

CRANFIELD UNIVERSITY

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THE ROLE OF ENVIRONMENTAL EFFECTS
AND *ACINETOBACTER* SPP.
IN ENHANCED BIOLOGICAL PHOSPHATE REMOVAL

SCHOOL OF WATER SCIENCES

PhD THESIS

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CRANFIELD UNIVERSITY

SCHOOL OF WATER SCIENCES

PhD THESIS

Academic Year 1995-1996

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The role of environmental effects and *Acinetobacter* spp.
in enhanced biological phosphate removal

Supervisor:

T Stephenson

FEBRUARY 1996



I Mam a Dad

ABSTRACT

One aim of this work was to define the role of *Acinetobacter* spp. in enhanced biological phosphate removal (EBPR). A culture enrichment and selective medium technique was developed to isolate *Acinetobacter* spp. from both conventional and EBPR activated sludges. The methodology proved to be successful and *Acinetobacter* spp. thus isolated were investigated for their enhanced phosphate removing abilities alongside reference cultures. The cultures were studied in shake flask batch investigations and in sequencing batch reactors (SBRs). None of the *Acinetobacter* spp. isolates studied exhibited EBPR.

A study of the effect of several environmental parameters; temperature, pH and influence of electron acceptors was the other aim of the work. Acclimatised EBPR sludges were used in these investigations which were studied in shake flask batch investigations and in SBRs. Temperature was shown to be an important variable; optimal wastewater temperature for anaerobic release and aerobic uptake of phosphate was found to be around 30°C. EBPR was still observed at extremes of 5 and 40°C and activation energies were also determined. pH levels also influenced EBPR. Batch studies showed different pH optima for anaerobic phosphate release and aerobic phosphate uptake. The presence of nitrates and nitrites in the anaerobic phase, thereby establishing anoxic as opposed to truly anaerobic conditions was evaluated. Under the operating conditions of the study, nitrate and nitrite concentrations up to 100 mgN l⁻¹ still showed ~ 70 % phosphate removal. During comparison of nitrate, nitrite and air as electron acceptors, aeration removed over 90% phosphate, nitrate addition 51-64% and nitrite addition <10%. Acetate was shown to be the preferred carbon substrate as opposed to glucose and methanol. Nitrate had less inhibitory effect on EBPR at the higher substrate concentration of 400 mg l⁻¹ (as COD).

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Prof Tom Stephenson for the opportunity to undertake this work. I am indebted to him for invaluable discussions, advice and direction throughout the course of this research and the preparation of this thesis.

I am also indebted to the EPSRC for their financial support via a Research Assistantship (Grant Ref. GR/H/14816).

Many thanks are extended to my colleagues at Water Sciences for their friendship and help. I am also grateful to the Biotechnology Centre at Cranfield for allowing me to use their facilities for certain procedures. I also wish to thank L Lötter (City of Johannesburg, Water and Wastes Directorate), Anglian Water and Severn Trent for kindly donating isolates and activated sludge samples.

Finally, sincerest thanks go to Marcus for his support and tolerance over the last few years.

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ABBREVIATIONS

3H2MB	3-hydroxy-2-methylbutyrate
3H2MV	3-hydroxy-2-methylvalerate
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
API	Analytical profile index
ATP	Adenosine triphosphate
BNR	Biological nutrient removal
BOD	Biological oxygen demand
C	Cytosine
COD	Chemical oxygen demand
DAPI	4',6-diamideino-2-phenylindole
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
EBPR	Enhanced biological phosphate removal
ED	Entner-Doudoroff
EDAX	Electron microscopy X-ray dispersive analysis
EMP	Embden-Meyerhof-Parnas
G	Guanine
HRT	Hydraulic retention time
MCRT	Mean cell residence time
MLSS	Mixed liquor suspended solids
MLTSS	Mixed liquor total suspended solids
MLVSS	Mixed liquor volatile suspended solids
N	Nitrogen
N _{Kj}	Kjeldahl nitrogen
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced form)
NCIMB	National Collections of Industrial and Marine Bacteria
NMR	Nuclear magnetic resonance

NO_2^-	Nitrite
NO_3^-	Nitrate
o-P	Orthophosphate
P	Phosphorus / phosphate
pe	Population equivalent
PHA	Poly- β -hydroxyalkanoate
PHB	Poly- β -hydroxybutyrate
PHV	Poly- β -hydroxyvalerate
P_i	Inorganic phosphorus / phosphate
pmf	Proton motive force
polyP	Polyphosphate
RAS	Return activated sludge
RNA	Ribonucleic acid
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
SBR	Stirred batch reactor
SCFA	Short chain fatty acid
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SOP	Soluble orthophosphate
SRT	Solids retention time
TCA	Tricarboxylic acid
TP	Total phosphorus / phosphate
TSS	Total suspended solids
UCT	University of Cape Town
UNC	University of North Carolina
VFA	Volatile fatty acid
VIP	Virginia Initiative Plant

NOTATION

ΔP	Amount of phosphate removed
ΔX	Amount of cell growth
θ	Temperature-activity coefficient
c	Proportionality constant
d	Day/s
e	Electron
E	Activation energy constant
E_a	Activation energy
h	Hour/s
k	Rate constant
k_c	Proportionality constant
M^+	Metal ion
min	Minute/s
n	Number
Q_{10}	Process rate increase produced by raising temperature 10°C
r	Reaction rate
R	Gas constant
t	Time
T	Temperature

CHAPTER 1.

1. GENERAL INTRODUCTION

1.1. EUTROPHICATION

One of the more serious water pollution problems currently causing concern is the eutrophication of water bodies due to nutrient enrichment by nitrogen (N) and phosphorus (as PO_4^{3-}) (Bowmer and Laut, 1990; Smalls *et al.*, 1990). Increased levels of these nutrients results in poor water quality due to growth of algal blooms and macrophytes; these pose a problem when they grow to excess causing blockage of waterways, flooding and interference with leisure activities. The main problem arises when the algae die since they place an enormous demand on the oxygen content of the water body as they are decomposed, this oxygen depletion can result in the death of fish. Blue-green algae can also produce scums and toxins (NRA, 1990). These can lead to skin irritation, gastroenteritis, liver damage and even death in humans. Fish and farm stocks can also be affected by liver or neuro-toxins leading to death (Kotak *et al.*, 1993). Phosphorus is generally recognised as the controlling nutrient for algal production in freshwaters (Vollenweider, 1985; Cullen, 1990) and if concentrations can be controlled, blue-green algae will be unable to grow excessively irrespective of the nitrogen concentration.

Phosphorus enters water bodies from point and non-point sources (Matthews, 1985; Artola *et al.*, 1995). Non-point (diffuse) sources are very difficult to control and include agricultural run-offs which may include high concentrations of phosphate-containing fertilisers. These fertilisers can give inefficient annual crop uptake values of only 20% for phosphorus (P) (Holford and Doyle, 1993). On the other hand, point sources such as industrial and domestic effluents containing detergents made with phosphate-builders are much easier to control by treatment in wastewater treatment plants. The control of phosphorus at

point source discharges is thought to be the best method of reducing and preventing eutrophication in water bodies (Vinçonneau *et al.*, 1985).

1.2. LEGISLATION

The recently introduced European Community urban wastewater treatment directive (UWWTD) sets effluent total phosphorus standards which must be achieved from wastewater treatment plants handling a population equivalent (pe) > 10 000 (CEC, 1991). The UK implements this directive by setting annual mean concentration standards for 'sensitive' water bodies. These standards are currently set at 2.0 mg l⁻¹ total P for treatment plants of 10 000 - 100 000 pe size and 1.0 mg l⁻¹ total P for plants treating a pe > 100 000. Only a few areas in the UK are currently classified as 'sensitive', namely the Norfolk Broads, the Lake District and Lough Neagh in Northern Ireland (Cooper *et al.*, 1995).

1.3. PHOSPHATE REMOVAL FROM WASTEWATER

Relatively little phosphate removal has been practised in the UK in the past (Farrimond and Upton, 1993), but countries such as South Africa, Canada and the US have extensive operational experience of the removal process (WRCSA, 1984; USEPA, 1987). Conventional activated sludge wastewater treatment processes can remove between 30-40% of the phosphorus content of municipal wastewater (Schaak *et al.*, 1985) but enhanced removal of around 90% to 0.5 - 1.0 mg phosphorus per litre is required to control the growth of algal blooms. Chemical precipitation can easily reduce phosphate concentrations to these levels by the addition of lime and metal salts of iron and aluminium (Yeoman *et al.*, 1993; Farrimond and Upton, 1993). However, disadvantages of precipitation are the high costs of chemicals and the accumulation of large quantities of waste sludge with associated disposal problems. These disadvantages led to studies on the potential of biological

phosphate removal as an alternative (Fuhs and Chen, 1975; Osborn and Nicholls, 1978). Biological wastewater treatment relies on a mixed culture of microorganisms breaking down and removing organic matter within a controlled environment. The wastewater must contain sufficient organic carbon, nitrogen, phosphorus and trace elements in available forms to maintain optimum microbial growth and preferred microbial consortia. Enhanced Biological Phosphate Removal (EBPR) is a process in which dissolved orthophosphate (as PO_4^{3-}) is accumulated from wastewater by microorganisms in excess of their normal metabolic requirements. Phosphate removal to concentrations $< 1.0 \text{ mg l}^{-1}$ and even as low as 0.3 mg l^{-1} have been achieved using EBPR (Arvin, 1985; Oldham, 1985; Draaijer *et al.*, 1993). The general theory of EBPR is that phase cycling between anaerobic and aerobic conditions promotes favourable conditions for the proliferation of enhanced phosphate accumulating microorganisms. In the UK, 23 small full-scale plants do remove phosphate - chemically. However, biological removal would be the preferred treatment method in large plants with a pe $> 100\,000$, since chemical removal would require many tankers to enter a plant each day (Cooper *et al.*, 1995).

1.3.1. Development of the Enhanced Biological Phosphate Removal Process

The initial discovery of EBPR in a wastewater treatment plant was quite accidental, and occurred when Srinath *et al.* (1959) were investigating the possibility of growing rice plants in hanging gardens on the surface of activated sludge. These plants formed poor rice grains and the vegetative growth was excessive - indicating phosphorus deficiency. This was subsequently found to be due to a lack of soluble phosphate being available to the plants, since the phosphate had been taken up in excess by the activated sludge.

Levin and Shapiro (1965) showed phosphate accumulation under aerobic conditions from a mixed microbial culture derived from activated sludge. They performed an experiment adding 2,4-dinitrophenol, which inhibits oxidative phosphorylation and hence phosphate removal, which indicated that the process was biological. In batch experiments, they showed that upon aeration phosphate uptake was observed, but without aeration, when the dissolved oxygen concentration decreased, phosphate was released into the bulk solution. They called the high phosphate removal 'luxury uptake'. Further work by Shapiro (1967) and Shapiro *et al.* (1967) demonstrated that the anaerobic release of phosphate was reversible on being subsequently aerated. Shapiro also suggested that phosphate release may be dependant on the prevailing redox conditions rather than on the dissolved oxygen concentration. Phosphate release seemed to be triggered when the redox potential dropped to -150 mV.

After these early observations, interest in EBPR processes intensified and observations of biological phosphate accumulation in activated sludge wastewater treatment plants were reported (Vacker *et al.*, 1967; Yall *et al.*, 1970; Milbury *et al.*, 1971). There was some debate as to whether the phenomenon was purely biological or due to a physico-chemical effect or even a combination of both (Menar and Jenkins, 1970; Arvin, 1983). In a biological mechanism, phosphate would be accumulated by biomass as polyphosphate, whereas a chemical removal mechanism would involve phosphate precipitation with calcium and magnesium. One reason why chemical removal gained support was that the high concentration of phosphate removed from the activated sludge would mean that the biomass contained inconceivably high levels of intracellular phosphate (Buchan, 1983). Witt *et al.* (1994) have recently suggested that physico-chemical mechanisms can complement biological phosphate removal. Barnard (1974) obtained nitrogen removal (between 90-95%) alongside excellent phosphate removal by precipitation (between 90-97%) without adding any chemicals.

In an attempt to clarify the biological / chemical mechanism debate Fuhs and Chen (1975) investigated phosphate uptake and release. They confirmed earlier observations (Shapiro *et al.*, 1967) that the process was biological. Their work also recognised the importance of anaerobic / aerobic phase cycling and also suggested that the reserve material, poly- β -hydroxybutyrate (PHB), played a role in polyphosphate accumulation. Barnard (1974) also emphasised the importance of the establishment of truly anaerobic conditions at the head of the process. He found that the presence of nitrates in the anaerobic zone created anoxic as opposed to anaerobic conditions which inhibited phosphate removal¹. This detrimental effect of nitrates in the supposedly anaerobic zone has since been observed in other investigations (Osborn and Nicholls, 1978; Hascoet and Florentz, 1985). Nicholls and Osborn (1979) proposed that a hydraulic retention time (HRT) of 2 hours was desirable for the anaerobic zone for good EBPR. They also suggested that anaerobic conditions prompted the storage of PHB which could be oxidised in the subsequent aerobic phase to provide energy for excess phosphate uptake. This excess phosphate was stored as polyphosphate. These early observations allowed the development of several EBPR process configurations.

1.3.2. Operating Configurations

Several operating configurations for activated sludge EBPR have been developed over the last 30 years and most are adaptations of processes initially designed for nitrogen removal. These adaptations are essentially the inclusion of a preliminary anaerobic zone before the aeration tanks and operating the process in a plug flow regime.

¹ Throughout this work, anaerobic refers to the absence of dissolved oxygen, nitrate and nitrite - or in fact any other electron acceptor, whereas anoxic refers to the absence of dissolved oxygen and presence of nitrate or nitrite.

EBPR processes are either main-stream processes which remove phosphate in the waste sludge of a plant, or side-stream processes which combine both biological and chemical treatment where phosphate is extracted from the main-stream sludge into a side-stream (5-25% of the main-stream) and is then precipitated (Arvin, 1985). Examples of main-stream processes are the Bardenpho, Modified Bardenpho / Phoredox, A/O, Modified Phoredox / A²/O, University of Cape Town (UCT), Modified UCT and Biodenipho (Arvin, 1985; U.S.E.P.A., 1987; van Starckenburg *et al.*, 1993). The Phostrip[®] process is an example of a side-stream process (Toerien *et al.*, 1990).

1.3.2.1. The Phostrip[®] Process

The Phostrip[®] side-stream process was the first process specifically designed for phosphate removal (Levin and Shapiro, 1965) and combines both biological and chemical phosphate removal. A fraction of the return activated sludge (RAS) from the biological process (10-30% of the influent flow) is diverted into a side-stream leading into an anaerobic stripping tank where phosphate is released from the sludge into the supernatant (Figure 1.). An overflow from the stripper tank leads to a separate tank where the supernatant is chemically treated with lime (or another coagulant). Phosphate is removed from the process in the chemical precipitant.

1.3.2.2. The Bardenpho Process

The four-stage main-stream Bardenpho process was first described by Barnard (1973) and was originally developed for nitrogen removal (Figure 2.). Influent wastewater first enters an anoxic zone, which also accepts the RAS and nitrate recycle from the first aerobic zone, where denitrification occurs. When influent BOD loading is very high compared to the oxygen equivalent of the nitrate recycle, anaerobic conditions may be established, when phosphate removal can occur.

Legend:  Anaerobic
 Anoxic
 Aerobic

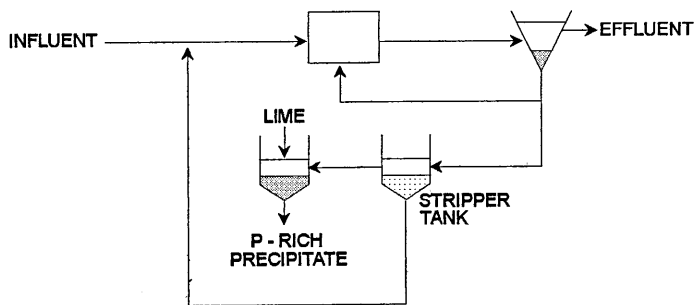


Figure 1. Schematic diagram of the Phostrip process

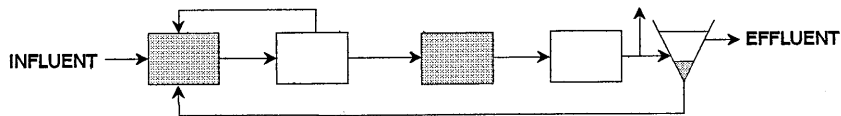


Figure 2. Schematic diagram of the Bardenpho process

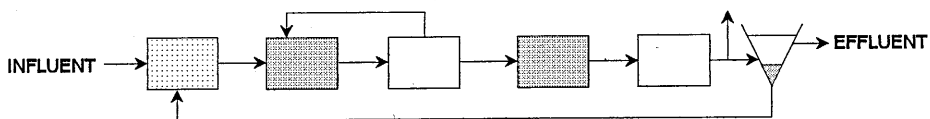


Figure 3. Schematic diagram of the Modified Bardenpho / Phoredox process

1.3.2.3. The Modified Bardenpho / Phoredox Process

This five-stage main-stream process is a modification of the original Bardenpho process and is designed for combined nitrogen and phosphorus removal (Figure 3.). The process is also referred to as the Phoredox process. Barnard (1976) realised that preventing nitrate from entering the first zone, therefore establishing anaerobic as opposed to anoxic conditions at the head of the plant, resulted in good EBPR. The first anoxic zone accepts the nitrate recycle from the first aerobic zone, where denitrification occurs. In the second anoxic zone, denitrification uses the nitrate produced in the first aerobic zone as electron acceptor. The second aerobic zone minimises release of phosphate during settling and strips any remaining nitrogen gas from solution.

1.3.2.4. The A/O Process

The A/O (Anoxic / Oxidic) process is a main-stream process, patented by Air Products, which removes phosphate from wastewater (Figure 4.). An anaerobic zone precedes the aerobic zone (Hong *et al.*, 1984). Influent wastewater first enters the anaerobic zone, which also accepts the RAS recycle, where phosphate is released into solution. Phosphate uptake by the biomass occurs in the subsequent aerobic zone. Phosphate leaves the system in the waste sludge.

1.3.2.5. The Modified Phoredox / A²/O Process

This three-stage main-stream process excludes the second anoxic and aerobic zones present in the Phoredox process (Figure 5.). The A²/O (Anaerobic/ Anoxic/ Oxidic) process is a modification of the A/O process, with the inclusion of an anoxic stage for denitrification, and is also patented by Air Products. Both nitrogen and phosphate can be removed by this process.

Legend:  Anaerobic
 Anoxic
 Aerobic

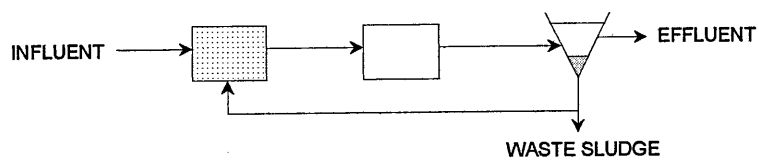


Figure 4. Schematic diagram of the A/O process

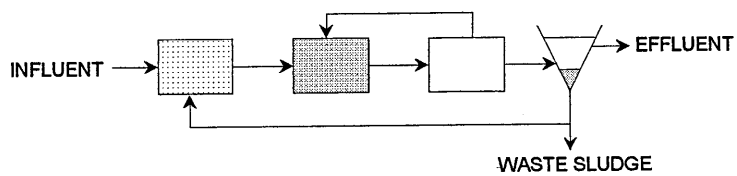


Figure 5. Schematic diagram of the Modified Phoredox (A2/O) process

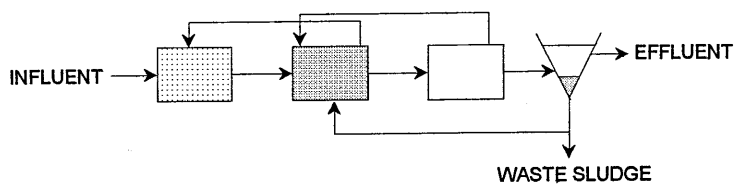


Figure 6. Schematic diagram of the UCT process

1.3.2.6. The UCT Process

The University of Cape Town (UCT) main-stream process is a modification of the three-stage Phoredox process designed to prevent nitrate from entering the anaerobic zone via the RAS recycle (Siebritz *et al.*, 1983). Instead, the RAS recycles into an anoxic zone (Figure 6.). An internal nitrate recycle from the aerobic zone also enters the anoxic zone, from which the anaerobic zone accepts another internal recycle.

1.3.2.7. The Modified UCT Process

The UCT process was modified slightly by Siebritz *et al.*, (1983) by subdividing the anoxic zone into two for improved anaerobic zone protection (Figure 7.). The RAS recycle enters the first anoxic zone, whereas the nitrate internal recycle from the aerobic zone enters the second anoxic zone. A further internal recycle from the anoxic zone enters the anaerobic zone, protecting the anaerobic zone from potential nitrate inhibition, still enabling both phosphorus and nitrogen removal.

1.3.2.8. The Bardenpho Process

The Bardenpho process uses one anaerobic reactor, where both the influent wastewater and RAS recycle are combined, alongside two further reactors which alternately operate as anoxic and aerobic reactors (to nitrify/denitrify) to remove both phosphorus and nitrogen (Bundgaard *et al.*, 1983). The wastewater flow pattern is anaerobic, anoxic, aerobic (Figure 8.).

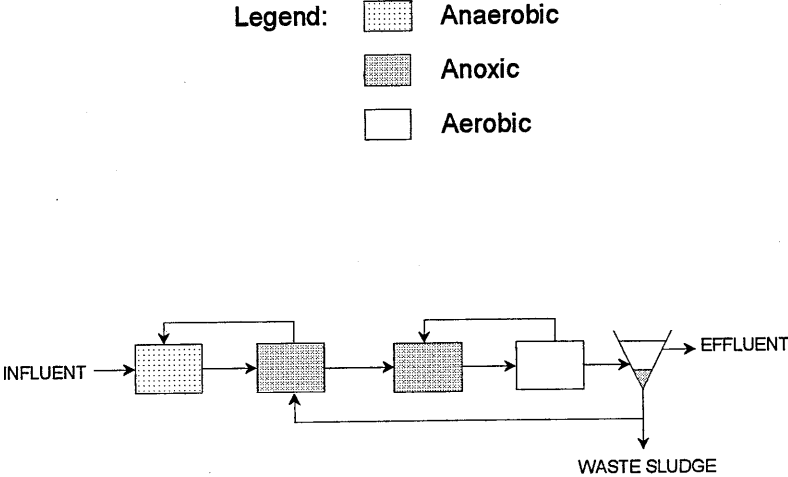


Figure 7. Schematic diagram of the Modified UCT process

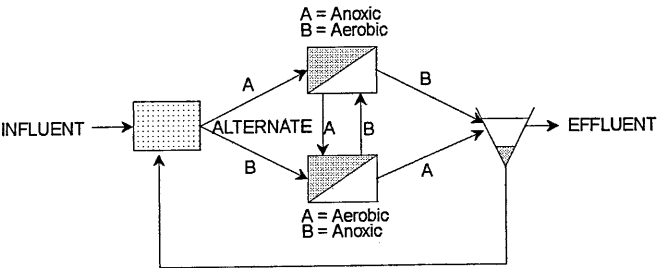


Figure 8. Schematic diagram of the Bardenpho process

1.3.2.9. Sequencing Batch Reactor

Sequencing Batch Reactor (SBR) technology has been used for EBPR since the late 1970s (Irvine *et al.*, 1979; Manning and Irvine, 1985; Okada and Sudo, 1986). The SBR is a fill and draw activated sludge system where each process step is undertaken in the same tank. An SBR designed for EBPR operates in anaerobic / aerobic cycles where each cycle has five separate phases: fill, react, settle, draw and idle. This set-up allows the process to operate within a time rather than a space sequence (Irvine and Ketchum, 1989). SBRs have been used at many full-scale small and medium sized wastewater treatment plants (Melcer *et al.*, 1987; Irvine *et al.*, 1987; Rusten and Eliassen, 1993; McClintock and Frazier, 1994).

1.3.3. Biochemistry of Enhanced Biological Phosphate Removal

Ever since the phenomenon of EBPR was first observed (Srinath *et al.*, 1959) researchers have attempted to model the biochemical mechanisms involved in the process (Comeau, 1990; Wentzel *et al.*, 1991; Wentzel *et al.*, 1992).

1.3.3.1. Fundamental Mechanism of EBPR

Early models of the mechanism of EBPR (Fuhs and Chen, 1975; Nicholls and Osborn, 1979; Marais *et al.*, 1983) developed from the observation that alternating anaerobic and aerobic conditions favoured the growth of phosphate accumulating bacteria (Barnard, 1976; Tomei *et al.*, 1993). These models, were however, mainly mechanistic, but they did indicate the essential features of the process. The basic mechanism is simple - phosphate is released from the biomass under anaerobic conditions and during aerobic conditions is taken up in excess and stored as polyphosphate granules in the cells (Figure 1.)(Heymann *et al.*, 1989; Vasiliadis *et al.*, 1990; Jing *et al.*, 1992).

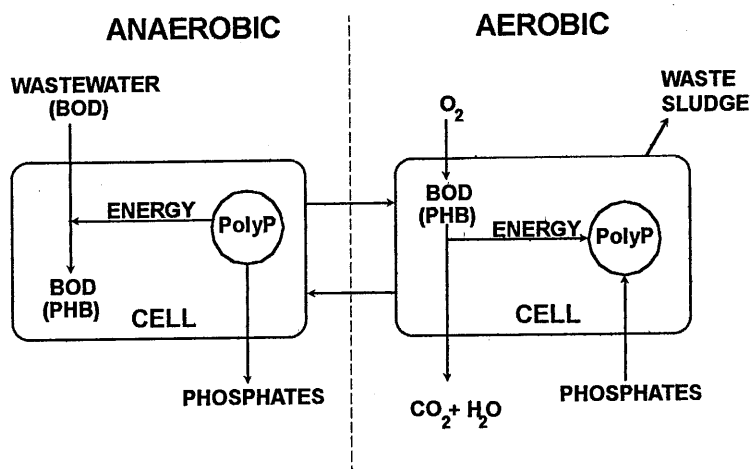


Figure 9. EBPR under alternating anaerobic / aerobic conditions

Repeated cycling between phosphate release under anaerobic conditions and phosphate uptake under aerobic conditions results in net intracellular phosphate accumulation. The amount of phosphate uptake is related to the extent of phosphate release in the previous anaerobic phase (Marais *et al.*, 1983). The accumulated phosphate is removed from the system via wasting of the phosphate rich biomass.

1.3.3.2. Polyphosphate

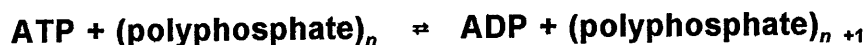
Microbial polyphosphates occur as cyclically condensed phosphates (metaphosphates), linearly condensed phosphates (polyphosphates) and as condensed cross-linked phosphates (ultraphosphates) (Harold, 1966). Schmidt *et al.* (1946) and Wiame (1947) first identified a basophilic substance first thought to be RNA as inorganic polyphosphate. Wiame (1947) also showed that volutin or metachromatic granules coincided with polyphosphate deposits. Polyphosphates (polyP) have the general formula $M_{(n+2)}P_nO_{(3n+1)}$. Bacterial polyphosphates range in the number of phosphate residues, for instance, in *Escherichia coli* polyphosphates of chain length 23 to 1000+ were detected (Rao *et al.*, 1985). The polyphosphate content of microorganisms is related to their growth conditions (Harold, 1966). Polyphosphates form complexes with other polymers such as proteins, nucleic acids, polypeptides, polysaccharides and phospholipids (Harold, 1966). Mono- and divalent- metal ions such as Mg^{2+} and Ca^{2+} can also form complexes with polyphosphates within cells (Kulaev, 1979). Van Groenestijn and Deinema (1985) showed that considerable amounts of K^+ , Mg^{2+} and Ca^{2+} were present in *Acinetobacter* 210A polyphosphate granules.

The role of polyphosphate (polyP) in bacterial metabolism is well researched (Harold, 1966; Kulaev and Vagabov, 1983; Kulaev, 1985). Polyphosphate can act as an energy reserve either via phosphorylation of AMP and ADP to ATP or more directly by phosphorylation of acetate, glucose, gluconate, fructose

etc. Two basic mechanisms by which polyP can be accumulated have been described, "luxury uptake" and "overplus accumulation" (Harold, 1966). Nutrient depletion e.g. nitrogen or sulphur, can result in polyP accumulation by "luxury uptake". Phosphate depletion followed by rapid phosphate exposure can cause polyP accumulation by the "overplus" mechanism. However, neither of these situations are likely to occur in municipal wastewater treatment plants (Siebritz, *et al.*, 1983; Arvin, 1985).

Generally, polyphosphate granules have been identified by staining cells using basic dyes such as methylene blue or toluidine blue (Fuhs and Chen, 1975; Kulaev, 1979; Deinema *et al.*, 1980). Newer methods have since been developed to identify polyphosphate deposits. These include: staining with the fluorescent dye 4',6-diamideino-2-phenylindole (DAPI); electron microscopy combined with X-ray dispersive analysis (EDAX) (Buchan, 1983); chemical fractionation (Mino *et al.*, 1984) and ^{31}P nuclear magnetic resonance (NMR) (Suresh *et al.*, 1985). Polyphosphate may be found at various cellular locations: such as a surface pool (Tijssen and van Steveninck, 1984; Halvorson *et al.*, 1987) and a cytoplasmic pool (volutin granules) (Harold, 1966). Polyphosphate accumulation has been found in many bacterial species typically found in activated sludge. Apart from bacteria, polyphosphates are also found in some species of protozoa, algae and fungi as well as mosses, flowering plants and animals (Kulaev, 1979).

One common way in which polyphosphate is synthesised is by the transfer of a terminal phosphoryl group of ATP to polyphosphate, catalysed by the enzyme polyphosphate: ADP phosphotransferase (polyphosphate kinase) (Kulaev and Vagabov, 1983):



This reaction is also reversible, polyphosphate kinase catalyses the formation of ATP from polyP and ADP, thus degrading polyphosphate. Polyphosphate kinase activity has been used to investigate the phosphate removing ability of *Acinetobacter* spp. in particular, with varying activity results (T'Seyen *et al.*, 1985b; Van Groenestijn *et al.*, 1989b; Bayly *et al.*, 1991; Bark *et al.*, 1992). Other enzymes such as 1,3-diphosphoglycerate : polyphosphate phosphotransferase (Kulaev and Vagabov, 1983) may also be involved in polyphosphate synthesis. Several enzymes other than polyphosphate kinase may be involved in polyphosphate degradation, such as, polyphosphate : AMP phosphotransferase, polyphosphate glucokinase, exopolyphosphatase, polyphosphate depolymerase, polyphosphate fructokinase, polyphosphate gluconatekinase and polyphosphate mannokinase. Van Groenestijn *et al.* (1987) found that ATP was produced in cell-free extracts of *Acinetobacter* strain 210A from bacterial and chemically prepared polyphosphate by the action of polyphosphate : AMP phosphotransferase and adenylate kinase. Bonting *et al.* (1991) also suggested that as a result of polyphosphate : AMP phosphotransferase and adenylate kinase activity polyphosphate acts as an energy reserve in *Acinetobacter* strain 210A. However, Bayly *et al.* (1990) investigated several enzymes previously thought to be involved in polyphosphate synthesis and measured the polyphosphate content in several strains of *Acinetobacter*. The enzymes studied were polyphosphate kinase, polyphosphate: AMP-phosphotransferase, 3-phosphoglycerate kinase and polyphosphate glucokinase. Their results suggested that polyphosphate synthesis in *Acinetobacter* does not involve any of these enzymes since their activities did not vary significantly between strains which do and do not accumulate polyphosphate to high levels. Wood and Clark (1988) review in detail the enzymes involved in polyphosphate metabolism.

1.3.3.3. Carbon Substrates

Volatile fatty acids (VFAs) / short chain fatty acids (SCFAs) are the preferred

carbon substrate for polyP accumulating organisms. They play an important role in the anaerobic phase of the process (Ghekiere *et al.*, 1991). Acetate is the VFA most commonly associated with EBPR (Wentzel *et al.*, 1988). Others such as propionate, butyrate, malate, pyruvate, lactate, valerate and succinate can also be utilised (Abu-ghararah and Randall, 1991; Satoh *et al.*, 1992) as can glucose (Potgieter and Evans, 1983). Uptake of VFAs occurs during the anaerobic phase (Somiya *et al.*, 1988) - a process which requires energy.

Municipal wastewaters do not generally contain high enough levels of acetate for efficient EBPR, therefore they have to be produced. Prefermentation, by increasing the solids retention time in an activated primary tank can achieve acetate production (Barnard, 1984; Bayly *et al.*, 1989). A separate side-stream fermenter feeding the anaerobic phase of the process with acetate has also been used to ensure an adequate supply of VFAs irrespective of influent flow and wastewater VFA concentration (T'Seyen *et al.*, 1985a; Rabinowitz and Oldham, 1986; Oldham and Abraham, 1994). Barnard (1994) and Rabinowitz (1994) discuss the prefermentation options available.

1.3.3.4. Carbon Storage

Polyphosphate accumulating bacteria utilise VFAs and convert them into poly- β -hydroxyalkanoates (PHAs) which are osmotically inert intracellular storage polymers. Poly- β -hydroxybutyrate (PHB) is the most commonly reported storage polymer since it is the main product of acetate feeding (Nicholls and Osborn, 1979; Comeau, *et al.*, 1985; Comeau, *et al.*, 1987b). PHB is a polymer of D(-)- β -hydroxybutyrate, its synthesis and degradation pathways are described elsewhere (Dawes and Senior, 1973; Comeau *et al.*, 1986). PHB accumulates under oxygen or nitrogen limitation when a carbon source is still available and under starvation conditions it can act as an energy source rather than a carbon source (Dawes and Senior, 1973). PHB accumulation to 50% of the cell dry weight has been reported (Dawes and Senior, 1973). PHB

accumulation by *Acinetobacter* sp. is well documented (Fuhs and Chen, 1975; Deinema *et al.*, 1980; Rees *et al.*, 1992a and b; Rees *et al.*, 1993; Vierkant *et al.*, 1990). Besides PHB other (PHAs) such as poly- β -hydroxyvalerate (PHV) can be stored intracellularly. VFAs with an even number of carbon atoms, such as acetate and butyrate favour PHB formation whereas an odd number, such as propionate, lactate and valerate tend to favour PHV formation (Comeau *et al.*, 1987b). The accumulation of 3-hydroxy-2-methylbutyrate (3H2MB) and 3-hydroxy-2-methylvalerate (3H2MV) has also been observed (Matsuo *et al.*, 1992; Satoh *et al.*, 1992).

Several methods are used to identify PHB in activated sludge. The most common of these is staining with Sudan Black (Fuhs and Chen, 1975; Lötter *et al.*, 1986). Rees *et al.* (1992b) developed a double-staining procedure for differentiating between PHB and polyphosphate in an *Acinetobacter* sp. isolate. They used Methylene blue for polyphosphate and Nile blue for PHB - Sudan Black would stain both. Gas chromatography can also be used (Deinema *et al.*, 1980; Comeau *et al.*, 1987b) as well as a spectrophotometric method (Somiya *et al.*, 1988).

Bacteria can also store carbon as glycogen which can be formed with glucose feeding (Dawes and Senior, 1973; Tracy and Flammino, 1987; Mino *et al.*, 1987). However, Nicholls and Osborn (1979) stated that glycogen accumulation was not observed in a full-scale EBPR plant.

1.3.3.5. Cations

Polyphosphate carries a large negative charge which is thought to be stabilised by the cations Mg^{2+} , K^+ and Ca^{2+} (Wentzel *et al.*, 1986). The availability of potassium and magnesium has been shown to be essential for successful EBPR, the process failed when either was excluded from the influent (Rickard and McClintock, 1992; Imai *et al.*, 1988; Imai and Endoh,

1990). Rickard and McClintock (1992) suggested that their role was involved with maintenance of cell electroneutrality, which had also been mentioned in an earlier study (Barnard, 1984). Calcium has been shown to be not needed for successful EBPR (Rickard and McClintock, 1992). Polyphosphate accumulation has been shown to be accompanied by Mg^{2+} and K^+ accumulation whilst phosphate release has been shown to be accompanied by Mg^{2+} and K^+ release (Arvin and Kristensen, 1985).

1.3.3.6. Energy Requirements

The energy required for uptake of acetate is provided by the breakdown of polyphosphate during the anaerobic phase, with phosphate being released to the bulk solution. In the aerobic phase the stored PHB is used as a carbon and energy source for cell metabolism and growth and as an energy source for polyphosphate formation which stimulates phosphate uptake. Provided that a source of acetyl CoA is available, PHB synthesis does not directly require ATP (Dawes and Senior, 1973).

1.3.3.7. Substrate and Phosphate Transport

The maintenance of a proton motive force (pmf) i.e. a chemiosmotic gradient across the cytoplasmic membrane, is important in bacterial energetics and plays a major role in ATP production and substrate transport (Comeau *et al.*, 1986). The pmf has both a pH and a charge component (Wentzel *et al.*, 1986). Bacteria try to keep a constant pmf value, so if the external pH changes, cations, e.g. K^+ , discharge from the cell (with a decrease in pH), or accumulate in the cell (with a pH increase) to maintain the pmf (Comeau *et al.*, 1986).

Acetate transport into the cell had been thought to occur via an active transport mechanism using ATP as an energy source (Marais *et al.*, 1983; Abu-ghararah and Randall, 1991). However, Wentzel *et al.* (1991) considers

acetate to be transported into the cell by passive diffusion.

Van Veen *et al.* (1993) found two phosphate transport systems in *Acinetobacter johnsonii* 210A, a high-affinity and a low-affinity system, both of which have also been determined in *A. lwoffii* (Yashphe *et al.*, 1992). The low-affinity system is constitutive and found in cells grown at low and high phosphate concentrations. The high-affinity system, on the other hand, is only activated when the cells have been starved of phosphate.

1.3.3.8. Detailed Biochemical Models

The more recent biochemical models of EBPR agree on all key points except on the generation of reducing equivalents, nicotinamide adenine dinucleotide (NADH), needed to convert acetate to PHB under anaerobic conditions (Comeau *et al.*, 1986; Wentzel *et al.*, 1986; Comeau *et al.*, 1985; del Carmen Doria-Serrano *et al.*, 1992; Mino *et al.*, 1987). The main models have been excellently reviewed by Wentzel *et al.* (1991), a summary only will be given here.

1.3.3.8.1. The Comeau / Wentzel Model

Comeau *et al.* (1985) first proposed this model which was slightly modified by Wentzel *et al.* (1986) to include several control mechanisms. This model specifies *Acinetobacter* sp. to be the typical microorganism responsible for EBPR although it recognises that other species have been implicated in the process. Polyphosphate is suggested as a source of energy for pmf renewal and substrate storage in Comeau's model under anaerobic conditions.

During the anaerobic phase, a high extracellular acetate concentration allows its passive diffusion into the cell where it is activated to acetyl Co-A by coupled adenosine triphosphate (ATP) hydrolysis which in turn releases cations i.e. Mg^{2+} or K^+ (expressed as M^+ in Figures 10. and 11.), and the anion H_2PO_4^- (Figure 10.). Regeneration of ATP from adenine diphosphate (ADP) occurs by transfer of a phosphoryl group from polyphosphate (PolyP_n) to the ADP which results in a decrease of the stored polyphosphate concentration and generation of ATP. Condensation of two acetyl-CoA molecules form acetoacetyl-CoA which is reduced by NADH to hydroxybutyryl-CoA, which is then polymerised to PHB. Acetate is metabolised via the tricarboxylic acid (TCA) cycle to supply the NADH.

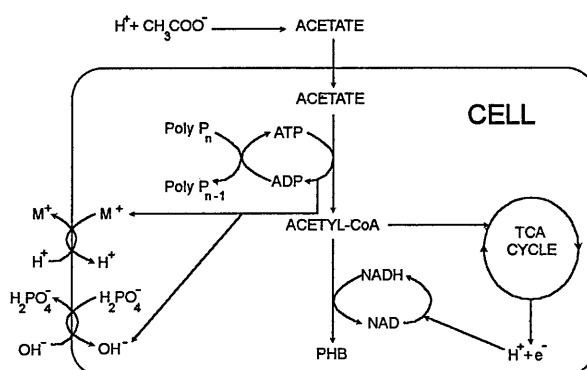


Figure 10. The Comeau / Wentzel model under anaerobic conditions

The model of Wentzel *et al.* (1986) suggested that the ATP/ADP and NADH/NAD ratios controlled polyphosphate and PHB synthesis and degradation. Under anaerobic conditions, i.e. no terminal electron acceptor, the NADH/NAD ratio increases, inhibiting the TCA cycle, thus increasing the amount of acetyl Co-A and stimulating PHB synthesis. Without oxidative phosphorylation the ATP/ADP ratio decreases, resulting in polyphosphate

degradation and a release of phosphate. Cations (M^+) are also released with phosphate, which occurs alongside the uptake of protons and hydroxyl ions from the bulk solution. This in turn restores the pmf which was originally dissipated during acetate (acetic acid) transport into the cell by the removal of H^+ ions.

Under aerobic conditions, consumption of PHB allows the cell to produce a pmf which can be used for phosphate transport and ATP production (Figure 11.). Under aerobic conditions the NADH/NAD ratio decreases allowing the TCA cycle and associated glyoxylate cycle to proceed enabling the oxidation of stored substrate (PHB). ATP is generated by simultaneous oxidative phosphorylation and phosphate is actively transported into the cell. The ATP generated can then be used for resynthesis of polyphosphate.

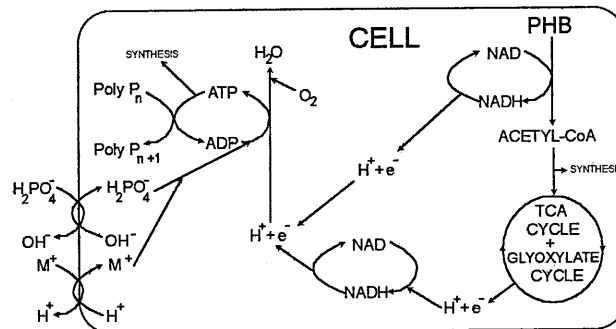


Figure 11. The Comeau / Wentzel model under aerobic conditions

1.3.3.8.2. The Mino Model

The Mino model (Mino *et al.*, 1987) describes the biochemistry involved for polyphosphate accumulating organisms which possess the Embden-Meyerhof-Parnas (EMP) pathway.

Under anaerobic conditions acetate is taken up and is activated to acetyl-CoA by coupled ATP hydrolysis, resulting in inorganic phosphate (P_i) being released to the bulk solution (Figure 12.). The accumulated polyphosphate supplies this ATP requirement. Carbohydrate (glucose, obtained from glycogen) is converted to pyruvic acid (pyruvate) via the EMP pathway producing NADH. The pyruvic acid is then converted to acetyl-CoA producing carbon dioxide. The acetyl-CoA is then converted to PHB using the NADH produced via the EMP pathway.

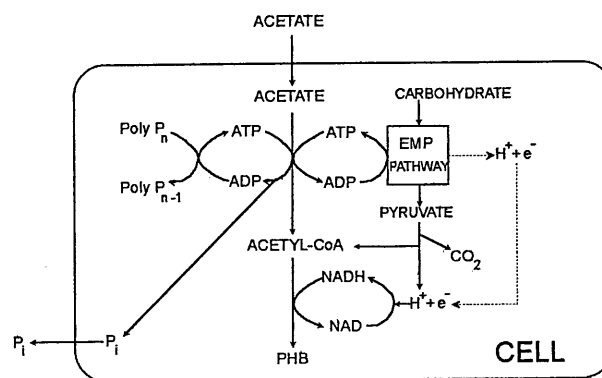


Figure 12. The Mino model under anaerobic conditions

Under aerobic conditions the Mino and Comeau / Wentzel models agree on most major points (Wentzel *et al.*, 1991).

Acinetobacter sp. do not possess the EMP (glycolytic) pathway (Mino *et al.*, 1987). However, if the Mino model is modified slightly then it can also be applied to *Acinetobacter* sp. (Wentzel *et al.*, 1991).

1.3.3.8.3. The Modified Mino Model

This model is similar to the Mino model except that under anaerobic conditions the reducing equivalents, NADH, needed to convert acetate to PHB are supplied by utilising carbohydrate via the Entner-Doudoroff (ED) pathway and not the EMP pathway (Wentzel *et al.*, 1991). This significantly affects the process because less energy is produced through the ED pathway. Some *Acinetobacter* sp. do possess the ED pathway (Juni, 1978) which can operate under anaerobic conditions. The result of this is that more polyphosphate breakdown will be needed to supply the energy to convert acetate to acetyl-CoA.

1.3.3.9. Feasibility of the Models

The molar ratio for acetate uptake : phosphorus released can be used to determine which of the models (if any) are feasible. Theoretically the values for this ratio are:-

Comeau / Wentzel Model	1:1
Mino Model	2:1
Modified Mino Model	1.5:1

Actual observed values have been reported by several studies: 0.7:1 (Comeau *et al.*, 1985; 1:1 (Fukase *et al.*, 1982; Wentzel *et al.*, 1985; Wentzel *et al.*, 1989); 1.3:1 (Iwema and Meunier, 1985; Mino *et al.*, 1987; Abu-ghararah and Randall, 1991); 3.4:1 (Mino *et al.*, 1987). This tends to suggest that all three models could be operational at any given time in an EBPR process depending on the bacterial population.

1.3.4. Environmental Factors Influencing EBPR

Many parameters can affect the performance of EBPR and the composition of the wastewater is crucial since there must be sufficient short chain fatty acids available for utilization by phosphate removing bacteria. Ideally an activated sludge process operates to simultaneously remove BOD, nitrogen and phosphates from wastewater. To achieve this a mixed culture containing all the required bacterial species for their physiological reactions must be maintained in harmony. This can present major problems if denitrification is not complete.

1.3.4.1. Oxygen Concentration

It has been observed that a low redox potential is required for phosphate release (Boughton, *et al.*, 1971) and values between -170 to -275 mV have been reported (Koch and Oldham, 1985). However, Schön *et al.* (1993) found a direct dissolved oxygen (DO) dependence for phosphate uptake and release but the redox potential affected the rate of phosphate release only under certain conditions. Pitman *et al.* (1983) observed that vigorous mixing action and vortexing should be avoided in anaerobic and anoxic zones. They also noted that oxygen entrainment by the action of screw pumps could oxygenate the RAS to levels greater than 3 mg l⁻¹, which when added to the influent at oxygen concentrations of around 4 mg l⁻¹ (as a result of hydraulic jumps and waterfalls in the feed channels), added a heavy oxygen load to the anaerobic zones. Toerien *et al.* (1990) advise that a DO concentration of 2-3 mg l⁻¹ in the aerobic zone is ideal, which should not be allowed to drop below 1 mg l⁻¹ and rise above 4-5 mg l⁻¹.

1.3.4.2. Temperature

Phosphate uptake has been shown to have an optimum temperature of 24-37°C (Boughton *et al.*, 1971). The anaerobic phosphate release rate has been

observed to increase by a factor of 2.1 to 2.6 with every 10°C rise between 10-30°C (Shapiro *et al.*, 1967). An optimal growth temperature of 34-35°C has been reported for *Acinetobacter* (Juni, 1978) as has 33°C (Hao and Chang, 1987). Within the range 16-40°C the highest maximum growth rates for *Acinetobacter calcoaceticus* were obtained at temperatures between 29-35°C, with no growth found at 41°C (du Preez and Toerien, 1978). However, Fuhs and Chen (1975) found an optimal temperature of 20-24°C for their *A. lwoffii* isolate. The effect of a range of temperatures on EBPR is discussed in detail in Chapter 6.

1.3.4.3. pH

For aerobic phosphate uptake an optimum pH range of 7-8 has been determined (Levin and Shapiro, 1965). In a similar study, an optimal pH range of 7.5-9.6 was observed (Boughton *et al.*, 1971). For *A. calcoaceticus* var. *lwoffii* the optimum pH for growth is 7.5 but the optimum pH for maximum phosphate uptake rate is 7 (Hao and Chang, 1987). The effect of a range of pH values on EBPR is discussed in detail in Chapter 7.

1.3.4.4. Nitrate Inhibition

EBPR is generally thought to be adversely affected by an excess of nitrates or oxygen (i.e. a terminal electron acceptor) in the anaerobic stage (Hascoet and Florentz, 1985). This prevents the formation of true anaerobic conditions needed for phosphate release and hence excess phosphate uptake in the subsequent aerobic stage. During denitrification, fatty acids are used to convert nitrate to N₂, N₂O and NO, decreasing substrate availability for EBPR bacteria. Production of VFAs by bacteria such as *Aeromonas* is also inhibited when nitrate is present in the anaerobic zone since the redox potential is too high (van Starckenburg *et al.*, 1993). The effect of nitrates on EBPR is discussed in detail in Chapter 8.

1.3.5. Microbiology

During their extensive EBPR studies Fuhs and Chen (1975) isolated various strains of the then named *Acinetobacter-Moraxella-Mima* group of bacteria and concluded that *Acinetobacter* spp. were most probably responsible for the observed biological phosphate removal. Since then many researchers have attempted to examine and elucidate the precise role of *Acinetobacter* spp. in the EBPR process.

A diverse range of polyphosphate-accumulating bacteria have been isolated from phosphate removing wastewater treatment plants (Table 1.). Apart from *Acinetobacter*, the most often cited are *Pseudomonas*, *Aeromonas*, *Achromobacter*, *Moraxella* and *Klebsiella* spp. (Florentz and Hartemann, 1984; Lötter and Murphy, 1985; Brodisch, 1985; Suresh *et al.*, 1985). However, by far the most commonly isolated and generally accepted microorganisms responsible for EBPR are members of *Acinetobacter* spp. (Fuhs and Chen, 1975; Ohtake *et al.*, 1985; Rensink *et al.*, 1985; Beacham *et al.*, 1989). Nevertheless, the overall role of *Acinetobacter* spp. in the process has often been questioned (Brodisch and Joyner, 1983; Stephenson, 1987; Cloete and Steyn, 1988a; Hiraishi *et al.*, 1989; Raper, 1990; Nakamura *et al.*, 1991; Auling *et al.*, 1991; Cloete *et al.*, 1992).

Brodisch (1985) investigated the possibility of a synergistic relationship between *Acinetobacter calcoaceticus* and *Aeromonas punctata* in co-culture, and found that upon the addition of the *A. punctata* isolate an improvement in the EBPR ability of the *A. calcoaceticus* isolate was observed. However, when Yeoman *et al.* (1988) attempted to bioaugment their laboratory scale EBPR process with *Acinetobacter lwoffii*, *Acinetobacter calcoaceticus* and *Aeromonas punctata* no improvement in phosphate release or uptake was detected.

Table 1. Polyphosphate accumulating bacteria found in activated sludge

Species	Reference
<i>Achromobacter</i>	Auling <i>et al.</i> (1991)
<i>Acinetobacter</i>	Bonting <i>et al.</i> , (1992); Van Groenestijn <i>et al.</i> , (1989a); Beacham <i>et al.</i> , (1992); Florentz and Hartemann (1984); Fuhs and Chen, (1975); Lötter and Murphy, (1985); Auling <i>et al.</i> (1991)
<i>Aeromonas</i>	Lötter and Murphy, (1985)
<i>Bacillus</i>	Florentz and Hartemann (1984)
<i>Citrobacter</i>	Auling <i>et al.</i> (1991)
Coryneform bacterium	Auling <i>et al.</i> (1991)
<i>Enterobacter</i>	Lötter and Murphy, (1985)
<i>Escherichia coli</i>	Kronberg, (1957)
<i>Klebsiella</i>	Gersberg and Allen, (1985)
<i>Micrococcus</i>	Harold, (1966); Nakamura <i>et al.</i> , (1991)
<i>Moraxella</i>	Lötter and Murphy, (1985); Auling <i>et al.</i> (1991)
<i>Nitrobacter</i>	Harold, (1966)
<i>Nocardia</i>	Kulaev and Vagabov, (1983)
<i>Pseudomonas</i>	Florentz and Hartemann (1984); Suresh <i>et al.</i> , (1985); Lötter and Murphy, (1985); Auling <i>et al.</i> (1991)
<i>Xanthobacter</i>	Auling <i>et al.</i> (1991)
<i>Xanthomonas</i>	Auling <i>et al.</i> (1991)

1.4. AIMS OF THIS INVESTIGATION

The objectives of the work described in this thesis are two-fold: to determine the importance and role of *Acinetobacter* spp. in the EBPR process and to investigate the effect of various levels of several environmental parameters on the overall process performance in mixed cultures.

Members of the *Acinetobacter* genus are generally thought to be the microorganisms responsible for EBPR. This study will investigate the EBPR ability of several different *Acinetobacter* spp. isolates which will be isolated from various activated sludge sources. Suitable isolation procedures will be developed for this purpose. The possibility of a synergistic relationship between *Acinetobacter* spp. and *Aeromonas* spp. will be investigated in co-culture fermentation studies.

Mixed culture studies will determine optimum conditions for maximum phosphate uptake under the influence of several environmental parameters. The effect of temperature on anaerobic phosphate release and aerobic phosphate uptake rates will be investigated within the range of 5-45°C. Similarly, the effect of various pH values on anaerobic phosphate release and aerobic phosphate uptake will be investigated between the range of pH 5-10.5. The significance of nitrate and nitrite in the anaerobic zone, creating anoxic as opposed to anaerobic conditions, will be determined. Nitrate, nitrite and air will also be compared as electron acceptors. The influence of the nature and concentration of the carbon substrate on the nitrate effects will also be examined.

CHAPTER 2.

2. MATERIALS AND METHODS

General materials and methods are mentioned here. Specific methods concerning particular chapters are described in those chapters.

2.1. EQUIPMENT

Two sequencing batch reactors (SBRs) were used for continuous operation at laboratory scale enabling the different environmental conditions required for EBPR to be attained in a time rather than a space sequence i.e. all the process phases occur sequentially in one reactor as opposed to using several tanks. The SBR is a fill-and-draw activated sludge system where the reactor is filled with wastewater feed, run as a batch system, allowed to settle, before treated effluent is withdrawn from the vessel (Irvine and Ketchum, 1989). Preliminary and additional investigations were undertaken in shake flask batch experiments.

2.1.1. Shake Flask Batch Experiments

Batch experiments were undertaken in various sized borosilicate glass Erlenmeyer flasks. Air and nitrogen were supplied via a gas distribution tube fitted with a borosilicate glass sinter (porosity No. 3., BDH, Poole). Temperature control and mixing were achieved by placing the flasks in a shaking water bath (Grant SS40-5 and CS25, Grant Instruments Ltd, Cambridge). When pH control was required, pH values were initially adjusted with the addition of 3 M HCl or NaOH solutions, after which values were maintained using 0.5 M solutions.

2.1.2. SBR Experiments

Acinetobacter spp., *Aeromonas caviae* and activated sludge were grown in fed batch culture in two SBRs. The SBRs simulated plug flow conditions and allowed straightforward comparisons to be drawn from results obtained from the shake flask experiments.

2.1.2.1. 5 l SBR

The SBR unit consisted of a 5 l flanged cylindrical vessel and headplate made from Nalgene (polycarbonate) with rubber gasket. A purpose-built fermentation controller comprising the interface, power switching system and software was commissioned for the study (Multiphase Fermentation Control Unit D453, Labcon, Co. Durham) and was controlled by a BBC Acorn Master computer (Acorn Computers Ltd, UK). The system allowed automatic control and monitoring of pH, temperature and redox potential alongside timed on / off switching of various pumps, valves and a stirrer. Relevant components could be sterilised by autoclaving at 121°C, 15 psi for 20 mins for aseptic operation. Influent, effluent, gas and control lines entered the fermenter via stainless steel tubing set into rubber bungs and septa in screw-in ports in the vessel headplate ensuring aseptic transfer into and out of the fermenter. Borosilicate glass tubing was used to transfer pH solutions. Silicone rubber tubing was used throughout except at pump heads where stronger viton tubing was used. Data was logged onto computer disk and/or printer at selected intervals. A schematic diagram of the fermentation system is shown in Figure 13.

The influent synthetic sewage feed was pumped to the vessel from a 10 l aspirator reservoir by a peristaltic pump (Schuco Scientific Ltd, London) at a rate of 5 l h⁻¹ through silicone tubing. Excess cells and clarified effluent were removed from the vessel to a 10 l aspirator waste reservoir by a peristaltic pump (Crouzet, UK) at a rate of 13.8 l h⁻¹ through silicone tubing.

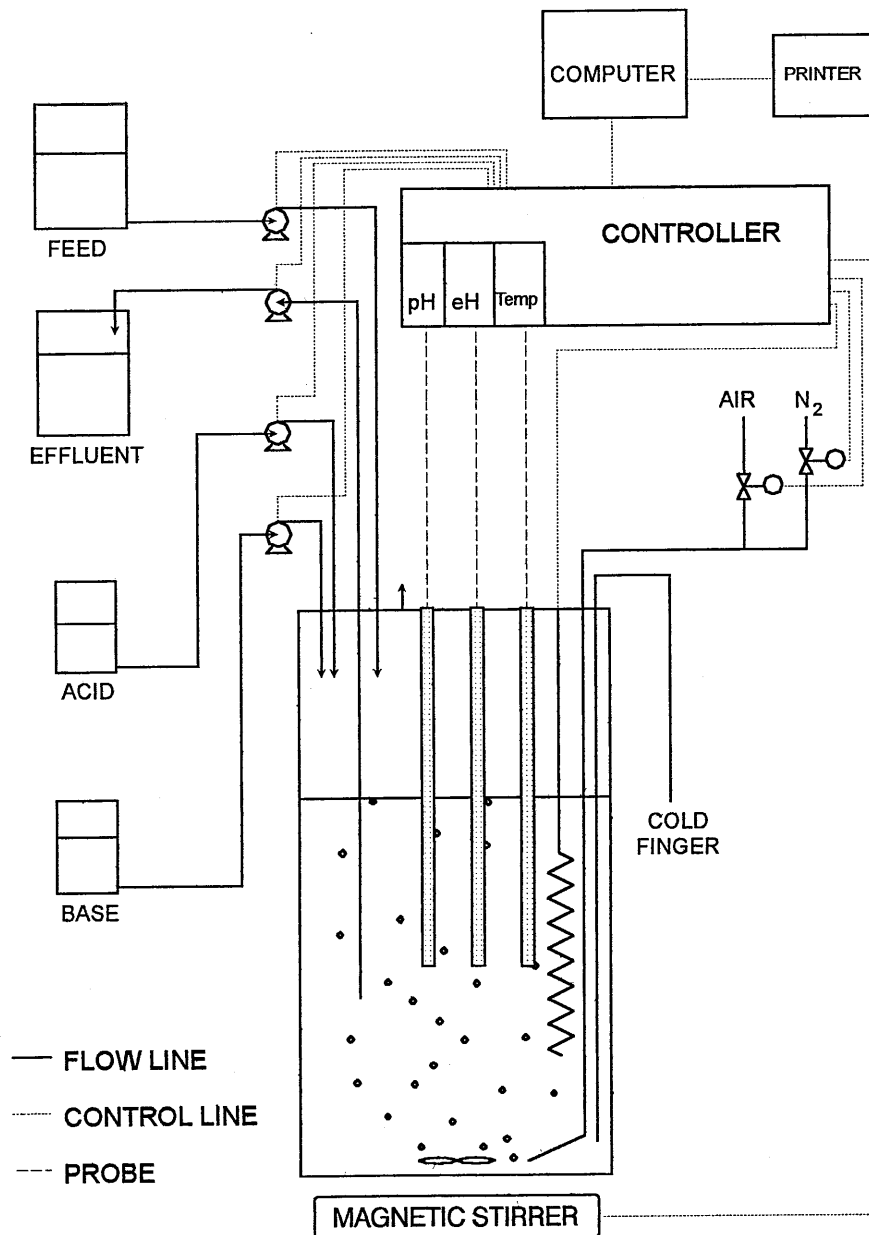


Figure 13. 5 l SBR System

Compressed air (mains supply) was delivered to the reactor via polyvinylchloride (PVC) tubing passing through a solenoid valve (Burkert Chromatic Ltd, Stroud) which switched flow on / off and was measured by a flow meter (Platon Flowbits Ltd, Basingstoke). Coarse air flow control was achieved by adjustment of an on-line mains regulator (Ingersoll-Rand, Bolton) whilst fine control was performed by a needle valve in the flow meter. The air entered the suspension through a sintered glass gas distribution tube (porosity No. 3., BDH, Poole) located at the base of the vessel which served as a fine bubble diffuser system. When aerating, a dissolved oxygen (DO) concentration of approximately 2 mg l^{-1} was sought. Nitrogen gas (oxygen-free grade, BOC, Guildford) was similarly controlled, measured and delivered to the vessel. The inlet gases were passed through an in-line $0.2 \text{ }\mu\text{m}$ bacteriological filter (Millipore, Watford) to ensure a sterile gas supply to the reactor. Off gases were vented via a condenser and similar bacteriological filter.

The temperature of the suspension was constantly monitored by an immersed thermocouple connected to the fermentation controller. The temperature could be controlled using two set-ups. A cold finger, containing constantly flowing cold tap water provided a background reduction in ambient temperature and temperatures below this were achieved by connecting the system onto a cooler unit (Grant Instruments Ltd., Cambridge). An immersion heater connected to the fermentation controller raised the temperature of the suspension to the set-point.

Mixing was achieved by a magnetic stirrer (LH Engineering 502, Stoke Poges) driving a flea located at the base of the vessel and was aided by four vertical stainless steel baffles. The air and nitrogen supply also helped to maintain completely mixed conditions in the vessel.

Solution pH was measured by an autoclavable glass combined reference electrode (Russell pH Ltd., Auchtermuchty) connected to the electrically

isolated interface. The pH was automatically maintained at the desired value by addition of 2 M NaOH or 2 M HCl delivered by peristaltic pumps (503U, Watson Marlow Ltd., Falmouth). Redox potential was monitored by an autoclavable combination platinum Ag / AgCl reference electrode (Russell pH Ltd., Auchtermuchty) connected to the controller. It was originally intended to use the redox potential values as a control mechanism between anaerobic and aerobic conditions, but the system proved unreliable in pure culture work due to fouling of the electrode.

Sampling from the fermenter was achieved by a sampling port off the effluent line designed to accept Universal bottles. To sample, the line to the effluent aspirator was clamped at a 'T' junction whilst solution was drawn from the fermenter. A volume several times greater than the dead space in the tubing was initially drawn before an actual sample was taken.

2.1.2.2. 10 l SBR

The second reactor consisted of a 10 l borosilicate glass flanged vessel with headplate and rubber gasket (Figure 14.). A working volume of 5 l was used for the investigations. This reactor was not designed for aseptic operation and was used solely for activated sludge investigations. A 4 channel programmable timer (RS Components Ltd, Corby) controlled the switching on / off of valves and pumps enabling plug flow conditions to be simulated.

Compressed air and nitrogen were delivered to the reactor in a similar manner to the 5 l reactor except that the solenoid valves were supplied by RS Components Ltd, Corby. Influent and effluent gases were also filtered similarly to the 5 l SBR.

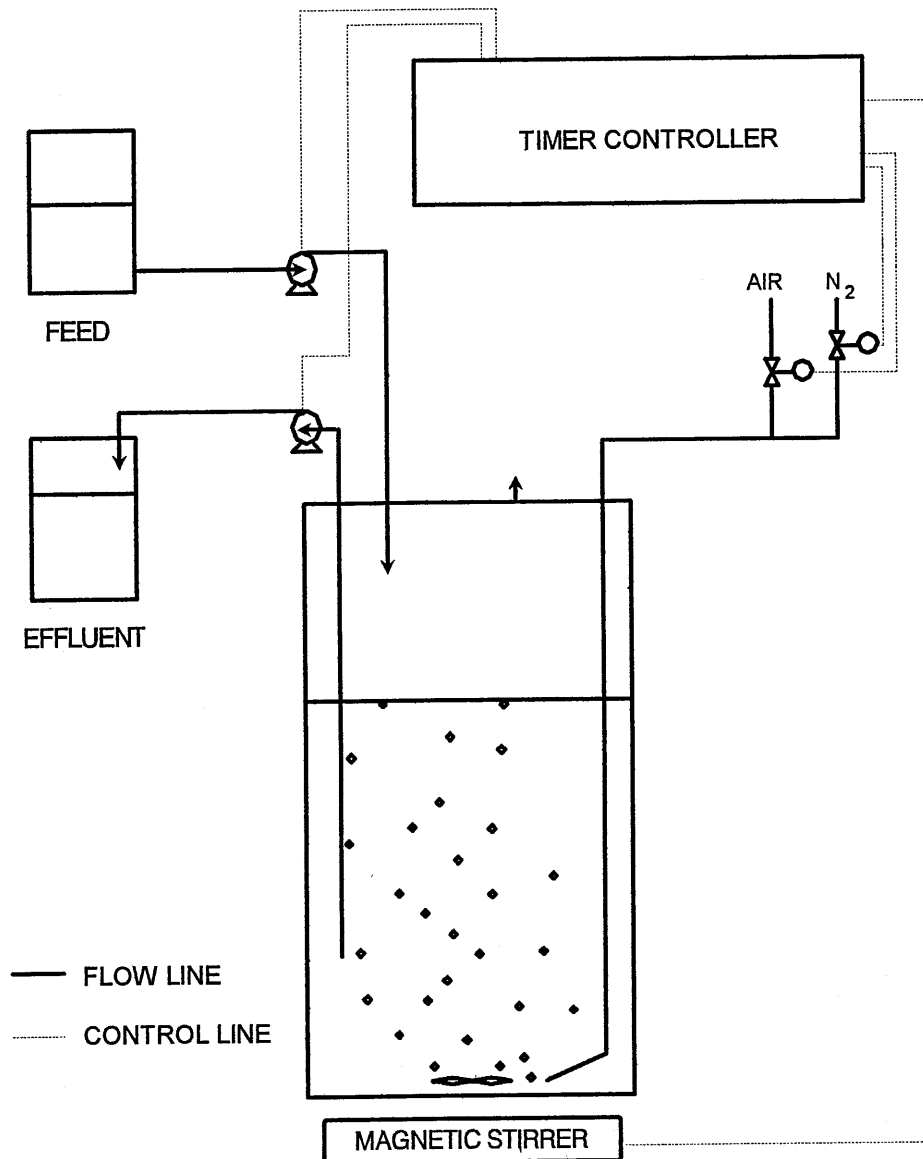


Figure 14. 10 l SBR system

The influent synthetic sewage feed was pumped to the vessel from a 10 l aspirator reservoir by a peristaltic pump (Schuco Scientific Ltd, London) at a rate of 7.1 l h^{-1} . Excess cells and clarified effluent were removed from the vessel to a 10 l aspirator waste reservoir by a peristaltic pump (Schuco Scientific Ltd, London) at a rate of 6.5 l h^{-1} .

Mixing was achieved using a magnetic stirrer (Ikamag Reo, Ika, Fisons, Loughborough) driving a flea located at the base of the vessel. Switching the stirrer on / off was controlled by a separate timer (Smiths Industries Environmental Controls Co. Ltd, London) to the main switch controller.

Solution pH could be monitored manually by inserting a pH probe (Russell pH Ltd., Auchtermuchty) into the reactor via a port in the headplate. The pH probe was connected to a Corning pH meter (Corning, Essex). Solution DO was similarly measured with a portable DO probe and meter (Jenway 9070, Essex). The redox potential was measured by a platinum electrode (Russell pH Ltd, Auchtermuchty) with a standard Calomel reference (Russell pH Ltd, Auchtermuchty) connected to a multimeter (Model 7040, Cirkit, Hong Kong). Temperature was measured with a standard mercury in glass thermometer. Samples were withdrawn from the vessel using pipettes via a port in the headplate.

2.2. PHASING

Design and operation of an EBPR process in the SBRs was made possible by the control mechanisms of the 5 l and 10 l reactors which allowed the systems to be programmed to sequence through various phases. Operation of the SBRs comprised a cycle consisting of a sequence of six distinct phases: feed, anaerobic react, aerobic react, excess sludge removal, settle and effluent removal phase. Figure 15. shows a single vessel in each phase of a complete cycle. This sequence resembles the standard fill-and-draw SBR configuration operation (Arora *et al.*, 1985; Irvine *et al.*, 1987; Irvine and Ketchum, 1989). The duration of each phase varied according to the operational requirements of each investigation.

The cycle starts with the feed phase where the reactor is filled with synthetic sewage feed during which the contents are stirred. This phase is short in duration compared to the react phases, enabling the SBRs to operate in a plug flow manner. If the time required for the feeding phase were relatively long the SBRs would operate as conventional activated sludge processes. The anaerobic react phase follows feeding where reactor contents are sparged with nitrogen and stirred, followed by the aerobic react phase where nitrogen sparging is replaced with compressed air. At the end of aerating, excess sludge is removed from the reactor, thereby withdrawing the accumulated phosphate in the biomass from the process, an action which also serves in controlling the mean cell residence time (MCRT). Stirring and gas sparging are switched off to allow the contents to settle for clarifying the effluent. The clarified effluent is removed from the reactor in the final phase of the cycle. The settled sludge and remaining clarified effluent acts as the RAS for the start of the following cycle. A new cycle immediately follows with the feed phase.

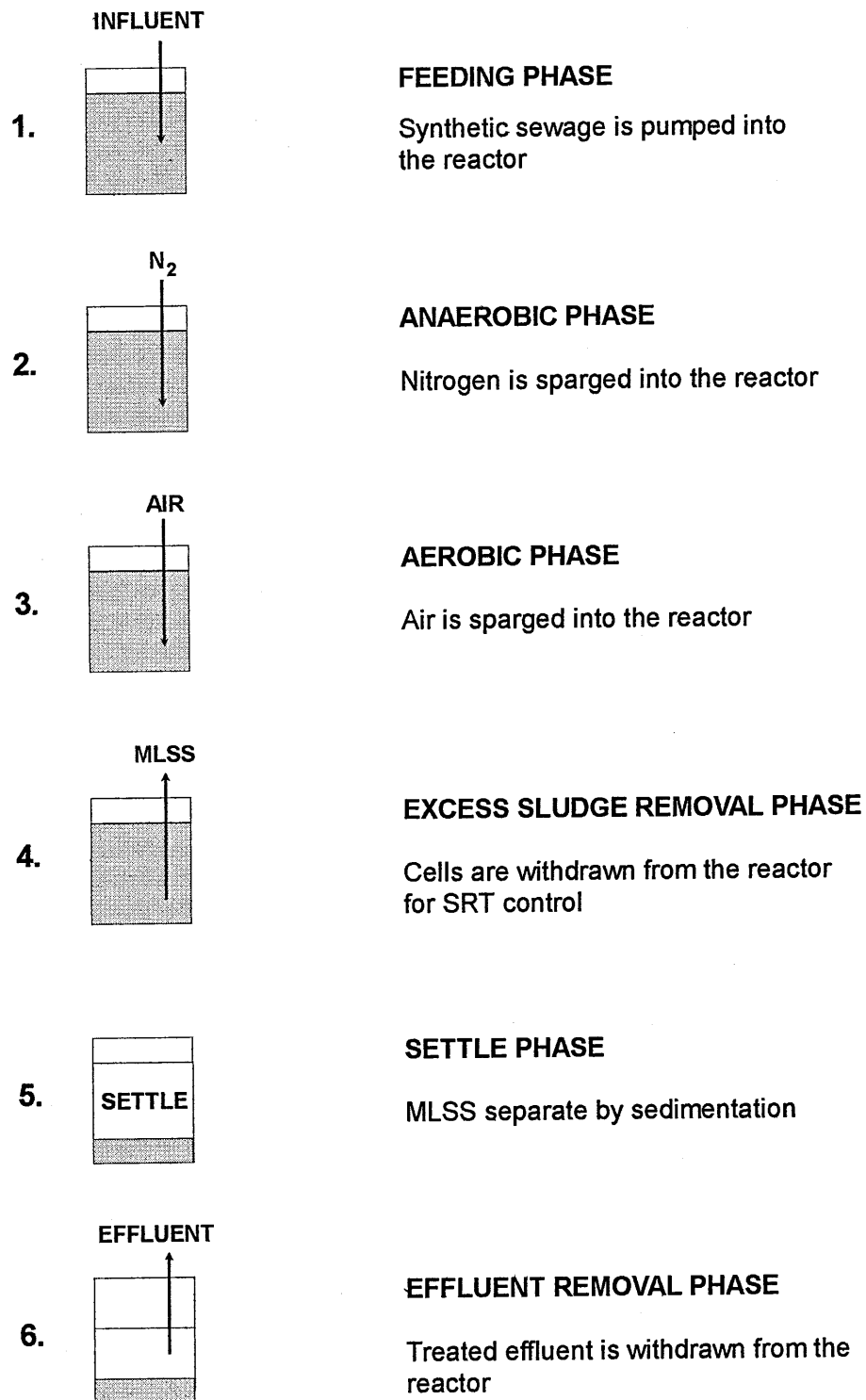


Figure 15. Phase sequence of one complete anaerobic / aerobic cycle

2.3 SYNTHETIC WASTEWATER

A synthetic sewage medium, allowing controlled substrate composition was designed to simulate influent wastewater and was used as the basic feed in all investigations. Two versions of the medium were used in the studies differing only in their carbon source, one being acetate, the other glucose (Table 2.). Tap water was used to prepare the feeds, securing the presence of micronutrients (Converti *et al.*, 1993). The yeast extract also provided essential growth factors and has been suggested to be essential for the growth of phosphate removing cultures and *Acinetobacter* sp. in particular (Warskow and Juni, 1972; Wentzel *et al.*, 1988). The cations K^+ and Mg^+ have been shown to be essential for EBPR and were therefore added as separate feed components to ensure adequate concentrations (Rickard and McClintock, 1992). The acetate based feed was used in most investigations whilst the glucose based feed was only used in the *Aeromonas* sp. pure culture and *Aeromonas* / *Acinetobacter* co-culture studies.

Table 2. Composition of synthetic sewage feed

Component	Acetate Feed Concentration (g l ⁻¹)	Glucose Feed Concentration (g l ⁻¹)
Sodium acetate	1.625	-
Glucose	-	3.7
NH ₄ Cl	0.267	0.267
MgSO ₄ ·7H ₂ O	0.125	0.125
KH ₂ PO ₄	0.0767	0.0767
Yeast Extract	0.05	0.05

The concentrated feed solution was made up in 10 l aspirators and sterilised by autoclaving at 121°C, 15 psi for 1 h. The KH_2PO_4 and glucose were sterilised separately by passing through a 0.2 μm filter (Whatman, Maidstone) and added to the aspirator after its contents had cooled. The pH was adjusted to $\text{pH } 7.2 \pm 0.1$.

2.4. ANALYTICAL METHODS

2.4.1. Chemicals

All chemicals were of Analar grade and were supplied either by BDH (Poole), Fisons (Loughborough) or Sigma (Poole). Culture media were supplied by Oxoid (Basingstoke).

2.4.2. Phosphorus

2.4.2.1. Glassware

All glassware was soaked overnight in sulphuric acid ($d_{20} \text{ 1.84}$) and rinsed several times with deionised water before use (Department of the Environment, 1980a).

2.4.2.2. Sample Storage and Preservation

When immediate analysis was not possible samples were preserved by the addition of a 400 mg l^{-1} mercuric chloride solution to 10 % (v/v) (Rossin and Lester, 1980). The preserved samples were stored at 4°C for a maximum of 4 days prior to analysis.

2.4.2.3. Sample Pretreatment

Samples for soluble ortho-phosphate (SOP) measurement (or more precisely 'reactive' phosphorus - see method in Department of the Environment, 1980a) were filtered through 0.2 μm filters (Whatman Ltd, Maidstone) before analysis. Total phosphate (TP) measurement was undertaken after a digestion step (Department of the Environment, 1980a).

2.4.2.4. Methodology

The standard spectrophotometric method (Department of the Environment, 1980a) based on the method of Murphy and Riley (1962) was employed. Absorbances were measured by a Philips PU 8620 UV/VIS/NIR spectrophotometer at a wavelength of 882 nm using 40 mm cells.

2.4.2.5. Interference

A complex and variable interference may be observed in the presence of excess nitrite. A slight excess of sulphamic acid is effective in overcoming the interference (details in Department of the Environment, 1980a). The procedure to overcome this potential interference was employed during comparison of electron acceptor investigations with nitrite.

2.4.3. Suspended Solids

Suspended solids were measured by a modification of the standard method (Department of the Environment, 1980b). Suspensions were filtered through a 0.2 μm filter (Whatman, Maidstone) and dried for 4 minutes in a 650 W microwave oven.

Mixed liquor volatile suspended solids (MLVSS) were determined according to 'Standard Methods' (APHA *et al.*, 1989) using a Gallenkamp Hotspot Furnace. Numerous samples were measured and a value of 85% ($\pm 2\%$) of the total suspended solids was determined.

2.4.4. Chemical Oxygen Demand

Two methods, the standard method (Department of the Environment, 1977) and the Hach kit system (Hach, Chemlab, Cambridge) were used to measure the Chemical Oxygen Demand (COD).

2.4.5. Nitrate

The standard spectrophotometric method was used (Department of the Environment, 1981). Absorbances were measured by a Philips PU 8620 UV/VIS/NIR spectrophotometer at a wavelength of 415 nm using 40 mm cells.

2.4.6. Nitrite

The standard spectrophotometric method was used (Department of the Environment, 1981). Absorbances were measured by a Philips PU 8620 UV/VIS/NIR spectrophotometer at a wavelength of 540 nm using 10 mm cells.

2.4.7. Glucose

Glucose was determined by an enzymic method using a D-Glucose Test Kit (Boehringer Mannheim, Cat. No. 716251). The manufacturer's recommended procedures were followed.

2.4.8. Acetic Acid / Acetate

Acetate was determined by an enzymic method using an Acetic Acid Test Kit (Boehringer Mannheim, Cat. No. 148261). The manufacturer's recommended procedures were followed.

2.4.9. Dissolved Oxygen

Dissolved oxygen (DO) was measured using a portable probe and meter (Jenway 9070, Essex).

2.4.10. pH

Off-line pH measurement was undertaken using a pH probe (Russell pH Ltd., Auchtermuchty) connected to a Corning pH meter (Corning, Essex).

2.4.11. Redox Potential

Off-line redox potential measurement was determined using a platinum electrode (Russell pH Ltd, Auchtermuchty) with a standard Calomel reference (Russell pH Ltd, Auchtermuchty) connected to a multimeter (Model 7040, Cirkit, Hong Kong).

2.4.12. Temperature

Off-line temperature measurements were undertaken using standard mercury in glass thermometers.

2.5. STATISTICAL ANALYSIS

All statistical analyses were calculated by the software program Excel 4.0 (Microsoft Corporation).

CHAPTER 3.

3. ISOLATION OF *ACINETOBACTER* SPP.

3.1. INTRODUCTION

Several genera may be involved in EBPR, but the general consensus is that members of the *Acinetobacter* genus are the main bacteria responsible for the process (Ohtake *et al.*, 1985; Beacham *et al.*, 1989). However, the actual role of *Acinetobacter* spp. in the EBPR process has often been questioned (Stephenson, 1987; Cloete and Steyn, 1988a and 1988b; Raper, 1990; Cloete *et al.*, 1992). Some of the doubt as to the role of *Acinetobacter* spp. in the EBPR process is partly due to early problems with classification alongside inconsistent methods employed in sampling, isolating, enumerating and identifying activated sludge bacterial populations which has led to conflicting observations (Venter *et al.*, 1989). Beacham *et al.* (1990) criticised traditional techniques used for sampling, isolating and identifying microorganisms in activated sludge since they tend to target single cells. This is a particular problem with *Acinetobacter* spp. as they often grow in large cell clusters and dispersal techniques risk damaging the cells (Cloete and Steyn, 1988a). One study estimated that the *Acinetobacter* spp. population was <10% of the total number of bacteria in a sludge sample, implying that the role of *Acinetobacter* spp. in EBPR had previously been overestimated (Cloete and Steyn, 1988b). Advances in classification and identification techniques, along with the realisation of the drawbacks of the older methods have recently contributed to a greater, if not yet thorough, understanding of the role of *Acinetobacter* spp. in EBPR.

3.1.1. Taxonomy of *Acinetobacter* Genus

Brisou and Prevot (1954) placed the non-motile group of *Achromobacter* into a new genus called *Acinetobacter*, whose nomenclature has had a complex and lengthy history (Vivian *et al.*, 1981). Strains now known to be *Acinetobacter* spp. have in the past been classified as *Achromobacter* sp.; *Bacterium anitratum*; *Diplococcus mucosus*; *Herella vaginicola*; *Mima polymorpha* and *Moraxella lwoffii* to name but a few (Baumann *et al.*, 1968). The genus had been assigned to the *Neisseriaceae* family (Lautrop, 1974). However, with recent changes in microbial nomenclature *Acinetobacter* has been placed in the gamma subclass of the class *Proteobacteria* based on 16S rRNA studies (Woese *et al.*, 1985). By rRNA:DNA hybridisation *Acinetobacter*, *Moraxella* and *Psychrobacter* are grouped in a separate branch of rRNA superfamily II (Van Landschoot *et al.*, 1986; Rossau *et al.*, 1989) which now form the *Moraxellaceae* family (Rossau *et al.*, 1991).

3.1.2. Classification and Identification of *Acinetobacter* spp.

The difficulty with *Acinetobacter* spp. classification is that they are very similar to other genera and have few unique phenotypic characteristics. Developments in techniques used to investigate genotypic characteristics have led to improvements. An important progress for the classification of *Acinetobacter* strains came with the development of a DNA transformation assay (Juni, 1972) which is still commonly used to identify and classify *Acinetobacter* to the genus level. Another study found that strains of *Acinetobacter* differ in DNA composition from 38-47 mol% Guanine + Cytosine (G+C) (Henriksen, 1976). DNA-DNA hybridisation has been used extensively in attempts to resolve the problems associated with the identification of *Acinetobacter* spp. to genospecies level. Initially, the genus was subdivided into 12 genospecies on the basis of DNA-DNA hybridisation (Bouvet and Grimont, 1986; Bouvet and

Grimont, 1987) which closely agreed with earlier work (Baumann *et al.*, 1968; Johnson *et al.*, 1970) (Table 3.). Four new genospecies - *A. baumanii*, *A. haemolyticus*, *A. johnsonii* and *A. juni*, were described alongside amended descriptions for *A. calcoaceticus* and *A. lwoffii*. Subsequently, a gamma radiation resistant species of *Acinetobacter* was isolated (Nishimura *et al.*, 1988a) and placed in a new species named *Acinetobacter radioresistens* based on outer membrane protein patterns, electrophoretic enzyme analysis, DNA-DNA hybridisations and phenotypic characteristics (Ino and Nishimura, 1989; Nishimura *et al.*, 1986; Nishimura *et al.*, 1987; Nishimura *et al.*, 1988a; Nishimura *et al.*, 1988b). Three new genospecies were classified and added to this scheme and numbered 13, 14 and 15, and *A. radioresistens* was shown to be identical to DNA group 12 (Tjernberg and Ursing, 1989). A further 5 genospecies have been added to the scheme and numbered 13 to 17 (Bouvet and Jeanjean, 1989). Of these, group 13 corresponds to group 14 previously described (Tjernberg and Ursing, 1989).

Ribotyping has also been used successfully for the identification of strains in the *Acinetobacter calcoaceticus* - *Acinetobacter baumanii* complex which consists of genospecies 1, 2, 3 and 13 (Gerner-Smidt, 1994). The use of any two of the three restriction enzymes used in the study showed banding patterns that could identify each of the 70 strains studied to the correct DNA group.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is another method which has been used to identify and study the taxonomy of the *Acinetobacter* population in activated sludge which suggested that *A. baumanii* and *A. haemolyticus* should be considered as subspecies of *A. calcoaceticus* rather than separate species (Bosch and Cloete, 1993). A separate study, using different isolates was in agreement with these findings (Nishimura *et al.*, 1988b).

Table 3. Subdivision of *Acinetobacter* into genospecies on the basis of DNA-DNA hybridisation

Genospecies (DNA Group)	Name	Comments G+C in mol %
1	<i>A. calcoaceticus</i>	40-42 G+C
2	<i>A. baumannii</i>	40-43 G+C (Bouvet & Grimont, 1986)
3		41-42 G+C
4	<i>A. haemolyticus</i>	40-43 G+C (Bouvet & Grimont, 1986)
5	<i>A. junii</i>	42 G+C (Bouvet & Grimont, 1986)
6		42 G+C (Bouvet & Grimont, 1986)
7	<i>A. johnsonii</i>	44-45 G+C (Bouvet & Grimont, 1986)
8/9	<i>A. lwoffii</i>	45-46 G+C
10		45 G+C
11		40 G+C
12	<i>A. radioresistens</i>	Isolated by Nishimura <i>et al.</i> , 1988
13		41 G+C (Tjernberg & Ursing, 1989)
14		Group 13 (Tjernberg & Ursing, 1989)
15		Tjernberg & Ursing, 1989
16		44 G+C (Bouvet & Jeanjean, 1989)
17		Bouvet & Jeanjean, 1989

An extensive physiological study attempted to improve identification between *Acinetobacter* genospecies where 211 strains were investigated for

329 biochemical characteristics (Kämpfer *et al.*, 1993). Two identification matrices covering most of the genospecies were constructed. One matrix correctly identified 98.0% of isolates over 22 tests, the other 90.8% over 10 tests using a Willcox probability >0.9 . However, DNA-DNA hybridisation or ribotyping was recommended to ensure correct identification of some genospecies. Another study investigated the reliability of phenotypic tests for the identification of *Acinetobacter* spp. (Gerner-Smidt *et al.*, 1991) and compared their results with earlier findings (Bouvet and Grimont, 1986; Bouvet and Jeanjean, 1989). Slight differences were observed, which were expected due to the use of different media and methodology. However, it has been suggested that the scheme of Bouvet and Grimont (1986) developed using clinical isolates, may well need modifying for samples from non-clinical environments (Beacham *et al.*, 1990). A recent study addressed this point and attempted to use and compare some of the identification schemes described above using non-clinical *Acinetobacter* isolates (Soddell *et al.*, 1993). These isolates were derived from a wastewater treatment pilot plant designed for EBPR described by Beacham *et al.* (1990). None of the identification schemes gave good results with the wastewater derived strains. They also showed some bias since different schemes tended to favour the correct identification of different genospecies. The wastewater derived genospecies identified with the most confidence were *A. johnsonii* (genospecies 7), *A. lwoffii* (genospecies 8) and genospecies 12.

Isolation and identification of *Acinetobacter* spp. in this laboratory can only be undertaken when the physiological characteristics of the genus have been considered. *Acinetobacter* spp. colonies are normally 2-3 mm in diameter after 24 h growth, are usually smooth, and can sometimes be very mucoid with a tendency to adhere to the growth substrate (Lautrop, 1974). The colonies appear mucoid when the cells are encapsulated. Pigmentation is not generally observed (Juni, 1984). They also often produce butyrous colonies (Henriksen,

1976). Cells are usually short, plump rods, 0.9-1.6 μm in diameter and 1.5-2.5 μm in length in the logarithmic phase, becoming coccoid in the stationary phase of growth. Characteristically they occur in pairs and variable length chains. They do not form spores (Juni, 1984). The genus is oxidase-negative, catalase-positive and considered Gram negative - although Baumann *et al.* (1968) noted that the rate of decolorisation by the alcohol is variable. Members are non-motile i.e. "swimming" motility does not occur, however "twitching" motility is observed on the surface of solid media (Juni, 1984). *Acinetobacter* spp. are found naturally in soil and water (Lautrop, 1974), sewage (Warskow and Juni, 1972) and can cause nosocomial infections in humans (Henriksen, 1976).

Acinetobacter spp. can be isolated on standard laboratory media such as nutrient agar (Towner *et al.*, 1991). Specific growth factors are not required by *Acinetobacter* spp., Baumann *et al.* (1968) repeatedly subcultured 106 strains in a mineral medium containing NH_4Cl as the nitrogen source and acetate as carbon source. All the strains grew "readily and abundantly". They are defined as chemoorganotrophic and are versatile in the utilisation of organic compounds as carbon and energy sources (Lautrop, 1974). Baumann *et al.* (1968) tested a wide range of organic compounds which could support their growth. Out of the 85 tested compounds, which could be used by one or more of the strains examined, only 5; acetate, butyrate, valerate, caproate and pyruvate could support the growth of every strain. Only a few strains can use glucose as the sole carbon source for growth and its metabolism occurs via the Entner-Doudoroff pathway (Juni, 1972). Both liquid and solid media are suitable for the growth of *Acinetobacter* spp. (Warskow and Juni, 1972; Henriksen, 1973).

The genus is defined as strictly aerobic, with oxygen as the terminal electron acceptor (Juni, 1978). However, some species can use nitrate when oxygen

is not available (Henriksen, 1976; Lötter, 1985). The precise nature of the electron transfer pathways are unclear (Towner *et al.*, 1991) but they are known to contain cytochromes *a* and *b* (Whittaker, 1971). They do not contain cytochrome *c*, a characteristic expected of oxidase-negative bacteria (Baumann *et al.*, 1968).

All strains grow between 20 and 30°C (Juni, 1984), but they do vary in their temperature optima (Henriksen, 1976). Generally, most strains are mesophilic and have temperature optima of 33-35°C. However, some strains are psychrophilic, failing to grow at 37°C and growing at 5°C (Breuil and Kushner, 1975). *A. johnsonii* strains are unable to grow at 37°C, and only strains of *A. baumannii* can grow at 44°C (Grimont and Bouvet, 1991). Du Preez and Toerien (1978) found that the highest maximum growth rates for *Acinetobacter calcoaceticus* were obtained at temperatures between 29-35°C.

Juni (1984) and Towner *et al.* (1991) note that the *Acinetobacters* have a pH optimum for growth which is slightly acid, and that aeration at a pH of 5.5-6 favours their enrichment from environmental samples. Lautrop (1974) notes that their optimum pH is around neutrality. Hao and Chang (1987) however, found an optimal pH for growth of pH 7.5 for *Acinetobacter calcoaceticus* var. *lwoffii*.

A valuable tool for the isolation of particular bacteria is the use of screening on selective media. The development of selective media favouring the growth and isolation of *Acinetobacter* spp. whilst suppressing other bacteria is not a new concept. Baumann (1968) used a mineral medium with acetate as a single source of carbon and energy for *Acinetobacter* spp. enrichment before their isolation. Grehn and von Graevenitz (1978) also used the ability of *Acinetobacter* spp. to grow on acetate as a sole carbon and energy source. A selective differential medium for the isolation of *Acinetobacter* spp. was

developed by Holton (1983), but the samples were derived from clinical sources. Even before isolation is attempted selective pressures can be imposed on mixed cultures to enhance the proliferation of certain species over others. Juni (1984) states that vigorous aeration at 30°C is favourable for the enrichment of the non-motile *Acinetobacter* spp. because members of the *Pseudomonas* genus in particular are prevented from moving to the surface layer, where they consume much of the available oxygen, therefore decreasing its availability to the bulk of the culture liquid. This methodology was first used by Baumann (1968) when *Acinetobacter* spp. were isolated from 28 out of 30 soil samples and 29 out of 30 water samples tested.

Since one aim of this work is to investigate the ability of various *Acinetobacter* spp. strains in removing phosphate from a synthetic sewage medium, attempts were made to isolate *Acinetobacter* spp. from several activated sludges for use in subsequent EBPR pure culture studies. Three activated sludges were used as potential sources of isolates. The sludges were derived from: a full scale phosphate removing plant in South Africa (from the aerated mixed liquor zone of Johannesburg Northern Wastewater Treatment Plant); a full scale conventional activated sludge plant not demonstrating EBPR (Anglian Water's Cotton Valley plant in Milton Keynes) and an EBPR pilot plant (located at Anglian Water's Cotton Valley plant in Milton Keynes).

3.2. EXPERIMENTAL

3.2.1. Development of an *Acinetobacter* spp. Identification Scheme

By considering the physiological characteristics of the genus, a relatively simple identification scheme was developed which could be used in a wastewater microbiology laboratory using standard techniques and methods. Characteristics were chosen which were either positive or negative for all

Acinetobacter spp.. The basic physiological criteria for presumptive identification as *Acinetobacter* spp. were selected as: aerobic growth on nutrient agar; colony pigmentation; Gram stain and oxidase test. The identification of isolates which did not meet these criteria was generally halted at this stage and the cultures were discarded. However, isolates which demonstrated the selected characteristics were subjected to biochemical tests using the Analytical Profile Index (API) system. API kits 20 E and 20 NE (Bio Merieux) were used in this study. Table 4. summarises the important identification criteria for *Acinetobacter* spp. based on the genus' physiological characteristics.

Table 4. Summary of important *Acinetobacter* spp. identification criteria

Test	<i>Acinetobacter</i> spp.
Aerobic growth on nutrient agar	+
Colony pigmentation	None
Gram stain	-
Cell morphology	Short rods / cocci
Oxidase	-
API 20 E and API 20 NE	<i>Acinetobacter</i> sp.

3.2.2. Identification Methods

Aseptic techniques were used throughout this work. The growth on nutrient agar test was always positive since isolates were originally isolated onto nutrient agar (Oxoid, CM3) plates and incubated at 30°C. Colony morphology was examined from nutrient agar plates. Gram stained smears were examined using a Swift M4000-D microscope. Originally, the oxidase test present in both

API 20 E and API 20 NE kits was used. However, conflicting results using duplicate isolates raised questions as to its suitability. Venter *et al.* (1989) also noted its unreliability. An alternative oxidase test was sought and the Oxidase Touch Sticks (Oxoid, BR64) test was found reliable and therefore employed for the majority of the work. Biochemical tests were undertaken using the API 20 E and API 20 NE Test Kits. All Gram positives were discarded, identification proceeded with Gram negatives only.

3.2.3. Resuscitation and Growth of Cultures

A general purpose medium, nutrient broth (fluid form) and agar (semi-solid form) was initially used for the revival, cultivation and maintenance of the microorganisms. As a guide for the isolation work a type strain culture was bought from The National Collections of Industrial and Marine Bacteria Ltd. (NCIMB Ltd., Aberdeen). An *Acinetobacter johnsonii* (NCIMB 12460) was selected. The isolate was delivered to the laboratory freeze-dried in a small glass ampoule and was revived according to NCIMB Ltd.'s recommendations. Nutrient broth and agar were used as revival media.

A freeze-dried activated sludge sample donated by L. Lötter (via the Cydna Laboratory, Scientific Services, Water and Waste Directorate, Johannesburg, S.A.) and sourced from an EBPR plant in South Africa was divided into two separate samples on arrival at the laboratory. The sample from one of the bottles was revived and used for the isolation work, the other was stored at 4°C. To revive the sludge, a small quantity of the freeze-dried sample was suspended in 10 ml of Ringers solution in an Universal bottle. The sample was shaken vigorously for several minutes and serial dilutions were prepared. Nutrient agar pour plates of the serial dilutions were made and incubated at 30°C for 2 d. In case of failure, a second resuscitation method was employed using liquid growth medium. Some of the freeze-dried sludge was placed in

two 500 ml Erlenmeyer flasks containing 200 ml of nutrient broth, stoppered with cotton wool, covered with aluminium foil, and incubated in an orbital shaker at ~ 70 rpm and 30°C. After 2 d growth loopfuls of the culture were streaked onto nutrient agar plates and incubated at 30°C for 2 d.

Two sloped *Acinetobacter* sp. isolates were also donated by L. Lötter which were isolated from the Johannesburg Northern Wastewater Treatment Plant. The isolation work was undertaken by the Cydna Laboratory, Scientific Services, Water and Waste Directorate, Johannesburg, S.A.. These isolates were referred to as SAA and SAB. On arrival at the laboratory they were streaked onto nutrient agar plates and incubated at 30°C for 2 d. Fresh nutrient agar slopes were then prepared and stored at 4°C for future use.

Two activated sludge samples were obtained from Anglian Water's wastewater treatment plant at Cotton Valley, Milton Keynes. One sample was taken from the conventional activated sludge plant, the other from the RAS line of their EBPR pilot plant. On arrival at the laboratory (transit time was around 30 min.) the sludge samples were shaken vigorously for several minutes before being streaked onto nutrient and acetate medium plates and incubated at 30°C. Some of the samples were also placed separately into 200 ml of nutrient broth and acetate medium in 500 ml Erlenmeyer flasks, stoppered with cotton wool, covered with aluminium foil and incubated in an orbital shaker at ~ 180 rpm and 30°C. After 2 d growth the cultures were streaked onto nutrient agar and acetate medium plates and incubated at 30°C for a further 2 d.

3.2.4. Isolation of *Acinetobacter* spp.

A random selection of well isolated 2 d old colonies were picked off the plates and subcultured onto fresh plates and incubated at 30°C for 2 d. Further subcultures were similarly performed to ensure purity of the isolates. These

isolates were then subjected to the *Acinetobacter* spp. presumptive tests.

3.2.5. Development of Culture Enrichment and a Selective Medium for the Isolation of *Acinetobacter* spp.

To aid *Acinetobacter* spp. isolation, enrichment and selective culture methodologies were developed. The mixed cultures were shaken vigorously to enhance aeration by increasing the speed of the orbital incubator to ~ 180 rpm for the isolations summarised in Tables 7. - 9. A selective medium was designed using known *Acinetobacter* spp. physiological characteristics and was a modification of Baumann's (1968) medium. The medium referred to as "acetate medium" was composed of: 2.3 g sodium acetate ($\text{NaCOOH} \cdot 3\text{H}_2\text{O}$); 0.5 g ammonium chloride (NH_4Cl); 0.05 g magnesium sulphate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$); 0.15 g potassium di-hydrogen orthophosphate (KH_2PO_4); 0.35 g Yeast Extract; made up to 1 l with tap water and adjusted to $\text{pH } 6 \pm 0.2$. For a solid medium, 10 g of Agar Bacteriological (Oxoid, L11) was added to the above.

3.2.6. Storage of Isolates

A nutrient agar slope of each isolate was maintained at 4°C for future reference. These were subcultured as infrequently as possible to avoid mutations - every three months was adequate to keep them viable. To subculture, the isolates were transferred onto fresh nutrient agar slopes, grown for 24 h at 30°C and then stored at 4°C.

3.3. RESULTS

3.3.1. Isolation of *Acinetobacter* spp. from Freeze-Dried South African Sludge

An *Acinetobacter* spp. isolation attempt was undertaken using the freeze-dried South African sludge (SAC). *Acinetobacter johnsonii* (NCIMB 12460) cultures were used as references. No *Acinetobacter* spp. were isolated (Table 5.).

Table 5. Identification of isolates from South African freeze-dried activated sludge. Attempt 1

Isolate	Gram Stain	Oxidase	Appearance	API 20 E	API 20 NE
SAC1	+				
SAC2	+				
SAC3	-	-	cream / white, oval cocci	<i>Erwinia</i>	<i>Pseudomonas</i> / <i>Aeromonas</i>
SAC4	+				
SAC5	+				
SAC6	-	-	cream / white, small cocci / rods	<i>Erwinia</i>	<i>Aeromonas</i>
SAC7	-	-	cream, feathery cocci / rods	<i>Erwinia</i>	<i>Xanthomonas</i> / <i>Aeromonas</i>
SAC8	-	-	cream, feathery cocci / rods	<i>Erwinia</i> / <i>Vibrio</i>	<i>Pseudomonas</i> / <i>Aeromonas</i>
SAC9	+				
SAC10	+				

During this isolation attempt tentative identification was established disregarding the oxidase test results. The oxidase test in the API kits gave

suspect results - the *Acinetobacter johnsonii* (NCIMB 12460) reference strains showed inconsistent oxidase test results. Since the oxidase test is one of the most important tests in the identification of *Acinetobacter* spp., a supplementary oxidase test was sought for use in future isolation attempts. A second attempt was made to isolate *Acinetobacter* spp. from the same South African sludge. *Acinetobacter johnsonii* (NCIMB 12460) isolates were again used as references. However, no *Acinetobacter* spp. were isolated, even with the added advantage of a reliable oxidase test result (Table 6.).

Table 6. Identification of isolates from South African freeze-dried activated sludge. Attempt 2

Isolate	Gram Stain	Oxidase	Appearance	API 20 E	API 20 NE
SAC11	-	+	cream, rods	<i>Pseudomonas</i> sp.	<i>Pasteurella</i>
SAC12	-	+	white, rods	<i>Pseudomonas</i> sp.	<i>Pseudomonas putida</i>
SAC13	-	+	white, rods	<i>Pseudomonas</i> sp.	<i>Pseudomonas vesicularis</i>
SAC14			contaminated		
SAC15	-	+	white, rods	<i>Pseudomonas</i> sp.	<i>Pseudomonas cepacia</i>
SAC16	-	+	white, rods	<i>Pseudomonas</i> sp.	<i>Aeromonas hydrophila / caviae</i>
SAC17	-	+	white, rods	<i>Pseudomonas</i> sp.	<i>Pseudomonas cepacia</i>
SAC18	-		contaminated		
SAC19	-	+	white, rods	<i>Pseudomonas</i> sp.	<i>Pseudomonas cepacia</i>
SAC20	-	+	white, rods	<i>Pseudomonas</i> sp.	<i>Pseudomonas vesicularis</i>
SAC21	-		pink colonies		

As a consequence of this second failure a rethink of the isolation methods was undertaken and a more selective approach was sought (see 3.2.5.). This led to the development of an *Acinetobacter* spp. culture enrichment and selective medium methodology. A third attempt was undertaken to isolate *Acinetobacter* spp. from the freeze-dried South African activated sludge sample using the new methodology. *Acinetobacter johnsonii* (NCIMB 12460) was used as a reference isolate. Excellent results were obtained using the improved methodology. Out of 24 randomly picked colonies 13 were identified as *Acinetobacter* spp. (Table 7.). These isolates were streaked onto nutrient agar slopes and stored at 4° C for future use.

Table 7. Identification of isolates from South African freeze-dried sludge using the selective acetate medium

Isolate	Gram Stain	Oxidase	Appearance	API 20 E	API 20 NE
SAC22	+				
SAC23	-	-	white, mucoid, short rods	<i>Acinetobacter</i> sp.	<i>Acinetobacter lwoffii</i>
SAC24	-		pink, rods		
SAC25	-	-	white, opaque, cocci	<i>Acinetobacter</i> sp.	<i>Acinetobacter lwoffii</i>
SAC26	-	-	white, mucoid, rods	<i>Acinetobacter</i> sp.	<i>Acinetobacter lwoffii</i>
SAC27			no growth		
SAC28	-	-	white, short rods	<i>Acinetobacter</i> sp.	<i>Acinetobacter lwoffii</i>
SAC29	-	-	white, cocci	<i>Acinetobacter</i> sp.	<i>Acinetobacter lwoffii</i>
SAC30	-	-	white, opaque, short rods	<i>Acinetobacter</i> sp.	<i>Acinetobacter lwoffii</i>
SAC31	-	-	white, cocci	<i>Acinetobacter</i> sp.	<i>Acinetobacter lwoffii</i>

SAC32			contaminated		
SAC33	-	+			
SAC34	-	+			
SAC35	-	-	white, mucoid, rods	<i>Acinetobacter</i> sp.	<i>Acinetobacter lwoffii</i>
SAC36			contaminated		
SAC37	-	-	white, short rods	<i>Acinetobacter</i> sp.	<i>Acinetobacter lwoffii</i>
SAC38	-	-	white, cocci	<i>Acinetobacter</i> sp.	<i>Acinetobacter lwoffii</i>
SAC39			pink rods		
SAC40	-	-	white, mucoid, short rods	<i>Acinetobacter</i> sp.	<i>Acinetobacter lwoffii</i>
SAC41			no growth		
SAC42	-	-	white, opaque, cocci	<i>Acinetobacter</i> sp.	<i>Acinetobacter lwoffii</i>
SAC43			no growth		
SAC44			contaminated		
SAC45	-	+	white, rods	?	<i>Pseudomonas</i> sp.
SAC46	-	-	white, mucoid rods	<i>Acinetobacter</i> sp.	<i>Acinetobacter lwoffii</i>

3.3.2. Isolation of *Acinetobacter* spp. from Cotton Valley Full Scale Conventional Activated Sludge Plant

The culture enrichment and selective medium methodology was used during this *Acinetobacter* spp. isolation attempt. In addition to *Acinetobacter johnsonii* (NCIMB 12460), the South African *Acinetobacter* sp. isolate SAA was used as a reference strain. Only limited success was obtained using the selective medium during this attempt, out of 18 isolates only 3 were identified as *Acinetobacter* spp. (Table 8.).

Table 8. Identification of isolates from Cotton Valley full-scale activated sludge plant using the selective acetate medium

Isolate	Gram Stain	Oxidase	Appearance	API 20 E	API 20 NE
MKA1	+				
MKA2	+				
MKA3	-	-	white, opaque, cocci	<i>Acinetobacter</i> sp.	<i>Acinetobacter lwoffii</i>
MKA4	-	-	white, mucoid, rods	<i>Acinetobacter</i> sp.	<i>Acinetobacter lwoffii</i>
MKA5	-	+	white, rods	<i>Pseudomonas</i> sp.	<i>Pseudomonas vesicularis</i>
MKA6			contaminated		
MKA7	+				
MKA8	+				
MKA9	+				
MKA10	+				
MKA11	-	-	white, opaque, rods	<i>Acinetobacter</i> sp.	<i>Acinetobacter johnsonii</i>
MKA12			pink colonies		
MKA13	+ / -				
MKA14	-	+	cream, rods	<i>Aeromonas hydrophila</i> / <i>caviae</i>	<i>Aeromonas hydrophila</i> / <i>caviae</i>
MKA15	-	+	white, rods	<i>Pseudomonas</i> sp.	<i>Pseudomonas vesicularis</i>
MKA16	+				
MKA17			contaminated		
MKA18	+				

3.3.3. Isolation of *Acinetobacter* spp. Isolates from Cotton Valley EBPR Pilot Plant

The culture enrichment and selective medium method was used during this isolation attempt. *Acinetobacter johnsonii* (NCIMB 12460) and South African isolates SAA and SAB were used as references. Out of the 15 original isolates, 5 were identified as *Acinetobacter* spp. (Table 9.).

Table 9. Identification of isolates from Cotton Valley EBPR pilot plant using the selective acetate medium

Isolate	Gram Stain	Oxidase	Appearance	API 20 E	API 20 NE
MKB1	+				
MKB2	-	-	white, mucoid, rods	<i>Acinetobacter</i> sp.	<i>Acinetobacter johnsonii</i>
MKB3	-	+			
MKB4			contaminated		
MKB5	+				
MKB6	-	+			
MKB7	+				
MKB8	-	-	white, rods	<i>Acinetobacter</i> sp.	<i>Acinetobacter lwoffii</i>
MKB9	-	-	white, rods	<i>Acinetobacter</i> sp.	<i>Acinetobacter lwoffii</i>
MKB10	-	-	white, mucoid, rods	<i>Acinetobacter</i> sp.	<i>Acinetobacter junii</i>
MKB11			contaminated		
MKB12	+				
MKB13	-	+			
MKB14	+				
MKB15	-	-	white, mucoid, rods	<i>Acinetobacter</i> sp.	<i>Acinetobacter johnsonii</i>

3.3.4. Independent *Acinetobacter* spp. Identification Verification

Two of the isolates were sent to the NCIMB Ltd. for independent identification. The isolates were SAC30 (see Table 7.) and MKA11 (see Table 9.). Both isolates were subjected to NCIMB Ltd.'s preliminary examination and were confirmed as *Acinetobacter* spp, possibly *Acinetobacter junii*. A copy of the NCIMB Ltd.'s report is shown in Appendix I.

3.4. DISCUSSION

On the whole, *Acinetobacter* spp. culture enrichment and selective acetate medium methodologies were very successful, since *Acinetobacter* spp. were isolated from each attempt undertaken using this improved methodology. In comparison, no *Acinetobacter* spp. were isolated using nutrient agar and broth at ~ pH 7.4 with moderate aeration. Composition of the selective acetate medium was designed with the needs for the growth of *Acinetobacter* spp. and EBPR requirements in mind. The medium composed of sodium acetate trihydrate as the main carbon and energy source - known to be a satisfactory carbon source (Baumann *et al.*, 1968); ammonium chloride as nitrogen source; potassium di-hydrogen ortho-phosphate for phosphate and the K⁺ ion; magnesium sulphate for the Mg²⁺ ion - the K⁺ and Mg²⁺ ions are considered essential for EBPR (Rickard and McClintock, 1992) and yeast extract for trace elements such as calcium, iron, copper, manganese and zinc, eliminating the requirement for a separate trace element solution. Warskow and Juni (1972) found that one *Acinetobacter* sp. strain which failed to grow on an acetate-mineral agar plate was able to do so when a trace of yeast extract was included in the medium. Wentzel *et al.* (1988) has also noted the importance of yeast extract for EBPR cultures fed with a single substrate.

The identification methods chosen for this study were selected on the basis of test selectivity, cost and time of response. Therefore the methods were employed in the following order: colony appearance, Gram stain, cell appearance, oxidase test, API 20 E and API 20 NE - the cheapest and quickest methods being tested first. Some care must be exercised in the interpretation of the API test results, particularly to species level, since the API 20 E and 20 NE kits have been designed for the identification of strains from clinical origins, and not from environmental samples. Towner and Chopade (1987) successfully used the API 20NE system to identify *Acinetobacter* spp. from a clinical environment. For this work, genus identification is sufficient and the API kits proved adequate with one reservation. Contradictory results were obtained using the API kits' oxidase test. On several occasions oxidase negative *Acinetobacter johnsonii* (NCIMB 12460) gave positive results. Venter *et al.* (1989) evaluated the accuracy of the API 20 E kit in the identification of activated sludge bacteria and also noted unreliability of the oxidase test. Another drawback of the API system is that once prepared, the oxidase reagents denature after a few weeks, therefore the kit must be used relatively quickly. Excellent reproducibility was found in the replacement test, Oxoid's Oxidase Touch Sticks test. Knight *et al.* (1993) assessed the ability of the Biolog Identification System to identify *Acinetobacter* spp. isolated from a wastewater treatment plant. This rapid identification method claims to be able to identify *Acinetobacter* to genospecies level, including isolates derived from non-clinical environments. From 234 isolates identified as *Acinetobacter* sp. according to Juni's transformation assay (Juni, 1972), 193 were also identified as *Acinetobacter* sp. by the Biolog system - 83% successful. However, identification to genospecies level was poor when results were compared to the phenotypic schemes of Bouvet and Grimont (1986); Bouvet and Grimont (1987); Bouvet and Jeanjean (1989) and Gerner-Smidt *et al.* (1991). Knight *et al.* (1995a) also found large differences in carbon utilisation profiles between individual *Acinetobacter* spp. genospecies

Ever since *Acinetobacter* sp. was first implicated with EBPR (Fuhs and Chen, 1975) investigations to determine the precise microbiology have been undertaken and as previously mentioned a great deal of the information gathered has been contradictory. Traditionally, the dynamics and diversity of the microbial population in activated sludge has been investigated by culture dependent techniques. However, these culture dependent methods for isolating, identifying and quantifying microorganisms from their natural environment have shortcomings (Banks and Walker, 1976). Beacham *et al.* (1990) criticised them since they tend to target single cells. This is a particular problem with *Acinetobacter* spp. since they often grow in large cell clusters and dispersal techniques risk damaging the cells (Cloete and Steyn, 1988b). A crude dispersal method was employed in this study in an attempt to separate flocs. However, since this study did not attempt to quantify *Acinetobacter* spp. in the activated sludges the simple isolation and identification methods employed are somewhat justified.

Acinetobacter spp. have been successfully isolated and identified from many EBPR wastewater treatment plants using various techniques (Table 10.). For instance, Beacham *et al.* (1990) investigated the diversity of *Acinetobacter* spp. isolated from a Modified UCT phosphorus removal plant and compared findings with similar work (Duncan *et al.*, 1988) using isolates from a Bardenpho type plant. Their method allowed for the presence of *Acinetobacter* spp. in clumps or clusters since the sludge was pre-treated in a number of ways - by sonication, stomaching (by a Colworth Stomacher), incubating with commercial polysaccharide degrading enzyme preparations and isolation by micromanipulation. This attempted to overcome the tendency of plating-out methods for favouring single cells. However, a greater genospecies diversity amongst isolates from the Modified UCT plant was found than from the Bardenpho type plant. In both investigations genospecies 5 (*A.junii*), 7 (*A.johnsonii*) and 8/9 (*A.lwoffii*) were the most common species isolated.

Table 10. *Acinetobacter* spp. isolated from conventional and EBPR wastewater treatment plants

Isolate	Plant Type	Notes	Reference
<i>A. calcoaceticus</i> var. <i>lwoffii</i>	Full Scale	Homogenised, then streaked on selective media. Identified with API 20NE	Hao & Chang (1987)
<i>A. lwoffii</i>	Full Scale Phostrip & Conventional	Plated out	Fuhs & Chen (1975)
<i>Acinetobacter</i> sp.	Pilot Scale Phostrip	Isolated by sonication & spread plates. Identified with API 20NE	Streichan <i>et al.</i> (1990)
<i>Acinetobacter</i> spp.	Pilot & Lab Scale Bardenpho	Various strains isolated	Bayly <i>et al.</i> (1990)
<i>Acinetobacter</i> spp.	Pilot Scale Modified UCT	Genospecies 2,3,5,6,7,8,9,10,11 & 12 isolated. <i>A. junii</i> dominant	Beacham <i>et al.</i> (1990)
<i>Acinetobacter</i> sp.	Full & Pilot Scale	Identified using biomarker, API 20NE, Enterotube, Oxiferm II & tests based on Juni (1984)	Auling <i>et al.</i> (1991)
<i>Acinetobacter</i> sp.	Pilot Scale	4 strains isolated, 2 accumulated >10x polyphosphate of other 2. Plated out & identified by Juni's transformation assay	Vasiliadis <i>et al.</i> (1990)
<i>Acinetobacter</i> / <i>Moraxella</i> sp.	Pilot & Lab Scale	1.5-10% of total population. Identified using API 20E	Brodisch & Joyner (1983)
<i>A. calcoaceticus</i> & <i>A. lwoffii</i>	Full Scale	Sonicated and plated out.	Suresh <i>et al.</i> (1985)
<i>A. lwoffii</i>	Pilot Scale with nitrification, denitrification	80% P uptake	Florentz & Hartemann (1984)
<i>A. calcoaceticus</i> var. <i>lwoffii</i>	Full Scale	60-70% of total population. 18-43% P removed. Identified using API 20E & fluorescent antibody	Lötter & Murphy (1985)
<i>A. calcoaceticus</i> var. <i>lwoffii</i>	Full Scale. Bardenpho	56-66% of total population. 9-43% P removal	Lötter (1985)

<i>Acinetobacter</i> spp.	Bardenpho	Genospecies 5, 7 and 8/9 commonest	Duncan <i>et al.</i> (1988)
<i>Acinetobacter</i> spp.	Full Scale and Pilot Plants	<i>in situ</i> identification with diaminopropane biomarker. <i>Acinetobacter</i> spp. dominant in low organic loading plants with nitrification, denitrification but not dominant in plants with high organic load	Auling <i>et al.</i> (1991)
<i>Acinetobacter</i> spp.	Full Scale. Phoredox	<i>in situ</i> identification with rRNA targeted oligonucleotide probes. <i>Acinetobacter</i> sp. <8% total active biomass	Wagner <i>et al.</i> (1994b)
<i>Acinetobacter</i> spp.	Conventional activated sludge plants. SBR & one-stage.	<i>n situ</i> identification with rRNA targeted oligonucleotide probes. <i>Acinetobacter</i> sp. <10% total active biomass	Manz, <i>et al.</i> (1994)

A large discrepancy has been observed between total microscopic counts and viable plate counts of activated sludge microorganisms. Even with optimised media, recoveries are only 5-15% and very biased and misleading results can be obtained, which often favour the gamma subclass, which includes *Acinetobacter* spp. (Wagner *et al.*, 1993). These observations highlight the need for the development of non-culture dependent techniques. A fluorescent labelled antibody against *Acinetobacter* spp. was successfully used to identify *Acinetobacter* spp. isolated from activated sludge and showed them to be dominant in the aerobic zone (Lötter and Murphy, 1985). However, in an aut-ecological study of *Acinetobacter* in activated sludge, the same fluorescent antibody technique showed that *Acinetobacter* were not present in high enough numbers to account for the observed phosphate removal (Cloete *et al.*, 1985). Also, a drawback of this method for microbial population studies is that a pure culture of the target microorganism is needed to produce the antibodies. Oligonucleotide probes on the other hand are more flexible for mixed population studies like activated sludge. The probes can be made

specific for different phylogenic levels, ranging from subspecies to kingdom level and can detect and identify previously uncultured bacteria (Wagner *et al.*, 1993). The further development and utilisation of *in situ* identification probes will be a major step forward in improving our knowledge of EBPR bacterial populations.

CHAPTER 4.

4. EBPR ABILITIES OF PURE *ACINETOBACTER* SPP. ISOLATES

4.1. INTRODUCTION

Attention was first focused on *Acinetobacter* spp. as important microorganisms in EBPR when Fuhs and Chen (1975) investigated activated sludge from the Seneca Falls, New York and Baltimore Back River treatment plants and concluded that the genus was responsible for the high levels of phosphate removal from the plants. Since then it has generally been believed that members of this genus play an important role in EBPR, and many researchers have supported their significance in the process (Buchan, 1983; Lötter and Murphy, 1985; Gersberg and Allen, 1985; Deinema *et al.*, 1985).

Others, however, have raised doubts as to the importance of the genus in EBPR. Stephenson (1987) has questioned the role of *Acinetobacter* spp. in the process and Lötter and Murphy (1985) showed that the mere presence of *Acinetobacter* spp. does not guarantee EBPR. They isolated *Acinetobacter* spp. from the aerobic zone of a five stage Bardenpho activated sludge plant which was not exhibiting EBPR at the time. Around 60% of the isolates which grew on their GCY agar plates were identified as *Acinetobacter* spp. Cloete and Steyn (1988a) have also cast a doubt. They determined that a maximum of only 34% of the phosphorus removed from activated sludge sampled from the primary aerobic zone of the Johannesburg Northern wastewater treatment plant could be attributed to removal by *Acinetobacter* spp. as polyphosphate. Other organisms and / or mechanisms had to be involved to account for the phosphate removal.

However, since significant levels of *Acinetobacter* spp. have often been reported in EBPR systems in the past (Lawson and Tonhazy, 1980; Buchan,

1981; and Lötter, 1985) a reasonable assumption might be drawn that *Acinetobacter* spp. may play an important role in the EBPR process. This study undertook to investigate the EBPR capabilities of several pure culture *Acinetobacter* spp. isolates obtained from various sources, including conventional and EBPR activated sludges from the UK and the Johannesburg Northern Works plant mentioned above. Their phosphate removal ability was determined under aerobic, single cycle anaerobic / aerobic and repeated cycling anaerobic / aerobic conditions.

4.2. EXPERIMENTAL

4.2.1. Cultures

Several *Acinetobacter* spp. isolates were investigated for their ability to demonstrate EBPR from a synthetic wastewater medium. These isolates were:

Acinetobacter johnsonii NCIMB 12460 (a type-strain isolate purchased from the National Collections of Industrial and Marine Bacteria Ltd.).

Acinetobacter sp. SAA (originally isolated from Johannesburg Northern Wastewater Treatment Plant, South Africa).

Acinetobacter sp. SAB (also isolated from Johannesburg Northern Wastewater Treatment Plant, South Africa).

Acinetobacter sp. SAC30 (isolated from a freeze-dried sample of South African activated sludge). The isolate was tentatively identified as *Acinetobacter lwoffii*.

Acinetobacter sp. MKA11 (isolated from activated sludge sampled from a full scale conventional activated sludge plant in the UK). The isolate was tentatively identified as *Acinetobacter johnsonii*.

Acinetobacter sp. MKB9 (isolated from the RAS line of an EBPR pilot plant in the UK). The isolate was tentatively identified as *Acinetobacter lwoffii*.

Isolates were revived and cultured as described in Chapter 3. A preculture of each isolate was prepared by transferring a loopful of cells from the stock slope to 200 ml of Acetate Medium in a 500 ml Erlenmeyer flask. The cultures were grown aerobically in an orbital incubator (Gallenkamp) at 150 rpm and 30°C for 48 hours.

4.2.2. Bench Scale Fermentation Unit

The 5 l SBR, with a working volume of 3 l was operated to investigate the EBPR capability of each isolate in turn. Operating conditions were chosen on consideration of a range of values found in the literature and on UK wastewater characteristics. Table 11. summarises the selected conditions.

4.2.3. Analytical Methods

Bacterial growth was monitored using a Philips PU 8620 UV/VIS/NIR spectrophotometer at 600 nm. Orthophosphate was determined using the standard method (Department of the Environment, 1980a).

4.2.4. Phosphate Uptake Shake Flask Batch Experiments

Relatively short term batch experiments were undertaken in shake flasks prior to longer term fermenter studies.

4.2.4.1. Aerobic Investigations

This initial experiment was undertaken to investigate the amount of phosphate metabolised by the *Acinetobacter* sp. isolates during normal aerobic growth. Acetate Medium agar plates were streaked with each isolate which had previously been maintained on nutrient agar slopes. Each isolate was incubated for 2 days at 25°C.

Table 11. Operating conditions for *Acinetobacter* spp. cyclic fermentations

<i>Acinetobacter</i> sp.	NCIMB 12460R1	NCIMB 12460R2	SAAR1	SAAR2	SABR1	SAC30R1	MKA11R1	MKB9R1
Working Volume (l)	2.7	2.7	3	3	3	3	3	3
MCRT (d)	d 1-8=14 d 9-19=8	9	d1-28=17 d29-45=10	10	10	9	10	10
Run Time (d)	19	23	45	35	28	29	33	27
Cycle Time (h)	8	12	8	8	8	8	8	8
Anaerobic Time (h)	d1-8=2 d9-19=1	2	2	2	d1-11=2 d12-28=1	1	1	1
Aerobic Time (h)	d1-8=5 d9-19=6	9	5	5	d1-11=5 d12-28=6	6	6	6
pH	No Control days 1-8 Control pH7 days 9-19	Controlled pH7.2	Controlled pH7	Controlled pH7	Controlled pH7.2	Controlled pH7.2	Controlled pH7.2	Controlled pH7
Temperature (°C)	Controlled 20	Controlled 25	Controlled 20	Controlled 20	Controlled 17.5	Controlled 20	Controlled 17.5	Controlled 17.5
Redox (max) Anaerobic (mV) Aerobic (mV)	Faulty probe	Faulty probe	- 175 + 80	- 185 + 85	- 165 + 70	- 180 + 70	-190 + 60	-185 + 70
Influent: COD (mg/l ¹) o-P (mg/l ¹) TSS(mg/l ¹)	450 20 200	350 15 150	350 20 250	400 15 250	300 13 200	300 12 150	300 12 200	300 11 150

After 2 days each isolate was subcultured - a loopful of the 2 day old culture was transferred to 500 ml Erlenmeyer flasks containing 200 ml of fresh Acetate Medium. The isolates were grown in an orbital incubator (Gallenkamp) at 130 rpm and 25°C for 24 hours. Phosphate uptake was determined by measuring the amount of phosphate removed from the supernatant over 24 hours. Cell growth was also monitored and each isolate was investigated in triplicate. The initial phosphate concentration was around 35 mg l⁻¹. Control experiments (A and B) were also undertaken for each isolate. Control A contained dead cells, control B contained no cells.

4.2.4.2. Anaerobic / Aerobic Investigations

These investigations involved incorporating a preliminary anaerobic step, as found in EBPR processes, into the experiment. It was hypothesised that phosphate release would occur during the anaerobic step followed by phosphate uptake during the subsequent aerobic step.

A loopful of a 2 day old culture of each isolate grown on Acetate Medium agar plates was subcultured into 1 l Erlenmeyer flasks containing 250 ml of Acetate Medium and grown in an orbital incubator (Gallenkamp) at 130 rpm and 25°C for 48 hours. After 2 days, 250 ml of fresh Acetate Medium was added to the flask. The flask was then transferred to a shaking water bath (Grant SS40-5, Grant Instruments Ltd., Cambridge) at 25°C and sparged with nitrogen for 2 hours followed by 6 hours aeration. Ortho-phosphate and cell concentration were measured every 15 minutes during the anaerobic phase and every hour during the aerobic phase. The batch tests for each isolate were carried out in triplicate unless otherwise stated. The initial ortho-phosphate concentration was around 15 mg l⁻¹. Control experiments (A and B) were also undertaken for each isolate. Control A contained dead cells, control B contained no cells.

4.2.5. Fermenter Experiments

Relatively long term fermentation runs were undertaken with each isolate. These allowed for acclimatisation to the cyclic anaerobic / aerobic conditions believed to be required for good EBPR.

4.2.5.1. Cyclic Anaerobic / Aerobic *Acinetobacter* spp. Fermentations

An inoculum was prepared by transferring a loopful of a two day old culture into a 500 ml Erlenmeyer flask containing 250 ml of Acetate Medium. This was incubated in an orbital shaker at 130 rpm and 25°C. After 2 days growth the culture was inoculated into the fermenter. Entire anaerobic / aerobic cycles were monitored periodically during the fermentation runs. Contamination checks were also undertaken on the fermenter culture. A sample was taken and streaked onto nutrient agar and acetate medium agar plates. If thought suspect, colonies from these plates were identified further (as described in Chapter 3.).

4.3. RESULTS

4.3.1. Aerobic Investigations

Aerobic batch experiments were undertaken on each *Acinetobacter* sp. isolate in triplicate. The amount of phosphate removed is expressed as ΔP mgP l⁻¹ and the amount of cell growth is expressed as ΔX mg cells l⁻¹. $\Delta P/\Delta X$ gives an indication of the amount of P metabolised by the isolates. The % removal is calculated as:

$$\frac{\text{Amount of phosphate removed}}{\text{Initial phosphate concentration}} \times 100\%$$

Out of the six isolates investigated, isolates SAB and SAC showed the highest amount of phosphate metabolised, expressed as $\Delta P/\Delta X$ (Table 12.). However the percentage phosphate removed from the supernatant was not very great and certainly fell short of the 90% levels required by the EC Urban Wastewater Directive (CEC, 1991).

Table 12. Phosphate uptake data under aerobic conditions for *Acinetobacter* spp. isolates

Isolate	ΔP (mg l ⁻¹) Mean	ΔP (mg l ⁻¹) SD	ΔX (mg l ⁻¹) Mean	ΔX (mg l ⁻¹) SD	$\Delta P/\Delta X$ Mean	% PO ₄ ⁻ Removed
<i>A. Johnsonii</i> NCIMB12460	3.53	0.38	103.67	4.73	0.034	10.1%
SAA	4.37	0.21	115.00	4.58	0.036	12.3%
SAB	4.63	0.32	113.67	5.69	0.042	13.3%
SAC30	4.00	0.17	96.00	7.00	0.042	11.6%
MKA11	3.83	0.12	111.30	5.51	0.034	10.6%
MKB9	3.87	0.12	107.30	5.03	0.036	11.1%

The highest percentage phosphate removed was shown by the South African isolates SAB, SAA and SAC respectively, followed by the EBPR pilot plant isolate, the full scale conventional activated sludge isolate and finally the NCIMB type strain. A similar trend was followed for the concentration of phosphate removed (ΔP). These results give an indication of the amount of phosphate needed for normal cell growth at these operating conditions over 24 hours. No phosphate uptake was measured in the controls.

4.3.2. Single Cycle Anaerobic / Aerobic Investigations

Mean results are shown for triplicate investigations for all six isolates (Figures 16. - 21.). None of the isolates showed phosphate curves typical of an EBPR process, since phosphate release was not observed during the anaerobic conditions. However, gradual phosphate uptake was detected for all isolates. A small increase in the absorbance of the bacterial cultures (all < 0.2 absorbance units) indicated slight bacterial growth during the course of a single cycle. Considering the levels of phosphate removed (Table 13.) it is clear that EBPR did not occur.

Table 13. Summary of single cycle anaerobic / aerobic shake flask batch investigations

Isolate	^a Initial P Transfer Rate - Anaerobic (mgP gTSS ⁻¹ , h)	^a Initial P Transfer Rate - Aerobic (mgP gTSS ⁻¹ , h)	ΔP (mgP l ⁻¹)	%P Removed
NCIMB 12460	-5.22	4.64	1.80	11.5
SAA	-5.57	6.96	2.03	15.5
SAB	-7.45	-2.22	2.20	14.0
SAC30	-11.88	3.94	2.14	14.4
MKA11	-5.33	2.61	3.97	24.0
MKB9	-0.89	3.18	3.33	21.9

^a Positive values indicate phosphate release, negative values indicate phosphate uptake.

Phosphate transfer rate is defined as the concentration of phosphate uptake or release (in mg) per gram of cells during the initial hour of the particular anaerobic or aerobic phase. ΔP and %P have been explained in 4.3.1.

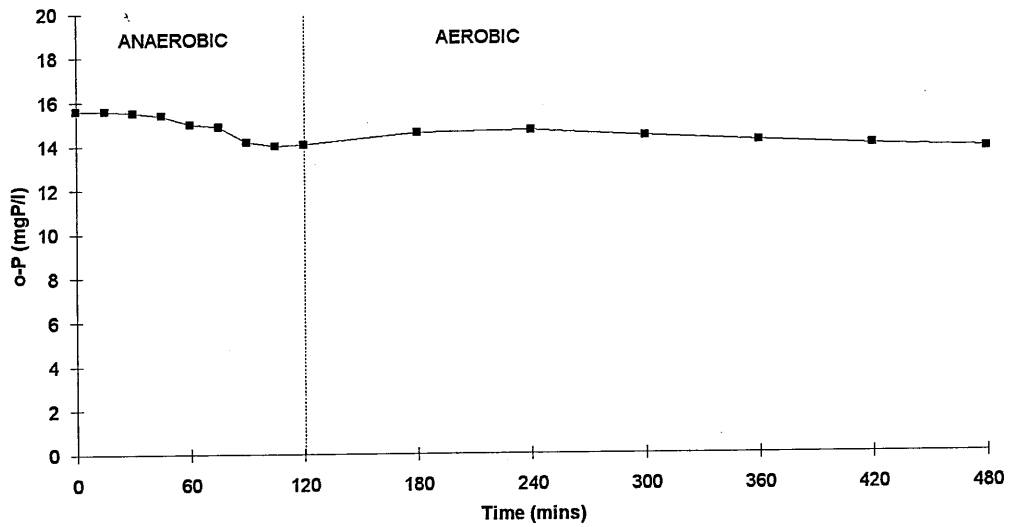


Figure 16. Anaerobic / aerobic single cycle profile for *Acinetobacter johnsonii* (NCIMB12460).

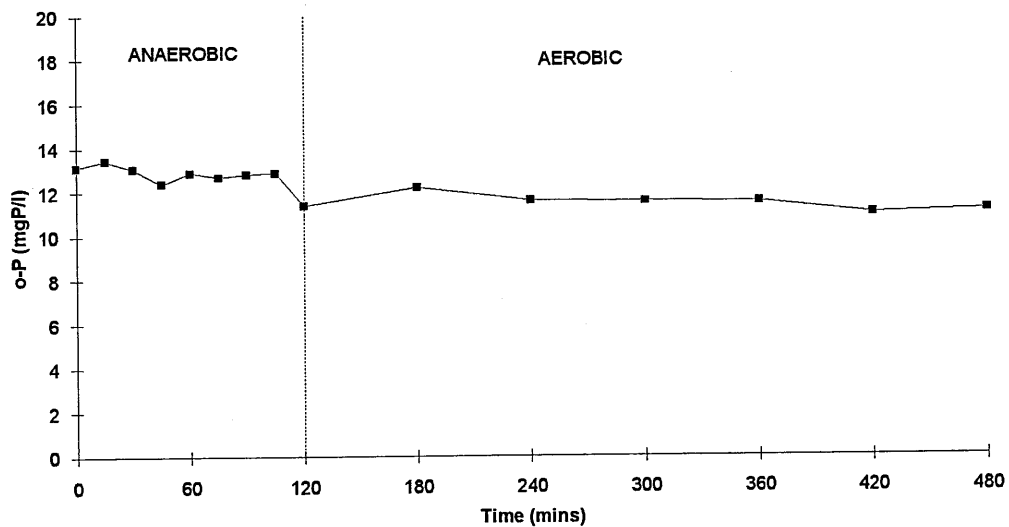


Figure 17. Anaerobic / aerobic single cycle profile for SAA

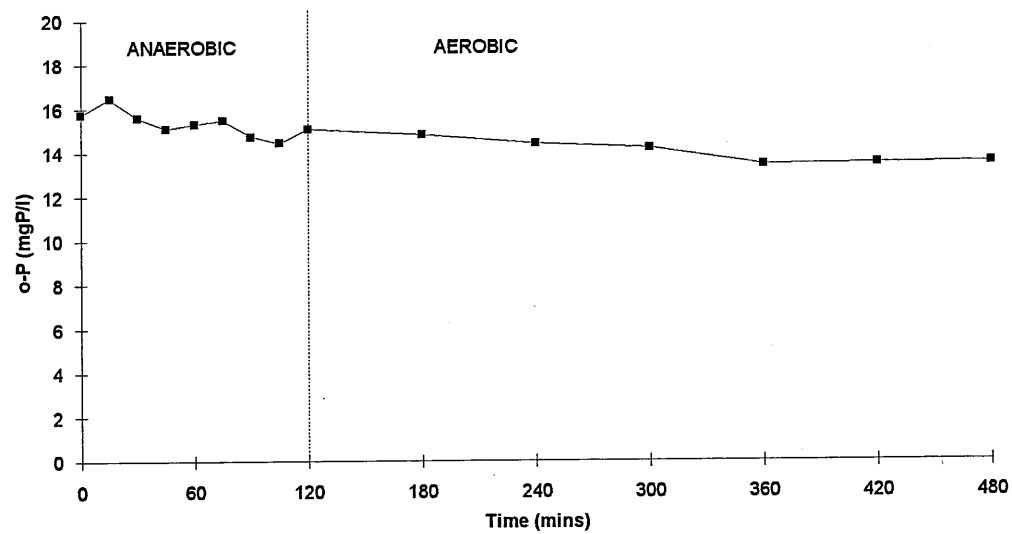


Figure 18. Anaerobic / aerobic single cycle profile for SAB

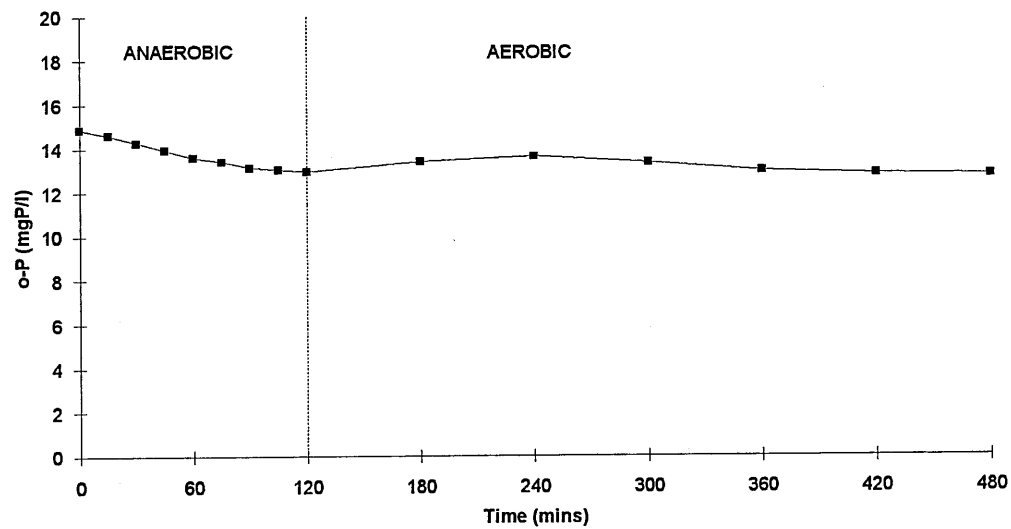


Figure 19. Anaerobic / aerobic single cycle profile for SAC

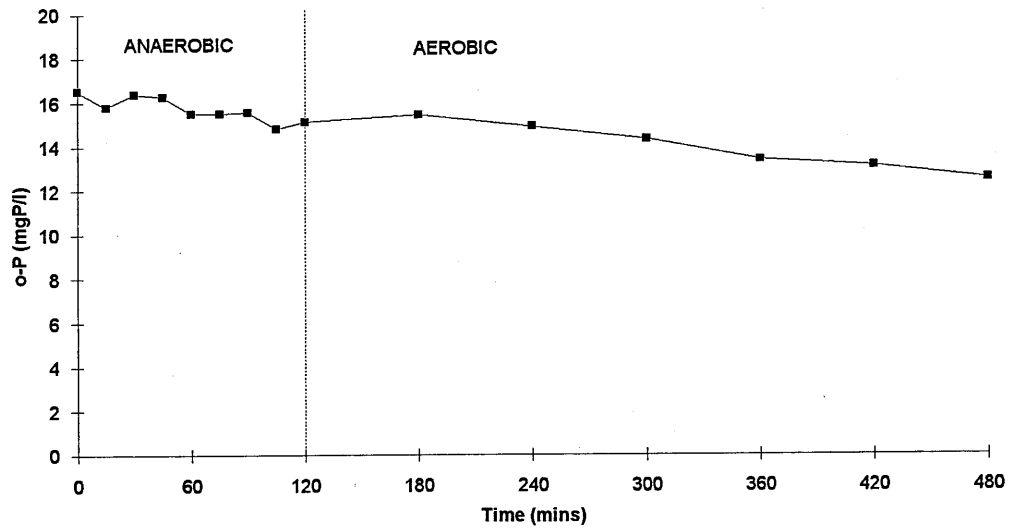


Figure 20. Anaerobic / aerobic single cycle profile for MKA

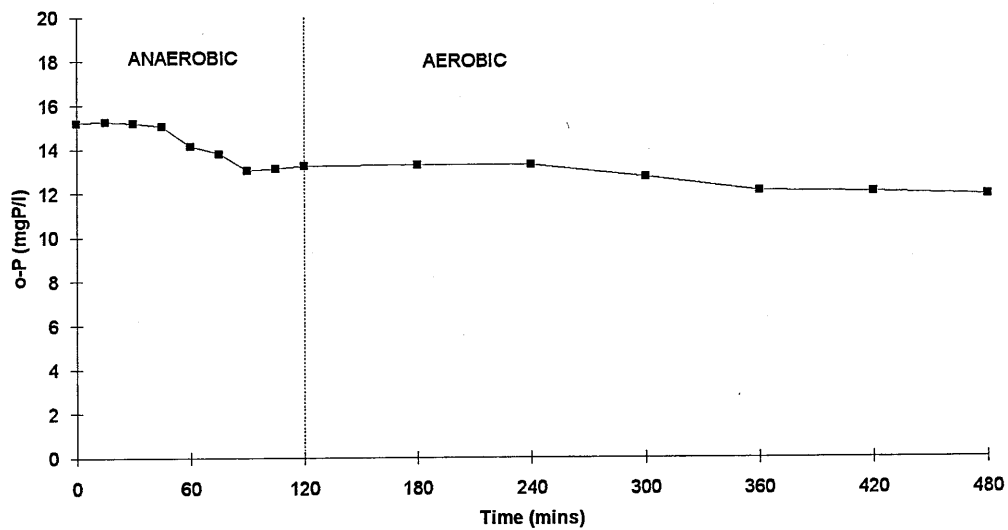


Figure 21. Anaerobic / aerobic single cycle profile for MKB

Isolate MKA11 (the conventional activated sludge isolate) showed the best percentage phosphate removal followed by MKB9 (the EBPR pilot plant isolate). Apart from these two isolates, the percentage phosphate removal was very similar to levels achieved in the aerobic study - the anaerobic phase seemed to have very little beneficial effect (Table 13.). However, comparing the concentration of phosphate removed in this, with the aerobic study, an interesting trend is shown. Apart from isolates MKA11 and MKB9, the concentration of phosphate removed was around twice as great in the aerobic study again indicating that the inclusion of the anaerobic phase in this study seemed to have no beneficial effect. Isolates MKA11 and MKB9 showed comparable phosphate concentration removal as in the aerobic study. However in this study their percentage phosphate removal was around twice that of the aerobic study indicating a slight improvement in their phosphate removal ability. In EBPR terms however, the observed phosphate removal from the supernatant by each isolate was not significant and was presumably the amount needed for normal metabolic requirements. The phosphate concentration remained constant in the controls during the experiment.

4.3.3. Cyclic Anaerobic / Aerobic *Acinetobacter* spp. Fermentation Investigations

Fermentation runs were undertaken with each isolate in turn. Details of the fermentation operating conditions are given in Table 11.

4.3.3.1. *Acinetobacter johnsonii* (NCIMB 12460)

The EBPR capability of this type strain isolate was investigated in two separate fermentation runs. The first fermentation (NCIMB12460R1) was undertaken for 19 days. Figures 22. - 24. show the phosphate curves on days 8, 12 and 19 of the fermentation.

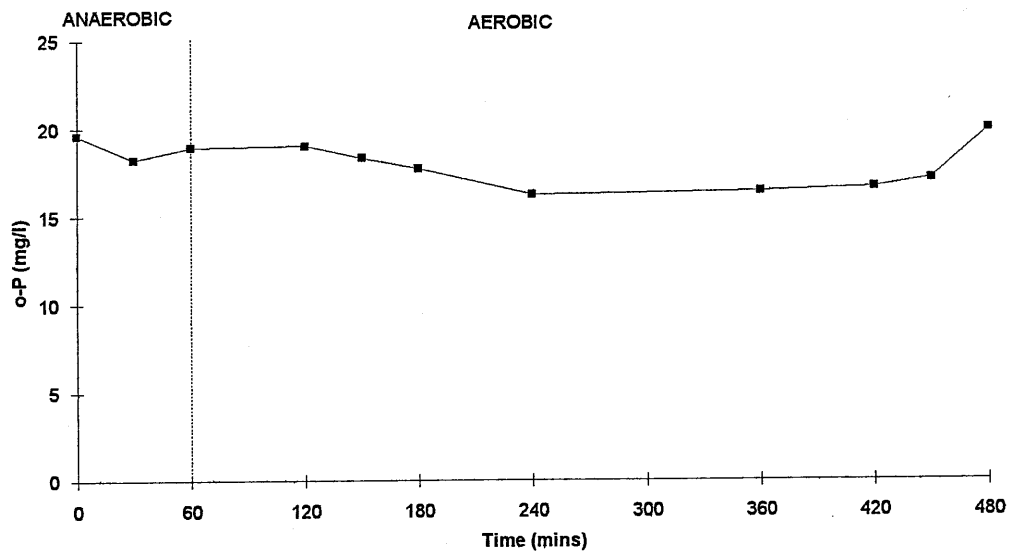


Figure 22. Cyclic fermentation profile for NCIMB12460R1 on day 8.
Where, time = 0 is 25 x 8 h cycles into the run

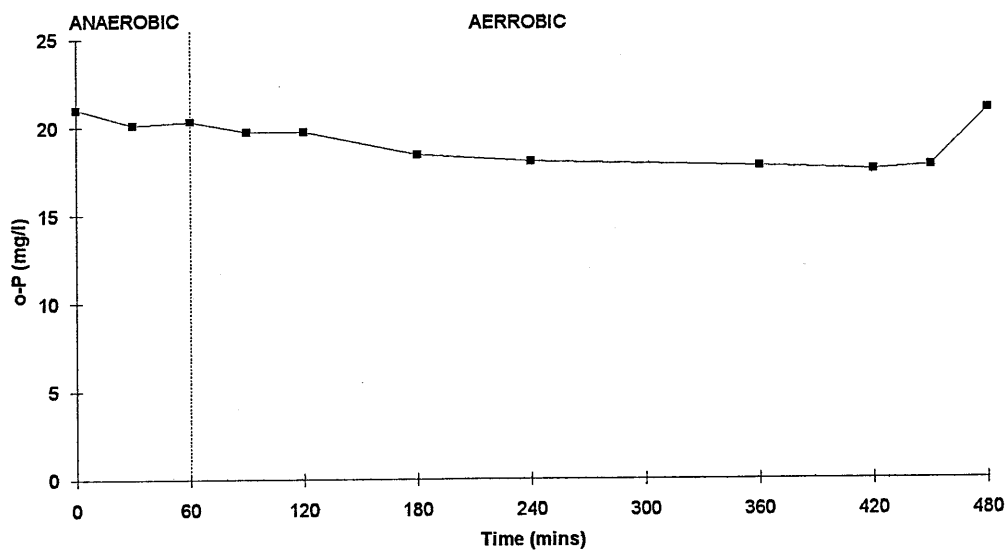


Figure 23. Cyclic fermentation profile for NCIMB12460R1 on day 12.
Where, time = 0 is 37 x 8 h cycles into the run

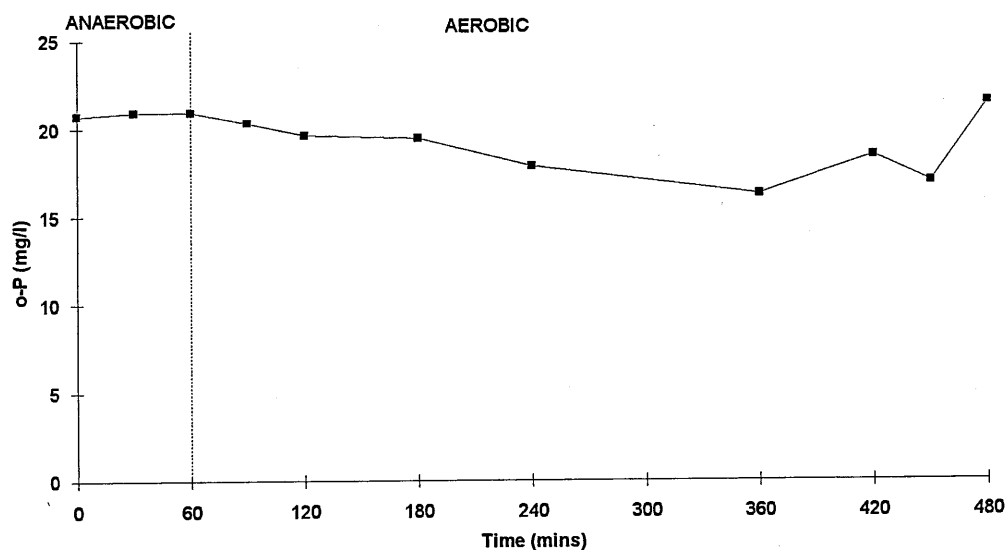


Figure 24. Cyclic fermentation profile for NCIMB12460R1 on day 19.
Where, time = 0 is 58 x 8 h cycles into the run

Throughout this fermentation run, poor phosphate release was observed during the anaerobic phase. However, some phosphate was removed during most complete anaerobic / aerobic cycles (Table 14.) The concentration and percentage of phosphate removal was disappointing throughout the fermentation run and levels comparable to those obtained in the single cycle anaerobic / aerobic study were achieved.

Table 14. Phosphate transfer during fermentation NCIMB12460R1

Isolate	^a Initial P Transfer Rate - Anaerobic (mgP gTSS ⁻¹ , h)	^a Initial P Transfer Rate - Aerobic (mgP gTSS ⁻¹ , h)	ΔP (mgP l ⁻¹)	%P Removed
Day 3	0.50	-14.00	3.0	14.8
Day 8	-3.50	-0.11	3.0	15.3
Day 12	-3.50	-3.00	3.5	16.7
Day 19	1.00	-6.50	2.3	11.1

^a Positive values indicate phosphate release, negative values indicate phosphate uptake.

The observed phosphate removal was thought to be the amount needed for normal metabolism, since the levels fell well short of those expected of an EBPR process.

As a result of these disappointing results a second fermentation run (NCIMB12460R2) was undertaken with the same isolate. A slightly higher temperature (25°C instead of 20°C) and a longer cycle time than the previous run was used in an attempt to stimulate EBPR. Otherwise fairly similar operating conditions were maintained for both runs. This second fermentation run was undertaken for 23 days. Figures 25. - 27. show the phosphate curves on days 8, 14 and 23 of the fermentation. Significant phosphate removal was not seen during the anaerobic phase on any day - indicating that EBPR was not occurring. Overall, however, an improved phosphate removal performance was observed during this second *Acinetobacter johnsonii* (NCIMB12460) isolate run, compared with the first fermentation.

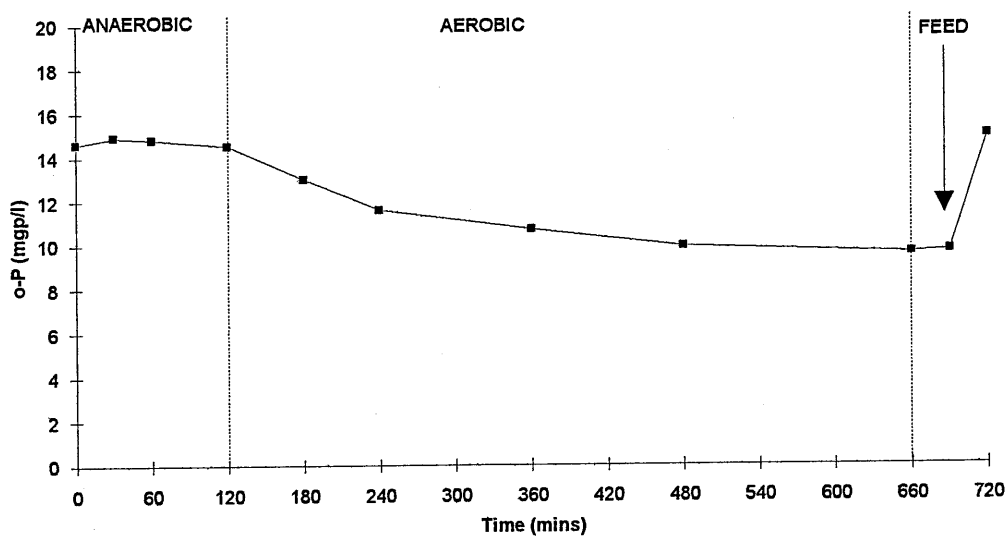


Figure 25. Cyclic fermentation profile for NCIMB12460R2 on day 8.
Where, time = 0 is 17 x 12 h cycles into the run

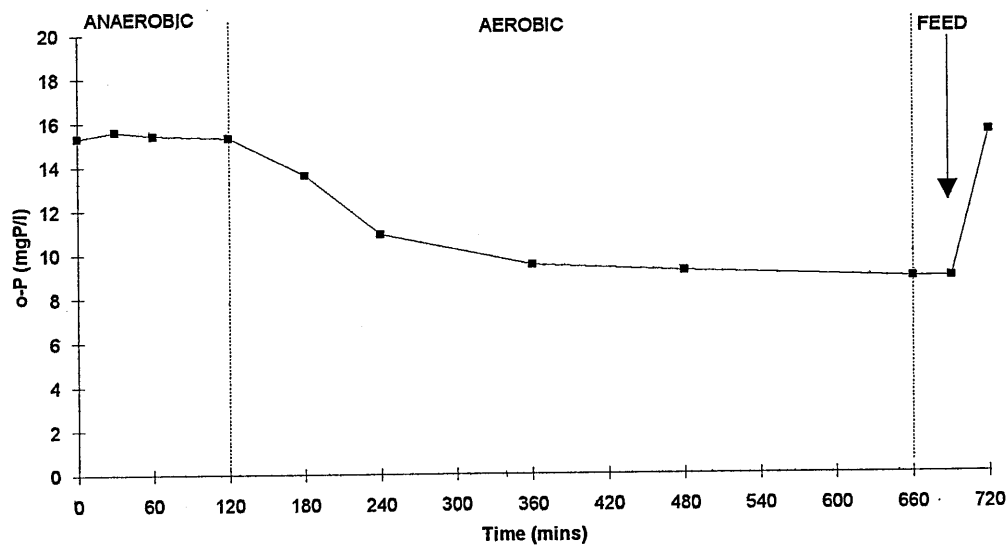


Figure 26. Cyclic fermentation profile for NCIMB12460R2 on day 14.
Where, time = 0 is 29 x 12 h cycles into the run

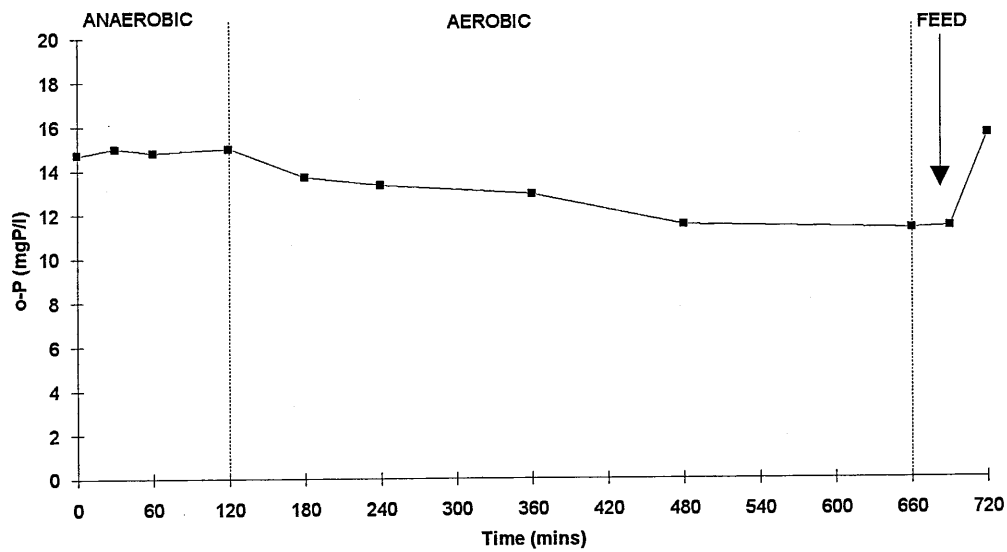


Figure 27. Cyclic fermentation profile for NCIMB12460R2 on day 23.
Where, time = 0 is 47 x 12 h cycles into the run

Phosphate removal (% and concentration) of this second run (Table 15.) was regularly around twice the levels found in the first run (Table 14.).

Table 15. Phosphate transfer during fermentation NCIMB12460R2

Isolate	^a Initial P Transfer Rate - Anaerobic (mgP gTSS ⁻¹ , h)	^a Initial P Transfer Rate - Aerobic (mgP gTSS ⁻¹ , h)	ΔP (mgP l ⁻¹)	%P Removed
Day 2	0.67	-6.00	4.9	32.2
Day 8	1.33	-10.00	5.0	34.2
Day 14	0.67	-11.33	6.4	41.8
Day 23	0.66	-8.67	3.4	23.1

^a Positive values indicate phosphate release, negative values indicate phosphate uptake.

The maximum initial phosphate removal rate during the aerobic phase showed a steady high value, and although up to 42% phosphate (6.4 mgP l^{-1}) was removed on day 14 of the run, these levels could not be improved upon or maintained. After day 23, when the maximum values were halved the run was terminated.

4.3.3.2. *Acinetobacter* sp. SAA

The EBPR capability of this South African isolate was investigated in two separate fermentation runs. The first fermentation run (SAAR1), details of which are described in Table 11., was undertaken for 45 days. Figures 28. - 30. show the phosphate curves on days 22, 37 and 45 of the run.

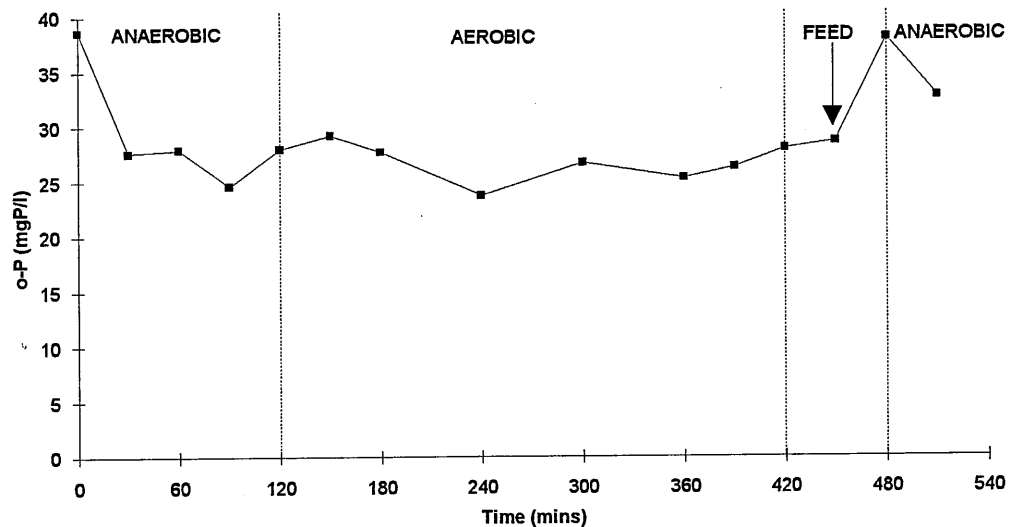


Figure 28. Cyclic fermentation profile for SAAR1 on day 22. Where, time = 0 is 67 x 8 h cycles into the run

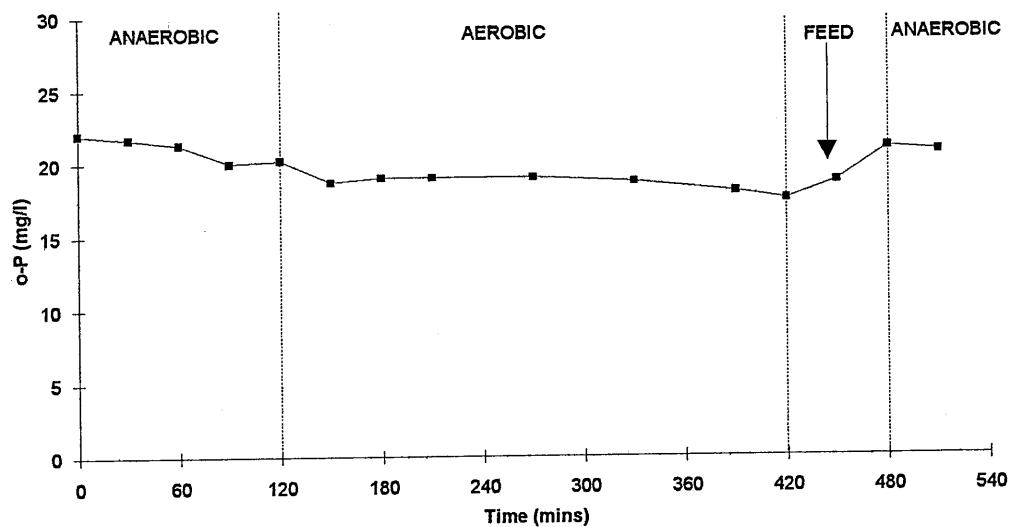


Figure 29. Cyclic fermentation profile for SAAR1 on day 37. Where, time = 0 is 112 x 8 h cycles into the run

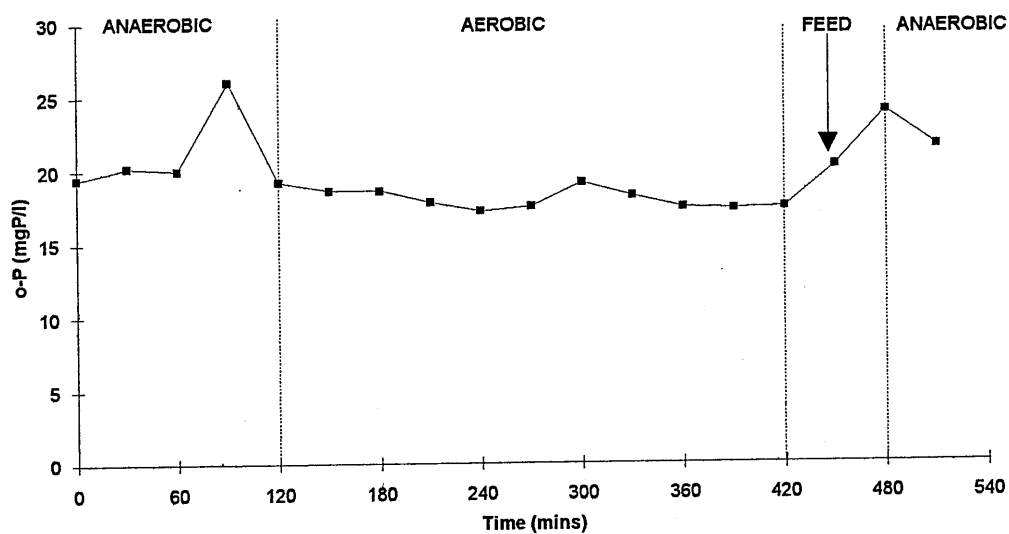


Figure 30. Cyclic fermentation profile for SAAR1 on day 45. Where, time = 0 is 136 x 8 h cycles into the run

Poor overall phosphate removal was observed during this run (Table 16.) and phosphate release was not generally observed during the anaerobic phase.

Table 16. Phosphate transfer during fermentation SAAR1

Isolate	^a Initial P Transfer Rate-Anaerobic (mgP gTSS ⁻¹ , h)	^a Initial P Transfer Rate - Aerobic (mgP gTSS ⁻¹ , h)	ΔP (mgP l ⁻¹)	%P Removed
Day 5	-0.80	-2.40	3.3	16.8
Day 13	-23.20	-4.00	1.4	6.5
Day 22	-42.80	-1.20	10.6	27.4
Day 29	12.00	-4.80	1.2	6.0
Day 37	-2.80	-4.80	4.5	20.5
Day 45	2.40	-2.40	2.0	10.3

^a Positive values indicate phosphate release, negative values indicate phosphate uptake.

Results of day 22 were odd, since unexplained sharp drops in the phosphate concentration were observed immediately after feeding and the commencement of the anaerobic phase, for the one complete cycle shown as well as the subsequent cycle. Consequently, the observed phosphate concentration removed from the supernatant, at 10 mgP l⁻¹, was extremely high. One explanation may be that the magnetic stirrer malfunctioned, resulting in poor mixing immediately after feeding resulting in the unexpected high phosphate results. Otherwise, apart from day 37 of the run, when 20% removal was obtained, very poor results were achieved. The phosphate removed from the supernatant was presumed to be needed for normal metabolic growth and not EBPR.

Due to the disappointing results obtained in fermentation run SAAR1 a second fermentation was undertaken with the same isolate.

This second fermentation (SAAR2) was undertaken for 35 days under similar conditions to SAAR1. Phosphate curves are shown on days 7, 28 and 35 of the run (Figures 31. - 33.).

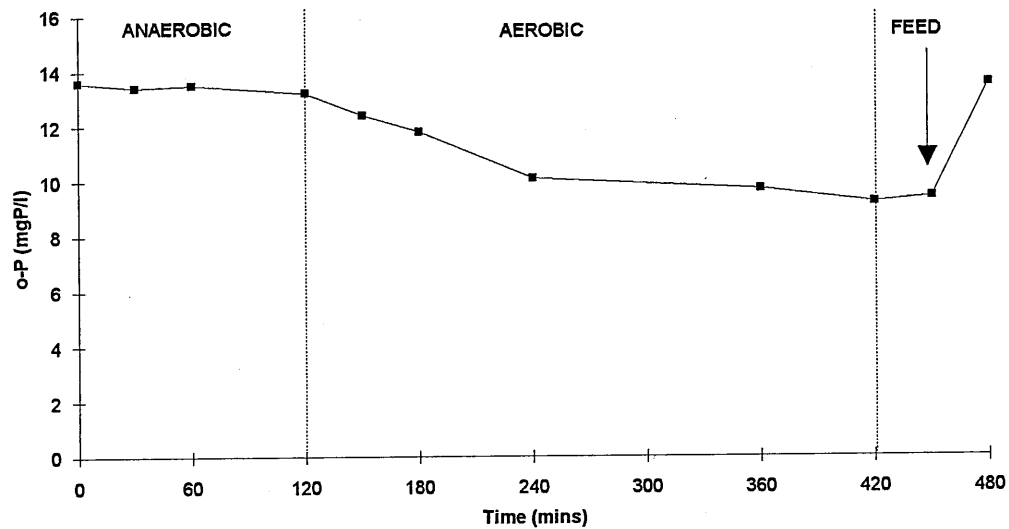


Figure 31. Cyclic fermentation profile for SAAR2 on day 7. Where, time = 0 is 22 x 8 h cycles into the run.

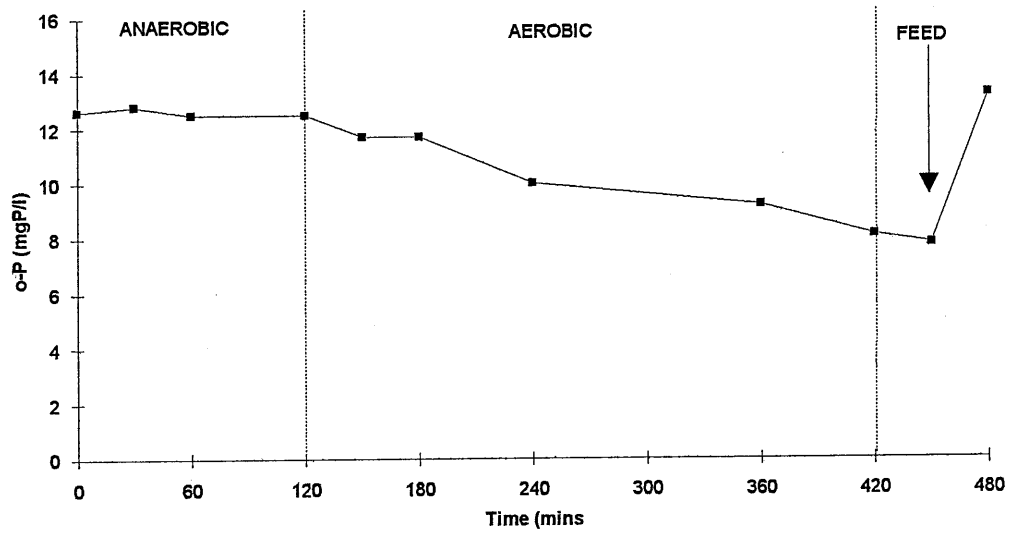


Figure 32. Cyclic fermentation profile for SAAR2 on day 28. Where, time = 0 is 85 x 8 h cycles into the run

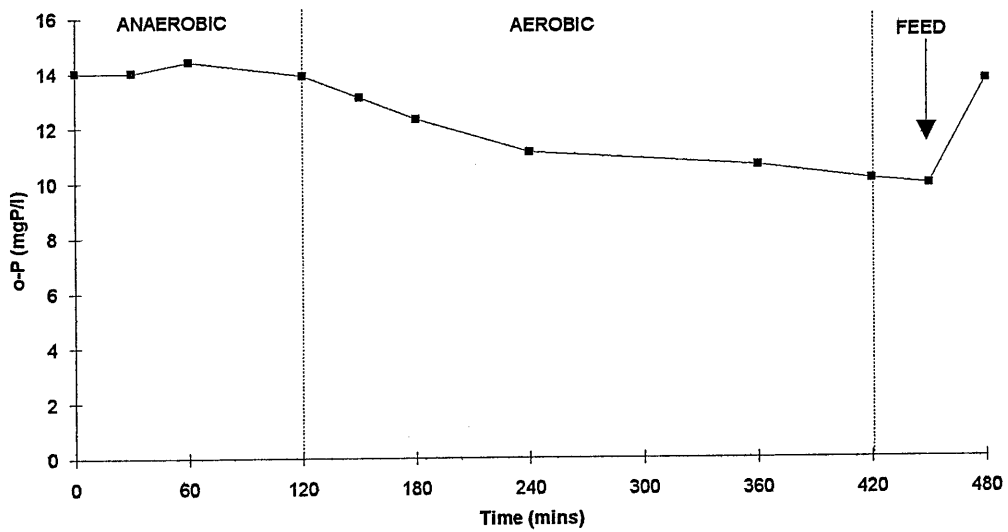


Figure 33. Cyclic fermentation profile for SAAR2 on day 35. Where, time = 0 is 106 x 8 h cycles into the run

Improved phosphate removal levels was achieved during this second fermentation run even though similar operating conditions to SAAR1 were employed. Again, phosphate release during the anaerobic phase was not observed. A maximum phosphate removal of 36% was achieved on day 28 of the run (Table 17.).

Table 17. Phosphate transfer during fermentation SAAR2

Isolate	^a Initial P Transfer Rate-Anaerobic (mgP gTSS ⁻¹ , h)	^a Initial P Transfer Rate - Aerobic (mgP gTSS ⁻¹ , h)	ΔP (mgP l ⁻¹)	%P Removed
Day 2	-0.80	-4.40	4.3	26.5
Day 7	-0.40	-5.60	4.4	32.4
Day 14	0.40	-4.80	4.4	29.3
Day 21	-2.40	-5.20	4.9	27.8
Day 28	-0.40	-3.20	4.5	35.7
Day 35	-1.60	-6.40	3.9	27.9

^a Positive values indicate phosphate release, negative values indicate phosphate uptake.

The levels achieved on days 21 and 28 could not be improved upon and by day 35 the levels had fallen back down to 28% and the fermentation was terminated.

4.3.3.3. *Acinetobacter* sp. SAB

The EBPR capability of this second South African isolate was investigated. This fermentation run (SABR1) was undertaken for 28 days. Operating conditions for the fermentation are described in Table 11. Figures 34. - 36. show the phosphate curves on days 2, 11 and 28 of the run.

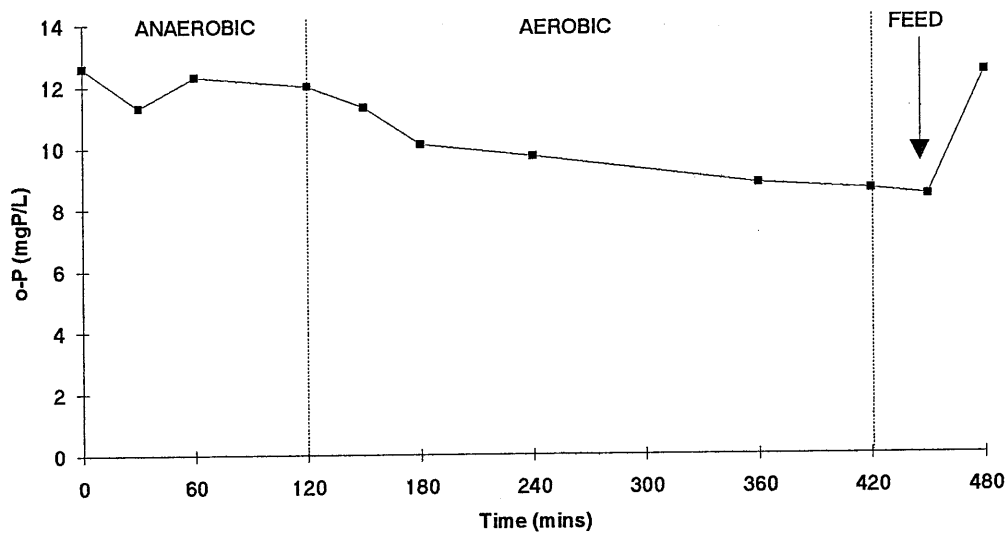


Figure 34. Cyclic fermentation profile for SABR1 on day 2. Where, time = 0 is 7 x 8 h cycles into the run

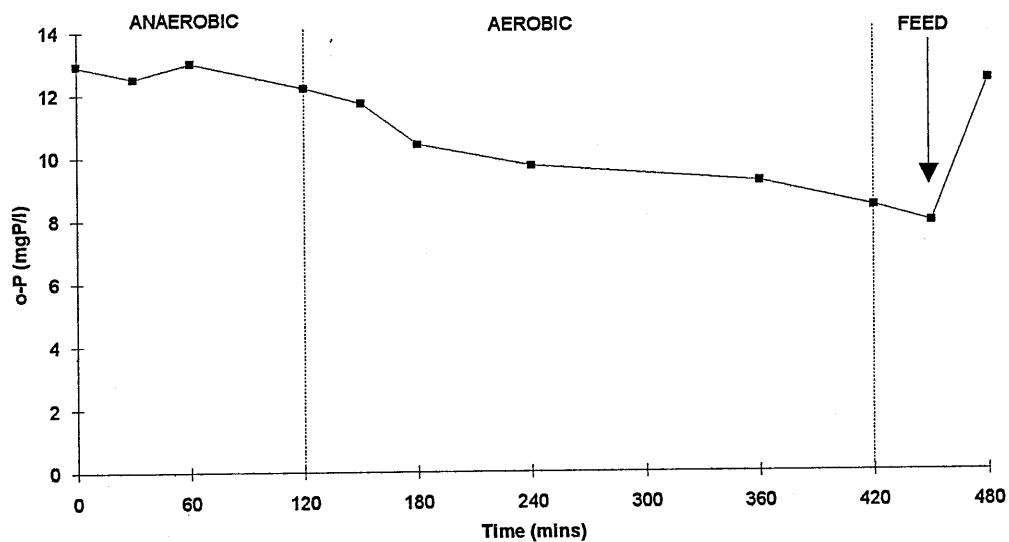


Figure 35. Cyclic fermentation profile for SABR1 on day 11. Where, time = 0 is 34 x 8 h cycles into the run

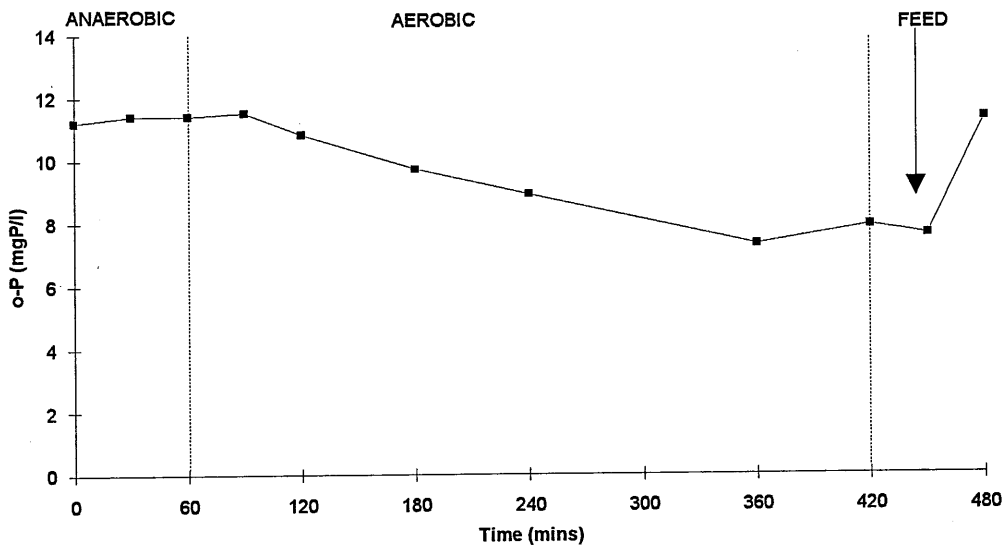


Figure 36. Cyclic fermentation profile for SABR1 on day 28. Where, time = 0 is 85 x 8 h cycles into the run

Steady phosphate removal was observed during this run, maintaining a level of around 30% or 4 mgP l⁻¹ throughout the fermentation (Table 18.). Anaerobic phosphate release characteristic of EBPR was not observed however.

Table 18. Phosphate transfer during fermentation SABR1

Isolate	^a Initial P Transfer Rate-Anaerobic (mgP gTSS ⁻¹ , h)	^a Initial P Transfer Rate - Aerobic (mgP gTSS ⁻¹ , h)	ΔP (mgP l ⁻¹)	%P Removed
Day 2	-1.50	-9.50	4.0	31.8
Day 7	1.00	-9.00	3.7	31.6
Day 11	0.50	-9.00	4.5	34.9
Day 18	1.00	-5.00	3.8	32.5
Day 25	-2.00	-4.00	4.2	32.0
Day 28	1.00	-3.00	3.3	29.5

^a Positive values indicate phosphate release, negative values indicate phosphate uptake.

On day 11, the highest phosphate removal at 34.9% and 4.5 mgP l⁻¹ was achieved, falling back to 29.5% and 3.3 mgP l⁻¹ on day 28 after which the fermentation run was terminated.

4.3.3.4. *Acinetobacter* sp. SAC30

The EBPR capability of this isolate tentatively identified as *Acinetobacter lwoffii* was investigated. The isolate originated from activated sludge sampled from the Northern Treatment Plant, Johannesburg, South Africa.

The fermentation run (SAC30R1) was undertaken for 29 days. Figure 37. shows the phosphate curve on day 20 of the fermentation.

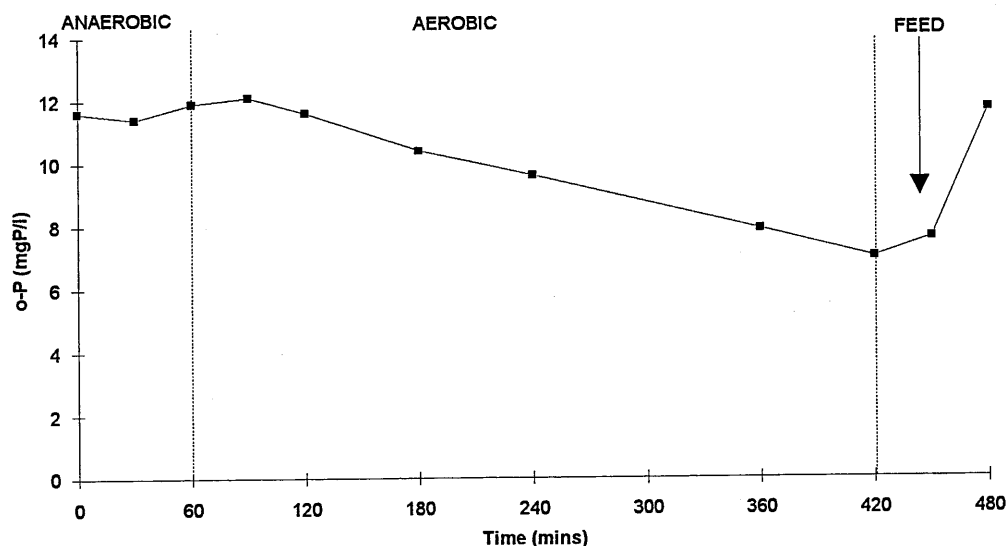


Figure 37. Cyclic fermentation profile for SAC30R1 on day 20.
Where, time = 0 is 61 x 8 h cycles into the run

Overall, only moderate results were achieved during this run (Table 19.).
Significant phosphate release was not observed during the anaerobic phase.

Table 19. Phosphate transfer during fermentation SAC30R1

Isolate	^a Initial P Transfer Rate-Anaerobic (mgP gTSS ⁻¹ , h)	^a Initial P Transfer Rate - Aerobic (mgP gTSS ⁻¹ , h)	ΔP (mgP l ⁻¹)	%P Removed
Day 3	1.33	-8.00	4.0	34.2
Day 8	1.33	-10.67	3.0	24.2
Day 20	2.00	-2.00	4.6	39.7
Day 29	2.00	-7.33	3.9	31.7

^a Positive values indicate phosphate release, negative values indicate phosphate uptake.

A maximum of 40% phosphate removal and 4.6 mgP l^{-1} was achieved on day 20 of the run but the initial aerobic phosphate removal rate was considerably less than on other days. These removal levels could not be maintained therefore the run was terminated after day 29 when phosphate levels had dropped off.

4.3.3.5. *Acinetobacter* sp. MKA11

The EBPR capability of this isolate tentatively identified as *Acinetobacter johnsonii* was investigated. The MKA11 isolate originated from conventional activated sludge not performing EBPR.

The fermentation run (MKA11R1) was undertaken for 33 days. Figures 38. - 40. show the phosphate curves on days 4, 20 and 33 of the fermentation.

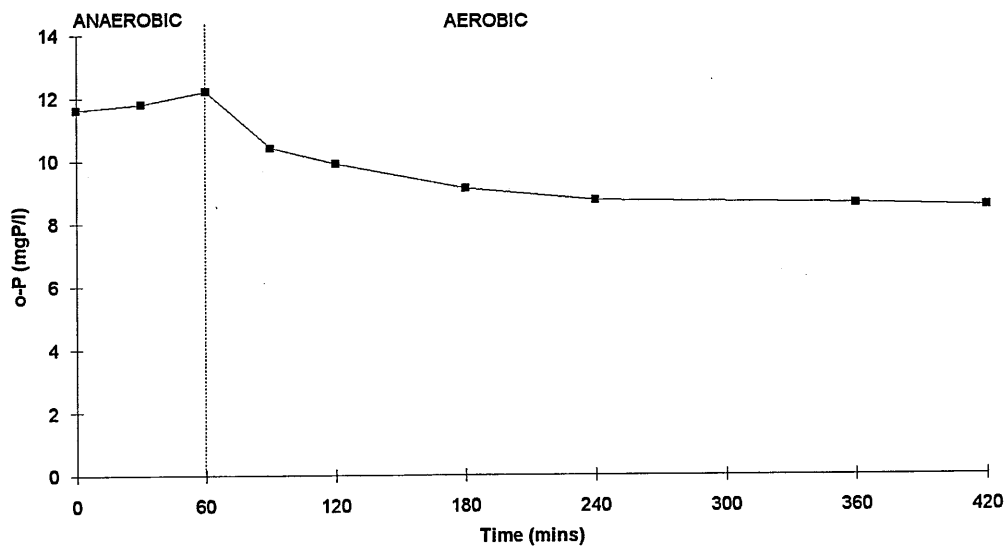


Figure 38. Cyclic fermentation profile for MKA11R1 on day 4. Where, time = 0 is 13 x 8 h cycles into the run

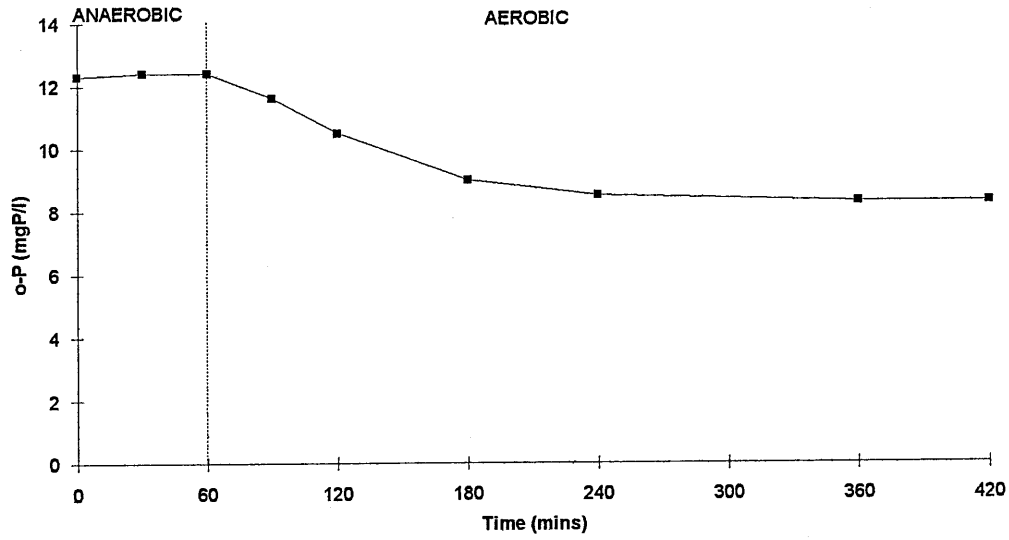


Figure 39. Cyclic fermentation profile for MKA11R1 on day 20.
Where, time = 0 is 61 x 8 h cycles into the run

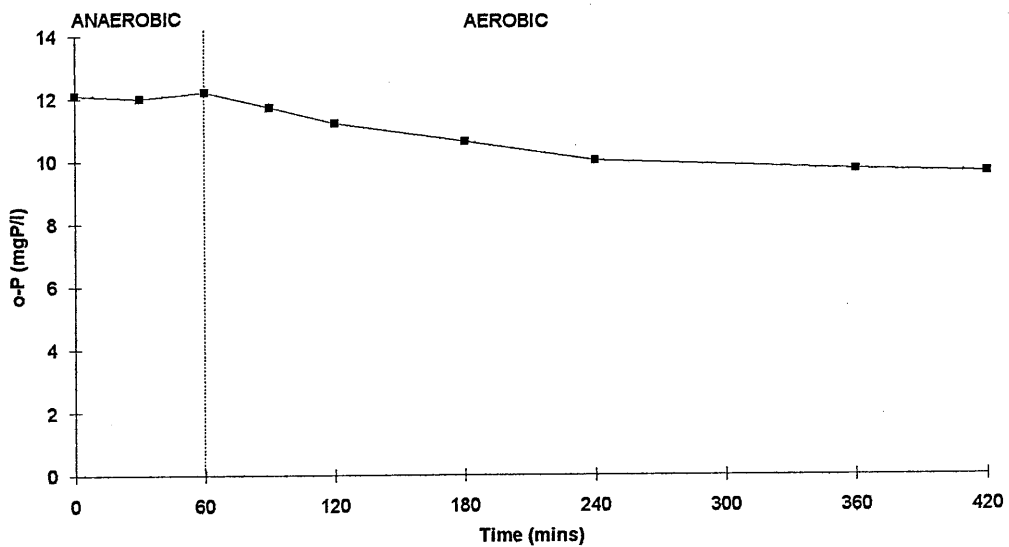


Figure 40. Cyclic fermentation profile for MKA11R1 on day 33.
Where, time = 0 is 100 x 8 h cycles into the run

Fairly respectable initial phosphate uptake rates were obtained for the aerobic phase of the process, which tended to decrease as the fermentation progressed (Table 20.).

Table 20. Phosphate transfer during fermentation MKA11R1

Isolate	^a Initial P Transfer Rate-Anaerobic (mgP gTSS ⁻¹ , h)	^a Initial P Transfer Rate - Aerobic (mgP gTSS ⁻¹ , h)	ΔP (mgP l ⁻¹)	%P Removed
Day 4	3.00	-11.50	3.1	26.7
Day 13	0.50	-10.50	3.1	26.1
Day 20	0.50	-7.00	4.0	32.5
Day 23	-0.50	-7.00	3.9	32.5
Day 26	0.50	-7.50	3.4	27.6
Day 33	0.50	-5.00	2.5	20.7

^a Positive values indicate phosphate release, negative values indicate phosphate uptake.

A maximum of 32.5% and 4 mgP l⁻¹ phosphate were removed on days 20 and 23 of this run. However, these levels decreased to 21% and 2.5 mgP l⁻¹ by day 33 after which the run was terminated.

4.3.3.6. *Acinetobacter* sp. MKB9

The EBPR capability of this isolate tentatively identified as *Acinetobacter lwoffii* was investigated. Isolate MKB9 originated from an EBPR pilot plant.

The fermentation run (MKB9R1) was undertaken for 27 days. Figure 41. shows the phosphate curve on day 9 of the fermentation.

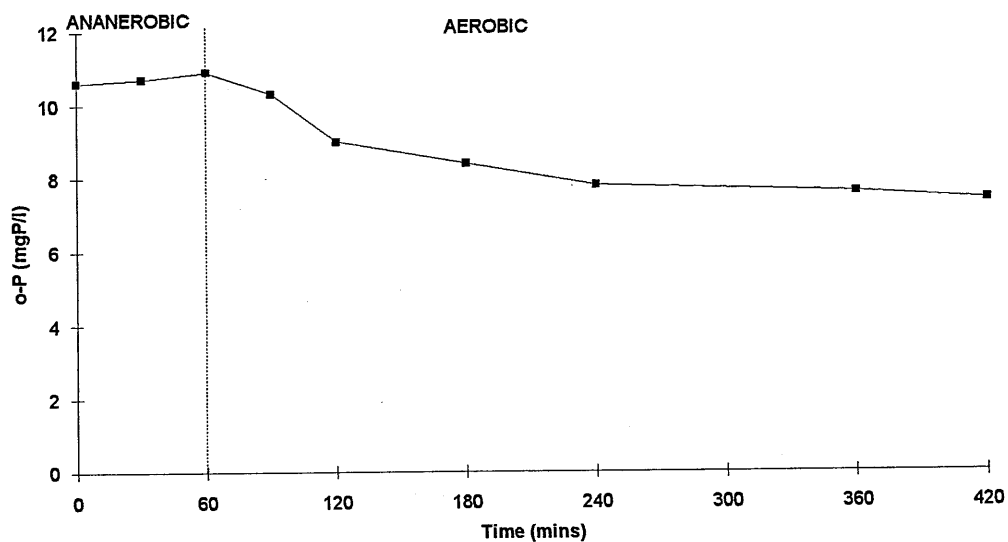


Figure 41. Cyclic fermentation profile for MKB9R1 on day 9.
Where, time = 0 is 28 x 8 h cycles into the run

Again, only moderate results were achieved during this fermentation run (Table 21.).

Table 21. Phosphate transfer during fermentation MKB9R1

Isolate	^a Initial P Transfer Rate-Anaerobic (mgP gTSS ⁻¹ , h)	^a Initial P Transfer Rate - Aerobic (mgP gTSS ⁻¹ , h)	ΔP (mgP l ⁻¹)	%P Removed
Day 3	1.33	-11.33	2.9	27.1
Day 9	2.00	-12.67	3.5	32.1
Day 20	-0.67	-10.67	3.5	30.4
Day 27	-0.67	-6.00	2.1	18.8

^a Positive values indicate phosphate release, negative values indicate phosphate uptake.

A maximum of only 32% and 3.5 mgP l⁻¹ phosphate was removed on day 9 of the run. The initial aerobic phosphate uptake rates were good at the start of the run but decreased as the fermentation progressed. The run was aborted after day 27 when only 2.1 mgP l⁻¹ or 18% phosphate was removed.

4.4. DISCUSSION

A number of researchers have investigated the phosphate removal ability of *Acinetobacter* spp. in pure cultures. However, these studies have concentrated on batch studies as opposed to anaerobic / aerobic cycling. Jenkins and Tandoi (1991) reported that no pure culture studies had been undertaken as a relatively long term alternating anaerobic / aerobic study mimicking EBPR process conditions. This study attempted to achieve that goal.

Introducing an anaerobic phase before the conventional aerobic phase did not have any beneficial effect on phosphate removal. Phosphate removal levels were comparable to those achieved in the aerobic investigation. None of the

six isolates were shown to be performing EBPR, and it was concluded that the modest phosphate uptake was the amount required for normal metabolic functions. However, good aerobic phosphate removal was demonstrated by several of the isolates, when percentage removal reached 40% on several occasions. However, the percentage levels improved when repeating anaerobic / aerobic cycles were initiated. Improved levels were achieved in as little as 6-8 cycles. Ubukata and Takii (1994) found that at least two anaerobic / aerobic cycles were required to induce the phosphate accumulating enzyme system in their EBPR cultures. Ye *et al.* (1988) undertook anaerobic / aerobic batch experiments with an *Acinetobacter calcoaceticus* pure culture, but found no EBPR, which is in agreement with the single cycle investigations conducted in this study. Lawson and Tonhazy (1980) investigated phosphate accumulation by four *Acinetobacter* strains isolated from the Johannesburg Northern Works pilot plant. They aerated their cultures for six hours and noted improved phosphate uptake under non-growth conditions (growth media had no nitrogen source) rather than during growth. In aerobic batch cultures, Deinema *et al.* (1985) found that only 10-20% of accumulated polyphosphate was released by *Acinetobacter* spp. containing 7-8% total phosphate. Ohtake *et al.* (1985) studied phosphate uptake and release in four *Acinetobacter calcoaceticus* strains in pure culture. They reported phosphate uptake and polyphosphate accumulation under aerobic conditions. Hao and Chang (1987) studied phosphate uptake by an *Acinetobacter* sp. isolated from an EBPR plant. A batch culture removed around 17 mgP l⁻¹ from an initial concentration of 34 mgP l⁻¹ within 6 hours. They also mentioned that prolonged nitrogen sparging (21-24 h) was required to induce even a slight phosphate release and they noted that this length of time would not be practical, since *Acinetobacter* spp. viability would be adversely affected. Lötter *et al.* (1986) isolated *Acinetobacter* strains from a Bardenpho EBPR plant and from a conventional activated sludge plant and compared their ability to accumulate polyphosphate. They concluded that isolates from both systems could accumulate polyphosphate, indicating that similar strains were present in both plant types

and that the conditions in the EBPR plant were not selective for polyphosphate accumulating *Acinetobacter* spp.

Although none of the *Acinetobacter* sp. isolates investigated in this study demonstrated EBPR, their potential to accumulate phosphate cannot be discounted. *Acinetobacter* spp. have been isolated from many full-scale and pilot-scale EBPR treatment plants. Beacham *et al.* (1990) investigated the diversity of *Acinetobacter* spp. isolated from a Modified UCT phosphorus removal plant. Their results were compared with similar work (Duncan *et al.*, 1988) which investigated isolates from a Bardenpho type plant. A greater genospecies diversity was found amongst isolates from the Modified UCT plant compared to the Bardenpho type plant. In both investigations genospecies 5 (*A.junii*), 7 (*A.johnsonii*) and 8/9 (*A.lwoffii*) were the most common species isolated. Further work by the same group (Beacham *et al.*, 1992) attempted to determine which genospecies were responsible for EBPR by investigating the polyphosphate accumulating abilities of 156 *Acinetobacter* isolates from media with high and low phosphate concentrations. They concluded that no single *Acinetobacter* genospecies was exclusively involved in EBPR and genospecies 8/9 dominated followed by genospecies 5 and then genospecies 7. However, some *Acinetobacter* isolates, including members of the above genospecies could not accumulate polyphosphate and only *A.calcoaceticus* (genospecies 1) and *A.haemolyticus* (genospecies 4) were not found. Streichan *et al.* (1990) studied and compared the polyphosphate-accumulating bacteria from pilot plants using different process configurations for EBPR. A great diversity was observed using an electron microscope and many contained polyphosphate granules. They found differences in the polyphosphate accumulating microbial population between various pilot plants indicating that the population depends on the process type and on sewage composition. Although various species were found, in every case "*Acinetobacter*-like" cells were identified in the bacterial population.

Duncan *et al.* (1990) compared the plasmid profiles of a good and a poor polyphosphate accumulating strain of *Acinetobacter* and found that the poor accumulators had lost two large plasmids. Since some strains seem to spontaneously lose the ability to accumulate polyphosphate this suggests that the polyphosphate accumulation property may be plasmid encoded. The polyphosphate accumulating properties of several isolates of *Acinetobacter* were described by Bayly *et al.* (1991) and Vasiliadis *et al.* (1990) Upon subsequent isolation from two of the strains, variants were found which no longer accumulate polyphosphate under the same growth conditions as the parent strains. Comparing a polyphosphate accumulating strain with its variant showed that 20kb of plasmid DNA had been deleted from the parent strain.

Bark *et al.* (1992) studied 10 *Acinetobacter* spp. isolates producing polyphosphate:AMP phosphotransferase for differences in polyphosphate accumulation and phosphate adsorption. A strain-specific difference was found in the size and number of polyphosphate granules which was independent of the age of the cultures. When the ability of certain *Acinetobacter* spp. to remove phosphate from mixed liquor was investigated, again the ability was found to be strain rather than species specific (Bosch and Cloete, 1993).

Bark *et al.* (1992) observed extensive phosphate adsorption at the outer cell wall surface of one *Acinetobacter* sp. isolate which indicated that phosphate precipitation may also be involved. This phenomenon of combined precipitation was also reported a few years beforehand (Arvin, 1985) and may explain why observed phosphate release cannot be accounted for by the lack in numbers of *Acinetobacter* spp. as in Cloete and Steyn (1988a). In fact, the early study suggested that the observed phosphate decrease in the wastewater was due to chemical precipitation possibly mediated by microbial metabolism. Streichan and Schön (1991) also considered the possibility of chemical precipitation of phosphate. Their study concluded that phosphorus is stored intracellularly in *Acinetobacter* as polyphosphate but also that insoluble phosphate is

precipitated by the metabolic activity of *Acinetobacter* spp. the extent of which was dependent on the amount of Ca^{2+} and Mg^{2+} in the wastewater and on the species of polyphosphate accumulating bacteria present. Phosphate removal from wastewater by combined biological and chemical precipitation has been investigated for several different sludges as well as pure cultures of polyphosphate accumulating *Acinetobacter* (Appeldoorn *et al.*, 1992). Under certain conditions the mixed culture sludges removed phosphate in a combined biological and chemical manner. However, the pure polyphosphate accumulating *Acinetobacter* isolate did not form precipitates.

An interesting hypothesis was proposed by Ye *et al.* (1988). They examined several pure type strains including *Acinetobacter calcoaceticus* as well as strains isolated from activated sludge for their ability to remove inorganic phosphate from synthetic wastewater. They concluded that EBPR from wastewater may actually be attributed to a symbiotic effect between anaerobic bacteria and aerobic bacteria. This agrees with the earlier work of Brodisch (1985) which found that in the presence of *Aeromonas punctata*, phosphate removal by *Acinetobacter calcoaceticus* increased. *A. punctata* converted carbohydrates present in the sewage to acetate, which is the favoured carbon source of *A. calcoaceticus*.

CHAPTER 5.

5. *AEROMONAS* AND *ACINETOBACTER* SP. CO-CULTURE INVESTIGATIONS

5.1. INTRODUCTION

Although members of the *Acinetobacter* genus are generally accepted to be responsible for EBPR (Fuhs and Chen, 1975; Ohtake *et al.*, 1985; Beacham *et al.*, 1989) the involvement of several other genera has also been reported. For instance, Kavanaugh and Randall (1994) stated that bacteria other than *Acinetobacter* spp. were involved in EBPR from their UCT type pilot plant. *Pseudomonas* sp., coliforms and the *Aeromonas* / *Vibrio* groups were found in much greater numbers than *Acinetobacter* spp.. Cloete and Steyn (1988a) found that *Acinetobacter* was not the dominant species in samples collected from the primary aerobic zone of a full scale EBPR plant. They found that only 34% of the phosphate removed from the activated sludge could be attributed to removal by *Acinetobacter* spp. as polyphosphate. In a pilot plant study, phosphate removal to less than 0.2 mgP l⁻¹ was observed where only minor proportions of *Acinetobacter* spp. were found (Brodisch and Joyner, 1983). Several studies have reported that the presence of other specific bacterial species may improve the EBPR capability of *Acinetobacter* spp.. Brodisch and Joyner (1983) found that *Aeromonas* was a major constituent of the microbial population of an EBPR pilot plant and two laboratory scale units. They suggested that *Aeromonas* spp. and *Pseudomonas* spp. were the main genera connected with EBPR and low numbers of *Acinetobacter* spp. were found in comparison. Other studies have also indicated that *Aeromonas* spp. may be connected with the process (Lötter and Murphy, 1985; T'Seyen *et al.*, 1985a).

The microbial population structure of a plant is very dependant on the process aeration regime. An anaerobic zone leads to the proliferation of fermentative

organisms such as *Aeromonas* spp. which can use organic compounds as electron donors and acceptors. *Aeromonas* spp., being facultative anaerobes can also respire aerobically and can tolerate the alternating anaerobic / aerobic conditions in an EBPR plant. During anaerobic conditions *Aeromonas* spp. can produce acetic acid, but cannot utilise it, resulting in its release to the surrounding medium. However, according to the Comeau / Wentzel model (1.3.3.8.1.) *Acinetobacter* spp. take up acetate into the cell during the anaerobic phase and store it as PHB. Under subsequent aerobic conditions, *Acinetobacter* spp. which are obligate aerobes, which do not grow under anaerobic incubation, can utilise the stored acetic acid as a carbon and energy source. T'Seyen *et al.* (1985a) identified bacteria which produced volatile fatty acids in a separate fermentation reactor, instead of the usual anaerobic zone found in EBPR plants, as *Aeromonas*, *Klebsiella* and *Escherichia*. Lötter and Murphy (1985) found that different species can dominate the bacterial population of an EBPR plant under the distinct and separate anaerobic, anoxic and aerobic conditions. They investigated the bacterial population present in the anaerobic, primary anoxic and aerobic zones of a full scale five stage Bardenpho activated sludge plant. *Acinetobacter* spp. dominated the aerobic zone. *Aeromonas* spp. were also present in significant numbers when good phosphate removal (48%) was recorded, but were absent at lower phosphate removal levels (18% and 31%). *Aeromonas* spp. dominated the anaerobic zone of the same plant when studied under anaerobic incubation. However, *Acinetobacter* spp. dominated the same anaerobic zone sample when studied under aerobic incubation. Larger numbers of *Pseudomonas* spp. were found in the anoxic zone, together with considerable numbers of *Acinetobacter* spp. and *Aeromonas* spp..

Brodisch (1985), using pure cultures, showed that *Aeromonas punctata* improved the EBPR capability of *Acinetobacter calcoaceticus* and suggested that *A. punctata* and *A. calcoaceticus* have a synergistic relationship in EBPR. Acetate is produced by *Aeromonas punctata* under anaerobic conditions.

Under subsequent aerobic conditions *Acinetobacter* sp. use the acetate as carbon source for the EBPR process. However, Yeoman *et al.* (1988) found no improvement in their EBPR system when augmented with *Acinetobacter calcoaceticus*, *Acinetobacter lwoffii* and *Aeromonas punctata*.

The presence of the correct *Aeromonas* species in the anaerobic zone of an EBPR plant is important to the type of fermentation produced. *Aeromonas punctata* breaks down glucose fermentatively via the Embden-Meyerhof pathway in a mixed acid fermentation yielding acetic, lactic, succinic and formic acids as well as ethanol as end products. However, *Aeromonas hydrophila* performs a butanediol fermentation resulting in 2,3-butanediol as a major end product alongside ethanol. In a butanediol fermentation the total amount of acidic compounds formed per mole of glucose is much less than in a mixed acid fermentation (Stanier *et al.*, 1976). Therefore from an EBPR point of view the presence of *Aeromonas punctata* is desirable over *Aeromonas hydrophila*. These two species cannot be distinguished using the API 20 E test kit, an additional test, the butanediol dehydrogenase test is commonly used for their differentiation (Schubert, 1974).

To complicate matters, according to the NCIMB Ltd. (personal communication), *Aeromonas punctata* has twice been renamed since the work of Brodisch (1985). Firstly, to *Aeromonas hydrophila* subsp. *anaerogenes* and secondly to *Aeromonas caviae*, the name by which the species is currently known. Kämpfer and Altwegg (1992) note that *A. caviae* and *A. punctata* share the same type strain (ATCC 15468).

The *Aeromonas* genus is defined as consisting of straight cells, rod shaped with rounded ends to coccoid. They are Gram negative, oxidase positive and catalase positive. They are generally motile by a single flagellum. Glucose metabolism is respiratory and fermentative. Carbohydrates are broken down to acid or acid and gas (CO₂ and H₂) and nitrate is reduced to nitrite. They

have an optimum temperature of 22-28°C and they are found in freshwater and sewage (Popoff, 1984; Joseph *et al.*, 1987).

5.2. EXPERIMENTAL

5.2.1. Cultures

An *Aeromonas caviae* (NCIMB 9235) isolate was selected for this study on the basis of its ability to breakdown glucose via a mixed acid fermentation yielding acetate as a major end product (Stanier *et al.*, 1976). The isolate was obtained from The National Collections of Industrial and Marine Bacteria Ltd. (NCIMB Ltd., Aberdeen) and was delivered to the laboratory in freeze-dried form. The isolate was revived according to NCIMB Ltd.'s recommendations. Nutrient broth and agar were used as revival media and a stock culture was stored at 4°C on a nutrient agar slope. A preculture of the isolate was prepared by transferring a loopful of cells from the stock slope to 200 ml of Glucose Medium in a 500 ml Erlenmeyer flask. The culture was grown aerobically in an orbital incubator (Gallenkamp) at 150 rpm and 30°C for 48 hours.

Acinetobacter sp. SAB was selected as the co-culture isolate on the basis of its better phosphate removal performance compared to the other *Acinetobacter* spp. isolates tested in pure culture (see 4.3.). A preculture was prepared similarly to the *Aeromonas* culture except that acetate medium was used instead of glucose medium.

For these studies the glucose medium comprised of: 0.375 g D-glucose, 0.0267 g NH₄Cl, 0.005 g yeast extract, 0.0125 g MgSO₄·7H₂O, 0.00767 g KH₂PO₄ and 1 l of deionised water. The acetate medium was made up similarly to the glucose medium except that the D-glucose was replaced with 1.7 g NaCH₃COOH. Glucose and acetate medium agar were prepared by adding 10 g of Agar Bacteriological (Oxoid, L11) to the above.

5.2.2. Bench Scale Fermentation Unit

The 5 l SBR with a working volume of 3 l was operated to investigate the co-culture relationship between *Aeromonas caviae* (NCIMB 9235) and *Acinetobacter* sp. SAB. Operating conditions are detailed in Table 22.

Table 22. *Aeromonas caviae* (NCIMB 9235) pure culture and *Aeromonas caviae* (NCIMB 9235) / *Acinetobacter* sp. co-culture fermentation operating conditions

Isolate	<i>Aeromonas caviae</i> (NCIMB 9235)	<i>Aeromonas caviae</i> (NCIMB 9235) / <i>Acinetobacter</i> sp.
Working Volume (l)	3	3
MCRT (d)	10	10
Fermentation Time (d)	days 1-9	days 10-23
Cycle Time (h)	12	12
Anaerobic Time (h)	4	4
Aerobic Time (h)	7	7
pH	not controlled days 1&2 controlled days 3-9 pH7	controlled at pH7
Temperature (°C)	25	25
Redox (max)(Anaerobic (mV) Aerobic (mV)	- 195 + 90	- 190 + 90
Influent: COD (mg l ⁻¹) o-P (mg l ⁻¹) TSS (mg l ⁻¹)	400 10 500	400 10 650

The synthetic sewage feed (glucose medium) contained D-glucose as the major carbon source. Stock solutions of the synthetic media were prepared for feeding into the fermenter at tenfold concentration.

5.2.3. Analytical Methods

Glucose was determined by an enzymic method using a D-Glucose Test Kit (Boehringer Mannheim, Cat. No. 716251). Acetic acid (acetate) was also determined via an enzymic method using an Acetic Acid Test Kit (Boehringer Mannheim, Cat. No. 148261). Bacterial growth was monitored using a Philips PU 8620 UV/VIS/NIR spectrophotometer at 600 nm. Orthophosphate was determined using the standard method (Department of the Environment 1980a). The COD, NO_3^- and NO_2^- were periodically determined.

5.2.4. Growth on Glucose and Acetate Medium

Acetate medium and glucose medium agar plates were streaked in triplicate with the *Aeromonas caviae* (NCIMB 9235) and *Acinetobacter* sp. SAB isolates which had previously been maintained on nutrient agar slopes. Each isolate was incubated for 2 days at 25°C.

5.2.5. Phosphate Uptake Shake Flask Batch Experiments

5.2.5.1. Aerobic Investigations

This initial experiment was undertaken to investigate the amount of phosphate metabolised by pure cultures of *Aeromonas caviae* (NCIMB 9235) and *Acinetobacter* sp. SAB during normal aerobic growth. Acetate medium agar plates were streaked with *Acinetobacter* sp. SAB and glucose medium agar plates were streaked with *Aeromonas caviae* isolates which had previously been maintained on nutrient agar slopes. Each plate was incubated for 2 days at 25°C. After 2 days each isolate was subcultured - a loopful of the culture was transferred and grown aerobically in 200 ml of glucose medium (*Aeromonas caviae*) and 200 ml of acetate medium (*Acinetobacter* sp. SAB) in 500 ml Erlenmeyer flasks. The isolates were grown in an orbital incubator

(Gallenkamp) at 130 rpm and 25°C for 24 hours. Phosphate uptake was determined by measuring the amount of phosphate removed from the supernatant over 24 hours. Cell growth was also monitored and each isolate was investigated in triplicate. The initial phosphate concentration was around 35 mg l⁻¹. Control experiments (A and B) were also undertaken for each isolate. Control A contained dead cells, control B contained no cells.

5.2.5.2. Anaerobic / Aerobic Investigations

These investigations involved incorporating a preliminary anaerobic step, as found in EBPR processes, into the experiment. Loopfulls of 2 day old cultures grown on acetate medium agar plates (*Acinetobacter* sp. SAB) and glucose medium agar plates (*Aeromonas caviae*) were subcultured into 1 l Erlenmeyer flasks containing 250 ml of acetate medium and glucose medium respectively and were grown in an orbital incubator (Gallenkamp) at 130 rpm and 25°C for 48 hours. After 2 days 250 ml of fresh acetate medium or glucose medium was added to the flasks. The flasks were then transferred to a shaking water bath (Grant SS40-5, Grant Instruments Ltd., Cambridge) at 25°C and sparged with nitrogen for 2 hours followed by 6 hours aeration.

Ortho-phosphate and cell concentration were measured every 15 minutes during the anaerobic phase and every hour during the aerobic phase. The batch tests for each isolate were carried out in triplicate unless otherwise stated. The initial ortho-phosphate concentration was around 15 mg l⁻¹. Control experiments (A and B) were also undertaken for each isolate. Control A contained dead cells, control B contained no cells.

5.2.5.3. Cyclic Anaerobic / Aerobic Fermentation Investigations

An *Aeromonas caviae* preculture was inoculated into the fermenter which contained 2.8 l of glucose medium. The *A. caviae* fermentation was operated

in a cyclic anaerobic / aerobic sequence from the outset details of which are outlined in Table 22. Since little was known about this fermentation the *A. caviae* was grown as a pure culture initially. After 9 days the *Acinetobacter* sp. preculture was added to the fermenter. The *Aeromonas* - *Acinetobacter* co-culture fermentation was undertaken for 14 days.

5.2.5.4. Concentrated Cell Studies

Since low cell concentrations were found in the pure culture studies compared to levels found in full scale EBPR plants, concentrated culture studies were undertaken. Phosphate removal comparisons were made of cells sampled directly from the fermenter and cells concentrated by centrifugation prior to the undertaking of shake flask experiments. The comparisons were undertaken in parallel investigations.

Towards the end of the aerobic phase of the cycle immediately prior to the one to be analysed ~ 1 l of culture was harvested from the fermenter and centrifuged. Centrifugation was undertaken in a Beckman J2-21 Centrifuge (using a JA-10 Rotor) at 7000 rpm (8670 g) for 15 minutes at 4°C. The resulting pellet was re-suspended in a distilled water wash and centrifuged again. The washed pellet was re-suspended in 100 ml of glucose medium feed in a 250 ml Erlenmeyer flask at the start of the shake flask study. The centrifugation process was timed so that the concentrated, washed pellet was ready for use a few minutes before the commencement of the anaerobic phase in the fermenter. The shake flask was placed in a shaking water bath at 20°C. The culture was sparged with nitrogen for 4 hours, followed by aeration - mimicking conditions in the fermenter. pH readings were taken at the start and end of the investigations. Samples were taken from the fermenter and shake flask within one minute of each other and were filtered immediately. Additionally, samples for glucose and acetate analysis were immediately heat treated to stop further enzymic activity.

Viability of the centrifuged culture was checked by streaking a loopful of cells onto nutrient agar plates and observing growth after 2 d incubation at 25°C.

5.3. RESULTS

5.3.1. Cell Growth on Glucose and Acetate Medium

Acinetobacter sp. SAB did not grow on two out of three glucose medium agar plates. Poor growth was observed on one plate - 5 distinct colonies were counted. The identification of two of these colonies was checked and confirmed as *Acinetobacter* sp.. The same isolate grew well on all three Acetate Medium agar plates. Three randomly chosen colonies were identified and confirmed as *Acinetobacter* spp.. *Aeromonas caviae* (NCIMB 9235) grew well on both the glucose and acetate medium agar plates. As a result of these observations, shake flask investigations were undertaken with the *Acinetobacter* sp. SAB isolate using acetate medium and with the *Aeromonas caviae* isolate using glucose medium.

5.3.2. Aerobic Investigations

Aerobic batch experiments of *Acinetobacter* sp. SAB and *Aeromonas caviae* (NCIMB 9235) isolates were each undertaken in triplicate. The amount of phosphate removed is expressed as ΔP mgP l⁻¹ and the amount of cell growth is expressed as ΔX mg cells l⁻¹. $\Delta P/\Delta X$ gives an indication of the amount of P metabolised by the isolates. The % phosphate removed is calculated as:

$$\frac{\text{Amount of phosphate removed}}{\text{Initial phosphate concentration}} \times 100\%$$

Results showed that *Acinetobacter* sp. SAB removed phosphate to a greater extent than *Aeromonas caviae* (Table 23.) possibly indicating a greater phosphate requirement for its normal metabolic growth.

Table 23. Phosphate uptake under aerobic conditions for *Acinetobacter* sp. SAB and *Aeromonas caviae* (NCIMB 9235) isolates

Isolate	ΔP (mg l ⁻¹) Mean	ΔP (mg l ⁻¹) SD	ΔX (mg l ⁻¹) Mean	ΔX (mg l ⁻¹) SD	$\Delta P/\Delta X$ Mean	% PO ₄ ⁻ Removed
<i>A.caviae</i>	2.10	0.26	110.33	11.93	0.019	6
<i>Acinet.</i> SAB	4.63	0.32	113.67	5.69	0.042	13

Acinetobacter sp. SAB showed the greatest amount of phosphate metabolised (expressed as $\Delta P/\Delta X$) as well as the highest percentage phosphate removal, although the percentage of phosphate removed from the supernatant was not significant in terms of EBPR for either isolate. Both isolates achieved similar concentrations of cell growth over the 24 hours, therefore indicating that *Acinetobacter* sp. SAB may require a greater phosphate concentration for its normal metabolism. No phosphate uptake was measured in the controls.

5.3.3. Single Cycle Anaerobic / Aerobic Investigations

Neither *Aeromonas caviae* isolate (grown in glucose medium) nor the *Acinetobacter* sp. SAB isolate (grown in acetate medium) demonstrated phosphate release and uptake curves typical of an EBPR process (Figure 42.) i.e. phosphate release was not shown by either isolate during anaerobic conditions. A gradual phosphate uptake trend was observed however for both isolates. A slight increase in the absorbance of the bacterial suspensions of 0.12 (*Aeromonas caviae*) and 0.14 (*Acinetobacter* sp. SAB) absorbance units indicated only slight bacterial growth. The phosphate concentration remained constant in the controls during the experiment. The absorbance of the control suspensions also remained constant, indicating no bacterial growth.

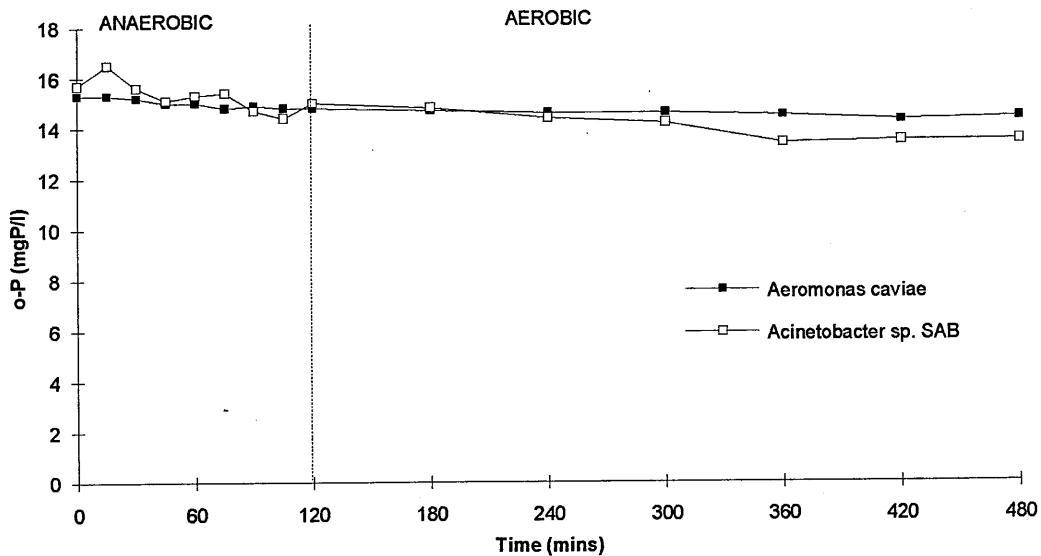


Figure 42. Single cycle anaerobic / aerobic profile for *Aeromonas caviae* (NCIMB 9235) and *Acinetobacter* sp. SAB

Taking into account the phosphate removal and transfer rates of both isolates (Table 24.) it is clear that EBPR did not occur and the percentage of phosphate removed was very similar to levels achieved in the aerobic study. However, the concentration of phosphate removed was around twice as great as in the aerobic investigation for both isolates. This may be due to the higher initial phosphate concentration at 35 mg l^{-1} in the aerobic study compared to 15 mg l^{-1} in this study. This may indicate that the initial phosphate concentration may be important to the phosphate removal concentrations which can be achieved.

Table 24. Phosphate transfer in single cycle anaerobic / aerobic shake flask batch investigations

Isolate	¹ Initial P Transfer Rate - Anaerobic (mgP gTSS ⁻¹ , h)	¹ Initial P Transfer Rate - Aerobic (mgP gTSS ⁻¹ , h)	ΔP (mgP l ⁻¹)	%P Removed
<i>A. caviae</i>	-3.42	-0.96	0.90	6
SAB	-7.45	-2.22	2.20	14.0

¹ Positive values indicate phosphate release, negative values indicate phosphate uptake.

Phosphate transfer rate is defined as the concentration of phosphate uptake or release (in mg) per gram of cells during the initial hour of the particular anaerobic or aerobic phase. ΔP and %P have been explained in 5.3.2.

Acinetobacter sp. SAB showed the higher percentage phosphate removal over the cycle. The *Aeromonas caviae* isolate removed only 6% phosphate, less than half the amount removed overall by the *Acinetobacter* sp. SAB isolates. The phosphate removed from the supernatant by each isolate was in all probability used for normal metabolic requirements and not EBPR.

5.3.4. Cyclic Anaerobic / Aerobic Fermentation Investigations

Since little was known about the *Aeromonas caviae* fermentation, pH was not controlled for the first 2 days. However, when the pH dropped to pH 3.5, presumably as a result of the formation of the desired acid end products, pH control was initiated. From day 3 pH was controlled and set to be maintained at pH 7.

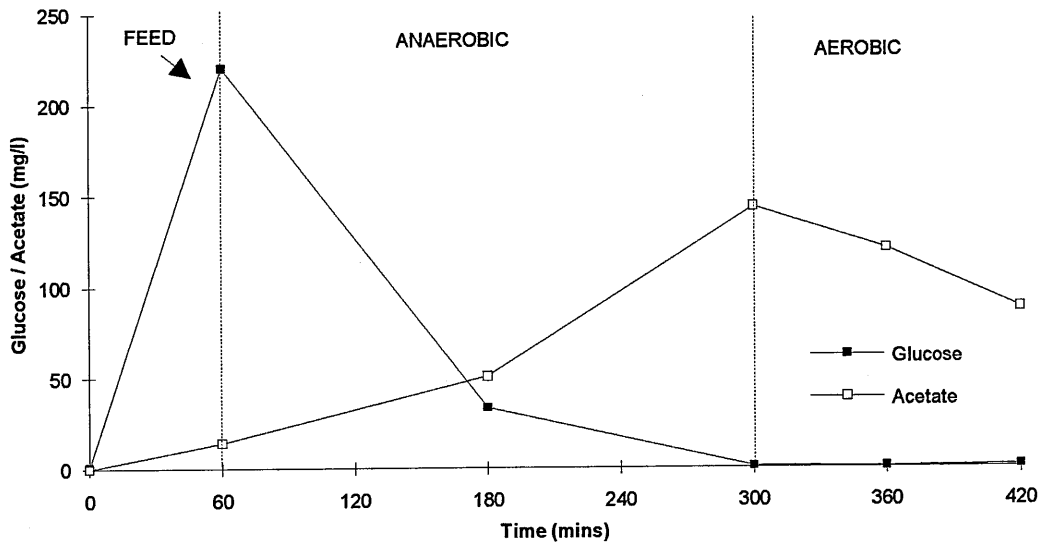


Figure 43. Glucose utilisation and acetate formation by *A. caviae* (NCIMB 9235) after 4 days. Where, time = 0 is 9 x 12 h cycles into the run

Glucose uptake, and acetate formation was clearly established four days after the onset of the *Aeromonas caviae* fermentation (Figure 43.). A longer anaerobic phase time of 4 h was undertaken in this fermentation run to allow sufficient time for the glucose to be converted to acetate. This optimum time span was determined in preliminary investigations on day 2 and 3 of the *Aeromonas* fermentation. No significant amount of acetate was formed at longer anaerobic phase times. Phosphate removal levels were at concentrations associated with normal metabolism, EBPR did not occur.

After 9 d, when the *Aeromonas* isolate was considered acclimatised, and glucose conversion to acetate / acetic acid was well established, a preculture of *Acinetobacter* sp. SAB was introduced into the fermenter. After only 2 d good percentage phosphate removal was observed in the co-culture (Figure

44.). A lower than usual concentration of phosphate in the synthetic glucose medium feed resulted in an influent phosphate concentration of only 5 mgP l⁻¹ instead of around 10 mgP l⁻¹. Consequently only 1.8 mgP l⁻¹ phosphate was removed.

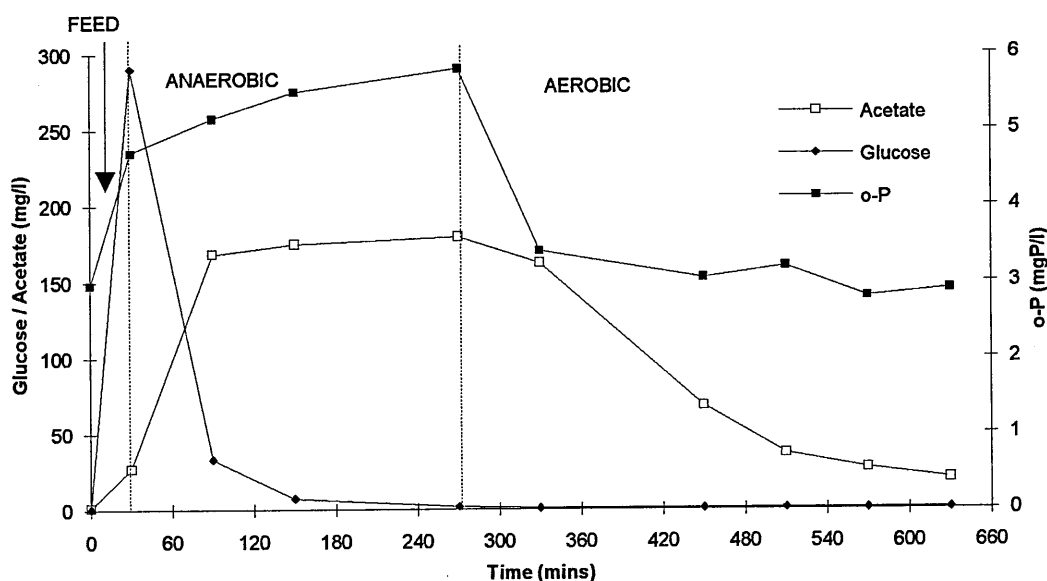


Figure 44. Glucose utilisation, acetate formation and phosphate removal by *Aeromonas / Acinetobacter* co-culture after 2 days. Where, time = 0 is 5 x 12 h into the co-culture run

The co-culture was allowed to develop and phosphate removal improved with fermentation time (Figures 45. - 48. and Table 25.).

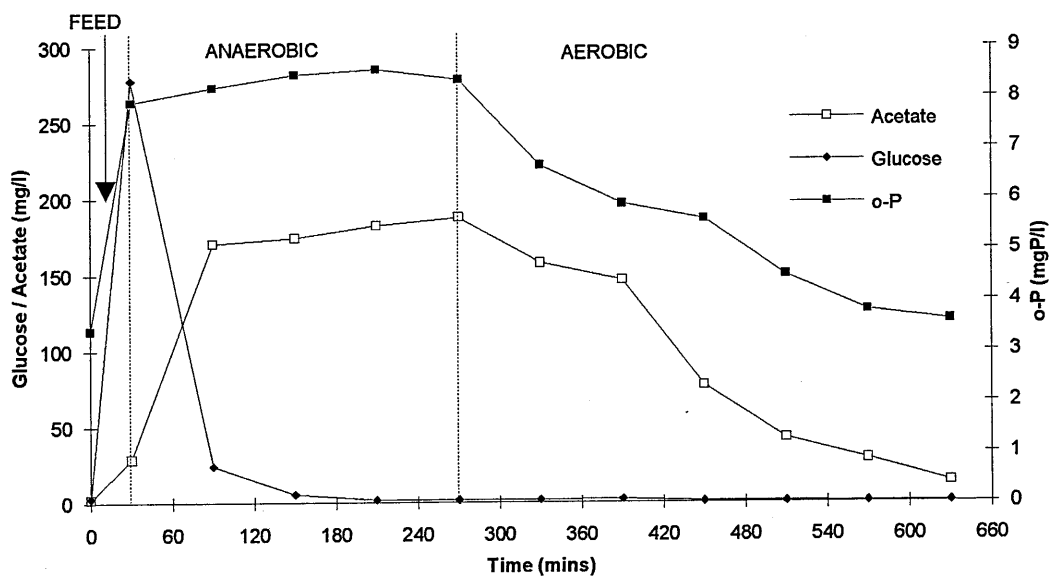


Figure 45. Glucose utilisation, acetate formation and phosphate removal by *Aeromonas* / *Acinetobacter* co-culture after 7 days. Where, time = 0 is 15 x 12 h cycles into the co-culture run

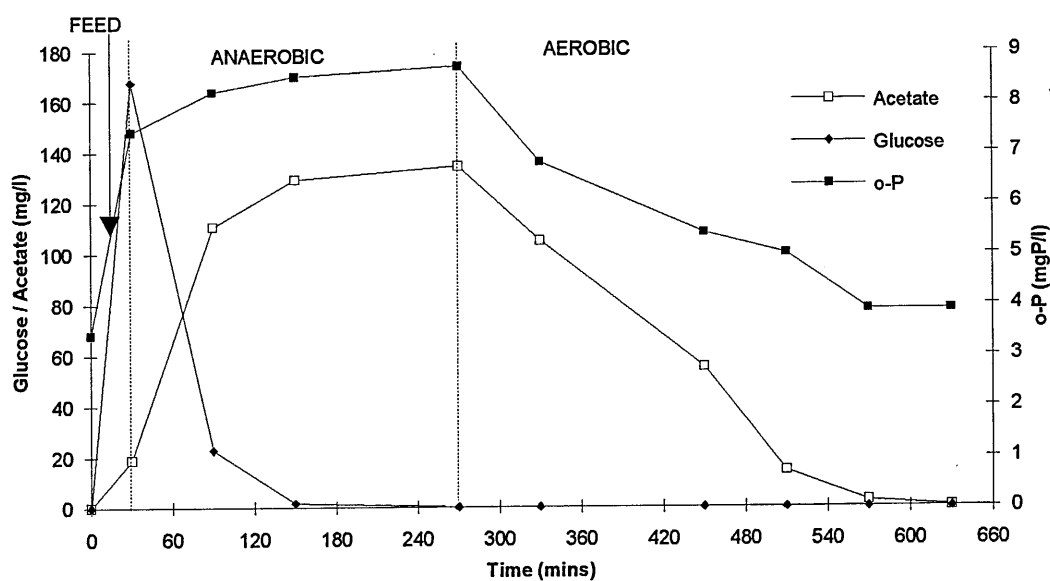


Figure 46. Glucose utilisation, acetate formation and phosphate removal by *Aeromonas* / *Acinetobacter* co-culture after 11 days. Where, time = 0 is 23 x 12 h cycles into the co-culture run

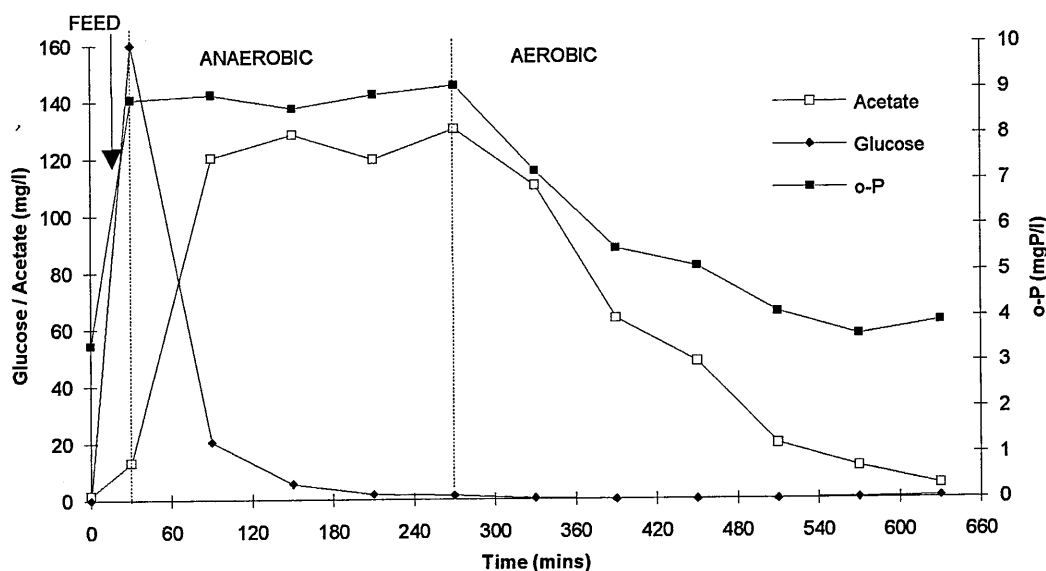


Figure 47. Glucose utilisation, acetate formation and phosphate removal by *Aeromonas / Acinetobacter* co-culture after 13 days. Where, time = 0 is 27 x 12 h cycles into the co-culture run

Greater percentage phosphate removal was attained by the *Aeromonas - Acinetobacter* co-culture than by a pure culture of *Acinetobacter* sp. SAB. A maximum of 35 % or 4.5 mgP l⁻¹ (see 4.3.3.3.) was removed by a pure culture of *Acinetobacter* sp. SAB whereas the co-culture achieved a maximum 56% phosphate removal on day 13 of the fermentation. A maximum concentration of 5.3 mgP l⁻¹ was removed on day 9 of the fermentation. Looking at the phosphate removal and transfer rates (Table 25.) it is possible that some excess phosphate, to that needed for normal metabolism, is being taken up by the co-culture. However, the EBPR characteristic of phosphate release during the anaerobic phase was not observed.

Table 25. Phosphate removal by co-culture fermentation

Day	¹ Initial P Transfer Rate - Anaerobic (mgP gTSS ⁻¹ , h)	¹ Initial P Transfer Rate - Aerobic (mgP gTSS ⁻¹ , h)	ΔP (mgP l ⁻¹)	%P Removed
2	0.75	-3.98	1.8	38.3
7	0.50	-2.82	4.3	54.4
9	-0.68	-2.94	5.3	54.6
11	1.04	-2.48	3.5	47.3
13	0.15	-2.48	4.9	55.7

¹ Positive values indicate phosphate release, negative values indicate phosphate uptake.

After a total of 23 days, 14 as a co-culture, the fermentation run was terminated.

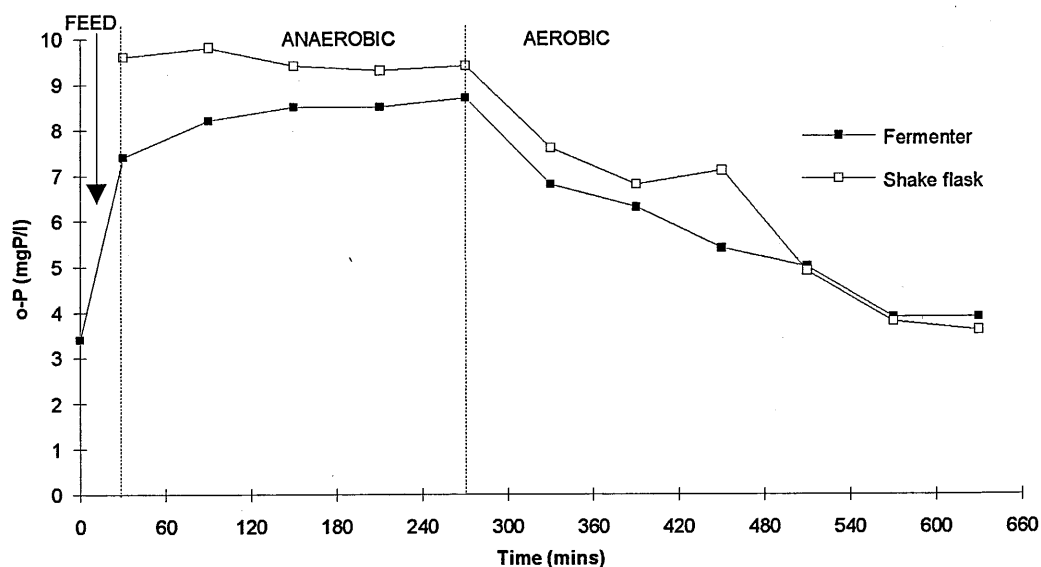


Figure 49. Comparison of phosphate removal by *Aeromonas* / *Acinetobacter* co-culture in shake flask and fermenter on day 11. Where, time = 0 is 23 x 12 h into the run

The phosphate transfer rates were therefore slightly different, due to the difference in cell concentrations, the shake flask culture had an aerobic initial phosphate transfer rate of less than half that of the fermenter culture. On day 11 of the fermentation, the shake flask culture removed more phosphate than the fermenter culture - although at a lower rate.

Overall, concentrating the co-culture prior to analysis did not significantly improve the amount of phosphate removed.

Table 26. Phosphate removal by co-culture fermentation in concentrated cell studies

Day	¹ Initial P Transfer Rate - Anaerobic (mgP gTSS ⁻¹ , h)	¹ Initial P Transfer Rate - Aerobic (mgP gTSS ⁻¹ , h)	ΔP (mgP l ⁻¹)	%P Removed
Fermenter Day 7	0.50	-2.82	4.3	54
Shake flask Day 7	-0.29	-1.09	5.4	58
Fermenter Day 11	1.04	-2.48	3.5	47
Shake flask Day 11	0.15	-1.36	6.0	62

¹ Positive values indicate phosphate release, negative values indicate phosphate uptake.

Although slightly increased phosphate removal was achieved by concentrating the co-culture cells prior to analysis, the levels were not as great as anticipated.

5.4. DISCUSSION

Overall, the presence of *Aeromonas caviae* (NCIMB 9235) appeared to improve the EBPR capability of *Acinetobacter* sp. SAB. This is in accordance with the observations of Brodisch (1985) but disagrees with the findings of Yeoman *et al.* (1988) who found no improvement in the performance of their system after bioaugmentation with an *Aeromonas* sp. isolate. Ye *et al.* (1988) have also suggested that there may be a symbiotic effect between anaerobic and aerobic bacteria in the EBPR process. Takeuchi (1991) suggested the existence of a commensal relationship between *Aeromonas* and *Acinetobacter*,

with *Aeromonas* producing acetate and *Acinetobacter* consuming it for survival and growth under anaerobic conditions.

Before addition to the *Aeromonas* culture, the *Acinetobacter* sp. had not been previously acclimatised to anaerobic / aerobic cycling in this study - the culture had been grown aerobically in an orbital incubator for two days. A period of co-culture acclimatisation was expected - during which the *Aeromonas* and *Acinetobacter* would hypothetically establish a synergistic relationship similar to that observed by Brodisch (1985). This acclimatisation period was unexpectedly short, phosphate removal was observed after only 2 days of the establishment of the co-culture.

When preparing isolates the almost complete failure of *Acinetobacter* sp. SAB to grow on glucose medium was not surprising since only a few *Acinetobacter* sp. can use glucose as a sole carbon source for growth (Juni, 1972). Presumably, *Acinetobacter* sp. SAB is not one of these species. Some carbon may be available as growth substrate in the small concentration of yeast extract in the glucose medium. However this is probably not sufficient to support much growth which may explain why a few *Acinetobacter* sp. SAB colonies grew on one of the plates.

For both isolates the single cycle anaerobic / aerobic investigations showed almost identical phosphate removal abilities as the aerobic investigations. Since improved phosphate removal levels were achieved in the cyclic anaerobic / aerobic fermentation studies, one explanation might be that the enzyme systems responsible for EBPR need more than one anaerobic phase before they are triggered. Ubukata and Takii (1994), in their mono-culture studies, found that at least two anaerobic / aerobic cycles were required to induce the excess phosphate accumulation enzyme system. In this co-culture study, not many anaerobic / aerobic cycles seem to be required for enzyme system induction, since after 2 days, or 5 cycles, a significant improvement in

the phosphate removal levels was observed.

One explanation why greater phosphate removal was not achieved in the cyclic fermentation studies (the phosphate concentration did not fall appreciably below 4 mgP l^{-1}) may be that the level of acetate, the carbon source for EBPR, may have become limiting (see Figures 45. - 47.). Phosphate removal declined rapidly as the acetate concentration decreased, presumably as it was metabolised by the *Acinetobacter* in the co-culture. However, the acetate concentration did not seem to decrease during the anaerobic phase, as would be expected from the biochemical models (1.3.3.8.). *Acinetobacter* sp. SAB, an obligate aerobe, has a competitive advantage over *Aeromonas caviae*, a facultative anaerobe, for carbon substrate utilisation under aerobic conditions. The switch from fermentative metabolism to respiratory metabolism is relatively slow. Therefore by the time the *Aeromonas* has switched its metabolism to respiration under aerobic conditions the carbon substrate (acetate) will have been used by the *Acinetobacter* sp., limiting and preventing the population dominance by the *Aeromonas* under aerobic conditions. However, the change from respiratory to fermentative metabolism is much quicker, since the biochemical pathways are already functional. This means that fermentation can start almost immediately under anaerobic conditions. The difference in the efficiency in switching from these two metabolic states enables the two isolates to survive as a co-culture. Neither isolate dominates overall, the *Aeromonas* isolate plays a dominant role under anaerobic conditions, whereas the *Acinetobacter* sp. isolate takes over under aerobic conditions.

CHAPTER 6.

6. MIXED CULTURE TEMPERATURE INVESTIGATIONS

6.1. INTRODUCTION

Excellent phosphate removal has been observed in EBPR plants operating over a wide range of temperatures. For instance, Krichen *et al.* (1985) and Sell *et al.* (1981) found good phosphate removal at 5°C whilst Jones *et al.* (1987) found improved anaerobic release and aerobic uptake of phosphate at the much higher temperature of 29°C. Temperature investigations on *Acinetobacter* sp. in pure culture have also been reported. From their studies, Du Preez and Toerien (1978) suggested that a temperature range of 29-35°C was optimal for EBPR plants utilising *Acinetobacter calcoaceticus* as a phosphate removing microorganism. However, Fuhs and Chen (1975) found maximum phosphate uptake at a slightly lower optimum temperature of 20-24°C for their *Acinetobacter lwoffii* isolate, a range which also corresponded to the isolates optimal growth temperature. Since only a few studies have investigated the effect of temperature on EBPR, and some of those are contradictory, this study was undertaken to investigate the effect of a comprehensive range of temperatures on the anaerobic release and aerobic uptake of phosphate during the process via a series of batch experiments. The effect of acclimatising the sludge at several temperatures was also investigated.

Wastewater temperature can significantly influence the EBPR process since the rate of biochemical reactions, and hence metabolism and growth of activated sludge organisms can be affected by temperature changes. Svante Arrhenius first applied an expression which simply explained why many reactions speed up with increasing temperature.

The Arrhenius equation defines the relationship between the value of the rate constant and the absolute temperature:

$$k = k_c e^{-\Delta E/RT} \quad (1.)$$

Where : k = rate constant
 T = temperature in degrees Kelvin
 R = gas constant
 k_c = a proportionality constant
 E = the activation energy constant

The relationship fails beyond a certain maximum temperature due to thermal denaturation of proteins. For instance Fuhs and Chen (1975) found that phosphate uptake of their *Acinetobacter lwoffii* isolate was significantly inhibited at 37°C and almost negligible at 45 °C.

The Arrhenius equation (1.) can also be written in logarithmic form:

$$\ln k = \ln k_c - \Delta E_a / RT \quad (2.)$$

or:

$$\ln k = -\Delta E_a / RT + c \quad (3.)$$

where c is a proportionality constant.

From this expression, a plot of the rate of reaction as a function of T^{-1} often gives a straight line with a negative slope. Such a plot can be used to determine the activation energy E_a (J mol^{-1}) value of a reaction since its slope is $-\Delta E_a / R$ and the constant c is the intercept on the y axis.

For the EBPR process, the temperature dependence of the initial maximum anaerobic soluble ortho-phosphate release rate can be described from the above equation as:

$$\ln (\text{init. max. sol. release rate}) = \Delta E_a / RT + c \quad (4.)$$

and similarly for the initial maximum aerobic soluble ortho-phosphate uptake rate:

$$\ln (\text{init. max. sol. uptake rate}) = \Delta E_a / RT + c \quad (5.)$$

The effect of temperature on the rate of a biological process can also be expressed by a simplified Arrhenius relationship:

$$r_T = r_{20} \theta^{(T-20)} \quad (6.)$$

Where: r_T = reaction rate at $T^\circ\text{C}$
 r_{20} = reaction rate at 20°C
 θ = temperature-activity coefficient
 T = temperature, $^\circ\text{C}$

For activated sludge typical values of θ range between 1.00 and 1.08 (Metcalf and Eddy, 1991).

The temperature coefficient Q_{10} indicates how many times the rate of a process increases if the temperature is raised by 10°C :

$$Q_{10} = \frac{\text{Rate at } T + 10^{\circ}}{\text{Rate at } T^{\circ}\text{C}} \quad (7.)$$

In general the Q_{10} value is considered to be around two for biological reactions (Du Preez and Toerien, 1978). The value of θ described in Equation 6. has been shown to be equal to $(Q_{10})^{1/10}$ (Novak, 1974).

This study investigates the effect of temperature on phosphate release and uptake by a mixed activated sludge culture over a range of $5\text{-}45^{\circ}\text{C}$ in shake flask batch experiments. Sludge acclimatised to particular temperatures was also investigated.

6.2. EXPERIMENTAL

6.2.1. Activated Sludge

Activated sludge acclimatised to EBPR was obtained from Severn Trent's EBPR pilot plant at Hinckley. A 5 l sample taken from the RAS line was transported back to the laboratory within two hours of sampling. The sludge was placed into the 10 l SBR which was immediately initiated on a programmed sequence of phase cycling, starting with the feed phase. After 24 hours some of the sludge was transferred to the 5 l SBR operating under a similar phase sequencing pattern. The suspended solids were manually adjusted from an original 6000 mg l^{-1} to a desired operating level of around $1800\text{-}2000 \text{ mg l}^{-1}$ in both reactors. The 10 l reactor was operated at room temperature ($20^{\circ}\text{C} \pm 3^{\circ}\text{C}$) and the 5 l reactor was initially operated at a temperature controlled at 17.5°C . Good EBPR was observed in both reactors 3 days after commencement of the run. The sludge was allowed to acclimatise

to the new conditions, in particular the synthetic sewage feed, for 19 days before shake flask batch temperature experiments were undertaken using sludge from the 5 l SBR. The 10 l SBR sludge was used as a seed source for each new temperature acclimatisation investigation.

6.2.2. Shake Flask Batch Experiments

Batch experiments to investigate the effect of temperature on EBPR were undertaken in 250 ml Erlenmeyer flasks placed in temperature controlled water baths (Grant SS40-5, Grant Instruments Ltd, Cambridge). The following temperatures were investigated for their effect on EBPR: 5, 7.5, 8, 10, 12, 15, 17.5, 18, 20, 22, 22.5, 25, 30, 35, 40, 42.5 and 45°C. For anaerobic investigations a volume of EBPR sludge was removed from the 5 l SBR at the start of a feeding phase (synthetic sewage feed was manually prevented from entering the vessel whilst the sample was removed) and placed in a flask. A N₂ line was introduced into the flask and a volume of diluted synthetic sewage feed was added to the sludge at the start of the experiment. For aerobic investigations a volume of sludge was removed from the 5 l SBR at the end of an anaerobic phase and transferred to a flask. An air line was placed in the flask and a volume of KH₂PO₄ solution was added to raise the ortho-phosphate concentration at the start of the study to around 50 mg l⁻¹. Samples were taken every 15 minutes and analysed for ortho-phosphate (o-P), total phosphate (TP), mixed liquor total suspended solids (MLTSS), chemical oxygen demand (COD), nitrate (NO₃⁻) and nitrite (NO₂⁻). From the results the phosphate release or uptake per unit MLTSS per hour was determined. Investigations were carried out in duplicate for each temperature. A control containing dead cells was set up for each investigation.

6.2.3. Fermenter Experiments

The 5 l SBR was used as a long term acclimatisation vessel for several temperatures. Activated sludge initially acclimatised to 17.5°C for 19 days was first investigated, followed by 10 and 25°C. After reseeded from the 10 l SBR and adjustment of the sludge temperature the vessel contents were allowed to acclimatise to the new conditions for at least 12 days following a temperature change before shake flask batch experiments were undertaken.

6.2.4. Analytical Methods

O-P, TP, TSS, NO_3^- -N, NO_2^- -N and COD were analysed as described in section 2.4.

Activation energies were determined by regression analysis using the Microsoft Excel version 4.0 software package.

6.3. RESULTS

6.3.1. Shake Flask Investigations

The effect both on anaerobic release and aerobic uptake was studied. The initial maximum phosphate release and uptake rates were determined over the first 60 minutes of the experiments.

6.3.1.1. Anaerobic Investigations

Shake flask batch investigations were undertaken to investigate the effect of temperature on anaerobic ortho-phosphate release. Figure 50. shows the means of duplicate experiments over a representative range of temperatures in the investigation.

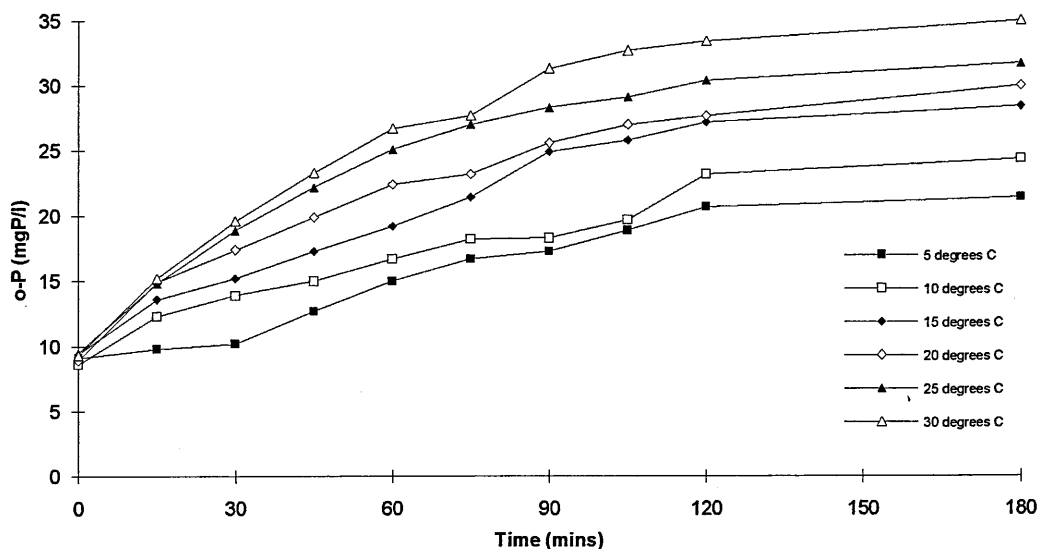


Figure 50. Anaerobic ortho-phosphate release at various temperatures in batch studies

The rate of anaerobic phosphate release increased steadily with increasing temperatures between 5°C and 30°C, with the optimum temperature for maximum phosphate release at 30°C. No phosphate was released at 45°C indicating that the phosphate removing microorganisms were dead.

The initial maximum anaerobic ortho-phosphate release rate complied with the Arrhenius relationship between 5 and 30°C (Figure 51.) with a resultant activation energy of 34.22 kJ mol⁻¹ with a regression coefficient of 0.97. At 35°C the rate of anaerobic phosphate release decreased and was further inhibited at 40°C with almost no release at 42.5°C.

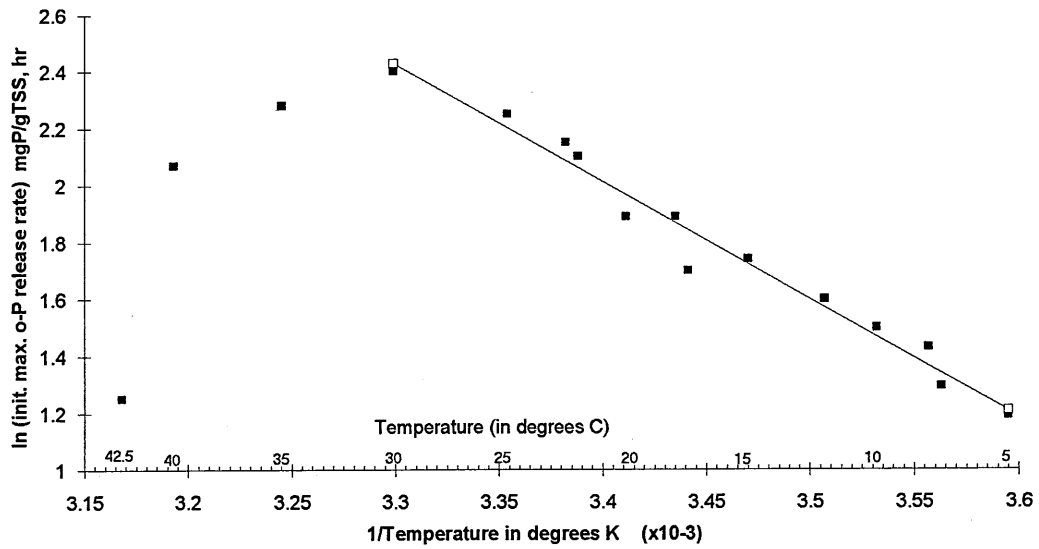


Figure 51. Arrhenius plot for initial maximum ortho-phosphate release rate under anaerobic conditions

No phosphate release was demonstrated in any of the controls.

6.3.1.2. Aerobic Investigations

Studies were undertaken in shake flask batch experiments to investigate the effect of temperature on aerobic ortho-phosphate uptake. Figure 52. shows the means of duplicate experiments over a representative range of temperatures in the investigation.

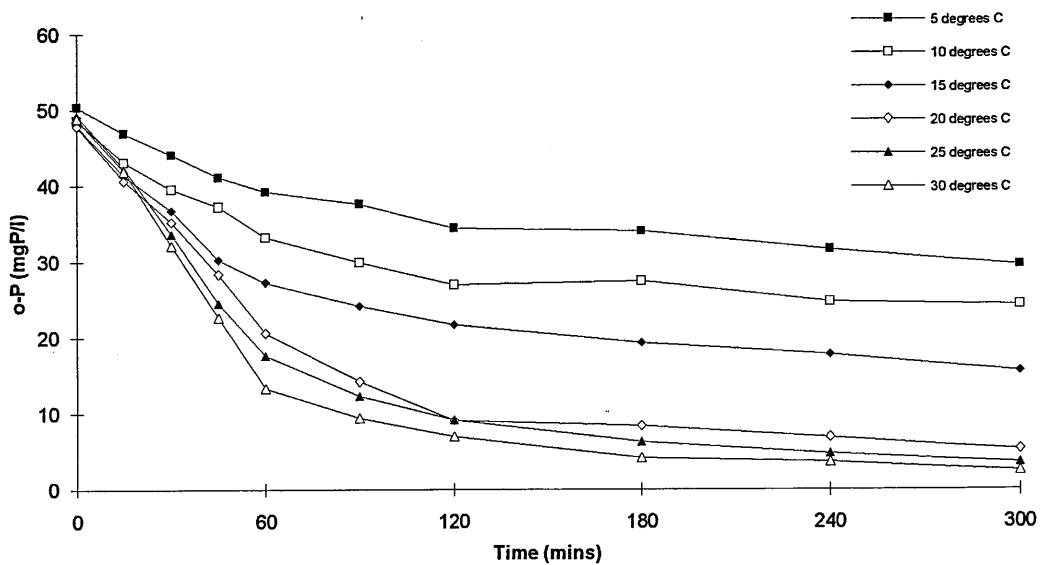


Figure 52. Aerobic ortho-phosphate uptake at various temperatures in batch studies

The rate of aerobic phosphate uptake increased with increasing temperatures between 5°C and 30°C. At temperatures greater than 15°C, improved and relatively similar removal was observed. The optimum temperature for phosphate uptake was 30°C.

The initial maximum aerobic ortho-phosphate uptake rate followed an Arrhenius relationship between 5 and 30°C (Figure 53.) with a resultant activation energy of 39.95 kJ mol⁻¹ with a regression coefficient of 0.96. At 35°C the aerobic uptake rate began to decline and at 42.5°C was significantly inhibited with negligible removal at 45°C indicating the phosphate removing cells were dead.

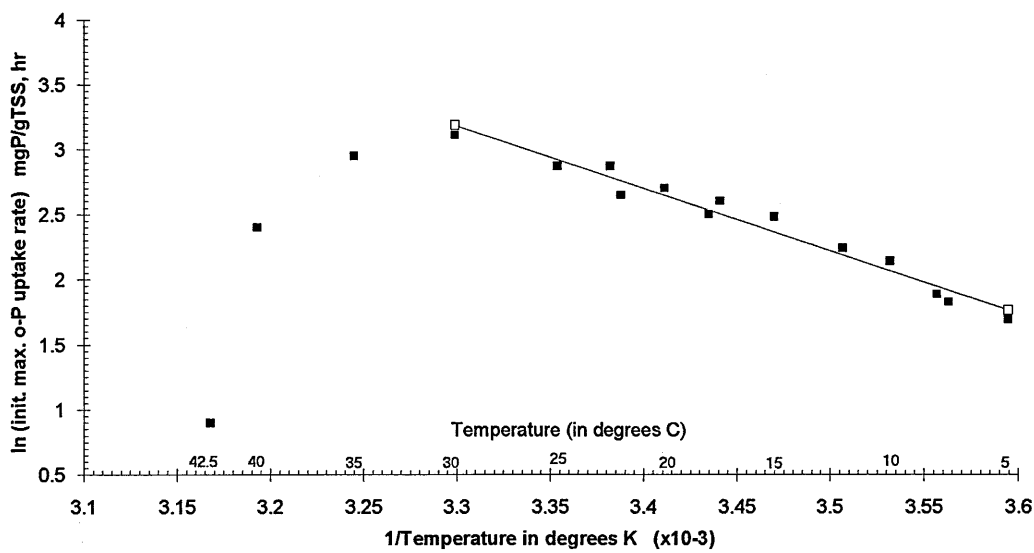


Figure 53. Arrhenius plot for initial maximum ortho-phosphate uptake rate under aerobic conditions

The controls showed no phosphate uptake.

6.3.2. Fermenter Investigations

The significance of a sludge temperature acclimatisation period prior to batch shake flask experiments was investigated in these studies. Acclimatisation temperatures of 10 and 25°C were investigated and compared alongside batch results of sludge acclimatised at 17.5°C described in section 6.3.1.

6.3.2.1. Anaerobic Investigations

The effect of temperature on the rate of anaerobic phosphate release was investigated in sludge acclimatised at 10°C for at least 12 days and at 25°C for at least 14 days prior to shake flask batch tests over a range of temperatures between 5 and 40°C. Both sludges followed an Arrhenius relationship between 5 and 30°C (Figure 54.).

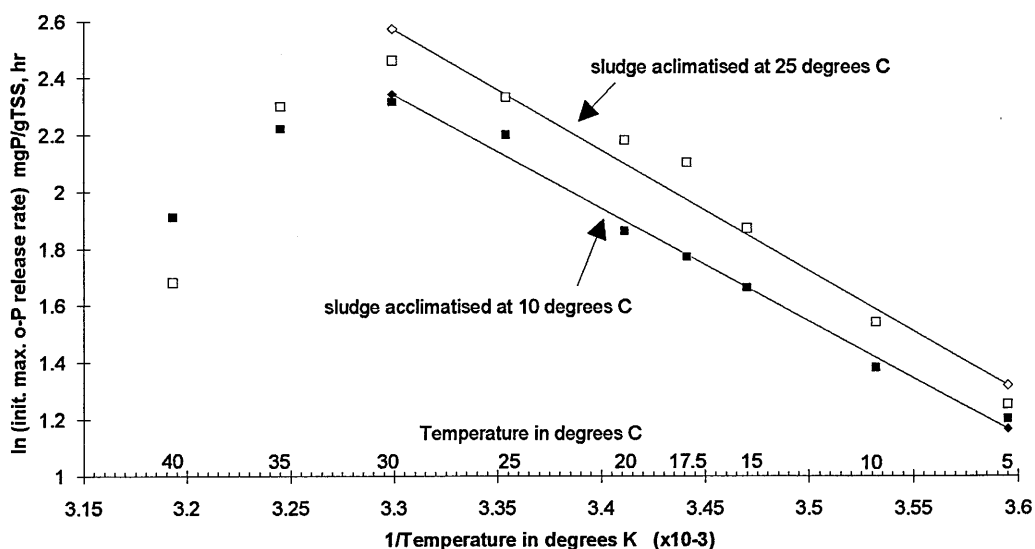


Figure 54. Arrhenius plots for initial maximum ortho-phosphate release rate under anaerobic conditions. Sludge acclimatised at 10°C & 25°C

The activation energy of the sludge acclimatised at 10°C was 33.01 kJ mol⁻¹ with a regression coefficient of 0.98. The sludge acclimatised at 25°C had an activation energy of 35.17 kJ mol⁻¹ with a regression coefficient of 0.96. Both plots showed similar trends with a maximum phosphate release achieved at 30°C and inhibition at 40°C. The controls showed no phosphate release.

6.3.2.2. Aerobic Investigations

The effect of temperature on the rate of aerobic phosphate uptake was investigated in sludge acclimatised at 10°C for at least 12 days and at 25°C for at least 14 days prior to shake flask batch tests over a range of temperatures between 5 and 40°C. Both sludges obeyed an Arrhenius relationship between 5 and 30°C (Figure 55.).

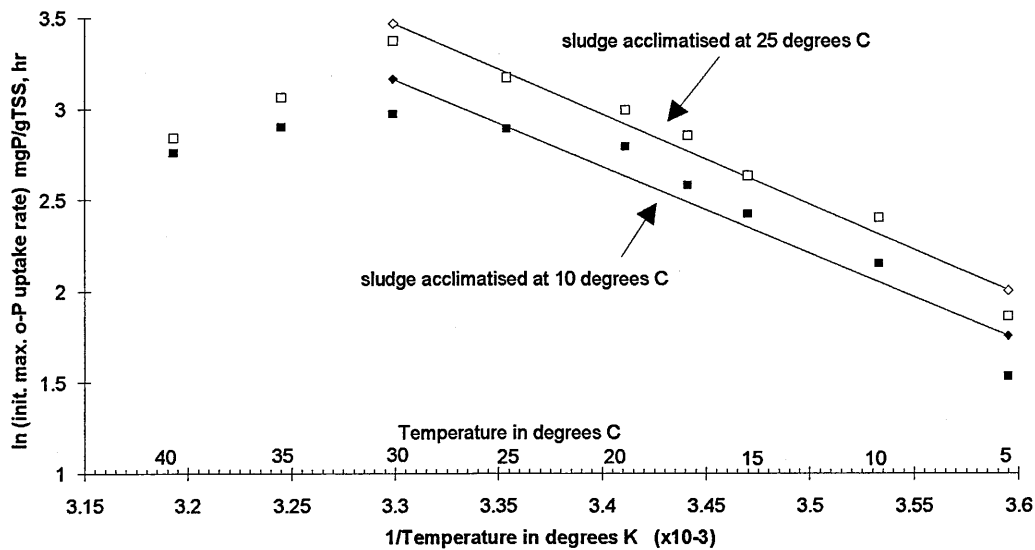


Figure 55. Arrhenius plots for initial maximum ortho-phosphate uptake rate under aerobic conditions. Sludge acclimatised at 10°C and 25°C

Both sludges showed similar trends, although actual rate values were slightly higher for the sludge acclimatised at 25°C. The initial maximum aerobic ortho-phosphate uptake rate followed an Arrhenius relationship between 5 and 30°C at both acclimatisation temperatures. The activation energy of the sludge acclimatised at 10°C was 39.51 kJ mol⁻¹ with a regression coefficient of 0.91, whilst the sludge acclimatised at 25°C had an activation energy of 41.08 kJ mol⁻¹ with a regression coefficient of 0.96. The optimum temperature for maximum phosphate uptake of both sludges was 30°C with uptake starting to be inhibited at 40°C. No phosphate uptake was observed in any of the controls.

6.4. DISCUSSION

The study showed that anaerobic release and aerobic uptake of phosphate was possible within an extensive temperature range of 5-40°C. At 42.5°C significant inhibition of release and uptake was observed whilst at 45°C the cultures were almost entirely inactive. Whilst phosphate release and uptake were observed at winter temperatures, the rates declined steadily with decreasing temperatures. This is in accordance with many other observations. Vassos *et al.* (1987) investigated the influence of low temperatures on EBPR at a modified Bardenpho or Phoredox plant at Kelowna, Canada. At around 15°C the plant efficiency altered significantly with changes in sludge temperature. At, or below 15°C around 68% overall phosphorus removal was achieved, but above 15°C around 85% removal was achieved. Ludwig *et al.* (1985) also found EBPR to be temperature dependant and phosphate removal decreased at lower temperatures. Hutton *et al.* (1994) evaluated two pilot biological nutrient removal (BNR) processes under winter conditions (12-13°C) and compared their ability to meet an effluent phosphate standard of 0.5 mg l⁻¹. Both the Virginia Initiative Plant (VIP) and Modified University of North Carolina (UNC) configurations were able to achieve the phosphate standard but a backup chemical precipitation capability was recommended for both systems

to ensure compliance. A Swedish study (Marklund, 1993) assessed the influence of low temperature wastewater on the efficiency of EBPR in a SBR where the temperature in the reactor varied between 3 and 8°C. The plant was designed to meet effluent standards of 0.5 mg l⁻¹ TP and 15 mg l⁻¹ BOD₇. Biological phosphate removal was observed even at the lowest temperatures, but with a significant increase in TP in the effluent at temperatures below 5°C. The activated sludge population was able to survive the lower temperatures and resumed normal functions when the temperature increased. However, the study found that effluent suspended solids were higher than expected and concluded that low water temperatures may have a harmful effect on sludge flocculation during the settlement phase. In addition to microbial metabolism temperature may affect other factors such as gas transfer rates and settling properties of activated sludge (Metcalf and Eddy, 1991). In the Marklund (1993) study the TP and BOD₇ effluent standards could only be met at the highest temperatures and in general better results were achieved at temperatures above 5°C. Sell *et al.* (1981) also investigated the effect of low temperatures of 5, 10 and 15°C in A/O configuration EBPR plants and found that even at temperatures as low as 5°C EBPR was not impaired. In fact, EBPR was enhanced as the temperature decreased which they explained by stating that the EBPR bacteria were psychrophilic. Above 10°C the non-EBPR mesophilic bacteria competed for substrates with the psychrophiles resulting in less phosphate removal at the higher temperatures. Krichen *et al.* (1985) also found that increased phosphate removal was observed at 5°C compared to 10 and 15°C and also suggested that facultative psychrophilic bacteria were responsible for increased removal at lower temperatures. However, the observations of Sell *et al.* (1981) and Krichen *et al.* (1985) do not agree with the results of this study.

Jones *et al.* (1987) compared both anaerobic phosphate release and aerobic phosphate uptake at two much higher temperatures of 24 and 29°C. At 29°C around 75% more TP was released under anaerobic conditions than at 24°C

and 33% less TP remained in the final aerobic reactor when operated at 29°C rather than at 24°C. The work of Converti *et al.* (1995) shows a similar temperature dependence on EBPR to that found in this study in that improved phosphate removal rates are observed at the higher temperatures. They investigated the effect of stressing an acclimatised EBPR anaerobic / aerobic biomass by changing the temperature from 20°C to 5, 15, 25, 30 and 35°C in turn. The batch tests were undertaken over 140 h under microaerophilic conditions. The tests at 5, 15 and 25°C showed a phosphorus release curve, with increasing release at decreasing temperatures, for the initial 50 h or so followed by phosphate uptake. The need to acclimatise the microbial population to the stress conditions was given as an explanation for this observation. However the overall phosphorus removal was almost identical at the end of the test for all temperatures. The main difference in performance was that final phosphorus removal concentrations were achieved in less time at the higher temperatures.

Boughton *et al.* (1971) investigated the effect of several temperatures on activated sludge within the range of 4 to 60°C. Batch studies indicated a wide optimum temperature range of 24-37°C for ^{32}P uptake, when phosphate removal was virtually 100%. At 45°C however, ^{32}P uptake had decreased to around 43% - a similar percentage removal to that found at 10°C. The effect of temperature on the production of VFAs, including acetate - a preferred EBPR substrate, has been investigated in a U.S. study (Skalsky and Daigger, 1995). They found that temperature had a significant effect, VFA production at winter wastewater temperatures of 14-16°C was around 40-50% less than that at summer temperatures of 21-24°C.

Hashimoto and Furukawa (1984) investigated the temperature dependency of phosphate release under anaerobic conditions. Four temperatures between 12 and 28°C were investigated in batch investigations. They found a very high activation energy of 16.4 kcal mol⁻¹ (68.7 kJ mol⁻¹) which is around twice as

high as the values of 33.01, 34.22 and 35.17 kJ mol⁻¹ determined for anaerobic release in this study. Mamais and Jenkins (1992) determined an activation energy value of 30 kJ mol⁻¹ in their anaerobic phosphate release study which is fairly similar to the values found in this study.

The Q_{10} and temperature-activity coefficient (θ) values for all anaerobic and aerobic investigations in this study were determined (Table 27.). The higher the Q_{10} (or θ) value, the more sensitive the reaction is to changes in temperature. The values also give an insight on the effect of acclimatising the sludge at the various temperatures.

In general, temperature acclimatisation of a mixed culture is considered important in establishing its temperature dependency. Benedict and Carlson (1973) studied the effect of temperature acclimatisation on a conventional activated sludge originally operating at 15-19°C by measuring the endogenous respiration rate. Three new temperatures were selected at 4, 19 and 32°C. The 19°C culture showed no temperature adaptation requirement over 52 days. However the 4°C culture showed the need for a 14 day acclimatisation period whilst the 32°C culture needed an unspecified period in the order of months. Although the minimum length of acclimatisation period was not determined in this study, 19 days for the 17.5°C sludge, 12 days for the 10°C sludge and 14 days for the 25°C sludge were sufficient to establish steady state conditions.

Table 27. The effect of temperature on anaerobic phosphate release and aerobic phosphate uptake in EBPR

EBPR Process Sludge	Temperature Range (°C)	Q_{10}	$\theta=(Q_{10})^{1/10}$
Anaerobic release rate - acclimatised at 10°C (mgP/gTSS, hr)	5-15	1.59	1.0475
	10-20	1.61	1.0488
	15-25	1.71	1.0551
	20-30	1.57	1.0461
	25-35	1.03	1.0030
	30-40	0.667	0.9603
Anaerobic release rate - acclimatised at 17.5°C (mgP/gTSS, hr)	5-15	1.78	1.0594
	10-20	1.48	1.0400
	15-25	1.65	1.0514
	20-30	1.65	1.0514
	25-35	1.03	1.0030
	30-40	0.72	0.9677
Anaerobic release rate - acclimatised at 25°C (mgP/gTSS, hr)	5-15	1.86	1.0640
	10-20	1.89	1.0657
	15-25	1.58	1.0468
	20-30	1.32	1.0282
	25-35	0.97	0.9970
	30-40	0.46	0.9253
Aerobic uptake rate - acclimatised at 10°C (mgP/gTSS, hr)	5-15	2.43	1.0929
	10-20	1.90	1.0663
	15-25	1.59	1.0475
	20-30	1.20	1.0184
	25-35	1.02	1.0020
	30-40	0.81	0.9791
Aerobic uptake rate - acclimatised at 17.5°C (mgP/gTSS, hr)	5-15	2.20	1.0820
	10-20	1.75	1.0576
	15-25	1.47	1.0393
	20-30	1.36	1.0312
	25-35	1.09	1.0087
	30-40	0.83	0.9815
Aerobic uptake rate - acclimatised at 25°C (mgP/gTSS, hr)	5-15	2.16	1.0801
	10-20	1.80	1.0605
	15-25	1.71	1.0551
	20-30	1.46	1.0386
	25-35	0.90	0.9895
	30-40	0.59	0.9486

For both anaerobic release and aerobic uptake of phosphate the Q_{10} value generally decreases with increasing temperature. This suggests that the EBPR process is more sensitive to temperature changes at lower temperatures. Anaerobic phosphate release from the sludge acclimatised at 10°C is slightly less sensitive to temperature changes between 5-15°C than the sludges acclimatised at 17.5 and 25°C suggesting a possible small shift in the microbial population. However the corresponding aerobic Q_{10} values suggest that phosphate uptake is significantly more sensitive to temperature changes within the 5-15°C range than anaerobic release even with acclimatisation to 10°C. It is clear from the Q_{10} values that temperature affects anaerobic release and aerobic uptake differently which is supported by the differences in anaerobic and aerobic activation energies determined from the Arrhenius plots.

Shapiro *et al.* (1967) investigated the effect of temperature on the anoxic release of phosphate in activated sludge. Freshly settled sludge was maintained at 10, 15, 20, 25 and 30°C and phosphate release was shown to be temperature dependant with around twice as much phosphate released at 30°C compared to 20°C over a 3.5 h experiment. Phosphate release at 10 and 15°C was much less, at around 25% of the 30°C concentration. They also determined Q_{10} values at the time interval between 60-180 minutes of the study of 2.1-2.6. Q_{10} values of several other investigations are given in Table 28. which can be compared with values determined in this study (Table 27.).

Mamais and Jenkins (1992) found $\theta = 1.043$ for maximum anaerobic release between 10-28°C, $\theta = 1.055$ for maximum aerobic uptake between 10-28°C and $\theta = 1.049$ for all EBPR processes between 10-30°C.

Table 28. Reported Q_{10} values for EBPR processes and *Acinetobacter* sp. investigations

Q_{10}	Process	Temperature Range °C	Reference
2.4	Anaerobic PO_4^{3-} release	20-30	Hashimoto & Furukawa (1984)
2.5	Anoxic PO_4^{3-} release	10-20	Shapiro <i>et al.</i> (1967)
2.6	Anoxic PO_4^{3-} release	15-25	Shapiro <i>et al.</i> (1967)
2.1	Anoxic PO_4^{3-} release	20-30	Shapiro <i>et al.</i> (1967)
3.8	Growth of <i>A. calcoaceticus</i> on TSB Medium	Below 23.5	Du preez & Toerien (1978)
1.7	Growth of <i>A. calcoaceticus</i> on TSB Medium	Above 23.5	Du preez & Toerien (1978)
2.1	Growth of <i>A. calcoaceticus</i> on Acetate Medium	10-29	Du preez & Toerien (1978)
2.8	Growth of <i>A. calcoaceticus</i> on Acetate Medium	Below 22	Du preez & Toerien (1978)
1.8	Growth of <i>A. calcoaceticus</i> on Acetate Medium	Above 22	Du preez & Toerien (1978)
1.52	Anaerobic PO_4^{3-} release	10-28	Mamais & Jenkins (1992)
1.7	Aerobic PO_4^{3-} uptake	10-30	Mamais & Jenkins (1992)

Some care must be exercised in comparing and interpreting Q_{10} and θ values of different studies since θ is a substrate-dependent variable (Novak, 1974) and the substrates in Table 28. varied between studies. However, they do indicate that the findings of this study are generally supported by others.

Yeoman *et al.* (1988) investigated the effect of temperature on EBPR in two laboratory scale plants fed with synthetic sewage, one of which was

bioaugmented with *Acinetobacter* sp. They found temperature to be a major influencing factor on EBPR. Between 12 and 25°C a 1°C rise in temperature improved phosphate removal by 2.5% to achieve around 75% phosphate removal in the control plant and over 80% removal in the bioaugmented plant at the highest temperature.

The effect of temperature on pure *Acinetobacter* sp. cultures has also been investigated over a wide temperature range. Du Preez *et al.* (1981) found that the optimum temperature for growth of *Acinetobacter calcoaceticus* was in the region of 29 - 36°C when grown in an acetic acid medium. Abbott *et al.* (1973) found a similar trend for the same species when grown in an ethanol medium. Van Groenestijn *et al.* (1989a) found the optimum temperature of growth of three *Acinetobacter* sp. strains varied within the temperature range of 25-33°C. Fuhs and Chen (1975) however, found a much lower optimum temperature for their *Acinetobacter lwoffii* isolate of 20-24°C, both for growth and phosphate uptake. Hao and Chang (1987) investigated phosphate uptake in *Acinetobacter* spp. and found the optimum temperature for growth to be 33°C, similar to the results of Abbott *et al.* (1973) and du Preez *et al.* (1981). From an Arrhenius plot, an activation energy of around 10 kcal mol⁻¹ (41.9 kJ mol⁻¹) was determined by Hao and Chang (1987). Their value was similar to those determined for aerobic phosphate uptake in this study which are within the range of typical activation energy values of around 8-12 kcal mol⁻¹ (33.5-50 kJ mol⁻¹) for the growth of bacteria (Humphrey, 1979).

The effect of temperature on polyphosphate accumulation alongside cell growth was investigated in three *Acinetobacter* sp. strains with varying results (van Groenestijn *et al.*, 1989a). Strain 210A accumulated most phosphate at a temperature as low as 5°C whilst strain B8 showed optimal accumulation at 27.5°C and accumulation in strain P was independent of temperature. Van Groenestijn and Deinema (1985) found the optimum temperature for growth of *Acinetobacter* strain 210A to be 25°C and found that the phosphorus

content of the cells was at a maximum of 10.1 % at 5°C compared to 1.4 % at 35°C. They suggested that faster growing cells (i.e. those at higher temperatures) may lose their ability to accumulate high concentrations of phosphate.

Temperature becomes an even more influential environmental factor in plants designed for biological nutrient removal (BNR) where nitrogen in addition to phosphate is biologically removed in a single sludge system. Randall *et al.* (1992) compared the performance of a conventional activated sludge system and a BNR system of a UCT configuration at 10, 15 and 20°C and several MCRTs. Complete nitrification in both plants and EBPR in the UCT plant was achieved at the three temperatures at a MCRT of 15 days. However, at the low temperature of 10°C and a low MCRT of 5 days EBPR failed in the UCT plant and nitrification was only partially complete or failed in both plants at lower temperatures and MCRTs. Generally, BNR can be successfully operated at low temperatures by increasing the MCRT.

If the temperature of an EBPR plant can be controlled this study suggests that the optimum operating wastewater temperature is around 30°C both for anaerobic release and aerobic uptake of phosphate. EBPR can also be observed at the two extremes, 5°C and 40°C but to a smaller extent and at a reduced rate.

CHAPTER 7.

7. MIXED CULTURE pH INVESTIGATIONS

7.1 INTRODUCTION

Wastewater pH is an important environmental consideration since most bacteria cannot tolerate pH values above 9.5 or below 4.0, and tend to have an optimum pH value for growth between pH 6.5 and 7.5 (Metcalf and Eddy, 1991). A variety of optimal pH values for EBPR have been reported. What seems clear however, is that above pH 12.5 and below pH 4.5 EBPR is inhibited (Boughton *et al.*, 1971). Optimum pH for overall phosphate removal seems to be slightly alkaline; Wentzel *et al.* (1988) suggested that controlling the pH at approximately pH 7.5 is essential for good phosphate removal. Differences have been found between the pH optima for the anaerobic and aerobic phases of the process. Smolders *et al.* (1994a) observed that anaerobic phosphate release rates increased with increasing pH values (values up to pH 8.2 were shown) whilst the optimum for aerobic phosphate uptake has been reported within several ranges such as 7.7 - 9.7 (Boughton *et al.*, 1971) and pH 7.0 - 8.0 (Levin and Shapiro, 1965).

Care should be taken in the interpretation of pH studies due to the varying operational conditions employed by different workers. In plants where anaerobic conditions are allowed to develop naturally as a result of oxygen consumption by respiration, CO₂ will dissolve into the wastewater resulting in a lowering of pH. However, if anaerobic conditions are achieved by N₂ sparging, as in this study, the CO₂ will be stripped out of solution by the inert gas minimising this effect (Fuhs and Chen, 1975). Ye *et al.* (1988) point out though, that in activated sludge, the greater diffusional resistance present in flocs will inhibit the release of CO₂, which in turn results in a lower pH inside the floc compared to the surrounding medium. Therefore, even N₂ sparging

may not completely displace the CO₂ around activated sludge flocs. When the system is subsequently aerated, a rise in the system pH may result due to air stripping of CO₂. Comeau *et al.* (1987a) observed an increase of 1.0 pH unit between anaerobic and aerobic conditions which they attributed to air displacing the CO₂. Converti *et al.* (1995) mention that the hydrolysis of polyphosphate during anaerobic conditions leads to the formation of phosphoric acid, resulting in the lowering of solution pH values.

7.2 EXPERIMENTAL

7.2.1. Activated Sludge

Acclimatised activated sludge was obtained from Anglian Water's EBPR pilot plant at Milton Keynes and used as an inoculum for this study. A 5 l sample taken from the RAS line of the plant was transported back to the laboratory within 30 minutes of sampling. The sludge was placed into the 10 l SBR which immediately commenced a programmed sequence of phase cycling as described in 2.2., starting with the feed phase. After 48 hours some of the sludge was transferred to the 5 l SBR operating under an identical phase sequencing pattern. Details of the 5 and 10 l SBRs are described in sections 2.1.2.1. and 2.1.2.2. respectively. The suspended solids were manually adjusted from an original 7500 mg l⁻¹ down to a desired operating concentration of around 2000-2500 mg l⁻¹ in both reactors. The two systems were fed with a synthetic sewage medium, as detailed in section 2.3., prepared at the appropriate concentration. pH was not controlled in the 10 l SBR, whereas pH control was implemented in the 5 l SBR as described in section 2.1.2.1. and set at pH 7.0. Both reactors operated at room temperature (20°C ± 3°C). Good EBPR was observed in both SBRs 5 days after commencement of the run. The sludge was allowed to acclimatise to the new conditions, in particular the synthetic sewage feed for 17 days before shake flask batch pH experiments were undertaken.

7.2.2. Determination of the Natural pH Profile of the Process

The 10 l SBR was operated without pH control to determine the natural pH pattern of the process under the various phases when fed with a synthetic sewage of constant composition and pH.

7.2.3. pH Control versus no pH Control

The effect on EBPR of controlling the pH of the sludge was investigated. The pH level was controlled in the 5 l SBR at a pre-set value of $\text{pH } 7.0 \pm 0.1$, whereas no pH control was applied in the 10 l SBR to allow for comparison. These investigations were undertaken 16 days into the run when 47 x 8 h cycles had been completed.

7.2.4. Shake Flask Batch Experiments

Batch experiments to investigate the effect of different pH values on EBPR were undertaken in 250 ml Erlenmeyer flasks placed in a shaking water bath (Grant SS40-5, Grant Instruments Ltd, Cambridge) controlled at 17.5°C . Values within the range of pH 5 - 10.5 were investigated. For anaerobic investigations a volume of EBPR sludge was removed from the 10 l SBR at the start of a feeding phase (synthetic sewage feed was manually prevented from entering the vessel whilst the sample was removed) and placed in a flask. A N_2 line was introduced into the flask and a volume of diluted synthetic sewage feed was added to the sludge at the start of the experiment. For aerobic investigations a volume of sludge was removed from the 10 l SBR at the end of an anaerobic phase and transferred to a flask. An air line was placed in the flask and a volume of KH_2PO_4 solution was added to raise the ortho-phosphate concentration at the start of the study to a nominal level of 25 mg l^{-1} . A pH probe was placed in each flask. The pH was manually adjusted to the experimental set-value using 3M HCl or NaOH solutions and was maintained

at the desired value using 0.5M solutions. Overshooting during pH adjustment was avoided as far as possible by careful addition of solutions. Samples were taken every 15 mins and analysed for o-P, TP, TSS, COD, NO_3^- and NO_2^- . From the results, the effects of various pH values on phosphate release and uptake were determined. Investigations were carried out in duplicate for each pH value. Two controls were set up for each investigation; one control contained dead cells, the other no cells.

7.2.5. Analytical Methods

Ortho-phosphate, TP, TSS, NO_3^- -N, NO_2^- -N and COD were analysed as described in section 2.4. The pH was measured in the shake flask experiments using pH probes (Russell pH Ltd., Auchtermuchty) connected to a Corning pH meter (Corning, Essex) and a Kent pH meter (Kent, Luton). The pH value was measured and monitored in the 5 l SBR as described in section 2.1.2.1.

7.3. RESULTS

7.3.1. Process pH Profile with no pH Control

Although not controlled, pH was monitored in the 10 l SBR during the acclimatisation period. Figure 56. shows typical pH plots over several complete cycles after 14 days acclimatisation.

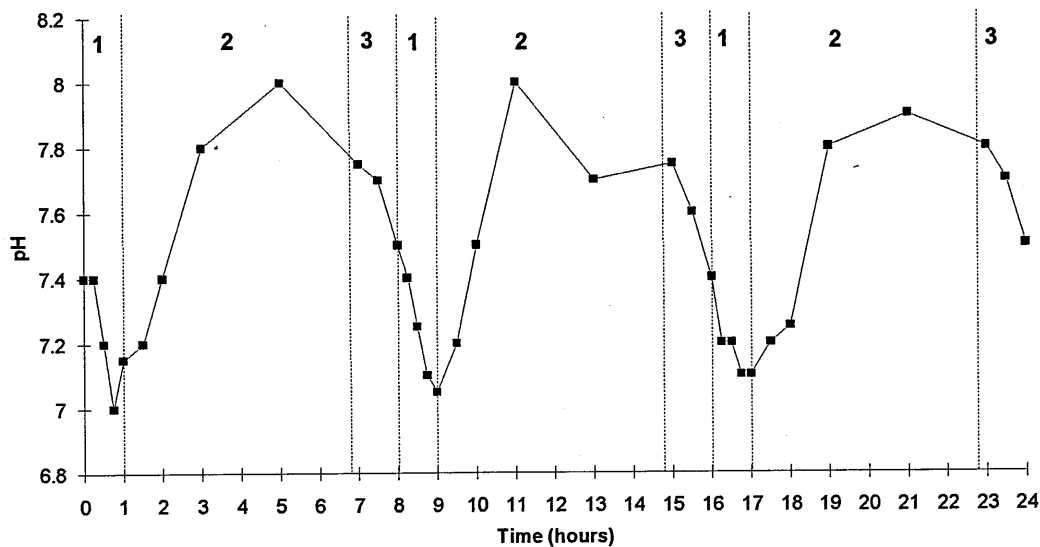


Figure 56. Typical pH profiles for anaerobic/aerobic cycling over 24 hours and three complete cycles. 1. - anaerobic phase, 2. - aerobic phase, 3.- harvesting, settle and feeding phases

The plot gives an indication of how the system settled to a fairly constant pH pattern with distinct differences in values between anaerobic and aerobic phases. The data shown was taken over a 24 h period when 41 x 8 h cycles had been completed prior to time = 0 when the sludge was being fed with a synthetic wastewater of pH 7.2 ± 0.1 . During the anaerobic phase (1. on the graph), the pH of the sludge drops from an initial value of approximately pH 7.4 immediately after feeding to a value of pH 7.0 - 7.1 at the end of the phase. Subsequently, after approximately 2 h aeration (2. on Figure 56.) the pH rises and remains at approximately pH 7.8 - 8.0 for the remainder of the phase. During the settle and harvesting phases (3. on Figure 1.) the pH drops again after the air is switched off as the solution becomes anaerobic. Evidently, the system has stabilised itself to a steady 0.8 - 1.0 pH unit difference between the anaerobic and aerobic phases.

7.3.2. pH Control versus no pH Control

Preliminary investigations were undertaken to compare the effect of controlling, and not controlling pH levels on phosphate removal. The pH of the sludge in the 5 l SBR was controlled at pH 7.0 whereas no pH control was applied to the sludge in the 10 l SBR. All other conditions had been maintained at similar levels. Figure 57. shows phosphate release and uptake over a complete 8 h cycle in the 10 l SBR with no pH control.

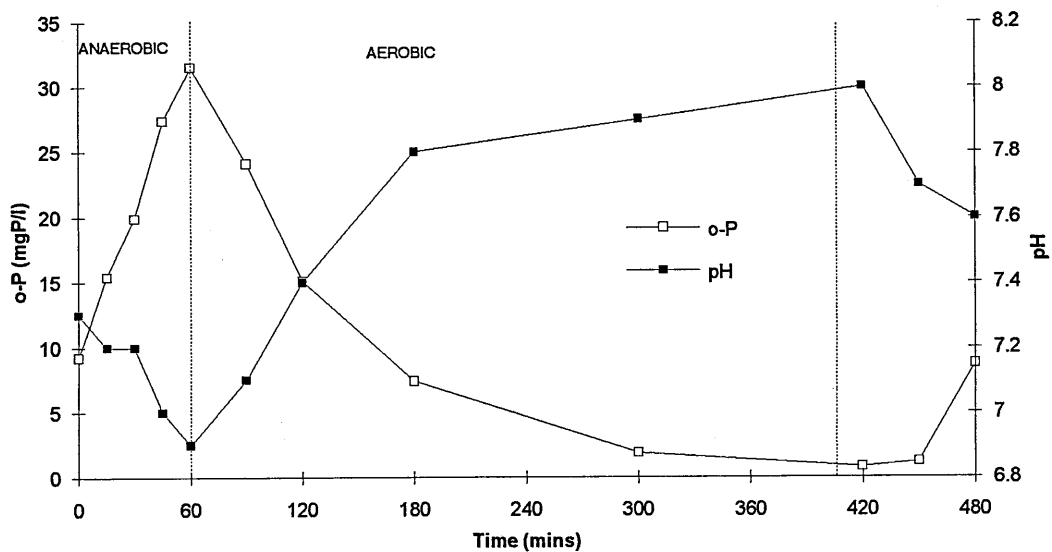


Figure 57. Phosphate release and uptake with no pH control. Where, time = 0 is 16 days (47 x 8 h cycles) into the run

Excellent EBPR is observed without pH control, where an influent phosphate concentration of 9.2 mg l^{-1} is reduced to 0.8 mg l^{-1} at the end of the aerobic phase, representing a reduction of 91% in phosphate levels. With the pH value controlled at pH 7.0, EBPR is again observed, but phosphate removal is lower (Figure 58.).

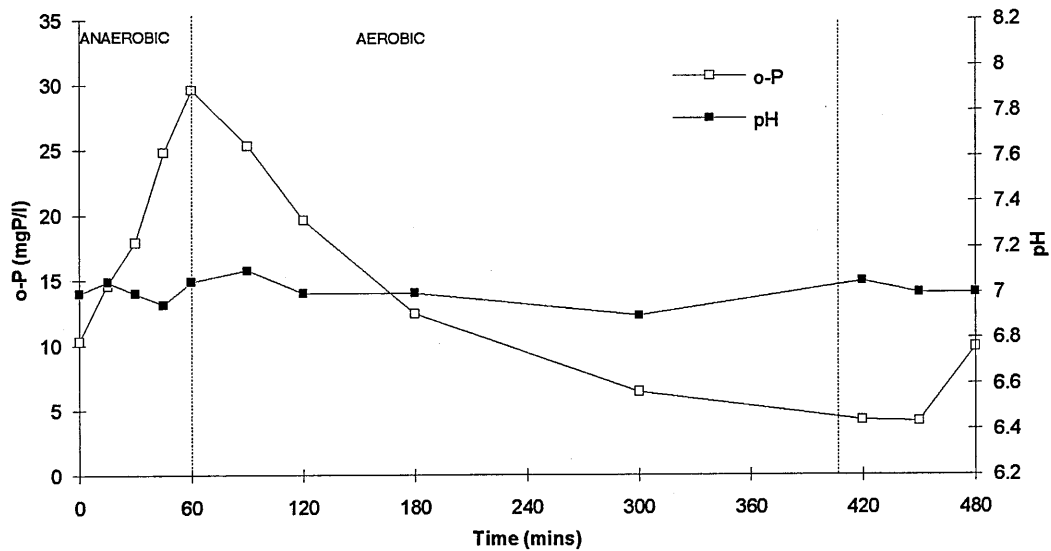


Figure 58. Phosphate release and uptake with pH controlled at pH 7.0.
Where, time = 0 is 16 days (47 x 8 h cycles) into the run

An influent phosphate concentration of 10.3 mg l^{-1} was reduced to 4.1 mg l^{-1} at the end of the aerobic phase, representing an overall reduction in phosphate concentrations of only 60%. In comparing the results from both systems, controlling the pH, at a value of pH 7.0 at least, seems to detrimentally affect aerobic phosphate uptake, whereas little difference is observed between the two systems in the concentration of phosphate released under anaerobic conditions.

7.3.3. SHAKE FLASK INVESTIGATIONS

The following pH values were investigated for their effect on EBPR: 5.0, 5.5, 6.0, 6.5, 7.0, 7.3, 7.5, 7.8, 8.0, 8.3, 8.5, 8.8, 9.0, 9.5, 10.0 and 10.5. The effect both on anaerobic release and aerobic uptake was studied.

7.3.3.1. Anaerobic Investigations

Shake flask batch investigations were undertaken to investigate the effect of pH on anaerobic ortho-phosphate release. Figure 59. shows the means of duplicate experiments of a representative selection of pH values in the investigation.

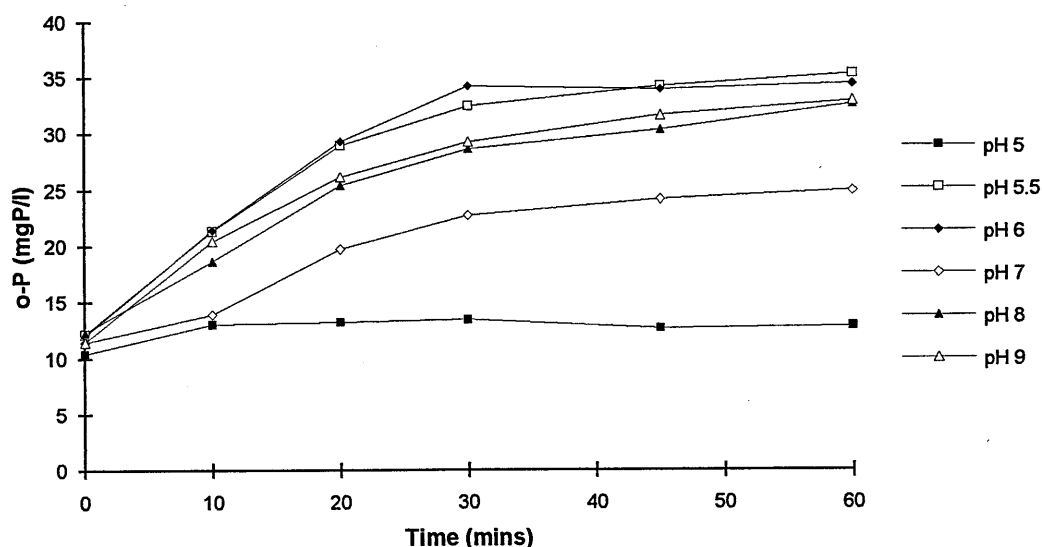


Figure 59. Effect of pH on anaerobic phosphate release rates, showing a representative selection of pH values in the study. Where time = 0 is at least 17 days (51 x 8 h cycles) into the run

From the results, optimal pH for anaerobic phosphate release seems to lie between pH 5.5 - 6.0, closely followed by values around pH 8.0 - 9.0, with much less release at pH 7.0 and negligible release at pH 5.0. The results of the study tends to point towards the existence of two optimal pH ranges. This is indicated more clearly in Figure 60. which shows the concentration of phosphate released in 1 h, from an initial nominal value of 10 mgP l^{-1} , for all the pH values investigated in the study. Duplicate experiments are shown.

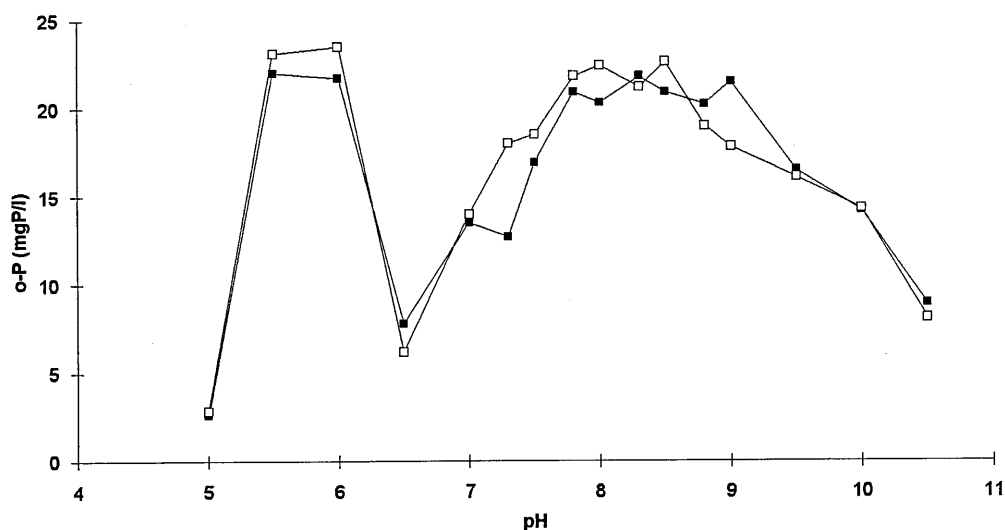


Figure 60. Effect of pH on anaerobic phosphate release after 1 h sparging with N_2

The study clearly shows two peaks for greatest anaerobic phosphate release - at two separate pH ranges of 5.5 - 6.0 and 7.8 - 9.0. Although similar concentrations were observed, the lower pH range showed slightly greater release than the higher range. The phosphate concentration remained unchanged at a nominal 10 mgP l^{-1} in all the controls during the anaerobic phase study.

7.3.3.2. Aerobic Investigations

Similar shake flask batch investigations to the anaerobic studies were undertaken to investigate the effect of pH on aerobic phosphate uptake. Duplicate experiments of a representative selection of pH values in the investigation are shown in Figure 61.

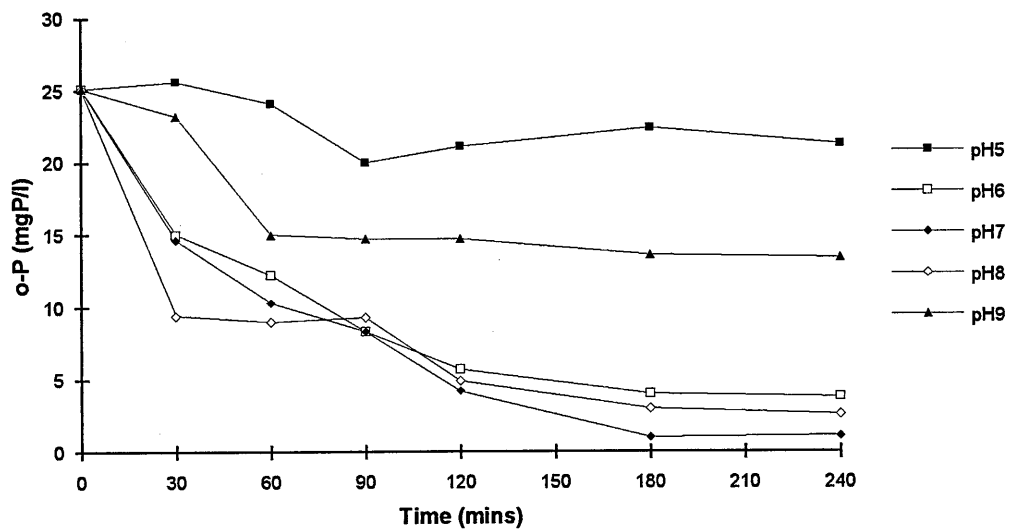


Figure 61. Effect of pH on aerobic phosphate uptake rates, showing a representative selection of pH values in the study. Where time = 0 is at least 17 days (51 x 8 h cycles) into the run

Optimal pH for aerobic phosphate uptake seems to lie between pH 7.0 - 8.0 with less uptake at pH 9.0 and negligible uptake at pH 5.0. Comprehensive results are shown in Figure 62. which indicates the concentration of phosphate uptake after 4 h aeration, from a nominal value of 25 mgP l⁻¹, for all pH values investigated.

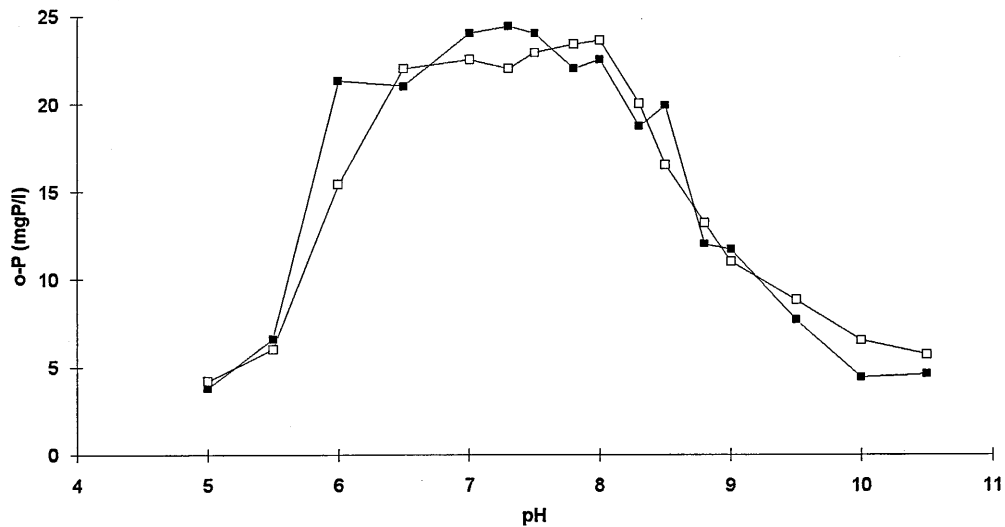


Figure 62. Effect of pH on aerobic phosphate uptake after 4 h aeration

Figure 62. shows good aerobic phosphate uptake within a wide pH range of pH 6.0 - 8.5, with excellent phosphate removal at levels greater than 90% within a narrower range of pH 7.0 - 7.5. Below pH 6.0 the process is inhibited by the acidic conditions, where < 30 % removal is observed. Similar inhibition and removal levels are observed at values greater than pH 9.5 at the other end of the scale.

The phosphate concentration remained unchanged at a nominal 25 mgP l^{-1} in all the controls up to pH 9.5. At, and above this value, a small decrease (< 25% of that observed in the experiment) in the soluble phosphate concentration was observed in both controls indicating a degree of precipitation at the higher values.

7.4. DISCUSSION

Overall, the study indicated that environmental pH may have a significant effect on the efficiency of the EBPR process. In full-scale treatment plants, extreme pH values derived from industrial effluents for instance, may have an adverse effect on the process. Smolders *et al.* (1994b) suggested that the population of polyphosphate microorganisms in the sludge may be affected by variations in the wastewater pH. They indicated that this could result in changes to the efficiency and economics of the operation, in particular the Phostrip process.

When the natural pH profile of the process was determined, a stable pattern of a 0.8 - 1.0 pH unit difference between anaerobic and aerobic phases was clearly established within 15 days of the commencement of the run. These results were similar to those obtained by Converti *et al.* (1995), where their sludge was fed with a synthetic wastewater feed at a constant pH value of 7.2. Their difference in pH values between anaerobic and aerobic phases was slightly narrower at 0.5 - 0.7 pH units. Their system established itself at a value of pH 7.1 under anaerobic conditions and pH 7.6 on aeration. The respective values observed in this study were pH 7.0 - 7.1 during anaerobic conditions and pH 7.8 - 8.0 during aerobic conditions. Converti *et al.* (1995) also investigated the effect of changing the pH of the influent synthetic wastewater from 7.2 down to 6.3, which resulted in a change in the phosphate removal efficiency and the need for 15 days to re-establish steady-state conditions. Their observations highlighted the importance of using a wastewater feed of a constant pH value for meaningful pH studies. A feed with a pH value of 7.2 ± 0.1 was used throughout this study. Table 29. summarises the effect of a range of pH values on phosphate release and uptake, both biological and chemical in pure and mixed cultures.

Table 29. Some reported effects of pH on phosphate release and uptake

pH	Effect	Study Conditions	Reference
<4.5	EBPR breakdown	Batch studies	Boughton <i>et al.</i> (1971)
< 5.5	Anaerobic release inhibition	Batch studies	This study
5.5 - 6.0	First optimum peak for anaerobic release	Batch studies	This study
< 6.0	Aerobic uptake inhibition	Batch studies	This study
7.0	Maximum phosphate uptake	<i>Acinetobacter</i> spp. in batch	Hao & Chang (1987)
7.0 - 7.5	Optimum aerobic phosphate uptake	Batch studies	This study
7.0 - 8.0	Optimum aerobic phosphate uptake	Batch studies	Levin & Shapiro (1965)
7.5	Optimum for growth	<i>Acinetobacter</i> spp. in batch	Hao & Chang (1987)
7.8 - 9.0	Second optimum peak for anaerobic release	Batch studies	This study
< 8.0	Negligible phosphate removal by precipitation	Full scale and batch	Olson and Connell (1971)
8.0	Maximum VFA production	At 23.5°C	Wedi (1992)
> 8.3	Chemical precipitation	Batch	Streichan and Schön (1991)
> 8.8	Aerobic uptake inhibition	Batch studies	This study
9.0	Optimum for growth <i>Acinetobacter</i> 210A	Grown on acetate medium	van Groenestijn <i>et al.</i> (1989a)
> 9.0	Detrimental for phosphate removal	Batch studies	Levin & Shapiro (1965)
> 10.5	Anaerobic release inhibition	Batch studies	This study
12.5	EBPR breakdown	EBPR studies	Boughton <i>et al.</i> (1971)

When controlling the pH of the process at pH 7.0 was compared alongside process performance with no pH control, improved phosphate uptake under aerobic conditions was observed in the system without pH control. The anaerobic release seemed unaffected whether pH was controlled or not. Hashimoto and Furukawa (1984) investigated the effect of pH on phosphate release from anoxic sludge. They found a 20% improvement in phosphate release when the pH was initially adjusted to pH 6.0 at the start of a batch investigation. However, when a similar investigation was undertaken but with periodic readjustment back down to pH 6 improved phosphate release was not observed. They did note however, that readjustment could improve release particularly if the pH rose above pH 8.0.

When Smolders *et al.* (1994a) investigated the effect of pH on phosphate release and acetate uptake during the anaerobic phase of EBPR they found that phosphate release was significantly affected by pH within the range pH 5.5 - 8.5. They showed that the phosphate release rate increased with increasing pH values between 5.5 and 8.2 which shows a similar trend to the observations found in this study if only the second peak (see Figures 59. and 60.) is considered. The detailed explanation for this observation given by Smolders *et al.* (1994a) tends to suggest that the first peak indicating an optimal pH range for anaerobic release at 5.5 - 6.0 is an anomaly, even though two separate investigations were undertaken at the pH values. Smolders *et al.* (1994a) explain in detail how acetate uptake by the sludge needed less energy at low pH compared to higher pH values, resulting in less phosphate being released at low pH compared to high pH. On the other hand, Osborn and Nicholls (1978) observed that within the range of pH 6.8 - 8.0 the pH value seemed to have little effect on anaerobic phosphate release from the cells.

Wentzel *et al.* (1988) recommend that system pH control at around pH 7.5 is essential to maintain EBPR. They found that anaerobic phosphate release had

a negligible effect on solution pH if the pH value was near 7.0, whereas aerobic uptake tended to increase pH which could cause system failure above pH 9.0. Their Phoredox system successfully achieved EBPR when maintaining pH at around 7.4 in the aerobic reactor and 7.1 in the anaerobic reactors which minimised calcium phosphate precipitation.

Boughton *et al.* (1971) investigated the effect of various pH values within the range pH 3.6 - 12.5 in their phosphate removing sludge. Their batch studies showed an optimal pH range of 7.7 - 9.7 for ^{32}P uptake when almost 100% phosphate removal was observed. Their values are slightly different to those obtained in this study, where a broad optimal pH range of 6.0 - 8.5 was observed for aerobic uptake. Boughton *et al.* (1971) determined that at a pH value of 4.5 less than 10% phosphate removal was observed whilst at pH 12.5 phosphate removal had decreased to around 15%. At pH 7.0 only 45% phosphate removal was observed. Levin and Shapiro (1965) investigated the effect of pH on phosphate removal in batch experiments at pH values controlled at 5.0, 6.0, 7.0, 8.0 and 9.0 and the optimum pH range for aerobic phosphate uptake was found to be within the range of pH 7.0 - 8.0 .

The effect of different pH values on phosphate release and uptake in pure cultures has also been reported. Hao and Chang (1987) investigated phosphate uptake in *Acinetobacter* spp. in batch culture and found the maximum phosphate uptake rate was attained at pH 7.0 whereas the optimum pH for growth was slightly higher at pH 7.5, similar to that found by Abbott *et al.* (1973). When Deinema *et al.* (1985) investigated the effect of pH on *Acinetobacter* 210A a pH value in the range pH 6.5 - 8.2 was found to be optimal both for phosphate uptake and growth of the strain in acetate medium. At pH 6.4 and pH 8.6 phosphate uptake was significantly inhibited. The effect of pH on the activity of the polyphosphate degrading enzyme polyphosphate:AMP phosphotransferase from *Acinetobacter* strain 210A has been investigated (van Groenestijn *et al.*, 1989b). The enzyme enables

Acinetobacter 210A to use polyphosphate as an energy reserve when other sources are not available. Enzyme activity was optimal at pH 8.5 with negligible activity at pH 6.2. When van Groenestijn and Deinema (1985) investigated the effect of pH in *Acinetobacter* strain 210A they found that the optimum pH for growth was 8.5, the minimum was 6.0 and the maximum was 9.5 and cell phosphate content was higher at lower pH values (~pH 6.2). In a later study, van Groenestijn *et al.* (1989a and 1989b) studied the effect of pH both on growth and polyphosphate accumulation in *Acinetobacter* strain 210A using three different carbon substrates, acetate, ethanol and lactate. An optimum pH for growth of pH 9.0 was determined when the strain was grown on acetate with no growth below pH 6.0. A broader optimum pH of around pH 5.5 - 9.0 was determined when the strain was grown on ethanol and lactate. Some growth was still observed at a pH of 4.7 on ethanol and all three substrates at pH 9.5. Above pH 6.5 phosphate accumulation was independent of the pH value. Significantly more phosphate was found in the biomass at pH 6.3 than at higher pH values when the strain was grown on acetate. Du Preez *et al.* (1983) also investigated the effect of pH on the growth rate of a pure culture of *Acinetobacter calcoaceticus*. They found an optimum pH for growth between 6.5 - 7.5 when the isolate was grown on ethanol, but when acetate was used as the carbon source a narrower range of 7.0 - 7.5 was observed, with a rapid decrease in growth rate below pH 7.0. When grown on ethanol, on the other hand, a reasonably high growth rate was observed down to pH 5.0. They suggested that this difference was due to the dissociation of acetate at low pH resulting in cell toxicity due to increased acid passing into the cells.

Fleit (1995) discusses the effect of VFA dissociation on the intracellular environment and how the cells regulate their pH in a paper evaluating the various EBPR process biochemical models. When VFAs such as acetate enter the cells an intracellular H^+ load arises as a result of VFA dissociation. pH and VFA are also considered by Wedi (1992), in an investigation on the effect of pH on VFA production for the EBPR process. At a sludge temperature of

23.5°C an optimum pH of 8.0 was observed for maximum VFA production. At pH 9.0 VFA production was significantly reduced with almost no production at pH 4.0. At lower sludge temperatures of 8.5 and 15°C VFA production increased steadily and in a similar manner as pH values rose from pH 4.0 to pH 9.0. However, maximum VFA production at the lower two temperatures was less than half the maximum achieved at 23.5°C and a pH of 8.0.

Controls were set up during this study to investigate whether phosphate removal could be attributed to chemical precipitation. The aerobic shake flask investigations indicated that at pH values of 9.5 and above some precipitation did occur. Streichan and Schön (1991) found that phosphate removal from a synthetic sewage medium containing no cells was due to chemical precipitation which only occurred at pH values greater than pH 8.3. Gerber *et al.* (1987a) even found the same phenomena at a pH of 7.0. Streichan and Schön (1991) suggested that differences in the synthetic sewages may account for this variation. Streichan *et al.* (1990) used pH as an identification criterion for polyphosphate accumulating bacteria in their studies. Phosphate removal from cultures growing at neutral pH was attributed to EBPR whilst at pH > 8.3 greater removal was observed but was due to chemical precipitation. Olson and Connell (1971) concluded that phosphate uptake as a result of calcium phosphate precipitation was negligible at pH levels below ~pH 8.0. The studies of Keurentjes and Iwema (1987) found that a biologically induced pH variation resulted in chemical phosphate removal. Levin and Shapiro (1965) however, concluded that in their studies phosphate removal was biological and not due to precipitation when they investigated the effect of pH on phosphate removal in batch experiments at pH values controlled at 5, 6, 7, 8 and 9. At the time they were actually investigating whether increased aeration encouraged phosphate precipitation due to CO₂ stripping and the associated rise in solution pH. They stated that the rate of phosphate removal should increase at higher pH values if the mechanism was precipitation, but they observed a lower rate at pH 9.0 than at pH 8.0.

A criticism of the methodology employed in this study may be drawn in that only the short term effects of different pH values on EBPR have been considered with no acclimatisation period to the set values (except at pH 7.0). However, the results of the investigation are in general agreement with several other published studies and an indication is given of the optimal and inhibitory pH values for anaerobic phosphate release and aerobic phosphate uptake. A suggestion for further work is that the EBPR process should be run in a longer-term study with pH controlled at 7.0 - 7.1 during the anaerobic phase and at 7.8 - 8.0 during the aerobic phase. The system should be allowed to acclimatise to the conditions, before investigating whether an increase in the overall phosphate removal rate can be achieved using the values obtained in the shake flask batch studies.

CHAPTER 8.

8. MIXED CULTURE NITRATE, NITRITE INVESTIGATIONS

8.1 INTRODUCTION

The presence of nitrate in the anaerobic phase of the EBPR process, is thought to be detrimental to overall phosphate removal, due to the establishment of anoxic as opposed to anaerobic conditions, which may interfere with the phosphate release associated with the anaerobic phase. Since the establishment of an anaerobic zone has been shown to be an essential prerequisite to successful EBPR, the consequence of potential nitrate interference may be very important. The extent of phosphate uptake during aerobic conditions has been correlated to the degree of phosphate released in the previous anaerobic phase (Barnard 1976; Nicholls and Osborn 1979; Marais *et al.* 1983). Therefore, the deleterious effect that the presence of nitrate may have on this release may be important when considering the overall phosphate removal efficiency of the process. The effect of the presence of nitrite on the other hand is much less well documented. Nitrite is fairly unstable, for example it is easily oxidised to nitrate, and consequently is usually present in wastewater at concentrations $< 1.0 \text{ mg l}^{-1}$. Nitrate, however, may be typically found in wastewater at concentrations up to around 20 mg l^{-1} (Metcalf and Eddy, 1991).

When the dissolved oxygen concentration of the wastewater is negligible, other electron acceptors such as nitrate, phosphate and sulphate may be utilised by the biomass, but the energy yielded from them is less than that with oxygen. After oxygen, nitrate is the most beneficial electron acceptor in terms of energy gain, the others are much less favourable (Barnes and Bliss, 1983). Since less energy is obtained by denitrification as opposed to oxygen respiration, nitrate only becomes an option as an electron acceptor when the dissolved oxygen

It is well established that phosphate is released from the biomass during anaerobic conditions and is taken up and stored as polyphosphate granules in the cells during aerobic conditions (Heymann *et al.*, 1989; Vasiliadis *et al.*, 1990; Jing *et al.*, 1992). Phosphate release and uptake is interrelated with the actions of electron donors i.e. organic substrates such as acetate. Under anaerobic conditions acetate is taken up by the biomass and stored as PHB, the metabolism of which results in phosphate release from the cells. Under subsequent aerobic conditions PHB is utilised by the cells, generating ATP which can be used for the re-synthesis of polyphosphate, resulting in phosphate uptake from the wastewater.

Several experimental investigations on the effect of nitrogen compounds on EBPR were undertaken in this study. The effect of anoxic (no oxygen, but presence of nitrate / nitrite) as opposed to anaerobic (no oxygen, no nitrate / nitrite) conditions on EBPR was studied; nitrate, nitrite and air were compared as electron acceptors; and the influence of the carbon source on nitrate effects was examined.

8.2 EXPERIMENTAL

8.2.1. Activated Sludge

Activated sludge, already acclimatised to an EBPR regime was obtained from Anglian Water's EBPR pilot plant at Milton Keynes and used as an inoculum for this study. A 5 l sample taken from the RAS line of the plant was taken to the laboratory within 30 minutes of sampling. The sludge was placed into the 10 l SBR and started on a programmed sequence of phase cycling as described in 2.2., starting with the feed phase. The suspended solids were manually adjusted from an original 6000 mg l⁻¹ down to a desired operating

concentration of around 2000-2500 mg l⁻¹. The system was fed with a synthetic sewage medium, as detailed in section 2.3., prepared at the appropriate concentration. The reactor operated at room temperature (20°C ± 3°C) and a MCRT of around 10 days. Good EBPR was observed in the SBR 5 days after commencement of the run. The sludge was allowed to acclimatise to the new conditions, in particular the synthetic sewage feed for 15 days before shake flask batch experiments were undertaken.

8.2.2. Determination of Typical Nitrate and Nitrite Process Profiles

The typical nitrate and nitrite plots for the process receiving the standard synthetic sewage feed were determined. These studies were undertaken after 14 d when 44 8 h cycles had been completed.

8.2.3. Shake Flask Batch Experiments

The presence of nitrate and nitrite in the anaerobic phase, resulting in the establishment of anoxic conditions was studied. Air, nitrate and nitrite were also investigated and compared for their effectiveness as electron acceptors. Several different carbon sources were studied for variations in nitrate effects. For all the tests, batch experiments were undertaken in 250 ml Erlenmeyer flasks placed in a shaking water bath (Grant SS40-5, Grant Instruments Ltd, Cambridge) with the temperature controlled at 17.5°C.

8.2.3.1. Effect of Nitrate in the Anaerobic Phase

This experiment was undertaken to investigate the effect of the presence of nitrate at various concentrations in the usually anaerobic phase of the process. A volume of EBPR sludge was removed from the 10 l SBR at the end of a feeding phase and placed in a flask. A N₂ line was introduced into the flask and a volume of sodium nitrate was added to give the desired initial nitrate

concentration of either 0, 0.2, 2.0, 5.0, 10.0, 25.0, 50.0 or 100 mg l⁻¹. The sludge was sparged with nitrogen for 2 h establishing anaerobic / anoxic conditions. Subsequently, the N₂ was switched off and the sludge was aerated for 5 h to investigate whether aerobic uptake in the ensuing phase was also affected.

8.2.3.2. Effect of Nitrite in the Anaerobic Phase

The effect of nitrite in the usual anaerobic phase was investigated in this study. The tests were undertaken as in 8.2.3.1., except that the sodium nitrate was replaced by sodium nitrite at the desired initial concentration.

8.2.3.3. Comparison of Nitrate, Nitrite and Air as Electron Acceptor

Nitrate, nitrite and air were compared as electron acceptors in this study. A volume of EBPR sludge was removed from the 10 l SBR at the end of a feeding phase and placed into flasks. The sludge was sparged with N₂ for 1 h establishing anaerobic conditions after which, either the N₂ was switched off and air was switched on or nitrate or nitrite were added with continued N₂ gassing.

8.2.3.4. Effect of Nitrate with Various Carbon Substrates

The effect of nitrate on the process using feeds containing different carbon sources was investigated. Acetate, glucose and methanol at 400 mg l⁻¹ (as COD - theoretically determined) were compared as process carbon sources under anaerobic and anoxic conditions. In addition, acetate at 200 and 100 mg l⁻¹ (as COD) was studied. The synthetic sewage feed was prepared at the desired concentration, replacing the usual sodium acetate with glucose and methanol when appropriate. A volume of sludge was removed from the 10 l SBR prior to a feeding phase and placed in flasks. The test feed solution was

added to the flask alongside a gas line. The sludge was sparged with N_2 for 1 h followed by 6 h aeration. For anoxic studies, nitrate was added with the feed solution. A nitrate concentration of 10 mg l^{-1} was selected - since this level might be found in typical wastewater.

For all investigations, samples were analysed for o-P, TP, TSS, COD, NO_3^- and NO_2^- . Redox potential and DO concentrations were monitored. The pH was also monitored in all investigations to ensure that pH levels were less than pH 9.0 (the level determined in 7.3.) above which precipitation may account for some phosphate removal. Investigations were carried out in duplicate for each test. Two controls were set up for each investigation. One control contained dead cells, the other no cells.

8.2.4. Analytical Methods

Ortho-phosphate, TP, TSS, NO_3^- -N, NO_2^- -N and COD were analysed as described in section 2.4. A slight excess of sulphamic acid was used to overcome nitrite interference of the determination of phosphate analytical method. pH was monitored using a Russell pH probe connected to a Corning pH meter, dissolved oxygen was measured using a portable Jenway probe and meter and redox potential was determined using a platinum electrode with a standard Calomel reference connected to a multimeter .

8.3. RESULTS

8.3.1. Determination of Typical Nitrate and Nitrite Plots of the Process

Nitrate and nitrite concentrations were monitored in the 10 l SBR during the acclimatisation period. Figure 63. shows typical nitrate and nitrite plots over several complete cycles after 14 days acclimatisation when 44 x 8 h cycles had been completed at $t = 0$.

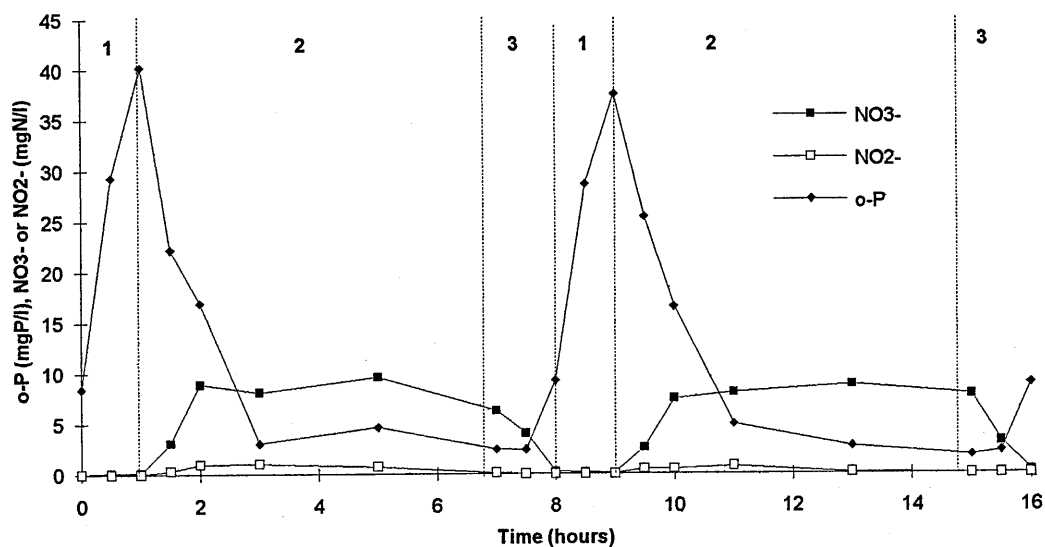


Figure 63. Typical phosphate, nitrate and nitrite profiles for anaerobic / aerobic cycling over two complete 8 h cycles. 1 - anaerobic phase, 2 - aerobic phase, 3 - harvesting, settle and feeding phases

The plot shows that a steady pattern with respect to phosphate release, phosphate uptake, nitrification and denitrification was well established by day 15 of the acclimatisation period. Good anaerobic phosphate release and aerobic phosphate uptake was observed, resulting in an overall phosphate removal of over 70 % in both cycles. The concentration of nitrate and nitrite was negligible during the anaerobic phase whereas nitrate was found in the aerobic phase (up to $\sim 10 \text{ mgN l}^{-1}$) as a result of nitrification of the ammonium chloride from the feed. Denitrification was observed during the harvesting and settle phases (3 in Figure 63.), reducing the nitrate, therefore resulting in a negligible carry-over into the anaerobic phase of the next cycle. Dissolved oxygen concentration was measured at $\sim 0.1 \text{ mg l}^{-1}$ during the anaerobic phase and $\sim 4.5\text{-}6.0 \text{ mg l}^{-1}$ during the aerobic phase. Redox potential was measured at $\sim -200 \text{ mV}$ during anaerobic conditions and up to $+150 \text{ mV}$ during aerobic conditions.

8.3.2. Shake Flask Batch Experiments

8.3.2.1. Effect of Nitrate in the Anaerobic Phase

The effect of nitrate on anaerobic phosphate release was investigated in this experiment. Several nitrate concentrations were studied, ranging from 0 to 100 mgN l⁻¹ (Figure 64.).

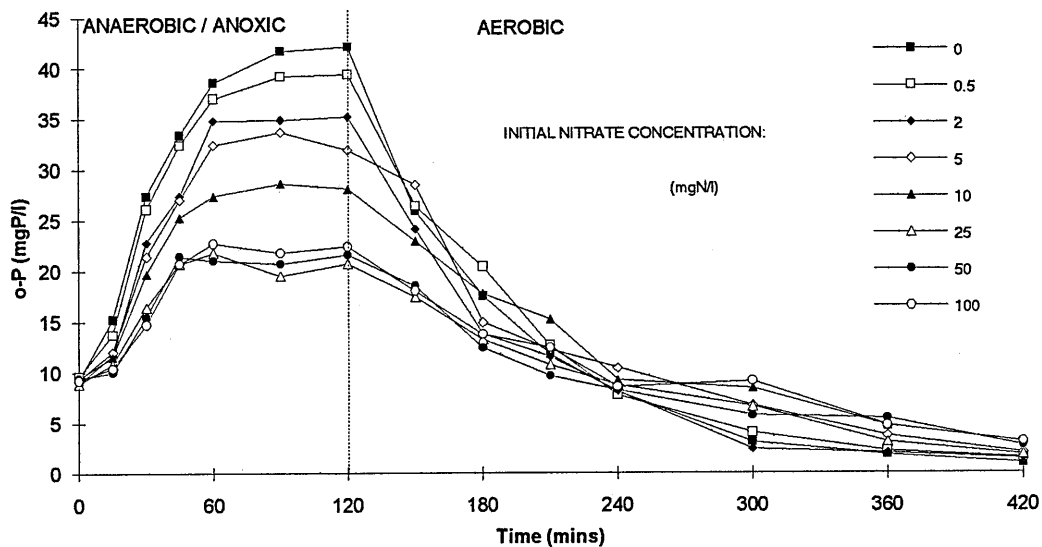


Figure 64. Effect of different initial nitrate concentrations on EBPR

The presence of nitrate, creating anoxic conditions, in the normally anaerobic phase did have some inhibitory effect on the process. The degree of inhibition was dependant on the concentration of nitrate added. At nitrate concentrations of 5 mgN l⁻¹ and under, anaerobic phosphate release was only slightly inhibited.

Denitrification almost completely reduced the nitrate within 30 minutes of the start of the experiment at these lower concentrations whereas nitrate was still present at the end of the 120 minute anaerobic / anoxic phase in the tests with an initial nitrate concentration of 25 mgN l⁻¹ and above (Figure 65.).

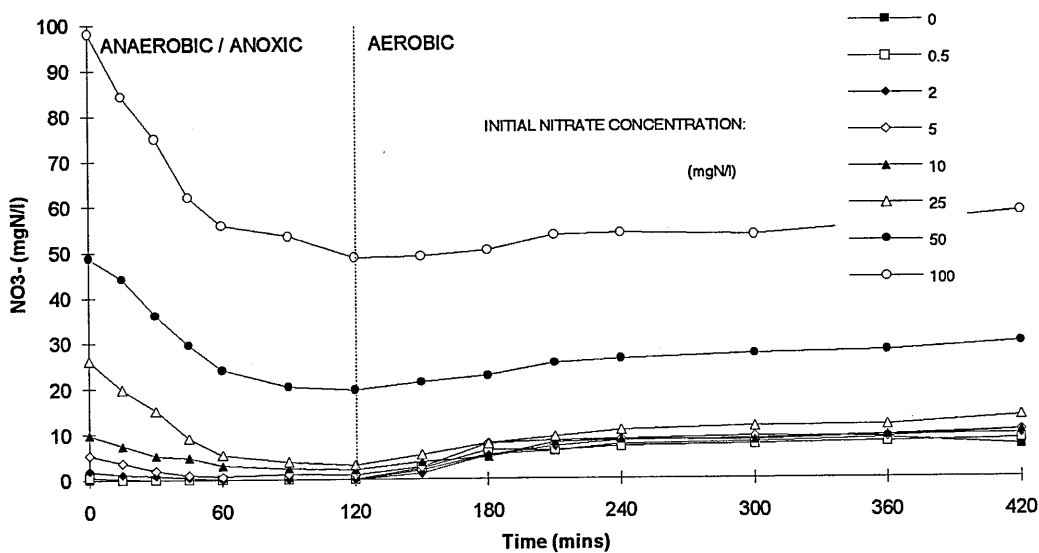


Figure 65. Denitrification and nitrification at different initial nitrate concentrations

At initial nitrate levels of 50 and 100 mgN l⁻¹ about half the initial concentration was still in solution at the end of the 120 minute anoxic phase. At these nitrate concentrations phosphate release stopped at around half the concentration of the maximum release achieved when no nitrate was added. During the following aerobic phase the nitrate concentration increased again as nitrification of the ammonium chloride in the feed occurred.

Phosphate release was observed to be affected by the initial nitrate concentration. Table 30. summarises the effects of the various nitrate concentrations.

Table 30. Effect of nitrate on phosphate release and uptake

Nitrate added (mgN l ⁻¹)	^a Initial P Transfer Rate - Anaerobic (mgP gTSS ⁻¹ , h)	^a Initial P Transfer Rate - Aerobic (mgP gTSS ⁻¹ , h)	ΔP (mgP l ⁻¹)	%P Removed
0.0	-12.5	10.7	8.25	90
0.5	-11.9	8.3	8.1	84
2.0	-11.5	9.5	7.5	84
5.0	-9.8	7.2	7.3	78
10.0	-7.8	4.5	6.3	67
25.0	-5.7	3.3	7.1	80
50.0	-5.4	4.3	6.7	71
100.0	-6.0	3.9	6.1	66

^a Negative values indicate phosphate release, positive values indicate phosphate uptake.

Phosphate transfer rate is defined as the concentration of phosphate uptake or release (in mg) per gram of cells during the initial hour of the particular anaerobic or aerobic phase. The amount of phosphate removed from the feed by the overall process is expressed as ΔP mgP l⁻¹ and the percentage removal is calculated as:

$$\frac{\text{Amount of phosphate removed}}{\text{Initial phosphate concentration}} \times 100\%$$

At nitrate concentrations below 5 mgN l^{-1} the process seemed little affected by nitrate addition when over 80% phosphate removal was observed. Generally, however, phosphate release and uptake rates were dependant on the initial nitrate concentration. The lower the initial nitrate concentration the greater the rate. A similar overall trend was observed for the concentration of phosphate removed from the wastewater after a single anaerobic / anoxic - aerobic cycle.

8.3.2.2. Effect of Nitrite in the Anaerobic Phase

The addition of nitrite to the normally anaerobic phase did not seem to significantly affect phosphate release, even at a concentration of 100 mgN l^{-1} (Figure 66.).

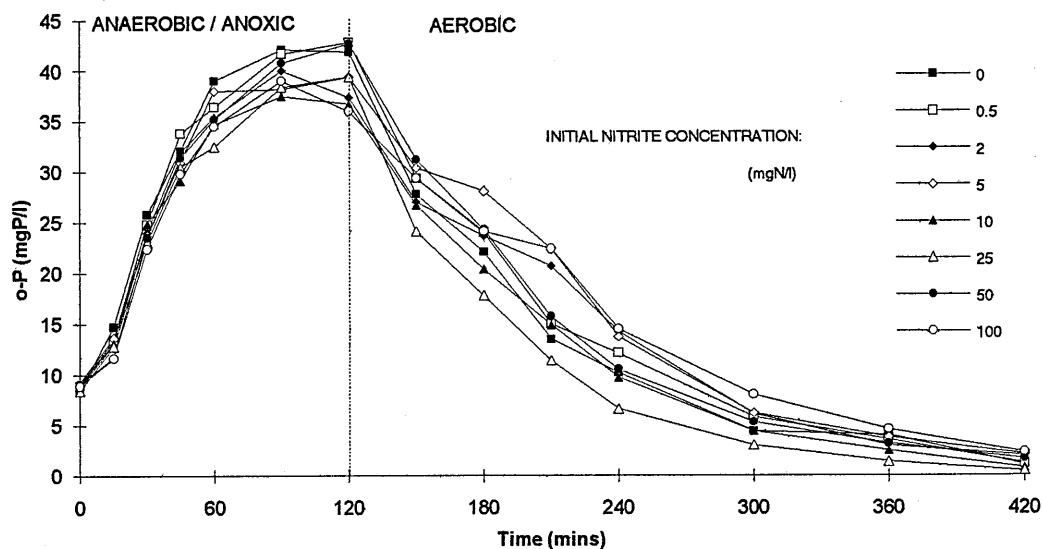


Figure 66. Effect of different initial nitrite concentrations on EBPR

This implies that very few bacteria can utilise nitrite, since the change in nitrite concentration during the study was small (Figure 67.).

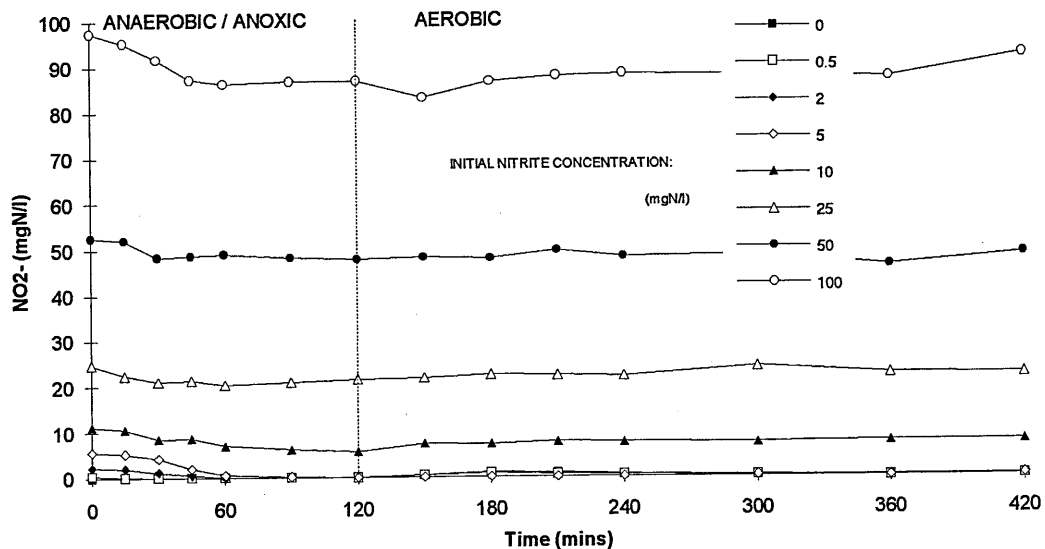


Figure 67. Change in nitrite concentration during anaerobic / anoxic and aerobic phases

Within 60 minutes of the start of the experiment, denitrification had reduced almost all the nitrite in the system when the initial nitrite concentrations were 5 mg N l⁻¹ and under. However, significant nitrite was still present at the end of the 120 minute anaerobic / aerobic phase in the tests with initial nitrite concentrations of 10 mgN l⁻¹ and above. At these higher concentrations, the amount of possible denitrification seemed to occur within the first 60 minutes.

The small difference in phosphate release rates between the lower and higher nitrite concentrations is shown in Table 31.

Table 31. Effect of nitrite on phosphate release and uptake

Nitrite added (mgN l ⁻¹)	^a Initial P Transfer Rate - Anaerobic (mgP gTSS ⁻¹ , h)	^a Initial P Transfer Rate - Aerobic (mgP gTSS ⁻¹ , h)	ΔP (mgP l ⁻¹)	%P Removed
0.0	-14.2	9.2	7.3	87
0.5	-12.5	8.5	7.7	86
2.0	-11.9	6.1	6.7	77
5.0	-13.1	5.1	7.1	77
10.0	-11.9	7.6	8.3	91
25.0	-11.5	10.5	7.9	94
50.0	-11.6	8.2	7.4	81
100.0	-11.6	5.4	6.6	74

^a Negative values indicate phosphate release, positive values indicate phosphate uptake.

The anaerobic / anoxic phosphate release rate was slightly greater at concentrations of 5 mgN l⁻¹ and under. At these values the nitrite concentration was negligible within 60 minutes of the start of the experiment. At nitrite concentrations of 10 mgN l⁻¹ a slightly lower anaerobic release rate was observed which did not decrease with further increase in initial nitrite concentration. At these concentrations nitrite was still abundant in the system. The level of nitrite addition seemed to have no effect on overall phosphate removal ability, since over 70% removal was observed in all the tests.

8.3.2.3. Comparison of Nitrate, Nitrite and Air as Electron Acceptor

Shake flask batch tests were undertaken to compare the effectiveness of nitrate, nitrite and air as electron acceptors in this experiment. Nitrate and nitrite were added at 10 and 25 mgN l⁻¹ (Figure 68.).

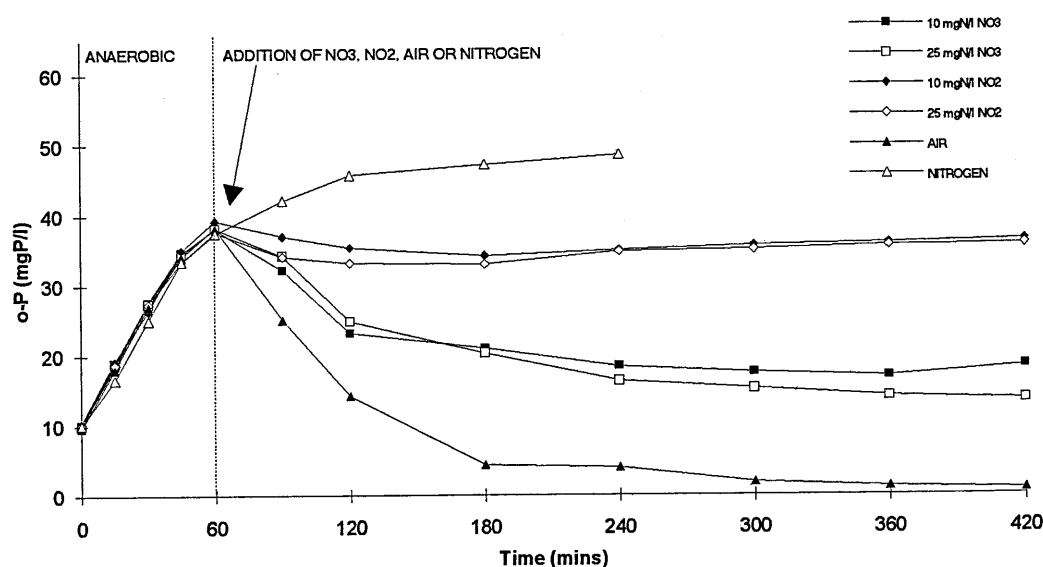


Figure 68. Phosphate release and uptake during comparison of nitrate, nitrite and air as electron acceptors

Air was by far the most effective as electron acceptor, followed by nitrate and nitrite respectively. With no addition of air, nitrate or nitrite the continuation of anaerobic conditions resulted in further phosphate release which levelled out after 120 minutes. The initial concentration of nitrate and nitrite did not seem to significantly affect phosphate removal. The behaviour of nitrate and nitrite was also monitored during the experiment (Figure 69.).

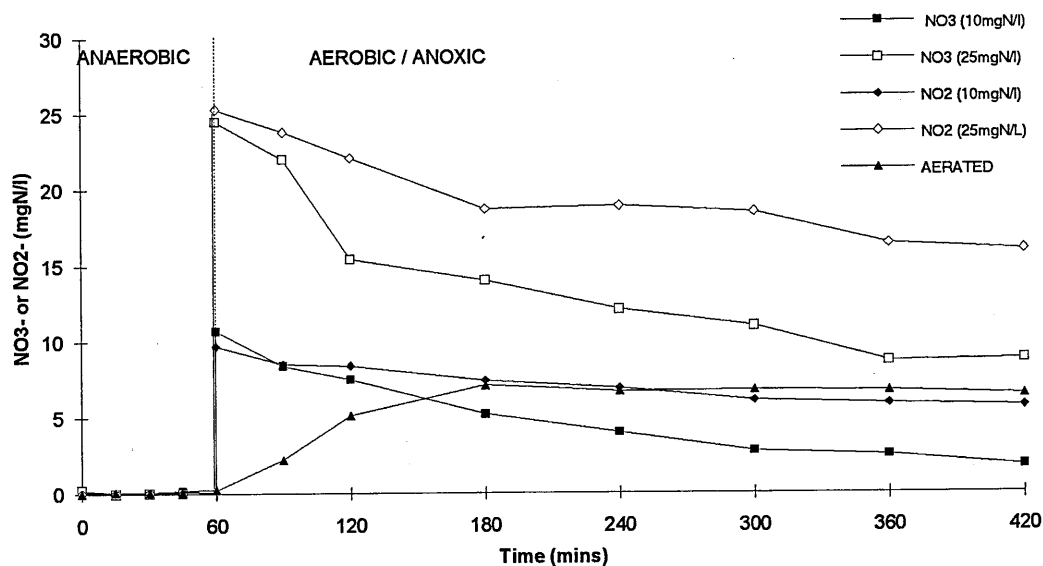


Figure 69. Nitrate and nitrite behaviour during comparison of nitrate, nitrite and air as electron acceptors

In Figure 69., nitrate concentration was followed in the nitrate and air tests whereas nitrite concentration was monitored in the nitrite tests.

Very little additional phosphate uptake was observed after 180 minutes (2 h after the addition of nitrate / nitrite) even though nitrate and nitrite were still present in the systems. Aeration resulted in over 90 % phosphate removal between the start and end of the aerobic phase removing 37 mgP l^{-1} , whereas nitrate removes 51-64 % and nitrite less than 10 % phosphate (Table 32.).

Table 32. Comparison of nitrate, nitrite and air as electron acceptor

Test	^a Initial P Transfer Rate - Anaerobic (mgP gTSS ⁻¹ , h)	^a Initial P Transfer Rate - Aerobic (mgP gTSS ⁻¹ , h)	ΔP (mgP l ⁻¹) - aerobic phase	%P Removed - aerobic phase
10 mgN l ⁻¹ nitrate	-12.4	6.6	19.6	51
25 mgN l ⁻¹ nitrate	-12.8	6.0	24.5	64
10 mgN l ⁻¹ nitrite	-13.1	1.7	2.8	7
25 mgN l ⁻¹ nitrite	-12.5	2.0	1.8	5
Aeration	-12.7	10.7	37.3	97

^a Negative values indicate phosphate release, positive values indicate phosphate uptake.

The initial aerobic phosphate uptake rate also follows a similar trend in that phosphate is taken up at a reduced rate with nitrate compared to air and further reduced with nitrite compared to air. The results indicate that nitrate is approximately half as effective as air as an electron acceptor whereas nitrite is only around 10 % as effective. No phosphate uptake was observed without the addition of nitrate, nitrite or aeration. Prolonged anaerobic conditions resulted in slightly further phosphate release which levelled out after 120 minutes.

8.3.2.4. Effect of Nitrate with Various Carbon Substrates

Shake flask batch investigations were undertaken to compare the effect of nitrate on acetate, glucose and methanol as the process carbon source. Additionally, acetate was investigated at a half and a quarter the normal feed strength (200 and 100 mg l⁻¹ as COD, as opposed to 400 mg l⁻¹). The carbon sources were initially assessed without nitrate addition (Figure 70.).

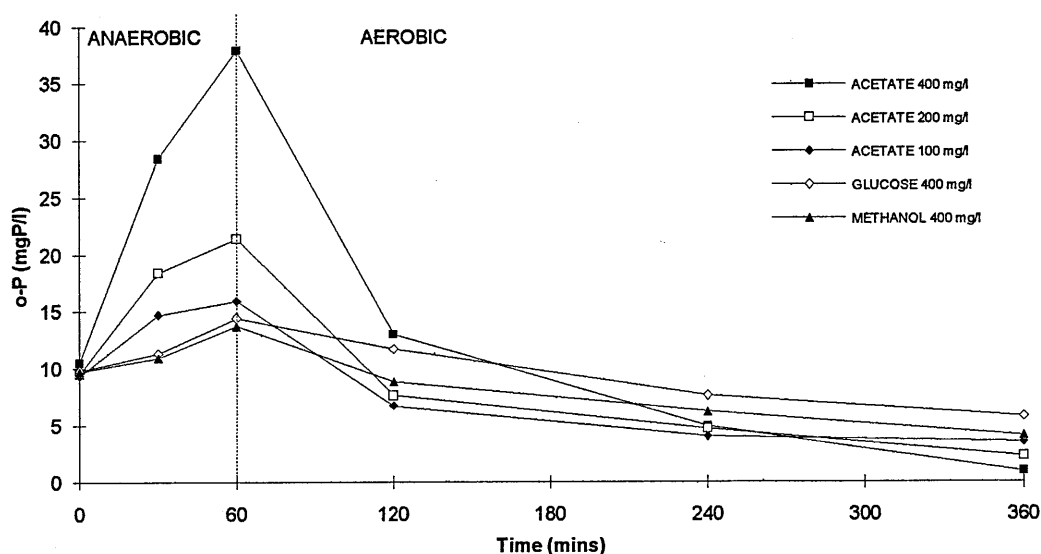


Figure 70. Comparison of acetate, glucose and methanol as carbon sources. With no nitrate addition

Acetate clearly seemed to be the preferred carbon source, resulting in the greatest anaerobic phosphate release and aerobic uptake. The concentration of acetate was also important, with better results at 400 mg l⁻¹ as opposed to 200 and 100 mg l⁻¹. Methanol and glucose performed very poorly in comparison.

A similar study was undertaken but with nitrate added, at a concentration of 10 mg l^{-1} at the start of the test (at $t = 0$) to create anoxic as opposed to anaerobic conditions (Figure 71.).

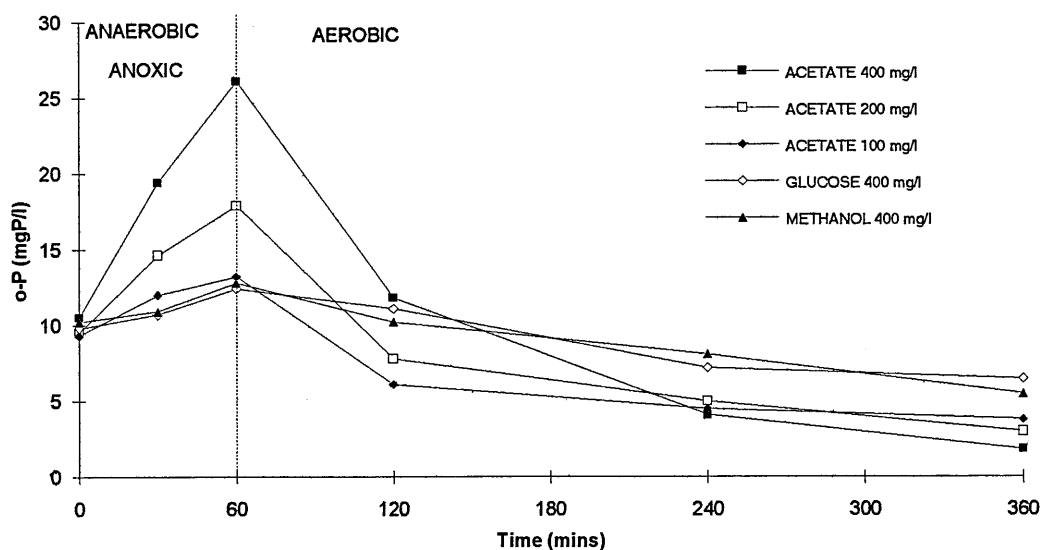


Figure 71. Comparison of acetate, glucose and methanol as carbon sources.
With nitrate addition at $t = 0$

Again, acetate resulted in the best phosphate release at the higher concentration. When the various carbon sources and acetate concentrations were compared for overall phosphate removal, acetate at the higher concentration of 400 mg l^{-1} without nitrate addition performed best and removed over 90 % of the initial phosphate concentration (Table 33.).

Table 33. Comparison of EBPR with acetate, glucose and methanol as carbon substrate, with and without the addition of nitrate

Test	^a Initial P Transfer Rate - Anaerobic (mgP gTSS ⁻¹ , h)	^a Initial P Transfer Rate - Aerobic (mgP gTSS ⁻¹ , h)	ΔP (mgP l ⁻¹)	%P Removed
400 mg l ⁻¹ acetate	-12.7	11.6	9.55	91
400 mg l ⁻¹ acetate + nitrate	-7.3	6.7	8.65	82
200 mg l ⁻¹ acetate	-5.7	6.6	7.2	76
200 mg l ⁻¹ acetate + nitrate	-3.9	4.7	6.5	68
100 mg l ⁻¹ acetate	-3.1	4.3	5.8	62
100 mg l ⁻¹ acetate + nitrate	-1.9	3.4	5.5	59
400 mg l ⁻¹ glucose	-2.1	1.2	4.0	41
400 mg l ⁻¹ glucose + nitrate	-1.2	0.6	3.3	34
400 mg l ⁻¹ methanol	-1.9	2.3	5.6	57
400 mg l ⁻¹ methanol + nitrate	-1.2	1.2	4.7	46

^a Negative values indicate phosphate release, positive values indicate phosphate uptake.

Whatever the carbon source, the addition of nitrate lowered the overall level of phosphate removed. Methanol seemed a slightly better carbon source for the process than glucose, but neither compared with acetate at a similar concentration. Both resulted in around half the phosphate removal of acetate.

8.4. DISCUSSION

Barnard (1976) first emphasised the importance of an anaerobic zone free of nitrates for successful EBPR. He found that the presence of nitrates hindered phosphate removal or even caused complete breakdown of the process and indicated that nitrate could act as an electron acceptor in the absence of oxygen impeding the establishment of anaerobic (as opposed to anoxic) conditions. In later work (Barnard, 1982), it was stipulated that the nitrate concentration should be well below 5 mgN l^{-1} to prevent process inhibition. Osborn and Nicholls (1978) also found that phosphate removal decreased in the presence of nitrate but that a nitrate concentration of 2 mgN l^{-1} seemed tolerable. The results of this study indicated that nitrate concentrations up to 5 mgN l^{-1} had very little deleterious effect on the overall process, whereas at concentrations of 50 and 100 mgN l^{-1} a drop in phosphate removal resulted, from levels over 80 % down to ~ 70 %. This lower level is still good EBPR - and the process did not breakdown. Several other reports have noted that when nitrates are present they should be denitrified so that an anaerobic zone does actually exist. Hashimoto and Furukawa (1984) found that when anoxic conditions (i.e. presence of nitrate) prevailed, phosphate release did not occur from their activated sludge. However, when the nitrate was completely removed by denitrification, phosphate release was observed. Comeau, *et al.* (1987a) also emphasised that nitrate in the anaerobic zone should be minimised and that return sludge should be denitrified. Lee *et al.* (1993) found that at concentrations greater than 2 mg l^{-1} , nitrate became inhibitory to phosphate release in a side stream BNR process.

Kuba *et al.* (1994) found that phosphate release was reduced with nitrate present. They partly attributed this to the presence of denitrifying bacteria, which consumed the carbon substrate, acetate, for denitrification as opposed to phosphate release. Takeuchi (1991) investigated the bacterial population dynamics in a conventional full scale activated sludge plant, with and without the inclusion of an anoxic zone receiving a high nitrate concentration wastewater. The study found that the addition of nitrate stimulated the growth of *Pseudomonas* spp., giving a competitive edge over *Acinetobacter* spp. under anoxic conditions. The failure of *Acinetobacter* spp. to dominate the anoxic zone may explain why phosphate removal was not significantly increased by the inclusion of a preliminary anoxic zone (presumably strictly anaerobic conditions were required). Conversely, upon the addition of acetate the advantage was reversed, stimulating *Acinetobacter* spp. growth over *Pseudomonas* spp.

The effect of nitrite on the process has not been previously reported in the literature. Results of this study indicate that the presence of nitrite at concentrations up to 100 mgN l^{-1} has very little effect on the EBPR process, which may suggest that very few microorganisms can use nitrite as a terminal electron acceptor - and those which can are probably not EBPR bacteria.

Whether or not nitrate can be used effectively as an electron acceptor in place of oxygen for EBPR may have important economic consequences. Results from this study indicate that nitrate can be used as an electron acceptor in place of oxygen (section 8.3.3.3.) but is less effective, and nitrite was shown to be much less effective than nitrate. McClintock *et al.* (1988) investigated and compared the growth and kinetic coefficients of two bench scale conventional activated sludge processes, one using oxygen, the other using nitrate as the terminal electron acceptor. They found that the maximum microbial yield coefficient and the endogenous decay coefficient were lower whilst the maximum substrate utilisation rate was higher for nitrate respiration

as opposed to oxygen respiration. They indicated that nitrate respiration may be an attractive economic option to conventional aerated activated sludge treatment due to lower sludge production leading to less costs involved in sludge handling and disposal in addition to the decrease in aeration energy costs. Vlekke *et al.* (1988) also investigated the possibility of using nitrate as the only electron acceptor for EBPR. They concluded that nitrate could be used, but suggested that it was not as efficient as oxygen for phosphate uptake. Comeau *et al.* (1987a) observed phosphate uptake in the presence of nitrate - suggesting that a proportion of the EBPR bacteria can use nitrate as an electron acceptor. Shin and Jun (1992) also investigated the feasibility of using nitrate in place of oxygen as the electron acceptor. They added nitrate (1000 mg l^{-1}) to their EBPR system at the end of the anaerobic phase instead of aerating, and continued sparging with nitrogen. Phosphate uptake was successfully achieved during this anoxic phase. However, when the system was compared with a normal aerated system run in parallel, phosphate uptake was lower in the anoxic process. Aeration reduced the phosphate concentration at the end of the anaerobic phase from around 50 mg l^{-1} to less than 1 mg l^{-1} whereas the anoxic system reduced a similar concentration down to around 15 mg l^{-1} . A study by Kerrn-Jespersen and Henze (1993) investigating anoxic and aerobic phosphate uptake, discovered that the phosphate accumulating bacteria could be separated into two groups. One group could use only oxygen as an electron acceptor whereas the other could use both oxygen and nitrate. A similar reasoning could explain why nitrate was less effective than oxygen in this study (section 8.3.3.3.). Simpkins and McLaren (1978); Hascoet *et al.* (1985); and Comeau *et al.* (1986) showed that nitrate could be used as an electron acceptor in place of dissolved oxygen for phosphate uptake. However, Arvin (1985) and Juni (1978) stated that *Acinetobacter* sp. should not be able to use nitrate as an electron acceptor. Lötter (1984) has shown however that this is not the case, *Acinetobacter* sp. can use nitrate in place of oxygen. When 100 *Acinetobacter* spp. isolates were tested for their ability to denitrify she found that 52 were able to reduce nitrate

to N_2 . Kuba *et al.* (1993) also investigated the possibility of using nitrate, instead of oxygen, as an electron acceptor in EBPR. Phosphate uptake was observed under anoxic conditions immediately upon inoculation from a Renpho EBPR plant. They stressed the need for careful nitrate addition to avoid re-release of phosphate as nitrate levels decreased and suggested measurement of the redox potential to monitor nitrate concentrations.

Some studies have concluded that phosphate release does not depend on the establishment of anaerobic conditions as such, but rather on the type and concentration of organic substrate (Deinema *et al.*, 1985; Gerber *et al.*, 1986; Gerber *et al.*, 1987b). Anaerobic conditions may only be essential for the EBPR process to allow the production of VFA's (such as acetate) by facultative anaerobic bacteria (such as *Aeromonas* spp.). Acetate has been found to be the preferred carbon substrate for EBPR (Wentzel *et al.*, 1988) and phosphate accumulating organisms such as *Acinetobacter* spp., which may explain why the presence of nitrate, at 10 mg l^{-1} , did not inhibit phosphate release at initial acetate concentrations of 400 mg l^{-1} in 8.3.3.4. Other carbon sources such as propionate, valerate and butyrate can be used, but are not as efficient in their conversion to PHB or PHV (Ghekiere *et al.*, 1991). This study compared acetate, glucose and methanol as a carbon substrate. Acetate, at 400 mg l^{-1} , with and without nitrate addition gave the best overall phosphate removal. Gerber *et al.* (1987b) also showed that phosphate release is influenced by the carbon substrate as opposed to the establishment of strictly anaerobic conditions. For instance, under anoxic conditions, acetate and propionate instigated phosphate release whereas methanol and glucose did not. Phosphate release was only observed with glucose and methanol after the onset of anaerobic conditions. When Hascoet and Florentz (1985) studied the influence of nitrates on EBPR they found that their system's nitrate concentration tolerance for good phosphate release depended on the COD concentration in the influent wastewater. They fed their system with a meat extract synthetic wastewater, which during storage resulted in some acetate

formation. At an initial COD of 400 mg l⁻¹, phosphate release was observed under anoxic conditions even at an initial nitrate concentration of 50 mgN l⁻¹. When the initial COD concentration was lowered to 300 mg l⁻¹ and 250 mg l⁻¹, phosphate release was only maintained with an initial nitrate concentration of 5 mgN l⁻¹ and under. However, at an initial COD concentration of 200 mg l⁻¹, phosphate release was only observed when no nitrate was present, and even then the release was less than at the higher initial COD concentrations. Whatever the initial nitrate and COD concentrations, good phosphate uptake was observed in the subsequent aerobic phase. Iwema and Meunier (1985) studied the competition between phosphate removing and denitrifying bacteria for acetic acid substrate. They showed that the phosphate accumulators could effectively compete with the denitrifiers for acetic acid even at low substrate concentration. Also, as long as acetic acid was present phosphate release was observed even at a nitrate concentration of 40 mgN l⁻¹. However, when all the acetate was utilised, phosphate release stopped, and if nitrate was present phosphate uptake was observed suggesting that nitrate may be used as an alternative electron acceptor. Ghekiere *et al.* (1991) concluded that an anaerobic zone devoid of nitrate is not really necessary for good EBPR and phosphate release and uptake was shown to occur simultaneously alongside denitrification. They found that nitrates become inhibitory as a result of competition between fermentative organisms and denitrifiers for organic substrates, when the anoxic conditions favour the denitrifiers. Gerber *et al.* (1986) found that phosphate release was not inhibited by an initial nitrate concentration of 10 mgN l⁻¹ with acetate, propionate or formate as carbon source. On the other hand, with glucose or methanol, negligible phosphate release was observed with the same initial nitrate concentration. However, as soon as the acetate was used up, nitrate became inhibitory, since phosphate release stopped and phosphate uptake commenced, but as soon as all the nitrate was used up, phosphate release was again observed.

The results of this study indicate that at adequate acetate concentrations, nitrate is not inhibitory to EBPR. However, in full-scale EBPR plants without direct acetate addition, nitrates may inhibit the overall process since the desired carbon source may not be produced by fermentation due to the lack of anaerobic conditions. Siebritz *et al.* (1983) proposed an EBPR process configuration, called a UCT (University of Cape Town) process to screen the anaerobic reactor from nitrate in the RAS lines. They diverted the settling tank underflow recycle in addition to the aerobic tank recycle to an anoxic tank from which they added a recycle line back to the anaerobic tank. This configuration ensured negligible amounts of nitrate entered the anaerobic reactor via effluent recycle lines. Good plant design and an adequate acetate supply (either produced by fermentation or dosed) can ensure that the presence of nitrate is not inhibitory to the EBPR process.

CHAPTER 9.

9. GENERAL DISCUSSION

The removal of phosphate from wastewater, rather than limiting the use of phosphates in detergents (the debate of the late 1980s), has been confirmed as the best economic and environmental option to reduce and eliminate water pollution problems due to eutrophication (Morse *et al.*, 1993). Legislation, in the form of the urban wastewater treatment directive (CEC, 1991) directly affects the UK, and has recently been introduced in an attempt to protect surface waters from inadequately treated wastewaters. In this current study, various aspects of the EBPR process were investigated and mixed cultures operating under different experimental conditions repeatedly met the directive standards.

In order to achieve the objectives outlined in Chapter 1. it was necessary to design a reliable system capable of exhibiting EBPR. The use of SBRs proved to be a good choice, since they offered considerable flexibility in operation. SBRs have also been used in several other laboratory and pilot-scale EBPR studies (Shin and Jun, 1992; Jing *et al.*, 1992; Okada *et al.*, 1992). Indeed, SBRs have been shown to be effective at full-scale, in small to medium sized municipal wastewater treatment plants (Melcer *et al.*, 1987; Irvine *et al.*, 1987; Rusten and Eliassen, 1993).

Excellent EBPR has been demonstrated in mixed culture investigations in this study under various conditions. For instance, phosphate removal as high as 97% was observed in one investigation, whilst in general phosphate removals > 80% were achieved. These high values compare favourably with many observations. For instance, Malnou *et al.* (1984) observed 77% phosphate removal with their Phoredox pilot-scale plant whilst Nicholls and Osborn (1979) observed 79% phosphate removal with a Modified Phoredox pilot-scale plant.

However, significant phosphate removal typical of an EBPR process was not observed with any of the 6 *Acinetobacter* spp. isolates investigated in pure culture. With the benefit of hindsight, this is not judged as a failure, rather an affirmation of the observations of other more recent studies. Since the start of this particular study, the evidence against *Acinetobacter* spp. being the major bacterial group responsible for EBPR has steadily accumulated. Advances in the development of *in situ* identification probes have played a significant role in gathering this evidence. These molecular-specific probes are ideal tools for investigating and characterising mixed bacterial populations such as activated sludge and have been used to estimate the role of *Acinetobacter* spp. in EBPR plants.

Wagner *et al.* (1993) first used group-specific oligonucleotide probes to investigate the microbial population of activated sludge *in situ*. These fluorescent rRNA targeted oligonucleotide probes have also been used to identify previously uncultured bacteria (Amann *et al.*, 1991). Wagner *et al.* (1993) investigated the activated sludge from the aeration zones of a large municipal wastewater treatment plant (München II, Gut Marienhof, Germany) using both conventional culture- dependent and *in situ* oligonucleotide probing techniques. Their studies concluded that *in situ* techniques improved the accuracy of bacterial quantification in activated sludge since culture-dependent methods produced biased results, favouring the growth of gamma-subclass proteobacteria such as enterobacteria whilst suppressing the beta-subclass proteobacteria.

A later study by the same group characterised the *in situ* microbial community of municipal wastewater treatment plants using oligonucleotide probes (Wagner *et al.*, 1994a; Wagner *et al.*, 1994b). Their investigations showed that bacteria other than *Acinetobacter* spp. must be responsible for EBPR in at least one of the plants (Hirblingen, Germany). In another plant showing efficient EBPR (Berlin-Ruhleben, Germany), preliminary studies showed that

Acinetobacter spp. comprised much less than 10% of the microbial population in both the anaerobic and aerobic reactors. Gram positive bacteria with a high G+C DNA content dominated the population of the Hirblingen plant. These bacteria also contained polyphosphate inclusions which indicate that they may play an important role in EBPR. Their study concluded that the possible role of *Acinetobacter* spp. in EBPR could only be of a secondary nature.

Wedi *et al.* (1995) confirmed the probability that *Acinetobacter* spp. play only a minor role in EBPR when they investigated the Hirblingen and Berlin-Ruhleben municipal wastewater treatment plants with group and genus specific fluorescently labelled rRNA targeted oligonucleotide probes. They reiterated earlier observations that Gram positive bacteria and the beta-subclass of *Proteobacteria* seemed to influence the EBPR process. They also noted that established EBPR biochemical models need to consider these bacterial groups and they refer that pure culture *Acinetobacter* spp. studies claiming EBPR need to be checked.

In addition to oligonucleotide probes, several other *in situ* techniques have been used to study the role of *Acinetobacter* spp. in EBPR. Hiraishi *et al.* (1989) and Hiraishi and Morishima (1990) investigated the bacterial population structure, in particular the distribution of *Acinetobacter* spp., of an anaerobic / aerobic EBPR system alongside a conventional aerobic activated sludge system, using respiratory quinone profiles. *Acinetobacter* spp. contain ubiquinone as the sole quinone, with Q-9 predominant (Collins and Jones, 1981). They concluded that Q-9 was only a minor component of both activated sludge systems, with Q-8 dominant followed by Q-10 and MK-7. When they isolated and identified bacteria grown on an acetate medium they failed to find any *Acinetobacter* spp. They mainly found Q-8 type bacteria such as certain *Pseudomonas* (non-typical) and *Comamonas* strains. Q-10 isolates were mainly *Paracoccus* whilst MK-7 isolates were mainly members of the *Flavobacterium-Cytophaga* complex. They concluded that EBPR does not

depend on the presence of *Acinetobacter* spp. and that many other activated sludge bacteria may be involved. They also mentioned the possibility of a symbiotic interrelationship amongst these bacteria.

Auling *et al.* (1991) investigated the microbial population of several full-scale and pilot-scale EBPR plants treating municipal wastewater using diaminopropane (DAP) as a biomarker. They proposed DAP as an excellent biomarker for *Acinetobacter* spp. since it generally constitutes around 90% of the total polyamines of the genus and it is absent in many non-*Acinetobacter* spp., thereby offering itself as a suitable tool for *in situ* *Acinetobacter* spp. enumeration. In contrast to Hiraishi *et al.* (1989), they found that *Acinetobacter* spp. constituted a major proportion of the microbial population in pilot plants with low organic loadings and nitrification and denitrification steps. However, in full-scale plants with high organic loading and without nitrification and denitrification steps, *Acinetobacter* spp. constituted only a minor fraction of the microbial population. In these plants they suggested that the dominant organisms were authentic pseudomonads, followed by the beta-subclass of *Proteobacteria*, the alpha-subclass of *Proteobacteria* and the *Flavobacterium-Cytophaga* complex.

Cloete and Steyn (1988b) estimated that *Acinetobacter* spp. enumerated *in situ* by a combined membrane filter-immunofluorescent technique were < 10% of the *Acinetobacter* spp. isolated from the same sample by a culture-dependent method and identified by the API 20E kit. They suggested that the numbers and hence role of *Acinetobacter* spp. in EBPR had probably previously been overestimated.

In addition, Manz *et al.* (1994) investigated the microbial population of a conventional full-scale activated sludge plant treating municipal wastewater and a full-scale SBR treating dairy wastewater using a combination of *in situ* techniques; DAPI staining and group-specific oligonucleotide probing. They

compared the findings of these techniques with culture dependent methods. The gamma-subclass of *Proteobacteria* (e.g. *Acinetobacter* spp.) was significantly overestimated with the culture dependent methodology.

Traditionally, however, culture-dependent techniques have been used to isolate, enumerate and identify EBPR bacteria. These culture-dependent methods are now known to significantly misrepresent the true microbial population composition of activated sludge. For instance, floc disruption methods such as sonication may result in non-viable, completely randomly distributed single cells within a sludge sample (Banks and Walker, 1977). Culture medium design and growth conditions are also important considerations, since no one particular medium will allow the growth of all activated sludge bacteria. Bacteria isolated by culture-dependent methods may therefore only represent a very small fraction of the actual bacterial population in the original sample (Prakasam and Dondero, 1967; Reasoner and Geldreich, 1985). Further progress in the development of *in situ* probing techniques, along with their more widespread utilisation, can avoid the biases associated with traditional culture-dependent methods which result in both over and under estimation of certain microbial species.

Using culture-dependent techniques, Kavanaugh and Randall (1994) observed that bacteria other than *Acinetobacter* spp., possibly *Aeromonas* / *Vibrio*, coliforms and *Pseudomonas* spp., were also involved in phosphate removal from their pilot-scale UCT configuration EBPR plant. They also suggested that *Pseudomonas*, *Aeromonas*, coliforms and *Acinetobacter* spp. may additionally be involved in denitrification.

Ubukata (1994) observed EBPR in pure culture with a Gram positive bacterium isolated from activated sludge. Interestingly, the isolate demonstrated true EBPR behaviour i.e. phosphate release was observed under anaerobic

conditions in addition to phosphate uptake under aerobic conditions. No indication was given as to the isolate's identification however.

In contrast to other recent studies, one study investigating the microbial population of a Modified UCT configuration full-scale plant during its start-up, did find significant numbers of *Acinetobacter* spp. in their system (Knight *et al.* (1995b). They also mentioned the shortcomings of their culture-dependent methodology and emphasised the necessity for the development and use of *in situ* molecular probes. Interestingly, they also detected significant numbers of *Aeromonas caviae* in the microbial population of the plant.

The vast majority of the most recent EBPR population studies support the findings of this current study. EBPR does not seem to result from the direct actions of *Acinetobacter* spp. in activated sludge. Other bacterial species must play important roles in the process. This study does not question however, that *Acinetobacter* spp. may play a secondary role in the process.

Bioaugmentation was attempted in this study in an effort to improve phosphate removal by an *Acinetobacter* sp. isolate which was also studied in pure culture. The bioaugmentation of the *Acinetobacter* sp. isolate with an *Aeromonas caviae* (NCIMB 9235) isolate seemed to improve overall phosphate removal. The co-culture system improved maximum phosphate removal from 36% removal in the *Acinetobacter* sp. mono-culture system to 56% removal in the co-culture system. In theory, the *Aeromonas* isolate was employed in the co-culture system to convert glucose substrate to acetate, thereby providing a suitable carbon substrate for the *Acinetobacter* sp. isolate to undertake EBPR. The improvement in phosphate removal by the co-culture system could not be easily explained however, since an adequate concentration of acetate was already provided in the synthetic sewage feed in the *Acinetobacter* sp. pure culture investigations. Some other, unknown factor may be responsible for the improved phosphate removal in this co-culture system.

Apart from bioaugmentation, theoretically, genetic engineering of certain strains may offer improvements in the phosphate removal ability of activated sludge cultures. Kato *et al.* (1993) and Ohtake *et al.* (1994) reported on the genetic improvement of *Escherichia coli* MV1184 for EBPR from wastewater. They attempted to enhance its phosphate accumulating ability by manipulating the genes involved in the transport and metabolism of inorganic phosphate. Various recombinant strains could remove two to three times more phosphate from a minimal medium than the control strain. One recombinant could remove ten times more phosphate than the control strain, reaching a maximum phosphorus content of 16% on a dry weight basis (49% as phosphate) with 65% of the cellular phosphorus estimated to be stored as polyphosphate. Typical phosphate content of activated sludge is 1-2% on a dry weight basis (Fuhs and Chen, 1975; Ohtake *et al.*, 1985) and *Acinetobacter calcoaceticus* has been shown to accumulate 5.3% phosphorus on a dry weight basis (16% as phosphate) (Ohtake *et al.*, 1985) whereas bacteria isolated from an EBPR process have been shown to accumulate 10% phosphorus on a dry weight basis (Deinema *et al.*, 1980). Genetic strain improvement could therefore, in theory, be used to improve EBPR process performance.

South Africa, North America, Scandinavia and Germany lead the world in EBPR technology. This is mainly as a consequence of the earlier introduction of phosphorus discharge standards in these countries, as far back as 1980 in some cases. In the UK, implementation of the European Community urban wastewater treatment directive (CEC, 1991) will result in the requirement for some wastewater treatment plants to remove phosphorus for the first time, possibly requiring extensive retrofitting of existing plants. As a result, several environmental parameters were investigated for their effect on the EBPR process, keeping in mind typical UK wastewater characteristics and operating conditions. An excellent account of the current state of EBPR in the UK has been presented by Cooper *et al.* (1995).

As Vlekke *et al.* (1988) and Kuba *et al.* (1993) suggested, microbiologically there is no theoretical reason why nitrate cannot be used as electron acceptor by EBPR bacteria, thereby offering a potential saving on aeration costs. This present study verified this observation, as long as sufficient VFAs (acetate) are present in the wastewater. Furthermore, this study showed that nitrite could also act as sole electron acceptor. This is in disagreement with the findings of Comeau *et al.* (1987b), the only other reference to nitrite within this context reported in the literature. They stated that nitrite did not act as an electron acceptor in their process.

A greater emphasis will be placed on EBPR in the UK in the future, since the number of 'sensitive' areas as classified under the EC urban wastewater treatment directive (CEC, 1991) are likely to increase. This study has indicated that phosphate removal can be undertaken satisfactorily under typical UK conditions.

CHAPTER 10.

10. CONCLUSIONS

1. Using culture enrichment and selective medium techniques, *Acinetobacter* spp. could be easily isolated from several sources: a freeze-dried sample of activated sludge originating from the Northern Wastewater Treatment Plant, Johannesburg; an activated sludge sample from a conventional wastewater treatment plant in Milton Keynes; and an activated sludge sample from an EBPR pilot plant in Milton Keynes.
2. Enhanced biological phosphate removal was not observed with any of the six *Acinetobacter* sp. isolates fed with an acetate based synthetic sewage feed. Aerobic phosphate uptake was observed, whereas anaerobic phosphate release was not.
3. When *Aeromonas caviae* (NCIMB 9235) and *Acinetobacter* sp. SAB (originating from the Northern Wastewater Treatment Plant, Johannesburg), were grown as a co-culture, improved phosphate removal was observed compared to when the isolates were cultured separately, suggesting an advantageous relationship between the two. Under anaerobic conditions the glucose feed was shown to be converted to acetate (presumably by the *Aeromonas*), which was utilised as a carbon substrate by the *Acinetobacter* sp. isolate.
4. Temperature had a significant effect on the anaerobic phosphate release and aerobic phosphate uptake ability of a EBPR activated sludge. Optimal wastewater temperature for both anaerobic release and aerobic uptake of phosphate was around 30°C. EBPR was still observed

at the extremes of 5°C and 40°C, but at a reduced rate. Activation energies ranging from 34.22 kJ mol⁻¹ to 41.08 kJ mol⁻¹ were determined depending on conditions.

5. In batch studies, pH affected anaerobic phosphate release and aerobic phosphate uptake at different pH values. Optimum aerobic phosphate uptake was observed at pH 7.0 - 7.5, whereas inhibition was detected at pH values > 8.8 and < 6.0. Two optimal peaks were observed for anaerobic phosphate release; one between pH 5.5 - 6.0, the other between pH 7.8 - 9.0 and inhibition was found at pH values > 10.5 and < 5.5.
6. Nitrate concentrations up to 5 mgN l⁻¹ had negligible deleterious effects on EBPR and concentrations up to 100 mgN l⁻¹ still resulted in ~ 70 % phosphate removal.
7. Nitrite concentrations up to 100 mgN l⁻¹ had negligible effect on EBPR.
8. Air, nitrate and nitrite were compared as terminal electron acceptors. Air was the most effective (removing over 90% phosphate), followed by nitrate (removing 51-64% phosphate) and then nitrite (removing <10% phosphate). No phosphate uptake was observed without the addition of one of the above.
9. Acetate at 400 mg l⁻¹ (as COD) was the preferred carbon substrate for EBPR and the presence of nitrate had very little effect on the process at this substrate concentration. Glucose and methanol performed poorly in comparison.

CHAPTER 11.

11. RECOMMENDATIONS

The following is a list of potential topics for further work:

1. The composition of the bacterial population of the various EBPR sludges should be investigated further. This could be undertaken by randomly selecting and identifying 100 colonies from each sludge source. (In this study only isolates suspected of being *Acinetobacter* spp. were identified). Furthermore, a media comparison could be undertaken by isolating and identifying colonies both on nutrient and acetate agar, and determining which species tended to be isolated on each media. This may result in indicating the predominance of certain species other than *Acinetobacter* spp. in the EBPR sludges.
2. Investigating and determining the optimum mean cell residence time for EBPR under UK conditions is a possible area of study.
3. The process could additionally be investigated using real (non-acetate supplemented) as opposed to synthetic wastewater, particularly the effect of the presence of nitrate in the anaerobic zone (thus establishing anoxic conditions).
4. VFA production in a separate prefermentation process could be investigated. This would ensure an adequate supply of VFAs irrespective of real wastewater influent flow and strength.
5. The presence of nitrate in such a VFA producing anaerobic fermentation zone could be established.

REFERENCES

- Abbott, B.J., Laskin, A.I. and McCoy, C.J. (1973) Growth of *Acinetobacter calcoaceticus* on ethanol. *Appl. Microbiol.* 25, 787-792.
- Abu-ghararah, Z.H. and Randall, C.W. (1991) The effect of organic compounds on biological phosphorus removal. *Water Sci. Technol.* 23, 1/3, 585-594.
- Amann, R., Springer, N., Ludwig, W., Görtz, H.-D. and Schleifer, K.-H. (1991) Identification *in situ* and phylogeny of uncultured bacterial endosymbionts. *Nature* 351, 161-164.
- APHA (American Public Health Association), AWWA (American Water Works Association) and WPCF (Water Pollution Control Federation). (1989) In: *Standard Methods for the Examination of Water and Wastewater*. (Clesceri, L.S., Greenberg, A.E. and Trussell, P.R., eds.) 17th Edition. APHA, Washington DC, USA.
- Appeldoorn, K.J., Boom, A.J., Kortstee, G.J.J. and Zehnder, A.J.B. (1992) Contribution of precipitated phosphates and acid-soluble polyphosphate to enhanced biological phosphate removal. *Water Res.* 26, 937-943.
- Arora, M.L., Barth, E.F. and Umphres, M.B. (1985) Technology evaluation of sequencing batch reactors. *J. Water Pollut. Control Fed.* 57, 867-875.
- Artola, C.G., Pareja, B.L. and Garcia, P.G. (1995) Impact on hydrology and nutrient movements of developments in river basins draining into reservoirs. *Water Res.* 29, 601-609.
- Arvin, E. (1983) Observations supporting phosphate removal by biologically mediated chemical precipitation - a review. *Water Sci. Technol.* 15, 3/4, 43-63.

Arvin, E. (1985) Biological removal of phosphorus from wastewater. *CRC Crit. Rev. Environ. Control* 15, 1, 25-64.

Arvin, E. and Kristensen, G.H. (1985) Exchange of organics, phosphate and cations between sludge and water in biological phosphorus and nitrogen removal processes. *Water Sci. Technol.* 17, 2/3, 147-162.

Auling, G., Pilz, F., Busse, H-J., Karrasch, S., Streichan, M. and Schön, G. (1991) Analysis of the polyphosphate-accumulating microflora in phosphorus-eliminating, anaerobic-aerobic activated sludge systems by using diaminopropane as a biomarker for rapid estimation of *Acinetobacter* spp. *Appl. Environ. Microbiol.* 57, 3585-3592.

Banks, C.J. and Walker, I. (1976) Sonication of activated sludge flocs and the recovery of their bacteria on solid media. *J. Gen. Microbiol.* 98, 363-368.

Bark, K., Sponner, A., Kämpfer, P., Grund, S. and Dott, W. (1992) Differences in polyphosphate accumulation and phosphate adsorption by *Acinetobacter* isolates from wastewater producing polyphosphate: AMP phosphotransferase. *Water Res.* 26, 1379-1388.

Barnard, J.L. (1974) Cut P and N without chemicals. *Water and Wastes Eng.* 11, 1, 33-36 and 11, 2, 41-44.

Barnard, J.L. (1976) A review of biological phosphorus removal in the activated sludge process. *Water S.A.* 2, 3, 136-144.

Barnard, J.L. (1982) The influence of nitrogen on phosphorus removal in the activated sludge process. *Water Sci. Technol.* 14, 1/2, 31-45.

Barnard, J.L. (1984) Activated primary tanks for phosphate removal. *Water S.A.* 10, 3, 121-126.

Barnard, J.L. (1994) Alternative prefermentation systems. In: *Proceedings of the Conference Seminar on Use of Fermentation to Enhance Biological Nutrient Removal. 67th Annual Water Environment Federation Conference & Exposition*. Chicago, Illinois. October 15. Water Environment Federation, Alexandria, VA, USA. 13-21.

Barnes, D, and Bliss, P.J. (1983) *Biological Control of Nitrogen in Wastewater Treatment*. E.& F.N. Spon Ltd, London.

Baumann, P. (1968) Isolation of *Acinetobacter* from soil and water. *J. Bacteriol.* 96, 39-42.

Baumann, P., Doudoroff, M. and Stanier, R.Y. (1968) A study of the Moraxella group. II Oxidase-negative species (Genus *Acinetobacter*). *J. Bacteriol.* 95, 1520-1541.

Bayly, R.C., May, J.W., Raper, W.G.C., Duncan, A., Pilkington, N.H. and Vasiliadis, G.E. (1989) The effect of primary fermentation on biological nutrient removal. In: *Proceedings of the 13th Federal Convention of Australian Water and Wastewater Association*. Canberra, Australia. 6-10 March. 162-166.

Bayly, R.C., May, J.W., Duncan, A., Vasiliadis, G. and Schembri, M. (1990) Physiology of polyphosphate accumulating *Acinetobacter* strains. In: *Proceedings of the 1st Australian Conference on BPR from Sewage*. Bendigo, Victoria, Australia. 107-115.

Bayly, R.C., Duncan, A., May, J.W., Schembri, M., Semertjis, A., Vasiliadis, G. and Raper, W.G.C. (1991) Microbiological and genetic aspects of the synthesis of polyphosphate by species of *Acinetobacter*. *Water Sci. Technol.* 23, 4/6, 747-754.

Beacham, A.M., Seviour, R.J. and Lindrea, K.C. (1989) Isolation of *Acinetobacter* spp. from the Bendigo Water Board pilot plant. In: *Proceedings of the 13th Federal Convention of Australian Water and Wastewater Association*. Canberra, Australia. 6-10 March. 456-460.

Beacham, A.M., Seviour, R.J., Lindrea, K.C. and Livingston, I. (1990) Genospecies diversity of *Acinetobacter* isolates obtained from a biological nutrient removal pilot plant of a modified UCT configuration. *Water Res.* 24, 23-29.

Beacham, A.M., Seviour, R.J. and Lindrea, K.C. (1992) Polyphosphate accumulating abilities of *Acinetobacter* isolates from a biological nutrient removal pilot plant. *Water Res.* 26, 121-122.

Benedict, A.H. and Carlson, D.A. (1973) Temperature acclimatisation in aerobic bio-oxidation systems. *J. Water Pollut. Control Fed.* 45, 10-24.

Bonting, C.F.C., Kortstee, G.J.J. and Zehnder, A.J.B. (1991) Properties of polyphosphate: AMP phosphotransferase of *Acinetobacter* strain 210A. *J. Bacteriol.* 173, 6484-6488.

Bonting, C.F.C., van Veen, H.W., Taverne, A., Kortstee, G.J.J. and Zehnder, A.J.B. (1992) Regulation of polyphosphate metabolism in *Acinetobacter* strain 210A grown in carbon- and phosphate-limited continuous cultures. *Arch. Microbiol.* 158, 139-144.

Bosch, M. and Cloete, T.E. (1993) The identification and classification of *Acinetobacter* strains exhibiting variations in phosphate accumulation by SDS-PAGE and numerical analysis. *Current Microbiol.* 27, 169-175.

Boughton, W.H., Gottfried, R.J., Sinclair, N.A. and Yall, I. (1971) Metabolic factors affecting enhanced phosphorus uptake by activated sludge. *Appl. Microbiol.* 22, 571-577.

Bouvet, P.J.M. and Grimont, P.A.D. (1986) Taxonomy of the genus *Acinetobacter* with the recognition of *Acinetobacter baumanii* sp. nov., *Acinetobacter haemolyticus* sp. nov., *Acinetobacter johnsonii* sp. nov., and *Acinetobacter junii* sp. nov. and emended descriptions of *Acinetobacter calcoaceticus* and *Acinetobacter lwoffii*. *Int. J. Syst. Bacteriol.* 36, 228-240.

Bouvet, P.J.M. and Grimont, P.A.D. (1987) Identification and biotyping of clinical isolates of *Acinetobacter*. *Ann. Inst. Pasteur/Microbiol.* 138, 569-578.

Bouvet, P.J.M. and Jeanjean, S. (1989) Delineation of new proteolytic genomic species in the genus *Acinetobacter*. *Res. Microbiol.* 140, 291-299.

Bowmer, K.H. and Laut, P. (1990) Biological nutrient recovery in Australian intensive rural industry. In: *Proceedings of the 1st Australian Conference on BPR from Sewage*. Bendigo, Victoria, Australia. 47-58.

Breuil, C., and Kushner, D.J. (1975) Lipase and esterase formation by psychrophilic and mesophilic *Acinetobacter* species, *Can. J. Microbiol.* 21, 423.

Brisou, J. and Prévot A.-R. (1954) Études de systématique bactérienne x. révision des espèces reunies dans le genre *Achromobacter*. *Ann. Inst. Pasteur Paris*, 86, 722-728.

Brodisch, K.E.U. and Joyner, S.J. (1983) The role of micro-organisms other than *Acinetobacter* in biological phosphate removal in activated sludge processes. *Water Sci. Technol.* 15, 3/4, 117-125.

Brodisch, K.E.U. (1985) Interaction of different groups of micro-organisms in biological phosphate removal. *Water Sci. Technol.* 17, 11/12, 89-97.

Buchan, L. (1981) The location and nature of accumulated phosphorus in seven sludges from activated sludge plants which exhibited enhanced phosphorus removal. *Water S.A.* 7, 1-7.

Buchan, L. (1983) Possible biological mechanism of phosphorus removal. *Water Sci. Technol.* 15, 3/4, 87-103.

Bundgaard, E., Kristensen, G.H. and Arvin, E. (1983) Full-scale experience with phosphorus removal in an alternating system. *Water Sci. Technol.* 15, 3/4, 197-217.

CEC - Council of the European Communities (1991) Directive concerning urban wastewater treatment (91/271/EEC). *Official Journal.* L135/40, 30 May.

Cloete, T.E. and Steyn, P.L. (1988a) The role of *Acinetobacter* as a phosphorus removing agent in activated sludge. *Water Res.* 22, 971-976.

Cloete, T.E. and Steyn, P.L. (1988b) A combined membrane filter-immunofluorescent technique for the *in situ* identification and enumeration of *Acinetobacter* in activated sludge. *Water Res.* 22, 961-969.

Cloete, T.E., Steyn, P. L. and Buchan, L. (1985) An aut-ecological study of *Acinetobacter* in activated sludge. *Water Sci. Technol.* 17, Paris, 139-146.

Cloete, T.E., Bosch, M. and Mienie, N.J.J. (1992) Organisms other than *Acinetobacter* capable of phosphorus removal from activated sludge mixed liquor. In: *Proceedings European Conference on Nutrient Removal from Wastewater.* Wakefield, U.K. September 1-4.

Collins, M.D. and Jones, D. (1981) Distribution of isoprenoid quinone structural types in bacteria and their taxonomic implications. *Microbiol. Rev.* 45, 316-354.

Comeau, Y. (1990) La déphosphatation biologique métabolisme microbien. *Sciences et Techniques de l'Eau* 23, 1, 47-60.

Comeau, Y., Hall, K.J. and Oldham, W.K. (1985) A biochemical model for biological enhanced phosphorus removal. *Water Sci. Technol.* 17, 11/12, 313-314.

Comeau, Y., Hall, K.J., Hancock, R.E.W. and Oldham, W.K. (1986) Biochemical model for enhanced biological phosphorus removal. *Water Res.* 20, 1511-1521.

Comeau, Y., Rabinowitz, B., Hall, K.J. and Oldham, W.K. (1987a) Phosphate release and uptake in enhanced biological phosphorus removal from wastewater. *J. Water Pollut. Control Fed.* 59, 707-715.

Comeau, Y., Oldham, W.K. and Hall, K.J. (1987b) Dynamics of carbon reserves in biological dephosphatation of wastewater. In: *Biological Phosphate Removal from Wastewaters. Advances in Water Pollution Control.* (Ramadori, R. ed.) Pergamon Press, Oxford. 39-57.

Converti, A., Zilli, M., Poloniecki, R.H., del Borghi, M. and Ferraiolo, G. (1993) Influence of nutrient concentration in new operating criteria for biological removal of phosphorus from wastewaters. *Water Res.* 27, 791-798.

Converti, A., Rovatti, M. and del Borghi, M. (1995) Biological removal of phosphorus from wastewaters by alternating aerobic and anaerobic conditions. *Water Res.* 29, 263-269.

Cooper, P., Upton, J.E., Smith, M. and Churchley, J. (1995) Biological nutrient removal: design snags, operational problems and costs. *J. Inst. Water Environ. Management* 9, 1, 7-18.

Cullen, P. (1990) Determining acceptable phosphorus discharges to freshwaters. In: *Proceedings of the 1st Australian Conference on BPR from Sewage*. Bendigo, Victoria, Australia. 37-46.

Dawes, E.A. and Senior, P.J. (1973) The role and regulation of energy reserve polymers in micro-organisms. *Adv. Microbial Physiol.* 10, 135-266.

Deinema, M.H., Habets, L.H.A., Scholten, J., Turkstra, E. and Webers, H.A.A.M. (1980) The accumulation of polyphosphate in *Acinetobacter* spp. *FEMS Microbiol. Letts.* 9, 275-279.

Deinema, M.H., van Loosdrecht, M. and Scholten, A. (1985) Some physiological characteristics of *Acinetobacter* spp. accumulating large amounts of phosphate. *Water Sci. Technol.* 17, Paris, 119-125.

del Carmen Doria-Serrano, Ma., González-Martínez, S. and Hernández-Esparza, M. (1992) Biochemical models for phosphate accumulating microorganisms. *Water Sci. Technol.* 26, 9-11, 2245-2248.

Department of the Environment (1977) *Methods for the Examination of Waters and Associated Materials. Chemical Oxygen Demand (Dichromate Value) of Polluted and Waste Waters*. HMSO, London.

Department of the Environment (1980a) *Methods for the Examination of Waters and Associated Materials. Phosphorus in Waters, Effluents and Sewages*. HMSO, London.

Department of the Environment (1980b) *Methods for the Examination of Waters and Associated Materials. Suspended, Settleable and Total Dissolved Solids in Waters and Effluents*. HMSO, London.

Department of the Environment (1981) *Methods for the Examination of Waters and Associated Materials. Oxidised Nitrogen in Waters*. HMSO, London.

Draaijer, H., Buunen-van Bergen, A.H.M., van't Oever, E. and Schellen, A.A.J.C. (1993) Full scale experiences with nutrient removal at two wastewater treatment plants in the Netherlands. *Water Sci. Technol.* 27, 5/6, 343-355.

Duncan, A., Vasiliadis, G.E., Bayly, R.C., May, J.W. and Raper, W.G.C. (1988) Genospecies of *Acinetobacter* isolated from activated sludge showing enhanced removal of phosphate during pilot-scale treatment of sewage. *Biotechnol. Letts.*, 10, 831-836.

Duncan, A., Bayly, R.C., May, J.W., Vasiliadis, G. and Semerjtis, A. (1990) Analysis of plasmids in *Acinetobacter* strains isolated from activated sludge. In: *Proceedings of the 1st Australian Conference on BPR from Sewage*. Bendigo, Victoria, Australia. 117-123.

Du Preez, J.C. and Toerien, D.F. (1978) The effect of temperature on the growth of *Acinetobacter calcoaceticus*. *Water S.A.* 4, 1, 10-13.

Du Preez, J.C., Toerien, D.F. and Lategan, P.M. (1981) Growth parameters of *Acinetobacter calcoaceticus* on acetate and ethanol. *European J. Appl. Microbiol. Biotechnol.* 13, 45-53.

Farrimond, M. and Upton, J. (1993) A strategy to meet the nutrient (N and P) standards of the urban wastewater directive. *Water Sci. Technol.* 27, 5/6, 297-306.

Fleit, E. (1995) Intracellular pH regulation in biological excess phosphorus removal systems. *Water Res.* 29, 1787-1792.

Florentz, M. and Hartemann, P. (1984) Screening for phosphate accumulating bacteria isolated from activated sludge. *Environ. Technol. Letts.* 5, 457-463.

Fuhs, G.W. and Chen, M. (1975) Microbiological basis of phosphate removal in the activated sludge process for the treatment of wastewater. *Microbial Ecology* 2, 119-138.

Fukase, T., Shibata, M. and Miyaji, Y. (1982) Studies on the mechanism of biological removal of phosphorus. *Jap. J. Water Pollut. Res.* 5, 309-317.

Gerber, A., Mostert, E.S., Winter, C.T. and de Villiers, R.H. (1986) The effect of acetate and other short-chain carbon compounds on the kinetics of biological nutrient removal. *Water S.A.* 12, 1, 7-12.

Gerber, A., de Villiers, R.H., Mostert, E.S. and van Riet C.J.J. (1987a) The phenomenon of simultaneous phosphate uptake and release, and its importance in biological nutrient removal. In: *Biological Phosphate Removal from Wastewaters. Advances in Water Pollution Control.* (Ramadori, R. ed.) Pergamon Press, Oxford. 123-134.

Gerber, A., Mostert, E.S., Winter, C.T. and de Villiers, R.H. (1987b) Interactions between phosphate, nitrate and organic substrate in biological nutrient removal processes. *Water Sci. Technol.* 19, 1/2, 183-194.

Gerner-Smidt, P. (1992) Ribotyping of the *Acinetobacter calcoaceticus*-*Acinetobacter-baumannii* complex. *J. Clin. Microbiol.* 2680-2685.

Gerner-Smidt, P., Tjernberg, I. and Ursing, J. (1991) Reliability of phenotypic tests for identification of *Acinetobacter* species. *J. Clin. Microbiol.* 29, 277-282.

Gersberg, R.M. and Allen, D.W. (1985) Phosphorus uptake by *Klebsiella pneumoniae* and *Acinetobacter calcoaceticus*. *Water Sci. Technol.* 17, Paris, 113-118.

Ghekiere, S., Bruynooghe, H., Van Steenberghe, K., Vriens, L., Van Haute, A. and Verachtert, H. (1991) The effects of nitrates and carbon compounds on enhanced biological phosphorus removal from wastewaters. *Europ. Water Pollut. Control* 1, 4, 15-24.

Grehn M. and von Graevenitz, A. (1978) Search for *Acinetobacter calcoaceticus* subsp. *anitratus* : Enrichment of faecal samples. *J. Clin. Microbiol.* 8, 342-343.

Grimont, P.A.D., and Bouvet, P.J.M. (1991) Taxonomy of *Acinetobacter*. In: *The Biology of Acinetobacter*. (Towner, K.J., Bergogne-Bérézin, E. and Fewson, C.A. eds.) Plenum Press, New York and London. 25-36.

Halvorson, H.O., Suresh, N., Roberts, M.F., Coccia, M. and Chikarmane, H.M. (1987) Metabolically active surface polyphosphate pool in *Acinetobacter lwoffii*. In: *Phosphate Metabolism and Cellular Regulation in Micro-organisms*. (Torriani-Gorini, A., Rothman, F.G., Silver, S., Wright, A. and Yagil, E. eds.) American Society for Microbiology, Washington. 220-224.

Hao, O.J. and Chang, C.H. (1987) Kinetics of growth and phosphate uptake in pure culture studies of *Acinetobacter* species. *Biotechnol. Bioeng.* 29, 819-831.

Harold, F.M. (1966) Inorganic polyphosphates in biology: structure, metabolism, and function. *Bacteriol. Revs.* 30, 772-794.

Hascoet, M.C. and Florentz, M. (1985) Influence of nitrates on biological phosphorus removal from wastewater. *Water S.A.* 11, 1, 1-8.

Hascoet, M.C., Florentz, M. and Granger, P. (1985) Biochemical aspects of enhanced biological phosphorus removal from wastewaters. *Water Sci. Technol.* 17, 11/12, 23-41.

Hashimoto, S. and Furukawa, K. (1984) Biological phosphorus release from activated sludge of sludge recycling nitrification-denitrification process. *J. Ferment. Technol.* 62, 437-444.

Henriksen, S.D. (1973) *Moraxella*, *Acinetobacter* and *Mimeae*. *Bacterial Rev.* 37, 522-561.

Henriksen, S.D. (1976) *Moraxella*, *Neisseria*, *Branhamella* and *Acinetobacter*. *Ann. Rev. Microbiol.* 30, 63-83.

Heymann, J.B., Eagle, L.M., Greben, H.A. and Potgieter, D.J.J. (1989) The isolation and characterization of volutin granules as subcellular components involved in biological phosphorus removal. *Water Sci. Technol.* 21, 4/5, 397-408.

Hiraishi, A. and Morishima, Y. (1990) Capacity for polyphosphate accumulation of predominant bacteria in activated sludge showing enhanced phosphate removal. *J. Ferment. Bioeng.* 69, 6, 368-371.

Hiraishi, A., Masamune, K. and Kitamura, H. (1989) Characterization of the bacterial population structure in an anaerobic-aerobic activated sludge system on the basis of respiratory quinone profiles. *Appl. Environ. Microbiol.* 55, 897-901.

Holford, I.C.R. and Doyle, A.D. (1993) The recovery of fertiliser phosphorus by wheat, its agronomic efficiency and their relationship to soil phosphorus. *Austr. J. Agric. Res.* 44, 1745-1756.

Holton, J. (1983) A note on the preparation and use of a selective differential medium for the isolation of *Acinetobacter* spp. from clinical sources. *J. Appl. Bacteriol.* 54, 141-142.

Hong, S.N., Krichen, D.J., Best, A. and Rachwal, A. (1984) Biological phosphorus and nitrogen removal via the A/O process; recent experiences in the United States and United Kingdom. *Water Sci. Technol.* 16, 10/11, 151-172.

Humphrey, A.E. (1979) Fermentation process modelling: an overview. *Ann. New York Acad. Sci.* 326, 17-33.

Hutton, R.H., Spach, R.A., Hedrick, L.G., Fiss, Jr., E.C., Francisco, D.E., Stein, R.M. and Wagoner, D.L. (1994) Performance of municipal biological nutrient removal systems under winter and storm flow conditions. In: *Proceedings of the 67th Annual Water Environment Federation Conference & Exposition*. Chicago, Illinois. October 15-19. Water Environment Federation, Alexandria, VA, USA. Vol. 1, 631-642.

Imai, H. and Endoh, K. (1990) Potassium removal accompanied by enhanced biological phosphate removal. *J. Ferment. Bioeng.* 69, 250-255.

Imai, H., Endoh, K. and Kozuka, T. (1988) Magnesium requirement for biological removal of phosphate by activated sludge. *J. Ferment. Technol.* 66, 657-666.

Ino, T. and Nishimura, Y. (1989) Taxonomic studies of *Acinetobacter* species based on outer membrane protein patterns. *J. Gen. Appl. Microbiol.* 35, 213-224.

Irvine, R.L., Miller, G. and Bhamrah, A.J. (1979) Sequencing batch treatment of wastewater in rural areas. *J. Water Pollut. Control Fed.* 51, 244-254.

Irvine, R.L., Murthy, D.V.S., Arora, M.L., Copeman, J.L. and Heidman, J.A. (1987) Analysis of full-scale SBR operation at Grundy Center, Iowa. *J. Water Pollut. Control Fed.* 59, 132-138.

Irvine, R.L. and Ketchum, L.H. (1989) Sequencing batch reactors for biological wastewater treatment. *CRC Crit. Revs. Environ. Control* 18, 4, 255-294.

Iwema, A. and Meunier, A. (1985) Influence of nitrate on acetic acid induced biological phosphate removal. *Water Sci. Technol.* 17, 11/12, 289-294.

Jenkins, D. and Tandoi, V. (1991) The applied microbiology of enhanced biological phosphate removal - accomplishments and needs. *Water Res.* 25, 1471-1478.

Jing, S.R., Benefield, L.D. and Hill, W.E. (1992) Observations relating to enhanced phosphorus removal in biological systems. *Water Res.* 26, 213-223.

Johnson, J.L., Anderson, R.S. and Ordal, E.J. (1970) Nucleic acid homologies among oxidase-negative *Moraxella* species. *J. Bacteriol.* 101, 568-573.

Jones, P.H., Tadwalkar, A.D. and Hsu, C.L. (1987) Enhanced uptake of phosphorus by activated sludge - effect of substrate addition. *Water Res.* 21, 301-308.

Joseph, S.W., Colwell, R.R. and MacDonell, M.T. (1987) *Aeromonas* taxonomy. *Experientia* 43, 349-350.

Juni, E. (1972) Interspecies transformation of *Acinetobacter*: genetic evidence for a ubiquitous genus. *J. Bacteriol.* 112, 917-931.

Juni, E. (1978) Genetics and Physiology of *Acinetobacter*. *Ann. Rev. Microbiol.* 32, 349-371.

Juni, E. (1984) *Neisseriaceae* Family, Genus III *Acinetobacter*. In: *Bergey's Manual of Systematic Bacteriology* (Krieg, N.R. and Holt, J.G. eds.) 14th edn. The Williams & Wilkins Co., Baltimore. Vol. 1., 303-307.

Kämpfer, P. and Altwegg, M. (1992) Numerical classification and identification of *Aeromonas* genospecies. *J. Appl. Bacteriol.* 72, 341-351.

Kämpfer, P., Bark, K., Busse, H.J., Auling, G. and Dott, W. (1992) Numerical and chemotaxonomy of polyphosphate accumulating *Acinetobacter* strains with high polyphosphate:AMP phosphotransferase (PPAT) activity. *System. Appl. Microbiol.* 15, 409-419.

Kämpfer, P., Tjernberg, I. and Ursing, J. (1993) Numerical classification and identification of *Acinetobacter* genomic species. *J. Appl. Bacteriol.* 75, 259-268.

Kato, J., Yamada, K., Muramatsu, A., Hardoyo and Ohtake, H. (1993) Genetic improvement of *Escherichia coli* for enhanced biological removal of phosphate from wastewater. *Appl. Environ. Microbiol.* 59, 3744-3749.

Kavanaugh, R.G. and Randall, C.W. (1994) Bacterial populations in a biological nutrient removal plant. *Water Sci. Technol.* 29, 7, 25-34.

Kerrn-Jespersen, J.P. and Henze, M. (1993) Biological phosphorus uptake under anoxic and aerobic conditions. *Water Res.* 27, 617-624.

Keurentjes, J.T.F. and Iwema, A. (1987) The effect of the pH variation between the anaerobic and the aerobic phase on biologically induced P-removal. In: *Biological Phosphate Removal from Wastewaters. Advances in Water Pollution Control*. (Ramadori, R. ed.) Pergamon Press, Oxford. 317-319.

Knight, G.C., McDonnell, S.A., Seviour, R.J. and Soddell, J.A. (1993) Identification of *Acinetobacter* isolates using the Biolog identification system. *Letts. Appl. Microbiol.* 16, 261-264.

Knight, G.C., Seviour, R.J., Soddell, J.A., McDonnell, S. and Bayly, R.C. (1995a) Metabolic variation among strains of *Acinetobacter* isolated from activated sludge. *Water Res.* 29, 2081-2084.

Knight, G.C., Seviour, E.M., Seviour, R.J., Soddell, J.A., Lindrea, K.C., Strachan, W., De Grey, B. and Bayly, R.C. (1995b) development of the microbial community of a full scale biological nutrient removal activated sludge plant during start-up. *Water Res.* 29, 2081-2084.

Koch, F.A. and Oldham, W.K. (1985) Oxidation-reduction potential - a tool for monitoring, control and optimization of biological nutrient removal systems. *Water Sci. Technol.* 17, 11/12, 259-281.

Kotak, B.G., Kenefick, S.L., Fritz, D.L., Rousseaux, C.G., Prepas, E.E. and Hrudey, S.E. (1993) Occurrence and toxicological evaluation of cyanobacterial toxins in Alberta lakes and farm dugouts. *Water Res.* 27, 495-506.

Krichen, D.J., Hong, S.N. and Tracy, K.D. (1985) Applied biological phosphorus removal technology for municipal wastewater treatment by the A/O process. In: *Proceedings of the International Conference on Management Strategies for Phosphorus in the Environment*. (Lester, J.N. and Kirk, P.W.W. eds.) Lisbon, Portugal. 1-4 July. Selper Ltd., London. 399-404.

Kronberg, S.R. (1957) Adenosine triphosphate synthesis from polyphosphate by an enzyme from *Escherichia coli*. *Biochim. Biophys. Acta*. 26, 294-300.

Kuba, T., Smolders, G., van Loosdrecht, M.C.M. and Heijnen, J.J. (1993) Biological phosphorus removal from wastewater by anaerobic-anoxic sequencing batch reactor. *Water Sci. Technol.* 27, 5/6, 241-252.

Kuba, T., Wachtmeister, A., van Loosdrecht, M.C.M. and Heijnen, J.J. (1994) Effect of nitrate on phosphorus release in biological phosphorus removal systems. *Water Sci. Technol.* 30, 6, 263-269.

Kulaev, I.S. (1979) *The Biochemistry of Inorganic Polyphosphates*. John Wiley and Sons. Chichester.

Kulaev, I.S. (1985) Some aspects of environmental regulation of microbial phosphorus metabolism. *FEMS Symposium* 23, 1-25.

Kulaev, I.S. and Vagabov, V.M. (1983) Polyphosphate Metabolism in Micro-Organisms. *Advances in Microbial Physiology*, Vol. 24. (Rose, A.H., Morris, J.G and Tempest, D.W. eds.) Academic Press, London. 83-171.

Lautrop, H. (1974) *Neisseriaceae* Family, Genus IV *Acinetobacter*. In: *Bergey's Manual of Determinative Bacteriology* (Buchanan, R.E. and Gibbons, N.E. eds.) 8th edn. The Williams & Wilkins Co., Baltimore. 436-438.

Lawson, E.N. and Tonhazy, N.E. (1980) Changes in morphology and phosphate-uptake patterns of *Acinetobacter calcoaceticus* strains. *Water S.A.* 6, 3, 105-112.

Lee, S.E., Kim, K.S., Kim, K.S. and Kim, C.W. (1993) Enhancement of phosphorus and nitrogen removal with a side stream biological nutrient removal process. *Water Sci. Technol.* 28, 7, 89-96.

Levin, G.V. and Shapiro, J. (1965) Metabolic uptake of phosphorus by wastewater organisms. *J. Water Pollut. Control Fed.* 37, 800-821.

Lötter, L.H. (1984) The role of bacterial phosphate metabolism in enhanced phosphorus removal from the activated sludge process. In: *EBPR from Wastewater. Post-Conference Seminar. IAWPRC.* Paris. 24-25 September. Vol. 1. 162-172.

Lötter, L.H. (1985) The role of bacterial phosphate metabolism in enhanced phosphorus removal from the activated sludge process. *Water Sci. Technol.* 17, 11/12, 127-138.

Lötter, L.H. (1991) Combined chemical and biological removal of phosphate in activated sludge plants. *Water Sci. Technol.* 23, 2, 611-621.

Lötter, L.H. and Murphy, M. (1985) The identification of heterotrophic bacteria in an activated sludge plant with particular reference to polyphosphate accumulation. *Water S.A.* 11, 4, 179-184.

Lötter, L.H. and Murphy, M. (1988) Microscopic evaluation of carbon and phosphorus accumulation in nutrient removal activated sludge plants. *Water Sci. Technol.* 20, 4/5, 37-49.

Lötter, L.H., Wentzel, M.C., Ekama, G. A. and Marais, G.v.R. (1986) A study of selected characteristics of *Acinetobacter* spp. isolated from activated sludge in anaerobic / anoxic / aerobic and aerobic systems. *Water S.A.* 12, 4, 203-208.

Ludwig, Ch., Spatzierer, G. and Matsché, N. (1985) Praktische anwendung der biologischen phosphorentfernung in kombination mit der simultanfällung. *gwf-wassser / abwasser* 126, H5, 257-263.

Malnou, D., Meganck, M., Faup, G.M. and du Rostu, M. (1984) Biological phosphorus removal: study of the main parameters. *Water Sci. Technol.* 16, 173-185.

Mamais, D. and Jenkins, D. (1992) The effects of MCRT and temperature on enhanced biological phosphorus removal. *Water Sci. Technol.* 26, 5/6, 955-965.

Manning, J.F. and Irvine, R.L. (1985) The biological removal of phosphorus in a sequencing batch reactor. *J. Water Pollut. Control Fed.* 57, 87-94.

Manz, W., Wagner, M., Amann, R. and Schleifer, K. H. (1994) *In situ* characterisation of the microbial consortia active in two wastewater treatment plants. *Water Res.* 28, 1715-1723.

Marais, G.v.R., Loewenthal, R.E. and Siebritz, I.P. (1983) Observations supporting phosphate removal by biological excess uptake - a review. *Water Sci. Technol.* 15, 3/4, 15-41.

Marklund, S. (1993) Cold climate sequencing batch reactor biological phosphorus removal - results 1991-92. *Water Sci. Technol.* 28, 10, 275-282.

Matsuo, T., Mino, T. and Sato, H. (1992) Metabolism of organic substances in anaerobic phase of biological phosphate uptake process. *Water Sci. Technol.* 25, 6, 83-92.

Matthews, P.J. (1985) Phosphate in water - is it really a problem? An English water authority over-view. In: *Proceedings of the International Conference on Management Strategies for Phosphorus in the Environment*. (Lester, J.N. and Kirk, P.W.W. eds.) Lisbon, Portugal. 1-4 July. Selper Ltd., London. 446-453.

McClintock, S.A., Sherrard, J.H., Novak, J.T. and Randall, C.W. (1988) Nitrate versus oxygen respiration in the activated sludge process. *J. Water Pollut. Control Fed.* 60, 342-350.

McClintock, S.A. and Frazier, J.A. (1994) Optimization of biological nutrient removal in sequencing batch reactors at the Dauphin Borough Wastewater Treatment Plant. In: *Proceedings of the 67th Annual Water Environment Federation Conference & Exposition*. Chicago, Illinois. October 15-19. Water Environment Federation, Alexandria, VA, USA. Vol 1. 585-593.

Melcer, H., Topnik, B.H. and Bedford, W.K. (1987) Conversion of small municipal wastewater treatment plants to sequencing batch reactors. *Environment Canada, Report EPS 3/NR/2*.

Menar, A.B. and Jenkins, D. (1970) Fate of phosphorus in waste treatment processes: enhanced removal of phosphate in activated sludge. *Environ. Sci. Technol.* 4, 1115-1121.

Metcalf and Eddy, (1991) *Wastewater Engineering. Treatment, Disposal and Reuse*. 3rd edn. McGraw-Hill, New York.

Milbury, W. F., McCauley, D. and Hawthorne, C.H. (1971) Operation of conventional activated sludge for maximum phosphorus removal. *J. Water Pollut. Control Fed.* 43, 1890-1901.

Mino, T., Kawakami, T. and Matsuo, T. (1984) Location of phosphorus in activated sludge and function of intracellular polyphosphates in biological phosphorus removal process. *Water Sci. Technol.* 17, 2/3, 93-106.

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

Morse, G.K., Lester, J.N. and Perry, R. (1993) *The Economic and Environmental Impact of Phosphorus Removal from Wastewater in the European Community.* Selper Publications, London.

Mostert, E.S., Gerber, A. and van Riet, C.J.J. (1989) A comparative study on the effects of feedstock composition on enhanced biological phosphate removal in modified activated sludge systems. *Environ. Technol. Letts.* 10, 9-22.

Murphy, J. and Riley, J. P. (1962) A modified single solution method for the determination of phosphate in natural waters. *Anal. Chim. Acta*, 27, 31-36.

Nakamura, K., Masuda, K. and Mikami, E. (1991) Isolation of a new type of polyphosphate accumulating bacterium and its phosphate removal characteristics. *J. Ferment. Bioeng.* 71, 4, 258-263.

Nicholls, H.A. and Osborn, D.W. (1979) Bacterial stress: prerequisite for biological removal of phosphorus. *J. Water Pollut. Control Fed.* 51, 557-569.

Nishimura, Y., Ino, T. and Iizuka, H. (1986) Isolation and characterization of the outer membrane of radiation-resistant *Acinetobacter* sp. FO-1. *J. Gen. Appl. Microbiol.* 32, 177-184.

Nishimura, Y., Kano, M., Ino, T., Iizuka, H., Kosako, Y. and Kaneko, T. (1987) Deoxyribonucleic acid relationship among the radiation-resistant *Acinetobacter* and other *Acinetobacter*. *J. Gen. Appl. Microbiol.* 33, 371-376.

Nishimura, Y., Ino, T. and Iizuka, H. (1988a) *Acinetobacter radioresistens* sp. nov. isolated from cotton and soil. *Int. J. Syst. Bacteriol.* 38, 209-211.

Nishimura, Y., Kanzaki, H. and Iizuka, H. (1988b) Taxonomic studies of *Acinetobacter* species based on the electrophoretic analysis of enzymes. *J. Basic Microbiol.* 28, 6, 363-370.

Novak, J.T. (1974) Temperature-substrate interactions in biological treatment. *J. Water Pollut. Control Fed.* 46, 1984-1994.

NRA - National Rivers Authority (1990) *Toxic Blue-Green Algae*. A report by the National Rivers Authority. Water Quality Series No. 2. NRA, London.

Ohtake, H., Takahashi, K., Tsuzuki, Y. and Toda, K. (1985) Uptake and release of phosphate by a pure culture of *Acinetobacter calcoaceticus*. *Water Res.* 19, 1587-1594.

Okada, M. and Sudo, R. (1986) Performance of sequencing batch reactor activated sludge processes for simultaneous removal of nitrogen, phosphorus and BOD as applied to small community sewage treatment. *Water Sci. Technol.* 18, 7/8, 363-370.

Okada, M., Murakami, A., Lin, C.K., Ueno, Y. and Okubo, T. (1991) Population dynamics of bacteria for phosphorus removal in sequencing batch reactor (SBR) activated sludge processes. *Water Sci. Technol.* 23, Kyoto, 755-763.

Okada, M., Lin, C.K., Katayama, Y. and Murakami, A. (1992) Stability of phosphorus removal and population of bio-P-bacteria under short term disturbances in sequencing batch reactor activated sludge process. *Water Sci. Technol.* 26, 3/4, 483-491.

Okada, H., Yamada, K., Hardoyo, Muramatsu, A., Anbe, Y., Kato, J. and Shinjo, H. (1994) Genetic approach to enhanced biological phosphorus removal. *Water Sci. Technol.* 30, 6, 185-192.

Oldham, W.K. (1985) Full scale optimization of biological phosphorus removal at Kelowna, Canada. *Water Sci. Technol.* 17, 11/12, 243-257.

Oldham, W.K. and Abraham, K. (1994) Overview of full-scale fermenter performance. In: *Proceedings of the Conference Seminar on Use of Fermentation to Enhance Biological Nutrient Removal. 67th Annual Water Environment Federation Conference & Exposition.* Chicago, Illinois. Oct 15. Water Environment Federation, Alexandria, VA. 81-86.

Olson, D.O. and Connell C.H. (1971) Co-uptake and co-release of phosphorus and magnesium by activated sludge. *A.I.Ch.E. Symposium Series, Water* 68, 124, 307-317.

Osborn, D.W. and Nicholls, H.A. (1978) Optimisation of the activated sludge process for the biological removal of phosphorus. *Prog. Water Technol.* 10, 1/2, 261-277.

- Pitman, A.R., Venter, S.L.V. and Nicholls, H.A. (1983) Practical experience with biological phosphorus removal plants in Johannesburg. *Water Sci. Technol.* 15, 3/4, 233-259.
- Popoff, M. (1984) Genus III *Aeromonas*. In: *Bergey's Manual of Systematic Bacteriology* (Krieg, N.R. and Holt, J.G. eds.) 14th edn. The Williams & Wilkins Co., Baltimore. Vol. 1., 545-548.
- Potgieter, D.J.J. and Evans, B.W. (1983) Biochemical changes associated with luxury phosphate uptake in a modified Phoredox activated sludge system. *Water Sci. Technol.* 15, 105-115.
- Prakasam, T.B.S. and Dondero, N.C. (1967) Aerobic heterotrophic populations of sewage and activated sludge. I. Enumeration. *Appl. Microbiol.* 15, 461-467.
- Rabinowitz, B. (1994) Criteria for effective primary sludge fermenter design. In: *Proceedings of the Conference Seminar on Use of Fermentation to Enhance Biological Nutrient Removal. 67th Annual Water Environment Federation Conference & Exposition*. Chicago, Illinois. Oct 15. Water Environment Federation, Alexandria, VA. 23-34.
- Rabinowitz, B. and Oldham, W.K. (1986) Excess biological phosphorus removal in the activated sludge process using primary sludge fermentation. *Can. J. Civ. Eng.* 13, 345-351.
- Randall, C.W., Pattarkine, V.M. and McClintock, S.A. (1992) Nitrification kinetics in single-sludge biological nutrient removal activated sludge systems. *Water Sci. Technol.* 25, 6, 195-214.
- Rao, N.N., Roberts, M.F. and Torriani, A. (1985) Amount and chain length of polyphosphates in *Escherichia coli* depend on cell growth conditions. *J. Bacteriol.* 162, 242-247.

Raper, W.G.C. (1990) Are new mechanisms involved in biological nutrient removal? In: *Proceedings of the 1st Australian Conference on BPR from Sewage*. Bendigo, Victoria, Australia. 149-156.

Reasoner, D.J. and Geldreich, E.E. (1985) A new medium for the enumeration and subculture of bacteria from potable water. *Appl. Environ. Microbiol.* 49, 1-7.

Rees, G.N., Vasiliadis, G., May, J.W. and Bayly, R.C. (1992a) Polyphosphate and poly- β -hydroxybutyrate production in *Acinetobacter* spp. isolated from activated sludge. *Water Sci. Technol.* 26, 9/11, 2213-2215.

Rees, G.N., Vasiliadis, G., May, J.W. and Bayly, R.C. (1992b) Differentiation of polyphosphate and poly- β -hydroxybutyrate granules in an *Acinetobacter* sp. isolated from activated sludge. *FEMS Microbiol. Letts.* 94, 171-174.

Rees, G.N., Vasiliadis, G., May, J.W. and Bayly, R.C. (1993) Production of poly- β -hydroxybutyrate in *Acinetobacter* spp. isolated from activated sludge. *Appl. Microbiol. Biotechnol.* 38, 734-737.

Rensink, J.H., Donker, H.J.G.W. and Simons, T.S.J. (1985) Phosphorus removal at low sludge loadings. *Water Sci. Technol.* 17, 11/12, 177-186.

Rickard, L.F. and McClintock, S.A. (1992) Potassium and Magnesium requirements for enhanced biological phosphorus removal from wastewater. *Water Sci. Technol.* 26, 9-11, 2203-2206.

Rossau, R., Vandenbussche, G., Thielemans, S., Segers, P., Grosch, H., Göthe, E., Mannheim, W. and De Ley, J. (1989) Ribosomal ribonucleic acid cistron similarities and deoxyribonucleic acid homologies of *Neisseria*, *Kingella*, *Eikenella*, *Simonsiella*, *Alysiella*, and Centers for Disease Control Groups EF-4 and M-5 in the emended family Neisseriaceae. *Int. J. Syst. Bacteriol.* 39, 185-198.

Rossau, R., van Landschoot, A., Gillis, M. and de Ley, J. (1991) Taxonomy of *Moraxellaceae* fam. nov., a new bacterial family to accommodate the genera *Moraxella*, *Acinetobacter*, and *Psychrobacter* and related organisms. *Int. J. Syst. Bacteriol.* 41, 310-319.

Rossin, A.C. and Lester, J.N. (1980) An evaluation of some of the methods available for the preservation of condensed phosphates in samples of waste water. *Environ. Technol. Letts.* 1, 9-16.

Rusten, B. and Eliassen, H. (1993) Sequencing batch reactors for nutrient removal at small wastewater treatment plants. *Water Sci. Technol.* 28, 10, 233-242.

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

Schaak, F., Boschet, A.-F., Chevalier, D., Kerlan, F. and Senelier, Y. (1985) Efficiency of existing biological treatment plants against phosphorated pollution. *Tech. Sci. Municip. L'Eau* 80, 173-181.

Schmidt, G., Hecht, L. and Thannhauser, S.J. (1946) The enzymic formation and the accumulation of large amounts of a metaphosphate in baker's yeast under certain conditions. *J. Biol. Chem.* 166, 775-776.

Schön, G., Geywitz, S. and Mertens, F. (1993) Influence of dissolved oxygen and oxidation-reduction potential on phosphate release and uptake by activated sludge from sewage plants with enhanced biological phosphorus removal. *Water Res.* 27, 349-354.

Schubert, R.H.W. (1974) *Aeromonas*. In: *Bergey's Manual of Determinative Bacteriology* (Buchanan, R.E. and Gibbons, N.E. eds.) 8th edn. Williams and Wilkins Co., Baltimore. 345-348.

Sell, R.L., Krichen, D.J., Noichl, O.J. and Hartzog, D.G. (1981) Low temperature biological phosphorus removal. In: *Proceedings of the Research Symposia 'Biological Treatment'. 54th Annual Water Pollution Control Federation Conference*. Detroit, Michigan. October 4-9. 1-16.

Shapiro, J. (1967) Induced rapid release and uptake of phosphate by microorganisms. *Science* 155, 1269-1271.

Shapiro, J., Levin, G.V. and Zea G., H. (1967) Anoxically induced release of phosphate in wastewater treatment. *J. Water Pollut. Control Fed.* 39, 1810-1818.

Shin, H.-S. and Jun, H. -B. (1992) Development of excess phosphorus removal characteristics in a sequencing batch reactor. *Water Sci. Technol.* 25, 4-5, 433-440.

Siebritz, I.P., Ekama, G.A. and Marais, G.v.R. (1983) A parametric model for biological excess phosphorus removal. *Water Sci. Technol.* 15, 3/4, 127-152.

Simpkins, M.J. and McLaren, A.R. (1978) Consistent biological phosphate and nitrate removal in an activated sludge plant. *Prog. Water Technol.* 10, 433-442.

Skalsky, D.S. and Daigger, G.T. (1995) Wastewater solids fermentation for volatile acid production and enhanced biological phosphorus removal. *Water Environ. Res.* 67, 230-237.

Smalls, I.C., Cattell, F.C.R., Bek, P.A. and Hatfield, E.R. (1990) Eutrophication criteria for water quality management in Australia. In: *Proceedings of the 1st Australian Conference on BPR from Sewage*. Bendigo, Victoria, Australia. 1-10.

Smolders, G.J.F., van der Meij, J. and van Loosdrecht, M.C. M. (1994a) Model of the anaerobic metabolism of the biological phosphorus removal process: stoichiometry and pH influence. *Biotech. Bioeng.* 43, 461-470.

Smolders, G.J.F., van Loosdrecht, M.C.M. and Heijnen, J.J. (1994b) pH: keyfactor in the biological phosphorus removal process. *Water Sci. Technol.* 29, 7, 71-74.

Soddell, J.A., Beacham, A.M. and Seviour, R.J. (1993) Phenotypic identification of non-clinical isolates of *Acinetobacter* species. *J. Appl. Bacteriol.* 74, 210-214.

Somiya, I., Tsuno, H. and Matsumoto, M. (1988) Phosphorus release-storage reaction and organic substrate behaviour in biological phosphorus removal. *Water Res.* 22, 49-58.

Srinath, E.G., Sastry, C.A. and Pillai, S.C. (1959) Rapid removal of phosphorus from sewage by activated sludge. *Experientia* 15, 339-340.

Stanier, R.Y., Adelberg, E.A. and Ingraham, J.L. (1976) In: *General Microbiology*. 4th ed. Prentice-Hall, USA. 615-617.

Stephenson, T. (1987) *Acinetobacter*: its role in biological phosphate removal. In: *Biological Phosphate Removal from Wastewaters. Advances in Water Pollution Control*. (Ramadori, R. ed.) Pergamon Press, Oxford. 313-316.

Streichan, M. and Schön, G. (1991) Periplasmic and intracytoplasmic polyphosphate and easily washable phosphate in pure cultures of sewage bacteria. *Water Res.* 25, 9-13.

Streichan, M., Golecki, J.R. and Schön, G. (1990) Polyphosphate-accumulating bacteria from sewage plants with different processes for biological phosphorus removal. *FEMS Microbiol. Ecol.* 73, 113-124.

Suresh, N., Warburg, R., Timmerman, M., Wells, J., Coccia, M., Roberts, M.F. and Halvorson, H.O. (1985) New strategies for the isolation of microorganisms responsible for phosphate accumulation. *Water Sci. Technol.* 17, 11/12, 99-111.

Takeuchi, J. (1991) Influence of nitrate on the bacterial flora of activated sludge under anoxic condition. *Water Sci. Technol.* 23, 4/6, 765-772.

Tijssen, J.P.F. and van Steveninck, J. (1984) Detection of a yeast polyphosphate fraction localized outside the plasma membrane by the method of phosphorus-31 nuclear magnetic resonance. *Biochem. Biophys. Res. Commun.* 119, 447-451.

Tjernberg, I. and Ursing, J. (1989) Clinical strains of *Acinetobacter* classified by DNA-DNA hybridization. *APMIS*, 97, 595-605.

Toerien, D.F., Gerber, A., Lötter, L.H. and Cloete, T.E. (1990) Enhanced biological phosphorus removal in activated sludge systems. In: *Advances in Microbial Ecology*. (Marshall ed.) New York, Plenum Press. Vol 11, 173-230.

Tomei, M.C., Di Pinto, A.C., Passino, R. and Ramadori, R. (1993) Biological phosphorus removal: a simplified design model. *Environ. Technol.* 14, 331-340.

Towner, K.J. and Chopade, B.A. (1987) Biotyping of *Acinetobacter calcoaceticus* using the API 20NE system. *J. Hosp. Infect.* 10, 145-151.

Towner, K.J., Bergogne-Bérézin, E. and Fewson, C.A. (1991) *Acinetobacter*: portrait of a genius. In: *The Biology of Acinetobacter* (Towner, K.J., Bergogne-Bérézin, E. and Fewson, C.A. eds.) Plenum Press, New York and London. 1-24.

Tracy, K.D. and Flammino, A. (1987) Biochemistry and energetics of biological phosphorus removal. In: *Biological Phosphate Removal from Wastewaters. Advances in Water Pollution Control.* (Ramadori, R. ed.) Pergamon Press, Oxford. 15-26.

T'Seyen, J.T., Le Flohic, P., Faup, G.M., Meganck, M. and Block, J.C. (1985a) A separate acetate producing reactor to improve biological phosphorus removal. In: *Proceedings of the International Conference on Management Strategies for Phosphorus in the Environment.* (Lester, J.N. and Kirk, P.W.W. eds.) Lisbon, Portugal. 1-4 July. Selper Ltd., London. 214-220.

T'Seyen, J., Malnou, D., Block, J.C. and Faup, G. (1985b) Polyphosphate kinase activity during phosphate uptake by bacteria. *Water Sci. Technol.* 17, 11/12, 43-56.

Tuft, R. (1990) Filamentous and phosphorus bacteria in biological nutrient removal systems : their relation to process performance. In: *Proceedings of the 1st Australian Conference on BPR.* Bendigo, Victoria, Australia. 125-133.

Ubukata, Y. (1994) Some physiological characteristics of a phosphate removing bacterium isolated from anaerobic / aerobic activated sludge. *Water Sci. Technol.* 30, 6, 229-235.

Ubukata, Y. and Takii, S. (1994) Induction ability of excess phosphate accumulation for phosphate removing bacteria. *Water Res.* 28, 247-249.

USEPA - US Environmental Protection Agency (1987) *Design Manual for Phosphorus Removal*. EPA/625/1-87/001. U.S. EPA, Center for Environmental Research Information, Cincinnati, OH, 45268.

Vacker, D., Connell, C.H. and Wells, W.N. (1967) Phosphate removal through municipal wastewater treatment at San Antonio, Texas. *J. Water Pollut. Control Fed.* 39, 750-771.

Van Groenestijn, J.W. and Deinema, M.H. (1985) Effects of cultural conditions on phosphate accumulation and release by *Acinetobacter* strain 210A. In: *Proceedings of the International Conference on Management Strategies for Phosphorus in the Environment*. (Lester, J.N. and Kirk, P.W.W. eds) Lisbon, Portugal. 1-4 July. Selper Ltd., London. 405-410.

Van Groenestijn, J.W., Deinema, M.H. and Zehnder, A.J.B. (1987) ATP production from polyphosphate in *Acinetobacter* strain 210A. *Arch. Microbiol.* 148, 14-19.

Van Groenestijn, J.W., Zuidema, M., van de Worp, J.J.M., Deinema, M.H. and Zehnder, A.J.B. (1989a) Influence of environmental parameters on polyphosphate accumulation in *Acinetobacter* sp. *Antonie van Leeuwenhoek* 55, 67-82.

Van Groenestijn, J.W., Bentvelsen, M.M.A., Deinema, M.H. and Zehnder, A.J.B. (1989b) Polyphosphate-degrading enzymes in *Acinetobacter* spp. and activated sludge. *Appl. Environ. Microbiol.* 55, 219-223.

Van Landschoot, A., Rossau, R. and De Ley, J. (1986) Intra- and intergeneric similarities of ribosomal ribonucleic acid cistrons of *Acinetobacter*. *Int. J. Syst. Bacteriol.* 36, 150-160.

Van Starkenburg, W., Rensink, J.H. and Rijs, G.B.J. (1993) Biological P-removal: state of the arts in the Netherlands. *Water Sci. Technol.* 27, 5-6, 317-328.

Van Veen, H.W., Abee, T., Kortstee, G.J.J., Konings, W.N. and Zehnder, A.J.B. (1993) Characterization of two phosphate transport systems in *Acinetobacter johnsonii* 210A. *J. Bacteriol.* 175, 200-206.

Vasiliadis, G., Duncan, A., Bayly, R.C. and May, J.W. (1990) Polyphosphate production by strains of *Acinetobacter*. *FEMS Microbiol. Letts.* 70, 37-40.

Vassos, T.D., Oldham, W.K. and Rabinowitz, B. (1987) The influence of low temperature on biological phosphorus removal at Kelowna, Canada. In: *Biological Phosphate Removal from Wastewaters. Advances in Water Pollution Control.* (Ramadori, R. ed.) Pergamon Press, Oxford. 343-347.

Venter, S.N., Lötter, L.H., de Haas, D.W. and MacDonald, L. (1989) The use of the analytical profile index in the identification of activated sludge bacteria: Problems and solutions. *Water S.A.* 15, 4, 265-267.

Vierkant, M.A., Martin, D.W. and Stewart, J.R. (1990) Poly- β -hydroxybutyrate production in eight strains of the genus *Acinetobacter*. *Can. J. Microbiol.* 36, 657-663.

Vinçonneau, J.C., Schaack, F., Boschet, A.F., Chevalier, D., Villesot, D., Jaubert, M., Laval, Ch. and Lambert, S. (1985) The problem of phosphorus in France - its presence in natural waters and biological phosphorus removal. *Water Sci. Technol.* 17, 11/12, 1-9.

Vivian, A., Hinchliffe, E. and Fewson, C.A. (1981) *Acinetobacter calcoaceticus*: some approaches to a problem. *J. Hosp. Infection*, 2, 199-203.

Vlekke, G.J.F.M., Comeau, Y. and Oldham, W.K. (1988) Biological phosphate removal from wastewater with oxygen or nitrate in sequencing batch reactors. *Environ. Technol. Letts.* 9, 791-796.

Vollenweider, R.A. (1985) Phosphorus, the key element in eutrophication control. In: *Proceedings of the International Conference on Management Strategies for Phosphorus in the Environment*. (Lester, J.N. and Kirk, P.W.W. eds.) Lisbon, Portugal. 1-4 July. Selper Ltd., London. 1-10.

Wagner, M., Amann, R., Lemmer, H. and Schleifer, K.-H. (1993) Probing activated sludge with oligonucleotides specific for Proteobacteria: inadequacy of culture-dependent methods for describing microbial community structure. *Appl. Environ. Microbiol.* 59, 1520-1525.

Wagner, M., Erhart, R., Manz, W., Amann, R., Lemmer, H., Wedi, D. and Schleifer, K.H. (1994a) Development of an rRNA-targeted oligonucleotide probe specific for the genus *Acinetobacter* and its application for in situ monitoring in activated sludge. *Appl. Environ. Microbiol.* 60, 792-800.

Wagner, M., Amann, R., Lemmer, H., Manz, W. and Schleifer, K.H. (1994b) Probing activated sludge with fluorescently labelled rRNA targeted oligonucleotides. *Water Sci. Technol.* 29, 7, 15-23.

Warskow, A.L. and Juni, E. (1972) Nutritional requirements of *Acinetobacter* strains isolated from soil, water and sewage. *J. Bacteriol.* 112, 1014-1016.

Wayne, L.G., Brenner, D.J., Colwell, R.R., Grimont, P.A.D., Kandler, O., Krichevsky, M.I., Moore, W.E.C., Murray, R.G.E., Stackebrandt, E., Starr, M.P. and Truper, H.G. (1987) Report of the Ad Hoc Committee on the reconciliation of approaches to bacterial systematics. *Int. J. Syst. Bacteriol.* 37, 463-464.

Wedi, D. (1992) Effects of an activated primary settling tank on biological phosphorus removal. *Water Sci. Technol.* 26, 9-11, 2199-2202.

Wedi, D., Wagner, M., Amann, R., Erhart, R., Lemmer, H. and Wilderer, P.A. (1995) In-situ-nachweis der untergeordneten rolle von bakterien der gattung *Acinetobacter* bei der erhöhten biologischen P-elimination. *gwf-wasser / abwasser* 136, 13, 17-23.

Wentzel, M.C., Dol, P.L., Ekama, G.A. and Marais G.v.R. (1985) Kinetics of biological phosphorus release. *Water Sci. Technol.* 17, 11/12, 57-71.

Wentzel, M.C., Lötter, L.H., Loewenthal, R.E. and Marais, G.v.R. (1986) Metabolic behaviour of *Acinetobacter* spp. in enhanced biological phosphorus removal - a biochemical model. *Water S.A.* 12, 4, 209-224.

Wentzel, M.C., Loewenthal, R.E., Ekama, G.A. and Marais, G.v.R. (1988) Enhanced polyphosphate organism cultures in activated sludge systems - Part 1: Enhanced culture development. *Water S.A.* 14, 2, 81-92.

Wentzel, M.C., Ekama, G.A., Loewenthal, R.E., Dol, P.L. and Marais, G.v.R. (1989) Enhanced polyphosphate organism cultures in activated sludge systems. Part II: Experimental behaviour. *Water S.A.* 15, 2, 71-88.

Wentzel, M.C., Lötter, L.H., Ekama, G.A., Loewenthal, R.E. and Marais, G.v.R. (1991) Evaluation of biochemical models for biological excess phosphorus removal. *Water Sci. Technol.* 23, 4/6, 567-576.

Wentzel, M.C., Ekama, G.A. and Marais, G.v.R. (1992) Processes and modelling of nitrification denitrification biological excess phosphorus removal systems - a review. *Water Sci. Technol.* 25, 6, 59-82.

Whittaker, P.A. (1971) Terminal respiration in *Moraxella lwoffii* (NCIB8250). *Microbios.* 4, 65.

Wiame, J.M. (1947) Etude d'une substance polyphosphoreé, basophile et metachromatique chez le levures. *Biochim. Biophys. Acta.* 1, 234-255.

Witt, P.Ch., Grabowski, F. and Hahn, H.H. (1994) Interactions between biological and physico-chemical mechanisms in biological phosphate elimination. *Water Sci. Technol.* 30, 6, 271-279.

Woese, C.R., Weisburg, W.G., Hahn, C.M., Paster, B.J., Zablen, L.B., Lewis, B.J., Macke, T.J., Ludwig, W. and Stackebrandt, E. (1985) The phylogeny of purple bacteria: the gamma subdivision. *Syst. Appl. Microbiol.* 6, 25-33.

Wood, H.G. and Clark, J.E. (1988) Biological aspects of inorganic polyphosphates. *Ann. Rev. Biochem.* 57, 235-260.

WRCSA - Water Research Commission (South Africa) (1984) *Theory, Design and Operation of Nutrient Removal Activated Sludge Processes*. WRC, Pretoria, SA.

Yall, I, Boughton, W.H., Knudsen, R.C. and Sinclair, N.A. (1970) Biological uptake of phosphorus by activated sludge. *Appl. Microbiol.* 20, 1, 145-150.

Yashphe, J., Chikarmane, H., Iranzo, M. and Halvorson, H.O. (1992) Inorganic phosphate transport in *Acinetobacter lwoffii*. *Curr. Microbiol.* 24, 275-280.

Ye, Q., Ohtake, H. and Toda, K. (1988) Phosphorus removal by pure and mixed cultures of microorganisms. *J. Ferment. Technol.* 66, 207-212.

Yeoman, S., Hunter, M., Stephenson, T., Lester, J.N. and Perry, R. (1988) An assessment of excess biological phosphorus removal during activated sludge treatment. *Environ. Technol. Letts.* 9, 637-646.

Yeoman, S., Lester, J.N. and Perry, R. (1993) Phosphorus removal and its influence on metal speciation during wastewater treatment. *Water Res.* 27, 389-395.

APPENDIX I

Our ref: ID 2595/NCID 3426

Your ref: 09/TS/666769

Date: 18 May 1994

Client: Ms. Marion Jones,
School of Water Sciences,
Cranfield Institute of Technology,
Cranfield,
Bedford.
MK43 0AL

IN CONFIDENCE

PRELIMINARY EXAMINATION

OF

TWO ISOLATES

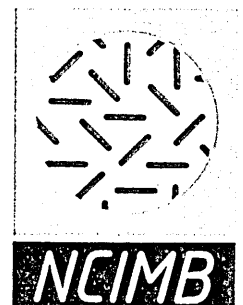
Signed: *Morag Hamilton*
Morag Hamilton

Authorised and approved on behalf of NCIMB Ltd: *Alison Baxter*
Alison J. Baxter

This report shall not be reproduced except in full, without the approval of NCIMB Ltd., and applies only to the cultures listed overleaf.

Furthermore, nothing in this report shall be taken to imply any endorsement by NCIMB Ltd as to the fitness for purpose of any product to which the report applies.

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75:DB10271



For: Ms. Marion Jones,
School of Water Sciences, 240
Cranfield Institute of Technology.

Source of Isolates: Isolate A Sewage
Isolate B Waste Water Treatment Plant

Cultures received 4 May 1994
Cultures passed for analysis 6 May 1994

Morphological descriptions are from growth on Lab M nutrient agar except as stated

Isolate Code	A	B
$^{\circ}\text{C}$ Incubation	30 $^{\circ}\text{C}$	30 $^{\circ}\text{C}$
Cell morphology X630	Photographs to follow	
Gram stain	-	-
Spores	-	-
Motility	+	+
Colony morphology 24 hours	colonies punctiform (<1mm in diameter), white, opaque, pulvinate, smooth, glossy, low convex.	colonies punctiform (<1mm in diameter), round, regular, entire, low convex, smooth, glossy, white, opaque.
$^{\circ}\text{C}$ Growth	37 $^{\circ}\text{C}$ (+) 45 $^{\circ}\text{C}$ - 41 $^{\circ}\text{C}$ +	37 $^{\circ}\text{C}$ + 45 $^{\circ}\text{C}$ - 41 $^{\circ}\text{C}$ +
Catalase	+	+
Oxidase	-	-
Fermentative in Glucose OF	- (Alkaline)	- (Alkaline)
Preliminary Identification	See attached sheet	

REPORT ON PRELIMINARY EXAMINATION NO:- 2595

Strain No.	Possible Identity	Proposed Further Tests	Possible Outcome	Estimated Cost of Further Work	Notes
A) B)	<u>Acinetobacter</u> species	Biochemical	genetic group of possibly species determined	£100.00-£125.00 per isolate + VAT	

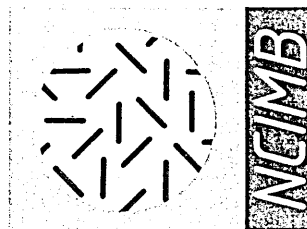
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with compliments

2595-A

2595-B



SERVICES TO
MICROBIOLOGY

Cells are 48 hrs on Lab 8 at 30°C by
Phase Contrast.