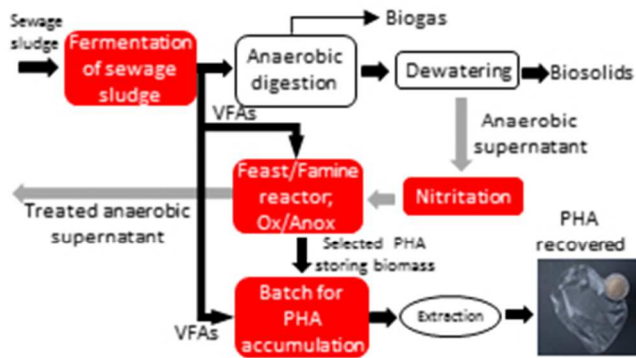


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Development of a novel process integrating the treatment of sludge reject water and the production of polyhydroxyalkanoates (PHAs)

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1 Title: Development of a novel process integrating
2 the treatment of sludge reject water and the
3 production of polyhydroxyalkanoates (PHAs)

4

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17

18 **ABSTRACT.** Polyhydroxyalkanoates (PHAs) from activated sludge and renewable organic
19 material could become an alternative product to traditional plastics since they are
20 biodegradable and biocompatible. In this work, the selection of PHA storing bacteria was
21 integrated to the side stream treatment for nitrogen removal via nitrite from sludge reject

22 water. A novel process was developed and applied where the alternation of *aerobic-feast and*
23 *anoxic-famine* conditions accomplished selection of PHA storing biomass and nitrogen
24 removal via nitrite. Two configurations were examined: in configuration 1 the ammonium
25 conversion to nitrite occurred in the same reactor where the PHA selection process occurred,
26 while in configuration 2 two separate reactors were used. The results showed the selection of
27 PHA storing biomass was successful in both configurations, while the nitrogen removal
28 efficiency was much higher (almost 90%) in configuration 2. The PHA selection degree was
29 evaluated by the volatile fatty acid (VFA) uptake rate (-qVFAs) and the PHA production rate
30 which were 182 ± 14 mgCOD/gX h and 89 ± 7 mgCOD/gX h respectively. The characterization
31 of biopolymers after the accumulation step, showed that it was composed of 3-
32 hydroxybutyrate (3HB) (60%) and 3-hydroxyvalerate (3HV) (40%). The properties
33 associated with the produced PHA suggest that they are suitable for thermoplastic processing.
34

35 **Keywords: polyhydroxyalkanoate (PHA); feast and famine regime; nitrogen removal**
36 **via nitrite; reject water**

37

38 INTRODUCTION

39 Polyhydroxyalkanoates (PHA) are biodegradable polymers that can be produced by many
40 different types of bacteria. Compared to conventional synthetic polymers, PHAs possess
41 obvious ecological advantages since they are completely biodegradable and nontoxic^{1,2} and
42 can be produced from a renewable source. The family of PHA polymers, including
43 polyhydroxybutyrate (PHB) and PHB-related copolymers, is versatile and thus, presents
44 significant opportunities for marketability. PHA production from mixed cultures and
45 renewable organic wastes as a carbon source^{3,4,5} has become very attractive over the last
46 years due to the decrease in the production cost of the process^{6,7}. Activated sludge from

47 wastewater treatment plants (WWTPs) is a well-known source of PHA-storing organisms that
48 store these polymers as carbon and energy reserve for biomass growth. PHA production from
49 mixed cultures are accomplished by a sequence of operations which are the following^{8,9}: i)
50 acidogenic fermentation to produce volatile fatty acids (VFAs) from biodegradable organics;
51 ii) selection of PHA storing biomass in a sequencing batch reactor (SBR); iii) batch step to
52 maximise PHA accumulation in the bacteria.

53 The carbon limitation strategy under feast and famine conditions has been found to be
54 favourable for the enrichment and long-term cultivation of PHA producing communities,
55 while nitrogen limitation is a successful strategy that can be employed to accomplish high
56 PHA contents during the PHA production step¹⁰. However, these processes are aerobic and
57 thus are energy intensive; it is estimated that approximately 39 MJ are needed to produce 1
58 kg of PHA when the aerobic accumulation step is employed^{11,12}.

59 The integration of nutrient removal in wastewater treatment systems with the PHA
60 production cycle is currently a challenge. Jiang et al. (2009)¹³, Morgan-Sagastume et al.
61 (2010)¹⁴ and Pittmann et al. (2014)¹⁵ evaluated the potential for the enrichment of PHA-
62 storing organisms and the PHA-storage capacity of fermented sludge under different
63 operational conditions. It was found that PHA producing organisms could be successfully
64 enriched using the fermented sludge as feedstock, although very high nutrient loads did limit
65 the maximum level of PHA accumulation¹⁴. Morgan-Sagastume et al. also examined a new
66 concept of WWTP operation, where the selection of PHA storing biomass was accomplished
67 based on the conversion of the readily biodegradable chemical oxygen demand (COD) from
68 municipal wastewater without its pre-conversion to VFAs¹⁶. However, post-treatment was
69 required, in particular for enhanced nutrient removal of the treated effluent. Anterrieu et al
70 (2014)¹⁷ studied the integration of PHA production with conventional nitrification and
71 denitrification for treating sugar beet factory waters, by operating with an anoxic feast and

72 aerobic famine phase. In the sludge treatment line of municipal wastewater treatment plants,
73 however, the nutrient loads are typically much higher, and side-stream treatment is an
74 attractive option to avoid recycling these nutrients to the main treatment line. Short-cut
75 nitrogen removal processes via the nitrite pathway have been found to provide an attractive
76 means of achieving nutrient removal from sludge reject waters at lower operating expenses
77 compared to conventional nitrification-denitrification¹⁸. Frison et al. (2014)¹⁹ compared the
78 potential PHA storage of sludge under nitrifying and non-nitrifying conditions in batch
79 reactors, and found PHA production yields of 0.6 Cmol PHA/Cmol VFA in both systems.
80 Nevertheless, the feasibility of integrating PHA production with nutrient removal via nitrite
81 has never been investigated.

82 In this work, a novel process for the potential integration of the side stream biological
83 nitrogen removal via nitrite with the selection of the PHA storing biomass from mixed
84 cultures treating anaerobic supernatant is examined. A modified pathway for nitrogen
85 removal was developed and applied in order to induce a feast and famine regime under
86 aerobic and anoxic conditions, respectively. The novel approach is based on (i) selection of a
87 PHA storing biomass in a sequencing batch reactor (SBR) by the alternation of aerobic feast
88 conditions for ammonia conversion to nitrite followed by anoxic famine (FF) conditions for
89 denitrification driven by internally stored PHA as carbon source; (ii) establishment of the
90 desired COD/N ratio for the selection of PHA storing biomass by controlling the dosing of
91 the external carbon source and the nitrogen contained in the anaerobic supernatant. A side-
92 stream treatment technology able to integrate the conversion of fermented sludge to PHAs
93 with nutrient removal via nitrite would enable the simultaneous reduction of nutrient loads to
94 the main line of the WWTPs, lowering operational costs, with the recovery of a valuable
95 resource, PHA, providing further economic advantages to the WWTP.

96

97 MATERIAL AND METHODS

98

99 2.1 Experimental set-up

100 Figure 1 shows two alternative configurations applied for nitrogen removal via nitrite and the
101 selection of PHA storing biomass downstream from the anaerobic digester. In configuration 1,
102 the nitrogen removal through nitrification/denitrification and the PHA selection process occurred
103 in a single SBR by applying a feast and famine regime. Specifically, during aerobic
104 conditions, ammonium was oxidized to nitrite, while during the anoxic famine conditions,
105 nitrite was reduced to nitrogen gas using the internally stored PHA as carbon source (Figure
106 1a). In configuration 2, two separate SBRs were used to accomplish the ammonium
107 conversion to nitrite and the selection of the PHA storing biomass (Figure 1b). Nitrification
108 was carried out in a dedicated SBR and then the liquid was fed to the selection reactor while
109 operated under famine conditions, accomplishing the denitrification using the stored PHA. In
110 the selection reactor, the COD/N ratio was adjusted by feeding the anaerobic supernatant as
111 main source of nutrients (nitrogen and phosphorus).

112 The PHA accumulation step of the examined process was performed in a fed-batch reactor to
113 maximize the cellular PHA content of the biomass harvested from the selection stage. The
114 volatile fatty acids (VFAs) that are required for the feast conditions in the selection reactor
115 and the PHA accumulation in the batch reactor were recovered from the acidogenic
116 fermentation of sewage sludge. Fermented liquid from sewage sludge was used as VFA
117 source. The process is described in detail in the work of Longo et al. (2015)²⁰. The technical
118 details of the applied configurations are given below.

119

120 *2.1.1 Configuration 1 (Figure 1a).* An SBR having a working volume of 26 L was used for
121 the selection of PHA storing bacteria. Full details of the SBR are reported in the supporting

122 information (SI). The cycles of the SBR consisted of 5 min of feeding, 300 min of reaction
123 phase (aerobic and anoxic), 15 min of settling and 5 min of discharge. Within the reaction
124 period, the aerobic phase was varied from 50 to 100 min during period 1, and from 100 to
125 150 min during period 2 (Configuration 1). This variation was introduced in order to compare
126 the effect of the availability of nitrite on the process. Two experimental periods were
127 performed using configuration 1 and adopting different COD/N ratios (Table 1). The target
128 COD/N was achieved by maintaining the volumetric organic loading rate (vOLR) stable at
129 674 ± 72 gCOD/m³d and altering the volumetric nitrogen loading rate (vNLR) by adjusting the
130 feeding of the anaerobic supernatant. Table 1 reports the operating conditions adopted during
131 the examined periods. In the first period, the feast conditions were established at the
132 beginning of the aerobic phase by applying a COD/N ratio of 5.6 ± 0.1 gCOD/gN, while in the
133 second period the COD/N was around 2.0 ± 0.02 gCOD/gN. The solids retention time (SRT)
134 was kept within the range of 12-15 days throughout each experimental period.

135 (Table 1)

136 (Figure 1a and 1b)

137

138 *2.1.2 Configuration 2 (Figure 1b)*. In a first step, a nitrification SBR (N-SBR) was applied as a
139 pre-treatment stage in order to enhance the conversion of ammonium to nitrite (nitrification). A
140 detailed description of the operating conditions of the N-SBR are given in the SI. The
141 anaerobic supernatant exiting from the N-SBR reactor contained ammonium in the range of
142 25 to 67 mgN/L, while the average level of nitrite was approximately 350 mgN/L. After
143 settling, the clarified effluent from the N-SBR was temporarily collected in a storage tank (80
144 L of volume) and then was fed to the selection SBR during the first 10-12 minutes of the
145 anoxic phase based on a volumetric nitrogen loading rate of 530 ± 11 gN/m³d, which
146 corresponded to a volumetric nitrite loading rate of 423 ± 95 gNO₂-N/m³d. The loading rate of

147 the carbon source during the feast was balanced with the nitrite loading rate applied during
148 the famine phase based on the ratio $2.2 \pm 0.1 \text{gCOD/gNO}_2\text{-N}$. The operation cycle of the
149 selection SBR consisted of 50 minutes of aerobic phase, followed by 250 minutes of anoxic
150 conditions. The length for the feeding, settling and discharging were respectively 15, 30 and
151 15 minutes. The SRT was kept in the same range as for Configuration 1, 12-15 days.

152

153 **2.2 PHA accumulation**

154 The biomass was collected under famine conditions (end of the anoxic phase from the
155 selection SBR) and was concentrated gravimetrically by applying 30 min of settling. The aim
156 was to reduce the level of nutrients contained in the biomass obtained from the selection SBR.
157 Then, the biomass was placed in triplicate fed-batch glass reactors with working volumes of
158 1 L that were equipped with blowers, diffusers and probes for the measurement of the
159 dissolved oxygen (DO, type WTW, CellOx® 325), the pH (Polyplast Pro) and the
160 temperature (PT100). The on line signals were automatically recorded. The DO level was
161 always maintained above 2 mg/L. The oxygen uptake rate (OUR) was determined using the
162 respirometer MARTINA (SPESS, Italy). The substrate was divided in fixed- volume aliquots
163 in order to dose manually each time approximately 1 gCOD/L of VFAs. New aliquots were
164 dosed when consecutive OUR values were $\sim 50\%$ less than the previously recorded values. In
165 order to test different COD/N/P ratios during the PHA accumulation, three different carbon
166 sources were applied; a synthetic mixture of VFA (COD/N/P 100:0:0), sewage sludge
167 fermentation liquid (SFL) (COD/N/P 100:9.7:2.1) and sewage sludge fermentation liquid
168 with wollastonite (WSFL) (COD/N/P 100:7.8:0.06). In the latter, the use of wollastonite
169 during fermentation improves the $\text{COD}_{\text{VFA}}/\text{NH}_4\text{-N}/\text{PO}_4\text{-N}$ ratio, since it limits the net release
170 of nutrients (ammonia and phosphates). The duration of the accumulation test was 6-8 h.

171

172 2.3 Calculations

173 In this study the VFAs concentration, expressed as mgCOD/L, was calculated as follows:

$$174 \quad \text{VFAs} = \sum (\text{HAc} + \text{HPr} + \text{HBt} + \text{iso-HBt} + \text{HPt} + \text{isoHPt} + \text{HHe} + \text{HHp}) \quad (1)$$

175 where: HAc is acetic, HPr is propionic, HBt is butyric, iso-HBt is iso-butyric, HPt is
176 pentanoic, isoHPt is iso-pentanoic, HHe is hexanoic and HHp is heptaonic acid.

177 The PHA monomeric concentrations were converted into COD units by following the
178 oxidation stoichiometry: 1.38 mgCOD/mg(3-hydroxybutyrate, HB), 1.63 mgCOD/mg(3-
179 hydroxyvalerate, HV) and 1.82 mgCOD/mg(3-hydroxyhexanoate, HH). The total PHA
180 concentration was calculated as follows:

$$181 \quad \text{PHA (mgCOD/L)} = \sum (\text{PHB} + \text{PHV} + \text{PHH}) \quad (2)$$

182 where: PHB is polyhydroxybutyrate; PHV is polyhydroxyvalerate; PHH is
183 polyhydroxyhexanoate.

184

185 The amount of PHA (g/L) was subtracted from the VSS (g/L) to calculate the concentration
186 of the active biomass (X, g/L). The latter was transformed as COD concentration by the
187 stoichiometric ratio of 1.42 gCOD/gVSS.

188 The fraction of the PHA in the biomass was calculated considering the following equation:

$$189 \quad \text{PHA}(\%) = \frac{\text{gPHA}}{\text{gVSS}} \times 100 \quad (3)$$

190 where the gPHA and the VSS were respectively the PHA and the volatile suspended solids of
191 the biomass. The specific VFA uptake rate ($-q_{\text{VFA}}$, mgCOD/gCOD_xh) and the PHA storage
192 rate (q_{PHA} , mgCOD/gCOD_xh), were determined by linear regression analysis by plotting the
193 concentration of VFAs and PHA as a function of time. The results were normalized for the
194 active biomass concentration. The rates were standardized at 20°C of temperature by the
195 Arrhenius equation²¹. The PHA storage yield ($Y_{\text{PHA/VFA}}$, gCOD_{PHA}/gCOD_{VFA}) and the growth

196 yield ($Y_{X/VFA}$, $gCOD_X/gCOD_{VFA}$) were calculated as the ratio between each maximum
197 specific rate (q_{PHA} and q_X , respectively) and the $-q_{VFA}$.

198

199 **2.4 Analytical methods**

200 The concentrations of the mixed liquor suspended solids (MLSS), mixed liquor volatile
201 suspended solids (MLVSS), chemical oxygen demand (COD), soluble chemical oxygen
202 demand (SCOD), total Kjeldahl nitrogen (TKN), ammonium (NH_4-N), phosphorous (TP)
203 were determined according to standard methods²². Nitrite (NO_2-N), nitrate (NO_3-N) and
204 phosphate (PO_4-P) concentration were determined by the ion chromatograph Dionex ICS-900
205 with AS14 as column, while the concentration of HAc, HPr, HBt, iso-HBt, HPt, isoHPt and
206 HHe and heptaoic acid was determined by liquid chromatography through a Dionex ICS-
207 1100 with IonPac ICE-AS1 as column. Samples of biomass were collected from the selection
208 and accumulation reactors and the liquor was removed through centrifugation. Then, the
209 thickened biomass was freeze-dried with a lyophilisation unit (Lio 5P, 5Pascal, Milano, Italy)
210 and analysed for the content of PHA using the method developed by Lanham et al., (2013)²³.
211 The specific ammonium uptake rate (sAUR) and specific nitrogen uptake rate (sNUR) were
212 determined in situ following the procedures that are given in SI. The nitrogen mass balances
213 were calculated for each experimental period (see SI).

214 The final biopolymers produced during the accumulation stage were also extracted from the
215 freeze dried biomass by applying the chloroform method (50 mL/g of freeze dried biomass)
216 followed by the addition of methanol as precipitation agent¹. Then, the biopolymers were
217 analysed through size exclusion chromatography (SEC, Polymer Lab) in order to determine
218 the molecular number (M_n), average molecular weights (M_w) and the polydispersity indices
219 (PDI). The glass transition temperature (T_g), melting temperature (T_m) and melting enthalpy

220 (DHm) were determined by differential scanning calorimetry (DSC, TA Instruments). More
221 information about the procedures are available on SI.

222

223 **RESULTS AND DISCUSSION**

224 **3.1 Efficiency of single stage reactor configuration**

225 The impact of combining the ammonium oxidation to nitrite, denitrification and selection of the
226 PHA storing biomass in a single reactor was evaluated by altering the percentage of the
227 aerobic versus the total cycle duration. The aerobic reaction duration varied from 17 to 30%
228 (Period 1, days 0 -50) and from 30 to 50% (Period 2, days 51 -108) of the total cycle duration.
229 In configuration 1, the main electron acceptor available during the feast conditions was
230 oxygen, while nitrite was the only electron acceptor present during the famine period. If no
231 additional external carbon source is present, an efficient famine condition under the anoxic
232 environment may occur when enough nitrite is available for the complete utilization of PHA.
233 Thus, the stoichiometry of the process imposes that the ratio of PHA stored (as COD) to the
234 nitrite denitrified should not exceed the stoichiometric value of $[1.72/(1-Y_{HD})]$
235 $(\text{gCOD}_{\text{PHA}}/\text{gNO}_2\text{-N})$, where Y_{HD} is the growth yield of the denitrifying bacteria using storage
236 compounds.

237

238 *3.1.1 Period 1.* At the beginning of period 1, the biomass showed a typical feast and famine
239 response (the duration of the feast phase was approximately 16% of the total cycle duration),
240 although the ammonium conversion to nitrite was $36\pm 13\%$, producing only $6.5 \text{ mgNO}_2\text{-N/L}$
241 at the completion of the aerobic phase (Figure 2 a and b). During feast conditions the $-q\text{VFA}$
242 was $96\pm 33 \text{ mgCOD/gX h}$ (Figure 5 and Table 2). Despite the relatively high DO
243 concentration during the aerobic phase, within the first 15 days (0-15 days) the nitrification
244 activity of the biomass seems to be negatively affected by the presence of VFA during the

245 feast phase, since the sAUR decreased from the initial value of 15-18 mgN/gX h (measured
246 in the inoculum) to 4.18 ± 1.93 mgN/gX h. This is likely due to the faster biomass growth rate
247 of VFA consuming heterotrophic organisms as compared to nitrifying autotrophs (Henze et al.
248 2000). As a consequence, the ratio between the PHA stored (COD based) and the nitrite
249 concentration at the beginning of the anoxic phase was 15.5 gCOD/gNO₂-N, which was too
250 high to allow for complete PHA degradation under famine conditions. The lack of nitrite
251 available under anoxic conditions resulted in poor nitrogen removal efficiency for this period
252 (Table 2). Under denitrifying famine conditions, the efficiency of PHA consumption was not
253 more than $41 \pm 1\%$ due to the shortage of nitrite as electron acceptor (Figure 5) and the PHA
254 accumulated in the selection reactor up to 0.21 gCOD_{PHA}/gCOD_X (Figure 2b). Maintaining
255 efficient famine conditions where the stored PHA is consumed has been found to be of high
256 importance in achieving a selected culture with a high PHA storage capacity²⁴.

257 During the first period, the $-q_{VFA}$ decreased down to 4 mgCOD/gX h (day 28, Figure 5) and
258 the $Y_{PHA/VFA}$ from 0.31 to 0.07gCOD/gCOD_{VFA} (Figure 5). To promote the growth of the
259 PHA storing bacteria during the anoxic/famine conditions, the ammonium conversion to
260 nitrite was enhanced by doubling the duration of the aerobic phase, while maintaining
261 constant the applied vNLR and the vOLR (days 29 to 50). The amount of nitrite increased up
262 to 26 mgNO₂-N/L and the nitrogen removal efficiency was 70.1%. The higher duration of the
263 aerobic phase favoured the degradation of the PHA up to 66% (Figure 5, day 39) under
264 famine conditions, due to the more abundant presence of electron acceptors. However, it was
265 found that ~30% of the previously stored PHA was consumed under aerobic/famine
266 conditions before the anoxic-famine period initiated. The increase in the aerobic period
267 resulted in an aeration time that exceeded the feast phase duration for the applied vOLR,
268 leading to some aerobic PHA degradation. The PHA content at the end of the anoxic famine
269 conditions was constantly below 0.01 gCOD/gCOD. Furthermore, at the end of period 1

270 (days 29-50), the $-q_{\text{VFA}}$ increased together with the $Y_{\text{PHA/VFA}}$ up to 108 ± 18 mgCOD/gX h
271 and 230 ± 150 mgCOD_{PHA}/gCOD_{VFA} (Figure 5), respectively.

272

273 *3.1.2 Period 2.* Once the feast and famine cycle and the nitrogen removal efficiency showed a
274 steady profile, the $v\text{NLR}$ was increased to 0.38 ± 0.03 kgN/m³d in order to enhance the
275 treatment capacity of the system, while maintaining constant the $v\text{OLR}$. The duration of the
276 aerobic reaction phase was slightly altered during this period in order to ensure sufficient
277 conversion of ammonium to nitrite and to control the $\text{COD}_{\text{PHA}}/\text{NO}_2\text{-N}$ ratio between 2.0 and
278 2.2 gCOD/gNO₂-N when the anoxic/famine condition took place. More specifically, aerobic
279 conditions were maintained for $45 \pm 12\%$ of the total cycle duration. The $s\text{AUR}$ slightly
280 increased up to 5.5 ± 0.5 mgN/gX h and within 100-150 min of aerobic conditions, the nitrite
281 concentration was 32 mgN/L. **As result, a stable feast-famine regime was established during**

282 days 51-108 (Figure 3 a and b). Under aerobic conditions, the average $-q_{\text{VFA}}$ was 136 ± 29
283 mgCOD/gX h (Figure 5), resulting in the decrease of the ratio of the feast to the total cycle
284 length from 18-19% (period 1) to 14-15% (period 2). The higher capacity of the biomass to
285 store PHA was confirmed by the $Y_{\text{PHA/VFA}}$, which increased from 230 ± 15 (period 1) to
286 371 ± 15 (period 2) mgCOD_{PHA}/gCOD_{VFA} (Figure 5). Furthermore, $72 \pm 16\%$ of the PHA was
287 degraded during the aerobic and anoxic famine conditions (Figure 5). Although the
288 denitrification efficiency of the denitrificable nitrogen was always higher than 94%, the
289 average nitrogen removal in the same period was low ($48.8 \pm 3.4\%$, average value, see SI)
290 since a significant residual ammonium concentration remained. Thus, an alternate process
291 configuration (Configuration 2) was studied in order to increase nitrogen removal and
292 augment PHA production further.

293 (Figure 2a and 2b)

294 (Figure 3a and 3b)

295 (Figure 4a and 4b)

296 (Figure 5)

297

298 3.2 Efficiency of the configuration with two separate reactors

299 To cope with the residual ammonium concentration, configuration 2 applied a two stage
300 process for nitrification and selection of PHA storing biomass. Ammonium was first oxidized
301 in the N-SBR (Figure 1b) and then the liquid was fed in the selection reactor when the anoxic
302 reaction phase started. Figure 4 (a and b) shows the typical profiles of nitrogen, PHA and
303 VFA concentration that were obtained in the selection SBR after adopting configuration 2
304 (day 108 to 150). The VFA were completely depleted within the first 30 min of the aerobic
305 phase, resulting in a $-q_{\text{VFA}}$ of $182 \pm 14 \text{ mgCOD/gX h}$ (Figure 5). Under feast conditions, the
306 PHA of the biomass rapidly increased at a q_{PHA} of $89 \pm 7 \text{ mgCOD/gX h}$, which is higher
307 compared to the respective value of configuration 1. The $Y_{\text{PHA/VFA}}$ increased as well, from
308 371 ± 15 to $433 \pm 10 \text{ mgCOD}_{\text{PHA}}/\text{gCOD}_{\text{VFA}}$ (Figure 5). After 50 min of aerobic phase, the
309 anoxic-famine phase started by switching off the blower and feeding the effluent of the N-
310 SBR. In configuration 2, nitrite was the only electron acceptor for the PHA degradation for
311 denitrification during the famine conditions. Furthermore, this strategy allowed a better control
312 of the applied $\text{COD}/\text{NO}_2\text{-N}$, avoiding nitrite limitation in the famine phase and enabling
313 higher nitrogen removal efficiency (Table 2). Figure 4(a) shows the increase of the nitrite
314 content during the anoxic phase (up to $75 \text{ mgNO}_2\text{-N/L}$). During the famine conditions, the
315 PHA was consumed, reaching a minimum concentration of $0.006 \text{ gCOD}_{\text{PHA}}/\text{gCOD}$ at the end
316 of the anoxic cycle. This fact indicates that almost all the stored PHAs were degraded. The
317 PHA consumption efficiency was indeed higher when compared with that of configuration 1
318 ($83 \pm 4\%$, Figure 5). Additionally, during the anoxic phase, the ammonium nitrogen decreased
319 from 28 to 21 mgN/L ; this reduction was correlated with the growth of the PHA storing

320 bacteria. The rate of PHA degradation during the famine phase is independent of the type of
321 electron acceptor present¹⁶. The famine (anoxic) duration and the carbon stored were enough
322 to achieve almost 90% of denitrification. The nitrite at the end of the cycle decreased to 15
323 mgNO₂-N/L. However, the nitrite concentration in the effluent was 7-10 mgNO₂-N/L,
324 indicating that denitrification occurred during the sedimentation but without any rising of
325 sludge. This was confirmed by the low content of solids in the effluent (<15 mg/L).

326 Configuration 2 is advantageous compared to configuration 1, since it enhances the overall
327 nitrogen removal efficiency up to 79±4.4%, when applying a vNLR of 0.53±0.11 kgN/m³d.
328 Furthermore, the PHA production rate and yield per VFA were enhanced. Coupling side-
329 stream nitrification/denitrification processes with PHA production through mixed microbial
330 cultures has the potential to be economically advantageous, since recovery of a valuable
331 resource can be incorporated into the wastewater treatment plant, while reducing nutrient
332 loads to the head of the plant, reducing operational costs in the mainstream. Furthermore, the
333 implementation of anoxic conditions during the famine phase of PHA production systems by
334 mixed cultures is a useful means of saving aeration energy.

335 (Table 2)

336

337 3.3. PHA accumulation

338 The PHA storage capacity gradually increased during the overall experimental period, as a
339 confirmation of a good acclimation response of the selected biomass. In this work, the
340 maximal capacity of biomass to store PHA was examined under the operation of
341 configuration 2, when better PHA productivity was obtained. After 8 hours, the biomass was
342 able to accumulate up to 19±2%, 21±5% and 41±4% (gPHA/gVSS x100) when sewage
343 sludge fermentation liquid (COD:N:P = 100:9.7:2.1, no N or P limitation), primary sludge
344 fermentation liquid with wollastonite (COD:N:P = 100:7.8:0.06, P- limited), and a synthetic

345 mixture of VFA (COD:N:P = 100:0:0, N and P-limited) were fed, respectively (Table 3). The
346 results were in the same range with other authors which used fermentation liquid from
347 sewage sludge¹⁴. The carbon source was also used as substrate for biomass growth. Despite
348 the fact that the synthetic mixture of VFAs presented a favourable ratio of COD:N:P to limit
349 the growth of new bacteria, the yield of active biomass per substrate consumed varied
350 between 0.20 and 0.28 gCOD/gCOD. This was due to the nitrogen and phosphorus present in
351 the liquor + biomass withdrawn from the selection reactor for the accumulation tests,
352 lowering the COD driven for PHA production. Limiting further the presence of nutrients
353 during the accumulation step would be of interest for process optimisation purposes in future
354 research in order to maximise PHA productivity.

355 The biopolymers that were produced with the WSFL and SFL had similar characteristics in
356 terms of 3HV and 3HB percentage (Table 3). With the use of the synthetic mixture of VFAs
357 as carbon source, the biopolymer was composed of 35% HB and 65% of HV and HH. This
358 correlates well with the VFA profile added during each test, where the synthetic VFA
359 mixture contained a higher fraction of HV precursors (Table 3).

360 (Table 3)

361 Characterisation of the recovered PHA supports their applicability for thermoplastic
362 processing. The analyses revealed that the biopolymers were composed of long molecular
363 chains, with a similar molecular weight varying between 6×10^5 and 8×10^5 g/mol and also a
364 similar chain length distribution (PDI 1.22-1.35). In general, the low crystallinity in
365 combination with a low Tg (between -1.1 to -0.5°C) indicate biopolymers with amorphous
366 characteristics¹⁴. Overall, the biopolymer characteristics observed were in the same range as
367 observed in other studies^{14,24}.

368 (Table 4)

369

370 **3.4 Aeration demand of the novel process and future perspectives**

371 The experimental results acquired in this study were used to estimate the potential aeration
372 savings (thus, decrease in energy costs) when the process for PHA production is integrated in
373 the side stream nitrogen removal via nitrite from the anaerobic supernatant. The calculations
374 are reported in detail in the SI (Table S5). The production of 1 kg of PHA with 60% HB and
375 40% HV, required the equivalent of 3.7 kgCOD of VFA, which are partially oxidized (1.3
376 kgCOD) and partially used to grow new bacterial cells (0.9 kgCOD, $Y_{X/VFA}=0.25$
377 kgCOD/kgCOD) during the accumulation stage. The amount of oxygen needed during the
378 accumulation can be estimated to be 1.7 kgO₂ considering that 1.3 kgO₂ is required for every
379 kg of COD that is oxidized²⁵. The percentage of PHA in the biomass could achieve 35%
380 (gPHA/gVSS), thus 2.6 kg of active biomass should be produced during the feast/famine
381 stage by providing 10.2 kg of COD (VFA). The oxygen required for the selection stage is the
382 oxygen provided only during the aerobic feast phase, which represents 20% of the reaction
383 phase duration of the SBR. The oxygen estimated during the aerobic feast phase is 4.6 kg of
384 O₂, meaning that the overall process required 6.4 kgO₂ for 1 kg of PHA. Compared with the
385 conventional aerobic PHA production (selection and accumulation phase), the aerobic and
386 anoxic feast/famine selection plus aerobic accumulation process could save approximately
387 58% of the oxygen requirement.

388 In the current work, the biological nitrogen removal via nitrite was integrated with the
389 selection of PHA storing biomass in the sludge treatment line. The integration of PHA
390 production within a WWTP at full scale was the driving force for the development of our
391 novel treatment scheme. A twofold objective is achieved in the novel process that is
392 proposed; enhanced selection of PHA stored biomass and wastewater and reject water
393 treatment for nitrogen removal. Thus, the examined process provides true added value
394 towards the effective treatment of nitrogen in highly contaminated effluents within WWTPs,

395 aiming at the same time to maximize resource recovery through the polymer production,
396 which could enhance the sustainability of the WWTP.

397

398 **ACKNOWLEDGMENT**

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401

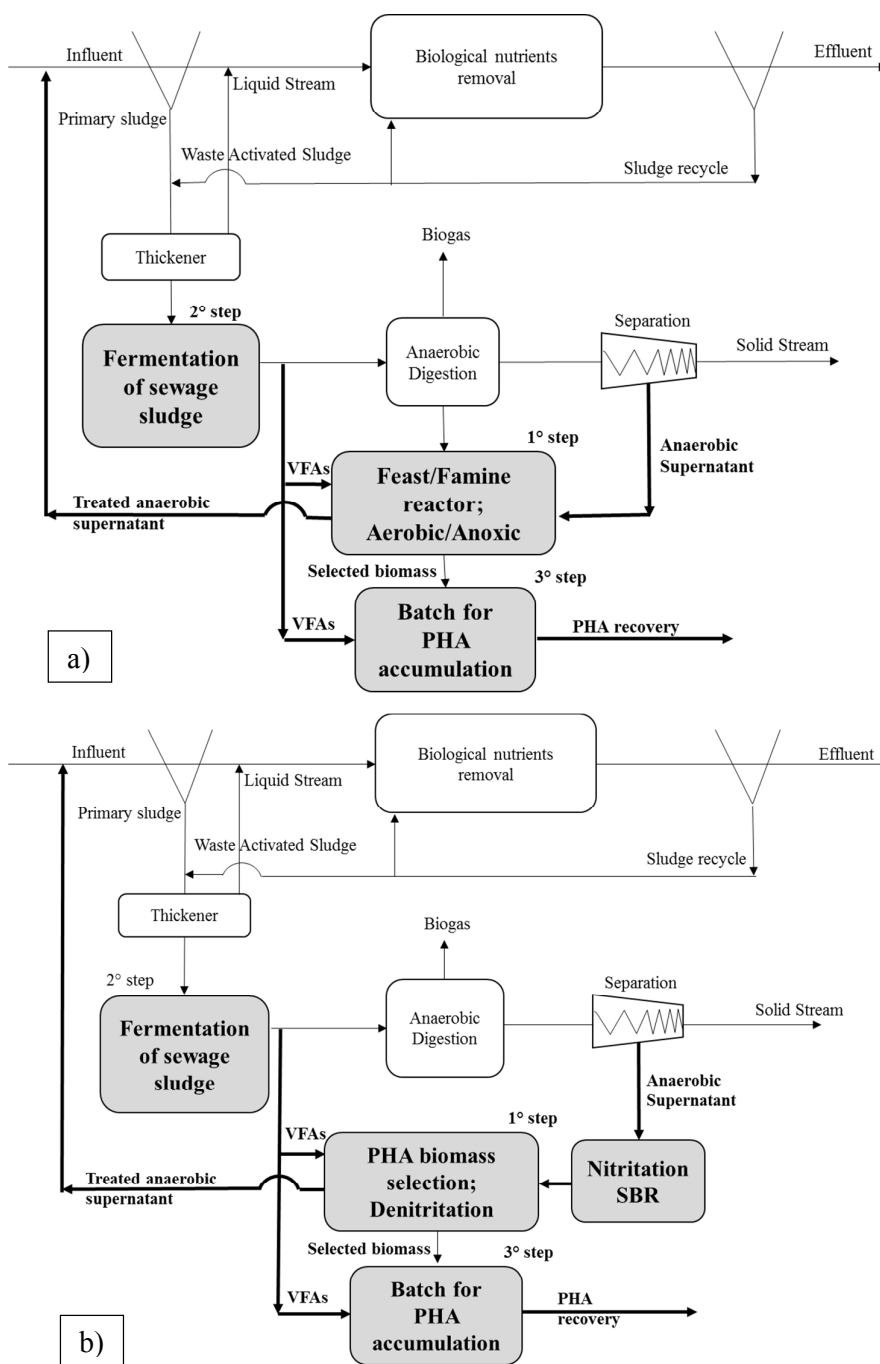
402 **4 References**

- 403 1. Madkour, M. H.; Heinrich, D.; Alghamdi, M. A.; Shabbaj, I. I.; Steinbüchel, A., PHA
404 Recovery from Biomass. *Biomacromolecules* **2013**, 14, 2963–2972.
- 405 2. Yu, J.; Chen, L. X. L., The Greenhouse Gas Emissions and Fossil Energy
406 Requirement of Bioplastics from Cradle to Gate of a Biomass Refinery. *Environ. Sci.*
407 *Technol.* **2008**, 42, 6961–6966.
- 408 3. Bengtsson, S.; Pisco, A. R.; Johansson, P.; Lemos, P. C.; Reis, M. A. M., Molecular
409 weight and thermal properties of polyhydroxyalkanoates produced from fermented sugar
410 molasses by open mixed cultures. *J. Biotechnol.* **2010**, 147 (3), 172-179.
- 411 4. Dionisi, D.; Caruccia, G.; Petrangeli Papinia, M.; Riccardi, C.; Majone, M.; Carrasco,
412 F., Olive oil mill effluents as a feedstock for production of biodegradable polymers. *Water*
413 *Res.* **2005**, 39, 2076–2084.
- 414 5. Yuan, H.; Chen, Y.; Zhang, H.; Jiang, S.; Zhou, Qi.; Gu, G. Improved Bioproduction
415 of Short-Chain Fatty Acids (SCFAs) from Excess Sludge under Alkaline Conditions. *Environ.*
416 *Sci. Technol.*, 2006, 40 (6), pp 2025–2029.
- 417 6. Braunegg, G.; Bona, R.; Koller, M., Sustainable polymer production. *Polymer-*
418 *Plastics Technol. Eng.* **2004**, 43 (6), 1779–1793.
- 419 7. Research Watch. *Environ. Sci. Technol.* **1996**, 30 (9), 1996.

- 420 8. Tamis, J.; Marang, L.; Jiang, Y.; van Loosdrecht, M. C. M.; Kleerebezem, R.,
421 Modeling PHA-producing microbial enrichment cultures—towards a generalized model with
422 predictive power. *New Biotechnol.* 2014, 31 (4).
- 423 9. Dionisi, D.; Majone, M.; Tandoi, V.; Beccari, M., Sequencing batch reactor: influence
424 of periodic operation on performance of activated sludge in biological wastewater treatment.
425 *Ind. Eng. Chem. Res.* **2001**, 40, 5110–5119.
- 426 10. Johnson, K.; Kleerebezem, R.; van Loosdrecht, M. C. M., Influence of the C/N ratio
427 on the performance of polyhydroxybutyrate (PHB) producing sequencing batch reactors at
428 short SRTs. *Water Res.* **2010**, 44, 2141–2152.
- 429 11. Meesters, K. H. P. *Production of poly (3 hydroxyalkanoates) from waste streams*;
430 Technical University of Delft: Delft, **1998**.
- 431 12. Ju, J.; Si, Y. A Dynamic Study and Modeling of the Formation of
432 Polyhydroxyalkanoates Combined with Treatment of High Strength Wastewater. *Environ. Sci.*
433 *Technol.* **2001**, 35, 3584-3588.
- 434 13. Jiang, Y.; Chen, Y.; Zheng, X., Efficient polyhydroxyalkanoates production from a
435 waste-activated sludge alkaline fermentation liquid by activated sludge submitted to the
436 aerobic feeding and discharge process. *Environ. Sci. Technol.* 2009, 43, 7734-7741.
- 437 14. Morgan-Sagastume, F.; Karlsson, A.; Johansson, P.; Pratt, S.; Boon, N.; Lant, P.;
438 Werker, A., Production of polyhydroxyalkanoates in open, mixed cultures from a waste
439 sludge stream containing high levels of soluble organics, nitrogen and phosphorus. *Water Res.*
440 **2010**, 5196-5211.
- 441 15. Pittmann, T., Steinmetz, H. Polyhydroxyalkanoate production as a side stream process
442 on a municipal waste water treatment plant. *Biores. Technol.* **2014**, 167, 297–302
- 443 16. Morgan-Sagastume, F.; Valentino, F.; Hjort, M.; Cirne, D.; Karabegovic, L.; Gerardin,
444 F.; Johansson, P.; Karlsson, A.; Magnusson, P.; Alexandersson, T.; Bengtsson, S.; Majone,

- 445 M.; Werker, A., Polyhydroxyalkanoate (PHA) production from sludge and municipal
446 wastewater treatment. *Water Sci. Technol.* 2014, 69, 177-184.
- 447 17. Anterrieu, S., Quadri, L., Geurkink, B., Dinkla, I., Bengtsson, S., Arcos-Hernandez,
448 M., Alexandersson, T., Morgan-Sagastume, F., Karlsson, A., Hjort, M., Karabegovic, L.,
449 Magnusson, P., Johansson, P., Christensson, M., Werker A. Integration of biopolymer
450 production with process water treatment at a sugar factory. *N Biotechnol.* **2014**, 31(4), 308-
451 323; DOI: 10.1016/j.nbt.2013.11.008.
- 452 18. Cervantes, F.J. *Environmental Technologies to Treat Nitrogen Pollution*. IWA
453 Publishing, London, 2009.
- 454 19. Frison, N.; Katsou, E.; Malamis, S.; Oehmen, A.; Fatone, F., Nutrient removal via
455 nitrite from reject water and polyhydroxyalkanoate (PHA) storage during nitrifying
456 conditions. *J. Chem. Technol. Biotechnol.* **2014**; DOI 10.1002/jctb.4487
- 457 20. Longo, S., Katsou, E., Malamis, S., Frison, N., Renzi, D., Fatone, F. Recovery of
458 volatile fatty acids from fermentation of sewage sludge in municipal wastewater treatment
459 plants. *Biores. Technol.* **2015**, 175, 436–444.
- 460 21. Carrera, J., Vicent, T., Lafuente, F.J. Influence of temperature on denitrification of an
461 industrial high-strength nitrogen wastewater in a two-sludge system. *Wat. SA.* **2003**, 29, 11–
462 16.
- 463 22. APHA, Standard Methods for the Examination of Water and Wastewater, 20th ed.;
464 American Public Health Association: Washington, DC, 1998.
- 465 23. Lanham, A.B., Ricardo, A.R., Albuquerque, M.G.E., Pardelha, F, Carvalheira, M.,
466 Coma, M., Fradinho, J., Carvalho, G., Oehmen, A., Reis, M.A.M. Determination of the
467 extraction kinetics for the quantification of polyhydroxyalkanoate monomers in mixed
468 microbial systems. *Proc. Bioch.* **2013**, 48, 1626–1634.

- 469 24. Albuquerque, M.G.E., Torres, C.A.V., Reis, M.A.M., Polyhydroxyalkanoate (PHA)
470 production by a mixed microbial culture using sugar molasses: Effect of the influent substrate
471 concentration on culture selection. *Wat. Res.* **2010**, 44, 3419-343318.
- 472 25. Gurieff, N.; Lant, P., Comparative life cycle assessment and financial analyses of
473 mixed culture polyhydroxyalkanoate production. *Bioresour. Technol.* **2007**, 3393-3403.
- 474
- 475

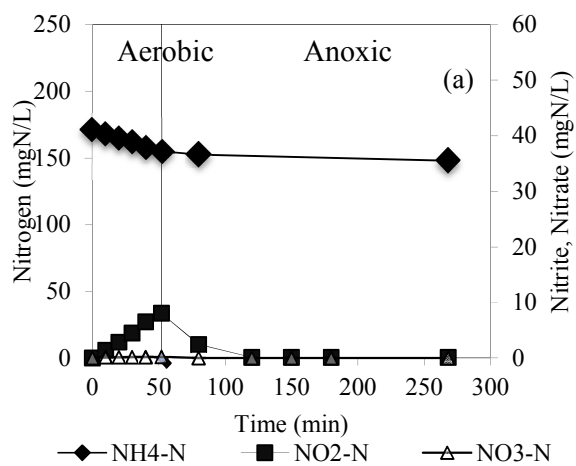


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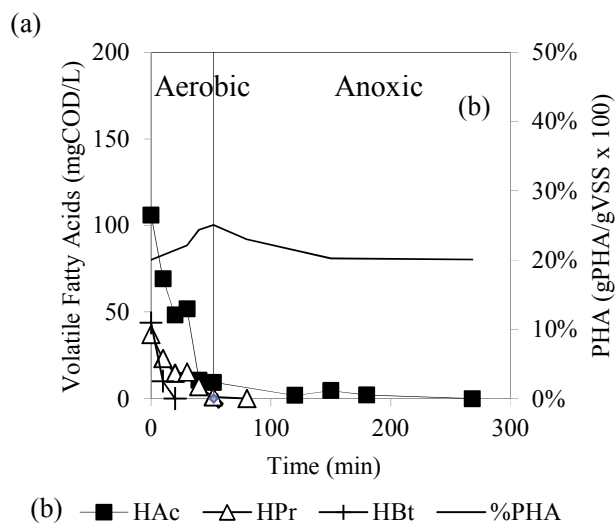
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478 **Figure 1.** Configurations for the combined selection of PHA storing biomass and nitrogen
 479 removal from sludge reject water by applying (a) the aerobic/anoxic feast/famine regime and
 480 nitrogen removal via nitrite in a single stage reactor and (b) a two-stage process of nitritation
 481 (first reactor for the production of electron acceptor) followed by an aerobic/anoxic
 482 feast/famine selection reactor.

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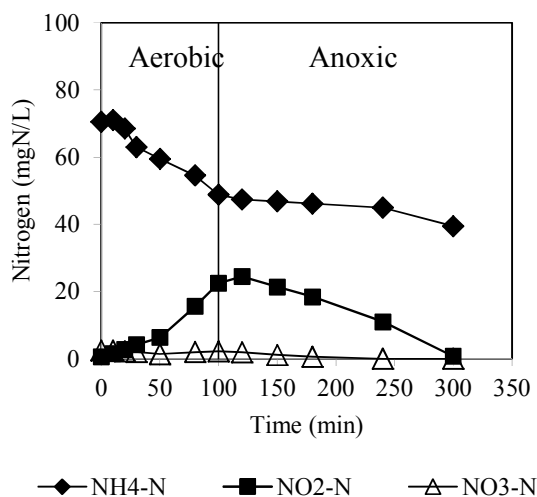
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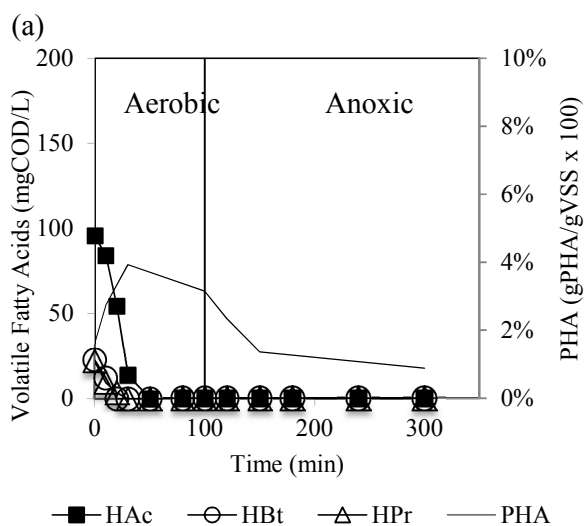
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Figure 2. Typical profiles of (a) nitrogen and (b) VFAs and PHA profile during the feast and famine phase of the SBR for period 1 of configuration 1.



489



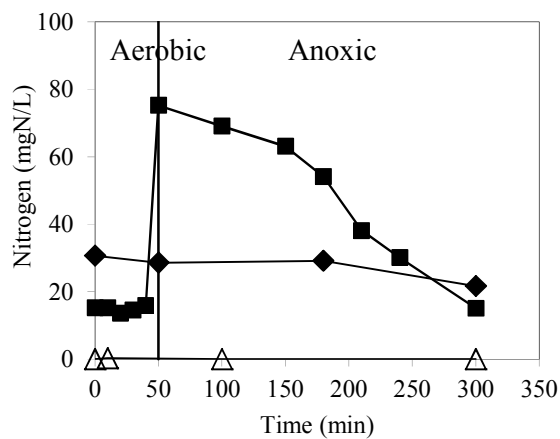
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(b)

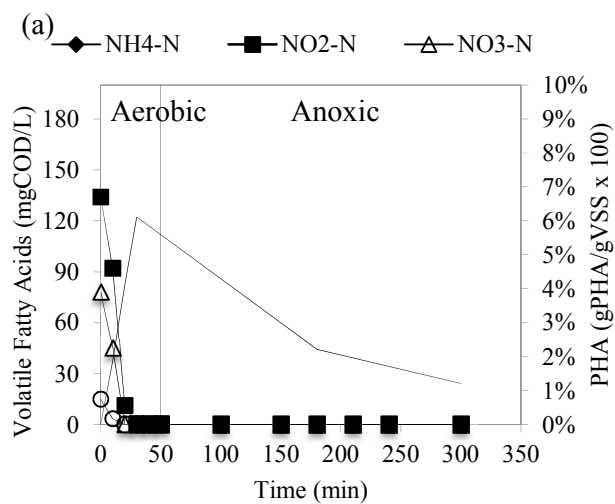
491 **Figure 3.** Typical profiles of (a) nitrogen and (b) VFAs and PHA during the feast and

492 famine phase in the selection reactor during period 2 of configuration 1.

493



494



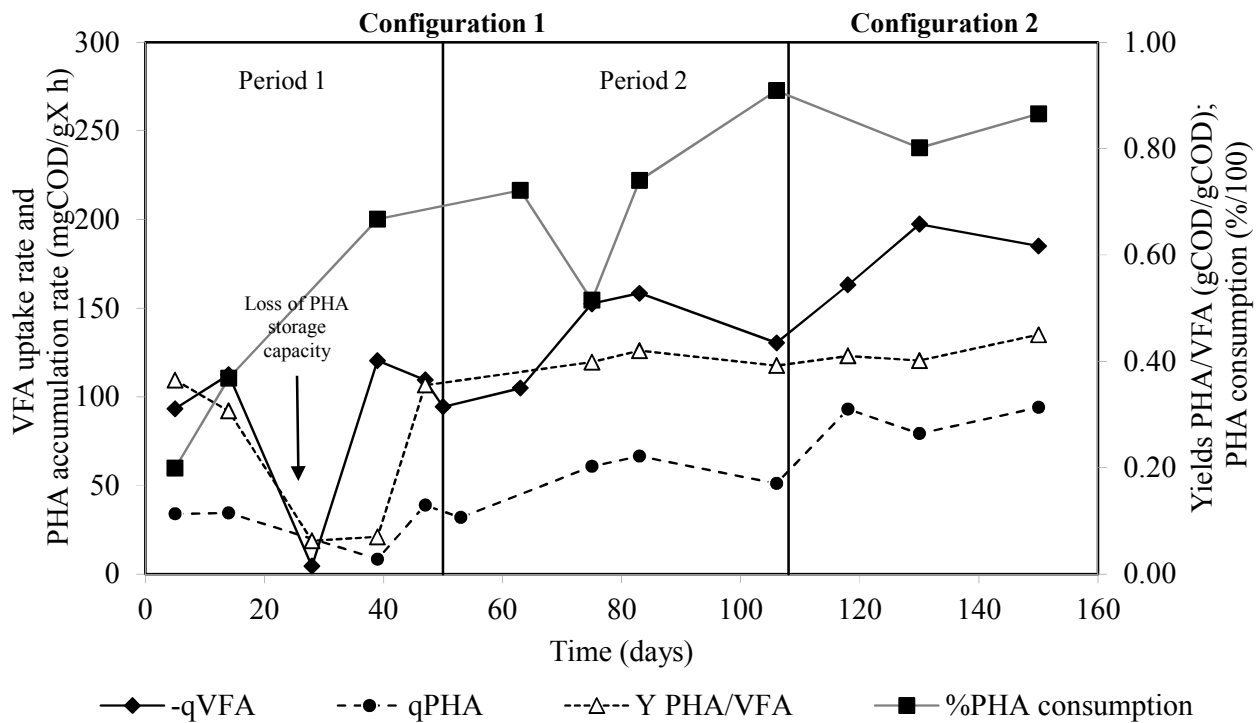
(b)

HAc
 HPr
 HBt
 — PHA

495

496 **Figure 4.** Typical profiles of (a) nitrogen and (b) VFAs and PHA during a cycle of the
 497 selection SBR in configuration 2.

498



499

500 **Figure 5.** $-qVFA$, $qPHA$, $Y_{PHA/VFA}$ and efficiency of PHA consumption during the overall

501 experimental periods of configurations 1 and 2

502

503

504 **Table 1.** Operating conditions for the ‘selection’ SBR that was applied for the examined
 505 configurations.

Parameter	Configuration 1		Configuration 2
	Period 1 (days 0-50)	Period 2 (days 51-108)	Period 3(days 109-138)
vNLR (gN/m ³ d)	110±27	380±29	530±11
OLR (gCOD/m ³ d)	623±55	760±43	1166±106
COD/N (gCOD _{VFA} :gNH ₄ -N)	5.6±0.06	2.0±0.02	2.2±0.1
F/M (gCOD:gX)	0.33±0.08	0.22±0.05	0.20±0.07
Aerobic reaction time/Anoxic reaction time	0.30±0.14	0.73±0.37	0.20

506

507

508 **Table 2.** Performance of configuration 1 & 2 for PHA biomass enrichment; -qVFA: VFA
 509 uptake rate, qPHA, PHA accumulation rate, Y_{PHA/VFA}, storage PHA yield based on the VFA.
 510 (*E_{nn}: nitrifying efficiency upon the nitrifiable incoming nitrogen;**E_{dd}: the denitrifying
 511 nitrogen efficiency upon the denitrifiable nitrogen).

Performance	Configuration 1		Configuration 2
	Period 1	Period 2	
Enn(%)*	39	49	87
Edd(%)**	94	97	89
-qVFA(mgCOD/gX h)	96±33	136±29	182±14
qPHA(mgCOD/gX h)	36±0.2	53±9	89±7
Y _{PHA/VFA} (mgCOD _{PHA} /gCOD _{VFA})	230±15	371±15	437±40

512

513

514 **Table 3.** Performance of the batch PHA accumulation using different types of carbon source.

Parameter	Synthetic mixture of VFA	WSFL	SFL
Duration of accumulation	8.5	8.5	8.5
COD(VFA):NH ₄ -N:PO ₄ -P	100:0:0	100:7.8:0.06	100:9.7:2.1
Initial/Final* NH ₄ -N (mgN/L)	35.7/27.2	20.1/185.5	35.2/146.5
Initial/Final* PO ₄ -P (mgP/L)	12.5/8.4	11.6/8.1	25.4/45.3
%PHAs (gPHA/gVSS x 100)	44±5%	21±2%	19±2%
HAc/HPr (gCOD/gCOD)	1.4	1.1	1.1
HV (%)	65 (HV+HH)	41	42
Y _{PHA/VFA} (gCOD/gCOD)	0.46±0.06	0.40±0.04	0.40±0.04
Y _{X/VFA} (gCOD/gCOD)	0.26±0.02	0.25±0.09	0.23±0.06

515

516 **Table 4.** Main properties of the biopolymers obtained with the different carbon sources after

517 the accumulation tests.

Carbon source	Mw (g/mol)	PDI (Mw/Mn)	T _g (°C)	T _{m1} (°C)	T _{m2} (°C)	ΔH _m (J/g)	T _{d-trans} (°C)
Synthetic mixture of VFA	6.2x10 ⁵	1.30	-1.1	138	147	21	267
SFL	6.5x10 ⁵	1.29	-0.5	136	144	24	275
WSFL	7.4x10 ⁵	1.25	-1.6	141	153	27	276

518 Mw: average molecular weight, PDI: polydispersity index, Mn: molar number, T_{d-trans}:

519 decomposition temperature (DSC analyses), T_g: glass-transition temperature, T_m: melting

520 temperature, ΔH_m: melting enthalpy

521

522