



Phycobiliprotein recovery coupled to the tertiary treatment of wastewater in semi-continuous photobioreactors. Tracking contaminants of emerging concern.

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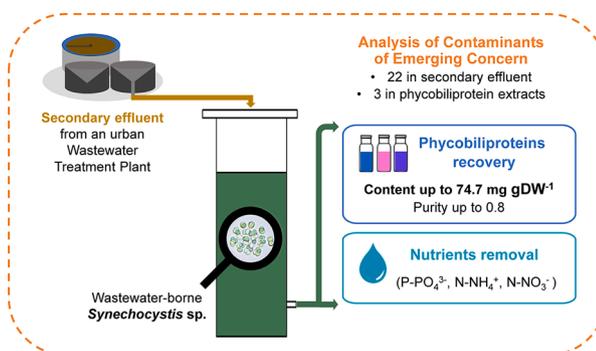
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HIGHLIGHTS

- *Synechocystis* sp. was used to recover pigments upon tertiary wastewater treatment.
- Nutrients were efficiently removed from secondary effluent by *Synechocystis* sp.
- Phycobiliprotein production potential in synthetic medium was 129.4 mg gDW⁻¹.
- Phycobiliprotein content in secondary effluent was stable, reaching 74.7 mg gDW⁻¹.
- Out of 22 organic microcontaminants, only 3 were detected in pigment rich extracts.

GRAPHICAL ABSTRACT



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ABSTRACT

This study evaluated a tertiary wastewater treatment technology using cyanobacteria to recover value-added phycobiliproteins. The presence of contaminants of emerging concern (CECs) in wastewater, cyanobacterial biomass and pigments recovered were also analyzed. For this, a wastewater-borne cyanobacterium (*Synechocystis* sp. R2020) was used to treat secondary effluent from a municipal wastewater treatment plant, with and without nutrients supplementation. Then, the stability of phycobiliprotein production was assessed by operating the photobioreactor in semi-continuous mode. Results showed similar biomass productivity with and without nutrients supplementation (153.5 and 146.7 mg L⁻¹ d⁻¹, respectively). Upon semi-continuous operation, the phycobiliprotein content was stable and reached up to 74.7 mg gDW⁻¹. The phycocyanin purity ratio ranged from 0.5 to 0.8, corresponding to food grade (>0.7). Out of 22 CECs detected in secondary effluent, only 3 were present in the phycobiliprotein extracts. In order to identify applications, prospective research should focus on CECs removal during pigment purification.

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1. Introduction

Most of the colorants available in the market are chemically derived, non-biodegradable and even hazardous to human health (Saini et al., 2018). This fact, along with society's increasing interest towards sustainable products, has been translated in a boosting demand of biobased pigments in the market (Christaki et al., 2015). Phycobiliproteins are natural pigments presenting tetrapyrrole chromophores (bilins), that differentiate them into three main groups: phycocyanins, allophycocyanins and phycoerythrins. From a commercial point of view, the intense colors and bioactivities (anti-cancer, anti-inflammatory, antioxidant) described in these pigments, enable their potential application in food, cosmetic, pharmaceutical and textile industries (Arashiro, 2020b). The main source of phycobiliproteins are cyanobacteria and certain algae. In fact, these compounds are valorized as a major source of income derived from cyanobacterial metabolism, being phycocyanin market size of about \$100 million (USD) (Pagels et al., 2021). Although using synthetic cultivation media under optimized conditions has been related to the production of high levels of phycobiliproteins using cyanobacterial biomass, its use is not yet cost-effective (Arashiro, 2020a; Khatoon et al., 2020).

Recycling nutrients from wastewater has been proposed as a sustainable, economical approach to recover cyanobacterial bioproducts by decreasing the use of freshwater and fertilizers, limiting environmental contamination caused by nutrient discharge, and reducing production costs by 50% (Shahid et al., 2021). Regarding phycobiliprotein production in waste-streams, different effluents have been investigated as nutrient source, such as aquaculture (Cardoso et al., 2021; Khatoon et al., 2020), dairy (Ma et al., 2023), tannery (Urbina-Suarez et al., 2022) or municipal (Arashiro et al. 2020b; Shahid et al., 2021) wastewaters. Secondary effluents (SE) from urban wastewater treatment plants (WWTPs) are promising for cyanobacterial culture as they: i) present low levels of organic carbon, but are rich in inorganic nutrients (N and P) (Ruiz et al., 2011), ii) are characterized by reduced odors, iii) are continuously generated, and iv) present low turbidity (Wang et al., 2017), enabling light transmission through the culture. The production of cyanobacterial high-value products in these streams may lead to higher productivities and to the generation of cleaner treated water; however, to the authors knowledge, there is a lack of research about phycobiliprotein recovery in SE.

Despite the potential of urban SE as a nutrient source, it is important to note that it may contain contaminants of emerging concern (CECs), including pharmaceuticals, personal care products, pesticides, and industrial chemicals. This is due to the fact that WWTPs have not been specially designed to effectively remove these pollutants (Petrie et al., 2015). Consequently, SE is also related to the discharge of CECs into the environment, potentially causing harmful effects in ecosystems (Vale et al., 2022). Similarly, if SE is used for cyanobacterial cultivation, CECs may accumulate in the biomass and pose a potential human or animal health threat if the resulting product is used in food or feed production. Regarding the monitoring and regulation of these substances, although it has not extensively been approached, the recast Drinking Water Directive included in its first watchlist two endocrine-disrupting CECs (17-beta-estradiol and nonylphenol) (Directive 2020/2184, 2020). Concerning water reuse, tracking CECs is of utmost importance not only to further develop regulations and establish realistic prospective applications for the recovered bioproducts, but also to enhance their social acceptance. Although the screening of CECs has been developed in microalgal bioproducts such as food supplements (Martín-Girela, 2020), it has never been examined in wastewater-derived pigment extracts.

The aim of this study is to assess the phycobiliprotein production capacity of the wastewater-borne cyanobacterium *Synechocystis* sp. R2020 in SE. This strain, may bring together the robustness of *Synechocystis* genera and the adaptation to wastewater conditions. The specific objectives are: (i) to evaluate the feasibility of using SE as source of nutrients, (ii) to assess the stability of pigments production of

Synechocystis sp. R2020 in a semi-continuous photobioreactor, and (iii) to evaluate the occurrence of CECs in SE, cyanobacterial biomass and pigment-rich extracts obtained, to foresee potential risks of their use. To the author's knowledge, this is the first time that these contaminants are analyzed in bioproducts derived from wastewater sources.

2. Material and methods

2.1. *Cyanobacterium inoculum*

Experiments were developed using an unicyanobacterial inoculum of the wastewater-borne strain *Synechocystis* sp. R2020 (GenBank database access number: MN493570.1), which was isolated from a microalgae-based WWTP by direct streaking or serial dilution in agar plates (Rueda et al., 2020). The strain used in this study was selected for its adaptability to wastewater (as it was isolated from waste streams), and for its proven capability to accumulate high-value bioproducts as polyhydroxybutyrate (Rueda et al., 2022). The inoculum was maintained in 1 L Erlenmeyer flasks at 25 °C, using sterile BG11 medium. Continuous agitation was ensured by sterile air pumping. Cool-white LED lamps were used to illuminate the cultures at an intensity of 36 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and a 15:9h light:dark cycle. This light:dark cycle mimics light regimes in natural habitats during the summer season, and was proposed in previous studies using this strain (Rueda et al., 2020; Rueda et al., 2022).

2.2. Experimental set-up and operation conditions

2.2.1. Phycobiliprotein production potential

Firstly, a batch experiment was developed out to estimate the endogenous phycobiliprotein production potential of *Synechocystis* sp. R2020 in unsterile conditions. For this, *Synechocystis* sp. R2020 was cultured in three 1 L Erlenmeyer flasks (triplicates), filled with 850 mL of unsterile modified BG11 (with a N-NO_3^- content of 160 mg N L^{-1}) for 6 days. The flasks were inoculated by measuring the turbidity of the inoculum at the beginning of the experiment, which was correlated to the concentration of volatile suspended solids (VSS) ($R^2 = 0.93$) (see supplementary material). The necessary amount of inoculum to reach 100 mg VSS L^{-1} in the flasks was retrieved by centrifuging the culture (3,300 g 10 min), and by diluting the pellet with 850 mL of modified BG11 medium. Continuous air pumping ensured culture agitation. Temperature was maintained at 25 °C, while light was provided by cool-white LED lamps at an intensity of 91 $\mu\text{mol m}^{-2} \text{s}^{-1}$. This irradiance was adopted for favouring protein and phycobiliprotein production in other cyanobacterial cultures (Ma et al., 2015). The light:dark cycle was maintained at 15:9h. In order to avoid phosphorus (P) precipitation, pH was daily adjusted to 7. For this purpose, carbon dioxide (CO_2) ($\geq 99.998\%$ (v/v), Nippon Gases) was pumped at a pressure of 0.2 MPa until the setpoint was reached. The position of Erlenmeyer flasks was changed daily to ensure homogeneous illumination.

2.2.2. Phycobiliprotein production in treated wastewater

Experiments using treated wastewater as nutrient source were carried out in cylindrical photobioreactors made of polymethyl methacrylate (PMMA) with a total volume of 3 L and a working volume of 2.5 L (Fig. 1). Temperature, light intensity and light:dark cycles were maintained as described previously (Section 2.2.1). Complete culture agitation at 200 rpm was achieved by magnetic stirring (VELP Scientifica, Usmate, Italy). pH was maintained between 7.5 and 9 with a pH controller (HI 8711, HANNA instruments, Italy). When pH was ≥ 9 , the controller opened an electro-valve, allowing for CO_2 ($\geq 99.998\%$ (v/v), Nippon Gases) diffusion into the culture. When the culture reached 7.5, the electro-valve was closed and the gas flow stopped. The position of the photobioreactors was changed daily to ensure homogeneous illumination.

The photobioreactors were fed with SE from an activated sludge WWTP in Barcelona Metropolitan Area. The effluent was weekly

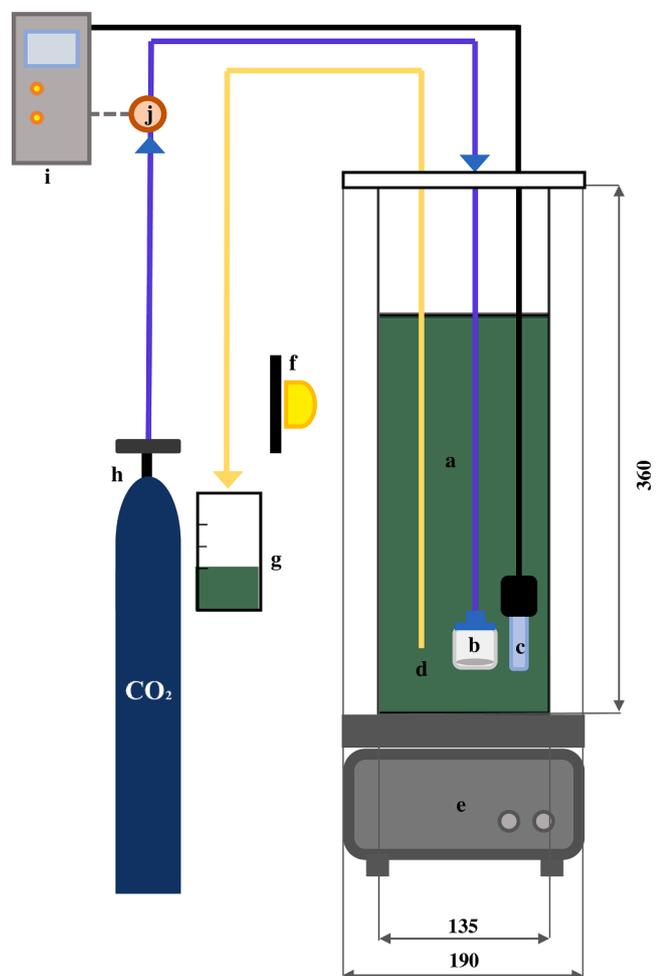


Fig. 1. Scheme of the experimental set-up. photobioreactor (a), CO₂ diffuser (b), pH sensor (c), sampling tube (d), magnetic stirrer (e), led lamp (f), harvesting vessel (g), CO₂ column (h), pH controller (i), electro-valve (j). Units in mm.

collected from the WWTP, filtered through a 0.7 μm pore glass microfiber filter, physico-chemically analyzed and stored (for a maximum of 4 days) at 4 $^{\circ}\text{C}$ until use. This WWTP performs a secondary treatment to remove organic matter but not nutrients, according to the limits of discharge in areas without eutrophication risk. Thus, SE was characterized by a low concentration of chemical oxygen demand (COD) and high concentration of ammonium (N-NH_4^+), as shown in Table 1. Although the SE was filtered, it was not sterilized nor disinfected prior to the experiments.

Firstly, a batch experiment was performed to assess whether the phycobiliprotein production could be enhanced by the supplementation of nutrients to SE. For this purpose, *Synechocystis* sp. R2020 was cultured using either SE or SE supplemented with N and P (SE + nutrients). In the latter case, the initial concentrations of nutrients were 100 mg L^{-1} of Total Inorganic Nitrogen (TIN) and 8 mg L^{-1} of phosphate. To obtain these conditions, 53.6 $\text{mg N-NO}_3^- \text{L}^{-1}$ were added to the SE as NaNO_3 and 6.7 $\text{mg P-PO}_4^{3-} \text{L}^{-1}$ as K_2HPO_4 . In order to inoculate the reactors with an initial biomass concentration of 100 mg VSS L^{-1} and remove any residual nutrients from BG11, *Synechocystis* sp. R2020 inoculum was previously centrifuged at 3,300 g for 10 min (as described in Section 2.2.1). This experiment lasted 4 days, until NH_4^+ and P-PO_4^{3-} were depleted from the SE culture.

Next, in order to assess whether the phycobiliprotein content was maintained over time, the photobioreactor was operated in semi-continuous mode, with a hydraulic retention time (HRT) of 5 days.

Table 1

Water quality parameters of the SE used in batch and semi-continuous experiments. SE; secondary effluent. SE + nutrients; secondary effluent supplemented with N and P. TSS; Total Suspended Solids. VSS; Volatile Suspended Solids. EC; Electrical Conductivity. TIN; Total Inorganic Nitrogen. DIC; Dissolved Inorganic Carbon. sCOD; Soluble Chemical Oxygen Demand.

Parameter	Batch experiment		Semi-continuous experiment
	SE	SE + nutrients	SE
TSS (mg L^{-1})	36.3	36.3	26.1
VSS (mg L^{-1})	30.3	30.3	21.8
Turbidity (NTU)	30.3	30.3	21.9
pH	7.6	7.6	7.3
EC (mS cm^{-1})	2.5	2.5	2.7
N-NH_4^+ (mg N L^{-1})	45.0	45.9	42.2
N-NO_3^- (mg N L^{-1})	0.7	56.2	1.2
N-NO_2^- (mg N L^{-1})	0.7	0.6	0.3
TIN (mg N L^{-1})	46.4	102.7	43.7
P-PO_4^{3-} (mg P L^{-1})	1.3	8.3	2.7
DIC (mg C L^{-1})	63.4	63.4	74.4
sCOD ($\text{mg O}_2 \text{L}^{-1}$)	58.0	58.0	53.5

This experiment lasted 12 days, following 4 previous days under batch conditions. The recovered biomass and the extracted pigments were analyzed for the determination of CECs.

2.3. Analytical methods

2.3.1. Physico-chemical parameters

Biomass growth was estimated by measuring the turbidity and the concentration of VSS of the culture. Turbidity was measured by placing a 10 mL sample in a turbidity meter (HI93703, HANNA Instruments). By filtering a 20 mL sample through a 0.7 μm pore glass microfiber by a vacuum system, VSS were determined.

In batch experiments, all physico-chemical parameters were measured at the beginning and at the end of the experiment (except for turbidity and pH, which were measured daily). In the semi-continuous experiment, all parameters were measured every 2–3 days, except for sCOD that was measured once a week. 0.7 μm pore glass microfiber filters were used to filter samples before measuring sCOD, nutrients (N and P) and alkalinity. Photometric test kits were used for alkalinity (513230BT and 513210BT, Lovibond) and sCOD (2420721, Lovibond) determination, and N-NH_4^+ was determined as described by Solorzano (1969). The remaining physico-chemical parameters were measured as described in Standard Methods (APHA-AWWA-WPCF, 2017). Total suspended solids (TSS) and VSS were determined following 2540C and 2540D methods. N-NO_2^- , N-NO_3^- and P-PO_4^{3-} contents were assessed by 4500- NO_2^- , 4500- NO_3^- and 4500-P methods, respectively. The addition of N-NO_3^- , N-NO_2^- and N-NH_4^+ contents was calculated to obtain TIN.

Alkalinity measurements were used to calculate the dissolved inorganic carbon (DIC) content according to Rueda et al. (2022). The duplication time (d), specific growth rate (d^{-1}), biomass to nutrients yields ($Y_{X/\text{N-NO}_3}$, $Y_{X/\text{P-PO}_4}$, $Y_{X/\text{N-NH}_4}$) and specific consumption rates (q_{P} , q_{N}) were calculated as described by Rueda et al. (2022).

2.3.2. Phycobiliprotein extraction and quantification

The biomass collected over the batch and semi-continuous experiments was centrifuged (3,300 g, 10 min), and pellets were frozen at -20 $^{\circ}\text{C}$ under dark conditions. Then, biomass was freeze-dried during 48 h under dark conditions (ScanVac CoolSafe, LaboGene), and weighed before phycobiliprotein extraction and CECs analysis. The methodologies for phycobiliprotein extraction and quantification were adapted from Arashiro et al. (2020b) and Zavřel et al. (2018).

Firstly, the addition of phosphate buffer (pH 7, 0.1 M) was performed at a proportion of 1:200 (w:w, freeze-dried biomass: solvent). Afterwards, a freeze-thawing process was developed. Following, bead beating was performed by adding glass beads to each sample tube (0.3 g of ϕ 0.1 mm and 0.7 g of ϕ 2 mm), and shaking them at 3,200 rpm for 10 min at

4 °C (Vortex-Genie™ 2, Scientific Industries SI™). Then, mixtures were centrifuged (9,500 g, 15 min) and supernatants collected. In order to assess the phycobiliprotein concentration, the supernatant's absorbances were measured by spectrophotometry (Lan Optics, UV-11) at OD_{562nm}, OD_{565nm}, OD_{615nm}, OD_{620nm}, OD_{652nm} and OD_{280nm}, and related to Equations (Eqs. (1)–(3) (Bennett and Bogorad, 1973):

$$\begin{aligned} \text{Phycocyanin (mg mL}^{-1}\text{)} &= \\ &= \frac{\text{OD}_{615\text{nm}} - 0.474 \cdot \text{OD}_{652\text{nm}}}{5.34} \end{aligned} \quad (1)$$

$$\begin{aligned} \text{Allophycocyanin (mg mL}^{-1}\text{)} &= \\ &= \frac{\text{OD}_{652\text{nm}} - 0.208 \cdot \text{OD}_{615\text{nm}}}{5.09} \end{aligned} \quad (2)$$

$$\begin{aligned} \text{Phycocerythrin (mg mL}^{-1}\text{)} &= \\ &= \frac{\text{OD}_{562\text{nm}} - 2.41 \cdot \text{phycocyanin} - 0.849 \cdot \text{allophycocyanin}}{9.62} \end{aligned} \quad (3)$$

The purity grade of phycobiliproteins is evaluated by the ratio between the amount of a certain phycobiliprotein and the total amount of proteins in the extract (Lauceri et al., 2019). Purity ratios were calculated as OD_{620nm}/OD_{280nm} for phycocyanin, OD_{652nm}/OD_{280nm} for allophycocyanin and OD_{565nm}/OD_{280nm} for phycocerythrin (Cuellar-Bermudez et al., 2015), since OD_{280nm} is related to the total amount of proteins. Extractions were developed in triplicate, under dark conditions.

The phycobiliproteins content (mg gDW⁻¹) was calculated from Eqs. (4)–(6), and the total phycobiliprotein content as the sum of phycocyanin, allophycocyanin and phycocerythrin contents.

$$\begin{aligned} \text{Phycocyanin content in biomass (mg gDW}^{-1}\text{)} &= \\ &= \frac{\text{phycocyanin (mg mL}^{-1}\text{)} \cdot \text{phosphate buffer (mL)}}{\text{DW(g)}} \end{aligned} \quad (4)$$

$$\begin{aligned} \text{Allophycocyanin content in biomass (mg gDW}^{-1}\text{)} &= \\ &= \frac{\text{allophycocyanin (mg mL}^{-1}\text{)} \cdot \text{phosphate buffer (mL)}}{\text{DW(g)}} \end{aligned} \quad (5)$$

$$\begin{aligned} \text{Phycocerythrin content in biomass (mg gDW}^{-1}\text{)} &= \\ &= \frac{\text{phycocerythrin (mg mL}^{-1}\text{)} \cdot \text{phosphate buffer (mL)}}{\text{DW(g)}} \end{aligned} \quad (6)$$

The ratio between the total phycobiliprotein recovered (mg L⁻¹) and TIN removed (mg N L⁻¹) was calculated as shown in Eq. (7):

$$\begin{aligned} \text{Total phycobiliprotein recovered / consumed TIN} &= \\ &= \frac{\text{Total phycobiliprotein content} \cdot X(t_2)}{\text{TIN}(t_1) - \text{TIN}(t_2)} \end{aligned} \quad (7)$$

where TIN(t₂) (mg L⁻¹) and TIN(t₁) (mg L⁻¹) are the final (t₂) and initial (t₁) TIN concentrations, and X(t₂) is the final biomass concentration, as g L⁻¹ of VSS.

2.3.3. Contaminants of emerging concern analysis

The presence of CECs was analyzed in the SE, biomass and in the pigment-rich extracts retrieved during the semi-continuous experiment. For SE, 3 samples were collected at the beginning, middle and end of the experimental period. Samples were filtered through a 0.7 µm pore GF/F filter (Whatman) and stored at -20 °C until analysis. SE samples were analyzed adapting a routine method (Matamoros et al., 2010). Briefly, to have all the samples under the same pH conditions and due to analytical requirements, 100 mL of filtered SE were adjusted to pH 2–3 and loaded in previously conditioned Solid Phase Extraction (SPE) cartridges. In the case of cyanobacterial biomass, 6 samples were collected twice a week during the semi-continuous experiment. Pigment-rich extracts obtained

from those biomass samples were integrated in 2 samples. The analyses of both, biomass and pigment-rich extracts, were made following the extraction method described by Tadić et al., (2019). Briefly, 10 mg of freeze-dried material were spiked with 25 µL of a 400-ppb solution containing four isotopically labeled surrogates (caffeine ¹³C, ibuprofen D₃, bisphenol A-D¹⁶ and atrazine D₅) (Sigma-Aldrich). Then, samples were subsequently extracted with methanol, evaporated and reconstituted in 20 mL of ultrapure water, which was loaded through previously conditioned SPE cartridges. All loaded cartridges from SE, biomass and pigments were cleaned, dried and eluted. All eluted fractions were evaporated, derivatized and injected in a GC-Orbitrap as described by Álvarez-González et al. (2023). Compound concentrations were quantified using the mass of the most abundant m/z fragment. The presence of qualifier ions was also verified (see supplementary material). Limit of Detection (LOD) and quantification ranged from 0.001 to 0.339 µg L⁻¹ and from 0.024 to 2.309 µg gDW⁻¹ for SE and biomass/pigment samples, respectively (see supplementary material).

3. Results and discussion

3.1. Phycobiliprotein production potential

The phycobiliprotein production potential of the wastewater-borne *Synechocystis* sp. R2020 was determined in a 6-days batch test with modified BG11 medium, reaching a total phycobiliprotein content of 129.4 ± 15.1 mg gDW⁻¹. A maximum biomass concentration of 579.1 ± 18.0 mg VSS L⁻¹ and phycobiliprotein production of 10.8 ± 1.4 mg phycobiliproteins L⁻¹ d⁻¹ were achieved by the end of the experiment. These results are in accordance with previous studies assessing the phycobiliprotein content of *Synechocystis* strains in synthetic media at lab-scale. For instance, Puzorjov et al. (2022) reached a maximum phycocyanin content of 111 mg gDW⁻¹ with a purity ratio of 0.83 by growing *Synechocystis* sp. PCC 6803 in BG11; while Touloupakis et al. (2015) reached a phycocyanin content of 196 mg gDW⁻¹ by growing *Synechocystis* sp. PCC 6803 in continuous mode. Thus, *Synechocystis* sp. R2020 appears as a potential candidate to assess the production of these pigments in waste streams.

3.2. Phycobiliprotein production in treated wastewater with and without nutrients supplementation

The phycobiliprotein production in treated wastewater was evaluated with and without nutrients supplementation, since phycobiliprotein storage in cyanobacteria highly depends on nitrogen availability, and N depletion can even lead to phycobiliprotein degradation (Zuorro et al., 2021).

3.2.1. Biomass growth and nutrients removal

Synechocystis sp. R2020 was able to successfully grow in SE with and without nutrients supplementation, reaching average biomass productivities of 153.5 and 146.7 mg VSS L⁻¹ d⁻¹, respectively (Table 2). Regarding biomass concentrations, 697.2 and 677.6 mg VSS L⁻¹ were reached for SE and SE + nutrients cultures, respectively (Fig. 2a). Previous studies showed how this strain was capable of growing even under N and P starvation, reaching maximum biomass concentrations close to those obtained in the present study (653 mg VSS L⁻¹). A possible reason for this is that under N limitation the cyanobacterium could continue growing on nutrients previously stored in the cell (Rueda et al., 2022). Furthermore, both *Synechocystis* sp. R2020 cultures produced biomass with similar specific growth rates (0.5 d⁻¹) (Table 2), which suggests that the nutrient content was not a limiting growth factor.

In the present study, nutrients were removed from the SE within 4 days (Fig. 2). Indeed, high nutrient removal efficiencies were observed with and without nutrients supplementation: 100% P-PO₄³⁻, 93 % N-NO₃⁻, 13% sCOD and 100 % N-NH₄⁺ in the case of SE; and 75% P-PO₄³⁻, 39 % N-NO₃⁻, 22% sCOD and 100 % N-NH₄⁺ in SE + nutrients. It has been

Table 2

Kinetic and stoichiometric parameters obtained in the batch experiment with SE. SE; secondary effluent. SE + nutrients; secondary effluent supplemented with N and P. VSS; Volatile Suspended Solids.

Parameter	SE	SE + nutrients
Biomass productivity (mg VSS L ⁻¹ d ⁻¹)	153.5	146.7
μ (d ⁻¹)	0.5	0.5
Duplication time (d)	1.3	1.4
q_p (mg P-PO ₄ ³⁻ gVSS ⁻¹ d ⁻¹)	1.2	5.2
$Y_{X/P}$	447.8	97.1
q_{NO_3} (mg N-NO ₃ ⁻ gVSS ⁻¹ d ⁻¹)	1.7	19.3
Y_{X/NO_3}	305.1	26.0
q_{NH_4} (mg N-NH ₄ ⁺ gVSS ⁻¹ d ⁻¹)	37.4	39.6
Y_{X/NH_4}	14.2	12.7

described that all forms of inorganic nitrogen are reduced to NH₄⁺ prior to cellular uptake. Thus, in order to save energy, cyanobacteria prefer N-NH₄⁺ over N-NO₃⁻ when these are supplied together (Ge and Champagne, 2016). The results of the present study support this fact, as both cultures (SE and SE + nutrients) showed similar yields ($Y_{X/N-NH_4^+}$ 14.2 and 12.7, respectively) (Table 2). However, it is important to take into account that, as pH was in average 8.5 and 8.3 for SE and SE + nutrients, respectively, some NH₄⁺ removal by NH₃ stripping was expected. According to the pH and the NH₄⁺-NH₃ equilibrium, NH₃ stripping was estimated as 18% of the total removal of NH₄⁺.

Other authors have pinpointed the potential of *Synechocystis* to adapt to streams containing high N-NH₄⁺ concentration (>200 mg N-NH₄⁺ L⁻¹), and reported high nutrient removal efficiencies similar to those obtained in the present study. For instance, 75.8% of N-NH₄⁺ and 71.4% of TP

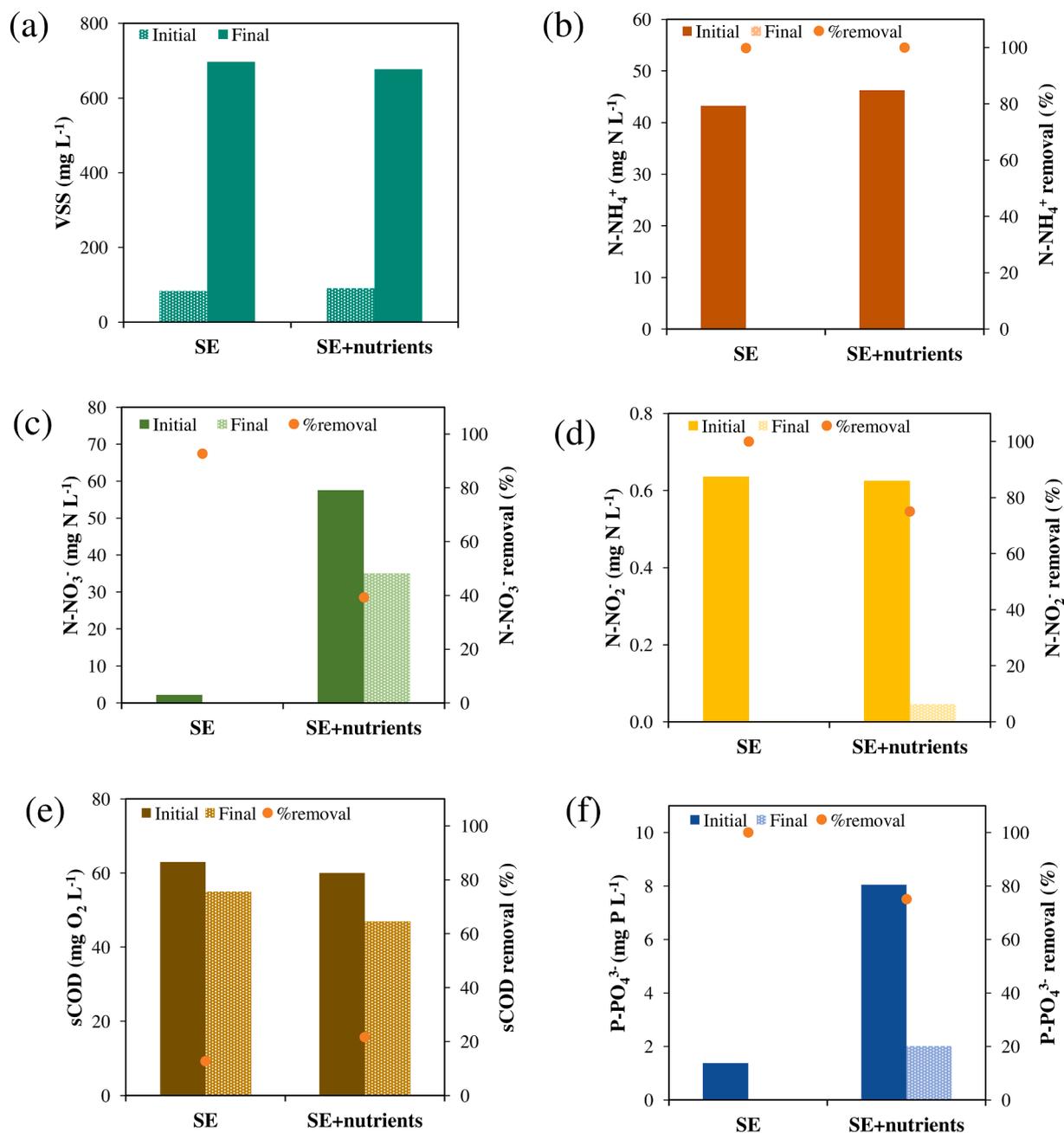


Fig. 2. *Synechocystis* sp. R2020 biomass growth and nutrient removal from the beginning till the end of the batch experiment with SE. Biomass growth is expressed as Volatile Suspended Solids concentration (mg VSS L⁻¹) (a). Inorganic nutrients (N-NO₃⁻, N-NO₂⁻, P-PO₄³⁻ and N-NH₄⁺) and soluble chemical oxygen demand (sCOD) are expressed as concentration (mg L⁻¹) and removal efficiency (% removal) (b, c, d, e, f). SE; secondary effluent. SE + nutrients; secondary effluent supplemented with N and P.

removal were achieved by growing *Synechocystis* sp. in anaerobically-digested swine wastewater (Cheng et al., 2020); and up to 66% of N and 96% of P by growing *Synechocystis* sp. in an unsterile effluent from acidogenic fermentation of sludge (Trentin et al., 2019).

The present study showed the capability of *Synechocystis* sp. R2020 to grow as efficiently in SE as in SE + nutrients, and removing all the nutrients from SE within 4 days. Therefore, this strain could potentially be implemented for tertiary wastewater treatment.

3.2.2. Phycobiliprotein production

The phycobiliprotein content of *Synechocystis* sp. R2020 was 25% higher in SE with nutrients supplementation (66.5 mg gDW^{-1}) than in SE (52.9 mg gDW^{-1}) (Fig. 3a). Purity ratios were also higher with nutrient supplementation, reaching a maximum value of 0.6 for phycocyanin. Please notice that purities above 0.7 meet the standards of food-grade pigment (Patil et al., 2006). In terms of content, it represents about 50% of the phycobiliprotein production potential of *Synechocystis* sp. R2020 grown in modified BG11 under the same light intensity ($129.4 \pm 15.1 \text{ mg gDW}^{-1}$). However, taking into account the biomass production ($153.5 \text{ mg VSS L}^{-1} \text{ d}^{-1}$ with SE, $146.7 \text{ mg VSS L}^{-1} \text{ d}^{-1}$ SE + nutrients and $83.1 \pm 4.0 \text{ mg VSS L}^{-1} \text{ d}^{-1}$ with BG11), the resulting total phycobiliprotein production is as follow: $8.2 \text{ mg phycobiliproteins L}^{-1} \text{ d}^{-1}$ with SE, $9.6 \text{ mg phycobiliproteins L}^{-1} \text{ d}^{-1}$ with SE + nutrients, and $10.8 \pm 1.4 \text{ mg phycobiliproteins L}^{-1} \text{ d}^{-1}$ with modified BG11; i.e. 76% and 89% of the production potential can be reached with SE and SE + nutrients, respectively (under the same light intensity).

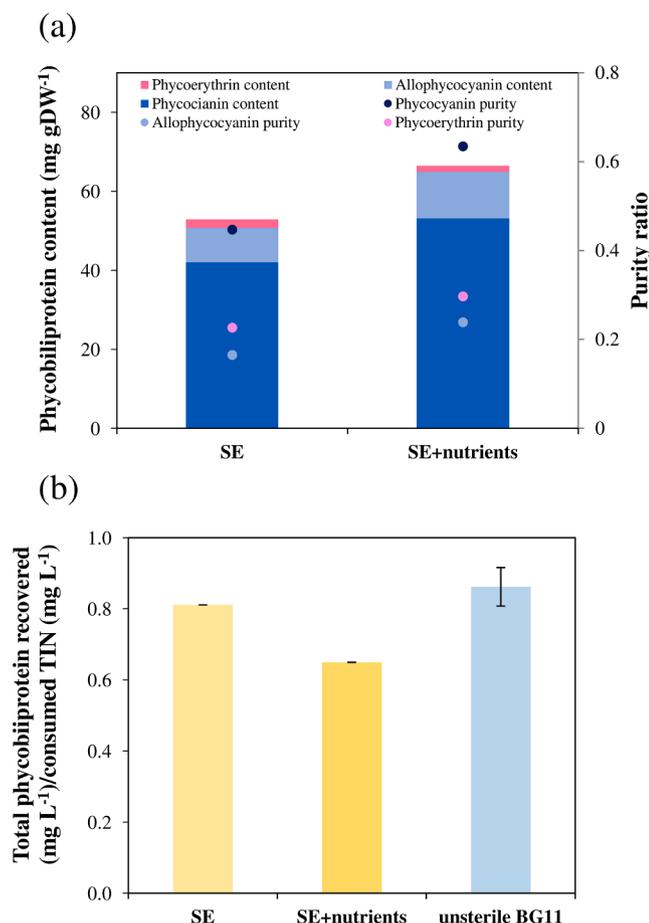


Fig. 3. *Synechocystis* sp. R2020 phycobiliprotein recovery. Content at the end of the batch experiment using SE and SE + nutrients (a), and ratio of phycobiliprotein recovery efficiency over the TIN consumed (b). SE; secondary effluent. SE + nutrients; secondary effluent supplemented with N and P. TIN; Total Inorganic Nitrogen.

N availability in the culture medium is crucial to ensure phycobiliprotein biosynthesis, as these pigments may act as a nitrogen storage molecule in cyanobacterial cells under stress conditions (Zhao et al., 2017). Taking into account the TIN utilization ratio (total phycobiliprotein recovered (mg L^{-1})/consumed TIN (mg N L^{-1})), the production of phycobiliproteins was most efficient in SE (0.81) and modified BG11 (0.86 ± 0.1), while SE + nutrients appeared to be least efficient (0.65) (Fig. 3b). Moreover, while TIN was completely removed from SE, it was not totally removed from SE + nutrients or synthetic medium (Fig. 2). More specifically, while only 0.5% of the initial TIN (0.25 mg N L^{-1}) remained in the SE, 34 % (35.1 mg N L^{-1}) and 46 % ($75.2 \pm 2.4 \text{ mg N L}^{-1}$) remained unutilized in the case of SE + nutrients and modified BG11, respectively. In a circular bioeconomy context, this situation is not desirable, as these nutrients would not be utilized for biomass growth and bioproduct recovery, and would be either discharged into the environment or treated in WWTPs, with the corresponding environmental and economic impacts. In fact, Arashiro et al. (2022) concluded that, only when residual nutrients from treated effluents are not discharged, recovering pigments from wastewater has a lower environmental impact than recovering them from synthetic growth medium.

Therefore, the results obtained pinpoint SE as a suitable culture medium for *Synechocystis* sp. R2020 to produce high value phycobiliproteins. Furthermore, the use of this effluent allows for the recovery of nutrients from waste streams in a circular bioeconomy approach.

3.3. Phycobiliprotein production in a semi-continuous photobioreactor

The production of phycobiliproteins in a semi-continuous photobioreactor treating SE was monitored to assess whether biomass production and phycobiliprotein content could be maintained over time, and to periodically recover pigment-rich extracts and cyanobacterial biomass in order to track the CECs.

3.3.1. Biomass growth and nutrients removal over time

The SE used to feed the photobioreactor was characterized by a high concentration of N-NH_4^+ and a very low concentration of N-NO_3^- , N-NO_2^- and P-PO_4^{3-} throughout the whole experimental period (Table 1). Biomass and nutrients concentration over the experiment are shown in Fig. 4. From operational day 3 on, *Synechocystis* sp. R2020 biomass in the reactor was sustained at a range from 444.5 to $610.0 \text{ mg VSS L}^{-1}$ (Fig. 4a). These results are in accordance with those reported in semi-continuous cultures of cyanobacteria treating effluents with similar nutrient composition. For instance, the average biomass content ranged from 390 to $840 \text{ mg VSS L}^{-1}$ in a mixed culture of cyanobacteria treating SE with digestate addition (TIN up to 27 mg N L^{-1} , P-PO_4^{3-} up to 3 mg P L^{-1}) (Arias et al., 2017).

Regarding nutrient removal, since the beginning of the semi-continuous operation, the P-PO_4^{3-} concentration in the photobioreactor was close to 0 mg P L^{-1} (Fig. 4c). Thus, almost complete removal of P-PO_4^{3-} was attained over the whole experiment. From operational day 5 on, N-NH_4^+ was removed at efficiencies $\geq 89\%$, but it was slightly accumulated during days 8 through 10 (Fig. 4e). This can be attributed to the slight decrease of biomass concentration in the photobioreactor on day 8 (Fig. 4a), and also to the low availability of P-PO_4^{3-} which could have become limiting. As P is metabolically essential, low concentrations of P may impede the removal of N when it is in excess (Ación et al., 2016). N-NO_3^- and N-NO_2^- concentrations in SE were very low ($\leq 1.2 \text{ mg N L}^{-1}$). Nevertheless, the removal efficiency of N-NO_3^- reached up to 70% from day 5 on (Fig. 4b). Regarding sCOD, it was measured on days 5 and 12. Compared to the sCOD concentration in SE (Table 1), the concentration in the culture slightly increased ($83.5 \text{ mg O}_2 \text{ L}^{-1}$), which may have been caused by the extracellular release of organic molecules (García et al., 2006).

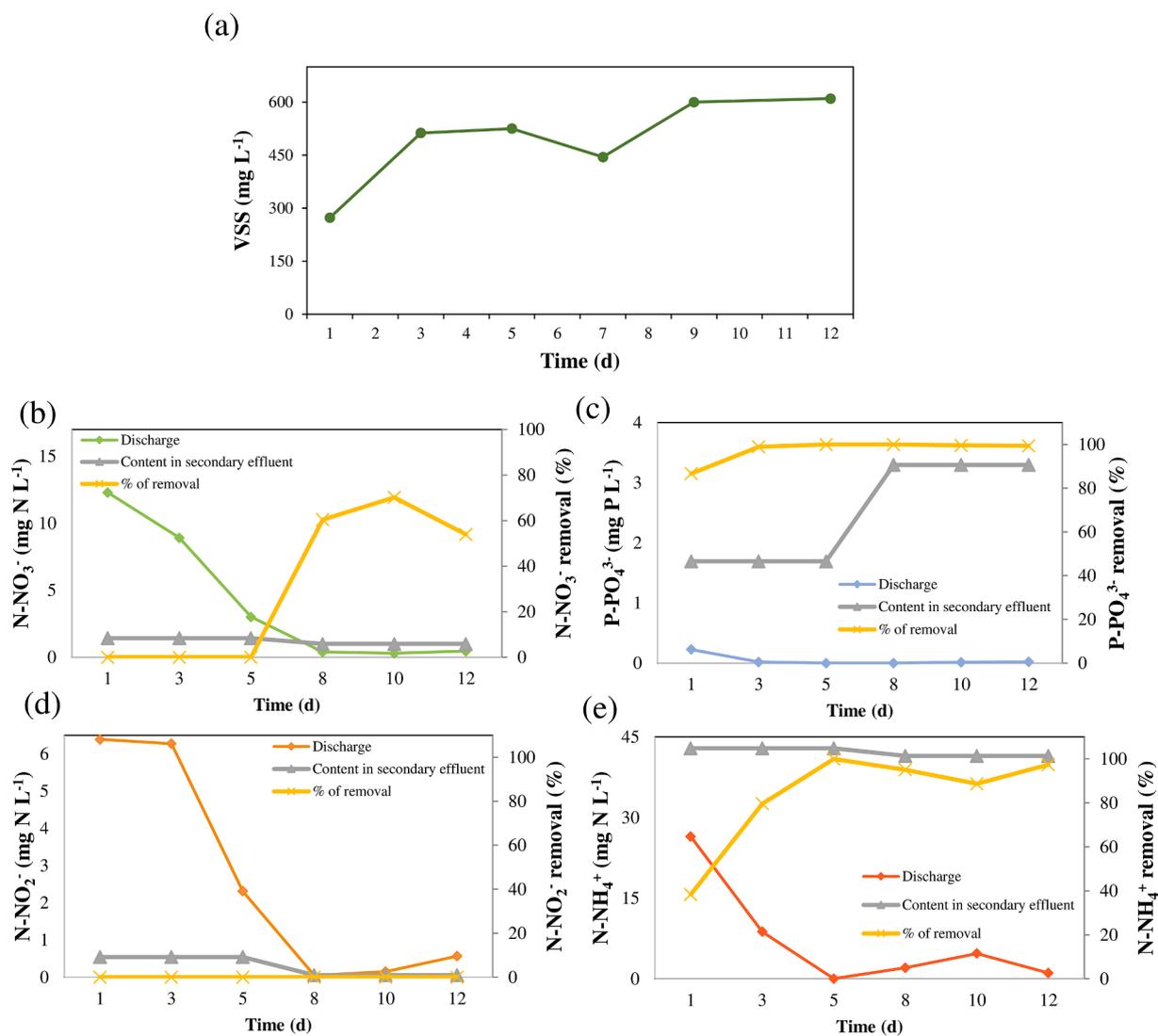


Fig. 4. *Synechocystis* sp. biomass growth and nutrient removal in a semi-continuous photobioreactor fed with treated wastewater. Biomass growth is expressed as the variation of Volatile Suspended Solids (mg VSS L^{-1}) over time (a). Inorganic nutrients (N-NO_3^- , N-NO_2^- , P-PO_4^{3-} and N-NH_4^+) are expressed as concentration (mg L^{-1}) and removal efficiency (% removal) (b, c, d, e).

3.3.2. Phycobiliprotein production over time

The phycobiliprotein content in biomass was maintained over the whole experimental period (Fig. 5) at similar values to those attained in the batch experiment with SE (Fig. 3a). A maximum total phycobiliprotein content of 74.7 mg gDW^{-1} was reached on day 5. The total phycobiliprotein content reached 58% of the production potential measured with modified synthetic growth medium ($129.4 \text{ mg gDW}^{-1}$). Food grade purity ratios (>0.7) were obtained for phycocyanin and maintained along the experiment (except in day 12, that was 0.5) (Fig. 5). Nevertheless, as phycobiliproteins were recovered from the SE of municipal wastewater treatment, other applications may be more appropriate for these high-value bioproducts.

Loss of phycobiliprotein content has been reported under the occurrence of different compounds in the medium. For instance, the presence of chromium, which can be found in tannery wastewater, induced a decrease in the total phycobiliprotein content of *Synechocystis* sp. (Shashirekha et al., 2015). Furthermore, phycobiliprotein synthesis inhibition has been related to the presence of organophosphorus insecticides in *Synechocystis* PCC6803 cultures (Kumar Mohapatra and Schiewer, 2000). Taking into account harmful compounds including CECs may be detected in treated wastewater, the presence of these molecules may have limited the phycobiliprotein production.

Phycobiliprotein synthesis in cyanobacteria not only depends on factors such as nutrient content in the medium, light intensity and light regime, but also on the biology of each cyanobacterium strain (Zuorro et al., 2021). Thus, comparing the phycobiliprotein production of different cyanobacteria genera in wastewater sources is challenging. The results of this study are within the values reported for cyanobacteria grown in consortia with wastewater bacteria. For instance, Shahid et al. (2021) reached a maximum phycobiliprotein content of 102 mg gDW^{-1} by growing the strain BERC06 with municipal wastewater in batch. In mixed cultures dominated by cyanobacteria grown in wastewater, 61 mg gVSS^{-1} (purity of 0.4) of phycocyanin were retrieved by Van den Hende et al. (2016); while Arashiro et al. (2020b) reported a phycocyanin content of 20 mg gDW^{-1} (purity of 2.1) treating SE from urban wastewater treatment supplied with 15% digestate (resulting in a nutrients concentration similar to the present study).

Overall, the studied *Synechocystis* sp. seems a promising strain capable of stably growing and synthesizing phycobiliproteins in treated wastewater. Nevertheless, in order to establish potential applications for these pigments, further tracking of contaminants in the recovered extracts is of great interest.

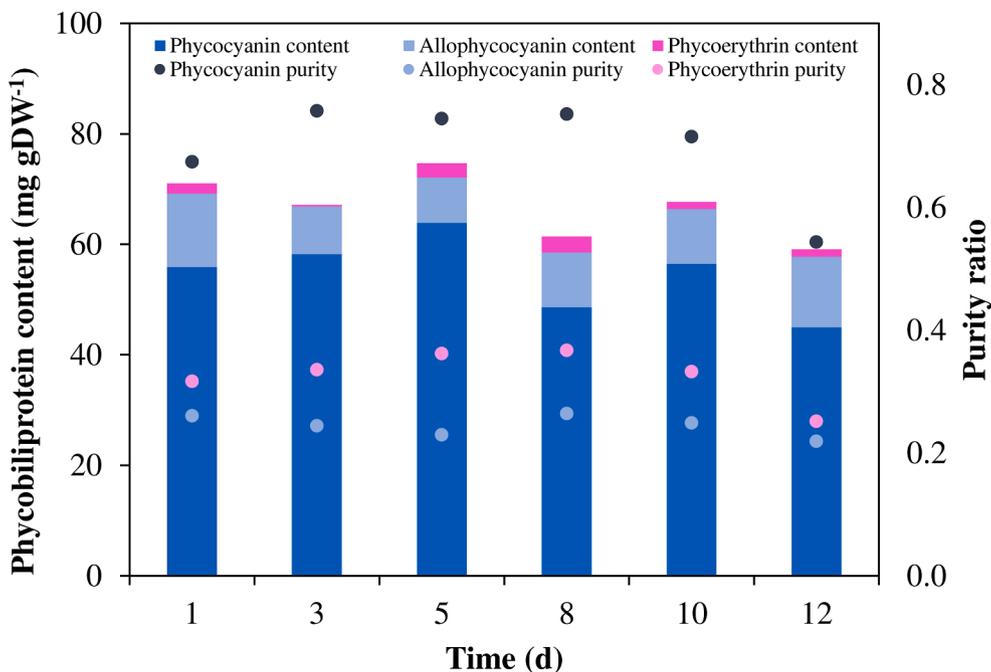


Fig. 5. *Synechocystis* sp. phycobiliprotein content and purity ratio over time during the semi-continuous experiment with treated wastewater.

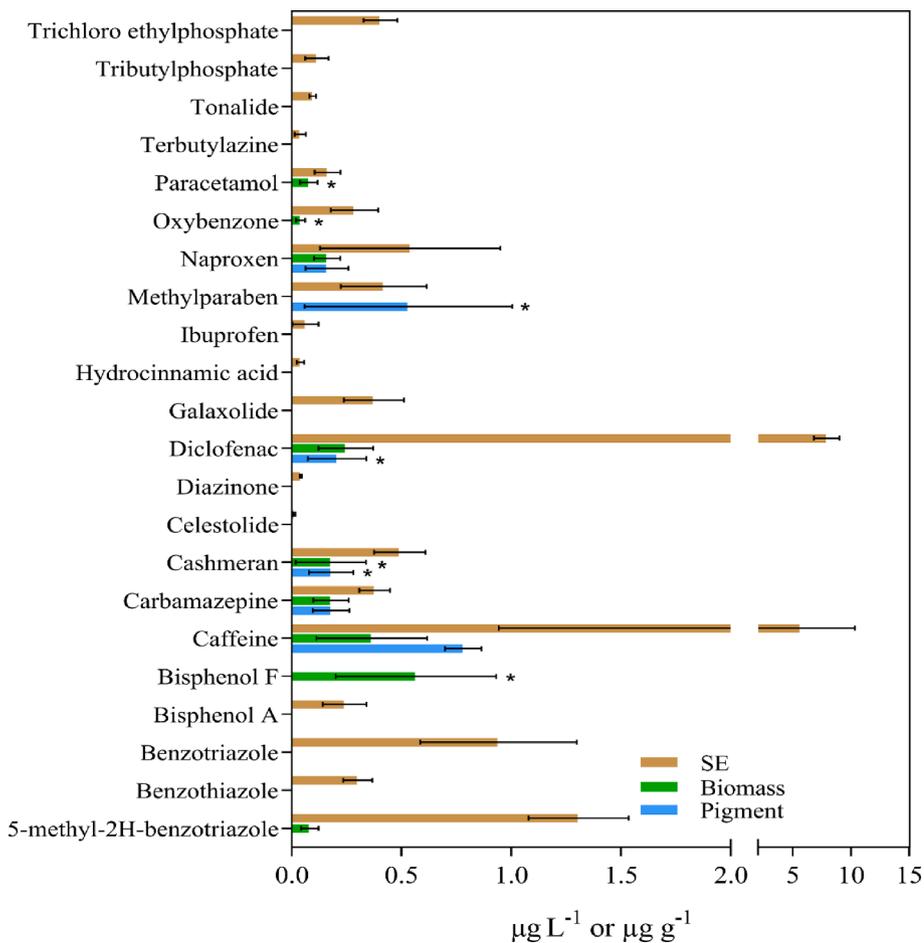


Fig. 6. Mean concentration of the detected CECs in the SE (n = 3), biomass and pigment-rich extracts (n = 5). Values are given in $\mu\text{g L}^{-1}$ for wastewater and $\mu\text{g gDW}^{-1}$ for biomass or pigment extracts. Compounds < LOD in all samples are not plotted. To calculate mean, values < LOD were considered to be LOD/2. Error bars are standard deviation. (*) indicates that the compound was detected in two or less samples. DW; Dry Weigh. LOD; Limit of detection. CECs; contaminants of emerging concern. SE; secondary effluent.

3.4. Occurrence of contaminants of emerging concern

The presence of CECs in the SE, biomass and pigment-rich extracts retrieved during the semi-continuous experiment is shown in Fig. 6 (see supplementary material). The major part of the analysed CECs was found in the treated wastewater (22 out of the 30 compounds analysed). Among them, there were 4 compounds with concentrations over $1 \mu\text{g L}^{-1}$ (diclofenac, caffeine, 5-methyl-2H-benzotriazole and benzotriazole). These findings are in agreement with a previous study carried out in secondary treated wastewater from the same area (Matamoros et al., 2017), where CECs such as diclofenac or benzotriazoles were found to be among the most abundant ones because they are known to be recalcitrant. On the other hand, caffeine, which in WWTPs is removed up to 80%, was also detected at elevated concentrations in SE (Buerge et al., 2003), and its concentration was higher than $15 \mu\text{g L}^{-1}$ in a previous study using microalgae to treat urban wastewater (Álvarez-González et al., 2023).

In the biomass, 5 compounds were found on at least one third of the samples. These compounds were caffeine, carbamazepine, diclofenac, naproxen and 5-methyl-2H-benzotriazole, and their concentrations ranged between 0.1 and $0.8 \mu\text{g gDW}^{-1}$. This indicates that CECs present in wastewater at the greatest concentration levels, were also those being incorporated into the cyanobacteria biomass. In fact, the concentration levels of CECs in biomass are in fair agreement with previous studies that evaluated the occurrence of CECs in microalgae biomass using primary treated wastewater (Matamoros et al., 2015; Álvarez-González et al., 2023).

Regarding the pigment-rich extracts, only caffeine was incorporated in all five pigment samples (at concentrations ranging from 0.70 to $0.84 \mu\text{g gDW}^{-1}$), while carbamazepine and naproxen were only found in more than two thirds of the samples at lower concentrations (from 0.16 to $0.2 \mu\text{g gDW}^{-1}$). The other three compounds (diclofenac, methylparaben and cashmeran), were detected only in less than two samples, and their concentrations were below the Limit of Quantification (LOQ). As the biomass extraction to obtain pigment extracts was developed with phosphate buffer, this could explain that only the more polar compounds (i.e. caffeine, carbamazepine and naproxen) were left on the pigment extract, while the less polar compounds remained in the biomass. However, due to the presence of remnants from phosphate buffer used in the pigment extraction process, it was not feasible to perform a mass balance of CECs transfer.

Overall, data indicates that while many compounds may be present in wastewater, only few of them would pass to the pigment-rich extracts. Furthermore, the pigment-rich extracts analysed here lack the last purification steps, and therefore even lower concentrations or no presence of CECs could be expected on the pure extract. To author's knowledge, this is the first study that assessed the incorporation of CECs on cyanobacterial wastewater-derived pigment extracts. Based on these preliminary results, the usage of these pigments should be excluded from food, medicine or cosmetic applications, as the bare presence of a few CECs may indicate that other unmonitored pollutants could also be incorporated in the extract. However, it is worth noticing that the concentration of CECs in the pigments observed in this study is negligible when compared to the levels anticipated in textiles manufactured from recycled plastics (Undas et al., 2023). Considering their low content, these pigments hold potential for utilization in the production of dyes. In this sense, prospective research should prioritize the evaluation of CECs and other contaminants removal during pigment purification, as this would help identify further potential applications for pigments produced in treated wastewater.

4. Conclusion

This study evaluated the tertiary treatment of urban wastewater using a wastewater-borne cyanobacterium (*Synechocystis* sp. R2020) to recover value-added natural pigments. Complete removal of N and P

was achieved in 4 days batch culture, not showing biomass growth limitations even without nutrients supplementation. The phycobiliprotein content and process performance were stable during semi-continuous operation, and the phycocyanin purity ratio achieved food grade. Out of the 22 CECs detected in the SE, only 3 were present in the phycobiliprotein extracts at very low concentrations. Further pigment purification steps may potentially decrease these concentrations.

CRedit authorship contribution statement

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

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biortech.2023.129287>.

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