Polyhydroxyalkanoate form and polyphosphate regulation: keys to biological phosphorus and glycogen transformations?

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Abstract Experimental studies with both synthetic and real domestic wastewater showed that poly-3-hydroxy-butyrate (3HB) and poly-3-hydroxy-valerate (3HV) formed in direct proportion to the acetate/propionate (Ace/Pro) ratio of the influent wastewater during Enhanced Biological Phosphorus Removal (EBPR). Acetic acid resulted in higher anaerobic phosphorus (P) release, polyhydroxyalkanoate (PHA) yield, 3HB content, and glycogen (CH) degradation. Linear regression showed that anaerobic P release (P_{rel}) and CH degradation (CH_{deg}) were both a function of Ace→3HB, but not of Pro→3HV. Aerobic P uptake (P_{up}) correlated best with preceding P_{rel} rather than PHA (but note P_{rel} correlated with Ace→3HB). Aerobic CH formation (CHform) correlated best with CH_{deg} and 3HB. The results imply the acetate/propionate content of influent has a major influence on PHA, CH, and P transformations. Short-term increases in acetic or propionic acid increased P_{rel}, but were always offset by corresponding changes in P_{up} to yield the same net P removal as the control reactor. Thus net P removal, and EBPR process performance, was probably a function of the population selected (i.e. XPAO fraction) during long-term cultivation.
Keywords Phosphorus; polyhydroxyalkanoate; wastewater

Introduction

Acetic and propionic acids are the two most common volatile fatty acids (VFAs) present in real septic domestic wastewaters (Naik, 1999; Shah, 2001). However in mechanistic modeling, design, and dynamic modeling of Enhanced Biological Phosphorus Removal (EBPR) systems, all VFAs are treated as acetic acid, and all polyhydroxyalkanoates (PHAs) formed from VFAs are treated as poly-3-hydroxy-butyric acid (3HB). Anaerobic processing of propionic acid has been discussed in only a few publications (Abu-Ghararah and Randall, 1989; Satoh *et al*., 1992) but the implications on aerobic transformations or overall process performance are largely unknown.

Most of this paper is focused on our current experiments which are investigating the effects of acetic and propionic acid ratio using a septic domestic wastewater from a full scale Bardenpho plant in Orange County, Florida. This plant received influent total VFA concentrations ranging from 34 to 73 mg/L over the last 2 years (Naik, 1999; Shah, 2001). This occurs year round in Florida due to warm, flat, sewer systems. Acetic and propionic acid were the two major VFAs, with acetic/propionic ratios of 2.0 to 5.4. In colder climates broad ranges of acetic/propionic ratio are encountered in deliberately prefermented wastewaters. VanMunch (1998), summarizing data from 4 full scale systems with prefermenters in Canada and Australia, observed acetic/propionic ratios of 1.5 to 3.0 in prefermented domestic wastewater. In temperate, non-prefermented wastewaters, there may be seasonal in-sewer fermentation, so propionic acid is potentially relevant to most EBPR plants.

Experimental results involving synthetic wastewater are also discussed. Synthetic wastewater data involved EBPR batch experiments for a number of substrates including acetic and propionic acid, but also including longer chain VFAs, succinic acid, and glucose. This data is analyzed with emphasis on the aerobic transformations and determining

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correlations with aerobic P uptake. There were also some synthetic wastewater experiments involving pH (HCl or NaOH addition) as a variable which are discussed when relevant.

Methods and materials

In this study septic (both acetic and propionic acids) domestic wastewater spiked with additional VFA (currently acetic) cultivated robust EBPR in two sequencing batch reactors (SBRs). In addition to characterization of SBR performance, periodic batch experiments were conducted in which the biomass from one of the SBRs was subdivided into 5 parallel reactors duplicating the 2 hour anaerobic/4 hour aerobic cycle of the SBRs. One reactor served as a control, while 2.5 mmol l^{-1}-C of VFA were added to each of the other 4 reactors. In the experiments discussed in this paper acetic/propionic acid combinations were used to total 2.5 mmol l^{-1}-C. One reactor received 2.5/0 (i.e. 2.5 mmol l^{-1}-C acetic acid/0 mmol l^{-1}-C propionic), the next 2/0.5, then 1.5/1, and finally 1/1.5. In the experiments with synthetic wastewater, the same SBRs were operated with a wastewater composed of nutrient broth, yeast extract, and inorganic nutrients (salts). The batch experiments were conducted in the same manner as current experiments, except that a single substrate was added. The substrates studied were acetic, propionic, valeric, isovaleric, and succinic acids, as well as glucose. Much of the synthetic wastewater study and analytical methods are detailed in another publication (Liu *et al*., in press).

In these experiments, so that 4 or 5 reactors could be operated with the same biomass simultaneously, it was not possible to obtain enough data points for kinetic analysis according to the models of Smolders *et al*. (1995) or Felipe and Daigger (1998). Thus multivariant linear regression was used to try and understand which parameters were related to each other, and the relationships obtained are entirely empirical rather than mechanistic. The emphasis in this publication is on developing an understanding of which transformations impact anaerobic and aerobic phosphorus (P) transformations most significantly rather than on developing the ability to model the systems dynamically, which is part of our ongoing research.

Results and discussion

Anaerobic transformations

The first significant observation from the domestic wastewater experiments was that poly-3-hydroxyvalerate (3HV) content of the PHA formed during anaerobiosis was proportional to the propionic acid content of the total VFAs received (Table 1). A second aspect of the changes in PHA biosynthesis as the fraction of propionic acid increased was a decrease in PHA yield (defined as the amount of PHA formed per unit VFA received), and thus the total amount of PHA formed (Table 1).

Table 1 Average effect of propionic acid content on anaerobic PHA form, quantity, and yield (relative to Control Reactor 1)

Reactor	Acetic fraction of total VFA (mmol l^{-1}-C/ mmol l^{-1}-C)	Propionic fraction of total VFA (mmol l^{-1}-C/ mmol l^{-1}-C)	3HB fraction of total PHA (mmol l^{-1}-C/ mmol l^{-1}-C)	3HV fraction of total PHA (mmol l^{-1}-C/ mmol l^{-1}-C)	Total PHA (mmol l^{-1}-C/ GMLSS)	Yield (PHA/VFA) (mmol l^{-1}-C/ mmol l^{-1}-C)
2	0.99	0.01	0.91	0.09	1.20	1.45
5	0.81	0.19	0.72	0.28	1.05	1.36
4	0.57	0.43	0.52	0.48	0.94	1.26
3	0.38	0.62	0.27	0.73	0.83	1.03

An additional observation was lower P release resulting from sequestration of propionic (Pro) versus acetic acid (Ace) during anaerobiosis (Table 2). pH values were very similar (± 0.15 pH units) in all reactors. Anaerobic CH degradation (CHdeg) was also observed to decrease as propionic acid content increased, probably due to a more positive redox balance than acetic acid during transformation to PHA (Satoh *et al*., 1992; Hood and Randall, 2001; Liu *et al*., 2002). When a carbon mass balance was conducted for VFAs sequestered + CH degraded = PHA formed it was noted that additional PHA that could not be explained solely from these two carbon sources was biosynthesized. It was also observed that this PHA formed from cryptic or unknown endogenous or exogenous sources was apparently associated with acetic acid rather than propionic acid. This phenomenon has been noted in several other studies (Louie *et al*., 2000; Satoh *et al*., 1996).

Multi-variant linear regression was conducted for anaerobic transformation of P and CH using absolute values of VFAs and intracellular inclusion (PHA and CH) data (absolute means not relative to the control so the control data was included). Eq. (1) shows the best model for P_{rel} with the most notable feature being that the acetic acid coefficient is higher than that of propionic acid. The data indicated that there was more P_{rel} from acetic→3HB transport/biosynthesis than from propionic→3HV transport/biosynthesis. CH_{deg} correlated with 3HB rather than the VFAs, 3HV, or interactive terms (Eq. (2)).

$$[P_{rel}, \text{mmol l}^{-1}\text{-P/gMLSS}] = 0.518\,[\text{Ace, mmol l}^{-1}\text{-C/gMLSS}] + 0.424\,[\text{Pro, mmol l}^{-1}\text{-C/gMLSS}] \quad (1)$$
$$R^2 = 0.69;\ F_{observed} = 14.7 > F_{significance} = 5.9 \times 10^{-4}$$

$$[CH_{deg}, \text{mmol l}^{-1}\text{-C/gMLSS}] = 0.200\,[3HB, \text{mmol l}^{-1}\text{-C/gMLSS}] \quad (2)$$
$$R^2 = 0.23;\ F_{observed} = 4.2 > F_{significance} = 6.1 \times 10^{-2}$$

The correlation for P_{rel} was also consistent with past data using synthetic wastewater (Liu *et al*., 2002), since acetic and isovaleric acids resulted in 3HB rather than 3HV, and the greatest P_{rel}. This may imply that the ATP/Poly-P utilization for VFAs that lead to 3HB is greater than that of VFAs leading to 3HV. Felipe and Daiggers' (1998) model describes P_{rel} as being a function of the energy requirements to replenish the proton motive force (PMF) due to its dissipation from transport of ionic VFAs across the cytoplasmic membrane into the PAO cell. In addition there is an ATP/Poly-P requirement for activation of acetyl or propionyl units with coenzyme A, a necessary step before PHA polymerization can take place. With some VFA→PHA pathways there can also be additional ATP driven reactions in the pathway. There must be some significant difference in one or more of these requirements (PMF maintenance/transport and activation/PHA polymerization) for Ace→3HB vs. Pro→3HV transport/biosynthesis.

Redox balances published by Hood and Randall (2001) indicated that both acetic and isovaleric acid would result in a negative contribution to cell redox balance and result in mainly 3HB. In contrast propionic and valeric acid would not have as negative, or even a

Table 2 Average anaerobic P release and CH degradation (relative to Control Reactor 1)

Reactor	Acetic fraction of total VFA (mmol l^{-1}-C/mmol l^{-1}-C)	Propionic fraction of total VFA (mmol l^{-1}-C/mmol l^{-1}-C)	Anaerobic phosphorus release (mmol l^{-1}-P/gMLSS)	CH degradation (mmol l^{-1}-C/gMLSS)	Yield (PHA/[VFA+CH]) (mmol l^{-1}-C/mmol l^{-1}-C)
2	0.99	0.01	0.35	0.18	1.22
5	0.81	0.19	0.33	0.13	1.17
4	0.57	0.43	0.30	0.11	1.11
3	0.38	0.62	0.27	0.02	1.02

positive, contribution to redox balance, and result in mainly 3HV. The correlation in Eq. (2) corresponds to the use of CH to balance the negative redox contribution from acetic acid transformation to 3HB, while the absence of any correlation with 3HV implies that cell redox balance during propionate conversion does not require the same amount of reducing equivalents. It is notable that the higher PHA yield from acetic acid (Table 1) revives the possibility that Hood and Randall's (2001) hypothesis based on negative redox balance resulting in greater P_{up} might have some validity, though Liu *et al's* (2002) data set did not show the increased yield under long term cultivation and implied PHA form rather than redox balance was significant.

Aerobic transformations

Analysis of aerobic transformations shows that acetic acid/3HB also resulted in greater aerobic P uptake (Table 3; note that all PHA was degraded by the end of aerobiosis). However there was no significant change compared to the behavior of the control with respect to net P removals. In each reactor polyP degradation and synthesis was significantly increased compared to the control reactor due to the additional VFAs added. But the additional release and uptake always offset each other (see $P_{uptake}/P_{release}$ ratio in Table 3).

Table 4 shows that net CH formation seemed to correspond with Ace/Pro and 3HB/3HV just as CH_{deg} did (Table 2), although the values were low compared to background CH and the trend was not statistically significant. Aerobic CH formation did not show this trend, however, since the initial CH content at the start of aerobiosis was very different in each reactor due to the differences in CH_{deg} shown in Table 2.

Linear regression of aerobic transformations (absolute values including control reactor) yielded more complex models than anaerobic transformations since there were many more independent variables and interactions to investigate. In order to make the models more illustrative of the fundamental driving forces of aerobic transformations, models regressed without interactions are shown here. However the models regressed with interactions show essentially the same thing.

Table 3 Aerobic P uptake and P uptake/P release ratio (relative to Control Reactor 1)

Reactor	Acetic fraction of total VFA (mmol l^{-1}-C/ mmol l^{-1}-C)	Propionic fraction of total VFA (mmol l^{-1}-C/ mmol l^{-1}-C)	3HB (mmol l^{-1}-C/gMLSS)	Total PHA (mmol l^{-1}-C/gMLSS)	Aerobic P Uptake (mmol l^{-1}-P/ gMLSS)	Aerobic P uptake/ anaerobic P release (mmol l^{-1}-P/ mmol l^{-1}-P)	Net P[1] removal (mmol l^{-1}-P/ gMLSS)
2	0.99	0.01	1.04	1.19	0.35	1.00	0.00
5	0.81	0.19	0.75	1.05	0.33	1.00	0.00
4	0.57	0.43	0.49	0.94	0.30	1.01	0.00
3	0.38	0.62	0.26	0.82	0.27	1.01	0.00

[1] Net removal relative to the control. All reactors, including the control, showed extremely robust net P removals of 0.12 mmol l^{-1}-P/gMLSS

Table 4 Aerobic CH tranformations (relative to Control Reactor 1)

Reactor	3HB (mmol l^{-1}-C/gMLSS)	Total PHA (mmol l^{-1}-C/gMLSS)	Aerobic P uptake (mmol l^{-1}-P/gMLSS)	Glycogen formation (mmol l^{-1}-C/gMLSS)	Net glycogen formation (mmol l^{-1}-C/GMLSS)
2	1.04	1.19	0.35	+0.12	–0.06
5	0.75	1.05	0.33	+0.11	–0.02
4	0.49	0.94	0.30	+0.19	+0.08
3	0.26	0.82	0.27	+0.14	+0.12

$$[P_{up}, \text{mmol } l^{-1}\text{-P/gMLSS}] = 1.144\,[P_{rel}, \text{mmol } l^{-1}\text{-P/gMLSS}] + 0.049\,[CH_{deg}, \text{mmol } l^{-1}\text{-C/gMLSS}] \quad (3)$$
$$R^2 = 0.96;\ F_{observed} = 161.5 > F_{significance} = 2.1 \times 10^{-9}$$

Eq. (3) is a surprising one in that all PHA terms, CH_{net}, and CH_{form} dropped out of the regression. A very similar model was obtained when interactions were included. The model in (3) is very robust with the F statistic indicating a much stronger model than was obtained in Eqs (1) and (2) for anaerobic transformations.

A similar regression analysis of Liu *et al.*'s (2002) data relative to the control showed that P_{up} correlated with 3HB, 3HV, and P_{rel} (Eq. (4)). Glycogen data was obtained with a method having much more significant error than the method in the current study so no statistically significant correlations for CH data could be obtained for Liu *et al.*'s (2002) data. The correlation in Eq. (4) was much poorer than that of Eq. (3), but also showed the importance of the preceding anaerobic P release on driving subsequent aerobic P uptake. Further examination of Liu *et al.*'s (2002) data showed that propionic acid produced more PHA and had a higher P_{rel} than isovaleric, and also had a higher P_{up}. Valeric acid made more PHA than isovaleric but had a lower P_{rel}, and had a lower P_{up}. This emphasizes the dependence of P_{up} on P_{rel} even more than PHA quantity or form.

$$[P_{up}, \text{mmol } l^{-1}\text{-P/gMLSS}] = 0.354\,[P_{rel}, \text{mmol } l^{-1}\text{-P/gMLSS}] + 0.187\,[3HB, \text{mmol } l^{-1}\text{-C/gMLSS}] + 0.122\,[3HV, \text{mmol } l^{-1}\text{-C/gMLSS}] \quad (4)$$
$$R^2 = 0.74\ F_{observed} = 28.6 > F_{significance} = 8.5 \times 10^{-9}$$

Kinetically increased P_{up} due to greater PHA formation and P_{rel} can be understood by referring to the rate equation for storage of poly-P in Filipe and Daiggers (1998). The high X_{PHA} and low X_{PP}, plus high S_{PO4}, caused by high acetic acid/3HB, drives a much higher rate of poly-P biosynthesis according to their model. The correlation of P_{up} with P_{rel} may imply that aerobic P_{up} is driven largely as a response to prior poly-P depletion. A review of the data from this and Liu *et al.*'s (2002) study shows that a key characteristic of VFAs yielding the highest P_{up} (e.g. acetic or isovaleric acid which both led to 3HB formation) was that they also drove the highest P_{rel}. Thus, in addition to Pup driven by PHA biodegradation, at least part of P_{up} (poly-P biosynthesis) may be due to biochemical regulation (maybe similar to poly-P accumulation after P starvation; Hardoyo *et al.*, 1994) in response to low intracellular poly-P levels.

The presence of poly-P biosynthesis as a response to poly-P depletion can also be seen by contrasting VFA driven P_{rel} with inorganic acid/base driven P_{rel}. In the synthetic wastewater study (Liu *et al.*, 2002) pH adjustment experiments were conducted both with and without VFA addition. P_{rel} and P_{up} consistently increased as the pH was lowered in 33 separate experiments ($R^2 = 0.55$ and 0.64 respectively; $F_{obs} = 38.4$ and 56.1 vs. $F_{sig} = 7.1 \times 10^{-7}$ and 1.9×10^{-8}). Note this was Prel driven by pH change from inorganic acid/bases and not the VFA→PHA driven P_{rel} of Smolders *et al.* (1994). Linear regression of the P data driven by inorganic acids/bases yielded Eq. (5) (relative to control reactor; $R^2 = 0.34$; $F_{obs} = 16.5$ vs. $F_{sig} = 3.1 \times 10^{-4}$).

$$[P_{uptake}, \text{mmol } l^{-1}\text{-P/gMLSS}] = 0.20\,[P_{release}, \text{mmol } l^{-1}\text{-P/gMLSS}] \quad (5)$$

Inorganic acid/base addition resulted in P_{rel} that did not stimulate very strong aerobic P_{up} compared to VFA driven P_{rel}. However it is notable that it did induce a significant increase in P_{up}, even though PHA content and form was entirely insensitive to changes in pH. This implies that there was some mechanism (presumably biochemical regulation) which induced aerobic P_{up} simply as a response to prior poly-P degradation.

Regression analysis of CH transformations during aerobiosis yielded very complex models when interactions were included. Eq. (6) shows the best model for regression without interactions. CH_{form} was dependent on the preceding CH_{deg}, again probably due to biochemical regulation or "memory". In addition 3HB rather than 3HV is significant, probably because 3HB correlated with higher CH_{deg} (Table 2).

$$[CH_{form}, \text{mmol } l^{-1}\text{-C/gMLSS}] = 0.697\,[CH_{deg}, \text{mmol } l^{-1}\text{-C/gMLSS}] + 0.207\,[3HB, \text{mmol } l^{-1}\text{-C/gMLSS}] \quad (6)$$
$$R^2 = 0.58;\ F_{observed} = 8.97 > F_{significance} = 4.15 \times 10^{-4}$$

The results of linear regression, particularly Eq. (3), implied PHA biosynthesis that is not accompanied by P release can be detrimental to P uptake. This seems to reflect the observation that even biomass that does not involve poly-P to a large extent still contains a large quantity of PHA (and glycogen) in anaerobic/aerobic systems. This is consistent with the hypothesis of Satoh *et al*. (1992) concerning the possibility of PHA/glycogen metabolism for GAOs that does not involve poly-P. Also, Punrattanasin *et al*. (2002) noted that the CH content of biological nutrient removal (BNR) processes are much higher than conventional, aerobic only, activated sludge systems. In this study control reactors had high quantities of anaerobic PHA formation and CH_{deg}, but much lower involvement of polyphosphate (control $P_{rel} = 0.35$ mmol l^{-1}-P/gMLSS vs. 0.7 in reactor 2; control $P_{up} = 0.47$ vs. 0.82). In all cases control P transformations were only 50% vs. reactor 2. In contrast control PHA was 75% of reactor 2 values at 3.34 mmol l^{-1}-C/gMLSS vs. 4.54 (thus yielding the 1.20 relative value in Table 1). Initial control CH (and all reactors at time zero) was 2.98 mmol l^{-1}-C/gMLSS, with CH_{deg} ranging from 0.56 mmol l^{-1}-C/gMLSS in the control to 0.74 in reactor 2. This indicated that PHA and CH data could also be significantly influenced by phenomena other than VFA driven EBPR. Anaerobic PHA formation does not inherently lead to EBPR if it is being formed inside competing populations, and this may explain the superior correlation with P release (i.e. involvement of polyP, which is unique to the PAOs) rather than PHA for P_{up}. It seems likely that anaerobic/aerobic sequencing selects for a population that stores PHA (and to some extent glycogen), while VFA drives selection for the subset of this population which also stores poly-P.

Conclusion

- Increases in influent propionic acid content resulted in higher 3HV, lower P release, and lower glycogen degradation during anaerobic transformations. Higher net glycogen formation occurred under aerobic conditions. The lower P release resulted in lower aerobic P uptake, but net P removal was not affected.
- Aerobic P uptake correlated more strongly with preceding anaerobic P release than with PHA forms, but P_{rel} was influenced by acetic acid (and thus 3HB) rather than propionic acid (and 3HV).
- Key features of acetic acid resulting in higher P uptake than propionic acid was that its transformation to 3HB resulted in greater P release than propionates transformation to 3HV. This implies that the poly-P/ATP requirement for acetate $\rightarrow$ 3HB was higher, presumably due to differences in PMF dissipation during transport, activation requirements for acetyl groups versus propionyl and acetyl groups, and ATP requirements during transformation to PHA.

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References

Abu-Ghararah, Z.H. and Randall, C.W. (1989). A proposed model for the anaerobic metabolism of short-chain fatty acids in enhanced biological phosphorus removal systems. *Research Journal WPCF*, 61, 1729–1730.

Felipe, C.D.M. and Daigger, G.T. (1998). Development of a Revised Metabolic Model for the Growth of Phosphorus-Accumulating Organisms, *Water Environment Research*, **70**(1), 67–79.

Hardoyo, K.Y., Muramatsu, A., Anbe, Y., Kato, J. and Ohtake, H. (1994). Molecular Genetics of Polyphosphate Accumulation in *Escherichia coli*, in *Phosphate in Microorganisms*, American Society for Microbiology, Washington, D.C., U.S.A.

Hood, C. and Randall, A.A. (2001). A Biochemical Hypothesis Explaining the Response of Enhanced Biological Phosphorus Removal Biomass to Organic Substrates, *Water Research*, **35**(11), 2758–2766.

Liu, Y.-H., Geiger, C. and Randall, A.A. (2002). The Role Of PHA Form In Determining The Response Of Enhanced Biological Phosphorus Removal Biomass To Volatile Fatty Acids, *Water Environment Research*, **74**, 57.

Louie, T.M., Mah, T.J., Oldham, W. and Ramey, W.D. (2000). Use of Metabolic Inhibitors and Gas Chromotography/Mass Spectrophotometry to Study Poly-β-Hydroxyalkanoates Metabolism Involving Cryptic Nutrients in Enhanced Biological Phophorus Removal Systems. *Water Research*, **34**(5), 1507–1514.

Naik, R.V. (1999). Enhancement of Denitrification Using Prefermenters in Biological Nutrient Removal Systems, Masters Thesis, University of Central Fla., Orlando, FL.

Punrattanasin, W., Randall, C.W. and Randall, A.A. (2002). The Utilization of Activated Sludge Polyhydroxyalkanoates for the Production of Biodegradable Plastics, *Poster at the IWA 3rd World Congress*, Australia, April, 2002.

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 process. *Water Science and Technology*, **26**(5–6), 933–942.

Satoh, H., Ramey, W.D., Koch, F.A., Oldham, W.K., Mino, T. and Matsuo, T. (1996). Anaerobic Substrate Uptake by the Enhanced Biological Phosphorus Removal Activated Sludge Treating Real Sewage, *Water Science and Technology*, **34**(1–2), 9–16.

Shah, R.R. (2001). Study of the Performance of Biological Nutrient Removal Systems With and Without Prefermenters, Masters Thesis, University of Central Fla., Orlando, FL.

Smolders, G.J.F., van der Meij, J., van Loodesrecht, M.C.M. and Heijnen, J.J. (1994). Model of the Anaerobic Metabolism of the Biological Phosphorus Removal Process: Stoichiometry and pH Influence, *Biotechnology and Bioengineering*, **43**, 461–470.

Smolders, G.J.F., van der Meij, J., van Loodesrecht, 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, *Biotechnology and Bioengineering*, **47**, 277–287.

VanMunch, E. (1998). *DSP Prefermenter Technology Book*, Science Traveller International, CRC WMPC Ltd., Brisbane, Old., Australia.