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

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Abstract

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

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

Keywords

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

1 Introduction

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

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

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

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

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

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

Only Ge et al. (2015) identified a novel PAO organism, belonging to the *Comamonadaceae* family, which became dominant and drove P-removal in a short-SRT EBPR treating abattoir wastewater. As a consequence, the available models, both ecological and mathematical, may lack relevant bacterial groups to properly describe short-SRT EBPR systems. Therefore, the main objectives of the present study are i) to describe the start-up and operation of a short-SRT EBPR system ; ii) to assess the microbial community dynamics and identify operation strategies promoting effective process performance; iii) to identify the limitations of available biochemical process models and propose potential extensions to model the short-SRT EBPR process.

2 Materials and Methods

2.1 Reactor description

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

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

<Table 1>

2.2 Analytical methods

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

2.3 Microbial analysis

Quantitative polymerase chain reaction (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*), *Thiothrix*, *Microthrix parvicella* and sulphate reducing bacteria (SRB). DNA was extracted according to Te-

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

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

3 Results and discussion

3.1 Reactor performance

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

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

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

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

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

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

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

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

<Figure 1>

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

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

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

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

3.2 Variation in microbial community composition and diversity

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

S1). The sample taken at day 30 was excluded from further analysis due to the low number of sequences available in the sample library. High-quality sequences were normalized by rarefying to 11,940 sequences per sample for further diversity and taxonomic comparisons.

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

<Figure 2>

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

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

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

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

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

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

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

<Figure 3>

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

3.3 Discrepancies between quantification methods

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

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

3.4 Synergies and competitions between bacteria in short SRT EBPR systems

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

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

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

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

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

SRB can interact with PAO in different ways. SRB produce sulphide, which can inhibit phosphorus release, thereby reducing it to 50% at concentrations of 60 mg-H_2S/L (Saa et al., 2013). Since phosphorus release is hindered under anaerobic conditions, PAO cannot grow under aerobic conditions, thus leading to their wash out from the system. Additionally, SRB take up organic carbon under anaerobic conditions. The most common SRB in wastewater treatment systems are incomplete carbon oxidizers, such as *Desulfobulbos* or *Desulfovivbrio* (Hao et al., 2014), both found via phylogenetic analysis (Fig. S14 and S15). The end product of incomplete oxidation process is acetate, which is finally available for other bacteria, such as PAO (Yamamoto-Ikemoto et al., 1996). Therefore, there is limited competition for carbon between incomplete oxidizing SRB and PAO. Other SRB, like *Desulfarculus* in this study, are able to completely oxidize organic carbon to carbon dioxide (Hao et al., 2014). Therefore, this second type of SRB may compete with PAO for organic carbon in the anaerobic phase, thus leading to incomplete phosphorus removal, as suggested by Fig. 3. To further support this hypothesis, the COD consumption was estimated, based on stoichiometric coefficients from literature (Yamamoto-Ikemoto et al., 1996), for the day with maximum reported SVI (SVI=1100 ml/g; sulphate reduction of 30%). About 35% of the influent COD was consumed for sulphate reduction. Surprisingly, sulphate reduction stopped before all COD was consumed, as comparably high COD levels and sulphate were detected at the end of

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

removal was restored, further supporting this hypothesis. These findings are relevant to those WWTPs located close to the coastline, where the sulphate content in wastewater is expected to be high (van den Brand et al., 2015). This is the case for Lundtofte WWTP, which is barely 5 km from the sea. A two-month sampling campaign was performed to further verify this. Influent sulphate concentrations measured in the influent to Lundtofte ranged from 35 to 162 mg-SO_4^-/L. It should be noted that there are also cases where the phosphorus and the sulphur cycle have been successfully combined, such as the SANI process (Wu et al., 2014). Nevertheless, phosphorus removal in this process is driven by microbes capable to oxidize sulphide rather than *Accumulibacter* (Guo et al., 2016), which makes it non comparable to our study.

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

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

<Figure 4>

3.5 Sludge settling properties

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

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

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

3.6 Relevance to available models

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

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

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

4 Conclusions

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

- EBPR process can be run at a system SRT of 3 days, effectively removing phosphorus and organic carbon. SVI was relatively high (SVI~200ml/g), but stable.
- NOB were effectively removed from the reactor at SRT below 3.5 days (corresponding to 1.75 days aerobic SRT). AOB were phased out from the SBR by lowering the SRT down to 3 days (i.e. 1.5 days of aerobic SRT) and controlling the oxygen level lower than 3 mg/L.
- Sulphate reduction was observed at SRT 3.5 days, producing sulphur reduced chemicals, which served as substrate for *Thiothrix*, thereby provoking filamentous bulking. SRB were shown to have a negative impact on phosphorus removal, although the inhibition mechanism could not be identified. SRB activity was suppressed by reducing the anaerobic SRT to 0.58 days and process performance was restored.

Models should include SRB related processes to properly address the competition between them and PAO. Furthermore, since bulking cannot be completely avoided, future research should address the inclusion of time varying parameters for settling models to properly track changes of or in sludge properties.

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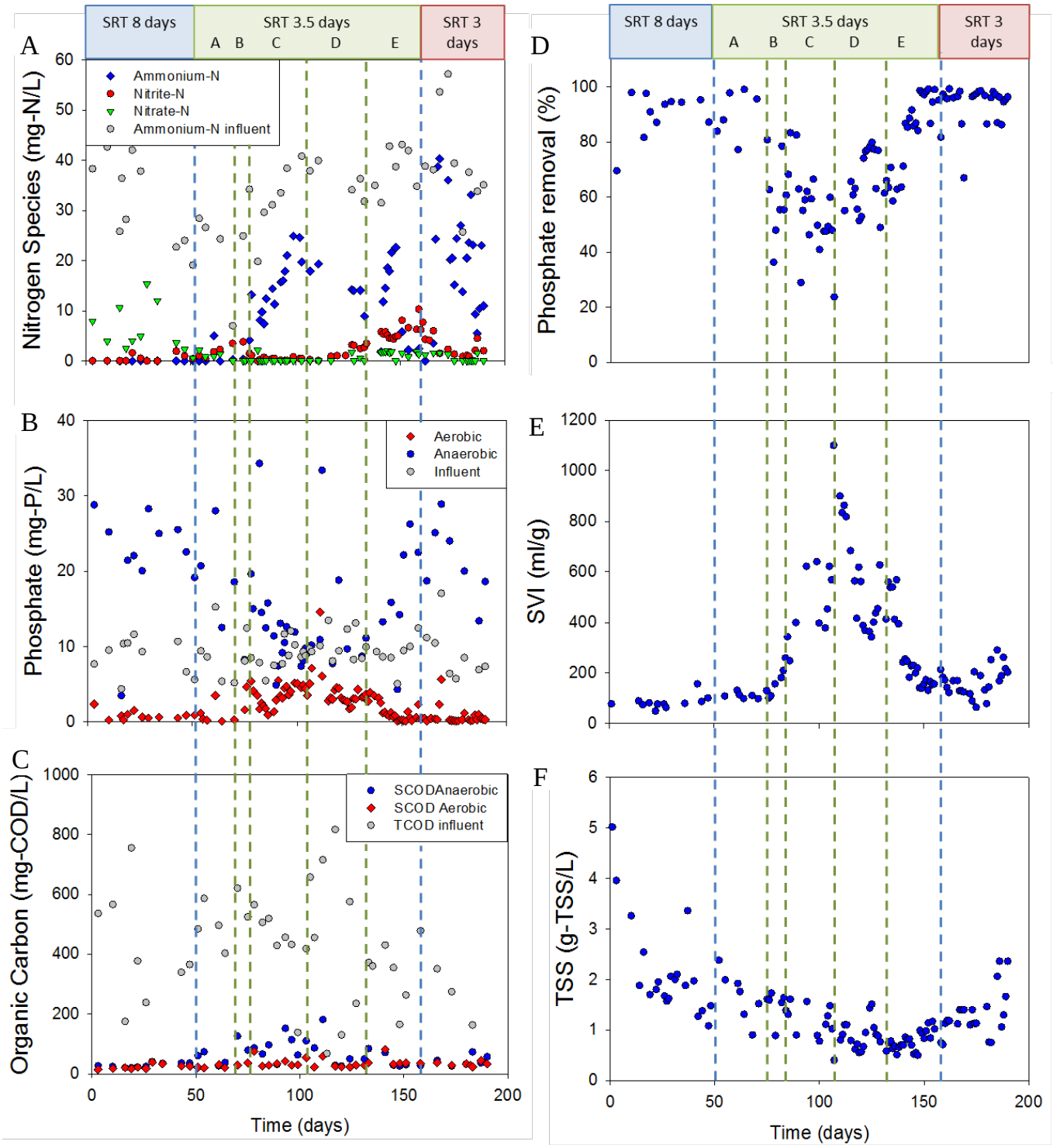

Figure 1: Reactor performance through 190 days a) Ammonia, nitrite and nitrate at the end of the aerobic phase and ammonia in the influent; b) phosphate at the end of the aerobic and anaerobic phases

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

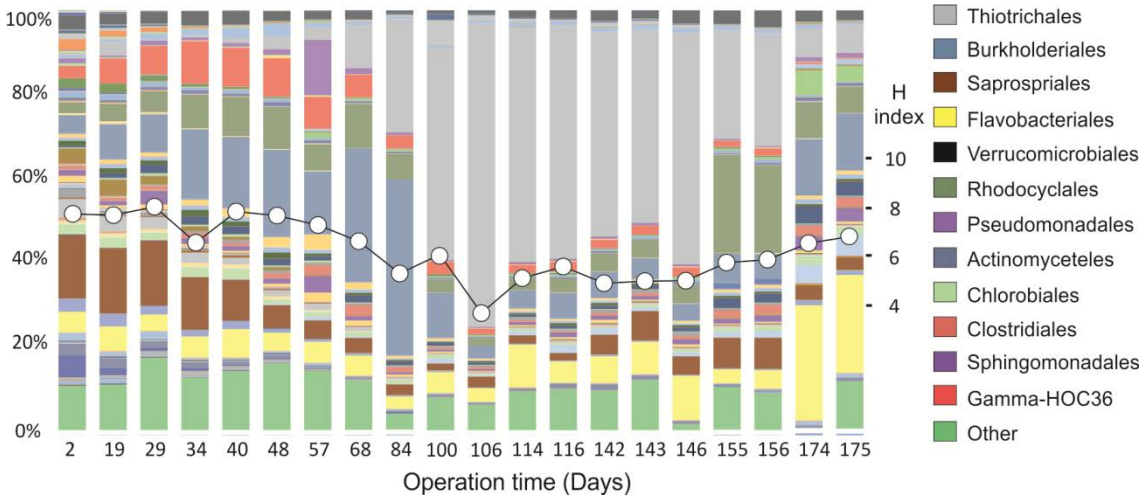

Figure 2: Order-level taxonomic classification of 16S rRNA amplicons at selected days of the reactor operation. Taxa abundance is expressed in percentage (left axis). Alpha-diversity at the order level measured as Shannon index (white dots, right axis).

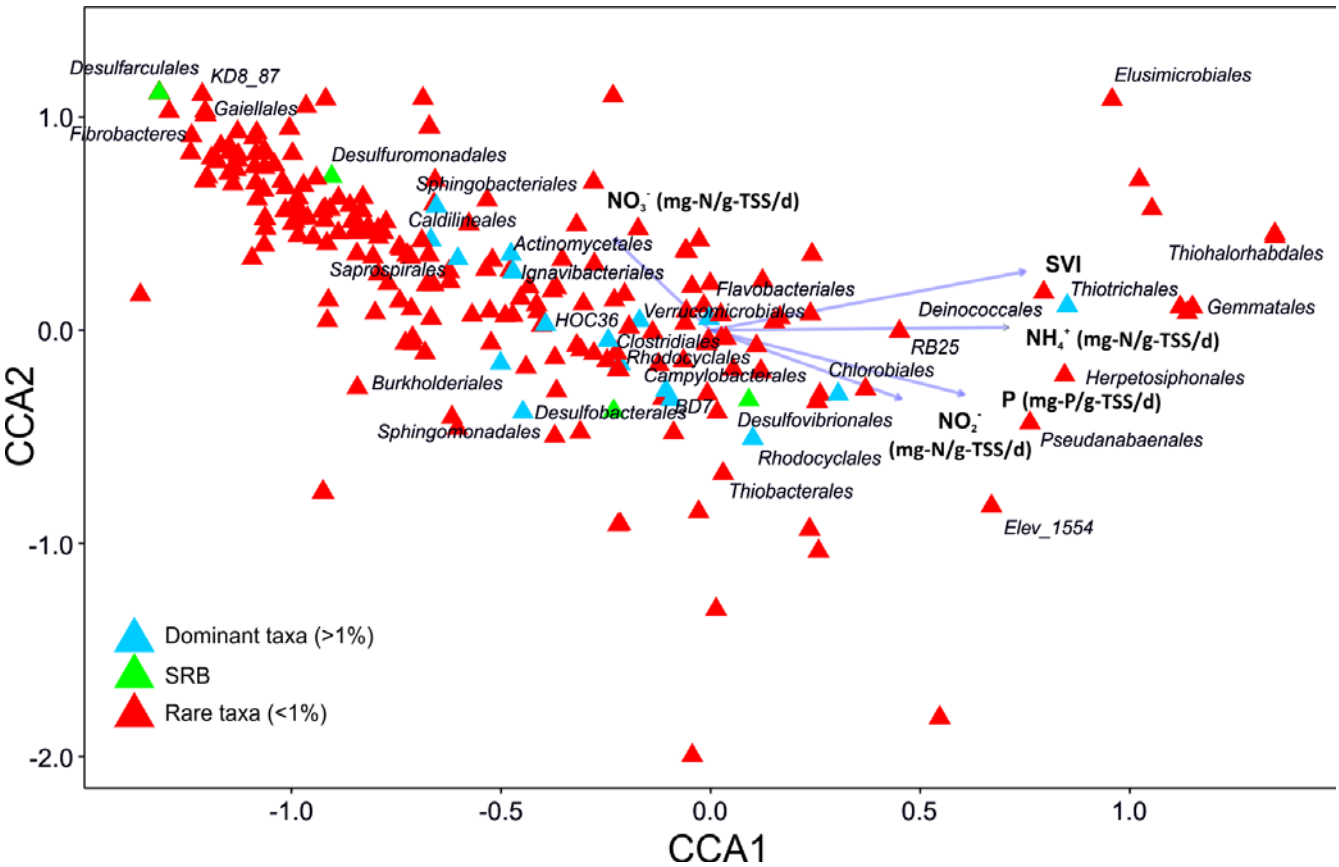

Figure 3: Canonical correspondence analysis (CCA) of order-level taxa within samples taken at time points during reactor operation constrained by five environmental variables: SVI (mL g^{-1}), ammonium removal (mg-N L^{-1} g-VSS^{-1}), nitrite accumulation (mg-N L^{-1} g-VSS^{-1}), nitrate accumulation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

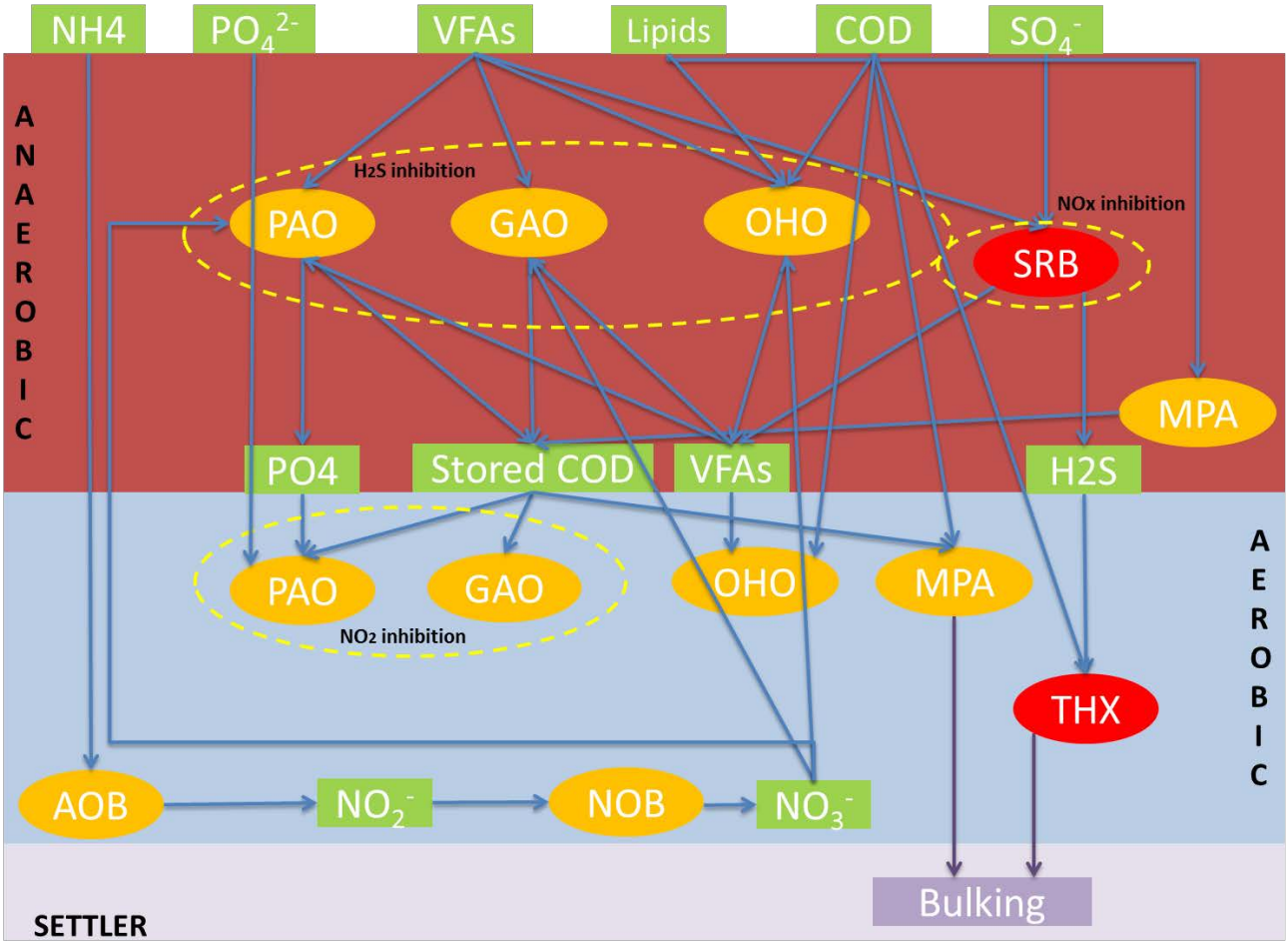

Figure 4: Ecological model proposed for the low-SRT EBPR system. Circled in yellow those microbial groups inhibited by chemicals (e.g. nitrite). Colored in red those microbial niches playing an important role in short-SRT EBPR systems but not acknowledged in previous studies. Blue arrows represent the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

Table 1: Operational conditions during the start-up and operation of the short-SRT 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

Supplementary information

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

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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 ^{o}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 ^{0}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	
Nitrospira genus NOB	16S-rRNA	Nspra-675f	GCGGTGAAATGCGTAGAKATCG	Graham et al. 2007
		Nspra-746r	TTTTTTGAGATTTGCTAG	
Nitrobacter 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
Thiothrix	16S-rRNA	21Nf	CGTAGGCGGCTCTTTAAGTCRGAT	Vervaeren et al. 2005
		21Nr	CCGACGGCTAGTTGACATCGTTTA	
Microthrix parvicella	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^{0}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 *Accumuli-bacter*	PAO462	Cy3 (red)	CCGTCATCTACWCAGGGTATTAAC	Crocetti et al., 2000
	PAO651		CCCTCTGCCAAACTCCAG	
	PAO846		GTTAGCTACGGCACTAAAAGG	
Actinobacteria (Tetrasphaera)	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	
Competibacter	GB	Cy5 (blue)	CGATCCTCTAGCCCACT	Kong et al., 2002
Defluviicoccus (cluser I)	TFO_DF218	Cy3 (red)	GAAGCCTTTGCCCCTCAG	Wong et al., 2004
	TFO_DF618		GCCTCACTTGTCTAACCG	
Defluviicoccus	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

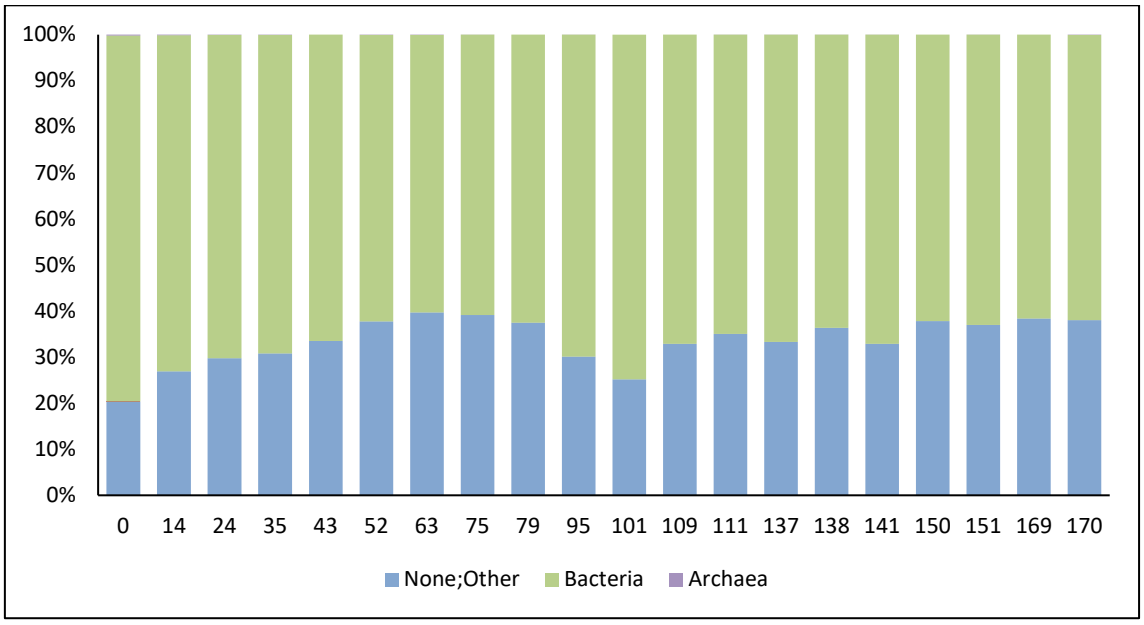

Figure S1: Domain-level taxonomic classification of 16S rRNA amplicons at selected time points of the reactor operation. Taxa abundance is expressed in percentage.

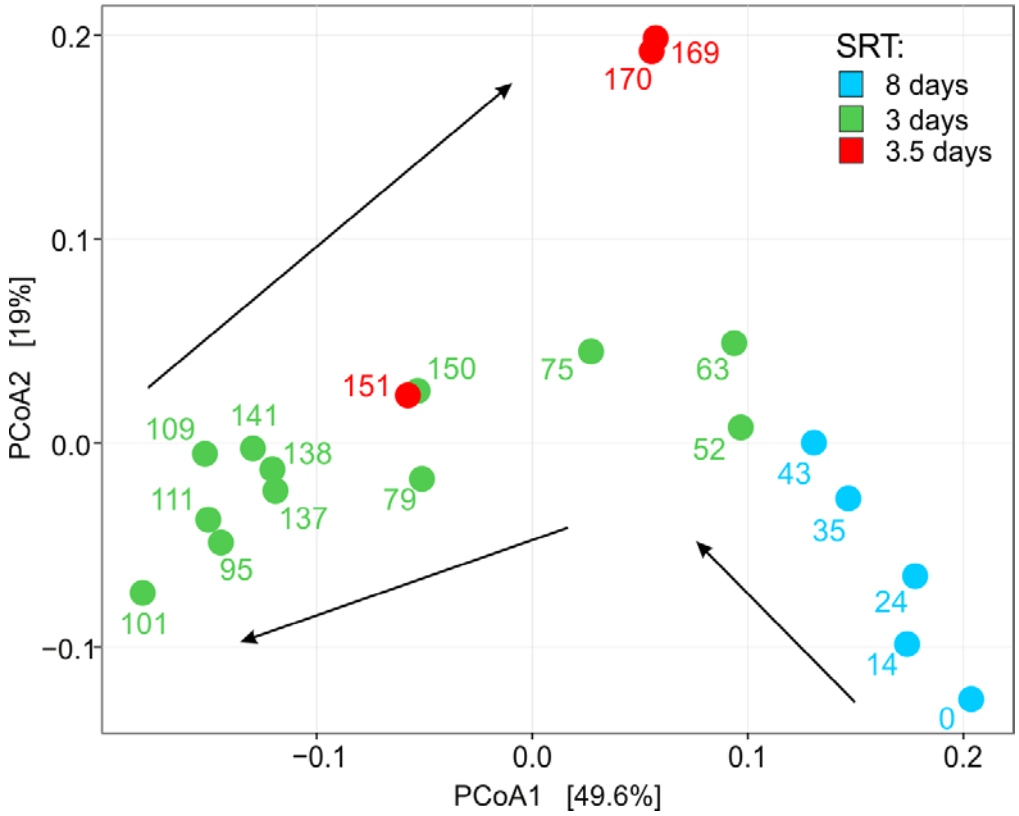

Figure S2: Phylogenetic distances between samples taken at different time points during reactor operation. The dissimilarity matrix was determined using Weighted UniFrac algorithm and plotted via principal coordinate analysis (PCoA). The percentage of variation explained by the principal coordinates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

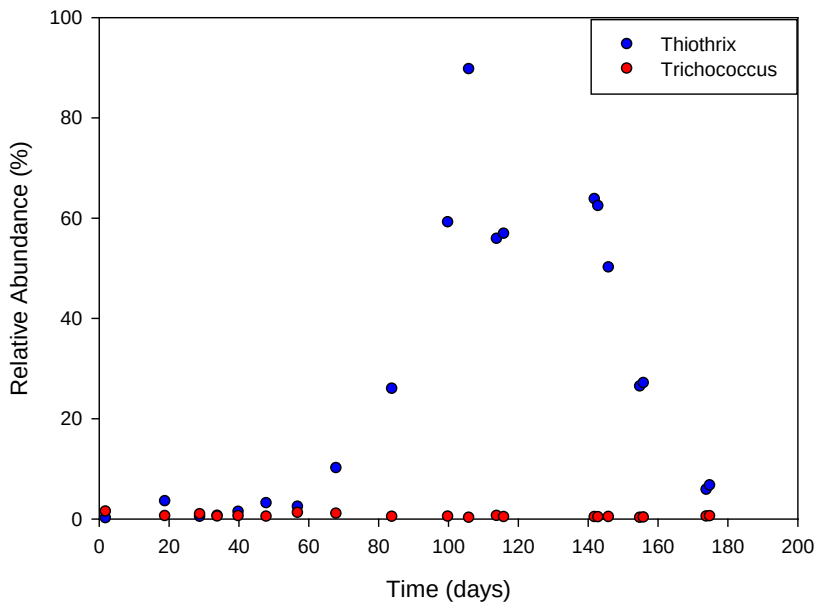

Figure S3: Relative abundance of filamentous bacteria along the operation of the short SRT EBPR. Only the 2 most abundant genera are shown.

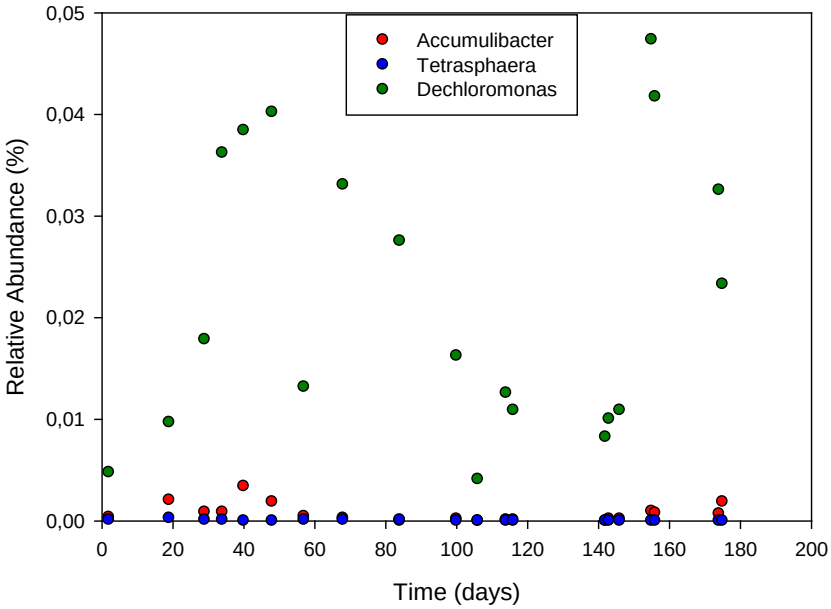

Figure S4: Relative abundance of PAO along the operation of the short SRT EBPR.

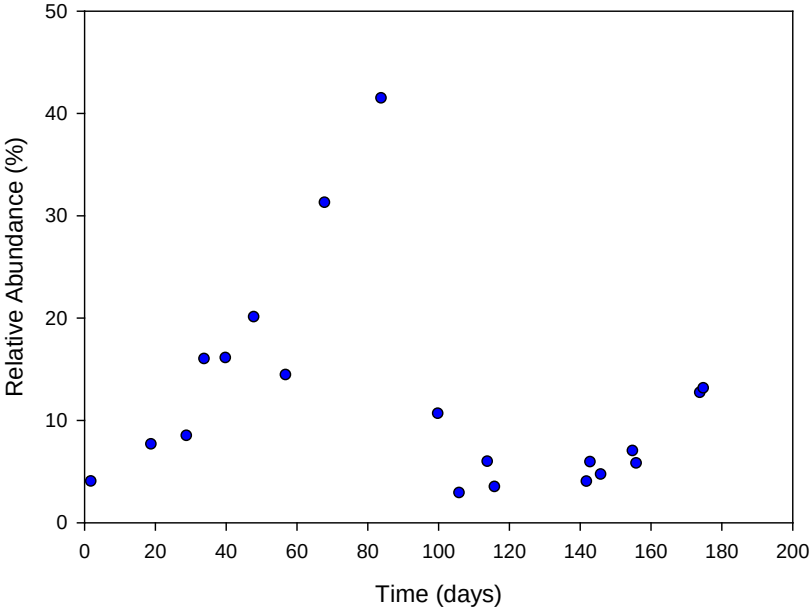

Figure S5: Relative abundance of *Comamonadaceae* along the operation of the short SRT EBPR.

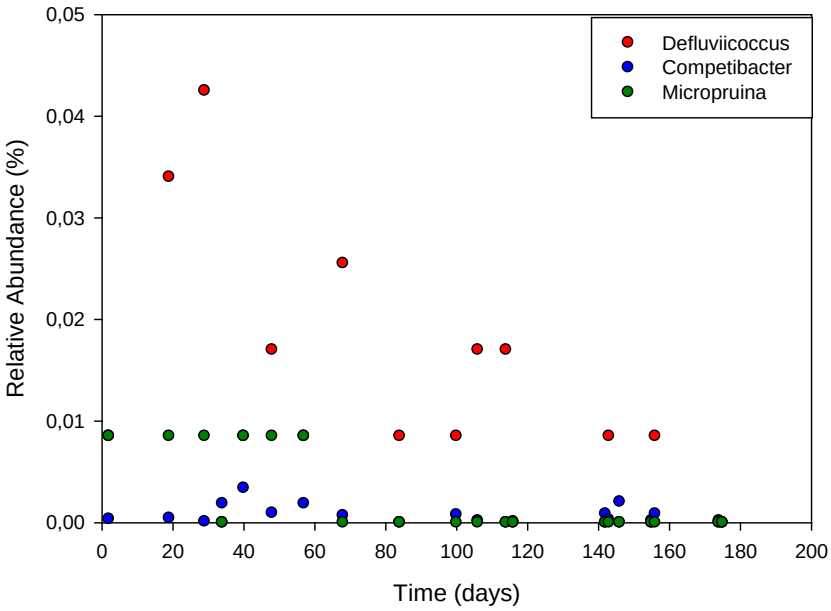

Figure S6: Relative abundance of GAO along the operation of the short-SRT EBPR.

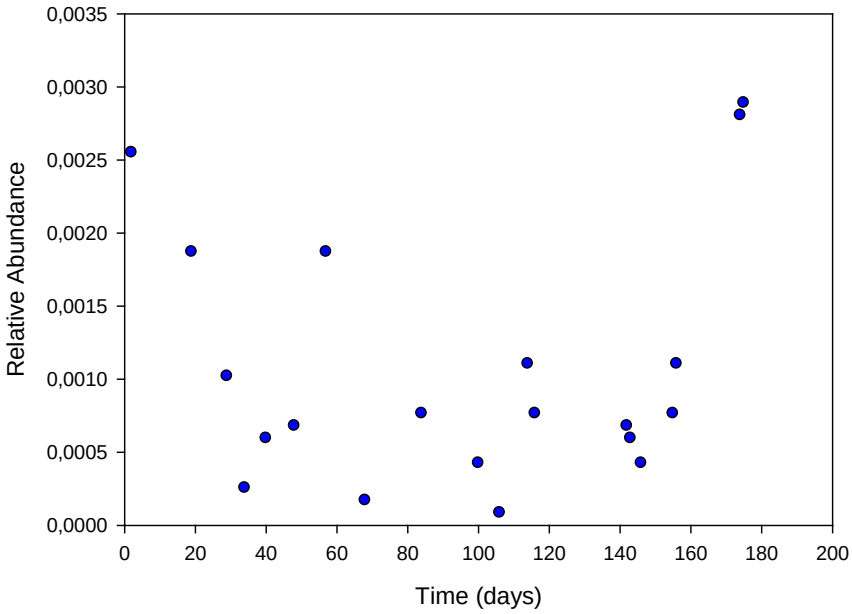

Figure S7: Relative abundance of SRBs along the operation of the short-SRT EBPR.

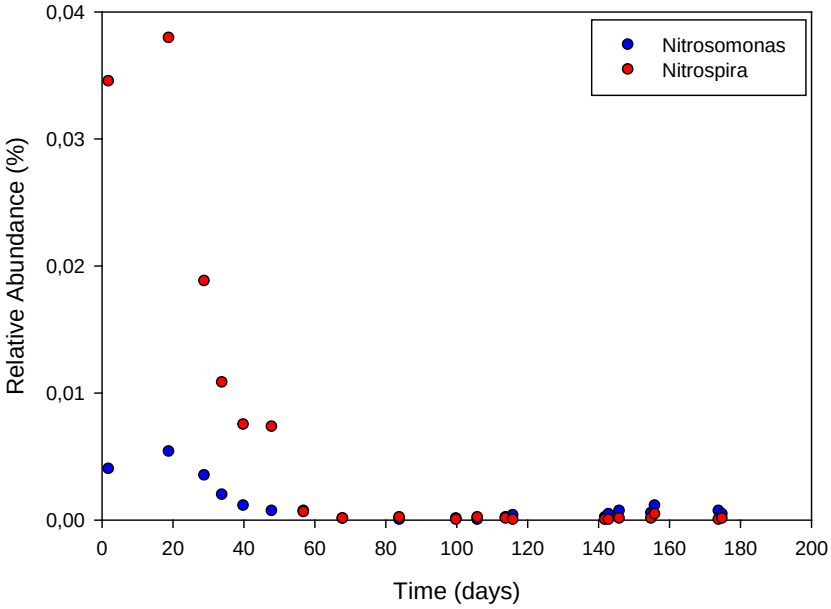

Figure S8: Relative abundance of AOB (*Nitrosomonas*) and NOB (*Nitrospira*) along the operation of the short-SRT EBPR.

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

Days	*Comamonadaceae*	*Trichococcus*	*Thiothrix*	*Accumulibacter*	*Tetrasphaera*	*Dechloromonas*	*Defluviicoccus*	*Competibacter*	*Micropurina*	*Nitrosomonas*	*Nitrospira*	*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

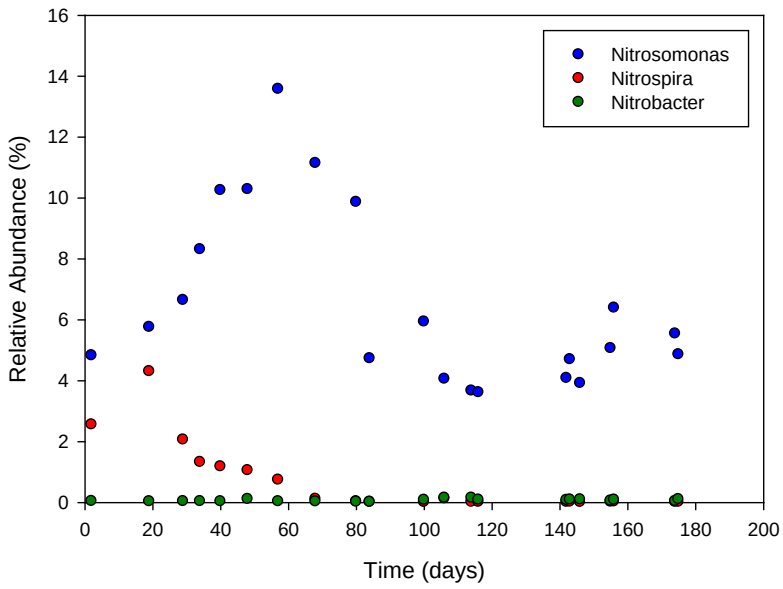

Figure S9: Relative abundance of AOBs along the operation of the short-SRT EBPR according to qPCR.

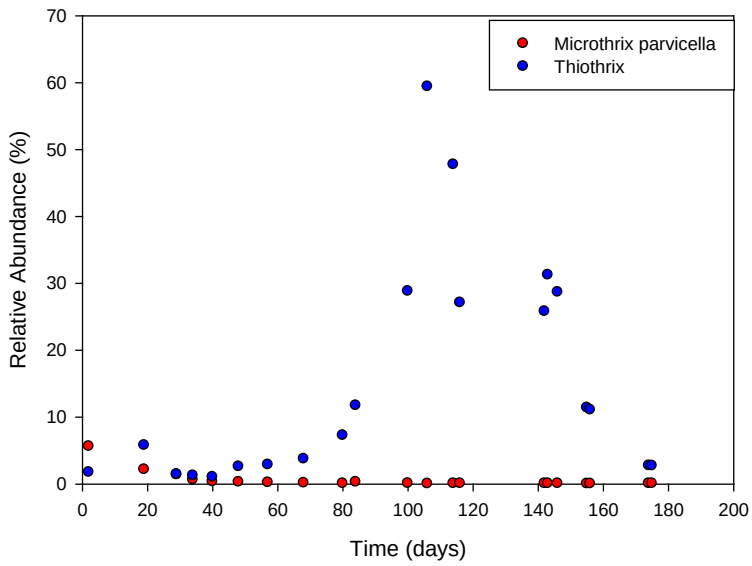

Figure S10: Relative abundance of filamentous bacteria along the operation of the short-SRT EBPR.

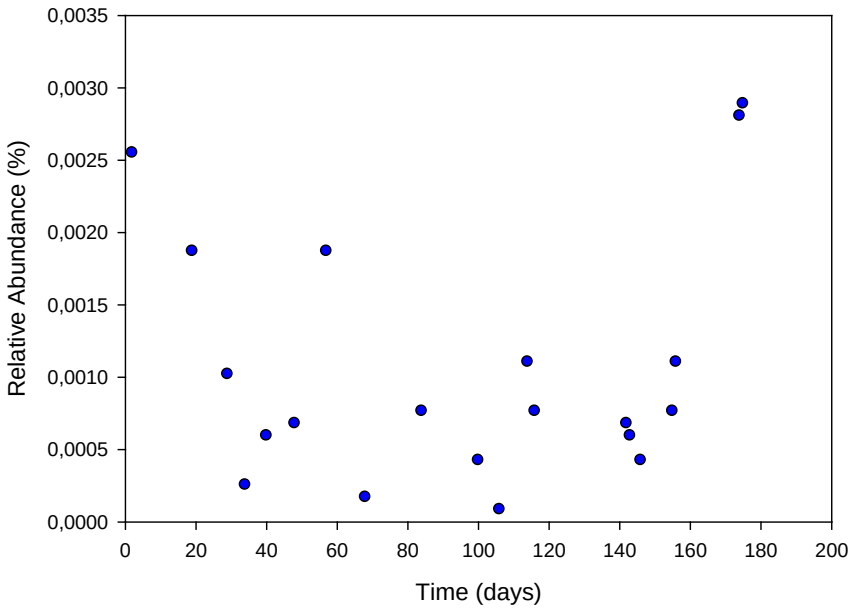

Figure S11: Relative abundance of SRB along the operation of the short-SRT EBPR.

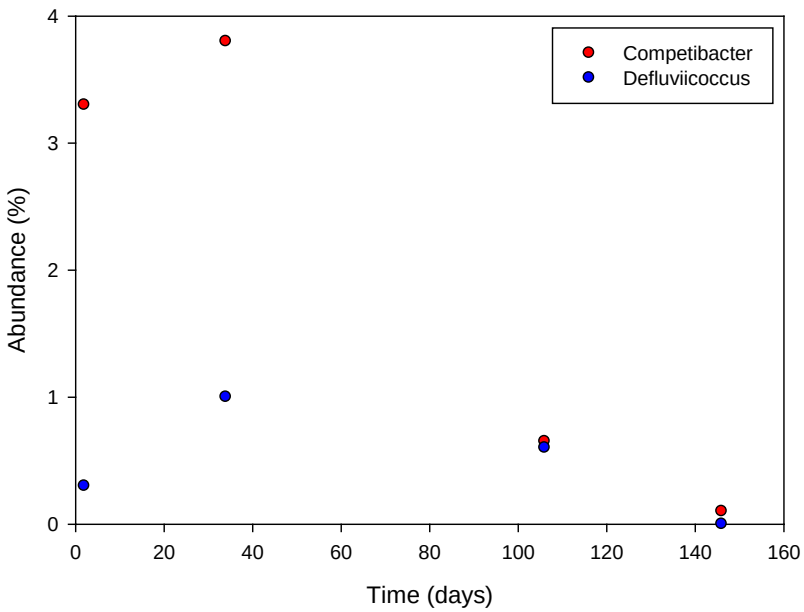

Figure S12: Relative abundance of GAO along the operation of the short-SRT EBPR.

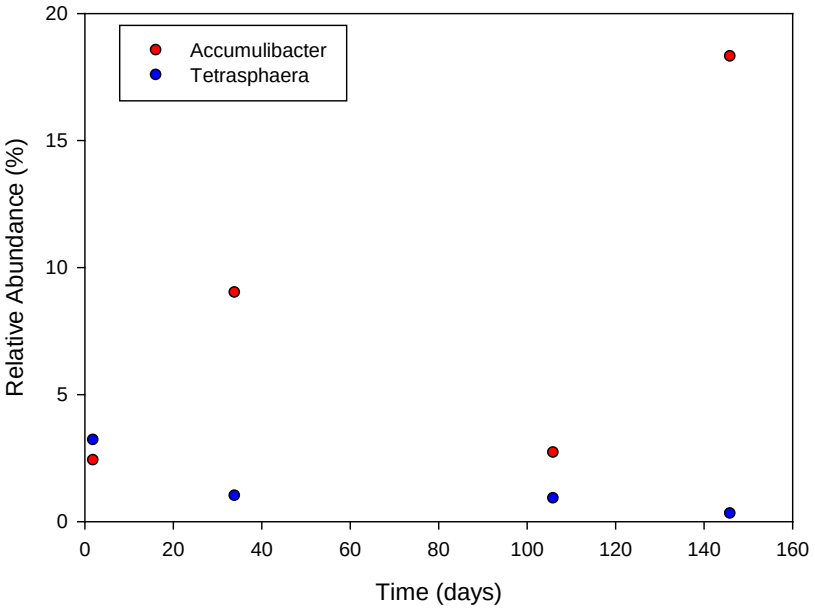

Figure S13: Relative abundance of PAO along the operation of the short-SRT EBPR.

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	*Nitrosomonas*	*Nitrospira*	*Nitrobacter*	*SRBs*	*Thiothrix*	*M. parvicella*	*Accumulibacter*	*Tetrasphaera*	*Competibacter*	*Defluviicoccus*
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

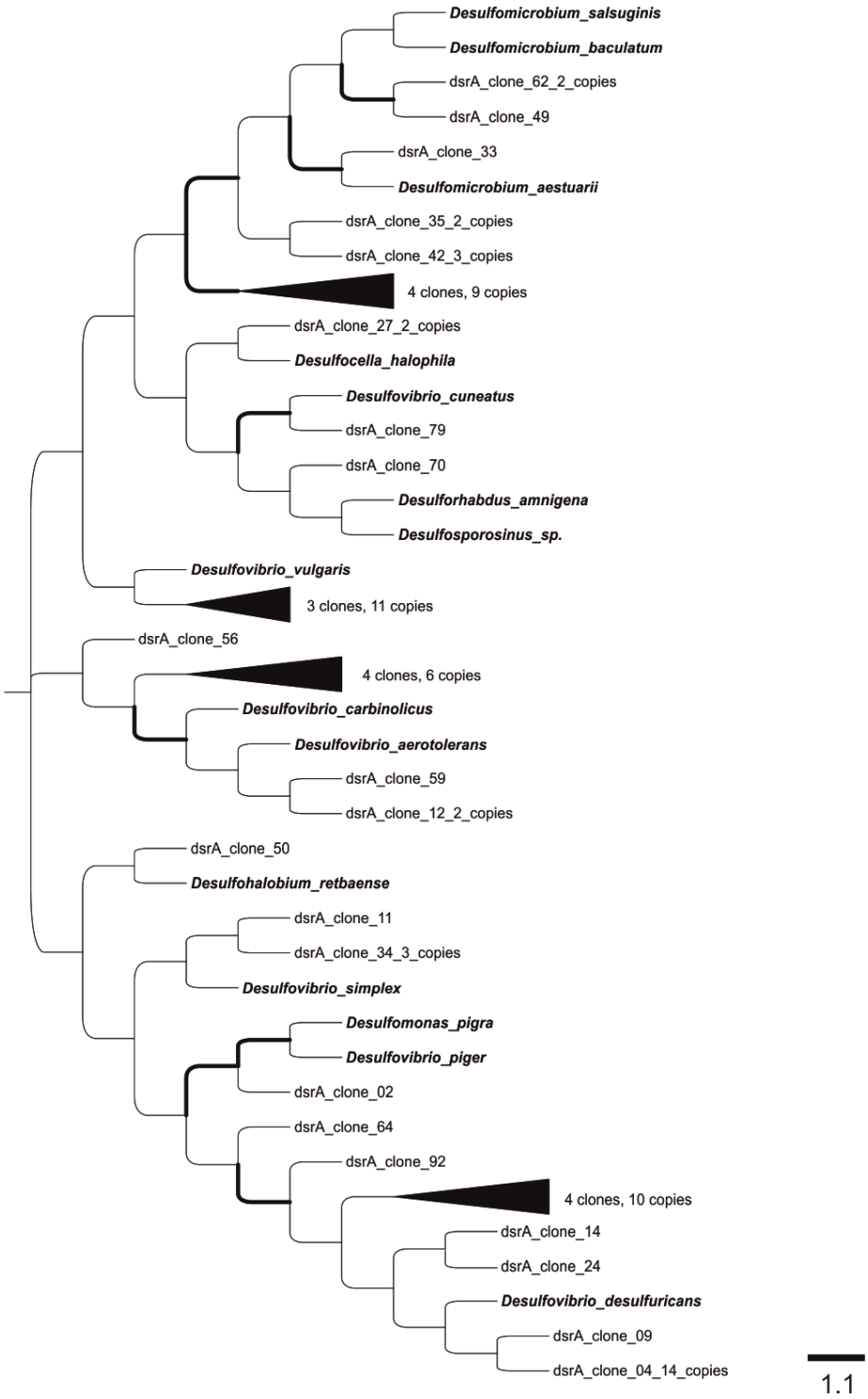

Figure S14: Phylogenetic tree of the cloned *dsrA* genes in the investigated reactor samples. The tree was constructed using the Kimura algorithm in MEGA with 1,000 bootstrap replicates. The percentage of replicate trees (>60%) are shown by thick branches. The scale bar represents 1.1 substitutions per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

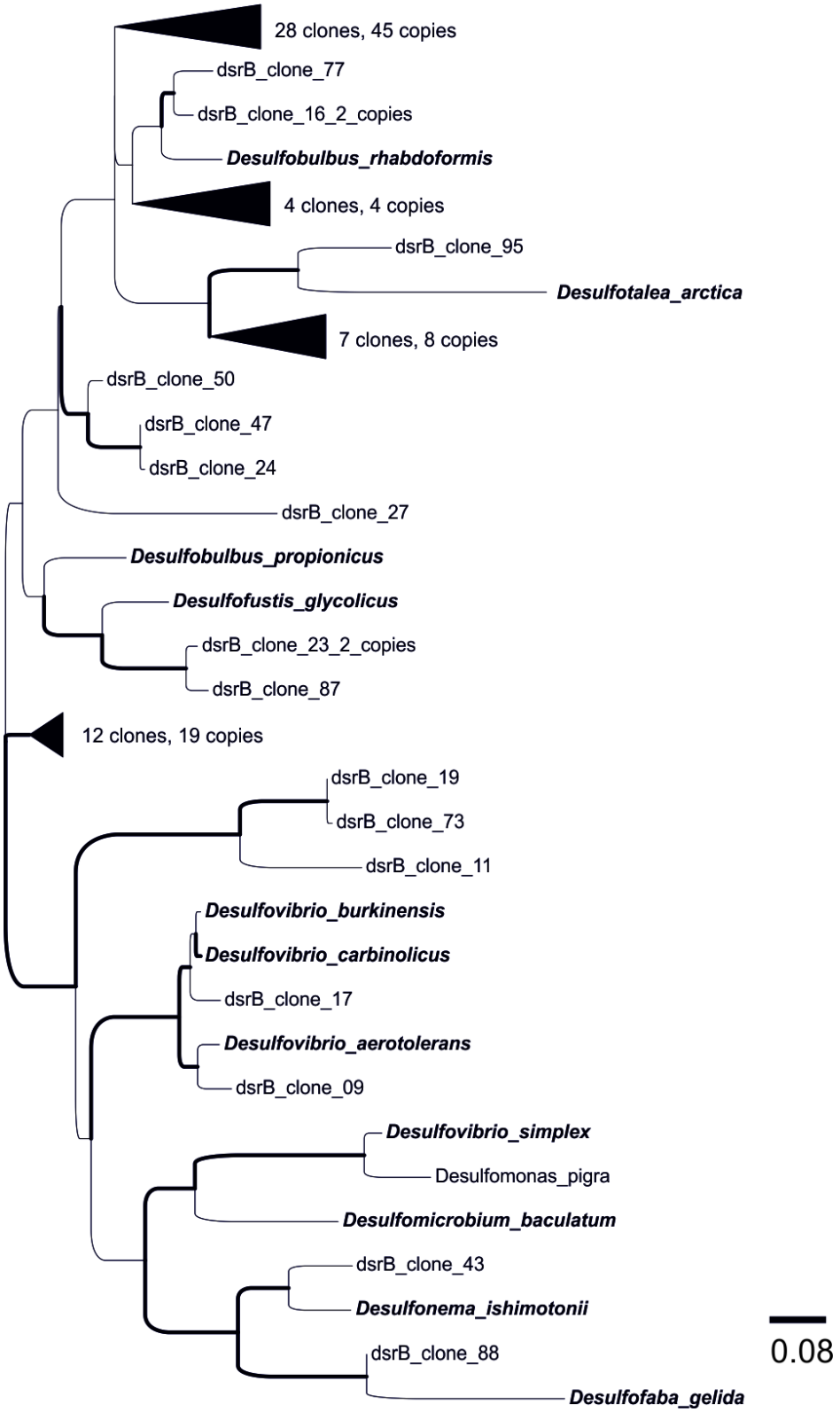

Figure S15: Phylogenetic tree of the cloned *dsrB* genes in the investigated reactor samples. The tree was constructed using the TN93 algorithm in MEGA with 1,000 bootstrap replicates. The percentage of replicate trees (>60%) are shown by thick branches. The scale bar represents 0.08 substitutions per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

11 Relation between sulphate reduction and phosphate removal

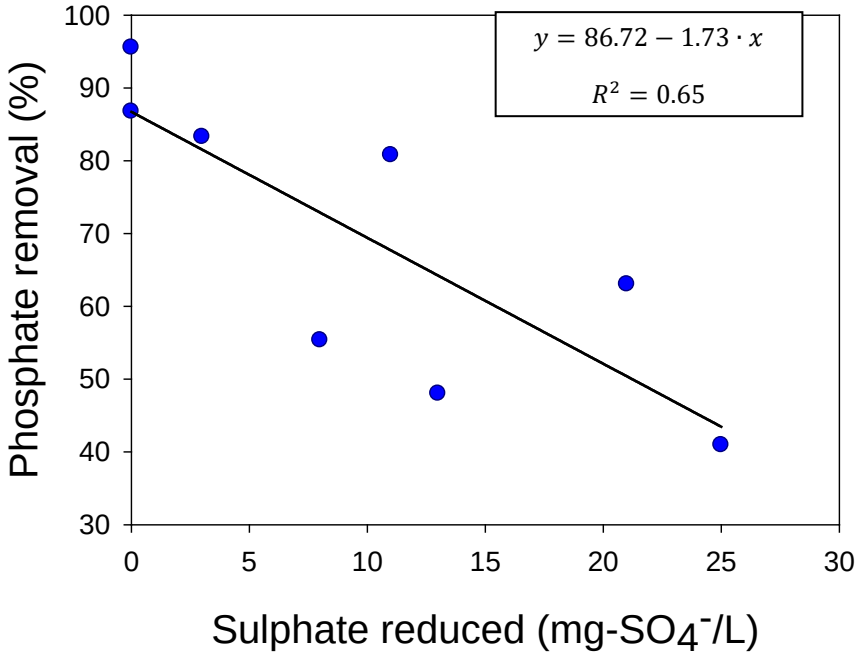

Figure S16: Correlation between sulphate reduced along the anaerobic phase and phosphorus removal.

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

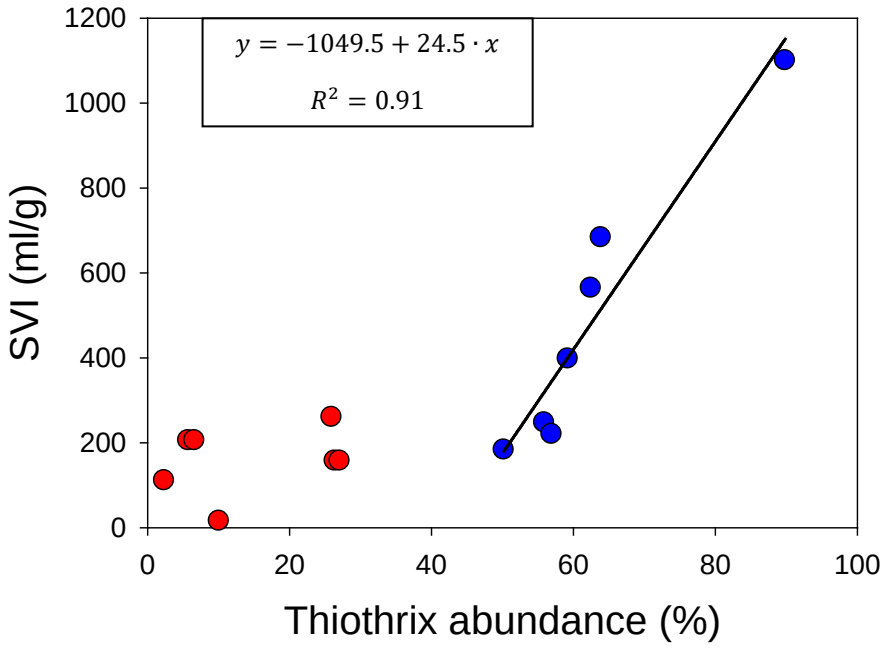

Figure S17: Correlation between SVI and *Thiothrix* abundance. Abundance lower than 50% is shown in red was excluded from the linear regression analysis.

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

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

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