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Dissolved organic matter stimulates N_2 fixation and *nifH* gene expression in *Trichodesmium*

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Abstract

Mixotrophy, the combination of heterotrophic and autotrophic nutrition modes, is emerging as the rule rather than the exception in marine photosynthetic plankton. *Trichodesmium*, a prominent diazotroph ubiquitous in the (sub)tropical oceans, is generally considered to obtain energy via autotrophy. While the ability of *Trichodesmium* to use dissolved organic phosphorus when deprived of inorganic phosphorus sources is well known, the extent to which this important cyanobacterium may benefit from other dissolved organic matter (DOM) sources is unknown. Here we provide evidence of carbon-, nitrogen- and phosphorus-rich DOM molecules enhancing N_2 fixation rates and

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nifH gene expression in natural *Trichodesmium* colonies collected at two stations in the western tropical South Pacific. Sampling at a third station located in the oligotrophic South Pacific Gyre revealed no *Trichodesmium* but showed presence of UCYN-B, although no *nifH* expression was detected. Our results suggest that *Trichodesmium* may behave mixotrophically in response to certain environmental conditions, providing them with metabolic plasticity and adding up to the view that mixotrophy is widespread among marine microbes.

Introduction

In remote ocean areas where nitrogen via riverine and atmospheric inputs is minimal, biological dinitrogen (N_2) fixation provides the main source of reactive nitrogen available for primary producers (Karl *et al.* 2002). N_2 fixation is carried out by a group of plankton called 'diazotrophs'. Diazotrophs play a crucial role in ocean biogeochemistry: they sustain ~50% of marine new primary production and may contribute >70% to carbon sequestration in the vast open ocean regions (Karl *et al.* 2012; Caffin *et al.* 2018). Diazotrophs encompass different groups of bacteria and archaea, organized in four clusters according to the phylogeny of their *nifH* genes (Zehr and Turner 2001). The geographical distribution of diazotrophs is greatly influenced by environmental factors and nutrient availability, that affect them differently according to their metabolism and nutritional requirements. For example, cyanobacterial diazotrophs such as *Trichodesmium* and *Crocosphaera* preferentially inhabit low inorganic nitrogen (sub)tropical waters >25ºC, while the symbiotic UCYN-A is usually more abundant in relatively colder (<25ºC) waters and can be found in both oligotrophic and nutrient-rich waters such as coastal upwelling systems (Sohm, Webb and Capone 2011; Agawin *et al.* 2014; Moreira-Coello *et al.* 2019).

Iron bioavailability affects the activity and distribution of diazotrophs to a great extent because the metalloproteins that make up the subunits of the nitrogenase enzyme include this trace metal (Berman-Frank *et al.* 2007). Diazotrophs need ~5 times more iron to fix N_2 than other phytoplankton

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need to assimilate ammonium (Berman-Frank *et al.* 2001; Kustka *et al.* 2003). Phosphorus is critically needed for the synthesis of membrane lipids and nucleotides, and, therefore, often limits diazotroph growth (Dyhrman, Ammerman and Van Mooy 2007). The extent to which iron or phosphorus limit diazotrophs varies geographically, e.g. *Trichodesmium* is phosphate-limited in the North Atlantic but iron-limited in the South Atlantic (Moore *et al.* 2009). However, *Trichodesmium* has the genetic capacity to metabolize dissolved organic phosphorus (DOP) molecules such as phosphomonoesters and phosphonates (Dyhrman and Haley 2006; Dyhrman *et al.* 2006), which allows it to grow in phosphate-depleted regions (Dyhrman *et al.* 2006; Orchard *et al.* 2010). Other diazotrophs such as *Crocosphaera* can use phosphomonoesters but not phosphonates, and therefore have access to a narrower range of DOP molecules for phosphorus acquisition (Dyhrman and Haley 2006).

Mixotrophy (the ability to use organic carbon and inorganic nutrients from organic matter compounds) is clearly emerging as the rule rather than as the exception in marine photosynthetic plankton nutrition (Moore 2013; Yelton *et al.* 2016). While chemolithoautotrophy is common in non-photosynthetic diazotrophs inhabiting soils and hot spring environments (Kwak and Shin 2016; Orlova *et al.* 2016), most marine pelagic non-photosynthetic diazotrophs are thought to depend on dissolved organic matter (DOM) for their growth (Benavides *et al.* 2015; Bombar, Paerl and Riemann 2016; Moisander *et al.* 2017). While the use of DOP by marine diazotrophic cyanobacteria has been documented, the potential benefits of other DOM compounds (their 'mixotrophic potential') for these microorganisms remains poorly understood. *Trichodesmium* and unicellular diazotrophs such as *Cyanothece* have been documented to take up DOM molecules such as carbohydrates and amino acids (Mulholland and Capone 1999; Feng *et al.* 2010; Benavides *et al.* 2017). In the ocean, the use of DOM by autotrophic phytoplankton is thought to alleviate inorganic nutrient and energy deficiency, potentially affecting their biogeography and/or seasonality (Barton *et al.* 2013). The extent to which diazotrophs rely on mixotrophy and its impact in their activity and distribution is unknown.

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During a cruise in the western tropical South Pacific (https://outpace.mio.univ-amu.fr/; Fig. 1) we used ^{13}C-labeled mixtures of organic carbon and amino acids to test the mixotrophic potential of diazotrophs using nanoscale secondary ion mass spectrometry (nanoSIMS) (Benavides *et al.* 2017). In that study we found that *Trichodesmium* was able to obtain carbon from DOM at rates comparable to previously published CO_2 fixation rates. These results indicated that DOM may provide an alternative source of carbon when environmental conditions discourage autotrophic carbon fixation via photosynthesis (Benavides *et al.* 2017). Here we investigate N_2 fixation and *nifH* gene expression of *Trichodesmium* in response to mixtures of organic carbon, amino acids, phosphomonoesters and phosphonates. We discuss the need to reconsider the physiology of *Trichodesmium* as well as its role in oceanic DOM dynamics.

Materials and Methods

Hydrography, inorganic and organic nutrients, and chlorophyll a

The experiments were conducted during the OUTPACE cruise (Oligotrophy to Ultraoligotrophy South Pacific Experiment, from 20 February to 2 April 2015 onboard the *R/V L'Atalante*; http://dx.doi.org/10.17600/15000900), at three long duration stations (6 days) named LDA, LDB and LDC (Fig. 1). Temperature, salinity, fluorescence and oxygen profiles were obtained by means of a SBE 9 plus CTD mounted on a rosette fitted with 24-12 L Niskin bottles. Chlorophyll *a,* inorganic nutrients and dissolved organic carbon, nitrogen and phosphorus (DOC, DON and DOP) concentrations were measured at each station as detailed in Moutin *et al.* (Moutin *et al.* 2017).

Experimental setup

At each station seawater was sampled from ca. 7-9 m depth, corresponding to 50% of the incident photosynthetically active radiation (PAR). Seawater was distributed in twenty-five 4.3 L polycarbonate bottles (5 sets of quintuplicates). Five bottles received a mixture of organic carbon

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molecules -DOC treatment- (sodium pyruvate, sodium acetate and glucose), another five bottles received a mixture of amino acids -DON treatment- (alanine, leucine and glutamic acid), and another five bottles received a mixture of phosphomonoesters and phosphonates -DOP treatment- (methylphosphonic acid, 2-aminoethylphosphonic acid and fructose 1,6-biphosphate). All treatments were supplied with the same amount of organic carbon (Benavides *et al.* 2015) and incubated for 36 h in deck incubators. The two remaining five bottle sets were kept unamended and treated as 'time zero' (immediately sampled at the beginning of the experiment to determine background $\delta^{15}N$ values), and 'control' (incubated for 36 h without DOM amendments). Of each set of five DOM-amended bottles, three were used for rate measurements (N_2 fixation, primary production -PP-, heterotrophic bacterial production -BP-) and heterotrophic bacterial abundance, while the fourth and fifth bottles were used for nucleic acid extractions (see below).

Primary and bacterial production rates

PP was measured in each triplicate DOM treatment by the ^{14}C-bicarbonate assay. For the control treatment, parallel incubations were used to determine ^{14}C assimilation in the dark and in a killed control (paraformaldehyde 0.5% final weight/volume for 30 min before the radioisotope was added). Briefly, incubations were done by adding ^{14}C-sodium bicarbonate (Perkin Elmer, Waltham, MA, USA) to a final radioactivity of approximately 2.96 kBq mL^{-1} (or 0.08 µCi mL^{-1}) from dawn to sunset (~8 h) in on-deck incubators. The total activity was measured from a subsample of 50 µL (dpm L^{-1}, in β-phenylethylamine). Total ^{14}C assimilation into particulate carbon was measured using glass fiber filters to harvest plankton biomass (dpm L^{-1}). The filters were inserted into 20 mL scintillation vials and acidified with 0.5 mL of 1N HCl for 24 h to remove any unincorporated ^{14}C-sodium bicarbonate. Radioactivity was measured with 10 mL scintillation cocktail (Ultima Gold LLT, Perkin Elmer) in a Packard Tri-Carb 3110 TR liquid scintillation counter. Radioactivity in the dark incubations was consistently greater than that in the killed control and therefore radioactivity in the dark incubation (dpm L^{-1}) was subtracted from that in the light. PP rates (mg C m^{-3} d^{-1}) were

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calculated using the ratio of radioactive to total dissolved inorganic carbon concentration measured at the respective station (Wagener *et al.* 2018) and were corrected for the preferential uptake of ^{12}C over ^{14}C.

BP (only measured at stations LDB and LDC) was determined by leucine incorporation (Smith and Azam, 1992) on analytical triplicates from each triplicate DOM amendment, time zero or control samples. A control killed with trichloroacetic acid (TCA, 5% final concentration) was also included in each sample batch. Samples and controls were amended with a mixture of [4,5-^{3}H]-leucine (4.14 TBq $mmol^{-1}$ or 112 Ci $mmol^{-1}$) at 7 nM and non-radioactive leucine at 13 nM. All samples were incubated in the dark for 1 h. The extraction procedure and assumptions for the conversion factor are detailed in Van Wambeke *et al.* (Van Wambeke *et al.* 2018). The incorporation of leucine was converted to carbon considering a conversion factor of 1.5 kg C mol $leucine^{-1}$ (Kirchman, Keil and Simon 1993). BP rates were corrected for unassimilated leucine in the DON mixture added.

Enumeration of heterotrophic bacteria

Samples of 1.8 mL were fixed with 0.25% (weight/volume) paraformaldehyde, incubated in the dark for 10-15 min at room temperature, flash-frozen and stored at -80°C. Before analysis, reference beads (Fluoresbrite, YG, 1 µm) were added to each sample. Bacteria were stained with SYBR Green I and counted on a BD Influx flow cytometer (BD Biosciences, San Jose, CA, USA) following the protocol of Bock *et al.* (Bock *et al.* 2018)

N_2 fixation rates

All bottles were amended with 6 mL of ^{15}N-labeled N_2 gas (98% Euriso-top), which is not contaminated with other nitrogenous forms (Dabundo *et al.* 2014; Benavides *et al.* 2015), following the methods of Montoya *et al.* ((Montoya *et al.* 1996), gently mixed for 5 min and incubated for 36 h

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(starting at ~22:00 and finishing around noon on the next day) in surface seawater flushed on-deck Plexiglas incubators fitted with blue screens to mimic the light intensity at the 50% PAR level as detailed in Benavides *et al.* (Benavides *et al.* 2017). After the incubation, control and DOM-amended bottles were filtered onto precombusted (6 h, 450ºC) GF/F filters (Whatman). The GF/F filters were stored in precombusted glass vials and kept at -20ºC until analysis. Isotope ratios were obtained with an Integra2 Analyzer, calibrated with reference material (IAEA-N1). N_2 fixation rates were calculated as described in Montoya *et al*. (Montoya *et al.* 1996). Rates were corrected by subtracting particulate nitrogen time zero $\delta^{15}N$ values and taking into account the dissolution of $^{15}N_2$ gas into the seawater sample by MIMS analyses (Kana *et al.* 1994) as detailed in Benavides et al. (Benavides *et al.* 2017).

Nucleic acid sampling and nifH gene sequencing

4.3 L samples for nucleic acid extractions (2-4 L depending on filter clogging) were filtered onto 0.2 µm Supor filters (Pall) with a peristaltic pump. Both filters for DNA and RNA were kept in sterile bead beater tubes, but RNA bead beater tubes contained a mix of 350 µL RLT buffer (Invitrogen) and 3.5 µL mercaptoethanol. All tubes were flash-frozen and stored at -80ºC until extraction. DNA was extracted using the DNeasy Plant Mini Kit (Qiagen), as detailed in Moisander et al. (Moisander *et al.* 2008). RNA extractions were performed with the RNeasy Minikit (Qiagen) as described in Moisander *et al.* (Moisander *et al.* 2014). cDNA was made using the Superscript III kit (Invitrogen). All reactions included parallel treatments with Reverse Transcriptase (RT) replaced with water (no-RT controls), and presence of genomic DNA in these samples was checked by *nifH* PCR. If bands were present on the 1.2% TAE gel in these no-RT treatments, the samples were DNAased again using the RNeasy columns, then checked again with *nifH* PCR. After removal of genomic DNA, cDNA from LDA and LDB showed bands in gel electrophoreses of *nifH* PCR, and were included in amplicon sequencing. cDNA samples from LDC did not show visible bands on the gel and were not included. One replicate for each treatment from LDA and LDB was included in amplicon sequencing. cDNA was amplified using

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the degenerate *nifH* primers following a nested PCR approach (Zehr, Waterbury and Turner 2001). The primers of the second nested round contained the Illumina overhang sequences (Shoemaker and Moisander 2017). Amplified products were purified using a magnetic bead protocol (Ampure) and then barcoded using the Illumina Nextera indexes. The barcoded products were again purified using magnetic beads, then quantified using the Picogreen protocol, and the quantity normalized to a multiplexed run on Illumina MiSeq (2x300) (Tufts University, Boston, MA). The obtained sequences were paired using Mothur (Schloss *et al.* 2009), and reads with ambiguities or more than eight homopolymers were not taken into account for further analyses. Operational taxonomic units (OTUs) were assigned the de novo picking method described in Edgar (Edgar 2010) in MacQIIME (Caporaso *et al.* 2010). OTUs containing less than fifteen sequences across all samples were not used. A sequence representing each OTU conceptually translated in Arb (Ludwig *et al.* 2004), and poor quality sequences and those that did not conceptually translate were discarded and not considered for further analysis. Amino acid sequences were aligned using the publicly available *nifH* database curated by the Zehr lab (https://www.jzehrlab.com/nifh, April 2015 database update). Sequences were classified with blastp run locally against the *nifH* database.

Trichodesmium qPCR counts

Trichodesmium DNA *nifH* gene copies (either DNA or cDNA) were quantified with TaqMan qPCR assays on a Stratagene Mx3005P thermal cycler (Applied Biosystems) with primers and probe for *Trichodesmium* (Church *et al.* 2005a): forward primer 5’- GAC GAA GTA TTG AAG CCA GGT TTC-3’, reverse primer 5’- CGG CCA GCG CAA CCT A-3’, and probe 5'-FAM-CAT TAA GTG TGT TGA ATC TGG TGG TCC TGA GC-3'-TAMRA-3'. *NifH* gene transcripts were normalized to *nifH* gene copies. Each reaction (12.5 µL) consisted of 6.25 µL TaqMan PCR Master Mix (Applied Biosystems), 0.25 µL of the forward and reverse primers (at 10 µM, HPLC purified, TAG Copenhagen, Denmark), 0.125 µL probe (at 10 µM, TAG Copenhagen), 4 µL PCR grade water, 0.63 µL bovine serum albumin (BSA, at 10.08 µg μL^{-1}), and 1 µL standard or sample (samples were pre-diluted to 5 ng μL^{-1} to add 5 ng DNA to all qPCR

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reactions). The qPCR program consisted of 2 min at 50ºC, 10 min at 95ºC continued by 45 cycles of 15 s at 95ºC and 1min at 60ºC. Standard dilutions (10^7-10^1) were run in duplicate, samples and no-template controls (NTCs) in triplicate. NTCs did not show any amplification. The efficiency was 110%. Inhibition tests were carried out on all samples and each primer-probe set by adding 2 µL of 10^5 standard to each sample. No inhibition was observed.

Results and discussion

The cruise transect crossed two distinct areas: (1) the Melanesian Archipelago west of the Tonga trench, where N_2 fixation rates were relatively high (Bonnet *et al.* 2018), dissolved iron concentrations substantial (Guieu, C., S., Bonnet, C., Petrenko, C., Menkes, V., Chavagnac, K., Desboeufs, Moutin 2018) and inorganic phosphate concentrations nearly undetectable (Moutin *et al.* 2017), and (2) the South Pacific Gyre waters where N_2 fixation rates were low (Bonnet *et al.* 2018) accompanied by low iron concentrations (Guieu, C., S., Bonnet, C., Petrenko, C., Menkes, V., Chavagnac, K., Desboeufs, Moutin 2018) and an accumulation of phosphate (Moutin *et al.* 2017). Station LDA was located in the Melanesian Archipelago area, LDB was located at the edge between the Melanesian Archipelago and the South Pacific Gyre, coinciding with a local high-chlorophyll accumulation (De Verneil *et al.* 2017), and LDC was situated in the ultraoligotrophic waters of the South Pacific Gyre (Fig. 1). DOC concentrations did not change along the cruise transect, but an accumulation of semi-labile DOC was observed in the South Pacific Gyre attributed to limited heterotrophic bacterial activity (Panagiotopoulos *et al.* 2019).

An enhancement in *Trichodesmium nifH* gene expression was detected in the DOM enrichment experiments from subsurface waters at stations LDA and LDB. However, no *nifH* mRNA was detected from at station LDC (Fig. 2; Table 2). The vast majority of the expressed *nifH* transcripts detected were from *Trichodesmium* (99.95-100%) in all stations and treatments. The remaining <0.05% of the expressed *nifH* reads were from other cyanobacteria, including UCYN-A, *Richelia*, and other

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unidentified cyanobacteria like Myxosarcina, Pleurocapsales and Nostocales (data not shown). The predominance of *Trichodesmium* in the detected *nifH* gene transcripts agrees with the results of Stenegren *et al.* (Stenegren *et al.* 2018), who reported that *Trichodesmium* was the most abundant diazotroph at stations LDA and LDB, with average abundances of 1.13 x 10^6 and 0.29 x10^6 *nifH* gene copies L^{-1}, respectively. N_2 fixation rates were thus mainly attributable to *Trichodesmium*, in agreement with single-cell nanoSIMS measurements of the same cruise (Bonnet *et al.* 2018). Therefore, qRT-PCR analyses were only performed for this cyanobacterium. *NifH* expression ranged from 1.2 x 10^3 to 3.1 x 10^3 and 6.2 x 10^4 to 5.8 x10^6 *nifH* gene transcripts L^{-1} at stations LDA and LDB, respectively.

In our experiments N_2 fixation rates ranged between 2.24 - 4.61, 10.79 - 29.21, and 0.32 - 0.58 nmol N L^{-1} d^{-1} at stations LDA, LDB and LDC, respectively (Fig. 2), and correlated significantly with *Trichodesmium nifH* gene expression at stations LDA and LDB ($r = 0.96$, Spearman rank $p<0.05$). Within each station, the level of response in terms of N_2 fixation rates was similar among the three types of DOM additions (Fig. 3A). The lack of a preferential response to any specific type of DOM was unexpected and suggests that *Trichodesmium* were not particularly limited by either organic nitrogen or organic phosphorus, but rather by organic carbon which is a component of all three types of DOM added to our incubations (note that the DOM mixtures added were equimolar in carbon). However, when comparing among stations the highest responses were observed upon the addition of the DON mixture (Fig. 3A), which was composed of amino acids. Both natural and cultured *Trichodesmium* colonies have been observed to take up amino acids, which may provide them with reduced nitrogen in periods where N_2 fixation is inhibited or energy limited (Paerl, Bebout and Prufert 1989; Mulholland and Capone 1999). Alternatively, amino acids may be used as a source of carbon. For example, amino acids are thought to sustain up to 40% of bacterial production in marine and freshwater ecosystems (Jorgensen *et al.* 1993). Primary production was only stimulated by organic molecule additions at station LDC, the most oligotrophic station (Fig. 3B; Table 1).

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Heterotrophic bacterial abundance and production were mainly stimulated by DON at station LDC (Figs. 3C-D).

Mixotrophy provides diazotrophs with alternative sources of nutrients and energy. For example, the unicellular diazotroph *Cyanothece* can use carbon-rich organic molecules to reduce energy investment in CO_2 fixation, which leaves more energy available for the energetically expensive process of N_2 fixation (Feng *et al.* 2010). *Trichodesmium* is often severely limited by phosphorus, but is capable of obtaining it from several organic and inorganic forms (Dyhrman *et al.* 2006, 2009; Polyviou *et al.* 2015). During the cruise, phosphate concentrations at the surface were low or undetectable even using nanomolar methods (Table 1), and its turnover rates fast (Frischkorn *et al.* 2018; Van Wambeke *et al.* 2018), suggesting strong competition for phosphorus in the microbial food web. In fact, *Trichodesmium* expressed genes related to phosphorus deficiency at several stations along the cruise transect (Frischkorn *et al.* 2018). In our study, the highest response to DOM additions in terms of N_2 fixation and *nifH* gene expression was observed at station LDB (Fig. 2). A large decaying phytoplankton bloom was detected at this station (de Verneil *et al.* 2017), which may explain the refractory nature of the local DOM (Panagiotopoulos *et al.* 2019). Under these conditions, it is not surprising that our addition of labile DOM enhanced diazotrophic activity at LDB. Despite this, N_2 fixation rates and *nifH* expression associated with *Trichodesmium* did not respond preferentially to DOP as compared to the other DOM mixtures added to our incubations (Fig. 2). The observed indifference in the use of organic carbon, amino acids or the phosphomonoesters and phosphonates (DOC, DON and DOP mixtures, respectively) suggests that *Trichodesmium* used the added DOM molecules as a source of carbon, and hence was not phosphorus-limited during our experiments. Iron-limited *Trichodesmium* cultures have been reported to express enzymes that oxidize trimethylamine (Walworth *et al.* 2018), which suggests that *Trichodesmium* can obtain nitrogen from DON as an alternative to N_2 fixation when experiencing micronutrient limitation.

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The stimulation of N_2 fixation rates by DON additions at LDC (where environmental DON concentrations were lowest; Table 1) was likely attributed to UCYN-B, which was the dominant diazotroph with 3.64 $x10^3$ *nifH* gene copies L^{-1} representing 81 to 100% of the total detected *nifH* genes (Stenegren *et al.* 2018). The undetectable *nifH* gene expression at this station is likely explained by the fact that our sampling took place at noon, while UCYN-B primarily express *nifH* at night (Church *et al.* 2005b). UCYN-B have been previously reported to fix more N_2 upon carbohydrate additions in the Southwest Pacific open ocean waters (Benavides *et al.* 2018), but not in similar experiments conducted at a coastal site (New Caledonian lagoon) where UCYN-B did not respond to neither DOC, DON nor DOP additions (Benavides *et al.* 2018).

As our ability to examine the metabolic potential of marine microbes increases, more and more species traditionally thought to depend solely on photosynthesis-derived compounds for their growth appear to use DOM compounds as for example glucose (Munoz-Marin *et al.* 2013; Duhamel *et al.* 2018). Non-cyanobacterial diazotrophs are widespread in the oceans (Farnelid *et al.* 2011) and are thought to use DOM (Bombar, Paerl and Riemann 2016), but the extent to which organic molecules are used by cyanobacterial photosynthetic diazotrophs in marine ecosystems is poorly constrained. Here we report an enhancement in N_2 fixation rates and *nifH* gene expression in natural *Trichodesmium* colonies at two stations in the Melanesian Archipelago region of the western tropical South Pacific, which suggests this cyanobacterium may benefit from mixotrophic nutrition under certain environmental or physiological conditions, an ability more widespread among marine microbes than previously thought (Yelton *et al.* 2016; Stoecker *et al.* 2017). *Trichodesmium nifH* gene expression was not detected at the third station sampled in the South Pacific Gyre, where N_2 fixation rates were much lower (Fig. 2; Table 2) and the dominant diazotroph was UCYN-B (Stenegren *et al.* 2018). Other autotrophic filamentous diazotrophs such as *Nodularia* have been reported to assimilate leucine (Hietanen *et al.* 2002). Unicellular diazotrophs such as *Crocosphaera* (Dyhrman and Haley 2006) and *Cyanothece* (Feng *et al.* 2010) can use phosphomonoesters and

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glycerol, respectively. Still, N_2 fixation models are mostly parameterized for *Trichodesmium* and its iron and phosphorus needs (Monteiro, Dutkiewicz and Follows 2011; Dutkiewicz *et al.* 2012). The present study, and other recent studies (Benavides *et al.* 2017, 2018; Rahav and Bar-Zeev 2017), indicate that taking the mixotrophic potential of diazotrophs into account will improve our understanding of their biogeography and seasonality. A development of biogeochemical models should in this context improve our ability to predict pools and fluxes of nitrogen in the global ocean.

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References

Agawin NSR, Benavides M, Busquets A *et al.* Dominance of unicellular cyanobacteria in the diazotrophic community in the Atlantic Ocean. *Limnol Oceanogr* 2014; **59:** 623-637.

Benavides M, Berthelot H, Duhamel S *et al*. Dissolved organic matter uptake by *Trichodesmium* in the Southwest Pacific. *Sci Rep* 2017; **7:** 1–6.

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Benavides M, Moisander PH, Berthelot H *et al*. Mesopelagic N_2 fixation related to organic matter composition in the Solomon and Bismarck Seas (Southwest Pacific). *PLOS ONE* 2015; **10:** 1–19.

Benavides M, Martias C, Elifantz H *et al.* Dissolved Organic Matter Influences N_2 Fixation in the New Caledonian Lagoon (Western Tropical South Pacific). *Front Mar Sci* 2018; **5:**89.

Berman-Frank I, Quigg A, Finkel ZV *et al.* Nitrogen-fixation strategies and Fe requirements in cyanobacteria. *Limnol Oceanogr* 2007; **52:** 2260–2269.

Bombar D, Paerl RW and Riemann L. Marine Non-Cyanobacterial Diazotrophs: Moving beyond Molecular Detection. *Trends Microbiol* 2016; **24:** 916-927.

Bonnet S, Caffin M, Berthelot H, Grosso O *et al.* In-depth characterization of diazotroph activity across the western tropical South Pacific hotspot of N_2 fixation (OUTPACE cruise). *Biogeosciences* 2018; **15:** 4215–4232.

Bonnet S, Caffin M, Berthelot H and Moutin T. Hot spot of N_2 fixation in the western tropical South Pacific pleads for a spatial decoupling between N_2 fixation and denitrification. *Proc Natl Acad Sci* 2017; **114:** E2800-E2801.

Church MJ, Short CM, Jenkins BD *et al.* Temporal patterns of nitrogenase gene (*nifH*) expression in the oligotrophic North Pacific Ocean. *Appl Environ Microbiol* 2005; **71**: 5362-5370.

Duhamel S, Van Wambeke F, Lefevre D *et al.* Mixotrophic metabolism by natural communities of unicellular cyanobacteria in the western tropical South Pacific Ocean. *Environ Microbiol* 2018; **20:** 2743-2756.

Dutkiewicz S, Ward BA, Monteiro F *et al.* Interconnection of nitrogen fixers and iron in the Pacific Ocean: Theory and numerical simulations. *Global Biogeochem Cycles* 2012; **26:** 1–16.

Dyhrman S, Ammerman J and Van Mooy B. Microbes and the Marine Phosphorus Cycle. *Oceanography* 2007; 20: 110–116.

Dyhrman ST, Benitez-Nelson CR, Orchard ED *et al.* A microbial source of phosphonates in oligotrophic marine systems. *Nat Geosci* 2009; **2:** 696–699.

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Dyhrman ST, Chappell PD, Haley ST *et al.* Phosphonate utilization by the globally important marine diazotroph *Trichodesmium*. *Nature* 2006; **439:** 68–71.

Dyhrman ST and Haley ST. Phosphorus scavenging in the unicellular marine diazotroph *Crocosphaera watsonii*. *Appl Environ Microbiol* 2006; **72:** 1452–1458.

Farnelid H, Andersson AF, Bertilsson S *et al.* Nitrogenase gene amplicons from global marine surface waters are dominated by genes of non-cyanobacteria. *PLOS ONE* 2011; **6:** e19223

Feng X, Bandyopadhyay A, Berla B *et al.* Mixotrophic and photoheterotrophic metabolism in *Cyanothece* sp. ATCC 51142 under continuous light. *Microbiology* 2010; **156:** 2566–2574.

Frischkorn KR, Haley ST and Dyhrman ST. Coordinated gene expression between *Trichodesmium* and its microbiome over day-night cycles in the North Pacific Subtropical Gyre. *ISME J* 2018; **12:** 997-1007.

Frischkorn KR, Krupke A, Guieu C, Louis J *et al. Trichodesmium* physiological ecology and phosphate reduction in the western tropical South Pacific. *Biogeosciences* 2018; **15:** 5761–5778.

Frischkorn KR, Rouco-Molina M, Van Mooy BAS *et al.* The *Trichodesmium* microbiome can modulate host N_2 fixation. *Limnol Oceanogr Lett* 2018; **3:** 401-408.

Guieu C, Bonnet S, Petrenko A *et al.* Iron from a submarine source impacts the productive layer of the Western Tropical South Pacific (WTSP). *Sci Rep* 2018; **8:** 9075.

Jørgensen N, Kroer N, Coffin R *et al.* Dissolved free amino acids, combined amino acids, and DNA as sources of carbon and nitrogen to marine bacteria. *Mar Ecol Prog Ser* 1993; **98:** 135–148.

Karl DM, Michaels A, Bergman B *et al.* Dinitrogen fixation in the world's oceans. *Biogeochemistry* 2002; **57-58:** 47-98.

Moisander PH, Benavides M, Bonnet S *et al.* Chasing after non-cyanobacterial nitrogen fixation in marine pelagic environments. *Front Microbiol* 2017; **8:**1736

Moisander PH, Serros T, Paerl RW *et al.* Gammaproteobacterial diazotrophs and *nifH* gene expression in surface waters of the South Pacific Ocean. *ISME J* 2014; **8:** 1962–1973.

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Moisander PH, Zhang R, Boyle EA *et al.* Analogous nutrient limitations in unicellular diazotrophs and *Prochlorococcus* in the South Pacific Ocean. *ISME J* 2011; **6:** 733–744.

Monteiro FM, Dutkiewicz S and Follows MJ. Biogeographical controls on the marine nitrogen fixers. *Global Biogeochem Cycles* 2011; 25: GB2003.

Moore CM, Mills MM, Achterberg EP *et al.* Large-scale distribution of Atlantic nitrogen fixation controlled by iron availability. *Nat Geosci* 2009; **2:** 867–871.

Moore LR. More mixotrophy in the marine microbial mix. *Proc Natl Acad Sci* 2013; **110:** 8323–8324.

Moreira-Coello V, Mouriño-Carballido B, Marañón E *et al.* Temporal variability of diazotroph community diversity in the upwelling region off NW Iberia. *Sci Rep* 2019; **9:** 3737.

Moutin T, Wagener T, Fumenia A, Gimenez A *et al.* Nutrient availability and the ultimate control of the biological carbon pump in the western tropical South Pacific Ocean. *Biogeosciences* 2017; **15:** 2961–2989.

Mulholland MR and Capone DG. Nitrogen fixation, uptake and metabolism in natural and cultured populations of *Trichodesmium* spp. *Mar Ecol Prog Ser* 1999;**188:** 33–49.

Muñoz-Marín MdC, Luque I, Zubkov MV *et al. Prochlorococcus* can use the Pro1404 transporter to take up glucose at nanomolar concentrations in the Atlantic Ocean. *Proc Natl Acad Sci* 2013; **110:** 8597–8602.

Orchard ED, Ammerman JW, Lomas MW *et al.* Dissolved inorganic and organic phosphorus uptake in *Trichodesmium* and the microbial community: The importance of phosphorus ester in the Sargasso Sea. *Limnol Oceanogr* 2010; **55:** 1390–1399.

Paerl HW, Bebout BM and Prufert LE Bacterial associations with marine *Oscillatoria* sp. (*Trichodesmium* sp.) populations ecophysiological implications. *J Phycol* 1989; **25:** 773–784.

Panagiotopoulos C, Pujo-Pay M, Benavides M *et al.* The composition and distribution of semi-labile dissolved organic matter across the southwest Pacific. *Biogeosciences* 2019; **16:** 105–116.

Polyviou D, Hitchcock A, Baylay AJ *et al.* Phosphite utilization by the globally important marine diazotroph *Trichodesmium. Environ Microbiol Rep* 2015; **7:** 824-30.

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Simon M. Isotope dilution of intracellular amino acids as a tracer of carbon and nitrogen sources of marine planktonic bacteria. *Mar Ecol Prog Ser* 1991; **75:** 295–301.

Sohm JA, Webb EA and Capone DG. Emerging patterns of marine nitrogen fixation. *Nat Rev Microbiol* 2011; **9:** 499–508.

Stenegren M, Caputo A, Berg C *et al.* Distribution and drivers of symbiotic and free-living diazotrophic cyanobacteria in the western tropical South Pacific. *Biogeosciences* 2018; **15:** 1559–1578.

Stoecker DK, Hansen PJ, Caron DA *et al.* Mixotrophy in the Marine Plankton. *Ann Rev Mar Sci* 2017; **9:** 311–335.

de Verneil A, Rousselet L, Doglioli AM *et al.* The fate of a southwest Pacific bloom: Gauging the impact of submesoscale vs. mesoscale circulation on biological gradients in the subtropics. *Biogeosciences* 2017; **14:** 3471–3486.

Walworth NG, Fu FX, Lee MD *et al.* Nutrient-colimited *Trichodesmium* as a nitrogen source or sink in a future ocean. *Appl Environ Microbiol* 2018; **84:** 1–14.

Van Wambeke F, Gimenez A, Duhamel S *et al.* Dynamics and controls of heterotrophic prokaryotic production in the western tropical South Pacific Ocean: Links with diazotrophic and photosynthetic activity. *Biogeosciences* 2018; **15:** 2669–2689.

Yelton AP, Acinas SG, Sunagawa S *et al.* Global genetic capacity for mixotrophy in marine picocyanobacteria. *ISME J* 2016; **10:** 2946–2957.

Zehr JP. Nitrogen fixation by marine cyanobacteria. *Trends Microbiol* 2011; **19:** 162–173.

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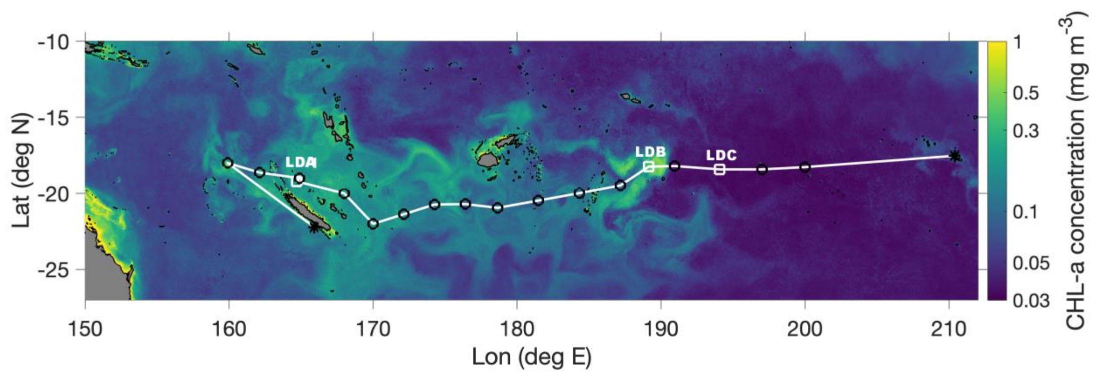

Fig. 1: Geographical location of the stations sampled during the OUTPACE cruise superimposed on a quasi-Lagrangian weighted mean of Chlorophyll *a* (from De Verneil *et al.* 2017).

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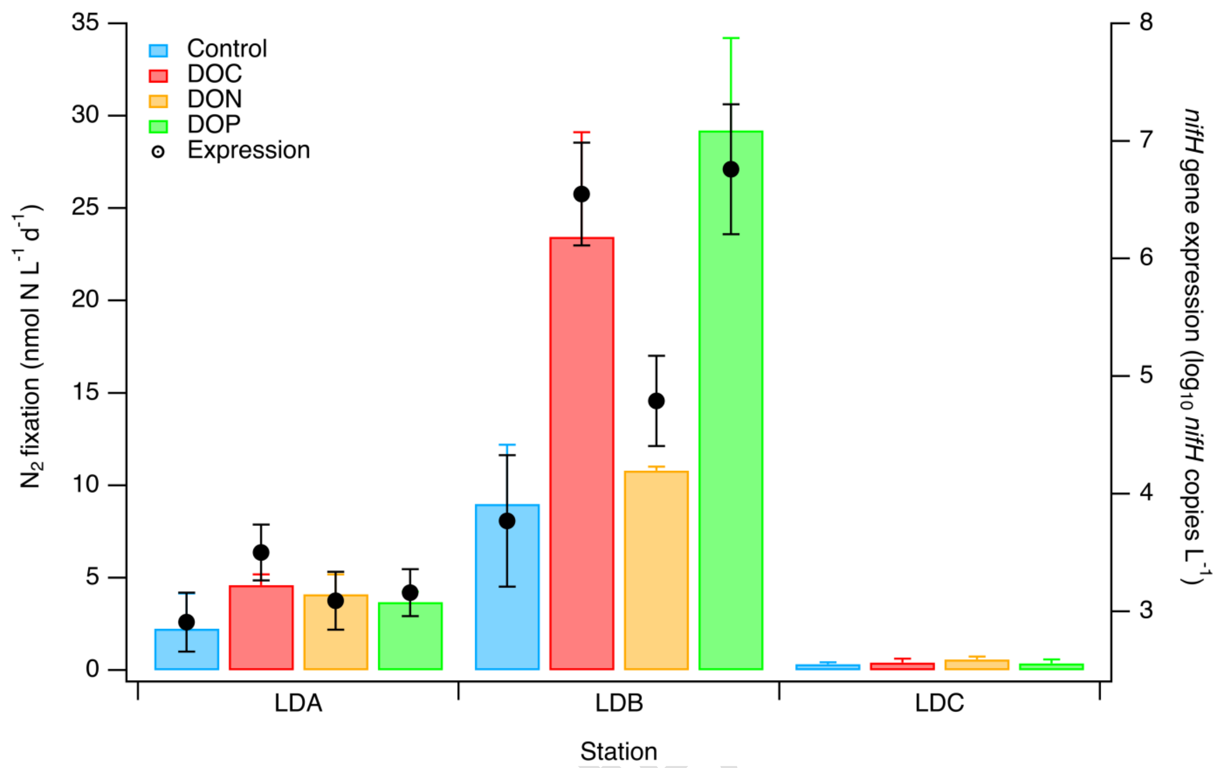

Fig. 2: N_2 fixation rates (bars; n=3) and *nifH* gene expression (dots; n=3) at noon after 36 h incubations with DOC, DON or DOP. Error bars represent ± 1 x standard deviation of the mean.

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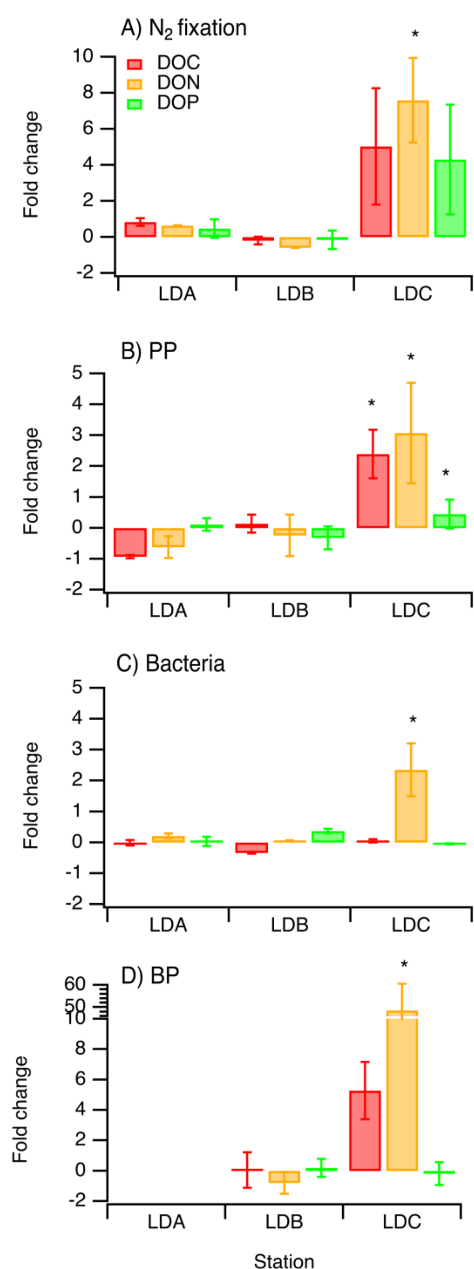

Fig. 3: Fold change responses to DOC, DON or DOP additions after 36 h incubations relative to controls without amendments at stations LDA, LDB and LDC. Fold change is given as (treatment − control)/control such that no change is equal to zero. (A) N_2 fixation rates, (B) primary production rates (PP), (C) bacterial abundance (bacteria), and (D) heterotrophic bacterial production rates (BP) (n=3 for all parameters). Asterisks denote statistically significant changes (Wilcoxon test, $p<0.05$). Error bars indicate the standard deviation of the mean in triplicate measurements.

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Table 1: Values of temperature, salinity, nitrate, phosphate, dissolved organic carbon, nitrogen and phosphorus (DOC, DON and DOP, respectively), Chlorophyll *a,* dissolved iron (dFe) measured at the start of the experiments ("time zero"). n/d means not analytically detectable.

Station	Temperature (ºC)	Salinity	Nitrate (nM)	Phosphate (nM)	DOC (µM)	DON (µM)	DOP (µM)	Chlorophyll *a* (µg L^{-1})	dFe (nM)
LDA	29.2	34.84	20	n/d	95.34 ± 2.81	6.20 ± 0.51	0.18 ± 0.02	0.36 ± 0.05	0.85 ± 0.05
LDB	29.9	35.05	15	7	70.65 ± 0.09	6.09 ± 0.58	0.18 ± 0.02	0.83 ± 0.07	0.71 ± 0.01
LDC	29.6	34.93	n/d	n/d	111.16 ± 121.75	5.32 ± 0.48	0.16 ± 0.03	0.09 ± 0.02	0.35 ± 0.01

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Table 2: *NifH* gene expression, N_2 fixation rates, primary production rates, bacterial cell counts and heterotrophic bacterial production rates (as leucine incorporation rates) in control samples (incubated without amendments), and samples incubated with DOC, DON and DOP, at stations LDA, LDB and LDC. n/a indicates not available data, n/d means not analytically detectable.

Station	Treatment	*nifH* gene expression (log_{10}*nifH*copies L^{-1})	N_2 fixation (nmol N L^{-1} d^{-1})	Primary production (mgC m^{-3} d^{-1})	Bacteria (cell mL^{-1})	Bacterial production (pmol Leu inc L^{-1} h^{-1})
LDA	Control	2.91 ± 0.25	2.24 ± 0.25	4.59 ± 0.57	6.39 ± 0.39 x10^5	n/a
	DOC	3.51 ± 0.24	4.61 ± 0.56	0.35 ± 0.25	6.11 ± 0.58 x10^5	n/a
	DON	3.09 ± 0.25	4.10 ± 1.07	1.77 ± 1.67	7.53 ± 0.49 x10^5	n/a
	DOP	3.16 ± 0.19	3.68 ± 0.57	5.29 ± 0.93	6.36 ± 1.01 x10^5	n/a
LDB	Control	3.77 ± 0.56	9.01 ± 3.21	4.28 ± 1.10	15.01 ± 1.39 x10^5	205 ± 10
	DOC	6.55 ± 0.44	23.45 ± 5.66	3.47 ± 0.87	9.15 ± 0.45 x10^5	216 ± 53
	DON	4.79 ± 0.38	10.89 ± 0.21	3.37 ± 1.19	14.5 ± 0.22 x10^5	41± 3
	DOP	6.76 ± 0.55	29.21 ± 5.01	2.08 ± 1.13	19.02 ± 1.05 x10^5	249± 4
LDC	Control	n/d	0.32 ± 0.1	0.29 ± 0.04	3.06 ± 0.05 x10^5	31 ± 13
	DOC	n/d	0.41 ± 0.22	0.89 ± 0.21	3.14 ± 0.16 x10^5	196 ± 102
	DON	n/d	0.58 ± 0.16	1.07 ± 0.43	10.01 ± 2.60 x10^5	155 ± 413
	DOP	n/d	0.36 ± 0.21	0.38 ± 0.12	2.84 ± 0.04 x10^5	25 ± 3

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