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Investigation of the colony size effect on light access and growth of the colonial microalga *Botryococcus braunii* in photobioreactor

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ABSTRACT

The colonial microalga *Botryococcus braunii* is recognized for its original hydrocarbon content, making it a promising candidate for the production of biofuels and other bio-based products. However, challenges related to its slow photosynthetic growth rate hinder economically viable processes and large-scale applications. This study investigates the effect of colony size on light absorption and growth efficiency in *B. braunii* BOT-22. To examine this, large colonies were disrupted into smaller ones using a high-pressure homogenizer, resulting in a tenfold increase in the mass absorption cross-section (from 8.0 to 79 m²·kg⁻¹), highlighting how colony size significantly affects light absorption. Next, cultivation in a photobioreactor allowed the calculation of the Mean Rate of Photon Absorption (MRPA), supporting a relationship between the colonial behavior of *B. braunii* and a photoprotective strategy. Higher incident light flux was associated with larger colonies, which is likely a mechanism to manage the light stress on embedded cells. Building on these results, medium-sized and larger colonies were successfully generated by applying different light conditions during pre-cultivation. Photobioreactor cultures of these medium-sized colonies (145 μm average diameter) showed 34 % higher biomass productivity (0.123 g·L⁻¹·d⁻¹ or 4.92 g·m⁻²·d⁻¹) and 74 % higher hydrocarbon productivity (0.033 g·L⁻¹·d⁻¹ or 1.30 g·m⁻²·d⁻¹) compared to the larger colonies (434 μm). These findings highlight the important role of optimizing colony size for better light absorption and productivity, providing valuable insights for improving photobioreactor performance in *B. braunii* cultivation.

1. Introduction

In the context of global energy and environmental transitions, the development of sustainable bioprocesses for producing biomolecules is crucial, particularly as alternatives to fossil-based resources [1]. The production of these biomolecules by microalgae offers key advantages, including high areal productivity, the use of non-arable land, efficient CO₂ fixation, flexible water requirements, and high lipid content, making them ideal for the sustainable production of biofuels and other compounds of interest [2]. Their ability to treat wastewater and avoid competition with food production further enhances their ecological and economic potential [3].

With its ability to accumulate hydrocarbons in its extracellular matrix, *Botryococcus braunii* holds great potential for producing biofuels and other petroleum-derived products. It also produces potentially recoverable and profitable products such as echinenone (carotenoid,

antioxidant) [4], exopolysaccharides (i.e., to potentially produce bio-sourced polyesters) [5], and nanoparticles with anti-microbial effects [6], possibly enabling better valorization of the biomass [7].

The colonial structure of *B. braunii* makes it a highly original subject for bioproduction. This configuration is closely linked to the accumulation of hydrocarbons in the extracellular matrix during growth [8]. Each colony consists of cells embedded in a polymerized aliphatic aldehydes network permeated by liquid hydrocarbons, all enclosed in a supporting wall adorned with a fibrillar colony sheath rich in exopolysaccharides [9–11]. Depending on the nature of these hydrocarbons, several races of *B. braunii* have been defined. Race A mainly produces alkadienes and alkatrienes [12]; race B is characterized by botryococcenes [13] and a small amount of methylated squalene [14]; race L by lycopadiene [15]; and finally, race S, not always accepted as a race, by alkanes [16]. A recent study proposed a reclassification of *B. braunii* into separate species using a comparative genomics analysis. They

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described the 3 following three species: *Botryococcus alkenealis* (A race), *Botryococcus braunii* (B race), and *Botryococcus lycopadienior* (L race) [17].

The specific growth rate of *B. braunii* is typically low, ranging from 0.09 to 0.5 d⁻¹ under controlled laboratory conditions, compared to 1 to 2 d⁻¹ for *Chlorella vulgaris* [18,19]. This slow growth rate results in low biomass and hydrocarbon productivities, increasing the overall production cost. To address this limitation, various approaches have been explored in the literature. This includes optimizing CO₂ supply, with studies showing that 2–5 % CO₂ enrichment of the gas phase can both enhance biomass yield and hydrocarbon production [18,20,21]. Nutrient manipulation, particularly the use of high bicarbonate concentrations (a similar strategy as enhancing CO₂ content in the gas phase), has been found to increase biomass and lipid productivity [22]. Moreover, the introduction of symbiotic bacteria, such as the alphaproteobacterial ectosymbiont BOTRYCO-2, has shown promise in promoting *B. braunii* growth [23]. Finally, temperature and light intensity optimizations have also been investigated [18,24–27].

However, while some studies have observed the impact of different parameters on colony size, to our knowledge, the specific effects and mechanisms by which colony size influences the growth of *B. braunii* have been explored only minimally, with very few studies addressing this aspect. In two studies, the discussion has primarily focused on how colony size responds to changes in light intensity and how this change in light supply influences growth and pigmentation [24,28]. Another study [29] reveals that colony formation significantly reduces the average mass absorption cross-section of the microalgae due to lower pigment concentrations, mutual shading among cells, and the presence of an extracellular matrix. Among these factors, nutrient limitation leading to reduced pigment levels was identified as the most significant contributor. This raises questions about the actual impact of colony structure independent of pigment concentration and the nutrient limitations used to generate the colonies in the study. Optimizing light supply for *B. braunii* cultivation in suspension within photobioreactors remains challenging. This is primarily due to the limited understanding of several key factors: the relationship between colony structure and size; the amount of light effectively absorbed by embedded cells; the light transfer conditions within suspensions of *B. braunii* colonies; and the resulting impact on photosynthetic growth and hydrocarbon production rates.

This work aims to investigate the role of colony size in determining light absorption in *B. braunii* culture and its correlation with the growth of *B. braunii* BOT-22 (race B). Initially, the differences in light absorption across two striking examples of colony sizes, produced using a high-pressure homogenizer (HPH), will be investigated, and the possible underlying mechanisms will be discussed. Subsequently, calculations of the Mean Rate of Photon Absorption (MRPA) will allow characterizing the relation between colony sizes, light effectively absorbed, and culture biological response. Finally, a clear correlation will be established between colony sizes and both biomass and hydrocarbon productivity. All of these results will underscore the critical role of colony size in optimizing light absorption in photobioreactors cultivating *B. braunii*, offering perspective for improving the culture of this particular strain.

2. Materials and methods

2.1. *Botryococcus braunii* strains

The strain used is *Botryococcus braunii* BOT-22 (race B), isolated from a reservoir in Okinawa Prefecture, Japan, and kindly provided by the University of Tsukuba. It is primarily known for producing botryococcene C₃₄H₅₈ [30]. Another strain, SAG 30.81 (race A), obtained from the Culture Collection of Algae at Göttingen University (Sammlung von Algenkulturen, SAG), was also used. This strain exhibited a much smaller colony size compared to the BOT-22 strain and was used solely to illustrate the case of *B. braunii* forming colonies with very small

diameters and the resulting impact on light capture efficiency. Unless otherwise specified, studies were conducted on the BOT-22 strain.

2.2. Culture medium

BOT-22 was grown in the following medium, adjusted for the biomass concentration reached in the present study to avoid nutrient limitation, leading to the following composition in mg·L⁻¹: 1680 NaNO₃; 180 MgSO₄·7H₂O; 20 CaCl₂·2H₂O; 4 Fe-Citrate; 4 Citric acid; 80 K₂HPO₄; 160 KH₂PO₄; 16 Na₂EDTA·2H₂O; 2 FeCl₃·6H₂O; 0.4 MnCl₂·4H₂O; 0.08 ZnSO₄·7H₂O; 0.016 CoCl₂·6H₂O; 0.04 Na₂MoO₄·2H₂O; 0.02 Thiamine (B1); 0.004 Biotin (H); 0.002 Cyanocobalamin (B12).

2.3. Culture systems

2.3.1. Pre-cultures

The microalgae BOT-22 and SAG 30.81 *B. braunii* were cultivated in flasks at an initial concentration of 0.15 g·L⁻¹ in a culture volume of 200 mL, in 500 mL flasks with air-CO₂ bubbling (approximately 33 mL·min⁻¹ and 1.5 % CO₂). The flasks were illuminated with incident LED white light at 50 or 80 μmol_{hv}·m⁻²·s⁻¹ and placed on an orbital shaker at 130 rpm for 19 to 30 days.

2.3.2. Photobioreactor cultivation to assess the effect of colony size on culture performance

Cultures were conducted in batch and continuous modes in a torus-shaped photobioreactor, described in detail elsewhere [31]. The culture volume was 1.35 L with a culture depth of 4 cm. Mechanical mixing was obtained using a marine impeller. A rotation speed of 250 rpm was applied, with periods at 500 rpm for 15 s every 15 min to prevent biomass sedimentation. An airflow rate of 50 mL·min⁻¹ was added to avoid oxygen accumulation with automated CO₂ injection regulated by pH value (set at pH = 7). The culture temperature was maintained at 20 °C ± 1 °C. Incident photon flux density (PFD) was obtained from an LED panel providing white light. The initial PFD value was set to 50 μmol_{hv}·m⁻²·s⁻¹ to avoid light stress and then increased to 100 μmol_{hv}·m⁻²·s⁻¹ 9 days after inoculation. Biomass concentration at inoculation was 0.1 and 0.2 g·L⁻¹ (expressed in dry-weight, denoted DW) for medium colonies (MC) and large colonies (LC), respectively. BOT-22 pre-cultures used for inoculation were exposed to two different light conditions, 50 ± 10 μmol_{hv}·m⁻²·s⁻¹ and 80 ± 10 μmol_{hv}·m⁻²·s⁻¹. These pre-cultures were cultivated for multiple generations under these conditions, leading to the formation of “large colony” and “medium colony” lineages. Nine days after inoculation, continuous cultivation was applied with constant dilution rates. A dilution rate value of 0.08 d⁻¹ was applied for MC colonies. Because of the lower growth rate, a value of 0.05 d⁻¹ was used to avoid culture washout for LC cultivation.

2.3.3. Photobioreactor cultivation to assess culture response to the mean rate of photon absorption (MRPA)

Cultivations were conducted in the torus-shaped photobioreactor following a similar procedure as described in Section 2.3.3, but with the aim to cover a larger range of PFD values to investigate the response of *B. braunii* under different MRPA conditions. The culture was then inoculated at 0.45 g·L⁻¹ to ensure a substantial biomass for a faster increase in incident PFD. The batch phase lasted for 26 days, followed by continuous cultivation at a dilution rate of 0.05 d⁻¹. Throughout the 46 days of cultivation, the PFD was gradually increased from 20 to 1000 μmol_{hv}·m⁻²·s⁻¹ to achieve progressively higher MRPA. Each PFD value was maintained for 3 days to allow sufficient time for stabilization of biomass concentration and pigment content through the photo-acclimation process, which typically occurs over several hours to days [32–35]. To assess pigment stabilization, pigment content measurements for a given PFD value were conducted over several days. Stabilization was found to occur in <3 days. Because of the larger biomass

concentration achieved in this experiment, a 2.5-fold concentrated medium was used to prevent nutrient depletion. The MRPA calculation method is detailed in Section 4.4.2.

2.4. Measurement of colony size, biomass concentration, and productivity

2.4.1. Microscopic analysis of colony size

Colony size was measured using a microscope (Zeiss, Germany) with Zen 3.0 software. Samples were placed on a slide with a 300 μm depth. This method, adapted from Tanoi et al. [37], is based on microscopy principles that distinguish colonies from the sample background. Microscopy produced 2D images, which were then analyzed using ImageJ software to measure colony size. The colony size distribution was generated using MATLAB software after processing the data obtained from ImageJ. Each category of colony diameter was represented as a percentage of the total biomass volume, using the approximation of spherical colonies, even though their actual shape deviates from perfect sphericity.

2.4.2. Dry weight biomass concentration measurement

The dry-weight biomass concentration, X , was determined by a gravimetric method. A volume of culture was filtered (VWR® glass fiber filter, 0.7 μm) and rinsed with at least three times the filtered volume of distilled water. The filters (in triplicate) were then placed in an oven at 105 °C for 24 h, cooled in a desiccator, and weighed using a precision balance (Mettler Toledo® XA105DU).

2.4.3. Pigment concentration measurement

The term “pigment” used in this article specifically refers to chlorophylls and carotenoids. Secondary carotenoids such as echinenone [4], botryoxanthins [37], and braunioxanthins [38] were not investigated. Therefore, the respective concentrations of primary and secondary carotenoids could not be determined. However, an estimate was made based on absorbance at 480 nm. Pigment concentrations were determined by spectrophotometry. Pigments were extracted by mixing culture and methanol in a 1:4 volume ratio (20 % culture). The samples were subjected to vortexing (10 s) and ultrasonication (30 s) three times, then incubated for 1–2 h at 50 °C in the dark until the colonies turned beige-white. After centrifugation (12,000 g for 5 min), the supernatant containing the pigments was analyzed at 480, 652, 665, and 750 nm (Jasco® V-630). The optical densities were used to determine the concentrations of chlorophyll *a*, chlorophyll *b*, and total primary carotenoids in the biomass using the equations of Ritchie [39,40] and Strickland and Parsons [41].

2.4.4. Measurement of chlorophyll *a*, leakage indicator

Chlorophyll *a* served as a marker for monitoring cell disruption in microalgae, as its release into the surrounding medium indicates disrupted cells and chloroplast membranes. Samples were taken and filtered (VWR® glass fiber filter, 0.7 μm) before and after treatment to assess if the mechanical treatment had a lytic effect on the cells. The filtrate was analyzed by spectrophotometry to determine pigment concentration as described in Section 2.4.3. Only chlorophyll *a* was used, as secondary carotenoids such as echinenone can be stored in the extracellular matrix [28].

2.5. Hydrocarbon analysis

2.5.1. Hydrocarbon extraction

Hydrocarbons were extracted from freeze-dried biomass or supernatant using the Folch method [42] with a 2:1 $\text{CHCl}_3/\text{MeOH}$ (v/v) mixture, involving vortexing (3×10 s), sonication (3×30 s), and a 2-h incubation on a shaker plate. The samples were then filtered through glass fiber columns, and the filtrate was evaporated at 40 °C under a nitrogen stream. The remaining residues were dissolved back in n-hexane for analysis by gas chromatography coupled with a flame ionization

detector (GC-FID).

2.5.2. GC-FID analysis

Hydrocarbon samples were analyzed by GC-FID [43]. Analysis was performed using a GC-8860 (Agilent Technologies, Santa Clara, CA, USA) equipped with an injector (injection volume: 2 μL , split set to 1:10, 10 $\text{mL}\cdot\text{min}^{-1}$), an HP-5 capillary column (non-polar phase, 5 % phenylmethylpolysiloxane, inner diameter 0.32 mm, film thickness 0.25 μm , length 30 m, Agilent Technologies, Santa-Clara, CA, USA), and a flame ionization detector. The hydrogen flow during analysis was maintained at 2 $\text{mL}\cdot\text{min}^{-1}$, and was produced using a COSMO H4000 hydrogen generator (F-DGSI, Evry, France). Analysis was performed with and without a measured addition of a squalene standard (99.8 %) to both serve as a quantitative reference and to isolate the quantity of squalene naturally produced by the BOT-22 strain. The chromatograph temperature program was as follows: injector temperature 280 °C, FID 340 °C, oven at 75 °C for 3 min, then ramped at 10 °C $\cdot\text{min}^{-1}$ to 300 °C for 28.5 min, and finally at 75 °C $\cdot\text{min}^{-1}$ to 340 °C for 4 min. The two main hydrocarbon peaks at retention times of 22.81 and 22.89 min corresponded to C_{34} and C_{32} botryococcenes. The identification of C_{34} and C_{32} botryococcenes was validated using GC–MS analysis. These two main hydrocarbons were combined and referred to as “hydrocarbons”. These “hydrocarbons” may also include small quantities of methylated squalenes, which are difficult to separate from botryococcenes by GC.

2.6. Cell disruption using a high-pressure homogenizer

The biomass obtained from the cultivation in a 220 mL flask, conducted at a PFD of 80 $\mu\text{mol}_{\text{hv}}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ with 0.2 vvm aeration and 1.5 % CO_2 sparging for 38 days, was processed using a high-pressure homogenizer (HPH, TS series 2.2 kW from Constant Systems Limited, Daventry Northants, UK) set at the lowest working pressure of 500 bar. The aim was to disrupt all the colonies without damaging individual cells.

2.7. Experimental measurements of radiation characteristics

2.7.1. Measurement of hemispherical normal transmittance and reflectance

Hemispherical normal transmittance and reflectance (denoted T_{NH} and R_{NH} , respectively) were measured for wavelengths ranging from 400 to 750 nm with 1 nm of spectral resolution for HPH-treated (HT) and untreated (NT) biomass adjusted to the same concentrations with PBS saline buffer at 0.82 $\text{g}\cdot\text{L}^{-1}$. Note that the samples were not washed with PBS, as biomass loss or colony disruption could occur during centrifugation due to the low density of some BOT-22 colonies. Moreover, T_{NH} values of the transparent culture medium were compared to PBS and showed no difference when used as a blank. Measurements were performed in a quartz cuvette with a 1 cm path length (110–10-40 Hellma Analytics, Müllheim, Germany) using an Agilent Cary 5000 series UV–Vis–NIR spectrophotometer (model AU12410027) equipped with an integrating sphere. The normal hemispherical transmittance was used because it was deemed more suitable for measuring the light transmission of diffused material, like microalgae [44]. It accounted for both the directly transmitted light and the forward-scattered light.

2.7.2. Estimation of the spectral mass absorption cross-section

A procedure developed and described by Kandilian et al. [44] using Normal-hemispherical transmittance and normal-hemispherical reflectance was employed to estimate the spectral absorption cross section ($A_{\text{abs},\lambda}$) of *B. braunii*. T_{NH} and R_{NH} were used following the inverse method to estimate the $A_{\text{abs},\lambda}$. Note, however, that the approach presented in Kandilian et al. was validated exclusively for non-colonial microalgae. The colonial nature of *B. braunii*, which results in significant heterogeneity, may introduce variations in effectively measured $A_{\text{abs},\lambda}$. Therefore, the calculated $A_{\text{abs},\lambda}$ should be considered as an approximation rather than a true radiative property of the microalga.

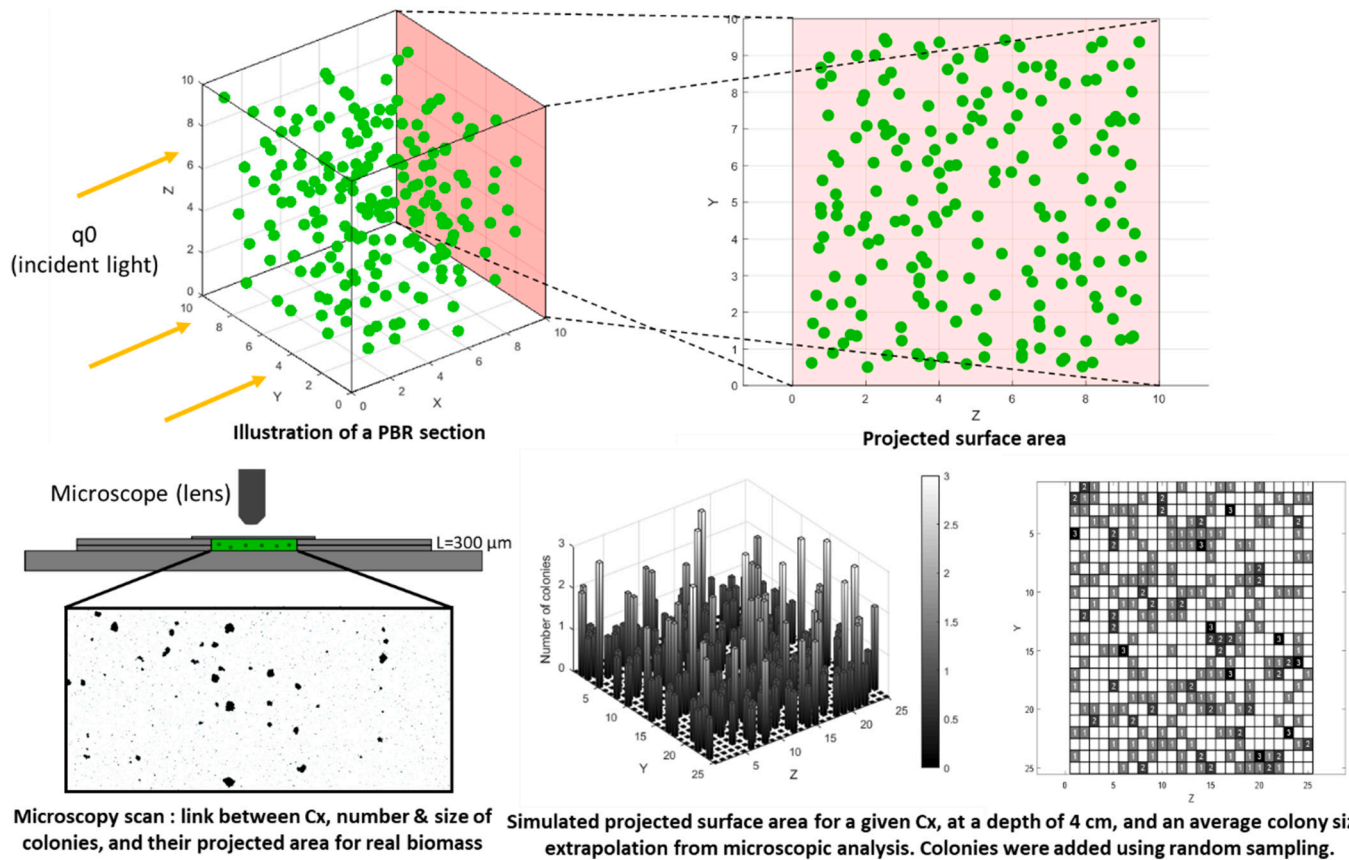


Fig. 1. Illustration of the method used to evaluate the probability of light hitting a colony when cultivated in a PBR. The simulated projected surface (grid) shown is a sample from the full analysis, which used at least 14,000 grid cells and 32 colonies, and up to several million grid cells and colonies in total, depending on the biomass concentration and colony size. The numbers on the scale and in the grid cells represent the number of stacked colonies. Cx: biomass concentration.

$A_{abs,\lambda}$ was then used to calculate the MRPA and the local fluence rate $G_{\lambda}(z)$. MRPA, denoted $\langle A \rangle$, represents the amount of photons absorbed in the photosynthetically active radiation (PAR) range (400–700 nm) per unit weight of biomass and per unit time, here averaged on the total culture volume, and is given for a photobioreactor presenting light attenuation only along the culture depth L by [45]:

$$\langle A \rangle = \frac{1}{L} \int_0^L \int_{400}^{700} A_{abs,\lambda} G_{\lambda}(z) d\lambda dz \quad (1)$$

$$G_{\lambda}(z) = q_{0,\lambda} e^{(-A_{abs,\lambda} X z)} \quad (2)$$

with L the depth of the PBR, $q_{0,\lambda}$ the spectral incident PFD, X the biomass concentration, and z the position along the PBR depth of culture.

2.8. Evaluation of the probability of light hitting a colony when cultivated in PBR

As a first attempt to compare the effect of cells growing in colonies on light penetration in PBRs, a simplified approach was employed. The objective was to estimate the percentage of photons that would reach the surface area representative of a colony, without assessing whether the light is subsequently scattered or absorbed (which would require more advanced approaches based on radiative transfer modeling, which was not the aim of this research).

For this purpose, the projected surface area of the colonies growing along the PBR depth (4 cm here) was used. An illustration to complement the description of this method is proposed in Fig. 1. To recreate the PBR and the projection of the surface area of the colonies suspended in

this PBR, a grid system and random sampling were implemented in MATLAB software. Based on the average area of a colony, squares having the same surface area were generated, forming the grid. Colonies were then added one by one with an equal probability of placement in each grid cell, using random sampling. Colonies could also overlap, thereby faithfully representing a projection of the surface area of all the colonies suspended in the PBR culture volume. The link between biomass concentration, average colony diameter, number of colonies, and their occupied areas was established based on image analysis (see Section 2.4.1.). All average sizes used were based on actual image analysis data. Note that one of the main limitations of this approach is that colony representation is simplified by using only the average size and not their size distribution.

An assessment of the approach was performed by considering a thickness of 300 μm , corresponding roughly to the slide used for image analysis. The difference between the actual occupied total area and the simulated area, corresponding to the fraction of photons reaching the colonies, did not exceed 3 %. This small discrepancy can be considered acceptable and is likely mainly due to the fact that no overlap is considered in the case of the experimental measurement. In the simulation, colonies were randomly positioned, and overlaps could occur, although they were relatively few for a 300 μm thickness. We then considered that this simple analysis thus enables, as a first approximation, the percentage of light reaching the colonies in culture as a function of biomass concentration, PBR thickness, and average colony size.

2.9. Statistical analysis

Error bars represent standard deviations of triplicate or quadruplicate measurements. Size distributions were compared using the Mann-

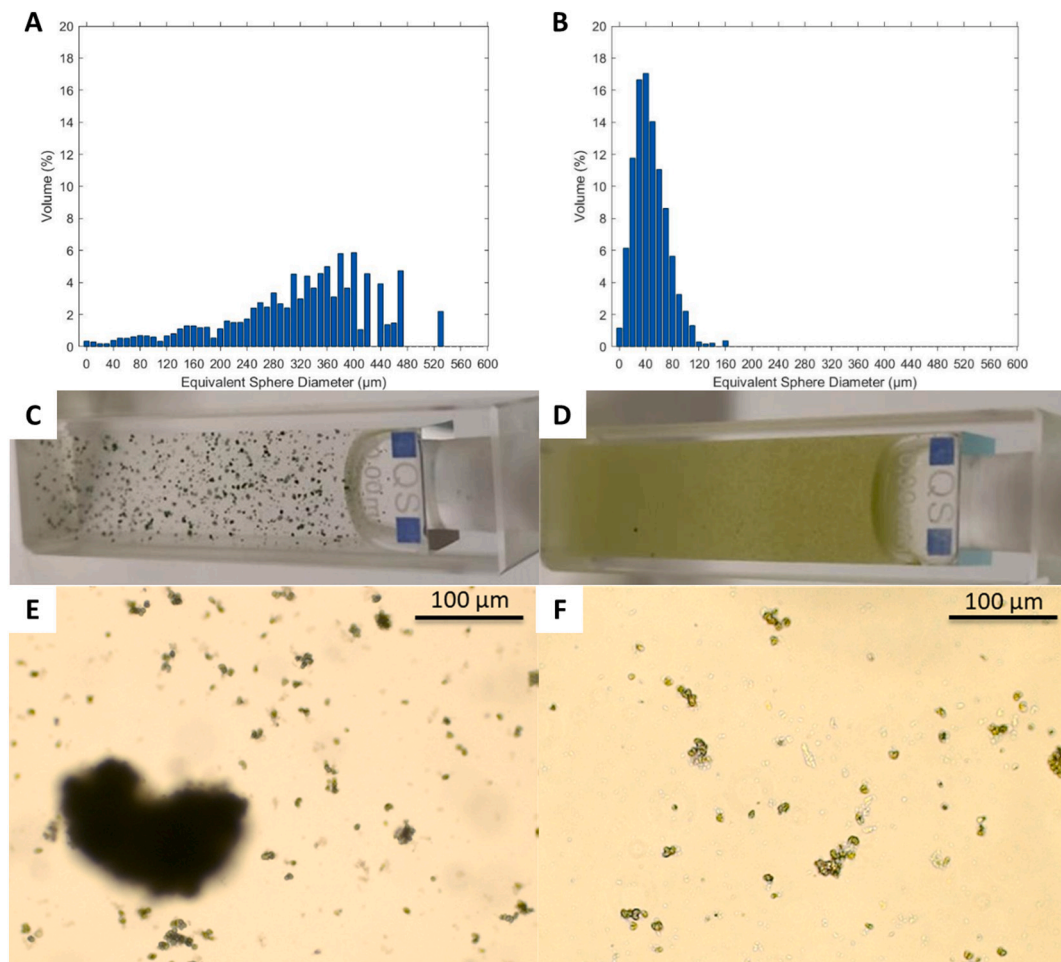


Fig. 2. Colony size distributions for a native BOT-22 population (A) and a disrupted population using HPH at 500 bar (B). The percentage of volume represents an approximation of the relative biomass quantity in each size category. The difference between the two populations, non-treated (C) and HPH-treated (D), is visible to the naked eye and in optical microscopy (E and F, respectively).

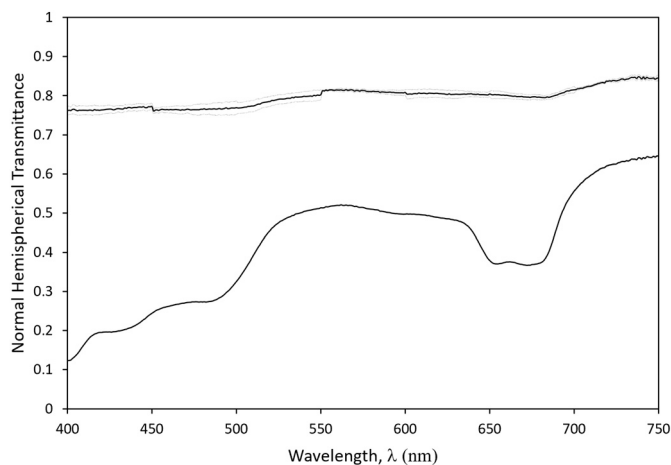


Fig. 3. Normal hemispherical transmittance for the untreated colony population (top line) and the HPH-treated colony population (bottom line). Standard deviations are shown as thin curves surrounding the main lines, based on at least three replicate measurements.

Whitney *U* test for non-normal distributions. Different letters indicate that the distributions are significantly different ($p < 0.05$).

3. Results & discussion

3.1. Effect on light absorption of intact full-sized colonies and of disrupted, smaller colonies

Previous research by Hoeniges et al. (2020) [29] on the impact of *B. braunii* colonial structure on light absorption demonstrated that mutual shading among cells reduces light absorption in colonies compared to the same number of individual free-floating cells. Additionally, it was concluded that the colony's extracellular matrix had a negligible effect on light absorption. However, in their study, nitrogen limitation was used to induce colony formation, which affected pigment content. Simulations using the Monte Carlo ray tracing method revealed that a substantial part of the observed decrease in the spectral average mass absorption could be directly attributed to reduced pigment concentrations. In the present study, we used native BOT-22 colonies and generated smaller colonies from the same biomass by treating it with a high-pressure homogenizer (HPH) at 500 bar. Thus, two biomass populations were compared: untreated native colonies and HPH-treated smaller colonies.

The biomass concentration was adjusted to $0.82 \text{ g} \cdot \text{L}^{-1}$, and the chlorophyll *a* content was very similar at $0.92 \pm 0.09\% \text{X}$ and $0.96 \pm 0.03\% \text{X}$, respectively, where %X refers to the percentage of pigment

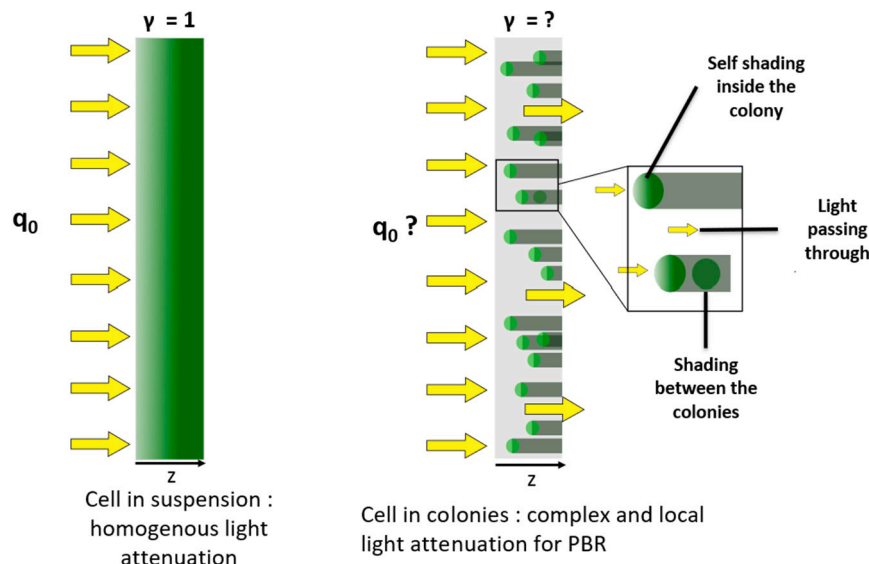


Fig. 4. Schematic representation of two cases of light attenuation. On the left, the light is homogeneously attenuated by single cells or small colonies. On the right, large colonies make light attenuation more complex. q_0 : incident light flux; z : PBR depth; $\gamma = 1$: full light attenuation to compensation point; $\gamma = ?$: unknown light attenuation.

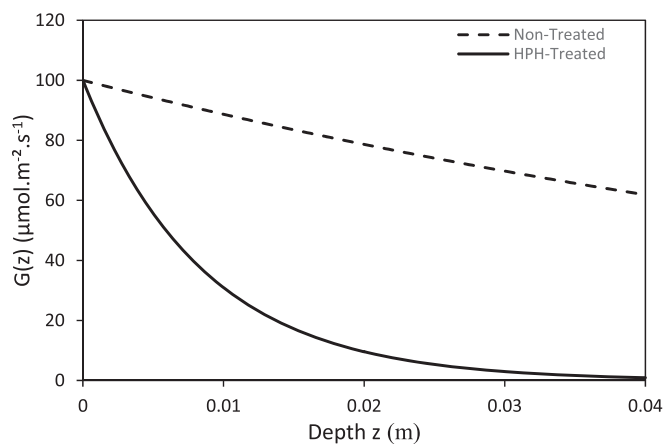


Fig. 5. Evolution of light flux density in a 4 cm depth flat panel PBR for the non-treated biomass (dotted line) and the HPH-treated biomass (solid line). The parameters used were: $PFD = 100 \mu\text{mol}_{\text{hv}}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, $X = 1.5 \text{ g}\cdot\text{L}^{-1}$, $\bar{A}_{\text{abs},\lambda} \text{ NT} = 8.0 \text{ kg}\cdot\text{m}^{-2}$ and $\bar{A}_{\text{abs},\lambda} \text{ HT} = 79 \text{ kg}\cdot\text{m}^{-2}$.

relative to biomass dry weight. Chlorophyll *a* was chosen as an intra-cellular marker to estimate cell disruption when it is released from the cells. Chlorophyll *a* leakage in the filtrate ($0.7 \mu\text{m}$ membrane) was measured at 0.04 % of the total chlorophyll *a* content in the untreated biomass and 3 % in the treated biomass. HPH successfully disrupted the vast majority of colonies into significantly smaller ones, while generating between 1 % ($0\text{--}10 \mu\text{m}$ in diameter) and 7 % ($10\text{--}20 \mu\text{m}$ in diameter) of single cells. If a direct relation between chlorophyll *a* leakage and cell disruption is assumed, only approximately 3 % of the biomass was found to be destroyed, which was considered acceptable for generating colonies of various sizes without significantly compromising the physical integrity of the embedded cells.

Fig. 2 highlights the clear difference in colony diameters between the two populations, resulting from the HPH treatment. Fig. 3 illustrates a large difference in T_{NH} between the two populations, with average T_{NH} values in the PAR of 0.80 for the untreated colonies and 0.42 for the HPH-treated colonies. This indicates the cells' ability to absorb light increases dramatically as the colony size is reduced, despite having the same amount of pigments. This finding is consistent with the simulation results obtained by Hoeniges et al. (2020). It is also consistent with the relationship established by Khatri et al. [46] between optical density measurements and the colony size of *B. braunii*.

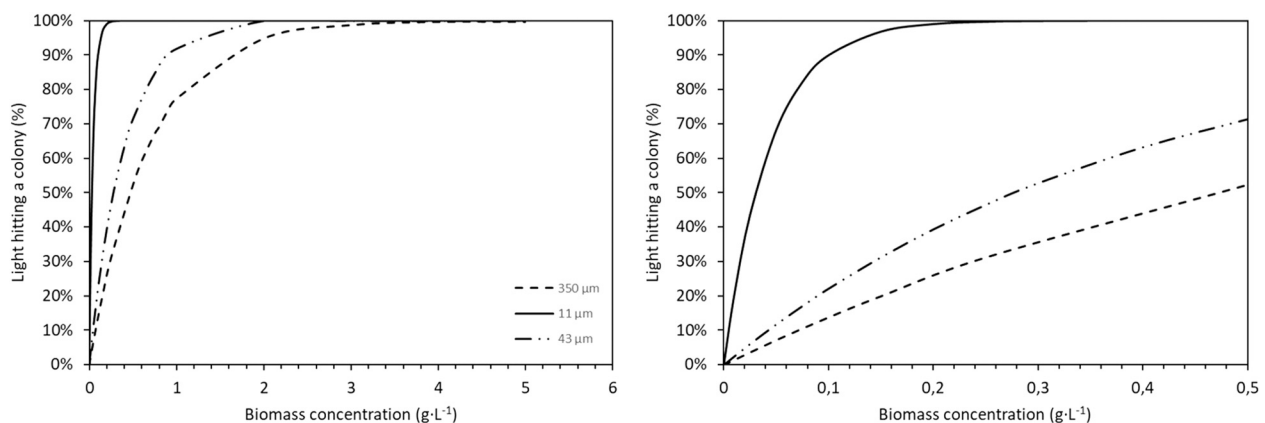


Fig. 6. Probability of light hitting a colony in a 4 cm thick flat-panel PBR as a function of biomass concentration, for mean colony diameters of $350 \mu\text{m}$ (dotted line), $43 \mu\text{m}$ (double dotted line), and $11 \mu\text{m}$ (solid line). The figure on the right is a zoomed-in view of the left figure.

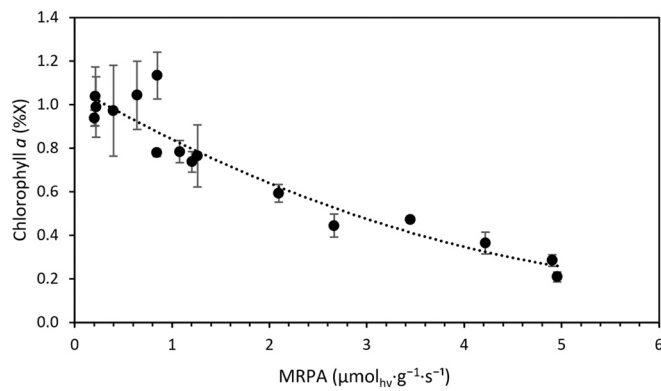


Fig. 7. Relationship between MRPA and chlorophyll *a* for different PDF applied in PBR. Error bars show standard deviations ($n = 3$).

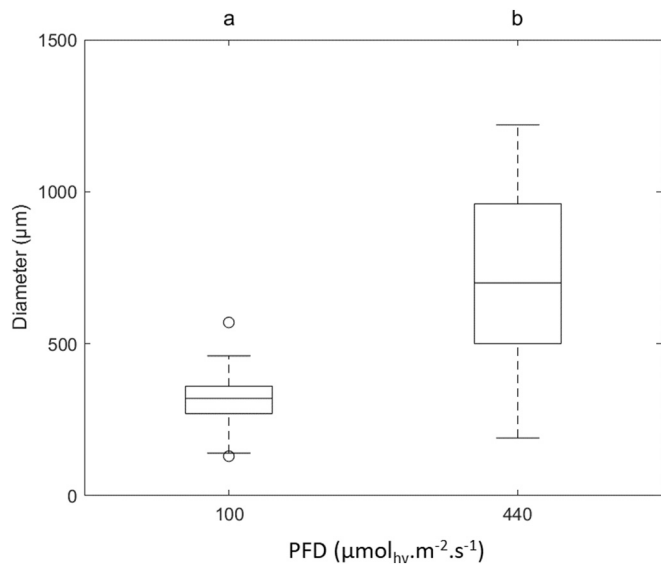


Fig. 8. Difference in diameter distribution for colonies cultivated in torus PBR under PFDs of 100 or 440 $\mu\text{mol}_{\text{hv}}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, shown as boxplots. Different letters indicate that the distributions are significantly different ($p < 0.05$, Mann-Whitney *U* test).

The effect on light absorption efficiency for a flat-panel photobioreactor (PBR) is illustrated in Fig. 4. In the case of small colonies or single cells, light attenuation occurs homogeneously. In contrast, as the colony size increases, biomass suspension becomes more heterogeneous. Three main phenomena are then identified: (i) self-shading occurs within the colony, leading to very strong local attenuation as discussed by [29]; (ii) light passes through the culture volume without interacting with a colony; and (iii) colonies themselves generate strong shading. Note that the light diffusion that can occur in such colony suspensions is not accounted for in this description.

The quantitative differences in light absorption and attenuation can also be estimated by calculating the strain's radiative properties, in this case, the spectral absorption cross-section ($A_{\text{abs},\lambda}$), using the method developed by Kandilian et al. (2016) [44]. By measuring T_{NH} and R_{NH} for specific biomass concentrations, $\bar{A}_{\text{abs},\lambda}$ was estimated for native colonies and HPH-treated colonies, yielding values of $8.0 \text{ m}^2\cdot\text{kg}_x^{-1}$ and $79 \text{ m}^2\cdot\text{kg}_x^{-1}$, respectively. Using these values and Eq. (1), it was possible to estimate the expected light attenuation in the 4 cm deep flat-panel PBR for the two populations, under a PDF of $100 \mu\text{mol}_{\text{hv}}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and for a biomass concentration of $1.5 \text{ g}\cdot\text{L}^{-1}$. For the non-treated colonies with a low $\bar{A}_{\text{abs},\lambda}$, light attenuation was incomplete, with a fluence rate value at

the back of the PBR $G(z = L)$ dropping to $63 \mu\text{mol}_{\text{hv}}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (Fig. 5). In contrast, for the treated colonies with the 10-fold higher $\bar{A}_{\text{abs},\lambda}$, $G(z = L)$ reached $1 \mu\text{mol}_{\text{hv}}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, indicating that nearly all incident light was absorbed over the culture depth.

Part of the explanation could be linked to light passing through the colonies without interacting with them, even though complex light diffusion and absorption are also likely at play. Some light paths along the culture depth undergo strong attenuation due to the presence of dense colonies, whereas others encounter no colonies at all along their trajectory. This effect can be illustrated by estimating the projected shaded area of the colonies, which corresponds to the percentage of incident light interacting with them. For that purpose, the equivalent of a PBR filled with colonies of either 11 μm , 43 μm , or 350 μm in diameter was simulated using a simple probabilistic approach. Results are given in Fig. 6.

The biomass concentration required to reduce the illuminated projected surface to $<1\%$ varied significantly depending on colony size. Note that this does not imply that all the light is absorbed, but rather that all the incident light enters a colony or a cell, and could therefore be attenuated. For the 350 μm colonies, a concentration of 3 to 4 $\text{g}\cdot\text{L}^{-1}$ is necessary, while for smaller colonies with an average radius of 43 μm (HPH-treated), around 2 $\text{g}\cdot\text{L}^{-1}$ is needed. Finally, for small colonies and single cells from the SAG 30.81 *B. braunii* strain with an average radius of 11 μm , a concentration of only 0.2 $\text{g}\cdot\text{L}^{-1}$ is required. This result highlights that, even without taking into account the complex light diffusion and absorption processes in colony cultivation, the heterogeneity of the colonies themselves plays a significant role in the reduced efficiency of light absorption in PBRs. The quantity of light inside and around colonies can differ greatly, with high light intensity available between colonies and substantial shading within them.

3.2. Light sensitivity of BOT-22 and colony size acclimation

Previous research on the relationship between light and colony formation in *Botryococcus braunii* has shown that, when exposed to high light intensities, colonies grow larger [47]. The increase in colony size, along with the accumulation of extracellular secondary carotenoids, reduces the light intensity experienced by most cells within the colony. The increase in colony size has been suggested as a photoprotective strategy [28]. In another study [46], colony size increased as the dilution rate increased. The rise in dilution rates logically reduced biomass concentration, affecting the amount of light received per cell and leading to a similar effect as exposure to higher light intensities. In light of this work and as suggested by the authors, this increase in colony size is likely explained by light stress induced by the decrease in biomass concentration.

The rate of photon absorption (RPA) is a key physical quantity in microalgae cultivation, especially in PBRs. It encompasses parameters such as the PFD and light attenuation within the culture volume, the latter depending on the radiative properties and biomass concentration of the microorganism. More importantly, RPA quantifies the rate at which photosynthetic microorganisms absorb light energy. It serves as a coupling quantity between light absorption and the microorganism's kinetic and metabolic responses, thereby enabling the link between biological performance and various light conditions or PBR designs [48].

In the usual case of a single-cell suspension, the local value of RPA (LRPA) decreases along the culture depth due to light attenuation within the PBR. However, at a given depth, the LRPA can be considered spatially uniform, which facilitates coupling with the kinetic response of the cultivated microalgae. For BOT-22 colonies, however, the situation is more complex. Indeed, as highlighted by Van den Berg et al. (2019) [28], the pigment content of cells within colonies is not uniform, with lower levels in the outer layers and higher levels in the interior, regardless of the colony's position in the photobioreactor depth. As a

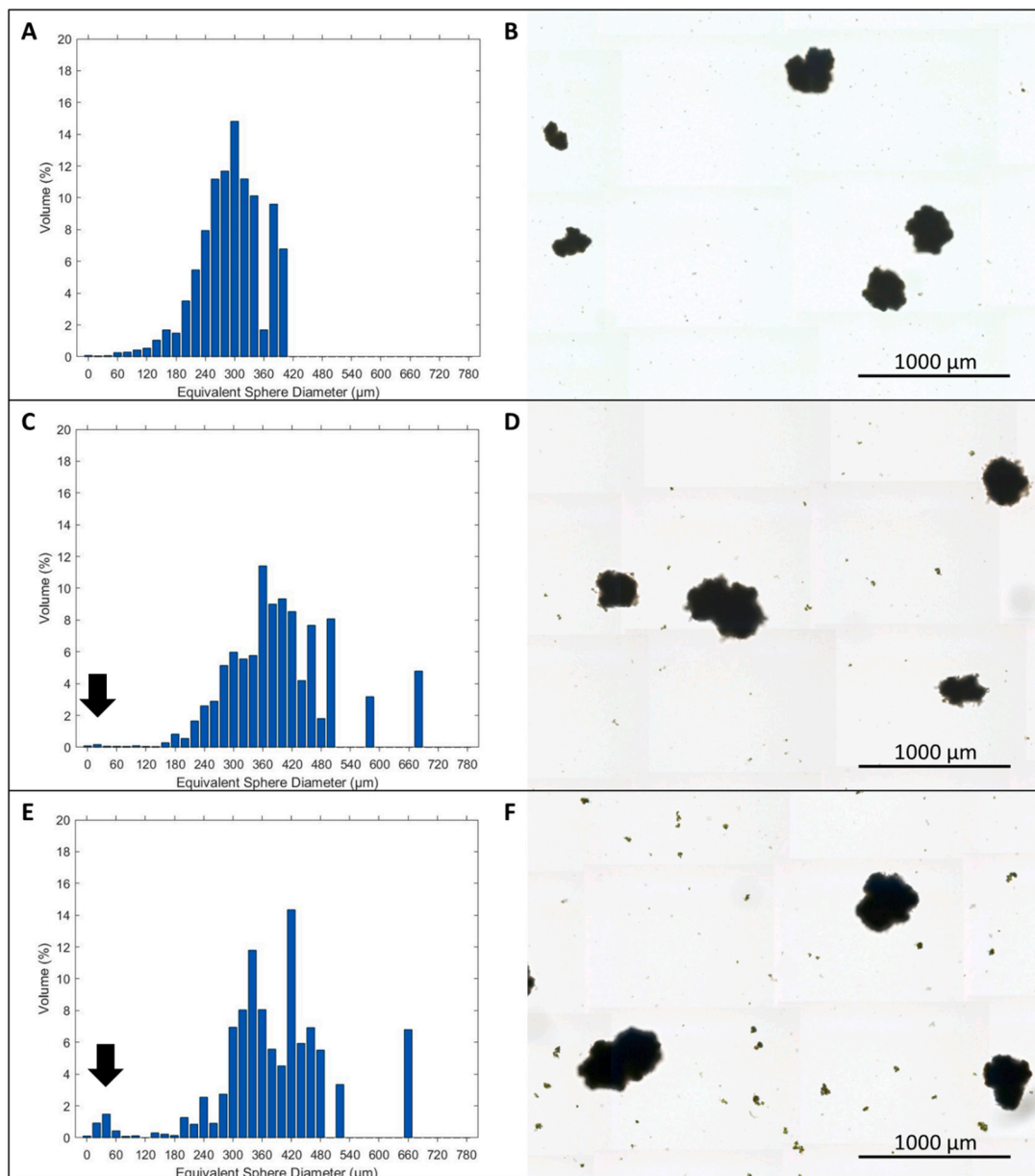


Fig. 9. Time evolution of colony diameter in flask batch culture on an orbital shaker, with aeration of 0.2 vvm and 1 % CO₂, under a PFD of 40 $\mu\text{mol}_{\text{hv}}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The black arrow indicates the presence of small colonies (20–60 μm in diameter). (A, B) day 4; (C, D) day 15; (E, F) day 21. B, D, and F are stitched optical microscope images (X100).

result, cells within a colony may exhibit varying photon absorption rates. In addition, smaller colonies (or single cells) receive less protection from available light and therefore exhibit higher RPA under the same PFD conditions compared to larger colonies.

It is well known that microalgae undergo photoacclimation, resulting, for example, in changes in pigmentation depending on the light received. Bonnanfant et al. (2021) [52] demonstrated that, in a PBR, the pigment content of *Chlorella vulgaris* was directly related to the MRPA value, thereby enabling the prediction of pigment acclimation as a function of light attenuation conditions (as represented by the MRPA). A similar investigation was then conducted here for the colonial strain *B. braunii*, for which the phenomena are certainly more complex. In this work, only chlorophyll *a* content was used, as the presence of secondary carotenoids in the extracellular matrix of *B. braunii* prevents reliable quantification of total carotenoids. The method used targets primary carotenoids, but their absorption spectra overlap with those of secondary carotenoids, making it difficult to distinguish and quantify each

fraction accurately. Different PFD values were therefore applied in the torus-shaped PBR during batch cultivation, with the PFD increased every 3 days, allowing for variations in MRPA. This gradual change in light intensity enabled the cells to acclimate over time, adjusting their pigment content accordingly.

Fig. 7 illustrates the relationship between chlorophyll *a* content and MRPA. Two distinct zones can be observed: the first zone, with a low MRPA ranging from 0 to 3 $\mu\text{mol}_{\text{hv}}\cdot\text{g}^{-1}\cdot\text{s}^{-1}$, emphasizes pigment decrease with increasing MRPA; the second zone, between 3 and 5 $\mu\text{mol}_{\text{hv}}\cdot\text{g}^{-1}\cdot\text{s}^{-1}$, where much more limited reduction in chlorophyll *a* content is observed despite increasing MRPA. A similar behavior was observed by Bonnanfant et al. for *Chlorella vulgaris*. This was attributed to increased light stress at high MRPA values, corresponding to lower pigment content (as seen in the second zone here). In the present study, a significant decrease in chlorophyll *a* content, as well as a moderate decline in biomass was observed in the PBR for MRPA values exceeding 5 $\mu\text{mol}_{\text{hv}}\cdot\text{g}^{-1}\cdot\text{s}^{-1}$, indicating that an MRPA above 5 $\mu\text{mol}_{\text{hv}}\cdot\text{g}^{-1}\cdot\text{s}^{-1}$ could

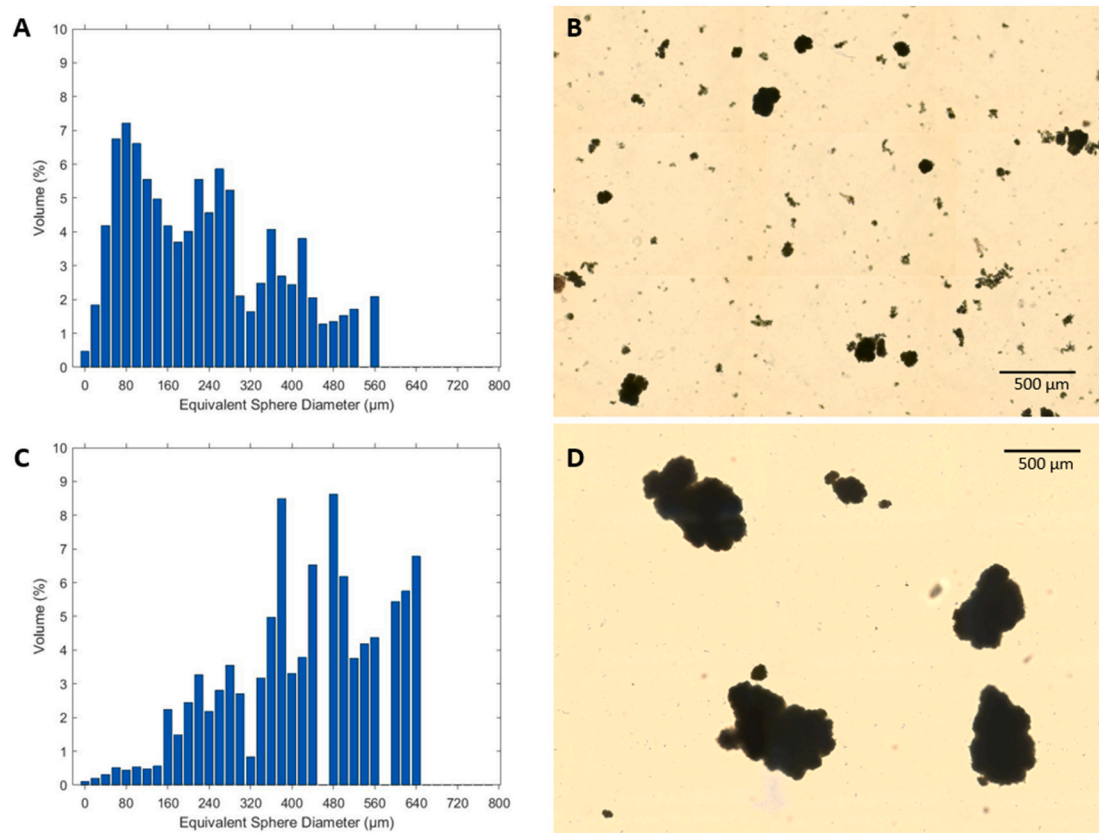


Fig. 10. Comparison of the colony size distributions and microscopic images of the two pre-cultures used for PBR inoculations. A, B: Inoculum cultivated at $50 \pm 10 \mu\text{mol}_{\text{hv}}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ over multiple passages. C, D: Inoculum cultivated at $80 \pm 10 \mu\text{mol}_{\text{hv}}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ over multiple passages.

lead to cell death in *Botryococcus braunii* BOT-22. This range of MRPA is low compared to *Chlorella vulgaris*, ranging from 0 to $25 \mu\text{mol}_{\text{hv}}\cdot\text{g}^{-1}\cdot\text{s}^{-1}$ as shown by Bonnanfant et al. (2021) [52]. This difference, along with the low maximum pigment content in the BOT-22 strain, estimated at around 1.8 % of biomass compared to other microalgae (usually $>5\%$, as in *Chlorella vulgaris*), supports the idea of a strong photosensitivity in *Botryococcus braunii* BOT-22. Consequently, it also supports the idea that the formation of colonies may be part of a protective strategy against light stress, allowing the cells to better manage exposure to variable light conditions due to the protective role of the colony structure. As shown in Fig. 8, applying both lower light ($100 \mu\text{mol}_{\text{hv}}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and higher light ($440 \mu\text{mol}_{\text{hv}}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) to the same PBR with similar biomass concentrations confirms that *Botryococcus braunii* increases its colony size as part of its acclimation strategy to manage increased light intensity.

As another illustration, Fig. 9 shows the evolution of colony size distribution under low light ($40 \mu\text{mol}_{\text{hv}}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). After 21 days of culture (i.e., flask orbital shaken batch culture with aeration of 0.2 vvm and 1 % CO_2), the appearance of small colonies measuring between 20 and 60 μm in diameter was observed. No nutrient limitation occurred (i.e., the medium used was designed to support up to $1.5 \text{ g}\cdot\text{L}^{-1}$ of biomass concentration, while only $0.37 \text{ g}\cdot\text{L}^{-1}$ was reached here). Furthermore, the pigment content remained stable throughout the cultivation, averaging around 1.8 % of dry biomass. These results tend to confirm that low-light acclimation leads to smaller colonies compared to medium- and high-light conditions, as also noted by Van der Berg et al. (2019) [28]. It tends to further demonstrate that this size acclimation can be reversible, at least over a period of 21 days.

It was observed that under low MRPA, small colonies could be generated even when starting from larger ones. In contrast, only large colonies were observed under high MRPA. This difference might be explained by heterogeneous light absorption within the culture volume.

As shown previously, this uneven light absorption resulted in higher RPA values along light paths with few or no colonies, thereby preventing the development of the more photosensitive, smaller colonies when the PFD was too high. Excessively high RPA values hindered the growth of small colonies due to photo-inhibition and could even lead to cell death, allowing only larger colonies to thrive. Conversely, when MRPA was sufficiently low, small colonies could develop successfully.

3.3. Comparison of cultivation performance between medium-sized colonies (MC) and large colonies (LC) in a torus photobioreactor

The pre-cultures of BOT-22 used were exposed to two different light conditions, $50 \pm 10 \mu\text{mol}_{\text{hv}}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and $80 \pm 10 \mu\text{mol}_{\text{hv}}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, generating different MRPA conditions. These pre-cultures were cultivated for multiple generations under these conditions, leading to the formation of “large colony” (LC) and “medium colony” (MC) lineages, as described in Fig. 10. These two populations are distinctly different, with the average size of the LC being 434 μm , and the MC 145 μm . These two pre-cultures, characterized by different colony sizes, were then used to inoculate two similar torus-shaped PBRs.

After inoculation, the PFD was gradually increased to $100 \mu\text{mol}_{\text{hv}}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ for both cultures, and continuous cultures were initiated after 9 days. As shown in Fig. 11, the two colony size distributions derived from the pre-cultures were maintained, as supported by the size distribution and photographs of both PBRs at similar biomass concentrations. In the case of MC, colony size appeared stable, whereas for LC, a progressive increase in size was observed.

When comparing culture performances (Table 1), the volumetric productivity of the MC PBR in both biomass and hydrocarbon content was approximately 34 % and 74 % higher, respectively, than in the LC PBR. This suggests that the difference in performance may be attributed to colony size and their capacity to absorb light efficiently. Another

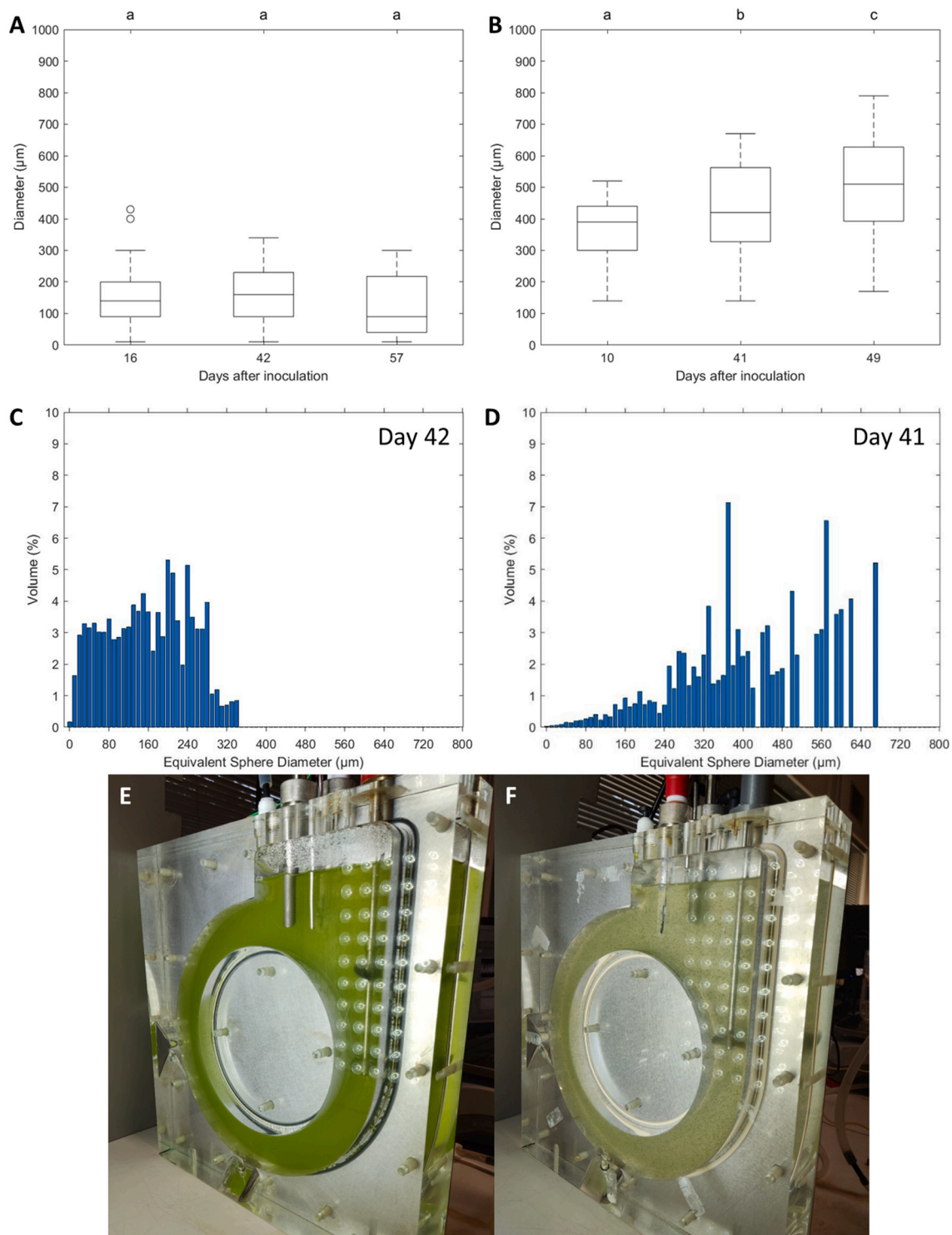


Fig. 11. Comparative analysis of the colony size distribution evolution in Torus PBRs at $100 \mu\text{mol}_{\text{phv}}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ inoculated with the MC pre-culture (A) and LC pre-culture (B). Histograms illustrate the size distribution differences between MC (C) and LC (D). E and F show photographs of the PBRs cultivating MC at $0.69 \text{ g}\cdot\text{L}^{-1}$ and LC at $0.74 \text{ g}\cdot\text{L}^{-1}$, respectively. Different letters indicate that the distributions are significantly different ($p < 0.05$, Mann-Whitney U test).

hypothesis could be a limitation of nutrient diffusion within the dense colonies, as is often observed in biofilms. The extracellular polymeric substances (EPS) matrix, composed mainly of polysaccharides and proteins, could indeed create barriers to nutrient transport [49].

One limitation in the comparison is in the application of different dilution rates, 0.08 d^{-1} for MC and 0.05 d^{-1} for LC. Although the exact impact on productivity cannot be determined, the 0.05 d^{-1} dilution rate was chosen to avoid the risk of washout, which had been observed at a

rate of 0.08 d^{-1} in another LC culture in the same PBR. Note that this last observation tends to support the direct relation between colony size, light absorption, and light sensitivity of *B. braunii*. For large colonies, the culture exhibits heterogeneity, with colonies providing substantial photoprotection while the inter-colony spaces remain highly exposed to light. Small colonies or single cells could then not thrive in these highly light-exposed inter-colony areas, leading to growth predominantly within the large colonies. This is also suggested by the progressive

Table 1

Cultivation performance in a photobioreactor illuminated at a PFD of 100 $\mu\text{mol}_{\text{hv}}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ for two continuous cultures of BOT-22 with different colony sizes. Identical culture conditions were applied. The dilution rate was maintained for at least one month. Errors represent the standard deviation of three technical replicates, with uncertainty propagation when applicable. MC: medium-sized colonies; LC: large colonies; MRPA: medium rate of photon absorption.

Photobioreactor	MC	LC
Average colony diameter (μm)	145	434
Hydrocarbon content ($\text{g}\cdot\text{gX}^{-1}$)	0.265 ± 0.007	0.203 ± 0.025
Biomass productivity ($\text{g}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$)	0.123 ± 0.004	0.092 ± 0.003
Hydrocarbon productivity ($\text{g}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$)	0.033 ± 0.001	0.019 ± 0.001
Biomass productivity ($\text{g}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$)	4.92 ± 0.16	3.68 ± 0.12
Hydrocarbon productivity ($\text{g}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$)	1.30 ± 0.04	0.76 ± 0.04
Dilution rate (d^{-1})	0.08	0.05
MRPA estimated ($\mu\text{mol}_{\text{hv}}\cdot\text{g}^{-1}\cdot\text{s}^{-1}$)	1.63 ± 0.06	2.03 ± 0.07

increase in colony size observed in the LC PBR over time in Fig. 11. As shown in Fig. 9, the reversion of large colonies to small ones was also observed under lower light conditions, supporting this hypothesis. As a consequence, cultures with large colonies cannot utilize light as efficiently as smaller colonies due to light losses between colonies and significant internal shading, leading to lower biomass productivity.

For the MC pre-culture, the lower light conditions seem more favorable for the emergence and growth of small colonies, which are then more efficient in light absorption in the PBR, resulting in better biomass and hydrocarbon productivities. However, as seen in Fig. 11, when MC was cultured under the same conditions as LC, the small colonies did not disappear. This is likely due to the more effective mutual shading effect generated by these small colonies, allowing for uniform light attenuation that provides photoprotection to the entire culture. Nonetheless, BOT-22 is likely a light-sensitive strain, requiring careful cultivation under low MRPA, typically between 1 and 3 $\mu\text{mol}_{\text{hv}}\cdot\text{g}^{-1}\cdot\text{s}^{-1}$.

It can be hypothesized that if the colony concentration reaches a sufficient level in a PBR (for a PBR with 4 cm thickness as used here, this would correspond to a minimum biomass concentration around 4 $\text{g}\cdot\text{L}^{-1}$) then the shading effect among the colonies may approach that of classic mutual shading, resulting in a relatively homogeneous light attenuation. Under these conditions, the re-emergence of small colonies, which are more efficient in light capture, is expected. Whether this phenomenon occurs due to selective effects or acclimation remains unclear.

3.4. Recommendation for the cultivation of light-sensitive colonial microalgae

As discussed, *Botryococcus braunii*, specifically the BOT-22 strain, presents unique characteristics that make its cultivation and the achievement of high productivity challenging. Although the primary aim of this work was not to optimize productivity directly, some recommendations can be made.

First, a significant difference in light absorption was observed, with smaller colonies absorbing light more effectively than larger colonies at equivalent biomass concentrations and pigment compositions. Furthermore, it was found that the biomass and hydrocarbon productivity of medium colonies (MC) were approximately 34 % and 74 % higher, respectively, than those of large colonies (LC). This suggests that cultivating BOT-22 in the form of small or medium colonies may be advantageous for maximizing productivity. It is worth noting, however, that cultivating larger colonies may also hold value for specific applications, such as enhanced resistance to predators [50] or specialized hydrocarbon extraction processes, such as the so-called milking process [51].

The low mean photon absorption rates observed in the culture, along with the low pigment content compared to other strains such as *Chlorella vulgaris*, suggest that this strain may be photosensitive. Therefore, maintaining either a sufficiently low light intensity or a high biomass

concentration is recommended to keep MRPA within the range of 1 to 3 $\mu\text{mol}_{\text{hv}}\cdot\text{g}^{-1}\cdot\text{s}^{-1}$, as identified in this article. Pre-culture conditions also appear to play a crucial role in this context. Indeed, maintaining this low MRPA throughout the pre-culture phase is necessary to ensure that light levels in the inter-colony zone do not become too high. Under such conditions, smaller colonies can grow effectively, providing even more light attenuation, which allows for increased PFD as biomass concentration rises. This enables the use of high PFD values that could lead to larger productivity without inducing light stress. Monitoring colony size could also serve as a useful indicator of the degree of light attenuation. Indeed, larger colonies are less likely to form, as internal colony shading becomes unnecessary when light is sufficiently attenuated within the culture depth.

4. Conclusion

This work has provided key insights into the relationship between light absorption, colony size, and growth in *Botryococcus braunii*. Managing colony size in photobioreactors through light intensity adjustments appears to be a promising strategy to optimize both biomass and hydrocarbon productivity. A deeper understanding of colony size control could also improve both destructive and non-destructive extraction of valuable compounds such as hydrocarbons, exopolysaccharides, and carotenoids. Further investigations into light absorption in colonial photosynthetic microorganism cultures may enable more accurate system modeling, facilitating the identification of optimal colony sizes and corresponding light intensities. Finally, future research could focus on the molecular mechanisms underlying colony size regulation and photoprotective responses, with the goal of improving cultivation processes and addressing the slow growth rate of *B. braunii*.

CRedit authorship contribution statement

Samy Kemel: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Luc Marchal:** Writing – review & editing, Supervision. **Widya Nur Habiba:** Writing – review & editing, Investigation. **Helene Marec:** Writing – review & editing, Resources. **Delphine Drouin:** Writing – review & editing, Resources, Formal analysis. **Olivier Gonçalves:** Writing – review & editing, Supervision, Methodology, Formal analysis. **Jeremy Pruvost:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Declaration of competing interest

Authors declare there are no potential financial or other interests that could be perceived to influence the outcomes of the research.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.algal.2025.104176>.

Data availability

Data will be made available on request.

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