



Short-sludge age EBPR process – Microbial and biochemical process characterisation during reactor start-up and operation

Valverde Pérez, Borja; Wágner, Dorottya Sarolta; Lóránt, Bálint ; Gülay, Arda; Smets, Barth F.; Plósz, Benedek G.

Published in:
Water Research

Link to article, DOI:
[10.1016/j.watres.2016.08.026](https://doi.org/10.1016/j.watres.2016.08.026)

Publication date:
2016

Document Version
Peer reviewed version

[Link back to DTU Orbit](#)

Citation (APA):
Valverde Pérez, B., Wágner, D. S., Lóránt, B., Gülay, A., Smets, B. F., & Plósz, B. G. (2016). Short-sludge age EBPR process – Microbial and biochemical process characterisation during reactor start-up and operation. *Water Research*, 104, 320-329. <https://doi.org/10.1016/j.watres.2016.08.026>

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

1 Short-sludge age EBPR process – microbial and biochem-
2 ical process characterisation during reactor start-up and
3 operation

4 Borja Valverde-Pérez*, Dorottya S. Wágner, Bálint Lóránt, Arda Gülay, Barth F.
5 Smets, Benedek Gy. Plósz*

6 Department of Environmental Engineering (DTU Environment), Technical University of Denmark, Miljøvej, Building 115, DK-
7 2800, Kgs. Lyngby, Denmark. E-mails: bvape@env.dtu.dk; beep@env.dtu.dk

8 *Corresponding authors

9

10

11 **Abstract**

12 The new paradigm for used water treatment suggests the use of short solid reten-
13 tion times (SRT) to minimize organic substrate mineralization and to maximize resource
14 recovery. However, little is known about the microbes and the underlying biogeochemi-
15 cal mechanisms driving these short-SRT systems. In this paper, we report the start-up
16 and operation of a short-SRT enhanced biological phosphorus removal (EBPR) system
17 operated as a sequencing batch reactor (SBR) fed with preclarified municipal
18 wastewater, which is supplemented with propionate. The microbial community was ana-
19 lysed via 16S rRNA amplicon sequencing. During start-up (SRT=8 d), the EBPR was

20 removing up to 99% of the influent phosphate and completely oxidized the incoming
21 ammonia. Furthermore, the sludge showed excellent settling properties. However, once
22 the SRT was shifted to 3.5 days nitrification was inhibited and bacteria of the *Thiothrix*
23 taxon proliferated in the reactor, thereby leading to filamentous bulking (sludge volume
24 index up to SVI=1100 mL/g). Phosphorus removal deteriorated during this period, like-
25 ly due to the out-competition of polyphosphate accumulating organisms (PAO) by sul-
26 phate reducing bacteria (SRB). Subsequently, SRB activity was suppressed by reducing
27 the anaerobic SRT from 1.2 day to 0.68 day, with a consequent rapid SVI decrease to
28 ~200ml/g. The short-SRT EBPR effectively removed phosphate and nitrification was
29 mitigated at SRT=3 days and oxygen levels ranging from 2 to 3 mg/L.

30

31 Keywords

32 Enhanced biological phosphorus removal; microbial diversity; resource recovery; short
33 solid retention time activated sludge systems; sulphate reducers.

34 1 Introduction

35 The conventional activated sludge (CAS) process has been used for more than 100 years
36 for wastewater treatment (Schneider 2014). The process relies on a microbial communi-
37 ty formed mainly by bacteria able to remove organic carbon, nitrogen and phosphorus
38 from sewage. Current research, however, proposes a new approach whereby wastewater
39 components are recovered rather than destroyed (Verstraete et al., 2009). This new par-

40 adigm suggests considering the wastewater treatment plant (WWTP) as a biorefinery,
41 whereby nutrients, energy, water, minerals or biopolymers can be separated and recov-
42 ered (Sheik et al., 2014).

43 Anaerobic digestion plays an important role in energy recovery from wastewater. One
44 option is to directly digest wastewaters with high organic carbon content (Shoener et
45 al., 2014). However, this alternative suffers from the loss of dissolved methane in the
46 effluent, which can be stripped to the atmosphere, thus contributing to climate change
47 (Verstraete et al., 2009). As an alternative, CAS can be combined with anaerobic diges-
48 tion, whereby the excess biomass wasted via solid retention time (SRT) control is an-
49 aerobically digested for biogas production. Operating CAS at long SRTs would lead to
50 the emission of large amounts of carbon dioxide in the aeration basins, thereby decreas-
51 ing the amount of organic carbon conveyed to the digester (Batstone et al., 2015). Short
52 SRT systems (i.e. $SRT < 4$ days) have been proposed as a means to promote carbon as-
53 similation (i.e. microbial growth) and accumulation as stored polymers rather than oxi-
54 dation (Jimenez et al., 2015). The effluent of these systems are rich in nitrogen and
55 phosphorus, which can be recovered through physicochemical processes (e.g. ammonia
56 stripping or struvite precipitation, Verstraete et al., 2009) or via biological assimilation
57 (e.g. green microalgae cultivation, Shilton et al., 2012).

58 Enhanced biological phosphorus removal (EBPR) systems operated at short SRT can
59 integrate phosphorus upconcentration with energy recovery (Ge et al., 2013 and 2015).
60 Growth of polyphosphate accumulating organisms (PAO) in EBPR systems is promoted
61 by circulating the mixed liquor through a sequence of anaerobic and aerobic environ-

62 ments (Mino et al., 1998). PAO release stored polyphosphate under anaerobic environ-
63 ments in the presence of bioavailable organic carbon, preferably volatile fatty acids
64 (VFA), which PAO take up and store as polyhydroxyalkanoates (PHA). PAO then use
65 the stored PHA under aerobic conditions as a carbon source for growth while accumu-
66 lating bioavailable phosphate in excess of their metabolic needs. Importantly, EBPR
67 systems can be used to create phosphorus rich effluent streams, optimal for P-recovery,
68 following two different strategies: either directly by partial diversion of the effluent
69 from the anaerobic reactor after phosphorus release (e.g. Barat and van Loosdrecht,
70 2006) or indirectly as a result of phosphorus release induced by anaerobic digestion of
71 EBPR sludge (Yuan et al., 2012).

72 EBPR systems are a mature technology widely studied both in laboratory- and full-scale
73 systems (Oehmen et al., 2007). The microbial communities in EBPR systems are also
74 very well-known as most of the full scale plants have a similar microbial diversity with
75 only the abundance of bacterial groups appearing to be plant specific (e.g. Nielsen et
76 al., 2010; Albertsen et al., 2012; Mielczarek et al., 2013). However, most of these stud-
77 ies are based on long SRT system observations, where nitrification also occurs. Only a
78 few studies have focused on short-SRT EBPR systems (Mamais and Jenkis, 1992;
79 Brdjanovic et al., 1998; Ge et al., 2015; Valverde-Pérez et al., 2015 and 2016). Short-
80 SRT activated sludge systems, i.e. A-stage systems, have different communities than
81 those reported for CAS (González-Martínez et al., 2016; Meerburg et al., 2016). Whilst
82 the main microbial groups and factors that affect their abundance in A-stage systems
83 have been widely reported, we lack the same information for short-SRT EBPR systems.

84 Only Ge et al. (2015) identified a novel PAO organism, belonging to the *Comamonada*
85 *daceae* family, which became dominant and drove P-removal in a short-SRT EBPR
86 treating abattoir wastewater. As a consequence, the available models, both ecological
87 and mathematical, may lack relevant bacterial groups to properly describe short-SRT
88 EBPR systems. Therefore, the main objectives of the present study are i) to describe the
89 start-up and operation of a short-SRT EBPR system ; ii) to assess the microbial com-
90 munity dynamics and identify operation strategies promoting effective process perfor-
91 mance; iii) to identify the limitations of available biochemical process models and pro-
92 pose potential extensions to model the short-SRT EBPR process.

93 2 Materials and Methods

94 2.1 Reactor description

95 The EBPR system was a sequenced batch reactor (SBR) with 8 L volume, operated at
96 hydraulic retention time (HRT) of 18 h and SRTs of 8, 3.5 and 3 days (operational con-
97 ditions are summarized in Table 1). The initial operation sequence was 2 hours of an-
98 aerobic phase, 3 hours of aerobic phase and 1 hour of settling and idle phase. The reac-
99 tor was fed during the first 2 minutes of the anaerobic phase. The SBR was fed with
100 pre-clarified wastewater from Lundtofte WWTP (Kgs. Lyngby, DK) and spiked with
101 synthetic wastewater supplemented with propionate and ortho-phosphate. 200 mg-
102 COD/ml of propionate were dosed to avoid organic carbon limitation, simulating propi-
103 onate dosing strategies based on primary sludge fermentation (Chanona et al., 2006). It
104 should be noted that Lundtofte WWTP relies on chemical precipitation for phosphorus

105 removal due to the low influent content on organic carbon. Phosphate was dosed to en-
106 sure that incoming phosphorus levels ranged between 6-10 mg-P/L. Oxygen was sup-
107 plied from a pressurized air-line and was manually controlled via needle valve manipu-
108 lation. The system was inoculated with biomass from a full-scale wastewater treatment
109 plant (Lynetten WWTP, Copenhagen, DK). The SBR operation was controlled using
110 LabView VI (National Instruments, Austin, USA).

111 <Table 1>

112 2.2 Analytical methods

113 Process performance was assessed by monitoring bulk liquid concentrations of ammo-
114 nia, nitrite, nitrate, phosphate and sulphate using test kits supplied by Merck© (USA)
115 after filtration through 0.2 µm filter. Soluble and total COD were measured with Hach-
116 Lange© test kits (USA). Dissolved oxygen (DO) and pH were monitored using FDO
117 925 and SenTix 980 probes, respectively (WTW, Germany). Sludge volume index (SVI,
118 Ekama et al., 1997) was monitored on a daily basis. Total suspended solids (TSS) were
119 measured using glass fibre filter (Advantec©, USA) with a pore size of 0.6 µm (APHA,
120 1995).

121 2.3 Microbial analysis

122 Quantitative polymerase chain reaction (qPCR) was carried out on all the extracted
123 DNA samples to determine the abundance of ammonia oxidizing bacteria (AOB) and
124 nitrite oxidizing bacteria (NOB, both *Nitrobacter* and *Nitrospira*), *Thiothrix*, *Microthrix*
125 *parvicella* and sulphate reducing bacteria (SRB). DNA was extracted according to Te-

126 rada et al. (2010). Detailed protocols on DNA extraction and qPCR are included in the
127 supplementary information (SI-1). Quantitative fluorescence *in situ* hybridization
128 (qFISH) was performed as specified in Nielsen et al. (2009). Details of the qFISH pro-
129 tocol are shown in SI-2.

130 Community 16S rRNA genes were subject to partial PCR amplification as suggested by
131 Gülay et al. (2016) and amplicons were sequenced using the Illumina MiSeq platform at
132 the DTU Multi Assay Core Center (Copenhagen, DK). Bioinformatic approaches from
133 Gülay et al. (2016) were applied in this study. Canonical correspondence analysis
134 (CCA) was used to examine the relationships between microbial community composi-
135 tion and system performance. Diversity of SRB was assessed via phylogenetic analysis
136 of clone libraries of genes involved in dissimilatory sulphite reductase: *dsrA* (Ben-Dov
137 et al., 2007) and *dsrB* (Geets et al., 2006). Further details can be found in SI-3.

138 3 Results and discussion

139 3.1 Reactor performance

140 The SBR system performance during the 190 days of operation is shown in Fig.1. Dur-
141 ing the operation at SRT 8 days (first 50 days), temperature varied between 16 and 19
142 °C, DO in the aerobic phase was always above 2 mg/L and pH ranged between 7.5 and
143 8. Under these conditions ammonia was fully oxidized. All non-discharged nitrate was
144 fully denitrified in the consecutive cycle (i.e. initial nitrate concentration up to 10 mg-
145 N/L; Fig. 1A). Nitrogen removal varied between 75 % and 91%. Phosphorus was effec-

146 tively removed and effluent concentrations were always below 1 mg-P/L (Fig. 1B). The
147 phosphorus removal varied between 80 and 98% (Fig. 1D). Phosphate concentration at
148 the end of the anaerobic phase dropped from 28 mg-P/L the first day down to 24 mg-
149 P/L after day 10, ranging from that day between 20 and 25 mg-P/L (Fig. 1B). Striking-
150 ly, phosphorus removal was poor (about 70% of phosphate removal) only during the
151 first week, when phosphate at the end of the anaerobic phase was highest. During that
152 period, most of the influent COD was removed in the anaerobic phase, whilst only 1 to
153 10 mg-COD/L were further removed along the aerobic phase (Fig. 1C). Likely, the ex-
154 tent of the anaerobic phase led initially to residual phosphorus release, due to PAO hy-
155 drolysing stored polyphosphate for maintenance under anaerobic conditions in absence
156 of available COD (Maurer and Gujer, 1995). Under aerobic conditions PAO could not
157 take up the released phosphorus, as they were limited by stored PHA. After an adapta-
158 tion period of 1 week, the internal PHA and polyphosphate storage were balanced and
159 all anaerobically released phosphorus was taken up in the subsequent aerobic phase.
160 TSS in the reactor decreased from 5 g/L to 1.8 g/L after 10 days, and then, depending
161 on the influent COD, varied between 1.5 and 2 g/L (Fig. 1F). The SVI was consistently
162 below 90 mL/g (Fig. 1E), indicating good sludge settling behaviour. Despite the fact
163 that nitrate was present at the beginning of the anaerobic phase, the SRT was high
164 enough to avoid PAO out-competition.

165 On day 50, the system SRT was reduced to 3.5 days. DO, temperature and pH remained
166 in the same ranges as in the previous period. Over a period of 28 days, nitrate in the ef-
167 fluent decreased, while nitrite accumulated as high as 4 mg-N/L (end of phase A, Fig.

168 1A). Nitrite accumulation suggests that nitrite oxidizing bacteria (NOB) were phased
169 out of the system, while ammonia oxidizing bacteria (AOB) were still active. Nitrite
170 was fully denitrified, thereby leading to a nitrogen removal higher than 80%. At tem-
171 peratures ranging between 16 and 19 degrees, AOB apparently grew faster than NOB,
172 and the 3.5 days SRT was insufficient to keep NOB in the reactor (Hellings et al.,
173 1998). Phosphate removal was comparable to the previous period, ranging between 83
174 and 99 % (Fig. 1D). Phosphate in the effluent was kept below 1 mg-P/L (Fig. 1B), ex-
175 cept for 2 days when the influent phosphate concentration was comparably higher (ap-
176 prox. 15 mg-P/L). Phosphate concentration at the end of the anaerobic phase (Fig. 1B)
177 decreased to 18 mg-P/L towards the end of this period. COD was also removed mainly
178 in the anaerobic phase. However, increasing amounts of COD were bleeding through to
179 the aerobic phase, thereby supporting aerobic heterotrophic growth (up to 97 mg-
180 COD/L when the influent TCOD was 620 mg-COD/L, Fig. 1C). TSS concentration
181 range decreased to 1-1.5 g/L. During this period, the SVI increased and ranged between
182 98 and 130 mL/g (Fig. 1E), indicating relatively good sludge settling properties.

183 The aerobic phase length was reduced from 3 to 2.5 hours for 5 days (phase B), thus
184 reducing the aerobic SRT from 1.75 to 1.45 days, to effectively wash-out AOB. Nitrite
185 concentrations immediately decreased and effluent ammonia concentration started to
186 increase. As a result of the shorter aerobic phase phosphorus removal also deteriorated
187 decreasing to a minimum value of 36% (Fig. 1D). Furthermore, the SVI increased up to
188 157 ml/g by the end of this period (Fig. 1E).

189 Although the reduced aerobic phase led to ammonia accumulation in the SBR, phospho-
190 rus removal was comparably low, likely due to a too short aerobic SRT for PAO
191 growth, and the 3 hours aerobic phase was restored and kept for another 26 days (phase
192 C). During the first week of this period, air supply failure led to oxygen concentrations
193 during the aerobic phase ranging from 0.5 to 2 mg/L. Ammonia was kept within the
194 same range as in the previous 5 days, whilst phosphate removal was restored to a range
195 of 55-80%. However, SVI increased up to a maximum of 343 ml/g, representative of
196 filamentous bulking, as consequence of comparably low oxygen levels in the aerobic
197 phase (Martins et al., 2003). Once the oxygen level was restored above 2 mg/L we ex-
198 pected that phosphate removal and SVI were restored. However, SVI further increased
199 to a maximum of 1100 mL/g, showing high variability along consecutive days (e.g. var-
200 iations of about 250 mL/g per day). Parallel, phosphorus removal decreased down to a
201 minimum of 23%, coincident with the maximum SVI. The decrease in PAO activity was
202 also reflected by much lower phosphate concentrations at the end of the anaerobic
203 phase, ranging from 7.4 to 13 mg-P/L (Fig. 1B). Since high oxygen levels are reported
204 to hinder filamentous bulking (Martins et al., 2003), oxygen was subsequently main-
205 tained higher than 4.5 mg/L (from day 102 to 109), without any positive impact on the
206 system performance.

207 Sulphate reduction during the anaerobic phase was 30% at the end of period C (Fig.
208 S16), suggesting that SRB were active in the EBPR. Previous studies have demonstrat-
209 ed the coexistence of sulphate reducing bacteria (SRB), *Thiothrix*, PAO and ordinary
210 heterotrophs in EBPR systems (Yamamoto-Ikemoto et al., 1996). *Thiothrix*, a filamen-

211 tous bacterium that contributes to filamentous bulking (Tandoi et al., 2006), can effec-
212 tively grow on the reduced sulphur compounds produced by the SRB under aerobic
213 conditions. To mitigate the growth of SRB, the anaerobic SRT was decreased from 1.2
214 days to 0.88 days for 23 days (phase D). SVI decreased to 363 ml/g within two weeks.
215 Phosphate removal was partially restored, ranging from 55 to 77%. During the aerobic
216 phase, to prevent the growth of other filamentous bacteria, DO was kept higher than 4.5
217 mg/L. Ammonia was the main nitrogen form in the effluent, although nitrite ranged be-
218 tween 0.1 and 3.2 mg-N/L.

219

<Figure 1>

220 Given the positive system response to the reduction of anaerobic SRT, we further re-
221 duced it to 0.68 days for the next 24 days (phase E). Within this period, after one week
222 of stable and high SVI (413-568 mL/g) and moderate phosphorus removal (58-70%),
223 the SVI progressively decreased to a minimum of 123 ml/g. Phase E was characterized
224 by limited filamentous bulking, with a SVI ranging between 123 and 213 ml/g, and a
225 sharp increase in phosphorus removal, restored to 85-99%. Indeed, after the first week,
226 the phosphate level in the effluent was below 0.8 mg-P/L. Sulphate reduction correlated
227 relatively well ($R^2=0.65$, Fig. S16) with phosphate removal, suggesting that as a conse-
228 quence of the sulphate reduction the PAO may have been outcompeted by SRB.

229

230 The phosphate concentration at the end of the anaerobic phase was as high as 18-24 mg-
231 P/L. Sulphate reduction was not observed from day 120 onward. DO was kept higher

232 than 4.5 mg/L to prevent bulking due to other filaments. However, AOB activity re-
233 turned, and nitrite was detected during the aerobic phase, reaching a maximum of 10.3
234 mg-N/L, which could potentially lead to phosphorus uptake inhibition. TSS varied be-
235 tween 0.5 and 1.2 g/L in this period. Towards the end of this period, the temperature
236 range slightly increased to 17-21 °C.

237 Finally, at day 150 the SRT was lowered to 3 days to repress AOB activity. Oxygen
238 level was kept between 2 and 3 mg/L, sufficiently high to support P uptake by PAO, as
239 suggested by previous model-based studies (Valverde-Pérez et al., 2015). As shown in
240 Fig. 1A, nitrite at the end of the anaerobic phase was reduced, whilst ammonia in-
241 creased again. Any nitrogen removal, about 40 % of the influent ammonia, observed at
242 this stage was likely due to assimilation, as neither nitrite nor nitrate were observed in
243 the effluent. Both, phosphorus removal kept high (85-99%) and SVI ranged between 90
244 and 290 ml/g. The SBR was kept stable at SRT=3 days, DO 2-3 mg/L and anaerobic
245 and aerobic phase lengths of 70 minutes (i.e. anaerobic SRT of 0.58 days) and 3 hours
246 (i.e. 1.5 day of aerobic SRT, above the minimum aerobic SRT reported by Brdjanovic et
247 al., 1998), respectively, for 35 days (> 3 SRTs).

248 3.2 Variation in microbial community composition and 249 diversity

250 Illumina MiSeq sequencing generated 1,745,605 raw assembled reads from 21 samples
251 with an average length of 420 bp. A total of 1,098,943 (63%) sequences passed quality
252 checks, and clustered across 167,622 OTUs, defined at 97% sequence similarity (Fig.

253 S1). The sample taken at day 30 was excluded from further analysis due to the low
254 number of sequences available in the sample library. High-quality sequences were nor-
255 malized by rarefying to 11,940 sequences per sample for further diversity and taxonom-
256 ic comparisons.

257 In Fig. 2, the distribution of the most abundant taxa is shown at the order level. It is
258 clear that from day 50, when the system SRT was reduced, the *Thiothrichales* order in-
259 creased in abundance, mostly comprising *Thiothrix spp.* This increase continued during
260 the bulking event. From a relative abundance of 50% onward (based on sequencing
261 analysis), the relative *Thiothrix* abundance strongly correlated with the extent of fila-
262 mentous bulking (characterised using SVI as surrogate – $R^2=0.92$; Fig. S17). From day
263 101 to 109 the anaerobic SRT was reduced to 0.88 day. These conditions resulted in a
264 reduction of *Thiothrix* abundance. However, no obvious patterns in known SRB taxa
265 (Fig. S11) – presumed to be responsible for *Thiothrix* growth by reducing sulphate to
266 sulphide – were found. After SVI correction and phosphorus removal restoration, the
267 microbial diversity became similar to the one by the end of the SRT=8-day phase based
268 on the H index (Fig. 3 and Fig. S2). Similar trends were confirmed via qPCR quantifica-
269 tion (Fig. S10).

270 <Figure 2>

271 *Rhodocyclales* were relatively abundant during good phosphorus removal and were only
272 displaced during filamentous bulking. *Rhodocyclales* include *Accumulibacter phosphat-*
273 *tis*, which is believed to be the main driver of phosphorus removal in EBPR systems
274 (Oehmen et al., 2007). However, *Accumulibacter* abundance was very low (<1%) com-

275 pared to previous studies (Fig. S4, Nielsen et al., 2010). Nevertheless, *Rhodocyclales*
276 positively correlated with phosphate removal suggesting that members other than *Ac-*
277 *cumulibacter* could have been involved in phosphorus removal (Fig. 6), e.g.,
278 *Dechloromonas* related PAO (Lv et al., 2015). However, qFISH analysis revealed high-
279 er abundance of *Accumulibacter* than that indicated by the sequencing data, especially
280 towards the end of the operational period (up to 18% of total bacteria, Fig. S13). This is
281 a major difference compared to the study by Ge et al. (2015), who did not find *Accumu-*
282 *libacter* in the short-SRT EBPR. Similar to the A-stage systems, it may be the case that
283 microbial communities are case specific, contrary to CAS, which contains a core com-
284 munity of abundant microorganisms (González-Martínez et al., 2016). *Tetrasphaera*
285 remained low during the operational period (Fig. S4 and S13).

286 *Saprospriales*, *Flavobacteriales* and *Burkholderiales* are heterotrophic microorganisms
287 that appeared at relatively high abundance along the operation of the reactor. In the case
288 of *Burkholderiales* the considerable abundance could be a consequence of their extreme
289 nutritional versatility and adaptability to different environmental conditions. *Burkhold-*
290 *eriales* are often found in EBPR systems (Sadaie et al., 2007), and include *Comamona-*
291 *daceae*, which have been reported as putative PAO (Ge et al., 2015). However, abun-
292 dance of *Comamonadaceae* did not follow the trends on phosphorus removal (Fig. S5).
293 *Saprospriales* are specialized in the hydrolysis of proteins and are abundant in conven-
294 tional EBPR systems (Nielsen et al., 2012), but also in short-SRT EBPR systems (Ge et
295 al., 2015). Finally, *Flavobacteriales* are rather nutritionally restricted, with the ability to
296 consume glucose and very few other carbon compounds. Nevertheless, they can hydro-

297 lyze several biopolymers and particulate products from bacterial decay (Kircham 2012).
298 They have also been found in Danish EBPR systems (Albertsen et al., 2012): they might
299 grow little in the actual treatment plant but are present due to their high abundance in
300 the influent sewage (Saunders et al., 2016). The higher abundance of *Flavobacteriales*
301 in the short-EBPR system compared to full scale EBPR (7% vs ~1% reported by Saun-
302 ders et al., 2016) suggests that microbial composition of short-SRT EBPR communities
303 is more closely related to the influent microbial communities, as suggested for the A-
304 stage systems (González-Martínez et al., 2016).

305 Following the trends in performance, NOB were washed out from the system from the
306 onset of SRT reduction (Fig. S8). AOB followed a similar trend, but their abundance in
307 the amplicon libraries was one order of magnitude lower. Targeted qPCR analysis was
308 conducted to quantify *Nitrosomonas*, *Nitrobacter* and *Nitrospira*. AOB guild ranged
309 between 3.6-13.6 % while *Nitrospira* ranged from 1 to 4.3% (Fig. S9, Table S12).

310 Despite their low abundance (Fig. S7 and S11), the role of SRB is also worth mention-
311 ing. Our results suggest that SRB can reduce sulphate at very low abundance (<0.15%),
312 as was the case for data presented by Pester et al. (2010). According to Fig. 3, the genus
313 *Desulfarculus* is strongly and negatively correlated with phosphorus removal. This is in
314 agreement with the process performance of the SBR, which showed very poor phospho-
315 rus removal when sulphate reduction was relatively high (Fig. SI-16). *Desulfarculus* is a
316 completely carbon oxidizing SRB, which is able to consume a wide range of organic
317 substrates. Furthermore, it is oxygen tolerant so it can survive during the aerobic phase
318 (Sun et al., 2010). *Desulfuromonas* also correlated negatively with phosphorus removal

319 (Fig. 3). Similarly to *Desulfarculus*, these bacteria can also oxidize propionate and ace-
320 tate to carbon dioxide. However, *Desulfuromonas* is only able to reduce sulphur and not
321 sulphate (Pfennig and Biebl, 1976).

322 <Figure 3>

323 Finally, GAO were barely detected in the libraries, indicating low abundance (<0.1%)
324 for both *Competibacter*- and *Defluviicoccus*- related GAO (Fig. S6). Despite the im-
325 portance of these organisms in EBPR systems, they may have been effectively outcom-
326 peted from the SBR due to the dosing strategy (propionate cannot be consumed by
327 *Competibacter*) and the relatively low temperatures and high pH, which give competi-
328 tive advantages to PAO over GAO (López-Vázquez et al., 2009). qFISH revealed higher
329 abundance of both *Competibacter* and *Defluviicoccus*, but always below 3.8% and 1%,
330 respectively (Fig. S12, Table S12). Other putative GAO, such as *Micropruina* (Kong et
331 al., 2001), were also present at low abundance (<0.1%, Fig. S6).

332 3.3 Discrepancies between quantification methods

333 There is a discrepancy between the results from the amplicon sequencing and data esti-
334 mated via qPCR or qFISH (Tables S11 and S12). For example, the abundance of *Nitro-*
335 *somonas* or *Nitrospira* determined via sequencing is comparably lower than the qPCR
336 estimate, as was observed by Pellicer-Nácher et al. (2014). A plausible explanation is
337 related to the understimation of low abundant bacteria by the amplicon sequencing
338 method. González et al. (2012) demonstrated that the amplification via PCR of low
339 abundant bacteria DNA is considerably less efficient than the amplification of highly

340 abundant bacteria DNA, thus leading to the overestimation of highly abundant microbi-
341 al groups. This is clearly the case for *Thiothrix* population, which has been underesti-
342 mated via sequencing at abundances below 5% and overestimated in other cases (Table
343 S11 and S12). When comparing sequencing and qFISH results other factors should be
344 taken into account, such as DNA extraction efficiency, which may introduce significant
345 bias into PCR-based estimates (Kim et al., 2013). As an example, Albertsen et al.
346 (2012) reports high discrepancy between qFISH based and sequencing based results for
347 Gram positive bacteria (e.g. *Tetrasphaera* or *Microthrix parvicella*) presumably, due to
348 inefficient DNA extraction. However, they found a good agreement between both data
349 sets for *Competibacter* or *Accumulibacter* bacteria, which suggests that discrepancies in
350 our study may have a different origin. Analyzing the correspondence between qFISH
351 probes targeting *Accumulibacter*, it can be seen that they detect low abundance within
352 the sequences amplified by the PCR. Therefore, the sequencing results may be biased
353 by the universal primer selection (Hong et al., 2009). To get a better overview of the
354 microbial diversity it is recommended to combine different techniques, preferably com-
355 bining methods that rely on DNA extraction (e.g. PCR based methods) with tools that
356 do not require this step (e.g. qFISH).

357 3.4 Synergies and competitions between bacteria in 358 short SRT EBPR systems

359 At SRT=3.5 days, NOB were effectively washed out from the system, thus leading to
360 nitrite accumulation (e.g. from day 50 to day 55). Likely, the operation of the EBPR

361 system at an aerobic SRT=1.75 days, combined with relatively high oxygen levels, had
362 a detrimental effect over *Nitrospira sp.* and not *Nitrosomonas*, thus leading to nitrite
363 rather than nitrate accumulation (Regmi et al., 2014). Additionally, high ammonia lev-
364 els at pH close to 8, e.g. phase E (Fig. 1A), may have inhibited NOB activity (Chandran
365 and Smets, 2000). Nitrite accumulation in this period could potentially lead to the inhi-
366 bition of PAO activity. Nitrite has been reported as a potent inhibitor for P-uptake under
367 both anoxic and aerobic conditions (e.g. $0.5 \cdot 10^{-3}$ mg-N- HNO_2 /L lead to 50% anabolic
368 activity inhibition; Meinhold et al., 1999; Pijuan et al., 2010). Indeed, previous experi-
369 mental runs at SRT=4-5 d lead to the wash out of PAO likely due to nitrite accumula-
370 tion in the aerobic phase. Therefore, these operational conditions were not tested in this
371 study (Valverde-Pérez et al., 2015b). Surprisingly, nitrite presence did not negatively
372 affect PAO performance. On the one hand, at the observed pH levels (ranging between
373 7.5 and 8), the concentration of free nitrous acid (FNA), which is the true inhibitor of
374 PAO activity, was varying between $0.8 \cdot 10^{-3}$ and $0.2 \cdot 10^{-3}$ mg-N /L, i.e., below the inhibi-
375 tion levels reported by Pijuan et al. (2010). On the other hand, PAO could have adapted
376 to the presence of nitrite (Wang et al., 2014). Additionally, if AOB or NOB are not ef-
377 fectively phased out at SRTs ranging from 4 to 6 days, PAO may be outcompeted by
378 denitrifying heterotrophs due to substrate competition during the anaerobic phase
379 (Guerrero et al., 2011). This has been demonstrated through dedicated simulation stud-
380 ies (Valverde-Pérez et al., 2015). Based on the stoichiometry reported on the activated
381 sludge model 2d (ASM2d, Henze et al., 1999), about 30% of the influent COD was con-

382 sumed by denitrifying bacteria, leaving enough COD (about 30mg-COD L⁻¹ per cycle)
383 available to replenish the PHA storage from PAO.

384 If nitrifiers are effectively washed out of the reactor, at the beginning of the anaerobic
385 phase there is no nitrate nor nitrite. The fully anaerobic phase could then promote sul-
386 phate reduction by SRB if sufficient sulphate is available. SRB were present in low
387 abundance along the study (Fig. S7 and S11) but sulphate reduction was not observed
388 when nitrate was recycled to the beginning of the anaerobic phase. SRB have been de-
389 scribed as tolerant bacteria, which can grow even in the absence of sulphate, showing
390 high metabolic flexibility (Plugge et al., 2011). Furthermore, SRB have a wide variety
391 of protection strategies that let them survive in adverse environments, such as those
392 characterized by aerobic or high salinity conditions (Zhou et al., 2011). Hence, SRB
393 could use their capabilities to survive in the reactor under unfavourable conditions and
394 only actively reduced sulphate once NO_x were not present at the beginning of the anaer-
395 obic phase of the SBR (i.e. when most of the propionate is available for their growth).
396 Alternatively, SRB could actively reduce sulphate in the presence of nitrate. However,
397 due to the prevalence of nitrate, reduced sulphur compounds could be oxidized back
398 via denitrification (Jensen et al., 2009). SRB may support the growth of *Thiothrix* by
399 producing sulphur reduced compounds available for their growth under aerobic condi-
400 tions. *Thiothrix* is an obligate aerobic mixotroph, which frequently appears in EBPR
401 reactors treating industrial wastewaters (Nielsen et al., 2010). However, the high abun-
402 dance found in this study has been only previously found in systems where SRB pro-
403 vided them with enough sulphur reduced chemicals (Yamamoto-Ikemoto et al., 1991,

404 1994, 1996). *Thiothrix* can significantly deteriorate settling properties of activated
405 sludge at relatively high relative abundance (Fig. S17), but do not seem to be related to
406 poor phosphorus removal (Fig. 3).

407 SRB can interact with PAO in different ways. SRB produce sulphide, which can inhibit
408 phosphorus release, thereby reducing it to 50% at concentrations of 60 mg-H₂S/L (Saa
409 et al., 2013). Since phosphorus release is hindered under anaerobic conditions, PAO
410 cannot grow under aerobic conditions, thus leading to their wash out from the system.
411 Additionally, SRB take up organic carbon under anaerobic conditions. The most com-
412 mon SRB in wastewater treatment systems are incomplete carbon oxidizers, such as
413 *Desulfobulbos* or *Desulfovivbrio* (Hao et al., 2014), both found via phylogenetic analy-
414 sis (Fig. S14 and S15). The end product of incomplete oxidation process is acetate,
415 which is finally available for other bacteria, such as PAO (Yamamoto-Ikemoto et al.,
416 1996). Therefore, there is limited competition for carbon between incomplete oxidizing
417 SRB and PAO. Other SRB, like *Desulfarculus* in this study, are able to completely oxi-
418 dize organic carbon to carbon dioxide (Hao et al., 2014). Therefore, this second type of
419 SRB may compete with PAO for organic carbon in the anaerobic phase, thus leading to
420 incomplete phosphorus removal, as suggested by Fig. 3. To further support this hypoth-
421 esis, the COD consumption was estimated, based on stoichiometric coefficients from
422 literature (Yamamoto-Ikemoto et al., 1996), for the day with maximum reported SVI
423 (SVI=1100 ml/g; sulphate reduction of 30%). About 35% of the influent COD was con-
424 sumed for sulphate reduction. Surprisingly, sulphate reduction stopped before all COD
425 was consumed, as comparably high COD levels and sulphate were detected at the end of

426 the anaerobic phase along the bulking event (Fig. 1C). Presumably, VFA, propionate in
427 this case, were taken up first by SRB or PAO, while the rest of the available COD needs
428 to be hydrolysed or fermented before it can be consumed by PAO and SRB. Since the
429 specific uptake rate of propionate by SRB is about 3 times higher than for PAO (calcu-
430 lations based on Henze et al., 1999 and Cassidy et al., 2015 are reported in SI-7), SRB
431 could outcompete PAO for VFA uptake at the beginning of the anaerobic phase. Sul-
432 phate reduction may stop due to self-inhibition of SRB with the produced sulphide
433 (Reis et al., 1992). Additionally, sulphide may also inhibit fermentative microorgan-
434 isms, thereby limiting the production of more easily biodegradable substrate available
435 for oxidation by SRB (McCartney and Oleszkiewicz, 1991). Several studies suggest that
436 sulphide is a strong inhibitor of microbial growth at relatively low pH levels, but others
437 observed that pH effect on inhibition was negligible (Chen et al., 2014). The COD
438 bleeding through from the anaerobic phase could enhance the growth of *Thiothrix* along
439 the aerobic phase (Vaiopoulou et al., 2007). Therefore, the most plausible explanation
440 for the inactivation of PAO is a combination of substrate competition at the beginning
441 of the cycle and inhibition due to sulphide. This could be the cause of poor phosphorus
442 removal of other systems where SRB coexisted with PAO (Yamamoto-Ikemoto et al.,
443 1991; Vaiopoulou et al., 2007). In this study, SRB activity was controlled by manipulat-
444 ing anaerobic SRT. When the anaerobic phase length was 2 hours, corresponding to 1.2
445 days - calculation without accounting for the 1 hour non aerated settling phase - those
446 SRB with a growth rate higher than 1 day^{-1} could grow in the reactor (Cassidy et al.,
447 2015). Once sulphate reduction was avoided during the anaerobic phase, phosphorus

448 removal was restored, further supporting this hypothesis. These findings are relevant to
449 those WWTPs located close to the coastline, where the sulphate content in wastewater
450 is expected to be high (van den Brand et al., 2015). This is the case for Lundtofte
451 WWTP, which is barely 5 km from the sea. A two-month sampling campaign was per-
452 formed to further verify this. Influent sulphate concentrations measured in the influent
453 to Lundtofte ranged from 35 to 162 mg-SO₄⁻/L. It should be noted that there are also
454 cases where the phosphorus and the sulphur cycle have been successfully combined,
455 such as the SANI process (Wu et al., 2014). Nevertheless, phosphorus removal in this
456 process is driven by microbes capable to oxidize sulphide rather than *Accumulibacter*
457 (Guo et al., 2016), which makes it non comparable to our study.

458 Interestingly, neither *Competibacter* nor *Defluviicoccus* seemed to affect phosphorus
459 removal during the operation. As stated before, the carbon source, propionate, dosed to
460 the system together with the relatively high pH and moderate temperature may have
461 outcompeted the GAO from the reactor (López-Vázquez et al., 2009). The low SRT
462 could also have contributed to the wash-out GAO. EBPR systems operated at SRT=3
463 days did not register instabilities due to the competition between GAO and PAO even at
464 30°C (Whang and Park, 2006). Nevertheless, should *Competibacter* grow at low SRTs,
465 attention should be paid to the incomplete oxidizing SRB. Since they can produce ace-
466 tate as a final product, this may become available for *Competibacter*, thereby making the
467 dosing strategies proposed in the literature unsuccessful (López-Vázquez et al., 2009).
468 Additionally, inhibition by sulphide may be another selective factor between PAO and
469 GAO, which should be subject of future study.

470 The proposed ecological model, based on the interactions between microbiological
471 guildts found in this study, is shown in Fig.4.

472 <Figure 4>

473 3.5 Sludge settling properties

474 Poor settling may compromise effluent quality by increasing the TSS in the effluent of
475 the plant, thereby compromising pathogen removal (Schuler and Jang, 2007). According
476 to Fig. 3, *Thiothrix* was the main taxon affecting the settling properties. This is in
477 agreement with previous studies where extreme filamentous bulking was provoked by
478 *Thiothrix* (SVI>600 mg/L, Yamamoto-Ikemoto et al., 1991; Miyazato et al., 2006). As
479 suggested by Fig. S17, there is linear correlation between *Thiothrix* abundance and SVI
480 only beyond 50% relative abundance. The poor fitting at lower abundances can be a
481 consequence of the synergic effect of a decrease in biomass density due to the coinci-
482 dent wash out of PAO, with filamentous bulking (Schuler and Jang, 2007). Since phos-
483 phorus uptake by PAO was hindered by SRB, the polyphosphate content in sludge was
484 expected to be lower, thereby producing low density sludge. Low density sludge is more
485 sensitive to filamentous bulking (Jassby et al., 2014), which could explain the highly
486 dynamic SVI found between days 80 and 150. On the other hand, previous studies have
487 shown poor correlation between filamentous bacteria abundance and SVI, suggesting as
488 an alternative to look for correlations with the hindered settling model parameters
489 (Wágner et al., 2015). Furthermore, Wágner et al. (2015) demonstrated that a specific
490 filamentous bacteria (*M. parvicella*), can drive bulking regardless of other filamentous

491 bacteria (e.g., *Chloroflexi*), prevailing at significantly higher abundance level. There-
492 fore, SVI dynamics found at relatively low abundance of *Thiothrix* could be driven by
493 other filaments which were much less abundant. As an example, filaments belonging to
494 the phylum *Firminicutes*, common filaments in EBPR systems (Mielczarek et al., 2012),
495 found at low abundance along the operation of the reactor, such as *Thrichococcus* (~1%
496 Fig. S3, Nielsen et al., 2009b), could affect the settling properties while *Thiothrix* were
497 not abundant. Strikingly, *M. parvicella* were found in low abundances (<1% at SRT<4
498 days). One plausible explanation is that these filaments are suppressed at SRTs lower
499 than 5.7 days (Noutsopoulos et al., 2006).

500 Low SRT activated sludge systems are characterized by relatively unstable microbial
501 communities, which facilitate filamentous bacteria proliferation (Liao et al., 2006). Pre-
502 vious work has demonstrated that SBRs are more robust than continuous flow systems
503 operated under the same conditions (Liao et al., 2006; Valverde-Pérez, 2015). This
504 work shows how, through careful control of the oxygen level and anaerobic SRT, fila-
505 mentous bulking can be controlled. Since the SBR operation leads to substrate gradi-
506 ents, flock forming bacteria are promoted over filamentous bacteria, thereby reducing
507 the risk of filamentous bulking event (Tandoi et al., 2006).

508 3.6 Relevance to available models

509 The findings of this study point out several limitations of available models to describe
510 phosphorus removal in EBPR systems, both activated sludge models (ASM-2d, Henze
511 et al., 1999) and metabolic models (Oehmen et al., 2010). Indeed, the latter has focused

512 on describing the competition between GAO and PAO for different substrates and under
513 different operational conditions (e.g. Carvalheira et al., 2014). Whilst this effort is very
514 valuable to model conventional EBPR systems, it seems incomplete when modelling
515 short SRT systems. The importance of the SRB in modelling anaerobic digestion pro-
516 cesses has already been acknowledged (Jeppsson et al., 2013), but, according to Fig. 4,
517 they should also be included in other process units across the WWTP. Otherwise, mod-
518 el-based optimization of EBPR systems may lead to model falsification (Sin et al.,
519 2006).

520 Even though the reactor operation became stable after regulating the anaerobic SRT, the
521 SBR still run under moderate bulking conditions (i.e. $SVI > 200$ ml/g). Low SRT systems
522 suffer from poor settling due to poor sludge flocculation (Meerburg et al., 2015). There-
523 fore, settling models should be improved by including time dependent parameters able
524 to capture the sludge properties dynamics (Jeppsson et al., 2013). Some efforts have
525 already been presented in the literature to reduce the uncertainty of settling parameters
526 due to filamentous bulking (Wágner et al., 2015), which up until now has been consid-
527 ered as a non-reducible uncertainty source (Belia et al., 2009).

528 4 Conclusions

529 This study describes the dynamics and successful start-up of a short-SRT EBPR system,
530 which is suitable for resource recovery, especially carbon and phosphorus. Our results
531 suggest that:

- 532 • EBPR process can be run at a system SRT of 3 days, effectively removing phospho-
533 rus and organic carbon. SVI was relatively high (SVI~200ml/g), but stable.
- 534 • NOB were effectively removed from the reactor at SRT below 3.5 days (correspond-
535 ing to 1.75 days aerobic SRT). AOB were phased out from the SBR by lowering the
536 SRT down to 3 days (i.e. 1.5 days of aerobic SRT) and controlling the oxygen level
537 lower than 3 mg/L.
- 538 • Sulphate reduction was observed at SRT 3.5 days, producing sulphur reduced chemi-
539 cals, which served as substrate for *Thiothrix*, thereby provoking filamentous bulking.
540 SRB were shown to have a negative impact on phosphorus removal, although the in-
541 hibition mechanism could not be identified. SRB activity was suppressed by reducing
542 the anaerobic SRT to 0.58 days and process performance was restored.
- 543 Models should include SRB related processes to properly address the competition be-
544 tween them and PAO. Furthermore, since bulking cannot be completely avoided, future
545 research should address the inclusion of time varying parameters for settling models to
546 properly track changes of or in sludge properties.

547 5 Acknowledgement

548 Borja Valverde-Pérez and Dorottya S. Wágner thank the Integrated Water Technology
549 (InWaTech) project (<http://www.inwatech.org>) for the financial support. We would like
550 to thank Ms. Lene Kirstejn Jensen and Dr. Marlene Mark Jensen for their assistance
551 conducting the qPCR measurements and Dr. A. Gizem Mutlu and Dr. Aranud Dechesne
552 for their guidance when conducting the qFISH analysis.

553 6 References

554 Albertsen, M., Hansen, L.B.S., Saunders, A.M., Nielsen, P.H., Nielsen, K.L., 2012. A
555 metagenome of a full-scale microbial community carrying out enhanced biological
556 phosphorus removal. *The ISME Journal*, 6, 1094-1106.

557

558 APHA, American Public Health Association. *Standard Methods for the Examination of*
559 *Water and Wastewater*. Washington DC, 1995.

560

561 Barat, R., van Loosdrecht, M.C.M., 2006. Potential phosphorus recovery in a WWTP
562 with the BCFS process: interactions with the biological process. *Water Research*, 40,
563 3507-3516.

564

565 Batstone, D.J., Hülsen, T., Mehta, C.M., Keller, J., 2015. Platforms for energy and nu-
566 trient recovery from domestic wastewater: A review. *Chemosphere*, 140, 2-11.

567

568 Belia, E., Amerlinck, Y., Benedetti, L., Johnson, B., Sin, G., Vanrolleghem, P.A.,
569 Gernaey, K.V., Guillot, S., Neumann, M.B., Rieger, L., Shaw, A., Villez, K., 2009.
570 *Wastewater treatment modelling: dealing with uncertainties*. *Water Science and Tech-*
571 *nology*, 60(8), 1929-1941.

572

573 Ben-Dov, E., Brenner, A., Kushmaro, A., 2007. Quantification of sulfate-reducing bacte-
574 ria in industrial wastewater, by real-time polymerase chain reaction (PCR) using *dsrA*
575 and *apsA* genes. *Microbial Ecology*, 54, 439-451.

576

577 Brdjanovic, D., van Loosdrecht, M.C.M., Hooijmans, M.C., Alaerts, G.J., Heijnen, J.J.,
578 1998. Minimal aerobic sludge retention time in biological phosphorus removal systems.
579 *Biotechnology and Bioengineering*, 60(3), 326-332.

580

581 Cassidy, J., Lubberding, H.J., Esposito, G., Keesman, K.J., Lens, P.N.L., 2015. Auto-
582 mated biological sulphate reduction: a review on mathematical models, monitoring and
583 bioprocess control. *FEMS Microbiology Reviews*, 39, 823-853.

584

585 Carvalheira, M., Oehmen, A., Carvalho, G., Reis, M.A.M., 2014. The impact of aeration
586 on the competition between polyphosphate accumulating organisms and glycogen ac-
587 cumulating organisms. *Water Research*, 66, 296-307.

588

589 Chandran, K., Smets, B.F., 2000. Single-step nitrification models erroneously describe
590 batch ammonia oxidation profiles when nitrite oxidation becomes rate limiting. *Biotech-
591 nology and Bioengineering*, 68(4), 396-406.

592

593 Chanona, J., Ribes, J., Seco, A., Ferrer, J., 2006. Optimum design and operation of pri-
594 mary sludge fermentation schemes for volatile fatty acids production. *Water Research*,
595 40, 53-60.

596

597 Chen, J.L., Ortiz, R., Steele, T.W.J., Stuckey, D.C., 2014. Toxicants inhibiting anaerobic
598 digestion: A review. *Biotechnology Advances*, 32, 1523-1534.

599

600 Ekama, G.A., Barnard, G.L., Gunthert, F.W., Krebs, P., McCorquodale, J.A., Parker,
601 D.S., Wahlberg, E.J., 1997. *Secondary Settling Tanks: Theory, Modelling, Design and*
602 *Operation*. International Association on Water Quality, London, UK.

603

604 Ge, H., Batstone, D.J., Keller, J., 2013. Operating aerobic wastewater treatment at very
605 short sludge ages enables treatment and energy recovery through anaerobic sludge di-
606 gestion. *Water Research*, 47, 6546-6557.

607

608 Ge, H., Batstone, D.J., Keller, J., 2015. Biological phosphorus removal from abattoir
609 wastewater at very short sludge ages mediated by novel PAO clade *Comamonadaceae*.
610 *Water Research*, 69, 173-182.

611

612 Geets, J., Borremans, B., Diels, L., Springael, D., Vangronsveld, J., van der Lelie, Van-
613 broekhove, K., 2006. DsrB gene-based DGGE for community and diversity surveys of
614 sulfate-reducing bacteria. *Journal of Microbial Methods*, 66, 194-205.

615

616 González, J.M., Portillo, M.C., Belda-Ferre, P., Mira, A., 2012. Amplification by PCR
617 artificially reduces the proportion of the rare biosphere in microbial communities. *Plos*
618 *One*, 7(1), 1-11.

619

620 González-Martínez, A., Rodríguez-Sánchez, A., Lotti, T., García-Ruíz, M.J., Osorio, F.,
621 González-López, J., van Loosdrecht, M.C.M., 2016. Comparison of bacterial communi-
622 ties of conventional and A-stage activated sludge systems. *Scientific Reports*, 6, 18786.

623

624 Guerrero, J., Guisasola, A., Baeza, J.A., 2011. The nature of the carbon rules the com-
625 petition between PAO and denitrifiers in systems for simultaneous biological nitrogen
626 and phosphorus removal. *Water Research*, 45, 4793-4802.

627

628 Gülay, A., Musovic, S., Alberchtsen, H.J., Al-Soud, W.A., Sørensen, S.J., Smets, B.F.,
629 2016. Ecological patterns, diversity and core taxa of microbial communities in ground-
630 water-fed rapid gravity filters. *The ISME journal*, 1-14.

631

632 Guo, G., Wu, D., Hao, T., Mackey, H.R., Wei, L., Wang, H., Chen, G., 2016. Function-
633 al bacteria and process metabolism of the Denitrifying Sulfur conversion-associated
634 Enhanced Biological Phosphorus Removal (DS-EBPR) system: an investigation by op-
635 erating the system from deterioration to restoration. *Water Research*, 95, 289-299.

636

- 637 Hao, T., Xiang, P., Mackey, H.R., Chi, K., Lu, H., Chui, H., van Loosdrecht, M.C.M.,
638 Chen, G.H., 2014. A review of biological sulfate conversions in wastewater treatment.
639 Water Research, 65, 1-21.
640
- 641 Hellinga, C., Schellen, A.A.J.C., Mulder, J.W., van Loosdrecht, M.C.M., Heijen, J.J.,
642 1998. The SHARON process: an innovative method for nitrogen removal from ammo-
643 nium-rich waste water. Water Science and Technology, 37(9), 135-142.
644
- 645 Henze, M., Gujer, W., Mino, T., Matsuo, T., Wentzel, M.C., Marais, G.V.R., Van
646 Loosdrecht, M.C.M., 1999. Activated sludge model n° 2d, ASM2d. Water Science and
647 Technology, 39, 165-182.
648
- 649 Hong, S., Bunge, J., Leslin, C., Jeon, S., Epstein, S.S., 2009. Polymerase chain reaction
650 primers miss half of rRNA microbial diversity. The ISME Journal, 3, 1365-1373.
651
- 652 Jasby, D., Xiao, Y., Schuler, A.J., 2014. Biomass density and filament length synergis-
653 tically affect activated sludge settling: systematic quantification and modeling. Water
654 Research, 48, 547-465.
655
- 656 Jeppsson, U., Alex, J, Batstone, D. J., Benedetti, L, Comas, J., Copp, J.B., Corominas,
657 L, Flores Alsina, X., Gernaey, K.V., Nopens, I., Pons, M.-N., Rodríguez-Roda, I.,

- 658 Rosen, C, Steyer, J.-P., Vanrolleghem, P.A., Volcke, E.I.P., Vrecko, D., 2013. Bench-
659 mark simulation models, quo vadis? *Water Science and Technology*, 68(1), 1-15.
660
- 661 Jensen, M.M., Petersen, J., Dalsgaard, T. Thamdrup, B., 2009. Pathways, rates and reg-
662 ulation of N₂ production in the chemocline of an anoxic basin, Mariager Fjord, Den-
663 mark. *Marine Chemistry*, 113, 102-113.
664
- 665 Jimenez, J., Miller, M., Bott, C., Murthy, S., Clippeleir, H., Wett, B., 2015. High-rate
666 activated sludge system for carbon management - Evaluation of crucial process mecha-
667 nisms and design parameters. *Water Research*, 87, 476-482.
668
- 669 Kim, J., Lim, J., Lee, C., 2013. Quantitative real-time PCR approaches for microbial
670 community studies in wastewater treatment systems: applications and considerations.
671 *Biotechnology Advances*, 31, 1358-1373.
672
- 673 Kirchman, D.L., 2002. The ecology of *Cytophaga-Flavobacteria* in aquatic environ-
674 ments. *FEMS Microbiology Ecology*, 39, 91-100.
675
- 676 Kong, Y.H., Beer, M., Seviour, R.J., Lindrea, K.C., Rees, G., N., 2001. Structure and
677 functional analysis of the microbial community in an aerobic:anaerobic sequencing
678 batch reactor (SBR) with no phosphorus removal. *Systematic Applied Microbiology*,
679 24, 597-609.

680

681 Liao, B.Q., Droppo, I.G., Leppard, G.C., Liss, S.N., 2006. Effect of solids retention
682 time on structure and characteristics of sludge flocs in sequencing batch reactors. *Water*
683 *Research*, 40, 2583-2591.

684

685 López-Vázquez, C.M., Oehmen, A., Hooijmans, C.M., Brdjanovic, D., Gijzen, H.J.,
686 Yuan, Z., van Loosdrecht, M.C.M., 2009. Modeling the PAO-GAO competition: ef-
687 fects of carbon source, pH and temperature. *Water Research*, 43, 450-462.

688

689 Lv, X.M., Shao, M.F., Li, J., Li, C.L., 2015. Metagenomic analysis of the sludge micro-
690 bial community in lab-scale denitrifying phosphorus removal reactor. *Applied Bio-*
691 *chemistry and Biotechnology*, 175, 3258-3270.

692

693 Mamais, D., Jenkins, D., 1992. The effect of MCRT and temperature on enhanced bio-
694 logical phosphorus removal. *Water Science and Technology*, 26(5-6), 955-965.

695

696 Martins, A.M.P., Pagilla, K., Heijnen, J.J., van Loosdrecht, M.C.M., 2003. Filamentous
697 bulking sludge – a critical review. *Water Research*, 38(4), 793-817.

698

699 Maurer, M., Gujer, W., 1995. Monitoring of microbial phosphorus release in batch ex-
700 periments using electric conductivity. *Water Research*, 29(11), 2613-2617.

701

702 McCartney, D.M., Oleszkiewicz, J.A., 1991. Sulfide inhibition of anaerobic degradation
703 of lactate and acetate. *Water Research*, 25(2), 203-209.

704

705 Meerburg, F.A., Boon, N., Van Winckel, T., Vercamer, J.A.R., Nopens, I., Vlaemink,
706 S.E., 2015. Toward energy-neutral wastewater treatment: a high-rate contact stabiliza-
707 tion process to maximally recover sewage organics. *Bioresource Technology*, 179, 373-
708 381.

709

710 Meerburg, F.A., Vlaeminck, S.E., Roume, H., Seuntjens, D., Pieper, D.H., Jauregui, R.,
711 Vilchez-Vargas, R., Boon, N., 2016. High-rate activated sludge communities have a
712 distinctly different structure compared to low-rate sludge communities, and are less
713 sensitive towards environmental and operational variables. *Water Research*, 100, 137-
714 145.

715

716 Meinhold, J., Arnold, E., Isaacs, S., 1999. Effect of nitrite on anoxic phosphate uptake
717 in biological phosphorus removal activated sludge. *Water Research*, 33(8), 1871-1883.

718

719 Mielczarek, A.T, Kragelund, C., Eriksen, P.S., Nielsen, P.H., 2012. Population dynam-
720 ics of filamentous bacteria in Danish wastewater treatment plants with nutrient removal.
721 *Water Research*, 46, 3781-3795.

722

- 723 Mielczarek, A.T., Nguyen, H.T.T., Nielsen, J.L., Nielsen, P.H., 2013. Population dy-
724 namics of bacteria involved in enhanced biological phosphorus removal in Danish
725 wastewater treatment plants. *Water Research*, 47, 1529-1544.
- 726
- 727 Mino, T., van Loosdrecht, M.C.M., Heijnen, J.J., 1998. Microbiology and biochemistry
728 of the enhanced biological phosphate removal process. *Water Research*, 32(11), 3193-
729 3207.
- 730
- 731 Miyazato, N., Yamamoto-Ikemoto, R., Takamatsu, S., 2006. Microbial community
732 change of sulfate reduction and sulfur oxidation in the activated sludge cultivated with
733 acetate and peptone. *Water Science and Technology*, 54(8), 111-119.
- 734
- 735 Nielsen, P.H., Daims, H., Lemmer, H., 2009. *FISH Handbook for Biological*
736 *Wastewater Treatment. Identification and Quantification of Microorganisms in Activat-*
737 *ed Sludge and Biofilms by FISH.* IWA Publishing, London, UK.
- 738
- 739 Nielsen, P.H., Kragelund, C., Seviour, R.J., Nielsen, J.L., 2009b. Identity and ecophysio-
740 logy of filamentous bacteria in activated sludge. *FEMS Microbiology Reviews*, 33,
741 969-998.
- 742
- 743 Nielsen, P.H., Mielczarek, A.T., Kragelund, C., Nielsen, J.L., Saunders, A.M., Kong,
744 Y., Hansen, A.A., Vollertsen, J., 2010. A conceptual ecosystem model of microbial

745 communities in enhanced biological phosphorus removal plants. *Water Research*, 44,
746 5070-5088.

747

748 Nielsen, P.H., Saunders, A.M., Hansen, A.A., Larsen, P., Nielsen, J.L., 2012. Microbial
749 communities involved in enhanced biological phosphorus removal from wastewater – a
750 model system in environmental biotechnology. *Current Opinion in Biotechnology*, 23,
751 452-459.

752

753 Noutsopoulos, C., Mamais, D., Andreakis, A., 2006. Effects of solids retention time on
754 *Microthrix parvicella* growth. *Water SA*, 32(3), 315-321.

755

756 Oehmen, A., Lemos, P.C., Carvalho, G., Yuan, Z., Keller, J., Blackhall, L.L., Reis,
757 M.A.M., 2007. Advances in enhanced biological phosphorus removal: from micro to
758 macro scale. *Water Research*, 41, 2271-2300.

759

760 Oehmen, A., Carvalho, G., Lopez-Vazquez, C.M., van Loosdrecht, M.C.M., Reis,
761 M.A.M., 2010. Incorporating microbial ecology into the metabolic modelling of poly-
762 phosphate accumulating organisms and glycogen accumulating organisms. *Water Re-*
763 *search*, 44(17), 4992-5004.

764

765 Pellicer-Nàcher, C., Frank, S., Gülay, A., Ruscadella, M., Terada, A., Al-Soud, W.A.,
766 Hansen, M.A., Sørensen, S.J., Smets, B.F., 2013. Sequentially aerated membrane bio-

767 film reactors for autotrophic nitrogen removal: microbial community composition and
768 dynamics. *Microbial Biotechnology*, 7, 32-43.

769

770 Pester, M., Bittner, N., Deevong, P., Wagner, M., Loy, A., 2010. A “rare biosphere”
771 microorganism contributes to sulfate reduction in a peatland. *The ISME Journal*, 4(12),
772 1-12.

773

774 Pfennig, N., Biebl, H., 1976. *Desulfuromonas acetoxidans* gen. nov. and sp. nov., a new
775 anaerobic, sulfur-reducing, acetate-oxidizing bacterium. *Archives of Microbiology*, 110,
776 3-12.

777

778 Pijuan, M., Ye, L., Yuan, Z., 2010. Free nitrous acid inhibition on the aerobic metabo-
779 lism of poly-phosphate accumulating organisms. *Water Research*, 44, 6063-6072.

780

781 Plugge, C.M., Zhang, W., Scholten, J.C.M., Stams, A.J.M., 2011. Metabolic flexibility
782 of sulfate-reducing bacteria. *Frontiers in Microbiology*, 2, 1-8.

783

784 Regmi, P., Miller, M.W., Holgate, B., Bunce, R., Park, H., Chandran, K., Wett, B.,
785 Murthy, S., Bott, C.B., 2014. Control of aeration, aerobic SRT and COD input for mai-
786 nstream nitrification/denitrification. *Water Research*, 57, 162-171.

787

788 Reis, M.A.M., Almeida, J.S., Lemos, P.C., Carrondo, M.J.T., 1992. Effect of hydrogen
789 sulfide on growth of sulfate reducing bacteria. *Biotechnology and Bioengineering*, 40,
790 593-600.

791

792 Saa, S., Welles, L., López-Vázquez, C., van Loosdrecht, M.C.M., 2013. Sulfide effects
793 on the anaerobic kinetics of phosphorus accumulating organisms. *Proceedings of the*
794 *World Congress on Anaerobic Digestion*, Santiago de Compostela, Spain.

795

796 Sadaie, T., Sadaie, A., Takada, M., Hamano, K., Ohnishi, J., Ohta, N., Matsumoto, K.,
797 Sadaie, Y., 2007. Reducing sludge production and domination of *Comamonadaceae* by
798 reducing the oxygen supply in the wastewater treatment procedure of a food-processing
799 factory. *Biosciences, Biotechnology and Biochemistry*, 71(3), 791-799.

800

801 Saunders, A.M., Albertsen, M., Vollertsen, J., Nielsen, P.H., 2016. The activated sludge
802 ecosystem contains a core community of abundant organisms. *The ISME Journal*, 10,
803 11-20.

804

805 Schneider, D.W., 2014. Who invented activated sludge? *Environmental Engineering*
806 *and Scientist*, 50(1), 8-11.

807

- 808 Schuler, A.J., Jang, H., 2007. Causes of variable biomass density and its effects on set-
809 tleability in full-scale biological wastewater treatment systems. *Environmental Science*
810 *and Technology*, 41, 1675-1681.
- 811
- 812 Shilton, A. N., Powell, N., Guieysse, B., 2012. Plant based phosphorus recovery from
813 wastewater via algae and macrophytes. *Current Opinion in Biotechnology*, 23, 884-889.
- 814
- 815 Shoener, B.D., Bradley, I.M., Cusick, R.D., Guest, J.S., 2014. Energy positive domestic
816 wastewater treatment: the roles of anaerobic and phototrophic technologies. *Environ-*
817 *mental Science Process and Impacts*, 16, 1204-1222.
- 818
- 819 Sheik, A.R., Muller, E.E.L., Wilmes, P., 2014. A hundred years of activated sludge:
820 time for a rethink. *Frontiers in Microbiology*, 47(5), 1-7.
- 821
- 822 Sin, G., Villez, K., Vanrolleghem, P.A., 2006. Application of a model-based optimisa-
823 tion methodology for nutrient removing SBRs leads to falsification of the model. *Water*
824 *Science and Technology*, 53(4-5), 95-103.
- 825
- 826 Sun, H., Spring, S., Lapidus, A., Davenport, K., Del Rio, T.G., Tice, H., Nolan, M.,
827 Copeland, A., Cheng, J.F., Lucas, S., Tapia, R., Goodwin, L., Pitluck, S., Ivanova, N.,
828 Pagani, I., Mavromatis, K., Ovchinnikova, G., Pati, A., Chen, A., Palaniappan, K.,
829 Hauser, L., Chang, Y.J., Jeffries, C.D., Detter, J.C., Han, C., Rohde, M., Brambilla, E.,

- 830 Göker, M., Woyke, T., Bristow, J., Eisen, J.A., Markowitz, V., Hugenholtz, P.,
831 Kyrpides, N.C., Klenl, H.P., Land, M., 2010. Complete genome sequence of *Desulfar-*
832 *culus baarsii* type strain (2st14^T). *Standards in Genomic Sciences*, 3, 276-284.
- 833
- 834 Tandoi, V., Jenkins, D., Wanner, J. 2006. *Activated sludge separation problems: theory,*
835 *control measurements, practical experiences.*, London, UK.
- 836
- 837 Terada, A., Lackner, S., Kristensen, K., Smets, B.F., 2010. Inoculum effects on com-
838 munity composition and nitrification performance of autotrophic nitrifying biofilm reactors
839 with counter-diffusion geometry. *Environmental Microbiology*, 12(10), 2858-2872.
- 840
- 841 Vaiopoulou, E., Melidis, P., Aivasidis, A., 2007. Growth of filamentous bacteria in an
842 enhanced biological phosphorus removal system. *Desalination*, 213, 288-296.
- 843
- 844 Valverde-Pérez, B., Ramin, E., Smets, B.F., Plósz, B.Gy., 2015. EBP2R – An innova-
845 tive enhanced biological nutrient recovery activated sludge system to produce growth
846 medium for green microalgae cultivation. *Water Research*, 68, 821-830.
- 847
- 848 Valverde-Pérez, B., Wágner, D.S., Cecchin, F., Jensen, C.J., Smets, B.F., Plósz, B. Gy.,
849 2015b. Impact of operational conditions and process configuration on process perfor-
850 mance and microbial community in short solid retention time EBPR systems. 1st IWA
851 Resource Recovery Conference, Ghent, Belgium.

852

853 Valverde-Pérez, B., 2015. Wastewater resource recovery via the enhanced biological
854 phosphorus removal and recovery (EBP2R) process coupled with green microalgae cul-
855 tivation. PhD thesis, Technical University of Denmark, Kgs. Lyngby, Denmark.

856

857 Valverde-Pérez, B., Fuentes-Martínez, J.M., Flores-Alsina, X., Gernaey, K.V., Huusom,
858 J.K., Plósz, B.Gy., 2016. Control structure design for resource recovery using the en-
859 hanced biological phosphorus removal and recovery (EBP2R) activated sludge process.
860 Chemical Engineering Journal, 296, 447-457.

861

862 Van den Brand, T.P.H., Roest, K., Chen, G.H., Brdjanovic, D., van Loosdrecht,
863 M.C.M., 2015. Occurrence and activity of sulphate reducing bacteria in aerobic activat-
864 ed sludge systems. World Journal on Microbiology and Biotechnology, 31, 507-516.

865

866 Verstraete, W., Van de Caveye, P., Diamantis, V., 2009. Maximum use of resources
867 present in domestic “used water”. Bioresource Technology, 100, 5537-5545.

868

869 Wágner, D.S., Ramin, E., Szabo, P., Dechesne, A., Plósz, B.Gy., 2015. *Microthrix par-*
870 *vicella* abundance associates with activated sludge settling velocity and rheology –
871 Quantifying and modelling filamentous bulking. Water Research, 78, 121-132.

872

873 Wang, Y., Zhou, S., Ye, L., Wang, H., Stephenson, T., Jiang, X., 2014. Nitrite survival
874 and nitrous oxide production of denitrifying phosphorus removal sludges in long-term
875 nitrite/nitrate-fed sequencing batch reactors. *Water Research*, 67, 33-45.

876

877 Whang, L.M., Park, J.K., 2006. Competition between polyphosphate and glycogen ac-
878 cumulating organisms in enhanced biological phosphorus removal systems: effect of
879 temperature and sludge age. *Water Environment Research*, 78, 4-11.

880

881 Wu, D., Ekama, G.A., Wang, H.G., Wei, L., Lu, H., Chui, H.K., Liu, W.T., Brdjanovic,
882 D., van Loosdrecht, M.C.M., Chen, G.H., 2014. Simultaneous nitrogen and phosphorus
883 removal in the sulfur cycle-associated Enhanced Biological Phosphorus Removal
884 (EBPR) process. *Water Research*, 49, 251-264.

885

886 Yamamoto-Ikemoto, R., Komori, T., Matsui, S., 1991. Filamentous bulking and hinder-
887 ance of phosphate removal due to sulfate reduction in activated sludge. *Water Science
888 and Technology*, 23, 927-935.

889

890 Yamamoto-Ikemoto, R., Matsui, S., Komori, T., 1994. Ecological interactions among
891 denitrification, poly-p accumulation, sulfate reduction, and filamentous sulfur bacteria
892 in activated sludge. *Water Science and Technology*, 30(11), 201-210.

893

894 Yamamoto-Ikemoto, R., Matsui, S., Komori, T., Bosque-Hamilton, E.J., 1996. Symbio-
895 sis and competition among sulfate reduction, filamentous sulfur, denitrification and
896 poly-P accumulation bacteria in the anaerobic-oxic activated sludge of a municipal
897 plant. *Water Science and Technology*, 34(5-6), 119-128.

898

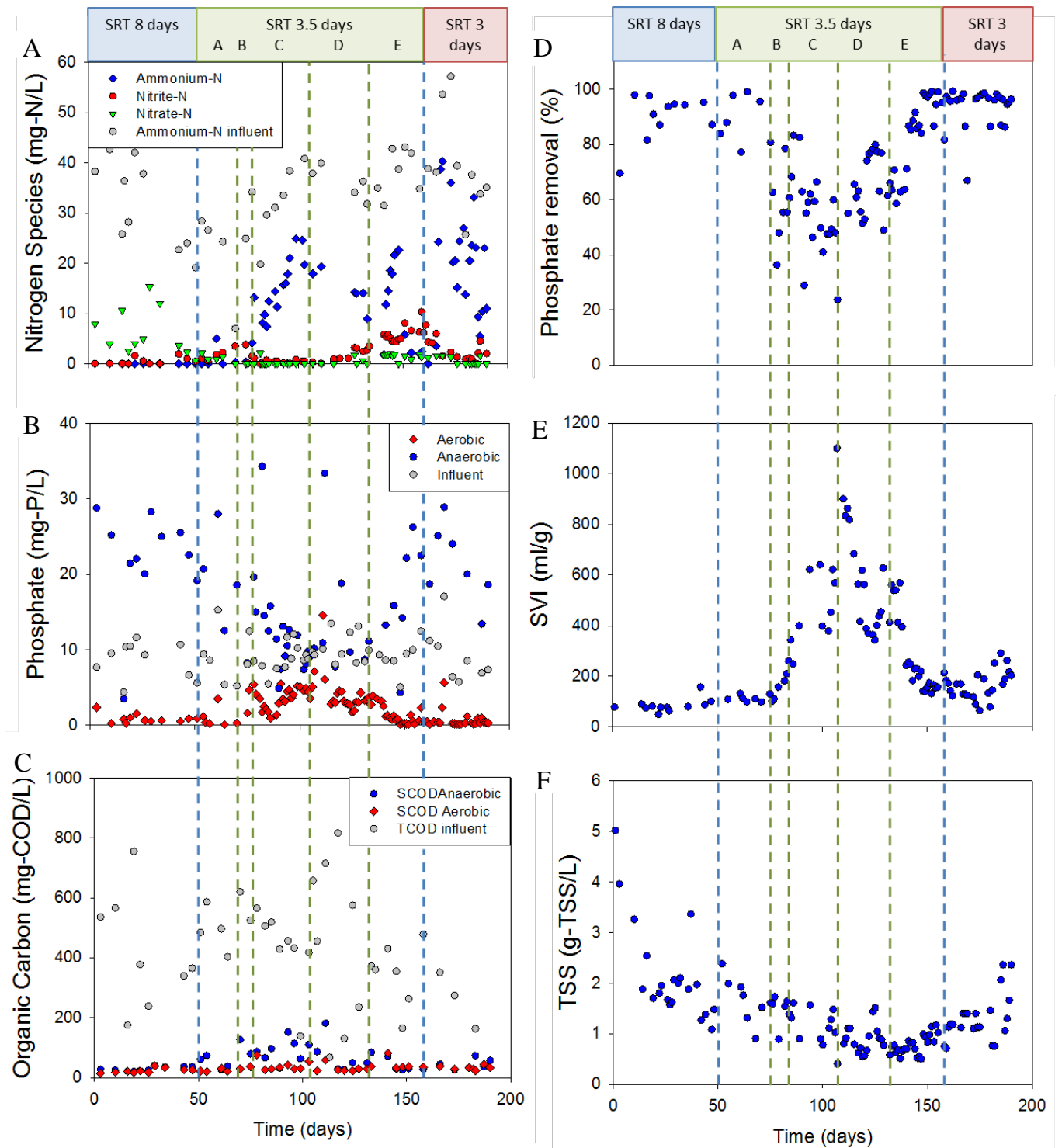
899 Yuan, Z., Pratt, S., Batstone, D.J., 2012. Phosphorus recovery from wastewater through
900 microbial processes. *Current opinion in biotechnology*, 23, 878-883.

901

902 Zhou, J., He, Q., Hemme, C.L., Mukhopadhyay, A., Hillesland, K., Zhou, A., He, Z.,
903 Van Nostrand, J.D., Hazen, T.C., Stahl, D.A., Wall, J.D., Arkin, A.P., 2011. How sul-
904 phate-reducing microorganisms cope with stress: lessons from systems biology. *Nature*,
905 9, 452-466.

906

907

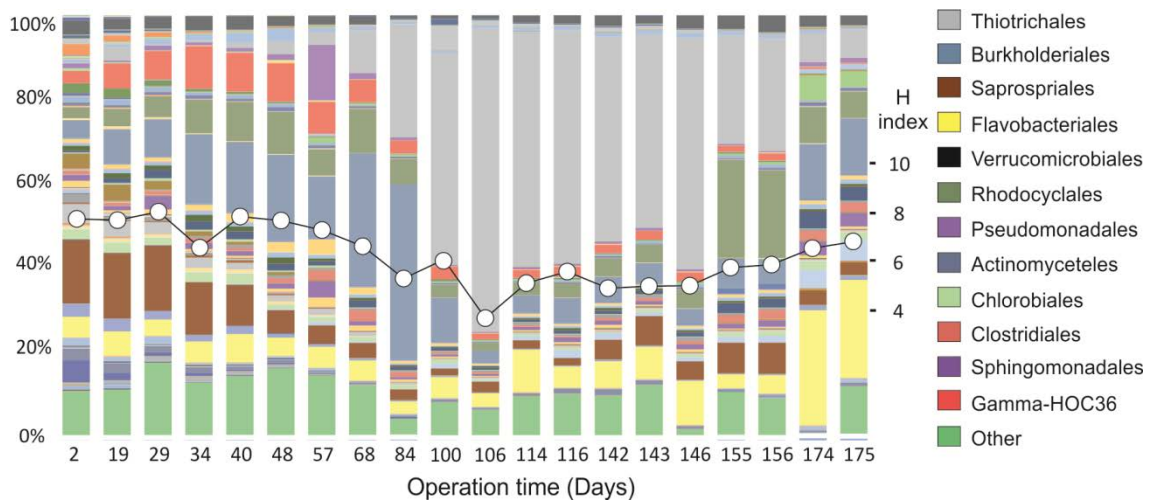


908

909 **Figure 1:** Reactor performance through 190 days a) Ammonia, nitrite and nitrate at the end of the aer-
 910 obic phase and ammonia in the influent; b) phosphate at the end of the aerobic and anaerobic phases

911 and influent; c) soluble COD at the end of the anaerobic and aerobic phases and total COD in the influ-
 912 ent; d) phosphate removal; e) sludge volume index; f) total suspended solids. Phase A: from day 50 to
 913 day 78 – anaerobic SRT=1.2 d and aerobic SRT=1.75 d; phase B: from day 78 to 83 – anaerobic
 914 SRT=1.2 d and aerobic SRT=1.45 d; phase C: from day 83 to 109 – anaerobic SRT=1.2 d and aerobic
 915 SRT=1.75 d; phase D: from day 109 to day 132 – anaerobic SRT=0.88 d and aerobic SRT=1.75 d;
 916 phase E: from day 132 to day 156 – anaerobic SRT=0.68 d and aerobic SRT=1.75 d.

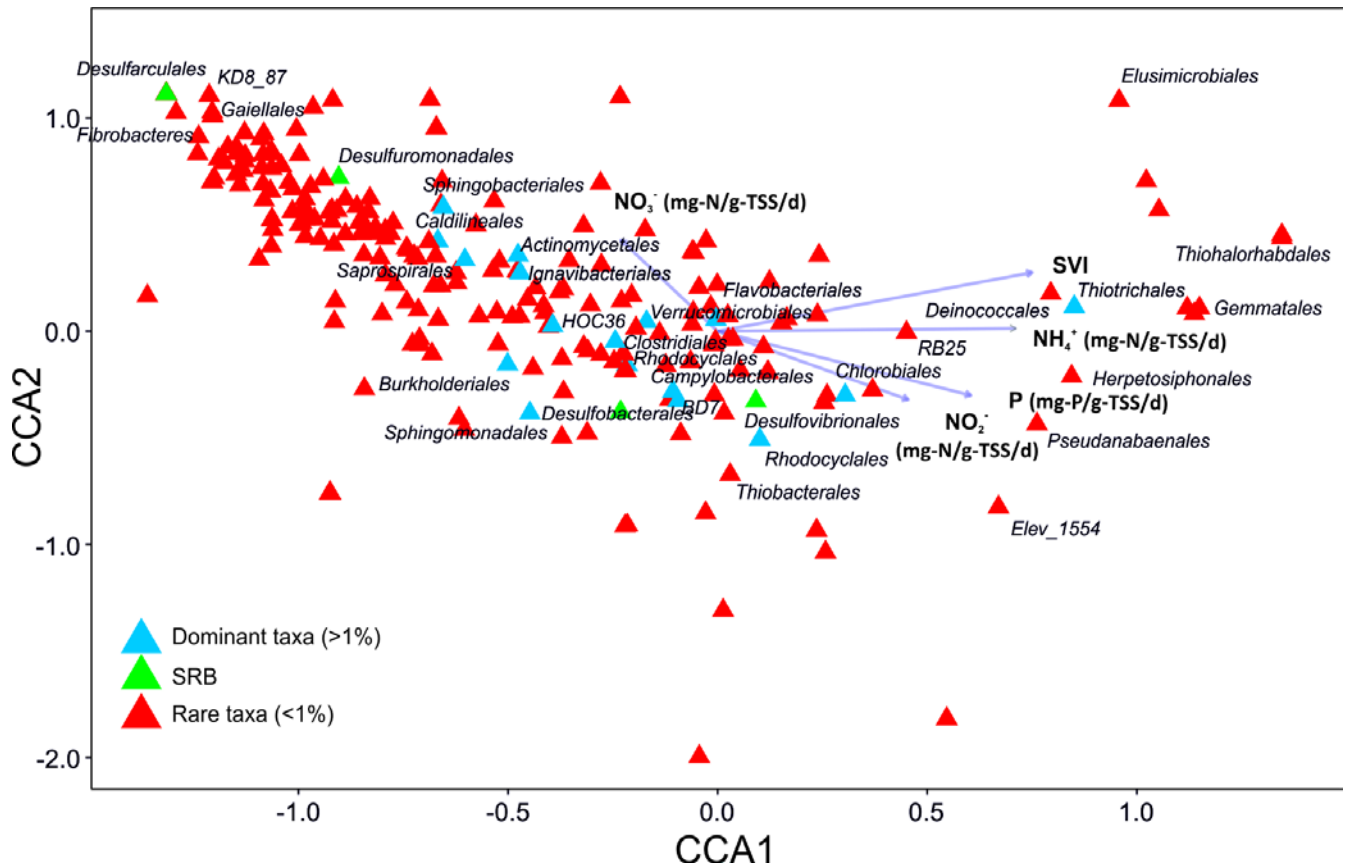
917
 918



919

920 **Figure 2:** Order-level taxonomic classification of 16S rRNA amplicons at selected days of the reactor
 921 operation. Taxa abundance is expressed in percentage (left axis). Alpha-diversity at the order level
 922 measured as Shannex index (white dots, right axis).

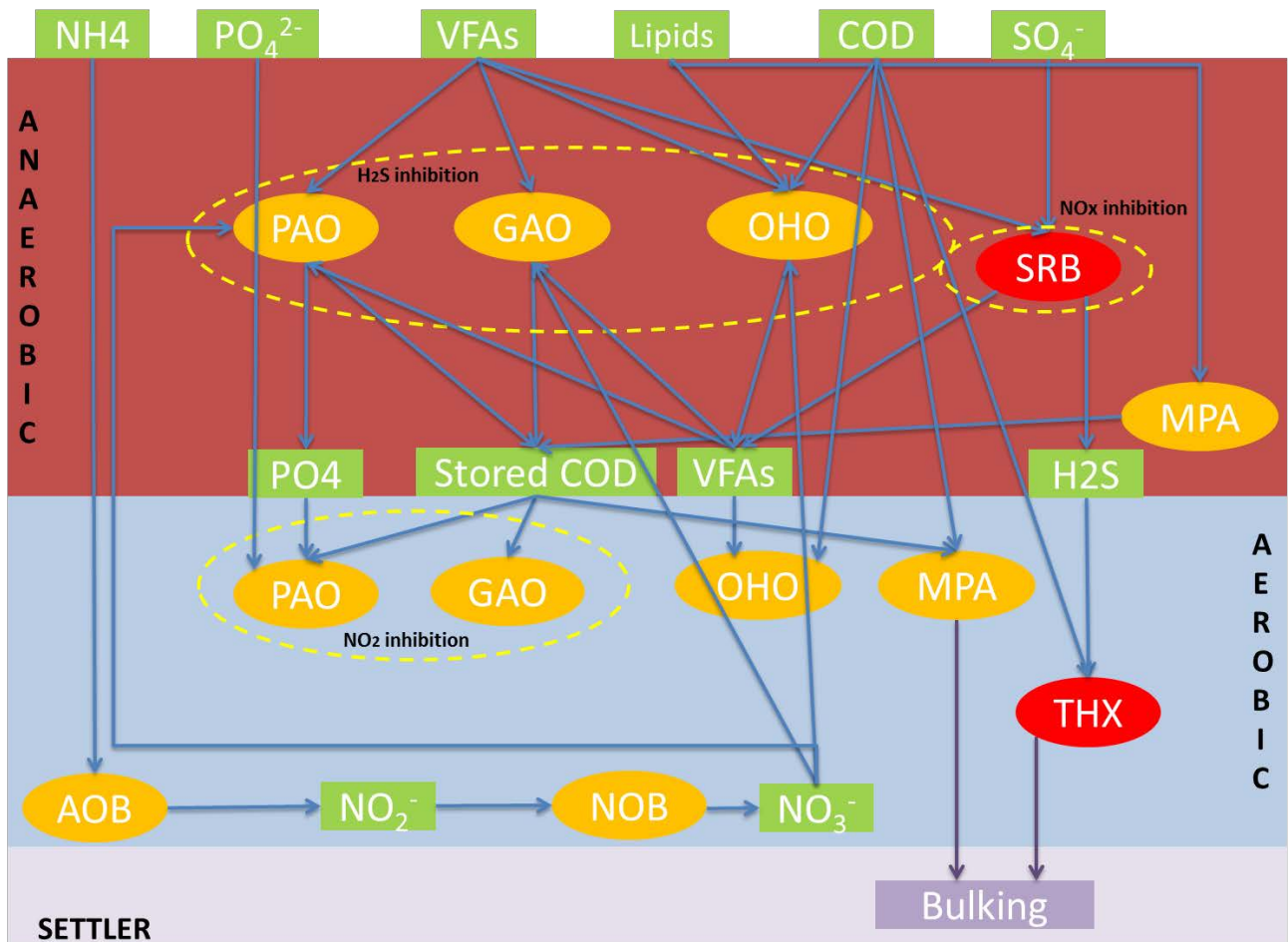
923



924

925 **Figure 3:** Canonical correspondence analysis (CCA) of order-level taxa within samples taken at time
 926 points during reactor operation constrained by five environmental variables: SVI (mL g^{-1}), ammonium
 927 removal ($\text{mg-N L}^{-1} \text{g-VSS}^{-1}$), nitrite accumulation ($\text{mg-N L}^{-1} \text{g-VSS}^{-1}$), nitrate accumulation (mg-N L^{-1}
 928 g-VSS^{-1}) and phosphorus removal ($\text{mg-N L}^{-1} \text{g-VSS}^{-1}$). The Bray Curtis algorithm was applied to calcu-
 929 late dissimilarity values.

930



931

932 **Figure 4:** Ecological model proposed for the low-SRT EBPR system. Circled in yellow
 933 those microbial groups inhibited by chemicals (e.g. nitrite). Colored in red those micro-
 934 bial niches playing an important role in short-SRT EBPR systems but not acknowledged
 935 in previous studies. Blue arrows represent the substrate and products flows. PAO: poly-
 936 phosphate accumulating organisms; GAO: glycogen accumulating organisms; OHO:
 937 ordinary heterotrophic organisms; SRB: sulphate reducing bacteria; MPA: *Microthrix*
 938 *parvicella*; THX: *Thiothrix*; AOB: ammonia oxidizing bacteria; NOB: nitrite oxidizing
 939 bacteria.

940

941 **Table 1:** Operational conditions during the start-up and operation of the short-SRT
 942 EBPR:

Period (day)	Global SRT (day)	Anaerobic SRT (day)	Aerobic SRT (day)	Oxygen level (mg L⁻¹)
1-50	8	2.67	4	2-3
51-78	3.5	1.2	1.75	
79-83			1.45	
84-109			1.75	2-3 (first week 0.5-2)
110-132		0.88	>4.5	
133-149		0.68		
150-190	3	0.58	1.5	2-3

943

944

945 **Supplementary information**

946

947 **Short-sludge age EBPR process – microbial and biochem-**
948 **ical process characterisation during reactor start-up and**
949 **operation**

950 Borja Valverde-Pérez*, Dorottya S. Wágner, Bálint Lóránt, Arda Gülay, Barth F.
951 Smets, Benedek Gy. Plósz*

952 Department of Environmental Engineering (DTU Environment), Technical University of Denmark, Miljøvej, Building 115, DK-
953 2800, Kgs. Lyngby, Denmark. E-mails: bvape@env.dtu.dk; beep@env.dtu.dk

954 *Corresponding authors

955

956

957

7 Quantitative polymerase chain reaction (qPCR)

Reactor biomass was collected as 2 mL samples, centrifuged at 13000xg, supernatants were removed and pellets were stored at -20 °C until further analysis. A 0.5 g subsample of reactor samples was subject to genomic DNA extraction using the MP FastDNA™ SPIN Kit (MP Biomedicals LLC., Solon, USA) per manufacturer's instructions. The concentration and purity of extracted DNA were checked by spectrophotometry (NanoDrop Technologies, Wilmington, DE, USA). Extracted DNA was stored at -20 °C in Tris-EDTA buffer until further processing. Quantitative PCR (qPCR) was carried out on all the extracted DNA samples to determine the abundance of ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB, both *Nitrobacter* and *Nitrospira*) as suggested by Terada et al., (2010), *Thiothrix* (protocol adapted from Vervaeren et al., 2005), *Microthrix parvicella* (protocol adapted from Kaetzke et al., 2005; Kumari et al., 2009; and Vanysacker et al., 2014) and sulphate reducing bacteria (SRB, protocol adapted from Wagner et al, 1998; and Geets et al., 2006). Primers used in this work to target the 16S rRNA genes and the functional gene *dsrB* for SRB are included in Table S1, together with the PCR protocols. For quantification, 1 copy of 16S rRNA gene for AOB and NOB, 1.33 copies of 16S rRNA gene for *Microthrix parvicella*, and 4.1 copies of 16S rRNA gene for total bacteria (Stoddard et al., 2014), 2 copies of 16S rRNA gene for *Thiothrix* (Lapidus et al., 2011) and 1 copy of the functional gene *dsrB* for SRB (Karkhoff-Schweizer et al., 1995) were considered.

Table S1: Primer sets for qPCR

Organism	Gene	Primers	Sequence (5' → 3')	Reference
Total Bacteria	16S- rRNA	1055f	ATGGCTGTCGTCAGCT	Ferris et al. 1996
		1392r	ACGGGCGGTGTGTAC	Lane 1991
β-proteobacterial AOB	16S- rRNA	CTO189fA/B	GGAGRAAAGCAGGGGATCG	Kowalchuk et al. 1997
		CTO 189fC	GGAGGAAAGTAGGGGATCG	Hermansson and Lindgren 2001
		RT1r	CGTCCTCTCAGACCARCTACTG	
<i>Nitrospira</i> genus NOB	16S- rRNA	Nspra-675f	GCGGTGAAATGCGTAGAKATCG	Graham et al. 2007
		Nspra-746r	TTTTTTGAGATTTGCTAG	
<i>Nitrobacter</i> genus NOB	16S- rRNA	FGPS872f	CTAAAACTCAAAGGAATTGA	Degrange and Bardin, 1995
		FGPS1269r	TTTTTTGAGATTTGCTAG	
Sulfate reducing bacteria	dsrA	RH1-dsr-F	GCCGTTACTGTGACCAGCC	Ben-Dov et al. 2007
		RH3-dsr-R	GGTGGAGCCGTGCATGTT	
	dsrB	DSRp2060F	CAACATCGTYCAYACCCAGGG	Geets et al. 2006
		DSR4R	GTGTAGCAGTTACCGCA	Wagner et al. 1998
<i>Thiothrix</i>	16S- rRNA	21Nf	CGTAGGCGGCTCTTTAAGTCRGAT	Vervaeren et al. 2005
		21Nr	CCGACGGCTAGTTGACATCGTTTA	
<i>Microthrix par- vicella</i>	16S- rRNA	S-S-M.par-0828- S-21f	GGTGTGGGGAGAACTCAACTC	Kaetzke, A. et al. 2005
		S-S-M.par-1018- A-17r	GACCCCGAAGGACACCG	Kumari, A. et al. 2009
				Vanysacker, L. et al. 2014

Table S2: Real time quantitative PCR conditions for “Most Bacteria”

	Temperature [°C]	Time [min:sec]	
1. Pre-heating	94	5:00	
2. Denaturation	94	0:30	
3. Annealing	55	0:30	
4. Elongation	72	1:00	
5. Plate read		0:01	Back to 2. Repeat 39 times
6. Melting curve	70-95	0:01	Gradient 0.2°C/s
7. Cooling	20	∞	

Table S3: Real time quantitative PCR conditions for AOB

	Temperature [°C]	Time [min:sec]	
1. Pre-heating	94	5:00	
2. Denaturation	94	0:30	
3. Annealing	60	0:30	
4. Elongation	72	1:00	
5. Plate read		0:01	Back to 2. Repeat 39 times
6. Melting curve	70-95	0:01	Gradient 0.2°C/s
7. Cooling	20	∞	

Table S4: Real time quantitative PCR conditions for *Nitrospira sp.*

	Temperature [°C]	Time [min:sec]	
1. Pre-heating	94	5:00	
2. Denaturation	94	0:30	
3. Annealing	64	0:30	
4. Elongation	72	1:00	
5. Plate read		0:01	Back to 2. Repeat 39 times
6. Melting curve	70-95	0:01	Gradient 0.2°C/s
7. Cooling	20	∞	

Table S5: Real time quantitative PCR conditions for *Nitrobacter*

	Temperature [°C]	Time [min:sec]	
1. Pre-heating	95	5:00	
2. Denaturation	94	0:45	
3. Annealing	50	1:00	
4. Elongation	72	1:00	
5. Plate read			Back to 2. Repeat 39 times
6. Melting curve	70-95	0:01	Gradient 0.2°C/s
7. Cooling	20	∞	

Table S6: Real time quantitative PCR conditions for Sulphate reducing bacteria *dsrA* gene

	Temperature [°C]	Time [min:sec]	
1. Pre-heating	95	5:00	
2. Denaturation	95	0:15	
3. Annealing	60	1:00	
4. Elongation	72	1:00	
5. Plate read			Back to 2. Repeat 40 times
6. Melting curve	70-95	0:01	0,2 °C/s
7. Cooling	20	∞	

Table S7: Real time quantitative PCR conditions for Sulphate reducing bacteria *dsrB* gene

	Temperature [°C]	Time [min:sec]	
1. Pre-heating	94	5:00	
2. Denaturation	94	1:00	
3. Annealing	56,9	1:00	
4. Elongation	72	1:00	
5. Plate read			Back to 2. Repeat 39 times
6. Melting curve	70-95	0:01	0,2 °C/s
7. Cooling	20	∞	

Table S8: Real time quantitative PCR conditions for *Thiothrix*

	Temperature [°C]	Time [min:sec]	
1. Pre-heating	95	5:00	
2. Denaturation	95	0:45	
3. Annealing	60	1:00	
4. Elongation	72	1:00	
5. Plate read			Back to 2. Repeat 39 times
6. Melting curve	70-95	0:01	0,2 °C/s
7. Cooling	20	∞	

Table S9: Real time quantitative PCR conditions for *Microthrix parvicella*

	Temperature [°C]	Time [min:sec]	
1. Pre-heating	94	5:00	
2. Denaturation	94	0:45	
3. Annealing	63 (-0,5°C per cycle)	0:45	Touchdown 63-54°C
4. Elongation	72	2:00	
5. Plate read			
6. Denaturation	94	0:45	
7. Annealing	54	0:45	
8. Elongation	72	0:45	
9. Plate read			Back to 6. Repeat 17 times
10. Melting curve	70-95	0:01	0,2 °C/s
11. Cooling	20	∞	

8 Quantitative fluorescence *in-situ* hybridization (qFISH) analysis:

The samples were pre-treated and fixed with 4% paraformaldehyde and stored at -20°C until qFISH analysis was done. 1-5µl of fixed sample were used for the analysis. The EUBmix probe, which comprises EUB338, EUB338-II and EUB338-III, was used to target total bacteria (Daims et al., 1999). *Accumulibacter* were targeted using PAOmix probes (PAO 651, 462 and 846, Crocetti et al., 2000) and *Tetrasphaera* were targeted with Actino-221a and Actino-658a, together with the competitors per Kong et al. (2005). *Competibacter* were targeted using the GB probe by Kong et al. (2002). *Defluviicoccus* cluser I was targeted using probes TFO_DF218 and TFO_DF618 (Wong et al., 2004) and cluster II was targeted using probes DF988 and DF1020 combined with the helper probes per Meyer et al. (2006). A Leica SP5 confocal laser scanning microscope with a 20x objective was used to image the samples. As suggested by Nielsen et al. (2009), 20 randomly chosen images were taken and analysed using daime (digital image analysis in microbial ecology) software (Daime et al., 2006).

Table S10: Probes for qFISH analysis

Organism	Probe	Dye	Sequence (5' → 3')	Reference
Most Eubacteria	EUB338		GCTGCCTCCCGTAGGAGT	Amann et al., 1990
Planctomycetales	EUB338-II	Fluo (green)	GCAGCCACCCGTAGGTGT	Daims et al., 1999
Verrumicrobiales	EUB338-III		GCTGCCACCCGTAGGTGT	Daims et al., 1999
Most <i>Accumuli-</i> <i>bacter</i>	PAO462		CCGTCATCTACWCAGGGTATTAAC	Crocetti et al., 2000
	PAO651	Cy3 (red)	CCCTCTGCCAAACTCCAG	
	PAO846		GTTAGCTACGGCACTAAAAGG	
<i>Actinobacteria</i> (<i>Tetrasphaera</i>)	Actino-221a	Cy5 (blue)	CGCAGGTCCATCCCAGAC	Kong et al., 2005
	c1Actino-221	-	CGCAGGTCCATCCCATAC	
	c2Actino-221	-	CGCAGGTCCATCCCAGAG	
	Actino-658a	Cy5 (blue)	TCCGGTCTCCCCTACCAT	
	c1Actino-658	-	TCCGGTCTCCCCTACCAC	
	c2Actino-658	-	ATTCCAGTCTCCCCTACCAT	
<i>Competibacter</i>	GB	Cy5 (blue)	CGATCCTCTAGCCCACT	Kong et al., 2002
<i>Defluviicoccus</i> (cluser I)	TFO_DF218		GAAGCCTTTGCCCTCAG	Wong et al., 2004
	TFO_DF618	Cy3 (red)	GCCTCACTTGTCTAACCG	
<i>Defluviicoccus</i>	DF988	Cy3 (red)	GATACGACGCCCATGTCAAGGG	Meyer et al., 2006

(cluser II)	H966	-	CTGGTAAGGTTCTGCGTTGC
	DF1020	Cy3 (red)	CCGGCCGAACCGACTCCC
	H1038	-	AGCAGCCATGCAGCACCTGTGTGGCGT

9 PCR amplification, pyrosequencing and bioinformatics analysis

9.1 PCR amplification and pyrosequencing

10 ng of extracted DNA were PCR amplified using Phusion (Pfu) DNA polymerase (Finnzymes, Finland) and 16S rRNA gene targeted modified universal primers PRK341F (5'- CCTAYGGGRBGCASCAG-3') and PRK806R (5'- GGACTACNNGGGTATCTAAT-3') to amplify the V3-V4 hypervariable region (Yu et al., 2005). PCR was performed as follows: an initial denaturation at 98 °C for 30 s, 30 cycles of denaturation at 98 °C for 5 s, annealing at 56 °C for 20 s and elongation at 72 °C for 20 s, and a final elongation step at 72 °C for 5 min. PCR products were analysed and cut from 1% agarose gel and purified by QIAEX II Gel Extraction Kit (QIAGEN). Sequencing was applied using the Illumina MiSeq platform at the DTU Multi Assay Core Center (Copenhagen, DK).

9.2 Bioinformatics analysis

The Paired End sequences from the raw fastq files supplied by Illumina MiSeq were assembled using the pandaseq software (Masella et al., 2012). All assembled sequences shorter than 200 nts and longer than 500 nts were removed. The primers were also removed from the sequences. Chimeras were removed with UCHIME (Edgar et al., 2011) using default settings. After quality checks, all analyses were performed using QIIME 1.4.0 software (Caporaso et al., 2010). High quality sequences were clustered into OTUs at 97% pairwise identity using the UCLUST algorithm (Edgar et al., 2011) with default settings, and representative sequences from each OTU were aligned against the Greengenes reference alignment (DeSantis et al., 2006) using PyNAST (Coporaso et al., 2010). Taxonomy assignment of each representative sequence was implemented using the BLAST algorithm (Altschul et al., 1997) against the Silva119 curated database. Unassigned sequences were re-

moved from the sequence library and subsampling at depth of 11,800 sequences was performed to equalize sample sizes.

Canonical correspondence analysis (CCA) was used to examine the relationships of microbial communities and system performance variables. Significant correlations between specific taxa and phosphorus removal were confirmed with Pearson's product-moment correlation analysis. All statistical analysis were performed with the vegan package in R (The R Foundation, Vienna, Austria).

9.3 Sulphate reducing bacteria diversity analysis

PCR was carried out with primers targeting dissimilatory sulphite reductase subunit-A (dsrA, Ben-Dov et al., 2007) and subunit-B (dsrB, Geets et al., 2006). PCR products were purified, after length verification, via agarose gel electrophoresis and cloned. 96 clones of both dsrA and dsrB PCR products were collected and grown. Finally, the plasmids containing the insert were isolated, purified and sequenced (Macrogen, Amsterdam, Nederland). Sequencing results were processed by locating inserts and eliminating repetitive sequences. Phylogenetic analysis was done using ClustalX multiple alignment software and MEGA phylogenetic tree construction software.

10 Microbial diversity analysis – pyrosequencing

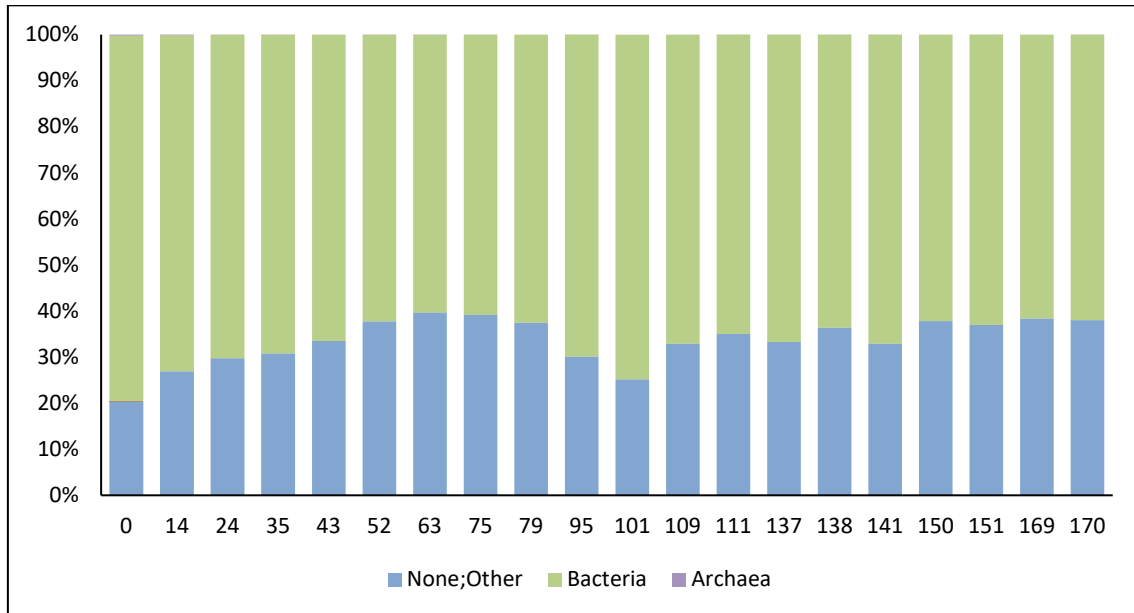


Figure S1: Domain-level taxonomic classification of 16S rRNA amplicons at selected time points of the reactor operation. Taxa abundance is expressed in percentage.

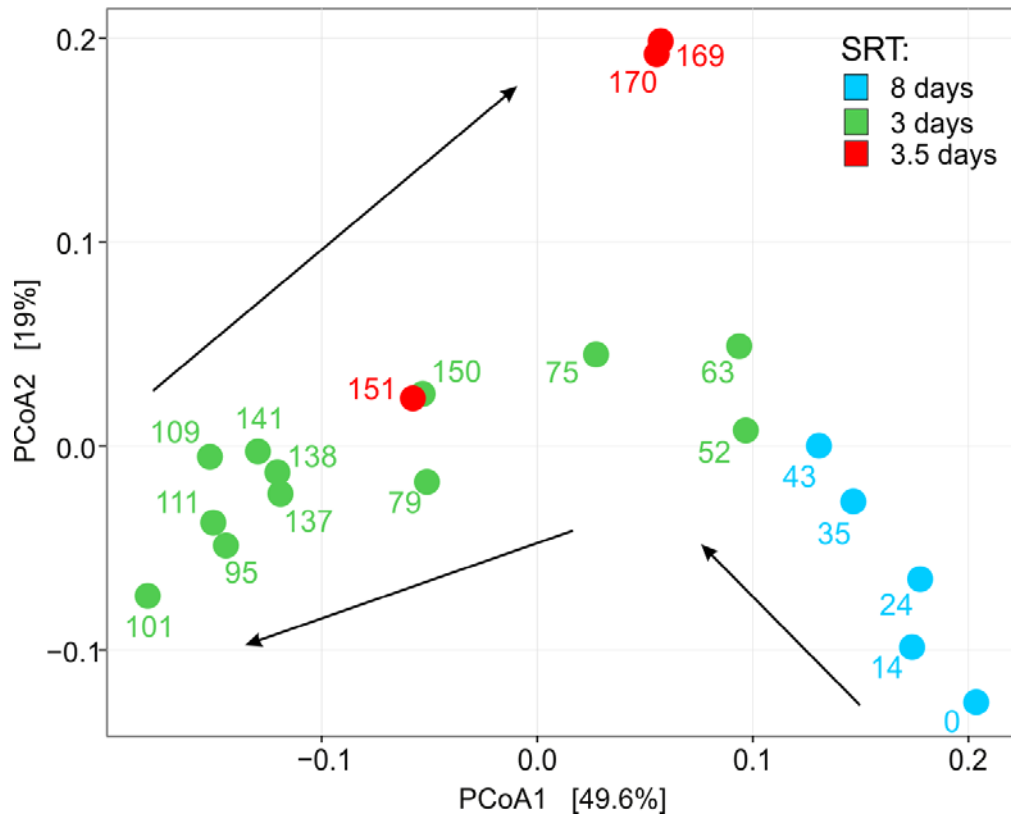


Figure S2: Phylogenetic distances between samples taken at different time points during reactor operation. The dissimilarity matrix was determined using Weighted UniFrac algorithm and plotted via principal coordinate analysis (PCoA). The percentage of variation explained by the principal coordinates is indicated on the axes.

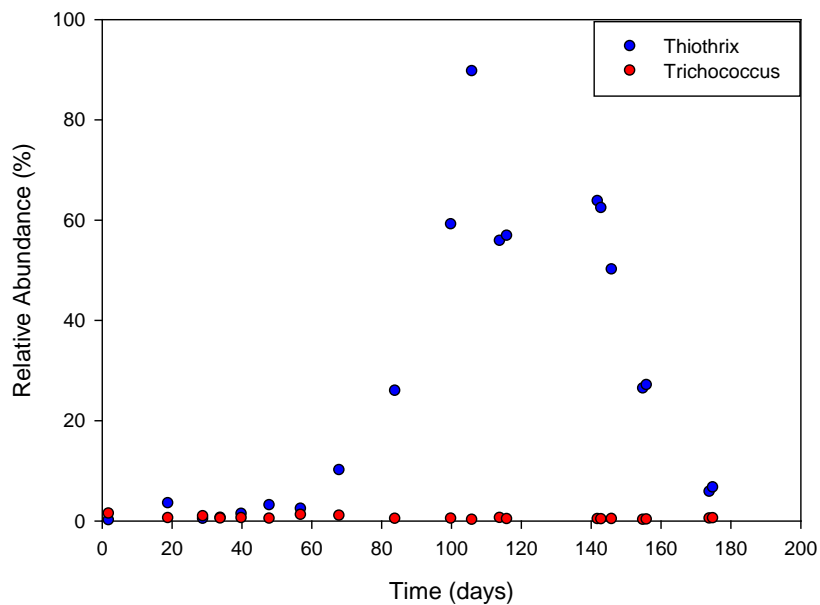


Figure S3: Relative abundance of filamentous bacteria along the operation of the short SRT EBPR. Only the 2 most abundant genera are shown.

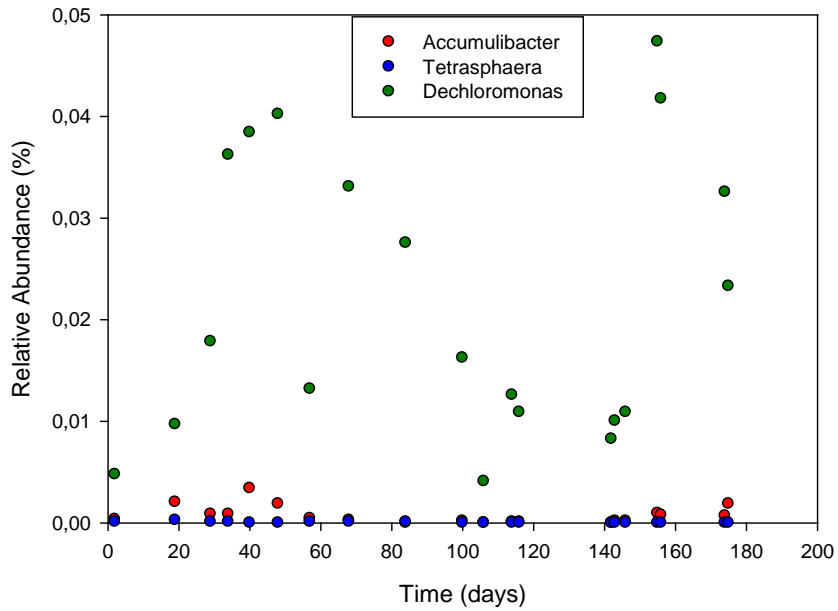


Figure S4: Relative abundance of PAO along the operation of the short SRT EBPR.

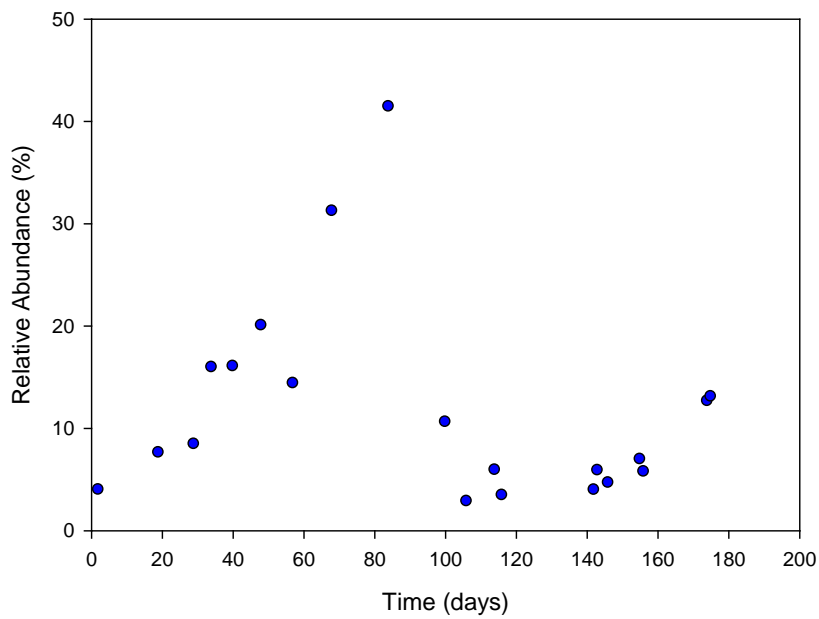


Figure S5: Relative abundance of *Comamonadaceae* along the operation of the short SRT EBPR.

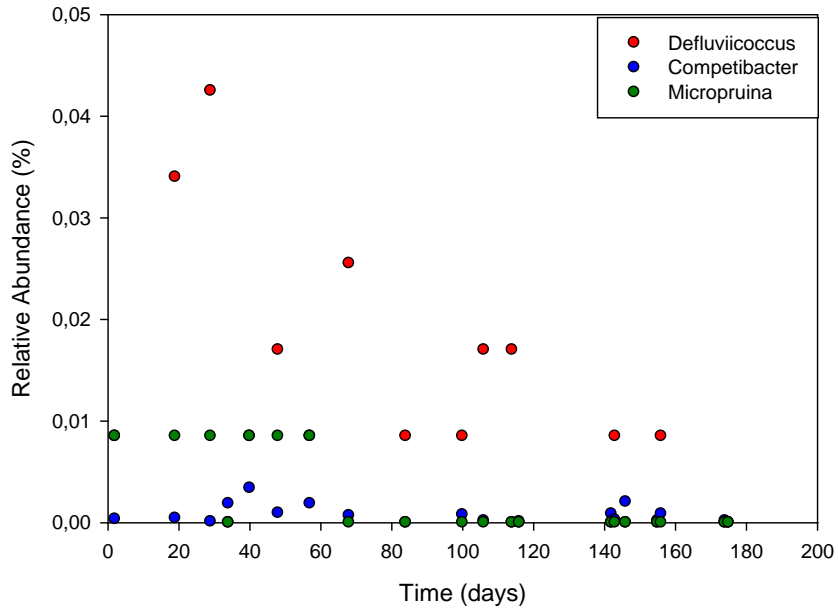


Figure S6: Relative abundance of GAO along the operation of the short-SRT EBPR.

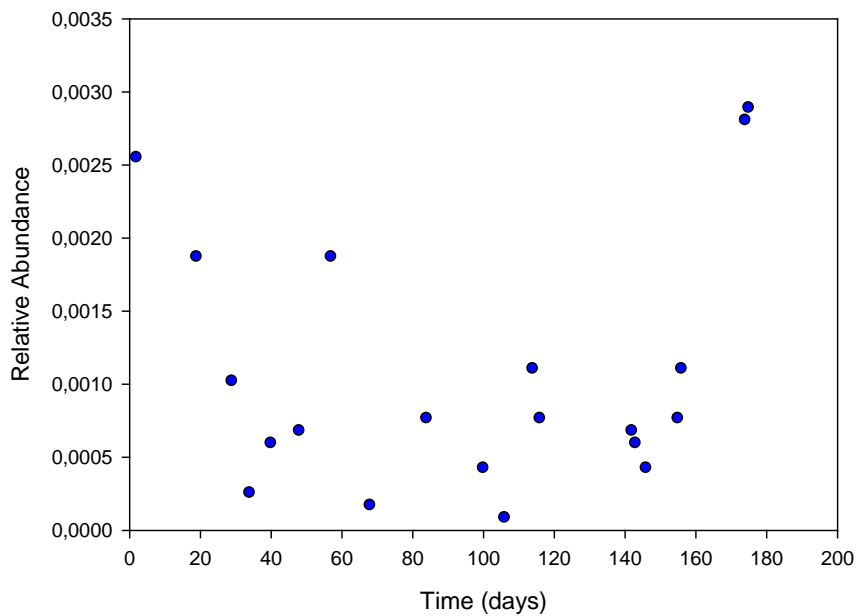


Figure S7: Relative abundance of SRBs along the operation of the short-SRT EBPR.

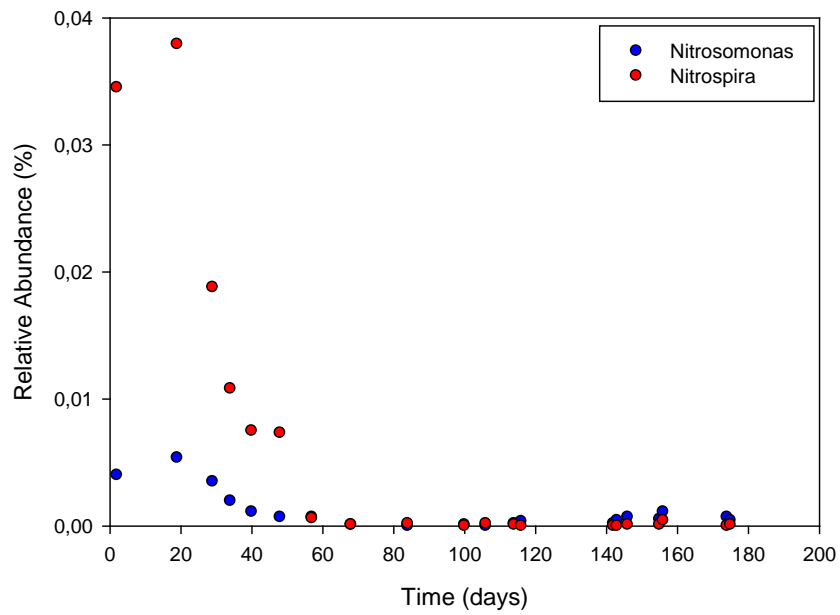


Figure S8: Relative abundance of AOB (*Nitrosomonas*) and NOB (*Nitrospira*) along the operation of the short-SRT EBPR.

Table S11: Relative abundance of different bacterial group (%) based on 16S rRNA gene amplicon sequencing results

Days	<i>Comamonadaceae</i>	<i>Trichococcus</i>	<i>Thiothrix</i>	<i>Accumulibacter</i>	<i>Tetrasphaera</i>	<i>Dechloromonas</i>	<i>Defluviicoccus</i>	<i>Competibacter</i>	<i>Micropurina</i>	<i>Nitrosomonas</i>	<i>Nitrospira</i>	SRB
2	3.9867	1.399	0.0594	3.40E-04	8.50E-05	4.76E-03	8.50E-03	3.40E-04	8.50E-03	4.00E-03	0.0345	2.55E-03
19	7.608	0.5003	3.4509	2.04E-03	2.55E-04	9.69E-03	0.034	4.25E-04	8.50E-03	5.36E-03	0.0379	1.87E-03
29	8.4495	0.8648	0.3392	8.50E-04	8.50E-05	0.0179	0.0425	8.50E-05	8.50E-03	3.49E-03	0.0188	1.02E-03
34	15.9555	0.3985	0.5596	8.50E-04	8.50E-05	0.0362	0	1.87E-03	0	1.96E-03	0.0108	2.55E-04
40	16.0575	0.5003	1.3481	3.40E-03	0	0.0384	8.50E-03	3.40E-03	8.50E-03	1.11E-03	7.48E-03	5.95E-04
48	20.0442	0.3815	3.0609	1.87E-03	0	0.0402	0.017	9.35E-04	8.50E-03	6.80E-04	7.31E-03	6.80E-04
57	14.3829	1.1446	2.3656	4.25E-04	8.50E-05	0.0132	8.50E-03	1.87E-03	8.50E-03	6.80E-04	5.95E-04	1.87E-03
68	31.2224	0.9751	10.056	2.55E-04	8.50E-05	0.0331	0.0255	6.80E-04	0	8.50E-05	8.50E-05	1.70E-04
84	41.423	0.3561	25.886	0	8.50E-05	0.0275	8.50E-03	0	0	0	1.70E-04	7.65E-04
100	10.6001	0.3731	59.0894	1.70E-04	0	0.0162	8.50E-03	7.65E-04	0	8.50E-05	0	4.25E-04
106	2.8562	0.1611	89.6134	0	0	4.08E-03	0.017	1.70E-04	0	0	1.70E-04	8.50E-05
142	3.9782	0.3052	63.7273	0	0	8.25E-03	0	8.50E-04	0	1.70E-04	0	6.80E-04
143	5.8824	0.2713	62.3368	1.70E-04	0	0.01	8.50E-03	2.55E-04	0	4.25E-04	0	5.95E-04
114	5.9164	0.5087	55.7741	8.50E-05	0	0.0126	0.017	0	0	1.70E-04	8.50E-05	1.11E-03
146	4.6668	0.3307	50.0933	1.70E-04	0	0.0109	0	2.04E-03	0	6.80E-04	8.50E-05	4.25E-04
116	3.4512	0.2968	56.8085	8.50E-05	0	0.0109	0	8.50E-05	0	3.40E-04	0	7.65E-04
155	6.9704	0.1526	26.3354	9.35E-04	0	0.0473	0	1.70E-04	0	5.10E-04	8.50E-05	7.65E-04
156	5.7548	0.2035	27.0307	7.65E-04	0	0.0417	8.50E-03	8.50E-04	0	1.11E-03	4.25E-04	1.11E-03
174	12.6488	0.407	5.7402	6.80E-04	0	0.0326	0	1.70E-04	0	6.80E-04	0	2.81E-03
175	13.0908	0.4579	6.622	1.87E-03	0	0.0233	0	0	0	4.25E-04	8.50E-05	2.89E-03

10.1 Complementary information about microbial diversity analysis – qPCR and qFISH

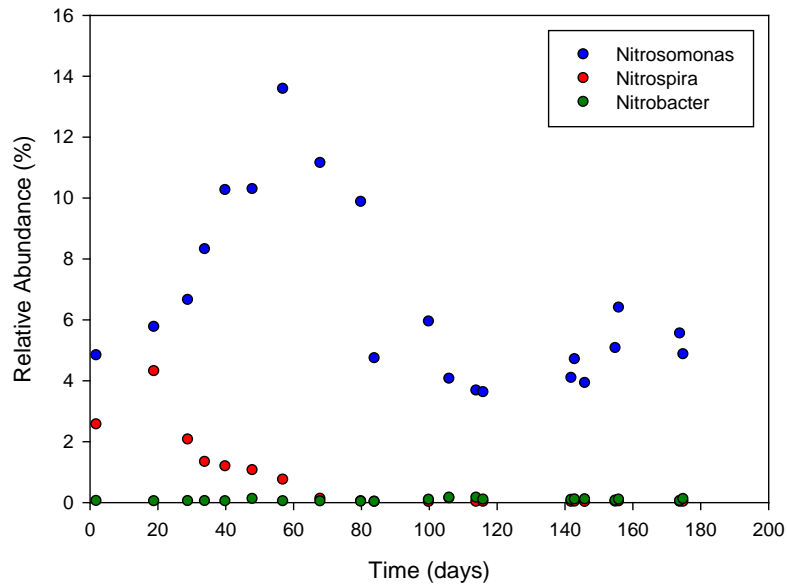


Figure S9: Relative abundance of AOBs along the operation of the short-SRT EBPR according to qPCR.

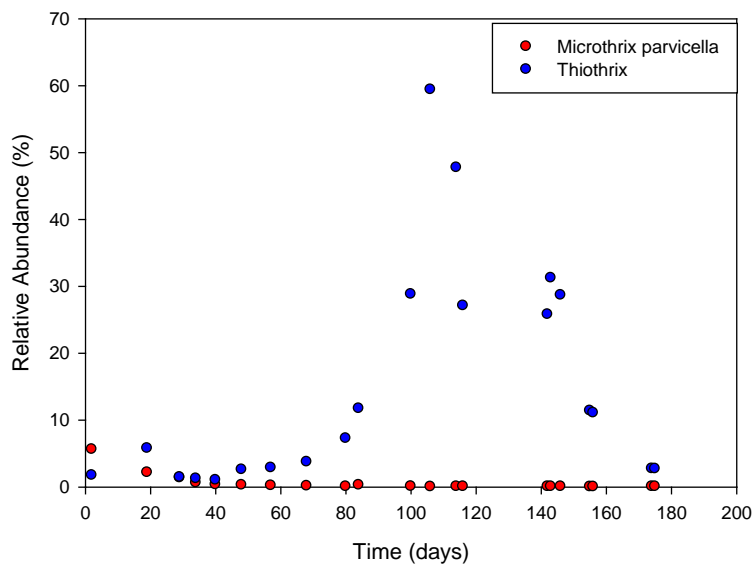


Figure S10: Relative abundance of filamentous bacteria along the operation of the short-SRT EBPR.

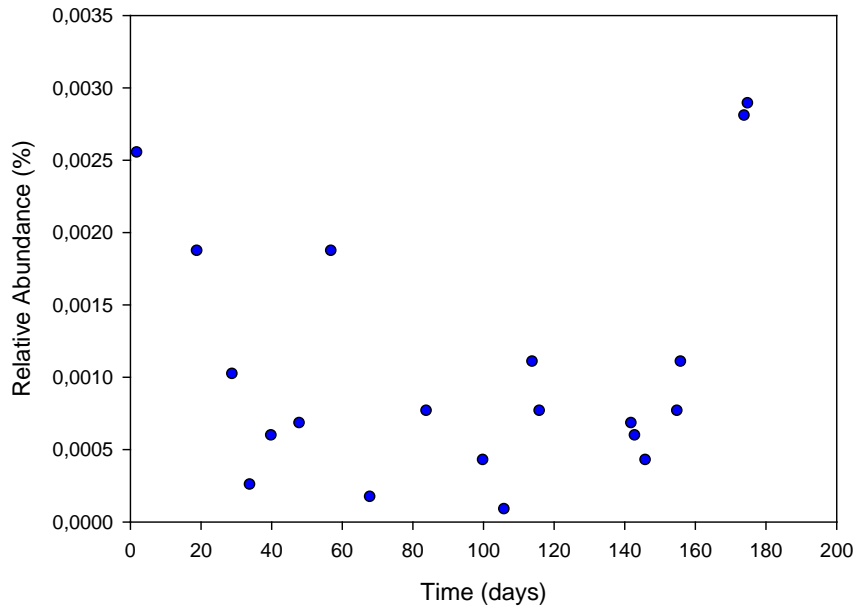


Figure S11: Relative abundance of SRB along the operation of the short-SRT EBPR.

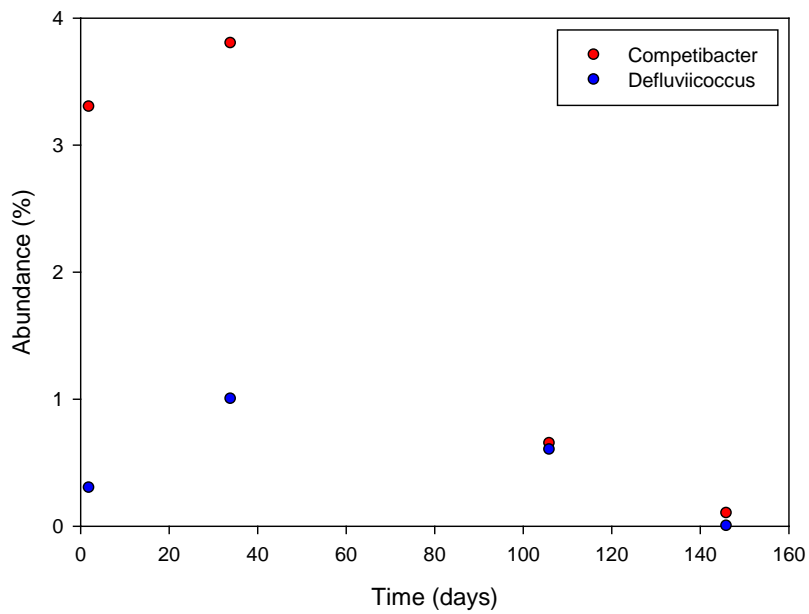


Figure S12: Relative abundance of GAO along the operation of the short-SRT EBPR.

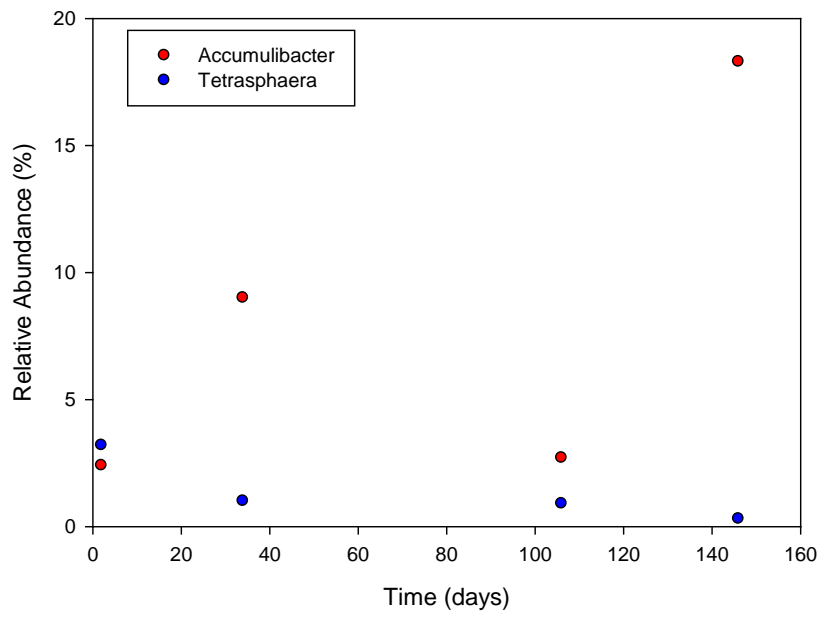


Figure S13: Relative abundance of PAO along the operation of the short-SRT EBPR.

Table S12: Relative abundance of different bacterial group (%) based on qPCR results (*Nitrosomonas*, *Nitrospira*, *Nitrobacter*, SRB, *Thiothrix* and *M. parvicella*) and qFISH (*Accumulibacter*, *Tetrasphaera*, *Competibacter* and *Defluviicoccus*)

Day	<i>Nitrosomonas</i>	<i>Nitrospira</i>	<i>Nitrobacter</i>	SRBs	<i>Thiothrix</i>	<i>M. parvicella</i>	<i>Accumulibacter</i>	<i>Tetrasphaera</i>	<i>Competibacter</i>	<i>Defluviicoccus</i>
2	4.823	2.5536	0.0392	0.0564	1.7425	5.6137	2.4	3.2	3.3	0.3
19	5.7574	4.2993	0.0313	0.0587	5.7705	2.1711				
29	6.6415	2.0573	0.0333	0.0505	1.446	1.3702				
34	8.3073	1.3198	0.0346	0.0319	1.263	0.6238	9	1	3.8	1
40	10.2485	1.1758	0.0304	0.0174	1.0369	0.3513				
48	10.2796	1.0503	0.1078	0.0261	2.5889	0.2784				
57	13.5748	0.7399	0.0314	0.0483	2.8757	0.2162				
68	11.1407	0.1112	0.0254	0.0812	3.7466	0.1475				
80	9.8615	0.0313	0.0166	0.0379	7.2613	0.0743				
84	4.7277	0.0131	0.0104	0.0377	11.7324	0.2807				
100	5.9333	5.97E-03	0.0807	0.053	28.8205	0.0879				
106	4.0536	0.1241	0.1455	0.0193	59.4082	0.0219	2.7	0.9	0.65	0.6
142	4.0811	0.0128	0.0765	0.0849	25.7995	0.0693				
143	4.6925	0.0149	0.087	0.0822	31.2367	0.0649				
114	3.6659	9.84E-03	0.1477	0.0439	47.7505	0.0698				
146	3.9116	7.46E-03	0.0932	0.0331	28.6741	0.0604	18.3	0.3	0.1	0
116	3.6107	0.0124	0.0856	0.0597	27.0951	0.0609				
155	5.0606	0.0163	0.0524	0.0384	11.3805	0.0364				
156	6.3885	0.0244	0.0843	0.0438	11.0835	0.0348				
174	5.5366	0.0131	0.0386	0.1249	2.732	0.0615				
175	4.855	0.0117	0.0998	0.1167	2.7143	0.0682				

10.2 Microbial diversity of sulphate reducing bacteria

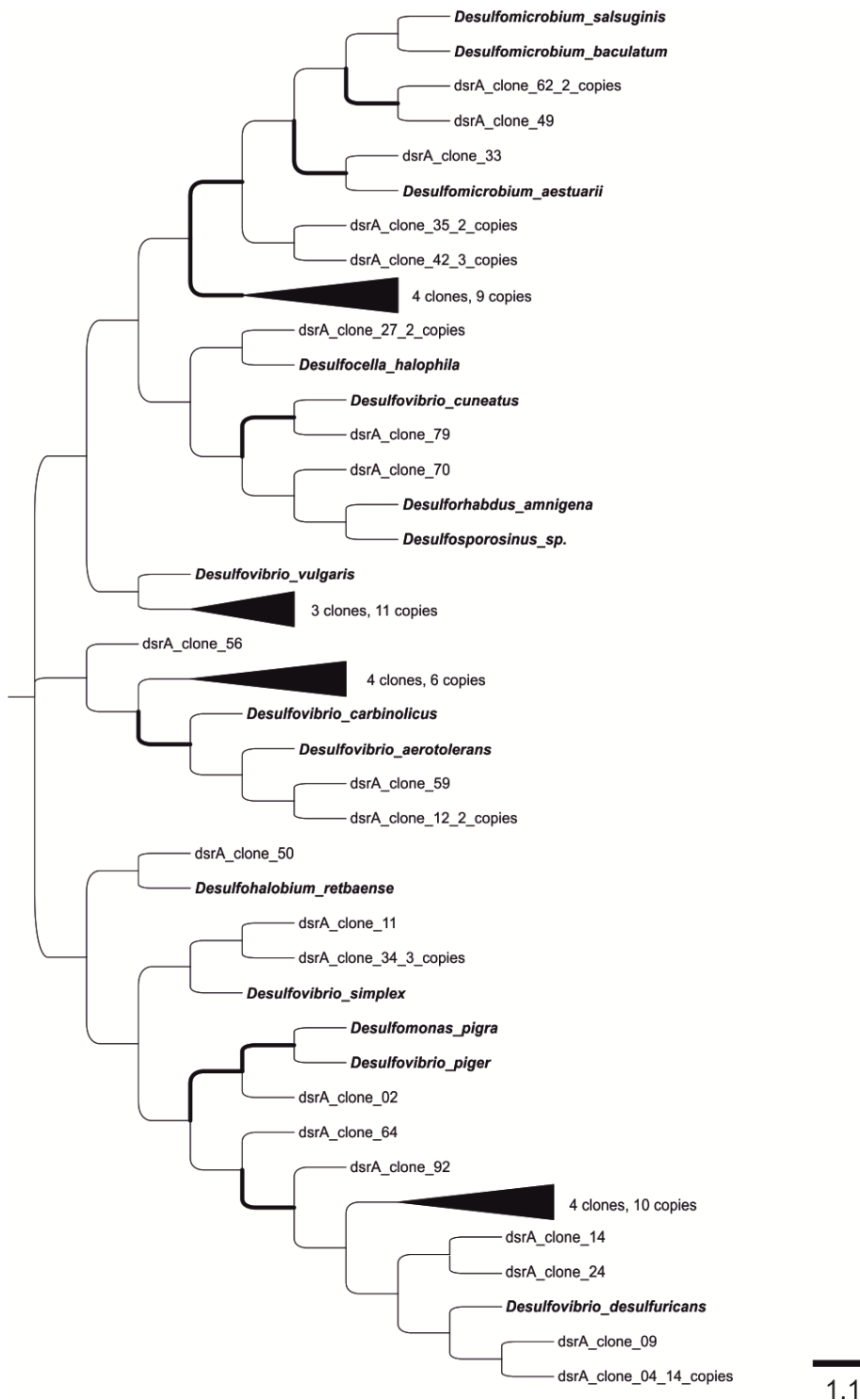


Figure S14: Phylogenetic tree of the cloned *dsrA* genes in the investigated reactor samples. The tree was constructed using the Kimura algorithm in MEGA with 1,000 bootstrap replicates. The percentage of replicate trees (>60%) are shown by thick branches. The scale bar represents 1.1 substitutions per nucleotide position.

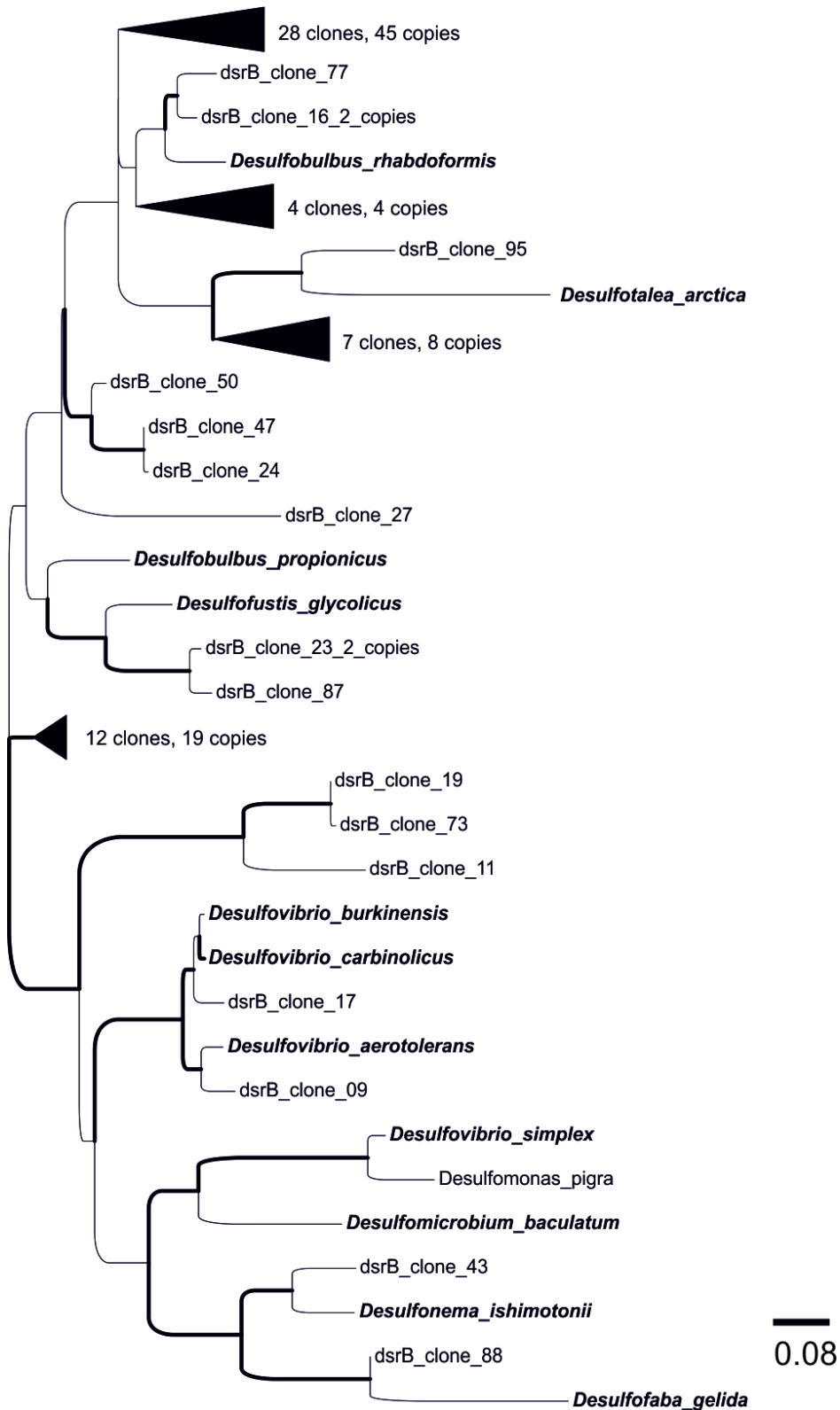


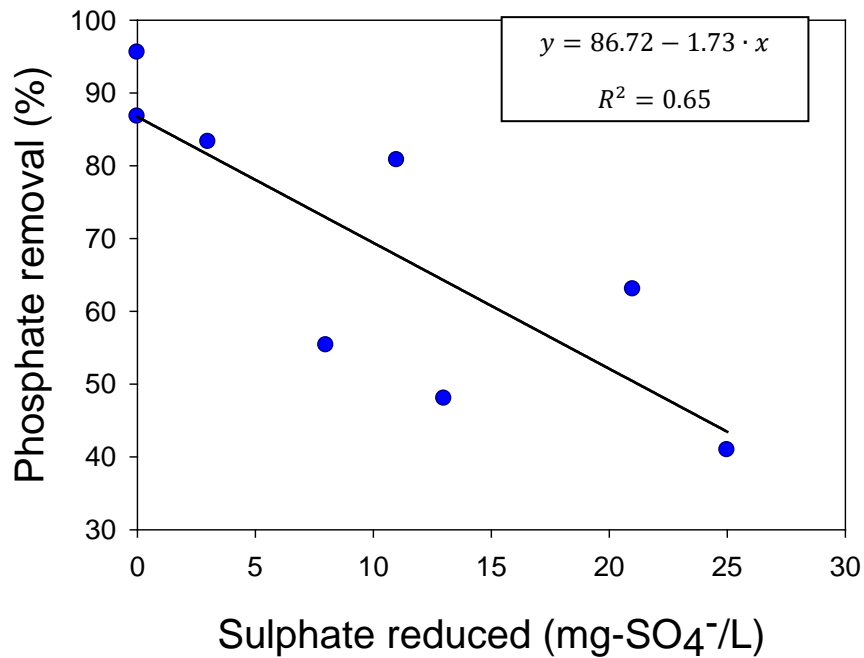
Figure S15: Phylogenetic tree of the cloned *dsrB* genes in the investigated reactor samples. The tree was constructed using the TN93 algorithm in MEGA with 1,000 bootstrap replicates. The percentage of replicate trees (>60%) are shown by thick branches. The scale bar represents 0.08 substitutions per nucleotide position.

10.3 Microbial community composition from sequencing quantified by targeting the 16S based primers and probes used for qFISH and qPCR

Table S13: Microbial community composition based on amplicon sequencing abundance inferred by targeting the 16S based primers and probes used for qFISH and qPCR

Days	PAO462	PAO651	PAO846	Actino-221a	Actino-658a	TFO_DF218	TFO_DF618	GB	DF988	DF1020
2	0.1759	0	0	0	0.5228	0	0	2.62	0	0
19	0.36954	0	0	0	0.2411	0	0	4.93	0	0
29	0.31114	0	0	0	0.1229	0	0	5.85	0	0
34	0	0	0	0	0	0	0	0	0	0
40	0.00861	0	0	0	0.0017217	0	0	1.19	0	0
48	0.70822	0	0	0	0.0605	0	0	5.97	0	0
57	0.2747	0	0	0	0.0201	0	0	5.05	0	0
68	0.04348	0	0	0	0.0072466	0	0.0036	4.54	0	0
80	0.01826	0	0	0	0.0104	0	0	2.26	0	0
84	0.01114	0	0	0	0	0	0	1.59	0	0
100	0.10559	0	0	0	0.0679	0	0	7.39	0	0
106	0.00889	0	0	0	0	0	0	0.32	0	0
142	0.05702	0	0	0	0	0	0.0023	0.66	0	0
143	0.06485	0	0	0	0	0	0	0.44	0	0
114	0.02416	0	0	0	0.0034508	0	0	0.37	0	0
146	0.04966	0	0	0	0	0	0	0.51	0	0
116	0.03791	0	0	0	0.0023692	0	0.0047	0.55	0	0
155	0.05344	0	0	0	0.0044536	0	0	0.47	0	0
156	0.06137	0	0	0	0	0	0	0.54	0	0
174	0.20401	0	0	0	0	0	0	0.14	0	0
175	0,34683	0	0	0	0.0074588	0	0	0.15	0	0

1 11 Relation between sulphate reduction and
2 phosphate removal

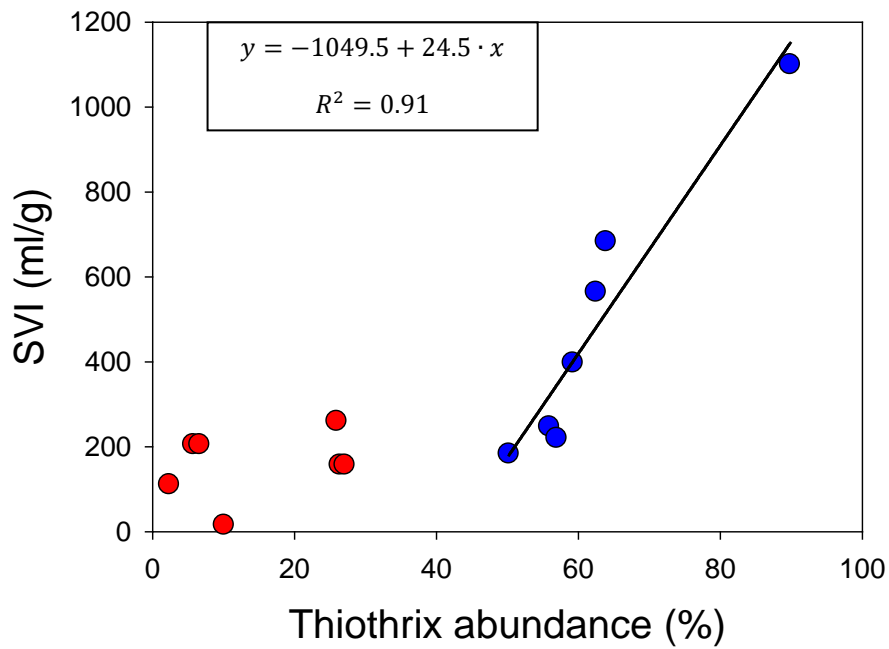


3

4 **Figure S16:** Correlation between sulphate reduced along the anaerobic phase and phosphorus removal.

5

6 12 Relation between *Thiothrix* and sludge
7 volume index (SVI)



8
9 **Figure S17:** Correlation between SVI and *Thiothrix* abundance. Abundance lower than 50% is shown
10 in red was excluded from the linear regression analysis.
11

12

13

14 **13 COD consumption by denitrifiers and SRB**
 15 **and uptake rates used in the discussion**

	Parameters	Calculation
COD consumption by denitrifiers	$Y_H=0.625 \text{ g-COD}\cdot\text{g-COD}^{-1}$ (Henze et al.1999)	$\Delta S_{NO3} \frac{1 - Y_H}{2.86 \cdot Y_H} \frac{1}{Y_H}$
COD consumption by SRB	$Y_{Pr/SO4}=1.333 \text{ mol-propionate/mol-SO}_4$ (Yamamoto-Ikemoto et al., 1996)	$\Delta S_{SO4} \cdot Y_{Pr/SO4}$
Specific uptake rate for SRB	$Y_{X/Pr}=0.026 \text{ g-COD}\cdot\text{g-COD}^{-1}$ $\mu_{max}=0.29 \text{ d}^{-1}$ (Cassidy et al., 2015)	$\frac{1}{Y_{X/Pr}} \mu_{max}$
Specific uptake rate for PAO under anaerobic conditions	$q_{PHA}=3 \text{ g-COD}\cdot\text{g-COD}$ (Henze et al., 1999)	-

16

17

18

19

20 14 References

- 21 Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman,
22 D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database
23 search programs. *Nucleic Acids Research* 25, 3389–402.
- 24 Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer
25 N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig
26 JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevins-
27 sky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J,
28 Knight R., 2010. QIIME allows analysis of high-throughput community sequenc-
29 ing data. *Nature Methods* 7, 335–336.
- 30 Caporaso JG, Bittinger K, Bushman FD, DeSantis TZ, Andersen GL, Knight R. 2010.
31 PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinforma-
32 tics* 26, 266–267.
- 33 Cassidy, J., Lubberding, H.J., Esposito, G., Keesman, K.J., Lens, P.N.L., 2015. Auto-
34 mated biological sulphate reduction: a review on mathematical models, monitoring
35 and bioprocess control. *FEMS Microbiology Reviews*, 39, 823-853.
- 36 Crocetti, G.R., Hugenholtz, P., Bond, P.L., Schuler, A., Keller, J., Jenkins, D., Blackall,
37 L.L., 2000. Identification of polyphosphate-accumulating organisms and design of
38 16S rRNA-directed probes for their detection and quantitation. *Applied Environ-
39 mental Microbiology*, 66 (3), 1175–1182.
- 40 Daims, H., Lücker, S., Wagner, M., 2006. Daime, a novel image analysis program for
41 microbial ecology and biofilm research. *Environmental Microbiology*, 8(2), 200-
42 213.
- 43 DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., Huber,
44 T., Dalevi, D., Hu, P., Andersen, G.L., 2006. Greengenes, a chimera-checked 16S

- 45 rRNA gene database and workbench compatible with ARB. *Applied Environmen-*
46 *tal Microbiology*, 72, 5069–5072.
- 47 Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME im-
48 proves sensitivity and speed of chimera detection. *Bioinformatics* 27, 2194–2200.
- 49 Ferris, M.J., Muyzer, G., Mard, D.M., 1996. Denaturing gradient gel electrophoresis
50 profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat
51 community. *Applied and Environmental Microbiology*, 62, 340-346.
- 52 Henze, M., Gujer, W., Mino, T., Matsuo, T., Wentzel, M.C., Marais, G.V.R., Van
53 Loosdrecht, M.C.M., 1999. Activated sludge model n° 2d, ASM2d. *Water Science*
54 *and Technology*, 39, 165-182.
- 55 Hermansson, A., Lindgren, P.E., 2001. Quantification of ammonia oxidizing bacteria in
56 arable soil by real-time PCR. *Applied Environmental Microbiology*, 67, 972-976.
- 57 Karkhoff-Scheizer, R.R., Huber, D.P.W., Voordouw, G., 1995. Conservation of the
58 genes for dissimilatory sulphite reductase from *Desulfovibrio vulgaris* and *Ar-*
59 *chaeoglobus fulgidus* allows their detection by PCR. *Applied and Environmental*
60 *Microbiology*, 61(1), 290-296.
- 61 Kaetzke, A., Jentsch, D., Eschrich, K., 2005. Quantification of *Microthrix parvicella* in
62 activated sludge bacterial communities by real-time PCR. *Letters in Applied Mi-*
63 *crobiology*, 40, 207-211.
- 64 Kong, Y.H., Ong, S.L., Ng, W.J., Liu, W.T., 2002. Diversity and distribution of a deep-
65 ly branched novel proteobacterial group found in anaerobic–aerobic activated
66 sludge processes. *Environmental Microbiology*, 4 (11), 753–757.
- 67 Kong, Y.H., Nielsen, J.L., Nielsen, P.H., 2005. Identity and ecophysiology of uncult-
68 tured actinobacterial polyphosphate-accumulating organisms in full-scale en-
69 hanced biological phosphorus removal plants. *Applied Environmental Microbiolo-*
70 *gy*, 71 (7), 4076–4085.

- 71 Kowalchuk, G.A., Stephen, J. R., De Boer, W., Prosser, J. I., Embley, T. M., Wolden-
72 dorp, J .W., 1997. Analysis of ammonia-oxidizing bacteria of the β subdivision of
73 the class Proteobacteria in coastal sand dunes by denaturing gradient gel electro-
74 phoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. Ap-
75 plied Environ Microbiology, 63, 1489–1497.
- 76 Kumari, S.K.S., Marrengane, Z., Bux, F., 2009. Application of quantitative RT-PCR to
77 determine the distribution of *Microthrix parvicella* in full-scale activated sludge
78 treatment systems. Applied Microbiology and Biotechnology, 83, 1135-1141.
- 79 Lane, D.J., 1991. 16S/23S rRNA sequencing. Nucleic Acid Techniques in Bacterial
80 Systematics, 125-175.
- 81 Lapidus, A., Nolan, M., Lucas, S., del Rio, T.G., Tice, H., Cheng, J.F., Tapia, R., Han,
82 C., Goodwin, L., Pitluck, S., Liolios, K., Pagani, I., Ivanova, N., Huntemann, M.,
83 Mavromatis, K., Mikhailova, N., Pati, A., Chen, A., Palaniappan, K., Land, M.,
84 Brambilla, E.M., Rohde, M., Abt, B., Verbarq, S., Göker, M., Bristow, J., Eisen,
85 J.A., Markowitz, V., Hugenholtz, P., Kyrpides, N.C., Klenk, H.P., Woyke, T.,
86 2011. Genome sequence of the filamentous, gliding *Thiothrix nivea* neotype strain
87 (JP2T). Standards in Genomic Sciences, 5, 398-406.
- 88 Masella AP, Bartram AK, Truszkowski JM, Brown DG, Neufeld JD. 2012. PANDAseq:
89 paired-end assembler for illumina sequences. BMC Bioinformatics 13, 31.
- 90 Meyer, R.L., Saunders, A.M., Blackall, L.L., 2006. Putative glycogenaccumulating or-
91 ganisms belonging to Alphaproteobacteria identified through rRNA-based stable iso-
92 tope probing. Microbiology, 152, 419–429.
- 93 Nielsen, P.H., Daims, H., Lemmer, H., 2009. FISH Handbook for Biological
94 Wastewater Treatment. Identification and Quantification of Microorganisms in Acti-
95 vated Sludge and Biofilms by FISH. IWA Publishing, London, UK.
- 96 Stoddard S.F, Smith B.J., Hein R., Roller B.R.K., Schmidt T.M., 2014. rrnDB: im-
97 proved tools for interpreting rRNA gene abundance in bacteria and archaea and a
98 new foundation for future development. Nucleic Acids Research, 43, 593-598.

- 99 Terada, A., Lackner, S., Kristensen, K., Smets, B.F., 2010. Inoculum effects on com-
100 munity composition and nitrification performance of autotrophic nitrifying biofilm re-
101 actors with counter-diffusion geometry. *Environmental Microbiology*, 12(10), 2858-
102 2872.
- 103 Vanysacker, L., Denis, C., Roels, J., Verhaeghe, K., Vankelecom, I.F.J., 2014. Devel-
104 opment and evaluation of a TaqMan duplex real-time PCR quantification method for
105 reliable enumeration of *Candidatus Microthrix*. *Journal of Microbiological Methods*,
106 97, 6-14.
- 107 Vervaeren, A., Wilde, K., Matthys, J., Boon, N., Raskin, L., Verstraete, W., 2005.
108 Quantification of an Eikelboom type 021N bulking event with fluorescence in situ
109 hybridization and real-time PCR. *Applied Microbiology and Biotechnology*, 68,
110 695-704.
- 111 Wagner, M., Roger, A., Flax, J.L., Brusseau, G.A., Stahl, D.A., 1998. Phylogeny of dis-
112 simulatory sulfite reductases supports an early origin of sulfate respiration. *Journal*
113 *of Bacteriology*, 11, 2975-2982.
- 114 Wong, M.T., Tan, F.M., Ng, W.J., Liu, W.T., 2004. Identification and occurrence of
115 tetrad-forming Alphaproteobacteria in anaerobic-aerobic activated sludge processes.
116 *Microbiology*, 150, 3741-3748.
- 117 Yamamoto-Ikemoto, R., Matsui, S., Komori, T., Bosque-Hamilton, E.J., 1996. Symbio-
118 sis and competition among sulfate reduction, filamentous sulfur, denitrification and
119 poly-P accumulation bacteria in the anaerobic-oxic activated sludge of a municipal
120 plant. *Water Science and Technology*, 34(5-6), 119-128.
- 121 Yu Y, Lee C, Kim J, Hwang S. 2005. Group-specific primer and probe sets to detect
122 methanogenic communities using quantitative real-time polymerase chain reaction.
123 *Biotechnology and Bioengineering*, 89, 670-679.
- 124