



**HAL**  
open science

## Dissolved organic matter stimulates N<sub>2</sub> fixation and nifH gene expression in *Trichodesmium*

Mar Benavides, Solange Duhamel, France van Wambeke, Katyanne Shoemaker, Pia Moisander, Ellen Salamon, Lasse Riemann, Sophie Bonnet

► **To cite this version:**

Mar Benavides, Solange Duhamel, France van Wambeke, Katyanne Shoemaker, Pia Moisander, et al.. Dissolved organic matter stimulates N<sub>2</sub> fixation and nifH gene expression in *Trichodesmium*. FEMS Microbiology Letters, 2020, 10.1093/femsle/fnaa034 . hal-02488660

**HAL Id: hal-02488660**

**<https://hal.science/hal-02488660v1>**

Submitted on 18 Oct 2024

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

RESEARCH LETTER – Environmental Microbiology

## Dissolved organic matter stimulates N<sub>2</sub> fixation and *nifH* gene expression in *Trichodesmium*

Mar Benavides<sup>1,\*</sup>, Solange Duhamel<sup>2,†</sup>, France Van Wambeke<sup>1</sup>, Katyanne M. Shoemaker<sup>3,‡</sup>, Pia H. Moisaner<sup>3</sup>, Ellen Salamon<sup>4</sup>, Lasse Riemann<sup>4,¶</sup> and Sophie Bonnet<sup>1</sup>

<sup>1</sup>Aix Marseille Univ., Université de Toulon, CNRS, IRD, MIO UM 110, 13288, Marseille, France, <sup>2</sup>Lamont-Doherty Earth Observatory, Columbia University, Palisades, NY 10964, USA, <sup>3</sup>Department of Biology, University of Massachusetts Dartmouth, 285 Old Westport Road, North Dartmouth, MA 02747, USA and <sup>4</sup>Department of Biology, University of Copenhagen, Strandpromenaden 5, 3000 Helsingør, Denmark

\*Corresponding author: Mediterranean Institute of Oceanography (MIO) Campus de Luminy Bâtiment Méditerranée 13288 Marseille cedex 09, France. Tel: +33 0 486090668; E-mail: [mar.benavides@ird.fr](mailto:mar.benavides@ird.fr)

**One sentence summary:** *Trichodesmium* generally considered an autotroph. This work shows an enhancement in N<sub>2</sub> fixation and *nifH* gene expression in *Trichodesmium* as a response to dissolved organic matter compounds, indicating a mixotrophic potential.

<sup>†</sup>Currently at: Graduate School of Oceanography, University of Rhode Island, Narragansett, RI, USA

<sup>‡</sup>Currently at: Department of Molecular and Cellular Biology, University of Arizona, Tucson, AZ 85721, USA

Editor: Euan James

<sup>§</sup>Mar Benavides, <http://orcid.org/0000-0001-9502-108X>

<sup>¶</sup>Lasse Riemann, <http://orcid.org/0000-0001-9207-2543>

### 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) resources is unknown. Here we provide evidence of carbon-, nitrogen- and phosphorus-rich DOM molecules enhancing N<sub>2</sub> fixation rates and *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* behaves 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.

**Keywords:** DOM; mixotrophy; western tropical South Pacific; diazotrophs

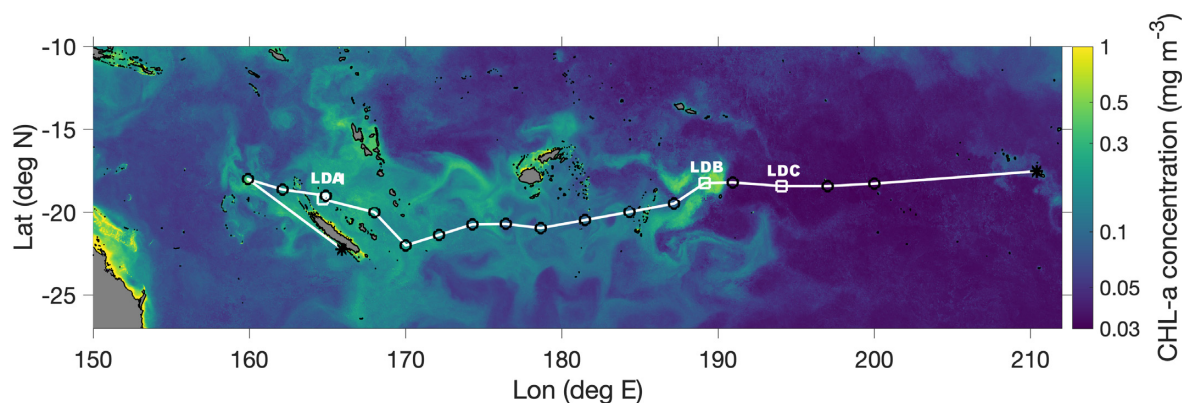
### INTRODUCTION

In remote ocean areas where nitrogen via riverine and atmospheric inputs is minimal, biological dinitrogen (N<sub>2</sub>) fixation provides the main source of reactive nitrogen available for primary producers (Karl et al. 2002). N<sub>2</sub> 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

Received: 20 December 2019; Accepted: 20 February 2020

© The Author(s) 2019. Published by Oxford University Press on behalf of FEMS. All rights reserved. For permissions, please e-mail: [journals.permissions@oup.com](mailto:journals.permissions@oup.com)



**Figure 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).

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 *Crocospaera* preferentially inhabit low inorganic nitrogen (sub)tropical waters  $>25$  °C, while the symbiotic UCYN-A is usually more abundant in relatively colder ( $<25$  °C) 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  $\sim 5$  times more iron to fix  $N_2$  than other phytoplankton 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 *Crocospaera* 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; Moisaner 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 *Cyanotheca* 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.

During a cruise in the western tropical South Pacific (<http://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). A total of five bottles received a mixture of organic carbon 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 (as in Benavides et al. 2015) and incubated for 36 h in on-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  $\delta^{15}\text{N}$  values), and 'control' (incubated for 36 h without DOM amendments). Of each set of five DOM-amended bottles, three were used for rate measurements ( $\text{N}_2$  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 samples using the  $^{14}\text{C}$ -bicarbonate assay. For the control treatment, parallel incubations were done to determine  $^{14}\text{C}$  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  $^{14}\text{C}$ -sodium bicarbonate (Perkin Elmer, Waltham, MA, USA) to a final radioactivity of approximately 2.96 kBq  $\text{mL}^{-1}$  (or 0.08  $\mu\text{Ci mL}^{-1}$ ) from dawn to sunset (~8 h) in on-deck incubators. The total activity was measured from a subsample of 50  $\mu\text{L}$  (dpm  $\text{L}^{-1}$ , in  $\beta$ -phenylethylamine). Total  $^{14}\text{C}$  assimilation into particulate carbon was measured using glass fiber filters to harvest plankton biomass (dpm  $\text{L}^{-1}$ ). The filters were inserted into 20 mL scintillation vials and acidified with 0.5 mL of 1 N HCl for 24 h to remove any unincorporated  $^{14}\text{C}$ -sodium bicarbonate. Radioactivity was measured with 10 mL of 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  $\text{L}^{-1}$ ) was subtracted from that in the light. PP rates ( $\text{mg C m}^{-3} \text{d}^{-1}$ ) were 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  $^{12}\text{C}$  over  $^{14}\text{C}$ .

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- $^3\text{H}$ ]-leucine (4.14 TBq/mmol or 112 Ci  $\text{mmol}^{-1}$ ) 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. (2018). The incorporation of leucine was converted to carbon considering a conversion factor of 1.5 kg C  $\text{mol}^{-1}$  leucine (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  $-80^\circ\text{C}$ . Before analysis, reference beads (Fluoresbrite, YG, 1  $\mu\text{m}$ ) 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. (2018)

## $\text{N}_2$ fixation rates

All bottles were amended with 6 mL of  $^{15}\text{N}$ -labeled  $\text{N}_2$  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. (1996), gently mixed for 5 min and incubated for 36 h (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. (2017). After the incubation, control and DOM-amended bottles were filtered onto precombusted (6 h,  $450^\circ\text{C}$ ) GF/F filters (Whatman). The GF/F filters were stored in precombusted glass vials and kept at  $-20^\circ\text{C}$  until analysis. Isotope ratios were obtained with an Integra2 Analyzer, calibrated with reference material (IAEA-N1).  $\text{N}_2$  fixation rates were calculated as described in Montoya et al. (1996). Rates were corrected by subtracting particulate nitrogen time zero  $\delta^{15}\text{N}$  values and taking into account the dissolution of  $^{15}\text{N}_2$  gas into the seawater sample by MIMS analyses (Kana et al. 1994) as detailed in 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  $\mu\text{m}$  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  $\mu\text{L}$  RLT buffer (Invitrogen) and 3.5  $\mu\text{L}$  mercaptoethanol. All tubes were flash-frozen and stored at  $-80^\circ\text{C}$  until extraction. DNA was extracted using the DNeasy Plant Mini Kit (Qiagen), as detailed in Moisander et al. (2008). RNA extractions were performed with the RNeasy Minikit (Qiagen) as described in 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 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 ( $2 \times 300$ ) (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 (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 GA A GTA TTG AAG CCA GGT TTC-3', reverse primer 5'-CGG CC A 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  $\mu$ L) consisted of 6.25  $\mu$ L TaqMan PCR Master Mix (Applied Biosystems), 0.25  $\mu$ L of the forward and reverse primers (at 10  $\mu$ M, HPLC purified, TAG Copenhagen, Denmark), 0.125  $\mu$ L probe (at 10  $\mu$ M, TAG Copenhagen), 4  $\mu$ L PCR grade water, 0.63  $\mu$ L bovine serum albumin (BSA, at 10.08  $\mu$ g  $\mu$ L<sup>-1</sup>) and 1  $\mu$ L standard or sample (samples were pre-diluted to 5 ng  $\mu$ L<sup>-1</sup> to add 5 ng DNA to all qPCR reactions). The qPCR program consisted of 2 min at 50 °C, 10 min at 95 °C continued by 45 cycles of 15 s at 95 °C and 1 min at 60 °C.

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  $\mu$ L 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 et al. 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 et al. 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 unidentified cyanobacteria like *Myxosarcina*, *Pleurocapsales* and *Nostocales* (data not shown). The predominance of *Trichodesmium*

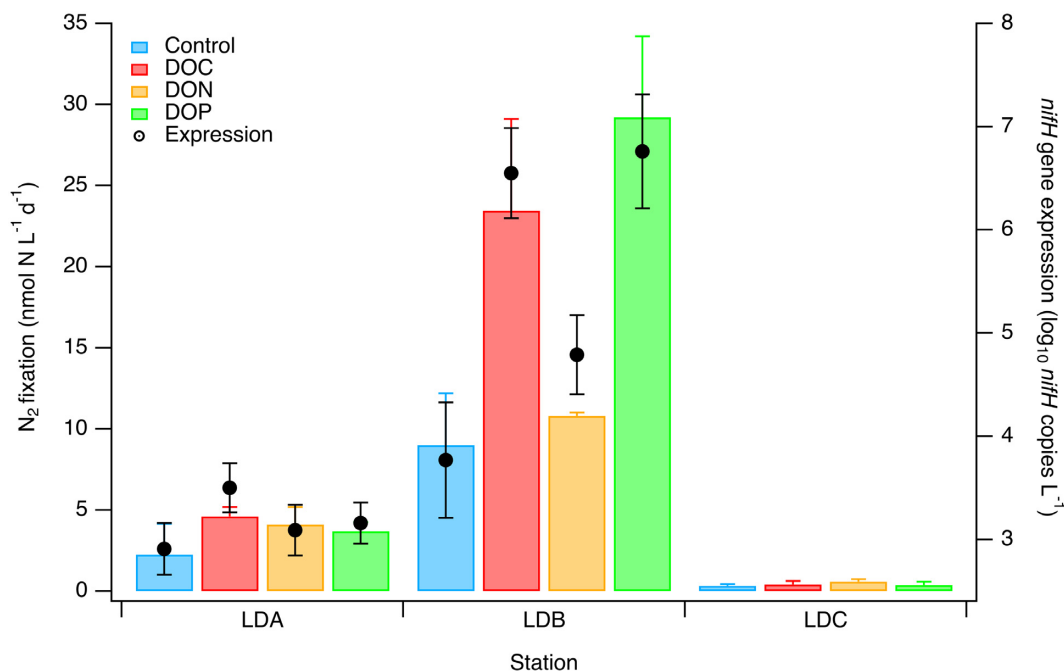


Figure 2.  $N_2$  fixation rates (bars;  $n = 3$ ) and *nifH* gene expression (dots;  $n = 3$ ) at noon after 36 h incubations with DOC, DON or DOP. Error bars represent  $\pm 1 \times$  standard deviation of the mean.

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  $1.13 \times 10^6$  and  $0.29 \times 10^6$  *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  $1.2 \times 10^3$  to  $3.1 \times 10^3$  and  $6.2 \times 10^4$  to  $5.8 \times 10^6$  *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). 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, Haley and Dyhrman 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, Haley and Dyhrman 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

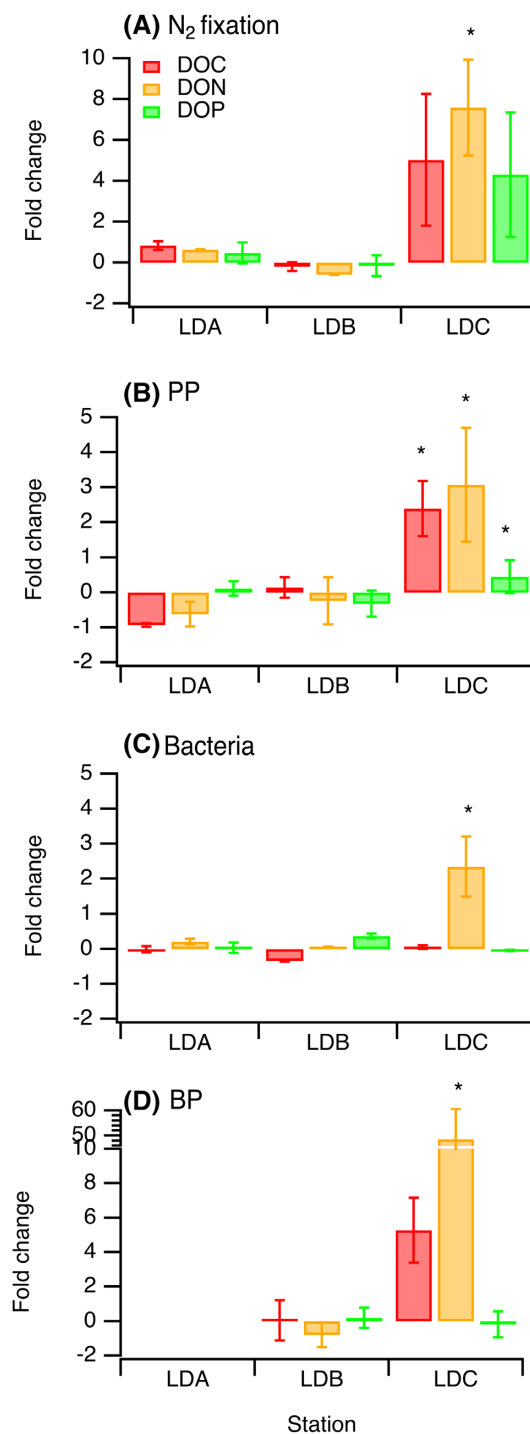


Figure 3. Fold change responses to DOC, DON or DOP additions after 36 h incubations relative to controls without amendments at stations LDA, LDB and LDC. Fold change is given as (treatment–control)/control such that no change is equal to zero. (A)  $N_2$  fixation rates, (B) primary production rates (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.

**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 (°C)	Salinity	Nitrate (nM)	Phosphate (nM)	DOC (μM)	DON (μM)	DOP (μM)	Chlorophyll <i>a</i> (μg/L)	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

**Table 2.** *nifH* gene expression, N<sub>2</sub> 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	<i>nifH</i> gene expression (log <sub>10</sub> <i>nifH</i> copies L <sup>-1</sup> )	N <sub>2</sub> fixation (nmol N L <sup>-1</sup> d <sup>-1</sup> )	Primary production (mgC m <sup>-3</sup> d <sup>-1</sup> )	Bacteria (cell mL <sup>-1</sup> )	Bacterial production (pmol Leu inc L <sup>-1</sup> h <sup>-1</sup> )
LDA	Control	2.91 ± 0.25	2.24 ± 0.25	4.59 ± 0.57	6.39 ± 0.39 × 10 <sup>5</sup>	n/a
	DOC	3.51 ± 0.24	4.61 ± 0.56	0.35 ± 0.25	6.11 ± 0.58 × 10 <sup>5</sup>	n/a
	DON	3.09 ± 0.25	4.10 ± 1.07	1.77 ± 1.67	7.53 ± 0.49 × 10 <sup>5</sup>	n/a
	DOP	3.16 ± 0.19	3.68 ± 0.57	5.29 ± 0.93	6.36 ± 1.01 × 10 <sup>5</sup>	n/a
LDB	Control	3.77 ± 0.56	9.01 ± 3.21	4.28 ± 1.10	15.01 ± 1.39 × 10 <sup>5</sup>	205 ± 10
	DOC	6.55 ± 0.44	23.45 ± 5.66	3.47 ± 0.87	9.15 ± 0.45 × 10 <sup>5</sup>	216 ± 53
	DON	4.79 ± 0.38	10.89 ± 0.21	3.37 ± 1.19	14.5 ± 0.22 × 10 <sup>5</sup>	41 ± 3
	DOP	6.76 ± 0.55	29.21 ± 5.01	2.08 ± 1.13	19.02 ± 1.05 × 10 <sup>5</sup>	249 ± 4
LDC	Control	n/d	0.32 ± 0.1	0.29 ± 0.04	3.06 ± 0.05 × 10 <sup>5</sup>	31 ± 13
	DOC	n/d	0.41 ± 0.22	0.89 ± 0.21	3.14 ± 0.16 × 10 <sup>5</sup>	196 ± 102
	DON	n/d	0.58 ± 0.16	1.07 ± 0.43	10.01 ± 2.60 × 10 <sup>5</sup>	155 ± 413
	DOP	n/d	0.36 ± 0.21	0.38 ± 0.12	2.84 ± 0.04 × 10 <sup>5</sup>	25 ± 3

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<sub>2</sub> fixation when experiencing micronutrient limitation.

The stimulation of N<sub>2</sub> 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 × 10<sup>3</sup> *nifH* gene copies L<sup>-1</sup> 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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 *Crocospaera* (Dyhrman and Haley 2006) and *Cyanothece* (Feng et al. 2010) can use phosphomonoesters and glycerol, respectively. Still, N<sub>2</sub> 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.

## ACKNOWLEDGMENTS

The authors thank the captain and crew of the R/V *L'Atalante* for assistance onboard.

## FUNDING

The OUTPACE (Oligotrophy from Ultra-oligoTrophy PACific Experiment) project was funded by the French national research agency (ANR-14-CE01-0007-01), the LEFE-CYBER program

(CNRS-INSU), the GOPS program (IRD) and the CNES (BC T23, ZBC 4500048836). M.B. received funding from the People Programme (Marie Skłodowska-Curie Actions) of the European Union's FP7/2007-2013 programme, REA grant agreement 625 185. P.M. and K.S. were supported by NSF OCE-1733610 award to P.M. S.D. was supported by National Science Foundation award OCE-1 434 916 and by the Vetlesen Foundation. E.S. and L.R. were supported the Danish Council for Independent Research (6108-00013).

**Conflicts of interests.** None declared.

## 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**:623–37.
- Barton AD, Pershing AJ, Litchman E et al. The biogeography of marine plankton traits. *Ecol Lett* 2013, DOI: 10.1111/ele.12063.
- Benavides M, Berthelot H, Duhamel S et al. Dissolved organic matter uptake by *Trichodesmium* in the Southwest Pacific. *Sci Rep* 2017;**7**:1–6.
- Benavides M, Martias C, Elifantz H et al. Dissolved organic matter influences N<sub>2</sub> fixation in the new caledonian lagoon (Western Tropical South Pacific). *Front Mar Sci* 2018;**5**:89.
- Benavides M, Moisaner PH, Berthelot H et al. Mesopelagic N<sub>2</sub> fixation related to organic matter composition in the solomon and bismarck seas (Southwest Pacific). *PLoS One* 2015;**10**: 1–19.
- Berman-Frank I, Cullen JT, Shaked Y et al. Iron availability, cellular iron quotas, and nitrogen fixation in *Trichodesmium*. *Limnol Oceanogr* 2001;**46**:1249–60.
- Berman-Frank I, Quigg A, Finkel ZV et al. Nitrogen-fixation strategies and Fe requirements in cyanobacteria. *Limnol Oceanogr* 2007;**52**:2260–9.
- Bock N, Van Wambeke F, Dion M et al. Microbial community structure along trophic and N<sub>2</sub> fixation gradients in the Western Tropical South Pacific. *Biogeosciences* 2018, DOI: 10.5194/bg-15-3909-2018.
- Bombar D, Paerl RW, Riemann L. Marine non-cyanobacterial diazotrophs: moving beyond molecular detection. *Trends Microbiol* 2016;**24**:916–27.
- Bonnet S, Caffin M, Berthelot H et al. In-depth characterization of diazotroph activity across the western tropical South Pacific hotspot of N<sub>2</sub> fixation (OUTPACE cruise). *Biogeosciences* 2018;**15**:4215–32.
- Caffin M, Moutin T, Foster RA et al. N<sub>2</sub> fixation as a dominant new N source in the western tropical South Pacific Ocean (OUTPACE cruise). *Biogeosciences* 2018, DOI:10.5194/bg-15-2565-2018.
- Caporaso JG, Kuczynski J, Stombaugh J et al. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 2010;**7**:335–6.
- 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**: 5362–70.
- Dabundo R, Lehmann MF, Treibergs L et al. The contamination of commercial <sup>15</sup>N<sub>2</sub> gas stocks with <sup>15</sup>N-labeled nitrate and ammonium and consequences for nitrogen fixation measurements. *PLOS ONE* 2014, DOI: 10.1371/journal.pone.0110335.
- de Verneil A, Rousselet L, Doglioli AM et al. The fate of a south-west Pacific bloom: gauging the impact of submesoscale vs. mesoscale circulation on biological gradients in the subtropics. *Biogeosciences* 2017;**14**:3471–86.
- 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**:2743–56.
- Dutkiewicz S, Ward BA, Monteiro F et al. Interconnection of nitrogen fixers and iron in the Pacific Ocean: theory and numerical simulations. *Glob Biogeochem Cycles* 2012;**26**:1–16.
- Dyhrman S, Ammerman J, Van Mooy B. Microbes and the marine phosphorus cycle. *Oceanography* 2007;**20**:110–6.
- Dyhrman ST, Benitez-Nelson CR, Orchard ED et al. A microbial source of phosphonates in oligotrophic marine systems. *Nat Geosci* 2009;**2**:696–9.
- Dyhrman ST, Chappell PD, Haley ST et al. Phosphonate utilization by the globally important marine diazotroph *Trichodesmium*. *Nature* 2006;**439**:68–71.
- Dyhrman ST, Haley ST. Phosphorus scavenging in the unicellular marine diazotroph *Crocospaera watsonii*. *Appl Environ Microbiol* 2006;**72**:1452–8.
- Edgar RC. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 2010, DOI: 10.1093/bioinformatics/btq461.
- 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 *Cyanotheca* sp. ATCC 51142 under continuous light. *Microbiology* 2010;**156**:2566–74.
- Frischkorn KR, Haley ST, 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 et al. *Trichodesmium* physiological ecology and phosphate reduction in the western tropical South Pacific. *Biogeosciences* 2018;**15**:5761–78.
- 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.
- Hietanen S, Lehtimäki JM, Tuominen L et al. *Nodularia* spp. (Cyanobacteria) incorporate leucine but not thymidine: Importance for bacterial-production measurements. *Aquat Microb Ecol* 2002;**28**:99–104.
- 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**:135–48.
- Kana TM, Darkangelo C, Hunt MD et al. Membrane Inlet Mass Spectrometer for Rapid Environmental Water Samples. *Anal Chem* 1994;**66**:4166–70.
- Karl DM, Church MJ, Dore JE et al. Predictable and efficient carbon sequestration in the North Pacific Ocean supported by symbiotic nitrogen fixation. *Proc Natl Acad Sci* 2012;**109**:1842–9.
- Karl DM, Michaels A, Bergman B et al. Dinitrogen fixation in the world's oceans. *Biogeochemistry* 2002;**57–58**:47–98.
- Kirchman DL, Keil RG, Simon M. Biomass and production of heterotrophic bacterioplankton in the oceanic subarctic Pacific. *Deep-Sea Res Part I* 1993;**40**:967–88.
- Kustka AB, Sañudo-Wilhelmy SA, Carpenter EJ et al. Iron requirements for dinitrogen- and ammonium-supported growth in cultures of *Trichodesmium* (IMS 101): Comparison with nitrogen fixation rates and iron:carbon ratios of field populations. *Limnol Oceanogr* 2003;**48**:1869–84.
- Kwak Y, Shin JH. First *Azospirillum* genome from aquatic environments: Whole-genome sequence of *Azospirillum*



- thiophilum BV-ST, a novel diazotroph harboring a capacity of sulfur-chemolithotrophy from a sulfide spring. *Mar Biol* 2016, DOI: 10.1016/j.margen.2015.11.001.
- Ludwig W, Strunk O, Westram R et al. ARB: A software environment for sequence data. *Nucleic Acids Res* 2004;32:1363–71.
- Moisander PH, Beinart RA, Voss M et al. Diversity and abundance of diazotrophic microorganisms in the South China Sea during intermonsoon. *ISME J* 2008;2:954–67.
- 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;8:1962–73.
- Monteiro FM, Dutkiewicz S, Follows MJ. Biogeographical controls on the marine nitrogen fixers. *Glob Biogeochem Cycles* 2011;25:GB2003.
- Montoya JP, Voss M, Kahler P et al. A Simple, High-Precision, High-Sensitivity Tracer Assay for N<sub>2</sub> Fixation. *Appl Environ Microbiol* 1996;62:986–93.
- Moore CM, Mills MM, Achterberg EP et al. Large-scale distribution of Atlantic nitrogen fixation controlled by iron availability. *Nat Geosci* 2009;2:867–71.
- Moore LR. More mixotrophy in the marine microbial mix. *Proc Natl Acad Sci* 2013;110:8323–4.
- Moreira-Coello V, Mouriño-Carballido B, Marañoń 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 et al. Nutrient availability and the ultimate control of the biological carbon pump in the western tropical South Pacific Ocean. *Biogeosciences* 2017;15:2961–89.
- Mulholland MR, Capone DG. Nitrogen fixation, uptake and metabolism in natural and cultured populations of *Trichodesmium* spp. *Mar Ecol Prog Ser* 1999;188:33–49.
- Munoz-Marin 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;110:8597–602.
- 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;55:1390–9.
- Orlova MV, Tarlachkov SV, Dubinina GA et al. Genomic insights into metabolic versatility of a lithotrophic sulfur-oxidizing diazotrophic Alphaproteobacterium *Azospirillum thiophilum*. *FEMS Microbiol Ecol* 2016, DOI: 10.1016/j.margen.2015.11.001.
- Paerl HW, Bebout BM, Prufert LE. Bacterial associations with marine *Oscillatoria* sp. (*Trichodesmium* sp.) populations ecophysiological implications. *J Phycol* 1989;25:773–84.
- 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;16:105–16.
- Polyviou D, Hitchcock A, Baylay AJ et al. Phosphite utilization by the globally important marine diazotroph *Trichodesmium*. *Environ Microbiol Rep* 2015;7:824–30.
- Rahav E, Bar-Zeev E. Sewage outburst triggers *Trichodesmium* bloom and enhance N<sub>2</sub> fixation rates. *Sci Rep* 2017, DOI: 10.1038/s41598-017-04622-8.
- Schloss PD, Westcott SL, Ryabin T et al. Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* 2009;75:7537–41.
- Shoemaker KM, Moisander PH. Seasonal variation in the copepod gut microbiome in the subtropical North Atlantic Ocean. *Environ Microbiol* 2017;19:3087–97.
- Smith DC, Azam F. A simple, economical method for measuring bacterial protein synthesis rates in seawater using tritiated-leucine. *Mar Microb Food Webs* 1992;6:107–14.
- Sohm JA, Webb EA, 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;15:1559–78.
- Stoecker DK, Hansen PJ, Caron DA et al. Mixotrophy in the marine plankton. *Ann Rev Mar Sci* 2017;9:311–35.
- 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;15:2669–89.
- Wagener T, Metzl N, Cuffin M et al. Carbonate system distribution, anthropogenic carbon and acidification in the western tropical South Pacific (OUTPACE 2015 transect). *Biogeosciences* 2018, DOI: 10.5194/bg-15-5221-2018.
- 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.
- Yelton AP, Acinas SG, Sunagawa S et al. Global genetic capacity for mixotrophy in marine picocyanobacteria. *ISME J* 2016;10:2946–57.
- Zehr JP, Turner PJ. Nitrogen fixation: Nitrogenase genes and gene expression. *Method Microbiol* 2001, DOI:10.1016/s0580-9517(01)30049-1.
- Zehr JP, Waterbury JB, Turner PJ. Unicellular cyanobacteria fix N<sub>2</sub> in the subtropical North Pacific Ocean. *Nature* 2001, DOI:10.1038/news010809-11.