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Production of phycobiliproteins, bioplastics and lipids by the cyanobacteria *Synechocystis* sp. treating secondary effluent in a biorefinery approach

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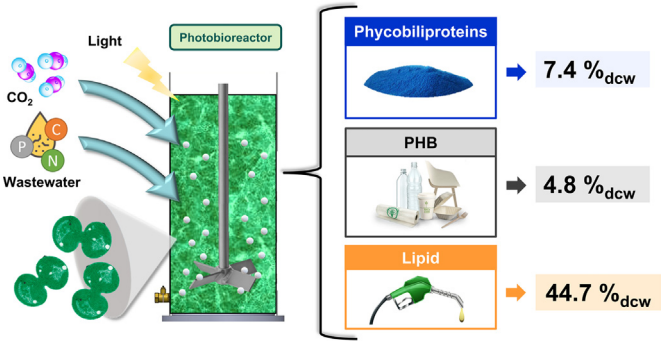
HIGHLIGHTS

- Cyanobacteria were successfully grown and kept dominant in secondary wastewater.
- Highest biomass productivity was reached with 8 days of hydraulic retention time.
- Maximum phycobiliproteins content of 7.4%$_{dcw}$ was achieved.
- A polyhydroxybutyrate content of 4.8% $_{dcw}$ was reached under N and P limitation.
- Lipid content of up to 44.7%$_{dcw}$ was achieved after 30 days of P starvation.

GRAPHICAL ABSTRACT



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ABSTRACT

Cyanobacteria have been identified as promising organisms to reuse nutrients from waste effluents and produce valuable compounds such as lipids, polyhydroxyalkanoates (PHAs), and pigments. However, almost all studies on cyanobacterial biorefineries have been performed under lab scale and short cultivation periods. The present study evaluates the cultivation of the cyanobacterium *Synechocystis* sp. in a pilot scale 30 L semi-continuous photobioreactor fed with secondary effluent for a period of 120 days to produce phycobiliproteins, polyhydroxybutyrate (PHB) and lipids. To this end, the harvested biomass from the semi-continuous photobioreactor was transferred into 5 L vertical column batch photobioreactors to perform PHB and lipid accumulation under nutrient starvation. Three hydraulic retention times (HRT) (6, 8 and 10 days) were tested in the semi-continuous photobioreactor to evaluate its influence on biomass growth and microbial community. A maximum biomass concentration of 1.413 g L^{-1} and maximum productivity of 173 mg L^{-1} d^{-1} was reached under HRT of 8 days. Microscopy analysis revealed a shift from *Synechocystis* sp. to *Leptolyngbya* sp. and green algae when HRT of 6 days was used. Continuous, stable production of phycobiliproteins in the semi-continuous photobioreactor was obtained, reaching a maximum content of 7.4%$_{dcw}$ in the biomass. In the batch photobioreactors a PHB content of 4.8%$_{dcw}$ was reached under 7 days of nitrogen and phosphorus starvation, while a lipids content of 44.7%$_{dcw}$ was achieved under 30 days of nitrogen starvation. PHB and lipids production was strongly dependent on the amount of nutrients withdrawn from the grow phase. In the case of lipids, their production was stimulated when there was only phosphorus depletion. While Nitrogen and phosphorus limitation

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was needed to enhance the PHB production. In conclusion, this study demonstrates the feasibility of cultivating cyanobacteria in treated wastewater to produce bio-based valuable compounds within a circular bioeconomy approach.

1. Introduction

Human population increase, economic development and the improvement of living standards have caused a tremendous rise in natural resources extraction and utilization (Frkova et al., 2020; González-Camejo et al., 2021). It is therefore essential to move towards a circular system paradigm in which resources are recovered and valorised from by-product streams such as wastewater (Ubando et al., 2020). In this context, cyanobacteria have been seen as a promising feedstock to be used in a wastewater biorefinery concept to recover nutrients from wastewater while producing bioproducts of interest such as polyhydroxybutyrate (PHB), lipids and phycobiliproteins (Arias et al., 2019; Yadav et al., 2021).

Cyanobacteria cultures have already been successfully applied to treat many types of wastewater such as municipal wastewater, industrial wastewater, agricultural run-off or digestate from anaerobic digestion (Arias et al., 2020; Li and Dittrich, 2019). Secondary effluents seem the most suitable streams to be used in a cyanobacteria wastewater biorefinery, given its low organic matter content and turbidity. Moreover, the use of cyanobacteria to treat secondary effluents has the potential to be cheaper than other tertiary treatments due to their ability to use the remaining nutrients from previous treatments even at extremely low concentrations (Arias et al., 2020).

Despite that, the use of wastewaters as a medium for cyanobacteria cultivation presents yet several challenges. One of the most important is the maintenance of cyanobacteria-dominated cultures during long-term periods and on large scales (Arias et al., 2020; López-Pacheco et al., 2021; Shahid et al., 2021). In fact, most of the studies regarding resource recovery from wastewater in a biorefinery concept have been limited to short cultivation periods in laboratory scale conditions (Arias et al., 2020). Moreover, in most of the cases, green microalgae rather than cyanobacteria have been used, meaning that specific cyanobacteria bioproducts (i.e. PHB or phycobiliproteins) cannot be produced (Hemalatha et al., 2019; Khan et al., 2019; Pérez-Rivero et al., 2019; Yadav et al., 2019). Other studies targeted just one specific product, failing in the efficient valorisation of the biomass. For instance, Krasaesueb et al. (2019) and Samantaray et al. (2011), used aquaculture wastewater to produce polyhydroxybutyrate (PHB) in 15 L and 112 L batch photobioreactors, respectively. Rueda et al. (2020b), used agricultural run-off to produce PHB with a cyanobacteria-rich mixed culture in a set of three 11 m^3 connected in series. Arashiro et al. (2020a), used anaerobically digested food industry effluents to produce phycobiliproteins using isolated cyanobacterial strains (*Nostoc* sp., *A. platensis* and *P. purpureum*) cultivated in 8 L plastic bags operated in batch for 10 days. Additionally, in many studies, to avoid contamination, wastewater was sterilized what has a negative economic and environmental impact (Shahid et al., 2021). For example, Meixner et al. (2018) used sterile digestate as a source of nutrients to grow *Synechocystis salina* and produce pigments and PHB.

Studies related to the simultaneous production of different bioproducts from waste effluents using cyanobacteria on a relatively big scale (i.e., pilot scale) are very few and in most of the cases the stability of the culture was evaluated only for short cultivation periods. Ashokkumar et al. (2019), studied the production of bioethanol and biodiesel using municipal wastewater with *Synechocystis* PCC6803 in an open raceway pond (1 m^3) operated semi-continuously for 40 days. Shahid et al. (2021) studied the production of lipids, carbohydrates, proteins, and pigments in three newly isolated cyanobacteria strains using urban wastewater as a nutrient source in a 10 L open pond operated in batch.

Therefore, the present study evaluates the stability, throughout 120 days, of the cyanobacteria *Synechocystis* sp. in a culture fed with secondary urban wastewater. Additionally, the simultaneous production of phycobiliproteins (which have a high commercial potential to be used as organic colorants in the nutraceutical, cosmetic, pharmaceutical and food industries, or as natural dyes in textile industries (Arashiro et al., 2020a), PHB (biodegradable thermoplastic) and lipids (feedstock for the generation of third generation biodiesel) was assessed. To do this, a 30 L vertical column photobioreactor was inoculated with the wastewater-borne cyanobacterium, *Synechocystis* sp. (Rueda et al., 2020a), which in principle is more adapted to this culture media and could potentially outcompete other microorganisms. Three different hydraulic retention times (HRT) of 10, 8 and 6 days were tested to evaluate their impact on biomass production and on *Synechocystis* sp. dominance. The phycobiliproteins content was assessed directly from the biomass grown in this photobioreactor. To reach nutrient starvation and thus stimulate the accumulation of PHB and lipids, a portion of the biomass was withdrawn from the growth photobioreactor and inoculated in a 5 L batch photobioreactor under nitrogen and phosphorus starvation.

Overall, this work evaluated the possibility to maintain during a long cultivation period a cyanobacteria-dominated culture using secondary effluent from urban wastewater treatment, while producing different molecules of interest in a biorefinery concept.

2. Materials and methods

2.1. Pre-culture of cyanobacteria

The wastewater-borne cyanobacterium monoculture of *Synechocystis* sp. used in this study was obtained as described elsewhere (Rueda et al., 2020a). The initial inoculum was pre-cultured in two flasks of 5 L for 7 days at 25 ± 2 °C and 30 μmol m^{-2} s^{-1} using sterile BG-11 medium. Then, a 30 L photobioreactor (PBR) was inoculated with the culture broth of the two 5 L flasks (inoculum concentration ≈ 0.6 g DW L^{-1}).

2.2. Experimental set-up

2.2.1. Growth photobioreactor

An airlift vertical PBR was used as growth reactor. This PBR was made of polymethyl methacrylate (PMMA), and had a diameter and height of 25 and 80 cm, respectively, corresponding to an effective volume of 30 L. Wall thickness was 5 mm (Fig. 1A). Four white light emitting diodes (LEDs) bars were placed at 15 cm from the reactor to provide homogeneous light with a PPFD (Photosynthetic Photon Flux Density) of 200–250 μmol m^{-2} s^{-1}. During the experiment, the PBR was maintained in alternate light:dark cycles of 15:9 h.

The PBR was mixed mechanically using an electric stirrer E-800-4 and operated at a frequency between 3.6 and 4.5 Hz, providing an agitation between 72 and 90 rpm (Damova, Spain). A continuous airflow of 5 L min^{-1} was injected using an air compressor at the bottom of the PBR to guarantee a better mixing during the last two periods (Table 1). To control the pH, high purity CO_2 (100 % *v*/v) (Hidrocarburos Metálicos, Spain) was injected by sparging it at a flow rate of 0.2 mL/min and a pressure of 0.3–0.5 MPa when pH was higher than the set-point. Temperature and pH were continuously recorded with a probe (Mettler Toledo, USA), and saved every 2 min on a computer with the software LabVIEW®. The pH was set at 8.4 based on previous studies that recommended a range from 8 to 9 (Arias et al., 2017). LabVIEW® software was employed for data acquisition (temperature, light and pH), monitoring and automatic control of the CO_2 injection, the feeding, and the harvesting pumps.

The PBR was continuously operated and monitored for the different periods during a total of 120 days (Table 1). The reactor was inoculated with 10 L of *Synechocystis* sp. inoculum (see Section 2.1) to have an initial

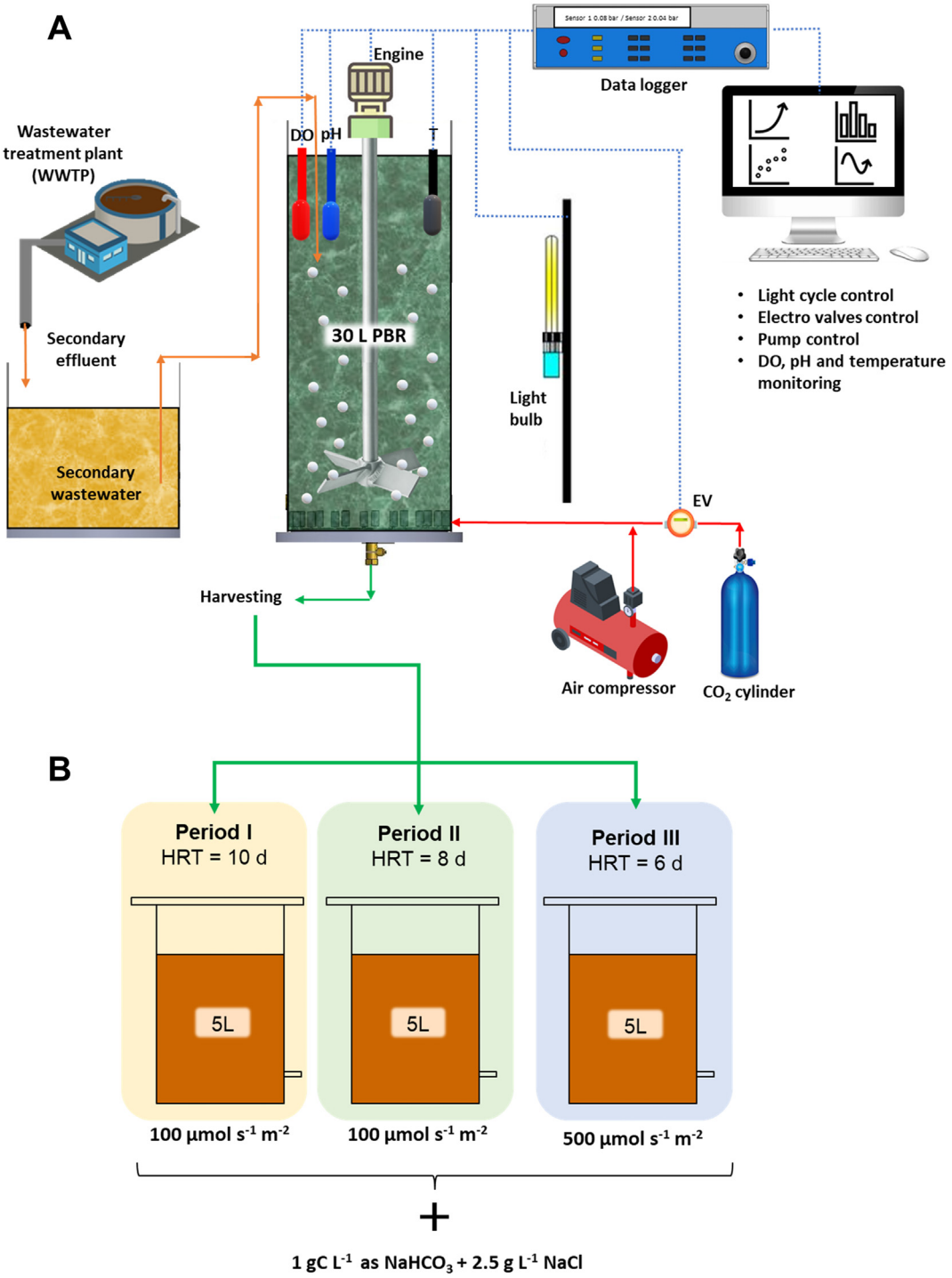

Fig. 1. Schematic diagram of the experimental set-up: growth photobioreactor (A) and accumulation photobioreactor (one batch reactor for each period) (B). Continuous orange lines represent the secondary effluent inflow. Continuous green lines represent the effluent outflow. Continuous red lines represent the gas line. The dotted blue line represents the wire connections between electrical equipment and the data logger. In each experimental period, the harvested culture broth (green line) is placed into the 5 L accumulation photobioreactors to enhance nutrient starvation and promote PHB and lipid accumulation. Abbreviations: EV: Electrovalve, DO: dissolved oxygen probe, HRT: Hydraulic retention time.

concentration in the reactor of 200 mg VSS L^{-1} and operated in batch during the first 20 experimental days using non-sterile BG-11. When biomass concentration reached 0.4–0.5 g DW L^{-1}, secondary effluent was fed semi-continuously to achieve a HRT of 10 days. The performance of the PBR was assessed under different HRT of 10, 8 and 6 days. Daily, at the beginning of the light cycle, a certain volume (depending on the HRT) of culture was harvested and collected in a 10 L Polyvinyl chloride (PVC) tank, and the same volume was gradually replaced by secondary effluent.

Table 2 shows the physicochemical characteristics of the secondary effluent used as growth medium. Secondary effluent was collected every week from a nearby municipal wastewater treatment plant (WWTP) and stored in 10 L PVC tanks at 4 °C (for a maximum of four days). The WWTP followed the European regulation (European Union Council, 1991) without nutrient removal before discharge. This secondary effluent had therefore a low content of COD and high content of ammonium. It should be noted that in this WWTP iron chloride is used in the primary

Table 1
Operating conditions of the photobioreactor during 120 days of semi-continuous cyanobacteria culture. Abbreviations: PPFD (Photosynthetic photon flux density); HRT (Hydraulic retention time).

Periods	Days	Medium	HRT	Influent flow rate	Airflow	Mixing	PPFD
			(days)	($L\ day^{-1}$)	($L\ min^{-1}$)	(rpm)	($\mu mol\ m^{-2}\ s^{-1}$)
Batch	20	BG-11	–	–	–	72	200
I	38	Secondary effluent	10	3.00	–	72	250
II	31	Secondary effluent + Nutrients	8	3.75	5	90	250
III	29	Secondary effluent +Nutrients	6	5.00	5	90	250

treatment to improve solids settleability. The addition of this chemical indirectly reduced the amount of P present in the effluent, which was low in all the experimental periods. During the first period (Table 1) limited growth was observed which was attributed to a low concentration of nutrients in the PBR. Therefore, during the second and third periods, NO_3^- and PO_4^{3-} were externally added when a reduction in the growth rate was detected. To this end, during the first 5 days of the second period (from days 58 to 62), NO_3^- and PO_4^{3-} were supplied to the PBR to reach a concentration of around 250 mg L^{-1} of $N\text{-}NO_3^-$ and 10 mg L^{-1} of $P\text{-}PO_4^{3-}$. During the third period, NO_3^- and PO_4^{3-} were added to get an average concentration in the PBR of around 50 mg L^{-1} of $N\text{-}NO_3^-$ and 2 mg L^{-1} of $P\text{-}PO_4^{3-}$. $N\text{-}NO_3$ was supplied from days 89 to 91, while $P\text{-}PO_4^{3-}$ was added on days 92–93 and 99–100. To further promote the growth, illumination was increased to 250 μmol $m^{-2}\ s^{-1}$ in the first, second and third periods (Table 1). The phycobiliprotein content was measured twice a week during the second and third experimental periods.

2.2.2. Accumulation photobioreactor

At the end of each experimental period, the effluent from the growth PBR was inoculated into a 5 L batch accumulation reactor, under nutrient starvation to promote the accumulation of PHB and lipids (Fig. 1B). This photobioreactor was operated in batch mode to deplete the remaining nutrients (N and P) from the growth reactor. In the accumulation reactor, $NaHCO_3$ (1 g C L^{-1}) and NaCl (2.5 g L^{-1}) were added to stimulate PHB production, as previously described in (Rueda et al., 2020a). Note that CO_2 was not supplemented in this reactor. After 2 and 3 weeks under these conditions, samples were taken and the PHB content analysed (except for the third period, when nutrients were depleted from the very beginning and samples were taken after 1 and 2 weeks). The lipid content was also quantified at the end of this period.

The accumulation photobioreactor was made of polymethyl methacrylate (PMMA), and was continuously agitated with a magnetic stirrer (VELP Scientifica, Usmate, Italy) ensuring complete mixing. It was continuously illuminated by three 14 W cool-white LED lights providing a PPFD of 100 μmol $m^{-2}\ s^{-1}$, except for the last period in which a higher irradiance of 500 μmol $m^{-2}\ s^{-1}$ was tested. This irradiance was provided by a 200 W LED floodlight with daylight colour (4200 K). Temperature (27 ± 2 °C) and pH (8.5 ± 1) were regularly measured.

2.3. Analytical procedures for physicochemical parameters

Dissolved oxygen (DO) and electrical conductivity were daily measured when light was switch on in the growth PBR with a HI94142 (HANNA Instruments) and Conductimeter GLP 31 (Crison, Spain), respectively. Light intensity in the PBR surface was measured with a light meter (HI 97500, HANNA instruments).

The culture broth harvested every day at the beginning of the light phase from the growth PBR was partially used to monitor the physicochemical parameters. In the period where the reactor was operated in batch, 200 mL of culture broth were collected for this purpose. Samples were filtered through glass fibre filters with a pore size of 0.7 μm. The filtrate was used to measure, once a week, alkalinity, chemical oxygen demand (COD). NH_4^+, NO_3^-, NO_2^- and PO_4^{3-} were also measured two/three times per week in the growth PBR and once a week in the accumulation photobioreactors. Total and carbonate alkalinity were measured by using a photometric kit (Tintometer, Amesbury, UK). Alkalinity was related to the dissolved inorganic carbon (DIC) content as described in Rueda et al. (2022a, 2022b). COD was analysed with a colorimetric method, using a kit from Lovibond (Tintometer, Amesbury, UK). $N\text{-}NO_3^-$, $N\text{-}NO_2^-$ and $P\text{-}PO_4^{3-}$ were measured by the colorimetric methods described in Standard Methods (methodologies 4500-NO_2^- and 4500-NO_3^- and 4500-P respectively) (APHA-AWWA-WPCF, 2017). Total suspended solids (TSS) and volatile suspended solids (VSS) in the growth reactor were measured two/three times per week

Table 2
Average pH, electrical conductivity (EC), dissolved oxygen (DO), temperature, dry weight (DW), dissolved inorganic carbon (DIC), chemical oxygen demand (COD), nitrogen and phosphorus concentrations in the influent and mixed liquor of the growth photobioreactor; maximum volumetric productivity (P_{max}) and average volumetric productivity (P_{mean}). Significant differences between samples are shown by letters a, b, and c (*p-value* < 0.05). HRT (d) corresponds to the hydraulic retention time (days) of the corresponding period.

	HRT 10 d		HRT 8 d		HRT 6 d	
Parameter	Influent	Effluent	Influent	Effluent	Influent	Effluent
pH	7.99 ± 0.38	8.71 ± 0.74	7.95 ± 0.67	8.77 ± 1.04	7.86 ± 0.46	8.54 ± 0.63
EC ($mS\ cm^{-1}$)	2.47 ± 0.08	2.44 ± 1.26 [a]	2.13 ± 0.09	3.29 ± 5.87 [b]	2.25 ± 0.26	2.45 ± 2.62 [c]
DO ($mg\ O_2\ L^{-1}$)	–	10.08 ± 2.17 [a]	–	13.28 ± 5.61[b]	–	8.27 ± 2.43 [a]
T (°C)	–	25.63 ± 1.69 [a]	–	26.57 ± 2.05 [a]	–	28.19 ± 1.24 [b]
DW ($mg\ L^{-1}$)	33.04 ± 4.14	366.90 ± 106.10 [a]	43.06 ± 14.29	830.10 ± 498.60 [b]	25.33 ± 3.15	447.50 ± 92.72 [a]
DIC ($mg\ C\ L^{-1}$)	71.32 ± 20.07	48.01 ± 24.74	71.22 ± 5.09	51.87 ± 24.58	63.72 ± 2.86	58.52 ± 21.23
COD ($mg\ O_2\ L^{-1}$)	96.65 ± 40.70	84.28 ± 31.36	105.83 ± 69.52	120.77 ± 41.61	66.50 ± 22.22	78.25 ± 21.65
$N\text{-}NH_4^+$ ($mg\ L^{-1}$)	43.14 ± 7.82	4.46 ± 7.35	43.61 ± 4.14	12.86 ± 18.78	48.89 ± 18.33	1.58 ± 3.13
$N\text{-}NO_3^-$ ($mg\ L^{-1}$)	0.49 ± 0.43	44.71 ± 41.88 [a]	74.56 ± 157.97	188.56 ± 70.53 [b]	12.18 ± 45.48	18.28 ± 11.67 [a]
$N\text{-}NO_2^-$ ($mg\ L^{-1}$)	0.62 ± 0.56	5.47 ± 5.10	1.02 ± 0.58	5.24 ± 4.93	0.41 ± 0.21	6.66 ± 5.84
$P\text{-}PO_4^{3-}$ ($mg\ L^{-1}$)	0.62 ± 0.40	1.91 ± 0.70 [a]	4.12 ± 5.96	3.41 ± 4.07 [a]	1.84 ± 2.42	0.16 ± 0.50 [b]
P_{max} ($mg\ L^{-1}\ day^{-1}$)		49		173		108
$P_{average}$ ($mg\ L^{-1}\ day^{-1}$)		34 ± 11		90 ± 59		70 ± 24

Note: Influent (A) and effluent (B) coated values indicate the average from the following number of experimental points (N). A) pH (N = 11), EC (N = 5), DW (N = 5), DIC (N = 4), COD (N = 5), $N\text{-}NH_4^+$ (N = 5), $N\text{-}NO_3^-$ (N = 3), $N\text{-}NO_2^-$ (N = 4), $P\text{-}PO_4^{3-}$ (N = 5), B) pH (N = 21), EC (N = 19), DO (N = 20), T (N = 20), DW (N = 12), DIC (N = 8), COD (N = 6), $N\text{-}NH_4^+$ (N = 11), $N\text{-}NO_3^-$ (N = 11), $N\text{-}NO_2^-$ (N = 11), $P\text{-}PO_4^{3-}$ (N = 8).

following gravimetric methods 2540C and 2540 D described in Standard Methods (APHA-AWWA-WPCF, 2017).

The daily volumetric biomass production P (g L^{-1} d^{-1}) was calculated using Eq. (1):

$$P\left(g\,L^{-1}d^{-1}\right) = \frac{(VSSr - VSSi)}{HRT} \quad (1)$$

where; VSSi and VSSr (g L^{-1}) are the volatile suspended solids measured in the influent and in the reactor, respectively. HRT (day) is the hydraulic retention time of the corresponding period. The average productivity for each period is the mean of the daily volumetric productivities; while the maximum productivity is the maximum daily productivity obtained for each period.

2.4. Identification of microorganisms and cell counts

The culture broth was observed under the microscope during each period to monitor the behaviour of growing cells, cell morphology and presence of other microorganisms (competition). Culture broth samples were observed using a bright light microscope (Motic, China) equipped with a camera (Fi2, Nikon, Japan) and a fluorescence microscope (Eclipse E200, Nikon, Japan) using the NIS-Element viewer® software. Cyanobacteria, microalgae, protozoa and rotifers were identified and classified following the morphological descriptions provided by the database CyanoDB (Komárek and Hauer, 2013) and a taxonomic book (Streble and Krauter, 2018).

The Neubauer chamber was used for cell counting following the methodology described by Nedbal (2020). To determine the number of filamentous species, all the filaments present in 0.1 μL sample were counted. Each filament was considered as 1 microorganism unit.

2.5. Phycobiliprotein extraction and quantification

During the second and third periods (HRT 8 days and HRT 6 days), and twice a week, the effluent from the growth PBR was centrifuged (3300 *g*, 10 min) and pellets (~ 1 g wet biomass) were frozen at −21 °C for phycobiliproteins extraction. The procedures reported by Arashiro et al. (2020b) and Zavřel et al. (2018) were combined and adapted to the conditions of this study. Firstly, a freeze-thaw cycle (−21 to 4 °C) was performed. Then, phosphate buffer (pH 7, 0.1 M) was added at a 1:10 (w:w, biomass: solvent) ratio. The resulting mixture was distributed in 1.5 mL tubes with glass beads (0.3 g of 0.1 mm ø beads, and 6 units of 2 mm ø beads), and cells were disrupted by bead beating (3200 rpm, 10 min) at 4 °C using a vortex (Vortex-Genie™ 2, Scientific Industries SI™). Finally, the disrupted sample was centrifuged (9500 rpm, 15 min), and the supernatant was collected and measured at OD_{562nm}, OD_{615nm} and OD_{652nm} wavelengths, which correspond to the maximum absorption of phycoerythrin, phycocyanin and allophycocyanin, respectively. The concentrations of phycobiliproteins were quantified according to Eqs. (2)–(4) (Bennett and Bogobad, 1973):

$$Phycocyanin\left(mg\,L^{-1}\right) = [OD_{615nm} - (0.474{*}OD_{652nm})]/5.34 \quad (2)$$

$$Allophycocyanin\left(mg\,L^{-1}\right) = [OD_{652nm} - (0.208{*}OD_{615nm})]/5.09 \quad (3)$$

$$Phycoerythrin\left(mg\,L^{-1}\right) = [OD_{562nm} - (2.41{*}PC) - (0.849{*}APC)\,]/9.62 \quad (4)$$

Supernatants were also measured at OD_{620nm}, OD_{652nm}, OD_{565nm} and OD_{280nm}, to calculate phycobiliprotein purity ratios, according to Eqs. (5)–(7) (Arashiro et al., 2020a):

$$Phycocyanin\ purity\ ratio = OD_{620nm}/OD_{280nm} \quad (5)$$

$$\text{Allophycocyanin}\ purity\ ratio = OD_{652nm}/OD_{280nm} \quad (6)$$

$$Phycoerythrin\ purity\ ratio = OD_{565nm}/OD_{280nm} \quad (7)$$

The DW of biomass was measured according to Standard Methods (2540 B - Total Solids Dried at 103–105 °C) (APHA-AWWA-WPCF, 2017), to express PBP content as $\%_{dcw}$. All the analyses were performed in triplicate and under dark conditions to avoid pigment degradation.

2.6. PHB extraction

Samples of 50 mL from the accumulation photobioreactor were harvested and centrifuged (4400 rpm, 10 min). Then, the pellet was washed with deionized water to remove residual salts. After that, the pellet was frozen at −80 °C for 12 h in an ultra-freezer (Arctiko, Denmark) and finally dried for 24 h in a freeze dryer (−110 °C, 0.049 hPa) (Scanvac, Denmark). PHB extraction was done based on the procedure reported by Lanham et al. (2012). Dried biomass was mixed with 1 mL of CH_3OH, acidified with H_2SO_4 (20 % v/v) and with 1 mL of $CHCl_3$ containing benzoic acid as internal standard. Then, samples were incubated in a dry-heat thermo-block (Selecta, Spain) for 5 h at 100 °C. After that, samples were refrigerated in iced freezing water for 30 min. Then 1 mL of deionized water was added and samples were vortexed during 1 min. The bottom $CHCl_3$ phase was removed, and introduced into chromatography vials. A gas chromatograph (GC) (7820A, Agilent Technologies, USA) with a DB-WAX 125–7062 column (Agilent, USA) was utilized for the quantification of PHB. Chromatography was performed using an injector split ratio of 5:1 and a temperature of 230 °C. The temperature of the FID was 300 °C. The estimation of PHB concentration was done by using the *co*-polymer of PHB-PHV as a standard. The carrier gas was He at a flow rate of 4.5 mL/min.

2.7. Lipid extraction

Harvested biomass from the accumulation reactor was centrifuged and the supernatant was removed. The pellet was frozen at −80 °C for 12 h in an ultra-freezer (Arctiko, Denmark) and frozen-dried for 24 h in a freeze dryer (−110 °C, 0.049 hPa) (Scanvac, Denmark). The lipids were extracted from lyophilized biomass by washing with chloroform:methanol solvent mixture in a 1:4 (*v*/v) ratio. A Soxhlet apparatus was used for the extraction of lipids. A rotary vacum evaporator was implemented for the evaporation of the solvents from the sample extracts. The residual oil was weighed on a calibrated analytical scale.

2.8. Data analysis

The normality of the data was assessed by using KS normality tests. To find significant differences between effluent physic-chemical parameters between experimental periods (HRT 10, 8 and 6), normal data sets were analysed by One-Way ANOVA with Tukey's posthoc tests. For data that did not pass the normality test, One-Way ANOVA with Kruskal-Wallis, and Dunn's posthoc tests were developed (p-*value* < 0.05). These analyses were conducted with the use of GraphPad Prism (version 5.1).

3. Results and discussion

3.1. Biomass concentration and production

During the first period (HRT of 10 days) the biomass concentration remained fairly stable at approximately 0.5 g L^{-1}. A maximum volumetric production of 49 mg DW L^{-1} day^{-1} was achieved (Table 2). The decrease in biomass concentration, hence in production, observed towards the end of this period was caused by the limited amount of nutrients in the influent wastewater, along with the formation of flocs in the mixed liquor and subsequent attachment as biofilm onto the walls of the PBR. Biofilm growth may have reduced light penetration in the photobioreactor, limiting biomass growth. To reduce such biofilm formation, the stirrer velocity was increased from 72 to 90 rpm in the subsequent periods.

During the second period (HRT of 8 days) an exponential growth of the biomass was observed (Fig. 2). The average DW was higher than in the first period, achieving values up to 1.4 g DW L^{-1} and maximum biomass productivity of 173 mg DW L^{-1} day^{-1}. This was linked to the addition of nutrients at the beginning of the second period.

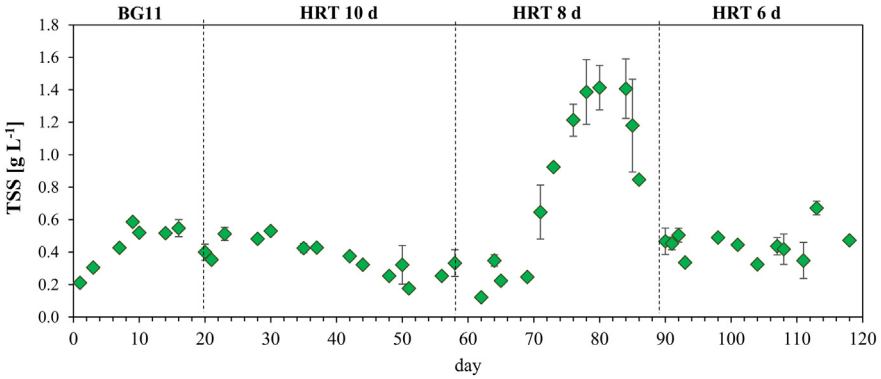

Fig. 2. Biomass concentration expressed as dry weight (DW) in the growth photobioreactor over 118 days of semi-continuous operation. *Synechocystis* sp. was initially cultivated in BG-11 and thereafter in secondary effluent at decreasing hydraulic retention time (HRT).

In the third period, the HRT was reduced to 6 days. The biomass concentration was much more stable than in previous periods (Fig. 2), reaching a concentration peak of 0.7 g DW L^{-1} with a maximum biomass productivity of 108 mg DW L^{-1} day^{-1}. Reduced biomass growth could be related to the concomitant presence of rotifers and high ammonium loads (see Sections 3.2 and 3.3).

Productivities found in this study are in the range obtained previously in other *Synechocystis* sp. experiments. Cai et al. (2013) cultured *Synechocystis* sp. in non-sterile conditions by using nutrients from anaerobic digestion effluents and obtained a productivity ranging from 41 to 151 mg L^{-1} day^{-1}. Troschl et al. (2018) obtained a maximum biomass productivity of 62 mg L^{-1} day^{-1} using a pilot photobioreactor fed with non-sterile BG-11. Singh and Kumar (2021) reported a biomass productivity of 56 mg L^{-1} day^{-1} for *Leptolyngbya* sp. cultivated in batch mode under non-sterile conditions. Note that *Leptolyngbya* sp. was the second most abundant cyanobacteria found in the present study (see Section 3.3).

3.2. Nutrients consumption

Fig. 3 shows the concentration of nutrients in the culture medium. The concentration of ammonia in secondary treated wastewater was similar during all the experimental periods, except for some peaks during days 60 and 90 (Fig. 3 D). Despite the progressive increase in ammonia loading rate along the periods, due to the decrease in HRT, the culture was able to eliminate all the ammonium added. Note that ammonium was the preferred N source for cyanobacteria due to its oxidation state, as it can be utilized without prior reduction for the synthesis of complex macromolecules such as amino acids (Jiang et al., 2016; Johnson et al., 2017).

Nevertheless, a high concentration of free ammonia (9.6–26.7 mgN-NH_3 L^{-1}) has been reported to be toxic for cyanobacteria (Rossi et al., 2020). Note that when secondary treated wastewater was added in the third period, the concentration of ammonium was on average 8.1 mg N-NH_4^+ L^{-1} and up to 12.5 mgN-NH_4^+ L^{-1} when there was a peak in the

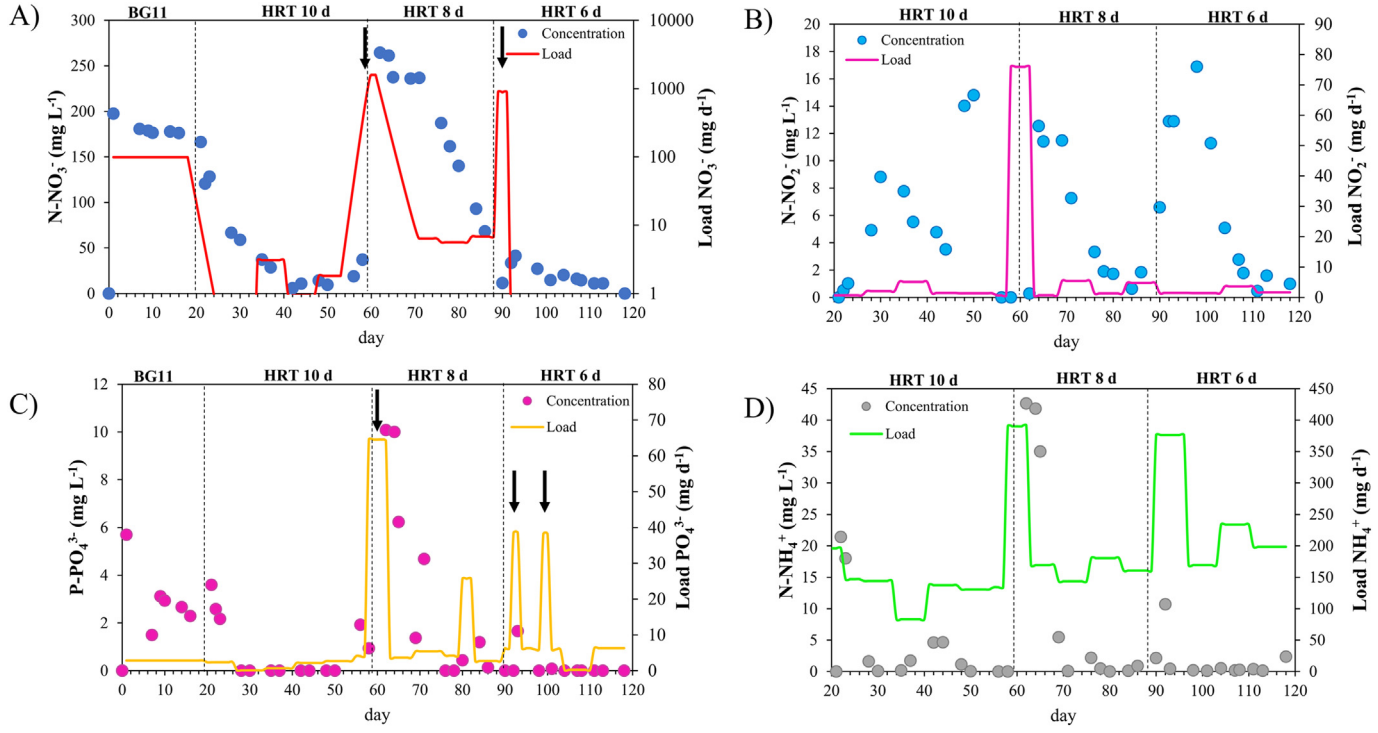

Fig. 3. Concentration of a) nitrate ($N\text{-}NO_3^-$), b) nitrite ($N\text{-}NO_2^-$), c) phosphate ($P\text{-}PO_4^{-3}$) and d) ammonium (NH_4^+) in the growth photobioreactor, along with the nutrients loading rate during 120 days of operation. Arrows indicate the points at which nutrients were added artificially.

treated wastewater concentration. Considering the pH and temperature in this study (8.4 and 28 °C, respectively), it is estimated from chemical equilibrium that on average 18 % of the ammonia N inside the photobioreactor was in the form of NH_3. From this value, and considering the respirometry results obtained by Rossi et al. (2020), a reduction from a 4–8 % of the photosynthetic activity during the different periods and up to 11 % in days with peaks of ammonium could be predicted. Indicating a certain inhibition of cyanobacteria growth. Ammonium inhibition together with the concomitant presence of rotifers may be responsible for the decrease in biomass concentration and productivity observed during this period.

In the first period, N-NO_3^- and P-PO_4^{3-} gradually decreased due to the utilization by cyanobacteria and dilution when secondary treated wastewater was added (note that the concentration of these nutrients in treated wastewater was really low). Thus, at the beginning of the second and third periods, nutrients (NO_3^- and PO_4^{3-}) were artificially supplied for 5 days (see Section 2.2). In both periods, the quick nutrients uptake and dilution (due to the low concentration of nutrients in treated wastewater) lead to almost no nutrients (1 mg NO_3^- L^{-1} and 0 mg PO_4^{-3} L^{-1}) at the end of each period (Fig. 3A, C).

Moderately high N:P molar ratios were found in the mixed liquor during the first two periods (30:1 and 80:1, respectively), which fit in the optimal N:P range suggested by Arias et al. (2020) to select cyanobacteria. However, in the third period a higher N:P ratio of 150:1 was detected in the culture medium, because of the lower HRT (6 days) and poor content of P- PO_4^{3-} in the treated wastewater source, which quickly diluted the synthetic P supplied to the PBR. This strong limitation of P may have stressed the cyanobacteria in the culture, explaining the lower productivity during this period.

3.3. Microorganism identification and cells count

Fig. 4 shows the relative abundance of different microorganisms identified in the culture broth for each experimental period. The culture was inoculated with *Synechocystis* sp., which was dominant during the batch period when non-sterile BG-11 was used as a nutrient source. During the first period, this initial *Synechocystis* sp. monoculture (99.6 %) turned into a consortium composed of *Synechocystis* sp. (84.8 %), the filamentous cyanobacteria *Leptolyngbya* sp. (12.6 %) and some green microalgae (1.6 %, mostly *Chlorella* sp. and *Scenedesmus* sp.). Diatoms (0.85 %) and protozoa (0.16 %) were also identified. In the second period, a slight increase in *Leptolyngbya* sp. (16.3 %) and green microalgae (2.2 %) was detected. In the third period, *Leptolyngbya* sp. increased up to 38.7 %, while *Synechocystis* sp. decreased to 42.6 %. Green microalgae, diatoms and protozoa were found at 14.7, 1.6 and 1.6 %, respectively. *Bdelloidea* (rotifers) (1.3 %) were also identified.

The decrease in cyanobacteria during the last period could be related to the higher ammonium load. Indeed, high concentrations of NH_4^+ may inhibit the photosynthetic activity of cyanobacteria and microalgae due to the toxicity of NH_3. Rossi et al. (2020) also reported a low tolerance to ammonia by the cyanobacteria *Leptolyngbya* sp. and *Synechocystis* sp., which showed a half inhibition concentration (EC_{50}) of 17.5 mg NH_3 L^{-1} and 11.7 mg NH_3 L^{-1}, respectively. Conversely, Rossi et al. (2020) estimated a much higher EC_{50} (60–96 mg NH_3 L^{-1}) for the green microalgae *Scenedesmus quadricauda* and *Chlorella sorokiniana*. *Chlorella* sp. and *Scenedesmus* sp. were the most relevant microalgae (green algae) species during the second and third periods (see Fig. S1). Considering the results by Rossi et al. (2020), the increase in the concentration of these microalgae could be attributed to their higher tolerance to NH_3.

In addition to ammonia inhibition, the shift from *Synechocystis* sp. to *Leptolyngbya* sp. may also be explained by the increase in the population of rotifers, coming from the secondary treated wastewater. As reported by Mialet et al. (2013), non-filamentous cyanobacteria (i.e., *Synechocystis* sp.) are among their preferred feed.

3.4. Phycobiliprotein content and purity

The phycobiliprotein content remained fairly stable during the second and third experimental periods, ranging between 4.8 and 7.4%$_{dcw}$. The maximum phycobiliprotein concentration (7.4%$_{dcw}$) was obtained during the third period (Fig. 5). Over the whole experiment, phycocyanin was the most abundant pigment in the biomass (up to 5.4%$_{dcw}$), followed by allophycocyanin (up to 2.1%$_{dcw}$) and phycoerythrin (up to 0.3%$_{dcw}$). The phycobiliprotein content decreased at the beginning of the third period, when *Synechocystis* sp. and *Leptolyngbya* sp. represented between 42.6 and 38.7 % of the biomass, respectively, but promptly reached values close to those obtained during the second period, dominated by *Synechocystis* sp. These results show the potential for sustainably recovering phycobiliproteins over time from cyanobacteria in secondary treated wastewater.

Regarding the phycobiliprotein average purity ratios, phycocyanin reached higher values during the second period (purity ratio = 0.6), dominated by cyanobacteria, as compared to the third period (purity ratio = 0.4), with a higher presence of rotifers and green microalgae. Indeed, a maximum phycocyanin purity ratio of 0.7 was measured during the second period (Fig. 5). Purity ratios for allophycocyanin and phycoerythrin were quite stable throughout both experimental periods (ranging from 0.2 to 0.3).

The phycobiliproteins content of this experiment is higher than the one obtained with cyanobacteria-dominated biomass grown in secondary effluent from urban wastewater treatment, with maximum concentrations of 2%$_{dcw}$ for phycocyanin and 0.8%$_{dcw}$ for phycoerythrin (Arashiro et al., 2020b) (see Table S1). Possible reasons for this could be the different phycobiliprotein extraction methodology used, the addition of nutrients (N and P) and the higher abundance of cyanobacteria in the extracted biomass (68 % vs 81 % in the third period of the present study).

Other studies on natural pigments production in monocultures of cyanobacteria, grown in several types of wastewaters, reached phycobiliproteins concentrations much more higher, up to 237 mg gDW^{-1} (Arashiro et al., 2020a; Khatoon et al., 2018). This may be attributed to the fact that wastewater was sterilized or diluted with synthetic growth media in these experiments, so contamination with grazers or other microorganisms was reduced. Moreover, the light intensities in these studies were lower (150–180 μmol m^{-2} s^{-1}) in comparison to the 250 μmol m^{-2} s^{-1} light

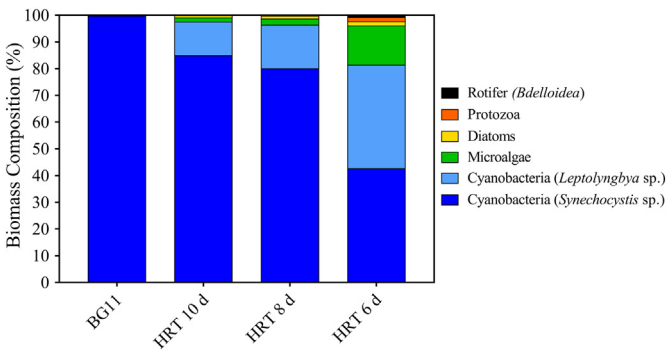

Fig. 4. Microorganisms quantification (cyanobacteria, green microalgae, diatoms, protozoa, and rotifers) during each operational period.

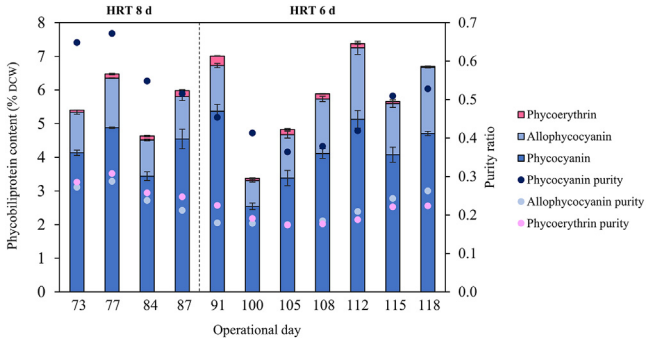

Fig. 5. Phycobiliprotein content and purity in the biomass from the growth photobioreactor over the experimental periods with a hydraulic retention time (HRT) of 8 and 6 days.

intensity in the present one. It has been described that at high light intensities cells lessen part of thylakoid membranes and phycobilisomes (protein complexes containing phycobiliproteins), as a protective mechanism to reduce the amount of radiant energy absorbed (Khatoon et al., 2018).

To enhance the production of pigments, further research should focus on the inoculation of the PBR with cyanobacteria strains which are described to present high phycobiliprotein content and control the light intensity of the culture.

3.5. PHB accumulation

At the end of each growth phase, a fraction of harvested biomass was placed in a 5 L photobioreactor to start the PHB accumulation phase. The maximum PHB content (4.8%$_{dcw}$) was obtained in the third experimental period, 7 days after the inoculation (Table 3). Interestingly, the highest content of PHB was achieved in the third period, when the population of *Synechocystis* sp. decreased to 42 % of the total population and *Leptolyngbya* sp. and green microalgae, which do not produce PHB, increased to 38 and 14 %, respectively. These results could be explained by the nutrient concentration in the 5 L accumulation photobioreactor. As shown in Table 3, although P was always limiting, there was N during the first and second periods. Conversely, in the last period, N and P were limited from the very beginning, which stimulated PHB production, even being cyanobacteria less dominant. These results suggest that N limitation is essential to stimulate PHB production. This is in accordance with previous findings, where PHB production was stimulated under N limitation and carbon excess (Price et al., 2020). In addition to this, in the last period, more light was added to the 5 L photobioreactor, which could have also affected the PHB production. This agrees with Gracioso et al. (2021), who observed that 300 μmol m^{-2} s^{-1} stimulated PHB production for *Synechocystis* sp. isolated from a mangrove. Conversely, Monshupanee and Incharoensakdi (2014) and Rueda et al. (2022b) did not observe any benefit in terms of PHB content by increasing light intensity. However, Rueda et al. (2022b) suggested that light intensity may increase PHB productivity.

The maximum PHB yields found in this study are within the range reported in the literature using several types of wastewaters as a nutrient source, in either mixed cultures or wild-type monocultures of cyanobacteria. For instance, a maximum PHB content of 4.5 % was reached in a 33 m^3 demonstrative PBR plant fed with agricultural run-off (Rueda et al., 2020b). A content of 6.2%$_{dcw}$ was obtained using sterile digestate in a 200 L photobioreactor (Troschl et al., 2018). Higher PHB values (32–80%$_{dcw}$) were obtained by other authors supplementing acetate and citrate to aquaculture wastewater or by genetically modifying strains (Krasaesueb et al., 2019; Samantaray et al., 2011) (see Supplementary Materials, Table S1 for further details).

3.6. Lipids production

Lipids accumulation was also conducted in the 5 L photobioreactor in which the concomitant PHB accumulation was done. Lipids accumulation was investigated for each experimental period and at the end of the accumulation phase. As shown in Table 3, the highest lipid content (44.7%$_{dcw}$) was obtained in period I, while a lower lipids content was obtained in the subsequent periods (12.6 and 12.2%$_{dcw}$ in periods I and II, respectively). These results suggest that the cyanobacteria culture was able to accumulate more lipids under phosphate starvation than under both nitrogen and phosphate starvation. In fact, Yang et al. (2018) confirmed that phosphorus limitation triggers the carbon fixation to lipid biosynthesis.

Table 3
PHB and lipids production with the biomass harvested from the growth photobioreactor in different experimental periods. Days after inoculation refers to the number of days the biomass was in the accumulation photobioreactor.

Experimental period	Days after inoculation of accumulation photobioreactors	PHB (%$_{dcw}$)	Lipids (%$_{dcw}$)	N (mg N L^{-1})	P (mg P L^{-1})
I	17	0.7	–	20.9	n.d.
	30	0.6	44.7	6.6	n.d.
II	13	1.6	–	1.9	0.6
	20	2.5	12.6	0.2	n.d.
III	7	4.8	–	0	n.d.
	19	3.1	12.2	0.1	n.d.

n.d. not detected.

Results found in this study are in the range of previous ones using BG-11 or cultivation media as a source of nutrients. For instance, Satpati and Pal (2021) and Singh and Kumar (2021) found a lipid content in *Leptolyngbya* sp. of 41.4 and 32 %, respectively. Lower results (11.4–13.5%$_{dcw}$) were obtained using artificial seawater with anaerobic digestate (Cai et al., 2013) (Supplementary Materials, Table S1).

4. Conclusion

In this study, the isolated *Synechocystis* sp. strain was tested for 120 days for multiproduct recovery in an integrated biorefinery approach using secondary effluent from urban wastewater treatment as a source of nutrients. The biomass produced from the 30 L growth PBR was able to accumulate up to 7.4%$_{dcw}$ of phycobiliproteins. The biomass produced in the growth PBR was subsequently inoculated in a 5 L batch photobioreactor to stimulate the PHB and lipids production. In the accumulation photobioreactor, a maximum PHB content of 4.8%$_{dcw}$ was achieved, while up to 44.7%$_{dcw}$ of lipids was obtained. The phycobiliprotein production was demonstrated to be constant during all the experimental periods. In the case of PHB and lipids, the concentration obtained was strongly dependent on the cultivation period and the amount of nutrients withdrawn from the growth PBR and fed to the accumulation photobioreactor. In the case of lipids, the maximum content was obtained in the period where there was presence of N, and only P was depleted (Period I). On the other hand, PHB production required both N and P limitation (Period III).

This study demonstrates the possibility to recover multiple bioproducts from the produced biomass. Nevertheless, further research should be done to increase the bioproducts' productivity by improving the photobioreactor performance and by searching for new strains with increased bioproduct production.

CRediT authorship contribution statement

Vincenzo Senatore: Conceptualization, Data curation, Methodology, Formal analysis, Investigation, Writing – original draft, Visualization. **Estel Rueda:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Visualization. **Marta Bellver:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – xoriginal draft, Visualization. **Rubén Díez-Montero:** Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Ivet Ferrer:** Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Tiziano Zarra:** Writing – review & editing, Supervision. **Vincenzo Naddeo:** Writing – review & editing, Supervision. **Joan García:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Data availability

Data will be made available on request.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.159343.

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