1 **Hydraulic retention time effects on wastewater nutrient**
2 **removal and bioproduct production via rotating algal biofilm**
3 **reactor**

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17 **Abstract**

18 Rotating Algal Biofilm Reactor (RABR) technology was successfully employed in an
19 effective strategy to couple the removal of wastewater nutrients with accumulation of
20 valuable bioproducts by grown algae. A secondary stage municipal wastewater was fed
21 to the developed system and the effects of the hydraulic retention time (HRT) parameter
22 on both nutrient removal and bioproduct production were evaluated under fed-batch
23 operation mode. Two sets of bench scale RABRs were designed and operated with
24 HRTs of 2 and 6 days in order to provide competitive environment for algal growth.
25 The HRT significantly affected nitrogen and phosphorus uptakes along with lipid and
26 starch accumulations by microalgae in harvested biofilms. Domination of nitrogen
27 removal in 2-day HRT with higher lipid accumulation (20% on dried weight basis) and
28 phosphorus removal in 6-day HRT with higher starch production (27% on dried weight
29 basis) was observed by comparing the performances of the RABRs in duplicate runs.

30 *Keywords:* Algal biofilm, Wastewater nutrient removal, HRT, Lipid, Starch

31 **1. Introduction**

32 Conventional wastewater treatment methods need to be implemented by post-treatment
33 approaches if they cannot fulfill the effluent standards. Algal systems associated with
34 conventional methods have the potential of improving quality in effluent of wastewater
35 treatment plant by reducing nutrient and metal loads into freshwater ecosystems
36 (Hoffmann, 1998).

37 Several efforts into nutrient removal via conventional mechanical or biological and also
38 chemical technologies along with their drawbacks in costs, energy consumptions,
39 maintenance and volume of produced waste sludge, have been reported (Lin et al.,
40 2002; Tchobanoglous & Burton, 1991). Among them, development of processes based
41 on the exploitation and recovery of microalgae under the physiological condition of
42 immobilized cells was proposed. In particular, nutrient removal from municipal
43 wastewater by immobilized *Chlorella vulgaris* and *S. obliquus* was studied (Ruiz-Marin
44 et al., 2010). Furthermore, an algal-based immobilization process in secondary
45 wastewater treatment (He & Xue, 2010) and application of different coagulants to
46 harvest microalgae uptaking nitrogen and phosphorus from municipal wastewater
47 (Udom et al., 2013) were developed. These studies demonstrated the feasibility of
48 nutrient removal by employment of different microalgae cultures, but challenges related
49 to process scaling up and costs have still to be faced up. Thus, incorporation of biofilm
50 photobioreactors in wastewater treatment processes to reduce the harvesting costs and
51 improve the removal efficiency can play a significant role in perspective of developing
52 economically feasible systems. In fact, removal of dissolved nitrogen and phosphorus is
53 improved by maximizing algae production in a biofilm system, which allows higher
54 concentrations and easier harvesting of valuable biomass (Christenson & Sims, 2012).
55 Further studies in this field were carried out with a) polymeric sheets for biofilm
56 generation in a flow cell biofilm reactor dedicated to nutrient removal in a post
57 treatment process (Boelee et al., 2011), b) horizontal flat panels in large scale
58 photobioreactors for phosphorus removal from tertiary step using microalgal biofilm
59 (Sukačová et al., 2015), c) algal-bacterial biofilm photobioreactor to eliminate carbon,
60 nitrogen and phosphorus from concentrate and domestic wastewater (Posadas et al.,

61 2013), d) submerged membrane module for algal biofilm growth and nutrient removal
62 using *Chlorella vulgaris* strain (Gao et al., 2015), and e) Twin-Layer photobioreactor
63 for separation of immobilized microalgae (Shi et al., 2014). Defects of these studies
64 might be found in culture medium and use of specific strain, installation of air
65 compressor, ecological and economic implications. On the other hand, concerns about
66 global energy demand, as well as negative impact of petroleum based energy on
67 environment, entice the attentions to the algal research as viable energy source (Quinn
68 & Davis, 2015).

69 Sustainable treatment process could be achieved by the exploitation of algae, which can
70 couple nutrient removal with production of biomass accumulating bioproducts, such as
71 lipids, proteins, carbohydrates and residual biomass for anaerobic digestion. In this
72 frame, this study covers lipid and starch production that can be converted to biodiesel
73 and bioethanol by trans-esterification and fermentation processes, respectively. Various
74 strategies have focused on high lipid and starch production from algae, both employing
75 single strains or mixed cultures, through the regulation and mutation of strains,
76 gravitation enrichment technique and inhibition of starch synthesis pathway (Goodson
77 et al., 2011; Hassanpour et al., 2015; Li et al., 2010; Mooij et al., 2015); however, those
78 approaches were carried out with genetically modified organisms or single strain of
79 microalgae fed with laboratory prepared water solutions which only simulated actual
80 wastewaters. Conversely, this study focused on the exploitation of a photoautotrophic
81 mixed culture made up of algae and bacteria capable of accumulating lipid and starch
82 while removing nutrients from secondary stage municipal wastewater. In particular,
83 RABR system was employed as algal photobioreactor. Needful substrate and nutrients

84 for biofilm formation were available from wastewater nitrogen and phosphorus sources,
85 without external nutrient addition.

86 Development of algal biofilm technology in wastewater treatment process faces
87 limitations of information about algal growth needs, biofilm formation, nutrient removal
88 efficiencies and standard operating conditions. In fact, investigation on performances
89 and processes related to algal biofilm technologies are needed, despite the occurrence of
90 established heterotrophic attached growth systems for wastewater treatment (Kesaano &
91 Sims, 2014). The aim of this study was finding out the role of HRT on nutrient removal
92 and bioproduct production in RABR systems, as a crucial parameter to distinguish
93 target systems depending on main process aim (nutrient removal or carbon storage). To
94 this aim, two sets of RABRs operated under fed-batch mode with HRTs of 2 and 6 days
95 on the mixed culture medium in order to obtain the dominant photobioreactor beside
96 effective operation of nutrient removal incorporation with bioproduct production.

97 **2. Materials and methods**

98 **2.1. Experimental setup and operation**

99 All experiments were performed in 8 L bench scale RABRs, operated at 4.8 rpm and
100 temperature of 21 | 25°C (dark | light). Each reactor was composed of a plastic
101 cylindrical wheel with 10 cm diameter and installed cotton cords onto wheel as the
102 biofilm substratum with average length of 28 inch (~71 cm) and 0.25 (~0.63 cm) inch
103 diameter. Cylinder rotated inside the 50% of reactor volume and light irradiance was on
104 the top led biofilm formation on cotton ropes. Also, reactors were illuminated under 8 h
105 dark and 16 h light period with sodium vaporizer lamp (1000W/ SWBL) at light
106 intensity of $200 \pm 20 \mu\text{mol m}^{-2} \text{s}^{-1}$ with Apogee Quantum Meter (MQ-200) at level of

107 biofilm surface. Also, UV filter sheets were installed over the RABRs in order to
108 prevent critical distribution of algae strains by UV irradiations from light (Holzinger &
109 Lütz, 2006). Biomass harvesting was applied via scraper after taking out the rope on
110 cylinder. The pH was measured every 2 days with transmitter (Mettler Toledo,
111 Switzerland). The RABRs were operated under fed-batch mode in two cases of, 2-day
112 HRT and 6-day HRT. Prior to fed-batch operation, the same conditions described above
113 were applied on the same reactors in batch mode of operation in order to specify the
114 biomass growth parameters, growth phases and process time period. Duplicate system
115 was considered to the whole reactors except microscopic qualification of growth media.

116 **2.2. Biomass growth analysis**

117 Municipal wastewater from the pond C, a secondary stage, in the city of Logan's
118 treatment facility, UT, USA, was prepared and preserved at 4°C before feeding into the
119 reactors. In order to find out the biomass seeding period and growth curves, duplicate
120 RABRs in batch operation was designed including biomass inoculation through adding
121 10 % (v/v) ratio of sludge from the same pond. Before adding, sludge properties of total,
122 fixed and volatile solid analysis were performed by means of oven at 103° C and muffle
123 furnace at 550° C for 12 h and 1 h respectively (USEPA Method 1684). The inoculation
124 samples involved mixture of various algae strains that microscopic analysis reported
125 based on the algal key and previous works (Bellinger & Sigee, 2015; Griffiths, 2009).

126 Biofilms harvesting were regulated for every 2 days and 4 hours after light period's
127 beginning. Removed biofilm from rope was collected into weighted aluminum bowl and
128 dried for 12 h at 103° C. Then after, dried biomass was cooled in desiccator and
129 weighted as biomass cell dried weight (CDW, g m⁻²). Fed-batch operation was

130 accomplished according to the results from batch mode to find out the growth phase
131 startup day. In order to perform 2-day HRT and 6-day HRT, liquid mediums were
132 drained and refilled as 1/2 and 1/6 of the reactor volume respectively, every 24 h with
133 light period initiation. Sampling from reactor content for biomass growth was just
134 focused for optical density (OD) by UV spectrophotometry (Shimadzu, UV 1800).

135 **2.3. Nutrient removal analysis**

136 Wastewater characterizations were analyzed before feeding to the RABR system. TSS
137 and VSS both were measured based on standard gravimetric method (USEPA Methods
138 8158 and 8164). During the experiment, samples were taken every 2 days from liquid
139 medium except total nitrogen (TN) and total phosphorus (TP) as daily inlet-outlet of the
140 cycle. Concentrations of TN, TP and Ammonium ($\text{NH}_4^+\text{-N}$) were determined
141 spectrophotometrically (Hach, DR 2700) using the persulfate digestion method (Hach
142 methods 10071-72), molybdovanadate with acid persulfate digestion (Hach method
143 10127) and salicylate method (Hach Method 10031) respectively. Nitrate (NO_3^-), Nitrite
144 (NO_2^-), orthophosphate (PO_4^{3-}) and Sulfate (SO_4^{2-}) concentrations in the broth medium
145 were monitored and measured by ion chromatography (IC) using a Dionex IonPac
146 AS12A anion-exchange column set at a flow rate of 1.5 ml min^{-1} . IC data also, were
147 analyzed via Chromeleon 7 Chromatography Data System (CDS) software.

148 **2.4. Bioproduct analysis**

149 Bioproduct analysis focused on carbon storages as lipid and starch. Harvested biofilms
150 in fed-batch mode as same as biomass growth analysis, were dried at 103°C then,
151 prepared for lipid determination procedure via colorimetric method the same as
152 described by Mishra et al. (Mishra et al., 2014) and starch determination using modified

153 methods (Asada et al., 2012; Mooij et al., 2014). A known amount of dried biomass was
154 sonicated inside 2 M HCl for about 30 min and the solution was heated for 3 h in
155 boiling water as hydrolyzation process. Afterward, neutralized by 2 M NaOH and
156 centrifuged for 15 min at 4000 rpm. Finally, after filtration by 0.2- μ m pore size filter,
157 dissolved glucose monomer concentration was determined by high-performance liquid
158 chromatography (Shimadzu Scientific) using BP-100H⁺ column from Benson Polymeric
159 (USA). Starch content was calculated by multiplying factor 0.9 to the glucose according
160 to starch determination method (McCready et al., 1950).

161 **3. Results and discussions**

162 **3.1. Biomass growth in batch operation of RABR**

163 In order to determine the whole batch process duration, a conventional biomass growth
164 curve as a function of the time was defined. This allowed preliminary considerations
165 about the HRT parameter, which was applied in the development of subsequent fed-
166 batch processes. Duplicate RABRs were fed with characterized municipal wastewater
167 and sludge for inoculation and seeding (Table 1). During 21 days of experiment, the lag,
168 growth and stationary phase were achieved through logarithmic areal density versus
169 time, (Fig. 1A). A 3-days long lag phase was observed and consecutive exponential
170 growth phase for microalgal biofilms occurred from day 3 to 11, after which the linear
171 part of natural log transformed growth data and stationary phase followed until day 21.
172 The maximum specific growth rate measured during the exponential phase was
173 0.149|006 day⁻¹ (mean | range) for algal biofilms. The maximum areal biomass density
174 measured through the stationary phases was 16.44 g m⁻² on average. Moreover, the
175 biofilm production rate as total accumulated biofilm's areal density over the time taken
176 to reach stationary phase, reached 0.96 g m⁻² d⁻¹ in batch mode. Growth of microalgae in

177 the bulk medium was analyzed via optical density at 750 nm wavelength (Fig. 1B).
178 Results from OD analysis demonstrate biofilms formation and biomass tendency to
179 grow on the cotton cord. The pH variations actuated the bulk medium to values over
180 8.5. High ranges of pH, indicates possibility of domination in bicarbonate uptake via
181 specific algae strain (Dubinsky & Rotem, 1974) and this can affect nutrient removal
182 during the experiment.

183 Table. 1

184 Fig. 1.

185 **3.2. Biomass growth and nutrient removal in fed-batch operation**

186 Fed-batch operation was accomplished as 2 sets of duplicate RABRs in 2 days and 6
187 days of HRT for 21 days of experiment. Draining and refilling were performed after 3
188 days of batch mode operation, according to results about lag phase of seeding period.
189 The CDW analysis of biofilm provided growth curve as natural logarithmic data versus
190 time for fed-batch operation to make the same diagram in batch operation. Maximum
191 growth rate in this operation mode was reached $0.2124 \pm 0.022 \text{ day}^{-1}$ (mean|range) for 2-
192 day HRT and $0.1924 \pm 0.018 \text{ day}^{-1}$ (mean|range) for 6-day HRT. Also maximum biofilm
193 production per area was obtained in 2-day HRT with 30.1 g m^{-2} which was 28 g m^{-2} for
194 6-day HRT. Furthermore, the biofilm production rates, calculated as the total biofilm
195 areal density accumulated divided by the time taken to reach stationary phase, were 1.74
196 and $1.64 \text{ g m}^{-2} \text{ day}^{-1}$ for biofilms in 2-day HRT and 6-day HRT respectively. Growth
197 curves for the algal biofilms and also in suspension for hydraulic retention times of 2
198 and 6 days are shown in Fig. 2. Prominent algal genera in 21 days of experiment

199 according to microscopic analysis of both HRTs with 40X magnification included
200 *Scenedesmus*, *Chlorella*, *Cyanobacteria*, *Oocystis*, *Ankistrodesmus* and *Synura*.

201 Fig. 2.

202 Removal of the nutrients, which are mostly nitrogen and phosphorus in algal research, is
203 a basic requirement of wastewater post-treatment to gain acceptable standards of
204 effluents. Using algal-based reactors improves nutrient uptake through the biomass
205 which could be harvested from the effluent. Initial total nitrogen and phosphorus
206 concentrations of the feed were 49.5 mg-N L⁻¹ (HRT 2) - 46.5 mg-N L⁻¹ (HRT 6) and
207 14.95 mg-P L⁻¹ (HRT 2) – 15.15 mg-P L⁻¹ (HRT 6) respectively. After 21 days of
208 treatment process beside of pH raising up to 9, broth medium reached 1.6 and 1.15 mg
209 L⁻¹ of TN and TP respectively in 2-day HRT, which were 1.85 and ~0 mg L⁻¹ of TN and
210 TP, respectively in 6-day HRT. Significant amounts of sulfate (SO₄²⁻), lead to consider
211 removal potentials in both HRTs. The reactor feed for 2-day HRT had 58.7 mg-S L⁻¹ of
212 sulfate and RABR decreased it by 30.6 mg-S L⁻¹ with average rate of 1.62 mg-S L⁻¹ d⁻¹,
213 which was 60.3 mg-S L⁻¹ to 29.24 mg-S L⁻¹ in 6-day HRT with average rate of 2.16 mg-
214 S L⁻¹ d⁻¹.

215 Nitrogen as ammonium (NH₄⁺), nitrate (NO₃⁻) and nitrite (NO₂⁻) were analyzed during
216 the experiment (Fig. 3), with initial concentrations of 5.2 mg L⁻¹, 0.08 mg L⁻¹ and 0.24
217 mg L⁻¹ respectively for 2-day HRT and subsequently 5.05 mg L⁻¹, 0.08 mg L⁻¹ and 0.25
218 mg L⁻¹ respectively for 6-day HRT. The removal efficiencies in overall process were
219 obtained 92% (NH₄⁺), 44% (NO₃⁻) and 57% (NO₂⁻) for 2-day HRT and subsequently,
220 100% (NH₄⁺), 47% (NO₃⁻) and 58% (NO₂⁻) for 6-day HRT. Phosphorus as
221 orthophosphate (PO₄³⁻) was also measured during the experiment with initial

222 concentrations of 3.52 mg L⁻¹ (HRT 2) and 3.33 mg L⁻¹ (HRT 6). Overall phosphate
223 removal efficiencies were reached to 97% and 79% for the HRT of 2 and 6 days
224 respectively. Nutrient concentrations in each two days were collected as soon as day 4
225 began with draining-refilling step. It is obvious that ammonium uptake is almost
226 complete in both HRTs despite of other nitrogen sources and this indicates preferential
227 utilization of ammonium by algae over nitrate (Eustance et al., 2013).

228 The pH variations in both HRTs were compatible with possibility of bicarbonate
229 domination chiefly, in the range of 8.5 to 9. On the other hand, nitrification process in
230 the reactor contains chemoautotrophic bacteria which need a carbon source such as CO₂
231 or HCO₃⁻, favorable initial conditions, lead bacteria to grow. However, pH ranges out of
232 8-8.5 effect on reaction rate and provide incomplete nitrification of ammonia (Clair N.
233 Sawyer, 1973). Thus; refilling the reactor with fresh feed, accumulates nitrite (NO₂⁻)
234 ions. This can be mentioned in both HRTs after day 9 that pH values had raising trend.

235 In order to find out the domination of nutrient removal in treatment process, the inlet
236 and outlet concentrations of total nitrogen, total phosphorus, were depicted for each
237 batch (Fig. 4). High amount of nitrogen uptake than phosphorus could be extracted
238 through the curves in 2-day HRT, unlike significant phosphorus removal rate than
239 nitrogen in 6-day HRT (Table. 2). This can be demonstrated better, through the uptake
240 rate's ratio as phosphorus over nitrogen (Fig. 5). During the fed-batch operation since
241 day 4, it is clear that phosphorus consumption rate in 6-day HRT is over than 2-day
242 HRT which means that phosphorus is finished prior than nitrogen. Likewise; nitrogen
243 consumption rate in 2-day HRT is more than 6-day HRT. Using the removal potentials
244 for both HRTs in each cycle (Table. 2), the maximum removal potential in 2-day HRT
245 is owned for nitrogen as 94 %, whereas in 6-day HRT is 99.5 % for phosphorus.

246 Fig. 4.

247 Table. 2

248 Fig. 5.

249 Both HRTs have increasing trend in nutrient removal potentials in conjunction with
250 biofilm growth but, RABR system with 2-day HRT prefers to remove nitrogen and with
251 6-day HRT prefers to use phosphorus according to results. With respect to the analysis
252 of TN and TP along with different nitrogen types in the bulk medium and also
253 orthophosphate concentrations, most of the nitrogen and phosphorus include the organic
254 types. On the other hand, long retention time gave higher removal capacity than short
255 one for NH_4^+ , NO_2^- and NO_3^- unlike better PO_4^{3-} removal in short HRT. This means that
256 there is sufficient time to nitrogen conversion and utilization in 6-day HRT and organic
257 nitrogen is assimilated with biofilm growth in 2-day HRT. Moreover, long retention
258 time led organic phosphorus conversion to the orthophosphate and assimilation,
259 probably due to the high activity of the phosphatase at the cell surface. In a mixed
260 culture of different strains, these can effect on carbon cycle to produce lipid or starch
261 with respect to the competitions to uptake nitrogen or phosphorus.

262 According to nitrogen and phosphorus removal efficiencies in this study, they both are
263 superior to most of previous reports as about 80% of TN and TP removal by twin layer
264 biofilm reactor (Shi et al., 2014), immobilized process for secondary stage municipal
265 wastewater that represents 52% of TN and 92% of TP removal efficiency (He & Xue,
266 2010) and also, 96% of TN and 99% of TP removal efficiency by cultivating *Chlorella*
267 *kessleri*, *Chlorella vulgaris* and marine algae species (Caporgno et al., 2015). Therefore,

268 it is a comparable strategy of treatment to reach optimal stage with no specified strain,
269 nutrient addition and costly harvesting method.

270 **3.3. Lipid and starch production**

271 In order to distinguish carbon flow into accumulation of molecules, bioproducts' results
272 for both HRTs were described versus time in case of lipid and starch (Fig. 6). The
273 maximum bioproduct production was obtained at the end of experiment as lipid in 2-day
274 of HRT and starch in 6-day HRT which are more than 20 % and 27 % of dried biofilm
275 respectively. This shows the preferability of 2-day and 6-day HRT in lipid and starch
276 production respectively. Regarding nutrient removal results, mixed culture's biology
277 and impacts of nutrient starvation and phosphorus ratio over nitrogen that can change
278 carbon flow's direction in lipid or starch production (Zhu et al., 2015), domination of
279 lipid production in short HRT than long, probably could be consequence of inhibition in
280 AGPase enzyme's activity to reduce starch accumulation. As a case study about
281 synthetic pathway of lipid and starch's accumulation, (Li et al., 2015) priority of lipid
282 production through a specific strain, *C. sorokiniana*, over starch in the N-depleted
283 condition have been discussed. Using nutrient removal capacities and domination of
284 organic nitrogen and organic phosphorus uptake in short and long retention times
285 respectively, daily improvement of removal capacities for both N and P along with
286 biofilm growth in both HRTs may give the possibility of specific genera for both algae
287 and bacteria in the mixed culture to provide nutrient starvation through the rough
288 removal process. Therefore; in the mix autotrophic environment several parameters can
289 influence on lipid or starch synthetic.

290 Fig. 6.

291 Similar to the nutrient removal, bioproducts analysis represented comparable results
292 considering lipid and hydrocarbon production by the rotating algal biofilm system and
293 flat panel photobioreactor as 7-22 % for lipid and 38-48 % for hydrocarbon using
294 *Chlorella Vulgaris* (UTEX #265) (Gross et al., 2013). In another study, Hassanpour et
295 al. investigated on gravitation enrichment of mixed culture to screen lipid and starch
296 producing species separately in a sequencing batch reactor using specific medium.
297 Maximum lipid and starch production reached by lipid producing photobioreactor with
298 values of 30% and 36% respectively on the basis of VSS.

299 In comparison with other investigations to improve the nutrient removal or carbon
300 storages, ecology-based environment which has been used in this study by HRT
301 variation and competitive conditions can represent advantages of biodiversity in the
302 mixed culture, compatibility with other variables and optimization possibility in process
303 design (Mooij et al., 2015).

304 Effective algal process that uses RABR to provide selective conditions by designing
305 suitable HRT for both nutrient removal and bioproduct production, offers a simple,
306 inexpensive and sustainable method. This can be applied on various wastewaters to find
307 out dominant biodiversity, and large scale optimization with aims of above.

308 **Conclusions**

309 Removal and utilization of municipal wastewater nutrients were established through
310 biofilm technology using microalgae to uptake them. Designed RABRs were operated
311 in fed-batch operation for two HRTs of 2 days and 6 days. Experiments during 21 days
312 demonstrated superiority of RABR with 2-day HRT in nitrogen removal and 6-day
313 HRT in phosphorus uptake. Notable results described advantage of 2-day HRT in lipid

and 6-day HRT in starch accumulation at the end of experiment. The HRT based sifting may represent new strategy to combine nutrient removal from wastewater with dominate lipid and starch synthesis to produce increased biodiesel and bioethanol respectively.

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415 **Figure Captions**

416 Fig.1. Algal biomass growth profile as natural log transformed data for attached biofilm
417 (A) showing the exponential phase (days 3-10) with equations and R^2 values describing
418 exponentially phase of biofilms and also, optical density @ 750 nm for suspension (B)
419 in batch mode during the experiment. Errors bars represent standard deviation (n=2).

420 Fig.2. Algal biomass growth profile in attached biofilm and broth medium for 2-day
421 HRT (A) and 6-day HRT (B) in cases of attached growth (solid lines) and suspended
422 growth (dotted lines). Errors bars represent standard deviation (n=2).

423 Fig.3. Nutrient concentration profile as ammonium ($\text{NH}_4^+\text{-N}$), nitrite ($\text{NO}_2^-\text{-N}$), nitrate
424 ($\text{NO}_3^-\text{-N}$), phosphate ($\text{PO}_4^{3-}\text{-P}$) in fed-batch mode during the experiment for 2-day HRT
425 (A) and 6-day HRT (B). Errors bars represent standard deviation (n=2).

426 Fig.4. Inlet and outlet concentrations of TN and TP in each batch of 2-day HRT (A) and
427 6-day HRT (B); Errors bars represent standard deviation (n=2).

428 Fig.5. Uptake rates of phosphorus over nitrogen, during the experiment in 2-day HRT
429 and 6-day HRT.

430 Fig.6. Accumulated lipid (A) and starch (B) profile during the experiment in 2-day HRT
431 and 6-day HRT. Errors bars represent standard deviation (n=2).

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437 **Tables**

438 Table 1. Specifications of the wastewater and sludge used for inoculation and seeding

Wastewater characteristics		Sludge characteristics	
TSS (mg/L)	53.3	Total solid (%WB)	7.7
VSS (mg/L)	46.6	Fixed solid (%WB)	70
COD (mg/L)	63.1	Volatile solid (%WB)	30
Nitrite as N (mg/L)	0.33		
Nitrate as N (mg/L)	0.45		
Ammonium as N (mg/L)	9.9		
Phosphate as P (mg/L)	3.49		
Sulfate (mg/L)	58.3		
TKN (mg/L)	49.2		
Total nitrogen (mg/L)	50		
Total phosphorus (mg/L)	15		

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442 Table 2. Removal capacities and removal rates of TN and TP in each batch for 2-day

443 HRT and 6-day HRT

Days	2-day HRT				6-day HRT			
	N		P		N		P	
	% Removal	mg L ⁻¹ d ⁻¹	% Removal	mg L ⁻¹ d ⁻¹	% Removal	mg L ⁻¹ d ⁻¹	% Removal	mg L ⁻¹ d ⁻¹
4	85.8	24.66	39.9	4.89	46.7	7.48	12.3	1.39
5	77.1	21.88	51.9	5.77	53.9	8.43	29.7	3.26
6	78.2	21.91	62.6	6.35	52.1	7.58	39.4	3.60
7	80.1	22.19	71.0	6.66	51.3	7.36	49.3	3.64
8	84.6	23.27	73.5	6.51	52.0	7.48	55.9	3.31
9	88.8	24.51	73.9	6.40	54.6	7.81	61.8	3.08
10	89.4	24.72	75.7	6.52	63.5	8.88	64.6	2.84
11	89.0	24.23	77.4	6.59	71.6	9.20	66.0	2.72
12	89.6	23.92	78.8	6.65	72.8	8.51	69.1	2.76
13	90.5	23.80	79.9	6.69	74.3	8.39	74.0	2.85
14	91.6	23.82	81.1	6.75	80.6	8.92	85.8	3.14
15	92.3	23.84	80.5	6.65	84.7	8.86	96.9	3.17
16	91.1	23.50	74.9	6.20	80.1	8.02	96.7	2.83
17	90.0	23.28	70.5	6.00	75.8	7.83	93.2	2.72
18	91.4	23.72	72.1	6.29	78.1	8.40	94.4	2.84
19	92.6	23.93	75.3	6.55	81.2	8.62	96.6	2.88
20	93.7	24.03	78.5	6.71	82.5	8.53	98.8	2.89
21	93.7	23.95	80.6	6.75	81.3	8.26	99.5	2.84

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447 **Figures**

448

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450 (A) showing the exponential phase (days 3-10) with equations and R^2 values describing

451 exponentially phase of biofilms and also, optical density @ 750 nm for suspension (B)

452 in batch mode during the experiment. Errors bars represent standard deviation (n=2).

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460 Fig.3. Nutrient concentration profile as ammonium (NH₄⁺-N), nitrite (NO₂⁻-N), nitrate
 461 (NO₃⁻-N), phosphate (PO₄³⁻-P) in fed-batch mode during the experiment for 2-day HRT
 462 (A) and 6-day HRT (B). Errors bars represent standard deviation (n=2).

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466 6-day HRT (B). Errors bars represent standard deviation (n=2).

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