Research Article

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Are cyanobacteria a nearly immortal source of high market value compounds?

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Abstract

BACKGROUND: When the human population increases, so does the need to explore a wider range of feedstocks and biomasses, such as cyanobacteria. However, a deeper understanding of the growth patterns and pigment production is required to support the selection of the most beneficial species and conditions for industrial production. The growth and pigment production (i.e., chlorophyll *a* and C-phycocyanin) of three cyanobacterium species were evaluated following a three-fold aim. The first goal was to compare among a species commonly selected for exploitation (*Arthrospira platensis*) and two alternative species (*Anabaena cylindrica* and *Nostoc muscorum*). The second goal was analyzing pigment production in the long-term. The last goal involved comparing different methods (spectrophotometry and fluorimetry) to understand whether there is an appropriate proxy of biomass increase and pigment production that can be used for monitoring purposes.

RESULTS: All species showed high longevity and proved capable of growing for more than 100 days without any additional supplementation. However, the maximum quantum yield of PS II (F_v/F_m) revealed that their photosynthetic efficiency varied over time with a clear decrease after 2 months. Pigment analysis showed a heterogeneous pattern during the growth periods of all three species that could only be captured by the parameter F_v/F_m, but the pattern was only present for *A. cylindrica* and *N. muscorum* in some stages of the culture period.

CONCLUSION: *N. muscorum* was found to be the best chlorophyll *a* and C-phycocyanin producer, with the production peaking for all species at defined time periods within the growth profile.

Supporting information may be found in the online version of this article.

Keywords: cyanobacteria; growth curves; pigment production; chlorophyll *a*; C-phycocyanin

ABBREVIATIONS

Chl *a*	Chlorophyll *a*
CO_2	Carbon dioxide
F_m	maximal fluorescence
F_o	minimum fluorescence
K	maximum growth
OD	optical density
PS II	Photosystem II
USD	United States Dollar

INTRODUCTION

The growing human population and diminishing global resources make it crucial to increase the range of feedstocks and biomasses available. Cyanobacteria are one of the microorganism groups whose exploitation can help fulfil some of this demand, while contributing to several of the Sustainable Development Goals (SDG) proposed by the United Nations concerning both environmental protection and sustainable production/consumption. In addition, worldwide consumers are becoming more aware of the resource scarcity and of the improved biological compatibility of natural products over their synthetic counterparts; this significantly support any endeavors aiming to fill gaps in these two fields. This context highlights the role of cyanobacteria as an important and sustainable player in the global economy today and in the future. Indeed, these organisms can be considered as a source of compounds with biological activity and as a promising feedstock for

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many applications. Lipids, pigments, and proteins from cyanobacteria are known for their antibacterial, antifungal, antiviral, and antitumor activities.[1,2] Moreover, their important roles as CO_2 mitigators, biofertilizers, food supplements (for human consumption and aquaculture), bioremediation agents, and bioenergy producers demonstrates the overall importance of cyanobacteria as a potential asset.[3,4] However, amongst more than 10 000 species of cyanobacteria, only *ca*. 1000 have been partly characterized and very few are currently cultured at an industrial scale.[5] This essentially means that research on the biotechnological potential of these organisms is still limited, but the market for cyanobacterial products is already large and its value has been increasing recently: USD 24 million in 2020 for phycobiliproteins, with industry expecting a consumption higher than USD 96 million by 2026 (https://www.businessresearchinsights.com, assessed on April 2022); and USD 463.7 million by 2025 for the chlorophylls market (https://www.valuemarketresearch.com, assessed on April 2022).

To date, pigments are the compounds produced by cyanobacteria with the highest commercial value, particularly carotenoids and phycobiliproteins.[6] These pigments have the potential to replace several synthetic dyes (that can induce toxic effects) and they can be used as natural colorants for textiles, food, drugs, and cosmetics.[1] Phycobiliproteins are water-soluble and fluorescent pigment-protein complexes that act as secondary light-harvesting components in the photosynthetic process.[3] Phycocyanin, a blue pigment-protein of the phycobiliprotein family, is mainly found in cyanobacteria and is conventionally defined as C-phycocyanin.[6] Aside from its use as natural dye, its biological activity also makes it suitable for pharmaceutical and biomedical applications. Its antitumor activity against several types of cancer cell lines,[7] along with its anti-oxidant nature,[8] anti-inflammatory activity,[9] and activity as a stimulator of the immune system,[10] render phycobiliproteins highly desirable in many industries. Like phycocyanin, chlorophyll *a* (Chl *a*) is a pigment that absorbs the light (mainly in the blue zone and, to a minor extent, in the red zone) of the electromagnetic spectrum. This pigment can be used as a food ingredient (E140),[11] in hygiene products as a deodorant, and in medical applications as a chemopreventive agent.[6,12,13]

The industrial cultivation of cyanobacteria is already taking place worldwide. However, its production is presently focused on a (too) short list of species (e.g., *Arthrospira* sp., *Aphanizomenon* sp., *Nostoc* sp.) that may not include the most adequate strains for the efficient extraction of the target molecules. Some studies have investigated the impacts of the culturing parameters, such as nutrient concentrations,[14] light[15] and temperature,[16] on cyanobacteria productivity. However, as to our knowledge, this is the first study investigating the longevity of cyanobacteria under straightforward culturing conditions with no nutrient re-supply along with the fluctuations in the production of pigments throughout the growth cycle. These are two clear variables for the optimization of cultures explored at larger scales for commercial purposes. In fact, nutrient supplementation is costly and can cause changes in physiology that will be reflected in an altered productivity. This includes the effect on the production of molecule(s) of interest and the corresponding expected extraction yield. In this context, the present study aimed at monitoring three filamentous cyanobacteria, *Anabaena cylindrica* (*A. cylindrica*), *Nostoc muscorum* (*N. muscorum*), and *Arthrospira platensis* (*A. platensis*), regarding their growth kinetics, photosynthetic efficiency, and pigment production. On one hand, this monitoring endeavor was expected to demonstrate that an anticipated knowledge of the pigment yield dynamics can support the optimization of exploitation routines at an industrial level; on the other hand, it serves as the basis for a detailed insight on whether pigment exploitation can be guided by easily acquirable monitoring parameters based on spectrophotometric measurements or fluorimetry. The widely used species *A. platensis* was included in the study to provide performance comparability with the less conventional alternative species, *A. cylindrica* and *N. muscorum*, under the hypothesis that the latter species may represent more efficient sources for pigment production, thus demonstrating the importance of a rational species selection when implementing exploitation settings.

MATERIALS AND METHODS

Cyanobacteria cultures and culturing conditions

Three species of filamentous cyanobacteria were used, *A. cylindrica* PCC 7122, *Nostoc muscorum* UTAD_N213, and *Athrospira platensis* UTEX LB 2340. Non-axenic cultures were maintained in borosilicate flasks, which were initially filled with 5 L of culture medium, in triplicate. For *A. cylindrica* and *N. muscorum*, the Woods Hole MBL synthetic medium was used[17] (Table S1) to provide its long-term support for the optimal growth of these cyanobacterial strains in our laboratory. *A. platensis* was cultured in Spirulina medium[18] (Table S2) because this medium was found to provide optimal growth for this strain in preliminary trials compared with MBL (it is worth noting that the aim was a comparison between the conventional and alternative species, hence the importance of establishing comparable growth conditions). Borosilicate flasks (5 L capacity) were inoculated using 150 mL of a 9 day-old inoculum to an initial optical density (OD) at 750 nm of 0.05 ± 0.01, then incubated at 26 ± 2 °C under a 16 h-light/8 h-dark photoperiod cycle, with a light intensity of 37 μmol photons m^{-2} s^{-1} (Quantum meter MQ-200, Apogee Instruments, Logan, Utah, USA), equal to 2300 lx, provided by cool white fluorescent tubes and constant aeration. Samples for growth rate, photosynthetic yield (2 mL), and pigment production (*ca*. 40 mL) were collected through the experimental period inside a flow chamber and using sterile material.

Spectrophotometric measurements

The culture growth was monitored three times a week by collecting optical density (OD) records at different wavelengths (440, 480, 620, 675, and 750 nm) to cover the absorption peaks of different pigments while controlling for total particulate matter. OD_{750} retrieved turbidity without the influence of pigments and, thus, was interpreted *a priori* as a proxy for cell suspension concentration. The other wavelengths gave insights regarding specific pigments: 440 and 675 nm for Chl *a*,[19] 480 nm for carotenoids,[20] and 620 nm for C-phycocyanin.[21]

Reliable endpoints to monitor culture growth and photosynthetic yield

An imaging chlorophyll fluorometer (Open FluorCAM 800-O/1010, Photon Systems Instruments; Brno, Czech Republic) was used to capture the maximum photosynthetic quantum yield of Photosystem II (F_v/F_m) after 15 min of dark adaptation. The excitation light peaked at 621 nm with a 40 nm band width, and saturating pulses were applied with an intensity of about 7000 μmol quanta m^{-2} s^{-1} and the duration of 0.8 s. Chl *a* fluorescence was captured by a CCD camera (CCD381) with a F1.2 (2.8–6 mm) objective, resulting in images with 512 × 512 pixels and a spectral range of 695–780 nm. Images were processed using the FluorCam7

software (Photon Systems Instruments; Brno, Czech Republic). Fluorometric measurements were made on each culture, three times a week, in triplicate. For this purpose, aliquots of 2 mL of each culture were transferred into 6-well plates. The ratio F_v/F_m was calculated following Eqn (1).

$$\frac{F_v}{F_m} = \frac{F_m - F_o}{F_m} \quad (1)$$

F_o is the minimum fluorescence in the dark-adapted sample and F_m is the maximal fluorescence after exposure to a saturating light pulse. F_o is also known as a proxy of photosynthetic biomass that allows for monitoring the culture growth over time. As biomass (and, hence, the fluorescence signal intensity) increased over time, the sensitivity settings of the instrument were adjusted correspondingly. Measurements of the fluorescence signal before and after the adjustment were corrected accordingly. The resulting growth curve was used to estimate species-specific growth curve parameters within defined time periods.

The growth curves and corresponding parameters were obtained by fitting a logistic function to biomass data, using MS Excel Solver (Eqn (2).

$$N(t) = \frac{k}{1 + \frac{k - N_0}{N_0} e^{-r(t)}} \quad (2)$$

N_0 is the initial cell density [F_o] at time 0, $N(t)$ is the cell density [F_o] at time t, k is the maximum density [F_o], r is the growth rate [d^{-1}], and lag phase [d] is the time before exponential growth starts. The

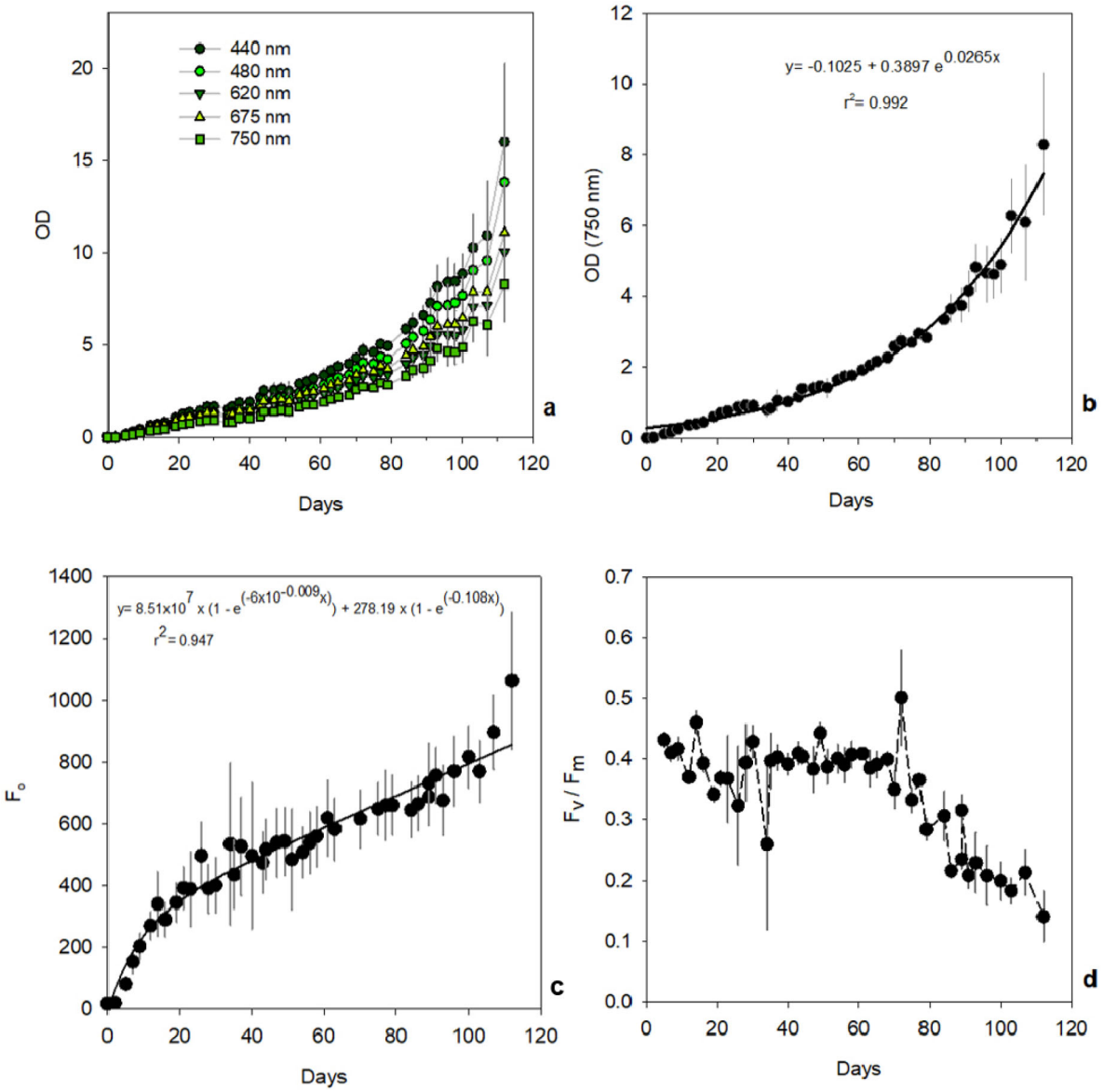

Figure 1. Profiling of *A. cylindrica* throughout a monitoring period of 112 days regarding: (a) multiple OD measurements; (b) best fitted curve modelling through the least-squares method, based on OD_{750}; (c) best fitted curve based on the F_o value; (d) dynamics of PS II efficiency (F_v/F_m) (only data with $F_o > 100$ are depicted). In all graphics, marks represent the mean of three replicates and the error bars represent the standard deviation. In panels (a) and (d), a line was added joining the marks within each series when deemed necessary for clarity purposes, not reflecting any fitted model. In panels (b) and (c), the solid black line represents the exponential model best fitted to the experimental data through the least-squares method.

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doubling time [d] (Eqn (3)) and doubling *per* day [d^{-1}] were then calculated (Eqn (4)).

$$\text{Doubling time} = \frac{\ln(2)}{r} \quad (3)$$

$$\text{Doubling per day} = \frac{1}{\text{Doubling time}} \quad (4)$$

Pigment quantification

The extraction and quantification of Chl *a* and C-phycocyanin were performed every 15 days for each cyanobacteria culture. Approximately 40 mL of each culture was recovered by centrifugation (4111 × *g* for 5 min; Eppendorf 5810 R). The pelleted biomass was stored at −20 °C until pigment quantification. Pure ethanol and 150 mmol L^{-1} of sodium phosphate buffer (pH = 7) was used to extract Chl *a* and C-phycocyanin, respectively.[22,23] These solid–liquid extractions (1:10, w:v) were performed at 35 °C for 50 min, with an agitation of 1500 rpm in a thermomixer (Eppendorf ThermoMixer® C). All extractions were performed in triplicate. For the extraction of C-phycocyanin from *N. muscorum*, a second extraction step was performed, using the biomass left overnight after the first extraction and applying the exact same conditions and the same amount of fresh solvent (150 mmol L^{-1} sodium phosphate buffer, pH 7).

At the end of the solid–liquid extraction, the cell suspensions were centrifuged at 9500 × *g* for 10 min in a VWR MicroStar 17 centrifuge; the supernatant was separated from the cell debris and used for pigment quantification. For each extract, the absorption spectra were collected between 200 and 700 nm using a UV-visible microplate reader (Synergy HT microplate reader – BioTek) in duplicate. The quantification of Chl *a* and C-phycocyanin was done using previously defined calibration curves (R^2 = 0.9805 and R^2 = 0.9912, built following optical density measurements

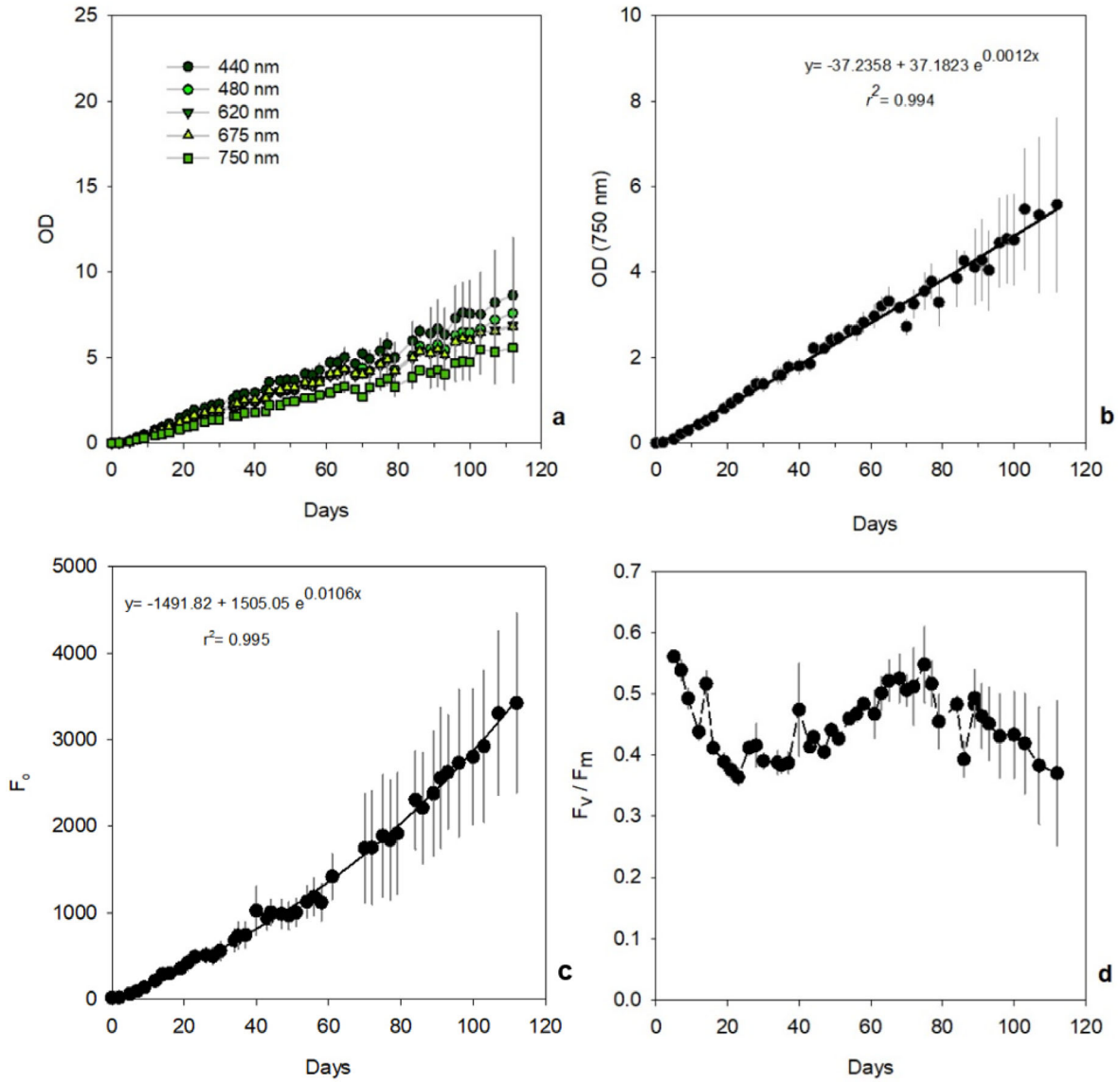

Figure 2. Profiling of *N. muscorum* throughout a monitoring period of 112 days regarding: (a) multiple OD measurements; (b) best fitted curve modelling through the least-squares method, based on OD_{750}; (c) best fitted curve based on the F_o value; d dynamics of PS II efficiency (F_v/F_m) (only data with F_o > 100 are depicted). In all graphics, marks represent the mean of three replicates and the error bars represent the standard deviation. In panels (a) and (d), a line was added joining the marks within each series when deemed necessary for clarity purposes, not reflecting any fitted model. In panels (b) and (c), the solid black line represents the exponential model best fitted to the experimental data through the least-squares method.

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at 667 nm and 615 nm, respectively). The yield of extraction ($mg_{pigment}\, g_{dry\ weight}^{-1}$) was calculated according to Eqn (5).

$$\text{Yield}\left(mg_{pigment} g_{dry\ weight}^{-1}\right) = \frac{\text{pigment concentration} \times \text{extract volume}}{\text{mass of drv cvanobacteria}} \quad (5)$$

RESULTS

Optical density measurements of cyanobacteria in the long-term

The profiles of the cultures monitored spectrophotometrically showed that all optical densities of the three cyanobacteria strains continuously and (generally) exponentially increased for more than 100 days, without reaching a stationary phase (panels (a) and (b) in Figs 1–3). Despite the use of different wavelengths for this monitoring stage, all showed the same tendency (Figs 1–3(a)). In this way, we considered the OD at 750 nm as a representative for further interpretation and detailed comparison among species. *A. cylindrica* and *A. platensis* showed similar $OD_{750\ nm}$ profiles. At the end of the experiment, *A. platensis* showed highest values of absorbance, followed by *A. cylindrica*, and finally *N. muscorum*. Adjusted exponential equations accurately described the increase in absorbance of these species, as denoted by the coefficients of determination higher than 0.98 that were obtained in all cases (Figs 1–3(b)).

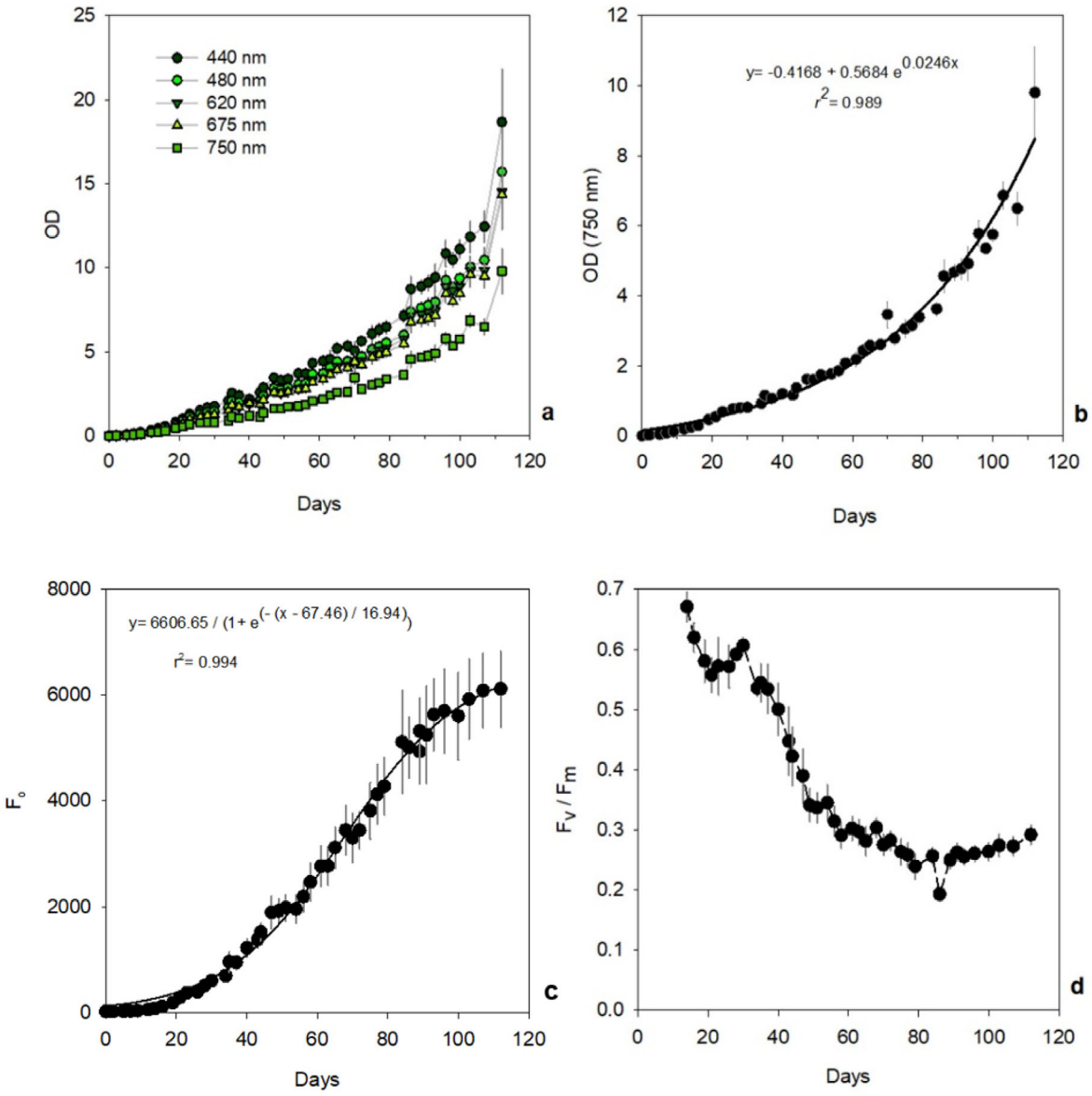

Figure 3. Profiling of *A. platensis* throughout a monitoring period of 112 days regarding: (a) multiple OD measurements; (b) best fitted curve modelling through the least-squares method, based on OD_{750}; (c) best fitted curve based on the F_o value; (d) dynamics of PS II efficiency (F_v/F_m) (only data with $F_o > 100$ are depicted). In all graphics, marks represent the mean of three replicates and the error bars represent the standard deviation. In panels (a) and (d), a line was added joining the marks within each series when deemed necessary for clarity purposes, not reflecting any fitted model. In panels (b) and (c), the solid black line represents the model best fitted to the experimental data through the least-squares method.

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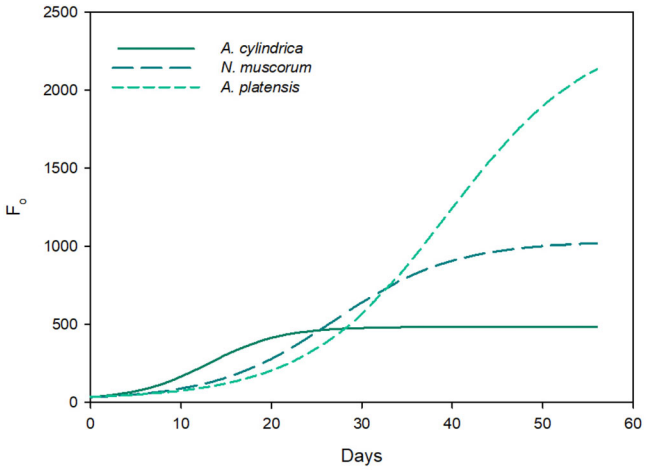

Figure 4. Growth kinetics estimated for the three species using F_o values following the fitting of logistic models to the experimental data retrieved through the first 56 days of the experience.

Growth and health status of cyanobacteria in the long-term

Despite the common use of optical density to describe cell growth, this technique does not differentiate living cells from dead cells. For this reason, other parameters (i.e., F_o and F_v/F_m) with better physiological relevance were also monitored during the experiments. The values of F_o were used as a proxy of the photosynthetically active biomass due to its high sensitivity and specificity towards Chl *a*. These data, illustrated in Figs 1–3(c), also showed that all the species continuously grew through 112 days. Despite fluctuations within and between species, all cyanobacteria were capable of withstanding growth for this long period of time without changing culture conditions and, remarkably, without nutrient supplementation. In the last days of the experiment, *A. platensis* started to stabilize its growth, indicating the realization of a stationary phase (Fig. 3(c)). However, the *A. platensis* species showed better biomass outcomes than the other two species. The *A. cylindrica* species reached lower values of F_o, denoting a flatter growth dynamics (Fig. 1(c)).

A detailed inspection of the F_o values through time (Figs 1(c), 2(c), and 3(c)) lead to the identification of what resembles a short plateau stage occurring between days 40 and 60 (especially visible in the case of *N. muscorum* and *A. platensis*), followed by a renewed acceleration of biomass production (Figs 1–3(c)). This specific feature found in the cyanobacteria growth data prevented the feasible adjustment of the appropriate logistic function for *A. cylindrica* and, consequently, the comparative analysis of growth dynamics. Therefore, to estimate growth parameters for species comparison, we artificially limited the fitting to data retrieved in the first half of the experimental period (up until day 56). In this first period, the growth of all cyanobacterium species is feasibly described by logistic equations ($r^2 = 0.919–0.993$; Fig. 4 and Table 1). Under these limited circumstances, *A. platensis* was the species with the longest lag phase of 1.73 days, while no lag phase was verified for the other two species. This resulted in the lowest growth rate during the first 23 days, which was then compensated for by a sharp acceleration (Fig. 4). As a result, *A. platensis* presented a doubling time of 5.57 days, allowing it to reach the highest value of maximum growth ($K = 2425.61$ [rel.unit]) out of the three species (Table 1). In contrast, *A. cylindrica* showed the highest increase in biomass during the first 20 days; but after that, the growth decelerated and the species recorded the lowest values of maximum growth ($K = 471.17$ [rel.unit]). This fast initial increase in biomass translated into the highest growth rate (0.24 d^{-1}) and doubling cells *per* day (0.34 d^{-1}). The behavior of *N. muscorum* was intermediate, between those of *A. platensis* and *A. cylindrica*.

The maximal PS II efficiency (F_v/F_m) was used as proxy for photosynthetic efficiency and, thus, as an indicator of the health status of the cultures. The F_v/F_m differed appreciably among the cultures and through the experiment (Figs 1–3(d)). *A. cylindrica* started with an F_v/F_m record of 0.43 at day 5 that remained stable until approximately day 70. After that day, the F_v/F_m started to decrease until reaching the value of 0.14 in the last day of the experiment. In contrast, *N. muscorum* showed a periodic pattern. While starting with F_v/F_m values of 0.56 at day 5, the records slowly decreased to 0.36 at day 23, then increased to a maximum value of 0.55 at day 75 and decreased again to 0.37. *A. platensis* was the species showing the most prominent declining of F_v/F_m throughout the experimental period: it recorded higher initial values of F_v/F_m (0.67) at day 14 that declined to average values of 0.27 during the last days.

Pigment production

Despite the continuous growth observed for the three cultures (Figs 1–3), this trend did not hold for pigment production (Fig. 5). Yields of C-phycocyanin and Chl *a* markedly fluctuated throughout the monitored period for all cyanobacteria but followed different patterns depending on the species. The concentration of C-phycocyanin was always higher than that of Chl *a*. More specifically, the production of C-phycocyanin by *A. cylindrica* was generally stable during the first half of the experiment (reaching 136.6 $mg_{C\text{-}phycocyanin}\ g_{dry\ weight}^{-1}$) and started to

Table 1. Growth parameters of the three species estimated following the fitting of logistic equations to experimental data retrieved through the first 56 days of the experiment. *K* stands for the maximum growth, *r* for growth rate, and *lag* for the lag phase; these parameters are expressed in days

	A. cylindrica	*N. muscorum*	*A. platensis*
***K* [rel. unit]**	471.17	1653.55	2425.61
***r* (d^{-1})**	0.24	0.10	0.12
***Lag* (d)**	0.00	0.00	1.73
Doubling time (d)	2.94	7.11	5.57
Doublings per day (d^{-1})	0.34	0.14	0.18
Model equation	$y = 471.17/(1 + 471.17–17.21/17.21\ e^{-0.24x})$	$y = 1653.55/(1 + 1653.55–16.78/16.78\ e^{-0.10x})$	$y = 2425.61/(1 + 2426.61–20.57/20.57\ e^{-0.12x})$
Model correlation (r^2)	0.919	0.956	0.993

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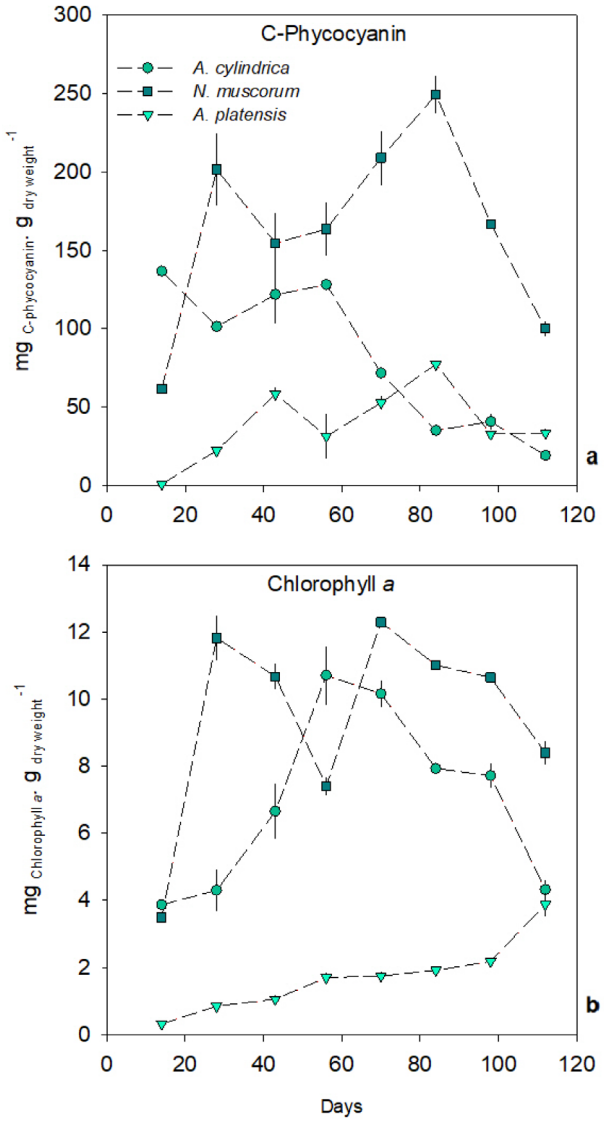

Figure 5. Yields of (a) C-phycocyanin and (b) chlorophyll *a* for the three species throughout a monitoring period of 112 days. In all, marks represent the mean of three replicates and the error bars represent the standard deviation. A line was added joining the marks within each series for clarity purposes.

decrease at day 56. In the first days, *A. cylindrica* was the best producer of C-phycocyanin but it was then surpassed by *N. muscorum*. C-phycocyanin production peaked in two specific moments of growth of the other two species: at day 30 and then more expressively at day 85 for *N. muscorum*; at day 45 and then more expressively at day 85 for *A. platensis*. The highest C-phycocyanin yield was recorded by *N. muscorum* throughout the culturing period, reaching a maximum of 249.4 $mg_{C\text{-}phycocyanin}\ g_{dry\ weight}^{-1}$. Clearly, *N. muscorum* was the best Chl *a* and C-phycocyanin producer, with a peak at around days 28 and 70 for Chl *a* (*ca.* 12 $mg_{Chlorophyll\ a}\ g_{dry\ weight}^{-1}$) and at days 28 and 84 for C-phycocyanin (*ca.* 201.5 and 249.4 $mg_{C\text{-}phycocyanin}\ g_{dry\ weight}^{-1}$). The C-phycocyanin production by *N. muscorum* started to decrease after day 84 and, at the end of the experiment, it reached a value similar to that achieved by day 14 (*ca.* 99.9 $mg_{C\text{-}phycocyanin}\ g_{dry\ weight}^{-1}$). *A. platensis* showed a similar profile for C-phycocyanin, with two moments of higher production (day 43 with *ca.* 61.4 $mg_{C\text{-}phycocyanin}\ g_{dry\ weight}^{-1}$ and day 84 with *ca.* 77.3 $mg_{C\text{-}phycocyanin}\ g_{dry\ weight}^{-1}$). However, the production of Chl *a* by *A. platensis* showed a different pattern, increasing continuously throughout time and reaching the highest yield at the end of the experiment of *ca.* 4.1 $mg_{Chlorophyll\ a}\ g_{dry\ weight}^{-1}$. *A. cylindrica* reached its maximum Chl *a* concentration after the first peak reached by *N. muscorum*, at day 56 (*ca.* 10.1 $mg_{Chlorophyll\ a}\ g_{dry\ weight}^{-1}$), and then the production decreased until reaching approximately 4.1 $mg_{Chlorophyll\ a}\ g_{dry\ weight}^{-1}$ by the end of the monitoring period; this value was approximately the same concentration that was observed at the beginning of the experiment, at day 14.

DISCUSSION

The ground explored in this work entails the interplay between the patterns of growth and pigment production in cyanobacteria. The production of C-phycocyanin and Chl *a* did not show a clear pattern during the growth period of each species, and the production dynamics was clearly different among species. This dynamic is of strategic importance to improve pigment production at a commercial scale because it would allow researchers to identify the growth state where compound production is the highest, thus leading to an improved harvesting efficiency. Despite the relevance of thoroughly understanding the growth dynamics of species exploited for biotechnological applications, very few studies have been published addressing cyanobacteria growth curves in detail. In this way, and to the best of our knowledge, this is one of the few works demonstrating the extraordinary longevity of cyanobacterium cultures (more than 100 days with no nutrient supplementation); this longevity is a promising feature for different areas where these organisms can be exploited. Another example of this longevity can be found for the terrestrial cyanobacterium *Nostoc* sp., which is able to grow for more than 140 days with no supplementation.[24] At an industrial scale, this feature is very useful because the culture medium does not need to be replaced or supplemented frequently, allowing for cost savings in consumables and labor.

As many studies have demonstrated, different species have different pigment contents and their production is dependent on the culture conditions[14,15,25] and the growth phase.[25] Some critical variables involve finding which species is the best producer of a specific compound of interest and understanding its growth phase when such production is maximal. However, it seems that such a systematic development of the exploitation process has been neglected, at least when considering the dedicated literature. Most of the existing studies do not evaluate the production of pigments through growth curves and instead only consider the specific days conveniently defined[16,26,27] or the exponential phase before a hypothesized stationary or decline phase, as would be typical for microalgae.[14,28] Interestingly, the species studied in the present work demonstrate a continuous increase in absorbance that lasts for at least the 112 days of the experiment. These optical density data are generally corroborated by the data from F_o (the relation between these two variables is depicted in Supporting Information, Fig. S1 and Table S3), which shows an increase in fluorescence during the whole experiment though with a growing trend, although *A. platensis* decelerated its growth in the last days (Fig. 3(c)) and *A. cylindrica* presented a less impressive cell biomass (Fig. 1(c)).

The minimal fluorescence, F_o, can be used as a proxy for microalgal biomass,[29,30] allowing for the feasible model of a curve that better resembles a biological growth curve (i.e., bearing an

exponential phase and then an approximation to a stationary phase) than that allowed by the data obtained from OD (showing a continuous growth acceleration, with no apparent biological significance). Indeed, high cell densities and particular cell morphologies can interfere with absorbance methods, and the data from F_o provided interesting results. Additionally, the growth curves of the three species showed a small plateau near days 40–60 and then restarted growing; this pattern was especially recognizable in *N. muscorum* and *A. platensis*.

This finding can be explained within two contexts. First, at a more practical level, it shows that there are two phases of accelerated growth (or exponential growth stages) within the growth curves of these cyanobacteria species. These are undistinguishable using typical monitoring tools, such as OD, but they can be accurately identified using fluorescence measurements, which greatly supports the sustainable large-scale exploitation of cyanobacteria. For example, we could identify *A. cylindrica* as the most demanding species in terms of initial nutrient supply because it showed the highest growth rates before reaching the first plateau and because it reached the plateau (or the system's carrying capacity) sooner. On the other hand, the most standard species, *A. platensis*, faced a *lag* phase (recognized only by using F_o measurements) before the first exponential phase. However, *A. platensis* was clearly the best performer in terms of biomass production in the long-term, as shown by the maximum growth rates and F_o records at the end of the experiment. It is worth noting that, despite the differences among species, the short-term growth rates found in this study's three species (0.10–0.24; Table 1) are lower than the typical records found in the literature for cyanobacteria (e.g., 0.89 for *A. flos-aquae*, 1.15 for *Microcystis* sp., 0.7 and 2.8 for *Synechocystis* sp. PCC6803).[31,32] Distinct species, culture conditions, and assessment periods likely explain the differences noted with the literature. The second arena where the intermediate plateau gains relevance is its biological or ecophysiological meaning. This intermediate plateau suggests that growth becomes limited at some point, likely due to essential nutrient limitation,[33,34] but then the cyanobacteria can overcome through some metabolic shift and restart growth; this was indeed confirmed in parallel metabolomic profiling studies,[35] further reinforcing the importance of using a monitoring tool that can identify the specific features of the growth and allow for predicting the production of target compounds. Indeed, specific compounds of interest can be preferably or exclusively available for exploitation before or after the metabolic shift indicated by the growth plateau. It is worth remarking at this point that *N. muscorum* was the best pigment producer but not the best biomass yielder; this demonstrates that biomass production is not a feasible proxy for pigment production. Thus, monitoring strategies intending to predict pigment production need to be carefully optimized to avoid lowering exploitation efficiencies.

Fluorimetry techniques can also give important insights for assessing photosynthetic efficiency. These techniques allow for an indication of the maximum quantum yield of the PS II and, consequently, insights on the photosynthetic efficiency, through the measurement of the parameter F_v/F_m. The F_v/F_m of the cultures tested herein was not constant during the entire experiment, with the exception of *N. muscorum*, where the yield remained quite stable (0.45 ± 0.05). Common values of F_v/F_m for cyanobacteria range within 0.4–0.6,[36] but the species of this study showed a higher range due to the long-term culturing (0.14–0.50 for *A. cylindrica*; 0.37–0.56 for *N. muscorum*; and 0.19–0.67 for *A. platensis*). The photosynthetic efficiency is a valuable parameter because it can assist the evaluation of the fitness of a culture, and if cells are experiencing stress, the yield decreases. Such stress conditions could be linked to the production of secondary metabolites or pigments, like carotenoids, with high commercial value.[37] However, this technique presents some limitations in the case of cyanobacteria because the absolute level of F_v/F_m is not a reliable indicator of PS II function.[38,39] This happens because, in cyanobacteria, the phycobiliprotein fluorescence also contributes to F_o, especially at high concentrations, and the PS II accounts for only a small proportion of total Chl *a*.[38] This can cause a downward distortion of the levels of F_v/F_m and, consequently, affect any conclusions regarding the photosynthetic activity.[38] Bearing in mind these limitations, fluorimetry could be a useful tool to compare cultures within the same group, and these observations may reflect different cellular strategies to cope with nutrient depletion.

Comparing the F_v/F_m data with the production of pigments, no relationship was found for *A. platensis*. However, some inferences could be made for *A. cylindrica* when comparing these two endpoints. The phycocyanin and Chl *a* from *A. cylindrica* started to decrease after approximately day 60, which was in accordance with a reduction in the values of F_v/F_m; however, until day 60 there were increases in Chl *a* that is not translate into F_v/F_m values. These data showed that, for *A. cylindrica*, F_v/F_m relates only to the production of C-phycocyanin. Regarding *N. muscorum*, there were two peaks in C-phycocyanin and Chl *a* production at around days 20 and 80, respectively, but only one peak was observed for F_v/F_m around day 80. These correlations between F_v/F_m data and pigment production were expected, as values of F_o, F_m, and F_v/F_m are influenced by the pigment concentration.[40] However, this correlation does not hold for *A. platensis*. Although presenting some limitations, F_v/F_m data showed that, despite the worst health status being observed in the last days (especially in the case of *A. cylindrica*, Fig. 1(d), and *A. platensis*, Fig. 3(d)), the cells of the three species were alive during the 112 days. This highlights the remarkable capability of the cyanobacteria to cope with nutrient limitations. In this arena, metabolomic studies could explain the mechanisms helping cyanobacteria thrive in the long-term under nutrient starvation and a potential disturbance in osmotic balance caused by the consumption of nutrients and the excretion of specific metabolites. Indeed, a possible path the organisms can trigger to tackle this challenge is the 'salt-out' strategy, i.e., they produce compatible solutes to lower the internal water potential in the presence of high external salinity.[41]

When looking specifically to the pigment production profiles, it is clear that higher concentrations of C-phycocyanin compared to Chl *a* are produced. This was expected since phycobiliproteins are the major constituents of cyanobacteria and can reach up to 60% of the total protein content.[42] *N. muscorum* was clearly the best Chl *a* and C-phycocyanin producer. Jaiswal *et al.*[25] quantified the pigment content of four cyanobacterium species over a period of 28 days and observed that the Chl *a* content decreased over time, while C-phycocyanin reached the highest level at day 14. In contrast with the present study, Jaiswal *et al.*[25] likely observed higher production of both Chl *a* and C-phycocyanin in *A. variabilis* (Chl *a* = 20–25 μg mg^{-1} DW; C-phycocyanin = 41.07 μg mg^{-1} DW) than in *Nostoc muscorum* (Chl *a* = 15–20 μg mg^{-1} DW; C-phycocyanin = 25–30 μg mg^{-1} DW), with lower C-phycocyanin concentration when compared with the present study. Similarly, Loaiza *et al.*[14] found that two strains of *Anabaena* are better Chl *a* and C-phycocyanin producers than two *Nostoc* strains. However, the values obtained in the exponential phase by Loaiza *et al.*[14] (*Nostoc* sp.: Chl *a* = 2.37–

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2.56 μg mL^{-1}, C-phycocyanin = 11.50–14.01 μg mL^{-1}; *Anabaena* sp.: Chl *a* = 15.04–18.09 μg mL^{-1}, C-phycocyanin = 85.46–102.90 μg mL^{-1}) are also much lower than those obtained here (maxima in the present study: 99 μg mL^{-1} Chl *a* and 1004 μg mL^{-1} C-phycocyanin for *N. muscorum*; 80 μg mL^{-1} Chl *a* and 1019 μg mL^{-1} C-phycocyanin for *A. cylindrica*), even in the first extraction corresponding to day 14. The increase in *A. platensis* pigment content during the first days was corroborated by the study of Kumar *et al.*,[16] although these authors only evaluated their growth during 25 days.

Arthrospira is one of the most well-known cyanobacteria, being widely cultured at the industrial scale and commercialized. It is a source of valuable products, such as proteins, essential amino-acids, vitamins (e.g., B12), C-phycocyanin, *β*-carotene, and *γ*-linolenic acid.[43] However, in our study, the Chl *a* production by *A. platensis* was the lowest throughout the experiment among the three species compared. Other works[27] also show that *Arthrospira* strains are worse phycobiliprotein producers when compared with *Anabaena* and *Nostoc* strains. C-phycocyanin from *A. platensis* is already widely used in several food products (e.g., gums, candies, frosting, ice cream and frozen desserts, coatings, and toppings) due to its blue color, which is very difficult to find in nature.[43] This means that there is a market demand for C-phycocyanin and that species like *N. muscorum* could be a valuable alternative to *Arthrospira*. Other interesting species to include in studies regarding pigment production are *Synechocystis* sp. and *Synechococcys* sp. *Synechocystis* sp. is a commonly used model in genetic editing studies and this technology can be applied to enhance its pigment production, namely of specific carotenoids (i.e., myxoxanthophyll and zeaxanthin).[44] The marine cyanobacterium *Synechococcys* sp. presents different phenotypes with dominance of different pigments.[45] Functional genomic information is available for this species and the genes involved in pigment production are also well known.[46] In this sense, we suggest that industry enlarges the scope of production to an array of species instead of focusing on a single one. The reasoning for this is two-fold. First, culturing conditions are mostly the same for the majority of cyanobacteria, and thus the logistics behind stepping into multi-species culturing within a production facility is certainly not complicated. Second, as proven by this limited comparison among three cyanobacteria species, a given compound of interest would be better produced by a specific species or strain, at a given phase of the culture growth profile under the defined culturing conditions. The results presented herein are consistent with those obtained by Simeunovic *et al.*,[27] who observed that the qualitative and quantitative contents of different types of phycobiliproteins in cyanobacteria are dependent on both the strain and culture media. This is exactly why the systematic assessment of yields of the strains of interest through time is a daunting, but critical task prior to the implementation of industrial exploitation processes to ensure their economic sustainability. In addition, selecting a parameter that correlates feasibly to the production of pigments is very important and would allow for better monitoring of the cultures to select the best period for the pigment extraction.

CONCLUSIONS

In this work, the growth and pigment production of three cyanobacterium species were assessed and compared. All three species showed impressive results, as they were able to grow without supplementation for more than 100 days, raising questions on whether the consumption of nutrients is very low or if there are some cells dying and providing nutrients for the remaining. The use of fluorometric tools allowed for the understanding that, although the cyanobacteria continuously grew, there was a degradation in their health status that was especially prominent for *A. platensis*. In terms of biomass outcome, *A. platensis* showed the best profile. However, *N. muscorum* was proven to be the most interesting species for industrial exploitation concerning pigments because it presented high growth rates along with the best production Chl *a* and C-phycocyanin.

During the growth period, pigment analysis showed a heterogeneous pattern, with peak moments of production for Chl *a* and C-phycocyanin. Unfortunately, the parameters used in this work (i.e., optical density, F_o, and F_v/F_m) did not consistently relate to pigment production, apart from F_v/F_m applying to *A. cylindrica* and *N. muscorum* in some stages of the culture period. Calibration studies involving these parameters and other biomass proxies (e.g., weight or cell number) with the goal of finding a platform for the feasible prediction of pigment production dynamics would be worth future investment with practical applications in the industry. Finally, it is worth remarking that optical density is a very commonly used parameter for these purposes, but the present study demonstrates the inadequacy of such a strategy.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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