Chlorella vulgaris cultivation under super high light intensity: an application of the flashing light effect

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Chlorella vulgaris **was grown under super high illumination (7000 µmolPhotonPAR/m^2/s). Continuous and flashing light patterns were applied to investigate potential gain originating from the flashing light effect (square pattern, duty cycle of 0.5). Three control configurations were tested, all under continuous light: 200 µmolPhotonPAR/m^2/s (conventional cultivation conditions), 4000 µmolPhotonPAR/m^2/s (about the same average amount of energy), and 7000 µmolPhotonPAR/m^2/s (maximum incident light intensity, cells did not grow). The experiments were conducted in ultra-thin flat-panel photobioreactors to ensure iso-actinic growth conditions. After acclimation, the monitored outcomes were: growth rate, macronutrient composition (proteins, carbohydrates, lipids), pigment content, and transient fluorescence (OJIP assays). The results showed that *Chlorella vulgaris* can grow at a similar rate under 200 µmolPhotonPAR/m^2/s of continuous light and under 7000 µmolPhotonPAR/m^2/s of flashing light for frequencies between 0.1 and 100 Hz. Acclimation mechanisms did not alter cell macronutrient composition, yet, chlorophyll contents decreased while carotenoids increased (allegedly linked to an increased expression of the VAZ cycle). OJIP tests also revealed potential up-regulation of the water-water cycle (featuring PTOX enzyme), which would allow faster repletion of the PQ pool, delaying photosynthetic apparatus saturation. Above 100 Hz (1000 Hz in this study), cells exhibited the same growth rate as under the equivalent amount of continuous light. It suggests that light alternation is too fast for the cells to perceive it. On the contrary, too low frequencies (0.01 Hz) showed lower performances than under the same average intensity. In this condition, during the light phase, cells are exposed to harmful conditions (continuous 7000 µmolPhotonPAR/m^2/s), and the too-long dark phase only allows the cells some rest but does not bring any benefits. In terms of applicability, these results pave the way for the use of super high light (either artificial or by sunlight concentration) to overcome the energy limitation burdening photoautotrophic microalgae cultivation.**

Microalgae | Flashing light | Super high illumination | Pigments | Macronutrients | Fluorescence assays

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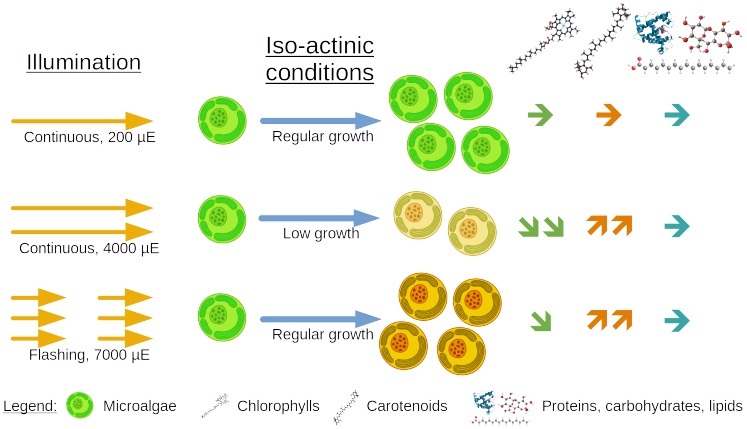


Graphical abstract

1. Introduction

Since the middle of the last century, humanity has started to put tremendous stress on its ecosystem. Despite technological progress, pressure on fossil fuels, water, arable lands, and biodiversity is peaking, inducing deleterious environmental consequences (1). In addition, the modern lifestyle (lack of physical activity and excessive consumption of transformed food products) causes adverse health effects, lumped under the term metabolic syndrome (2). Facing this dire situation, mankind regards microalgae as part of the solution. Indeed microalgae photoautotrophic cultivation produces quality food / feed (3, 4) and high-value bio-sourced molecules (5, 6). Besides, it comes with environmental benefits: carbon dioxide capture, water pollutions remediation (nitrogen, phosphorous) (7, 8), and possible valorization as biofuel of extraction processes leftovers (9). Finally, it can be led on non-arable lands avoiding competition with current food-producing cultures.

Still, the biomass yields obtained by photoautotrophic cultivation are low (from less than 1 g/L up to 30 g/L depending on the optical length and the incident illumination) (10). This limitation can be explained by the light supply's dilute nature leading to an energy limitation. In this view, heterotrophy appears an appealing alternative for microalgae cultivation capable of expressing this metabolism. The supply of a substrate (mainly glucose or acetate) serving both as carbon and energy source allows reaching higher cell density in a shorter time than photoautotrophy (11). In addition, it enables fed-batch strategies and the use of classical fermentors, which are less expensive than photobioreactors. Nevertheless, this way of bypassing microalgae cultivation energy limitation has two main drawbacks. First, the use of sugar induces a non-negligible cost (one-third of the total production costs (12)) and requires strict axenic operation as potential

contamination would proliferate much faster than microalgae. Second, the produced cells are of lower quality (lower protein, pigments, and vitamins contents) than their photoautotroph counterparts (11, 13, 14).

Acknowledging this analysis, this work explores a new paradigm to ensure a high energy supply to microalgal cultures of exclusive photoautotrophs and optional heterotrophs. This proposed method is based on the use of the flashing light effect. While much debated, the general idea behind this term originates from Kok and Myers' pioneering works (15, 16). In a nutshell, it consists in manipulating light delivery by alternating light and dark periods at frequencies unlikely to occur naturally (1 to 100 Hz)(17, 18). The light phase allows the cells to capture light energy and stock it into a pool of energy-storing molecules. This phase should be long enough to charge the pool but short enough to avoid its saturation and Reactive Oxygen Species (ROS) generation. The dark phase allows cells to use the stored energy to turn CO_2 into carbohydrates. It has to be long enough to process the pool of energy-storing molecules but short enough to prevent the cell from becoming idle. It was long hypothesized that, under certain conditions, this mechanism could increase the efficiency of the photosynthetic apparatus.

In this regard, several studies are to be acknowledged as they deciphered part of the intertwined parameters: frequency, light intensity, and duty cycle. Among these parameters, frequency is maybe the one for which a consensus has been reached. Authors agree that a frequency below 0.1 Hz (10 s cycle time) generally induces a lowered growth because of dark respiration contribution (19–22). On the contrary, a frequency above 1000 Hz produces results equivalent to a continuous illumination of the same average intensity, as cells cannot perceive the too-fast cycle (23). Other parameters effects are more challenging, if not impossible, to isolate. Nevertheless, other authors demonstrated that the duty cycle has a non-linear effect modulated by the cycle frequency (20). However, light intensity also comes into play. Several studies showed that for frequency of 5 Hz under high illumination (more than 500 µmolPhotonPAR/m^2/s), lowering the duty cycle from 0.5 to 0.1 improves photosynthetic oxygen production (23–25), while the opposite is observed under low illumination (23). Two facts impair further in-depth analysis. First, cells were not acclimated to the same starting conditions in-between studies, and it can affect the monitored outcomes (19). Second, for some cultures, signs of acclimation have been reported during treatment (21) while, for others, authors waited several days for measurement stabilization (26). Nevertheless, by taking a step back, recent reviews showed that net gains in photosynthetic efficiency, in the long run, could be ruled out (27, 28).

Still, one avenue remains open and relatively unexplored: the use of the flashing light effect to maintain photosynthetic efficiency under extremely high illuminations, typically several thousands of µmolPhotonPAR/m^2/s (µE on the figures for the sake on readability). This approach would represent a shift in paradigm as 500 µmolPhotonPAR/m^2/s are classically referred to as *high* (29) and 1300 as *excessive* (30). If valid, it would allow microalgae cultivation in a light-intensified manner. Three studies seem to point out that this type of cultivation is possible. They were conducted on well-stirred optically dense cultures under 3000, 7000, and 8000 µmolPhotonPAR/m^2/s respectively (31–33). In these cases, the combination of the light gradient inside of the culture and the constant mixing supposedly induced an adequate perceived light pattern allowing the cells to grow.

This work aimed at validating the aforementioned hypothesis under controlled conditions. To do so, green microalgae *Chlorella vulgaris* cultures were led under iso-actinic conditions under increasing alternate light frequencies (0.01 to 1000 Hz) with a constant duty cycle of 0.5. The incident illumination was set at 7000 µmolPhotonPAR/m^2/s (hence 3500 µmolPhotonPAR/m^2/s on average). Three control conditions were used: a conventional cultivation illumination (200 µmolPhotonPAR/m^2/s, continuous), a similar average amount of continuous light, and continuous 7000 µmolPhotonPAR/m^2/s lighting. The monitored parameters were: culture growth rate, photosynthetic apparatus status (trough transient fluorescence readings, also called OJIP assays), and cell composition in terms of pigments and macronutrient contents (to detect if, as for heterotrophy, high energy supply lowers cell quality).

2. Materials and Methods

2 1. Strain, growth medium, and subculturing

The strain used for this study was *Chlorella vulgaris* (CV 211-11b) obtained from SAG Culture Collection, Germany. Cells were subcultured using B3N medium (autoclaved) (34). The passaging procedure was characterized by 1/100 sampling, 250 mL flasks, 50 mL culture medium, repeated every two weeks. Flasks were placed in an orbitally shaking incubator (Infors HT Minitron, 100 rpm, 30 µmolPhotonPAR/m^2/s, 25 °C, under air with 1 % CO_2).

2 2. Culture vessel

Investigations were conducted in V-shaped ultra-thin flat-panel photobioreactors (working volume 135 mL, total volume 140 mL) (Fig. 1). These photobioreactors and their instrumentation were specifically designed to ensure iso-actinic growth conditions (27). Their main features are a 6 mm deep culture compartment combined with inline monitoring of the transmitted spectrum and special care in choosing wide flat glass sheets to enclose the culture (as they avoid potential optical artifacts or curvature lens effect). Together, they allow conducting of the culture in turbidostat mode at a specific wavelength. In this case, light transmission was chosen at 463 nm (maximum culture absorption over the 300 to 1000 nm range), corresponding to the chlorophyll peak in the blue region. The spectrophotometer readings were used to order dilution when light intensity exiting the culture dropped below 80 % of the incident illumination. Therefore microalgae proliferated under a light intensity whose value ranges from at least 80 up to 100 % of the incident light intensity. This regulation protocol is often encountered in experiments

where the objective is to ensure that the light absorption is the same between all the tested conditions, which was the case here. The advantage is that it does not require sampling the culture, extracting, and quantifying chlorophyll on a regular basis as an inline proxy is provided by the spectrophotometer (26).

Furthermore, the spatial distribution of the incident light was measured (LICOR LI 250 A & LI-190R sensor) to draw a map of the illumination cast onto the photobioreactor surface. The measurements showed that the variation over the photobioreactor surface did not exceed 10 % of the spatially averaged incident light intensity (for example, for an average value of 200 µmolPhotonPAR/m^2/s, the minimum over the surface was at least 190 µmolPhotonPAR/m^2/s, while the maximum did not exceed 210 µmolPhotonPAR/m^2/s). In the coming sections, the *spatially averaged incident light intensity* will be simply referred to as *light intensity*.

In addition to the former precautions, classical photobioreactor utilities were also mounted on the culture system. Temperature control (25 °C) was ensured by the second compartment hosting a water circulation. Injected gas was a mixture of air and CO_2 (2.5 %w) regulated by mass flow controllers (Brooks Instrument SLA5800 Series). The flowrate was set to 200 NmL/min, allowing a mixing time below 10 seconds. Numerical simulations and experimental tests were carried out to validate that cell did not experience mechanical stress (35). A custom-made Arduino architecture controlled the medium injection. For more details, the reader can refer to (36) who developed the device. Photobioreactors were checked twice daily to detect potential biofilm proliferation. In such rare cases, the system was stopped, dismantled, and cleaned.

The lighting device was made of high-power white LEDs (Liili 2400 2ft 4000K) combined with a MOSFET array operating as a power switch. This system allowed to create flashing light up to 10 kHz with a perfect square-shaped signal. The maximum incident light intensity achievable was 7000 µmolPhotonPAR/m^2/s by placing one panel on each side of the photobioreactor (2 panels in total, the front one operating at 4000 µmolPhotonPAR/m^2/s, the back one operating at 3000 µmolPhotonPAR/m^2/s, as it had to be placed somewhat further away). The whole system (photobioreactor, lighting device, and instrumentation) was placed in a dark enclosure to avoid external perturbations.

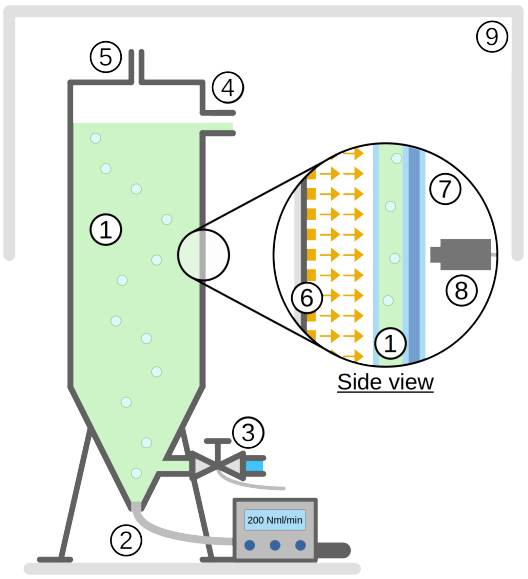


Fig. 1. Culture device schematic. Left - front view, encircled - side view. 1 - culture compartment, 2 - gas injection, 3 - computer-regulated valve controlling fresh medium injection, 4 - overflow, 5 - vent, 6 - LED panel & incident light rays, 7 - water circulation connected to a heat-chiller, 8 - optical fiber connected to the spectrophotometer, 9 - enclosure protecting from external light (not entirely represented)

2 3. Tested conditions

Chlorella vulgaris cultures were led under light frequencies ranging from 0.01 to 1000 Hz with a constant duty cycle of 0.5 and a square shape light pattern (Table 1). The incident illumination was set at 7000 µmolPhotonPAR/m^2/s (hence 3500 µmolPhotonPAR/m^2/s on average). A first set of control runs was performed under 200 µmolPhotonPAR/m^2/s of continuous lighting. It is a commonly used light intensity for photoautotrophic cultivation (37–39). Furthermore, it lies outside of the photolimitation (around 150 µmolPhotonPAR/m^2/s) for *Chlorella vulgaris* (CV 211-11b) (40). The second set of control runs was conducted under a constant illumination of 4000 µmolPhotonPAR/m^2/s because it is close to the averaged intensity of the tested light pattern (for technical reasons achieving precisely 3500 µmolPhotonPAR/m^2/s was not possible). Finally, for the sake of completeness, runs under constant maximum illumination were also led. Each of the tested conditions was duplicated biologically.

Experiment	Incident illumination (µmolPhotonPAR/m^2/s)	Duty cycle (-)	Frequency (Hz)
Control 1	200	1	-
Control 2	4000	1	-
Control 3	7000	1	-
0.01 Hz	7000	0.5	0.01
0.1 Hz	7000	0.5	0.1
1 Hz	7000	0.5	1
10 Hz	7000	0.5	10
100 Hz	7000	0.5	100
1000 Hz	7000	0.5	1000

Table 1. Summary of the tested conditions. Each was duplicated biologically

2 4. Cell cultivation

The whole setup was sterilized chemically and thoroughly washed with autoclaved MilliQ water. Then, rinsing water was replaced by the culture medium. Cells were inoculated to reach a light transmission around 80 %. The culture was autonomously conducted by turbidostat retroaction. Classically, it took three days for the culture to enter a steady state (stabilization of the dilution rate). Once stabilized, the culture was continued for three days, then harvested. Fresh cells (circa 3 mL) were used for photosynthetic apparatus status qualification. The remaining cells were washed twice by centrifugation (4 °C, 11000 rpm, 10 minutes). Biomass was then

frozen and freeze-dried (1-day primary drying, 1-day secondary drying, Christ alpha 1-2 LD +). Biomass powder was stored in the dark at -20 °C before being used for pigment and macronutrient composition assays.

2 5. Growth rate measurement

The growth rate was measured continuously. In this configuration, the growth rate equals the photobioreactor dilution rate. The latter was obtained using the volume of culture overflowing from the photobioreactor over one day. This measurement method features two main advantages: very easily conducted and nullifies the perturbation originating from evaporation. Its main drawback is the measurement frequency: once a day. The resulting flowrate (expelled volume over one day) was divided by the photobioreactor working volume to yield the dilution rate, hence the growth rate. Reported measurements are the stabilized value over three consecutive days. Usually, the cultures took three days to stabilize, leading to experiments lasting between six and seven days.

2 6. Photosynthetic apparatus qualification - OJIP assays

Fresh samples were placed in a dark enclosure for 15 minutes immediately after their withdrawal from the culture vessel. Once dark-adapted, photosynthetic apparatus status was qualified using transient variable chlorophyll fluorescence readings (AquaPen 110-C), also referred to as OJIP tests. Before processing them, the signals were checked for potential saturation (never encountered). Then, the readings were processed following Strasser's guidelines (41). First, the general dynamic of the fluorescence signal was analyzed (succession of OJIP stages). Afterward, the focus was directed toward the Reaction Centers (RC) condition. The three primary parameters of this stage of the analysis were: absorption per reaction center (ABS/RC), trapping per reaction center (TR_0/RC), and transfer per reaction (ET_0/RC). The first one (ABS/RC) accounts for the quantity of energy captured by antennae associated with a reaction center. The second one (TR_0/RC) focuses on the fraction of this energy that is directed toward the core of the photosystem II (PSII). Consequently, the dissipated amount of energy can be computed as ABS/RC - TR_0/RC. The last one (ET_0/RC) relates to the amount of excitation leaving the PSII down the electron chain (towards the PQ pool, the cytochrome $b_{6/f}$, and the PSI). Finally, as unclassical results arose, an in-depth analysis of the overall system dynamic was conducted. It was based on the total number of turnover required to reach saturation (N), the amount of energy required to saturate the system (Sm), and the system energy capture rate (M_0, scaled on J stage (41)). One should note that N is obtained by dividing Sm by M_0.

2 7. Pigment extraction and quantification

1 mg of freeze-dried microalgae powder was homogenized in 5 mL pure methanol using MP Biomedicals FastPrep42 bead miller. The suspension was cooked for 20 minutes at 60 °C (shaded from light) (42). The liquid was then filtered (0.22 µm) and stored in dark vials at 4 °C before quantification.

Quantification of pigments was carried out on an Ultima 3000 HPLC (Thermo Fisher Scientific) coupled with a UV Detector. Separation was achieved on an Acclaim Polar Advantage II C18 column (4.6 × 150 mm, 3 µm, 120 Å) from Thermo Fisher Scientific. The column temperature was maintained at 30 °C. Pure methanol was the mobile phase. The flow rate was 0.5 mL/min, and the elution was set in isocratic mode. Injection volume was 5 µL, and the total run analysis was 40 minutes. Compounds were identified by comparing their retention time and their UV spectra with standard solutions. UV spectra were recorded from 200 nm to 700 nm. Absorbance was recorded at 400, 450, 500, and 650 nm. Pigments quantifications were led using the area of the peaks in external calibration for the most sensible of the recorded wavelength. External calibration concentrations ranged from 0.25 to 5 mg/L. Pigment standards and methanol were purchased from Sigma-Aldrich. Standards had a purity greater than 97 %.

2 8. Macronutrient quantification

4 to 5 mg of freeze-dried microalgae powder was resuspended in 4.5 mL of 75 %vol 1 N NaOH / 25 %vol methanol (43). The cells were homogenized using MP Biomedicals FastPrep42 bead miller. 200 µL of this homogenized suspension were used for total carbohydrates determination using anthrone blue method (calibration curve realized twice at 630 nm using glucose, linearity range 0.05 g/L to 0.5 g/L, 5 points, R^2 = 0.999) (44). Another aliquot of 200 µL was processed with the same protocol without anthrone to nullify potential chlorophyll contribution at 630 nm. Then, the homogenized solution was cooked for 30 min at 90 °C (with frequent mixing) to break down triglycerides and saponify fatty acids (43). Two aliquots of 600 µL were used to quantify proteins using micro-biuret method (calibration curve realized twice at 310 nm using bovine serum albumin, linearity range 0.05 g/L to 1 g/L, 6 points, R^2 = 0.999) (45). Finally, 400 µL were used for lipids quantification by charring method (46) after improved Bligh and Dyer lipids extraction (47, 48) (calibration curve realized twice at 375 nm using palmitic acid, linearity range 0.04 g/L to 0.4 g/L, 6 points, R^2 = 0.975).

2 9. Statistical analysis

Statistical significance was assessed using the ANOVA test. When the null hypothesis was rejected ($p < 0.05$), data were further analyzed using Tukey's Honestly Significant Difference test. The following results are presented as the mean of the replicate, while the error bars account for the spread. Indeed two tests are too few to draw meaningful standard deviations.

3. Results

All the runs were conducted with success. The main qualitative observation is their color change. The control cultures under 200 µmolPhotonPAR/m^2/s of constant light were of classical deep green color, while the others turned orange. This color change and the associated pigment composition

evolution were also observed by other authors who exposed *Chlorella vulgaris* to high light (19). Finally, the cultures under continuous illumination of 7000 µmolPhotonPAR/m^2/s did not grow, and the cultures exhibited an unambiguous yellow color.

3 1. Growth rate

Figure 2 presents the growth rates obtained under the different conditions. The results can be classified into four groups. The first one (lettered a, group averaged growth rate 2.11 ± 0.12 1/day) gathers the control run under moderate illumination and the runs with frequencies from 0.1 to 100 Hz under 7000 µmolPhotonPAR/m^2/s. The observed value aligns well with authors' observations for the same strain under non-stressful conditions (1.92 1/day (40)). Finding these runs in the same group is surprising as the cells under flashing light were exposed, on average, to 17.5 times more light than the control. However, they exhibited a similar growth rate. Thus further analysis is required to decipher the coping mechanisms at play. The second group consists of the cultures under a constant illumination of 4000 µmolPhotonPAR/m^2/s and the ones under the illumination of 7000 µmolPhotonPAR/m^2/s with a frequency of 1000 Hz (b, group averaged growth rate 1.29 ± 0.08 1/day). One should note that the cultures were exposed to similar average light intensities 4000 and 3500 µmolPhotonPAR/m^2/s, respectively. The third group (c) was made of the runs under 7000 µmolPhotonPAR/m^2/s of light flashed at the lowest tested frequency (0.01 Hz). It exhibited a growth rate of 0.77 ± 0.04 1/day. Finally, as aforementioned, the cultures under 7000 µmolPhotonPAR/m^2/s of constant light (lettered d) did not grow (over 7 days). In this latter configuration, the controller ordered medium injection sporadically only compensated evaporation. A simple test was carried out to infer a potential loss of these last cultures. Once the cultures were stopped, 1 mL of culture was transferred into fresh medium (50 mL) and placed back in the incubator used for subculturing. After two weeks, the flasks turned green. While this test does not have the robustness of classical viability assay (49), such as flow cytometry, it seems to suggest that cells would have been in a stasis phase and not necessarily lost, at least for some of them.

3 2. Macronutrient composition

Cells proteins, carbohydrates, and lipids fractions are reported in Figure 3. First of all, the average sum of the fraction is 93 % (minimum 82 % and maximum 102 %), which correlated with the reported 6.30 % ash content for *Chlorella vulgaris* grown under classical photoautotrophic conditions (50). Then, it can be noted that cell proteins (p=0.245, 45.7 % on average) and lipids (p=0.398, 25.4 % on average) fractions did not change significantly over the tested conditions. The carbohydrate fraction showed some difference between the tested conditions. The reported values vary between 14.7 % and 28.0 %. Yet, it seems difficult to establish, and therefore comment, a clear trend as all statistically relevant groups are intertwined.

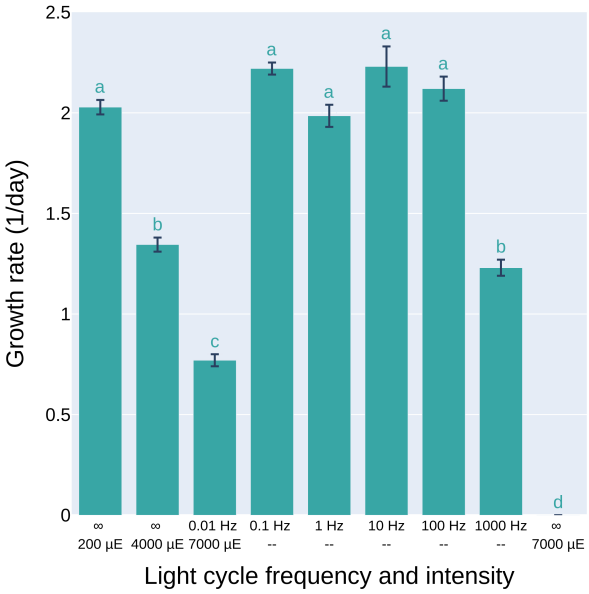


Fig. 2. Growth rate for tested conditions. ∞ - continuous light. Error bars - spread. Compact letter display based on Tukey's HSD test (p<0.05)

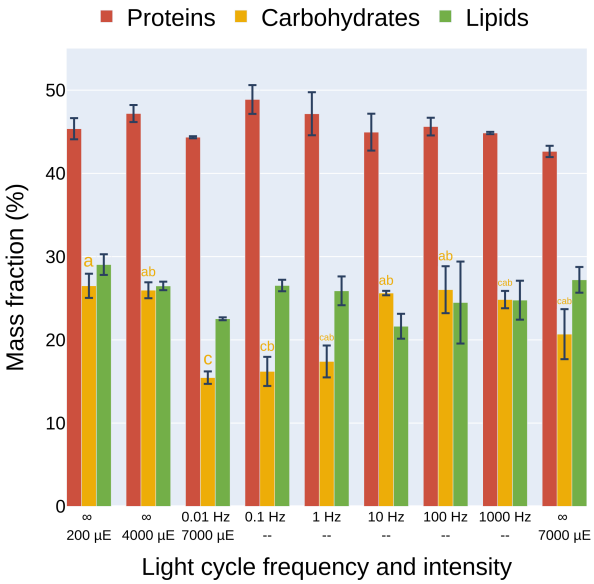


Fig. 3. Cell composition in proteins, carbohydrates, and lipids. ∞ - continuous light. Error bars - spread. Compact letter display based on Tukey's HSD test (p<0.05)

3 3. Pigment contents

Figure 4 reports the cells' pigment contents for all the tested growth conditions. The first comment is that, while detected, violaxanthin concentrations were below the quantification limit for most tests. Hence they are not reported here. Focusing on the reported values, one can see that the runs performed under moderate (200 µmolPhotonPAR/m^2/s) continuous illumination exhibits substantially higher chlorophyll *a* and *b* contents and lower lutein and zeaxanthin contents than the other runs. This correlates well with this the previous qualitative observation that culture turned from green to orange or even yellow for the one under continuous exposure to 7000 µmolPhotonPAR/m^2/s.

In more details, exposure to high light lowered chlorophyll

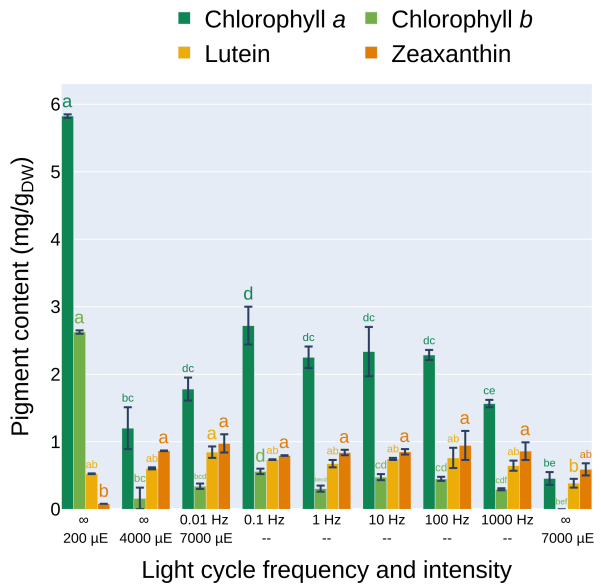


Fig. 4. Cell pigment contents. While detected, violaxanthin cell content was for most tests below the lower quantification limit; hence it is not reported. ∞ - continuous light. Error bars - spread. Compact letter display based on Tukey's HSD test ($p<0.05$)

a content by a factor 2.4 (from 5.8 to 2.4 mg/g$_{DW}$) for runs (0.1 to 100 Hz) yielding a growth rate comparable to the 200 µmolPhotonPAR/m^2/s control. The other runs showed an even lower chlorophyll *a* content (3 to 12-fold decrease). A similar trend is observed for chlorophyll *b*, with an average 7-fold decrease and a disappearance for the runs performed under continuous illumination of 7000 µmolPhotonPAR/m^2/s. Taken together, these results point toward a dramatic reduction in the cell antenna size and PSU expression.

Focusing on carotenoids, the exposure to high light triggered a massive 10-fold increase in zeaxanthin content as well as a moderate one for the lutein content (+39 %, for the 0.1 to 100 Hz group with respect to 200 µmolPhotonPAR/m^2/s control runs). Zeaxanthin increase can be explained by its role in the safe-dissipation of excess energy (quenching of excited chlorophyll ^{1}Chl*) as well as its antioxidant role (removing $^1O_2$ and related products) (51, 52). Lutein increase requires further discussion to be understood. Indeed, lutein has three biological roles: ensuring proper cohesion/folding of the antennae (53), transferring light excitation to chlorophyll, and triplet state chlorophyll (^{3}Chl*) quenching directly in the antennae (52, 54). High light intensity should decrease lutein content because of the two first functions of lutein. Indeed, chlorophyll expression is downregulated, and additional light harvesting could be harmful. This expected behavior was observed for the same microalgae strain under moderate illumination when increasing light intensity from 25 to 800 µmolPhotonPAR/m^2/s (36). Nevertheless, in the case of super high illumination, the third role of lutein seems to be the driver for its expression. Indeed, direct quenching of triplet state chlorophyll in the antennae lowers the chance of creating singlet state oxygen (a potent ROS species).

Taking a step back, the overall increase in excess light dissipation capability can be assessed by computing the ratio for the total carotenoid content over the total chlorophyll content (Figure available in supplementary materials). The value of the ratio starts at 0.07 for the control runs under 200 µmolPhotonPAR/m^2/s (already encountered value for this strain grown under non-stressful light conditions (8)), rises to 0.57 for the 0.1 to 100 Hz to then get close or even surpass 1 for the other tested configurations. This reinforces the assumption of safe dissipation mechanisms over-expression under super high flashing light.

3 4. Photosynthetic apparatus qualification

On top of being of great biotechnological value, cell pigment contents delivered valuable insights about the mechanisms the cells deployed to acclimate to high illumination. The following section dives deeper and examines *in vivo* photosynthetic apparatus response thanks to OJIP tests. The transient readings of variable fluorescence are reported on Figure 5 for the control runs under 200 µmolPhotonPAR/m^2/s, the runs under 4000 µmolPhotonPAR/m^2/s of constant light, and the runs performed under 7000 µmolPhotonPAR/m^2/s of incident illumination at a frequency of 100 Hz. Two qualitative comments have to be drawn. The readings from the cells cultivated under high light intensity showed a higher noise-to-signal ratio than the ones of those cultivated under moderate illumination. This can be correlated with the lower chlorophyll contents reported in the previous section. This comment is all the more true for the cells cultivated under continuous illumination of 7000 µmolPhotonPAR/m^2/s. In their case, signals were not exploitable and were excluded from the statistical analysis, as well as the graphs. The second comment relates to the general trend of the plot. The control runs exhibited classical OJIP rise with easily distinguishable phases. The other runs showed a delayed J phase, no I phase inflection and even a decrease in intensity after the J phase. Furthermore, J phase intensity and saturation intensity are close.

Figure 6 b reports indicators characterizing the photosynthetic apparatus functioning as a whole. As one can see, the number of turnovers required to reach system saturation (N) is much higher for the high light acclimated cells. An explanation could come from a lower rate of reaction center closure (M_0), which would allow more time for QA^- to be oxidized back into QA. However, this hypothesis is invalided by the observations. Indeed, all the runs' closure rates were comparable to the controls ($p=0.373$). The other explanation is a very fast regeneration of QA originating from other mechanisms, as pointed out by Sm parameter values. Two studies support this latter explanation. Bonnanfant reported that *Chlorella vulgaris* managed short burst of high light (10 s or less) by quickening QA regeneration (22). Furthermore, Kedem recently exposed the role of PTOX (Plastid Terminal OXidase, an enzyme reverting PQH_2 to PQ in the water-water cycle) in the mechanisms deployed by a *Chlorella* species to cope with high light (31). Hence, intense conversion of PQH_2 to PQ regenerates the QA pool, mechanically increasing the needed turnovers to reach saturation, delay-

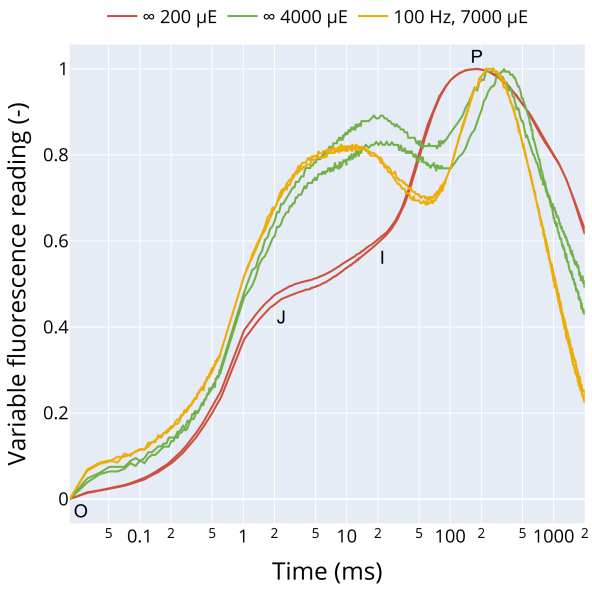
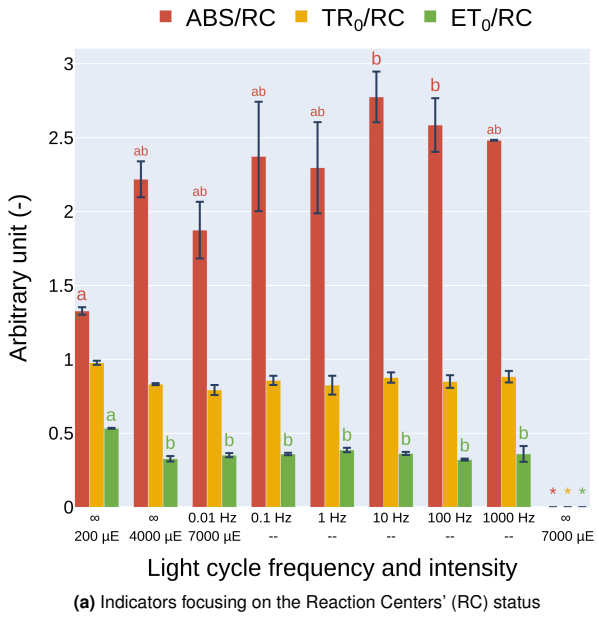
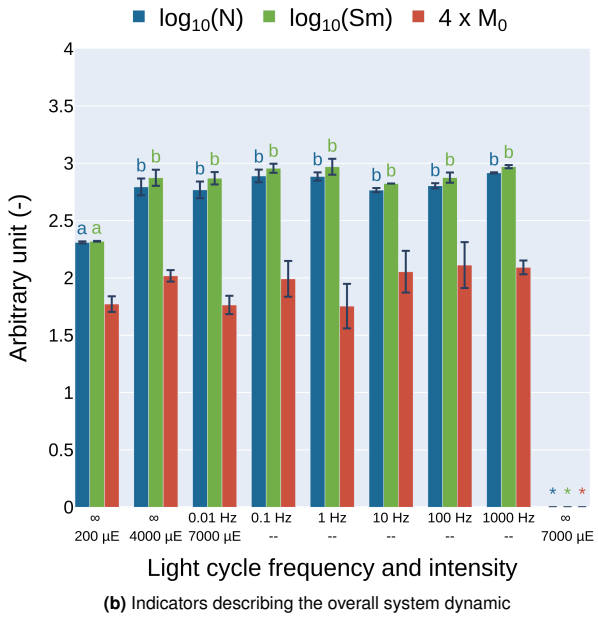


Fig. 5. Variable fluorescence signals (biological replicates). Noise, allegedly linked to low chlorophyll content, is noticeable on runs under continuous illumination (∞) of 4000 µmolPhotonPAR/m^2/s, and the run under flashing light (100 Hz, duty cycle of 0.5) with an incident light of 7000 µmolPhotonPAR/m^2/s



(a) Indicators focusing on the Reaction Centers' (RC) status



(b) Indicators describing the overall system dynamic

Fig. 6. OJIP tests results. * not reported, not included in the statistical analysis, because of erratic measurements, allegedly linked to extremely low cell chlorophyll content of the cell produced under continuous illumination (∞) of 7000 µmolPhotonPAR/m^2/s. ∞ - continuous light. Error bars - spread. Compact letter display based on Tukey's HSD test (p<0.05)

ing the J phase, and even lowering the variable fluorescence signal at I phase. This mechanism explains well the observed variable fluorescence trend while correlating with N, Sm, and M_0 values and the proper functioning of the reaction centers.

This unusual behavior raised the question of a potential malfunctioning of the reaction centers. Figure 6 a reports the indicators associated with reaction centers' operation. These results clearly showed that the magnitude of energy captured by antennae per reaction center (ABS/RC) was substantially higher for the cells cultivated under high illumination. Nevertheless, the amount that was funneled towards the core (TR_0/RC) was similar for all configurations (p=0.148). These findings lead to two conclusions. Under very high light, the cells acclimated by reducing their number of cores in a larger proportion than their antenna size (n modulation strategy favored over σ modulation strategy). In addition, they strongly increased their safe dissipation capabilities (ABS/RC - TR_0/RC) as suggested by the results mentioned above (pigment contents). Going further down the electron transport chain, ET_0/RC indicates the amount of energy transferred to QA (semiquinone, PSII primary acceptor). This parameter is slightly lower for the cells cultivated under high light. In more detail, the electron transfer efficiency from donor to acceptor side can be estimated with the ET_0/TR_0 ratio (or Ψ_0). This indicator appeared to be similar (0.46 on average) among the runs exhibiting a nominal growth rate (lettered a on Figure 2). In comparison, it is slightly lower (0.39 on average) for the runs yielding a lower growth rate. Still, overall, all these parameters indicate healthy reaction centers, with a somewhat higher tendency to dissipation for those acclimated to high light intensity. Therefore, the explanation of the substantial difference in the observed variable fluorescence trends (Fig. 5) has to be searched elsewhere.

4. Discussion

Taking a step back, it can be hypothesized that the successful maintenance of growth performance under super high flashing light resulted from: augmented safe dissipation (managing incoming energy) and high PTOX activity (quickening the regeneration of QA, hence avoiding system overload). Yet, this statement has to be modulated by the cycle frequency, which dramatically impacts cell growth (Fig. 2). The overall trend elucidation will be carried out using the framework of the light integration concept (55). Figure 7 presents 5 hypothesized configurations, each illustrating a possible explanation for the outcomes encountered in the reported ex-

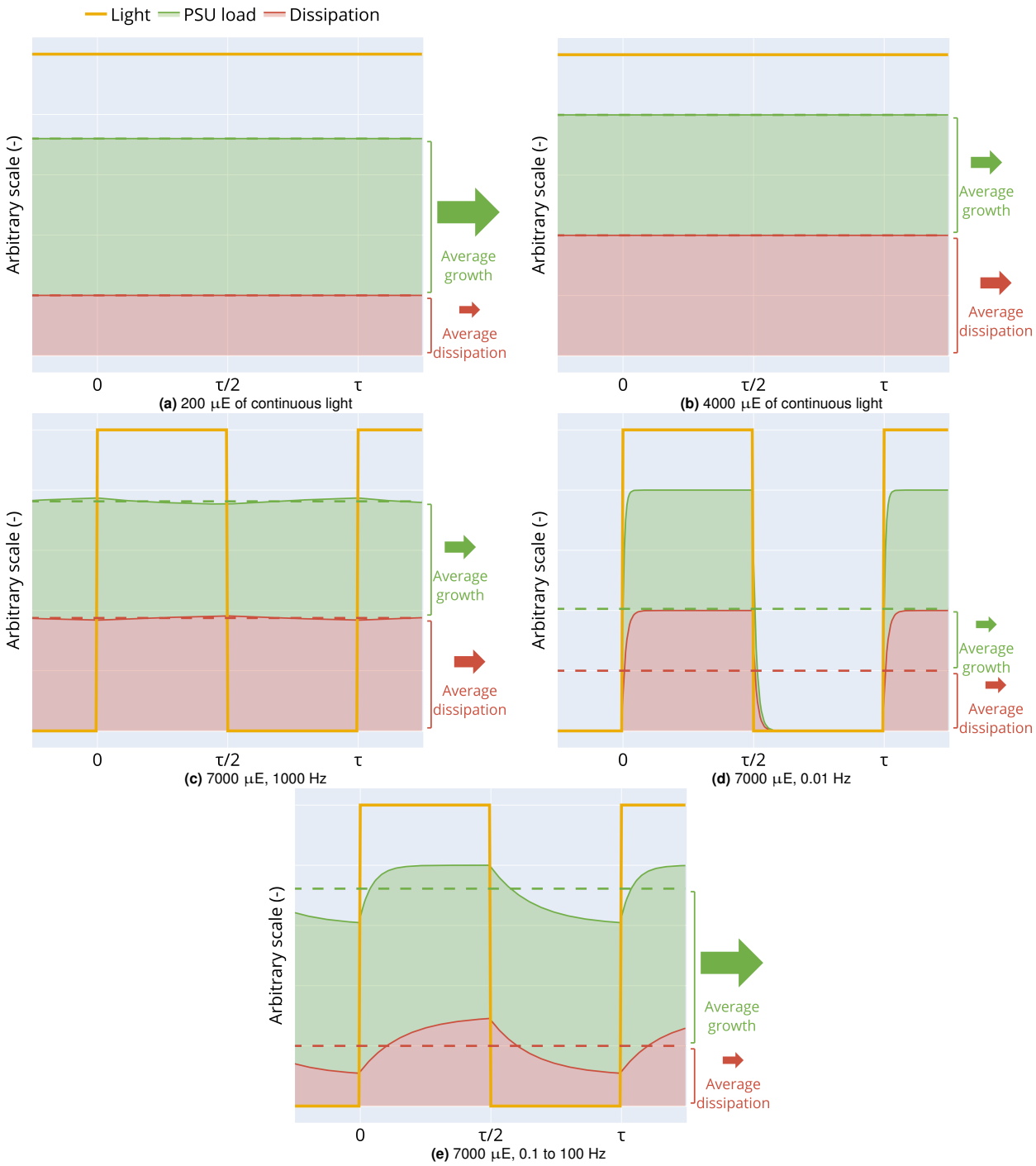


Fig. 7. Schematics illustrating the different light integration patterns associated to the reported growth rate performance categories. Abscissa: τ - cycle time

periments. The borderline configurations are first explored, before tackling the case of moderate frequency flashing high light.

The control runs configuration is presented in Figure 7 a. Under moderate continuous light intensity, the photosynthetic system functions at its nominal level (high output towards anabolism and low dissipation rate). Increasing light to high continuous intensity (Fig. 7 b) pushes the cells to deploy safe dissipation mechanisms to cope with the environment. Hence, the contribution to growth is hindered. The system behaves similarly in the case of high frequency flashing light (Fig. 7 c), as already reported by other authors (23). Indeed, the applied solicitation is too fast for the system to perceive it otherwise than by its average value. Hence, it stabilizes around the same state as the one under the equivalent continuous light intensity. This is all the more striking when one examines the reported growth rates: they are comparable under 4000 µmolPhotonPAR/m^2/s of continuous light and 7000 µmolPhotonPAR/m^2/s of light flashed at 1000 Hz (Fig. 2). Similarly, a very low light frequency (Fig. 7 d) makes the system alternate between a harmful state and a respiratory phase. Under 0.01 Hz, high light exposure lasts 50 seconds, which was shown to be long enough to induce photodamage to the studied strain (22) as corroborated by the fact that no growth was observed under constant light of 7000 µmolPhotonPAR/m^2/s. Furthermore, the dark phase is long

enough to halt the Calvin cycle (a deactivation time of light-induced RuBP regeneration is around 40 s (56)) and leave respiration, further lowering the observed growth (19, 25). Consequently, the resulting growth is even lower than the one obtained under the same average amount of continuous light. Finally, under moderate light frequencies (Fig. 7 e), light is delivered in such a way that it triggers QA reduction while limiting the extent of safe dissipation. Therefore the contribution to cell growth is fostered while dissipation is kept under control. All in all, it allows the cell to grow at a nominal rate (Fig. 2) after adequate photosynthetic apparatus acclimation.

5. Applicability

Two broad applications can be foreseen for super high light cultivation. The first is the production of carotenoids enriched biomass. Indeed, on top of classical carotenoid antioxidant effects and under investigation anticancer potency (57, 58), lutein (+39 % production) and zeaxanthin ($\times$10 production) have demonstrated benefits for human eye and brain health. Indeed, they both can cross the blood-brain barrier and contribute to eye protection (59, 60) (filtering blue light) and cognitive capabilities preservation (61–63) (by preventing oxidative damages in synapses).

In addition to this high added-value molecules production, which could be led indoors using LED panels, super high flashing light opens a second avenue. Thanks to it, massive photoautotrophic cultivation could be led outdoor using concentrated sunlight. To do so, engineers will have to take advantage of the light gradient created by high cell density within cultures (mutual shading). In this configuration, mixing shuttles cells back and forth from light to dark zones, forcing them to experience light/dark cycles, while allowing to maintain a constant illumination onto the surface of the photobioreactor. Luckily, light cycle frequencies encountered in photobioreactors (ranging from 0.1 and 10 Hz (64, 65)) are compatible with the ones required by cthe cells to manage super high illumination. Therefore, finely tuned photobioreactors could be deployed outside on a relatively small area as inexpensive mirrors would handle light capture over a larger area. As candidate designs, one could think of tubular photobioreactors with static mixers or baffled air-lift flat-panel photobioreactors (Fig. 8).

Going one step further, the proposed paradigm can be exemplified by comparing two continuous cultures of *Chlorella vulgaris*: one exposed to conventional illumination, the other under super high light. Let us assume two vertical photobioreactors located in Almeria, Spain. On the 30th of July, under a clear sky, the average incident illumination between 10 am and 2:30 pm is 198 µmolPhotonPAR/m^2/s (66). This period of time was selected for two reasons. First, it represents the best-case scenario for conventional illumination. Second, the average light intensity is the same as in our first control run.

Assuming the cell density (g_{DW}/mL) is the same in the two cultures, the pigment density ([Chl] in $g_{Chlorophyll}$/mL) should be different. Based on our results, the factor should be at least 3. Indeed, cells exposed to 200 µmolPhotonPAR/m^2/s in a dense culture are likely to express a higher chlorophyll content than in our optically thin setup (36). Therefore, assuming a value of 3 for this ratio represents the worst-case scenario to assess super high light relevance. In any case, this pigment density difference would result in a much lower light attenuation in the second case (Fig. 9, top). With this information, it is possible to compute the width of the photic zone within the photobioreactors using Beer-Lambert law (Eq. 1). The photic is defined here as the zone where illumination is high enough to allow photosynthesis to surpass respiration. For *Chlorella vulgaris*, the minimum light intensity required to observe growth (I_c) is around 10 µmolPhotonPAR/m^2/s (40).

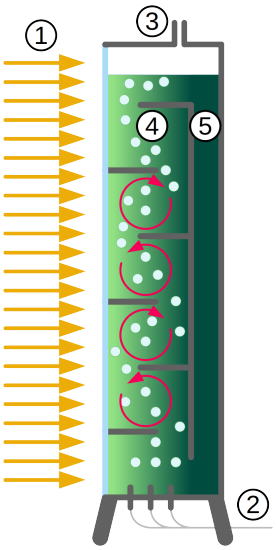


Fig. 8. Baffled air-lift flat-panel photobioreactor illuminated by super high light. 1 - High-intensity continuous light, 2 - Gas line feeding the culture and ensuring mixing, 3 - Gas outlet, 4 - Riser, hosting a high cell density culture, allowing mutual shading, 5 - Downcomer

$$L = -\frac{1}{\sigma[Chl]} ln \frac{I_c}{I_0} \quad \textbf{(1)}$$

where σ is the *in vivo* optical cross-section of chlorophyll (relatively similar between low and high light acclimated cells (26)) and I_0 the incident intensity (200 µmolPhotonPAR/m^2/s for the Conventional run - C -, 7000 µmolPhotonPAR/m^2/s for the Super High Light run - SHL -).

$$\frac{L_{SHL}}{L_C} = \frac{[Chl]_{SHL}}{[Chl]_C} \frac{ln \frac{I_c}{I_{0,C}}}{ln \frac{I_c}{I_{0,SHL}}} \quad \textbf{(2)}$$

Therefore, the ratio of the photic zone widths between the two photobioreactors can be computed (Eq. 2) and yields 6.6 for the given parameters. It is important to note that, in the super high light part of the photic zone, the growth rate is equal to the nominal growth rate (it does not overpass this value) (Fig. 9, middle). The volumetric productivity can therefore be assumed to be proportional to the width of the photic zone. Thus, the super high light operation is 6.6 times more productive than conventional operation at the price of using 35 times more light. Therefore, super high light is 5.3 times less efficient than conventional illumination. Yet, these figures

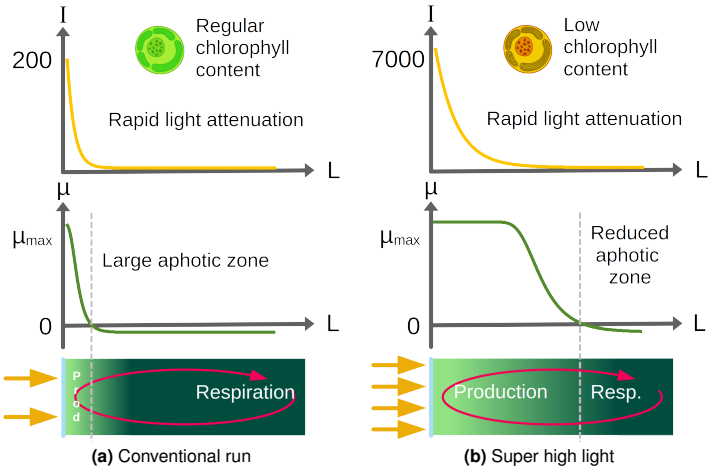


Fig. 9. Illustration of the light flied and its consequence for two photobioreators. Left - conventional illumination, right - super high illumination. Top - light field, middle - cell growth and photic zone, bottom - illustration of the cell motion

misleadingly advocate against super high light utilization. Indeed, for the same areal productivity (the relevant indicator for industrial solar processes), the mirrors would collect the extra light are much cheaper than 6 additional conventional photobioreactors.

Of course, the challenges are still numerous. Among them, the most acute ones are proper tailoring of the duty cycle (Fig. 8 and 9, bottom) and heat. Regarding cell trajectories, the computational fluid dynamic can be of assistance (67). Heat could be mitigated with adequate means (UV-IR reflecting films (68), for example) as it could lead to culture loss (32).

6. Conclusion

Taking advantage of the flashing light effect, *Chlorella vulgaris* was successfully cultivated under 7000 μmolPhotonPAR/m^2/s of incident light (duty cycle of 0.5) in an iso-actinic environment. The results showed that the cells could grow at a rate similar to the one obtained under 200 μmolPhotonPAR/m^2/s of continuous light. Acclimation mechanisms did not alter cell macronutrient composition, yet, chlorophyll contents decreased while carotenoid ones increased (allegedly linked to an increased expression of the VAZ cycle). OJIP tests also revealed potential up-regulation of the water-water cycle (featuring PTOX enzyme), which would allow faster repletion of the PQ pool, delaying photosynthetic apparatus saturation. From a biological perspective, those results align with Kok and Myer's conclusion: the flashing light effect does not increase photosynthetic efficiency but maintains it under extreme light. From a biotechnological perspective, compared to the use of glucose as substrate, super high light maintains cells' protein content and increases their carotenoid contents while also lowering their chlorophyll one. All this while contributing to fixing carbon dioxide. Furthermore, the required flashing frequencies are compatible with those encountered in photobioreactors. Therefore, this proof-of-concept of the use of super high light (either artificial or by sunlight concentration) paves the way to overcome the energy limitation burdening photoautotrophic microalgae cultivation.

7. Acknowledgements

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