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Cultivation and selection of cyanobacteria in a closed photobioreactor used for secondary effluent and digestate treatment

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Abstract

The main objective of this study 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 (PO_4^{3-}), phosphorus volumetric load (L_V-P) and carbon limited/non-limited conditions leaded to different species composition, nutrients removal and biomass production in the culture. High TIN/ PO_4^{3-} concentrations in the influent (36 mg N L^{-1}/3 mg P L^{-1}), carbon limitation and an average L_V-P 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/ PO_4^{3-} 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 P-PO_4^{3-} $L^{-1}d^{-1}$. However, although cyanobacteria predominance was also favored with a L_V-P 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.

Keywords: high rate algal ponds, algae, centrate, bioproducts, bioenergy.

1. 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 polyhydroxyalkanoates (Stal, 1992).

Studies related to the production of cyanobacteria and their metabolites 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, 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). i. e. in lakes Total Inorganic Nitrogen TIN=1.167 and inorganic phosphorus concentration $P\text{-}PO_4^{3-}$=0.107 mgL^{-1} corresponds to 2.15 mgL^{-1} of biomass concentration (Beaulieu et al., 2013), while open ponds with a secondary effluent from industrial wastewater (TIN=9.31 and $P\text{-}PO_4^{3-}$=2.37) reach an average of 668 mgL^{-1} (Van Den Hende et al., 2016a). Therefore it seems reasonable that cyanobacteria selection in wastewater cultures should be conducted considering the same determining factors, especially in terms of nutrients interactionIn the field of wastewater technology, several species of cyanobacteria have been successfully cultivated at experimental scale using both primary and secondary treated

111 wastewaters (urban and industrial) as feedstock (Kamilya et al., 2006; Renuka et al., 2013; Van
112 Den Hende et al., 2016a; Vijayakumar, 2012). The use of anaerobic digestate as nutrient source
113 has also been evaluated (Markou and Georgakakis, 2011). However its use is conditioned by their
114 high ammonium (NH_4^+), organic carbon and solids content, and most the studies included a
115 dilution with tap water (Prajapati et al., 2014). Hence, the use of digestate diluted in another
116 minor nutrient source (e.g. secondary effluent) could provide enough nutrients to fullfil the
117 requeriments of cyanobacteria production and the possibility of their selective growth.

118 All in all, the objective of this study was to select and grow wastewater-borne cyanobacteria
119 from a consortium of microalgae in a closed photobioreactor (PBR) fed with a mixture of
120 secondary effluent and digestate. This work aimed to a dual benefit, considering the concomitant
121 treatment of these waste streams. The study was carried out throughout 1 year in order to
122 consider all the potential variations and variables affecting the PBR during a long term operation
123 and therefore obtaining a realistic knowledge of the system functioning.

124

125 **2. Methodology**

126 *2.1 Experimental set-up*

127 The closed PBR was located indoors and consisted in a cylindrical tube made of
128 polymethyl methacrylate (5 mm thickness) with a total volume of 35.8 L and a culture working
129 volume of 30 L (Fig. 1). It was initially filled with 20 L of tap water and 10 L of inoculum
130 obtained from the mixed liquor of an experimental high rate algal pond (HRAP) (1.54 m^2, 470 L)
131 treating primary settled urban wastewater. A detailed description of the high rate algal pond
132 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., 2016; 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. Illuminance 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 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 Figure 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 by means of CO_2 (100% v) (Carbonos 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

157 previous literature that reported a pH preference of cyanobacteria ranging from 8 to 9 (Ahn et al.,
158 2002; Reynolds, 1987; Unrein et al., 2010; Yamamoto and Nakahara, 2005). Furthermore,
159 different experiments demonstrated that when the pH reached values around 10, the green algae
160 *Scenedesmus* sp. outcompeted cyanobacteria (in preparation). Both air and CO_2 were injected in
161 the PBR similarly.

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

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

180

181 *2.2 Experimental procedure*

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

195 *2.3 Analytical methods*

196 All parameters were determined in triplicate and analyzed from the PBR influent
197 (digestate and secondary effluent) and effluent (equivalent to the mixed liquor of the culture). All
198 samples were taken and analyzed at the end of the dark phase. Analyses for orthophosphate
199 (dissolved reactive phosphorus) ($P\text{-}PO_4^{3-}$), nitrite ($N\text{-}NO_2^-$), nitrate ($N\text{-}NO_3^-$), Total Ammoniacal
200 Nitrogen (TAN)and alkalinity were performed twice a week (two different days). $P\text{-}PO_4^{3-}$, $N\text{-}NO_2^-$, $N\text{-}NO_3^-$ 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.

On the other hand, Total Kjeldahl Nitrogen (TKN) and Total Phosphorus (TP) were occasionally measured following methodologies 4500 B and 4500 P, respectively, of Standard Methods (APHA-AWWA-WPCF, 2001). Organic Nitrogen (ON) was calculated as the difference between TKN and TAN, whereas Organic Phosphorus (OP) was determined as the difference between TP and $P\text{-}PO_4^{3-}$. Due to the low levels registered for ON and OP in the influent (ranging from 2.8-5.7 mg L^{-1} and 0.9-1.2 mg L^{-1}, respectively), during the three periods they were not considered to determine N:P ratios. TIN and $P\text{-}PO_4^{3-}$ were used instead, as they represented the direct available N and P for cyanobacteria (Pick and Lean, 1987). Although it is known that certain cyanobacteria species are able to produce extracellular polyphosphatase enzymes to release dissolved phosphorus from organic phosphorus (Cottingham et al., 2015), in our case this could be a minor source for P because most of it was in the form of $P\text{-}PO_4^{3-}$.

Applied P volumetric load (L_V-P) (given in mg $P\text{-}PO_4^{3-}L^{-1}d^{-1}$) was calculated following equation 1:

$$\mathrm{Lv-P} = \frac{Q * P - \mathrm{PO_4}^{3-}}{V} \quad [1]$$

where *Q* is the flow ($L^{-1}d^{-1}$), $P\text{-}PO_4^{3-}$ is the influent concentration (mg P L^{-1}) and V (L^{-1}) is the volume of the PBR.

223 Total suspended solids (TSS) and volatile suspended solids (VSS) were measured once a
224 week in the influent following the gravimetric method 2540 C and 2540 D in Standard Methods
225 (APHA-AWWA-WPCF, 2001). Biomass concentration in the PBR culture was measured 3-5
226 times per week through turbidity. At the start of the study, a 4-points calibration curve was
227 performed in triplicate between turbidity and dry weight (0.460-1.45 g TSS L^{-1}, corresponding to
228 0.4-1.2 g VSS L^{-1}), having a correlation coefficient R^2 = 0.997. This correlation was checked
229 once a week by comparing turbidity values with TSS and VSS concentrations. Through this data,
230 biomass production in the PBR (given in g VSS $L^{-1}d^{-1}$) was estimated following equation 2:

231 $$Biomass\ production = \frac{Q*VSS}{V}$$ [2]

232 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
233 the volume of the PBR.

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

239 Culture pH and temperature were continuously measured with probes inside the PBR.
240 Both pH and temperature were measured with a pH meter with a temperature sensor (Mettler
241 Toledo, USA). Light intensity was also continuously measured with a probe attached to the PBR
242 and determined by means of a light meter (Hanna (USA)). Results of the probes were continually
243 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).

3. 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 1 for the different three periods, thus leading to different conditions in the PBR throughout the experiment (Table 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. 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. 3). This microbial community remained the same from the first days of operation till the end of the period.

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. 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. 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 $N\text{-}NO_3^-$. $P\text{-}PO_4^{3-}$ concentration in the PBR was slightly greater than 1 mg L^{-1}, and the average removal was 44% (Fig. 4b). During period 1 the highest N:P ratio in the PBR mixed liquor of the whole experiment was registered (27:1). This value was due to the high amount of TIN remaining in the culture in the form of $N\text{-}NO_3^-$. 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.

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. 2b). This biofilm

was mainly constituted by large populations of the cyanobacterium cf. *Oscillatoria* sp. (Fig. 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 $N\text{-}NO_3^-$ content (Fig. 5). This is due to the low $N\text{-}NO_3^-$ concentration registered in the effluent of the HRAP at this stage (Table 1). PBR influent and effluent $N\text{-}NO_3^-$ concentrations were very similar, indicating a lower nitrification activity in the PBR in comparison to period 1 (Fig. 5b). Regarding TAN, it was also completely removed (<96 %) (Fig. 4). $N\text{-}NO_2^-$ concentration was also very low, usually below the limit of quantification (see Table 2). $P\text{-}PO_4^{3-}$ average concentration was slightly lower than 1 mg L^{-1} and its average removal efficiency fairly similar to that of period 1 (47%) (Fig. 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.

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. 2c). The biomass production (0.039 g VSS L^{-1} d^{-1}) was also lower than that in

312 period 2. These trends were related with nutrient depletion in the culture. Indeed, this period was
313 characterized by the lowest values of TIN in the PBR mixed liquor, mostly due to low $N\text{-}NO_3^-$ in
314 the culture (see Table 2). In contrast, $N\text{-}NO_3^-$ concentration in the PBR influent was higher than
315 in period 2 (Fig. 5). Average $N\text{-}NO_3^-$ removal was 91%, while in the other periods it was
316 negligible or even negative (period 1). Similarly to the other periods, TAN was also completely
317 removed (>95 %) (Fig. 4). $P\text{-}PO_4^{3-}$ concentration had the lowest influent and effluent values of
318 the whole experiment, showing very high removal rates (>95%) (see Figure 4b). The high
319 nutrient removal in this period was related to P limitation, which led to an increase of the N
320 uptake and the subsequent lowest N:P ratio estimated during the three periods. Alkalinity content
321 was higher in both PBR influent and effluent than in the other periods (ranging from 174 and 330
322 mg $CaCO_3$ L^{-1} equivalent to 20.88 and 39.6 mg C L^{-1}).

323 In this last period, most of the algae community was dominated by the cyanobacteria
324 *Chroococcus* sp., cf. *Aphanocapsa* sp., and some filaments of cf. *Oscillatoria* sp., which formed
325 large flocs (Fig. 7).

326

327

328 **4. Discussion**

329 Biomass concentration and composition changed during the three periods according to the
330 nutrients input in the PBR influent and the corresponding N:P ratios. Table 3 summarizes influent
331 and effluent N:P ratios, the L_V-P and the main dominant algae in each period. In period 1,
332 microalgae community was dominated by *Chlorella* sp. with a relatively low abundance of
333 cyanobacteria, considering the high influent concentrations of TIN, TAN and $P\text{-}PO_4^{3-}$ (see Table

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 1 and 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 $P\text{-}PO_4^{3-}$ concentrations were slightly lower (see Tables 1 and 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 3). These conditions shifted the algae community favoring cyanobacteria, being cf. *Oscillatoria* 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 $P\text{-}PO_4^{3-}$ 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.

356 *Oscillatoria* and *Chroococcus* were almost the only microalgae in the culture. However, biomass
357 concentration and production were lower than in the previous period due to P limitation.

358 All in all, the results obtained indicate that cyanobacteria can be selected from mixed algae
359 consortia grown in completely stirred PBRs fed with treated wastewater and digestate and under
360 pH control (lack of C limitation), when the L_V-P is approximately 0.23 mg $P\text{-}PO_4^{3-}$ $L^{-1}d^{-1}$ (the
361 average corresponding to period 2). Specifically in a PBR with 30 L and HRT=10 days, this
362 would be equivalent to a $P\text{-}PO_4^{3-}$ influent concentration of approximately 2 mg L^{-1}. This P load
363 together with a N concentration which gives a 10:1 ratio, leads to an increase in the biomass
364 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
365 these conditions, the remaining N:P ratio in the culture would be similar or lower with decreasing
366 influent loads, as it can be seen in period 3 when compared to the two other periods. However,
367 when the L_V-P decreased to values under 0.16 mg $P\text{-}PO_4^{3-}$ $L^{-1}d^{-1}$ in period 3, the biomass
368 production was reduced to 0.039 g $L^{-1}d^{-1}$ as a direct consequence of the acute P limitation.

369 Results on adaptation of cyanobacteria to low P concentration and limitation found in the
370 present study are comparable to those previously reported on cultivation of cyanobacteria in
371 wastewaters (see Table 4). Nevertheless, it should be taken into account that the majority of these
372 studies were performed using pure cultures in batch lab scale experiments. The results by
373 Kamilya et al. (2006) and Su et al. (2012) indicated that cyanobacteria can be successfully
374 cultivated with low concentrations of P, as observed in our study. In contrast, other studies
375 revealed that cyanobacteria can also be cultivated with higher concentrations and even with very
376 high L_V-P values. For instance, Pouliot et al., (1989) and (Van Den Hende et al., 2016a) worked
377 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.
378 However, with the exception of the study by Renuka et al. (2013), all the other studies had low

379 N:P ratios, and therefore P limitation. In spite of being a relevant issue, the predominance of the
380 same species or the potential contamination during the culture was not mentioned in the most of
381 these studies. Only the studies performed by Pouliot et al., (1989) and Van Den Hende et al.,
382 (2016a) revealed the occurrence of other green algae species during the cultivation. On the other
383 hand, one study concerning microalgae dynamics in a photobioreactor fed with secondary
384 effluent obtained a culture largely dominated by *Chlorella*. This fact could be attributed to the
385 low nutrient concentration and nitrogen limitation (0.13 mgL^{-1} of TIN and 1.3 mgL^{-1} of $P\text{-}PO_4^{3-}$)
386 (Marchello et al., 2015).

387 Considering that the PBR of this study was used as an additional wastewater treatment system
388 for the mixture of secondary effluent and digestate, results obtained can be compared with other
389 studies focused in microalgae biomass production in PBRs using treated effluents as nutrient
390 sources. Although the biomass depends on the influent characterization and the different
391 operation modes, SST concentration and biomass production in this study were comparable to
392 those of other studies (Table 5). These results also reveal that recycling of nutrients from
393 secondary effluents and, in the particular case of this study the addition of digestate, can be
394 positively used to grow valuable biomass, obtaining at the same time a further treatment of the
395 wastewater used. Therefore, it could become a feasible alternative to conventional wastewater
396 with a double benefit, as this microalgae bioremediation is highly efficient in nutrients removal
397 and it wouldn´t require any chemical input. Furthermore, the potential of cyanobacteria to
398 produce and accumulate added-value products such as antibacterial substances, glycogen or
399 polyhydroxyalcanoates (PHAs) (bioplastics) could counterbalance the maintenance and operation
400 costs of closed PBRs. The data provided highlights the need of further studies regarding the
401 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.

5. Conclusions

In this study, 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. *Oscillatoria* sp., cf. *Aphanocapsa* sp. and *Chroococcus* sp. were found to dominate the culture with an increasing biomass content. 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. This information can contribute to future studies on further production of valuable by-products from cyanobacterial biomass in wastewater treatment systems.

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622 Table 1. Average (standard deviation) of the main quality parameters of the digestate, secondary
623 effluent and the influent PBR (mixture of digestate and secondary effluent) during the three
624 experimental periods (n = 15-20).

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})	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})	<LOQ	1.44 (0.69)	1.44 (0.69)	<LOQ	0.09 (0.22)	0.09 (0.22)	<LOQ	1.17 (0.82)	1.17 (0.82)
N-NO_3^- (mg L^{-1})	<LOQ	22.55 (6.5)	22.55 (6.55)	<LOQ	14.40 (4.57)	14.37 (4.57)	<LOQ	20.91 (6.12)	20.91 (6.12)
TIN (mg L^{-1})[c]	-	-	35.73 (5.62)	-	-	21.22 (4.43)			26.98 (8.5)
P-PO_4^{3-} (mg L^{-1})	<LOQ	2.96 (0.83)	2.96 (0.83)	<LOQ	2.19 (0.65)	2.19 (0.65)	<LOQ	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]TIN: total inorganic carbon.

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628 Table 2. Average (standard deviation) of the main quality parameters of the effluent of the photobioreactor
629 during the three experimental periods. *n*= 15-20 except for SST, SSV and biomass production (*n*= 37-60)
630 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})	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})[a]	31.94 (5.01)	23.41-44.08	12.88 (4.82)	5.04-20.62	4.12 (1.33)	0.78-6.54
P-PO_4^{3-} (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

631 [a]*TIN: total inorganic carbon.*
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634 Table 3. Influent and effluent N:P values, L_V-P and main dominating microalgae during the 3
635 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 P L^{-1} d^{-1})	
1	13:1	6-30:1	27:1	14-42:1	0.28	*Chlorella* sp., cf. *Oscillatoria* sp.
2	10:1	6-17:1	12:1	7-22:1	0.23	cf. *Oscillatoria* sp., cf. *Aphanocapsa* sp., C*hroococcus* sp.
3	18:1	11-29:1	5:1	2-15:1	0.16	cf. *Aphanocapsa* sp., *Chroococcus* sp., cf. *Oscillatoria* sp.

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638 Table 4. Summary of the average values of TIN and $P\text{-}PO_4^{-3}$ and N:P ratios of the periods with
639 cyanobacteria dominance of this study compared with other cyanobacteria culture studies fed with
640 wastewaters.

Microalgae cultivated	Cultivation mode	Influent	TIN (mg L^{-1})	$P\text{-}PO_4^{3-}$ (mg L^{-1})	N:P ratio	L_V-P (mg $P\text{-}PO_4^{3-}$ L^{-1} d^{-1})	Reference
Cyanobacteria dominated mixed culture	Semi-continuous	Secondary effluent and digestate	21.22	2.19	10:1	0.23	This study[a]
Cyanobacteria dominated mixed culture	Semi-continuous	Secondary effluent and digestate	26.97	1.41	18:1	0.16	This study[b]
Spirulina platensis	Batch	Fish culture effluent	6.62	0.67	10:1	NA[c]	Kamilya et al. (2006)
Nostoc muscorum	Batch	Fish culture effluent	6.62	0.67	10:1	NA[c]	Kamilya et al. (2006)
Phormidium sp.	Batch	Secondary effluent	26	1.8	14:1	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	4:1	NA[c]	Talbot and de la Noüe (1993)
Phormidium sp.	Semi-continuous	Swine manure	50	17.5	2:1	2.64	Pouliot et al. (1989)
Phormidium sp.	Batch	Digestate	8.8	6.4	1.4:1	NA[c]	Cañizares-Villanueva et al. (1994)
Geminocystis sp., *Aphanocapsa* sp.	Sequencing batch	Secondary effluent	4.65	2.37	1.9:1	1.15	(Van Den Hende et al., 2016a)
Arthospira platensis	Batch	Diluted olive-oil mill wastewater	564	65.22	8:1	NA[c]	(Markou et al., 2012)

[a] *culture corresponding to period 2 of this study.*
[b] *culture corresponding to period 3 of this study.*
[c] *not applicable.*

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642 Table 5. Average of TSS and biomass production in the three periods of the study compared with other

643 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.*

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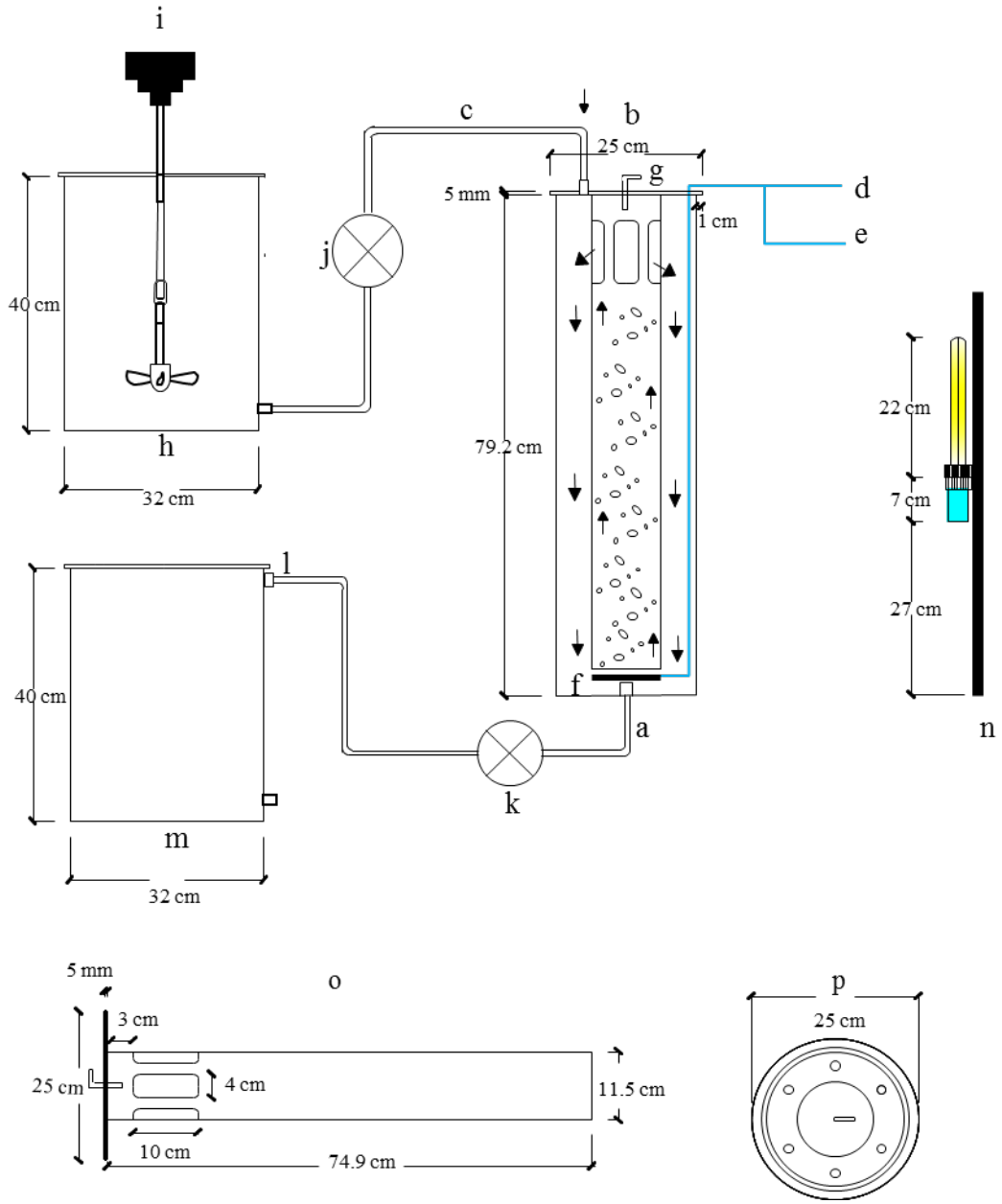

Fig. 1 Schematic diagram of the photobioreactor (PBR) set-up: a) PBR body with the internal cylinder inside, b) PBR cover, c) feeding tube, d) air tube, e) CO_2 tube, f) air sparger, g) gas outlet, h) feeding tank, i) electric stirrer, j,k) pumps, l) effluent tube, m) harvesting tank, n) external lamp, o) detail of internal cylinder, p) top view of the photobioreactor. Arrow's path follows the continuous culture movement inside the PBR.

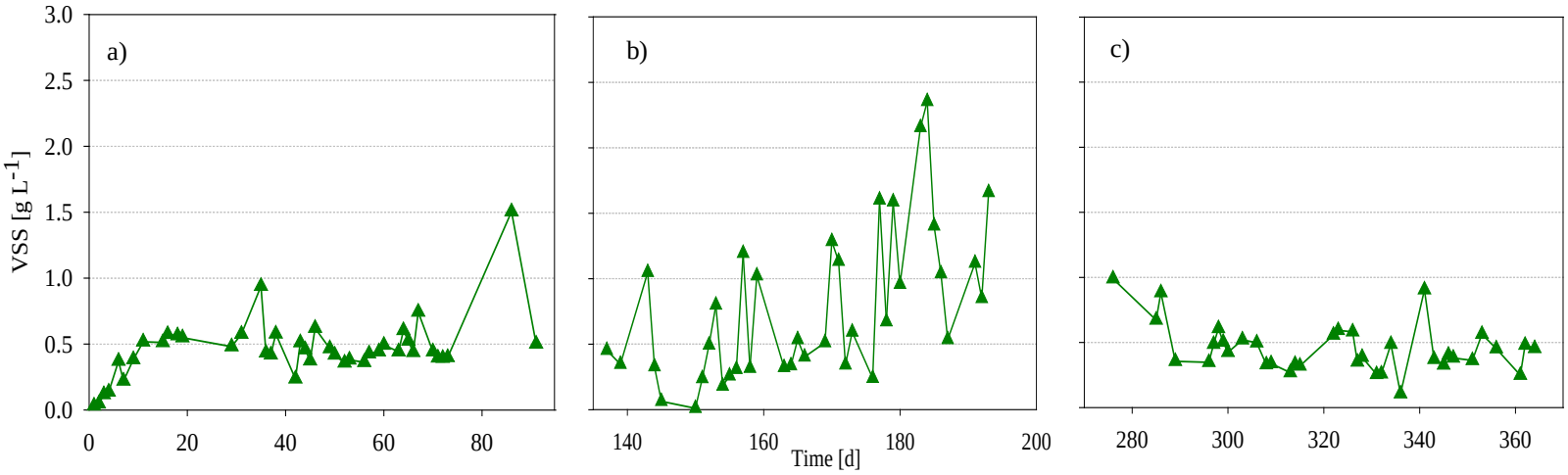

Fig. 2. Changes in the biomass contained in the PBR during the three periods. a) period 1, b) period 2, c) period 3. Biomass is given as volatile suspended solids (g VSS L^{-1}).

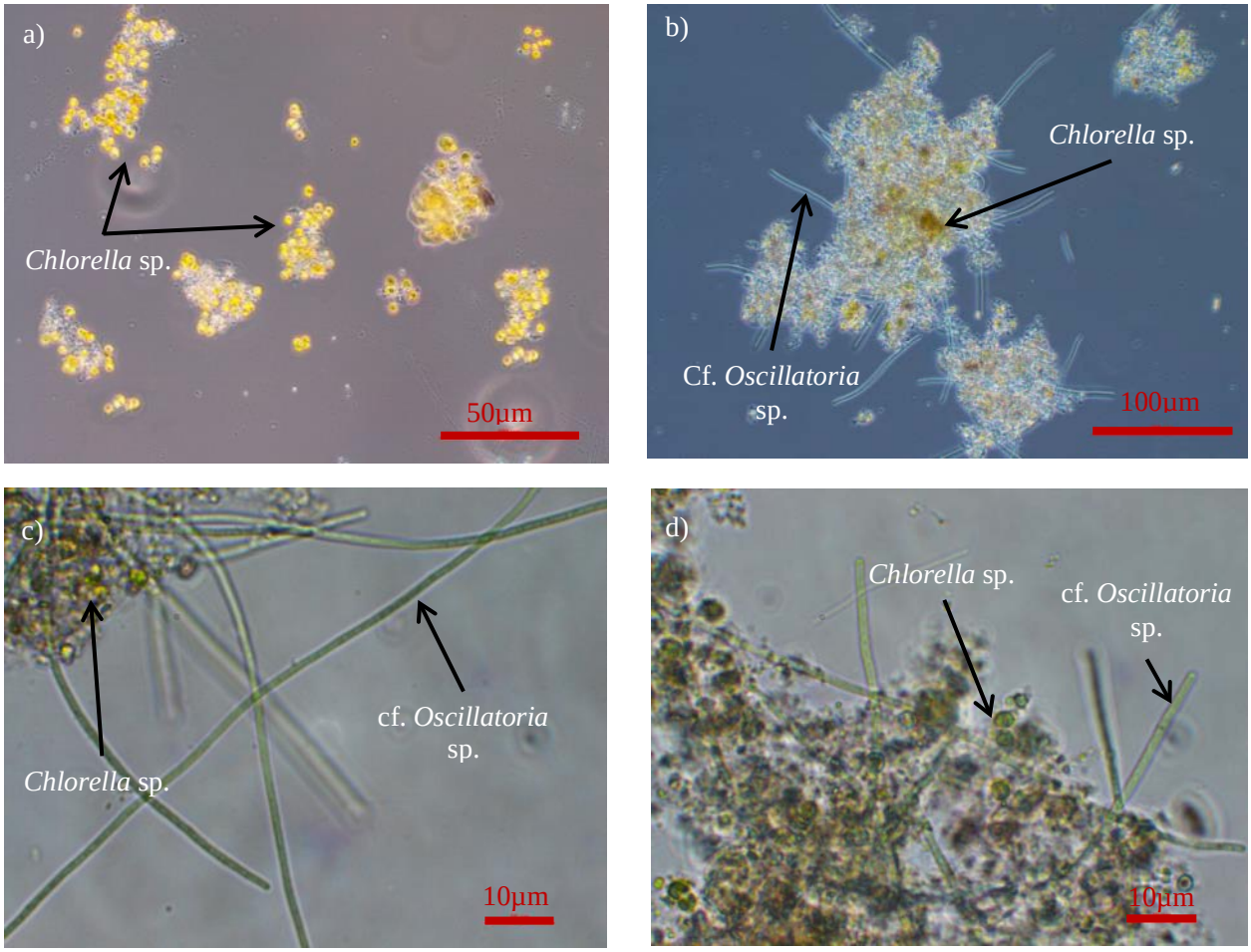

Fig. 3. Microscopic images illustrating the dominant algae during period 1. a) Initial mixed culture dominated by *Chlorella* sp. immersed in flocs with some dispersed individuals observed in phase contrast microscopy (400X); b) Algal flocs mostly composed of *Chlorella* sp. with some filaments of cf. *Oscillatoria* sp., observed in phase contrast microscopy (200X); c) Detail of a lateral side of an algal floc with filaments of cf. *Oscillatoria* 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. *Oscillatoria* sp., observed in bright light microscopy.

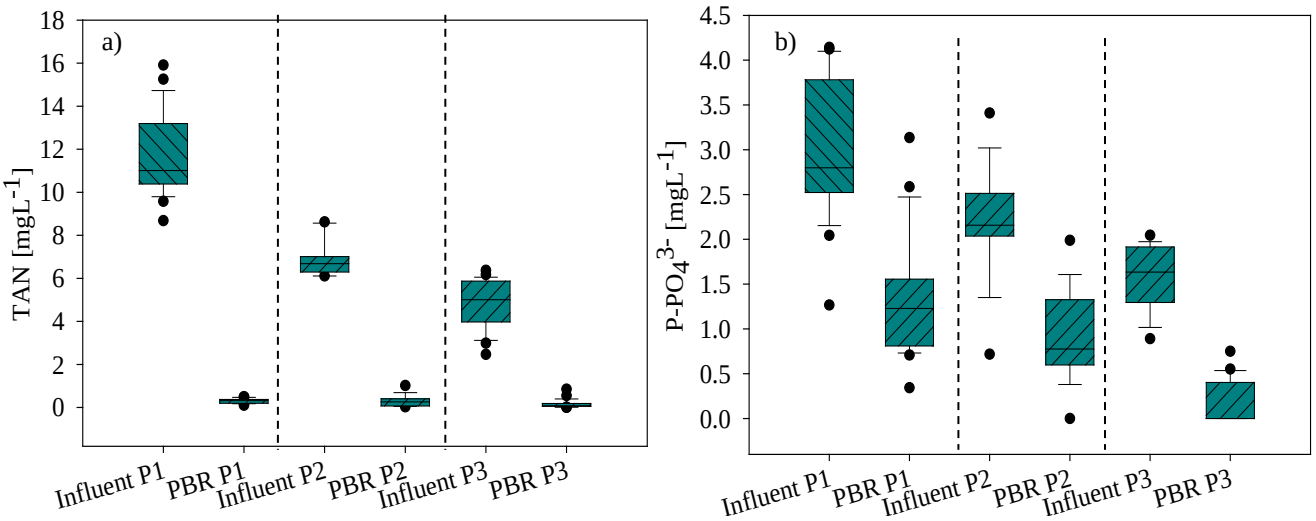

Fig. 4. Box-plot of the PBR influent and effluent a) ammonium (TAN) and b) orthophosphates ($P\text{-}PO_4^{3-}$) concentration. P1, P2 and P3 mean period 1, 2 and 3. Discontinued vertical lines separate the 3 operational periods.

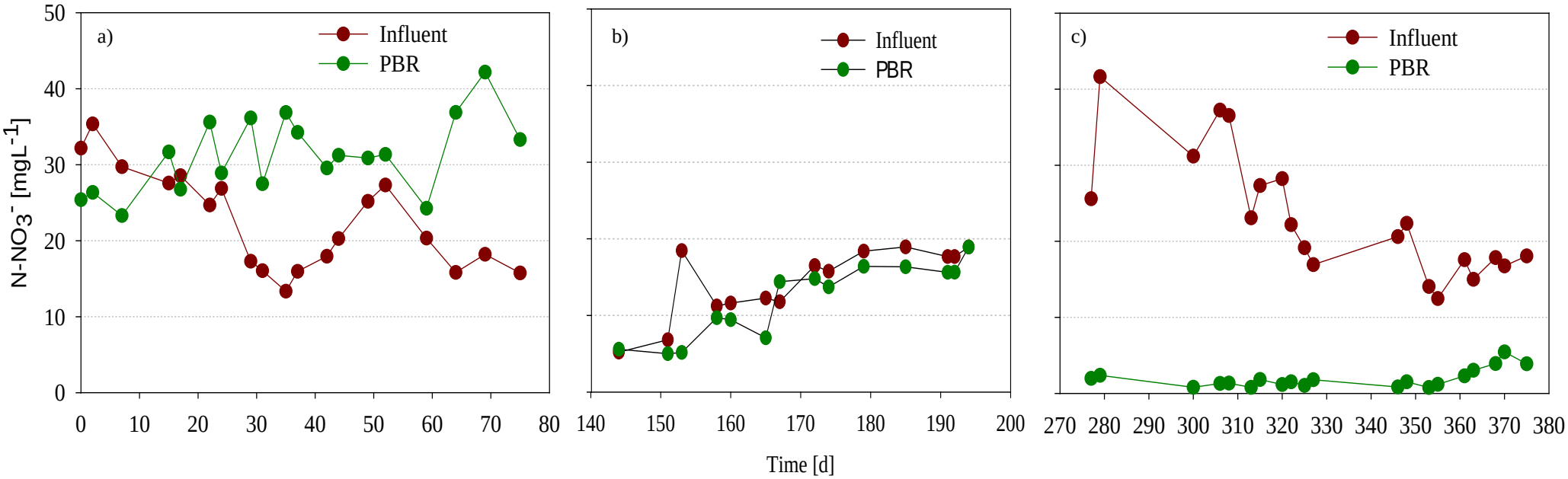

Fig. 5. Changes in nitrate ($N\text{-}NO_3^-$) concentration in the influent and the effluent of the PBR during the three periods. a) period 1, b) period 2 and c) period 3.

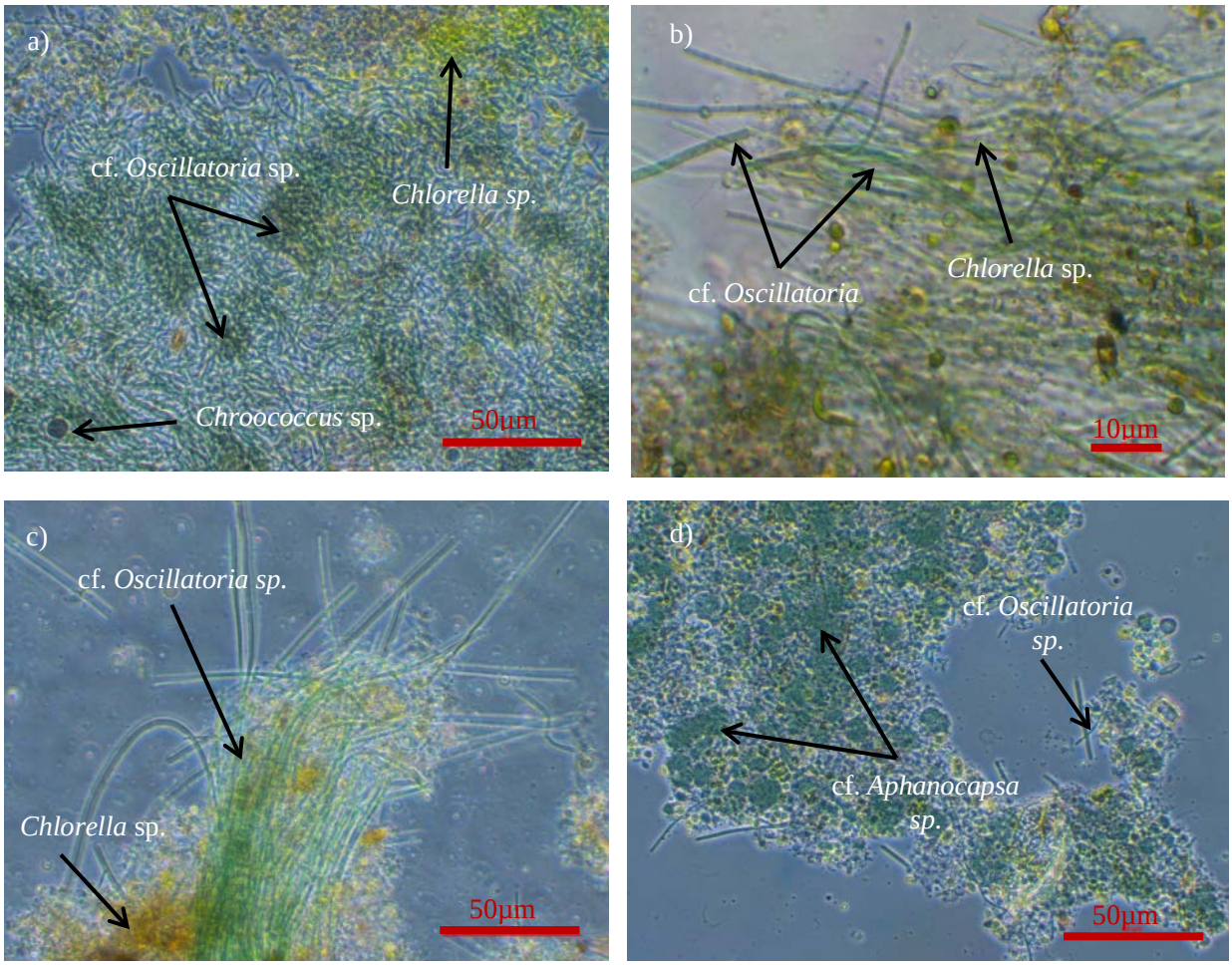

Fig. 6. Microscopic images illustrating the dominant algae during period 2; a) Algal floc largely dominated by filamentous cf. *Oscillatoria* sp., groups of *Chlorella* sp. and dispersed individuals of *Chroococcus* sp. observed in phase contrast microscopy (400X); b) Detail of an algal floc dominated by cf. *Oscillatoria sp*. with some dispersed *Chlorella* sp. observed in bright field microscopy (1000X); c) Grouped filaments of cf. *Oscillatoria* 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. *Oscillatoria* sp. observed in phase contrast microscopy (400X).

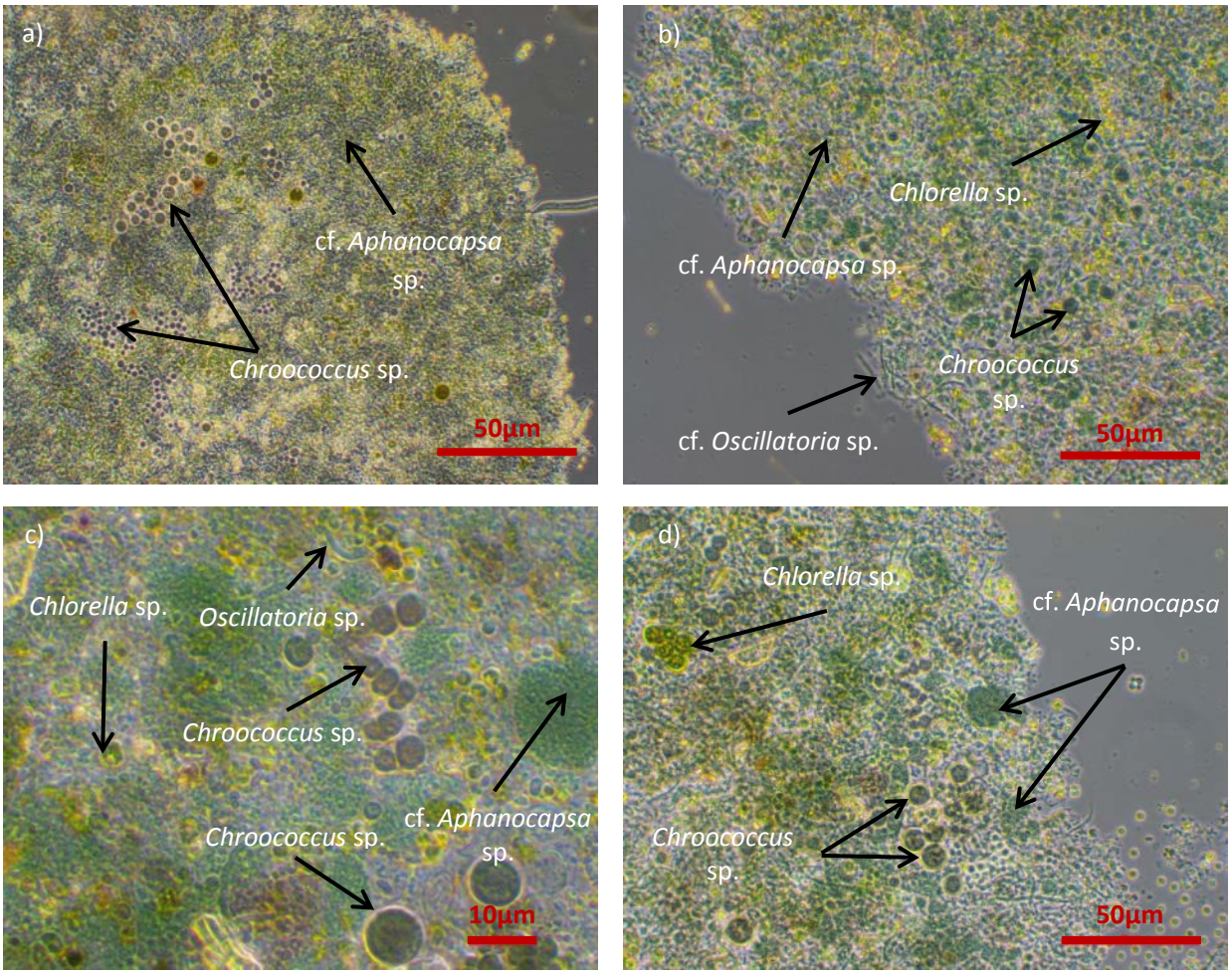

Fig. 7. Microscopic images illustrating the microbial composition along period 3. a) Algal floc largely dominated by cyanobacteria cf. *Aphanocapsa* sp., cf. *Chroococcus* sp. and dispersed *Chlorella sp.* observed in phase contrast microscopy (400X); b) Algal floc dominated by cf. *Aphanocapsa* sp. with some dispersed *Chlorella* sp. and cf. *Oscillatoria* 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).