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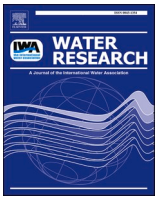

Natural pigments and biogas recovery from cyanobacteria grown in treated wastewater. Fate of organic microcontaminants

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ABSTRACT

Cyanobacterial wastewater-based biorefineries are a sustainable alternative to obtain high-value products with reduced costs. This study aimed to obtain phycobiliproteins and carotenoids, along with biogas from a wastewater-borne cyanobacterium grown in secondary effluent from an urban wastewater treatment plant, namely treated wastewater. For the first time, the presence of contaminants of emerging concern in concentrated pigment extracts was assessed. Tertiary wastewater treatment was conducted in a 3 L photobioreactor inoculated with *Synechococcus* sp., and operated in semi-continuous regime with a hydraulic retention time of 6 days. The carotenoid content was stable (reaching up to 4 mg g DW^{-1}) regardless of the wastewater composition, while the phycobiliprotein content (up to 214 mg g DW^{-1}) varied according to nitrogen availability. In concentrated pigment extracts, only 3 (out of 20) organic microcontaminants were detected. The biochemical methane potential of pigment-extracted biomass (222 NL CH_4 kg VS^{-1}) was still 72 % of raw biomass. In conclusion, a cyanobacteria culture rich in *Synechococcus* sp. appears as a promising source of bio-based products in a circular economy approach.

1. Introduction

Cyanobacteria are photosynthetic microorganisms characterized by a rich metabolism, which can be exploited to recover a wide diversity of high-value bio-based products. Indeed, cyanobacteria are a promising source of natural pigments such as phycobiliproteins and carotenoids, with market values of 1.5 billion USD (Pagels et al., 2021). The most abundant auxiliary pigments contained in cyanobacterial cells are phycobiliproteins, which can be classified depending on their absorption peaks into: phycocyanins (λ_{max}= 610–625 nm), allophycocyanins (λ_{max}= 650–660 nm), and phycoerythrins (λ_{max}= 490–570 nm). In fact, cyanobacteria are mainly cultured to produce these natural pigments, with intense colorations and a wide variety of bioactivities, which are gaining market value in industries such as food, cosmetics, and textile (Arashiro et al., 2020a).

Nevertheless, the economic feasibility of microalgal pigment recovery technologies is still limited, due to the high energy demand and costs of: i) standard culture media components (Arashiro et al., 2020a), and ii) biomass downstream processing (harvesting, dewatering and extraction) (Kouhia et al., 2015). This, together with the threat that the spill of nutrient-rich wastewater can cause to ecosystems (Yen et al., 2013), has led to the development of alternative strategies such as cyanobacterial production in waste streams for biomass valorization in the framework of a circular economy. A biorefinery is based in the production of diverse products, with the generation of minimum residues from a source of raw biomass (Monlau et al., 2021). In the context of wastewater bioremediation, the recovery of pigments and biogas (bioenergy) from cyanobacteria treating wastewater, would contribute to both the profitability and sustainability of the process. Indeed, the use of residual biomass after phycobiliprotein extraction as a substrate for anaerobic digestion is feasible in terms of solvents involved (i.e., distilled water or phosphate buffer) (Ruiz-Domínguez et al., 2019). Furthermore, anaerobic digestion leads to the mineralization of organic nitrogen and phosphorus, which could be recovered in the digestate and applied as biofertilizer in a

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circular bioeconomy approach (Monlau et al., 2021).

To date, most studies on the recovery of pigments from wastewater have used either sterile or diluted wastewater sources (Babu Balaraman et al., 2021; Khatoon et al., 2018; Narindri Rara Winayu et al., 2021). Concerning the recovery of phycobiliproteins along with other co-products from unsterile wastewater, Shahid et al. (2021) obtained 102 mg phycobiliproteins per g dry mass along with lipids by growing cyanobacteria in municipal wastewater, while van den Hende et al. (2016) digested pigment-extracted biomass to recover biogas. In order to assess the stability of the process, which is one of the main challenges of bioproduct recovery from wastewater, Arashiro et al. (2020b) and Senatore et al. (2023) developed pilot-scale semi-continuous experiments coupling tertiary wastewater treatment to the recovery of pigments from cyanobacteria.

The development of a successful cyanobacterial-based biorefinery treating wastewater has to overcome barriers as contamination by green microalgae and wastewater composition fluctuation over time. In this context, there is a need to find strains that: i) accumulate high concentrations of target bioproducts, ii) present faster growth rates than those of the outcompeting microorganisms, to remain the predominant species (Shahid et al., 2021), and iii) are able to cope with changing conditions over time. Thus, wastewater-borne strains are pinpointed as potential candidates, as they are more adapted to wastewater conditions than strains from culture collections.

Among the waste streams that may be used cyanobacterial-biorefineries, the secondary effluent from urban wastewater treatment plants (WWTPs) fulfills the requirements in terms of physicochemical characteristics and nutrient composition. In comparison with raw sewage, this treated effluent promotes the growth of cyanobacteria, given the low chemical oxygen demand (COD), high ammonium ($N—NH_4^+$) and low phosphate ($P\text{-}PO_4^{3-}$) concentrations (Senatore et al., 2023). However, this stream contains harmful compounds such as contaminants of emerging concern (CECs), that are not removed in conventional activated sludge systems. The study of these compounds has for years been focused on environmental contamination, meaning that most of the effort has been put to improve their removal from water bodies. However, their persistence in microalgal biomass and bio-products has barely been assessed. The increasing importance of microalgae in the food sector, has led to the proposal of prospective quality control strategies and regulations (Salehipour-Bavarsad et al., 2024). Thus, novel methodologies for screening CECs in microalgal food supplements have been proposed (Martín-Girela et al., 2020). Despite being crucial for improving the social acceptance of wastewater bio-refineries, the analysis of CECs in wastewater-derived products is almost inexistent. Recent studies have analyzed the presence of CECs in wastewater-based microalgal bio-stimulants (Ruales et al., 2024) and pigment-rich extracts (Bellver et al., 2023). In fact, a preliminary study recovering phycobiliproteins from *Synechocystis* sp. grown in secondary effluent from urban wastewater treatment, showed that only 3 out of 22 CECs detected in this stream were found in crude phycobiliprotein extracts. In particular, only caffeine, carbamazepine and naproxen were detected. Since these compounds are polar, they may remain in the phosphate buffer used for phycobiliproteins extraction (Bellver et al., 2023). However, it remained unclear whether further pigment purification steps could reduce their concentration in the final product.

The aim of the present study is to evaluate the potential of urban secondary effluent as a source of nutrients for the recovery of natural pigments from a wastewater-borne *Synechococcus* sp. in a circular economy approach. This general objective was achieved by the following specific objectives: i) assessing the pigment production potential of *Synechococcus* sp. in unsterile synthetic media, ii) studying *Synechococcus* sp. pollutant removal capacity, biomass growth and pigments production over time in urban secondary effluent, iii) evaluating the biogas production potential of raw and pigment-extracted biomass, and iv) analyzing the persistence of CECs from secondary effluent samples to in concentrated pigment extracts. To the author's knowledge, this is the first time that the presence of these contaminants in concentrated and dialyzed wastewater-derived phycobiliproteins is analyzed.

2. Materials and methods

2.1. Cyanobacterial biomass and pigments production

2.1.1. Strain selection and culture

A strain of *Synechococcus* sp. isolated from photobioreactors treating wastewater, that resembled to *Synechococcus* sp. PCC8966 according to NCBI Genebank database (Rueda et al., 2020), was used in the present study. The inoculum was maintained under sterile conditions in a 1 L photobioreactor at room temperature (25 °C). The culture was grown in BG11, mixed continuously by air bubbling, and kept under white-LED lamps with a light irradiance of 36 μmol m^{-2} s^{-1} and a light:dark cycle of 15:9 h This culture was used as inoculum for batch and semi-continuous experiments (Sections 2.1.2 and 2.1.3, respectively).

2.1.2. Phycobiliprotein production potential

In order to determine the phycobiliprotein production potential of this *Synechococcus* sp. strain under optimum conditions, batch trials in standard culture medium were developed 1 L Erlenmeyer flasks (in triplicate) during 6 days. Non-sterile BG11 (modified with 158 mg $NaHCO_3$ L^{-1} and 971.4 mg $NaNO_3$ L^{-1}) was used as culture medium. Temperature and agitation were the same as described previously (Section 2.1.1). However, light irradiance was fixed at 91 μmol m^{-2} s^{-1}, and pH was daily adjusted to 7.0 by CO_2 injection using a pH meter (GLP 21, CRISON). Cultures were inoculated with an initial volatile suspended solid (VSS) concentration of 0.1 g L^{-1} Inoculation volume was calculated with a regression curve correlating turbidity and the concentration of VSS (g L^{-1}) (R^2= 0.95) (see supplementary material).

2.1.3. Phycobiliprotein production in secondary effluent

The phycobiliprotein production in secondary effluent was quantified in semi-continuous assays, in order to determine the stability of the process over time. In this case, 2.5 L of culture were grown in a 3 L polymethyl methacrylate (PMMA) cylindrical photobioreactor, under the light intensity and light:dark cycle described in 2.1.2. Biomass was continuously stirred at 200 rpm (VELP Scientifica, Usmate, Italy). The pH was maintained between 7.5–9.0 by automatic injection of CO_2, by means of an electro-valve connected to a pH sensor and controller (HI 8711, HANNA instruments, Italy). The standard culture medium was here replaced by unsterile and filtered (0.7 μm) secondary effluent from a municipal WWTP located in Barcelona (Spain).

Initially, biomass grew in batch until a concentration of 0.4 g VSS L^{-1} was achieved. Then, secondary effluent was added (and culture retrieved) in a semi-continuous mode, to maintain a hydraulic retention time (HRT) of 6 days. Culture retrieval was carried out under complete mixing conditions, maintaining the same HRT and cellular retention time in the photobioreactor. The reactor was operated during 3 HRTs (18 days). Over this period, culture broth was retrieved three times per week, after which it was centrifuged at 3300 g for 10 min (centrifuge 5702, Eppendorf). Pellets were preserved in 15 mL Falcon tubes at −21 °C, and freeze-dried for 48 h (ScanVac CoolSafe, LaboGene). This protocol was applied prior to pigment extraction, biogas production tests and CECs analyses for all the biomass samples analyzed. The main physico-chemical parameters of the studied secondary effluent are shown in supplementary material.

2.2. Analytical methods

2.2.1. Physic-chemical analyses

Culture growth/ biomass production was estimated every other day by both measuring turbidity and VSS concentration. Turbidity was measured in aliquots of 10 mL with a nephelometer (HI-93,703, HANNA instruments). VSS were determined following Standard Methods (2540C

and 2540D) (APHA-AWWA-WPCF, 2017). N—NO_3^-, N—NO_2^- and P-PO_4^{3-} contents were measured following Standard Methods (4500-NO_3^-, 4500-NO_2^- and 4500-P), while N—NH_4^+ was measured as described by Solorzano (1969). Nutrient concentrations were measured at the beginning and end of the batch experiment; and every other day during the semi-continuous experiment.

2.2.2. Phycobiliproteins, carotenoids and chlorophyll-a extraction and quantification

The phycobiliprotein extraction protocol was adapted from Arashiro et al. (2020b). 3 mg of dry biomass were added to 1 mL of phosphate buffer, and two freeze-thaw cycles (−21 °C to 4 °C) were developed to disrupt the cells. After disruption, slurries were poured into 1.5 mL Eppendorf tubes and centrifuged at 9500 g for 15 min (centrifuge 5702, Eppendorf). Then, supernatants were recovered and measured at $OD_{562\ nm}$, $OD_{565\ nm}$, $OD_{615\ nm}$, $OD_{620\ nm}$, $OD_{652\ nm}$ and $OD_{280\ nm}$ in a UV–VIS spectrophotometer (UV-11, LanOptics). Analyses were developed in triplicate and phosphate buffer was used as a blank. While phycobiliprotein concentrations were calculated according to Bennett and Bogorad (1973), phycobiliprotein purity ratios were calculated as described by Cuellar-Bermudez et al. (2015) (Eqs. (1)-3):

$$\text{Phycocyanin purity ratio} = OD_{620nm}/OD_{280nm} \quad (1)$$

$$\text{Allophycocyanin purity ratio} = OD_{652nm}/OD_{280nm} \quad (2)$$

$$\text{Phycoerythrin purity ratio} = OD_{565nm}/OD_{280nm} \quad (3)$$

After phycobiliproteins extraction, crude phycobiliprotein extracts were freeze-dried (48 h, under dark conditions) (ScanVac CoolSafe, LaboGene) and stored at −21 °C until CECs analysis. The same protocol was applied to pigment-extracted biomass prior to biochemical methane potential tests. CECs were analysed from pigment crude extracts, concentrated extracts and raw biomass samples.

Carotenoids and chlorophyll-*a* extraction procedures were adapted from Zavřel et al. (2015). Freeze-dried biomass samples (3 mg) were placed in 1.5 mL Eppendorf tubes, and extracted with 1 mL of methanol (4 °C). Pigment concentrations were measured and quantified in UV–VIS spectrophotometer (UV-11, LanOptics) at $OD_{470\ nm}$, $OD_{665\ nm}$ and $OD_{720\ nm}$ following the equations described by Zavřel et al. (2015). Analyses were developed in triplicate and methanol was used as a blank.

All the extractions were developed in triplicate, and the pigment content was expressed as mg of pigment per g of dry weigh (mg g DW^{-1}).

2.2.3. Pigment concentration

To obtain the concentrated phycobiliprotein extracts, crude extracts were resuspended in 25 mL of distilled water, and a two-step precipitation with ammonium sulfate was developed by adapting the methodology described by Burgess (2009). For the first precipitation (proteins other than phycobiliproteins), 20 % ammonium sulfate was added to the mixture under continuous stirring and incubated at 4 °C (30 min). Then, samples were centrifuged at 4 °C (1200 g, 20 min) (centrifuge 5424 R, Eppendorf), supernatants recovered, and phycobiliproteins further precipitated with 60 % ammonium sulfate overnight. Afterwards, pellets were obtained by centrifugation (1200 g, 10 min) (centrifuge 5424 R, Eppendorf), resuspended in distilled water (25 mL) and dialysed (4 °C) against the same solvent using dialysis membranes (Ø 12,000 Da, Sigma-Aldrich). At the end of the procedure, dialyzed and concentrated pigment samples were poured into 15 mL Falcon tubes, freeze-dried (48 h, under dark conditions) (ScanVac CoolSafe, LaboGene) and stored at −21 °C until CECs analysis.

2.2.4. Contaminants of emerging concern analysis

CECs were analysed in secondary effluent, freeze-dried cyanobacterial biomass and pigment samples collected over the experimental period. In order to assess how a precipitation with ammonium sulphate of the pigment extracts could affect the accumulation of CECs, both crude and precipitated phycobiliprotein samples were analysed. For secondary effluent, 3 samples were collected at the beginning, mid and end of the experimental period. The analytical methodologies for quantifying CECs in secondary effluent, biomass, crude and concentrated phycobiliprotein extracts were developed as described by Bellver et al. (2023). Limits of detection (LOD) and quantification for secondary effluent, biomass and crude pigment extract samples are reported in Bellver et al. (2023), while LODs for precipitated pigment extracts, as well as concentrations of CECs for secondary effluent, biomass, crude and precipitated pigment extracts are reported in supplementary material.

2.3. Biogas production from raw and phycobiliprotein-extracted biomass

The anaerobic biodegradability and biogas production potential of cyanobacterial biomass, before (raw biomass) and after (residual biomass) phycobiliprotein extraction, was determined by biochemical methane potential (BMP) tests.

2.3.1. Biochemical methane potential tests

Batch reactors were inoculated with mesophilic, digested sludge from a municipal WWTP located in Barcelona (Spain). Tests were carried out in 160 mL serum bottles, with a working volume of 50 mL (in duplicate). Bottles were inoculated with 5 g volatile solids (VS) L^{-1} of substrate ($VS_{substrate}$) and a $VS_{substrate}$:$VS_{inoculum}$ of 0.5, as described by Arashiro, et al. (2020b). Additionally, blank samples without substrate were run to determine the inoculum-produced background methane; while microcrystalline cellulose (CEL) (Thermo scientific, Germany) was used as a positive control. After filling each reactor with the corresponding volumes of inoculum and substrates, bottles were flushed with helium gas, sealed with butyl rubber stoppers and placed in a platform shaker incubator (OPAQ, Ovan, Spain) at 90 rpm and 35 ± 2 °C. Pressure in each batch reactor was periodically measured with a digital manometer (GMH 3151 Gresinger™, Germany). Biogas composition was determined by calculating the percentage of methane and carbon dioxide in the digester's headspace. Gases were analysed using a gas chromatograph (GC- Trace Thermo Finnigan, U.S.A) equipped with a Thermal Conductivity Detector, which involved injecting the gas samples into a packed column (Hayesep 3 m 1/8 in 100/120). Helium was used as the carrier gas in split less mode, with a flow rate of 19 mL min^{-1}. The oven temperature was set to 35 °C, resulting in a retention time of 2.0 min. The injector and detector temperatures were set at 150 °C and 250 °C, respectively. The system was calibrated using standard methane (99.9 % CH_4, Messer-Griesheim, Germany) and carbon dioxide (99.9 % CO_2, Messer-Griesheim, Germany) by injecting duplicate samples to create a six –point standard curve in the range of 10–100 % for each gas. Measurements were performed until the daily methane production was <1 % of the total accumulated methane production in all batch reactors. Methane yields were corrected to standard conditions (0 °C and 101,3 kPa) over the substrate concentration (VS).

2.3.2. Statistics and kinetics data analysis

The cumulative biochemical methane yield (BMY, NL CH_4 kg VS^{-1}) was modelled using a first-order kinetic model as a function of time t (d), as described in Eq. (4):

$$B = B_0 + [1 - \exp(-kt)] \quad (4)$$

where: B_0 stands for the methane production potential (NL CH_4 kg VS^{-1}), k is the first order kinetic rate constant (d^{-1}), B is the accumulated methane production at time t (NL CH_4 kg VS^{-1}) and t is time (d). The pair of experimental data (B, t) was adjusted by the least-square method using the SOLVER function from Excel. This allows the determination of parameters k and B_0 of each assay.

The error variance (s^2) is estimated by the following Eq. (5):

$$S^2 = \frac{\Sigma(y - x)}{N - k} \quad (5)$$

where: y is the experimental value, x is the value estimated by the model, N is the number of samples and K is the number of model parameters ($K = 2$).

3. Results and discussion

3.1. Phycobiliprotein production potential of Synechococcus sp.

The phycobiliprotein production potential of the wastewater-borne *Synechococcus* sp. grown in standard culture medium was 273.7 mg g DW^{-1} of total phycobiliprotein, corresponding to a phycobiliprotein productivity of 16.5 ± 2.7 mg L d^{-1}. This content was regarded as the phycobiliprotein production potential of this *Synechococcus* sp. strain in unsterile conditions, and used for further comparison with the results obtained in secondary effluent.

Phycocyanin was the most abundant (79 % of the total phycobiliprotein content) and had the highest purity (ratio of 1.3), followed by allophycocyanin (16 %, purity ratio of 0.5) and phycoerythrin (5 %, purity ratio of 0.6). The results obtained fall within the range reported in the literature for *Synechococcus* sp. For instance, a concentration around 100 mg gDW^{-1} of high-purity phycocyanin was achieved by growing *Synechococcus elongatus* in BG11 (Tan et al., 2023), and up to 439 mg g DW^{-1} by growing *Synechococcus* sp. PCC7002 in a culture where N was supplied in form of nitrite and ammonia ions (Lin et al., 2022). The differences between these results may be attributed not only to differences in the extraction methods, but also to the distinctive biology of the strain used.

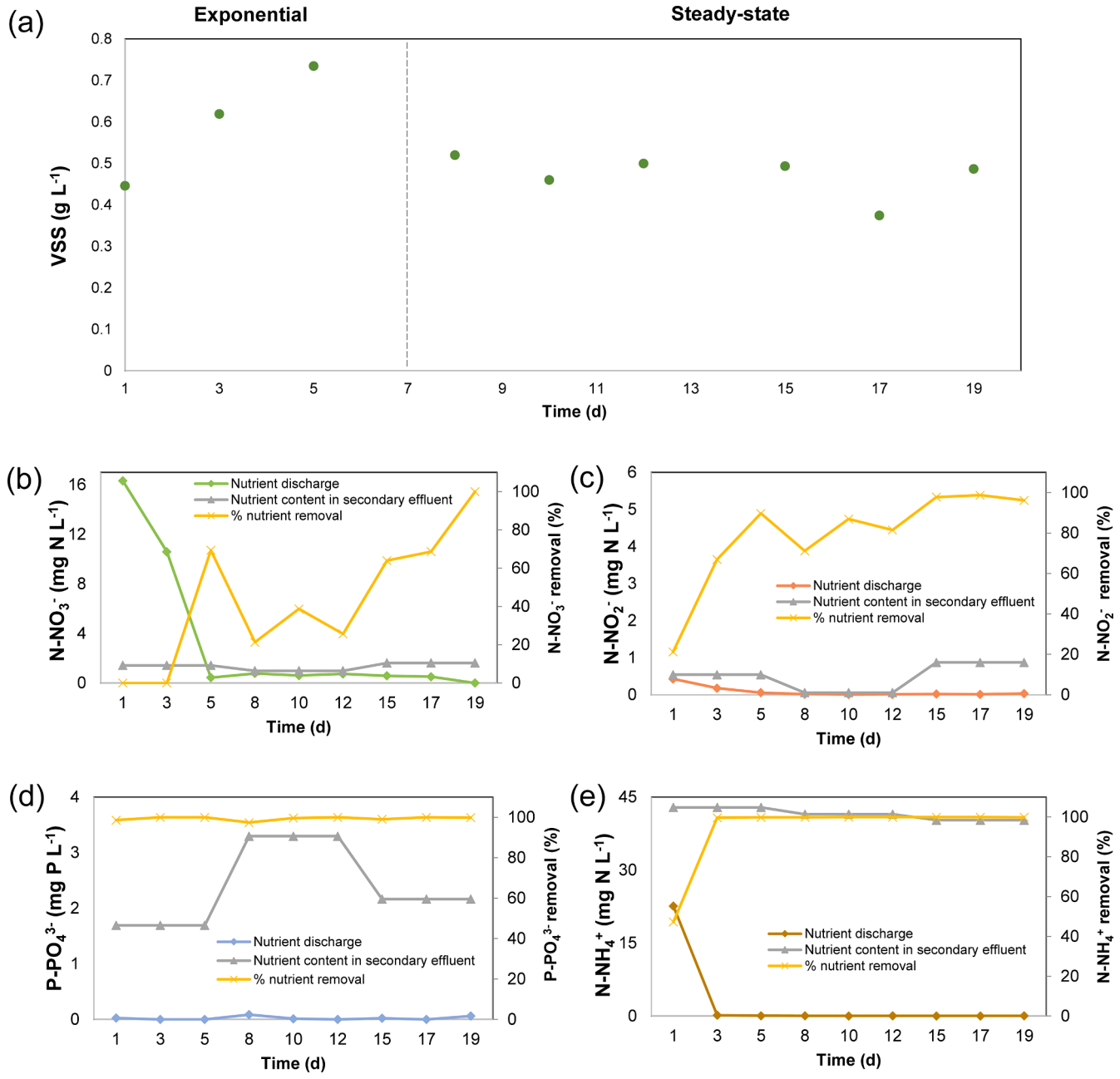

Fig. 1. *Synechococcus* sp. biomass growth (expressed as Volatile Suspended Solids (VSS) concentration) in the semi-continuous reactor (a). Discharge, secondary effluent and removal (%) of nutrients ($N—NO_3^-$, $N—NO_2^-$, $P-PO_4^{3-}$ and $N—NH_4^+$) over time (b, c, d, e).

3.2. *Synechococcus sp. biomass production in secondary effluent*

Upon tertiary wastewater treatment, *Synechococcus* sp. biomass concentration reached a maximum of 0.7 g VSS L^{-1} at operational day 5 (Fig. 1, a). However, from operational day 8 onwards, it stabilized around 0.5 g VSS L^{-1}, and biomass productivity around 0.1 g VSS L^{-1} d^{-1} (Table 1).

Differences between biomass production and growth kinetics before and after reaching the steady state may be attributed to the decrease in nutrient availability, since both N and P were very limited by the time when steady state was reached. These results are in accordance with those described by Senatore et al. (2023), who reached productivities of 108 mg DW L^{-1} d^{-1} by treating secondary effluent with *Synechocystis* sp. (HRT of 6 days), which increased to 173 mg DW L^{-1} d^{-1} by increasing the concentration of nutrients (HRT of 8 days).

Nutrients content and removal efficiencies are shown in Fig. 1. Secondary effluent was characterized by a relatively high and stable concentration of $N—NH_4^+$ over the whole experiment (41.5 ± 1.3 mg N L^{-1}), which was steadily removed with high efficiency (> 99.9 % from day 3 on). Regarding $N—NO_3^-$, $N—NO_2^-$ and $P\text{-}PO_4^{3-}$, secondary effluent had very low concentrations of these nutrients (< 3.5 mg L^{-1}), and removal efficiencies up to 100 and 99 % were attained for $N—NO_3^-$ and $N—NO_2^-$ respectively. Variations on the removal of $N—NO_3^-$ from day 5 to day 15 may be related to the fact that, over this period, the concentration of this nutrient was maintained at very low values. A possible reason for this is that all forms of inorganic nitrogen are reduced to $N—NH_4^+$ prior to cellular uptake. So when both $N—NH_4^+$ and $N—NO_3^-$ are supplied together, cyanobacteria prefer the uptake of former over the latter (Arashiro et al., 2020b). In the case of $P\text{-}PO_4^{3-}$, high removal efficiencies (99–100 %) were sustained over time.

Inhibition by free ammonia in wastewater treatment systems has been related to reduced efficiencies, especially in the case of cyanobacterial monocultures (Rossi et al., 2020). Regarding domestic wastewater characteristics, a promising cyanobacterial candidate for tertiary treatment must have tolerance to high $N—NH_4^+$ concentrations. In fact, results on the growth of *Synechococcus* sp. in $N—NH_4^+$ rich streams are diverse. Indeed, while concentrations above 20 mg $N—NH4^+$ L^{-1} had a negative effect on *Synechococcus* sp. growth (Srimongkol et al., 2019), *Synechococcus* sp. MK568070 showed a stable growth in oil refinery wastewater with concentrations up to 47.6 mg $N—NH_4^+$ L^{-1} (Blažina et al., 2019). Besides, a biomass concentration of 2 g DW L^{-1} was reached by culturing *Synechococcus* sp. NKBG042902 in municipal wastewater with a concentration of 100 mg $N—NH_3^+$ L^{-1} (Aketo et al., 2020). In the present study, the $N—NH_4^+$ concentration in the reactor ranged between 10.3 and 20.6 mg L^{-1}, which enabled a stable cyanobacterial growth over time.

Table 1
Kinetic parameters, biomass and pigment productivities in the semi-continuous experiment. VSS; Volatile Suspended Solids, μ; specific growth rate.

Parameter	Exponential phase (day 1–5)	Steady-state (day 8–19)
VSS (g L^{-1})	0.60 ± 0.14	0.47 ± 0.05
Biomass productivity (g L^{-1} d^{-1})	0.16 ± 0.04	0.08 ± 0.02
μ (d^{-1})	0.36 ± 0.19	0.20 ± 0.06
Duplication time (d)	2.26 ± 1.20	3.79 ± 1.55
Phycobiliprotein productivity (mg L^{-1} d^{-1})	31.12 ± 14.65	6.58 ± 1.61
Chlorophyll-*a* productivity (mg L^{-1} d^{-1})	2.43 ± 0.81	0.83 ± 0.02
Carotenoid productivity (mg L^{-1} d^{-1})	0.62 ± 0.17	0.26 ± 0.07

Thus, the studied cyanobacteria culture seems promising for nutrients removal upon tertiary wastewater treatment.

3.3. *Natural pigments production by Synechococcus sp. in secondary effluent*

The production of phycobiliproteins, chlorophyll-*a* and carotenoids was monitored during the semi-continuous operation of the photobioreactor. Average pigment contents are shown in Fig. 2, while average productivities and purities are shown in Table 1.

Higher values were obtained during the initial exponential phase, as compared to the subsequent steady-state. Specifically, the maximum phycobiliprotein content (214.3 mg g DW^{-1}), phycocyanin purity ratio (1.1) and total phycobiliprotein productivity (41.5 mg phycobiliproteins $L^{-1}d^{-1}$), were achieved at operational day 3. Such high phycobiliprotein content is close to the phycobiliprotein production potential achieved in unsterile synthetic media (273.7 mg g DW^{-1}). However, this elevated phycobiliprotein content was not stable over the experimental period (Fig. 2, a), as it decreased once the $N—NO_3^-$ content in the reactor was depleted. Once the reactor reached the steady-state (day 5), the phycobiliprotein content, productivity and phycocyanin purity ratio stabilized at values around 91 mg gDW^{-1}, 6.6 mg L^{-1} d^{-1} and 0.5, respectively (Table 1).

Cyanobacterial pigment loss (chlorosis) under diverse stress conditions has been previously described, and specifically when induced by N

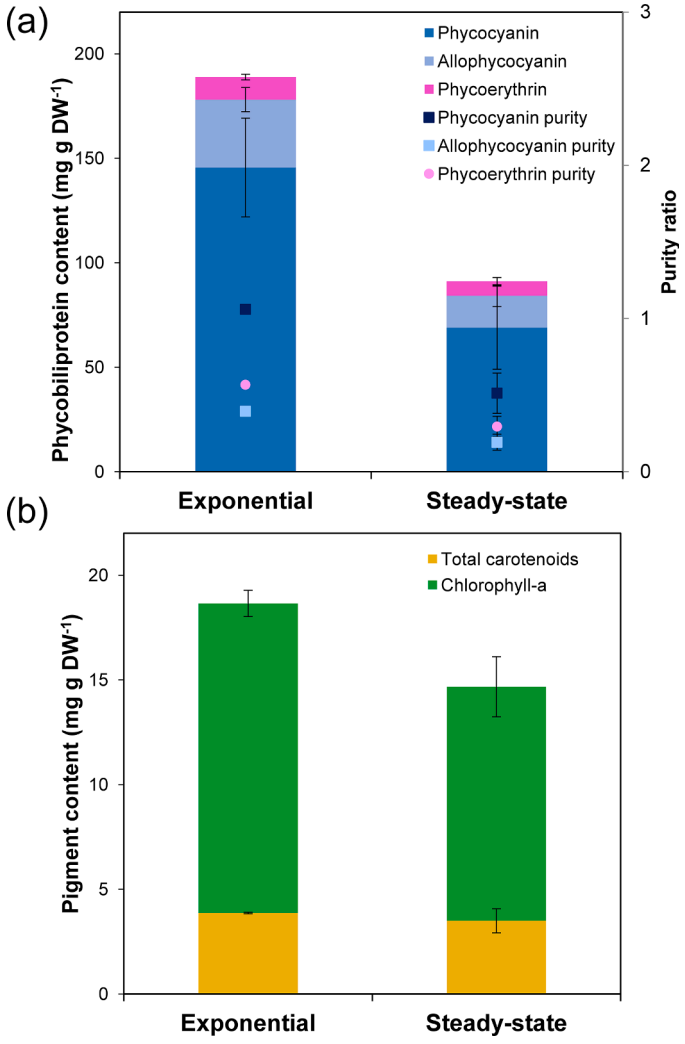

Fig. 2. *Synechococcus* sp. phycobiliprotein (a), carotenoid and chlorophyll-a (b) content over the exponential and steady-state periods.

starvation. Thus, under N stress, cyanobacteria conduct an immediate degradation of the phycobilisomes (which contain the phycobiliproteins), as so as to release amino acids to maintain the protein synthesis (Forchhammer and Schwarz, 2019). In fact, in N-depleted cultures, this process is characterized by rapid phycocyanin loss, that may be followed by chlorophyll-*a* decay. In the present work, this photosynthetic pigment showed a similar trend as phycobiliproteins, but attenuated with a lower decay (Fig. 2, b). Chlorophyll-*a* content in the biomass reached a maximum value of 15.5 mg g DW^{-1} at day 3, which progressively decreased and stabilized at values ranging from 9.3 to 13.6 mg g DW^{-1}. Conversely, the carotenoids content was maintained fairly stable over the whole experiment (2.5–4.1 mg g DW^{-1}).

Even if the photobioreactor was operated in a semi-continuous mode maintaining an HRT of 6 days, inorganic N was depleted. Thus, by the time the culture was retrieved and replaced by secondary effluent, cells could have been N-starved for some hours. In fact, this phenomenon has also been found in microalgae consortia grown in food-processing effluents, in which $N—NH_4^+$ was depleted within 2 days of culture (Amadu et al., 2023). Furthermore, phycobiliprotein degradation to fulfill the nutritional requirements of the wastewater-grown strains BERC03 and BERC04, was associated to the low phycobiliprotein yields achieved (Shahid et al., 2021). Culture bleaching has also been related to different environmental stresses, such as sulphur, iron and P deprivation (Hemlata and Fatma, 2009), as well as salinity increase (Samiotis et al., 2022) or toxicity caused by the presence of herbicides (González-Barreiro et al., 2004). Even though the pigment content reduction in this study is mainly attributed to N scarcity, the toxicity of the wide variety of CECs detected in the secondary effluent, as well the low P supply (Fig. 1, d), may have also contributed to the pigment content evolution over time.

Concerning phycobiliprotein recovery from wastewater reported in the literature, contents up to 237 mg g DW^{-1} with purities reaching 1.14 were obtained in sterile wastewater (Khatoon et al., 2018). On the other hand, total phycobiliprotein and carotenoid contents up to 102 mg g DW^{-1} and $<$ 2 mg g DW^{-1} were recovered from unsterile urban wastewater (Shahid et al., 2021). These values are within those reported in the present study (Fig. 2). The results obtained with *Synechoccoccus* sp. in treated wastewater are close to those obtained in treated swine wastewater, where up to 13 % DW of phycocyanin and allophycocyanin, and up to 2 mg g DW^{-1} of β-carotene were recovered (Narindri Rara Winayu et al., 2021). Additionally, phycocyanin purities showed a maximum of 0.97 with a tendency to decline over time. It is important to note that the aforementioned study only lasted 12 h vs. 18 days in the present one, which was performed in semi-continuous operation and treating undiluted secondary effluent. In the present work, the phycocyanin content in the biomass during the steady-state operation (day 8–19), was on

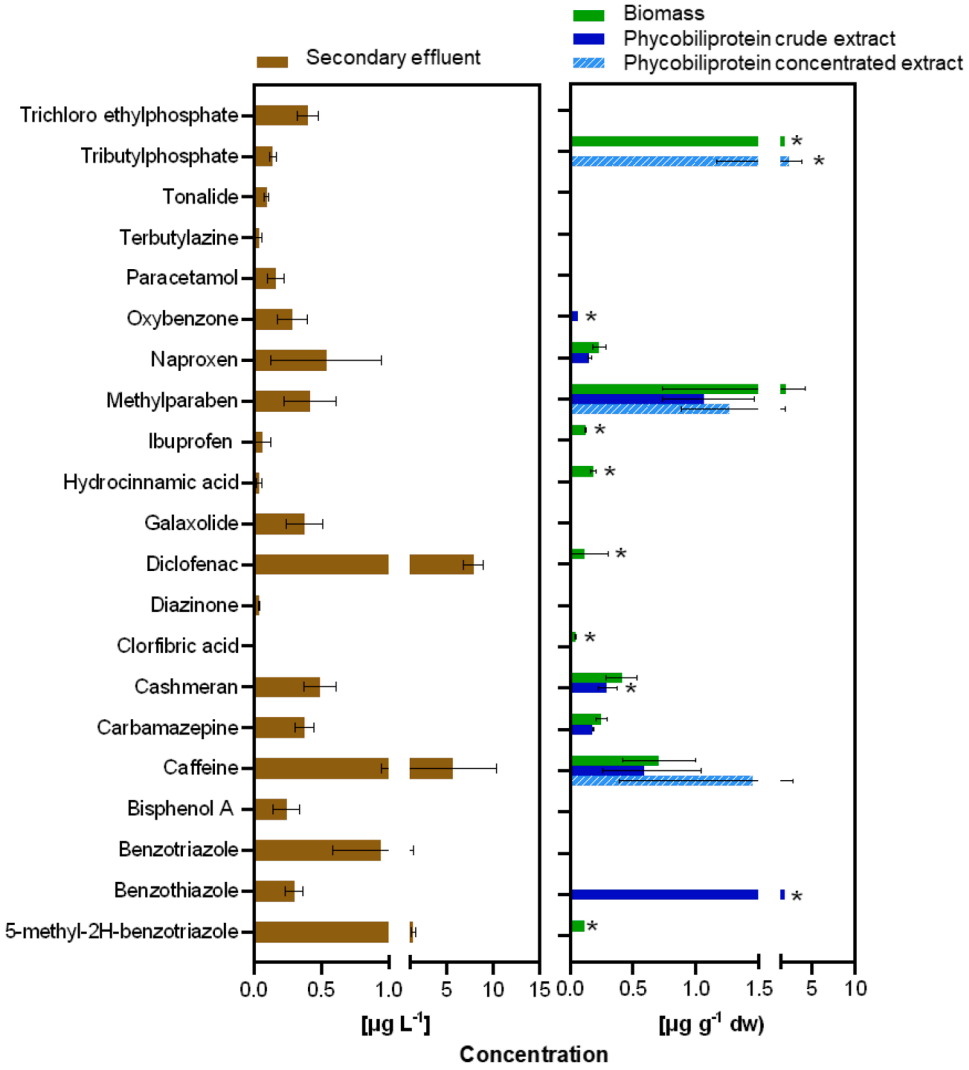

Fig. 3. Mean concentrations of the detected CECs in secondary effluent ($n = 3$). Error bars indicate SD (left). Mean concentrations of the detected CECs in biomass ($n = 5$), pigment extracts ($n = 5$) and phycobiliprotein rich extracts ($n = 3$). Error bars indicate the concentration ranges (right). To calculate means, values <LOD were not considered. (*) indicates that the compound was detected in two or less samples. CECs; Contaminants of emerging concern, LOD; Limit of Detection.

average 68.9 ± 19.9 mg g DW^{-1}. This is in line with the phycocyanin content (74 mg g DW^{-1}) achieved with *Synechocystis* sp. in a 30 L photobioreactor treating secondary effluent in semi-continuous mode (Senatore et al., 2023). As may be expected, higher values (91 mg g DW^{-1}) were achieved in semi-continuous experiments with synthetic media (Zanolla et al., 2022).

To summarise, the studied wastewater-borne *Synechococcus* sp. strain appears as a promising source of natural pigments in a circular bioeconomy approach. According to the results obtained under the operational conditions assayed, it is expected that higher pigment productivities could be achieved by increasing the nutrient loading rate. Thus, further efforts such as reducing the HRT or testing more highly loaded waste streams should be addressed in the future in order to increase phycobiliprotein recovery over time.

3.4. Fate and concentration of contaminants of emerging concern

The concentrations of CECs in the secondary effluent, cyanobacterial biomass, crude and concentrated phycobiliprotein extracts is shown in Fig. 3 and supplementary material. In global, data indicates that while many compounds were present in secondary effluent, only 2 of them passed to the concentrated phycobiliprotein extract.

In the secondary effluent, most of the analysed CECs were detected (20 out of the 29 analysed compounds) (Fig. 3). Moreover, 4 of these compounds presented concentrations higher than 1 μg L^{-1} (namely diclofenac, caffeine, 5-methyl-2H-benzotriazole and benzotriazole). Such concentration levels were also found in a previous study (Bellver et al. 2023). Regarding cyanobacterial biomass, 5 compounds (caffeine, carbamazepine, naproxen, methylparaben and cashmeran) were detected on > 60 % of the samples, and their concentrations ranged from 0.2 to 1.0 μg gDW^{-1}. Interestingly, while some compounds highly present in wastewater, such as caffeine and naproxen, were clearly incorporated into the biomass, other abundant compounds in wastewater, like diclofenac or 5-methyl-2H-benzotriazole, were scarcely detected in the biomass. In the case of diclofenac, photodegradation is the most plausible explanation for its limited presence in cyanobacterial biomass, as this compound has been proven to be light-sensitive in previous studies (Zhang et al., 2017; Wu et al., 2022). Conversely, for 5-methyl-2H-benzotriazole, the low concentration detected in biomass may be explained by limited sorption. Previous studies have also reported low adsorption, minimal bioaccumulation and partial removal of benzotriazole by microalgae (Matamoros et al., 2015; Wu et al., 2022). In contrast, compounds such as cashmeran or methylparaben were found in biomass, even if they presented lower concentrations in treated wastewater (Fig. 3). In the case of cashmeran, a synthetic musk, this was most likely due to its high hydrophobicity (log Kow of 4.5 (Rimkus, 1999), which may have triggered the sorption on the biomass. In fact, musks are known to partition into wastewater solids (Smyth et al., 2007), and a previous study found that they bioaccumulated in microalgal biomass (Matamoros et al., 2015). For methylparaben, its presence in biomass may be explained by its moderate biodegradability (Matamoros et al., 2015), its continuous presence in treated wastewater, and a potential microalgae bioaccumulation from the water fraction (Mustafa et al., 2021).

Regarding crude pigment extracts, only caffeine, methylparaben and naproxen were incorporated in >60 % of the samples, while the rest of detected compounds were only detected on <40 % of the samples. The concentrations of these three compounds on the extracts (0.1 to 1 μg g DW^{-1}) were close to the ones found on the biomass. As the extraction was made with phosphate buffer at pH = 7, a possible explanation would be that only compounds with polar properties at that pH were extracted. In this sense, caffeine and methylparaben are polar in its neutral state and naproxen is ionized at pH=7 (pK_a=4).

In a previous study, it was hypothesized that, as the crude pigment extracts analysed (obtained from *Synechocystis* sp.) missed the precipitation steps, even lower concentrations or no presence of CECs could be expected on the concentrated extract (Bellver et al., 2023). Thus, in the present study, phycobiliprotein precipitation was tested (see Section 2.2.3). It was observed that caffeine and methylparaben still remained in the concentrated extracts in similar or even higher concentration ranges (0.4 to 3.0 μg g DW^{-1}). This could be related to the fact that caffeine and methylparaben were specifically bound on the phycobiliproteins, while naproxen –which is known to bind strongly to serum protein (Mortensen et al., 1979)- probably remained with the rest of proteins that were removed in the first precipitation step (20 % of ammonium sulfate). The presence these two CECs on the concentrated pigments indicates that other non-measured contaminants could also be present, which could limit the usage of those pigments for human applications. Nonetheless, the concentrations of the measured compounds were very similar to the ones found in crops grown in peri-urban agriculture, which were assessed to not pose a human health risk for consumption (Margenat et al., 2019). This suggests that such contaminants concentrations were low enough to allow the usage of the concentrated pigments for the formulation of dyes, among other potential uses. In any case, the tracking of other contaminants together with further purification techniques could be explored in order to clarify the potential market for these pigments.

3.5. Biogas production by anaerobic digestion of pigment-extracted biomass

The production of biogas from residual biomass after the extraction of phycobiliproteins was studied to evaluate the potential recovery of bioenergy along with the pigments. The results of BMP tests with raw and pigment-extracted biomass are shown in Table 2 and Fig. 4.

Piment-extracted biomass methane yield was 222.1 NL CH_4 kg VS^{-1}, being 72 % of the methane yield from unextracted (raw) biomass (314.9 NL CH_4 kg VS^{-1}). As phycobiliproteins are anaerobically biodegradable, the lower methane yield achieved after pigment extraction may be related to the decrease in biodegradable organic matter. Indeed, the extraction method may have resulted in the release of both soluble and membrane-bound proteins, reducing the concentration of soluble organic matter. Nevertheless, the methane yield was still 72 % that of raw biomass, even after the recovery of a value-added bio-based product along with biogas.

Regarding the kinetics of biogas production, the anaerobic digestion of pigment-extracted biomass was significantly faster than raw biomass (> 19 %), indicating significantly higher degradation rates (p-value = 0.005). Indeed, pigment-extracted biomass reached 96 % of the final methane yield after 8 days, compared to raw biomass that achieved the same methane yield after 10 days. This has practical implications upon full-scale operation in terms of HRT, hence anaerobic digester volume and costs.

The results of this study are in accordance with other biochemical methane potential tests using microalgal biomass as a substrate (Passos and Ferrer (2015); Ansari et al. (2017)). However, in some cases the

Table 2
Final methane yield, methane content and kinetic constant (k) of *Synechococcus* sp. biomass grown in secondary effluent, before (raw biomass) and after phycobiliprotein extraction (pigment-extracted biomass). Significant differences between samples are shown by letters a and b (p-value < 0.05). NL; Normal Liters.

	Sample	Final methane yield (NL CH_4 kg VS^{-1})	Methane content (%)	First-order kinetics constant k (d^{-1})
Raw	biomass	314.9 ± 7.0*	75.5 ± 0.48ns	0.337 ± 0.03 (R^2 = *0.992*)**
Pigment-extracted	biomass	222.1 ± 10.2*	76.3 ± 0.08ns	0.402 ± 0.04 (R^2 = *0.989*)**

* Significant at $p < 0.05$; ** highly significant at $p < 0.01$; ns = non significance.

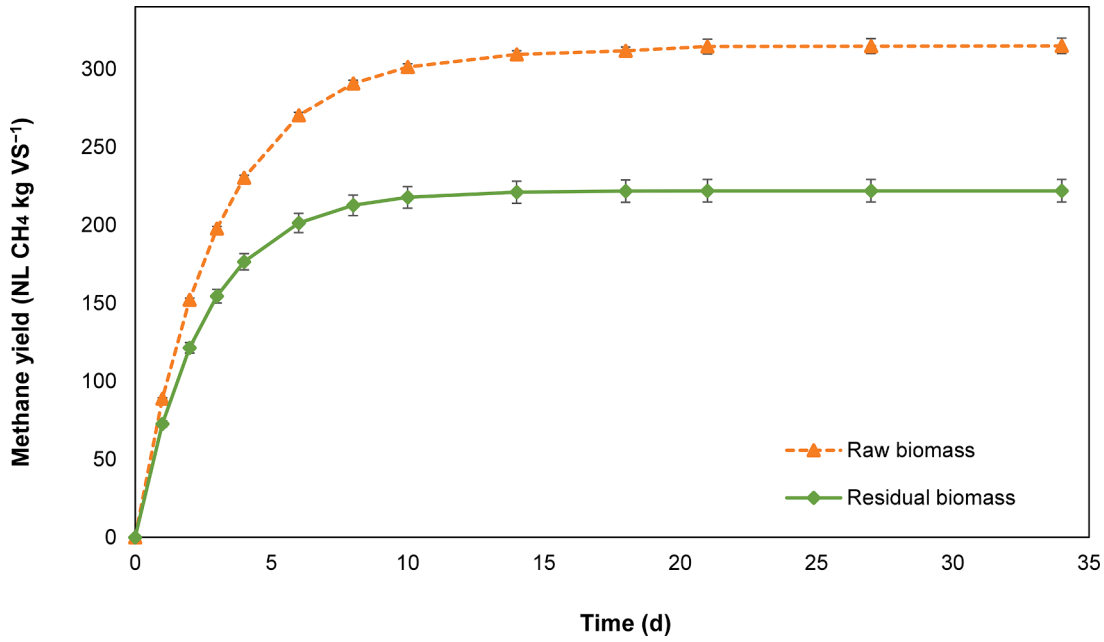

Fig. 4. Methane yield of *Synechococcus* sp. biomass grown in secondary effluent, before (raw biomass) and after phycobiliproteins extraction (pigment-extracted biomass). NL; Normal Liters.

methane yield increased after pigments extraction. For instance, Meixner et al. (2018), van den Hende et al. (2016) and Arashiro et al. (2020b) reported 81 %, 89 % and 6.7 % higher methane yield from pigment-extracted biomass in comparison with raw biomass (348 vs. 429 NL CH_4 kg VS^{-1}, 272 vs. 305 NL CH_4 kg VS^{-1} and 187 vs. 153 NL CH_4 kg VS^{-1}, respectively). This could be related to the fact that the cyanobacterial peptidoglycan layer is highly variable depending on the strain and highly dependent on the specific downstream processes applied. On the one hand, downstream processing can break down the structure of the cyanobacteria biomass and increase the soluble organic matter, making the residual biomass more digestible and increasing the methane yield. On the other hand, the removal of compounds upon downstream processing can decrease the organic matter availability, hence the methane yield. This may explain the higher methane yield obtained from *Synechococcus* sp. biomass before than after pigment extraction (314.9 vs 222.1 NL CH_4 kg VS^{-1}). However, the process kinetics were enhanced, meaning that pigment extraction enhanced the hydrolysis of biomass.

To date, some cyanobacterial biorefineries treating waste streams have been tested at pilot or demonstration scale. Díez-Montero et al. (2020) run a demonstrative-scale biorefinery with a mixed culture dominated by *Synechococcus* sp. treating agricultural runoff. Harvested biomass underwent a thermal pretreatment and achieved an average methane yield (240 NL CH_4 kg VS^{-1}) close to those obtained in the present study. However, the commercial-scale implementation of cyanobacterial biorefineries is still in its early stages, because of challenges associated with production processes and downstream strategies. In this context, the valorization of residual biomass after bio-based products extraction for biogas production may lead to more efficient utilization of the resources. The combination of both bio-based products and bioenergy recovery may improve the sustainability of the process from a technical, economic and environmental point of view.

4. Conclusions

The studied cyanobacteria culture successfully reduced the nutrients concentration, while producing biomass for natural pigments and biogas recovery. Promising results were achieved over the semi-continuous operation of the photobioreactor (18 days), with a total carotenoid content that was stable over time, reaching up to 4 mg g DW^{-1}. The maximum phycobiliprotein content was 214 mg g DW^{-1}, which was limited by nutrients depletion over time. Out of 20 CECs detected in the secondary effluent, only caffeine, methylparaben and tributylphosphate remained in the concentrated extracts. Still, it should be noticed that their presence may restrict the commercial application to markets other than food. Finally, the methane yield of phycobiliprotein-extracted biomass (72 % of raw biomass) showed how it may contribute to the sustainability of the biorefinery. To increase biomass production, future studies could focus on the supplementation of the secondary effluent with other nutrient-rich residues, such as digestate.

CRediT authorship contribution statement

Marta Bellver: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Evelyn Ruales:** Writing – original draft, Methodology, Investigation, Formal analysis. **Rubén Díez-Montero:** Writing – review & editing, Resources, Conceptualization. **Mónica Escolà Casas:** Writing – original draft, Validation, Formal analysis, Conceptualization. **Víctor Matamoros:** Writing – review & editing, Resources, Conceptualization. **Ivet Ferrer:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

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.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2024.123005.

Data availability

Data will be made available on request.

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