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Dissolved Organic Matter Influences N₂ Fixation in the New Caledonian Lagoon (Western Tropical South Pacific)

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Specialized prokaryotes performing biological dinitrogen (N₂) fixation (‘diazotrophs’) provide an important source of fixed nitrogen in oligotrophic marine ecosystems such as tropical and subtropical oceans. In these waters, cyanobacterial photosynthetic diazotrophs are well known to be abundant and active, yet the role and contribution of non-cyanobacterial diazotrophs are currently unclear. The latter are not photosynthetic (here called “heterotrophic”) and hence require external sources of organic matter to sustain N₂ fixation. Here we added the photosynthesis inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) to estimate the N₂ fixation potential of heterotrophic diazotrophs as compared to autotrophic ones. Additionally, we explored the influence of dissolved organic matter (DOM) on these diazotrophs along a coast to open ocean gradient in the surface waters of a subtropical coral lagoon (New Caledonia).

Total N₂ fixation (samples not amended with DCMU) ranged from 0.66 to 1.32 nmol N L⁻¹ d⁻¹. The addition of DCMU reduced N₂ fixation by >90%, suggesting that the contribution of heterotrophic diazotrophs to overall N₂ fixation activity was minor in this environment. Higher contribution of heterotrophic diazotrophs occurred in stations closer to the shore and coincided with the decreasing lability of DOM, as shown by various colored DOM and fluorescent DOM (CDOM and FDOM) indices. We tested the response of diazotrophs (in terms of nifH gene expression and bulk N₂ fixation rates) upon the addition of a mix of carbohydrates (“DOC” treatment), amino acids (“DON” treatment), and phosphonates and phosphomonesters (“DOP” treatment). While nifH expression increased significantly in Trichodesmium exposed to the DOC treatment, bulk N₂ fixation rates increased significantly only in the DOP treatment. The lack of nifH expression by gammaproteobacteria, in any of the DOM addition treatments applied, questions the contribution of non-cyanobacterial diazotrophs to fixed nitrogen inputs in the New Caledonian lagoon. While the metabolism and ecology of heterotrophic diazotrophs is currently elusive, a deeper understanding of their ecology and relationship with DOM is needed in the light of increased DOM inputs in coastal zones due to anthropogenic pressure.

Keywords: non-cyanobacterial diazotrophs, nifH, DCMU, DOM, CDOM, FDOM
INTRODUCTION

Biological dinitrogen (N$_2$) fixation provides an important source of fixed nitrogen to fuel primary production in the oceans (Karl et al., 2002), being especially critical in areas devoid of other significant fixed nitrogen sources such as the oligotrophic subtropical gyres. N$_2$ fixation is performed by specialized prokaryotic microbes called “diazotrophs.” These include cyanobacterial and non-cyanobacterial (bacteria and archaea) groups, that are commonly detected and quantified via the presence of the nifH gene, which encodes for a subunit of the nitrogenase enzyme system (Zehr, 2011). Traditionally, marine diazotrophs were hypothesized to be constrained by warm, low inorganic nitrogen waters (Sohm et al., 2011). Yet, several filamentous cyanobacteria are able to obtain energy from dissolved organic matter-DOM (Rippka et al., 1979), and several reports have shown that the most abundant photoautotrophs in the world’s oceans (Prochlorococcus and Synechococcus) harbor the genes necessary to use amino acids, peptides and sugars (Yelton et al., 2016). As evidence accumulates, it seems that the ability of using DOM as an alternative source of nutrients under limiting situations is common in various planktonic groups (Stoecker et al., 2017). Among marine diazotrophs, unicellular cyanobacteria such as Cyanothecce have been reported to grow on glycerol (Feng et al., 2010), and filamentous diazotrophs such as Trichodesmium may obtain as much carbon from amino acids assimilation as from CO$_2$ fixation in the field (Benavides et al., 2017). Non-cyanobacterial and non-photosynthetic diazotrophs require external sources of DOM as energy sources (Riemann et al., 2010). The globally widespread non-cyanobacterial diazotroph Gamma A (Langlois et al., 2015) upregulates nifH gene expression upon glucose + mannitol additions (Moisander et al., 2011). Previous work has also shown that N$_2$ fixation activity is stimulated by DOM additions (Bonnet et al., 2013; Rahav et al., 2013, 2015), and correlates with labile DOM compounds in environments where non-cyanobacterial (non-photosynthetic) diazotrophs predominate, such as the aphytic mesopelagic layer (Benavides et al., 2015), or the surface ultraoligotrophic eastern Mediterranean Sea (Rahav et al., 2016).

N$_2$ fixation and diazotroph community studies have mostly focused on populations in the North Atlantic (Benavides and Voss, 2015) and North Pacific Oceans, while other areas such as the Indian and the South Atlantic and Pacific Oceans have been chronically under-sampled (Luo et al., 2012). The western tropical Southwest Pacific (WTSP) Ocean comprises warm, oligotrophic, trace metal rich waters that harbor among the high N$_2$ fixation rates measured at sea (>500 µmol N m$^{-2}$ d$^{-1}$; Bonnet et al., 2017). The activity and distribution of diazotrophs in the WTSP has been related to temperature, iron and inorganic nutrient availability thresholds (e.g., Bonnet et al., 2009; Moisander et al., 2010). In austral summer conditions, the relatively warmer and productive waters like the Arafura, Timor and Solomon Seas (∼26.5–30°C) are dominated by Trichodesmium and UCYN-B (Messer et al., 2016), while UCYN-A remains confined to the colder and more oligotrophic Coral Sea (∼22–26.5°C; Moisander et al., 2010; Bonnet et al., 2015; Messer et al., 2016). Together with the high abundances of diazotroph phylotypes (Moisander et al., 2010; Bonnet et al., 2015), the WTSP has been recently acknowledged as a hotspot of global importance for N$_2$ fixation activity (Bonnet et al., 2017). Thus, understanding how environmental factors (besides temperature and inorganic nutrients) shape the distribution and activity of diazotrophs in the WTSP is of utmost importance to assess their potential to fuel primary production in these oligotrophic waters and their contribution to the oceanic nitrogen budget.

New Caledonia, situated at the eastern edge of the WTSP, is surrounded by a reef enclosing one of the largest coral lagoons in the world. Here, primary production is nitrogen-limited throughout the year (Torretón et al., 2010), prompting diazotrophic activity (Garcia et al., 2007; Biegala and Raimbault, 2008) and the presence of various cyanobacterial diazotroph phylotypes including Trichodesmium (often occurring as large blooms; Rodier and Le Borgne, 2008), diatom-diazotroph symbioses (DDAs) as well the unicellular cyanobacteria UCYN-A1, UCYN-A2, UCYN-B, and UCYN-C (Turk-Kubo et al., 2015). However, non-cyanobacterial diazotrophs have been far less studied, and despite representatives such as the putative gammaproteobacterium y-24774A11 that has been detected at abundances of $10^2$–$10^3$ nifH copies L$^{-1}$ within the lagoon (Turk-Kubo et al., 2015), their contribution to N$_2$ fixation in this environment remains unquantified. Moreover, the importance of DOM as a source of energy and nutrition for both cyanobacterial and non-cyanobacterial diazotrophs has never been explored in this environment. In this study we assess if the inhibition of photosynthesis triggers DOM nutrition in pelagic diazotrophs, and whether the N$_2$ fixation activity and/or nifH expression of these pelagic diazotrophs is enhanced by different model DOM molecules.

MATERIALS AND METHODS

Hydrography, Oxygen, Inorganic Nutrients, Chl α and DOC Measurements

Sampling took place on 13 January 2016 approximately from 8:00 a.m. to noon at four stations along an anthropogenic pressure gradient in the New Caledonian lagoon onboard the R/V Archamia. Station 1 was close to the rim of the coral reef barrier and Station 4 near the harbor of the city of Nouméa (Figure 1, Table 1). This transect connects the passage of Dumbêa (the main canal for the entrance of ships into the lagoon) with the harbor. Sampling started at Station 1 at high tide and proceeded toward the coast as the tide ebbed.

At each station, temperature and salinity were measured by casting a SeaBird SB19 plus CTD probe from the surface to the bottom. Water samples for the measurement of oxygen, nutrients, chlorophyll α (Chl α) and DOC concentrations, and colored DOM (CDOM) and fluorescent DOM (FDOM) parameters (see below) were collected from the subsurface (∼3 m), using a 5 L Niskin bottle and transported to the lab for analysis in coolers equipped with ice packs within 2 h. Samples for the measurement of dissolved oxygen were taken in triplicate and analyzed ashore using an automated
titration system with a photometric endpoint (Langdon, 2010). Samples for the determination of nitrate plus nitrite (NO$_x$) and phosphate (PO$_4^{3-}$) concentrations were taken on acid-washed 20 mL polyethylene tubes, poisoned with 1% HgCl$_2$, and stored at 4°C until analysis. The samples were analyzed on a AA3 Bran+Luebbe autoanalyzer following JGOFS recommendations (JGOFS, 1994). The detection limits were 50 and 10 nM for NO$_x$ and PO$_4^{3-}$, respectively. Chl $a$ concentrations were estimated from seawater samples (500 mL) filtered through 25 mm GF/F filters (Whatman) and stored at −80°C until analysis, extracted in methanol on a AU Turner Designs bench fluorometer, previously calibrated with pure Chl $a$ (Herbland et al., 1985).

For the analysis of DOM, samples for FDOM and CDOM were filtered through 0.2 µm polycarbonate filters in a custom-made filtration system pre-cleaned with 1 M HCl and Milli-Q water. Samples for DOM analysis were stored at 4°C until analysis (within 1 month), while samples for FDOM absorption analyses were directly measured on a liquid waveguide capillary flow cell (WPI) according to the method of Kowalczyk et al. (2013). Excitation-emission matrix (EEM) spectra for analysis of FDOM were determined using a Hitachi F-7000 spectrofluorometer (Tedetti et al., 2011). FDOM components were identified by parallel factor analysis (PARAFAC), a statistical decomposition technique used to extract the most representative fluorescent components within EEMs dataset. To increase the validity of the PARAFAC analysis, data from the present experiment were pooled with 39 other samples obtained from the same waters, 3 months before and after the experiment. Indeed, to avoid systematic biases, remove signals unrelated to fluorescence and to validate the number of FDOM components, a large dataset was needed (Stedmon et al., 2003). For example, in the case where two of the components had very similar fluorescence characteristics and/or were highly correlated in their appearance among samples, the PARAFAC approach would have difficulties to discern between the two. We then determined several spectral indices: the absorption at 442 nm as a proxy of phytoplankton production and the absorption at 350 nm as an indicator of terrestrial inputs in the bay (Passow and Alldredge, 1995), a biological index (BIX) of recent autochthonous FDOM contribution (Huguet et al., 2009), a specific ultraviolet absorbance at 254 nm of DOC (SUVA$_{254}$), which is an indicator of aromatic DOM (Weishaar et al., 2003), and the ratio of spectral slopes ($S_r$) at 275–295 nm ($S_{275-295}$) and 350–400 nm ($S_{350-400}$) ranges, related to the molecular weight shift of DOM (Helms et al., 2008; Table 2).

**FDOM and CDOM Analyses**

Samples for the analysis of FDOM and CDOM were filtered through 0.2 µm polycarbonate filters in a custom-made filtration system pre-cleaned with 1 M HCl and Milli-Q water. Samples for FDOM analyses were stored at 4°C until analysis (within 1 month), while samples for CDOM absorption analyses were directly measured on a liquid waveguide capillary flow cell (WPI) according to the method of Kowalczyk et al. (2013). Excitation-emission matrix (EEM) spectra for analysis of FDOM were determined using a Hitachi F-7000 spectrofluorometer (Tedetti et al., 2011). FDOM components were identified by parallel factor analysis (PARAFAC), a statistical decomposition technique used to extract the most representative fluorescent components within EEMs dataset. To increase the validity of the PARAFAC analysis, data from the present experiment were pooled with 39 other samples obtained from the same waters, 3 months before and after the experiment. Indeed, to avoid systematic biases, remove signals unrelated to fluorescence and to validate the number of FDOM components, a large dataset was needed (Stedmon et al., 2003). For example, in the case where two of the components had very similar fluorescence characteristics and/or were highly correlated in their appearance among samples, the PARAFAC approach would have difficulties to discern between the two. We then determined several spectral indices: the absorption at 442 nm as a proxy of phytoplankton production and the absorption at 350 nm as an indicator of terrestrial inputs in the bay (Passow and Alldredge, 1995), a biological index (BIX) of recent autochthonous FDOM contribution (Huguet et al., 2009), a specific ultraviolet absorbance at 254 nm of DOC (SUVA$_{254}$), which is an indicator of aromatic DOM (Weishaar et al., 2003), and the ratio of spectral slopes ($S_r$) at 275–295 nm ($S_{275-295}$) and 350–400 nm ($S_{350-400}$) ranges, related to the molecular weight shift of DOM (Helms et al., 2008; Table 2).

**N$_2$ and Carbon Fixation Rates**

Seawater for the quantification of N$_2$ and carbon fixation rates was sampled using a Teflon pump connected to polyethylene tubing. Bulk N$_2$ and carbon fixation rates were measured at each transect station on triplicate 2.3 L transparent polycarbonate bottles filled to overflow and closed air-free with septum screwcaps. Once in the lab (within ~2h) the bottles were amended with stable isotopes to assay N$_2$ and carbon fixation simultaneously. Each bottle was amended with NaH$^{13}$CO$_3$ 99 atom% $^{13}$C to a final ~10 atom% enrichment, and 2.5 mL 98.9 atom% $^{15}$N$_2$ gas (both isotopes acquired from Cambridge Isotope Laboratories) injected through the septum using a gas-tight syringe (Montoya et al., 1996). Commercial $^{15}$N$_2$ gas stocks have been recently reported to be contaminated with varying amounts of other nitrogenated forms such as nitrous oxide and nitrite (Dabundo et al., 2014). The $^{15}$N$_2$ gas batch used here was
tested for contamination levels at J. Granger’s lab (University of Connecticut, CT, USA), which were found to be negligible and hence did not affect our N$_2$ fixation rates (Benavides et al., 2015).

After isotope additions, the incubation bottles were inverted 20 times, transferred to running seawater incubators covered with blue light screening reproducing the light intensity at the corresponding sampling depth, and incubated for 24 h. To test for non-cyanobacterial (non-photoautotrophic) N$_2$ fixation activity, a second set of incubation bottles was amended with DCMU to a final concentration of 50 µM (Rahav et al., 2015). These bottles were covered with black fabric bags to keep them in the dark at the same conditions as described above.

After incubation, the samples were filtered through precombusted GF/F filters (Whatman), and the filters were stored at -20°C until analysis. Background (time zero δ$^{15}$N and δ$^{13}$C) samples were taken at every station to allow an accurate calculation of N$_2$ and carbon fixation rates. The samples were analyzed by continuous-flow isotope ratio mass spectrometry (IRMS) using an Integra2 Analyser (Integra CN), calibrated with International Atomic Energy Agency standards IAEA 310-A for nitrogen (47.2%o) and IAEA 303-B for carbon (466%o). The minimum quantifiable N$_2$ fixation rate was 0.0041 nmol N L$^{-1}$ d$^{-1}$, as determined by error propagation analysis (Gradoville et al., 2017). We note that the use of the traditional $^{15}$N$_2$ “bubble method” (Montoya et al., 1996) instead of the “dissolved method” (Mohr et al., 2010) may have resulted in an underestimation of the actual rates (Grokopf et al., 2012), although the long incubation time (24 h) likely reduces this possibility.

**DOM Addition Experiments**

To test for the enhancement of N$_2$ fixation rates and/or nifH gene expression as a response to DOM enrichments, we sampled seawater in 25 extra 2.3 L polycarbonate bottles at Station 2. Five bottles were amended with a mix of carbohydrates —“DOC treatment”- (sodium pyruvate, sodium acetate and glucose), another five with a mix of amino acids —“DON treatment”- (alanine, leucine, and glutamic acid), and another five were amended with a mix of phosphonates —“DOP treatment”- (methylphosphonic acid, 2-aminomethylphosphonic acid and fructose 1,6-biphosphate). All DOM mixtures were prepared to be equimolar in carbon (all contained 4 µM carbon; Benavides et al., 2015). Five bottles were used as a control (no DOM additions), and another five bottles were used as a “time zero” (i.e., sacrificed at the start of the experiment to know the baseline conditions). All bottles were spiked with 2.5 mL $^{15}$N$_2$ and incubated for 24 h as described above. At the end of the incubation period, samples for NO$_3$.
PO₄³⁻ and DOC analyses were taken from three bottles of each treatment (i.e., DOC, DON, DOP) as described above. The fourth and fifth bottles from each treatment were kept for RNA analyses, respectively (see below).

**RESULTS**

**Hydrography, Nutrients and in Situ DOM**

The temperature of surface waters increased from 26.97°C to 28.23°C moving onshore from Station 1 toward Station 4, while salinity remained close to 35.6 at all stations (Table 1). The concentration of oxygen decreased onshore from 201 to 194 μmol kg⁻¹, while inorganic nutrient concentrations (NO₃ and PO₄) did not vary significantly along the transect, and neither did Chl a concentrations, with the exception of Station 3 where Chl a was slightly lower (Table 1). DOC concentrations remained between 80 and 90 μM along the transect, showing a peak of ~98 μM at Station 2 (Table 1).

CDOM absorption at 442 nm and 350 nm (a₄₄₂ and a₃₅₀) presented lower values at station 2 and 3 as compared to the values recorded at Stations 1 and 4 (Table 1) which indicated a decrease in CDOM concentrations. Moreover, the spectral slope S₂₇₅−₂₉₅ and the spectral ratio S₂₇₅ was high with values >1 and >0.0.3 nm⁻¹, respectively, which indicated the presence of photodegraded DOM with low molecular weight. In addition, the SUVA₂₅₄ indices were <4 (this reference value is indicative of DOM with low aromaticity) with an average value of 1.09 ± 0.44. Then CDOM at 442 nm and 350 nm is strongly photodegraded with a low aromaticity. Three fluorescent DOM compounds were identified by the PARAFAC model: terrestrial humic-like (compound 1), tyrosine-like (compound 2) and tryptophan-like (compound 3; Table 3). Humic-like compound signatures were higher closer to the coast (Station 4) and decreased to values around ~5 QSU at the other stations. This pattern is opposite to that of tyrosine-like compounds signals, which increased toward the barrier reef. Tryptophan-like compound concentrations did not follow any coast-open ocean pattern but presented the highest concentrations at Station 2. The BIX indices were homogenous and >1 (this value is indicative of an enrichment in DOM from bacterial origin) along the transect (Table 1). Overall, the optical analyses of DOM suggest that while river-derived refractory DOM was more abundant close to the coast, biological-derived labile DOM compounds were present homogeneously at all stations along the transect.

**N₂ and Carbon Fixation Activity and Diazotroph Abundance**

No clear spatial trends were observed in either N₂ or carbon fixation rates along the transect. N₂ fixation rates in samples not amended with DCMU ranged from 0.66 to 1.32 nmol N L⁻¹ d⁻¹, with the highest rates measured at Station 3 (Figure 2A). Carbon fixation rates in samples without DCMU ranged from 1.50 to 2.14 μg C L⁻¹ d⁻¹ (Figure 2B). Both N₂ and carbon fixation were effectively inhibited in DCMU-amended samples (>90%; Figures 2A,B).

Among the diazotroph phylotypes quantified by qPCR, *Trichodesmium* and gammaproteobacteria showed the highest abundance, ranging between 8.4 × 10⁴ and 2 × 10⁵, and 7.5 × 10² and 1.8 × 10⁶ nifH copies L⁻¹ respectively (Table 1). UCYN-A and UCYN-B phylotypes were one to two orders of magnitude lower, and undetectable at station 3 (Table 1).

**N₂ Fixation, Diazotroph Abundance and Optical Response to Model DOM Compound Additions**

At Station 2 we tested whether the addition of DOC, DON or DOP upregulated *nifH* expression and/or enhanced N₂ fixation rates. N₂ fixation rates were similar in the control, DOC and DON treatments (~0.8 nmol N L⁻¹ d⁻¹), yet they doubled to ~2 nmol N L⁻¹ d⁻¹ when DOP was added (Figure 3). However, due to the variability among replicates, the enhancement of either N₂ fixation or expression of *nifH* genes was not statistically significant in any treatment (Kruskal-Wallis, p > 0.05).

The DOC, DON, and DOP treatments yielded slightly higher abundances of UCYN-A *nifH* transcripts compared to transcripts measured in the controls, yet similar transcript...
numbers were with obtained in each of the three treatments (~5–6 × 10^5 nifH transcripts L^-1; Figure 3). UCYN-B did not show differential expression among treatments, while *Trichodesmium* increased by one order of magnitude when exposed to the DOC treatment (Figure 3). Despite being the most abundant ambient group as determined by DNA qPCR counts, gammaproteobacteria did not show detectable *nifH* gene expression in our DOM enrichment experiments (including control bottles). The prolonged incubation (24 h) in polycarbonate bottles could have affected *nifH* expression in this group (Moisander et al., 2014).

Regarding optical parameters, the DOC treatment resulted in a peak of tyrosine-like compounds concentrations (+51%) whereas no changes were recorded in humic-like and tryptophan-like compounds concentrations (Table 4). The effect of DON and DOP treatments on concentrations of fluorescent compounds are not shown as FDOM results were considered outliers in the validation of PARAFAC model.

**DISCUSSION**

**Spatial Variability of N2 Fixation Rates and Diazotrophic Phylotypes**

Gammaproteobacteria and *Trichodesmium* were the most abundant diazotrophs among the four phylotypes quantified (Table 1). The *nifH* copies of these two phylotypes were higher than in a previous study performed in the lagoon during the austral summer. The abundances, reported by Turk-Kubo et al. in *nifH* copies, ranged from 2.8 × 10^3 to 6.4 × 10^4 (UCYN-A), 3.5 × 10^2 to 2.7 × 10^3 (UCYN-B), 3.4 × 10^2 and 1.4 × 10^5 (*Trichodesmium*), and 4 × 10^4 to 1.9 × 10^5 (gammaproteobacteria) *nifH* copies L^-1. Typically, the *nifH* copy numbers reported here are similar to published numbers from Berthelot et al. (2017) who reported abundances up to 1.3 × 10^5 (UCYN-A), 7.5 × 10^4 (UCYN-B), 1.6 × 10^5 (*Trichodesmium*), and 2.7 × 10^4 (gammaproteobacteria) *nifH* copies L^-1 from the Solomon Sea.

DCMU blocks photosynthesis and thus the energy source required by autotrophic cyanobacterial diazotrophs to fix N2. Hence, measured N2 fixation rates from DCMU-amended samples should be representative of non-cyanobacterial N2 fixation. The lack of *nifH* expression in gammaproteobacteria in our DOM addition experiments (see below) questions whether this specific group was active. Nevertheless, other non-cyanobacterial diazotrophs such as *Bradyrhizobium* and *Mesorhizobium*, previously detected in the lagoon (Pfreundt et al., 2016), could be responsible for the measured N2 fixation rates in DCMU-amended bottles. N2 fixation rates in DCMU-amended incubations represented 4–7% of N2 fixation measured without amendments. The New Caledonian lagoon is dominated by autotrophic cyanobacterial diazotrophs (Rodier and Le Borgne, 2008; Turk-Kubo et al., 2015), where non-cyanobacterial phylotypes have been reported to represent <2% of the diazotrophic community (Turk-Kubo et al., 2015; Pfreundt et al., 2016). Hence, such a low contribution to total N2 fixation rates may not be surprising, but deserves further study in other parts of the lagoon and other times of the year. Still, N2 fixation rates in DCMU-amended bottles ranged between 0.04 and 0.11 nmol N L^-1 d^-1, which is in the range of N2 fixation rates measured in environments where non-cyanobacterial diazotrophs prevail (Moisander et al., 2017).

It has to be noted that putative non-cyanobacterial N2 fixation was higher at stations closer to the coast (Stations 3 and 4), which may be linked to the Dumbéa river inputs importing bioavailable CDOM at 350 nm and terrestrial humic-like DOM compounds into the bay. Spectral indices (Sr and S275–295) revealed low molecular weight and photodegraded terrestrial CDOM/DFOM at Station 4 (the most impacted by the river), which has been seen to enhance its availability.

**TABLE 3 | Excitation and Emission maxima (Ex/Em) of three CDOM fluorescent components validated by PARAFAC model and identification by comparison with the literature data.**

<table>
<thead>
<tr>
<th>Comp.</th>
<th>Ex(nm)</th>
<th>Em(nm)</th>
<th>Comp.</th>
<th>Ex(nm)</th>
<th>Em(nm)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>COMPOUND 1 Humic like</td>
<td>&lt;220</td>
<td>454(322)</td>
<td>Humic-like (A)</td>
<td>230–260</td>
<td>400–480</td>
<td>Coble, 1996</td>
</tr>
<tr>
<td>Terrestrial humic-like</td>
<td>&lt;260</td>
<td>454</td>
<td>Yamasita et al., 2010</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HUmC-like (A)</td>
<td>230</td>
<td>476</td>
<td>Tedetti et al., 2016</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COMPOUND 2 Tyrosine like</td>
<td>225(275)</td>
<td>304</td>
<td>C7</td>
<td>240(300)</td>
<td>338</td>
<td>Murphy et al., 2007</td>
</tr>
<tr>
<td>Component 8 Tyrosine-like (B)</td>
<td>275</td>
<td>304</td>
<td>Stedmon and Markager, 2005</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>225(275)</td>
<td>304</td>
<td>Tedetti et al., 2016</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COMPOUND 3 Tryptophane like</td>
<td>270(335)</td>
<td>340</td>
<td>Tryptophane (T1)</td>
<td>220–235</td>
<td>334–360</td>
<td>Coble, 1996</td>
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<tr>
<td>230(300)</td>
<td>352</td>
<td>Martias et al., 2018</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

**FDOM sources**

Allocotchnous: terrestrial inputs from river

Autochtonous: heterotrophic bacterial degradation of DOM

Autochtonous: coral production, heterotrophic bacterial degradation of DOM
to heterotrophic bacteria in oligotrophic waters (Su et al., 2017). At Station 1, DOM sources come from autochthonous phytoplankton production), which is also available for bacterial growth (based on $a_{442}$ values >0.1 as compared to Stations 2 and 3; Steinberg et al., 2004). The observed relationship between DOM lability proxies and non-cyanobacterial N$_2$ fixation activity in the present study support the findings of previous studies in the WTSP and the Mediterranean Sea (Benavides et al., 2015, 2016; Rahav et al., 2016). Nevertheless, a mechanistic understanding of how non-cyanobacterial diazotrophs benefit from different DOM molecules (e.g., metabolic pathways, affinity for different substrates) is still lacking (Bombar et al., 2016).

**Diazotrophic Responses to DOM Additions**

The influence of the three different DOM treatments applied (DOC, DON, and DOP) differentially influenced nifH gene expression of different phylotypes detected, with transcript abundance increasing similarly in UCYN-A for all amendments as compared to the controls (from $\sim$3 to $5\times10^3$ nifH transcripts L$^{-1}$), while no differences were observed between controls and amendments for UCYN-B transcripts (Figure 3). UCYN-B fixes carbon during the day and N$_2$ at night to avoid nitrogenase enzyme inhibition by photosynthetically derived oxygen. Thus, nifH expression in UCYN-B is typically higher at night (Church et al., 2005; Zehr et al., 2007). Hence, the absence of nifH expression in UCYN-B during the day (when our RNA samples were taken) is not surprising, even in the presence of external DOM sources. Similarly, Moisander et al. (2011) observed that nifH expression was not upregulated in UCYN-B when incubated with a mixture of glucose and mannitol. UCYN-A do not have a tricarboxylic acid pathway (Tripp et al., 2010), and thus require reduced organic carbon sources provided by their hosts (Thompson et al., 2012). It is therefore plausible that DOM additions in our experiments enhanced the diazotrophic activity of UCYN-A, similar to the experiments of Moisander et al. (2011), who demonstrated upregulated nifH gene expression in UCYN-A upon the addition of a glucose and mannitol mixture. In our experiments, however, a preference for DOC, DON or DOP was not observed and the nifH expression response was similar across treatments (Figure 3).

Active assimilation of both DOC and DON was measured in natural *Trichodesmium* colonies from the WTSP open ocean waters in similar experiments (Benavides et al., 2017). However,
in that previous study only the addition of DON resulted in an increase of filament-specific N₂ fixation rates (Benavides et al., 2017). The bulk water N₂ fixation measurements presented here do not allow us to discern the contribution of each diazotroph phylotype. Nevertheless, while bulk N₂ fixation rates were highest in the DOP treatment, nifH gene expression by *Trichodesmium* was highest in the DOC treatment (Figure 3). The peak of tyrosine-like compounds coinciding with the highest nifH expression of *Trichodesmium* observed in the DOC treatment may be related to the production of CDOM by this cyanobacterium (Steinberg et al., 2004). Surprisingly, DOM additions did not result in any nifH expression in the group of non-cyanobacterial diazotrophs tested in this study. In marine waters, maximum nifH expression of gammaproteobacteria typically reaches $\times 10^4$ nifH transcripts L⁻¹ (Luo et al., 2012; Langlois et al., 2015). For example, Church et al. (2005) reported an average of $4 \times 10^2$ nifH transcripts L⁻¹ for gammaproteobacteria in a diel cycle study at station ALOHA in the North Pacific Ocean, which is in the same order of magnitude as in a study in the WTSP by Moisander et al. (2014). Nevertheless, a study including samples from a wide range of oceanographic cruises across the North and South Atlantic and Pacific Oceans reported up to $\times 10^5$ gammaproteobacterial nifH transcripts per liter in sunlit surface waters (Langlois et al., 2015). In offshore waters of the WTSP, the addition of glucose and mannitol upregulated nifH expression in gammaproteobacteria (Moisander et al., 2011).

The lack of significant responses to DOM additions in this study could reflect sufficient DOM availability (based on SR$_{275-295}$ and SUVA$_{254}$ indexes) potentially provided by corals in the lagoon which are known for producing tryptophane-like compounds (available DOM sources for bacteria; Weishaar et al., 2003). However, the absence of detected gammaproteobacterial nifH transcripts, also in the control bottles, suggests that bottle confinement effects may have inhibited this group (Moisander et al., 2014). In addition to the diel changes in nifH gene expression between phylotypes (Church et al., 2005; Turk et al., 2011), it is possible that the nifH expression measured in our samples does not reflect the immediate response of diazotrophs to DOM additions. It must be noted that while RNA samples represent a snapshot of nifH expression at the time of sampling, N₂ fixation rates integrate the nitrogenase activity that has taken place over a period of 24 h. Such differences probably also account for the uncoupling observed between N₂ fixation rates and nifH gene expression.

**N₂ Fixation Rates Enhanced by DOP Additions**

Diazotrophs differ in how they access DOP. For example, *Crocosphaera watsonii* (here represented by the UCYN-B phylotype) obtains inorganic phosphorus using high affinity phosphate binding systems, and organic phosphorus sources such as phosphomonoesters utilizing alkaline phosphatases (Dyhrman and Haley, 2006). In addition to these phosphorus sources, *Trichodesmium* is also able to grow on phosphonates and use phosphate (Dyhrman et al., 2006; Polivyiu et al., 2015). It is intriguing why the nifH gene expression of *Trichodesmium* or of any of the other diazotroph phylotypes tested- was not enhanced when DOP was enriched, and deserves further study. However, an enhancement of N₂ fixation activity was observed in the DOP treatment (Figure 3), and could be due to inorganic phosphorus limitation alleviation. Unfortunately, we do not have alkaline phosphatase activity data to confirm whether the DOP added to our incubations was ultimately transformed to inorganic phosphorus compounds, but PO$_4^{3-}$ concentrations nearly doubled in DOP-amended samples with respect to the control (data not shown), suggesting that active alkaline phosphatases were functioning. The fact that N₂ fixation rates enhanced by DOP additions was not reflected in a significant enhancement of nifH expression of any of the diazotroph phylotypes tested suggests that the N₂ fixation activity measured may be provided by a diazotroph not accounted for in our nifH gene q-RT-PCR assays. Alternatively, transcription may be interrupted when the number of transcripts is enough to activate the nitrogenase protein, resulting in an uncoupling between expression and nitrogenase activity, as previously reported in *Trichodesmium* cultures (Levitan et al., 2010).

**CONCLUSIONS**

Although cyanobacterial diazotrophs predominate in the New Caledonian lagoon, here we demonstrate that non-cyanobacterial diazotrophs contribute ~10% of total N₂ fixation rates. Comparable to previous findings (see Moisander et al., 2017) gammaproteobacteria were the most abundant based on DNA-derived qPCR nifH gene counts, yet we did not detect any nifH gene expression for this group throughout the experiments. This lack of detectable expression together with the low N₂ fixation rates measured in DCMU-treated samples questions the contribution of non-cyanobacterial diazotrophs to biologically fixed nitrogen inputs in the lagoon. Yet, the response of the

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**TABLE 4 | NO₃, PO₄³⁻, DOC concentrations and values of FDOM-related ratios for the different DOM additions (DOC, DON, and DOP treatments) performed at Station 2.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NO₃ (µM)</th>
<th>PO₄³⁻ (µM)</th>
<th>DOC (µM)</th>
<th>Humic-like (QSU)</th>
<th>Tyrosine-like (QSU)</th>
<th>Tryptophan-like (QSU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.1 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>90.6 ± 15.5</td>
<td>6.54</td>
<td>44.79</td>
<td>11.71</td>
</tr>
<tr>
<td>DOC</td>
<td>0.2 ± 0.1</td>
<td>0.1 ± 0.0</td>
<td>98.6 ± 3.3</td>
<td>6.57</td>
<td>67.45</td>
<td>11.53</td>
</tr>
<tr>
<td>DON</td>
<td>0.2 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>104.1 ± 30.6</td>
<td>n/d</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>DOP</td>
<td>0.2 ± 0.0</td>
<td>0.3 ± 0.0</td>
<td>107.3 ± 7.5</td>
<td>n/d</td>
<td>n/d</td>
<td>n/d</td>
</tr>
</tbody>
</table>

*Standard deviation values (± SD) are provided where available.*
cyanobacterial diazotrophic phenotypes to the amended DOM treatments suggests that mixotrophic nutrition may be important in these organisms and should be investigated further.

**AUTHOR CONTRIBUTIONS**

Conceived and designed the experiments: MB, CM, SB; Performed the experiments: MB, CM; Analyzed the data: MB, CM, HE; Contributed reagents, materials, analysis tools: CM, SB, HE, CD, IB-F; Wrote the paper: MB, CM, CD, HE, IB-F, SB.

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**REFERENCES**


**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer AF-C and handling Editor declared their shared affiliation.