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David G. Welkie

Debby Sherman
*Purdue University*, dsherman@purdue.edu

William B. Chrisler

Galya Orr

Louis A. Sherman
*Purdue University*, lsherman@purdue.edu

# Analysis of carbohydrate storage granules in the diazotrophic cyanobacterium *Cyanothece* sp. PCC 7822

David G. Welkie[1], Debra M. Sherman[2], William B. Chrisler[3], Galya Orr[3], and Louis A. Sherman[1*]

[1]Department of Biological Sciences, Purdue University, West Lafayette, IN 47907
[2]Life Science Microscopy Facility, Purdue University, West Lafayette, IN 47907
[3]Environmental Molecular Sciences Laboratory, Pacific Northwest National Laboratory, Richland, Washington

*corresponding author: Louis Sherman. email: lsherman@purdue.edu.

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Abbreviations: polyhydroxybutyrate, PHB; transmission electron microscopy TEM; periodic acid-thiocarbohydrazide-osmium, PATO.

**Abstract**

The unicellular diazotrophic cyanobacteria of the genus *Cyanothece* demonstrate oscillations in nitrogenase activity and $H_2$ production when grown under 12h light-12h dark cycles. We established that *Cyanothece* sp. PCC 7822 allows for the construction of knock-out mutants and our objective was to improve the growth characteristics of this strain and to identify the nature of the intracellular storage granules. We report the physiological and morphological effects of reduction in nitrate and phosphate concentrations in BG-11 media on this strain. We developed a series of BG-11-derived growth media and monitored batch culture growth, nitrogenase activity and nitrogenase-mediated hydrogen production, culture synchronicity, and intracellular storage content. Reduction in $NaNO_3$ and $K_2HPO_4$ concentrations from 17.6 and 0.23 mM to 4.41 and 0.06 mM, respectively, improved growth characteristics such as cell size and uniformity, and enhanced the rate of cell division. Cells grown in this low NP BG-11 were less complex, a parameter that related to the composition of the intracellular storage granules. Cells grown in low NP BG-11 had less polyphosphate, fewer polyhydroxybutyrate granules and many smaller granules became evident. Biochemical analysis and transmission electron microscopy using the histocytochemical PATO technique demonstrated that these small granules contained glycogen. The glycogen levels and the number of granules per cell correlated nicely with a 2.3 to 3.3-fold change from the minimum at L0 to the maximum at D0. The differences in granule morphology and enzymes between *Cyanothece* ATCC 51142 and *Cyanothece* PCC 7822 provide insights into the formation of large starch-like granules in some cyanobacteria.

## Introduction

Cyanobacteria are important model organisms for key biological processes such as carbon sequestration, nitrogen fixation, and hydrogen production. Much of the current interest in cyanobacteria and their potential to produce biofuels is based upon the relationship of fossil fuels and human activity—the large increase of energy-related $CO_2$ emissions in the atmosphere and the potential scarcity or high cost of fossil fuels. Photosynthesis results in the production of energy and reducing equivalents that can be used for the fixation of $CO_2$ into reduced carbon compounds and for processes such as $N_2$ fixation in diazotrophic cyanobacteria. All cyanobacteria contain hydrogenases and diazotrophic strains also contain nitrogenases that can generate $H_2$ and this capability has recently been exploited in both heterocystous and unicellular strains (Ananyev et al. 2008; Bandyopadhyay et al. 2010; Min and Sherman 2010b). There are obvious reasons for making cyanobacteria a production organism. Their ability to directly convert solar energy into liquid or gaseous fuels, minimal nutrient requirements (Ruffing 2011), and the use of atmospheric $CO_2$ as a carbon source provide economic and environmental incentives. Advances in areas such as metabolic modeling (Knoop et al. 2010; Saha et al. 2012) and cultivation (Burrows et al. 2008), have prompted advances in engineering cyanobacteria for biofuel production. Recent research on cyanobacteria has yielded successful production systems for various bioenergy compounds including fatty acids (Liu et al. 2011), isoprene (Lindberg et al. 2010), 1-butanol (Lan and Liao 2012), and hydrogen (Bandyopadhyay et al. 2011; Min and Sherman 2010b).

*Cyanothece* spp. are unicellular diazotrophic cyanobacteria that temporally separate photosynthesis and nitrogen fixation(Bandyopadhyay et al. 2013). During the day, light energy is utilized via photosynthesis to generate energy and reductant. During this process, $CO_2$ is fixed via the Calvin cycle and stored in glycogen granules that form between the thylakoid membranes. During the night, photosynthesis shuts down and respiration utilizes the stored glycogen as a substrate to produce additional cellular energy and to remove molecular oxygen from the cell. The lack of intracellular $O_2$ permits the oxygen sensitive nitrogenase enzymes, which are expressed at the onset of the dark, to enzymatically fix atmospheric nitrogen (Sherman et al. 1998). This process also

produces hydrogen as a by-product and is responsible for the majority of the $H_2$ evolved (Bandyopadhyay et al. 2011; Min and Sherman 2010b). *Cyanothece* sp. PCC 51142 (henceforth *Cyanothece* 51142) has been studied extensively due to its well-defined diurnal cycles between phototrophy and diazotrophy and because it has particularly impressive hydrogen production rates. Although *Cyanothece* sp. PCC 51142 has provided considerable insight into how major processes of the cell relate to one another, it has proven recalcitrant for the development of a genetic system due to its tendency for illegitimate recombination and the resulting difficulty in generating mutations (Min and Sherman 2010a).

The genomic sequences of seven members of the *Cyanothece* genus have been finished and all can produce reasonably high levels of $H_2$ under suitable conditions. However, a stable genetic system only has been developed for *Cyanothece* sp. PCC 7822 (henceforth *Cyanothece* 7822 (Min and Sherman 2010a). This cyanobacterium also has a propensity to form intracellular granules of polyhydroxyalkonoate (PHA) (excess carbon storage and electron sink), polyphosphate (phosphate storage), and cyanophycin (nitrogen storage as a co-polymer of the amino acids asp and arg). Recent proteomic analysis has yielded insights into how *Cyanothece* 7822 differs from *Cyanothece* 51142 (Aryal et al. 2013) under both nitrogen-replete and nitrogen-deficient conditions and highlighted pathways that may provide suitable targets for genetic manipulation. Due to the availability of genomic sequence information, capacity for genetic modifications, and aforementioned metabolic characteristics, *Cyanothece* 7822 is a prime candidate to use a chassis for biofuel production.

While efforts to improve the facility of genetics and mutant segregation in *Cyanothece* 7822 are ongoing, the aim of this study is to improve the physiological and growth characteristics and to gain a better understanding of global cellular processes. The growth medium used for cultivation of many cyanobacteria, BG-11 (Allen 1968), is a relatively rich media for phototrophic organisms and provides nitrate and phosphate in excess (Ananyev et al. 2008; Burrows et al. 2008; Chen et al. 2008). The molar nitrate and phosphate ratio is much higher than the canonical Redfield ratio (16:1) (Ernst et al. 2005), as well as the proposed range of nitrate:phosphate (N:P) appropriate for nitrogen fixing species suggested by (Klausmeier et al. 2004). Therefore, adjustments to BG-11

started with reductions in nitrate and phosphate both in the amount and in the relative molar abundance, using the Redfield ratio as a starting point. We demonstrate that the revised media led to improved growth and to a reduction in intracellular storage granules. In turn, the less complex morphology enabled us to determine that glycogen was stored in granules that are distinct in size and shape from those found in *Cyanothece* 51142.

## Materials and Methods

### Cultivation

*Cyanothece* 7822 was cultured in BG-11 medium (Allen 1968) in 100 ml cultures in 250 ml volume Erlenmeyer flasks at 30°C. Shaking was maintained at 125 rpm with 30-40 μmoles photons $m^{-2}s^{-1}$ of light from cool white fluorescent bulbs. For experimental procedures, cultures were inoculated in modified BG-11 media with nitrate and phosphate adjustments as listed in Table 1. Stock cultures were grown in continuous light conditions for 7 days and sub-cultured to ~1.5 x $10^6$ cells $ml^{-1}$ (OD730 = 0.12, Chl = ~0.5 μg $ml^{-1}$). When indicated, cultures were grown under 12h light-12h dark light regimen to induce diurnal cycling in nitrogen-deficient media.

### Measurement of growth and cellular size and shape

Growth was measured by optical density ($A_{730}$) with a Perkin-Elmer Lambda 40 spectrophotometer and through direct cell counting using a Petroff-Hauser counter. The chlorophyll (Chl) concentration of the cells was determined by measuring light absorbance at 750, 678, and 620 nm and using the equation: [Chl] = $14.97(A_{678} - A_{750}) - 0.615(A_{620} - A_{750})$ (Min and Sherman 2010b).

In addition, the population structure of cultures grown in various media and conditions was investigated using a BD influx cell sorter (BD Biosciences, San Jose, CA, USA) measuring individual cell size (forward scatter, FSC) and internal complexity (side scatter, SSC). Samples were analyzed at the Environmental Molecular Sciences Laboratory (EMSL) at Pacific Northwest National Laboratory (Richland,WA, USA). At

137 times, cells were transported at 4°C and briefly thawed at room temperature prior to
138 analysis.
139
140 Analysis of intracellular storage granules
141
142 Intracellular glycogen content was measured quantitatively using a colorimetric
143 biochemical assay and histocytochemically on the transmission electron microscope
144 (TEM). For quantitative measurements, 1 ml of culture was harvested, resuspended in 0.2
145 ml of deionized water, and digested with 0.4 ml of 40% (wt/vol) KOH at 90 °C for 1 h.
146 The digestion was allowed to cool and two volumes of cold 100% ethanol was added and
147 the mixture was stored overnight at -20°C allowing for carbohydrate precipitation. The
148 precipitate was centrifuged for 30 min at 12,000 rpm in a microfuge and 0.1 ml of
149 concentrated $H_2SO_4$ was added and the solution was further incubated at room
150 temperature for 10 min. Samples were diluted with 0.9 ml of deionized water and
151 carbohydrates were measured according to the anthrone technique described by
152 Schneegurt (1994). Aliquots of 0.5 ml of the dissolved precipitates were added to test
153 tubes containing 1ml of freshly prepared anthrone reagent, consisting of concentrated
154 $H_2SO_4$ with 2.0 g/L anthrone, and immediately vortexed. The reaction was refluxed for
155 10 min at 90°C, cooled, and the resulting solution was used to acquire the $A_{625}$
156 absorbance value and compared to that of a standard curve.
157 For TEM analysis, cells from cultures used for the physiology measurements were
158 concentrated via centrifugation and thin sections were prepared for intracellular glycogen
159 detection through application of a specific post staining procedure based on the PATO
160 method (Hanker et al. 1964; Sherman and Sherman 1983). Cells were sampled at four
161 time points throughout the 12h light-12h dark period after 72 h growth in nitrogen-
162 deficient BG-11 and low NP BG-11 media. Cells were pre-stained with 0.15% ruthenium
163 red in 0.1 M Na-cacodylate buffer, pH 7.0 (NaCaB), for 1h at room temperature and
164 subsequently washed. The primary staining consisted of 3.6% glutaraldehyde with 0.15%
165 ruthenium red in NaCaB, incubated for 1 h at room temperature; this was followed by a
166 wash with NaCaB, and then a secondary staining procedure with 1% $OsO_4$ with 0.15%
167 ruthenium red in NaCaB, for 1 h at 4°C. Fixed samples were pelleted, enrobed in 2%

agarose, and resulting pellets were cut into blocks for dehydration and embedding. The samples were dehydrated via a graded ethanol series and embedded in fresh Spurr resin. Thin sections were cut and retrieved with a 100-mesh Nickel grid coated with a formvar + carb on film. Grids were post stained by floating on droplets of 1% periodic acid for 1 h at room temperature, rinsed with water, floated on droplets of 1% thiosemicarbazide (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at room temperature, rinsed again with water, and allowed to air dry. Subsequently, the sections were exposed to $OsO_4$ vapors in a small, closed, Petri plates for 7 hours at room temperature, stained with 2% aqueous uranyl acetate for 5 min, rinsed with water, and air-dried. A control for each time point was prepared by replicate preparation minus the periodic acid and thiosemicarbazide prior to final osmium vapor exposure. Samples were imaged with a Philips CM-100 TEM operated at 100 kV, Spot 3, 200 µm condenser aperture, and with a 50 µm objective aperture. Images were captured on Kodak SO-163 film.

Measurement of nitrogenase activity and hydrogen evolution

Nitrogenase activity was assayed via a modified acetylene reduction technique, whereby 12-30 ml of culture was sealed in 66-ml clear glass bottles in air or sparged with argon for 1 min to generate an anoxic environment and immediately sealed with a rubber stopper. To each sample bottle, 3 ml of acetylene gas was injected. Bottles were incubated in conditions consistent with their respective time points; D0 representing the end of the 12h light period, D3 representing 3 h into the 12 h dark period, and 3 other time points in the light (L0, L3, and L11) representing 0 h, 3 h, and 11 h into the light period, respectively. After incubation for 24 h, a 0.2 ml headspace gas was used to analyze the amount of acetylene reduced to ethylene using a HP 5890 Series II gas chromatograph (Hewlett Packard, Ltd.) equipped with a 6-ft Poropak N column and a flame ionization detector using nitrogen as a carrier gas. The temperature of the injector, detector and oven were 100, 150 and 100°C, respectively. Duplicate samples were analyzed for each set of conditions. To calculate the amount of acetylene reduced, the peak area values were divided by the calculated conversion factor of 13,736.77, duration of incubation, and amount of chlorophyll to result in units of µmol $C_2H_2$ reduced per mg

199 chlorophyll per hour (Colon-Lopez et al. 1997; Min and Sherman 2010b; Schneegurt et
200 al. 1994).
201 For hydrogen evolution, 12-30 ml of culture was transferred to 66-ml glass
202 bottles similar to the preparation for the nitrogenase assays and measured in air or
203 sparged with argon for 1 min to provide an anoxic environment. Bottles were
204 immediately sealed and incubated under continuous light at 30°C, shaking at 125 rpm.
205 After 24 hours, 0.2 ml of headspace gas was injected into a HP 5890 Series II gas
206 chromatograph (Hewlett Packard, Ltd.) outfitted with a thermal conductivity detector and
207 a molecular sieve column (HP Molesieve, catalog number 19095P-MS9). Nitrogen was
208 used as a carrier gas. The molar quantity of $H_2$ produced was calculated by the equation
209 determined via standard curve: [moles $H_2$ = (peak area + 10774) / (889044)]. The rate of
210 $H_2$ production was calculated based on moles of $H_2$ produced per mg chlorophyll per
211 hour of incubation period (µmol $H_2$ / mg Chl.h) (Min and Sherman 2010b).
212
213 Phylogenetic analysis of glycogen branching enzymes in *Cyanothece* spp.
214
215 Sequence alignment was performed with MacVector™ (Oxford Molecular Ltd.,
216 Cary, NC, USA) software (v 12.6.0) with the ClustalW algorithm using the default
217 settings. A neighbor-joing tree was generated and bootstrap analysis with 1000 replicates
218 was performed. Scores >50% are indicated at the branch points. The GenBank accession
219 numbers for the sequences are as follows: *Cyanothece* 51142 cee_2248 (YP_001803664),
220 cce_4595 (YP_001806009), cce_1806 (YP_001803222); *Cyanothece* sp. CCY 0110
221 CY0110_25931 (ZP_01730776), CY0110_29634 (ZP_01728659), CY0110_28144
222 (ZP_01728224); *Cyanothece* 7822 cyan7822_2889 (YP_003888122), cyan7822_1547
223 (YP_003886814); *Cyanothece* sp. PCC 8801 PCC8801_0452 (YP_002370704),
224 PCC8801_2353 (YP_002372522); *Cyanothece* sp. PCC 8802 Cyan8802_0465
225 (YP_003136257), Cyan8802_2403 (YP_003138111); *Cyanothece* sp. PCC 7424
226 PCC7474_1226 (YP_002376542), PCC7424_4362 (YP_002379596); *Cyanothece* PCC
227 7425 Cyan7425_4120 (YP_002484795); *Synechocystis* sp. PCC 6803 sll0158
228 (NP_442003), slr0237 (NP_440018); *Synechococcus elongates* PCC 7942

Synpcc742_1085 (YP_400102); At time of publication *Cyanothece* BH63E ATCC 51472 was still in DRAFT without GenBank accession identities.

## Results

### Effects of media modifications on growth

*Cyanothece* 7822 was grown in a series of BG-11 derived media with various changes in macronutrients (Table 1). Decreasing the available nitrogen source from 17.65 mM to 4.41 mM and reducing the phosphate from 0.23 mM to 0.06 mM (low NP BG-11) resulted in improved growth, as measured by cell counting, optical density and chlorophyll content at the end of 168 h incubation under continuous light (Table 2). Adjustments to the nitrate:phosphate ratio (N:P) had an insignificant effect on growth once N and P levels were decreased to 25% of normal BG-11. When the N:P was cut in half (from 77:1 to 37:1) by further reducing the nitrate content an additional 2-fold, no significant change in growth was observed when compared to low NP BG-11 (data not shown). Furthermore, cultures grown in reduced nitrogen-deficient BG-11 displayed no significant difference in nitrogen fixation and hydrogen evolution capabilities compared to cells grown in regular nitrogen-deficient BG-11 (Table 3). The results for nitrogenase activity and $H_2$ production are virtually identical for cells grown in the different media. In addition, cultures grown in both media had identical variations in the peak of activity of about 2 h from experiment to experiment. The peak of activity varied from L11 to D3, which is typical for such circadian oscillations and which is the reason for the high standard deviation. These results prompted us to standardize on this low NP BG-11 (containing 4-fold reduction in N and P) for further investigations on intracellular storage content.

### Effects of $NaNO_3$ and $KH_2PO_4$ reduction on cellular state homogeneity in the population

Flow cytometry analysis of *Cyanothece* 51142 cell populations in batch culture displayed a homogeneous composition with a single, tight population. On the contrary,

*Cyanothece* 7822 grown in regular BG-11 was composed of two distinct populations that differed in terms of both individual cell size and internal complexity (Fig. 1A). This characteristic of *Cyanothece* 7822 was lost when grown in the low NP BG-11. The 4-fold reduction in N and P shifted the cells in batch culture to become a single tight population, similar to that seen with *Cyanothece* 51142 (Fig. 1B). This observation was confirmed by light microscopic observations; cells of *Cyanothece* 7822 grown in low NP BG-11 were smaller, rounder, and more uniform in size (Fig. 1D), whereas cells grown in BG-11 *Cyanothece* 7822 cells were larger, more egg-shaped, and more variable in size (Fig. 1C).

Culture population analysis was also performed on *Cyanothece* 7822 cultures grown in nitrogen-deficient low NP BG-11 at various time points during a 12 h light-12h dark diurnal cycle to induce synchronized photosynthesis and nitrogen fixation. Samples displayed consistent similarity in cell size across the four time points tested, L0, L3, D0, and D3 (Fig. 2A), but slight variations in internal complexity were observed (Fig. 2B). These slight variations in complexity, along with the stability in cell size, demonstrate the dynamic nature of the ultrastructure across the diurnal cycle when the organism shifted metabolically from photosynthesis to respiration within a culture population that is well synchronized.

Effects of $NaNO_3$ and $KH_2PO_4$ reduction on ultrastructure and storage granules

Results from flow cytometry analysis were verified and extended using transmission electron microscopy. Electron micrographs of *Cyanothece* 7822 grown in BG-11 had shown the presence of numerous storage granules in the cell, including those composed of polyphosphate, polyhydroxybuturate and cyanophycin (Bandyopadhyay et al. 2013). *Cyanothece* 7822 cultures were studied by both high pressure freezing (HPF) and chemical fixation to generate a better understanding of intracellular ultrastructure. High pressure freezing preserves cellular structure the best, because the flash freezing prevents loss of intracellular material. However, we also needed to utilize histochemical techniques that depend upon chemical fixation, so we compared cells prepared by the two techniques. HPF was performed on cells grown in regular BG-11 (Fig. 3A) and those grown in low NP BG-11 (Fig. 3B). In many of the BG-11 cells, there was dark material

291 in the nucleoplasmic area and some cells contained many dark granules, both of which
292 we interpret as polyphosphate. The cells grown in low NP BG-11 showed this dark
293 material far less frequently and generally had fewer granules present. The population of
294 cells seen in the electron microscope were much more similar to each other in the low NP
295 BG-11 than in regular BG-11, consistent with the flow cytometry results.
296 The comparison of HPF vs. the microwave chemical fixation procedure for
297 *Cyanothece* 7822 is shown in Figure 4. The cells were from a culture that had been
298 grown in regular BG-11 and the cells were taken from late-log phase. The major
299 differences routinely observed between the two techniques can be seen in the
300 nucleoplasmic region. In the microwave procedure, there are lighter areas that we
301 interpret were caused by the loss of intracellular material (compare Fig. 4B vs. 4A). This
302 leads to slight shrinkage in the chemically fixed cell and the relative sizes were typical of
303 what was seen in such comparisons. This led to a slightly higher density of material in the
304 photosynthetic membrane regions. In addition, there were typically more "holes" in place
305 of granules; the results suggested that many of these holes were caused by the loss of
306 polyphosphate from granules during microtomy. There were typically more presumptive
307 polyhydroxybutyrate (PHB) granules in the chemically-fixed cells (fig. 4B), presumably
308 because the PHB was kept in a more native form when fixed by high pressure freezing
309 (Fig. 4A).
310 In order to detect the glycogen granules *in situ* within *Cyanothece* 7822, we
311 utilized a well-understood histochemical procedure, the PATO technique that depends
312 upon periodic acid, thiosemicarbazide, and osmium tetroxide to generate a black deposit
313 after reaction with glycogen (Hanker et al. 1964; Sherman and Sherman 1983). We had
314 demonstrated some years ago that this procedure could quantitatively identify glycogen
315 granules in *Synechococcus* sp. PCC 7942 (Sherman and Sherman 1983) and it was also
316 successful in *Cyanothece* 7822 (Fig. 5 and 6). Figure 5 compares the PATO stained
317 granules in *Cyanothece* 7822 cells grown under $N_2$-fixing conditions in a 12h light-12h
318 dark diurnal cycle at L0 and D0 time points (Fig. 5). All of the dark staining granules are
319 composed of glycogen and it is obvious that these granules are more numerous and
320 densely packed in cells at D0, which is at the end of the photosynthetic period, compared
321 to L0, which is at the end of the 12 hour dark period. Figure 5C represents the D0 control

without the addition of periodic acid and thiosemicarbazide; we consider the white patches to represent areas that contain glycogen granules.

This procedure was then used to quantitate glycogen granule composition when cells were grown under $N_2$-fixing conditions so that we could compare granules to the amount of glycogen detected via biochemical procedures. Figure 6 shows cells from L0, L3, D0 and D3 after PATO treatment. It is once again clear that cells at L0 have the lowest number of glycogen granules, whereas cells at the other times contain more granules. Micrographs from this experiment were used for quantitative analysis of glycogen content. The results indicated that the glycogen content peaked at the end of the light period with ~5000 individual granules (per TEM section) and successively decreased to a minimum of ~1500 granules at the end of the dark period. Biochemical analysis correlated these observations with a maximum glycogen content of 11 µg/mL at the end of the light period and a minimum of 4.8 µg/mL at the end of the dark period. In both cases, a similar fold change of approximately 3.3- to 2.3-fold was observed for both granule number and glycogen composition, respectively (Fig. 7). We conclude from this correlation and the strong staining using the PATO technique that these small granules contain glycogen.

## Discussion

The goal of this study was to obtain culture conditions for *Cyanothece* 7822 that would improve the growth properties and minimize the amount of macronutrients (C, N and P) that were stored in large intracellular inclusion bodies. This was done successfully, with the bonus of identifying glycogen granules quite different from those seen in *Cyanothece* 51142. The composition of the growth medium is an often-overlooked parameter, but it can have major implications on the cultivation of cyanobacteria. Whether it is for biomass production, influencing natural processes like nitrogen fixation, or stimulating the production of valuable pathway intermediates, the composition of the growth medium can have as much an impact as genetic modifications and the induction of metabolic pathways. *Cyanothece* 7822 is a phycoerythrin (PE) rich cyanobacterium isolated from rice-field soil in India (Min and Sherman 2010a). PE-rich

353 cyanobacteria dominate in low nutrient environments, where nutrient levels are quite
354 different that that found in BG-11, and have developed highly efficient uptake and
355 retention mechanisms for nitrogen (Herrero et al. 2001), carbon (Badger and Price 2003),
356 and phosphorous (Ritchie et al. 2001). In fact, the use of BG-11 has been reported to be
357 inhibitory to growth for PE-rich species like *Synechococcus* spp. (Ernst et al. 2005).
358 Specifically, BG-11 is rich in both nitrate and phosphate and has a N:P far higher than the
359 Redfield ratio (16:1). The Redfield concept is a species-specific biogeochemical
360 phenomenon (Rhee and Gotham 1980) that links the cellular mole ratio of carbon,
361 nitrogen, and phosphorous to that found in the environment and is typically used to
362 estimate ocean productivity and nutrient status of aquatic systems (Bertilsson et al. 2003).
363 This ratio can also be considered the biochemical optimum for phytoplankton and ranges
364 from ~10:1 to the ~40:1 for cyanobacteria (Klausmeier et al. 2004).

365 Based on these findings, various reductions of both nitrate and phosphate were
366 made. Although altering the N:P ratio had little effect on the growth, a overall four-fold
367 reduction in nitrate and phosphate did have a positive effect on growth and lowered flask-
368 to-flask variability (the standard deviation of OD measurements among replicate cultures
369 grown in the reduced media was cut in half). In addition, cells continued to display high
370 nitrogenase activity and good capacity for hydrogen production. Since the low NP BG-11
371 media included reduction in nitrate from ~17.65 mM to 4.41 mM, the transition from
372 nitrogen-replete to nitrogen-deficient media, and attaining cultures growing
373 diazotrophically, required less time and in our experience no prior growth in nitrogen-
374 free media was necessary before measuring nitrogenase activity. Cultures of *Cyanothece*
375 7822 achieved high biomass more rapidly by growing in low NP BG-11, followed by
376 transition to nitrogen-deficient BG-11.

377 The reduction in N and P concentrations directly affected the internal storage
378 content of the cells. The excess N and P in BG-11 resulted in nitrogen storage in
379 cyanophycin granules, carbon into PHB granules and polyphosphate into excess material
380 seen in the nucleoplasmic region or in polyphosphate granules. Indeed, one of the
381 important findings from this study was the different appearance of polyphosphate in
382 electron micrographs of cells prepared by HPF (more dark staining material in the
383 nucleoplasm) or by chemical fixation (more and larger granules, some containing dark

material, but most observed as holes). Growth in low NP BG-11 had much less polyphosphate and the nucleoplasm was generally free of the dark-staining material. This explained a long-standing puzzle as to why some cells contained this material and others did not. Cells from cultures grown in low NP BG-11 also had fewer large granules, such as cyanophycin and PHB, thus indicating that cells did not need to store as much nitrogen or carbon under these conditions.

The relative lack of these large granules revealed a large number of smaller granules between the photosynthetic membranes. These had been seen previously, but it was assumed that one type of the larger granules was composed of glycogen, as in *Cyanothece* 51142 (Schneegurt et al. 1994; Schneegurt et al. 1997). These granules resembled the *β*-particles of glycogen that we had identified in *Synechococcus* sp. PCC 7942 many years earlier (Sherman and Sherman 1983) and we immediately demonstrated that they could be stained by the PATO technique. In addition, glycogen granules fluctuated under $N_2$-fixing conditions during the light-dark periodicity and correlated quite nicely with the total glycogen content that was determined biochemically.

It was surprising that a species within the *Cyanothece* genus contained different morphological types of glycogen granules. The assembly and degradation of the glycogen granules in *Cyanothece* 51142 is an important feature for the storage of potential energy and carbon from the light to the dark—the granules are formed via photosynthesis and are mobilized in the dark as a substrate for respiration to make ATP and to reduce intracellular oxygen to protect nitrogenase (Schneegurt et al. 1994; Schneegurt et al. 1997). The highly synchronized pattern of formation and mobilization of these granules is critical for the highly synchronized pattern of photosynthesis and $N_2$ fixation. The smaller glycogen granules in *Cyanothece* 7822 also demonstrated a synchronized formation and degradation as cells went from light to dark under $N_2$-fixing conditions, such that the underlying protective mechanism for nitrogenase was present.

Nonetheless, the structure of the two types of granules is of interest and has been studied in some detail by Ball and collaborators (Ball et al. 2011). Most cyanobacteria make soluble glycogen that is stored in *β*-granules. Some strains, such as *Cyanothece* 51142, were shown to store the material as semi-amylopectin. Amylopectin is composed of highly ordered, repeated clusters of glucose moieties due to densely localized α-1,6

branch points along glucan chains with intervals of 9-10 nm. This type of structure is responsible for the water-insoluble characteristics of these starch-like granules (Suzuki et al. 2013). However, in glycogen, the α-1,6 branch points are uniformly distributed and the density of the branches becomes very high at the periphery of the molecule, thus limiting their size to about 42 nm in diameter, compared to over 100 nm in diameter for the starch-like granules (Ball et al. 2011; Schneegurt et al. 1994).

The genes for many of the biochemical processes have been identified (Suzuki et al. 2013), and the differences between the two *Cyanothece* strains is notable. The storage of α-glucan occurs through the sequential actions of ADP-glucose pyrophosphorylase (AGPase), glycogen/starch synthase (GS/SS) and a branching enzyme (BE) (Preiss 1984; Suzuki et al. 2010). Investigations of the genomic sequences of both species revealed that both contain two genes for GS/SS (cyan7822_4734 and cyan7822_2570 in *Cyanothece* 7822, and cce_0980 and cce_3396 in *Cyanothece* 51142), but vary slightly in the number of AGPase and branching enzymes genes. *Cyanothece* 51142 contains two AGPase genes (cce_0987 and cce_2658) and three BE genes (cce_1806, cce_2248, and cce_4595), whereas *Cyanothece* 7822 has one AGPase gene (cyan7822_2049) and two BE genes (cyan7822_2889 and cyan7822_1547).

Genomic sequence analysis has determined that the number of genes coding for enzymes in the glycogen biosynthesis pathway is variable among cyanobacterial species. *Synechococcus elongatus* PCC 7942 has the simplest composition with just one of each gene. Others, like *Synechocystis* sp. PCC 6803 and *Anabaena* sp. PCC 7120, are similar to *Cyanothece* 7822, having one gene for AGPase and two genes for GS/SS (Suzuki et al. 2010). We suggest that the different forms of polysaccharide storage observed in *Cyanothece* 7822 and *Cyanothece* 51142 are due the actions of the branching enzymes. It is likely that the concerted actions of the three BE isoforms in *Cyanothece* 51142 give rise to the starch-like granules observed, as postulated by (Suzuki et al. 2013). Moreover, the sequence relationship among branching enzymes found in members of the *Cyanothece* genus, *Synechocystis* and *Synechococcus* show a correlation between species that harbor three BE isoforms with the formation of semi-amylopectin and species that have either only BE1 or both BE1 and BE2 (Fig. 8). At the moment, only the unicellular diazotrophic cyanobacteria found in benthic or oceanic regions seem to produce the semi-

amylopectin granules and all have the 3 BE enzymes, as shown in Figure 8. From analysis of the amino acid sequences, the most likely scenario is that, in *Cyanothece* 51142, BE1 (773 aa) duplicated to form BE2 (651 aa) and that either BE1 or BE2 then duplicated again to form BE3 (647 aa). The major differences among the 3 proteins are a large N-terminal deletion of 111 amino acids for BE2 and 97 amino acids for BE3. There are also various length deletions at the C terminus. BE1 and BE2 demonstrated 58% identity and 74% similarity, whereas BE1 vs. BE3 or BE2 vs. BE3 were only 29% and 44% identical and conserved, respectively. Obviously, BE3 has changed the most and is likely critical for the formation of the large starch-like granule formation. Further biochemical and molecular analysis will be required to determine the enzymatic basis for the different forms of glycogen storage among these strains. Although it is likely easier to synchronize the degradation of a smaller number of large granules (as in *Cyanothece* 51142), compared to a larger number of small granules (as in *Cyanothece* 7822), both approaches seem to work well. Nevertheless, *Cyanothece* 51142 shows greater synchrony and higher peaks for both nitrogenase activity and $H_2$ production, indicating a value for the large starch-like granules.

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**Table 1**

BG-11 media modified by lowering N and P concentrations

| Media | $NaNO_3$ | $K_2HPO_4$ | N:P |
|---|---|---|---|
| BG-11 | 17.65 mM | 0.23 mM | 77 |
| Low NP BG-11 (1) | 4.41 mM | 0.06 mM | 74 |
| Low NP BG-11 (2) | 2.2 mM | 0.06 mM | 37 |

**Table 2**

Effects of BG-11 and low NP BG-11 on growth of *Cyanothece* 7822

| Media | Cell No. | OD730 | [Chl] |
|---|---|---|---|
| BG-11 | $6.5 \pm 2.8 \times 10^6$ | $0.58 \pm 0.1$ | $4.62 \pm 1.5$ |
| Low NP BG-11 | $1.3 \pm 1.6 \times 10^7$ | $0.70 \pm 0.05$ | $6.01 \pm 0.7$ |

Growth measurements were made after incubation in continuous light for 168h.

**Table 3**

Specific activities for hydrogen evolution and nitrogenase activity

| | NF BG11 | | Reduced NF BG11 | |
|---|---|---|---|---|
| | $H_2$ µmol/mg Chl/h | $N_2$ µmol reduced/mg Chl/h | $H_2$ µmol/mg Chl/h | $N_2$ µmol reduced/mg Chl/h |
| L11 | $46.3 \pm 25.8^A$ | $28.2 \pm 7.5^a$ | $48.3 \pm 28.8^A$ | $36.7 \pm 12.8^a$ |
| D0 | $34.5 \pm 18.1^A$ | $35.0 \pm 11.8^a$ | $37.8 \pm 11.1^A$ | $42.4 \pm 8.9^a$ |
| D3 | $9.4 \pm 9.1^A$ | $0.9 \pm 0.6^a$ | $14.8 \pm 13.9^A$ | $0.8 \pm 0.5^a$ |
| L0 | $0.0 \pm 0.0^A$ | $0.0 \pm 0.0^a$ | $0.0 \pm 0.0^A$ | $0.0 \pm 0.0^a$ |
| L3 | $2.5 \pm 4.9^A$ | $5.2 \pm 6.3^a$ | $6.6 \pm 7.2^A$ | $4.0 \pm 4.6^a$ |

Cells were grown in nitrogen-deficient regular NF and modified NF BG-11 for 3 days under 12h light-12h dark conditions. Experimental error was based on 4 technical replicates. One-way ANOVA and comparison of means using Tukey method of 95% confidence intervals indicate that there is no significant difference in activities between cells grown in the different nutrient conditions.

## Figure Legends

**Figure 1**

(A) Distribution of cells in batch culture by individual size (x-axis) and internal complexity (y-axis) of individual *Cyanothece* 51142 (dark grey) and *Cyanothece* 7822 cultures grown in BG-11 (light grey); (B) *Cyanothece* 7822 grown in low NP BG-11 (dark grey) exhibiting a population architecture shift to a single population compared to *Cyanothece* 7822 grown in regular BG-11 (light grey); (C) High magnification light micrographs of *Cyanothece* 7822 grown in regular BG-11 and (D) low NP BG-11 media. Error bars represent standard deviation from 3 replicate samples

**Figure 2**

(A) Individual cell size of *Cyanothece* 7822 as measured by forward scattering; (B) Internal complexity in *Cyanothece* 7822 cells grown in nitrogen-deficient low NP BG-11 media during the 12h light-12h dark cycle as measured by side scattering. Culture samples were taken at four time periods: D0 (beginning of dark period), D3, L0 (end of dark period) and L3.

**Figure 3**

Electron micrographs of *Cyanothece* 7822 cells grown in BG-11 medium (A) and low NP BG-11 medium (B). Cultures for both types were fixed by high pressure freezing for best preservation of cellular ultrastructure. PP, polyphosphate; PL, photosynthetic lamellae.

**Figure 4**

Electron micrographs of *Cyanothece* 7822 grown in regular BG-11 and prepared by high pressure freezing (A) or by the microwave chemical fixation procedure (B).

**Figure 5**

Electron micrographs of *Cyanothece* 7822 stained using the PATO technique with the microwave chemical fixation procedure. Cells were grown under $N_2$-fixing conditions with a 12h light-12h dark cycle. (Left) L0, the end of the dark period; (Center) D0, the end of the light period; and (Right) Untreated control from D0.

**Figure 6**

Comparison of PATO-treated *Cyanothece* 7822 grown under $N_2$-fixing conditions with a 12 h light- 12 h dark diurnal pattern. L0 to D3 represent the time period during $N_2$-fixing conditions at which samples were taken. PL, photosynthetic lamellae; PP, polyphosphate.

**Figure 7**

Comparison of glycogen content and glycogen granules in *Cyanothece* 7822 grown under $N_2$-fixing conditions in 12h light-12h dark. Error bars represent standard deviations from replicate samples. ■---■ Glycogen content; ▲—▲ Glycogen granules measured from PATO-stained cells. Error bars represent standard deviation from 3 replicate samples.

**Figure 8**

Neighbor-joining tree showing amino acid sequence relationships among branching enzymes in the *Cyanothece* spp. with sequenced genomes.

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