Response of an EBPR population developed in an SBR with propionate to different carbon sources

M. Pijuan, J.A. Baeza, C. Casas and J. Lafuente

Departament d'Enginyeria Química, ETSE, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain (E-mail: *Maite.Pijuan@uab.es*; *JuanAntonio.Baeza@uab.es*; *Carles.Casas@uab.es*; *Javier.Lafuente@uab.es*)

Abstract The effect of different carbon sources (propionate, acetate, butyrate and glucose) on an enhanced biological phosphorus removal biomass developed with propionate as the sole carbon source was studied. Firstly, a group of different cycle studies was carried out using each substrate independently and then, another cycle study was performed with a mixture of substrates. Propionate was found to be the substrate with the highest substrate uptake rate in both sets of experiments. It was also the volatile fatty acid (VFA) which required less reducing power and less P-release to be uptaken. Four different polyhydroxyalkanoate (PHA) monomers produced during the anaerobic phase were detected, and PHB, PHV and PH2MV were quantified. Significant differences in PHA composition were obtained depending on the carbon source. The carbon recovery ratio for the anaerobic phase was also evaluated. The lowest value observed among the different cycle studies was obtained for butyrate, while the highest value was obtained for acetate.

Keywords Carbon source; enhanced biological phosphorus removal; phosphorus accumulating organisms; polyhydroxyalkanoates; volatile fatty acids.

Introduction

The enhanced biological phosphorus removal (EBPR) process is based on the enrichment of activated sludge with polyphosphate accumulating organisms (PAO) and details of their biochemical pathways are still only hypotheses without confirmation. Under anaerobic conditions, PAO take up organic substrates (preferably volatile fatty acids (VFA)) and store them as polyhydroxyalkanoates (PHA), while the reducing equivalents are provided by glycolysis of internally stored glycogen (Mino *et al*., 1987; Satoh *et al*., 1992; Smolders *et al*., 1994a,b). The energy for this process is obtained partly from the glycogen utilisation but mostly from the hydrolysis of the intracellular stored polyphosphate (polyP), resulting in an orthophosphate release into solution. In the subsequent aerobic phase, PAO take up extra amounts of orthophosphate to recover the intracellular polyP levels by oxidising the stored PHA. Meanwhile they grow and replenish the glycogen pool using PHA as both carbon and energy sources (Smolders *et al*., 1995). Net phosphorus removal is achieved by wasting sludge after the aerobic period, when the biomass contains a high level of polyP.

Sometimes, deterioration of the EBPR in laboratory-scale systems has been observed (Cech and Hartman *et al*., 1990) and it has been attributed to the carbon source supplied during the anaerobic phase of the EBPR process. More attention has to be paid to the type of carbon source in the wastewater and its effect on EBPR due to the heterogeneous organic matter in wastewater which often reaches the treatment plant without complete acid fermentation. At this point, research in the response of EBPR systems to different substrates has been increased in recent years (Hood and Randall, 2001; Levantesi *et al*., 2002; Randall and Liu, 2002). Aiming to understand the different responses of an EBPR biomass developed using propionate as the sole carbon source in front to different substrates, two different sets of experiments were conducted. Propionate, acetate, butyrate and glucose

Water Science and Technology **Vol 50 No 10 pp 131–138** © IWA Publishing 2004

were used as sole carbon sources and in combination as a mixed substrate. The synthesis of different PHA depending on the substrate used as well as the reducing power required to store these substrates was analysed in each cycle study to contribute to a deeper knowledge of PAO behaviour.

Materials and methods

Reactor

The experiments were performed in a 10 L sequencing batch reactor (SBR) seeded with activated sludge from a non-EBPR plant (Granollers Wastewater Treatment Plant, Catalonia, Spain). It was operated with four cycles per day with a controlled temperature of 25°C. Each cycle consisted of 2 h anaerobic react, 3.5 h aerobic react, 25 min of settling and, in the last 5 min, extraction of 5 L of the supernatant. A volume of 5 L of synthetic wastewater (composition below) was added during the first 5 min of the subsequent cycle, producing a hydraulic residence time (HRT) of 12 h. The sludge residence time (SRT) was kept at 9 d by periodic sludge wastage during the end of the aerobic period but before the mixing was stopped. The pH was controlled during the aerobic period at 7.0 ± 0.1 with 1M HCl. These experiments were conducted after 90 days of SBR operation, when the reactor was working with an EBPR population in steady state and the biomass concentration was around 4,000 mg $VSS \cdot L^{-1}$, with a VSS/TSS ratio of 0.65 at the end of the aerobic period.

Synthetic wastewater

Two separate solutions called "concentrated feed" (constituting 0.25 L per 5 L synthetic wastewater) and "P-water" (constituting 4.75 L per 5 L synthetic wastewater) collectively formed the synthetic wastewater used in this study. The composition of the synthetic wastewater used in this study was the same as the one used in Pijuan *et al*. (2004). The EBPR population of the reactor was developed using propionate as the sole carbon source and the initial concentration in the SBR (after the feeding phase) was increased progressively over a 20 d period from 20 mg to 160 mg/L (1.62 mmol C/g VSS).

Chemical analyses

Analyses of phosphate in filtered samples were performed with an electrophoresis capillary system (Quanta 4000E CE – WATERS). The electrolyte used was a commercial solution (Ionselect high mobility anion electrolyte). The VFA (propionate, acetate and butyrate) were measured by gas chromatography (GC). Glycogen was determined by a modification of the method of Smolders *et al*. (1994a). An amount of 30 mg of lyophilised sludge samples was digested in a water bath with 0.6 N HCl aqueous solution at 100°C during 5 h. After cooling and filtering through a 0.22 µm filter (Millipore), the concentration of glucose was measured using a Yellow Spring Instrument (2700 Select). Triplicates of each sample were done. PHA was measured according to a modification of the method of Comeau *et al*. (1988). 30 mg of lyophilised sludge samples were digested and methylated with 4 ml of acidulated methanol (3% H_2SO_4) and 4 ml of chloroform during 6 h at 100°C. Benzoic acid was used as the internal standard. The calibration of the method was performed using as standard a 3-hydroxybutyric acid and 3-hydroxyvaleric acid copolymer (7:3) (Fluka). 3-hydroxy-2-methylvalerate (3H2MV) was quantified using 2-hydroxycaproic acid (Aldrich) as standard. Another non-identified monomer unit was detected when butyrate was used but not quantified because no standards were available. The analyses were performed in a GC system (Hewlett Packard 5890). Triplicates of each sample were done. Finally, total suspended solids (TSS) and volatile suspended solids (VSS) were done according to Standard Methods (APHA, 1995).

Cycle studies

After obtaining a steady-state population in the SBR, two sets of cycle studies were conducted to investigate the response of the biomass to different substrates. The experiments were performed in the SBR with the same operational conditions. Since cycle studies were generally carried out once per day, at least three 6 h SBR normal cycles elapsed between each study. The first set of experiments consisted of four different cycle studies carried out with four different carbon sources. Propionate, acetate and butyrate were studied separately as different VFA and glucose was also tested as a carbon source different from VFA. Also, an additional experiment with a mixture of all the substrates used previously was carried out in the SBR in order to study possible inhibitory or synergistic effects between them.

Microbial analyses

Fluorescence *in situ* hybridization (FISH) was performed with Cy5-labelled EUBMIX probes (for most bacteria; Daims *et al*. 1999), Cy3-labelled PAOMIX (for *Accumulibacter*, Crocetti *et al*., 2000) and Cy3-labelled GAOMIX (for *Competibacter*, Crocetti *et al*., 2002). FISH preparations were visualised with a Leica DMIRB confocal laser scanning microscope (CLSM) and quantification of *Accumulibacter* as a proportion of all bacteria was done using methods previously reported (Zeng *et al*., 2003).

Results and discussion

Normal reactor operation

It has been suggested that propionate could selectively enrich PAO over other microorganisms like glycogen-accumulating organisms (GAO). The use of propionate, instead of acetate, seems to be beneficial in obtaining a highly enriched PAO culture as observed by Pijuan *et al*. (2004), and for this reason, this carbon source was used to develop the PAO population used in this study. The reactor achieved an EBPR population in steady state after one month of operation. In the inoculum sludge, the PAOMIX-probes bound 4% of all bacteria (*Accumulibacter*). After the initial 30 days period of operation, the PAOMIX-binding cells proportion increased to 45% of all bacteria. This PAO percentage was maintained in all the cycles studies performed with different substrates. No GAOMIX-binding cells (*Competibacter*) were detected after 30 days of the SBR operation, even though some of them were present in the inoculum sludge. This result agrees with the hypothesis that propionate favours the enrichment of PAO and it is detrimental for GAO (Hood and Randall, 2001).

Cycle studies with single substrates

The responses of an EBPR population to four different individual substrates (propionate, acetate, butyrate and glucose) were analysed. The same amount of COD (approximately 280 mg COD·L^{-1}) was added in the cycle studies performed with VFA, while in the cycle study performed with glucose, less concentration was added (approximately 200 mg COD·L^{-1}) to avoid the presence of this substrate under aerobic conditions. These concentrations are slightly higher than the normal propionate working concentration in the reactor (approximately 240 mg COD·L^{-1}) and for this reason the levels of glycogen at the end of the different cycles studies used to be slightly higher than the initial ones.

The experimental profiles obtained in the cycle studies are shown in Figure 1. Although all the carbon sources were depleted during the anaerobic period, propionate and acetate were consumed during the first 50 min of the cycle and butyrate and glucose needed more time to be depleted. Substrate uptake rates (mmol substrate/g VSS min) for propionate and acetate were similar and higher than those obtained for butyrate and glucose (Table 1). It is difficult to determine if the lack of acclimation of the biomass to these substrates was the

reason for these low values or if they were the normal consumption rates for these substrates. Similar results were obtained by Lemos *et al*. (1998) who found the highest consumption rate for acetate followed by propionate and butyrate, using a biomass adapted to consume simultaneously these different fatty acids.

Two ratios for P-release were calculated (Table 2): one versus substrate uptake and another one versus PHA accumulated. A higher P-release versus substrate uptake was observed for acetate (0.319 mmol P/mmol C), followed by propionate, butyrate and glucose. These results are comparable with values found in the literature (Liu *et al*., 2002; Pijuan *et al*., in press). Regarding P-release versus PHA accumulated, a higher ratio was observed when propionate was used (0.711 mmol P/mmol C) followed by butyrate, acetate and glucose. This different trend was observed because the ratio PHA produced versus substrate uptake was very different depending on the carbon source (Table 3).

Glycogen degradation versus substrate uptake is shown in Table 2. When propionate was consumed, less reducing power was needed to uptake VFA and therefore, less glycogen was degraded. On the contrary, when acetate was used, the highest glycogen degradation was detected per VFA uptake. When glucose was used as carbon source, instead of

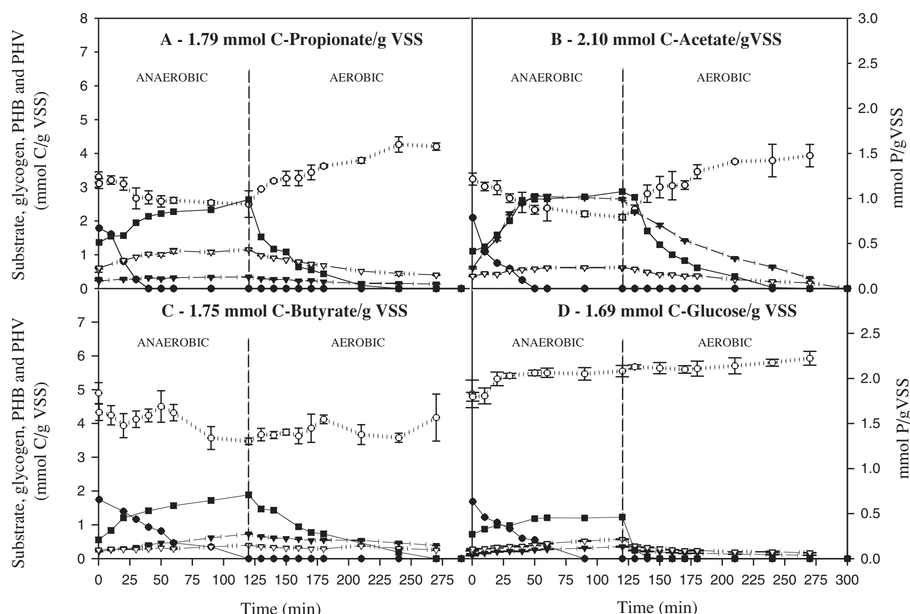

Figure 1 Experimental profiles of different compounds during the anaerobic/aerobic cycles of the SBR with propionate, acetate, butyrate and glucose. (● substrate; ■ phosphorus; ○ glycogen; ▼ PHB; ▽ PHV)

Table 1 Rates obtained in the cycle studies performed with propionate, acetate, butyrate and glucose as a single substrate and when they were used in a mixed substrate

Rates	Single substrate				Mixed substrate	
	Propionate	Acetate	Butyrate	Glucose		
P-release* (mmol P/gVSS min)	0.0073	0.0142	0.0035	0.0017	0.0117	
Substrate-uptake (mmol C/gVSS min)	0.051	0.047	0.016	0.022	0.057 (Prop) 0.015 (Acet)	0.020 (Buty) 0.018 (Gluc)
Substrate-uptake (mmol subs./gVSS min)	0.017	0.023	0.004	0.004	0.019 (Prop) 0.007 (Acet)	0.005 (Buty) 0.003 (Gluc)
P-uptake (mmol P/gVSS min)	0.010	0.010	0.007	0.039	0.006	

* This rate was calculated when there was still substrate in the mixed liquor

Table 2 Ratios obtained in the cycle studies performed with propionate, acetate, butyrate and glucose as a single substrate and when they were used in a mixed substrate. These ratios were calculated using the initial and final concentration of each compound in the corresponding phase

Ratios	Single substrate				Mixed substrate
	Propionate	Acetate	Butyrate	Glucose	
P-release/subs.-uptake (mmol P/mmol C)	0.268	0.319	0.217	0.053	0.199
P-release/PHA-prod. (mmol P/mmol C)	0.425	0.248	0.466	0.147	0.438
Gly-degra./subst.-upt. (mmol C/mmol C)	0.447	0.628	0.491	0.278*	0.295
P-upt./PHAoxidized (mmol P/mmol C)	0.721	0.384	1.291	0.552	0.356

* When glucose was used as substrate, glycogen was synthesized not degraded

degradation, glycogen production was observed. This carbon source was not comparable with the VFA because it followed a different pathway to be stored and part of it was stored as glycogen.

With respect to the aerobic phase, PAO sequestered phosphorus at the same rate when they used propionate or acetate as the carbon source (Table 1). These values are similar to previous results (Pijuan *et al*., 2004). When butyrate was used, this rate was smaller and more time was required for total P-uptake (Figure 1).

The effect of the different carbon sources on PHA production was also assessed. Tables 3 and 4 show the different PHA monomers quantified for each substrate and its molar percentages. Acetate uptake resulted in the highest accumulation and PHB was the polymer mainly formed. Similar results were obtained by Satoh *et al*. (1992) and Lemos *et al*. (1998). When propionate was used as the sole carbon source, three monomer units were detected: 3HV, 3HB and 3H2MV. When butyrate was used as substrate, a different unidentified monomer was detected with the chromatographic analysis. This monomer only appeared when butyrate was used as carbon source and it seemed the main monomer synthesized using butyrate because of the high area detected in the analysis. Glucose utilisation produced a relative lower amount of stored PHA, because part of it was stored as glycogen instead of PHA.

The lowest carbon recovery ratio was observed when butyrate was used, while acetate presented the highest one. The low recovery ratios detected with some substrates imply a low efficiency of PHA storage. Nevertheless, it is important to remark that the butyrate carbon recovery ratio can be higher if the contribution of the PHA monomer detected but not quantified could be taken into account.

Cycle study with mixed substrate

A cycle study with a mixture of propionate, acetate, butyrate and glucose was carried out to study PAO behaviour in front of different substrates, knowing its response to these

Table 3 Accumulated PHA (PHB+PHV+PH2MV) during the anaerobic phase in the different cycle studies performed with propionate, acetate, butyrate and glucose as single substrates or when they were used as mixed substrate

Ratios	Single substrate				Mixed substrate
	Propionate	Acetate	Butyrate	Glucose	
PHB-prod./subst-upt. (mmol C/mmol C)	0.04	1.15	0.36	0.16	0.29
PHV-prod./subst-upt. (mmol C/mmol C)	0.34	0.13	0.08	0.20	0.16
PH2MV-prod/subst-upt (mmol C/mmol C)	0.26	0	0.02	0.0	0.0
PHA total-prod./subst-upt. (mmol C/mmol C)	0.64	1.28	0.46	0.36	0.45
Carbon recovery ratio (mmol C/mmol C)	0.44*	0.81*	0.31*	0.64**	0.35

* (PHA produced)/(substrate taken up + glycogen degraded)
** (PHA produced + glycogen produced)/(substrate taken up)

Table 4 Molar percentages of PHB, PHV and PH2MV production during the anaerobic period, obtained in the cycle studies using propionate, acetate, butyrate and glucose as single substrates and when they were used as mixed substrate

	Single substrate				Mixed substrate
	Propionate	Acetate	Butyrate	Glucose	
% PHB	6.2	89.6	78.5	44.1	64
% PHV	53.4	10.4	16.4	55.9	36
% PH2MV	40.4	0	5.1	0	0

substrates individually (cycles studies described before). The initial substrate concentrations for this cycle study were (mmol C/g VSS): 0.93 acetate, 0.68 propionate, 1.27 butyrate, 0.63 glucose. Figure 2A shows the experimental profiles obtained for the different substrates and phosphate. All substrates were completely exhausted during the first 60 min of the anaerobic period, but phosphate was not totally sequestered under aerobic conditions. Glycogen, PHB and PHV experimental data are presented in Figure 2B.

Tables 1 and 2 show also the different rates and ratios obtained for this cycle study. The substrate uptake rates obtained for propionate and butyrate were very similar to those obtained in the cycle studies using single substrates, meanwhile, acetate and glucose uptake rate decreased significantly. The cycles studies performed with different substrates added individually, showed that PAO could uptake propionate and acetate at a very similar rate (mmol substrate/g VSS min). Nevertheless, when this population had the mixed substrate, substrate uptake rate for propionate was almost maintained, while for acetate it was significantly reduced.

When the mixture of substrates was used, the ratio of P-uptake in the aerobic period versus PHA oxidised was lower than those obtained in the cycle studies with single substrates. One possible explanation for this ratio could be the composition of the intracellular PHA. Randall and Liu (2002) observed a correlation between aerobic P-uptake and PHA composition, but taking into account only the PHB and PHV. Nevertheless, when butyrate was used as the only carbon source or even when it was used in combination with other substrates, a different PHA was detected. The fraction of this non-identified monomer unit seemed to be important and might determine the P-uptake rate which presented a similar value when butyrate was used individually (0.007 mmol P/g VSS min) and when it was used in a mixed substrate (0.006 mmol P/g VSS min).

Some differences were observed in the comparison of the ratios of glycogen utilised versus substrate uptake obtained in the cycle studies performed with individual substrates

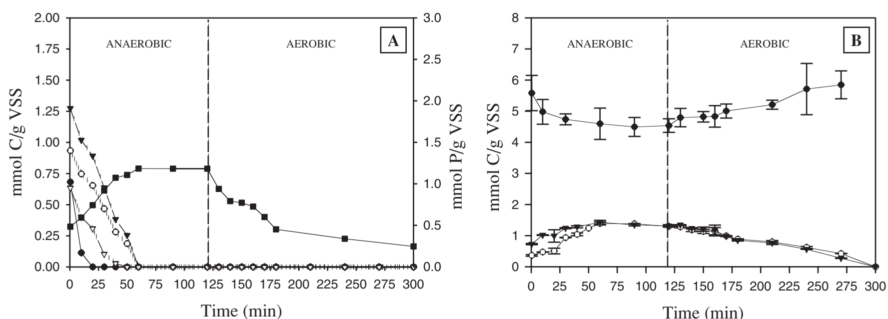

Figure 2 Experimental profiles of different compounds during the cycle study of the SBR performed with a mixture of four substrates. **A** – Substrates and phosphorus: ○ acetate; ● propionate; ▼ butyrate; ▽ glucose; ■ phosphorus. **B** – Intracellular compounds: ● glycogen; ○ PHB; ▼ PHV

and that obtained with the mixed substrate. The cycle study carried out with mixed substrate presented the lowest glycogen degradation (0.297 mmol C/mmol C). This could be due to the fact that the glucose present in the mixed substrate was acting as a source of reducing power as suggested by Jeon *et al*. (2001) and therefore, less glycogen had to be degraded to store the different VFA as PHA.

Conclusions

The main conclusions obtained from this study are:

- PHA composition was influenced strongly by the carbon source. Acetate was mainly stored as PHB, meanwhile propionate was mainly stored as PHV and PH2MV.
- When butyrate was used as substrate, another monomer unit was detected in the chromatographic analysis. This monomer only appeared when butyrate was used as carbon source and it seemed to be the main monomer synthesized.
- Some PHB and PHV storage was observed when glucose was used.
- The ratio of glycogen utilized versus substrate uptake in the cycle studies performed with individual substrates was higher than that obtained with the mixed substrate. This could be due to the fact that glucose present in the mixed substrate was acting as a source of reducing power and therefore, less glycogen had to be degraded to store the different VFA as PHA.
- Acetate presented the highest carbon recovery ratio, followed by glucose, propionate and butyrate. Nevertheless, it is important to remark that butyrate recovery ratio can be higher if the contribution of the PHA monomer detected but not quantified could be taken into account.

References

Cech, J.S. and Hartman, P. (1990). Glucose induced breakdown of enhanced biological phosphate removal. *Environ. Technol.*, **11**, 651–658.

Comeau, Y., Hall, K.J. and Oldham, W.K. (1988). Determination of poly-hydroxybutyrate and poly-hydroxyvalerate in activated sludge by gas-liquid chromatography. *Appl. Environ. Microbiol.*, **54**, 2325–2327.

Crocetti, G.R., Hugenholtz, P., Bond, P.L., Schuler, A., Keller, J., Jenkins, D. and Blackall, L.L. (2000). Identification of polyphosphate accumulating organisms and design of 16S rRNA-directed probes for their detection and quantitation. *Appl. Environ. Microbiol.*, **66**, 1175–1182.

Crocetti, G.R., Banfield, J.F., Keller, J., Bond, P.L. and Blackall, L.L. (2002). Glycogen-accumulating organisms in laboratory-scale and full-scale wastewater treatment processes. *Microbiology*, **148**, 3353–3364.

Daims, H., Bruhl, A., Amann, R., Schleifer, K.H. and Wagner, M. (1999). The domain-specific probe EUB338 is insufficient for the detection of all bacteria: Development and evaluation of a more comprehensive probe set. *Syst. Appl. Microbiol.*, **22**, 434–444.

Hood, C.R. and Randall, A.A. (2001). A biochemical hypothesis explaining the response of enhanced biological phosphorus removal biomass to organic substrates. *Wat. Res.*, **35**, 2758–2766.

Jeon, C.O., Lee, D.S. and Park, J.M. (2001). Enhanced biological phosphorus removal in an anaerobic-aerobic sequencing batch reactor: characteristics of carbon metabolism. *Water Environ. Res.*, **73**(3), 295–300.

Lemos, P.C., Viana, C., Salgueiro, E.N., Ramos, A.M., Crespo, J.P.S.G. and Reis, M.A.M.(1998). Effect of carbon source on the formation of polyhydroxyalkanoates (PHA) by a phosphate-accumulating mixed culture. *Enz. Microb. Technol.*, **22**, 662–671.

Levantesi, C., Serafim, L.S., Crocetti, G.R., Lemos, P.C., Rossetti, S., Blackall, L.L., Reis, M.A.M. and Tandoi, V. (2002). Analysis of the microbial community structure and function of a laboratory scale enhanced biological phosphorus removal reactor. *Environ Microb.*, **4**, 559–569.

Liu, Y., Geiger, C. and Randall, A.A. (2002). The role of poly-hydroxy-alkanoate form in determining the response of enhanced biological phosphorus removal biomass to volatile fatty acids. *Water Environ. Res.*, **74**, 57–67.

Mino, T., Arun, V., Tsuzuki, Y. and Matsuo, T. (1987). Effect of phosphorus accumulation on acetate metabolism in the biological phosphorus removal. In *Biological Phosphate Removal from Wastewaters (Advances in Water Pollution Control 4).* Ramadori, R. (ed.), Pergamon Press, Oxford, pp. 27–38.

Pijuan, M., Saunders, A.M., Guisasola, A., Baeza, J.A., Casas, C. and Blackall, L.L. (2004). Enhanced biological phosphorus removal in a sequencing batch reactor using propionate as the sole carbon source. *Biotechnol. Bioeng.*, **85**, 56–67.

Randall, A.A. and Liu, Y. (2002). Polyhydroxyalkanoates form potentially a key aspect of aerobic phosphorus uptake in enhanced biological phosphorus removal. *Wat. Res.*, **36**, 3473–3478.

Satoh, H., Mino, T. and Matsuo, T. (1992). Uptake of organic substrates and accumulation of polyhydroxyalkanoates linked with glycolysis of intracellular carbohydrates under anaerobic conditions in the biological excess phosphate removal processes. *Wat. Sci. Tech.*, **26**(5–6), 933–942.

Smolders, G.J.F., van der Meij, J., van Loosdrecht, M.C.M. and Heijnen, J.J. (1994a). Model of the anaerobic metabolism of the biological phosphorus removal process: Stoichiometry and pH influence. *Biotechnol. Bioeng.*, **42**, 461–70.

Smolders, G.J.F., van der Meij, J., van Loosdrecht, M.C.M. and Heijnen, J.J. (1994b). Stoichiometric model of the aerobic metabolism of the biological phosphorus removal process. *Biotechnol. Bioeng.*, **44**, 837–848.

Smolders, G.J.F., van der Meij, J., van Loosdrecht, M.C.M. and Heijnen, J.J. (1995). A structured metabolic model for anaerobic and aerobic stoichiometry and kinetics of the biological phosphorus removal process. *Biotechnol. Bioeng.*, **47**, 277–87.

Standard methods for the examination of water and wastewater (1995). 19th edn. American Public Health Association, Washington, DC, USA.

Zeng, R.J., Saunders, A.M., Yuan, Z., Blackall, L.L. and Keller, J. (2003). Identification and comparison of aerobic and denitrifying polyphosphate-accumulating organisms. *Biotechnol. Bioeng.*, **83**, 140–148.

Reproduced with permission of copyright owner. Further reproduction prohibited without permission.