Please cite this article as: Arias, D. M., Uggetti, E., García-Galán, M. J., García, J. 2018. Production of polyhydroxybutyrates and carbohydrates in a mixed cyanobacterial culture: effect of nutrients limitation and photoperiods. *New BIOTECHNOLOGY, 42,* 1-11

Production of polyhydroxybutyrates and carbohydrates in a mixed cyanobacterial culture: effect of nutrients limitation and photoperiods

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Abstract

In the present study, different photoperiods and nutritional conditions were applied to a mixed wastewater-borne cyanobacterial culture in order to enhance the intracellular accumulation of polyhydroxybutyrates (PHBs) and carbohydrates. Two different experimental set-ups were used. In the first, the culture was permanently exposed to illumination, while in the second, it was submitted to light/dark alternation (12h cycles). In both cases, two different nutritional regimes were also evaluated, N-limitation and P-limitation. Results showed that the highest PHB concentration (104 mg L^{-1}) was achieved under P limited conditions and permanent illumination, whereas the highest carbohydrate concentration (838 mg L^{-1}) was obtained under N limited condition and light/dark alternation. With regard to bioplastics and biofuel generation, this study demonstrates that the accumulation of PHBs (bioplastics) and carbohydrates (potential biofuel substrate) is favored in wastewater-borne cyanobacteria under conditions where nutrients are limited.

Keywords: mixed culture, green algae, biorefinery, bioproducts, bioenergy.

Introduction

In recent decades, alternative energy sources such as biofuels, biogas and value-added products such as bioplastics have received considerable attention for their potential to replace petroleum-based products and all their known drawbacks. Thus, the development of new, sustainable and cost-effective technologies to obtain carbon neutral bio-products has now become a priority [1]. In this context, special attention has been paid to cyanobacteria, due to their capacity to synthesize a large variety of bioactive compounds and other valuable metabolites. Similar to eukaryotic microalgae that accumulate starch, they can synthesize and store polysaccharides such as glycogen, but more interestingly they also have the capacity to accumulate polyhydroxybutyrates (PHBs) [2]. PHBs are polyesters synthesized as intracellular carbon and energy reserves. This family of polymers is characterized by plastic-like chemical and physical properties which, in combination with biodegradability and biocompatibility, make them promising alternatives to plastics derived from the petrochemical industry [3]. Currently, PHBs can be obtained by a number of different chemical and biotechnological means, with fermentative bacterial processes being the most frequently used to produce and commercialize them [4]. However, these fermentative processes require addition of a large amount of exogenous organic carbon and a continuous oxygen supply, which nowadays makes production of bacterial PHBs much more expensive than that of traditional plastics [5].

Glycogen, on the other hand, is a water-soluble α-polyglycan which constitutes the primary and most suitable substrate for biofuel generation, mainly via anaerobic fermentation, anaerobic digestion and bio-hydrogen technologies [6,7]. The use of cyanobacteria to produce glycogen is advantageous compared with other higher plants or green algae producing carbohydrates [8], mainly due to their lack of a hard cellulose cell wall, which typically requires additional pretreatment and further expensive conversion

processes to extract the product [9,10].

Most studies related to the production of PHBs and glycogen from cyanobacteria have been based on pure or genetically modified cultures [6,11–14], using sterile medium substrates in expensive and highly controlled processes, which keep the cost of the products too high to compete with their petroleum-based counterparts. In this respect, a more sustainable alternative approach for the production of metabolites could be the use of mixed wastewater-borne cultures dominated by cyanobacteria. The possibility of maintaining a cyanobacteria dominated culture in a pilot photobioreactor fed with wastewater was recently demonstrated [15,16]. However, the production of metabolites in this type of culture is still limited and accumulation strategies should be further investigated.

Recent studies have demonstrated that intracellular concentrations of either PHB or glycogen in cyanobacteria could be enhanced by modifying environmental and cultivation factors such as temperature, pH, inorganic carbon availability, nutrient concentration (N and P) and light availability (photoperiod and intensity) [17]. Among these conditions, nutrient limitation is the approach most frequently used [7,11,12]. Indeed, it has been demonstrated that the lack of N and P in the feeding media leads to an increase of both PHB (up to 20% in terms of dry cell weight (dcw)) and glycogen content (up to 60% (dcw)) [7,18–21]. Other important factors to consider in metabolite production are the photoperiods and the light intensity, which affect crucial physiological processes such as photosynthesis, respiration, cell division and the intracellular carbon components [22,23].

Taking the above into consideration, the aim of the present work was to apply different photoperiods and nutrient limitation conditions to a mixed cyanobacterial culture in order

to improve PHB and carbohydrate accumulation. The wastewater consortium was inoculated into a synthetic growth medium in order to evaluate PHB production under N and P limiting media separately. To the authors' knowledge, this is the first report of a cyanobacteria-dominated mixed culture subjected to different conditions, in this case considering different photoperiods paired with nutrient limitation, to enhance production of target metabolites.

Materials and methods

Reagents and chemicals

K_2HPO_4, $NaNO_3$, $NaHCO_3$, $CaCl_2 \cdot 2H_2O$, and Na_2EDTA were obtained from Panreac (Barcelona, Spain), $MgSO_4 \cdot 7H_2O$, $C_6H_8FeNO_7$, $C_6H_8O_7$, HCl, NaOH, chloroform ($CHCl_3$) and D-glucose were purchased from Scharlau (Barcelona, Spain). CH_3OH, H_2SO_4, $C_{17}H_{36}$ (heptadecane) and PHB-PHV co-polymer standard were purchased from Sigma-Aldrich (St. Louis, US). Glass microfiber filters (1 μm) were provided by Whatman (Maidstone, UK). **Give common names for C6H8O7 (citric acid?) and C6H8FeO7 (Ammonium ferric citrate?)**

Experimental set-up

Cyanobacteria dominated biomass

Experiments were performed at the laboratory of the GEMMA research group (Universitat Politècnica de Catalunya BarcelonaTech, Spain). Previous to the experimental set up, a microbial consortium mostly formed by cyanobacteria (abundance 60-70%) cf. *Aphanocapsa* sp. and cf. *Chroococcidiopsis* sp. was selected and cultivated in a pilot-scale closed photobioreactor (PBR). The PBR (30L) was used as a tertiary wastewater treatment system fed with secondary urban wastewater and liquid digestate, with a hydraulic retention time of 10 d. Detailed characteristics of this system can be

found elsewhere [15]. Cyanobacteria-dominated biomass was collected from a harvesting tank connected to the PBR and thickened by gravity in Imhoff cones for 30 min before its use in this study.

Experimental photobioreactor set-up

Four batch tests were performed during two consecutive weeks (15 days) in order to improve intracellular PHB and glycogen production. They were carried out in four closed cylindrical PBRs of polymethacrylate, with an inner diameter of 11cm, a total volume of 3 L and a working volume of 1 L (Fig. 1).

Experiments were carried out in two sets of two reactors each. In the first set, the effect of N and P limitation was tested under permanent illumination; in the second set, the same nutrient conditions were tested under light/dark alternation (12h dark/12h light). Right before the start of the experiments, 60 mL of settled biomass from the pilot-scale PBR was suspended in 1 L of growth medium in each of the four reactors. Microscopic images of the initial biomass are shown in Figure 2 and characteristics of the inoculum after suspension in the growth media are given in Table 1.

In order to achieve N and P limitation, two different growth media were used:

- The two reactors with N limitation contained N-free BG-11 growth medium consisting of: 0.04 g L^{-1} K_2HPO_4, 0.036 g L^{-1} $CaCl_2 \cdot 2H_2O$, 0.001 g L^{-1} Na_2EDTA, 0.075 g $MgSO_4 \cdot 7H_2O$, 0.01 g $C_6H_8FeNO_7$, 0.001 g $C_6H_8O_7$, and 1ml L^{-1} of trace elements.
- The two reactors with P limitation contained P-free BG-11 growth medium consisting of: 1.5 g $NaNO_3$, 0.036 g L^{-1} $CaCl_2 \cdot 2H_2O$, 0.001 g L^{-1} Na_2EDTA, 0.075 g $MgSO_4 \cdot 7H_2O$, 0.01 g $C_6H_8FeNO_7$, 0.001 g $C_6H_8O_7$ and 1.0 ml L^{-1} of trace elements.

Reactors were continuously agitated with a magnetic stirrer (Selecta, Spain) at 250 rpm. Temperature was continuously measured by a probe inserted in the PBR (ABRA, Canada) and kept constant at 27 (±2) °C by means of a water jacket around the reactor. Continuous monitoring of pH was carried out with a pH sensor (HI1001, HANNA, USA) and kept at 8.7 with a pH controller (HI 8711, HANNA, USA) by the automated addition of HCl 0.1 N and NaOH 0.1 N. Light intensity was set at 220 μmol m^{-2} s^{-1} and provided by means of two external halogen lamps (60 W) placed in opposite sides of each PBR.

In both experimental set-ups, $NaHCO_3$ was added manually to the cultures as the only soluble inorganic carbon (IC) source, in order to provide enough carbon to be transformed into PHB/carbohydrate. Availability of $NaHCO_3$ was monitored by daily analyses of IC.

Analytical methods

The cultures in the reactors were analyzed for total inorganic carbon (TIC), total organic carbon (TOC), orthophosphate (dissolved reactive phosphorus) ($P\text{-}PO_4^{3-}$), nitrite ($N\text{-}NO_2^-$) and nitrate ($N\text{-}NO_3^-$) on alternate days, 3 days per week. IC and soluble organic carbon (OC) were measured daily (5 days per week), after filtering the samples through 1 μm pore glass microfiber filters. Total nitrogen (TN) and total phosphorus (TP) were measured 2 days per week. Dissolved organic nitrogen (DON) and dissolved organic phosphorus (DOP) were determined by the analysis of filtered samples following the same procedure used for TN and TP analysis respectively, and subtracting the value of $N\text{-}NO_2^-$ and $N\text{-}NO_3^-$, in the case of DON, or the value of $P\text{-}PO_4^{3-}$ in the case of DOP. Organic Nitrogen (ON) was calculated as the difference between TN and $N\text{-}NO_2^-$ and $N\text{-}NO_3^-$, whereas organic phosphorus (OP) was determined as the difference between TP and $P\text{-}PO_4^{3-}$. $P\text{-}PO_4^{3-}$, $N\text{-}NO^{2-}$, $N\text{-}NO^{3-}$ concentrations were analyzed using an ion chromatograph DIONEX ICS1000 (Thermo-scientific, USA), and TOC, TIC, OC, IC and

TN using a C/N analyzer (21005, Analytikjena, Germany). TP was analyzed following the method described in 4500 B and 4500 P, respectively, of Standard Methods (APHA-AWWA-WPCF, 2001). **If this is a reference it should go into the list.**

Total suspended solids (TSS) and volatile suspended solids (VSS) were measured in the culture 3 days per week following the gravimetric method 2540 C and 2540 D in Standard Methods (APHA-AWWA-WPCF, 2001). Chlorophyll *a* was measured two days per week using the procedure 10200 H described in the Standard Methods (APHA-AWWA-WPCF, 2001). Dissolved oxygen (DO) was measured daily with a dissolved oxygen-meter (Thermo-scientific, USA)directly in each PBR, inserting the sensor into the mixed liquor.

PHB and carbohydrate analysis

PHB and carbohydrate content were measured daily in the constant illumination experiments, and at the end of both the light phase and the dark phase during the first week of the 12h light/dark experiments. 50 ml of mixed liquor were collected and centrifugated (4200 rpm, 10 min), frozen at -80 ºC overnight in an ultra-freezer (Arctiko, Denmark) and finally freeze-dried for 24h in a lyophilizer (-110 ºC, 0.049 hPa) (Scanvac, Denmark).

PHB extraction protocol was adapted from the methodology described by Lanham et al. [24]. Briefly, approximately 2 mg of freezed-dried biomass were weighed in a glass tube with a Teflon liner screw cap, where 1 mL of MeOH acidified with H_2SO_4 (20% v/v) and 1 mL of $CHCl_3$ containing 0.5 mg mL^{-1} heptadecane were added as internal standards. The tubes were then incubated at 100 °C in a dry-heat thermo-block (Selecta, Spain) during 5 h. After this period, the tubes were cooled on ice for 30 min. Thereafter, 0.5 mL of deionized water was added and the tube was vortexed during 1 m to aid the two phase separation (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 water. The co-polymer of PHB-PHV (86:14% wt, CAS 80181-31-3) was used as a standard for HB and HV. A sixpoint calibration curve was prepared at different concentrations of PHB-PHV and processed in the same way as the real samples. PHB was determined by means of GC (7820A, Agilent Technologies, USA). **Define HB and HV**

Carbohydrate content was extracted following the method described by Lanham et al. [25]. Briefly, approximately 2 mg of freeze-dried biomass were weighted and placed in glass tubes with Teflon liner screw caps, where 2mL of a diluted solution of 0.9 N HCl was added. The tubes were then incubated in a heating-block at 100 °C during 2h. The samples were cooled in an ice bath, the supernatant extracted and then the carbohydrates content was determined following the phenol–sulfuric acid method described in [26], using *D*-glucose as a standard.

Microbial evolution

Since the biomass was initially composed of a mixed culture, composition changes within the reactors were examined microscopically once a week for qualitative evaluation of microalgae populations.

Heterotrophic bacteria monitoring was not considered due to the pure autotrophic condition to which the cultures were submitted. Daily values of organic carbon content were used as indicator of a mostly autotrophic activity in the reactor.

Microbial visualization was performed in an optical microscope (Motic, China) equipped with a camera (Fi2, Nikon, Japan) connected to a computer (software NIS-Element viewer®). Cyanobacteria and microalgae species were identified *in vivo* using conventional taxonomic books [27], as well as a database of cyanobacteria genus [28].

Kinetic and stoichiometric parameters

The active biomass concentration (X), given in [g L^{-1}], was calculated as follows:

$$\mathbf{X = (VSS - Carbohydrates - PHB)} \quad [1]$$

The yields of PHB per substrate consumed ($Y_{PHB/S}$) and carbohydrates per substrate consumed ($Y_{carbs/S}$), were calculated using the following equation:

$$\mathbf{YPHB/S\ or\ Y}\text{carbohydrates}\mathbf{/S} = \frac{\mathbf{PHB\ or\ Carbohydrates\ accumulated}}{\mathbf{IC\ consumed}} \quad \mathbf{[2]}$$

And expressed as mgPHB or Carbohydrates·mg IC.

The maximum specific substrate uptake rate (q_S) [mg IC·g X d^{-1}], maximum specific polymer production rate (q_P) [mg PHB or Carbs·g X d^{-1}], were determined dividing the slope of the linear function of experimental results during the experiments by the average of the resulting active biomass.

Results and Discussion

Biomass growth

All cultures remained in oxygenic conditions throughout the experiments, thus similar DO average values were found in the lluminated phase in both set ups: 7.1±1.2 mg L^{-1} and 7.6±1.9 mg L^{-1} for N-limited and P-limited conditions respectively during permanent illumination, and 7.3±0.5 mg L^{-1} and 7.8±0.6 mg L^{-1} for N-limited and P-limited conditions respectively during the light-dark alternation. Only a slight decrease was

observed at the end of the dark phase (6.2±0.5 mg L^{-1} and 6.6±0.5 mg $L^{-1}$for N-limited and P-limited conditions, respectively).

Under constant illumination, the initial concentration of biomass (0.35 g VSS L^{-1}) reached values of up to 0.97 g VSS L^{-1} on day 8 of operation in the N-limited culture, decreasing to 0.86 g VSS L^{-1} by the end of the experiment (Figure 3a). Results in the P-limited culture indicated a higher growth rate, achieving a concentration of 1.62 g VSS L^{-1} also on day 8 of operation, decreasing afterwards to 1.38 g VSS L^{-1}. Conversely, under alternate illumination, the initial biomass concentration of 0.33 g VSS L^{-1} reached values up to 0.99 g VSS L^{-1} on day 12, remaining stable until day 15 (0.76 g VSS L^{-1}) in the N-limited culture (Figure 3b). Meanwhile, the P-limited culture showed an increasing trend over the length of the experiment, achieving 1.35 g VSS L^{-1} on day 15.

Chlorophyll *a* content in N-limited cultures showed a similar pattern under both illumination conditions, having a clear decreasing trend (Figures 3c and 3d). Furthermore, these cultures developed a yellowish color during the experimental period, indicating the decay of pigments inside the reactors. In contrast, chlorophyll *a* content in the P-limited culture under permanent illumination increased from 1.00 mg L^{-1} (initial chlorophyll *a* content) to a maximum of 2.61 mg L^{-1} on day 10, following a very similar pattern to the biomass content. On the other hand, under alternate illumination the initial concentration increased from 0.95 mg L^{-1} until a maximum of 3.10 mg L^{-1} on day 12. It is important to highlight that under P limitation in both photoperiods, chlorophyll *a* content was higher under alternate illumination than under permanent illumination. This can be associated to the disruption of chorophyll *a* biosynthesis caused by continued illumination periods as previously documented [29,30].

Regarding biomass composition, under permanent illumination in the experiment with

N-limitation, the initial composition remained constan during the entire experimental period. In contrast, an increase in the amount of cf. *Aphanocapsa* sp. over the other species of green algae as well as other cyanobacteria genus was observed from day 8 of operation onwards in the P-limitation experiment, as can be seen in Figure 4a. and Supplementary Figure S1. Under alternate illumination, the evolution of the biomass composition in both N and P limitation experiments was very similar to that observed in the constant illumination set-ups. Hence, during N limitation, the biomass composition throughout the experimental time was constant, whereas in the culture submitted to P-limitation, an evident increase of cyanobacteria cf. *Aphanocapsa* sp. over the other species from day 8 onwards was observed (Figure 4b).

These results demonstrate that, in both experimental set-ups, the initial biomass concentrations under both illumination conditions increased 2.3-2.4-fold with N limitation, and 4-fold with P limitation, as a consequence of the light periods and unlimited carbon supply. Furthermore, despite the evident increase in biomass, the observed growth was higher in both P limited cultures than in the N limited conditions.

Nutrients concentration

The IC and OC concentrations in the cultures during the experiments are shown in Figure 5. It can be observed that maximum values of OC in all the cultures were low during the first days of operation (5-9 mg L^{-1}), and subsequently nearly undetectable. Thus, the only relevant carbon source was the IC added in the form of $NaHCO_3$. This fact highlights the impossibility of contamination by heterotrophic bacterial activity in the culture and their contribution to PHB production. Indeed, such microorganisms have high organic carbon requirements to synthesize PHBs and usually the enrichment of mixed bacterial cultures is performed in fermentation processes with organic loads >300 mg C L d^{-1} [31–33].

Concerning IC uptake dynamics, under permanent illumination, IC was consumed from the second day of operation in both N and P limitation experiments, until values were lower than 50 mg C L^{-1}. In fact, in the N-limited culture (Figure 5a), addition of $NaHCO_3$ was needed during the first six days of operation, after which IC started to be consumed at a lower rate until the final addition of carbon (day 11). In the P-limited experiment (Figure 5b), IC was consumed at a faster rate and the addition of $NaHCO_3$ was required until day 12 of operation.

In contrast, under alternate light/dark cycles, IC had to be added during the first 4 days of operation in both nutrients limitation conditions (peaks in Figure 5c). During the first week, the samples collected at the end of the dark phase revealed a minimal or even null carbon uptake during night. After this period, carbon was added on days 8 and 12 in the N-limited condition and three more additions on days 8, 10 and 12 in the P-limited condition. A rapid consumption was registered until day 9, decreasing and starting to accumulate in the culture after that day.

As shown in Table 2, the initial TN concentrations ranged between 22 and 27 mg L^{-1} in both N-limited experiments (under constant illumination and alternate, respectively) and between 339 and 344 mg L^{-1} in the P-limited cultures. These values remained stable until the end of the experiments. Similar results were observed with the initial concentrations of TP, 14.7 mg L^{-1} and 13.4 mg L^{-1} in the N-limited cultures and 12.9 mg L^{-1} and 10.6 mg L^{-1} in the P-limited cultures. However, variations in the organic forms present in the reactors were observed according to the addition of IN or IP. In both set-ups under P-limitation, with no additional IP in the medium, the initial concentration of OP, averaging 11.8 mg L^{-1}, remained the same until the end of the experiment. A similar trend was observed in the N-limitation experiments, in which the final values remained close to the initial values (22.24 mg L^{-1}). In contrast, when IN or IP were supplied to the cultures, an

increase in ON and OP was observed in the corresponding reactors. Taken these points into consideration, it can be assumed that the nutrients supplemented to the culture were consumed and transformed into biomass. It is important to point out that the DON ranged from 1.03 to 1.66 mg L^{-1} in all the experiments, whereas DOP showed values below 0.67 mg L^{-1} as noted for these types of systems [34]. The ON and OP content mostly corresponded to the active biomass.

Regarding the ON/TOC ratio, the intial value of 3.8±0.3 increased only in the cultures under N limitation conditions in both set-ups, reaching values of 8.56 and 6.8 under permanent illumination and light/dark alternation, respectively. These results probably led to the reduction of chlorophyll *a* content in these reactors observed from day 3 of operation until the last day, as shown in Figures 3c and 3d. Furthermore, these cultures developed a yellowish color throughout the experimental time, an evident sign of chlorosis or bleaching, a process characterized by the degradation of pigments such as chlorophyll *a* [35]. Previous studies associated this process only with N limitation [36], although this phenomenon was also observed in the study by Markou et al. [37] in *Arthrospira* sp. submitted to P limited conditions. In this study, the different colors observed were confirmed in the microscopic images, showing an evident discoloration with respect to the initial culture when submitted to N limitation in both photoperiods (Figures 4a and 4b).

PHB production

Under permanent illumination, PHB concentration in the N-limited culture increased slowly until day 9, when it reached a value of approximately 50 mg L^{-1}. It remained constant until the end of the experiment (Figure 6a); in contrast, PHB concentration in the P-limited culture reached a maximum of 104 mg L^{-1} on day 8, which

decreased to 90 mg L^{-1} on day 9 and to 69 mg L^{-1} on day 10. Concentrations oscillated between 60 and 80 mg L^{-1} from that day until the end of the experiment.

In the case of light/dark alternation experiments, the concentration of PHB in the culture submitted to N-limited conditions slowly increased until day 12, achieving a concentration of 61 mg L^{-1} which remained nearly constant until day 15 (Figure 6b). A similar trend was observed in the P-limited culture, reaching the same PHB concentration on day 12 that continued to increase until day 15 (76 mg L^{-1}).

Regarding intracellular content, under permanent illumination, the highest values observed were 5.4% dcw and 5.7% dcw with N limitation and P limitation conditions, reached on days 9 and 8, respectively. After that, the intracellular content of PHB gradually decreased to 5.2% and 4.8% respectively, on the last day of incubation. In contrast, the maximum content observed during the 12h light/dark periods were of 6.5% dcw under N limitation and 5.6% dcw under P limitation. Both values were obtained on days 12 and 15 for N-limited and P-limited, respectively.

Decay in the PHB percentages and concentrations might be influenced by the change of dominating species, as noted above. Thus, the possibility of having *cf. Aphanocapsa* sp. as dominant species in the culture can be associated to a lower PHB accumulation in comparison with other species submitted to P limitation under permanent illumination. However, PHB content and concentrations were not affected when the culture was submitted to the alternation of light and dark under P-limitation, although this culture presented the dominance of this species on the same day of operation (day 12).

Despite these percentages, the results indicate that the highest concentrations of PHBs were reached when the culture was submitted to P limitation in both photoperiods, especially under permanent illumination. Such high concentrations were a consequence

of the high biomass concentrations observed under this condition. In comparison to other studies, the highest concentrations of PHB were reached in the cultures submitted to P limitation, as previously observed in the studies by Meixner et al. [14] and Nishioka et al. [39], in which values of 123 mg L^{-1} and 1400 mg L^{-1} were reached, respectively. In a different study, a culture under N limitation only reached 67.2 mg L^{-1}, in spite of having 21% of PHB [39]. The lower concentrations observed in the N-limited experiments can be also attributed to the lower biomass concentration but additionally to the influence of the chlorosis observed in both photoperiods. This was also detected in the study by Jau et al. [40], who associated the delay of PHB accumulation in the cyanobacteria *Spirulina platensis* to factors such as chlorosis influencing the pigment synthesis. In the present study, although the culture submitted to light/dark alternation and N limitation also presented chlorosis, similar and even higher PHB contents were reached (6.5% dcw) with respect to the P-limited conditions tested (5.6%-5.7% dcw) (Figure 6b, Table 3), when considering the intracellular content (% dcw) and not the concentration. This fact suggest that dark periods could improve PHB accumulation as previously found for *Nostoc muscorum* [41] and *Synechocystis* sp. PCC 6803 [42]. In these studies, the increase of PHB during dark periods was associated with the conversion of glycogen to PHB.

Carbohydrate production

Regarding carbohydrates, under constant illumination the N-limited culture reached a maximum concentration of 641 mg L^{-1} on day 8, whereas for the P-limited culture reached a maximum of 552 mg L^{-1} on the same day. From that point onwards, concentrations decreased in both experiments, more markedly in the N-limitation set-up. (Figure 7a). Under alternate illumination, the N-limited culture accumulated a maximum concentration of 838 mg L^{-1} on day 12, which rapidly decreased to 430 mg L^{-1} by the end of the experiment, whereas the P-limited culture reached a maximum concentration of

only 432 mg L^{-1} on day 12 of operation, which also decreased until day 15 (Figure 7b).

Regarding intracellular contents, the highest carbohydrate accumulation was observed under N limitation, reaching a concentration of 63% dcw under permanent illumination, and 74% under 12h light/dark periods, in contrast to the maximum content of 46% and 35% dcw achieved with P limitation under permanent light and 12h light/dark respectively. As it is mentioned by Markou et al. [7], when cyanobacteria are submitted to N starvation, the flow of the photosynthetically fixed carbon is turned from the protein synthesis metabolic pathway to the lipid or carbohydrate synthesis pathways. In the present study, carbohydrates represented the major carbon storage form in the cultures compared to PHB. Indeed, the concentration as well as the percentage (dcw) of carbohydrates reached are in the order of 8 times higher than those obtained of PHB. This higher accumulation of carbohydrates was also observed in the studies of [43,44].

Metabolite production achievements

The accumulation of carbohydrates and PHB in cyanobacteria submitted to nutrient limitation is a response to this stress condition. Thus, both glycogen and PHB act as buffers to avoid useless metabolic cycles, especially during dark–light transitions, regulating the switch between photosynthetic and catabolic pathways in the cells [45]. In the present study, biomass as well as metabolite concentrations reached the highest values under light/dark alternation, with the sole exception of PHB concentration under P limitation, which was higher under constant illumination. The use of alternating cycles is more representative of natural conditions and implies an advantage for further escalation of the process to outdoors systems, avoiding additional energy costs for illumination.

As far as we are aware, this is the first study enhancing the accumulation of metabolites such as PHB and carbohydrates in mixed cyanobacteria-microalgae wastewater borne

cultures. For comparison purposes, literature results on PHB and carbohydrates accumulation in photoautotrophic conditions on cultures of cyanobacteria are summarized in Tables 3 and 4. As can be seen, PHB and carbohydrate accumulation is species-dependent and in cases where the content of both polymers was evaluated (i.e. this study and the studies by de Philippis et al. and Monshupanee et al. [43,44]), the accumulation of both seemed to follow different trends, as maximum concentrations were achieved after different incubation periods. In the case of PHB production, most of the studies reached values within a range of 0.2-8.5% dcw, which are near to those found in this study. In some cases, as in the study by Sharma et al. [41], a slightly higher accumulation percentage was obtained, but after a longer time of incubation (21 days). In the study by Monshupanee et al. [44], the strain *Synechocystis* sp. PCC6803 reached values above 13% PHB (dcw) after 12 days of experiments under both N and P starvation conditions, much higher than the maximum values of 5.4% and 5.7% obtained in this study under N and P limitation respectively, and during the same incubation period. Only Nishioka et al. and Miyake et al. [38,39] obtained the highest percentages with cyanobacteria *Synechococcus* sp. MA19 submitted to nutrients limited conditions with IC as the carbon source.

Regarding carbohydrates, it is important to highlight that they can be accumulated by both cyanobacteria and green algae. Thus, carbohydrates measured in this study included both glycogen accumulated by cyanobacteria and starch accumulated by green algae. Generally, maximum carbohydrate content was obtained under N-limitation in both photoperiods, with values of 63% dcw and 75% dcw under constant illumination and light/dark alternation respectively. These results are similar and even higher than the maximum values found in other studies carried out under the same nutritional conditions and similar period of incubation, with the only exception being the study of de Philippis

et al. [43], who obtained up to 70% dcw in 2.7 days. In contrast, the carbohydrate content reached in this work under P limitation (46% and 36% under constant illumination and light/dark alternation, respectively) are higher than those obtained in the afore-mentioned studies (23% dcw after 2.7 d [43], 28.9% dcw after 12 d[44]). Only the strain *Spirulina platensis* was able to accumulate more than 60% dcw of carbohydrates in a P-limited culture [13][46].

Observing the kinetic specific rates of all tests (Table 5), the highest specific consumption rate of IC per g of biomass was observed under permanent illumination in both limitation conditions, reaching rates of 182.52 and 183.61 mg IC·X d^{-1}, in N-limited and P-limited cultures, respectively. This implies that those cultures had higher IC consumption than under conditions of light alternation. On the other hand, the highest maximum specific PHB production was reached under permanent illumination with P limitation and under light/dark alternation with N limitation, in accordance with the highest percentages reached by these two conditions. Likewise, the maximum specific production rate of carbohydrates was reached in N limitation under both illumination conditions in accordance with the highest percentages achieved during that limitation.

In all the experiments, a low formation of PHB compared to the IC consumed was observed, with maximum yields below 0.1 in all the conditions. Notwithstanding, the highest yields were reached in the culture submitted to light/dark alternation. This pattern was also observed in the carbohydrate yields. This fact represents an important issue in terms of economic feasibility, since the use of alternating cycles is more representative of natural conditions and implies an advantage for further escalation of the process to outdoor systems, avoiding additional energy costs for illumination.

In general, results obtained in this study reveal that cyanobacteria-dominated cultures

grown in wastewater effluents can be used as PHB and carbohydrate producers. It should be mentioned that changes in biomass composition due to the lack of unsterile conditions cannot be avoided. In this particular case, the unicellular cyanobacteria *cf. Aphanocapsa* sp. revealed a stronger dominance over other cyanobacteria with P-limitation under both illumination conditions. However, it only presented a possible interference in biomass and PHB production when submitted to permanent light. The presence of these phenomena is inevitable with real unsterile processes. Hence, the results of this investigation provide a solid foundation for further studies in the field.

It is important to remark that the production of these valuable metabolites from wastewater native microorganisms could be a cost-effective alternative to pure cultures. Indeed, in this case the additional costs of biomass production and chemical inputs to maintain sterile conditions can be avoided if using waste streams as substrates. In such case, variables of the processes as the inclusion of organic carbon and their effect on heterotrophic bacterial activity should be considered.

All in all, the use of this technology represents a promising approach of biorefinery to produce either bioplastics or biofuels. The results highlight the need for further studies regarding the enhancement of the production of these by-products using these cultures.

Conclusion

This study demonstrated the enhanced accumulation of both bioplastics (PHB) and a potential biofuel substrate (carbohydrate), in a mixed cyanobacterial culture used for wastewater bioremediation. The effect of N and P limitation during two different photoperiods on metabolite production was evaluated for two weeks. Results showed that the highest PHB concentration (104 mg L^{-1}) was reached under P limitation and constant illumination, whereas the highest carbohydrate concentration (838 mg L^{-1}) was obtained

in N limitation under light/dark alternation. With regard to bioplastics and biofuel generation, this study highlights and demonstrates that nutrient limitation could be a good approach to enhance PHB and carbohydrate accumulation in wastewater-borne cyanobacteria.

Acknowledgments

The authors would like to thank the European Commission [INCOVER, GA 689242] for their financial support. Dulce Arias kindly achnowledge her PhD scholarship funded by the National Council for Science and Technology (CONACYT) [328365]. M.J. García and E. Uggetti would like to thank the Spanish Ministry of Industry and Economy for their research grants [IJCI-2014-22767 and IJCI-2014-21594, respectively]. Authors appreciate Estel Rueda and Laura Torres for their contribution during the experiment deployment. Authors thank Alice King for her help with the manuscript review.

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Table 1. Characterization of the inoculum (biomass) taken from the 30L PBR and added in the four experimental PBR containing growth medium (n=4). Values are given as mean values (standard deviation).

Parameter	Mean value (Standard deviation)
Temperature (ºC)	24
pH	8.2 (0.2)
TSS (g L^{-1})	0.43 (0.05)
VSS (g L^{-1})	0.34 (0.06)
Chlorophyll *a* (mg L^{-1})	1.00 (0.08)
TOC (mg L^{-1})	84.6 (7.5)
ON (mg L^{-1})	22.2 (1.8)
OP (mg L^{-1})	11.8 (1.6)
PHB (% (w/gTS))	3.4 (0.3)
Carbohydrates (% (w/gTS))	7.4 (0.4)

Table 2. N and P values of the culture at the end of the experiment (day 15).

	Permanent illumination		Light/dark alternation	
Parameter [mg L^{-1}]	N-limited	P-limited	N-limited	P-limited
TN	22.28	339.22	27.22	344.05
ON	22.28	154.22	27.22	238.05
IN	0	185	0	106
TP	14.73	12.9	13.36	10.62
OP	12.13	12.9	11.66	10.62
IP	2.65	0	1.7	0

Table 3. Summary of the maximum percentages and concentration values of PHB in the experiments performed in this study compared with other cyanobacteria culture studies.

Cyanobacteria cultivated	Nutrient limited	Photoperiod Light:dark (h)	Maximum concentration (mg L^{-1})	Maximum (% dcw)	Day of incubation (d)	Reference
Cyanobacteria dominated mixed culture	N	24:0	51.6	5.4	9	This study
Cyanobacteria dominated mixed culture	P	24:0	104.23	5.7	8	This study
Cyanobacteria dominated mixed culture	N	12:12	61.61	6.5	12	This study
Cyanobacteria dominated mixed culture	P	12:12	76.36	5.6	15	This study
Nostoc muscorum	N	14:10	-	6.4	21	[18]
Anabaena cylindrica	N	24:0	-	0.2	21	[47]
Synechococcus sp. MA19	N	0:24	67.2	21	6	[39]
Synechocystis sp. PCC6803	N	24:0	-	14.6	12	[44]
Synechocystis sp. PCC6803	P	24:0	-	13.5	12	[44]
Synechocystis salina	P	16:8	123.2	6	30	[14]
Synechococcus sp. MA19	P	24:0	1400	62	4	[38]
Spitulina maxima	N	24:0	-	0.7	4	[43]
Spitulina maxima	P	24:0	-	1.2	4	[43]
Nostoc muscorum	P	14:10	-	8.5	21	[41]
Spitulina platensis	P	14:10		3.5	60	[5]

Table 4. Summary of the maximum percentages and concentration values of carbohydrates in the experiments performed in this study compared with other cyanobacteria culture studies.

Cyanobacteria cultivated	Nutrient limited	Photoperiod	Maximum concentration (mg L^{-1})	Maximum (% dcw)	Days of incubation (d)	Reference
Cyanobacteria dominated mixed culture	N	24:0	641.30	62.71	8	This study
Cyanobacteria dominated mixed culture	P	24:0	662.38	46.05	12	This study
Cyanobacteria dominated mixed culture	N	12:12	838.05	74.76	12	This study
Cyanobacteria dominated mixed culture	P	12:12	432.13	35.98	12	This study
Arthrospira platensis	N	24:0	800	65	3.5	[48]
Spitulina platensis	P	24:0	-	65	-	[37]
Spitulina platensis	P	24:0	-	63	9	[46]
Synechocystis sp. PCC 6803	N	24:0	-	36.8	12	[44]
Synechocystis sp. PCC 6803	P	24:0	-	28.9	12	[44]
Spitulina maxima	N	24:0	-	70	2.7	[43]
Spitulina maxima	P	24:0	-	23	2.7	[43]
Arthrospira platensis	N	24:0	-	65	7	[49]

Table 5. Kinetic and stoichiometric parameters

	Permanent illumination		Light/dark alternation	
Parameter	N-limited	P-limited	N-limited	P-limited
qS [mg IC·g X d^{-1}]	182.52	183.61	124.33	80.43
qPHB[mg PHB·g X d^{-1}]	8.08	10.18	12.17	7.09
qcarbs [mg carbs·g X d^{-1}][a]	204.99	95.47	200.36	60.41
Yphb/S[mg PHB·mg IC d^{-1}][b]	0.04	0.05	0.10	0.08
Ycarbs/S[mg carbs·mg IC d^{-1}][a,b]	0.86	0.46	1.29	0.48

[a] Carbohydrates

[b] Yields calculated with the maximum PHB/carbohydrates concentration obtained and the corresponding IC consumed at that day.

List of figures

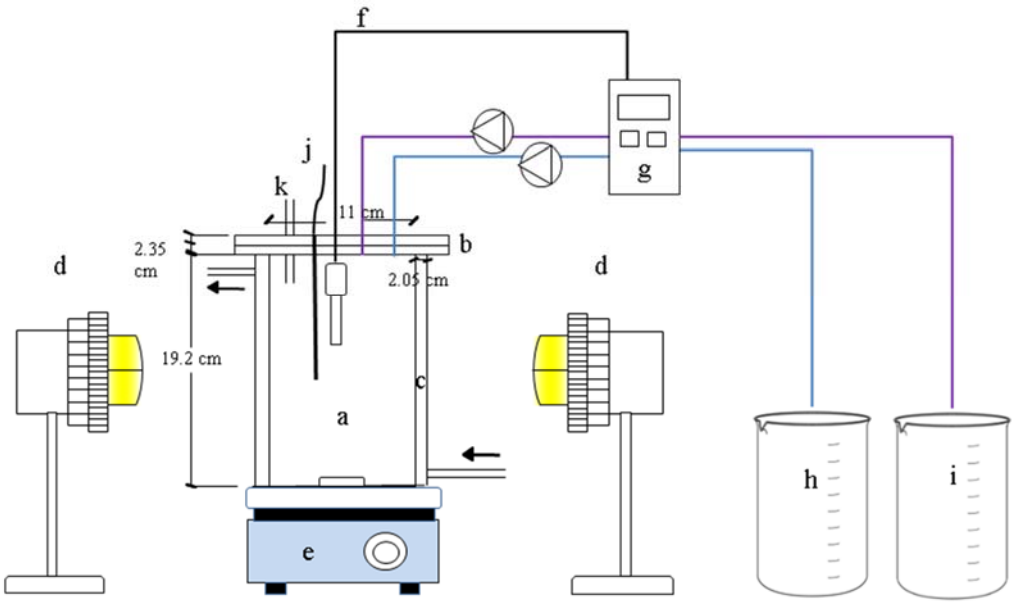


Fig. 1

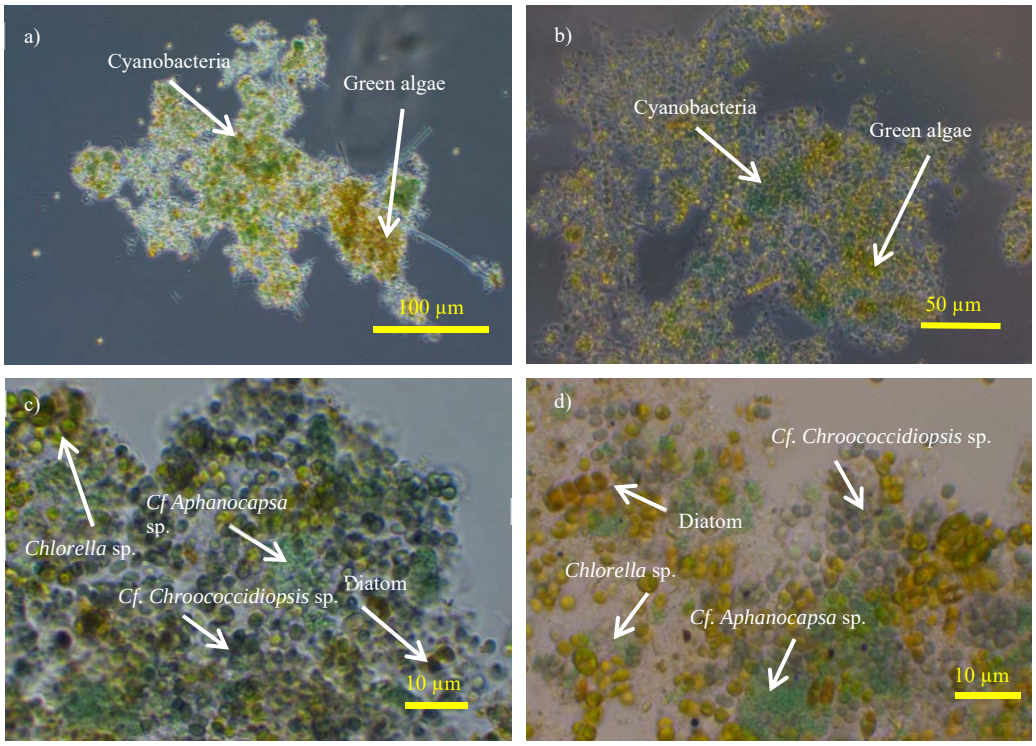

a)
Cyanobacteria
Green algae
100 µm
b)
Cyanobacteria
Green algae
50 µm
c)
Chlorella sp.
Cf Aphanocapsa sp.
Cf. Chroococcidiopsis sp.
Diatom
10 µm
d)
Cf. Chroococcidiopsis sp.
Diatom
Chlorella sp.
Cf. Aphanocapsa sp.
10 µm

Fig. 2

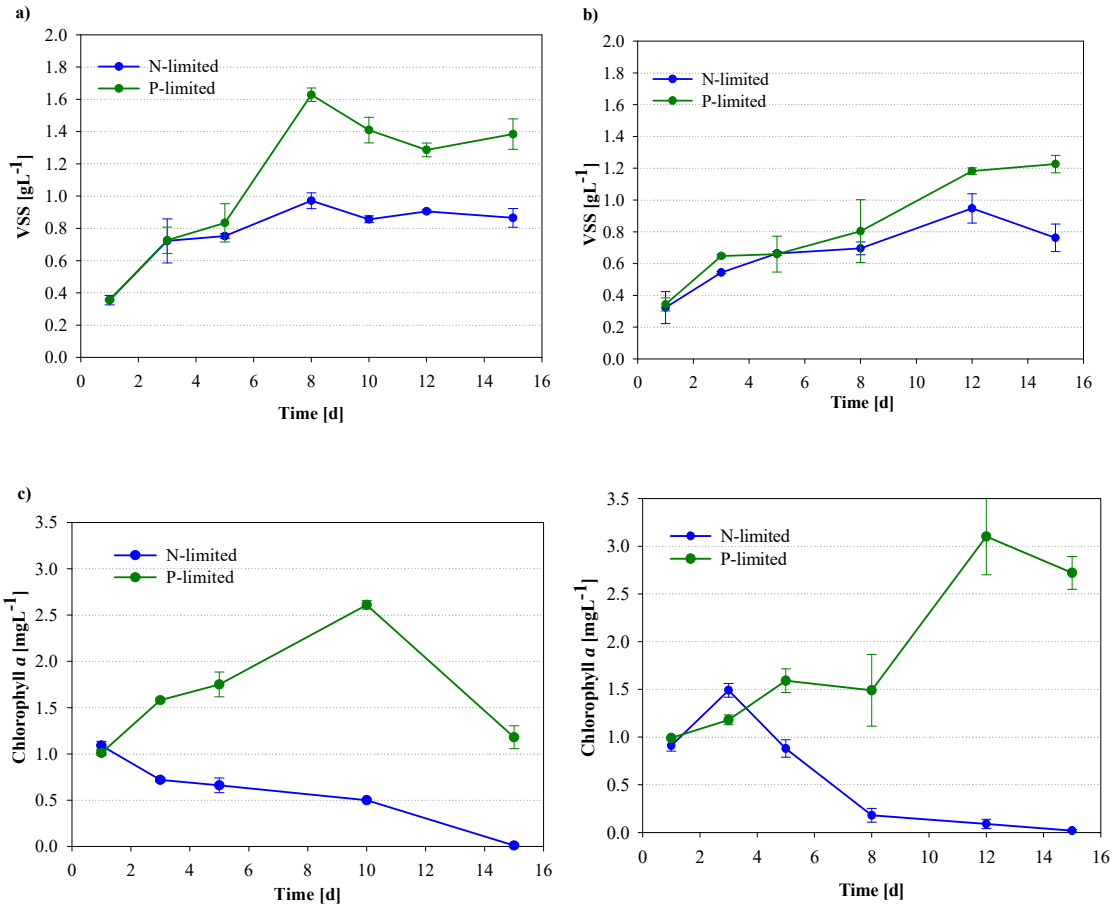
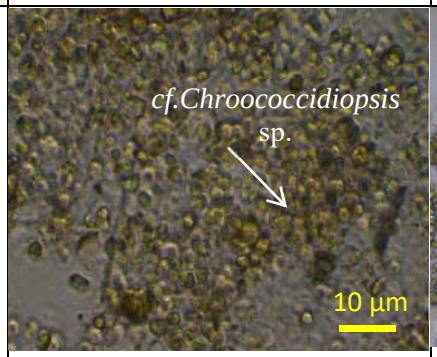
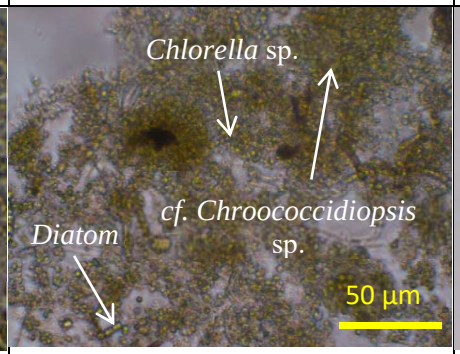
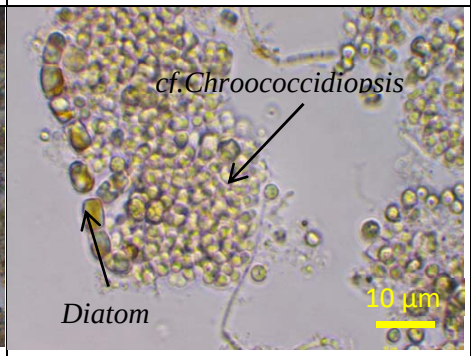
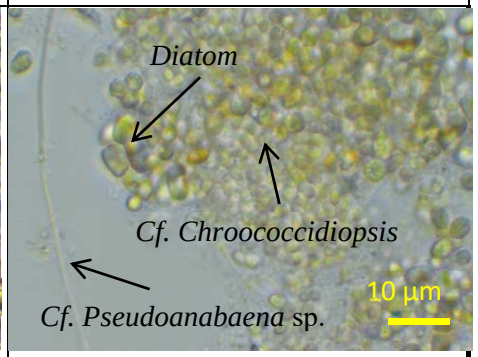


Fig. 3

a)		Day			
		3	8	12	15
Constant illumination	N-limited				
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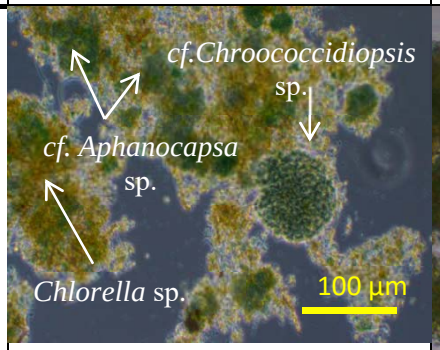
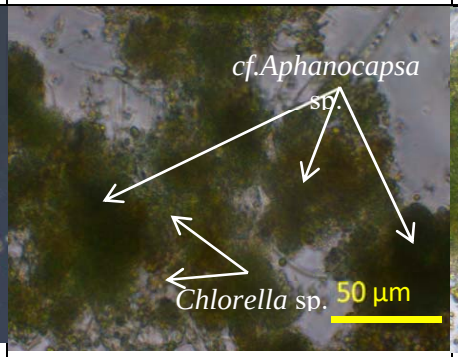
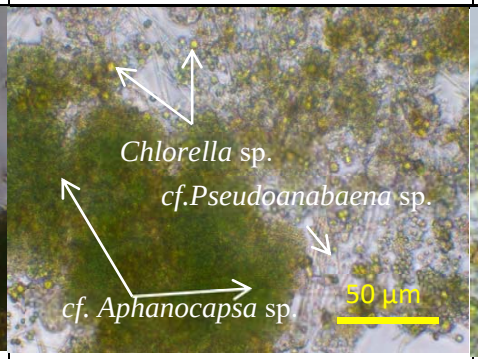
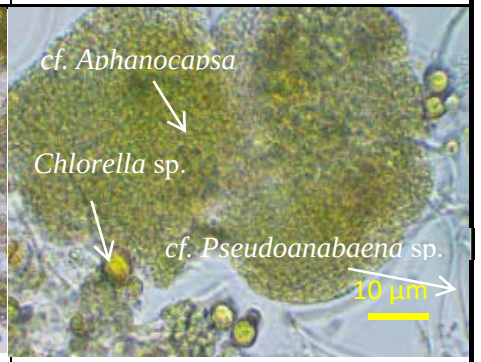
	P-limited				
		Phase contrast microscopy (200x)	Bright light microscopy (400x)	Bright light microscopy (400x)	Bright light microscopy (1000x)

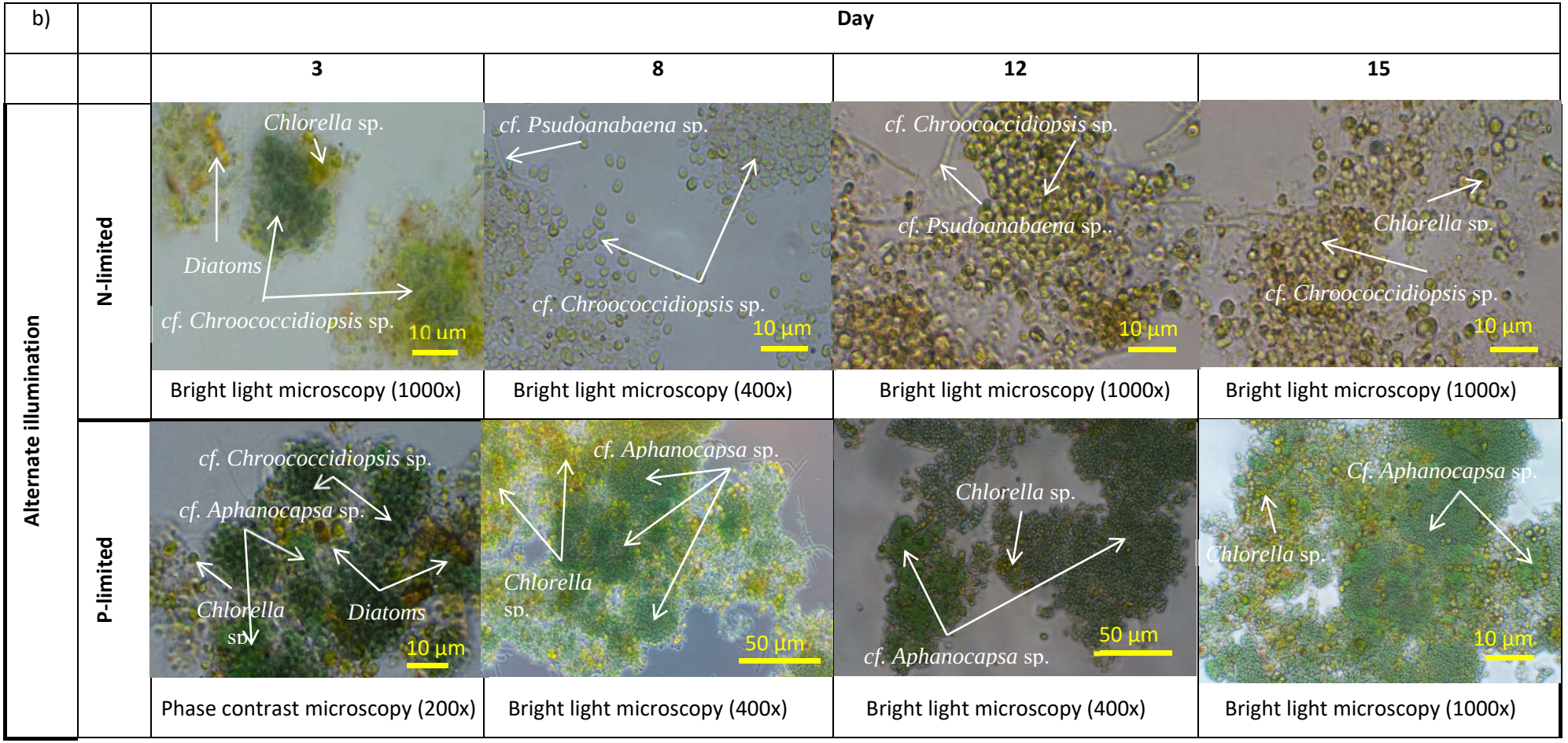

b)
Day
3
8
12
15
Alternate illumination
N-limited
P-limited
Chlorella sp.
Diatoms
cf. Chroococcidiopsis sp.
10 µm
cf. Psudoanabaena sp.
cf. Chroococcidiopsis sp.
10 µm
cf. Chroococcidiopsis sp.
cf. Psudoanabaena sp..
10 µm
Chlorella sp.
cf. Chroococcidiopsis sp.
10 µm
Bright light microscopy (1000x)
Bright light microscopy (400x)
Bright light microscopy (1000x)
Bright light microscopy (1000x)
cf. Chroococcidiopsis sp.
cf. Aphanocapsa sp.
Chlorella sp.
Diatoms
10 µm
cf. Aphanocapsa sp.
Chlorella sp.
50 µm
Chlorella sp.
cf. Aphanocapsa sp.
50 µm
Cf. Aphanocapsa sp.
Chlorella sp.
10 µm
Phase contrast microscopy (200x)
Bright light microscopy (400x)
Bright light microscopy (400x)
Bright light microscopy (1000x)

Fig 4

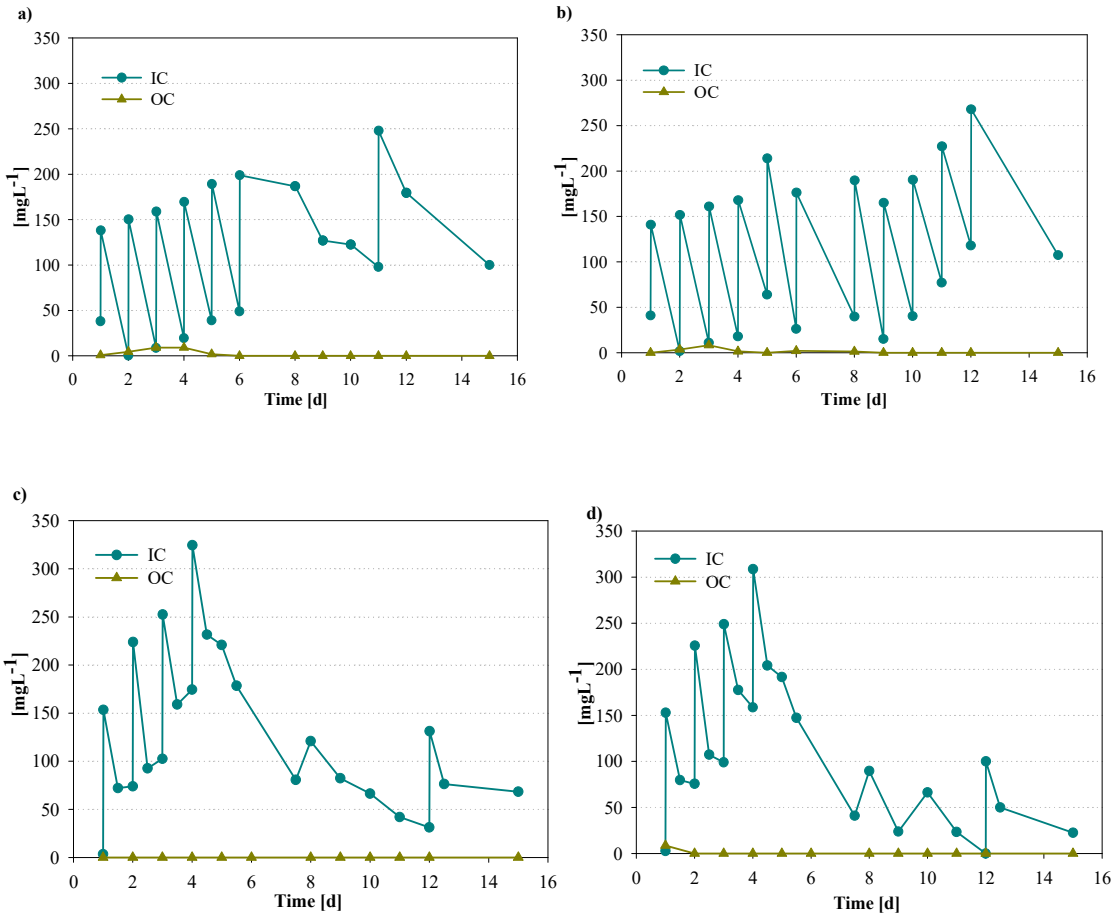


Fig. 5.

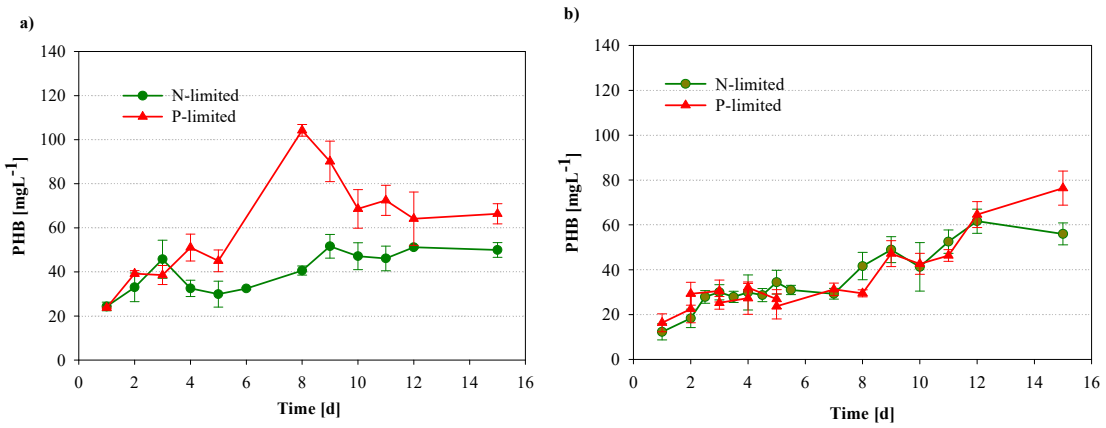

a)
b)
N-limited
P-limited
PHB [mgL-1]
Time [d]
0
20
40
60
80
100
120
140
0
2
4
6
8
10
12
14
16

Fig. 6

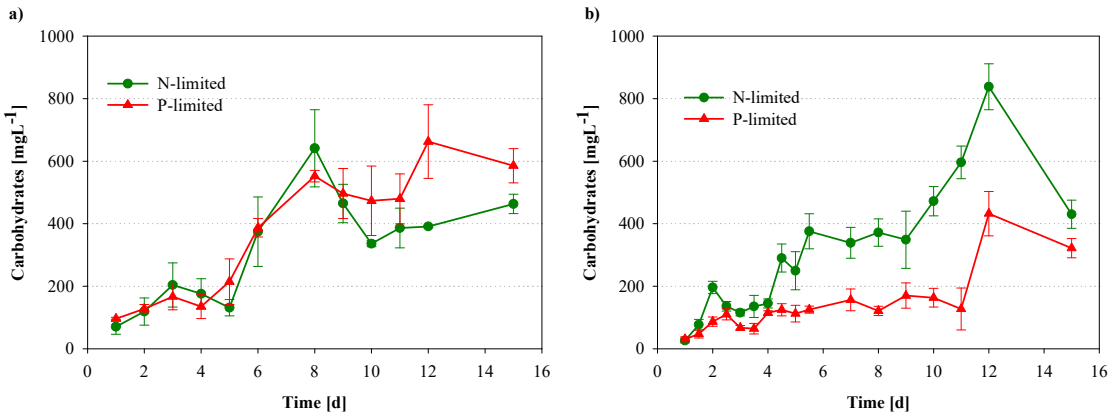

a)
b)
N-limited
P-limited
Carbohydrates [mgL-1]
Time [d]
0
200
400
600
800
1000
0
2
4
6
8
10
12
14
16

Fig. 7

Supplementary material

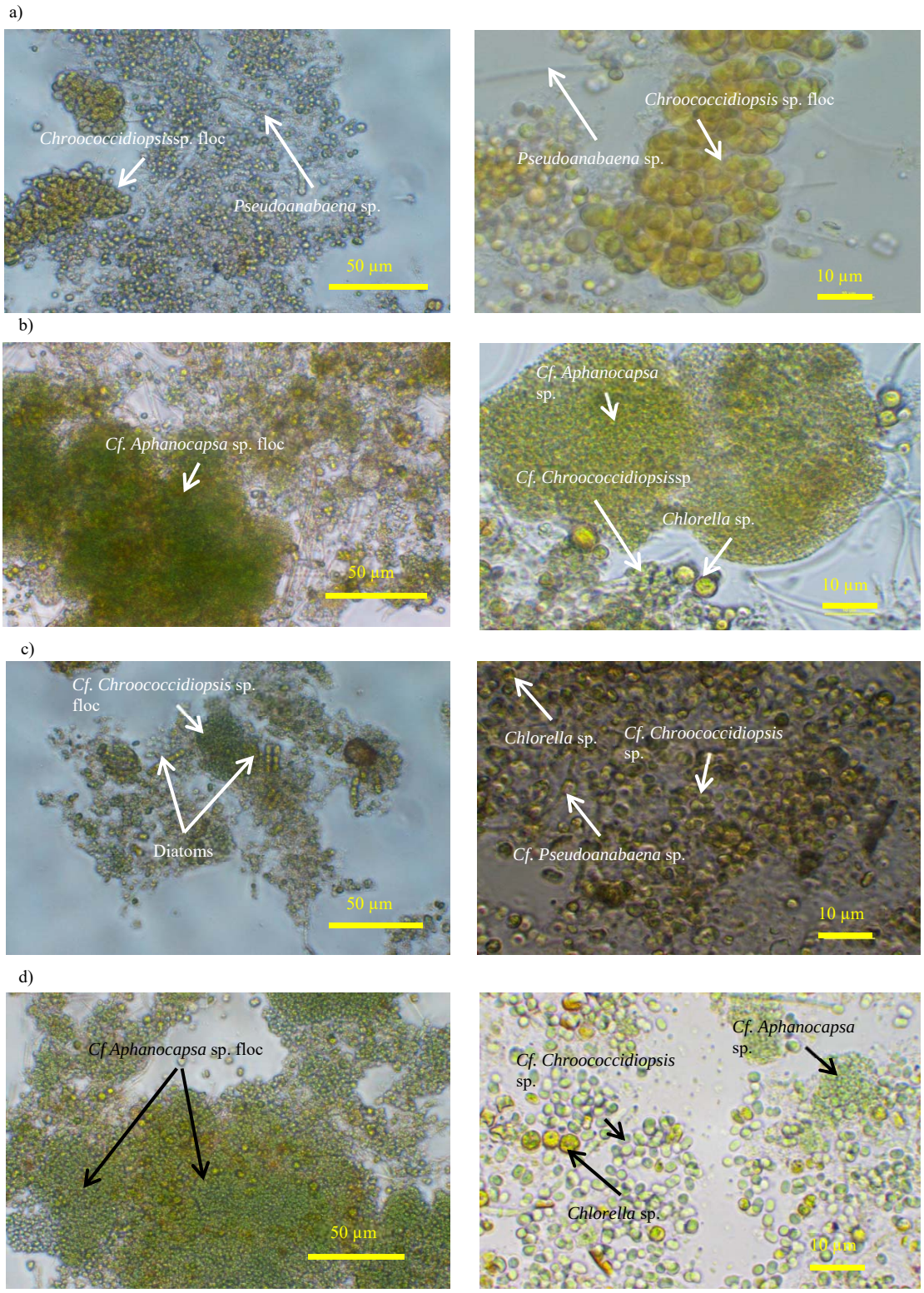


Fig. S1. Microscopic images illustrating the general view (left) at 400x and detailed view (right) at 100x of the microbial composition of the culture in the end of the experimental

time. a) N-limited culture under permanent illuminance showing cyanobacteria floc with cf. *Chroococcidiopsis* sp. and a filaments of cf. *Pseudoanabaena* sp.; b) Culture submitted to phosphorus limitation under permanent illuminance showing large colonies of cf. *Aphanocapsa* sp. immersed in flocs, with some filaments of cf. *pseudoanabaena* sp. and dispersed *Chlorella* sp.; c) Nitrogen-limited culture under light/dark alternation showing cyanobacteria dominated floc composed by cf. *Chroococcidiopsis* sp. and some filaments of cf. *Pseudoanabaena* sp. and diatoms immersed; d) phosphorus-limitated culture under light/dark alternation showing large flocs composed by cf. *Aphanocapsa* sp. and cf. *Chroococcidiopsis* sp.