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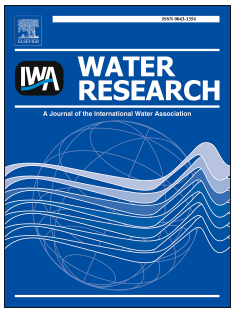

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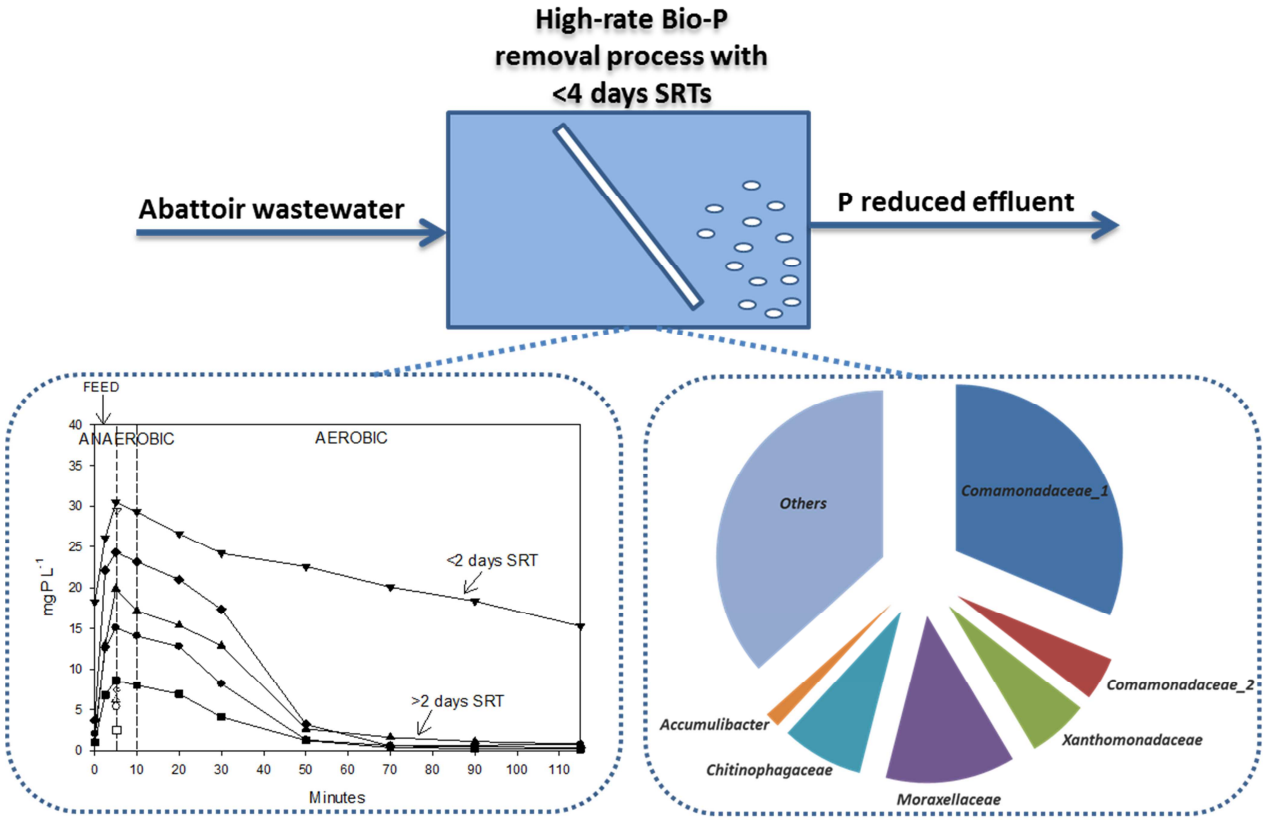

Biological phosphorus removal from abattoir wastewater at very short sludge ages mediated by novel PAO clade *Comamonadaceae*

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Abstract:

Recent increases in global phosphorus costs, together with the need to remove phosphorus from wastewater to comply with water discharge regulations, make phosphorus recovery from wastewater economically and environmentally attractive. Biological phosphorus (Bio-P) removal process can effectively capture the phosphorus from wastewater and concentrate it in a form that is easily amendable for recovery in contrast to traditional (chemical) phosphorus removal processes. However, Bio-P removal processes have historically been operated at medium to long solids retention times (SRTs, 10-20 days typically), which inherently increases the energy consumption while reducing the recoverable carbon fraction and hence makes it incompatible with the drive towards energy self-sufficient wastewater treatment plants. In this study, a novel high-rate Bio-P removal process has been developed as an energy efficient alternative for phosphorus removal from wastewater through operation at an SRT of less than 4 days. The process was most effective at an SRT of 2-2.5 days, achieving >90% phosphate removal. Further reducing the SRT to 1.7 days resulted in a loss of Bio-P activity. 16S pyrotag sequencing showed the community changed considerably with changes in the SRT, but that *Comamonadaceae* was consistently abundant when the Bio-P activity was evident. FISH analysis combined with DAPI staining confirmed that bacterial cells of *Comamonadaceae* arranged in tetrads contained polyphosphate, identifying them as the key polyphosphate accumulating organisms at these low SRT conditions. Overall, this paper demonstrates a novel, high-rate phosphorus removal process that can be effectively integrated with short SRT, energy-efficient carbon removal and recovery processes.

Keywords: Biological phosphorus removal; Short sludge retention time; Polyphosphate accumulating organisms; *Comamonadaceae*; Tetrad-forming bacteria

1. Introduction

Phosphorus is an important element to agricultural and industrial activity, and there are concerns over its depletion as a non-renewable resource (Yuan et al., 2012). Thus there is growing interest in capturing and recycling phosphorus from other sources, such as wastewater. Phosphorus removal is essential in advanced wastewater treatment to meet reduced discharge limits on effluent phosphorus concentrations. Phosphorus removal results in a solids stream rich in phosphorus that has the potential for recovery. Phosphorus removal via biological processes is more economical and environmentally sustainable compared to traditional (chemical) phosphorus removal processes for a number of reasons, including chemical costs, the ability to subsequently release and recover phosphorus, and plant-availability of phosphorus where waste activated sludge (WAS) is used directly (Shu et al., 2006).

So far, Bio-P removal processes have been widely applied to treat domestic wastewater (<10 mg P L^{-1} in influent) (Pijuan et al., 2008), abattoir wastewater (20-40 mg P L^{-1}) (Lemaire et al., 2009), and high-strength industrial wastewater (60-100 mg P L^{-1}) (Broughton et al., 2008). Effective phosphorus removal (>90%) has been achieved in most cases, resulting in a final phosphorus concentration generally below 1 mg L^{-1} phosphate (PO_4^{3-})-P. The typical process configuration for the Bio-P process is either alternating anaerobic/aerobic phase in one reactor (e.g. sequencing batch reactor (SBR)) or recirculating sludge through anaerobic and aerobic zones, often with anoxic reactor(s) in between for denitrification (e.g., Bardenpho, modified University of Cape Town (UCT), etc.) (Oehmen et al., 2007). Generally, an overall sludge retention time (SRT) in the range of 8-30 days is applied (Oehmen et al., 2007) to retain key organisms, e.g. nitrifiers for nitrification. This relatively long SRT can introduce challenges to maintain a stable Bio-P removal process, as some organisms (e.g. denitrifiers

and glycogen accumulating organisms (GAOs)) can outcompete polyphosphate accumulating organisms (PAOs) for available carbon (Oehmen et al., 2007), resulting in a decreased Bio-P activity and diminished phosphorus removal efficiency. Therefore, the focus of previous work has been on the investigation of PAOs and their competitors, and process optimisation to avoid competition. Novel processes, such as high-rate activated sludge (A-stage) processes focus on carbon accumulation in an initial short-SRT process (Jimenez et al., 2005), and achieving phosphorus removal in the same stage would be highly beneficial (Ge et al., 2013). However, investigation of the minimal SRT required for Bio-P removal processes has been limited so far. There are two papers most relevant in this regard. Mamais and Jenkins (1992) evaluated the effect of SRT (2-4 days) on Bio-P removal, and reported that the Bio-P removal functioned efficiently at the SRT above 2.9 days, and deteriorated once the SRT decreased to 2.6 days. Brdjanoyic et al. (1997) used a model-based approach to identify the minimum SRT required for the stable Bio-P removal process, which was estimated as 8 days at 20℃, extending to 16 days at 10℃ and further to 32 days at 5℃. These SRTs are far beyond the SRT range (0.5-2 days) normally applied in the A-stage processes.

The most well known group of PAOs is the *Rhodocyclus*-related "*Candidatus Accumulibacter phosphatis*" (referred to as *Accumulibacter* hereafter), which has been commonly found in many full-scale Bio-P removal plants, with a typical abundance of 5-20% of the bacterial community (Yuan et al., 2012). The metabolism of *Accumulibacter* has been studied extensively, and includes carbon (i.e., volatile fatty acids (VFAs)) uptake and polyhydroxyalkanoates (PHA) formation under anaerobic conditions, followed by the oxidation of PHA to provide energy for phosphate uptake and cell growth under subsequent aerobic conditions (Oehmen et al., 2007). Several other bacteria have also been identified as PAOs (referred to as non-*Accumulibacter* PAOs hereafter), including *Tetrasphaera* (Kong et

al., 2005; Nguyen et al., 2011), *Pseudomonas* (Günther et al., 2009), *Microlunatus phosphovorus* (Nakamuka et al., 1995), *Halomonas* (Nguyen et al., 2012), and some members of *Actinobacteria* (Beer et al., 2006). Some non-*Accumulibacter* PAOs (e.g. *Tetrasphaera*) have been found to be more abundant in certain non-domestic full-scale Bio-P removal plants than *Accumulibacter* (Nguyen et al., 2011). Metabolic processes of non-*Accumulibacter* PAOs are also reported to be different from *Accumulibacter*, where some can take up carbon sources other than VFAs, such as glucose and amino acids, with unknown storage compounds formed instead of PHA (Nakamura et al., 1995; Nguyen et al., 2011). The diversity, metabolism, and function of non-*Accumulibacter* PAOs have not been investigated to the same extent as *Accumulibacter*. In particular, phylogeny and function in non-domestic and high-rate systems such as short SRT Bio-P systems (Ge et al., 2013) have not been investigated, with most analysis having been done in domestic or synthetic feed systems.

This paper describes the development and characterisation of a high-rate Bio-P removal process with an operating SRT of less than 4 days, focusing on the capability and differentiation of the microbial agent compared to Bio-P processes in conventional, longer-SRT activated sludge systems.

2. Material and Methods

2.1. Abattoir wastewater

Wastewater was collected from a local abattoir (following dissolved air flotation and solid paunch separation) in Queensland, Australia on a fortnightly basis and stored at 4°C. Wastewater was diluted with tap water to a total chemical oxygen demand (TCOD) of 2-3 g L^{-1} to dampen variations of the wastewater strength and composition due to the intermittent collection schedule. Characteristics of the wastewater feed (after the dilution) were analysed

125 regularly, and the results are summarised in Table 1. The average ratio of SCOD to total
126 phosphorus in the wastewater feed was approximately 40.
127
128 **2.2. Sequencing batch reactor (SBR) setup and operation**
129 A lab-scale SBR with a working volume of 5 L was used. The SBR was inoculated with
130 activated sludge from a non-enhanced biological phosphorus removal (non-EBPR) full-scale
131 wastewater treatment plant located in Brisbane, Australia. The SBR was operated with eight
132 cycles (3 h per cycle) per day in a temperature controlled room (20-22°C). Each cycle
133 consisted of a 10 min anaerobic period, a 105 min aerobic period, and a 65 min settle/decant
134 period. The wastewater was fed into the SBR in the first 5 min of the anaerobic period and
135 discharged in the decant period (5 min) to maintain the HRT. The SRT of the SBR was
136 maintained by wasting sludge during the last 5 min of the aerobic period. pH was measured
137 using a glass body pH probe (TPS, Australia), ranging between 7.0-7.8, but not controlled.
138 The dissolved oxygen (DO) concentration in the aerobic period was maintained between 1.5
139 and 2 mg O_2 L^{-1} by providing air intermittently with an on/off controller and using a DO
140 membrane probe (YSI, Australia). Both DO and pH probes were calibrated regularly and
141 connected to a process logic control system for data recording.
142
143 The SBR was operated for over 10 months where the HRT and SRT were varied to evaluate
144 the phosphorus removal performance. Table 2 summarized the HRT and SRT applied in each
145 operating period. It should be noted that the real SRT in some periods differed slightly from
146 the target SRT due to varying solids concentrations in the effluent.
147
148 **2.3. Chemical analysis**

Mixed liquor samples were taken regularly and analysed for TCOD, soluble COD (SCOD), total suspended solids (TSS), volatile suspended solids (VSS), total Kjeldahl phosphorus (TKP) and PO_4^{3-}. Before measuring PO_4^{3-} and SCOD concentrations, the mixed liquor samples were filtered through Millipore filter units (0.45 µm pore size). TSS, VSS, and COD were analysed based on Standard Methods (APHA, 1998). PO_4^{3-} and TKP were measured using a Lachat Quik-Chem 8000 Flow Injection Analyser (Lachat Instrument, Milwaukee).

2.4. Calculation methods

$$Specific\quad \mathrm{Re}moval\quad Efficiency\,(mgP\quad gVSS^{-1}\quad d^{-1}) = \frac{(PO_{4\ in}^{3-} - PO_{4\ out}^{3-}) \times SBR\quad loading}{Biomass\quad concentration \times \mathrm{Re}actor\quad volume}$$

Where $PO_{4\ in}^{3-}$ = PO_4^{3-}-P concentration in the influent (mg L^{-1});

$PO_{4\ out}^{3-}$ = PO_4^{3-}-P concentration in the effluent (mg L^{-1});

SBR loading = 9 $L_{wastewater}$ d^{-1};

Biomass concentration = Biomass concentration in the SBR (g VSS L^{-1});

Reactor volume = 5 L.

2.5. Characterisation of microbial communities

2.5.1. 16S rRNA gene amplicon pyrosequencing

Genomic DNA was extracted from the biomass samples collected in the SBR by using a FastDNA Spin Kit for soil (MP Biomedicals, USA) according to the manufacturer's protocol. The quantity and quality of the extracted DNA was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) and agarose gel (1%, weight/volume) electrophoresis. The extracted DNA was submitted to the Australian Centre for Ecogenomics (ACE) for 16s rRNA gene pyrotag sequencing on the Genome Sequencer FLX Titanium

platform (Roche, USA). The primers used for pyrotag sequencing were modifications of the 926F (5'-AAACTYAAAKGAATTGACGG-3') and 1392wR (5'-ACGGGCGGTGWGTRC-3').

Pyrotag sequence analysis was performed as described previously (Ge et al., 2013) using ACE Pyrosequencing Pipeline (Imelfort and Dennis, 2011a) developed based on QIIME (Caporaso et al., 2010) and ACACIA (Bragg et al., 2012). The generated operational taxonomic units (OTUs) table was then normalised by using Nomaliser (Imelfort and Dennis, 2011b).

2.5.2. Fluorescence *in situ* hybridization (FISH) and polyphosphate (polyP) staining

FISH and polyP staining by DAPI (4´, 6´- diamidino-2-phenylindole) is incompatible in a single preparation, and these two must be done sequentially. FISH was performed according to Amann (1995). Table 3 summarizes the oligonucleotide probes used in this study, along with the hybridization conditions and related references. Slides were viewed using a Zeiss LSM 510 Meta Confocal laser scanning microscope (Zeiss, Germany) and the location of important fields noted after the image was acquired. Subsequent polyP staining was conducted by incubation with 1 µg mL^{-1} DAPI in dark for 60 min (Serafim et al., 2002). The fields from which FISH images had been collected were located, and images of DAPI stains were also recorded by the confocal microscope with filter sets that allow the emission wavelength of 450-520 nm to pass through.

2.5.3. Scanning electron microscopy (SEM) analysis

Biomass samples were collected from the SBR, freeze-dried and analysed using a SEM with a back-scattered electron detector (excitation voltage of 12 kV) to investigate the morphology

198 of bacteria cells. Energy dispersive X-ray spectroscopy (EDX) together with a SEM was used
199 for elemental (phosphorus) analysis.
200

201 # 3. Results

202 **3.1. Phosphorus removal performance**

203 Fig. 1 shows PO_4^{3-}-P present in the influent and effluent of the SBR during all operating
204 periods. Bio-P removal was present in the SBR at the end of the start-up period, and the
205 removal efficiency was consistently near or above 90% in the subsequent operating periods
206 (1-4), where the SRT was varied from 2 days to 4 days (HRT maintained at 0.5 day). The
207 specific removal efficiency was highest at 2 days SRT (Table 4), and slightly decreased with
208 increasing the SRT to 2.5 days and further to 3 and 4 days. Typical Bio-P removal profiles
209 obtained in the cycle studies performed during these periods are shown in Fig. 2. Based on
210 the PO_4^{3-}-P concentrations of the raw wastewater fed into the SBR and the dilution within the
211 SBR, the calculated PO_4^{3-}-P concentrations in the SBR bulk liquid after the feeding were
212 much lower than the PO_4^{3-}-P concentrations measured at the end of the feeding period (Fig.
213 2). This clearly indicates that PO_4^{3-} was released to the bulk liquid during the anaerobic phase
214 and taken up from the liquid in the subsequent aerobic phase, with < 3 mg PO_4^{3-}-P L^{-1}
215 remaining in the effluent. Phosphate release was strongly linked to the uptake of the available
216 carbon (represented by SCOD) in the SBR, and an example of the SCOD uptake profile in a
217 SBR cycle during Period 2 (2 days SRT, 0.5 day HRT) is shown in Fig. 3. The majority of
218 SCOD was consumed during the anaerobic phase (including the feeding period), with a small
219 fraction of SCOD uptake during the subsequent aerobic phase, indicating the residual SCOD
220 was non-biodegradable. Moreover, the removal efficiency was not influenced by extending
221 the HRT to 1 day (Period 5), but dropped during Period 6 and Period 7, likely due to
222 variations in the feed composition. At the end of Period 7, the Bio-P removal efficiency

returned to the previous level (Fig. 1), and was maintained when the HRT was extended to 1 day again in Period 8 with doubling the COD feeding (to 4000 mg COD L^{-1}) to maintain a similar COD load as previously. Again, this indicated the HRT did not have a substantial impact on the Bio-P removal efficiency, but instead that the Bio-P removal capability was mainly driven by SRT. Additionally, the SRT was reduced to 1.7 day to determine the minimum SRT for achieving Bio-P removal. As shown in Fig. 2, the Bio-P removal was lost, as reflected by the lack of PO_4^{3-} release during the anaerobic phase, ultimately resulting in a decreased removal efficiency.

3.2. Changes in microbial community composition for different SRTs

Fig. 4 shows average abundances of the major bacterial groups in the SBR under operations with 2-4 days SRTs (0.5 day HRT), with additional information provided in Supplementary Information Fig. S1. The phylogenetic relationship of these bacterial groups is also shown in Fig. 4 (bottom). Generally, the SBR microbial communities changed considerably with changes in SRT, except *Comamonadaceae* and *Xanthomonadaceae*, which were present consistently. The family *Comamonadaceae* in this case was primarily represented by two genera, one predominant in all the tested periods (approximately 30%), and the other emerging at 2.5 and 4 days SRTs (approximately 8%), possibly induced by variations in the feed composition. Members affiliated with *Moraxellaceae* were also consistently present at 2, 2.5 and 3 days SRTs, but disappeared at 4 days SRT, where increased abundances of *Sphingomonadaceae, Flexibacteraceae* and *Accumulibacter* were observed. Among these bacterial groups, *Comamonadaceae* was most closely related to *Accumulibacter* compared to other abundant groups, nominating it as an organism of interest for further investigation.

3.3. Identification of putative PAOs in the high-rate Bio-P removal process

The ability to form and store polyP (a key feature of PAOs) by *Comamonadaceae* was further investigated using FISH analysis and DAPI staining. There is no FISH probe available to target the entire family of *Comamonadaceae*, but a previously published Cte probe is targeting several prevalent genera in this family, including *Comamonas spp.*, *Acidovorax spp.*, *Hydrogenophaga spp.*, and *Aquaspirillum spp.* (Schleifer et al., 1992). Bacterial cells hybridized with the Cte probe were present in all biomass samples of 2-4 days SRTs. They existed in three different morphotypes, including small rods, often present as single cells that were either within or attached to the flocs, small cocci formed in tetrads occurring singly or in clusters, and filaments often arranged in chains (an example is shown in Supplementary Information Fig. S2). However, polyP was found only in tetrads according to the DAPI stain, which showed intracellular yellowish granules suggesting polyP storage. Fig. 5 shows an example of FISH images collected from a biomass sample of 2 days SRT, with the tetrads hybridized with Cte and Eubmix probes displaying DAPI-positive polyP in the tetrads. This strongly indicates that the Cte probe-defined *Comamonadaceae* with a tetrad morphology (here called tetrad-*Comamonadaceae*) were putative PAOs. FISH hybridisation simultaneously with DAPI (including the EUBmix probe) generally indicated no probe binding in contrast with literature methods (Serafim et al., 2002), which may be due to the high polyP content in the cells interfering with binding. Thus the microscopic examination of FISH and DAPI staining were conducted separately in this study to obtain the photos shown in Fig. 5.

In general, a small portion of short-rod shaped cells were also observed to be DAPI positive in the samples at 4 days SRT, and identified as *Accumulibacter* by FISH analysis using PAOmix probes (photos not shown).

As the tetrad-forming bacteria containing polyP at short SRTs (e.g. 2 days) were of particular interest, they were further investigated by SEM combined with EDX elemental analysis. Fig. 6 shows SEM images and spot EDX analysis of tetrad-forming bacteria at 2 days SRT. Strong phosphorus peaks were observed in all the cocci of the tetrads (one example shown in Fig. 6A), along with some other peaks (the carbon and oxygen peaks originated from the organic biomass matrix), while much lower levels of phosphorus were detected from the biomass background (one example shown in Fig. 6B). The cellular phosphorus observed with EDX was most likely polyP, which is a major component of intracellular phosphorus pools stored in bacterial cells (Ohtake et al., 1994).

4. Discussion

4.1. Impact of SRT on the high-rate Bio-P removal performance

Complete phosphorus removal was achieved in the high-rate SBR with 2-4 days SRT, and the specific removal rate appeared to increase with a decrease in SRT from 4 to 2 days. However, further decreasing the SRT to 1.7 days resulted in the cessation of Bio-P removal, probably due to wash out of functional PAOs from the SBR. This indicates an optimum of 2-2.5 days SRT. In practise, a high-rate Bio-P removal process with such short SRTs can strongly enhance the competitiveness of Bio-P removal process and expand applications over other phosphorus removal methods, as this process allows substantial process intensification through reducing reactor and clarifier volumes (correspondingly lower construction costs). It should also be noted that in this high-rate Bio-P removal process, there was no nitrification/denitrification for nitrogen removal due to the inability of nitrifiers to grow at such short SRTs. If the practical treatment target is the combined nitrogen and phosphorus removals, this high-rate Bio-P removal process can achieve only partial nitrogen removal due to absorption and assimilation, rather than nitrification (Ge et al., 2013). It means the SBR

effluent may be required to be post-treated for residual nitrogen removal e.g. via an anammox process (a low energy-demand nitrogen removal process with no carbon requirement). This combined treatment process could still offer a number of benefits in terms of costs, energy demand and space requirements compared to conventional nutrient removal processes (see the detailed evaluation in Ge et al. (2013)), particularly where a high-nitrogen, low carbon effluent can be utilised for irrigation, and where sludge can be digested and phosphorus recovered from centrate.

There are extensive studies that evaluate Bio-P removal efficiencies at different SRTs, with the majority focusing on SRTs between 8-12 days, which also enable nitrogen removal. A shorter SRT of 5 days was tested in the studies of Choi et al. (1996) and Chang et al. (1996). Bio-P removal was achieved in both cases but the phosphorus removal efficiency of approximately 85% was slightly lower than the efficiency (>90%) obtained at 10 days SRT. The SRT was further reduced to 3.2 days in the study of Mamais and Jenkins (1992), which achieved a removal efficiency of approximately 90% at 3.2 days and 2.9 days SRTs. However, the removal efficiency decreased to approximately 80% with further reducing the SRT to 2.6 days, and to 40% at 2.3 days SRT, which is not consistent with the observations in this study. This difference was likely due to the relatively low COD loading rate of the SBR in their studies, which may not have provided sufficient carbon for PAOs. This need for organic carbon is a challenge in applying short SRT Bio-P removal in domestic wastewater, which has lower COD (300-500 mg COD L^{-1}). However, the results here indicate the essential metabolic capability existing for successful phosphorus removal exists at SRTs down to 2 days, and can likely be applied to emerging low-energy, assimilative-adsorptive-accumulative high-rate aerobic wastewater treatment processes (Batstone et al., 2014), if organic carbon can be suitably retained or supplied (e.g., from primary sludge hydrolysis).

4.2. Dynamic nature of microbial communities and correlations with the high-rate Bio-P removal process

This is the first molecular study to determine bacterial community diversities and dynamics of Bio-P removal processes at short SRTs (<4 days). Generally, the bacterial communities are very dynamic under different SRTs in the SBR, but the population of *Comamonadaceae* was consistently present in the functionally stable SBR. *Comamonadaceae* has been commonly detected in activated sludge wastewater treatment processes at both full and lab scales, and is capable of consuming a wide variety of organic acids, including amino acids (Willems, et al., 1991). The versatile nature of *Comamonadaceae* may have given them a competitive advantage to become abundant in the high-rate SBR, as proteins that are among the main components in the abattoir wastewater used in this study and could be broken down to amino acids, could allow *Comamonadaceae* to outcompete organisms that are only capable of utilising VFAs. Moreover, altering SRT in the SBR resulted in changes of the reactor performance, which may have also influenced the SBR communities. For example, the population of *Moraxellaceae* was nearly eliminated at 4 days SRT compared with 2-3 days SRTs, possibly because *Moraxellaceae* were inhibited by high-ammonia present at a 4 day SRT (resulting from an increase in protein hydrolysis).

Variations in the feed composition (organics in abattoir wastewater) were also observed to affect the bacterial community structure in the SBR. For example, *Saprospiraceae* (Fig. S1) emerged in an operating period with an SRT of 2.5 days, but was not present in other periods at SRTs of 2 and 3 days. This indicates that the selection pressure due to changes in the feed composition may have had further influence on the bacterial community compositions in addition to the SRT. Variations in the compositions of abattoir wastewater across different

collections (e.g. containing more proteins) may have given *Saprospiraceae* a transient advantage over the rest of the mixed microbial community, as *Saprospiraceae* has been reported to be capable of hydrolysing proteins (Xia et al., 2008). Similarly, *Chitionphagaceae* became more abundant during the two subsequent operating Periods 2-3 (2 and 4 days SRTs), which could also be for the same reason.

Although SRT was a dominant driver for microbial community, the Bio-P removal was functionally very stable in the SBR during Periods 1-4 for over 100 days. It suggests that population shifts or community fluctuations under different stress conditions caused by environmental/operational changes (e.g. variations of wastewater compositions, HRT, etc.) still allowed functional stability for Bio-P removal. This indicates that different PAO populations appeared in the community (see e.g. the different *Comamonadaceae* genera present at 4 days SRT) with similar capability, thereby ensuring the stability of the Bio-P removal process.

4.3. Putative PAOs responsible for the high-rate Bio-P removal process

This study has shown that the Cte probe-defined tetrad-*Comamonadaceae* is likely to be an important group of PAOs present in the high-rate Bio-P removal process, as they were present in large amounts and accumulated polyP as indicated by DAPI staining. Although the family of *Comamonadaceae* contains numerous genera, they have diverse ecophysiological behaviours. For example, genus *Curvibacter* is likely to be involved in protein hydrolysis and denitrification (Nielsen et al., 2010) and genus *Malikia* has been identified as a PHA and polyP accumulating bacterium (Spring et al., 2005). This may indicate that different genera of *Comamonadaceae* as identified here may play different roles in the high-rate Bio-P removal process, such as proteolysis to supply soluble carbon and/or polyP accumulation. We

attempted to utilise more specific FISH probes, including the ACI208 probe for *Acidovorax spp.* (Amann et al., 1996). However, this did not bind to tetrad-*Comamonadaceae*.

Bacterial cells of *Comamonadaceae* with a tetrad morphology were observed for the first time in this study. Tetrad-forming organisms have been observed in Bio-P removal systems before, which have been shown, phylogenetically, to be members of the *Alphaproteobcteria* (e.g. *Amaricoccus spp.*, and '*Defluvicoccus vanus*'), *Betaproteobacteria* (*Quadracoccus sp.*), *Gammaproteobacteria* and *Actinobacteria* (*Tetrasphaera spp.*, *Micropruina glycogenica* and *Kineosphaera limosa*) (Maszenan et al., 2005; Oehmen et al., 2007; Nguyen et al., 2011). Understanding of the relevance of this morphology to Bio-P is limited, although preliminary investigations have been made, such as '*Defluvioccus*'-related tetrad-forming organisms showing phenotypic traits similar to GAOs (Wong et al., 2004), and *Tetrasphaera* forming clusters of tetrads identified as a putative PAO (Nguyenn et al., 2011). Moreover, it is still unknown what factors (e.g. process operation, wastewater composition, etc.) induce bacterial cells to arrange themselves in tetrads, and what benefits can be obtained from this arrangement. The need for future studies may therefore mainly be related to the understanding of key factors that affect the morphotypes of *Comamonadaceae* and the relations to their phenotypes.

5. Conclusions

Bio-P removal can be achieved in the high-rate SBR with short SRTs (<4 days) treating abattoir wastewater, with the specific removal efficiency being highest at 2-2.5 days SRTs. Reducing the SRT further to 1.7 days resulted in a drastic decrease of Bio-P removal performance in the SBR. Varying SRTs in the SBR also influenced the bacterial community compositions, but the population of *Comamonadaceae* was consistently dominant in the

functionally stable SBR with 2-4 days SRTs. Bacterial cells of *Comamonadaceae* (Cte probe-defined) arranged in tetrads contained positive DAPI stained polyP, indicating *Comamonadaceae* was a strong candidate as the responsible PAO in this high-rate Bio-P removal process.

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List of tables and figures

Table 1 – Characteristics of the wastewater used in this study.

Characteristic	Wastewater
TCOD (g L^{-1})	2.9 (0.2)[a]
SCOD[b] (g L^{-1})	1.0 (0.1)
pH	7-7.5
TKP[b] (mg L^{-1})	28.2 (1.1)
PO_4^{3-}-P (mg L^{-1})	23.4 (0.6)

[a]: Standard deviations shown in parenthesis are based on 17 different wastewater samples collected over a 10 months period.

[b]: SCOD: Soluble COD; TKP: Total Kjeldahl phosphorus.

Table 2 – Summary of the SBR operating conditions in this study.

Operating period	HRT (day)	Target SRT (day)	Actual SRT (day)
Start-up (55 days)	0.5	2	2.1
Period 1 (33 days)	0.5	3	2.8
Period 2 (21 days)	0.5	2	1.9
Period 3 (28 days)	0.5	4	3.8
Period 4 (27 days)	0.5	2.5	2.3
Period 5 (25 days)	1	2.5	2.4
Period 6 (27 days)	1	2	2
Period 7 (39 days)	0.5	2	2
Period 8 (15 days)	1	2	2
Period 9 (9 days)	1	1.7	1.7

Table 3 – Oligonucleotide probes used for FISH.

Probe	Specificity	Sequence (5´-3´)	Formamide%	References
ACI208	*Acidovorax spp.*	CGCGCAAGGCCTTGC	20	Amann et al. (1996)
Cte	*Comamonas spp., Acidovorax spp., Hydrogenophaga spp., Aquaspirillum spp.*	TTCCATCCCCCTCTGCCG	20	Schleifer et al. (1992)
EUB338	*Most bacteria*	GCTGCCTCCCGTAGGAGT	0-50	Amann et al. (1990)
EUB338 II		GCAGCCACCCGTAGGTGT	0-50	Daims et al. (1999)
EUB338 III		GCTGCCACCCGTAGGTGT	0-50	Daims et al. (1999)
PAO462	*Most Accumulibacter*	CCGTCATCTACWCAGGGTATTAAC	35	Crocetti et al. (2000)
PAO651		CCCTCTGCCAAACTCCAG	35	Crocetti et al. (2000)
PAO846		GTTAGCTACGGCACTAAAAGG	35	Crocetti et al. (2000)

EUB338, EUB338II and EUB338III were applied simultaneously as EUBmix.
PAO264, PAO651, PAO846 were used together in a mixture called PAOmix.

Table 4 – A Summary of the phosphate (PO_4^{3-}-P) removal performance in the high-rate SBR.

Operating period	HRT	SRT	PO_4^{3-}-P removal (%)	Specific PO_4^{3-}-P removal (mg $gVSS^{-1}d^{-1}$)
Start-up	0.5d	2.1d	58.7±6.3	4.8±0.6
Period 1	0.5d	2.8d	95.8±2.3	8.9±0.2
Period 2	0.5d	1.9d	90.7±3.1	11.4±0.2
Period 3	0.5d	3.8d	88.8±2.1	7.2±0.1
Period 4	0.5d	2.3d	91.3±3.6	9.4±0.3
Period 5	1d	2.4d	89.4±1.5	5.8±0.1
Period 6	1d	2.0d	77.6±4.1	7.8±0.2
Period 7	0.5d	2.0d	65.2±4.9	9.4±0.3
Period 8	1d	2.0d	96.1±1.6	8.0±0.1
Period 9	1d	1.7d	51.3±3.5	7.0±0.3

Error margins indicate 95% confidence intervals across different measurements over each period.

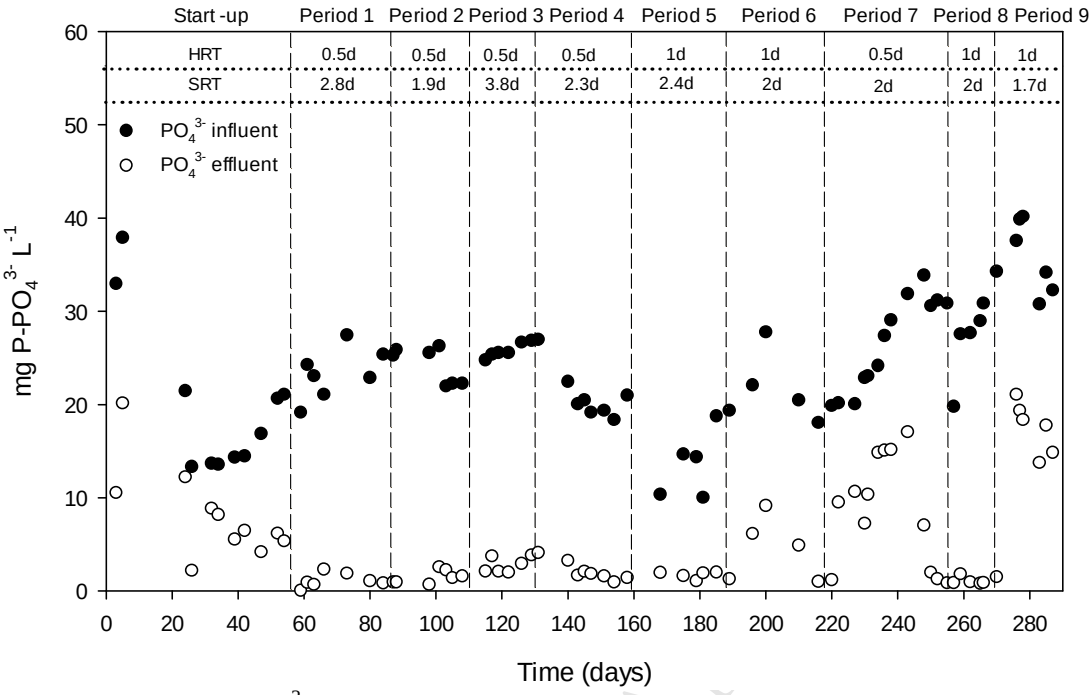

Fig. 1 – Phosphate (PO_4^{3-}) concentrations in influent and effluent across the whole operating period.

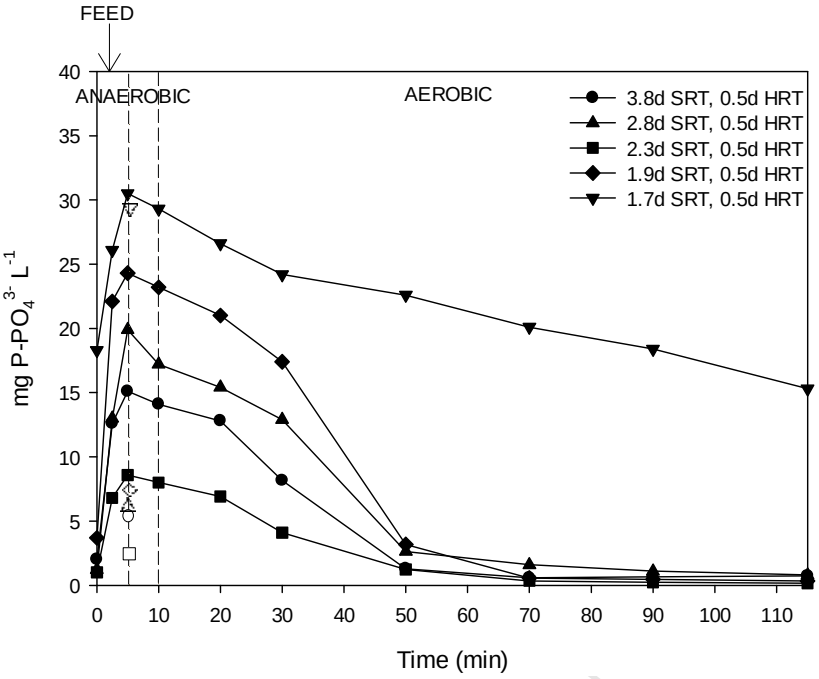

Fig. 2 – Phosphate (PO_4^{3-}) profiles during the SBR cycle studies performed in the different operating periods. Open symbols shown at 5 min represent the dilution-based, calculated PO_4^{3-} concentrations in the SBR after feeding, corresponding to the equivalent closed symbol operations (the calculation considers the PO_4^{3-} concentrations of the raw wastewater fed into the SBR and the dilution within the SBR).

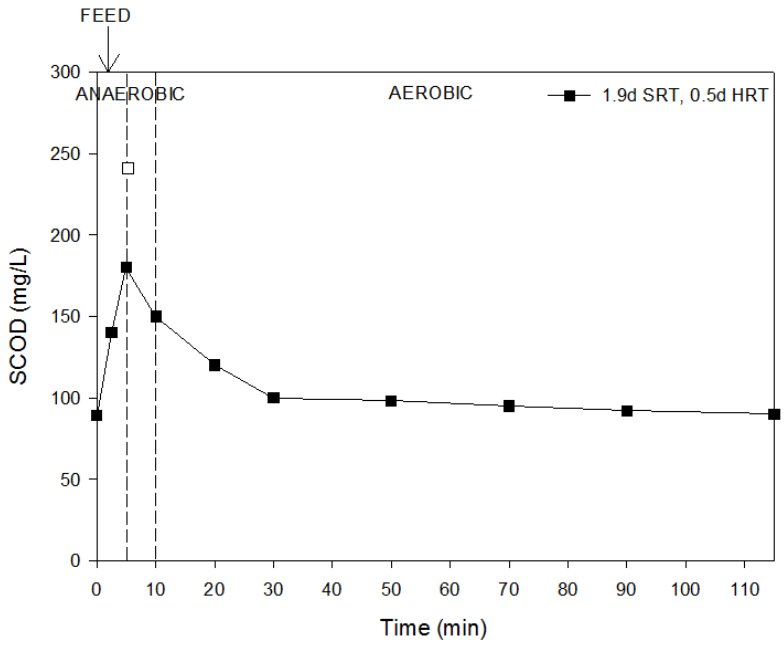

Fig. 3 – SCOD profiles in a SBR cycle under the SBR operation of 0.5 day HRT and 1.9 days SRT. Open symbols shown at 5 min represent the dilution-based, calculated SCOD concentration in the SBR after feeding, corresponding to the equivalent closed symbol operation (the calculation considers the SCOD concentration of the raw wastewater fed into the SBR and the dilution within the SBR).

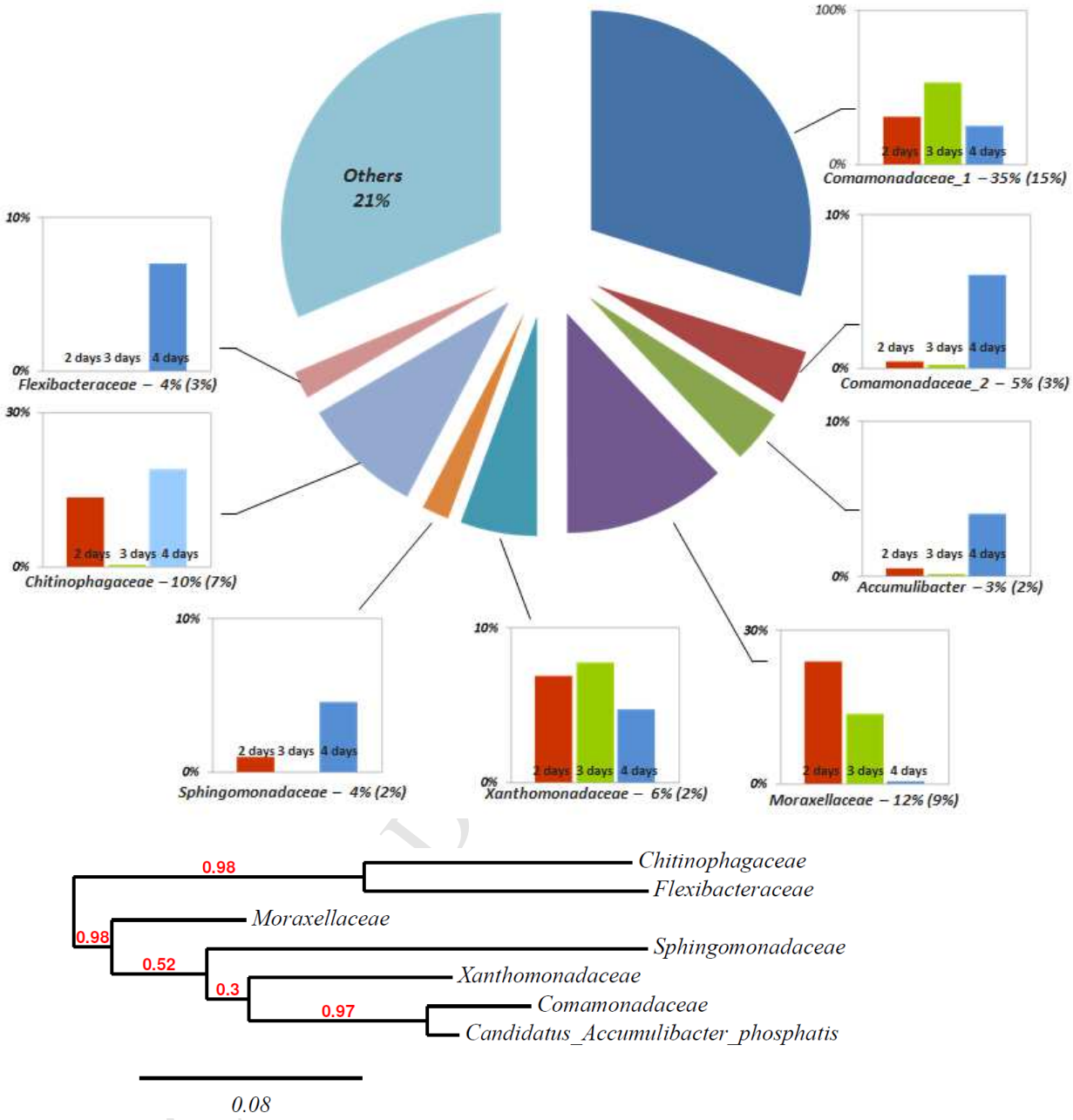

Fig. 4 – Average abundances of the major bacterial groups (family level, except the genus of *Accumulibacter*) in the SBR operated at 2-4 days SRTs and their maximum likelihood phylogenetic relationship according to Dereeper et al. (2008) (*Comamonadaceae_1* and *Comamaonadaceae_2* mean different genera within the family *Comamonadaceae;* Standard deviations shown in parenthesis are based on three sequential samples analysed at each of the three SRTs).

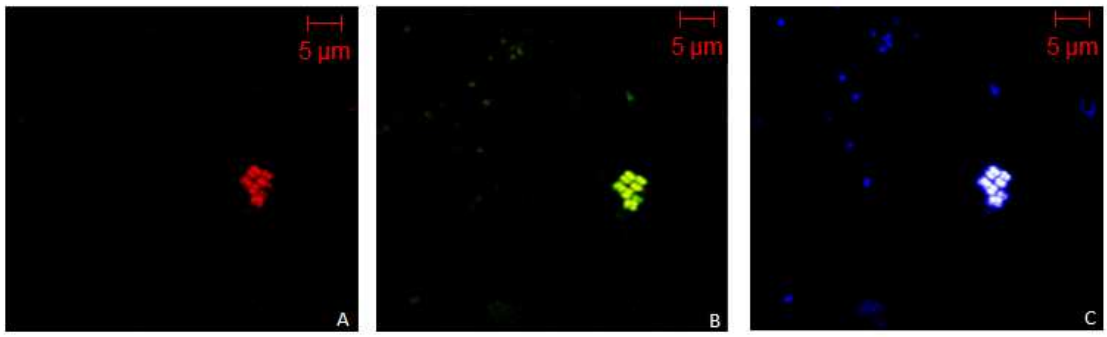

Fig. 5 – FISH and DAPI staining images of tetrad-forming bacteria in the high-rate SBR with 2 days SRT. (A) FISH image shows tetrad-forming bacteria hybridizing with bacterial probe Cte (red). (B) FISH image shows tetrad-forming bacteria hybridizing with bacterial probes EUBmix (green) and Cte (red). The overlay of red and green is yellow. (C) Tetrad-forming bacteria containing polyphosphate stained by DAPI. Polyphosphate emits a bright yellow colour and bacterial cells emit blue colour. The overlay of yellow and blue is bright white.

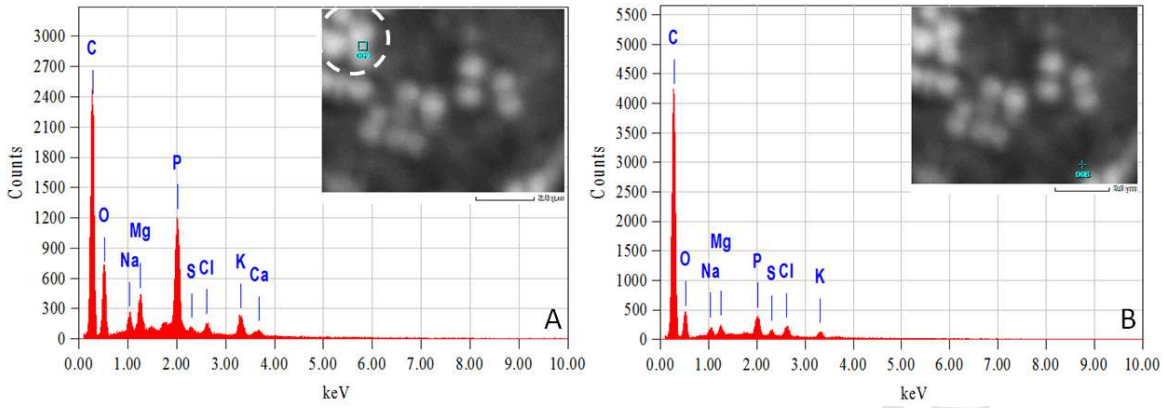

Fig. 6 – Characterisation of tetrad-forming bacteria using scanning electron microscope (SEM). (A) SEM image of typical tetrad-forming bacteria. The energy dispersive X-ray (EDX) spectrum shown below was collected from the specific spot within tetrad-forming bacteria indicated in the above image. (B) shows the EDX spectrum collected from the specific spot outside of tetrads-forming bacteria.

Biological phosphorus removal from abattoir wastewater at very short sludge ages mediated by novel PAO clade *Comamonadaceae*

Research Highlights:

► Bio-P removal was achieved in a SBR based activated sludge process with < 4 days SRTs.

►The process was most effective at 2-2.5 days SRTs, but inhibited at 1.7 days SRT.

► Ability of polyphosphate storage was found in a novel PAO clade *Comamonadaceae*.

►The process can be integrated with short SRT, energy-efficient carbon removal processes.

Supplementary Information

Manuscript title: Biological phosphorus removal from abattoir wastewater at very short sludge ages mediated by novel PAO clade *Comamonadaceae*

Authors: Huoqing Ge, Damien J. Batstone, Jürg Keller*

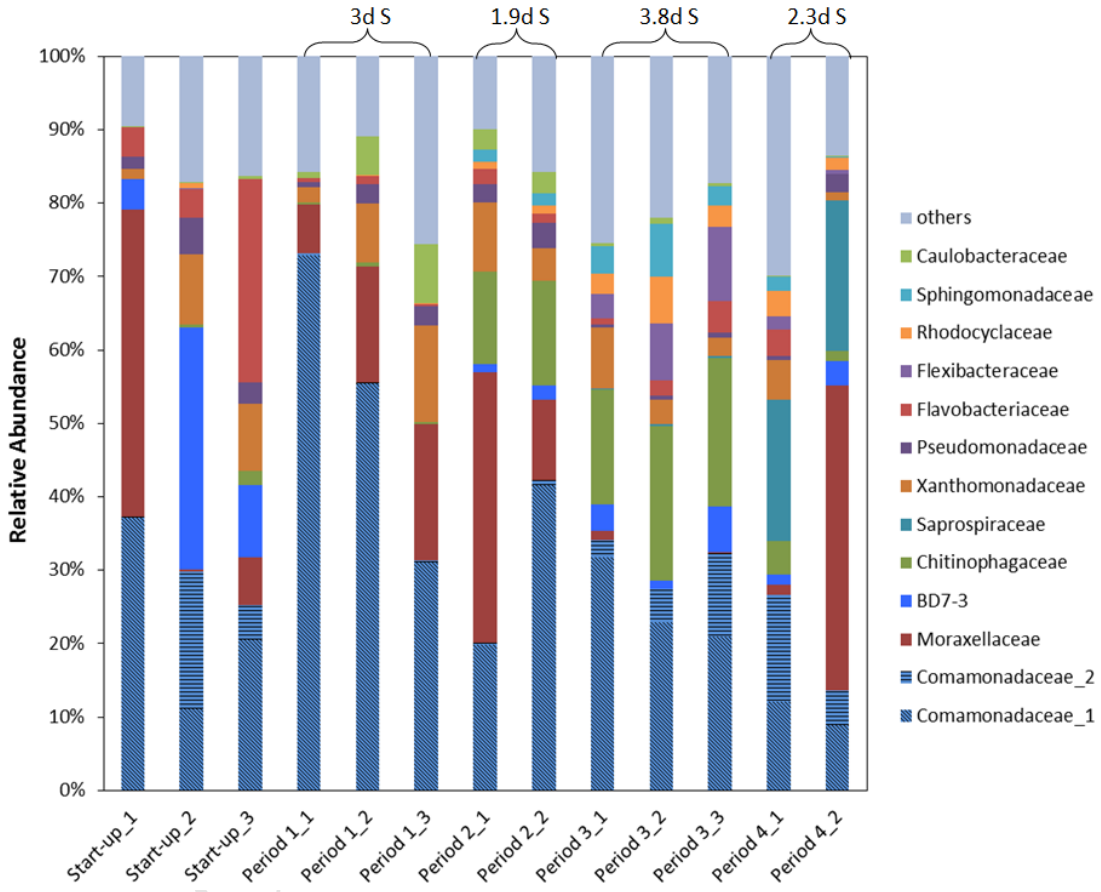

Fig. S1 – Microbial communities (family level, expect the order of *BD7-3*) identified by 16S rRNA gene Pyrotag sequencing in the SBR in the start-up period (Day 34, Day 40, Day 52) and Periods 1-4 (Day 62, Day 72, Day 80, Day 98, Day 105, Day 110, Day 116, Day 128, Day 145 and Day 158). (S represents SRT, *Comamonadaceae__1* and *Comamaonadaceae__2* mean different genera within the family *Comamonadaceae*)

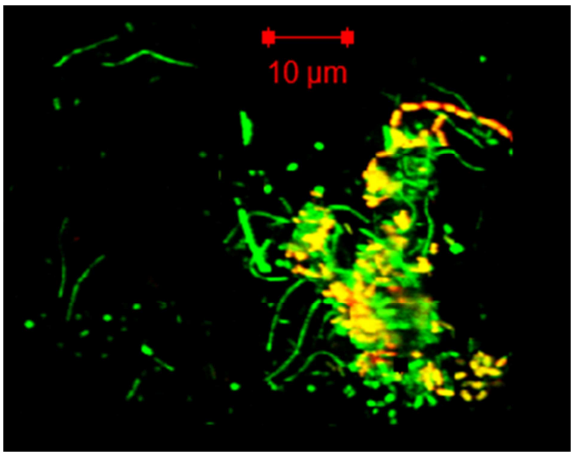

Fig. S2 – The FISH image of probe Cte-defined bacteria in activated sludge from the high-rate SBR at 2 days SRT. It shows bacteria hybridizing with the bacterial probes Eubmix (green) and probe Cte (red) with different morphologies: small rods and filaments. The overlay of red and green is yellow.

Table S1 – Nitrogen concentrations in the abattoir wastewater used in this study.

Characteristic	Wastewater
TKN[a] (mg L^{-1})	110 (15)[b]
NH_4^+-N (mg L^{-1})	65 (9)
Particulate N (mg L^{-1})	43 (1.1)

[a]: TKP: Total Kjeldahl nitrogen; NO_3^- and NO_2^- were also detected at much lower levels (data now shown).

[b]: Standard deviations shown in parenthesis are based on 17 different wastewater samples collected over a 10 months period.