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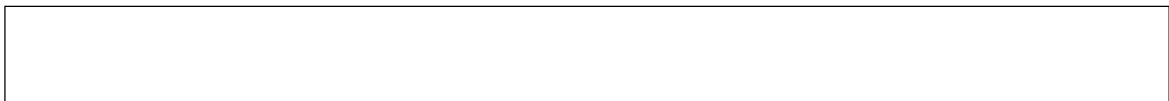
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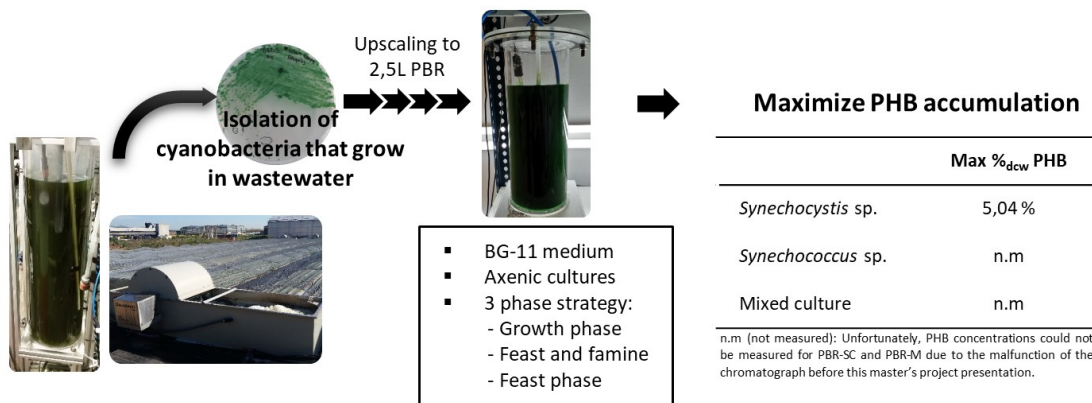


Photosynthetic production of polyhydroxybutyrates (PHB) by cyanobacteria isolated from wastewater treatment processes.

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Graphical abstract



Highlights

- 4 cyanobacteria species were isolated from wastewater
- High concentrations of N and P enhanced the cyanobacteria dominance
- N limitation reduced the %N/VSS which may indicate inhibition of protein synthesis.
- Feast and famine allowed to transform glycogen into PHB in *Synechocystis* sp.
- High concentrations of C boosted both PHB and glycogen accumulation.

Keywords

PHB · glycogen · isolated cyanobacteria · bioproducts · biorefinery · feast-famine strategy

Abstract

Accumulation of non-degradable plastics in the environment has become a problem of worldwide concern. In this context, polyhydroxybutirates (PHB) have arisen as a promising alternative as they have similar characteristics to ordinary plastics but these polymers can be biodegraded. PHB production by cyanobacteria has the potential to reduce the production costs of these bioplastics whereas a carbon neutral production process is achieved. However, new approaches enhancing the accumulation yields should be investigated to attain a competitive production process. The main objectives of this work were 1) to isolate different cyanobacterial species growing in different photobioreactors fed with wastewater and 2) to study the PHB accumulation in the isolated species using a three-stage feeding strategy (growth phase, feast-famine phase and feast phase). *Synechocystis* sp, *Synechococcus* sp, *Leptolyngbya* sp, and *Microcoleus* sp, were isolated from samples taken from wastewater treatment photobioreactors, which demonstrates the high competitiveness of these species in this media. During the feast and famine phase, a reduction in the glycogen content was observed in *Synechocystis* sp and an increase in *Synechococcus* sp and mixed culture reactors, whereas PHB continued increasing. During the feast phase, a clear increase in both glycogen and PHB was observed in all the reactors, reaching a maximum of 5.04% dcw in *Synechocystis* sp. These results demonstrate that the interchange of the various carbon accumulation forms can be controlled by the carbon content and feeding strategy followed

INTRODUCTION

During the last 50 years, plastics production has increased twenty fold (322 million tonnes in 2015) and is expected to keep growing, doubling the current production [1]. The use of plastics has become essential in several sectors, such as automobiles, home appliances, computer equipment, packaging, medical applications etc [2]. Polypropylene and Polyethylene are the two polymers most frequently used [3]. Approximately the 60% of the ever-produced plastics worldwide ($4900 \cdot 10^6$ tonnes) is discarded to landfill or natural ecosystems. For instance, about $5-13 \cdot 10^6$ tonnes of plastics (1.5-4% of the annual production) end up in the oceans every year, where they are transported by marine currents around the world. It is estimated that 500,000 tonnes of plastics are already accumulated in the oceans [1]. Considering that these compounds are barely degraded, environmental issues and serious pollution problems are arising: Some examples of the current problems are serious injuries, drowning, choking or the lack of appetite caused by entanglement or ingestion of plastics by marine and terrestrial animals [4] or the presence of micro and nanoplastics in the environment that can be ingested by the lower trophic levels or they can even penetrate the cell membrane inducing toxic effects [5]. In consequence, there is a growing interest in finding sustainable alternatives to mitigate the environmental problems aforementioned, such as the use of alternative materials like biodegradable plastics. Since Lemoigne isolated Polyhydroxybutyrate (PHB) from *Bacillus megaterium* in the 1920s [2], this biopolymer has gained interest as a solid alternative to conventional plastics. PHBs are bioplastics that can be biodegraded and mineralized by microorganisms and they have similar characteristics to ordinary plastics (such as polypropylene), including an equal response to extrusion, injection moulding, or fibre

spinning [6–10]. Metabolix was the first company to commercialize PHB in 2004. Since then, the PHB market has progressively grown and many other manufacturers have arisen. However, its price is still far from being competitive with conventional plastics (PHA is 18-fold costlier than polypropylene) [8].

PHBs can be produced by more than 300 different species of prokaryotes as an energy and carbon storage polymers [11,12], such as heterotrophic bacteria, like *Cupriavidus necator* or several species of *Pseudomonas*, autotrophic bacteria such as cyanobacteria or purple bacteria and many species of Archaea. The production of PHB by eukaryotic organisms, such as plants or algae, requires a previous genetic transfer of the corresponding genes [10]. Currently, the already established biotechnological processes uses heterotrophic biomass to produce PHB, as the cultivation of this biomass in big amounts is easier and the PHB production is much more efficient [6,10,13,14]. However, heterotrophic bacteria typically requires large amounts of organic carbon from vegetal origins, such as beet, cane molasses, corn or starch [6,12,15], which leads to competition for arable land used to produce crops for human consumption [11,16,17]. The use of these carbon sources together with the oxygenation requirements of heterotrophic bacteria accounts for approximately the 30-50% of the PHB biosynthesis costs, being one of the main bottleneck in industrial production of bioplastics [6,13,16]. Cyanobacteria have become a promising alternative to heterotrophic bacteria as they are able to produce PHB by oxygenic photosynthesis, meaning that they can use sunlight and recycle CO₂ from industrial effluents to produce bioplastics. Hence, the production costs, in terms of growth media, decreases substantially whereas a carbon neutral production process is achieved. Furthermore, they avoid the ethical conflicts of using arable areas to produce non-edible products, as they can be cultivated in many non-

agricultural places such as in building roofs or desertic regions [10,13,16,17]. However, the PHB production by cyanobacteria face some challenges:

- They need long time periods to grow and accumulate PHB
- The harvesting of the biomass could be complicated, what could mean up to a 30% of the biomass production costs.
- PHB productivity by direct photosynthesis is significantly lower than that of heterotrophic bacteria, almost always below 10% DCW [10,13,17].

Therefore, cyanobacterial biosynthesis of PHB needs to be optimized and new approaches focusing on enhancing the accumulation yields and the minimization of the production costs, should be investigated to attain a cost-effective production process.

One alternative to produce cyanobacterial biomass in a cost-effective and eco-friendly manner is to use wastewater effluents as a nutrient source [18]. Acién *et al.*, concluded that using wastewater as a nutrient feedstock could reduce the production costs of microalgae biomass from 3€/kg to 1.8€/kg [18]. This cost reduction is related with the lack of sterilization of substrates or reactors, the use of cheaper equipment and the exploitation of low cost nutrient sources when using wastewater instead of pure cultures [19,20]. However, although wastewater is a promising alternative to grow cyanobacterial biomass it still has to overcome different problems. Mixed wastewater-borne cultures are composed by different species of cyanobacteria, each one with different PHB accumulation rates, other bacteria that also can accumulate PHB and eukaryotic microorganisms, such as green algae, diatoms, protozoa, yeasts, etc. that are unable to accumulate PHB [19]. The presence of non-PHB accumulating microorganisms and microorganisms with low accumulating rates causes an important reduction of the

dry cell weight accumulation percentages in comparison with those reported for pure culture and synthetic substrates (Table 1).

Table 1. PHB Production in non-engineered cyanobacteria under photosynthetic metabolism

Type of culture	Limitation	Medium	Photoperiod light:dark (h)	Incubation period (days)	Maximum PHB (% dcw)	Reference
Cyanobacterial dominated mixed culture	N	BG-11	12:12	8	6.5	[21]
Cyanobacterial dominated mixed culture	N	Specified in the reference	24:24	2	3.8	[19]
A mixed culture of green algae, cyanobacteria, bacteria and protozoa	No limitation	Digestate and wastewater secondary effluent	12:12	35	< 0.5	[22]
<i>Synechocystis</i> sp. PCC 6714	N,P	BG-11 (x2 P)	24:0	16	23	[23]
<i>Nostoc muscorum</i>	P	ES	16:8	8	21.5	[24]
<i>Synechococcus</i> sp. MA19	P	BG-11	24:0	10.8	55	[25]
<i>Spirulina subsalsa</i>	N (increased NaCl)	ASNII	14:10	15	14.7	[26]

It is well known that a lack of nitrogen (N) and phosphorus (P) in the growth media together with an imbalance in the C:N ratio enhances the PHB and glycogen accumulation [8,13,19,21]. Another strategy to enhance the carbon uptake efficiencies is to add the carbon in a feast and famine regime. This strategy consist in repeatedly alternate the full availability (feast phase) with the complete absence (famine phase) of carbon [19,27]. The feast and famine regime has been widely applied in the production of PHB by mixed bacterial cultures, as it causes a selective pressure over the PHB storing microbial population [27]. Furthermore, this regime not only select PHB storage microorganisms, but it also generates an unbalanced growth, that stimulates the polymer storage (which can be used as a carbon source when this nutrient is exhausted). On the contrary when the substrate is present in the media for a long time, bacteria

adapt their metabolism and growth become more important than storage [28]. Arias *et al.* applied this strategy in a cyanobacterial dominated wastewater-borne culture for the first time, using bicarbonate as inorganic carbon source. The authors observed that with transient carbon regimes the carbon uptake efficiency improved, and thus, if a subsequent accumulation process (feast phase) was performed a higher polymer content could be obtained [19].

Based on this previous work, the first objective of the present work is to isolate different cyanobacterial species growing in systems fed with wastewater, which are expected to be the best adapted and most competitive in that environment. The second objective is, to study each of the isolated species separately using defined and sterilized culture mediums in order to select the one that accumulates more PHB. A three stages strategy will be applied: a first stage in which all the nutrients necessary for cells growth will be added; a second stage that will start when C, N, P are depleted. A feast-famine strategy will be used in this phase, aiming to improve the C uptake efficiency of the species; and eventually, a third stage in which inorganic C (IC) will be continuously supplied in order to boost the PHB accumulation. The present work aims to increase the limited knowledge about PHB accumulation in cyanobacterial growing in wastewater.

MATERIALS AND METHODS.

Chemicals and reagents

K_2HPO_4 , $NaNO_3$, $NaHCO_3$, $CaCl_2 \cdot 2H_2O$, $NaOH$, Na_2EDTA , $NaHCO_3$ and the reagents needed for NO_3^- , NO_2^- , NH_3 and P analysis were obtained from Panreac (Barcelona, Spain). $MgSO_4 \cdot 7H_2O$, $C_6H_8FeNO_7$ (ammonium ferric citrate), $C_6H_8O_7$ (citric acid), HCl , chloroform ($CHCl_3$) and D-glucose were purchased from Scharlau (Barcelona, Spain). Prepared BG-11 broth, CH_3OH , H_2SO_4 , $C_{17}H_{36}$ (heptadecane) and PHB-PHV (86:14% wt, CAS 80181-31-3) copolymer standard were purchased from Sigma-Aldrich (St. Louis, US).

Description of the sampling site.

Microalgae samples were taken from two different photobioreactors (PBRs), where a mixed consortium of cyanobacteria and microalgae were cultivated using water from the sewage treatment as feedstock. Both photobioreactors were chosen because the species present there, were able to grow and eliminate nutrients from wastewater.

The first sampling site was a full-scale tubular horizontal semi-closed photobioreactor located at the UPC experimental area, in Viladecans (41.288 N, and 2.043 E UTM). This PBR had a total useful volume of 11.7 m^3 and was fed daily with 2.3 m^3 of a mix of water from a drainage channel (agricultural run-off of the different fields in the area and the discharge of the Gavà-Viladecans WWTP) and treated domestic water from an aerated septic tank (ratio 6:1). More information about the operation and design of this PBR can be found in [29,30].

The second sampling site was a 30L closed photobioreactor. Inoculated with *Nostoc* sp. (soil origin) and daily fed on a semi-continuous mode with 3L of digestate diluted in

secondary effluent from high-rate algal pond in a ratio 1:50 as described previously by Arias *et al.* [31].

Media and culture conditions.

Three different BG11 concentrations (BG11-1, BG11-2, and BG11-3) were used to evaluate the effect of nutrient concentration in the isolation. BG11-1 growth medium consists of: 1500 mg·L⁻¹ NaNO₃, 31.4 mg·L⁻¹ K₂HPO₄, 36 mg·L⁻¹ MgSO₄, 36.7 mg·L⁻¹ CaCl₂·2H₂O, 20 mg·L⁻¹ Na₂CO₃, 1 mg·L⁻¹ NaMgEDTA, 5.6 mg·L⁻¹ citric acid, 6 mg·L⁻¹ Ferric ammonium citrate and 120 mg·L⁻¹ NaHCO₃. BG11-2 and BG11-3 are a 1:2.5 dilution and a 1:5 dilution of BG11-1 respectively.

The plates were prepared with 1% bacteriological agar supplemented with the corresponding BG11 medium. The BG11 medium and the Bacteriological agar should be autoclaved separately (at double strength) and mixed when cooled at 50°C, to avoid the formation of toxic decomposition products [32]. The inoculated plates were incubated at 30 ± 2 °C under 15 hours light: 9 hours dark cycles and were illuminated with a 14W cool-white LED. Two illuminances of 1 or 0.1 klx were tried.

Isolation and purification.

After sample collection, different plating methods were applied to isolate axenic cultures. The isolation strategy consisted of three basic steps:

1. **Disaggregation of the biomass flocs:** It was used to destroy the flocs and facilitate the manipulation of the microalgae samples. Two disaggregation techniques were evaluated:

- Sonication of the biomass samples in an ultrasonic bath at 360 W during 45s and vortexing the sample 10s after that. This cycle was repeated nine times as described by Abzazou *et al.* [33].
- Homogenization of the sample with a Polytron™ PT 2500E (Polytron, Montreal, Canada) at 10000 rpm during 1min.

Both techniques are useful for disaggregating the samples. The first one was milder, even so it was able to break the flocs. The second one was much faster and no loss of cells viability was observed in this case. However, the second technique could be harmful for cells with low resistance to shear stresses.

2. **Application of the plating method:** streak plating and spread plating were used to get rid of the unwanted microorganisms (e.g. microalgae, bacteria, fungus). Streak plate isolation consisted in streaking the sample across the agar surface using a bacterial loop. As the distance from the origin increases single cell colonies are formed. In the spread plate method, 100 µL of sample were added with a micropipette and dispersed over the surface with a sterile cell spreader [34]. In this method three sample dilution were done to the sample to ensure that single colonies were formed. This way, the first sample was the undiluted sample (C1), the second dilution (C2) was a 1 to 100 dilution and the third (C3) a 1 to 10⁴ dilution.

In both methods three different concentrations of the culture media were used in order to see the effect of nutrients concentration in the isolation (see Media and culture conditions section). After plating, the agar plates were incubated until single colonies were observed (approximately 2 weeks).

- 3. Isolation from the agar plate and Re-plating:** once single colonies were formed, they were isolated from the agar plate using a platinum bacterial loop. If a single cyanobacterial species from a colony was observed in the bright light microscope, an equal colony was placed into 2 mL of liquid BG11 medium. On the other hand, if more than one cyanobacterial genus was identified or the presence of green algae or other microorganisms were identified in a colony, this colony was re-plated in another agar plate with the same medium concentration as the initial plate. This process was repeated as many times as needed to obtain an axenic culture.

Once axenic plates have been obtained, it is necessary to preserve them in order to pick up single colonies of a defined species whenever it is necessary. To do that, an axenic colony is spread over a 1% bacteriological agar plate supplemented with BG11 medium and incubated at 30 ± 2 °C and 1 klx for proper growth. When new colonies are formed, the plate is stored at 4°C for 1 month. This process should be done periodically to maintain the isolated species.

Identification of cyanobacteria

The isolated cyanobacteria were observed by both a bright light microscope (Motic, China) equipped with a camera (Fi2, Nikon, Japan) and a fluorescence microscope (Eclipse E200, Nikon, Japan) connected to a computer (software NIS-Element viewer®). Cyanobacteria were identified and classified following the morphological descriptions of a cyanobacteria database [35] and a reference taxonomic book [36]. The morphological characteristics include cell size and shape, trichomes, thickness, formation of colonies, amongst others.

Upscaling: from plate to a laboratory scale photobioreactor.

As mentioned previously, once cyanobacteria were isolated in plate, they were transferred to liquid BG-11. To do that, few colonies are picked up with a platinum loop and transferred to 2 mL sterile BG-11 medium contained in a 15mL test tube. When the color of the culture changed to a blue-green color, the biomass was transferred to a larger volume of medium. From 2mL to 10mL, from 10 to 50mL, from 50 to 250mL and from 250 to 1000mL. Eventually, the 1000 mL was the inoculum for the 2,5L lab scale photobioreactors. A small scale-up ratio of 1:5 is needed to achieve proper cyanobacterial growth. Test tubes were used for volumes from 2 to 10mL. These cultures were kept at 30 ± 2 °C under 1 klx illumination and are shaken manually once a day. Erlenmeyer flasks were used for volumes from 50 mL to 1000 mL. These cultures were continuously agitated either by means of sterile air bubbling or by magnetic agitation, and were kept under 2.1 klx illumination at 30 ± 2 °C. From 250 mL onwards, aeration with compressed air was needed to keep the pH in a proper level (pH 7-10). It took about 15 days to scale up from one volume to another.

Photobioreactors experimental set up

Three different types of culture were tested for PHB and glycogen accumulation, two from the isolated species (*Synechocystis* sp. and *Synechococcus* sp.) and one mixed culture obtained from the first sampling site. Each reactor was inoculated with 0,5 L of the pure culture of the studied strain. In the case of the mix culture 2.5 L obtained from the first sampling site were directly added to the reactor. Two replicates were done for each of the isolates.

Three different phases were applied to each of the replicates in order to stimulate the PHB and glycogen accumulation:

1. *Growing phase*: N, P and C were added in the BG-11 medium in a concentration that cells could consume when biomass reached a concentration of $1 \text{ g} \cdot \text{L}^{-1}$ VSS (no extra media was added to the mix culture). N, P and C were periodically controlled; if N was depleted before the other nutrients, a small amount of N- NaNO_3 ($4.6 \text{ g N-NaNO}_3 \cdot \text{L}^{-1}$) was added to provide the necessary amount of N for cell growth until the other nutrients were completely consumed. Only extra N was supplied, since C was already supplied as CO_2 by the pH controller and cyanobacteria can resist long periods of time without P [37].
2. *Feast-Famine phase*: the aim of this phase is to improve C uptake efficiency of the studied cyanobacteria. It started when N, P and C from the previous phase were depleted and it lasted 1 week. During this phase C was available during 6 h per day (feast) and non-available during the rest of the day (famine). In the case of the experiment with *Synechocystis* sp., C was added as CO_2 during 3h by the pH controller, and it was expected to be consumed after 3h, so after 6h no more C would be available. Due to the difficulty of controlling the C addition by CO_2 injection, bicarbonate was used for the feast and famine phase in the *Synechococcus* sp. and mixed culture reactors. In both cases about 5 mg L^{-1} of bicarbonate were supplied at the beginning of the feast and famine phase. This amount of bicarbonate was expected to be consumed after 6h before the famine stage started.
3. *Feast phase*: approximately $120 \text{ mg} \cdot \text{L}^{-1}$ of bicarbonate were added to each culture at the beginning of the feast phase whereas CO_2 was also injected to control the pH. The continuous presence of C was expected to boost the PHB and glycogen accumulation.

The PHB and glycogen accumulation experiments were carried out in closed cylindrical PBR made of polymethacrylate with a diameter of 11 cm and a total volume of 3L (see Figure 1). Two replicates were performed for each of the previously isolated species, *Synechocystis* sp. (PBR1-S), *Synechococcus* sp. (PBR-SC) and a unique replicate for the Mixed culture (PBR-M), which was filled with mixed liquor from the hybrid PBR of the first sampling site. All the reactors were continuously agitated with a magnetic stirrer (VELP scientifica, Usmate, Italy) at a speed high enough to ensures complete mixing. Reactors were submitted to 15:9 h light:dark phases and were illuminated by two external 14W cool-white LED providing a medium illuminance of 2.1 klx. Temperature was regularly measured and maintained at 30 (± 2) °C. The pH was continuously measured by a pH probe (HI1001, HANNA, USA). A set point of 7.5 was used when CO₂ was injected to control the pH while a set point of 9 was set when HCl was used in order to prevent a massive injection of acid and the degradation of the alkalinity. Both pH values were selected taking into account the pH at which microalgae thrive (between 6.5-10) [38]. The pH was controlled by an automatic pH controller (HI 8711, HANNA, USA) adding CO₂ during the growing and the feast phases and HCl 0.5M during feast-famine phase (except for the *Synechocystis* sp. experiment where CO₂ was used as inorganic carbon source instead of bicarbonate).

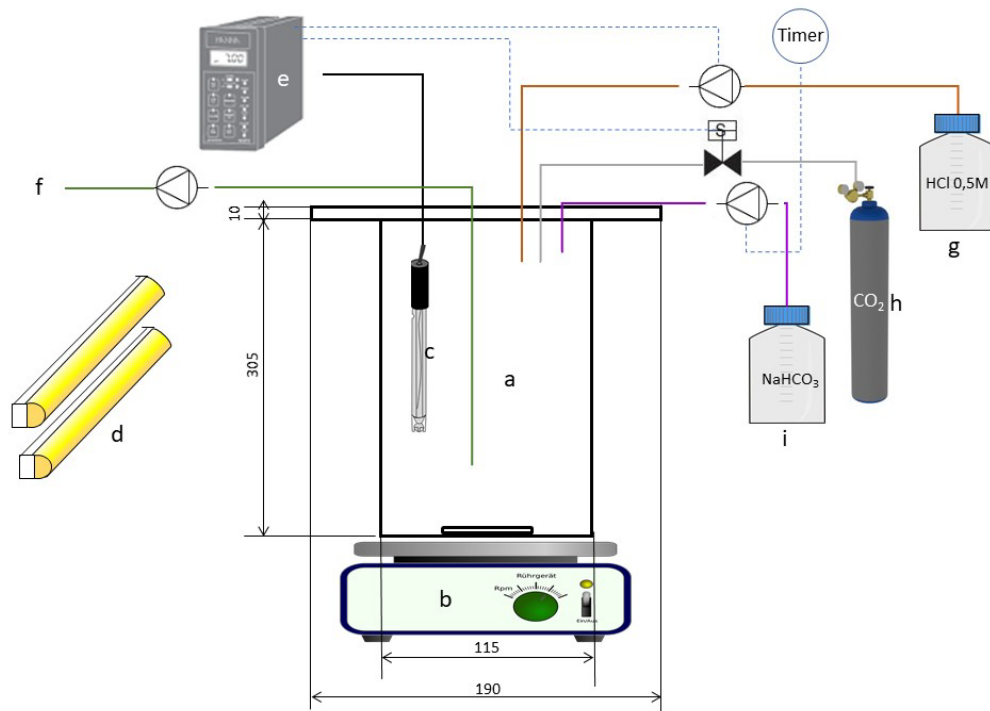


Figure 1. Scheme of the photobioreactors (PBR) set up: a) body of the photobioreactor, b) Magnetic stirrer, c) pH probe, d) cool-white LED lamps, e) pH controller, f) sample extraction system, g) HCl automatic addition system, h) CO₂ automatic injection system, i) NaHCO₃ automatic addition system.

Analytical methods

Physicochemical properties

The cultures were periodically analyzed to evaluate the nutrients uptake and the growth rate of the different species. Dissolved and total inorganic carbon (DIC and TIC) as well as the total dissolved carbon (TDC) and the total carbon (TC) were measured at the beginning of the experiment and after each of the three experimental phases (growing phase, feast-famine phase and feast phase). The other C forms can be calculated as follows:

- Total organic carbon (TOC) = TC-TIC
- Dissolved organic carbon (DOC) = TDC - DIC
- Particulate organic carbon (POC) = TOC-DOC
- Particulate inorganic carbon (PIC) = TIC-DIC

DIC or TIC (in case PIC was present in an important amount) were measured once a week during the whole experiment.

Total nitrogen (TN), total dissolved nitrogen (TDN), nitrite (N-NO₂), nitrate (N-NO₃) and ammonia (N-NH₃) were measured similarly. The other N forms can be calculated as:

- Dissolved organic nitrogen (DON) = TDN - N-NO₂ - N-NO₃ - N-NH₃
- Particulate nitrogen (PN) = TN - DON - N-NO₂ - N-NO₃ - N-NH₃

Nitrate, which is the most important form of inorganic nitrogen in the medium, was measured twice per week during the growing phase to control the N uptake.

Total phosphorus (TP), total dissolved phosphorus (TDP) and orthophosphate (P-PO₄) were also measured at the beginning of the experiment and after each of the three phases. Dissolved organic phosphorus and particulate phosphorus were calculated as:

- Dissolved organic phosphorus (DOP) = TDT - P-PO₄
- Particulate phosphorus (PP) = TDP - DOP - P-PO₄

Orthophosphates were measured twice per week to follow the P uptake.

TC, TDC, TIC, DIC, and TN concentrations were analyzed using a C/N analyzer (21005, Analytikjena, Germany). N-NO₂ and N-NO₃ were measured by the colorimetric methods described in Standard Methods (methodologies 4500-NO₂⁻ and 4500-NO₃⁻, respectively) [39]. P-PO₄, TP and TDP were analyzed following the methodologies 4500-P E and 4500-P described in Standard Methods [39]. All the dissolved forms were previously filtered through 1-3 μm pore glass microfiber filter.

Total suspended solids (TSS) and volatile suspended solids (VSS) were measured 2 days per week following the gravimetric method 2540C and 2540 D described in Standard Methods [39].

PHB and carbohydrates analysis

PHB and carbohydrates were measured after each of the experimental phases. Samples were taken at the beginning of the 15h light cycle and before C addition. 50 mL of mixed liquor were collected and centrifugated (4200 rpm, 10 min), frozen at $-80\text{ }^{\circ}\text{C}$ overnight in an ultra-freezer (Arctiko, Denmark) and finally freeze-dried for 24 h in a lyophilizer ($-110\text{ }^{\circ}\text{C}$, 0.049 hPa) (Scanvac, Denmark).

PHB extraction and analysis were adapted from the methodology described by Lanham *et al.* [40]. Briefly, 1 mL of MeOH acidified with H_2SO_4 (20% v/v) and 1mL of CHCl_3 containing benzoic acid as internal standard were added to between 2-3 mg of lyophilized biomass and then the tubes were incubated in a dry-heat thermo-block (Selecta, Spain) during 5 h at 100°C . Then, the tubes were rapidly cooled on ice for 30 min, and 0.5 mL of deionized water were added to the tubes and they were vortexed during 1 minute to separate the different solvents by density (MeOH and water in the upper phase and CHCl_3 in the lower phase). CHCl_3 was removed with a Pasteur pipette and placed into a gas chromatography (GC) vial with molecular sieves to remove the water that could remain in the sample. The co-polymer of PHB-PHV was used as a standard for hydroxybutyrate (HB) and hydroxyvalerate (HV). A calibration curve with six points was prepared and processed in the same way as the samples. PHB was determined by means of GC (7820A, Agilent Technologies, USA).

Carbohydrates extraction was done following the methodology described by Lanham *et al.* [41] and the glycogen concentration was measured using the phenol-sulfuric acid method described by Dubois *et al.* [42]. Briefly, 2 mL of 1N HCl was added to 2 mg of lyophilized biomass, and then the tubes were incubated in a dry-heat thermo-block (Selecta, Spain) at 100°C during 2h. Samples were left to cool down to room temperature (approximately 15 min) and then 0.5 mL of 5%_{w/v} phenol solution and 2.5 mL of H₂SO₄ were added to the tubes. They were vortexed and 10 min after they were placed in a bath at 35°C for 15min. Eventually the absorbance at 492 nm was measured by means of a spectrophotometer (Spectronic Genesys 8, Spectronic instrument, UK).

Calculations

The growth rate was calculated by the following general formula:

$$\mu_X (d^{-1}) = \frac{\ln(X)_{t2} - \ln(X)_{t1}}{t2 - t1} \quad (1)$$

Where $\ln(X)_{t2}$ and $\ln(X)_{t1}$ are the natural logarithm of the biomass concentration given in mg VSS·L⁻¹ at the end of the growing phase (t2) and at the beginning of the growing phase (t1) respectively. The term t2-t1 is the period of time (in days) in which the formation of biomass takes place (growth phase).

The doubling time was calculated as follows:

$$t2 (d) = \frac{\ln(2)}{\mu_X (d^{-1})} \quad (2)$$

The biomass to nutrients yield were calculated for the growing phase as follows:

$$Y_{X/N-NO3,P-PO4} = \frac{VSS (mg \cdot L^{-1})_{t2} - VSS (mg \cdot L^{-1})_{t1}}{N,P (mg \cdot L^{-1})_{t1} - N,P (mg \cdot L^{-1})_{t2}} \quad (3)$$

Where VSS (mg·L⁻¹)_{t2} and VSS (mg·L⁻¹)_{t1} are the biomass concentration expressed as m VSSg·L⁻¹ at the end of the studied period (t2) and at the beginning of the period (t1).

N, P (mg·L⁻¹)_{t2} and N, P (mg·L⁻¹)_{t1} are the nutrient concentration (N-NO₃, P-PO₄) at the end and the beginning of the period.

The specific consumption rate was calculated as follows:

$$q_{X/N-NO_3, P-PO_4} (\text{mg } N, P \cdot \text{mg VSS}^{-1} \cdot \text{d}^{-1}) = \frac{\mu_X (\text{d}^{-1})}{Y_{X/N-NO_3, P-PO_4}} \quad (4)$$

The glycogen and PHB content were calculated as follows:

$$\%DCW_{Gly, PHB} = \frac{\text{mg } (gly, PHB)}{\text{mg } (DCW)} \cdot 100 \quad (5)$$

The average volumetric and specific production rate of glycogen and PHB in each of the three phases (growth, feast and famine and feast phases) was calculated as follows:

$$\bar{r}_{PHB, gly} (\text{mg} \cdot \text{L}^{-1} \cdot \text{d}^{-1}) = \frac{PHB, gly_{\text{end of the phase}} (\text{mg} \cdot \text{L}^{-1}) - PHB, gly_{\text{begining of the phase}} (\text{mg} \cdot \text{L}^{-1})}{\text{duration of the phase } (d)} \quad (6)$$

$$\bar{q}_{PHB, gly} (\text{mg} \cdot \text{mg VSS}^{-1} \cdot \text{d}^{-1}) = \frac{\bar{r}_{PHB, gly} (\text{mg} \cdot \text{L}^{-1} \cdot \text{d}^{-1})}{\bar{X}_{\text{phase}} (\text{mg VSS} \cdot \text{L}^{-1})} \quad (7)$$

where \bar{r} is the average volumetric production rate in the specific phase and \bar{X} is the average biomass concentration during the phase.

RESULTS AND DISCUSSION

Isolation and purification

Four different species were isolated, two of them from the 30L vertical column photobiorrector sample and the other two from the HTH-PBR sample. As it can be observed in Fig. 1A, the sample taken from the 30L vertical PBR was mainly dominated by *Nostoc* sp. and *Microcoleus* sp., but also there is a high presence of green algae as *Chlorella* sp. From this initial sample *Microcoleus* sp. and *Synechocystis* sp. could be

isolated (Fig. 1B,C). *Microcoleus* sp. can be morphologically distinguished by its densely packed and parallelly arranged trichomes of about 5 μm width (Fig. 1 B1, B2) [35]. *Synechocystis* sp. can be recognized by its spherical-oval shape of around 3-4 μm . They are usually found in pairs or single cells, but never forming colonies (Fig. 1 C1, C2) [35]. As it can be observed in Fig. 1 B3 *Microcoleus* sp. form long and eye visible filaments that ends covering all the plate surface. On the contrary, *Synechocystis* sp. form punctiform colonies (Fig 1C3).

Although the initial culture was dominated by *Nostoc* sp. it was not possible to isolate them. *Nostoc* sp. belongs to the Nostocales order, constituted by filamentous cyanobacteria with specialized cells known as the heterocytes (cells specialized in fixing atmospheric nitrogen) [43]. Different authors have found that N_2 -fixing cyanobacteria are able to displace non- N_2 -fixing cyanobacteria only when low N:P ratios and low TN concentrations are present [44], [45], [46]. Komárek *et al.* found out that a N:P ratio of 11.5 and TN concentration ranging between 0.2-1.3 $\text{mg N} \cdot \text{L}^{-1}$ could lead to N_2 -fixing cyanobacterial bloom [43]. The N:P ratio of the BG-11 medium was 40 and the nitrogen was over 50 mg L^{-1} in all the medium dilutions. Meaning that, BG-11 media is not appropriate to isolate N_2 -fixing cyanobacteria such as *Nostoc* sp. Instead, a modified BG-11 without NaNO_3 should be used if the isolation of this species is required. In this work, no modifications of the BG-11 medium were done, as the isolation of species favoured in nitrogen rich waters is required, as the long-term purpose of this study is to use wastewater, which is usually rich in nitrogen, as a feedstock for cyanobacteria cultivation and biopolymers accumulation. *Synechocystis* sp., on the other hand, could be isolated although its presence was really low in the initial sample. Falkowski *et al.* suggested that when nutrient concentration and mixing are low the smallest cells are

the most probably selected, as they have the highest surface area to volume ratio, which favours the nutrients diffusion from the bulk to the cell membrane [47]. Since there is no mixing when isolating cyanobacteria in agar plates, the smaller cells, such as *Synechocystis* sp. are the ones that develop the most.

Regarding the initial sample from hybrid PBR (first sampling site) (Fig. 2A), the mixed culture was dominated by cyanobacteria mainly belonging to the *Synechococcus* sp. and *Leptolyngbia* sp. However, there is also a big presence of green algae such as *Chlorella* sp. and *Ulothrix* sp. Two different species were finally isolated from that sample, *Synechococcus* sp. and *Leptolyngbia* sp. *Synechococcus* sp. can be morphologically distinguished by its small (about 0.5 μm of diameter) long-oval cells grouped in 2 to 4 cells chains [35]. They form punctiform colonies (Fig. 2B). *Leptolyngbia* sp. are waved filaments composed of shortly cylindrical cells. They form filamentous colonies (Fig. 2C).

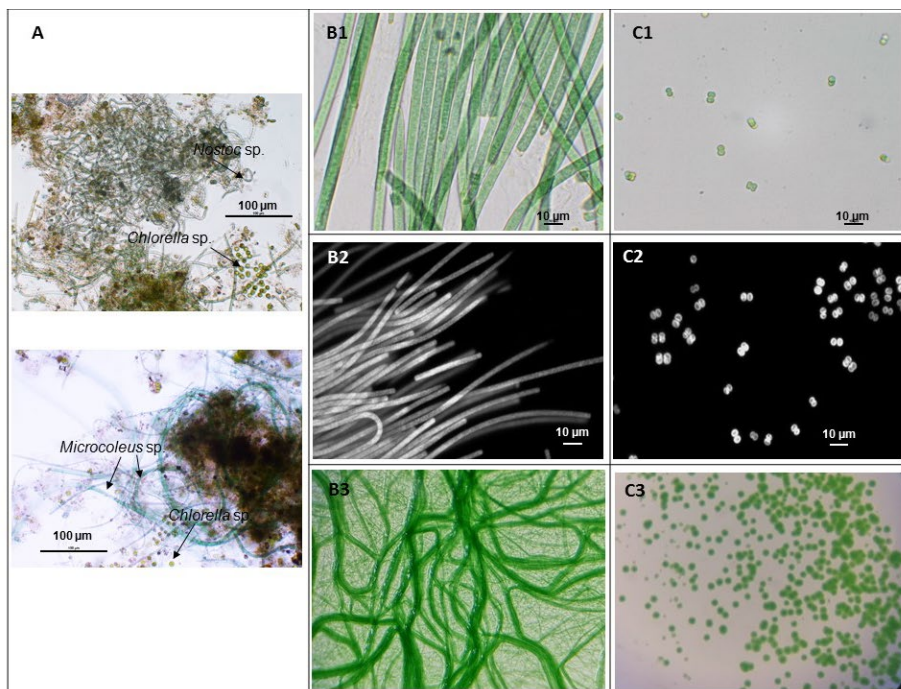


Figure 2. Microscope images of the initial sample and the strains isolated from the 30L vertical column photobioreactor A) Initial sample observed in bright light microscopy. B) Isolated *Microcoleus* sp. C) Isolated *Synechocystis* sp. B1 and C1 are observed at bright light microscopy (x1000) and B2 and C2 in fluorescence microscopy (x1000) and B3, C3 are images of colonies observed under the stereo microscope (x40).

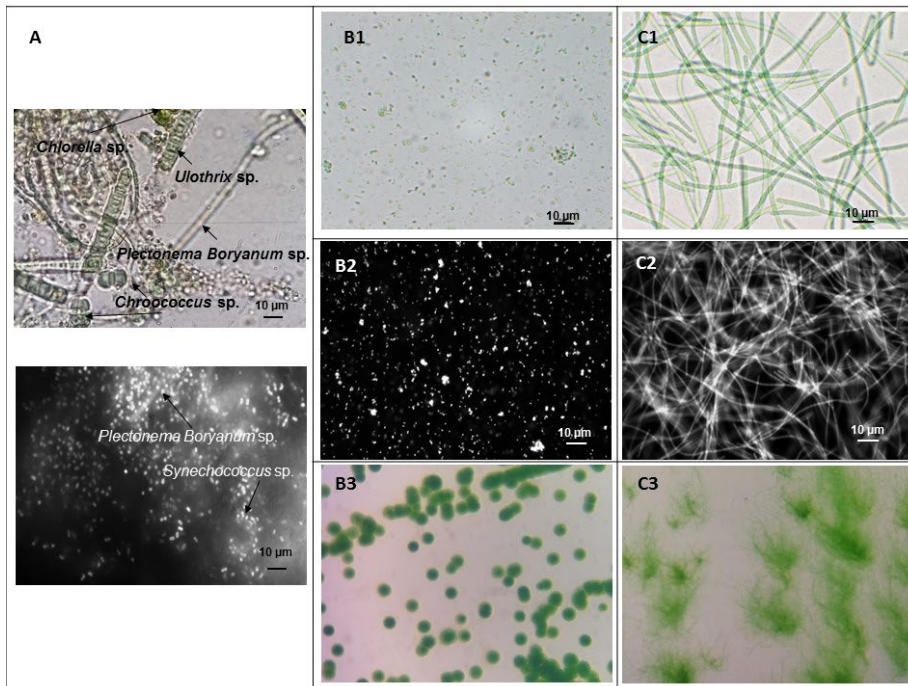


Figure 3. Microscope images of the initial sample and the isolated strains from the HTH-PBR A) Initial sample observed in bright light microscopy and in fluorescence microscopy. B) Isolated *Synechococcus* sp. C) Isolated *Leptolyngbia* sp. B1, C1 are observed at bright light microscopy (x1000), B2, C2 fluorescence microscopy (x1000) and B3, C3 are images of colonies observed under the stereo microscope (x40).

A total of 36 assays were evaluated during the isolation process: two different plating techniques, and for each of them, two different illumination conditions, 3 concentrations of the medium and 3 dilutions of the initial sample. Both isolation techniques were useful to isolate any of the species. However, spread plating needed a dilution of the initial sample of at least 1:100 to obtain isolated colonies, whereas a dilution higher than 1:10⁴ is too big when using streak plating, and no colony formation were observed in that case.

Illuminances of 100 lux and 1000 lux were tried. No colonies were formed when culturing the plates at 100 lux, whereas 1000 lux seemed to enhance the cyanobacterial growth. It was also observed that colonies were formed after moving the plates placed at 100 lux to 1000 lux after 15 days under low illumination, which demonstrates that there is a minimum illumination for the cyanobacteria to start growing and that higher

illumination enhanced the colonies formation. Conversely, Rippka, suggested that light should be kept low (200-500 lux) if isolation is done without air enriched with CO₂[48]. Therefore, a deeper study in illumination should be done if the optimal illumination must be found for each of the different species.

Regarding the different BG-11 medium concentrations tested, it was observed that higher concentration of nutrients (247 mg N-NO₃⁻·L⁻¹ and 5.6 mg P-PO₄³⁻·L⁻¹) enhanced the cyanobacteria dominance over green algae. Ferragut *et al.* found that nitrogen addition and P limitation (N:P ratio > 16) promoted the cyanobacteria dominance [49]. These authors attributed this dominance to the capacity of cyanobacteria to accumulate P internally. This was also observed in a recent study by Arias *et al* [31], in which a N:P ratio leading to P limitation stimulated the cyanobacterial growth. Bista *et al.* observed that an increase in N and temperature favoured the cyanobacteria dominance over green algae and diatoms [50]. In opposition to these studies, Ma *et al.* found that chlorophytes grew more rapidly than cyanobacteria when N and P were in high concentrations [51]. These authors attribute the dominance of chlorophytes to their smaller cell size. Larger species have a large storage capacity, but their growth is slower, whereas smaller cells are capable to quickly take up the nutrients and displace the bigger species. Loza *et al.* demonstrated that cyanobacteria preferences for different nutrient condition (low nutrients, eutrophic and hypertrophic conditions) is species dependant rather than something general from the entire phylum [52]. For instance, non-heterocystous cyanobacteria like *Leptolyngbya boryana* are favoured with increasing ammonium or NO₃⁻ levels, whereas N scarcity facilitates the development of heterocystous cyanobacteria [52].

PHB and glycogen accumulation experiments

Growth rate

Two of the four isolated species were evaluated for PHB and glycogen accumulation and were compared with a mixed culture from the photobioreactor in Viladecans (fed with agricultural runoff from a drainage channel). The other two species were not evaluated, as their growth was too slow, so they were difficultly scalable from an industrial point of view. Only one of the two replicates of *Synechococcus* sp. PBR reached the end of the experiment, as the other one died after contamination with protozoa. Growth rate and nutrient consumption were evaluated for each of the reactors.

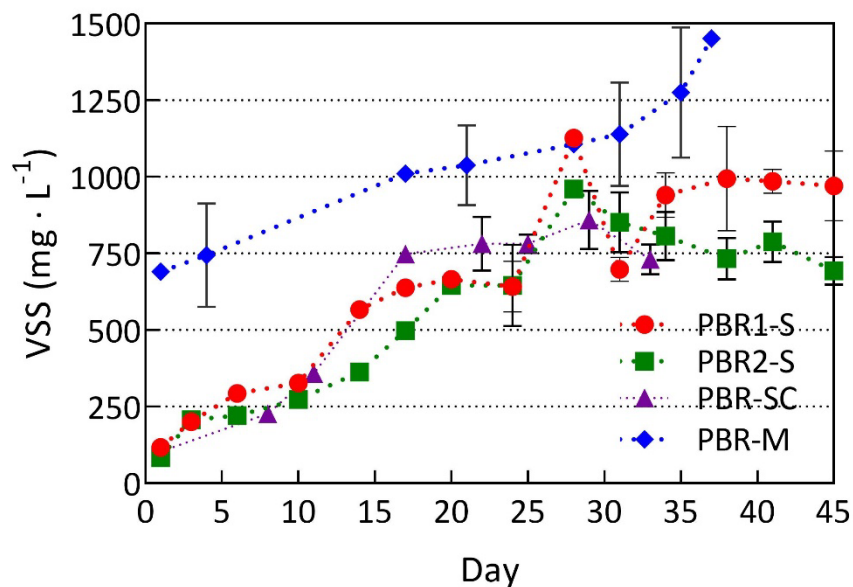


Figure 4. Evolution of the volatile suspended solids ($\text{mg VSS} \cdot \text{L}^{-1}$) during the experimental time in the different photobioreactors.

As observed in Figure 4, the PBRs inoculated with pure cultures started with a biomass concentration of around $100 \text{ mg VSS} \cdot \text{L}^{-1}$. These cultures grew until reaching values between $750 \text{ mg} \cdot \text{L}^{-1}$ and $1000 \text{ mg} \cdot \text{L}^{-1}$. In the case of PBR-M the biomass growth was less pronounced, starting with a biomass concentration of $690 \text{ mg VSS} \cdot \text{L}^{-1}$ and reaching a value of $1037 \text{ mg VSS} \cdot \text{L}^{-1}$ at the end of the growth phase. The sample taken from the first sampling site was directly inoculated in the reactor without adding any extra nutrient.

The biomass had already grown in the sampling site and nearly all the nutrients from the wastewater influent had been consumed there, so when the sample was introduced in the lab reactor, low concentrations of nutrients were left to be consumed, explaining the higher initial biomass concentration and subsequent slower growth rate. Actually, the biomass in this reactor was reaching the stationary phase when it was inoculated to the lab scale reactor.

The biomass composition remained constant during the whole experiment in both replicates of the *Synechocystis* sp. experiment. For the *Synechococcus* sp. reactors, as mentioned before, the two replicates were contaminated by protozoa from the very beginning and just one of them survived. Firstly, many protozoa in the cyst form were observed and almost none in the proliferative stage. As the experimental time advanced, some of the protozoa evolved to the active form. These protozoa can engulf the *Synechococcus* sp. biomass leading to a marked decrease in the growth and abundance of the *Synechococcus* sp. population. At this point, a clear aggregation of the biomass was observed. This flocculation process may be considered as a defence strategy, induced by the presence of protozoa, to prevent further predation [53,54]. In spite of the protozoa presence, *Synechococcus* sp. were able to grow and dominate the culture during all the experimental time, what is more, a clear decrease in the protozoa cyst form were observed and the active protozoa were maintained stable (Figure 5. B1, B2). This kind of contaminations is practically unavoidable if these processes are scaled up to industrial scales [55], so strategies to minimize the cyanobacterial predation should be further studied.

In the mixed culture the population was mainly dominated by cyanobacteria of the *Synechocystis* sp. and *Leptolyngbya* sp, and the percentages of these two genera remained constant for the entire experimental time (Figure 5. A1, A2)

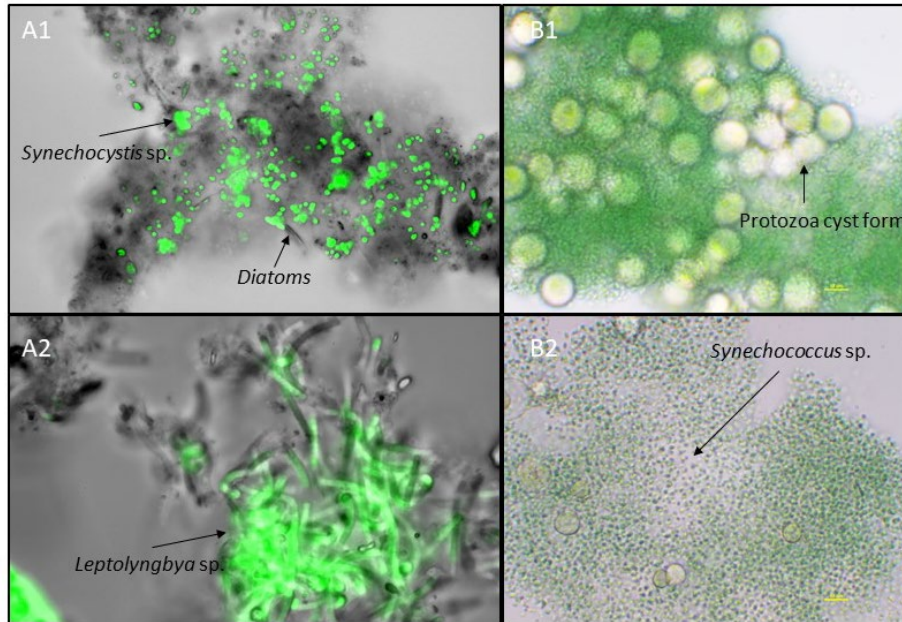


Figure 5. Images of the Mixed culture (A) observed with fluorescence microscope (Eclipse E200, Nikon, Japan) in the optical mode and the fluorescence mode and merged with the image processing software ImageJ. The green part indicates autofluorescence generated by the cyanobacteria presence. Images of the *Synechococcus* sp. culture (B) taken under light microscope (x1000). A1 and A2 are image of the Mixed culture at days 14 and 3 respectively. B1 and B2 are image of the *Synechococcus* sp. culture at days 8 and 26 respectively.

The growth phase lasted 31 days in the *Synechocystis* sp. experiment (PBR1-S and PBR2-S), 22 days in the *Synechococcus* sp. experiment (PBR-SC) and 23 days in the mixed culture (PBR-M). The duration of the growth phase depended on the growth rate of each specie and the nutrient consumption rate. As shown in Table 2, PBR-SC was the culture with a higher growth rate (0.131 d^{-1}) leading to a duplication time of 5.3 days. Despite the effect of the contamination by protozoa, *Synechococcus* sp. were able to grow faster than the *Synechocystis* sp. or the mix culture. Probably, if there had not been contamination the growth rate would had been even higher. Values between 0.3 and 2.8 d^{-1} for *Synechocystis* sp. [56–60] and between 0.3 and 1.23 d^{-1} for the *Synechococcus*

sp. [59–61] have been reported in the literature, which are about one order of magnitude higher than the ones observed in the present study. This slower growth may be related with the medium light intensity applied to the PBRs. Gonçalves *et al.*, studied the effect of light and temperature on the growth rate of two species of cyanobacteria, concluding that the optimal irradiance and temperature for *Synechocystis Salina* was 180 $\mu\text{mol photon}\cdot\text{m}^{-2} \text{ s}^{-1}$ PAR and 25°C [57]. In the present study, an average illumination of 2.1 klx (about 36 $\mu\text{mol photon}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PAR) and a temperature of 30 ± 2 °C were applied. Further optimization of the illumination should be performed in order to increase the growth rate and the nutrients consumption rate. This way, less time will be needed during the growth phase, speeding up the whole PHB production process.

Table 2. Growth rate, duplication time, nutrients uptake rate, yields in each of the experiments.

Studied Genus	PBR	μ_x (d ⁻¹)	t2 (d)	$q_{\text{N-NO}_3}$ (mg N·g VSS ⁻¹ ·d ⁻¹)	$Y_{\text{X/N-NO}_3}$	$q_{\text{P-PO}_4}$ (mg P·g VSS ⁻¹ ·d ⁻¹)	$Y_{\text{X/P-PO}_4}$
<i>Synechocystis</i> Sp.	PBR1-S	0.083	8.4	5.89	14.07	0.43	191.03
	PBR2-S	0.077	9.0	10.20	7.53	0.45	170.22
<i>Synechococcus</i> Sp.	PBR-SC	0.131	5,3	8.69	15,07	0.27	492.94
Mixed culture	PBR-M	0.021	32,6	0.85	25.10	0.05	433.02

Nutrients uptake

N and P uptake

Nutrients consumption profiles for the different PBR are represented in Figure 6. As can be observed PBR2-S, exhausted all the NO_3^- within the first 4 days of experiment, what means a specific uptake rate of 10.2 mg N·g VSS⁻¹ d⁻¹. Authors attribute this quick consumption to a period of instability caused by the presence of organic carbon (Figure 8. C2). The presence of organic carbon can change the cyanobacteria metabolism to mixotrophy, which in turn can reduce photoinhibition and increase the metabolic

activity. Given that NO_3^- uptake is energy dependant [62], the presence of organic carbon may have supplied extra energy for the NO_3^- reduction. In the 6th day of the experiment, NO_3^- was added to PBR2-S reactor to equalise the concentration with the other replicate of *Synechocystis* sp. From that point onward, the NO_3^- consumption followed the same pattern in both replicates. If the instable period of PBR2-S is not taken into account, PBR-SC was the reactor with a higher NO_3^- uptake (Table 2), followed by the mixed culture, and the two replicates of *Synechocystis* sp. cultures.

The culture with the highest N yield was the mixed culture, followed by PBR-SC, PBR1-S and PBR2-S (Table 2). This means that PBR-M was the most efficient using the NO_3^- to grow. In contrast, PBR2-S had a much lower yield than the other reactors, which is again related with the quick consumption of NO_3^- at the beginning of the experiment, that was not translated in a faster growth rate. A yellowish colour was detected in PBR-M from day 31, indicating a clear chlorosis process. Chlorosis is generally related to a lack of nutrients, and consists basically of a degradation of the pigments of the cyanobacteria [63]. No colour changes were observed in the other reactors. The differences between cultures may be related to the previous growth phase, since the isolated cultures grew in a higher NO_3^- concentration medium than the mixed culture, what led to a higher accumulation of nitrogen in cells. Figure 8. B 1-4 shows this observation, where it can also be seen that the %N/VSS after the growth phase was higher than a 9% in all the isolated cultures and below the 7% in the mixed culture.

Figure 6. b) shows the PO_4^{3-} consumption. In all the isolated reactors a PO_4^{3-} release was observed in the 4 first days of the experiment. This release of PO_4^{3-} can be directly related to pH. During the upscaling process the pH can reach values of 10, which can

have caused the precipitation of dissolved PO_4^{3-} present in the inoculated biomass. When the pH was maintained below 9, the precipitated forms of P were dissolved again, accounting for the increase in soluble PO_4^{3-} . No PO_4^{3-} were detected in PBR-M until day 23, when an unexpected drop in the pH caused by malfunction in the peristaltic pump occurred. This pH drop may have caused the death of some cells explaining the increase in organic P and its following mineralization (Figure 8. A4). The two PBRs with *Synechocystis* sp. were the reactors with a higher specific P consumption rate ($0.43 \text{ mg P g VSS}^{-1}\cdot\text{d}^{-1}$ and $0.45 \text{ mg P}\cdot\text{g VSS}^{-1}\cdot\text{d}^{-1}$ respectively, see Table 2). This rate seemed to be related to the initial concentration, since the higher it became, higher the consumption rate was.

Much higher specific consumptions rates were observed by other authors, which is again related to the slow growth rate due to the lack of light. For instance, a specific N uptake rate of $242 \text{ mg N}\cdot\text{g}^{-1}\cdot\text{d}^{-1}$ for *Synechocystis* sp. PCC 6714 MT were found by Kamravamanesh *et al.* [64] and $460 \text{ mg N}\cdot\text{g}\cdot\text{d}^{-1}$ for *Synechocystis* sp. PCC6803 by Kim *et al.* [65]

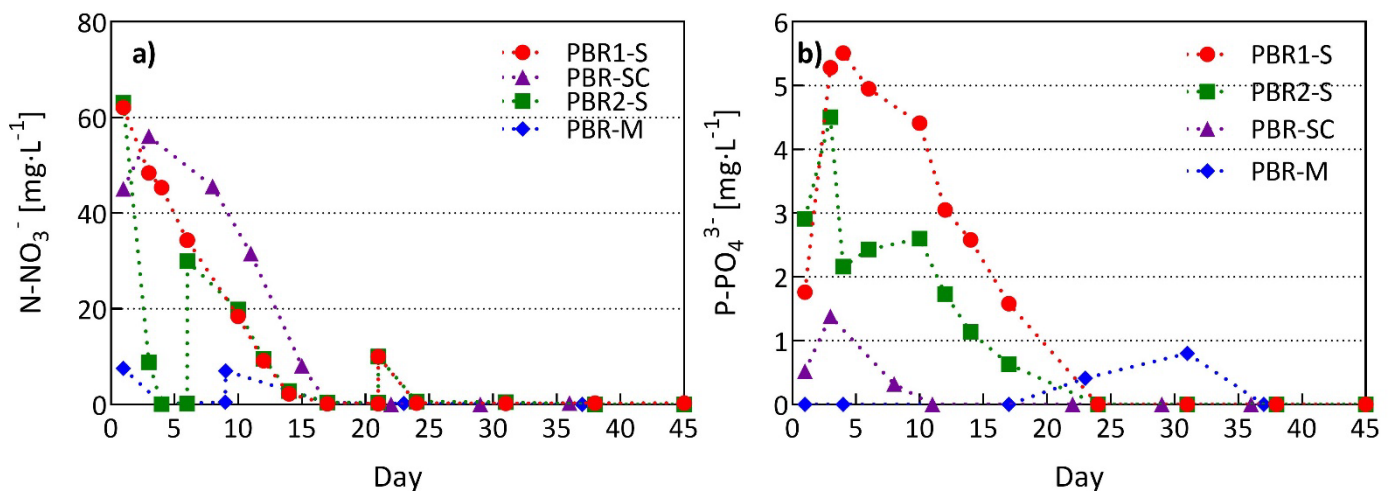


Figure 6. N-NO_3^- (a) and P-PO_4^{3-} (b) profile for all the photobioreactors.

Carbon uptake

The three experimental phases can be clearly distinguished in the carbon profiles of all the reactors (Figure 7.). During the growth phase, IC increased due to the CO₂ injection by the pH controller. When N and P were almost depleted, the pH control done by CO₂ was replaced for HCl additions. At this point IC started to decrease until reaching the feast-famine phase (C, N, P exhaustion). At the feast-famine phase small C additions were done. Inset A.1 in Figure 7. shows a feast cycle for the *Synechocystis* sp. PBRs. In these reactors the feast-famine phase was done by CO₂ additions during 3h by the pH controller. Two additions were done in PBR1-M, one at the beginning of the famine cycle and the other after 3h; one addition was done in PBR2-M after 3h from the beginning of the cycle. Not all the C added was consumed during the 6h that the feast cycle lasted. On the contrary, C accumulated as feast-famine were performed. This means that there was not a complete depletion of the IC throughout the famine cycle. Seeing that IC control by means of CO₂ addition was complicated, NaHCO₃ was used for the other two experiments. In insets B.1 and C.1 of Figure 7, the feast cycle of PBR-SC and PBR-M are shown. In this case, one addition of NaHCO₃ was done every day at the beginning of the feast-famine cycle, resulting in an IC concentration of 3.53 mg C·L⁻¹ for PBR-SC and 7.23 mg C·L⁻¹ for PBR-M. At the end of the feast cycle an IC concentrations of 1.97 mg C·L⁻¹ and 1.4 mg C·L⁻¹ were found in PBR-SC and PBR-M respectively. In both reactors a really low concentration was left at the end of the feast cycle, so it can be concluded that there will be C limitation during the famine phase. When comparing the PBRs performance it was clear that PBR-M had a higher IC consumption capacity than PBR-SC and PBR-S, being the replicates of PBR-S the ones with lower IC consumption rate. Although there is not a general affinity for one C form, it seemed that bicarbonate uptake is faster than

CO₂. According to Benschop *et al.* preference for a specific C form depends on the concentration, at low IC concentrations, HCO₃⁻ supports the majority of the photosynthesis, whereas at higher IC concentrations the CO₂ uptake become more important [66]. After the feast-famine phase HCO₃⁻ was added reaching values of 173 mg C·L⁻¹, 129.8 mg C·L⁻¹, 81,5 mg C·L⁻¹ and 96.79 mg C·L⁻¹ for the PBR1-S, PBR2-S, PBR-SC and PBR-M respectively. In the case of PBR-M and PBR-SC a precipitation of IC was observed during the Feast phase (Figure 8. C3 and C4). The precipitated IC represented a 63% and a 33% of the added IC at the beginning of the phase for PBR-M and PBR-SC respectively. Therefore, the quick drop in IC observed in Figure 7. C was mainly due to C precipitation rather than consumption, whereas IC consumption was the main cause of the drop in PBR-SC.

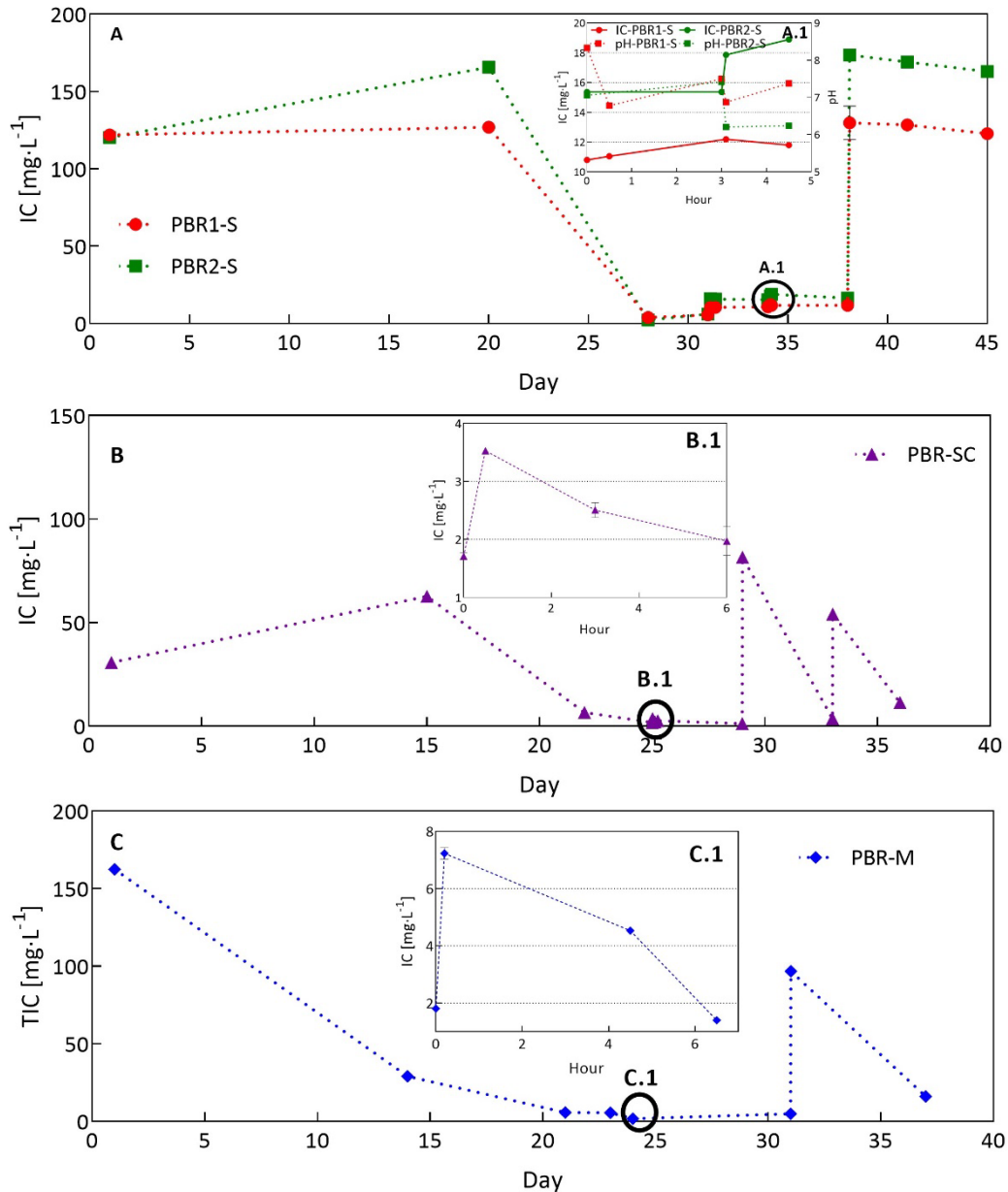


Figure 7. A and B are the inorganic carbon concentration profiles for the *Synechocystis* sp. and *Synechococcus* PBR respectively. C is the total inorganic carbon (dissolved + particulate inorganic carbon) profile of the mixed PBR. Insets A.1, B.1 and C.1 are a feast-famine profile of days 34, 25 and 24 respectively.

P, N and C mass balances

The mass balances of P, N and C are shown in Figure 8. The sum of all the different forms of N and P should be held constant during the whole experiment. The small oscillations observed should be attributed to experimental errors. In the case of C, CO₂ was not considered in the balance as dissolved CO₂ concentration was not measured. So, an increase in C was expected during the phases where CO₂ was used to

control the pH (in all the phases for reactors PBR1-S and PBR2-S, during the growth and feast phases in PBR-SC and during the feast phase in PBR-M). CO₂ was added only in the feast phase in PBR-M as high presence of particulated inorganic carbon (PIC), was observed at the beginning of the experiment (approximately 126 mg C·L⁻¹). Hence, pH control was done with HCl addition at the initial phases to get rid of all the IC forms.

Regarding the evolution of the concentration of the different forms of P and N forms, it is evident that all the dissolved forms were fixed in the biomass, with the exception of PBR-M reactor, where the amount of NO₃⁻ gained for cell growth was equal to the dissolved organic nitrogen (DON) released due to an important rate of cellular lysis. Figure 8. A1, A2 and A3 show that the %P in the biomass was higher the first day of the experiment and below 1% during the rest of the experiment for PBR1-S, PBR2-S and PBR-SC. This decrease may be attributed to the dissolution of the precipitated forms of P. This is further supported by the fact that PBR1-S, which is the reactor with the highest difference between the %P at first day and day 31, was also the one with a higher P release (Figure 6. B) whereas PBR-SC, which was the one with less P release is also the one with less %P difference. Regarding the % N, the N content in the cell decreased in all the PBRs (except for PBR2-S). N is a major constituent of aminoacids and nitrogenous bases. A decrease in the % N may be related to an inhibition in the biosynthesis of proteins due to a lack of N, which in turn enhances the NADPH pool. This surplus of reducing power has been observed by many authors as the responsible for the increase in the PHB and glycogen accumulation in the cell [8,13,21,38].

A small concentration of dissolved organic carbon (DOC) was observed in all the reactors at some point. However, in all the cases, excluding the DOC value of PBR2-M at day 1

(Figure 8. C2), the maximum DOC concentrations reached were below $65 \text{ mg C} \cdot \text{L}^{-1}$. This fact ensures that the PHB production was photoautotrophic, since heterotrophic bacteria need OC loads $> 300 \text{ mg C} \cdot \text{L}^{-1} \cdot \text{d}^{-1}$ to synthesize PHB [21]. Concerning the %C, in Figure 8. C1 and C2 it can be observed that in PBR1-S and PBR2-S it increased over the experimental time. This is an indicator that cells were accumulating C, reaching values of 68% and 69.5 % of C per mg of VSS in PBR1-S and PBR2-S respectively. On the contrary, in the other two PBR a decrease in the %C was observed during growth phase. Part of the %C was recovered in PBR-SC whereas it continued decreasing in PBR-M.

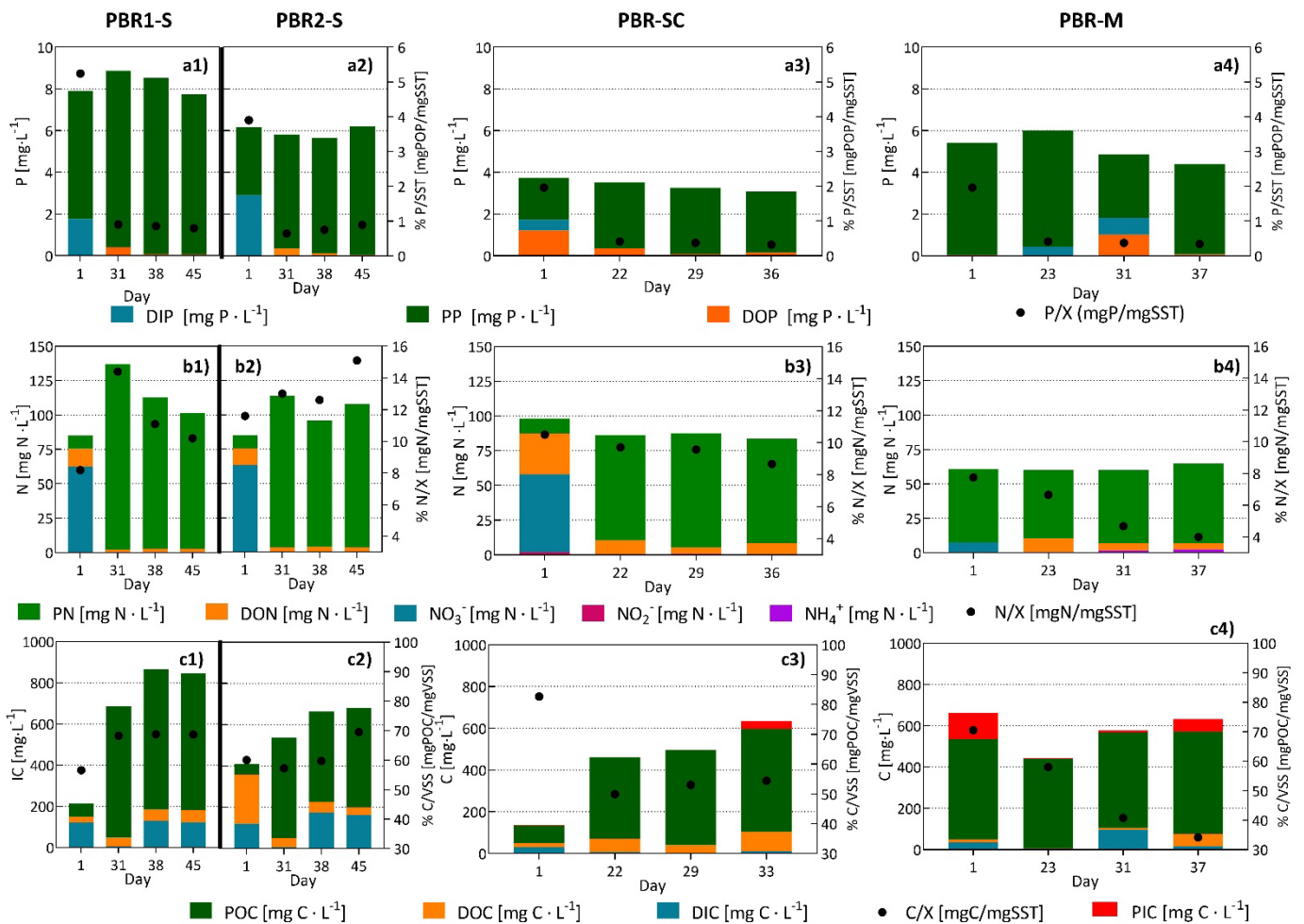


Figure 8. Balances of the different studied forms of phosphorus (a), nitrogen (b) and carbon (c) in $[\text{mg} \cdot \text{L}^{-1}]$ and the percentages of particulate organic phosphorus, nitrogen and carbon in the biomass at the beginning of the experiment, after the growth phase, after the fast-famine phase and at the end of the experiment. A1, b1, c1 correspond to PBR1-S, a2, b2, c2 to PBR2-S, a3, b3, c3 to PBR-SC and a4, b4, c4 to PBR-M. DIP, DIN and DIC are the dissolved inorganic P, N and C, PP and PN are the particulate P and N, DOP, DON and DOC are the dissolved organic forms of P, N and C, PIC is the particulate inorganic carbon and POC the particulate organic carbon.

PHB and glycogen accumulation

The main objective of this work was to study the effect of a feast-famine phase and a posterior feast phase in the PHB and glycogen accumulation in cyanobacteria isolated from wastewater treatment. Figure 9 shows the PHB and glycogen percentages profiles during the experimental time.

At the beginning of the growth phase, glycogen content was about a 10% in both PBR-SC and PBR-M, and PHB was not detected in none of them (Fig. 9b and 9c). This demonstrates that glycogen, differently than PHB, can be accumulated even without N and P limitation, what demonstrates that glycogen is the main C storage polymer [67].

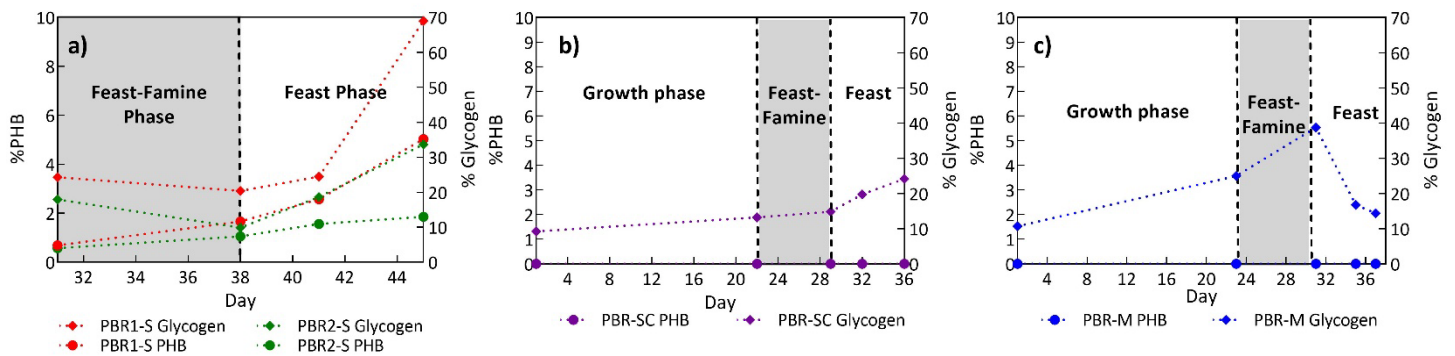


Figure 9. PHB and glycogen profiles for a) *Synechocystis* sp. reactors (PBR1-S and PBR2-S) b) *Synechococcus* sp. reactor (PBR-SC) and c) Mixed culture reactor (PBR-M).

Throughout the Feast-famine phase, the PHB percentage increased gradually in all PBRs of *Synechocystis* sp. with a specific productivity of $3.32 \cdot 10^{-3}$ mg PHB·mg VSS⁻¹·d⁻¹ and $3.14 \cdot 10^{-4}$ mg PHB·mg VSS⁻¹·d⁻¹ for PBR1-S and PBR2-S, respectively (see Table 3). However, glycogen percentages decreased in PBR1-S and PBR2-S reactors in this phase, implying that there was some interconversion from glycogen to PHB formation. On the contrary, in PBR-SC and PBR-M, glycogen concentration increased at a specific rate of $4.25 \cdot 10^{-3}$ mg Gly·mg VSS⁻¹·d⁻¹ and $3.37 \cdot 10^{-2}$ mg Gly·mg VSS⁻¹·d⁻¹ for PBR-SC and PBR-M, respectively (see Table 3).

Table 3. Kinetic and stoichiometric parameters of the feast-famine and the feast phase of the different reactors.

	PBR1-S	PBR2-S	PBR-SC	PBR-M
Feast-Famine phase				
\bar{r}_{PHB} (mg PHB·L ⁻¹ ·d ⁻¹)	1.667	0.405	n.m	n.m
\bar{q}_{PHB} (mg PHB · mg VSS ⁻¹ ·d ⁻¹)	1.90 · 10 ⁻³	5.09 · 10 ⁻⁴	n.m	n.m
Max %PHB	1.66%	1.05%	n.m	n.m
\bar{r}_{gly} (mg Gly·L ⁻¹ ·d ⁻¹)	-0.620	-11.486	3.488	31.769
\bar{q}_{gly} (mg Gly · mg VSS ⁻¹ ·d ⁻¹)	-7.06 · 10 ⁻⁴	-1.44 · 10 ⁻²	4.25 · 10 ⁻³	3.37 · 10 ⁻²
Max % Gly	24,30%	17,96%	14.83 %	38.75 %
Feast phase				
\bar{r}_{PHB} (mg PHB·L ⁻¹ ·d ⁻¹)	4.627	0.731	n.m	n.m
\bar{q}_{PHB} (mg PHB · mg VSS ⁻¹ ·d ⁻¹)	4.7 · 10 ⁻³	9.9 · 10 ⁻⁴	n.m	n.m
Max %PHB	5.03%	1.85%	n.m	n.m
\bar{r}_{gly} (mg Gly·L ⁻¹ ·d ⁻¹)	66.740	23.006	11.747	-38.746
\bar{q}_{gly} (mg Gly · mg VSS ⁻¹ ·d ⁻¹)	6.79 · 10 ⁻²	3.12 10 ⁻²	1.43 · 10 ⁻²	-19.701
Max % Gly	68.97%	33.68%	24.20 %	38.75 %

n.m (not measured): Unfortunately, PHB concentrations could not be measured for PBR-SC and PBR-M due to the malfunction of the chromatograph before this master's project presentation.

These differences can be related with the differences in the C addition. As explained in the previous section, IC addition with CO₂ does not allow to reach a complete depletion of IC, whereas bicarbonate additions were completely depleted after sometime. When IC was added in the feast phase, a fast increase in both PHB and glycogen were observed in PBR1-S, PBR2-S and PBR-SC (see Figure 9a and 9b) being more important the glycogen boost. In the case of PBR-M, the glycogen concentration decreased up to 14.4% at a specific rate of -19.7 mg Gly·mg VSS⁻¹·d⁻¹. This decrease is explained by the high C precipitation rate in this PBR. This precipitation probably occurred at the beginning of the feast phase, so C was not completely available during this period. The maximum PHB and glycogen accumulation percentage were a 5% and 68.9%, respectively, and were reached in PBR1-S. These percentages of PHB are quite low compared to previous studies (see Table 1), whereas the glycogen accumulation achieved was in accordance with previous results [21,23].

These outcomes seem to indicate that small amounts of IC need to be provided so the C interconversion can occur. As observed in the previous section, CO₂ was more slowly consumed than bicarbonate, therefore a small amount of IC was always present in the PBR1-S and PBR2-S reactors, whereas in the other two reactors C was completely depleted. This small amount of C left in these reactors may allow the cell to generate a higher glycogen pool during the light phase and maintain a certain internal IC level necessary to convert glycogen to PHB. On the contrary, the quick consumption of bicarbonate in PBR-SC and PBR-M may have generated a higher glycogen pool during the feast cycle, but part of this glycogen pool was probably consumed in the famine cycle for the cell maintenance when IC was depleted in the media. When C was present during the dark cycle, it could be used-up to generate the essential molecules for the cell maintenance, whereas glycogen could be converted to PHB in order to gain energy and recover the NADP⁺ needed in other metabolic processes [55]. If C was completely depleted, the glycogen and PHB reserves would be used to generate the essential molecules instead of convert glycogen to PHB. Something similar was observed in a previous study of Arias *et al*, where a clear decrease in PHB was observed during the dark phase when IC was completely depleted, whereas a rise in PHB when a small C concentration was present in the media was found. Glycogen, instead, was reduced in any case [19]. On the other hand, as observed during the feast phase, if an excess of C was added, a boost in both glycogen and PHB was observed, rather than a carbon interconversion.

C interconversion has been observed in many recent studies [19,21,23,55,67]. For instance, Kamravamanesh *et al*, observed that when polyphosphate (PolyP) was no longer available, glycogen conversion to PHB occurred [23]. And Troschl *et al*, described

that PHB accumulation occurred in 3 consecutive phases: a growth phase, a subsequent N limitation and glycogen and PHB production from CO₂ and a final interconversion of glycogen to PHB [55]. However, none of them has studied the effect of IC concentrations on the C metabolic pathway. Therefore, in order to verify the previous hypothesis, it will be necessary to further study the metabolic pathways of C with C starvation, low C concentrations or high C concentrations.

Conclusions

In this study, PHB and glycogen accumulation were studied in two different species of cyanobacteria under a three-phase strategy (growth phase, feast and famine phase and feast phase). *Synechocystis* sp, *Synechococcus* sp, *Leptolyngbia* sp. and *Microcoleus* sp. were isolated from wastewater-borne cultures from two photobioreactors fed with different types of wastewater. During the isolation procedure it was observed that:

- High N and P concentrations promoted the cyanobacteria dominance over green algae.
- There was a minimum illumination for cyanobacteria to start growing.
- Small cells were selected when isolation was done in agar plates as they have a highest surface area to volume ratio.

Optimization of the growing conditions is needed to minimize the time consumed for the growth phase during the PHB and glycogen accumulation experiments. The maximum PHB and glycogen accumulation percentage reached were 5% and 68.9%, respectively. Moreover, it was observed that glycogen conversion to PHB depended on the C concentration in the media. These results demonstrate the necessity to

further study the mechanism of glycogen conversion to PHB and the effect of C in this process. This mechanism could be considered an opportunity to reach high PHB contents that would be impossible if PHB was synthesised only by means of CO₂ uptake.

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