Synthetic biology of cyanobacterial cell factories
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Dynamics of the physiology, metabolome and proteome during the circadian cycle of *Synechocystis* sp. PCC 6803 under culturing conditions relevant for mass culturing

Investigating the diurnal oscillations of cyanobacterial physiology is relevant for a better understanding of the fundamental mechanism of circadian control, and for society because of the many biotechnological applications that are foreseen for those organisms. Depending on the growth conditions (e.g. outdoor growth in a large scale photobioreactor) the growth and production efficiency will be governed by the availability of nutrients. This includes the intermittent availability of light and the efficient usage of that light.

Here, we present the results of a study of the model cyanobacterium *Synechocystis* sp. PCC 6803, cultured in a lab-scale photobioreactor while simulating physiological conditions present during large-scale culturing. The culture is sparged with N₂ and CO₂ without additional O₂. This leads to an anoxic environment during the dark period. A circadian illumination regime of 12 h light/12 h dark is applied. We show that formation of biomass strictly follows the availability of light. The storage compound glycogen reached a maximum level of ~40% (w/w) already before the end of the light period. The subsequent consumption of glycogen during the dark period essentially only started in the last quarter of the dark period, which suggests anticipatory mechanisms in cellular regulation, possibly to prime the cellular machinery for efficient energy usage.

The metabolite levels, as determined by Nuclear Magnetic Resonance analysis, show slight but significant changes that allow the grouping of samples from different time points according to the metabolome profile. Likewise, proteome analysis by mass spectrometry shows slight changes in protein levels.


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10.1 Introduction

Cyanobacteria, such as *Synechocystis* sp. PCC 6803 (hereafter: *Synechocystis*) are oxygenic phototrophic microorganisms that can be used for various biotechnological applications. Consequently, CO₂ can be utilized via the cyanobacterial metabolism as the carbon substrate for a sustainable source of bulk chemicals, biofuels, etc. – thus generating CO₂-neutral energy sources and commodity chemicals (11, 279). Specifically, there is an interest in the formation of biomass, compounds contained in the biomass, and/or compounds excreted into the growth medium. Excretion of products, achieved by engineering microbial cell factories, typically involves precursors to bioplastics (e.g. lactic acid), biofuels (e.g. ethanol) or drop-in biofuels (e.g. butanol) (215) but also secondary metabolites such as vitamins (32).

In this study, cells were grown in a continuous culture in a turbidostat-controlled lab-scale photobioreactor, sparged with N₂ and CO₂ (280). The culture was subjected to a circadian rhythm of 12 h light/12 h dark, resulting in oxygen depletion during the dark period. These are conditions that mimic the conditions in a large scale photobioreactor that can be equipped with a degassing system and that can be sparged with off-gases originating from the combustion of fossil fuel (81). A changing environment, especially with respect to the primary source of energy (light), and the lack of oxygen in the dark period, has a strong influence on the metabolic state of cyanobacteria; they can react to an anoxic environment by fermentation for cellular maintenance (19, 281).

Cyanobacteria have adapted to their changing environment by evolving regulation systems governed by a circadian clock. With the clock cells possess the means to react in a timed manner to use energy efficiently, i.e. to anticipate a synchronization of their physiology with the availability of light (277). The circadian clock of cyanobacteria is entrained by light/dark periods and allows synchronizing of the clock between cells in a population (278).

The dynamic behavior of *Synechocystis* cultured at high density and under a continuous 12 h high light/12 h dark cycle, without the addition of oxygen, results in growth exclusively during the light period. As expected, and in accordance with the experimental setup, the O₂ levels are zero during the dark period.

We set out to investigate the metabolome and proteome of such cells from samples taken at time points close to the change of light-availability, to study the immediate response and subsequent adaption to the oscillating conditions. Samples from different time points are separated with respect to their metabolite profile. This allows the identification of select metabolites that correlate with the differentiated physiology at the different time points. Most protein levels change only slightly, except for the levels of proteins involved in the high-light response; the latter increase after the shift to a light period. Both analyses suggest that in order to change the flux through a metabolic network the relative amounts of the metabolites
and proteins do not need to change drastically, at least for the time points close to the change of the light regime.

Next to the investment in growth, the cells utilize the fixed internal carbon compounds for the synthesis of storage compounds in the light period, to allow survival and maintenance in the dark period (e.g. glycogen) (185). Here we show the dynamic behavior of glycogen accumulation, which peaks already before the end of the light period and shows a non-linear degradation profile during the dark period. The rapid degradation at the end of the dark period suggests preparation in anticipation of the upcoming light period, possibly governed by the circadian clock.

For an educated biotechnological application it is of importance to understand the regulation of the metabolic network of *Synechocystis* and the flux distribution therein. The results of this study contribute to this understanding.

10.2 Materials and Methods

10.2.1 Strain and pre-culture Conditions

A pre-culture of the glucose tolerant wild type strain of *Synechocystis* sp. PCC 6803 (obtained from D. Bhaya, Stanford) was incubated in BG-11 medium (Sigma) at 30°C in a shaking incubator at 120 rpm (Innova 43, New Brunswick Scientific). Cultures were illuminated with constant moderate light provided by 15W cool fluorescent white light (F15T8-PL/AQ, General Electric, CT, USA). The light intensity was 30 \( \mu \text{E}/\text{m}^2/\text{s} \) measured with a LI-250 light meter (LI-COR, NE, USA). Prior to the experiment in the photobioreactor (see below), a 100 ml pre-culture was cultivated in BG-11 medium in a 300 ml Erlenmeyer flask. To assure that an axenic culture was used, an aliquot of the culture was spread on BG-11 plates and on LB plates (solidified with 1.5% w/v agar). Plates were incubated at 30°C and screened for three consecutive days for potential contaminants. BG-11 plates were supplemented with 10 mM TES-KOH (pH 8), 5 mM glucose and 0.3% sodium thiosulfate.

10.2.2 Photobioreactor (PBR)

The *Synechocystis* pre-culture (see above) was used to seed two FMT-150 photobioreactors (Photon System Instruments, Brno, Czech Republic, hereafter PBR) with 100 ml exponentially growing culture, each. This study makes use of the ~1000 ml vessel model FMT-150, that is temperature controlled and illuminated from one side by blue- and red-light emitting diodes (LED) (280). Growth was monitored by the integrated densitometer at OD 735. Culture medium was BG-11 (Sigma). Continuous gas mixing was provided by the Gas Mixing System GMS150 (Photon System Instruments, Brno, Czech Republic) set to 0.5% \( \text{CO}_2 / 99.5% \text{N}_2 \), coupled to mass flow controllers (Smart Mass Flow Model 5850S, Brooks Instruments, PA, USA) to supply 0.5 l/min to each PBR. The \( \text{CO}_2 \) influx provided an excess of (inorganic) carbon and clamped the pH in the preferred range of 7.5-8.0. The temperature was kept at 30 ± 1°C. The lid of the PBRs accommodated a pH electrode, a Clarktype dO2 electrode and a pCO2 electrode (Mettler-Toledo). The light regime applied by the LED board provided 12 hours light and 12 hours darkness. The culture was kept at optical density 0.8 ± 0.01 as measured by the integrated densitometer at 735 nm by turbidostat control. This value corresponds to approx. 2.6 as determined at OD 730 in a bench-top spectrophotometer with 1 cm path length (Lightwave II, Biochrom, Cambridge, UK). The pump coupled to the turbidostat control attached to the medium reservoir was set to 5.6 ml/min. The two vessels were illuminated with LED arrays consisting of blue (445 nm, 18 nm full width at half maximum (FWHM)), and 458 nm, 16 nm FWHM, respectively) and red (for both PBRs: 636 nm, 20 nm FWHM)(280). The LED boards provided 500 \( \mu \text{E}/\text{m}^2/\text{s} \) to both cultures. Less than 5% of the light-input was
measured at the opposite side of the vessel, probably implying a light-limited regime of the whole population (282). The 12 hours light and 12 hours dark rhythm was applied over a period of one week, after a ‘quasi-steady state’ was reached, judged from the optical density measurements and visual inspection of the recurring patterns of OD\textsubscript{735} and OD\textsubscript{680} (Supplemental material Figure S1). Next, over a period of 10 consecutive days, samples were taken for metabolite analysis. Thereafter, within three consecutive 24-hour periods samples for protein analysis, glycogen determination, dry cell weight (gram dry weight, gDW) determination were taken (for sampling time points see Figure 1). The repetitive signal-output patterns of the PBR system (OD\textsubscript{735}, OD\textsubscript{680}, pH, dO\textsubscript{2}, and dCO\textsubscript{2}) were essentially identical for the whole duration of the experiment (Supplemental material Figures S1 to S4). Solely the dO\textsubscript{2} signal showed slightly decreasing amplitude during the initial 1-week period that was used to establish a ‘quasi-steady state’. This signal stabilized, however, before the sampling took place (Supplemental material Figure S2). For growth rate determination a best fit based on an exponential equation was derived from the OD\textsubscript{735} signal in between the pre-set thresholds for the turbidostat control which controls the supply of fresh medium (Supplemental material Figures S1 and S4). For the identification of the first actual growth phase after the shift to the light period the lower threshold effectively indicates the end of the lag-phase. It should be noted that the rapid drop of the OD\textsubscript{735} signal at the onset of the dark period is an artifact of the optical-density measurement, caused by the temperature change of the equipment, due to the switching-off of the light. Likewise, the rapid increase of the OD\textsubscript{735} signal at the onset of illumination is caused by this.

**Figure 1:** Experimental setup and sampling scheme. OD\textsubscript{735}, OD\textsubscript{680}, pH, dO\textsubscript{2}, and dCO\textsubscript{2} signals were automatically recorded over the whole duration with an interval of 1 min. Samples for glycogen were taken every hour. Samples for gDW, metabolite analysis and proteome analysis were taken at 5 time points (TP1: 1 hour after the onset of light, TP2: 3 hours, TP3: 11.75h, TP4: 13h, and TP5: 15 hours). The number of sampling time points for the latter three analyses was limited by the volume that could be taken from the culture without disturbing the continuous behavior of the system. A higher resolution of sampling time points around the time point of shift from the light period to the dark period was motivated by the findings by Kucho et al. who indicated significant changes in gene expression at the subjective day to night transition (272).

### 10.2.3 Metabolite detection by NMR and their statistic evaluation

Rapid sampling from the PBRs for metabolite determination was performed as follows: 50 ml of cell suspension from the photobioreactor vessel were harvested through a sampling port with a syringe into a 50 ml tube. For samples from the dark period the tubes were covered with aluminium foil to avoid unwanted illumination. Cells were pelleted by centrifugation at 6,000 rpm at 4°C for 5 min (in a pre-chilled centrifuge, Eppendorf centrifuge 5403, 16F6-38 rotor). The supernatant was removed carefully and the remaining wet pellet was snap-frozen in liquid N\textsubscript{2} and stored at -80°C. After collection of all samples from the whole time period the tubes were collected on liquid N\textsubscript{2}, caps were replaced with caps containing holes and samples were freeze-dried. After 24 to 48 hours the tubes were collected on liquid N\textsubscript{2} and stored at -80°C. This protocol aims for the recovery of a dry cell pellet of about 25 mg for \textsuperscript{1}H Nuclear Magnetic Resonance (NMR) spectroscopy analysis. The NMR-based metabolite-analysis method followed the method as described earlier (283). Briefly, \textsuperscript{1}H NMR was acquired by the analysis of the extract of the freeze-dried cell material in KH\textsubscript{2}PO\textsubscript{4} buffer in D\textsubscript{2}O (pH 6). As internal standard for concentration determination 0.54 mM trimethylsilyl propionic acid solution salt-d\textsubscript{4} (TMSP-
Circadian dynamics

d4 was used with pre-saturating residual HDO signal. Before multivariate data analysis evaluation of the metabolite profile, the NMR spectrum was divided into a series of bins, generating a profile with about 256 peaks using AMIX (ver. 3.6, Bruker, Karlsruhe, Germany), as detailed in Kim et al. (2010). After baseline correction, a multivariate data analysis using partial least square discriminant analysis (PLS-DA) was used (SIMCA 13.0, Umetrics) with the pareto scaling method. The supervised method allowed incorporation of the a priori knowledge we had from the sample history (such as the time point (TP), whether it was a sample from the light or the dark, which sample from the 10 consecutive days it was), etc. This allows discrimination between which of those factors result in separation of the profiles and suggests correlations. The evaluation according to the variable importance in the projection (VIP) score (from the SIMCA software package) allows ranking according to the highest VIP score (compare also Figure S5A in the supplemental material). The compounds responsible for separation in the PLS-DA were then manually identified comparing the relevant peak-pattern to a database.

10.2.4 Proteome determination by mass spectrometry (MS) analysis

Sampling from the PBRs for protein analysis was performed as follows: 10 ml of cell suspension from the vessel were harvested via a sampling port with a syringe into a 15 ml tube (on ice) that already contained 5 ml of ice-cold 50 mM ammonium bicarbonate (Fluka BioUltra >99.5%). For samples from the dark period the tubes were thoroughly covered with aluminium foil. Cells were pelleted by centrifugation at 6,000 rpm at 4°C for 10 min (in a pre-chilled centrifuge, Eppendorf centrifuge 5403, 16F6-38 rotor). The supernatant was discarded completely and cells were re-suspended in 1 ml of ice-cold 50 mM ammonium bicarbonate and snap-frozen in liquid N2 and stored at -20°C. Prior to the disruption of the cells by sonication, the cell suspension was supplemented with 0.1% (w/v) Rapigest SF (Waters). Cell lysis was performed by 1 round of sonication with a micro-tip (duration for 5 min, output strength of 5, and a 50% duty cycle by a probe-sonicator (Branson) on melting ice. Cell debris was pelleted by centrifugation at 14,000 rpm at 4°C for 30 min (in a pre-chilled table top-centrifuge). The supernatant content of the supernatant, containing the soluble fraction of the crude cell-free extract, was immediately determined by a BCA assay, using bovine serum albumin as the standard, according to the manufacturers protocol (Pierce). The protein concentrations of the lysates range from ~0.5 mg/ml to ~1.0 mg/ml (Figure S5B in the supplemental material). Lysates were brought to same protein concentrations (0.5 mg/ml) by diluting with 0.1% Rapigest SF in 50 mM ammonium bicarbonate. A preliminary investigation of the crude cell-free extract prepared in an identical manner on SDS-PAGE showed no significant protein degradation at room temperature for up to 3 hours (data not shown). Subsequently, samples were reduced with 5 mM dithiothreitol (Sigma-Aldrich) at 60°C for 30 min and alkylated with 15 mM iodoacetamide (Sigma-Aldrich) at ambient temperature in the dark for 30 min. Proteolytic digestion was initiated by addition of modified trypsin gold grade (Promega, Madison WI) at a 1:50 protease:protein (ratio by weight), followed by overnight incubation at 37°C. Following digestion, breakdown of the acid-labile detergent (Rapigest SF) was induced by the addition of 1% trifluoroacetic acid to achieve a pH < 2 and subsequent incubation at 37°C for 45 min. The peptide solutions were centrifuged at 13,000 rpm for 10 min and supernatant was collected. Prior to analyses a protein digest MassPREP Protein Digestion Standard (Waters Corporation) was added, at 1:1 dilution to the sample (50 fmol/µl alcohol dehydrogenase (ADH1) and enolase (ENO) from Saccharomyces cerevisiae).

Nanoscale LC separations of tryptic peptides were performed with a NanoAcquity system (Waters Corporation). Samples were loaded onto a Symmetry C18 5 µm, 2 cm x 180 µm trap column (Waters Corporation) at a flow rate of 5 µl/min prior to separation on a Bridged Ethyl Hybrid C18 1.7 µm, 25 cm x 75 µm analytical reversed phase column (Waters Corporation) by application of a 90 minute gradient from 1% acetonitrile, 0.1% formic acid to 40% acetonitrile, 0.1% formic acid at a column flow rate of 0.300 µl/min. Analysis of eluting tryptic peptides was performed using a Synapt G2 quadrupole time-of-flight mass spectrometer (Waters Corporation, Manchester, UK) equipped with a nanolock spray source (Waters Corporation), fitted with a pico-tip emitter (New Objective, Woburn, MA). Operated values were: around 3 kV
capillary voltage, cone voltage of 40 eV, a source temperature of 90°C and TOF-voltage set at 7 kV. The collision gas used was argon, maintained at a constant pressure of 2 µbar in the collision cell. The lock mass, [Glu1]-Fibrinopeptide B, was delivered from the auxiliary pump of the NanoAcquity system at a concentration of 100 fmol/µl and a supply rate of 0.5 µl/min to the reference sprayer of the nanolock spray source which was sampled every 120 seconds. The data were post-acquisition lock-mass corrected using the mono-isotopic mass of the doubly charged precursor of [Glu1]-Fibrinopeptide B. Accurate mass precursor and fragment ion LC-MS data were collected in data independent LCMS² mode of acquisition (284, 285). Samples of the two Bioreactors, each sampled over 2 days at five time-points (total of 20 samples) were injected twice (technical replicates) to ascertain the reproducibility of the mass spectrometric analysis.

10.2.5 Analysis and evaluation of proteomics data

Continuum LC-MS data were processed using ProteinLynx GlobalSERVER version 2.5 (PLGS 2.5, Waters Corporation). Parameter settings: digest reagent: trypsin, allow 1 ‘missed cleavage’, search tolerances: automatic, typically 5 ppm for precursor and 15 ppm for product ions, fixed modification cysteine: carbamidomethylation, and variable modification: methionine oxidation. Protein identifications were obtained searching the entries of a *Synechocystis* proteome database (UniProt), with ADH1 and ENO1 of *S. cerevisiae* appended as internal standard to address technical variation and allow for absolute quantitation of protein amounts. Estimation of false-positive identification rates was done by searching a randomized version of the abovementioned human protein database generated within PLGS 2.5. HI3 peptide quantitation was used to quantify fmols (label free absolute quantitation) of each protein measured by use of the known amounts of the added Massprep standards (286). Data were exported as csv-files and fmols determined by HI3 peptide quantitation were normalized by summed fmols determined for each LC-MS run to adjust for variability in sample content and instrument performance. Any protein was required to be detected a minimum of 9 (out of 40) injections, in order to be regarded as reliably identified and quantified, which lead to identification of 357 *Synechocystis* proteins with a false discovery rate of <1% as determined by the number of identifications from the randomized database (data not shown). As the data acquired needed to reflect the protein profile over the whole time series, to probe the diurnal rhythm of *Synechocystis*, we applied a second round of selection criteria: i.e. proteins needed to be detected over all time points measured, and be detected in at least two technical replicates for both the PBRs. This provided a list of 176 proteins for which the normalized fmols (nfmol) are shown in the supplemental material (Table S1).

10.2.6 Glycogen analysis

Rapid sampling from the PBRs for glycogen determination was performed as follows: ~5 ml of cell suspension from the vessel were harvested through a sampling port with a syringe into a 15 ml tube. For samples from the dark period the tubes were covered with aluminium foil. Aliquots of 2 ml cell suspension (technical duplicates for each PBR) were pelleted by centrifugation at 14,000 rpm at 4°C for 5 min (in a pre-chilled centrifuge). The supernatant was removed carefully and the remaining wet pellet was stored at -20°C. Glycogen was determined essentially as described before (287), employing the D-Fructose/D-Glucose assay kit (Megazyme) adapted for use in a 96-well plate reader. Cell pellets were re-suspended in 200 µl KOH (5.35 M) and hydrolyzed for 90 min at 95°C in a thermo shaker at 500 rpm. For glycogen precipitation 600 µl of cold ethanol (absolute, Scharlau) were added to previously cooled samples and placed on ice for 2 hours. The insoluble glycogen was pelleted by centrifugation at 14000 rpm at 4°C for 5 min (in a pre-chilled centrifuge), and washed twice with cold ethanol. The remaining pellet was supplemented with 300 µl acetate buffer (200 mM, pH 5.2) and 50 µl amylglucosidase (Roche) dissolved in acetate buffer and incubated overnight at 55°C under constant agitation. Samples were centrifuged at 5,000 rpm at room temperature for 1 min and the glucose concentration was determined with the D-Fructose/D-Glucose assay kit (Megazyme) according to the manufacturers’ instructions. The amount of glycogen was normalized to the gDW.
10.2.7 Dry cell weight measurements

For the determination of the dry cell weight 2 subsequent aliquots of 25 ml of culture were harvested from the PBRs into the same 30 ml pre-weight glass tube. The cell suspension was pelleted by centrifugation at 10,000 rpm at room temperature for 10 min. The supernatant was removed carefully without disturbing the pellet after which the cell pellet was dried in a stove at 110°C overnight. The tube was subsequently weighted.

10.3 Results

10.3.1 Growth characteristics of a turbidostat culture in a diurnal rhythm

Wild-type *Synechocystis* was used in an experimental setup which mimics culturing conditions relevant to biotechnological applications. Sampling time points were chosen such that we could investigate dynamic changes, specifically those around the transition from a light to dark period (shortly after the onset of light, at the end of the light period, and shortly after the onset of the dark period) (Figure 1). Figure 2 shows the summary of the physiological parameters that were followed and derived from the online measurements of the photobioreactors (PBRs). The data in Figure 2 visualize one representative day of the multi-day experiment. The reproducibility of the physiological data is presented in the supplemental material (Figures S1 to S4). Figure 2 shows the data of day 7. After the onset of illumination (at 0 hours), the culture shows a lag-phase of ~2 hours before entering the more rapid growth phase (Figure 2A). A lag phase after the shift from the dark period into a light period has been observed before in a light/dark experiment with *Synechocystis*, utilizing roughly the same maximal light intensity, but simulating an actual diel cycle by a gradual increase of light intensity to a maximum at subjective noon followed by a gradual decline (288).

Over the course of a whole light period the OD\textsubscript{680} decreases relative to OD\textsubscript{735}, thus Chlorophyll a (Chl a) content per cell seems to decrease, indicating an adaptation to the high light intensity and/or photobleaching, or a regulation governed by the circadian clock (Figure 2A) (289). From the 735 nm absorption values we determine the growth rate (Figure 2B), which shows the lack of growth during the first ~2 hours of the light period and in the dark period. Further, it shows that growth rate increases until the ‘subjective noon’, and slows down again after that peak, when advancing into the ‘subjective evening’. Figure 2C shows the oxygen production during the light period, essentially following the growth and dilution pattern and the completely anoxic environment in the dark period. Likewise, the utilization pattern of CO\textsubscript{2} indicates growth and dilution. Both pH and temperature show the influence of the dripping-in of fresh medium during the pumping-phases as well, and are held rather constant during the course of the experiment (Figure 2D).
Figure 2: Physiological data during the light/dark rhythm. (A) Optical density (OD) readings from the PBR. OD<sub>735</sub> in black, and OD<sub>680</sub> in gray. (B) Growth rates calculated from fitting an exponential equation to the OD<sub>735</sub> as shown in (A) during the growth phase in the light period. Error bars represent the standard deviation; if the error bars are not visible they are smaller than the data point symbol. For panel A and B the sampling time points for metabolite and protein analysis are indicated (TP1 to TP5); the vertical dashed line indicates the end of the lag-phase (see materials and methods section for details). (C) Dissolved oxygen (dO<sub>2</sub>) and dCO<sub>2</sub> recording. (D) pH and temperature recording.

10.3.2 Glycogen storage dynamics and constant cell dry weight

As expected, the glycogen content of the cells increases during the light period, demonstrating that cells accumulate this internal carbon storage compound for the upcoming dark period. Surprisingly, the maximum glycogen levels of ~40% of the cell DW are reached already at the subjective ‘afternoon’, 9.5 hours after the onset of illumination, thus 2.5 hours before onset of the dark period (Figure 3).

Throughout the rest of the subjective day period and the major part of the night period the glycogen content does not change much. Essentially, it is decreasing to 35% of the cell DW before the glycogen-degradation rate increases significantly.
Circadian dynamics

Figure 3: Glycogen synthesis and degradation pattern during the light/dark rhythm. Average and standard deviation (n = 2) are shown.

On average, only 0.5% Glycogen/g cell DW per hour is degraded between reaching the peak level and the onset of faster degradation. During the last hours of the dark period (~2 to 3 hours before the onset of illumination) the majority of the glycogen is consumed rapidly, resulting in a glycogen content of $21.73 \pm 5.06\%$ at the last time point in the dark period and even $14.67 \pm 7.03\%$ at the first time point of the light period (Figure 3). After that and coinciding with the start of the rapid growth phase, the glycogen content increases again. The cell DW determined at the 5 defined time points as well as the OD shows a constant value (Table 1).

Table 1: Correlation between Glycogen content, gDW and OD$_{735/730}$. OD and gDW remain stable; the glycogen content shows fluctuations (compare also Figure 3).

<table>
<thead>
<tr>
<th>Hours after light-onset</th>
<th>1.5</th>
<th>3.5</th>
<th>11.50</th>
<th>13.5</th>
<th>15.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light/dark period</td>
<td>Light</td>
<td>Light</td>
<td>Light</td>
<td>Dark</td>
<td>Dark</td>
</tr>
<tr>
<td>OD$_{735}^a$</td>
<td>0.785 ± 0.010</td>
<td>0.798 ± 0.006</td>
<td>0.805 ± 0.004</td>
<td>0.780 ± 0.000</td>
<td>0.776 ± 0.001</td>
</tr>
<tr>
<td>OD$_{730}^b$</td>
<td>2.54 ± 0.11</td>
<td>2.66 ± 0.11</td>
<td>2.61 ± 0.11</td>
<td>2.64 ± 0.12</td>
<td>2.57 ± 0.13</td>
</tr>
<tr>
<td>mgDW/ml</td>
<td>0.370 ± 0.014</td>
<td>0.367 ± 0.019</td>
<td>0.367 ± 0.028</td>
<td>0.343 ± 0.005</td>
<td>0.353 ± 0.009</td>
</tr>
<tr>
<td>Glycogen content (% of gDW)</td>
<td>15.26 ± 4.59</td>
<td>20.59 ± 6.43</td>
<td>35.35 ± 2.89</td>
<td>35.84 ± 4.10</td>
<td>39.09 ± 4.25</td>
</tr>
</tbody>
</table>

* as determined by the densitometer of the PBR
* as determined by the bench-top spectrophotometer

This is certainly expected for the OD$_{735}$ as it was used for the turbidostat controlled growth regime, hence was held constant by the input of fresh medium. It further shows that the cell DW remains constant. The composition of the enclosed biomass however, seems to change, at least with respect to the glycogen content. Significantly, various cyanobacteria show anaerobiosis-induced fermentation capacity in the dark (19). However, an HPLC analysis on a Rezex ROA Organic Acid column (Phenomnex) of the external medium,
aiming at the detection of common excreted organic acids and other fermentation products, shows none of these prominent (fermentation) excretion products (such as lactate, acetate, formate, etc.) (data not shown, detection limits ~50 µmol/L).

10.3.3 Metabolite profiling

Next, we investigated whether the dynamic behavior of the glycogen pool is reflected in an equally dynamic metabolite pattern. Multivariate data analysis employing partial least square discriminant analysis (PLS-DA) of the metabolite signals collected by NMR show that there are significant changes over time of the signals of the NMR profile. The samples dedicated for the metabolite analysis stem from 10 consecutive days (for both PBRs), to allow a statistically meaningful evaluation. Visualization of the first two components of the PLS-DA (Figure 4) shows that the samples from the same time points (TP) group together based on their metabolite profile. It is evident that the samples from the first two time points (during the early light period, TP 1 and 2) share a common profile in contrast to the samples from the three remaining time points (TP 3 to 5). Significantly, the third time point (from the light period, shortly before the onset of darkness, TP 3), although grouping closer with the samples from the dark period (TP 4 and 5), is located between the two pairs, which is in accordance to the actual time profile. As is evident from the grouping of the data from the 5 different sampling time points, the effects on the metabolite profile imposed by the periodic light regime are larger than the differences imposed by two PBRs and the actual day of sampling (over the 10 consecutive days), suggesting a quasi-steady state for the whole course of the experiment.

Figure 4: The effect of the light/dark rhythm on the metabolite profile of the cells. Visualization of the PLS-DA along the axis of components one and two of the PLS analysis. The sampling time points (TP) are color coded as indicated. Samples separate on the basis of different metabolite(s) (levels). Samples from the same time points group together by reason of a similar metabolite profile.

Those metabolites from the first two components that resulted in the grouping of the metabolite profiles by the PLS-DA were ordered according to their variable importance in the
projection (VIP) scores (283). This shows that ~40% of the metabolite signals from the NMR profile contribute to the separation of the profiles because those have a VIP-score >1 (Supplemental material Figure S5). Based on that finding we identified the compounds corresponding to the most highly ranking NMR signals and additionally some other compounds from the spectrum (Table 2). Compounds that rank high in the list of VIP scores are thus changing over the 24 hours light/dark period, whereas those that rank low probably remain rather stable.

Table 2: Metabolites that contribute to the separation of samples in the PLS-DA. The identified compounds are ordered according to their rank in the VIP-score analysis. A total of 237 signals (bins) were recorded by the NMR analysis. A VIP-score >1 shows that the compound had a strong influence on the separation in the PLS-DA, whereas a VIP-score <1 shows the opposite. Mind that we identified only selected signals out of the total of 237 signals. See Figure S5 in the supplemental material for an overview of all VIP-scores.

<table>
<thead>
<tr>
<th>Rank</th>
<th>VIP score</th>
<th>Shift δ (p.p.m.)</th>
<th>Metabolite</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.091</td>
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Glutamate, the metabolite showing the largest dynamic range (Table 2), is an amino acid that can originate from 2-oxoglutarate in the TCA cycle and ammonia, catalyzed by a glutamate dehydrogenase. Thus, glutamate is a key player in protein synthesis and also part of the biosynthesis routes of several other amino acids. Protein biosynthesis is directly correlated to intensive translation, which is needed for growth. Another growth-related functionality of glutamate is that it is a component of the peptidoglycan layer. Furthermore, glutamate is the precursor of glutamine, which is also identified as a compound showing significant temporal dynamics. Both glutamate and glutamine show higher levels during the light period than in the dark period (Figure 5).

Alanine, an amino acid derived from pyruvate metabolism, shows a high VIP score and mildly changing levels (Figure 5). The main routes for alanine consumption are towards protein biosynthesis and incorporation into the cell wall, by crosslinking the sugar polymers of the peptidoglycan layer. Aspartate and methionine, linked to the central carbon metabolism via oxaloacetate from the TCA cycle, also show levels that follow the temporal dynamics (Figure 5).

Acetate shows a dynamic pattern (Figure 5). In catabolism acetate can be formed through Acetyl-CoA and acetylphosphate and has been identified as a fermentation product of several cyanobacterial species (19). It is also a by-product of the methionine biosynthesis, coupling it to amino acid and protein biosynthesis, hence growth. However, we do not identify cysteine, and the intracellular methionine decreases in the dark period whereas acetate increases. The pathways to/from acetate are ATP-dependent and thus could play a role in the ATP-homeostasis.

We further identify a change of fructose-1,6-bisphosphohate (FBP) levels during the diurnal rhythm (Figure 5). Interestingly, the level of fructose-6-phosphate (F6P), the product of FBP in the photosynthetic Calvin-Benson-Bassham (CBB) cycle, and precursor of FBP in glycolysis, does not show changing levels (Figure 5).

Additionally identified metabolites that lack distinct dynamical change in their levels, are lactate, from the pyruvate metabolism, fumarate and citrate from the TCA cycle (Figure 5).

Pool sizes, as determined here for intracellular compounds, are good indications for build-up or consumption of metabolites present at a certain time point, especially when the environment changes. However, they cannot show the actual flux through a pathway.
Figure 5: Identified metabolites as listed in Table 2. Mean and SEM are shown.
10.3.4 Proteomic changes induced by the day-night regime

Changes in glycogen and metabolite pool sizes (but also changes in the flux through a pathway) can originate from increased or decreased enzyme activity. The enzymatic activity in a metabolic network can be altered in a variety of ways. The amount of protein to carry out an enzymatic reaction can be altered, e.g. through regulation of transcription and/or translation, while the number of active enzymes or the rate of the enzymatic reaction can also be manipulated by post-translational or allosteric regulation. The enzymes involved in the central carbon metabolism of photosynthetic organisms are often regulated by the availability of light, or reduction/oxidation of thiol-groups (mediated by thioredoxin), or by phosphorylation (290). In chloroplasts, the majority of the enzymes of the CBB cycle are regulated by the action of thioredoxin. In *Synechocystis*, only a few enzymes have been identified to be thioredoxin-controlled. Additionally, the enzymes involved in glycogen synthesis and degradation and some proteins from the carbon concentrating mechanism (CCM) are potentially regulated by thioredoxin (291). We evaluated changes in protein levels of 176 proteins from the soluble fraction of *Synechocystis* (Supplemental material Table S1). The major constituents of the photosynthetic machinery, the proteins composing the phycobilisomes, PSII, PSI and the cytochrome *b*6f complex, did not show very large changes during the changes from light to dark and vice versa. High light after the dark period at 1 h and 3 h, can be expected to induce up-regulation of proteins involved in photo-protection or protection from reactive oxygen species (compare also (63, 264)). As shown before, the treatment with high light results in a quick response of a wide array of transcripts as shown by Hihara et al. (63). We found a significant up-regulation for a few proteins that are coded from transcripts involved in the high light and/or the stress response (63). The phycobilisome-associated non-photochemical quencher, orange carotenoid protein (OCP, Slr1963), was up-regulated at the time points 1 and 3 h after the onset of illumination (Figure 6). OCP is involved in photoprotection and the dissipation of excess energy. Superoxide dismutase (SodB, Slr1516), an enzyme assisting in the scavenging of reactive oxygen species, shows a high level at the first time point (Figure 6). A similar trend, thus a high level at early time points, is observed for the chaperon GroES (Slr2075), GroEL (Slr2076) and GroEL2 (Sll0416) (Figure 6). However, most of the proteins detected showed little or no changes during the different time points analyzed of the day-night regime.
10.3.5 Integration of metabolomic and proteomic data of light-dark changes in Synechocystis

As we collected data both for proteins and metabolites during the light/dark experiment, this provides us with a unique dataset to probe the systematic changes in Synechocystis of both metabolites and enzymes during diurnal growth. To this end, the quantitative metabolite data that showed the highest changes were compared to the quantitative data of the proteins involved in their metabolism. In addition, for the few metabolites that did not show significant changes the same comparison was done.

The comparison shows that from the glutamate and glutamine metabolism only two enzymes have been identified in our proteome study: GlnA (encoded by slr1756) and GlnN (encoded by slr0288). Both enzymes fulfill the function of a glutamate-ammonia ligase, hence are considered a glutamine synthase (GS). It has been shown in earlier work that the transcript of glnA is present in the presence of a fixed nitrogen source and is additionally up-regulated when cells are nitrogen starved, whereas glnN was only detected upon nitrogen starvation, thus differential behavior of glnA and glnN has been elucidated under nitrogen depletion conditions (292). In addition the GlnN protein was found to be down-regulated in response to carbon limitation, but GlnA did not show a significant change upon the shift to a low-carbon condition (264). However, transcript analysis showed that upon carbon limitation both, glnA and glnN, together with the transcripts for the glutamine-oxoglutarate aminotransferase (GOGAT), are down-regulated. This suggests that a reduction of the rate of nitrogen assimilation is a response to carbon limitation, to balance carbon and nitrogen fluxes (208). Here we find that upon a shift to the dark, GlnA is down-regulated, whereas GlnN shows no significant change (Figure 6). The down-regulation of GlnA correlates with the trend of decreasing glutamate and glutamine concentrations (Figure 5). It should be noted that
we assume that nitrogen in the medium is not limiting as during the growth phase fresh BG-
11 medium is supplied. Furthermore, in batch cultures significantly higher cell densities are
reached with BG-11 medium, without showing signs of nitrogen limitations. A decrease of
carbon influx into the metabolism in the dark might result in the need to adjust the nitrogen
assimilation. Consistent with our finding is the decreased expression of glnA in the dark
period of a diel experiment, investigating the influence of a gradually fluctuating daily light
cycle on Synechocystis growth and transcription (288).

The physiological data presented above suggests that the rate of carbon assimilation
decreases in the dark period, because the glycogen storage is already full, and cells do not
grow any more. However, availability of carbon (dCO₂) is not limiting (Figure 2C). The
amount of Ribulose-1,5-bisphosphat carboxylase/oxygenase (RuBisCO) shows slightly lower
levels (~10%) at the time point close to the shift to dark and at the two time points from the
dark period (Figure 7). Here, we do not identify any of the prominent players of the CCM
e.g. ndhR, etc.) (208), except CcmK (coding from sll1029) and CcmM (coding from
sll1029). It has been shown before that CcmK protein levels are stable in a carbon limited
environment (264) and it is stable also here. Interestingly CcmM, a putative structural protein
of the carboxysomes (52), has been shown to interact with thioredoxin (TrxA) (291). Several
proteins that have been shown to be differentially regulated upon carbon limitation (264) are
found here (e.g. Ssr1528, Gpx2, OCP, GlnN, ChlP, NirA, etc.), but, except OCP, they are all
stable here (Table S1). Thus we conclude that cells are not carbon limited, but rather stop
fixation of significant amounts of carbon and need to adjust nitrogen assimilation accordingly
to keep the carbon and nitrogen fluxes balanced.

Figure 7 shows the change of the levels of FBP and the relatively stable signal of F6P,
the two metabolites identified from the central carbon metabolism, and those proteins that are
identified from the CBB cycle, the pentose phosphate pathway (PPP) and glycolysis.
Significantly, the lower levels of FBP coincide with the light phase, when the flux through
the photosynthesis-driven CBB cycle is high (293). GlpX, a FBP 1-phosphohydrolase, and
FbaA, an FBP aldolase increase from the 1 hour time point to the 3 hour time point and
decrease to a lower level again in the three remaining sampling time points. The amount of
Gap2, the prime glyceraldehyde-3-phosphate dehydrogenase in the fixation-direction of the
CBB cycle is about 10-fold higher than the amount of Gap1, which, in turn, is mainly active
in glycolysis (294). Both proteins seem to be at a rather stable level during the light/dark
cycle that is imposed on them here. The enzymes catalyzing the preceding steps in the CBB
cycle, namely phosphoglycerate kinase (Pgk) and RuBisCO (RbcL and RbsS), show slightly
higher amounts during the early light period and a lower (10%) amount in the three
remaining sampling time points, close to and in the dark period. The enzymes of the CBB
cycle that are shared with the Pentose Phosphate Pathway (PPP) do not show a significantly
altered pattern. The first steps towards glycogen synthesis, the phosphoglucomutase (Pgm),
and the ADP-glucose pyrophosphorylase (Agp), also show mild up-regulation in the early light period.

**Figure 7:** Calvin-Benson-Bassham (CBB) cycle and part of the central carbon metabolism. The majority of the proteins in the CBB cycle could be identified. Larger insets on the right show the two identified metabolites from the CBB cycle, FBP and F6P. PPP: Pentose phosphate pathway; F6P: Fructose-6-phosphate; FBP: Fructose-1,6-bisphosphate; G3P: Glyceraldehyde-3-phosphate; DHAP: Dihydroxyacetone phosphate; BPG: 1,3-bisphosphoglycerate; 3PG: 3-phosphoglycerate; PG2: 2-phosphoglycerate; PEP: Phosphoenolpyruvate; PYR: Pyruvate; AcCoA: Acetyl-CoA; RuBP: Ribulose-1,5-bisphosphate; 2PG: 2-Phosphoglycerate; Ru5P: Ribulose-5-phosphate; X5P: Xylulose-5-phosphate; S7P: Sedoheptulose-7-phosphate; Ri5P: Ribose-5-phosphate; 6PG: 6-Phospho-gluconate; 6PGL: 6-Phospho-gluconolactone; G1P: Glucose-1-phosphate; ADPGlc: ADP-glucose; Amy: Amylose; ENO: Enolase; PDH: Pyruvate dehydrogenase (complex); Mean and SEM are shown.

### 10.4 Discussion

Here, we observe that growth is a consequence of availability of light and before growth starts during the light period, a lag-phase of ~2 hours can be identified. The
concomitant gradual decrease in OD$_{680}$ suggests a lowering of the Chl $a$ content of the cells during the light period. Glycogen is accumulating in the light period, reaching a maximum already before the end of the subjective day. A fast degradation is triggered in the dark period only shortly before the onset of the subjective day. Several intracellular metabolites allow the differentiation of the separate time points, however only a few have significantly changed in their levels. Notably, as mentioned before, the pool sizes do not necessarily reflect the flux through a biochemical network. Metabolic control through enzymatic activity is governed by the level of a respective enzyme but also by other means, such as allosteric regulators or light dependent regulation by the thioredoxin-system.

Here, we apply a circadian illumination regime of 12 h light/12 h dark in a ‘square-wave’ cycle, hence an abrupt change from dark to ~500 µE/m$^2$/s red plus ~500 µE/m$^2$/s blue light. In contrast to moderate light/dark cycles or sinusoidal light/dark cycles, a high-intensity light/dark cycle can lead to photobleaching and loss of Chl $a$ (289). Here we observe the recurring lowering of the ratio of OD$_{680}$ over OD$_{735}$, but the ‘quasi-steady state’ is stable over the whole course of the experiment lasting >2 weeks (compare also supplemental material Figures S1 to S4). Consistent with a high-light stress response (63) we find several proteins up-regulated in the time points after the shift to the light period (e.g. Ocp and SodB in Figure 6). Significantly, this ‘high light response’ was not evident from a comparable diel study by Labiosa et al. (288) with gradually increasing and decreasing light conditions. In that study the gradual increase and decrease of light intensity correlates with the observed growth rate. During the dark (aerated) period a low growth rate was observed, which was minimal in the first ~2 hours of the light period (288).

Studies on the loss of respiration and fermentation in cyanobacteria are rare (19, 289). It is suggested that cyanobacteria experience dark and anoxic environments in microbial mats or in dense algal blooms, often coupled to the availability of organic carbon sources (19). Studies dealing with anoxic hydrogen production provide some information on the physiology during fermentation in cyanobacteria (281, 295–297). Lactate, acetate and hydrogen have been identified as fermentation products in Synechococcus sp. PCC 7002 (281). Hydrogen production by Synechocystis is hypothesized to be supported by NADH and reduced ferredoxin, both originating from the route of glucose towards acetate (with coupled ATP generation), essential to maintain the cellular redox balance (295, 298). Here we suggest that Synechocystis ferments in the dark and anoxic period, leading to acetate and lactate formation. Interestingly only acetate levels are increased 1 and 3 hours after the shift to the dark, whereas lactate levels remain constant over the five sampling points. This, apparently minimal, fermentation activity seems not to be enough to support growth in the dark anoxic period when respiration is not possible. Recently it has been shown that lack of respiration, by deletion of the terminal oxidases in Synechocystis, results in a phenotype that can recover from a moderate light/dark change, but does not withstand high light/dark cycles. This suggests that respiratory chain oxidases and the photosynthetic machinery are interlinked.
and that functional terminal oxidases are necessary for the recovery of high light stress periods or the dissipation of energy during the high light period (289).

Glycogen accumulation starts at the same time point as growth sets in, that is ~2 hours after the onset of light. Thereafter, a linear increase of glycogen content is observed until a maximum is reached in the subjective afternoon. The different dynamics during the dark period (Figure 3), together with the constant cell dry weight suggest an investment of carbon storage into growth related compounds in the last hours of the respective dark period. This is a clear indication of an anticipatory effect, to prepare the cells for the upcoming light period when it is likely necessary to possess machinery for the harvesting of light energy and subsequently the machinery for growth, possibly including DNA replication, transcription, translation and protein synthesis. It should be noted that for *Synechococcus* sp. PCC 7942 it has been shown that cell growth and genome replication can be uncoupled (277). Interestingly, the carbon storage dynamics for cyanobacteria (*Oscillatoria agardhii*, (300, 301)) as well as plants (*Arabidopsis thaliana*, (302)) cultivated in the presence of oxygen suggest a linear build-up and linear degradation pattern, even adjusted to the duration of the light and dark periods. This linear degradation has been modelled by flux balance analysis for *Synechocystis* by Knoop et al. (168). Although minor, more rapid glycogen degradation in the last ~2 hours of the dark period is evident in this model. The detailed analysis of the participating enzymes in the flux balance analysis shows a higher flux through phosphoglucomutase (Pgm) exactly in those 2 hours. Consistent with all studies mentioned, also in our study *Synechocystis* seems to aim at a low glycogen content at the start of the light period. Two of the enzymes in the glycogen synthesis and degradation pathway, phosphoglucomutase and ADP-glucose pyrophosphorylase are substrates for thioredoxin regulation, thus subject to a redox-dependent regulation essentially achieved by the presence and absence of light (291). The rapid glycogen degradation in the last hours of the dark period observed here suggests either direct regulation of the enzymes involved by the circadian clock, or by a change in the redox status of the cytosol prior to the onset of light (which might also be under the control of the circadian clock).

The observed fluctuations in FBP level support the notion that the flux through FBP is high during the photosynthetic activity of the CBB cycle, whereas and accumulation of FBP is possible during the dark when glycolysis is active, and fueled by the breakdown of glycogen (293). Although the majority of the CBB cycle enzymes do not change levels much (e.g. even RuBisCO levels only decrease by 10% in the dark period) it is evident that other regulation systems must lead to the regulation of its activity, such as the ferredoxin/thioredoxin system (291, 303). The FBP dynamics observed here further suggest that the breakdown of glycogen through glycolysis is possible without respiration in *Synechocystis*, presumably through fermentation pathways. We do not observe growth during the dark anoxic phase, but, as mentioned before, slow growth is observed in a similar study where oxygen is provided (288). We thus hypothesize that fermentation, or the redox-neutral
Chapter 10

supply of carbon from glycogen to intermediary metabolism, is not enough to support growth but rather only for maintenance.

All in all, the dynamic behavior observed in this study suggests low maintenance costs for the majority of the subjective night period and a rapid release of carbon into the metabolism shortly prior to the onset of the light period, possibly to prime the cells for light harvesting and growth. The culture conditions chosen might lead to a sort of ‘hibernation’ in most of the dark period. Also the slight drop in both OD$_{735}$ and OD$_{680}$ suggest cell lysis, possibly of cells that cannot maintain their integrity. The OD drop does not directly correlate with the pattern of glycogen degradation, which could otherwise have been a reason for differential scattering in the OD determination.

This study emphasizes that the culturing conditions have a strong impact on the physiological response of cyanobacteria. However, cyanobacteria have means to adapt to a different environment by different modes of growth.

10.5 Acknowledgments

We are indebted to Naira Quintana (presently at Rousselot, Belgium) for the initiative to enable the collaborative endeavor reported here. We are very grateful to Timo Maarleveld from CWI/VU (Amsterdam) for a custom made a Python™ script handling the output from the NMR analysis, and evaluating and visualizing the separate metabolites. We thank Rob Verpoorte from Leiden University (metabolome analysis) and Hans Aerts from the AMC (proteome analysis) for lab space and equipment. We thank Hans Matthijs from IBED for inspiring dialogues and insightful thoughts on continuous culturing of cyanobacteria. We are grateful to Sandra Waaijenborg, Johan Westerhuis and Huub Hoefsloot from BDA for helpful discussions. We are very grateful to Wilmar van Grondelle and R. Milou Schuurmans for help with sampling and glycogen determination. SAA, PvA, and KJH are supported by the research program of BioSolar Cells, co-financed by the Dutch Ministry of Economic Affairs, Agriculture and Innovation.

10.6 Supplemental material

Supplemental material for this chapter may be requested per Email for personal use: angermayr@gmail.com.