Chlorella vulgaris acclimated cultivation under flashing light: an in-depth investigation under iso-actinic conditions

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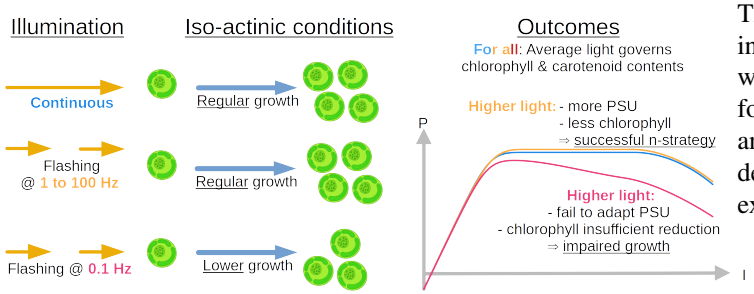
Chlorella vulgaris **was cultivated under continuous and flashing light. Special care was taken in designing a culture device ensuring iso-actinic conditions, and a protocol ensuring acclimation. Continuous light cultures were carried out from 25 to 800 µmolPhotonPAR/m^2/s. They served as control. Flashing light (square shape, duty cycle of 0.5) was applied with four frequencies: 0.1, 1, 10, and 100 Hz. The monitored outcomes were the culture growth rate, cell pigment content (chlorophylls, lutein, violaxanthin, and zeaxanthin), and photosynthetic apparatus (Fv/Fm and light curve). Acclimated cultures under continuous light showed photolimitation and photosaturation phases, without sign of photoinhibition, even under 800 µmolPhotonPAR/m^2/s. Frequencies of 1 Hz and above induced little to no difference between cells acclimated under flashing light and their control control (same amount of continuous light). Indeed, cells exhibited the same growth rate, similar absolute pigment composition, and light curve characteristics. They only differ by a lower chlorophyll *b* to chlorophyll *a* ratio at high intensity, suggesting an acclimation strategy favoring an increased number of photosystems instead of an increased light-harvesting capability. Conversely, cells cultivated at 0.1 Hz showed a lower growth rate and an incapacity to adapt efficiently to high incident illumination. From a biotechnological perspective, these results support the idea that high frequency flashing light, in the tested conditions, does not bring benefits with respect to the same average amount of continuous light. Consequently, it may ease the design of large scale photobioreactors, where light/dark cycles are inevitable.**



Graphical abstract

Microalgae | Acclimated | Light | Intermittent light | Iso-actinic

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1. Introduction

Photosynthetic microalgae are considered biological factories capable of producing many molecules with advanced applications in the cosmetic and pharmaceutical sectors, but also quality food for humans and animals (1, 2). In addition to producing natural molecules, they provide ecosystem services, including water and atmospheric pollutions control. To a certain extent, their valorization is possible as biofuel (3). Finally, they do not compete with food crops because they can grow on non-arable land. For all these reasons, microalgae have emerged as promising candidates to meet societies' current challenges: climate change and scarcity of fossil resources.

Microalgae can be cultivated in two ways: via photosynthesis (for photoautotrophs) or sugar respiration (for optional and strict heterotrophs). The first approach ensures higher-quality biomass (high protein, pigment, and vitamin contents (4)) than the second, avoids the use of an expensive carbon source, and eases the production process as bacterial contamination does not necessarily lead to the loss of the culture. However, the use of light always raises the questions of its perception by the microalgae. Indeed, no matter how well-mixed a dense culture is, cells experience an alternation of light and dark phases. These light/dark cycles originate from the combination of two phenomena: cell mutual shading - inducing a light gradient within the photobioreactor - and mixing - which shuttles cells back and forth between lit and dark zones -. The question of how light/dark cycles affect microalgae biotechnological performances has been of interest to the community for a long time and is still much debated. This introduction does not aim at reviewing this field. The interested reader can refer: to Kok and Myers' pioneering works (5, 6), opinions on how the flashing light effect could foster microalgae growth (7, 8), and recent survey or meta-analyze leading to believe that net gains in performances are debatable (9, 10). Part of the observed dispersion in reported experimental data can be attributed to four main factors:

- the optical thickness of the culture. Some authors led their analysis on very dense culture (often associated to batch operation) (11, 12). This procedure intertwines the effects of the externally applied light cycle and the one induced by mutual shading and mixing. Therefore it makes difficult drawing clear conclusions on the sole effect of the applied light pattern,
- the type of culture vessel. Sometimes, cultures were led on flasks (13) or bubble columns (14) for which glass curvature induces a lens effect, inducing a heterogeneous light field even within dilute cultures,

- the measurement method. Short-term (minutes) oxygen production (15, 16) and long-term (days) growth rate (17, 18) are the two main ways of reporting biotechnological performances (pigment production is rarer (19)). Yet, the first can be biased by a potential lack of acclimation of the cells. They could therefore express positive outcomes, which would sadly not last long in a steady production environment,
- the strain used for the study. Some authors worked with cyanobacteria, others with eukaryotic microalgae, the two being hardly comparable (20).

Acknowledging this state of facts, this work aimed to investigate the effect of flashing light on microalgae in tightly controlled conditions (21). Therefore, a dedicated culture vessel was developed to allow homogeneous illumination of the cells. It is made of an ultra-thin flat-panel photobioreactor operated at a constant and low optical density. *Chlorella vulgaris* was chosen as the model for this study because of its ubiquity and usage in both industrial and scientific communities. Cells were cultivated long enough to ensure their acclimation to the applied light pattern. Once acclimated, the culture was analyzed comprehensively. This work reports growth rate, cells' photocollective and photoprotective pigment contents, and photosynthetic apparatus status. The tested incident illumination ranged from 25 to 800 µmolPhotonPAR/m^2/s (referred to as µE/m^2/s for the sake of compactness in this article). Four frequencies were tested (0.1, 1, 10, and 100 Hz) to simulate what can be encountered in classical photobioreactors and reproduced with artificial lighting (22).

2. Materials and Methods

2 1. Strain, growth medium, and subculturing

Chlorella vulgaris SAG 211-11b was purchased from the Culture Collection of Algae at Göttingen University (SAG), Germany. The strain was maintained in liquid culture before being used as an inoculum for the experimental campaign test. To this end, the cells were cultured over B3N medium (whose composition is given in (23)) in 250 mL flasks in an incubator (INFORS HT Minitron) at 25°C on an orbital shaker (100 rpm). The culture was exposed to continuous low light intensity (30 µmolPhotonPAR/m^2/s) and air-enriched with 1% CO_2.

All abiotic parameters except light (intensity and frequency) were kept constant and non-limiting throughout the experiment for each culture. In that respect, the strain was cultured over B3N medium and maintained at 20 °C using a heater-chiller. Aeration was set at 1.5 vvm, with 2.5 % CO_2-enriched air for mixing and growth purposes. Constant availability of nitrogen, potassium, phosphorous, calcium, and magnesium was confirmed thanks to ionic chromatography analyses. All the cultures were conducted in triplicate.

2 2. Cell cultivation

The cultures were grown in an experimental device (Fig. 1) composed of three 135 mL working volume ultra-thin flat-panel photobioreactors and a lighting device (LED panel Lili 2400 2ft 4000K - neutral white light - Liite), ensuring a homogeneous lighting, stable in time and which spectrum was insensitive to the applied electric power. These two parts were enclosed in an aluminum chamber to prevent perturbation by external light.

Throughout the runs, the culture optical density was kept constant at 0.10 +0.00/-0.01 (over 6 mm light path) by dynamic adjustment of the dilution rate. The setpoint triggering the dilution order was set at a transmitted light corresponding to 80 % of the incident light (spectrophotometer OceanOptics FlAME-T-XR1-ES - wavelength range: 200 to 1025 nm). However, as microalgae do not absorb light uniformly over the visible spectrum, the question of the wavelength for optical density monitoring arises. It was chosen to perform the monitoring on the wavelength corresponding to the maximum absorption peak of the spectrum (463 nm, chlorophyll *a*). Thus, it ensured that the absorption of light was lower than 20 % in all the other regions of the spectrum. All these precautions allow to consider that the cultures were conducted under iso-actinic conditions (10). Therefore, all cells within the culture device experienced the same light history.

Finally, the runs were conducted in a steady state to avoid the gradual obscuring induced by a batch cultivation protocol. First, the cells were exposed to the desired light conditions for 3 days. Then, the runs were continued, and the growth rate was measured until stabilization, *i.e.*, no significant difference, supported by a statistical test. Finally, the cells were drained and analyzed. For more details, the reader can refer to (24).

2 3. Tested conditions

The tested conditions are summarized in Table 1. For runs under flashing light, a square shape light pattern with a duty cycle of 0.5 was applied. The tested frequencies ranged from 0.1 to 100 Hz. Indeed, several authors showed that a frequency below 0.1 Hz (10 s cycle time) generally induces a lowered growth because of dark respiration contribution (25–28). 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 (11). Finally, three incident illuminations were chosen to investigate flashing light: 200 µmolPhotonPAR/m^2/s (100 µmolPhotonPAR/m^2/s in average, lying within the photolimitation zone), 400 µmolPhotonPAR/m^2/s (200 in average, within the photosaturation zone), and 800 µmolPhotonPAR/m^2/s (400 in average, the maximum accessible with the setup).

2 4. Growth rate measurement

The first parameter to be monitored was the growth rate as it is an indicator of cells' general well-being. In turbidostat mode, the growth rate is equal to the dilution rate once a steady state has been reached. Therefore, the growth rate was obtained using Eq. 1, where V_2 - V_1 is the difference in volume discharged between times t_1 and t_2, and V_{PBR} the

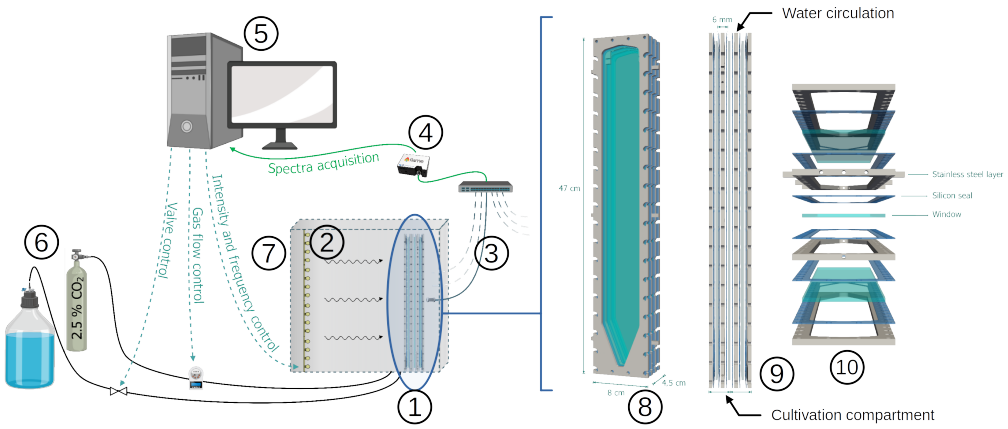

Fig. 1. Culture device schematic. 1 - ultra-thin flat-panel photobioreactor, 2 - LED panel, 3 - optical fiber collecting transmitted light, 4 - spectrophotometer, 5 - computer storing data and controlling the whole setup, 6 - fresh medium tank and gas injection line, 7 - enclosure preventing external light perturbation, 8 - front view of the photobioreactor, 9 - side view of the photobioreactor, 10 - top exploded view of the photobioreactor

Incident illumination (µmolPhotonPAR/m^2/s)	Duty cycle (-)	Average illumination (µmolPhotonPAR/m^2/s)	Frequency (Hz)
25	-	25	0
50	-	50	0
80	-	80	0
120	-	120	0
160	-	160	0
200	-	200	0
240	-	240	0
280	-	280	0
320	-	320	0
360	-	360	0
400	-	400	0
500	-	500	0
600	-	600	0
700	-	700	0
800	-	800	0
200	0.5	100	0.1
200	0.5	100	1
200	0.5	100	10
200	0.5	100	100
400	0.5	200	0.1
400	0.5	200	1
400	0.5	200	10
400	0.5	200	100
800	0.5	400	0.1
800	0.5	400	1
800	0.5	400	10
800	0.5	400	100

Table 1. Summary of the tested conditions. All were carried out in biological triplicate. For runs under flashing light a square shape light pattern was applied

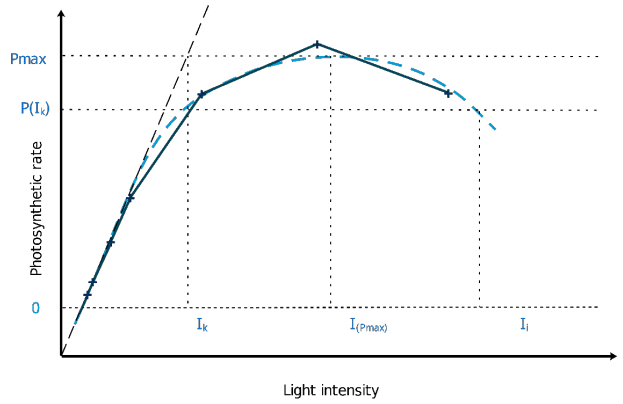

Fig. 2. Illustration of an instantaneous light curve obtained with by fluorometric assay and the associated key values

photobioreactor working volume.

$$D = \mu = \frac{V_2 - V_1}{t_2 - t_1} \times \frac{1}{V_{PBR}} \quad \textbf{(1)}$$

2 5. Photosystem II status qualification

Upon withdrawing from the photobioreactor, fresh samples were washed twice by centrifugation (4 °C, 11000 rpm, 10 minutes), normalized at an optical density of 0.5 (750 nm), and placed in a dark enclosure for 15 minutes. Once dark-adapted, photosynthetic apparatus status was qualified using light curve assay (AquaPen 110-C), also referred to as PI or PE curve. To obtain the curves, quantum yield under different illuminations was multiplied by the incident light intensity (29). This method relies on two assumptions: proper functioning of the PSII (valid up to 7000 µmolPhotonPAR/m^2/s for this strain under flashing light (30)) and similar light absorption per reaction between compared samples. The fluorometer parameters were set as follows: measuring color: 630 nm, measuring flash pulse: 900 µmolPhotonPAR/m^2/s, saturating pulse: 1500 µmolPhotonPAR/m^2/s, measuring pulse: 1000 µmolPhotonPAR/m^2/s, actinic light: 300 µmolPhotonPAR/m^2/s. From these analyses, several parameters were recovered (Fig. 2). They include the Fv/Fm ratio, P_{max} - the maximum rate of photosynthesis -, $I_{P_{max}}$ - the intensity for which the photosynthesis rate is maximum -, I_k - the light intensity corresponding to the intersection point between the theoretical linear relationship and P_{max} (delimiting the end of the photolimitation zone) -, P_{I_k} - the photosynthetic rate at the intensity I_k -, and finally I_i - the intensity at which the depression of photosynthesis occurs defined when $P = P_{I_k}$ in the descending phase of the light curve (delimiting the entry in the photoinhibition zone) -.

2 6. Pigment extraction and quantification

In parallel to photosynthetic apparatus qualification, the remaining cells were washed a second time 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 assays.

To quantify cell pigment content, 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) (31). 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 7. 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 (HSD) test. The following results are presented as the average of the replicate (n = 3), while the error bars account for the standard deviation.

3. Results

All the runs were led successfully. A minor mishap led to the loss of the photosynthetic apparatus data for the two configurations (320 and 360 µmolPhotonPAR/m^2/s under continuous illumination). Nevertheless, it was not deemed critical. Qualitatively, the cultures under continuous high light (above 500 µmolPhotonPAR/m^2/s) required a longer time to acclimate (4 to 5 days instead of 3).

The generated data are summarized in two tables. Table 2 reports growth rates and pigments contents. Table 3 displays values associated with photosynthetic apparatus analyses. A compact letter display was not adopted on the figures for the sake of readability. Instead, qualitative p-value matrices are proposed, except when no statistical difference was reported (for violaxanthin and zeaxanthin, allegedly because of the high noise on the latter). These matrices reports Tukey's Honestly Significant Difference tests results in a graphical manner (4 levels: $p < 0.05$ - statistically different -, $0.05 \leqslant p < 0.10$ - reasonable doubt -, $0.10 \leqslant p < 1$ - similar -, $p = 1$ - diagonal term -). The tested conditions correspond to cultures under 100, 200 and 400 µmolPhotonPAR/m^2/s of average incident light for continuous lighting (linearly interpolated for 100 µmolPhotonPAR/m^2/s) and cycle frequency of 0.1, 1, 10, 100 Hz.

Average intensity ($\mu E/m^2/s$)	Frequency (Hz)	Growth rate (1/day)	Chlorophyll *a* (mg/g_{DW})	Chlorophyll *b* (mg/g_{DW})	Lutein (mg/g_{DW})	Violaxanthin (mg/g_{DW})	Zeaxanthin (mg/g_{DW})
25	0	0.09 (0.03)	17.83 (0.56)	8.69 (0.51)	2.44 (0.06)	0.24 (0.01)	ND
50	0	0.22 (0.10)	9.36 (0.20)	4.95 (0.10)	1.24 (0.06)	0.13 (0.01)	ND
80	0	1.50 (0.04)	9.77 (1.86)	5.24 (0.98)	1.19 (0.26)	0.08 (0.02)	0.04 (0.00)
120	0	2.01 (0.05)	8.10 (0.72)	3.86 (0.04)	0.92 (0.16)	0.11 (0.05)	0.06 (0.02)
160	0	2.11 (0.04)	6.39 (0.82)	3.28 (0.11)	0.63 (0.00)	0.06 (0.00)	0.06 (0.01)
200	0	2.21 (0.05)	5.68 (0.24)	2.60 (0.05)	0.52 (0.01)	0.06 (0.00)	0.08 (0.00)
240	0	2.30 (0.04)	4.52 (0.18)	2.25 (0.09)	0.45 (0.02)	0.04 (0.00)	0.08 (0.00)
280	0	2.29 (0.10)	3.64 (0.47)	1.73 (0.16)	0.39 (0.03)	0.05 (0.00)	0.07 (0.00)
320	0	2.19 (0.05)	5.08 (0.68)	2.55 (0.17)	0.64 (0.07)	0.09 (0.01)	0.12 (0.01)
360	0	2.30 (0.05)	4.83 (0.93)	2.38 (0.26)	0.52 (0.07)	0.05 (0.01)	0.15 (0.01)
400	0	2.36 (0.05)	4.53 (0.32)	2.25 (0.13)	0.46 (0.04)	0.06 (0.00)	0.12 (0.01)
500	0	2.30 (0.04)	3.27 (0.72)	1.75 (0.26)	0.32 (0.07)	NQ	0.07 (0.02)
600	0	2.28 (0.10)	2.84 (0.31)	1.36 (0.12)	0.45 (0.03)	0.05 (0.00)	0.16 (0.02)
700	0	2.24 (0.17)	2.37 (0.07)	1.12 (0.00)	0.27 (0.02)	0.05 (0.01)	0.05 (0.00)
800	0	2.38 (0.05)	1.42 (0.23)	0.84 (0.14)	0.21 (0.04)	NQ	0.14 (0.04)
100	0.1	1.66 (0.01)	6.66 (0.13)	4.27 (0.15)	0.75 (0.06)	0.06 (0.01)	ND
100	1	1.90 (0.01)	8.27 (0.37)	3.81 (0.09)	1.08 (0.06)	0.08 (0.01)	0.08 (0.01)
100	10	1.87 (0.10)	7.97 (0.22)	3.79 (0.14)	0.80 (0.04)	0.06 (0.01)	0.03 (0.01)
100	100	1.87 (0.04)	8.97 (0.30)	4.55 (0.21)	1.05 (0.04)	0.10 (0.01)	0.06 (0.01)
200	0.1	1.81 (0.03)	9.14 (2.09)	4.43 (0.70)	0.85 (0.15)	0.08 (0.01)	0.04 (0.01)
200	1	1.96 (0.07)	7.60 (1.16)	3.10 (0.07)	0.71 (0.02)	0.07 (0.01)	0.07 (0.01)
200	10	2.06 (0.04)	5.19 (0.40)	2.18 (0.10)	0.40 (0.03)	0.04 (0.01)	0.02 (0.00)
200	100	2.18 (0.09)	5.05 (0.63)	1.98 (0.11)	0.36 (0.06)	0.05 (0.01)	0.03 (0.01)
400	0.1	1.86 (0.03)	6.75 (0.32)	2.75 (0.13)	0.36 (0.02)	NQ	NQ
400	1	2.21 (0.04)	5.42 (0.22)	2.23 (0.02)	0.62 (0.09)	0.04 (0.01)	0.14 (0.05)
400	10	2.27 (0.03)	4.63 (0.10)	1.91 (0.04)	0.58 (0.02)	0.05 (0.01)	0.11 (0.02)
400	100	2.34 (0.06)	2.92 (0.21)	1.47 (0.08)	0.36 (0.03)	0.04 (0.00)	0.08 (0.01)

Table 2. Results associated to growth rate measurement and pigments quantification. Reported as average (standard deviation, n = 3). ND - Not Detected, NQ - detected but Not Quantifiable

Furthermore, to ease graphs analysis, control runs results (under continuous light) are presented in the beam instead of a line with markers and error bars. In addition, to facilitate the distinction between the frequency, the points abscissas have been artificially shifted to the left and the right. Finally, the average light intensity over a light/dark cycle was chosen as abscissa as it allows for directly evaluating the amount of light integration (32).

3 1. Growth rate

Figure 3 presents the growth rates obtained under the different conditions. Under continuous light, the culture shows a photolimitation phase (up to 160 µmolPhotonPAR/m^2/s, above p = 0.190) followed by a photosaturation phase featuring a stable growth rate around 2.28 $\pm$ 0.09 1/day. The reported growth rate under 25 µmolPhotonPAR/m^2/s of constant light shows a discrepancy with the general trend. It is thought to be because our method of growth rate measurement (volume overflowing from the photobioreactor) is not capable of measuring maintenance, *i.e.* negative growth rate. Surprisingly, no photoinhibition was evidenced, as the growth rate remained stable even under 800 µmolPhotonPAR/m^2/s. Other authors reported an onset of photoinhibition as early as 500 µmolPhotonPAR/m^2/s for *Chlorella* species (33). This discrepancy is attributed to the fact that, in this work, cells were allowed enough time to acclimate and cope with high light. A qualitative hint of this need for acclimation is the longer time it took for the cultures to enter a steady state.

Out of the photolimitation zone, applying a flashing light pattern induced two different behaviors. For high frequencies (10 and 100 Hz), the observations align well with the reports under continuous light. For lower frequencies, somewhat lower growth rates were found, with a clear drop for 0.1 Hz ($\simeq$ -20 %). These observations correlate with the idea that a too-long dark phase (5 seconds for 0.1 Hz) prevents proper light integration by the microalgae. Indeed, a too-long dark phase has two adverse effects. On the one hand, it stalls the photosynthetic units in an idle state (the system stops after exhaustion of energy-rich intermediates such as PQH_2 and PC^+, in some hundreds of milliseconds (34)). On the other hand, it decreases the activity of light-mediated enzymes in the Calvin cycle (35). One can compare characteristic times to determine which of the two phenomena hinders growth. For energy-rich intermediates, hundreds of milliseconds can be considered. However, in the case of the 0.1 Hz cycle, a -20 % decrease in growth rate implies that 40 % (duty cycle of 0.5) of the dark phase does not contribute to growth. This leads to a photosynthetic deactivation time of about 3 seconds. This duration is much higher than the interme-

Average intensity ($\mu E/m^2/s$)	Frequency (Hz)	Fv/Fm (-)	I_k ($\mu E/m^2/s$)	I_{max} ($\mu E/m^2/s$)	I_i ($\mu E/m^2/s$)	P_{I_k} (α ETR)	P_{max} (α ETR)
25	0	0.820 (0.026)	169 (10)	375 (19)	834 (90)	79 (4)	102 (4)
50	0	0.843 (0.006)	200 (8)	425 (28)	902 (85)	99 (3)	126 (5)
80	0	0.837 (0.006)	217 (8)	448 (15)	927 (31)	107 (4)	136 (5)
120	0	0.840 (0.010)	241 (4)	504 (8)	1054 (18)	113 (1)	144 (1)
160	0	0.843 (0.006)	271 (10)	568 (18)	1192 (28)	129 (3)	165 (4)
200	0	0.837 (0.012)	273 (9)	568 (17)	1184 (33)	130 (3)	165 (3)
240	0	0.840 (0.010)	288 (11)	605 (22)	1272 (42)	127 (8)	162 (10)
280	0	0.833 (0.006)	300 (16)	626 (38)	1308 (86)	135 (4)	171 (6)
320	0	NA	NA	NA	NA	NA	NA
360	0	NA	NA	NA	NA	NA	NA
400	0	0.777 (0.006)	291 (22)	624 (40)	1337 (72)	129 (12)	166 (14)
500	0	0.780 (0.010)	319 (13)	673 (32)	1423 (80)	121 (6)	154 (8)
600	0	0.777 (0.021)	302 (13)	634 (33)	1333 (84)	115 (5)	147 (7)
700	0	0.777 (0.012)	318 (8)	661 (17)	1374 (36)	137 (3)	174 (4)
800	0	0.760 (0.020)	330 (16)	701 (39)	1491 (94)	126 (5)	162 (7)
100	0.1	0.777 (0.021)	255 (43)	540 (80)	1144 (146)	101 (19)	129 (23)
100	1	0.837 (0.006)	217 (13)	454 (34)	950 (86)	104 (5)	133 (7)
100	10	0.840 (0.010)	235 (11)	498 (15)	1058 (32)	106 (12)	136 (15)
100	100	0.800 (0.017)	243 (7)	507 (14)	1059 (27)	113 (5)	144 (6)
200	0.1	0.797 (0.012)	225 (22)	489 (15)	1068 (72)	103 (11)	133 (11)
200	1	0.813 (0.006)	238 (11)	501 (19)	1057 (36)	119 (8)	152 (10)
200	10	0.750 (0.026)	264 (23)	556 (49)	1168 (105)	117 (9)	149 (11)
200	100	0.833 (0.006)	276 (14)	574 (27)	1194 (54)	128 (9)	163 (11)
400	0.1	0.777 (0.015)	240 (20)	494 (40)	1019 (82)	111 (7)	141 (8)
400	1	0.790 (0.010)	303 (5)	639 (17)	1351 (52)	137 (2)	175 (4)
400	10	0.790 (0.000)	306 (29)	646 (64)	1366 (139)	119 (13)	152 (17)
400	100	0.780 (0.010)	323 (11)	676 (18)	1416 (28)	133 (5)	169 (6)

Table 3. Results associated to photosynthetic apparatus analyses. Reported as average (standard deviation, n = 3). NA - Not Available

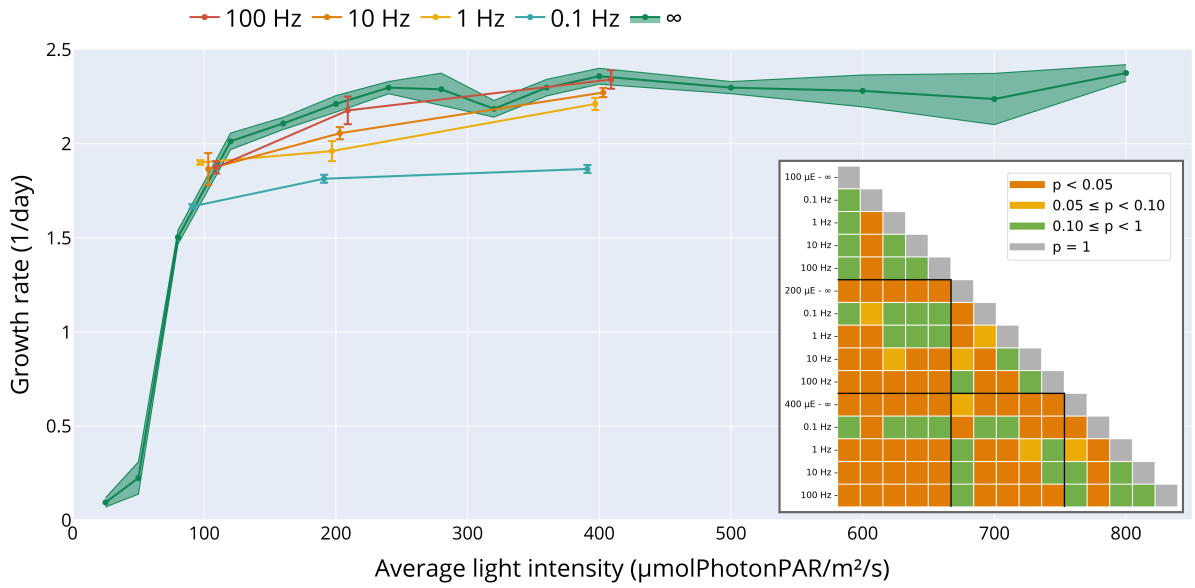

Fig. 3. Growth rate for all the tested conditions. ∞ - continuous light. Light pattern: square signal with a duty cycle of 0.5. Error bars - standard deviation (n = 3). Nested matrix, p-values based on Tukey's HSD test. Black lines delimit the blocks with the same average light intensity

diate/exhaustion of the energy-rich intermediate/exhaustion characteristic time. Hence, the observed growth rate decrease is likely due to Calvin cycle enzyme deactivation, which correlates with other authors findings (36).

The same comment could be drawn within the photolimitation zone. However, the smaller magnitude of the growth rates combined with measurement uncertainty yield statistically overlapping results. It would therefore be risky to make

the same assertion.

3 2. Pigment content

Microalgae pigment contents are of interest for two reasons: they constitute added-value molecules of significant industrial interest (especially carotenoids (37)), and deliver insights into the strategy deployed by the cells to harvest light. First, we will focus on photocollective pigments (chlorophyll *a* and *b*). Then, we will move on to the photoprotective ones: violaxanthin, zeaxanthin, and lutein.

3.2.1. Photocollective pigments. Cell chlorophyll contents are presented in Figure 4 (chlorophyll *a* on the top, chlorophyll *b* on the middle). In the case of continuous illumination, both pigments exhibit a substantial downward trend with increasing light intensity (from 17.83 $\pm$ 0.56 to 1.42 $\pm$ 0.23 mg/g_{DW} for chlorophyll *a* and 8.69 $\pm$ 0.51 to 0.84 $\pm$ 0.23 mg/g_{DW} for chlorophyll *b*). The downregulation of chlorophyll production as intensity increases is a well-known mechanism (38). Under photolimited conditions, cells upregulate their chlorophyll production to capture as much light as possible. Conversely, in photosaturated conditions, microalgae do not need much chlorophyll to collect the amount of light necessary for photosynthesis. Thus, they reduce their chlorophyll content to avoid excess energy entering the electron transport chain that may damage the photosynthetic apparatus. The effect is particularly marked here because the culture was acclimated. In terms of absolute values, observations agree with other results reported on the same strain using the same medium (39).

Flashing light does not seem to alter microalgae pigment production, with the exception of one case: low frequency and low light (0.1 Hz under 100 µmolPhotonPAR/m^2/s). The latter condition exhibits lower chlorophyll *a* and *b* contents than all its counterparts. Such a downregulation is challenging to explain. As these cells were exposed to a low amount of light, with long periods in between, they should thrive to increase their light harvesting capabilities. Therefore, both an increased absolute number of photosynthetic units (chlorophyll *a* as proxy) and collection capabilities (chlorophyll *b* as proxy) should be anticipated. Additional analysis via fluorometry will help to analyze this enigmatic observation further.

Going one step further, chlorophyll *a* over chlorophyll *b* ratio was analyzed (Fig. 4 bottom). The first comment is that under continuous light, despite changes in absolute values, this ratio remains constant (around 2, p = 0.45) over the tested conditions. This finding is somewhat contradictory to other studies which report an increase to 3.3 of this ratio under 400 µmolPhotonPAR/m^2/s for the same strain (40). The factors potentially explaining this discrepancy are: 1. batch mode in their case (maybe not achieving full acclimation) and 2. the use of BBM medium, which might have induces a nitrogen limitation. Interestingly, low and moderate (below 10 Hz) frequencies of flashing light induced an increase of this ratio by a sizable amount (circa +25 %) for the highest light intensity. It suggests that to counterbalance intermittent light availability, microalgae favor a strategy where they generate more photosystems to create more energy-rich photosynthesis intermediates. At the same time, they reduce their photocollection capability to prevent excessive energy collection during the light phase (a strategy referred to as n-strategy (41)). Growth rate results show that this strategy efficiently maintains nominal photosynthesis output. To further analyze this hypothesis, it may be relevant to investigate photoprotective pigment content to assess its efficiency in protecting from photodamage.

3.2.2. Photoprotective pigments. Cell violaxanthin and zeaxanthin contents are graphed in Figure 5 (top and bottom, respectively). Two qualitative comments have to be drawn first. Firstly, cell violaxanthin content was below the quantification level for the runs under 800 µmolPhotonPAR/m^2/s of continuous light, and it is therefore not present in the figure. Secondly, zeaxanthin quantification exhibited a high noise level and failed for the two of the configurations under 0.1 Hz. While an increasing trend with increasing light intensity can be guessed, the noise level prevents statistical analyses from asserting any differences.

Nevertheless, it can be affirmed that with increasing light intensity, violaxanthin turned into zeaxanthin (Fig. 6 top). This is the sign of the expression of the VAZ cycle. Indeed, to prevent the damages that could be induced by excessive light, microalgae transform violaxanthin into antheraxanthin then zeaxanthin, incidentally triggering light-harvesting complex aggregation (42). Antenna aggregation brings closer chlorophyll and carotenoid molecules and eases the transfer of excess excitation from the first to the second, ultimately dissipating energy overload as heat (as of the NPQ mechanism). The dynamic of the conversion of violaxanthin to zeaxanthin also correlates well with the observed growth rate. No zeaxanthin is expressed under photolimited conditions (below 80 µmolPhotonPAR/m^2/s). It appears at the beginning of the transition between photolimitation and photostaturation (80 to 120 µmolPhotonPAR/m^2/s) and increases steadily afterward.

In addition to this interconversion, microalgae also increased the total amount of violaxanthin and zeaxanthin with respect to their chlorophyll content (Fig. 6 bottom). This ratio compares the safe dissipation capacity to the light collection one. As one can see on the matrix, the flashing light did not induce any significant difference from its continuous counterpart. Still, light intensity had a clear effect. While xanthophyll to chlorophyll ratios are similar under 100 and 200 µmolPhotonPAR/m^2/s, it is markedly higher for cells cultivated under 400 µmolPhotonPAR/m^2/s (800 µmolPhotonPAR/m^2/s incident illumination during light phase). Therefore, the deployed strategy (lowering chlorophyll content) was efficient enough not to require the upregulation of the safe dissipation mechanism, until incident illumination became too high. This can be further investigated by monitoring photosynthetic apparatus status via fluorometry, as featured in the next section.

Finally, lutein follows the same trend as chlorophylls (see Supplementary materials). This observation can be explained by lutein's biological role. Indeed, its main function is to en-

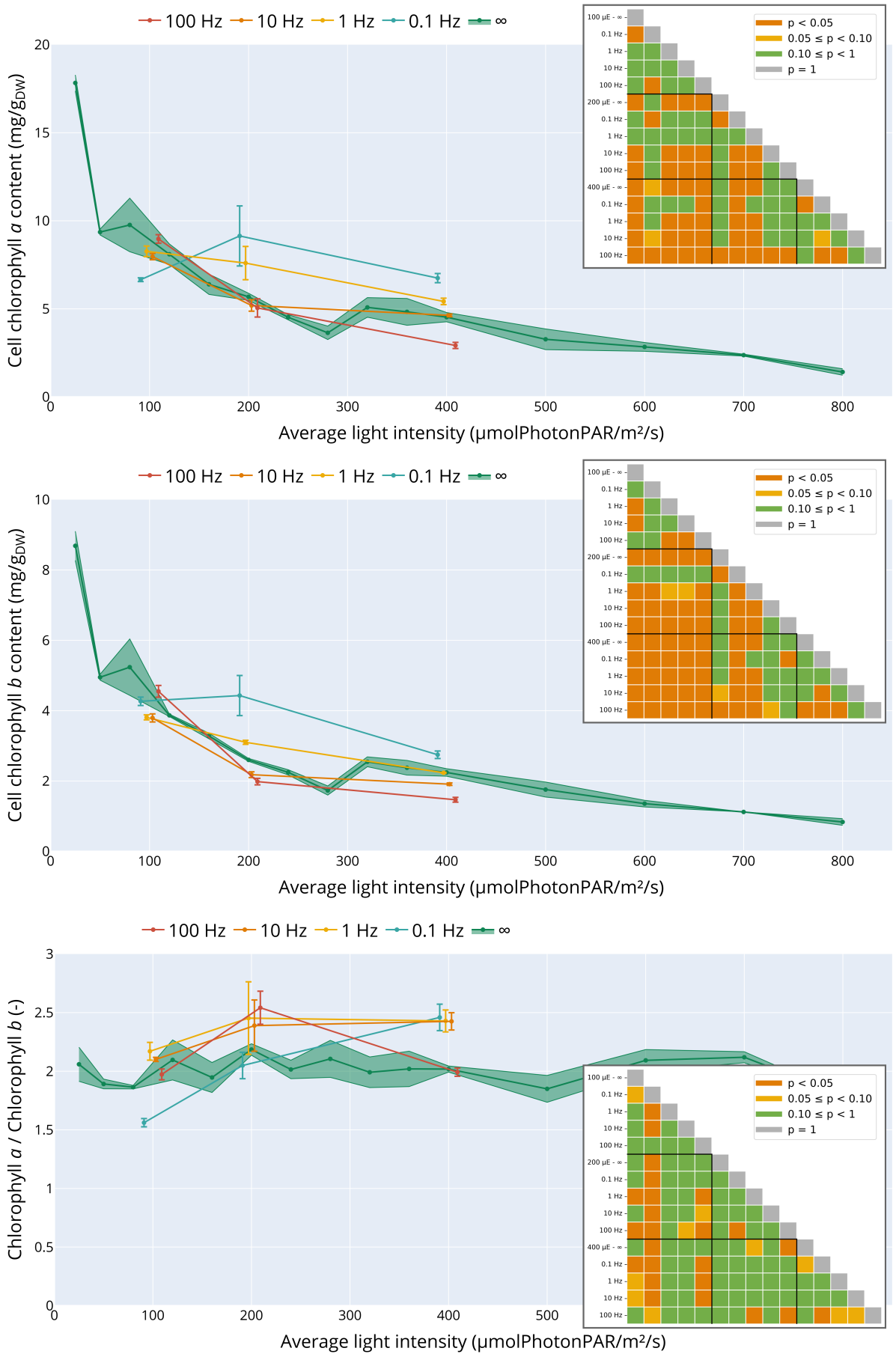

Fig. 4. Cell chlorophyll contents and ratio for all the tested conditions. Top - chlorophyll *a*, middle - chlorophyll *b*, bottom - chlorophyll *a* over chlorophyll *b* ratio. ∞ - continuous light. Light pattern: square signal with a duty cycle of 0.5. Error bars - standard deviation (n = 3). Nested matrices, p-values based on Tukey's HSD test. Black lines delimit the blocks with the same average light intensity

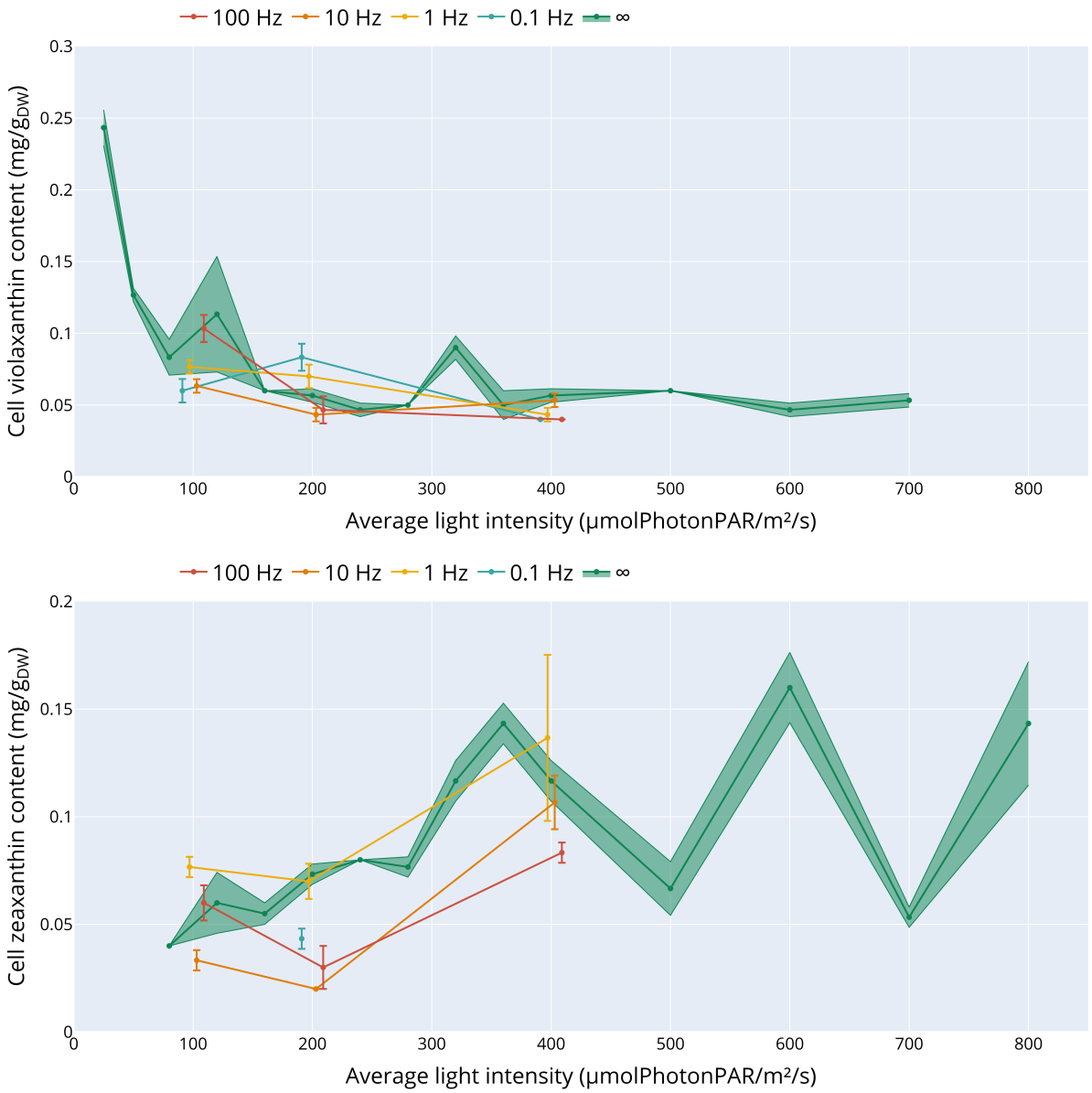

Fig. 5. Cell violaxanthin and zeaxanthin (key VAZ cycle pigment) contents for all the tested conditions. Top - violaxanthin (below quantification level for the runs under 800 µmolPhotonPAR/m^2/s of continuous light), bottom - zeaxanthin. ∞ - continuous light. Light pattern: square signal with a duty cycle of 0.5. Error bars - standard deviation (n = 3). No statistically difference between the different light patterns was detected, allegedly because of the high level of noise

sure proper folding of the antennae (43). Its secondary roles as accessory light-harvesting pigment and reactive oxygen species quencher (44) do not appear to be needed in the tested conditions. Therefore its quantity is closely tied to the one of chlorophylls.

3 3. Effect on photosynthetic apparatus

Fv/Fm ratio, the first proxy photosynthetic apparatus stress, is reported in Figure 7. As one can see, all the reported values lie around 0.8 $\pm$ 0.05, which indicates a healthy functioning of the photosynthetic apparatuses (45). While some statistically significant variations exist (*e.g.*, under 200 µmolPhotonPAR/m^2/s for a frequency of 10 Hz, Fv/Fm = 0.750 $\pm$ 0.010), their amplitude is deemed low enough to conclude that, once acclimated, the cells did not experience heavy stress for any of the tested conditions.

After analyzing this marker of potential light stress, the next step is to examine how acclimated cells manage light of increasing intensity. It can be done by inspecting the shape of the light curve (or PI, PE curve). One should note the critical difference between these curves and the reported growth rate (Fig. 3). These curves represent the instantaneous response of the cells to increasing light after acclimation to a specific light pattern. Consequently, they deliver insight into the photosynthetic apparatus but should not be used to derive potential outcomes of a long-running production process. Conversely, the growth rate reported in this work represents the acclimated cell growth performance and can serve as valid data to design a process.

Key values that characterize these curves are reported in

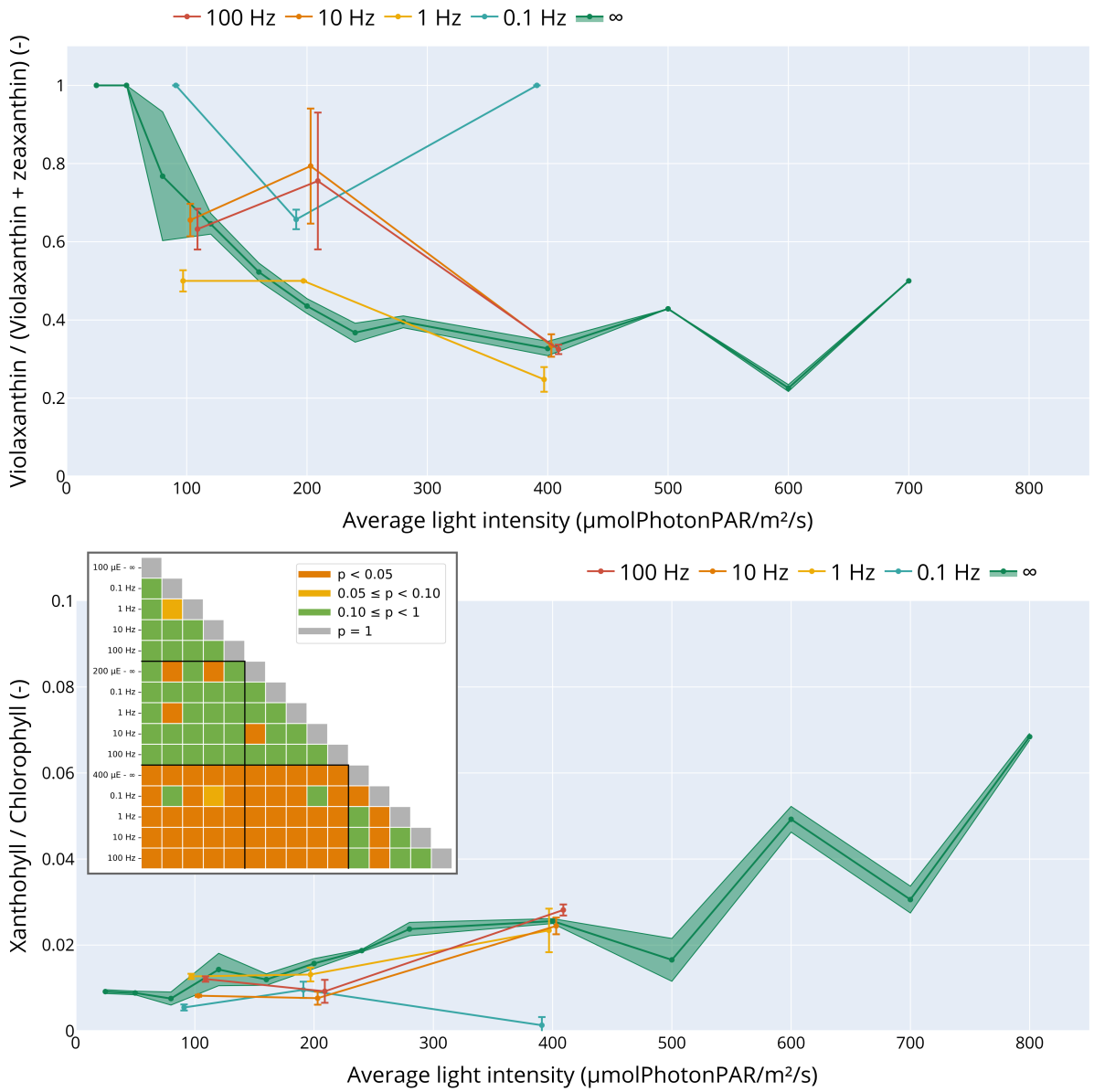

Fig. 6. Top - VAZ cycle epoxidation state - violaxanthin / (violaxanthin + zeaxanthin) - for all the tested conditions. Bottom - Xanthophyll over chlorophylls ratio. ∞ - continuous light. Light pattern: square signal with a duty cycle of 0.5. Error bars - standard deviation (n = 3). Top - no value included when zeaxanthin was not detected. No statistically difference between the different light patterns was detected, allegedly because of the high level of noise. Bottom - nested matrix, p-values based on Tukey's HSD test. Black lines delimit the blocks with the same average light intensity

Table 3 and graphed in supplementary materials. From the table data, one can conclude that the application of flashing did not induce a significant change in most cases with respect to continuous light (except for 400 µmolPhotonPAR/m^2/s at 0.1 Hz). For the sake of readability, Figure 8 (left) only displays pooled curves (continuous and flashing, min/max spread) for 100 and 400 µmolPhotonPAR/m^2/s average light intensities (lowest and highest tested values). As one can see, increasing acclimation light intensity increased the possible instantaneous output (P_{max}) of the curve. Furthermore, it delayed the onset of photoinhibition (I_i) and the end of the photolimitation phase (I_k). This strategy is well-known for continuous light (Fig. 8 - right (46)). When facing increasing continuous light, microalgae decrease their light-collection capability (increasing I_i and I_k) while upregulating their safe dissipation mechanisms (allowing them to achieve a higher P_{max} at high intensity and further delaying I_i). These observations also correlate very well with our cell pigment content results (lower chlorophyll and higher xanthophyll contents). In addition, cells acclimated under 400 µmolPhotonPAR/m^2/s of averaged flashing light intensity express an instantaneous photosynthetic output (under 800 µmolPhotonPAR/m^2/s) that represents 97 % or more of their maximum (over the range analyzed by the Aquapen - 10 to 1000 µmolPhotonPAR/m^2/s -). This last observation is another token of the efficiency of the deployed acclimation strategy. Nevertheless, going further in depth in the general analysis of the difference between low and high-light acclimated cells is complicated. For example, proper comparison of absolute P_{max} values is hampered by the fact that one needs to be sure that the captured

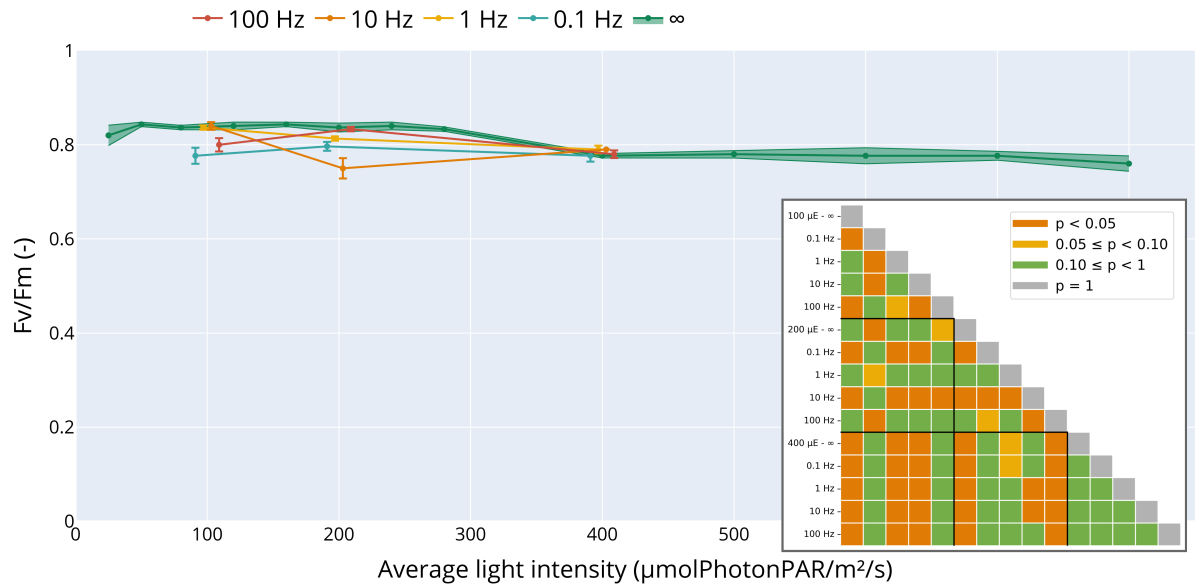

Fig. 7. Fv/Fm ratio all the tested conditions. ∞ - continuous light. Light pattern: square signal with a duty cycle of 0.5. Error bars - standard deviation (n = 3). Nested matrix, p-values based on Tukey's HSD test. Black lines delimit the blocks with the same average light intensity

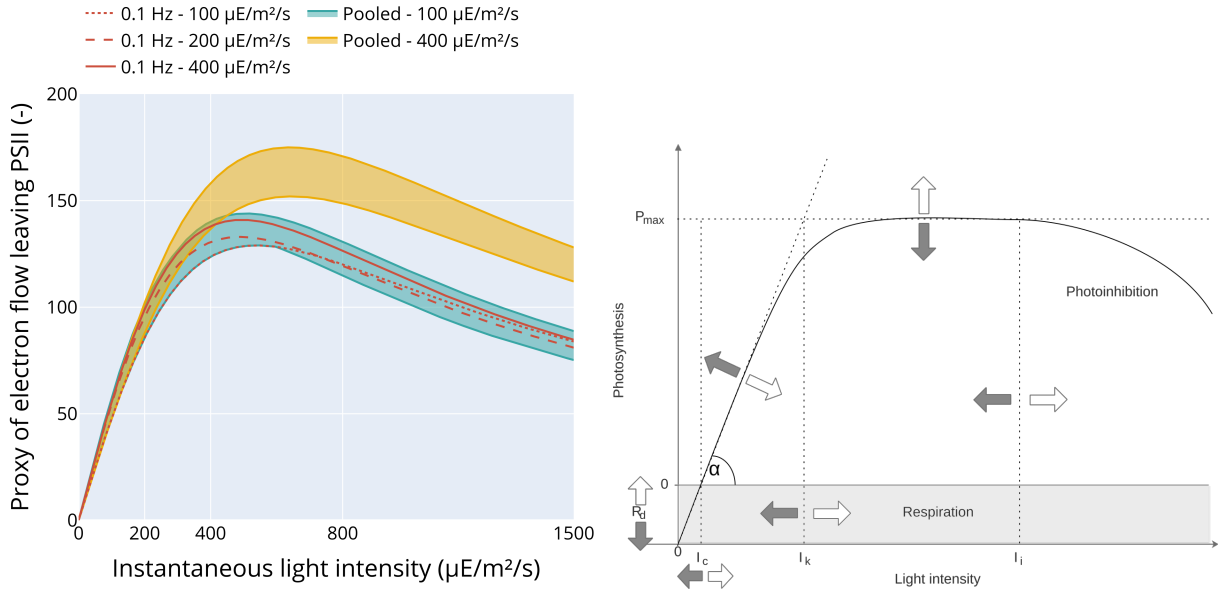

Fig. 8. Left - Light curves pooled (continuous and flashing, beam covering the min/max spread) over all frequencies for 100 and 400 µmolPhotonPAR/m^2/s. Right - classical evolution of the light curve shape with increasing (open arrows) or decreasing (closed arrows) acclimation light (46)

amount of light per reaction center is the same between samples. To get such information, OJIP tests might be required. In a more general sense, the fluorometric assays deployed in this work are focused on PSII functioning. Yet, it is only one of the many actors at stake in the light reactions of photosynthesis. Probing PSI expression level (via protein expression assays or PSI-focused fluorometry) may also unravel insights on cell acclimation strategy, at least for increasing light intensity (47). Going one step further, protein assays could also yield PSII and Light Harvesting Complex expression levels and confirm the deployment of a n over σ-strategy by the cells.

The observed behavior under 0.1 Hz is surprising. Under 100 µmolPhotonPAR/m^2/s of average light intensity (photolimitation zone), microalgae behave similarly to other frequencies. However, they show discrepancies under 200 and 400 µmolPhotonPAR/m^2/s of average illumination. The cells grown under 0.1 Hz show similar photosynthetic apparatus functioning over the tested illuminations. This lack of adaption renders the photosynthetic apparatuses inadequate to face the high illumination during the light phase of the cycle. Therefore it is not surprising to observe a loss in terms of photosynthetic output (-10 % under 800 µmolPhotonPAR/m^2/s of incident illumination compared to their maximum), which can in part explain the lower growth rate observed for such conditions (-20 %, Fig. 3). Therefore, this decrease in growth performance can be attributed to both a too-long dark phase and inefficient use of light during the light phase. Further

investigations (e.g., different duty cycles) could be of interest to better discriminate the two effects and draw guidelines to avoid such inadequate configuration in production photobioreactors.

4. Conclusion

Iso-actinic cultures of *Chlorella vulgaris* were acclimated to a wide range of light intensities (up to 800 µmolPhotonPAR/m^2/s) and frequencies (continuous illumination, and from 0.1 to 100 Hz, duty cycle of 0.5). Their responses were monitored at three different levels: 1. at the culture level with the growth rate, 2. at the cells level with their content in photocollective and photoprotective pigments, 3. at the photosynthetic apparatus level with Fv/Fm ratio and light curve response. Cultures acclimated under continuous light showed photolimitation and photosaturation phases but no photoinhibition phase, even under 800 µmolPhotonPAR/m^2/s. Yet, under high light (500 µmolPhotonPAR/m^2/s and above), they required more time to enter a steady state. This observation highlights the need to wait long enough to ensure proper acclimation to obtain reproducible and comparable measurements. Nevertheless, pigment and photosynthetic apparatus measurements aligned well with established literature under continuous illumination. Regarding the potential effect of flashing light, frequencies of 1 Hz and above induced little to no difference between cells cultivated under flashing light and those under the same average amount of light. Indeed, cells exhibited the same growth rate, similar absolute pigment composition, and light curve characteristics. They only differ by a lower chlorophyll *b* to chlorophyll *a* ratio under high illumination, suggesting an acclimation strategy favoring an increased number of photosystems instead of an increased light-harvesting capability. Conversely, cells cultivated at 0.1 Hz showed a lower growth rate and an incapacity to adapt efficiently to high incident illumination. From a biotechnological perspective, these results support the idea that, in the tested conditions, flashing light does not bring benefits with respect to the same average amount of continuous light. Nevertheless, they advocate for the use of flashing light, artificial or from mixing in optically dense culture, to increase incident light intensity cast onto photobioreactors. Indeed, after proper acclimation, potentially inhibitory incident illuminations (*e.g.*, 1600 µmolPhotonPAR/m^2/s) could see their harmful effects nullified as it is the average illumination (*e.g.*, 800 µmolPhotonPAR/m^2/s with a duty cycle of 0.5) that drives cell response. Furthermore, this work could be extended by investigating the acclimation temporal dynamic (not only the achieved steady-state) as well as other values of the duty cycle.

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6. CRediT author statement

Conceptualization PP, WL, VP. Methodology WL, VP. Software VP. Validation WL. Formal analysis WL, VP. Investigation WL. Writing - Original Draft VP. Writing - Review & Editing WL, PP. Supervision PP, VP. Funding acquisition PP.

7. Conflicts of interest

Authors have no conflict of interest to disclose.

8. Statement of Informed Consent, Human/Animal Rights

No conflicts, informed consent, human or animal rights applicable.

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