Unicellular Cyanobacteria Exhibit Light-Driven, Oxygen-Tolerant, Constitutive Nitrogenase Activity Under Continuous Illumination

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Abstract

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

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[6]. 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[7]. 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[5]. 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.[8]

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[9,10] 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-)[11].

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[12]. 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.

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[13].

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[14].

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[15]. Further supporting our prediction of physiological conservation, *Crocasphaera*

112   *watsonii* WH8501 has been reported to grow diazotrophically under continuous light for

113   weeks[16]. 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[17]. 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[18]. 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[19] 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.

Discussion

149

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. *Gloeothece* 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[15]. One of the few papers to-date citing the *Gloeothece* 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[20]. 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[21]. 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[22] and Cretaceous[23] periods. The oceans were also highly nutrient limited in the

196   Jurassic[24] 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[18]. 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[18].

203   UCYN-A became endosymbiotic in pelagic prymnesiophytes approximately 91 million

204   years ago[18]. Recent research has found that UCYN-A is actively fixing nitrogen in the Arctic

205   Ocean[10] and that UCYN-A preferentially expresses nitrogen fixation genes in the light[25] 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[9]. With continuing loss of icecaps causing an amplification of surface temperature

210   warming[26], the growth of continuous light adaptable diazotrophs toward the poles may become

211   increasingly relevant to marine ecosystem function considering their primary production role[27]

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[28,29]. 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

Methods

Culture Conditions

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

Continuous Nitrogenase Activity Measurements

Five mL of the three to eight-day old cultures growing under continuous light ($\mu E\ m^{-2}\ s^{-1}$. , 120 rpm, 30°C) or 12 h light/12 h dark cycle in ASPII N+ or ASP II N- (nitrogen-containing or nitrogen-free media) were transferred into a 20-ml glass serum bottle (Wheaton) respectively. The bottle was sealed with a red rubber stopper (Wheaton) and injected with 0.5 ml of acetylene. The bottle was incubated under light or dark for one hour. Five mL of headspace gas sample from the 20 mL culture bottle was administered via a 1 mL GSV Loop to the GC-MS (Agilent 7890A/5975C). The volatile compounds were separated by CP7348 column (Agilent PoraBOND Q 25 m x 250 μm x 3 μm) with Pulsed Split Mode at 100:1 ratio. The carrier gas was hydrogen at a flow rate of 0.8 mL/min, and the supply of Ultra-pure N_2 and Ultra-Zero air for the FID were

244   15 and 200 ml/min respectively. The GC program was initiated at 32⁰C 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 $\mu E\ m^{-2}\ s^{-1}$ 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_2 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_4, 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[19] 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.

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330 **Figures**
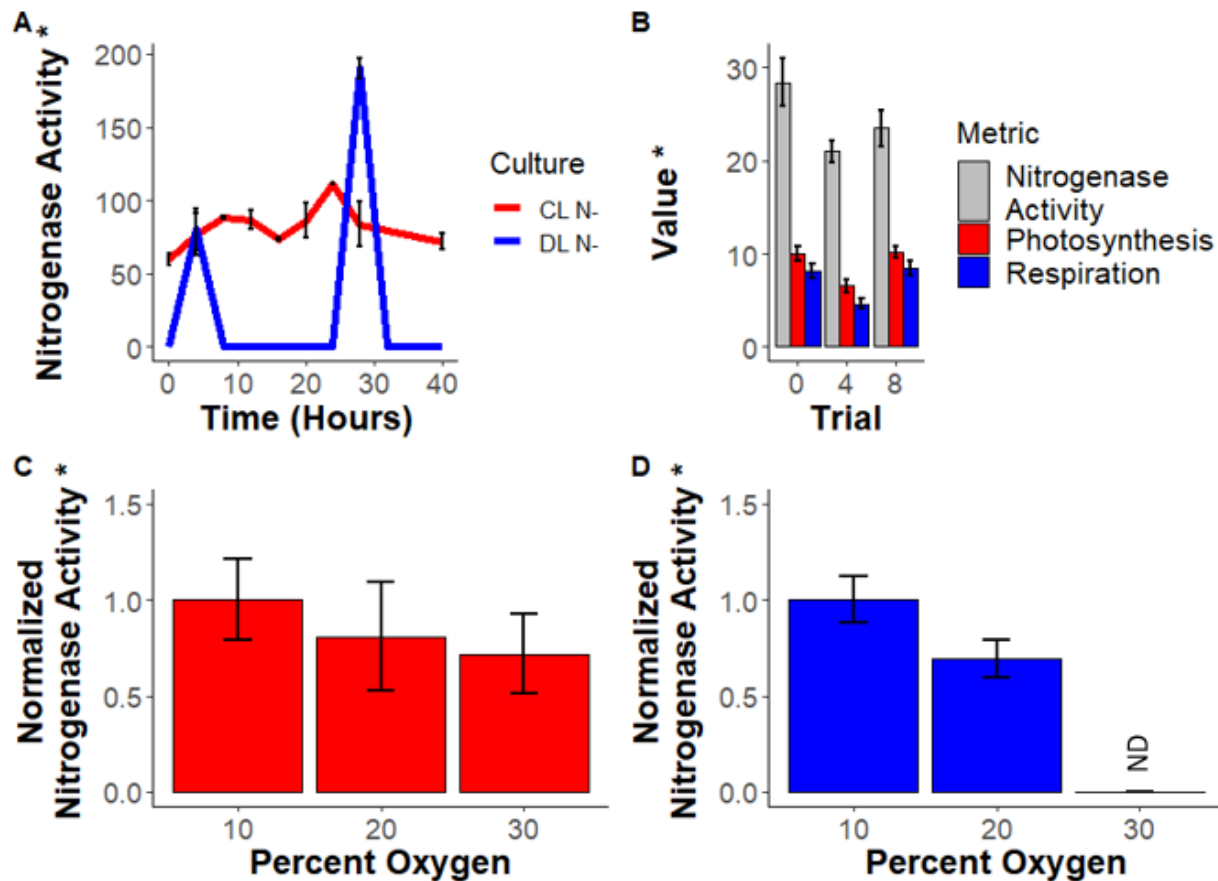
332 **Figure 1**: Nitrogenase activity is constitutive and concurrent with oxygenic photosynthesis in

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 nmol $O_2 \cdot$ (mL$\cdot$ µg ChlA $\cdot$ min)$^{-1}$ and acetylene reduction assay units

336 are (integrated units $\cdot 10^{-6}$) $\cdot$ (µg ChlA $\cdot$ mL$\cdot$ hour)$^{-1}$, activity in 1A is measured in (nmol ethylene

337 $\cdot 10^{-6}$) $\cdot$ (OD$_{720} \cdot$ mL$\cdot$ hour)$^{-1}$. ND = Not Detected. Bars are standard deviation based on n=3.

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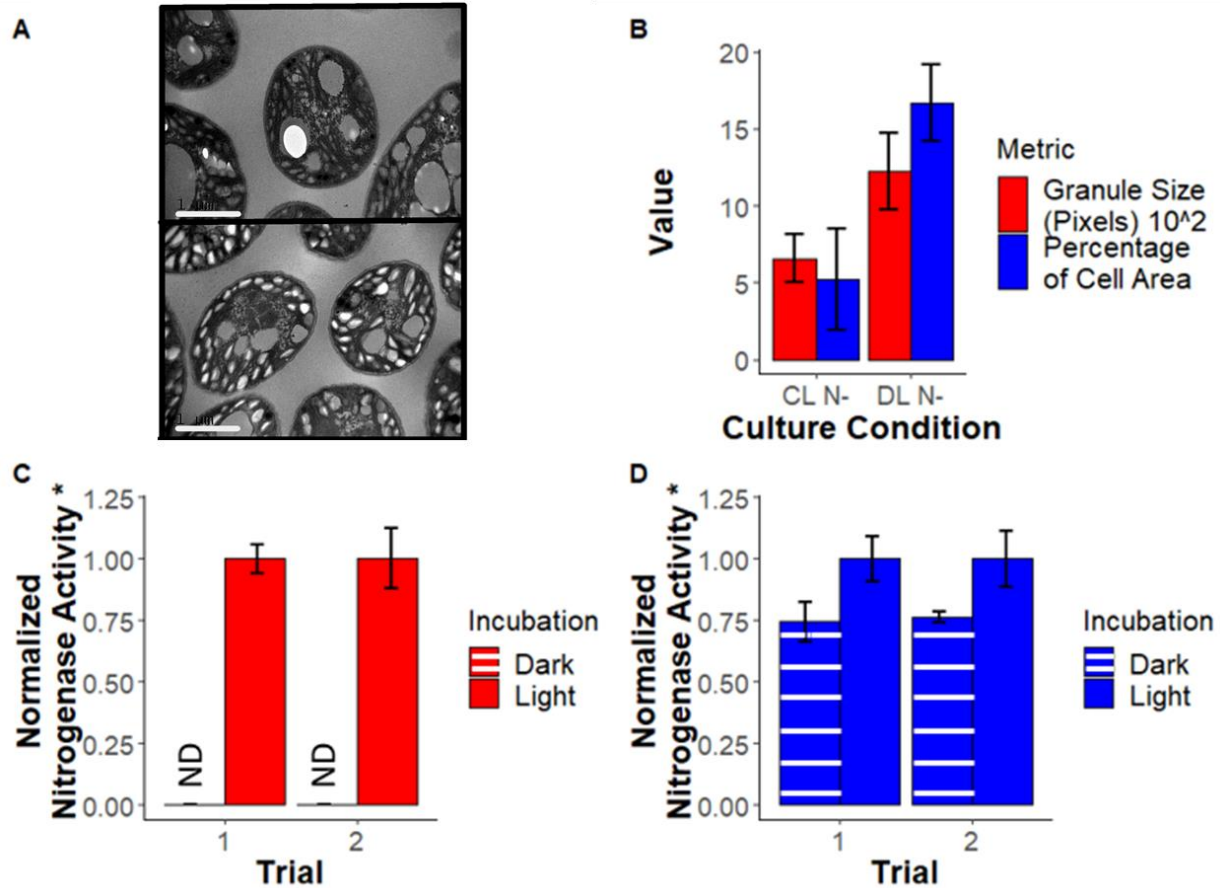
Figure 2: Bacterial glycogen granules (smaller white granules toward cell periphery) occupy less

space in cells from CL N- culture (2A top) than DL N- culture (2A bottom) with the difference

being quantified using ImageJ (2B). White bars represent 1μm (2A). CL N- culture nitrogenase

activity is dependent on illumination (2C) while DL N- exhibits nitrogenase activity regardless

of illumination (2D). Acetylene reduction units are (integrated units $\cdot$ 10^{-6}) $\cdot$ (μg ChlA $\cdot$ mL $\cdot$

hour)$^{-1}$. ND = Not Detected. Bars for 2C and 2D are standard deviation based on n=3. Bars for

2B are standard deviation based on cells analyzed (CL N- n=5, DL N- n=6)

A

B

C

D
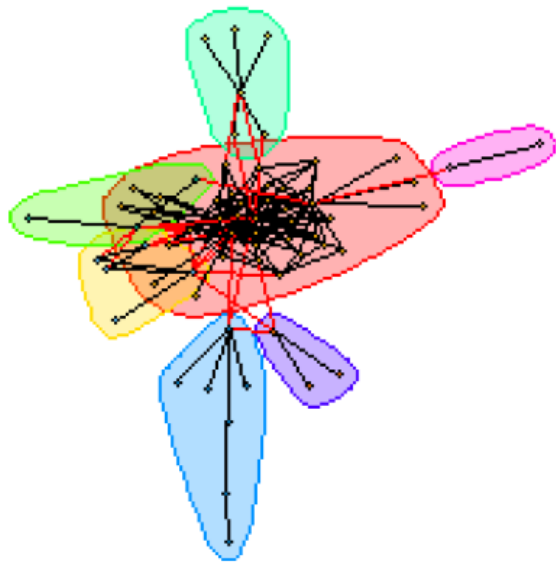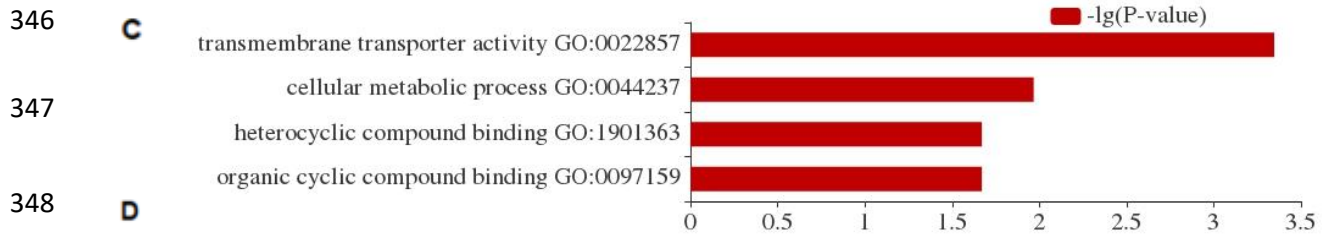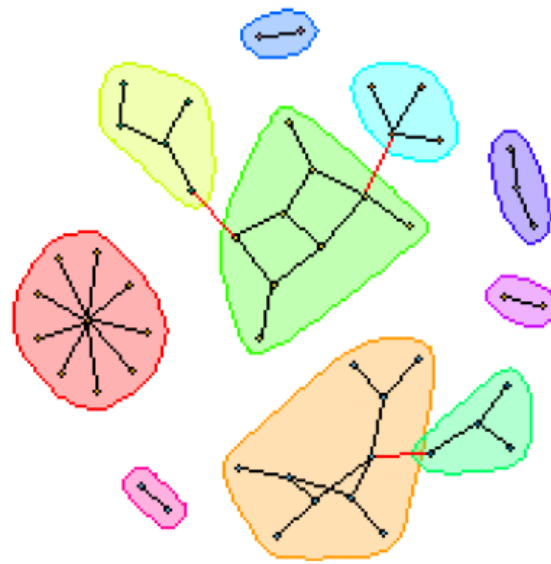
| Differentially Correlated CL N- Genes of Interest	
Gene	**Predicted Function**
cce_1857	NifU-like protein, Fe-S cluster assembly/repair
cce_2926	Transmembrane helix, mediates protein-protein interaction and multiprotein complexes
cce_0574, cce_0575, cce_0576	Ferrous iron transport
cce_0470	Antisigma factor antagonist
cce_3724	Protein folding acceleration

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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).

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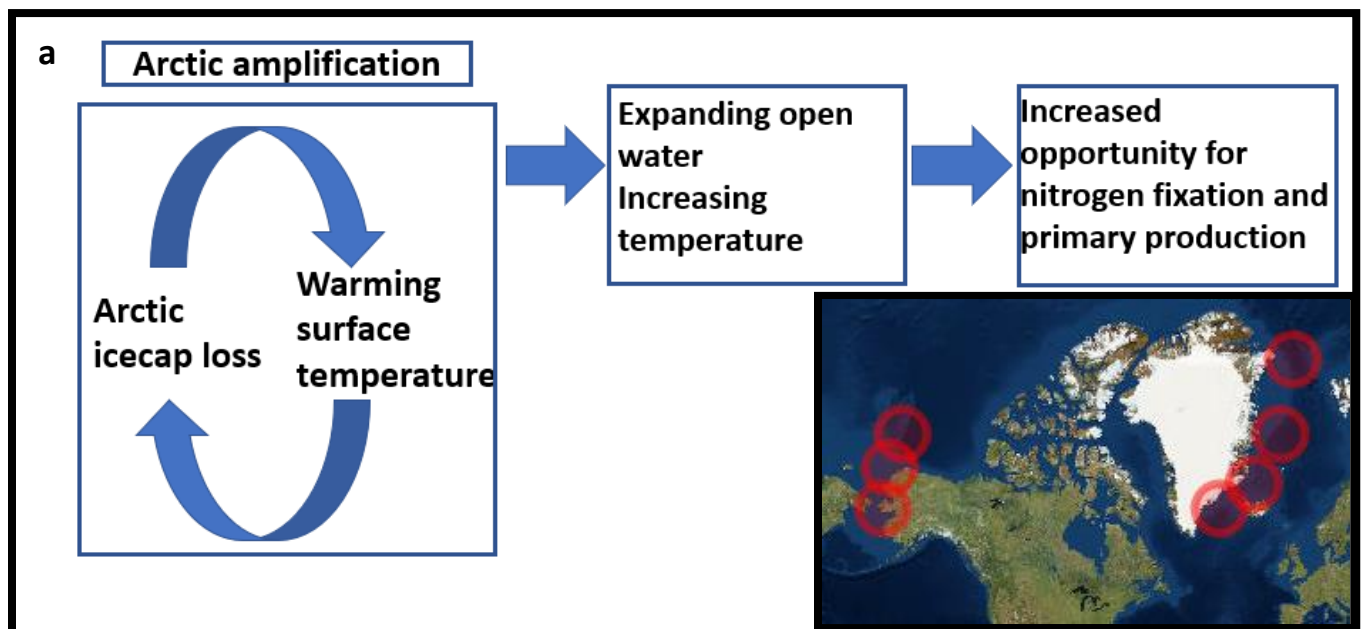
364

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[9,10] (4B).

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Acknowledgements

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376 Citations

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