Appl Microbiol Biotechnol (2017) 101:8607–8619
https://doi.org/10.1007/s00253-017-8571-3

ENVIRONMENTAL BIOTECHNOLOGY

Effects of different carbon sources on enhanced biological phosphorus removal and "*Candidatus* Accumulibacter" community composition under continuous aerobic condition

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Received: 6 July 2017 / Revised: 20 September 2017 / Accepted: 28 September 2017 / Published online: 24 October 2017

Abstract Previous studies have shown that enhanced biological phosphorus removal (EBPR) performance under continuous aerobic conditions always eventually deteriorates; however, the speed at which this happens depends on the carbon source supplied. The published data suggest that propionate is a better carbon source than acetate is for maintaining operational stability, although it is not clear why. A lab-scale sequencing batch reactor was run initially under conventional anaerobic/aerobic conditions with either acetate or propionate as the carbon source. Chemical and microbiological analyses revealed that both sources performed as expected for such systems. When continuous aerobic conditions were imposed on both these established communities, marked shifts of the "*Candidatus* Accumulibacter" clades were recorded for both carbon sources. Here, we discuss whether this shift could explain the prolonged EBPR stability observed with propionate.

Keywords "*Candidatus* Accumulibacter" · Enhanced biological phosphorus removal (EBPR) · Polyhydroxyalkanoates (PHA) · Polyphosphate kinase gene (*ppk*) · Real-time qPCR

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Introduction

Enhanced biological phosphorus removal (EBPR) is an economically and environmentally attractive process to remove phosphorus (P) from wastewater. In the conventional processes, the biomass is cycled through carbon-rich anaerobic and carbon-deficient aerobic phases. These treatment systems, in principal, enrich the environment for growth of the so-called polyphosphate accumulating organisms (PAO) (Mino et al. 1998; Blackall et al. 2002; Seviour et al. 2003; Oehmen et al. 2006) by providing conditions that encourage the anaerobic production of polyhydroxyalkanoate (PHA) and its subsequent aerobic oxidation, thus supplying energy for the accumulation and synthesis of intracellular polyphosphate (polyP) granules. Net P removal is achieved by wasting sludge after the aerobic phase (Mino et al. 1998; Blackall et al. 2002; Seviour et al. 2003; Oehmen et al. 2006).

The traditional metabolic models for the PAO in EBPR systems involve the anaerobic uptake of organic substrates, preferably volatile fatty acids (VFAs), from wastewater and their intracellular storage as PHA. The reducing equivalents and energy to synthesize PHA are provided mainly by glycolysis of internally stored glycogen reserves and hydrolysis of intracellular polyP (Mino et al. 1987). In the subsequent aerobic zone, PAO assimilate orthophosphate from the bulk

liquid to synthesize intracellular polyP in excess of that hydrolyzed under anaerobic conditions. The PAO grow aerobically and replenish their glycogen stores, using their PHA stores as carbon and energy sources (Arun et al. 1988; Smolders et al. 1995).

Thus, PAO are thought to achieve dominance in this anaerobic-aerobic process because they can grow aerobically in the absence of any exogenous carbon and energy sources by using the PHA accumulated anaerobically (Seviour et al. 2003). Higher and more stable EBPR performance is usually achieved with propionate rather than acetate as the anaerobic supplied substrate, (Seviour and Nielsen 2010), although some claim the opposite (Shen et al. 2017). Although the anaerobic phase is considered essential for conventional EBPR, some studies have reported successful removal of P under strictly aerobic conditions in lab-scale reactors (Ahn et al. 2002; Guisasola et al. 2004; Pijuan et al. 2005; Ahn et al. 2007). Intermittent supply of carbon creates feast-famine phases (Beun et al. 2000; Dircks et al. 2001) analogous to those imposed in traditional EBPR processes (Guisasola et al. 2004). However, the successful removal of P under such conditions appears unsustainable over long periods (Pijuan et al. 2006).

Glycogen accumulating organisms (GAO) are also often present in EBPR systems and can store organic substrates as PHA under anaerobic/feast conditions. Unlike in the PAO, the reducing equivalents and energy to synthesize PHA in GAO are provided mainly by glycolysis of their internally stored glycogen reserves, and not from hydrolysis of intracellular polyP (Liu et al. 1996; Oehmen et al. 2006). Their glycogen reserves are replenished from stored PHA under aerobic/famine conditions, under which they grow, but no intracellular polyP is produced. Therefore, GAO may compete with PAO for organic substrates as carbon sources under anaerobic/feast conditions (Liu et al. 1996), and their proliferation and dominance lead to a corresponding deterioration in biomass EBPR capacity (Crocetti et al. 2002; Meyer et al. 2006; Schroeder et al. 2008; Schroeder et al. 2009).

Carbon source composition is one factor suggested to have considerable impact on EBPR stability (Vargas et al. 2009). Propionate is considered a better carbon source than acetate for conventional EBPR, and is thought to provide PAO with a competitive advantage over GAO (Oehmen et al. 2006). Furthermore, propionate-enriched PAO populations developed under conventional EBPR conditions appeared to maintain biological phosphate removal under strict aerobic conditions for a longer period (46 days) than acetate-enriched PAO populations did (11 days) (Vargas et al. 2009; Pijuan et al. 2006). However, the advantages, if any, that propionate confers on the PAO under continuous aerobic conditions are still unclear (Vargas et al. 2009).

Improvements in our understanding of the mechanisms underlying such advantages in the propionate system may lead to improved knowledge-based control of EBPR systems and their development as "add-ons" to treatment plants that do not remove P. This would represent a cost-effective and environmentally friendly alternative to the construction of new EBPR plants or the currently applied chemical removal methods (Ahn et al. 2007). Several studies suggest that a phylogenetic diversity exists among the PAO population (Seviour and Nielsen 2010). In particular, PAO belonging to "*Candidatus* Accumulibacter" (hereafter *Accumulibacter*) fall into two types (types I and II) (McMahon et al. 2007), which are further grouped into 14 clades (IA-E and IIA-I), based on their polyphosphate kinase gene (*ppk*) sequences (He et al. 2007; Peterson et al. 2008; Mao et al. 2015; Skennerton et al. 2015; Zhang et al. 2016). Recent studies have also described quantitative PCR (qPCR) assays for members of each current clade of *Accumulibacter* (He et al. 2007; Ong et al. 2014; Zhang et al. 2016; Camejo et al. 2016). In this study, we operated a lab-scale EBPR reactor, using either acetate or propionate as the major carbon source, under anaerobic-aerobic and subsequent continuous aerobic conditions, and any population shifts among individual *Accumulibacter* clades associated with changes in EBPR performance were analyzed using qPCR assays.

Materials and methods

Sequencing batch reactor operation

A lab-scale sequencing batch reactor (SBR) with a working volume of 4 L was operated with separate dosing of acetate and propionate as carbon sources. The activated sludge inoculum for the SBR was obtained from an anaerobic-anoxic-aerobic (A_2O) biological nutrient removal plant (Yokohama, Japan) treating municipal wastewater. Note that the acetate-fed sludge was discharged before propionate-fed operation. The SBR was first run under alternating anaerobic-aerobic conditions for 33 days with acetate (Run A1) and 46 days with propionate (Run P1) to stabilize EBPR performance. It was then operated under strictly aerobic conditions for the next 20 days with acetate (Run A2) and 39 days with propionate (Run P2). The SBR was operated in a water bath at 20 °C on a 6-h cycle consisting of feed (10 min), alternating anaerobic feast phase (120 min) and aerobic famine phase (190 min) or a single aerobic famine phase (310 min), settling (30 min), and discharge of supernatant (10 min). SRT and HRT were controlled at 8 days and 12 h, respectively. The same reactor volume was maintained by discarding 125 mL of mixed liquor during the aerobic reaction period of each cycle before settling, and 1.75 L of supernatant was removed at the discharge period of each cycle. Bulk liquid dissolved oxygen (DO) concentrations were measured using a galvanic DO probe (DO-24P, DKK-TOA, Tokyo, Japan) and controlled at < 0.10 mg L^{-1} during the anaerobic feast phase, by turning nitrogen gas on at

the beginning of the phase, and at > 2.0 mg L^{-1} during the aerobic phase, by turning the air from the diffuser on and off as required. The pH was controlled at 7.0 ± 0.1 with 0.5 M HCl/H_2SO_4 and 0.5 M NaOH, using a pH controller (TPX-98 with FX-300H probe, TOKO, Tokyo, Japan).

Synthetic wastewater

The synthetic wastewater had a similar composition to that used by Nittami et al. (2011), consisting of two separate concentrated solutions, "C-solution" and "P-solution." The C-solution contained the following ingredients (g L^{-1} milli-Q water): acetate (CH_3COONa), 10.9 or propionate (CH_3CH_2COONa), 8.51; peptone, 0.5; NH_4Cl, 0.84; $MgSO_4{\cdot}7H_2O$, 1.8; $MgCl_2{\cdot}6H_2O$, 3.2; $CaCl_2{\cdot}2H_2O$, 0.84; Bacto yeast extract, 0.4; allylthiourea, 0.01; and a nutrient solution (12 mL) that consisted of (g L^{-1} milli-Q water): EDTA, 10; $ZnSO_4{\cdot}7H_2O$, 0.12; $MnCl_2{\cdot}4H_2O$, 0.12; $CoCl_2{\cdot}6H_2O$, 0.15; $CuSO_4{\cdot}5H_2O$, 0.03; $FeCl_3{\cdot}6H_2O$, 1.5; H_3BO_4, 0.15; KI, 0.18; $NaMoO_4{\cdot}2H_2O$, 0.06. The P-solution contained only KH_2PO_4, 2.46 g L^{-1} in milli-Q water. The C-solution and P-solution were sterilized separately by autoclaving. At the beginning of the feed period, 100 mL of C-water and 100 mL of P-water were added together to the SBR with 1.8 L of tap water (1:20 dilution). Thus, the initial acetate and P concentration in each cycle were set to 79.8 mg-C L^{-1} and 14.0 mg-P L^{-1}.

Chemical analysis

CH_3COO^- and $CH_3CH_2COO^-$ in filtered activated sludge samples were measured using an LC-10A HPLC with a Shim-pack IC-A3 column (Shimadzu, Kyoto, Japan) and Shim-pack SPR-H column (Shimadzu, Kyoto, Japan), respectively. P was analyzed as phosphate in filtered samples and total P (TP) concentration in the mixed liquor after persulfate digestion, using the ascorbic acid method (APHA 1998). MLSS was also determined according to standard methods (APHA 1998). PolyP content of activated sludge biomass during each cycle was also calculated as follows: (average TP concentration during one cycle − phosphate concentration at the end of the aerobic famine phase)/MLSS. Concentrations of biomass PHAs (PHB, PHV, and PH2MV) were quantified using gas chromatography/mass spectrometry (GC/MS), as in a previous study (Mikami et al. 2013). Biomass glycogen levels were also quantified, using the Glucose CII-test kit (Wako, Osaka, Japan), as in the previous study (Mikami et al. 2013).

Activated sludge samples, fixation, and DNA extraction

Activated sludge mixed liquor samples were collected from the acetate- and propionate-fed SBRs on days 1 and 11 in Run A2, 33 in Run P1, and 39 in Run P2. The samples were fixed on site in 4% (*w*/*v*) paraformaldehyde (PFA), and then stored at − 20 °C before FISH analysis (in the "Fluorescence in situ hybridization" section). A 1-mL aliquot of mixed liquor collected from the acetate reactor on days 1, 33 (Run A1), and 8 (Run A2) and the propionate reactor on days 1, 46 (Run P1), 21, and 39 (Run P2) were washed with Tris-EDTA buffer ($T_{10}E_1$, pH 8.0) and stored at − 20 °C prior to DNA extraction. DNA extractions were performed using the FastDNA SPIN Kit for soil (Qbiogene, CA, USA) and stored at − 20 °C prior to real-time PCR analysis after measuring the DNA concentration using a spectral photometer NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) was performed as detailed by Daims et al. (2005), using the oligonucleotide probes listed in Table 1. The probes were labeled with Cy3 or Fluos fluorochromes purchased from Proligo (Sydney, NSW), Nippon Gene (Tokyo, Japan), and Rikaken (Nagoya, Japan). Slides were air dried, mounted in Vectashield® (Vectashield Laboratories, Burlingame, CA), and then viewed on a BX51 epifluorescence microscope (Olympus, Tokyo, Japan).

Real-time PCR

Real-time PCR targeting the 16S rRNA genes of general bacteria and *Accumulibacter* as well as *ppk1* of *Accumulibacter* clades I, IIA, IIC, IID, and IIF was performed according to a previous study (Ong et al. 2014), using DNA extracted from activated sludge samples. Reactions were performed on a Thermal Cycler CFX 96 Real-Time PCR Detection System (BioRad, USA). Amplification reactions were carried out using the iQ™ SYBR®Green Supermix (BioRad, USA) in a total volume of 20 μL with primer sets targeting the general bacteria 16S rRNA gene, *Accumulibacter* 16S rRNA gene, and *ppk1* genes of *Accumulibacter* clades I, IIA, IIC, IID, and IIF (Table 1). Each reaction mixture (20 μL) contained 5 ng of template DNA, 10 μL of iQ™ SYBR®Green Supermix, and 0.5 μM of each primer. The optimized temperature programs were as follows: a denaturation (95 °C for 3 min), and 40 cycles of amplification and quantification (94 °C for 30 s, annealing temperature as listed in Table 1 for 45 s, and 72 °C for 30 s). All measurements were performed in triplicate for each run. Standard curves were constructed using the plasmids extracted from *Accumulibacter* clones belonging to the clades I, IIA, IIC, IID, and IIF obtained in a previous study (Ong et al. 2014). The initial concentrations of the plasmid DNA were measured using a NanoDrop ND-2000 spectrophotometer, and the copy numbers of the 16S rRNA genes of general bacteria and *Accumulibacte*r as well as *ppk1* of *Accumulibacte*r clades I,

Table 1 Overview of the oligonucleotide probes and primers applied for FISH and real-time PCR analyses, respectively

Probe/primer name	Specificity/target	Sequence (5′-3′)	FA conc. (%)/ annealing temp. (°C)	Reference
EUB338[a)]	Most bacteria	GCT GCC TCC CGT AGG AGT	0–50[c)]	Amann et al. (1990)
EUB338-II[a)]	*Planctomycetales*	GCA GCC ACC CGT AGG TGT	0–50[c)]	Daims et al. (1999)
EUB338-III[a)]	*Verrucomicrobiales*	GCT GCC ACC CGT AGG TGT	0–50[c)]	Daims et al. (1999)
PAO462[b)]	*Ca.* Accumulibacter phosphatis	CCG TCA TCT ACW CAG GGT ATT AAC	35	Crocetti et al. (2000)
PAO651[b)]	Most members of the *Ca.* Accumulibacter cluster	CCC TCT GCC AAA CTC CAG	35	Crocetti et al. (2000)
PAO846[b)]	*Ca.* Accumulibacter phosphatis	GTT AGC TAC GGC ACT AAA AGG	35	Crocetti et al. (2000)
518f	*Ca.* Accumulibacter 16S rRNA genes	CCA GCA GCC GCG GTA AT	65	Muyzer et al. (1993)
PAO-846r		GTT AGC TAC GGC ACT AAA AGG		He et al. (2007)
341f	Bacterial 16S rRNA genes	CCT ACG GGA GGC AGC AG	60	Muyzer et al. (1993)
534r		ATT ACC GCG GCT GCT GG		Muyzer et al. (1993)
Acc-ppk1-763f	Acc-I *ppk1*	GAC GAA GAA GCG GTC AAG	61	He et al. (2007)
Acc-ppk1-1170r		AAC GGT CAT CTT GAT GGC		He et al. (2007)
Acc-ppk1-893f	Acc-IIA *ppk1*	AGT TCA ATC TCA CCG AGA GC	61	He et al. (2007)
Acc-ppk1-997r		GGA ACT TCA GGT CGT TGC		He et al. (2007)
Acc-ppk1-254f	Acc-IIC *ppk1*	TCA CCA CCG ACG GCA AGA C	66	He et al. (2007)
Acc-ppk1-460r		CCG GCA TGA CTT CGC GGA AG		He et al. (2007)
Acc-ppk1-375f	Acc-IID *ppk1*	GGG TAT CCG TTT CCT CAA GCG	63	He et al. (2007)
Acc-ppk1-522r		GAG GCT CTT GTT GAG TAC ACG C		He et al. (2007)
Acc-ppk1-355f	Acc-IIF *ppk1*	CGA ACT CGG CGA AAG CGA GTA	70	Ong et al. (2014)
Acc-ppk1-600r		ATC GCC TCC GAG CAA CTG TTC		Ong et al. (2014)

[a] EUB338, EUB338-II, and EUB338-III were used as a mixture probe "EUBmix"

[b] PAO462, PAO651, and PAO846 were used as a mixture probe "PAOmix"

[c] 35% was applied in the present study

IIA, IIC, IID, and IIF in the plasmid DNAs were calculated as reported previously (Dhanasekaran et al. 2010).

Results

EBPR in acetate-fed reactor

Anaerobic-aerobic operation (Run A1)

The SBR was run under an anaerobic-aerobic operation regime using acetate as the major carbon source for 33 days, before switching to continuous aerobic operation (Run A2). Figure 1a shows the reactor performance on day 33. In the anaerobic period, the dosed acetate was completely consumed, and 36 mg-P (as phosphate) g-MLSS^{-1} was released into the bulk water. In the following aerobic period, 43 mg-P g-MLSS^{-1} was removed from the bulk liquid and net P removal was achieved. These transformations are typical of an EBPR biomass and were observed after day 16.

Continuous aerobic operation (Run A2)

The acetate SBR was then run under continuous aerobic operation for 20 days. Figure 1b shows the reactor performance on day 1, where the dosed acetate was completely consumed within the initial 10 min of the cycle. After 20 min, 10 mg-P (as phosphate) g-MLSS^{-1} had been released into the bulk

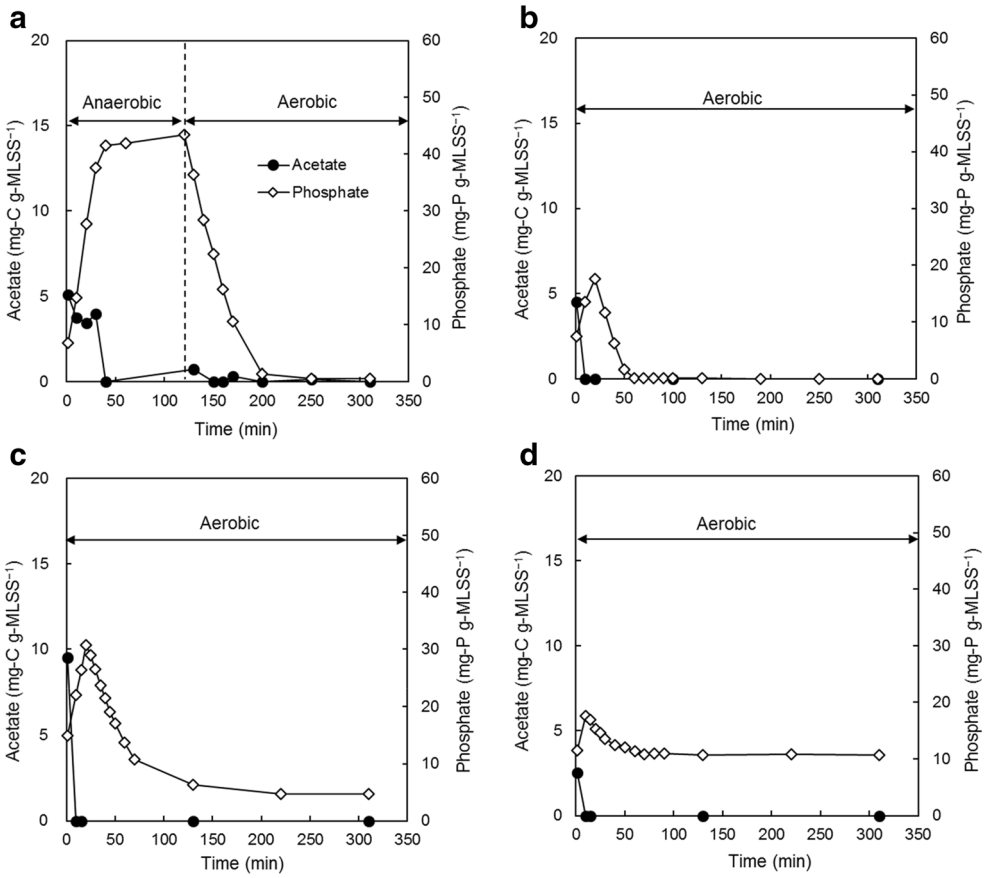

Fig. 1 Performance of sequencing batch reactor using acetate during one batch cycle **a** on day 33 in Run A1, **b** on day 1 in Run A2, **c** on day 11 in Run A2, and **d** on day 20 in Run A2

water, 18 mg-P g-MLSS^{-1} was removed from the bulk liquid, and net P removal was achieved. This trend was similar to that observed with acetate under continuous aerobic conditions in previous studies (Guisasola et al. 2004; Pijuan et al. 2005, 2006), and analogous to that in the anaerobic-aerobic periods of Run A1 (Fig. 1a).

However, the chemical transformations after 11 days showed marked differences compared to those for day 1 in Run A2 (Fig. 1c). In both cycles, the acetate consumption ratios were similar, but on day 11, 4.7 mg-P g-MLSS^{-1} remained at the end of the cycle, despite the feed P release (16 mg-P g-MLSS^{-1}) and famine uptake (26 mg-P g-MLSS^{-1}) during the first 20 min of the cycle being higher than those recorded on day 1.

The last cycle under continuous aerobic conditions on day 20 (Fig. 1d) showed further changes. Similar acetate consumption rates to those seen on days 1 and 11 (Fig. 1b, c) were seen. However, P release during the feast phase (6.2 mg-P g-MLSS^{-1}) and P uptake during the famine phase (7.0 mg-P g-MLSS^{-1}) were both lower than those recorded on days 1 and 11. In addition, the switch from P release to P uptake, which occurred after approximately 10 min, occurred earlier than that on days 1 and 11 (Fig. 1b, c). Moreover, no net P removal was achieved and > 10 mg-P g-MLSS^{-1} remained at the end of the cycle on day 20.

EBPR in propionate-fed reactor

Anaerobic-aerobic operation (Run P1)

After the acetate-fed sludge in Run A2 (in the "EBPR in acetate-fed reactor" section) was removed from the SBR, anaerobic-aerobic operation using a new inoculum and propionate as the major carbon source was performed for 46 days (Run P1). Figure 2a shows the reactor performance on day 46. In the anaerobic feed cycle period, the dosed propionate was completely consumed, and 14 mg-P g-MLSS^{-1} of phosphate was released into the bulk water. In the following aerobic famine cycle period, 19 mg-P g-MLSS^{-1} of phosphate was removed from the bulk liquid. These typical trends of EBPR were also observed after day 20 in Run P1.

Continuous aerobic operation (Run P2)

The propionate SBR was then run under continuous aerobic operation for 39 days. Figure 2b shows the reactor performance on day 7, where the dosed propionate was completely consumed within the initial 20 min. P release (16 mg-P g-MLSS^{-1}) was completed within 10 min, although the unassimilated propionate remained. This trend was consistent

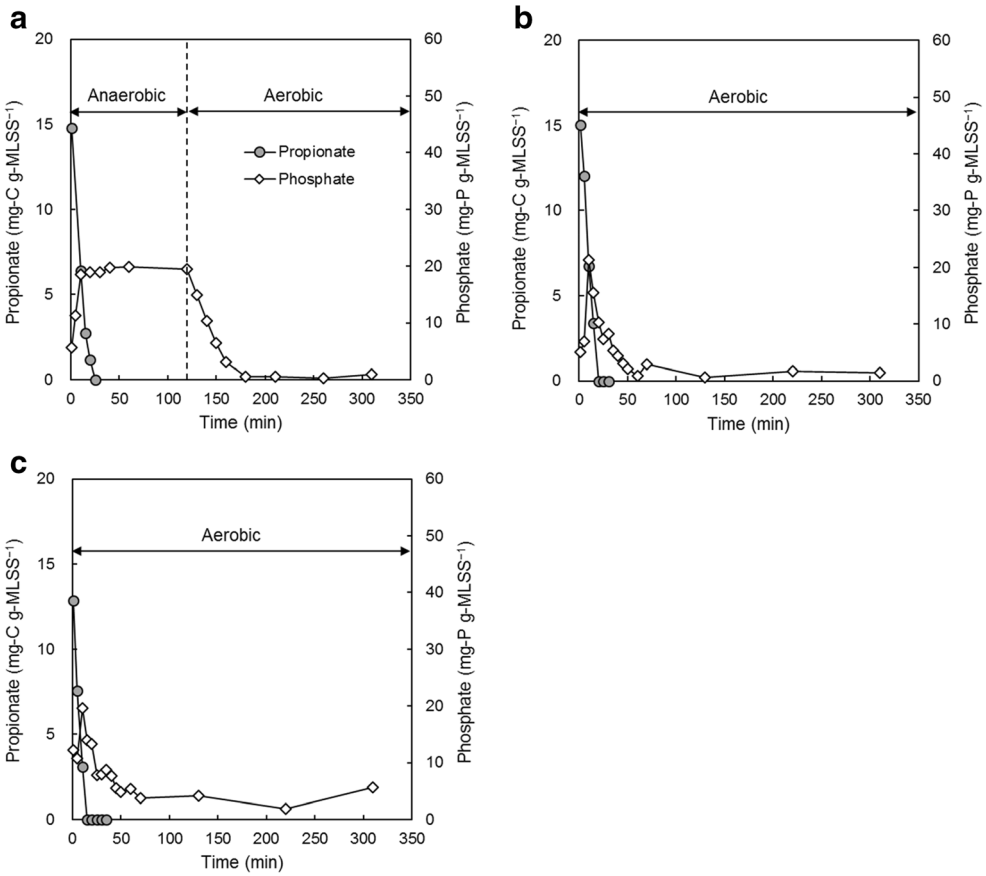

Fig. 2 Performance of sequencing batch reactor using propionate during one batch cycle **a** on day 46 in Run P1, **b** on day 7 in Run P2, and **c** on day 39 in Run P2

throughout reactor operation in Run P2, similar to that reported in previous studies (Vargas et al. 2009).

However, there were marked differences from when acetate was used in Run A2 (Pijuan et al. 2005, 2006), where P release in the feed stage continued until or after acetate depletion. In the following Run P2 famine stage, phosphorus was uptaken (20 mg-P g-MLSS^{-1}) and a net P removal was achieved, although 1.4 mg-P g-MLSS^{-1} remained at the end of the cycle. This P removal performance was observed until day 39, which is similar to that observed in a previous study (Vargas et al. 2009). However, the performance on day 39 of Run P2 (Fig. 2c) showed marked changes to those on day 7 (Fig. 2b); a higher propionate consumption ratio and incomplete P removal were recorded, with > 5 mg-P g-MLSS^{-1} of P remaining at the end of the cycle.

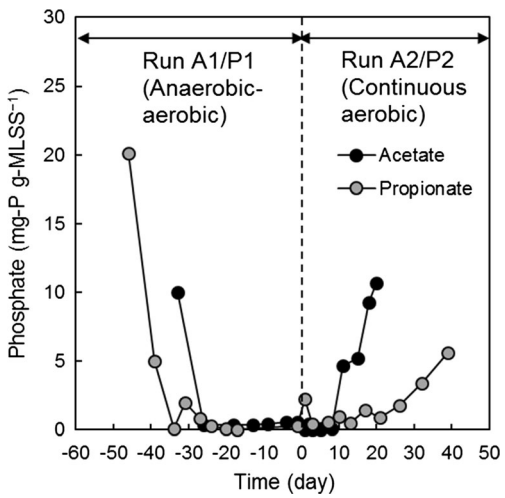

Fig. 3 Phosphorus removal performance of the acetate- and propionate-fed sequencing batch reactors at the end of operational period

Figure 3 shows the mixed liquor P concentrations at the end of the cycles in both the acetate- and propionate-fed SBRs. Once the systems had switched from anaerobic-aerobic operations (Runs A1 and P1) to continuous aerobic operations (Runs A2 and P2), P removal in the acetate-fed reactor (Run A2) deteriorated more rapidly than it did in the propionate-fed reactor (Run P2). The mixed liquor P concentration reached 10 mg-P g-MLSS^{-1} on day 20 in the acetate SBR (Run A2), whereas it remained at ≤ 3.4 mg-P g-MLSS^{-1} until day 32 in the propionate SBR (Run P2).

Biomass storage polymer levels

To investigate whether biomass storage compound levels corresponded with EBPR performance, glycogen and PHA

Table 2 Changes of biomass storage polymers and MLSS during the operation

Substrate	Run	Time [day]	PHB Upper stand: concentration [mg-C g-MLSS^{-1}][1)], lower stand: ratio to total PHA [%][2)]	PHV	PH2MV	PH2MB	Glycogen [mg-C g-MLSS^{-1}][3)]	PolyP content [%][5)]	MLSS [g L^{-1}]
Acetate	A1	33	7.86 87.9	0.890 9.95	0.103 1.15	0.0911 1.02	7.69	8.77	3.20
	A2	1	11.9 93.7	0.537 4.23	0.168 1.32	0.0978 0.770	11.6	10.0	2.86
		11	4.27 90.2	0.464 9.80	0.00 0.00	0.00 0.00	3.75	15.8	2.59
		20	1.92 88.9	0.239 11.1	0.00 0.00	0.00 0.00	3.10	9.55	2.88
Propionate	P1	1	0.923 6.63	8.81 63.3	4.19 30.1	n.a.[4)] n.a.[4)]	15.4	2.02	1.98
		46	1.49 5.88	9.06 35.8	14.8 58.3	n.a.[4)] n.a.[4)]	39.7	13.5	3.84
	P2	1	0.476 4.41	4.06 37.5	6.28 58.0	n.a.[4)] n.a.[4)]	21.0	10.2	4.06
		7	0.385 4.85	3.33 42.0	4.22 53.2	n.a.[4)] n.a.[4)]	19.0	9.21	5.02
		13	0.820 12.8	2.83 44.1	2.76 43.1	n.a. [4)] n.a.[4)]	15.6	11.3	4.97
		21	0.387 4.80	3.82 47.4	3.84 47.8	n.a.[4)] n.a.[4)]	12.6	9.60	5.76
		39	0.518 8.02	3.24 50.2	2.70 41.7	n.a.[4)] n.a.[4)]	17.1	9.38	5.46

[1)] PHA concentrations were measured at 120 min in Run 1 and 10/20 min in Run 2

[2)] Each PHA component (mg-C g-MLSS^{-1}) was divided by total PHA (mg-C g-MLSS^{-1}) and centupled

[3)] Glycogen concentrations were measured at 310 min both in Runs 1 and 2. Note that it was measured at 260 min only on day 1 in Run 1 of propionate

[4)] n.a. means not assessed

[5)] PolyP (g-P L^{-1}) was divided by MLSS (g L^{-1}) and centupled

levels were measured periodically in both the acetate- and propionate-fed SBR biomasses (Table 2).

In the acetate-fed SBR, total PHA, glycogen, and polyP levels rose from 9.94 mg-C g-MLSS^{-1}, 7.69 mg-C g-MLSS^{-1}, and 8.77% (day 33, Run A1) to 12.7 mg-C g-MLSS^{-1}, 11.61 mg-C g-MLSS^{-1}, and 10.0% (day 1, Run A2), respectively, after switching from anaerobic-aerobic operation to continuous aerobic operation. However, subsequently, PHA and glycogen levels fell to 2.16 and 3.10 mg-C g-MLSS^{-1}, respectively, under continuous aerobic conditions on day 20. Only biomass polyP levels maintained a concentration of more than 9.55% under continuous aerobic operation. The main PHA component synthesized in the acetate-fed SBR was PHB, irrespective of operational conditions (i.e., anaerobic-aerobic or continuous aerobic), which is in agreement with all previous data obtained with acetate as the carbon source (Table 3). The proportion of PHB in the total PHA accounted for 87.9 to 93.7% (Table 2).

In the propionate-fed SBR, total PHA and glycogen levels rose from 13.92 and 15.4 mg-C g-MLSS^{-1} (day 1), respectively, to 25.4 and 39.7 mg-C g-MLSS^{-1} (day 46) in Run P1, but immediately fell to 10.82 and 21.0 mg-C g-MLSS^{-1} after switching to continuous aerobic operation on day 1 in Run P2. Both then fell gradually to 6.46 and 17.1 mg-C g-MLSS^{-1}, respectively, by day 39. Biomass polyP content also rose from 2.02% (day 1) to 13.5% (day 46) in Run P1, but immediately fell to 10.2% on day 1 after switching to continuous aerobic operation, and stayed at > 9.21% until the end of the continuous aerobic operation. Thus, total PHA and glycogen levels were generally higher than those detected in the acetate-fed SBR biomass, although biomass polyP contents were similar under both feeding regimes. Moreover, the reduction rates of biomass PHA and glycogen levels in Run P2 (0.11 and 0.10 mg-C g-MLSS^{-1} day^{-1}) were clearly lower than those in Run A2 of the acetate-fed SBR (0.55 and 0.45 mg-C g-MLSS^{-1} day^{-1}). Such trends indicate a more stable EBPR performance under continuous aerobic conditions with propionate than with acetate. The main PHA components synthesized with propionate were always PH2MV and PHV again, irrespective of the operational conditions,

Table 3 Data from the literature concerning the PHA accumulated and form produced in EBPR systems using acetate and propionate as carbon sources

Carbon source	Operation	PHB	PHV	PH2MV	Others	References
Acetate	Anaerobic-aerobic	90.2	9.8	–	–	Smolders et al. (1994)
Acetate	Anaerobic-aerobic	69.4	30.6	–	–	Pereira et al. (1996)
Acetate	Anaerobic-aerobic	70.3	20.7	–	–	Hesselmann et al. (2000)
Acetate	Anaerobic-aerobic	46.6	22.2	31.2	–	Puiga et al. (2008)
Acetate	Anaerobic-aerobic	69.9	30.1	0.0	–	Pijuan et al. (2009)
Propionate	Anaerobic-aerobic	4.0	46.5	49.0	0.5	Pijuan et al. (2004)
Propionate	Anaerobic-aerobic	2.0	45.0	53.0	–	Oehmen et al. (2005a)
Propionate	Anaerobic-aerobic	2.1	50.7	47.2	–	Puiga et al. (2008)
Propionate	Anaerobic-aerobic	6.2	53.4	40.4	–	Pijuan et al. (2009)
Propionate[1)]	Anaerobic-aerobic	3.9	43.7	52.4	–	Vargas et al. (2009)
Propionate[1)]	Continuous aerobic	2.3	37.2	60.5	–	Vargas et al. (2009)

[1)] Those two data sets were collected from a same reactor, which was first operated under anaerobic-aerobic condition and then continuous aerobic condition

which is in agreement with all previous data obtained with propionate as the carbon source (Table 3). The contributions of PH2MV and PHV to the total PHA were 30.1 to 58.3% and 35.8 to 63.3% (Table 2), respectively.

Microbial community composition

Acetate-fed reactor

FISH was performed on biomass samples collected during continuous aerobic operation after days 1 and 11 (Run A2) using the PAOmix probe (Cy3) binding to cells of *Accumulibacter* and the EUBmix probe (Fluos) targeting almost all bacterial cells (Fig. 4a, b). Yellow cells showing hybridization with both the PAOmix and EUBmix probes, and identified as *Accumulibacter*, were observed on both sampling days 1 (Fig. 4a) and 11 (Fig. 4b), and the cell morphologies appeared to be similar in each sample. Real-time PCR was performed on DNA samples extracted from biomass samples collected on days 1, 33 (Run A1), and 8 (Run A2), using the primer sets targeting the 16S rRNA gene of almost all bacteria and *Accumulibacter* and *ppk1* genes of *Accumulibacter* clades I, IIA, IIC, IID, and IIF (Table 1). Figure 5a shows the quantified copy numbers of each clade gene.

As for the 16S rRNA genes, the copy number of *Accumulibacter* increased from 2.67×10^6 copies ng-DNA^{-1} to 5.11×10^7 copies ng-DNA^{-1} during 32 days in Run A1 and was maintained at 3.83×10^7 copies ng-DNA^{-1} until day 8 in Run A2. On the other hand, the total bacterial copy number continued to rise from 3.98×10^7 to 2.66×10^8 to 1.13×10^9 copies ng-DNA^{-1} during the 40 days of operation from Run A1 to Run A2. Consequently, the ratio of copy numbers of *Accumulibacter* to those of total bacteria changed from 6.7 to 19%, and then to 3.4% (Fig. 5b).

Previous studies have reported that the *Accumulibacter* genome and other bacterial genomes have 2 and 4.2 (on average) copies of the rrn operon (Garcia et al. 2006; Větrovský and Baldrian 2013), respectively. Therefore, based on the copy numbers of 16S rRNA genes determined by qPCR and the rrn operon, the estimated *Accumulibacter* abundances were 14, 40, and 7.1% of the total microbial cells on days 1, 33 (Run A1), and 8 (Run A2), respectively. This relative

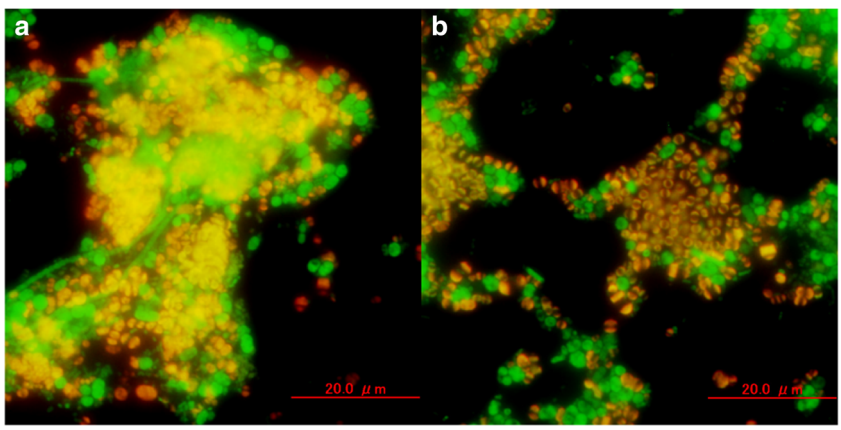

Fig. 4 Composite FISH images of the biomass samples of the acetate-fed reactor showing yellow or orange cells hybridizing both with the EUBmix (green) and PAOmix (red) probes on day 1 (**a**) and day 11 (**b**) in Run A2

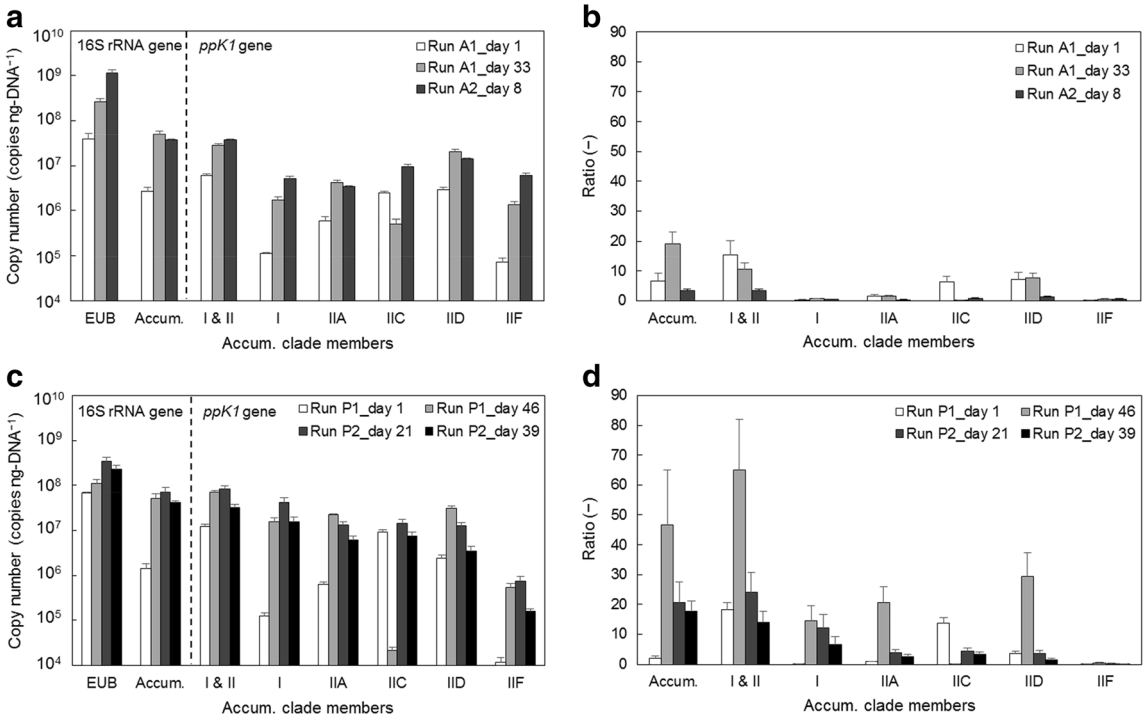

Fig. 5 Absolute gene copy numbers (copies ng-DNA^{-1}) of the 16S rRNA and *ppk1* gene determined by qPCR assay ((**a**) and (**c**), respectively) and the ratios (%) of the 16S rRNA/*ppk1* gene copy number of *Candidatus* Accumulibacter to the total bacterial 16S rRNA gene copy number ((**b**) and (**d**)). EUB and Accum. represent total eubacteria and *Candidatus* Accumulibacter, respectively

abundance value on day 33 is similar to that reported by other studies using lab-scale SBR sludges enriched with acetate under anaerobic-aerobic conditions (e.g., 50% in Pijuan et al. 2006).

Figure 5a shows the quantified absolute copy numbers of *ppk* genes. Those of all clades, except for clade IIC, increased after 32 days incubation in Run A1, and maintained this level until day 8 in Run A2, following a similar trend to the copy numbers of the *Accumulibacter* 16S rRNA gene. This trend suggests that no drastic population changes occurred in the PAO community composition after the initial switch from anaerobic-aerobic operation (Run A1) to continuous aerobic operation (Run A2). However, one striking change was that *ppk* copy numbers for clade IIC members, minor components of the total *Accumulibacter* populations under anaerobic/aerobic EBPR conditions, increased markedly under continuous aerobic conditions in Run A2.

Propionate-fed reactor

FISH was performed on biomass samples collected during both anaerobic-aerobic operation (Run P1, at day 33) and continuous aerobic operation (Run P2, day 39), using the PAOmix (Cy3) and EUBmix (Fluos) probes (Fig. 6a, b). Yellow or orange cells (i.e., *Accumulibacter*) were observed in both samples (Fig. 6a, b), and their morphologies appeared to be similar. The coccoid cells were similar to those seen in the acetate-fed reactor, but their cell diameters appeared to be

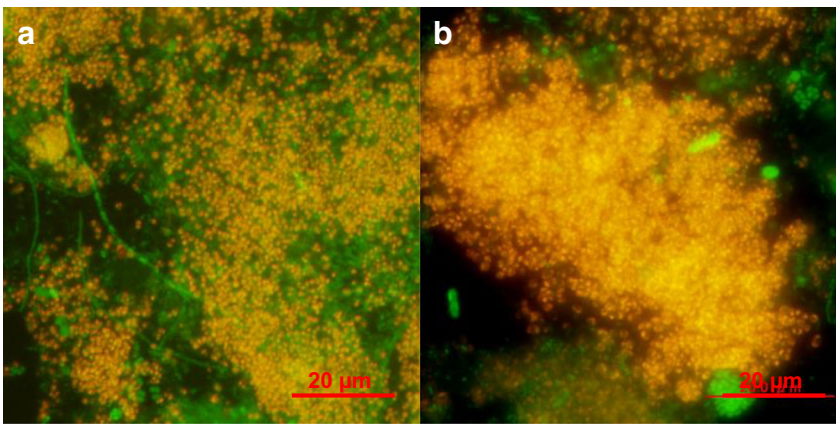

Fig. 6 Composite FISH images of the biomass samples of the propionate-fed reactor showing yellow or orange cells hybridizing both with the EUBmix (green) and PAOmix (red) probes on day 33 in Run P1 (**a**), and on day 39 in Run P2 (**b**)

smaller (Fig. 4). Real-time PCR was performed again on DNA samples extracted from biomass samples collected on days 1, 46 (Run P1), 21, and 39 (Run P2), using the primer sets shown in Table 1. Figure 5c shows the quantified copy numbers of each gene. As with the 16S rRNA genes, the copy numbers of those of Accumulibacter increased from 1.40×10^6 to 5.05×10^7 copies ng-DNA^{-1} after 45 days in Run P1 and were maintained at 7.09×10^7 and 4.12×10^7 copies ng-DNA^{-1} on days 21 and 39, respectively, in Run P2. The total bacterial copy numbers also increased from 6.76×10^7 to 1.08×10^8 copies ng-DNA^{-1} after 45 days in Run P1 and were maintained at 3.44×10^8 and 2.32×10^8 copies ng-DNA^{-1}, respectively, on days 21 and 39 in Run P2. Consequently, the ratio of copy numbers of *Accumulibacter* and the estimated *Accumulibacter* abundances were 2.1, 47, 21, and 18% (Fig. 5d) and 4.4, 99, 44, and 38% of the total microbial cells on days 1, 46 (Run P1), 21, and 39 (Run P2), respectively. The *Accumulibacter* relative abundances (Run P1) on day 46 (99%) are higher than those obtained in other studies using lab-scale SBR sludges fed with propionate under anaerobic-aerobic conditions (e.g., 55% in Pijuan et al. 2004; 65% in Oehmen et al. 2005b; 70% in Vargas et al. 2009; and 90% in Lu et al. 2006).

These abundance were subsequently reduced considerably to approximately half, under continuous aerobic conditions in Run P2, but the *Accumulibacter* abundance ratio was 5–6 times higher than that determined for the acetate-fed SBR in Run A2 (7.1%). The copy numbers of all clades, except those of clade IIC (Fig 5c), increased after the 45 days acclimation in Run P1, and these numbers were maintained until day 21/39 in Run P2, following a similar trend to the copy numbers of the *Accumulibacter* 16S rRNA gene. This result also indicates that there were no drastic changes in the A*ccumulibacter* percentage relative abundances in the propionate-fed reactor after switching from anaerobic-aerobic to continuous aerobic operation. However, only the clade IIC members increased during Run P2 more than they did with acetate-fed communities during Run A2. Thus, it seems that the *Accumulibacter* members of this clade may be favored under aerobic feed conditions irrespective of the carbon source.

Discussion

This study sets out to compare the stability of EBPR performance under continuous aerobic conditions in response to feeding acetate or propionate as the supplied carbon source. Changing the operating conditions of this EBPR process from anaerobic/aerobic to continuously aerobic led to a rapid deterioration in EBPR capacity, as measured by P release/uptake ratios, levels of intracellular glycogen and PHA, and percentage relative abundances of *Accumulibacter* PAO in the respective communities. Such trends were seen with both acetate- and propionate-fed communities; however, they were much less marked and occurred more slowly with propionate than with acetate as the carbon source. In particular, the aerobic *Accumulibacter* population relative abundances remained markedly higher with propionate than with acetate (Fig. 5b, d).

As stated earlier, in conventional anaerobic/aerobic EBPR systems, PAO have a selective advantage over other chemoorganoheterotrophic bacteria in the anaerobic feed stage (Seviour et al. 2003; Oehmen et al. 2007). The carbon source is rapidly assimilated, but instead of supporting anaerobic cell replication, it is used to synthesize PHA or other storage compounds, thus providing the PAO with a carbon and energy source that allows them to grow and assimilate and synthesize polyP. Cells with no storage compounds are outcompeted under aerobic conditions because little or no carbon source is now available to them.

Under aerobic feed conditions, the selective factors imposed on the community are likely to be quite different. It is likely that other aerobic chemoorganotrophs may eventually outcompete the PAO for the carbon source, which is used to support aerobic respiration. Consequently, levels of PAO intracellular storage compounds inevitably decrease. Hence, the PAO no longer have the same selective advantage in the famine stage of the process. The data presented here and in previous studies are consistent with this suggestion. As already stated, the relative abundances of *Accumulibacter*, expressed as percentages of total bacterial cells, fell markedly under continuous aerobic conditions (Fig. 5b, d), but these organisms persisted after prolonged aerobic periods, especially with a propionate feed. The remarkable metabolic versatility and flexibility of *Accumulibacter* are well documented (He and McMahon 2011), but the data presented here (Fig. 5a, c) suggest that members of clade IIC are able to adapt to the aerobic conditions used in this study.

There are several explanations as to why propionate might support a more stable aerobic EBPR than acetate might. These include the ability of the same PAO populations to assimilate propionate more efficiently than they do acetate and faster than most other chemoorganoheterotrophs in the aerobic feed stage, and to retain the ability to store some as PHA. Alternatively, propionate may select for other *Accumulibacter* populations from clades with a more efficient and adaptable PAO metabolism. Vargas et al. (2009) claimed that the only difference between continuously aerobic and anaerobic/aerobic EBPR communities with either acetate or propionate feed was the composition of the stored PHA (i.e., PHB or PHV and PH2MV, respectively). They also suggested, without saying how, that this attribute alone may be responsible for the EBPR stability observed with propionate. Pijuan et al. (2009) also suggested that EBPR microbial communities exposed to acetate and propionate mainly produced PHV and both PHV and PH2MV from propionate, respectively.

16S rRNA gene copy numbers of members of *Accumulibacter* clade IID had increased by the end of the anaerobic/aerobic operation, regardless of whether acetate or propionate was used as the carbon source (Fig. 5a, c), suggesting that the added carbon source played no role in their enrichment. However, 16S rRNA gene copy numbers of clade I and IIA members had increased substantially more within the propionate-fed community than within the acetate-fed community (Fig. 5a, c). If the earlier suggestions that the PHA composition ultimately decides the performance of continuous aerobic EBPR and only propionate-fed bacteria can synthesize PH2MV are correct, then our qPCR data might suggest that *Accumulibacter* members of clades I and IIA are responsible for much of the PH2MV accumulation and are thus key players in preserving EBPR capacity under continuous aerobic conditions with propionate. Equally feasible is that other bacterial populations in these communities were responsible for substantial PHA storage; alternatively, members of *Accumulibacter* clades yet unrecognized may have been responsible.

Not all the published data report the same population trends. For example, Gonzalez-Gil and Holliger (2011) found that *Accumulibacter* clade IIA members were abundant in both acetate and propionate EBPR reactors, whereas clade I members were abundant only in the acetate-fed reactor. However, no explanations were provided for this observation, and no information was supplied on the chemical components of the PHA, except that their aerobic biomass was organized as granules, whereas flocs were used in the present study and by Pijuan et al. (2009).

The most striking shift in the populations was the increase under continuously aerobic conditions in the relative abundances of *Accumulibacter* clade IIC members with both acetate and propionate; however, the reason for this enrichment is not clear. Camejo et al. (2016) have investigated the affinity of clade IIC members to oxygen as an electron acceptor; DO levels during active P uptake differing markedly in their lab- and pilot-scale reactors (0.05 versus 0.2 mg L^{-1}) were thought to explain the dominance of clade IIC members in their pilot-scale reactor. Slater et al. (2010) suggested that clade IIC members had a higher affinity for phosphate than clade IA members did, which may give them a competitive edge at low phosphate concentrations. However, whether members of other clades are also less competitive for phosphate under aerobic conditions remains to be determined. Shen et al. (2017) also showed that members of clade IIC were highly abundant in EBPR processes run at high temperature. Moreover, Welles et al. (2015, 2017) suggested that clade IIC members are distinctively metabolically flexible, being able to switch to a mixed PAO-GAO metabolism where both glycogen and polyP are degraded to provide energy for the sequestration of acetate anaerobically when polyP is not stoichiometrically limiting the VFA uptake. From whole genome sequence data, Skennerton et al. (2015) demonstrated that clade IIC members were alone in encoding a gene for nitrate reductase and, hence, capable of denitrification. This observation could explain the enrichment of clade IIC members in reactors capable of nitrate reduction (Kim et al. 2013).

However, the present reactor was operated under high DO concentrations during P uptake (> 2.0 mg L^{-1}) and normal temperature (20 °C). The influent contained a high phosphate concentration and allylthiourea to inhibit nitrification, which indicates poor denitrifying activity. Moreover, it is difficult to see how possessing nitrate reductase could provide a competitive advantage to clade IIC members under the conditions used here. Whether by acting as a redox-balancing complex, as suggested by Skennerton et al. (2015), it could assist *Accumulibacter* in coping with these conditions is also unknown.

Zhang et al. (2016) reported that clade IIC members are well represented in nine geographically and operationally distinct activated sludge WWTPs. Mao et al. (2015) also detected clade IIC members in the majority of full-scale activated sludge WWTPs in six countries and found them to be the most common of the five clades (I, IIA, IIB, IIC, and IID) examined. Moreover, the inoculates used in the present study were collected from municipal WWTPs, which contained all the clades examined (I, IIA, IIC, IID, and IIF); however, only clades IIC and IID showed high *ppk1* copy numbers (Fig. 5a, c). Consequently, every result supports that clade IIC was well adaptable in full scale WWTPs, and thus, the reason behind *Accumulibacter* clade IIC members having a selective advantage in municipal WWTPs should clarify why they have selective advantage under continuously aerobic conditions. The authors hypothesized that this clade could not adapt under strictly controlled long anaerobic conditions during P release, such as those present in lab-scale reactors.

One problem with studies of this kind is the assumption that all members of each *Accumulibacter* clade share the same metabolic traits. However, as Albertsen et al. (2016) and Saad et al. (2016) reminded us, the foundation of identifying *Accumulibacter* members of each individual clade is based on the sequences of a single gene, *ppk*, and its sequence diversity within each clade is approximately 12%. Thus, there is evidence that differences in its sequence embrace Accumulibacters probably with markedly different ecophysiologies.

Acknowledgements The authors express their gratitude to Mr. Shizuo Furukawa, who helped set up the stirred tank system, and Dr. Mamoru Oshiki and Mr. Satoru Motooka, who helped measure the PHA. The Environmental Planning Bureau of the Yokohama City Government is acknowledged for the provision of activated sludge.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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