The rate and fate of N$_2$ and C fixation by marine diatom-diazotroph symbioses

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N$_2$ fixation constitutes an important new nitrogen source in the open sea. One group of filamentous N$_2$ fixing cyanobacteria (Richelia intracellularis, hereafter Richelia) form symbiosis with a few genera of diatoms. High rates of N$_2$ fixation and carbon (C) fixation have been measured in the presence of diatom-Richelia symbioses. However, it is unknown how partners coordinate C fixation and how the symbiont sustains high rates of N$_2$ fixation. Here, both the N$_2$ and C fixation in wild diatom-Richelia populations are reported. Inhibitor experiments designed to inhibit host photosynthesis, resulted in lower estimated growth and depressed C and N$_2$ fixation, suggesting that despite the symbionts ability to fix their own C, they must still rely on their respective hosts for C. Single cell analysis indicated that up to 22% of assimilated C in the symbiont is derived from the host, whereas 78–91% of the host N is supplied from their symbionts. A size-dependent relationship is identified where larger cells have higher N$_2$ and C fixation, and only N$_2$ fixation was light dependent. Using the single cell measures, the N-rich phycosphere surrounding these symbioses was estimated and contributes directly and rapidly to the surface ocean rather than the mesopelagic, even at high estimated sinking velocities (<10 m d$^{-1}$). Several eco-physiological parameters necessary for incorporating symbiotic N$_2$ fixing populations into larger basin scale biogeochemical models (i.e., N and C cycles) are provided.

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INTRODUCTION

In large expanses of the sunlit open ocean, concentrations of bioavailable nitrogen (N) are low to below analytical detection. In these regions the primary source of new N comes from biological N$_2$ fixation (BNF) [1], or the reduction of di-nitrogen (N$_2$) to ammonia [2]. Historically, the larger colonial filamentous cyanobacteria, Trichodesmium spp., has been considered the dominant N$_2$ fixer (diazotroph) [3], whereas recent work has highlighted the equally significant role of smaller unicellular N$_2$ fixing cyanobacteria, i.e., Candidatus Atelocyanobacterium thalassa (UCYN-A1 and UCYN-A2), which live symbiotically with a eukaryotic alga [4–6]. Seldom is the co-occurring heterocystous symbiotic cyanobacteria Richelia intracellularis (hereafter Richelia) and Calothrix rhizosoleniae (hereafter Calothrix) studied or reported in a similar context [7]. Heterocysts are specialized thick-walled cells that are differentiated to isolate the oxygen (O$_2$) sensitive nitrogenase enzyme for N$_2$ fixation from O$_2$ produced in the neighboring photosynthetic vegetative cells [8, 9] (Supplementary Fig. 1). Richelia and Calothrix are the few heterocystous cyanobacteria commonly found in the oligotrophic oceans, where they form highly specific relationships with eukaryotic diatoms: Hemiaulus spp., Rhizosolenia spp. and Chaetoceros compressus, respectively [10–12].

Cyanobacteria, especially heterocystous types, have a high propensity for symbioses, i.e., the diverse group of land plants (liverworts, hornworts, cycads, ferns, angiosperms, and cyanolichens) that enter partnerships with cyanobacteria [13]. Unlike the marine diatoms, these terrestrial systems are better characterized. A clear distinction in the land plant-cyanobacterial symbioses is the investment by the plant for specialized chambers and cavities to hold their symbionts. In most, the cavities are darkened and therefore the symbiotic cyanobacteria live heterotrophically by reduced C from their respective hosts [14–16]. In many terrestrial examples, the heterocystous cyanobacteria are also intercalary types and differentiate multiple heterocysts along the filament [17]. The heterocysts of Richelia and Calothrix symbions are single and terminal, and therefore filament length increases/decreases by changing the number of vegetative cells. After establishment in land plants, symbiotic N$_2$ fixation rates increase [18], whereas both C and ammonia assimilation by the symbionts decrease, as does the symbiont growth rate [15–16; 18]. Thus, in the terrestrial examples, both partners adapt to the partnership, and in the case of the symbiont, multiple metabolic changes provide direct benefit to their respective hosts.

Draft genomes of the Richelia symbiont strains from three diatoms: H. hauckii (RintHH01), H. membranaceus (RintHM01) and R. clevi (RintRC01), are available [19, 20]. The Calothrix genome (CalSC01) was also sequenced after its isolation from a chain of Chaetoceros compressus diatoms (19; 21). Collectively, the diatom-heterocystous cyanobacterial symbioses are a unique system to
study because the symbiont cellular location varies from internal, to "partial", to fully external (Supplementary Fig. 1). Moreover, the symbiont cellular location is also related to the symbiont genome size, content, and age of the partnerships. For example, the more internal Richelia symbions have smaller draft genomes (RintHM01, 2.21 Mbp; RintHH01, 3.24 Mbp) and are more ancient, while external symbionts possess larger genomes (RintRC01, 5.48 Mbp; CaliSC01, 5.97 Mbp) and more recently emerged [19–22]. The RintHM01 genome was poorly sequenced and has not been fully analyzed [19], and genomes RintHH01 and RintRC01 are shown to lack several N assimilatory pathways common to cyanobacteria, including nitrogen fixers (e.g., ammonium), and N reductases (e.g., nitrate, urease) [19, 20]. Thus, Richelia’s N assimilation is largely restricted to the energy-demanding N₂ fixation process, whereas the Calothrix symbiont draft genome (CaliSC01) is more similar in size and content to other free-living heterocystous cyanobacteria [19, 20, 23]. The diversity of the host diatoms is limited to a few phylogenetic markers and two field transcriptomic studies [22, 24, 25]. High rates of N₂ and C fixation have been measured in bulk water field incubations when Richelia symbioses are present [26–29]. Both partners are photosynthetic organisms that possess ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) for carbon (C) fixation [19, 20], yet which partner, if not both, fixes C is unknown. Recently, Nieves-Morión et al. [23] reported the presence and absence of a number of membrane transporters potentially required for metabolic exchanges between the partners. An interesting absence in the RintHH01 and RintRC01 genomes are ABC bicarbonate (HCO₃⁻; C) transporters common to other cyanobacteria for acquiring inorganic C for CO₂ fixation [23]. Alternatively, the genomes of the Richelia symbionts, encode genes for the NDH-1 type of CO₂ uptake and genes similar to the SulP-family HCO₃⁻ transporter BicA [23]. Interestingly, the bicA in RintHH01 is fragmented into four consecutive sequences reflecting incomplete sequencing or gene erosion, whereas in RintRC01 it has remained a full sequence [23]. Hence an open question in the diatom-Richelia symbioses is which partner is fixing C and whether the partners compete for CO₂. In heterocystous cyanobacteria, photosynthesis results in reduced C in the form of sugars which functions as the primary energy source for N₂ fixation. Recently, a hypothetical flux model based on four metabolic processes (photosynthesis, N₂ fixation, biosynthesis, respiration) was applied to the Hemiaulus-Richelia symbiosis in order to predict which partner supports another one another [30]. The model predicted that 25% of the host diatom C fixation was provided to the symbiont to drive N₂ fixation, and the symbiont supplied a majority (up to 82%) of its fixed N to the host Hemiaulus [30]. Hence determining the rate and role of C fixation is important for sustaining the symbioses itself, but also impacts to what extent these symbioses contribute to primary and new production. Despite their ubiquitous distribution and biogeochemical significance, there are few reported N₂ and C fixation rate measurements for these symbiotic diatoms [26–29, 31]. In fact, with the exception of a few single cell N₂ fixation rates reported in Foster et al. [32], all measurements are from bulk and/or cell concentrates (i.e., plankton tows), and therefore include the activities of co-occurring populations or other N₂-fixers such as Trichodesmium spp. and unicellular diazotrophs. In the recent work presented by Pyle et al. [33], rates of N₂ fixation were estimated on non-anoxic enrichment H. hauckii-Richelia cultures using indirect acetylene reduction assays. Here, using stable isotope labeling experiments with and without eukaryotic inhibitors and secondary ion mass spectrometry (SIMS) measures, we report a high number of single cell rate measurements of both C and N₂ fixation for Hemiaulus- and Rhizosolenia-Richelia symbioses. These represent the largest number of field measurements for these important populations. Because our dataset is from wild populations, we provide several eco-physiological parameters necessary for modeling metabolite exchanges between two partners which have not been well-studied, modeled or understood. These parameters are additionally directly to larger scale models that attempt to predict basin scale N₂ and C fixation.

MATERIALS AND METHODS

Experiments and sampling

Two expeditions to the western tropical North Atlantic (WTNA) were made between 23 May–21 June 2010 and 9 September–6 October 2011 for sampling and performing the stable isotope incubation experiments described herein (Supplementary Fig. 2; Supplementary Table 1). The stations for incubation experiments were selected based on an initial screening of the surface plankton for the presence of symbiotic diatoms by microscopy (Supplementary Materials). In 2010, stations 2, 4, 5, 9, and 10 had densities of the symbiotic diatoms (>100 cells/filter) deemed suitable for the incubation experiments, while in 2011 cell densities were lower. In 2011, the pre-dawn cast was not always feasible due to time constraints, and only stations 10, 24, and 29 were sampled for incubation experiments for bulk and single cell activity measurements.

Symbiotic diatom cell counts

Symbiotic cells for microscopy counts were collected directly from the conductivity, temperature, depth (CTD) rosette at the same or similar depths as water collected for the incubation experiments (Supplementary Fig. 2; Supplementary Table 1). Briefly, aliquots of the Niskin bottles were gravity filtered onto a 47 mm diameter Poretics membrane 10.0 µm pore size filter held within a Swinnex filter holder (Millipore, Billerica, MA USA). Gravity filtration varied from 1 to 2 h. If the filter clogged after 2 h, the remaining volume in the Niskin was measured using a graduated cylinder (1 L) and the volume filtered was corrected. The filter was mounted onto an oversized glass slide (75 mm × 50 mm × 1 mm), examined and symbiotic diatoms were identified using blue (450–90 nm) and green (510–60 nm) filter sets on 400 X. The diatoms with Richelia were identified as H. hauckii, H. membranae, and R. clevei, based on ultrastructure and size. When symbiotic cells were in high densities (2010 cruise), several smaller grid areas (62.5 µm²) of the filter were scanned; otherwise, the whole filter was viewed for counting. Usually, at least 500 symbiotic cells were enumerated, and corrections to the cell abundances were made by the area scanned. The abundances were normalized to the volume filtered and reported as diatom host-Richelia L⁻¹⁻¹.

Incubation experiments

For incubation experiments, whole water was collected from two-six depths at select stations using the CTD rosette (Supplementary Materials; Supplementary Fig. 2; Supplementary Table 1). Whole water was transferred directly into acid-rinsed, transparent, 2.75 L polycarbonate bottles. Bottles were filled without headspace and each bottle amended with 1.5 mL of 0.5 M NaHCO₃ and 2 mL 99% ¹⁵N₂ (Cambridge isotopes, Andover MA USA) through the septa cap using gas tight syringes (Hamilton, Reno, NY USA). The bottles were manually inverted several times to mix the isotopes and placed on their sides in an on-deck incubator with continuously flowing surface seawater. Bottles were incubated during daylight hours (~12 h) and screen shading was used to simulate the 0.1, 1, 25, 50, 75% incident surface irradiance. Water collected from the near surface (0–5 m) were incubated without screening. Surface irradiance was measured as average surface radiation (PAR; µe m⁻² s⁻¹) at the time of sampling using the light sensor mounted on the CTD package. In 2010, due to sampling limitations 1 bottle was collected for whole water bulk analysis, and 1 bottle for SIMS analysis. In 2011, 3 replicate bottles were incubated for whole water bulk analysis and 1 bottle for SIMS analysis. Time points included the time of injection (time 0), and time end (~12 h). At sundown, using a peristaltic pump (Cole-Parmar, Vernon Hills, PA USA), the entire contents of 1 or 3 bottles (2010 or 2011, respectively) were filtered onto a pre-combusted GFF (25 mm diameter) filter held in a Swinnex filter holder for bulk analysis. The filters were placed in sterile Eppendorf tubes (1.5 mL), acidified using nitric acid (a digestion solution with an open beaker of 37% hydrochloric acid (HCl) to remove inorganic C from the filters, then placed in a 60 °C oven to complete dehydration for 12–24 h. The dried filters were weighed on a microbalance and prepared for combustion analyses in tin capsules.
Measurements of particulate nitrogen (N; µg) and carbon (C; µg) and atom % $^{15}$N and atom % $^{13}$C were made with an automated elemental analyzer (Thermo Flash EA, 1112 Series) coupled to a Delta Plus Advantage isotope ratio mass spectrometer (Thermo Finnigan, Dreieich Germany; EA-IRMS). Instrument accuracy and precision were estimate at 0.3652 ± 0.0002$^2$ N atom % and 1.0660 ± 0.0010 $^{13}$C atom % based on the mean and standard deviation of caffeine standards measured in conjunction with the samples. Fixation rates were calculated as a function of the change in the tracer concentration of the particulate pool relative to the size of the pool between time 0 and the time end [34].

In 2011, incubations with the eukaryotic inhibitor treatments were set up from populations collected at station 25. Similar procedures as described above were used, but on smaller volume bottles and on concentrated plankton. In these experiments 5 L of surface seawater was collected by bucket and gravity filtered onto a 10 µm (Poretics) pore size 47 mm diameter membrane filter held in a filter tower. The filter was directly placed in a 250 mL acid-washed polycarbonate Nalgene bottle filled with 0.2 µm FSW and capped without air bubbles. When filled without air, these bottles held 2.75 mL volume. Cycloheximide was amended to a final concentration of 0.05 mg/mL in the treated bottles. No amendments or un-treated bottles were used as the controls; four bottles were set up for controls, and four for cycloheximide. All bottles were amended with 180 µL of 0.5 M NaH$_2$CO$_3$ and 250 µL of 99% $^{15}$N$_2$ (Cambridge isotopes) in order to have an expected labeling percent of 3% and standard deviation of caffeine standards measured in conjunction with the samples. Fixation rates were calculated as a function of the change in the tracer concentration of the particulate pool relative to the size of the pool between time 0 and the time end [34].

RESULTS AND DISCUSSION

Abundances of N$_2$ fixing symbioses in the WTNA

To date, the various marine symbiotic diatoms are notoriously understudied, and hence our understanding of their abundances and distribution patterns is limited [7]. In general, these symbiotic plankton are capable of forming expansive blooms, but largely co-occur at low densities in tropical and subtropical waters with a few rare reports in temperate waters [26–29, 39–42]. The Rhizosolenia-Richelia symbioses have been more commonly reported in the North Pacific gyre [26, 27, 31], and the western tropical North Atlantic (WTNA) near the Amazon and Orinoco River plumes is an area where widespread blooms of the H. hauckii-Richelia symbioses are consistently recorded [28, 29, 42–47].

In the summer of 2010, bloom densities (10$^8$–10$^9$ cells L$^{-1}$) of the H. hauckii-Richelia symbioses were encountered at multiple stations with mesohaline (30–35 PSU) surface salinities (Supplementary Table 1). The R. clevei-Richelia symbioses were less abundant (2–30 cells L$^{-1}$). Similar densities of H. hauckii-Richelia have been reported in the WTNA during spring (April–May) and summer seasons (June–July) (28–49; 46). In fall 2011, less dense
symbiotic populations (0–50 cells L$^{-1}$) were observed, and the dominant symbioses was the larger cell diameter (30–50 µm) $H.$ membranaeus associated with Richelia. Previous observations of $H.$ membranaeus-Richelia in this region are limited and reported as total cells (i.e., 12-218 cells) and highest numbers recorded in Aug–Sept in waters near the Bahama Islands [43]. On the other hand, Rhizosolenia-Richelia are even less reported in the WTNA, and most studies by quantitative PCR assays based on the nifH gene (for nitrogenase enzyme for N$_2$ fixation) of the symbiont (44; 46–7). Unlike qPCR which cannot resolve if the populations are symbiotic or active for N$_2$ fixation, the densities and activity reported here represent quantitative counts and measures of activity for symbiotic Rhizosolenia.

The WTNA is largely influenced by both riverine and atmospheric dust deposition (e.g., Saharan dust) [48], including the silica necessary for the host diatom frustules, and trace metals (e.g., iron) necessary for photosynthesis by both partners and the nitrogenase enzyme (for N$_2$ fixation) of the symbiont. We observed similar hydrographic conditions (i.e., low to unmeasurable concentrations of dissolved N, sufficient concentrations of dissolved inorganic P and silicates, and variable surface salinities; 22; 28–29; 40–47) as reported earlier that favor high densities of $H.$ hauckii-Richelia blooms. Unfortunately our data is too sparse to determine if these conditions are in fact priming and favoring the observed blooms of the $H.$hauckii-Richelia symbioses in summer 2010, and to a lesser extent in the Fall 2011.

![Fig. 1 Representative epi-fluorescent micrographs and NanoSIMS imaging of wild collected symbiotic diatom-Richelia symbioses incubated with $^{15}$N$_2$ and $^{13}$C-bicarbonate for 12 h under in situ conditions. Images from left to right include: epi-fluorescent image taken prior to NanoSIMS analyses and correspond to the parallel NanoSIMS imaging of total secondary ion count (0.001 x Esi), enrichment of $^{15}$N ($^{15}$N/$^{14}$N), and enrichment of $^{13}$C ($^{13}$C/$^{12}$C). The epi-fluorescent images show excitation patterns expected for the symbiont and host chloroplast. The secondary ion content images show the host cellular boundaries and destructive nature of the NanoSIMS analyses. (A) Symbiotic $H.$ hauckii-Richelia cell collected from 2 m with 2 Richelia filaments emitting a bright orange-red fluorescence under green excitation (510–60 nm). The strong fluorescence associated with the $Richelia$ filaments corresponds to a high $^{15}$N enrichment ($^{15}$N/$^{14}$N ratio image), whereas cellular $^{13}$C enrichment ($^{13}$C/$^{12}$C) is above background but uniformly low. (B) A larger $H.$ membranaeus-Richelia cell collected from 25 m with two clearly fluorescent filaments of $Richelia$. Note the terminal heterocysts on either end of the two filaments, indicating recent or in situ cellular division of the $Richelia$. NanoSIMS images show uniform high enrichment of both $^{15}$N and $^{13}$C ($^{15}$N/$^{14}$N and $^{13}$C/$^{12}$C ratio images respectively) in areas of both symbiont and host chloroplast with the exception of one heterocyst designated with an arrow. (C) The blue excitation (459–90 nm) micrograph of the apical end of a symbiotic $R.$ clevei-Richelia cell shows a fluorescent yellow $Richelia$ and corresponds to high $^{15}$N enrichment ($^{15}$N/$^{14}$N ratio image) in the heterocyst whereas the corresponding $^{13}$C enrichment ($^{13}$C/$^{12}$C ratio image) is low. Here, the secondary ion content image distinguishes the remnants of the diatom frustule. The enclosed markings in the NanoSIMS images define the regions of interest (ROIs), which were used to determine the $^{13}$C/$^{12}$C and $^{15}$N/$^{14}$N ratios. Scale bars are 5 µm.](image-url)
A biometric relationship between C and N activity and host biovolume

The diatom-Richelia symbioses are considered highly host specific [10, 11], however, the driver of the specificity between partners remains unknown. We initially hypothesized that host selectivity could be related to the N₂ fixation capacity of the symbiont. Moreover, it would be expected that the larger H. membranaceus and R. clevei hosts which are ~2–2.5 and 3.5–5 times, respectively, larger in cell dimensions than the H. hauckii cells would have higher N requirements (Supplementary Table 2). In fact, recently it was reported that the filament length of Richelia is positively correlated with the diameter of their respective hosts [22]. Thus, to determine if there is also a size dependent relationship between activity and cell biovolume, the enrichment of both ¹⁵N and ¹³C measured by SIMS was plotted as a function of symbiotic cell biovolume.

Given the long incubation times (12 h) and previous work [32] that show fixation and transfer of reduced N to the host is rapid (i.e., within 30 min), we expected most if not all of the reduced N, or enrichment of ¹⁵N, to be transferred to the host diatom during (i.e., within 30 min), we expected most if not all of the reduced N, or enrichment of ¹⁵N, to be transferred to the host diatom during the experiment (Fig. 1). Therefore, we measured and report the enrichment for the whole symbiotic cell, rather than the enrichment in the individual partners (Supplementary Table 2; Fig. 2). The enrichment of both ¹³C/¹²C and ¹⁵N/¹⁴N was significantly higher in the larger H. membranaceus-Richelia cells (atom % ¹³C: 1.5628–2.0500; atom % ¹⁵N: 0.8645–1.0200) than the enrichment measured in the smaller H. hauckii-Richelia cells (atom % ¹³C: 1.0700–1.3078; atom % ¹⁵N: 0.3642–0.7925) (Fig. 2) (¹³C, Mann–Whitney p = 0.009; ¹⁵N, Mann–Whitney p = 0.001). In addition, the higher enrichment also corresponded to higher rates of N₂ fixation (e.g., 12.6–28.4 fmol N cell⁻¹ h⁻¹ for H. membranaceus-Richelia compared to 0.1–4.29 fmol N cell⁻¹ h⁻¹ for H. hauckii-Richelia; Supplementary Table 2) and C fixation (219–685 fmol C cell⁻¹ h⁻¹ compared to 2.74–59.8 fmol C cell⁻¹ h⁻¹, respectively; Supplementary Table 2).

Similar findings of higher ¹⁵N enrichment and N₂ fixation rates were also reported by Martínez-Pérez et al. [5], when comparing the larger N₂ fixing UCYN-A2-haptophyte symbioses to the smaller UCYN-A1-haptophyte symbiotic cells. Here, however, pooling all the SIMS measurements and biovolume estimates for the three different symbioses, we present a robust relationship between enrichment (atom % ¹³C or atom % ¹⁵N) and biovolume for both C and N (Fig. 2). It suggests that the larger size demands a higher activity for both N and C, which would be expected from allometric theory. Here, allometry refers to the relationship that biological processes scale by body-size [49].

Light dependence of N₂ fixation by diatom-Richelia symbioses

In order to determine if metabolic activity for the various symbioses is light dependent, the stable isotope incubation experiments were set up under simulated light conditions of the water column (i.e., 0.1–100% incidence surface light). Activity rates are reported for both bulk (see below) and single cells.

Although the data is more limited for the two larger symbiotic diatoms: H. membranaceus and R.clevei-Richelia symbioses, both follow the expected trend for phototrophic organisms to have higher C fixation in cells collected nearer to the surface and incubated under higher light intensities [50]. For example, the H. membranaceus-Richelia symbioses collected nearer to the surface had higher ¹³C enrichment and rates of C fixation than those collected from a deeper depth (25 m). However, ¹⁵N enrichment and corresponding N₂ fixation rates for the H. membranaceus-Richelia symbioses were similar regardless of their depth of collection. The two R. clevei-Richelia symbioses measured high and comparable enrichment of ¹³C and to a lesser extent ¹⁵N (atom % ¹³C = 1.2053 and 1.2346; atom % ¹⁵N = 0.7543 and 0.4480) to that measured in the H. membranaceus-Richelia symbioses (Supplementary Table 2).

Given the higher number of measurements for the H. hauckii-Richelia symbioses at multiple light levels, we plotted the individual rates of N₂ and C fixation as a function of photosynthetically active radiation (PAR) measured at the time of collection (Fig. 3). A hyperbolic tangent model [33] fit well for the N₂ fixation rate (adjusted R² = 0.56). This indicates that N₂ fixation followed a light dependence and saturation kinetics of maximum N₂ fixation (N₂fixmax) and enrichment (¹⁵Nfixmax) at the highest irradiances. Unexpectedly, the light response curve for C fixation could not be fit with the same or any saturation model. In fact, the ¹³C enrichment and corresponding estimated C fixation rates were highly variable (Supplementary Table 2). N₂ fixation in heterocystous cyanobacteria is fueled by C fixation and thus one expects parallel trends in activity for C and N₂ fixation [31]. We hypothesize that the fixed C of both partners is partitioned into growth and respiration to fuel other metabolic activities (i.e., N₂ fixation), and resulted in highly variable enrichment patterns. The estimated N- and C- based growth rates (see below) indicated that the cells were also growing at different rates and additionally could influence the observed variation. Further experimentation would be required to test these hypotheses and both require shorter incubations and multiple time points than presented here.

Similar lab experiments for determining the light dependency on N₂ fixation have been reported in the facultative Calothrix SC01 strain that forms symbioses with Chaetoceros diatoms, in a
C-based growth rates (Supplementary Table 2) identified Kranz cells collected at the three stations (C-based growth rate: the maximum rate of N₂ fixation and/or at different stages of the growth cycle, and still the populations studied here were likely different subpopulations stations ~700 km apart (Supplementary Fig. 2). The wild measurements are pooled from three different stations with SC01 was growing asymbiotic (21; 52–53). In these enrichment cultures [21, 33, 52, 53], in these H. hauckii-Richelia few older lab studies of R. clevei-Richelia and a recent report on H. hauckii-Richelia enrichment cultures [21, 33, 52, 53]. In these earlier works, the same light dependent N₂ fixation is shown. The strong light dependent activity shown here, however, was somewhat surprising given that measures were derived from field populations, unlike these other experiments which were done in controlled laboratory settings and additionally Calothrix SC01 was growing asymbiotic (21; 52–53). Furthermore, our measurements are pooled from three different stations with experiments that span approximately four weeks and two stations ~700 km apart (Supplementary Fig. 2). The wild populations studied here were likely different subpopulations and/or at different stages of their growth cycle, and still the response in their N₂ activity fit well to the curve. In fact, N and C-based growth rates (Supplementary Table 2) identified significant differences in estimated growth rates between the cells collected at the three stations (C-based growth rate: Kruskal–Walls, p = 0.09; N-based growth rate: Kruskal–Walls, p = 0.004). These growth rates were calculated from the SIMS derived cellular ¹³C/¹⁵N enrichment and initial N/C content based on biovolume (see Supplementary Materials). Moreover, both N-based and C-based growth was depth dependent, where cells nearer to the surface (stations 2 and 19) had higher estimated growth rates (Supplementary Table 2; Kruskal–Walls, p = 0.002 and p = 0.008, respectively). Hence, genetically distinct symbiotic strains have a similarly robust N₂ fixing activity response to light despite varying growth states.

Increased N₂ fixation by symbiotic cyanobacteria is largely under the host control in many terrestrial symbioses (17; 54). A similar scenario was suggested in the early work of applying NanoSIMS to field collected symbiotic diatoms because higher rates of N₂ fixation were reported in the symbiotic populations compared to asymbiotic ones [32]. However, in terrestrial symbiotic examples, the number of symbionts per host is dramatically different. Typically, one to two Richelia filaments associate with a Hemiaulus spp. host, whereas in plants, a symbiotic chamber houses 100’s to 1000’s of symbionts [17, 54]. Moreover, in most terrestrial examples, the number of heterocysts per symbiont filament increases after establishment [17, 18], however in Richelia (and Calothrix), the heterocysts are single and terminal. Thus, Richelia sustains a high N₂ fixing capacity by maintaining short filaments and a high heterocyst to vegetative cell ratio [23]. It is plausible and hypothesized that Richelia acquires energy from their hosts, e.g., in the form of C substrates. This would be a particularly attractive strategy for the internal Richelia symbionts of Hemiaulus spp. because they reside in close proximity to the host photosynthetic machinery [22].

**Reduced C and N₂ fixation when host is inhibited**

To better understand the potential control and role of C fixation mediated by the host diatoms, ¹⁵N₂ and ¹³C bicarbonate incubation experiments were treated with cycloheximide, a eukaryotic cytosolic protein translation inhibitor. Here, the enrichment of ¹⁵N and ¹³C were visualized and measured by SIMS in both the symbiont and the host separately. DCMU (3-(3,4-dichlorophenyl)-1, 1-dimethylurea) and chloramphenicol are other common inhibitors, however, both have been shown to inhibit several cyanobacteria strains, including heterocystous types [55]. Cycloheximide is not inhibitory to cyanobacteria and often applied as a cultivation strategy to avoid enrichment of eukaryotes [56]. Earlier lab studies on diatoms (and other eukaryotic algae, i.e., dinoflagellates) have shown that at similar concentrations (0.1–10 μg/ml) of cycloheximide, as used here, can reduce key metabolic processes, including photosynthesis and energy generation [57–59]. Hence, cycloheximide seemed to be an attractive and appropriate inhibitor for shutting down the diatom host photosynthesis, because the cyanobacterial symbiont would not be influenced.

The enrichment of both ¹⁵N/¹⁴N and ¹³C/¹²C was significantly reduced in the inhibited cells (Fig. 4; Supplementary Table 3; T test; p < 0.03). The decreased enrichment was measured in the host cells and only the heterocysts of Richelia filaments compared to the respective control cells. In fact, in the inhibited cells, the enrichment for ¹⁵N/¹⁴N was clearly localized to the symbiont filament, whereas ¹³C/¹²C was reduced overall without an apparent localization (Fig. 4B). The average decrease in ¹³C/¹²C and ¹⁵N/¹⁴N enrichment was one to two times, or 16–78% and 76% reduction in estimated rates of C and N₂ fixation, respectively, when the cells were inhibited. These results are congruent with studies on terrestrial-based symbioses, where the CO₂ (and NH₄⁺) assimilation rates are measurably depressed in symbiotic cyanobacteria (17–18; 54). Increased N₂ fixation is expected when the host is present, as it is the basis of the partnership and has been previously reported [32]. Moreover, it could be that the increased C fixation by the host leads to C:N ratio favoring increased N₂ fixation. The latter hypothesis remains to be tested.
transported to the heterocysts from vegetative cells, and N2 fixation energy, organic C and N derived from the host.

Richelia could be that reduced C substrates derived from the host are transferred to the heterocysts. Encoded in the Hemiaulus-Richelia RintHH01 genome is an invertase (InvB), an enzyme used to irreversibly split sugar [23]. In Anabaena sp. 7120, a related heterocystous cyanobacteria, InvB is heterotrophic and sucrose functions as a reduced C substrate for heterocysts that fix N2 from the host to symbiont. Shorter time incubations akin to a pulse-chase experiment would be desirable for estimating the gradual transfer, or perhaps a dual label incubation experiment of 13C and 15N distribution in Hemiaulus-Richelia symbioses, which were incubated with 13C-bicarbonate and 15N2 and untreated (A) or treated with a eukaryotic inhibitor (B). Images from left to right include: Epi-fluorescent image taken prior to NanoSIMS analyses and correspond to the parallel NanoSIMS imaging of total secondary ion count (0.001 x Esi), enrichment of 15N (15N/14N), and enrichment of 13C (13C/12C). Epi-fluorescent images (A panel, green excitation: 510–60 nm; B panel, blue excitation: 459–90 nm) taken prior to analyses are used to approximate the cellular location of the symbiont and host chloroplast. The emission visible in the epi-fluorescent micrographs correspond to the filaments of Richelia and chloroplast of the host diatoms. Each host cell has two symbiotic filaments, and the white arrows designate the terminal heterocysts of the Richelia filaments, which emit red and yellow-orange under green (A) and blue excitation (B), respectively. Note that in the control cells (untreated, A or top images), enrichment of 15N and 13C is uniformly high in both host and symbiont, whereas the cell treated with eukaryotic inhibitor has localized 15N enrichment to the two symbiont filaments and generally low enrichment of 13C in whole cell (B, or bottom images). The enclosed markings in the NanoSIMS images define the regions of interest (ROIs), which were used to determine the 13C/12C and 15N/14N ratios. Scale bars are 5 μm.

The reduction in both C and N2 fixation in inhibited cells indicates that the host is required, and likely controls the symbiont’s metabolism. In heterocystous cyanobacteria, there is a coordinated effort between vegetative cells and heterocysts to exchange energy, organic C and fixed N. Reduced C is rapidly transported to the heterocysts from vegetative cells, and N2 fixed in heterocysts is exported to vegetative cells [60–62]. The 13C/12C of vegetative cells was similar between inhibited and control cells (t test, p = 0.08); hence C fixation by Richelia persisted in the absence of the host.

The 13C/12C in terminal heterocysts of untreated cells was, however, significantly higher than the inhibited cells indicating that the host must also contribute to the increased 13C/12C enrichment measured in the heterocysts. One plausible scenario could be that reduced C substrates derived from the host photosynthesis are transferred to the Richelia vegetative cells and subsequently transported to the heterocysts. Encoded in the Richelia RintHH01 genome is an invertase (InvB), an enzyme used to irreversibly split sugar [23]. In Anabaena sp. 7120, a related heterocystous cyanobacteria, InvB is heterotrophic and sucrose functions as a reduced C substrate for heterocysts that is transported from vegetative cells [63, 64]. Unfortunately, our incubation period was too long to clearly resolve the transfer of C from the host to symbiont. Shorter time incubations akin to a pulse-chase experiment would be desirable for estimating the gradual transfer, or perhaps a dual label incubation experiment of 14C- and 13C-labels combined with NanoSIMS to trace the fate of labeled carbon [65].

Using the quantitative measures by SIMS in the control cells, we estimate that 8–22% of assimilated 13C in the symbiont is derived from the host, and 78–91% of the host 15N is derived from the symbiont. Our estimates are consistent with a recent cellular model of Hemiaulus-Richelia that reported 25% of fixed C in the symbiont is derived from the host, and 82% of fixed N in the host is from the symbiont [30]. In the cellular model, however, input values were largely derived from literature values of biovolume, biovolume to C relationships, and assumed stoichiometry [30]. The variation we measured here in the host derived C (8–22%) could be attributed to our long incubation times and thus “bottle effects” [66]. It is also likely that some fixed C was respired in order to fuel N2 fixation. Moreover, we have little knowledge of the life history of the cells prior to collection, hence we cannot discount that the population of cells were in varying growth states and contributed to the observed variation. In fact, the estimated growth (both N- and C-based) was higher in the control cells compared to the inhibited cells (p = 0.009). The latter is expected because activity was largely inhibited in the treated cells. However, in both the control and inhibited cells, the symbiont (vegetative cells) had higher estimated growth than the respective host cell (Supplementary Table 3; p = 0.02). In plant-cyanobacteria symbioses, slower growth is reported in the symbionts [18]; hence our results are unexpected and suggest uncoordinated growth cycles between the partners.

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Impact of symbiotic diatoms on bulk N2 and C fixation

Studies on rate and fate of N2 and C fixation by the symbiotic diatoms are fewer compared to investigations on other N2 fixers i.e., *Trichodesmium*, unicellular diazotrophs. In fact, searching the open world data archive PANGEA for datasets reported for the latter groups, and *Richelia* finds 1564, 126, and 35 datasets, respectively. Thus, the data presented here and motivation for the work was to contribute new rate information for these biogeochemical relevant yet under-reported populations.

To date, there are a few estimates of N2 fixation by the *Hemiaulus-richelia* populations from the same WTNA region. These earlier studies attribute a large source of new N to the photic zone derived from these symbiotic diatom populations [28, 29, 67, 68]. Moreover, this new N contributes significantly to C export in the region [29, 67, 68] and likewise in other regions of the world where diatom-*Richelia* symbioses persist (i.e., N. Pacific: 26–27; 31). However, the previous measurements of N2 fixation in the WTNA were indirect because acetylene reduction assays were used and applied to cell concentrates (i.e., plankton “slurries”). Thus, the individual cell activity cannot be distinguished. Combining the single cell measurements by SIMS with cell abundances by microscopy (Supplementary Table 1, 2) we could more accurately estimate the fraction of N2 fixation attributable to the symbiotic populations.

It is important to note that although our measures are on individual cells, the populations were incubated in whole water bottle experiments. Hence, we cannot discount that some enrichment in the individual cells is derived from the transfer of reduced substrates from co-occurring populations of other N2 fixers. In 2010, however, incubations were performed at stations almost entirely dominated by *H. hauckii-richelia* cells (>104 cells L\(^{-1}\)), whereas in 2011, biomass in general was low, and observations of other N2 fixers i.e., *Trichodesmium* spp., *Crocosphaera watsonii*, were rare in general and in particular at the stations assayed for N2 and C fixation measurements.

In 2010, we estimate that between 12–53% of the bulk N2 fixation was accounted for by the *H. hauckii-richelia* populations. Although the symbiotic cell densities at depth (11–21 m) of station 2 were slightly higher than the populations in the surface (2–4 m), the surface *H. hauckii-richelia* cells had higher individual N2 fixation rates (0.86–3.58 fmol N cell\(^{-1}\) h\(^{-1}\) and therefore made a larger contribution (53%) to the total fixed N2. Meanwhile, cell densities at station 25 (106 cells L\(^{-1}\)) were similar to station 2, however rates of N2 fixation (0.10–0.35 fmol N cell\(^{-1}\) h\(^{-1}\) ) were reduced, and therefore the estimated contribution (12–17%) to bulk N2 fixation was lower at station 25. The latter findings are directly relevant to models that use abundance estimates (including mRNA abundances of *nifH* genes for nitrogenase), rather than in situ activity to estimate new N contributing to N budgets [e.g., 69, 70].

The contribution to total C fixation by the symbioses was estimated to be far less than their N contribution. For example, *H. hauckii-richelia* accounts for only 2–5% of bulk C fixation at stations 2 and 25, and is comparatively less than the contribution of 12–53% to bulk N2 fixation. Observations of the *H. hauckii-richelia* populations during the bloom reported that one or both partners possessed variable cell integrity [46]. For example, long diatom chains (8–12 symbiotic cells per chain and >50 symbiotic cells in a chain) were reported at station 2 with fully intact symbiotic *Richelia* filaments (2–3 vegetative cells and terminal heterocyst), and at station 25 chains were short (1–2 symbiotic cells) and associated with short *Richelia* filaments (only terminal heterocyst). Moreover, the symbiotic *H. hauckii* hosts possessed poor chloroplast auto-fluorescence at station 25 [46]. Given that the cells selected for NanoSIMS were largely single cells, rather than chains, we suspect that these cells were in a less than optimal cell state, which was also reflected in the low 14C/12C enrichment ratios and low estimated C-based growth rates (0.30–57 div d\(^{-1}\)).

These are particularly reduced compared to the growth rates recently reported for enrichment cultures of *H. hauckii-richelia* (0.74–93 div d\(^{-1}\)) (Supplementary Table 2) [33].

In 2011, higher cellular N2 fixation rates (15.4–27.2 fmols N cell\(^{-1}\) h\(^{-1}\) ) were measured for the large cell diameter *H. membranaceus-richelia*, symbioses. Despite high rates of fixation, cell abundances were low (4–19 cells L\(^{-1}\)), and resulted in a low overall contribution of the symbiotic diatoms to the whole water N2 (>1%) and C fixation (>0.01%). The estimated C-based growth rates for *H. membranaceus* were high (1.9–3.5 div d\(^{-1}\) ), whereas estimated N-based growth rates (0.3–4 div d\(^{-1}\) ) were lower than previously published [33; 52–53]. Hence the populations in 2011 were likely in a pre-bloom condition given the low cell densities.

Estimating symbiotically derived reduced N to surface ocean

To date, determining the fate of the newly fixed N from these highly active but fragile symbiotic populations has been difficult. Thus, we attempted to estimate the excess N fixed and potentially available for release to the surface by using the numerous single cell-specific rates of N2 fixation determined by SIMS on the *Hemiaulus* spp.-*Richelia* symbioses (Supplementary Materials). Because the populations form chains during blooms and additionally sink, we calculated the size-dependent sinking rates for both single cells and chains (50 cells). Initially we hypothesized that sinking rates of the symbiotic associations would be more rapid than the N excretion rates, such that most newly fixed N would contribute less to the upper water column (sumit).

The sinking velocities were plotted (Fig. 5) as a function of cell radius at a range (min, max) of densities and included two different form resistance values (\(\phi = 0.3\) and 1.5). As expected, the combination of form resistance and density has a large impact on the sinking velocity. For example, a *H. hauckii* cell of similar radius (10 μm) and density (3300 kg m\(^{-3}\)) but higher form resistance (0.3 vs. 1.5) sinks twice as fast at the lower form resistance (Fig. 5).

This points to chain formation (e.g., increased form resistance) as a potential ecological adaptation to reduce sinking rates. Recently, colony formation was identified as an important phenotypic trait that could be traced back ancestrally amongst both free-living and symbiotic diatoms that presumably functions for maintaining buoyancy and enhancing light capture [22].

The concentration of fixed N surrounding a *H. hauckii* and *H. membranaceus* cell were modeled (Supplementary Materials; Supplementary Table 4; Fig. 6). First, the cellular N requirement (\(Q_n\) mol N cell\(^{-1}\)) for a cell of known volume, \(V\), as per the allometric formulation of Menden-Deuer and Lessard [71] is

\[
Q_n = \frac{V}{6} \times 10^{-15} \text{ mol N cell}^{-1}
\]

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The concentration of dissolved N (nmol L$^{-1}$) is presented at of varying cell sizes (3 µm and 30 µm) for H. hauckii-Richelia (A and B, respectively) and H. membranaceus-Richelia (C and D, respectively) growing at specific growth rates of 0.4 d$^{-1}$ (dashed red lines) or 0.68 d$^{-1}$ (solid black lines). Exudation follows the same principle as diffusive uptake as per Kiorboe [72] in the absence of turbulence.

The concentration of fixed N surrounding the cell ($C_r$) was iteratively calculated by the following:

$$C_r = \frac{E_N}{(4\pi D + 4r_x)} + C_i$$  \hspace{1cm} (3)

The concentric radius ($r_x$) as per Kiorboe [72] uses a diffusivity of N assumed to be $1.86 \times 10^{-5}$ cm$^2$ sec$^{-1}$ and the background concentration of N ($C_i$) is assumed to be negligible. Figure 5 presents the results for the two symbioses: H. membranaceus and H. hauckii at the two growth rates and as chains or singlets. Mean sinking rates for cells with a high form resistance (e.g., chains) are <10 m d$^{-1}$ (Fig. 5). Simplified exudation calculations assuming no motility suggest that the phycosphere surrounding individual cells in these chains would range from ~3–70 nmol N L$^{-1}$ at the cell surface and ~1–25 nmol N L$^{-1}$ at a distance of twice the equivalent spherical radius (Fig. 6). Sinking would deform these plumes and increase flux away from the cellular boundary layer so these are considered maximal near-cell concentrations of exudate. More complex mechanistic models of diffusive fluid dynamics (e.g., x, y, z) would be needed to simulate deformation of exudate plumes as a function of sinking speeds and the flow field (laminar or turbulent) [73–75].

In oligotrophic regimes such as WTNA, where N concentrations in the surface mixed layer are often below detection, the symbiotically derived reduced N serves as an important source of new N to the surface ocean. Based on our calculations, we would predict the phycosphere surrounding these symbioses to be N-rich, and given sinking speeds <10 m d$^{-1}$, these exudates retain the availability of limiting nutrients in the surface waters and fuel regional primary production rather than contributing to the mesopelagic nutrient inventory.

**Conclusions**

Despite widespread distributions and occasional large-scale blooms, diatom-Richelia symbioses have been largely understudied. Our understanding of their metabolic activities and partner interactions has been limited to $N_2$ fixation and the exchange of fixed N, respectively. Here, we report both the $N_2$ and C fixation activity of single cells from three different diatom-Richelia symbiotic populations collected and assayed in the wild. A robust biometric relationship was identified where larger symbiotic cells have higher activity. $N_2$ fixation by one symbiotic population appears to be light dependent, whereas unexpectedly
C fixation is highly variable and independent of light. Inhibitor experiments designed to shut down the host photosynthesis and communication resulted in depressed C and N2 fixation activity suggesting that the hosts are the primary C fixing partner and likely control their symbionts N2 fixing activity. Single cell rates and estimated sinking velocities were combined with a simplified model to predict that most of the fixed N is released in the upper water column by the symbiotic diatoms. In summary, the observations reported here for both abundance and in situ activity contributes to an improved understanding of symbiotic diatom distribution, ecology, and contribution to N/C cycling.

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