Towards stable cyanobacterial cell factories

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3 Photonfluxostat: A method for light-limited batch cultivation of cyanobacteria at different, yet constant, growth rates

The growth rate and physiology of photoautotrophic bacteria are dependent on the incident light color and intensity. Here we report a widely applicable and straightforward method for light-limited batch cultivation of phototrophic bacteria at different, yet constant, growth rates. We illustrate its usage with *Synechocystis* sp. PCC6803, a model cyanobacterium used as a chassis for sustainable cell-factories and capable of turning CO₂ into commodity products. The cultivation method we developed resembles a ‘photonfluxostat’. It enables the setting of the growth rate of phototrophs during batch cultivation by adjustment of the illumination intensity (‘photon dosing’). Using this method to study the growth-rate response of *Synechocystis*, we found that while the cell volume increased, the chlorophyll a content and the PSI/PSII ratio decreased, as growth rate increased. This method allows for a quantitative and controlled study of the light-dependent physiology of phototropic bacteria, a highly relevant group of bacteria for modern biotechnology.

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3.1 Introduction

Growth rate is an important characteristic of the physiological state of a bacterial population. Understanding how cells achieve balanced growth by coordinating the expression of thousands of genes for a variety of cellular processes while competing for resources under fluctuating environmental conditions, is still one of the most fundamental challenges in cell physiology. From an evolutionary point of view, it is very intriguing to understand how and why cells have different metabolic strategies with respect to catabolic efficiency at different growth rates. Such phenotypic traits have now been observed across all domains of life. From an application point of view, control of microbial growth rate by modulation of nutrient availability can have a huge impact on product yield in industrial settings. Therefore, detailing the correlation of growth rate with various cellular responses is of paramount importance for better understanding of cell physiology.

Cyanobacterial research has attracted considerable attention recently. Not only are cyanobacteria used as model organisms for understanding basic cellular processes, such as oxygenic photosynthesis, the circadian clock, and various stress responses, but also for the design of green cell factories for the sustainable production of compounds of interest. This latter aspect is very pertinent as it aims to contribute to the transition towards a bio-based economy that would make our societies much more sustainable. Dissecting the specific growth rate-dependent responses of cyanobacteria should be further explored, to allow further optimization of such sustainability applications. Unlike chemotrophic organisms, cyanobacteria use light as their primary energy source during autotrophic growth. Experiments in which growth rate is systematically varied as a function of light availability will help increase our insight into cyanobacterial physiology.

So far, in microbial physiology, the chemostat and the turbidostat have been the most widely applied cultivation devices in studies that involved the systematic variation of growth rate, both for chemotrophic and photoautotrophic organisms. For photo(auto)trophic organisms this implies the use of a constant incident light intensity, which, if intended to be non-limiting, will restrict workable cell densities to very low values, since light has to travel a considerable distance through most planes of the culturing vessel. Occasionally, dynamic illumination regimes have been used, for instance to simulate a natural diel regime and to maximize photosynthetic activity. Recently, the turbidostat was used to characterize growth of Synechocystis sp. PCC6803 (hereafter Synechocystis) in a flat-panel
photobioreactor, where the effects of varying light quantity and color on cell growth were explored. In addition, using a similar set up, the parameters related to the growth rate of *Synechocystis* with respect to circadian rhythms were systematically investigated. The successful application of these cultivation strategies indeed broadens our understanding of cyanobacterial cell physiology, and inevitably, also raises relevant new questions. Turbidostats inevitably take longer than standard batch cultivations and require many additional control units. Thus, the number of conditions and strains that can be investigated are limited. In this contribution we describe how to overcome this limitation of current cultivation methods.

Light supply, because of its inherent physical properties, and unlike conventional nutrients such as carbon or nitrogen sources, can be accurately manipulated dynamically with respect to color and intensity. This results in additional ways to achieve steady state exponential growth, for instance, via the possibility of a dynamic adjustment of the incident light intensity. We have explored this property to devise a simple and convenient cultivation strategy, which combines the flexibility of batch culturing with the ability of the chemostat to obtain stable physiological states (i.e. ‘steady-states’). It allows for light-limited batch cultivation of cyanobacteria at different, yet constant, growth rates. The method can be understood in terms of the Herbert-Pirt equation, a classical relation in bacterial physiology. We applied this new cultivation method to study the relationship in *Synechocystis* between growth rate and relevant physiological parameters such as average cell size, chlorophyll a (Chl a) content and PSI/PSII ratio. The results obtained indicate that average cell size positively correlates with growth rate, whereas the cellular contents of Chl a and the PSI/PSII ratio, do not. These results illustrate the feasibility and advantages of this new method as an easier mean, in comparison with continuous cultivation systems, to obtain controlled phototrophic growth in the lab. Moreover, our method can be directly applied for the physiological characterization of other photo(auto)troths in the future.

### 3.2 Mathematical framework

In microbiology, the Herbert-Pirt equation expresses a relation between the specific uptake rate (*q_s*) of a limiting nutrient, the biomass-yield (*Y_{x/s}*), the organism on this nutrient, the organism’s specific growth rate (*μ*) and the maintenance requirement (*m_s*) of the cells for the growth-limiting nutrient to maintain cellular integrity,

\[
q_s = \frac{1}{Y_{x/s}} \mu + m_s,
\]

with *q_s* and *m_s* in \(\text{mol substrate/hr} \times \text{gram biomass}\), *Y_{x/s}* in \(\text{gram biomass/mol substrate}\) and *μ* in \(\text{hr}^{-1}\). 

29
Equation (1) expresses a steady-state flux relation: the specific substrate uptake rate ($q_s$) equals the substrate-uptake rate leading to growth, which amounts to $\frac{1}{Y_{x/s}} \mu$ moles of substrate per unit time per gram biomass, and the rate required for cellular maintenance, which is growth-rate independent, equals $m_s$ moles of substrate per unit time per unit biomass.

We can extend this relation to the uptake of photons, which we study in this work. We choose to denote photons as substrate by $h$, such that equation (1) becomes

$$q_h = \frac{1}{Y_{x/h}} \mu + m_h,$$

(2)

The total uptake rate of photons by a culture then equals $J_h = l \times q_h \times X$ with assumed $l$ as a constant for photon loss specific to the cultivation set-up (absorption by medium, scattering, etc.) and $X$ as the amount of biomass in grams, such that,

$$\frac{J_h}{X} = l \times q_h = \frac{l}{Y_{x/h}} \mu + l \times m_h$$

$$\Rightarrow \frac{J_h}{X} = q'_h = \frac{1}{Y_{x/h}} \mu + m'_h$$

(3)

This equation relates the biomass specific photon uptake rate, $\frac{J_h}{X}$, by a culture (e.g. cyanobacteria), controlled by the light-intensity ($I_h$) set by the experimentalist, to the specific growth rate of the organism, $\mu$, the apparent (i.e. corrected for losses by scattering, absorption by medium components, etc.) photon yield, $Y'_{x/h}$, and the apparent (idem) maintenance requirement of photons, $m'_h$.

We use equation (3) to describe how the growth rate depends on the specific photon uptake rate by the culture, $\frac{J_h}{X}$. We tailor this by setting light intensity, $I_h$ in $\mu$mol photons m$^{-2}$ s$^{-1}$, that we expose the culture to; hence, we assume that they are proportional (i.e. $J_h \propto I_h$). So, by setting the incident light intensity, corrected for the biomass density in the reactor, we also set the growth rate – as long as the culture is light-limited.

3.3 Practical implementation

The practical implementation of the mathematical framework presented above requires a cultivation set-up in which biomass density (here, optical density at 720 nm, OD$_{720}$) can be monitored frequently (within a few minutes); and
subsequently, light intensity \( I_h \) can be modulated such that \( q'_h = \frac{J_h}{OD_{720}} = \) \textit{Constant}. We decided to implement this new cultivation regime, here termed “photonfluxostat”, in a Multi-Cultivator (MC1000-OD, Photon Systems Instruments, Czech Republic, Fig. S3.1) controlled remotely by a newly developed computer program. This device enables multiple parallel cultivations (up to 8) to run simultaneously, testing different strains and/or conditions independently under photonfluxostat conditions. The newly developed software was written in Python. It is completely open-source and made available at https://gitlab.com/mmp-uva/pyCultivator along with extensive documentation. We wrote a set of software packages to monitor and control a Multi-Cultivator. Its built-in OD sensor is capable of measuring at 680 nm and 720 nm and can be used via a serial link, which also supports changes in various settings such as light intensity and gas flow. Software configurations are stored in a XML file to enable automatic triggering of the software.

Generally, once all measurements are stored, the software calculates whether adjustments need to be made to the light settings. The light state can be configured to be conditional on time, to simulate day/night regimes, and the light intensity can be configured to be dependent on the measured OD values. The new light intensity is calculated such that \( q'_h = constant \), while changes are only made when the difference between current and desired intensity is > 1 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \).

### 3.4 Results and discussion

We used the cultivation set-up described above to test specific growth-rate dependent responses of \textit{Synechocystis}. We ran a total of 39 completely independent (parallel) photonfluxostat cultivations at different growth rates by varying the light intensity per \( OD_{720} \). The \( OD_{720} \) was monitored every 5 min and the light flux \( (J_h, \mu \text{mol photons m}^{-2} \text{s}^{-1}) \) was adjusted such that the \( q'_h = \frac{I_h}{x} \) (i.e. \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \text{OD}_{720}^{-1} \)) remained constant. The photonfluxostat regime was maintained until the maximum irradiation capacity of the LED panel, equipped with “cool-white” LED (PSI, CZ), was reached. This means, depending on the photonfluxostat light regime imposed, which ranged from 20 up to 200 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \text{OD}_{720}^{-1} \), that at least an \( OD_{720} \) of 1.5 was reached. Cultivations began with an initial \( OD_{720} \) of around 0.05, illuminated with a constant light intensity until \( OD_{720} \) reached 0.6, except for the light regime at 20 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \text{OD}_{720}^{-1} \), for which we waited until \( OD_{720} \) 0.825. At this point, the different photonfluxostat regimes were imposed by adjusting the light intensity to the actual \( OD_{720} \) as explained above, and as
exemplified for one light regime in Fig. 3.1A. Samples for offline measurements were collected at OD$_{720} \approx 1$.

The OD$_{720}$ data recorded (Fig. 3.1B) under the photonfluxostat conditions were used to calculate the growth rate based on fitting the entire range with a linear model, for which $R^2$ was also calculated (Fig. 3.1B, Fig. S3.2), or based on a sliding window (Fig. S3.3). The offline measurements included: (i) dry weight using a gravimetric method $^{105}$; (ii) average cell volume and cell counts using a CASY counter; (iii) Chl a content based on the OD$_{680}$ and OD$_{730}$; and (iv) the PSI/PSII ratio by 77K fluorescence spectroscopy $^{13}$. All have been determined independently as a function of growth rate, which was modulated by the light regime imposed.

Based on the relationship between $q'_h$ and $\mu$, two distinct states can be observed. We find that indeed $q'_h$ and $\mu$ are linearly dependent when the light regimes imposed in the photonfluxostat mode are below 100 $\mu$mol photons m$^{-2}$ s$^{-1}$ OD$_{720}^{-1}$ (Fig. 3.1B). Beyond this point, the cultures are no longer strictly light-limited, and hence, an increase in $q'_h$ does not result in a linear increase in $\mu$. We named these two states as ‘light-limited’ and ‘light-excess’, respectively. It is important to note that Eq. (3) predicts that the growth rate will vary linearly with $q'_h$, only under the assumption that the $Y'_x/h$ and the $m'_h$ remain constant at all growth rates under light-limited conditions. As counter-intuitive as this may appear, we observed that irrespective of the biomass specific photon flux (i.e. $\mu$mol photons m$^{-2}$ s$^{-1}$ OD$_{720}^{-1}$), as long as cells are light-limited, these do seem to stay relatively constant. By fitting a linear model through the growth rate data collected at different biomass specific photon fluxes during light-limitation (Fig. 3.1B, filled black line, $R^2 = 0.95$), we could estimate these parameters and compare them to previously published data (Fig. 3.1B, dashed line $^{106}$).

The $m'_h$ in our case resulted in a slightly negative number ($-0.0185$ mol photons gDW$^{-1}$ h$^{-1}$). Although disconcerting, this result does highlight the otherwise neglected technical difficulties involved in the estimation of maintenance, which can be more accurately determined using for instance a retentostat $^{107}$. Regarding the biomass yield on photons ($Y'_x/h$), we found it to be $0.876$ gDW mol photons$^{-1}$. Given the uncertainties surrounding how much photons are actually absorbed by cells depending on light sources and experimental set-ups $^{108}$, we consider this value to be comparable with the value obtained in this study using turbidostat data [$0.628$ gDW mol photons$^{-1}$ (Fig. S3.4)], and with previous reports based on data from continuous cultivations of Synechocystis: $1.237$ gDW mol photons$^{-1}$$^{106}$ using “cool-white” LED light and $1.724$ gDW mol photons$^{-1}$$^{109}$ using red monochromatic light (659...
nm, half width 16.3 nm). Furthermore, it is also comparable to the $Y_{x/h}$ determined in continuous cultivations of *Dunaliella tertiolecta* and *Chlorella sorokiniana*, 0.78 and 0.75 gDWmol photons$^{-1}$, respectively.

Fig. 3.1 Schematic overview comparing two different cultivation strategies: photonfluxostat (A, B) and batch (C, D). (A) Different regions with archetype biomass (left), incident irradiance (middle), and light irradiance per OD$^{720}$ (right) as a function of cultivation time. Different colors in the background show different light regimes applied. (B) The inset shows the growth curves under all light regimes that range only from the data point when light regimes were switched on to the data point when sample was taken (reflecting the intensities of all the light regimes in color gradient). The graph below indicates the relationships between light regimes and growth rates calculated based on the growth curves (reflecting $R^2$ of the fitting from the growth rate calculation in gradient). The filled black line is the fitting for growth rates at different light regimes, while the dash line originates from published data$^{106}$. Filled circles and triangles indicate light-limited state and light-excess state, respectively. (C) The inset shows the light regimes for batch cultivation, where fix light intensity was given after inoculation and then were switched to target different fixed light intensities after OD reached the set threshold. The main graph in panel C indicates the light intensity per OD along the whole cultivation. (D) Growth curves beyond the point at which different fixed target light intensities were imposed. Each filled circle, triangle in (B), and each line in (C and D) represents a single observation.
The light-excess phase clearly falls outside the boundaries of the framework with which the photonfluxostat was conceived, since then, light is no longer the limiting substrate by definition. However, by taking into account the high $R^2 (> 0.98)$ of the growth rates estimated by fitting a linear model over the whole range (Fig. 3.1B), one might consider that the stable relationship between $q'/r$ and $\mu$ might still then hold. Although this is still the case to a certain extent, the estimation of growth rate using a sliding window of 20 consecutive data points throughout this range, clearly shows a slowly decaying pattern for these high-intensity cultivations (Fig. S3.3). This is in stark contrast to what is observed for the light-limited cultures, during which it is indeed remarkably constant (idem). This suggests that in the photonfluxostats set to light-excess conditions, as the incident light is further increased to keep up with the growing culture, the cells may begin to experience increasing levels of photoinhibition and/or exciton dissipation. The steadily dropping growth rate could be a consequence of the increasing fitness costs associated with a higher demand for PSII repair mechanisms, or the increasing rate of orange carotenoid protein mediated non-photochemical quenching expected under these conditions. The latter could possibly be minimized if the photonfluxostat regimes were to be set using, not the cool white light source used here, but instead, monochromatic red light. Significantly, the limit value at the highest light intensities gives rise to the conclusion that the cells may grow temporarily with an apparent rate as high as 0.15 h$^{-1}$.

To further explore how far along in photonfluxostat mode a constant growth rate could be maintained, we performed additional experiments in which we let the cultures grow until the maximum irradiation capacity of the LED panel was reached. Meanwhile, a common batch culture (Fig.3.1C&D) was also carried out in the same set-up to enable a direct comparison between these two cultivation strategies. In both cases, fixed light intensity was given after inoculation, and then switched to corresponding target light regimes after $OD_{720}$ reached 0.6. Because the light intensities were fixed in batch culture and the biomass concentration kept accumulating, the light intensity per OD there was not constant (Fig.3.1C). Hence, the cultures failed to maintain a constant growth rate in batch mode under light limited conditions (Fig.3.1D and Fig. S3.5A), due to the varying photon flux reaching the cells. In contrast, under photonfluxostat conditions, where fixed light intensity per OD was ensured, relatively constant growth rates were achieved (Fig. S3.5B). These were maintained throughout the entire span of two to three population doublings, depending on the light regimes used in photonfluxostat, and limited by the maximum capacity of the LED panel. The higher biomass concentration that is then obtained would be suitable for sampling for physiological studies that
require a larger amount of biomass (e.g. proteomics, metabolomics, amongst others).

Concerning the average cell volume, faster growing *Synechocystis* cells were found to be larger than slower growing cells (Fig. 3.2A). As the growth rate increased from 0.04 to 0.11 h\(^{-1}\) (corresponding to a doubling time of 6.3 h), the average cell volume significantly increased from 5 to 11 fL (p-value < 0.01). This is accompanied by a proportional drop in cell numbers per volume per OD\(_{730}\) (Fig. 3.2B) that results in a relatively stable gDW L\(^{-1}\) OD\(_{730}\)\(^{-1}\) under all conditions tested. The mechanism and explanation behind the correlation between growth rate and cell size remains elusive and the topic of much debate \(^{115}\). It has, however, been observed for organisms across all phylogenetic kingdoms \(^{115,116}\). Here, we show that the positive correlation between cell volume and growth rate also holds for *Synechocystis* under photonfluxostat conditions. It has been suggested that due to the accumulation of sufficient chaperones required to trigger cell division; or because cells have to accumulate more newly synthesized biomass components for proper resource allocation; either may require larger cell size \(^{115-117}\). A recent study on marine *Synechococcus* has also reported this growth-rate dependency for cell size during phosphate and nitrate limited growth \(^{118}\). To the best of our knowledge, here we provide for the first time systematic evidence that this relationship also holds under light limitation.

As mentioned above, we also investigated how Chl a content and the PSI/PSII ratio varies with growth rate under both light-limited and light excess conditions. Chl a is one of the two main antenna pigments for light harvesting in *Synechocystis* along with phycobilisomes \(^{119}\). The PSI/PSII ratio is of paramount importance for generating and modulating a balanced ATP and NADPH supply in order to ensure biomass formation under varying light conditions \(^{119}\). The Chl a content tends to decrease as the light intensity increases under light-limitation (Fig. 3.2C). This suggests that cells might fine tune Chl a levels to ensure optimal growth by minimizing the threats posed by photoinhibition, for instance through the excessive formation of reactive oxygen species (ROS), while assuring that sufficient photons are absorbed. In the light-excess state, Chl a content remains relatively stable (Fig. 3.2C). The reason behind this may simply be that cells require a minimum amount of Chl a to ensure photosynthetic capacity. Alternatively, it may be that photoautotrophs have been selected to maintain a certain over-capacity that grants them with a competitive advantage when faced with fluctuations in light availability. Irrespective of the underlying explanation, the different patterns of Chl a
content for the light-limited and light-excess states, results in a linear relationship between Chl a content and $\mu$ (Fig. 3.2D).

Fig. 3.2 Dependence of several phenotypic traits on growth rate (set with different light regimes). The relationships of (A) the average (mean) cell volume and (B) cell numbers are as a function of growth rate, while (C) and (D) shows the trends of Chl a content at different light regimes and growth rates, respectively. Samples were taken from each independent cultivation as depicted (OD$_{720}$ $\approx$ 1). Each filled circle represents a single observation.

The PSI/PSII ratio during the light-limited state as inferred from 77K fluorescence spectroscopy (Fig. 3.3A and B), tends to decrease as the light intensity increases (Fig. 3.3C), as also observed for the Chl a content (Fig. 3.2C). Considering earlier reports, the latter is not unexpected. Most of the Chl a is attached to PSI, and it has been suggested that stable PSI complex formation may be controlled by regulation of Chl a synthesis$^{120}$. In such a case, a decreased Chl a content results in a decreased PSI/PSII ratio, as corroborated by our observations (Fig. S3.6), since this would lead to a decreased amount of functional PSI. This effect may be reinforced by the increasingly reduced state of the electron pool that comes with a higher $q'_{h}$, which results in the suppression of PSI formation$^{121}$. Furthermore, minimization of harmful ROS production can also be attained through the decrease of the amount of active PSI, making cells more resistant to increasing light intensities$^{122}$. Under the light-excess state, and in alignment with the
observations made for Chl a content, the PSI/PSII ratio was relatively stable. Once again, this may simply mean that either a minimum ratio should be kept for cellular activities, or that cells are conditioned to anticipate certain environmental dynamics in respect to light availability. However, it can also be a consequence of the photo-damage caused by higher light intensities that starts to affect the function of PSII, counteracting the decreased flux through PSI, and hence, maintaining the ratio stable. Although no mechanistic explanation has been proposed, we observe that the PSI/PSII ratio and μ do correlate linearly (Fig. 3.3D).

Fig. 3.3 The PSI/PSII ratio under photonfluxostat conditions. (A) The raw emission signals were fitted through normal distributions, and further normalized by the maximum fluorescence value of the PSII peak (left one). Growth rate (in gradient grey scale) is reflected in each curve. The PSI/PSII ratios were plotted against (C) the different light regimes used and (D) growth rate. Samples were taken from each independent cultivation as depicted (OD$_{720}$ ≈ 1). Each filled circle represents a single observation.

The data collected here as a function of growth rate regarding biomass concentration (independently measured in gDW L$^{-1}$ and OD$_{730}$), cell numbers per volume per OD$_{730}$, and average cell volume, can be further explored. As mentioned above, average cell volume increased as cells grew faster (Fig. 3.2A), while cell numbers decreased constantly (Fig. 3.2B). The total amount of
dry weight concentration per OD\textsubscript{730} remained relatively constant (148±39 mg L\textsuperscript{-1} OD\textsubscript{730}\textsuperscript{-1}), implying that the single cell weight increase (likely caused by the increased cell volume, Fig. S3.7), is counterweighed by the decreased cell numbers per volume per OD\textsubscript{730}. This indicates that both the gDW L\textsuperscript{-1} and the optical density are suitable indicators of the biomass concentration, despite the variations of the cell volumes and cell numbers per volume observed at the different growth rates. Still, relative to gravimetric methods, OD\textsubscript{730} is a much more convenient indicator of biomass concentration, because it is much easier and quick to measure, and much less subject to large technical variations. The relationships between OD, cell number, cell volume and cell dry weight such as the ones established here from data collected at different growth rates, are very useful for further modeling efforts\textsuperscript{124}. This can be illustrated by the popularity of databases where such numbers are collected (e.g. BioNumbers\textsuperscript{125}), and for which additional \textit{Synechocystis} specific data can now be added, reducing the need for extrapolation across distantly related organisms.

3.5 Conclusions

Cyanobacteria are currently being explored to answer fundamental questions in physiology and as a potential chemical production platform. This renders the characterization of growth rate-dependent phenomena, such as cellular responses or fitness costs associated with product formation, highly pertinent. In this context, a simple method for cyanobacterial cultivation at controlled growth rates is urgently required.

During phototrophic growth, one of the biggest advantages when considering light as a substrate, in comparison to all other nutrients, is that it can be adjusted bidirectionally in a virtually instantaneous fashion. Differently put – one might say that a ‘spoonful of photons’ will disappear from solution at the speed of light. This underlying property is what makes photonfluxostat cultivations possible, enabling experimentalists to obtain cultures at different, yet constant, growth rates, in a batch-like cultivation set-up. We have implemented this new cultivation method relying on tailor-made software, which is being freely distributed. We used it to investigate the relationship between growth rate and different basic physiological parameters. The method devised and implemented here can be directly applied to other single-cell photoautotrophic organisms.

3.6 Materials and methods

3.6.1 Strains and cultivation conditions
**Batch photocultivation at controlled growth rates**

*Synechocystis* sp. strain PCC6803 (a glucose-tolerant derivative, obtained from D. Bhaya, University of Stanford, Stanford, CA) was cultivated in BG-11 medium in a Multi-Cultivator. The cultures were enriched with 10 mM TES-NaOH (pH 8.0) at 30 °C and bubbled at a flow of ~100 ml/min a mix (v/v) of 99% N₂ and 1% CO₂, with a working volume of 60 mL (projected area exposed to light = 0.0028 m²).

### 3.6.2 On-line OD measurements

When an OD measurement is started, the software starts preparing the Multi-Cultivator, by making sure there is no gas flow and that most gas bubbles have left the culture, and that the LED panels have been switched off. If this runs smoothly, the OD is measured at the desired wavelength(s) for each (active) channel. To improve the reliability of the measurement, the software does multiple readings and averages them (detailed in the supplemental material). After the results of all measurements have been obtained, the pre-measurement situation is restored and the results of the measurements are processed for later use and storage. A typical measurement routine, where the OD of all eight channels at both wavelengths (680 and 720 nm) is measured, takes about 10 to 15 seconds to complete, including the time to wait until the gas bubbles have left the culture.

### 3.6.3 Turbidostat

The setup used in this experiment is based on a modified Multi-Cultivator, with additional pumps (Reglo ICC, ISMATEC, Germany) transferring fresh medium to the cultures, and from there to the waste. All is controlled using the in-house software package controlling the Multi-Cultivator hardware. This enables measurements of the cell density (720 nm) at regular intervals, and switching on, or off, the pumps to dilute the cultures when the selected OD₇₂₀ threshold is reached. Cells from pre-cultures in shake flask were inoculated at OD₇₂₀ about 0.05 in 6 independent cylindrical vessels. When the OD₇₂₀ threshold of 0.5 was reached, as chosen by others, cultures were diluted to 8% (v/v) of the total work volume with fresh BG-11. Growth rate was calculated by fitting a linear function through the natural logarithm of the OD₇₂₀ during each cell ‘growth-dilution’ cycle.

Different fixed light intensities (10, 15, 20, 30, 40, 50 µmol photons m⁻² s⁻¹) were set for each vessel at the beginning of the experiment. In order to increase the number of light regimes tested, we slightly altered the light settings (maximum change = 10 µmol photons m⁻² s⁻¹) once the growth rates were maintained stable at a certain light intensity. In this way we tested a total of 11 light intensities [5, 7, 10, 15, 20, 30, 40 (twice), 45, 50 and 60 µmol photons m⁻² s⁻¹]. For each culture and applied light intensity, we averaged the growth rates in the given time interval and estimated the light regime in mol photons gDW⁻¹ h⁻¹ based on the mean OD₇₂₀ and light intensity.

### 3.6.4 Batch cultivation

For batch cultivation, light intensity was set to 30 µmol photons m⁻² s⁻¹ after inoculation at OD₇₂₀ of ~0.05 in 8 independent cylindrical vessels. Analogous to the procedure used
during photonfluxostat cultivation, when OD<sub>720</sub> reached above 0.6, light intensity was switched to different fixed values (30, 40, 50, 60, 70, 80, 90, 100 µmol photons m<sup>-2</sup> s<sup>-1</sup>). Light intensity per OD<sub>720</sub> was calculated based on the light intensity and OD<sub>720</sub> values automatically recorded every 5 minutes.

### 3.6.5 Dry cell weight measurement

Cellulose acetate membranes (0.45 µm, Whatman) were washed twice using Mill Q water and immediately dried in a stove (Electrolux) for at least 4 hours at 90 °C. The weight of each membrane was measured with an analytical balance (AB204, METTLER TOLEDO). When the OD of the cultures reaches around 1, a 30 mL sample was taken from each culture, and filtered through the pre-weighted membrane. After washing with Mill Q water, the membrane with cells was dried for at least 4 hours in a 90 ºC stove, before weighing again. The dry cell weight per volume was calculated, and normalized by the OD<sub>730</sub> measured from a spectrophotometer (Lightwave II, Biochrom) in parallel.

### 3.6.6 Cell volume measurement

Cell volume was measured through a CASY counter (Roche), following the Operation Manual. Briefly, after the samples were taken, around 10 µL (depending on the total counts for each measurement) of each sample was mixed with 10 mL CASY ton in a CASY cup. The machine is blanked by CASY ton before every measurement, to eliminate interference by small particles that may be present in the cups.

### 3.6.7 Calculation of chlorophyll a content

Generally, OD<sub>680</sub> and OD<sub>730</sub> of the samples were measured immediately after samples were taken, via use of a spectrophotometer (Lightwave II, Biochrom). Then, Chl a content was calculated based on an empirical formula, 10.186(OD<sub>680</sub> − OD<sub>730</sub>) − 0.08 = mg Chl a L<sup>-1</sup>, deduced from a published method<sup>126</sup>.

### 3.6.8 Measurement of the PSI/PSII ratio

77 K fluorescence analysis was used to measure the cellular PSI/PSII ratio<sup>127</sup>. Briefly, samples were taken from different illumination conditions, diluted four times in ice-cold medium with glycerol (final concentration, 30% [v/v]), and immediately frozen in liquid nitrogen. The samples were analyzed in an OLIS 500 spectrofluorimeter equipped with a Dewar cell. With excitation at 440 nm, the fluorescence emission spectra were recorded between 600 and 750 nm, a wavelength domain in which PSII (685 nm), and PSI (720 nm) show well-separated emission peaks (Fig 3.3A).

The peak area for PSII and PSI emission were calculated as follows. Baseline correction was done using the “Fill Peaks” algorithm from the baseline R package<sup>128</sup> with 150 points as the half width of the local window. For deconvoluting adjacent peaks, the maximum and minimum wavelength where each peak lies was determined by visual inspection of the spectra. For PSII these values were set to 680 and 705 nm and 705 and 780 nm for PSI. These regions were extracted from the dataset and used to fit a
normal distribution by means of a non-linear least square method using the \textit{nls} function from \textit{R} \cite{R29}. As initial parameters for this algorithm, the standard deviation was set to 5 and the mean to 730 and 694 for PSI and PSII, respectively. The parameters estimated by the \textit{nls} function were used to draw the fitted normal distribution from (Fig 3.3B) which the area was integrated by the trapezoidal rule. The ratio was then calculated by dividing the estimated areas.

3.6.9 Experimental replication and statistical treatment

In the cultivations carried out in this study, since we were interested in the dynamics of the relationships between variables, we decided to spread our experimental conditions throughout the whole target range rather than perform multiple replicates for a few illumination conditions. This ensured that the fits carried out were based on multiple observations ranging up to 39 independent photonfluxostats. Statistical testing was performed using the software package \textit{R}, as indicated when the data is presented \cite{R29}.

3.7 Acknowledgements

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3.8 Supplementary data

Supplementary data to this article can be found online \textit{https://doi.org/10.1016/j.algal.2016.10.004}