundances did not increase in response to any nutrient, but addition of N resulted in a decrease. When the easternmost stations 17 and 21 were pooled, there were no significant differences among treatments for *C. watsonii*.

γ-24774A11 remained at relatively stable ambient concentrations in the study area. Iron and PFe resulted in significantly increased abundances in the westernmost part of the study area, compared with treatments with N (Figure 4 and Supplementary Figure S1). Similarly to *C. watsonii*, none of the nutrients appeared to be limiting γ-24774A11 at station 21, but in contrast, negative impacts of N were observed at this station. The reduced abundances under N treatment were significant in the northeastern part of the study area (Supplementary Figure S2).

Net growth rates were estimated based on the change in gene copies in station 8 water (Figure 4).

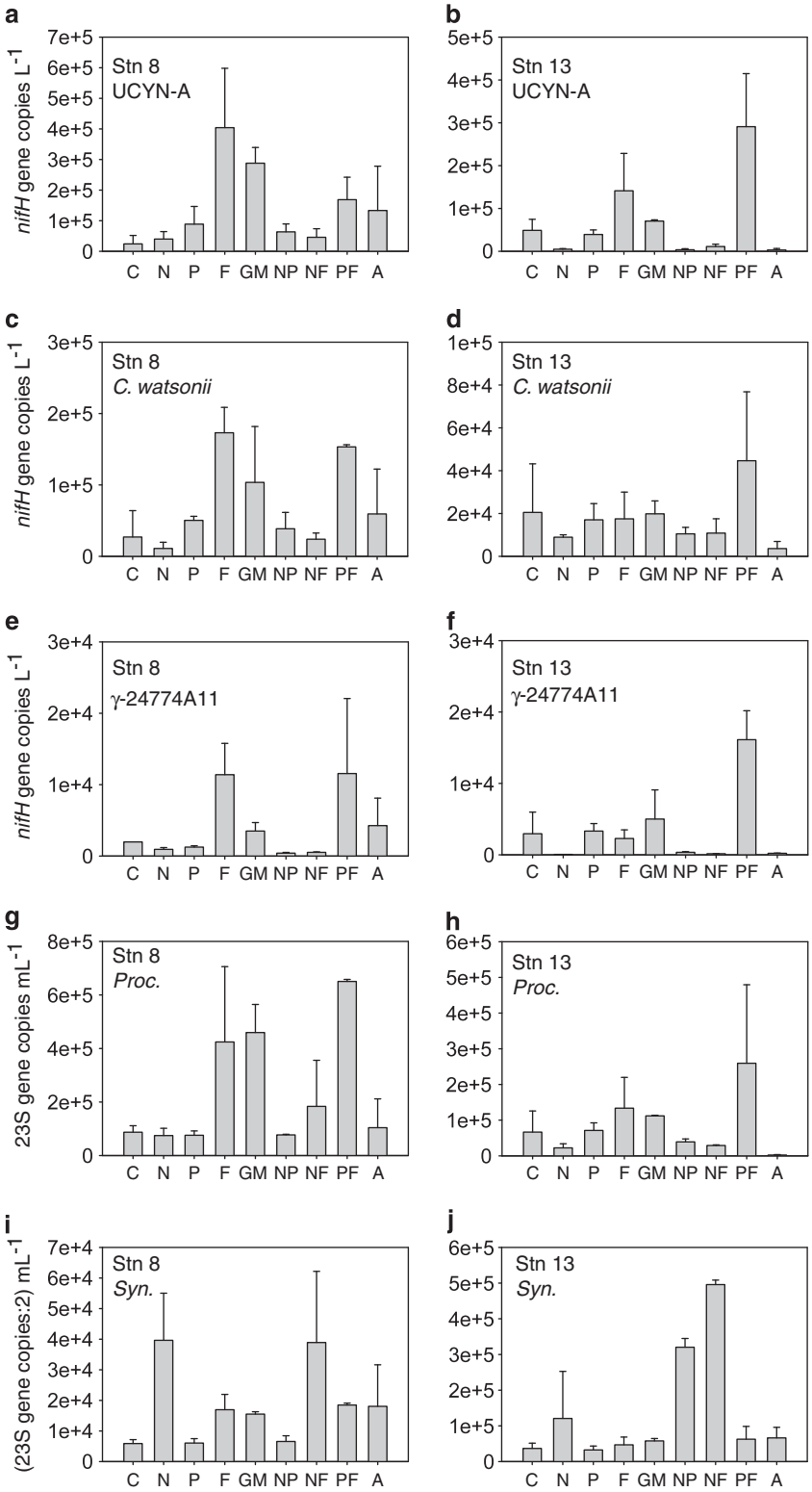

Figure 4 Abundances of UCYN-A (**a**, **b**), *C. watsonii* (**c**, **d**), γ-24774A11 (**e**, **f**) (*nifH* gene copies l^{-1}), *Prochlorococcus* (**g**, **h**) and *Synechococcus* (**i**, **j**) (23S copies ml^{-1}) in response to nutrients at stations 8 (**a**, **c**, **e**, **g**, **i**) and 13 (**b**, **d**, **f**, **h**, **j**). C, control; N, nitrogen; P, phosphorus; F, iron; GM, glucose + mannitol; NP, nitrogen + phosphorus; NF, nitrogen + iron; PF, phosphorus + iron; A, nitrogen-phosphorus + iron + glucose + mannitol.

The maximum net growth rates for UCYN-A, *C. watsonii* and γ-24774A11 were 0.19, 0.61 and 0.52 d^{-1}, respectively. Maximum net growth rates were observed for all diazotrophs under Fe or PFe additions, and elevated rates compared with control were also observed in response to organic carbon

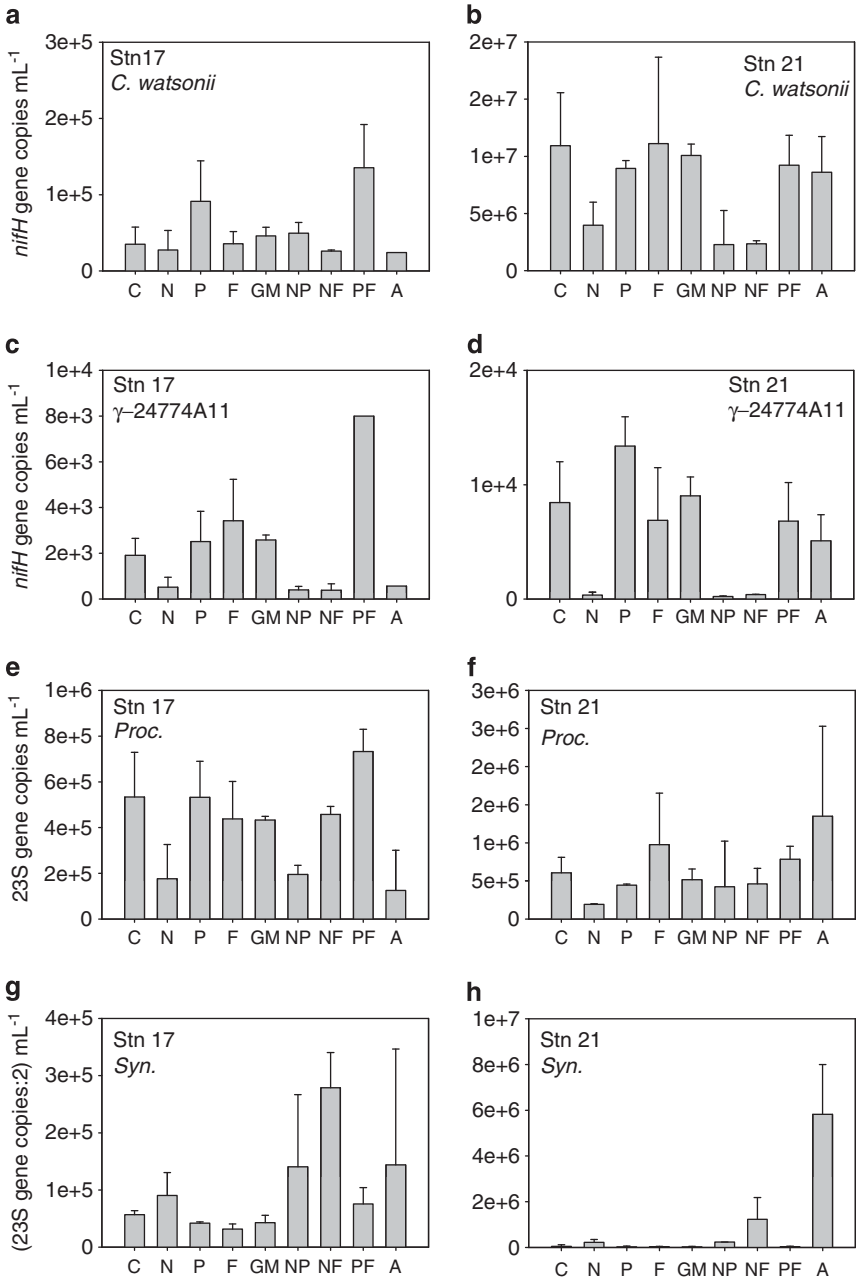

Figure 5 Abundances of *C. watsonii*, (**a**, **b**) γ-24774A11 (**c**, **d**) (*nifH* gene copies L^{-1}) and *Prochlorococcus* (**e**, **f**) and *Synechococcus* (**g**, **h**) (23S copies ml^{-1}) in response to nutrients at stations 17 (**a**, **c**, **e**, **g**) and 21 (**b**, **d**, **f**, **h**). C, control; N, nitrogen; P, phosphorus; F, iron; GM, glucose + mannitol; NP, nitrogen + phosphorus; NF, nitrogen + iron; PF, phosphorus + iron; A, nitrogen + phosphorus + iron + glucose + mannitol.

additions. Positive net growth was not detected in any of the other conditions for UCYN-A. For *C. watsonii*, there was no positive net growth in control, N and NFe treatments. For the γ-24774A11, growth was observed under Fe, GM and 'All' treatments.

Total bacterial abundances were remarkably stable in all treatments except in the 'All' treatment. The only exception was the NP treatment at station 8 that also had a slightly elevated bacterial abundance. At stations 8, 17 and 21, bacterial abundances at the end of the experiment were approximately an order of magnitude greater in the 'All' treatment than in any of the other treatments (Figure 6). Owing to the skewed nature of the data, it remained slightly off normal distribution in spite of transformations. One-way analysis of variance ($P = 0.000$) indicated a significant difference between 'All' and the other treatments, and the Kruskal–Wallis nonparametric test indicated significant differences among treatments ($P = 0.044$). We observed the appearance of small pennate diatoms and unidentified nanoflagellates in response to some nutrient treatments. At station 21, abundances of the small diatoms varied from non-quantifiable (P, Fe, GM, PFe and 'All') up to 10^3–10^4 ml^{-1} (control, N, NP and NFe).

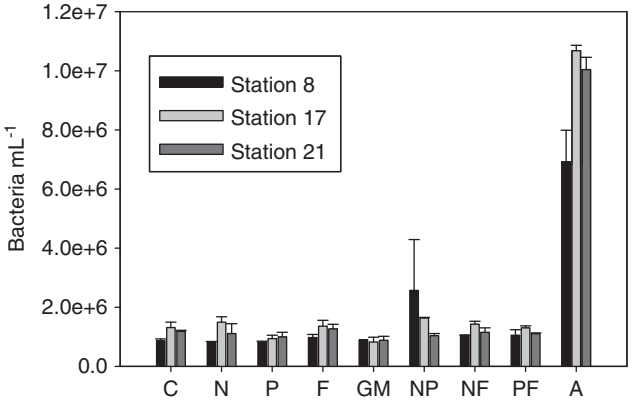

Figure 6 Abundances of bacteria in response to nutrients at stations 8, 17 and 21 (cells ml^{-1}) determined by epifluorescence microscopy.

All incubation bottles were washed with 10% HCl, but the experiments were not carried out under strict trace element clean conditions. Measurements were made to investigate concentration of background iron in the incubations. The data showed background iron was approximately an order of magnitude greater than the levels detected in the upper mixed layer, with an average of 5.7 nM total dissolvable Fe in the incubation bottles at the end of the experiment ($n = 5$).

Discussion

Responses in unicellular diazotrophs

There was no previous information on empirical growth rates of the uncultivated unicellular diazotrophs, however, these data are essential for estimating and mathematical modeling of growth of N_2-fixing microbial populations and their contribution to N budgets. We expected to possibly see differences among the groups in responses to iron, because iron requirements may vary among diazotrophs and with environmental factors. It was recently shown that elevated CO_2 concentrations may reduce Fe requirements in *Crocosphaera* (Fu *et al.*, 2008). UCYN-A does not have Photosystem II (Zehr *et al.,* 2007a), which has three Fe atoms (Raven, 1990), thus it may require less iron than the photoautotrophic diazotrophs *Crocosphaera* and *Trichodesmium*. Iron requirements of heterotrophic diazotrophs such as γ-Proteobacteria are unknown. Diazotrophs also differ in how they are able to access dissolved organic phosphorus sources, which may cause differences in their phosphorus requirements and responses in bioassays. *Trichodesmium* can grow on phosphonates (Dyhrman *et al.*, 2009; Beversdorf *et al.*, 2010) and refractory organic phosphorus compounds, and *Crocosphaera* has alkaline phosphatase activity for dissolved organic phosphorus utilization (Dyhrman *et al.*, 2007). For UCYN-A and other heterotrophic diazotrophs, ability to utilize organic P sources has not been characterized. In spite of these known and potential differences, responses to iron and phosphate detected in the experiments were generally similar among the three unicellular diazotrophs.

For *C. watsonii*, the estimated maximum net growth rate was 0.61 d^{-1}, while previous laboratory experiments with *C. watsonii* isolates reported maximum growth rates of 0.46–0.49 d^{-1} (Tuit *et al.*, 2004; Goebel *et al.*, 2010). A maximum growth rate of 0.77 d^{-1} was approximated theoretically for the uncultivated UCYN-A, based on cell size (Goebel *et al.*, 2010), while the maximum net growth rate in our experiment was 0.19 d^{-1}. Growth rate of γ-24774A11, 0.52 d^{-1}, falls in this range. A major difference to laboratory culture experiments from these data is that the apparent growth rates measured in the field likely were affected by losses due to grazing. Owing to their difference in size, *C. watsonii* and UCYN-A are probably targeted by different grazers, and the faster apparent growth rates of *C. watsonii* than by UCYN-A could potentially reflect lower grazing pressure. UCYN-A may also be selected against when kept in sealed bottles, and although temporarily enriched in our experiments, may have suffered from 'bottle effects' more than *C. watsonii* that can be isolated and grown on rich media. Our data suggest, however, that relatively fast *in situ* growth rates are possible in bottle incubations in the open ocean if suitable conditions are present. The results also suggest that nutrients rather than grazing pressure were limiting the growth of diazotrophs *in situ*, as differential responses were observed among nutrient treatments.

The results suggested that all diazotrophs were either Fe or organic carbon limited. Our incubation experiments were not carried out under strictly trace element clean conditions and we did detect elevated Fe in the bottles that received no intentional Fe additions. In spite of the presence of this background Fe in incubations, there were increases in diazotroph abundances in response to additions of Fe or carbon. While P added alone did not result in elevated diazotroph abundance (in spite of the background Fe in the bottles), when P was combined with Fe (always added with EDTA), numbers of all diazotrophs as well as *Prochlorococcus* were enhanced. Thus, the stimulatory effect was clearly linked with the Fe + EDTA addition. We do not know whether the background Fe was bioavailable, so the positive responses to Fe + EDTA may still be true responses to Fe. Independent positive responses seen under either Fe + EDTA or carbon additions, however, suggest that the limiting factor may not have been Fe but something else that was associated with addition of Fe, such as the chelator (Burns *et al.*, 2006). It is also possible that the results reflect limitation by a trace element other than Fe, as the addition of a chelator may have improved bioavailability of other trace elements besides iron. Another alternative explanation is that EDTA was broken down by some microbes, making it available as carbon, energy or nutrient resource for the diazotrophs.

Responses in Prochlorococcus

The dominant oceanic *Prochlorococcus* MIT9312 genotype was very abundant in our study area (Hewson *et al.*, 2009). This genotype is part of a *Prochlorococcus* clade termed high light ecotype (Moore *et al.*, 1998) and several other *Prochlorococcus* strains with unique characteristics share closely similar ribosomal RNA genes with this type strain (Rocap *et al.*, 2002; Zinser *et al.*, 2006).

While some uncultivated *Prochlorococcus* are thought to acquire nitrate (Martiny *et al.*, 2009), none of the cultivated strains have this capability. In the Sargasso Sea, *Prochlorococcus* pigment content responded positively to NH_4NO_3 additions, although nitrate alone or iron had no effect (Davey *et al.*, 2008; Moore *et al.*, 2008). In contrast, in our experiments, the HL *Prochlorococcus* abundances remained stable or were reduced under N (nitrate + ammonium) additions, and responded positively to iron and organic carbon additions. There was a remarkable similarity in *Prochlorococcus* responses at stations 8 and 13 with those in the unicellular diazotrophs. The data suggest that *Prochlorococcus* and diazotrophs were limited by the same nutrients and that the net growth of *Prochlorococcus* was not primarily N limited, inspite the fact that overall N limitation was seen at the total phytoplankton biomass level. The results appear to differ from those from the North Atlantic (Bell *et al.*, 2002; Moore *et al.*, 2008), but the types of nutrient response measures used (pigment content vs cell abundance) may not be comparable, as per cell Chl *a* content potentially increases relatively more than cell abundance in response to N (Davey *et al.*, 2008).

Prochlorococcus may utilize several strategies to alleviate N limitation. HL *Prochlorococcus* may induce N stress proteins that have reduced N content, reducing overall N requirements, while downregulating high N content ribosomal proteins (Gilbert and Fagan, 2011), thus providing a mechanism for N conservation. *Prochlorococcus* may also have benefited from recycled N fixed by diazotrophs.

Responses in Synechococcus

As the similar trends that were observed for *Prochlorococcus* and unicellular diazotroph responses by qPCR were unexpected, we investigated the possibility that this might have been caused by a DNA sample processing bias. As *Synechococcus* CC9605 has assimilatory nitrate reductase, we hypothesized that this genotype would positively respond to nitrate additions in our treatments, unlike what was observed for *Prochlorococcus* and diazotrophs. Our results from FCM and qPCR provided strong independent evidence for N, NP and NFe limitation of *Synechococcus*. The distinct patterns detected by qPCR for *Synechococcus* in comparison with the other qPCR targets suggest that sample processing biases cannot explain the remarkable similarity in *Prochlorococcus* and diazotroph responses. We cannot, however, explain the absence of elevated biomass of *Synechococcus* in 'All' treatment at stations 8, 13 and 17, except to speculate that competition with bacteria or eukaryotic phytoplankton, or top–down effects may have had a role.

Several factors potentially contribute to variation between the *Synechococcus* counting methods. A major difference is that FCM counts cells while qPCR targets gene copies from DNA extracts, and the comparisons may be skewed by the fact that at times each cell may have more than one copy of the genome. Further, cells that have low pigmentation (and may be dead) are not counted by FCM but are included in the qPCR numbers. On the other hand, FCM doesn't discriminate *Synechococcus* genotypes, while the qPCR assay was *Synechococcus* 9605-specific. Additional discrepancies may be caused by differences in the sampling volume, sample losses or degradation, and accuracy of the qPCR standards.

Responses to dissolved organic carbon

Positive responses to dissolved organic carbon were observed in the unicellular diazotrophs and *Prochlorococcus*. Oceanic picocyanobacteria can take up leucine (Church *et al.*, 2004), and several *Prochlorococcus* strains take up glucose (Gomez-Baena *et al.*, 2008). It would be expected *Prochlorococcus* doesn't often encounter high concentrations of glucose in its primary habitat in the oligotrophic open ocean, but positive responses to glucose + mannitol additions in our study suggest *Prochlorococcus* in the oligotrophic South Pacific are capable of actively taking advantage of available sugars to support their growth. This capacity may allow preservation of energy by rapid uptake and recycling of sugars that are lost in cell exudates and sloppy feeding by zooplankton.

UCYN-A and *C. watsonii* may benefit from similar strategies. UCYN-A and *C. watsonii* have many ABC transporters, and for UCYN-A several of these are specialized in carbohydrate transport (Tripp *et al.*, 2010). UCYN-A requires external electron donors for photophosphorylation and glycolysis, which is an important respiratory energy generation pathway in UCYN-A as it doesn't have the TCA pathway. Highest abundances of UCYN-A are often found near the Chl *a* maximum where carbohydrates originating from elevated phytoplankton biomass may be more readily available, providing an available carbon source for UCYN-A. On the other hand, *C. watsonii* primarily grows closer to the surface (Moisander *et al.*, 2010) and relies on photosynthesis for energy, yet our results suggest it may also supplement its energy resources with external sugars if they are available. In contrast, *Synechococcus* was primarily N limited with the rest of the phytoplankton and did not respond to the organic carbon additions.

Bacterioplankton in our study area appeared co-limited by several nutrients, observed as a dramatic increase in total bacterioplankton abundances if N, P, Fe and carbon were added ('All' treatment), which also generally had the highest Chl *a*. Van Wambeke *et al.*, 2008 also reported bacterial production increases in the South Pacific Gyre when glucose was added alone or in combination with other nutrients. We did not observe evidence for negative impacts of carbon addition to Chl *a* that might have been caused by more efficient microbial uptake (thus competition) of N and P after their carbon limitation was relieved (Joint *et al.*, 2002); however, abundances of Synechococcus were possibly affected, as numbers were reduced in 'All' treatment compared with N, NP or NFe in some experiments.

Spatial and temporal differences in nutrient limitation
We observed variability in nutrient limitation patterns across the study area. Stimulation of diazotrophs and *Prochlorococcus* by Fe and carbon was observed in the western part of the transect (stations 8 and 13), and not in the northeastern end (stations 17 and 21), while N addition had a negative impact at the latter stations. Availability of iron and DOC in the study area varied, with highest concentrations in the northeast where iron and carbon stimulation were not observed. Although bioavailability of the DOC in our study area is unknown, the lowest DOC concentrations coincided where we saw responses to organic carbon additions.

Nutrient-enrichment bioassays are notorius for enriching growth of certain copiotrophic bacteria such as *Alteromonas* (Ivars-Martinez *et al.*, 2008) at the expense of dominant marine bacteria, highlighting the difficulty to isolate dominant marine microorganisms using rich media. The nutrient response we observed in bacterial abundance in the 'All' treatment is a net community response and likely dominated by these opportunistic species, whose growth is potentially masking nutrient limitations by other, non-cultivable members of the community. Growth of these groups appeared limited in other treatments, however, besides the 'All' treatment. Such biases in community, as well as potential nutrient remineralization over time may have contributed to the observed responses in the diazotroph and picocyanobacterial abundances. Influence of top–down effects through grazing and viral lysis were also not quantified but must have influenced the accumulated cell numbers, thus the growth rates presented here are considered net rates.

Conclusion

Our enrichment experiments suggest that organic carbon and a combination of iron and an organic chelator positively influence growth of unicellular diazotrophs and HL *Prochlorococcus* populations in the Southwest Pacific Ocean. While in some previous studies, enrichment of unicellular diazotrophs in response to nutrients did not occur (Zehr *et al.*, 2007b), the results from this study show it is possible to enrich heterotrophic and autotrophic unicellular diazotrophs in microcosm incubations, thus encouraging future cultivation efforts. The empirical net growth rates reported here should be useful for mathematical modeling of oceanic unicellular diazotroph distributions.

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