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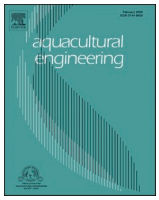

Chlorella vulgaris cold preservation (4°C) as a means to stabilize biomass for bioreactor inoculation: A six-month study

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ABSTRACT

Chlorella vulgaris cells were maintained over six months (or tentatively) using three protocols: two-week subculturing (positive control), storage at 4°C, and simple abandonment (negative control). Cultures were monitored by their optical and cell densities over the trial period. Cells were characterized by their size, pigment profile, photosystem II status (OJIP test), electron transport rate assay (light curve), and lag phase duration when regrown. The abandoned cultures quickly showed cells deviating from their nominal state (increased size, a loss of their pigments, a negative alteration of their photosynthetic capacity, and an extended lag phase when inoculated into fresh medium). Frequent subculturing yielded reasonably stable performances. Yet, our experience showed that uncontrollable factors (human errors, lack of communication between teams) could expose the cultures to unfortunate incidents. 4°C preservation allowed the cells to have a constant size and a slightly increased, yet stable, pigment profile associated to a dark acclimation (+12 % total chlorophyll). Finally, regrowth tests demonstrated that 4°C preservation induces slightly improved performance (lag phase duration reduced by 9.5 %) than frequent subculturing. Those findings advocate for the use of 4°C preservation to reduce cell maintenance work and conserve a pool of cells in a similar state to be used as repeatable inoculum for larger-scale experiments while nullifying otherwise batch-to-batch variation effects. Subculturing work can be reduced from once every two weeks to once every six months at least.

1. Introduction

Over the past century, the human population has dramatically increased, and the quality of life made substantial improvements. Nevertheless, these evolutions have put great stress on our ecosystem (Díaz et al., 2019), among which pressures on fossil fuels, water, arable lands, and biodiversity might be the most dire. With the objective of mitigating this situation, microalgae emerge as a tool capable of helping humanity reduce its footprint. Indeed, they are able to produce many molecules with applications ranging from food and feed to advanced compounds used in the cosmetic and pharmaceutical industries (Rizwan et al., 2018; Levasseur et al., 2020), while delivering ecosystemic benefits (CO_2 fixation (Molitor et al., 2019), phosphate fixation (Brown and Shilton, 2014), nitrogen fixation (Hellebust and Ahmad, 1989), effluent bioremediation (Sasi et al., 2020) …). Still, before they realize the full extent of their promises, numerous scientific challenges remain to be addressed.

Acknowledging this need for research, scholars strive to create new knowledge aiming at fostering microalgal biotechnology. In this view, laboratory-scale trials are still the basis of most scientific investigations. This type of trial implies several tedious steps, only supporting the investigations, with limited added value that represents a burden for scientists. Among them, culture maintenance is of note. Maintaining always available, ideally in the exponential phase, cultures of microalgae represents a sizable load (financial and human cost). Indeed, one has to constantly prepare fresh medium, sterilize glassware, passage culture, and discard the cells. Therefore, substantial gains are to be harnessed by limiting this activity. This view is shared by many researchers (Abreu et al., 2012; Sánchez-Saavedra et al., 2019), and mollusk cultivators who also have to maintain a neverending fresh stock of microalgae (Núñez-Zarco and Sánchez-Saavedra, 2011; Sánchez-Saavedra, 2006).

On the academic side, laboratory scientists are often left with cryopreservation as the only option backed by the literature. In this

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perspective, the tremendous work of Day is to be acknowledged (Day, 2007). Cryopreservation is a long and species-dependent process. To ensure its success, one should first subculture the cells in low temperature and low light conditions before harvesting (and optionally concentrating) them in the exponential phase. Then, a cryoprotectant (precise quantities of methanol or DMSO) is to be added to limit osmotic pressure and preserve the cell membrane over the course of the process (Cañavate and Lubian, 1994). In the next step, the cells are to be cooled at a precise rate in a single or dual-step protocol (sometimes an intermediary phase is added around −40°C for 15 min to allow dehydration before rapid cooling to −196°C). In these conditions, the cells can be stored for an extensive period of time, usually several years. Upon need, they are thawed, following an adequate procedure (5 min in 40°C water, classically), and subcultured again. Yet, the first stage of subculturing can be long as the cells must be allowed some time to recover (at least one day in the dark before exposure to dim light) (Buhmann et al., 2013; Abreu et al., 2012). While this protocol is economically efficient and avoids genetic drifting, it requires specific equipment and particularly skilled technicians. Furthermore, the cells cannot be considered as readily available.

Acknowledging this limitation, Chen developed a method to immobilize *Scenedesmus quadricauda* cells in alginate beads, which can be stored in a regular fridge (4°C) for three years (Chen, 2001). Even after this storage duration, the cells showed limited morphological evolution (reversible disappearance of their pyrenoid) and were able to regrow to provide satisfactory phycoremediation performances (pH, dissolved oxygen, and ammonium stabilization in a fish tank). Expanding this work, Sánchez-Saavedra *et al.* optimized alginate beads preservation (3.4 years at 4°C) for cyanobacterium *Synechococcus elongatus*, with the aim to reduce laboratory maintenance work and limit genetic drifting (Sánchez-Saavedra et al., 2019).

Moving away from academic investigations, another microalgal biotechnology field that developed positive low-temperature preservation of microalgae is fish and mollusk cultivation. The motivation originates from the fact that live cells have a higher nutritional potential than frozen alternatives. Here again, Sánchez-Saavedra's pioneering work is to be acknowledged. Preservation durations are usually a few months (4–16 weeks at 4°C), as younglings rearing lasts up to two months, and studied species are mainly diatoms. Findings are diverse and sometimes contradictory, highlighting that, like cryopreservation, low-temperature procedures are also species-dependent. Some exhibit nominal regrowth (Sánchez-Saavedra, 2006), while others have a rate divided by two to six (Sánchez-Saavedra and Núñez-Zarco, 2012). In terms of macronutrient composition, both stable (Welladsen et al., 2014; Sánchez-Saavedra, 2006) and time-fluctuating protein contents are observed (Sánchez-Saavedra and Núñez-Zarco, 2012). Diving deeper, the amino acid composition itself can be altered, as the cells could use methionine to synthesize dimethylsulfoniopropionate, a natural cryoprotectant. In the same sense, diatom lipid contents have been reported to occasionally increase (Sánchez-Saavedra, 2006; Sánchez-Saavedra and Núñez-Zarco, 2012), allegedly to foster poly-unsaturated fatty acids, which help to maintain membrane fluidity. Still, some findings contradict this view, with a 70 % drop for the diatom *Melosira dubia* (Welladsen et al., 2014).

While the economic rationale behind aquafeed led to substantial research on diatoms, some authors also worked with green microalgae. Among them, two reports are of note. Welladsen *et al.* showed that *Dunaliella tertiolecta* and *Nannochloropsis* sp. handled relatively well an 8-week storage period at 4°C (Welladsen et al., 2014). Focusing on *Nannochloropsis gaditana*, Camacho-Rodriguez *et al.* demonstrated that, after process optimization, low-density (5 g/L) and concentrated (150 g/L, paste-like) culture could be stored for 4 months at 4°C without significant evolution (excluding pigment content and photosystem II quantum yield which evolves within weeks).

Finally, as the application of microalgae as commercial food emerges need to increase food-intended microalgae shelf-life arises. Castelló *et al.* evaluated the conservation of microalgae as a food product over 2 months Castelló et al. (2018)). They tested several food quality indicators, such as bacterial concentration (CFU/mL) or color (in CIE L*a*b* color space). Nevertheless, the fact that the authors advise storage at 4°C storage further suggests cold storage as a potential means to ensure stability of the cells.

Going back to the first analysis that live and readily available microalgal cells maintenance is tedious and expensive and acknowledging that, despite satellite works having been led, no easy-to-implement solution has been documented to date, this work investigates the conservation at a positive low-temperature (4°C) of *Chlorella vulgaris* for scientific research purposes. *Chlorella vulgaris* was chosen as the model organism for this study as it is Generally Recognized As Safe (GRAS status) by the US FDA and considered safe food by the European Food Safety Authority. Furthermore, its wide biotechnological potential has been acknowledged (Safi et al., 2014), and, from a down-to-earth perspective, it might be the most widespread strain in laboratory microalgal studies. This work has two objectives. The first one is a reduction of cells maintenance work by deploying an extremely easy and readily implementable technique, *i.e.*, fridge storage. The second is the conservation of a pool of cells in a similar state so that different trials could be led with this biomass at different times while avoiding batch-to-batch variation (*e.g.*, in the case of several serial tests in a photobioreactor system).

2. Materials and methods

2.1. Microalga strain & culture medium

The strain used in the study was *Chlorella vulgaris* (species SAG 211–11b, purchased from the Culture Collection of Algae at Göttingen University, Göttingen, Germany). The cells were maintained, amplified, and cultivated in suspension. The medium used for cell maintenance was a Bold Basal Medium with three times the nitrogen load (referred to as B3N medium (Andersen and America, 2005)). This medium was chosen because it is chemically defined and rich in nitrogen while not inducing substrate inhibition. Finally, cell culture was conducted in an orbitally shaking incubator (Infors HT Minitron, 100 rpm, 30 μmolPhotonPAR/m^2/s - continuous, measured with LICOR LI 250 A & LI-190R sensor -, 25°C, under air with 1 % CO_2 - continuously supplied -). The light intensity was intentionally chosen at a low value to increase cell pigment content and ease the detection of potential differences arising over time. In addition, CO_2 was added to ensure high cell density and, therefore, limit the volume to be sampled to reach the cell mass requirement for pigment assays.

2.2. Tested conditions

Three preservation conditions were tested. The first one was the classical maintenance protocol in our laboratory: every two weeks, cells are passaged into fresh medium (1/100 passaging, 50 mL medium in 250 mL Erlenmeyer) and placed back in the incubator for two weeks (conditions described above). This two-week period was chosen as, from our experience and under the abovementioned conditions, it allows passage cells in their exponential phase (cell concentration of 0.3 g_{DW}/L), as advised in the literature (Welladsen et al., 2014; Day, 2007). This condition is referred to as *subcultured* hereinafter (positive control).

The second procedure was placing the cells in a sealed bottle (50 mL of culture in 60 mL glass bottles shaded from light using aluminum foil) two weeks after their passaging and storing them at 4°C in the dark. Harvesting cells in the exponential phase ensures that they have excess nutrients, allowing them to acclimate to cold conditions (Welladsen et al., 2014). This condition is referred to as *4 ° C* hereinafter.

The third condition represents an extreme procedure in which the intent was to serve as a negative control. Like the two other conditions, the cells were passaged from a two-week-old mother culture. Still, cells

were not further maintained and left within the incubator until sampling and evaporation exhausted the flask contents. This condition is referred to as *neglected* hereinafter (negative control).

Sampling (3 mL) was carried out on a weekly basis at the beginning, then frequency was adjusted to extend the trial duration. For each sample, cell count, cell size, optical density at 750 nm, and photosynthetic apparatus status were analyzed. Once every two weeks, cells pigment profile was analyzed. Once every six weeks, regrowth tests were carried out. The three conditions were carried out in biological duplicate (A and B). For each line, cells dispatched between the three tested procedures originated from the very same culture to ensure an identical starting point. One should note that the choice of resorting to biological duplicate was born of the will to keep the time committed to cells manageable. Indeed, performing the subsequently introduced assays is relatively time-consuming. Nevertheless, this choice prevents the use of statistical testing (such as ANOVA tests) in a rigorous manner. Therefore, the results are presented either with two curves (replicate A and B) or as the average of the two duplicates. The associated error bars represent the spread and not the standard deviation, which would have been smaller.

2.3. Cell count and size

Negative (cell lysis) and positive (allegedly because of heterotrophy) cell count evolutions, as well as size changes, have been reported by various authors over the course of their 4˚C preservation protocols (Sánchez-Saavedra, 2006; Sánchez-Saavedra and Núñez-Zarco, 2012; Camacho-Rodríguez et al., 2016). Two monitoring methods have therefore been deployed on a weekly basis. First, cell suspension absorbance at 750 nm has been recorded as it is an easy-to-acquire proxy of cell density (Griffiths et al., 2011). Second, the suspensions were analyzed using a particle counter (Beckman Coulter Multisizer 4), which determines both suspension cell density and cell size distribution.

2.4. Cell pigment profile

The second outcome that was monitored was the cell pigment profile as some authors reported its evolution (Camacho-Rodríguez et al., 2016; Sánchez-Saavedra et al., 2019). Therefore, once every two week, the cells pigment content was analyzed. To do so, the cells were washed twice by centrifugation (4˚C, 11,000 rpm, 10 min). Biomass was then frozen and freeze-dried (1-day primary drying, 1-day secondary drying, Christ alpha 1–2 LD +, condenser temperature −40˚C). Biomass powder was stored in the dark at −20˚C before pigment extraction.

Extraction was carried out by homogenizing 1 mg of freeze-dried microalgae powder in 5 mL pure methanol using MP Biomedicals Fast-Prep42 bead beater. The suspension was cooked for 20 min at 60˚C (shaded from light) (Porra, 1990). The liquid was then filtered (0.22 μm). 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 $\AA$) 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. The injection volume was 5 μL, and the total run analysis was 40 min. 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. Pigment 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 %. For each sample, the five pigments of interest (chlorophyll *a*, *b*, lutein, violaxanthin, and zeaxanthin) were reported systematically. 'N.A.' was used whenever one of them could not be detected or quantified.

2.5. Photosynthetic apparatus status

2.5.1. Transient fluorescence assay

Every two weeks, photosynthetic apparatus status was qualified at two levels, first at the photosystem II (PSII) level and second at the whole electron transport chain level. To do so, fresh samples were placed in a dark enclosure for 15 min immediately after their withdrawal from the flasks. Once dark-adapted, photosynthetic apparatus status was qualified using transient variable chlorophyll fluorescence readings (AquaPen 110-C), also referred to as OJIP tests. Before processing them, the signals were checked for potential saturation. Then, the readings were processed following Strasser's guidelines (Strasser et al., 2000). First, the general dynamic of the fluorescence signal was analyzed (succession of OJIP stages). Afterward, the focus was directed toward the Reaction Centers (RC) condition. The three primary parameters of this stage of the analysis were: absorption per reaction center (ABS/RC), trapping per reaction center (TR_0/RC), and transferring per reaction (ET_0/RC). The first one (ABS/RC) accounts for the quantity of energy captured by antennae associated with a working reaction center. The second one (TR_0/RC) focuses on the fraction of this energy that is directed toward the core of the photosystem II. Consequently, the dissipated amount of energy can be computed as ABS/RC - TR_0/RC. The last one (ET_0/RC) relates to the amount of excitation leaving the PSII down the electron chain (towards the PQ pool, the cytochrome $b_{6/f}$, and the PSI).

2.5.2. Light curve assay

Following the OJIP tests, samples were allowed another 15-min period to dark-adapt again. Then, a light curve assay, also referred to as PI or PE curve, was led. To obtain the curves, quantum yield under different illuminations was multiplied by the incident light intensity (Genty et al., 1989). This method relies on two assumptions: proper functioning of the PSII (assessed by Fv/Fm ratio here) and similar light absorption per reaction between compared samples (validated by the pigment content similarity). As it exposes microalgae to different light intensities for a longer period of time, this assay allows to probe the functioning and output of the photosynthetic units as a whole, not only the PSII. Using a mathematical model, it is possible to identify several biological parameters using the experimental points (Fig. 1). 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) -.

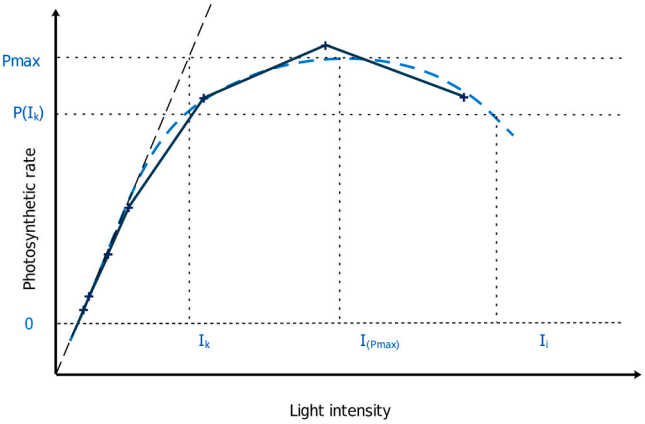

Fig. 1. Illustration of an instantaneous light curve obtained with by fluorometric assay and the associated key values (Levasseur et al., 2023).

2.6. Regrowth tests and lag phase determination

Every six weeks, the cells were inoculated into fresh B3N medium (1/100 passaging - ensuring similar initial cell count, as shown by the results on preserved cultures cell density -, 50 mL medium in 250 mL Erlenmeyer) and placed back in the incubator. These cultures were monitored twice daily by optical density at 750 nm readings. Two outcomes were tracked: regrowth (yes or no) and the duration of the lag phase with respect to control (cells subcultured every two weeks). The lag phase durations were obtained by using the duration the cultures needed to reach an optical density of 1.0 (linearly interpolated between the two closest readings).

3. Results and discussion

Cultures were sampled on a weekly basis for the first twelve weeks.

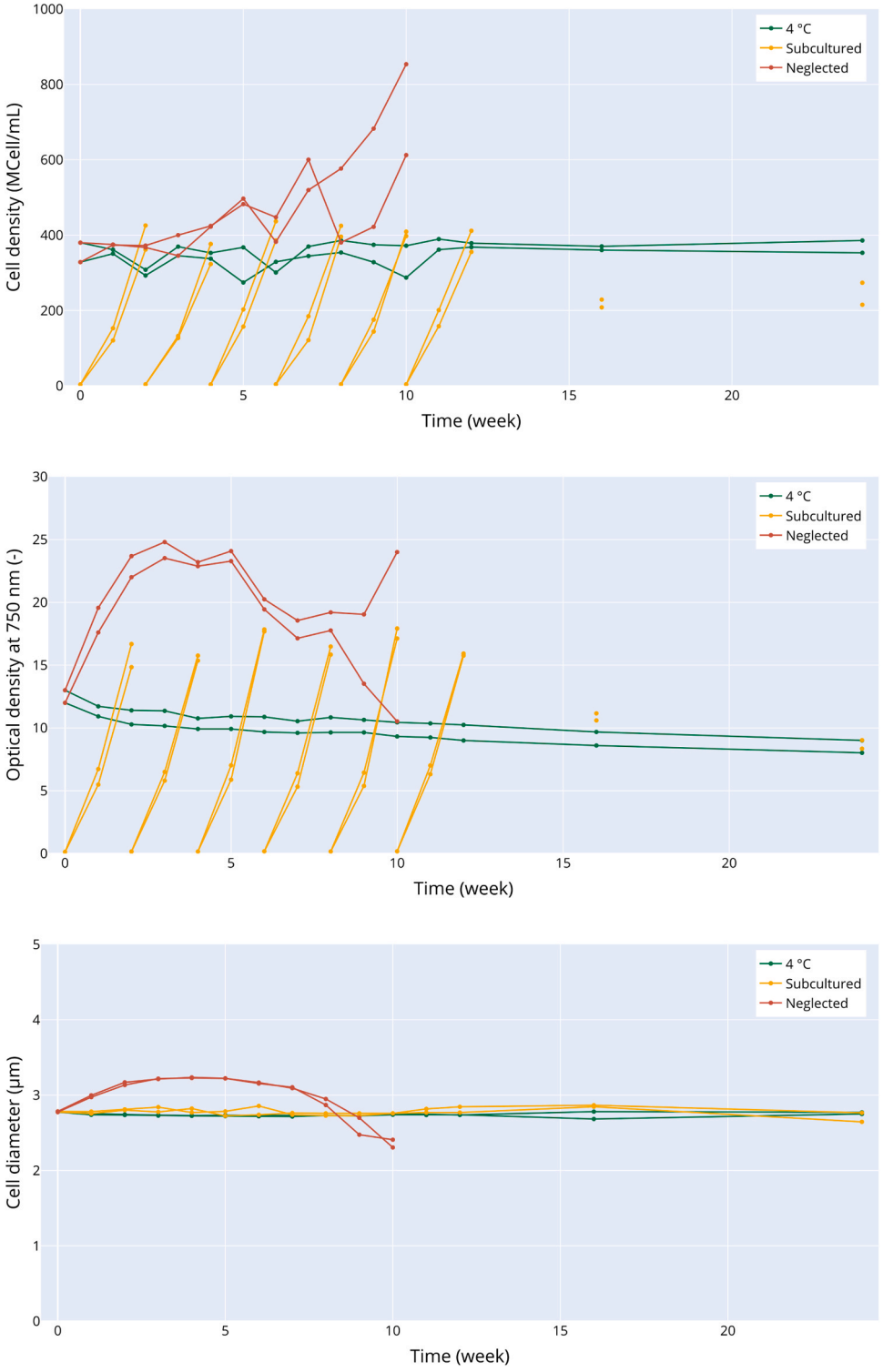

Fig. 2. Top - Cell density. Middle - Culture optical density at 750 nm. Bottom - Cell size. Discontinued lines and points are used to present the results for the subcultured, as passaging was carried out every two weeks. Each duplicate graphed individually.

As no major discrepancy was observed between the cells stored at 4°C and the subcultured ones, the sampling frequency was subsequently decreased to extend the trial duration (sampling on weeks 16 and 24). On the contrary, the neglected cultures experienced evaporation (about 5 % per week), which limited the trial duration to ten weeks for this condition. In addition, the last samples withdrawn from these cultures could be considered of questionable representativity as some cells adhere to the flask walls.

3.1. Cell count and size

Fig. 2 presents the cell density (in MCell/mL), the culture optical density at 750 nm, and the cell size. The first comment is qualitative. The cultures preserved at 4°C showed a remarkably stable cell density, with a slightly decreasing optical density. The subcultured cells exhibit a large range of variation in terms of cell and optical densities. It is inherent to this maintenance protocol, which implies regular dilution by

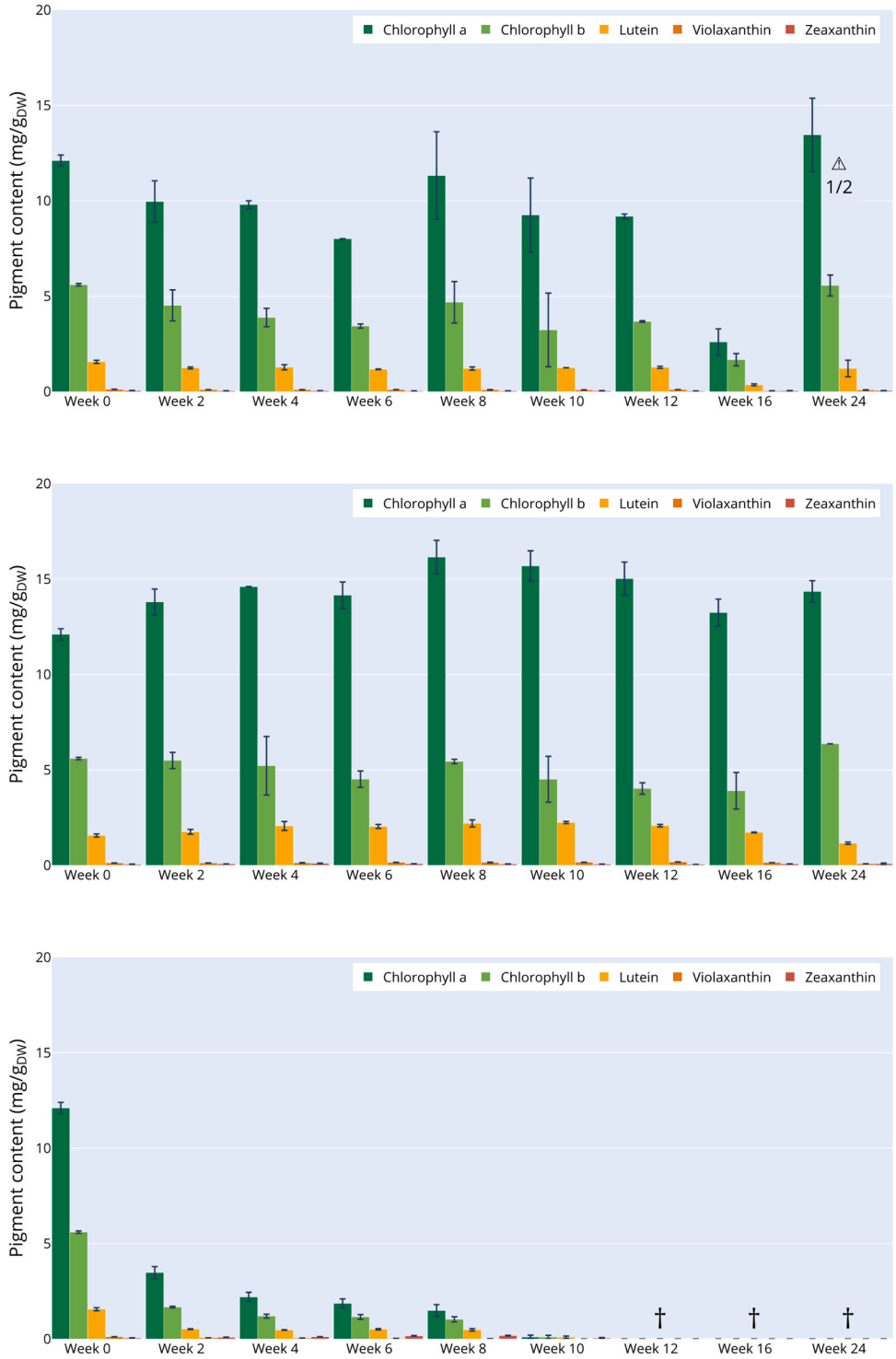

Fig. 3. Top - Pigment profiles of the subcultured cells. May the kind reader please note that the values on week 24 were divided by 2 for aesthetic reasons. Middle - Pigment profiles of the cells stored at 4°C. Bottom - Pigment profiles of the neglected cells. † data missing because of culture exhaustion. Data presented as the average of the two replicates, error bar covering the spread.

1/100 of the culture. Still, cell count, and optical densities are also quite repeatable but marginally higher than the one at 4°C at the end of the amplification. Regarding the size of the cells, it is remarkably stable over time for both maintenance protocols.

Moving to the neglected cultures, their cell density increases first and stabilizes after three weeks. Their optical density exhibits an evolution with the same trend. The final values of these indicators are thought to be quite extreme due to the combination of evaporation, flocculation, and cell lysis (see below for cell poor condition indicators). They are, therefore, quite dispersed as those phenomena may not be highly repeatable from flask-to-flask. The size of the cell follows the same trend as the optical density: a rise and a fall.

The desynchrony between cell density and optical density readings can be explained by a change in cell morphology and composition. For the neglected cells, the size increase can explain the optical density dynamic in part, and the rest could be linked to lipid accumulation. In the same manner, the discrepancy between cell count and optical density for the cold-preserved cultures could originate from a change in cell composition, as reported by other authors (Sánchez-Saavedra, 2006; Sánchez-Saavedra and Núñez-Zarco, 2012; Welladsen et al., 2014). Still, for the subcultured cells, this explanation does not stand as cell size is at a nominal value, and lipid accumulation cannot be invoked. Another explanation can be drawn. Indeed, the cells might still be dividing, altering the cell wall thickness and the size of the chloroplasts, which, in turn, modulates the cell optical cross-section, hence the culture optical density signal (Baránková et al., 2020).

Finally, from a biotechnological point of view, among the three qualified indicators, ensuring stable cell size is paramount as cell and optical densities can be adjusted (by dilution or gentle centrifugation) to ensure repeatable photobioreactor inoculation.

3.2. Pigment contents

After cell size, culture cell count, and optical density, pigment contents are the next parameters describing the biomass. Fig. 3 presents the cells pigment contents for the three maintenance protocols over time. The first comment is that the reported values are similar to what was already observed for this strain under low light (Levasseur et al., 2023). Still, as one can see, subculturing every two weeks (top) induces some batch-to-batch variation. Yet values are reasonably close. Interestingly, on week 16, the cell had a surprisingly low pigment contents. The *post hoc* explanation could be an error in the medium formulation (presumably on the nitrogen content). Another artifact is to be reported on week 24. It this case, the cell pigment contents were doubled compared to the other cultures (pigment extraction was repeated for confirmation). Here, the explanation was that the incubator illumination had been halved by other scientists in our laboratory. These two incidents, while unfortunate, provide vivid examples of uncontrollable factors that can alter a cell maintenance protocol based on frequent subculturing.

The 4°C preservation protocol (middle) yielded biomass with a consistent pigment profile, after a slight increase after week 2 and on. While the time pattern was not described by other authors, they also reported an increase in pigment contents (Camacho-Rodríguez et al., 2016; Sánchez-Saavedra et al., 2019). This increase can be hypothesized to originate from the fact that cells are stored in the dark. Hence, they would thrive to produce pigment in an effort to harvest more light. This also correlates with the change in culture optical density, while cell count and size remain constant, supporting the explanation of a change in optical properties.

Finally, neglecting cultures (bottom) induces a constant decrease in their pigment contents. This observation echoes nitrogen reallocation strategies by the cell. Indeed, under nutrient-replete and low-light conditions, they produce a high quantity of pigments, especially chlorophyll. Then, when facing nitrogen limitation, they break down chlorophyll to recover the nitrogen it contains (Ishida et al., 2014; Pancha et al., 2014; Pozzobon et al., 2020).

Apart from the absolute values and their trends, it is also interesting to analyze the ratio between photoprotective carotenoids (violaxanthin and zeaxanthin) and total chlorophyll. This ratio is classically used to quantify light treatment harshness by probing the expression of the VAZ cycle (Pozzobon, 2022). In the present work, its value oscillates around 0.010 ± 0.002 for the subcultured and the cold-preserved cells. The observed values are in line with the one reported for the same strain under 100 μmolPhotonPAR/m^2/s (Levasseur et al., 2023). On the opposite, the value of the violaxanthin and zeaxanthin over chlorophyll a and b ratio increases up to 0.085 for the neglected ones. This increase is driven by both chlorophyll content reduction and zeaxanthin production. The final value is even higher than the one reported for *Chlorella vulgaris* exposed to 800 μmolPhotonPAR/m^2/s (0.07). Yet, in the present case, light cannot be responsible for this increase as it is quite limited. Therefore, it can be explained by the general antioxidant properties of those molecules at stake (especially zeaxanthin, but also lutein (Camarena-Bernard et al., 2024; Choudhury and Behera, 2001; Jahns and Holzwarth)), which may be expressed by the cells as a defense mechanism against increasing reactive oxygen species level.

Therefore, it can be concluded that in terms of pigment profile, subculturing or preserving *Chlorella vulgaris* at 4°C yields similar cells. The latest has the advantage of limiting the potential occurrence of problems originating from frequent passaging (*e.g.*, mistakes in the formulation of the culture medium).

3.3. Photosynthetic apparatus status

3.3.1. Transient fluorescence assays

As transient fluorometric assays (OJIP tests) yield an extensive amount of indicators, only a subset was graphed in Fig. 4. The selected weeks were: 0 as it is the starting point, week 4 as the neglected culture Fv/Fm ratio dropped below 0.5 (indicating stress), week 8, week 12, and week 24.

As one can see, the subcultured cells exhibit very stable and coherent functioning of the PSII, with values of 1.38 ± 0.11, 1.06 ± 0.08, and 0.50 ± 0.09 for ABS/RC, TR_0/RC, and ET_0/RC, respectively. Those values are in close agreement with the ones reported for healthy *Chlorella vulgaris* cells grown under 200 μmolPhotonPAR/m^2/s (Pozzobon, 2022). Still, the kind reader will note that the analysis performed on week 24 is not different from the others. This is because OJIP test focuses on PSII, and, in all cases for the subcultured cells, PSIIs are healthy. Therefore, it will be necessary to complement this analysis by a more global assay, such as the light curve.

The cells preserved at 4°C showed a slight deviation over time. Indeed, after 24 weeks of storage, the values of those indicators increased to 2.00 ± 0.11 (+45 %), 1.34 ± 0.00 (+ 26 %), and 0.70 ± 0.07 (+ 40 %), respectively. Two mechanisms can be hypothesized to explain this observation. First, a moderate reduction in the number of functioning reaction centers could be at play. Hence, for the given amount of captured light, the values of the indicators increase (by lowering the denominator value in the ratios). This assumption is supported by the lowered, while still reasonable, value of the Fv/Fm ratio. Alternatively, an increase in light capture efficiency for a similar number of active PSII would lead to the same evolution (by increasing the numerator values in the ratios). This hypothesis is supported by the slightly increasing pigment content. Further analyses are therefore required to arbitrate between the two candidate explanations.

Finally, in a much easier analysis, these explanations can be transposed to the case of the neglected cultures. In their case, after 4 weeks, the values of the indicators increased dramatically even though cell chlorophyll content decreased. These observations indicated a severe loss of functioning reaction centers, which is supported by the diving Fv/Fm values.

3.3.2. Light curves

While transient fluorescence assays and Fv/Fm ratios are informative

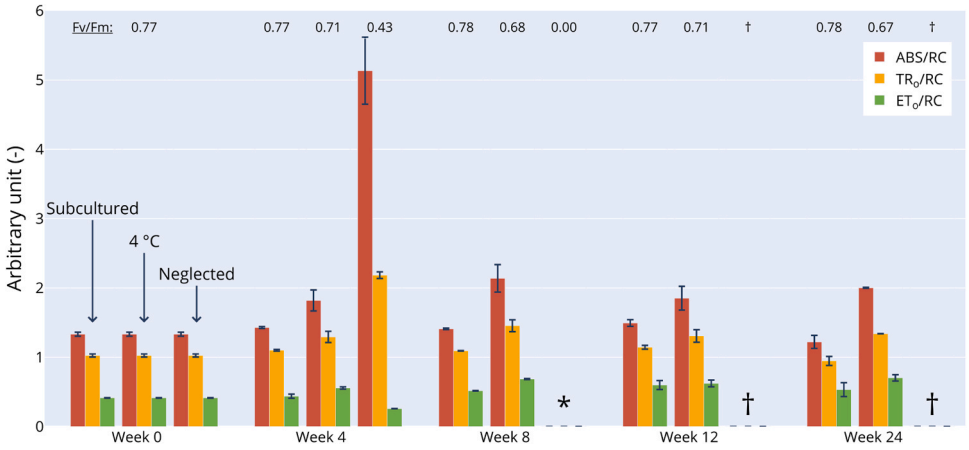

Fig. 4. OJIP and Fv/FM assays results. * analytical artifact allegedly due to poor sample condition. † data missing because of culture exhaustion. Data presented as the average of the two replicates, error bar covering the spread.

on the PSII status, they only last two seconds and 300 ms, respectively, and are conducted under super saturating light. Hence, additional information is to be gathered by analyzing the photosynthetic apparatus on a longer time scale and under different illuminations. This is what light curve assays do. Fig. 5 presents the light curve readings for the three tested protocols. As for PSII functioning indicators, the subcultured cells performances are stable. Consistent with the observation for the pigment profile, the performance of the week 24 samples can be explained by the fact that the cells were grown under a halved illumination. Therefore, they have a higher amount of chlorophyll, allowing them to capture more light and exhibit a higher output. This mechanism is only valid during the test period, as, in the long run, under high light, they would experience photodamage and acclimate by lowering their pigment content.

Before moving on to analyzing the behavior of the cells stored at 4˚C, it is interesting to focus on the neglected cells. Indeed, they provide a valid point of comparison as negative control. As one can see, after two weeks, cells exhibit a dramatically reduced output and an incapability to manage high light (curve reaching 0 under 1000 μmolPhotonPAR/m^2/s) despite having lowered their chlorophyll content and increase their xanthophyll one. It is important to note that this negatively altered behavior is observed for a Fv/Fm ratio of 0.71. Week 3 and on sees a rapidly decaying activity, which is coherent with dwindling Fv/Fm ratio and diving PSII performance indicators.

Over the course of their storage, the cells maintained at 4˚C display progressively downscaled light curves. Even though, the evolution form one measurement to the next (*e.g.*, week 0 and week 4) may show some statistical overlap, the overall trend is clear when comparing more distant data (*e.g.*, week 0 and week 8). Despite this decrease, the light curves still overpass the one of the neglected biomass on week 2, while they have similar values of the Fv/Fm. Furthermore, Han's model fitting procedure reveals that the evolution is characterized by a lowering of the output under saturating light (P_{max} - from 59 to 36) but also a lowering of the saturating intensity (I_k - from 106 to 83 μmolPhotonPAR/m^2/s) and the inhibition light intensity (I_i - from 578 to 529 μmolPhotonPAR/m^2/s). These traits altogether are the token of acclimation to low light conditions (Grobbelaar, 2013). Therefore, it can be concluded that the lowered Fv/Fm ratio more likely originates from pigment expression than actual damage to the photosynthetic units.

From this analysis, it can be concluded that, as indirectly suggested by Camacho-Rodriguez Fv/Fm measurement (Camacho-Rodríguez et al., 2016), fluorometry allows for the detection of early signs of cell alteration during a preservation procedure. In the context of this work, results showed that 4˚C preservation in the dark induced a slight acclimation of the cells. From a biotechnological point of view, this might have a consequence if one aims to inoculate a photobioreactor with a 4˚C-preserved culture. Yet, the only way to decipher this potential effect is by leading actual regrowth tests.

3.4. Lag phase determination

Following the conclusion of the previous section, regrowth tests are of peculiar interest. First of all, all the inoculated cultures grew. Still, differences in the lag phase duration could be noticed (Table 1). As one can see, the neglected cells required far more time than the others to reach an optical density of 1.0 (205 h versus 107 and 118). Here, observations are consistent. Those cells are in poor condition and require a long time to recover. Focusing on the two other protocols, it is interesting to note that the cells conserved at 4˚C showed a shorter lag phase (by about 10 h) than the subcultured ones. This shows that while informative, the implication of fluorometric assays must always be confirmed by cell growth experiments. Indeed, one could have expected that because of their slight dark acclimation, the 4˚C-preserved cells would not have been optimally acclimated to the regrowth test conditions. Therefore, it would have been logical to anticipate the opposite observation (4˚C cells exhibiting a longer lag phase). In addition to a shorter lag phase, the 4˚C-preserved cells exhibit lower dispersion than their subcultured counterparts. This is an advantageous trait from a biotechnological point of view as it would reduce batch-to-batch variation.

3.5. Overall mechanism and applicability

Overall, it is possible to propose a mechanism that would describe 4˚C-preserved cells behavior. First of all, as the cells are harvested in the exponential phase, they have extra nutrient stores, which represent means for a potential acclimation strategy. Second, while stored at 4˚C, their metabolism is not completely halted, and storing them in the dark triggered a slow acclimation process. Consequently, they increased their pigment content to be able to harvest more light upon re-illumination. Regarding the characteristic time of the process, the acclimation lasted two to four weeks. This time scale contrasts with de Mooji's team whose results shows an acclimation time of 9.85 ± 0.18 h for *Chlorella sorokiniana* at normal temperature (de Mooij et al., 2017), and Cullen and Lewis who reported a characteristic time of 8.06 ± 0.07 h for *Thalassiosira pseudonana* - clone 3H - (a diatom) (Cullen and Lewis, 1988). This difference in the dynamic is thought to originate directly from the low temperature. In addition to preparing the cell to harvest more light, the results reported in this work show that the evolution of pigment contents slightly alters the photosynthetic apparatus function (detected by fluorometry). Our observations on this aspect of cold preservation corroborate those of Camacho-Rodriguez (Camacho-Rodríguez et al.,

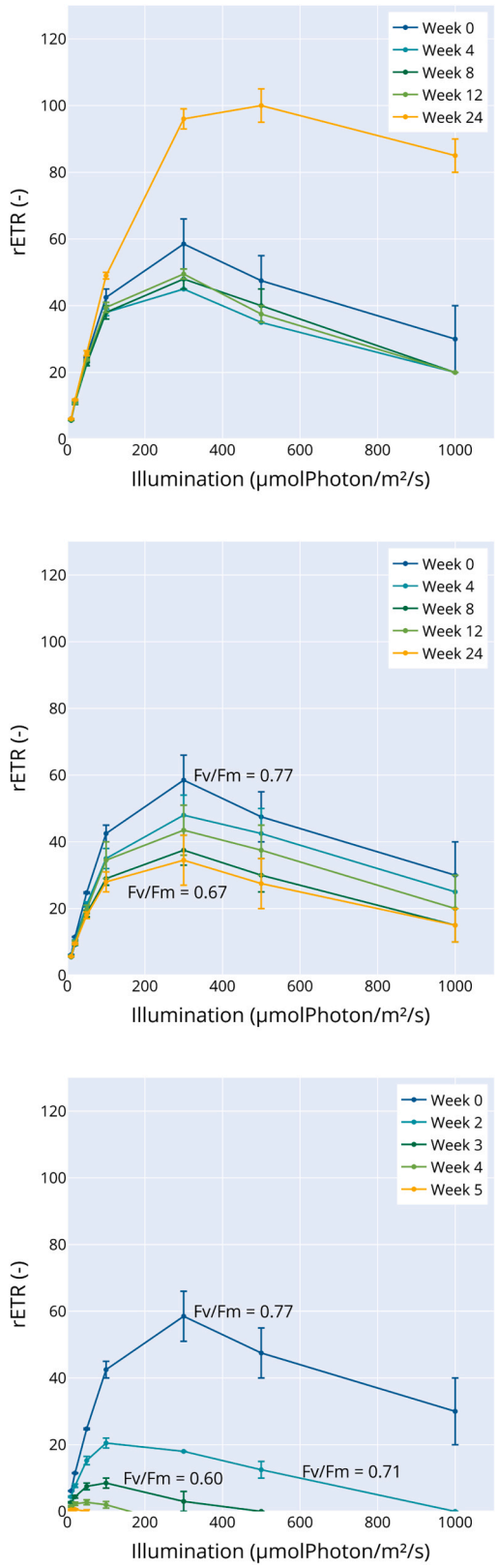

Fig. 5. Top - Electron Transport Rate (ETR) of the subcultured cells. May the kind reader please remember that the cells produced on week 24 were exposed to an illumination twice as low as the other ones. Middle - Electron Transport Rate of the cells stored at 4°C. Bottom - Electron Transport Rate of the neglected cells, no valid data for week 1 (saturated signal). Data presented as the average of the two replicates, error bar covering the spread.

Table 1
Time required for the cultures to reach an optical density at 750 nm of 1.0. † data missing because of culture exhaustion.

Week	Lag phase duration(h)		
	Subcultured	4°C	Neglected
6	118 ± 5	107 ± 0	205 ± 8
12	119 ± 1	114 ± 3	†
18	124 ± 5	110 ± 0	†
24	123 ± 2	108 ± 5	†

2016). It was, therefore, important to evaluate the consequence of the pigment contents up-regulation by regrowing the cells. Regrowth tests showed that the 4°C-preserved cells have the edge over the subcultured cells and develop slightly faster, allegedly, thanks to their higher pigment contents, which allow them to harvest more light. This mechanism holds for at least six months for *Chlorella vulgaris*. Still, as the cells evolve even at 4°C, one cannot expect to store them as long as he could with a proper cryopreservation procedure. Therefore, extrapolation beyond six months requires proper scientific documentation.

From a biotechnological point of view, those findings advocate for the use of 4°C preservation to reduce cell maintenance work and conserve a pool of cells in a similar state to be used as repeatable inoculum for larger-scale experiments while nullifying otherwise batch-to-batch variation effects. Yet, one should be aware of the dark acclimation that the cells will undergo. Therefore, in order to further limit run-to-run variation, it is advisable to leave the cells to rest for two weeks (pigments expression characteristic time) before inoculating the first photobioreactor.

Finally, in addition to question of the species specificity of the reported observation and the lack macronutrient monitoring, this work raises the question of storing the culture in an illuminated cold chamber to investigate if light acclimation can also be induced. Yet, it would surely remain a purely intellectual question as fridges are dark environments.

4. Conclusions

Chlorella vulgaris cells were maintained over six months (or tentatively) using three protocols: frequent subculturing, storage at 4°C, and simple abandonment. The last one quickly showed cells deviating from their nominal state. Indeed, they exhibited an increased size, a loss of their pigments, allegedly because of nitrogen depletion, a negative alteration of their photosynthetic capacity, and a 5-day lag phase when inoculated into fresh medium. Frequent subculturing yielded stable performances. Yet, our experience showed that uncontrollable factors (human errors, lack of communication between teams) could expose the cells to unfortunate incidents (altered medium composition, illumination change). 4°C preservation allowed the cells to have a constant size and a slightly increased, yet stable, pigment profile associated to a dark acclimation. Finally, regrowth tests demonstrated that 4°C preservation induces slightly improved performance (lag phase duration and dispersion) than frequent subculturing. Those findings advocate for the use of 4°C preservation to reduce cell maintenance work and conserve a pool of cells in a similar state to be used as repeatable inoculum for larger-scale experiments while nullifying otherwise batch-to-batch variation effects.

CRediT authorship contribution statement

Victor Pozzobon: Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Wendie Levasseur:** Writing – review & editing, Methodology, Formal analysis, Conceptualization. **Cristobal Camarena-Bernard:** Writing – review & editing, Methodology, Investigation, Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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References

Abreu, L., Borges, L., Marangoni, J., Abreu, P.C., 2012. Cryopreservation of some useful microalgae species for biotechnological exploitation. J. Appl. Phycol. 24 (6), 1579–1588. https://doi.org/10.1007/s10811-012-9818-0.

Andersen, R.A., Andersen and Phycological Society of America, 2005. Algal Culturing Techniques. Academic Press. Google-Books-ID: 9NADUHyFZaEC.

Baránková, B., Lazár, D., Nauš, J., Solovchenko, A., Gorelova, O., Baulina, O., Huber, G., Nedbal, L., 2020. Light absorption and scattering by high light-tolerant, fast-growing Chlorella vulgaris IPPAS C-1 cells. Algal Res. 49, 101881 https://doi.org/10.1016/j.algal.2020.101881. ⟨https://www.sciencedirect.com/science/article/pii/S2211926419310963⟩.

Brown, N., Shilton, A., 2014. Luxury uptake of phosphorus by microalgae in waste stabilisation ponds: current understanding and future direction. Rev. Environ. Sci. Bio/Technol. 13 (3), 321–328. https://doi.org/10.1007/s11157-014-9337-3.

Buhmann, M.T., Day, J.G., Kroth, P.G., 2013. Post-cryopreservation viability of the benthic freshwater diatom Planothidium frequentissimum depends on light levels. Cryobiology 67 (1), 23–29. https://doi.org/10.1016/j.cryobiol.2013.04.005. ⟨https://www.sciencedirect.com/science/article/pii/S0011224013001235⟩.

Camacho-Rodríguez, J., Cerón-García, M.C., Macías-Sánchez, M.D., Fernández-Sevilla, J. M., López-Rosales, L., Molina-Grima, E., 2016. Long-term preservation of concentrated Nannochloropsis gaditana cultures for use in aquaculture. J. Appl. Phycol. 28 (1), 299–312. https://doi.org/10.1007/s10811-015-0572-y.

Camarena-Bernard, C., Jullien, T., Pozzobon, V., 2024. Heterotrophic culture of Scenedesmus almeriensis for lutein productivity enhancement. J. Appl. Phycol. In press.

Cañavate, J.P., Lubian, L.M., 1994. Tolerance of six marine microalgae to the cryoprotectants dimethyl sulfoxide and methanol. J. Phycol. 30 (3), 559–565. https://doi.org/10.1111/j.0022-3646.1994.00559.x.

Castelló, M.L., Pariente, G., Andrés, A., Ortolá, M.D., 2018. Evaluation of strategies for preservation of microalgae Chlorella. J. Food Process. Preserv. 42 (2), e13518 https://doi.org/10.1111/jfpp.13518.

Chen, Y.-C., 2001. Immobilized microalga Scenedesmus quadricauda (Chlorophyta, Chlorococcales) for long-term storage and for application for water quality control in fish culture. Aquaculture 195 (1), 71–80. https://doi.org/10.1016/S0044-8486(00)00540-8. ⟨https://www.sciencedirect.com/science/article/pii/S0044848600005408⟩.

Choudhury, N.K., Behera, R.K., 2001. Photoinhibition of photosynthesis: role of carotenoids in photoprotection of chloroplast constituents. Photosynthetica 39 (4), 481–488. https://doi.org/10.1023/A:1015647708360.

Cullen, J.J., Lewis, M.R., 1988. The kinetics of algal photoadaptation in the context of vertical mixing. J. Plankton Res. 10 (5), 1039–1063. https://doi.org/10.1093/plankt/10.5.1039.

Day, J.G., 2007. Cryopreservation of Microalgae and Cyanobacteria. In: Day, J.G., Stacey, G.N. (Eds.), Cryopreservation and Freeze-Drying Protocols, Methods in Molecular Biology™. Humana Press, Totowa, NJ, pp. 141–151. https://doi.org/10.1007/978-1-59745-362-2_10.

S. Díaz, J. Settele, E. Brondizio, H.T. Ngo, M. Guèze, J. Agard, and C. Zayas.IPBES global assessment: summary for policymakers. Retrieved from IPBES website: ⟨https://www.ipbes.net/news/ipbes-global⟩ …, 2019.

Genty, B., Briantais, J.-M., Baker, N.R., 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochim. Biophys. Acta (BBA) - Gen. Subj. 990 (1), 87–92. https://doi.org/10.1016/S0304-4165(89)80016-9. ⟨https://www.sciencedirect.com/science/article/pii/S0304416589800169⟩.

Griffiths, M.J., Garcin, C., van Hille, R.P., Harrison, S.T.L., 2011. Interference by pigment in the estimation of microalgal biomass concentration by optical density. J. Microbiol. Methods 85 (2), 119–123. https://doi.org/10.1016/j.mimet.2011.02.005. ⟨http://www.sciencedirect.com/science/article/pii/S016770121100056X⟩.

Grobbelaar, J.U., 2013. Mass Production of Microalgae at Optimal Photosynthetic Rates. Photosynthesis. IntechOpen. ⟨https://www.intechopen.com/chapters/45158⟩, 10.5772/55193.

Hellebust, J.A., Ahmad, I., 1989. Regulation of nitrogen assimilation in green microalgae. Biol. Oceanogr. 6 (3-4), 241–255. https://doi.org/10.1080/01965581.1988.10749529.

Ishida, H., Izumi, M., Wada, S., Makino, A., 2014. Roles of autophagy in chloroplast recycling. Biochim. Biophys. Acta (BBA) - Bioenerg. 1837 (4), 512–521. https://doi.org/10.1016/j.bbabio.2013.11.009. ⟨http://www.sciencedirect.com/science/article/pii/S0005272813002016⟩.

Jahns, P., Holzwarth, A.R., 2012. The role of the xanthophyll cycle and of lutein in photoprotection of photosystem II. Biochim. Biophys. Acta (BBA) - Bioenerg. 1817 (1), 182–193. https://doi.org/10.1016/j.bbabio.2011.04.012. ⟨https://www.sciencedirect.com/science/article/pii/S0005272811000971⟩.

Levasseur, W., Perré, P., Pozzobon, V., 2020. A review of high value-added molecules production by microalgae in light of the classification. Biotechnol. Adv. 41, 107545 https://doi.org/10.1016/j.biotechadv.2020.107545. ⟨http://www.sciencedirect.com/science/article/pii/S0734975020300422⟩.

Levasseur, W., Perré, P., Pozzobon, V., 2023. Chlorella vulgaris acclimated cultivation under flashing light: an in-depth investigation under iso-actinic conditions. Algal Res. 70, 102976 https://doi.org/10.1016/j.algal.2023.102976. ⟨https://www.sciencedirect.com/science/article/pii/S2211926423000097⟩.

Molitor, H.R., Moore, E.J., Schnoor, J.L., 2019. Maximum CO2 utilization by nutritious microalgae. ACS Sustain. Chem. Eng. 7 (10), 9474–9479. https://doi.org/10.1021/acssuschemeng.9b00656.

de Mooij, T., Nejad, Z.R., van Buren, L., Wijffels, R.H., Janssen, M., 2017. Effect of photoacclimation on microalgae mass culture productivity. Algal Res. 22, 56–67. https://doi.org/10.1016/j.algal.2016.12.007. ⟨https://www.sciencedirect.com/science/article/pii/S2211926416307342⟩.

Núñez-Zarco, E., Del Pilar Sánchez-Saavedra, M., 2011. Cold storage of six marine benthic diatoms native to the Mexico Pacific coast. J. World Aquac. Soc. 42 (4), 530–538. https://doi.org/10.1111/j.1749-7345.2011.00495.x.

Pancha, I., Chokshi, K., George, B., Ghosh, T., Paliwal, C., Maurya, R., Mishra, S., 2014. Nitrogen stress triggered biochemical and morphological changes in the microalgae Scenedesmus sp. CCNM 1077. Bioresour. Technol. 156, 146–154. https://doi.org/10.1016/j.biortech.2014.01.025. ⟨http://www.sciencedirect.com/science/article/pii/S0960852414000431⟩.

delPilarSánchez-Saavedra, M., 2006. The effect of cold storage on cell viability and composition of two benthic diatoms. Aquac. Eng. 34 (2), 131–136. https://doi.org/10.1016/j.aquaeng.2005.06.004. ⟨https://www.sciencedirect.com/science/article/pii/S0144860905000749⟩.

delPilarSánchez-Saavedra, M., Molina-Cárdenas, C.A., Castro-Ochoa, F.Y., Castro-Ceseña, A.B., 2019. Protective effect of glycerol and PEG-methyl ether methacrylate coatings on viability of alginate-immobilized Synechococcus elongatus after cold storage. J. Appl. Phycol. 31 (4), 2289–2297. https://doi.org/10.1007/s10811-019-1756-7.

Porra, R.J., 1990. A simple method for extracting chlorophylls from the recalcitrant alga, Nannochloris atomus, without formation of spectroscopically-different magnesium-rhodochlorin derivatives. Biochim. Biophys. Acta (BBA) - Bioenerg. 1019 (2), 137–141. https://doi.org/10.1016/0005-2728(90)90135-Q. ⟨http://www.sciencedirect.com/science/article/pii/000527289090135Q⟩.

Pozzobon, V., 2022. Chlorella vulgaris cultivation under super high light intensity: an application of the flashing light effect. Algal Res. 68, 102874 https://doi.org/10.1016/j.algal.2022.102874. ⟨https://www.sciencedirect.com/science/article/pii/S2211926422002454⟩.

Pozzobon, V., Levasseur, W., Guerin, C., Gaveau-Vaillant, N., Pointcheval, M., Perré, P., 2020. Desmodesmus sp. pigment and FAME profiles under different illuminations and nitrogen status. Bioresour. Technol. Rep. 10, 100409 https://doi.org/10.1016/j.biteb.2020.100409. ⟨https://www.sciencedirect.com/science/article/pii/S2589014X2030030X⟩.

Rizwan, M., Mujtaba, G., Memon, S.A., Lee, K., Rashid, N., 2018. Exploring the potential of microalgae for new biotechnology applications and beyond: a review. Renew. Sustain. Energy Rev. 92, 394–404. https://doi.org/10.1016/j.rser.2018.04.034. ⟨http://www.sciencedirect.com/science/article/pii/S1364032118302557⟩.

Safi, C., Zebib, B., Merah, O., Pontalier, P.-Y., Vaca-Garcia, C., 2014. Morphology, composition, production, processing and applications of Chlorella vulgaris: a review. Renew. Sustain. Energy Rev. 35, 265–278. https://doi.org/10.1016/j.rser.2014.04.007. ⟨https://www.sciencedirect.com/science/article/pii/S1364032114002342⟩.

Sánchez-Saavedra, M.D.P., Núñez-Zarco, E., 2012. Photosynthetic and biochemical effects of cold storage on marine benthic diatoms of the Mexican Pacific coast. J. World Aquac. Soc. 43 (2), 249–258. https://doi.org/10.1111/j.1749-7345.2012.00553.x.

Sasi, P.K.C., Viswanathan, J.M.A., Thomas, D.M., Jacob, J.P., Paulose, S.V., 2020. Phycoremediation of Paper and Pulp Mill Effluent using Planktochlorella nurekis and Chlamydomonas reinhardtii-a comparative study. J. Environ. Treat. Tech. 8 (2), 809–817.

Strasser, R.J., Srivastava, A., Tsimilli-Michael, M., 2000. The fluorescence transient as a tool to characterize and screen photosynthetic samples. Probing photosynthesis: mechanisms, regulation and adaptation 445–483.

Welladsen, H., Kent, M., Mangott, A., Li, Y., 2014. Shelf-life assessment of microalgae concentrates: effect of cold preservation on microalgal nutrition profiles. Aquaculture 430, 241–247. https://doi.org/10.1016/j.aquaculture.2014.04.016. ⟨https://www.sciencedirect.com/science/article/pii/S0044848614001823⟩.