The Trichodesmium microbiome can modulate host N$_2$ fixation

Kyle R. Frischkorn, Mónica Rouco, Benjamin A. S. Van Mooy, Sonya T. Dyhrman

1Department of Earth and Environmental Sciences and the Lamont-Doherty Earth Observatory, Columbia University, Palisades, New York; 2Department of Marine Chemistry and Geochemistry, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts

Scientific Significance Statement

Trichodesmium is a marine N$_2$ fixing cyanobacterium that fuels primary productivity in the nutrient-poor oligotrophic ocean by supplying biologically available nitrogen. Although colonies of Trichodesmium ubiquitously co-occur with a microbiome of heterotrophic bacteria, the influence these organisms have on Trichodesmium N$_2$ fixation is uncertain. Here, we show that the rate of Trichodesmium N$_2$ fixation in samples from the western North Atlantic can be significantly altered by manipulating the microbiome with signaling molecules. These observations indicate that microbiome dynamics are an important and previously overlooked control on Trichodesmium N$_2$ fixation in the global ocean.

Abstract

Trichodesmium is a marine, diazotrophic cyanobacterium that plays a central role in the biogeochemical cycling of carbon and nitrogen. Colonies ubiquitously co-occur with a diverse microbiome of heterotrophic bacteria. We show that manipulation of the microbiome with quorum sensing acyl homoserine lactones (AHLs) significantly modulated rates of N$_2$ fixation by Trichodesmium collected from the western North Atlantic, with positive and negative effects of varied magnitude. Changes in Trichodesmium N$_2$ fixation did not correlate with changes in microbiome composition or geochemistry. With AHL addition, a subset of the significantly differentially expressed genes was related to known quorum sensing responses in model bacteria. However, there was little overlap in specific microbiome transcriptional responses to AHL addition between stations. Overall, these host-microbiome interactions reflect a complex interplay of biotic and environmental factors that together form an overlooked mechanism modulating Trichodesmium N$_2$ fixation.

Trichodesmium is a keystone member of marine environments because of its ability to provide fixed N$_2$ that fuels primary productivity in otherwise nutrient poor regions (Capone et al. 1997). Some estimates predict Trichodesmium accounts for approximately half of the total oceanic fixed N$_2$ (Bergman et al. 2013). In oligotrophic regions, Trichodesmium N$_2$ fixation is strongly affected by the availability of nutrients in the water column (Sohm et al. 2011). With the high-iron quotas

*Correspondence: sdyhrman@ldeo.columbia.edu

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Data Availability Statement: Data is available in the NCBI SRA under accession code PRJNA450995, the BCO-DMO data repository at https://www.bco-dmo.org/dataset-deployment/716824, and FigShare at https://figshare.com/articles/Trichodesmium_NATL_AHL_experiment_OG_counts_and_annotations/7051589.

Additional Supporting Information may be found in the online version of this article.

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associated with N₂ fixation and photosynthesis, iron limitation
is the canonical constraint on diazotrophy in *Trichodesm-i um* (Berman-Frank et al. 2001). In the oligotrophic western
North Atlantic however, high-iron concentrations relative to
phosphorus lead to phosphorus depletion, and *Trichodesmium*
distribution and N₂ fixation is thought to be more strongly
influenced by phosphorus availability, relative to other sys-
tems (Sañudo-Wilhelmy et al. 2001; Moore et al. 2013; Rouco
et al. 2014, 2018). Despite the fact that resource controls on
*Trichodesmium* eco-physiology are well established, using geo-
chemistry to predict and model the distribution and activities
of this organism remains challenging (Capone et al. 2005;
McGillivray 2014; Snow et al. 2015). Recent studies have
suggested that some of the challenges associated with model-
ing *Trichodesmium* dynamics are in part due to the fact that its
physiology is tightly linked to that of its microbiome
(Frischkorn et al. 2017; Lee et al. 2017).

*Trichodesmium* ubiquitously co-occurs with a microbiome
of epibiotic microorganisms (Paerl et al. 1989; Hewson
et al. 2009; Hmelo et al. 2012; Rouco et al. 2016; Frischkorn
et al. 2017; Lee et al. 2017), yet the role of this microbiome in
modulating *Trichodesmium* physiological ecology is still poorly
understood. These epibions are tightly associated with colo-
nyes of *Trichodesmium* filaments, and together make up a con-
served community that is unique from planktonic microbes
in the surrounding water column (Hmelo et al. 2012; Rouco
et al. 2016). In other systems, such communities of bacteria
are known to regulate physiological activities on a popula-
tion-wide scale through cell–cell signaling called quorum
sensing (Miller and Bassler 2001). Quorum sensing mole-
cules in the acyl homoserine lactone (AHL) family modulate a
range of activities in bacteria by altering gene expression and
subsequently behavior (Waters and Bassler 2005), thus alter-
ing physiology through a mechanism that is decoupled from
population-wide scale through cell–cell signaling called quorum
sensing. Previous

Field incubations showed microbiome activity was specifically altered by the three AHL compounds used here and not by N-3-oxoocatonyl
homoserine lactone (Van Mooy et al. 2012). Selective manipu-
lation of the microbiome was possible because natural, non-
deuterated forms of these molecules have been observed in
*Trichodesmium* colonies, but *Trichodesmium* itself cannot
produce or respond to AHAs (Van Mooy et al. 2012). We
incubated the control and +AHL bottles for 4 h in on-deck
flow-through incubators shaded with blue film to mimic
situ conditions. A 4h incubation was chosen as it was short
enough to capture +AHL induced transcriptional changes in
the microbiome, and long enough to have induced N₂
fixation changes in *Trichodesmium*. Biomass limitations and
destructive sampling precluded time series analysis. The
experiments were designed to selectively alter microbiome

Methods

We collected *Trichodesmium* colonies from surface water
along a cruise transect in the western North Atlantic (Fig. 1a)
aboard the *R/V Atlantic Explorer* (AE1409) during May 2014, as
previously described (Frischkorn et al. 2017). We focused on
field studies with freshly isolated colonies because the clade of
the cultured strain (*Trichodesmium erythraeum* IMS101) is not
dominant in the environment (Rouco et al. 2014), and it
likely has a derived microbiome. Briefly, we conducted six
hulls of a net tow (mesh size of 130 μm) at ~ 07:00 h in the
upper 20 m to collect samples for experiments. We isolated
*Trichodesmium* colonies from the net tow and washed them
times with fresh 0.2 μm sterile-filtered local surface sea-
water, a method that prevents contamination by planktonic
microbe carryover and has reproducibly yielded the stable
*Trichodesmium* microbiome (Rouco et al. 2016; Frischkorn
et al. 2017, 2018). After washing, we transferred approxi-
mately 30 cleaned colonies of similar sizes and morphologies
into acid-clean, polycarbonate bottles filled with 30 mL of
sterile-filtered seawater. Efforts were made to place colonies in
bottles to mimic colony morphology distribution found in
net tow samples. To minimize handling effects, time from ini-
tial sampling to incubation was less than 15 min. We spiked
the six experimental bottles (+AHL) with a cocktail of three
di-deuterated AHs (N-(decanoyl)homoserinelactone, N-(dode-
canoyl)homoserinelactone, and N-(tetradecanoyl)homoserine-
lacton) in dimethylsulfoxide (DMSO) to a final concentration of
500 nmol L⁻¹, and the six control bottles with only DMSO.

*Trichodesmium* consortia members are known to produce and
metabolize dimethylsulfide products (Lee et al. 2017), and
additional negative controls may be warranted for future work
focused on quorum sensing. Previous field incubations
showed microbiome activity was specifically altered by the three AHL compounds used here and not by N-3-oxoocatonyl
homoserine lactone (Van Mooy et al. 2012). Selective manipu-
lation of the microbiome was possible because natural, non-
deuterated forms of these molecules have been observed in
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enough to capture +AHL induced transcriptional changes in
the microbiome, and long enough to have induced N₂
fixation changes in *Trichodesmium*. Biomass limitations and
destructive sampling precluded time series analysis. The
experiments were designed to selectively alter microbiome
activity, rather than isolate quorum sensing pathways, and we discuss details regarding the experimental approach and operational negative controls in prior studies (Van Mooy et al. 2012; Krupke et al. 2016).

After 4 h of incubation, we assayed three of the bottles in each condition for N$_2$ fixation rate using the acetylene reduction technique as previously described (Capone 1993). Briefly, approximately 30 *Trichodesmium* colonies were placed in each 60 mL polycarbonate bottle containing 30 mL of filtered seawater as described above. We injected a 1 mL aliquot of acetylene into the bottle through a septum cap, gently inverted the bottle, and incubated in an on-deck incubator as previously described. We then analyzed bottle headspace for ethylene at time-points approximately every 30 min by gas chromatography (Capone 1993). Triplicate daily analysis of a 17 ppm ethylene standard was used to calibrate the gas chromatograph. The average daily coefficient of variation for analysis of the ethylene headspace standard was 4.9%. Linear regressions of headspace ethylene concentration vs. time and appropriate Bunsen coefficients were used to determine the total ethylene production rate (Breitbarth et al. 2004). The limit of detection for ethylene production, defined as the accumulation of ethylene through the course of the incubation to a level three times greater than detected at the first time-point, was 8.1 pmol colony$^{-1}$ h$^{-1}$. The estimated N$_2$ fixation rate was calculated by dividing the total ethylene production rate by 4 (Capone 1993). Average coefficient of variation of N$_2$ fixation rates in replicate incubations was 10.2%.

Chauvenet’s criterion (95% confidence interval) was used to remove occasional outlying rates among replicate incubations. Student’s t-tests were used to identify significant differences in N$_2$ fixation rates between replicate control incubations and replicate incubations receiving an AHL amendment.

For gene expression analysis, we filtered each of the remaining three bottles of each treatment onto 5 μm pore size polycarbonate filters after the 4 h incubation period and immediately stored them in liquid nitrogen until sample processing. Three bottles set up as controls as previously described were used to measure in situ N$_2$ fixation rates at stations where metatranscriptome sequencing experiments were not carried out. Total dissolved phosphorus (TDP) was determined on 0.2 μm filtrates of surface water (~ 5 m depth) samples collected via CTD into acid-clean polycarbonate bottles. Samples were processed at the SOEST Laboratory for Analytical Biogeochemistry at the University of Hawaii, according to facility protocols.

We extracted prokaryotic RNA from triplicate control and +AHL samples, pooling together triplicate samples, sequencing 60 million paired end reads, and trimming as previously described (Frischkorn et al. 2018). To obtain read counts for each sample, we mapped trimmed forward and reverse reads to metagenome assemblies from the same sampling locations that were previously characterized and clustered into orthologous groups (OGs) (Frischkorn et al. 2017). OGs were annotated (Frischkorn et al. 2017) and sorted into metabolic functional groups of both known quorum sensing responses and other functions (available as a supplemental dataset hosted on FigShare at 10.6084/m9.figshare.7051589). We carried out mapping using RSEM with the paired-end and
Table 1. Number of total OGs from individual microbiome members that had significant differential expression (DE) after +AHL amendment. Counts were tabulated for each gene in a microbiome genome bin that belongs to an OG that was found to be significantly higher or lower in response to +AHL amendment. Bin number and total OGs reflect findings in Frischkorn et al. (2017). OGs total refers to the total number of OGs found in each genome bin.

| Bin | Identity       | OGs total | Sta. 2: Contribution to DE OGs | Sta. 10: Contribution to DE OGs | Sta. 17: Contribution to DE OGs | Avg. % (St. Dev) of DE OGs per genome |
|-----|----------------|-----------|-------------------------------|-------------------------------|-------------------------------|--------------------------------------|
| 4   | Bacteroidetes  | 8354      | 121                           | 61                            | 247                           | 1.71 (1.1)                           |
| 5   | Rhodospirillales | 2787      | 26                            | 32                            | 48                            | 1.27 (0.4)                           |
| 6   | Bacteroidetes  | 6016      | 41                            | 26                            | 155                           | 1.23 (1.2)                           |
| 7   | Gammaproteobacteria | 4195   | 105                           | 55                            | 163                           | 2.57 (1.3)                           |
| 8   | Rhodobacterales | 2693      | 75                            | 15                            | 102                           | 2.38 (1.7)                           |
| 10  | Rhodospirillales | 4206      | 53                            | 34                            | 106                           | 1.53 (0.9)                           |
| 11  | Rhodospirillales | 1766      | 51                            | 13                            | 60                            | 2.34 (1.4)                           |
| 12  | Rhodospirillales | 1884      | 29                            | 24                            | 34                            | 1.54 (0.3)                           |

St. Dev, standard deviation.

Bowtie2 parameters (Li and Dewey 2011) and tabulated counts across only OGs from the eight core epibiont genome bins (Table 1, supplemental dataset 10.6084/m9.fgshare.7051589). We determined significant changes in OG expression between control and +AHL samples using a stringent empirical Bayes approach called “analysis of sequence counts (ASC)” (Wu et al. 2010). This approach evaluates the posterior probability associated with a given fold change across the pooled triplicates, and performs similarly, but conservatively, on replicated and unreplicated sample datasets (Wu et al. 2010). OGs were considered significantly higher or lower if they had a 95% or higher posterior probability of a fold change greater than 2 between treatment and control. Taxonomic relative abundance estimates for metagenome samples were previously calculated (Frischkorn et al. 2017).

**Results and discussion**

**Biological interactions are a driver of *Trichodesmium* N₂ fixation**

*Trichodesmium* in situ N₂ fixation rates increased from north to south, with stations north of 20° latitude having significantly lower rates of N₂ fixation than those in the south (p = 0.02, one-way ANOVA; Fig. 1a; Supporting Information Table S1). Phosphorus is known to be a limiting nutrient for N₂ fixation in this region (Sáruñdo-Wilhelmy et al. 2001; Sohm et al. 2011), and surface TDP concentration in the stations north of 20° latitude differed significantly from those collected to the south (p < 0.03, one-way ANOVA; Fig. 1a; Supporting Information Table S1). Although the changes in N₂ fixation and TDP between the northern and southern stations are consistent with phosphorus being a strong driver of N₂ fixation across the transect, the in situ rates of N₂ fixation were not significantly correlated with TDP (R² < 0.319, p = 0.186, one-way ANOVA; Fig. 1a). This suggests that factors other than phosphorus concentration might also influence *Trichodesmium* N₂ fixation.

At all stations where we performed experiments (Fig. 1a), N₂ fixation was significantly changed in response to microbiome manipulation with the +AHL amendment, with significant decreases in rate at Sta. 2 and 10 (p = 0.04 and 0.03, respectively), and a significant increase at Sta. 17 (p = 0.01) (Fig. 1b). Although *Trichodesmium* has co-occurred with a N₂ fixing epibiont in the North Pacific Ocean (Momper et al. 2014; Gradoville et al. 2017), herein *Trichodesmium* colonies did not show evidence of non-*Trichodesmium* nif genes (Frischkorn et al. 2017), indicating that *Trichodesmium* was the only diazotrophic organism within these samples. The magnitude and direction of microbiome-induced changes in host N₂ fixation ranged from significantly decreased at the northernmost station to significantly increased at the southernmost station, despite similarities in TDP between proximal stations (Fig. 1a,c). This suggests that biotic interactions within colonies can act independently of geochemistry to influence *Trichodesmium* physiology.

Extrapolating the range of changes we observed in +AHL treatments to in situ N₂ fixation rates, we illustrate the potential influence of the microbiome on host physiology (Fig. 2). This theoretical N₂ fixation range was determined from the maximum (+20%) and minimum (−41%) percent change after +AHL amendment to contextualize the potential for biological interactions to alter observed N₂ fixation rates (Fig. 2). Although this visualization should be interpreted with caution, the hypothetical 61% range of variation reflects a scenario where biological interactions drive subsequent N₂ fixation higher or lower than would otherwise be expected, even given noted uncertainty in these measurements (coefficient of variation = 10.2%). Such changes are modulated on
The microbiome can respond to AHLs, which is consistent with the fact that all genome bins were found to possess putative luxR genes from canonical quorum sensing operons (Frischkorn et al. 2017). The Gammaproteobacterium (bin 7) contributed to 60% of the significantly responsive metabolic functional groups across all three stations tested (Supporting Information Fig. S1). Notably, similar Gammaproteobacteria are ubiquitously found in association with colonies across multiple environments and in culture (Hmelo et al. 2012; Rouco et al. 2016; Frischkorn et al. 2017; Lee et al. 2017) and biologically relevant concentrations of AHLs have been found in Trichodesmium colonies (Van Mooy et al. 2012). Taken together, it is likely that the microbiome could ubiquitously influence Trichodesmium N₂ fixation via quorum sensing pathways.

Little is known about the quorum sensing and quenching pathways in the Trichodesmium microbiome, but in model systems these pathways are complex, operating in circuits that can influence each other in distinct ways and lead to a cascade of unique transcriptional responses and resulting shifts in activity (e.g., Wagner et al. 2004; Schuster and Greenberg 2006). Amendment with AHLs induced significant changes in OG expression at each station (Fig. 3), and some of these OGs encoded metabolic functions that were the same as quorum sensing-induced shifts in model organisms such as Pseudomonas aeruginosa (Wagner et al. 2003, 2004; Schuster and Greenberg 2006). OGs in the energy metabolism, chemotaxis, motility and attachment, and nitrogen and amino acid metabolism functional groups were significantly differentially expressed across all three stations (Fig. 3). Similar to model organisms (Wagner et al. 2003), changes in these functional groups highlight how basic metabolic processes and lifestyle can be modulated in response to signaling molecules like AHLs. Transposon-related OGs were also differentially expressed at all stations (Fig. 3). AHLs are known to stimulate gene transfer agents and increase transposon mobility (Schaefer et al. 2002; Auchtung et al. 2005), and many quorum sensing genes are adjacent to transposons or encoded within them (Thomson et al. 2000; Wei et al. 2006). Although there were similarities in the metabolic functional groupings of differentially expressed OGs (Fig. 3), at the gene family level there was little overlap between specific OGs that contributed to those categories across the three stations (Supporting Information Fig. S2). The five OGs making up the conserved AHL response were all annotated as putative, uncharacterized proteins (Supporting Information Fig. S2). Sampling a time course in AHL addition experiments would help identify more quorum sensing pathways and any conserved responses that occurred earlier than the 4 h time point. In summary, microbiome transcriptional responses to AHLs varied inconsistently between stations and additional experiments are required to evaluate how different microbiome activities drive the observed variation in Trichodesmium N₂ fixation.

Previous work found that community composition of the microbiome varied significantly between northern stations (e.g., Sta. 2) and the southern stations (e.g., Sta. 10 and 17),
but that the \textit{Trichodesmium} host did not (Frischkorn et al. 2017). However, in the southern stations where community composition and geochemistry were similar, transcriptional responses in the microbiome were dissimilar and yielded different effects on N$_2$ fixation. For example, although relative abundances of the core microbiome members between Sta. 10 and 17 were not significantly different (Frischkorn et al. 2017), and TDP was similar, variable epibiont transcription forced by quorum sensing elicited different \textit{Trichodesmium} N$_2$ fixation responses. In sum, N$_2$ fixation responses are not predictable from microbiome community composition alone. Variability in AHL responses in model systems can vary as much as 100-fold due to nutrient status, oxygen availability, and whether cultures were planktonic or growing as a biofilm (Schuster and Greenberg 2006; Duan and Surette 2007). Furthermore, the transcriptional regulators that are activated by quorum sensing molecules do not exist in isolation, but rather interact with a web of regulators and quorum quenching molecules that affect physiology on a genome-wide scale—a finding that has been used as an explanation for the rapid adaptability of bacteria to fluctuating environments (Schuster and Greenberg 2006). The concentrations of the AHLs experienced by the epibionts could also affect the direction, magnitude, and characteristics of gene expression, as hydrolytic enzyme activity in marine particulate matter is strongly affected by the concentration of AHLs added (Krupke et al. 2016). Similarly, in \textit{P. aeruginosa}, different concentrations of the same quorum sensing signaling molecule result in different responses that subsequently elicit opposite host physiological responses (Williams and Cámara 2009). In the \textit{Trichodesmium} holobiont, the effects of the microbiome on N$_2$ fixation likely reflect a complex interplay of environment, community composition, chemical signaling, and metabolic functional response, and a more mechanistic understanding of activities in the microbiome is needed to model how biological interactions modulate \textit{Trichodesmium} N$_2$ fixation.

\section*{Conclusions}

Here, we show that selective manipulation of microbiome activities can alter the N$_2$ fixation rate of the \textit{Trichodesmium} host over short time scales, expanding the suite of factors that are known drivers of marine N$_2$ fixation. If the observed interplay between host and microbiome holds true across the full range of oligotrophic environments \textit{Trichodesmium} inhabits, then these interactions are likely an overlooked factor that influences \textit{Trichodesmium} N$_2$ fixation, and future ecological studies of \textit{Trichodesmium} should take into account the activities of the microbiome.

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