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Sinking *Trichodesmium* fixes nitrogen in the dark ocean

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Abstract

The photosynthetic cyanobacterium *Trichodesmium* is widely distributed in the surface low latitude ocean where it contributes significantly to N₂ fixation and primary productivity. Previous studies found *nifH* genes and intact *Trichodesmium* colonies in the sunlight-deprived meso- and bathypelagic layers of the ocean (200-4 000 m depth). Yet, the ability of *Trichodesmium* to fix N₂ in the dark ocean has not been explored. We performed ¹⁵N₂ incubations in sediment traps at 170, 270 and 1 000 m at two locations in the South Pacific. Sinking *Trichodesmium* colonies fixed N₂ at similar rates than previously observed in the surface ocean (36-214 fmol N cell⁻¹ d⁻¹). This activity accounted for 40 ± 28 % of the bulk N₂ fixation rates measured in the traps, indicating that other diazotrophs were also active in the mesopelagic zone. Accordingly, cDNA *nifH* amplicon sequencing revealed that while *Trichodesmium* accounted for most of the expressed *nifH* genes in the traps, other diazotrophs such as *Chlorobium* and Deltaproteobacteria were also active. Laboratory experiments simulating mesopelagic conditions confirmed that increasing hydrostatic pressure and decreasing temperature reduced but did not completely inhibit N₂ fixation in *Trichodesmium*. Finally, using a cell metabolism model we predict that *Trichodesmium* uses photosynthesis-derived stored carbon to sustain N₂ fixation while sinking into the mesopelagic. We conclude that sinking *Trichodesmium* provides ammonium, dissolved organic matter and biomass to mesopelagic prokaryotes.

Introduction

Dinitrogen (N_2) fixing prokaryotes (diazotrophs) supply bioavailable nitrogen to planktonic communities, fueling primary production and contributing to carbon export in the ocean [1]. Nitrogen inputs by diazotrophs may become even more important in the future ocean, as global warming enhances water column stratification constraining nitrogen availability for primary producers [2]. Early studies suggested that diazotrophs were only present in low latitude warm oligotrophic waters of the (sub)tropical ocean. However, over the past decade it has become clear that diazotrophs are also found in cold and nutrient-rich environments such as estuaries, shelf seas, polar regions and the ocean's dark pelagic realm [3]. Devoid of light, N_2 fixation in the dark ocean has been attributed to heterotrophic non-cyanobacterial diazotrophs presumably relying on reduced organic compounds for energy and carbon supply [4–6]. However, cyanobacterial diazotrophs have been repeatedly observed in mesopelagic to bathypelagic depths (200 to 4 000 m; Table S1; [7]).

The filamentous cyanobacterium *Trichodesmium* thrives in tropical and subtropical ocean's photic zone where it can introduce 60-80 Tg $N\ y^{-1}$, representing roughly half of the reactive nitrogen input to the global ocean [8]. *Trichodesmium* has gas vesicles which confer cells with buoyancy, restricting their vertical distribution in the water column [9, 10]. Accordingly, *Trichodesmium* biomass is thought to be fully remineralized within the photic zone [11]. Recently, however, molecular and imaging studies have documented intact *Trichodesmium* cells and expression of nitrogenase genes down to 4 000 m depth across the world's oceans (Table S1). The mechanisms through which *Trichodesmium* sinks into the dark ocean may include gravitational sinking, downwelling

70 events [12], mineral ballasting [13], or sudden autocatalytic cell death in response to
71 nutrient limitation [14]. Sinking velocities can be fast enough for surface photoautotrophic
72 cells to reach the dark ocean while remaining viable [15–18], but whether sinking
73 *Trichodesmium* remains metabolically active and carries out N₂ fixation in the dark ocean
74 is not known.

75
76 If diazotrophically active *Trichodesmium* fixes N₂ in the dark ocean it would not only
77 affect the global marine nitrogen inventory but conceivably also stimulate microbial
78 processing of particulate organic matter in the (sub)tropical regions where it thrives,
79 affecting vertical carbon export and remineralization in the dark ocean [19]. In the
80 present study we combine ¹⁵N₂ incubation on sediment traps, sinking simulation
81 laboratory experiments and cell metabolism modeling to document that *Trichodesmium*
82 can fix N₂ while sinking far below the photic zone, constituting a hitherto unaccounted
83 reactive nitrogen and organic matter source in the dark ocean.

84 85 **Materials and Methods**

86 87 *Sinking particle sampling in the South Pacific Ocean*

88
89 Sinking particles were collected in the western tropical South Pacific during the TONGA
90 cruise (doi: [10.17600/18000884](https://doi.org/10.17600/18000884)) onboard the R/V *L'Atalante* from November 1st to
91 December 5th 2019. Surface tethered mooring lines (~1 000 m long) were deployed at
92 two stations: S05M (21.157°S, 175.153°W, 5 days) and S10M (19.423°S, 175.133°W, 4
93 days). The mooring lines featured three sediment traps placed at 170, 270 and 1 000 m.
94 These depth levels were chosen to match the base of the photic layer, the usual depth to

95 calculate flux attenuation (100 m deeper than the photic layer) and the base of the
96 mesopelagic layer, respectively. Each trap consisting of four particle interceptor tubes
97 mounted on an articulated cross-frame. Out of the four tubes deployed per depth, two
98 were used for this study. The first tube was filled with 6 l of 0.2 μm filtered seawater
99 followed by 2.5 l of a 50 g l⁻¹ saline brine labeled with ¹⁵N₂ gas. The brine was prepared
100 in a 4.5 l polycarbonate bottle fitted with a septum screwcap and labeled with high-purity
101 ¹⁵N₂ gas (Euroiso-top) injected through the septum and the bubble thoroughly mixed with
102 the brine for several hours using a magnetic stirring plate. The brine had an enrichment
103 ~55 ¹⁵N atom % as determined by membrane inlet mass spectrometry [20]. The second
104 tube was filled with 6 l RNAlater solution [21] and 2.5 l of non-labeled brine.
105
106 Immediately upon recovery of the traps onboard, the upper layer of the tubes was
107 carefully removed with a peristaltic pump until the density gradient of the brine was
108 reached. The seawater layer overlying the brine prevented potential intrusions of surface
109 seawater when recovering the trap line onboard. We confirmed that no surface
110 contamination occurred since the particulate organic carbon fluxes measured in brine-
111 filled traps agreed with those measured in parallel traps kept closed during recovery
112 onboard ('RESPIRE' traps [22]; M. Bressac, personal communication). The brine of the
113 ¹⁵N₂-labeled traps was transferred to magnetic stirrer plates to ensure homogeneous
114 aliquot sampling. Three aliquots of 50 ml were filtered onto precombusted 25 mm GF/F
115 filters (GE Healthcare, Little Chalfont, UK) for bulk elemental analysis coupled isotope
116 ratio mass spectrometry (EA-IRMS), and another three were filtered onto 1 μm
117 polycarbonate filters (Nucleopore, Whatman, Maidstone, UK) and fixed with 2%
118 microscopy grade paraformaldehyde for single-cell nanoscale secondary ion mass
119 spectrometry (nanoSIMS) analyses (see below). Samples integrating biomass between

2 000 and 200 m (bottlenet [16]) were used to measure natural ^{15}N atom % enrichment of bulk biomass and *Trichodesmium* cells. In parallel, the whole brine volume of the tube filled with RNA later was immediately filtered onto 0.2 μm polysulfone filters (Supor, Pall Gelman, Port Washington, NY, USA). The filters were transferred to bead beater tubes containing a mix of 0.1 mm and 0.5 mm silica beads, flash-frozen in liquid nitrogen and stored at -80°C until RNA extractions (see below).

Bulk and *Trichodesmium*-specific N_2 fixation rates

The $^{15}\text{N}/^{14}\text{N}$ ratio of dissolved nitrogen was measured by membrane inlet mass spectrometry and the bulk $^{15}\text{N}/^{14}\text{N}$ ratio and particulate nitrogen concentration of particles with an Integra CN EA-IRMS (SerCon Ltd, Cheshire, UK) as described elsewhere [23].

The ^{15}N atom % enrichment of *Trichodesmium* filaments was analyzed on a nanoSIMS 50L (CAMECA, Gennevilliers, France) at the Leibniz Institute for Baltic Sea Research (IOW, Germany). Sample filters were mounted with conductive tape on 10 x 5 mm aluminum stubs (Ted Pella Inc., Redding, CA) and gold-coated to a thickness of ca. 30 nm (Cressington auto sputter coater). A 1 pA 16 keV Cesium (Cs^+) primary beam was scanned on a 512 x 512 pixel raster with a raster area of 15 x 15 μm , and a counting time of 250 μs per pixel. Samples were pre-sputtered with 600 pA Cs^+ current for 2 min in a raster of 30 x 30 μm to remove the gold and surface contaminants and reach the steady state of ion formation. Negative secondary ions $^{12}\text{C}^-$, $^{13}\text{C}^-$, $^{12}\text{C}^{14}\text{N}^-$, $^{12}\text{C}^{15}\text{N}^-$ and $^{31}\text{P}^-$ were detected with electron multiplier detectors, and secondary electrons were simultaneously imaged. Sixty serial quantitative secondary ion mass planes were generated, drift corrected and accumulated to the final image. Mass resolving power was >8 000 to resolve isobaric interferences. Data was processed using the

Look@nanoSIMS software [24]. Isotope ratio images were generated by dividing the $^{13}\text{C}^-$ ion count by the $^{12}\text{C}^-$ ion count, and the $^{12}\text{C}^{15}\text{N}^-$ ion count by the $^{12}\text{C}^{14}\text{N}^-$ ion count pixel by pixel. Individual *Trichodesmium* filaments (trichomes) were identified in nanoSIMS secondary electron $^{12}\text{C}^-$, $^{12}\text{C}^{14}\text{N}^-$ images. These images were used to define regions of interest (ROIs). For each ROI, the $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ ratios were calculated based on the ion counts averaged over the ROIs.

Bulk N_2 fixation rates were calculated following the equations of Montoya et al. [25]. *Trichodesmium*-specific volumetric N_2 fixation rates were calculated using the following equation:

$$\text{Volumetric } \text{N}_2 \text{ fixation rate} = \frac{(A_{\text{Tricho}} - A_{\text{TrichoNat}})}{(A_{\text{N}_2} - A_{\text{TrichoNat}})} \times \frac{PN_{\text{Tricho}}}{t}$$

where A_{Tricho} is the ^{15}N atom% enrichment of individual *Trichodesmium* cells incubated with $^{15}\text{N}_2$, $A_{\text{TrichoNat}}$ is the natural ^{15}N atom% enrichment of *Trichodesmium* as analyzed by nanoSIMS (see above), A_{N_2} is the ^{15}N atom% enrichment of dissolved N_2 measured by membrane inlet mass spectrometry (see above), PN_{Tricho} is the nitrogen biomass of *Trichodesmium* and t is the incubation time. PN_{Tricho} is calculated by converting *Trichodesmium nifH* gene copies l^{-1} (as provided by Bonnet et al. [26]) to carbon considering the average of the mmol C : *nifH* ratio provided in Meiler et al. [27]. Carbon is converted to nitrogen considering a C:N ratio of 6:1 [28]. *Trichodesmium* abundance is calculated by converting *Trichodesmium nifH* gene copies l^{-1} into cells l^{-1} considering a ratio of 12 and 103 *nifH* gene copies per *Trichodesmium* cell, obtained empirically for stations S05M and S10M, respectively, by comparing quantitative PCR and microscopy *Trichodesmium* counts [26]. Finally, *Trichodesmium* cell-specific N_2 fixation rates (fmol N

cell⁻¹ d⁻¹) are calculated by dividing volumetric rates by *Trichodesmium* abundance (cells l⁻¹).

RNA extractions, nifH gene sequencing and bioinformatics

RNA was extracted using the RNeasy mini kit (Qiagen) including a 1 h on-column DNase digestion. PCRs on extracted RNA controlled for complete DNA digestion. Reverse transcription (RT) were performed with TaqMan Reverse Transcription (Applied Biosystems) using reverse primer nifH3 (5'-ATR TTR TTN GCN GCR TA-3') and 5 µl of RNA extract. Triplicate nested PCR reactions were conducted using degenerate *nifH* primers nifH1 (5'-TGYGAYCCNAARGCNGA-3'write here), nifH2 (5'-ADNGCCATCATYTCNCC-3'), *nifH3* and *nifH4* (5'-TTYTAYGGNAARGGNGG-3') [29]. The PCR mix was composed of 5 µl of 5X MyTaq red PCR buffer (Bioline), 1.25 µl of 25 mM MgCl₂, 0.5 µl of 20 µM forward and reverse primers, 0.25 µl Platinum Taq and 5 µl of cDNA (1 µl from the first PCR reaction was used as template in the second reaction). The reaction volume was adjusted to 25 µl with PCR grade water. Triplicate PCR products were pooled and purified using the GeneClean Turbo kit (MP Biomedicals). Samples were sequenced using the Illumina MiSeq platform with 2 × 300 bp paired-end reads. Demultiplexed paired-end sequences were dereplicated, denoised, assembled and chimeras discarded using the DADA2 pipeline [30]. This generated 6 929 ASVs (146 000 ± 18 000 reads per sample). Sequences have been deposited in the Sequence Read Archive under accession number PRJNA742179. The ASVs were translated to amino acid sequences using FrameBot [31] and filtering for homologous genes was done following the NifMAP pipeline [32]. This reduced the number of ASVs to 6 503 accounting for 842 515 reads (96% of all reads). Taxonomic ranks were assigned

according to the *nifH* gene reference database collated and maintained by the Zehr research group (v. June 2017; <https://www.jzehrlab.com/nifh>).

Hydrostatic pressure experiments with *Trichodesmium* cultures

The effect of increasing hydrostatic pressure and decreasing temperature on *Trichodesmium* was tested using a sinking particle simulator [33]. Exponentially growing cultures of *Trichodesmium erythraeum* IMS101 grown at 27°C under a 12h:12 light:dark cycle on YBCII medium [34] were transferred to four autoclaved 500 ml high pressure titanium bottles (HPBs) placed inside incubators (Mettmert IPP 750+, Schwabach, Germany) programmed to decrease temperature at the desired pace. For 360 h, the pressure within the HPBs was increased from 0 to 3 MPa using a piloted pressure generator based on a motorized syringe controlled by a computer. Pressure was logged continuously by means of a Metrolog (Metro-Mesures, Mennecy, France) and controlled by the software with a precision of 0.2%. Concomitantly, temperature of the HPBs was decreased from 27 to 14°C. These conditions simulate those experienced by cells when sinking from the surface to ~300 m depth with a conservative sinking speed of 20 m d⁻¹ (at the lower end of empirically measured *Trichodesmium* sinking velocities; Table S2). Triplicate analytical culture aliquots were sampled from two HPBs at 192 and from another two HPBs at 360 h, corresponding to simulated depths of 160 and 300 m, respectively. N₂ fixation incubations at these time points were done under the corresponding hydrostatic pressure conditions by 100 ml culture aliquots to pressurized titanium flasks containing 30% volume of culture medium previously enriched with ¹⁵N₂ gas (prepared as explained above) and incubated for 24 h. Particulate and dissolved samples were analyzed by EA-IRMS and MIMS as described above. Cultures under a

12h:12h dark:dark cycle and temperature decrease pace identical to that of pressurized cultures were used as a control.

Sinking *Trichodesmium* cell metabolism model

We hypothesized that *Trichodesmium* fixes N_2 while sinking into the dark ocean at the expense of carbon accumulated from photosynthesis before starting to sink. Sinking *Trichodesmium* can thus only fix N_2 until reaching the depth where its carbon storage is depleted. To compute how much carbon storage *Trichodesmium* needs to acquire before sinking to be able to fix N_2 at 1 000 m depth, we adapted a previously published coarse-grained cell metabolism model [35]. The model simulates photosynthesis, N_2 fixation, and respiration for the entire trichome, resolving diffusion boundary layers for oxygen transport and distinguishing between photosynthetic and non-photosynthetic cells (Fig. S1). Photosynthetic cells fix carbon with harvested light energy, accumulate carbon, and synthesize new biomass, whereas non-photosynthetic cells use carbon stored by other cells to fix N_2 (Fig. S1).

To adapt the model to *Trichodesmium* colonies sinking into the mesopelagic layer we considered the variability in temperature and oxygen observed from the ocean surface to 1 000 m in our study (Fig. S2) as well as the inhibiting effect of increasing nitrate concentrations on N_2 fixation. Walden's rule [47] was used to simulate the variation of oxygen diffusivity, as in previous studies [37], and the Arrhenius equation was used to simulate the temperature dependencies of metabolisms [35]. The adapted model was applied to *Trichodesmium* sinking from the bottom of the mixed layer (~40 m; Fig. S2) over a wide range of calculated velocities (ranging from 12 to ~600 m d⁻¹;

Supplementary Methods) considering a ratio of initial carbon storage level relative to non-storage biomass (R_{Sto}) ranging between 0 and 2. This value of R_{Sto} range is conservative, since it varies between 1 and 10 according to carbohydrate and lipid to protein ratios in various phytoplankton species [38]. To test the effect of nitrate inhibition on *Trichodesmium*'s N_2 fixation in the mesopelagic, we considered a decrease in the diazotrophically active cells by 70 and 50% in each trichome. All model equations are detailed in Inomura et al. [35] and the adapted code is fully available in Zenodo (<https://zenodo.org/record/5153594>; doi: 10.5281/zenodo.5153594).

Statistical analyses

^{15}N atom % enrichment values were checked for normality using a Shapiro-Wilk test and significant differences between samples tested with Wilcoxon test, using R software package dplyr in RStudio Version 1.2.5033.

Results and Discussion

Trichodesmium fixes N_2 and expresses *nifH* in the mesopelagic ocean

The isotopic enrichment of single *Trichodesmium* filaments in the traps ranged between 0.428 ± 0.002 and 0.463 ± 0.033 ^{15}N atom % (Fig. 1). The ^{15}N atom % enrichment of all *Trichodesmium* filaments analyzed was significantly higher than that of filaments not incubated with $^{15}N_2$ collected over the same depth range (0.364 ± 0.006 ^{15}N atom %; Wilcoxon test $p = 0.004$ and $p = 0.014$ for S05M and S10M, respectively; Fig. 1). The derived cell-specific N_2 fixation rates ranged between 36 and 214 $fmol\ N\ cell^{-1}\ d^{-1}$ (Table

S3), in the same range of previous *Trichodesmium* cell-specific N₂ fixation measurements in surface waters of the South Pacific [39, 40]. While the salinity in the traps (~50 ppt) was higher than that of ambient waters (~34 ppt), previous studies have shown that increased salinity reduces but does not impair *Trichodesmium* growth up to 42 ppt [41]. Comparing in *Trichodesmium* cultures grown on 34 and 50 ppt showed a decrease in N₂ fixation rates by 63 ± 15 % (Supplementary Methods). Hence, the high salinity of the ¹⁵N₂-labeled brine may have reduced but did not completely inhibit N₂ fixation in *Trichodesmium*, implying that in situ rates could be higher than measured in our traps. Overall, these cell-specific rates indicate that *Trichodesmium* sinking into the mesopelagic zone can fix N₂ at rates comparable to the surface.

Amplicon sequencing revealed that *nifH* gene transcripts annotated as *Trichodesmium* were present in all sediment trap samples, accounting for 26-84% (average 56%) of the sequence reads (Fig. 2). This indicates that *Trichodesmium* constituted a substantial fraction of the diazotroph community that actively transcribed the *nifH* gene in all samples (Fig. 2). However, comparing the ¹⁵N₂ atom % enrichment of bulk sediment trap material and individual filaments, *Trichodesmium* accounted for 1 to 70% of the N₂ fixation activity measured in the traps (Table S3). Hence, other organisms contributed to N₂ fixation within the traps, which could be driven by either surface diazotrophs attached to particles or by true mesopelagic N₂ fixation driven by diazotrophs residing in deep waters. The contribution of *Trichodesmium* to mesopelagic N₂ fixation in the subtropical region studied here is notorious but likely restricted to the (sub)tropical regions where this cyanobacterium abounds. The predicted warming and expansion of oligotrophic subtropical gyres towards higher latitudes may expand the effect of sinking *Trichodesmium* to higher latitudes in the future [42].

295
296 *Crocospaera*, the only other cyanobacterial group present in the cDNA sequence data,
297 comprised 10% of the cDNA sequence reads of station S05M at 270 m, but less than
298 0.1% in the other samples. Sequences annotated as the genus *Chlorobium*
299 (Bacteroidota) comprised 60% of cDNA sequences recovered from 1 000 m depth at
300 station S05M and constituted a substantial fraction of the expression profiles of station
301 S10M at 170 m and 270 m, comprising 16% and 20% of cDNA sequences, respectively,
302 but their contribution was less than 1% in the rest of the samples. This indicates that,
303 when specific conditions are met, *Chlorobium* can contribute substantially to the *nifH*
304 transcript pool. A noteworthy contribution to the expression profiles was
305 Alphaproteobacteria classified as the genus *Yangia* (Rhodobacteraceae [43]), which
306 comprised approximately 10% of cDNA sequences of S05M but were virtually
307 undetected at S10M. Both Bacteroidota and Rhodobacteraceae have been identified as
308 epibionts of *Trichodesmium* [44] and were likely attached to the colonies as they sunk
309 from the surface ocean into the traps. The other most prevalent group was
310 Deltaproteobacteria, with genera such as *Desulfatibacillum*, *Desulfobacter*,
311 *Desulfolobus*, and *Desulfovibrio* together comprising 12.5% of cDNA reads on average.
312 This group has not been previously identified as an epibiont of *Trichodesmium* but is
313 commonly observed in sinking particles intercepted with sediment traps in the dark
314 ocean [21].

315

316 *Sinking Trichodesmium simulation experiments*

317

318 We confirmed the ability of *Trichodesmium* to fix N₂ under mesopelagic conditions using
319 a particle sinking simulator (see Methods; [42]). *Trichodesmium* cultures submitted to

increasing hydrostatic pressure for 192 and 360 h in the dark (equivalent to 150 and 300 m simulated depth) had ^{15}N atom % enrichment values of 0.375 ± 0.012 and 0.370 ± 0.002 atom %, respectively (Fig. 3A), indicating low but measurable N_2 fixation. Control *Trichodesmium* cultures kept at ambient lab pressure conditions had ^{15}N atom % enrichments of 0.390 ± 0.012 and 0.393 ± 0.008 after 192 and 360 h of incubation, respectively (Fig. 3B). This difference in pressurized (Fig. 3A) and non-pressurized (Fig. 3B) N_2 fixation rates is in agreement with previous metabolic rate slowdown in epipelagic prokaryotes submitted to mesopelagic pressure levels [45] and indicates that increasing pressure reduces but does not completely incapacitate N_2 fixation in *Trichodesmium*. We however note that while the sinking simulator allows changing pressure and temperature along the simulated sinking process, it does not allow changing nutrient concentrations. The increasing concentrations of nitrate with depth in the mesopelagic may inhibit N_2 fixation. Thus, the rates measured in these sinking simulations represent a non-inhibited upper limit of diazotrophic activity.

How does *Trichodesmium* obtain energy to fix N_2 at depth?

Trichodesmium uses photosynthetically fixed carbon to fuel the energetically expensive process of N_2 fixation [46]. Hence, the dark conditions of the mesopelagic ocean should be expected to halt photosynthesis and N_2 fixation in *Trichodesmium*, eventually leading to its death. We hypothesized that *Trichodesmium* can fix N_2 in the dark ocean using stored intracellular carbon acquired by photosynthesis before starting to sink. To test how much carbon storage *Trichodesmium* would need to fix N_2 until reaching 1 000 m of depth (corresponding to our field observations), we adapted a published cell metabolism model [47] (Fig. S2). The model simulates changes in intracellular carbon storage (the

ratio of carbon storage to biomass or R_{Sto}) while sinking linearly in the water column over a wide range of sinking velocities. The model output provides a relationship between the cell's initial R_{Sto} (R_{Sto} value before starting to sink) and the depth at which carbon storage depletes (Fig. 4).

Considering that *Trichodesmium* starts sinking below the mixed layer depth (~40 m; Fig. S2), the cell's metabolism becomes dependent on intracellular carbon storage when light is extinguished (~170 m; Fig. S2). Below that depth, R_{Sto} decreases (Fig. 4). When the initial $R_{Sto} = 1$ (which is at the lower end of the range measured in phytoplankton, see Methods), the cell's carbon storage is depleted at around 750 m assuming a medium sinking velocity of 375 m d⁻¹ (yellow dot in Fig. 4). With a higher initial R_{Sto} of 1.3 and the same sinking velocity, carbon storage is depleted at 1 000 m. This suggests that an initial R_{Sto} of at least 1.3 is required for *Trichodesmium* cells to reach 1 000 m of depth with enough carbon to sustain N₂ fixation (pink dot in Fig. 4) and be consistent with our field data (Fig. 1). We also note that *Trichodesmium* may obtain carbon from dissolved organic matter available in their surrounding medium [40], which is not modeled here. This could decrease the required initial R_{Sto} to sustain N₂ fixation at 1000 m depth.

Trichodesmium invests a significant part of its carbon storage in maintaining low intracellular oxygen concentrations through respiratory protection [47, 48]. The decrease in temperature with depth slows down *Trichodesmium*'s metabolism, decreasing the speed of oxygen diffusion (Walden's rule [47]), which reduces the level of respiratory protection needed to fix N₂ and decreasing and thus carbon storage consumption. While the decrease in temperature with depth also increases the saturated concentration of oxygen, the concentration of oxygen below the mixed layer in our field experiment was

under-saturated (Fig. S2), with oxygen deficit particularly pronounced below 600 m. The combined effects of declining temperatures and oxygen under-saturation contribute to the reduction in carbon storage consumption and are reflected as an increasing slope in the non-linear curve in Fig. 4.

The high nitrate concentrations of the mesopelagic ocean could inhibit N_2 fixation in *Trichodesmium* [49]. While no studies have explicitly tested the inhibition of N_2 fixation by nitrate in the mesopelagic, we explored this effect by considering two constant levels of inhibition (70% and 50%; Fig. S3), which are in the upper range of previous culture and field studies [50]. The model predicts that under 70% and 50% inhibition the required initial R_{Sto} decreases to ~ 0.5 and ~ 0.8 , respectively (Fig. S3). This indicates that when nitrate inhibits N_2 fixation less carbon storage is consumed, allowing *Trichodesmium* to remain metabolically active at deeper depths (Fig. S3). Previous studies have shown that *Trichodesmium* can fix N_2 in the presence of up to 20 μM nitrate provided phosphate concentrations are high enough to sustain growth [50]. At the depths where traps were deployed during our field experiment, excess phosphate with respect to nitrate according to the Redfield stoichiometry of 16:1 (P^* parameter; Fig. S2) likely permitted N_2 fixation (Fig. 1). Finally, the increased partial pressure of CO_2 with depth could also favor N_2 fixation in *Trichodesmium* as previously shown in culture experiments [51].

Is fast sinking necessary for *Trichodesmium* to fix N_2 in the mesopelagic?

Previous studies have suggested that viable and/or active growing cyanobacteria in the dark ocean are associated with fast-sinking particles, mineral ballasting, or episodic flux

events [17, 18, 52]. While the gas vesicles of *Trichodesmium* may prevent it from sinking, a previous study found that sinking *Trichodesmium* can contribute importantly to organic matter export during atmospheric dust ballasting events [50]. Three months before our cruise, a volcano erupted over the Tonga-Kermadec volcanic arc [53]. This volcano was particularly close to station S10M (~144 km). Measurements of lithogenic silica in the trap material (Supplementary Methods) showed that the lithogenic to particulate organic matter ratio of particulate matter was maximal at station S10M (Table S4). Scanning electron microscopy images (Supplementary Methods) showed volcanic materials intermingled with *Trichodesmium* filaments (Fig. S4), particularly at the 1 000 m trap of station S10M where *Trichodesmium nifH* gene transcripts were more abundant (Fig. 2). Hence, the eruption may have stimulated fast sinking by *Trichodesmium*, particularly at the S10M site. However, both our laboratory sinking simulations and the results of the cell metabolic model indicate that *Trichodesmium* can fix N₂ within the mesopelagic depth range even when sinking at low velocity (e.g. 20 m d⁻¹).

Potential impact of sinking *Trichodesmium* on mesopelagic prokaryotic communities

Trichodesmium releases up to 19% of the N₂ fixed as ammonium [54], which is the main energy source for dark CO₂ fixation by the abundant Thaumarchaeota [55]. Considering the *Trichodesmium*-specific N₂ fixation rates measured here and a ratio of dark CO₂ fixed per ammonium oxidized of 0.1 [56, 57], the ammonium released by sinking *Trichodesmium* may sustain CO₂ fixation rates of up to 0.2 μmol C m⁻³ d⁻¹. This represents ~13% of previous dark CO₂ fixation rates measured in the mesopelagic ocean [58]. However, to date very few dark CO₂ fixation measurements are available

and their magnitude in *Trichodesmium*-dominated regions (particularly in the South Pacific Ocean) is unknown.

More importantly, *Trichodesmium* releases ca. 50% of its fixed N₂ as dissolved organic nitrogen [59], which mostly composed of labile amino acids [60]. The mycosporine and tryptophan-like compound optical signatures observed at >1 000 m in our study are consistent with previous measurements of natural [61–63] and cultured *Trichodesmium* colonies (Supplementary Methods; Fig. S6). These signals were particularly strong at S10M (Fig. S5) coinciding with highest *Trichodesmium nifH* transcripts (Fig. 2) and supporting the active release of amino acids by sinking *Trichodesmium*. Amino acids released by active sinking *Trichodesmium* could add up to the labile compounds released by sinking particles in the dark ocean [64–67], contributing to the prokaryotic respiration organic matter [68] in the subtropical and tropical regions where *Trichodesmium* occurs.

Conclusions

This study provides the first cell-specific N₂ fixation rates, nitrogenase expression and metabolic mechanistic understanding of *Trichodesmium* sinking into the mesopelagic zone, representing a step forward from early findings of viable surface ocean phytoplankton at depth. Diazotrophically active *Trichodesmium* can provide mesopelagic bacteria and archaea with ammonium and labile amino acids, contributing to chemolithoautotrophy and organic matter remineralization in the mesopelagic zone, respectively. The balance between these two processes sets the ultimate role of the dark ocean in carbon sequestration with consequences for global climate. Given the

widespread blooms of *Trichodesmium* in the surface ocean, which are predicted to expand due to climate change [42], we contend that the impact of sinking *Trichodesmium* on the biogeochemistry of the dark ocean needs to be considered in carbon sequestration models.

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Figure legends

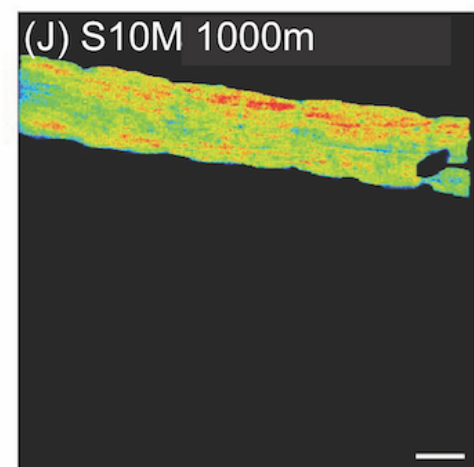
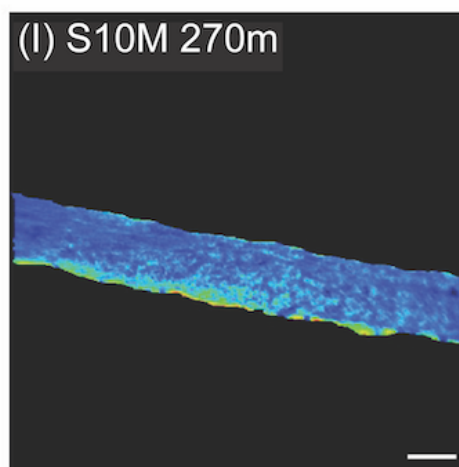
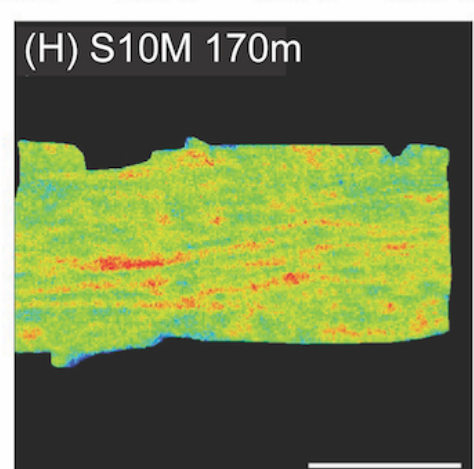
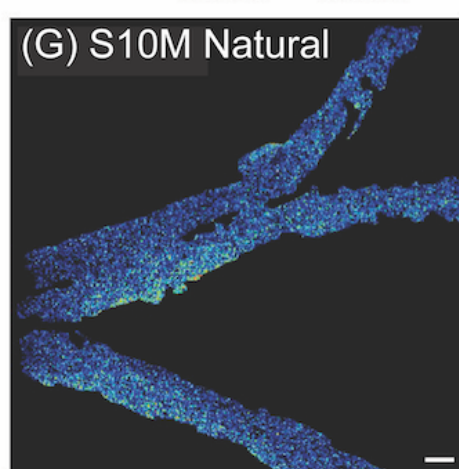
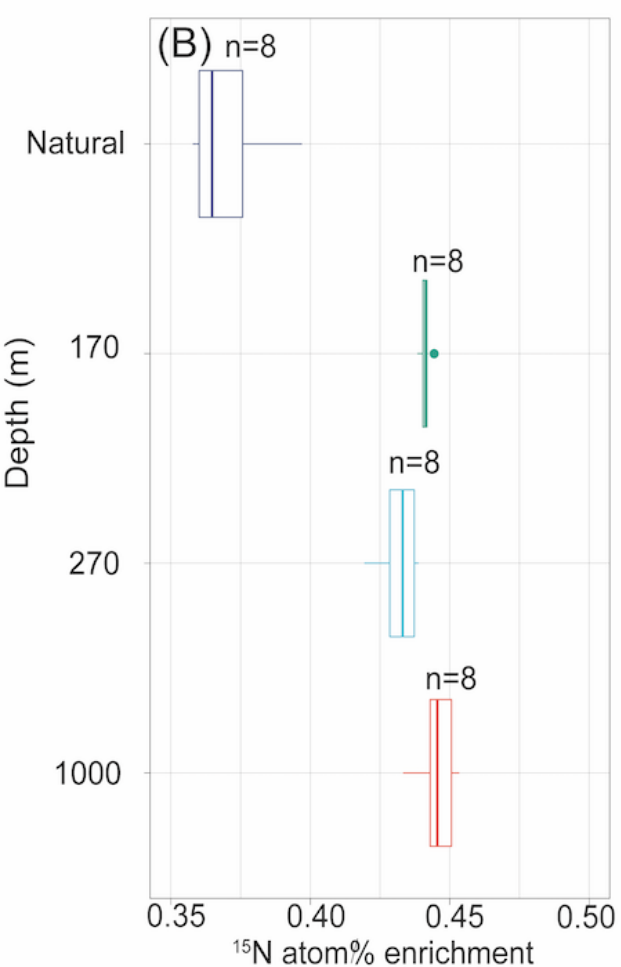
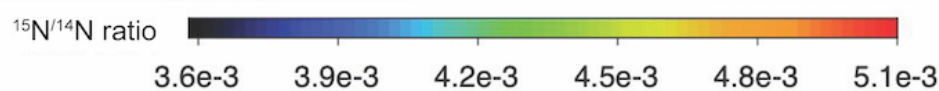
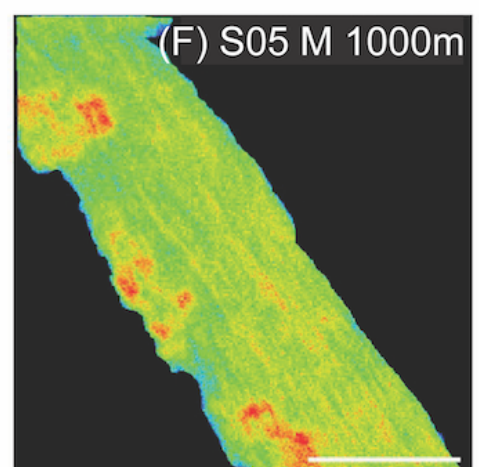
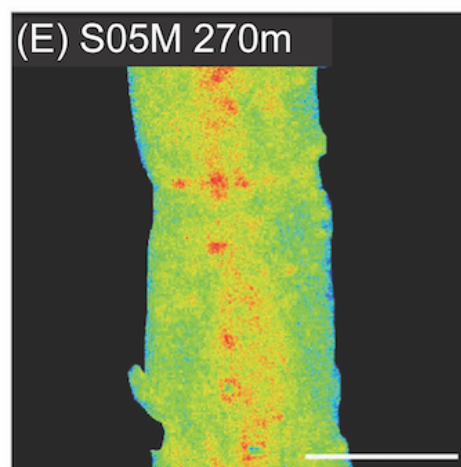
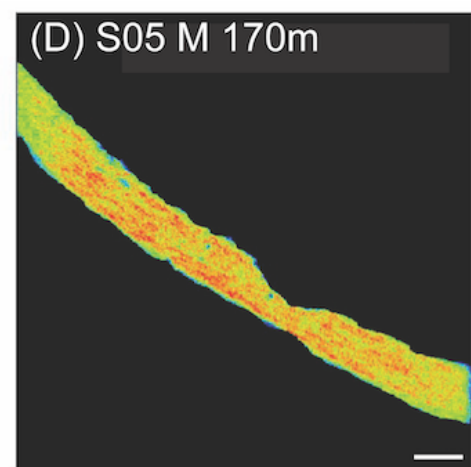
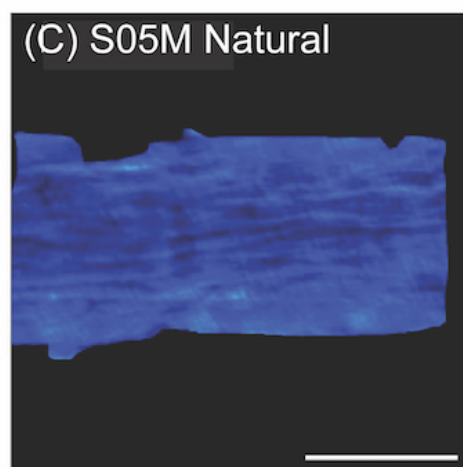
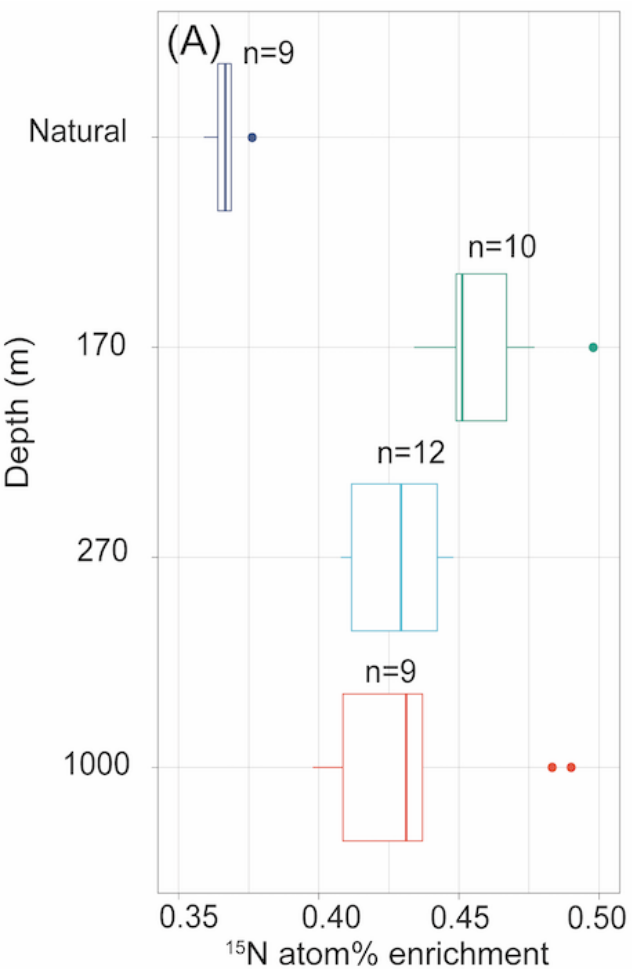
Figure 1. *Trichodesmium*-specific ^{15}N atom % enrichment. (A, B) Boxplots showing the range, average and outliers of $^{15}\text{N}/^{14}\text{N}$ ratios measured in natural (non $^{15}\text{N}_2$ -labeled) and $^{15}\text{N}_2$ -labeled samples from 170 m, 270 m and 1 000 m depth at stations S05M and S10M, respectively. The number of trichomes scanned per depth is shown over each box (n values). All *Trichodesmium* filaments analyzed were significantly enriched in ^{15}N . Examples of nanoSIMS $^{15}\text{N}/^{14}\text{N}$ ratio images of *Trichodesmium* filaments sampled at station S05M showing the $^{15}\text{N}/^{14}\text{N}$ isotopic ratio enrichment of *Trichodesmium* filaments according to the color bar. Filaments shown in panel (C) are those not incubated with $^{15}\text{N}_2$ (natural). The filaments shown in panels (D-F) are those incubated with $^{15}\text{N}_2$ and collected from sediment traps deployed at 170, 270 and 1 000 m, respectively. The same pattern is repeated for station S10M in panels (G-J). The scale bar in nanoSIMS $^{15}\text{N}/^{14}\text{N}$ ratio images is 5 μm .

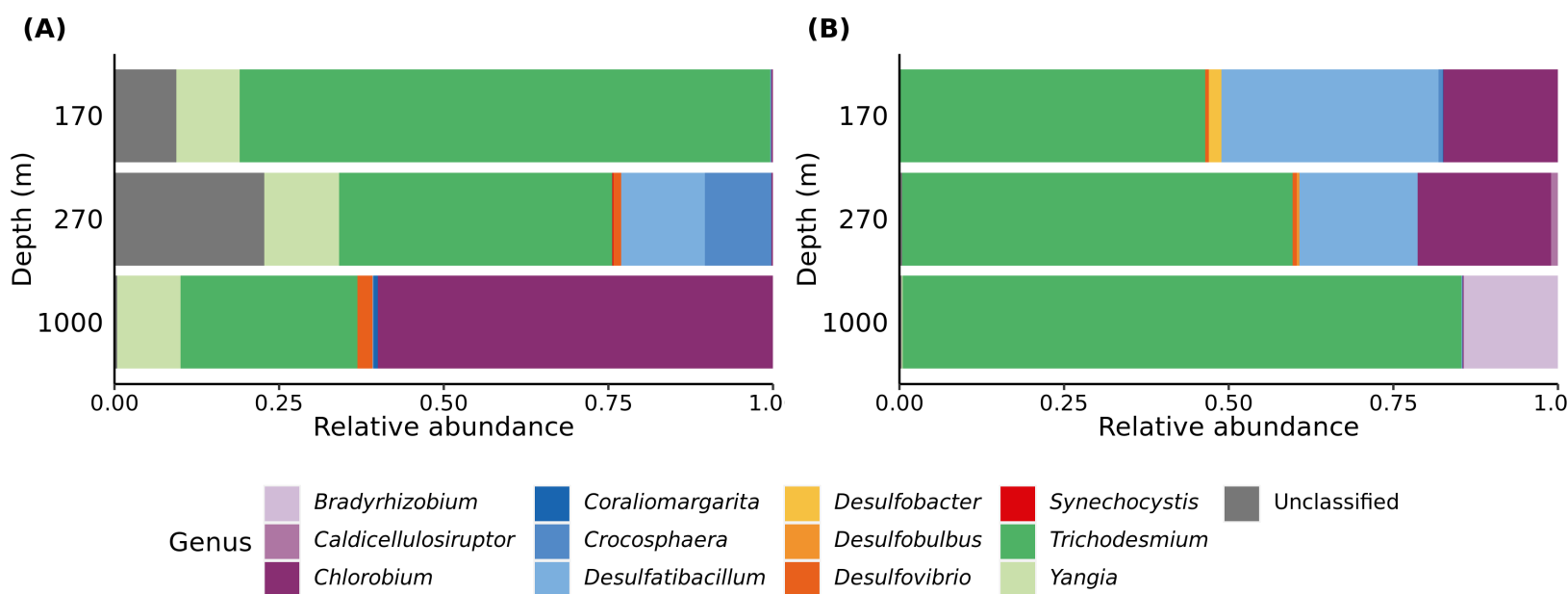
Figure 2. Diazotroph community expression profiles. Relative abundance of *nifH* cDNA reads originating from sediment traps at 170 m, 270 m, and 1 000 m at stations S05M (A) and S10M (B). Relative abundances were calculated based on $146\,000 \pm 18\,000$ reads per sample.

Figure 3. (A) ^{15}N atom % enrichment values of triplicate *Trichodesmium* cultures submitted to a 12h:12h dark:dark light cycle under increasing hydrostatic pressure (0-3 MPa) and decreasing temperature (27 to 14°C) on a sinking particle simulator for 192 and 360 h (simulating 160 and 300 m depth, respectively). (B) ^{15}N atom % enrichment

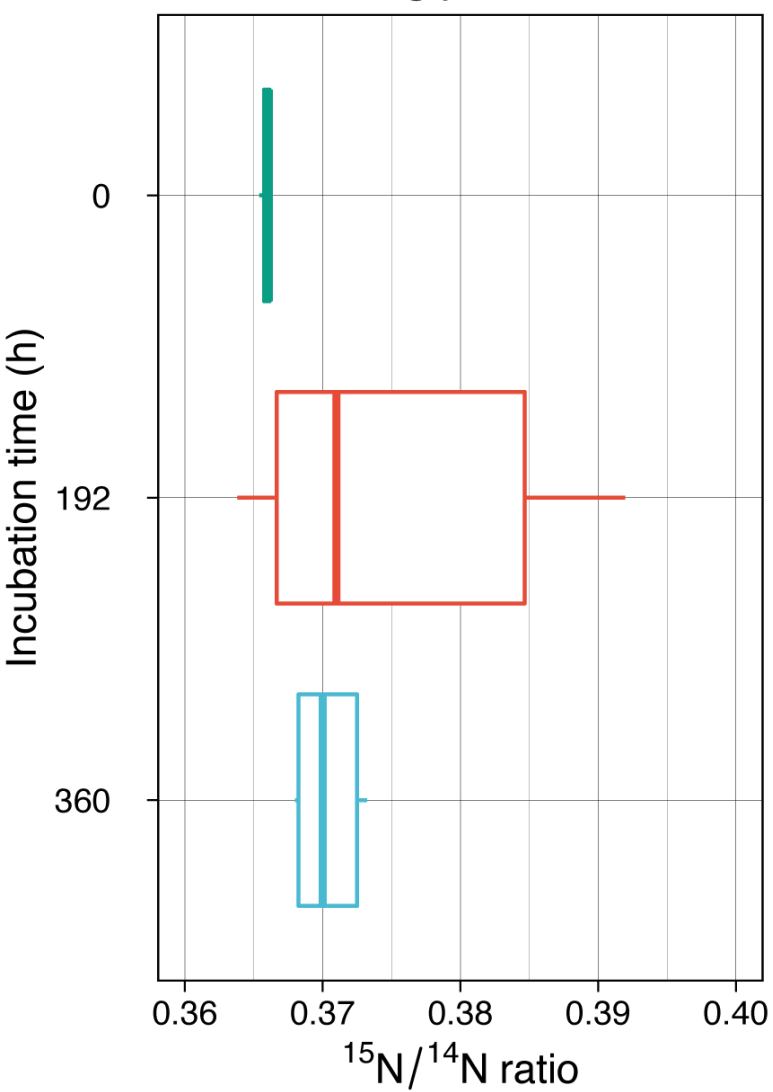
values of triplicate *Trichodesmium* cultures submitted to a 12h:12h dark:dark at ambient laboratory pressure and decreasing temperature (27 to 14°C) for 192 and 360 h.

Figure 4. Relationship between the initial carbon storage to biomass ratio (initial R_{Sto}) of *Trichodesmium* cells and the depth at which cell carbon storage is depleted. The x-axis represents the initial R_{Sto} , i.e. the carbon storage to biomass ratio of *Trichodesmium* at 40 m (the bottom of the mixing layer, where cells start to sink). The y-axis represents the depletion depth, i.e. the depth at which carbon storage becomes zero. The blue line represents the threshold depth below which cell carbon storage is depleted (and thus N_2 fixation is no longer possible) for various initial R_{Sto} values. The shaded area indicates the depletion depth range considering the variability in empirical and theoretical sinking velocities of *Trichodesmium* (Supplementary Methods; Table S2). The yellow and pink dots indicate the depletion depth for initial R_{Sto} values of 1 and 1.3, respectively. The blue line represents the simulation based on the median sinking velocity. The dashed line indicates the depletion depth of 1 000 m depth. The depletion depth must be below 1 000 m for cells to fix N_2 at 1000 m depth and explain our field observations.





(A) Increasing pressure



(B) Lab ambient pressure

