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PRODUCTION OF CARBOHYDRATES AND POLYHYDROHYBUTYRATE BY CYANOBACTERIA GROWN IN WASTEWATER

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THESIS DISSERTATION OF THE PHD TITLE

PRODUCTION OF CARBOHYDRATES AND POLYHYDROHYBUTYRATE BY CYANOBACTERIA GROWN IN WASTEWATER

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A mi madre

Preface

The current thesis is framed within the context of two Spanish National Projects and one European project: the DIPROBIO project "Algal biomass production and digestion from wastewater" (CTM2012-37860), financed by the Spanish Ministry of Science and Innovation; the project entitled FOTOBIOGAS "Biogas production from microalgae-bacteria grown in closed photobioreactors for wastewater treatment (CTQ2014-57293-C3-3-R), financed by the Spanish Ministry of Economy and Competitiveness; and the project INCOVER (INCOVER, GA 689242), financed by the European Union's Horizon 2020 Research and Innovation Programme.

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Summary

Cyanobacteria are prokaryotic aerobic photosynthetic microorganisms with the capacity to synthesize a large variety of bioactive compounds and valuable byproducts. Among those valuable byproducts, glycogen (carbohydrates) and polyhydroxybutyrate (PHB) are receiving increasing interest due to their potential as biofuel substrate and as bioplastic, respectively. However, its production is associated with high operational costs, caused by the use of pure cultures with controlled sterile conditions, which increases byproducts prices and limits its marketability. A sustainable alternative could be the use of wastewater-borne cyanobacteria cultivated in wastewater. Notwithstanding, in spite of being an attractive alternative, the utilization of cyanobacterial cultures grown in wastewater to produce biomass and byproducts strictly depends on biomass composition and cyanobacteria dominance over other microalgae. Thus far, there are only few studies focusing on the growth of cyanobacteria in wastewater, and factors controlling cyanobacteria competence relationships are still not completely understood. Therefore, the main objective of this PhD thesis was to select and cultivate cyanobacteria in wastewater effluents for carbohydrates and PHB production.

The thesis was divided in two main parts. The first consisted in evaluating the effect of nutritional and operational conditions on the selection and grow of wastewater-borne cyanobacteria. While the second part was focused on the accumulation of carbohydrates and PHB throughout different strategies based on nutrients and carbon limitation.

In the first part, the effect of nutritional and operational conditions on the selection and growth of wastewater-borne cyanobacteria cultivated in secondary wastewater and digestate was considered. In this part cyanobacteria were selected from an initial mixed microalgae consortium using treated municipal wastewater and digestate from an anaerobic digester (mixed 50:1, treated wastewater:digestate) as nutrient source.

Firstly, nutrients effects (e.g. concentrations, ratios and loads) were evaluated in a long term study performed in a pilot scale photobioreactor (PBR) (30 L) operated at semi-continuous mode. Results of this section evidenced that wastewater-borne cyanobacteria species *Aphanocapsa* sp., *Chrooccocus* sp. and *Pseudanabaena* sp. were able to dominate over green algae *Chlorella* sp. and *Stigeoclonium* sp. in a pilot semi-continuous PBR. Their dominance was achieved under non-limited carbon conditions, N:P ratios between 16:1-49:1 (molar basis) and low inorganic phosphorus (P) loads (~0.25 mg L d^{-1}) and concentrations.

Under these conditions, a culture dominated by cyanobacteria was able to reach an average biomass production of 0.08 g $L^{-1}d^{-1}$.

Lastly, short term studies in lab-scale PBRs (3L) were carried out to evaluate the effect of operational conditions (e.g. hydraulic regimes, solids retention time (SRT), hydraulic retention time (HRT)). Results indicated that HRT of 6 days and SRT of 10 days provided suitable conditions to remove unsettled low P tolerant green algae (e.g. *Scenedesmus* sp.) and to improve cyanobacteria concentration from 2% until 70% of the total population, while increasing the biomass production to 0.12 g L^{-1} d^{-1}. Conversely, the reduction of HRT and SRT negatively affected cyanobacteria dominance, favoring green algae dominance and a higher presence of heterotrophic bacteria.

In the second part of the thesis, the cyanobacteria dominated culture previously obtained was submitted to different nutritional and light conditions to accumulate carbohydrates and PHB. Initially, the effect of nutrients limitation (N and P limitation) and photoperiods (permanent light and light/dark alternation) was tested. Experiments were performed in lab-scale PBRs (3L) operated in batch conditions during two weeks. In these first tests, results indicated that PHB content up to 6.5% and a carbohydrates content up to 75% can be reached under N limitation and light/dark alternation in twelve days of incubation. Later, the effect of the carbon feast and famine strategy on the production of carbohydrates and PHB was evaluated. This strategy consists in the alternation of periods with carbon availability and a subsequent absence of carbon during the cultivation. Feast and famine strategy was assessed along with the effect of nutrients ratios and loads in a lab-scale (2.5 L) sequencing batch reactors. With the application of this strategy, the culture accumulated almost 4% of PHB after a complete depletion of nitrogen, while carbohydrates reached the highest content (43%-48%) under P limitation. Such contents were obtained in only 24h of incubation under aerobic illuminated conditions.

In conclusion, the results obtained in this PhD thesis demonstrated that the dominance of cyanobacteria in microalgal-based wastewater treatment systems can be achieved by controlling operational and nutritional conditions. Concerning polymers production, results indicated that carbon uptake, and the consequent polymers production from cyanobacteria can be enhanced through carbon and nutrients feeding strategies.

Resumen

Las cianobacterias son microorganismos fotosintéticos aeróbicos procarióticos con la capacidad de sintetizar una gran variedad de compuestos bioactivos y subproductos valiosos. Entre ellos, el glucógeno (carbohidratos) y el polihidroxibutirato (PHB) están recibiendo un creciente interés debido a su potencial como sustrato de biocombustible y como bioplástico, respectivamente. Sin embargo, su producción está asociada a altos costos operacionales, causados por el uso de cultivos puros con condiciones estériles controladas, lo que aumenta los precios de subproductos y limita su comerciabilidad. Una alternativa sostenible podría ser el uso de cianobacterias de aguas residuales cultivadas en aguas residuales. No obstante, a pesar de ser una alternativa atractiva, la utilización de cultivos de cianobacterias cultivadas en aguas residuales para producir biomasa y subproductos depende estrictamente de la composición de la biomasa y del dominio de las cianobacterias sobre otras microalgas. Hasta ahora, hay pocos estudios que se centren en el crecimiento de las cianobacterias en las aguas residuales, y los factores que controlan las relaciones de competencia con las cianobacterias aún no se conocen por completo. Por lo tanto, el objetivo principal de esta tesis doctoral fue seleccionar y cultivar cianobacterias en efluentes de aguas residuales para producción de PHB y carbohidratos.

La tesis se dividió en dos partes principales. La primera consistió en evaluar el efecto de las condiciones nutricionales y operativas en la selección y el crecimiento de cianobacterias nativas de aguas residuales. Mientras que la segunda parte se centró en la acumulación de carbohidratos y PHB a través de diferentes estrategias basadas en limitación de nutrientes y de carbono.

En la primera parte, se consideró el efecto de las condiciones nutricionales y operativas en la selección y el crecimiento de cianobacterias nativas de aguas residuales cultivadas en aguas residuales secundarias y en digestato. En esta parte, las cianobacterias se seleccionaron de un consorcio inicial mixto de microalgas que utilizaba aguas residuales municipales tratadas y digestato de un digestor anaeróbico (agua residual tratada 50:1: digestato) como fuente de nutrientes.

En primer lugar, se evaluaron los efectos de los nutrientes (e.g., concentraciones, ratios y cargas) en un estudio a largo plazo realizado en un fotobiorreactor (FBR) a escala piloto (30 L) operado en modo semicontinuo. Los resultados de esta sección evidenciaron que las especies de cianobacterias nativas de aguas residuales *Aphanocapsa* sp., *Chrooccocus* sp. y *Pseudanabaena* sp. fueron capaces de dominar sobre las algas verdes *Chlorella* sp. y *Stigeoclonium* sp. en un FBR piloto semicontinuo. Su dominio se logró bajo condiciones de carbono no limitado,

relaciones N:P entre 16: 1-49: 1 (base molar) y bajas cargas de fósforo inorgánico (P) (~ 0.25 mg L d^{-1}). Bajo estas condiciones, un cultivo dominado por cianobacterias fue capaz de alcanzar un promedio de producción de biomasa de 0.08 g $L^{-1}d^{-1}$.

Por último, se llevaron a cabo estudios a corto plazo en PBRs a escala de laboratorio (3L) para evaluar el efecto de las condiciones operacionales (e.g., regímenes hidráulicos, tiempo de retención de sólidos (TRS), tiempo de retención hidráulica (TRH)). Los resultados indicaron que el un TRH de 6 días y un SRT de 10 días proporcionaron condiciones adecuadas para eliminar las algas verdes suspendidas, tolerantes a bajos niveles de P (e.g., *Scenedesmus* sp.), y mejorar la concentración de cianobacterias de 2% hasta 70% de la población total, mientras que aumenta la producción de biomasa a 0.12 g L^{-1} d^{-1}. Por el contrario, la reducción de TRH y TRS afectó negativamente a la dominancia de las cianobacterias, favoreciendo el dominio de las algas verdes y una mayor presencia de bacterias heterótrofas.

En la segunda parte de la tesis, el cultivo dominado por cianobacterias previamente obtenido se sometió a diferentes condiciones nutricionales y lumínicas para acumular carbohidratos y PHB. Inicialmente, se probó el efecto de la limitación de nutrientes (limitación de N y P) y fotoperiodos (iluminación constante y alternancia luz/oscuridad). Los experimentos se realizaron en FBRs a escala de laboratorio (3L) operados en condiciones batch durante dos semanas. En estas primeras pruebas, los resultados indicaron que un contenido de PHB de hasta 6.5% y un contenido de hasta 75% de carbohidratos puede alcanzarse con limitación de N y alternancia de luz/oscuridad, en doce días de incubación. Más tarde, se evaluó el efecto de la estrategia de festin y hambre de carbono sobre la producción de carbohidratos y PHB. Esta estrategia consiste en la alternancia de períodos con disponibilidad de carbono y una posterior ausencia de carbono durante el cultivo. La estrategia de festin y el hambre se evaluó junto con el efecto de las proporciones de nutrientes y las cargas en un reactor de lotes secuenciales a escala de laboratorio (2.5 L). Con la aplicación de esta estrategia, el cultivo acumuló casi 4% de PHB después de una reducción completa de nitrógeno, mientras que los carbohidratos alcanzaron el contenido más alto (43%-48%) con la limitación de P. Dichos contenidos se obtuvieron en solo 24 h de incubación bajo condiciones aeróbicas iluminadas.

En conclusión, los resultados obtenidos en esta tesis doctoral demostraron que el dominio de las cianobacterias en los sistemas de tratamiento de aguas residuales a base de microalgas se puede lograr mediante el control de las condiciones operativas y nutricionales. Con respecto a la producción de polímeros,

los resultados indicaron que la absorción de carbono y la consecuente producción de polímeros a partir de cianobacterias pueden mejorarse mediante estrategias de alimentación de carbono y nutrientes.

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Acronyms and abbreviations

DO	Dissolved Oxygen
HB	Hydroxybutyrate
HV	Hydrohyvalerate
HRAP	High rate algal ponds
HRT	Hydraulic retention time
IC	Soluble inorganic carbon
IP	Inorganic phosphorus
LOD	Low of detection
Lv	Volumetric load
N	Nitrogen
ND	Not detected
NA	Not applicable
P	Phosphorus
PBR	Photobioreactor
PHB	Polyhydroxybutirate
PP	Particulate phosphorus
PON	Particulate organic nitrogen
PSBR	Photo-sequencing batch reactor
Q	Flow rate
Qm	Mixed liquor discharge flow
Qs	Supernatant discharge flow
-qS	Maximum specific substrate uptake rate
qcarbs	Maximum specific carbohydrate production rate
qPHB	Maximum specific polyhydroxybutyrate production rate
S	Substrate
SC	Semi-continuous reactor
SOC	Soluble organic carbon
SRT	Solids retention time
TAN	Total ammoniacal nitrogen
TC	Total carbon
TIN	Total inorganic nitrogen
TON	Total organic nitrogen
TOP	Total organic phosphorus
TSS	Total suspended solids
TSS_m	Mixed liquor total suspended solids
TSS_x	Supernatant total suspended solids
V	Volume
VSS	Volatile suspended solids
TOC	Total organic carbon
TIC	Total inorganic carbon
TN	Total nitrogen
TP	Total phosphorus
X	Active biomass

Y Yield

Chapter 1

Introduction

In recent decades, alternative energy sources (e.g. biofuels and biogas) and valuable products (e.g. 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 (Lau et al., 2015). In this context, the cultivation and use of microalgae and cyanobacteria as feedstock to obtain biofuels, bioproducts and bioenergy has become a relevant research topic. Increasing scientific interest has been devoted to cyanobacteria (blue-green algae) due to their ability to grow in wastewater effluents and to their capacity to produce and accumulate intracellular bioactive compounds of interest for non-food purposes. Such as, phycobiliproteins, polyhydroxyalkanoates (PHA) e.g., poly (3-hydroxyalkanoate) (PHB) and carbohydrates in form of glycogen (Abed et al., 2009; Shalaby, 2011). Carbohydrates and PHB are attracting increasing interest due to their potential as a biofuel substrate and as a bioplastic, respectively. The main interest in cyanobacteria producing these polymers is based on carbon storage through oxygenic photosynthesis, implying simple requirements for cultivation and the utilization of CO_2 as carbon source (Stal, 1992). 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.

Studies related to the production of cyanobacteria and polymers generally employ pure or genetically modified cultures (Miyake et al., 2000). Such strictly controlled processes lead to high production costs, and subsequently expensive products (Samantaray and Mallick, 2012). In this context, a more sustainable alternative for the production of polymers from cyanobacteria could be the use of wastewater-borne mixed 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. Cyanobacteria cultivation in such a variable media implies certain disadvantages related to the competition with other microorganisms, especially with green microalgae (Chlorophyta) (Drosg, 2015).

Although several studies in lakes and reservoirs have dealt with the importance of nutrients' interaction with algal composition (Dolman et al., 2012), there are comparatively few studies focusing on the relation between nutritional conditions in wastewater and the dominance of cyanobacteria (Van Den Hende et al., 2016a). Hence, the factors controlling these competence relationships are still not completely understood.

Chapter 2

Objectives and thesis outline

2.1 Objectives

The main objective of this PhD thesis was to select and cultivate cyanobacteria in wastewater effluents for carbohydrates and polyhydrohybutyrates (PHB) production.

To fulfil this main objective, this thesis was divided in two parts:

1. The first part consisted in evaluating the effect of nutritional and operational conditions on the selection and growth of wastewater-borne cyanobacteria cultivated in secondary wastewater and digestate. The specific objectives were:

   - To evaluate nutritional conditions in the cultivation and selection of wastewater-borne culture dominated by cyanobacteria in a semi-continuous photobioreactor.
   - To evaluate the effect of low hydraulic retention time and high nutrients loads in the cultivation and selection of a wastewater-borne culture dominated by cyanobacteria in a photo-sequencing batch reactor.
   - To evaluate the effect of high hydraulic retention time and low nutrients loads in the cultivation and selection of a wastewater-borne culture dominated by cyanobacteria in a photo-sequencing batch reactor.

2. The second part was focused on the enhancement of carbohydrates and PHB accumulation in a wastewater-borne culture dominated by cyanobacteria throughout different strategies based on nutrients and carbon limitation. The specific objectives were:

   - To evaluate the effect of photoperiods and nutrient limitation in the production of carbohydrates and PHB.
   - To evaluate the effect of the feast and famine strategy and the effect of nutrients ratios and loads in the production of carbohydrates and PHB.

2.2 Thesis outline

This PhD thesis is based on scientific articles sequentially ordered according the specific objectives. In **Chapter 3**, a review of the state of the art of challenges in cyanobacteria cultivation in wastewater for carbohydrates and polyhydrohyalkanoates (PHA) production is presented. This chapter critically analyzes and compares the most relevant results obtained in this PhD thesis and in other studies about the conditions favouring cyanobacteria growth in mixed-culture reactors and their potential use for carbohydrates and PHA production.

In **Chapter 4,** the effect of nutrients concentrations and ratios on the culture composition and biomass concentration of a mixed green algae/cyanobacteria consortium was evaluated. This experiment was carried out in a semi-continuous photobioreactor fed with secondary wastewater and digestate in an one-year long experiment.

However, nutrients concentration is not the only factor controlling competition of cyanobacteria with green algae. Thus, short-term experiments were carried out analyzing the effect of photo-sequencing batch operation, hydraulic retention time, solids retention time and nutrients loads on cyanobacteria dominance (**Chapter 5 and 6**).

As a first essay on the possible factors affecting the production of polymers in wastewater-borne cultures dominated by cyanobacteria, a mixed cyanobacterial culture obtained from soil was cultivated in wastewater at different hydraulic retention times (**Chapter 7**). Since it was observed that polymers are affected mainly by nutrients limitation, posterior essays on wastewater borne cultures were focused on the evaluation of N or P limitations and photoperiods in batch operation along 15 days (**Chapter 8**).

In **Chapter 9** feast and famine strategy, until now only used in bacterial culture for polymers production, was proposed for the optimization of polyhydroxybutyrate and carbohydrates production in wastewater-borne cyanobacterial cultures. Carbon feast and famine and effect of nutrients ratios and loads was evaluated in a photo-sequencing batch reactor.

Chapter 10 presents the discussion of the results obtained in this thesis and proposes operational criteria for a full-scale case study in order to achieve cyanobacteria dominance and PHB accumulation.

Chapter 11 draws the conclusions based on the obtained results.

Chapter 3

State of the art

The contents of this chapter were adapted from the publication: Arias, D.M., García, J. and Uggetti, E. (in preparation) Production of polymers by cyanobacteria grown in wastewater: current status, challenges and future perspectives.

3.1 Introduction

Cyanobacteria, also known as blue-green algae, have been around on our planet during more than 3.5 billion years. They were the first organisms to form molecular oxygen and to contribute changing the biosphere from anaerobic to largely aerobic (Oren, 2014). They are by far the largest group of photosynthetic prokaryotic organisms as judged by their widespread occurrence, frequency, abundance and morphological diversity (Vijayakumar et al., 2012; Whitton and Potts, 2012). Moreover, when growth conditions are not suitable, some species can survive adverse conditions for long periods (Whitton, 2002).

In general, efficient adaptation of cyanobacteria to almost any environment implies that their cultivation does not require energy rich compounds like other non-photosynthetic microorganisms (Lau et al., 2015). Such simple requirements of nutrients give them a great potential to create low cost eco-friendly technologies for wastewater treatment and bioproducts generation (Lau et al., 2015). Indeed, effectivity of cyanobacteria to grow in different effluents such as municipal and industrial wastewater has been already documented (Parmar et al., 2011; Vijayakumar, 2012a).

This strategy offers a double advantage since wastewater treatment can result into clean water while high value byproducts can be also obtained (Markou et al., 2014; Parmar et al., 2011). In fact, cyanobacteria are known to storage polymeric compounds as polyhydroxyalkanoates (PHAs) and carbohydrates in certain nutrient deficient conditions (Samantaray et al., 2011). PHAs and carbohydrates production from cyanobacteria have received much attention because their potential use as bioplastics and biofuels substrate, respectively.

However, bioplastics and biofuels obtained from cyanobacteria have gone through many years of efforts towards commercialization with limited successes (Chen et al., 2013; Drosg et al., 2015). Since the main limitation concerning cyanobacteria growth for bioplastics production is the high cost of the source of nutrients, wastewater may overcome such bottleneck by offering a cheap substrate. Though cyanobacteria have demonstrated their capacity to treat wastewater effluents, their cultivation in such variable environment involves certain challenges that need to be considered. The main challenge is linked to contamination by other species, specially by green algae (Hashimoto and Furukawa, 1989). This fact would represent a serious drawback during cyanobacterial biomass production, and affects further PHAs and carbohydrates production.

This review considers the characteristics of cyanobacteria, their potential to grow in wastewater effluents and their use for polymers production, specially carbohydrates and PHAs. A critical analysis on potential interesting aspects as the conditions favouring cyanobacteria in mixed-culture reactors are also provided and discussed. In addition, a view of current advances and future prospects in polymers production from mixed cyanobacterial cultures is also included.

3.2 Overview of cyanobacteria

Cyanobacteria are prokaryotic oxygenic phototrophs found in almost every conceivable habitat on earth (Abed et al., 2009). They constitute one of the largest groups of gram-negatives prokaryotes that involves a wide diversity in morphology, physiology, cell division patterns, cell differentiation and habitats (Rippka et al., 1979). Cyanobacteria are also unified by the ability to carry out a plant like oxygen photosynthesis, being chlorophyll *a* and phycobiliproteins their main photosynthetic pigments (Stal and Moezelaar, 1997). Additionally, all cyanobacteria are autotrophs that fix CO_2 through the reductive pentose phosphate cycle or Calvin cycle (Flores and Herrero, 2005).

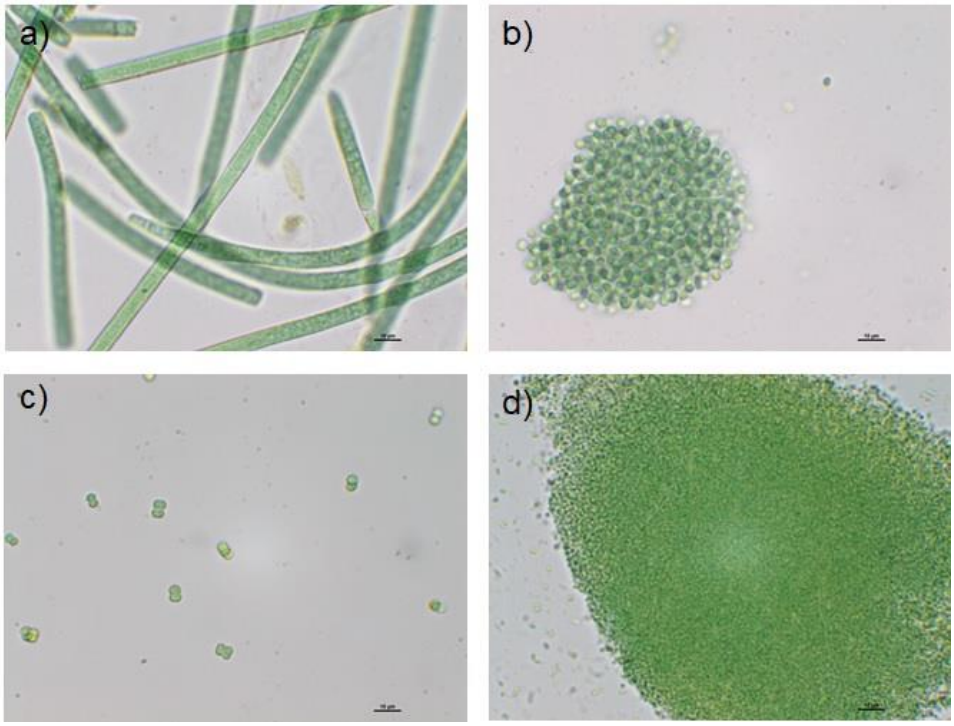

Fig. 3.1 Cyanobacteria species; a) *Microcoleus* sp., b) *Aphanocapsa* sp., c) *Synechocystis* sp., d) *Synechococcus* sp.. Observed in bright light microscopy at 1000X.

Cyanobacteria also make an important contribution to the Earth's nitrogen cycle by incorporating nitrogen into the biosphere through assimilatory processes (Abed et al., 2009). Many species live in the soil and other terrestrial habitats, where they are important in the functional processes of ecosystems and the cycling of nutrient elements (Wynn-Williams, 2002).

Due to their high protein and vitamin content providing a good source of fiber and for their good digestibility, cyanobacteria are used for a variety of purposes including food and feed supplements (Bohutskyi and Bouwer, 2013; Vijayakumar, 2012a). Other current and prospective applications of cyanobacteria include the production of pharmaceuticals due to the range of biological activity of secondary metabolites isolated that includes antibacterial, antifungal, antialgal, antiprotozoan, and antiviral activities (Abed et al., 2009; Rosgaard et al., 2012). Some of the marine cyanobacteria constitute potential sources for large-scale production of vitamins, such as vitamins B and E. Other applications widely recognized are as biofertilizers, hydrogen and amino acid production, light energy photo conversion and for the control of algal blooms (Vijayakumar, 2012a). Furthermore, they are utilized to prevent the expansion of desertification in soils (Xu et al., 2013). In this process, cyanobacteria improve the mineralization of nutrients, changing the surface soil properties by trapping and retaining water in sandy soils, reducing water infiltration and protecting the soil from erosion (Colica et al., 2014). Since cyanobacteria are naturally transformable, they can be genetically manipulated relatively easily. Thus, their employment for genetic engineering has been wide and successful (Machado and Atsumi, 2012; Sarsekeyeva et al., 2015). Genetic engineering of cyanobacteria has allowed the production of a number of value-added compounds (*e.g.* ethanol, isobutyraldehyde, isobutanol, 1-butanol, isoprene, ethylene, hexoses, cellulose, mannitol, lactic acid, fatty acids), however, all these findings have been found in lab-scale experiments (Nozzi et al., 2013; Rosgaard et al., 2012).

Recent studies have demonstrated that cyanobacteria form ideal consortia with hemotrophic bacteria and can effectively be used to cleanup oil contaminated sediments and wastewaters (Abed et al., 2009). In this process wastewater streams have been frequently used as a readily available and low-cost substrate for microalgal growth, biomass production and nutrient removal (Rawat et al., 2011). On the other hand, wastewater has been recognized as the only economic and sustainable source of nutrient for biomass cultivation (Park et al., 2011).

During the last decades, cyanobacteria have received much attention as a rich source of bioactive compounds and have been considered as one of the most promising group of organisms to produce them (Abed et al., 2009). However, the

use of waste streams as nutrients source reduces the range of biomass applications due to its possible contamination by various pollutants present in wastewater (Markou et al., 2014). Therefore, cyanobacteria grown in wastewater should mainly be used for the production of non-food applications (Talbot and de la Noüe, 1993), such as for bioenergy, biofuels and bioplastics generation.

3.3 Cyanobacteria cultivation in wastewater treatments systems for nutrients removal

The utilization of microalgae and cyanobacteria for wastewater treatment was firstly proposed by Oswald and Golueke (1960) through an implementation study for the treatment of domestic wastewater using open raceway ponds in California. In this process, cyanobacteria as well as other microalgae grow in association with aerobic heterotrofic bacteria (Abed et al., 2009; Borde et al., 2003). Indeed, photosynthetic microorganisms produce molecular oxygen that is used as electron acceptor by bacteria to degrade organic matter. In return, bacteria release carbon dioxide during the mineralization process and complete the photosynthetic cycle (Muñoz and Guieysse, 2006). Cyanobacteria can be used in wastewater treatment for a range of purposes such as the removal of nutrients, the reduction of both Chemical Oxygen Demand (COD) and Biological Oxygen Demand (BOD and also for the removal of heavy metals (Abdel-Raouf et al., 2012; de Godos et al., 2009; Honda et al., 2012; Wang et al., 2010). This kind of wastewater treatment is regarded as an economical and environmentally friendly process with no secondary pollution as long as the biomass produced can be reused and allows efficient nutrient recycling (Rawat et al., 2011; Honda, et al., 2012).

Cyanobacteria based technologies have been successfully applied to the treatment of domestic, livestock and industrial wastewaters (Muñoz and Guieysse, 2006). Efficiencies found are often superior to those achieved in conventional aerobic activated sludge processes in terms of nutrient assimilation and pathogen removal, with lower energy demands (García et al., 1998). However, wastewater treatment with cyanobacteria have been performed with monocultures, mostly in lab scale studies (Table 3.1).

3.3.1 Municipal wastewater

Nitrogen and phosphorous removal has been the main objective of cyanobacteria cultures for municipal wastewater systems, since nutrient enrichment and the consequent eutrophication can alter the structure and function of aquatic ecosystems, potentially endangering human health, biodiversity and ecosystem

sustainability (Wang et al., 2012). Cyanobacteria are indicated for this purpose since they can assimilate different forms of nitrogen and phosphorus for growth (i. e. NH_4^+ (ammonium), NO_2^- (nitrite), NO_3^-(nitrate) and PO_4^{3-} (orthophosphate)) (Abdel-Raouf et al., 2012). Different species of cyanobacteria, mostly filamentous species like *Phormidium sp.* (Pouliot et al., 1989; Boelee et al., 2014), *Spirulina platensis* (Magro et al., 2012) have been successfully cultivated using municipal wastewater, not only for the removal of nitrogen and phosphorous (>60%) from municipal wastewater but also for the reduction of COD and BOD (>80%) (Table 3.1).

3.3.2 Industrial wastewater

The diversity and dominance of cyanobacteria species in different aquatic bodies demonstrate the potential tolerance of this group to a wide variety of contaminants (Sood et al., 2015). Through several studies, cyanobacteria have demonstrated their ability of biodegradation and biosorption of persistent chlorinated hydrocarbons and heavy metals (El-Bestawy, 2008; Kirkwood et al., 2001). Filamentous *Oscillatoria brevis*, *Westiellopsis prolifica, Anabaena sp.* and *Nostoc muscorum* have been utilized with encouraging results for the treatment of several industrial effluents. Those include cheese factory effluents (Blier et al., 1995), oil, soap and fodder industry effluents (El-Sheekh et al., 2014), dry industry effluent (Vijayakumar, 2012b), distillery effluents (Ganapathy et al., 2011), and effluents from aquaculture (Kamilka et al., 2006), removing nutrients and organic matter by 50-100% (Table 3.1). Other studies also include the biodegradation of phenol and dichloroacetate from pulp-and-paper wastewater (almost 100%) (Kirkwood et al., 2005) and the removal of copper (75%), cobalt (11.8%), lead (100) and manganese (61.5%) from mixed domestic-industrial effluent (El-Bestawy, 2008). It is noted that these studies have been performed in lab-sacale studies and until now this technology have not been applied to large scale systems.

3.3.3 Secondary effluents

Tertiary wastewater processes aim at removing ammonia, nitrate and phosphate remaining in secondary effluents, and generally are more expensive than secondary treatments. For this reason, cyanobacteria cultures could offer an efficient solution for tertiary treatment due to their ability to use remaining nutrients from previous wastewater treatments as a result of their low nutrient requirements. Previous studies have documented the utilization of cyanobacteria for tertiary treatment of different secondary effluents by filamentous species as *Oscillatoria* sp. (Hashimoto and Furukawa, 1989), *Phormidium* sp. (Cañizares-Villanueva et al., 1994; Blier et al., 1995;) and *Spirulina maxima* (Olguín et al., 2003;

Cheunbarn and Peerapornpisal, 2010) and mixed cultures dominated by cyanobacteria *Aphanocapsa* sp. and *Phormidium* sp. (Van Den Hende et al., 2016a) (Table 3.1).

3.3.4 Digestates

Recent research has been focused on the utilization of the liquid part of effluent from anaerobic digestion for microalgae and cyanobacteria growth. Due to the characteristics of digestate, which is rich in ammonia, phosphate and acids this is a potential option for accomplishing cyanobacteria requirements (Cheng et al., 2014; Bjornsson et al., 2013). It has been found that several species of microalgae and cyanobacteria can grow on diluted and undiluted digestates from various sources, including swine slurry (Cheng et al., 2014), municipal organic waste (Uggetti et al., 2014), abattoir digestate (Bchir et al., 2011), swine manure (Cheunbarn and Peerapornpisal, 2010; Hu et al., 2012), poultry manure (Iyovo et al., 2010) and microalgal anaerobic digestate (Arias et al., 2017; Prajapati et al., 2014). Thus, recycling nutrients digestates could also integrate an economic and ecological option to grow cyanobacterial biomass for obtaining nonfood byproducts.

Table 3.1 Wastewater treatment performances obtained with different cyanobacteria.

Wastewater influent	Species of cyanobacteria	Biomass concentration/or production	Organic matter and/or nutrients removal (%)	Reference
Wastewater mixed with growth medium	*Spirulina platensis*	2.76 g L^{-1}	COD 81	Magro et al. (2012)
Municipal wastewater	*Mixed cyanobacteria dominated consortium*	1.7 g L^{-1}	BOD 99 COD 87 PO_4^{3-}-P 97 NH_4^+-N 90 NO_3^--N 100	Renuka et al. (2013)
Municipal wastewater diluted with swine manure	*Phormidium* spp.	0.074 g $L^{-1}.d^{-1}$	NH_4^+-N: 95 PO_4^{3-}-P: 62	Pouliot et al. (1989b)
Municipal wastewater with industrial effluent	*Nostoc muscorum and Anabaena subcylindrica*	ND	COD 67 P 95 NH_4^+-N 80 NO_3-N 96	El-Sheekh et al. (2014)
Municipal wastewater diluted with industrial wastewater	*Anabaena oryzae, A. variabilis, TolypothrIx ceytonica*	ND	BOD 89 COD 74	El-Bestawy, (2008)
Distillery effluent	*Nostoc muscorum*	ND	BOD 54 COD 69 TP 56 NH_4^+-N 59 NO_3^--N 83	Ganapathy et al. (2011)

Table 3.1 (continue)

Wastewater influent	Species of cyanobacteria	Biomass concentration/or production	Organic matter and/or nutrients removal (%)	Reference
Aquaculture effluent	*Nostoc muscorum*	ND	PO_4^{3-}-P 47.76 NH_4^+-N 50.39 NO_3^--N 50.39	Kamilya et al. (2006)
Dye industry effluent	*Oscillatoria brevis and Westiellopsis prolifica*	ND	COD~100 PO_4^{3-}-P~100 NH_4^+-N~100 NO_3^--N~100	Vijayakumar, (2012b)
Secondary effluent from activated sludge	*Oscillatoria* sp.	1.6 g L^{-1}	PO_4^{3-}-P >90 NH_4^+-N >90	Hashimoto and Furukawa (1989)
Secondary effluent	*Phormidium* sp.	ND	PO_4^{3-}-P >42 NH_4^+-N >84 NO_3^--N >44	Su et al. (2012)
Secondary effluent	*Mixed culture dominated by cyanobacteria Geminocystis sp., Aphanocapsa sp.*	0.32 g $L^{-1}.d^{-1}$	BOD 33 COD 45 TN 37 TP 20	Van Den Hende et al. (2016)
Secondary effluent and digestate	Mixed /cyanobacteria dominated culture	0.08 g $L^{-1}.d^{-1}$	NH_4-N 95.6 PO_4^{3-}-P 58.9	Arias et al. (2017) (Chapter 4)
Secondary effluent and digestate	Mixed microalgae/cyanobacteria dominated culture	0.12 g $L^{-1}.d^{-1}$	TOC 85 TN 58 TP 83	Arias et al. (n.d.a) (Chapter 6)

Table 3.1 (continue)

Wastewater influent	Species of cyanobacteria	Biomass concentration/or production	Organic matter and/or nutrients removal (%)	Reference
Swine manure digestate	*Spirulina platensis*	17.8 x 10^4 cells.mL	BOD 45 COD 23 PO_3^4-P 67 NH_4-N 92 NO_3-N 49	Cheunbarn and Peerapornpisal (2010)
Swine manure digestate	*Phormidium* sp.	ND	PO_3^4-P 48 NH_4-N 30 NO_3-N 100 TP 63	Cañizares-Villanueva et al. (1994)
Microalgae digestate	*Chroococcus* sp.	0.8 g L^{-1}	NH_4-N 85 NO_3-N 77 TDP 89 sCOD 70	Prajapati et al. (2014)

ND: Not detected

3.4 Maintenance of cyanobacteria dominated culture in wastewater treatment systems

Cyanobacteria cultivation in such a variable media implies the competition with other microorganisms, especially with green microalgae. Thus, one of the challenges facing cyanobacteria cultivation in large scale open (e.g., high rate algal ponds (HRAP)) or close (e.g., photobioreactors (PBR)) systems is maintaining the dominance of these microorganism throughout long periods of time. Dominance of individual species typically occurs when that species possesses adaptations that provide an advantage over other species under certain environmental conditions (Sutherland et al., 2017).

As the most of the studies on cyanobacteria interactions and dominance have occurred in lakes and reservoirs, the first knowledge about this topic is based on those environments. Based on those studies, cyanobacteria growth has been related to complex interactions among biotic (competition for resources and selective predation by zooplankton grazers) (Reynolds, 1987) and abiotic factors, (e.g. light intensity, temperature, turbulence, pH and nutrients (Ahn et al., 2002; Dolman et al., 2012; Levich, 1996; Marinho and Azevedo, 2007; Reynolds, 1987)). Thus, some cyanobacteria are able to compete at wide range of water temperatures, light, oxygen, pH and nutritional conditions (Ahn et al., 2002; Chevalier et al., 2000; Reynolds, 1987; Unrein et al., 2010; Yamamoto and Nakahara, 2005). However, among all these factors, most of the studies agree that the nitrogen and phosphorus ratio (N:P), and their absolute concentration levels are the two key factors determining the competition capacity of cyanobacteria (Ahn et al., 2002; Cai et al., 2013; Cottingham et al., 2015; Levich, 1996; Levine and Schindler, 1999; Pick and Lean, 1987; Pinto and Litchman, 2010; Talbot and de la Noüe, 1993). Cyanobacteria behave in a similar way in wastewater treatment systems carried out in HRAP and PBR. In fact, the feasibility of controlling dominant cyanobacteria or microalgae in open or close wastewater treatment systems is still limited to few studies focusing on the relation among nutritional conditions in wastewater, competition with other microorganisms and the presence of cyanobacteria (Arias et al., 2017 (Chapter 4); Van Den Hende et al., 2016a). Hence, the factors controlling these competition relationships are still poorly understood (Park et al., 2011; Sutherland et al., 2017).

From the first studies in open ponds systems treating wastewater and in contaminated aquatic ecosystems an enormous diversity of microalgae, cyanobacteria, protozoa and small metazoan have been pointed out (Palmer, 1969). Cyanobacteria *Oscillatoria* sp. and *Phormidium* sp., together with some species of

green algae, such as, *Chlorella* sp., *Scenedesmus* sp., *Chlamydomonas* sp., *Stigeoclonium* sp., the diatom *Nitzschia* sp., and the protozoa *Euglena* sp., were stated as the most tolerant species to polluted environments (Oswald et al., 1953; Palmer, 1969). From those first studies and on, small and large scale wastewater treatment systems have been inoculated with pure or mixed cultures of photosynthetic microorganisms. In the case of cyanobacterial pure cultures, many species, especially filamentous species were cultivated in batch lab scale experiments in the studies of Cañizares-Villanueva et al., (1994), Pouliot et al., (1989) and Talbot and de la Noüe, (1993). However, in spite of being a relevant issue, the contamination of other microorganisms in the culture have not been mentioned in these studies.

In the last years, several studies using wastewater-borne mixed cultures in open and closed systems have been performed. The type of influent, total concentration of nutrients and type of operation of the system have defined the dominance of green algae or cyanobacteria in the culture as it can be observed in Table 3.2.

Table 3.2 Mixed liquor total inorganic nitrogen (TIN), total inorganic phosphorus (TIP) and the main dominating species in mixed microalgae/cyanobacterial cultures treating wastewater in high rate algal ponds (HRAP) and photobioreactor (PBR).

Dominant specie (percentage)	Wastewater	System	Hydraulic regime	TIN (mg L^{-1})	IP (mg L^{-1})	N:P ratio[a]	Reference
Geitlerinema sp. 60%, over *Pseudoanabaena* sp., *Ulothrix* sp., *Stiegeoclonium* sp., *Leptolyngbya* sp., *Planktolymgbya* sp..	Diluted digestate vinasse	HRAP	Continuous	49	1	109	Posadas et al. (2015)
Stigeoclonium sp. dominated *consortium*	Municipal	HRAP	Continuous	ND	10	-	Arcila and Buitrón (2016)
Stigeoclonium sp. dominated consortium	Municipal wastewater	HRAP	Continuous/ biomass recirculation	4.7	-	-	Gutiérrez et al. (2016a)
Micractinium pusillum and Pediastrum boryanum dominated consortium	Municipal wastewater	HRAP	Continuous	15	6	6	Sutherland et al. (2017)
Pseudoanabaena sp. (50%) dominated consortium	Centrate	HRAP	Continuous/ biomass recirculation	175	10	39	Posadas et al. (2017)

Table 3.2 (continue)

Dominant specie (percentage)	Wastewater	System	Hydraulic regime	TIN (mg L^{-1})	IP (mg L^{-1})	N:P ratio[a]	Reference
Chlorella sp. (60%), *Pseudoanabaena* (15%)	Digestate	HRAP	Continuous/ biomass recirculation	100	14.5	15	Marín et al. (2018)
Pseudanabaena sp. dominated consortium	Digestated diluted with secondary effluent	PBR	Semi-continuous	12.88	0.90	32	Arias et al. (2017) (Chapter 4)
Aphanocapsa sp. dominated consortium	Digestated diluted with secondary effluent	PBR	Semi-continuous	4.12	0.20	46	Arias et al., (2017) (Chapter 4)
Chlorella sp. dominated consortium	Digestate diluted with secondary effluent	PBR	Semi-continuous	32	1.4	51	Arias et al., (2017) (Chapter 4)
Scenedesmus sp. dominated consortium	Digestate diluted with secondary effluent	PBR	Semi-continuous	5.5	ND	-	Arias et al., (2018)
Chlorella sp. and *Scenedesmus* sp. dominated consortium	Municipal wastewater	HRAP	Semi-continuous	8	2	9	Cho et al. (2015)

Table 3.2 (continue)

Dominant specie (percentage)	Wastewater	System	Hydraulic regime	TIN (mg L^{-1})	IP (mg L^{-1})	N:P ratio[a]	Reference
Phormidium sp. dominated consortium	Aquaculture	PBR	Sequencing batch	14.2	3.6	9	Van Den Hende et al. (2014)
Eukaryotic microalgae dominated consortium with some species of *Phormidium* sp. and *Oscillatoria* sp.	Manure	PBR	Sequencing batch	37.1	2.1	39	Van Den Hende et al. (2014)
Stigeoclonium sp. dominated consortium	Chemical	PBR	Sequencing batch	44	2.69	36	Van Den Hende et al. (2014)
Aphanocapsa sp. dominated consortium	Secondary effluent	HRAP	Sequencing batch	2.99	2.17	3	Van Den Hende et al. (2016)
Green algae dominated consortium	Digestate diluted with secondary effluent	PBR	Sequencing batch	22	2	24	Arias et al. (2018b) (Chapter 5)
Aphanocapsa sp. dominated consortium	Digestate diluted with secondary effluent	PBR	Sequencing batch	13.82	0.89	34	Arias et al., (n.d.a) (Chapter 6)

[a] Ratio considering inorganic species given in molar bases
Not applicable

3.4.1 Role of nitrogen and phosphorus

Filamentous cyanobacteria *Pseudanabaena* sp. and colonial *Aphanocapsa* sp. are the genus most frequently cited in waste streams in last years, competing over green algae species such as *Chlorella* sp. and *Sigeoclonium* sp. These two cyanobacteria have demonstrated resistance to different types of wastewater and to develop in a wide rage of nutrients concentrations. For instance, in the study of Posadas et al., (2017), a 50% dominance of *Pseudanabaena* sp. was achieved in a HRAP with high N and P concentrations in the mixed liquor (TIN 100 mg L^{-1}, TIP 14.5 mg L^{-1}), while Arias et al. (2017) (Chapter 4) observed the same organisms N and P concentrations (TIN 12.88 mg L^{-1}, TIP 0.90 mg L^{-1}). Similarly, in the case of *Aphanocapsa* sp., the studies of Arias et al. (2017) (Chapter 4) and Arias et al., (n.d.a) (Chapter 6). found this organism dominating in TIN and TIP concentrations of 4.12-13.82 and 0.20-0.89 mg L^{-1}, respectively, while the study of Van Den Hende et al. (2016) achieved the majority of this organism in TIN and TIP values of 2.99 and 2.17 mg L^{-1}.

One remarkable property of these studies cited above is that, in spite of having different N and P concentrations, all presented a N:P ratio higher than 32:1. The only exception was the work of Van Den Hende et al. (2016). Therefore the N:P ratio was in general more than two times greater than the Redfield ratio (16:1 in molar bases), considered the standard ratio for microalgae growth (Redfield, 1958). On the contrary, in the study of Marchello et al. (2015), lower N with respect to P (TIN 0.13 mg L^{-1} and TIP 1.3 mg L^{-1}) (nitrogen limitation) promoted green algae *Chlorella* sp. competition over *Pseudanabaena* sp. This fact suggests that very low phosphorus concentration or phosphorus limitation with respect to nitrogen is a determinant fact on the predominance of cyanobacteria growing in wastewater systems. This ability to out-compete other microalgae under conditions of P limitation may be related to higher affinity to phosphorus than many other photosynthetic organisms (Monchamp et al., 2014; Mur et al., 1999). Due to their capacity to store that nutrient as polyphosphate and perform luxury uptake (Cottingham et al., 2015; Flores and Herrero, 2014).

3.4.2 Role of operational conditions

In spite that nutrients play a crucial role in species dominance in cultures, this factor cannot be considered as the only aspect favoring the competition of cyanobacteria. As observed in Table 3.2, some systems presented n the studies by Van Den Hende et al. (2014), Posadas et al. (2015), Arias et al. (2018) and Arias et al., (2017) also experienced high N:P ratios and low or lack of cyanobacteria presence. This implies that other factors might have prevented their dominance over green algae. In fact, the change in operational conditions, such as, hydraulic

regimes (continuous, sequencing batch, semi-continuous), solids retention time (SRT), hydraulic retention time (HRT), organic loadings, incorporation of a settling period or recirculation of biomass, also have impact on the microbial dynamics.

3.4.2.1 Hydraulic regimes (continuous, sequencing batch, semi-continuous)

In general, closed photobioreactors are considered the most appropriated operation for a better species control because open systems are more susceptible to changes in environmental conditions (i. e. pH and temperature). In fact, dominance of cyanobacteria has been mainly obtained in closed systems (Table 3.2). Only in the case of Posadas et al. (2017) and Van Den Hende et al. (2014) *Pseudanabaena* sp. dominance is achieved in HRAP, favoured by the hydraulic regime.

Continuous feeding and semi-continuous feeding are the most conventional hydraulic regimes used in closed and open systems. Continuous feeding procedure is the most utilized hydraulic regime in HRAP at full scale (Park et al., 2011). This strategy consists on a constant feeding of the reactor during time. Such type of feeding usually promotes the growth and predominance of green algae species as *Chlorella* sp. and *Stigeoclonium* sp., and usually show strong microbial variability depending on seasonality (Gutiérrez et al., 2016b; Passos et al., 2015). Such changing conditions make difficult the selection of determined species. On the other hand, photobioreactors operated in a semi-continuous feeding, consisting in a single feeding per day, results advantageous for microorganisms as cyanobacteria prsenting higher affinity to nutrients in comparison to many other photosynthetic organisms (Monchamp et al., 2014; Mur et al., 1999). A successful dominance of cyanobacteria under this type of operation have been achieved by Arias et al. (2017) under high N:P ratios and unlimited carbon conditions.

Another less conventional hydraulic regime in green algae/cyanobacteria based wastewater treatment systems is the case of sequencing batch operation. This type of hydraulic regime has been widely used for activated sludge systems (Kapley et al., 2007; Lee et al., 2015; Puigagut et al., 2005). In green algae/cyanobacteria cultures, it have been performed in both HRAP and PBRs (Van Den Hende et al., 2016a, 2014), and basically consists on a single feeding per day working at uncoupled SRT and HRT. This means that certain volume of mixed liquor is retired of the reactor, followed by a settling period and a posterior supernatant removal. Such operation provides several advantages over semi-continuous operation since nutrients loads can be easily manipulated, while maintaining a SRT adequate for specific species besides the fact that fast-uptake microorganisms are selected. Furthermore, the settling period included in this operational strategy encourage floc formation that settle faster, whereas unsettled cells are removed from the

supernatant (Valigore et al., 2012). Contrary to continuous and semi-continuous hydraulic regimes, which do not promote extensive spontaneous flocculation, this approach can improve subsequent harvesting processes. This operation implies an advantage for microorganisms able to easily form aggregates as cyanobacteria (Arcila and Buitrón, 2016; de Godos et al., 2014), while removing non-settling green algae species as *Chlorella* sp., *Scenedesmus* sp., *Chlamydomonas* sp. *Desmodesmus* sp., *Micractinium* sp., which present poor settling capacities (Van Den Hende et al., 2014).

3.4.2.2 Other operational factors

An important factor also affecting cyanobacteria selection is the solids retention time. In general, long solids retention time higher than 10 days are adequate for cyanobacteria cultures mainly conformed by filamentous species such as *Phormidium* sp. and *Pseudanabaena* sp. (Arias et al., 2017, n.d.a; Cromar and Fallowfield, 1997; de Godos et al., 2014; Posadas et al., 2014; Sutherland et al., 2017). Especially in HRAP cyanobacteria species usually appeared in winter season, when solids retention time increase and slow grow is registered. While SRT lower than 8 days promote species as *Scenedesmus* sp. *Chlorella* sp. and *Stigeoclonium* sp**.** (Arias et al., 2018; Gutiérrez et al., 2016a; Wood, 1987).

Other type of operation process which has been reported in literature is the recirculation from settlers to reactors in order to maintain biomass production and composition of microalgae in the reactors. So far, this type of operation only have improved the selection and maintenance of green algae (Gutiérrez et al., 2016a; Marín et al., 2018; Park et al., 2013, 2011), but failed when have been used for cyanobacteria dominance maintenance in HRAP (Benemann et al., 1980). However, in the recent study by Posadas et al., 2015 it was maintained the dominance of *Oscillatoria* sp. (90%) in a closed PBR during 90 days performing a recirculation flow of 4.2 L min^{-1} and long SRT. That dominance declined when that flow was increased and the SRT was decreased.

On the other hand, in the study by Sutherland et al. (2017), in spite that green algae species dominated the most of the time in a year long performance of a full-scale HRAP, the cyanobacteria *Microcystis aeruginosa* became the dominant species when the influent had the presence of this organism. This same situation was observed in the study by Cho et al. (2015). It is important to notice that this species belongs to a category of toxic cyanobacteria and is frequently found in cyanobacteria blooms.

3.5 Polymers accumulation in cyanobacteria

During the last decades cyanobacteria have received much attention as a rich source of bioactive compounds and have been considered as one of the most promising group of organisms to produce them (Abed et al., 2009). However, the use of waste streams as nutrients source reduces the range of biomass applications due to its possible contamination by various pollutants present in wastewater (Markou et al., 2014). Therefore, cyanobacteria grown in wastewater should mainly be used for the production of non-food applications such as for bioenergy, biofuels and bioplastics generation (Talbot and de la Noüe, 1993). In this case, cyanobacteria cultivation has arisen interest in the production of polyhydroxyalkanoates (PHAs) and carbohydrates, which are used as a bioplastic and a biofuel substrate, respectively. The main knowledge about cyanobacteria producing both polymers have been mainly studied in pure cultures but recently, investigations have been directed to the utilization of mixed non-sterile cultures.

3.5.1 Production of polyhydroxyalkanoate (PHAs) in pure cyanobacterial cultures

Polyhydroxyalkanoates (PHAs) are polyesters of 2-, 3-, 4-, 5- or 6-hydroxyacids currently receiving much attention because of their potential application as renewable and biodegradable plastics (Kessler and Witholt, 2001; Scheller and Conrad, 2005; Queirós et al., 2014). PHAs are accumulated as carbon storage from a wide variety of substrates such as renewable resources (sucrose, starch, cellulose, triacylglycerols), fossil resources (methane, mineral oil, lignite, hard coal), byproducts (molasses, whey, glycerol), chemicals (propionic acid, 4-hydroxybutyric acid) and carbon dioxide (Sudesh et al., 2000). These compounds are also produced and stored as intracellular granules by a large variety of prokaryotes. Most bacteria and cyanobacteria accumulate PHAs as carbon and energy storage material (Stal, 1992).

Cyanobacteria have special interest as low-cost PHAs producers because of their minimal nutrient requirements for growth and their capability of accumulating PHAs by oxygenic photosynthesis (Abed et al., 2009; Panda and Mallick, 2007; Wu et al., 2002). Several studies have already demonstrated the capacity of cyanobacteria to accumulate bioplastics, mainly in form of polyhydroxybutirate (PHB), but also as co-polymers of PHAs as poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (P(3HB-co-3HV)) (Bhati and Mallick, 2015). Similar to other prokaryotes, cyanobacteria assimilate and store nutrients for future consumption in conditions of essential nutrients excess (Bhati and Mallick, 2015). Table 3.3 shows that some cyanobacteria species have been assessed for the presence of PHAs,

mostly in form of PHB. Moreover, in most of the studies PHAs accumulation is species dependent and is a function of operational and nutritional conditions, such as parameters related to the growth phase (e.g. light-dark cycles, temperature, pH and nutrients limitation) and the type of carbon source (organic or inorganic carbon), Among all these conditions, nutrient limitation is the most common approach. As shown in Table 3.3, nutrients limitation, along with organic carbon sources (as glucose or acetate) have increased PHB content to values ranging from 10 to 46% in terms of dry cell weight (dcw) in cyanobacteria such as *Synechoccocus* sp., *Spirulina* sp. and *Nostoc* sp., and reaching the maximum 85% (dcw) of PHB in the specific case of *Aulosira fertilissima*. On the other hand, the use of inorganic carbon source with N or P limitation have increased PHB concentration up to 20% (dcw). In this case, the specific strain used in the study of Nishioka et al. (2001) (*Synechocystis sp. MA19*) was able to reach the highest accumulation of 60% of PHB.

Table 3.3 Summary of the maximum percentages of PHAs in cyanobacteria pure cultures performed in batch tests.

Species of cyanobacteria	Type of PHA	Content (% dry cell weight)	Days of incubation (d)	PHA optimization process	Reference
Synechococcus sp. *MA19*	PHB	18	9	Acetate addition/N deficiency	Miyake et al. (1997)
Spirulina platensis	PHB	10	15	Acetate/N deficiency	Jau et al. (2005)
Synechocystis sp.	PHB	38	10	Acetate/P deficiency/gas exange limitation	Panda and Mallick, 2007)
Nostoc muscorum	PHB	46	7	Acetate/glucose/dark incubation/N-P deficiency	Sharma et al. (2007)
Synechocystis sp. *PCC6803*	PHB	5	21	Acetate/pH/T/P deficiency	Panda and Mallick, (2007)
Aulosira fertilissima	PHB	85	5	Acetate/P deficiency	Samantaray and Mallick, (2012)
Aulosira fertilissima	P(3HB-co-3HV)	77	14	Fructose/valerate/P deficiency	Samantaray and Mallick, (2014)
Nostoc muscorum	P(3HB-co-3HV)	60	7	Glucose/acetate/N deficiency	Bhati and Mallick, (2015)
Synechocystis sp.	PHB	4	7	Inorganic carbon/N-P deficiency	Wu et al. (2001)
Spirulina subsalsa	PHB	7.45	15	Inorganic carbon/N deficiency/increased salinity	Shrivastav et al. (2010)
Nostoc muscorum	PHB	6.4	21	Inorganic carbon/N deficiency	Ansari and Fatma, (2016)
Anabaena cylindrical	PHB	0.2	21	Inorganic carbon/N deficiency	Lama et al. (1996)
Synechococcus sp. MA19	PHB	21	6	Inorganic carbon/N deficiency	Miyake et al. (1996)

Table 3.3 (continue)

Species of cyanobacteria	Type of PHA	Content (% dry cell weight)	Days of incubation (d)	PHA optimization process	Reference
Synechocystis sp. PCC6803	PHB	14.6	12	Inorganic carbon/N deficiency	Monshupanee and Incharoensakdi, (2014)
Synechocystis sp. PCC6803	PHB	13.5	12	Inorganic carbon/P deficiency	Monshupanee and Incharoensakdi, (2014)
Synechocystis salina	PHB	6	30	Inorganic carbon/P deficiency	Meixner et al. (2016)
Synechococcus sp. MA19	PHB	62	4	Inorganic carbon/P deficiency	Nishioka et al. (2001)
Spirulina maxima	PHB	0.7	4	Inorganic carbon/N deficiency	De Philippis et al. (1992)
Spirulina maxima	PHB	1.2	4	Inorganic carbon/P deficiency	De Philippis et al. (1992)
Nostoc muscorum	PHB	8.5	21	Inorganic carbon/P deficiency	Sharma and Mallick, (2005)
Spirulina platensis	PHB	3.5	60	Inorganic carbon/P deficiency	Panda et al. (2006)

3.5.2 Production of carbohydrates in pure cyanobacterial cultures

Carbohydrates are a wide category encompassing monosaccharides and glucose-based polymers (di-, oligo-, and poly- saccharides). These compounds act as major energy-storage and cell wall components in green algae/cyanobacteria cells. Cyanobacteria are able to synthesize glycogen (α- 1,4 linked glucan) during photosynthesis and deposit it as granules between the thylakoid membrane (Shively, 1988). Glycogen in cyanobacteria has the physiological function to provide maintenance energy for cell integrity and viability in dark periods (Suzuki et al., 2010).

The accumulation of carbohydrates by cyanobacteria have driven attention as an alternative to first generation biodiesel feedstock (e.g.. sugar, grain and oleaginous crops) that have significant drawbacks and limitations related to food versus fuel (Aikawa et al., 2012; Chen et al., 2013; Markou, 2012; Markou et al., 2013). On the contrary, the use of cyanobacterial or microalgal carbohydrates provide several advantages, including their high growth rate and high areal (or volumetric) productivity, and their capacity to grow in wastewater effluents. Besides their harvesting cycles are shorter (~1–10 days), compared with other feedstock (harvest once or twice a year). These advantages improve suitability for ethanol production (Harun et al., 2010). Furthermore, the particular use of glycogen from cyanobacteria compared with carbohydrates obtained from other higher plants or green algae is also advantageous (Nozzi et al., 2013), mainly due to their lack of a hard cellulose cell wall, which typically requires additional pretreatments and further expensive conversion processes to extract the product (Bohutskyi and Bouwer, 2013; Mendez et al., 2015; Nozzi et al., 2013).

Similar to the accumulation of PHAs, intracellular accumulation of carbohydrates in cyanobacteria has been promoted by the modification of environmental and cultivation factors, mainly N and P deficiency using inorganic carbon (Table 3.4). In fact, maximum carbohydrates content and periods of accumulation depends on the species. In general, the highest carbohydrates content is obtained under N limitation (65-70%). Observing for instance in *Spirulina maxima,* higher accumulation (70%) when submitted to N deficiency, but lower content when submitted to P deficiency in a period of 2.7 days (De Philippis et al. 1992). On the other hand, the strain of *Synechocystis sp. PCC 6803* achieved the higher carbohydrates content (36.8) in N deficiency but in a period of 12 days (Monshupanee and Incharoensakdi, 2014).

Table 3.4 Summary of the maximum percentages of carbohydrates in cyanobacteria pure cultures performed in batch tests.

Species of cyanobacteria	Maximum (% dcw)	Days of incubation (d)	Optimization process	Reference
Arthrospira platensis	65	3.5	Inorganic carbon/ N deficiency	Aikawa et al. (2012)
Spitulina platensis	65	ND	Inorganic carbon/ P deficiency	Markou et al. (2012)
Spitulina platensis	63	9	Inorganic carbon/P deficiency	Markou et al. (2013)
Synechocystis sp. PCC 6803	36.8	12	Inorganic carbon/N deficiency	Monshupanee and Incharoensakdi, (2014)
Synechocystis sp. PCC 6803	28.9	12	Inorganic carbon/P deficiency	Monshupanee and Incharoensakdi, (2014)
Spitulina maxima	70	2.7	Inorganic carbon/N deficiency	De Philippis et al. (1992)
Spitulina maxima	23	2.7	Inorganic carbon/P deficiency	De Philippis et al. (1992)
Arthrospira platensis	65	7	Inorganic carbon/N deficiency	Sassano et al. 2010)

3.5.3 Polyhydroxybutyrate and carbohydrates production in cyanobacterial cultures using wastewater substrates

Few studies focused on continuous processes of wastewater treatment and simultaneous polymer production. The first investigation so far,was the study of Samantaray et al. (2011), who grew *Aulossira fertilissima* with wastewater from an aquaculture system operating in batch condition. In that study, promising results of 200 mg L^{-1} of PHB were obtained after 15 days of incubation, while P-PO_4, N-NO_3 and N-NH_4 were completely depleted. Later, in the study by Meixner et al. (2016), *Syncechocystis salina* was cultivated in a pilot scale PBR (200 L) using diluted digestate (1:3 in distilled water) as feeding. After 40 days of incubation in batch conditions, this strain was able to accumulate 88 mg L^{-1} of PHB while removing 86%, 50% and 60% of COD, TN and TP, respectively. Recently, in the study of Arias et al., (n.d.b) a soil cyanobacterial dominated consortium was cultivated in a PBR (2.5) operated in a semi-continuous mode, fed with municipal wastewater. Results from this study evidenced that, with high HRT (10 days), biomass production of 0.05-0.07 mg L^{-1} d^{-1} 90% dominated by N-fixing cyanobacteria was achieved. This high HRT and low nutrients load achieved removals efficiencies of TN >95%, TP 35-78%, TOC >93% and TIC >82%. Moreover, N limitation in the culture stimulated a continuous carbohydrates accumulation up to 48%. However, PHB showed low percentages (<1%) of accumulation. It should be notice that in spite of being a

relevant issue, the predominance of the same species or the potential contamination during was only mentioned in this latter studies.

On the whole, promising results in terms of wastewater treatment and PHB and/or carbohydrates led to conclude that waste streams could be used as substrate to obtain valuable products from cyanobacteria without the additional costs related to growth medium.

3.5.4 Polyhydroxybutyrate and carbohydrates production in mixed wastewater-borne cyanobacterial cultures.

As previously mentioned, the potential production of PHB and carbohydrates from cyanobacteria have mostly been based on pure strains fed with sterile growth mediums (Miyake et al., 2000; Nishioka et al., 2001). These facts, associated with high operational costs, have increased polymer prices, prevented further scale-up of the technology and limited its marketability. In this respect, a more sustainable alternative approach for the production of PHB and carbohydrates could be the use of mixed wastewater-borne cultures dominated by cyanobacteria. Although previous works about PHB production in pure cultures have used organic carbon, the use of this substrate in mixed cultures would prejudice cyanobacterial dominance and increase heterotrophic bacteria activity. Thus, the use of inorganic carbon is clearly the option in the case of mixed cultures. Furthermore, the use of inorganic carbon is nowadays significantly attractive due to the worldwide concern with the CO_2 impact in climate change. Recent studies have tested the availability of cyanobacteria dominated cultures, instead of pure cultures, for producing PHBs and carbohydrates using inorganic carbon as substrate. In the study by Arias et al. (2018c), mixed wastewater-borne cyanobacteria were submitted to N and P deficiency and different photoperiods (24 h light, 12h light dark) during 15 days of incubation. The maximum PHB content obtained in that study was 6.5%, with 75% (dcw) of carbohydrates in 12 days when submitted to N deficiency and 12h light/dark. In a continuing study by Arias et al. (2018a), this long time of incubation was reduced by employing the carbon feast and famine strategy, mostly used in bacterial fermentation systems (Bengtsson et al., 2008; Oliveira et al., 2017). This strategy consisted in transient carbon availability during a first stage process (cultivation period) along with nutrients deficiency. The use of this strategy in the first stage led to 3.8% dcw of PHB and 53% dcw of carbohydrates in less than 1.2 days of incubation in a second stage (consisting in batch tests).

Concerning PHB content, results obtained with the wastewater-borne cyanobacteria in both studies are within the range of PHB production from pure cultures using inorganic carbon as substrate (0.2-8.5%) (De Philippis et al., 1992;

Lama et al., 1996; Meixner et al., 2016; Miyake et al., 2000; Monshupanee and Incharoensakdi, 2014; Panda and Mallick, 2007). In exceptional cases, a slightly higher accumulation percentage was obtained, but after a longer periods of time (21 days) (Sharma and Mallick, 2005). Only the studies of Miyake et al. (1996) and Nishioka et al. (2001) were able to obtain higher PHB content (55%) in only 5 days with specific strains of cyanobacteria *Synechococcus* sp. MA19 submitted to nutrients limited conditions with inorganic carbon as the carbon source (Table 3.3). With regards to carbohydrates content, the percentages obtained with wastewater-borne cyanobacteria by Arias et al. (2018c) in N limitation are similar to those obtained in most of the previous studies carried out in pure cultures of *Spirulina*/*Arthrospira* sp. (Table 3.4) (De Philippis et al., 1992; Markou et al., 2013; Sassano et al., 2010).

It is important to highlight that, although in the study by Arias et al. (2018a) percentages of both PHB and carbohydrates are slightly lower, they correspond to only 1.2 day of incubation. This means that the introduction of feast and famine strategy during the cultivation phase of cyanobacteria can reduce the prolonged periods necessary to accumulate both polymers, resulting in incubation even shorter than the ones required by pure cultures (Table 3.4).

3.6 Aspects to consider to produce polymers from cyanobacteria cultivated in wastewater and future approach

Through the results of several studies analyzed and discussed in this review, it is clear that the production of polymers from cyanobacteria cultivated in wastewater is a promising issue, but still has challenges to be overcome prior its scale-up.

The first one is the possibility of maintaining a cyanobacteria dominated culture during prolonged periods. The key for the control and maintenance of the culture is to establish which is the most adequate medium for growing cyanobacteria and hinder other microorganisms. The control of nutrients concentrations and ratio, as well and operational conditions are crucial in order to maintain cyanobacteria dominance in mixed culture, and also to prevent any possible contamination. In term of nutrients, the best approach to select cyanobacteria seems to be the maintenance of high N:P ratios (>32:1) in the culture. Indeed, cyanobacteria high affinity to P could increase their dominance over green algae species as *Chlorella* sp. and *Stigeoclonium* sp.. Reviewing all the different influent sources, it seems that the utilization of influents with stable characteristics (e.g. digestate) or low nutrients sources (e.g. secondary effluents) can be the most appropriate nutrient source for cyanobacteria selection. Indeed, in such influents nutrients loading can be more easily controlled than in other more

variable sources (e.g. municipal effluents). Another factor to consider are operational conditions. Results suggest that the sequencing batch operation is appropriate for the selection of easy settleable cyanobacteria. However, the aspects concerning nutrients loads and adequate solids retention time should be carefully tested, mostly due to the appearance of other easy settleable microorganisms like *Stigeoclonium* sp. or *Pediastrum* sp. .

Another bottleneck concerns carbohydrates and PHB production from mixed wastewater-borne cyanobacteria. Results provided in this state of the art evidence that mixed cyanobacterial cultures can compete with pure cultures. Indeed, the occurrence of both polymers has been confirmed in mixed cultures dominated by several species. The utilization of this kind of cultures can avoid the high production costs derived by controlled sterile conditions used in pure cultures, which still limit their large scale production. On the whole, mixed cyanobacteria-bacteria growing in wastewater treatment systems for polymers production offers an alternative to significantly enhance the environmental and economic benefits of bioplastics and biofuels production. Notwithstanding, the efficient production of PHAs (bioplastics) and carbohydrates (biofuel substrate) using cyanobacteria cultivated in wastewater is still limited to few studies carried out at lab and pilot scale under controlled conditions. Further research needs to focus on scaling-up the technology and carefully analyze the effect of outdoor conditions (e.g. direct sunlight and temperature). If is demonstrated the viability of using cheap wastewater sources as feedstock for polymers in natural conditions, then this alternative would constitute a sustainable approach for bioplastics or biodiesel.

Chapter 4

Selection of cyanobacteria in a semi-continuous reactor

The contents of this chapter were adapted from the publication: Arias, D.M., Uggetti, E., García-Galán, M.J., García, J., 2017. Cultivation and selection of cyanobacteria in a closed photobioreactor used for secondary effluent and digestate treatment. Sci. Total Environ. 587–588, 157–167. doi:10.1016/j.scitotenv.2017.02.097

4.1 Abstract

The main objective of this chapter was to select and grow wastewater-borne cyanobacteria in a closed photobioreactor (PBR) inoculated with a mixed consortium of microalgae. The 30 L PBR was fed with a mixture of urban secondary effluent and digestate, and operated in semi-continuous mode. Based on the nutrients variation of the influent, three different periods were distinguished during one year of operation. Results showed that total inorganic nitrogen (TIN), inorganic phosphorus concentration (IP), phosphorus volumetric load (L_V-IP) and carbon limited/non-limited conditions led to different species composition, nutrients removal and biomass production in the culture. High TIN/IP concentrations in the influent (36 mg N L^{-1}/3 mg P L^{-1}), carbon limitation and an average L_V-IP of 0.35 mg P $L^{-1}d^{-1}$ were negatively related to cyanobacteria dominance and nutrients removal. On the contrary, cyanobacteria predominance over green algae and the highest microbial biomass production (averaging 0.084 g Volatile Suspended Solids (VSS) $L^{-1}d^{-1}$) were reached under TIN/IP concentrations of 21 mg N L^{-1}/2 mg P L^{-1}, no carbon limitation and an average L_V-P of 0.23 mg IP $L^{-1}d^{-1}$. However, although cyanobacteria predominance was also favored with a L_V-IP 0.15 mg$L^{-1}d^{-1}$, biomass production was negatively affected due to a P limitation in the culture, resulting in a biomass production of 0.039 g VSS $L^{-1}d^{-1}$. This study shows that the dominance of cyanobacteria in a microalgal cyanobacterial community in an agitated PBR using wastewater as nutrient source can be obtained and maintained for 234 days. These data can also be applied in future biotechnology applications to optimize and enhance the production of added value products by cyanobacteria in wastewater treatment systems.

4.2 Introduction

Cyanobacteria (blue-green algae) are prokaryotic aerobic photosynthetic microorganisms with a long history of adaptive and evolutionary diversification, which has also conferred them the capacity to synthesize a large variety of bioactive compounds and other valuable by-products (Mimouni et al., 2012). During the last two decades, the industrial production of cyanobacteria has arisen special interest since they have been identified as one of the most promising group of organisms for the isolation of novel and biochemically active natural products such as antibiotics, antifungal or antivirus (Abed et al., 2009; Shalaby, 2011). Unlike eukaryotic algae, cyanobacteria have also the potential to assimilate and store glycogen, cyanophycin, polyphosphates and polyhydroxyalkanoate (Stal, 1992).

Studies related to the production of cyanobacteria and their polymers generally employ pure or genetically modified cultures (Miyake et al., 2000). However, cultivation of cyanobacteria is not easy, even if pure cultures are submitted to strictly controlled processes using sterile medium substrates. In most of the cases contamination with other types of algae, in particular green algae (*Chlorophyta*), cannot be avoided (Drosg et al., 2015). Moreover, the use of these strictly controlled pure cultures in industrial applications lead to high production costs, and subsequent relatively expensive products (Samantaray and Mallick, 2012). Indeed, medium and pure culture expenses corresponds to 33% of the operational costs (Piccolo, 2012)).

However, in the case of non-food applications such as the production of bioenergy or biofuels production, or bioplastics generation, a strict sanitary control is not required. In these cases, an alternative approach for the production of cyanobacteria could be the use of wastewater-borne cyanobacteria cultures, using non-sterile waste streams as substrate. In fact, wastewater treatment technologies are considered as the most promising and sustainable alternative to reduce additional production costs associated with nutrients and water in cyanobacteria cultures (Samantaray et al., 2011; Zhou et al., 2012). Indeed, the use of inexpensive substrates requiring lower energy inputs and cheaper equipment could reduce the production costs compared to pure culture processes. However, maintaining a dominant population of cyanobacteria in wastewater treatment systems is still limited to a few successful case-studies ((Van Den Hende et al., 2016a, 2016b)and therefore remains as a challenging task (de Godos et al., 2014). Certainly, one of the problems most frequently encountered is that of cyanobacteria being out-competed by green algae in wastewater borne cultures; the factors that control these competence relationships are not well understood.

Most of the information available regarding the different factors that control growth and predominance of cyanobacteria found in literature comes from fresh water ecosystems, such as lakes and reservoirs. Cyanobacteria development in these environments depends on complex interactions among a great number of physical and chemical factors such as light intensity, temperature, turbulence, pH, and other biotic factors (Ahn et al., 2002; Dolman et al., 2012; Levich, 1996; Marinho and Azevedo, 2007; Reynolds, 1987). However, among all these factors, most of the studies agree that the nitrogen and phosphorus ratio (N:P) and their absolute concentration levels are the two key factors determining the competition capacity of cyanobacteria (Cai et al., 2013; Cottingham et al., 2015; Levich, 1996; Levine and Schindler, 1999; Pinto and Litchman, 2010; Talbot and de la Noüe, 1993). In this context, because cyanobacterial blooms frequently develop in eutrophic water

ecosystems, it was firstly assumed that they required high N and P concentrations (Pick and Lean, 1987; Reynolds, 1987). However, later studies demonstrated that their dominance was related to a higher affinity than that of many other photosynthetic organisms for N and P (Monchamp et al., 2014; Mur et al., 1999). In addition to this high nutrient affinity, cyanobacteria have a substantial storage capacity for both these nutrients (Flores and Herrero, 2014), and some types of cyanobacteria have the capacity of fixing atmospheric N (Levine and Schindler, 1999; Schindler, 1977). This way, they can out-compete other microalgae under conditions of N and/or P limitation (Cottingham et al., 2015; Kim et al., 2007; Marinho and Azevedo, 2007). For this reason, cyanobacteria dominance has been reported under a wide range of N:P ratios, from 0.5:1 (N limitation) to >64:1 (P limitation) (Chislock et al., 2013; Levine and Schindler, 1999; Pick and Lean, 1987; Stocknerl and Shortreed, 1988). Even though natural concentrations of nutrients found in fresh water ecosystems are usually at least three orders of magnitude lower than those found in urban, agricultural or industrial wastewaters (de la Noüe et al., 1992). In this context, higher nutrient concentrations in wastewater promote higher algal photosynthesis, oxygen production and biomass concentration (Ahmadi et al., 2005). For instance, in the study of Beaulieu et al. (2013) in lakes ecosystems, Total Inorganic Nitrogen TIN=1.167 and inorganic phosphorus concentration IP=0.107 mgL^{-1} corresponds to 2.15 mgL^{-1} of biomass concentration, while in the study of Van Den Hende et al. (2016a) using open ponds with a secondary effluent from industrial wastewater (TIN=9.31 and IP=2.37) reach an average of 668 mgL^{-1}. Therefore, it seems reasonable that cyanobacteria selection in wastewater cultures should be conducted considering the same determining factors, especially in terms of nutrients interaction. In the field of wastewater technology, several species of cyanobacteria have been successfully cultivated at experimental scale using both primary and secondary treated wastewaters (urban and industrial) as feedstock (Kamilya et al., 2006; Renuka et al., 2013; Van Den Hende et al., 2016a; Vijayakumar, 2012b). The use of anaerobic digestate as nutrient source has also been evaluated (Markou and Georgakakis, 2011). However its use is conditioned by their high ammonium (NH_4^+), organic carbon and solids content, and most the studies included a dilution with tap water (Prajapati et al., 2014). Hence, the use of digestate diluted in another minor nutrient source (e.g. secondary effluent) could provide enough nutrients to fullfil the requeriments of cyanobacteria production and the possibility of their selective growth.

All in all, the objective of this chapter was to select and grow wastewater-borne cyanobacteria from a consortium of microalgae in a closed photobioreactor (PBR) fed with a mixture of secondary effluent and digestate. This work aimed to a dual benefit, considering the concomitant treatment of these waste streams. The

study was carried out throughout 1 year in order to consider all the potential variations and variables affecting the PBR during a long term operation and therefore obtaining a realistic knowledge of the system functioning.

4.3 **Methodology**

4.3.1 Experimental set-up

The closed PBR was located indoors and consisted in a cylindrical tube made of polymethyl methacrylate (5 mm thickness) with a total volume of 35.8 L and a culture working volume of 30 L (Fig. 4.1). It was initially filled with 20 L of tap water and 10 L of inoculum obtained from the mixed liquor of an experimental high rate algal pond (HRAP) (1.54 m^2, 470 L) treating primary settled urban wastewater. A detailed description of the high rate algal pond system can be found elsewhere (García et al., 2006; Gutiérrez et al., 2015). The inoculum (105 mg TSS L^{-1}) consisted in a community of microalgae, bacteria, protozoa and small metazoa. Microscope observations (not shown) indicated that most of the biomass corresponded to microalgae, which is in accordance with previous publications (García et al., 2006; Gutiérrez et al., 2016b; Nurdogan and Oswald, 1996). Microalgae consortium was mostly composed by green algae (genus *Chlorella* and *Stigeoclonium*) and cyanobacteria (cf. *Oscillatoria*).

The culture in the PBR was continuously maintained in alternate light:dark periods of 12 h. Illumination during the light phase was supplied by a 600 W external metal halide lamp equipped with a digital ballast (model 5500k, Sunmaster, USA) placed at a 70 cm distance from the PBR. This lamp, working with a wavelength spectrum 380-780 promoting the photosynthetically active radiation, provided approximately 14500 lux (204 μmol m^{-2} s^{-1}), which corresponds to the irradiance recommended to increase algal activity (200-400 μmol m^{-2} s^{-1}) (Huesemann et al., 2016; Wang et al., 2015). Due to the lamp type (cool blue light), culture temperature was minimally influenced during the experimental time, ranging from 22 to 29 ºC throughout the study and changing 1 to 2 °C between light:dark cycles. Complete culture mixing in the PBR was achieved by continuous air injection at a flow of 10 L min^{-1} and a pressure of

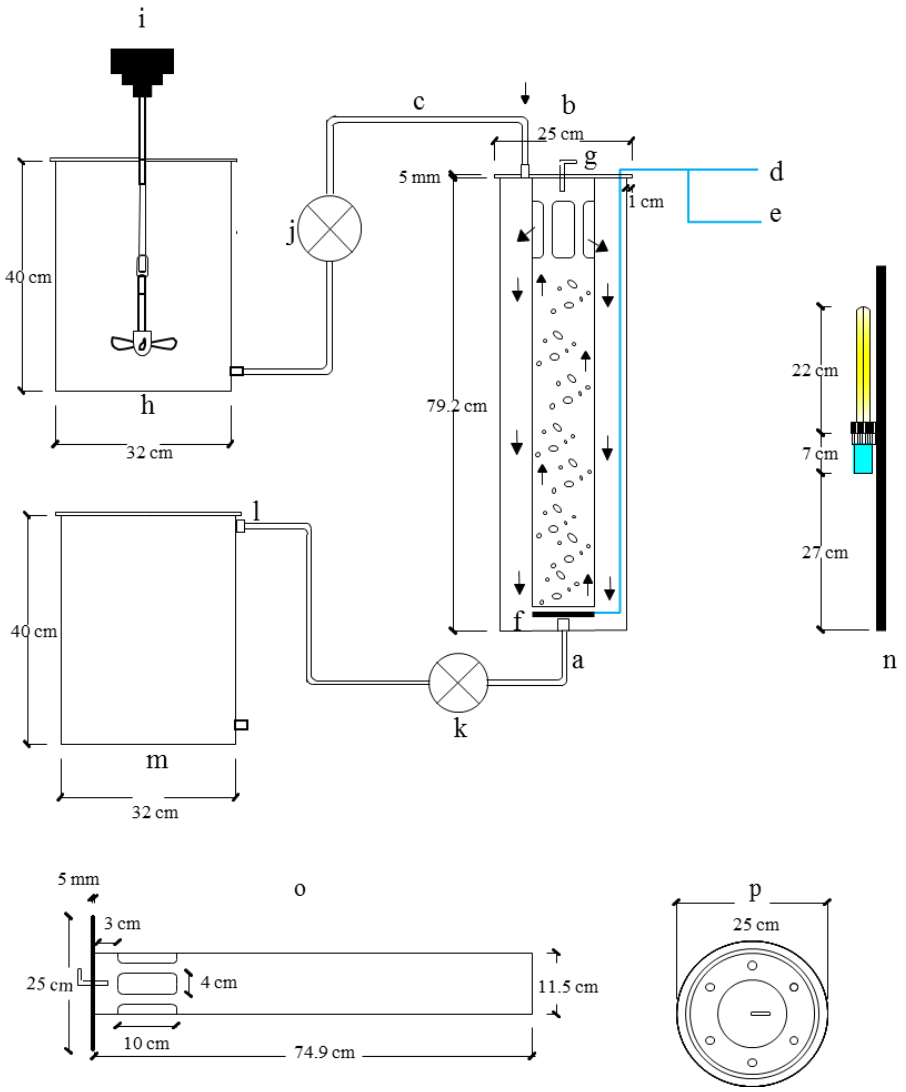

Fig. 4.1 Schematic diagram of the photobioreactor (PBR) set-up: a) PBR body with the internal cylinder inside, b) PBR cover, c) feeding tube, d) air tube, e) CO2 tube, f) air sparger, g) gas outlet, h) feeding tank, i) electric stirrer, j,k) pumps, l) effluent tube, m) harvesting tank, n) external lamp, o) detail of internal cylinder, p) top view of the photobioreactor. Arrow's path follows the continuous culture movement inside the PBR.

0.034 MPa, using a 105 W air compressor (model ACQ-012, JAD, China). Air was distributed to the culture with an air sparger placed on the bottom of the PBR that ensured complete mixing of the culture, creating a circulation from the internal methacrylate cylinder to the external part (arrow's path in Fig. 4.1). Additionally, the total capacity of the PBR body allowed a free space on the surface connected to the degassing port placed on top. The pH of the culture was constantly controlled in two of the periods by means of CO_2 (100% v) (Carburos Metalicos, Spain) injection when necessary, at a flow of 0.3 L min^{-1} and a pressure of 0.3-0.5 MPa. The pH set point value was 8.3, ranging from 7.5 to 8.9 (note that values higher than 8.3 were reached until CO_2 had a homogenous contact with the medium culture). This pH set point of 8.3 was selected based on previous literature that reported a pH preference of cyanobacteria ranging from 8 to 9 (Ahn et al., 2002; Reynolds, 1987; Unrein et al., 2010; Yamamoto and Nakahara, 2005). Furthermore, different experiments demonstrated that when the pH reached values around 10, the green algae *Scenedesmus* sp. outcompeted cyanobacteria (Arias et al., 2018). Both air and CO_2 were injected in the PBR similarly.

The culture was fed on a semi-continuous mode (once a day) and operated with a hydraulic retention time (HRT) and solids retention time (SRT) of 10 days. Thus, each day at the end of the dark phase, 3 L of the culture were harvested (effluent) and collected in a plastic harvesting tank (32 L). This volume was then restituted by 3 L of digestate diluted in secondary effluent from the HRAP in a ratio of 1:50. Digestate was obtained daily from a lab-scale anaerobic digester with a capacity of 2 L and a useful volume of 1.5 L (flow of 0.075 $L^{-1}d^{-1}$)., operated under mesophilic conditions (35°C) and with a SRT (or HRT) of 20 days. This digester was fed with thickened microalgae biomass obtained from the HRAPs aforementioned. Detailed characteristics and operation conditions of the digester are described elsewhere (Passos and Ferrer, 2014; Passos et al., 2013). The secondary effluent was obtained from the same HRAP (after gravity biomass separation in a settler (see Passos et al., 2014)). Table 4.1 shows the properties of the digestate, the secondary effluent and the influent PBR mixture. The dilution ratio of 1:50 was chosen to low the Total Ammoniacal Nitrogen (TAN) to values under 15mgL^{-1} in the influent. Digestate and secondary effluent were collected daily in enough quantity and mixed in a plastic feeding tank (32 L) with an electric stirrer (620 rpm, 1600 W, Rubi, UK) during the harvesting process (approximately 5 min) and posteriorly pumped into the PBR.

CO_2 injection control, culture feeding and harvesting pumps, as well as temperature, light and pH monitoring were controlled and registered by using LabVIEW ® software.

Table 4.1 Average (standard deviation) of the main quality parameters of the digestate, secondary effluent and the influent PBR (mixture of digestate and secondary effluent) during the three experimental periods (n = 15-20). LOD is limit of detection.

Parameter	Period 1			Period 2			Period 3		
	Digestate	Secondary effluent	PBR influent[a]	Digestate	Secondary effluent	PBR influent[a]	Digestate	Secondary effluent	PBR influent[a]
pH	-	-	7.9 (0.3)	-	-	8.1 (0.4)	-	-	8.2 (0.7)
TSS (g L^{-1})	13.4 (8.5)	-[b]	0.26 (0.17)	18.5 (4.2)	-[b]	0.39 (0.084)	18.5 (7.5)	-[b]	0.37 (0.15)
VSS (g L^{-1})	12.3 (6.5)	-[b]	0.24 (0.13)	14.1 (5.1)	-[b]	0.28 (0.11)	12.5 (3.5)	-[b]	0.25 (0.69)
Alkalinity (mg $CaCO_3$ L^{-1})	-	-	153 (40.6)	-	-	147 (22.6)	-	-	290 (66.2)
TAN (mg L^{-1}) [c]	587 (96.2)	0.21 (0.84)	11.74 (1.93)	337 (44.5)	0.52 (0.67)	6.76 (0.89)	245 (110)	0.26 (0.8)	4.9 (2.2)
N-NO_2^- (mg L^{-1})	<LOD	1.44 (0.69)	1.44 (0.69)	<LOD	0.09 (0.22)	0.09 (0.22)	<LOD	1.17 (0.82)	1.17 (0.82)
N-NO_3^- (mg L^{-1})	<LOD	22.55 (6.5)	22.55 (6.55)	<LOD	14.40 (4.57)	14.37 (4.57)	<LOD	20.91 (6.12)	20.91 (6.12)
TIN (mg L^{-1})[d]	-	-	35.73 (5.62)	-	-	21.22 (4.43)	-	-	26.98 (8.5)
IP (mg L^{-1})	<LOD	2.96 (0.83)	2.96 (0.83)	<LOD	2.19 (0.65)	2.19 (0.65)	<LOD	1.41 (0.44)	1.41 (0.44)

[a]*PBR influent was prepared as a dilution of digestate within secondary effluent in a 1:50 ratio.*
[b]*TSS and VSS in the secondary effluent corresponded to values <0.02 g L^{-1} during the three periods.*
.[c]*TAN: Total ammoniacal nitrogen.*
[d]*TIN: total inorganic carbon.*

4.3.2 Experimental procedure

The PBR was continuously operated and periodically monitored during 1 year from March 2015 until April 2016. In order to evaluate its performance, this year was divided in three periods ranging from 50 to 90 days. In these periods the quality of the secondary effluent and the digestate changed according to seasonal variations of the HRAP and microalgae anaerobic digestion performance, as previously described by García et al. (2000) and Passos et al. (2014). Period 1 extended from May to July. During this period, CO_2 injection was unnecessary as pH values remained close to the set point value. After this period the culture was fed only once per week during the month of August in order to decrease nitrification activity in the culture by reducing nutrients availability. After this month, feeding conditions were reestablished. Period 2 extended from September to November. In this period, CO_2 was injected to maintain the pH near the set point value, as well as during Period 3 (comprising the months February to April). The months between period 2 and 3 were operated in the same conditions but they were not monitored.

4.3.3 Analytical methods

All parameters were determined in triplicate and analyzed from the PBR influent (digestate and secondary effluent) and effluent (equivalent to the mixed liquor of the culture). All samples were taken and analyzed at the end of the dark phase. Analyses for inorganic phosphorus (IP), nitrite ($N\text{-}NO_2^-$), nitrate ($N\text{-}NO_3^-$), Total Ammoniacal Nitrogen (TAN) and alkalinity were performed twice a week (two different days). IP (measured as orthophosphate ($P\text{-}PO_4^{3-}$)), $N\text{-}NO_2^-$, $N\text{-}NO_3^-$ concentrations were measured using an ion chromatograph DIONEX ICS1000 (Thermo-scientific, USA). TAN (consisting on the sum of $N\text{-}NH_4^+$ + $N\text{-}NH_3^-$) was determined using the colorimetric method indicated in Solorzano (1969). Alkalinity was determined using the titration method *2320 B* of Standards Methods (APHA-AWWA-WPCF, 2001). Total inorganic nitrogen (TIN) was calculated as the sum of $N\text{-}NO_2^-$, $N\text{-}NO_3^-$ and TAN. TIN and IP were used to calculate N:P ratios, as they represented the direct available N and P for cyanobacteria (Pick and Lean, 1987).

Applied P volumetric load (L_V-P) (given in mg IP $L^{-1}d^{-1}$) was calculated following equation 1:

$$\mathrm{Lv-P} = \frac{Q*IP}{V} \quad [1]$$

where Q is the flow ($L^{-1}d^{-1}$), IP is the influent concentration (mg P L^{-1}) and V (L^{-1}) is the volume of the PBR.

Total suspended solids (TSS) and volatile suspended solids (VSS) were measured once a week in the influent following the gravimetric method 2540 C and 2540 D in Standard Methods (APHA-AWWA-WPCF, 2001). Biomass concentration in the PBR culture was measured 3-5 times per week through turbidity. At the start of the study, a 4-points calibration curve was performed in triplicate between turbidity and dry weight (0.460-1.45 g TSS L^{-1}, corresponding to 0.4-1.2 g VSS L^{-1}), having a correlation coefficient $R^2 = 0.997$. This correlation was checked once a week by comparing turbidity values with TSS and VSS concentrations. Through this data, biomass production in the PBR (given in g VSS $L^{-1}d^{-1}$) was estimated following equation 2:

$$Biomass\ production = \frac{Q*VSS}{V} \quad [2]$$

where Q is the flow ($L^{-1}d^{-1}$), VSS is the biomass concentration in the PBR (g L^{-1}) and V (L^{-1}) is the volume of the PBR.

Chlorophyll *a* was measured in the culture once a week using the procedure 10200 H described in the Standard Methods (APHA-AWWA-WPCF, 2001). Turbidity and dissolved oxygen (DO) were measured with a turbidity-meter (Hanna, USA) and a dissolved oxygen-meter (Thermo-scientific, USA) respectively. DO was measured directly in the PBR, inserting the sensor in the mixed liquor.

Culture pH and temperature were continuously measured with probes inside the PBR. Both pH and temperature were measured with a pH meter with a temperature sensor (Mettler Toledo, USA). Light intensity was also continuously measured with a probe attached to the PBR and determined by means of a light meter (Hanna (USA)). Results of the probes were continually stored in periods of 2-3 minutes in a computer with the software LabVIEW®.

Effluent samples were examined under an optic microscope (Motic, China) once a week for qualitative evaluation of microalgae populations and to determine the cyanobacteria abundance. Note that microalgae were uncountable due to the presence of flocs. The microscope was 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 (Bourrelly, 1985; Palmer, 1962) as well as a database of cyanobacteria genus (Komárek and Hauer, 2013).

4.4 **Results**

The experiment focused in the selection of cyanobacteria from an initial mixed green algae-cyanobacteria consortium. It should be taken into account that the term "selection" is referred to the transformation of the culture into a culture "mostly" dominated by cyanobacteria species, not a pure cyanobacteria culture. This transformation from the initial consortium was directly related to the characteristics summarized in Table 4.1 for the different three periods, thus leading to different conditions in the PBR throughout the experiment (Table 4.2). However, DO, temperature and pH had quite similar average values and ranges during the three periods (note that only the pH was controlled). Therefore, main differences were observed in the biomass concentration and nutrients content in the culture.

During the first days of operation biomass concentration increased very quickly, reaching a value of 0.37 g VSS L^{-1} after only 6 days (Fig. 4.2a) and indicating that the digestate mixed with the secondary effluent was an appropriate feed medium to support photosynthetic growth. During the following days, the biomass remained relatively constant with an average concentration of VSS 0.49 g L^{-1} (Chlorophyll *a* of 3.9 mg L^{-1}) and a production rate of 0.048 g L^{-1} d^{-1}. The variations observed in the measured concentrations were caused by the occasional detachment of biofilm growing on the walls of the PBR. In this first period, the initial mixed consortia turned into a culture mainly composed by the green algae *Chlorella* sp. with the presence of filamentous cyanobacteria cf. *Oscillatoria* sp. (Fig. 4.3). This microbial community remained the same from the first days of operation till the end of the period.

Table 4.2 Average (standard deviation) of the main quality parameters of the effluent of the photobioreactor during the three experimental periods. *n*= 15-20 except for SST, SSV and biomass production (*n*= 37-60) and temperature and pH (*n*= 50000-75000).

	Period 1		Period 2		Period 3	
Parameter	Average	Range	Average	Range	Average	Range
Temperature (ºC)	26.1 (1.9)	20.0-30.7	26.2 (1.5)	19.3-30.1	23.6 (2.9)	17.1-27.4
pH	8.1 (2.3)	7.5-8.6	8.4 (1.4)	7.8-8.8	8.6 (1.1)	7.1-8.4
DO (mg L^{-1})	8.8 (1.8)	4.5-7.8	6.8 (1.2)	3.9-8.8	6.4 (1.5)	4-7.5
TSS (g L^{-1})	0.52 (0.26)	0.035-2.79	1.05 (0.84)	0.031-2.55	0.49 (0.29)	0.062-1.41
VSS (g L^{-1})	0.41 (0.24)	0.013-1.503	0.84 (0.71)	0.011-2.36	0.39 (0.23)	0.049-1.14
Biomass production (g VSS L^{-1} d^{-1})	0.041 (0.024)	0.013-0.15	0.084 (0.06)	0.011-0.23	0.039 (0.02)	0.0049-0.11
Chlorophyll *a* (mg L^{-1})	3.9 (1.3)	1.7-5.6	4.5 (1.2)	3.1-6.2	3.7 (1.7)	1.2-6.7
Alkalinity (mg $CaCO_3$ L^{-1})	92.5 (18.5)	62.7-136.3	112.3 (15.5)	95.0-143	224.4 (42.9)	174.2-330.1
TAN (mg L^{-1})a	0.30 (0.11)	0.10-0.50	0.30 (0.27)	0.03-1.02	0.16 (0.21)	0.01-0.85
N-NO_2^- (mg L^{-1})	0.41 (0.48)	<LOQ-1.39	0.09 (0.22)	<LOQ-0.70	0.06 (0.07)	<LOQ-0.19
N-NO_3^- (mg L^{-1})	31.18 (5.01)	23.31-42.19	12.49 (4.82)	5.01-18.90	3.95 (1.25)	0.77-5.45
TIN (mg L^{-1})[b]	31.94 (5.01)	23.41-44.08	12.88 (4.82)	5.04-20.62	4.12 (1.33)	0.78-6.54
IP (mg L^{-1})	1.36 (0.70)	0.30-3.13	0.90 (0.52)	<LOQ-1.98	0.20 (0.25)	<LOQ-0.75

.[a]*TAN: Total ammoniacal nitrogen.*

[b]*TIN: total inorganic nitrogen.*

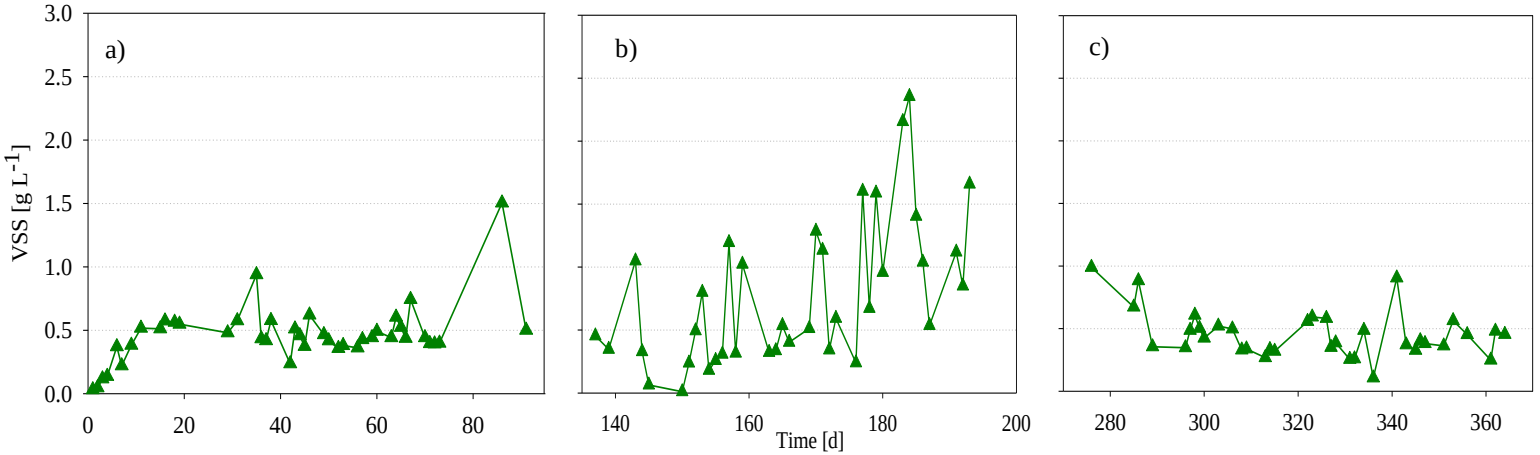

Fig. 4.2 Changes in the biomass contained in the PBR during the three periods. a) period 1, b) period 2, c) period 3. Biomass is given as volatile suspended solids (g VSS L^{-1}).

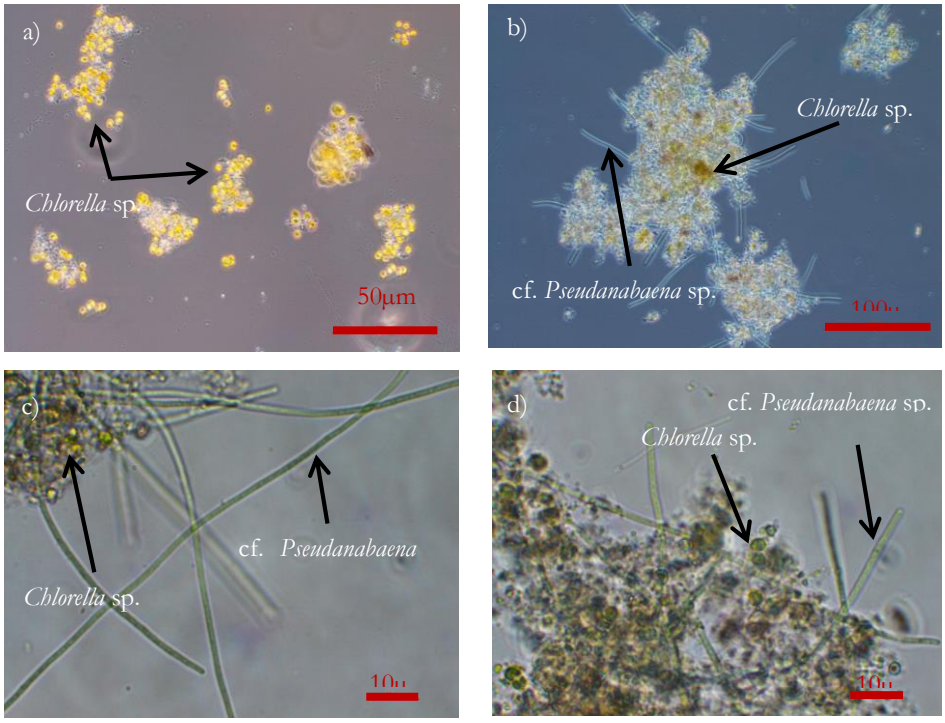

Fig. 4.3 Microscopic images illustrating the dominant algae during period 1. a) Initial mixed culture dominated by *Chlorella* sp. immersed in flocs with some dispersed individuals observed in phase contrast microscopy (400X); b) Algal flocs mostly composed of *Chlorella* sp. with some filaments of cf. *Pseudanabaena* sp., observed in phase contrast microscopy (200X); c) Detail of a lateral side of an algal floc with filaments of cf. *Pseudanabaena* sp., and *Chlorella* sp., observed in bright light microscopy; d) Detail of a lateral side of an algal floc with immersed *Chlorella* sp. and filaments of cf. *Pseudanabaena* sp., observed in bright light microscopy.

Regarding the concentration of TAN in the culture, it was consistently very low, meaning its almost complete removal in the PBR (>95%). This was also observed in the other two periods, although influent concentrations were gradually lower (Fig. 4.4a). $N\text{-}NO_3^-$ showed a very different behavior, and its concentration in the culture gradually increased during the first 15 days of operation, leading to concentrations in the PBR higher than those in the influent (average values of 32.9±4.8 mg L^{-1} and 18.6±4.1 mg L^{-1}, respectively) (Fig. 4.5a). This trend was indicative of a conspicuous nitrification activity during this period. $N\text{-}NO_2^-$ concentration was usually quite low in comparison to $N\text{-}NO_3^-$. IP concentration in the PBR was slightly greater than 1 mg L^{-1}, and the average removal was 44% (Fig. 4.4b). During period 1 the highest N:P ratio in the PBR mixed liquor of the whole experiment was registered (60:1 in molar bases). This value was due to the high

amount of TIN remaining in the culture in the form of N-NO_3^-. On the other hand, alkalinity values in this period ranged from 60-130 mg $CaCO_3$ L^{-1} (equivalent to 7.2-15.6 mg C L^{-1}). This fact also caused that pH values remained stable around the set value (8.3) without CO_2 injection. Thus, the low carbon content in the culture and the lack of CO_2 injection due to the pH stability clearly suggest carbon limitation in this period.

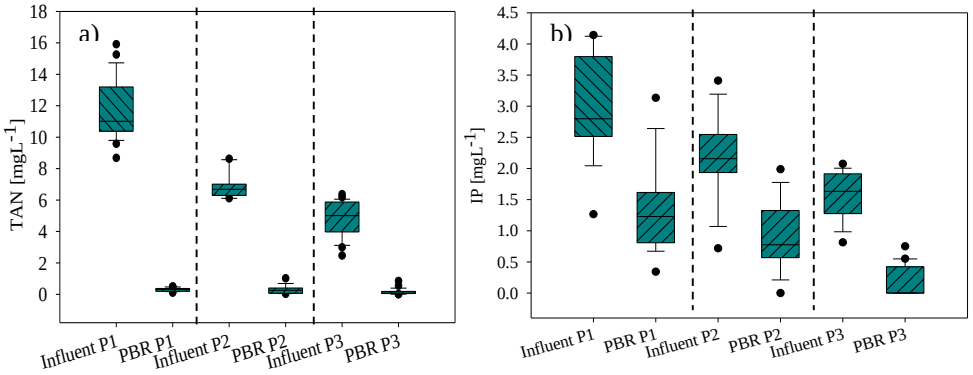

Fig. 4.4 Box-plot of the PBR influent and effluent a) ammonium (TAN) and b) inorganic phosphorus (IP) concentration. P1, P2 and P3 mean period 1, 2 and 3. Discontinued vertical lines separate the 3 operational periods.

In period 2, the average biomass concentration was higher than in the other two periods (average VSS of 0.84 g L^{-1} and Chlorophyll *a* of 4.5 mg L^{-1}), but it also had a higher variability mostly due to the detachment of biofilm growing on the walls of the PBR (Fig. 4.2b). This biofilm was mainly constituted by large populations of the cyanobacterium cf. *Pseudanabaena* sp. (Fig. 4.6). In fact, this cyanobacterium also formed big flocs in the mixed liquor culture that included other cyanobacteria such as cf. *Aphanocapsa* sp., *Chroococcus* sp., and green algae *Chlorella* sp. Biomass production during this period was also higher than in the other two periods, with an average value of 0.084 g VSS L^{-1} d^{-1} and a maximum value of 0.24 g VSS L^{-1} d^{-1}.

At the beginning of period 2, the concentration of nutrients in the culture was generally low, in particular N-NO_3^- content (Fig. 4.5). This is due to the low N-NO_3^- concentration registered in the effluent of the HRAP at this stage (Table 4.1). PBR influent and effluent N-NO_3^- concentrations were very similar, indicating a lower nitrification activity in the PBR in comparison to period 1 (Fig. 4.5b). Regarding TAN, it was also completely removed (<96 %) (Fig. 4.4).

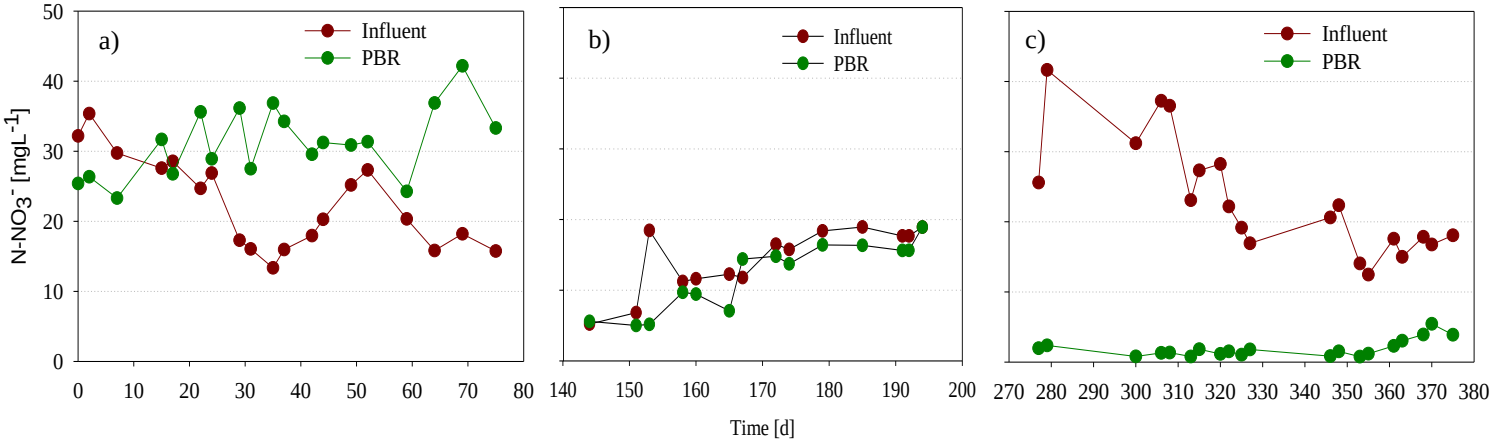

Fig. 4.5 Changes in nitrate ($N\text{-}NO_3^-$) concentration in the influent and the effluent of the PBR during the three periods. a) period 1, b) period 2 and c) period 3.

$N\text{-}NO_2^-$ concentration was also very low, usually below the limit of quantification (see Table 4.2). IP average concentration was slightly lower than 1 mg L^{-1} and its average removal efficiency fairly similar to that of period 1 (47%) (Fig. 4.4b). However, N:P ratio in the culture was lower than in period 1 (12:1) due to the lower $N\text{-}NO_3^-$ concentration. In the case of alkalinity content, the values were higher than those of period 1, ranging from 95-143 mg $CaCO_3$, equivalent to 11.4-17.6 mg C L^{-1}. In this period, CO_2 was sparged in the culture and therefore carbon was not a limiting factor.

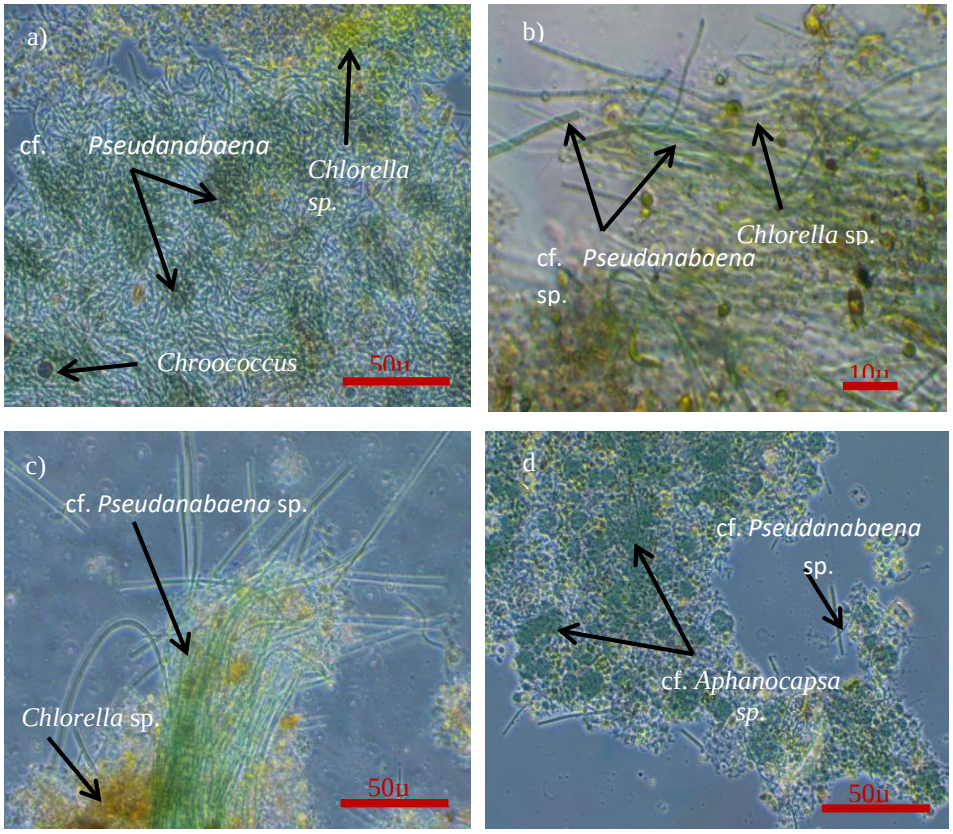

Fig. 4.6 Microscopic images illustrating the dominant algae during period 2; a) Algal floc largely dominated by filamentous cf. *Pseudanabaena* sp., groups of *Chlorella* sp. and dispersed individuals of *Chroococcus* sp. observed in phase contrast microscopy (400X); b) Detail of an algal floc dominated by cf. *Pseudanabaena sp.* with some dispersed *Chlorella* sp. observed in bright field microscopy (1000X); c) Grouped filaments of cf. *Pseudanabaena* sp. with some dispersed *Chlorella* sp., observed in phase contrast microscopy (400X); d) Algal floc largely dominated by cyanobacteria cf. *Aphanocapsa* sp. with some filaments of cf. *Pseudanabaena* sp. observed in phase contrast microscopy (400X).

After finishing the monitoring of period 2, no additional change was performed to the culture because cyanobacteria continued dominating the culture. Therefore, the culture followed a normal operation until the start of period 3. During this period, the biomass concentration decreased from 1.1 g VSS L^{-1} in the first day to an average of 0.56 g VSS L^{-1} (Chlorophyll *a* of 3.7 mg L^{-1}) in the following 75 days of operation, which was lower than the average in period 2 (0.84 g L^{-1}) (Fig. 4.2c). The biomass production (0.039 g VSS L^{-1} d^{-1}) was also lower than that in period 2. These trends were related with nutrient depletion in the culture. Indeed, this period was characterized by the lowest values of TIN in the PBR mixed liquor, mostly due to low $N\text{-}NO_3^-$ in the culture (see Table 4.2). In contrast, $N\text{-}NO_3^-$ concentration in the PBR influent was higher than in period 2 (Fig. 4.5). Average $N\text{-}NO_3^-$ removal was 91%, while in the other periods it was negligible or even negative (period 1). Similarly, to the other periods, TAN was also completely removed (>95 %) (Fig. 4.4). IP concentration had the lowest influent and effluent values of the whole experiment, showing very high removal rates (>95%) (see Fig. 4.4b). The high nutrient removal in this period was related to P limitation, which led to an increase of the N uptake and the subsequent lowest N:P ratio estimated during the three periods. Alkalinity content was higher in both PBR influent and effluent than in the other periods (ranging from 174 and 330 mg $CaCO_3$ L^{-1} equivalent to 20.88 and 39.6 mg C L^{-1}).

In this last period, most of the algae community was dominated by the cyanobacteria *Chroococcus* sp., cf. *Aphanocapsa* sp., and some filaments of cf. *Pseudanabaena* sp., which formed large flocs (Fig. 4.7).

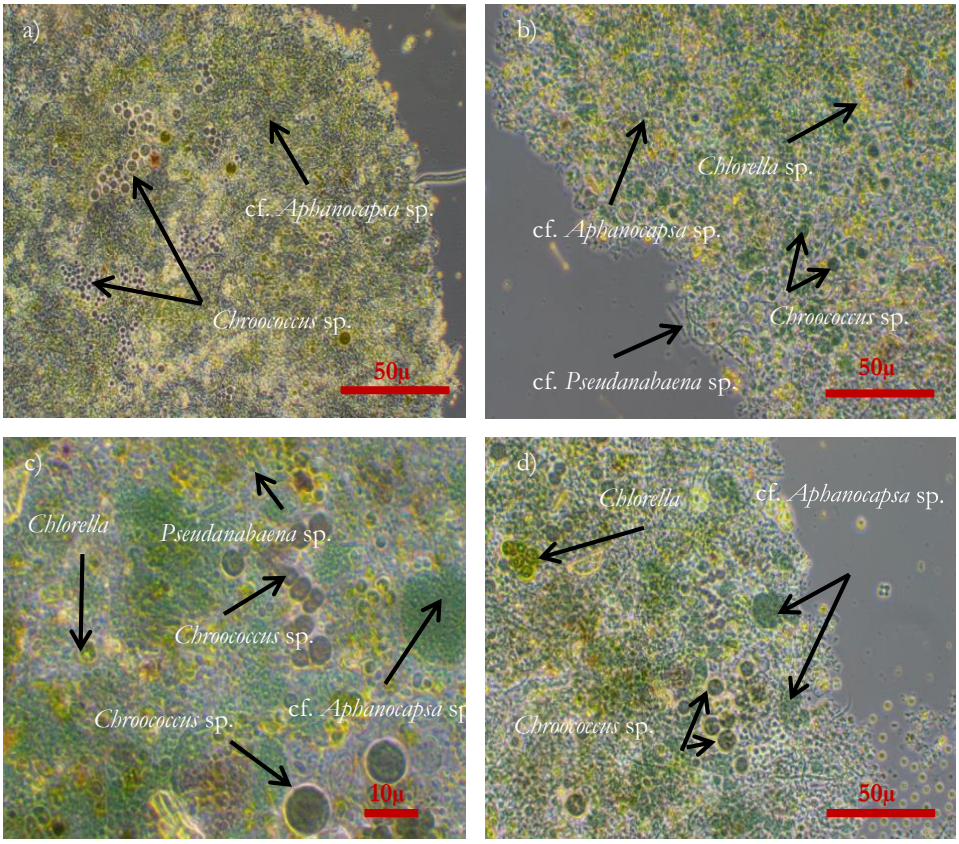

Fig. 4.7 Microscopic images illustrating the microbial composition along period 3. a) Algal floc largely dominated by cyanobacteria cf. *Aphanocapsa* sp., cf. *Chroococcus* sp. and dispersed *Chlorella sp.* observed in phase contrast microscopy (400X); b) Algal floc dominated by cf. *Aphanocapsa* sp. with some dispersed *Chlorella* sp. and cf. *Pseudanabaena* sp. observed in phase contrast microscopy (400X); c) Detail of an algal floc dominated by cyanobacteria *Aphanocapsa* sp. with immersed *Chroococcus* sp. and cf. *Oscillatoria* sp., observed in phase contrast microscopy (1000X); d) Algal floc largely dominated by cyanobacteria (cf. *Aphanocapsa*), observed in phase contrast microscopy (400X).

4.5 **Discussion**

Biomass concentration and composition changed during the three periods according to the nutrients input in the PBR influent and the corresponding N:P ratios. Table 4.3 summarizes influent and effluent N:P ratios, the L_V-P and the main dominant algae in each period. In period 1, microalgae community was dominated by *Chlorella* sp. with a relatively low abundance of cyanobacteria, considering the high influent concentrations of TIN, TAN and IP (see Table 4.1). Due to the absence of CO_2 injection during this period, nitrifying bacteria

competed with algae for inorganic C as well as for TAN (Markou and Georgakakis, 2011; Villaverde et al., 1997), and released high amounts of $N\text{-}NO_3^-$, accounting for the higher concentrations detected in the effluent than in the influent (31.2 mg L^{-1} and 22.5 mg L^{-1}, respectively, see Tables 4.1 and 4.2), and also the higher N:P ratios. In fact, the amount of carbon available for nitrification and photosynthesis was mostly that corresponding to alkalinity. Carbon limitation contributed to the relatively poor nutrients uptake and removal in this period, and favored the dominance of the green algae *Chlorella* sp.

In period 2, influent N:P ratio was similar to that of period 1, but TIN and IP concentrations were slightly lower (see Tables 4.1 and 4.3). However, there wasn't C limitation due to pH control by means of CO_2 injection, leading to a lower N:P ratio in the culture compared to that of period 1 (Table 4.3). These conditions shifted the algae community favoring cyanobacteria, being cf. *Pseudanabaena* sp. the dominant photosynthetic microorganism. The trend observed corroborates that cyanobacteria show a higher affinity for nutrients than other types of algae (Monchamp et al., 2014). Despite the biomass fluctuations observed in period 2, a clear increasing tendency was observed as well as a higher biomass concentration and production than in period 1. These results are directly related to the absence of carbon limitation and similar influent and effluent N:P ratios, which are indicative of lack of nutrient limitation.

In period 3 TIN concentration in the PBR influent was higher than in period 2, and IP concentration was lower, obtaining the highest N:P ratio of the three periods. On the other hand, N:P ratio in the effluent was the lowest, indicating P limitation related to the low concentration detected in the influent and N depletion. In these conditions, cyanobacteria cf. *Aphanocapsa*, cf. *pseudanabaena* and *Chroococcus* were almost the only microalgae in the culture. However, biomass concentration and production were lower than in the previous period due to P limitation.

Table 4.3 Influent and effluent N:P values (in molar basis), L_V-P and main dominating microalgae during the 3 experimental periods.

	Influent		Efluent			Main algae
Period	N:P ratio average	N:P ratio range	N:P ratio average	N:P ratio range	L_V-P (mg IP L^{-1} d^{-1})	
1	29:1	13-66:1	59:1	31-93:1	0.28	*Chlorella* sp., cf. *Oscillatoria* sp.
2	22:1	13-37:1	27:1	16-49:1	0.23	cf. *pseudanabaena* sp., cf. *Aphanocapsa* sp., C*hroococcus* sp.
3	40:1	24-64:1	11:1	4-33:1	0.16	cf. *Aphanocapsa* sp., *Chroococcus* sp., cf. *Oscillatoria* sp.

All in all, the results obtained indicate that cyanobacteria can be selected from mixed algae consortia grown in completely stirred PBRs fed with treated wastewater and digestate and under pH control (lack of C limitation), when the L_V-P is approximately 0.23 mg IP $L^{-1}d^{-1}$ (the average corresponding to period 2). Specifically, in a PBR with 30 L and HRT=10 days, this would be equivalent to a IP influent concentration of approximately 2 mg L^{-1}. This P load together with a N concentration which gives a 10:1 ratio, leads to an increase in the biomass production up to an average of 0.084 g $L^{-1}d^{-1}$, with a maximum yield of 0.23 mg $L^{-1}d^{-1}$. Within these conditions, the remaining N:P ratio in the culture would be similar or lower with decreasing influent loads, as it can be seen in period 3 when compared to the two other periods. However, when the L_V-P decreased to values under 0.16 mg IP $L^{-1}d^{-1}$ in period 3, the biomass production was reduced to 0.039 g $L^{-1}d^{-1}$ as a direct consequence of the acute P limitation.

Results on adaptation of cyanobacteria to low P concentration and limitation found in the present study are comparable to those previously reported on cultivation of cyanobacteria in wastewaters (see Table 4.4). Nevertheless, it should be taken into account that the majority of these studies were performed using pure cultures in batch lab scale experiments. The results by Kamilya et al. (2006) and Su et al. (2012) indicated that cyanobacteria can be successfully cultivated with low concentrations of P, as observed in our study. In contrast, other studies revealed that cyanobacteria can also be cultivated with higher concentrations and even with very high L_V-P values. For instance, Pouliot et al., (1989) and (Van Den Hende et al., 2016a) worked with a L_V-P which was 13-16 and 5-7 times higher than the value of period 2 and 3 of this study. However, with the exception of the study by Renuka et al. (2013), all the other studies had low N:P ratios, and therefore P limitation. In spite of being a relevant issue, the predominance of the same species or the potential contamination during the culture was not mentioned in the most of these studies. Only the studies performed by Pouliot et al., (1989) and Van Den Hende et al., (2016a) revealed the occurrence of other green algae species during the cultivation. On the other hand, one study concerning microalgae dynamics in a photobioreactor fed with secondary effluent obtained a culture largely dominated by *Chlorella*. This fact could be attributed to the low nutrient concentration and nitrogen limitation (0.13 mgL^{-1} of TIN and 1.3 mgL^{-1} of IP) (Marchello et al., 2015).

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Table 4.4 Summary of the average values of TIN and IP and N:P ratios of the periods with cyanobacteria dominance of this study compared with other cyanobacteria culture studies fed with wastewaters.

Microalgae cultivated	Cultivation mode	Influent	TIN (mg L^{-1})	IP (mg L^{-1})	N:P ratio[a]	L_V-P (mg IP L^{-1} d^{-1})	Reference
Cyanobacteria dominated mixed culture	Semi-continuous	Secondary effluent and digestate	21.22	2.19	22:1	0.23	This study[b]
Cyanobacteria dominated mixed culture	Semi-continuous	Secondary effluent and digestate	26.97	1.41	40:1	0.16	This study[c]
Spirulina platensis	Batch	Fish culture effluent	6.62	0.67	22:1	NA[c]	Kamilya et al. (2006)
Nostoc muscorum	Batch	Fish culture effluent	6.62	0.67	22:1	NA[c]	Kamilya et al. (2006)
Phormidium sp.	Batch	Secondary effluent	26	1.8	31:1	NA[c]	Su et al. (2012)
Cyanobacteria dominated mixed culture	Batch	Raw urban wastewater	104	3.1	34:1	NA[c]	Renuka et al. (2013)
Phormidium sp.	Batch	Secondary effluent	26	6	9:1	NA[c]	Talbot and de la Noüe (1993)
Phormidium sp.	Semi-continuous	Swine manure	50	17.5	4:1	2.64	Pouliot et al. (1989)
Phormidium sp.	Batch	Digestate	8.8	6.4	3.1:1	NA[c]	Cañizares-Villanueva et al. (1994)
Geminocystis sp., *Aphanocapsa* sp.	Sequencing batch	Secondary effluent	4.65	2.37	4.2:1	1.15	(Van Den Hende et al., 2016a)
Arthospira platensis	Batch	Diluted olive-oil mill wastewater	564	65.22	18:1	NA[c]	(Markou et al., 2012c)

[a] *Ratio in molar basis*
[b] *culture corresponding to period 2 of this chapter.*
[c] *culture corresponding to period 3 of this chapter*
[d] *not applicable.*

Table 4.5 Average of TSS and biomass production in the three periods of the study compared with other long term studies using secondary effluents.

Microalgae cultivated	Cultivation mode	Influent	Duration of the study (days)	TSS ($g\ L^{-1}$)	Biomass productivity ($g\ L^{-1}\ d^{-1}$)	Reference
Mixed microalgae/cyanobacteria dominated culture	Semi-continuous	Secondary effluent and digestate	375	0.67[a]	0.05[b]	This study
Microalgae	Alternating batch and continuous	Secondary effluent	157	0.7	0.08[c]	Arbib et al. (2013)
Mixed microalgae and cyanobacteria	Continuous	Secondary effluent	160	0.87	0.02[c]	Honda et al. (2012)
Microalgae	Continuous	Secondary effluent	190	0.2	0.05[c]	Takabe et al. (2016)
Microalgae	Alternating batch, continuous and semi-continuous	Secondary effluent	195	0.2-0.3[d]	0.03-0.04[b]	Viruela et al. (2016)
Geminocystis sp., *Aphanocapsa* sp.	Sequencing batch	Secondary effluent	122	0.67	0.32	(Van Den Hende et al., 2016a)

[a] *Average of the three periods monitored in this study.*
[b] *Biomass productivity presented as g VSS L^{-1} d^{-1}.*
[c] *Biomass productivity presented as g TSS L^{-1} d^{-1}.*
[d] *Values presented in terms of VSS.*

Considering that the PBR of this study was used as an additional wastewater treatment system for the mixture of secondary effluent and digestate, results obtained can be compared with other studies focused in microalgae biomass production in PBRs using treated effluents as nutrient sources. Although the biomass depends on the influent characterization and the different operation modes, SST concentration and biomass production in this study were comparable to those of other studies (Table 4.5). These results also reveal that recycling of nutrients from secondary effluents and, in the particular case of this study the addition of digestate, can be positively used to grow valuable biomass, obtaining at the same time a further treatment of the wastewater used. Therefore, it could become a feasible alternative to conventional wastewater with a double benefit, as this microalgae bioremediation is highly efficient in nutrients removal and it wouldn´t require any chemical input. Furthermore, the potential of cyanobacteria to produce and accumulate added-value products such as antibacterial substances, glycogen or polyhydroxyalcanoates (PHAs) (bioplastics) could counterbalance the maintenance and operation costs of closed PBRs. The data provided highlights the need of further studies regarding the enhancement of the production of these by-products.

From an engineering point of view, PBRs could be integrated into a real wastewater treatment plant in order to treat both wastewater treatment effluent and digestate, while producing valuable products. Following the encouraging results obtained from this study, further research could be addressed in order to the scale-up of the technology. Indeed, the effect of outdoor conditions (e.g. direct sunlight and temperature) should be carefully assessed.

4.6 **Conclusions**

In this chapter, digestate diluted with secondary effluent wastewater was used to select a culture dominated by cyanobacteria from an initial mixed microalgae consortium. During approximately one year of operation, the nutrient variations in the influent and their ratios played a key role in the culture composition and biomass concentration. The results evidenced that cyanobacteria species dominated over green algae when the influent had non-limited carbon conditions and low phosphorus content. Under these conditions, cf. *Pseudanabaena* sp., cf. *Aphanocapsa* sp. and *Chroococcus* sp. were found to dominate the culture with an increasing biomass content. This chapter shows that the dominance of cyanobacteria in a microalgal cyanobacterial community in an agitated PBR using wastewater as nutrient source can be obtained and maintained for 234 days. This

information can contribute to future studies on further production of valuable by-products from cyanobacterial biomass in wastewater treatment systems.

Chapter 5

Selection of cyanobacteria in a photo-sequencing batch reactor operated at high loads

The contents of this chapter were adapted from the publication: Arias, D.M., Uggetti, E., García-Galán, M.J., García, J., 2018. Nutrients and biomass dynamics in photo-sequencing batch reactors treating wastewater with high nutrients loadings. Ecol. Eng. 119, 35–44. doi:10.1016/j.ecoleng.2018.05.016

5.1 Abstract

In this chapter, different strategies for the treatment of a mixture of digestate from an anaerobic digester diluted and secondary effluent from a high rate algal pond were investigated. To this aim, the performance of two photo-sequencing batch reactors (PSBRs) operated at high nutrients loading rates and different solids retention times (SRTs) were compared with a semi-continuous photobioreactor (SC). Performances were evaluated in terms of wastewater treatment, biomass composition and biopolymers accumulation during 30 days of operation. PSBRs were operated at a hydraulic retention time (HRT) of 2 days and SRTs of 10 and 5 days ($PSBR_{2\text{-}10}$ and $PSBR_{2\text{-}5}$, respectively), whereas the semi-continuous reactor was operated at a coupled HRT/SRT of 10 days ($SC_{10\text{-}10}$). Results showed that $PSBR_{2\text{-}5}$ achieved the highest removal rates in terms of TN (6.7 mg $L^{-1}\cdot d^{-1}$), TP (0.31 mg $L^{-1}\cdot d^{-1}$), TOC (29.32 mg $L^{-1}\cdot d^{-1}$) and TIC (3.91 mg $L^{-1}\cdot d^{-1}$). These results were in general 3-6 times higher than the removal rates obtained in the $SC_{10\text{-}10}$ (TN 29.74 mg $L^{-1}\cdot d^{-1}$, TP 0.96 mg $L^{-1}\cdot d^{-1}$, TOC 29.32 mg $L^{-1}\cdot d^{-1}$ and TIC 3.91 mg $L^{-1}\cdot d^{-1}$). Furthermore, both PSBRs were able to produce biomass up to 0.09 g L^{-1} d^{-1}, more than twofold the biomass produced by the semi-continuous reactor (0.04 g L^{-1} d^{-1}), and achieved a biomass settleability of 86-92%. This chapter also demonstrated that the microbial composition could be controlled by the nutrients loads, since the three reactors were dominated by different species depending on the nutritional conditions.

5.2 Introduction

Wastewater treatment with microalgae is regarded as an economical and environmentally friendly process with the additional advantage that the biomass produced can be reused, allowing an efficient nutrient recycling (Rawat et al., 2011; Honda, et al., 2012). In this process, microalgae work in association with aerobic heterotrophic bacteria so that photosynthetic microorganisms produce molecular oxygen that is used as electron acceptor by bacteria to degrade organic matter (Abed et al., 2009; Borde et al., 2003). In return, bacteria release carbon dioxide during the mineralization process and complete the photosynthetic cycle (Muñoz and Guieysse, 2006). This wastewater treatment process has been successfully used for a range of purposes such as removal of nutrients and other compounds (e.g. heavy metals) and also to reduce the load of organic matter (Abdel-Raouf et al., 2012; de Godos et al., 2009; Honda et al., 2012; Wang et al., 2010). Furthermore, wastewater is nowadays considered the only economically viable source of water

and nutrients for the production of microalgae biomass that can then be used for valuable by-products generation (Pittman et al., 2011; Uggetti et al., 2014).

In spite of the benefits, microalgae-based wastewater treatment technologies face operational limitations and challenges, such as the high costs derived from biomass separation from the treated wastewater (Renuka et al., 2013; Trivedi et al., 2015; Udom et al., 2013). Indeed, an efficient separation requires the use of biomass harvesting processes which can increase the production cost by 20–30% (Molina-Grima et al., 2003; Renuka et al., 2013; Yaakob et al., 2014). Recently, several studies have proposed to include a sedimentation period in the operational mode in order to increase spontaneous flocculation and the subsequent formation of large flocs (Valigore et al., 2012; Van Den Hende et al., 2016a, 2014). This process can be carried out in a photo-sequencing batch reactor (PSBR), where hydraulic retention time (HRT) and solids retention time (SRT) are uncoupled, similarly to activated sludge systems (Wang et al., 2015). This way, the cells are forced to form flocs that settle faster, whereas unsettled cells are removed from the supernatant (Valigore et al., 2012). Contrary to conventional operations, which do not promote extensive spontaneous flocculation (e.g. continuous, semi-continuous and batch), this approach can avoid additional intensive harvesting process. In addition, uncoupled HRT/SRT could influence nutritional dynamics and biomass composition. This can cause biochemical changes in microalgal biomass, affecting the accumulation of valuable biopolymers such as carbohydrates, lipids and, in the case of cyanobacteria, polyhydroxybutyrate (PHB) (Arcila and Buitrón, 2016; Arias et al., 2018a). All these compounds have obtained an increasing attention due to their potential use as biodiesel substrate and as bioplastics in the case of PHBs. The information of such promising alternative is still insufficient and all the aspects concerning nutrients dynamics in this kind of systems need to be addressed.

In the previous chapter, it was demonstrated that nutrients dynamics in a semi-continuous reactor used for a wastewater tertiary treatment played an important role in the biomass composition during a long term study. In that study, the use of digestate from an anaerobic digester diluted with secondary wastewater from a high rate algal pond proved to be suitable for the growth of a selective culture of cyanobacteria. All in all, the present chapter aims to evaluate the performance of different photo-sequencing batch reactors during tertiary treatment of digestate diluted with secondary wastewater, comparing the dynamics with a conventional semi-continuous reactor (SC) in terms of wastewater treatment, biomass composition and biopolymers accumulation.

5.3 **Material and methods**

5.3.1 Inoculum

A mixed culture composed by green algae, cyanobacteria, bacteria, protozoa and small metazoa was used as inoculum. It was collected as thickened biomass (100 mL) from a harvesting tank connected to a pilot closed-photobioreactor (30 L) already used as tertiary wastewater treatment (Arias et al., 2017).

5.3.2 Experimental set-up

Experiments were performed at lab-scale in three photobioreactors consisting of a closed polymethacrylate cylinder with an inner diameter of 11 cm, a total volume of 3 L and a working volume of 2.5 L each. Experiments were carried out during 30 days, and all of them were submitted to light/dark cycles of 12 h each. Illumination during the light phase was supplied by two external halogen lamp (60W) placed at opposite sides of each reactor and providing 220 μmol m^{-2} s^{-1} of light. Reactors were continuously agitated (with the exception of settling periods) with a magnetic stirrer (Selecta, Spain) set 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. pH was continuously monitoring with a pH sensor (HI1001, HANNA, USA) and kept at 8.5 with a pH controller (HI 8711, HANNA, USA) by the automated addition of HCl 0.1 N or NaOH 0.1 N. A diagram of the process of each reactor is presented in Fig. 5.1.

Two of the reactors were operated in a sequencing batch operation mode at a HRT of 2 days. One of these photo-sequencing batch reactors (PSBR), named $PSBR_{2\text{-}10}$, was operated at a SRT of 10 days. This means that 0.25 L of mixed liquor were discharged at the end of the dark phase, then the agitation was stopped and biomass was allowed to settle during 30 minutes. After this period, 1 L of the supernatant was withdrawn and then the total volume discharged (1.25 L) was replaced with the same volume of wastewater influent (Fig. 5.1a). The other sequencing batch reactor (named $PSBR_{2\text{-}5}$) was operated with a SRT of 5 days. Thus, 0.5 L of the mixed liquor were withdrawn at the end of the dark phase before the subsequent settling time of 30 minutes. After the settling period, 0.75 L of the supernatant was withdrawn and then the total volume retired (1.25 L) was replaced with the same volume of wastewater influent (Fig. 5.1b). The operation of these PSBRs was compared with that of a semi-continuous reactor named $SC_{10\text{-}10}$ (control reactor). This reactor was fed once a day and operated at a HRT and SRT of 10 days. This means that each day at the end of the dark phase, 0.2 L of the mixed

liquor were withdrawn and subsequently this volume was replaced by 0.2 L of wastewater influent (Fig. 5.1c).

The influent treated in the reactors consisted on uncentrifuged digestate diluted in secondary effluent in a ratio of 1:50 (characteristics are shown in Table 5.1). The secondary effluent was obtained from a pilot system treating municipal wastewater which comprised a primary settler, a high rate algal pond (HRAP) and a secondary settler (Gutiérrez et al., 2016). The digestate was obtained from lab-scale anaerobic digesters (1.5 L) that produced biogas from microalgae biomass harvested from the HRAP. A detailed description of the system may be found in (Arias et al., 2018b). Mixed liquor and supernatant withdrawal, and feeding were performed by the automatic peristaltic pumps.

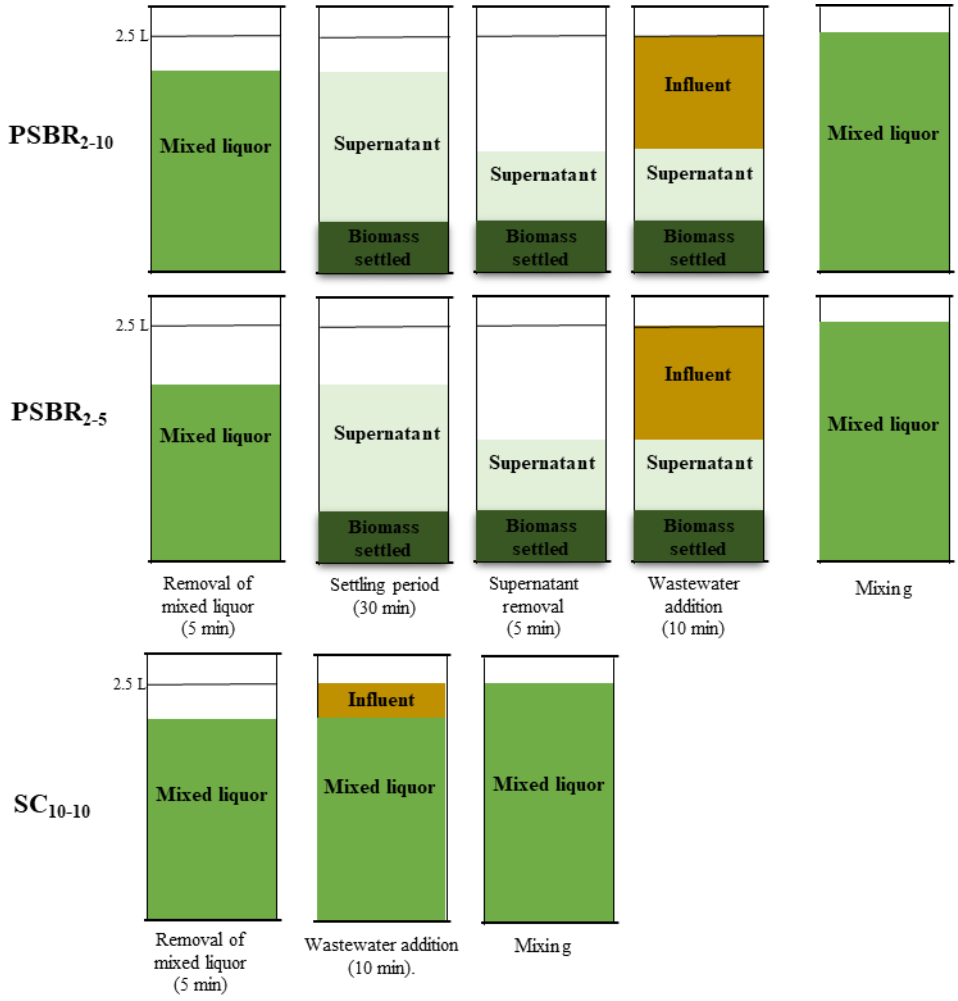


Fig. 5.1 Daily operation process of $PSBR_{2\text{-}10}$, $PSBR_{2\text{-}5}$ and $SC_{10\text{-}10}$.

Table 5.1 Average (standard deviation) of the main water quality parameters of digestate, secondary effluent and the influent wastewater (constituted by digestate diluted in a ratio 1:50 with secondary effluent) (n=4).

Parameter	Digestate	Secondary effluent	Influent wastewater
pH	-	-	7.1 (0.8)
SST [g·L^{-1}]	21.85 (1.80)	-[a]	0.44 (0.04)
SSV [g·L^{-1}]	17.90 (2.21)	-[a]	0.36 (0.04)
TC [mg·L^{-1}]	20638.50 (1145.00)	38.54 (6.00)	413.23 (23.02)
TOC [mg·L^{-1}]	16993.5 (382.30)	18.01 (3.20)	340.23 (7.71)
TIC [mg·L^{-1}]	3645.00 (762.70)	20.53 (2.8)	73.31 (15.31)
TN [mg·L^{-1}]	4685.41 (678.52)	25.51 (5.98)	83.35 (13.69)
TAN [mg·L^{-1}]	1020.45 (233.99)	0.045 (0.00)	20.41 (4.68)
N-NO_3^- [mg·L^{-1}]	<LOD	8.99 (1.24)	8.99 (1.24)
N-NO_2^- [mg·L^{-1}]	<LOD	1.22 (0.29)	1.22 (0.29)
TIN [mg·L^{-1}]	1020.45 (306.55)	10.25 (3.45)	30.62 (6.20)
TON [mg·L^{-1}]	2644.51 (373.52)	5 (1)	52.99 (7.49)
TP [mg·L^{-1}]	402 (115)	3.22 (1.02)	11.26 (1.63)
IP [mg·L^{-1}]	<LOD	1.72 (0.13)	1.72 (0.13)
TOP [mg·L^{-1}]	402 (115)	1.51 (0.60)	9.54 (2.35)

[a] TSS and VSS in the secondary effluent corresponded to values lower than 0.07 g L^{-1}.

5.3.3 Analytical methods

5.3.3.1 Nutrients concentrations

Nutrients monitoring was carried out by analyzing samples taken from the reactors at the end of the dark phase, after settling. All parameters were determined in triplicate and analyzed from the influent (mixed digestate and secondary effluent) and the supernatant of each reactor. Note that in the case of the reactor $SC_{10\text{-}10}$, the supernatant sample was taken from the mixed liquor withdrawn and submitted to a separation process. Samples from the influent were measured once per week, and samples of supernatant were analyzed three days per week.

Nitrogen was measured as total ammoniacal nitrogen (TAN), nitrite ($N\text{-}NO_2^-$), nitrate ($N\text{-}NO_3^-$), total nitrogen (TN) and total phosphorus (TP). TAN (sum of $N\text{-}NH_3$ and $N\text{-}NH_4^+$) was determined using the colorimetric method indicated in Solorzano (1969). $N\text{-}NO_2^-$ and $N\text{-}NO_3^-$ concentrations were analyzed using an ion chromatograph DIONEX ICS1000 (Thermo-scientific, USA), while TN was analyzed by using a C/N analyzer (21005, Analytikjena, Germany). Total inorganic nitrogen (TIN) was calculated as the sum of $N\text{-}NO_2^-$, $N\text{-}NO_3^-$ and TAN. Total organic nitrogen (TON) (in dissolved and particulate form) was calculated as the difference between TN and TIN.

Phosphorus compounds analyzed were inorganic phosphorus (IP) measured as orthophosphate (dissolved reactive phosphorus) ($P\text{-}PO_4^{3-}$) and total phosphorus (TP). IP concentrations were analyzed using an ion chromatograph DIONEX ICS1000 (Thermo-scientific, USA) and total phosphorus (TP) was analyzed following the methodology described in Standard Methods (APHA-AWWA-WPCF, 2001). Total organic phosphorus (TOP) forms (dissolved and particulate) were calculated as the difference between TP and IP.

Total organic carbon (TOC), Total inorganic carbon (TIC), soluble organic carbon (SOC) and soluble inorganic carbon (IC) were measured from raw and filtered samples using a C/N analyzer (21005, Analytikjena, Germany).

The volumetric load (Lv-Z) of each nutrient (TOC, TIC, TAN, NO_2^-, $N\text{-}NO_3^-$, TIN, TON, TN, IP, TOP and TP) was calculated in [mg Z $L^{-1}d^{-1}$] as shown in eq. 1:

$$\mathbf{Lv - Z} = \frac{\boldsymbol{Q * X}}{\boldsymbol{V}} \quad (1)$$

Where Q is the flow [$L^{-1}d^{-1}$], Z is the nutrient influent concentration [mg Z L^{-1}] and V [L^{-1}] is the volume of the reactor.

5.3.3.2 Biomass concentration

Total suspended solids (TSS) and volatile suspended solids (VSS) were measured in the mixed liquor at the end of the dark phase three days per week. In $PSBR_{2\text{-}10}$ and $PSBR_{2\text{-}5}$, two samples were taken; one from the mixed liquor right before stopping the agitation in order to evaluate the biomass production, and one from the supernatant after the sedimentation to evaluate the biomass settleability. Chlorophyll *a* was analyzed twice per week in the mixed liquor. Both analytical procedures were performed using the methodology described in Standard Methods (APHA-AWWA-WPCF, 2001).

Biomass production of each reactor in [g VSS $L^{-1}d^{-1}$] was estimated as follows:

$$\text{Biomass production} = \frac{Q*VSS}{V} \quad (2)$$

where Q is the flow [$L^{-1}d^{-1}$], VSS is the biomass concentration in the reactor [g L^{-1}] and V [L^{-1}] is the volume of the reactor.

Settleability [%] was determinate according to the following formula:

$$\text{Settleability} = 100 * \left[1 - \left(\frac{TSSs}{TSSm}\right)\right] \quad (3)$$

Where TSS_m [mg L^{-1}] is the mixed liquor suspended solids concentration and TSS_s [mg L^{-1}] is the supernatant suspended solids concentration.

Microalgae composition was qualitatively evaluated by means of microscope observations twice per week using an optic 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 (Bourrelly, 1985; Palmer, 1962), as well as a database of Cyanobacteria genus (Komárek and Hauer, 2013b).

5.4 **Results and discussion**

5.4.1 Nutrients dynamics and removal efficiency

Due to the different HRT, nutrients volumetric load applied to $PSBR_{2\text{-}10}$ and $PSBR_{2\text{-}5}$ was five times higher than the load applied to $SC_{10\text{-}10}$ (Table 5.2). Furthermore, it is noticeable that the organic forms of nitrogen and phosphorus (TON and TOP) provided by the digestate (Table 5.1) were the main sources of nutrients. This fact influenced the TN and TP uptake and removals efficiencies.

As it can be observed in Fig. 5.2, TN in the effluent (without the biomass) showed similar concentrations in the three reactors. However, when comparing the semi-continuous reactor with the sequencing batch it is noticeable that the best performance in terms of nutrients assimilation and removal was reached by the sequencing batch operation ($PSBR_{2\text{-}10}$ and $PSBR_{2\text{-}5}$). Indeed, considering the higher load applied to the sequencing batch reactors (Fig. 5.2b and 5.2c), these showed a higher removal rates of TN (>29 mg L^{-1} d^{-1}) than semi-continuous reactor (6.70 mg L^{-1} d^{-1}) (Fig. 5.2a). It is important to remark that Lv-TN was constituted by 63% of TON and 37% of TIN (Table 5.1). Since it is impossible for microalgae to uptake organic nitrogen, TON should have been mineralized to TAN before being consumed by microalgae (Pehlivanoglu and Sedlak, 2004). As observed in the three reactors, TON was almost totally transformed, whereas TAN presented a high variability during the experimental period, ranging from 0 to 13.45 mg L^{-1} (Fig. 5.2). This suggests that high concentrations of TAN could be derived from the mineralization of TON. Regarding $N\text{-}NO_3^-$, it can be seen that the three reactors showed similar concentrations during the experiment (around 12 mg L^{-1}) (Table 5.3). In this case, similar concentrations in $N\text{-}NO_3^-$ were indicative of a higher removal. On the contrary, $N\text{-}NO_2^-$ showed higher values in the reactors than in the influent, (3.84±3.33 mg L^{-1} in $SC_{10\text{-}10}$, 6.08±4.52 in $PSBR_{2\text{-}10}$ and 6.63±4.28 mg L^{-1} in $PSBR_{2\text{-}5}$), suggesting the inhibition of the nitrification process in the three reactors (Pollice et al., 2002).

Within the wastewater treatment context, due to the similar TN concentrations in the three reactors (Table 5.3), similar removal percentages were obtained (Table 5.4). Furthermore, higher removals were observed in TAN (>80%) and TON (99%), while $N\text{-}NO_2^-$ and $N\text{-}NO_3^-$ were not removed in any reactor. Despite such similarities in the general performance, the removal rates achieved in the two PSBRs for TN, TAN and TON were more than 4 times higher than those of the SC reactor (Table 5.4).

Table 5.2 Nutrients volumetric load (Lv) in each reactor according to the hydraulic retention time (n=4).

Parameter	$SC_{10\text{-}10}$[a]	$PSBR_{2\text{-}10}$[b]	$PSBR_{2\text{-}5}$[c]
Lv-TC [$mg \cdot L^{-1} \cdot d^{-1}$]	41.35 (2.3)	186.10 (9.36)	186.10 (9.36)
Lv-TOC [$mg \cdot L^{-1} \cdot d^{-1}$]	34.02 (0.77)	153.11 (3.47)	153.11 (3.47)
Lv-TIC [$mg \cdot L^{-1} \cdot d^{-1}$]	7.33 (1.53)	32.99 (6.89)	32.99 (6.89)
Lv-TN [$mg \cdot L^{-1} \cdot d^{-1}$]	8.65 (1.99)	37.60 (8.95)	37.60 (8.95)
Lv-TAN [$mg \cdot L^{-1} \cdot d^{-1}$]	2.04 (0.47)	9.18 (2.10)	9.18 (2.10)
Lv-N-NO_3^- [$mg \cdot L^{-1} \cdot d^{-1}$]	0.90 (0.12)	4.04 (0.55)	4.04 (0.55)
Lv-N-NO_2^- [$mg \cdot L^{-1} \cdot d^{-1}$]	0.12 (0.03)	0.55 (0.13)	0.55 (0.13)
Lv-TIN [$mg \cdot L^{-1} \cdot d^{-1}$]	3.06 (0.62)	13.77 (2.79)	13.77 (2.79)
Lv-TON [$mg \cdot L^{-1} \cdot d^{-1}$]	5.29 (0.75)	23.82 (3.37)	23.82 (3.37)
Lv-TP [$mg \cdot L^{-1} \cdot d^{-1}$]	1.13 (0.16)	5.63 (0.82)	5.63 (0.82)
Lv-IP [$mg \cdot L^{-1} \cdot d^{-1}$]	0.17 (0.01)	0.86 (0.07)	0.86 (0.07)
Lv-TOP [$mg \cdot L^{-1} \cdot d^{-1}$]	0.95 (0.24)	4.77 (1.18)	4.77 (1.18)

[a]Reactor operated at a coupled HRT and SRT of 10 d.
[b]Reactor operated at an uncoupled HRT of 2 days and SRT of 10 d.
[c]Reactor operated at an uncoupled HRT of 2 days and SRT of 5 d.

Table 5.3 Average (standard deviation) of the main nutrients concentrations of the supernatant of SC10-10, PSBR2-10 and PSBR2-5 during the experiment (n=9-15).

	$SC_{10\text{-}10}$[a]	$PSBR_{2\text{-}10}$[b]	$PSBR_{2\text{-}5}$[c]
Parameter	Average	Average	Average
TIC [mg·L^{-1}]	28.61 (23.69)	39.60 (18.99)	47.47 (20.77)
SOC [mg·L^{-1}]	47.41 (9.80)	54.50 (23.46)	49.84 (10.76)
TN [mg·L^{-1}]	21.66 (7.12)	23.59 (6.89)	21.92 (4.96)
TAN [mg·L^{-1}]	4.10 (5.08)	3.71 (4.19)	2.82 (3.25)
N-NO_2^-[mg·L^{-1}]	3.85 (3.33)	6.08 (4.52)	6.63 (4.28)
N-NO_3^-[mg·L^{-1}]	13.53 (4.78)	12.33 (3.43)	12.12 (4.48)
TIN [mg·L^{-1}]	21.47 (7.20)	22.12 (8.07)	21.57 (5.62)
TON [mg·L^{-1}]	0.19 (0.63)	1.47 (2.93)	0.035 (1.17)
TP [mg·L^{-1}]	10.88 (2.89)	14.63 (5.71)	9.33 (6.69)
IP[mg·L^{-1}]	1.37 (1.05)	1.13 (1.41)	2.90 (2.90)
TOP [mg·L^{-1}]	6.89 (8.48)	13.5 (4.30)	6.43 (6.61)

[a]Reactor operated at a coupled HRT and SRT of 10 d.

[b]Reactor operated at an uncoupled HRT of 2 days and SRT of 10 d.

[c]Reactor operated at an uncoupled HRT of 2 days and SRT of 5 d.

Table 5.4 Nutrients removal performances and removal rate of the effluent of the three reactors during the experiment (n=9-15).

	$SC_{10\text{-}10}$[a]		$PSBR_{2\text{-}10}$[b]		$PSBR_{2\text{-}5}$[c]	
Parameter	Removal percentage [%]	Removal rate [$mg \cdot L^{-1} \cdot d^{-1}$]	Removal percentage [%]	Removal rate [$mg \cdot L^{-1} \cdot d^{-1}$]	Removal percentage [%]	Removal rate [$mg \cdot L^{-1} \cdot d^{-1}$]
TOC	86	29.32	84	128.78	85	130.81
TIC	53	3.91	40	13.13	35	11.63
TN	64	6.70	63	29.82	63	29.74
TAN	80	1.63	82	7.51	86	7.91
$N\text{-}NO_3^-$	-	-	-	-	-	-
$N\text{-}NO_2^-$	-	-	-	-	-	-
TIN	32	0.98	30	4.10	29	4.02
TON	99	5.29	99	23.58	99	23.58
TP	27	0.31	-	-	17	0.96
IP	20	0.03	34	0.29	-	-
TOP	29	0.27	-	-	33	1.56

[a]Reactor operated at a coupled HRT and SRT of 10 d.
[b]Reactor operated at an uncoupled HRT of 2 days and SRT of 10 d.
[c]Reactor operated at an uncoupled HRT of 2 days and SRT of 5 d.

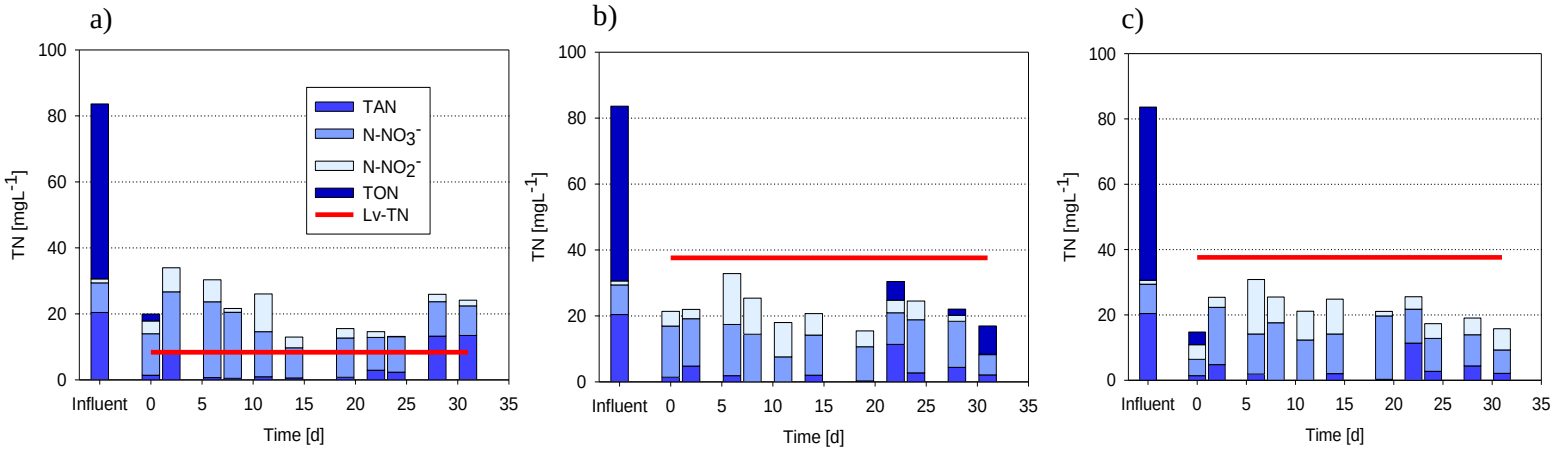


Fig. 5.2 Average influent and effluent TN concentrations during the experiment in a) $SC_{10\text{-}10}$, b) PSBR2-10 and c) $PSBR_{2\text{-}5}$. The average Lv-TN is presented in mg L^{-1} d^{-1}.

On the other hand, TP in the effluent showed different patterns than those observed for TN. In general, the best performance was obtained in the semi-continuous reactor ($SC_{10\text{-}10}$) where the Lv-TP was very low (1.13±0.16 mg L^{-1} d^{-1}) and was removed at a rate of 0.30 mg L^{-1} d^{-1}. TP concentration in $PSBR_{2\text{-}10}$ showed an increasing pattern along the experimental time and values up to 15 mg L^{-1} were reached in the last week of operation (Fig. 5.3b). In the case of $PSBR_{2\text{-}5}$, concentrations of TP remained higher than 10 mg L^{-1} and then decreased to 6 mg L^{-1} in the two last weeks of operation (Fig. 5.3c).

These patterns in the three reactors depended on the mineralization of TOP in all the reactors (Fig. 5.3). As for TON, microalgae are also unable to uptake organic phosphorus, then it was necessary that a mineralization process took place to transform it to inorganic phosphorus species (Donald et al., 2017; Rodríguez and Fraga, 1999). High IP concentrations observed in the last two weeks in $SC_{10\text{-}10}$ and in the $PSBR_{2\text{-}5}$ indicated that TOP transformed to IP was not consumed. A better mineralization of TOP was observed in $PSBR_{2\text{-}5}$ even though both PSBRs received the same Lv-TP. This result could be related to the SRT of the reactors, since the best mineralization of TOP was obtained in the reactor operating at 5 days. It is known that the mineralization process is microorganism dependent (Rodríguez and Fraga, 1999), meaning that microalgae and bacteria growing under lower SRT conditions were able to consume more P than microorganisms growing in a SRT of 10 days.

Regarding wastewater treatment, the best TP removal efficiencies were achieved in $SC_{10\text{-}10}$ and $PSBR_{2\text{-}5}$ (Table 5.4). $PSBR_{2\text{-}5}$ showed a removal rate of TP of 1.56 mg L^{-1} d^{-1}, which is six times higher the removal rate of $SC_{10\text{-}10}$. Due to the increase in TOP concentration in $PSBR_{2\text{-}10}$, no net removal was observed.

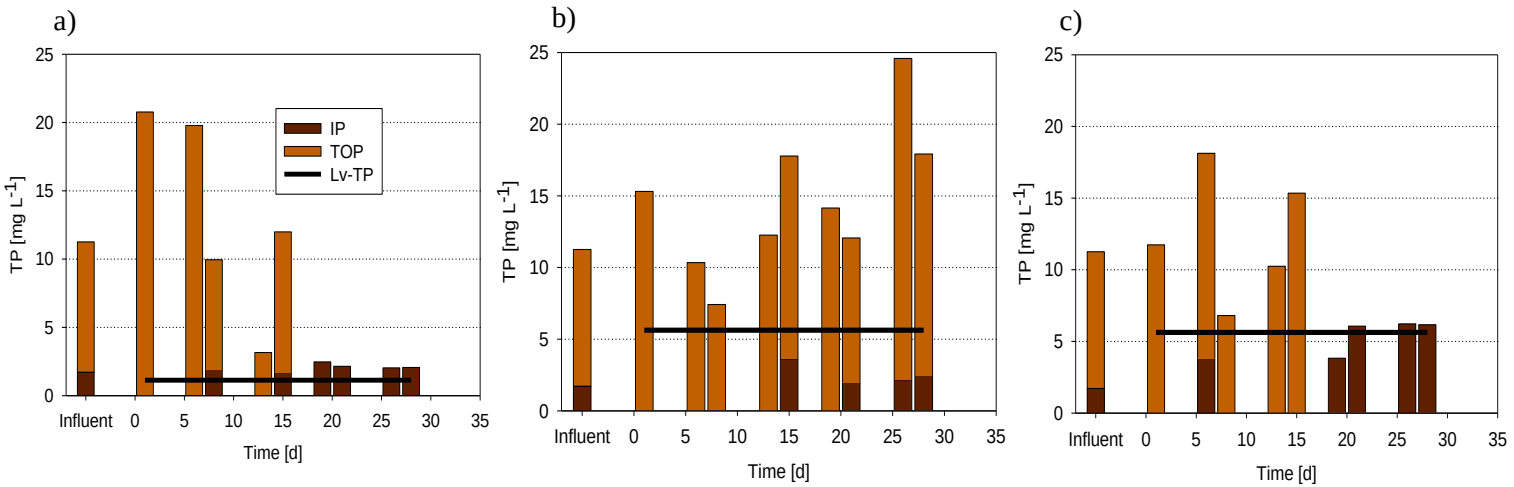


Fig. 5.3 Average influent and effluent TP concentration during the experiment in a) $SC_{10\text{-}10}$, b) PSBR2-10 and c) $PSBR_{2\text{-}5}$. The average Lv-TP is presented in mg L^{-1} d^{-1}.

Regarding the uptake of the different carbon species, although the effluent in three reactors averaged similar concentrations along the experimental time (Table 5.3), they showed differences in the removals regarding the Lv-TOC (Fig. 5.4). Due to the similar concentrations of TOC in the three reactors, removal efficiencies were similar (85±1%) regarding the influent wastewater total content. However, removal rates in both PSBRs (128.78 mg L^{-1} d^{-1} for $PSBR_{2\text{-}10}$ and 130.81 mg L^{-1} d^{-1} for $PSBR_{2\text{-}5}$) were 4 times higher than that of the semi-continuous reactor (29.32 mg L^{-1} d^{-1}) (Table 5.4). Similarly, $PSBR_{2\text{-}10}$ and $PSBR_{2\text{-}5}$ also reached up to three times higher removal rates of TIC (Table 5.4).

Given the results obtained, it is clear that the treatment efficiency of both $PSBR_{2\text{-}10}$ and $PSBR_{2\text{-}10}$ is high enough to become a feasible alternative to treat uncentrifugued digestate diluted with secondary effluent in microalgal wastewater treatment systems. According to the removal rates obtained, both PSBRs achieved the highest removals of TN, TOC and TIC, and TP (with the exception of TP in $PSBR_{2\text{-}10}$). According to the higher transformation of TOP to IP in $SC_{10\text{-}10}$, the increase of the HRT in the PSBRs could be a good strategy to achieve better removal efficiencies. In the case of other nutrients and organic matter removal, it was demonstrated that the PSBRs showed a better performance in relation to the load applied. Moreover, such systems have the advantage that higher wastewater volumes can be treated per day.

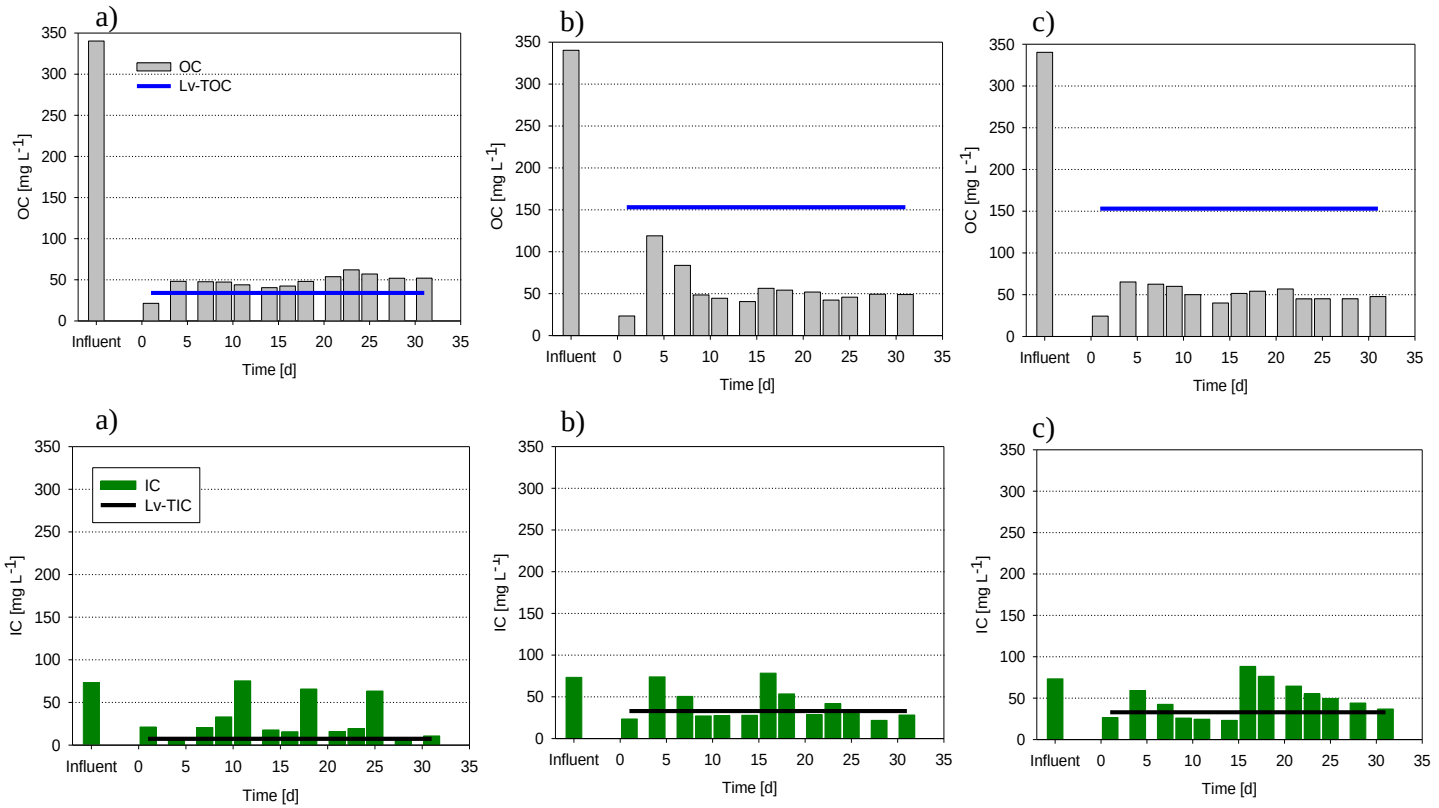


Fig. 5.4 Average TOC and TIC influent and effluent SOC and TIC concentration during the experiment in a) $SC_{10\text{-}10}$, b) $PSBR_{2\text{-}10}$ and c) $PSBR_{2\text{-}5}$. The average Lv-TOC/TIC is presented as mg L^{-1} d^{-1}.

5.4.2 Biomass production

Regarding the concentration of biomass, all the reactors showed an exponential increase during the first two weeks of operation. In the $SC_{10\text{-}10}$ reactor, it increased from an initial concentration of 0.207±0.081 mg L^{-1} to 0.451 mg L^{-1} in day 15, and after that a constant biomass concentration of approximately 0.420 mg L^{-1} was maintained. $PSBR_{2\text{-}10}$ showed an increasing pattern until day 27, achieving then the highest concentration of 0.910 mg L^{-1}. In $PSBR_{2\text{-}5}$, the concentration increased from 0.207 mg L^{-1} to 0.652 mg L^{-1} on day 13 and then it decreased and oscillated between 0.434 and 0.586 mg L^{-1} during the rest of the experiment. Regarding the chlorophyll *a* content, it remained constant in $SC_{10\text{-}10}$ and $PSBR_{2\text{-}5}$ during the experiment (0.597±0.091 and 0.829±0.279 mg L^{-1}, respectively) (Fig. 5.5), while $PSBR_{2\text{-}10}$ showed and increase from the initial concentration of 0.633 mg L^{-1} to 2.82 mg L^{-1} on the day 30.

In spite of the clear increase patterns registered in the biomass concentration, the highest biomass production was achieved in $PSBR_{2\text{-}5}$ (Fig. 5.5), due to the highest volume withdrawn. Thus, the biomass production reached by this reactor was 0.135 mg L^{-1} d^{-1} in day 15, and similarly to biomass concentration, it decreased during the following days, maintaining a quite constant production of approximately 0.11 g VSS $L^{-1}\cdot d^{-1}$. Despite that a lower mixed liquor volume was extracted in $PSBR_{2\text{-}10}$, the biomass production achieved was similar to that reached in $PSBR_{2\text{-}5}$ on day 27. On the contrary, the biomass production in $SC_{10\text{-}10}$ only increased from 0.021 to 0.04 g L^{-1} d^{-1} on day 10, maintaining similar values from that point on.

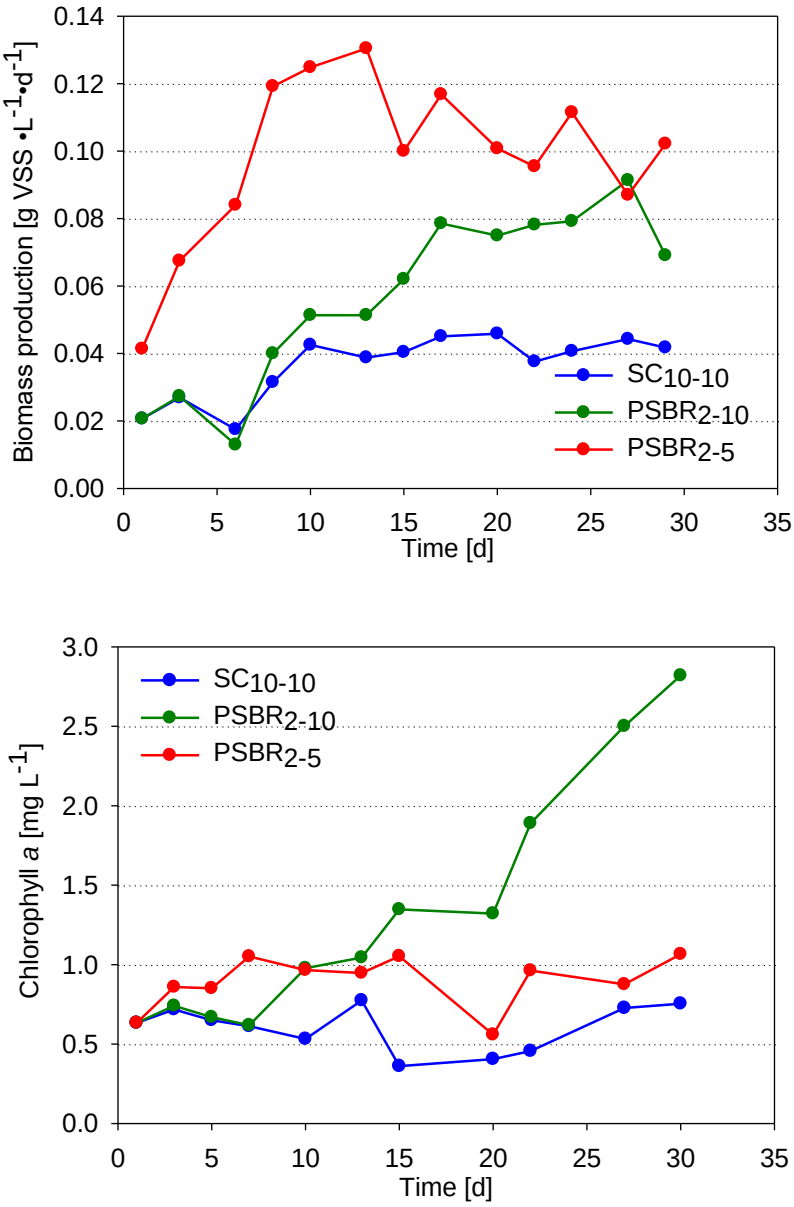


Fig. 5.5 Time course of biomass production and chlorophyll *a* content.

According to the microscopic monitoring, the microbial composition in $SC_{10\text{-}10}$ was similar during the whole experiment (Fig. 5.6). The biomass was composed mostly by microalgal mixed flocs containing diatoms, unicellular cyanobacteria cf. *Aphanocapsa* sp., green algae species as *Chlorella* sp. and dispersed *Scenedesmus* sp., and rotifers protozoa. Bacterial colonies were also observed mostly during the last ten days of operation.

In $PSBR_{2\text{-}10}$, a culture with the same composition observed in $SC_{10\text{-}10}$ was observed, with mixed flocs composed by green algae, some cyanobacteria and the presence of diatoms. However, microbial composition in the following days showed an increasing presence of bacterial colonies (Fig. 5.7). Contrary to the $SC_{10\text{-}10}$ reactor, green algae *Chlorella* sp increased in $PSBR_{2\text{-}10}$, whereas dispersed cells of *Scenedesmus* sp. were not observed. Protozoa species as *Vorticella* sp. were frequently visualized.

On the other hand, $PSBR_{2\text{-}5}$ showed a different microbial evolution compared to the other reactors. As observed in Fig. 5.8, algal flocs were rarely observed, whereas bacterial flocs were observed from the first days of operation onwards. In this reactor, the green algae present in the culture belonged to species of *Chlorella* sp. and *Stigeoclonium* sp. Other species of protozoa, cyanobacteria and diatoms were rarely observed in the culture.

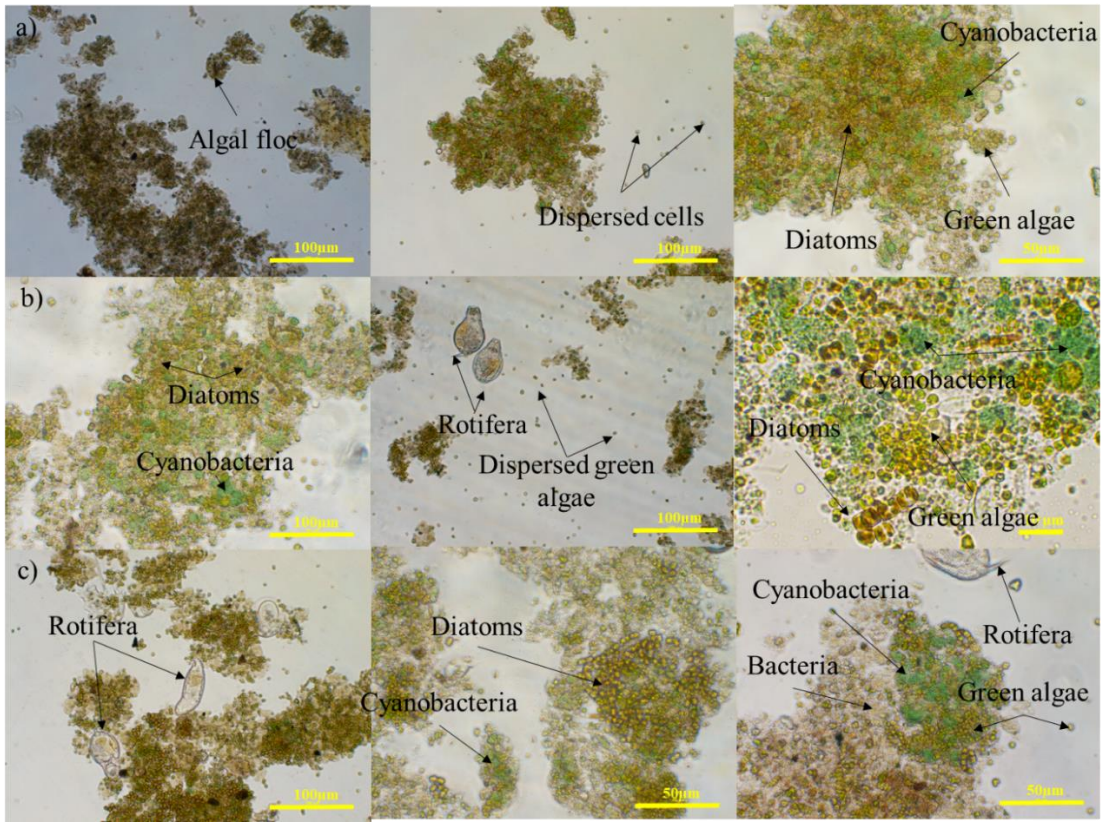


Fig. 5.6 Microscopic images illustrating microbial composition in $SC_{10\text{-}10}$ during the periods; a) days 1-10, b) days 11-20 and c) days 21-30.

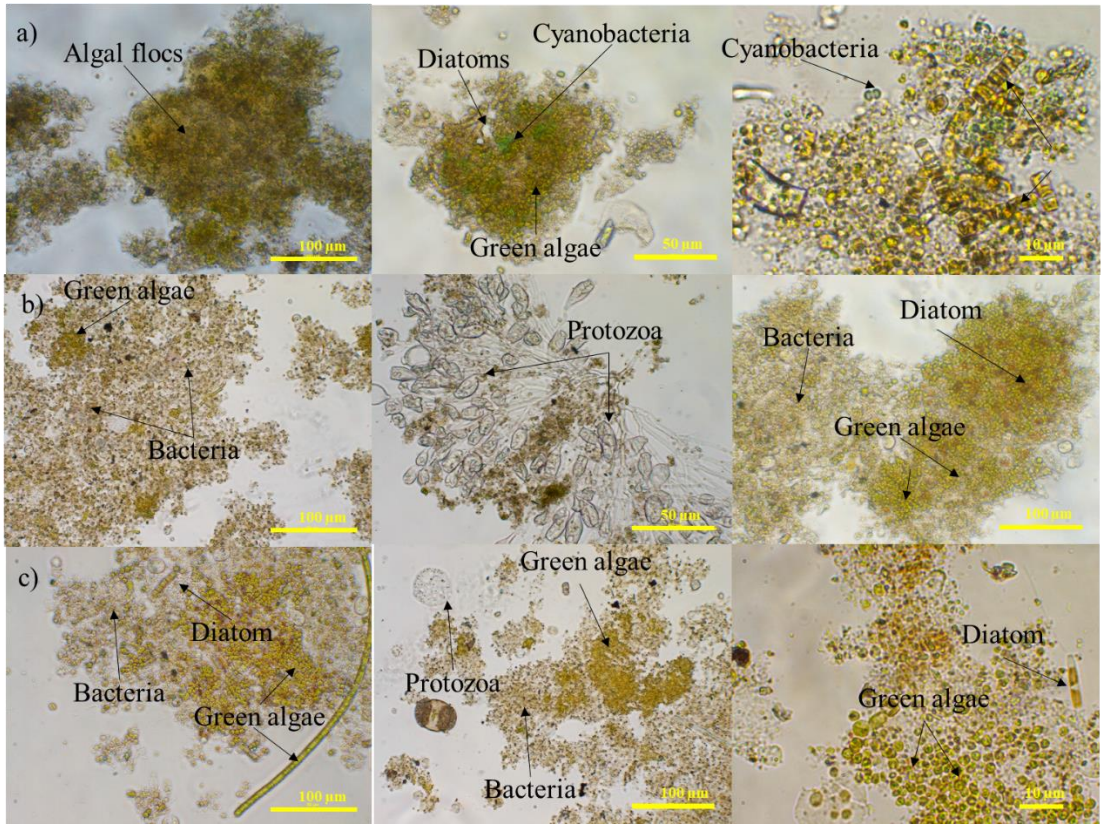


Fig. 5.7 Microscopic images illustrating microbial composition in $PSBR_{2\text{-}10}$ during the periods; a) 1-10 days, b) 11-20 days and c) 21-30 days.

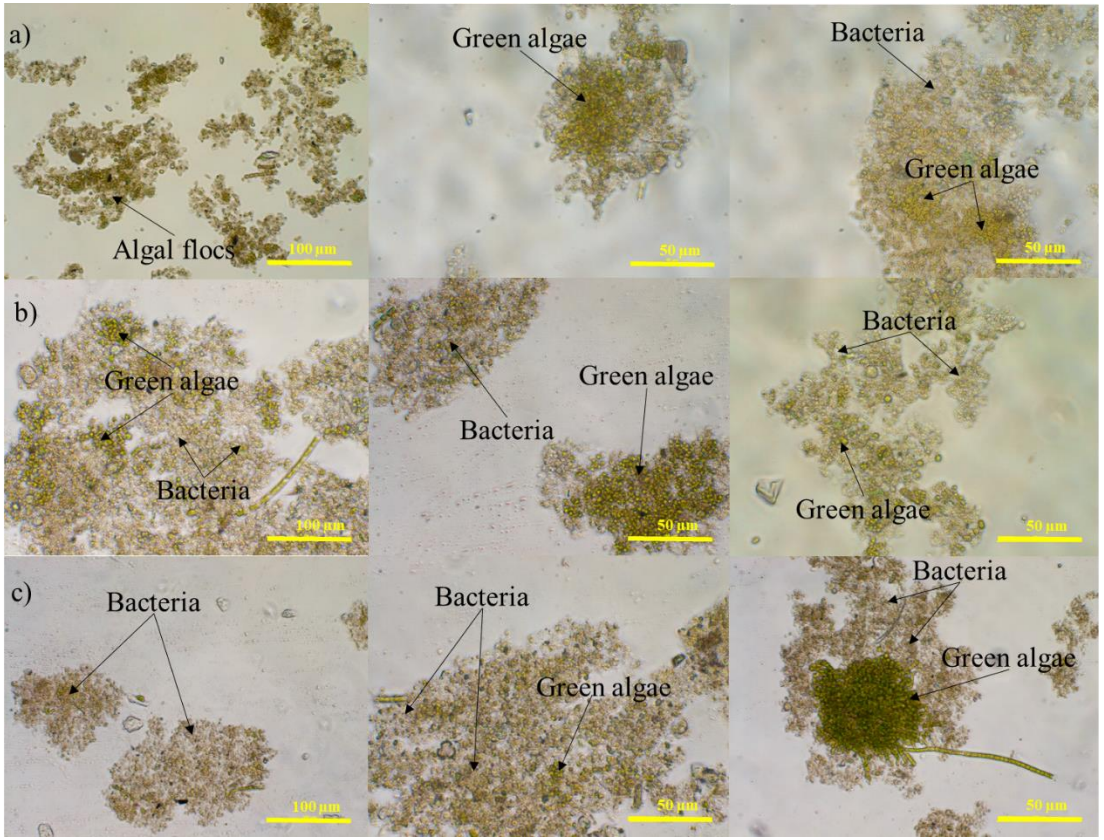


Fig. 5.8 Microscopic images illustrating microbial composition in $PSBR_{2\text{-}5}$ during the periods; a) days 1-10, b) days 11-20 and c) days 21-30.

In addition to the lack of dispersed cells observed by microscopy in $PSBR_{2-10}$ and $PSBR_{2-5}$, the concentration of SSV in the supernatant was 0.075±0.021 mg L^{-1} and 0.072±0.003 mg L^{-1}, respectively, from the first days of operation and remained quite constant during the experimental time. Such values implied a settleability of 86 to 92%. When comparing the biomass composition of the three reactors, it is clear that the strategy of operating $PSBR_{2-10}$ and $PSBR_{2-5}$ applying uncoupled SRT and HRT improved the capacity of the microorganisms to form flocs and perform a fast settling process, which are good results regarding the achievement of a feasible the harvesting process.

Considering these systems for biomass production, this chapter demonstrated that microbial composition could be controlled by nutrients loads and, at the same time, influenced by the SRT. For instance, it was shown that protozoa and diatoms can survive in a wide range of nutrients loads since their presence was observed in either low loads (SC_{10-10}) or high loads ($PSBR_{2-10}$). However, their presence was conditioned by long SRT (10 days), as it is usually observed in this type of systems (Shariati et al., 2011). On the other hand, the fact that the presence of cyanobacteria occurred in SC_{10-10} (low loads) but not in $PSBR_{2-10}$ (high loads), even if they operated at the same SRT, showed that nutritional conditions highly affect cyanobacteria presence. It would be important to improve the competition capacity of these species in microalgae-based wastewater treatments, since they are potential PHB and carbohydrates producers.

Another important result to consider is that bacterial presence increased more in $PSBR_{2-10}$ and $PSBR_{2-5}$ than SC_{10-10}. This suggests that the introduction of high loads of nutrients, especially TOC in the PSBRs, promoted the growth of heterotrophic bacteria. Another important fact to consider is that the influent used in this experiment (secondary effluent and digestate) contained high TOC:TIC ratio (4.64). In a previous study of Van Den Hende et al., (2014), it was shown that TOC:TIC ratios higher than 2.39 improved heterotrophic bacteria domination in PSBRs operated at 2 days of HRT. The semi-continuous reactor (using the same influent but a lower nutrient load) showed a dominance of microalgae, confirming that the load applied to the reactors played an important role in the microbial community composition.

It is important to highlight that although microalgae based wastewater treatments similar to the ones described in this chapter, have been successfully used for the treatment of digestate from different sources, the majority of the studies until now have employed batch or semi-continuous operation (R. Cañizares-Villanueva et al., 1994; Pouliot et al., 1989a; Ruiz-Marin et al., 2010; Sepúlveda et al., 2015; Uggetti et al., 2014; Viruela et al., 2016). However, such operations

presented limitation in nutrients removal rates, biomass production and the possibility to produce an easily settling culture. The strategy of using sequencing batch operation of photobioreactors for digestate removal is still limited to only a few studies. In the study of Van Den Hende et al., (2014), a 4 L PSBR operated at an HRT of 2 d to treat manure digestate was used. The authors obtained removal rates of TN and TP of 4.5 mg L^{-1} d^{-1} and 0.11 mg L^{-1} d^{-1}, respectively, producing 0.068 g L^{-1} d^{-1} of biomass. Remarkably, the results of $PSBR_{2-5}$ in this study reached higher removals rates (29.82 mg L^{-1} d^{-1} and 1.05 mg L^{-1} d^{-1} of TN and TP were removed, respectively) and, at the same time, a higher biomass production was achieved (0.11 g L^{-1} d^{-1}). On the other hand, the removal rate of TN of the present study was lower than that obtained by Wang et al., (2015), who used a 8 L PSBR operated at an HRT of 4 d to remove of diluted digestate. These authors obtained a removal rate of 71 mg L^{-1} d^{-1} of TN, applying nitrification and denitrification strategies in the PSBR, and at the same time producing 0.15 g L^{-1} d^{-1} of biomass.

5.5 Conclusions

In this chapter, nutrients removal and biomass growth were analyzed in photosynthetic sequencing batch reactors (PSBR) treating digestate diluted with secondary effluent. Two PSBRs were operated at hydraulic retention time (HRT) of 2 days and solids retention time (SRT) of 10 and 5 days, comparing the results obtained with those of a semi-continuous (SC) reactor operating at HRT and SRT of 10 days. PSBR showed removals rates of 30 mg L^{-1} d^{-1} of total nitrogen and up to 1 mg L^{-1} d^{-1} of total phosphorus. Concerning inorganic carbon and organic carbon uptake, PSBRs achieved removal rates of 128-130 mg TOC L^{-1} d^{-1} and 12-13 mg TIC L^{-1} d^{-1}. These results were in general 1-5 times higher than the removal rates obtained in the semi-continuous reactor. PSBRs were able to produce biomass up to 0.09 g L^{-1} d^{-1}, more than two fold the biomass produced by SC, obtaining also a biomass settleability of 86-92%. Furthermore, this chapter demonstrated that microbial composition could be controlled by nutrients loads, since the three reactors were dominated by different species depending on the nutrients concentrations.

Chapter 6

Selection of cyanobacteria in a photo-sequencing batch reactor operated at low loads

The contents of this chapter were adapted from the publication: Arias, D.M., Rueda, E., Uggetti, E., García-Galán, M.J., García, J., Submitted. Selection of cyanobacteria over green algae in a photo-sequencing batch bioreactor fed with wastewater. Sci. Total Environ.

6.1 Abstract

Cyanobacteria constitute a group of microorganisms able to produce valuable bioactive compounds. Their cultivation in wastewater is the most promising, cost-effective and eco-friendly strategy for biomass production. However, the maintenance of a cyanobacteria dominated culture in wastewater depends on the competition with other microorganisms (e.g. green algae) and the nutrients concentration dynamics. In this chapter, photo-sequencing batch operation was used as strategy to select cyanobacteria over unsettling green algae in a wastewater treatment system, considering for the first time the effect of operational conditions on nutritional dynamics and microorganisms' competition. During 30 days of operation, an initial microalgae mixed consortia dominated by *Scenedesmus* sp. was cultivated in two different photo-sequencing batch reactors (PSBRs) operated at HRT of 6 days ($PSBR_6$) and 4 days ($PSBR_4$) at a theoretical SRT of 10 d. Both PSBRs were compared with a semi-continuous photobioreactor (SC_{10}) operated at 10 d of HRT and 10 days of SRT. The results indicated that $PSBR_6$ and $PSBR_4$ decreased *Scenedesmus* sp. population by 88% and 48%, respectively. However, only $PSBR_6$ provided suitable conditions to select cyanobacteria from a green algae dominant culture. These conditions included volumetric loads of 11.72 mg TN L^{-1} d^{-1}, 2.04 mg TP $L^{-1}d^{-1}$ and 71.81 mg TC $L^{-1}d^{-1}$. The remaining nutrients in the culture led also to a phosphorus limiting N:P ratio (34:1) that improved the increase of cyanobacteria from an initial 2% until 70% of the total population. In addition, $PSBR_6$ reached a biomass production of 0.12 g L^{-1} d^{-1}, while removing TN, TP and TOC by 58%, 83% and 85%, respectively. Conversely, the introduction of higher nutrients loads caused by lower HRT ($PSBR_4$) led to an increase of only 13% of cyanobacteria while SC_{10} remained with the same biomass composition during all the experimental time. Thus, this chapter showed that the dominance of cyanobacteria in microalgal-based wastewater treatment systems can be controlled by the operational and nutritional conditions. This knowledge could contribute to control microalgae contamination from up-scaling cyanobacterial biomass production in wastewater treatment systems.

6.2 Introduction

Nowadays, the cultivation and use of microalgae and cyanobacteria as feedstock to obtain biofuels, bioproducts and bioenergy has become a

relevant research topic. Increasing scientific interest has been devoted to cyanobacteria (blue-green algae) due to their ability to grow in wastewater effluents and to their capacity to produce and accumulate intracellular bioactive compounds of interest for food and non-food purposes, such as, phycobiliproteins, polyhydroxybutyrate and glycogen (Abed et al., 2009; Shalaby, 2011). These bioproducts can be used as pigments, bioplastics and a biofuel substrates, respectively (Markou et al., 2013; Samantaray and Mallick, 2014; Stal, 1992; Van Den Hende et al., 2016a).

The alternative use of wastewater effluents as nutrients source for cyanobacteria growth represents the most promising, cost-effective and eco-friendly strategy to reduce production costs associated with nutrients and water input (Samantaray et al., 2011; Zhou et al., 2012). However, cyanobacteria cultivation in such a variable media implies certain disadvantages related to the competition with other microorganisms, especially with green microalgae. Certain studies have related competition relationships to abiotic factors such as temperature, nutrients and pH (Tang et al., 1997). Although several studies in lakes and reservoirs have dealt with the importance of nutrients' interaction with algal composition (Dolman et al., 2012), there are comparatively few studies focusing on the relation between nutritional conditions in wastewater and the presence of cyanobacteria (Arias et al., 2017; Van Den Hende et al., 2016a). Hence, the factors controlling these competition relationships are still not completely understood. Recently, Arias et al. (2017) successfully selected and maintained a dominant population of cyanobacteria from an initial green algae dominated consortium under a long term operation. The dominance of cyanobacteria over green algae as *Chlorella* sp. and *Stigeoclonium* sp. was associated to the total nutrients concentration controlling competence relationships, in particular low inorganic phosphorus loads (<0.23 mg IP L d^{-1}) and N:P ratios between 16:1-49:1 (molar basis).

However, this factor cannot be considered as the only aspect favoring the competition of cyanobacteria since other green microalgae as *Scenedesmus* sp. can also tolerate low phosphorus content and high N:P ratios (Arias et al., 2018; Gantar et al., 1991; Xin et al., 2010). Indeed, *Scenedesmus* sp. has been widely reported in different types of wastewater treatment systems (e.g. waste stabilization ponds and high rate algal ponds) because of its high tolerance to a wide range of N and P loads (Xin et al., 2010); besides, it is considered as one of the best competitors for inorganic carbon in comparison to cyanobacteria and other green algae (Ji et al., 2017). These similar optimal nutrient conditions for *Scenedesmus* sp. and cyanobacteria

cultures suggests that the latter could be highly exposed to contamination and competence. This fact would represent a serious drawback during cyanobacterial biomass production, and production strategies should be developed in order to improve cyanobacteria competition over this kind of organisms.

Contrary to *Scenedesmus* sp. and other eukaryotic microalgae, many species of cyanobacteria have the ability to easily form aggregates in the culture and promote a high settleability and natural gravity harvesting (Arcila and Buitrón, 2016; de Godos et al., 2014). This advantage can be used as a strategy to select cyanobacteria over unsettling green algae. Thus, the use of optimized hydraulic retention time (HRT) and solids retention time (SRT) during biomass production can be used under continuous operation to select microorganisms able to form flocs fast, while unsettling microorganisms will be continuously removed. However, despite being a promising alternative, uncoupled HRT and SRT can also lead to changes related to nutrients loads and their consequences on microbial changes need to be addressed.

Taking all this into consideration, the main objective of this chapter is to select cyanobacteria from an initial consortium dominated by unsettled green algae (*Scenedesmus* sp.), by means of applying uncoupled HRT and SRT in a photo-sequencing batch reactor (PSBR) using secondary effluent and digestate as feedstock. This work aims to evaluate the effect of batch operation (including a settling phase), nutrients loads and ratios, and SRT and HRT conditions on the dominance of cyanobacteria in mixed cultures.

6.3 Material and methods

6.3.1 Experimental set up

6.3.1.1 Inoculum

The experimental set-up was located at the laboratory of the GEMMA research group (Universitat Politècnica de Catalunya. BarcelonaTech, Spain). A mixed culture was used as inoculum; its composition was evaluated by microbial counting and revealed a consortium mostly dominated by *Scenedesmus sp.* (93±2%), with other species of green algae (4±1%), cyanobacteria (2±1%) and diatoms (1±0.01%). Microscopic images of the initial culture biomass are shown in Fig. 6.1. It was obtained from a pilot tertiary wastewater treatment system consisting of a closed photobioreactor (PBR) (30 L) fed with urban secondary wastewater and

liquid digestate (not centrifuged), operated at a HRT of 10 days. Operational details and other characteristics of this system can be found in detail elsewhere (Arias et al., 2018a). The biomass was collected from a harvesting tank connected to the PBR and it was thickened by gravity in laboratory Imhoff cones during 30 min before inoculation.

6.3.1.2 Experimental set-up

The experiments were carried out indoors. Three experimental closed PBRs of polymethacrylate and cylindrical shape, with an inner diameter of 11 cm, a total volume of 3 L and a working volume of 2.5 L were installed (Fig. 6.2). Each PBR was inoculated with 100 mL of thickened biomass and filled up to 2.5 L with deionized water.

All the PBRs were continuously maintained in alternate light:dark phases of 12 h and continuously agitated (with the exception of the settling periods) with a magnetic stirrer (Selecta, Spain) set 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 PBR. Illumination during the light phase was supplied by two external halogen lamp (60W) placed 11 cm from each PBR. Each two sets provided 220 μmol m^{-2} s^{-1} of light. pH was continuously measured with a pH sensor (HI1001, HANNA, USA) and kept at 8.5 with a pH controller (HI 8711, HANNA, USA) by the automated addition of HCl 0.1 N and NaOH 0.1 N. Automatic addition and withdrawal of the mixed liquor, the supernatant and the feeding in the PBRs was carried out automatically by peristaltic pumps.

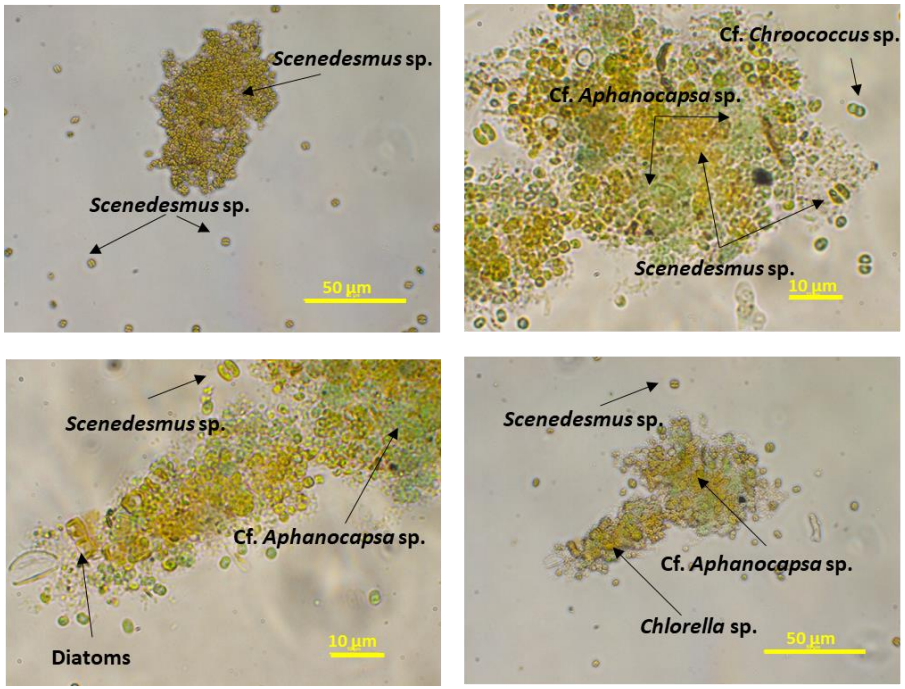


Fig. 6.1 Microscopic images of the inoculum observed in bright light microscopy at 500x and 1000x. Algal flocs and dispersed cells are composed of green algae *Scenedesmus* sp. and *Chlorella* sp., cyanobacteria cf. *Aphanocapsa* sp., cf. *Chroococcus* sp. and diatoms.

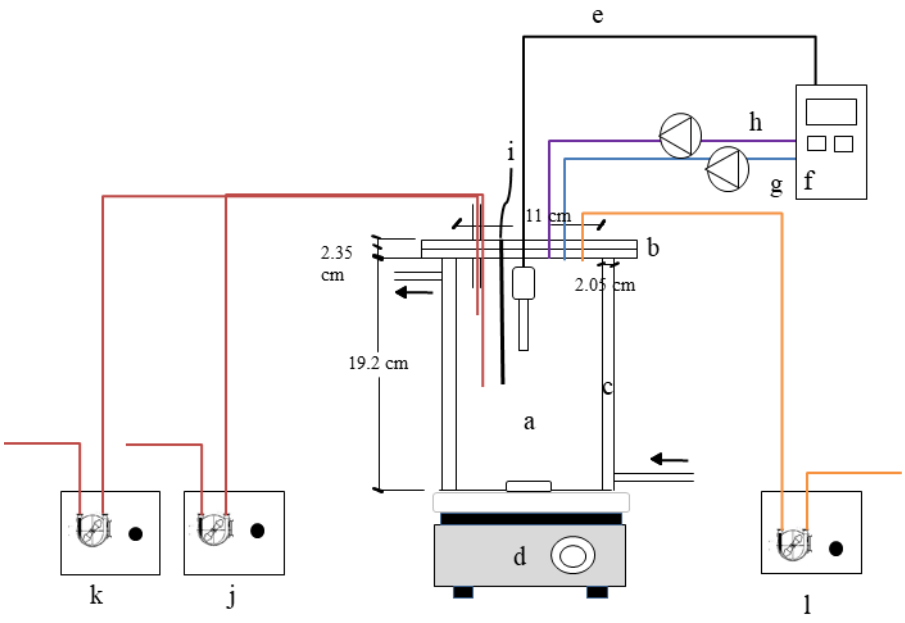


Fig. 6.2 Schematic diagram of each photobioreactor set-up: a) Body of the PBR, b) cover, c) water jacket, arrows indicate the water flux by the water jacket around the PBR, d) magnetic stirrer, e) pH sensor, f) pH controller, g) acid solution input, h) Basic solution input, i), temperature sensor, j) peristaltic pump controlling mixed liquor withdrawal, k) peristaltic pump controlling supernatant withdrawal (only for sequencing batch operation) and l) peristaltic pump controlling influent input.

Different uncoupled and coupled hydraulic retention time (HRT) and solids retention time (SRT) conditions were tested during an experimental time of 30 days. Under all the conditions, theoretical SRT was fixed to 10 d. Two PBRs were operated as a sequencing batch reactors (PSBR) with different HRT. Thus, one PBR, named $PSBR_6$, was operated with an HRT of 6 days, while the other, named $PSBR_4$, was operated at an HRT of 4 days. A semi-continuous photobioreactor (namely SC_{10}) was operated with coupled HRT/SRT of 10 days and used as control. Operational diagram of each PBR can be observed in Fig. 6.3. In $PSBR_6$, 0.250 L of the mixed liquor were withdrawn at the end of the dark phase. Posteriorly, the agitation was stopped and biomass was allowed to settle during 30 minutes, followed by the withdrawal of 0.167 L of the supernatant.

Total volume retired of 0.417 mL was replaced by the same volume of wastewater influent. Otherwise, in $PSBR_4$, 0.250 L of the mixed liquor were withdrawn at the end of the dark phase, followed by 30 min of biomass settling and a subsequent withdrawal of 0.375 of the supernatant. Total volume retired of 0.625 L was replaced by the same volume of wastewater influent. All the experiments were submitted to light/dark cycles of 12 h each. It should be noticed that real SRT calculated in the PSBRs was conditioned by the solids withdrawn in the mixed liquor prior to sedimentation and the solids contained in the supernatant. On the other hand, in SC_{10} 0.250 L of the mixed liquor was withdrawn at the end of the dark phase and subsequently replaced by the same volume of wastewater influent.

Wastewater influent consisted in digestate diluted in secondary effluent in a ratio of 1:50. Digestate was obtained daily from a lab-scale microalgae anaerobic digester (1.5 L, flow of 0.075 L^{-1} d^{-1}), operated at 35 °C and a SRT and HRT of 20 days. While secondary effluent was obtained from a secondary settler after a high rate algal pond (HRAP) treating urban wastewater (Gutiérrez et al., 2016a; Passos et al., 2014b). Dilution of 1:50 was chosen according to a previous study by Arias et al., (2017) (Chapter 4), which showed the suitability of this dilution rate for cyanobacteria growth. Characteristics and operation of the anaerobic digester are detailed in Passos et al. (2013). Characteristics of the digestate, the secondary effluent and the wastewater influent mixture are shown in Table 6.1.

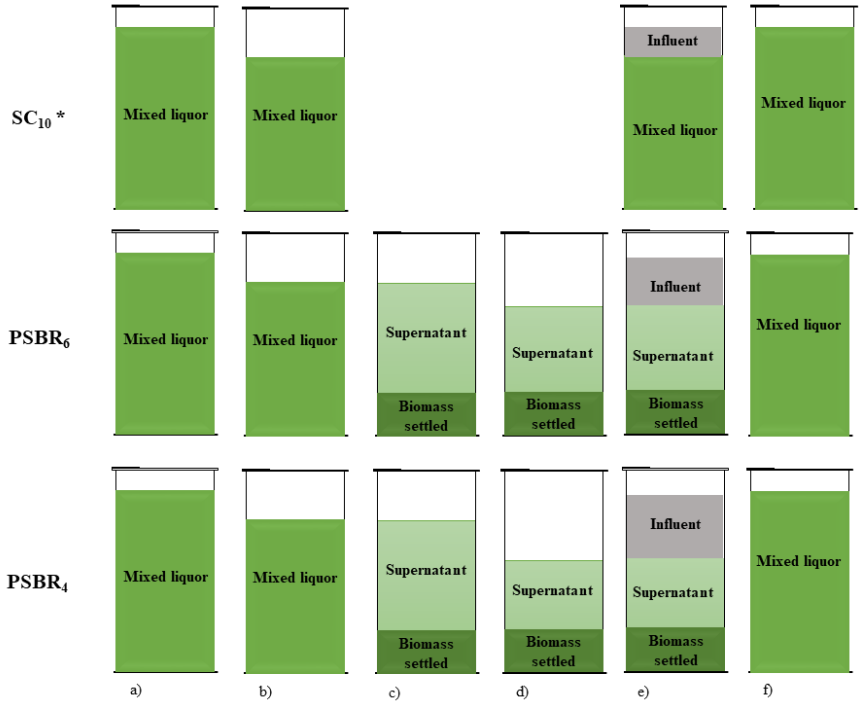


Fig. 6.3 Scheme of operation of the photobiorreactors showing the process during the last minutes of the dark phase; a)-b) biomass removal (5 min); c) biomass settling (30 min); d) supernatant removal (10 min); e-f) effluent addition (10 min) and mixing. Biomass separation in SC_{10} was performed in an independent process.

Table 6.1 Average (standard deviation) of the main quality parameters of the digestate, secondary effluent and the influent (mixture of digestate and secondary effluent) (n=5–10).

Parameter	Digestate	Secondary effluent	Influent[a]
TSS [g·L^{-1}]	28.09 (6.15)	-[b]	0.56 (0.12)
VSS [g·L^{-1}]	21.50 (4.45)	-[b]	0.43 (0.09)
TOC [mg·L^{-1}]	15999.74 (1337.55)	18.35 (2.06)	320.36 (26.79)
SOC [mg·L^{-1}]	3117.68 (315.51)	18.37 (2.05)	62.72 (6.35)
TIC [mg·L^{-1}]	5538.51 (845.8)	21.50 (1.78)	111.19 (16.95)
TN [mg·L^{-1}]	3555.50 (1036.51)	23.50 (1.54)	71.58 (20.76)
TAN [mg·L^{-1}]	1020 (93.95)	-[c]	20.41 (1.88)
N-NO_2^-[mg·L^{-1}]	<LOD	1.73 (0.29)	1.73 (0.29)
N-NO_3^-[mg·L^{-1}]	<LOD	8.12 (3.07)	8.12 (3.07)
TIN [mg·L^{-1}]	1020.02 (257.05)	9.85 (4.94)	30.25 (5.24)
TON [mg·L^{-1}]	2535.02 (779.45)	13.65 (3.91)	40.18 (12.06)
TP [mg·L^{-1}]	1000.8 (93)	0.69 (0.52)	20.03 (1.87)
IP [mg·L^{-1}]	<LOD	1.97 (3.41)	1.97 (3.41)
TOP [mg·L^{-1}]	920 (90)	-[d]	18.40 (1.80)
TOC:TIC[e]	-	-	2.88:1
TN:TP[e]	-	-	7.9:1

<LOD Limit of detection.

[a] The influent was prepared as a dilution of digestate within secondary effluent in a 1:50 ratio.

[b] TSS and VSS in the secondary effluent corresponded to values <0.03 g L^{-1}.

[c] TAN in the secondary effluent corresponded to values <0.002 mg L^{-1}.

[d] TOP in the secondary effluent corresponded to values <0.09 mg L^{-1}.

[e] Ratio in molar bases

6.3.2 Microbial evolution

Quantitative analysis of microalgae and cyanobacteria was performed once a week by microscopic area counting (cells·mL^{-1}) (Guillard and Sieracki, 2005). To this aim, 20 μL of mixed liquor were added to a slide with a coverslip and individual cells were counted per field until reach ~400 cells. Microalgae were quantified in bright field microscopy at 40X, while cyanobacteria species were counted using fluorescence microscopy with the operation of filters containing an excitation filter (510-560 nm), emission filter (590 nm) and dichroic beam splitter (575 nm). Both bright field and

fluorescence microscopy were performed using a fluorescence microscope (Eclipse E200, Nikon, Japan).

Qualitative evaluation of microalgae composition changes within each PBR was monitored by microscopy once a week. Microbial visualization was performed in an optic 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 (Bourrelly, 1985; Palmer, 1962), as well as a database of cyanobacteria genus (Komárek and Hauer, 2013b).

6.3.3 Analytical methods

Samples taken from influent wastewater (digestate + secondary effluent) and effluent of the three PBRs (supernatant after settling) were analyzed for nutrients concentration. It should be noticed that nutrients in the effluent are equivalent to the nutrients contained in the mixed liquor of the culture. Note that in the case of SC_{10}, the supernatant sample was taken from the mixed liquor withdrawn and subsequently submitted to a separation process (30 minutes biomass settling) in order to remove biomass from the effluent.

Nutrients analysis were performed three times per week for total organic carbon (TOC), total inorganic carbon (TIC) and soluble organic carbon (SOC), inorganic phosphorus (IP) measured as $P\text{-}PO_4^{3-}$, nitrite ($N\text{-}NO_2^-$), nitrate ($N\text{-}NO_3^-$) and total ammoniacal nitrogen (TAN). Total nitrogen (TN) and total phosphorus (TP) were measured two days per week. Total ammoniacal nitrogen (TAN) (sum of $N\text{-}NH_3$– and $N\text{-}NH_4^+$) was determined using the colorimetric method indicated in Solorzano (1969). Ion chromatography was used to measure concentrations of IP, $N\text{-}NO^{2-}$ and $N\text{-}NO^{3-}$ by means of a DIONEX ICS1000 (Thermo-scientific, USA), whereas TOC, TIC and TN were analyzed by using a C/N analyzer (21005, Analytikjena, Germany). TP was analyzed following the methodology described in Standard Methods (APHA-AWWA-WPCF, 2001). Total inorganic nitrogen (TIN) was calculated as the sum of $N\text{-}NO_2^-$, $N\text{-}NO_3^-$ and TAN. Total organic nitrogen (TON) (the sum of dissolved and particulate form) was calculated as the difference between TN and $N\text{-}NO_2^-$ and $N\text{-}NO_3^-$, whereas total organic phosphorus (TOP) (the sum of dissolved and particulate form) was determined as the difference between TP and IP.

Total suspended solids (TSS) and volatile suspended solids (VSS) were measured in the mixed liquor (in all the PBRs) and in the supernatant (only in $PSBR_6$ and $PSBR_4$) three days per week while Chlorophyll *a* concentration was measured in the cultures once per week. Those procedures were based in the methodology described in Standard Methods (APHA-AWWA-WPCF, 2001). Dissolved oxygen (DO) was measured daily in the mixed liquor of each PBR, using a dissolved oxygen-meter (Thermo-scientific, USA). All parameters defined above were determined in triplicate, and measured in samples taken at the end of the dark phase.

6.3.4 General calculations

Real SRT [d^{-1}] was calculated as follows in eq. 1:

$$\mathbf{SRT} = \frac{V}{[Qm + Qs(\frac{TSSx}{TSSm})]} \quad (1)$$

Where: V [L^{-1}] is the volume of the PBR, Q_m [L^{-1}] is the mixed liquor discharge flow, Q_s is the supernatant discharged flow, TSS_m [mg L^{-1}] is the mixed liquor suspended solids concentration and TSS_x [mg L^{-1}] is the supernatant suspended solids concentration.

Settleability [%] was determinate according to the following formula eq. 2:

$$\mathbf{Settleability} = \mathbf{100} * \left[\mathbf{1} - \left(\frac{\boldsymbol{TSSs}}{\boldsymbol{TSSx}}\right)\right] \quad (2)$$

Where TSS_m [mg L^{-1}] is the mixed liquor suspended solids concentration and TSS_x [mg L^{-1}] is the supernatant suspended solids concentration.

Biomass production of each PBR in [g VSS $L^{-1}d^{-1}$] was estimated following eq. 3:

$$\mathbf{Biomass\ production} = \frac{\boldsymbol{Q*VSS}}{\boldsymbol{V}} \quad (3)$$

where Q is the flow [$L^{-1}d^{-1}$], VSS is the biomass concentration in the PBR [g L^{-1}] and V [L^{-1}] is the volume of the PBR.

Applied nutrients (TOC, TIC, TAN, N-NO_2^-, N-NO_3^-, TIN, TON, TN, IP, TOP and TP) volumetric load (Lv-Z) in [mg Z $L^{-1}d^{-1}$] was calculated as follows:

$$\mathbf{Lv-Z} = \frac{\boldsymbol{Q}*\boldsymbol{X}}{\boldsymbol{V}} \quad (3)$$

Where Q is the flow [$L^{-1}d^{-1}$], Z is the corresponding nutrient influent concentration [mg Z L^{-1}] and V [L^{-1}] is the volume of the PBR.

6.4 **Results**

6.4.1 Settling efficiencies and real SRT

In this chapter the introduction of a settling phase to the PBRs operation was assessed in order to select flocs forming biomass and remove the unsettled biomass, composed mostly by green algae *Scenedesmus* sp., achieving an increase in the abundance of cyanobacteria from an initial mixed green algae-cyanobacteria consortium. Indeed, the settling phase tested promoted cyanobacteria dominance, but the relative abundance of cyanobacteria in the different PSBRs was also influenced by the SRT and nutritional loads.

As observed in Fig. 6-S1, settleability increased after the settling phase from 38% to 90% in $PSBR_6$ and from 24% to 77% in $PSBR_4$. This increase was caused by the natural gravity harvesting of the cultures. These changes in settleability affected the SRT in the cultures. In $PSBR_6$, the calculated SRT at day 1 was 7.1 d, increasing until reaching a SRT of 9 days, which remained constant after day 12 until the end of the experiment. On the other hand, the operation of $PSBR_4$ started with a SRT of 6.6 d that increased until reaching a SRT of approximately 8 d after the day 8 of operation. On the contrary, in SC_{10} the operation consisted in a coupled HRT/SRT of 10 days, and the theoretical SRT remained as absolute value, since no sedimentation was performed.

6.4.2 Biomass production and microbial evolution

The changes in settleability and SRT also had an impact in the biomass concentration, as shown in Fig. 6.4. The biomass concentration in SC_{10} (with a HRT/SRT of 10 d) showed a quite constant pattern during all

the experimental time (0.56±0.05 g VSS L^{-1}). $PSBR_6$ and $PSBR_4$ showed an increase in the biomass during the first 12 days of operation, until reaching 0.57±0.09 g VSS L^{-1} and 0.65±0.072 g VSS L^{-1}, respectively, from day 15 onwards. The highest biomass production was achieved in $PSBR_4$ (0.16 g $VSS \cdot L^{-1} \cdot d^{-1}$), higher than those observed in SC_{10} (0.06g $VSS \cdot L^{-1} \cdot d^{-1}$) and $PSBR_6$ (0.12g $VSS \cdot L^{-1} \cdot d^{-1}$).

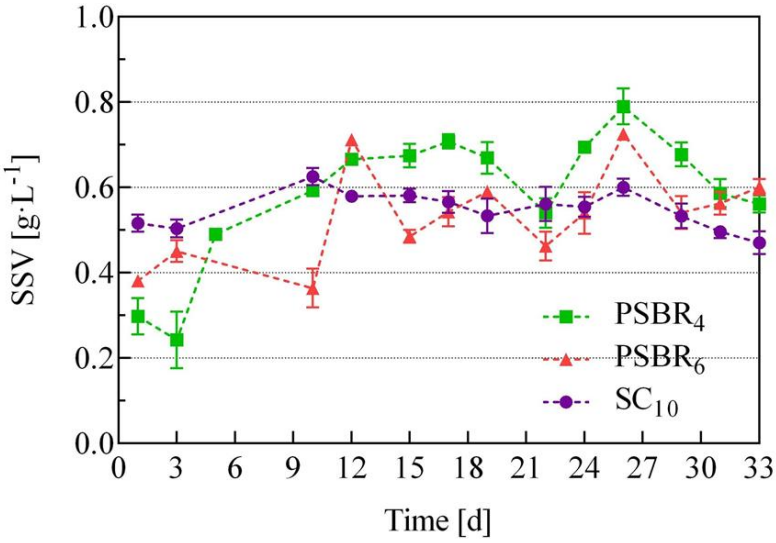


Fig. 6.4 Biomass concentration contained in SC_{10}, $PSBR_6$ and $PSBR_4$ during the experimental time. Biomass is given as volatile suspended solids (g VSS L^{-1}).

Microscopic observations during the last 10 days of operation are provided in Fig. 6.5 and clearly show how the culture in the SC_{10} remained mostly dispersed and with a population dominated by Scenedesmus sp. On the contrary, the cultures in $PSBR_6$ were turned into a culture dominated by flocs throughout the experimental time. More interestingly, a more predominant presence of flocs formed by cyanobacteria Aphanocapsa sp. was observed. In the case of $PSBR_4$, dispersed cells of Scenedesmus sp. were observed in the culture, while other green algae as Stigeoclonium sp. were also present.

These observations agreed with the microbial counting of the biomass in the three PBRs. Biomass in SC_{10} showed the presence of microalgae Scenedesmus sp. throughout the experimental time with a slow

decreasing trend, from an initial abundance of 91% to 74%. While other species such as cyanobacteria and green algae as Chlorella sp. and Stigeoclonium sp. increased from the initial 4% to 7% and 14%, respectively (Fig. 6.6a). In this case, only diatoms and protozoa remained with the same abundance than in the beginning (1-2%) during all the experimental time.

On the other hand, the initial Scenedesmus sp. abundance in $PSBR_6$ was reduced from the initial 95% to only 7% (88% *Scenedesmus* sp. decrease) at the end of the experiment (Fig. 6.6b). Interestingly, as *Scenedesmus* sp. population lowered, cyanobacteria abundance increased with respect to other microorganism from an initial 2% until up to 70% from day 9 onwards. The abundance of other green algae than Scenedesmus sp. also gradually increased from 3% to 28% on day 19 and then decrease to 21% on day 33. Other microorganisms as diatoms and protozoa remained constant (2% and 1%, respectively) during all the experimental time.

In $PSBR_4$, *Scenedesmus* sp. abundance was gradually reduced from the initial 94% until reaching 52% (48% *Scenedesmus* sp. removal). On the contrary, cyanobacteria abundance increased, reaching 15% in the last day of operation from an initial 2%. Other green algae abundance also increased from 4% until 29% in the last day of operation (Fig. 6.6c). Other microorganisms as diatoms and protozoa where also observed in all the experimental time, remaining an abundance of 1-2%.

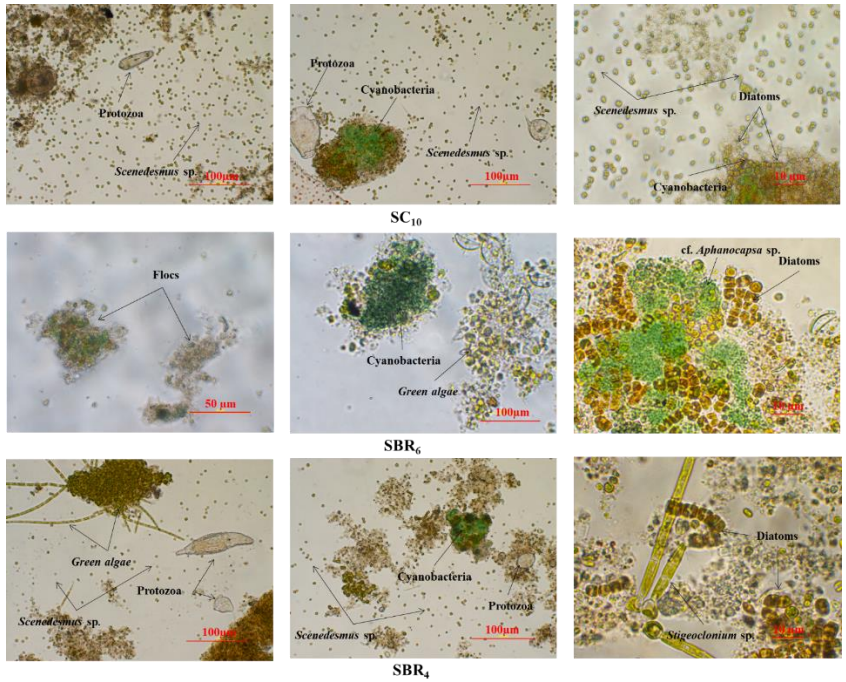


Fig. 6.5 Microscopic images illustrating the microbial composition of SC_{10}, operated with a HRT 10d, $PSBR_6$ operated with a HRT of 6 d and $PSBR_4$ operated with a HRT of 4 d, during the last ten days of operation. Observed in bright light microscopy at different scales.

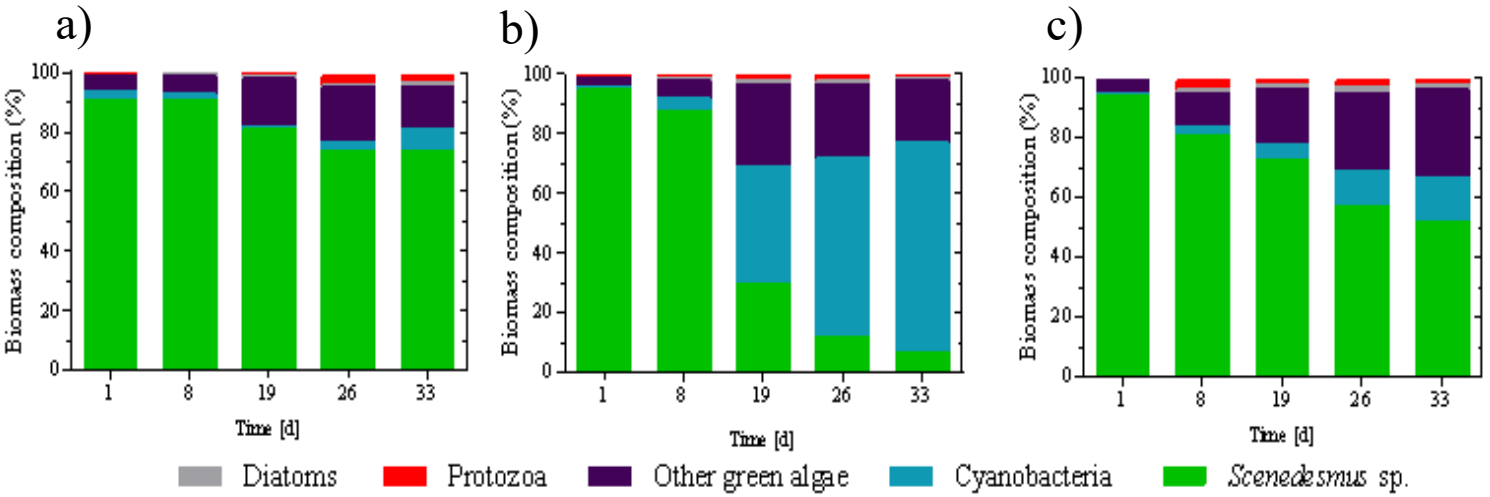


Fig. 6.6 Biomass composition of a) SC_{10}, b) $PSBR_6$ and c) $PSBR_4$. Percentages were calculated considering the total cells mL^{-1}.

Although the presence of heterotrophic bacteria was not directly quantified in this chapter, it was indirectly evaluated through the ratio of chlorophyll a mass (implying green algae and cyanobacteria) divided by total biomass (VSS) (sum of green algae and/or cyanobacteria and hetherotrophic bacteria). Thus, the highest content of photosynthetic microorganism was found in SC_{10} control, with a Chlorophyll *a*/VSS ratio of 1.03 mg Chl *a*/g VSS, whereas $PSBR_6$ and $PSBR_4$ reached values of 0.56 and 0.53 mg Chl *a*/g VSS, respectively. Lower values in PSBRs indicate highest content of bacteria, also according to the higher organic applied.

6.4.3 Nutritional conditions

6.4.3.1 Nutrients uptake and wastewater treatment

Unsettling and settling operation performed in this chapter led to different nutritional loads in the PBRs by means of the operational HRT (Table 6.2). In general, the volumetric loads Lv-TIC/Lv-TOC, as well as all the nitrogen and phosphorus forms, increased in each PBR in relation to the decrease in HRT. These differences in the loads caused different culture compositions (Table 6.3) and led a selective pressure on microbial populations, as has been shown in previous Sections.

Table 6.2 Average (standard deviation) of the nutrients volumetric loading (Lv) in each photoreactor.

Parameter	SC_{10}	$PSBR_6$	$PSBR_4$
Lv-TOC [mg·L^{-1}·d^{-1}]	32.04 (2.68)	53.31 (4.46)	80.09 (6.70)
Lv-TIC [mg·L^{-1}·d^{-1}]	11.12 (1.70)	18.50 (2.82)	27.80 (4.24)
Lv-TN [mg·L^{-1}·d^{-1}]	7.04 (2.08)	11.72 (3.45)	17.61 (5.19)
Lv-TAN [mg·L^{-1}·d^{-1}]	2.04 (0.19)	3.39 (0.31)	5.10 (0.47)
Lv-N-NO_3^- [mg·L^{-1}·d^{-1}]	0.81 (0.31)	1.35 (0.51)	2.03 (0.77)
Lv-N-NO_2^- [mg·L^{-1}·d^{-1}]	0.17 (0.03)	0.29 (0.05)	0.43 (0.07)
Lv-TIN [mg·L^{-1}·d^{-1}]	3.03 (0.52)	5.03 (0.87)	7.56 (1.31)
Lv-TON [mg·L^{-1}·d^{-1}]	4.02 (1.21)	6.69 (2.01)	10.05 (3.02)
Lv-TP [mg·L^{-1}·d^{-1}]	1.24 (0.45)	2.06 (0.75)	3.09 (1.12)
Lv-IP [mg·L^{-1}·d^{-1}]	0.20 (0.34)	0.33 (0.57)	0.49 (0.85)
Lv-TOP [mg·L^{-1}·d^{-1}]	1.04 (0.11)	1.73 (0.18)	2.60 (0.26)

Table 6.3 Average values (standard deviation) of the main parameters in the effluent samples (n=8-11).

Parameter	SC_{10}	$PSBR_6$	$PSBR_4$
TIC [mg·L^{-1}]	63.16 (47.65)	50.85 (16.53)	96.15 (51.09)
SOC [mg·L^{-1}]	61.83 (12.96)	48.84 (8.03)	55.67 (8.68)
TN [mg·L^{-1}]	7.65 (7.13)	17.07 (14.1)	8.02 (17.18)
TAN [mg·L^{-1}]	0.07 (0.09)	2.064 (2.75)	0.21 (0.16)
N-NO_2^-[mg·L^{-1}]	0.35 (0.66)	0.67 (1.27)	1.49 (1.29)
N-NO_3^-[mg·L^{-1}]	1.03 (0.85)	11.10 (8.44)	5.08 (4.89)
TIN [mg·L^{-1}]	1.24 (0.84)	13.82 (8.45)	6.79 (6.85)
TON [mg·L^{-1}]	6.41 (6.29)	3.25 (5.65)	1.23 (10.33)
TP [mg·L^{-1}]	0.39 (0.69)	3.35 (2.66)	2.35 (2.33)
IP [mg·L^{-1}]	0.38 (0.67)	0.89 (0.94)	0.96 (0.92)
TOP [mg·L^{-1}]	0.01 (0.02)	2.46 (1.72)	1.39 (1.41)

Total nitrogen, phosphorus and carbon concentrations in the effluent (therefore, concentrations in the PBRs) with respect to the load applied are shown in Fig. 6.7. As it can be observed, TN and TP in SC_{10} were completely consumed during the experiment (Fig. 6.7a). TIN was almost completely eliminated during the experiment, whereas TON decreased gradually. Conversely, IP was completely removed during the experimental time. Likewise, TOP value was completely consumed along the experiment.

In the case of the effluent of $PSBR_6$, TN was not completely eliminated due to an increase in TIN (Fig. 6.7b). It should be noticed that the Lv-TIN was composed of 60% TAN and 30% N-NO_2^-+N-NO_3^- (Table 6.1), and in the effluent, 98% of the TIN concentration in the PSBR effluent was based on N-NO_3^- values. This result suggests a high nitrification activity. On the other hand, IP was completely consumed during the first 15 days of operation, and afterwards it occasionally showed concentrations above 1.5 mg L^{-1} in the effluent. This increase in the IP concentration can be associated also to the mineralization of OP in this PSBR, since TOP concentration in the effluent maintained values between 3.02 to 4.2 mg L^{-1} (Table 6.3). Note that part of TON and TOP concentration is related by particulate forms caused by intracellular algae. Thus, concentrations of TOP may be associated to intracellular polyphosphates accumulated in unsettling biomass.

$PSBR_4$ effluent showed a similar pattern to that found in $PSBR_6$ regarding TIN, TON, IP and TOP concentrations. However, it is clear that $PSBR_4$ showed a better TN uptake despite the higher loads applied. Hence, a complete

transformation of TON to TAN was observed along the experiment. While an efficient removal of the influent TIN was achieved during the first half of the experiment (Fig. 6.7c). After that period, TIN values remained around 10 mg L^{-1} (mostly in the form of $N\text{-}NO_3^-$), that are lower than those observed in $PSBR_6$. Similarly, IP was also completely eliminated during the first half of the experiment and then the effluent showed values around 2 mg L^{-1}, mostly due to the mineralization of the accumulated TOP, that was more pronounced in the second half of the experiment (Fig. 6.7c).

On the other hand, according to the SOC concentrations in the PBRs, a better removal of TOC was observed with high loads. For instance, SC_{10} showed higher concentrations (61.8±12.0 mg L^{-1}) than $PSBR_6$ (48.8±8.0 mg L^{-1}), indicating a better elimination of organic matter in spite of having and initial higher loads than that of the SC_{10} (Fig. 6.7b). In the case of $PSBR_4$, it showed similar concentrations than $PSBR_6$. As observed in Fig. 6.6c, with the exception of the results obtained on day 4, the concentrations of TOC along the experimental time showed values of approximately 50 mg L^{-1}.

Main nutrients and organic matter removals are presented in Table 6.4. In general, SC_{10} showed the highest removal percentages of all N and P forms (>80%), whereas $PSBR_6$ and $PSBR_4$ reached values between 54% and 78% of TN and TP removal. Indeed, previous studies have related high nutrients removal efficiencies in photobioreactors operated under long HRT (Muñoz and Guieysse, 2006). Remarkably, in spite that the PSBRs showed lower removal percentages, they reached higher removal rates than SC_{10} due to the higher volume of wastewater treated.

Conversely, in the case of organic matter removal, SC_{10} showed the lower removal efficiency, achieving only 41% of TOC removal, while both PSBRs doubled that removal percentage (Table 6.4). Similarly, higher removal rates were observed in both PSBRs with respect to $SC_{10,}$ more specifically $PSBR_4$ showed the highest removal rates of all the PBRs. These high performances observed in both PSBRs for TOC elimination can be associated to the appearance of heterotrophic bacteria in the culture as a consequence of the high loads of TOC introduced to the PBRs (Van Den Hende et al., 2014).

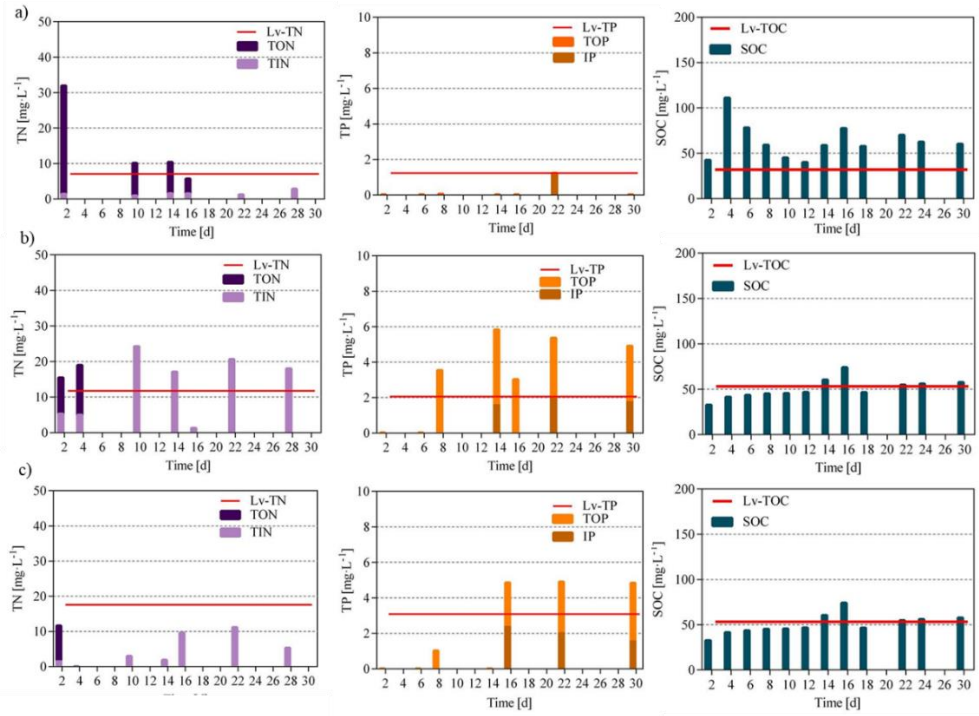


Fig. 6.7 Time course of effluent concentration for total nitrogen (TIN), total phosphorus (TP) and total organic carbon (TOC) in [mg L^{-1}]. a) SC_{10}, b) $PSBR_6$ and c) $PSBR_4$. Lines represents the average volumetric load in [mg L^{-1} d^{-1}.

Table 6.4 Removal performance and nutrients removal rate of the effluent of SC_{10}, $PSBR_6$ and $PSBR_4$ during the experimental time (n=8-11).

Parameter	SC_{10}		$PSBR_6$		$PSBR_4$	
	Removal [%]	Removal rate [mg L^{-1} d^{-1}]	Removal [%]	Removal rate [mg L^{-1} d^{-1}]	Removal [%]	Removal rate [mg L^{-1} d^{-1}]
TIC [mg·L^{-1}]	43.17 (39.60)	4.8 (4.40)	54.95 (26.91)	10.17 (4.98)	13.40 (15.99)	3.72 (4.45)
TOC [mg·L^{-1}]	40.71 (15.68)	25.85 (1.74)	84.77 (6.11)	45.19 (1.13)	82.61 (12.88)	66.1 (3.58)
TAN [mg·L^{-1}]	99.64 (0.97)	2.03 (0.02)	89.88 (26.25)	3.05 (0.89)	98.97 (1.42)	5.05 (0.07)
N-NO_2^-[mg·L^{-1}]	79.77 (69.48)	0.14 (0.12)	61.27 (7.36)	0.18 (0.02)	13.87 (16.76)	0.06 (0.08)
N-NO_3^-[mg·L^{-1}]	87.34 (26.73)	0.71 (0.22)	-	-	-	-
TIN [mg·L^{-1}]	95.90 (6.55)	2.90 (0.20)	54.31 (57.39)	2.73 (2.89)	77.55 (44.09)	5.87 (3.33)
TON [mg·L^{-1}]	91.04 (15.42)	6.52 (1.10)	95.46 (14.04)	11.37 (1.67)	98.29 (9.69)	17.5 (1.73)
TN [mg·L^{-1}]	80.96 (32.40)	3.25 (1.30)	57.52 (68.21)	3.85 (4.56)	80.05 (50.45)	8.04 (5.07)
IP [mg·L^{-1}]	80.71 (34.01)	0.16 (0.07)	54.82 (47.72)	0.18 (0.16)	51.27 (46.70)	0.25 (0.23)
TOP [mg·L^{-1}]	99.95 (0.11)	1.04 (0.00)	86.63 (9.35)	1.50 (0.16)	92.45 (7.66)	2.89 (0.20)
TP [mg·L^{-1}]	98.05 (3.44)	1.22 (0.04)	83.28 (13.28)	1.72 (0.27)	88.27 (11.63)	3.06 (0.36)

On the other hand, TIC, required by autotrophic microorganisms, specially by green algae and cyanobacteria, was unlimited in the three PBRs and concentrations presented different patterns. In the case of SC_{10}, TIC concentration was accumulated from day 2 to day 14, when it reached a concentration of 147.5 mg L^{-1}, higher than that of the influent (111.20±16.95 mg L^{-1}) After that day, TIC concentration decreased to values lower than 10 mg L^{-1} in the last days of operation. In the $PSBR_6$, IC concentration increased from 25.73 mg L^{-1} in the day 2 until 64.63 in day 11. After that day, the concentration decreased until day 24 to values close to 50 mg L^{-1}; the average uptake of this compound was 55%. In $PSBR_4$, high concentrations of TIC were observed during the first half of the experimental time, achieving concentrations up to 150 mg L^{-1}. After this period, TIC concentrations maintained a generally stable pattern of approximately 50 mg L^{-1} until the last days of operation. The uptake of TIC during all the period in $PSBR_6$ and during the second half of $PSBR_4$ agreed with the nitrification occurring in these PBRs.

6.5 **Discussion**

According to the results obtained, the competition dynamics of Scenedesmus sp., other green algae and cyanobacteria in SC_{10}, $PSBR_6$ and $PSBR_4$ were defined by the pressure created by the coupling and uncoupling HRT/SRT. It is clear that the SC_{10}, employing a coupled SRT/HRT, maintained the same biomass concentration and population during most of the experiment. However, the settling phase produced by the uncoupling of HRT and SRT employed in $PSBR_6$ and $PSBR_4$ affected the biomass growth and competition dynamics of the microorganisms present in the culture. It is interesting to note that $PSBR_6$ showed a higher efficiency in removing unsettled Scenedesmus sp. than $PSBR_4$, despite that the latter had a highest unsettled volume withdrawn. This clearly imply that other factors as nutrients content and changes in SRT than the washing-out of unsettled microorganisms provided by the uncoupled SRT/HRT played an important role in microorganism's dynamics.

Biomass composition and growth presented different patterns according to the N and P concentrations and ratios in the influent and effluent of each PBR. Even though influent TN:TP ratio was the same for all the PBRs (7.9:1), nutrient loads were different (Table 6.2) and led to different influent and effluent N:P ratios, biomass production and the main dominant algae (Table 6.5). It should be noticed that N:P ratios in the influent includes all dissolved and particulate forms, whereas the ratio in the effluent only includes TIN:TIP ratio, since it represents the direct available nutrients for microalgae/cyanobacteria (Pick and Lean, 1987). In SC_{10}, the

dominance of *Scenedesmus* sp. over other species can be associated to the low loads of N and P introduced to the culture (Table 6.2) in addition to the absence of a settling phase. Low nutrient loads promoted nitrogen limitation in the culture (low TIN:TIP ratio). That limitation, coupled with the lack of washing-out of *Scenedesmus* sp. reduced the possibilities of other species to grow and compete with the initial culture.

On the other hand, $PSBR_6$ showed the highest TIN:TIP ratio (mainly caused by the nitrification process), suggesting P limitation in the culture. This ratio favoured the increase in cyanobacteria population, in particular the dominance of unicellular cf. Aphanocapsa sp.. In addition, the washing-out of other possible unsettling P tolerant microorganisms such as Scenedesmus sp. contributed to improve the dominance of cyanobacteria. It should be noted that, despite of P limitation, cyanobacteria dominated culture in $PSBR_6$ achieved a higher biomass production than SC_{10}. This fact can be associated to the capability of cyanobacteria to accumulate phosphorus as polyphosphates and perform luxury uptake (uptake of P in excess of their need for growth) (Cottingham et al., 2015). High biomass production of cyanobacteria under limiting P conditions has also been observed in previous studies (Arias et al., 2017; Arias et al., 2018). Furthermore, the ratio obtained in $PSBR_6$ is within the optimum range of 16:1 - 49:1, proposed in a previous study by Arias et al., (2017) (Chapter 4) and that related the dominance of wastewater borne cyanobacteria in competition to green algae to high IN:IP values.

In the case of $PSBR_4$, high nutrients loads (Table 6.2) led to a TIN:TIP ratio in the influent similar to the Redfield ratio (16:1 in molar basis), considered the optimum ratio for microalgae growth (Redfield, 1958). This absence of nutrients caused a fast growth and reestablishment of unsettling Scenedesmus sp., causing a lower settleability in $PSBR_4$ than in $PSBR_6$ (Fig. 6-S1), in spite of removing higher volume of supernatant. Furthermore, the high Lv-TOC added to this PBR also improved the heterotrophic bacterial activity, contributing to the highest biomass production (Table 6.5).

Table 6.5 Effluent N:P (molar basis) values, biomass production and main dominant microalgae.

Photobioreactor	TIN:TIP	TOC:TIC	Biomass production [g VSS L^{-1}]	Dominant species
SC_{10}	7:1	5:1	0.06	*Scenedesmus* sp.[a]
$PSBR_6$	34:1	4:1	0.12	*Aphanocapsa* sp.[b]
$PSBR_4$	16:1	17:1	0.16	*Scenedesmus* sp.[a]

[a]Green algae

[b]Cyanobacteria

Another factor that should be considered regarding the dominance of cyanobacteria in $PSBR_6$ is the calculated SRT. The results of calculated SRT for $PSBR_6$ were always above 9 days, and in consequence slow growing microorganisms such as cyanobacteria were favoured. On the contrary, calculated SRT of $PSBR_4$ led to an HRT close to 8 days, which could have influenced the predominance of microorganisms able to perform a faster growth rate. In this case, some cells of Scenedesmus sp. and other green algae that were unsettled could have been able of reestablish (at least, partially) the cell concentration that was daily retired from the supernatant. This same response was observed in previous studies, in which either cyanobacteria or green algae dominance was enhanced depending on the SRT and under similar nutritional conditions (e.g. cyanobacteria in SRT of 10 d (Arias et al., 2017; Hu et al., 2017) and Scenedesmus sp. in 8 d (Arias et al., 2018; Gutiérrez et al., 2016; Passos et al., 2014)). It should be highlighted that the lower biomass production of SC_{10} and $PSBR_6$ could also be related to the higher SRT registered (9-10 days); such high values are associated to a slow growth rate in the culture (de Godos et al., 2014; Valigore et al., 2012).

In summary, the results indicate that sequencing batch operation can be used to select cyanobacteria from a green algae dominant culture. The conditions for improving cyanobacteria dominance include the use of a closed stirred PSBR fed with secondary effluent, when operated at an HRT of 6 d and a theoretical SRT of 10 d ($PSBR_6$), thus, introducing loads of 11.72 mg TN $L^{-1}d^{-1}$, 2.04 mg TP $L^{-1}d^{-1}$ and 71.81 mg TC $L^{-1}d^{-1}$. Under these conditions, the culture would be able to have residual nutrients concentrations which allow for a phosphorus limiting N:P ratio in the culture (34:1) and improve the increase of the total cyanobacteria populations from an initial 1% until 70%. Additionally, this operation led to a biomass production up to an average of 0.12 g $L^{-1}d^{-1}$ while removing 3.85 mg L^{-1} d^{-1} of TN, 1.81 mg L^{-1} d^{-1} of TP and 55.38 mg L^{-1} d^{-1} of TC. Although the decrease in HRT to 4 d ($PSBR_4$) led to a slightly higher removal rates of TP and TN and TC (Table 6.4), high nutrients loads cannot improve cyanobacteria dominance in the culture. Indeed, this operation promotes green algae dominance (81% of the total population) and biomass growth up to 0.16 g L^{-1} d^{-1}.

To the authors knowledge, the strategy of uncoupling SRT and HRT has been employed mostly to promote the formation of green-algae and cyanobacteria aggregates and the enhancement of nutrients rates removal, as shown in the studies of Van Den Hende et al., (2014; 2016), and Wang et al., (2015). However, in these studies, the effect of operational conditions on nutritional dynamics and the competition of specific microorganisms were not considered. Thus, this work

addresses this issue for the first time, as well as the influence of factors such as nutrients loads, settleability and microbial evolution in the selection process.

6.6 **Conclusions**

In this chapter, different strategies based on uncoupling SRT and HRT were used to select cyanobacteria over unsettling green algae *Scenedesmus* sp.. During 30 days, an initial microalgae mixed consortia dominated by *Scenedesmus* sp. was cultivated in two different photo-sequencing batch reactors (PSBRs) operated at HRT of 6 and 4 days and at a theoretical SRT of 10 d. Both PSBRs were compared with a semi-continuous photobioreactor (SC) operated at 10 d of HRT/SRT. The results indicated that the PSBR operated at 6 days of HRT provided suitable conditions to select cyanobacteria from a green algae dominant culture. These conditions included volumetric loads of 11.72 mg TN L^{-1} d^{-1}, 2.04 mg TP $L^{-1}d^{-1}$ and 71.81 mg TC L^{-1} d^{-1}. The remaining nutrients in the culture led also to a phosphorus limiting N:P ratio in the culture (34:1) that improved the cyanobacteria population increase from an initial 2% until 70% of the total population. In addition, this chapter showed that the dominance of cyanobacteria in microalgal-based wastewater treatment systems can be controlled by the operational and nutritional conditions. This knowledge could contribute to control microalgae contamination from up-scaling cyanobacterial biomass production in wastewater treatment systems.

Chapter 7

Assessing the potential of soil cyanobacteria for wastewater treatment and polymers production

The contents of this chapter were adapted from the publication: Arias, D.M., Uggetti, E., García, J. In preparation. Assessing the potential of soil cyanobacteria for wastewater treatment and polymers production.

7.1 Abstract

In this chapter, a mixed N-fixing soil cyanobacterial culture was cultivated in municipal wastewater to produce polymers in a one-stage operation. Four photobioreactors (PBRs) were operated in semi-continuous mode during a period of 30 days, evaluating the effect of different hydraulic retention time (HRT) and nutrients concentration on nutrients and organic matter removal, biomass composition and polymers accumulation. Two PBRs were operated at a HRT of 10 d with undiluted wastewater (A_{10full}) and diluted 2:1 wastewater with distillated water ($A_{10diluted}$). Other two PBRs were operated with undiluted wastewater at HRT of 8 d (A_{8full}) and 6 d (A_{6full}). The results evidenced that high HRT and low nutrients load achieved the highest removals efficiencies in TN >95%, TP 35-78%, TOC >93% and TIC >82%. These high removals led to N limitation that stimulated a continuous carbohydrates accumulation up to 48%. Furthermore, under those conditions a biomass production of 0.05-0.07 mg L^{-1} d^{-1} dominated of N-fixing cf. *Nostoc* sp. and cf. *Oscillatoria* sp. was achieved. Otherwise, lower HRT and thus high nutrients loads promoted carbon limitation, which led to lower N and P removals (TN 66-75%, TP 27-58%), poor carbohydrates contents (<14%) and low biomass production (0.05-0.07 mg L^{-1} d^{-1}). Besides the fact that these conditions favored contamination of green algae species. This chapter provides important information about cyanobacteria cultivation control and further production of valuable by-products from biomass in wastewater treatment systems.

7.2 Introduction

Cyanobacteria are a diverse group of prokaryotic microorganisms with a large ecological and physiological variety. As the most of cyanobacteria species live in aquatic environment, many others are capable of living in soil and other terrestrial habitats, where they are important in the functional processes of ecosystems and the cycles of nutrient elements (Patzelt et al., 2014; Wynn-Williams, 2002). Besides, their long history of adaptive and evolutionary diversification has also conferred them the capacity to synthesize a large variety of bioactive compounds and other valuable by-products (Mimouni et al., 2012). In fact, in the last two decades there has been increasing interest in cyanobacteria cultivation recognizing their great potential as a source of fine chemicals, as bio-fertilizers, and as source of renewable fuel (El-Bestawy, 2008).

Recently, the utilization of cyanobacteria for polyhydroxybutyrate (PHB) and glycogen (carbohydrates) production have become an interesting topic due to their application as a bioplastic and biofuel substrate respectively (Arias et al., 2018c;

Markou et al., 2012b; Stal, 1992). Studies carried out in this field have led to conclude that a good number of cyanobacteria have natural capabilities to store both polymers. The presence of such compounds is species dependant and can be stimulated by nitrogen or phosphorus deficient conditions and presence of excess amount of carbon (Saharan et al., 2014). Indeed, the most of the studies carried out in batch tests have demonstrated an increase of both PHB (up to 20% in terms of dry cell weight (dcw)) and glycogen content (up to 60% dcw) under N and P limiting conditions (Asada et al., 1998; De Philippis et al., 1992; Masato Miyake et al., 1997; Panda et al., 2006). Interestingly, the highest values in polymer production have been found in isolated N-fixing cyanobacteria in the case of PHB (Panda et al., 2005; Sharma and Mallick, 2005), and in filamentous cyanobacteria in the case of carbohydrates (Aikawa et al., 2012).

Most of the studies of cyanobacteria producing polymers have been based on isolated pure cultures, using sterile medium substrates in expensive and highly controlled processes (Asada et al., 1999; Masato Miyake et al., 1997; Samantaray and Mallick, 2014, 2012). These processes involve high production costs that commit the economical viability of the bioproducts. In this context, an approach for the cultivation of cyanobacteria producing polymers could be the use of wastewater as growth medium. Indeed, this promising and sustainable alternative could reduce production costs associated with pure cultures control and nutrients and water supply (Honda et al., 2012).

Wastewater treatment has been tested with many cyanobacteria species as *Arthorspira* sp. (Cheunbarn and Peerapornpisal, 2010; Magro et al., 2012; Olguín et al., 2003), *Oscillatoria* sp. (Hashimoto and Furukawa, 1989; Vijayakumar, 2012b), *Phormidium* sp. (Blier et al., 1995; Cañizares-Villanueva et al., 1995; Pouliot et al., 1989a) and *Nostoc* sp. (Ganapathy et al., 2011). Such studies found that cyanobacteria possess a great wastewater treatment efficiency due to their photoautotrophic nature and their affinity to affinity for nutrients (El-Bestawy, 2008). However, so far the studies of cyanobacteria cultivation in wastewater are limited to biomass production and contaminants' removal while the analysis of polymers during this process are limited to few studies (Olguín et al., 2001; Zhou et al., 2012). The most of them carried out in mono-cultures, without paying attention on factors that may affect polymers production in the process. Thus, all the aspects concerning operational conditions and nutrients dynamics in this kind of systems still need to be addressed. Therefore, the main objective of this work was to asses the cultivation of mixed soil crusts dominated by cyanobacteria in a one-stage operation to produce polymers while treating municipal wastewater. To the authors' knowledge this is the first time that a mixed soil culture is employed to produce

valuable polymers within a wastewater treatment process. In the present chapter, a soil cyanobacterial consortium was cultivated in four semi-continuous closed photoreactors (PBRs) evaluating the effect of different hydraulic retention times (HRTs) on nutrients and organic matter removal, biomass composition and polymers production.

7.3 **Materials and methods**

7.3.1 Inoculum

Cyanobacteria obtained from dry soil crusts was used as inoculum. Dry soil crusts were taken in September, 20th 2016, from a wastewater treatment plant at located in Saint-Étienne-de-Tulmont, France. The dry biomass was first hydrated and agitated in an Erlenmeyer with BG-11 growth medium during two days until the cells were suspended. The culture consisted on a mixed culture of nitrogen-fixing cyanobacteria of the genus cf. *Nostoc* sp. and cf. *Tolypothrix* sp. and cf. *Pseudoanabaena* sp. (Fig. 7.1), which are among the species mostly found in terrestrial environments (Garca-Pichel et al., 2001; Patzelt et al., 2014). Suspended biomass was subsequently introduced in a 30 L agitated closed photobioreactor (PBR) that was operated as mother reactor. PBR was maintained with nitrogen-free BG-11 growth medium and air-sparing, in order to maintain a nitrogen-fixing dominated culture along the experiment. Growth medium was changed twice a week by settling the biomass during 30 minutes and manually retiring 28 L of supernatant. The culture in the PBR was continuously maintained in alternate light:dark periods of 12 h. Illumination during the light phase was supplied by a 600W external metal halide lamp equipped with a digital ballast (model 5500k, Sunmaster, USA) placed at a 70 cm distance from the PBR. This lamp provided approximately 14,500 lx (204 μmol m−2 s−1), which corresponds to the irradiance recommended to enhance algal activity (200–400 μmol m−2 s−1) (Huesemann et al., 2016; Wang et al., 2010). Due to the lamp type (cool blue light), culture temperature was minimally influenced during the experimental time, ranging from 16 to 20 °C throughout the study and changing 1 to 2 °C between light:dark cycles. Agitation was provided by a rotary paddle placed in the placed in the middle of the cylinder, rotating at 40 rpm. The pH of the culture was constantly controlled at 7.5, by means of CO_2 (100% v) (Carbonos Metálicos, Spain) injection when necessary, at a flow of 0.3 L min^{-1} and a pressure of 0.3–0.5 MPa.

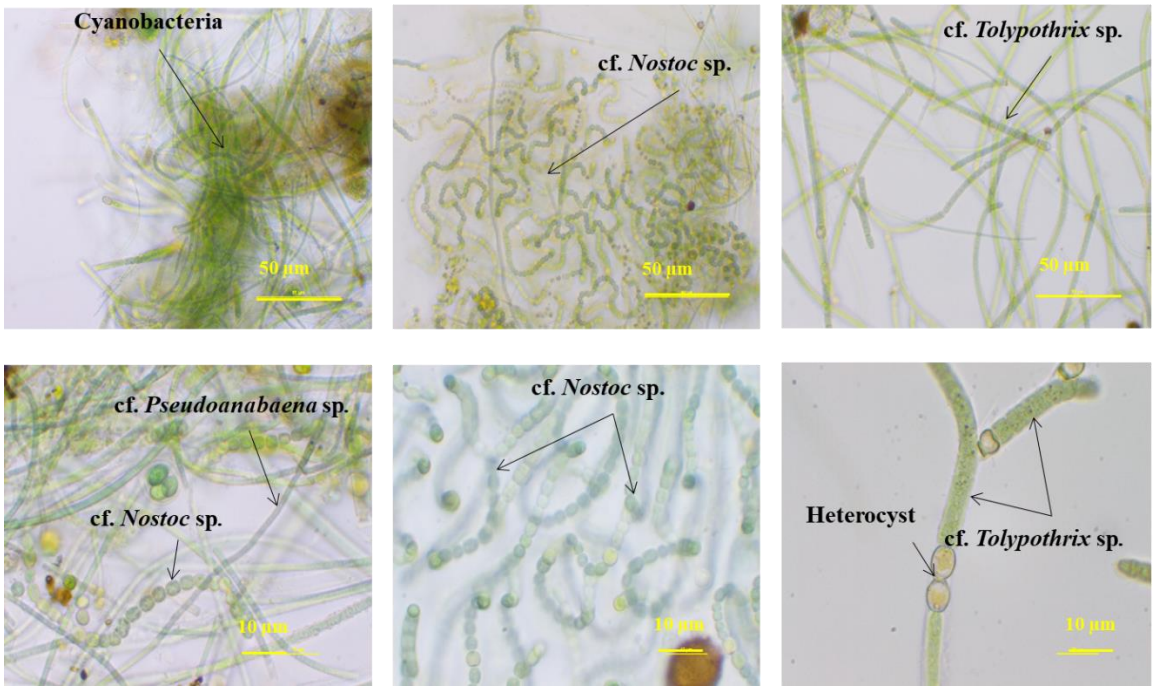


Fig. 7.1 Microscopic images illustrating the microbial diversity of biomass maintained in the mother reactor; a), b), c) general view of the culture observed at 500x; d) detail of the lateral side of a filamentous floc with cf. *Pseudoanabaena* sp. and cf. *Nostoc* sp.; e) detail of a floc of cf. *Nostoc* sp.; f) detail of cf. *Tolypothrix* sp. All the observations were performed in bright field microscopy.

7.3.2 Experimental set-up

Lab scale experiments were performed in two different periods carried out in two consecutive months (Period 1 in October 2017 during 30 days and period 2 in November 2017 during 25 days) Experiments were carried out in 4 lab scale closed polymethacrylate photobioreactors (PBRs) with a total volume of 3 L and a working volume of 2.5 L. The four PBRs were operated in semi-continuous mode using municipal wastewater as feeding. The municipal wastewater used in this experiment was the effluent from a primary settler with a useful volume of 7 L, a surface area of 0.03 m^2, hydraulic retention time (HRT) in the range of 0.7–1.4 h, and an hydraulic surface loading rate of 11.3 $m^3/m^2 \cdot d^{-1}$ treating raw urban wastewater from a nearby municipal sewer placed at the laboratory of the GEMMA research group (Universitat Politècnica de Catalunya·BarcelonaTech, Barcelona, Spain) (Gutiérrez et al., 2016a). Wastewater influent characteristics of the two periods are summarized in Table 7.1.

After the mother PBR reached a minimum biomass concentration of 0.15 g VSS L^{-1}, 3 L of mixed liquor was collected and thickened by gravity in Imhoff cones for 30 min before its inoculation. Inoculation all the reactors was performed by suspending 0.2 L of settled biomass in the PBRs with approximately 2.3 L nitrogen-free BG-11 growth medium. The growth medium consisted in a solution composed of: 0.04 g L^{-1} K_2HPO_4, 0.072 g L^{-1} $CaCl_2 \cdot 2H_2O$, 0.001 g L^{-1} Na_2EDTA, 0.075 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.6 g L^{-1} $C_6H_8FeNO_7$ (ammonium ferric citrate), 0.006 g L^{-1} $C_6H_8O_7$ (citric acid), and trace elements: 0.00286 g L^{-1} H_3BO_3, 0.00039 g L^{-1} $Na_2Mo_4 \cdot 2H_2O$, 0.0018 g L^{-1} $MnCl_2 \cdot 4H_2O$, 0.00008 g L^{-1} $CuSO_4 \cdot 5H_2O$, 0.00022 g L^{-1} $ZnSO_4 \cdot 7H_2O$ and 0.00005 g L^{-1} $Co(NO_3)_2.6H_2O$.

In period 1, two PBRs were operated at an hydraulic retention time (HRT) of 10 days. Which means that feeding was realized once a day by removing 0.25 L of the mixed liquor and subsequently replacing this volume with 0.25 L of wastewater effluent. One of those PBRs, named A_{10full}, was operated with undiluted municipal effluent, while the other, named $A_{10diluted}$, was operated with the wastewater influent diluted with deionized water in a ratio 2:1. In period 2 one of the PBRs (named A_{8full}) was operated with 8 days of HRT and the other, named A_{6full}, was operated with 6 days of HRT, both PBRs were fed with undiluted wastewater. In the case of A_{8full}, approximately 0.313 L of the mixed liquor were withdrawn and subsequently this volume was then replaced with approximately 0.313 L of wastewater effluent. Whereas in reactor A_{6full}, approximately 0.416 L of the mixed liquor were withdrawn and subsequently this volume was replaced with

approximately 0.416 L of undiluted wastewater. All PBRs were operated during at least three solids retention time and the biomass was constant.

All PBRs were continuously maintained in alternate light:dark phases of 12 h. Illumination during the light phase was supplied by two external halogen lamps (60W) placed at opposite sides of each reactor and providing 220 μmol m^{-2} s^{-1} of light. PBRs were continuously agitated with a magnetic stirrer (Selecta, Spain) set at 250 rpm. Temperature was continuously measured by a probe inserted in the PBRs (ABRA, Canada) and kept constant at 27 (±2) °C by means of a water jacket around the reactors. pH was continuously monitored with a pH sensor (HI1001, HANNA, USA) and kept at 7.5 with a pH controller (HI 8711, HANNA, USA) by the automated addition of HCl 0.1 N or NaOH 0.1 N (depending on pH). Mixed liquor harvesting and feeding were performed each day at the end of the dark phase by the automatic addition/withdrawn accomplished by peristaltic pumps (Arias et al., 2018c) (Fig. 7.2).

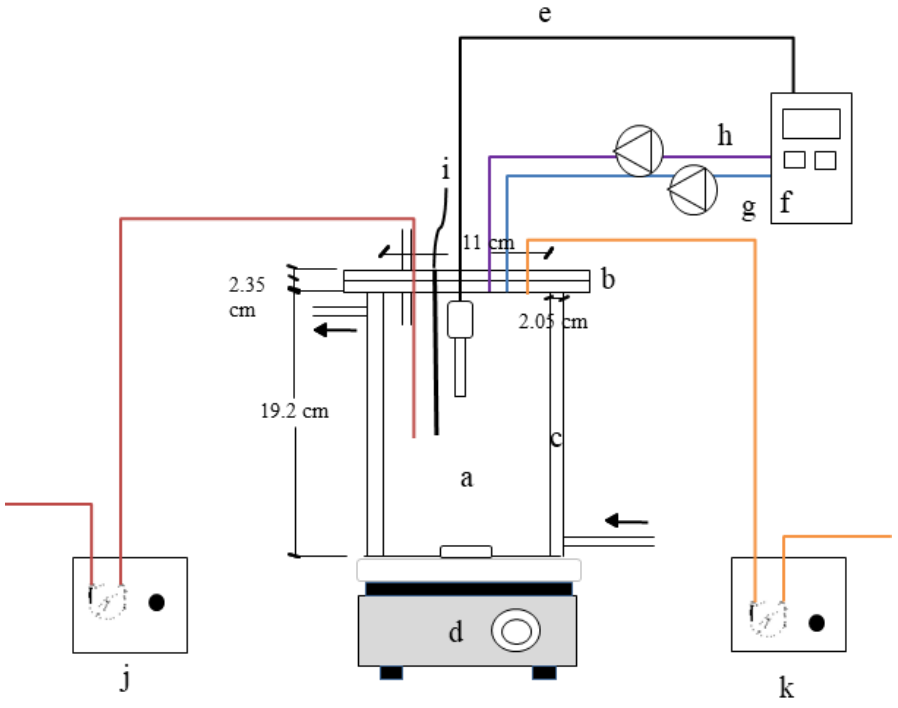


Fig. 7.2 Schematic diagram of each photobioreactor (PBR) set-up: a) Body of the PBR, b) cover, c) wáter jacket, Arrows indicate the wáter flux by the wáter jacket around the PBR, d) magnetic stirrer, e) pH sensor, f) pH controller, g) acid solution , h) Basic solution, i),

temperature sensor, j) Peristaltic pumb controling mixed liquor withdrawal, k) Peristaltic pumb controling influent introduction.

Table 7.1 Average (standard deviation) of the main quality parameters of the municipal wastewater used as influent (n=6–12).

Parameter	Period 1[a c]	Period 2[b]
pH	7.2 (0.5)	7.4 (0.6)
SST [$g \cdot L^{-1}$]	0.31 (0.17)	0.17 (0.07)
SSV [$g \cdot L^{-1}$]	0.29 (0.14)	0.15 (0.08)
TOC [$mg \cdot L^{-1}$]	179.28 (61.54)	121.54 (24.04)
SOC [mg·L-1]	31.49 (18.11)	51.17 (16.99)
TIC [$mg \cdot L^{-1}$]	71.46 (12.25)	85.09 (5.82)
TN [$mg \cdot L^{-1}$]	70.71 (14.47)	66.74 (6.70)
TAN [$mg \cdot L^{-1}$]	35.44 (11.02)	46.26 (6.97)
$N\text{-}NO_3^-$ [$mg \cdot L^{-1}$]	<LOD	<LOD
$N\text{-}NO_2^-$ [$mg \cdot L^{-1}$]	<LOD	<LOD
TIN [$mg \cdot L^{-1}$]	35.44 (11.02)	46.26 (6.97)
TON [$mg \cdot L^{-1}$]	35.27 (17.33)	20.48 (9.19)
TP [$mg \cdot L^{-1}$]	6.99 (3.57)	5.22 (1.41)
IP [$mg \cdot L^{-1}$]	2.91 (0.39)	2.97 (0.33)
TOP [$mg \cdot L^{-1}$]	4.08 (4.19)	2.25 (1.31)

LOD: Limit of detection (0.05 mgL^{-1}).
[a] Samples taken along 30 days.
[b] Samples taken along 25 days.
[c] Municipal wastewater used in one of the PBRs of this period ($A_{10diluted}$) was diluted in a ratio 2:1 with deionized water.

7.3.3 Analysis procedures

Parameters concerning water quality and biomass production were determined in triplicate and analyzed from the influent and effluent (equivalent to the mixed liquor of the culture) twice a week at the end of the dark phase. Nutrients analyzed were total ammoniacal nitrogen (TAN), total organic carbon (TOC), total inorganic carbon (TIC) and soluble organic carbon (SOC), inorganic phosphorus (IP), nitrite ($N\text{-}NO_2^-$), nitrate ($N\text{-}NO_3^-$), total nitrogen (TN) and total phosphorus (TP). TAN (sum of $N\text{-}NH_3$ and $N\text{-}NH_4^+$) was determined using the colorimetric method indicated in Solorzano (1969). IP (measured as orthophosphate $P\text{-}PO_4^{3-}$), $N\text{-}NO^{2-}$ and $N\text{-}NO^{3-}$ concentrations were analyzed using

an ion chromatograph DIONEX ICS1000 (Thermo-scientific, USA), while TOC, TIC, OC, IC and TN were analyzed by using a C/N analyzer (21005, Analytikjena, Germany). Total phosphorus (TP) was analyzed following the methodology described in Standard Methods (APHA-AWWA-WPCF, 2001). Total inorganic nitrogen (TIN) was calculated as the sum of $N\text{-}NO_2^-$, $N\text{-}NO_3^-$ and TAN. Total organic nitrogen (TON) (in dissolved and particulate form) was measured in the effluent supernatant and calculated as the difference between TN and TIN, whereas total organic phosphorus (TOP) (in dissolved and particulate form) was also measured in the effluent supernatant and determined as the difference between TP and IP.

Total suspended solids (TSS) and volatile suspended solids (VSS) were measured in the mixed liquor three days per week. While Chlorophyll *a* was analyzed twice a week. These parameters were analysed using procedures described in the Standard Methods (APHA-AWWA-WPCF, 2001).

Dissolved oxygen (DO) was measured directly in each reactor, inserting a sensor in the mixed liquor during the second half light phase with a dissolved oxygen-meter (Thermo-scientific, USA).

7.3.4 Microbial characterization

Quantitative analysis of microalgae and cyanobacteria was performed by microscopic area cells counting ($cell \cdot mL^{-1}$) three times a week. To this aim, 20 μL of mixed liquor were added to a slide with a coverslip and individual cells were counted per field until reaching ~400 cells. Microalgae were quantified in bright field microscopy at 40X, while cyanobacteria species were counted using fluorescence microscopy with the operation of filters containing an excitation filter (510-560 nm), emission filter (590 nm) and *dichroic* beam splitter (575 nm). Both bright field and fluorescence microscopy were performed using a fluorescence microscope (Eclipse E200, Nikon, Japan).

Identification of microalgae species within each reactor was monitored by microscopy once a week. Microbial visualization was performed in an optic 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 (Bourrelly, 1985; Palmer, 1962), as well as a database of cyanobacteria genus (Komárek and Hauer, 2013b).

7.3.5 Polymers quantification

Carbohydrates and polyhydroxybutyrate (PHB) content were measured twice per week in all the PBRs at the end of the dark phase. Then, 50 mL of mixed liquor were collected and centrifuged (4200 rpm, 10 min), frozen at −80 °C overnight in an ultra-freezer (Arctiko, Denmark) and finally freeze-dried for 24 h in a lyophilizer (−110 °C, 0.049 hPa) (Scanvac, Denmark). PHB and carbohydrates extraction and quantification was conducted using the methodology described in Arias et al. (2018).

Carbohydrates and PHB content were calculated according to Fradinho et al. (2016), in terms of percentage of VSS:

$$\%\text{Carbohydrates or PHB} = \frac{\text{gPHB or gCarbohydrates}}{\text{gVSS}} * 100$$

7.4 Results and discussion

7.4.1 Wastewater treatment

Throughout the experimental time, the municipal wastewater used as influent in the two periods showed similar characteristics in pH, TN and IP (Table 7.1). Period 1 showed higher TSS, TOC, TIC and TON with higher variability than period 2. Otherwise, period 2 showed higher TIN, and doubled the TIN:TON ratio (2:1) in comparison with Period 1 (1:1). Such differences between influents were caused by the variability of the wastewater. The wastewater treatment performance of the PBRs was evaluated in terms of organic carbon, nitrogen, and phosphorus removals. Effluent characteristics are summarized in Table 7.2.

The PBRs operated in period 1, A_{10full} and $A_{10diluted}$, averaged 86 and 82% in the removal of TOC, respectively. Both PBRs showed better TOC removal in the first fifteen days of operation. Subsequently, TOC concentration in the PBRs increased in relation with the increment in influent TOC (Fig. 7.3). In addition, almost all the TIC content (>93%) was eliminated during all the experimental time. Regarding the PBRs operated in period 2, A_{8full} and A_{6full}, both showed higher removal efficiencies than the PBRs of Period 1, removing TOC by 89% (Fig. 7.3). While TIC was almost completely consumed (>96%) in both reactors. These high removals in TC suggest carbon limitation in the cultures.

Concerning nitrogen species, A_{10full} and $A_{10diluted}$ removed more than 98% of TAN and TON, while the total removal of TN in these PBRs were 95 and 98%,

respectively (Fig.7.4). As in most of the time very low values of $N\text{-}NO_2^-$ and $N\text{-}NO_3^-$ were obtained (Table 7.2), it is assumed that all the TAN and TON (mineralized to TAN) was consumed by eukaryotic microalgae/cyanobacteria. Otherwise, A_{8full} and A_{6full} showed very low concentrations (<2 mg L^{-1}) of $N\text{-}NO_2^-$, $N\text{-}NO_3^-$ (thus, low nitrification) and TON, while presenting TAN accumulation until achieving more than 20 mg L^{-1} in the last two weeks of operation (Fig. 7.4c y 7.4d). This increase in TAN concentration likely occurred due to the carbon limitation occurring during period 2. In spite of those increments in TAN concentrations, the general TN removal in A_{8full} and A_{6full} were 75 and 66% respectively.

As already explained in Section 7.3.2, PBRs were firstly inoculated in N-free BG-11 growth medium containing phosphorus. This caused an initial concentration approximated of 5 mg L^{-1} of IP in the cultures. In A_{10full}, the general TP removal averaged 53% (Fig.7.4a). Whereas high values of IP the effluent along the experiment caused a removal efficiency of only 8%, while TOP was removed by 82%. Similarly, $A_{10diluted}$ maintained TP concentration mostly in form of IP around 2.5 mg L^{-1} (Fig. 3b). This reactor achieved 55% of IP and 91% of TOP removal efficiency, while the general TP removal was 78%. On the other hand, A_{8full} presented high IP concentrations despite that the influent contained lower TP concentration than in period 1 (Table 7.1). Until day 20, TP (almost composed by IP) maintained concentrations closed to the influent (5.22 ± 1.41 mg L^{-1}) (Fig.7.4c). Notwithstanding, the average removal percentages were TP 59%, IP 7% and TOP 56%. In the case of A_{6full}, it presented a better performance than the reactor aforementioned (Fig.7.4d). IP was removed by 57%, TOP by 65% and TP by 62%.

Table 7.2 Average (standard deviation) of the main quality parameters of the effluent (supernatant after biomass settling) of the PBRs during the experiment (n=6-12).

Parameter	A_{10full}[a]	$A_{10diluted}$[a]	A_{8full}[b]	A_{6full}[b]
pH	7.56 (0.11)	7.82 (0.14)	7.32 (0.05)	7.36 (0.16)
OD	6.03 (0.28)	7.01 (0.51)	7.59 (1.81)	10.34 (10.35)
TSS [$g \cdot L^{-1}$] [c]	0.51 (0.21)	0.40 (0.14)	0.54 (0.14)	0.32 (0.05)
VSS [$g \cdot L^{-1}$] [c]	0.44 (0.20)	0.38 (0.19)	0.46 (0.06)	0.31 (0.05)
Biomass production [$g \cdot L^{-1}d^{-1}$] [c]	0.04 (0.02)	0.04 (0.01)	0.06 (0.01)	0.05 (0.02)
TOC [$mg \cdot L^{-1}$][c]	256 (140)	258 (112)	244 (40)	187 (56)
SOC [$mg \cdot L^{-1}$]	25.5 (11.5)	31.7 (14.5)	13.5 (3.3)	13.5 (2.8)
TIC [$mg \cdot L^{-1}$]	5.17 (1.88)	3.81 (0.94)	3.15 (2.22)	3.96 (4.03)
Chlorophyll a [$mg \cdot L^{-1}$] [c]	1.13 (0.58)	1.10 (0.66)	0.69 (0.32)	0.53 (0.19)
TN [$mg \cdot L^{-1}$]	3.05 (4.49)	1.21 (2.50)	16.29 (9.56)	22.19 (8.76)
TAN [$mg \cdot L^{-1}$]	0.68 (1.01)	0.71 (1.07)	16.09 (10.32)	17.92 (9.55)
$N\text{-}NO_3^-$ [$mg \cdot L^{-1}$]	0.12 (0.31)	0.08 (0.25)	0.15 (0.35)	0.38 (0.54)
$N\text{-}NO_2^-$ [$mg \cdot L^{-1}$]	2.25 (3.17)	0.42 (1.18)	<LOD	1.32 (1.85)
TIN [$mg \cdot L^{-1}$]	3.05 (4.49)	1.21 (2.50)	16.24 (10.65)	19.62 (12.94)
TON [$mg \cdot L^{-1}$]	<LOD	<LOD	0.05 (0.16)	2.57 (3.70)
TP [$mg \cdot L^{-1}$]	3.45 (2.23)	1.68 (2.08)	3.73 (3.89)	2.08 (1.64)
IP [$mg \cdot L^{-1}$]	2.67 (1.38)	1.31 (1.20)	2.75 (1.47)	1.29 (0.47)
TOP [$mg \cdot L^{-1}$]	0.74 (0.83)	0.37 (0.83)	0.98 (1.88)	0.79 (1.23)

LOD: Limit of detection (0.05 mgL^{-1}).

[a] A_{10full} and $A_{10diluted}$ corresponded to the PBRs operated during period 1 along 30 days.

[b] A_{8full} and A_{6full} corresponded to the PBRs operated during period 2 along 25 days.

[c] Parameters measured in the mixed liquor.

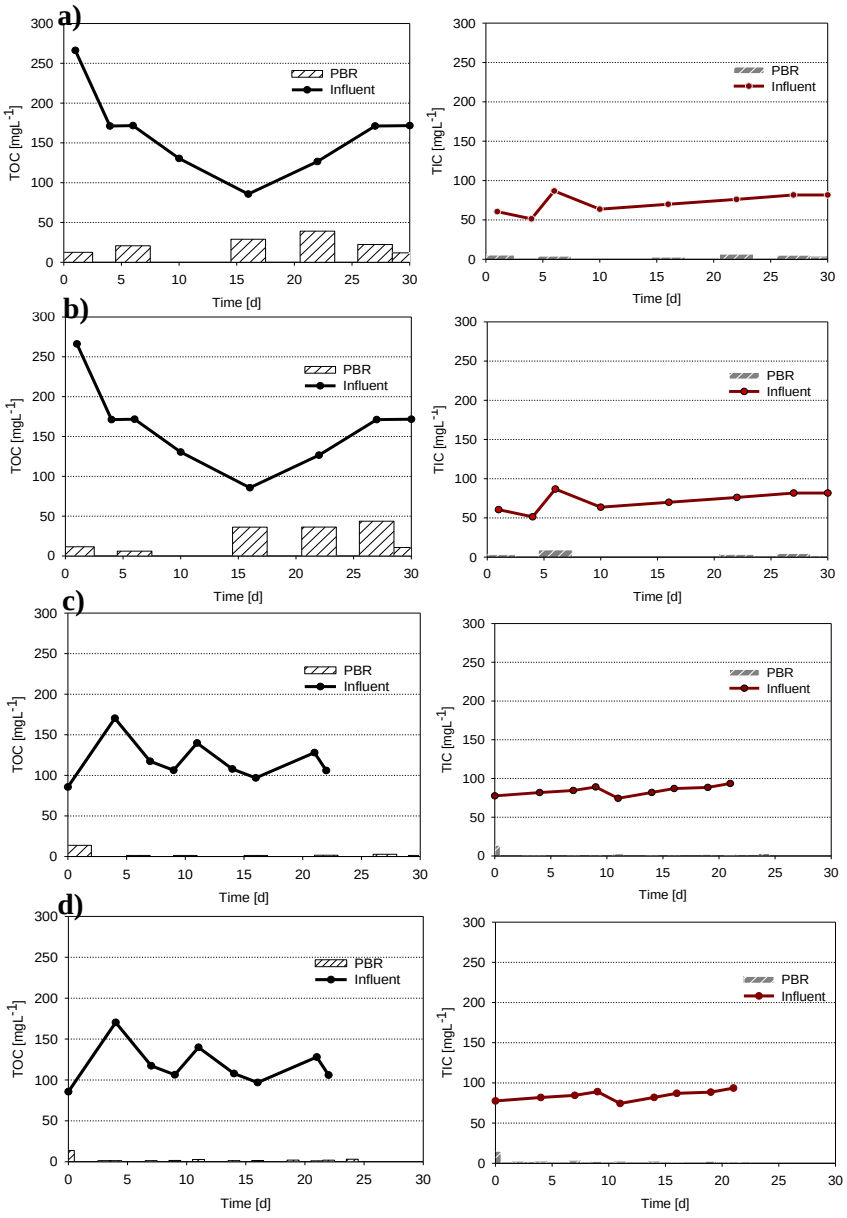


Fig. 7.3 Time course of influent and PBR (effluent) TOC (left) TIC (right) during Period 1: a) A_{10full}, b) $A_{10diluted}$, and Period 2: c) A_{8full} and c) A_{6full}.

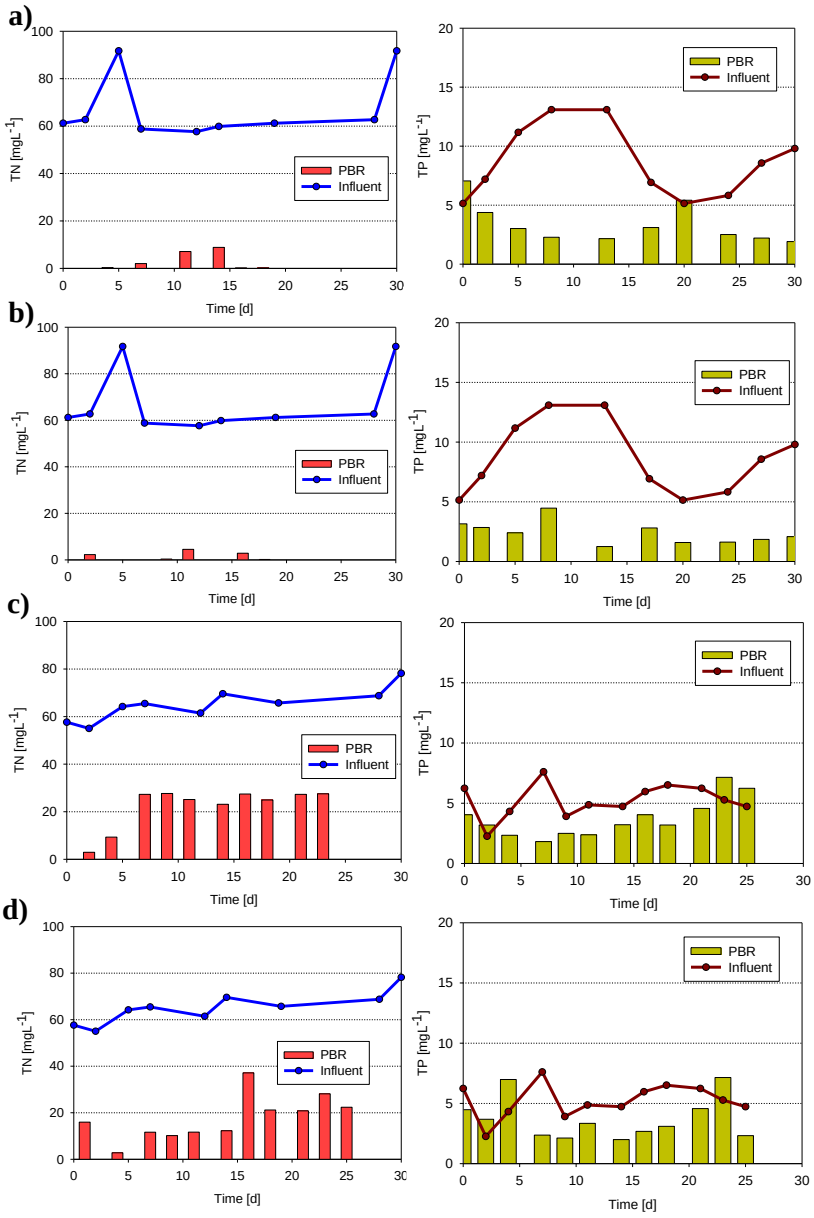


Fig. 7.4 Time course of influent and PBR (effluent) TN (left) and TP (right) during Period 1 a) A_{10full}, b) $A_{10diluted}$ and Period 2 c) A_{8full} and c) A_{6full}.

7.4.2 Biomass production

In spite of the N limitation observed in A_{10full} and $A_{10diluted}$, the biomass concentration in these PBRs presented an increasing pattern along the experiment maintaining similar trends. Both PBRs reached a stable condition between day 20 and 30 with biomass concentrations oscillating among 0.55 and 0.75 g L^{-1}. In this case, biomass production followed the same trend, with an average value of 0.07±0.01 mg L^{-1} d^{-1} in A_{10full} and 0.05 mg L^{-1} d^{-1} in $A_{10diluted}$ from day 20 to day 30. A similar trend was observed in chlorophyll *a* content, thus, after day 15, chlorophyll *a* maintained constant values of 1.55±21 mg L^{-1} (A_{10full}) and 1.58 mg L^{-1} ($A_{10diluted}$) (Fig. 7.5).

Biomass production in period 2 reached lower values than the PBRs of period 1, the average production between day 8 and the end of the experiments was 0.06±0.01 mg L^{-1} d^{-1} in A_{8full} and 0.04 mg L^{-1} d^{-1} in A_{6full} (Fig, 7.5). Differently to A_{10full} and $A_{10diluted}$, A_{8full} and A_{6full} did not present N limitation but carbon limitation. This fact could lead to low nutrients removal, decrease in biomass and chlorophyll *a* concentration. Furthermore, A_{10full} and $A_{10diluted}$ biomass increase was not affected by N limitation since soil cyanobacteria are able to grow with reduced N content thanks to their ability to uptake atmospheric nitrogen (Patzelt et al., 2014; Rajeev et al., 2013; Xu et al., 2013).

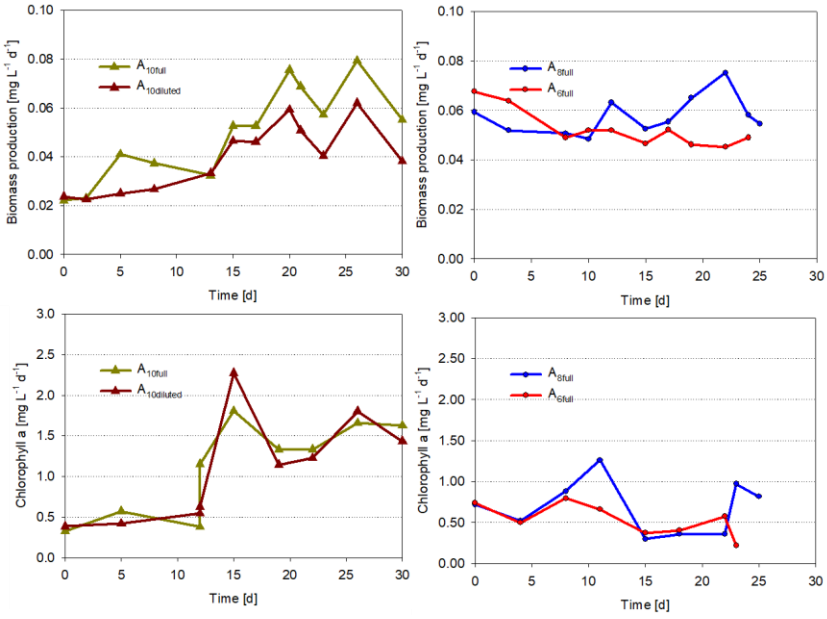


Fig. 7.5 Time course of biomass production and chlorophyll *a* content.

7.4.3 Biomass composition

Since all the cultures were performed in non-controlled and non-sterile conditions, contamination by other microorganisms was expected along the time. All the cultures had a cyanobacteria dominance by 99.88% at the beginning of the experiments. During the period of cultivation, an increase in other species was observed in different quantities depending on the HRT and the nutrients in the cultures. However, cyanobacteria dominance maintained during the experimental time (>98%), and only the PBRs with lower HRT, A_{8full} and A_{6full}, showed a slightly higher incidence of other microorganisms as, green algae, diatoms and protozoa (Fig. 7.6).

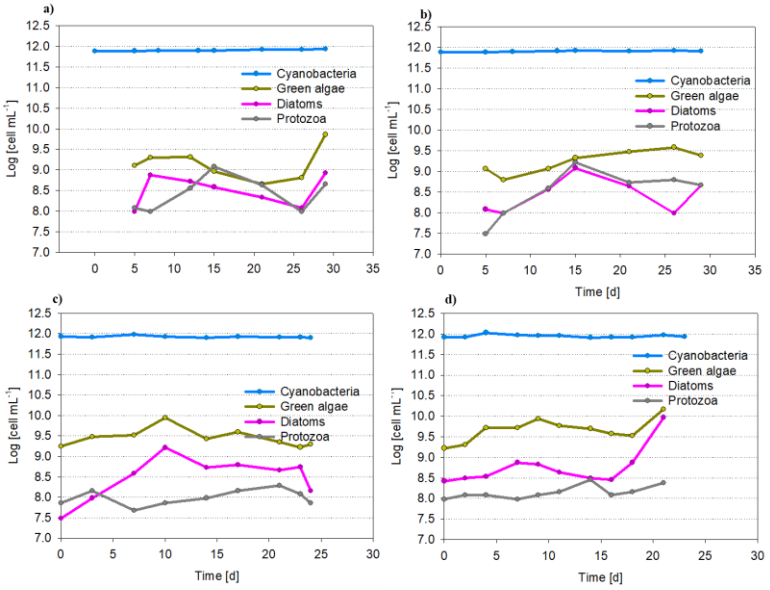


Fig. 7.6 Biomass composition in a) A_{10full}, b) $A_{10diluted}$, c) A_{8full} and c) A_{6full}.

Microbial observations during the experiment revealed that the main cyanobacteria species observed in all the cultures were filamentous *Pseudoanabaena* sp. and *Tolypothrix* sp. (Supplementary figures 7-S1, S2, S3 and S4). A_{10full} showed a strong domination by those species grouped in large flocs with the appearance of green algae species as cf. *Westella* sp. and cf. *Planktospheria* sp. *Nostoc* species inoculated in the culture were barely observed in this reactor (Fig. 7-S1). Conversely, $A_{10diluted}$ showed more presence of *Nostoc* sp., but not in the filamentous shape that this species showed in the beginning (Fig. 7.1). Along the experiment *Nostoc* sp. were grouped rounded by a gelatinous exopolysaccharide mass (Fig. 7-S2). Along with *Nostoc* sp., colonies of cf. *Aphanocapsa* sp. and cf. *Chroococcus* sp.

were also observed. Filamentous cyanobacteria cf. *Tolypothrix* sp. was also observed, and also filaments from the family of cf. *Rivularia* sp.. In this reactor also green algae cf. *Westella* sp. and big colonies of cf. *Planktospheria* sp. were found. Similarly, A_{8full} and A_{6full} also presented a dominant presence of filamentous N-fixing *Rivularia* sp. and *Pseudoanabaena* sp., grouped cf. *Nostoc* sp. and *Chrooccocus* sp. (Figs. 7-S3 and 7-S4). In the particular case of A_{6full}, this reactor showed the presence of green algae *Chlorella* sp..

In general, all the cultures presented good natural sedimentation since filamentous cyanobacteria species are recognized as easy-settling species (Arcila and Buitrón, 2016; de Godos et al., 2014). In addition, unicellular species as cf. *Aphanocapsa* sp. and cf. *Chrooccocus* sp. remained in big colonies. While species of green algae observed in the cultures were generally observed inside the flocs formed by cf. *Pseudoanabaena* sp. Only A_{6full} presented turbidity due to the presence of *Chlorella* sp.

7.4.4 Polymers production

Along the time, two clear patterns in carbohydrates intracellular content were observed (Fig. 7.7). A_{10full}, $A_{10diluted}$, A_{8full} and A_{6full} presented different initial carbohydrates content according to the period in which they were inoculated. As explained in section 7.3.2, biomass was maintained in a stock reactor under N-limited conditions prior their inoculation. A_{10full}, $A_{10diluted}$ were firstly inoculated (Period 1) while A_{8full} and A_{6full} started their operation 30 days after (Period 2). Thus, the PBRs operated in Period 1 showed an initial carbohydrates content of 15 % while A_{8full} and A_{6full} contained an intracellular content of 30 % at the day of inoculation.

In the case of A_{10full} and $A_{10diluted}$, both PBRs showed first a slight decrease from 15% to 5 and 10%, respectively in the first days of operation. Subsequently, they gradually increased their intracellular content to 34 and 48%, respectively. This trends were consequence of the nutrients behavior previously described in section 7.3.1. Increasing carbohydrates in A_{10full} and $A_{10diluted}$ can be associated to N limitation occurring during most of the experimental time (Fig. 7.3). Likewise, accumulation percentages reached in both PBRs during a continuous operation are comparable to previous studies performed in long batch operation testing N limitation in *Synechocystis* sp. (Monshupanee and Incharoensakdi, 2014), *Spirulina maxima* (De Philippis et al., 1992) and *Arthrospira* sp. (Aikawa et al., 2012; Sassano et al., 2010). These results suggest that N limitation led to a very fast carbon transformation to carbohydrates in the species of cyanobacteria used in this study.

However, it should be highlighted that those studies were carried out in growth medium under a 24h light cycle, while in this study, municipal wastewater was used as nutrient source under a circadian cycle.

On the other hand, A_{8full} and A_{6full} halved the initial intracellular content of 30% during the first four days of operation. A_{8full} continue a decreasing pattern until reaching 14% in day 25 while A_{6full} maintained a constant carbohydrates content of approximately 12%. This decreasing trend might be due to the availability of nutrients along with carbon limitation observed from the first days of operation (Fig. 7.4). Furthermore, due to the high initial biomass inoculated, higher carbon requirements were needed, thus inorganic carbon from the influent and carbohydrates accumulated in the biomass were both used as carbon source for cell maintenance. The carbohydrates content obtained in these two PBRs are close to the ones obtained by Arcila and Buitrón (2016) in a high rate algal pond (HRAP) treating wastewater operated at hydraulic and solids retention times of 2 and 6 d of HRT, obtaining 12 and 16 %, respectively.

With regards to PHB accumulation, values below 1% were registered in all PBRs even if N limitation was occurring. In the previous studies of Bhati and Mallick, (2015), Panda et al. (2005) and Sharma and Mallick, (2005) have related the accumulation of polyhydroxyalkanoates in N-fixing microorganisms to P limiting conditions. Thus, the availability of that nutrient in all the cultures could likely influence the poor accumulation of this polymer in this study..

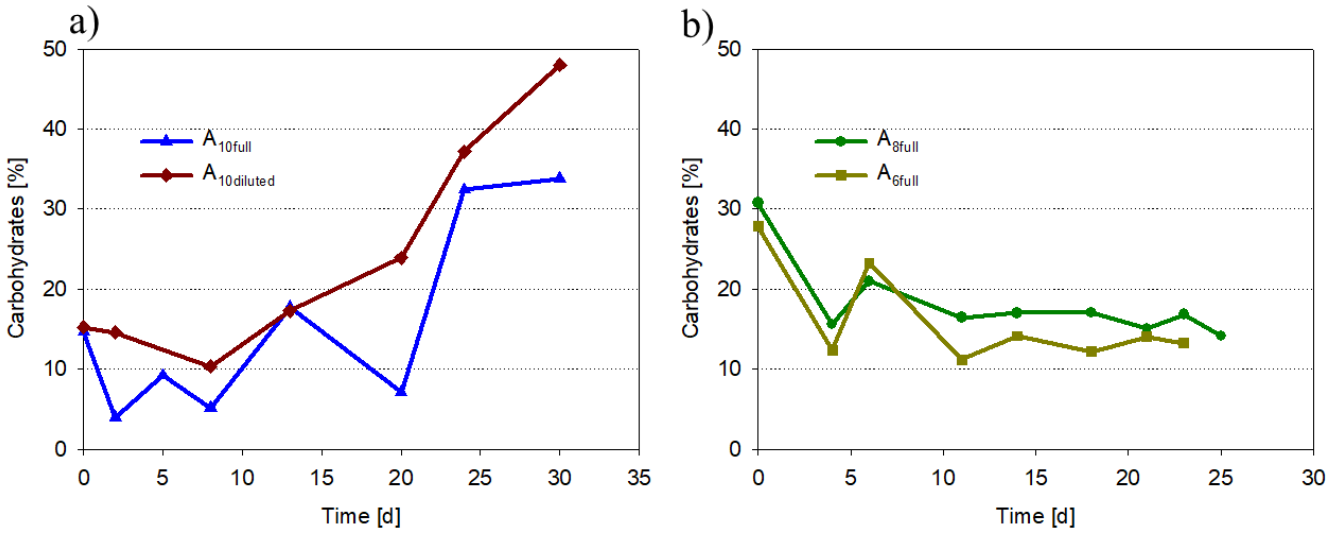


Fig. 7.7 Time course accumulation of carbohydrates.

7.5 **Conclusions**

In this chapter, a mixed N-fixing soil cyanobacterial culture was cultivated in municipal wastewater to produce polymers in a one-stage operation. The results evidenced that the cultivation of soil cyanobacterial at high HRT and low nutrients load led the highest removals efficiencies in TN >95%, TP 35-78%, TOC >93% and TIC >82%. These high removals led to N limitation that stimulated a continuous carbohydrates accumulation up to 48%. Furthermore, under those conditions a biomass production of 0.05-0.07 mg L^{-1} d^{-1} dominated of N-fixing cf. *Nostoc* sp. and cf. *Oscillatoria* sp. was achieved. Otherwise, lower HRT and thus high nutrients loads promoted carbon limitation, which led to lower N and P removals (TN 66-75%, TP 27-58%), poor carbohydrates contents (<14%) and low biomass production (0.05-0.07 mg L^{-1} d^{-1}). Besides the fact that these conditions favored contamination of green algae species. This study provides important information about cyanobacteria cultivation control and further production of valuable by-products from biomass in wastewater treatment systems.

Chapter 8

Accumulation of polymers in mixed cyanoacterial cultures: nutrients limitation and photoperiods in batch experiments

The contents of this chapter were adapted from the publication: Arias, D.M., Uggetti, E., García-Galán, M.J., García, J., 2018c. Production of polyhydroxybutyrate and carbohydrates in a mixed cyanobacterial culture: effect of nutrients limitation and photoperiods. N. Biotechnol. 42, 1–11. doi:https://doi.org/10.1016/j.nbt.2018.01.001

8.1 Abstract

In this chapter, different photoperiods and nutritional conditions were applied to a mixed wastewater-borne cyanobacterial culture in order to enhance the intracellular accumulation of polyhydroxybutyrate (PHB) 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 chapter demonstrates that the accumulation of PHBs (bioplastics) and carbohydrates (potential biofuel substrate) is favored in wastewater-borne cyanobacteria under conditions where nutrients are limited.

8.2 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 (Lau et al., 2015). 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 polymers. 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 polyhydroxybutyrate (PHB) (Stal, 1992). 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 (Reis et al., 2003). 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 (Steinbüchel and Füchtenbusch, 1998). 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 (Panda et al., 2006).

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 (Aikawa et al., 2015; Markou et al., 2012a) . The use of cyanobacteria to produce glycogen is advantageous compared with other higher plants or green algae producing carbohydrates (Nozzi et al., 2013), 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 (Bohutskyi and Bouwer, 2013; Mendez et al., 2015).

Most studies related to the production of PHBs and glycogen from cyanobacteria have been based on pure or genetically modified cultures (Aikawa et al., 2015; Drosg et al., 2015; Koller and Marsalek, 2015; Markou, 2012; Meixner et al., 2016), 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 polymers 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 (Arias et al., 2017; Van Den Hende et al., 2016a). However, the production of polymers 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) (Khajepour et al., 2015). Among these conditions, nutrient limitation is the approach most frequently used (Drosg et al., 2015; Koller and Marsalek, 2015; Markou et al., 2012a). 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)) (Ansari and Fatma, 2016; Kaewbai-ngam et al., 2016; Markou et al., 2012a; Miyake et al., 2000; Panda et al., 2005). Other important factors to consider in polymer production are the photoperiods and the light intensity, which affect crucial physiological processes such as photosynthesis, respiration, cell division and the intracellular carbon components (Krzemińska et al., 2014; Renaud et al., 1991).

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 polymers.

8.3 Materials and methods

8.3.1 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).

8.3.2 Experimental set-up

8.3.2.1 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 (Arias et al., 2017). 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.

8.3.2.2 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. 8.1).

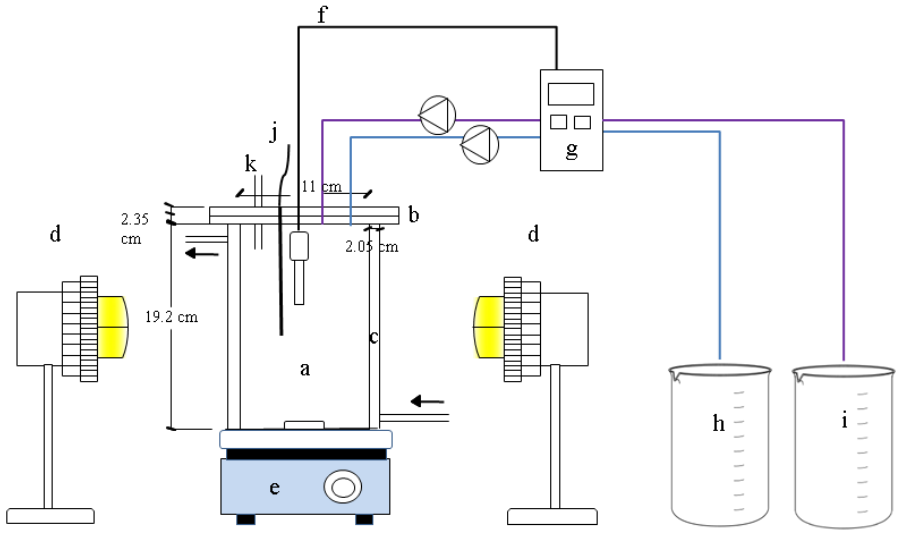


Fig. 8.1 Schematic diagram of each photobioreactor (PBR) set-up: a) body of the PBR, b) cover, c) water jacket; arrows indicate the water flux around the PBR, d) external lamps, e) magnetic stirrer, f) pH sensor, g) pH controller, h) acid solution, i) basic solution, j, temperature sensor, k) port for manual addition of carbon.

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 were suspended in 1 L of growth medium in each of the four reactors. Microscopic images of the initial biomass are shown in Fig. 8.2 and characteristics of the inoculum after suspension in the growth media are given in Table 8.1.

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.

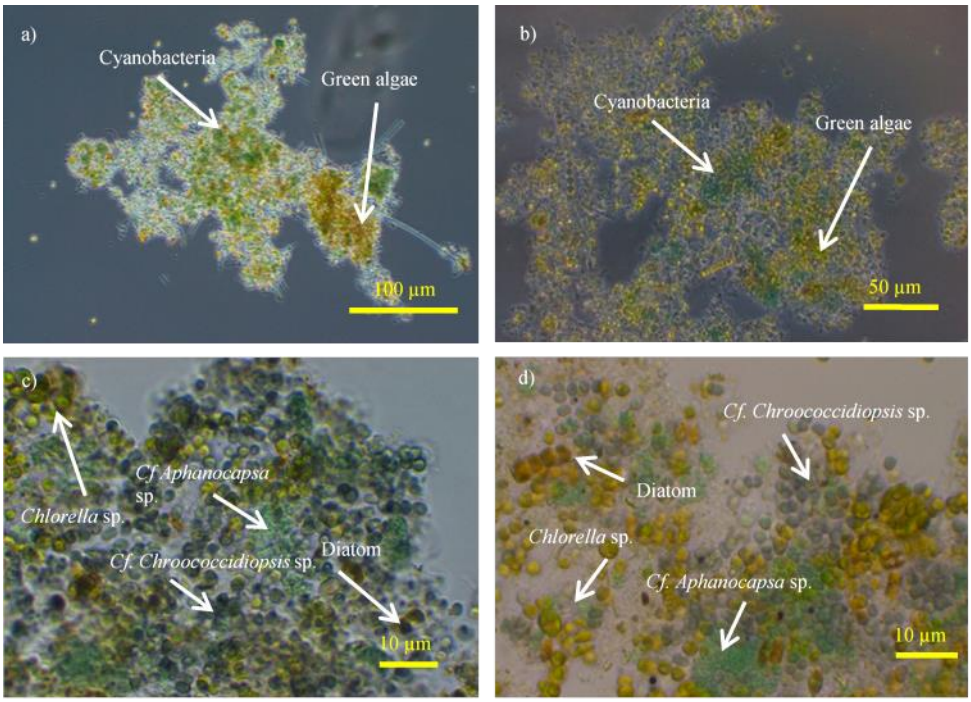


Fig. 8.2 Microscope images illustrating the initial microbial composition of the culture. a), b) mixed culture dominated by cyanobacteria immersed in flocs observed in phase contrast microscopy (200X) and (400X) respectively; note darker cyanobacteria aggregates; c), d) detail of floc composed by cyanobacteria *Aphanocapsa* sp. and *Chroococcidiopsis* sp. (bigger and darker cells than *Aphanocapsa* sp.), green algae *Chlorella* sp., and diatoms observed in bright light microscopy (1000X).

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 approximately 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.

Table 8.1 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)
TON (mg L^{-1})	22.2 (1.8)
TOP (mg L^{-1})	11.8 (1.6)
PHB (% (W/gTS))	3.4 (0.3)
Carbohydrates (% (W/gTS))	7.4 (0.4)

8.3.3 Analytical methods

The cultures in the reactors were analyzed for total inorganic carbon (TIC), total organic carbon (TOC), Inorganic phosphorus (IP) (measured as orthophosphate $P\text{-}PO_4^{3-}$), nitrite ($N\text{-}NO_2^-$) and nitrate ($N\text{-}NO_3^-$) on alternate days, 3 days per week. IC and soluble organic carbon (SOC) 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 IP in the case of DOP. Total organic nitrogen (TON) was calculated as the difference between TN and $N\text{-}NO_2^-$ and $N\text{-}NO_3^-$, whereas Total organic phosphorus (TOP) was determined as the difference between TP and IP. IP, $N\text{-}NO^{2-}$, $N\text{-}NO^{3-}$ concentrations were analyzed using an ion chromatograph DIONEX ICS1000 (Thermo-scientific, USA), and TOC, TIC, SOC, 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).

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.

8.3.4 PHB and carbohydrate analysis

PHB and carbohydrate content were measured daily in the constant illumination experiments, and measured daily at the end of the light phase in the 12h light/dark experiments. In this last experiment, polymers were also measured in samples taken at the dark phase during the first week. 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. (2013). 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 min 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% W, CAS 80181-31-3) was used as a standard for hydroxybutyrate (HB) and hydroxyvalerate (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).

Carbohydrate content was extracted following the method described Lanham et al. (2012). Briefly, approximately 2 mg of freeze-dried biomass were weighted and placed in glass tubes with Teflon liner screw caps, where 2 mL 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 (DuBois et al., 1956), using *D*-glucose as a standard.

8.3.5 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 indicators of autotrophic organisms 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 (John et al., 2002), as well as a database of cyanobacteria genus (Komárek and Hauer, 2013b).

8.3.6 Kinetic and stoichiometric parameters

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} (Cornet et al., 1998). Thus, the active biomass (X) was calculated as:

$$X = (VSS - Carbohydrates - PHB) \quad (1)$$

Carbohydrates and PHB were calculated according to (Fradinho et al., 2016a), in terms of percentage of VSS:

$$\%Carbohydrates\ or\ PHB = \frac{gPHB\ or\ gCarbohydrates}{gVSS} * 100 \quad (2)$$

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. (3):

$$YPHB/S\ or\ Ycarbohydrates/S = \frac{PHB\ or\ Carbohydrates\ accumulated}{IC\ consumed} \quad (3)$$

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.

8.4 **Results and Discussion**

8.4.1 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 (Fig. 8.3a). 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 (Fig. 8.3b). 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 (Fig. 8.3c and 8.3d). 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 (Li et al., 2009; Sforza et al., 2012).

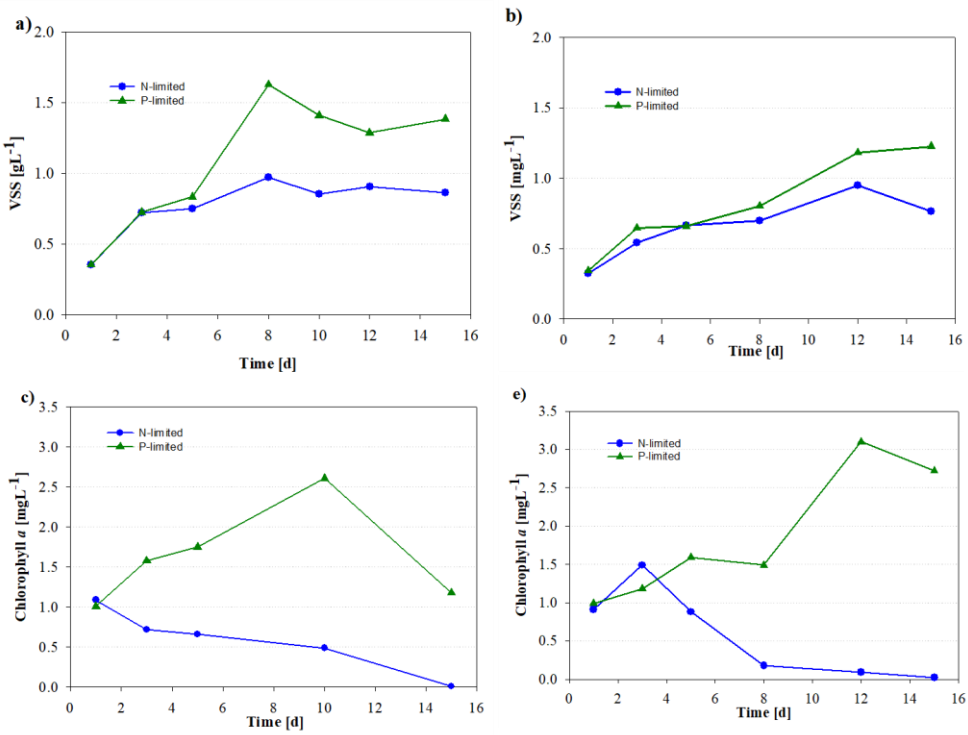
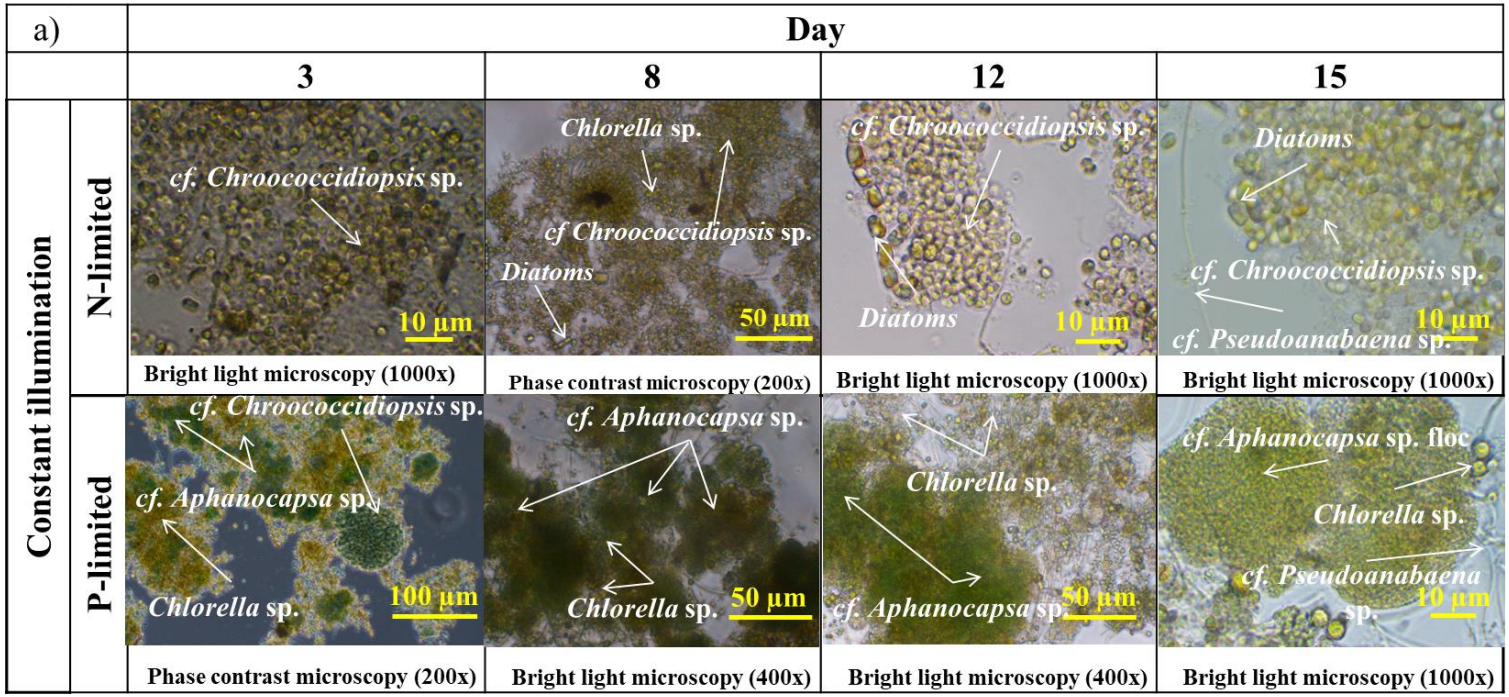


Fig. 8.3 Biomass (VSS) and Chlorophyll a concentration under nitrogen and phosphorus limitation in the cultures submitted to a) and c) permanent illumination and b) and d) light/dark alternation.

Regarding biomass composition, under permanent illumination in the experiment with N-limitation, the initial composition remained constant 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 Fig. 8.4a. and Supplementary Figure 8-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 (Fig. 8.4b).


a)
Day
3
8
12
15
Constant illumination
N-limited
P-limited
cf. Chroococcidiopsis sp.
10 µm
Bright light microscopy (1000x)
Chlorella sp.
cf Chroococcidiopsis sp.
Diatoms
50 µm
Phase contrast microscopy (200x)
cf. Chroococcidiopsis sp.
Diatoms
10 µm
Bright light microscopy (1000x)
Diatoms
cf. Chroococcidiopsis sp.
cf. Pseudoanabaena sp.
10 µm
Bright light microscopy (1000x)
cf. Chroococcidiopsis sp.
cf. Aphanocapsa sp.
Chlorella sp.
100 µm
Phase contrast microscopy (200x)
cf. Aphanocapsa sp.
Chlorella sp.
50 µm
Bright light microscopy (400x)
Chlorella sp.
cf. Aphanocapsa sp.
50 µm
Bright light microscopy (400x)
cf. Aphanocapsa sp. floc
Chlorella sp.
cf. Pseudoanabaena sp.
10 µm
Bright light microscopy (1000x)

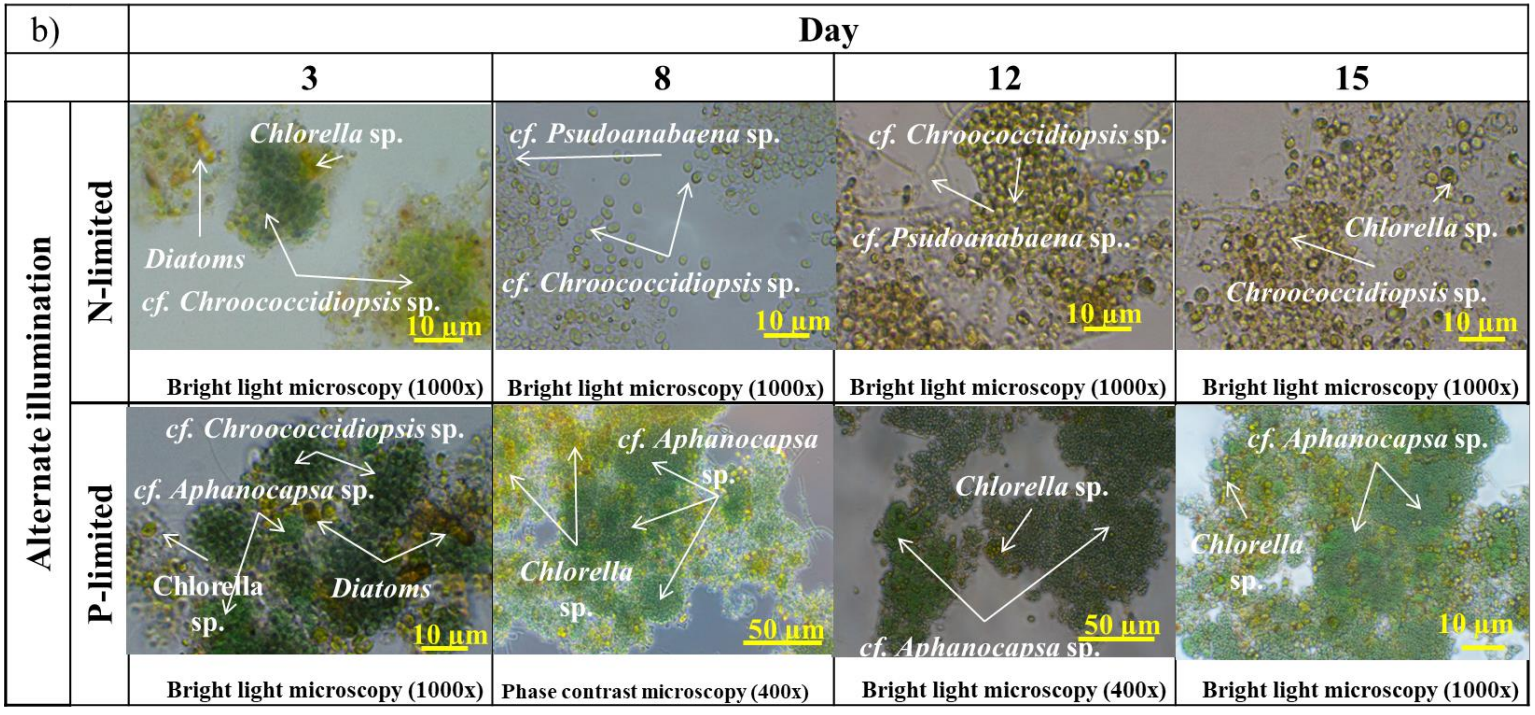


Fig. 8.4 Microscope images illustrating the microbial composition evolution of the culture submitted to a) permanent illumination and b) alternate illumination under nitrogen and phosphorus limitation trough the time. Microscopy technique used is indicated below each picture.

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.

8.4.2 Nutrients concentration

The IC and SOC concentrations in the cultures during the experiments are shown in Fig. 8.5. It can be observed that maximum values of SOC 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 conspicuous 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 $L\ d^{-1}$ (Albuquerque et al., 2010; Castro-Mayorga et al., 2014; Fradinho et al., 2016b).

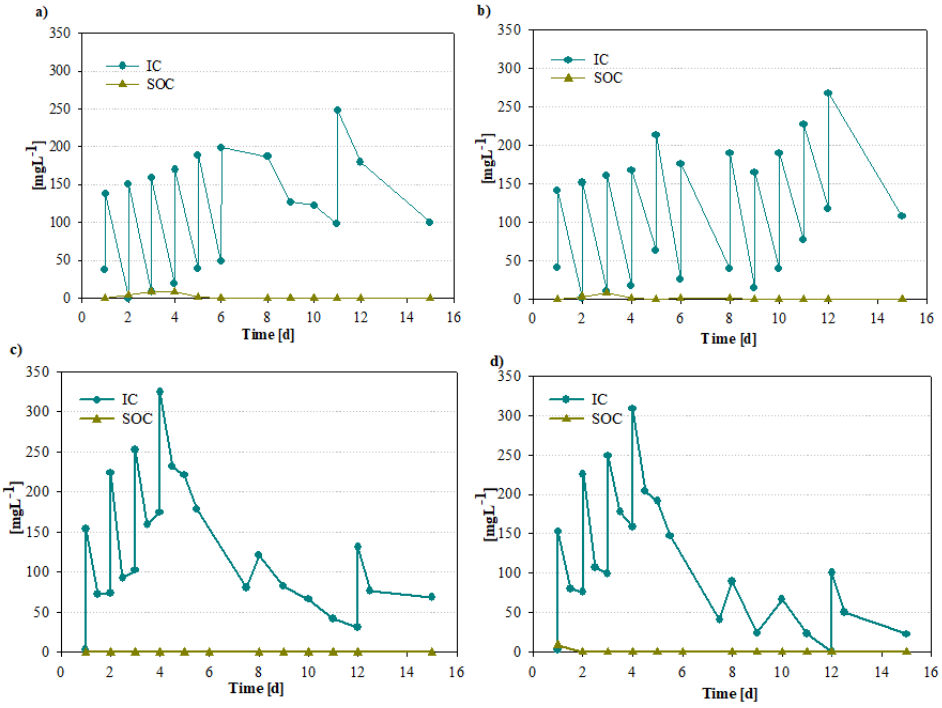


Fig. 8.5 Inorganic carbon and organic carbon dynamics for a) nitrogen and b) phosphorus limited conditions in the cultures submitted to permanent illumination; c) nitrogen and d) phosphorus limited conditions in the cultures submitted to light/dark alternation. The highest peaks indicate $NaHCO_3$ additions.

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 (Fig. 8.5a), 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 (Fig. 8.5b), 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 Fig. 8.5c). 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 8.2, the final 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 final 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 TOP, 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 TON and TOP 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}, whereas DOP showed values below 0.67 mg L^{-1} in all the experiments. However, these values can be considered low and relatively nigligibles, as stated in García et al., (2002). The ON and OP content therefore mostly corresponded to the active biomass.

Table 8.2 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
TON	22.28	154.22	27.22	238.05
IN	0	185	0	106
TP	14.73	12.9	13.36	10.62
TOP	12.13	12.9	11.66	10.62
IP	2.65	0	1.7	0

Regarding the TOC/TON 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. N limitation in the culture also led to a decrease of chlorophyll *a* content in these reactors observed from day 3 of operation until the last day, as shown in Figs. 8.3c and 8.3d. 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* (Collier and Grossman, 1992). Previous studies associated this process only with N limitation (Sauer et al., 2001), although this phenomenon was also observed in the study by Markou et al. (Markou et al., 2012b) 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 (Fig. 8.4a and 8.4b).

8.4.3 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 (Fig. 8.6a); 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 (Fig. 8.6b). 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}).

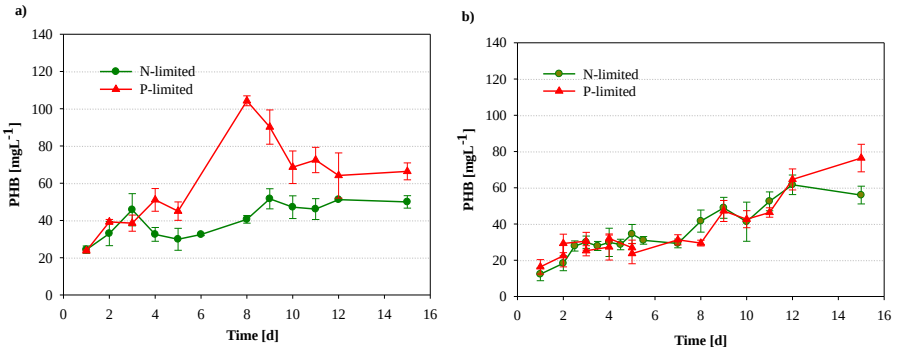


Fig. 8.6 PHB concentration under nitrogen and phosphorus limitation in the cultures submitted to a) permanent illumination and b) light/dark alternation.

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, (2016) and Nishioka et al, (2001), 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 (Miyake et al., 1996). 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. (Jau et al., 2005), 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) (Figs. 8.6b, Table 8.3), 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* (Sharma and Mallick, 2005) and *Synechocystis* sp. PCC 6803 (Wu et al., 2001). In these studies, the increase of PHB during dark periods was associated with the conversion of glycogen to PHB.

Table 8.3 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 chapter
Cyanobacteria dominated mixed culture	P	24:0	104.23	5.7	8	This chapter
Cyanobacteria dominated mixed culture	N	12:12	61.61	6.5	12	This chapter
Cyanobacteria dominated mixed culture	P	12:12	76.36	5.6	15	This chapter
Nostoc muscorum	N	14:10	-	6.4	21	(Ansari and Fatma, 2016)
Anabaena cylindrica	N	24:0	-	0.2	21	(Lama et al., 1996)
Synechococcus sp. MA19	N	0:24	67.2	21	6	(Miyake et al., 1996)
Synechocystis sp. PCC6803	N	24:0	-	14.6	12	(Monshupanee and Incharoensakdi, 2014)
Synechocystis sp. PCC6803	P	24:0	-	13.5	12	(Monshupanee and Incharoensakdi, 2014)
Synechocystis salina	P	16:8	123.2	6	30	(Meixner et al., 2016)
Synechococcus sp. MA19	P	24:0	1400	62	4	(Nishioka et al., 2001)
Spitulina maxima	N	24:0	-	0.7	4	(De Philippis et al., 1992)
Spitulina maxima	P	24:0	-	1.2	4	(De Philippis et al., 1992)
Nostoc muscorum	P	14:10	-	8.5	21	(Sharma and Mallick, 2005)
Spitulina platensis	P	14:10		3.5	60	(Panda et al., 2006)

8.4.4 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. (Fig. 8.7a). 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 (Fig. 8.7b).

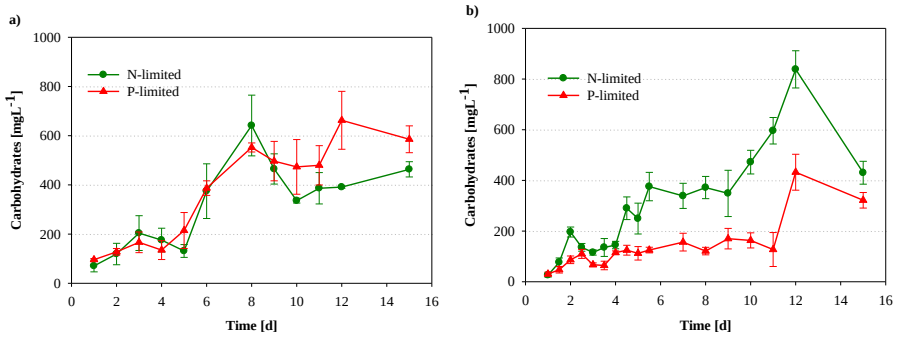


Fig. 8.7 Carbohydrates concentration under N and P limitation in the cultures submitted to a) permanent illumination and b) light/dark alternation.

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. (2012a), 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 chapter, 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 De Philippis et al. (1992) and Monshupanee and Incharoensakdi (2014).

8.4.5 Polymer 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 (Gründel et al., 2012). In the present chapter, biomass as well as polymer 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 polymers such as PHB and carbohydrates in mixed cyanobacteria-microalgae (eukariothic) wastewater borne cultures. For comparison purposes, literature results on PHB and carbohydrates accumulation in photoautotrophic conditions on cultures of cyanobacteria are summarized in Tables 8.3 and 8.4. As it can be seen, PHB and carbohydrate accumulation is species-dependent and in cases where the content of both polymers was evaluated (e.g. this study and the studies by De Philippis et al. (1992) and Monshupanee and Incharoensakdi, (2014), 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 chapter. In some cases, as in the study by Sharma and Mallick, (2005), a slightly higher accumulation percentage was obtained, but after a longer time of incubation (21 days). In the study by Monshupanee et al. (2014), 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 Miyake et al. (1996) and Nishioka et al. (2001) obtained the highest percentages with cyanobacteria *Synechococcus* sp. MA19 submitted to nutrients limited conditions with IC as the carbon source.

Table 8.4 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 chapter
Cyanobacteria dominated mixed culture	P	24:0	662.38	46.05	12	This chapter
Cyanobacteria dominated mixed culture	N	12:12	838.05	74.76	12	This chapter
Cyanobacteria dominated mixed culture	P	12:12	432.13	35.98	12	This chapter
Arthrospira platensis	N	24:0	800	65	3.5	Aikawa et al., 2012)
Spitulina platensis	P	24:0	-	65	-	Markou et al., 2012b)
Spitulina platensis	P	24:0	-	63	9	Markou et al., 2013)
Synechocystis sp. PCC 6803	N	24:0	-	36.8	12	Monshupanee and Incharoensakdi,
Synechocystis sp. PCC 6803	P	24:0	-	28.9	12	2014)
Spitulina maxima	N	24:0	-	70	2.7	De Philippis et al. (1992)
Spitulina maxima	P	24:0	-	23	2.7	De Philippis et al. (1992)
Arthrospira platensis	N	24:0	-	65	7	Sassano et al. (2010)

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. (1992), 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) De Philippis et al. (1992), 28.9% dcw after 12 d Monshupanee and Incharoensakdi, (2014). Only the strain *Spirulina platensis* was able to accumulate more than 60% dcw of carbohydrates in a P-limited culture (Markou, 2012; Markou et al., 2013).

Observing the kinetic specific rates of all tests (Table 8.5), the highest specific consumption rate of IC per g of biomass was observed under permanent illumination in both limitation conditions, reaching rates of 0.351 and 0.353 Cmol IC·Cmol X^{-1} 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.

Table 8.5 Kinetic and stoichiometric parameters.

	Permanent illumination		Light/dark alternation	
Parameter	N-limited	P-limited	N-limited	P-limited
-qS [Cmol IC·Cmol X^{-1} d^{-1}]	0.3510	0.3531	0.2391	0.1547
qPHB[Cmol PHB·Cmol X^{-1} d^{-1}]	0.0005	0.0007	0.0008	0.0005
qcarbs [Cmol carbs·Cmol X^{-1} d^{-1}][a]	0.0044	0.0020	0.0043	0.0013
Yphb/S[Cmol PHB·Cmol IC^{-1} d^{-1}][b]	0.0014	0.0017	0.0035	0.0028
Ycarbs/S[Cmol carbs·Cmol IC^{-1} d^{-1}][a,b]	0.0096	0.0051	0.0143	0.0053

[a] Carbohydrates

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

In general, results obtained in this chapter 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 polymers 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.

8.5 **Conclusion**

This chapter 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 polymer 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 chapter highlights and demonstrates that nutrient limitation could be a good approach to enhance PHB and carbohydrate accumulation in wastewater-borne cyanobacteria.

Chapter 9

Enhancing PHB and carbohydrates production using the feast and famine strategy

The contents of this chapter were adapted from the publication: Arias, D.M., Fradinho, J.C., Uggetti, E., García, J., Oehmen, A., Reis, M.A.M., 2018. Polymer accumulation in mixed cyanobacterial cultures selected under the feast and famine strategy. Algal Res. 33, 99–108. doi:https://doi.org/10.1016/j.algal.2018.04.027

9.1 Abstract

In this chapter, a photo-sequencing batch reactor (PSBR), operated with transient carbon availability (feast and famine) and different nutrients loads, was used to select cyanobacteria accumulating polyhydrohybutyrate (PHB) and carbohydrates from a mixed wastewater-borne microbial culture. The PSBR 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 PSBR, 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 PSBR 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.

9.2 Introduction

Cyanobacteria are prokaryotes capable to perform oxygenic photosynthesis and they can be found in almost every environment on earth (Whitton and Potts, 2012). 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 (Abed et al., 2009). Cyanobacteria are able to accumulate both carbohydrates in form of glycogen and and polyhydroxyalkanoates (PHA), e.g., polyhydroxybutyrate (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 (Panda

and Mallick, 2007). 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 (Stal, 1992). 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 (Aikawa et al., 2015; Drosg et al., 2015; Koller and Marsalek, 2015; Markou, 2012; Meixner et al., 2016), implying strictly controlled processes leading to high production costs, and subsequently expensive products (Samantaray and Mallick, 2012). 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 (Cottingham et al., 2015; Havens et al., 2003; Konopka et al., 1978; Levich, 1996), but also in wastewater systems (Arias et al., 2017; Van Den Hende et al., 2016b), highlighted that absolute nutrients concentration and ratio (N:P) are the two most important factors influencing the competition of cyanobacteria with other species (e.g., green algae) (Cottingham et al., 2015; Talbot and de la Noüe, 1993). More specifically, the dominance of cyanobacteria has been related to their high affinity for N and P and capacity to store them intracellularly (Flores and Herrero, 2014).

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 (De Philippis et al., 1992). 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 (Markou et al., 2013; Monshupanee and Incharoensakdi, 2014; Sharma and Mallick, 2005). 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 (Reis et al., 2003). 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 chapter, a mixed wastewater consortium was cultivated in a sequencing batch reactor (PSBR), 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.

9.3 Material and methods

9.3.1 Photo-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 photo-sequencing batch reactor (PSBR). The inoculum utilized consisted of a consortium of green algae, cyanobacteria, bacteria and protozoa, obtained from a pilot photobioreactor described elsewhere (Arias et al., 2017).

The PSBR 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. 9-S1):

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 PSBR was continuously illuminated with two external LED lamps (23W) placed on the two sides of the PSBR 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 PSBR was operated for 6 months during which three different conditions were tested as shown in Table 8.1. 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 to be in steady state when the concentration of total suspended solids (TSS) at the end of the cycle showed stable results or after the system reached three SRTs.

The growth solution added at the beginning of the light phase in each cycle consisted in growth medium containing: 0.049 g L^{-1} NH4Cl, 0.072 g L^{-1} $CaCl_2 \cdot 2H_2O$, 0.001 g L^{-1} Na_2EDTA, 0.075 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.6 g L^{-1} $C_6H_8FeNO_7$ (ammonium ferric citrate), 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 $Na_2Mo_4 \cdot 2H_2O$, 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 $Co(NO_3)_2.6H_2O$. Depending on the operation of the PSBR, K_2HPO_4 concentration varied 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

independently as the inorganic carbon source by the addition of 1 pulse of 6 mL containing 0.442 g L^{-1} (0.050 g C L^{-1}) at the beginning of the carbon phase during operations 1 and 2, and 2 pulses of 0.442 g L^{-1}, one at the beginning and the other in the middle of the light phase in condition 3.

Table 9.1 Experimental operating conditions of the PSBR.

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]
Condition 1	50	12.5	4.2
Condition 2	50	12.5	1
Condition 3	100	12.5	1

[a] Volumetric load per volume of reactor.

9.3.2 Batch test for polymer accumulation

For each operation period, when steady state was reached, 200 mL of mixed liquor were collected and placed in a separate batch reactor, filled to 400 mL with deionized water and used for polymer production batch experiments. In these tests, the operation of the 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 (Fradinho et al., 2013a; Oehmen et al., 2005).

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-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 PSBR cycle, leading to nutrient limitation in all the experiments.

9.3.3 Analytical Methods

Each time that the PSBR 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 (SOC), Inorganic phosphorus (IP) (measured as orthophosphate P-PO_4^{3-}), nitrite (N-NO_2^-), nitrate (N-NO_3^-), and total ammoniacal nitrogen (sum of N-NH_4^+ and N-NH_3^+). Unfiltered samples were used to analyze total nitrogen (TN) and total phosphorus (TP). Particulate Organic Nitrogen (PON) was calculated as the difference between TN and NH_4^+, N-NO_2^- and N-NO_3^-, whereas particulate phosphorus (PP) was determined as the difference between TP and IP, it should be notice that PP is composed of intracellular particulate inorganic P (phosphate, pyrophosphate, and polyphosphate) and organic P. SOC and IC were measured by means of the TOC analyzer (Shimadzu, Japan) while IP, N-NH_4^+, N-NO^{2-}, N-NO^{3-} concentrations were measured by colorimetry using a segmented flow analyzer (Skalar, The Netherlands). TN was analyzed spectrophotometrically with total nitrogen kits (Hach, Germany), and TP was analyzed following the methodology described in Standard Methods (APHA-AWWA-WPCF, 2001).

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

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

Carbohydrates and PHB content were measured according to the methods described by (Lanham et al., 2012) and (Lanham et al., 2013) with minor modifications. Thus, the biomass collected was centrifuged, ultra-frozen and finally freeze dried. Carbohydrates were determined by mixing the freeze-dried biomass with 2 mL of 0.9 M HCl and digested for 2 h at 100 ºC. The supernatant was filtered (0.45 μm membrane) and glucose was analyzed by HPLC using D-glucose as a standard. For PHB determination, freeze-dried biomass was digested for 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 PSBR 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).

9.3.4 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 IP L^{-1} d^{-1}] and N volumetric load (N load) [mg TAN L^{-1} d^{-1}] were calculated following Eq. (1):

$$\text{C load, P load, N load} = \frac{\text{C-}Na_2CO_3 \text{ or IP or N-}NH_4^+ * Q}{V} \quad (1)$$

Where Q is the flow [L^{-1} d^{-1}], IP^- or TAN is the influent concentration [mg L^{-1}] and V [L^{-1}] is the volume of the PSBR.

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 $Cmol^{-1}$ (Cornet et al., 1998). Thus, the active biomass (X) was calculated as:

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

Carbohydrates and PHB were calculated according to (Fradinho et al., 2016a), in terms of percentage of VSS:

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

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):

$$Y_{PHB/S \text{ or } Y_{carbohydrates/S}} = \frac{\text{PHB or Carbohydrates accumulated}}{\text{IC consumed}} \quad (4)$$

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

9.4 **Results and discussion**

9.4.1 Photo-sequencing batch reactor (PSBR) performance

9.4.1.1 General conditions

Throughout the PSBR 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 PSBR. In Fig. 9.1, 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 9.1), in spite of not having carbon addition in the first 6 hours of light, photosynthetic activity was registered due to the residual IC present in the bulk culture at the start of the light phase (Fig. 9.1a). Hence, during the first 2 hours of the light phase and until all IC was consumed, oxygen increased to 12.5 mg L^{-1}. After that time, a gradual decrease of dissolved oxygen was observed until reaching 9.8 mg L^{-1}. Once the carbon was supplied to the culture, an increasing pattern in the oxygen concentration was observed, until reaching a concentration around 40 mg L^{-1}. At the same time, the IC content in the medium declined reaching a specific uptake rate of 0.019±0.004 Cmol IC/Cmol X^{-1} d^{-1} (Table 9.2). As soon as the IC was completely consumed (approximately after 4.5 hours), the oxygen gradually decreased until the dark phase started. Then argon was supplied to the culture, and oxygen quickly reached zero. During the addition of carbon and IC uptake, pH tended to increase due to the photosynthesis performed by microalgae/cyanobacteria and during the dark phase, pH tended to decrease likely because of the organic acids released by the culture (Atteia et al., 2013; Bouteleux et al., 2005). This is a plausible assumption since a concentration of 7.8 mg L^{-1} of SOC was measured at the end of the dark phase. In both cases, when pH tended to increase or decrease, there was an addition of acid or base solutions to maintain pH value around 8.2, which can be observed in the oscillations in Fig. 9.1.

Concerning condition 2, where low carbon load was coupled with low phosphorus, a profile similar to condition 1 was observed. Thus, oxygenic activity

was observed from the start of the light phase as consequence of the residual IC (around 2.7 mg L^{-1}) (Fig. 9.1b), following the same trend on pH, oxygen and IC as condition 1. However, a faster uptake of IC 0.039±0.002 Cmol IC/Cmol X^{-1} d^{-1} (Table 9.2) and higher concentrations of oxygen (up to 45 mg L^{-1}) were reached. During condition 2, pH oscillations were less frequent than condition 1 during the dark phase. Indeed, SOC released during the dark phase was slightly lower than in condition 1, reaching 6.5 mg L^{-1}.

Contrary to the conditions previously mentioned, differences in the oxygen profiles were observed in condition 3. In this case, due to the addition of IC from the start of the light phase, the oxygen started increasing until reaching values of 34.42 mg L^{-1} after 8 hours and maintaining constant values (±0.29 mg L^{-1}) during the rest of this period. According to these results, pH oscillated along the entire light phase. Like under the previous conditions, oxygen was always present in the culture while most of the total IC consumption occurred in the light phase, and only 4% of IC was consumed during the dark period. In spite of having more IC availability, the IC uptake rate was lower when compared with conditions 1 and 2, having only a rate of 0.016±0.002 Cmol IC/Cmol X^{-1} d^{-1} (Table 9.2). Also, several oscillations in pH values were observed during the dark phase, recording high concentrations of SOC (~18 mg L^{-1}) at the end of the dark phase.

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

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

It has to be considered that oxygen reached very high levels in the PSBR due to the reduced headspace of the reactor and the poor oxygen transfer between the reactor and the atmosphere. In this chapter, values up to 560% were reached, considering that 100% of air 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 (Peng et al.,

2013; Solimeno et al., 2015). In general, it is stated that the maximum tolerable dissolved oxygen level is 400% of air saturation (Chisti, 2007). 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

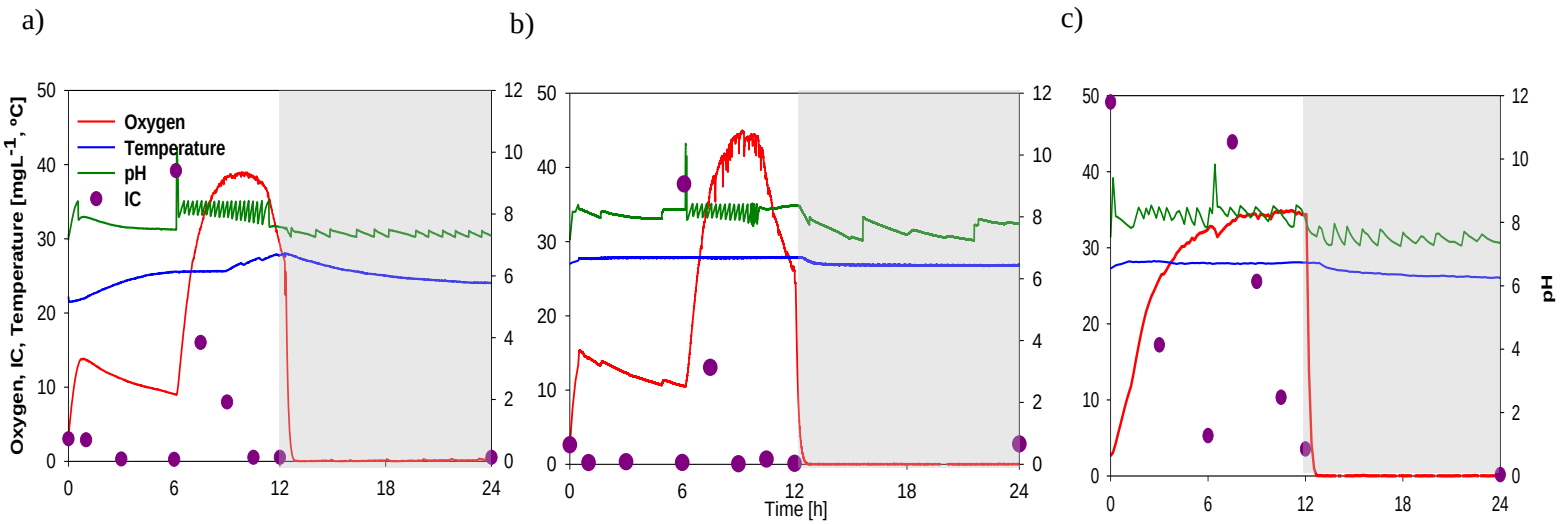


Fig. 9.1 Oxygen, temperature, pH and inorganic carbon (IC) patterns during one cycle of operation of a) carbon limitation (Condition 1) b) carbon and phosphorus limitation (condition 2), and c) phosphorus limitation (condition 3). White zone represents the light phase and grey zone represents the dark anaerobic phase.

9.4.1.2 Nutrient profiles

During steady-state operation in condition 1, the profile of TAN showed a higher consumption than the uptake of IP, as it can be seen in Fig. 9.2a. 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 TAN·g X^{-1} d^{-1} and 0.22 mg IP·g X^{-1} 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 9.3). 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.

In condition 2, the N-NH_4^+ profile showed a similar pattern as in condition 1 (60% of removal in the light phase), although the N-NH_4^+ specific uptake rate was slightly higher (1.24 mg TAN·g X^{-1} d^{-1}). However, the highest uptake of TAN was found after the pulse of carbon in the middle of the light phase, where 53% of the TAN was achieved. On the other hand, contrarily to condition 1, the phosphate uptake rate was lower (0.103 mg IP·g X^{-1} d^{-1}) and phosphate was completely removed after 9 hours (Fig. 9.2b). In this condition, PON and PP showed similar values and percentages in terms of active biomass than condition 1 (65±17 mg L^{-1} and 3.7±1.8 mg L^{-1}, respectively, Table 9.3). The biomass concentration was slightly higher than condition 1, (0.74±0.05 g VSS L^{-1}). The active biomass profile was constant during the first eight hours of the light phase (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.

Table 9.2 Kinetic and stoichiometric parameters of the PSBR 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^{-1} d^{-1}]	0.019 (0.004)	0.039 (0.002)	0.016 (0.002)
q_{PHB}[Cmol PHB·Cmol X^{-1} d^{-1}]	-	-	0.00032 (0.0003)
q_{carbs} [Cmol Carbs·Cmol X^{-1} d^{-1}]	0.011 (0.002)	0.049 (0.003)	0.0085 (0.003)
$Y_{PHB/S}$[Cmol PHB·Cmol IC^{-1}]	-	-	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.

Table 9.3 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)

Finally, in condition 3, the simultaneous addition of carbon and nutrients led to the maximum N and P removals observed in the PSBR. Thus, after adding the nutrients to the culture, $N\text{-}NH_4^+$ and IP were almost completely consumed. With respect to the specific uptake rates, N uptake almost doubled the value of the previous conditions with 2.3 mg $N\text{-}NH_4^+$·g X^{-1} d^{-1} and P uptake showed similar uptake than condition 1 with 0.22 mg IP·g X^{-1} d^{-1}. In this condition, the VSS concentration raised to an average of 1.2±0.1 g VSS L^{-1} during the three cycles, while the active biomass averaged 0.99±0.09 g X L^{-1}, approximately 50% higher than the values of condition 1 (0.66 g VSS L^{-1}). Considering the removal of N and P, it can be assumed that this condition led to nitrogen and phosphorus limitation during most of the operational time. As shown in Fig. 9.2c, the nutrients supplied at the beginning of the light phase were coupled with the carbon addition. During steady-state operation, as it can be seen in Fig. 9.2c, 84.7% of $N\text{-}NH_4^+$ removal was found during the first hour of the light phase, and was completely removed after 4 hours. Concurrently, IP was completely consumed as soon as it was added. Due to the increment in inorganic nitrogen removal, PON concentration increased to 81±9.06 but maintaining similar percentages than the other conditions (8.2±0.9 % g·g X^{-1}, while OP registered a concentration of 15±1.8 mg L^{-1} and a percentage of 1.5 % g·g X^{-1} (Table 9.3).

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 (Cuéllar-bermúdez et al., 2017). Considering these ratios and biomass concentrations, and the formation of PON and PP (Table 9.3), 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 (Cottingham et al., 2015).

9.4.1.3 Polymer accumulation in the photo-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 9.2); while PHB content only 0.3±0.1% at the end of the light phase. As shown in Fig. 9.3a, 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 (Atteia et al., 2013; Miyamoto and Miura, 1987). 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 (Stal and Moezelaar, 1997), then, without the presence of an electron acceptor, as occurred in this case, energy generation occurs during glycogen conversion to PHA (Zhou et al., 2008). 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) (Bengtsson, 2009; Oehmen et al., 2006, 2005; Zhou et al., 2008), and also occurring in anoxygenic photosynthetic bacteria (Fradinho et al., 2013b). 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%).

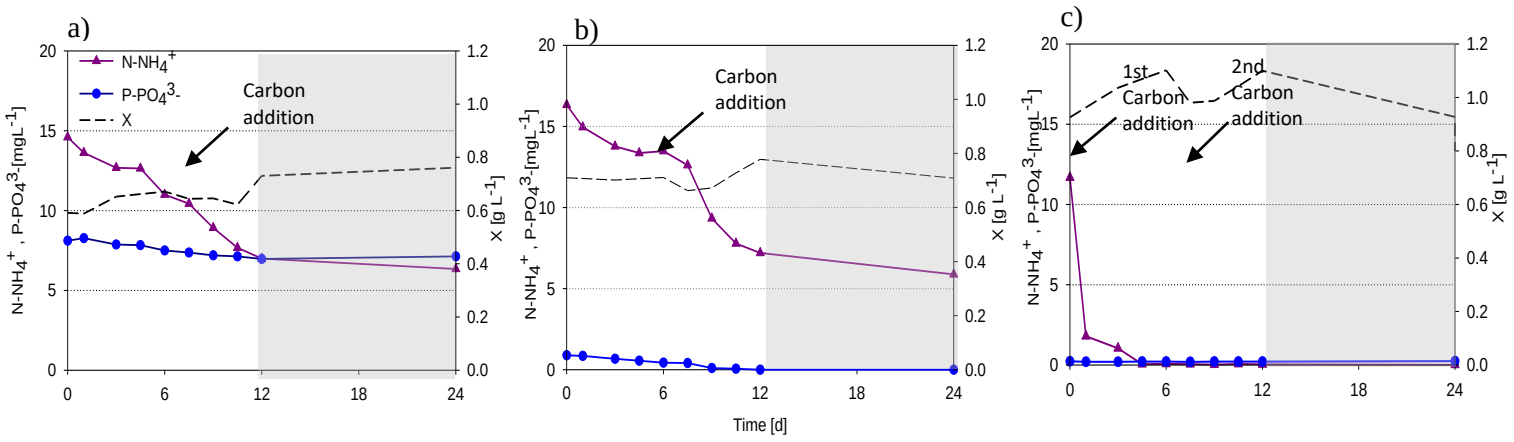


Fig. 9.2 Nutrient concentrations and active biomass (X) during one cycle of operation of a) carbon limitation (Condition 1) b) carbon and phosphorus limitation (condition 2), and c) phosphorus limitation (condition 3). White zone represents the light phase and grey zone represents the dark anaerobic phase.

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 9.2). After finishing the IC available, carbohydrates were quickly consumed before the end of the light phase, but slightly consumed during the dark phase (Fig. 9.3b). 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 PSBR, 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. 9.3c). 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.

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^{-1} 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 9.2). 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^{-1}). 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 $carbs^{-1}$) than by means of IC uptake (0.009 Cmol PHB·Cmol IC^{-1}). This was likely due to the highest carbohydrate concentration in condition 3, in comparison with other conditions.

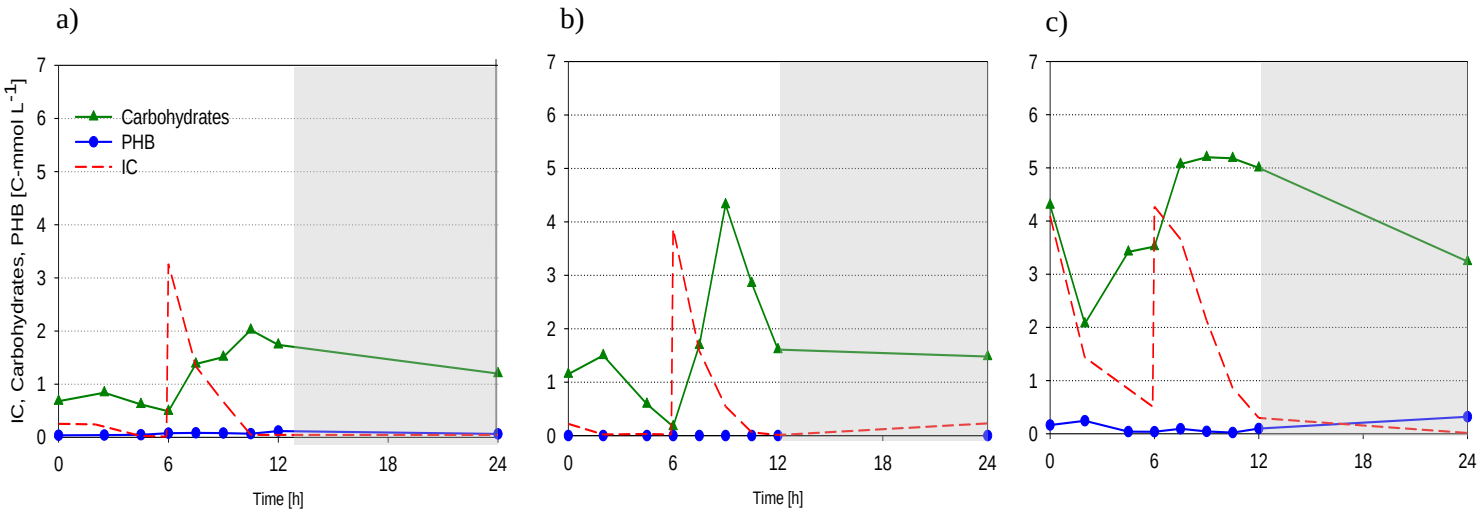


Fig. 9.3 Inorganic carbon (IC) consumption profile and transformation of polyhydroxybutyrate (PHB) and carbohydrates during one cycle of operation of a) carbon limitation (Condition 1) b) carbon and phosphorus limitation (condition 2), and c) phosphorus limitation (condition 3). The white zone represents the light phase and grey zone represents the dark anaerobic phase.

9.4.1.4 Biomass microbial composition

Throughout the operation of the PSBR, 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. 9.4).

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. 9.5). 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., 2017 (Chapter 4) (Arias et al., 2017), 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. 9.6).

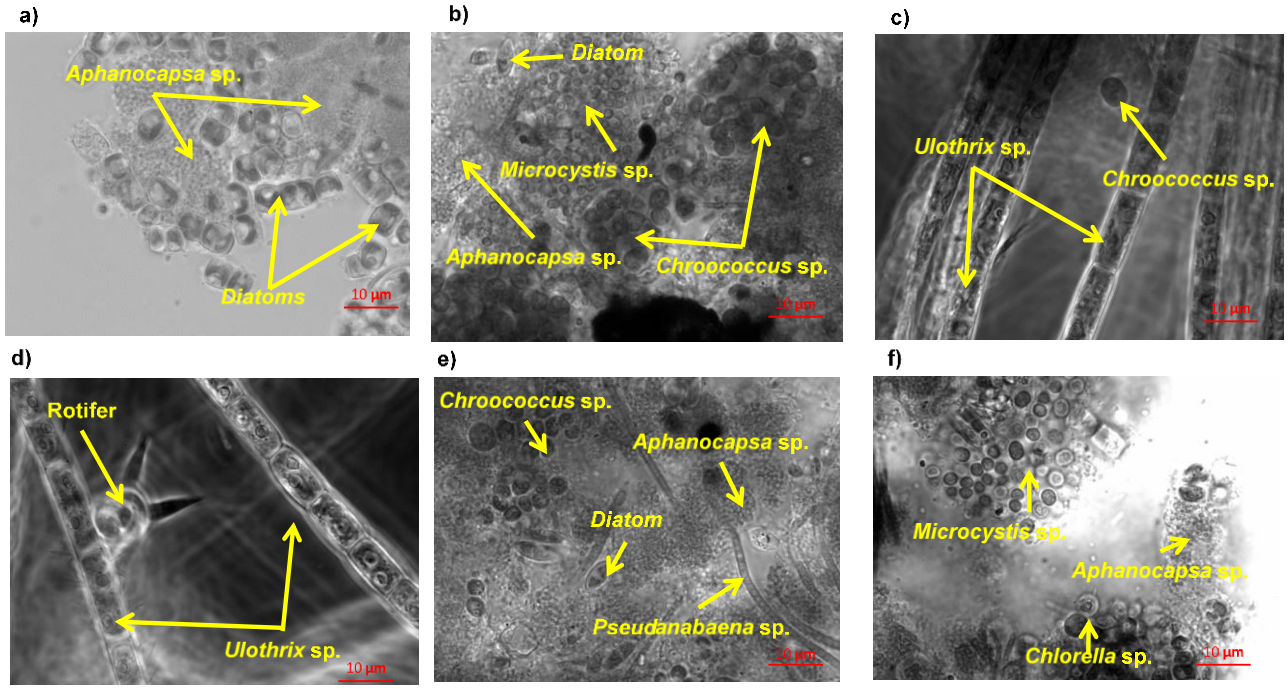


Fig. 9.4 Microscopic images illustrating the microbial diversity during condition 1 (carbon limitation); a) lateral side of an algal floc composed by cyanobacteria *Aphanocapsa* sp. and diatoms; b) algal floc composed by *Aphanocapsa* sp., *Microcystis* sp., *Chroococcus* sp. and some diatoms immersed; c) filaments of green algae *Ulothrix* sp. and a cell of *Chroococcus* sp.; d) rotifer's tail attached to a filament of green algae *Ulothrix* sp.; e) algal floc largely dominated by *Aphanocapsa* sp. and with *Chroococcus* sp. and *Pseudanabaena* sp. and diatoms immersed; f) flocs of *Chroococcus* sp. and *Aphanocapsa* sp. and a floc of green algae *Chlorella* sp. All the observations were performed in bright field microscopy (1000×).

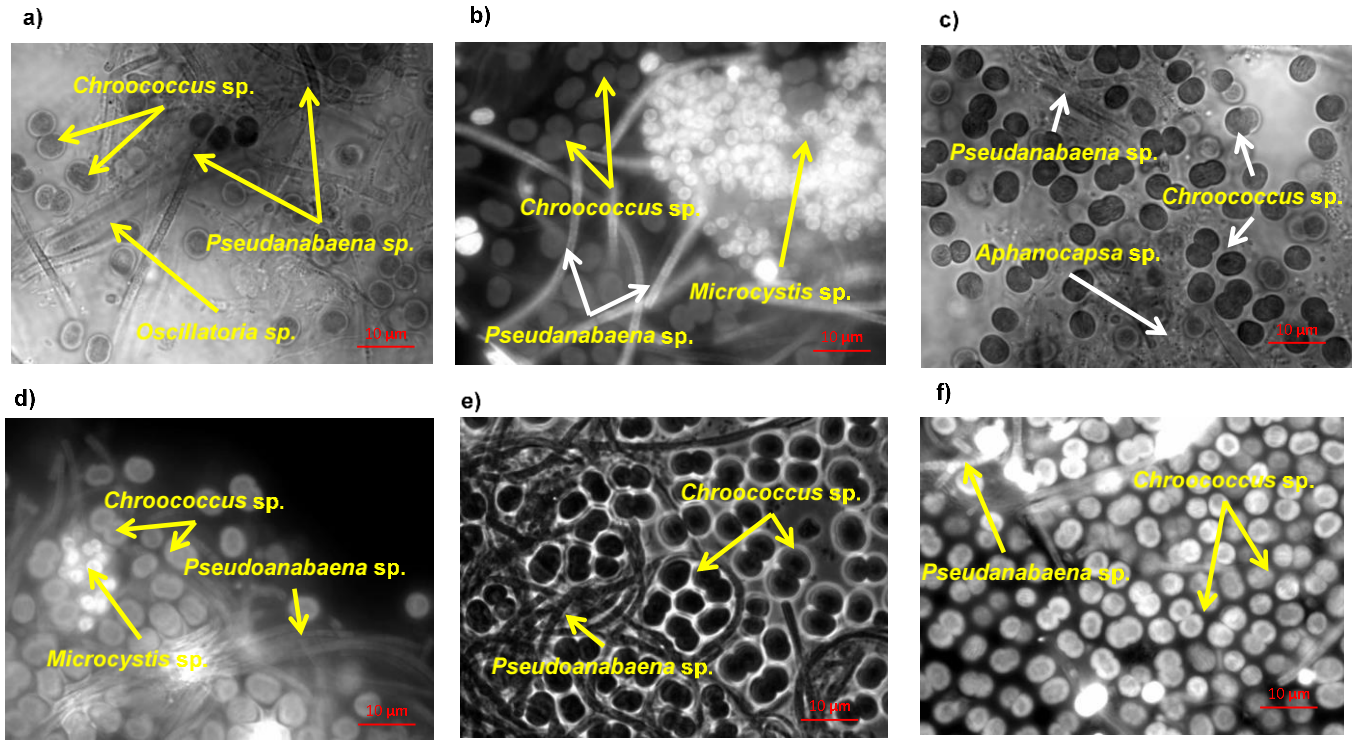


Fig. 9.5 Microscopic images illustrating mainly the dominance of the cyanobacteria *Chroococcus* sp. in condition 2 (phosphorus and carbon limitation); *Aphanocapsa*, *Microcystis* sp. and some filaments of *Pseudanabaena* sp. are also observed. The observations were performed in a, c, e) bright field microscopy (1000×) and c, d, f) fluorescence microscopy (1000×).

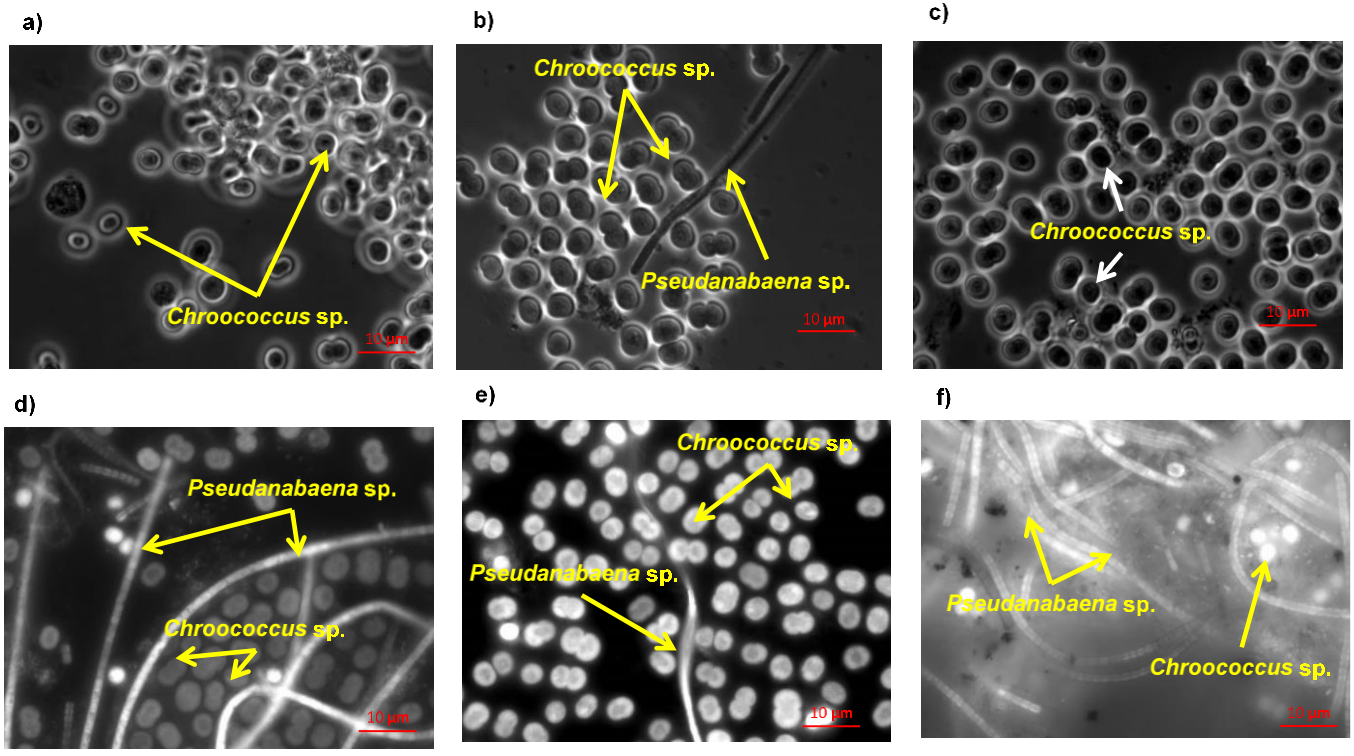


Fig. 9.6 Microscopic images illustrating the dominance of cyanobacteria *Chroococcus* sp. with some filaments of *Pseudanabaena* sp. during condition 3 (phosphorus limitation). The observations were performed in a, b, c) bright field microscopy (1000×) and c, d, f) fluorescence microscopy (1000×).

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

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

9.4.2 Polymers accumulation in batch test experiments

A batch test was performed after reaching the steady state of each operational condition tested in the PSBR in order to assess the polymer (PHB and carbohydrates) storage capacity. These experiments started with biomass concentrations of 0.46, 0.61 and 1.51 $g \cdot VSS \cdot L^{-1}$, for conditions 1, 2 and 3, respectively. Since no additional nutrient source was supplied to the biomass, the remaining nitrogen ($N\text{-}NH_4^+$ of 1.8 and 1.1 mg L^{-1} for condition 1 and 2, respectively) was consumed in the first hours of the tests, while no remaining N was observed in the batch test corresponding to condition 3. In the case of IP, this

nutrient was only observed in the first hours of condition 1, at 1.15 mg IP L^{-1} and not observed in any of the other accumulation tests. Furthermore, other parameters, such as dissolved oxygen, did not reach the levels achieved in the continuous operation during the light phase due to a greater oxygen transfer, since it was an open system (9.6 mg O_2 L^{-1}, 28ºC).

The accumulation of carbohydrates was achieved during the light aerobic phase in all the conditions. Hence, 6.62, 11.41 and 15.57 Cmmols Carbs L^{-1}, corresponding to percentages of 29, 48 and 43%, were obtained in the batch test of conditions 1, 2 and 3, respectively, after 24h of incubation (Fig. 9.7). Although a slight increase on carbohydrate content was observed in the first 4 hours of the dark anaerobic phase in condition 2 (from 48 to 53%), the carbohydrates content declined in all the conditions during the dark anaerobic phase. On the other hand, PHB started increasing in the light phase, as long as the N-NH_4^+ was depleted in the medium. Contrary to carbohydrate patterns, PHB accumulation also continued in the dark anaerobic phase for batch tests of conditions 1 and 2 likely occurring by means of carbohydrate conversion to PHB. In this case, PHB reached values of 1.63 and 1.08 Cmmol PHB L^{-1}, corresponding to percentages of 3.9 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 PSBR 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. from the photo-sequencing batch reactor (PSBR) 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.

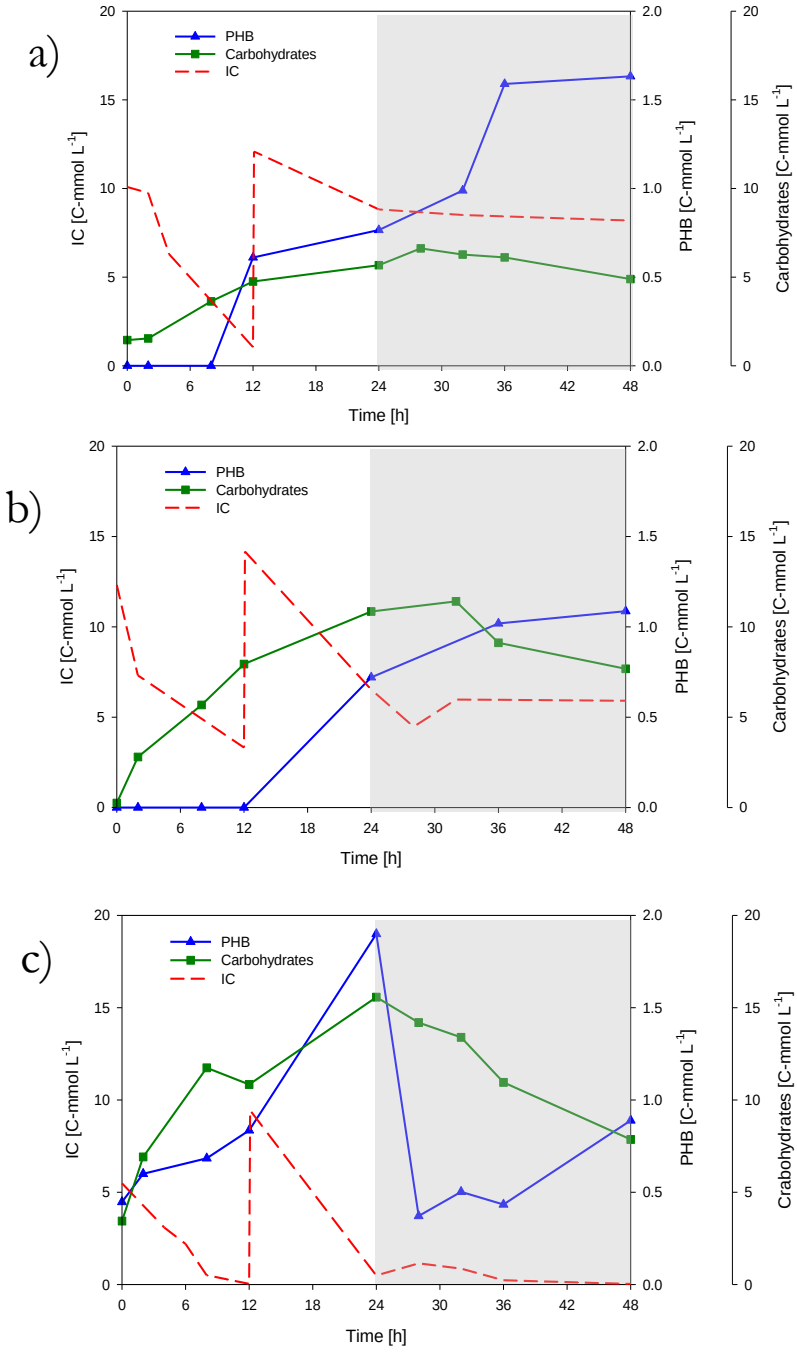


Fig. 9.7 Inorganic carbon (IC) consumption profile, poly (3-hydroxyalkanotes) (PHB) and carbohydrates transformation in batch tests performed with biomass collected

Reviewing the specific kinetic rates in all the batch tests (Table 9.4), 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 PSBR (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 PSBR (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 PSBR 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 PSBR (~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 9.4). 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 PSBR (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 PSBR, 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 9.4), this value is lower than the yield achieved by other anoxygenic photosynthetic bacteria 0.94 Cmol PHB/Cmol carbs (Fradinho et al., 2013a). 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 (e.g., 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 9.4 Kinetic and stoichiometric parameters of the batch tests performed during a steady state of the PSBR for each operational condition.

		$-q_s$ [Cmol IC·Cmol X^{-1} d^{-1}]	$q_{carbohydrates}$ [Cmol Carbs ·Cmol X^{-1} d^{-1}]	q_{PHB} [Cmol PHB ·Cmol X^{-1} d^{-1}]	Y_{carbs} [Cmol Carbs·Cmol IC^{-1}]	Y_{PHB} [Cmol PHB·Cmol IC^{-1}]	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 (Reis et al., 2003). 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 (Stal and Moezelaar, 1997). On the other hand, PHB accumulated under both light and dark conditions during the aerobic light phase as long as IC was available (and N was limited), and during the anaerobic dark phase from the carbohydrates stored in the previous illuminated phase.

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

Considering that the main goal of his study was to produce both polymers; this research is contributing to the advance in the limited knowledge of feast and famine application to cyanobacteria producing PHB and carbohydrates. Among the

possible applications for both polymers produced, carbohydrates can be used as substrate to obtain biodiesel, bioethanol and biomethane, while PHB can be directed for bioplastics generation. The application of either of the two polymers will depend on the economic feasibility of each process and the commercial interest of the final product.

9.4.2.1 Comparison of a mixed cyanobacterial culture enhancement in a photo-sequencing batch reactor (PSBR) with other studies on polymer accumulation in batch tests

For comparison purposes, the percentages obtained in this work are here compared with batch experiments performed with cyanobacteria cultures submitted to aerobic illuminated autotrophic conditions (Table 9.5). In general, carbohydrate content obtained in the batch test of condition 1 (29%) was similar to those obtained by most of the previous studies carried out in pure cultures (De Philippis et al., 1992; Markou et al., 2013; Sassano et al., 2010). However, most of the other studies are surpassed by the values obtained in the batch tests of conditions 2 and 3. Only the study of Aikawa et al., (Aikawa et al., 2012) obtained a percentage of 47% in the first 24 h using a specific strain (*Arthrospira platensis*) that already contained 17% of carbohydrates.

Concerning the PHB content, results obtained with the wastewater-borne cyanobacteria utilized in this study, for all the conditions, achieved a higher PHB content in shorter time in comparison with pure cultures and even with the same type of culture (Arias et al., 2018; Chapter 8). Generally, most of the studies showed a very low process of PHB accumulation, reaching percentages lower than 1% in the first 24h. In the particular case of Monshupanee et al., (Monshupanee and Incharoensakdi, 2014), PHB accumulation started after 120h of incubation achieving a maximum of 13% after 288h. The results of this study are only similar to those of Nishioka et al., (Nishioka et al., 2001), whose specific strain (*Synechocystis sp. MA19*) was able to start PHB accumulation after 10 h and reached 2% in 24 h. Remarkably, these authors reached 55% PHB content after an incubation of 120 h.

Table 9.5 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]	-	(Aikawa et al., 2012)
Cyanobacteria dominated mixed culture	16-22	0.7-0.9	(Arias et al., 2018; Chapter 8)
Spirulina maxima	34[e]-35[f]	0.4[f]-0.9[f]	(De Philippis et al., 1992)
Anabaena cylindrica	-	0.1[d]	(Lama et al., 1996)
Spitulina platensis	30 [f]	-	(Markou et al., 2013)
Synechocystis sp. PCC 6803	0.5[d]	0[d]	(Monshupanee and Incharoensakdi, 2014)
Synechocystis sp. MA19	-	2[d]	(Nishioka et al., 2001)
Arthrospira platensis	32.5[f]	-	(Sassano et al., 2010)

[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.

9.5 Conclusions

In this chapter, carbohydrate (biofuel substrates) and PHB (bioplastics) accumulation in wastewater-borne cyanobacteria was enhanced through transient carbon regimes in a photo-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.

Chapter 10

Discusion

In this chapter, the most relevant findings obtained from the experiments carried out during this thesis are gathered and discussed. The two main points discussed here concern: 1) the maintenance of cyanobacteria dominated cultures in wastewater treatment system, and 2) polyhydroxybutyrate and carbohydrate production in such cultures. The comparison of the results with previous studies is not carried out here, since it was part of the discussion of the state of the art (**Chapter 3**).

The knowledge acquired is used to propose operational criteria for a full-scale case study in order to achieve cyanobacteria dominance and PHB accumulation.

10.1 Maintenance of cyanobacteria dominated cultures in wastewater treatment systems

Cyanobacteria cultivation in a variable media, as is the case of wastewater, implies the competition with other microorganisms, especially with green microalgae (*Chlorophyta*). One of the main challenges in this thesis was to find key factors that favour the selection and dominance of cyanobacteria species. It should be taken into account that the term "selection" is referred to the transformation of the culture into a culture "mostly" dominated by cyanobacteria species, not a pure cyanobacteria culture. The most important factors concerned nutritional and operational conditions.

10.1.1 Nutritional conditions

Nitrogen and phosphorus ratios (N:P) and their absolute concentration in the culture were two key factors in the competition of cyanobacteria over other microorganisms. Nutritional patterns observed in **Chapters 4, 5, 6 and 9**, suggest that cyanobacteria abundance and dominance was mainly controlled by those factors. Indeed, cyanobacteria species of cf. *Aphanocapsa* sp., cf. *Chroococcus sp.* and cf. *Pseudanabaena* sp. (Fig. 10.1) dominated over green algae when the culture showed a very low P concentration (lower than 1 mg, in the range from 0.20 to 0.90 mg P L^{-1}), and N:P ratios of 32-46, in semi-continuous and sequencing batch operation. In addition, the best nutrients loading to achieve cyanobacteria dominance were TN <11.72 mg N L d^{-1}, TP < 2.06 mg P L d^{-1}. While the best load was TOC < 53 mg C L d^{-1}. These conditions were evidenced when cyanobacteria was cultivated in wastewater (**Chapter 4 and 6**), as well as in synthetic growth medium (**Chapter 9**).

One nutritional factor preventing cyanobacteria dominance was the inorganic carbon (IC) limitation. For instance, in the results showed in **Chapter 4,** *Chlorella* sp. maintained the same initial dominance in the culture due to IC and N limitation. In this experiment, IC was not added, and the high nitrites concentration in the culture indicated that the most of the ammonium in the influent was consumed by nitrifying bacteria. Therefore, the N and C limitation in this experiment restricted cyanobacteria or any other microorganism growth.

Another nutritional factor that prevented cyanobacteria dominance was the high nutrients and organic carbon loads. High concentrations, as the ones observed in **Chapter 6** in the PBR operated at 4 d HRT (TN 17.61 mg N L d^{-1}, TP 3.09 mg P L $d^{-1}$and TOC 80 mg C L d^{-1}) led to green algae dominance. Moreover, the increase of these values led to an increment in heterotrophic bacteria **Chapter 5**.

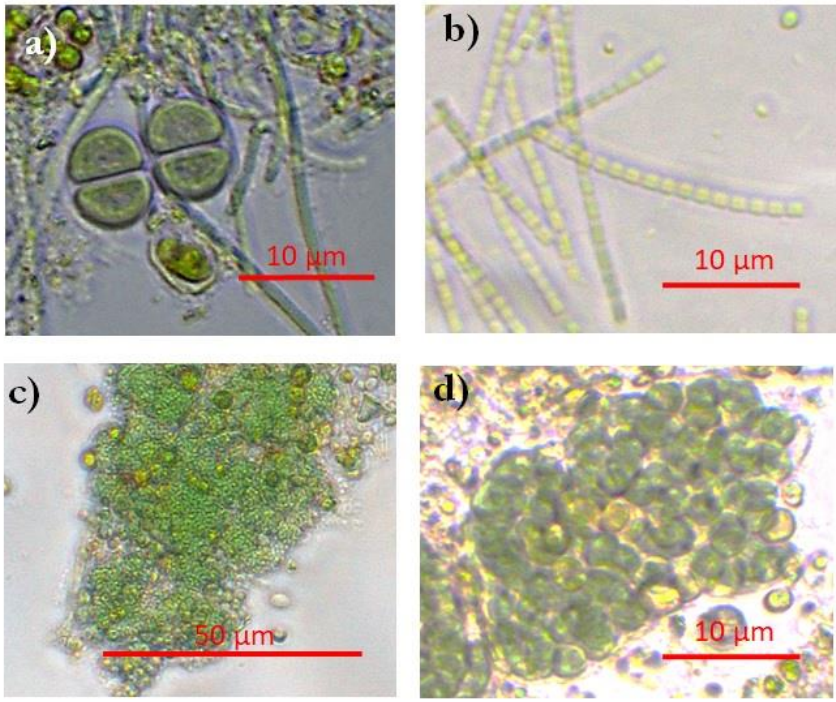


Fig. 10.1 Main cyanobacteria species observed in the systems operated in this thesis, observed in bright light microscopy at different scales. a) cf. *Chroococcus* sp., b) cf. *Pseudanabaena* sp., c) cf. *Aphanocapsa* sp., d) cf. *Chroococcidiopsis* sp..

10.1.2 Operational conditions

Together with nutrients ratio and concentrations, operational conditions (e.g. solids retention time and hydraulics regimes) also played an important role in cyanobacteria dominance.

10.1.2.1 Solids retention time (SRT)

Among the different solids retention times (SRT) tested in this thesis, 10 days was the optimal for increasing cyanobacteria dominance (Table 10.1). Although not included in the thesis, other study (Arias et al., 2018) was made following the same methodology proposed in **Chapter 4**, but using a hydraulic retention time (HRT) of 8 days. In this case, in spite of similar nutritional conditions as in **Chapter 4**, cyanobacteria were not dominating the culture. In addition, **Chapter 5**, working at SRT of 5 days, showed a complete absence of cyanobacteria.

10.1.2.2 Hydraulic regimes

Hydraulic regime played a crucial role in the selection of cyanobacteria. In this thesis, two hydraulic regimes were used and compared, semi-continuous operation and sequencing batch.

The semi-continuous operation was suitable to select cyanobacteria under the nutritional conditions previously mentioned (detailed in **Chapter 4**). This type of operation resulted beneficial when cyanobacteria were competing with green algae species as *Chlorella* sp. and *Stigeocloneum* sp.. On the contrary, it was inefficient when competing with low P tolerant species such as *Scenedesmus* sp. Therefore, sequencing batch operation was tested as strategy to face this issue (**Chapter 5 and 6**). In general, several benefits were found with this type of operation. One of the most important was the possibility of controlling nutritional conditions through changing HRT without altering the SRT. Moreover, the uncoupled HRT/SRT operation allows to continuously select microorganisms able to form flocs faster (e.g. cyanobacteria), while unsettling microorganisms are removed. In the case of the population of *Scenedesmus* sp., that is an unsettled microorganism, it was decreased or considerably removed in all the experiments using this operational strategy. In case that other microorganisms than cyanobacteria with fast settleability (e.g. green algae *Stigeoclonium* sp. and diatoms) were present, nutritional conditions were used to enhance cyanobacteria proliferation (**Chapter 6**). In this thesis, it was observed that, under the nutritional conditions assessed, only the operation at 6 d HRT and 10 d SRT was suitable to enhance cyanobacteria dominance. On the contrary, the

decrease of HRT favoured the presence of filamentous green algae, diatoms and heterotrophic bacteria.

Table 10.1 Main dominating green algae (*Chlorella* sp., *Scenedesmus* sp.) or cyanobacteria (cf. *Pseudanabaena* sp., cf. *Aphanocapsa* sp.) in wastewater cultures under different operational and nutritional conditions applied in this thesis. Dominant specie (percentage).

	Hydraulic regime	HRT	SRT	TIN[a] (mgL-1)	IP[b] (mgL-1)	N:P ratio[c]	Reference
cf. *Pseudanabaena* sp. dominated consortium	Semi-continuous	10	10	12.88	0.9	32	Chapter 4
cf. *Aphanocapsa* sp. dominated consortium	Semi-continuous	10	10	4.12	0.2	46	Chapter 4
Chlorella sp. dominated consortium	Semi-continuous	10	10	32	1.4	51	Chapter 4
Scenedesmus sp. dominated consortium	Semi-continuous	10	10	21.47	1.37	24	Chapter 5
Scenedesmus sp. dominated consortium	Semi-continuous	10	10	1.24	0.38	7	Chapter 6
Green algae/bacteria dominated consortium	Sequencing batch	2	10	22.12	1.13	43	Chapter 5
Green algae/bacteria dominated consortium	Sequencing batch	2	5	21.57	2.9	16	Chapter 5
cf. *Aphanocapsa* sp. dominated consortium	Sequencing batch	6	10	13.82	0.89	34	Chapter 6
Scenedesmus sp. dominated consortium	Sequencing batch	4	10	6.79	0.96	16	Chapter 6

[a] Total inorganic nitrogen
[b] Total inorganic phosphorus
[c] Ratio considering inorganic species given in molar basis.

10.2 **Polyhydroxybutyrate and carbohydrates production in mixed wastewater-borne cyanobacterial cultures.**

As occurring in pure cultures producing polymers, wastewater-borne cyanobacteria cultures also need optimized conditions to accumulate PHB and carbohydrates. In this thesis, three different approaches for polymers accumulation were tested: 1) N and P limitation during permanent availability of carbon, 2) different photoperiods and 3) feast and famine of inorganic carbon. The main accumulation achieved are summarized in Table 10.2.

10.2.1 Nutrients deficiency

The results obtained in **Chapters 7, 8 and 9** revealed the capacity of PHB and carbohydrates accumulation of wastewater-borne cyanobacteria. The maximum PHB accumulation was observed in complete absence of nitrogen. Otherwise, carbohydrates showed different behaviours. In **Chapter 7 and 8**, the best accumulation was performed during N deficiency when filamentous cf. *Pseudoanabaena* sp., cf. *Rivularia* sp. and cf. *Chroococcidiopsis* sp. were dominating, while in **Chapter 9**, the best performances were achieved in absence of P, when cf. *Chroococcus* sp. were dominant in the culture. Such differences can be attributed to the different species dominating the culture.

Table 10.2 Summary of the maximum percentages of PHBs and carbohydrates obtained in mixed wastewater-borne cyanobacterial cultures performed in batch studies of this thesis.

Cyanobacteria dominating specie	Maximum PHB content (% dcw)	Days of incubation (d)	Maximum Carbohydrates content (% dcw)	Days of incubation (d)	Optimization process	Reference
Chroococcidiopsis sp.	5.4	9	62.7	8	N deficiency + 24 h photoperiod	Chapter 8
Aphanocapsa sp.	5.7	8	46.1	12	P deficiency + 24 h photoperiod	Chapter 8
Chroococcidiopsis sp.	6.5	12	74.8	12	N deficiency + alternated 12h light-dark photoperiod	Chapter 8
Aphanocapsa sp.	5.6	15	35.9	12	P deficiency + alternated 12h light-dark	Chapter 8
Not clear dominance between cyanobacteria (*Chroococcus* sp., *Pseudanabaena* sp. *Aphanocapsa* sp.) and green algae (*Chlorella* sp. *Ulothrix* sp.)	3.9	2	29	1	24 h photoperiod + Previous cultivation with feast and famine process	Chapter 9
Chroococcus sp., *Pseudanabaena* sp. *Aphanocapsa* sp.)	3.5	2	53	1.2	24 h photoperiod + Previous cultivation feast and famine process and P deficiency	Chapter 9
Chroococcus sp.,	3.8	1	43	1	24 h photoperiod + Previous cultivation with P and N deficiency	Chapter 9

10.2.2 Feast and famine strategy

Table 10.3 highlights that all the conditions tested in **Chapter 8** (N and P limitation during different luminic conditions, permanent light and light/dark alternation) showed the highest maximum rates of carbon uptake (-qS). However, this high uptake of carbon did not lead to higher rates or yields of PHB or carbohydrates. This implies that the uptake of carbon was mainly used for biomass growth instead of being used for polymers accumulation, and this inefficient use of carbon led to prolonged periods (9-12 days) for polymers accumulation. In **Chapter 9**, feast and famine was proposed as a strategy for the optimization of carbon uptake for polymers accumulation. This strategy, until now only performed in mixed bacterial cultures, has been one of the most promising strategies applied in this thesis to select specific accumulating microorganisms and to improve PHB and carbohydrate production. This process consisted on a transitory carbon supply during cultivation, in which the biomass is subjected to a period of carbon availability and a subsequent absence of carbon. Then, subsequent accumulation tests would show a fast and efficient carbon transformation to polymers.

As it can be observed in Table 10.3, the carbon uptake (–qS) of the conditions assessed in **Chapter 9** were considerably lower than the ones observed in **Chapter 8**. While the maximum rates of PHB (qPHB) and carbohydrates (qcarbs) were higher when submitted to feast and famine operation (**Chapter 9**). This means that this strategy indeed increased the velocity of carbon uptake and carbon was used more efficiently for polymers accumulation. This efficient uptake of carbon led to similar accumulation percentages than the maximum accumulations observed in **Chapter 8** but in shorter periods of time (24h) (Table 10.2).

It should be noticed that in **Chapter 8** that the alternation of light/dark periods during 2 weeks improved the capacity of PHB accumulation (Table 10.2). On the other hand, the accumulation contents obtained in the batch tests in **Chapter 9** were limited to only 24h of incubation in aerobic light phase and subsequent 24h of accumulation in dark anaerobic phase. Then, it is possible that PHB and carbohydrates accumulation in the batch tests of **Chapter 9** would increase if the dark phase was followed by another period of light aerobic phase.

This assumption is also encouraged by the capacity of cyanobacteria to accumulated PHB under both light and dark conditions (**Chapter 9**). PHB accumulated during the aerobic light phase were conditioned to the

availability of inorganic carbon and the depletion of nitrogen. While its accumulation during the dark phase was performed by means of carbohydrates conversion.

In the case of carbohydrate content, the accumulation process was stopped during the dark period because carbohydrate accumulation depended on the CO_2 uptake, which in turn depends on the light availability. However, this compound showed a fast accumulation once light phase and carbon was available.

Having said that, values here obtained suggest that PHB and carbohydrates can be produced efficiently in the light phase, but PHB accumulation can continue by means of carbohydrate conversion during the dark phase. Moreover, the possibility of employing a light/dark process implies a reduction of the cost related to artificial illumination in a full scale process.

Considering that the main goal of this study was to produce both polymers; this research is contributing to improve the limited knowledge of feast and famine application to produce PHB and carbohydrates from cyanobacteria. Among the possible applications of those polymers, carbohydrates can be used as substrate to obtain biodiesel, bioethanol and biomethane, while PHB can be directed for bioplastics generation. The application of either of the two polymers will depend on the economic feasibility of each process and the commercial interest of the final product.

Table 10.3 Stoichiometric and kinetic parameters of mixed wastewater-borne cyanobacterial cultures performed in batch studies of this thesis.

Optimization process	-qS [Cmol IC·Cmol X^{-1} d^{-1}]	qPHB[Cmol PHB·Cmol X^{-1} d^{-1}]	qCarbs [Cmol carbs·Cmol X^{-1} d^{-1}]	Y_{PHB}/S[Cmol PHB·Cmol IC^{-1} d^{-1}]	Ycarbs/S[Cmol carbs·Cmol IC^{-1} d^{-1}]	Reference
N deficiency + 24 h photoperiod	0.351	0.0005	0.0044	0.0014	0.0096	Chapter 8
P deficiency + 24 h photoperiod	0.3531	0.0007	0.002	0.0017	0.0051	Chapter 8
N deficiency + alternated 12h light-dark photoperiod	0.2391	0.0008	0.0043	0.0035	0.0143	Chapter 8
P deficiency + alternated 12h light-dark	0.1547	0.0005	0.0013	0.0028	0.0053	Chapter 8
24 h photoperiod + Previous cultivation with feast and famine process	0.023a	0.0019	0.0079	0.062	0.34	Chapter 9
24 h photoperiod + Previous cultivation feast and famine process and P deficiency	0.034a	0.0016	0.023	0.043	0.64	Chapter 9
24 h photoperiod + Previous cultivation with P and N deficiency	0.0054a	0.0015	0.012	0.071	0.87	Chapter 9

-qS Maximum specific substrate uptake rate; qPHB Maximum specific polyhydroxybutyrate production rate; qcarbs Maximum specific carbohydrate production rate, Y_{PHB}/S Yield of polyhydroxybutyrate; Ycarbs/S Yield of carbohydrate.

10.3 **Operational criteria for PHB production from wastewater-borne cyanobacteria in a full scale hybrid photobioreactors placed in Barcelona**

Based on the results obtained in this thesis, some strategies and recommendations concerning operational designs are provided for the study-case of full scale PBRs located at the Agròpolis campus, Barcelona. These PBRs were built as part of project INCOVER: "Innovative Eco-technologies for Resource Recovery from Wastewater", in which one of the goals is the use of wastewater for PHAs production trough cyanobacteria selection and accumulation.

The plant consists in three horizontal hybrid (semi closed) tubular photobioreactors PBRs. Each reactor is growing a mixed culture containing mostly green algae and cyanobacteria, using a combination of agricultural runoff and domestic wastewater as carbon and nutrients source (Fig. 10.2). A detailed description of the plant is provided in Uggetti et al. (2018). Briefly, each PBR consists of two open tanks made of polypropylene (5 m long x 1 m width x 0.6 m height) connected between them through sixteen low density polyethylene tubes (125 mm diameter and 47 m length). PBR operate with a useful volume of approximately 11.7 m^3. Both open tanks ensure and favour the homogenous distribution and mixing of the liquor and also the release of the exceeding dissolved oxygen accumulated along the closed tubes. In each open tank, a paddle-wheel with six blades (1 m width x 0.35 m long) is installed 1.8 m away from the external edge and at 3 cm height from the bottom. An engine (0.35 kW) connected to each paddle wheel provides a turning speed which can be changed from 0 to 12 rpm.

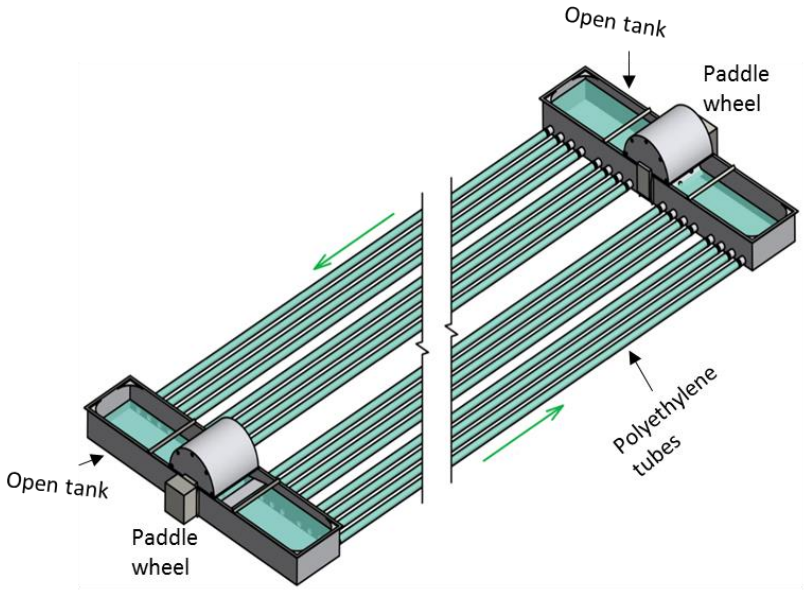


Fig. 10.2 Scheme of one hybrid horizontal tubular photobioreactor located in the INCOVER plant located in Barcelona, Spain.

10.3.1 Operation until May 2018

These photobioreactors were firstly inoculated with 10 L (each PBR) of a mixed culture of green algae and bacteria grown in experimental high rate algal ponds treating domestic wastewater. From that moment on, PBRs were operating in continuous strategy, fed with 2.3 m^3 d^{-1} each (6.9 m^3 d^{-1} in total) of a mixture of agricultural and domestic wastewater at a ratio of approximately 10:1 (Fig. 10.3). This mixture had a nutrient concentration of TIN 13.1±6.9 mg L^{-1} and IP 0.8±1.1 mg L^{-1}. Every day, 6.4 m^3 of agricultural wastewater and 0.5 m^3 of domestic wastewater (the latter previously treated in an aerated septic tank) were pumped into a homogenization tank, where they were mixed. Then, 2.3 m^3 d^{-1} of mixed liquor were pumped out from each PBR to one storage tank. Subsequently, the same volume of influent from the homogenization tank was pumped into each PBR. This operation took place in the early mornings in order to have nutrients available for biomass growth during the day. No CO_2 was added in the system.

Separation of the biomass and treated water (effluent) was conducted in a lamella settler installed after the 3 PBRs. The water stored in the 3 storage tanks was then pumped to a lamella settler were coagulation and flocculation was performed using (polyaluminum chloride liquid, with a 9% of aluminum (PAX-18) (provided by Kemira Water Solutions, Spain).

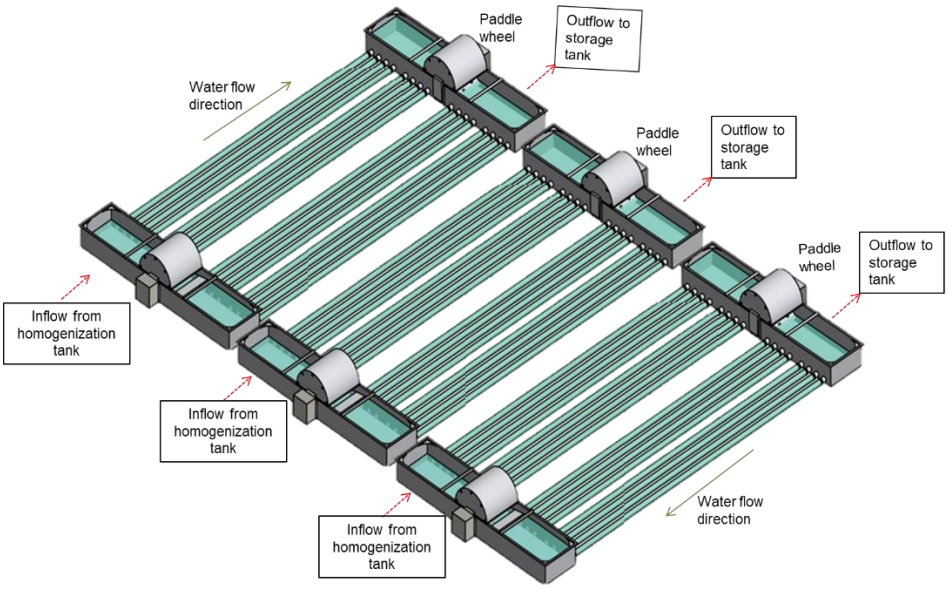


Fig. 10.3 Scheme of the operation until May, 2018 of the three full scale photobioreactors operated in continuous feeding, located in the INCOVER plant in Barcelona, Spain.

Under this operation, a complete depletion of nutrients was achieved, while up to 0.8 g VSS L^{-1} of biomass concentration were achieved. In addition, this operation allowed the presence of diatom cf. *Cyclotella* sp., the green algae cf. *Oocystis* sp. and the cyanobacteria cf. *Synechocystis* sp. but without a clear dominance of any of them. While, there were small amounts of other photosynthetic microorganisms. Based on the results of the INCOVER plant it can be deduced that they presented nutrients limitation. This same microbial dynamic is observed in the semi-continuous reactor tested in this thesis in **Chapter 5**, the same biomass composition was maintained thanks to the low concentration of nutrients in the influent, causing N and P limitation.

10.3.2 Recommendations for optimizing polymers production from wastewater-borne cyanobacteria

10.3.2.1 Reactors configuration

In order to select and cultivate cyanobacteria and to accumulate PHB, it is recommended to use one of the reactors for the cultivation and selection phase, while the second and the third reactor could be configured to enhance PHB accumulation.

A change in the current operation is recommended, from parallel feeding to in serial feeding. This means that the first reactor (PBR 1) would be fed directly with 2.3 m^3 of mixed agricultural wastewater and domestic wastewater previously aerated as explained in section 10.3.1., while the second reactor (PBR 2) would be fed with the effluent from the first reactor and finally, the third reactor (PBR 3) would receive the mixed liquor from the second reactor (Fig. 10.4).

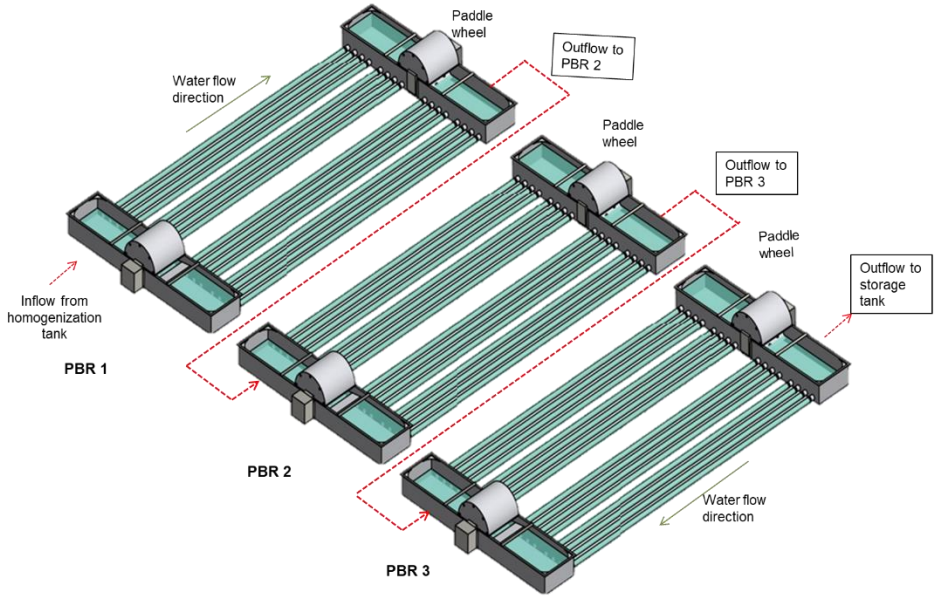


Fig. 10.4 Scheme of the recommended operation in serial feeding for maximizing PHB production from cyanobacteria in the INCOVER plant located in Barcelona, Spain.

10.3.2.2 Selection of cyanobacteria

In order to achieve the dominance of cyanobacteria in this system some operational strategies are recommended for PBR 1 according to the results obtained in **Chapter 4, 5 and 6**;

- To keep the pH around 8 by means of CO_2 injection, this pH would enhance cyanobacteria production and would prevent carbon limitation.

- A SRT higher than 5 days is recommended.

- It is advised to increase the influent N and P concentration. TP should be between 1-2 mg L d^{-1}. According to that concentration, TN concentration should correspond to N:P ratio of 32-42 (in molar basis).

10.3.2.3 Production of PHB

After achieving a cyanobacterial dominated culture and a steady state condition in PBR 1, mixed liquor from this reactor containing biomass and remaining nutrients should be used as feeding for PBR 2. In PBR 2, remaining nutrients would be removed, while feast and famine strategy (**Chapter 9**) can be applied. It should be noticed that the periods of light/dark suggested will correspond to a circadian cycle, with 12 h of light during the day and 12 h of dark during the night. This process can be applied as follows;

- Day (light phase)

-Period of famine (6 h): this period starts at the first hour of sunrise, with the feeding of mixed liquor from PBR 1. It would be characterised by the continuous CO_2 injection. During this period, the remaining nutrients in the culture would be consumed and a complete depletion of N would cause a fast PHB and glycogen accumulation.

-Period of feast (6 h): this period would take place during the following 6 h. during this period the culture would be maintained without CO_2 injection or any additional carbon source. In these conditions, the culture would be maintained in carbon limitation, thus, the remaining nutrients would be uptake using the intracellular carbon accumulated.

- Night (dark phase)

-Dark phase (12h): in this period, the culture should be maintained without CO_2 injection. In this period, the PHB accumulated will be partially consumed as energy source during the dark. As observed in **Chapter 9**, PHB accumulation by means of glycogen conversion occurred during the dark phase with availability of inorganic carbon. Therefore, it is expected that PHB and glycogen will be partially consumed due to the lack of CO_2.

Subsequently, mixed liquor with selected biomass from PBR 2 would be transferred to PBR 3. In this latter PBR, the culture would be submitted to a continuous CO_2 injection during all the day and ensure that the culture present availability of IC during the night. Due to the expected lack of N, PHB would be accumulated during the day along with glycogen, and then, during the night, PHB accumulation would continue by means of glycogen conversion. As discussed in **Chapter 9**, IC availability during the dark phase would be determinant in terms of glycogen conversion. The biomass obtained in PBR 3 would be then transferred to the storage tank and finally harvested in the lamellar settler.

10.3.2.4 First results of the application of recommended configuration to the full scale hybrid photobioreactors

From June 2018, part of the methodology proposed in this thesis was applied in the INCOVER plant. The feeding was changed from continuous to serial feeding. PBR 1 influent was modified by the addition of potassium nitrate for having approximated concentrations of TN 10 mg L^{-1} and TP 1 mg L^{-1}. This configuration led to a culture dominated by cyanobacteria belonging to cf. cf. *Synechococcus* sp. and cf. *Pseudanabaena* sp. with some cf. *Plectonema* sp. and cf. *Boryanum* sp. (Fig. 10.5). Future directions would be to configure PBR 2 and PBR 3 according to the recommended in section 10.3.2.3.

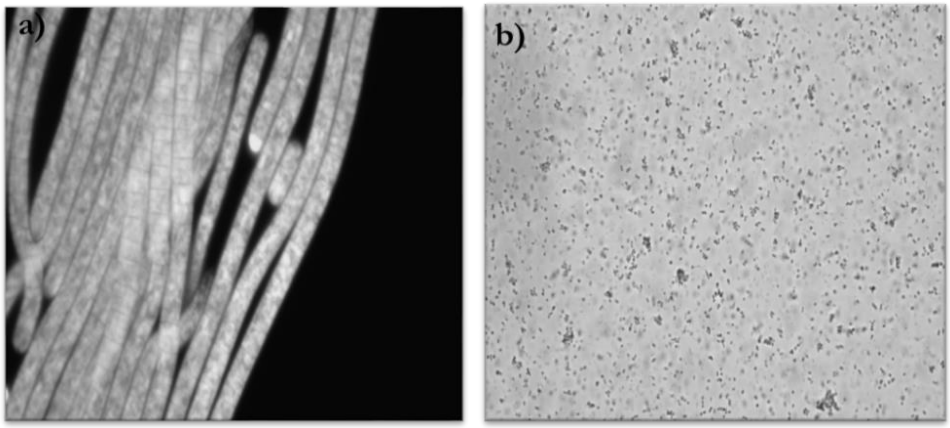


Fig. 10.5 Microscopic images of the main species of cyanobacteria dominating in photobioreactor 1 (PBR 1) of the INCOVER plant, b) cf. *Phormidium* sp. observed in fluorescence microscopy (1000x), and c) cf. *Synechococcus* sp. observed in bright light microscopy (1000x).

Chapter 11

Conclusions

This thesis addresses important aspects to be considered for the production of carbohydrates and polyhydroxybutyrate (PHB) from cyanobacteria cultivated in wastewater. Initially, the effect of nutritional (e.g. concentrations, ratios and loads) and operational (e.g. hydraulic regimes, solids retention time (SRT), hydraulic retention time (HRT)) conditions on the selection and growth of wastewater-borne cyanobacteria was assessed.

Secondly, carbohydrates and PHB accumulation in wastewater-borne cultures dominated by cyanobacteria was assessed. Experiments were performed firstly by testing the effect of nutrients limitation and photoperiods. Then, by evaluating the effect of the carbon feast and famine strategy and the effect of nutrients ratios and loads on the production of carbohydrates and PHB.

In this chapter, the overall conclusions of the thesis are grouped in two main parts. Finally, perspectives of future works are presented.

11.1 Selection and growth of cyanobacteria in a wastewater treatment system

Results from a first experiment evidenced that culture composition and biomass concentration strictly depend on nutrient variations in the influent (composed of digestate diluted with secondary wastewater effluent) and nutrients ratios. A positive response in wastewater-borne cyanobacteria dominance was observed when the culture had N:P ratios between 16:1-49:1 (molar basis), low inorganic phosphorus loads (~0.25 mg L d^{-1}) and carbon availability. Under these conditions, species *Aphanocapsa* sp., *Chrooccocus* sp. and *Pseudanabaena* sp. dominated over green algae *Chlorella* sp. and *Stigeoclonium* sp.. Additionally, an average biomass production of 0.08 g $L^{-1}d^{-1}$ was achieved. With these first results, this work demonstrated the possibility of selecting cyanobacteria from mixed microalgae consortium.

However, similar to cyanobacteria, other green algae such as *Scenedesmus* sp. (widely reported in wastewater treatment systems) have a high tolerance to low phosphorus content and high N:P ratios. Further experiments tested cyanobacteria cultivation in photo-sequencing batch reactors. When operated at a hydraulic retention (HRT) of 6 days and solids retention time (SRT) of 10 days, the system was able to remove unsettled green algae *Scenedesmus* sp., while improving cyanobacteria concentration from an initial 2% until 70% of the total population. Conversely, the reduction of HRT and SRT negatively affected cyanobacteria dominance, favoring green algae competition.

Nutrient's volumetric loads applied during the sequencing batch operation also played an important role in the dominance of cyanobacteria. Results from last experiments confirmed that volumetric loads around 11.72 mg TN L^{-1} d^{-1}, 2.04 mg TP $L^{-1}d^{-1}$ and 71.81 mg TC L^{-1} d^{-1} led to a limiting N:P ratio in the culture (34:1). Such conditions improved the dominance of *Aphanocapsa* sp. while increasing the biomass production to an average of 0.12 g L^{-1} d^{-1}. On the contrary, higher volumetric loading, specially the introduction of high loads of TOC (>96.15 mg L^{-1} d^{-1}), increased the presence of heterotrophic bacteria.

Although the cultivation and selection of cyanobacteria was the main goal, all the cyanobacteria dominant cultures demonstrated promising removal efficiencies in a wastewater treatment context. Indeed, removal efficiencies >95% in terms of TAN and IP, >60% of TN and >80% of TP were achieved in the treatment of digestate and secondary wastewater effluent. These results reveal that the recycling of nutrients from secondary effluents and digestate can be positively used to grow valuable biomass, obtaining at the same time a further treatment of the effluents used.

11.2 **Carbohydrates and polyhydroxybutyrate accumulation in wastewater-borne cultures dominated by cyanobacteria**

The culture obtained in previous experiments was then submitted to different conditions in order to maximize polymers' accumulation. Initially, the effect of nutrients limitation (N and P limitation) and photoperiods (permanent light and light/dark alternation) with excess of carbon was tested. During the subjection of the culture to different conditions, two patterns in microbial composition were observed. Firstly, the exposition to N limitation in both permanent light and light/dark alternation improved *Chroococcidiopsis* sp. competition over *Aphanocapsa* sp. and beaching of the cells, while P limitation led to an inversed pattern favouring the dominance of *Aphanocapsa* sp.. In spite of the different species dominance, intracellular polymers accumulation of PHB showed values up 5% in all the conditions, with a maximum of 6.5% under P limitation and constant illumination. Otherwise, carbohydrates intracellular content variated depending on the nutrient limitation, lower values (36-46%) were achieved during P limitation, while the highest contents (63-75%) were reached under N limitation. Remarkably, the maximum polymers contents were achieved after an incubation of 9-12 days.

Although these results represent encouraging values in comparison with polymers obtained in pure cultures, the introduction of feast and famine emerged as an attractive approach to considerably reduce those prolonged accumulation

periods. Unlike the previous batch test, where the culture was submitted to N or P limitation with permanent availability of carbon, feast and famine strategy consisted in a cultivation phase characterised by the alternation of carbon availability and carbon limitation. With this strategy, along with nutrients limitation, the culture was forced to increase the velocity of carbon consumption. Therefore, batch accumulation tests performed after submitting the culture to feast and famine, result in PHB concentration of 4% after a complete depletion of nitrogen, while carbohydrates reached the highest content (43%-48%) under phosphorus limitation. Such contents were obtained in only 24h of incubation under aerobic illuminated conditions.

11.3 **Perspectives of future works**

Results obtained in this PhD thesis suggest that the production of polymers from cyanobacteria cultivated in wastewater can have a promising and attractive application. However, some challenges still need to be overcome prior to the scale-up of the technology.

In terms of selection and cultivation of cyanobacteria, the main parameters to optimize are related to operational conditions. For instance, all the experiments were performed indoors under controlled light, temperature and pH conditions. Further research could be addressed in order to convert this technology to outdoors conditions. Therefore, future cyanobacteria cultivation systems should have to be tested outdoors, under less controlled conditions. Indeed, the effect of outdoor conditions (e.g. seasonality, direct sunlight and temperature variations) should be carefully assessed.

With regards to perspectives on polymers accumulation from wastewater-borne cyanobacterial cultures, certain operational conditions also need to be optimized. Among these, temperatures and pH during the accumulation tests were always controlled at 27-30 ºC and pH ~8. Such parameters not only influence cyanobacteria competition over other microorganisms, but also influence metabolic paths of PHB and carbohydrates accumulation. Therefore, it would be important test other range of values in order to evaluate its effects in the polymers accumulation capacity and production.

Due to the encourage polymers accumulation obtained under feast and famine regimes, future works should be addressed to integrate this strategy into the cultivation phase in a full-scale system. The application of this strategy to the cultivation process would integrate both steps, the selection of cyanobacteria and

the conditioning of the culture to an efficient use of carbon able to increase polymers accumulation.

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Supplementary material

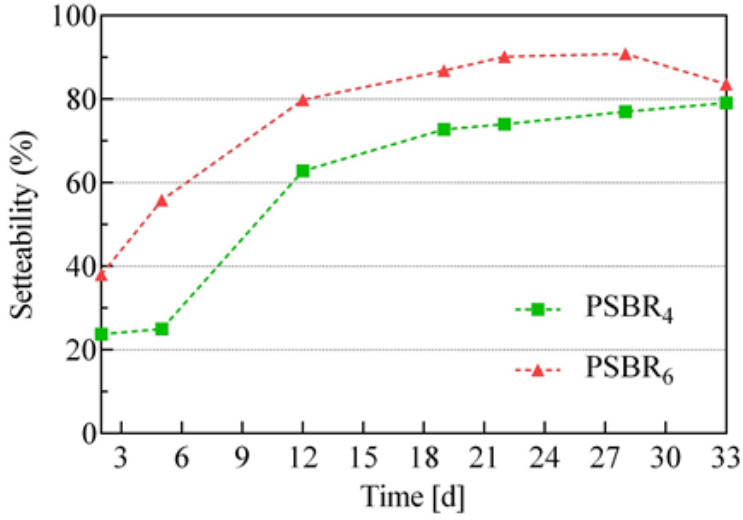

Fig. 6-S1. Settleability along the experimental time obtained in $PSBR_6$ and $PSBR_4$ after 30 minutes of settling.

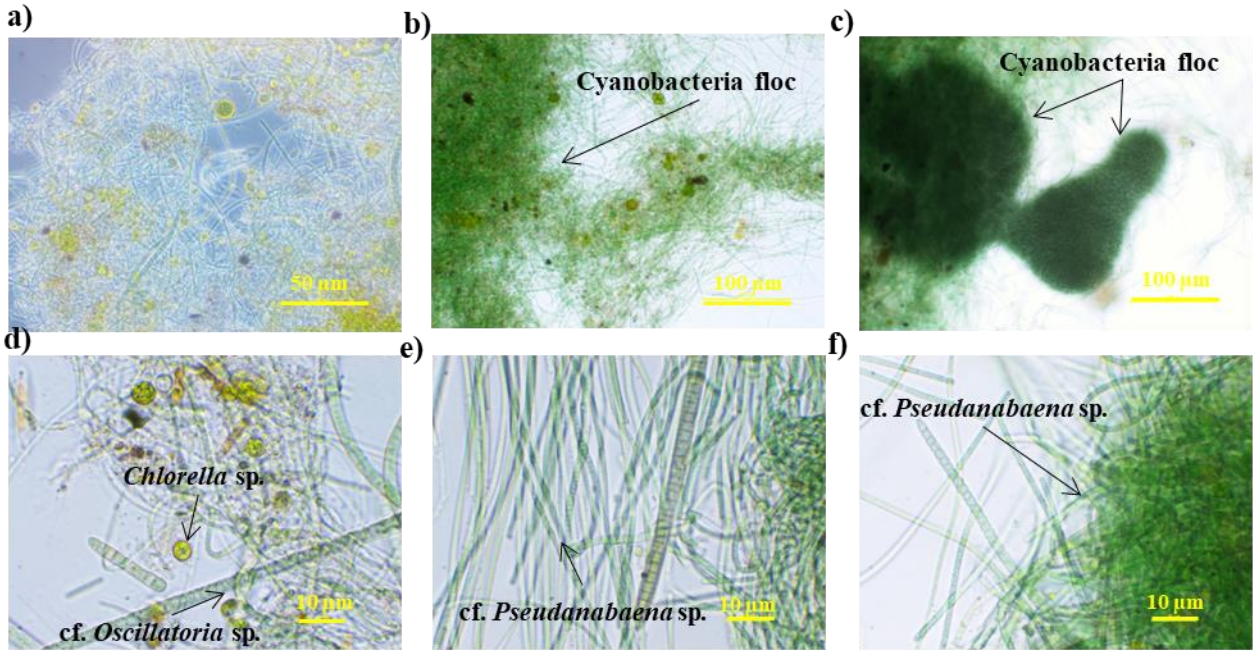

Fig. 7-S1 Microscopic images illustrating the microbial diversity in A10full; a), b) c) general view of the culture showing a high dominance of filamentous cyanobacteria with some unicellular green algae, observed in a) contrast microscopy (400X), b) and c) bright light microscopy (200X). d) detail of a lateral side of a floc composed by cf. Oscillatoria sp., and green algae chlorella sp. and e) and f) detail of a lateral side of a floc composed by filamentous cf. pseudanabaena sp., observed in bright field microscopy (1000×).

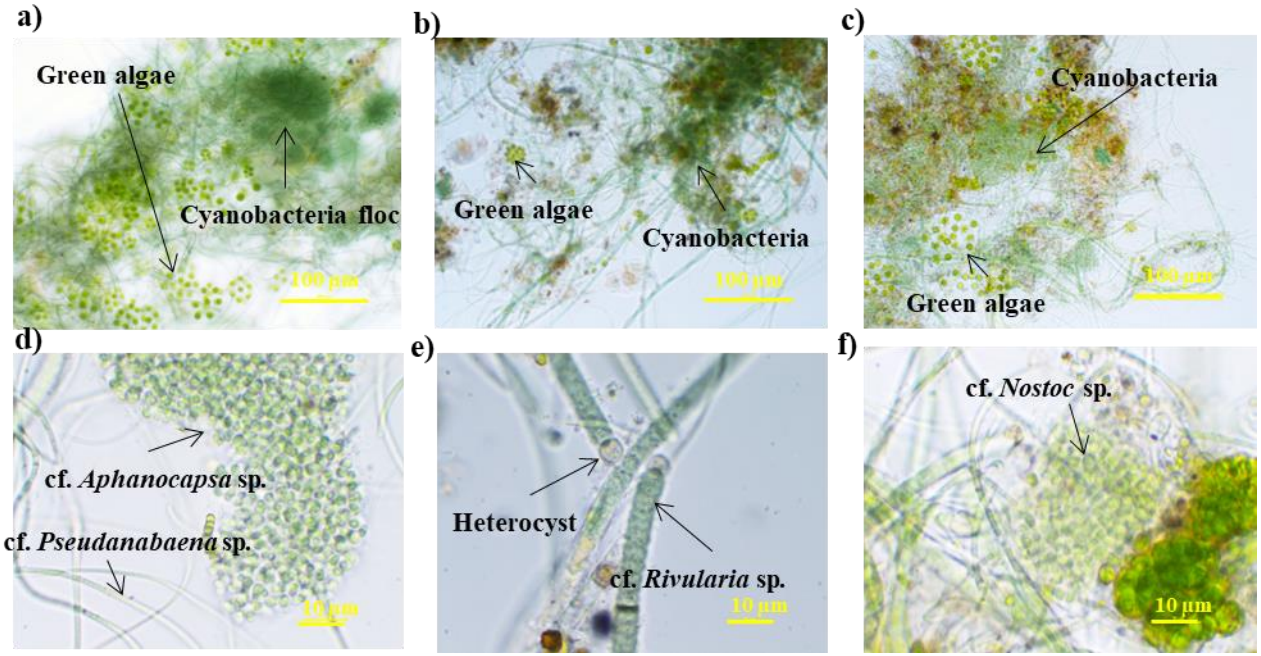

Fig. 7-S2 Microscopic images illustrating the microbial diversity during in A10diluted; a), b) c) general view of the culture showing floc composed by filamentous and unicellular cyanobacteria and colonies of green algae cf. Westella sp. observed in bright light microscopy (200X). d) detail of colonies of cf. Aphanocapsa sp., with filaments of Pseudanabaena sp.; e) detail N-fixing cf. Rivularia sp., f) detail of grouped colonies of cf. Nostoc sp. rounded by filamentous cyanobacteria and green algae observed in bright field microscopy (1000×).

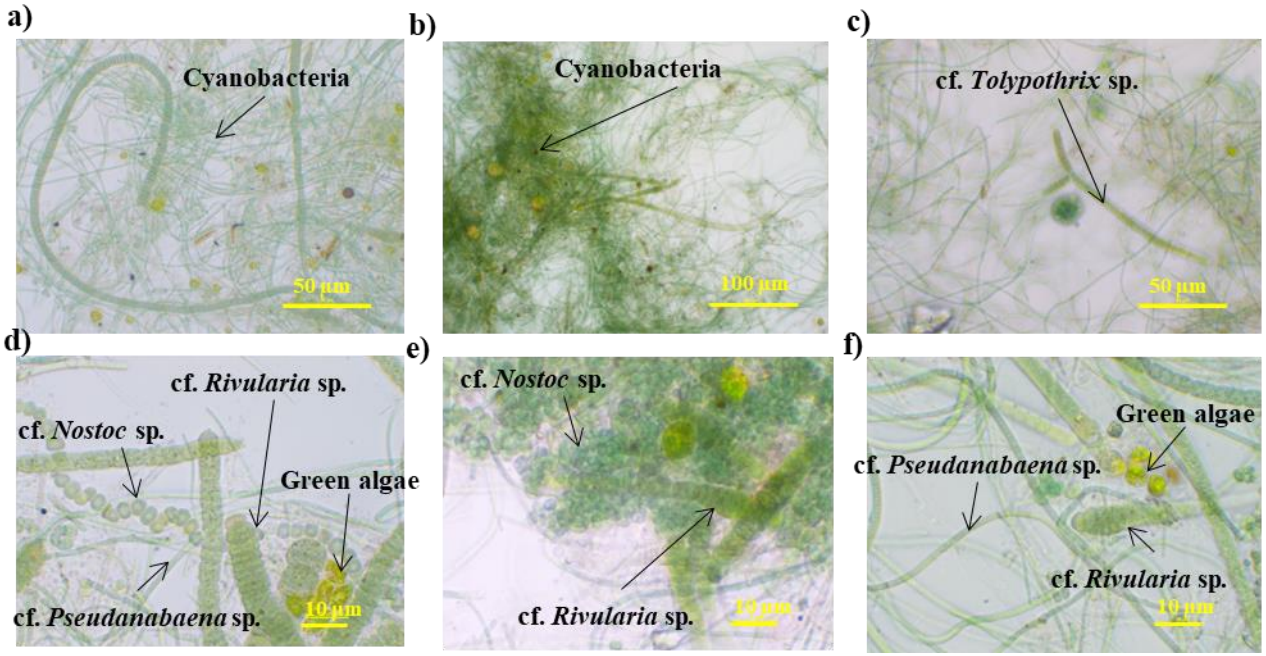

Fig. 7-S3 Microscopic images illustrating the microbial diversity in A_{8full}; a), b) c) general view of the culture showing a high dominance of filamentous cyanobacteria, observed in bright light microscopy at a) and c) 400X, and b) (200X). d) detail of a lateral side of a floc composed by cf. *Rivularia* sp., cf. *Nostoc* sp. and green algae *Chlorella* sp.; e) detail of a floc composed by agglomerates of cf. *Nostoc* sp. with cf. *Rivularia* sp. f) detail of a mixed culture composed by cf. *Rivularia* sp., cf. *Pseudanabaena* sp. and cf. *Chlorella* sp. observed in bright field microscopy (1000×).

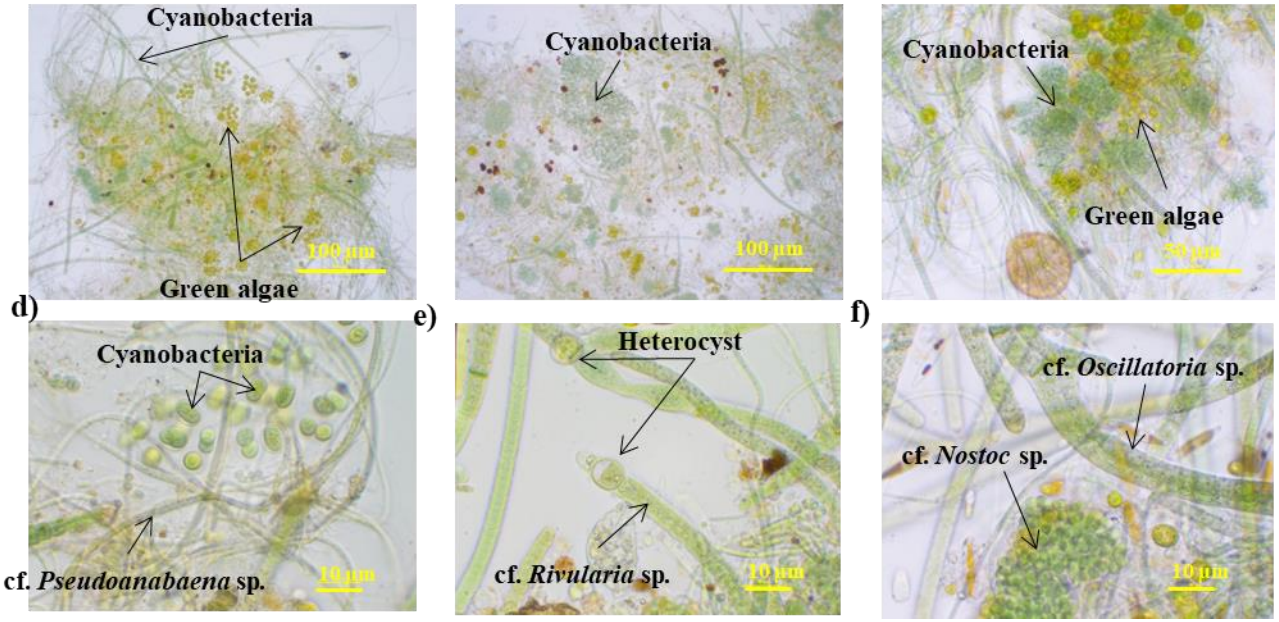

Fig. 7-S4 Microscopic images illustrating the microbial diversity in A_{8full}; a), b) c) general view of a mixed culture cyanobacteria and green algae, observed in bright light microscopy at a), b) 200X, and c) 400X). d) detail of a lateral side of a floc composed by unicellular cyanobacteria and cf. *Pseudoanabaena* sp.; e) detail of N-fixing cf. *Rivularia* sp., f) detail of agglomerate of cf. *Nostoc* sp. and cf. *Oscillatoria* sp., observed in bright field microscopy (1000×).

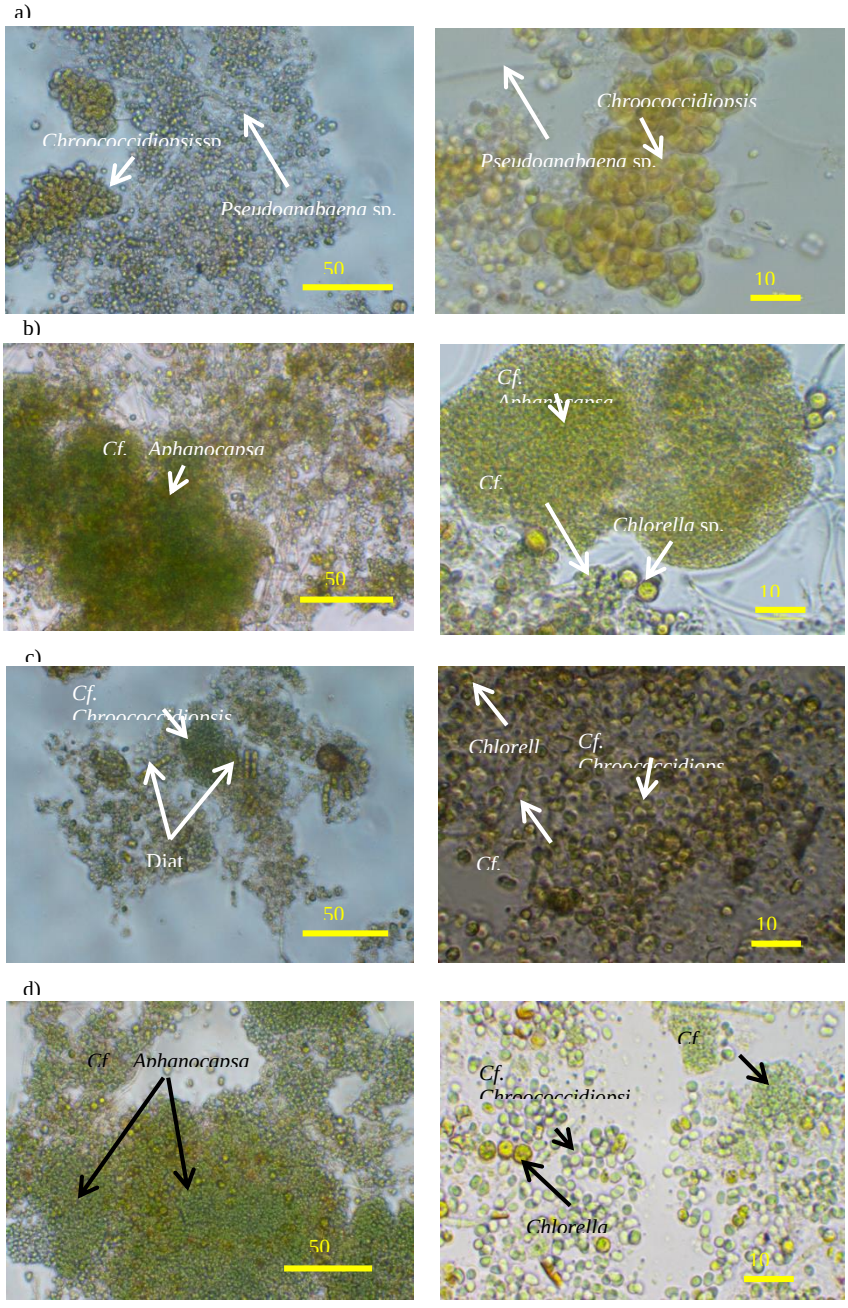

Fig. 8-S1. Microscopic images illustrating the general view (left) at 400x and detailed view (right) at 100x of the microbial composition of the culture in the end of the experimental time. a) N-limited culture under permanent illumination showing cyanobacteria floc with cf.

Chroococcidiopsis sp. and a filaments of cf. *Pseudoanabaena* sp.; b) Culture submitted to phosphorus limitation under permanent illumination 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.

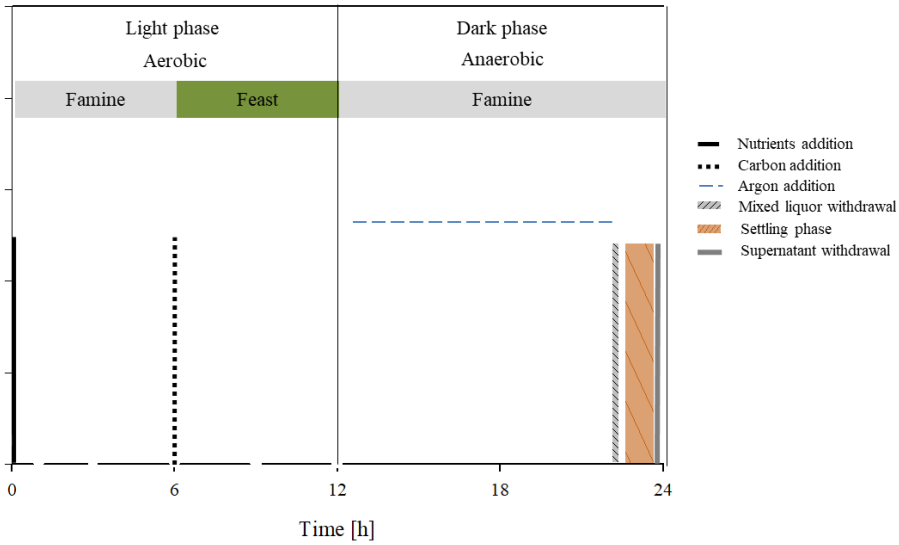

Fig. 9-S1. Scheme of the operation of the PSBR in a 24h cycle.