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1 **Sinking *Trichodesmium* fixes nitrogen in the dark ocean**

2

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23 interests.

24 **Abstract**

25

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

45 **Introduction**

46

47 Dinitrogen (N₂) fixing prokaryotes (diazotrophs) supply bioavailable nitrogen to
48 planktonic communities, fueling primary production and contributing to carbon export in
49 the ocean [1]. Nitrogen inputs by diazotrophs may become even more important in the
50 future ocean, as global warming enhances water column stratification constraining
51 nitrogen availability for primary producers [2]. Early studies suggested that diazotrophs
52 were only present in low latitude warm oligotrophic waters of the (sub)tropical ocean.
53 However, over the past decade it has become clear that diazotrophs are also found in
54 cold and nutrient-rich environments such as estuaries, shelf seas, polar regions and the
55 ocean's dark pelagic realm [3]. Devoid of light, N₂ fixation in the dark ocean has been
56 attributed to heterotrophic non-cyanobacterial diazotrophs presumably relying on
57 reduced organic compounds for energy and carbon supply [4–6]. However,
58 cyanobacterial diazotrophs have been repeatedly observed in mesopelagic to
59 bathypelagic depths (200 to 4 000 m; Table S1; [7]).

60

61 The filamentous cyanobacterium *Trichodesmium* thrives in tropical and subtropical
62 ocean's photic zone where it can introduce 60-80 Tg N y⁻¹, representing roughly half of
63 the reactive nitrogen input to the global ocean [8]. *Trichodesmium* has gas vesicles
64 which confer cells with buoyancy, restricting their vertical distribution in the water column
65 [9, 10]. Accordingly, *Trichodesmium* biomass is thought to be fully remineralized within
66 the photic zone [11]. Recently, however, molecular and imaging studies have
67 documented intact *Trichodesmium* cells and expression of nitrogenase genes down to 4
68 000 m depth across the world's oceans (Table S1). The mechanisms through which
69 *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

120 2 000 and 200 m (bottlenet [16]) were used to measure natural ^{15}N atom % enrichment
121 of bulk biomass and *Trichodesmium* cells. In parallel, the whole brine volume of the tube
122 filled with RNAlater was immediately filtered onto 0.2 μm polysulfone filters (Supor, Pall
123 Gelman, Port Washington, NY, USA). The filters were transferred to bead beater tubes
124 containing a mix of 0.1 mm and 0.5 mm silica beads, flash-frozen in liquid nitrogen and
125 stored at -80°C until RNA extractions (see below).

126

127 *Bulk and Trichodesmium-specific N_2 fixation rates*

128

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

132 The ^{15}N atom % enrichment of *Trichodesmium* filaments was analyzed on a nanoSIMS
133 50L (CAMECA, Gennevilliers, France) at the Leibniz Institute for Baltic Sea Research
134 (IOW, Germany). Sample filters were mounted with conductive tape on 10 x 5 mm
135 aluminum stubs (Ted Pella Inc., Redding, CA) and gold-coated to a thickness of ca. 30
136 nm (Cressington auto sputter coater). A 1 pA 16 keV Cesium (Cs^+) primary beam was
137 scanned on a 512 x 512 pixel raster with a raster area of 15 x 15 μm , and a counting
138 time of 250 μs per pixel. Samples were pre-sputtered with 600 pA Cs^+ current for 2 min
139 in a raster of 30 x 30 μm to remove the gold and surface contaminants and reach the
140 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 $^{31}\text{P}^-$
141 were detected with electron multiplier detectors, and secondary electrons were
142 simultaneously imaged. Sixty serial quantitative secondary ion mass planes were
143 generated, drift corrected and accumulated to the final image. Mass resolving power was
144 >8 000 to resolve isobaric interferences. Data was processed using the

145 Look@nanoSIMS software [24]. Isotope ratio images were generated by dividing the $^{13}\text{C}^-$
146 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
147 pixel. Individual *Trichodesmium* filaments (trichomes) were identified in nanoSIMS
148 secondary electron $^{12}\text{C}^-$, $^{12}\text{C}^{14}\text{N}^-$ images. These images were used to define regions of
149 interest (ROIs). For each ROI, the $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ ratios were calculated based on
150 the ion counts averaged over the ROIs.

151

152 Bulk N_2 fixation rates were calculated following the equations of Montoya et al. [25].

153 *Trichodesmium*-specific volumetric N_2 fixation rates were calculated using the following
154 equation:

155

156
$$\text{Volumetric } \text{N}_2 \text{ fixation rate} = \frac{(A_{\text{Tricho}} - A_{\text{TrichoNat}})}{(A_{\text{N}_2} - A_{\text{TrichoNat}})} \times \frac{PN_{\text{Tricho}}}{t}$$

157

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

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

172

173 RNA extractions, *nifH* gene sequencing and bioinformatics

174

175 RNA was extracted using the RNeasy mini kit (Qiagen) including a 1 h on-column
176 DNase digestion. PCRs on extracted RNA controlled for complete DNA digestion.
177 Reverse transcription (RT) were performed with TaqMan Reverse Transcription (Applied
178 Biosystems) using reverse primer *nifH3* (5'-ATR TTR TTN GCN GCR TA-3') and 5 µl of
179 RNA extract. Triplicate nested PCR reactions were conducted using degenerate *nifH*
180 primers *nifH1* (5'-TGYGAYCCNAARGCNGA-3'write here), *nifH2* (5'-
181 ADNGCCATCATYTCNCC-3'), *nifH3* and *nifH4* (5'-TTYTAYGGNAARGGNGG-3') [29].
182 The PCR mix was composed of 5 µl of 5X MyTaq red PCR buffer (Bioline), 1.25 µl of 25
183 mM MgCl₂, 0.5 µl of 20 µM forward and reverse primers, 0.25 µl Platinum Taq and 5 µl
184 of cDNA (1 µl from the first PCR reaction was used as template in the second reaction).
185 The reaction volume was adjusted to 25 µl with PCR grade water. Triplicate PCR
186 products were pooled and purified using the GeneClean Turbo kit (MP Biomedicals).
187 Samples were sequenced using the Illumina MiSeq platform with 2 × 300 bp paired-end
188 reads. Demultiplexed paired-end sequences were dereplicated, denoised, assembled
189 and chimeras discarded using the DADA2 pipeline [30]. This generated 6 929 ASVs
190 (146 000 ± 18 000 reads per sample). Sequences have been deposited in the Sequence
191 Read Archive under accession number PRJNA742179. The ASVs were translated to
192 amino acid sequences using FrameBot [31] and filtering for homologous genes was
193 done following the NifMAP pipeline [32]. This reduced the number of ASVs to 6 503
194 accounting for 842 515 reads (96% of all reads). Taxonomic ranks were assigned

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

197

198 *Hydrostatic pressure experiments with Trichodesmium cultures*

199

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

220 12h:12h dark:dark cycle and temperature decrease pace identical to that of pressurized
221 cultures were used as a control.

222

223 *Sinking Trichodesmium cell metabolism model*

224

225 We hypothesized that *Trichodesmium* fixes N₂ while sinking into the dark ocean at the
226 expense of carbon accumulated from photosynthesis before starting to sink. Sinking
227 *Trichodesmium* can thus only fix N₂ until reaching the depth where its carbon storage is
228 depleted. To compute how much carbon storage *Trichodesmium* needs to acquire
229 before sinking to be able to fix N₂ at 1 000 m depth, we adapted a previously published
230 coarse-grained cell metabolism model [35]. The model simulates photosynthesis, N₂
231 fixation, and respiration for the entire trichome, resolving diffusion boundary layers for
232 oxygen transport and distinguishing between photosynthetic and non-photosynthetic
233 cells (Fig. S1). Photosynthetic cells fix carbon with harvested light energy, accumulate
234 carbon, and synthesize new biomass, whereas non-photosynthetic cells use carbon
235 stored by other cells to fix N₂ (Fig. S1).

236

237 To adapt the model to *Trichodesmium* colonies sinking into the mesopelagic layer we
238 considered the variability in temperature and oxygen observed from the ocean surface to
239 1 000 m in our study (Fig. S2) as well as the inhibiting effect of increasing nitrate
240 concentrations on N₂ fixation. Walden's rule [47] was used to simulate the variation of
241 oxygen diffusivity, as in previous studies [37], and the Arrhenius equation was used to
242 simulate the temperature dependencies of metabolisms [35]. The adapted model was
243 applied to *Trichodesmium* sinking from the bottom of the mixed layer (~40 m; Fig. S2)
244 over a wide range of calculated velocities (ranging from 12 to ~600 m d⁻¹;

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

253

254 Statistical analyses

255

256 ^{15}N atom % enrichment values were checked for normality using a Shapiro-Wilk test and
257 significant differences between samples tested with Wilcoxon test, using R software
258 package dplyr in RStudio Version 1.2.5033.

259

260 **Results and Discussion**

261

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

263

264 The isotopic enrichment of single *Trichodesmium* filaments in the traps ranged between
265 0.428 ± 0.002 and 0.463 ± 0.033 ^{15}N atom % (Fig. 1). The ^{15}N atom % enrichment of all
266 *Trichodesmium* filaments analyzed was significantly higher than that of filaments not
267 incubated with $^{15}N_2$ collected over the same depth range (0.364 ± 0.006 ^{15}N atom %;
268 Wilcoxon test $p = 0.004$ and $p = 0.014$ for S05M and S10M, respectively; Fig. 1). The
269 derived cell-specific N_2 fixation rates ranged between 36 and 214 $fmol\ N\ cell^{-1}\ d^{-1}$ (Table

270 S3), in the same range of previous *Trichodesmium* cell-specific N₂ fixation
271 measurements in surface waters of the South Pacific [39, 40]. While the salinity in the
272 traps (~50 ppt) was higher than that of ambient waters (~34 ppt), previous studies have
273 shown that increased salinity reduces but does not impair *Trichodesmium* growth up to
274 42 ppt [41]. Comparing in *Trichodesmium* cultures grown on 34 and 50 ppt showed a
275 decrease in N₂ fixation rates by 63 ± 15 % (Supplementary Methods). Hence, the high
276 salinity of the ¹⁵N₂-labeled brine may have reduced but did not completely inhibit N₂
277 fixation in *Trichodesmium*, implying that in situ rates could be higher than measured in
278 our traps. Overall, these cell-specific rates indicate that *Trichodesmium* sinking into the
279 mesopelagic zone can fix N₂ at rates comparable to the surface.

280

281 Amplicon sequencing revealed that *nifH* gene transcripts annotated as *Trichodesmium*
282 were present in all sediment trap samples, accounting for 26-84% (average 56%) of the
283 sequence reads (Fig. 2). This indicates that *Trichodesmium* constituted a substantial
284 fraction of the diazotroph community that actively transcribed the *nifH* gene in all
285 samples (Fig. 2). However, comparing the ¹⁵N₂ atom % enrichment of bulk sediment trap
286 material and individual filaments, *Trichodesmium* accounted for 1 to 70% of the N₂
287 fixation activity measured in the traps (Table S3). Hence, other organisms contributed to
288 N₂ fixation within the traps, which could be driven by either surface diazotrophs attached
289 to particles or by true mesopelagic N₂ fixation driven by diazotrophs residing in deep
290 waters. The contribution of *Trichodesmium* to mesopelagic N₂ fixation in the subtropical
291 region studied here is notorious but likely restricted to the (sub)tropical regions where
292 this cyanobacterium abounds. The predicted warming and expansion of oligotrophic
293 subtropical gyres towards higher latitudes may expand the effect of sinking
294 *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

320 increasing hydrostatic pressure for 192 and 360 h in the dark (equivalent to 150 and 300
321 m simulated depth) had ^{15}N atom % enrichment values of 0.375 ± 0.012 and $0.370 \pm$
322 0.002 atom %, respectively (Fig. 3A), indicating low but measurable N_2 fixation. Control
323 *Trichodesmium* cultures kept at ambient lab pressure conditions had ^{15}N atom %
324 enrichments of 0.390 ± 0.012 and 0.393 ± 0.008 after 192 and 360 h of incubation,
325 respectively (Fig. 3B). This difference in pressurized (Fig. 3A) and non-pressurized (Fig.
326 3B) N_2 fixation rates is in agreement with previous metabolic rate slowdown in epipelagic
327 prokaryotes submitted to mesopelagic pressure levels [45] and indicates that increasing
328 pressure reduces but does not completely incapacitate N_2 fixation in *Trichodesmium*. We
329 however note that while the sinking simulator allows changing pressure and
330 temperature along the simulated sinking process, it does not allow changing nutrient
331 concentrations. The increasing concentrations of nitrate with depth in the mesopelagic
332 may inhibit N_2 fixation. Thus, the rates measured in these sinking simulations represent
333 a non-inhibited upper limit of diazotrophic activity.

334

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

336

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

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

349

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

362

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

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

374

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

390

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

392

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

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

409

410 Potential impact of sinking *Trichodesmium* on mesopelagic prokaryotic communities

411

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

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

421

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

433

434 **Conclusions**

435

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

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

448

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450

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463

464 **Competing Interests:** Authors declare that they have no competing interests.

465

466 **Data Availability Statement:** All data are available in the main text or the
467 supplementary materials. The model code for this study can be found in

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469 been deposited in the Sequence Read Archive under accession number PRJNA742179.

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- 664

665 **Figure legends**

666

667 **Figure 1.** *Trichodesmium*-specific ^{15}N atom % enrichment. (A, B) Boxplots showing the
668 range, average and outliers of $^{15}\text{N}/^{14}\text{N}$ ratios measured in natural (non $^{15}\text{N}_2$ -labeled) and
669 $^{15}\text{N}_2$ -labeled samples from 170 m, 270 m and 1 000 m depth at stations S05M and
670 S10M, respectively. The number of trichomes scanned per depth is shown over each
671 box (n values). All *Trichodesmium* filaments analyzed were significantly enriched in ^{15}N .
672 Examples of nanoSIMS $^{15}\text{N}/^{14}\text{N}$ ratio images of *Trichodesmium* filaments sampled at
673 station S05M showing the $^{15}\text{N}/^{14}\text{N}$ isotopic ratio enrichment of *Trichodesmium* filaments
674 according to the color bar. Filaments shown in panel (C) are those not incubated with
675 $^{15}\text{N}_2$ (natural). The filaments shown in panels (D-F) are those incubated with $^{15}\text{N}_2$ and
676 collected from sediment traps deployed at 170, 270 and 1 000 m, respectively. The
677 same pattern is repeated for station S10M in panels (G-J) The scale bar in nanoSIMS
678 $^{15}\text{N}/^{14}\text{N}$ ratio images is 5 μm .

679

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

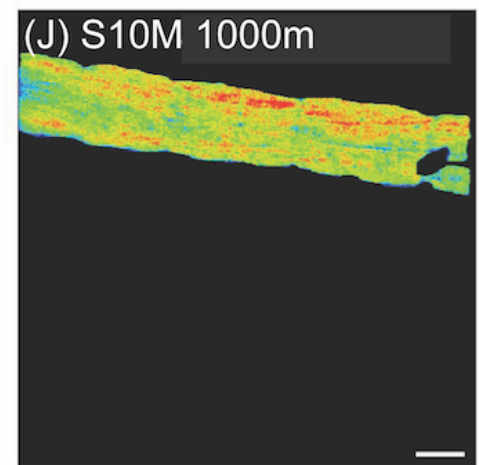
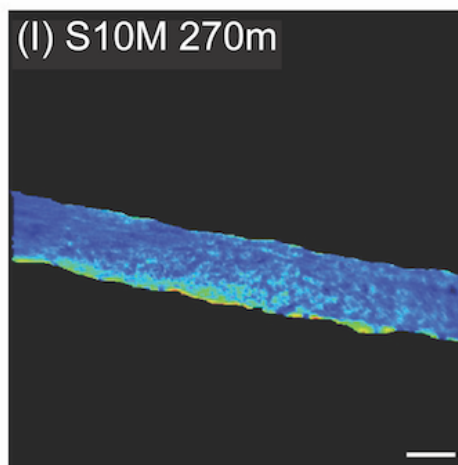
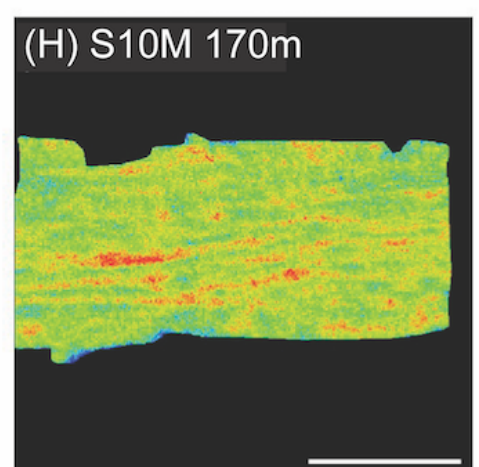
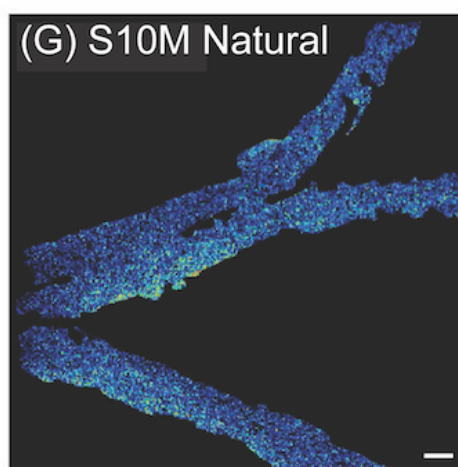
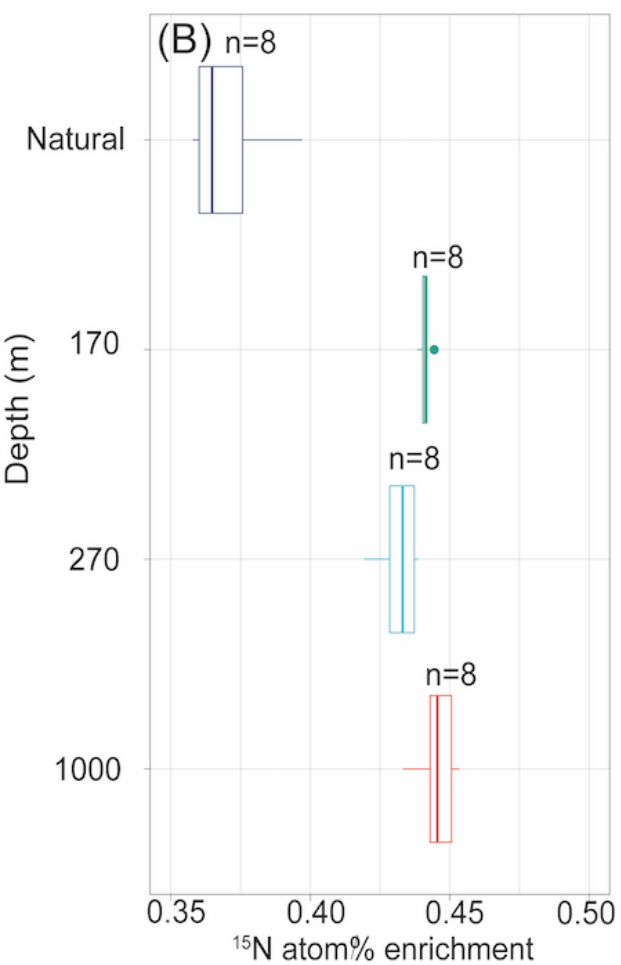
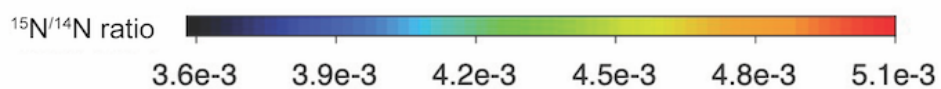
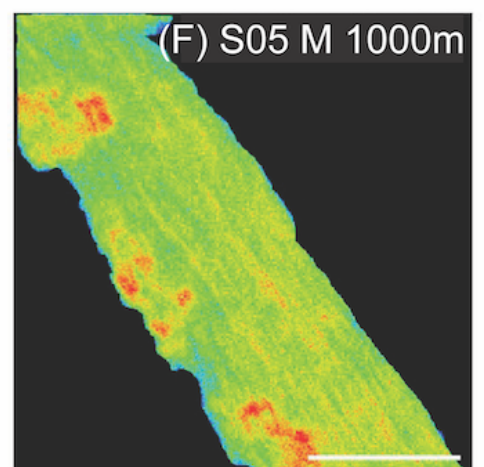
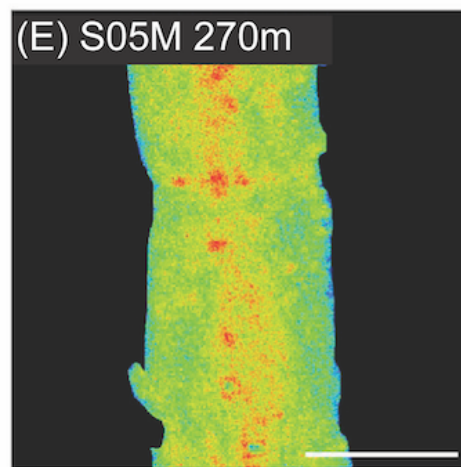
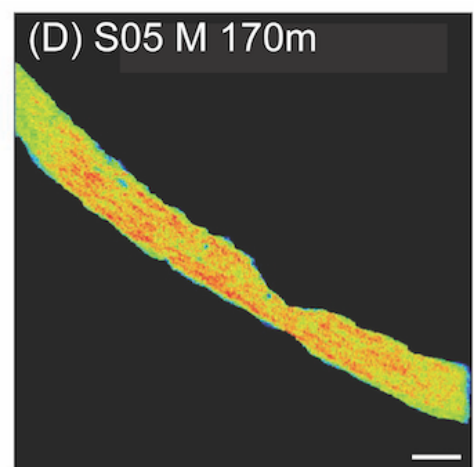
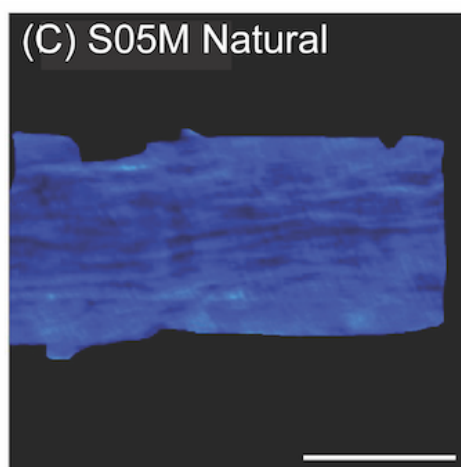
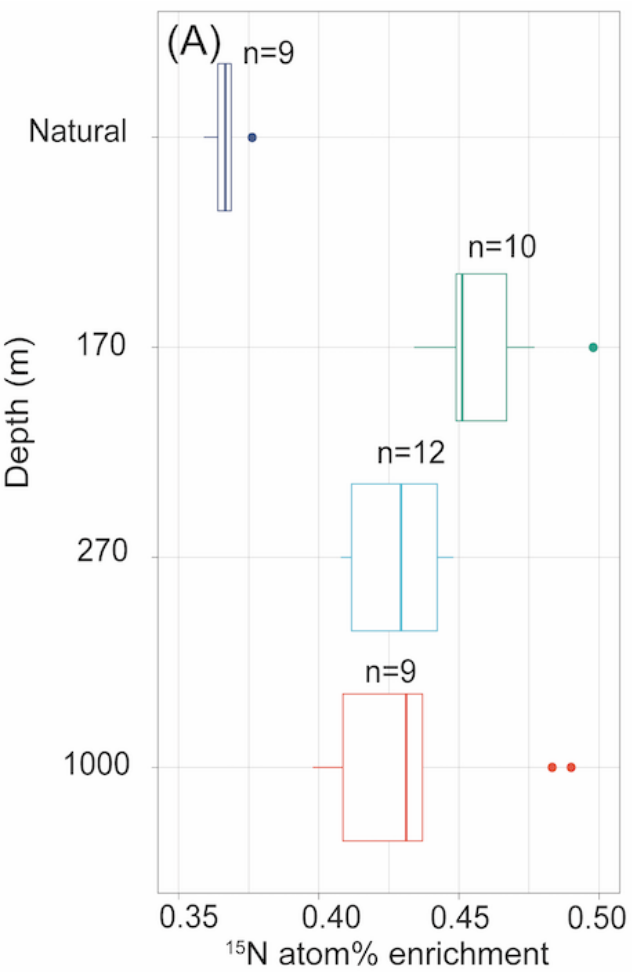
684

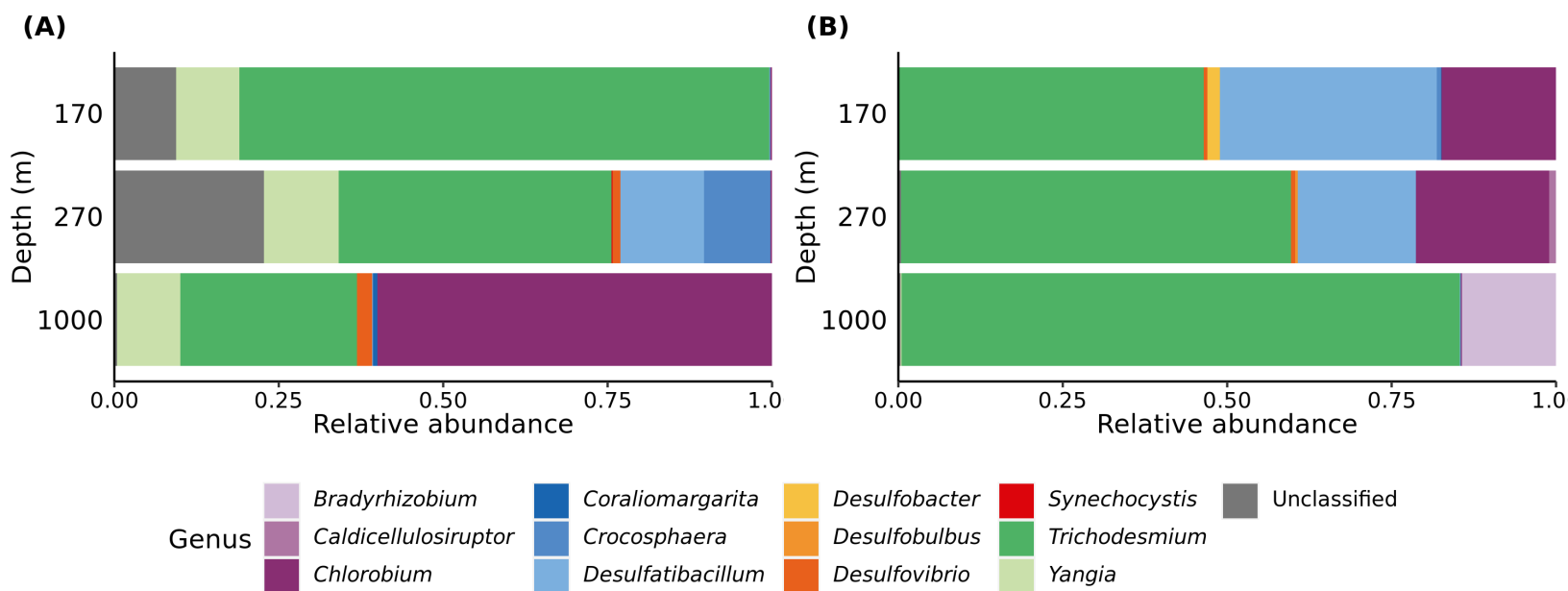
685 **Figure 3.** (A) ^{15}N atom % enrichment values of triplicate *Trichodesmium* cultures
686 submitted to a 12h:12h dark:dark light cycle under increasing hydrostatic pressure (0-3
687 MPa) and decreasing temperature (27 to 14°C) on a sinking particle simulator for 192
688 and 360 h (simulating 160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

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

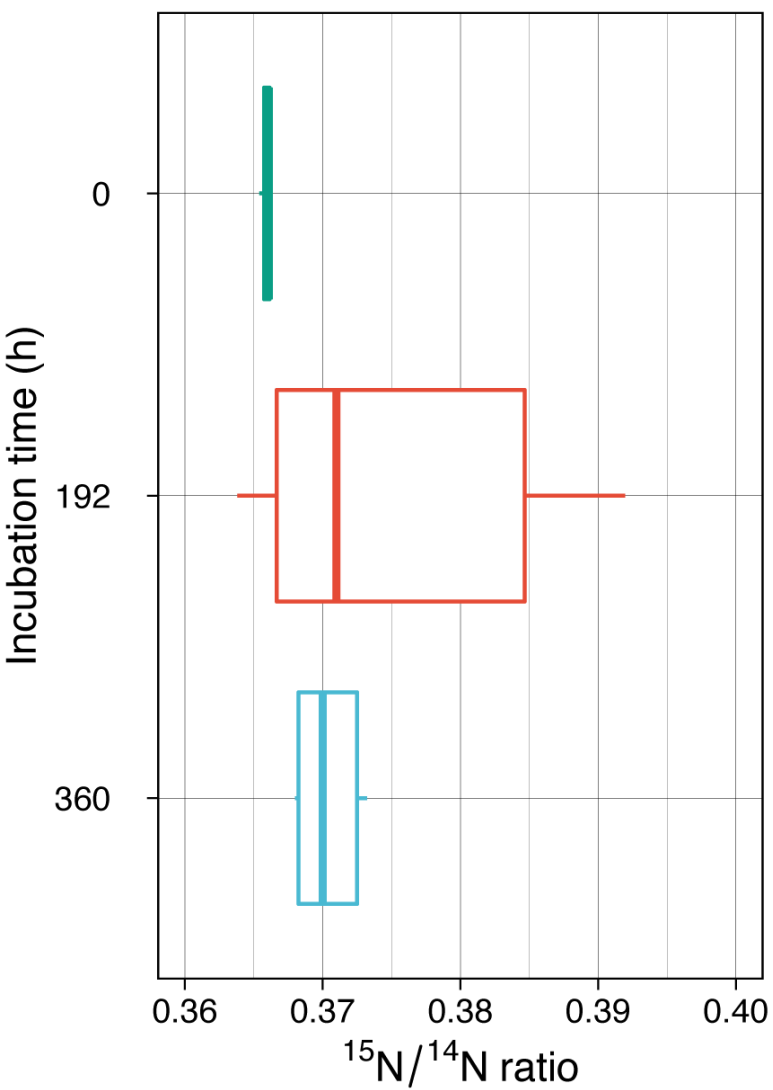
691

692 **Figure 4.** Relationship between the initial carbon storage to biomass ratio (initial R_{Sto}) of
693 *Trichodesmium* cells and the depth at which cell carbon storage is depleted. The x-axis
694 represents the initial R_{Sto} , i.e. the carbon storage to biomass ratio of *Trichodesmium* at
695 40 m (the bottom of the mixing layer, where cells start to sink). The y-axis represents the
696 depletion depth, i.e. the depth at which carbon storage becomes zero. The blue line
697 represents the threshold depth below which cell carbon storage is depleted (and thus N_2
698 fixation is no longer possible) for various initial R_{Sto} values. The shaded area indicates
699 the depletion depth range considering the variability in empirical and theoretical sinking
700 velocities of *Trichodesmium* (Supplementary Methods; Table S2). The yellow and pink
701 dots indicate the depletion depth for initial R_{Sto} values of 1 and 1.3, respectively. The
702 blue line represents the simulation based on the median sinking velocity. The dashed
703 line indicates the depletion depth of 1 000 m depth. The depletion depth must be below
704 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

