



## 19 **Abstract**

20           Cyanobacteria have played a profound role in shaping the biosphere, most notably  
21 through the Great Oxygenation Event (GOE) with the advent of photosynthesis<sup>1</sup>. Cyanobacteria  
22 also contribute to global primary production through biological nitrogen fixation (BNF) using  
23 nitrogenase<sup>2,3</sup>, an oxygen-labile enzyme complex that evolutionarily predates the GOE<sup>4</sup>. Current  
24 literature reports nitrogenase activity in unicellular cyanobacteria is protected from oxygen  
25 through diurnal separation of photosynthesis and BNF<sup>5</sup>. However, historic conditions of  
26 continuous-light and warm temperature at polar latitudes during the Triassic and Cretaceous may  
27 have created a selective advantage amongst unicellular cyanobacteria for non-temporal  
28 mechanisms of maintaining nitrogenase activity in the presence of oxygen. Here we report  
29 constitutive nitrogenase activity concurrent with a net-gain of oxygen through photosynthesis in  
30 a continuous-light adapted culture of the unicellular cyanobacteria, *Cyanothece* sp. ATCC  
31 51142. Nitrogenase activity in the adapted culture exhibited dependence on light and an  
32 increased resilience to artificially raised oxygen-tension compared to traditional culture. We  
33 predict cyanobacteria closely related to *Cyanothece* sp. ATCC 51142 also possess this  
34 physiology and found an accessory predicted proteome with functional relevance. This work  
35 provides a model of light-driven, oxygen-tolerant, constitutive nitrogenase activity and suggests  
36 this physiology may be conserved in closely related unicellular diazotrophic cyanobacteria with  
37 implications for primary production in polar ecosystems and potential biotechnological  
38 application in sustainable agriculture production.

## 39 **Introduction**

40 Cyanobacteria are reported to overcome the oxygen sensitivity of nitrogenase by  
41 employing spatial or temporal mechanisms to create aerobic, micro-oxic, or anaerobic  
42 environments<sup>6</sup>. For instance, filamentous *Anabaena* sp. PCC 7120 forms heterocysts to protect  
43 nitrogenase in a micro-oxic environment that lacks oxygen-producing photosystem II and  
44 performs high respiration<sup>7</sup>. The unicellular cyanobacterium, *Cyanothece* sp. ATCC 51142, was  
45 previously reported to separate the two processes of oxygen-producing photosynthesis and  
46 oxygen-labile nitrogen fixation temporally within the same cell using a diurnal rhythm<sup>5</sup>. Other  
47 unicellular cyanobacteria, like *Cyanothece* sp. PCC 7425 (formerly *Synechococcus* sp. PCC  
48 7425), are dependent on an externally controlled anaerobic environment to fix nitrogen.<sup>8</sup>

49 Currently reported mechanisms of nitrogen fixation are useful under the prevailing  
50 environmental constraints of cyanobacterial growth in dark/light cycles in waters at temperate  
51 latitudes. However, recent discoveries of free-living and symbiotic unicellular cyanobacteria in  
52 polar regions exposed to continuous-light<sup>9,10</sup> raises the possibility of non-temporal mechanisms  
53 of nitrogenase protection in unicellular cyanobacteria. While filamentous cyanobacteria can fix  
54 nitrogen in continuous light within the protective heterocyst, there may be a selective advantage  
55 amongst diazotrophic unicellular cyanobacteria capable of non-temporal protection of  
56 nitrogenase when exposed to continuous light.

57 In addition to fixing nitrogen, cyanobacterial nitrogenase also produces hydrogen gas,  
58 resulting in overlapping research between these two areas. Recent experiments maintained under  
59 a micro-oxic atmosphere suggest a link between photosystems and nitrogenase-catalyzed  
60 hydrogen production when a *Cyanothece* sp. ATCC 51142 culture was transitioned from  
61 nitrogen-replete (CL N+) to nitrogen-deplete conditions under continuous-light (CL N-)<sup>11</sup>.  
62 *Cyanothece* sp. ATCC 51142 was also reported to have high hydrogen production under light

63 when transitioned from the dark phase of a dark-light cycle, concomitant with a net loss of  
64 oxygen in both media and headspace when the experiment was run in a closed, non-sparged  
65 system<sup>12</sup>. While these experiments focus on hydrogen production under micro-oxic conditions,  
66 they hint that unicellular cyanobacteria may be able to harness light to drive nitrogenase activity,  
67 consistent with our hypothesis.

## 68 **Results**

69 We found sub-culturing *Cyanothece* sp. ATCC 51142 in ASPII N- media in continuous  
70 light for many generations (CL N-) resulted in constitutive nitrogenase activity under normal  
71 atmospheric conditions. Previous studies with *Cyanothece* sp. ATCC 51142 in CL N- under  
72 normal atmospheric conditions only allowed short periods of adaptation, or did not take time-series  
73 measurements, likely explaining why this physiology has yet to be reported for *Cyanothece* sp.  
74 ATCC 51142.

75 Following the discovery of constitutive nitrogenase activity, we took oxygraphy  
76 measurements of the adapted culture, demonstrating a net gain of oxygen concurrent with  
77 nitrogenase activity in our CL N- adapted culture (Figure 1B). While previous reports have  
78 shown nitrogenase activity in a non-adapted culture of CL N- *Cyanothece* sp. ATCC 51142, the  
79 cultures were still entrained in a cycle, resulting in separation of photosynthesis from nitrogen  
80 fixation. We separately validated the oxygraphy data using a secondary experiment measuring  
81 nitrogenase activity and oxygen changes over time in non-sparged, closed vials (data not shown  
82 here).

83 Next, we aimed to determine the effect of oxygen on nitrogenase activity in this adapted  
84 culture. Increasing oxygen produced a small negative effect on nitrogenase activity in the  
85 adapted CL N- culture relative to the DL N- culture (Figure 1C and 1D, respectively). This

86 suggests, as expected, oxygen increase in the oxygraphy and headspace experiment were not  
87 causal for increased nitrogenase activity, but more likely an artefact of the increased photosystem  
88 activity that generates the reductive energy and ATP for nitrogenase activity. This also shows  
89 that the CL N- culture has an increased tolerance to oxygen relative to the DL N- culture.

90 The building experimental evidence suggests a photosystem driven nitrogenase activity,  
91 leading us to compare the effect of light on nitrogenase activity between CL N- and DL N-  
92 cultures. Our CL N- culture exhibited nitrogenase activity in illuminated incubation while the  
93 dark incubated CL N- culture had undetectable nitrogenase activity (Figure 2C) which is  
94 indicative that this physiology is photosystem driven. DL N- culture tested during peak  
95 nitrogenase activity exhibited nitrogenase activity in both illuminated and dark incubation, with  
96 slightly higher activity in under illumination (Figure 2D), possibly due to photo-cyclic  
97 phosphorylation while PSII was shut-down by the PsbA sentinel protein<sup>13</sup>.

98 In addition to undetectable nitrogenase activity in the dark, CL N- cells had less bacterial  
99 glycogen granules than DL N-, as observed by TEM imaging (Figure 2A and 2B). The DL N-  
100 culture presented many bacterial glycogen granules, consistent with previous literature<sup>14</sup>.  
101 Bacterial glycogen granules are produced and stored during the day to fuel the respiration  
102 necessary for dark constrained nitrogen fixation in DL N- cultured *Cyanothece* sp. ATCC 51142.  
103 The lower presence of bacterial glycogen granules in the CL N- culture adds to the evidence  
104 suggesting this adapted CL N- physiology is more dependent on photosynthesis than respiration  
105 for nitrogenase activity. However, the oxygraphy tests show respiration still occurs at high levels  
106 relative to the CL N+ culture, indicating that relatively high respiration is still associated with  
107 this physiology (data not shown here).

108           After discovering oxygen-tolerant nitrogenase activity in *Cyanothece* sp. ATCC 51142,  
109 we further investigated the literature for closely related cyanobacteria that might suggest  
110 conservation of this physiology. One paper reported similar physiology in *Gloeothece* sp.  
111 68DGA<sup>15</sup>. Further supporting our prediction of physiological conservation, *Crocasphaera*  
112 *watsonii* WH8501 has been reported to grow diazotrophically under continuous light for  
113 weeks<sup>16</sup>. However, the data was not made available and the relation of photosynthesis and  
114 nitrogen fixation was not investigated. *Cyanothece* sp. PCC 8801, formerly *Synechococcus* sp.  
115 RF-1, has also been reported to perform continuous nitrogenase activity in continuous light, but  
116 its photosynthetic regime was not investigated<sup>17</sup>. We found the cyanobacteria predicted to share  
117 this physiology share a most recent common ancestor that is absent from other *Cyanothece*, such  
118 as *Cyanothece* sp. PCC 7425<sup>18</sup>. This information indicates that *Gloeothece* sp. 68DGA,  
119 *Cyanothece* sp. ATCC 51142, *Cyanothece* sp. PCC 8801, and *Crocasphaera watsonii* WH8501  
120 may all share this ability, despite a current lack of data.

121           Supporting this prediction, analysis of UniProt predicted proteomes revealed a unique  
122 accessory proteome common between *Cyanothece* sp. ATCC 51142, *Crocasphaera watsonii*  
123 WH8501, and *Cyanothece* sp. PCC 8801 (CL N- group) but absent from the control group. The  
124 control group consisted of the pan-proteome constructed from the UniProt predicted proteome of  
125 anaerobic diazotroph *Cyanothece* sp. PCC 7425 and closely related but non-diazotrophic  
126 *Synechocystis* sp. PCC 6803. These comparisons were made with *Cyanothece* sp. ATCC 51142  
127 as the reference database with a 50% amino acid sequence similarity as the cutoff. We found 431  
128 unique proteins in the CL N- group and 686 predicted proteins unique to the control group. To  
129 gain an understanding of the functional significance of these predicted proteins we used public  
130 data from a recent transcriptome project<sup>19</sup> to analyze differential correlation of these genes

131 between DL N- and CL N- conditions over time. While the transcriptome project did not observe  
132 the physiology we are reporting, likely due to the short transition time from DL N-, the start of  
133 the adaptive response to continuous light still gives valuable insights that can be expounded on in  
134 later experiments. We followed the differential correlation analysis with a network analysis and  
135 GO enrichment analysis to allow functional inferences.

136         The accessory proteome of the CL N- group had higher network inter-connectivity  
137 compared to the control (Figure 3A and 3B, respectively) indicating these genes are more  
138 orchestrated in expression changes between dark/light cycled and continuous light conditions.  
139 The CL N- group had significantly higher GO enrichment for transmembrane transport and  
140 cellular metabolic processes (Figure 3C). Genes unique to the CL N- group with significant  
141 differential correlation ( $p < 0.001$ ) between DL N- and CL N- were investigated for functional  
142 implications. A three-gene operon (*cce\_0574-cce\_0576*) located near the *nif*-operon is predicted  
143 to facilitate ferrous ion uptake. A predicted NifU-like gene (*cce\_1857*) was also found to be  
144 unique to the CL N- group. Iron is an important part of the nitrogenase Fe protein and NifU  
145 proteins are involved in Fe-S cluster construction and repair. Other genes of interest are  
146 predicted to be involved in transcription and translation regulation or protein-protein interactions.  
147 Most predicted genes found to be significantly differentially expressed were hypothetical  
148 proteins, many of which were predicted to be membrane proteins.

## 149 **Discussion**

150         This work establishes a model culture for oxygen-tolerant nitrogenase activity as  
151 evidenced by comparative measurements of CL N- and DL N- nitrogenase activity with  
152 complementary oxygraphy, headspace oxygen, and manipulated oxygen concentration  
153 experiments. We began elucidation of the mechanisms through acetylene reduction assays and

154 TEM ultrastructure analysis. We predict this adaptive ability may be conserved in cyanobacteria  
155 originating from a shared common ancestor. The foremost question we interrogate below is if  
156 nitrogen fixation and photosynthesis are occurring simultaneously in single cells. Regardless of  
157 the culture's tendency towards physiologic homogeneity or heterogeneity, there is nitrogenase  
158 activity concurrent with a net gain of oxygen, suggesting some mechanism of oxygen-tolerance.  
159 We also discuss the possible conservation of this ability and its implications for ecosystem  
160 function and biotechnological application.

161         There have been limited previous attempts to determine if an individual cell can fix  
162 nitrogen and produce oxygen simultaneously, likely due to the counter-intuitive nature of the  
163 proposition. *Gloeotheca* sp. 68DGA is reported to have a homogenous and time-tolerant  
164 distribution of NifH protein throughout the culture when adapted to continuous light, suggesting  
165 all cells are fixing nitrogen<sup>15</sup>. One of the few papers to-date citing the *Gloeotheca* sp. 68DGA  
166 paper observed *Crocasphaera watsonii* WH 8501 and determined individual cells could not fix  
167 carbon and nitrogen simultaneously to a large extent<sup>20</sup>. However, this *Crocasphaera watsonii*  
168 WH 8501 culture was only exposed to continuous light for 24 hours, the acetylene reduction  
169 assays indicated the culture was still entrained in a rhythm, and oxygen production was not  
170 measured.

171         We propose two explanations for our observed physiology. The first possible explanation  
172 is that the culture is physiologically heterogenous where a portion of the cells are performing  
173 high respiration to protect nitrogenase from oxygen and provide reducing equivalents while the  
174 other portion of the culture is performing photosynthesis. Our second explanation is that the  
175 culture is homogenous and most of the cells are fixing nitrogen and performing photosynthesis



176 simultaneously. Our results, when evaluated together, are not consistent with physiological  
177 heterogeneity and indicate the higher likelihood of a physiologically homogenous culture.

178         If individual cells in the CL N- culture were relying solely on high respiration to protect  
179 and support nitrogenase activity, they should still exhibit nitrogenase activity when incubated in  
180 the dark. However, we observed undetectable levels of nitrogenase activity in this scenario  
181 (Figure 2C) indicating the necessity of light and implicating photosystems as a driving force for  
182 this physiology. We also observed a net gain of oxygen concurrent with nitrogenase activity in  
183 the CL N- adapted culture (Figure 1B), indicating that a heterogenous culture would need  
184 individual cells with respiration rates high enough to decrease their local oxygen without  
185 decreasing the total oxygen. This seems unlikely, especially considering the culture is planktonic  
186 and shaken. The lower content of bacterial glycogen granules in CL N- relative to DL N- also  
187 contradicts the proposition of individual cells with abnormally high respiration in CL N-,  
188 assuming the hypothetical “high respiration” cell would fuel respiration with stored  
189 carbohydrates in the bacterial glycogen granules consistent with previous literature<sup>21</sup>. The results  
190 of this multi-faceted interrogation indicate the higher likelihood of a physiologically  
191 homogenous culture than a heterogenous culture.

192         This physiological adaptation, which we predict is conserved in the CL N- group (Figure  
193 3) may have originated from a selective advantage in response to environmental parameters  
194 present in the warm, open waters at polar latitudes that were exposed to continuous light during  
195 the Triassic<sup>22</sup> and Cretaceous<sup>23</sup> periods. The oceans were also highly nutrient limited in the  
196 Jurassic<sup>24</sup> likely flowing over into the Cretaceous and creating a demand for nitrogen fixation as  
197 has been proposed for the symbiotic UCYN-A, which is closely related to *Cyanothece* sp. ATCC  
198 51142<sup>18</sup>. Recent phylogenetic work indicates *Cyanothece* sp. ATCC 51142 and *Crocasphaera*

199 *watsonii* WH8501 diverged from their most recent common ancestor during the early Triassic  
200 and *Cyanothece* sp. ATCC 51142 from *Cyanothece* sp. CCY 0110 occurred near the beginning  
201 of the Cretaceous, preceding the divergence of UCYN-A1 and UCYNA-2 from their respective  
202 common ancestor<sup>18</sup>.

203 UCYN-A became endosymbiotic in pelagic prymnesiophytes approximately 91 million  
204 years ago<sup>18</sup>. Recent research has found that UCYN-A is actively fixing nitrogen in the Arctic  
205 Ocean<sup>10</sup> and that UCYN-A preferentially expresses nitrogen fixation genes in the light<sup>25</sup> but it  
206 doesn't produce an oxygen since it has lost PSII throughout coevolution with its symbiotic  
207 partners. *Cyanothece*-like free living cyanobacteria from the Chroococcales order, which may  
208 share this physiological adaptation, have also been found actively expressing *nifH* genes in arctic  
209 latitudes<sup>9</sup>. With continuing loss of icecaps causing an amplification of surface temperature  
210 warming<sup>26</sup>, the growth of continuous light adaptable diazotrophs toward the poles may become  
211 increasingly relevant to marine ecosystem function considering their primary production role<sup>27</sup>  
212 (Figure 4).

213 In addition to environmental implications, *Cyanothece* sp. ATCC 51142 provides a  
214 model of oxygen-tolerant nitrogenase activity, which upon elucidation can be emulated in  
215 attempts at engineering BNF into crops. Recent attempts to transfer a functional complement of  
216 nitrogenase genes to *Synechocystis* sp. 6803 have demonstrated the complexities engineering  
217 BNF into a non-diazotrophic phototroph<sup>28,29</sup>. The engineered cells exhibited nitrogenase activity  
218 at anaerobic levels, but cells exposed to 1% oxygen quickly lost ~90% of activity. The genes  
219 identified in our bioinformatic analysis (Figure 3D) mark a starting point for further inquiry into  
220 the mechanism of this physiology which may lend further insights to BNF engineering projects.

221

## 222 **Methods**

### 223 **Culture Conditions**

224 *Cyanothece* sp. ATCC 51142 cultures were cultivated batch style and sub-cultured for at  
225 least ten generations in ASPII N- (nitrogen deplete) or ASP II N+ (nitrogen replete) media. The  
226 continuous light cultures were grown at 30°C on a circular shaker at 120rpm under continuous  
227  $\mu\text{E m}^{-2} \text{s}^{-1}$  light. Cultures were refreshed weekly leading up to experiments. The 12-hour dark /  
228 12-hour light cycled cultures were grown in an Innova 44® Incubator set at 30°C on a circular  
229 shaker at 120rpm. The light period for the 12-hour dark / 12-hour light cycled culture was grown  
230 under GE gro-lights. When preparing culture for acetylene incubation to determine nitrogenase  
231 activity, 5 mL's of culture was transferred to a 20 mL glass tube. The seal used on the glass tube  
232 was dependent on the experiment. The continuous nitrogenase activity experiment used a red cap  
233 while the headspace oxygen experiment used a blue air-impermeable cap.

### 234 **Continuous Nitrogenase Activity Measurements**

235 Five mL of the three to eight-day old cultures growing under continuous light ( $\mu\text{E m}^{-2} \text{s}^{-1}$ .  
236 , 120 rpm, 30°C) or 12 h light/12 h dark cycle in ASPII N+ or ASP II N- (nitrogen-containing or  
237 nitrogen-free media) were transferred into a 20-ml glass serum bottle (Wheaton) respectively.  
238 The bottle was sealed with a red rubber stopper (Wheaton) and injected with 0.5 ml of acetylene.  
239 The bottle was incubated under light or dark for one hour. Five mL of headspace gas sample  
240 from the 20 mL culture bottle was administered via a 1 mL GSV Loop to the GC-MS (Agilent  
241 7890A/5975C). The volatile compounds were separated by CP7348 column (Agilent PoraBOND  
242 Q 25 m x 250  $\mu\text{m}$  x 3  $\mu\text{m}$ ) with Pulsed Split Mode at 100:1 ratio. The carrier gas was hydrogen  
243 at a flow rate of 0.8 mL/min, and the supply of Ultra-pure N<sub>2</sub> and Ultra-Zero air for the FID were

244 15 and 200 ml/min respectively. The GC program was initiated at 32°C held for 4 min, and  
245 ramped at 110°C to reach 232°C. The temperatures of the valve box heater and flame ionization  
246 detector (FID) heater were 100°C and 250°C, respectively. The scanning mass range of MSD  
247 was between 10 to 50  $m/z$  and the inlet temperature was set at 250°C. All measurements were  
248 performed in triplicate.

249

## 250 **Oxygraphy Measurements**

251 Oxygraphy measurements were taken using a Hansa Oxygraph II and Hansa Light  
252 Source. The light source was set at  $\mu\text{E m}^{-2} \text{ s}^{-1}$  and the temperature of the chamber was kept  
253 constant using a circulating chiller set at 30°C. Cultures were concentrated from the 3 replicate  
254 tubes used for each acetylene reduction, 15 mL's total, down to 3 mL's total by centrifugation  
255 and removing supernatant. Before the concentrated samples were transferred to the oxygraphy  
256 chamber, sodium bicarbonate was added and brought to a final concentration of 10 mM. The  
257 addition of sodium bicarbonate served to make sure there was an adequate supply of carbon  
258 dioxide to the cyanobacteria since there is no headspace in the prepared oxygraphy chamber.  
259 Samples came to relative stability (steady rate of respiration) in the dark before the light source  
260 was turned on, photosynthesis was measured for at least two minutes or until the rate stabilized,  
261 then the light source was turned off and respiration was measured for at least 2 minutes or until  
262 stabilized. The stir bar setting was set to 75% and the equilibration of the device was completed  
263 with distilled deionized water at 30°C that was sparged by 99% nitrogen gas.

## 264 **Simultaneous Measurement of Nitrogenase Activity and Headspace Oxygen**

265 For each experimental replication, all samples were sealed in air impermeable glass tubes  
266 (20 mL) at the zero hour with 5 mL of culture. Each tube had 0.5 mL of 99% carbon dioxide  
267 added to prevent a loss of photosynthesis over the 12 to 16 hours incubation period. Carbon  
268 dioxide was added to all tubes, including the control and zero to one-hour incubation samples for  
269 consistency. Tubes had 0.5 mL's of pure acetylene added immediately preceding their designated  
270 1-hour incubation period. After acetylene incubation, 5 mL's of headspace were withdrawn for  
271 determination of nitrogenase activity (ARA). An additional 5 mL was taken from the same tube  
272 and injected into a GC autosampler vial for oxygen content measurement. The GC autosampler  
273 vials were degassed preceding the experiment by filling and withdrawing the vials with 80%  
274 hydrogen, 20 % carbon dioxide gas to 20 psi. Each vial underwent 6 rounds of 30 seconds gas  
275 and degas, ending with a gas fill. The headspace oxygen analysis was performed using GC  
276 coupled with thermal conductivity detector (TCD) (Shimadzu GC 14B equipped with a  
277 CombiPal AOC-5000 auto-sampler and an MSH 02-00B injector needle with a 2 mL injection  
278 loop, SHIMADZU Corp.). The carrier gas was Helium at 275 kPa, and the supply of Ultra-Zero  
279 Air, 95% Argon- 5% Methane, and H<sub>2</sub> were 177.8, 167.5, and 510 kPa respectively. The column  
280 had a total flow rate of 20 mL/min and a purge flow rate of 1.0 mL/min and a pressure of 323.0  
281 kPa. The temperatures of the injection loop, oven, and TCD were 100, 90, and 100°C  
282 respectively. GC-TCD data were obtained by passing sample through four columns in series  
283 (Hayesep N 80/100 mesh 1.50 m × 1/8 IN × 2.1 mm SS, Hayesep D 80/100 mesh 2.50 m × 1/8  
284 IN × 2.1 mm SS, Hayesep D 80/100 mesh 2.50 m × 1/8 IN x 2.1 mm SS, and Supelco 60/80  
285 Molecular Sieve 5 Å 3.0 m × 1/8 IN × 2.1 mm SS) for 13-min isothermal period at 90 °C. For  
286 quality assurance, all measurements were performed in triplicate.

## 287 **Exogenous Oxygen Manipulation**

288 To control the oxygen content, a combination of withdrawing headspace and adding  
289 volumes of gas were employed. Tubes were then incubated for an hour in their native  
290 environment, light or dark, then sampled for acetylene reduction as mentioned previously. All  
291 measurements were performed in triplicate.

## 292 **Light Dependent Incubation**

293 Continuous-light and 12-hour dark / 12-hour light cycled culture was added to sealed  
294 glass tubes (20 mL) as described above and had 0.5 mL acetylene added. The tubes were  
295 incubated either under illumination or the dark with temperature and shaking speed held constant  
296 at 30°C and 120 rpm. Headspace was withdrawn and analyzed for acetylene reduction as a  
297 representation of nitrogenase activity as described above using the Agilent GCMS.

## 298 **TEM microscopy**

299 Triplicate acetylene reduction assays were measured to quantify nitrogenase activity.  
300 Twenty mL glass tubes containing five mL culture each were combined and centrifuged at  
301 12,000xg for 10 minutes. Supernatant was removed and the pellet was washed twice in 5mL of  
302 0.1M Millonig's phosphate buffer, 7.4 pH, centrifuged at 12,000g for 5 minutes. After two  
303 washes the pellet was resuspended once more in 1 mL of 0.1M Millonig's phosphate buffer, 7.4  
304 pH, and transferred to a 1.25 mL capacity, externally threaded, conical bottom, free-standing  
305 tube. This tube was centrifuged at 12,000xg for 10 minutes and had the supernatant removed and  
306 was then covered with 2.5% glutaraldehyde in 0.1M Millonig's phosphate buffer, 7.4 pH. The  
307 tube was incubated at room temperature for 1 hour then stored at 4°C for less than 6 hours before  
308 embedding.

309           After this time period the samples were washed twice in the buffer, post-fixed with 2%  
310 OsO<sub>4</sub>, buffer rinsed twice and refrigerated overnight. The samples were then rinsed twice in  
311 water, washed twice each with 30% acetone, 50% acetone, 70% acetone and uranyl acetate, 90%  
312 acetone, and 100% acetone. The samples were then embedded in Epon plastic, sectioned using a  
313 diamond knife and ultra-microtome, transferred to copper grids, and stained with lead citrate.

314           Images were captured on JEOL JEM-100CX II tungsten-filament 100kV transmission  
315 electron microscope. Digital imaging was conducted with Gatan Erlangshen ES500W camera,  
316 using Gatan Digital Micrograph software. ImageJ was used for extracting bacterial glycogen  
317 granule size using thresholding set at 0 and between 110 and 120.

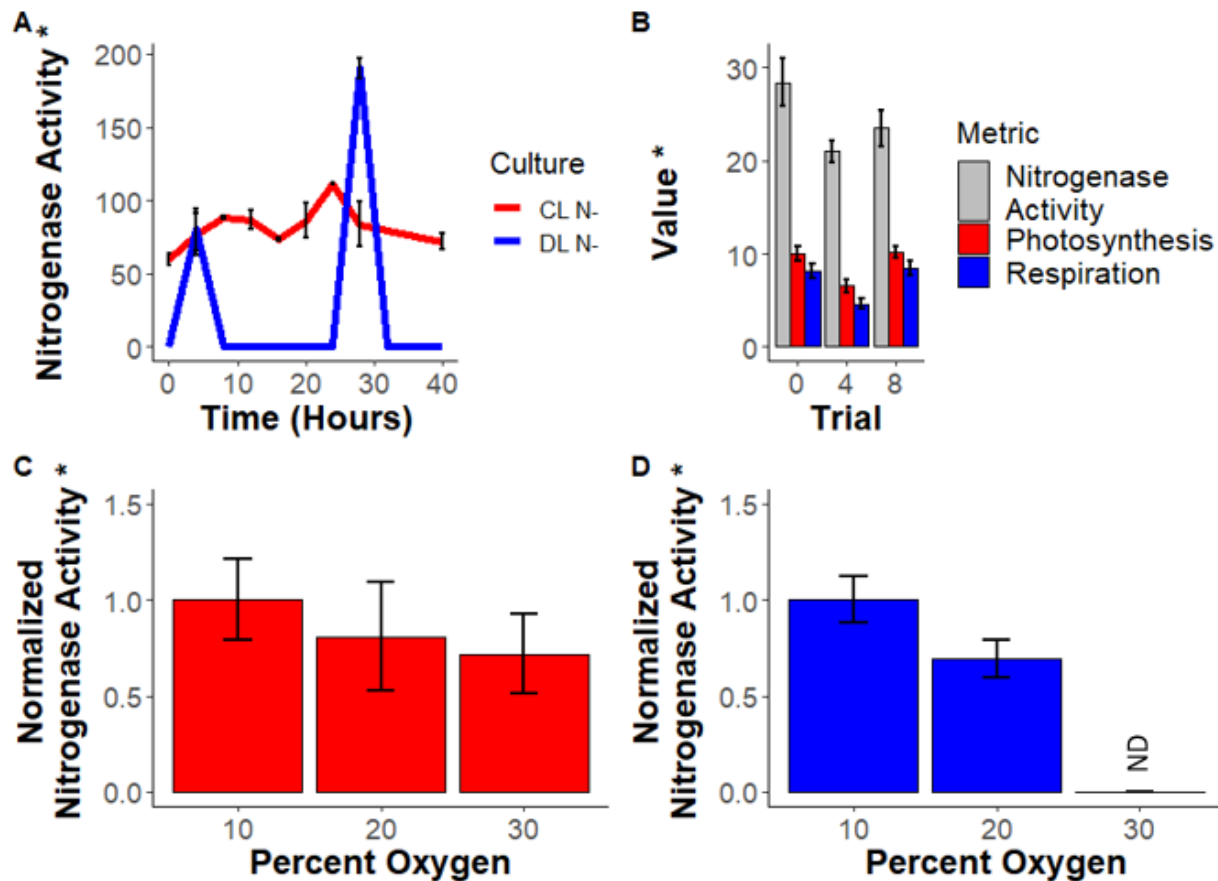
### 318 **Bioinformatic Analysis**

319           UniProt predicted proteomes were used for selected bacterial strains and compared to  
320 *Cyanothece* sp. ATCC 51142 using BLAST+. Proteins were found to be unique to the “CL N-”  
321 group accessory proteome or the control group pan-proteome at 50% amino acid sequence  
322 similarity to *Cyanothece* sp. ATCC 51142. These predicted proteins were then interrogated at the  
323 transcriptome level from a recent experiment<sup>19</sup> for differential correlation between lighting  
324 conditions using DiffCor in R. There were 11 time points taken from the DL N- cycle and 9 time  
325 points taken from the CL N- cycle. Gene pairs with highly significant differential correlation ( $p <$   
326 0.001) were visualized as networks using MEGENA in R. We also looked for enrichment of  
327 gene ontology annotation to make inferences of functional roles of the genes using UniProt.

328

329

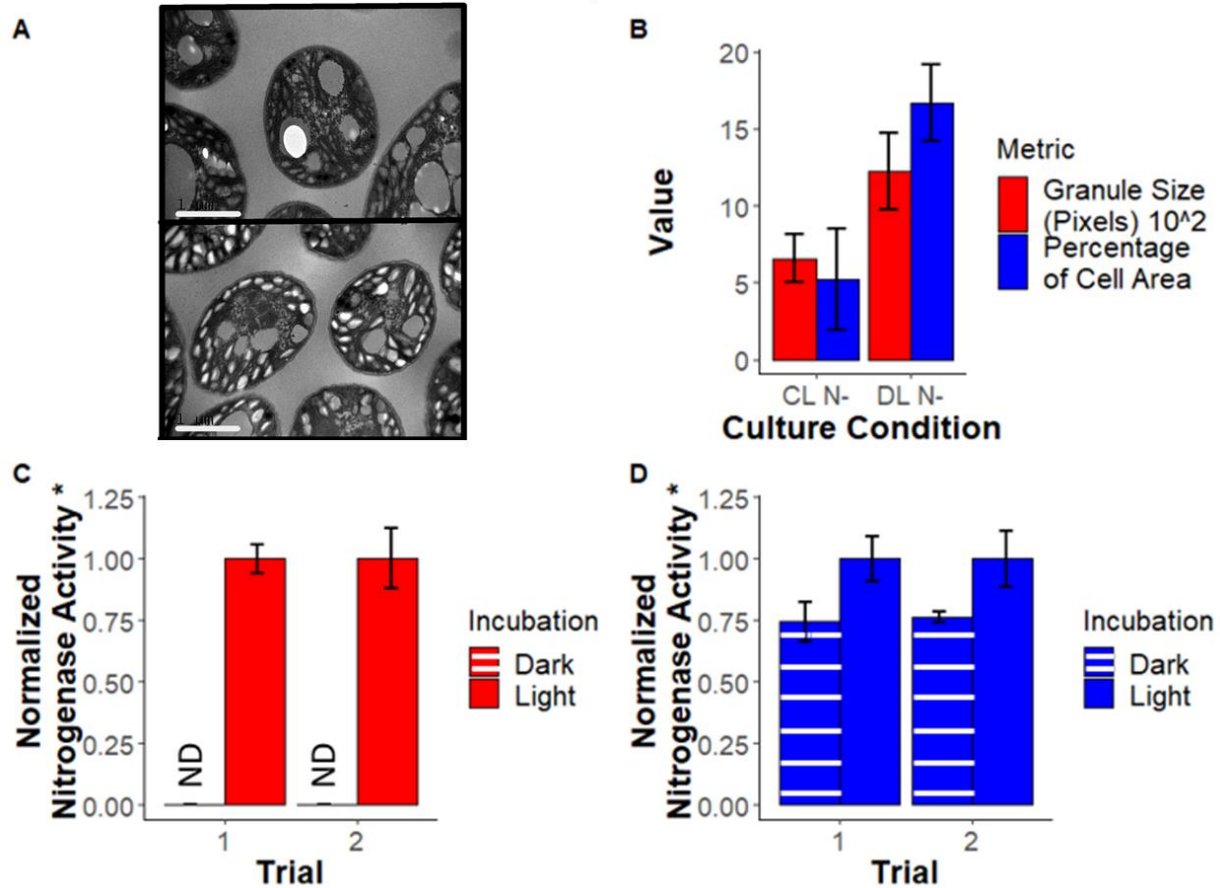
330 **Figures**



331

332 **Figure 1:** Nitrogenase activity is constitutive and concurrent with oxygenic photosynthesis in  
 333 adapted CL N- culture (1A, 1B). Nitrogenase activity in the CL N- culture (1C) is more resilient  
 334 in increasing oxygen concentration compared to DL N- culture (1D). \* = Photosynthesis and  
 335 respiration (1B) units are  $\text{nmol O}_2 \cdot (\text{mL} \cdot \mu\text{g ChlA} \cdot \text{min})^{-1}$  and acetylene reduction assay units  
 336 are  $(\text{integrated units} \cdot 10^{-6}) \cdot (\mu\text{g ChlA} \cdot \text{mL} \cdot \text{hour})^{-1}$ , activity in 1A is measured in  $(\text{nmol ethylene}$   
 337  $\cdot 10^{-6}) \cdot (\text{OD}_{720} \cdot \text{mL} \cdot \text{hour})^{-1}$ . ND = Not Detected. Bars are standard deviation based on n=3.

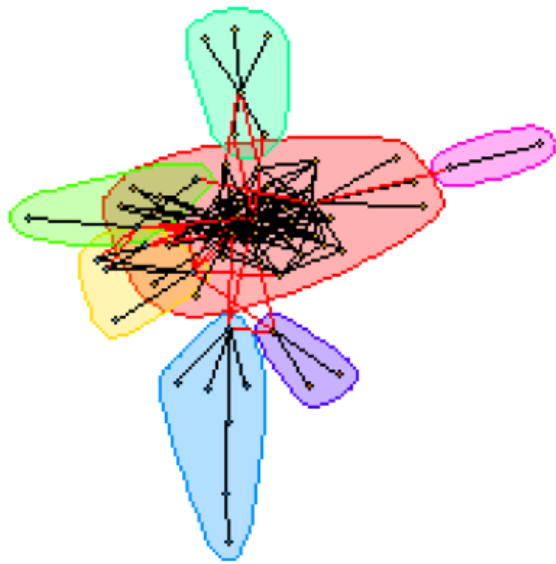




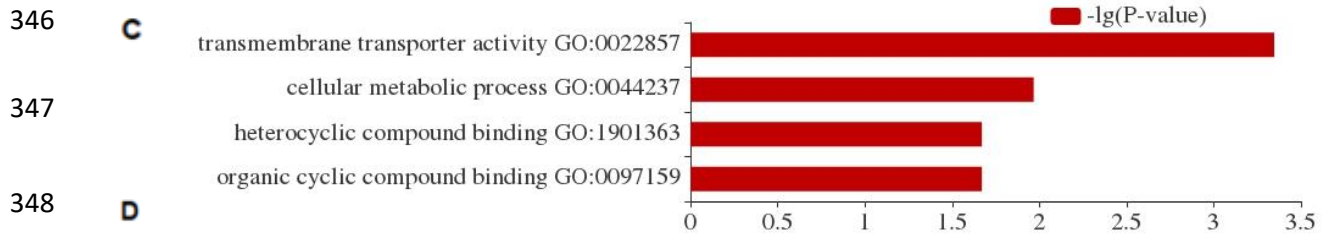
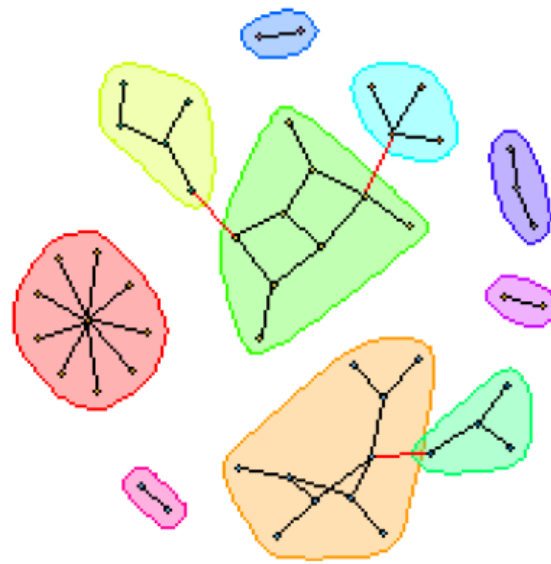
338

339 **Figure 2:** Bacterial glycogen granules (smaller white granules toward cell periphery) occupy less  
340 space in cells from CL N- culture (2A top) than DL N- culture (2A bottom) with the difference  
341 being quantified using ImageJ (2B). White bars represent  $1\mu\text{m}$  (2A). CL N- culture nitrogenase  
342 activity is dependent on illumination (2C) while DL N- exhibits nitrogenase activity regardless  
343 of illumination (2D). Acetylene reduction units are  $(\text{integrated units} \cdot 10^{-6}) \cdot (\mu\text{g ChlA} \cdot \text{mL} \cdot$   
344  $\text{hour})^{-1}$ . ND = Not Detected. Bars for 2C and 2D are standard deviation based on  $n=3$ . Bars for  
345 2B are standard deviation based on cells analyzed (CL N-  $n=5$ , DL N-  $n=6$ )

A



B



349

### Differentially Correlated CL N- Genes of Interest

350

**Gene**

**Predicted Function**

351

*cce\_1857*

NifU-like protein, Fe-S cluster assembly/repair

352

*cce\_2926*

Transmembrane helix, mediates protein-protein interaction and multiprotein complexes

353

*cce\_0574, cce\_0575, cce\_0576*

Ferrous iron transport

354

*cce\_0470*

Antisigma factor antagonist

355

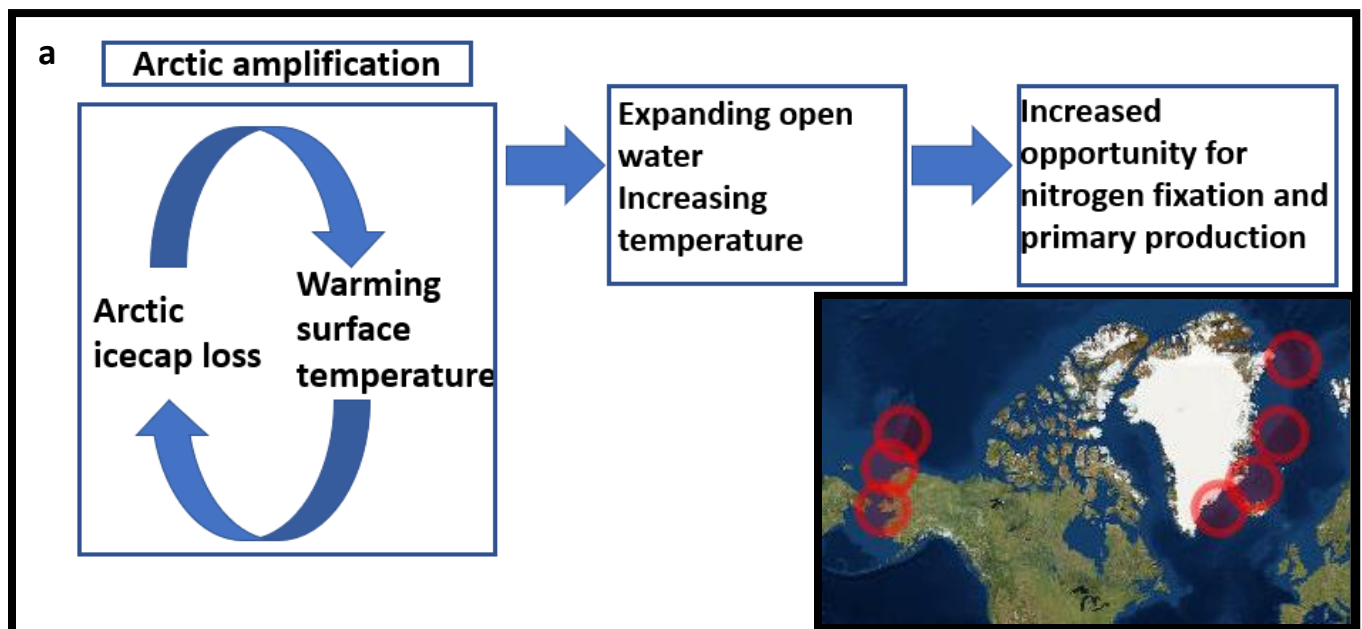
*cce\_3724*

Protein folding acceleration

356

357 **Figure 3:** The genes with differential correlations between DL N- and CL N- with adjusted p-  
358 values  $<0.001$ , the predicted accessory proteome had higher connectivity (3A) while the control  
359 group had lower connectivity (3B). The predicted accessory proteome with differential  
360 correlation with adjusted p-value  $< 0.001$  was enriched for various gene ontologies compared to  
361 the control group (3C). Genes of interest were found based on predicted function that could play  
362 a role in our observed physiology (3D).

363



364

365 **Figure 4:** Primary production of unicellular diazotrophic cyanobacteria in the arctic has potential  
366 to increase due to increasing open water and rising temperatures at latitudes that experience  
367 continuous light for months (4A). Cyanobacteria closely related to *Cyanothece* have been  
368 recently discovered in the Arctic Ocean<sup>9,10</sup> (4B).

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