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Polymer accumulation in mixed cyanobacterial cultures selected under the feast and famine strategy

[1]Dulce María Arias, [2]Joana C. Fradinho, [1]Enrica Uggetti, [1]Joan García, [2]Adrian Oehmen, [2]Maria A. M. Reis*.

[1]GEMMA – Group of Environmental Engineering and Microbiology, Department of Civil and Environmental Engineering, Universitat Politècnica de Catalunya•BarcelonaTech, c/ Jordi Girona 1-3, Building D1, E-08034, Barcelona, Spain.

[2]UCIBIO-REQUIMTE, Department of Chemistry, Faculty of Sciences and Technology, Universidade NOVA de Lisboa, 2829-516 Caparica, Portugal.

* Corresponding author:

Tel.: +351 212948385

Fax: +351 212948385

E-mail address: amr@fct.unl.pt

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Abstract

In this study, a sequencing batch reactor (SBR), operated with transient carbon availability (feast and famine) and different nutrients loads, was used to select cyanobacteria accumulating poly (3-hydroxyalkanoate) (PHB) and carbohydrates from a mixed wastewater-borne microbial culture. The SBR was operated with 12h aerobic light and 12h anaerobic dark phases, evaluating the effect of three different operational conditions consisting on; 1) carbon limitation, 2) carbon and phosphorus limitation and 3) phosphorus limitation. Once a steady state was reached in each operational period of the SBR, part of the biomass was collected and submitted to separate batch tests in order to investigate the maximum PHB and carbohydrates accumulation levels. Batch tests were performed during 24h of illuminated aerobic condition and 24h of dark anaerobic condition, while inorganic carbon was constantly present. During the SBR operation, inorganic carbon was mostly used for biomass and carbohydrate production, showing very low PHB accumulation levels (<1%). Notwithstanding, in subsequent batch tests, PHB was accumulated after a complete depletion of nitrogen, reaching almost 4%. Concerning carbohydrates, it was found that phosphorus limitation (with and without carbon limitation) led to a culture mostly dominated by cyanobacteria and higher levels of carbohydrate content (43%-48%) than the culture with carbon limitation and high loads of nitrogen and phosphorus (29%). Such contents were obtained in only 24h of incubation under aerobic illuminated conditions. Hence, these encouraging results indicate that carbon uptake and the consequent polymers production from cyanobacteria can be enhanced through carbon and nutrient feeding strategies.

Keywords: Algae, wastewater-borne cyanobacteria, bioproducts, wastewater.

1. Introduction

Cyanobacteria are prokaryotes capable to perform oxygenic photosynthesis and they can be found in almost every environment on earth [1]. During the last decades, they have received much attention as a rich source of polymers, being considered as one of the most promising group of organisms to produce them [2]. Cyanobacteria are able to accumulate both carbohydrates in form of glycogen and and polyhydroxyalkanoates (PHA), e.g., poly (3-hydroxyalkanoate) (PHB). Carbohydrates and PHB are attracting increasing interest due to their potential as a biofuel substrate and as a bioplastic, respectively. Although those polymers are also accumulated in other photosynthetic and non-photosynthetic bacteria, the studies that have been done thus far have based their polymers production on the utilization of organic molecules as C source [3]. In the case of cyanobacteria, their mechanism for polymer production is based on carbon storage through oxygenic photosynthesis implying simple requirements for cultivation and the utilization of CO_2 as carbon source [4]. This ability for CO_2 fixation and conversion into biopolymers is nowadays significantly attractive due to the worldwide concern with the CO_2 impact in climate change.

Until now, experiments on carbohydrates and PHB production from cyanobacteria have been performed through pure strains and genetically modified species [5–9], implying strictly controlled processes leading to high production costs, and subsequently expensive products [10]. In this context, a more sustainable alternative for the production of polymers from cyanobacteria could be the use of wastewater-borne cultures. This approach implies the lack of sterilization of substrates or reactors and cheaper equipment that could reduce the production costs compared to pure culture processes. Nevertheless, in spite of being an attractive alternative, the utilization of mixed cyanobacterial cultures to produce biomass and polymers strictly depends on the composition of the culture.

Indeed, a conventional mixed wastewater-borne culture is composed by a mixture of cyanobacteria, other bacteria (which also could accumulate carbohydrates and PHB) and eukaryotic microorganisms, such as green algae, diatoms, metazoa and protozoa, which are unable to produce both polymers. Hence, a certain control of the consortium composition would be necessary in order to achieve favorable yields.

Previous studies, mainly carried out in lakes and reservoirs [11–14], but also in wastewater systems [15,16], highlighted that absolute nutrients concentration and ratio (N : P) are the two most important factors influencing the competition of cyanobacteria with other species (i.e., green algae) [12,17]. More specifically, the dominance of cyanobacteria has been related to their high affinity for N and P and capacity to store them intracellularly [18].

Concerning the polymer accumulation capacity of cyanobacteria, it has been demonstrated that nutrient limitation coupled with carbon excess are determining factors to increase polymer accumulation [19]. Thus, due to their high tolerance to nutrient changes and carbon availability, cyanobacteria polymer production usually requires prolonged periods. The low carbon uptake efficiency turns the polymer production into a slow process compared with processes involving heterotrophic bacteria. Indeed, the maximum accumulation of polymers in cyanobacteria usually takes more than 9 days of incubation for carbohydrates and more than 11 days for PHB accumulation [20–22]. This fact highlights the need for new strategies to improve the efficiency of inorganic carbon (IC) uptake in cyanobacteria and its transformation into polymers.

Considering the example of mixed bacterial cultures, one of the most feasible strategies to select specific accumulating microorganisms and improve PHB and carbohydrate production is the application of unbalanced growth, also called feast and famine [23].

This process consists of a transitory carbon supply, in which the biomass is subjected to a period of carbon availability and a subsequent absence of carbon. With this process, cell growth and storage products are enhanced while the microorganisms able to store carbon and utilize their own reserves are selected.

In this work, feast and famine is proposed as a strategy for the selection of autotrophic cyanobacteria accumulating polymers. To the authors' knowledge this is the first time that this strategy is employed to select cyanobacteria from a wastewater-borne culture using inorganic carbon as substrate to produce value-added polymers. In the present study, a mixed wastewater consortium was cultivated in a sequencing batch reactor (SBR), evaluating the effect of different nutrients ratios and loads under transient carbon availability on polymer production during the intercalation of aerobic and anaerobic phases. In addition to the effect of those factors on polymer production, the effects of other parameters such as microbial composition, nutrient uptake and oxygen production are also considered and discussed.

2. Material and methods

2.1 Sequencing batch reactor set-up

For the enrichment of cyanobacteria producing PHB and carbohydrates, a double jacket acrylic reactor with a working volume of 2 L was used. The reactor was operated as a non-sterile sequencing batch reactor (SBR). The inoculum utilized consisted of a consortium of green algae, cyanobacteria, bacteria and protozoa, obtained from a pilot photobioreactor described elsewhere [16].

The SBR was operated with a hydraulic retention time (HRT) of 2 days and sludge retention time (SRT) of 10 days. The reactor operation was based on 24h cycles according to the following scheme (Fig. A1):

1. Light aerobic phase (12h): i) 9:30 am, nutrient uptake period, starting with the addition of 1000 mL of growth medium to the reactor (6h), ii) 3:00 pm, carbon uptake period (carbohydrate accumulation), starting with a pulse of 6 mL of 0.442 g L^{-1} of Na_2CO_3 (0.050 g C L^{-1}).

2. Dark anaerobic phase (12h): iii) 9:30 pm, start of the anaerobic phase in which argon was sparged into the culture (250 mL min^{-1} for 20 min every 2 hours) in order to remove oxygen, iv) 8:50 am, effluent withdrawal phase (2 min) in which 200 mL of the mixed liquor was removed from the culture, v) 9:00 am, settling phase (30 min without stirring), vi.) Supernatant withdrawal phase in which 800 mL were removed from the medium.

During the light phase, the SBR was continuously illuminated with two external LED lamps (23W) placed on the two sides of the SBR giving a light intensity of 91 W/m^2, corresponding to a volumetric light intensity of 2.1 W/L.

Throughout the whole cycle (with the exception of the settling and supernatant withdrawal phases), the medium was stirred using a magnetic stirrer (VELP scientific, USA). The reactor temperature was controlled at 27 ºC by means of a water jacket and a thermostat bath (Julabo, Germany). The pH of the reactor was maintained at 8.2 using dosages of 0.01 M NaOH and 0.05 M HCl.

The SBR was operated for 6 months during which three different conditions were tested as shown in Table 1. In the first condition high N and P loads were applied to the culture while it was exposed to a low carbon load. In the second condition a low carbon load was coupled with a low phosphorus load. Lastly, in the third condition, the effect of only low phosphorus load was tested. Those conditions tested the influence of loads on cyanobacteria dominance, growth and production of PHB and carbohydrates. Each condition was tested when the reactor reached steady state. The reactor was considered

149 to be in steady state when the concentration of total suspended solids (TSS) at the end of
150 the cycle showed stable results or after the system reached three SRTs.

151 The growth solution added at the beginning of the light phase in each cycle consisted in
152 growth medium containing: 0.049 g L^{-1} NH_4Cl, 0.072 g L^{-1} $CaCl_2 \cdot 2H_2O$, 0.001 g L^{-1}
153 Na_2EDTA, 0.075 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.6 g L^{-1} $C_6H_8FeNO_7$ (ammonium ferric citrate),
154 0.006 g L^{-1} $C_6H_8O_7$ (citric acid), and 1.0 ml L^{-1} of trace elements: 2.86 g H_3BO_3, 0.39 g
155 $Na_2Mo_4 \cdot 2H_2O$, 1.8 g $MnCl_2 \cdot 4H_2O$, 0.08 g $CuSO_4 \cdot 5H_2O$, 0.22 g $ZnSO_4 \cdot 7H_2O$ and 0.05 g
156 $Co(NO_3)_2.6H_2O$. Depending on the operation of the SBR, K_2HPO_4 concentration varied
157 from 0.24 g L^{-1} in operation 1 and 0.0056 g L^{-1} for operations 2 and 3. Na_2CO_3 was added
158 independently as the inorganic carbon source by the addition of 1 pulse of 6 mL
159 containing 0.442 g L^{-1} (0.050 g C L^{-1}) at the beginning of the carbon phase during
160 operations 1 and 2, and 2 pulses of 0.442 g L^{-1}, one at the beginning and the other in the
161 middle of the light phase in condition 3.

162 Table 1. Experimental operating conditions of the SBR.

Operation period	C load (mg L^{-1} d^{-1})[a]	N load (mg L^{-1} d^{-1})[a]	P load (mg L^{-1} d^{-1})[a]	C:N:P ratio
Condition 1	50	12.5	4.2	12:3:1
Condition 2	50	12.5	1	50:12.5:1
Condition 3	100	12.5	1	100:12.5:1

163 [a] Volumetric load per volume of reactor.

164 **2.2 Batch test for polymer accumulation**

165 For each operation period, when steady state was reached, 200 mL of mixed liquor were
166 collected and placed in a separate batch reactor, filled to 400 mL with deionized water
167 and used for polymer production batch experiments. In these tests, the operation of the
168 reactor was based on a 48 h cycle, starting with 24h of light aerobic condition followed

by 24h of dark anaerobic condition. During the light aerobic phase, the reactor worked as an open photoreactor while in the anaerobic phase the reactor was hermetically closed. This strategy was performed in order to accumulate high carbohydrate content during the light aerobic phase and to enhance the possibility of a further conversion of the carbohydrates to PHB during the anaerobic dark phase. Similar behavior can be found in other microorganisms, such as polyphosphate accumulating organisms (PAOs), glycogen accumulating organisms (GAOs) and other photosynthetic bacteria [24,25].

During the light phase of these batch tests, the reactor was illuminated by two external halogen lamps (100W) placed on the two sides of the reactor at a light intensity of 343 W/m^2, corresponding to a volumetric light intensity of 2.2 W/L.

At the beginning of the light phase, a pulse of 10 mL containing 100 mg of carbon in the form of Na_2CO_3 ($C\text{-}Na_2CO_3$) was fed to the reactor to support polymer accumulation. Further carbon addition was supplied manually, as pulses of Na_2CO_3, to prevent carbon depletion. No N and P sources were added, thus, the only amount of nitrogen and phosphorus was the one remaining from the previous SBR cycle, leading to nutrient limitation in all the experiments.

2.3 Analytical Methods

Each time that the SBR reached steady state, three cycles of the reactor were monitored. Biomass was harvested from the reactor at different time points (9-11 samples per cycle) in order to analyze biomass concentration and composition, nutrients concentration, and PHB/carbohydrate production capacity of the biomass. The samples taken from the mixed liquor were filtered (~0.2 μm glass microfiber filter, Whatman, UK) and used to analyze soluble inorganic carbon (IC), soluble organic carbon (OC), orthophosphate (dissolved reactive phosphorus) ($P\text{-}PO_4^{3-}$), nitrite ($N\text{-}NO_2^-$), nitrate ($N\text{-}NO_3^-$), and ammonium (N-

193 NH_4^+). Unfiltered samples were used to analyze total nitrogen (TN) and total phosphorus
194 (TP). Particulate Organic Nitrogen (PON) was calculated as the difference between TN
195 and NH_4^+, $N\text{-}NO_2^-$ and $N\text{-}NO_3^-$, whereas particulate phosphorus (PP) was determined as
196 the difference between TP and $P\text{-}PO_4^{3-}$, it should be notice that PP is composed of
197 intracellular particulate inorganic P (phosphate, pyrophosphate, and polyphosphate) and
198 organic P. OC and IC were measured by means of the TOC analyzer (Shimadzu, Japan)
199 while $P\text{-}PO_4^{3-}$, NH_4^+, $N\text{-}NO^{2-}$, $N\text{-}NO^{3-}$ concentrations were measured by colorimetry
200 using a segmented flow analyzer (Skalar, The Netherlands). TN was analyzed
201 spectrophotometrically with total nitrogen kits (Hach, Germany), and TP was analyzed
202 following the methodology described in Standard Methods (APHA-AWWA-WPCF,
203 2001).

204 Dissolved oxygen (DO), pH and temperature in the reactor were measured *in situ* with a
205 DO electrode (Mettler Toledo, USA) and a pH electrode (Mettler Toledo, USA). On line
206 measurements (DO, pH, and temperature) were acquired with the software BIOCTR17.

207 The biomass concentration was measured as total suspended solids (TSS) and volatile
208 suspended solids (VSS) by filtration according to standard methods (APHA-AWWA-
209 WPCF, 2001).

210 Carbohydrates and PHB content were measured according to the methods described by
211 [26] and [27] with minor modifications. Thus, the biomass collected was centrifuged,
212 ultra-frozen and finally freeze dried. Carbohydrates were determined by mixing the
213 freeze-dried biomass with 2 mL of 0.9 M HCl and digested for 2 h at 100 ºC. The
214 supernatant was filtered (0.45 μm membrane) and glucose was analyzed by HPLC using
215 D-glucose as a standard. For PHB determination, freeze-dried biomass was digested for
216 2h at 100 °C with 1 mL of chloroform and 1 mL of methanol with 20% sulfuric acid. 1

ml of the organic phase was extracted into GC vials and measured by gas chromatography. The gas chromatograph used was coupled to a Flame Ionization Detector (GC-FID Varian CP-3800) and equipped with a Stalbiwax column (Resek, USA). Helium was utilized as the carrier gas at a flow rate of 1 mL/min. The co-polymer Poly (3-hydroxybutyric acid-co-3-hydrohyvaleric acid) (86:14 wt) (Sigma-Aldrich) was used as a standard for hydroxybutyrate (HB) and hydroxyvalerate (HV), and heptadecane was used as the internal standard. Standards with six different concentrations were processed in the same way as the samples.

Biomass composition within the SBR was monitored under microscopy for qualitative observation of microscopic populations. Microbial observation was performed in an epifluorescence microscope (Olympus BX51, Japan). Abundance of the different organisms was determined qualitatively with microscopic observations of the biomass. Cyanobacteria and microalgae species were identified *in vivo* using conventional taxonomic books (Bourrelly, 1985; Palmer, 1962), as well as a database of cyanobacteria genus (Komárek and Hauer, 2013).

2.4. General calculations and kinetic and stoichiometric parameters

Nutritional parameters applied as C volumetric load (C load) [mg C-Na_2CO_3 L^{-1} d^{-1}], P volumetric load (P load) [mg P-PO_4^{3-} L^{-1} d^{-1}] and N volumetric load (N load) [mg N-NH_4^+ L^{-1} d^{-1}] were calculated following Eq. (1):

$$\text{C load, P load, N load} = \frac{\text{C-Na}_2\text{CO}_3 \text{ or P-PO}_4^{3-} \text{ or N-NH}_4^+ * Q}{V} \qquad (1)$$

Where Q is the flow [L^{-1} d^{-1}], P-PO_4^{3-} or N-NH_4^+ is the influent concentration [mg L^{-1}] and V [L^{-1}] is the volume of the SBR.

The active biomass concentration was calculated by assuming a composition of $CH_{1.566}O_{0.405}N_{0.192}S_{0.005}P_{0.06}$ with a molecular weight of 23.08 g $Cmol^{-1}$ [31]. Thus, the active biomass (X) was calculated as:

$$X = (VSS - Carbohydrates - PHB) \quad (2)$$

Carbohydrates and PHB were calculated according to [32], in terms of percentage of VSS:

$$\%Carbohydrates\ or\ PHB = \frac{gPHB\ or\ gCarbohydrates}{gVSS} * 100 \quad (3)$$

The yields of PHB ($Y_{PHB/S}$) and carbohydrates ($Y_{carbs/S}$) per substrate consumed [Cmol PHB or carbohydrates/Cmol IC] were calculated following eq. (4):

$$YPHB/S\ or\ Ycarbohydrates/S = \frac{PHB\ or\ Carbohydrates\ accumulated}{IC\ consumed} \quad (4)$$

The maximum specific substrate uptake rate ($-q_S$) [Cmol IC/Cmol *X* d^{-1}], maximum specific polymer production rate (qPHB, qCarbs) [Cmol PHB or carbohydrates/Cmol *X* d^{-1}], were determined by dividing the slope of the linear function of experimental results along the cycle by the average of active biomass.

3. Results and discussion

3.1 Sequencing batch reactor (SBR) performance

3.1.1 General conditions

Throughout the SBR operation, the main goal was the selection and enrichment of polymer producing cyanobacteria from the initial mixed culture. Along this selection phase, three different conditions were imposed on the SBR. In Figure 1, pH, oxygen and temperature profiles during the three operating conditions of the PBR are shown. In the first operating condition (high N and P loads and low carbon loads - Table 1), in spite of not having carbon addition in the first 6 hours of light, photosynthetic activity was

261 registered due to the residual IC present in the bulk culture at the start of the light phase
262 (Fig. 1a). Hence, during the first 2 hours of the light phase and until all IC was consumed,
263 oxygen increased to 12.5 mg L^{-1}. After that time, a gradual decrease of dissolved oxygen
264 was observed until reaching 9.8 mg L^{-1}. Once the carbon was supplied to the culture, an
265 increasing pattern in the oxygen concentration was observed, until reaching a
266 concentration around 40 mg L^{-1}. At the same time, the IC content in the medium declined
267 reaching a specific uptake rate of 0.019±0.004 Cmol IC/Cmol X d^{-1} (Table 2). As soon as
268 the IC was completely consumed (approximately after 4.5 hours), the oxygen gradually
269 decreased until the dark phase started. Then argon was supplied to the culture, and oxygen
270 quickly reached zero. During the addition of carbon and IC uptake, pH tended to increase
271 due to the photosynthesis performed by microalgae/cyanobacteria and during the dark
272 phase, pH tended to decrease likely because of the organic acids released by the culture
273 [33,34]. This is a plausible assumption since a concentration of 7.8 mg L^{-1} of OC was
274 measured at the end of the dark phase. In both cases, when pH tended to increase or
275 decrease, there was an addition of acid or base solutions to maintain pH value around 8.2,
276 which can be observed in the oscillations in Figure 1.

277 Concerning condition 2, where low carbon load was coupled with low phosphorus, a
278 profile similar to condition 1 was observed. Thus, oxygenic activity was observed from
279 the start of the light phase as consequence of the residual IC (around 2.7 mg L^{-1}) (Fig.
280 1b), following the same trend on pH, oxygen and IC as condition 1. However, a faster
281 uptake of IC 0.039±0.002 Cmol IC/Cmol X d^{-1} (Table 2) and higher concentrations of
282 oxygen (up to 45 mg L^{-1}) were reached. During condition 2, pH oscillations were less
283 frequent than condition 1 during the dark phase. Indeed, OC released during the dark
284 phase was slightly lower than in condition 1, reaching 6.5 mg L^{-1}.

285 Contrary to the conditions previously mentioned, differences in the oxygen profiles were
286 observed in condition 3. In this case, due to the addition of IC from the start of the light
287 phase, the oxygen started increasing until reaching values of 34.42 mg L^{-1} after 8 hours
288 and maintaining constant values (±0.29 mg L^{-1}) during the rest of this period. According
289 to these results, pH oscillated along the entire light phase. Like under the previous
290 conditions, oxygen was always present in the culture while most of the total IC
291 consumption occurred in the light phase, and only 4% of IC was consumed during the
292 dark period. In spite of having more IC availability, the IC uptake rate was lower when
293 compared with conditions 1 and 2, having only a rate of 0.016±0.002 Cmol IC/Cmol X d^{-1}
294 (Table 2). Also, several oscillations in pH values were observed during the dark phase,
295 recording high concentrations of OC (~18 mg L^{-1}) at the end of the dark phase.

296 Interestingly, the patterns observed in pH and oxygen with relation to IC profiles, enabled
297 an non-direct determination of the periods of IC consumption and the moments when the
298 IC was depleted, all without the need of measuring the carbon in the medium.

299 Comparing the three conditions, it can be observed that condition 2 led to higher oxygen
300 concentrations and faster IC uptake (0.039±0.002 Cmol IC/Cmol X d^{-1}). This fact could
301 be attributed to a more efficient photosynthetic performance when the culture was under
302 low P and C loads in comparison with the other conditions. On the contrary, the lowest
303 values were reached under condition 3, when the carbon was present during the entire
304 light phase.

305 It has to be considered that oxygen reached very high levels in the SBR due to the reduced
306 headspace of the reactor and the poor oxygen transfer between the reactor and the
307 atmosphere. In this study, values up to 560% were reached, considering that 100% of air
308 saturation at the average temperature in the three conditions (8 mg O L^{-1}, 26.9 ºC). In

closed reactors, microalgae and cyanobacteria deal with high oxygen levels due to the photosynthetic process. In most of the cases, the high concentrations of dissolved oxygen causes damage to cellular metabolism and thus, causes inhibition depending on the species and the time of exposure [35,36]. In general, it is stated that the maximum tolerable dissolved oxygen level is 400% of air saturation [37]. In this work, although the culture was exposed to a maximum of 560% of air saturation (condition 2), the three reactors maintained higher values than 400% and the time of exposure to such values was only 4-6 hours per day, thus no toxic effects were noticed.

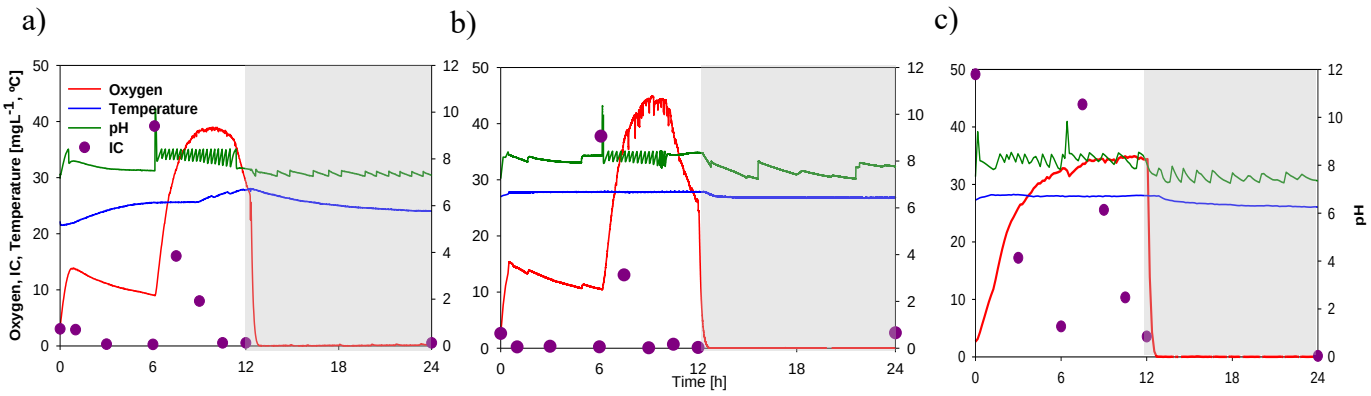

Fig. 1. Oxygen, temperature, pH and inorganic carbon (IC) patterns during one cycle of operation of a) carbon limitation (Condition 1) b) carbon and phosphorus limitation (condition 2), and c) phosphorus limitation (condition 3). White zone represents the light phase and grey zone represents the dark anaerobic phase.

Table 2. Kinetic and stoichiometric parameters of the SBR performance during the different operational conditions. Average and standard deviations (in parentheses) calculated from the three repeated cycles.

Operation	Condition 1	Condition 2	Condition 3
$-q_s$ [Cmol IC/Cmol X d^{-1}]	0.019 (0.004)	0.039 (0.002)	0.016 (0.002)
q_{PHB}[Cmol PHB/Cmol X d^{-1}]	-	-	0.00032 (0.0003)
q_{carbs} [Cmol Carbs/Cmol X d^{-1}]	0.011 (0.002)	0.049 (0.003)	0.0085 (0.003)
$Y_{PHB/S}$[Cmol PHB/Cmol IC]	-	-	0.009 (0.004)[a]
$Y_{PHB/S}$[Cmol PHB/Cmol carbs]	-	-	0.018 (0.015)[b]
$Y_{Carbs/S}$[Cmol Carbs/Cmol IC]	0.4 (0.2)	0.93 (0.3)	0.4 (0.2)

[a] yield calculated within 12 h of illumination.
[b] yield calculated within 12 h of dark.

3.1.2 Nutrient profiles

During steady-state operation in condition 1, the profile of $N\text{-}NH_4^+$ showed a higher consumption than the uptake of $P\text{-}PO_4^{3-}$, as it can be seen in Fig. 2a. Along the light phase, the initial nitrogen source was reduced by about 60%, while P source was consumed by 16%. On the other hand, a removal lower than 0.5% was found for both nutrients during the night. Specific uptake rates during the light phase were 1.08 mg $N\text{-}NH_4^+$/g X d^{-1} and 0.22 mg $P\text{-}PO_4^{3-}$/g X d^{-1}, respectively. Meanwhile, particulate organic nitrogen (PON) and particulate phosphorous (PP) concentrations, during this condition, had an average of 53±22 mg L^{-1} and 3.1±0.8 mgL^{-1} respectively, corresponding to 0.6 and 9.6 % g·g X^{-1} (Table 3). The biomass concentration present in the culture was on average 0.66±0.06 g VSS L^{-1} in the three cycles monitored. The active biomass maintained a constant concentration during the first eight hours of the light phase (0.55±0.04 g X L^{-1}), and posteriorly gradually increased to 0.76 g X L^{-1} after the addition of carbon, and even after IC was completely removed from the medium.

Table 3. Particulate phosphorus (PP) and organic nitrogen (PON) during the different operational conditions. Average and standard deviations calculated from the three repeated cycles.

	PP [% g·g X^{-1}]	PON [% g·g X^{-1}]
Condition 1	0.6 (0.1)	9.6 (4)
Condition 2	0.5 (0.2)	8.7 (2.3)
Condition 3	1.5 (0.2)	8.2 (0.9)

In condition 2, the $N\text{-}NH_4^+$ profile showed a similar pattern as in condition 1 (60% of removal in the light phase), although the $N\text{-}NH_4^+$ specific uptake rate was slightly higher (1.24 mg $N\text{-}NH_4^+$/g X d^{-1}). However, the highest uptake of $N\text{-}NH_4^+$ was found after the pulse of carbon in the middle of the light phase, where 53% of the $N\text{-}NH_4^+$ was achieved.

353 On the other hand, contrarily to condition 1, the phosphate uptake rate was lower (0.103
354 mg $P\text{-}PO_4^{3-}$/g X d^{-1}) and phosphate was completely removed after 9 hours (Fig. 2b). In
355 this condition, PON and PP showed similar values and percentages in terms of active
356 biomass than condition 1(65±17 mg L^{-1} and 3.7±1.8 mg L^{-1}, respectively, Table 3). The
357 biomass concentration was slightly higher than condition 1, (0.74±0.05 g VSS L^{-1}). The
358 active biomass profile was constant during the first eight hours of the light phase
359 (0.71±0.04 g X L^{-1}) and posteriorly increased to 0.78 g X L^{-1} in the last 4 hours of light.

360 Finally, in condition 3, the simultaneous addition of carbon and nutrients led to the
361 maximum N and P removals observed in the SBR. Thus, after adding the nutrients to the
362 culture, $N\text{-}NH_4^+$ and $P\text{-}PO_3^{4-}$ were almost completely consumed. With respect to the
363 specific uptake rates, N uptake almost doubled the value of the previous conditions with
364 2.3 mg $N\text{-}NH_4^+$/g X d^{-1} and P uptake showed similar uptake than condition 1 with 0.22
365 mg $P\text{-}PO_4^{3-}$/g X d^{-1}. In this condition, the VSS concentration raised to an average of
366 1.2±0.1 g VSS L^{-1} during the three cycles, while the active biomass averaged 0.99±0.09
367 g X L^{-1}, approximately 50% higher than the values of condition 1 (0.66 g VSS L^{-1}).
368 Considering the removal of N and P, it can be assumed that this condition led to nitrogen
369 and phosphorus limitation during most of the operational time. As shown in Fig.2c, the
370 nutrients supplied at the beginning of the light phase were coupled with the carbon
371 addition. During steady-state operation, as it can be seen in Fig. 2c, 84.7% of $N\text{-}NH_4^+$
372 removal was found during the first hour of the light phase, and was completely removed
373 after 4 hours. Concurrently, $P\text{-}PO_4^{3-}$ was completely consumed as soon as it was added.
374 Due to the increment in inorganic nitrogen removal, PON concentration increased to
375 81±9.06 but maintaining similar percentages than the other conditions (8.2±0.9 % g·g X^{-1}
376 , while OP registered a concentration of 15±1.8 mg L^{-1} and a percentage of 1.5 % g·g X^{-1}
377 (Table 3).

It should be noticed that other nitrogen forms, as $N\text{-}NO_2$ and $N\text{-}NO_3$, showed values <0.05 mg L^{-1} under all the conditions, which implies a lack of nitrification or denitrification processes in the mixed liquor.

The N/P ratio of conditions 1, 2 and 3 were 4.9, 12.03 and 10.45 mg $N\text{-}NH_4^+$/mg $P\text{-}PO_3^{4-}$, respectively, which are among the lowest values of N/P ratio in microalgae/cyanobacteria species of 8 to 45 mg N/mg P [38]. Considering these ratios and biomass concentrations, and the formation of PON and PP (Table 3), it is clear that the low load of $P\text{-}PO_3^{4-}$ and the availability of carbon in conditions 3 pressured the culture to be transformed into a consortium able to perform luxury uptake and lead to polyphosphate accumulation [12].

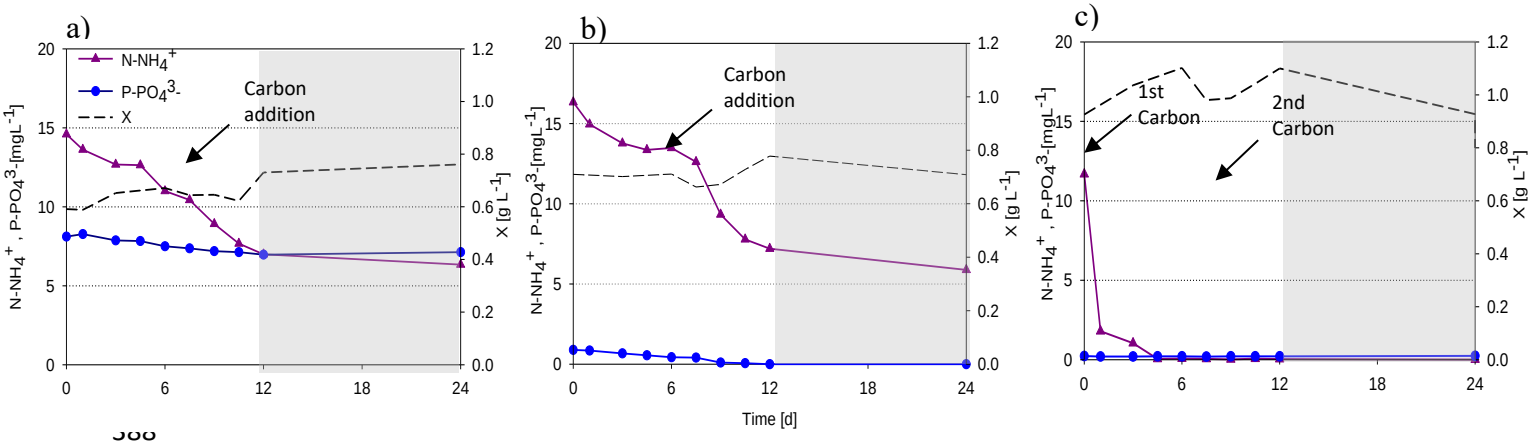

Fig. 2. Nutrient concentrations and active biomass (X) during one cycle of operation of a) carbon limitation (Condition 1) b) carbon and phosphorus limitation (condition 2), and c) phosphorus limitation (condition 3). White zone represents the light phase and grey zone represents the dark anaerobic phase.

3.1.3 Polymer accumulation in the sequencing batch reactor operation

Due to the different operating strategies to which the culture was submitted (combinations of nutrients and carbon limitation), differences in active biomass, PHA and carbohydrates between each operational condition were observed. It should be noted that throughout this

study only hydroxybutyrate monomers were detected, indicating the sole production of a PHB homopolymer. During the steady-state of condition 1, a maximum of 10.3±3.0 % of carbohydrate was reached after 4.5 h of the addition of carbon with a maximum specific carbohydrates rate of 0.011 Cmol carbs/Cmol X d^{-1} (Table 2); while PHB content only 0.3±0.1% at the end of the light phase. As shown in Fig. 3a, carbohydrates were accumulated after the pulse of IC to the medium, and increased until reaching 2.02 C mmol L^{-1}. Afterwards, a decline in carbohydrates was observed for the last 2 hours of the light phase (after IC depletion) and posteriorly in the dark phase. This decrease is associated with the stored carbon consumption by cyanobacteria (and other microorganisms in the culture) when carbon is limited in the medium. In the case of green algae metabolism, they ferment starch during anaerobic dark conditions and transform into reducing equivalents for posterior byproducts formation and release, such products are mainly acetate and ethanol, formate, glycerol, lactate, H_2 and CO_2 [34,39]. Otherwise, in the particular case of cyanobacteria and bacteria metabolism during the dark, energy generation can occur from glycogen respiration in the presence of an electron acceptor [40], then, without the presence of an electron acceptor, as occurred in this case, energy generation occurs during glycogen conversion to PHA [41]. This conversion from glycogen to PHAs is a type of metabolism also occurring during the anaerobic phase in certain types of microorganisms, such as glycogen accumulating organisms (GAOs) and (PAOs) [24,41–43], and also occurring in anoxygenic photosynthetic bacteria [44]. In condition 1, due to the anaerobic conditions in the dark phase, carbohydrates were consumed but only marginally, which can explain why PHB maintained the same low values reached in the light phase (0.3±0.1%).

In condition 2, when the culture was submitted to low carbon and P loads, a more efficient utilization of carbon transformation into carbon storage was observed. A maximum

carbohydrates accumulation of 18.87% was achieved after 3 hours from the carbon pulse reaching 5.2±1.0 Cmmol Carbs L^{-1} and a maximum specific carbohydrate rate of 0.049 Cmol Carbs/Cmol X d^{-1}, more than four times the rate obtained in condition 1 (Table 2). After finishing the IC available, carbohydrates were quickly consumed before the end of the light phase, but slightly consumed during the dark phase (Fig. 3b). This slight consumption led again to a lack of carbohydrate conversion to PHA during the night; PHA were not observed in any phase of this operational condition.

Unlike the previous operations in the SBR, in the steady-state of condition 3, in spite of achieving a lower carbohydrate content (13.8 %), a higher concentration was reached, 5.7±0.5 Cmmol carbs L^{-1}, in the last hours of the light phase (Fig. 3c). This is likely due to the high biomass concentration reached under this condition (1.2 g VSS L^{-1}). Additionally, PHB accumulation of 1.03±0.07% was achieved at the end of the dark anaerobic phase. This accumulation of PHB was likely performed by means of carbohydrate conversion since IC was not available.

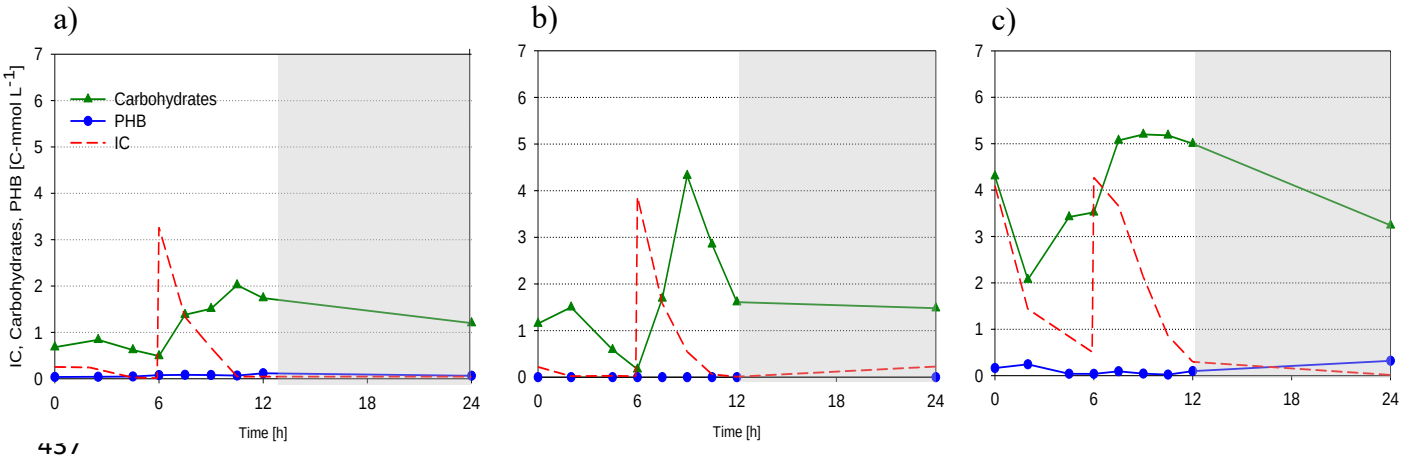

Fig. 3. Inorganic carbon (IC) consumption profile and transformation of poly (3-hydroxyalkanotes) (PHB) and carbohydrates during one cycle of operation of a) carbon limitation (Condition 1) b) carbon and phosphorus limitation (condition 2), and c) phosphorus limitation (condition 3). The white zone represents the light phase and grey zone represents the dark anaerobic phase.

In general, results indicated that the persistent availability of N and P coupled with low loads of carbon in condition 1 did not substantially improve carbohydrate accumulation. Under this condition only a low yield of carbohydrates of 0.04 Cmol Carbs/Cmol IC was obtained. Additionally, under this condition the culture was unable to accumulate PHB. Meanwhile, when the culture was submitted to low carbon loads with P limitation in condition 2, the carbohydrate accumulation efficiency was improved, reaching yields of 0.93 Cmol Carbs/Cmol IC. However, PHB was not accumulated (Table 2). On the other hand, when carbon was always available and the culture had P limitation (condition 3), nitrogen also became limiting and the efficiency of carbohydrate production decreased (Ycarbs 0.093 Cmol Carbs/Cmol IC). However, the culture was able to produce PHB with a higher efficiency by means of carbohydrates conversion during the dark anaerobic phase (0.018 Cmol PHB/Cmol carbs) than by means of IC uptake (0.009 Cmol PHB/Cmol IC). This was likely due to the highest carbohydrate concentration in condition 3, in comparison with other conditions.

3.1.4 Biomass microbial composition

Throughout the operation of the SBR, changes in the culture's composition were observed in each operation mode. While the culture was originally composed by a consortium mostly formed by cyanobacteria (abundance ~60-70%), their dominance in the culture depended on the limitation of P in the culture. Hence, during the steady state of condition 1, where N and P were not limited in the culture, the mixed culture was composed by ~50% cyanobacteria and ~50% of other Phyla such as Chlorophyta (green algae) and Bacillariophyta (Diatoms). Due to their morphologic characteristics, the cyanobacteria present in the culture were identified to genus level as *Aphanocapsa* sp., *Chroococcus* sp., *Microcystis* sp., *Pseudoanabaena* sp. and *Oscillatoria* sp.. Likewise, it was identified species belonging to Chlorophyta were identified, such as *Chlorella* sp., *Ulothrix* sp. and

Scenedesmus sp.. Other species of diatoms and animals like Rotifera were frequently observed in the culture during this operation mode (Fig. A2).

When changing to condition 2, the biomass turned to an almost complete cyanobacteria dominated culture (abundance ~90%), mainly by *Chroococcus* sp., with some *Pseudoanabaena* sp., and *Aphanocapsa* sp. (Fig. A3). Green algae *Chlorella* sp. and diatoms were occasionally present, while rotifers were rarely observed in the culture. Hence, it is assumed that non-cyanobacteria microorganisms cannot grow so effectively in low P volumetric loads (1 mg L^{-1} d^{-1}) and P limitation. It should be noticed that under this condition the highest carbohydrate conversion efficiency was achieved, implicating that *Chroococcus* sp. are able to achieve high amounts of carbohydrates when is submitted to low carbon loads during the aerobic illuminated conditions.

In condition 3, a microbial consortium similar to condition 2 was observed, however, *Aphanocapsa* sp. was rarely observed, which suggests that appearance and dominance of this cyanobacteria depends not only on the phosphorus limitation, but also on the availability of nitrogen in the culture. This fact was also observed in a previous study by Arias et al., [16], where this organism dominated over green algae and other cyanobacteria when phosphorus was limiting and nitrogen was available. On the contrary, in the present work *Chroococcus* sp. was dominated the culture (abundance ~80%) while *Pseudoanabaena* sp. (abundance ~10%) was frequently observed. This fact implies that *Chroococcus* sp. can tolerate both nutrients limitation, however, the lack of nitrogen restricted carbohydrate accumulation. Species belonging to other Phyla were rarely observed under this condition (Fig. A4).

493 The activity of other microorganisms, such as nitrifying and denitrifying bacteria, as well
494 as other heterotrophic microorganisms, was discarded due to the low values registered in
495 $N\text{-}NO_2$ and $N\text{-}NO_3$ as well as the lack of external sources of organic carbon.

496 In summary, general results obtained in the continuous operation of the SBR indicated
497 that the application of different operating strategies can be applied in order to select
498 different cultures. Hence, the application of low carbon loads during the light phase
499 coupled with high load of nitrogen and phosphorus (50/12.5/4.2 mg $C\text{-}Na_2CO_3 \cdot N\text{-}$
500 $NH_4^+ \cdot P\text{-}PO_3^{4-}$ d^{-1}), promoted low biomass concentration (0.6 mg VSS L^{-1}), low N and P
501 uptake, non-efficient carbohydrate production, and no PHB production. Moreover, this
502 condition cannot be used for a cyanobacteria dominated culture selection, since the
503 growth of other species, like green algae, diatoms and protozoa, is also promoted. When
504 a culture is operated under both low carbon and phosphorus loads with high nitrogen
505 (50/12.5/1 mg $C\text{-}Na_2CO_3 \cdot N\text{-}NH_4^+ \cdot P\text{-}PO_3^{4-}$ d^{-1}), a culture dominated by cyanobacteria was
506 selected. Furthermore, although the low load of P was completely consumed, nitrogen
507 uptake was not improved as consequence of carbon limitation, causing the utilization of
508 the carbon available for carbohydrate production instead of biomass production, while no
509 PHB was accumulated. On the other hand, when the culture had high loads of carbon and
510 nitrogen and low loads of phosphorus (100/12.5/1 mg $C\text{-}Na_2CO_3 \cdot N\text{-}NH_4^+ \cdot P\text{-}PO_3^{4-}$ d^{-1}), a
511 selected cyanobacteria dominated culture could take up more nitrogen until the point of
512 being depleted few hours after its addition. Therefore, the carbohydrates accumulation
513 performed during the light phase was not efficient, due to the utilization of nitrogen and
514 carbon for biomass growth (0.99 g X L^{-1}, ~40% more than in previous conditions).
515 Nevertheless, under this operating condition the total amount of carbohydrates
516 accumulated during the light phase increased and could, therefore, be slightly converted
517 into PHB during the dark phase due to the anaerobic conditions.

518 **3.2 Polymers accumulation in batch test experiments**

519 A batch test was performed after reaching the steady state of each operational condition
520 tested in the SBR in order to assess the polymer (PHB and carbohydrates) storage
521 capacity. These experiments started with biomass concentrations of 0.46, 0.61 and 1.51
522 $g \cdot VSS \cdot L^{-1}$, for conditions 1, 2 and 3, respectively. Since no additional nutrient source was
523 supplied to the biomass, the remaining nitrogen ($N\text{-}NH_4^+$ of 1.8 and 1.1 mgL^{-1} for
524 condition 1 and 2, respectively) was consumed in the first hours of the tests, while no
525 remaining N was observed in the batch test corresponding to condition 3. In the case of
526 $P\text{-}PO_4^{3-}$, this nutrient was only observed in the first hours of condition 1, at 1.15 mg $P\text{-}PO_4^{3-}$ L^{-1}
527 and not observed in any of the other accumulation tests. Furthermore, other
528 parameters, such as dissolved oxygen, did not reach the levels achieved in the continuous
529 operation during the light phase due to a greater oxygen transfer, since it was an open
530 system (9.6 mg O_2 L^{-1}, 28ºC).

531 The accumulation of carbohydrates was achieved during the light aerobic phase in all the
532 conditions. Hence, 6.62, 11.41 and 15.57 Cmmols Carbs L^{-1}, corresponding to
533 percentages of 29, 48 and 43%, were obtained in the batch test of conditions 1, 2 and 3,
534 respectively, after 24h of incubation (Fig. 4). Although a slight increase on carbohydrate
535 content was observed in the first 4 hours of the dark anaerobic phase in condition 2 (from
536 48 to 53%), the carbohydrates content declined in all the conditions during the dark
537 anaerobic phase. On the other hand, PHB started increasing in the light phase, as long as
538 the $N\text{-}NH_4^+$ was depleted in the medium. Contrary to carbohydrate patterns, PHB
539 accumulation also continued in the dark anaerobic phase for batch tests of conditions 1
540 and 2 likely occurring by means of carbohydrate conversion to PHB. In this case, PHB
541 reached values of 1.63 and 1.08 Cmmol PHB L^{-1}, corresponding to percentages of 3.9
542 and 3.5%, respectively, after 48h of incubation. This pattern was not observed in

condition 3, in which 1.89 Cmmol PHB L^{-1}, corresponding to 3.8 %, were obtained after 24h of light aerobic incubation. In this case, the culture already started with 1% of PHB from the SBR operation. After changing the condition to dark anaerobic phase, PHB decreased from 3.8% to less than 1% in the first 4 hours. It should be noticed that during the batch tests for conditions 1 and 2, IC was available during the dark phase, while it was almost depleted in condition 3. Hence, the decrease in PHB after finishing the light phase can be attributed to the lack of IC in the medium. This suggests that the presence of IC must be rigorously controlled during PHB production trials, otherwise this polymer may be quickly consumed by organisms in famine.

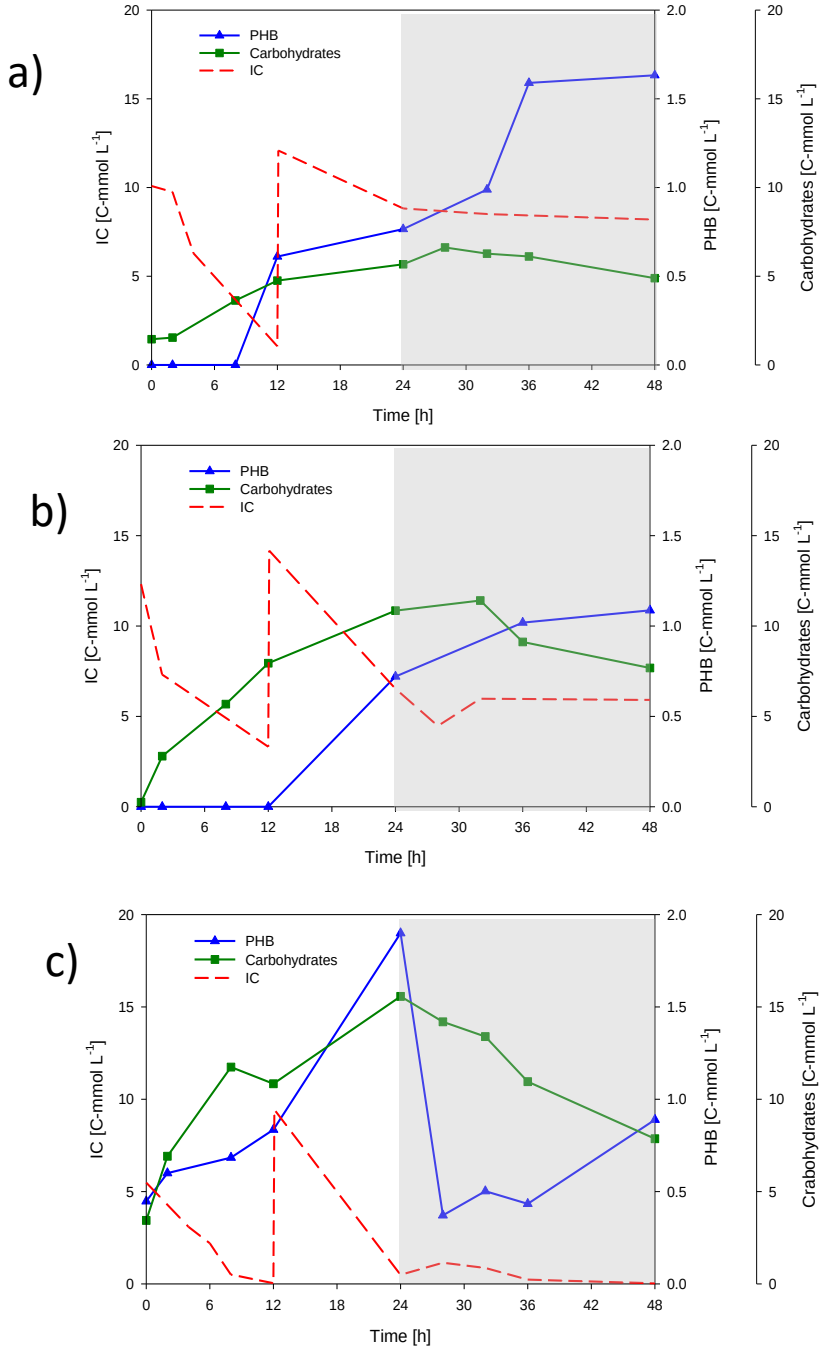

Fig. 4. Inorganic carbon (IC) consumption profile, poly (3-hydroxyalkanotes) (PHB) and carbohydrates transformation in batch tests performed with biomass collected from the sequencing batch reactor (SBR) during steady-state operation under a) carbon limitation (Condition 1) b) carbon and phosphorus limitation (condition 2), and c) phosphorus limitation (condition 3). zone represents the light phase and grey zone represents the dark anaerobic phase.

Reviewing the specific kinetic rates in all the batch tests (Table 4), the highest consumption of IC was obtained with sludge selected under condition 3, with 0.0054 Cmol IC/Cmol X d^{-1}. However, this value and the rates observed in conditions 1 and 2

were lower than the ones obtained in the SBR (0.016-0.039 Cmol IC/Cmol X d^{-1}). Likewise, the maximum specific carbohydrate production rate was reached under condition 2, notwithstanding, it was achieved two times lower values than that obtained in the SBR (0.049 Cmol IC/Cmol X d^{-1}). The explanation for such results may be the change of conditions in the batch test, for example, the light type. Since the type of light provided in the SBR corresponded to a wavelength spectrum of 380–780 nm, the photosynthetically active radiation is promoted. On the other hand, the light used in the batch tests was halogen light. Although the same volumetric light intensity was provided as in the SBR (~2.2 W/L), its spectrum spanned from 380 to ~1000. Thus decreasing the energy available in the PAR region, and likely leading to a decrease in the metabolic rates. Nevertheless, despite the fact that these rates decreased, the batch tests enabled the understanding of the most favorable conditions for polymer production. This fact can be seen in the percentages of polymer content and polymer yields reached (Table 4). Thus, although carbohydrate yields reached lower values in biomass taken from conditions 1 and 2, the biomass from condition 3 increased more than twice the values obtained in the SBR (0.5 Cmol Carbs/Cmol IC). Likewise, PHB yields considerably increased as well. Although no PHB was detected for conditions 1 and 2 in the operation of the SBR, the batch test allowed those cultures to accumulate PHB in the light and dark anaerobic phase. This results in the highest yields during the dark anaerobic phase, when PHB accumulation occurred by means of carbohydrate conversion. Although the yield $Y_{PHB/carbs}$ reached in the batch test of condition 1 was the highest of all conditions for this study (0.37 Cmol PHB/Cmol carbs) (Table 4), this value is lower than the yield achieved by other anoxygenic photosynthetic bacteria 0.94 Cmol PHB/Cmol carbs [25]. It is noteworthy that the highest PHB yield during the dark phase was achieved in the biomass from condition 1, which was composed by 50% of other microorganisms (i.e., green

algae, diatoms, protozoa) unable to produce PHB and 50% of cyanobacteria able to accumulate PHB. This fact indicates that the cyanobacteria community from that culture with a diverse species, such as, *Aphanocapsa* sp. and *Microcystis* sp., was more efficient accumulating PHB than the other cyanobacteria dominated consortiums, mainly composed by *Chroococcus* sp..

Table 4. Kinetic and stoichiometric parameters of the batch tests performed during a steady state of the SBR for each operational condition.

		$-q_s$ [Cmol IC/Cmol X d^{-1}]	$q_{carbohydrates}$ [Cmol Carbs /Cmol X d^{-1}]	q_{PHB} [Cmol PHB /Cmol X d^{-1}]	Y_{carbs} [Cmol Carbs/Cmol IC]	Y_{PHB} [Cmol PHB/Cmol IC]	PHB [%]	Carbs [%]
Condition 1	Light aerobic	0.023[a]	0.0079	0.0019	0.34	0.062	2.6	27.3
	Dark anaerobic	0.0011	-0.002	0.0016	-	0.37 [b]	3.9	16.0
Condition 2	Light aerobic	0.034[a]	0.023	0.0016	0.64	0.043	2.04	47.3
	Dark anaerobic	0.0034	-0.0081	0.001	-	0.098 [b]	3.5	29.9
Condition 3	Light aerobic	0.0054[a]	0.012	0.0015	0.87	0.071	3.8	43.2
	Dark anaerobic	0.0002	-0.0083	0.0005	-	0.081[b]	1.9	23.7

[a] value calculated considering the two pulses of carbon as one sole dosage of carbon.
[b] Y_{PHB} [Cmol PHB/Cmol carbs].

The process of transient carbon availability has been widely employed in diverse bacterial cultures and has demonstrated to improve polymer production efficiencies mainly in terms of PHB [23]. However, the use of this process to improve polymer production in cyanobacteria has not been previously performed. Given the experimental design of the batch tests, the maximum polymer accumulation capacity of the culture is uncertain. In the case of carbohydrate content, it is clear that the dark condition stopped the accumulation process because carbohydrate accumulation depends on the CO_2 uptake, which in turn depends on the light availability. Once in the dark phase, and due to the lack of oxygen (electron acceptor), cyanobacteria were forced to perform anaerobic dark energy generation using carbohydrates as endogenous compounds [40]. On the other hand, PHB accumulated under both light and dark conditions during the aerobic light

609 phase as long as IC was available (and N was limited), and during the anaerobic dark
610 phase from the carbohydrates stored in the previous illuminated phase.

611 Results obtained in the batch tests indicate, in general, that the best condition to
612 accumulate PHB and carbohydrates is to use a culture previously adapted to carbon
613 availability during the light phase along with high N and P limitation (Condition 3). Under
614 these conditions, microorganisms obtained the highest Y_{PHB} and Ycarbs. Furthermore,
615 the highest PHB accumulation occurred in a shorter period in comparison to other
616 conditions. From all the batch tests it is clear that high carbohydrate production depends
617 on phosphorus limitation coupled with IC availability and the presence of light, while
618 PHB can be accumulated during N limitation through IC uptake, during the presence of
619 light, and by carbohydrates conversion, during the dark anaerobic phase. In terms of PHB,
620 IC availability during the transition from light to dark conditions, when anaerobic
621 condition is not well established, is necessary to avoid PHB consumption as a source of
622 energy, as it was observed in condition 3. Having said that, values here obtained suggest
623 that PHB and carbohydrates can be produced efficiently in the light phase but also PHB
624 accumulation can continue by means of carbohydrate conversion during the dark. The
625 possibility of employing a light/dark process implies a reduction on the cost related to
626 artificial illumination in a full scale process. In such case, future studies should be
627 conducted to evaluate PHB and carbohydrate accumulation in batch tests during 12h/12h
628 light/dark periods for several days, to simulate real daylight illumination.

629 Considering that the main goal of his study was to produce both polymers; this research
630 is contributing to the advance in the limited knowledge of feast and famine application to
631 cyanobacteria producing PHB and carbohydrates. Among the possible applications for
632 both polymers produced, carbohydrates can be used as substrate to obtain biodiesel,

633 bioethanol and biomethane, while PHB can be directed for bioplastics generation. The
634 application of either of the two polymers will depend on the economic feasibility of each
635 process and the commercial interest of the final product.

636 *3.2.1 Comparison of a mixed cyanobacterial culture enhancement in a sequencing batch*
637 *reactor (SBR) with other studies on polymer accumulation in batch tests*

638 For comparison purposes, the percentages obtained in this work are here compared with
639 batch experiments performed with cyanobacteria cultures submitted to aerobic
640 illuminated autotrophic conditions (Table 5). In general, carbohydrate content obtained
641 in the batch test of condition 1 (29%) was similar to those obtained by most of the
642 previous studies carried out in pure cultures [19,20,45]. However, most of the other
643 studies are surpassed by the values obtained in the batch tests of conditions 2 and 3. Only
644 the study of Aikawa et al., [46] obtained a percentage of 47% in the first 24 h using a
645 specific strain (*Arthrospira platensis*) that already contained 17% of carbohydrates.

646 Concerning the PHB content, results obtained with the wastewater-borne cyanobacteria
647 utilized in this study, for all the conditions, achieved a higher PHB content in shorter time
648 in comparison with pure cultures and even with the same type of culture [47]. Generally,
649 most of the studies showed a very low process of PHB accumulation, reaching
650 percentages lower than 1% in the first 24h. In the particular case of Monshupanee et al.,
651 [22], PHB accumulation started after 120h of incubation achieving a maximum of 13%
652 after 288h. The results of this study are only similar to those of Nishioka et al., [48],
653 whose specific strain (*Synechocystis sp. MA19*) was able to start PHB accumulation after
654 10 h and reached 2% in 24 h. Remarkably, these authors reached 55% PHB content after
655 an incubation of 120 h.

Table 5. Summary of the carbohydrate and PHB contents obtained in accumulation tests performed in this study compared with other aerobic batch studies performed with 24 h illumination.

Cyanobacteria cultivated	Carbohydrates percentage obtained in 24h (% VSS)	PHB percentage obtained in 24h (% VSS)	Reference
Cyanobacteria dominated mixed culture	29	2.6	This study[a]
Cyanobacteria dominated mixed culture	48	2.04	This study[b]
Cyanobacteria dominated mixed culture	43	3.8	This study[c]
Arthrospira platensis	47[d]	-	[46]
Cyanobacteria dominated mixed culture	16-22	0.7-0.9	[47]
Spirulina maxima	34[e]-35[f]	0.4[f]-0.9[f]	[19]
Anabaena cylindrica	-	0.1[d]	[49]
Spitulina platensis	30 [f]	-	[20]
Synechocystis sp. PCC 6803	0.5[d]	0[d]	[22]
Synechocystis sp. MA19	-	2[d]	[48]
Arthrospira platensis	32.5[f]	-	[45]

[a] Results obtained in condition 1
[b] Results obtained in condition 2
[c] Results obtained in condition 3
[d] Values estimated from the figures in the original reference
[e] Value obtained in 9 h of incubation
[f] Values assumed as the half of the maximum content.

From a general view, this study highlighted that the improvement of PHB and carbohydrate accumulation in wastewater borne cyanobacteria can be achieved. The continuous process of cultivation and selection of specific microorganisms, through transient carbon regimes, enhanced the carbon uptake efficiency and the accumulation capacity of polymers. Thus, when the culture is submitted to a posterior accumulation process, a higher polymer content can be obtained. According to the encouraging results found in this study, further research should be directed to batch/fedbatch experiments testing 12h light/dark cultivation under controlled conditions, and then a posterior

enrichment of the microorganisms with real wastewater streams can be performed. In this last case, a double benefit could be achieved by producing valuable products while wastewater treatment is performed. Moreover, the production of polymers from wastewater-borne cyanobacterial cultures could be a cost-effective alternative to controlled pure cultures.

4. Conclusions

In this study, carbohydrate (biofuel substrates) and PHB (bioplastics) accumulation in wastewater-borne cyanobacteria was enhanced through transient carbon regimes in a sequencing batch reactor. During the continuous operation of the reactor, inorganic carbon was mostly used for biomass and carbohydrate production, showing very low PHB accumulation levels. Notwithstanding, in subsequent batch tests, PHB was accumulated after a complete depletion of nitrogen, reaching almost 4% of PHB. Concerning carbohydrates, it was found that phosphorus limitation (with and without carbon limitation) led to a culture mostly dominated by cyanobacteria, and to higher levels of carbohydrates content (43%-48%) than the culture with carbon limitation and high loads of nitrogen and phosphorus (29%). Such contents were obtained in only 24h of incubation under aerobic illuminated conditions. Hence, these encouraging results indicate that carbon uptake and the consequent polymers production can be enhanced through carbon and nutrient feeding strategies.

Authors' contribution

D.M. Arias performed the experiments, analysis and interpretation of the data, and drafted the manuscript. J.C. Fradinho supervised the experiments, contributed to the interpretation of the data and critically reviewed the manuscript. E. Uggetti, J. García, A.

Oehmen and M. A. M. Reis contributed to the interpretation of the data and critically reviewed the manuscript. All the authors read, edited and approved the manuscript.

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Conflict of Interest Statement

-The authors declare no conflicts of interest including any financial or other interests that could be perceived to influence the outcomes of the research.

-No conflicts, informed consent, human or animal rights applicable.

-All authors have agreed to authorship and submission of the manuscript for peer review.

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