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COD BALANCES IN BIOLOGICAL NUTRIENT (NITROGEN AND PHOSPHORUS) REMOVAL ACTIVATED SLUDGE SYSTEMS

JOHN B. COPP

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AUTHOR: John B. Copp, B.Sc. (McMaster University)
M.Eng. (McMaster University)

SUPERVISOR: Professor P. L. Dold

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ABSTRACT

This research set out to investigate reported anomalies in the calculation of COD balances in biological nutrient (nitrogen and phosphorus) removal (BNR) activated sludge systems. For non-BNR systems, accurate COD balances are consistently attainable from experimental measurements. That is, influent COD can be accounted for in the effluent flow, waste sludge stream, and mass of oxygen utilised for carbonaceous oxidation. For BNR systems, in a number of instances where COD balances have been performed, the balances do not close. That is, the sum of the COD leaving the system is as much as 20% less than the COD entering the system, and thus there is an apparent COD 'loss'. In attempting to explain the problem, a laboratory based experimental program was developed which isolated specific areas of interest within BNR systems. The study involved two main experimental phases.

In the first experimental phase, a closed denitrification assay technique was developed. This assay technique allowed specific aspects which have relevance to be evaluated: the nitrate-to-oxygen conversion factor, and the yield of activated sludge organisms under anoxic conditions. The advantages of the closed assay were that all of the needed COD balance terms were independently measurable, and the denitrification system was sealed which isolated the assay environment from the surrounding environment and minimised the effects of external inputs (i.e. oxygen transfer from the air to the liquid).

In the second experimental phase, an excess biological phosphorus removal (EBPR) system was operated to investigate the influence of the influent COD to phosphorus ratio on EBPR biological activity and COD balance calculations. The EBPR system was a laboratory–scale sequencing batch reactor (SBR) which was

extensively monitored over an eight month period. This allowed for the investigation of several factors such as, the rate of anaerobic phosphorus release and COD uptake, the ratio of anaerobic phosphorus released to COD taken up, and the rate of aerobic phosphorus uptake. The system also allowed for the calculation of COD balances.

The main body of this thesis is presented as a series of five papers. The first paper (Chapter 3) presents a study on the nitrate-to-oxygen conversion factor for denitrification which theoretically is 2.86 gO_2/gNO_3-N. That is, when nitrate replaces oxygen as electron acceptor, the mass of nitrate reduced can be converted to oxygen equivalence - for purposes of COD balancing - through the use of this conversion factor. Calculations from a series of denitrifying experiments resulted in an observed conversion factor of 2.96 gO_2/gNO_3-N. However, the observed factor could not be distinguished statistically from the theoretical value, hence confirming the theoretical factor.

The second paper presents a study of sludge production under anoxic (denitrifying) and aerobic conditions. Decreased sludge production and COD 'losses' are confounded in nutrient removal activated sludge systems. Hence this portion of the investigation was initiated to separate these two influences. Batch tests were performed under anoxic (denitrifying) and aerobic conditions using various organisms. The results show that COD balances were achieved, but differences in sludge production under the two conditions indicate a difference in true yield between the environments. An anoxic yield of 0.402 mg particulate COD / mg consumed COD was determined and compared with an observed aerobic yield of 0.645 mg particulate COD / mg consumed COD. These results dispel the assertion that less sludge production results because of COD 'losses'. That is, decreased sludge production in BNR systems can at least in part be explained by a lower yield during unaerated periods.

The third and fourth papers (Chapters 5 & 6) present the results from the EBPR system. The third paper concentrates on the results from the SBR and the influence of the various influent COD to phosphorus ratios. Five different influent ratios were investigated ranging from 8 to 98 (mgCOD/mgP) using a synthetic feed with acetate as the sole carbon source. The phosphorus content of the waste sludge increased from 4 to 17 per cent of the total solids as the influent COD:P ratio decreased. However, complete anaerobic COD uptake was observed irrespective of the phosphorus content of the sludge. Also, COD balances on the reactor averaged 1.04 indicating no apparent COD 'loss' in the system. The fourth paper presents the results of batch tests performed on the waste sludge from the SBR. Anaerobic batch tests were designed to determine the influence of the SBR steady state influent COD to phosphorus ratio on the kinetics and stoichiometry of the system's microbial community. In particular, the rate of substrate uptake increased from 104 to 211 mgCOD/gVSS/hr as the influent ratio decreased. Similarly, the rate of phosphorus release increased from 17 to 166 mgP/gVSS/hr as the influent ratio decreased. The batch test results showed that the ratio of COD taken up to phosphorus released under anaerobic conditions varies with the phosphorus content of the sludge. The observed release ratio increased from 0.17 to 0.79 mgP/mgCOD as the phosphorus content increased.

The final paper (Chapter 7) presents a theoretical biochemical model for EBPR systems. The proposed model makes a distinction between glycogen accumulating organisms (GAOs) and polyphosphate accumulating organisms (PAOs) but suggests that significant populations of both types of organisms exist in most EBPR systems. That is, it is proposed that only in systems that are stressed does one type of organism dominate the mixed community. The biochemical models of Comeau *et al.* (1986) and Wentzel *et al.* (1986) were merged with the biochemical model of Satoh *et al.* (1994) to form a

comprehensive model for EBPR system activity. The two-organism model provides several advantages over single organism models including the ability to predict variable rates of reaction and a variable anaerobic COD uptake to phosphorus release ratio.

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GLOSSARY OF TERMS AND SYMBOLS

BNR	Biological Nutrient Removal
COD	Chemical oxygen demand
DO	Dissolved oxygen
EBPR	Excess biological phosphorus removal
EMP	Emden-Meyerhoff-Parnas (glycolytic pathway)
GAO	Glycogen accumulating organism
$M_{COD,denit}$	Mass of COD oxidised through denitrification
$M_{COD,o}$	Initial mass of COD
$M_{COD,t}$	Mass of COD at time,t
$M_{NO2,o}$	Initial mass of nitrite
$M_{NO2,t}$	Mass of nitrite at time,t
$M_{NO2produced}$	Mass of nitrite produced through denitrification
$M_{NO3,o}$	Initial mass of nitrate
$M_{NO3,t}$	Mass of nitrate at time,t
$M_{NO3removed}$	Mass of nitrate denitrified
N	Nitrogen
NADH	Nicotinamide adenine dinucleotide
ORP	Oxidation reduction potential
OUR	Oxygen utilisation rate
P	Phosphorus
PAO	Polyphosphate accumulating organism
PHA	polyhydroxyalkanoate
PHB	poly – β – hydroxybutyrate
PHV	poly – β – hydroxyvalerate
PMF	Proton motive force
Pv	Phosphorus content of sludge as a percentage of volatile solids

Px	Phosphorus content of sludge as a percentage of total solids
SBR	Sequencing batch reactor
SCVFA	Short chain volatile fatty acids
TCA	Tricarboxylic acid
TEM	Transmitting electron microscope
TKN	Total Kjeldahl nitrogen
TP	Total phosphorus
TSS	Total suspended solids
VSS	Volatile suspended solids
Y	Yield

CHAPTER ONE

INTRODUCTION

1.1 INTRODUCTION

Recent interest in the quality of surface waters has resulted from an increased public environmental awareness. Emphasis has been placed on remediation of existing problem areas and strict regulations have been proposed to eliminate discharges that have the potential to affect water quality. Eutrophication is one of several conditions which causes poor quality in surface waters and generally occurs when waters have high nutrient (nitrogen and phosphorus) levels. Control strategies to limit nutrients in receiving waters have concentrated primarily on point source contributors such as wastewater treatment facilities. Biological control of nutrient discharges from activated sludge systems has evolved over the years and now includes such processes as nitrification (nitrate production from ammonia), denitrification (nitrogen removal) and excess biological phosphorus removal (EBPR). Of particular interest to this investigation is the biological control of nitrogen and phosphorus discharges from activated sludge wastewater treatment facilities.

For those facilities that discharge into waters which are not nitrogen sensitive, there is no need to eliminate nitrogen from the effluent. However, these facilities must avoid causing toxicity in the receiving water due to un-ionised ammonia. To eliminate the toxicity associated with high effluent ammonia, biological nitrification is most commonly utilised. Nitrification is the biologically mediated oxidation of ammonia to nitrate which can be carried out by both autotrophic and heterotrophic organisms (van Loosdrecht and Jetten, 1998). Although

nitrification reduces effluent toxicity, it does not reduce the total nitrogen discharged from the treatment facility.

For those facilities that discharge into nitrogen sensitive streams, reduction or elimination of nitrogen in the effluent has been the aim. Biological nitrogen removal (denitrification) is the biologically mediated process through which nitrate is reduced to nitrogen gas, thereby removing it from the liquid stream. Denitrification occurs when nitrate is utilised as a terminal electron acceptor, and was thought to occur only in the absence of oxygen. However, recent findings suggest that aerobic denitrification also can occur (van Loosdrecht and Jetten, 1998). Nevertheless, in activated sludge, aerobic denitrification is thought to have a minimal impact on nitrogen removal. In practise, denitrification is attained through including unaerated zones within the activated sludge process.

Excess biological phosphorus removal (EBPR) also is a biologically mediated process used within activated sludge systems to achieve phosphorus removal from wastewater. The process involves cultivating within the mixed community, microorganisms (termed polyphosphate accumulating organisms – PAOs) which have the ability to take up more phosphorus than they require for growth. The net effect of this uptake is a reduced wastewater concentration to a level of residual phosphorus which can be less than 1mg/L.

Experience has shown that significant BNR activity does not occur in strictly aerobic systems. Rather, BNR behaviour is achieved by incorporating an unaerated zone into the process design. For denitrification, an anoxic stage (nitrate present, no oxygen) is included and for EBPR, an anaerobic stage (neither nitrate nor oxygen present) must be included in the reactor configuration. One consequence of BNR activated sludge systems with unaerated zones is an apparent 'loss' of influent chemical oxygen demand (COD). Analyses have shown

this 'loss' to be as much as 20% of the influent COD for EBPR systems with both nitrogen and phosphorus removal and slightly less for denitrifying systems (Barker and Dold, 1995). Several recent studies on denitrification have demonstrated COD 'losses' (McClintock *et al.*, 1988; Power *et al.*, 1992; Smyth, 1994).

The phenomenon of COD 'loss' is of interest from both a theoretical and a practical point of view.

- On a theoretical basis, COD balances often are used as a means to validate experimental data. The implicit assumption is that the balance should close. That is, it should be possible to account for input COD in various output terms. The fact that balances on EBPR systems do not close points to a deficiency in understanding of system behaviour. For example, the reported balance calculations may be overlooking an output term such as COD in evolved or stripped gasses [Wable (1992) investigated this aspect, and concluded that this accounted for only a minimal amount of COD]. Another possibility would be that the balance calculations incorporate flawed assumptions. For example, the assumed nitrate-to-oxygen conversion factor of 2.86 gO_2/gNO_3-N may be incorrect.

- On a practical basis, a consequence of COD 'loss' is a reduction in both sludge production and oxygen demand. These two aspects impact treatment plant costs significantly (both capital and operating costs). Therefore, a full understanding of the mechanisms causing the observed 'loss' may provide the means for maximising the 'loss', thereby optimising costs. Regarding the reduced sludge production specifically, this characteristic of EBPR systems appears to have attained quite wide acceptance. In addition, in certain instances reduced sludge production has been assumed to directly confirm

COD 'loss'. This is not the case necessarily. For example, it may be that the reduction reflects a decreased true growth yield which is offset by increased electron acceptor consumption. If this were the case, the COD balance may still close. Therefore, data for evaluating potential COD 'losses' should include information on both sludge production and electron acceptor consumption.

Experimental studies into the EBPR process have culminated in the development of a number of biochemical models (Comeau *et al.*, 1986; Wentzel *et al.*, 1986; Mino *et al.*, 1987; Arun *et al.*, 1988; Wentzel *et al.*, 1991; Satoh *et al.*, 1994; Smolders *et al.*, 1994). The original models were based on the use of acetate or acetic acid as a substrate. However, studies conducted since the introduction of the original models have shown that various other short chain volatile fatty acids (SCVFA) can be used as substrates for EBPR organisms (Arun *et al.*, 1989). EBPR organisms sequester substrate anaerobically and store the material as polyhydroxyalkanoates (PHA). It has been postulated that in doing so, the EBPR organisms gain a competitive advantage over other heterotrophs under aerobic conditions. One of the most significant features in all of the EBPR models is the recognition that adenosine triphosphate (ATP) and reducing equivalents (NADH) are required for the anaerobic conversion of SCVFA substrates to PHA. However, each of the proposed models differs in its approach to achieve the production of these compounds. Similarly, each of the models provide different explanations when addressing experimental results. In terms of this study it should be noted that none of the biochemical models address the possibility of COD 'loss'.

1.2 OBJECTIVE

The primary objective of this study was to investigate the phenomenon of COD imbalances in biological nutrient removal (BNR) activated sludge systems. A secondary objective was to evaluate the various biochemical models for EBPR behaviour.

The approach to addressing these objectives was to design experimental systems which would provide data allowing a comprehensive analysis of aspects which impact COD balances in BNR systems. One of the difficulties in performing and interpreting COD balance data from BNR systems is that there are many interactions and linkages between various biological processes. This often confounds the analysis. Therefore, the purpose of the experimental design was to separate the confounding influences as far as possible. Two experimental systems were designed and operated. The objective of the first experimental phase was to evaluate (i) the true growth yield under anoxic as opposed to aerobic conditions, and (ii) the nitrate-to-oxygen conversion factor used in calculating the denitrification term of COD balances. A necessary part of the experimental design was to monitor all the parameters required to perform a rigorous COD balance. This would identify if denitrification is implicated in the COD balance problem.

The objective of the second experimental phase was to operate an EBPR system to (i) identify if excess biological phosphorus removal behaviour is associated with the COD 'loss', and (ii) provide new data for evaluating the biochemical models. In an effort to isolate EBPR activity the experimental design involved minimising the influent nitrogen content so as to reduce the role of nitrate. At the outset it was decided that a sequencing batch reactor (SBR) system should be used rather than a continuous flow EBPR system. The advantages of the SBR

system included: (i) simplified operation and data collection; (ii) obviating the need to control and measure inter-reactor recycle flow rates and composition; and (iii) a simple means for generating dynamic process response data.

Background for this thesis is provided in Chapter 2. Included in this section is background on BNR activated sludge processes and behaviour as well as a short description of the predominant biochemical models for EBPR. Chapter 2 also provides a brief description of COD mass balances and COD 'losses' within BNR systems. The main body of the thesis is presented as a series of five papers (Chapters 3 to 7). Results of the denitrification experiments are presented in Chapters 3 and 4 ("Confirming the Nitrate-to-Oxygen Conversion Factor for Denitrification" and "Comparing Sludge Production Under Aerobic And Anoxic Conditions" respectively). Chapters 5 to 7 present the results from the EBPR sequencing batch reactor as a three part paper series. Chapter 5 presents the results for the reactor and examines the influence of the influent P:COD ratio on the EBPR behaviour. Chapter 6 presents the results from batch tests using the SBR waste sludge and Chapter 7 presents a conceptual biochemical model for the observed behaviour in EBPR systems.

CHAPTER TWO

BACKGROUND ON BIOLOGICAL NUTRIENT REMOVAL IN ACTIVATED SLUDGE SYSTEMS

2.1 INTRODUCTION

Conventional activated sludge systems have traditionally capitalised on the aerobic growth of various microorganisms to facilitate the removal of organic material from wastewater. However, as nitrogen and phosphorus are both essential for microbial growth, nutrients are removed from the wastewater as a consequence of growth. It is generally suggested that a microbial cell can be empirically represented by the chemical formula $C_5H_7O_2N$. Implied in this empirical representation is that 12% of the cell mass is nitrogen. The nitrogen content is approximately four to five times greater than the phosphorus content by weight; that is, phosphorus is approximately 2.5% of the total mass of the cell.

Once incorporated into biomass, nitrogen and phosphorus are removed from the liquid stream *via* the waste sludge. Ultimately, the mass of nutrients incorporated into the biomass and removed from the wastewater is a function of the net growth occurring in the system. This, in turn, is affected by the system configuration and the biomass retention time in the system. However, because municipal wastewater generally contains more nitrogen and phosphorus than are required for growth processes, conventional activated sludge systems do not normally attain low nutrient concentrations (<1 mg/l) in the effluent.

The need to achieve consistently high nutrient removal efficiencies has resulted in various changes over the years to the conventional aerobic suspended growth process; such that today, advanced activated sludge systems frequently are designed for biological nutrient (nitrogen and phosphorus) removal (BNR).

2.2 REACTOR TYPES

A number of BNR activated sludge treatment schemes have developed utilising a variety of configurations and implementing aerated and unaerated zones. Three different types of reactors (aerobic, anoxic and anaerobic) predominate in BNR systems. The reactors are classified as aerobic, anoxic or anaerobic depending on the availability of specific terminal electron acceptors. Under *aerobic* conditions, oxygen is available to the mixed microbial community for use as a terminal electron acceptor. *Anoxic* conditions occur when oxygen is not available and nitrate is substituted as the electron acceptor. Under *anaerobic* conditions, neither oxygen nor nitrate is available and there is no energy yield from the terminal electron transport system.

2.3 BIOLOGICAL DENITRIFICATION

Biologically eliminating nitrogen from wastewater involves implementing conditions conducive to nitrate reduction and nitrogen gas evolution. This is achieved by including an anoxic stage in the treatment design which, due to the lack of oxygen, encourages the biological reduction of nitrate according to the sequence:

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

Denitrifying organisms oxidise readily biodegradable substrate and utilise nitrate as an electron acceptor. Therefore, both the availability of organic substrate and the availability of nitrate impact on biological denitrification. Without a generous supply of available substrate and the associated electrons, the need for an electron acceptor is diminished resulting in less nitrogen removal. The substrate requirements may be met internally from components in the wastewater or externally through the addition of substrates such as methanol. Recognising the need for utilisable substrate, several nitrification/denitrification treatment schemes including pre- and post- denitrification have developed to optimise nitrogen removal.

Pre-denitrification refers to a treatment scheme in which the anoxic denitrification reactor is placed upstream of the aerobic reactor. Nitrate produced through nitrification in the aerobic reactor is recycled to the head of the plant where its reduction is coupled to the oxidation of influent substrate. Post-denitrification refers to a process configuration in which the anoxic reactor is positioned downstream of the aerobic reactor. Nitrification in the initial aerobic tank produces nitrate for denitrification, but carbon removal in the same tank eliminates much of the biodegradable material necessary for denitrification. To overcome this shortcoming, substrate (e.g. methanol) may be added to the anoxic reactor in a stoichiometric amount [approximately 4.5:1 methanol to nitrogen in units of COD:N (Sadick *et al.*, 1998)]. Denitrifying organisms use the methanol as a source of electrons and reduce nitrate to nitrogen gas.

2.4 EXCESS BIOLOGICAL PHOSPHORUS REMOVAL

In a conventional activated sludge system, it is expected that 2.5% of the dry microbial mass will be phosphorus. However, it is well known that some bacterial species in the mixed microbial population of activated sludge can be

induced to accumulate phosphorus in excess of their physiological requirements. The activated sludge process used to achieve this type of removal is called excess biological phosphorus removal (EBPR).

2.4.1 Observed EBPR Behaviour

Figure 2.1 is a simplified schematic representation of the heterotrophic behaviour that occurs during the anaerobic, anoxic and aerobic stages of an EBPR system.

Figure 2.1 shows a University of Cape Town (UCT) process which incorporates an anoxic zone to facilitate denitrification and an anaerobic zone to encourage EBPR behaviour. According to current understanding, the EBPR system is mediated by two distinct groups of heterotrophic organisms - termed EBPR and non-EBPR organisms. The EBPR organisms actually remove the phosphorus from the wastewater while the non-EBPR organisms are believed to play a role in the hydrolysis and fermentation of particulate organic material. The transformation of this complex matter by the non-EBPR organisms results in the production of short-chain volatile fatty acids (SCVFA) which are necessary for EBPR activity.

- Under anaerobic conditions, non-EBPR organisms enzymatically break down both complex soluble and particulate substrates and produce SCVFA. The EBPR organisms sequester the SCVFA and store the substrate as organic polymers (PHA). A significant increase in the wastewater soluble phosphorus concentration occurs during this period due to the release of inorganic phosphate from the EBPR organisms. The mechanism and driving force for this release is not entirely clear although it is apparent that without phosphorus release in the anaerobic zone, excess phosphorus removal will not occur (Lotter *et al.*, 1986).

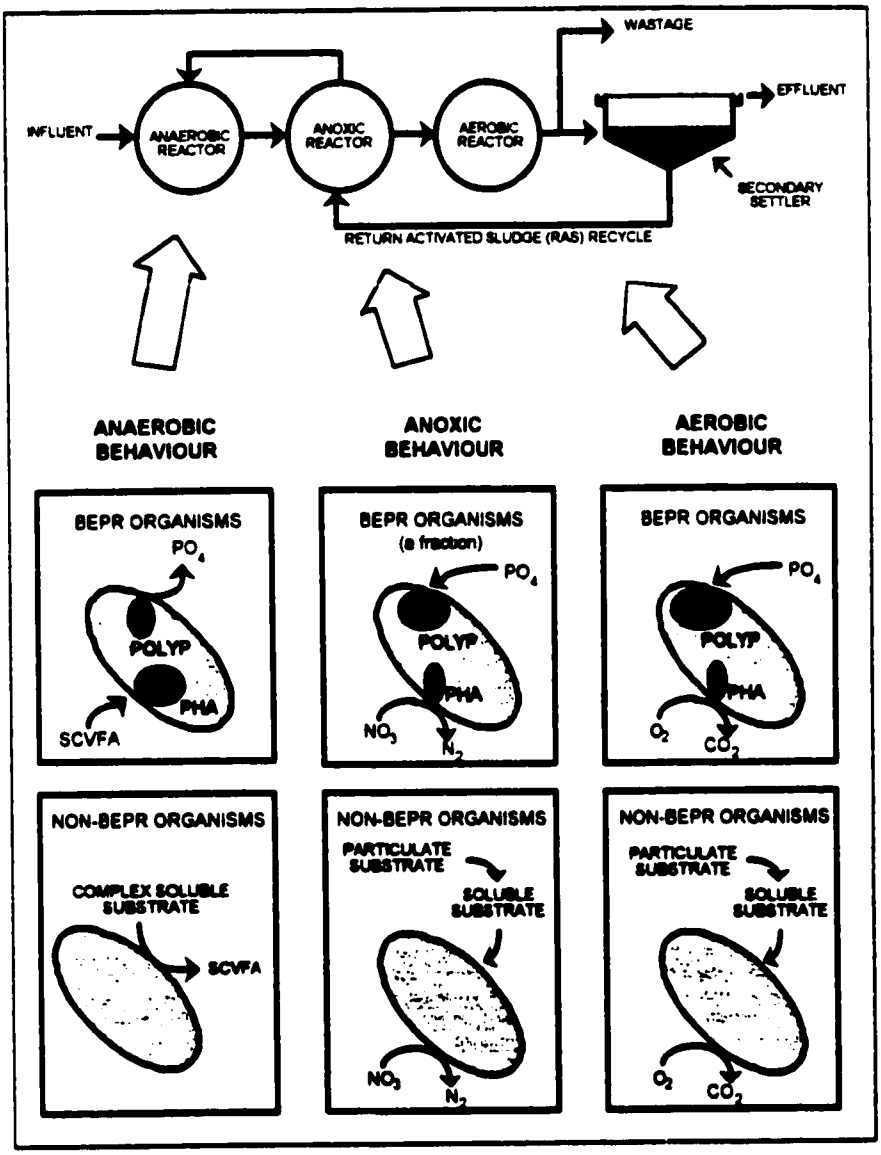

Figure 2.1: Schematic representation of the microbial behaviour in a EBPR system for both EBPR and non-EBPR organisms (Barker, 1995).

- An anoxic zone is not required in a EBPR system even though one is often incorporated into full-scale nutrient removing designs. Under anoxic conditions, it is believed that non-EBPR organisms continue to breakdown particulate material, but the availability of nitrate (acting as an electron

acceptor) during this stage reduces the production of fermentation products and SCVFA. Minimal denitrification was exhibited in enhanced cultures of EBPR organisms suggesting that these organisms are unable to use nitrate as an electron acceptor (Wentzel *et al.*, 1989). However, other findings suggest that the availability of nitrate can induce phosphorus uptake and PHB degradation (Gerber *et al.* 1987, Barker and Dold, 1996). It is apparent from a review of the EBPR literature that at least some EBPR organisms have the ability to utilise nitrate as an electron acceptor. Therefore, in the presence of nitrate, those EBPR organisms that can denitrify take up phosphorus. However, batch tests indicate that phosphorus uptake is less efficient per unit of PHB oxidised under anoxic conditions as compared to uptake under aerobic conditions (Barker and Dold, 1996).

- Under aerobic conditions, hydrolysis of particulate matter by non-EBPR organisms continues and both types of organisms utilise oxygen as an electron acceptor. PHA degradation occurs as the EBPR organisms take up phosphorus and synthesise polyphosphate. By synthesising polyphosphate granules, the cell generates a sink for phosphate energy and a non-toxic phosphorus storage compound. It also has been suggested that polyphosphate accumulation reflects an imbalance in the energy generation and biosynthesis pathways that are ongoing (van Groenestijn *et al.*, 1989).

2.4.2 EBPR Biochemical Models

The complexity of the EBPR process clouds our understanding of experimental observations. However, in an attempt to explain the process, a number of biochemical models have been proposed: the Comeau model (Comeau *et al.*, 1986), the Wentzel model (Wentzel *et al.*, 1986) and the Mino model (Mino *et al.*, 1987). Over the years, various changes to the original models have been proposed, but recognising the need for anaerobic/aerobic cycling and SCVFA

(originally acetate) as a competent substrate for the process has not changed. Each gives theoretical explanations for anaerobic substrate uptake, PHA (originally PHB) synthesis and phosphate release. Also, each provides possible mechanisms for phosphorus uptake and PHA degradation under aerobic conditions. However, there are several notable differences in the models.

Anaerobic Comeau Model

In the Comeau model, acetate is 'transported' into the cell where it is converted to acetyl-CoA, transformed into PHB, and stored within the cell. These processes result in the hydrolysis of high energy phosphate molecules and the release of inorganic phosphate to the bulk medium. Several factors are thought to contribute to the control of these processes including the membrane potential and the concentrations of acetyl-CoA and nicotinamide adenine dinucleotide (NADH). The main anaerobic points described by the Comeau model involve acetate uptake, PHB synthesis, reducing power production and inorganic phosphate release. These points are outlined below:

- The flow of acetic acid into the cell is driven by the pH differential across the membrane. Immediately after entry into the cell, the acid dissociates and the resulting proton dissipates a portion of the proton motive force (pmf) across the membrane. Internal polyphosphate is used as a source of energy to counteract the effects of the acid influx. Because the 'transport' of acetic acid into the cells is thought to be driven by the pH gradient across the membrane, the upper limit to the acetic acid accumulation within the cell is determined indirectly by the amount of polyphosphate that is available to restore the pH gradient and 'allow' more acid uptake.

- Two molecules of acetate are converted to one monomer unit of PHB through a multi-step synthesis pathway. Acetate is first activated to acetyl-CoA

utilising energy from ATP hydrolysis. High concentrations of acetyl-CoA and NADH induce the synthesis of PHB through this mechanism.

- PHB production from acetyl-CoA requires an input of electrons from NADH. Production of NADH for this process is achieved by oxidation of acetyl-CoA through the tricarboxylic acid cycle (TCA). One mole of acetate oxidised through the TCA cycle produces 4 moles of NADH. Hence, for each mole of acetate taken up, 0.11 moles of acetate is diverted to the TCA cycle where sufficient necessary reducing power is produced to convert the remaining acetate to PHB.

- ATP is required for two processes. It is needed to restore the pmf and it is needed in the acetate 'activation' step. The TCA cycle provides some of that phosphate energy and the remainder is provided by the hydrolysis of polyphosphate granules. From polyphosphate, a terminal phosphate is transferred to an ADP molecule resulting in the production of ATP and the capturing of the phosphate energy. The resulting ATP is consumed in the activation step or is used to pump a proton out of the cell *via* a reversal of the ATPase protein. It is postulated that inorganic phosphate accumulates inside the membrane due to the hydrolysis of the ATP and is released from the cell due to the action of an unknown carrier protein which 'senses' the pH gradient. Comeau stated: " With a reduced pH gradient (due to the influx of organic acid), as is the case for bio-P (EBPR) bacteria under conditions of energy limitation, inorganic phosphate could not be used for synthetic processes and would be released from the cell if the intracellular concentration exceeded a certain level." Thus the release of phosphate is not necessary for excess phosphorus removal but is postulated to reflect the rate of polyphosphate hydrolysis.

Anaerobic Wentzel Model

The anaerobic model proposed by Wentzel *et al.* (1986) does not differ significantly from the model proposed by Comeau *et al.* (1986). However, the Wentzel model also describes ion transport mechanisms. Wentzel *et al.* (1986) proposed that acetic acid enters the cell, is transformed into PHB, and stored within the cell. Although the mechanisms used are very similar, some of the driving forces are described differently in each of these models. The main ideas concerning acetic acid uptake, PHB synthesis and phosphorus release are outlined below:

- Acetic acid diffuses into the cell passively without the expenditure of energy and is stored as PHB.

- Synthesis of PHB occurs *via* the pathway proposed by Comeau and requires the reducing energy from NADH. The synthesis of PHB is stimulated by high concentrations of acetyl-CoA and a high NADH:NAD^+ ratio.

- The NADH needed for the production of PHB is produced in the TCA cycle. As in the Comeau model, for each mole of acetate which enters the cell, sufficient NADH is produced by diverting 0.11 moles of acetyl-CoA through the TCA cycle.

- ATP hydrolysis is required for the 'activation' of acetate to acetyl-CoA. The intracellular inorganic phosphate concentration increases due to ATP hydrolysis until it exceeds the physiological requirements of the cell and then is released. The release of phosphorus is mediated through a $H_2PO_4^-$ / OH^- antiport system located in the cell membrane. In addition to phosphate release, a concomitant release of metal ions (Mg^{2+},K^+) occurs though a similar proton antiport system. These mechanisms result in a charge balance such that

one mole of metal associated positive charges is released for each mole of phosphate released.

Like the Comeau model, the Wentzel model concedes that diffusion of acetic acid into the cell increases the proton concentration in the cell and dissipates the pH gradient. However, the Wentzel model postulates that phosphate expulsion (as $H_2PO_4^-$) from the cell restores the proton motive force. The driving force for this phosphate expulsion according to the Wentzel model is the internal phosphate concentration.

Combined Comeau/Wentzel Anaerobic Model

The similarities in the Comeau and Wentzel models allows them to be combined. A schematic representation of the Comeau/Wentzel model is presented in Figure 2.2.

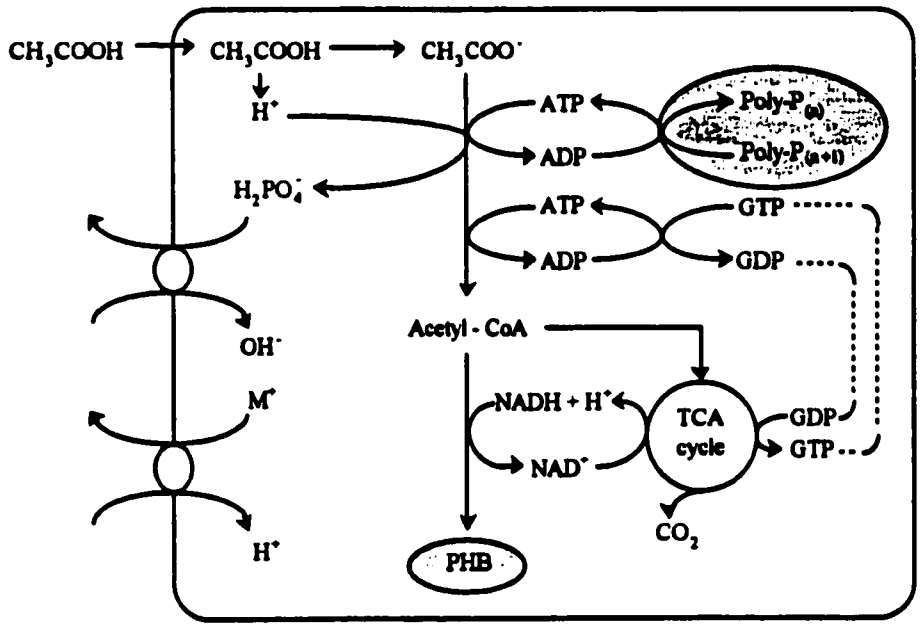

Figure 2.2: Schematic representations of the Comeau/Wentzel model for the anaerobic stage of a biological excess phosphorus removal process.

Anaerobic Mino Model

The biochemical model proposed by Mino *et al.* (1987) and reiterated by Arun *et al.* (1988) to explain the mechanisms of substrate utilisation in the anaerobic stage differs only slightly from those proposed by Comeau and Wentzel. In particular, the Mino model explains observations on changes in intracellular carbohydrates. The main points regarding the fate of acetate, PHB synthesis and NADH production in the Mino model are as follows:

- Acetate is taken up and stored as PHB.

- PHB production occurs through the same series of steps and intermediate compounds as described by the Comeau/Wentzel model, and similarly requires ATP and NADH.

- Glycogen oxidation through the Emden-Meyerhoff-Paranas (EMP) pathway provides both the ATP and NADH necessary for the production of PHB. [Mino *et al.* (1987) found a higher concentration of intracellular carbohydrate in the aerobic zone than in the anaerobic zone of an EBPR system and concluded that carbohydrates in EBPR organisms were being oxidised under anaerobic conditions].

- The Mino model does not specifically address orthophosphate release. However, it is believed that as the intracellular phosphate concentration rises, due to the hydrolysis of ATP and/or polyphosphates, it is released. No mechanism for its expulsion from the cell is offered.

A schematic representation of the anaerobic stage of the Mino model is presented in Figure 2.3.

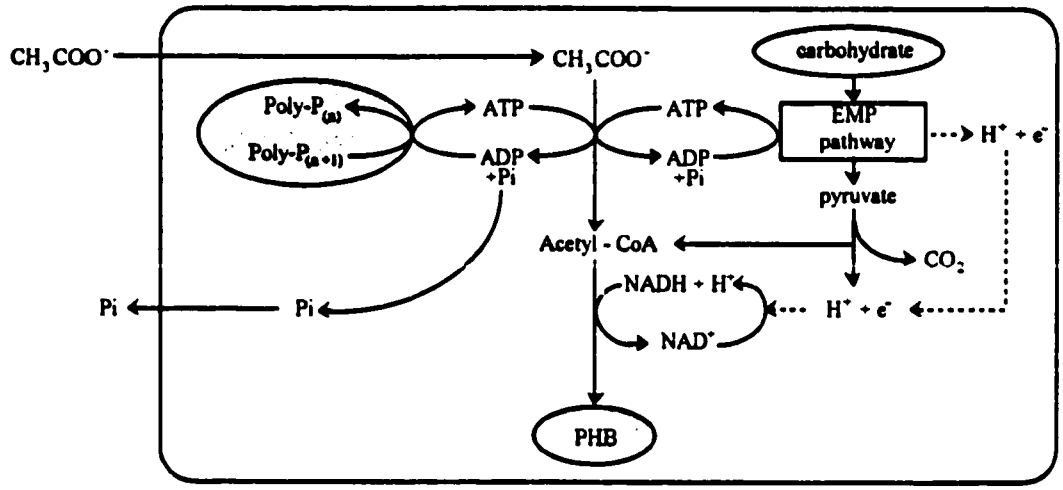

Figure 2.3: Schematic representations of the Mino model for the anaerobic stage of a biological excess phosphorus removal process.

Combined Aerobic Models

Unlike the biochemical models proposed for anaerobic conditions, the aerobic models do not differ significantly from each other and have been combined in this discussion.

All of the models make several assumptions - based on the proposed anaerobic models and experimental observations eluded to above - with regard to the state of the EBPR organisms upon entering the aerobic stage of the process. For instance, it is assumed that little readily biodegradable substrate remains in the wastewater, and that within the EBPR organisms there is a significant storage of PHB and NADH which were synthesised during the anaerobic stage.

- Under aerobic conditions, protons and electrons from the electron carriers (NAD(P)H, $FADH_2$) are transferred to the electron transport system (ETS) where molecular oxygen is reduced to water. The flow of electrons decreases

the internal NADH concentration thereby decreasing the NADH:NAD^+ ratio. As proposed by Wentzel *et al.* (1986), the reduced ratio signals for the degradation of PHB and the production of acetyl-CoA by the mechanism proposed in the Comeau model. A portion of the resulting acetyl-CoA is used in anabolic processes for cell synthesis while another portion enters the TCA cycle where it is oxidised to carbon dioxide and water. Oxidation of acetyl-CoA, coupled to the electron transport system, results in the generation of ATP.

- The proposed mechanisms would normally result in an increase in the ATP concentration causing a slow down in ATP production through feedback inhibition. However, the synthesis of polyphosphate from ATP, in EBPR organisms, decreases the ATP concentration within the cell so that inhibition does not occur. In addition, by generating polyphosphate, a sink for inorganic phosphate is created. Hence, to generate more ATP from ADP, phosphate from the external environment is used. This results in phosphorus uptake and excess phosphorus removal.

- The models agree on most points related to the aerobic stage of the EBPR process. However, the models differ slightly with respect to energy storage compounds. The Comeau/Wentzel model proposes that only polyphosphate is created under aerobic conditions in EBPR organisms. This contrasts the Mino model which predicts that both polyphosphate and glycogen are synthesised under aerobic conditions. The mechanism for glycogen synthesis is not specifically addressed, but carbohydrate synthesis is critical for the proposed anaerobic behaviour.

Figure 2.4 shows a schematic representation of the aerobic stage as proposed by the Comeau/Wentzel and Mino models.

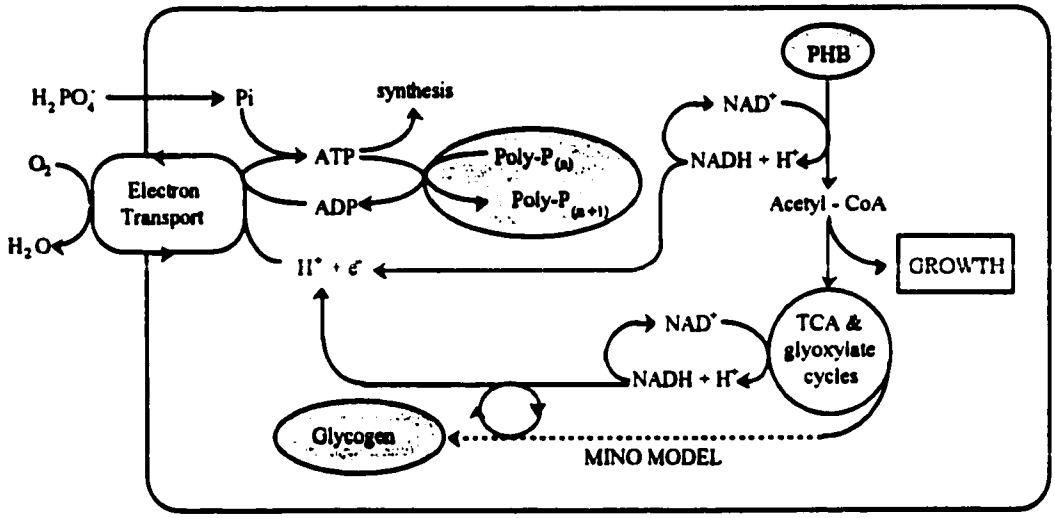

Figure 2.4: Schematic representations of the combined Comeau, Wentzel and Mino models for the aerobic stage of a biological excess phosphorus removal process.

2.5 SCVFA PRODUCTION

EBPR behaviour is induced by incorporating an anaerobic zone into the reactor configuration, yet the presence of an anaerobic stage does not guarantee enhanced phosphorus removal (Cech and Hartman, 1993; Matsuo, 1994; Satoh *et al.*, 1994). However, it is apparent that excess phosphorus uptake by EBPR organisms does not occur in strictly aerobic systems (Lotter *et al.*, 1986). Incorporation of an anaerobic zone into the configuration induces some of the microorganisms in the mixed community to produce intracellular polyphosphate granules. Observations of these granules have shown that they can occupy up to 60% of the cell volume and in activated sludge systems designed for excess phosphorus removal, phosphorus has made up as much as 38% of the VSS mass (Lotter *et al.*, 1986, Wentzel *et al.*, 1989). It is unclear how the anaerobic zone induces EBPR

activity. However, certain biochemical reactions are known to occur in the anaerobic zone and are believed to be essential to the process.

The biochemical models which describe the EBPR process have one common feature and that is the use of SCVFA as substrate for the EBPR organisms in the anaerobic zone. Although it is accepted that SCVFA makes up a small portion of the biodegradable organic matter in municipal wastewater, the majority of the organic matter (approximately 70%) consists of dissolved and colloidal macromolecular material and dispersed fine particulate material. Thus, the raw wastewater provides insufficient SCVFA for EBPR activity. Instead, SCVFA must be synthesised within the system, and it has been postulated that that occurs through fermentation in the anaerobic zone.

2.5.1 Fermentation

It has been established that EBPR systems require an anaerobic zone in addition to an aerobic zone in the reactor configuration. In some cases, an anoxic zone is also included although this is not necessary to induce EBPR activity. The occurrence of the aerobic tank in the system essentially eliminates the possibility that excess phosphorus removal is mediated by strict anaerobes as the aerobic regime would be toxic to these anaerobic bacteria. Instead, the requirement for the anaerobic zone suggests that facultative anaerobes play an important role in the process. However, it does not suggest that EBPR organisms are facultative anaerobes.

Facultative anaerobes are microorganisms that have the ability to alter their oxidative processes depending on the availability of an electron acceptor. During periods of oxygen deprivation these organisms uncouple oxidative phosphorylation and switch to a fermentative metabolism. Fermentation in these organisms results in the production of various organic metabolites through the

activation of metabolic pathways that do not operate under aerobic conditions. These pathways generate energy for the cell and metabolites that are released from the cell as by-products. In the case of EBPR, it is believed that those organisms that can ferment do so to satisfy their energy requirements during anaerobic conditions. The products from fermentation then act as substrates for EBPR organisms.

The property of facultative anaerobiosis distinguishes members of the *enteric* group of bacteria from other Gram-negative groups (Stainer *et al.*, 1976). The *Enterobacteriaceae* family of bacteria include *Escherichia, Salmonella, Shigella, Vibrio, Aeromonas, Enterobacter, Serratia* and *Proteus*. Under aerobic conditions, enteric bacteria utilise organic acids, amino acids and carbohydrates in respiratory metabolism and store reserve carbon material almost entirely through the production of glycogen. Under anaerobic conditions, energy in provided by fermentation of carbohydrates producing a number of small organic acids and ethanol. It is believed that all members of the group have the ability to ferment mono- and polysaccharides as well as polyalcohols. It is likely that facultative anaerobes play an important role in excess phosphorus removal systems and that anaerobic fermentation in these bacteria is key to the process.

Sugar fermentation in enteric bacteria during periods of oxygen deprivation occurs through the EMP pathway and produces pyruvic acid. In some enteric bacteria, further fermentation results in the production of 2,3 butanediol. However, pyruvic acid is most frequently transformed into a mixture of ethanol and organic acids. Such acids include lactic, acetic, succinic and formic to name a few. Another characteristic feature of these bacteria is the ability to cleave pyruvic acid with the addition of coenzyme A to form formic acid and acetyl-CoA. In this pathway, formic acid is the predominant end product. However, some bacteria have the enzyme formic hydrogenlyase which cleaves the formic

acid to carbon dioxide and hydrogen gas. The fermentation pathways are shown in Figure 2.5.

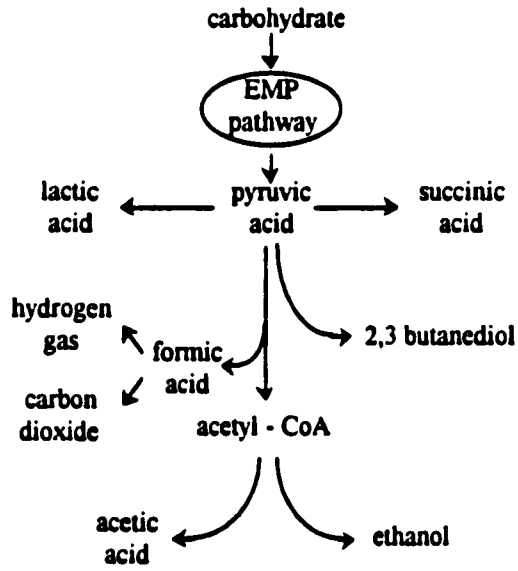

Figure 2.5: Fermentation by-products known to be produced by members of the enteric group of facultative anaerobes.

2.5.2 Metabolite Production

The biochemical models are dependent on the production of SCVFA through fermentation under anaerobic conditions. Numerous authors have suggested that non-EBPR organisms play a critical role in EBPR activity (Brodisch, 1985; Cloete *et al.*, 1985; Wentzel *et al.*, 1985; Lotter *et al.*, 1986). For instance, Brodisch (1985) found that the presence of *Aeromonas* punctata - a facultative anaerobe whose fermentative by-product is acetate from carbohydrate - was required to get excess phosphorus removal, and Wentzel *et al.* (1985) concluded that readily biodegradable material in the influent is converted to lower fatty acids by non-EBPR organisms.

Even though SCVFA have not been detected at appreciable concentrations under anaerobic conditions, mounting indirect evidence provides support for this theory. In particular, results have shown that intermediates of the tricarboxylate acid (TCA) cycle are excreted from aerobic bacteria when grown under anaerobic conditions (Vollbrecht, 1982). The enzymes required for degradation of the intermediates are inhibited by high concentrations of NADH caused by a lack of oxygen. Vollbrecht (1982) used the aerobic bacteria, *Alcaligenes eutrophus*, and showed that during periods of oxygen deprivation various metabolites were released including, acetate, ethanol, formate and hydrogen gas. Extrapolating these results to the anaerobic stage of an EBPR system confirms that the anaerobic stage provides an ideal environment for excretion of a number of these metabolites.

Further evidence in support of SCVFA production is provided by Iversen (1987), who reported that production of metabolites by facultative anaerobes is not limited to periods of oxygen limitation. In cultures of *Klebsiella pneumoniae* excretion of acetate and pyruvate to the bulk liquid was noted with excess glucose under aerobic conditions and oxidation of the pyruvate and acetate only occurred following the exhaustion of the glucose supply.

2.6 COD BALANCES

COD balances can be used to check the validity of experimental data in activated sludge systems. The balances are comprised of input and output COD terms and the balance reflects a comparison between the totals. Although the input COD can be determined directly, the output COD is calculated through the addition of three output terms: total COD in the effluent stream, total COD of the waste stream, and total COD oxidised.

- COD in the effluent and waste streams, like the influent, is determined directly.
- The mass of COD oxidised is indirectly calculated from the mass of electron acceptor consumed. In aerobic systems, this comes from the oxygen utilisation rate (OUR).
- In nitrifying systems, the OUR is a measure of the oxygen used for nitrification and for the oxidation of organic material. As the balance is only concerned with the carbonaceous oxygen requirement, the oxygen utilised for nitrification must be subtracted from the total mass of oxygen utilised.
- In denitrifying systems, nitrate replaces oxygen as electron acceptor for substrate oxidation. This process is included in the balance by converting the reduced nitrate to oxygen equivalents. The mass of nitrate-nitrogen reduced is converted to COD units using a proportionality constant.

The total COD leaving the system is the sum of these three terms and the COD mass balance is calculated by comparing the output COD to the input COD.

2.7 COD 'LOSS'

Much of the research into EBPR has concentrated on the anaerobic zone and its biochemical complexity. However, a much more fundamental problem is of interest to this study. Of particular importance to this investigation is a literature review and COD balance investigation by Barker and Dold (1995). In this review, the authors reported that systems incorporating anaerobic regions exhibited COD imbalances of approximately 20%. That is, less than 80% of the influent COD could be accounted for in the effluent, waste, and oxidised COD streams. The authors performed COD balances on the data of eight strictly aerobic activated sludge systems. The results varied between 98.4 and 100.4% with an average of 99.7%. These results were contrasted with the balances

obtained on 21 aerobic/anoxic/anaerobic systems which showed balances between 60.7 and 89.7% with an average of 78.1%.

Barker and Dold (1995) also examined data from anoxic/aerobic systems (McClintock *et al.*, 1988) and found COD balances ranging from 85-95% with an average of 92%. Although these systems provided better mass balances than those under aerobic/anoxic/anaerobic conditions, there apparently is a significant difference compared to aerobic-only systems, suggesting that COD can be 'lost' under anoxic conditions as well as under anaerobic conditions. This conclusion is supported by the results of other anoxic studies (Power *et al.*, 1992; Smyth, 1994).

Power *et al.* (1992) studied chemical phosphorus removal using alum in systems with two reactors in series (70% anoxic and 30% aerobic). The results indicated that with or without alum, the COD balances were not 100%, but varied between 70 and 85% in the system without alum and 75 and 95% in the system with alum. Excess nitrate was supplied to the anoxic reactors in both systems. This ensured that the reactors remained anoxic and did not become anaerobic. In another study, Smyth (1994) operated a strictly anoxic reactor and also found a COD imbalance. In that study, two sludge ages were tested (9.6 and 7.9 days) and the calculated COD balances were 85% at both retention times. These results indicate that COD 'losses' are prevalent under anoxic conditions as well as anaerobic conditions.

2.7.1 Potential Causes of COD 'Loss'

For COD to be unaccounted for in a COD balance, it likely must be 'lost' in one of three ways. The first and most readily apparent mechanism for COD 'loss' involves the experimental procedures used to determine the amount of COD which exits the system. As stated previously, COD leaving the system is

accounted for in one of three terms (COD of waste, COD of effluent, or COD oxidised) all of which have some degree of experimental error.

The COD of the waste and effluent streams are determined using a closed reflux method. Some volatile organic compounds are not oxidised using this procedure even though they have an associated COD. Thus, the presence of these compounds would be interpreted as a COD 'loss' upon analysis. The only problem with this scenario is that COD 'losses' have been noted in a number of systems under varied operating conditions and with a variety of wastewaters. Therefore, the material not oxidised would have to be unbiodegradable. If it were biodegradable, it would be degraded within the system, resulting in an increased OUR measurement and a closed balance.

The OUR measurements pose other problems as experimental interpretation is inherent in the OUR technique. Nevertheless, the magnitude of the consistently negative COD balances, suggests that the OUR technique is an unlikely sink for the 'lost' COD. Similarly, because the nitrogen balances around these systems are very good, it is not expected that the change in nitrate is in error. Although experimental errors might contribute to the COD imbalances a closer examination reveals that it is unlikely that all of the 'losses' can be accounted for in this manner.

Another potential cause of COD 'losses' is the mathematical assumption used to convert the nitrate-nitrogen denitrified to oxygen equivalents. COD balancing involves converting the mass of nitrate reduced to oxygen equivalents using a proportionality constant, 2.86 gO_2/gNO_3-N. This constant has never been challenged and some consideration should be given to verifying this value experimentally. If for some reason the experimental constant was found to be different from 2.86, alterations to the balance would occur.

The last explanation for the COD 'loss' involves the microbial production of volatile compounds. For instance, many microorganisms in activated sludge can produce a number of reduced gases which have an associated COD (methane, hydrogen gas, gaseous sulphides and volatile organics). The evolution of any of these gaseous compounds from the system would result in a calculated COD 'loss'. Nevertheless, reduced gas production in these systems has not been shown experimentally and as such has not been linked as yet to COD 'losses' (Wable, 1992).

2.7.2 Studies into COD 'Loss'

As described earlier, one known by-product of enteric bacteria fermentations is hydrogen gas. Hydrogen gas has an associated COD and thus its production during an anaerobic stage and emission from the system would result in an apparent COD 'loss'. Tanisho *et al.* (1987) used continuous cultures of *Enterobacter aerogenes* to study hydrogen gas production under fermentative conditions. Extrapolating the results to activated sludge, predicts an expected 'loss' in COD of approximately 8%. These 'losses' are not as great as those predicted by Barker and Dold (1995). However, if hydrogen gas production is the cause of COD 'loss', then influent characteristics and process configuration may impact on observed gas production and overall COD 'loss'.

One specific study attempted to test the hydrogen production hypothesis in activated sludge (Wable, 1992; Wable and Randall, 1992; Wable and Randall, 1994). The investigation was designed to test the off-gasses from the anaerobic stage through the use of a sealed anaerobic reactor. Analysis of the gasses present in the head space and dissolved in the liquid showed negligible hydrogen production. In fact, it was reported that the head space above the anaerobic reactor was under negative pressure, indicating no net gas production at all. In

all, less than 1% of the observed COD 'losses' could be explained through hydrogen production. Although, hydrogen production may be a plausible explanation for a portion of the COD 'loss', as yet no experimental observations have been obtained which confirms this theory and thus it remains only a theory.

2.7.3 Importance of COD 'loss'

In the activated sludge process, where a significant part of the operational costs are associated with aeration and the handling of solids produced in the system, any reduction in aeration requirements or yield of biosolids results in a significant cost savings. The 'loss' of COD accomplishes both of these cost saving results. Also, because there is the potential for significant cost savings, optimising the 'loss' of COD should maximise these savings. To do this, the COD 'loss' phenomenon needs to be thoroughly investigated so that the mechanisms of COD 'loss' can be understood. However, the flow of COD in these biological systems is confounded by a number of factors. Therefore, if this phenomenon is to be understood then studies which separate these confounding factors must be developed.

CHAPTER THREE

CONFIRMING THE NITRATE-TO-OXYGEN CONVERSION FACTOR FOR DENITRIFICATION

This chapter contains the complete text of a paper published in *Water Research*. The full reference is:

Copp J. B. and Dold P. L. (1998) Confirming the nitrate-to-oxygen conversion factor for denitrification, *Water Research*, **32**, 1296-1304.

CONFIRMING THE NITRATE-TO-OXYGEN CONVERSION FACTOR FOR DENITRIFICATION

John B. Copp & Peter L. Dold

Department of Civil Engineering, McMaster University
1280 Main Street West, Hamilton, Ontario, Canada L8S 4L7

ABSTRACT

Numerous studies have demonstrated the phenomenon of COD 'loss' in nutrient removal activated sludge systems incorporating unaerated zones. One potential cause for the calculated 'loss' is in the mathematical assumption made with regards to nitrate reduction in denitrification. In the COD balance calculation a factor is used to convert the amount of nitrate used as a terminal electron acceptor to an equivalent amount of oxygen. The theoretical conversion factor of 2.86 gO_2 per gNO_3-N denitrified is derived from oxidation/reduction half-reactions. Data from twelve 1L denitrification batch tests in a closed reactor system were used to confirm this factor. A value of 2.96 was observed but could not be distinguished statistically from the theoretical value. Interpretation of the data also revealed that no COD was 'lost' in these dominant denitrifier experiments with a simple soluble substrate.

Key words: activated sludge, nutrient removal, COD balances, denitrification, nitrate-to-oxygen equivalents

INTRODUCTION

The phenomenon of chemical oxygen demand (COD) 'loss' in nutrient removal activated sludge systems incorporating unaerated zones has been reported in a number of studies (Power *et al.*, 1992; Wable and Randall, 1992; Smyth, 1994; Barker and Dold, 1995). In contrast, this 'loss' is not observed in aerobic-only systems. For nutrient removal systems with unaerated zones, not all of the influent COD is accounted for in the effluent flow, waste sludge stream, oxygen utilisation and the oxygen equivalence of nitrate-nitrogen reduction. Analysis has shown this 'loss' to be in the range of 20% of the influent COD. A consequence of the 'loss' is reduced aeration requirements and/or biosolids production in nutrient removal systems compared to aerobic-only systems.

Initial concern over COD imbalances was raised in studies on biological excess phosphorus removal (EBPR) systems with anaerobic zones. However, recent analyses on denitrification data also indicate that COD 'loss' may occur in anoxic/aerobic and anoxic-only systems (Power *et al.*, 1992; Smyth, 1994). Barker and Dold (1995) performed COD balances on 21 anaerobic/anoxic/aerobic systems and a series of anoxic/aerobic systems. The balances averaged 78% and 92%, respectively. Although the anoxic/aerobic balances are better than those for anaerobic/anoxic/aerobic systems, the balances do not close. The 'loss' of COD under anoxic conditions has been supported further by various studies using different municipal wastewaters (Power *et al.*, 1992; Smyth, 1994). Power *et al.* (1992) found COD balances ranging between 70 and 95% while Smyth (1994) operated anoxic-only reactors and found COD balances of 85%. It would appear from these studies that COD 'loss' can be attributed to both anoxic and anaerobic conditions.

Figure 3.1 provides a simplified representation of substrate consumption. A portion (Y) of the consumed substrate is converted to microbial mass and the remainder (1-Y) is oxidised to CO_2 and H_2O. For an electron balance, the sum of electrons directed to microbial mass plus the electrons absorbed by the terminal electron acceptor should equal the number of electrons transferred from the substrate. The COD test provides a measure of potential reduction electrons. Hence, for an electron balance:

COD of consumed substrate = COD of microbial mass generated
+ oxygen equivalence of electron acceptor reduced

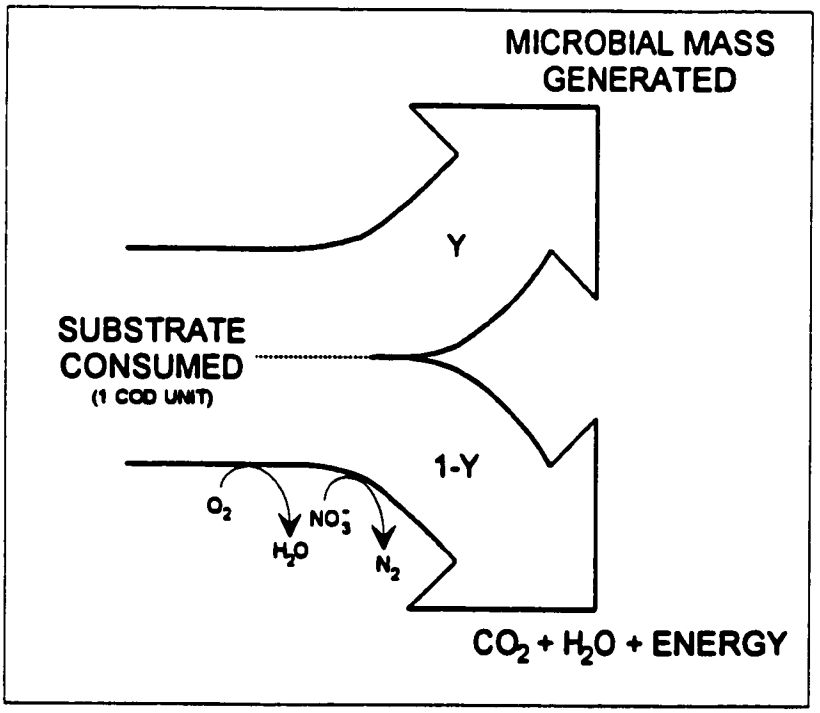

Figure 3.1: Schematic representation of COD flow incorporating microbial mass production, substrate oxidation and electron acceptor reduction.

COD balances for activated sludge systems are comprised of input and output COD mass terms. The output COD is calculated through the addition of three terms and the balance reflects a comparison between the input and output totals. The generalised balance can be written as:

Input COD = COD (unfiltered) in the influent stream

Output COD = COD (unfiltered) in the effluent stream
+ COD in the waste stream
+ amount of COD oxidised (oxygen equivalence of electron acceptor consumed)

$$\text{COD balance} = \left(\frac{\text{Output COD}}{\text{Input COD}}\right) \times 100\% \qquad (1)$$

A number of factors should be noted:

- COD in the influent, effluent and waste streams can be determined directly.
- The term for the amount of COD oxidised is measured indirectly by equating to the oxygen equivalents of electron acceptor consumed. In an aerobic system, this comes from the oxygen utilisation rate (OUR) measurement.
- In nitrifying systems, the OUR is a measure of the oxygen used for nitrification and the oxidation of organic material. As the balance is concerned only with the carbonaceous oxygen requirement, the oxygen utilised for nitrification must be subtracted from the total mass of oxygen utilised.
- In systems incorporating denitrification, nitrate replaces oxygen as the electron acceptor for part of the substrate oxidation. This process is included in the balance by converting the reduced nitrate to oxygen equivalents. To

perform this calculation, the input and output nitrate (and nitrite) concentrations for unaerated reactors must be known.

- The studies on anoxic and anaerobic/anoxic/aerobic systems have demonstrated lesser volatile solids production per unit influent COD compared to aerobic-only systems. Possibly this is a direct consequence of COD 'loss' in the systems incorporating unaerated zones; that is, less COD is available. Based on theoretical considerations, less solids generation might be explained by lower yields under anoxic and/or anaerobic conditions. The literature supports this idea as aerobic yields have been reported to be 40% greater than anoxic yields (Koike and Hattori, 1975). Nevertheless, regardless of the yield, there must be an equivalent amount of electron acceptor consumed to accept the electrons liberated during substrate oxidation and a balance on COD should be attained unless there is some unidentified sink [i.e. $Y + (1-Y) = 1$ in Fig. 1].

There are only a limited number of ways that COD can be 'lost' in these systems. For instance, experimental error which is inherent in any laboratory setting, could be the cause, but consistency in the 'loss' of COD suggests that this is at best a minor contributor. A frequently proposed explanation for the COD 'loss' involves the microbial production of a gaseous compound(s). Evolution of reduced gaseous compounds would result in a COD 'loss' across the system. Many microorganisms in activated sludge may produce a number of reduced gases (methane, hydrogen, gaseous sulphides and volatile organics) but gas production in these systems has not been demonstrated (Wable, 1992; Wable and Randall, 1992; Wable and Randall, 1994) and as such has not been linked, as yet, to COD 'loss'.

An alternative explanation for the calculated COD 'loss' in the systems with unaerated zones simply could be a mathematical artefact if the assumption used to

convert reduced nitrogen to oxygen equivalents were not correct. If the conversion factor was greater than 2.86 gO_2/gNO_3-N (see below) then the calculated balance would be closer to 100% and the 'loss' smaller. In systems incorporating denitrification, the mass of COD oxidised through denitrification can be accounted for by estimating the equivalent amount of oxygen which would have been needed if oxygen had been the electron acceptor instead of nitrate. It has been proposed that denitrification essentially is a four step process (Payne, 1981).

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \qquad (2)$$

Each step may be represented by a half-reaction where e^- denotes electron equivalents (COD) transferred from the organic substrate:

$$2e^- + NO_3^- + 2H^+ \rightarrow NO_2^- + H_2O \qquad (3)$$

$$e^- + NO_2^- + 2H^+ \rightarrow NO + H_2O \qquad (4)$$

$$2e^- + NO + 2H^+ \rightarrow N_2O + H_2O \qquad (5)$$

$$2e^- + N_2O + 2H^+ \rightarrow N_2 + H_2O \qquad (6)$$

The net reaction for complete denitrification is obtained by combining the four equations:

$$10e^- + 2NO_3^- + 12H^+ \rightarrow N_2 + 6H_2O \qquad (7)$$

or equivalently,

$$e^- + \tfrac{1}{5}NO_3^- + \tfrac{6}{5}H^+ \rightarrow \tfrac{1}{10}N_2 + \tfrac{3}{5}H_2O \qquad (8)$$

Similarly, the half-reaction for the reduction of oxygen is:

$$e^- + \frac{1}{4}O_2 + H^+ \rightarrow \frac{1}{2}H_2O \qquad (9)$$

The above two equations imply that the transfer of one electron equivalent requires the reduction of 1/4 mol of oxygen or 1/5 mol of nitrate, i.e.:

$$\begin{aligned} \tfrac{1}{5} \text{ mol nitrate} &\equiv \tfrac{1}{4} \text{ mol oxygen} \\ \tfrac{14}{5} \text{ g } NO_3^- \text{-N} &\equiv \tfrac{32}{4} \text{ g oxygen} \\ 1 \text{ g } NO_3^- \text{-N} &\equiv 2.86 \text{ g oxygen} \end{aligned} \qquad (10)$$

Thus, if $M_{NO3removed}$ represents the mass of nitrate denitrified to nitrogen gas, then the COD oxidised through denitrification is given by:

$$M_{COD,denit} = 2.86 \times M_{NO3removed} \qquad (11)$$

The assumption here is that nitrate denitrified is converted to nitrogen gas (N_2), and that there is no release of intermediates (NO_2, NO, N_2O). If intermediates were released the factor of 2.86 would be lower. For example, if nitrite were produced and the reduction proceeded no further, the overall equation would be written as follows:

$$e^- + \frac{1}{2}NO_3^- + H^+ \rightarrow \frac{1}{2}NO_2^- + \frac{1}{2}H_2O \qquad (12)$$

As above, this reduction would imply that an electron equivalent transfer requires the reduction of 1/4 mol of oxygen or 1/2 mol of nitrate:

$$\frac{1}{2} \text{ mol nitrate} \equiv \frac{1}{4} \text{ mol oxygen}$$

$$1 \text{ g } NO_3^- \text{-N} \equiv 1.14 \text{ g oxygen} \quad (13)$$

Similarly:

$$M_{COD,denit} = 1.14 \times M_{NO2produced} \quad (14)$$

In systems with nitrate disappearance and some nitrite accumulation (but no other intermediate accumulation), the nitrite must be accounted for in the oxidised COD calculation:

$$M_{COD,denit} = 2.86 \times M_{NO3removed} - (2.86 - 1.14) \times M_{NO2produced} \quad (15)$$

which can be simplified as follows:

$$M_{COD,denit} = 2.86 \times [M_{NO3removed} - 0.6 \times M_{NO2produced}] \quad (16)$$

EXPERIMENTAL DESIGN

Consider a batch test in which a soluble organic substrate is combined with a small seed of denitrifying organisms. Assume that the following parameters are measured at intervals: total COD, NO_3 and NO_2. Figure 3.2 shows the type of response that could be expected. Note however, that Figure 3.2 is for illustrative purposes only and shows straight lines where in practice, non-linear responses would be anticipated. In the test, total COD (CODt) measures COD of the residual substrate plus COD of the biomass. Hence, the decline in CODt is a measure of the COD oxidised.

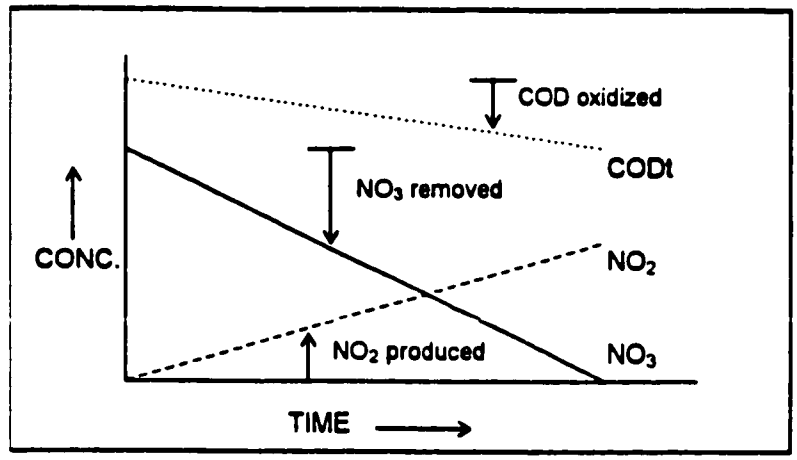

Figure 3.2: Qualitative response in batch denitrification test.

The data collected over the course of the experiment, in conjunction with Eqn. 16, can be used to generate a plot of COD oxidised *versus* electron acceptor reduced. This should have a slope of 2.86 and pass through the origin; that is (where the subscripts o and t represent the initial conditions and the conditions at time t):

$$M_{COD,o} - M_{COD,t} = 2.86 \times [M_{NO3,o} - M_{NO3,t} - 0.6 \times (M_{NO2,t} - M_{NO2,o})] \qquad (17)$$

However, because the masses are calculated by difference from the initial conditions, the error in each calculation becomes the sum of the errors in the initial measurement and the timed measurement. Alternatively, a similar expression, which maintains the absolute error, can be generated by rearranging Eqn. 17:

$$M_{COD,t} = 2.86 \times [M_{NO3,t} + 0.6 \times M_{NO2,t}] + M_{COD,o} - 2.86 \times M_{NO3,o} - 1.72 \times M_{NO2,o} \qquad (18)$$

Using this simplification, data pairs of corrected nitrate values ($M_{NO3,t} + 0.6 \times M_{NO2,t}$) are plotted against the observed total COD at time t and, as the initial

conditions are fixed, analysis should give a slope of 2.86 [but not an intercept of zero].

In using this procedure, two approaches could be used to interpret the results:

- Should the slope not be 2.86, then the results could be interpreted as meaning that the slope represents the true nitrate to oxygen equivalence factor. That is [if there is a nitrogen balance and therefore no accumulation of NO or N_2O intermediates], there is some error in the assumed redox half-reactions. The implicit assumption here is that there is no COD 'loss' from the system.
- On the other hand, one might accept that the redox half-reactions are correct so that the factor 2.86 cannot be changed. In that case, if the observed slope is greater than 2.86, it would imply that COD 'loss' occurs over the course of the experiment. Similarly, however, if the observed slope is 2.86, it would have to be concluded that there is no COD 'loss' occurring in the system.

Although, the equivalence factor has never been challenged, the objective of this study was to design an experimental protocol using a dominant culture of denitrifiers and a soluble substrate (citrate) to test this factor. Aside from testing the factor the experimental approach also would identify or rule out a potential cause of the COD 'loss' observed in activated sludge systems incorporating unaerated zones.

METHODOLOGY

A culture of *Pseudomonas denitrificans* was obtained from American Type Culture Collection, Rockville, Maryland. The freeze-dried culture was revived in the prescribed media and then streaked on Difco Pseudomonas Isolation Agar (PIA) using glycerol as the carbon source. The culture was stored on PIA at 4°C

and transferred monthly to new agar because colony growth was apparent at this temperature.

Culture Medium

Early results (data not shown) suggested that *Pseudomonas denitrificans* growth in reverse osmosis/deionised water (RO/DI) was severely retarded when the media was not supplemented with calcium and molybdenum. As such, over the course of this investigation the culture medium changed several times with respect to these constituents. Of the six RO/DI experiments, three had no molybdenum, and three had molybdenum of 54 μg/L. Similarly, four of these experiments had calcium of 72 mg/L and in the other two there was calcium of 8 mg/L. Note that neither calcium nor molybdenum were added to the tap water experiments.

Table 3.1: Batch test growth media for *Pseudomonas denitrificans*.

	Medium 1		Medium 2	
Autoclaved tap water	1000	mL		
KNO_3	670	mg	670	mg
$(NH_4)_2SO_4$	200	mg	200	mg
KH_2PO_4	63	mg	63	mg
$MgSO_4 \bullet 7H_2O$	50	mg	50	mg
$FeCl_3 \bullet 6H_2O$	1.2	mg	1.2	mg
$Na_3C_6H_5O_7 \bullet 2H_2O$	1000	mg	1000	mg
$CaCl_2$			variable	
$(NH_4)_6Mo_7O_{24} \bullet 4H_2O$			variable	
$CuSO_4$			80	μg
$ZnSO_4$			80	μg
$MnSO_4 \bullet H_2O$			80	μg
Reverse osmosis / deionised water			1000	mL

Assay Technique

Using a sterile platinum loop, 200 mL of medium (Table 3.1) in a 250 mL Erlenmeyer flask was inoculated with a colony from the agar plate. A foam stopper was placed in the flask opening and covered with aluminum foil. Parafilm was then stretched over the neck of the flask. The flask was secured in a reciprocating shaker at room temperature (22°C +/- 2°C) and 65 rpm for between 12 and 48 hours prior to the start of the batch tests. It was not anticipated that the shaker speed would induce a lot of oxygen transfer thus no attempt beyond these measures was made to further exclude oxygen from the culture or air space above the liquid.

Following the initial inoculation period, the contents of the flask were transferred to a 1 L (1.15 L total capacity) round-bottomed three-necked flask which had been flushed with nitrogen gas (Figure 3.3). To the 200 mL of starter culture, 900 mL of newly prepared medium was added. The contents were mixed using a magnetic stir bar and stirring plate, and an initial 20 mL sample was withdrawn. Dissolved oxygen (≈5-7 mg O_2/L) was present initially, but was below a measurable limit within 2 hours after the reactor was sealed. The reactor was sealed at each neck using rubber stoppers with various sized holes to accommodate pH, ORP and dissolved oxygen probes, a sampling port and a gas outlet port. Where the holes could not be made gas tight, a silicone based sealer was used. On occasion the mineral oil manometer showed upwards of a 1 m height differential indicating that the apparatus was gas tight. ORP and dissolved oxygen concentration were monitored continuously and recorded on a plotter. Solution pH (7.5 +/- 0.1) was not recorded but was monitored continuously and controlled using a Cole-Parmer model 5997-20 pH controller attached to a metering pump with a reservoir of 3% phosphoric acid (H_3PO_4). This acid was

selected to avoid peak interference on the ion chromatographic equipment used for anion analysis. Less than 2 mL of acid was added per experiment.

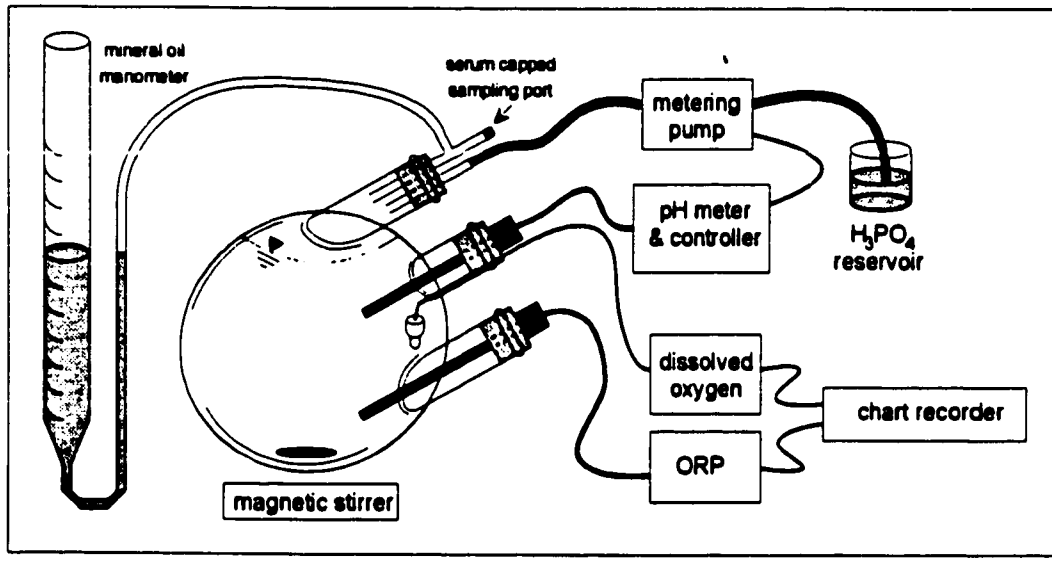

Figure 3.3: Batch test apparatus.

Gas production was monitored using the mineral oil manometer. As gas was produced in the reactor an increase in pressure caused the oil level in the burette to rise. By moving the Tygon, however, the oil levels in the tubing and the burette could be made equal. This method equalised the pressure at the liquid surfaces and thereby maintained atmospheric pressure in the flask headspace and ensured accurate gas production measurements. After such adjustments, the change in oil level in the burette provided a volumetric measure of the gas produced since the previous adjustment (correcting for volume of liquid samples removed).

During batch tests, samples of the liquid were withdrawn when at least 8 mL of gas had been produced or when there was sufficient capacity in the manometer to allow for a sample. Samples were analysed for COD, both total and filtered (0.1 μm). Additionally, filtered samples were injected into a Dohrmann DC-190

carbon analyser for total carbon and inorganic carbon measurements as well as a Dionex Ion Chromatograph model AI450 for anion analysis (nitrite and nitrate). A Technicon Traacs 800 colourometric auto analyser was used to verify nitrite and nitrate values and obtain an ammonia plus ammonium concentration. The number of samples varied between 5 and 8 per experiment depending on the rate of reaction and the volume of gas produced between samplings. The end of the batch tests was signified by a decreased rate of gas production which generally coincided with a drop in ORP.

RESULTS AND DISCUSSION

Twelve batch tests were performed giving three characteristic responses depending on the initial soluble COD:NO_3^- ratio at the onset of anoxic conditions. Figures 4 to 6 show typical results.

High initial COD:NO_3^- ratio (Figure 3.4):

- Nitrate decreased to zero while nitrite first accumulated and then also decreased to zero.
- Soluble COD (CODs) decreased but remained in excess of 200 mg/L at the end of the test.

Intermediate initial COD:NO_3^- ratio (Figure 3.5):

- Nitrate again decreased to near zero. However, nitrite accumulated, but did not decrease appreciably following nitrate disappearance.
- Soluble COD decreased to a level less than 100 mg/L, and then remained relatively constant.

Low initial COD:NO_3^- ratio (Figure 3.6):

- Nitrate decreased to a value of approximately 20 mg/L and very little nitrite accumulated.
- Soluble COD decreased to a level of approximately 30 mg/L.

Several features were characteristic of all of the batch tests including a decrease in total COD (CODt) and ammonium, and an increase in particulate COD (CODp).

As would be expected in batch tests of this type, an increase in the microbial mass was noted during the course of each experiment. It follows that as growth occurs there should be an increase in bacterial activity. Increased activity was observed in the nitrate and soluble COD disappearance profiles as each of these curves showed an increasing slope with time. Similarly, in those experiments where nitrite accumulated, the rate of nitrite appearance increased over the initial phase of the experiments.

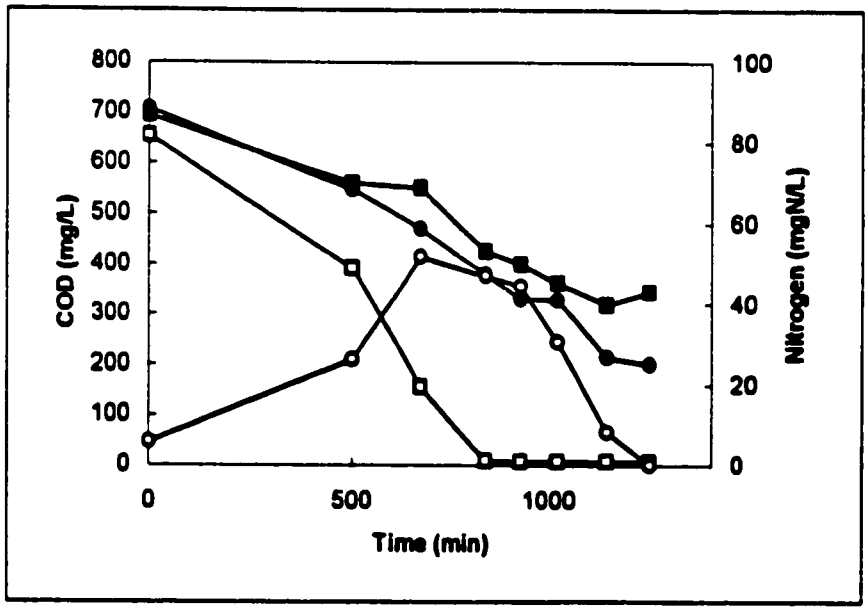

Figure 3.4: Typical result of batch test with high initial COD:NO_3^- ratio [■ CODt, ● CODs, □ Nitrate-N, ○ Nitrite-N].

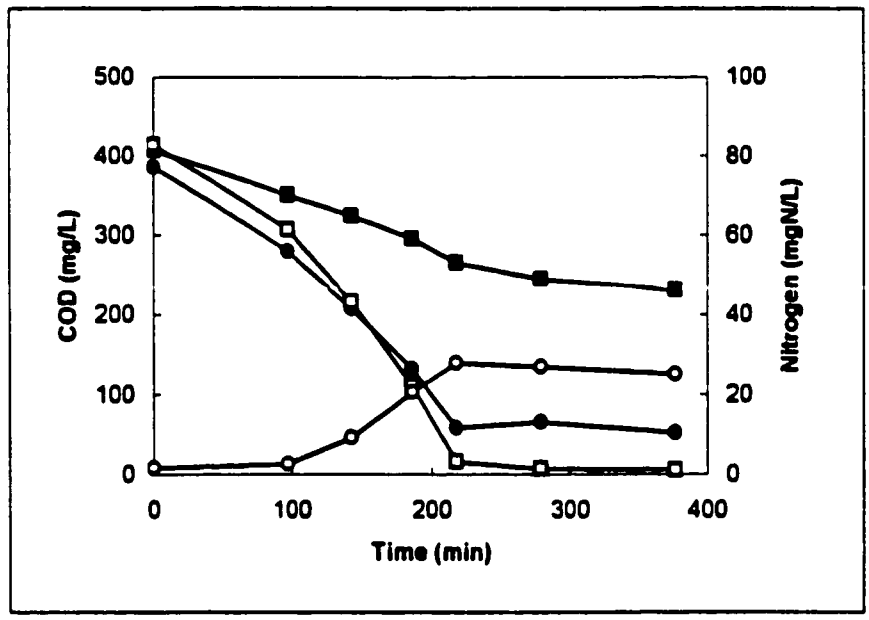

Figure 3.5: Typical result of batch test with intermediate initial COD:NO_3^- ratio [■ CODt, ● CODs, □ Nitrate-N, ○ Nitrite-N].

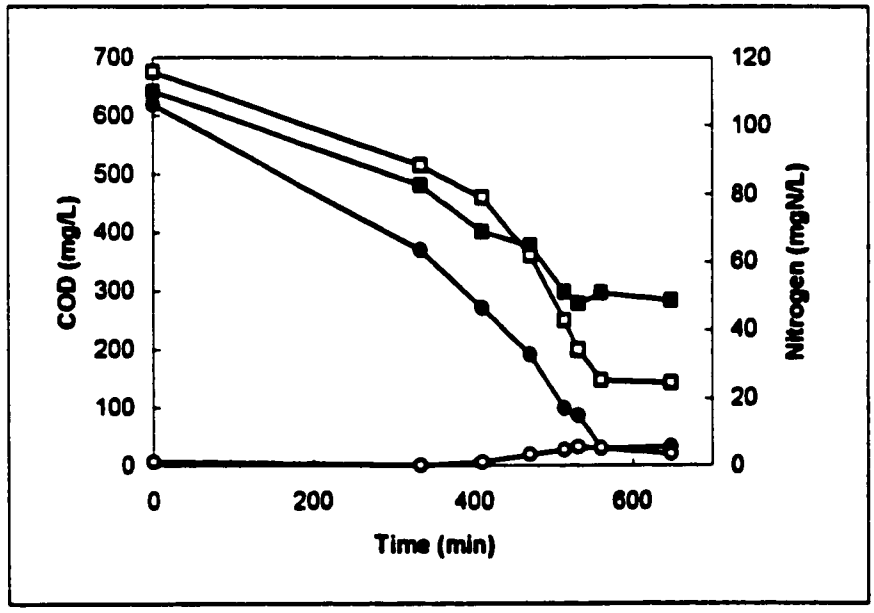

Figure 3.6: Typical result of batch test with low initial COD:NO_3^- ratio [■ CODt, ● CODs, □ Nitrate-N, ○ Nitrite-N].

The data collected in the twelve experiments was used to calculate a number of dependent mathematical relationships but of particular interest to this study is Figure 3.7 which shows an example of the correlation between COD oxidised and nitrate reduced (Eqn. 18). As noted earlier, nitrite which accumulated during the tests was converted to nitrate equivalents. For the plot shown in Figure 3.7 (from Figure 3.5), the data gave a slope of 2.38 gO_2/gNO_3-N which is somewhat lower than the expected value of 2.86. However, Figure 3.7 shows a correlation based on only 6 observations in one batch test. The small number of observations and the associated errors in measured nitrate and COD values introduces a degree of uncertainty. To decrease the uncertainty in the slope and better estimate this parameter, the results of the twelve batch tests were combined. This was achieved by coding the data from each experiment. Coding involves subtracting from each observation the average of all observations for that experiment. This then allows all the data to be placed on one graph centred at (0,0) and puts a greater emphasis on the experiments with more observations. There is a statistical advantage to this procedure over averaging the values from different tests. The method increases the degrees of freedom in the least squares regression analysis and decreases the influence of poor data points. A plot of the coded data is shown in Figure 3.8. Here, the best-fit slope is 2.96, and statistically, it is not possible to say that the best-fit slope fits the data better than the theoretical value of 2.86.

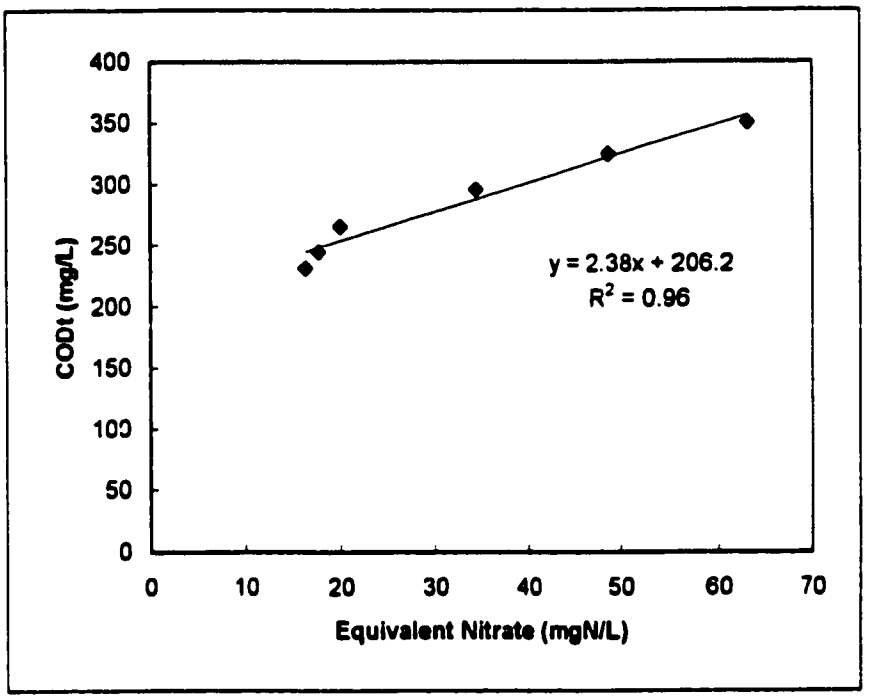

Figure 3.7: Total COD *versus* equivalent nitrate for one batch test.

Instead of combining the results in a single plot, the data for the twelve experiments can be examined individually (e.g. Figure 3.7). Table 3.2 presents the data as calculated for each batch test. The observed conversion factors vary somewhat but the average value of 2.90 gO_2/gNO_3-N is in good agreement with the theoretical value and the value calculated by coding the data. COD balances also were calculated for each experiment. To calculate the balances, the theoretical conversion factor (2.86 gO_2/gNO_3-N) was used and in each case a very good COD balance was observed. The apparent variation in the calculated values obtained in the experiments with tap water was attributed to variations in the tap water from day to day, although this was not verified. The results of each test combined with the results obtained by coding the data confirm that the theoretical conversion factor adequately describes the relationship between reduced nitrate and oxygen equivalents in these batch tests.

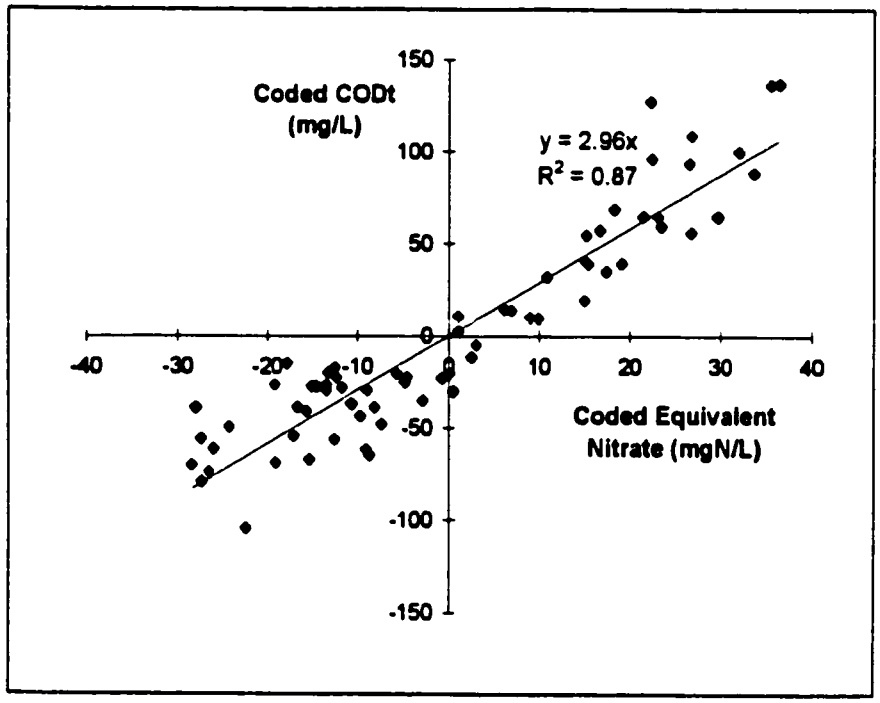

Figure 3.8: Coded data from all twelve batch tests used to determine the reduced nitrate to oxygen equivalents conversion factor.

Table 3.2: Observed nitrate to oxygen equivalents conversion factors and COD balances using a conversion factor of 2.86 gO_2/gNO_3-N for each of the twelve experiments.

Batch Test #	Media Basis	Conversion Factor (# of observations)	COD Balance
1	tap water	2.38 (6)	1.04
2	tap water	2.98 (7)	0.95
3	tap water	2.46 (6)	1.04
4	tap water	1.80 (4)	1.16
5	tap water	2.15 (5)	1.06
6	tap water	4.08 (7)	0.94
7	RO/DI	2.58 (6)	1.02
8	RO/DI	3.29 (7)	0.94
9	RO/DI	3.81 (6)	0.94
10	RO/DI	2.86 (5)	1.01
11	RO/DI	2.61 (4)	1.03
12	RO/DI	3.81 (4)	0.93
averages		2.90	1.01

If it is assumed that 2.86 gO_2/gNO_3-N represents the true factor between COD oxidised and nitrate reduced as demonstrated in these experiments, then two conclusions can be reached. One conclusion is arrived at by assuming that no COD was 'lost' in these experiments. Hence, the calculated factor is interpreted as the true conversion factor. In this case, as the theoretical and experimental conversion factors are in agreement, it can be concluded that the redox half-reactions used in the theoretical derivation are correct and are applicable. Alternatively, a second conclusion can be reached if it is assumed that the theoretical factor 2.86 is correct. Using this assumption, an observed slope greater than 2.86 would suggest COD 'loss'. However, the results confirm the 2.86 factor and indicate that there was no COD 'loss' in these experiments. Both approaches lead to the same conclusions. The conversion factor between oxidised COD and reduced nitrate is 2.86 gO_2/gNO_3-N and COD 'loss' did not occur. This result contrasts earlier studies where COD 'loss' did occur under anoxic conditions.

In this study, a dominant culture of denitrifiers was used with a readily biodegradable soluble substrate, but in the anoxic only and anoxic/aerobic systems where COD 'losses' were reported this was not the case. In those systems, several differences are apparent: not all the organisms were denitrifiers and not all the municipal wastewater substrate was soluble. Therefore, presumably the COD disappearance is linked to either the behaviour of the non-denitrifiers or to the hydrolysis of complex substrate.

In nutrient (N & P) removal systems, with anaerobic, anoxic and aerobic zones a further degree of complexity is added by the anaerobic stage. For instance, under these conditions, both the denitrifiers and non-denitrifiers are exposed to an environment without one of the traditional terminal electron acceptors, NO_3 or O_2

(note that in anoxic/aerobic systems, the denitrifiers are always exposed to an electron acceptor). Investigations of N & P systems are further complicated by biological excess phosphorus removal behaviour, where in the anaerobic zone there is apparent COD uptake and storage without the requirement for an electron acceptor.

CONCLUSION

Many recent studies on nutrient removal activated sludge systems have revealed that COD in the influent cannot be accounted for in the effluent stream, waste sludge, oxygen utilisation or nitrogen reduction. That is, COD balances on these systems are less than 100%. Although the 'loss' of COD in these systems is advantageous because it results in the treatment of this 'lost' COD at no cost, it introduces a degree of uncertainty in process design. In this study, one possible sink for the 'lost' COD, the reduced nitrate to oxygen equivalent conversion factor 2.86 gO_2/gNO_3-N, was investigated.

A closed reactor system was developed for this purpose. Twelve batch tests were performed using dominant cultures of *Pseudomonas denitrificans* utilising sodium citrate as substrate (electron donor) and potassium nitrate as electron acceptor. The results of the twelve trials were combined by coding the data. The constructed plot indicated a best-fit slope of 2.96 gO_2/gNO_3-N. However, the theoretical value, 2.86, also adequately described the relationship between nitrate reduction and oxygen equivalents. Two approaches for interpretation of the data provided the same conclusion. That is, in this system of dominant denitrifiers, and soluble substrate, there was no 'loss' of COD. Nevertheless, the differences between this investigation and previous denitrification studies, where COD 'losses' were exhibited, suggest that COD may be 'lost' due to the action of non-

denitrifiers in an unaerated environment or possibly during hydrolysis of complex substrate.

ACKNOWLEDGEMENTS

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CHAPTER FOUR

COMPARING SLUDGE PRODUCTION UNDER AEROBIC AND ANOXIC CONDITIONS

This chapter contains the complete text of a paper to be published in *Water Science and Technology* (accepted November, 1997). The full reference is unknown at this time.

COMPARING SLUDGE PRODUCTION UNDER AEROBIC AND ANOXIC CONDITIONS

John B. Copp & Peter L. Dold

Department of Civil Engineering, McMaster University
1280 Main Street West, Hamilton, Ontario, Canada L8S 4L7

ABSTRACT

The results of 32 batch tests demonstrate a significant difference in biomass yield under anoxic and aerobic conditions using a variety of soluble substrates. An anoxic yield of 0.402 mg particulate COD / mg consumed COD was calculated which is 62% of the corresponding aerobic yield of 0.645 mg particulate COD / mg consumed COD. Batch tests were performed under anoxic and aerobic conditions using a seed of either activated sludge from one of two local municipal wastewater treatment facilities or a prepared sample of *Pseudomonas denitrificans*. Irrespective of the test organism, the calculated yields were consistent for each electron acceptor. A novel approach to quantifying the consumption of electron acceptor was employed in these batch tests. COD balance calculations were performed and confirmed that all of the initial COD could be accounted for in terms of residual soluble COD, biomass production and electron acceptor consumption.

Key words: activated sludge, anoxic, aerobic, respiration, denitrification, yield, sludge production

INTRODUCTION

An objective of many activated sludge municipal wastewater treatment applications is that of biological nitrification – the microbially-mediated oxidation of ammonia to nitrate. Both organic substrate degradation and nitrification require oxygen, supply of which is the single largest operating expense in wastewater treatment. In the absence of oxygen, a significant fraction of the mixed heterotrophic population in activated sludge is able to use nitrate as the terminal electron acceptor in place of oxygen. This mechanism for oxidation of organics (*anoxic respiration* or *denitrification*) has led to the development of a number of activated sludge system configurations which incorporate aerated zones for nitrification and unaerated zones for denitrification. Denitrification has a two-fold impact: (1) reduced aeration requirements, and (2) reduced effluent nitrogen content. Furthermore, a number of studies (e.g. McClintock *et al.*, 1988; Barker and Dold, 1996) have observed lower sludge production in systems incorporating denitrification. This study was directed at quantifying the difference in sludge production that can be expected when substrate is oxidised under anoxic or aerobic conditions.

Bauchop and Elsden, (1960) proposed that cell yield from substrate oxidation is related to the yield of high energy compounds such as ATP from catabolic processes. That is, the amount of ATP generated by the cell and hence the amount of sludge production reflects catabolic efficiency as well as thermodynamic differences. The exact stoichiometry of ATP generation per unit substrate oxidised in respiring cells is not known, but it has been observed that less ATP is generated under denitrifying conditions as compared to aerobic conditions with several microbial species. For example, Koike and Hattori (1975) reported an anoxic yield which was 66 percent of the aerobic yield for

Pseudomonas denitrificans. Nevertheless, the yield of organisms on a dry weight basis per ATP generated is presumed to be constant (Hadjipetrou *et al.*, 1964). The lower ATP production under anoxic conditions should result in a lower cell yield for organisms utilising nitrate as compared to oxygen as an electron acceptor.

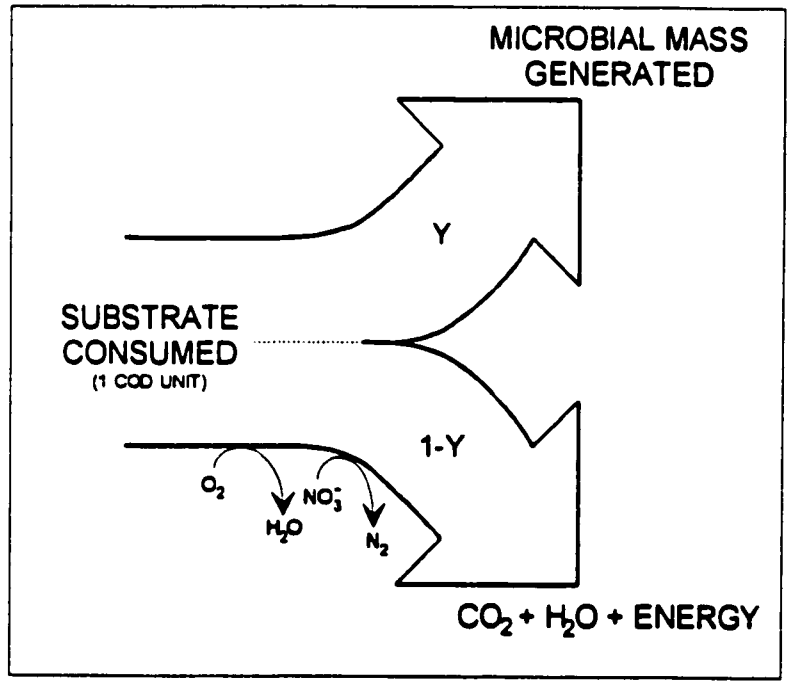

Figure 4.1: Schematic representation of COD flow due to growth processes.

The overriding factor involved in the amount of sludge production is cell yield. That is, the amount of sludge produced is a direct consequence of the efficiency of the cell to convert organic substrate into biomass. Figure 4.1 schematically represents the flow of COD during growth. A portion (1-Y) of the consumed COD is oxidised to CO_2 and water, providing energy for converting the remaining COD (Y) to biomass. COD oxidation is balanced by the reduction of an equivalent mass of electron acceptor. As Figure 4.1 shows, true growth yield (Y) is defined as the biomass COD produced per unit substrate COD consumed:

$$Y = \frac{g\ biomass\ COD\ produced}{g\ substrate\ COD\ consumed} \qquad (1)$$

Alternatively, the yield may be expressed in terms of biomass COD produced per unit of electron acceptor reduced. This is equivalent to:

$$\frac{g\ biomass\ COD\ produced}{g\ COD\ oxidised} = \frac{g\ biomass\ COD\ produced}{g\ oxygen\ consumed} = \frac{Y}{1-Y} \qquad (2)$$

In denitrification systems the yield often is expressed in units of g biomass COD produced per g NO_3-N reduced. To calculate Y in this case, the mass of nitrate must be converted to an equivalent mass of oxygen using the nitrate-to-oxygen conversion factor (Copp and Dold, 1997):

$$\frac{g\ biomass\ COD\ produced}{g\ NO_3 - N\ reduced} = \frac{2.86Y}{1-Y} \qquad (3)$$

In terms of the earlier ATP discussion, the ratio of biomass produced to substrate oxidised [Y/(1-Y)] should be less with nitrate as electron acceptor as opposed to oxygen. However, a COD balance must be maintained regardless of the electron acceptor [i.e. 1 = Y + (1-Y)]. Conducting a COD balance on experimental data for yield calculations is important for checking the integrity of the data. Unfortunately this check has seldom been applied.

This study was undertaken to investigate growth yields under anoxic and aerobic conditions. A closed system was designed such that the components involved in the COD balance check could be quantified. Experiments were conducted under aerobic and anoxic conditions using either *Pseudomonas denitrificans* or activated

sludge from full-scale treatment plants, each grown on a number of defined substrates.

EXPERIMENTAL DESIGN

Consider a batch test in which a soluble organic substrate is combined with a small seed of heterotrophic organisms. Assume that the following parameters are measured at intervals: total COD and soluble COD. Figure 4.2 shows the type of response that could be expected. Note however, that Fig. 4.2 is for illustrative purposes only and shows straight lines; in practice non-linear responses would be anticipated. In the test, total COD (CODt) measures COD of the residual substrate plus COD of the biomass (CODp). Hence, the decline in CODt is a measure of the COD oxidised, and CODp is calculated as the difference between CODt and the residual substrate (CODs).

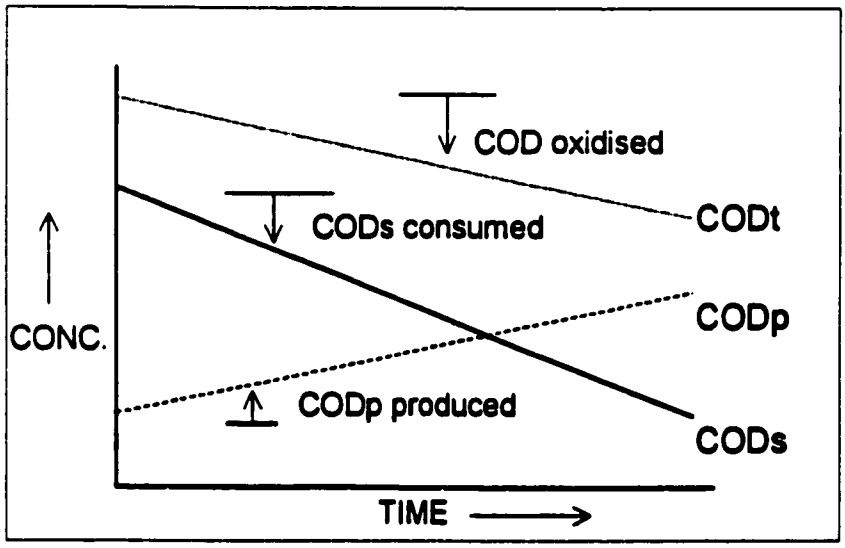

Figure 4.2: Qualitative response in batch test.

The data collected over the course of the batch experiment can be used to generate a time-paired plot of CODp *versus* CODs. The slope of the plot is an estimate of yield (Y). Alternatively, the experimental data can be plotted as CODp *versus* electron acceptor utilisation (nitrate concentration for anoxic tests or oxygen consumed for aerobic tests). The slope of this plot estimates the quotient Y/(1-Y), from which Y can be calculated. In this way the data provide two independent estimates of Y. Several assumptions must be made, however, for this to hold true. For instance the experiment must occur over a short period of time so that endogenous decay can be neglected. Non-limiting substrate and electron acceptor conditions are desirable so as to ensure the maximum growth rate and shortest duration. Additionally, in analysing the data in this fashion, the assumption is made that the measured decrease in soluble COD is converted to either biomass (CODp) or is oxidised to CO_2 and water. That is, there is no production of soluble COD as a by-product of metabolism.

METHODOLOGY

Test Organisms

A culture of *Pseudomonas denitrificans* was obtained from American Type Culture Collection, Rockville, Maryland. The freeze-dried culture was revived in the prescribed medium and then streaked on Difco Pseudomonas Isolation Agar (PIA) using glycerol as the carbon source. The culture was stored on PIA at 4°C and transferred monthly to new agar because colony growth was apparent at this temperature. Samples of activated sludge were obtained from two local municipal treatment facilities: one a pre-denitrification configuration and the other a strictly aerobic system.

Batch Test Medium

Table 4.1 shows the macro and micro components of the culture medium. Early results (data not shown) suggested that anoxic growth of *Pseudomonas denitrificans* in reverse osmosis/deionized water (RO/DI) was severely retarded when the media was not supplemented with calcium. As such, over the course of this investigation the culture medium changed several times with respect to calcium. Of the fifteen anoxic *Pseudomonas denitrificans* experiments, four were performed using tap water in which calcium was not supplemented, five had a calcium concentration of 8 mg/L and the remaining six had calcium of 72 mg/L. All aerobic experiments had calcium of 72 mg/L. Nitrate was added to the anoxic media at a concentration of 93 mgNO_3-N/L but was not added to aerobic experiments.

Table 4.1: Batch test growth media for *Pseudomonas denitrificans*.

	Medium 1	Medium 2
Autoclaved tap water	1000 mL	
$(NH_4)_2SO_4$	200 mg	200 mg
KH_2PO_4	63 mg	63 mg
$MgSO_4 \bullet 7H_2O$	50 mg	50 mg
$FeCl_3 \bullet 6H_2O$	1.2 mg	1.2 mg
$CaCl_2$		variable
$(NH_4)_6Mo_7O_{24} \bullet 4H_2O$		54 µg
$CuSO_4$		80 µg
$ZnSO_4$		80 µg
$MnSO_4 \bullet H_2O$		80 µg
Reverse osmosis / deionized water		1000 mL

All substrates used in this study were soluble. Several different simple substrates were used including sodium citrate, dextrose and sodium acetate. A few more complex substrates also were used including nutrient broth, yeast extract and

Bacto-peptone. Table 4.2 shows the approximate masses of substrate added to 1L of culture medium when each was used as a substrate.

Table 4.2: Mass of substrate added to 1L of medium when each was used as carbon and energy source.

Substrate	Mass (g) / 1L of Media
Sodium citrate	1.0
Dextrose	0.50
Sodium acetate	1.1
Nutrient broth	0.43
Yeast extract	0.45
Bacto-peptone	0.47

Assay Technique

For experiments involving *Pseudomonas denitrificans*, 200 mL of medium (Table 4.1) in a 250 mL Erlenmeyer flask was inoculated with a colony from the agar plate using a sterile platinum loop. A foam stopper was placed in the flask opening and covered with aluminum foil. Parafilm was then stretched over the neck of the flask. The flask was secured in a reciprocating shaker at room temperature (22°C +/- 2°C) and 65 rpm for between 12 and 48 hours prior to the start of the batch tests. For the anoxic tests, it was not anticipated that the shaker speed would induce a lot of oxygen transfer thus no attempt beyond these measures was made to further exclude oxygen from the culture or air space above the liquid. For experiments involving activated sludge samples, a fresh sludge sample was obtained from the treatment facilities on the morning of each test. Upon returning to the laboratory, the activated sludge experiments were started within a half hour.

Anoxic Tests

Following the initial inoculation period, the contents of the flask were transferred to a 1 L (1.15 L total capacity) round-bottomed three-necked flask which had been flushed with nitrogen gas. To the 200 mL of *P. denitrificans* starter culture (or 60 mL of activated sludge mixed liquor) newly prepared medium was added to a final volume of 1100 mL. The contents were mixed using a magnetic stir bar and stirring plate, and an initial 20 mL sample was withdrawn. Dissolved oxygen (≈5-7 mg O_2/L) was present initially, but was below a measurable limit within 2 hours after the reactor was sealed. The reactor was sealed at each neck using rubber stoppers with various sized holes to accommodate pH, ORP and dissolved oxygen probes, a sampling port and a gas outlet port. Where the holes could not be made gas tight, a silicone based sealer was used. Gas production was monitored using a mineral oil manometer (see Copp and Dold, 1997). As gas was produced in the reactor an increase in pressure caused the oil level in the burette to rise. By adjusting the vertical position of the manometer the pressure in the flask could be maintained at atmospheric. After such adjustments, the change in oil level in the burette provided a volumetric measure of the gas produced since the previous adjustment (correcting for volume of liquid samples removed). On occasion the mineral oil manometer showed upwards of a 1 m stable height differential prior to adjustment indicating that the apparatus was gas tight. During batch tests, samples of the liquid were withdrawn when at least 8 mL of gas had been produced or when there was sufficient capacity in the manometer to allow for a sample. ORP and dissolved oxygen concentration were monitored continuously and recorded on a plotter. Solution pH (7.5 +/- 0.1) was not recorded but was monitored continuously and controlled using a Cole-Parmer model 5997-20 pH controller attached to a metering pump with a reservoir of 3% phosphoric acid (H_3PO_4). This acid was selected to avoid peak interference on the ion chromatographic equipment used for anion analysis. Less than 2 mL of acid

was added per experiment. The end of the anoxic batch tests was signified by a decreased rate of gas production which generally coincided with a drop in ORP.

Aerobic Tests

The aerobic testing procedure was similar to the anoxic test procedure in most respects. Two hundred (200) mL of *P. denitrificans* starter culture (or 60 mL of activated sludge) was topped up to 1100 mL with newly prepared medium in a round-bottomed flask. No attempt to purge the apparatus of oxygen was made nor was the reactor sealed. As air was to be intermittently introduced into the reactor over the course of the experiment, the manometer was disconnected for these trials. Dissolved oxygen (DO) and pH were monitored throughout. A UCT Chemical Engineering dissolved oxygen controller (Hitech Micro Systems) was used to continuously monitor and control the reactor DO concentration. The controller was operated using a high set point of 4.0 mg O_2/L and a low set point of 2.0 mg O_2/L. When the DO in the reactor dropped to 2.0 mg O_2/L the controller switched on a small aquarium pump which pumped air into the reactor. The air remained on until the reactor DO reached 4.0 mg O_2/L at which time the controller switched off the pump. Normal aerobic microbial activity in the reactor utilised the DO for cellular function resulting in a linear decrease in DO following the pump being shut off. The cyclical rise and fall of DO occurred many times over the course of the experiments. An internal microprocessor in the controller calculated and stored the rate of oxygen utilisation (OUR) for each drop in DO. The OUR data were downloaded from the controller to a PC at the end of the experiment through a cable link. A DO plotter attached to the controller was used to manually verify the accuracy of the OUR measurement. A precipitous drop in OUR signalled the end of the aerobic test.

Sample Analyses

The number of samples varied between 5 and 8 per experiment depending on the rate of reaction and in the case of the anoxic tests, the volume of gas produced between samplings. Samples from all experiments were analysed for COD, both total and filtered (0.1 μm). Additionally, filtered samples were injected into a Dionex Ion Chromatograph model AI450 for anion analysis (nitrite and nitrate). A Technicon Traacs 800 colourometric auto analyser was used to verify nitrite and nitrate values and obtain an ammonia plus ammonium concentration.

RESULTS AND DISCUSSION

Anoxic Experiments

In total, 26 batch tests were performed under anoxic conditions. Figure 4.3 and Figure 4.4 show typical results from one of these batch tests. This particular experiment used *P. denitrificans* in tap water with citrate as substrate. The figures illustrate several characteristic features including non-linear changes in all of the measured data indicating that microbial growth was occurring during the course of the experiments. The rate of substrate and electron consumption increased throughout the experiments indicating increased biological activity as the test progressed. Also, the S-curve response noted in the plot of gas production (Figure 4.4) is indicative of an activity increase under non-limiting conditions at the start of the test. The top of the curve signifies a decreasing rate of activity which is consistent with a substrate limitation at the end of the experiment.

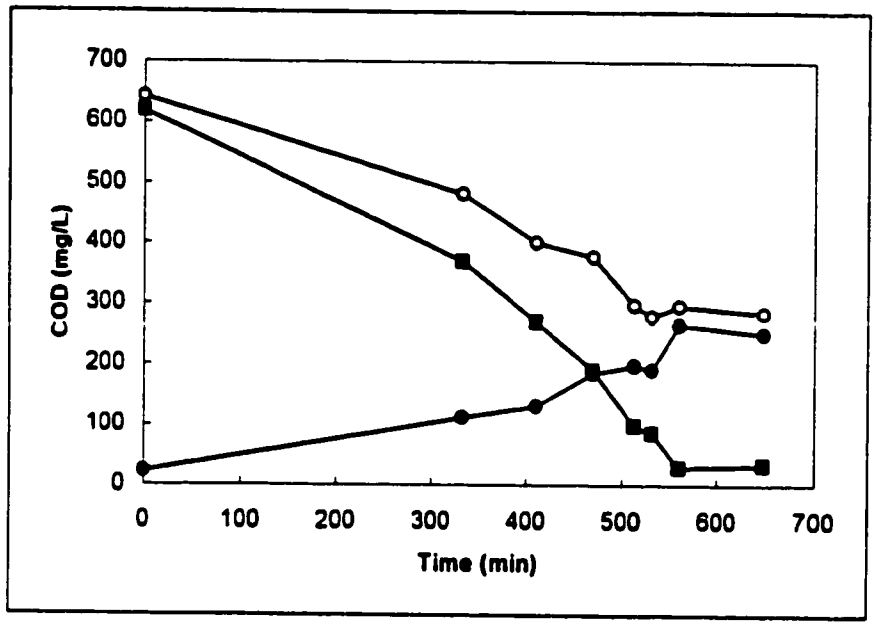

Figure 4.3: Typical COD profiles for anoxic tests [O CODt, ■ CODs, ● CODp].

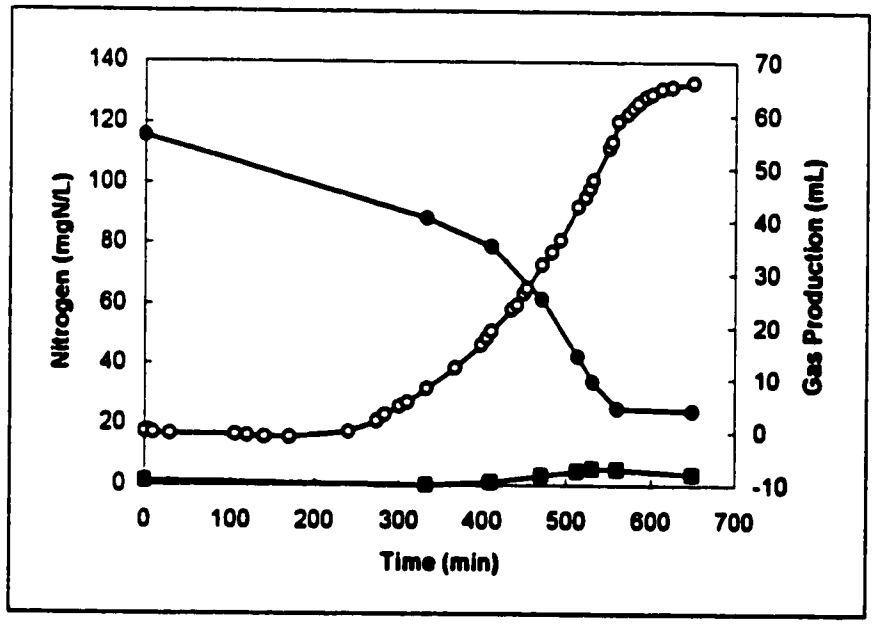

Figure 4.4: Typical nitrogen and gas production profiles for anoxic tests [O gas prod., ■ nitrite-N, ● nitrate-N].

To perform a COD balance check, the sum of the final total COD and the equivalent mass of electron acceptor reduced (in oxygen units) should equal the initial total COD. As these anoxic tests were performed in a closed system essentially devoid of oxygen, the amount of electron acceptor reduced was equal to the drop in nitrate corrected for any nitrite production. [It should be noted that for all of the anoxic experiments, COD balances and yield calculations were made from the second data point. Recall that the reactor contained oxygen at the start of these tests but the oxygen had been depleted prior to taking the second sample]. For example, Fig. 4 shows a nitrate decrease of 64 mgN/L and a nitrite increase of 3.6 mgN/L. Correcting for the nitrite production and multiplying by the nitrate-to-oxygen conversion factor of 2.86 gives an equivalent oxygen consumption of approximately 176 mgO_2/L (Copp and Dold, 1997). Adding this value to the total COD at the end of the experiment and dividing by the initial total COD gives a COD balance of 96% for this particular experiment. The COD balances from all of the anoxic tests were similarly close to unity and resulted in an average COD balance of 99% (Table 4.3).

Figure 4.5 results from plotting particulate COD at time *t* against the corresponding nitrate and soluble COD also at time *t*. The slopes, as explained in the introduction, are the calculated yields based on COD and nitrate. That is, the data in this experiment gave a yield of 0.416 mg particulate COD / mg consumed COD based on the soluble COD data and 2.16 mg particulate COD / mg reduced nitrate based on the nitrogen data. The yield based on nitrate can be converted to COD units according to Equation 3 giving a yield of 0.430 mg particulate COD / mg consumed COD. Agreement between these independent measures of yield further supports the validity of the experimental results. Table 4.3 summarises the yield and COD balance results for all 26 anoxic tests.

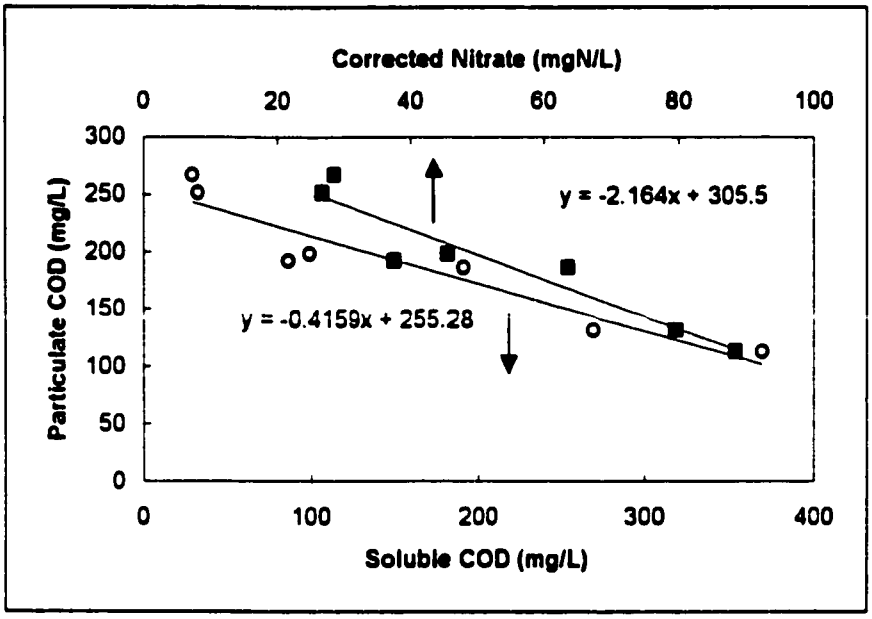

Figure 4.5: Results of yield plot for the data presented in Figure 4.3 and Figure 4.4 [O CODs, ■ nitrate-N].

Table 4.3: Yield as calculated under anoxic conditions using various soluble substrates.

Seed Organism(s)	Soluble Substrate	# of Expts	Yield			Average COD Balance
			COD based (g COD/g COD)	Nitrogen based (g COD/g COD)	(g COD/g N)	
P. denitrificans	citrate	12	0.424	0.433	2.202	0.95
P. denitrificans	acetate	2	0.192	0.196	0.696	1.04
P. denitrificans	glucose	1	0.382	0.299	1.220	1.06
Mixed liquor	citrate	4	0.512	0.451	2.35	1.09
Mixed liquor	glucose	2	0.365	0.349	1.53	1.01
Mixed liquor	nutrient broth	2	0.366	0.371	1.69	0.98
Mixed liquor	Bacto-peptone	2	0.363	0.379	1.75	0.95
Mixed liquor	yeast extract	1	0.363	0.351	1.55	1.02
Weighted averages		26	0.402	0.394	1.92	0.99

Aerobic Experiments

Six (6) batch tests were performed under aerobic conditions and Figure 4.6 and Figure 4.7 show a typical result. In this case a sample of municipal activated sludge was used with citrate as substrate. Here again, non-linear responses were

noted suggesting that microbial growth was occurring over the course of the experiment. The increase in activity is best illustrated by the increase in oxygen utilisation rate (OUR) when conditions were non-limiting and the steep decline in OUR as soluble substrate became limiting (Figure 4.7).

The area under the OUR curve [represented by the cumulative oxygen utilised curve – Figure 4.7] is the amount of oxygen utilised by the respiring cells. In the tests involving activated sludge samples, nitrification occurred. Hence, the amount of oxygen utilised in the test was adjusted to account for the mass of oxygen required for nitrification. In this case, slightly more than 13 mgN/L of nitrate was produced, requiring approximately 60 mgO_2/L. This requirement was subtracted from the total oxygen used, to calculate the oxygen utilised for COD oxidation. Figure 4.7 shows that approximately 165 mgO_2/L was utilised in this experiment of which approximately 105mg/L was used for COD oxidation. As there was no waste sludge in these batch tests, COD balances were calculated by adding the oxygen used for COD oxidation to the total COD at the end of the experiment and dividing by the initial total COD in the reactor. The experiment shown in Figure 4.6 and Figure 4.7 had a COD balance of 0.98 or 98%. Similar COD balances were calculated for all of the aerobic experiments and the average COD balance was 99% (Table 4.4).

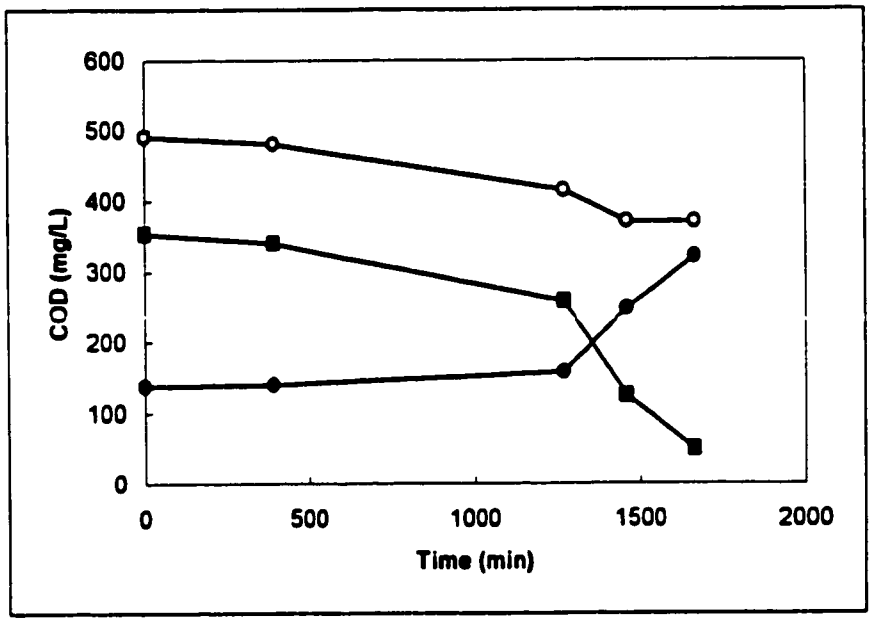

Figure 4.6: Typical COD profiles for aerobic tests [O CODt, ■ CODs, ● CODp].

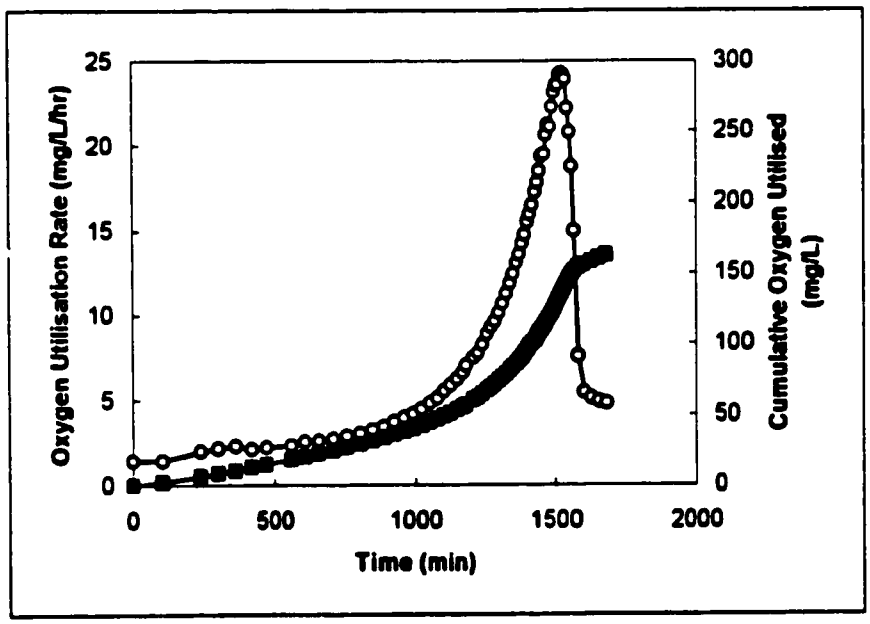

Figure 4.7: Typical OUR and cumulative oxygen profiles for aerobic tests [O OUR., ■ cumulative oxygen utilised].

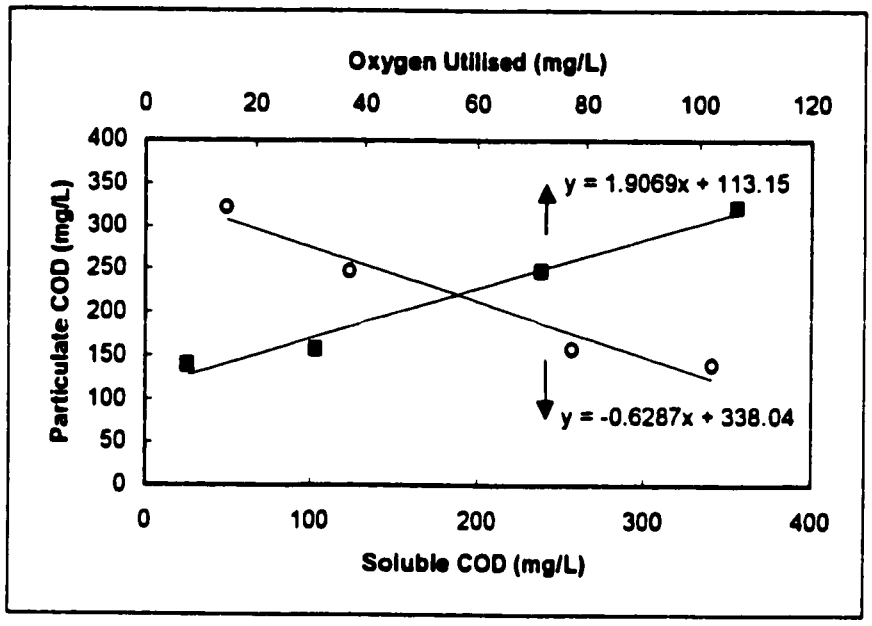

Figure 4.8: Results of yield plots for the data presented in Figure 4.6 & Figure 4.7 [O CODs, ■ oxygen utilised].

The aerobic yield was calculated from plots of particulate COD *versus* soluble COD and particulate COD *versus* the amount of oxygen utilised for COD oxidation (Figure 4.8). Figure 4.8 shows that the yield from this test was 0.629 mg particulate COD / mg consumed COD based on the soluble COD data and 1.91 mg particulate COD / mg of oxygen utilised based on the OUR data. As with nitrate, the latter yield was converted to more conventional units according to Equation 2 resulting in a yield of 0.656 mg particulate COD / mg consumed COD. Again, agreement between these independent measures of yield strengthens this experimental evidence. The yield and COD balance results of the aerobic tests are compiled in Table 4.4.

Table 4.4: Yield as calculated under aerobic conditions using various soluble substrates.

Seed Organism(s)	Soluble Substrate	# of Expts	Yield		Average COD Balance
			COD based (g COD/g COD)	OUR based (g COD/g COD)	
P. denitrificans	citrate	3	0.637	0.687	0.97
P. denitrificans	acetate	1	0.669	0.557	1.09
Mixed liquor	citrate	2	0.645	0.680	0.98
Weighted averages		6	0.645	0.663	0.99

CONCLUSION

A number of recent studies on nutrient removal activated sludge systems have demonstrated lower than expected sludge production. Because these studies also have exhibited COD 'losses' [COD in the influent cannot be accounted for in the effluent stream, waste sludge, oxygen utilisation or nitrogen reduction] there is uncertainty as to the cause of the lower sludge production. Is the lower sludge production the direct result of COD 'loss' or is the lower sludge production caused by a lower growth yield under anoxic conditions as compared to the yield under strictly aerobic conditions?

A novel reactor system was developed to investigate the anoxic *versus* aerobic yield problem. Thirty-two (32) batch tests were performed using a number of soluble substrates. A facultative denitrifying organism and samples of activated sludge were subjected to both anoxic and aerobic conditions. The test results indicate that regardless of the seed organism there is a significantly smaller growth yield under anoxic conditions (0.402 mg particulate COD / mg consumed COD) as compared to aerobic conditions (0.645 mg particulate COD / mg consumed COD). The amount of electron acceptor consumed in these experiments was measured to facilitate the calculation of COD balances. The balances approached unity and thus confirmed that all of the initial COD was

accounted for. These batch tests have confirmed that less sludge production can be expected when anoxic zones are present in a system configuration. This factor should be accounted for in the design of nutrient removal activated sludge configurations with unaerated zones to avoid over-estimating the amount of sludge production in the system.

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CHAPTER FIVE

INFLUENCE OF INFLUENT PHOSPHORUS CONCENTRATION ON AN EXCESS BIOLOGICAL PHOSPHORUS REMOVAL SEQUENCING BATCH REACTOR

PART I: EXPERIMENTAL BEHAVIOUR

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INFLUENCE OF INFLUENT PHOSPHORUS CONCENTRATION ON AN EXCESS BIOLOGICAL PHOSPHORUS REMOVAL SEQUENCING BATCH REACTOR

PART I: EXPERIMENTAL BEHAVIOUR

John B. Copp & Peter L. Dold

Department of Civil Engineering, McMaster University
1280 Main Street West, Hamilton, Ontario, Canada L8S 4L7

ABSTRACT

Results are presented for an eight month excess biological phosphorus removal (EBPR) study examining the influence of influent phosphorus on the kinetics and stoichiometry of the system's microbial community. This paper presents the experimental results. A sequencing batch reactor (SBR) was operated using four 6-hr cycles per day at a relatively constant influent COD of 430 to 470 mg COD/L, using sodium acetate as the sole source of substrate. Five stable EBPR experimental periods with influent phosphorus concentrations ranging from 4.5 to 55.5 mgP/L were studied. The phosphorus content of the sludge varied with influent phosphorus from 4.0 to 17.0 per cent of the TSS. Complete anaerobic substrate uptake was consistently achieved irrespective of the influent phosphorus level and filament growth in the reactor was negligible. A novel approach for measuring the daily mass of oxygen utilised was employed in this study which allowed for the independent calculation of COD mass balances across the system.

COD mass balances for the system varied from 0.91 to 1.12 and averaged 1.04 indicating that no COD 'loss' was observed in the system.

Key words: activated sludge, anaerobic, anoxic, aerobic, excess biological phosphorus removal, biological nutrient removal, sequencing batch reactor, COD balance

INTRODUCTION

Excess biological phosphorus removal (EBPR) activity in activated sludge provides the means for eliminating phosphorus from wastewater and preventing phosphorus influx to receiving surface waters. EBPR is a microbial process which involves the cultivation, within the mixed community, of microorganisms that have the ability to take up phosphorus in excess of that required for growth. The net effect of this uptake is a reduced phosphorus concentration which can be less than 1mg/L in a well-operated system. The removal of phosphorus is achieved through the microbially mediated storage of polyphosphate granules which can occupy up to 60% of the cell volume with a phosphorus mass as much as 38% of the volatile suspended solids (Lotter *et al.*, 1986; Wentzel *et al.*, 1989). Polyphosphate storage is encouraged in these systems by exposing the biomass to anaerobic and aerobic stages sequentially, favouring the growth of polyphosphate-accumulating facultative anaerobes.

Within the stages of an EBPR system, characteristic behaviour is observed in each stage of the process (Figure 5.1). During the anaerobic stage, substrate is sequestered by the biomass and converted internally to polyhydroxyalkanoates (PHAs) while phosphorus is released from the biomass causing an increase in soluble phosphorus. During the aerobic stage, PHA degradation occurs and

soluble phosphorus is accumulated as polyphosphate within the biomass, leaving a reduced level of phosphorus in the effluent.

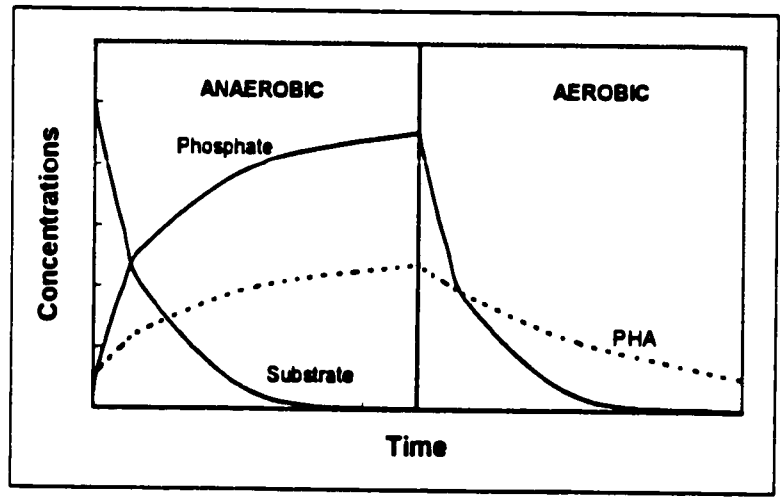

Figure 5.1: Schematic illustration of characteristic EBPR behaviour in an anaerobic/aerobic sequenced bioreactor.

A substantial amount of data on EBPR has been accumulated over the last number of years, yet questions remain with respect to biochemical mechanisms and the development of accurate mathematical simulation models. Much of the biochemical supposition around EBPR has been based on known pathways in *Acinetobacter spp.*, which was shown to exhibit EBPR activity and dominate systems with aerated and unaerated stages (Lotter, 1985). Although several organisms isolated from activated sludge are reported to accumulate excess phosphorus, much of the early research concentrated on *Acinetobacter spp.* as the principal organism responsible for this phenomenon in activated sludge. However, more recent evidence disputes early conclusions on the bacterial make-up of systems with aerated and unaerated stages (Cech and Hartman, 1993; Kavanaugh and Randall, 1994; Satoh *et al.*, 1994; Ubukata and Takii, 1994; Blackall *et al.*, 1997; Bond *et al.*, 1997). These latest studies suggest that

Acinetobacter spp only plays a limited role in these systems, and hence casts some doubt on the active biochemical mechanisms. Nevertheless, several biochemical EBPR models have been developed over the years (Comeau *et al.*, 1986; Wentzel *et al.*, 1986; Mino *et al.*, 1987; Arun *et al.*, 1988) and modifications to these models have since been suggested (Wentzel *et al.*, 1991; Matsuo *et al.*, 1992; Satoh *et al.*, 1994; Smolders *et al.*, 1994).

The biochemical models have been used successfully to provide a biological explanation for much of the experimental data in the literature. However, questions still exist regarding the formation of mechanistic process models implemented in simulators used for process design and optimisation. In particular, a number of studies have identified COD mass balance problems in nutrient removal sludge systems (McClintock *et al.*, 1988; Power *et al.*, 1992; Randall *et al.*, 1992; Wable *et al.*, 1992; Smyth, 1994; Wable *et al.*, 1994; Barker and Dold, 1995). Throughout this study, emphasis was placed on COD mass balances. That is, all of the terms required for the mass balance calculations were independently measured, including a daily measurement of the mass of oxygen utilised. By placing an emphasis on these mass balance terms, a database of observations which could be used to meet modelling objectives was generated.

In the development of a mechanistic model, it is necessary to understand the behavioural and physical responses that can be expected given a change in influent characteristics. To this end, the impact of influent phosphorus was the focus of this study. A sequencing batch reactor (SBR) exhibiting EBPR activity was operated over an extended period at various influent COD:phosphorus ratios. The SBR behaviour was monitored closely and following acclimation at each influent COD:phosphorus ratio, a period of intensive monitoring was initiated. During these periods, profiles of reactor behaviour were monitored and waste sludge from the SBR was subjected to batch tests to further probe areas of interest

(*Part II*). The results of a similarly study were recently reported (Liu *et al.*, 1997), and provided a basis for comparison; however, the work in this study covered a wider range of ratios.

The objective of this paper (*Part I*) is to present the experimental data from the laboratory-scale EBPR reactor in its entirety. The SBR protocol and start-up problems will be discussed. Also, steady state data are presented along with nitrogen and phosphorus profiles through the cycle. Finally, oxygen utilisation rates and mass balances are presented and discussed. Part II in this series focuses on the periods of intense monitoring including presentation of the results from batch tests and comparison of the results to results published in the literature. Finally, Part III addresses the biochemical aspects of the experimental observations and provides a biochemical explanation of the observed behaviour.

METHOD

SBR Operation

A sequencing batch reactor (SBR) was operated in an Erlenmeyer flask (operating volume 4L). The fill and draw system was operated using four 6-hour cycles per day and controlled using a ChronTrol model XT4 control timer. Each cycle involved adding 1.8 litres of influent to the 2.2 litres of mixed liquor in the SBR, followed by 2.25 hours unaerated (and mixed), 2.25 hours aerated (and mixed) and 1.5 hours of settling (not mixed). Over the last 10 minutes of the settling phase in each cycle, supernatant was withdrawn down to a volume of 2.2 litres. To simplify sludge age control in the reactor, waste sludge (target sludge age of 10 days) was removed once per day during the last ten minutes of the aerated and fully mixed period in the fourth cycle.

Each day, a single eight (8) litre batch of synthetic wastewater (sodium acetate with a small amount of yeast extract and trace minerals) was prepared from stock

solutions according to Table 5.1. The basis for the media was water which had been treated using a reverse osmosis process and a deionising column (RO/dI). At the start of each cycle, 1.8 litres of the synthetic feed solution (Table 5.1) was added to the settled sludge leftover from the previous cycle (target hydraulic retention time of 13.3 hours). The filled volume in the reactor was controlled by a float switch which turned off the feed pump once the reactor contained a liquid volume of 4L. This feeding process took less than ten minutes.

Over the eight months, a total of 5 experimental periods were examined, each characterised by a different influent COD to influent phosphorus ratio. The various ratios were achieved by varying the influent phosphorus while maintaining a relatively constant influent COD (440 mgCOD/l). Influent phosphorus levels were varied from 4.5 mgP/L to 55.5 mgP/L (Table 5.2).

Table 5.1: Composition of the influent synthetic wastewater and stock solutions used to make up the influent.

Compound Source	Compound	Stock Solution Concentration (g compound/L)	Target Feed Concentration
$MgSO_4 \cdot 7H_2O$	Mg	56	42 mg/L
$CaCl_2$	Ca	63	16 mg/L
NH_4Cl	N	46	23 mg/L
$FeCl_3 \cdot 6H_2O$	Fe	1.5	390 µg/L
$ZnSO_4 \cdot 7H_2O$	Zn	0.53	130 µg/L
$MnSO_4 \cdot H_2O$	Mn	0.72	180 µg/L
$CuSO_4 \cdot 5H_2O$	Cu	0.14	35 µg/L
$CoCl_2 \cdot 6H_2O$	Co	0.13	34 µg/L
H_3BO_3	B	0.08	19 µg/L
KI	I	0.15	36 µg/L
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	Mo	0.14	35 µg/L
Yeast Extract		13.5	3.4 mg/L
$CH_3COONa \cdot 3H_2O$ (Sodium Acetate)		235.3 (as COD)	440 mg/L
KH_2PO_4 & K_2HPO_4	K	224	variable
KH_2PO_4 & K_2HPO_4	P	110	variable

The first phase in each cycle after feeding was unaerated. Several procedures were used to minimise oxygen transfer into the liquid during feeding. For instance, continuous mixing of the reactor began ten minutes into each cycle and ceased at the start of the settling stage. It was found that if mixing began at the start of each cycle a significant amount of splashing was created by the partially submerged mixing blades and increased the oxygen transfer into the reactor liquid. By delaying the onset of mixing by ten minutes, splashing did not occur. Similarly, to avoid the introduction of oxygen through the feed, the feed solution was purged with nitrogen gas for the 30 minutes immediately prior to the start of each cycle.

Aeration of the reactor in phase two of each cycle was accomplished using pure oxygen because better oxygen transfer could be achieved with a lower gas flow rate (as compared to air). It was found that when air was used a significant gas flow rate was required and the rise of bubbles inside the reactor resulted in the deposition of solids in the neck of the flask above the liquid surface. During the settle period the reactor was neither aerated nor mixed. Decant was pumped from the reactor during the final ten minutes of the settling stage and the volume of decant removed was controlled by setting the depth of the decant tubing. This procedure ensured that a similar liquid volume remained in the reactor, following the decant being pumped out, irrespective of whether or not waste sludge had been removed during that cycle.

The system design required minimal maintenance. Any solids attached to the walls of the reactor were scraped loose and the feed tubing was cleaned regularly to minimise microbial growth.

SBR Monitoring

The reactor was monitored continuously for pH and ORP. The pH was controlled above 6.8 (solution pH was never observed above 7.5) using a Cole-Parmer model 5997-20 pH controller attached to a metering pump with a reservoir of sodium carbonate (Na_2CO_3). This base was selected to avoid peak interference on the ion chromatographic equipment used for anion analysis. Reactor ORP was monitored continuously using a Fisher Accumet pH/Ion meter model 230 and plotted on a Kipp and Zonen chart recorder model #BD112.

A UCT Chemical Engineering dissolved oxygen controller (Hitech Micro Systems) was used to monitor and control the reactor dissolved oxygen (DO) concentration and measure the oxygen utilisation rate (OUR) during the aerated phase in each cycle. The controller was operated using a high set point of 5 mg O_2/L and a low set point of 2 mg O_2/L. During the aerated stage, when the DO in the reactor dropped to 2 mg O_2/L the controller switched open a solenoid valve resulting in the flow of oxygen from a gas cylinder into the reactor. The oxygen flowed through an aspirator and into the SBR until the reactor DO reached 5 mg O_2/L. Once the controller sensed a DO of 5 mg O_2/L the solenoid was closed. Normal aerobic microbial activity in the reactor utilised the DO for cellular function resulting in a linear decrease in DO following the solenoid being closed. An internal microprocessor in the controller calculated and stored the rate of oxygen utilisation (OUR) for each drop in DO which occurred many times over the course of each cycle. The OUR data was downloaded from the controller to a PC at the end of each day through a cable link. A DO plotter attached to the controller was used to manually verify the accuracy of the OUR measurement. From the area under the OUR *versus* time curve a daily estimate of the mass of oxygen utilised was calculated.

SBR Analysis

On a daily basis, waste and decant samples were analysed for both filtered and unfiltered COD (1.5μm Whatman 934-AH glass fibre filters) according to *Standard Methods* (APHA, 1992). Filtered samples of the waste and decant were injected into a Dionex Ion Chromatograph model AI450 for anion analysis (nitrite, nitrate and phosphorus) and a Technicon Traacs 800 colourometric auto analyser was used to obtain an ammonia plus ammonium concentration. As the feed was a clear colourless liquid devoid of solids, sample analysis on the feed was restricted to unfiltered samples only. That is, the feed samples were analysed for total COD and unfiltered samples were analysed for anions and ammonia. Frequently, although not every day, samples were removed from the completely mixed reactor at the end of the anaerobic period and filtered before measuring the concentration of anions, or more specifically, the concentration of soluble phosphorus following the release phase. During 'steady state' periods, waste samples were regularly analysed for TSS, VSS, TKN and TP (both filtered and unfiltered) also in accordance with *Standard Methods* (APHA, 1992). As part of this comprehensive testing period profiles of the reactor activity over the cycle were measured. Samples were removed from the reactor at timed intervals, filtered immediately, and analysed for anions and ammonia. Additionally, samples removed at the end of the anaerobic period, at the end of the aerobic period and at the end of the settle period were analysed for TKN and TP, both filtered and unfiltered.

Electron Microscope Sample Preparation

A waste sample (i.e. after the aerated period) from experimental period V was fixed, embedded and sectioned, then examined with a transmitting electron microscope. The following steps outline the embedding procedure.

Fixation: A 1.5 mL waste sludge sample was centrifuged and 1 mL of liquid was discarded. The sample was then mixed with 1 mL of 2% glutaraldehyde in 0.1M cacodylate buffer (pH 7.1) and placed in the fridge for 3 hours where it was mixed occasionally. The sample was then centrifuged and rinsed three times with 0.1M cacodylate buffer before it was centrifuged again and placed into 1% osmium (OsO_4) in 0.1M cacodylate buffer for 1 hour on ice (mixed every 15 minutes).
Dehydration: The sample was centrifuged and placed into 50% ethanol for 1 hour on ice. The sample was then subjected to a series of ethanol solutions (70%, 90%, 95%, 100%, 100%) each for 1 hour and centrifuged between steps. Finally the sample was again centrifuged and placed into 100% propylene oxide.
Infiltration and Embedding: Infiltration with Spurr's resin was accomplished through a propylene oxide:Spurr's resin series (2:1 v/v, 1:1, 1:2, 100% Spurr's, 100% Spurr's) for 1 hour each with samples rotated during each step. The sample was then placed into an embedding mould and polymerised at 70°C for 8 hours.

Initial Attempt at 24 Hour Cycle Operation

Prior to settling on four 6-hour cycles per day, the reactor was set up to run using a 24 hour cycle. Synthetic feed was added during the unaerated (and mixed) stage over a 2 hour period. The total length of the unaerated stage was 6 hours and the aerated stage was 17.5 hours with 0.5 hours for settling. In total, five start-up attempts at a 24 hour cycle were tried over a four month period, but all attempts ultimately failed to produce a viable EBPR system.

Figure 5.2 shows the influent COD and phosphorus for one of these attempts. For this attempt, the sludge was acquired from a full-scale EBPR facility. After receiving a 1L sample of sludge, it was immediately aerated for just over 1 hour during which time the soluble phosphorus decreased from approximately 43 mgP/L to less than 1 mgP/L. The sludge was then placed into the SBR with 3L of synthetic feed and the cycle stages began. Figure 5.2 shows how the influent

characteristics were changed during the first few days of operation before finally settling on an average influent COD of 565 mg/L and an average influent phosphorus of 34.7 mgP/L.

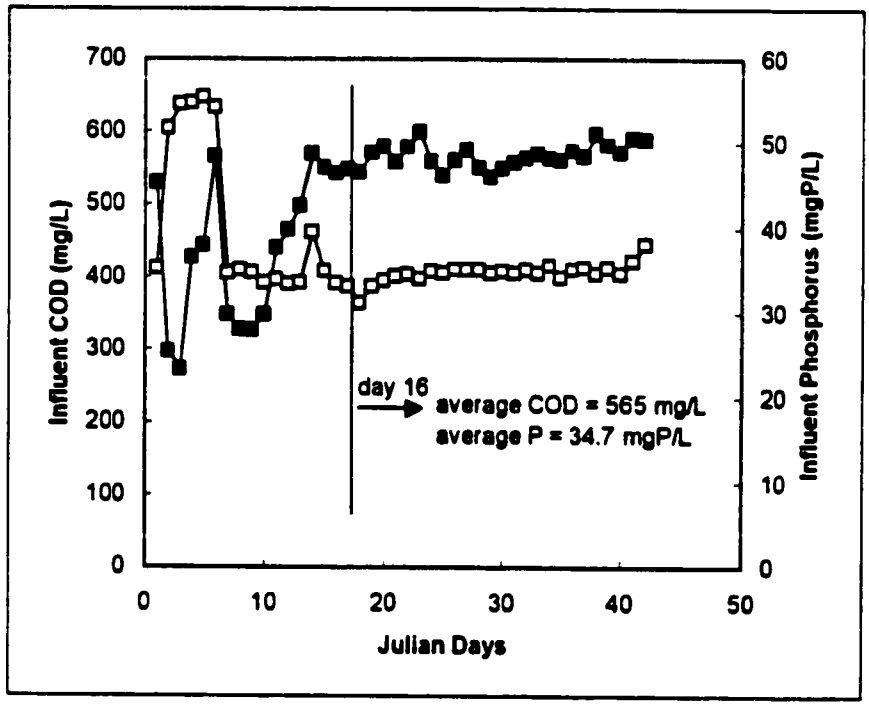

Figure 5.2: Influent characteristics of the feed used during one attempt at a 24 hour cycle (■ - influent COD; □ - influent phosphorus).

Over the initial 15 days, the influent was manipulated in an attempt to control and stabilise the reactor behaviour. In particular there were two areas of concern: readily biodegradable substrate bleed-through to the aerobic stage, and effluent phosphorus. The results of Randall and Chapin (1997) suggest that bleed-through of readily biodegradable substrate from the unaerated stage into the aerobic stage can be detrimental to EBPR stability. Hence, when bleed-through was detected – through soluble COD measurement and high OUR detection - the influent COD was decreased in the following cycle. Similarly, when effluent phosphorus increased, the influent phosphorus for the next cycle was decreased.

Nevertheless, this control strategy was abandoned on day 15 when very little EBPR activity was detected. It was reasoned that if a stable culture was to be produced then the feed should be equally stable. However EBPR activity did not redevelop.

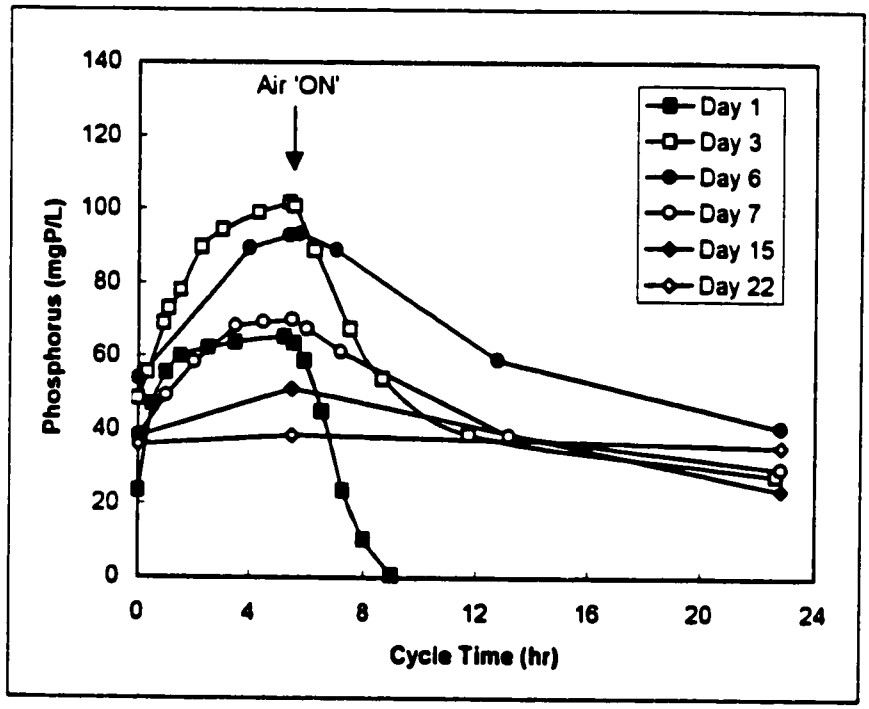

Figure 5.3: Examples of the soluble phosphorus profiles showing anaerobic release and aerobic uptake over the first 22 days during one attempt at achieving EBPR activity using an SBR operating on a 24 hour cycle.

During the initial start-up of this reactor, the EBPR activity was monitored closely. Samples taken from the reactor at the end of the unaerated stage were analysed, and on several days timed samples were taken so that the phosphorus profile through the cycle could be observed. Figure 5.3 shows examples of those results. It is clear from the figure that phosphorus release increased over the first few days but then rapidly decreased such that after two sludge ages very little

release or uptake was occurring. The maximum phosphorus released as measured by the phosphorus concentration at the end of the unaerated stage is shown in Figure 5.4. Figure 5.4 shows the decline in anaerobic phosphorus release over the course of this attempt.

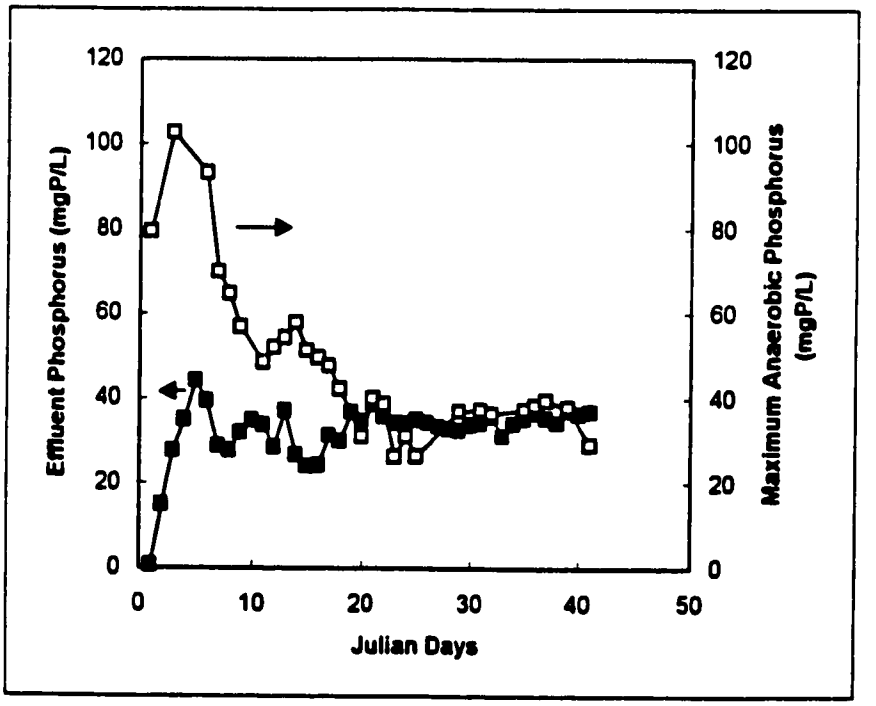

Figure 5.4: Effluent phosphorus and anaerobic phosphorus release for one attempt at a 24 hour cycle: (■ - effluent phosphorus; □ - anaerobic phosphorus release).

It also is interesting to note that during these attempts at a 24 hour cycle, the effluent phosphorus increased almost immediately (Figure 5.4). That is, the effluent phosphorus was <1 mgP/L at the end of day 1, 14 mgP/L at the end of day 2, and 28 mgP/L at the end of day 3. Figure 5.4 shows that the effluent phosphorus increased to approximately the same level as the influent then remained constant. Comparing the release and uptake data for this particular attempt reveals that uptake of phosphorus occurred, but only the mass released

was taken up. That is, there was no indication of excess phosphorus removal during this period. Following the failure to establish EBPR behaviour in a system operated on a single 24-hour cycle, the system operation was modified to incorporate four 6-hour cycles per day. In this mode, stable EBPR performance was established very satisfactorily.

RESULTS AND DISCUSSION

SBR Operation

The sequencing batch reactor (SBR) was operated successfully using four 6-hour cycles per day with aerated and unaerated stages to encourage biological excess phosphorus removal (EBPR) activity. Sludge for starting up the SBR was obtained from a full-scale biological nutrient removing (BNR) facility and phosphorus release and uptake activity was observed from the first day. However, initially uptake was not complete and phosphorus levels in the effluent were greater than 7 mgP/L. After an acclimation period of approximately 27 days, complete uptake was observed and effluent phosphorus concentrations decreased to near zero. Figure 5.5 shows the progression in effluent phosphorus during this initial start-up phase and the rapid increase in anaerobic phosphorus release after day 10. The difference in behaviour between this start-up phase and the start-up phase using a 24 hour cycle is striking. Where there was an immediate trend towards increased effluent phosphorus and decreased anaerobic phosphorus release using a 24 hour cycle, here the effluent phosphorus was relatively stable (below the influent level) and anaerobic phosphorus release increased rapidly.

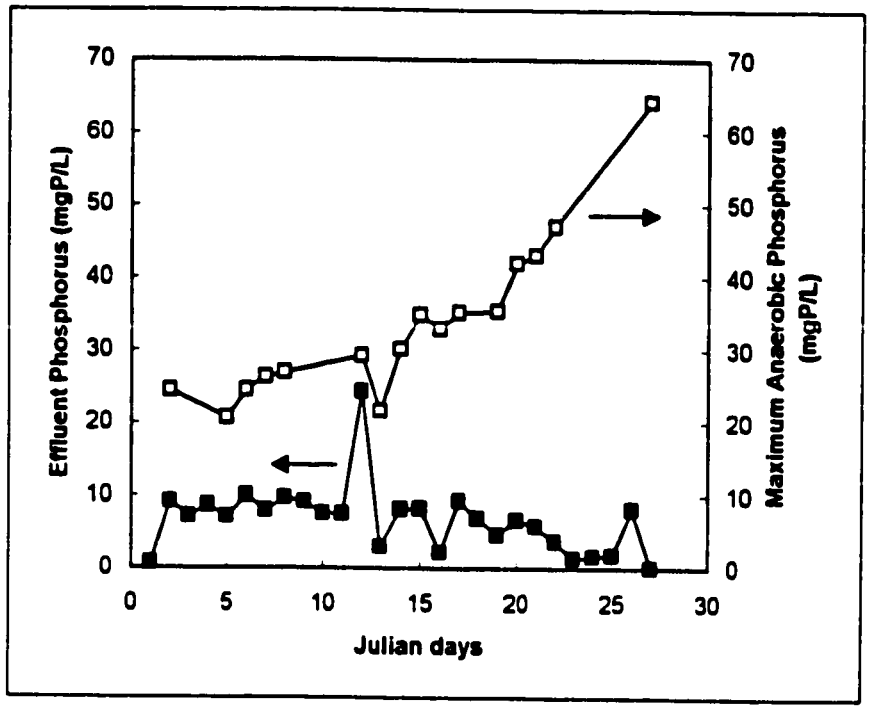

Figure 5.5: Effluent phosphorus and maximum anaerobic phosphorus during the first 27 days of operation using four 6-hour cycles per day (■ - effluent phosphorus; □ - anaerobic phosphorus release).

Although 1.5 hours per cycle was allotted to settling, most of the settling process was complete within 10-15 minutes. For all periods, the sludge in the system settled very well, but a minor amount of pin-floc remained in the supernatant. The decant VSS (Table 5.3) was limited to between 16 mg/L (experimental period I) and 24 mg/L (experimental period IV) on average during the stable operating periods. Periodic examination of the waste mixed liquor solids under a light microscope at 1000x magnification (wet mounts using phase contrast, dry mounts with Gram and Neisser staining) showed no presence of filamentous organisms. This was in spite of the fact that the original sludge sample from the full-scale facility was bulking and contained significant numbers of *M. parvicella* and Type 0041 filamentous bacterial types. Microscopic monitoring revealed the steady decline in these filamentous organisms. These results contrast the work of

Randall and Chapin (1997) who found that filamentous organisms out-competed EBPR organisms at high influent acetic acid concentrations. In that system the filamentous organisms caused bulking problems and quickly disabled EBPR activity. However, it should be noted that proliferation of the filamentous organisms in that system followed acetic acid 'breaking through' from the anaerobic stage, and entering the anoxic and aerobic zones. In contrast, 'break through' did not occur during this study. Acetate was measured regularly at the end of the unaerated stage and was never detected.

In total, five experimental periods were investigated, each characterised by a different influent COD to phosphorus ratio (COD:P). The different COD:P ratios were achieved by varying the influent phosphorus concentration while maintaining the feed COD at a relatively constant level. Figure 5.6 shows the different influent phosphorus concentrations *versus* time and Table 5.2 lists the average influent COD values, influent phosphorus values and COD:P ratios.

Table 5.2: Chronologically ordered, sequencing batch reactor experimental periods

Experimental Period	Influent COD (mg/L)	Average Daily COD Load (mg/d)	Influent Phosphorus (mg/L)	Influent COD:P Ratio (mg/mg)	Average Daily F:M (mg/mg/d)
I	440	3012	10.6	42	0.60
II	470	3517	25.0	19	0.47
III	430	3307	12.5	34	0.43
IV	440	3335	4.5	98	0.41
V	440	3233	55.5	8	0.43

As evident in Figure 5.6, two attempts were made using an influent phosphorus concentration of greater than 50 mgP/L. On the first attempt, when the influent phosphorus was increased from 25 to 55 mgP/L, the system response was promising over the first few days after the change. That is, the amount of anaerobic phosphorus release increased and the effluent phosphorus remained low. However, after 10 days the response suddenly became unstable (data not

shown). On one particular day, an effluent phosphorus concentration of 191 mgP/L was observed. That is, less phosphorus was being taken up aerobically than being released anaerobically. Also, effluent ammonia levels which had been <0.1 mgN/L rose to between 2 and 4 mgN/L and effluent nitrate decreased to below a detectable limit. Lowering the feed phosphorus concentration to 12.5 mgP/L solved the problem as stable conditions returned over the next sludge age. Stable nitrification was restored as was EBPR activity with low effluent phosphorus levels. The reasons for the unstable activity are unknown especially as the reactor was stable during period V when the influent phosphorus was 55.5 mgP/L (COD:P of 8). Similar unstable EBPR activity was reported by Liu *et al.* (1997) when they attempted to operate a system with an influent COD:P ratio of 13.3 (mg COD/mg P).

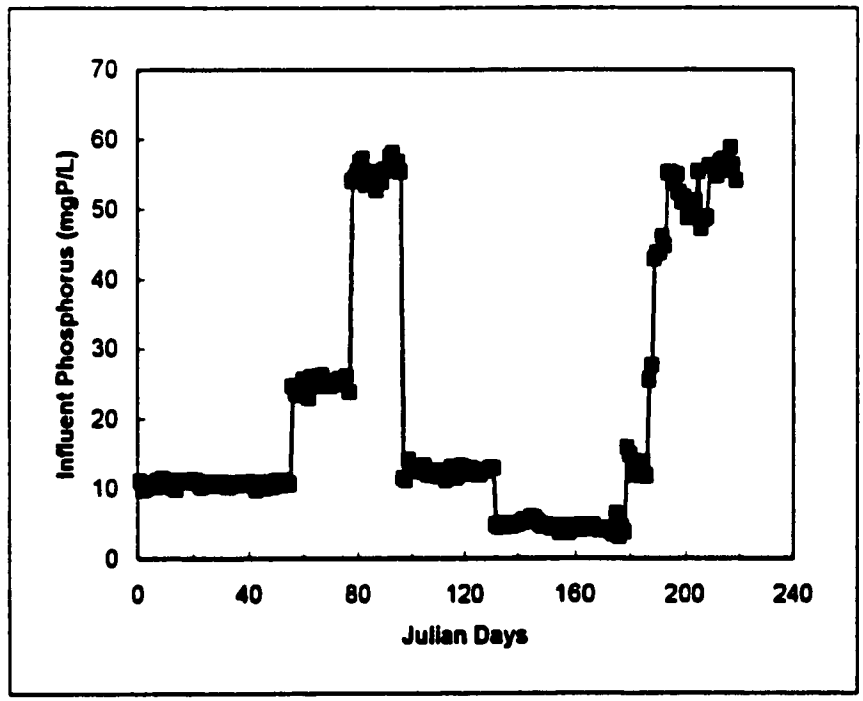

Figure 5.6: Influent phosphorus over the course of the study.

Table 5.3: Average composition of effluent samples from the SBR for the five experimental periods during 'steady state' operation.

Experimental Period	Influent Phosphorus (mgP/L)	Effluent Phosphorus (mgP/L)	Effluent VSS (mg/L)	Effluent NH_3 (mgN/L)	Effluent NO_x (mgN/L)
I	10.6	0.3	16	2.6	3.5
II	25.0	0.5	16	0.0	5.5
III	12.5	0.5	16	0.1	4.5
IV	4.5	0.1	24	0.6	4.8
V	55.5	1.5	23	0.1	5.1

Phosphorus in the effluent was relatively constant during each stage. However, Table 5.3 shows that there was an increasing trend in effluent phosphorus as the influent phosphorus increased. Nevertheless, effluent phosphorus levels generally remained low for the entire period of operation, and the average values during steady state operation reflect typical values (Table 5.3). However, there were times when the effluent phosphorus did increase (Figure 5.7). Mechanical problems for instance, occasionally disrupted the SBR cycle and caused an increase in effluent phosphorus. Low effluent phosphorus levels normally returned within 24 hours of the problem detection. Also, a brief increase in effluent phosphorus was observed during the transition from experimental period IV to experimental period V. Unlike the transition from period I to II (influent phosphorus 10.6 to 25.0) when effluent phosphorus remained <1mgP/L, the transition from IV to V (influent phosphorus 4.5 to 55.5) caused a temporary rise in effluent phosphorus for three days and reached a maximum of 18 mgP/L. Effluent phosphorus decreased over the next few days and was below a detectable limit by the sixth day.

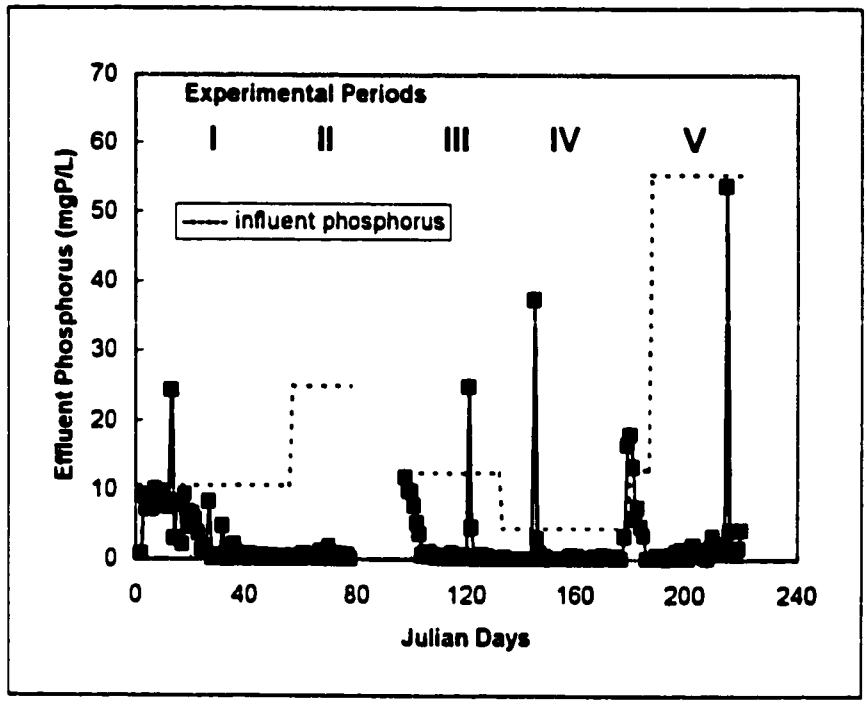

Figure 5.7: Effluent phosphorus for all experimental periods showing four process upsets due to mechanical problems and the increase in effluent phosphorus during the transition from experimental period IV to V.

During each experimental period near-complete nitrification was observed as indicated by the very low average concentrations of effluent ammonia (Table 5.3). It should be noted that nitrate was the dominant product in three of the five periods, but a persistent concentration of nitrite was observed in the experimental periods with the lowest concentrations of influent phosphorus. In periods II, III, and V, nitrate was the dominant nitrification end-product and nitrite approached or was below the detectable limit. However, in periods I and IV, nitrite predominated. Nitrate was produced in these periods (1.1 and 1.9 mgN/L respectively) but a consistent and greater concentration of effluent nitrite was detected. This is an interesting observation, but an explanation of these results is not offered.

Further results of the investigation are listed in Table 5.4. The SBR was maintained at a reasonably constant solids retention time (SRT) during all of the periods (8.2 to 10.2 days when accounting for solids in the decant), but as expected, the volatile suspended solids to total suspended solids ratio (VSS:TSS) varied inversely with influent phosphorus. That is, as influent phosphorus increased, so did the mass of inorganic polyphosphate in the biomass. In the final stage, fully 60% of the mass in the reactor was inorganic material (VSS/TSS = 0.41), and expressed in terms of phosphorus content, 17% of the TSS was phosphorus (Px). Alternatively, expressed in terms of VSS, the mass of phosphorus was 42% of the mass of VSS (Pv).

Table 5.4: SBR steady state results for the five experimental periods.

Influent Phosphorus (mgP/L)	Influent COD:P (mg/mg)	SRT (days)	VSS (mg/L)	TSS (mg/L)	VSS:TSS (mg/mg)	Px – P Content (% P of TSS)	Pv – P Content (% P of VSS)
4.5	98	10.2	2041	2448	0.83	4.0	5.1
10.6	42	8.2	1456	2062	0.71	7.6	10.7
12.5	34	10.0	2068	2953	0.70	8.4	12.0
25.0	19	9.3	1892	3347	0.57	10.4	18.8
55.5	8	9.1	2146	5241	0.41	17.0	41.6

Electron Microscope Images

The supposition throughout the investigation was that phosphorus storage was being achieved through synthesis of polyphosphate granules inside the cells. To test this hypothesis, a waste sludge sample (i.e. end of the aerobic phase) from experimental period V (influent P = 55.5 mgP/L) was embedded, sectioned and examined with a transmitting electron microscope (TEM). Polyphosphate granules are electron dense which makes them easily visible with the electron microscope [electron dense bodies are dark when viewed in a TEM]. Figure 5.8 shows a polaroid photograph of a typical sample of cells viewed with the TEM. The black circular bodies inside the cells are polyphosphate granules [arrow 1] which was confirmed through spectral analysis of a sampling of those bodies in

various cells [data not shown]. The spectral analysis also indicated a high concentration of magnesium in these black inclusions.

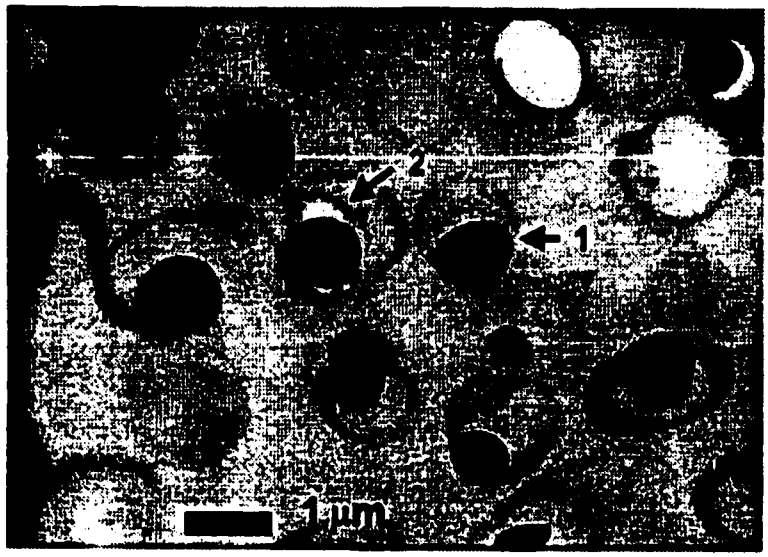

Figure 5.8: Polaroid photograph of electron microscope image taken while viewing a 900nm thick section of an embedded sample of waste sludge from experimental period V.

The sections prepared for the TEM were 900nm thick which should explain why very little detail can be seen in the photograph. Originally, thinner sections were prepared, but when viewed with the microscope most of the cells had large holes in them which indicated that the sectioning procedure had torn something from the cells. This was confirmed when thicker sections were prepared and the black bodies were seen. It was later reasoned that the polyphosphate granules were so dense that it was not possible to cut through a granule without tearing it loose from the cell. Hence, it was concluded that the holes seen in the thinner sections were created when the polyphosphate granules were removed during sectioning. Evidence of this can be seen in the photograph [arrow 2] as the start of a hole can be seen around one of the polyphosphate granules. Unfortunately, TEM sampling was not done during any of the periods with a low influent phosphorus

concentration as comparison of the samples may have provided some useful insights. Nevertheless, viewing a sample from experimental period V did confirm that polyphosphate granules were being synthesised by the biomass in the SBR.

Nutrient Profiles

The SBR was allowed to stabilise for a minimum of two sludge ages after a change in influent phosphorus. Following this period of acclimation, an intensive period of monitoring was undertaken, and as part of this monitoring, profiles of the reactor response were generated. Figure 5.9 (experimental period II) and Figure 5.10 (experimental period IV) illustrate typical examples. At the start of the unaerated stage (after influent addition), nitrate and nitrite (carried over from the previous cycle) were denitrified, and ammonia, supplied in the influent initially increased (mostly likely due to hydrolysis of particulate material in the reactor) then stayed relatively constant until the end of the unaerated stage. Further, acetate was sequestered by the biomass removing it from the bulk solution and the concentration of soluble phosphorus increased. The two figures demonstrate the differences in phosphorus release observed during the two periods. With an influent phosphorus of 4.5 mgP/L phosphorus release is significantly less than the release when the influent was 25.0 mgP/L even though there was no appreciable difference in influent COD. These figures also show that the influent phosphorus concentration impacts on the rate of anaerobic phosphorus release. Figure 5.9 (influent P = 25 mgP/L) shows phosphorus release in the order of 100mgP/L in 0.5 hours yet Figure 5.10 (influent P = 4.5 mgP/L) shows phosphorus release of approximately 40mgP/L over 2.25 hours.

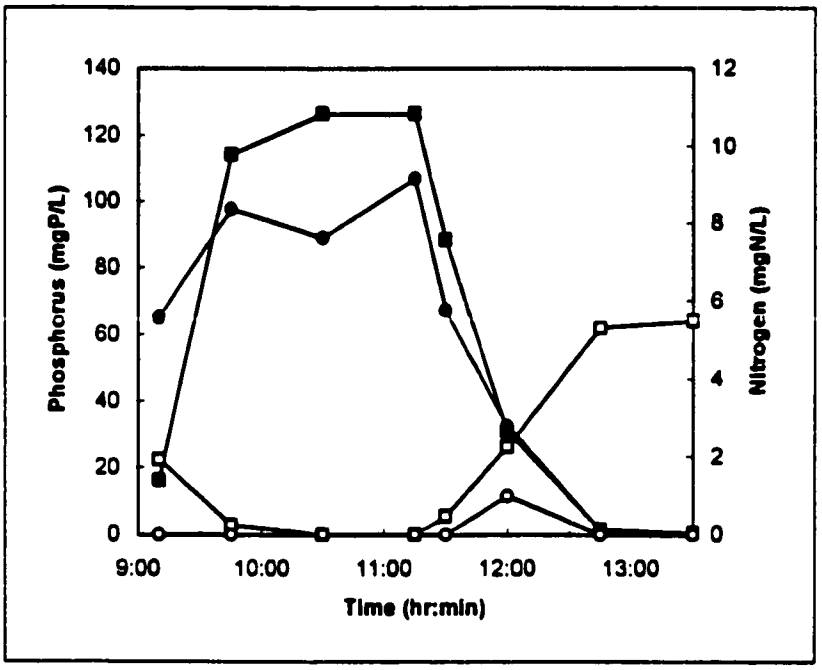

Figure 5.9: Typical nutrient profile observed over one 6-hr cycle when influent phosphorus was 25.0 mgP/L: phosphorus (■); ammonia (●); nitrite (○) and nitrate (□).

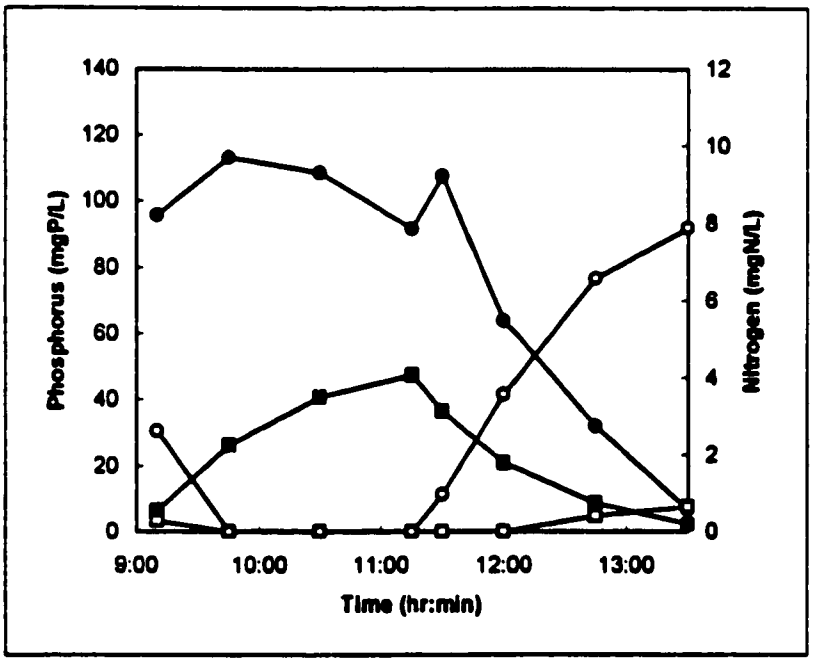

Figure 5.10: Typical nutrient profile observed over one 6-hr cycle when influent phosphorus was 4.5mgP/L: phosphorus (■); ammonia (●); nitrite (○) and nitrate (□).

Following the onset of aeration, there was a rapid decrease in soluble phosphorus and ammonia while the production of nitrite and nitrate indicated nitrification. Sample analysis revealed the presence of nitrite and nitrate immediately after starting the aerobic stage, but in all but two experimental periods (I & IV), the nitrite was converted to nitrate by the end of the aerobic stage. Figure 5.9 and Figure 5.10 illustrate these differences. When the influent phosphorus was low, nitrite was produced and not completely oxidised to nitrate, but when influent phosphorus was high, minimal nitrite was produced and nitrate predominated in the effluent. Nevertheless, nitrogen at the end of the aerobic stage was relatively constant for all experimental periods, averaging between 3.5 and 5.5 mgNO_x-N/L while very little ammonia was detectable at the end of the aerobic stage during all of the experimental periods.

One of the issues investigated in this study was the release of phosphorus at the end of the anaerobic stage. The experimental results revealed that the degree with which the phosphorus was released from the biomass varied for each experimental period. Figure 5.11 shows the observed relationship between influent phosphorus and the anaerobic phosphorus release. This cannot be represented by a single linear relationship over the entire range of influent phosphorus concentrations. Rather, two distinct regions can be seen. At an influent phosphorus concentration greater than 10 mgP/L, Figure 5.11 shows that the anaerobic phosphorus release varied linearly with influent phosphorus (slope of 2.2 mgP released/mg influent P). However, it would appear that at some point between 5 mgP/L and 10 mgP/L, this relationship breaks down possibly reflecting the balance between phosphorus for growth and phosphorus for polyphosphate synthesis. Assuming that there is a minimum phosphorus requirement for growth, then at a influent phosphorus level below that, all available phosphorus is incorporated into the biomass leaving none for polyphosphate synthesis. But,

once that growth requirement is met, presumably all remaining phosphorus is converted to polyphosphate and is available for release. Therefore, it was not unexpected that at low influent phosphorus concentrations [but greater than required for growth], a slight increase in influent phosphorus resulted in a large increase in released phosphorus. For instance with this reactor set-up, for each mgP/L in the influent (and zero in the effluent), beyond the growth requirement, the sludge mass stored 18 mgP/L of polyphosphate, all of which is available for release. The exact experimental relationship for an influent phosphorus less than 10 mgP/L is not obtainable using this data as only one point lies within the critical area. However, a relationship close to 18 mgP released/mg influent P is expected.

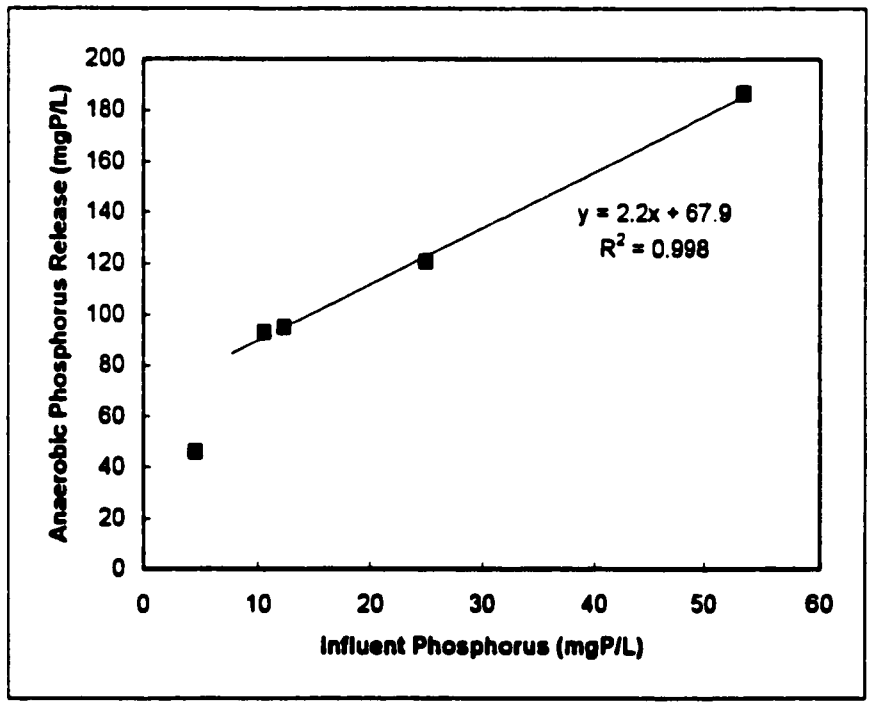

Figure 5.11: Average observed anaerobic phosphorus release in the SBR plotted against the average concentration of influent phosphorus for that period.

Monitoring of the SBR included semi-continuous measurements of oxygen utilisation rate (OUR) during the aerobic stage in every cycle. Figure 5.12 illustrates a typical OUR profile series for a 24 hour period. Excellent agreement in the measured rates between cycles was consistently achieved on a daily basis, but the profiles varied considerably between experimental periods (Figure 5.13). For illustrative purposes, a profile from experimental period IV (inf P = 4.5 mgP/L) is plotted with a profile from experimental period V (inf P = 55.5 mgP/L). This figure shows that although the influent COD was similar and soluble COD entering the aerobic stage was negligible during both experimental periods, the OUR response and hence the biological activity in the periods was very different. The difference in response is addressed in Part III.

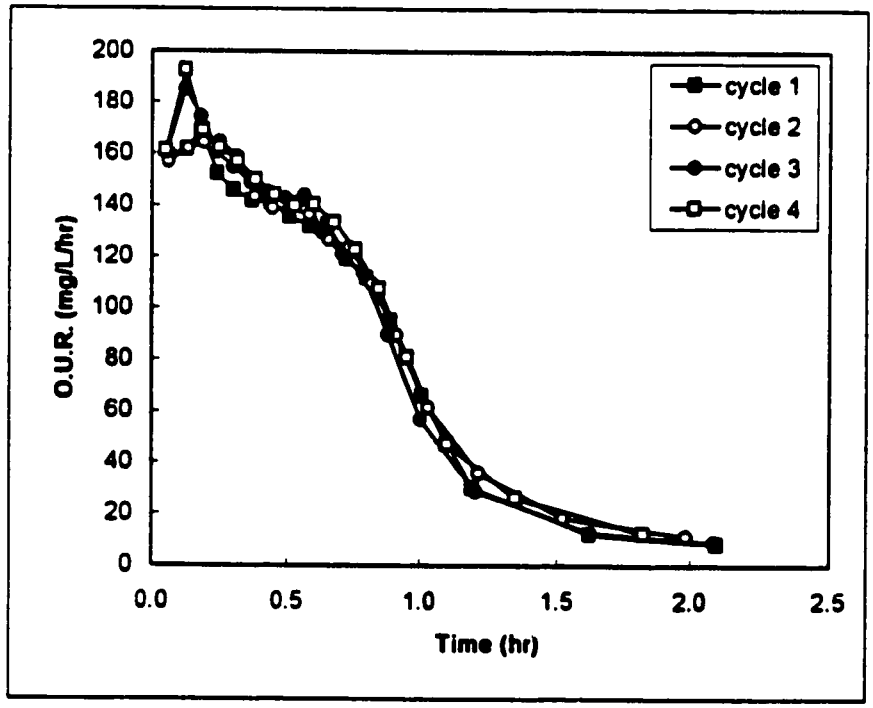

Figure 5.12: A comparison of oxygen utilisation rate profiles during the aerated period over 4 cycles for one 24 hour period.

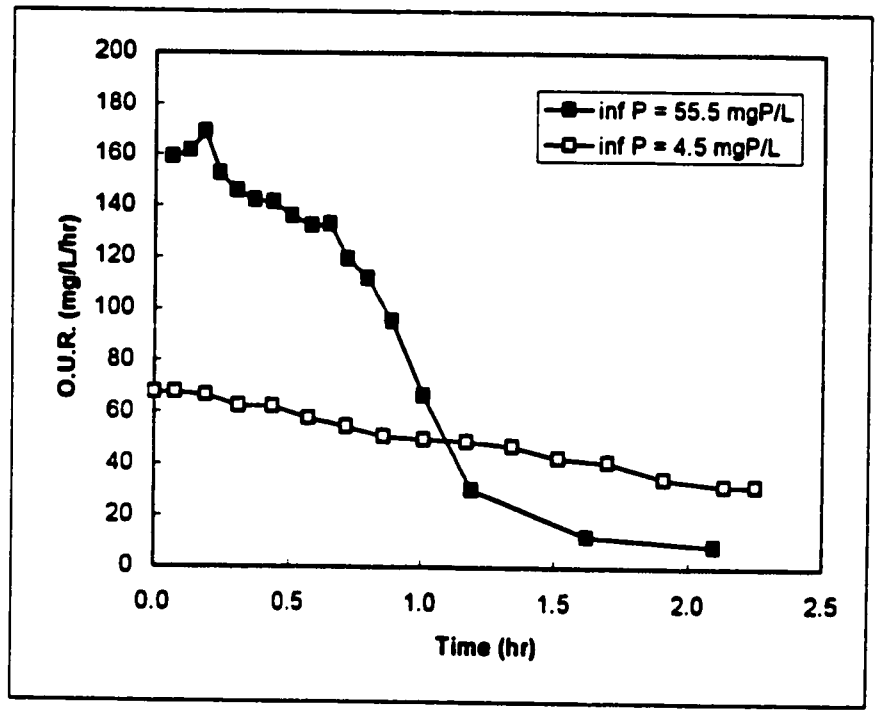

Figure 5.13: Typical observed oxygen utilisation rate profiles over one aerated period during experimental periods IV (inf P = 4.5 mgP/L) and V (inf P = 55.5 mgP/L).

COD Mass Balances

In this SBR system, the components required to calculate the COD mass balances were either measured or independently calculable from the daily data. In general, COD which enters the system must be accounted for in the effluent flow, waste sludge stream, oxygen utilisation or oxygen equivalence of an alternate electron acceptor reduction i.e. nitrate.

The total mass of COD in the waste and decant streams was measured daily, and by measuring the oxygen utilisation during each aerobic period on a daily basis, the total mass of oxygen utilised each day also was known. However, the total mass of oxygen utilised each day includes a significant amount of oxygen for

nitrification. As the COD balance is concerned with carbonaceous oxygen use only, the mass used for nitrification is subtracted from the total. That is, 4.57 mgO_2 / $mgNO_3$-N produced is required to oxidise ammonia to nitrate. So, multiplying the mass of nitrate produced by 4.57 gives the mass of oxygen utilised for nitrification which is then subtracted from the total to give the carbonaceous oxygen use. A similar operation must be performed to account for the oxygen equivalence of the nitrate used in denitrification. A conversion factor of 2.86 mgO_2 / mg NO_3-N denitrified (Copp and Dold, 1998) is used to convert the mass of nitrate denitrified to an equivalent mass of oxygen.

Input COD = COD (unfiltered) in the influent stream

Output COD = COD (unfiltered) in the effluent stream
+ COD (unfiltered) in the waste stream
+ carbonaceous O_2 utilised [total O_2 used – O_2 for nitrification]
+ COD oxidised for denitrification

$$\text{COD balance} = \left(\frac{\text{Output COD}}{\text{Input COD}}\right)$$

Table 5.5 lists the terms used to calculate the COD mass balances around this SBR. It should be noted that where nitrite was produced or denitrified the data was converted to nitrate units to simplify the table. COD balances were calculated daily and Table 5.5 shows the average values for each period along with the calculated COD balances. It is interesting to note that four of the five periods exhibit balances greater than 1.0. That is, in these systems, the data suggests that more COD was exiting the system than entering it in the influent. Nevertheless, all the balances were within 12% of expected and two of the systems had very good balances at 1.02 (experimental period II) and 1.04

(experimental period IV). Several factors may have contributed to the poorer balances obtained in the other periods. For instance, the poor balance obtained during experimental period I might reflect the start-up period and transition to artificial feed. The high concentration of inorganic solids during experimental period V made the task of obtaining representative samples for COD measurements more difficult. However, the exact cause of the poorer COD balances is unknown.

Table 5.5: Average COD mass balance data for the five experimental periods.

Influent Phosphorus (mgP/L)	Influent COD (mg/day)	Effluent COD (mg/day)	Waste COD (mg/day)	Oxygen utilised (mgO/day)	Equivalent NO_3 denitrified (mgN/day)	Equivalent NO_3 produced (mgN/day)	COD Balance
4.5	3335	501	947	2205	31	66	1.04
10.6	3012	382	808	1680	23	47	0.91
12.5	3307	516	1004	2402	38	72	1.12
25.0	3429	533	1065	2129	47	88	1.02
55.5	3233	458	923	2458	43	81	1.11

CONCLUSION

A sequencing batch reactor (SBR) exhibiting excess biological phosphorus removal (EBPR) activity was operated over eight months using a synthetic feed with acetate as the source of carbon and COD. The reactor was operated using four 6-hour cycles per day. Initial attempts to operate with a 24-hour cycle failed to produce a viable EBPR culture. In total, five experimental periods were investigated with each characterised by a different influent COD:phosphorus ratio ranging from 8 to 98 mg COD:mg phosphorus. Nitrification was achieved in all periods and filament growth was negligible throughout the study. Nutrient profiles determined during steady state operation of the SBR showed characteristic behaviour during each period, but highlighted the differences in activity between the periods. Specifically, the nutrient profiles revealed that the rate of anaerobic phosphorus release and the mass of phosphorus released varied

with the influent COD:P ratio. Similarly, oxygen utilisation data showed that the influent COD:P ratio had an impact on the biological activity within the reactor. COD mass balances were calculated for each period and revealed balances within 12% of unity, with four of the five experimental periods exhibiting COD mass balances greater than 100%. The accumulated data from the SBR provided the basis for further investigation and analysis which is presented in Part II and Part III of this series.

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CHAPTER SIX

INFLUENCE OF INFLUENT PHOSPHORUS CONCENTRATION ON AN EXCESS BIOLOGICAL PHOSPHORUS REMOVAL SEQUENCING BATCH REACTOR

PART II: BATCH TESTS

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INFLUENCE OF INFLUENT PHOSPHORUS CONCENTRATION ON AN EXCESS BIOLOGICAL PHOSPHORUS REMOVAL SEQUENCING BATCH REACTOR

PART II: BATCH TESTS

John B. Copp & Peter L. Dold

Department of Civil Engineering, McMaster University
1280 Main Street West, Hamilton, Ontario, Canada L8S 4L7

ABSTRACT

The results of batch tests designed to examine the anaerobic behaviour of sludge in a excess biological phosphorus removal (EBPR) system are presented. Waste sludge samples from a sequencing batch reactor (SBR) exhibiting EBPR activity were subjected to anaerobic batch tests (with acetate addition) to determine the influence of the SBR steady state influent COD:phosphorus ratio on the kinetics and stoichiometry of the system's microbial community. Five stable SBR experimental periods with influent COD:phosphorus ratios ranging from 8 to 98 mg COD:mg phosphorus were studied (see *Part I*). Within the SBR, complete anaerobic substrate uptake was consistently achieved irrespective of the influent phosphorus level. Observations on the reactor and during batch tests demonstrated that the anaerobic rates of substrate uptake and phosphorus release were not constant. The rates of substrate uptake and phosphorus release increased linearly with the sludge phosphorus content. In addition, the batch test results showed that the ratio of phosphorus released to acetate taken up was not constant, but increased linearly with the phosphorus content of the sludge sample.

Key words: activated sludge, anaerobic, anoxic, aerobic, excess biological phosphorus removal, biological nutrient removal, sequencing batch reactor, COD balance

INTRODUCTION

The microbial behaviour in EBPR systems has long been the source of controversy as a definitive biochemical model has yet to be developed which satisfactorily describes all the experimental observations. That is, a consensus has not been arrived at with respect to an applicable biochemical model. In particular, the anaerobic stage of the EBPR process has been the focus of a substantial amount of research, but still is not fully understood. During the anaerobic stage of a EBPR system, soluble phosphorus increases and substrate in sequestered by the biomass which stores it internally as polyhydroxyalkanoates (PHAs). The release of phosphorus and uptake of substrate are believed to be linked and dependent on one another. It has been suggested that in the absence of stored phosphorus, substrate uptake will be inhibited. That is, there will be no accumulation of PHAs unless there is phosphorus available for release.

During the anaerobic stage of a biological phosphorus removal system, it is typical to observe an increase in soluble phosphorus. The orthophosphate comes at the expense of intracellular phosphates which are known to decrease during the anaerobic stage (Lotter, 1985). Wentzel *et al.* (1985) found that without phosphorus release in the anaerobic stage excess phosphorus was not taken up in the aerobic stage. They also found that phosphorus uptake by EBPR organisms was linearly related to the amount of phosphorus released in the anaerobic stage. However, this was contradicted by Lotter (1985) who found no correlation between the amount of phosphorus released and the amount removed. Wentzel *et al.* (1985) used batch tests with acetate as substrate to determine that the rate of

release was first order with respect to organism concentration and zero order with respect to substrate concentration. In the same tests, it was found that approximately 1mg of phosphorus was released for every 2 mg of acetate COD sequestered. Since that result was reported, the ratio of phosphorus released to COD taken up has served as the defining stoichiometric relationship in the anaerobic stage. Numerous studies have investigated and reported on this ratio, but the variable nature of the ratio in the literature has not allowed for a conclusion as to whether the ratio is a constant or if constant, what the true value of the constant is (Fukase *et al.*, 1984; Arvin and Kristensen, 1985; Wentzel *et al.*, 1985; Mino *et al.*, 1987; Wentzel *et al.*, 1989; Cech and Hartman 1993; Satoh *et al.*, 1994; Smolders *et al.*, 1994; Liu *et al.*, 1997).

To investigate the variability in published kinetic and stoichiometric results related to the anaerobic stage of EBPR systems, a series of anaerobic batch tests were undertaken using sludge from an SBR exhibiting EBPR activity. The purpose of the testing was to determine the impact of the steady state influent COD:phosphorus ratio [fed to the SBR] on the kinetic and stoichiometric behaviour of the microbial population under anaerobic conditions. In particular, the rates of phosphorus release and COD uptake were investigated as well as the ratio of phosphorus released to COD taken up under anaerobic conditions. The objective of this paper is to present the results of the batch tests and compare the results to the rates and ratios published in the literature.

METHOD

SBR Operation

A sequencing batch reactor (SBR) was operated in a 4L Erlenmeyer flask as fully described in *Part I*. Influent to the SBR contained sodium acetate as the only organic substrate. The fill and draw system was operated as 2.25 hours unaerated,

2.25 hours aerated and 1.5 hours of settling during each of four 6-hour cycles per day. Sludge age was controlled by wasting sludge once per day during the last ten minutes of the aerated and fully mixed period in the fourth cycle (target sludge age of 10 days). The waste sludge was subsequently used in the batch tests outside of the reactor.

Batch Tests

Five SBR experimental periods characterised by different influent COD:phosphorus ratios were investigated and for each of the five periods, the reactor was allowed to stabilise for not less than two sludge ages. After allowing the reactor to stabilise, a period of intense monitoring was undertaken which included a series of batch tests on the waste sludge. For the batch tests, the waste sludge was divided up into six aliquots of 50mL and each placed into 60ml glass jars with screw-top lids and magnetic stir bars. To mimic the activity in the SBR during cycle 1, the 50mL aliquots were diluted with 6mL of liquid made up of an acetate stock solution and a test solution (Figure 6.1). This dilution reflected the lower concentration of solids in the SBR following the removal of the waste sludge (i.e. 1/10 of the solids were removed during wasting resulting in a new concentration 90% of the old. Similarly, the solids concentration in the batch test vessels was 50/56 or 90% of the waste sludge concentration.). The test solution used to dilute the samples, which was prepared just prior to setting up the assay, was based on feed media, but did not contain any phosphorus.

Of the six vessels, two were used as controls and the final four reaction vessels each received the same mass of acetate and volume of test solution. To vessel 1, no acetate was added, but the sludge was diluted with 6 mL of test solution. To vessel 2, twice the acetate of the 4 reaction vessels was added with sufficient test solution to make up the final volume to 56 mL. Reaction vessels 3 through 6 received the same volume of acetate and test solution. The concentration of

sodium acetate stock was adjusted by trial, depending on the amount of phosphorus to be released. For example, if the anticipated release in the test was 600 mgP/L then the initial concentration of acetate in the sample bottle may have been as high as 1000 mgCOD/L.

Immediately following the addition of acetate and test solution, an initial sample was taken, the vessels were sealed and placed on a 6-position magnetic stirring apparatus which mixed all of the vessels at the same speed. At timed intervals only one of the four vessels was opened, a sample was taken and then analysed. The five timed samples (initial + 4 samples, one from each vessel) were then combined giving the release profile that would be expected under anaerobic conditions. The assumption made here was that the four reaction vessels would react in the same manner and that each vessel received a consistent mixture of the waste sludge. The data obtained did not contradict this assumption.

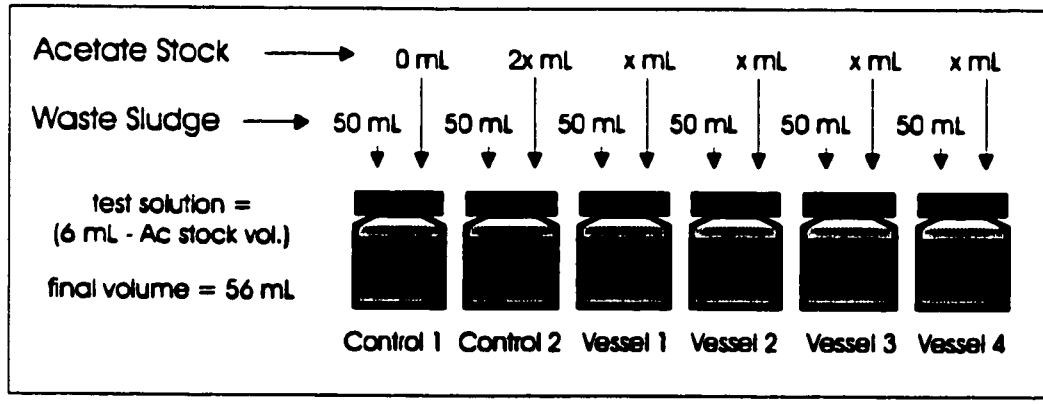

Figure 6.1: Batch test set-up using two control vessels and four reaction vessels with the same mass of sludge and acetate.

Approximately 400 mL of sludge was wasted from the reactor each day and each batch test required 300 mL of sludge. Hence, only one batch test could be performed daily. However, one of the purposes of the batch tests was to

investigate the effect of acetate concentration on the anaerobic behaviour. To do this, batch tests were performed on subsequent days during periods when the SBR was operating in a steady state. That is, different acetate concentrations were investigated on different days using the same procedure.

Sample Analysis

The timed samples were filtered (1.5μm Whatman 934-AH glass fibre filters) according to *Standard Methods* (APHA, 1992) and injected into a Dionex Ion Chromatograph model AI450 for anion analysis (nitrite, nitrate and phosphorus) and a Technicon Traacs 800 colourometric auto analyser was used to obtain an ammonia plus ammonium concentration. Also, a COD measurement was made on the filtered samples.

RESULTS

Batch Test Protocol Development

Batch tests on the waste sludge from the SBR were developed for a more comprehensive analysis of phosphorus release and acetate uptake under anaerobic conditions. In the procedure outlined above, for each batch test the waste sludge sample was augmented with a set concentration of COD (as sodium acetate). Each vessel was opened for sampling only once to provide data on the time sequence of P release. Initially in the batch test design, each of the six vessels was set up with a different concentration of acetate and several batch tests were performed at the same time. That is, each vessel was opened and sampled multiple times to provide P release data for different initial acetate concentrations. The procedure was designed in this way so that the impact of acetate

concentration on the anaerobic behaviour could be investigated using the same waste sludge sample.

At the start of the first test procedure, a sample was taken from each vessel, the vessels were sealed and placed on the mixing apparatus. Then, at timed intervals each of the vessels was opened and a sample was taken, filtered and analysed. This design required that all the vessels be opened to the air each time a sample was taken thus rejuvenating the headspace gases with oxygen. Although this was a relatively quick exchange, it caused significant problems in the batch test results and in fact substantial phosphorus uptake was observed when this procedure was used (Figure 6.2).

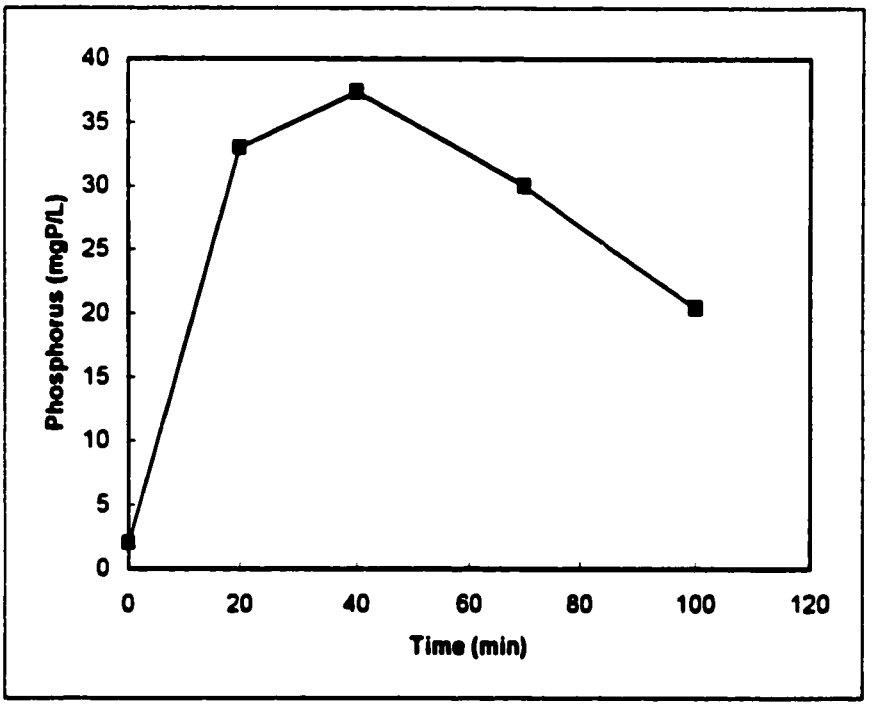

Figure 6.2: Time course of anaerobic phosphorus release experiment showing phosphorus uptake due to infiltration of oxygen from the reaction vessel headspace.

Figure 6.2 shows a typical batch test result in which the reaction vessel was opened on more than one occasion. Note that phosphorus release is followed by a period of phosphorus uptake which is inconsistent with the expected behaviour under anaerobic conditions. The figure shows that the rate of phosphorus release was the greatest prior to opening the reaction vessel, but the rate immediately decreased once the vessel had been opened and the first sample taken. As the vessels were resealed after each sample was taken, it was concluded that the uptake behaviour was an artefact caused by the infiltration of oxygen into the headspace during the sampling periods.

To eliminate this problem, a second batch test procedure (see Method) was developed. This procedure involved a series of batch tests performed over a number of days. Each batch test examined only one acetate concentration and analysis of the series of tests was used to determine the impact of acetate concentration on the anaerobic behaviour. Each batch test resulted in the collection of five timed samples (initial + 4 samples, one from each vessel) from the four sealed vessels. To test the effect of opening the vessels more than once, a sample was removed from the first vessel in the series each time one of the other vessels was sampled and the results were compared. Figure 6.3 illustrates this comparison. The data shows that opening the first vessel multiple times reduced the amount of phosphorus released as compared to the other vessels in the series that were opened only the one time. That is, the phosphorus release observed in the first vessel was always less than the phosphorus released in the last vessel which was opened only once (Figure 6.3). These results confirmed our earlier findings that by opening the vessels more than once, oxygen entered the headspace and disrupted the rate and mass of phosphorus released. Utmost care should be taken to ensure that the internal reaction vessel environment remains anaerobic when performing these types of experiments, because it is apparent that

even a small mass of oxygen infiltration has a large impact on the observed behaviour.

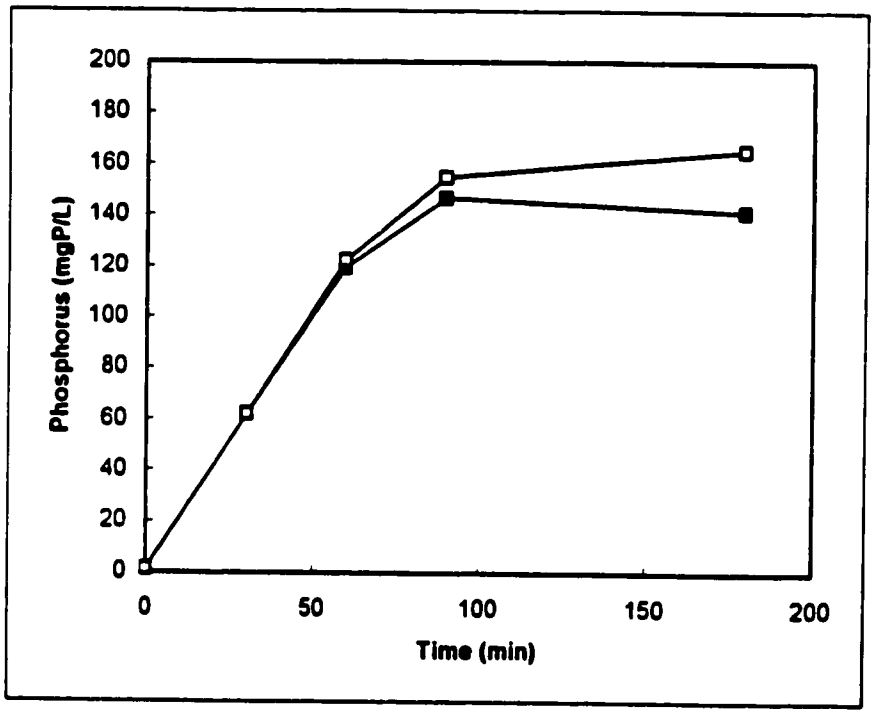

Figure 6.3: Observed anaerobic phosphorus release in a single vessel opened several times (■) and in a series of vessels each opened only once (□).

Comparing the Batch Tests to the SBR

The results obtained in the batch tests were compared to the observed anaerobic behaviour in the SBR and several interesting observations were apparent. For instance, the release of phosphorus observed in the SBR was only a fraction of the total releasable phosphate. That is, at the end of the anaerobic period in the SBR, a substantial mass of phosphorus remained in the biomass and was not released.

This conclusion was reached from the results of batch tests in which excess COD was provided. Figure 6.4 compares a profile of phosphorus release in the SBR and the results of a batch test on the same sludge, but with an excess of COD. Note the slight delay in the SBR phosphorus release while denitrification occurred. It is clear from these results that the SBR biomass retained stored phosphorus at the end of the anaerobic period, yet that phosphorus was releasable. The results from each experimental period showed the same trend. That is, even when the influent COD:phosphorus ratio was 98 (influent P = 4.5mgP/L), the mass of phosphorus released in the SBR was only a fraction of the phosphorus released in batch tests with excess COD.

Figure 6.5 shows the maximum concentration of phosphorus observed in batch tests during each experimental period when excess COD was provided and also shows the average anaerobic phosphorus release observed in the SBR for the same periods. Using an excess of COD in some of these batch tests also revealed that essentially all of the stored polyphosphate was releasable. This contradicts the findings of Wentzel *et al.* (1989) who found that approximately 70% of the phosphorus contained in the sludge was releasable. They hypothesised that the releasable mass corresponded to low molecular weight polyphosphate and that the remaining portion of the polyphosphate was stable and unreleasable.

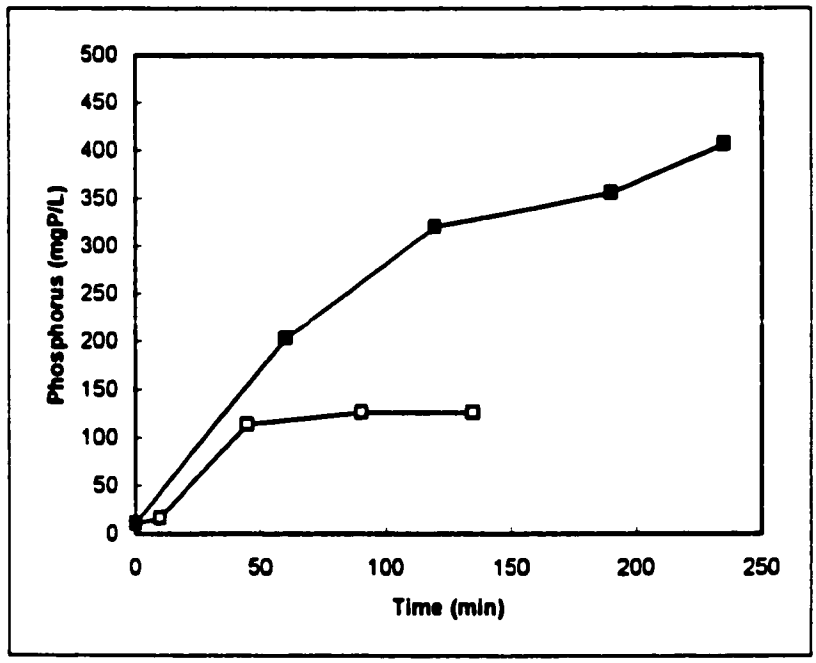

Figure 6.4: Observed anaerobic SBR phosphorus release profile (□) compared to the phosphorus release profile observed the next day in an anaerobic batch test with excess acetate-COD provided (■).

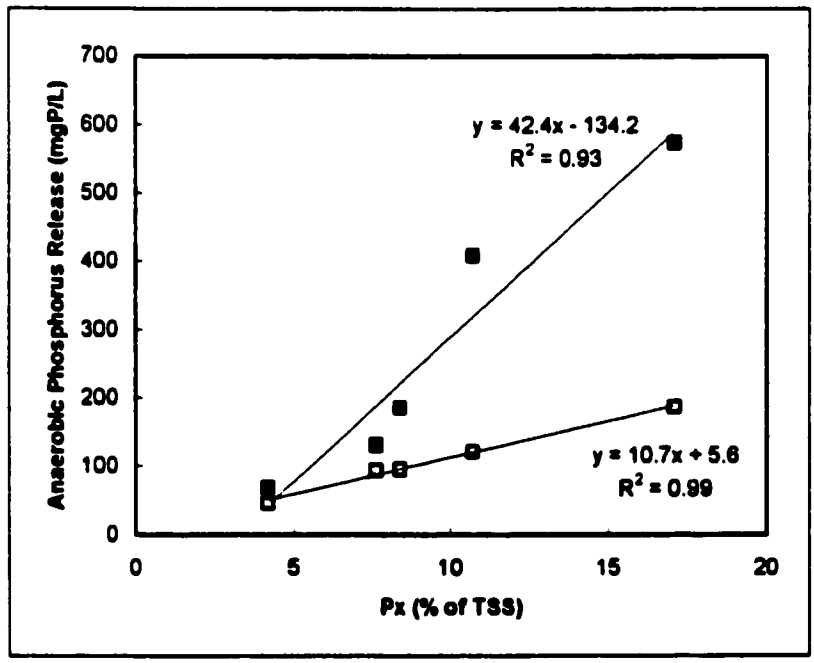

Figure 6.5: Observed anaerobic phosphorus release in the SBR (□) plotted with the maximum phosphorus released during batch testing (■) against the sludge phosphorus content expressed as a function of TSS.

The kinetics of anaerobic phosphorus release and COD uptake at the start of the batch tests also were of interest to this study (Table 6.1). The batch test data showed a general increasing trend in the rate of COD uptake and phosphorus release as the phosphorus content of the sludge (Px) increased. However, the data does not provide a direct cause-and-effect relationship. Nevertheless, the data is in good agreement with previously published data (Table 6.2) and confirms that these rates are not constant but reflect the microbial community which in turn is influenced by the steady state influent characteristics. Figure 6.6 illustrates how the observed rates increased linearly with the phosphorus content of the sludge.

Table 6.1: Rates of COD uptake and phosphorus release observed during batch tests [ND – not determined].

Phosphorus Content - Px (% of TSS)	Maximum P Released (mgP/L)	Maximum Rate of COD Uptake (mgCOD/L/hr)	Maximum Rate of COD Uptake (mgCOD/gVSS/hr)	Maximum Rate of P Release (mgP/L/hr)	Maximum Rate of P Release (mgP/gVSS/hr)	P Release to COD Uptake Ratio (mgP/mgCOD)
4.0	68.0	209.6	103.5	35.3	17.4	0.17
7.6	130.0	ND	ND	86.4	64.6	ND
8.4	185.0	283.2	142.3	100.6	50.6	0.36
10.4	406.8	384.3	201.7	192.7	101.1	0.50
17.0	573.6	417.7	211.4	328.8	166.4	0.79

Table 6.2: Rates of COD uptake and phosphorus release reported in the literature.

Reference (adapted from Liu *et al.* 1997)	Px – Phosphorus Content (% of TSS)	Maximum Rate of COD Uptake (mgCOD/gVSS/hr)	Maximum Rate of Phosphorus Release (mgP/gVSS/hr)
Fukase *et al.* (1984)	2.0	160	3
Mino *et al.* (1987)	3.0	112	14
Mino *et al.* (1987)	6.1	189	52
Smolders *et al.* (1994)	7.0-7.5	109	42-130
Fukase *et al.* (1984)	9.6-10.0	280-370	100-110
Wentzel *et al.* (1988)	14.6-15.4	128-299	114-155

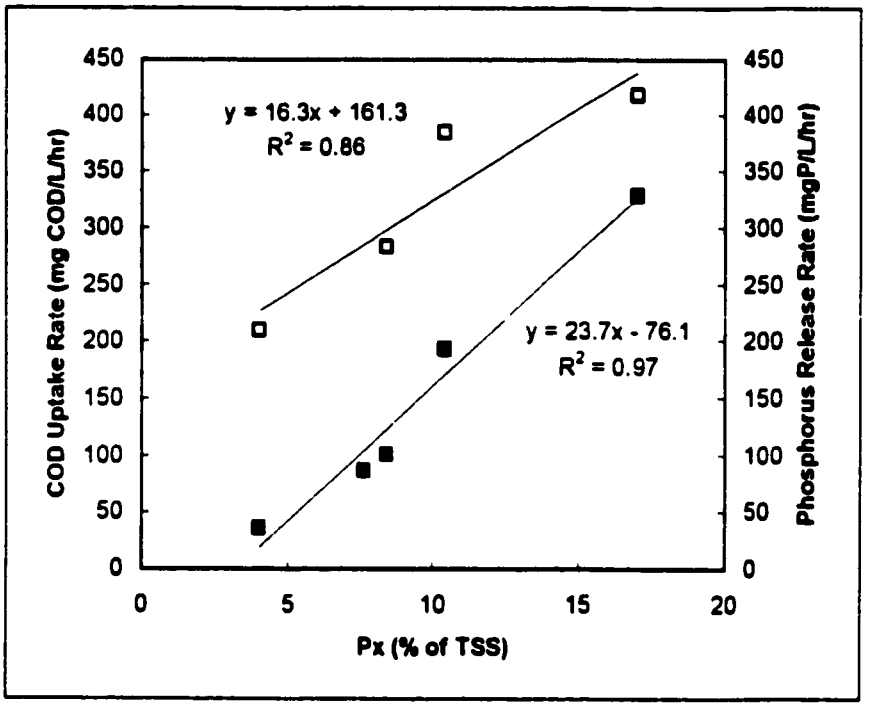

Figure 6.6: Observed initial rate of phosphorus release (■) and COD uptake (□) plotted against the phosphorus content of the sludge.

From the data obtained during the batch tests, the ratio of phosphorus released to COD taken up was calculated. This was accomplished by plotting soluble phosphorus *versus* soluble COD for each sample obtained in the batch test. The regression analysis then gave the observed phosphorus release to COD uptake ratio for a particular batch test. However, recall that different acetate-COD concentrations were studied on subsequent days. This provided several similar but different estimates of the required ratio. Figure 6.7 shows the results of four batch tests performed on four different days with different initial COD concentrations. Note that the slopes of the curves are similar demonstrating that the batch tests were repeatable and that the initial COD concentration had a minimal impact on the observed ratio. The linear response also demonstrates that the decreasing acetate concentration over the course of each test had a negligible impact on the observed ratio. That is, the ratio of phosphorus released to COD

taken up in a batch test remained constant even when the soluble COD decreased to less than 50 mg/L.

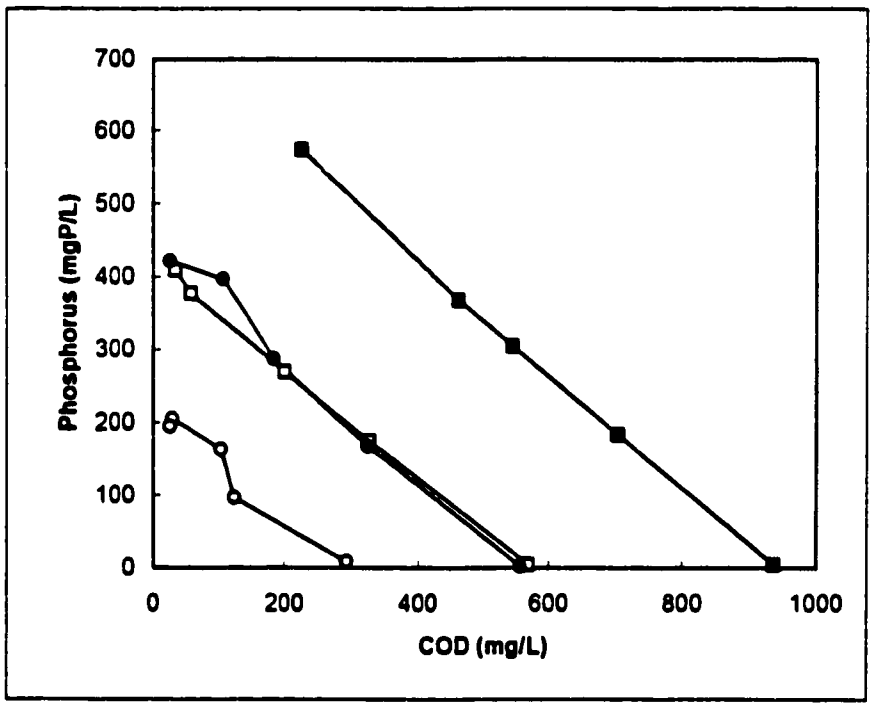

Figure 6.7: Results of four batch tests performed on four separate days during one stable SBR operating period (influent P = 55.5 mgP/L).

One option for the data analysis was to average the slopes calculated on the different days. However several advantages could be gained by creating one data series from all the data. To combine the data from different days, the data were coded (Figure 6.8). Coding involves subtracting from each observation the average of all observations for that experiment. That is, the average phosphorus and average COD concentrations were subtracted from each observation on each day. This then allows all the data to be placed on one graph centred at (0,0) and one best-fit curve to be calculated for all the data. Figure 6.8 shows a schematic illustration of the coding procedure. Two of the four days shown in Figure 6.7 are used to demonstrate. For one test the average phosphorus concentration for the five observations was 286 mgP/L and the average COD was 575 mg/L. These

values were then subtracted from each observation individually. For example, the first sample on that day had a COD of 950 mg/L and a phosphorus concentration of 0 mgP/L. The coded data point then has a COD of 375 mg/L [950 – 575] and a phosphorus of –286 mgP/L [0 – 286]. A similar procedure is followed for each of the points. For instance, the second sample on that day is coded to 125 mg COD/L [700-575] and –104 mgP/L [190 – 286]. For another test the average COD and phosphorus were both approximately 240 mg/L. To code the data on that day 240 mg/L was subtracted from the observed phosphorus and COD concentrations for each sample. The result is that all the data from different tests is centred around the origin. This allows for the calculation of one best-fit curve. Note that one coded relationship was developed for each steady state SBR period. That is, five different coded plots were developed from the batch test data, each associated with a different influent COD:phosphorus ratio being fed to the SBR.

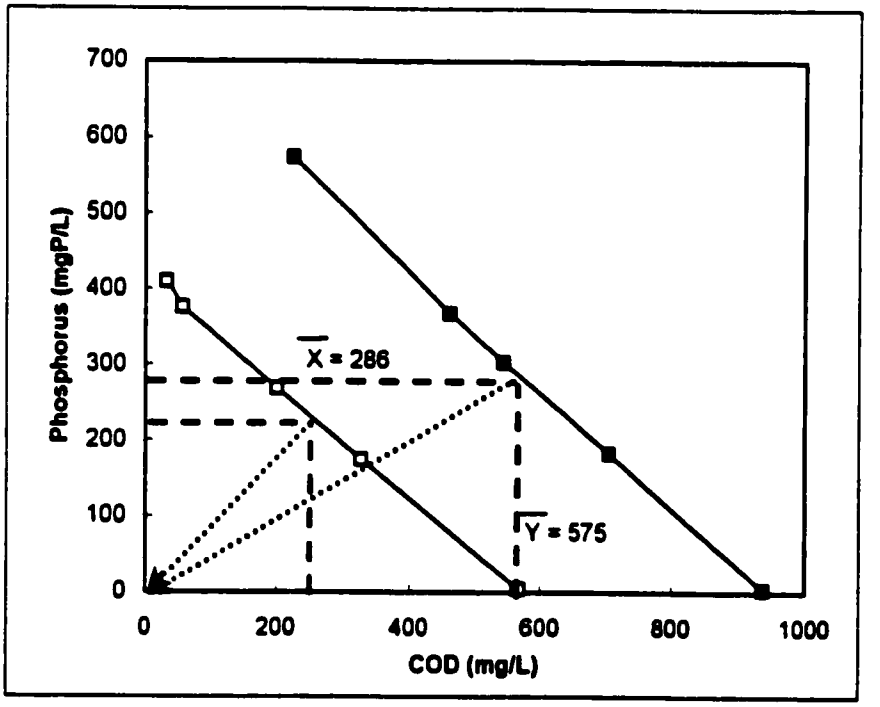

Figure 6.8: Schematic illustration of the data coding procedure.

There is a statistical advantage to this procedure over averaging the values from different tests in that coding increases the degrees of freedom in the least squares regression analysis and decreases the influence of poor data points. Plots of the coded data for experimental periods IV and V are shown in Figure 6.9 and Figure 6.10.

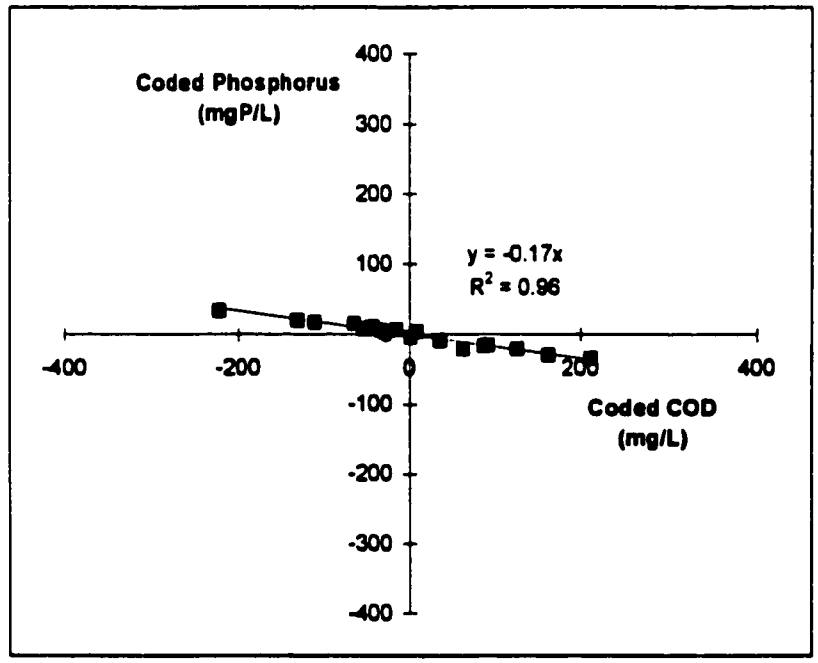

Figure 6.9: Observed anaerobic phosphorus release to COD uptake ratio (0.17 mgP/mgCOD) calculated using coded data from batch tests performed during experimental period IV when the influent phosphorus to the SBR was 4.5mgP/L.

The figures show that the phosphorus and COD values are highly correlated as indicated by the high correlation coefficient. They also illustrate the value of coding the data and reinforce the conclusion that the batch tests done on different days were repeatable and resulted in similar slopes. However, the plots, shown using the same scale, clearly demonstrate that the release/uptake ratios were not the same in these two periods. A different ratio was calculated from the batch test data in each of the periods. In experimental period IV when the SBR received

influent phosphorus at 4.5 mgP/L, the release/uptake ratio was calculated as 0.17 mgP released/mg COD taken up or 0.35 moles of P released/mole of Ac-COD taken up. This compares to 0.79 mgP released/mg COD taken up or 1.63 moles of P released/mole of Ac-COD taken up during experimental period V when the SBR received an influent with 55.5 mgP/L.

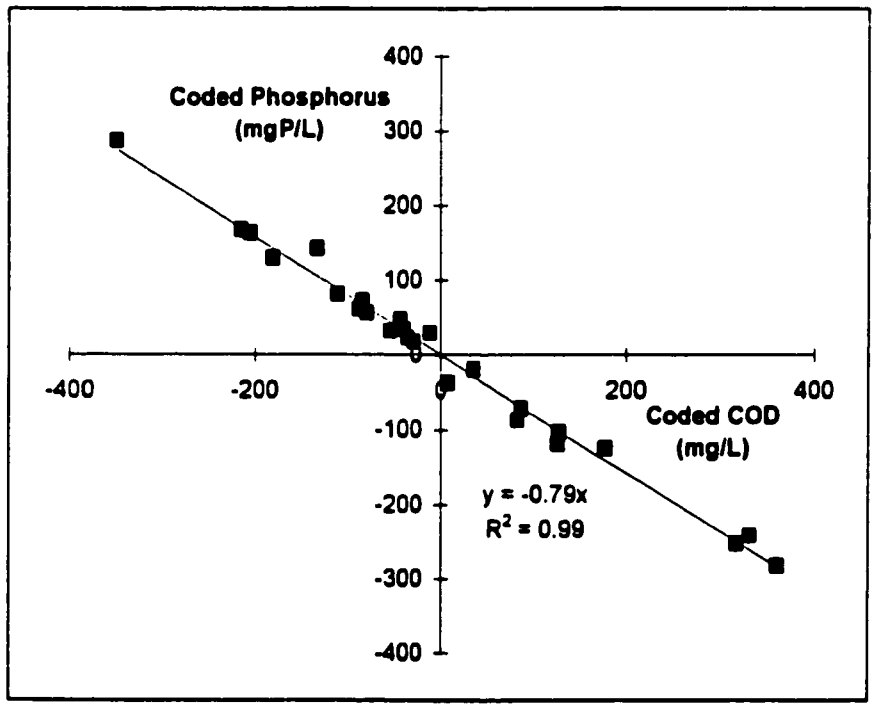

Figure 6.10: Observed anaerobic phosphorus release to COD uptake ratio (0.79 mgP/mgCOD) calculated using coded data from batch tests performed during experimental period V when the influent phosphorus to the SBR was 55.5mgP/L.

Table 6.3 presents the ratios for all of the experimental periods and shows that the data is in good agreement with published ratios (Fukase *et al.*, 1984; Mino *et al.*, 1987; Wentzel *et al.*, 1988; Cech and Hartman 1993; Liu *et al.*, 1997). However, the data tends to run contrary to conventional thinking and the biochemical models which are presently used to describe EBPR activity (Comeau *et al.*, 1986; Wentzel *et al.*, 1986; Mino *et al.*, 1987; Arun *et al.*, 1988). In both of the predominant biochemical EBPR models the ratio of phosphorus release to acetate

uptake is a constant. Each of the models predicts a different ratio but the ratios are constant within each model. That is, the biochemical models as presently understood do not allow for a variable ratio as observed in this and many other studies. Nevertheless, there is mounting evidence to suggest that the ratio is variable and that these biochemical models inadequately predict this behavioural aspect.

Table 6.3: Phosphorus release to acetate uptake ratios reported in the literature and observed in this study.

Reference	Px – Phosphorus Content (% of TSS)	Phosphorus Release to Acetate Uptake Ratio (mol P/mol Ac)
Liu *et al.* (1997)	1.5	0.04
Mino *et al.* (1987)	3.0	0.29
this study	**4.5**	**0.35**
Liu *et al.* (1997)	5	0.57
Liu *et al.* (1997)	6	0.91
Mino *et al.* (1987)	6.1	0.78
Liu *et al.* (1997)	8	0.94
this study	**8.4**	**0.74**
Liu *et al.* (1997)	9.1	1.07
Fukase *et al.* (1984)	9.4	0.84
Cech and Hartman (1993)	9.6	0.80
Liu *et al.* (1997)	10	1.01
this study	**10.4**	**1.03**
Liu *et al.* (1997)	11	1.23
Liu *et al.* (1997)	12.1	1.32
Wentzel *et al.* (1988)	14.6-15.4	1.07-1.84
this study	**17.0**	**1.63**

To illustrate the variable nature of this ratio more clearly, the data in Table 6.3 is plotted in Figure 6.11 and a regression line is drawn through the data points determined in this study. Plotted in this manner, the data shows a high correlation coefficient and demonstrates the linear relationship between the sludge's phosphorus content and the release/uptake ratio.

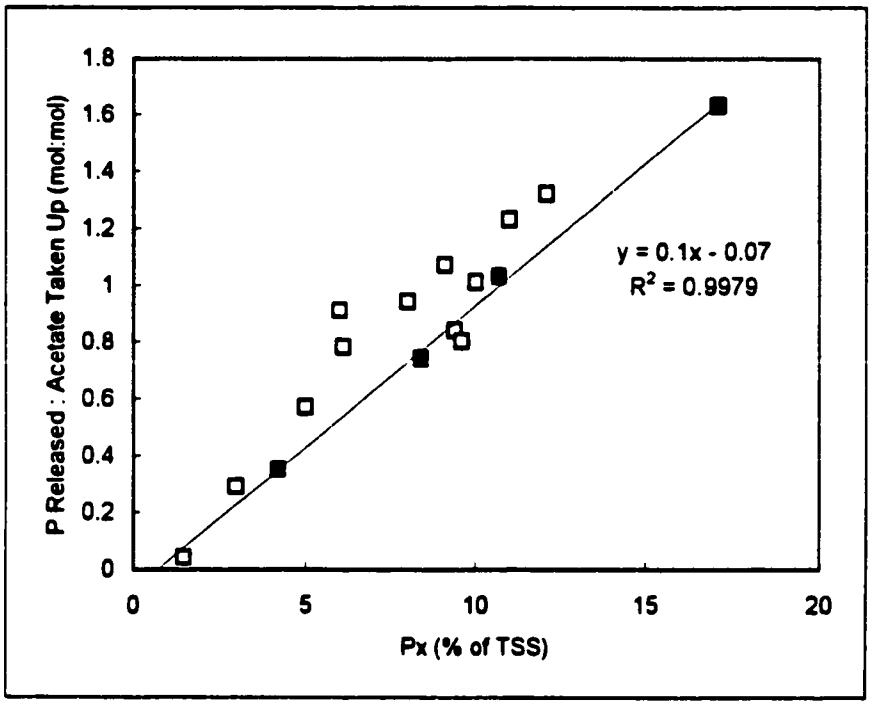

Figure 6.11: Phosphorus release to acetate uptake ratios as reported in the literature (□) and observed in this study (■). Regression line drawn through the ratios observed in this study only.

CONCLUSION

A sequencing batch reactor (SBR) was operated over an eight month period at five different influent COD:phosphorus ratios (the complete operation is described in *Part I* of this paper series). The SBR exhibited EBPR activity for the entire experimental period and served as the source of sludge for a series of batch tests which were designed to investigate the anaerobic behaviour of the microbial population in a EBPR system. The results of the anaerobic batch tests have been presented and clearly show that the steady state ratio of influent phosphorus to influent COD has a direct influence on both the stoichiometric and kinetic microbial behaviour within a EBPR system. An anaerobic batch test protocol was developed with special attention given to maintaining an anaerobic environment

in the testing vessels because it was demonstrated that even a small infiltration of oxygen was sufficient to disrupt the phosphorus release behaviour. The results showed that anaerobic rates of COD uptake and phosphorus release increase linearly with the phosphorus content of the sludge. The ratio of phosphorus released to COD taken up also increases linearly with the sludge phosphorus content.

Analysis of the results of this study indicate that the biochemical models presently used to describe EBPR activity alone are inadequate and are unable to predict bulk solution observations. That is, these models are unable to predict variable rates of uptake and release based solely on a change in influent characteristics. Similarly, the models do not predict variable ratios of phosphorus release to COD uptake. These conclusions strongly suggest that changes be made to the way in which EBPR activity is viewed, and specifically anaerobic substrate uptake and phosphorus release. Part III of this paper series addresses the inadequacies in the biochemical models and describes a potential alternative view. Specifically, a two organism mechanism is described and provides the most plausible explanation for the changing rates and ratio.

ACKNOWLEDGEMENTS

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CHAPTER SEVEN

INFLUENCE OF INFLUENT PHOSPHORUS CONCENTRATION ON AN EXCESS BIOLOGICAL PHOSPHORUS REMOVAL SEQUENCING BATCH REACTOR

PART III: BIOCHEMICAL EXPLANATION

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INFLUENCE OF INFLUENT PHOSPHORUS CONCENTRATION ON AN EXCESS BIOLOGICAL PHOSPHORUS REMOVAL SEQUENCING BATCH REACTOR

PART III: BIOCHEMICAL EXPLANATION

John B. Copp & Peter L. Dold

Department of Civil Engineering, McMaster University
1280 Main Street West, Hamilton, Ontario, Canada L8S 4L7

ABSTRACT

A biochemical explanation of excess biological phosphorus removal (EBPR) in anaerobic-aerobic activated sludge systems is presented. The biochemical model is closely based on previously reported mechanisms, but proposes that significant populations of glycogen accumulating organisms (GAOs) and polyphosphate accumulating organisms (PAOs) co-exist in these systems. It is proposed that observed behaviour in EBPR systems is the net result of the combined behaviour of both GAOs and PAOs and that both types of organisms exist in the mixed microbial community. It is also proposed that pH regulation plays an important role in observed anaerobic behaviour and should be accounted for in the analysis of experimental data.

Key words: activated sludge, excess biological phosphorus removal, biological nutrient removal, sequencing batch reactor, biochemical model, polyphosphate, glycogen, polyhydroxyalkanoate

INTRODUCTION

Since Comeau *et al.* (1986) first introduced their biochemical model of excess biological phosphorus removal (EBPR), a substantial amount of research has resulted in various changes to the original model (Wentzel *et al.*, 1986; Mino *et al.*, 1987; Arun *et al.*, 1988; Wentzel *et al.*, 1991; Satoh *et al.*, 1994; Smolders *et al.*, 1994). However, the essence of the original model remains. The model described the anaerobic uptake of acetate and storage as polyhydroxybutyrate (PHB). However, other substrates and storage compounds have since been identified. More recent models recognise that many short chain volatile fatty acids (SCVFA) are sequestered anaerobically by the organisms in EBPR systems and stored as many different polyhydroxyalkanoates (PHA) including PHB. Figure 7.1 illustrates the steps in the conversion of acetate to PHB, but similar steps are involved in the conversion of other substrates and in the production of other PHAs.

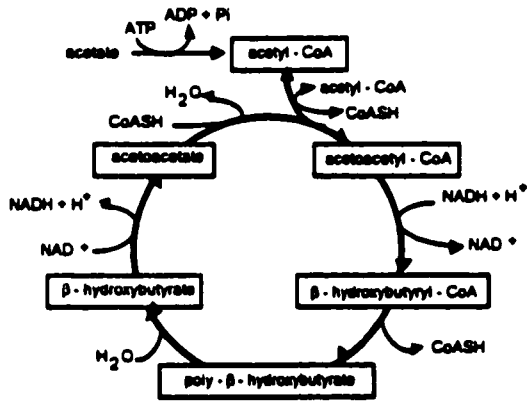

Figure 7.1: Biochemical pathway for the production of poly-β-hydroxybutyrate (PHB) from acetate showing acetate activation with ATP and NADH sequestration (Gottschalk, 1986).

Figure 7.1 demonstrates that the conversion to PHA occurs in a multi-step reaction sequence that requires reducing equivalents. By sequestering reducing equivalents through this process, PHA acts as an electron sink and energy storing compound.

As Figure 7.1 shows, one of the most significant features in all of the EBPR models is the recognition that adenosine triphosphate (ATP) and reducing equivalents (NADH) are required for the anaerobic conversion of acetate (SCVFA) to PHB (or PHA). In this process, ATP is required for SCVFA activation. Activation converts the SCVFA into useful biochemical pathway intermediates by combining the SCVFA with coenzyme A (CoA) through an ATP requiring reaction. Figure 7.1 shows this conversion for acetate which results in the production of acetyl-CoA. This conversion, in addition to activating the SCVFA, also traps the substrate inside the cell. That is, once activated in this manner, the coenzyme A prevents that molecule's transport across the membrane.

Comeau/Wentzel Anaerobic Model

The biochemical explanations for the anaerobic stage in EBPR systems have focused on the production of ATP and NADH necessary to satisfy the requirements for PHA synthesis. The Comeau and Wentzel models (Wentzel *et al.*, 1986) – hereinafter referred to as the Comeau/Wentzel model (Figure 7.3) – both proposed that an active tricarboxylic acid (TCA) cycle provides both the reducing equivalents for PHA synthesis and a portion of the required ATP (Figure 7.2). This follows from the TCA cycle stoichiometry that the oxidation of one mole of acetyl-CoA produces four moles of reducing equivalents. It was postulated that a portion of the SCVFA taken up anaerobically (for acetate, 0.11 moles per mole taken up) is shunted to the TCA cycle for oxidation to CO_2. The diversion of this substrate to the TCA cycle produces the reducing equivalents needed to convert the remaining SCVFA to PHA. That is, in the case of acetate,

for each mole sequestered, 0.11 moles is diverted to the TCA cycle where the equivalent of 0.44 moles of NADH are produced. The NADH is then available for the conversion of the remaining acetate (0.89 moles) to PHB *via* the cycle shown in Figure 7.1.

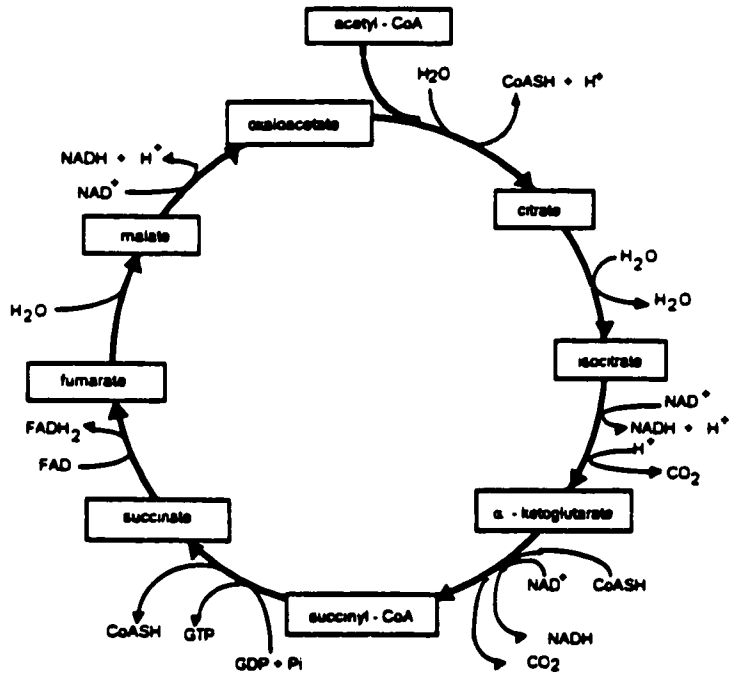

Figure 7.2: Biochemical reactions of the tricarboxylic acid (TCA) cycle.

The diversion of substrate to the TCA cycle also results in the production of ATP. Guanine triphosphate (GTP) is generated in the succinyl-CoA to succinate step [one mole of GTP is produced for each mole of acetate oxidised in this process], and ATP is known to be produced from GTP in a neutral phosphorus transfer mechanism between adenosine diphosphate (ADP) and GTP. However, the TCA cycle provides only a fraction of the required ATP. The remaining ATP is provided by polyphosphate cleavage. In this reaction, ATP is generated through another phosphorus transfer mechanism in which one monomer unit of polyphosphate is transferred to ADP according to the following reaction:

$$PolyP_{(n+1)} + ADP \leftrightarrow ATP + PolyP_{(n)}$$

A characteristic of the anaerobic stage in EBPR systems is an increase in the bulk concentration of soluble phosphorus and a decrease in the concentration of internal polyphosphate which is consistent with the model described by Comeau and Wentzel. The model recognises that following ATP synthesis, the subsequent use of ATP for substrate activation results in the production of the cleavage products, ADP and inorganic phosphate. It is believed that the build-up of phosphate inside the cell triggers the release of phosphorus into the bulk liquid. According to Figure 7.1, one mole of ATP is required for each mole of acetate internalised. This suggests that for each mole of acetate taken up, one mole of phosphorus should be released to the bulk liquid. However, the ATP produced as a result of the TCA cycle is internally regenerated and will not add to the pool of internal inorganic phosphorus. Therefore, only the mass of inorganic phosphorus produced as a result of polyphosphate cleavage will accumulate in the cell. Hence, for each mole of acetate taken up anaerobically, the Comeau/Wentzel model suggests a release of 0.89 moles of phosphorus (0.89 x 31/64 mgP/mgCOD). Combining the reactions for the synthesis of a mole of PHB gives:

$2\ C_2H_4O_2 + 2\ ATP\ (polyP) + NADH$	$\rightarrow C_4H_6O_2 + 2\ ADP + 2\ Pi + NAD + 2\ H_2O$
$0.25\ C_2H_4O_2 + 0.25\ ATP + 0.5\ H_2O + NAD$	$\rightarrow 0.5\ CO_2 + 0.25\ ATP\ (TCA\ cycle) + NADH$
$2.25\ C_2H_3O_2 + 2\ ATP\ (polyP)$	$\rightarrow C_4H_6O_2 + 2\ ADP + 2\ Pi + 0.5\ CO_2 + 1.5\ H_2O$

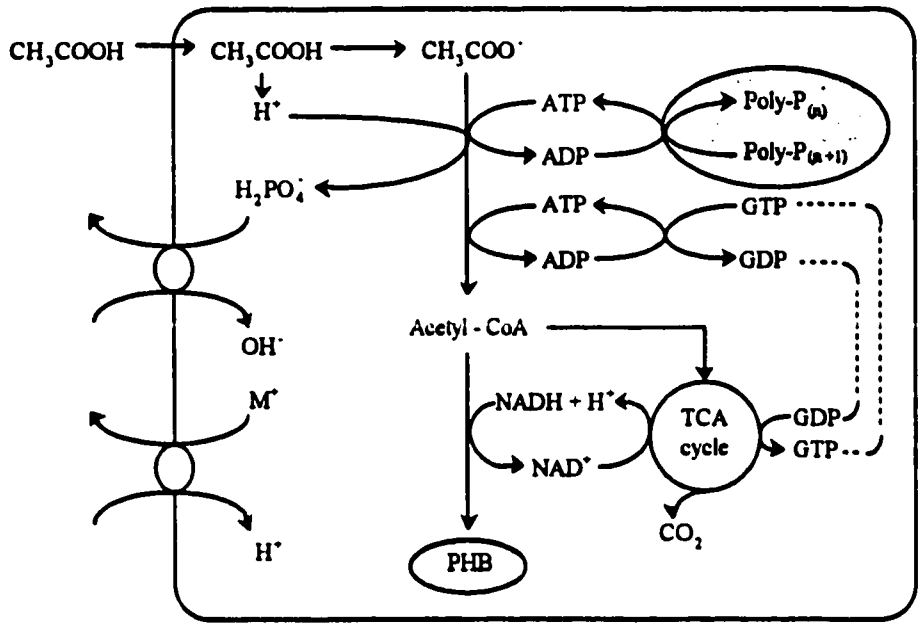

Figure 7.3: Schematic representations of the Comeau/Wentzel model for the anaerobic stage of a biological excess phosphorus removal process.

Mino Anaerobic Model

In more recent EBPR models, the production of NADH through the TCA cycle has been challenged as researchers have postulated inhibition of this cycle under anaerobic conditions (Arun *et al.*, 1988). Also, observations of intracellular carbohydrate reductions during the anaerobic stage are not explained by the Comeau/Wentzel model. Consequently, the Mino model (Figure 7.4) proposed the oxidation of internal carbohydrate stores (i.e. glycogen), through the Emden-Meyerhoff-Parnas (EMP) – glycolytic – pathway, (Mino *et al.*, 1987; Arun *et al.*, 1988) as the source of reducing equivalents. From a stoichiometric analysis, it can be shown that oxidation of a mole of glycogen to two moles of acetyl-CoA through the EMP pathway produces four moles of NADH; enough to reduce eight moles of acetyl-CoA to PHB.

By oxidising carbohydrates through the EMP pathway the cell also gains a source of ATP as three moles of ATP are generated for each mole of glycogen oxidised. Also, ATP produced from glycogen oxidation results in the recycling of inorganic phosphate in much the same way as phosphate is recycled in the TCA cycle of the Comeau/Wentzel model. Therefore, by producing ATP through carbohydrate oxidation, the cell reduces its dependence on polyphosphate as a source of energy and reduces the build-up of inorganic phosphate. Anaerobically then, for each mole of acetate taken up, the Mino model predicts the release of 0.50 moles of phosphorus (0.5 x 31/64 mgP/mgCOD). Combining the reactions of the Mino model to give one mole of PHB gives:

$$1.5\ C_2H_4O_2 + 0.75\ ATP\ (glycolysis) + 0.75\ ATP\ (polyP) + 0.75\ NADH \rightarrow 0.75\ C_4H_6O_2 + 1.5\ ADP + 1.5\ Pi + 0.75\ NAD + 1.5\ H_2O$$

$$0.25\ C_6H_{10}O_5 + 0.75\ ADP + 0.75\ Pi + 0.75\ NAD + 0.25\ H_2O \rightarrow 0.25\ C_4H_6O_2 + 0.75\ ATP + 0.75\ NADH + 0.5\ CO_2$$

$$1.5\ C_2H_4O_2 + 0.25\ C_6H_{10}O_5 + 0.75\ ATP\ (polyP) \rightarrow C_4H_6O_2 + 0.75\ ADP + 0.75\ Pi + 0.5\ CO_2 + 1.25\ H_2O$$

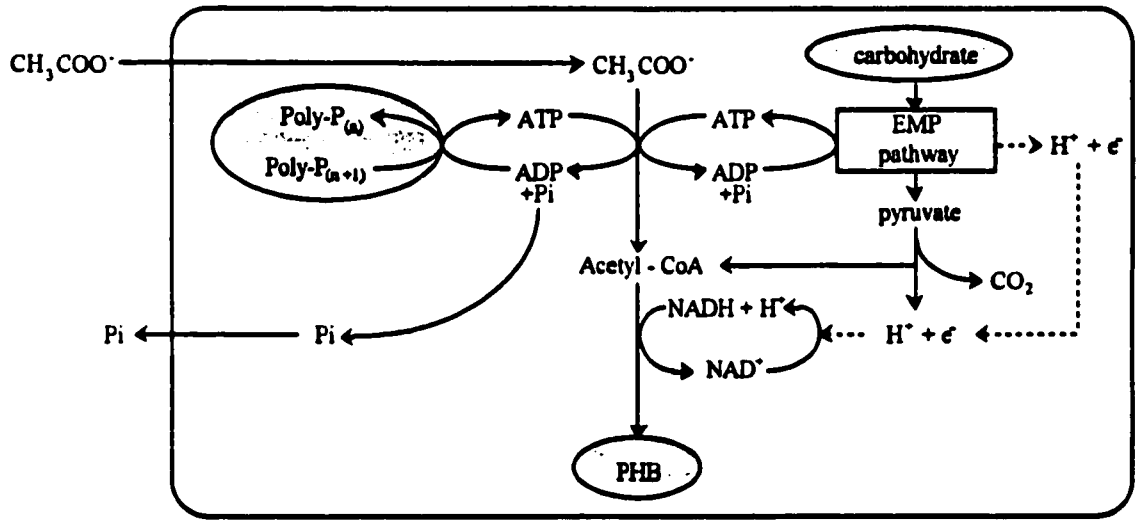

Figure 7.4: Schematic representations of the Mino model for the anaerobic stage of a biological excess phosphorus removal process.

Carbohydrate oxidation through the EMP pathway as proposed in the Mino model was modified to the Entner-Douforoff (ED) pathway by Wentzel *et al.* (1991). Citing microbiological evidence, Wentzel *et al.* (1991) concluded that the enzymes required for the EMP pathway were not produced in *Acinetobacter spp.* – the organism believed to be responsible for EBPR activity (Lotter *et al.*, 1985). Rather, carbohydrate oxidation in *Acinetobacter spp.* occurs through the ED pathway, hence the modification to the model. However, there is mounting evidence which suggests that *Acinetobacter spp* plays only a limited role in EBPR activity (Brodisch, 1985; Ubukata and Takii, 1994; Blackall *et al.*, 1997; Bond *et al.*, 1997), thus calling into question the basis for changing the carbohydrate oxidation pathway as described in the original Mino model.

From an analysis of the two models, the impact of carbohydrate oxidation can be calculated. The Mino model predicts 0.50 moles of phosphorus released for each mole of acetate taken up (Mino *et al.*, 1987; Arun *et al.*, 1988), while the Comeau/Wentzel model predicts 0.89 moles of phosphorus released for each mole of acetate taken up (Comeau *et al.*, 1986; Wentzel *et al.*, 1986). Numerous studies have been initiated to determine which model best describes experimental data and much of the literature focuses on this ratio as a defining aspect. That is, if the observed ratio approximates 0.5, then the authors generally support the Mino model and similarly if the observed data suggests a ratio of 0.89 then support for the Comeau/Wentzel model is reported. However, rarely does the data present a clear ratio. Rather, a range of ratios have been observed with no definitive conclusion as to the correct model (Fukase *et al.*, 1984; Arvin and Kristensen, 1985; Wentzel *et al.*, 1985; Mino *et al.*, 1987; Wentzel *et al.*, 1989; Cech and Hartman 1993; Satoh *et al.*, 1994; Smolders *et al.*, 1994; Liu *et al.*, 1997; Schuler and Jenkins, 1997; Copp and Dold, 1998b). The literature describes anaerobic

molar phosphorus release/COD uptake ratios from 0 to 1.84 which spans well beyond the ratios predicted by either biochemical model.

The issue of COD uptake and phosphorus release is further complicated by observations of stable anaerobic COD uptake without phosphorus release or excess phosphorus removal. Both Satoh *et al.* (1994) and Cech and Hartman (1993) operated anaerobic/aerobic systems which demonstrated anaerobic substrate uptake without phosphorus release – essentially a molar ratio of 0. These systems did not exhibit EBPR activity as polyphosphate was not accumulated; however, intracellular carbohydrates were consumed during the uptake of substrate.

To explain the results, Satoh *et al.* (1994) proposed the production of a mixture of PHB and polyhydroxyvalerate (PHV) from acetate and glycogen making use of the succinate-propionate fermentation pathway (SPF). Stoichiometrically, this pathway addresses several important considerations. Recall that NADH and ATP are required for PHA synthesis and to explain anaerobic decreases in intracellular carbohydrates, Mino proposed the use of the EMP pathway with additional ATP being provided by polyphosphate hydrolysis. However, polyphosphate was not present in the cultures studied by Satoh *et al.* (1994) or Cech and Hartman (1993). Use of the SPF pathway alleviates this problem. That is, a stoichiometric balance both in terms of ATP and NADH can be attained by combining the SPF pathway with those previously discussed without the need for polyphosphate energy (Satoh *et al.*, 1992; Satoh *et al.*, 1994).

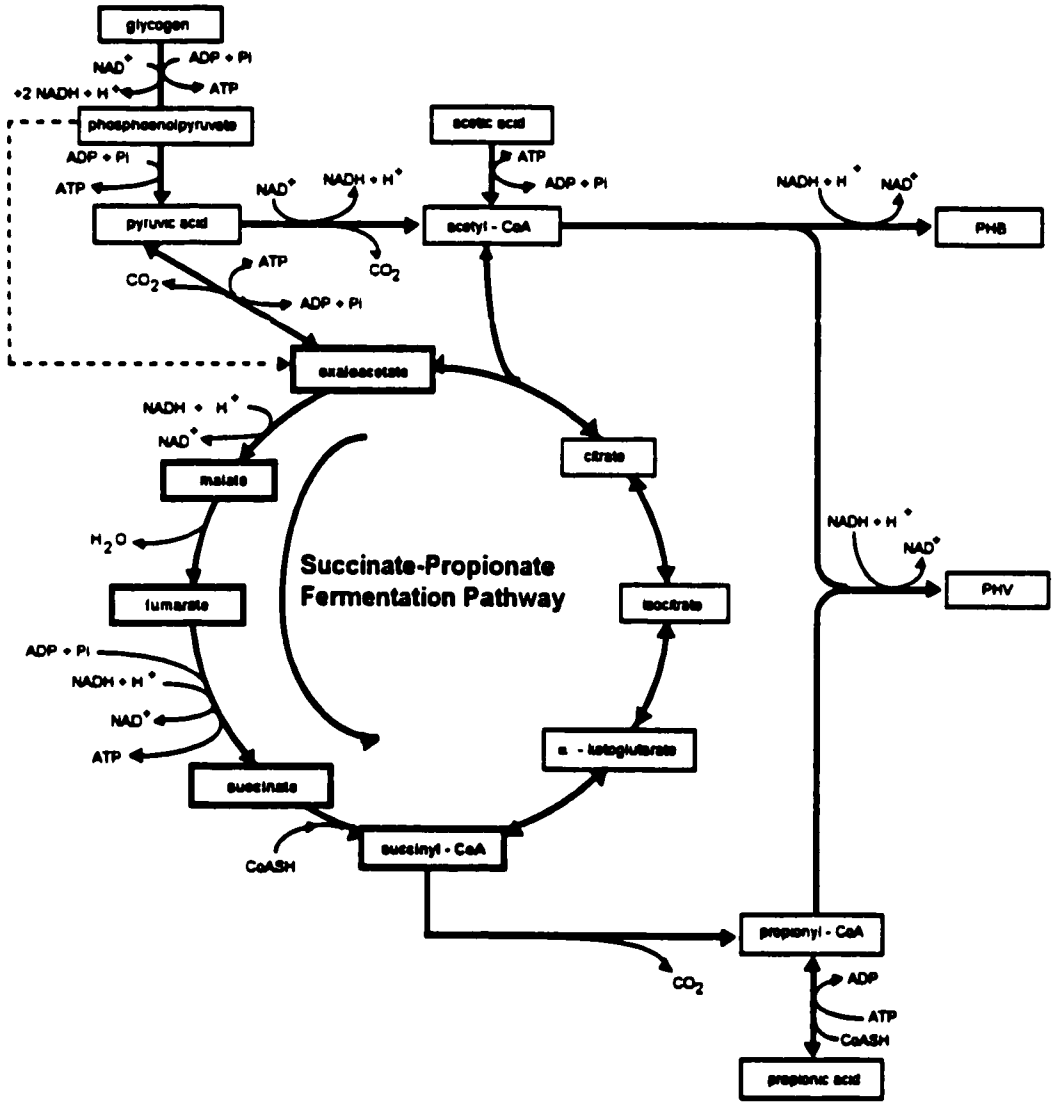

Figure 7.5: Satoh *et al.* (1994) model for PHB and PHV synthesis in the absence of polyphosphate energy.

In the model, Satoh *et al.* (1994) proposed that glycogen is oxidised through the EMP pathway producing phosphoenolpyruvate and pyruvic acid as intermediates. These compounds are subsequently converted to oxaloacetate which enters the SPF pathway as shown in Figure 7.5. The proposed fermentation pathway is essentially a reversal of several steps in the TCA cycle and analogous to the reactions that occur in propionibacteria (propionate producing bacteria).

Oxaloacetate is reduced to succinyl-CoA through malate, fumarate and succinate utilising two moles of NADH in the process. During these steps, the reduction of fumarate to succinate results in the production of ATP through electron transport phosphorylation (Gottschalk, 1986). Satoh *et al.* (1994) suggest that ATP is utilised in the production of succinyl-CoA from succinate, but it is unclear if ATP is required for this step and hence has not been included in Figure 7.5 (Sokatch, 1973; Gottschalk, 1986).

Anaerobic pH Regulation

Within the literature on EBPR systems, observed ratios of anaerobic phosphorus release to COD taken up suggest that EBPR systems are more complicated than predicted by the Mino or Comeau/Wentzel biochemical models alone. The Comeau/Wentzel model predicts a ratio of 0.89 moles of phosphorus released per mole of acetate taken up, but ratios in excess 1.8 have been observed experimentally (Wentzel *et al.*, 1989). To explain observed ratios larger than predicted by the models, researchers have developed theories around other cellular ATP uses. For instance, it has been suggested that the EBPR release/uptake ratio may be confounded by pH regulation and membrane transport considerations as the role of ATP in these processes is in dispute (Wentzel *et al.*, 1986; Matsuo *et al.*, 1992; Smolders *et al.*, 1994; Fleit, 1995). Smolders *et al.* (1994) found that the uptake/release ratio varied with pH and attributed the differences to a variable energy requirement for acetate transport across the membrane in the dissociated and negatively charged form. However, in a biochemical review of EBPR results, Fleit (1995) disputed this theory. Fleit (1995) pointed out that if acetate is actively transported into the cell then it should be possible to isolate mutants which are unable to transport acetate. However, acetate transport mutants have not been described in the literature. Alone, the lack of a mutant does not rule out the possibility that acetate is actively transported but Filipe and Daigger (1998) point out that acetate uptake does not

exhibit enzyme saturation kinetics which does suggest that acetate [likely in the undissociated form] passively diffuses through the membrane. Also, the range of uptake/release ratios in the literature can not be explained solely by a transport model based of pH considerations as suggested by Smolders *et al.* (1994).

Fleit (1995) postulated that internal pH regulation in EBPR organisms may play a crucial role in anaerobic phosphorus release. If it is assumed that substrate uptake occurs through passive SCVFA diffusion, then an examination of the biochemical models reveals a potential pH problem. In the case of acetic acid, one proton enters the cells with each acetate internalised. Once inside the cell, the acetic acid will immediately dissociate releasing a proton. This in turn will decrease the intracellular pH and reduce the proton motive force (PMF) across the cell membrane. To maintain the PMF and prevent a drop in intracellular pH, the cell must expel the proton. Figure 7.3 demonstrates that one potential expulsion mechanism may be as phosphate ($H_2PO_4^-$), but this requires that proton expulsion be dependent on the activation of acetate to acetyl-CoA and ultimately on the kinetics of PHA production. In this scenario, SCVFA diffuses into the cell unhindered, irrespective of cellular activity. Hence, if PHA production exceeds or equals that diffusion then this mechanism provides a satisfactory result. However, it is conceivable that SCVFA diffusion will exceed PHA production causing a decrease in intracellular pH and inhibition of cell functions. Also, phosphate transport studies suggest that the preferred mechanism of phosphate transport is in the divalent form (HPO_4^{2-}) associated with a divalent metal cation (van Veen *et al.*, 1993; van Veen *et al.*, 1994). Alternatively, Fleit (1995) identified specific pH control mechanisms to translocate protons out of the cell, and pertinent to this discussion is the H^+/ATPase enzyme complex.

Normal aerobic operation of the H^+/ATPase complex results in the production of ATP from the energy stored in the proton gradient across the membrane [high H^+

outside]. However this ATPase complex can be used in reverse if necessary to generate a proton gradient by coupling proton expulsion to ATP hydrolysis. Anaerobic decay of the PMF and proton gradient due to SCVFA influx in EBPR systems may result in such an instance and cause a reversal of the complex. Hence, ATP would be hydrolysed to pump protons from the cell and would act as an intracellular pH and PMF control mechanism. Recently, Filipe and Daigger (1998) theoretically quantified the moles of ATP required for PMF maintenance and presented modelling results which supported the concept that anaerobic acetate uptake by EBPR organisms requires ATP hydrolysis for PMF maintenance. In EBPR systems, the ATP required for cell maintenance is generated from polyphosphate hydrolysis and therefore adds to the load of inorganic phosphate inside the cell. Experimental observations suggest that for each mole of COD taken up anaerobically, as much as 1 mole of ATP is hydrolysed in excess of the requirements for PHA synthesis (Wentzel *et al.*, 1989).

In EBPR systems, the direct consequence of this added ATP hydrolysis is an increase in released phosphorus and an increased ratio of phosphorus released to COD taken up under anaerobic conditions. However, in systems exhibiting anaerobic substrate uptake without the production of polyphosphate, the consequences are more complicated. The stoichiometric analysis by Satoh *et al.*, (1994) reveals that anaerobic uptake of acetate without polyphosphate yields:

12 acetate + 5 glycosyl units → 11 3-hydroxyalkanoate units (8 – PHB + 3 – PHV)

Recall, that ATP hydrolysis in the production of succinyl-CoA is in dispute; hence a similar analysis can be done assuming no hydrolysis of ATP in the production of succinyl-CoA from succinate. That analysis results in the following:

6 acetate + 6 ATP	→	6 acetyl-CoA + 6 ADP
1 glycosyl unit + 3 ADP	→	2 acetyl-CoA + 3 ATP + 8 (H)
1 glycosyl unit + 3 ADP	→	acetyl-CoA + propionyl-CoA + 3 ATP + 2 (H)
9 acetyl-CoA + propionyl-CoA + 10 (H)	→	5 hydroxyalkanoate units
6 acetate + 2 glycosyl units	→	5 hydroxyalkanoate units (4 – PHB + 1 – PHV)

The significance of such an analysis relates to the relative proportions of hydroxyalkanoate units produced. On a molar basis, the model proposed by Satoh *et al.*, (1994) predicts the accumulation of 27% PHV and 73% PHB from anaerobic acetate uptake without polyphosphate energy input. This contrasts the second case where 20% PHV is predicted. The uncertainty in the actual biochemical stoichiometry of these steps makes an accurate prediction impossible without further study. Complicating this discussion is the issue of pH regulation within the cell and the role, if any, that ATP plays in pH maintenance.

Table 7.1 shows the stoichiometric impact that pH regulation (through ATP hydrolysis) has on the proportional production of PHB and PHV using the two models described above. The results show that a wide range of potential proportions can be attained depending on the requirement for cellular pH regulation. As expected, both models predict that as the requirement for pH maintenance energy increases, so does the proportion of PHV produced.

Table 7.1: Theoretical effect of ATP hydrolysis for pH regulation on the proportion of PHB and PHV produced in a system without polyphosphate accumulation under anaerobic conditions.

Satoh Model for GAOs

pH control ratio (mol P/ mol Ac Upt)	Resulting Equation	Molar Ratios			mol %	
		Glycogen consumed	Acetate Uptake	PHA produced	PHB	PHV
0	5 Gly + 12Ac --> 3 PHV + 8 PHB	1	2.4	2.2	73	27
0.5	4 Gly + 6 Ac --> 3 PHV + 4 PHB	1	1.5	1.8	57	43
0.8	49 Gly + 60 Ac --> 39 PHV + 40 PHB	1	1.2	1.6	51	49
1	11 Gly + 12 Ac --> 9 PHV + 8 PHB	1	1.1	1.5	47	53

Modified Satoh Model for GAOs

pH control ratio (mol P/ mol Ac Upt)	Resulting Equation	Molar Ratios			mol %	
		Glycogen consumed	Acetate Uptake	PHA produced	PHB	PHV
0	2 Gly + 6 Ac --> 1 PHV + 4 PHB	1	3	2.5	80	20
0.5	3 Gly + 6 Ac --> 2 PHV + 4 PHB	1	2	2	67	33
0.8	18 Gly + 30 Ac --> 13 PHV + 20 PHB	1	1.7	1.83	61	39
1	4 Gly + 6 Ac --> 3 PHV + 4 PHB	1	1.5	1.75	57	43

PROPOSED MODEL

Observations of anaerobic COD uptake with and without phosphorus release have complicated understanding of EBPR systems such that a uniformly applicable biochemical model has not been developed. The models presented above have been calibrated to numerous studies but each is dependent on the system(s) to which they are being applied. As yet, no biochemical model has been applied successfully to all of the data. The biochemical model proposed in this paper is a combination of many aspects previously proposed and verified. The objective was to develop one comprehensive biochemical explanation.

Conceptually, the most significant feature of this model is that it is a two-organism model. That is, a separation between glycogen accumulating organisms (GAOs) and polyphosphate accumulating organisms (PAOs) is proposed. For several years researchers have reported observations of 'G' bacteria or alternatively GAOs. These GAOs take up substrate and synthesise PHA anaerobically in competition with PAOs. However, GAOs do not store polyphosphate and hence do not contribute to the removal of excess phosphorus. It is postulated that a competitive advantage over GAOs is gained by the PAOs in the presence of excess phosphorus. But, as the influent phosphorus decreases in relation to the available readily biodegradable COD, GAOs proliferate on anaerobically available COD. This in itself is not original, but it is proposed that the organisms co-exist in anaerobic-aerobic systems and that it is the interaction and kinetics of these organisms which dictate observed behaviour. It is proposed that significant populations of GAOs and PAOs exist in most anaerobic-aerobic systems and only in systems which are stressed does one of the populations become negligible. This suggestion runs contrary to conventional thinking - and the premise on which most biochemical models are based - in which well operating EBPR systems are believed to be almost entirely populated by polyphosphate accumulating organisms (Brdjanovic *et al.*, 1997). Recently, debate on the denitrifying ability of PAOs has surfaced, but that particular aspect is beyond the scope of this preliminary model.

Anaerobic Behaviour

Figure 7.6 presents the biochemical behaviour of PAOs under anaerobic conditions. The model closely resembles those of Comeau/Wentzel (Comeau *et al.*, 1986; Wentzel *et al.*, 1986) with a minor variation for proton transport and pH regulation. For the PAOs, it is proposed that SCVFA diffuses into the cell without the expenditure of energy and is converted to PHA making use of NADH generated by the TCA cycle and ATP produced as a consequence of the TCA

cycle and through hydrolysis of polyphosphate. The proposed mechanisms are analogous to those described in the Comeau/Wentzel model, and it is accepted that the hydrolysis of ATP results in an intracellular increase in inorganic phosphorus which is subsequently released from the cell along with a metal counter ion. Based on the empirical evidence presented previously, the proposed model also accepts that ATP hydrolysis is required for proton expulsion and pH regulation within the cells. Therefore, to prevent internal acidification, protons which diffuse into the cell with the SCVFA are pumped out of the cell through a reversal of the H^+/ATPase proton pump. The proposed model attributes added ATP hydrolysis to pH regulation processes and suggests that approximately 1 mole of ATP is required per mole of COD taken up, but further study will be necessary to determine the extent to which ATP is used for this purpose.

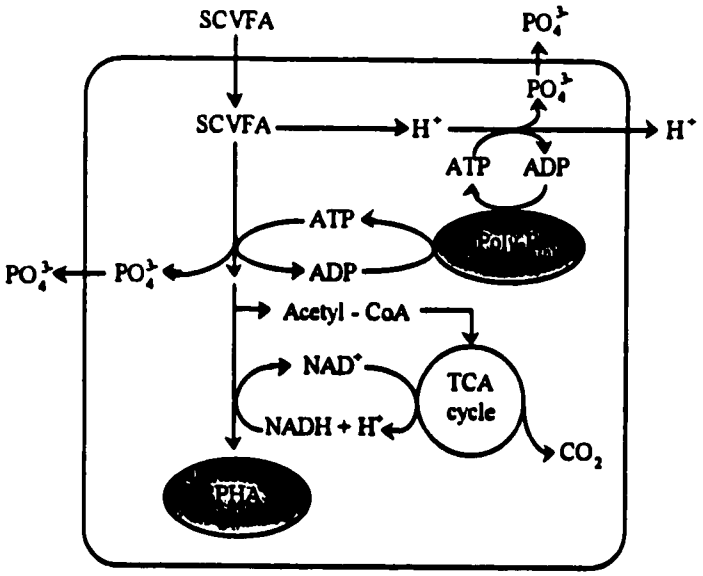

Figure 7.6: Biochemical explanation of anaerobic SCVFA uptake and PHA synthesis in polyphosphate accumulating organisms.

For the GAOs, SCVFA enters the cell and is converted to PHA utilising the energy and reducing equivalents generated by carbohydrate oxidation. It is

proposed that carbohydrate oxidation through the EMP pathway results in the generation of NADH and ATP which are subsequently used by the cell for PHA production. The use of the succinate-propionate fermentation pathway ensures that a stoichiometric balance is achieved for NADH and ATP during PHA production. As with the PAOs, SCVFA diffuses into the GAO cell in an undissociated form. The protons generated by the dissociation of the SCVFA once inside the cell are pumped from the cell utilising ATP in a similar fashion to that described for the PAOs. However, recall that the cleavage of ATP generated from carbohydrate oxidation does not add to the intracellular inorganic phosphate pool; thus no phosphorus is released from the GAOs under anaerobic conditions. Figure 7.7 schematically illustrates the proposed mechanisms.

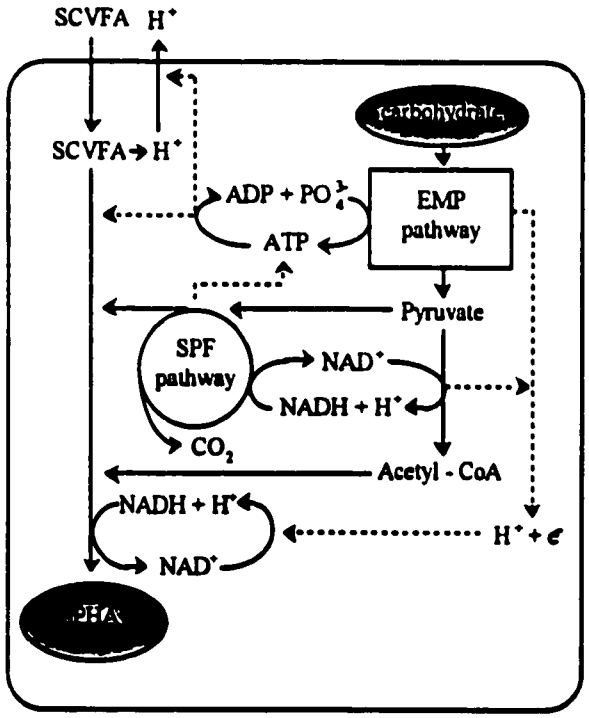

Figure 7.7: Biochemical reactions involved in the anaerobic uptake of SCVFA and synthesis as PHA in glycogen accumulating organisms.

In recent years, several studies have differentiated PHA production into its component parts and have attempted to biochemically explain the results based on reported stoichiometry (Liu *et al.*, 1996; Sudiana *et al.*, 1997; Liu *et al.*, 1997). Results of this type of analysis show good agreement with expected ratios. However, any analysis of proportions of PHA produced in anaerobic-aerobic systems must take into consideration, not only the proportion of PAOs and GAOs in the culture but also possible ATP uses within the cell.

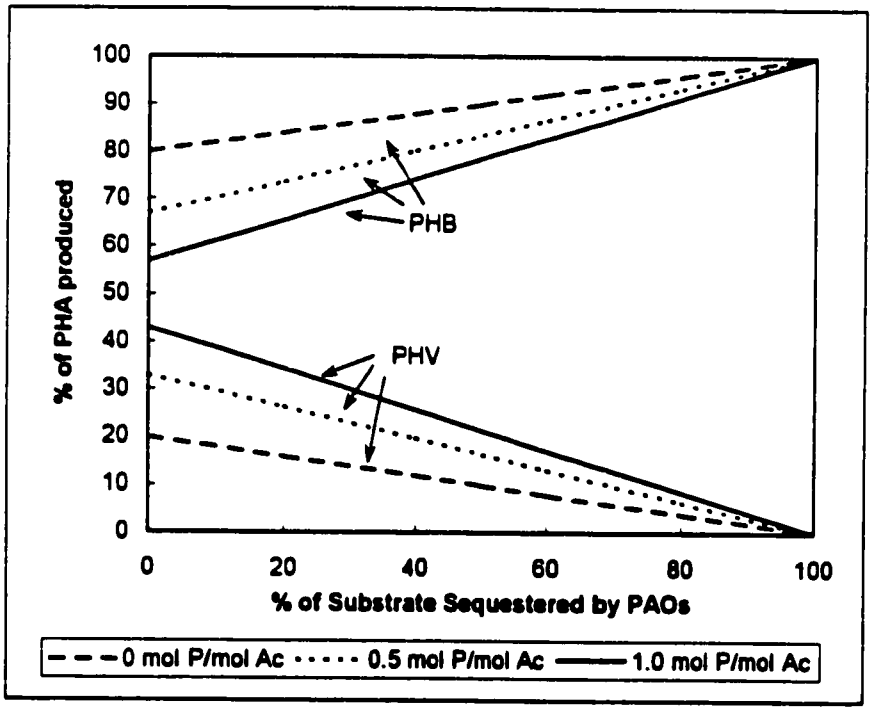

Figure 7.8: Implications on the anaerobic production of PHA in a EBPR system taking into account pH regulation and the populations of both GAOs and PAOs.

Figure 7.8 graphically illustrates the overall impact of PAOs and ATP use for pH regulation on the relative production of PHB and PHV in a culture being fed acetate. Three molar pH control ratios were used ranging from no pH control to one mole of ATP per mole of acetate sequestered [in excess of the ATP required for acetate activation]. The result of this analysis reveals that not only does ATP

hydrolysis for pH regulation affect the mass of phosphorus anaerobically released, but ATP hydrolysis also substantially affects the proportions of PHA produced. This analysis is consistent with the findings of Schuler and Jenkins (1997) who observed that, in acetate fed SBRs, the PHV fraction of the PHA produced decreased as the phosphorus content of the sludge increased.

The co-existence of the GAOs and PAOs in one system also has several kinetic and stoichiometric implications, but is consistent with the data obtained as part of this study. It is proposed that PAOs are kinetically favoured over GAOs in the anaerobic stage in terms of substrate sequestration rate. Thus, the tendency would be for PAOs to out-compete GAOs under unlimited conditions. However, it is proposed that PAO proliferation is limited by the availability of phosphorus in the system. That is, under phosphorus limited conditions the population of PAOs reaches a maximum which allows GAO proliferation. GAOs which are not limited by the same constraint, co-exist with the PAOs and compete for available substrate with the PAOs under anaerobic conditions.

The suggestion that a significant population of PAOs and GAOs co-exist in these systems also has implications in terms of experimentally observed behaviour. For instance, observed rates of reaction, whether they be COD uptake rates, phosphorus release rates, phosphorus uptake rates or oxygen utilisation rates, reflect the combined rates of the organisms present. Take for example two hypothetical systems which have only PAOs and GAOs present (Figure 7.9). In one of the systems [system A] the population of PAOs is greater than in the other [system B] but the combined population is the same in each system. The basis of the proposed model is that the PAOs are kinetically superior at sequestering substrate under anaerobic conditions. Hence, it is expected that as the population of PAOs increases in relation to the GAOs, the rate of COD uptake will increase. Therefore, for the hypothetical example, system A with the greater population of PAOs would be expected to have a greater rate of anaerobic substrate uptake than

system B. Similarly, the rate of phosphorus release would be greater in system A with the larger PAO population.

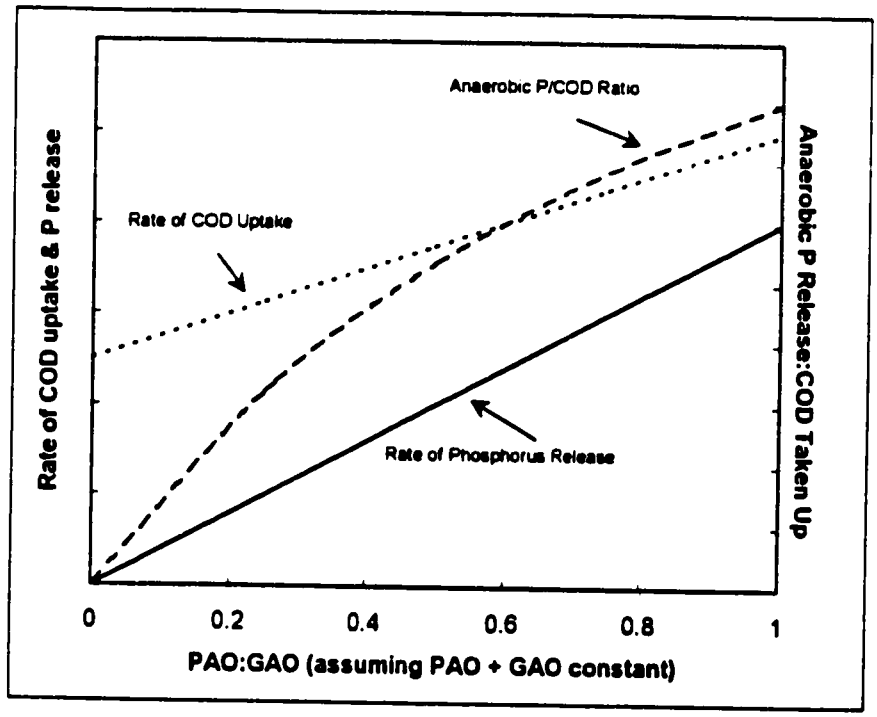

Figure 7.9: Schematic illustration of changes in rates of COD uptake, phosphorus release and the ratio of phosphorus released to COD taken up as the proportion of PAOs changes in a hypothetical PAO/GAO only system where phosphorus for growth is neglected.

Observed stoichiometric relationships are also affected in a two organism model. The phenomenon of phosphorus release under anaerobic conditions is unique to the PAOs, but anaerobic substrate uptake is not. If it is assumed that the ratio of phosphorus released to COD taken up is constant for PAOs then the relative population sizes of PAOs and GAOs in a system will dictate the observed overall ratio for that system. For example, in the hypothetical systems, PAOs in system A take up COD and release phosphorus at a constant ratio. Meanwhile, GAOs in the same system also take up substrate but do not release phosphorus. In system

B, a smaller population of PAOs takes up COD and releases phosphorus at the same ratio as the PAOs in system A, while the GAOs simply take up substrate. In this scenario the observed ratios of anaerobically taken up COD to phosphorus released will not be the same in the two systems. The observed ratio in system A will be larger even though the actual ratio attributed to the PAOs is the same. In essence, the model predicts that the ratio of anaerobic COD uptake to phosphorus released will vary depending on the relative population sizes of the PAOs and GAOs in a particular system.

The results described in *Part I* of this paper series show these trends (Copp and Dold, 1998a). When the influent phosphorus concentration was high (55.5 mgP/L – influent COD/P, 8), the ratio of phosphorus released to COD taken up was also high (1.63 mol P/mol Ac-COD) as was the rate of phosphorus release (330 mgP/L/hr). As the influent phosphorus decreased (4.5 mgP/L – influent COD/P, 98), there was an apparent change in population dynamic. The observed phosphorus release to COD uptake ratio decreased (0.35 mol P/mol Ac-COD) as did the rate of phosphorus release (35 mgP/L/hr). Similarly, the rates of substrate uptake varied with changes in influent phosphorus. These results are consistent with a number of recent studies. Lui *et al.* (1997) observed a variable anaerobic ratio of COD taken up to phosphorus released and concluded that the change in the observed ratio was a reflection of a kinetic competition within the microbial community. Similarly, Schuler and Jenkins (1997) observed that the rate of anaerobic acetate uptake increased as the phosphorus content of the sludge increased.

When the SBR in this system was fed a high concentration of phosphorus, the organism population present was kinetically faster at anaerobic substrate sequestration which is consistent with the proposed model. At high influent phosphorus:COD levels, PAOs are expected to dominate the mixed culture. As the influent phosphorus:COD decreases, growth of PAOs becomes limited which

allows the GAOs to proliferate. As the population of PAOs decreases, the effective PAO:GAO ratio changes which impacts on the anaerobic rate of substrate uptake, phosphorus release and the anaerobic ratio of COD taken up to phosphorus released.

In generating this biochemical explanation, the Mino model (Mino *et al.*, 1987; Arun *et al.*, 1988; Arun *et al.*, 1989) was not embraced for several reasons. In particular, the Mino model is unable to predict the anaerobic behaviour in a number of recent EBPR studies (Lui *et al.*, 1997; Copp and Dold, 1998b). As well, inhibition of the TCA cycle under anaerobic conditions has not been shown, hence it can not be concluded that the TCA cycle is not a source of NADH in these systems. Rather, it is believed that the Mino model reflects the combination of biochemical activity taking place in both the PAOs and GAOs in anaerobic/aerobic activated sludge systems.

The Mino model predicts that in PAOs, NADH is produced through the oxidation of glycogen based on the premise that the TCA cycle is inhibited under anaerobic conditions (Arun *et al.*, 1988). From their observations, Arun *et al.* (1988) concluded that NADH production through the EMP pathway exceeded the stoichiometric requirement for PHB synthesis. It was suggested in support of the Mino model that the resulting increase in the intracellular NADH concentration would inhibit the production of NADH through the TCA cycle. Arun *et al.* (1989) confirmed that the observed reduction in intracellular carbohydrate and thus the theoretical production of NADH exceeded the requirement for the observed PHB production. However, Arun *et al.* (1989) also observed that polyhydroxyvalerate (PHV) was produced during the anaerobic stage of an aerobic/anaerobic activated sludge system and concluded that the additional uses for NADH would maintain the NAD^+ concentration and ensure the continued operation of the glycolytic pathway. Matsuo *et al.* (1992) found other sinks for NADH as they discovered that in addition to PHV, other polyhydroxyalkanoates

(PHA) such as 3-hydroxy-2-methylbutyrate and 3-hydroxy-2-methylvalerate are synthesised during the anaerobic stage.

In light of these observations, the Mino model was modified so that the NADH produced through the oxidation of carbohydrates was utilised in the production of an array of PHA. However, this modification tends to contradict one of the previous assumptions that was made in formulating the original Mino model (Mino *et al.*, 1987). Recall, that it was assumed that excess production of NADH would cause an increase in the cellular NADH stores and inhibit NADH production through the TCA cycle by inhibiting succinate dehydrogenase, a key metabolic enzyme in the TCA cycle. However, it has been demonstrated that NADH is used in the synthesis of many PHA polymers. This type of behaviour was observed by Dawes (1981) who noted an immediate increase in intracellular NADH following the abrupt imposition of an oxygen limitation on an aerobically growing culture. However, adaptation to the anaerobic conditions within 90 minutes resulted in a decrease in the NADH concentration to a level only slightly higher than observed under aerobic conditions. Further, Pereira *et al.* (1996) concluded that the TCA cycle is a source of reducing equivalents under anaerobic conditions. Thus, without evidence to the contrary, it can not be assumed that under anaerobic conditions the NADH concentration increases to a level that inhibits its production through the TCA cycle. Many recent observations related to EBPR cannot be explained by the Mino or Comeau/Wentzel models alone, but the Mino model does provide an explanation of bulk observations of a system with both PAOs and GAOs.

Aerobic Behaviour

For aerobic conditions, it is proposed that similar growth reactions occur in both PAOs and GAOs (Figure 7.10 & Figure 7.11). Stored PHA, accumulated during the anaerobic stage, provides the carbon and energy for growth under aerobic

conditions. Energy for growth is produced through substrate level phosphorylation as well as oxidative phosphorylation. It is believed that a portion of the stored PHA is transformed into new cellular components utilising the energy generated by PHA oxidation to CO_2 and water. Oxidation produces reducing equivalents which are subsequently utilised by the electron transport system and H^+/ATPase membrane complex for ATP production. It is believed that in both PAOs and GAOs, PHA accumulation exceeds that which is required for growth in the aerobic stage. Hence, energy regulation mechanisms within the cell transform the stored PHA into stable high energy biodegradable polymers for future use.

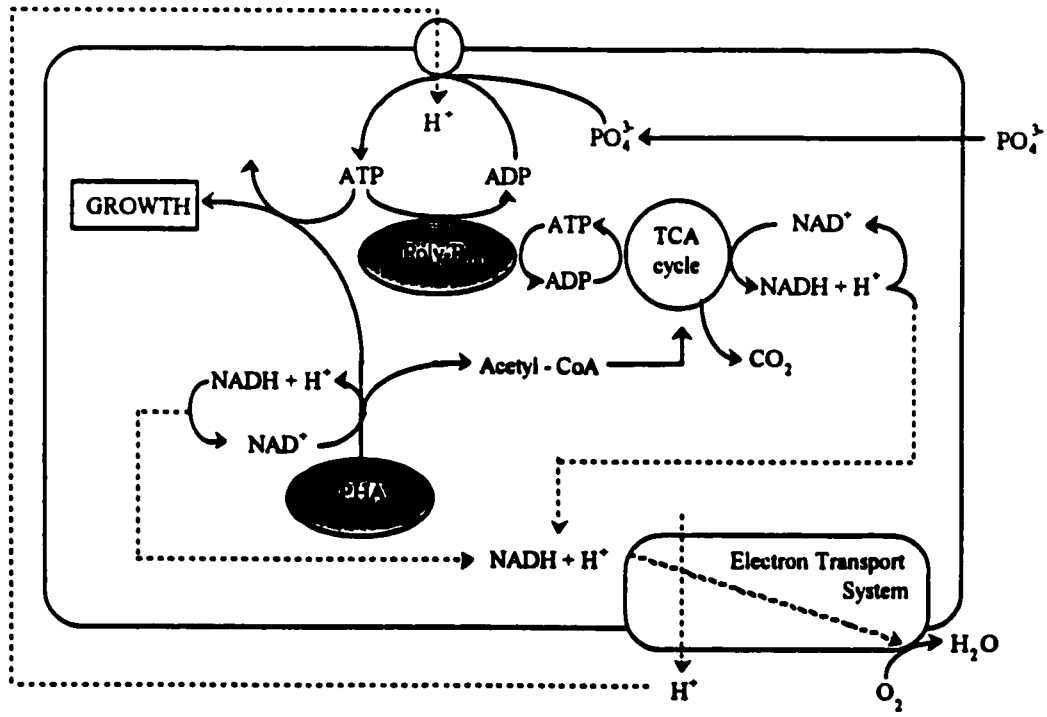

Figure 7.10: Biochemical reactions of PAOs under aerobic conditions.

In PAOs the polymer is polyphosphate. In GAOs the polymer is carbohydrate – believed to be glycogen. Aerobic conditions induce the breakdown of PHA which in turn produces reducing equivalents which must be utilised so as to not

inhibit other cellular processes. In PAOs it is believed that the reducing equivalents enter the electron transport system where protons are translocated out of the cell. The potential energy gained by this translocation and resultant PMF generation is utilised by the H^+/ATPase complex for ATP production. Excess ATP production subsequently induces the excess uptake of phosphorus and production of polyphosphate which acts as a storage compound for high energy phosphate bonds. In GAOs glycogen acts as the energy storing compound. Instead of being oxidised through the electron transport system, excess reducing equivalents in GAOs are used to reduce precursors to glycogen which acts as an electron sink under aerobic conditions. Unlike storage in PAOs, the production of glycogen in GAOs does not require excess phosphorus, hence GAOs do not take up phosphorus in excess of growth requirements.

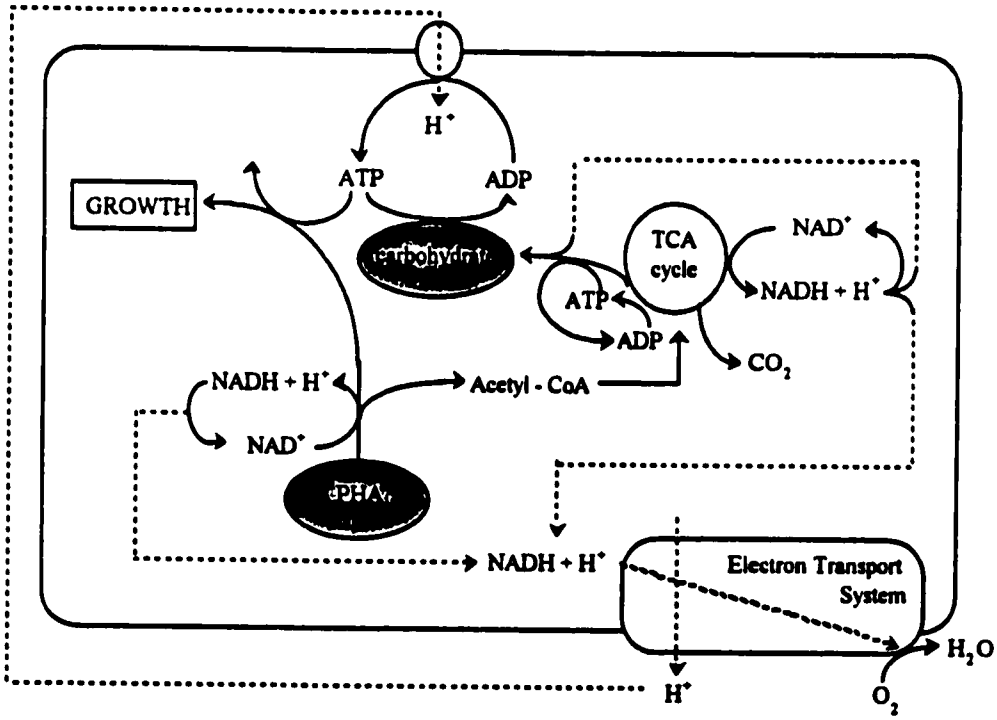

Figure 7.11: Biochemical reactions of GAOs under aerobic conditions.

The aerobic results of this study are consistent with the proposed model in that the rate of aerobic phosphorus uptake increased as the influent phosphorus increased. Recall that the model predicts a kinetic advantage for PAOs in the presence of sufficient phosphorus and that PAOs are expected to dominate the mixed culture at high influent phosphorus. A comparison of the rates of anaerobic phosphorus release and aerobic uptake demonstrate a linear agreement suggesting consistency in the population activities under anaerobic and aerobic conditions (Figure 7.12). A deviation in the linearity can be seen at lower rates; however, this is attributed to phosphorus uptake by GAOs for growth. The cultures exhibiting lower rates of phosphorus movement are expected to be dominated by GAOs that will aerobically take up phosphorus for growth but not exhibit phosphorus release under anaerobic conditions. Hence, the linear relationship between the rates breaks down as the GAOs begin to dominate.

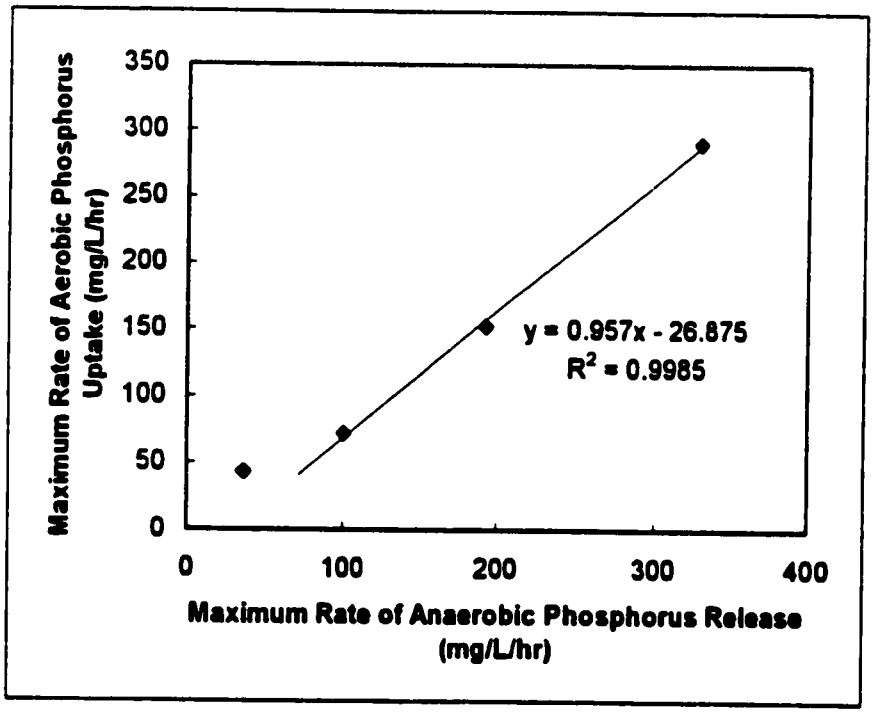

Figure 7.12: Comparison of the rate of aerobic phosphorus uptake to the rate of anaerobic phosphorus release.

Other evidence in support of the proposed model is provided by the oxygen utilisation rate data in conjunction with the phosphorus uptake profiles under aerobic conditions. In particular, the proposed model suggests that oxygen utilisation by PAOs will substantially decrease following the depletion of phosphate. It is believed that the availability of phosphate allows for the continued synthesis of ATP and subsequent polyphosphate. In the absence of phosphate, neither ATP nor polyphosphate will be generated due to phosphorus limitation. For illustrative purposes, Figure 7.13 shows the results of two tests from this study – one [a] when influent phosphorus was high (55.5 mgP/L) and one [b] when the influent phosphorus was low (4.5 mgP/L). Clearly, the aerobic activity when the influent phosphorus was high far exceeds the activity when phosphorus was low. Yet in both cases, complete COD uptake was achieved in the anaerobic stage prior to aeration. System [a] with high influent phosphorus is expected to be dominated by PAOs and shows a large drop in oxygen utilisation rate (OUR) as phosphorus approaches zero. This indicates that oxygen utilisation and hence substrate oxidation in system [a] is dependent on the availability of soluble phosphorus. However, system [b] which is predicted to be dominated by GAOs shows a steady drop in OUR seemingly less affected by the phosphorus concentration. When soluble phosphorus exceeds approximately 20mgP/L, the OUR in system [a] (PAO dominated) is far larger than in system [b] (GAO dominated). Below this apparent threshold, phosphorus limitation seems to significantly affect the activity of system [a] but has less of an impact on the activity in system [b]. This effect is consistent with the prediction that phosphorus uptake in system [b] is dominated by GAO growth whereas phosphorus uptake in system [a] reflects the storage of energy in PAOs.

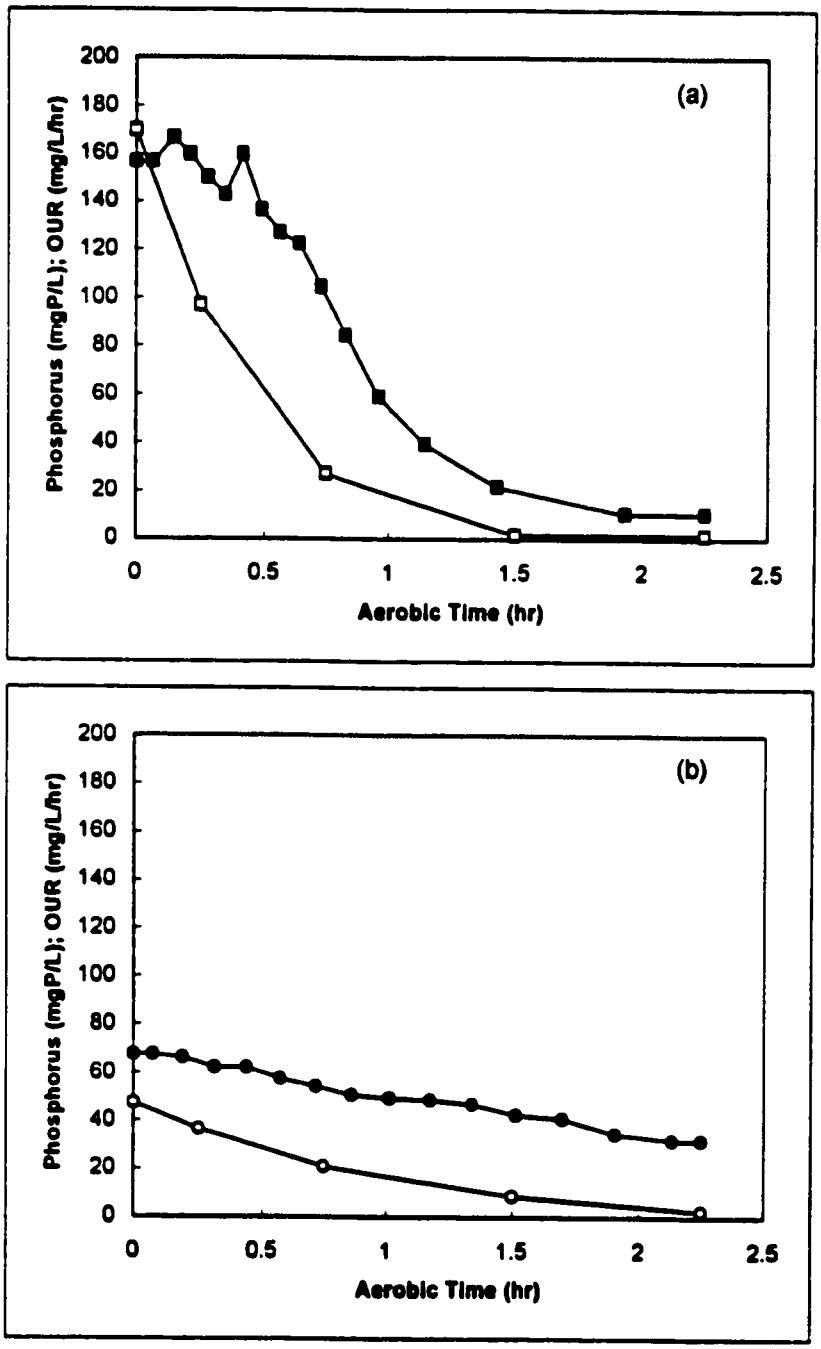

Figure 7.13: Comparison of oxygen utilisation rates (OUR) and phosphorus uptake profiles for two systems at steady state receiving different influent concentrations of phosphorus [(a) – influent P = 55.5 mgP/L, ■ - OUR, □ - phosphate; (b) – influent P = 4.5 mgP/L, ● - OUR, ○ - phosphate].

The model suggests that PAOs do not aerobically synthesise glycogen for energy storage. Hence, it is implied that the relative concentration of PAOs and GAOs in a culture will impact on the observed intracellular carbohydrate concentration (ICC). That is, in cultures dominated by GAOs, a higher ICC would be expected. The lower relative PAO concentration, in turn, suggests that the culture will have a decreased capacity for phosphorus removal. Observations by Liu (1998) support this theory. Using SBRs which were cycled through unaerated and aerated stages, Liu (1998) observed that decreasing phosphorus removal occurred as ICC increased. Stable phosphorus removal was observed when the ICC was 9-10% of the MLSS, but phosphorus removal efficiency decreased to 50% when the ICC reached 16% of the MLSS and 25% when the ICC reached 19% of the MLSS. These results support the theory that the increased ICC suggests a population shift in the culture. That is, GAOs began to dominate with the shift resulting in a decrease in phosphorus removal efficiency and an increase in ICC. Similarly, Schuler and Jenkins (1997) observed a decrease in ICC as the phosphorus content of the sludge increased. Schuler and Jenkins (1997) also concluded that less carbohydrate was consumed anaerobically as the phosphorus content of the sludge increased, again consistent with the proposed model.

CONCLUSION

The results of a biological phosphorus removing sequencing batch reactor study were used as the basis for the development of a biochemical explanation of biological phosphorus removal in anaerobic-aerobic activated sludge systems. The biochemical model is closely based on previously reported mechanisms, but proposes that GAOs and PAOs co-exist in these systems. Observations of EBPR systems have revealed variable rates of reaction and stoichiometry which can be explained by the proposed model. It is proposed that observed behaviour in

EBPR systems is the net result of the combined behaviour of both GAOs and PAOs and that the proportion of GAOs and PAOs in a single culture can significantly effect the behaviour of the mixed microbial community. It is proposed that PAOs possess a selective advantage in the presence of sufficient phosphorus, but limiting PAO growth by limiting the available phosphorus allows for the proliferation of GAOs. Both PAOs and GAOs anaerobically sequester substrate which is stored internally as PHA. Under aerobic conditions, the breakdown of PHA provides the carbon and energy for growth utilising oxygen as a terminal electron acceptor. In PAOs, excess energy generation is stored as polyphosphate whereas GAOs store excess energy as carbohydrate (glycogen). Also, it is proposed that pH regulation plays an important role in anaerobically observed behaviour and should be accounted for in the analysis of experimental data.

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CHAPTER EIGHT

CONCLUSIONS AND RECOMMENDATIONS

8.1 OVERVIEW

This research set out to investigate reported anomalies in the calculation of COD balances in biological nutrient (nitrogen and phosphorus) removal (BNR) activated sludge systems. In a number of BNR systems, where COD balances have been performed, the balances do not close. That is, approximately 20% of the COD entering the system can not be subsequently accounted for in the balance calculations. Hence, there is an apparent COD 'loss'. A two-phased experimental plan encompassing the separate investigation of denitrification and excess biological phosphorus removal (EBPR) was devised. Some of the results confirmed theoretical understanding while others represent new findings. Each experimental phase involved a number of findings with respect to COD balances in BNR systems. In addition, the results from the EBPR system were used as a basis for formulating a new biochemical model to explain EBPR system activity.

8.1.1 Denitrification Assay Phase

- A closed denitrification apparatus was developed which completely isolated the denitrifying environment from external stimuli. The apparatus allowed for comprehensive monitoring during batch testing, and provided flexibility in experimental design. Batch tests, performed in the apparatus, were designed to eliminate the effects of confounding influences which normally interfere with the clear interpretation of denitrification data.

- A series of denitrification batch tests revealed a variation in the production of nitrite based on the initial COD: NO_3^- ratio at the onset of anoxic conditions. Nitrite was not produced when the initial COD: NO_3^- ratio was low but was produced when the ratio was high. The exact cause of nitrite production was not determined.

- Time paired plots of the batch test data were used to confirm the theoretical nitrate-to-oxygen conversion factor of 2.86 gO_2/gNO_3-N. Confirming this theoretical factor essentially eliminates this mathematical manipulation as a source of COD 'loss' in denitrifying systems.

- An estimate of the organism yield under anoxic conditions was determined (0.402 mg particulate COD / mg consumed COD) and similar aerobic experiments were used to determine the yield under aerobic conditions (0.645 mg particulate COD / mg consumed COD). A comparison reveals that the anoxic yield is 62% of the corresponding aerobic yield. This has particular significance with respect to sludge production. That is, as the yield is the overriding factor involved in the amount of sludge produced, significantly less sludge production can be expected in treatment systems incorporating anoxic zones.

- COD balances revealed no COD 'losses' during the batch tests, yet less sludge was produced under anoxic conditions. These results indicate that less sludge production can be expected under anoxic conditions irrespective of whether or not COD is 'lost'. That is, observations of decreased sludge production in BNR systems can at least in part be explained by a lower yield under anoxic conditions.

- Further, in these batch tests without COD 'loss', soluble (and readily biodegradable) substrates were used, but in the denitrifying systems where COD 'losses' were reported this was not the case. Therefore, potentially the COD 'losses' in those systems is linked to the hydrolysis of complex substrate.

8.1.2 Excess Biological Phosphorus Removal Phase

- A sequencing batch reactor (SBR) was operated using four 6-hour cycles per day over an eight month period. Each cycle had an unaerated, aerated and settling stage which encouraged stable EBPR activity. The SBR was fed a synthetic influent wastewater incorporating acetate as the sole source of carbon and substrate. Five experimental periods were investigated, each characterised by a different influent COD:phosphorus ratio. Influent ratios varied from 8 to 98 and were achieved by manipulating the influent phosphorus concentration while maintaining the influent COD concentration approximately constant.

- Semi-continuous measurements of oxygen utilisation rate (OUR) during each aerated period consistently showed excellent agreement in the OUR profiles over a 24-hour period. However, the OUR data also highlighted substantial differences in biological activity between experimental periods. When the influent COD:P ratio was low (i.e. high influent P), a high initial OUR was observed following the onset of aeration, but the OUR rapidly decreased as phosphorus became limiting. In contrast, when the influent COD:P ratio was high, the initial OUR was lower and the slow, uniform rate of decline in the observed OUR was unaffected by the phosphorus concentration.

- Data from the SBR clearly demonstrated that the mass of phosphorus released during the unaerated stage increases as the influent COD:P ratio decreases.

That is, as the proportion of phosphorus in the influent increased relative to influent COD, an increase in anaerobic phosphorus release was observed. This increase in phosphorus release was in spite of the fact that similar masses of acetate were sequestered. Measurements of soluble COD and acetate confirmed that essentially all of the influent substrate was taken up during the unaerated stage irrespective of the influent wastewater phosphorus content.

- Batch tests performed on the waste sludge from the SBR were used to determine that essentially all of the internally stored phosphorus was releasable. However, a comparison of these results with those from the SBR revealed that, in the SBR, only a fraction of the releasable phosphorus was released during the unaerated stage.

- Maximum anaerobic phosphorus release rates were determined for each experimental period and showed that the rate of phosphorus release increased as the influent COD:P ratio decreased. Similarly, the rate of COD uptake also increased as the influent ratio decreased. Time paired plots of the batch test data were used to calculate the ratio of phosphorus released to COD taken up. The data revealed that the ratio was constant during each experimental period, but varied between periods. That is, as the influent COD:P ratio decreased, the ratio of phosphorus released to COD taken up increased. A comparison of the data with rates and ratios published in the literature showed excellent agreement.

- The SBR data did not show any COD 'losses'. COD balances for the five experimental periods averaged 104%, indicating that all of the influent COD was adequately accounted for in the waste stream, the effluent stream and the mass of electron acceptor consumed. As COD 'losses' were not observed during this study, it is concluded that COD 'losses' are not inherently

attributable to the biochemical reactions associated with EBPR activity. However, the defined medium and soluble substrate used in this study does not preclude COD 'loss' through hydrolysis, fermentation or sequestration of particulate substrate in EBPR systems treating municipal wastewater.

- A biochemical explanation was formulated to explain the experimental data in terms of a two-organism model. The biochemical models of Comeau *et al.* (1986) and Wentzel *et al.* (1986) were merged with the biochemical model of Satoh *et al.* (1994) to form a comprehensive model of EBPR system activity. It is proposed that in most EBPR systems, significant populations of both glycogen accumulating organisms (GAOs) and polyphosphate accumulating organisms (PAOs) co-exist and, only in systems that are stressed does one type of organism dominate the mixed community. Explaining the data using a two-organism model provides several advantages over single organism models including the ability to predict variable rates of reaction and a variable anaerobic COD uptake to phosphorus release ratio.

8.2 RECOMMENDATIONS FOR FURTHER RESEARCH

This thesis has reported the results of an experimental study on BNR biological activity. Focussing on COD balances in BNR, the results provide a basis for further investigation of the COD 'loss' phenomenon which has been observed in BNR systems. Clarification of this phenomenon no doubt will occur as further experimental work is undertaken. However, several areas specific to the experimental design require further study both in terms of understanding BNR biological activity and COD 'losses' in these systems; for example:

- The influence of hydraulic and solids retention times on BNR activity in an SBR.

- SBR cycle time and the impact of altering the length of aerated and unaerated stages within each cycle.

- The impact of particulate substrate (and raw wastewater) on denitrification and EBPR behaviour.

- The development of a mechanistic mathematical model incorporating PAOs and GAOs along with their associated polymers, so that the data accumulated through experimental investigation can be simulated and predicted.

The COD 'loss' phenomenon is confounded in most BNR systems by the complexity of the biological activity and the experimental design. A biochemical model - without COD 'loss' - has been proposed which explains the activity in this study and is consistent with the data on EBPR systems. However, several aspects need to be investigated in more detail so that improvements in the model can be developed; for example:

- The impact of the influent COD to phosphorus feeding ratio on polymer (PHA, glycogen and polyphosphate) production.

- The influence of the influent ratio on the proportions of PHA produced under anaerobic conditions.

- ATP use for pH regulation under anaerobic conditions.

The objective of this study was to investigate COD 'loss' in BNR systems. However, COD 'losses' were not observed in either of the two systems operated here. In terms of the objective, this was a disappointment as the study was not

able to solve the identified research problem. Nevertheless, the results of the study have excluded a number of possible reasons for COD 'loss' in BNR systems. An obvious requirement to allow further investigation is being able to design an experimental BNR system which does in fact exhibit COD 'loss'. To this end, perhaps the approach should be to repeat experiments on BNR systems identical to those which have shown calculated COD 'losses' previously (e.g. systems analysed by Barker and Dold, 1995).

8.3 CONTRIBUTION TO KNOWLEDGE

The objective of this research was to investigate the impact of biological nutrient removal activity on the calculation of COD balances around BNR activated sludge systems. In addressing this objective, the research contained in this thesis makes a contribution to the knowledge through an increased understanding of issues relating to BNR activity; these include:

- The 'loss' of COD observed in activated sludge systems is not inherently induced by BNR activity. This was demonstrated by closed COD balances in both denitrification assays and in an SBR exhibiting EBPR activity.

- A bioassay method was developed as a means for comparing stoichiometry of aerobic *versus* anoxic growth. The system provides an accurate method for comparing nitrate-to-oxygen equivalence. Also, the assay provided a means for comparing yields through two independent yield calculation methods.

- It has previously been reported that, in EBPR systems, the ratio of COD taken up to phosphorus released under anaerobic conditions is constant. However, from the operation of an SBR exhibiting EBPR activity and associated batch tests, it was found that anaerobic substrate uptake, phosphorus release and the

resultant release ratio were not constant, and could be correlated to the phosphorus content of the sludge.

- The results from the SBR were used as the basis for the development of a biochemical explanation of biological phosphorus removal in anaerobic-aerobic activated sludge systems. The biochemical model is closely based on previously reported mechanisms, but proposes that GAOs and PAOs co-exist in these systems. It is proposed that observed behaviour in EBPR systems is the net result of the combined behaviour of both GAOs and PAOs and that the proportion of GAOs and PAOs in a single culture can significantly effect the behaviour of the mixed microbial community. Also, it is proposed that pH regulation plays an important role in anaerobically observed behaviour and should be accounted for in the analysis of experimental data.

In addition, the results of this study have a significant practical benefit in terms of the design and the simulation of full-scale nutrient removal activated sludge systems. The results from this study can be used as a basis for improving existing mechanistic models incorporated in simulation software packages. This should lead to better estimates of sludge production in BNR facilities as well as increased predictive capabilities with respect to nutrient cycling.

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