Anaerobic Phosphate Release from Activated Sludge with Enhanced Biological Phosphorus Removal. A Possible Mechanism of Intracellular pH Control

Philip L. Bond, Jürg Keller, Linda L. Blackall

Advanced Wastewater Management Centre, Departments of Chemical Engineering and Microbiology, The University of Queensland, Brisbane, 4072, Australia; telephone: +61 7 3365 4645; fax: +61 7 3365 4726; e-mail: blackall@biosci.uq.edu.au

Received 17 March 1998; accepted 2 November 1998

Abstract: The biochemical mechanisms of the wastewater treatment process known as enhanced biological phosphorus removal (EBPR) are presently described in a metabolic model. We investigated details of the EBPR model to determine the nature of the anaerobic phosphate release and how this may be metabolically associated with polyhydroxyalkanoate (PHA) formation. Iodoacetate, an inhibitor of glycolysis, was found to inhibit the anaerobic formation of PHA and phosphate release, supporting the pathways proposed in the EBPR metabolic model. In the metabolic model, it is proposed that polyphosphate degradation provides energy for the microorganisms in anaerobic regions of these treatment systems. Other investigations have shown that anaerobic phosphate release depends on the extracellular pH. We observed that when the intracellular pH of EBPR sludge was raised, substantial anaerobic phosphate release was caused without volatile fatty acid (VFA) uptake. Acidification of the sludge inhibited anaerobic phosphate release even in the presence of VFA. From these observations, we postulate that an additional possible role of anaerobic polyphosphate degradation in EBPR is for intracellular pH control. Intracellular pH control may be a metabolic feature of EBPR, not previously considered, that could have some use in the control and optimisation of EBPR. © 1999 John Wiley & Sons, Inc. *Biotechnol Bioeng* **63**: 507–515, 1999.

Keywords: phosphorus removal; activated sludge; metabolic model; pH regulation

INTRODUCTION

For the biological removal of phosphorus (P) from wastewater, an activated sludge process known as enhanced biological phosphorus removal (EBPR) is used. In the design of EBPR systems, there are various operational configurations that basically comprise some version of an anaerobic/aerobic treatment of the wastewater (Toerien et al., 1990). While there are many full-scale EBPR activated sludge plants in operation worldwide, optimal P removal is not always acheved. Considerable effort has focused on understanding the biochemical nature of EBPR in activated sludge systems, although details of the metabolic pathways involved are still incomplete.

Biological models to explain the characteristic anaerobic and aerobic biochemical transformations that occur in EBPR have been proposed largely from measurements in mixed culture activated sludge systems. Organisms that accumulate phosphorus in the form of polyphosphate are selected for in these systems. The transformations occurring under the anaerobic conditions are thought to be critical for the selection of polyphosphate accumulating organisms (PAO) in these anaerobic/aerobic systems. In the anaerobic stage, a considerable amount of phosphate is released by the sludge, and biological models link this metabolically with the events of intracellular polyphosphate degradation, polyhydroxyalkanoate (PHA) accumulation, and carbohydrate (glycogen) utilisation (Arun et al., 1988; Satoh et al., 1992; Smolders et al., 1994; Wentzel et al., 1991). It is thought that this energy production from polyphosphate degradation provides PAO with a selective advantage in EBPR. The metabolic link suggested in the models is that polyphosphate degradation provides ATP which is utilised in the production of PHA (Arun et al., 1988; Satoh et al., 1992; Smolders et al., 1994; Wentzel et al., 1991). Mechanisms for the production of ATP from polyphosphate degradation are suggested and include (1) direct catalysis by polyphosphate kinase (Kornberg, 1957; Kulaev and Vagabov, 1983), (2) production of ADP and subsequent ATP by the combined action of polyphosphate:AMP phosphotransferase and adenylate kinase (van Groenestijn and Deinema, 1987), and (3) by the efflux of an uncharged metal–phosphate complex coupled with a proton, resulting in the generation of a proton motive force that could drive production of ATP (van Veen et al., 1994).

In laboratory reactors operating with the anaerobic/aerobic configuration for P removal, sludges have been ob-

Correspondence to: L. L. Blackall

© 1999 John Wiley & Sons, Inc. CCC 0006-3592/99/050507-09

tained that have very poor P removal capabilities. Such sludges show minimal phosphorus compound transformations but carry out the carbon compound transformations thought to be inseparable from EBPR (Bond et al., submitted to Appl. Environ. Microbiol.; Fukase et al., 1984; Liu et al., 1994; Satoh et al., 1994). In P-removing sludge it is thought the ability of PAO to take up volatile fatty acids (VFA) in the anaerobic stage provides the selective advantage for these bacteria in EBPR reactors. However, in these non-P-removing sludges PAO may have no competitive advantage. If these sludges can accumulate PHA anaerobically without phosphate release, then what do the polyphosphate degradation and phosphate release do for PAO?

Recently, it has been observed that anaerobic phosphate release is affected by pH. During the anaerobic uptake of VFA in EBPR sludge, raising the mixed liquor pH resulted in increased phosphate release (Liu et al., 1996b; Smolders et al., 1994). It is suggested that the extra phosphate release results from the bacteria requiring more energy at the higher pH for uptake of the acetate anion (Smolders et al., 1994). In contrast to the observations above, it has also been hypothesised that the anaerobic release is a consequence of intracellular acidification of EBPR sludge, caused by the passive diffusion of indissociated VFA across bacterial membranes (Fleit, 1995).

There is a poor understanding of the metabolic details of EBPR. While it is probable that polyphosphate degradation results in the production of energy in the anaerobic stages, investigations have failed to show that this actually occurs in EBPR sludge. Also, the occurrence of non-P-removing sludges in the anaerobic/aerobic systems obscures the metabolic picture of EBPR. Opposing explanations for the effect of pH on the anaerobic phosphate release are proposed in the literature. However, in our investigations anaerobic phosphate release from the sludge was stimulated by alkalisation of the mixed liquor (Bond et al., submitted to Appl. Environ. Microbiol.), similar to the observations described above (Liu et al., 1996b; Smolders et al., 1994). This study aimed to investigate the nature of the anaerobic events in EBPR sludge to improve the understanding of the anaerobic metabolism. In particular, we investigated the influence of pH on anaerobic phosphate release by EBPR sludge.

MATERIALS AND METHODS

Source of EBPR Sludge

To measure the anaerobic transformations of EBPR, batch experiments were carried out on sludge samples taken from a sequencing batch reactor (SBR) operating in the anaerobic/aerobic arrangement for P removal. The operation and high EBPR performance of the reactor is described in a previous paper (Bond et al., submitted to Appl. Environ. Microbiol.). Samples for the batch experiments were taken from the SBR during a period when extremely high and stable P removal occurred (stage B, from day 145 to day 181) (Bond et al., submitted to Appl. Environ. Microbiol.). During this period, the P content of the mixed liquor suspended solids (Px) measured between 8.5% and 12.5% at the end of the aeration stages.

Anaerobic transformations were also measured directly in the SBR during an earlier period of operation when intermediate P removal performance occurred (Bond et al., submitted to Appl. Environ. Microbiol.). This was performed on day 83 of operation stage A, when the Px measured 4.0%.

Chemical Analyses

Analyses of phosphate and acetate in filtered samples were performed as previously described (Bond et al., submitted to Appl. Environ. Microbiol.). Total phosphorus of the mixed liquor was determined in duplicate 10 mL samples by the sulphuric acid–nitric acid digestion method (APHA, 1992). Mixed liquor suspended solids (MLSS), cellular polyhydroxyalkanoate (PHA), and mixed liquor carbohydrate were all determined as previously described (Bond et al., submitted to Appl. Environ. Microbiol.).

Batch Experimental Setup

Batch tests were used to investigate aspects of anaerobic phosphate release from samples of EBPR sludge over a 2-h period. For each batch test a 110 to 130 mL sample of mixed liquor was taken from the SBR near the end of the aerobic stage, when the cells have accumulated most of the phosphate. After the sludge was gravity settled (5 to 10 min), the supernatant was withdrawn from the sample and the agent affecting phosphate release was added to the supernatant. The effectors used were acetate, diethanolamine, formate, or iodoacetic acid. The pH of the amended supernatant was quickly adjusted to the experimental level and this was added back to the settled sludge. The amended mixed liquor was then placed in a 250-mL Dreschel flask which was gassed with N_2 to expel air and keep the mixture suspended.

At intervals throughout the batch experiment, samples of the mixed liquor were taken by syringe through a supraseal and immediately filtered for phosphate and acetic acid analyses. Mixed liquor samples were taken before the addition of the effector and after the 2-h test, to determine the glycogen utilised and PHA accumulated throughout the anaerobic period. In each flask, the pH was maintained manually by frequent measurement with a pH electrode and adjustment with NaOH or HCl solutions (approximately every 10 min).

RESULTS

To investigate the details of the anaerobic events of EBPR the transformations of phosphate release, PHA production and carbohydrate utilisation were measured in batch samples of P removing activated sludge. The results of the final transformations over the 2-h period for the experi-

Table I. Transformations of phosphate-P, PHA, and carbohydrate measured in batch samples of mixed liquor after 2 h of anaerobic incubation at various pH and with various effectors. Experiments were carried out on different days, and Px is the phosphorus content of the MLSS at the time of the experiment.

Experimental conditions		Phosphate released (mg P/g MLSS)	PHA produced (mg/g MLSS)	Carbohydrate utilised (mg/g MLSS)
	Batch number and effector added			
Experiment 1:	(1) No addition control	1.7	0	2
pH 7.0 ± 0.2,	(2) 5 m*M* acetate	47.7	106	40
Px = 8.5%	(3) 5 m*M* acetate and 5 m*M* iodoacetate	8.8	0	0
Experiment 2:	(1) No addition control	3.8	−7	3
pH 8.5 ± 0.2,	(2) 5 m*M* acetate	67.0	69	36
Px = 8.6%	(3) 100 m*M* DEA	49.0	−7	4
	(4) 5 m*M* acetate and 100 m*M* DEA	60.5	−7	8
Experiment 3:	(1) No addition control	1.0	0	0
pH 6.0 ± 0.2,	(2) 5 m*M* acetate	39.3	86	41
Px = 8.8%	(3) 50 m*M* acetate	16.1	12	3
	(4) 5 m*M* acetate and 50 m*M* formate	16.5	0	3
Experiment 4:	(1) No addition control	1.6	−3	3
pH 6.0 ± 0.2,	(2) 4 m*M* acetate	38.0	76	42
Px = 8.6%	(3) 10 m*M* acetate	17.2	29	18
	(4) 20 m*M* acetate	15.1	26	19
Experiment 5:	(1) pH 6.0 ± 0.2	17.8	17	13
10 m*M* acetate,	(2) pH 6.4 ± 0.2	39.1	51	26
Px = 12.5%	(3) pH 6.8 ± 0.2	59.7	100	40
	(4) pH 7.2 ± 0.2	81.0	135	44

ments are given in Table I. Details of each of these experiments are explained in the text.

Effect of Iodoacetate on Anaerobic Phosphate Release

Initially the existence of a metabolic link between anaerobic phosphate release and PHA accumulation as suggested in EBPR biological models (Wentzel et al., 1991) was investigated. Anaerobic transformations were observed in batch samples of EBPR activated sludge in the presence of iodoacetate over the 2-h period. Iodoacetate is an inhibitor of glycolysis (Webb, 1966), and when carbohydrate utilisation was inhibited in the presence of iodoacetate and acetate, there was no PHA production and phosphate release was severely diminished (Fig. 1 and Table I, experiment 1). When the sludge was incubated with acetate alone, during experiment 1, there was considerable phosphate release as well as carbohydrate utilisation and PHA production. These results support the suggestion in the biological model that the events of phosphate release, PHA accumulation, and glycogen utilisation are metabolically linked.

Effect of Intracellular Alkalisation on Anaerobic Phosphate Release

Previously, the performance of an extremely poor P-removing sludge, labelled the Q sludge, was described (Bond et al., submitted to Appl. Environ. Microbiol.). During that period of SBR operation, it was noticed that when the supernatant pH was permitted to rise in the anaerobic stages, the anaerobic phosphate release increased. This effect was measured in subsequent anaerobic stages of the SBR cycles shortly after the appearance of the Q sludge (on day 83 of operation stage A) (Bond et al., submitted to Appl. Environ. Microbiol.) and the results are shown here (Fig. 2). In the first cycle, the anaerobic pH was permitted to rise, while in the subsequent cycle it was controlled at pH 7.0. More phosphate release into the supernatant was detected in the first cycle, when the pH reached approximately 8.2 (Fig. 2). It is likely that this rise in mixed liquor pH was accompanied by some rise in intracellular pH. This prompted our

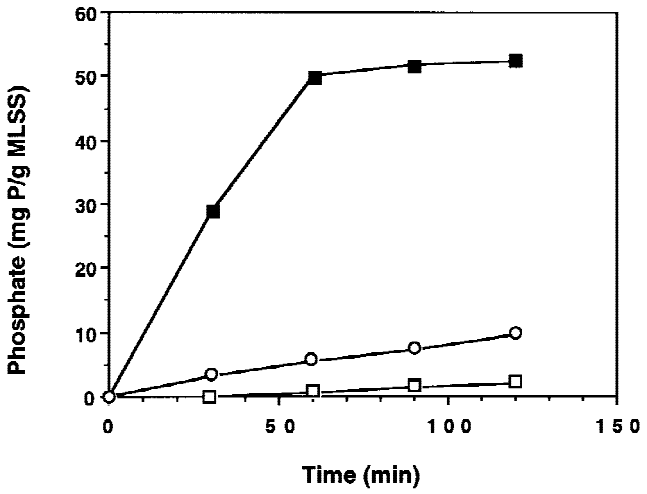

Figure 1. Anaerobic release of phosphate from batch samples of EBPR mixed liquor with a Px of 8.5%, at pH 7.0 ± 0.2, in the presence of no addition control (□), 5 m*M* acetate (■), and 5 m*M* acetate plus 5 m*M* iodoacetate (○).

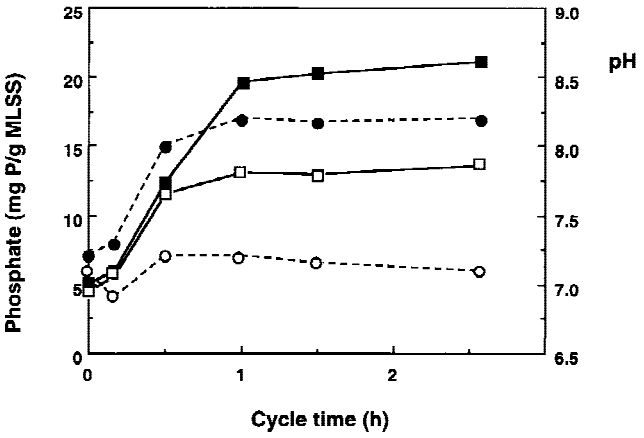

Figure 2. Release of phosphate during subsequent anaerobic stages in the SBR, while there was no pH control (cycle 1, closed symbols), and while the pH was controlled between pH 7.2 and 6.8 (cycle 2, open symbols). This was observed during a period of SBR operation when intermediate P removal occurred, as described in a previous study (Bond et al., submitted to Appl. Environ. Microbiol.). Cycle 1, phosphate-P (–■–) and pH (-●-); cycle 2, phosphate-P (–□–) and pH (-○-).

investigations into the effect of deliberately raising intracellular pH on the anaerobic phosphate release.

To investigate the effect of raising the intracellular pH on anaerobic phosphate release, batch samples of good P-removing EBPR sludge (Px of 8.6%) were incubated in the presence of the weak base diethanolamine (DEA) at pH 8.5. Batch samples taken from the reactor were prepared as described in the methods section and the phosphate release was measured. In the presence of DEA, with or without acetate, and with acetate alone, substantial phosphate release from the sludge was observed (Fig. 3). After 2 h, the release observed with DEA alone was 73% of that observed by incubation with acetate alone. The release with no addition to the sludge was only 6% of the release in the presence of acetate alone. In a shorter time frame, up to 1 h, the phosphate release caused by DEA even exceeded the acetate alone initiated release (Fig. 3). The phosphate release in the presence of DEA occurred without significant utilisation of carbohydrate or PHA production (Table I, experiment 2). Thus, the anaerobic events of EBPR that are suggested to be linked in the biological model could be separated by intracellular alkalisation.

The effect of DEA at various pH values on the anaerobic phosphate release was also investigated (Fig. 4). Large phosphate release only occurred at pH 8.5, while minor release occurred at the lower pH values. Indicating that both the presence of the weak base and elevated pH were required to cause this large phosphate release.

Effect of Intracellular Acidification on Anaerobic Phosphate Release

Experiments were carried out to investigate the effect of lowering the intracellular pH on the anaerobic phosphate release. The phosphate release was detected in batch samples of sludge in the presence of excess amounts of acetic and formic acids at pH 6.0. Significant inhibition (approximately 60%) of the phosphate release was observed in the presence of excess acid, even when the acidification was caused by acetic acid (Fig. 5). Carbohydrate utilisation and PHA production wear nearly completely inhibited by this internal acidification, as shown in Table I, experiment 3. We also observed significant inhibition of phosphate release at concentrations of acetate as low as 10 m*M* (Table I, experiment 4). However, at the concentrations of 10 and 20 m*M* acetic acid, the PHA production and the carbohydrate

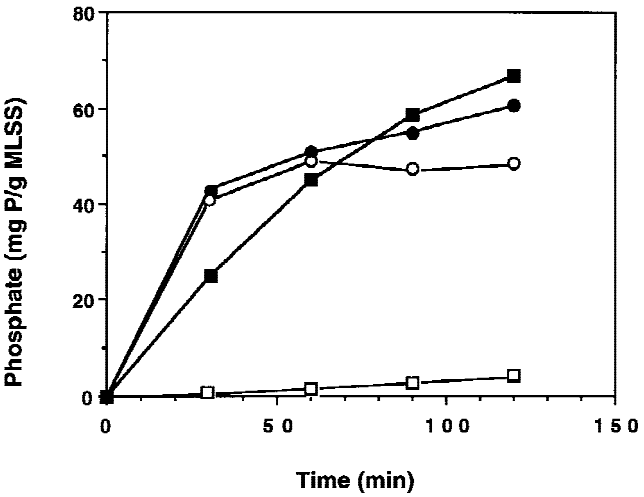

Figure 3. Anaerobic release of phosphate from batch samples of EBPR mixed liquor with a Px of 8.6%, at pH 8.5 ± 0.2, in the presence of no addition control (□), 5 m*M* acetate (■), 100 m*M* DEA (○), and 100 m*M* DEA with 5 m*M* acetate (●).

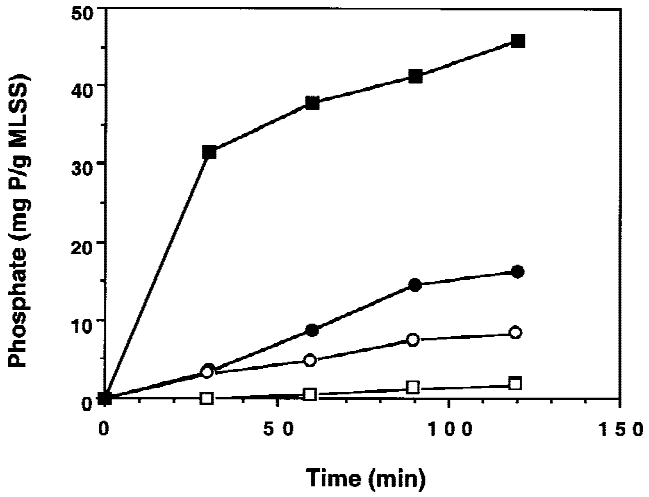

Figure 4. Anaerobic release of phosphate from batch samples of EBPR mixed liquor with a Px of 8.6%, in the presence of DEA at various pH. Release from sample with no addition control (□), 100 m*M* DEA at pH 6.0 ± 0.2 (○), 100 m*M* DEA at pH 7.0 ± 0.2 (●), and 100 m*M* DEA at pH 8.5 ± 0.2 (■).

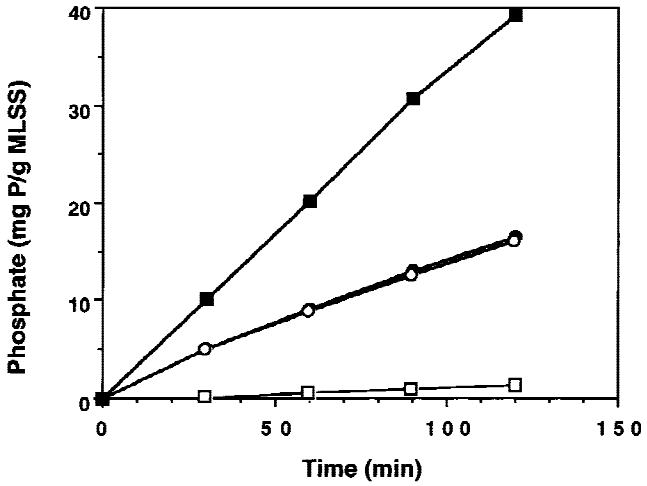

Figure 5. Anaerobic release of phosphate from batch samples of EBPR mixed liquor with a Px of 8.8%, in the presence of acid at pH 6.0 ± 0.2. Release from sample with no addition control (□), 5 m*M* acetate (■), 50 m*M* acetate (○), and 50 m*M* formate with 5 m*M* acetate (●).

utilisation were considerably less inhibited than at 50 m*M* acid (Table I, experiments 3 and 4), while the phosphate release was similar in these cases.

The effect of acidification on the anaerobic metabolism was studied over a range of pH values. Inhibition of the phosphate release was greatest at the low pH values and was proportionally less as the pH increased (Fig. 6). Similarly the inhibition of PHA production and carbohydrate utilisation was greatest at the low pH and this inhibition was less with increasing pH (Table I, experiment 5). These results suggest that the inhibition of the anaerobic processes was a result of internal acidification of the cells, as inhibition occurred only when excess acid was present and the pH was lowered.

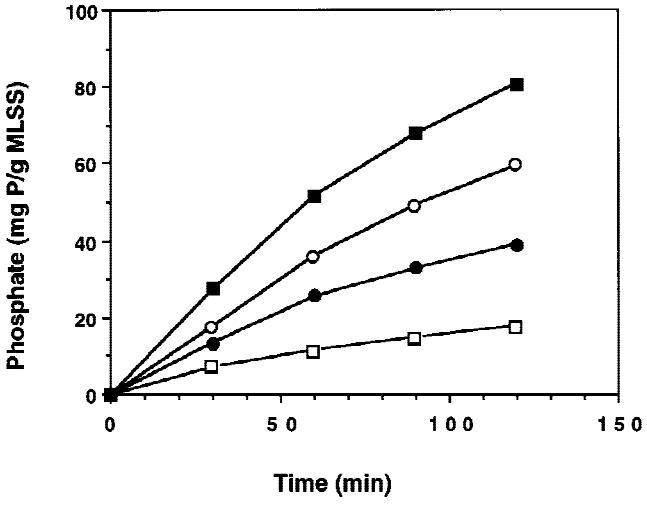

Figure 6. Anaerobic release of phosphate from batch samples of EBPR mixed liquor with a Px of 12.5%, in the presence of 10 m*M* acetate at pH 6.0 ± 0.2 (□), pH 6.4 ± 0.2 (●), pH 6.8 ± 0.2 (○), and at pH 7.2 ± 0.2 (■).

DISCUSSION

Methods to study EBPR

The events that are observed in EBPR activated sludge are very unusual microbiological transformations. According to the biological model, the bacteria responsible for the EBPR process have to be able to store and utilise considerable amounts of polyphosphate, PHA, and glycogen. To date, pure cultures of organisms that perform according to the EBPR model have not been obtained, thus there is a lack of relevant isolates available for investigations into the metabolic details of EBPR. However, details of EBPR can be determined from experiments carried out on enhanced, mixed culture EBPR sludge, which was the approach taken here.

EBPR sludge from the laboratory scale reactor had extremely stable P removal capabilities and a high phosphorus content (Px of up to 12.8%), which is considerably higher than that observed in typical domestic EBPR plants, in which Px may be 3–5%. This demonstrates that the reactor sludge was highly specialised for P removal and should be most appropriate for investigations of EBPR metabolism.

To study the effects of intracellular pH on the anaerobic metabolism of EBPR sludge during the batch tests, the intracellular pH was perturbed by incubating the sludge with excessive amounts of membrane-permeable acids or base. Acidification was achieved with acetic acid or formic acid, and alkalisation was achieved with diethanolamine (DEA). These have dissociation constant (pK_a) values of 4.74, 3.74 (Serjeant and Dempsey, 1979), and 8.88 (Perrin, 1965), respectively. Bacterial intracellular pH has been observed to be manipulated by the addition of such weak acids or weak bases in appropriate conditions (Bakker and Mangerich, 1983; Nakamura et al., 1984; Repaske and Adler, 1981). The permeable nature of membranes to these acids and bases is employed in methods used to measure bacterial intracellular pH (Booth, 1985). At low extracellular pH, the undissociated weak acids, acetic or formic acid, will cross cell membranes and acidify the cytoplasm. At high extracellular pH, the weak base, DEA, will cross cell membranes and raise the cytoplasmic pH.

Biological Models for EBPR

There is strong evidence that the anaerobic carbon compound transformations in the EBPR process are metabolically linked. Nonradioactive ^{13}C-labelled VFA has been observed to be incorporated into PHA in EBPR sludge during anaerobic conditions (Satoh et al., 1992). It is also quite feasible that glycolysis of intracellular carbohydrate (glycogen) provides reducing power and ATP for the production of PHA (Arun et al., 1988; Mino et al., 1987; Satoh et al., 1992). Recent models explaining these anaerobic events have been proposed and the theoretical molar ratios of carbohydrate consumption, PHA production, acetate uptake and CO_2 production correspond well with that observed in

EBPR sludge (Satoh et al., 1992; Smolders et al., 1994). Polyphosphate degradation is also included in these models, as it is suggested that this is linked to the carbon compound transformations. However, details of polyphosphate degradation in EBPR sludge have not been elucidated.

In the batch experiments carried out in this study, incubation of the EBPR sludge with 5 m*M* acetate was used as the positive control for the anaerobic transformations. In all these controls, anaerobic transformations typical of high performance EBPR were detected (Satoh et al., 1992; Smolders et al., 1994). This demonstrates the suitability of the batch experiments for the proposed study.

Iodoacetate is a known inhibitor of glycolysis (Webb, 1966), and we used it to study its effect on anaerobic transformations in the EBPR sludge. The results show that iodoacetate inhibited glycolysis of carbohydrate and, in turn, PHA production and phosphate release were diminished. This confirms that the anaerobic processes of phosphate release and PHA production are linked to the carbohydrate degradation. While this provides further support for the EBPR biological model, no further understanding of the biochemical pathways is gained from this observation. In particular, it is not clear what advantage polyphosphate degradation provides this sludge, as non-P-removing sludges have also been observed to carry out similar carbon compound transformations in the anaerobic stage (Liu et al., 1994; Satoh et al., 1994). Similar inhibiting effects by iodoacetate on these carbon compound transformations have also been detected in non-EBPR sludge (Liu et al., 1994). It is likely that the anaerobic PHA production in non-EBPR sludge is also linked to the glycolysis of carbohydrate.

Alkalisation and Anaerobic Phosphate Release in EBPR Activated Sludge

Other investigators have observed that by raising the extracellular pH in the presence of VFA, an increase in the phosphate release occurs (Liu et al., 1996a; Smolders et al., 1994). This was also seen in experiments described in this study. Progressively more phosphate release occurred as the extracellular pH was increased, in the presence of 5 m*M* acetate (compare the release in Table I, experiments 1, 2, and 3, batch number 2 for each experiment) and 10 m*M* acetate (Fig. 6). It has been speculated that the extra phosphate release was due to the higher energy demand for active uptake of the acetate anion at the higher pH (Smolder et al., 1994). However, other researchers have suggested that acetate uptake occurs by passive diffusion of the undissociated acid (Fleit, 1995). Observations made during operation of the SBR suggest that it is the undissociated acid that is taken up by the sludge biomass (Bond et al., submitted to Appl. Environ. Microbiol.). These suggestions of undissociated acid uptake contradict the above explanation for the pH effect by Smolders et al. (1994). However, higher energy demand for cell maintenance, also suggested by Smolders et al. (1994), is a possible explanation for the observed pH effect. This is discussed further below.

It is possible that intracellular alkalisation may be a metabolic feature of anaerobic phosphate release from EBPR sludge. Our results suggest that raising the intracellular pH alone can cause anaerobic phosphate release of a similar magnitude to that caused by acetate uptake by EBPR sludge. The initial rate of phosphate release by alkalisation is greater than the corresponding reaction with acetate (Fig. 6). It was seen that alkalisation completely inhibits PHA production and glycolysis in the presence of acetate, while causing phosphate release (Table I, experiment 2). This alkalisation was therefore able to completely uncouple the carbon-related reactions (glycolysis and PHA production) from phosphate release in EBPR sludge. It is possible that the anaerobic polyphosphate degradation may be a mechanism for which the cells buffer intracellular pH. Here we discuss two possible mechanisms that cells may use to maintain intracellular pH. These mechanisms are represented in Fig. 7, and they are not necessarily mutually exclusive.

Anaerobic phosphate release caused by intracellular alkalisation has been observed in eukaryotic cells. Some yeast and algae that accumulate polyphosphate have been reported to hydrolyse this in response to alkaline stress under anaerobic conditions (Castro et al., 1995; Pick et al., 1990). In those studies, it is suggested that the polyphosphate hydrolysis is a mechanism to maintain cytoplasmic pH. Polyphosphate hydrolysis generates $H_2PO_4^-$ which has a dissociation constant pK_a of 7.2, and high intracellular concentrations of this would buffer cytoplasmic pH during alkalisation. According to investigations into condensed polyphosphates (van Wazer and Holst, 1950), bacterial polyphosphate (Harold and Harold, 1965; van Groenestijn et al., 1989), and activated sludge granules (Heymann et al., 1989), the suggested structure of activated sludge polyphosphate is that it is a polyanion, negatively charged for each

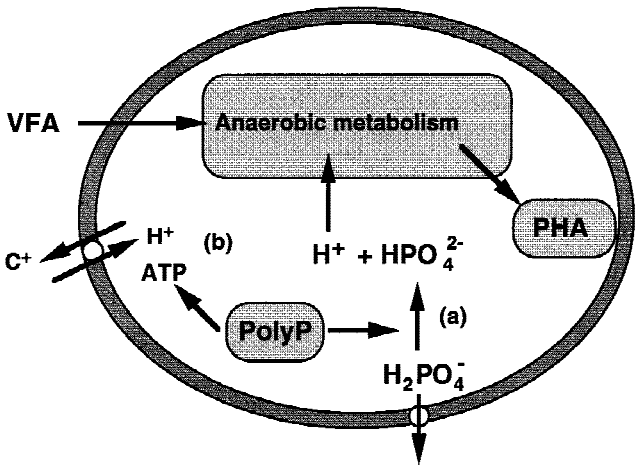

Figure 7. Simple representation of the possible occurrence of anaerobic events in EBPR activated sludge. Anaerobic metabolism of carbon compounds causes alkalisation of cell, while degradation of polyphosphate provides protons to maintain intracellular pH by two possible mechanisms: (a) by direct buffering by phosphate and (b) by providing ATP for energy requiring systems that ultimately use a cation (C^+)–proton antiport.

phosphorus, complexed with Mg^{2+} and K^+ ions. Hydrolysis of polyphosphate with this structure, possibly mediated by a polyphosphatase, would result in the generation of $H_2PO_4^-$ and metal cations. Therefore, the anaerobic polyphosphate degradation in EBPR sludge could result in hydrolysis products that buffer intracellular pH. This is represented as mechanism (a) (Fig. 7).

The other mechanism to maintain intracellular pH, Fig. 7(b), is to use energy, derived from polyphosphate degradation (or phosphate efflux), to drive homeostatic mechanisms. This hypothesis is consistant with the present EBPR biological model, in that the link between the anaerobic carbon compound transformations and polyphosphate degradation is by energy demands, and has been suggested previously (Smolders et al., 1994).

We used the weak base DEA to cause the alkalisation and looked for other effects of DEA on the sludge. It is unlikely that the base was utilised as substrate by the sludge as no carbohydrate utilisation or PHA accumulation was measured in its presence. Considerable cell lysis was not detected during the batch tests by performing cell counts on samples (results not shown). It is possible that the presence of DEA affected the permeability of the cell membrane, causing the large phosphate release (Fig. 3). However, the large phosphate release was not observed at low pH in the presence of DEA (Fig. 4), supporting our postulation that intracellular alkalisation stimulated the phosphate release.

An alternative experimental approach was undertaken to investigate intracellular pH changes during the anaerobic transformations of EBPR sludge. We attempted to directly measure intracellular pH using ^{31}P NMR. However, conducting useful experiments to observe intracellular pH changes is not trivial. An indicator that locates in the cytoplasm and shows shift in the appropriate range is desired. During anaerobic transformations of EBPR sludge, the large flux of intracellular phosphate makes this an unsuitable indicator. We have not yet generated useful data with this approach.

Inhibition of Phosphate Release by Acidification

It has been postulated, but not supported by experimental data, that uptake of VFA causes intracellular acidification and this drives phosphate release (Fleit, 1995). This hypothesis was not supported by data obtained in these experiments. We found that intracellular acidification of the sludge, in the presence of acetate, inhibited the anaerobic phosphate release as well as the PHA production and carbohydrate utilisation.

The anaerobic phosphate release with 4 or 5 m*M* acetate at pH 6.0 was inhibited by approximately 60% when acetate was increased to 10, 20, or 50 m*M* at pH 6.0. However, the inhibition was approximately the same for all the increased concentrations of acid (Table I, experiments 3 and 4). Acidification completely inhibited PHA formation and carbohydrate utilisation, although some phosphate release still occurred. It appeared that the acidification led to leakage of phosphate from the cells, but this is different from the phosphate release caused by PHA formation in EBPR sludge.

The effect of changing the pH in the presence of low levels of acetic acid produced strikingly different levels of phosphate release that appeared directly related to the pH (Fig. 6). This indicates that the inhibition of phosphate release is directly related to intracellular acidification and not just the presence of acetate. The levels of PHA accumulation and carbohydrate utilisation also decreased with increasing acidification, although these changes were not as constant as the decreases in phosphate release (Table I, experiment 5). Further studies are required to identify possible explanations for these observations.

Theoretical Considerations of Phosphate Release and Intracellular pH Control

We hypothesise that anaerobic degradation of polyphosphate is a mechanism of intracellular pH control required to buffer alkalisation caused by anaerobic carbon compound transformations in EBPR. This requires further supporting evidence. However, here we discuss additional aspects of the proposed EBPR metabolic model affecting this hypothesis.

The models proposed for EBPR anaerobic metabolism have been generated by linking known bacterial biochemical pathways with the observed stoichiometry of PHA formation and carbohydrate glycolysis (Arun et al., 1988; Satoh et al., 1992; Smolders et al., 1994). In contrast to the models our hypothesis suggests that polyphosphate degradation may not directly produce ATP. However, ATP production resulting from carbohydrate glycolysis, phosphate efflux, or even from a balance of mechanisms (a) and (b) occurring (Fig. 7), may satisfy cell requirements. Regardless, details of the EBPR anaerobic metabolism are yet to be confirmed in isolated bacteria from EBPR sludge.

Other pathways not yet investigated may be involved in the anaerobic reactions. For example, it is quite feasible in this complex arrangement of metabolic pathways that a proton imbalance may occur. Therefore, one possible explanation of the observed pH effect on phosphate release is that anaerobic PHA production/glycogen utilisation causes intracellular alkalisation. This coincides with the increased phosphate release we detect with increasing intracellular pH, and with the inhibitory effect of acidification on the phosphate release. However, if the anaerobic carbon compound transformations utilize protons, as we postulate, it may be expected that acidification of the cells would permit the anaerobic production of PHA and utilisation of carbohydrate. The results obtained in these experiments did not clearly indicate this. Thus, the effect of pH on anaerobic metabolism in EBPR sludge is likely more complex than represented in this simple model.

It would seem that the present theories of phosphate transport in EBPR are unsupportive to the postulated mechanism of pH control, mechanism (a) (Fig. 7). Although the details of this phosphate transport are not known, a

low-affinity phosphate transporter has been studied in activated sludge isolates and in *Acinetobacter* (van Veen et al., 1994). This transporter would carry a diprotonated metal–phosphate complex (van Veen et al., 1994). This is supported by the anaerobic release of phosphate and cations by EBPR sludge, detected to have a molar ratio of approximately 3:1:1 for P:Mg:K (Wentzel et al., 1991). If the efflux of phosphate in EBPR sludge were of the diprotonated form, this would contradict our postulation that phosphate is acting to buffer intracellular pH. According to mechanism (a) this phosphate species is the buffering anion (Fig. 7). However, phosphate concentrations in the anaerobic stage of SBRs are observed to rise to several millimolar, and if the efflux is merely diffusion-driven then intracellular concentrations would be high and may still be adequate for this to act as an effective pH buffer. Clearly, further investigations are required to determine details of the transport mechanisms of acetate and phosphate, and the postulated pH link between the anaerobic metabolism of EBPR.

Also contradictory to our hypothesis is the possibility that the increased phosphate release detected when raising the pH can be explained by the present EBPR model. As previously mentioned, higher energy requirements by the cell at higher pH could be necessary for the uptake of acetate, the maintenance of pH, or for maintaining proton motive force. Therefore, it may be argued that the increased polyphosphate degradation detected with increasing pH is an artifact not relevant to EBPR metabolism, and the observed dependence can be explained by the current EBPR biological model. However, this current model does not explain the nature of the response we cause by intracellular alkalisation. The phosphate release, in response to the raised intracellular pH, is rapid and immediate. If the cells were not experiencing intracellular pH perturbations, as the EBPR model suggests, it is unlikely that they would have the machinery to respond to the alkalisation in such a rapid manner. For example, if energy were used to maintain the intracellular pH, neutrophiles may do this by activation of sodium–proton (or potassium–proton) antiport systems (Kroll, 1990). Such homeostatic mechanisms would require gene expression, and therefore a longer time scale than observed here, to be effective (Booth, 1985). This could only occur if the cells were experiencing previous intracellular pH stress, as suggested in our hypothesis.

CONCLUSIONS

The present biological model for EBPR activated sludge suggests that the anaerobic events of phosphate release and PHA accumulation are metabolically linked. In the established model, it is proposed that polyphosphate degradation results in production of ATP. This is supported by our observation of the inhibition of the anaerobic processes in EBPR sludge by the glycolysis inhibitor iodoacetate.

Results of this study suggest that the metabolic link between polyphosphate degradation and PHA accumulation is more complex than indicated in the biological EBPR model. Anaerobic phosphate release was caused by intracellular alkalisation, without the occurrence of typical EBPR carbon compound transformations. From the results of this study, a model is postulated suggesting that the anaerobic events of VFA uptake, PHA production, and glycolysis result in a net alkalisation in the cells. On this basis, the model suggests that polyphosphate degradation is a mechanism by which the cells maintain intracellular pH in the anaerobic conditions.

These findings provide a possible new concept in the proposed anaerobic metabolism of EBPR. This has ramifications on the current mechanism proposed in the EBPR biological model, by which PAO maintain a selective advantage in EBPR activated sludge systems. Clearly, further investigations are required to corroborate the model that is postulated here. Ultimately, this information may prove useful in developing strategies to ensure that PAO are selected for in EBPR reactors.

This work was funded by the CRC for Waste Management and Pollution Control Ltd., a centre established and supported under the Australian Government's Cooperative Research Centres Program. We thank Dr. Al McEwan for useful discussions on microbial physiology.

References

APHA. 1992. Standard methods for the examination of water and wastewater. 18th ed. In: Greenberg AE, Clesceri LS, Eaton AD, editors. American Public Health Association, Washington, DC.

Arun V, Mino T, Matsuo T. 1988. Biological mechanism of acetate uptake mediated by carbohydrate consumption in excess phosphorus removal systems. Water Res 22:565–570.

Bakker EP, Mangerich WE. 1983. The effects of weak acids on potassium uptake by *Escherichia coli* K-12. Biochim Biophys Acta 730:379–386.

Booth IR. 1985. Regulation of cytoplasmic pH in bacteria. Microbiol Rev 49:359–378.

Castro CD, Meehan AJ, Koretsky AP, Domach MM. 1995. In situ ^{31}P nuclear magnetic resonance for observation of polyphosphate and catabolite responses of chemostat-cultivated *Saccharomyces cerevisiae* after alkalinization. Appl Environ Microbiol 61:4448–4453.

Fleit E. 1995. Intracellular pH regulation in biological excess phosphorus removal systems. Water Res 29:1787–1792.

Fukase T, Shibata M, Miyaji Y. 1984. The role of an anaerobic stage on biological phosphorus removal. Water Sci Technol 17:69–80.

Harold FM, Harold RL. 1965. Degradation of inorganic polyphosphate in mutants of *Aerobacter aerogenes.* J Bacteriol 89:1262–1270.

Heymann JB, Eagle LM, Greben HA, Potgieter DJJ. 1989. The isolation and characterization of volutin granules as subcellular components involved in biological phosphorus removal. Water Sci Technol 21: 397–408.

Kornberg SR. 1957. Adenosine triphosphate synthesis from polyphosphate by an enzyme from *Escherichia coli.* Biochim Biophys Acta 26: 294–300.

Kroll RG. 1990. Alkalophiles. In: Edwards C, editor. Microbiology of extreme environments. New York: McGraw-Hill, p 55–92.

Kulaev IS, Vagabov VM. 1983. Polyphosphate metabolism in microorganisms. Adv Microb Physiol 24:83–171.

Liu WT, Mino T, Matsuo T, Nakamura K. 1996a. Biological phosphorus removal processes. Effect of pH on anaerobic substrate metabolism. Water Sci Technol 30:25–32.

Liu WT, Mino T, Nakamura K, Matsuo T. 1994. Role of glycogen in acetate uptake and polyhydroxyalkanoate synthesis in anaerobic–aerobic activated sludge with a minimized polyphosphate content. J Ferment Bioeng 77:535–540.

Liu WT, Mino T, Nakamura K, Matsuo T. 1996b. Glycogen accumulating population and its anaerobic substrate uptake in anaerobic–aerobic activated sludge without biological phosphorus removal. Water Res 30:75–82.

Mino T, Arun V, Tsuzuki Y, Matsuo T. 1987. Effect of phosphorus accumulation on acetate metabolism in the biological phosphorus removal process. In: Ramadori R, editor. Biological Phosphate Removal from Wastewaters. Rome, Italy: Pergamon Press. p. 27–38.

Nakamura T, Tokuda H, Unemoto T. 1984. K^+/H^+ antiporter functions as a regulator of cytoplasmic pH in a marine bacterium *Vibrio alginolyticus.* Biochim Biophys Acta 776:330–336.

Perrin DD. 1965. Dissociation constants of organic bases in aqueous solution. London: Butterworths.

Pick U, Bental M, Chitlaru E, Weiss M. 1990. Polyphosphate-hydrolysis: A protective mechanism against alkaline stress. FEBS Lett 274:15–18.

Repaske DR, Adler J. 1981. Change in intracellular pH of *Escherichia coli* mediates the chemotactic response to certain attractants and repellents. J Bacteriol 145:1196–1208.

Satoh H, Mino T, 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. Water Sci Technol 26:933–942.

Satoh H, Mino T, Matsuo T. 1994. Deterioration of enhanced biological phosphorus removal by the domination of microorganisms without polyphosphate accumulation. Water Sci Technol 30:203–211.

Serjeant EP, Dempsey B. 1979. Ionisation constants of organic acids in aqueous solution. Oxford: Pergamon Press.

Smolders GJF, van der Meij J, van Loosdrecht MCM, Heijnen JJ. 1994. Model of the anaerobic metabolism of the biological phosphorus removal process: Stoichiometry and pH influence. Biotechnol Bioeng 43:461–470.

Toerien DF, Gerber A, Lötter LH, Cloete TE. 1990. Enhanced biological phosphorus removal in activated sludge systems. Adv Microb Ecol 11:173–230.

van Groenestijn JW, Bentvelsen MMA, Deinema MH, Zehnder AJB. 1989. Polyphosphate-degrading enzymes in *Acinetobacter* spp. and activated sludge. Appl Environ Microbiol 55:219–223.

van Groenestijn JW, Deinema MH. 1987. The utilization of polyphosphate as an energy reserve in *Acinetobacter* sp. and activated sludge. In: Ramadori R, editor. Biological Phosphate Removal From Wastewaters. Rome, Italy: Pergamon Press. p 1–6.

van Veen HW, Abee T, Kortstee GJJ, Pereira H, Konings WN, Zehnder AJB. 1994. Generation of a proton motive force by the excretion of metal phosphate in the polyphosphate-accumulating *Acinetobacter johnsonii* strain 210A. J Biol Chem 269:29509–29514.

van Wazer JR, Holst KA. 1950. Structures and properties of condensed phosphates. I. Some general considerations about phosphoric acids. J Am Chem Soc 72:639–644.

Webb JL. 1966. Enzyme and metabolic inhibitors. New York: Academic Press.

Wentzel MC, Lötter LH, Ekama GA, Loewenthal RE, Marais GVR. 1991. Evaluation of biochemical models for biological excess phosphorus removal. Water Sci Technol 23:567–576.