J Ind Microbiol Biotechnol (2009) 36:885–893
DOI 10.1007/s10295-009-0566-3

ORIGINAL PAPER

Biomass granulation in an aerobic:anaerobic-enhanced biological phosphorus removal process in a sequencing batch reactor with varying pH

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Received: 14 January 2009 / Accepted: 16 March 2009 / Published online: 7 April 2009
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Abstract Long-term influences of different steady-state pH conditions on microbial community composition were determined by fluorescence in situ hybridization (FISH) in a laboratory scale reactor configured for enhanced biological phosphorus removal (EBPR). Chemical profiles were consistent with shifts in populations from polyphosphate-accumulating organisms (PAO) to glycogen-accumulating organisms (GAO) when pH fell from pH 7.5 to 7.0 and then to 6.5. While biomass was both dispersed and flocculated at pH 7.5, almost complete granulation occurred gradually after pH was dropped to 7.0, and these granules increased in size as the pH was reduced further to 6.5. Reverting back to pH 7.5 led to granule breakdown and corresponding increases in anaerobic phosphate release. Granules consisted almost entirely of *Accumulibacter* PAO cells, while putative GAO populations were always present in small numbers. Results suggest that low pH may contribute to granulation under these operational conditions. While chemical profiles suggested the PAO:GAO balance was changing as pH fell, FISH failed to reveal any marked corresponding increase in GAO abundances. Instead, TEM evidence suggested the *Accumulibacter* PAO phenotype was becoming more like that of a GAO. These data show how metabolically adaptable the *Accumulibacter* PAO can be under anaerobic:aerobic conditions in being able to cope with marked changes in plant conditions. They suggest that decreases in EBPR capacity may not necessarily reflect shifts in community composition, but in the existing population metabolism.

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Keywords Enhanced biological phosphorus removal · Glycogen accumulating organisms · Aerobic granules · *Accumulibacter*

Introduction

All current enhanced biological phosphorus removal (EBPR) processes work on the principle of cycling the microbial community alternatively through aerobic and anaerobic conditions. In these, the betaproteobacterial *Candidatus* "Accumulibacter phosphatis" phosphate-accumulating organisms (PAO) are considered largely responsible for EBPR [9, 20, 43]. Under anaerobic conditions, they take up volatile fatty acids (VFA) to produce polyhydroxyalkanoates (PHA), while the polyphosphate (polyP) is used for the energy-consuming steps of VFA assimilation and PHA synthesis. Reducing equivalents for PHA production are provided by catabolism of internal glycogen stores [39]. Under subsequent aerobic conditions, the PAOs through PHA utilization assimilate phosphates into polyP, replenish their glycogen stores and grow.

However, EBPR processes behave unreliably. One possible reason is that another physiological group, the glycogen-accumulating organisms (GAO), take over the community by out-competing the PAO for substrates anaerobically [39, 44]. These GAOs have phenotypes similar to PAO, except they accumulate glycogen instead of polyP aerobically. So far, the GAO identified include members of the *Alphaproteobacteria* (*Defluviicoccus vanus*) [36, 47] and *Gammaproteobacteria* (*Candidatus* "Competibacter phosphatis") [8, 25, 26].

Controlling the GAO by manipulating pH was suggested by Filipe et al. [18, 19]. They showed the P release to acetate uptake ratio changed with operating pH and suggested that

at pH 7.5 and above, the GAOs assimilate acetate more slowly than the PAO, and so cannot compete with them [39, 41]. In the absence of polyP reserves, the energy for GAO acetate assimilation has to come from glycogen, whose anaerobic consumption therefore increases. Such behavior requires a greater amount of PHA for glycogen replenishment whose biomass levels should therefore increase under subsequent aerobic conditions [39]. At lower pH, the GAOs are claimed to assimilate acetate faster anaerobically than do the PAOs, thus becoming dominant, and EBPR performance fails. However, they did not identify their GAO populations, and in fact there are very few microbiological data available to support this proposal [39, 40].

Thus, we have run a sequencing batch reactor (SBR) EBPR process operating under different pH conditions to obtain a better insight into how the microbial community composition might shift by using fluorescence in situ hybridization (FISH) with probes targeting the known PAO and GAO populations.

During these experiments the biomass organization shifted almost entirely from a floc structure to a granular organization, a change that seemed to parallel falls in operational pH. Whether granules always arise from pre-existing flocs is unclear from the literature because the early stages in granule formation have not always been followed. Encouraging slow growth rates of populations with pre-existing high cell co-aggregation propensities may assist them [16, 30, 32], but less certain is whether imposed cell starvation [33] leading to increased exocellular polymeric substances (EPS) hydrophobicity in the floc matrix does [31]. Short SBR settling times [21], where more loosely aggregated biomass is lost from the reactor, may also influence biomass aggregation [35] and operating temperature [15], shear [7] and dissolved oxygen concentrations [38] all seem to determine granule stability. However, explanations for these events, where given, are not always persuasive, and there may be several quite different mechanisms involved in granule production.

This paper suggests a mechanism for biomass granulation under the conditions used here and presents data to support the view that the phenotype of the *Accumulibacter* PAO may shift partially to that of a GAO, where cells store increasing amounts of glycogen during the famine stage at the expense of polyP synthesis.

Materials and methods

Reactor operation

A sequencing batch reactor (SBR) with a 1.0 l working volume (B. Braun) was operated at controlled temperature (about 20°C). It was fed with a synthetic wastewater of the following composition (per liter): 512.5 mg of $CH_3COO{\cdot}Na$, 85.3 mg of $NaH_2PO_4{\cdot}2H_2O$, 100.8 mg of NH_4Cl, 180 mg of $MgSO_4{\cdot}7H_2O$, 72 mg of KCl, 14 mg of $CaCl_2{\cdot}2H_2O$, 2 mg of *n*-allylthiourea to inhibit nitrification, 5 mg of yeast extract, 5 mg of peptone and 0.3 ml of trace elements [45]. Mixed liquor taken from the aerobic reactor of a modified University of Cape Town (UCT) full-scale EBPR process in Castlemaine, Victoria, Australia was washed, and 500 ml settled biomass was used as seed to inoculate the SBR. The reactor was operated on a cycle of 6 h, consisting of a 5-min filling phase, a 115-min anaerobic phase, a 195-min aerobic phase, a 30-min settling phase, and a 15-min withdrawal phase. Biomass was mixed during the anaerobic and aerobic phases by a single agitator with six Rushton turbine blades (6-cm diameter) at 300 revolutions min^{-1}. To maintain aerobic conditions, about 450 ml min^{-1} of air was bubbled through a stainless steel diffuser. The reactor pH was strictly controlled at 7.5 for 27 days, at 7.0 for 49 days, at 6.5 for 109 days and then at 7.5 for 70 days, with an Ingold pH electrode and pH controller (LH505, LH Fermentation) by adding 0.25 N HCl or 0.25 N NaOH when required. At the end of the aerobic phase, about 35.7 ml of excess sludge was discarded to maintain a mean cell retention time of 7 days. After settling, about 464.3 ml of the effluent was discarded.

Chemical analysis

Analysis of phosphorus, biomass polyphosphate and total suspended solids was performed according to standard methods [3]. Acetate was determined with a high performance liquid chromatograph (LC-10Ai, Shimadzu) equipped with an anion column (Shodex kC-811, Shodex Denko) as described by Ahn et al. [1]. To determine intracellular levels of PHA and glycogen, biomass samples were collected and immediately frozen in a mixture of dry ice and methanol, followed by lyophilization. For PHA, biomass was incubated at 100°C for 24 h with chloroform and acidified methanol [10% (v/v) sulfuric acid] [1]. Identification and quantification of PHA methyl esters were conducted by a gas chromatograph (3900, Varian) equipped with a Chrompack capillary column (CP-Sil5CB, Varian) and a flame ionization detector. Poly (3-hydroxybutyric acid-*co*-3-hydroxyvaleric) acid (Aldrich) and sodium 3-hydroxybutyrate (Lancaster) were used as standards. Further details are given in Ahn et al. [1]. No poly β-hydroxyvalerate (PHV) was detected in any of the reactor samples. For glycogen, sludge samples were autoclaved with 0.6 N HCl at 121°C for 1 h. After cooling to room temperature, glycogen concentrations were measured as glucose equivalents using a hexokinase enzymatic glucose kit (Thermo).

Table 1 rRNA targeted probes used for FISH detection of organisms in this study

Probe name	Sequence (5′–3′)	Target	Form (%)	Reference
EUB338-I[a]	GCTGCCTCCCGTAGGAGT	Most bacteria	35	[2]
EUB338-II[a]	GCAGCCACCCGTAGGTGT	*Planctomycetales*	35	[10]
EUB338-III[a]	GCTGCCACCCGTAGGTGT	*Verrucomicrobiales*	35	[10]
PAO462b[b]	CCGTCATCTRCWCAGGGTATTAAC	*Candidatus* 'Accumulibacter phosphatis'	35	[49]
PAO651[b]	CCCTCTGCCAAACTCCAG	Most members of the *Candidatus* 'Accumulibacter'	35	[9]
PAO846b[b]	GTTAGCTACGGYACTAAAAGG	*Candidatus* 'Accumulibacter phosphatis'	35	[49]
RHC439	CNA TTT CTT CCC CGC CGA	*Rhodocyclus* spp., most members of the *Candidatus* 'Accumulibacter' cluster, *Azospira* lineage	30	[20]
Actino-221	CGCAGGTCCATCCCAGAC	*Actinobacterial* PAO	30	[24]
C1-Actino-221	CGCAGGTCCATCCCATAC	Competitor probe 1 for Actino-221	30	[24]
C2-Actino-221	CGCAGGTCCATCCCAGAG	Competitor probe 2 for Actino-221	30	[24]
Actino-658	TCCGGTCTCCCCTACCAT	*Actinobacterial* PAO	40	[24]
C1-Actino-658	TCCGGTCTCCCCTACCAC	Competitor probe 1 for Actino-658	40	[24]
C2-Actino-658	ATTCCAGTCTCCCCTACCAT	Competitor probe 2 for Actino-658	40	[24]
GB[c]	CGATCCTCTAGCCCACT	GAO – group GB	35	[25]
GB_G1(GAOQ989)[c]	TTCCCCGGATGTCAAGGC	*Candidatus* 'Competibacter Phosphatis'/ subgroup G1 in group GB	35	[8]
GB_G2[c]	TTCCCCAGATGTCAAGGC	Subgroup G2 in group GB	35	[25]
TFO_DF618[d]	GCCTCACTTGTCTAACCG	*Defluvicoccus vanus*-related organisms, cluster 1	35	[47]
TFO_DF218[d]	GAAGCCTTTGCCCCTCAG	*Defluvicoccus vanus*-related organisms, cluster 1	35	[47]
DEF988	GATACGACGCCCATGTCAAGGG	*Defluvicoccus vanus*-related organisms, cluster 2	35	[36]
DEF1020	CCGGCCGAACCGACTCCC	*Defluvicoccus vanus*-related organisms, cluster 2	35	[36]
H966	CTGGTAAGGTTCTGCGCGTTGC	Helper probe for DEF988		[36]
H1038	AGCAGCCATGCAGCACCTGTGTGGCGT	Helper probe for DEF988 and DEF1020		[36]
CFX1223[e]	CCATTGTAGCGTGTGTGTMG	Phylum 'Chloroflexi'	35	[6]
GNSB-941[e]	AAACCACACGCTCCGCT	Phylum 'Chloroflexi'	35	[6]

[a] Equimolar concentrations of each probe were mixed together and applied as EUBmix

[b] Equimolar concentrations of each probe were mixed together and applied as PAOmix

[c] Equimolar concentrations of each probe were mixed together and applied as GAOmix

[d] Equimolar concentrations of each probe were mixed together and applied as DF1mix

[e] Equimolar concentrations of each probe were mixed together and applied as CFXmix

FISH analyses

Biomass samples were collected at the end of the aerobic phase for FISH analysis and immediately fixed in 4% (w/v) formaldehyde for gram-negative and 50% (v/v) ethanol for gram-positive cells. Granules were homogenized with a pellet pestle prior to FISH, which was carried out as described by Daims et al. [12] with the probes listed in Table 1. Slides were mounted in VectaShield (Vector Laboratories, USA) and examined with either an epifluorescence microscope (Eclipse 800, Nikon) or a confocal laser scanning microscope (CLSM) (TCS SP2, Leica).

For FISH analysis on granule sections, 30-μm-thick sections were prepared from fixed granules embedded in Tissue-Tek OCT Compound (4583, ProSciTech) using a cryostat (CM1850, Leica) and placed on gelatin-coated microscopic slides. The OCT compound was removed by soaking the slides in distilled water and FISH performed as described earlier.

Populations were quantified (qFISH) from micrographs taken with the CLSM using daime software [11]. Values are expressed for each probe as a percentage of the total area fluorescing with the EUBmix probes [2, 10], based on 40 fields of view at ×630 magnification. All qFISH errors are represented as standard errors.

To detect polyphosphate and PHA in the cells, 4′,6-diamidino-2-phenylindol (DAPI) [23] and Nile blue A [42], respectively, were used.

Electron microscopic analysis of granules

For scanning electron microscopy (SEM) and transmission electron microscopy (TEM), granules were fixed in 2.5% (v/v) glutaraldehyde solution and 1% (w/v) osmium tetroxide for 2 h each at room temperature and then dehydrated through an acetone series [0–100% (v/v)]. For SEM, granules were dried with a critical point dryer (CPD 030, Bal-Tec) and gold/palladium coated using a sputter coater (E5100, Polaron). Coated specimens were examined in a SEM microscope (S150, Cambridge) at 10–20 kV.

For transmission electron microscopy (TEM), granules were infiltrated with Spurr's epoxy resin and left to harden overnight at about 65–70°C. They were sectioned with a diamond knife (DDK) on an LKB Bromma ultramicrotome, and then stained with 2% (w/v) uranyl acetate and lead citrate for 15 min each. Specimens were examined by a Jeol JEM100CX electron microscope at 100 kV.

Results

Effect of pH on EBPR performance

The daily variations in final effluent P levels in samples taken at the ends of the anaerobic and aerobic stages of the SBR operating at pH 7.5, 7.0 and 6.5 are given in Fig. 1. Initially the SBR was operated at pH 7.5. The amount of anaerobic P release increased steeply over the first 20 days to about 110.7 mg-P l^{-1}, and the polyP biomass content reached about 9.7% (w/w) after day 27. These values are typical for EBPR processes with acetate as the carbon source and consistent with a microbial community dominated by PAO [39]. When the pH was changed from 7.5 to 7.0 on day 28, the P released under anaerobic conditions fell initially to about 62.1 mg-P l^{-1} on day 47, but then gradually increased to 82.6 mg-P l^{-1}. Biomass polyP content also decreased to about 6.8% (w/w). On day 77, operating pH was again lowered from 7.0 to 6.5, a shift that led to a slight decrease in the level of P released anaerobically to 71.1 mg-P l^{-1}. Biomass P content had also fallen to 6.0% (w/w) at pH 6.5. This trend is consistent with an increasing presence of GAO not accumulating polyphosphate in their cells and agrees with those seen by Filipe et al. [18] and Jeon et al. [22].

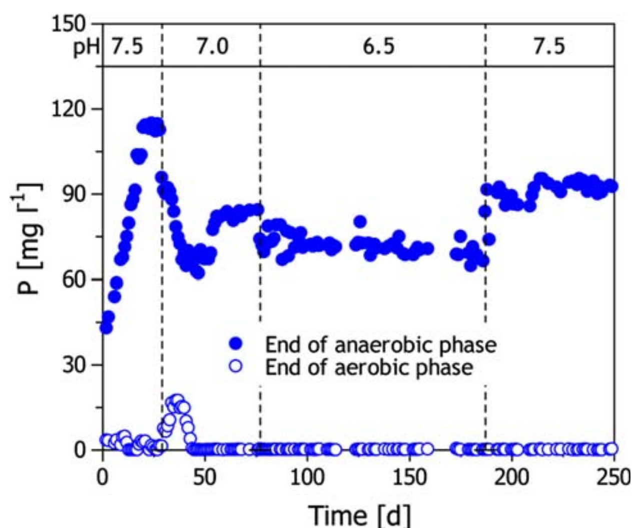

Fig. 1 Daily effluent P concentrations at the end of anaerobic and aerobic stages of the SBR under different operational pH conditions

On the other hand, a marked improvement in EBPR capacity was observed when the pH was changed back from 6.5 to 7.5, where 90.9 mg-P l^{-1} of anaerobic P release and 7.6% (w/w) biomass P content were measured, increasing from 6% at pH 6.5. The MLSS values changed from 2,900 (±25) mg l^{-1} at initial pH 7.5 (day 27), rising to 4,500 (±50) mg l^{-1} at pH 7 (day 76) and 5,600 (±50) mg l^{-1} at pH 6.5 (day 183), before falling to 3,570 (±30) mg l^{-1} when pH was increased to 7.5 (day 218).

Effect of operating pH on reactor chemical profiles

The chemical profiles exhibited by the communities grown at these different pH values are given in Fig. 2. They show the typical patterns for EBPR processes carried out in SBR systems fed acetate [37, 39]. Thus, under anaerobic conditions, P release corresponded to acetate assimilation and an increase in biomass PHA content and glycogen utilization, while complete P uptake and glycogen replenishment paralleled PHA utilization in the subsequent aerobic stage. These observations are consistent with the existing EBPR-activated sludge models. When operational pH was decreased, anaerobic biomass PHA production fell from 4.0 mM-C at pH 7.5 to 2.2 mM-C at pH 7.0, and 2.0 mM-C at pH 6.5. On the other hand, glycogen utilization levels increased markedly from 1.9 mM at pH 7.5 to 3.1 mM-C at pH 7.0 and then 8.1 mM-C at pH 6.5. These data again strongly support the view that that the GAO phenotype became more dominant in the community as operational pH decreased.

Effect of operating pH on biomass organization and community structure

Changing pH also had a marked influence on the organization of the biomass in the SBR (Fig. 3). Thus, at pH 7.5 and early stages at pH 7.0, biomass appeared as loosely associated

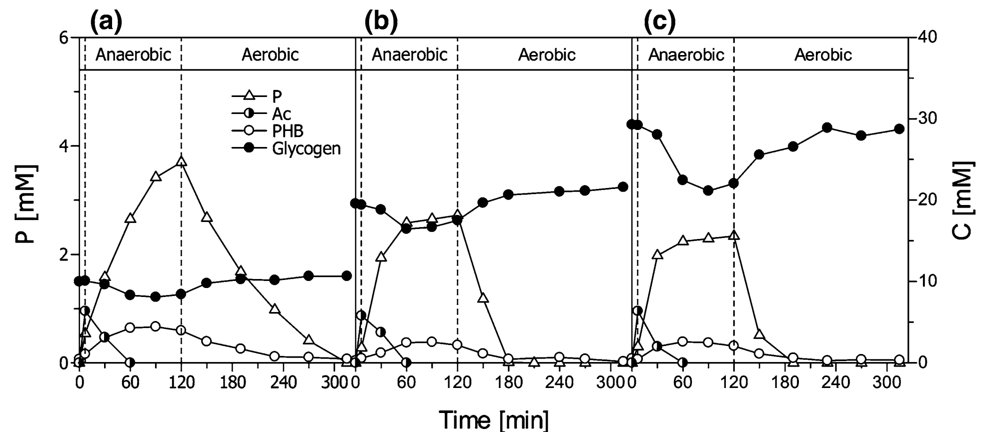

Fig. 2 Typical profiles of the EBPR chemical transformations occurring in the SBR under different operational pH conditions. **a** pH 7.5; **b** pH 7.0; **c** pH 6.5. Samples were taken after 27 days (pH 7.5), 76 days (pH 7.0) and 133 days (pH 6.5). Glycogen, PHA and acetate are all expressed as mM carbon equivalents

flocs (Fig. 3a–c) with visible filamentous matrix. FISH analyses showed they contained large numbers of often spherical and densely packed microcolonies of *Accumulibacter* cells containing polyP by DAPI staining (Fig. 3a–c). Although both *Competibacter* and cluster II *Defluviicoccus* microcolonies were present, they were there in small numbers (Table 2). Then at pH 7.0, the biomass changed, and from a visual assessment gradually became granulated, a transformation coinciding with the recovery of EBPR performance following the pH change (Fig. 1). Far fewer flocs were visible. These granules (up to 1-mm diameter) were regularly spherical with smooth outer surfaces (Fig. 3d), and FISH analysis at pH 6.5 showed that they contained much larger aggregates of *Accumulibacter* cells (Fig. 3j–l) and a few '*Chloroflexi*' filamentous bacteria (Fig. 3j).

Actinobacterial PAO responding to the Actino-658 probe were detected in the early pH 7.5 FISH sample, but were rarely seen in subsequent samples, probably due to their inability to utilize acetate as a carbon source [24]. *Accumulibacter* PAOs were always the dominant organisms in the reactor. When the pH was decreased from 7.5 to 7.0, the *Accumulibacter* PAO levels increased and remained relatively unchanged with a subsequent decrease in pH to 6.5 (Table 2). Although the levels of the cluster II *Defluviicoccus* increased slightly with this fall in pH, their numbers remained low (Table 2), while cluster I *Defluviicoccus* and *Competibacter* were rarely seen by FISH probing at any pH (Table 2). Some of the larger clusters in individual granules appeared to be aggregates of smaller microcolonies of *Accumulibacter*, indicating a possible starting point for their formation (Fig. 3f–i).

The granules had increased in size to about 2–3 mm diameter, and FISH of thin sections revealed they consisted almost entirely of *Accumulibacter* cells (Fig. 3j–l). TEM of these revealed they contained sparse numbers of small electron-dense polyP granules, but most of the cell was filled with electron transparent material (Fig. 3e). Occasional larger polyP granules appeared to have become dislodged during sectioning, resulting in obvious holes in the sections. Only occasional small clusters of *Competibacter* (Fig. 3k) and cluster II *Defluviicoccus* (Fig. 3l) were observed, but these were randomly distributed with no evidence of population stratification.

When the pH was then increased to 7.5, the granules appeared to become less stable and began to disintegrate within 24 h, so that they gradually became much less common, being replaced after 7 days with loosely aggregated biomass. Closer examination revealed that the commonly seen filamentous bacteria were largely restricted to members of the '*Chloroflexi*' and were prevalent.

Discussion

Because of their operational attractions, interest in using granulated biomass instead of flocs in SBR systems is increasing. Their rapid settling rates provide an opportunity to generate and sustain very high biomass levels [13, 14], allowing shorter treatment times in smaller reactors.

Granule formation is still a poorly understood process, and the literature is often contradictory, suggesting that several factors probably contribute towards granulation and that multiple mechanisms, which may be application specific, are likely to be involved. For example, the observations of Weber et al. [46], suggesting that protozoa and fungi were crucial initially in providing the matrix for bacterial growth and EPS production in their granules, are quite different from the situation with those examined here. Consequently, it may not be possible to sustain a unified mechanistic theory for granulation, although its common feature is that it seems to occur mainly in SBR systems [34].

This paper presents evidence to suggest that granulation of biomass in an SBR configured for EBPR and operating with Rushton turbines at a relatively high shear seems to be influenced by operating pH. We know only of one other report where pH has been reported to influence EBPR granule stability [28], with an abattoir waste feed. In this study

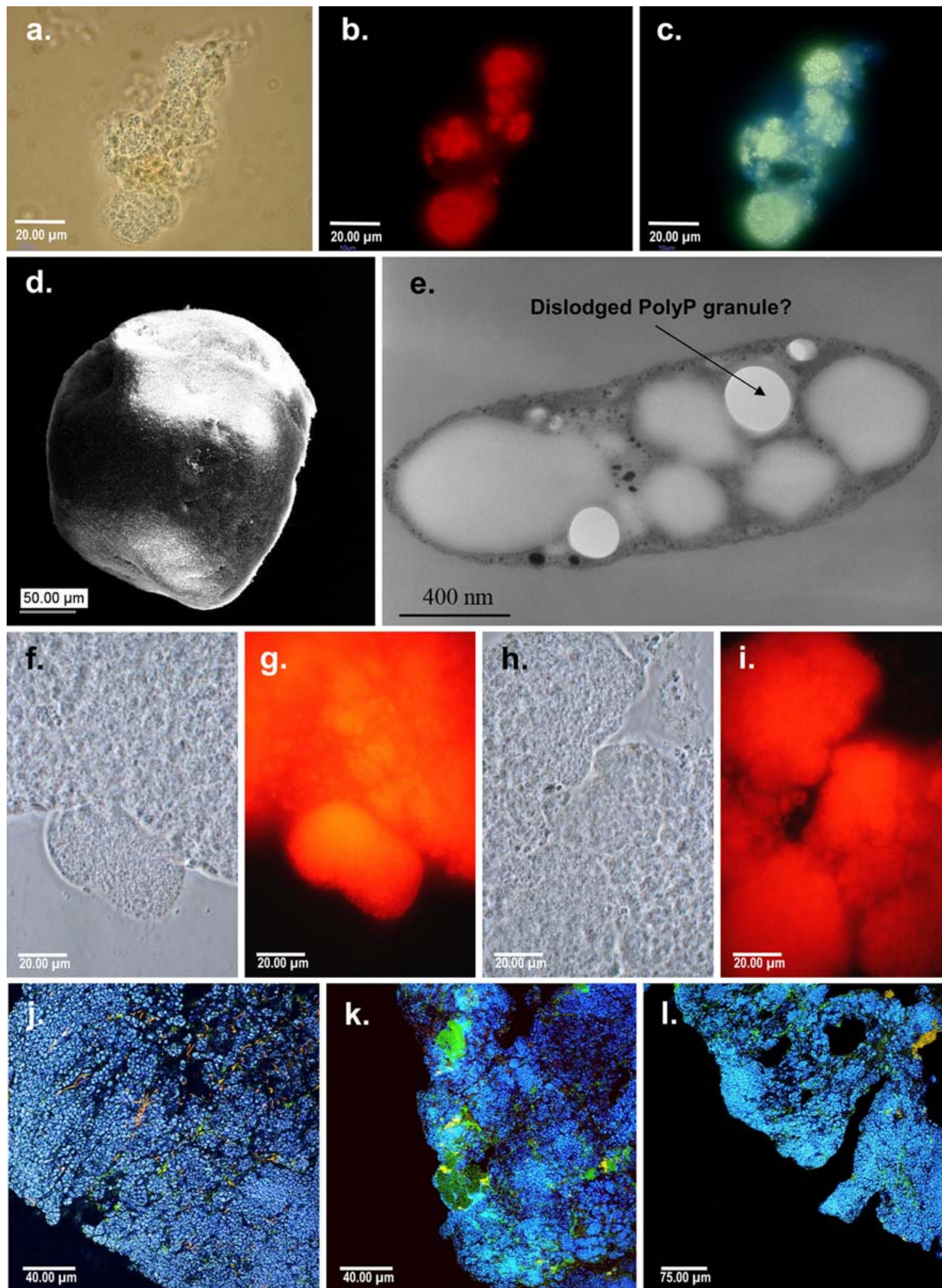

Fig. 3 **a**–**c** FISH micrographs for the same biomass sample taken at pH 7.5 (day 17). **a** Phase constrast; **b** after FISH with RHC439 probe; **c** after DAPI staining showing polyP fluorescence (yellow); **d** SEM of a granule produced at pH 7.0; **e** TEM of a PAO cell in a sample taken at the end of the aerobic stage at pH 6.5. **f**–**i** FISH images of biomass at pH 7.0 (day 72); **f** and **h** phase contrast images; **g** and **i** corresponding fields of view after FISH probing with PAOmix probes. **j**–**l** CLSM fluorescent images of sections of granules produced at pH 6.5 (day 180). FISH image overlays showing bacteria hybridized with **j** EUBmix only (green), PAOmix and EUBmix (blue + green = light blue) and CFX1223/GNSB-941 and EUBmix (red + green = yellow-orange); **k** EUBmix only (green), PAOmix and EUBmix (light blue) and GBmix and EUBmix (yellow); **l** EUBmix only (green), PAOmix and EUBmix (light blue) and DEF988 and EUBmix (yellow)

Table 2 Semi-quantitative FISH data for selected populations in communities generated in the EBPR SBR operating at different pH values

Sample		FISH probe			
Time (days)	pH	PAOmix	DEF988[a]	GBmix	CFXmix
17	7.5	40.4 ± 1.6	2 ± 0.2	−/+	10 ± 0.8
72	7.0	67.7 ± 3.5	6 ± 0.7	−/+	3.6 ± 0.6
96	6.5	65.2 ± 3	5.7 ± 0.6	<1%	1.9 ± 0.4
179	6.5	68.7 ± 2.7	4.7 ± 0.5	<1%	3 ± 0.5

± Rarely observed

[a] All organisms responding to DEF1020 probe were covered by DEF988 probe

granules became less stable at pH 6.5 than they were at pH 7.5, which are trends opposite to ours. However, the possible influence of long-term exposures on granular stability to variation in pH was not followed [28]. This illustrates the point made above about the inappropriateness of assuming a single mechanism is responsible for all granulation processes. Our flocs were also much more regularly spherical and smoother than theirs [28], and no surface-attached protozoa were seen, possibly because of the higher shear used in our study.

Thus, biomass in the reactor operating at pH 7.5 and the early stages of pH 7.0 was organized almost entirely as small flocs containing multiple microcolonies of densely packed *Accumulibacter* cells staining positively for polyP. These occupied a large portion of the floc volume (Fig. 3a–c), suggesting that they were able to persist there under the imposed shear forces better than most other floc-associated populations, which became physically dislodged as flocs eroded. The ability of PAO microcolonies to withstand high shear and tolerate physical stress better than other floc populations has been documented [27]. Consequently, under our conditions persisting flocs eventually became highly enriched with *Accumulibacter* microcolonies, and the granules then formed, possibly by their subsequent co-aggregation (Fig. 3f–i).

Such a mechanism is not inconsistent with the TEM data of Lemaire et al. [28], which show clearly delineated and spatially related heterogeneities in cell density and EPS matrix appearance within individual granules. This is exactly what might be expected if aggregation of different individual microcolonies was involved in their formation. It might also explain the presence of regular cell-free channels, which were also seen in our granules (Fig. 3l). Furthermore, this proposal provides a mechanism for the reported increases in their diameter, which may also involve increases in sizes of individual co-aggregated microcolonies from PAO replication.

Which mechanisms initiate granulation as pH decreases with our SBR conditions are not clear. Whether an increasing negative surface charge might facilitate ionic bridging between cells in the microcolonies or increase the adhesive properties of the EPS is unclear, but the mechanism appeared to be reversible. Thus, when pH was switched back to pH 7.5, granules became structurally less stable and readily disintegrated. Lowering the pH down to 7 led to regeneration of granules over a few days (data not presented).

Chemical data (Figs. 1, 2) obtained from the SBR reactors showed that the P release:acetate assimilation ratios fell as the operating pH was lowered, but then rose after reversion to pH 7.5. Biomass P levels in samples taken at the end of the aerobic stages at pH 7 and 6.5 also fell while their glycogen contents increased. Both observations are consistent with a population shift from a PAO- to GAO-dominated community [39]. Yet by FISH analyses there were no such major shifts in community composition, and the granules formed at both pH 7.0 and 6.5 were always dominated by *Accumulibacter* PAO, with very few *Competibacter* or *Defluviicoccus* GAO cells. Furthermore, TEMicrographs of individual cells in such samples showed they always contained large amounts of electron transparent material (Fig. 3e). This was unlikely to be PHA, since none could be detected chemically at the end of the aerobic stage (Fig. 2), and was confirmed by Nile blue A staining, which showed only a few of the cells taken then contained PHA granules (data not shown). So this suggests that *Accumulibacter* PAOs, not the putative *Defluviicoccus* or *Competibacter* GAOs, are the cells storing the high amounts of intracellular glycogen responsible for the chemical patterns shown (Fig. 2). In fact, it is difficult to interpret these trends any other way. The question is whether this glycogen as well as the polyP stores is used by the PAOs as their major energy source for anaerobic acetate transport and PHA synthesis, a possibility that receives some support from other studies.

Thus, it now appears that *Accumulibacter* can shift its phenotype from that of a typical PAO to a GAO in response to a changing environment. For example, Zhou et al. [48] showed under extended P limitation that *Accumulibacter* no longer synthesized polyP aerobically for use as the energy source for acetate assimilation and PHA synthesis anaerobically, and the P release:acetate uptake ratios fell. Instead, these PAOs switched to using glycogen as the major anaerobic energy source, therefore behaving essentially as a GAO. A similar shift was also suggested by Barat et al. [4, 5], who recorded differences in the anaerobic P release: acetate uptake ratios with changes in Ca^{2+} concentrations. These too could not be explained in terms of shifts in community composition. They postulated that a PAO metabolism occurred with *Accumulibacter* at low Ca^{2+} and a GAO

phenotype at high Ca^{2+}, from Ca^{2+} either complexing with the intracellular polyP granules or precipitating P. In either case polyP availability as energy source would be reduced, and glycogen was used instead. However, Falkentoft et al. [17] have suggested that the biofilm thickness may create limitations in phosphate diffusion to the PAO, which Lemaire et al. [29] suggested may have contributed to the higher levels of PAO located in outer sections of their granules. Further work is needed to examine this possibility more fully.

In conclusion, smooth regular granules consisting almost exclusively of *Accumulibacter* PAO were generated in an anaerobic: aerobic EBPR SBR operating at high shear rate coinciding with a drop in operating pH from 7.5 to 7.0 and then 6.5. Increasing pH to 7.5 saw them partially disintegrate. It is proposed that these granules form from existing flocs, consisting mainly of *Accumulibacter* PAO microcolonies, which co-aggregate initially into small spherical granules that increase in size, probably by the same process. Chemical profiles suggest that the GAO phenotype becomes more dominant as pH falls. The recorded increases in consumption of glycogen to replace polyP (Fig. 2) as an anaerobic energy source as seen by decreases in the level of P release, are consistent with this proposal (see [39]), but FISH data reveal little or no change in granule community composition. Instead, the PAOs store little intracellular polyP but considerable glycogen aerobically, consistent with a partial switch in phenotype from a PAO to GAO.

Acknowledgments This work was supported by the Australian Research Council (ARC) Discovery (DP0557646DS) and Victorian State Government Smartwater research grants. J. Ahn was funded as a La Trobe Postdoctoral Fellow and S. Schroeder supported by a PhD scholarship from La Trobe University. S. McIlroy received an ARC APA PhD scholarship.

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