IDENTIFICATION OF POLYPHOSPHATE ACCUMULATING BACTERIA FROM PILOT- AND FULL-SCALE NUTRIENT REMOVAL ACTIVATED SLUDGES

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September 1999

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Dissertation submitted in compliance with the requirements for the Master's Degree in Technology in the Department of Biotechnology, Technikon Natal, Durban

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BLAISE WILLIAM ATKINSON

I hereby declare that the dissertation represents my own work. It has not been submitted before for any diploma/degree or examination at any other Technikon/University.

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BLAISE ATKINSON

I hereby approve the final submission of the following dissertation.

_____

MR FÁIZAL BUX
SUPERVISOR
M.TECH: BIOTECHNOLOGY (TECHNIKON NATAL)

this 23 day of SEPTEMBER, 1999, at Technikon Natal.

DEDICATION

This dissertation has been prepared and written with new students to the field of activated sludge biological nutrient removal in mind.

I hope you find this work informative and wish you the best with your studies.

....and, of course, Mom and Dad....

BW ATKINSON

ABSTRACT

General removal of phosphorus (P) from wastewater was introduced in Scandanavia in the late 1960's. At that time it was believed that P alone was limiting to algal growth and that the sole removal of P would solve the problem of eutrophication. However, we now know that both P and nitrogen (N) contribute to this deleterious effect and as such, much research has been conducted concerned with both the biological and chemical removal of these nutrients from sewage effluents. Enhanced biological phosphorus removal (EBPR), which is basically the biological accumulation of soluble P (as polyphosphate or poly-P) from the bulk liquid in excess of normal metabolic requirements, still tends to be sensitive to many external parameters and, as such, is subject to fluctuations. This makes it extremely difficult for wastewater treatment installations to achieve and maintain full compliance with strict discharge regulations. A more comprehensive understanding of the microbial community within the mixed liquor of a wastewater treatment system is therefore required which will ultimately assist in improving system design and performance.

Chemical and civil engineers, when designing biological wastewater treatment systems, consider only the processes (biological or chemical) taking place within the reactor/s with little or no regard for the individual microbial species or the entire microbial community involved. Process design appears to be tackled empirically from a 'black box' approach; biological reactions or processes occurring within a system such as wastewater treatment are all lumped together and attributed to a single surrogate organism ie., the response of the surrogate to certain stimuli accounts for the total system response. This is similar to an analogy which Professor George Ekama (Dept of Civil Engineering, UCT), a leading scientist in wastewater treatment and process design, refers to where engineers, if, for example, are confronted with modelling the dynamics of carbon dioxide utilisation of a forest, would recognise the accumulative system response and not give cognisance to each individual tree's contribution. It is true that if one had to consider every microbial species present in a highly organised community such as activated sludge, process models, designed to make quantitative and qualitative predictions as to the expected effluent quality from a particular design, would become increasingly complex and superfluous. It is evident from the countless accomplishments that engineers have succeeded, to a certain degree, in modelling wastewater treatment systems. One only has to consider the tremendous success of biological P (bio-P) removal and nitrification/denitrification processes at full-scale. However, there are limitations to this empirical approach and EBPR processes occasionally deteriorate in phosphate removal efficiency. In order to further optimise biological processes, whether they be organics oxidation, bio-P removal, nitrification or denitrification, biological community analyses will have to play a more significant role in design. The better microbial community structure and function is understood, the better the control and management of the system. With the advent of improved microbial identification and enumeration (to a certain extent) techniques (*in situ*), it was considered significant to investigate the mechanism of bio-P removal and to elucidate which bacteria are actively responsible for this process.

To this end, experimental work was conducted in two phases: ① laboratory, where samples of mixed liquor were obtained from a full-scale wastewater treatment facility exhibiting biological nutrient removal (BNR) characteristics and ② pilot plant, where an enhanced culture of polyphosphate accumulating organisms (PAO's) was developed and probed using molecular identification and enumeration techniques (as well as a cultivation-dependent approach). During phase ① of experimentation, mixed liquor samples were taken from the aerobic zone of Darvill Wastewater Works (WWW, Pietermaritzburg, Kwa-Zulu Natal, South Africa) and cultivated on solid Casitone Glycerol Yeast Autolysate (CGY) agar. Isolation of the various bacterial components ensued using conventional plating techniques. All isolates were screened for their ability to accumulate poly-P through successive anaerobic/aerobic incubation and only those isolates displaying the desirable P transformations ie., anaerobic P release and aerobic P uptake, were subsequently identified. Identification was achieved using the Gram stain, various qualitative biochemical tests and the API 20NE identification system. Results showed unequivocal dominance of the gamma subclass of the *Proteobacteria* cluster (*Proteobacteria* contains the majority of the traditional Gram negative organisms). At genus level, the Pseudomonads appeared to dominate the PAO bacterial community of the full-scale plant under investigation. Although *Acinetobacter* spp. accumulated large quantities of poly-P from the phosphate enriched liquid media, their low recovery rates on solid agar media did not warrant their implication as the organisms central to phosphate uptake and removal from the activated sludge system in

question.

An enhanced culture of PAO's, at pilot-scale, was successfully developed during phase ❷ of experimentation by incrementally increasing the acetate fraction whilst concomitantly decreasing the settled sewage fraction to the feed influent of the laboratory unit. The pilot plant, modelled on the 3-stage Phoredox process, was seeded with mixed liquor obtained from a non-EBPR single aerobic full-scale activated sludge plant (Amanzimtoti WWW, Kwa-Zulu Natal South Coast, South Africa) in order to primarily investigate population dynamics and to monitor changes (if any) in the bacterial community structure when subjected to EBPR conditions. Community analyses of both activated sludge systems were performed using both conventional cultivation and molecular techniques, involving rRNA-targeted fluorescently-labelled oligonucleotide probes (fluorescent *in situ* hybridization or FISH). The probes were complementary to conserved regions of the rRNA of the alpha (ALF), beta (BET), gamma (GAM) subclasses of the *Proteobacteria*, gram positive bacteria with a high G + C DNA content (GPBHGC), the *Cytophaga-Flavobacterium* (CF) cluster and *Acinetobacter* (ACA). Total cell counts using membrane filtration and the specific DNA stain 4',6-diamidino-2-phenylindole (DAPI) were also determined.

Due to the controversy regarding the bias (in terms of the resulting microbial community structure) imposed by cultivation techniques (viable but non-culturable organisms; nutrient rich media selection of fast growing organisms; spatial distribution of organisms in the natural environment cannot be elucidated), a direct comparison between plating and whole cell hybridization was done to assess the degree of variation with respect to the community structure. Application of FISH to the enhanced culture resulted in the dominance of the alpha subclass of the *Proteobacteria*, followed by the gamma and beta *Proteobacteria* subclasses. The *Cytophaga-Flavobacterium* and GPBHGC clusters were also detected but their counts were much lower than those of the above-mentioned groups. *Pseudomonas* spp., using plating, appeared to dominate the bacterial community in the enhanced culture. Plating also resulted in the total dominance of the gamma subclass of *Proteobacteria* in all the activated sludge mixed liquors investigated. *Acinetobacter* spp. constituted *ca.* 3% of all bacteria in the aerobic zone of the enhanced culture using FISH (anaerobic and anoxic reactor results were very similar). Total cell counts between direct and indirect methods of analysis resulted in differences of three orders of magnitude, emphasising the inability of cultivation techniques to recover all bacteria from environmental samples.

Results of this study indicated the importance of the *Proteobacteria* (namely the alpha subclass) to EBPR operations. The dominance of *Acinetobacter* spp. in EBPR activated sludge was never demonstrated, even when using plating techniques. It therefore stands to reason that the role of this genus in EBPR operations has been exaggerated and that bacteria other than *Acinetobacter* are involved. Due to its ubiquity and diverse metabolic ability, it appears that *Pseudomonas* spp. are responsible for a significant fraction of phosphate removal from the EBPR activated sludge plants investigated ie., Darvill WWW and the developed enhanced PAO culture.

PREFACE

Some of the material presented in this dissertation has/will been/be published and presented elsewhere:

Atkinson, BW, Mudaly, DD and Bux, F (1999) Influencing activated sludge community structures to enhance the biological phosphorus removal mechanism - Part 1: Culture development and other considerations. *Water SA* (submitted).

Mudaly, DD, Atkinson, BW and Bux, F (1999) Influencing activated sludge community structures to enhance the biological phosphorus removal mechanism - Part 2: Community analysis using a culture-independent approach. *Water SA* (submitted).

Water Research Commission (1999) Investigation of the microbial contribution to nutrient removal in activated sludge wastewater treatment processes. *WRC Report No.* **K5/822** (in prep.).

Sidat, M, Bux, F and Kasan, HC (1999) Laboratory-scale investigation of biological phosphate removal from municipal wastewater. *Water SA* (in press).

Sidat, M, Bux, F and Kasan, HC (1999) Polyphosphate accumulation by bacteria isolated from activated sludge. *Water SA* **25**(2): 175-179.

Atkinson, BW, Bux, F and Kasan, HC (1997) Characterisation and function of phosphate accumulating bacteria present in an activated sludge plant modelled upon the NDBEPR JHB system. Oral presentation at Joint Natal Biochemistry and Microbiology Symposium, 15th - 17th October, 1997, Innovation Centre, University of Natal, Durban, South Africa.

Atkinson, BW, Bux, F and Kasan, HC (1998) The contribution of *Acinetobacter* spp and other poly-P organisms to biological phosphorus removal operations. Poster presentation at The Water Institute of Southern Africa Biennial Conference and Exhibition, 4th - 7th May, 1998, Baxter Theatre Complex, Cape Town, South Africa.

Atkinson, BW, Bux, F and Kasan, HC (1999) Enhancement of polyphosphate accumulating bacteria in an activated sludge system. Oral presentation at the African International Environmental Protection Symposium, 4th - 8th July, 1999, Imperial Hotel, Pietermaritzburg, South Africa.

Mudaly, DD, Atkinson, BW, Bux, F, Brözel, VS and Kasan, HC (1999) Determination of bacteria predominating in a pilot-scale enhanced biological phosphorus removal activated sludge process using a cultivation-independent approach. Oral presentation at the African International Environmental Protection Symposium, 4th - 8th July, 1999, Imperial Hotel, Pietermaritzburg, South Africa.

ACKNOWLEDGEMENTS

I would like to sincerely thank the following people for their assistance in the completion of this project:

- Professor Hamanth Kasan and Mr Faizal Bux for guiding, understanding and supporting me over the years which we have worked together;
- Mohammed Sidat and Delon Mudaly for their assistance with certain aspects of the research;
- Paul Gaydon, Dave de Haas and management of Umgeni Water for their permission to allow me to complete my studies with the use of the laboratory-scale unit at Darvill Wastewater Works. Special thanks to Paul and Dave for your invaluable assistance and advice;
- The Steering Committee appointed by the Water Research Commission for your invaluable guidance. A special mention must be made of Dr Mjoli (Chairperson, Steering Committee) for her support and encouragement. Thank you to the Water Research Commission (Pretoria, South Africa) for funding the project and the National Research Foundation (NRF, formerly the FRD) for the personal bursary;
- Melanie Bekink, Patti Joyce, Merle Smuts and Simon Makhavhu of Umgeni Water (Analytical Services, Darvill Wastewater Works, Pietermaritzburg) for all the time spent on the completion of the various analyses. Brian and Melanie - thanks for your great help looking after the unit for me on those many weekends. Thank you to Sue Freese and Rose Smith for your help and patience. Thanks must also be extended to Zakhele Jikazi who guided me through the setup of the unit;
- Prof. Ekama (Dept of Civil Engineering, UCT) and Prof. Cloete (Dept of Microbiology and Plant Pathology, UP) for their invaluable comments and assistance. Their insight and ability in the field of wastewater treatment is held in the highest regard by the author; and
- Lastly, but definitely not least, my friends and family for your support over the duration of this particular project and my studies. In particular, my mother, sister and brother-in-law but especially to you Mum for giving me the opportunity to get this far. This work is also dedicated to my Dad. My love and special thanks to Hayley for your encouragement and support through the tough times. You took the brunt of my frustration, accepted it and returned only compassion. Thank you!

BW ATKINSON

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CHAPTER TWO

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ACA	oligonucleotide probe specific for *Acinetobacter* spp.
ADP	adenosine diphosphate
AE	aerobic reactor
ALF	oligonucleotide probe specific for the alpha subclass of the *Proteobacteria*
AN	anaerobic reactor
API	Analytical Profile Index
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
AX	anoxic reactor
BEPR	biological excess phosphorus removal
BET	oligonucleotide probe specific for the beta subclass of the *Proteobacteria*
bio-P	biological phosphorus
BNR	biological nutrient removal
BPR	biological phosphorus removal
CF	oligonucleotide probe specific for the *Cytophaga-Flavobacterium-Bacteroides*
CFU	colony forming unit
CGY	casitone glycerol yeast autolysate agar
COD	chemical oxygen demand
COD_{sol}	total soluble COD
CWWR	Centre for Water and Wastewater Research
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
DO	dissolved oxygen
DSVI	dilute sludge volume index

DWA	Department of Water Affairs
EBPR	enhanced biological phosphorus removal
EFF	effluent
EUB	oligonucleotide probe specific for a conserved region on the 16S rRNA of *Bacteria*
f_{bs}	reactor biomass fraction
f_{bsi}	biodegradable, soluble influent COD fraction
f_{bpi}	biodegradable, particulate influent COD fraction
f_{ts}	total readily biodegradable COD fraction
f_{usi}	unbiodegradable, soluble influent COD fraction
f_{upi}	unbiodegradable, particulate influent COD fraction
f_{xa}	anaerobic mass fraction
FISH	fluorescent *in situ* hybridization
F/M	food:microorganism ratio
fMLTP	filtered mixed liquor total phosphorus
GAM	oligonucleotide probe specific for the gamma subclass of the *Proteobacteria*
GAO	glycogen accumulating organism
GPBHGC	Gram positive bacteria with high G + C DNA content
GPBLGC	Gram positive bacteria with low G + C DNA content
HAc	acetate
HGC	oligonucleotide probe specific for Gram positive bacteria with high G + C DNA content
HRT	hydraulic retention time
INF	influent
MCRT	mean cell retention time
ML	mixed liquor
MLOSS	mixed liquor organic suspended solids
MLSS	mixed liquor suspended solids

MLTP	mixed liquor total phosphorus
MLVSS (VSS)	mixed liquor volatile suspended solids (volatile suspended solids)
N	nitrogen
N_{ti}	total influent nitrogen
NaAc	sodium acetate
NDBEPR	nitrification denitrification biological excess phosphorus removal
ortho-P	orthophosphorus (PO_4^{3-})
OUR	oxygen utilization rate
P	phosphorus
P_{ti}	total influent phosphorus
PAO	phosphorus accumulating organism
PC	personal computer
PCR	polymerase chain reaction
PHA	polyhydroxyalkonoates
PHB	poly-β-hydroxybutyrate
poly-P	polyphosphate
Q	flow rate
Q_i	influent flow rate
R_s	sludge age
RBCOD	readily biodegradable COD
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
S_{bpi}	biodegradable, particulate influent COD
S_{bsi}	biodegradable, soluble influent COD
S_i	unbiodegradable soluble COD
S_{te}	total effluent COD

S_{ti}	total influent COD
SBCOD	slowly biodegradable COD
SCFA	short chain fatty acid
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SRP	soluble reactive phosphorus
SRT	solids retention time
SV_{30}	sludge volume after 30 min settling
SVI	sludge volume index
TCA	tricarboxylic acid
TKN	Total Kjeldahl Nitrogen
TP	total phosphorus
TSS	total suspended solids
UCT	University of Cape Town
V	volume
VFA	volatile fatty acid
VM	vanadate-molybdate
WWW	wastewater works

CHAPTER ONE

GENERAL INTRODUCTION AND SCOPE OF RESEARCH

1.1 DEATH OF A DAM

Eutrophication is a natural ageing process which usually occurs in lakes and other quiescent bodies of water through the introduction of the plant nutrients, phosphorus (P) and nitrogen (N), to the impoundment. Without human intervention, the process takes place over hundreds of years, but is greatly accelerated by various human activities in sensitive areas. Eutrophication of natural and man-made water impoundments is a problem encountered in many countries, including South Africa. However, problems experienced in South Africa which promulgate eutrophication are long storage times of dams and reservoirs, high summer temperatures and long daylight hours (Bolitho, 1976).

A water impoundment can be classified according to its trophic status. 'Trophy' of a lake or dam basically describes the rate at which organic matter is supplied to or by the relevant impoundment per unit time (Wetzel, 1983). The term therefore refers to the 'productivity' of a water body ie., the amount of new organic biomass formed per unit time within the aquatic ecosystem. The trophic status of water is directly influenced by the concentration of P and N in solution (TABLES 1.1 and 1.2).

In contrast to the other nutritional components in the hydrosphere, P is the least abundant and usually limits biological productivity. The most significant form of P in lake systems is inorganic or orthophosphorus (ortho-P or PO_4^{3-}), the uptake of which satisfies metabolic requirements for living organisms (Muyima *et al.*, 1997). Organic P is then synthesised intracellularly. The sum of inorganic and organic forms of P in a body of water, both in particulate and solubilised form, is known as total phosphorus (TP). Release of phosphate from aquatic sediments is accomplished primarily by bacterial metabolism of organic matter at the bottom of the lake. This metabolism usually creates conditions conducive to the resolubilisation of P (decrease in ambient pH through production of

acid) (Wetzel, 1983). Low trophic states in an impoundment can exist despite high P loadings, however, provided water residence times are low (Thornton, 1980).

TABLE 1.1 Relationship of lake trophy status (productivity) to average concentrations of N (Wetzel, 1983).

Lake productivity	Inorganic N (μg/L)	Organic N (μg/L)
Ultra-oligotrophic	<200	<200
Oligo-mesotrophic	200-400	200-400
Meso-eutrophic	300-650	400-700
Eutrophic	500-1 500	700-1 200
Hypereutrophic	>1 500	>1 200

TABLE 1.2 Relationship of lake trophy status (productivity) to average concentrations of P (Wetzel, 1983).

Lake productivity	Total P (μg/L)
Ultra-oligotrophic	<5
Oligo-mesotrophic	5-10
Meso-eutrophic	10-30
Eutrophic	30-100
Hypereutrophic	>100

A classic example of eutrophication in the South African context is the deterioration of the Hartbeespoort Dam. Constructed 66 years ago, the dam became so overgrown with water hyacinth (60% of surface or 1 500 hectares) during the mid 1970's, that the Department of Water Affairs (DWA) was compelled to spend R200 000 on various rehabilitation programmes[1] (Rudd, 1979). Constant clearing of water weeds from the canal system amounted to

[1] Spraying the dam surface with copper sulphate and decreasing the pH of the water with sulphuric acid inhibits the growth of algae and other problematic plants.

R40 000 per annum. Hypereutrophic conditions intensified during the 1980's, resulting in algal hyperscums (crusts of algal biomass) which were concentrated up to a metre thick at the dam wall (Haarhoff *et al.*, 1992). However, through greater understanding of the prevailing conditions and implementation of intensive remedial programmes, the dam now enjoys oligotrophic status although speculators agree that the improvement may only be temporary.

Enrichment of water in an impoundment results in many undesirable effects, the primary effect being the establishment of profuse algal blooms and excessive growth of nuisance aquatic plants. These include several species of blue-green (*Cyanobacteria*) and green (*chlorophyta*) algae, the diatoms and flagellates, water grasses, rooted broad leaved plants and floating water plants (hyacinth) (Rudd, 1979). Joska and Bolton (1994) found *Cladophora glomerata* to be the major problem algal weed in South Africa. Secondary effects, which are a direct result of weed and algal growth, include rapid oxygen uptake from the water causing the lower water to become anaerobic; stratification (with respect to oxygen and temperature) of the water occurs due to the exclusion of sunlight and heat from the lower waters; production of methane and sulphides from the anaerobic digestion of dead plants which sink to the bottom of the dam; these acid anaerobic conditions which occur cause the release of bound phosphates, iron and manganese from sediments; production of sulphides results in fish kills (especially in winter) and the negative aesthetic appeal of the dam due to overturning of the water and the appearance that the water is black; sulphides also impart a strong rotten egg smell to the water; and the water can no longer be used for potable purposes as certain species of the algae are toxic (Rudd, 1979). Costs of purification of such over-fertilized waters escalate dramatically and the chemical quality of these waters becomes altered, resulting in the need for alternative treatment methods as well as a high chlorine demand. Many limnological studies have been conducted concerning eutrophication, concerned primarily with its causes and effects (Walmsley and Thornton, 1984; Chutter, 1990; Dillon and Molot, 1996) and the results have conclusively indicated that eutrophication is promoted if P and N are released into a reservoir or catchment area. These studies have also shown that eutrophication can be effectively controlled and curtailed if the P load to receiving waters is controlled. Gross eutrophication becomes marked when the inorganic soluble N and P concentrations in waters are in excess of 0.3 mgN/L and 0.015 mgP/L, respectively (Lilley *et al.*, 1997). It is virtually impossible to control eutrophication by limiting nitrogen due to the ability of the causative agents (algae) to fix and assimilate atmospheric N. Assimilated N is then made available to other aquatic life forms when these cells die and assimilated N is released. The increased awareness that P is the limiting nutrient

has led to the introduction of more stringent legislation governing the discharge of P to receiving waters.

It is not necessary to enforce nutrient limitation when effluents are to be used directly for irrigation purposes, industrial use or to be discharged directly to sea. Only if the effluent is to be discharged to a lake or impoundment where the effects of eutrophication are undesirable ie., sensitive catchment areas, is nutrient removal strictly enforced. Areas which, in 1979, were considered sensitive included the Vaal River from its catchment area to Bloemhof Dam; the upper Limpopo-Crocodile catchment; the catchments of the following dams: Rietvlei, Hartbeespoort, Roodeplaat, Klipvoor, Bon Accord, Vaalkop and Boskop; the Great Olifants River above Loskop Dam; the Umgeni; the Umlaas River; the Buffalo River from King William's Town to Bridle Drift Dam; the Great Berg River; and Atlantis (Rudd, 1979). In 1980, the DWA introduced legislation limiting the dissolved ortho-P content of point source discharges to seven sensitive catchments to 1 mgP/L (Government Gazette, 1984) [2]. However, a five year period of grace was permitted before legislation enforcement in order to encourage development of P removal technology and implementation. Excessive chemical P precipitation was not encouraged due to its mineralising effect on water. The mechanism of biological P removal (BPR) was studied intensely due to its attractive features, especially the low operating costs. During this period many existing wastewater treatment plants in South Africa were modified or new plants constructed to incorporate biological excess phosphorus removal (BEPR) [3]. Although the initial capital expenditure is high, BEPR operations are significantly lower (on a cost basis) to operate and maintain than chemical dosing plants, purely because chemical precipitants cannot be recovered and are expensive. However, although the BPR mechanism is now understood to a certain extent, it is still difficult to achieve full compliance with the Special P Standard and the effects of BPR often have to be complemented through simultaneous chemical dosing to remove residual P in the effluents.

1.2 REDUCING EFFLUENT NUTRIENT LOADS

In order to sustain life, the following elements are constantly required by organisms in assimilable quantities:

[2] This became known as the Special Phosphate Standard.
[3] Also referred to as enhanced biological phosphorus removal (EBPR).

hydrogen (H); oxygen (O); carbon (C); nitrogen (N); phosphorus (P); and sulphur (S) (Marais and Ekama, 1984). The chemical state of these elements in the earth's atmosphere is as a direct result of the metabolism of living organisms. Hydrogen and oxygen are readily available from water whilst carbon from carbon dioxide (CO_2). In the context of wastewater technology, limiting nutrients for growth are considered to be N, P and C (Lilley *et al.*, 1997).

1.2.1 Biogeochemical cycles

The pathways by which the nutrients mentioned above are circulated within an ecosystem are referred to as biogeochemical cycles. For each element, the cycling process basically involves (1) a reservoir, where the source normally remains unavailable to the surrounding biota ie., rocks and sediments; assimilable forms of the element are only released by various geological components; (2) an exchange pool, represented by an elemental source from which organisms are able to accumulate nutritional components ie., hydrosphere and atmosphere; and (3) a biotic community through which elements move along either simple or elaborate food chains (Mader, 1998). Nitrogen is involved in a gaseous cycle where the element is assimilated from and returned to the atmosphere. Phosphorus, however, is sedimentary where the element is absorbed from the soil by plant roots, exchanged to heterotrophs and returned to the soil by decomposers (Mader, 1998). As FIGS 1.1 and 1.2 show, both P and N are interchangable with reference to the ecosystems they inhabit, as both elements are capable of moving between terrestrial and aquatic systems.

1.2.1.1 The phosphorus cycle

FIGURE 1.1 refers. The weathering and mining of P-containing rocks makes phosphate ions (PO_4^{3-}) available to plants which accumulate the inorganic ion from the soil (FIG. 1.1; 1). Orthophosphate is the only directly utilisable form of soluble inorganic P. Some of the solubilised P is transferred from the terrestrial environment to the aquatic environment as a result of leaching. Aquatic algae take the ortho-P ion up (FIG. 1.1; 2a) before sedimentation can occur, converting it to the organic form (FIG. 1.1; 2b).

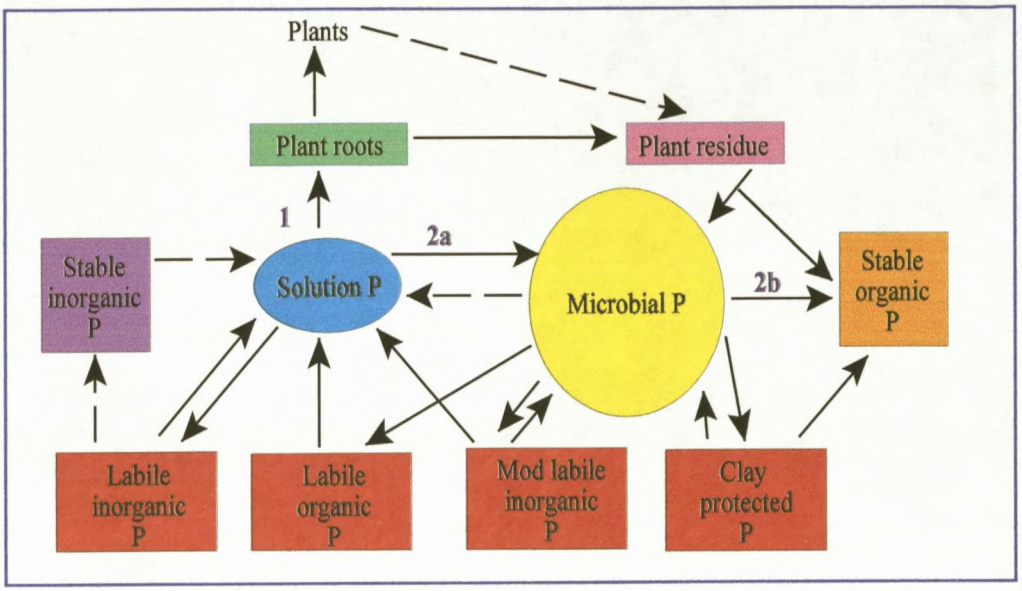

FIGURE 1.1 Schematic representation of the phosphorus cycle (adapted from Muyima *et al.*, 1997).

Organically bound P can then be utilised by heterotrophic organisms which feed off plants and algae, returning P to the extracellular environment once they die and are decomposed (Mader, 1998). Phosphate is referred to as a limiting nutrient because many of the sources consist of insoluble complexes such as calcium, iron and aluminium salts (Muyima, *et al.*, 1997). At any one time, freshwater supplies usually only contain trace amounts of phosphate ion. Human intervention which increases the quantity of phosphate in the environment includes the mining of phosphate ores for fertiliser production, animal feed supplementation and detergent production.

1.2.1.2 The nitrogen cycle

FIGURE 1.2 refers. Although N is in abundant supply, constituting 78-79% of the earth's atmosphere, it remains chemically inert as nitrogen gas (N_2), requiring tremendous amounts of energy to be utilised directly as a utilisable source of nutrient. The nitrogen cycle is a complex biochemical process in which N is structurally altered by fixation, assimilation and reduction (Wetzel, 1983). The majority of living organisms require a source of combined N products such as ammonia, nitrates and organic N for their nutrition. However, combined forms of N in soil and water are scarce.

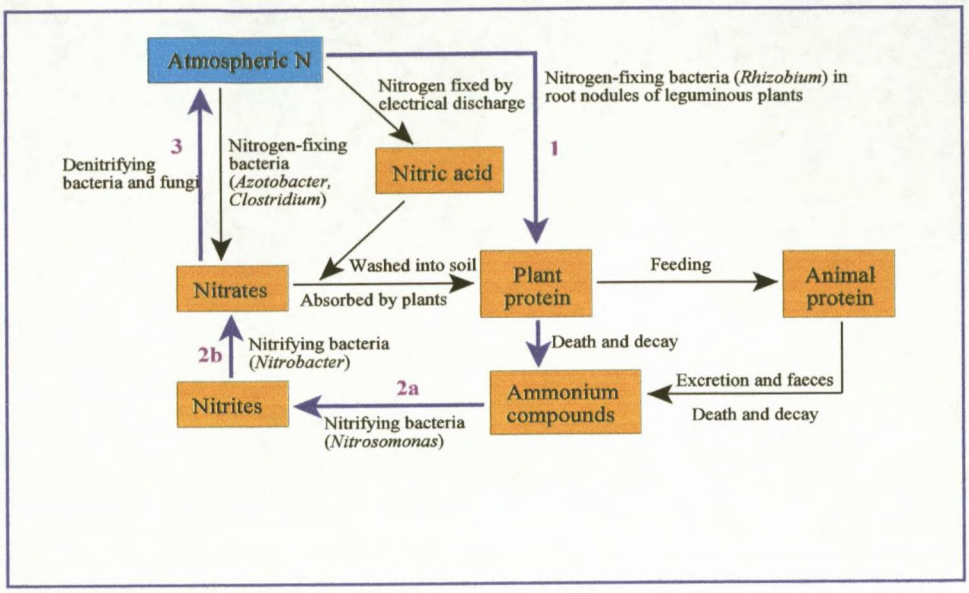

FIGURE 1.2 Schematic representation of the nitrogen cycle (adapted from Muyima *et al.*, 1997). Coloured arrows represent processes directly involved in eutrophication.

Dominant forms of N in fresh water include dissolved N_2, free or saline ammonia (NH_3/NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-) and various organic compounds such as amino acids, amines, nucleotides and proteins (Wetzel, 1983). Plants depend on various genera of bacteria which are capable of fixing atmospheric N to manufacture organic compounds (Mader, 1998). Various aquatic cyanobacteria are able to reduce N_2 directly to saline ammonia although fixation will only occur in any appreciable amounts once soluble inorganic compounds are severely depleted (Mader, 1998). In fact, the complete N cycle of water impoundments is microbial in nature.

Nitrogen fixation occurs when N_2 is reduced, making N and other organic products available to plants and microorganisms (FIG. 1.2; 1). The product of fixation, ammonia, enters the environment and is oxidised to nitrite and nitrate through a highly specialised process known as nitrification. It is only in these oxidised states that the majority of bacteria and plants are able to assimilate N. Nitrification is catalysed by two highly specialised groups of obligatory aerobic chemoautotrophic bacteria [4], commonly referred to as nitrifying bacteria.

[4] Chemoautotrophs derive energy for growth from the oxidation of inorganic compounds. In wastewater microbiology, the principal species of interest are the nitrifying bacteria which derive energy by oxidising saline ammonia to nitrite and nitrate.

Nitrification occurs in two stages: production of NO^-_2 from NH^+_4 (FIG. 1.2; 2a); and production of NO^-_3 from NO^-_2 (FIG. 1.2; 2b). As can be seen from the following equations, nitrification does not depend on the presence of N_2 at all:

$NH^+_4 + 1.5O_2 \rightarrow NO^-_2 + H_2O + 2H^+ + 250$ kJ (reaction 1)

$NO^-_2 + 0.5O_2 \rightarrow NO^-_3 + 75$ kJ (reaction2) (Wanner, 1997).

The autotrophic bacteria responsible for the oxidation of ammonia to nitrite [reaction 1] belong to the genera *Nitrosomonas*, *Nitrospira*, *Nitrosococcus* and *Nitrosolobus* (Bosch and Cloete, 1993). When applying *in situ* genome specific molecular techniques to an activated sludge displaying high nitrifying-denitifying capacity, Wagner *et al.*, (1998) found *Nitrosococcus mobilis* to be the dominant ammonia oxidiser. Constituting the nitrite oxidisers [reaction 2] are *Nitrobacter*, *Nitrospira* and *Nitrococcus* (Bosch and Cloete, 1993). Due to their high solubility, these oxidised forms of N are transported to water bodies and courses through terrestrial leachates. Nitrate, under anoxic conditions, can be used by various aerobic bacteria as an alternative terminal electron acceptor to oxygen. The reduction process of denitrification converts NO^-_3 back to N_2 (FIG. 1.2; 3). Denitrification usually counterbalances N fixation in a natural situation but with the advent of fertiliser production from N_2, fixation and therefore N availability to organisms far outweighs denitrification rates (Mader, 1998). The various oxidation states of N during nitrification/denitrification processes are shown in FIG. 1.3.

1.2.2 Origins of P

As this study is concerned primarily with different aspects of bio-P removal and to a much lesser degree, the processes of nitrification and denitrification, it will suffice to detail the sources of P contamination alone. Sources and repercussions of N contamination on the environment will only be discussed in the broad sense as it will often parallel that of P.

The primary sources of P pollution entering the environment can be categorised as either diffuse or point. Diffuse sources basically originate from rural and urban areas. Agricultural fertilizers contribute the greatest amount to

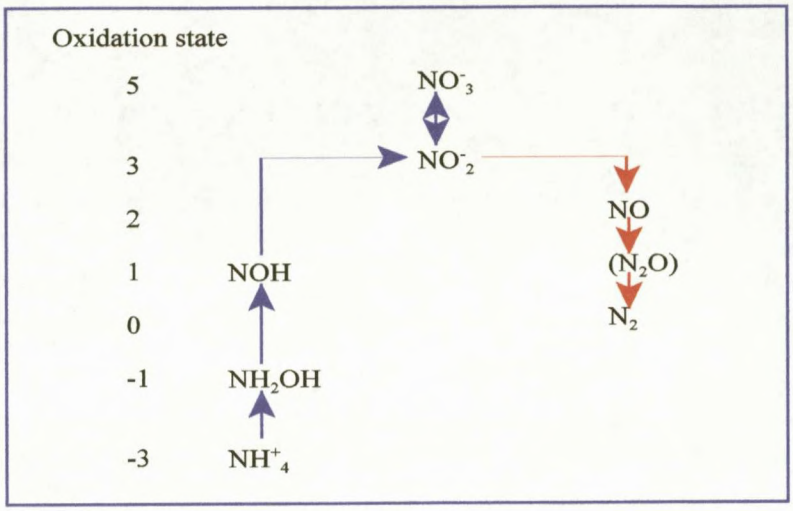

FIGURE 1.3 Changes in the oxidation state of nitrogen during the processes of nitrification and denitrification (Wanner, 1997).

diffuse source pollution. As the name suggests, this pollution problem cannot be targeted to a specific area and treatment to remove the P content from diffuse sources is not usually economically feasible. However, quantification of the P load to an impoundment can be achieved. Meyer and Harris (1991) developed an improved method for estimating and modelling P loads to rivers such that reliable environmental impact studies can be conducted. Atmospheric precipitation and dry fall-out are also classified as diffuse pollution (Rudd, 1979). Point source pollution is due to industrial and domestic effluents [5]. Point source pollution contributes the highest P load to the environment but methods to treat the water do exist (Wentzel, 1992).

The difficulty in consistently achieving the mandatory effluent P standard in wastewater treatment has led to alternatives being considered. One option to reduce P loads to the environment is to eliminate P from detergents. It has being estimated that P in detergents only constitutes approximately 40% of the total P load in domestic wastes (Wentzel, 1992). The most common alternative detergent builder to phosphate is zeolite, the mining of which is

[5] Effluents emanating from wastewater treatment plants are also regarded as a source of point pollution.

considered to be equivalent to mining for raw phosphate-containing minerals (De Haas, 1998). In-depth feasibility studies rendered little economic incentive to remove P from detergents. The decision was therefore taken in South Africa not to introduce a P ban but rather to discover methods of improving existing P removal technologies or to develop new technologies (De Haas, 1998).

1.3 BIOLOGICAL WASTEWATER TREATMENT

Biological treatment of wastewater can be accomplished in a number of ways; the determining factors depend upon efficiency and economics of wastewater contact with the microorganisms concerned. Some technologies depend upon immobilisation techniques such as biofilm systems, suspended-growth systems such as activated sludge and lagoon systems such as aerobic lagoons, anaerobic lagoons, maturation ponds and high-rate algal ponds (Muyima *et al.*, 1997). The scope of this introduction will, however, only deal with suspended-growth systems, namely the activated sludge process.

1.3.1 Conventional activated sludge systems

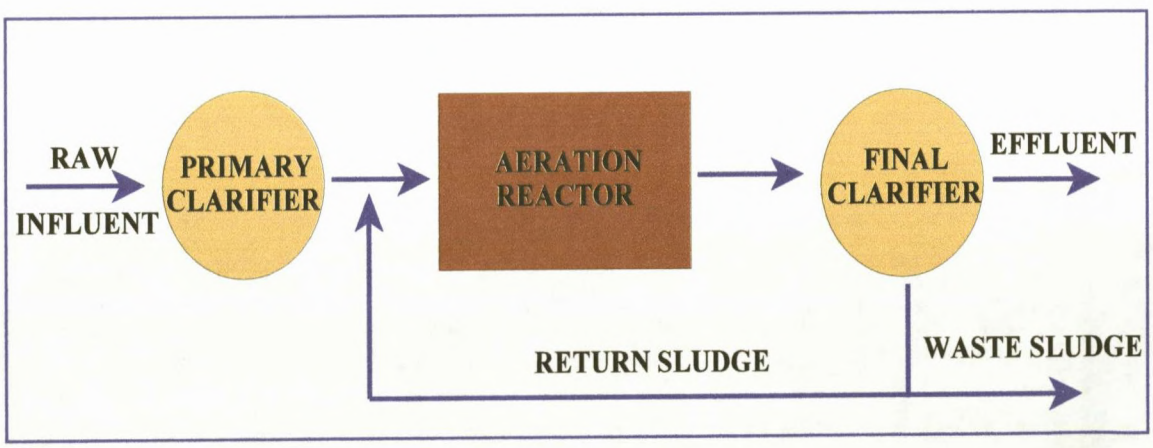

FIGURE 1.4 Conventional activated sludge system incorporating a single aerobic zone.

FIGURE 1.4 refers. Activated sludge systems are based on suspended-growth processes and have become an integral part of municipal wastewater treatment. The process relies on the dense growth of microorganisms in a reactor where air is continuously supplied to allow for carbonaceous oxidation. The term 'activated sludge' refers to an aerobic slurry of microorganisms which can be removed from the process through sedimentation and returned, in quantifiable amounts, to the wastewater stream (Grady and Lim, 1980). All activated sludge systems operate with the following characteristics in common: utilization of a flocculent slurry of microorganisms to remove organic matter from the surrounding wastewater; prior to effluent discharge from the plant, microorganisms are removed through sedimentation thereby reducing outgoing solids loads; settled microorganisms are recycled to the biological reactor via a clarifier underflow; and dependency of plant performance on the mean cell residence time (MCRT or sludge age) in the system (Grady and Lim, 1980). In the presence of nutrients and oxygen, a high rate of microbial growth is achieved. Microbial metabolism of the organic matter present results in the production of oxidised end-products such as carbon dioxide, nitrates, sulphates and phosphates, as well as the biosynthesis of new microbial biomass (Gray, 1989; Horan, 1990; Bitton, 1994; Muyima *et al.*, 1997). In conventional activated sludge systems, three aspects of the process can be varied independently in order to achieve a different response. These are process layout, loading rate and method of aeration. Process configuration can include batch, completely mixed, gradient in substrate and aeration supply (such as plug flow processes) and step feeding (Horan, 1990). Loading rate can be high, low, very low or extended aeration. Aeration systems can be surface or submerged (Gray, 1989).

The basic ecological unit of activated sludge is the floc. Microbial floc formation is essential to the success of activated sludge processes as it allows for rapid and efficient separation of sludge from treated wastewater in the sedimentation tank. Although the exact mechanism of floc formation is not well understood it seems to be almost entirely bacterially mediated (Muyima *et al.*, 1997). The model proposed by Forster and Dallas-Newton (1980), explaining the structure of the floc, has gained acceptance and forms the basis of our conception of floc arrangement. According to this model, filamentous microorganisms form the matrix or backbone of the structure to which zoogleal (floc-forming) microorganisms attach (Bitton, 1994). This attachment is thought to be brought about by exopolysaccharides, present in the form of a capsule or discrete slime layer (Horan, 1990). These polymers, composed of sugars, amino sugars, uronic acids and amino acids, are produced during the endogenous

phase of growth and form the three-dimensional matrix (Bitton, 1994). As extracellular polysaccharide production gradually continues, other microorganisms and colloidal material become entrapped in the matrix and the floc diameter increases. Surface charges on the microbial cells and bridge formation by polyvalent cations also contribute to flocculation (Gray, 1989). This very rigid floc structure has impeded the quantitative analysis of the activated sludge community structure as complete dispersion of the floc is extremely difficult. Clumping of cells in the floc leads to an underestimation of the number of active cells present in the mixed liquor when using viable plate count techniques. Problems attributable to poor floc formation can be one of two causes: (1) nonfilamentous bulking which describes flocs that do not settle well due to excessive production of extracellular polysaccharides and the formation of loose flocs ie., excess of zoogleal organisms; and (2) filamentous bulking, caused by excessive growth of filamentous bacteria (Bitton, 1994). These undesirable characteristics will have negative economic consequences to the plant in question.

Since its inception in the early 20th century, the activated sludge process has been widely adopted and further developed to cope with increased effluent loads and contaminants. The greatest feature of the process has proven to be its flexibility and its ability to deal with the ever increasing stresses placed upon it such as biological nutrient removal (BNR) operations.

1.3.2 Modified activated sludge systems

Increasing industrial and population demands on water supplies have ensured that the activated sludge process has had to undergo various modifications in order to cope with the increase in carbonaceous and nutrient loadings. Single aerobic systems are no longer sufficient to treat domestic and industrial wastes and changes incorporated into the process relate to size, number and configuration of the reactors; flow direction and mixed liquor recycle; and flow regime within the reactors (Wentzel *et al.*, 1992a) . Phosphorus and nitrogen removal from wastewater is essential to ensure the sustainability of potable water supplies. However, in conventional systems, phosphate is only taken up in quantities which satisfy basic bacterial metabolic requirements. Nutrient loads to receiving waters would subsequently remain excessive, the negative implications of which have already being discussed. Therefore,

in order to encourage the resident microflora in the system to accumulate P in excess of basic metabolic requirements, modifications and extensions to conventional reactor configurations have being implemented.

Nitrification denitrification biological excess phosphorus removal (NDBEPR) activated sludge systems were developed in 1974 by Barnard who recognised the importance of an anaerobic zone at the head of the biological reactor to encourage excess biological P uptake (Wentzel *et al.*, 1992a). He realised that bio-P removal was induced if the organism mass was stressed by subjecting it to conditions of anaerobiosis, thereby releasing P to the bulk liquid (Ekama *et al.*, 1984). This particular system was named the Bardenpho system and since its inception, a number of further modifications have taken place to enhance biologically mediated nutrient removal. Other configurations include the Phoredox system, also referred to as the modified Bardenpho or 5-stage Bardenpho system; the 3-stage Phoredox system (FIG. 1.5); the modified UCT (University of Cape Town) process (FIG. 1.6); and the Johannesburg system. As can be seen from FIGS 1.5 and 1.6, BPR processes are all configured upon the same basis ie., anaerobic zone at head of the aerobic reactor, but vary in the number of reactors and type of mixed liquor recycle flows required for efficient nutrient removal. It must be noted that the processes mentioned above were designed to incorporate both P and N removal; systems removing solely N also exist eg., modified Ludzack-Ettinger and Wuhrmann processes. These systems, however, are beyond the scope of this study and will not be discussed further.

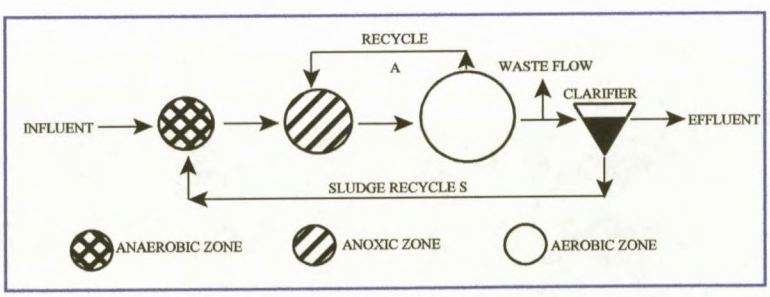

FIGURE 1.5 The 3-stage Phoredox system incorporating NDBEPR processes.

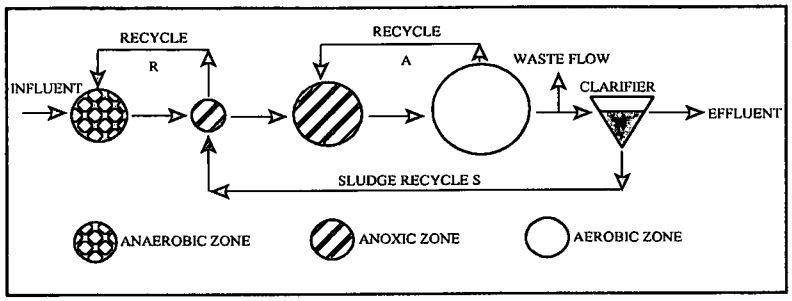

FIGURE 1.6 The modified UCT system incorporating NDBEPR processes.

1.3.3 Biological phosphorus removal

Although P can be removed from a wastewater by chemical precipitation [6], the main emphasis of this study is concerned with BPR. Reference can be made to Brodisch *et al.*, (1987) and De Haas (1998) for a more definitive and comprehensive description of chemical P removal operations. A distinction must be made, however, between P removal via intentional, simultaneous chemical addition by the plant operator and P removal via chemical changes of the wastewater as a result of biological action eg., changes in alkalinity, acidity and pH. It can be assumed, however, that when treating municipal wastewater by an appropriately designed activated sludge process without chemical addition, excess P removal is principally mediated by the biological mechanisms of absorption and adsorption (Ekama *et al.*, 1984).

Biological phosphorus removal operations are increasingly gaining support over pre- and post-precipitation due to: (1) the low sludge production of the process; (2) the fertiliser value of bio-P sludge; (3) although capital outlay may be intensive (especially for existing plants which require modifications), they are generally less expensive to operate; and (4) the avoidance of anion enrichment of the treated water (Wentzel, 1992; Henze, 1996; Romanski *et al.*, 1997). Although BPR can effectively compete with chemical removal in terms of cost, the characteristics of the influent wastewater to the plant will dictate the performance of the removal mechanism.

[6] Chemical precipitants include ferrous and ferric ions, aluminium salts, lime and polyelectrolytes which can either be dosed independently or simultaneously with biological P removal operations.

There exists within the activated sludge mixed liquor a population of organisms which are capable of accumulating soluble P as polyphosphate (poly-P) granules (referred to as volutin granules). They are commonly termed phosphorus accumulating organisms [PAO's] (Wentzel *et al.*, 1988) and are able to take up P in excess of normal metabolic requirements. The process is therefore termed biological excess P removal. These organisms will, however, only proliferate in the system and the mechanism of poly-P accumulation will only be induced if certain structural alterations to the aeration basin are made ie., an anaerobic zone at the head of the basin. Previous research has shown that the anaerobic zone leads to the enrichment of fermentative organisms such as *Enterobacter*, *Klebsiella*, *Citrobacter*, *Pasteurella*, *Proteus* and *Aeromonas* (Lötter and Murphy, 1985). The PAO's are then able to accumulate the fermentation products viz., acetic, lactic, succinic, propionic and butyric acids, and store them as intracellular carbon and energy reserves ie., poly-β-hydroxyalkonoates (PHA's) of which poly-β-hydroxybutyrate (PHB) is an example (Satoh *et al.*, 1992; Lilley *et al.*, 1997). As a result of internal carbohydrate accumulation, the PAO's release P back into solution and the ortho-P concentration in the anaerobic zone increases. In the subsequent aerobic reactor the PAO's utilise the internally stored carbon for growth which increases their biomass in the sludge. PHB is also used as an energy source in the aerobic reactor to take up P from the bulk solution and to re-synthesise the poly-P degraded in the anaerobic reactor. P uptake, together with an increase in the quantity of PAO's in the system, leads to a nett removal of P from the wastewater (Wentzel, 1992). Soluble P, accumulated as biological poly-P in the solid phase, is then removed from the system via the waste sludge stream.

According to the current EBPR models, uptake of P in the aerobic zone is directly related to the quantity of P released in the anaerobic zone ie., the more P released in the anaerobic zone, the greater the ability of the biomass to take up P under aerobic conditions (Helmer and Kunst, 1998). The amount of P which can be removed by bio-P activity is also directly coupled to the amount of volatile fatty acid (VFA) that the PAO's accumulate in the anaerobic tank (Henze, 1996).

Although the process of enhanced biological P removal is well understood from a technical aspect, it remains difficult to achieve consistent and reproducible removal rates at full-scale due to our lack of understanding of the process from a biochemical and microbiological point of view (Satoh *et al.*, 1996; Wang and Park, 1998). Without

understanding the correlation between the PAO community structure within activated sludge mixed liquor and the wastewater plant's performance, reliable and efficient biological phosphorus removal operations will remain difficult to design.

1.4 MAIN OBJECTIVES OF THE STUDY

The aim of this study was to ascertain the incidence of *Acinetobacter* (a known PAO) in EBPR activated sludges and to determine which other bacteria contribute to this phenomenon.

To this end, the objectives of the research were as follows:

1. operate a laboratory-scale BNR activated sludge plant modelled on the 3-stage Phoredox process;
2. evaluate unit efficiency based on biological phosphorus uptake and release;
3. characterise influent to and monitor effluent emanating from the laboratory-scale unit ie., COD fractionation, phosphorus (total P and ortho-P) and nitrogen (TKN, ammonia and nitrate) concentrations;
4. identify and enumerate *Acinetobacter* spp. present in sludge mixed liquors of both a full- and laboratory-scale EBPR activated sludge process;
5. through sample manipulation, identify and enumerate other dominant heterotrophic bacteria (PAO's) present in the mixed liquor of the processes;
6. assess the phosphorus removal capacity of *Acinetobacter* and other dominant bacterial species in axenic cultures; and
7. using a cultivation-independent approach, identify and enumerate those bacterial phylogenetic groups (as well as enumeration of *Acinetobacter* spp.) present in the laboratory-scale activated sludge unit exhibiting a strong EBPR mechanism.

CHAPTER TWO

LITERATURE REVIEW

2.1 WATER - THE FUNDAMENTAL BUILDING BLOCK OF LIFE

Water is the essence of life. Yet despite its paramount value to the sustenance of life it is often a grossly ignored resource, only considered when a new related crisis evolves. Of dire concern is the manner and extent to which fresh water supplies are exploited by mankind. One only has to examine the actual water available for human consumption to realise what a precious commodity it is (FIG. 2.1).

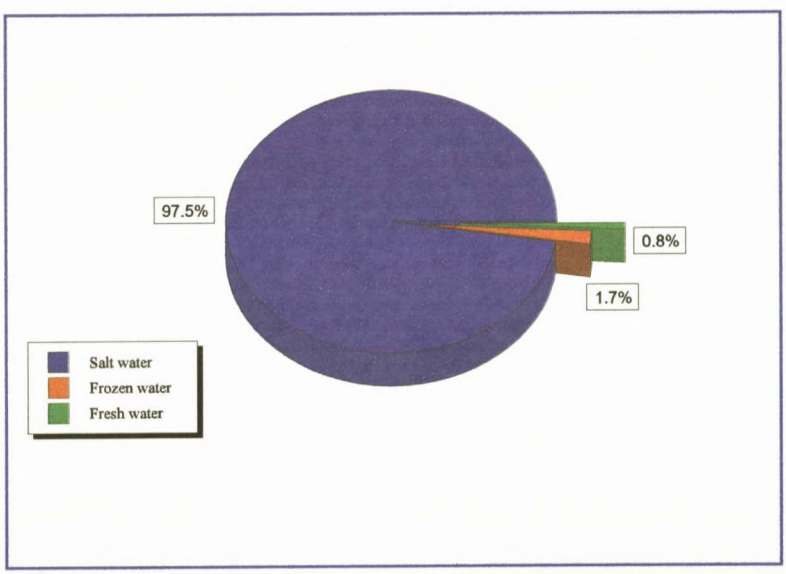

FIGURE 2.1 Availability of worlds water resources (Umgeni Water, 1996). Of the 0.8% fresh water available, only 0.05% can actually be utilised for human consumption.

Only 2.5% of the world's water is not contained in the oceans, and of that, two-thirds is reserved in the polar ice-caps and glaciers. Of the remaining amount (0.8%), two-thirds is lost due to evapotranspiration (approximately 0.3% remaining). Some 20% of the remaining amount of potentially potable water is located in areas too remote for human access (0.06%) and three-quarters of the remaining 80% is not effectively captured for use as it arrives

in the form of monsoons and floods. We therefore only get to use less than 0.05% of the total water on the planet (Serageldin, 1998).

Fresh water available for human consumption is rapidly becoming scarcer due to massive amounts of pollutants being discharged directly into waterways. These pollutants subsequently impregnate ground water supplies and aquifers, diminishing reserves further. The number of countries confronted by a water deficit in the world today is 26 (equivalent to a total population of 300 million). This figure will, however, increase drastically to approximately two-thirds of the world's population by the year 2050 if pro-active steps to curb the downward trend are not implemented (Abu-Zeid, 1998). It has been estimated that South Africa alone already utilises approximately 60% of its usable water supplies, a demand which will increase tremendously as the country strives for first-world status (Asmal, 1998). One only has to try to find a non-polluted river or body of water to realise how critical the situation has become. It is evident that the demand on water resources has escalated dramatically with population explosions, urbanisation and the subsequent growth of mega-cities. The concept of sustainability of our natural resources has now become a matter of urgency and should no longer be viewed as an ideological philosophy.

As discussed in Chapter 1, N and P are the two key nutrients determining the productivity (trophy) of a lake or other water impoundment. The greater the human activity in a catchment area, whether by urban development or agriculture, the greater the annual loads of N and P entering an impoundment. Phosphorus is usually the limiting nutrient since the planet's supply of P is generally much lower than that of N. Phosphorus compounds also tend to be more insoluble than N compounds. The following Vollenweider model (Vollenweider, 1975) describes the consequences of nutrient infiltration into deep lake systems:

$$[M] = \sum L / z\,(s + r)$$

where: $[M]$ = concentration of nutrient in the system, (mg/L);
$\sum L$ = loads from all possible sources (soil, effluent, rain);
z = mean depth of impoundment;
s = net rate at which nutrient is exchanged with the sediment; and
r = flushing rate of impoundment.

From the above equation, it is evident that two possible sinks for P exist in lakes and dams: (1) fixation of P in bottom sediments which therefore makes it unavailable for algal growth (s); and (2) loss of nutrient to overflow (r).

Phosphorus from point source loads is therefore strictly monitored in order minimise the negative impacts of nutrient enrichment. A particular area of interest is the reduction of P concentrations emanating from wastewater treatment operations, one in which this study is primarily concerned.

2.2 MECHANISMS OF BIOLOGICAL PHOSPHORUS REMOVAL

Excess biological phosphate removal by bacteria present in the mixed liquor of activated sludge was first observed by Vaker *et al.*, (1967). Since then, this phenomenon has gained world-wide support and is utilised in both new and existing wastewater treatment plants which are either constructed or upgraded to accommodate biological nutrient removal. The tendency to opt for bio-P removal over chemical precipitation is due to the low sludge production of the system, the fertiliser value of the resultant waste sludge and the use of wastewater components ie., influent COD and its various fractions, as process chemicals to control the mechanism (Henze, 1996).

Bio-P removal occurring in activated sludge is a direct result of the ability of certain microorganisms, termed polyphosphate accumulating organisms (PAO's), to accumulate large quantities of poly-P intracellularly. The phrases 'enhanced' and 'excess' are often incorporated to emphasise the ability of these organisms to accumulate poly-P in excess of their normal metabolic requirements. In order to encourage the growth and proliferation of these organisms, as well as to induce the bio-P mechanism, two conditions are essential: sequential anaerobic and aerobic reactors; and the presence of volatile fatty acids in the anaerobic reactor (Wentzel *et al.*, 1990). A description of the various biological processes and mechanisms occurring in the different zones of EBPR processes follows.

2.2.1 Anaerobic zone

In wastewater technology, anaerobiosis describes an environment in which both dissolved oxygen and oxidised forms of nitrogen ie., nitrates and nitrites, are absent (Jenkins and Tandoi, 1991; Muyima *et al.*, 1997). The function of the anaerobic zone in EBPR operations is two-fold: (1) the reduced redox potential induces conversion of the influent readily biodegradable COD (RBCOD) to short chain fatty acids (SCFA's), also known as volatile fatty

acids (VFA's) via acidogenisis by non-PAO heterotrophs, and (2) it provides an ideal environment where PAO's are able to take up the VFA's and accumulate them intracellularly as PHA's, the most common of which is PHB (Ekama and Wentzel, 1997). The function of the anaerobic zone was originally thought to be one of stimulation of P release; it has, however, now been realised that the zone is solely responsible for the production of suitable substrate/s ie., VFA, through fermentation which allows for the proliferation of PAO's in the system (due to lack of substrate competition from non-PAO's) (Fuhs and Chen, 1975; Muyima *et al.*, 1997). Due to the absence of terminal electron acceptors, PAO's are able to utilise the VFA's exclusively through the energy generated from poly-P hydrolysis. This zone is essential for the nett removal of P from wastewater as it preconditions the PAO's to take up excess P under aerobic conditions ie., results in diminished intracellular poly-P storage granules or volutins which must be replenished in the following aerobic zone. It is essential that neither nitrates nor dissolved oxygen (DO) are recycled to or enter the anaerobic zone as the effects on P removal are adverse. This is due to one of three possible mechanisms: (1) competition for VFA between PAO's and other 'normal' heterotrophs; (2) reduction in PAO activity due to reduced fermentation of RBCOD ie., reduction in VFA synthesis; and (3) some species of PAO's are able to denitrify (in the case of nitrate infiltration) and all are aerobic (in the case of DO) so the organisms will preferably utilise these terminal electron acceptors to obtain energy rather than switching their metabolism to poly-P hydrolysis (which directly affects P accumulation in the subsequent aerobic zone) (Henze *et al.*, 1995).

The three principal processes implicated in EBPR which occur in the anaerobic zone and which are discussed in further detail include: (1) VFA synthesis and accumulation; (2) PHB synthesis; and (3) P (as ortho-P) release.

2.2.1.1 Volatile fatty acid synthesis and sequestration

In the anaerobic reactor, facultative organisms (non-PAO's) are able to derive a small amount of energy (sufficient for survival alone) through the generation of intracellular electron acceptors. Through the Embden-Meyerhof pathway, they are able to degrade high molecular readily biodegradable organics (such as glucose) to low order fatty acids ie., acetate, lactate, butyrate, succinate and fumarate. However, under anaerobic conditions these acids cannot enter the Krebs cycle and are subsequently released into the bulk liquid (Ekama *et al.*, 1984). PAO's have a distinct

advantage over other normal heterotrophic organisms as they are able to utilise accumulated polyphosphate stores to supply the energy required to activate the low molecular organics for accumulation (Satoh *et al.*, 1992). Wentzel *et al.*, (1990) found that the rate of acetate sequestration by PAO's is zero order with respect to soluble acetate and occurs very rapidly. Although VFA's are known to be the primary carbon source accumulated by PAO's in the anaerobic zone, other short chain organic compounds are also known to be taken up and accumulated as PHA (Satoh *et al.*, 1997).

Volatile fatty acid concentration in the influent to wastewater treatment plants can be increased through the installation of primary sludge fermenters at the head of the activated sludge process. Acid-phase anaerobic digestion of primary sludge is used to boost the RBCOD and VFA fractions in the feed wastewater and can either be included as an in-line or side-stream facility (Banister and Pretorius, 1998). Primary sludge fermentation is therefore a very practical solution for those plants which experience erratic bio-P removal as a result of weak influent COD and resultant low RBCOD and VFA concentrations. Seeding the fermenters with partially digested sludge ie., 1 to 3 d digested, has been shown to improve VFA production efficiency with maximum VFA yields of 10% of the total influent COD been recorded after 6 d (Banister and Pretorius, 1998).

2.2.1.2 Poly-β-hydroxybutyrate synthesis

Refer to FIG. 2.2 for a schematic representation of the synthesis and degradation of PHB in the anaerobic and aerobic zones, respectively. A consequence of the high organic carbon concentration in the anaerobic zone is that provided both an electron and energy source are available ie., NADH/NAD and ATP/ADP, carbon can be taken up intracellularly and accumulated as PHB. Due to the inhibition of the tricarboxylic acid (TCA) cycle in the anaerobic zone (due to high NADH/NAD ratio), the synthesis of PHB acts as an electron sink, decreasing the NADH/NAD ratio (Wentzel *et al.*, 1986). This promotes the TCA cycle which generates more electrons. The interaction occurring between the TCA cycle and PHB synthesis, mediated by the NADH/NAD ratio, ensures that all VFA taken up is stored as PHB. Reducing equivalents such as $NAD(P)H_2$ are also essential for PHB synthesis. Stoichiometrically, one $NAD(P)H_2$ molecule is required for the conversion of 2 mol acetate to PHB (Appeldoorn *et al.*, 1992). Before acetate can be stored as PHB, the molecule needs to be activated to acetyl-CoA. This is done

at the expense of 1 mol ATP per mol acetate if acetate kinase is involved or 2 mol ATP/mol acetate if the enzyme, acetyl-CoA synthetase, catalyses the reaction (Appeldoorn *et al.*, 1992).

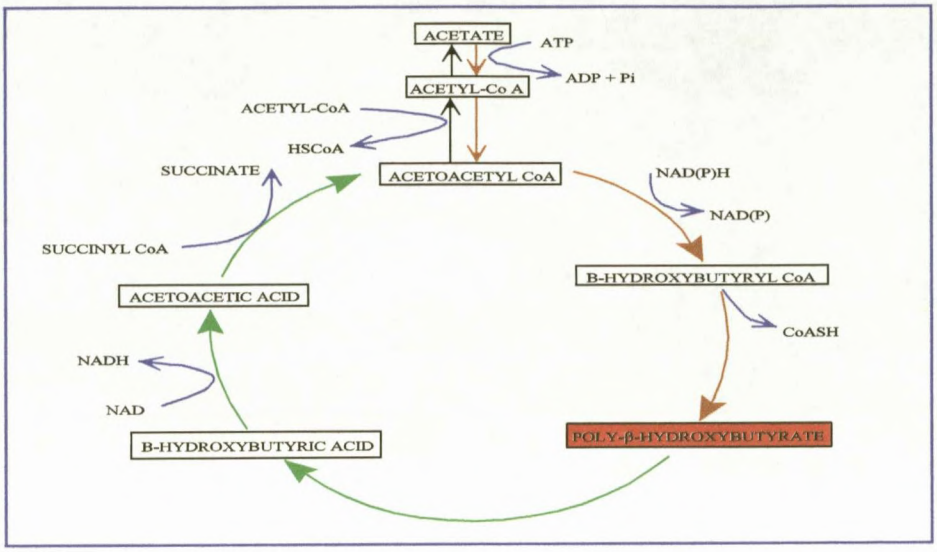

FIGURE 2.2 Biochemical model depicting synthesis and degradation of poly-β-hydroxybutyrate in the anaerobic and aerobic zones respectively (adapted from Wentzel *et al.*, 1986).

2.2.1.3 Orthophosphate release

A high external concentration of low organic molecules such as acetate allows for passive diffusion into the cell ie., without expenditure of energy. Activation of sequestered acetate to acetyl-CoA by coupled ATP hydrolysis has the effect of decreasing the ATP/ADP ratio to such a degree that ATP synthesis is stimulated via poly-P degradation (Wentzel *et al.*, 1986). On a molar basis, it has been found that 1 mol intracellular P is released per mol VFA sequestered (Wentzel *et al.*, 1990). Therefore, if acetate is used as substrate, *ca.* 0.5 mgP is released per mg acetate (as COD) sequestered. The mass of P released will always be proportional to the VFA feedstock in solution ie., the greater the VFA concentration the greater the mass of P released. A minimum prerequisite for P release in the anaerobic reactor is a biodegradable COD concentration of 60 mgCOD/L (Ekama *et al.*, 1983, as cited by Punrattanasin and Randall, 1998) or a RBCOD fraction of 25 mgCOD/L surrounding the organisms (Ekama *et al.*, 1984).

Compounds such as acetate, formate and propionate are capable of inducing P release from phosphate-laden sludge under anaerobic, anoxic and aerobic conditions which has compelled the belief that the release of P is primarily dependent on the nature of the substrate interacting with the biomass and not the prevailing anaerobic conditions (Gerber *et al.*, 1987a).

2.2.2 Anoxic zone

Anoxia refers to an environment in which nitrates (and nitrites) are present yet dissolved oxygen is absent (Muyima *et al.*, 1997). The anoxic zone is responsible for denitrification in the activated sludge system and will only be discussed in cognisance of its significance in reducing the level of nitrates recycled to the anaerobic zone. The anoxic zone is fed by the effluent from the anaerobic zone and by the mixed liquor recycle from the aerobic zone (a-recycle). The variable nature of the a-recycle ensures that no dissolved oxygen enters the anoxic zone which results in the reduction and removal of NO_x from the system.

It has been found that the PAO community can be divided into two fractions - those that are only capable of aerobic respiration ie., use oxygen as an electron acceptor, and those that are capable of utilising both oxygen and nitrate as electron acceptor (Kerrn-Jespersen and Henze, 1993). Phosphorus will therefore be taken up in the anoxic zone if no VFA or other low molecular organic compound is available for assimilation ie., if acetate leaks from the anaerobic zone into the anoxic zone, P release and PHB accumulation will continue (Artan *et al.*, 1997). Although considerable, anoxic P uptake is quantitatively lower than aerobic P uptake and is a first order reaction with respect to the size of anaerobically accumulated PHB stores (as is aerobic P uptake) (Kerrn-Jespersen and Henze, 1993; Artan *et al.*, 1997). Kerrn-Jespersen and Henze (1993) found that a linear relationship existed between accumulated PHB concentration, denitrification rates and P uptake rates under anoxia. Anoxic P uptake has the potential to reduce costs of wastewater treatment considerably due to simultaneous P removal and denitrification occurring in one zone.

2.2.3 Aerobic zone

The primary function of the aerobic zone is to oxidise organic material in the sewage (Muyima *et al.*, 1997). When a suitable sludge age is selected and the autotrophic organisms are able to establish themselves in the system, oxidation of ammonia N to nitrite and nitrate ie., nitrification, occurs simultaneously. Aerobiosis also provides an environment where the PAO's are able to take up the P released in the anaerobic zone as well as the P entering the system through the feed sewage (Muyima *et al.*, 1997).

2.2.3.1 Poly-β-hydroxybutyrate degradation

The degradation of PHB proceeds via its hydrolysis to free β-hydroxybutyrate, oxidation of the acid to acetoacetate and finally activation of acetoacetate to acetoacetyl-CoA (FIG. 2.2) (Wentzel *et al.*, 1986). Two molecules of acetyl-CoA are then formed via the activated cleavage of acetoacetyl-CoA and these end products enter the TCA cycle (Wentzel *et al.*, 1986). The degradation pathway is regulated by high concentrations of pyruvate and/or a high NADH/NAD ratio and it can therefore be concluded that the pathway will only be functional in an environment where concentrations of extracellular organic substrate is low and a terminal electron acceptor is present ie., in the aerobic zone (Wentzel *et al.*, 1986). Degradation of PHB to acetate provides a carbon and energy source for cell metabolism and function.

2.2.3.2 Phosphorus uptake and poly-P synthesis

Translocation of extracellular soluble phosphate ions occurs via the hydroxyl mediated antiport. The cations required for neutralising the charge imbalance and stabilising the phosphoryl bonds ie., Mg^{2+}, Ca^{2+} and K^{+}, are taken up by the cell via the proton mediated antiport (Wentzel *et al.*, 1986). A consequence of the presence of an external electron acceptor in the aerobic zone is a reduction in the NADH/NAD ratio and an increase in the ATP/ADP ratio. A high and non-limiting ATP/ADP ratio stimulates poly-P synthesis. The principal mechanism of poly-P synthesis is via the phosphorylation of accumulated phosphate by ATP, the reaction of which is as follows:

$$ATP + (PO_4)_n \rightleftharpoons ADP + (PO_4)_{n+1}$$

It is evident that this pathway controls both poly-P synthesis and degradation, the direction of which is regulated entirely by intracellular ATP/ADP ratios. Poly-P synthesis will be promoted by high concentrations of ATP, a condition likely to be encountered in the aerobic zone where oxidative phosphorylation is able to proceed (Wentzel *et al.*, 1986).

2.3 TECHNICAL CONSIDERATIONS OF BIOLOGICAL PHOSPHORUS REMOVAL

Man has through his ingenuity and absolute necessity to maintain secure, potable water supplies been able to exploit naturally occurring phenomena to achieve favourable end results. An example of this is the process of biomanipulation, a phrase coined to basically describe the manipulation of a naturally occurring biological community to achieve a desirable and calculated change in a particular biocenosis (Moss, 1998). Nowhere is this more evident than in the process of wastewater purification. As mentioned previously, municipal wastewater can be treated by a number of biological means viz., fixed-film reactors, suspended growth reactors and stabilization ponds. The activated sludge process has received the most attention from microbiologists and engineers alike yet the intricate microbiological interactions occurring within the reactor vessel remain elusive due to limitations of the various analytical procedures and techniques presently available (see section 2.6). The following section deals with the technical aspects of BPR design and operation and describes how these operational parameters contribute to the maintenance of an active and functional biological community.

2.3.1 Operational parameters governing the efficiency of activated sludge systems

When designing and operating activated sludge systems, cognizance must be taken of a number of key parameters to ensure that secondary biological treatment and bio-P removal operates at maximum efficiency. These parameters include: loading rates to the system; sludge age; mixed liquor suspended solids concentration; internal recycle rates and anaerobic mass fractions.

2.3.1.1 Loading rate

Loading rate is the term applied to the rate at which sewage (raw or settled) enters the activated sludge reactor (Horan, 1990). The rate at which the plant is loaded with carbonaceous compounds directly influences effluent quality, settling properties of the sludge and overall plant performance. Loading rates also affect the solids retention time (SRT or MCRT, see 2.3.1.2 below) and thus the daily rate of sludge wastage from the system. A balancing tank, situated at the inlet to the works, is usually constructed to regulate variations in diurnal flow rate and organic loads (Lilley *et al.*, 1997). The four basic parameters that describe loading rate are volumetric loading; organic loading; and sludge loading or food to microorganism ratio (F/M) (Horan, 1990).

2.3.1.1.A Volumetric loading

Volumetric loading is a description of the flow of wastewater in relation to the reaction tank capacity (Gray, 1989). Hydraulic retention time (HRT) determines the total amount of time that a volume of sewage will be contained in the system and is defined as:

$$\mathbf{HRT\ (d) = \frac{V}{Q}}$$

where: V = reactor volume (L)
Q = influent flow rate (L/d)

The reactor volume must therefore be of sufficient magnitude to allow for adequate contact time between the sludge flocs and the sewage. Overly large reaction zones will, however, result in secondary P release[1] which will obviously impair a plant's bio-P removal efficiency (Rabinowitz and Wilson, 1998). The secondary clarifier/settler must also be suitably designed to ensure minimal SRT as activated sludge which has aerobically accumulated P will release P when an oxygen deficiency occurs (Schön *et al.*, 1993). In conventional systems, HRT is usually operated at 6-10 hours (Horan, 1990) whilst EBPR systems require maximum 2 h anaerobiosis and 4 - 12 h aerobic retention times (Muyima *et al.*, 1997).

[1] Mechanism of P release which is not associated with VFA uptake.

2.3.1.1.B Organic loading

Horan (1990) refers to organic loading as a measure of the amount of biological oxygen demand (BOD) applied per unit volume of reactor tank capacity. However, as stated by Marais and Ekama (1984), describing organic loading in terms of chemical oxygen demand (COD) offers a more quantitative representation of the electron donor capacity and therefore the amount of energy contained within a wastewater sample. Daily COD loads should therefore be determined, according to the following equation, to obtain a more holistic impression of organic loading to the biological reactor:

$$\textbf{Organic load (g/L/d)} = \frac{Q \times COD}{V}$$

where: Q = influent flow rate (L/d)
COD = COD of influent (g/L)
V = reactor volume (L)

2.3.1.1.C Sludge loading or F/M ratio

This is the most practical of the loading parameters as it is the only form over which the plant operator has any control through maintenance of a fixed concentration of mixed liquor suspended solids (MLSS) in the aeration basin (Horan, 1990). The ratio basically describes the carbonaceous load (COD) entering the plant which is available to the active biomass (Gray, 1989). The F/M ratio influences COD removal rates, sludge settleability and nitrification in the aerobic vessel. As the following equation indicates, adjusting sludge wastage rates directly affects the load:

$$\textbf{F/M ratio} = \frac{Q \times COD}{V \times X}$$

where: Q = influent flow rate (L/d)
COD = COD of influent (g/L)
V = reactor volume (L)
X = reactor suspended solids (g/L)

A high F/M ratio indicates exponential growth of the activated sludge biomass. Although a large reduction in COD will be observed, the microorganisms do not form flocs and are generally dispersed which causes settleability problems. However, low F/M ratios ensure oligotrophic conditions (with respect to substrate) in the reactor and bacterial metabolism enters the endogenous respiration phase with cell lysis and resynthesis continuously occurring. Under these conditions almost complete organics oxidation can be expected, resulting in an effluent of high quality and sludge flocculating and settling well (Gray, 1989).

2.3.1.2 Sludge age

The performance and efficiency of an activated sludge system depends to a large extent on the MCRT. MCRT is also referred to as solids retention time[2] (SRT) or sludge age (R_s). Once mixed liquor has moved to the secondary clarifiers, activated sludge with well formed flocs will settle and separate from the effluent. A portion of this settled sludge will be wasted from the system whilst the remaining fraction will be returned to the inlet of the biological process via the clarifier underflow. The fraction of solids which are wasted will determine the average amount of time which the biomass will occupy the reactor. Sludge age can therefore be defined as the mass of sludge in the reactor (both aerated and unaerated reactors) divided by the mass of sludge wasted per day (Lilley *et al.*, 1997) ie.,

$$R_s = \frac{V \times X}{X_w}$$

where: Rs = sludge age (d)
V = reactor volume (L)
X = reactor suspended solids (g/L)
Xw = daily wasted sludge suspended solids (g/L/d)

Sludge can either be wasted as thickened sludge from the clarifier underflow or as TSS directly from the aeration basin (Horan, 1990). Precise control of R_s can be maintained through the latter method (hydraulic control) as the underflow concentration, which varies considerably, does not need to be calculated. The technique of hydraulic control allows an operator to alter R_s without complications simply by increasing or decreasing the mass of sludge wasted.

Sludge age of a process must be strictly monitored as it has a direct influence on BPR (Wentzel, 1990). EBPR depends on R_s as the PAO active mass fraction in the mixed liquor has been found to decrease as R_s is increased (Rodrigo *et al.*, 1996). The effect of R_s on P removal is complex. When $R_s < 3$ d, P removal increases as R_s increases; yet for $R_s > 3$ d, increasing R_s will result in a decrease in P removal (Wentzel *et al.*, 1990). Increasing R_s for the former effect ie., $R_s < 3$ d, will result in an increase in RBCOD conversion to VFA and therefore an increase in P release and uptake[3]. Increasing R_s further, however, will result in a P removal decline due to a decreased active biomass fraction present in the waste mixed liquor (only active biomass is associated with P uptake) (Wentzel *et al.*, 1990).

[2] Solids retention time must not be confused with hydraulic retention time. Both parameters can be adjusted independently of one another.
[3] P release and subsequent P uptake are proportional; an increase in P release in the anaerobic zone will result in increased P uptake rates in the aerobic zone.

Rodrigo *et al.*, (1996) , when studying the effect of various sludge ages on an EBPR system, found that higher values of P removal were recorded as R_s was decreased. Wentzel *et al.*, (1988), after developing an enhanced culture of PAO's at 10 and 20 d sludge ages, reported P removals of 60 and 50 mgP/L respectively, indicating higher P removal rates at lower sludge ages (due to higher active biomass fractions). Wentzel *et al.*, (1990) also found that R_s had a minor effect on P release in the anaerobic zone. It must be noted that nett P removal is regarded as the difference between P release in the anaerobic zone and P uptake in the aerobic zone.

2.3.1.3 Mixed liquor suspended solids

The concentration of suspended solids in the system, which to a large extent constitutes the resident biomass, is referred to as the MLSS. This value offers the system operator a crude measure of the biomass contained within the process. With the advent of steady state and kinetic design modelling (Wentzel *et al.*, 1990), the accurate determination of biomass in activated sludge systems has become a significant criterion. Historically, sludge biomass and specific growth rates were calculated on the basis of MLSS, volatile suspended solids (VSS) or COD of the activated sludge (Liebeskind and Dohmann, 1994). In NDEBPR systems the mixed liquor organic suspended solids (MLOSS, also referred to as mixed liquor VSS or MLVSS) is made up of four components: heterotrophic active biomass; endogenous residue; inert material; and autotrophic active biomass (Ubisi *et al.*, 1997; Wentzel *et al.*, 1998). The heterotrophic active biomass component arises from the synthesis of heterotrophic organisms on influent biodegradable COD and is lost via endogenous respiration or death processes; the autotrophic active biomass arises from the synthesis of autotrophic organisms in the nitrification of ammonia and is also lost via endogenous respiration processes; endogenous residue is generated as a result of the unbiodegradable fraction from heterotrophic and autotrophic biomass death processes; and inert material represents the unbiodegradable particulate COD fraction entering the system via the influent (Ubisi *et al.*, 1997). All four MLOSS components are returned to the anaerobic reactor (in bio-P operations) via the clarifier s-recycle and are removed via the waste flow. Reference can be made to Ubisi *et al.*, (1997) and Wentzel *et al.*, (1998) for a simple batch test procedure for heterotrophic biomass determination in activated sludge mixed liquors. Liebeskind and Dohmann (1994) formulated an improved method of DNA extraction from activated sludge for quantitative biomass determination. Although the proposed method showed close correlation to the kinetic models when applied to pure cultures and

high-rate aeration sludges, the full DNA complement could not be extracted from sludges containing precipitating agents (ie., ferric iron). Unfortunately, the procedures are unable to distinguish between heterotrophic PAO and non-PAO's in mixed liquor samples originating from EBPR operations. The requirement for a simple method to determine the active PAO fraction therefore still exists and must be investigated.

2.3.1.4 Control of internal recycle rates (refer to FIGS 1.5 and 1.6 for mixed liquor recycle flows)

There are three different recycle flows available to plant designers; the number of recycles will depend entirely upon wastewater characteristics/objectives of treatment and subsequent process configuration (Lilley *et al.*, 1997). The more recycles employed will obviously increase the complexity of the operation and greater control will have to be exercised. Conventional, fully aerobic systems have only a clarifier return or underflow stream, commonly referred to as the s-recycle. As implied, the s-recycle returns final clarifier underflow or thickened activated sludge to the inlet of the activated sludge process. In BNR operations, control of this stream is vitally important as it will contain similar nitrate concentrations to that of the effluent. The s-recycle returns mixed liquor to the anaerobic zone in the Phoredox system (3- or 5-stage); the anoxic zone of the UCT or modified UCT systems downstream of the anaerobic zone; and the pre-anoxic zone of the Johannesburg system (Lilley *et al.*, 1997).

The internal a-recycle or aerobic-anoxic recycle, returns nitrates which are formed during nitrification in the aerobic zone to the anoxic zone for denitrification. The a-recycle can be monitored and governed according to TABLE 2.1 (Lilley *et al.*, 1997).

TABLE 2.1 Optimisation of a-recycle rate.

Nitrate concentration	Remedial action to be taken
• Effluent - high [nitrate] • End of anoxic zone - zero nitrate	Increase recycle rate as further denitrification may be possible
• End of anoxic zone - high [nitrate] • UCT process - decrease in P removal • Bulking sludge	Reduce recycle rate - the anoxic zone is operating at full denitrification potential and no further denitrification is possible

The r-recycle, which is only applicable to the UCT or modified UCT processes, returns mixed liquor from the anoxic zone to the anaerobic zone to ensure that the design anaerobic mass fraction is maintained (Lilley *et al.*, 1997).

2.3.1.5 Anaerobic mass fractions

To obtain good excess P removals from wastewater, the anaerobic mass fraction (f_{xa}) plays an integral part in ensuring sufficient VFA is synthesised and accumulated and ortho-P is released. Determining f_{xa} is based solely upon influent total COD (S_{ti}) as this will dictate the readily biodegradable COD (RBCOD) fraction entering the plant. The anaerobic reactor is sized according to the following formula (Wentzel, 1992):

$$f_{xa} = \frac{\textbf{mass of sludge in anaerobic reactor}}{\textbf{mass of sludge in system}}$$

Ekama *et al.*, (1984) present the following guidelines for estimating f_{xa}:

- $S_{ti} < 400$ mgCOD/L, $f_{xa} = 0.20 - 0.25$;
- $400 < S_{ti} < 700$ mgCOD/L, $f_{xa} = 0.15 - 0.20$; and
- $S_{ti} > 700$ mgCOD/L, $f_{xa} = 0.10 - 0.15$.

Increasing f_{xa} above 15 to 20% does not appear to be justified as it must be remembered that increasing f_{xa} will result in an obligatory reduction in mass fractions of the other reactors in the system (Wentzel, 1992).

2.3.2 Wastewater characterisation

Wastewater is the source of substrate and nutrients for the microbial consortium of activated sludge and, as such, its composition must be regularly monitored to obtain maximum process efficiency. When designing an activated sludge process, the chemical characteristics of the wastewater to be treated are perhaps the most significant factors to consider (Ekama and Marais, 1984a). These characteristics dictate the selection of the process to be employed as well as the degree of P and N removal which will be attainable. It is crucial to determine the following characteristics of the influent wastewater when one requires NDEBPR:

- mean daily influent flow (Q_i);
- mean total influent COD (S_{ti}) and TKN (N_{ti}) concentrations as well as the readily biodegradable COD (RBCOD or S_{bsi}) fraction;
- TKN/COD and Total P/COD concentration ratios;
- if nitrification is desired, the maximum specific growth rate of the nitrifiers at the reference temperature of 20°C; and
- average minimum and maximum temperatures at which the process operates (Ekama and Marais, 1984a).

Carbon, nitrogen and phosphorus are required by microorganisms in balanced amounts although in activated sludge processes this will hardly be possible. A reasonable estimate for the C:N:P ratio is 100:6:1.5 (Buchan, 1984). Nitrogen can only be considered available for biological activity when present as ammonia and phosphorus when present as soluble phosphate. The nutrient content of wastewater will be discussed in further detail.

2.3.2.1 COD fractionation

Urbain *et al.*, (1998) were able to successfully design a multispecies model without initially fractionating the influent to predict steady-state and transient conditions describing BNR processes. Historically, the models which have been proposed to predict NDBEPR performance (Henze *et al.*, 1987; Dold *et al.*, 1991; Wentzel *et al.*, 1992b) require the division of influent COD into its constituent fractions to ensure accuracy (FIG. 2.3). It is, however, accepted that influent COD is the principal factor which governs BNR performance. Municipal wastewaters can be divided into three main COD fractions viz., unbiodegradable, degradable and heterotrophic active biomass. The unbiodegradable fraction can be divided into two further subfractions, termed unbiodegradable particulate and unbiodegradable soluble (Ekama *et al.*, 1992; Wentzel *et al.*, 1995). The soluble component will pass unaltered through the system and be discharged with the effluent but the particulate portion will be absorbed to the sludge floc surface and removed from the system via sludge wastage (Ekama *et al.*, 1992). The two subfractions of the biodegradable COD, however, play the most significant role in NDEBPR plants and their quantification is deemed absolutely essential to ensure maximum plant efficiency and monitoring of system response. Slowly or particulate biodegradable COD (SBCOD or S_{bpi}) consists of particulate organic matter which requires extracellular hydrolysis

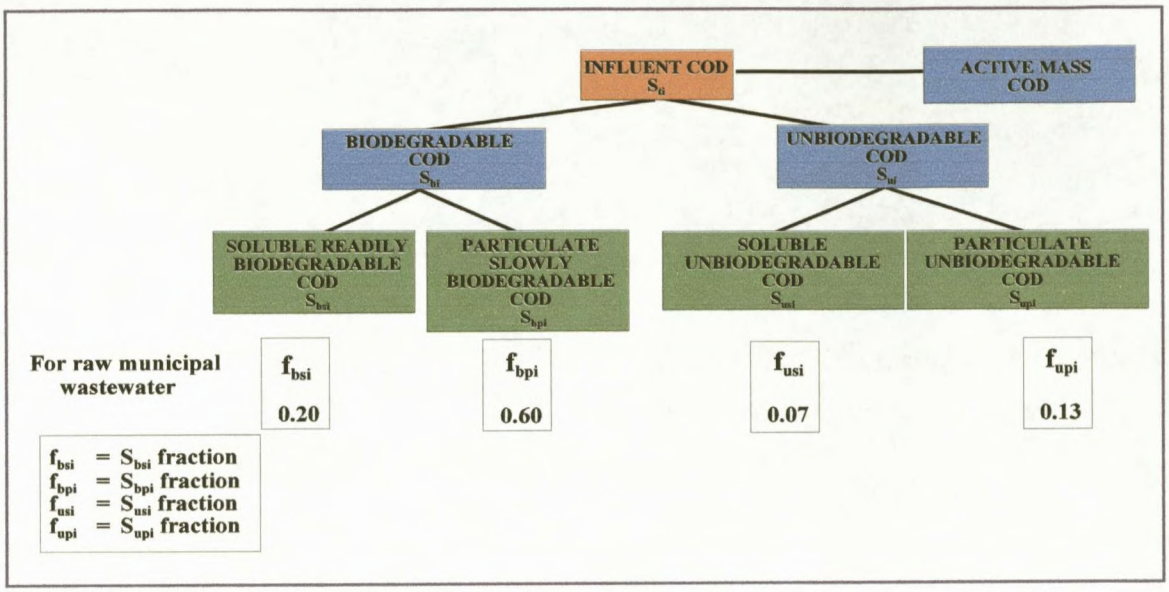

FIGURE 2.3 Total influent COD division of municipal wastewaters into various fractions (Ekama and Marais, 1984b).

(a rate limiting process) before it can be incorporated into the cell mass; RBCOD (S_{bsi}), however, consists of soluble, low molecular organics which can be utilised directly and rapidly (in the space of minutes) by the active biomass and plays a vital role in BEPR, denitrification and aerobic kinetics (Ekama *et al.*, 1992). It is this fraction of influent wastewater that engineers and microbiologists are most concerned with as it is of fundamental importance to BPR operations. The magnitude of both N and P removal has been directly linked to the magnitude of influent RBCOD in contact with the biomass in the anaerobic zone ie., the higher the VFA concentration as a result of RBCOD fermentation, the greater the amount of P released and consequently, the greater the amount of P taken up in the aerobic zone. According to Randall *et al.*, (1992, as cited by Punrattanasin and Randall, 1998), an influent COD concentration of 50 mg/L is required to biologically remove 1 mgP/L from municipal wastewater. The RBCOD fraction can be calculated by one of two methods; one is based on a bioassay method (Wentzel *et al.*, 1995) and the other on physical-chemical separation (Mamais *et al.*, 1993). The physical method is based on the hypothesis that the distinction between RBCOD and SBCOD can be attributable to differences in molecule size ie., RBCOD consists of small molecules which can be readily transported into microbial cells; conversely, SBCOD

consists of larger molecules requiring extracellular hydrolysis before uptake. Mamais *et al.*, (1993) found that filtration through a 0.45 μm filter (the inclusion of a flocculation step prior to filtration overcomes the problem of correct pore size selection) gave satisfactory results when compared to biological assays. Wentzel *et al.*, (1997) improved upon the method by reducing time consumption and costs considerably. They found that by replacing zinc sulphate flocculant with aluminium sulphate negated the need for pH adjustment and the 0.45 μm filters recommended by Mamais *et al.*, (1993) can be replaced with glass fibre filters (Whatman GF/C) without any loss in accuracy.

2.3.2.2 Phosphorus fractionation and determination

Total phosphorus (TP) in municipal wastewaters consists of a soluble ortho-P fraction and an organically bound P fraction which may be soluble or particulate in form (Ekama and Marais, 1984b). Through numerous batch studies, it has been shown that in both raw and settled municipal wastewaters the ortho-P fraction predominates, ranging between 70 to 90% of the TP. Organically bound P is converted to ortho-P through biological activity which indicates higher ortho-P concentrations in the activated sludge process required for removal than in the influent. Ekama and Marais (1984b) therefore recommend the TP test for influent P determination and the filtered ortho-P test for effluents.

In order to gain a more comprehensive understanding of the BPR mechanism occurring in activated sludge, it has become crucial to distinguish biologically-stored forms of phosphate (mainly poly-P) from chemically precipitated forms (mainly ortho-P; chemical precipitation albeit from biological processes) (De Haas, 1998). Psenner *et al.*, (1984) proposed a method of sequential extraction steps to quantify ecologically significant phosphate fractions in lake sediments. Uhlmann *et al.*, (1990) expanded upon this method to include the extraction and quantification of the poly-P fraction in EBPR activated sludge. However, the methods described above both include complicated extraction procedures and analysis techniques (nuclear magnetic resonance spectroscopy). Blonda *et al.*, (1994) proposed an indirect method of activated sludge ortho-P determination by means of the conventional method. The essence of this method is that an unfiltered sludge sample is subjected to the colour-forming reaction. Prior to spectrophotometry, the sludge residue is filtered off. The ortho-P content of a filtered sample is concurrently

determined and the difference between the two samples yields the sludge ortho-P content. This is summed up in the following formula (Blonda *et al.*, 1994):

Solid phase ortho-P = Total ortho-P (unfiltered) - Liquid phase ortho-P (filtered).

Activated sludge, particularly that from BNR plants, contains upwards of 1% P on a dry weight basis of the MLSS (De Haas *et al.*, 1990a). Although poly-P is an important component of stored P, it occurs in varying degrees together with nucleic acids, ortho-P and phospholipids in chemical extracts of activated sludge. It is common to find poly-P concentrations of cell extracts in the literature determined as the difference between ortho-P concentration before and after acid hydrolysis [1 M hydrochloric or 1 M sulphuric acid] (De Haas *et al.*, 1990b). Reference can be made to De Haas *et al.*, (1990a) for an evaluation of various techniques for ortho-P and TP determination in activated sludge extracts. A significant conclusion stated, however, was that it is inadvisable to determine ortho-P (especially at low concentrations) in the presence of acid-hydrolysable (condensed) phosphates such as poly-P and nucleotides. Due to the strongly acidic conditions imposed by the ortho-P method, other phosphate fractions present in solution will result in either positive or negative interference (De Haas *et al.*, 1990a).

2.3.2.3 Nitrogen fractionation

Similar to the various COD fractions, influent N consists of both biodegradable and unbiodegradable fractions. Nitrogen entering a wastewater treatment installation will invariably consist of an ammonia fraction (as free, NH_3, or saline NH_4^+ ammonia) and a proteinaceous fraction. The biodegradable organic N is ammonified in the anaerobic zone of the biological system ie., converted to ammonia N through various hydrolytic reactions, where it is made available for immediate incorporation into the bacterial protoplasm (Ekama and Marais, 1984b; Wanner, 1997). Due to the relatively low oxygenated state of sewer systems, nitrates and nitrites are likely to be denitrified before the wastewater reaches the plant and concentrations can be considered negligible (Ekama and Marais, 1984b).

Characterisation of influent nitrogenous material is made possible with two individual tests viz., the Total Kjeldahl Nitrogen (TKN) which measures organically bound N and free/saline ammonia. A separate Kjeldahl free and saline ammonia (KA) test can be performed if the organic N fraction is desired ie., TKN - KA = organic N. Nitrates and

nitrites in treated effluents can, however, be determined in separate tests of the same name (Standard Methods, 1989).

Concentration of the various influent N fractions influences the efficiency of both denitrification and EBPR rates alike. In normal municipal wastewaters where the RBCOD fraction of the influent is *ca.* 15 to 20%, denitrification is affected by the TKN/COD ratio in the following way:

- complete nitrate removal will be possible if the TKN/COD < 0.08 mgN/mgCOD in the Phoredox process;
- complete nitrate removal will not be possible if 0.08 < TKN/COD < 0.11; and
- EBPR will cease to occur if TKN/COD > 0.14 (Ekama *et al.*, 1984).

2.4 MICROBIOLOGY OF BIOLOGICAL PHOSPHORUS REMOVAL

Cloete (1997) proposed a simple model (FIG. 2.4) to deal and gather information regarding species diversity and function within a complex microbial ecosystem such as activated sludge. The model correlates species diversity with the various microbial habitats which are normally encountered in nature, allowing qualitative conclusions to be extrapolated concerning the microbial community. Category A indicates either a sterile situation or an environment where both species diversity and cell numbers are low. An increase in species diversity yet low cell numbers of each species is indicated by category B. Nutrient limitation accounts for the low number of individual cells found in the community. Category C represents a stable community where both the number of species and cell numbers of the representative species are high. Activated sludge is an example of a category C community and will be best suited to overcome environmental stress ie., toxic shock in which case the sludge will more than likely initially move towards a category A community; after a certain stabilisation (acclimation) period, it will shift towards category D (see later) and finally return to category C. Category D indicates an extreme environment where few species with a high number of individuals exist. Wentzel *et al.*, (1988), when identifying the organisms from an enhanced PAO culture (originating from laboratory-scale UCT and 3-stage Bardenpho /Phoredox systems), discovered that > 90% of the bacterial community constituted the genus *Acinetobacter*.

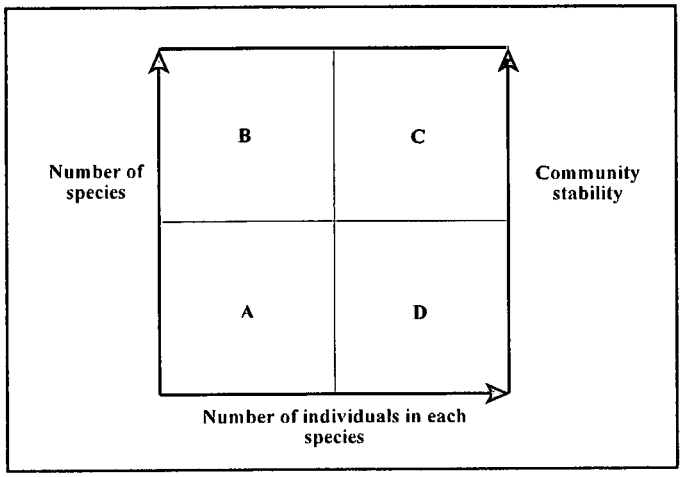

FIGURE 2.4 Theoretical model proposed by Cloete (1997) indicating various microbial habitats based on species diversity.

Enhanced cultures therefore appear to be best represented by a category D community due to the environmental stresses imposed on the resident microflora ie., sequencing anaerobic/aerobic zones and the gradual introduction of a single carbon source such as a low molecular organic compound viz., acetate. However, according to Bond *et al.*, (1997), operating a sequenced batch BPR reactor in order to achieve a culture displaying a strong EBPR mechanism resulted in the detection of very low numbers of *Acinetobacter* cells[4]. They concluded that bacteria other than *Acinetobacter* are responsible for EBPR. Results of these findings suggest that the development of enhanced cultures of PAO's should therefore be classified in either category B or C.

The biomass of activated sludge is the active agent of biological wastewater treatment, responsible for carbonaceous material oxidation and nutrient removal. To date, process engineering has received the greatest attention and in the interim, has practically become optimised. Yet systems based on NDEBPR principles still regularly fail to achieve the desired end result. This is due to limitations in our current understanding of the complexities of microbiological

[4] Wentzel *et al.*, (1988) used culture dependent methods of isolation and the API procedure for subsequent identification. Bond *et al.*, (1997) used *in situ* molecular techniques for bacterial identification.

interactions occurring within the sludge as well as our inadequate knowledge of microbial community structure-function correlations (Wagner *et al.*, 1993). To describe and control these microbial processes and mechanisms, Wanner (1997) suggests that activated sludge should be characterised from the following viewpoints:

- characterisation and quantification of microbial constituents according to metabolic activities;
- identification and classification of microorganisms; and
- activated sludge quality ie., settleability, dewaterability.

Ecological studies of activated sludge are integral to creating a complete and more definitive understanding of the process, diversity and various functions performed by the constituent microflora. The microbial community of activated sludge consists of bacteria, protozoa, fungi, algae and filamentous organisms although species types from different sludges will vary considerably, depending on process design and influent wastewater characteristics (Bux *et al.*, 1994; Muyima *et al.*, 1997). Since organic carbon is the most important energy source entering these systems, it can be expected that the heterotrophic bacteria will dominate the community structure (Kämpfer *et al.*, 1996). Probing activated sludge with fluorescently labelled oligonucleotide probes specific for the alpha, beta and gamma subclasses of the *Proteobacteria* (see section 2.5) has revealed that the microbial consortia are dominated by the *Proteobacteria* (approximately 80%), a phylum containing the majority of the traditional Gram negative bacteria [the majority of which are heterotrophic] (Wagner *et al.*, 1993). A number of heterotrophic organisms have been investigated intensively for their involvement in processes such as BNR. These organisms include *Acinetobacter*, *Moraxella*, *Pseudomonas*, *Microlunatus*, *Achromobacter*, *Aeromonas*, *Vibrio*, *Citrobacter*, *Pasteurella*, *Enterobacter*, *Proteobacter*, *Klebsiella*, *Bacillus* and coliforms (*Escherichia coli* and *E. intermedium*) (Hart and Melmed, 1982; Brodisch and Joyner, 1983; Cloete *et al.*, 1985a,b; Lötter, 1985; Lötter and Murphy, 1985; Lötter *et al.*, 1986a,b; Cloete and Steyn, 1988a,b; Kavanaugh and Randall, 1994; Wagner *et al.*, 1994a,b; Momba and Cloete, 1996a,b; Ubukata and Takii, 1998).

Many of these studies have implicated *Acinetobacter* as the principal agent responsible for BPR in activated sludge systems. Although Koch's principles for other nutrient removal processes viz., N assimilation in biomass, nitrification and denitrification as well as ammonia release from organic N, have been satisfied, the same cannot

be said for EBPR processes (Jenkins and Tandoi, 1991). Due to *Acinetobacter's* inability to fulfill the Koch-Henle postulates adapted to microbial ecology, the exact role of the organism in BPR has become somewhat dubious (Cloete *et al.*, 1985a; Steyn and Cloete, 1989). These revised postulates state that the microorganism in question must be associated with a certain phenomenon (BPR) under all circumstances; the organism must be isolated and studied in pure culture; pure culture studies must render similar results to those found in the natural habitat; and the microorganism must be present in sufficiently large numbers to warrant its association with the particular function (Steyn and Cloete, 1989). The primary concern amongst wastewater biologists is that not all *Acinetobacter* strains exhibit P release concomitant with substrate uptake under anaerobic conditions, a prerequisite for classification as a PAO.

2.4.1 Microbial growth and dominance

The ability of a microbial species to grow efficiently in an environment is dependent on two factors: (1) it must be capable of successfully competing for the limited supply of nutrients entering the system and of transporting them across the cell wall, and (2) it must then metabolise the compounds in order to produce energy (Horan, 1990). The greater the yield of energy from a given nutrient, the greater the ability of the bacterium to outgrow its competitors and achieve dominance in the habitat. Microorganisms are able to generate energy through intracellular catabolic processes in one of two ways, either by substrate-level or oxidative phosphorylation (Horan, 1990). Substrate-level phosphorylation allows organisms to generate ATP molecules in the absence of terminal electron acceptors through the catalysis of high energy containing compounds (such as PAO's in the anaerobic zone). Oxidative phosphorylation allows the reducing equivalent acceptors, NAD and NADP, to be reoxidised through the presence of a terminal electron acceptor (such as dissolved oxygen). This process is referred to as respiration and involves an electron transport chain which produces energy in the form of ATP as reducing equivalents are passed along the chain.

Wilkinson and Munro (1967, as cited by Pirt, 1975) discovered that when an energy source is supplied in excess ie., VFA's in the anaerobic zone, it may be stored as an energy reserve ie., PHB, constituting a high proportion of

the biomass dry weight. However, only if an organism contains a substrate specific permease[5] in its cell wall will it be able to actively take up and utilise the energy source. If a microorganism lacks the permease for a specific substrate, it will not be able to utilise the substrate for growth even if it is present at high extracellular concentrations (Horan, 1990).

According to Monod's equation, the specific growth rate of an organism (μ) is a function of its maximum specific growth rate (μ_m)[6], the saturation coefficient (K_s)[7] and the concentration of the growth limiting substrate (S). When assessing microbial competition for a limited foodstock ie., RBCOD in EBPR, K_s is a very important parameter to consider. K_s basically describes the affinity which an organism has for a growth-limiting substrate; the lower the value for K_s, the greater the affinity for substrate (Horan, 1990). Therefore, in growth-limiting environments such as wastewater treatment processes, those organisms which display low K_s values will have the propensity to grow rapidly and achieve dominance in the system. A high K_s value may therefore explain why the PAO community in activated sludge is unable to achieve dominance in the absence of a selector reactor. Conversely, in excess substrate environments, those organisms possessing higher μ_m values will grow successfully and attain dominance.

The effects of growth phase and initial cell concentration of *Acinetobacter* spp. on P release and uptake have also been investigated (Momba and Cloete, 1996a,b; Rustrian *et al.*, 1997). As can be expected, Momba and Cloete (1996b) found a linear relationship existed between initial biomass and phosphate uptake and that at low cell densities (10^2-10^5 per mL) there was a nett release of P rather than uptake. The physiological state of the cells also determines the response of the biomass to P accumulation. Cloete and Bosch (1994) have observed that P is accumulated at the end of log and during stationary phase once active growth has ceased and concluded that a maximum number of cells in the stationary phase of growth should be accumulated in the aerobic zone to optimise bio-P removal. Rustrian *et al.*, (1997) found that *Acinetobacter* released more P under anaerobic conditions when in the stationary phase of growth yet P uptake was equally efficient with cells in the log and stationary phase. These results can have a direct implication on EBPR operations as growth conditions for the PAO community can be

[5] Permeases are extremely selective carrier enzymes located in bacterial cell walls which recognise and transport specific or structurally related substrates across the cell membrane.
[6] μ_m is the growth rate of an organism which would be observed with no substrate limitation.
[7] K_s is the growth-limiting substrate concentration which allows an organism to grow at half μ_m.

better optimised to improve and promote the mechanism.

2.4.2 *Acinetobacter* - model polyphosphate accumulating organism?

Since enhanced phosphate removal was first postulated to be mediated by a biological mechanism (Srinath *et al.*, 1959; Fuhs and Chen, 1975), much research has centred around identifying and elucidating those organisms responsible for the process (Barnard, 1976; Hart and Melmed, 1982; Buchan, 1983; Cloete and Steyn , 1988a,b; Bosch and Cloete, 1993; Kavanaugh and Randall, 1994; Nakamura *et al.*, 1998). At the beginning of the decade, *Acinetobacter* came to be regarded as the model organism in biochemical models describing the mechanism of EBPR (Wentzel *et al.*, 1986) due mainly to its presence in high numbers and its favourable physiological characteristics when isolated from BPR treatment plants using conventional plating techniques. Bacteriological studies of biological nutrient removal (BNR) systems have emphasised the functional role of *Acinetobacter* in enhanced P removal (Lötter, 1985). Development of enhanced cultures of PAO's has shown tremendous specificity in the population structure when one considers that more than 90% of the organisms cultured aerobically from laboratory-scale UCT and 3-stage Bardenpho systems were identified as *Acinetobacter* (Wentzel *et al.*, 1988; Wentzel *et al.*, 1989a).

2.4.1.1 Characteristics and taxonomy

The type strain of the genus *Acinetobacter* viz., *A. calcoaceticus* (Bergey's, 1984), derived its name from a conclusive study by Baumann *et al.*, (1968). The investigators were looking to characterise 106 strains from the oxidase negative moraxella group (the *Mima-Herellea-Acinetobacter* group of bacteria) and concluded that all the strains investigated were members of one genus. They proposed that the name *Acinetobacter* be adapted for members of the group. They also suggested that the type species be named *A. calcoaceticus*, based on findings by Beijerinck in 1909 who isolated and named the bacterium *Micrococcus calcoaceticus* due to its ability to grow on media containing calcium acetate as the sole carbon source.

Acinetobacter strains have a wide distribution in nature and can readily be isolated from soil, water and sewage samples. Due to their ubiquity, classification of isolates by various researchers has proven very confusing and demanding. During earlier times, classification of the genus was extremely haphazard due to similarities of *Acinetobacter* to the genera *Moraxella*, *Neisseria* and *Branhamella*. Phenotypic similarities included the appearance of plump cells of coccal or coccobacillary shape which were arranged in pairs and which stained Gram negative (Henriksen, 1976). Bacteria belonging to the genus *Acinetobacter* have been difficult to classify due to their lack of unique phenotypic properties which enable them to be differentiated with any certainty from other similarly appearing organisms (Juni, 1978).

According to classification in Bergey's Manual (1984), distinguishing phenotypic characteristics of *Acinetobacter* are Gram negative reactions, an aerobic metabolism, and oxidase negative[8] and catalase positive biochemical assay. They appear as plump rods, commonly occurring in pairs, in the logarithmic phase of growth but become spherical (coccoid) in the stationary phase (Lötter and Murphy, 1985). Dimensions of individual cells are 0.9-1.6 μm in diameter and 1.5-2.5 μm in length. They are mesophilic organisms, growing between 20-30°C but most strains grow optimally at 33-35°C. Many strains are encapsulated and do not form endospores. Although they do not have flagella, species do show twitching motility [those strains exhibiting motility have been shown to have polar fimbriae, 50 Å in diameter] (Henriksen, 1976; Juni, 1978). Most members of the genus grow well in defined media containing a single carbon and energy source. Most *Acinetobacter* spp. are unable to reduce nitrate to nitrite although those strains which are capable of nitrate reduction are unable to utilise the compound as an alternate electron acceptor in anaerobic respiration processes (Juni, 1978). *Acinetobacter* spp. are unable to obtain energy for growth by fermentation. When grown on solid media, *Acinetobacter calcoaceticus* (*A. calcoaceticus*) colonies are domed and mucoid with a viscous or gluey consistency. They are whitish to cream in colour and have a musky smell (Lötter and Murphy, 1985). After 20 h of growth, colonies are approximately 1-2 mm in diameter.

2.4.1.2 *Microlunatus phosphovorus* - the new model organism?

Due to the controversy surrounding the role of *Acinetobacter* in P removal operations, microbiologists have, for

[8] Indicates a lack of cytochrome C.

the past decade, attempted to isolate other bacteria in activated sludge upon which the mechanism can be modelled. Recently, a new P removing bacterium from a laboratory-scale activated sludge system in Japan was isolated and identified as *Microlunatus phosphovorus* (*M. phosphovorus*) (Nakamura *et al.*, 1995, as cited by Ubukata and Takii, 1998). The bacterium shows all the physiological traits characteristic of PAO's ie., P accumulation mechanism is only induced in sequential anaerobic/aerobic systems, and has the propensity to accumulate P to a maximum of 23% dry weight (luxury uptake). Its carbon and phosphorus transformation patterns coincide with those of EBPR sludge and, as such, the bacterium has been considered as a candidate for the model PAO in EBPR processes. *M. phosphovorus* is a Gram positive coccus (diameter = 1.7 - 2.1 μm) and sequencing shows a high G+C genomic DNA content [65.6 mol%; phylogenetically belongs to the Gram positive high GC (HGC) bacteria] (Ubukata and Takii, 1998). The excess P accumulation mechanism is inducible in the bacterium, a useful feature when examining and attempting to define bio-P removal processes. However, the bacterium's dominance in EBPR processes has yet to be demonstrated and because it cannot readily be isolated from activated sludge (as opposed to *Acinetobacter*), its application to BPR studies may be limited.

2.4.1.3 The 'G' bacteria

When assessing EBPR efficiency, cognisance must be taken of the 'G' bacteria, a group of normal inhabitants found in activated sludge mixed liquor. There is a microbial community in activated sludge which is capable of organic substrate uptake and assimilation in the anaerobic zone with subsequent metabolism of these storage granules in the aerobic zone (Maszenan *et al.*, 1998). This community is composed of two distinct groups of organisms with distinctly different modes of substrate uptake and synthesis of storage granules. The first group are the PAO's, the metabolism of which has been previously discussed. The second group are the 'G' or glycogen accumulating organisms (GAO's). In conventional systems GAO's are involved in normal organic oxidation processes but in selector systems such as BPR, their presence and impact to system efficiency can become more prominent. These Gram negative cocci grow as tetrads and are able to out-compete PAO's in anaerobic/aerobic systems by accumulating polysaccharide and not polyphosphate in the aerobic zone (Cech and Hartman, 1993; Cech *et al.*, 1994; Maszenan *et al.*, 1998). The 'G' bacteria are thought to be able to effectively compete with fermentative organisms for RBCOD and PAO's for VFA in the anaerobic zone as they are able to obtain the necessary reducing

power and energy required for uptake through glycolysis ie., the Embden-Meyerhof pathway. Their proliferation in an EBPR system will eventually lead to a decline as far as phosphate removal is concerned. Cech and Hartman (1993) found that the 'G' bacteria were able to dominate in an anaerobic-oxic system when the influent consisted of an acetate-glucose mixture yet when acetate was used as substrate alone, the PAO's were able to dominate their competitors. Influent P/COD (P_{ti}/S_{ti}) ratios also affects the microbial community structure of EBPR activated sludge (Liu *et al.*, 1997). A low P_{ti}/S_{ti} ratio enriches for GAO's whilst high P_{ti}/S_{ti} ratios promote the growth of PAO's and suppress the proliferation of GAO's in the process (Liu *et al.*, 1997). Competing with the non-PAO's ie., fermentative bacteria, for RBCOD in the anaerobic zone reduces VFA production which ultimately influences P release by the PAO's. With the advent of molecular techniques for elucidating bacterial identification and relatedness, it has been shown that all the Gram negative tetrad cocci in activated sludge are closely related taxonomically, belonging to the alpha subdivision of the *Proteobacteria* [see section 2.5] (Maszenan *et al.*, 1998). According to Blackall *et al.*, (1997), independently isolated 'G' bacterial strains from the Cech and Hartman (1993) study and from full-scale plants in Italy showed near identical homogeneity to one another and 93% homogeneity to *Rhodobacter* (alpha subdivision of *Proteobacteria*), based on 16S rDNA sequences. They proposed the generic and species name, *Tetracococcus cechii*, for the two strains. Maszenan *et al.*, (1998) proposed a new genus (*Amaricoccus*) to house four 'G' bacterial isolates from Australia, Italy and Macau. Phylogenetic classification of these isolates in the *Proteobacteria* phylum of the domain *Bacteria* shows their relatedness to many of the PAO's (see section 2.5), indicating that some relationship between the two competing bacteria does exist. This implies that an identical or similar mode of metabolism between the two bacterial types may have existed at some stage of their evolution.

2.5 TAXONOMICAL STATUS OF THE *PROTEOBACTERIA* (PURPLE BACTERIA)

All life on earth is comprised of three domains ie., the *Bacteria* (eg., *Acinetobacter*, *Pseudomonas*), the *Archaea* (microorganisms thought to live in extreme environments ie., anaerobic, heat and salt) and the *Eukarya* (animals, fungi, plants, protists) (Woese *et al.*, 1990). This phylogenetic system is based on comparative analyses of sequences of the small subunit (16S) rRNA gene. The taxonomic name *Bacteria* refers only to the previously

named *Eubacteria*, a super-kingdom quite distinct from the *Archaebacteria*. The explicit distinction made between the *Bacteria* and *Archaea* illustrates how bacteria can no longer be classified in terms of morphological and/or biochemical characteristics. The majority of microorganisms are morphologically indistinct; only in rare cases does this method of characterisation allow the microbiologist to definitively identify individual species through microscopy (Woese, 1987; Manz *et al.*, 1992). Relationships based on nucleotide sequencing now have to be considered in order to gain a more definitive method of classification and phylogeny (Woese, 1987). As the majority of bacteria from natural communities remain unculturable [9], it has been suggested that phenotypic methods of identification ie., culture dependent methods, should be complemented by *in situ* methods of bacterial cell identification and enumeration (Wagner *et al.*, 1993; Wagner *et al.*, 1994a).

The domain (super-kingdom) *Bacteria* presently consists of eleven characterised bacterial phyla (divisions) (TABLE 2.2, Woese, 1987; Wagner and Amann, 1997). It is evident from TABLE 2.2, that when using the 16S rRNA molecule to phylogenetically structure the domain *Bacteria*, the arrangement within the various phyla becomes somewhat confusing ie., photosynthetic species grouped with non-photosynthetic species; anaerobes are paired with aerobes and heterotrophs with chemolithotrophs. Most of the taxa are defined by unique, conserved oligonucleotide sequences or signatures ie., rRNA sequence positions which are not found in the rRNA of other groups (Manz *et al.*, 1992). Many of the traditional Gram-negative bacteria are contained in the *Proteobacteria* class (Woese, 1987; Manz *et al.*, 1992). At least four distinct subdivisions exist in the purple bacteria, designated alpha (α), beta (β), gamma (γ) and delta (δ) (Woese, 1987).

2.6 THE "GREAT PLATE COUNT ANOMALY" AND THE REVOLUTION

A revolution is occurring in the field of microbiology. For too long, microbiologists concerned with analysing various microbial communities and population dynamics within a specific biocenosis have relied heavily upon conventional plating techniques for isolation purposes. Although the determination of bacterial numbers in a given sample is a basic prerequisite for any microbiologist, attempts at understanding microbial diversity have been

[9] When plating on solid media, the number of colony forming units represents only a fraction (< 10% or even < 1%) of the viable cell counts made by direct microscopic techniques (De Haas, 1998).

severely limited due to a lack of suitable isolation and identification techniques (Brözel and Cloete, 1992). The main concern amongst wastewater scientists is that many of the microbial constituents of the activated sludge

TABLE 2.2 Phyla and subdivisions (including representative examples) of the domain *Bacteria* (blue coloured rows indicate those subdivisions known to be implicated in EBPR).

***Proteobacteria* (Purple bacteria)**
α subdivision (*Nitrobacter*)
β subdivision (*Alcaligenes*, *Pseudomonas testoteroni*, *P. cepacia*, autotrophic nitrifiers)
γ subdivision (*Acinetobacter*, *Aeromonas*, fluorescent pseudomonads, Enterics, *Vibrio*)
δ subdivision (sulphate reducing bacteria and myxobacteria)
***Firmicutes* (Gram positive bacteria)**
A. *Actinobacteria* (High G+C species) (*Arthrobacter*, *Micrococcus, Microlunatus*)
B. *Bacillus/Clostridium* group (Low G+C species) (*Clostridium*, *Bacillus*)
C. Photosynthetic species (*Heliobacterium*)
D. Species with Gram negative walls (*Megasphaera*)
Cyanobacteria and chloroplasts
Nostoc, *Oscillatoria*
Spirochetes and relatives
A. Spirochetes (*Spirochaeta*)
B. Leptospiras (*Leptospira*)
Green sulfur bacteria
Chlorobium
Bacteroides, flavobacteria and relatives
A. Bacteroides (*Bacteroides*)
B. Flavobacterium group (*Flavobacterium*, *Cytophaga*)
Planctomyces and relatives
A. Planctomyces (*Planctomyces*)
B. Thermophiles (*Isocystis*)
Chlamydiae
Chlamydia

TABLE 2.2 ***continued***

Radioresistant micrococci and relatives
A. Deinococcus group (*Deinococcus radiodurans*)
B. Thermophiles (*Thermus aquaticus*)
Green non-sulfur bacteria and relatives
A. Chloroflexus group (*Chloroflexus*)
B. Thermomicrobium group (*Thermomicrobium roseum*)
Thermotogoa

community are viable yet non-culturable (Wagner *et al.*, 1994a,b; Amann *et al.*, 1995) which inevitably results in extremely biassed assumptions regarding biodiversity. Examination of TABLE 2.3 reflects our present level of understanding of microbial diversity (adapted from Bull *et al.*, 1992).

TABLE 2.3 Known and estimated numbers of microbial species.

Group	Discovered	Estimated total	Percentage of discovered species
Viruses	5 000	130 000	4
Bacteria	4 760	40 000	12
Fungi	69 000	1 500 000	5
Algae	40 000	60 000	67

The activated sludge process is one such habitat which consists of a complex mixture of generalist and specialist microorganisms. In an attempt to obtain a fundamental understanding and to optimise the biological component of key processes such as EBPR occurring within sludge, isolation (or lack thereof) and identification techniques have, of necessity, progressed to the molecular level of organisation. The conventional method of dilution and spread plating is the main reason why, for years, *Acinetobacter* has been implicated as the primary active agent in BPR operations.

2.6.1 Conventional isolation and identification techniques

A wide range of bacteria can be isolated from activated sludge mixed liquors using conventional microbiological techniques such as sample dilution and spread plate inoculation. A number of methods such as the Most Probable Number (MPN) technique, pour plate method, surface plate method and membrane filtration are available for estimating numbers of selected metabolic groups in sludge (Schade and Lemmer, 1994). However, these methods (either the composition of the media or the incubation protocol) have been shown to be extremely selective as to which organisms are allowed to grow and form visible colonies. The two different types of cells which remain viable yet non-culturable in environmental samples can either be: (1) known species for which the cultivation conditions are not suitable for growth or which have entered a non-culturable state, and (2) unknown species that have not yet been cultured for lack of suitable techniques (Amann *et al.*, 1995). Sufficient documentation of the true community structure is therefore not made available (Snaidr *et al.*, 1997). This becomes evident when one considers EBPR. The most significant effect of cultivation of activated sludge mixed liquor on nutrient rich solid media is an underestimation of bacteria belonging to the beta subclass of *Proteobacteria* and a gross overestimation of members of the gamma subclass of *Proteobacteria* (Wagner *et al.*, 1994b). Standard plate counts do not reveal the bacterial community structure of activated sludge; they rather reflect the selectivity of the growth media for certain bacteria (Wagner and Amann, 1997). In effect, typical isolation media and conditions give rise to typical bacteria whilst the unculturable fraction of the population, which may also be functionally active, are overlooked.

As far as solid media is concerned, Casitone Glycerol Yeast Autolysate Agar (CGY) has been shown to yield the highest plate counts for activated sludge samples (Pike *et al.*, 1972; Bux *et al.*, 1994). However, due to its high nutrient content the suitability of the medium to isolate viable bacteria from activated sludge is questionable. Solid cultivation media should, as far as possible, emulate the environment from which the bacteria of interest originate ie., bacteria from oligotrophic systems are best enumerated with low nutrient agars eg., R2A and R3A agars; conversely, bacteria from eutrophic systems are enumerated with high nutrient agars eg., CGY, trypticase soybroth (TS), nutrient and plate count agar (Osborn *et al.*, 1989; Brözel and Cloete, 1992; Kämpfer *et al.*, 1996). Nutrient rich media, when applied to population community analyses of activated sludge will render erroneous results as it

will support the growth of faster growing bacteria ie., higher μ_m, which may not actually play a major role in the system (Osborn *et al.*, 1989). When isolating bacteria from an EBPR activated sludge system, Kämpfer *et al.*, (1996) found that recovery rates using R2A agar were usually in the region of one order of magnitude higher than the nutrient rich medium, TS agar. Osborn *et al.*, (1989) found that diluted CGY agar yielded the highest recoveries from activated sludge, when compared with dilute Fuhs and Chen media, balance tank effluent and settled sewage. Yet none of the media investigated isolated the total bacteria present in the mixed liquor samples. Different media, however, result in different population compositions, so direct comparisons of the various culture-dependent methods available to evaluate isolation efficiency are not beneficial. Although no medium can be expected to recover all viable cells from wastewater samples, it seems appropriate to use either chemically defined or custom manufactured media designed to yield the highest counts (Reasoner and Geldreich, 1985).

Some of the commercially available biochemical test procedures aimed at speeding up the process of identification from cell cultures have been found to be potentially unreliable when identifying *Acinetobacter*. It is possible that the assays confuse *Acinetobacter* with other closely related genera such as *Pseudomonas*, *Moraxella* and *Alcaligenes* (Osborn *et al.*, 1989; Venter *et al.*, 1989). The analytical profile index (API) has been one of the most widely used identification tools for isolates obtained from various activated sludge community studies (Hart and Melmed, 1982; Brodisch and Joyner, 1983; Lötter, 1985; Lötter and Murphy, 1985; Osborn *et al.*, 1989; Bosch and Cloete, 1993; Kavanaugh and Randall, 1994). Although these studies used either the API 20E or API 20NE test kits, the accuracy of identification was apparently not examined. The system was originally designed for use with clinical isolates and those exhibiting strongly fermentative metabolism (Knight *et al.*, 1993). Some degree of discrepancy can therefore be expected when attempting to identify isolates from environmental samples or those exhibiting aerobic oxidative metabolism. Venter *et al.*, (1989) evaluated the accuracy of isolate identification by the API 20E system and found it to be reliable provided the following precautions are realised:

- fermentation tests are checked 24 h after the first reading;
- inoculum must be in the logarithmic phase of growth; and
- the API oxidase test is not conclusive and must be supplemented by a standard oxidase test.

Venter *et al.*, (1989) concluded that the high numbers of *Acinetobacter* in previous studies may have been due to the unreliable API oxidase test. When identifying strictly aerobic isolates from a full-scale activated sludge plant using both the API 20E system (in conjunction with the standard oxidase test) and conventional biochemical assays, Venter *et al.*, (1989) found a strong correlation in the numbers of *Acinetobacter* spp. between the two methods, at values (expressed as a percentage of the total CFU count) considerably lower than those reported elsewhere when using the API system alone (10% of total colonies isolated). Osborn *et al.*, (1989) also found that the API system was not reliable at the species level of identification and that additional biochemical tests had to be conducted to confirm identity. Nevertheless, the API identification system remains a rapid tool for bacterial identification from environmental samples provided the manufacturers instructions are followed precisely and the necessary precautions mentioned above are taken.

The Biolog and Microbact identification systems have also been used in various activated sludge diversity studies (Osborn *et al.*, 1989; Beacham *et al.*, 1990; Knight *et al.*, 1993; Cloete, unpublished data). As mentioned above, many of the commercially available rapid identification systems have been designed for use with clinical isolates and those with strongly fermentative metabolism. However, manufacturers of the Biolog Identification System have claimed the system is capable of identifying nonclinical isolates of organisms such as *Acinetobacter* (oxidative metabolism). To appraise the claim, Knight *et al.*, (1993) isolated a large number of *Acinetobacter* spp. from a full-scale BNR plant in Australia (confirmed as *Acinetobacter* by the transformation assay of Juni, 1972). They had a high identification success rate at genus level (82.8%) but reported a certain degree of difficulty in reading the plates (can be overcome when using the manufacturers autoanalyser). They also attributed incorrect identification to an insufficient data base and claim that although the system is a valuable tool for environmental microbiologists, it will only become recognised once the data base is updated. Osborn *et al.*, (1989), whilst evaluating mixed liquor bacteria recovery rates of various nutrient media, concurrently compared the competency of the Microbact and API systems for identification purposes. They found that the Microbact nitrate reduction test was more reliable than the API test and the incorporation of more tests ie., 24 tests for Microbact as opposed to 20 for API, makes the Microbact system more reliable and species specific than the API system. The lower cost of Microbact also makes it a more attractive identification tool than API.

2.6.2 Direct enumeration and identification techniques

A means to overcome the quantitative and qualitative biases imposed by cultivation-dependent methods is the use of chemotaxonomic and molecular techniques for *in situ* analyses of microbial communities (Amann, 1995). The term *in situ* indicates that all microbial cells in an environmental sample are not isolated prior to investigation but are rather studied intact, within the original sample (Snaidr *et al.*, 1997). Techniques employed include application of biomarker approaches, immunofluorescence and sequence-based molecular methods, otherwise referred to as fluorescently labelled *in situ* hybridization (FISH). These methods have proven to be very powerful tools in their ability to quantitatively enumerate specific species in diverse microbial communities and many mainstay perceptions (ie., *Acinetobacter* as model poly-P organism) have been shattered due to their accurate and reliable identification abilities. TABLE 2.4 illustrates the variance in results when different identification techniques are applied to activated sludge in an effort to enumerate *Acinetobacter* in EBPR mixed liquor samples.

TABLE 2.4 Numbers of *Acinetobacter* spp. in activated sludge using different enumeration techniques.

Number of *Acinetobacter* cells (% of total community)	Enumeration/identification technique	Reference
54 - 66%	API 20E	Hart and Melmed, 1982
90%	total plate counts and API 20E	Lötter *et al.*, 1986a
5 - 11%	MPN and API 20E	Kavanaugh and Randall, 1994
< 10%	fluorescent antibodies	Cloete and Steyn, 1987; 1988b
3 - 6%	quinone profiles	Hiraishi *et al.*, 1989
< 1%	16S rRNA oligonucleotide probes	Bond *et al.*, 1997
6%	16S rRNA oligonucleotide probes	Wagner *et al.*, 1994b

2.6.2.1 Flourescent *in situ* hybridization (FISH)

There are three categories of RNA in prokaryotes viz., messenger (m) RNA responsible for transmitting sequence information from the chromosome to the ribosome; transfer (t) RNA which decodes the message and ribosomal (r) RNA involved in the structure of the ribosome and reading of the genetic message (Priest and Austin, 1993).

Ribosomes within biological cells are the sites of protein synthesis. They are composed of a mixture of nucleic acids (ribosomal RiboNucleic Acids - rRNA) and proteins and in bacterial cells, have an average size of 70S (Blackall *et al.*, 1998a). Because of their central role in cell survival, maintenance and reproduction, rRNA's and their genes are almost universally present in cellular life forms, are functionally constrained and therefore evolutionally conserved which makes them valuable indicators of identity and relatedness (Olsen and Woese, 1993). Perhaps their greatest quality as far as spatial distribution and identification in activated sludge (or any environmental sample) is concerned is their natural amplification and high copy number per cell (usually >10 000/cell) (Amann, 1995). Three sub-units ie., the 5S, 16S and 23S molecules, constitute an intact rRNA molecule. An average bacterial 16S rRNA molecule is approximately 1 500 nucleotides long whilst that of 23S rRNA molecules is 3 000 nucleotides (Amann *et al.*, 1995). There is therefore sufficient information contained within these molecules to establish reliable phylogenetic analyses, even if they are not fully sequenced (>1 000 nucleotides should be determined for accuracy and confidence) (Amann *et al.*, 1995).

Each rRNA-targeted oligonucleotide is chemically linked to a fluorochrome molecule which allows cells hybridised with the fluorescently labelled oligonucleotide to be directly visualised using epifluorescent microscopy or scanning confocal laser microscopy (Amann, 1995; Amann *et al.*, 1995). The choice of fluorochrome used in fluorescent studies (immunofluorescence or probe hybridization) is dependent upon availability in a stable, purified form, the ease with which it can be coupled to a carrier molecule (antibody or oligonucleotide probe) without influencing biological activity and the availability of optical apparatus that enable detection of the emitted fluorescent light (Bosch and Cloete, 1993). When excited with light at the appropriate wavelength, probes produce a dye conferred fluorescence which allows cells below the limit of resolution of light microscopy to be visualised (Porter and Feig, 1980).

The general procedure of bacterial cell quantification, using FISH, from an environmental sample is shown in FIG. 2.5. The process usually incorporates a total cell count using the DNA intercalating dye 4,6-diamidino-2-phenylindole (DAPI) followed by hybridization with the universal bacterial probe, EUB (the fluorescing stain 3,6-tetramethyl diaminoacridine or acridine orange can also be used to microscopically visualise DNA molecules but

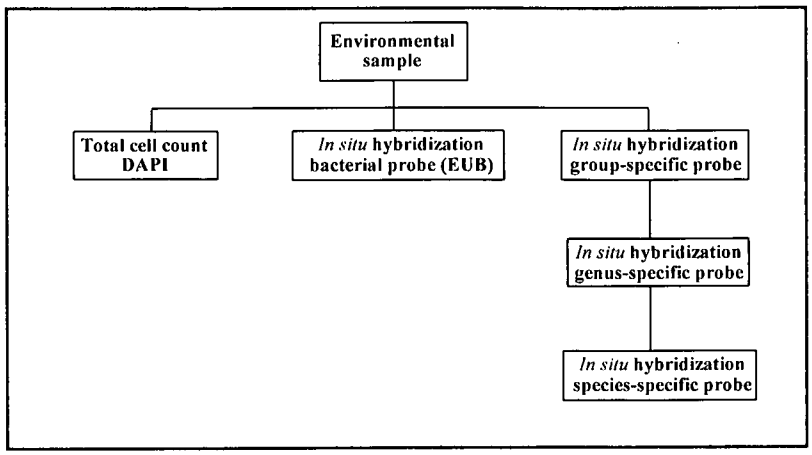

FIGURE 2.5 General flow sheet for the rRNA approach to community analyses (Wagner and Amann, 1997).

there are a number of difficulties inherent to its application; refer to Porter and Feig (1980) for a brief review). DNA-DAPI complexes fluoresce bright blue when visualised at wavelengths of > 390 nm whilst unbound DAPI and non-DNA-DAPI complexes fluoresce a weak yellow which enhances the visibility of DAPI fluorescence (Porter and Feig, 1980). The EUB/DAPI ratio gives an indication as to the bacterial composition of the sample. Dual EUB/DAPI staining of activated sludge samples (Hicks *et al.*, 1992, as cited by Wagner and Amann, 1997) has revealed that approximately 80% of the microbial cells present in the samples were metabolically active bacteria, of which only 3-19% could be cultivated on optimised media. The specificity of oligoprobes is freely adjustable, dependent upon the requirements of the user. Different phylogenetic levels ranging from kingdom eg., *Bacteria* (EUB), to subclass eg., gamma (GAM) subclass of *Proteobacteria*, to genus eg., *Acinetobacter* (ACA), species and subspecies can be probed, the degree of organization required been the only limitation (Wagner *et al.*, 1994a).

In situ identification of the organisms in a batch-type EBPR sludge has revealed that the four major bacterial groups present were the alpha and beta subclass of *Proteobacteria*, Gram positive bacteria with a high G + C content and bacteria belonging to the *Cytophaga-Flavobacterium* cluster of the *Cytophaga-Flavobacterium-Bacteroides* phylum (Kawaharasaki *et al.*, 1999). However, Wagner *et al.*, (1994a), when characterising the Proteobacterial microbial

consortia in municipal mixed liquor from Hirblingen, Germany, found that the beta subclass dominated over the alpha and gamma subclasses. Simultaneous plating of the sludge on nutrient rich medium showed dominance of the gamma subclass of *Proteobacteria* emphasising the bias introduced with cultivation techniques. *In situ* hybridization of mixed liquor samples at the family level of organization from the anaerobic and aerobic zones of the same plant in Germany have revealed the following trends (Snaidr *et al.*, 1997):

•	anaerobic basin	α *Proteobacteria*	=	11%
		β *Proteobacteria*	=	24%
		γ *Proteobacteria*	=	5%
		GPBHGC	=	24%
		Cytophaga-Flavobacterium	=	9%
•	aerobic basin	α *Proteobacteria*	=	9%
		β *Proteobacteria*	=	26%
		γ *Proteobacteria*	=	10%
		GPBHGC	=	19%
		Cytophaga-Flavobacterium	=	8%

These results show conclusively that there is no markable population shift between zones in an EBPR process when characterised at family level. Whether a shift occurs at species level requires verification, but regardless of any shifts which may occur, the significance of the *Proteobacteria* to efficient EBPR operations is evident.

Staining activated sludge samples with DAPI at elevated concentrations to those used for DNA staining - referred to as the polyphosphate-probing concentration - results in the fluorescence of intracellular volutin and lipid inclusions (Nakamura *et al.*, 1998; Streichan *et al.*, 1990, as cited by Kawaharasaki *et al.*, 1999). Bacteria which accumulate large quantities of poly-P are easily distinguished by colour and intensity of fluorescence due to the following DAPI stain characteristics: (1) DNA-DAPI fluorescence is blue-white; (2) polyphosphate-DAPI fluorescence is bright yellow; and (3) lipid-DAPI fluorescence is weak yellow and fades rapidly (in the space of seconds) (Kawaharasaki *et al.*, 1999). Dual staining of samples with DAPI at elevated concentrations and EUB will result in the determination of the PAO population in EBPR sludges ie., PAO/EUB ratio. *In situ* identification of

those bacteria exhibiting strong poly-P accumulation (identified through DAPI staining) can also be achieved through dual staining with class ie., *Proteobacteria*, and subclass ie., alpha, beta or gamma, oligonucleotide probes which totally negates culture-dependent methods of isolation and/or Neisser (poly-P) staining procedures.

For all its promise and potential, however, there are still technical problems inherent to FISH when applying the technology to microbial systems such as activated sludge. Qualitatively, the protocol of probe hybridization and detection has been optimised at all levels of organisation but due to the nature of activated sludge, quantitative results are often difficult and limited ie., complete dispersion of sludge flocs remains a technical problem which limits the application. Other problems include DNA retrieval for sequence determination, PCR biases when amplifying the sequence of interest and an imposed selection of the retrieved or target sequences (Hiraishi *et al.*, 1998). One of the possible solutions directed towards these problems is the combination of molecular and biomarker methods.

2.6.2.2 Immunofluorescence

The immunofluorescence approach was introduced as an *in situ* identification technique, prior to FISH, in an attempt to avoid culture-dependent techniques and has been used effectively to identify *Acinetobacter* in activated sludge samples (Cloete et al., 1985a,b; Lotter and Murphy, 1985; Cloete and Steyn, 1987; Cloete and Steyn, 1988b). Although the technique is highly specific for the bacterium in question, there are a number of limitations associated with it (Wagner and Amann, 1997). Firstly, the presence of extracellular polymeric substances in activated sludge flocs can inhibit the penetration of antibodies to the target cells; the method of raising antibodies in host animals requires initially culturing the bacterium of interest; and cross-reaction of antibody with contaminants does occur, resulting in high levels of background fluorescence (Wagner and Amann, 1997).

2.6.2.3 Quinone profiles

Respiratory or isoprenoid quinones are a class of lipids which are constituents of bacterial plasma membranes. They play important roles in electron transport, oxidative phosphorylation and active transport across the membrane (Collins and Jones, 1981). The numerical analysis of lipoquinone profiles has offered an effective method for

monitoring population shifts and for classifying bacterial communities in wastewater sludges (Hiraishi *et al.*, 1991). Quinones are usually extracted from an environmental sample using an organic solvent. After evaporation and re-extraction, the concentrated quinone is applied to column chromotography to separate menaquinone and ubiquinone (Hiraishi *et al.*, 1998). Municipal sludges are usually characterised according to their menaquinone and ubiquinone components (Hiraishi *et al.*, 1989; Hiraishi *et al.*, 1998). Quinone components are then identified and quantified using spectrochromotography and mass spectrometry. Numerical analyses of quinone profiles can enhance the information regarding bacterial community dynamics in wastewater ecosystems. The strength of the technique lies not only in its ability to assess taxonomic structure of bacterial communities but also in that variations in bacterial population structure over space and time can be quantified (Hiraishi *et al.*, 1991).

2.6.2.4 Microautoradiography

Autoradiography has classically been used in the medical field but has recently been introduced to environmental sample analyses to determine microbial community structures. Typically, a radiolabelled compound appears in the cell or biological structure of interest through adsorption of a tracer or labelled substrate uptake. The radiolabelled sample is then placed in contact with a radiosensitive emulsion and the emissions from the radioactive sample interact with silver bromide crytals in the emulsion. The emulsion is then developed using standard photographic procedures and the silver grains appear on top of the radioactive structure which can then be viewed microscopically (Nielsen *et al.*, 1998; Nielsen *et al.*, 1999a). Although autoradiography can successfully be applied to study the *in situ* physiology of various microorganisms, it is limited by its lack of proper identification of the organisms in question. However, Nielsen *et al.*, (1998), through simultaneous use of autoradiography and FISH, were able to correlate function/activity with identification which is a tremendous breakthrough for activated sludge identification-diversity-physiological studies.

2.7 CONCLUSIONS FROM REVIEW OF LITERATURE

Although there is a general consensus that *Acinetobacter* spp. is not the model PAO, there still exists a large degree of doubt as to exactly which bacterial organisms are contributing to the process. The fact that existing models are

been applied to design new or upgrade existing wastewater treatment installations and that these plants sometimes fail to achieve the primary objective of BNR seems to bear witness to this fact. A more comprehensive understanding of the microbial community/biocenosis of a biological wastewater treatment system will assist in improving system design and performance. The organisms are, after all, the primary catalysts responsible for the various biodegradation processes which occur to remediate the wastewater in question. With the advent of more conclusive and qualitative techniques for microbial community analyses, it seems likely that many of these issues will be addressed in the near future. Accordingly, a thorough investigation of the microbial community in both a full-scale biological nutrient removal plant and enhanced poly-P culture seems warranted to attempt to elucidate those organisms which are actively involved BPR.

CHAPTER THREE

ISOLATION AND IDENTIFICATION OF POLYPHOSPHATE ACCUMULATING ORGANISMS FROM A FULL-SCALE NDBEPR WASTEWATER TREATMENT PLANT USING CONVENTIONAL PLATING TECHNIQUES

3.1 INTRODUCTION

When Darvill Wastewater Works (WWW) was purchased by Umgeni Water in 1992, a large capital investment was injected into the plant in order to address the need, amongst others, for:

- a formal anaerobic zone;
- the introduction of a pre-anoxic zone to protect the anaerobic zone from the recycle of nitrates; and
- a primary sludge fermentation-thickener system for generation of VFA's to be pumped directly to the anaerobic zone (De Haas, 1998).

The activated sludge process at Darvill WWW consists of conventional primary treatment and sedimentation, followed by secondary biological treatment. The activated sludge process has been designed to conform closely to the Johannesburg process configuration (FIG. 3.1).

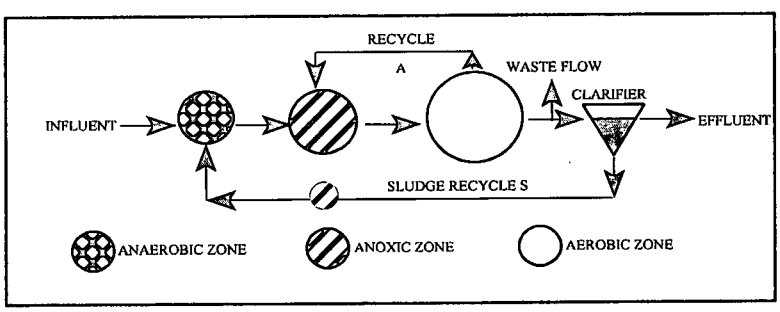

FIGURE 3.1 The Johannesburg system incorporating NDBEPR features.

The activated sludge plant consists of three aeration basins (total volume = 19 600 m^3) with five surface aerators

(75 kW) in each basin. There were originally six aerators but stirrers have replaced the first row of aerators in order to create an informal anoxic zone ie., no partitioning walls exist between the aerobic and anoxic zones (De Haas, 1998). The vertical pumping action of the surface aerators also create anoxic zones at the bottom of the aeration basins. Refer to FIG. 3.2 for a diagrammatic description of the Darvill WWW activated sludge plant.

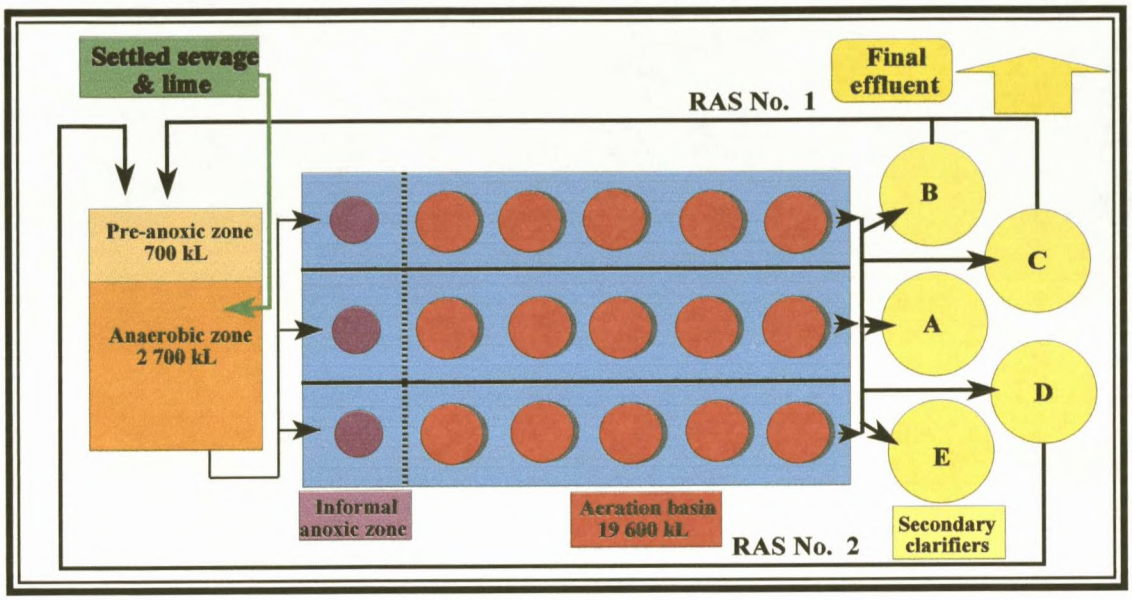

FIGURE 3.2 Schematic representation of Darvill activated sludge plant modified for bio-P removal.

Settled sewage flow is fed directly to the anaerobic zone together with a VFA containing stream originating from the supernatant of the primary sludge fermenters. Denitrification of the return sludge is encouraged by feeding a minor fraction (*ca.* 4%) of the settled sewage into the pre-anoxic zone. Flows in excess of the average dry weather flow (*ca.* 60 mL/d) are diverted to the anoxic/aerobic zones in order to ensure a minimum hydraulic retention time for mixed liquor passing through the anaerobic zone (De Haas, 1998). TABLE 3.1 provides actual operating ranges of Darvill WWW. A description of settled sewage composition data flowing into the activated sludge plant over a two year period (January 1995 to February 1997) is shown in TABLE 3.2. Chemical data is based on the average of two daily grab samples taken 12 h apart (except for TKN, weekly grab) (De Haas, 1998).

TABLE 3.1 Operating features of Darvill secondary biological treatment process.

Design average dry weather inflow	60 ML/d
Design maximum wet weather inflow	160 ML/d
Design MLSS	3 800 mg/L
Mean cell retention time	7.4 d

TABLE 3.2 Settled sewage composition to Darvill activated sludge plant (average for a two year period; January 1995 to February 1997).

Determinand	Mean value
COD	242.9 mg/L
TKN	24.8 mgN/L
NH3	18.2 mgN/L
Alkalinity	141 mg/L as CaCO3
Ortho-P	5.6 mgP/L
TP	8.5 mgP/L

Darvill WWW is also prone to trade effluent problems, primarily in the form of illegal dumping of vegetable oil waste to the sewer by local producers of cooking oil, margarine and soap. These wastes cause serious primary sedimentation and scum removal problems, as well as being directly implicated in drastic increases in secondary effluent ortho-P concentrations. High influent and effluent ortho-P concentrations are as a direct result of these liquid wastes entering the plant as the industries concerned use phosphoric acid in their refining process (De Haas, 1998). Oily emulsions in the aerobic basins diminish aeration efficiency which sometimes leads to poor nitrification rates with resulting high secondary effluent ammonia concentrations.

At this stage of research, laboratory equipment for the molecular *in situ* identification of mixed liquor bacterial cells was not available at the Centre for Water and Wastewater Research (CWWR). Plating and identification methods of the Darvill BNR plant were therefore employed in an attempt to elucidate those organisms which were dominant in the system and to evaluate the P uptake capacity of the various presumptive PAO isolates.

3.2 MATERIALS AND METHODS

3.2.1 Mixed liquor sampling

Grab samples of mixed liquor were obtained from the aerobic zone of the full-scale activated sludge system at Darvill WWW. Samples were collected in sterile 1 L Schott bottles containing glass beads to enhance disruption of the floc structure. Floc disruption would theoretically increase the number of freely-suspended cells and therefore increase viable cell counts. Mixed liquor was stored on ice during transit and processed immediately upon return to the laboratory.

3.2.2 Isolation of bacteria

Serial dilutions (10^{-2} - 10^{-8}), using sterile distilled water, were made of a well-mixed sample of mixed liquor. 0.1 mL of each dilution was spread on individual casitone glycerol yeast autolysate (CGY) agar plates (APPENDIX 1) (Pike *et al.*, 1972; Osborn *et al.*, 1989; Lötter, 1989; Bux *et al.*, 1994). Plates were incubated at 20°C for 5 d. Plates which contained between 30 - 100 colony forming units (CFU's) were retained for further study and the other plates were discarded. Each colony was restreaked on solid isolation media (CGY) and the dilution from which it was obtained recorded. After further incubation at 20°C for 5 d, plates were checked for purity and isolates screened for poly-P accumulation.

3.2.3 Screening of isolates for poly-P accumulation

All isolates obtained from Darvill WWW were screened for their ability to take up soluble P prior to identification. Isolates which did not show propensity to take up P in any significant amount were subsequently discarded. The mixed liquor culture medium (ML medium) in which the screening assay was conducted was formulated according to similar studies conducted by Bosch and Cloete (1993). Mixed liquor obtained from the anaerobic zone of Darvill WWW was allowed to settle for 2 h, after which the supernatant was centrifuged at 5 000 g for 20 min using a

Beckman J6-MC centrifuge. Supernatant was filtered through Whatman No. 1 filter paper to remove any remaining suspended particles. Filtrate was supplemented with 5 g/L sodium acetate, 0.5 g/L $MgSO_4.7H_2O$ and 0.18 g/L KNO_3 and the pH adjusted to 7.0 with 2 N HCl before autoclaving at 121°C for 15 min.

Inoculum for the study was prepared through inoculation of universals containing nutrient broth medium (10 mL) with well-formed colonies from individual agar plates and incubated at 30°C ± 2°C for 24 h. Cell counts and viability were checked by serially diluting 1 mL inoculum and plating onto Nutrient Agar. Plates were subsequently incubated at 30°C for 24 h and CFU's counted and recorded. Phosphorus uptake was assessed using an anaerobic/aerobic cyclic system. Four millilitres inoculum was aseptically transferred to 96 mL sterile ML medium. Nitrogen gas was bubbled into the suspension and head space for 10 mins to purge the flask and contents of oxygen. Flasks were sealed with cotton wool and aluminium foil and incubated anaerobically at ambient temperature (22°C ± 2°C) for 2 h on an orbital shaker at 80 rpm ± 5 rpm. Aeration then commenced using fish pumps and air diffusion stones for 5 h. The medium was agitated (120 rpm ± 5 rpm) to ensure maximum diffusion of DO throughout the bulk liquid and to ensure the maintenance of a homogenous suspension. Upon cessation of anaerobiosis, 10 mL medium was extracted and filtered through 0.22 μm syringe filter pieces. The pH of the medium was continuously monitored during aerobiosis (maintained between 7.0 to 7.5) to ensure that chemical P precipitation, through the formation of calcium or magnesium salts, did not occur. When necessary, pH was adjusted using a 0.1 M HCl solution. Orthophosphate concentration in the medium was determined using the Merck SQ 118 photometer; test kit P(VM) 14842. Uninoculated ML medium was used as control, the concentration of which represented the initial ortho-P concentration. The amount of phosphate removed from the medium by the respective isolates was determined according to the following formula (Bosch and Cloete, 1993):

PO_4^{3-} taken up by isolate = [control PO_4^{3-} after 7 h incubation] - [test PO_4^{3-} after 7 h exposure to isolate]

The ATCC bacterium, *Acinetobacter calcoaceticus* (ATCC# 23055) was used as a reference strain for all P uptake studies.

3.2.4 Identification of isolates

All isolates showing propensity to take up soluble phosphate were Gram stained and identified using the API 20NE system. Isolates unable to accumulate substantial quantities of P (with respect to the *A. calcoaceticus* reference strain) were discarded. Bacterial isolates were initially differentiated according to the oxidase biochemical test using oxidase touch sticks (Oxoid). Where necessary, additional biochemical tests were performed to elucidate identification to species level (including cases of unacceptable/doubtful identification profiles). Neisser and PHB stains were done according to Jenkins *et al.*, (1984) to confirm the presence of volutin and PHB granules, respectively (APPENDICES 2 and 3). The *A. calcoaceticus* ATCC culture was treated in the same manner to validate the identification procedure.

3.3 RESULTS

3.3.1 Polyphosphate accumulating organisms

Once monocultures of the total number of viable and culturable mixed liquor isolates were obtained and poly-P accumulation screening assays complete, bacteria showing the propensity to take up excess quantities of soluble P were routinely Gram, Neisser and PHB stained and identified. Thirty nine isolates were initially subjected to phosphate uptake studies of which 16 showed the ability to take up P in any appreciable amount (TABLE 3.3; see section 3.3.2). This amounted to 41% of the total recovered isolates which were presumed to be poly-P positive. Ten of the poly-P positive bacteria were Gram negative (*Proteobacteria*) whilst six were Gram positive, relating to 63 and 37% of the total PAO population, respectively (FIG. 3.3). The PAO isolates were subsequently identified and expressed as a percentage of their relevant Gram reactions (FIGS 3.4 and 3.5). Percentage composition of the individual isolates within the total cultured PAO population is shown in FIG. 3.6.

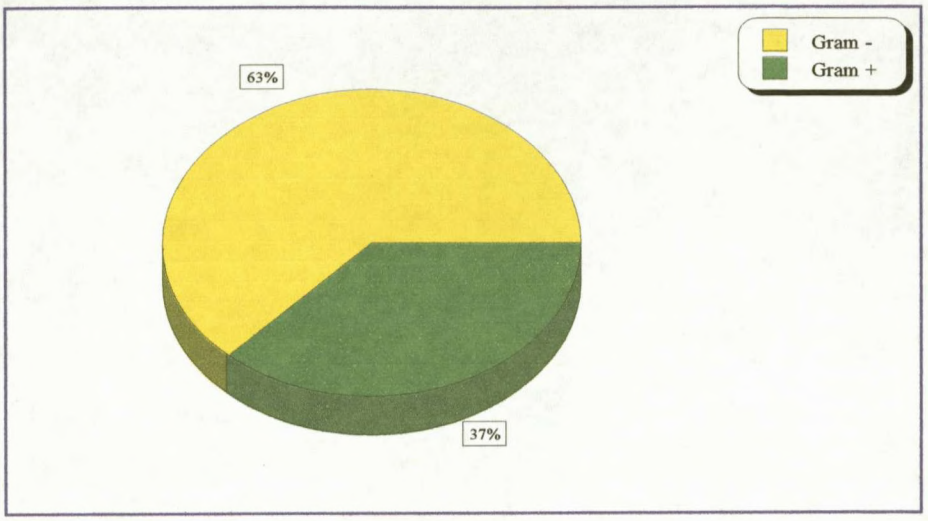

FIGURE 3.3 Percentage PAO bacterial population according to Gram reaction.

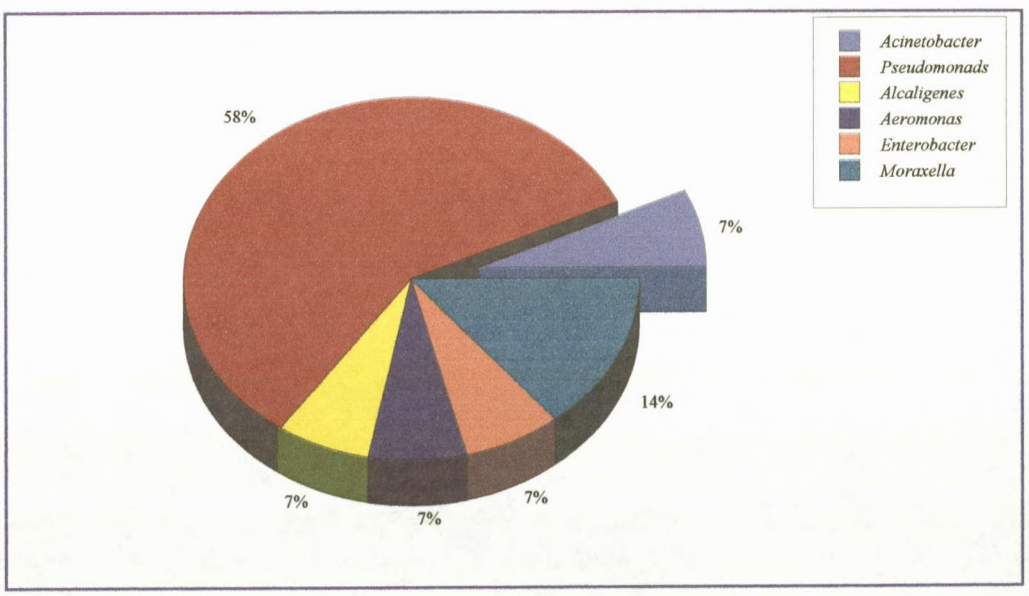

FIGURE 3.4 Percentage heterotrophic Gram negative PAO genera isolated from the aerobic zone of Darvill WWW. Cultures grown and maintained on CGY agar.

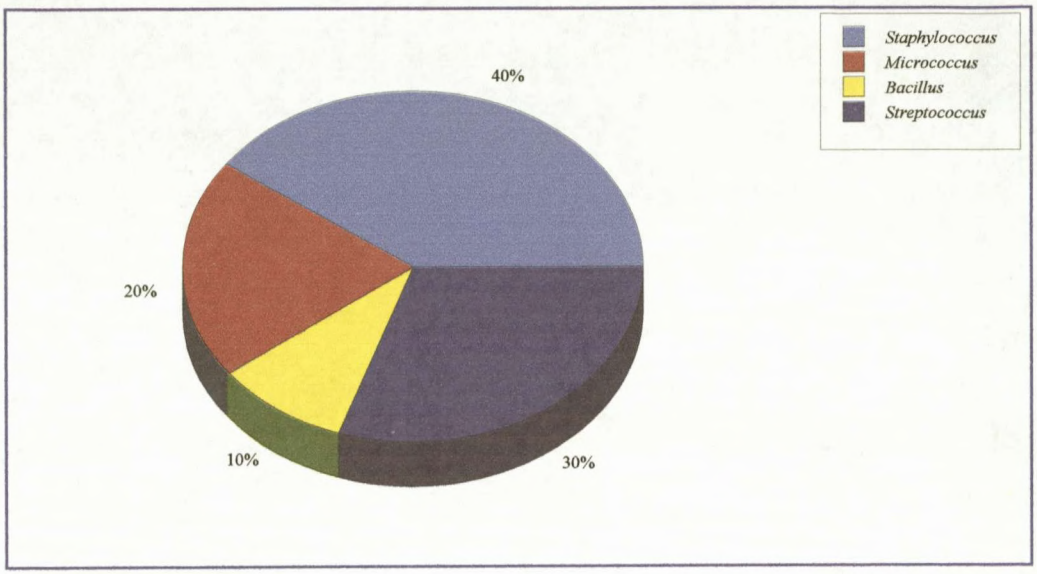

FIGURE 3.5 **Percentage heterotrophic Gram positive PAO genera isolated from the aerobic zone of Darvill WWW. Cultures grown and maintained on CGY agar.**

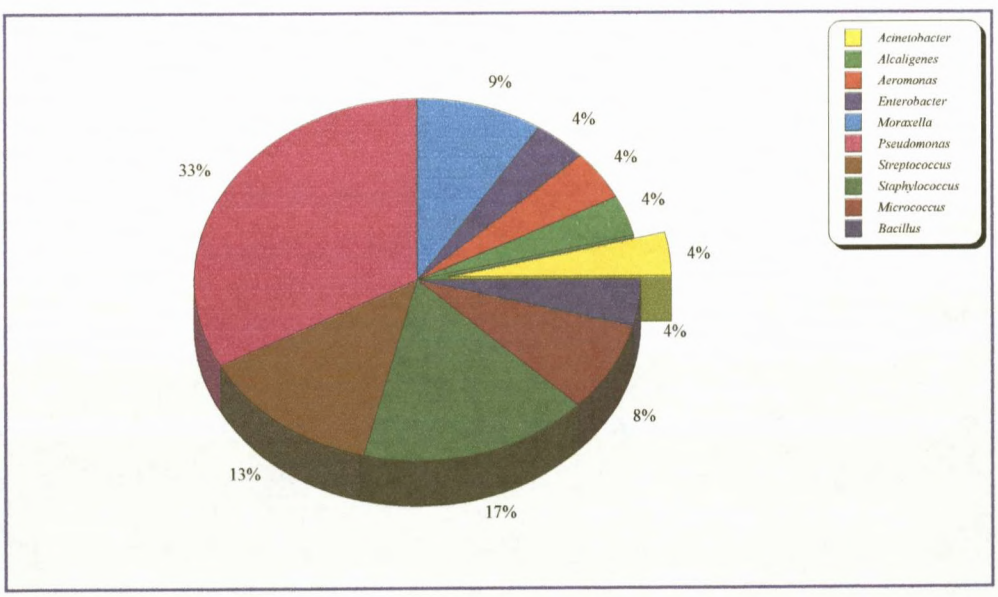

FIGURE 3.6 **Percentage composition of total PAO population recovered from Darvill WWW mixed liquor on CGY agar. Total cell count from aerobic zone on CGY agar was 1.7 x 10^6 CFU/mL.**

It is evident from FIGS 3.4 and 3.6 that either the incidence or recovery rate of *Acinetobacter* spp. in the mixed liquor was very low with respect to the other poly-P isolates in the Darvill WWW activated sludge system. In either instance, *Acinetobacter* spp. only amounted to 7 and 4% of the Gram negative and total PAO community, respectively. *Pseudomonas* spp. appeared to dominate the heterotrophic PAO population (33%) followed by the Gram positive organisms, *Staphylococcus* spp. and *Streptococcus* spp. (17 and 13%), respectively (FIG. 3.6).

3.3.2 Phosphate uptake by isolates

The phosphate accumulating capacity of the various isolates is presented in TABLE 3.3. It was interesting to note that there was reasonable correlation between the reference and wild *Acinetobacter* strains in P uptake capacity based on cellular phosphate uptake (column 4; TABLE 3.3), although the difference in uptake remained quite marked. Never the less, both *Acinetobacter* isolates showed the highest P uptake rates when compared to the other isolates. The Proteobacterial gamma subclass *Pseudomonas* spp. (*P. putrefaciens*, *P. mendocina* and *P. fluorescens*) showed the ability to take up reasonably large quantities of PO_4-P from the bulk liquid ie., *ca.* 1 to 2 x 10^{-11} mgP per cell. *Bacillus cereus*, a low G + C bacterium, showed the highest P uptake rates when compared to the other Gram positive organisms (1.0 x 10^{-11} mgP/cell). *Alcaligenes denitrificans* showed lowest phosphate uptake at 1.4 x 10^{-12} mgP/cell.

3.3.3 Phylogenetic distribution and classification of PAO's

With the advent of improved methods of determining the relatedness of bacteria ie., rRNA sequencing, the entire prokaryotic taxonomic structure has been reorganised with many bacteria been classified under new genera and species names. This becomes especially evident when one considers the genus *Pseudomonas* where it has since been discovered that many individual species of this genus are in fact more related (on a genotypic basis) to species from other, more physiologically and phenotypically distinct genera. Classification of the Darvill WWW PAO population using the API 20NE system of identification shows a narrow phylogenetic distribution of functional organisms with the *Proteobacteria* dominating this fraction of the total heterotrophic community (TABLE 3.4 and

FIG. 3.7). The most prominent PAO isolates proved to be the gamma subclass members of *Proteobacteria* (47%), followed by the *Firmicutes Bacillus/Clostridium* (previously referred to as the Gram positive low G + C bacteria; GPBLGC) cluster (29%). The beta subclass of *Proteobacteria* (18%) and the *Firmicutes Actinobacteria* (formerly known as the Gram positive high G + C bacteria; GPBHGC) (6%) constituted the remainder of the PAO community (FIG. 3.7). No representative species from the *Cytophaga-Flavobacterium-Bacteroides* family of organisms were isolated (a bacterial cluster thought to be implicated in EBPR).

TABLE 3.3 Phosphate accumulating capacity shown by bacterial monocultures (in descending order of magnitude) isolated from the aerobic zone of Darvill WWW using conventional isolation and identification techniques.

Organism	PO_4-P uptake (mgP/L)	Initial viable cell counts (CFU [a]/mL)	Cellular PO_4-P (mgP/CFU [a])
ATCC reference strain[b]	5.8	7.04×10^{7}	8.2×10^{-11}
Acinetobacter calcoaceticus	6.1	1.00×10^{8}	6.1×10^{-11}
Aeromonas hydrophila	2.8	5.20×10^{7}	5.4×10^{-11}
Pseudomonas putrefaciens	1.1	5.50×10^{7}	2.0×10^{-11}
Pseudomonas mendocina	6.5	4.60×10^{8}	1.4×10^{-11}
Pseudomonas fluorescens	6.8	6.00×10^{8}	1.1×10^{-11}
Bacillus cereus	4.5	4.30×10^{8}	1.0×10^{-11}
Micrococcus spp.	3.4	3.70×10^{8}	9.2×10^{-12}
Moraxella phenylpyruvica	5.2	7.40×10^{8}	7.0×10^{-12}
Staphylococcus epidermidis	3.4	6.00×10^{8}	5.7×10^{-12}
Streptococcus spp.	1.7	3.20×10^{8}	5.3×10^{-12}
Pseudomonas testosteroni	4.4	8.40×10^{8}	5.2×10^{-12}
Staphylococcus aureus	1.8	4.80×10^{8}	3.8×10^{-12}
Pseudomonas acidovorans	2.9	8.90×10^{8}	3.3×10^{-12}
Staphylococcus spp.	1.5	7.90×10^{8}	1.9×10^{-12}
Enterobacter agglomerans	2.0	1.12×10^{9}	1.8×10^{-12}
Alcaligenes denitrificans	4.6	3.20×10^{9}	1.4×10^{-12}

[a]: CFU = colony forming unit; [b]: ATCC reference strain = *Acinetobacter calcoaceticus* (ATCC # 23055)

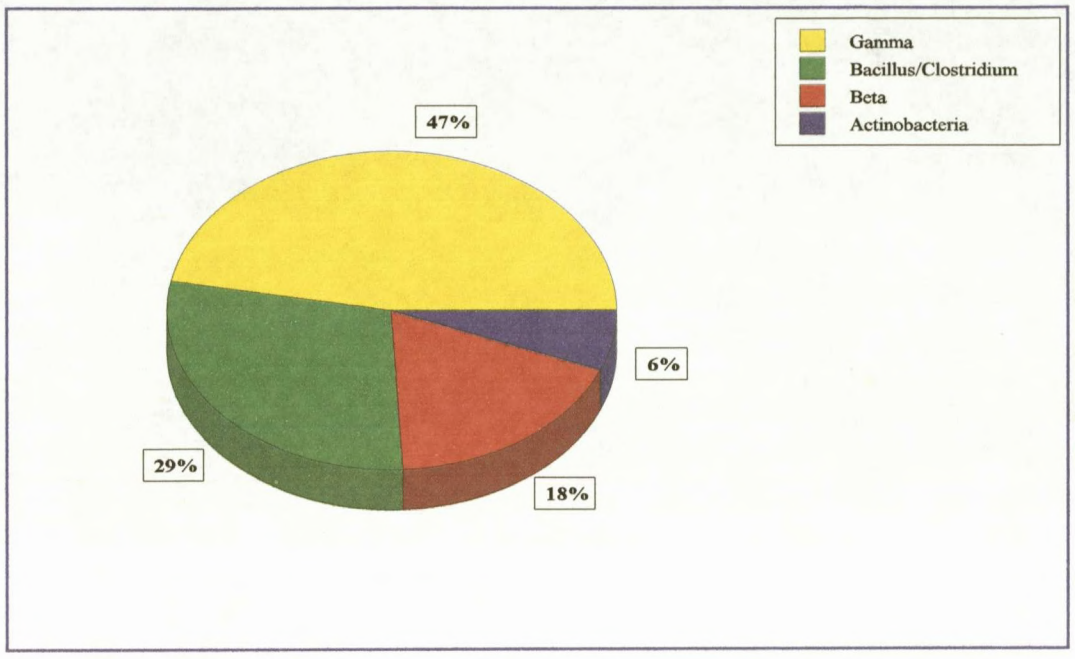

FIGURE 3.7 **Graphical representation of isolated PAO's from Darvill WWW mixed liquor at the family level of classification.**

TABLE 3.4 **Phylogenetic classification and current bacterial names of PAO's cultivated from Darvill WWW (see section 2.5; TABLE 2.2).**

Organism	Synonym	Phylogenetic classification
ATCC reference strain	NC	*Proteobacteria* (γ subdivision)
Acinetobacter calcoaceticus	NC	*Proteobacteria* (γ subdivision)
Aeromonas hydrophila	NC	*Proteobacteria* (γ subdivision)
Pseudomonas putrefaciens	*Shewanella putrefaciens*	*Proteobacteria* (γ subdivision)
Pseudomonas mendocina	NC	*Proteobacteria* (γ subdivision)
Pseudomonas fluorescens	NC	*Proteobacteria* (γ subdivision)
Bacillus cereus	NC	*Firmicutes* (*Bacillus/Clostridium* group)
Micrococcus spp.	NC	*Firmicutes* (*Actinobacteria*)
Moraxella phenylpyruvica	NC	*Proteobacteria* (γ subdivision)

TABLE 3.4 *continued*

Organism	Synonym	Phylogenetic classification
Staphylococcus epidermidis	NC	*Firmicutes* (*Bacillus/Clostridium* group)
Streptococcus spp.	NC	*Firmicutes* (*Bacillus/Clostridium* group)
Pseudomonas testosteroni	*Comamonas testosteroni*	*Proteobacteria* (β subdivision)
Staphylococcus aureus	NC	*Firmicutes* (*Bacillus/Clostridium* group)
Pseudomonas acidovorans	*Comamonas acidovorans*	*Proteobacteria* (β subdivision)
Staphylococcus spp.	NC	*Firmicutes* (*Bacillus/Clostridium* group)
Enterobacter agglomerans	NC	*Proteobacteria* (γ subdivision)
Alcaligenes denitrificans	*Achromobacter xylosoxidans* subsp. *denitrificans*	*Proteobacteria* (β subdivision)

NC = no change

3.4 DISCUSSION

Many studies have previously been conducted to elucidate those heterotrophic PAO's actively functional in various activated sludge mixed liquors (Hart and Melmed, 1982; Brodisch and Joyner, 1983; Cloete *et al.*, 1985a,b; Lötter, 1985; Lötter and Murphy, 1985; Osborn et al., 1989; Kavanaugh and Randall, 1994). Since the discrepancy surrounding many *Acinetobacter* spp. regarding their incorrect carbon and phosphorus transformations (as far as phosphate release and uptake is concerned) has become more prominent during the latter years of investigation, research from a microbiological perspective has become very focussed in attempting to elucidate exactly which organisms are responsible for EBPR. This, however, remains an extremely daunting task due to the vast microbial diversity which exists in activated sludge and various abiotic parameters ie., influent characteristics, which affect the BPR mechanism and activated sludge process as a whole.

When applying standard microbiological techniques to isolate organisms from activated sludge samples, literature shows a tremendous degree of variation as far as the dominance of *Acinetobacter* spp. is concerned. Brodisch and Joyner (1983), when isolating and identifying mixed liquor bacteria on CGY agar and API 20E, respectively, found a 10% distribution of the bacterium in the aerobic zone during pilot plant studies. In contrast, Lötter and Murphy

(1985) found that *Acinetobacter* totally dominated the bacterial population from a full-scale plant exhibiting EBPR using chemifluorescent and API 20E techniques of identification. Lötter (1985), studying a full-scale plant and using a fluorescein isothiocyanate-labelled fluorescent antibody against *Acinetobacter* spp., followed by the API 20E identification system, indicated that 56% of the bacterial colonies that grew on basic medium with acetate as the sole carbon source were identified as *Acinetobacter*. Cloete *et al.*, (1985a,b), when applying fluorescent antibody techniques to estimate numbers of *A. calcoaceticus* and *A. phosphodevorans* in activated sludge, found that the number of *Acinetobacter* exceeded 10^6 cells/mL, counts of which were highest in the aerobic zone. High counts of *Acinetobacter* in the aerobic zone can be expected due to its purely aerobic metabolism. Lötter *et al.*, (1986a) found that *Acinetobacter* strains metabolise carbon via the Entner-Doudoroff pathway which is inoperative in the absence of a terminal electron acceptor ie., anaerobic zone. An interesting finding from their study was that none of the *Acinetobacter* strains investigated possessed the glycolytic (Embden-Meyorhof) pathway ie., *Acinetobacter* is therefore unable to produce reducing equivalents in the absence a terminal electron acceptor indicating that the organism should, theoretically, not be capable of PHB synthesis in the anaerobic zone. The major contributing factors to discrepancies between authors when conducting bacterial community analyses of BPR plants are perhaps the problems inherent to the scaling up and/or down of operations, using sludge from structurally different processes and the points selected for mixed liquor sampling. Direct comparisons between results from various authors must therefore be approached with caution. During this study, the low numbers of *Acinetobacter* (7 and 4%; FIGS 3.4 and 3.6, respectively) can only suggest that the genus is not the dominant PAO in the Darvill activated sludge process although a number of reasons which may account for its low recovery rate are offered during the course of this discussion.

The dominance of *Pseudomonas* spp. in the aerobic sample (FIGS 3.4 and 3.6) is in agreement with Brodisch and Joyner (1983) who found that *Pseudomonas*, together with *Aeromonas*, constituted more than 50% of the total aerobic microbial population (albeit samples were obtained from pilot- and laboratory-scale units). Wentzel *et al.*, (1988), conducting research using activated sludge laboratory-scale units, found that a distinct shift in the microbial community structure occurred if the anaerobic PAO mass fraction was not sufficient to utilise all the fermented product. Leakage of VFA (acetate in this instance) and RBCOD to the subsequent anoxic and/or aerobic zones

allowed *Pseudomonas* spp. to obtain dominance over organisms such as *Acinetobacter* spp. in their systems. Although *Pseudomonas* spp. do exhibit excess P removal, the magnitude and rate of P uptake is much lower and slower than that exhibited by *Acinetobacter* spp. (Wentzel *et al.*, 1988). However, if a particular organism such as *Pseudomonas* is present in a BNR treatment plant in sufficient numbers and nutrient availability continually supports biomass production, the genus can account for the majority of P removal from the system. Indeed, Bosch and Cloete (1993) found that P removal occurs by virtue of sufficient biomass in a system and not through an enhanced accumulation capacity per cell. The diversity of *Pseudomonas* with respect to its metabolic capability becomes evident when one considers the findings of Kavanaugh and Randall (1994) where mixed liquor samples were cultivated on four different carbon sources viz., acetate, propionate, butyrate and D,L,-3-hydroxybutyrate, and denitrification medium. *Pseudomonas* and *Aeromonas/Vibrio* isolates were found to constitute the largest proportion of recovered cells on all media used although the coliforms dominated growth on propionate medium. The growth of *Pseudomonas* and *Acinetobacter* on denitrification and fatty acid medium was significant in that it indicated an overlap in their roles in activated sludge ie., P uptake and denitrification.

The majority of pseudomonads isolated from Darvill belong to the gamma-subclass of *Proteobacteria* (TABLE 3.4). According to literature, plating of mixed liquor samples on nutrient rich media such as CGY can lead to a gross overestimation of the gamma subclass of *Proteobacteria* (Wagner *et al.*, 1994a). If this was the case, however, *Acinetobacter* (another gamma *Proteobacteria* organism) numbers should have amounted to more than 4% of the total isolated PAO population (FIG. 3.6). The dominance of the gamma subclass of *Proteobacteria* is evident in FIG. 3.7 and TABLE 3.4. However, the major contribution to gamma subclass dominance derived from *Pseudomonas* spp. A possible explanation for this could be the method of identification employed for this study ie., the API 20NE system. The majority of the studies mentioned above used the API 20E system of identification. It is possible that the API 20NE system is incapable of discerning the various *Acinetobacter* strains found in activated sludge and therefore incorrectly identified the genus during the course of this study. Bosch and Cloete (1993), using the API 20NE system, found the index to be unsatisfactory due to their type strain of *Acinetobacter calcoaceticus* been incorrectly identified as *A. baumannii.* The reference strain used during this study was *A. calcoaceticus* obtained from the ATCC and although the species was correctly identified using the API 20NE system

(82.8% probability), reasonable doubt does exist as to the correct identification of other members of this genus. Beacham *et al.*, (1990), investigating *Acinetobacter* genospecies diversity in a modified UCT pilot plant using CGY isolation media and both the Microbact 24E identification system and transformation assay of Juni (1972), found the dominant species isolated was *A. junii*. Other species isolated included *A. lwoffi*, *A. johnsonii*, and *A. baumannii*. *Acinetobacter haemolyticus* and *A. calcoaceticus* were not found at all. During this study, however, only *A. calcoaceticus* was isolated from the mixed liquor. This discrepancy may be contributed to the fact that the API 20NE test kit may only be suitable for identifying *A. calcoaceticus* when considering identification of *Acinetobacter* at species level. The system does, however, incorporate six *Acinetobacter* species in the profile. If they were present in any appreciable amounts in the mixed liquor samples, more *Acinetobacter* isolates should have been correctly identified at genus level.

Momba and Cloete (1996a), using various size and volume calculations, found that due to its small cell size, *Acinetobacter* is capable of removing a maximum of 10^{-10} mgP/cell. The control and wild *A. calcoaceticus* strains investigated during this study showed P removal capacities of 8.2×10^{-11} and 6.1×10^{-11} mgP/L, respectively (TABLE 3.3). Bosch and Cloete (1993) recorded similar uptake rates of *ca.* 10^{-11} to 10^{-12} mgP/cell for *Acinetobacter* isolates from activated sludge mixed liquors. This reduced P removal may have been due either to the physiological condition or phase of growth of the cells or initial biomass concentrations when they were introduced to surplus phosphate conditions. The fact that metabolic properties of isolates in pure culture may differ significantly from those in mixed cultures must also not be excluded. Momba and Cloete (1996b) found a strong correlation between phosphate removal, biomass concentration, phase of growth and bacterial species. Their studies showed that *Acinetobacter radioresistens*, at high initial cell densities (10^7), removed most of the phosphate during the first hour of lag phase. Minimal quantities of phosphate were removed during stationary phase of growth. During the present study, it is unlikely that the 24 h pre-incubation period prior to inoculation in the phosphate containing medium (see section 3.2.3) seriously disadvantaged the cells physiologically. However, the low aeration period of 5 h during poly-P uptake studies would not have permitted the cells to reach the stationary phase of growth. Bosch and Cloete (1993), conducting similar studies, observed a five hour lag phase for all their *Acinetobacter* isolates investigated. They also found that stationary phase was only reached after 10 and 14 h of growth. The phosphate which would

potentially have been removed during the stationary phase of this study is therefore excluded when determining the phosphate uptake capacity of the various isolates. Momba and Cloete (1996b) allowed for a 24 h period for full duration of experimentation. It is therefore envisaged that if the duration of poly-P uptake studies was extended, the quantity of phosphate removed from solution would increase. The *Pseudomonas fluorescens* isolate which Momba and Cloete (1996b) used for their studies showed maximum phosphate uptake during the lag and logarithmic phase of growth. The five hour aerobic period during this study would have been sufficient for the *Pseudomonas* isolates to achieve logarithmic growth. This could account for the comparable phosphate uptake rates of the *Pseudomonas* and *Acinetobacter* isolates (TABLE 3.3).

Phosphate release in the anaerobic zone by PAO's is directly proportional to the amount of extracellular organic substrate ie., the more substrate available for bacterial sequestration, the more P will be released. This is significant as the mechanism of P release and uptake, itself, is proportional. As discussed earlier, the primary objective of the anaerobic zone in EBPR is to encourage the maximum release of intracellular P so the PAO's are 'starved' once they enter the aerobic state. It has been reported that the physiological state of PAO's in the anaerobic zone also affects P release (Rustrian *et al.*, 1997). They found that *Acinetobacter* cells in the stationary phase of growth were able to release more accumulated P (18-58% poly-P) than those in exponential growth (5-38%). It is possible that the PAO isolates used during this study were not properly conditioned before P uptake experiments commenced which would have affected P release and subsequently, P uptake capacity (TABLE 3.3). During experimentation in the present study, ML medium (supplemented with 5 g/L sodium acetate) was seeded with 24 h (T=30°C) nutrient broth cultures (see section 3.2.3). Turbidity suggested that the isolates were in log phase of growth prior to seeding. However, introduction of the cultures to ML medium (new organic substrate) implies that the cells may have 'regressed' to a period of adjustment or lag phase. Although growth phase was never monitored during the course of this study, it is hypothesised that the 2 h period of anaerobiosis would not have been sufficient time for the cells to advance to stationary or perhaps even log phase of growth. According to findings by Rustrian *et al.*, (1997) P release (and P uptake) would therefore be reduced. Phosphate uptake by the various isolates may therefore be in excess of those values reported in TABLE 3.3. It is recommended that when similar studies are conducted, inoculum bulking media should resemble very closely the experimental media to maintain a steady growth curve.

When considering biodiversity in activated sludge, cognisance must be taken of the isolation medium used for cell recovery. When assessing four different media for activated sludge bacteria isolation viz., dilute CGY and Fuhs and Chen media, balance tank effluent and settled sewage, Osborn *et al.*, (1989) found that dilute CGY agar gave the highest counts (1.5×10^6 CFU/mL). These results compare favourably with the total plate counts of 1.7×10^6 CFU/mL (see legend of FIG. 3.6) obtained during this study. Pike *et al.*, (1972) previously recommended CGY agar as the medium resulting in the highest plate counts for activated sludge samples. However, the high nutrient content of this medium supports the growth of faster growing organisms which rapidly outcompete their slower growing counterparts resulting in limited cell recovery and low cell counts. Osborn *et al.*, (1989), simultaneously examining mixed liquor samples using plating and the Acridine Orange technique, counted 2.03×10^8 bacterial cells/mL using the direct microscopy technique. Bitton (1994) estimates that total aerobic bacterial counts in standard activated sludge are in the order of 10^8 CFU/mg sludge. When assessing the most quantitative method for heterotrophic microorganism counts from activated sludge, Schade and Lemmer (1994) found that the surface plate method yielded significantly higher counts when compared to the pour plate method. The use of CGY agar during this study would definitely account for the reduced cell count and limited distribution of bacterial cells at genus level. It is unfortunate that no single medium can be expected to recover all viable cells from activated sludge mixed liquor samples. The low *Acinetobacter* cell counts could maybe be attributable to the organism having a low specific growth rate, therefore been rapidly outgrown by the other isolates. Oerther *at al.*, (1997) found that when augmenting sequence batch reactors, fed with primary effluent and supplemented with sodium acetate, with pure cultures of *Acinetobacter*, the bacterium decreased in number over the course of the experiment suggesting that it was unable to successfully compete with other organisms. Although EBPR was successfully reported, the dilution of *Acinetobacter* from the system indicates that it has a low maximum specific growth rate with respect to other heterotrophic PAO's in mixed liquor. Polyphosphate accumulating organisms, as a group, are generally considered to be relatively slow growing, usually requiring one week or more of cultivation time before forming visible colonies (Smolders *et al.*, 1994). The findings of Oerther *et al.*, (1997) also suggest that bioaugmentation of microbial systems does not necessarily guarantee a desirable result and that other, more subtle techniques such as biomanipulation should be implemented to enhance the microbial community. It was also shown that no positive correlation between numbers of *Acinetobacter* in mixed liquor and successful EBPR existed (Oerther *et al.*, 1997).

The incubation temperature of 20°C may also have been a limiting factor to the successful growth and establishment of visible colonies of *Acinetobacter*. According to Bergey's Manual (1984) all *Acinetobacter* strains are able to grow between 20 and 30°C although most strains have a temperature optima of 33-35°C. Pike *et al.*, (1972) concluded that incubation on CGY agar for 6 d at 22°C resulted in the highest total plate counts for activated sludge mixed liquors. The study did not, however, offer any indication of the recovery success of specific bacterial species at this temperature.

Another area of concern was that the sensitivity of the oxidase touch stick reaction, used to characterise the isolates on the basis of their oxidase biochemical reaction, was not extensively verified during the course of this study. Although the reference *Acinetobacter* strain was correctly characterised on the basis of its oxidase reaction, the presence of weakly oxidase positive organisms may have been incorrectly reported. Due to phenotypic similarities existing between *Acinetobacter* spp. and *Moraxella* spp. (Juni, 1978), the oxidase reaction has become the only qualitative method of discerning between the two genera ie., *Acinetobacter* is oxidase negative; *Moraxella* is oxidase positive. A qualitative oxidase biochemical assay is therefore of utmost importance to ensure correct identification of the two closely related genera. Venter *et al.*, (1989) have questioned the applicability of the API oxidase test and suggested that it be supplemented by a standard oxidase test. The possibility that incorrect oxidase reactions may have been entered into the API profile due to reduced touch stick sensitivity cannot be ignored.

An interesting finding by Brodisch (1985) is that the genetic information coding for enhanced phosphate removal in PAO's can be lost during passage on artificial media and can become even more prominent if the information is coded on a plasmid (still to be clarified). PAO isolate counts in this study may have therefore been underestimated due to the loss of relevant genetic information. If the information was lost during isolation and monoculture development, the isolate would have been discarded if it did not show the propensity to take up phosphate during polyphosphate accumulation studies even though it was expressing the function in the original sample.

When identifying organisms from nutrient removing plants, many authors proceeded to discard the Gram positive isolates before identification which, to a degree, may have retarded development and growth in the area of nutrient

removal (Brodisch and Joyner, 1983; Lötter, 1985; Osborn *et al.*, 1989). However, the contribution of the Gram positive bacterial population cannot be ignored when considering P removal. It is evident from FIGS 3.4 - 3.7 and TABLE 3.4 that this group is playing a significant role in bio-P removal operations. Of the total PAO population isolated, 42% proved to be Gram positive (FIG. 3.5), the majority of which were members of the Bacillus/Clostridium group (formerly referred to as low G + C or LGC species) (FIG. 3.7). The remaining Gram positive organisms were from the Actinobacteria group (formerly referred to as high G + C or HGC bacteria). The HGC bacteria have been implicated in the removal of phosphate in BPR processes (Wagner *et al.*, 1994a; Kämpfer *et al.*, 1996). Liu *et al.*, (1997), however, using PCR-amplified bacterial 16S rDNA sequences, found that HGC bacteria were not a major component of the total PAO microbial biomass. Present research substantiates the findings of the latter study where only one isolate, subsequently identified as *Micrococcus* spp., was recovered belonging to the HGC class (FIG. 3.4 and TABLE 3.4). Other isolates viz., *Staphylococcus*, *Streptococcus* and *Bacillus* spp., belonged to the LGC class of Gram positive organisms.

The low number of isolates recovered during this study may also have been due to insufficient floc disruption. When interested with total plate counts and maximum viable cell recovery, mixed liquor samples essentially require stringent techniques of cell dispersion due to the structural stability of the floc. Cloete and Steyn (1988a) and Beacham *et al.*, (1990) applied sonication and stomaching, respectively, in attempt to improve cell recovery. Beacham *et al.*, (1990) also used various commercial enzyme preparations viz., dextranase, amyloglycosidase, and Novozym 234, which are known to contain active polysaccharide degrading enzymes yet no improvement in floc disintegration was noted. Hart and Melmed (1982) found grapelike clusters of *Acinetobacter* cells in nutrient removing sludges by light microscopy. Using electron microscopy, however, they found that these clusters contained up to 1 000 individual cells. Lack of suitable disruption and dispersion techniques would, therefore, result in grossly underestimated *Acinetobacter* numbers as one cluster growing on solid agar media could be misinterpreted as one CFU and enumerated as such.

3.5 CONCLUSIONS

Acinetobacter spp. were not the dominant PAO's isolated from the Darvill WWW activated sludge plant when using CGY agar as isolation medium. The high uptake rate yet low incidence of the species could not account for the entire P removal mechanism occurring at the full-scale plant. The ubiquitous nature of *Pseudomonas* suggests that this genus is playing a vital role in P removal due to its presence in extremely high numbers. The fact that *Pseudomonas* spp. are able to synthesise intracellular organic granules during anaerobiosis, denitrify during anoxia and respire during aerobiosis can only suggest that the genus will achieve a distinct competitive advantage over other bacteria present in the BNR activated sludge biocenosis.

The dominance of the gamma subclass of *Proteobacteria* can be attributed to the bias imposed by serial dilution and plating on nutrient rich solid medium. The contribution of the gamma subclass to EBPR operations must not be underestimated, however, as the magnitude of P cumulatively taken up by these organisms is in excess of the other PAO's which were isolated. Other genera isolated during this stage of experimentation, showing the ability to accumulate P when studied in homogenous cultures include *Aeromonas*, *Bacillus*, *Micrococcus*, *Moraxella*, *Staphylococcus*, *Streptococcus*, *Alcaligenes* and *Enterobacter*. Bacteria from HGC and LGC were found to be very prominent in the Darvill PAO community, indicating their significance to the EBPR process.

It is evident from the above discussion that conventional methods of serial dilution and plating are not sufficient when investigating biodiversity from environmental samples. Problems inherent to these techniques of isolation and identification which must be considered include:

- sampling bias - is the sample representative of the entire microbial community and is it prepared sufficiently ie., disruption and dispersion;
- plating bias - will the isolation medium employed recover the maximum number of viable cells and will the incubation temperature be optimal for the majority of cells;

- counting bias - are serial dilutions executed quantitatively; are all the isolates (CFU's) included when counting or passaging for monoculture development; and
- identification bias - is the database sufficient to include all isolates; when reading results, can colour or turbidity changes (in the case of API 20NE) be misinterpreted.

Conventional methods are sufficient when investigating specific clinical isolates, where the number of interfering organisms are minimised. However, if one considers environmental samples such as activated sludge where the number of different bacterial species involved is large, alternative methods of spatial distribution and community analyses are required. Microbial community analyses are now moving to a lower level of organisation with the advent of non-cultivation and molecular techniques ie., immunofluorescence, quinone profiles and FISH. This is not to say that microbiologists must rebuke conventional plating methods for it remains a powerful tool and our level of understanding would not be where it is today without it.

CHAPTER FOUR

ENHANCED CULTURE DEVELOPMENT

4.1 INTRODUCTION

In an attempt to formulate system response data on P release and uptake, Wentzel *et al.*, (1988) developed an enhanced PAO culture by incrementally decreasing the sewage fraction to an activated sludge pilot plant whilst simultaneously increasing the feed acetate fraction to a maximum of 500 mgCOD/L as sodium acetate. They defined an enhanced culture system as one in which the system response could be accredited to the resident PAO's, through selection of a suitable substrate (sodium acetate) and set of environmental conditions (sequencing anaerobic/aerobic reactors) which promoted their dominance. This approach theoretically allowed for more qualitative results to be construed than artificially cultivated pure bacterial cultures grown in chemostat systems. Growth of other normal competing heterotrophs and natural predation would be curtailed but not totally excluded from the system. PAO behaviour in these systems could therefore be expected to conform closely to their behaviour in normal mixed culture systems. When aerobic cultures were subsequently identified using the API system, it was found that greater than 90% of the bacterial population consisted of *Acinetobacter* spp. (Wentzel *et al.*, 1988). This finding compelled the researchers to accept the notion that *Acinetobacter* was indeed the principal organism responsible for poly-P accumulation and P removal from municipal wastewater. Subsequent models were therefore formulated using *Acinetobacter* as the surrogate PAO.

The objective of this phase of the study was to establish an enhanced culture of PAO's using similar techniques and methods to those employed by Wentzel *et al.*, (1988). Cultures were subsequently isolated and identified using both conventional plating (including API) and molecular techniques (FISH, see CHAPTER 5). It must be emphasised that the enhanced culture was not used further to study kinetics or other process parameters affecting poly-P accumulation and bio-P removal. From the literature review it appears that although molecular techniques of identification had been applied to normal mixed liquor cultures from full-scale treatment installations, probing of

specially developed enhanced cultures was limited. It was envisaged that present research findings would offer an intrinsic understanding of the microbial community displaying strongly enhanced bio-P removal mechanisms.

4.2 MATERIALS AND METHODS

4.2.1 Laboratory-scale unit description

4.2.1.1 Unit set-up

An insulated shipping container (6 m long) was used as an external laboratory to house the pilot plant unit (manufactured by Department of Civil Engineering, University of Cape Town). A submersible pump (50 L/min) was suspended in the sump where settled sewage from the balancing tank enters the head of the full-scale activated sludge system at Darvill WWW (Pietermaritzburg, South Africa). The pump ensured that settled sewage was continuously supplied to the pilot plant. Settled sewage was pumped directly to a second shipping container which housed a refrigerated tank (500 L, 2-4°C). The tank was filled once or twice weekly and rinsed at least once a month. A motorised impellor in the tank ensured that the sewage was continuously mixed. De Haas (1998), when positioning and designing the layout of the units in 1993, considered pumping raw sewage but the layout of the Works and the fact that it was undergoing a major capital upgrade at the time made this logistically very difficult. Raw sewage would also have required maceration to break up solids, rags and other debris prior to pumping to the pilot plant. The pump was connected to the container in a closed system so when the tank was full and the valve closed, sewage was diverted back to the sump for treatment in the full-scale biological reactor.

4.2.1.2 Unit configuration and layout

The pilot plant was designed and modelled upon the 3-stage Phoredox configuration and operated at 20°C (±1°C). A heater (during winter months) or airconditioner (during summer months) was used to regulate the ambient temperature within the container. A schematic design of the pilot plant is given in FIG. 4.1. The reactor configuration consisted of the following: an anaerobic (AN) zone (V = 8 L); an anoxic (AX) zone (V = 4 L); first aerobic (AE1) zone (V = 10 L); and second aerobic (AE2) zone (V = 10 L). The clarifier (2.5 L), downstream of

the reactors, was positioned at a 60° angle to the perpendicular. Target influent flow rate (Q_i) was set at 36 L/d; settled sewage was fed directly to the AN zone using a peristaltic pump (Gilson). The s-recycle, pumped from the clarifier to the AN zone, was set at a ratio of 1:1, with respect to Q_i. The a-recycle, from AE2 to the AN zone, was set at a ratio of 3:1, with respect to Q_i.

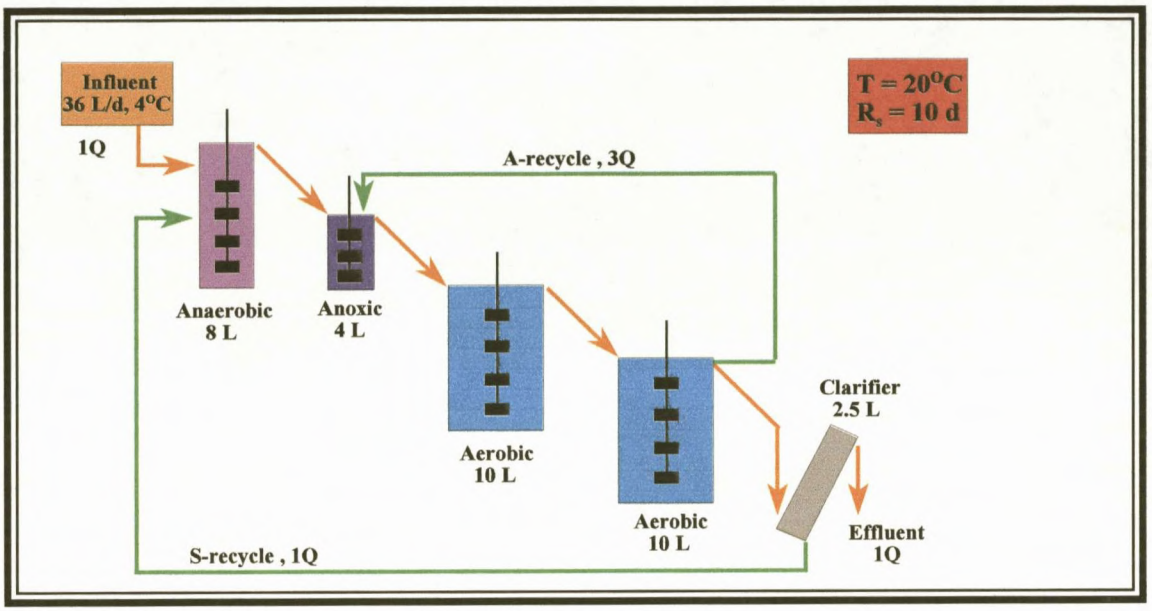

FIGURE 4.1 Schematic representation of the laboratory-scale unit modelled upon the 3-stage Phoredox process.

Influent to the unit consisted of settled sewage pumped directly from the sump at the head of the full-scale activated sludge process at Darvill WWW. Batches of sewage (80 L) were prepared every second day by pumping sewage from the 500 L cold storage tank into a 160 L tank, housed in the same container as the pilot plant. The 160 L tank was constructed of stainless steel and equipped with an insulated jacket containing ethylene glycol which was chilled via an immersed copper coil from a refrigerator. Influent (in the 160 L tank) was maintained at 4°C which curtailed biological accumulation of COD and nutrients by microorganisms entering the system via the sewage.

Sludge was wasted from the system by daily withdrawal of the appropriate volume of mixed liquor (3.2 L) from AE2. The system sludge age (R_s) was therefore maintained at 10 d for the duration of experimentation ie., unit

volume capacity = 32 L; sludge wasted = 3.2 L. Oxygen uptake rate (OUR) was measured in AE2 by means of a dissolved oxygen (DO) probe and meter (Dept of Civil Engineering, UCT). Air supply to the aerated zones (AE1 and AE2) was controlled such that the aquarium air pumps switched on at a DO concentration of 2.0 mg/L and switched off at DO concentrations of 5.0 mg/L. The meter, operating in OUR mode, recorded OUR over a 24 h period. Temperature and OUR results were then downloaded onto PC using an RS 232 adapter and data were subsequently manipulated using Lotus 1-2-3 software.

4.2.2 Unit operation

4.2.2.1 Acquisition of seed inoculum

Mixed liquor (25 L) was obtained from the return sludge stream of Amanzimtoti WWW (South Durban, South Africa). The plant treats both industrial (automobile, chemical and beer manufacturers) (50%) and domestic waste (50%). South African Breweries constitutes the bulk of the industrial flow to the plant (90%). Secondary biological treatment at the plant consists of two parallel activated sludge units, the east bank and west bank. Each bank is divided into six aeration bands, each consisting of four surface aeration units (Pillay, 1998). Various operating and wastewater characteristics pertaining to the activated sludge process at Amanzimtoti WWW are given in TABLE 4.1. As the treated water from the plant is pumped to river and directly out to sea, the plant does not need to comply with the Special P Standard (<1 mgP/L). TP's and SRP's are therefore not routinely monitored.

Since the primary objective of this project was to identify organisms in activated sludge responsible for poly-P accumulation, it seemed appropriate to inoculate the pilot plant with activated sludge biomass from a non-BNR plant ie., Amanzimtoti WWW, and monitor changes in the microbial community as the culture became more 'enhanced' ie., as the culture, through manipulation of certain abiotic parameters, shifted towards accumulating P in excess of normal metabolic requirements.

Mixed liquor seed inoculum was decanted into the pilot plant and the volume made up to 32 L using settled sewage. This rendered a final suspended solids concentration of approximately 1 500 mg/L. The inoculum was fed 100%

settled sewage from Darvill WWW ie., no supplementation, for one week until the biomass became acclimatised to the particular sewage. Feed supplementation to the unit then commenced for the duration of the project.

TABLE 4.1 Operating parameters and average feed composition to Amanzimtoti Wastewater Treatment Plant activated sludge process.

Total reactor volume - east plant west plant	4 680 m^3 9 366 m^3
Maximum wet weather flow	36 000 m^3/d
Minimum wet weather flow	21 000 m^3/d
Operating MLSS	3 500 - 5 000 mg/L
Mean cell residence time	20 - 25 d
Average COD (influent)	1 000 mg/L
Average NH_3 (influent)	18 mgN/L
Average pH (influent)	6.8
Average alkalinity (influent)	220 mg/L as $CaCO_3$

4.2.2.2 Feed supplementation for enhanced culture development

For the duration of pilot plant operation, it was essential to attain a constant influent COD of 500 mg/L in order to simulate steady-state conditions. However, during periods 4.1 - 4.5 (TABLE 4.2) this proved difficult since Darvill settled sewage is relatively weak ie., average of 250 to 350 mg/L and <100 to 250 mg/L COD under dry and wet weather conditions, respectively. However, it was decided to accept a lower influent COD concentration for the periods mentioned, using sodium acetate as the sole organic supplement. Sewage supplements consisted of the following (refer also to TABLE 4.2):

- orthophosphate - the required volume was added to the feed (80 L) from a concentrated stock solution containing 450 g/L K_2HPO_4 ie., 1 mL stock solution per 80 L was equivalent to 1 mgP/L;
- magnesium chloride - the required volume of a concentrated stock solution containing 189.7 g/L $MgCl_2.6H_2O$ was added per 80 L batch;
- sodium bicarbonate for alkalinity - 100 mg/L as $CaCO_3$ or 13.45 g $NaHCO_3$ added per 80 L for entire

experimental period; and

- sodium acetate - stoichiometrically, 1 g sodium acetate added to the feed is equivalent to 0.78 g COD (eg., 100 mgCOD/L is equivalent to 10.3 g anhydrous sodium acetate per 80 L batch).

TABLE 4.2 Sewage supplement composition (excluding micronutrient supplementation).

Period Date range	No. of days	NaAc mg COD/L	K_2HPO_4 mg P/L	$MgCl_2$ mg Mg/L	$NaHCO_3$ mg/L as $CaCO_3$
4.1 19/6/98 - 25/6/98	7	0	0	0	100
4.2 26/6/98 - 03/8/98	39	100	5	8.5	100
4.3 04/8/98 - 22/8/98	19	150	5	11.3	100
4.4 23/8/98 - 11/9 98	20	200	23/8 - 02/9 = 5 03/9 - 09/9 = 10 10/9 - 11/9 = 20	11.3 11.3 11.3	100
4.5 12/9/98 - 02/10/98	21	300	20	25.5	100
4.6 03/10/98 - 22/10/98	20	400	20	25.5	100
4.7 23/10/98 - 21/11/98	30	500	23/10-28/10=30 29/10-31/10=40 01/10-04/11=50 05/11-21/11=50	25.5 25.5 25.5 42.5	100

Anhydrous sodium acetate (NaAc; ACE, South Africa) concentrations were incrementally increased to the feed at regular time intervals (TABLE 4.2). At concentrations of 400 mgNaAc/L as COD (period 4.6), settled sewage in the feed was diluted with tap water, maintaining a total COD concentration to the unit of approximately 500 mg/L. At this stage, macro- and micronutrient supplementation to the feed commenced in accordance with the recipe suggested by Wentzel *et al.*, (1988) (TABLE 4.3). Nutrient concentrations in the feed were becoming limiting due to dilution which negatively affects bacterial growth and metabolism. Ammonium chloride was also added to the feed stock to maintain a TKN value of approximately 25 mgN/L.

TABLE 4.3 Influent macro- and micronutrient supplementation per 100 mgCOD sodium acetate (adapted from Wentzel *et al.*, 1988).

Chemical	mg added per 100 mgCOD as sodium acetate	
	Compound	Element
	MACRONUTRIENTS	
$CaCl_2.2H_2O$	11.7	3.2
Yeast extract	1	
	MICRONUTRIENTS	
$FeSO_4.7H_2O$	0.525	0.105
$ZnSO_4.7H_2O$	0.15	0.034
$MnSO_4$	0.15	0.055
$CuSO_4.5H_2O$	0.03	0.008
$CoCl_2.6H_2O$	0.03	0.007
$Na_2MoO_4.2H_2O$	0.015	0.006
H_3BO_3	0.03	0.005
KI	0.008	0.006

4.2.2.3 Unit maintenance

The refrigerated tank (160 L) was cleaned using a brush and warm water every second day before refilling. The larger tank (500 L) was cleaned in the same manner once a month. Agitation in the reactors was briefly halted and the interior surfaces brushed daily. Pump tubing lines were cleaned daily by means of squeezing and brushing. Soft silicone tubing proved easiest to keep clean. Marprene tubing (internal diameter of 4.8 mm and wall thickness of 1.6 mm, Aeromix) was used to connect the peristaltic pumps to the silicone tubing due to its anti-abrasive properties and long working life (*ca.* 10 000 h). All T-pieces and weirs were cleaned daily to prevent blockages and spillages. The DO probe was cleaned and calibrated daily (using a saturated sodium sulphite solution) and the membrane changed every two weeks.

4.2.2.4 Acid dosing

Wentzel *et al.*, (1988) found that P uptake in the aerobic reactors results in a drastic increase in the pH of the mixed liquor. They found that when operating an enhanced culture, the pH could increase above 9 which results in operational problems and potential collapse of the whole system. This loss of H^+ ions has previously being described biochemically in Wentzel *et al.*, (1986). It was therefore anticipated that pH control would become increasingly significant and critical with increasing acetate dosing. Mixed liquor pH in all four reactors was monitored daily using a portable probe and meter (Beckman). Dilute hydrochloric acid (20 - 300 mL of a 1M HCl solution was diluted to 1 L) was dosed to AE1 at a rate of 500 mL/d (TABLE 4.4). For the majority of experimentation, the addition of acidity maintained the mixed liquor pH below 8.

TABLE 4.4 Experimental periods for acid dosing to laboratory-scale unit.

Period	No. of days	Acid dosage (mmol/d)	Zone dosed with acid
4.1	7	0	-
4.2	39	0	-
4.3	19	0	-
4.4	20	0	-
4.5	21	Dosing commenced on 30/9 = 20 to 40	AE1
4.6	20	40 to 300	AE1
4.7	30	300	AE1

4.2.3 Determinand analysis

4.2.3.1 Preparation of glassware

In order to avoid P contamination and subsequent erroneous results, all glassware underwent a stringent washing procedure. Test tubes and beakers were rinsed in tap water and soaked overnight in a phosphate free cleaning solution (7X®-PF, Polychem). Glassware was then scrubbed with a brush and soaked overnight in a 20% (v/v) sulphuric acid solution, followed by a triple rinse with deionised water. The glassware was then dried in an oven. The affinity of the phosphate ion for glass is well documented and should be avoided to achieve qualitative results.

4.2.3.2 Collection and preservation of samples

Samples extracted from the pilot plant were decanted into pre-washed and dried 250 mL plastic honey jars. The honey jars were triple rinsed with the sample of interest and samples were preserved according to APPENDIX 4.

4.2.3.3 Parameters measured

The parameters routinely measured, hydraulic flow of interest and methods of analysis included:

- COD (influent and effluent): SQ 118 (Merck, Method No. 14690) and closed reflux microwave digestion (APPENDIX 5);
- Readily biodegradable COD (influent): SQ 118 (Merck, Method No. 14690) and microwave digestion (APPENDIX 6);
- Total P (influent, effluent, mixed liquor, filtered AN, AX, AE1 and AE2 zones): Skalar auto-analyser/ spectrophotometrically (APPENDIX 7);
- Soluble reactive P (influent and effluent): Skalar auto-analyser/spectrophotometrically (APPENDIX 8);
- TKN (influent and effluent): Skalar autoa-nalyser;
- Ammonia (influent and effluent): SQ 118 (Method No. 14752) and Skalar auto-analyser;
- Nitrates (effluent): SQ 118 (Method No. 14773) and Skalar auto-analyser;
- MLSS and VSS (mixed liquor sample routinely taken from AE2 zone but periodically taken from all zones to calculate mass fractions): APPENDIX 9;

- SVI and occasionally DSVI (mixed liquor from AE2): APPENDIX 10;
- pH (from AN, AX, AE1 and AE2 zones); and
- OUR (AE2 zone).

Due to phosphate supplementation to the pilot plant influent feed, concentrations of P in the effluent were usually >1 mgP/L. As a result, the vanadate-molybdate (VM) method was selected for P determination due to the ability of the procedure of working in higher P concentration ranges (as opposed to the molybdate-ascorbic acid method which is usually employed to operate in P concentration ranges of <1 mgP/L). For ortho-P determinations, 5 mL sample (pre-diluted if necessary into the range 1 - 100 mgP/L) was mixed with 5 mL colour reagent. Samples were left to stand for 30 mins at room temperature to allow for full colour development. Absorbance was read at 470 nm using a UV/VIS spectrophotometer with a flow cell of 1 cm pathlength against a distilled water blank (APPENDICES 7 and 8). Standards were included in each run for TP and SRP determination. A typical standard curve for these determinations is given in FIG. 4.2.

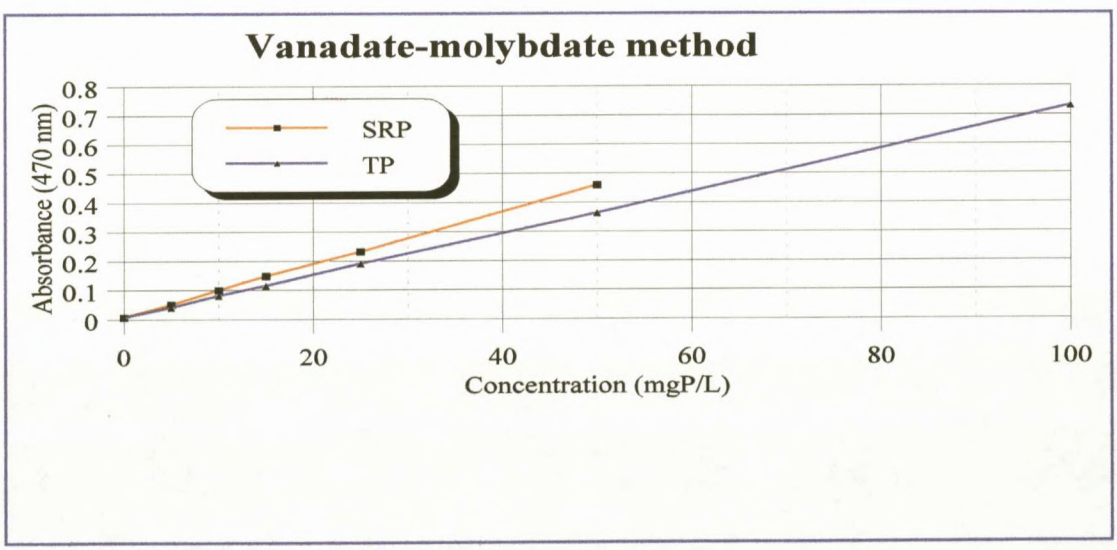

FIGURE 4.2 **Typical calibration curve for total P and soluble reactive P determination by the vanadate-molybdate method. Path length = 1 cm.**

4.3 RESULTS

4.3.1 Pilot plant biological P uptake performance

Total phosphorus entering the pilot plant was, as far as possible, fractionated in an effort to quantify and evaluate the biological P uptake mechanism in its entirety. Filtered mixed liquor total phosphorus (fMLTP) samples were taken from AN, AX, AE1 and AE2 and analysed to obtain a more holistic impression of P release and uptake occurring throughout the system (FIGS 4.3A - 4.3E). Phosphorus release and uptake and total soluble phosphate removal results were then linearised to determine the extent of correlation which existed between the mechanisms (FIG. 4.4). The transient behaviour of the P removal mechanism is shown in FIG. 4.5, depicting soluble reactive phosphorus (SRP, as P) removal as a function of time. TABLE 4.5 shows actual influent and effluent SRP concentrations and percentage SRP removal for the experimental periods. Suspended solids TP concentration (mgP/L) and the P content of the active biomass (mgP/gMLVSS) are shown simultaneously in FIG. 4.6. FIG. 4.7 gives a graphical description of the poly-P content of the active biomass (mgP/gMLVSS) and the mass (m/m) of accumulated poly-P with respect to the total active biomass concentration (dry weight).

4.3.1.1 Total phosphate release and uptake in the various redox zones

For the duration of pilot plant operation, the SRP fraction of TP was only measured in the influent and effluent streams. However, the biological P release and uptake mechanism was constantly monitored throughout the system by taking filtered mixed liquor samples ie., AN, AX, AE1 and AE2 mixed liquor was filtered through Whatman No. 1 (or equivalent) filter paper, and recording changes in soluble TP. Results of these analyses are shown graphically in FIGS 4.3A - 4.3E which represent soluble fMLTP concentrations in the various zones (reported as mgP/L). It must be noted that the figures only report experimental periods 4.3 to 4.7 as during periods 4.1 and 4.2 the correct protocol for fMLTP determination was still under investigation. Phosphorus removal ie., $\Delta P = 13.9$ mgP/L in FIG. 4.3A, is reported as the difference between influent and effluent TP concentrations.

It is evident from FIGS 4.3A to 4.3E that the bio-P mechanism was operational due to the expected curve

representing anaerobic P release and subsequent uptake in the following reactors. An unexpected feature, however, was the extent of anoxic P uptake which occurred throughout enhanced culture development. A concomitant increase in anaerobic P release was noted as enhanced culture development progressed and initial TP concentration in the feed was incrementally increased. It became necessary to increase initial P concentrations in the feed to ensure P was never limiting in the system ie., present in effluent at concentrations > 1 mgP/L. The increase in P release can be attributed to increased poly-P accumulation although, during period 4.6 where a sudden decline in the influent TP concentration can be noted ie., 33 mgP/L during period 4.5 to 25 mgP/L during period 4.6 (FIG. 4.3D), high P release rates were continually recorded. Nett P uptake from the system gradually increased as the culture became enhanced. Initial nett P uptake during period 4.3 was ca. 14 mgP/L (FIG. 4.3A) which increased to a maximum of ca. 37 mgP/L during period 4.7 (FIG. 4.3E). A slight increase in effluent soluble phosphate concentration was noted during periods 4.4 (FIG. 4.3B) and 4.6 (FIG. 4.3D) which was attributed to prolonged solids retention times in the clarifier which promoted the onset of anaerobiosis.

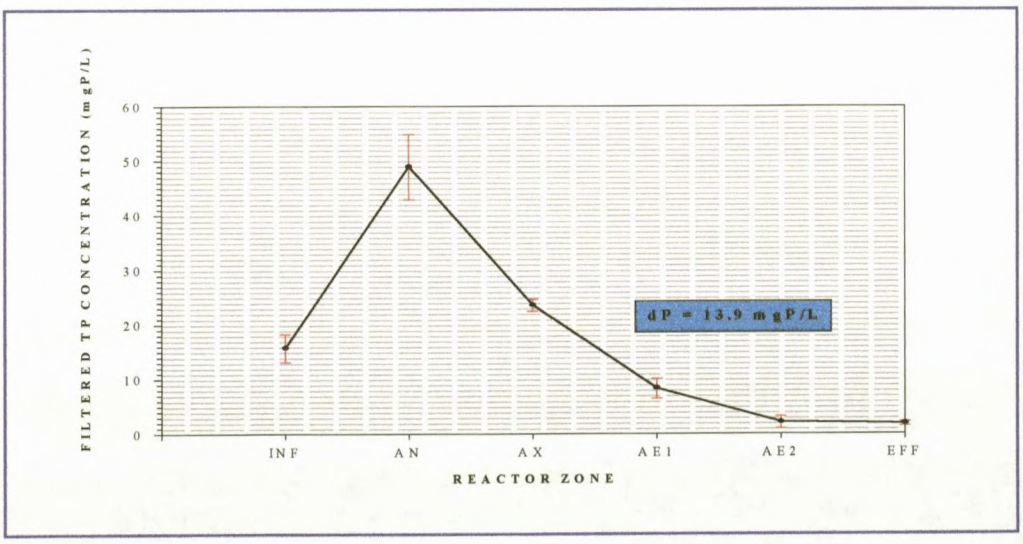

FIGURE 4.3A Average total phosphorus concentrations at various stages of pilot plant (INF=influent; AN=anaerobic; AX=anoxic; AE1=aerobic 1; AE2=aerobic 2; EFF=effluent) during period 4.3 (150 mgCOD/L as sodium acetate). Standard deviations are indicated at each point.

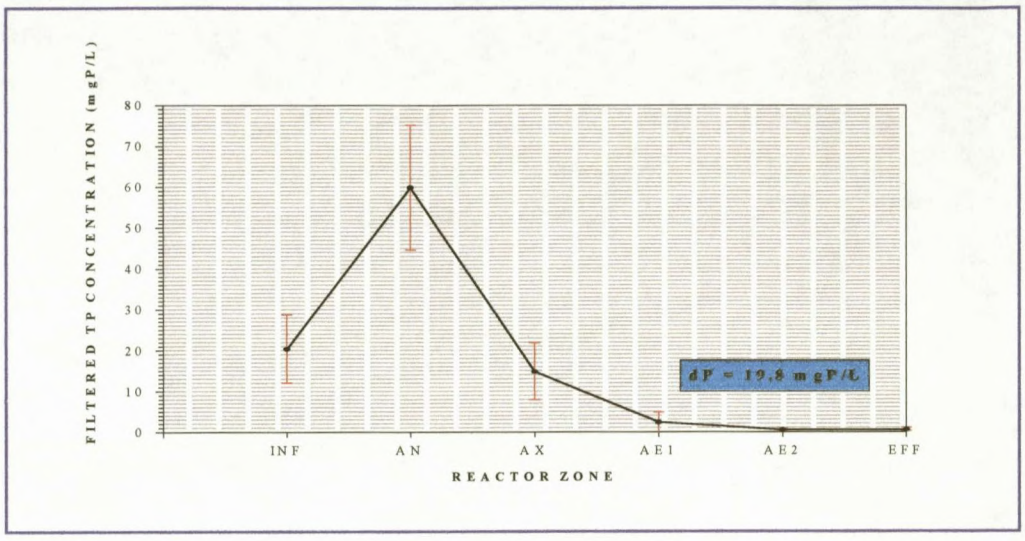

FIGURE 4.3B **Average total phosphorus concentrations at various stages of pilot plant (INF=influent; AN=anaerobic; AX=anoxic; AE1=aerobic 1; AE2=aerobic 2; EFF=effluent) during period 4.4 (200 mgCOD/L as sodium acetate). Standard deviations are indicated at each point.**

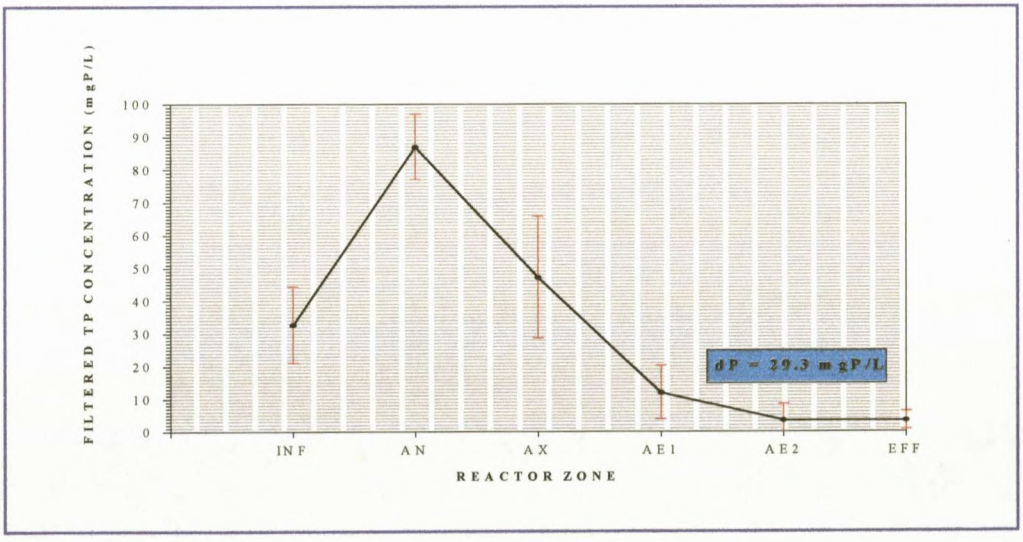

FIGURE 4.3C **Average total phosphorus concentrations at various stages of pilot plant (INF=influent; AN=anaerobic; AX=anoxic; AE1=aerobic 1; AE2=aerobic 2; EFF=effluent) during period 4.5 (300 mgCOD/L as sodium acetate). Standard deviations are indicated at each point.**

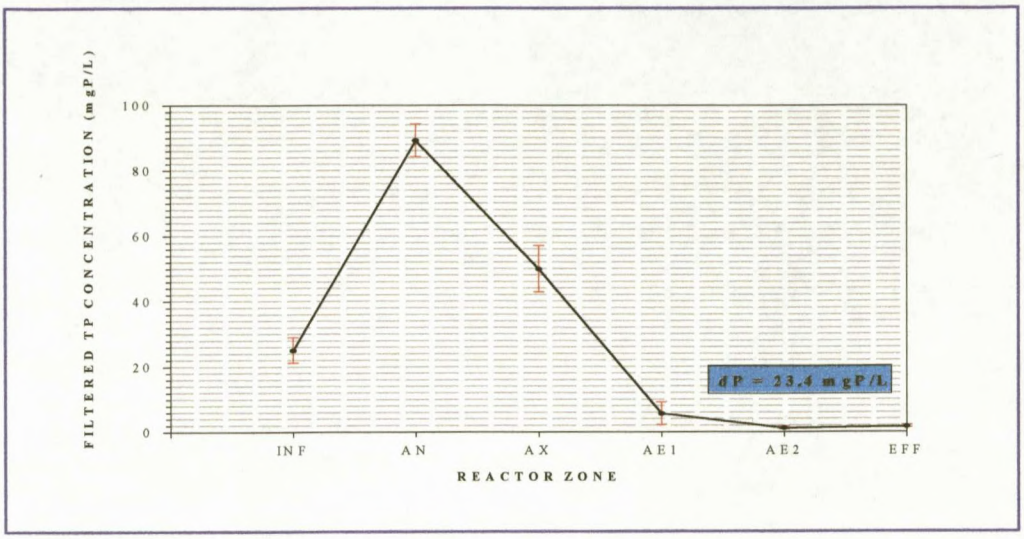

FIGURE 4.3D **Average total phosphorus concentrations at various stages of pilot plant (INF=influent; AN=anaerobic; AX=anoxic; AE1=aerobic 1; AE2=aerobic 2; EFF=effluent) during period 4.6 (400 mgCOD/L as sodium acetate). Standard deviations are indicated at each point.**

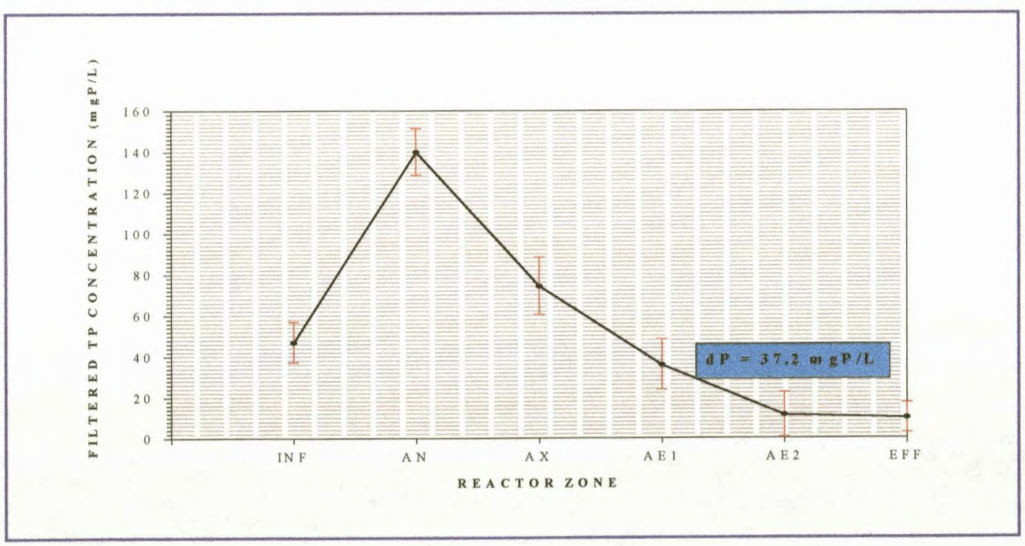

FIGURE 4.3E **Average total phosphorus concentrations at various stages of pilot plant (INF=influent; AN=anaerobic; AX=anoxic; AE1=aerobic 1; AE2=aerobic 2; EFF=effluent) during period 4.7 (500 mgCOD/L as sodium acetate). Standard deviations are indicated at each point.**

A linear relationship was found to exist between anaerobic P release and aerobic P uptake for the entire duration of pilot plant operation (FIG. 4.4). The values depicted in FIG. 4.4 are based on differences between mean influent and effluent TP (bottom regression) and anaerobic fMLTP and effluent (top regression) results obtained for periods 4.3 to 4.7. This was done to distinguish between the phrases P uptake and P removal. During this study, P uptake reflected the difference between anaerobic P release ie., difference between P concentration in anaerobic reactor and influent, and effluent P concentration. Phosphate removal was used to describe total system response ie., difference between influent and effluent P concentrations. Anaerobic P release ranged from 33.2 mgP/L during period 4.3 to 92.9 mgP/L during period 4.7.

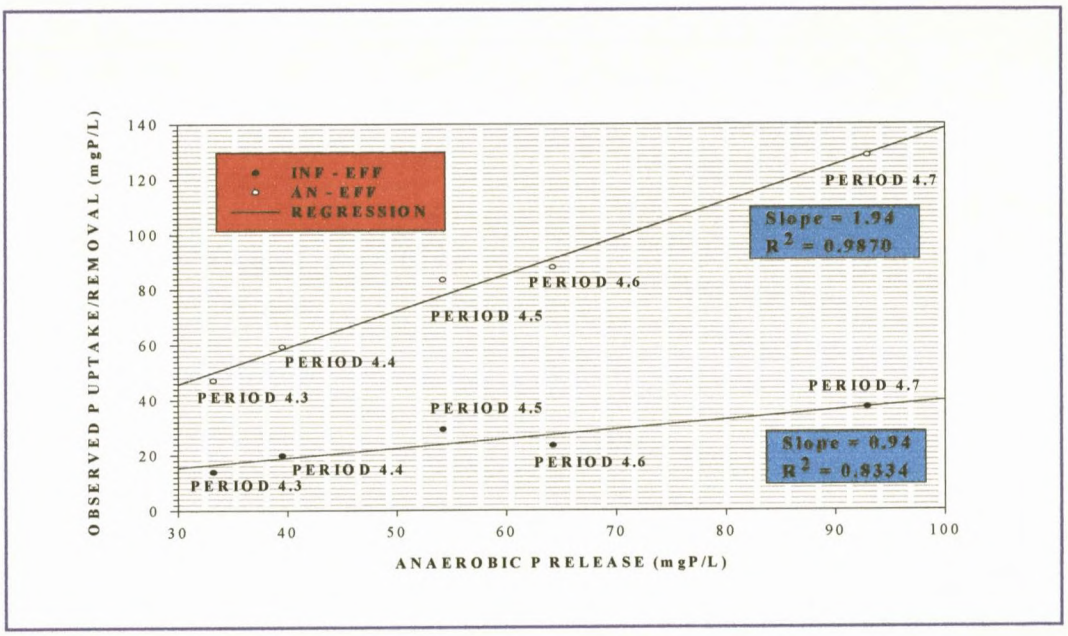

FIGURE 4.4 **Total phosphate (as P) removal (top regression) and aerobic total phosphate (as P) uptake (bottom regression) plotted as a function of anaerobic total soluble phosphate release (as P).**

4.3.1.2 Total soluble phosphate removal from the system

Soluble phosphorus removal versus time data, for the full extent of unit operation, is shown in FIG. 4.5. A gradual increase in the biological phosphorus removal mechanism can be noted as the acetate fraction in the feedstock was

increased although it seems that steady-state, as far as phosphate removal is concerned, was never achieved. The decline in phosphate removal during periods 4.4 and 4.5 can be attributed to operational problems which were experienced where power failures affected agitation and aeration (sometimes for up to a day), resulting in the onset of total system anaerobiosis. According to FIG. 4.5, inducement of the excess biological phosphate uptake mechanism seems to have developed by day 52 of operation. At this stage it was assumed that the microbial community in the sludge had shifted supporting PAO dominance.

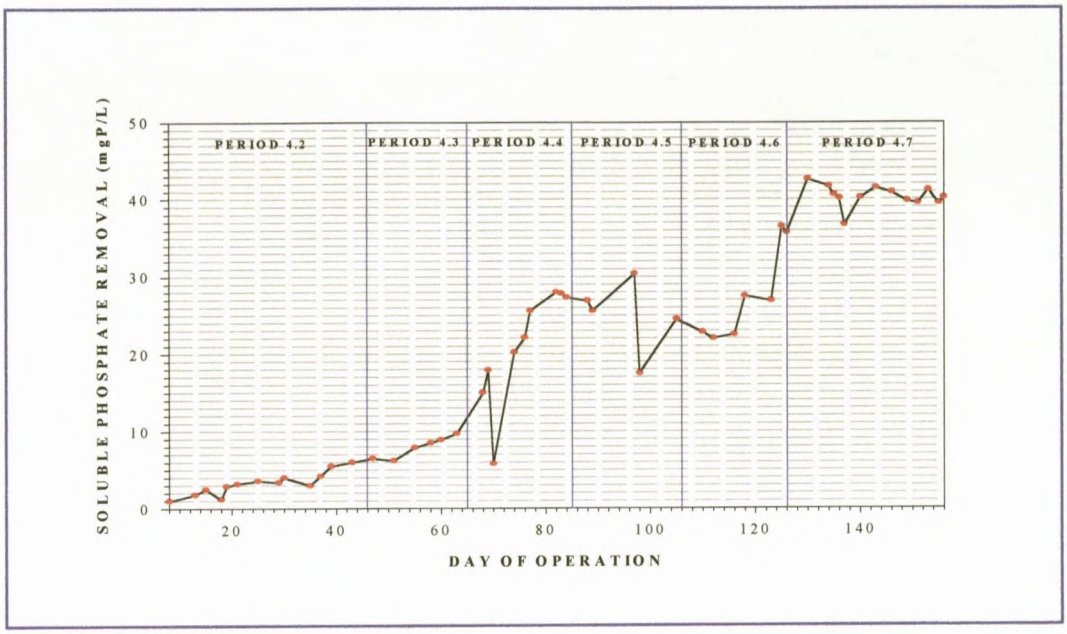

FIGURE 4.5 Total soluble phosphate (as P) removal plotted as a function of time.

Average influent and effluent SRP concentrations and percentage SRP removal for periods 4.1 to 4.7 are given in TABLE 4.5. During the experimentation period, soluble P was supplemented to the influent feed (in the form of K_2HPO_4) to ensure P was not limiting. However, during periods 4.4 and 4.6 the bio-P removal mechanism increased dramatically and effluent ortho-P concentrations decreased below 1 mgP/L (TABLE 4.5). This in turn may have negatively affected the accuracy and sensitivity of the VM method of ortho-P determination due to effluent concentrations decreasing below the procedural lower detection limit. The decrease in influent SRP concentrations during period 4.6 is as a result of dilution of the settled sewage fraction to the feed. Phosphate supplementation, however, did not increase concomitantly and remained constant at 20 mgP/L (refer to TABLE 4.2). A decrease in

P uptake, based on percentage removal, was recorded during period 4.7 (85.4% as opposed to 98.8% during period 4.6) but actual P removal continued to increase (TABLE 4.5). Although this doesn't reflect a decreasing removal mechanism, it may have been an indication that the active biomass fraction in the system was no longer sufficient to cope with higher influent phosphate concentrations.

TABLE 4.5 Mean soluble influent and effluent P concentrations (mg P/L) and percentage removal for periods 4.1 to 4.7.

Results expressed as mean values with sample standard deviations (SD) in parentheses.

Period	Average soluble P concentration (mgP/L)			
	Influent	Effluent	Removal	% P_{sol} removal
4.1	ND	ND	ND	ND
4.2	12.2 (1.47)	9.9 (0.91)	2.3	18.9
4.3	12.7 (0.21)	7.4 (0.39)	5.3	41.7
4.4	16.7 (6.15)	0.4 (0.20)	16.3	97.6
4.5	27.0 (3.80)	2.5 (2.53)	24.5	90.7
4.6	24.1 (2.16)	0.3 (0.35)	23.8	98.8
4.7	41.2 (7.00)	6.0 (5.60)	35.2	85.4

ND = not determined

4.3.1.3 Phosphate content of mixed liquor solids

Suspended solids TP in FIG. 4.6 was recorded as the difference between unfiltered and filtered mixed liquor TP concentrations in AE2. Due to strict pH control in the aerobic reactors, chemical precipitation of P was regarded as negligible for the duration of the study and any P removed was therefore assumed to be associated with biological mechanisms. It must be emphasised that P fractionation was not conducted (Psenner *et al.*, 1984; De Haas *et al.*,

1990a; Uhlmann *et al.*, 1990) and it is therefore not evident in which form phosphate was stored. This was not considered limiting to evaluating system performance as an estimation of the P content (whether surface bound or intracellularly accumulated) of the sludge was considered sufficient for evaluating enhanced culture development. Phosphate uptake based on solids was only measured during periods 4.5 to 4.7 (FIGS 4.6 and 4.7). The P content of the active mass (based on MLVSS; RHS axis; blue line) in FIG. 4.6 was recorded as the suspended solids TP divided by mean MLVSS concentration for the period. The sudden increase in both TSS and VSS phosphate during period 4.7 (FIG. 4.6) indicates that a strong bio-P mechanism was induced through increasing the TP load to the system rather than increasing the RBCOD load (refer to TABLES 4.2 and 4.5 for period loading rates).

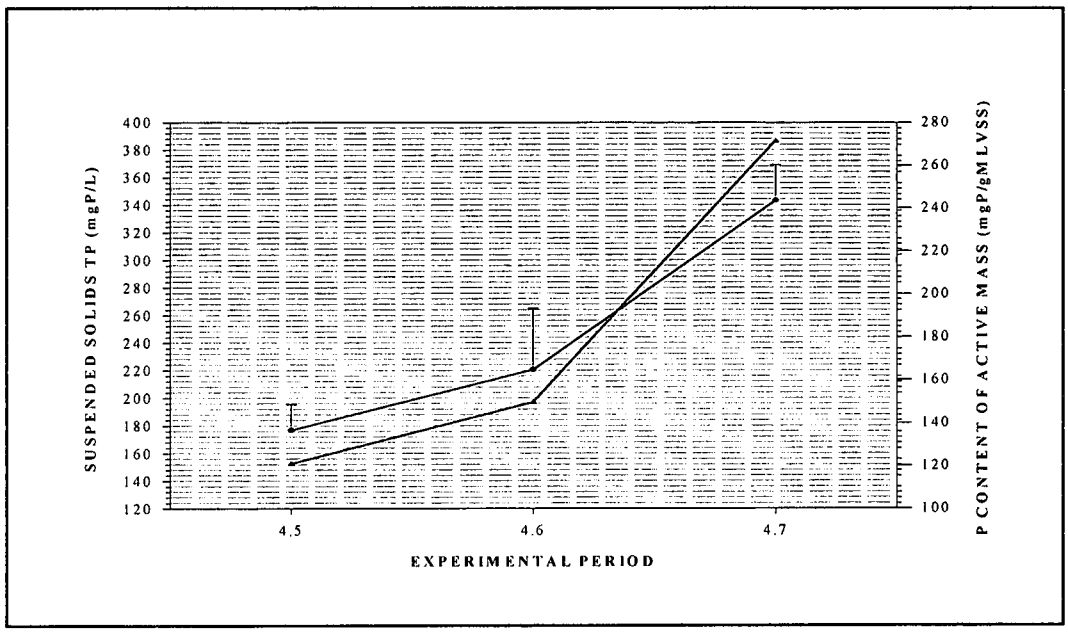

FIGURE 4.6 Mixed liquor suspended solids TP concentration and P content of volatile suspended solids for experimental periods 4.5 to 4.7.

The approximate poly-P content of the sludge active mass (based on MLVSS; LHS axis) and poly-P mass fraction (mgP/mgMLVSS; dry weight; RHS axis) are shown in FIG. 4.7. Poly-P content of the active mass was estimated at TP of suspended solids (FIG. 4.6; LHS axis) less 3% P content of MLVSS (FIG. 4.6; RHS axis) (Romanski *et al.*, 1997). Of particular interest in FIG. 4.7 is the high P content of the system MLVSS (*ca.* 27 mgP/mgVSS) during period 4.7. As suggested in FIG. 4.6, the increase in accumulated poly-P during period 4.7 can be attributed

to the increase in influent phosphate and COD loads.

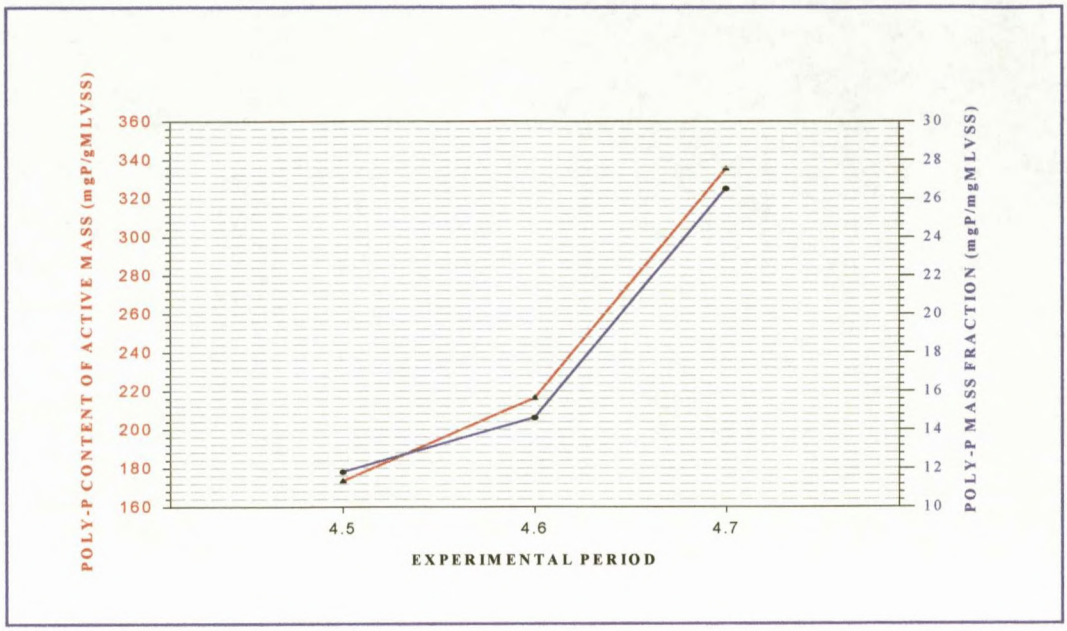

FIGURE 4.7 Polyphosphate content of volatile suspended solids and polyphosphate mass fraction with respect to volatile suspended solids for experimental periods 4.5 to 4.7.

4.3.2 Influent and effluent monitoring

4.3.2.1 Influent characterisation and mass loads

In order to achieve steady-state conditions, target influent flow rates and COD loads to the pilot plant were 36 L/d and 18 gCOD/d (equivalent to 500 mgCOD/L), respectively. However, the influent COD target proved difficult to achieve due to the weak COD load of Darvill settled sewage. Periods prior to 4.5 (when acetate supplementation increased to 300 mg/L as COD) were therefore deficient of the target COD load (TABLE 4.6). The contribution of supplemented NaAc (based on stoichiometry) to the various period COD loads was recorded as follows: period 4.2 = 3.5 gCOD/d; period 4.3 = 5.5 gCOD/d; period 4.4 = 7.1 gCOD/d; period 4.5 = 11.2 gCOD/d; period 4.6 = 14.6 gCOD/d; and period 4.7 = 18.1 gCOD/d. At 100 per cent acetate (HAc) supplementation during period 4.7, theoretical COD (as acetate) addition based on reaction stoichiometry was never achieved which led to lower

recorded COD and RBCOD loads for the period (TABLE 4.6).

TABLE 4.6 Average influent flow rate (Q_i), hydraulic retention time (HRT) and mass loading rate of the pilot plant for experimental periods 4.1 to 4.7.

Results expressed as mean values with sample standard deviations (SD) in parentheses.

Period	Flow rate Q_i (L/d)	HRT (h)	COD load (gCOD/d)	RBCOD load (gCOD/d)	TP load (gP/d)	TKN load (gN/d)
4.1	ND	ND	ND	ND	ND	ND
4.2	34.8 (2.4)	AN: 5.5 AX: 2.8 AE: 13.8 TOT: 22.1	15.14	4.52	0.59	1.04
4.3	36.3 (4.62)	AN: 5.3 AX: 2.6 AE: 13.2 TOT: 21.1	16.52	5.05	0.59 0.47*	1.23
4.4	35.5 (4.08)	AN: 5.4 AX: 2.7 AE: 13.5 TOT: 21.6	15.27	6.39	0.92 0.6*	1.35
4.5	37.3 (2.16)	AN: 5.2 AX: 2.6 AE: 12.9 TOT: 20.7	22.68	11.34	1.23 1.0*	1.38
4.6	36.6 (2.8)	AN: 5.3 AX: 2.6 AE: 13.1 TOT: 21.0	19.73	12.77	0.92 0.89*	1.1
4.7	36.2 (2.75)	AN: 5.3 AX: 2.7 AE: 13.3 TOT: 21.3	17.81	13.61	1.74 1.48*	0.83

ND = not determined; * period mass loading rate of SRP (gP/d).

The sudden increase in COD entering the system during period 4.5 (22.7 mgCOD/d) was due to the infiltration of industrial effluent from a local vegetable oil refinery into the sewage system. Organic and TP loads entering Darvill WWW therefore increased drastically which effected the operation of the pilot plant. Total phosphorus and TKN

entering the system was also routinely monitored to ensure phosphate was not limiting ie., always detectable in the clarified effluent, and the denitrification capacity of the anoxic zone was never over-extended. The strength of the bio-P removal mechanism became especially evident when one considers that influent TP loads had to be increased from 0.92 g/d during period 4.6 to 1.74 g/d during the latter half of period 4.7 (TABLE 4.6). Hydraulic retention time in the plant and individual reactors remained fairly constant for the duration of the operation and seemed to be fairly independent of Q_i. The HRT of the system ranged from 21 to 22 h with HRT values of *ca.* 5 h and 13 h recorded in the anaerobic and aerobic reactors, respectively.

4.3.2.2 Carbonaceous material

Total mean influent (S_{ti}) and effluent COD (S_{te}) concentrations, as well as influent RBCOD (S_{bsi}) concentrations, were determined in order to monitor organic substrate utilization (TABLE 4.7 and FIG. 4.8).

TABLE 4.7 **S_{ti} concentrations, corresponding S_{bsi} fractions (f_{ts}), percentage COD removal, F/M ratios and TP removal based on S_{ti} and S_{bsi} for experimental periods 4.1 to 4.7.**

Results expressed as mean values with sample standard deviations (SD) in parentheses.

Period	S_{ti} (mgCOD/L)	S_{bsi} fraction (f_{ts})	% COD removed	F/M ratio[a] (d^{-1})	$\Delta P/\Delta S_{ti}$ (mgP/mgCOD)	$\Delta P/\Delta S_{bsi}$ (mgP/mgCOD)
4.1	ND	ND	ND	ND	ND	ND
4.2	435 (37.74)	0.30	87.4	0.31	0.03	0.09
4.3	455 (124.96)	0.31	91.9	0.33	0.03	0.09
4.4	430 (88.31)	0.42	91.0	0.28	0.07	0.14
4.5	608 (171.33)	0.50	91.0	0.29	0.05	0.09
4.6	539 (59.26)	0.65	93.0	0.25	0.05	0.07
4.7	492 (51.3)	0.76	94.2	0.23	0.08	0.10

[a] F/M ratio based on g COD per d/ g MLSS; ND = not determined

An unusual observation during period 4.7 was that at a theoretical HAc supplementation of 500 mg/L as COD, the RBCOD fraction (f_{ts}) amounted to only 76% of S_{ti} which itself amounted to 492 mg/L (TABLE 4.7). The mean RBCOD concentration achieved during period 4.7 therefore only amounted to 374 mgCOD/L (TABLE 4.7 and FIG. 4.8). The same pattern was also recorded during period 4.6, where the mean RBCOD concentration amounted to 350 mgCOD/L as opposed to the target concentration of 400 mgCOD/L. Due to the maintenance of the system on HAc alone during period 4.7, the convergence of S_{ti} and S_{bsi} during the latter stages of system operation was expected (FIG. 4.8). High COD removal was recorded throughout experimentation ie., 87 to 94% removal (TABLE 4.7). It is evident from FIG. 4.8 that S_{te} remained fairly constant throughout experimentation and that steady-state, as far as COD removal was concerned, had been achieved.

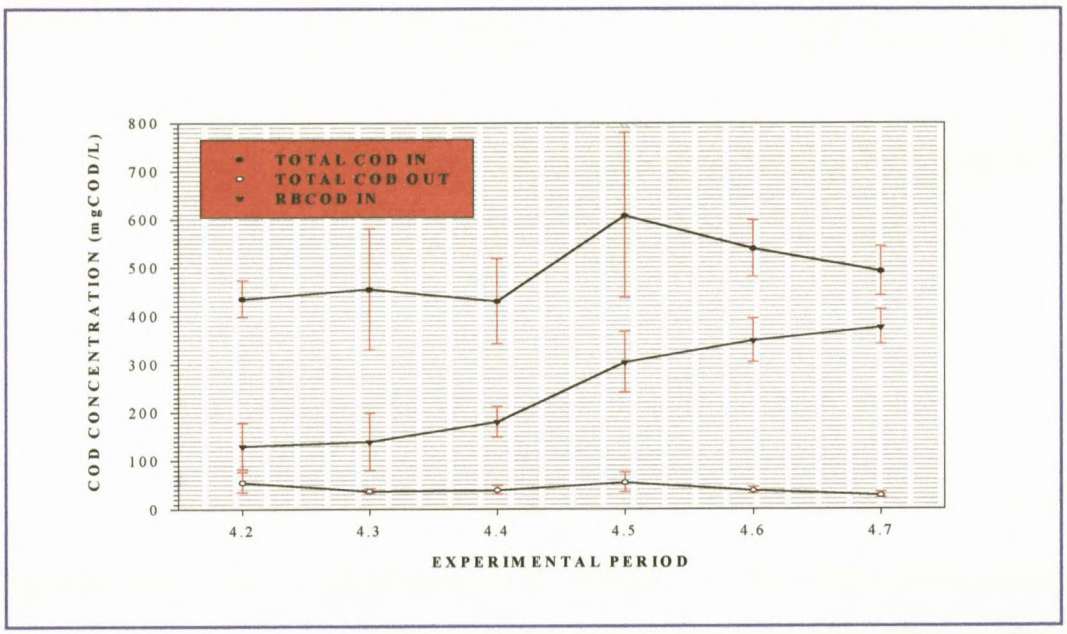

FIGURE 4.8 Influent total COD (S_{ti}), total effluent COD (S_{te}) and influent readily biodegradable COD (S_{bsi}) during experimental periods 4.2 to 4.7.

TABLE 4.7 also summarises the results of the Food/Microorganism (F/M) ratio as well as TP removal based on S_{ti} and S_{bsi} (mgP/mgCOD) for total system operation. Due to a reasonably stable influent organic load and constant sludge age, the F/M ratio was controlled within the range of 0.23 to 0.33 gCOD per d/g MLSS (TABLE 4.7). When calculating phosphate removal based on S_{bsi}, it was assumed that all the RBCOD was removed in the system due

to the extended anaerobic HRT and reasonably limiting conditions as far as organic substrate was concerned ie., low F/M ratio. These values give an indication of COD utilisation efficiency with regards to phosphate uptake and removal in the aerobic reactor.

The positive influence of influent RBCOD on soluble phosphate (as P) removal is shown in FIGS 4.9 and 4.10. FIGURE 4.9 clearly shows a concomitant increase in SRP removal (to a maximum of *ca.* 35 mgP/L during period 4.7) as influent RBCOD concentrations increased. This axiom is reiterated by FIG. 4.10 where SRP removal is plotted as a function of RBCOD. When soluble phosphorus removal was plotted as a function of the influent RBCOD concentration (mgCOD/L), a correlation was shown to exist in that an increase in phosphate uptake rate was recorded as RBCOD concentrations in the influent increased (FIG. 4.10). It can therefore be assumed that P removal would have increased if the maximum HAc supplementation of 500 mgCOD/L had been achieved. During period 4.6, soluble phosphate proved to be limiting with only 0.3 mgP/L recorded in the effluent (refer to TABLE 4.5). This may explain the slightly reduced phosphate removal recorded for the period (FIGS 4.9 and 4.10).

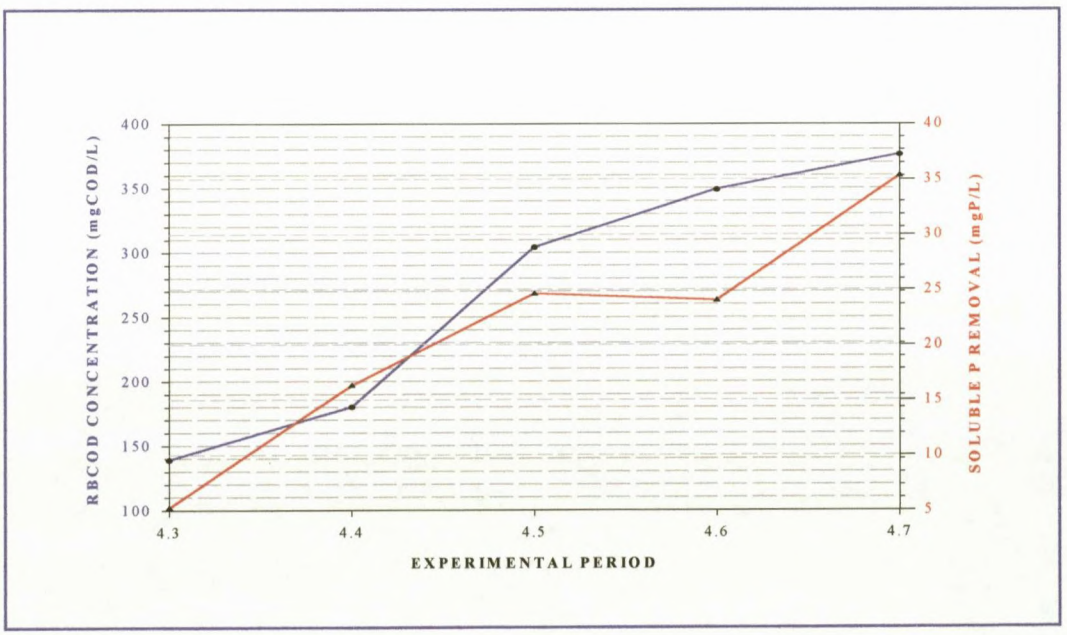

FIGURE 4.9 Influent readily biodegradable COD plotted simultaneously with biological soluble phosphate (as P) removal during experimental periods 4.3 to 4.7.

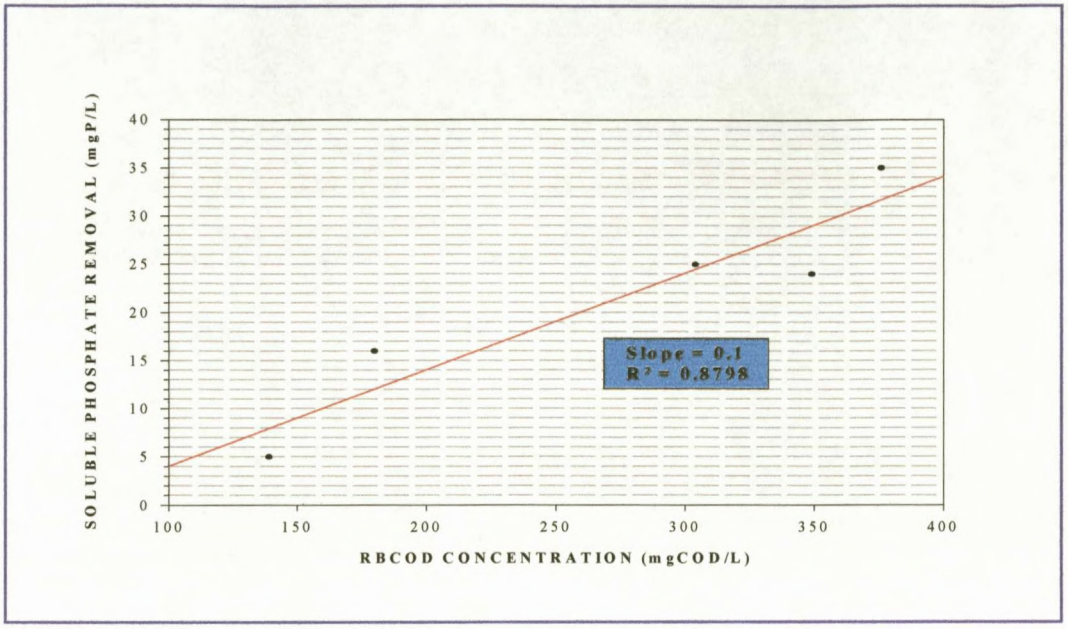

FIGURE 4.10 Soluble phosphate (as P) removal plotted as a function of influent readily biodegradable COD.

4.3.2.3 Phosphorus and nitrogen

Results of mean influent TKN/COD and TP/COD ratios for periods 4.2 to 4.7 are summarised in TABLE 4.8. The TKN/COD ratio was, as far as possible, maintained at 0.06 mgN/mgCOD.

TABLE 4.8 Mean influent TKN/COD (N_{ti}/S_{ti}) and TP/COD (P_{ti}/S_{ti}) ratios for experimental periods 4.2 to 4.7.

Period	N_{ti}/S_{ti} (mgN/mgCOD)	P_{ti}/S_{ti} (mgP/mgCOD)
4.2	0.07	0.04
4.3	0.07	0.03
4.4	0.09	0.06
4.5	0.06	0.05
4.6	0.06	0.05
4.7	0.05	0.10

During the latter stages of experimentation (periods 4.6 and 4.7), influent required supplementation with ammonium chloride to regulate the ratio. The high TKN/COD ratios, recorded during periods 4.2 to 4.4, are mainly due to the reduced COD strength of the influent entering the plant. During period 4.7, the TP/COD ratio increased to a mean value of 0.1 due to influent supplementation of up to 50 mgP/L. Mean TKN/COD and TP/COD ratios, for the total duration of system operation, amounted to 0.07 and 0.06, respectively.

Data regarding the TKN/COD ratio (TABLE 4.8) are represented graphically in FIG. 4.11. The increase in S_{ti} during period 4.5 and concomitant decrease in N_{ti} managed to reduce the ratio considerably from 0.09 during period 4.4 to 0.06 where the ratio was stabilised through constant N supplementation (as NH_4Cl) to the influent. To achieve the desired TKN/COD ratio of 0.06 for uniform S_{ti} of 500 mg/L, N_{ti} should remain constant at 30 mgN/L. A TKN removal rate of *ca.* 90% was generally reported for the duration of pilot plant operation and nitrification/denitrification mechanisms appeared to be operational (FIG. 4.12).

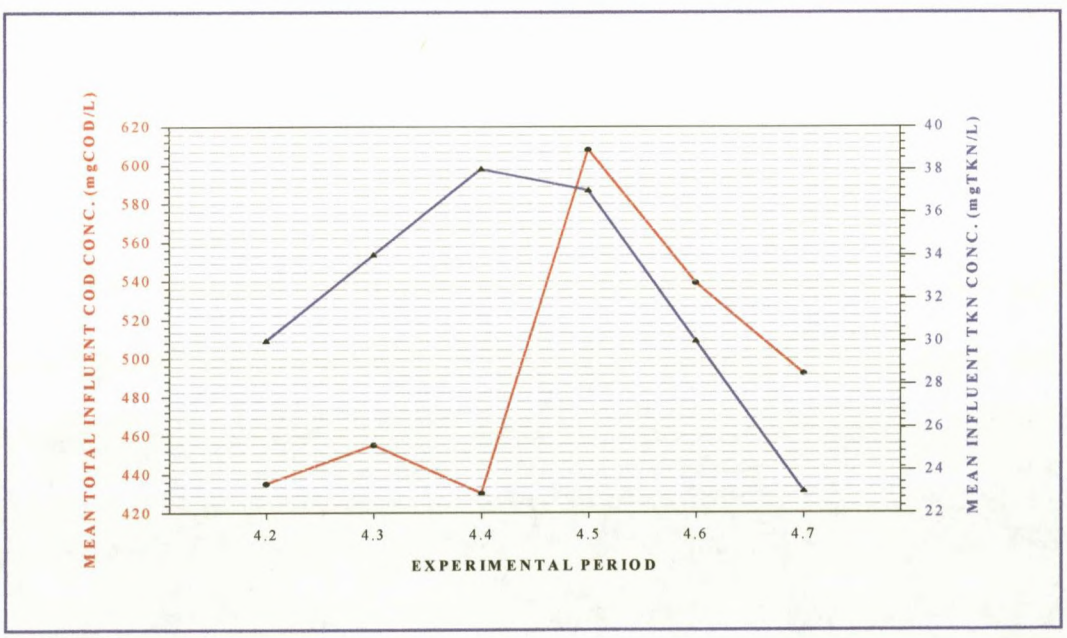

FIGURE 4.11 Mean influent total COD and influent TKN concentration for experimental periods 4.2 to 4.7.

Influent and effluent ammonium (NH_4) and effluent nitrate concentrations for periods 4.2 to 4.7 are shown in FIG. 4.12. Nitrification appeared to proceed at a steady rate with a maximum of *ca.* 2 mg NH_4-N/L (period 4.3) appearing in the effluent. Generally, nitrification processes were responsible for removing between 90 to 99% of influent ammonia. The high concentration of nitrates in the effluent during period 4.4 (3.4 mgNO_3-N/L) is primarily due to the high TKN (FIG. 4.11) and high TKN/COD ratio (TABLE 4.8) recorded for the same period (FIG. 4.12). Effluent nitrates, however, were generally below 2 mgN/L, indicating optimal performance of the anaerobic zone as far as recycled nitrates were concerned. It is evident that as the TKN/COD ratio approached 0.06, denitrification of the system improved with a steady decline of effluent nitrate been recorded (FIG. 4.12).

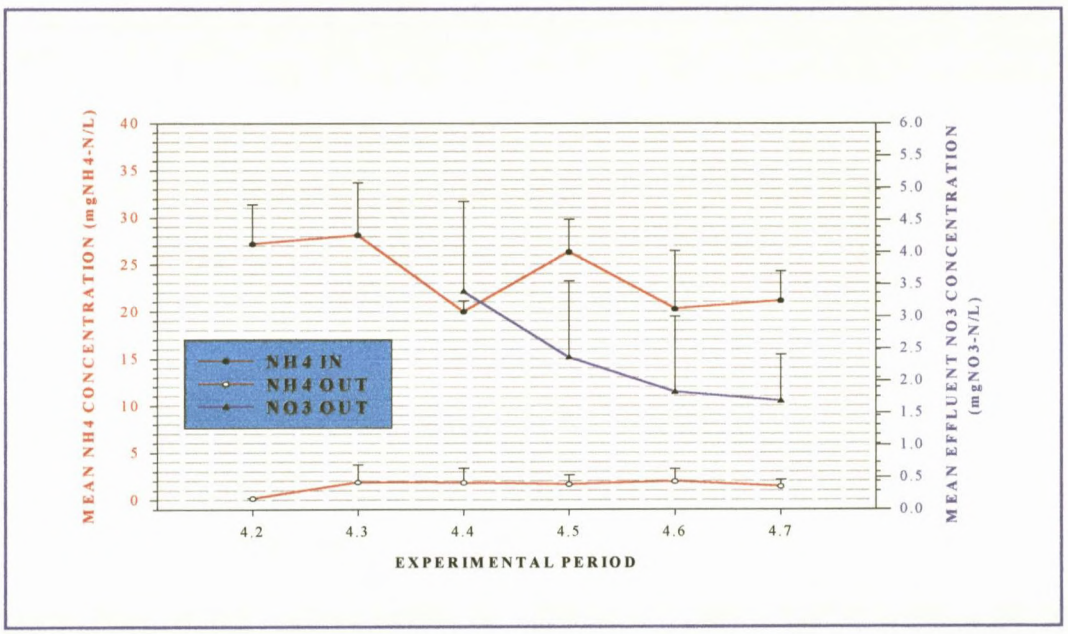

FIGURE 4.12 Mean influent and effluent saline ammonium and effluent nitrate concentration for experimental periods 4.2 to 4.7.

4.3.3 RBCOD batch tests

Due to the significance of the RBCOD fraction of S_{ti} to EBPR processes, it is necessary to ensure reproducibility and accuracy of results in order to obtain a qualitative impression of the system bio-P mechanism (due to the fact that S_{bsi} basically governs the mechanism within the anaerobic reactor). Batch experiments were therefore conducted to determine the stringency required for pH adjustment during RBCOD determination. Mamais *et al.*, (1993) recommend pH adjustment to 10.5 after addition of the zinc sulphate flocculant and prior to filtration. Three pH values were investigated viz., 10.0, 10.5 and 11.0, on two different influent and effluent samples to observe the effects on COD_{sol}, S_i and S_{bsi} (TABLE 4.9). No significant difference in total RBCOD between the various adjusted pH values of the two samples could be observed even though independent COD_{sol} and S_i values varied considerably.

TABLE 4.9 RBCOD determination at different pH values to evaluate effect of pH fluctuations on reproducibility of results.

Sample	pH	COD_{sol} (influent) (mgCOD/L)	S_i (effluent) (mgCOD/L)	RBCOD (mgCOD/L)
1	10.0	431	28	403
	10.5*	431	26	405
	11.0	435	28	407
2	10.0	446	37	409
	10.5*	445	41	404
	11.0	448	46	402

* pH recommended for flocculation by Mamais *et al.*, (1993).

4.3.4 Sludge settleability, system solids and mass fractions

Although system MLSS concentrations increased dynamically (until steady-state was achieved during period 4.5), the active portion or MLVSS of TSS showed a distinct decline from an initial value of 77% to 53% during period 4.7 (TABLE 4.10). The increase in MLSS during period 4.5 corresponds directly to the increased COD load during

the same period (TABLES 4.6 and 4.10). Data regarding SV_{30} and SVI are also given in TABLE 4.10. The high SVI value recorded during period 4.4 may be as a consequence of operational malfunction where aeration and agitation to the unit ceased for approximately 2 d, allowing for the proliferation of filamentous organisms (low DO filaments). No visual sludge bulking in the clarifier was observed. However, a problem which did arise during period 4.5 was the emergence of a pin-floc sludge. Although this did not adversely impact the SVI results, effluents tended to be turbid, presumably due to the high incidence of free bacteria (resulting in higher S_{te} values).

TABLE 4.10 Mean MLSS, MLVSS/MLSS ratios and SV_{30} and SVI values for experimental periods 4.1 to 4.7.

Results expressed as mean values with sample standard deviations (SD) in parentheses.

Period	MLSS (g/L)	MLVSS/MLSS * (mgVSS/mgTSS)	SV_{30} (mL/L)	SVI (mL/g)
4.1	ND	ND	ND	ND
4.2	1.532 (0.38)	0.77	141.8 (15.80)	92.6 (16.98)
4.3	1.587 (0.25)	0.71	167.0 (27.00)	105.2 (12.45)
4.4	1.689 (0.28)	0.69	213.3 (43.87)	126.3 (35.92)
4.5	2.420 (0.50)	0.61	206.9 (17.10)	85.5 (14.25)
4.6	2.429 (0.23)	0.61	208.3 (11.69)	85.8 (6.96)
4.7	2.421 (0.22)	0.53	201.1 (26.12)	83.1 (11.42)

ND = not determined; * calculated in aerobic zone

During experimental periods 4.4 to 4.7, mixed liquor mass fractions (APPENDIX 11) were routinely monitored to ensure the anaerobic fraction did not fluctuate significantly with subsequent negative effects (TABLE 4.11). Anaerobic mass fractions of 10% or lower can result in acetate leakage from the reactor ie., bacterial mass in the reactor is too small to accumulate all the acetate efficiently, which apparently stimulates the excessive growth of

Pseudomonas spp. (Wentzel *et al.*, 1988). During the experimental periods mentioned above, anaerobic mass fractions ranged between *ca.* 20 - 24%. The aerobic zone, due to its higher working volume (20 L) contained approximately 64 - 68% total suspended solids. It is evident from the results that the system had achieved steady-state during periods 4.4 to 4.7 with regards to mass fractions in the respective reactors (TABLE 4.11). Any changes in process efficiency could therefore be attributed to abiotic parameters such as influent feed composition and DO concentration.

TABLE 4.11 Mixed liquor mass fractions of individual reactors during experimental periods 4.4 to 4.7.

Period	AN	AX	AE*
4.4	0.24	0.12	0.64
4.5	0.23	0.13	0.64
4.6	0.20	0.12	0.68
4.7	0.23	0.12	0.65

* includes combined AE1 and AE2 reactor zones

4.3.5 System pH, temperature and oxygen utilization rate

The pH of the reactor zones was monitored daily to ensure severe fluctuations in mixed liquor proton concentrations did not occur. Although alkalinity was continuously fed to the reactor (refer to TABLE 4.2) it has been reported that inducing the bio-P removal mechanism can cause the pH of the bulk liquid to increase drastically, potentially resulting in total collapse of the system (Wentzel *et al.*, 1988). Analysis of TABLE 4.12 shows a gradual increase in the pH value of AE1 and AE2 as the bio-P mechanism became stronger. The higher pH of AE2 caused the pH of AX to gradually increase due to the A-mixed liquor recycle flow. Acid dosing with dilute HCl to AE1 commenced during the latter stages of period 4.5 (refer to TABLE 4.4) and a decrease in the pH of AX, AE1 and AE2 was observed during periods 4.6 and 4.7. The anaerobic zone remained within a relatively constant pH range for the duration of experimentation ie., *ca.* 7.0 - 7.2. Of significance in TABLE 4.12 was that the pH of the aerobic reactors was maintained below pH 8.0 at all times in order to discourage chemical precipitation of phosphate and to exclusively observe the biological EBPR mechanism.

TABLE 4.12 Reactor pH values during experimental periods 4.1 to 4.7.

Results expressed as mean values with sample standard deviations (SD) in parentheses.

Period	pH - AN	pH - AX	pH - AE1	pH - AE2
4.1	ND	ND	ND	ND
4.2	6.99 (0.14)	7.09 (0.12)	7.16 (0.13)	7.29 (0.18)
4.3	7.01 (0.09)	7.15 (0.09)	7.30 (0.03)	7.43 (0.06)
4.4	7.18 (0.15)	7.42 (0.10)	7.49 (0.04)	7.68 (0.03)
4.5	7.11 (0.12)	7.30 (0.09)	7.69 (0.08)	7.87 (0.15)
4.6	7.16 (0.06)	7.26 (0.07)	7.48 (0.26)	7.75 (0.28)
4.7	7.14 (0.07)	7.23 (0.13)	7.22 (0.15)	7.63 (0.28)

ND = not determined

A progressive increase in oxygen utilization rate (OUR) was noted as S_{ti} and TSS in the system increased (FIG. 4.13). A surprising feature though was that OUR continued to increase although the MLVSS (indicative of active biomass) decreased. As expected, the OUR curve achieved a maximum value of 23.5 mgO/L.h during period 4.5 when the maximum COD load entered the plant. It was encouraging to note that OUR values remained consistently elevated during periods 4.6 and 4.7 when the settled sewage fraction was diluted and the system was sustained on acetate alone, indicating that oxygen was required for aerobic metabolism of intracellular organic storage granules ie., PHB. This assumption can be made due to the lack of a slowly biodegradable substrate source in the bulk liquid as well as assuming that all the acetate substrate was utilised in the unaerated zones prior to aeration. Due to the monitoring and measurement of OUR in AE2 alone, it seems likely that no extracellular organic substrate was available to the microbial biomass in the AE2 bulk liquid. A certain fraction of the oxygen requirement would also have been due to nitrification functions and endogenous respiration mechanisms. The oxygen demand for nitrification processes has been calculated as 4.57 mgO/mgNH_4-N (Ekama *et al.*, 1992). Ekama *et al.*, (1992) also state that in order for nitrification to proceed efficiently, the DO concentration in the aerobic reactor should

essentially be maintained above 1.0 mgO/L. This was successfully achieved during this study due to automation of the aeration which was switched off and on at DO concentrations of 5 and 2 mgO/L, respectively. It must be emphasised that for this study, OUR was routinely monitored to observe system response to various parameters ie., acetate and COD supplementation, only. Carbon (COD) mass balances (or N or P mass balances) were not conducted which may have limited many of the assumptions based on the fate of these nutrients.

In addition, FIG. 4.13 shows the temperature of the mixed liquor for the entire duration of system operation. Temperature fluctuations were minimised using an airconditioning unit and heater. Mean temperature for each experimental period was successfully maintained at 20°C ensuring successful growth of nitrifiers within the system and to promote steady-state regarding the kinetics and stoichiometry of the anaerobic and aerobic phases of the biological phosphate removal. Maintenance of a steady temperature assisted interpretation of results in that other temperature dependent metabolic processes such as PHB and extracellular COD consumption, OUR and bacterial cell growth could be observed without attributing conversion rates to this parameter.

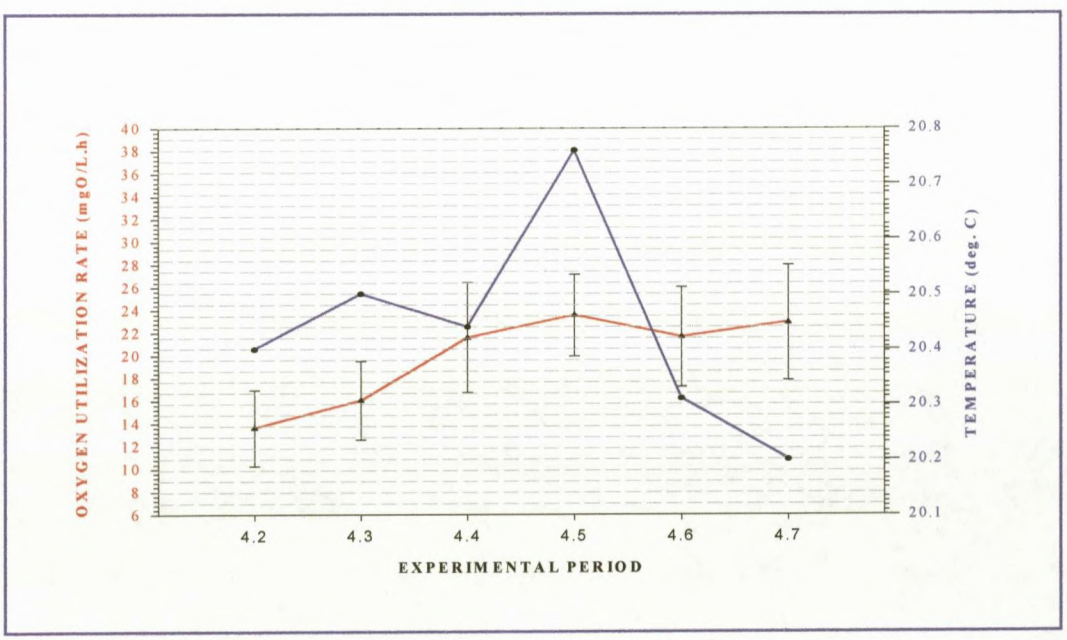

FIGURE 4.13 Oxygen utilization rate and temperature of mixed liquor reported for experimental periods 4.2 to 4.7.

4.4 DISCUSSION

It is evident from FIGS 4.3A to 4.3E that the biological phosphate removal mechanism was functional for the entire duration of pilot plant operation. The characteristic curve of increasing soluble phosphate concentrations in the anaerobic reactor and decreasing phosphate concentrations in the aerobic reactors coincides with existing EBPR biochemical and kinetic models. The observed increase in phosphate is indicative of the hydrolysis of intracellular poly-P to provide the energy required for short chain fatty acid translocation and PHB synthesis (Stephenson, 1987). A striking feature of the enhanced culture was the magnitude of P release, uptake and removal in the respective reactors. During period 4.7 (FIG. 4.3E), anaerobic P release amounted to 93 mgP/L (difference between anaerobic phosphate release and initial influent phosphate concentration) and P uptake amounted to 130 mgP/L (anoxic and aerobic reactors) resulting in a nett P removal of *ca.* 37 mgP/L. Wentzel *et al.*, (1988) and Ekama *et al.*, (1992), however, reported a P release of 250 mgP/L (anaerobic and anoxic) and a P uptake of 310 mgP/L (aerobic reactor), resulting in a nett removal of 60 mgP/L for 500 mgCOD/L acetate feed (theoretically the same as period 4.7). The microbial community of the two above-mentioned studies proved to be very specific as far as dominance was concerned as 90% of the enhanced culture, using the API system, was identified as *Acinetobacter*. Differences in phosphate removal capacities may suggest that a different microbial community was developed during the present study (identification results for the enhanced culture are presented in CHAPTER 5).

Of further interest during enhanced culture development was the degree to which phosphate was taken up under anoxic conditions, indicating the presence of PAO's in the mixed liquor capable of utilising nitrate as oxidant. Although not always well accepted, anoxic phosphate uptake has been reported on numerous occasions (Artan *et al.*, 1997; Šorm *et al.*, 1997). Phosphate taken up in the anoxic zone also indicated that all the HAc supplemented in the feed liquor, as well as products resulting from the fermentation of S_{bsi}, were sequestered in the anaerobic zone. According to Gerber *et al.*, (1987b) PAO's release P under any redox condition ie., anaerobic, anoxic or aerobic, provided acetate or propionate are present in the bulk liquid. However, anoxic phosphate uptake will occur if no external substrate is available, the bacteria responsible utilising intracellular storage granules synthesised in the preceding anaerobic zone (Meinhold *et al.*, 1998). Conversely, in the presence of external substrate, the fate of P

(uptake or release) depends on substrate type (Artan *et al.*, 1997). In a more recent study conducted by Artan *et al.*, (1997) which confirms the findings of Gerber *et al.*, (1987b), it was found that phosphate was consistently released in the presence of nitrate (and oxygen; see Wentzel *et al.*, 1989a) if acetate was present.

Phosphorus accumulating bacteria can be divided into two groups: one group is capable of utilising only oxygen as an external terminal electron acceptor whilst the other group is capable of utilising both oxygen and nitrate (Kerrn-Jespersen and Henze, 1993). Lower energy production from the metabolism of intracellular PHB stores and resulting lower yields (with reference to biomass production) under anoxic conditions results in lower phosphate uptake rates under these conditions (Wentzel *et al.*, 1989a; Artan *et al.*, 1997). During the present study, however, anoxic phosphate uptake proved to be as efficient as aerobic uptake (TABLE 4.13). If PHB storage granules were utilised as a source of substrate under anoxic conditions (as found by Meinhold *et al.*, 1998), aerobic phosphate uptake would have been compromised due to decreased intracellular PHB concentrations. The appearance of reduced aerobic phosphate uptake during period 4.4 (14.3 mgP/L) was due to excessive anoxic phosphate uptake (45.1 mgP/L) (TABLE 4.13). Effluent soluble phosphate concentrations during this period were generally below 1 mgP/L, illustrating the lack of residual phosphate in the aerobic reactors for EBPR.

TABLE 4.13 Anoxic and aerobic phosphate uptake during experimental periods 4.3 to 4.7.

(Reference can be made to FIGS 4.3A to 4.3E)

Period	Anoxic P uptake (mgP/L)	Aerobic P uptake* (mgP/L)
4.3	25.4	21.5
4.4	45.1	14.3
4.5	39.8	43.6
4.6	39.3	48.7
4.7	65.6	63.0

* includes both AE1 and AE2

Prolific phosphate uptake in the anoxic zone (TABLE 4.13) suggests that a significant fraction of the PAO population consisted of denitrifying bacteria which were able to utilise nitrate to generate sufficient energy for poly-

P accumulation (Kerrn-Jespersen and Henze, 1993). It was therefore anticipated that the majority of denitrification occurring in the system was as a result of phosphate uptake activity. The fact that phosphate uptake in the anoxic zone was, to a certain extent, limited (in that residual soluble phosphate was recorded in the aerobic reactors), however, signifies that many of the PAO's were unable to denitrify and could only take up the balance of the residual phosphate once they entered the aerobic zone. Therefore, competition for soluble phosphate exists amongst the PAO community within activated sludge, with the bulk of P made available for the denitrifiers (if actual MCRT in the anoxic zone allows for it). Many of the PAO's will then become redundant (with reference to poly-P accumulation) once they enter the aerobic zone due to limited availability of substrate (both intra- and extracellular). Monitoring of nitrites in the anoxic mixed liquor was not conducted and as such, the extent of denitrification ie., nitrates reduced partially to nitrites or nitrates reduced completely to molecular nitrogen, was not recorded which may have proved limiting as far as controlling the recycle of oxidised forms of nitrogen back to the anaerobic reactor was concerned. Lötter *et al.*, (1986a) have shown that a number of *Acinetobacter* strains are only capable of incomplete denitrification whilst the majority are incapable of denitrification at all. This suggests that PAO's other than *Acinetobacter* were dominant in the EBPR system during the present investigation.

The length of transition from a purely aerobic sludge to one exhibiting EBPR could be attributable to a number of factors. One could assume that PAO's are either not present (unlikely) or are metabolically restrained (low specific growth rates or μ) in conventional aerobic operations and are therefore present in very low numbers with high dilution rates from the system. The PAO's are relatively slow growing compared to the other heterotrophs which places them at a distinct disadvantage when competing for COD in the aerobic zone (Ekama *et al.*, 1984; Mino *et al.*, 1998). This may explain why PAO's have rarely been reported in any substantial numbers in purely aerobic or anoxic-aerobic systems. Another possibility is that the lag period required for the PAO's to switch from aerobic organic metabolism to anaerobic assimilation may be rate limiting and a lengthy process. FIGURE 4.5 indicates improved phosphate removal during period 4.4 at 200 mgCOD/L acetate supplementation. This suggests that the PAO's may require a specific concentration of RBCOD entering the anaerobic zone before they are able to establish themselves and dominate the microbial community. Although Ekama *et al.*, (1984) stipulate that an anaerobic f_{bs} of 25 mgCOD/L is required before P release is observed, it is possible that a small quantity of nitrates may be

returned to the anaerobic zone if using the 3-stage Phoredox process and if complete denitrification does not occur. A certain fraction of the S_{bsi} will then be used for denitrification in the anaerobic reactor until the nitrates have been reduced ie., reaction stoichiometry appears as such - 8 mgCOD is required to reduce 1 mg NO_3-N (Ekama, 1999). Only once this has occurred will the S_{bsi} become available for the sole use of the PAO's. It seems essential, however, that the PAO's must constitute a certain fraction of the system MLVSS before their presence and activity becomes apparent. This finding supports investigations of others where it was found that initial biomass concentration and bacterial phase of growth play significant roles in determining the extent to which P will be removed from waste liquors (Momba and Cloete, 1996a,b).

In conventional activated sludge processes, the amount of P incorporated in the sludge is normally of the magnitude of 0.02 mgP/mgVSS (Wentzel, 1992). In EBPR processes, this value can be increased significantly to approximately 0.06-0.15 mgP/mgVSS (Wentzel, 1992). Wentzel *et al.*, (1988), using fully enhanced conditions, were able to obtain P/VSS ratios of 0.38. An increase in sludge P accumulation is indicative of PAO dominance over other 'normal' heterotrophs in a system and the response for the present investigation is shown in FIGS 4.6 and 4.7. The lower P/VSS ratio of *ca.* 0.26 (when compared to Wentzel *et al.*, 1988) is attributable to the lower P removal which was achieved (FIG. 4.7). The value does, however, indicate that a strong EBPR mechanism was operative.

For successful P removal in the Phoredox process, near complete denitrification is essential to prevent infiltration of nitrates into the anaerobic zone. The extent of dentrification is controlled by influent TKN/COD ratios (discussed later) but of further interest is that readily biodegradable COD fractions should exceed 0.20 (Ekama *et al.*, 1984). This criterion was maintained throughout experimentation with f_{ts} values ranging from 0.30 through to 0.76 for experimental periods 4.2 and 4.7, respectively. The minimum RBCOD concentration in the anaerobic reactor required to stimulate P release is approximately 25 mgCOD/L (Ekama *et al.*, 1984). The degree of P release in the anaerobic reactor will subsequently increase as the RBCOD fraction increases. This, in turn, will allow for stronger EBPR in the aerobic reactor to occur as P removal is proportional to P release. Randall *et al.*, (1992) reported that approximately 50 mgCOD/L is required to remove 1 mgP/L from municipal wastewater. They did not,

however, specify relative RBCOD or SBCOD (slowly biodegradable COD) fractions. If this was the case, the enhanced culture receiving 500 mgCOD/L as HAc during period 4.7 would theoretically only be capable of removing 10 mgP/L. Analysis of FIGS 4.3E to 4.5 and TABLE 4.5 shows that a maximum of *ca.* 37 mgTP-P/L and *ca.* 35 mgPO_4-P/L were removed from the system during period 4.7. A more relevant assumption was that of Daigger and Bowen (1994) who concluded that 7 g of VFA are required per gram of P to be removed. This implies that a fully optimised system receiving 500 mgHAc/L would be capable of removing approximately 71 mgP/L. Gerber *et al.*, (1987a) also found that the release of phosphate from sludge exhibiting EBPR is primarily dependent on the nature and concentration of the substrate interacting with the biomass and not the creation of anaerobic conditions. When developing an enhanced culture of PAO's, care must be exercised if supplementing the feed liquor with compounds such as acetate or propionate as these VFA's are capable of inducing P release from phosphate-laden sludge under anaerobic, anoxic and aerobic conditions (Gerber *et al.*, 1987a). Incremental addition of acetate, as was performed during the present research, should ensure that the anaerobic zone does not become over loaded.

Sewage characteristics have a significant influence on biological nutrient removal. Assuming a sufficient active organism mass constituting the system MLVSS ie., PAO's, nitrifiers and 'normal' heterotrophs, the TKN/COD and TP/COD ratios will dictate the quantity of N and P which can be removed from the process. If the TKN/COD ratio is low, excess COD becomes available to allow for complete denitrification (Pitman, 1982). However, when the ratio is high, denitrification will not be complete and residual nitrates infiltrating the anaerobic zone will have a marked effect on the anaerobic conditioning of the sludge. At fixed sludge ages viz., 10 d for this research, the N removal potential of a process is fixed. This implies that the effluent quality will vary, depending on the magnitude of the influent TKN/COD ratio (Ekama and Marais, 1984c). Influent TKN/COD ratios will also dictate the process type to be employed. Wentzel *et al.*, (1988) maintained a TKN/COD ratio of 0.06 during enhanced culture development using both 3-stage Bardenpho and UCT processes. Likewise, during the present research, TKN/COD ratios were maintained below 0.08 mgN/mgCOD (except for period 4.4) which, according to Ekama *et al.*, (1984), is suitable for complete nitrate removal when employing the Phoredox (3-stage Bardenpho) process (TABLE 4.8).

As stated earlier, 1 mg P removed requires approximately 50 mg COD in the bulk liquid (Randall *et al.*, 1992). This correlates to a TP/COD ratio of 0.02 mgP/mgCOD (assuming TP = SRP). It is therefore likely that the system under present investigation was operating under COD limiting conditions and that the full P removal capacity was not realised. The high TP/COD ratio of 0.10 mgP/mgCOD during period 4.7 (TABLE 4.8) may have limited P removal considerably which would account for the lack of substantial improvement during the same period (see FIG. 4.5).

Effluent TKN values centred around a mean value of *ca.* 3.5 mgN/L for the duration of experimentation (except for period 4.7 where TKN effluent values fell below the lower detection limit of 3 mgN/L). The effluent TKN consists of free and saline ammonia, biodegradable soluble organic N, unbiodegradable soluble organic N and TKN in the effluent volatile solids (Ekama and Marais, 1984c). During the present study, saline ammonia constituted approximately 4% during period 4.2 to *ca.* 100% during period 4.7 (see FIGS 4.11 and 4.12). This was due to the dilution of the settled sewage fraction (with subsequent dilution of sewage TKN) and supplementation of the influent with ammonium chloride in order to maintain a relatively constant influent TKN concentration.

The settling properties of activated sludge in the system were measured by means of SVI (TABLE 4.10). Except for periods 4.3 and 4.4, SVI values indicated that the sludge was settling well. At no stage during culture development did it appear as though filamentous organisms were becoming dominant and settling in the clarifier proceeded well. Activated sludge flocs also appeared to be large and compact. The maximum recorded SVI value of 126 mL/g during period 4.4 may have indicated the onset of dispersed growth which became evident during period 4.5, resulting in comparatively high S_{te} values. The formation of a pin-point floc may have been due to the gradual increase in COD loads to the system which suppresses the production of glycocalyx [1] (Wanner, 1997). Excessive organic loading seems to be responsible for the high SVI values during periods 4.3 and 4.4 as a simultaneous decrease in SVI to 85.5 mL/g was recorded during period 4.5 as the system MLSS increased to 2.420 g/L (TABLE 4.10). The gradual decrease in activated sludge MLVSS from 0.77 (normal range for activated sludge) during period 4.2 to 0.53 mgMLVSS/mgMLSS during period 4.7 (TABLE 4.10) is indicative of an enhanced culture

[1] Glycocalyx is an extracellular biopolymer which creates the matrix for firm activated sludge flocs.

of PAO's. Wentzel *et al.*, (1988) were able to obtain VSS/TSS values of 0.46 mgVSS/mgTSS. Decreasing MLVSS concentrations as the EBPR mechanism became stronger indicates that the P content of the organic material was increasing.

When conducting EBPR pilot-scale studies, Wentzel *et al.*, (1988) suggest two operational procedures which prevent overloading of the PAO's in the anaerobic reactor with acetate (resulting in acetate leakage). These include limiting the acetate load increments and enlarging the anaerobic reactor. They also suggest an anaerobic mass fraction of 30% and an anoxic mass fraction of 8%. During the present study, anaerobic mass fractions fluctuated between 20 - 24% whilst the anoxic fraction remained reasonably stable at 12% (TABLE 4.11). Acetate increments were increased every two to four sludge ages ie., every 20 to 40 d, to ensure that sufficient time was permitted for maximum acetate sequestration in the anaerobic zone and to allow for significant growth (yield) of PAO's in the system (TABLE 4.2). As discussed earlier, it appeared that acetate was successfully sequestered in the anaerobic zone due to anoxic phosphate uptake. However, a limitation to the study was that acetate in the effluent from anaerobic zone was not monitored. It is hypothesised that if acetate was present in the bulk liquid entering the anoxic zone, concentrations would have been minimal due to the observed nett phosphate uptake.

Increasing pH values in both aerobic reactors, until acid dosage commenced during experimental period 4.6, becomes evident in TABLE 4.12. At one stage during period 4.5, the mixed liquor pH in AE1 increased to *ca.*9 pH units, which would have obviously detrimentally affected the performance of the system. The biochemical model of Wentzel *et al.*, (1986) proposes that phosphate uptake in the aerobic zone occurs via the hydroxyl mediated antiport and cation uptake (Mg^{2+}, K^{+} and Ca^{2+}), required for stabilisation of the poly-P chain, occurs via the proton mediated antiport. Although charge neutrality is maintained both extra- and intracellularly, extracellular alkalinity and acidity decrease by one and two moles respectively for every mole phosphate taken up. Implications of the loss of both alkalinity and acidity are that mixed liquor pH from the anaerobic zone will increase in the aerobic zone if the anaerobic pH $\geq$6.8 (Wentzel *et al.*, 1986). Liu *et al.*, (1996), investigating the effect of pH on anaerobic substrate metabolism, suggest that an anaerobic pH value of 6.8 $\pm$ 0.7 results in the most efficient metabolism of acetate and PHB synthesis by PAO's which therefore results in higher phosphate uptake rates. The pH of anaerobic

mixed liquor during the present study remained fairly neutral, in the region of 7.0 - 7.2 pH units (TABLE 4.12). Maintaining the mixed liquor pH below 7.8 was also crucial in order to attribute phosphate removal to the biological mechanism of EBPR. Meinhold *et al.*, (1998), through a number of batch tests, found that phosphate precipitation is minimal if pH does not rise much above 7. Romanski *et al.*, (1997) state that phosphate precipitation with Ca^{2+} will only occur at pH > 8.0 and that if the system pH is maintained below this value, only biological phenomena will be observed. Although one can argue that precipitation of phosphate due to pH changes in the mixed liquor brought about by PAO metabolism can be regarded as biological, the objective of this work was to define the system according to actual physical microbial phosphate uptake and not biologically mediated precipitation.

Although temperature fluctuations in the container housing the pilot plant did occur, mean ambient temperature was successfully maintained at 20°C (FIG. 4.13). Higher operating temperatures would have placed more stress on the system due to higher oxygen requirements for oxidation of the influent organic fraction and excessive biomass production which may have resulted in operational problems. The reference temperature of 20°C is used for many of the existing nitrification-denitrification models describing the kinetics of the process (Ekama *et al.*, 1992; Ekama and Wentzel, 1997) and is used as the standard temperature when estimating maximum specific growth rates of the nitrifiers in a system ie., μ_{nm} or μ_{nm20}. The growth rate of the nitrifiers is very temperature sensitive and halves every 6°C decrease in temperature (Ekama *et al.*, 1992). It was therefore decided that selection of a suitable temperature of 20°C and R_s of 10 d would improve nitrification efficiency.

4.5 CONCLUSIONS

During the course of this study, it became apparent that a laboratory-scale activated sludge process, displaying a strong EBPR mechanism, had been successfully developed using acetate as the sole supplemented RBCOD source. Care must be exercised when supplementing laboratory-scale systems with either acetate or propionate, however, as both compounds induce P release under any redox condition ie., anaerobic, anoxic, aerobic. Incrementally increasing this fraction of S_{ti} will ensure the anaerobic zone does not exceed the VFA accumulation capacity and no VFA will 'leak' to the following reactors.

Increasing the RBCOD fraction and thereby increasing the VFA concentration in the anaerobic zone of an activated sludge EBPR process will increase the P removal efficiency significantly. This was demonstrated by a concomitant increase in P removal as acetate to the pilot plant increased. It also appears that the PAO's require a certain S_{bsi} concentration in the bulk liquid of the anaerobic zone viz., *ca.* 200 mgCOD/L, before they are able to release significant quantities of phosphate as well as displaying enhanced phosphate uptake. This may be due to certain metabolic requirements for PHB synthesis or the utilization of sequestered VFA for cellular maintenance functions prior to PHB synthesis. Recycling oxidised forms of nitrogen to the anaerobic zone (via the s-recycle) will further limit efficient RBCOD and VFA utilization by the PAO's and encourage growth of denitrifiers.

The physical-chemical method proposed by Mamais *et al.*, (1993) for S_{bsi} determination does not appear to be pH sensitive. The authors recommend pH adjustment to 10.5 for flocculation but results of this study show that sample pH can be adjusted to within the range of 10.0 to 11.0 without any loss in accuracy.

A primary objective of enhanced culture development was to simulate conditions proposed by Wentzel *et al.*, (1988) to provide samples for microbial community characterisation. It was hoped that if API results confirmed *Acinetobacter* spp. to be the dominant bacterial genus in the present culture, a direct comparison between the two enhanced cultures could be made. The use of FISH would then conclusively demonstrate any bias imposed when employing conventional techniques of isolation and identification. However, it became evident that the two cultures differed significantly in their microbial community structures. One of the more significant differences was that of the anoxic reactor response to EBPR. The culture of Wentzel *et al.*, (1988) demonstrated anoxic phosphate release for the duration of experimentation. The culture in the present study invariably demonstrated anoxic phosphate uptake implying that a population of denitrifying PAO's had been developed. If *Pseudomonas* spp. (a denitrifying PAO) had become dominant in the system, as suspected, it was possible that the anaerobic zone was over-extended and that acetate was leaking to the anoxic zone. Results of identification of the enhanced culture using both conventional microbiological and molecular techniques follow in CHAPTER 5.

CHAPTER FIVE

IDENTIFICATION OF ACTIVATED SLUDGE BACTERIA USING CONVENTIONAL AND MOLECULAR TECHNIQUES

5.1 INTRODUCTION

With the establishment of a molecular laboratory at the CWWR during the latter half of 1998 and beginning of 1999, culture-independent microbial community analyses became possible using fluorescent *in situ* hybridization (FISH) techniques. Two of the biological sludge types mentioned during the course of this study viz., Amanzimtoti WWW (full-scale plant) and the enhanced PAO culture (pilot plant), were subsequently analysed using both conventional (serial dilution and plating on solid media) and molecular (rRNA hybridization with fluorescently labelled oligonucleotide probes) techniques to obtain a more holistic impression of the bacterial community structure from two vastly different processes [1]. The primary objective at this stage was to assess the magnitude of bias imposed by plating using the results of FISH as a reference for the total bacterial community. If predominance could be associated with function, one would be capable of discerning which bacterial species/strains were responsible for the key activities of interest ie., poly-P accumulation in the enhanced culture sludge. Initiating pilot plant studies with a non-nutrient removal sludge (Amanzimtoti WWW) would permit certain assumptions to be made regarding the effect of selection factors known to induce EBPR ie., presence of an anaerobic zone and increased acetate (VFA) concentration in the bulk liquid, on the microbial community structure. One would therefore be able to semi-qualitatively attribute function ie., EBPR, to microbial dominance.

The study of microbial ecology essentially requires the accurate and specific detection and enumeration of populations and their changes over time (Zarda *et al.*, 1991). Over the last decade, molecular methods based on

[1] Amanzimtoti WWW is a single reactor aerobic process with no BNR (perhaps a certain degree of nitrification due to the long sludge age ie., 20-25 d); the enhanced culture was developed using a NDBEPR pilot plant modelled upon the 3-stage Phoredox process.

DNA or rRNA sequence analysis have become accepted as the archetype for conducting microbial ecological studies primarily due to the limitations of culturability been negated, carving the way from phenotypic to genotypic characteristics for evolutionary inference. Beneficiaries of this relatively new approach to ecological studies include those scientists interested in EBPR in activated sludge and, more specifically, interested in those organisms responsible for the process. The *Acinetobacter* enigma began in 1975 when Fuhs and Chen isolated the organism from an EBPR plant and implied that, due to its high recovery rates on solid media, it was playing an important role in the process. Since then, many studies have been conducted on the genus and results were such that they coincided with those of Fuhs and Chen (1975) ie., due to its high recovery rates, *Acinetobacter* was implicated as the main catalyst responsible for EBPR (Buchan, 1981; Hart and Melmed, 1982; Brodisch, 1985; Lötter, 1985; Lötter and Murphy, 1985; Wentzel *et al.*, 1988). It must be emphasised that although the references indicated above confirmed the dominance of *Acinetobacter* on solid media, other studies, using similar techniques, have been conducted which prove the contrary ie., *Acinetobacter* spp. have only constituted a small proportion of total recovered cells (Brodisch and Joyner, 1983). With the advent of *in situ* whole cell hybridization techniques and nucleic acid sequencing, a drastically different picture regarding the microbial community structure in EBPR operations has been painted.

Within the 16S and 23S rRNA molecules of bacteria there are regions of different evolutionary conservation. Using oligonucleotide probes complementary to these unique sequences it is possible to identify individual or groups of microorganisms from environmental samples *in situ* (Braun-Howland *et al.*, 1992). The analysis is based on nucleotide sequence comparison of rRNA's or their genes extracted from naturally occurring biomass or from previously isolated organisms. Characterisation of unknown organisms by rRNA sequences requires a reference collection of sequences from known organisms. Although the reference sequence collection can only be regarded as substantial and is far from comprehensive, it is anticipated that with the refining of techniques the collection will experience a rapid expansion. A major advantage of probe hybridisation is that because molecules rather than organisms are isolated, the method is not only limited to species that are amenable to laboratory cultivation. In addition, the oligonucleotide probes are freely adjustable to incorporate different phylogenetic levels ranging from the subspecies level to the kingdom level of organisation (Manz *et al.*, 1992; Wagner *et al.*, 1993; Wagner *et al.*,

1994b).

The primary objective of this phase of the research was therefore twofold: (1) to assess the microbial community structure of two significantly contrasting activated sludge processes using both conventional microbiological and direct molecular techniques, and to obtain semi-qualitative data regarding population dynamics (population shift over time) when EBPR selection is introduced to a non-EBPR sludge; and (2) to compare community structure results of both cultivation and *in situ* identification methods to assess the magnitude of bias inferred through the plating of mixed liquors.

5.2 MATERIALS AND METHODS

5.2.1 Mixed liquor sampling

Grab samples of mixed liquor were collected from the anaerobic, anoxic and aerobic zones of the enhanced pilot plant culture. In addition, samples were also collected from the thickened sludge recycle flow at Amanzimtoti WWW. Sampling bottles contained glass beads (3 mm) to enhance disruption of the floc structure. For *in situ* hybridizations, 10 mL mixed liquor samples from both activated sludge processes were immediately fixed with 30 mL 4% (w/v) paraformaldehyde solution (1:3, v/v), kept at 4°C for 2 h and stored in a 1:1 mixture of phosphate buffered saline (PBS) and 96% ethanol at -20°C (Amann, 1995) (APPENDIX 12).

5.2.2 Cultivation and plate counts

Serial dilutions (10^{-2} - 10^{-8}), using 9 mL sterile distilled water, were done on homogenised (1 mm diameter glass beads) mixed liquor samples. Igepal CA-630 (synonym: nonidet; Sigma) was added to further disperse sludge flocs and cells. Aliquots of each dilution (0.1 mL) were spread on individual CGY agar plates (APPENDIX 1) and incubated at 20°C for 5 d. Plates were scored ($30 \leq n \leq 300$) and those which contained between 30-100 CFU's were retained for further study. The other plates were subsequently discarded. Well developed, individual colonies were

re-streaked on solid isolation media (CGY) and incubated further at 20°C for 5 d. Passaging (sub-culturing) of isolates continued until monocultures of each isolate were obtained. Isolates were then identified according to the API 20NE identification system.

5.2.3 Staining techniques

Neisser and PHB staining of the activated sludge samples for microscopic observation of intracellular lipophilic and metachromatic granules was done according to Jenkins *et al.*, (1984) (APPENDICES 2 and 3).

5.2.4 Cell fixation and immobilization on glass slides

Bacteria were fixed according to section 5.2.1 above, washed in PBS and finally resuspended to a final cell concentration of *ca.* 10^8-10^9 cells/mL (Amann, 1995; APPENDIX 12). Microscope slides were cleaned and prepared before 3 μL fixed cell suspension was spotted onto slide surface (APPENDIX 13). After allowing the suspension to air dry, the cells were dehydrated with successive passaging through an ethanol series (APPENDIX 13). An alternative fixation protocol was utilised to enhance probe penetration for *in situ* detection of Gram positive bacteria. Activated sludge mixed liquor samples were fixed by addition of absolute ethanol to a final concentration of 50% (v/v). Prior to hybridization, ethanol fixed cells were treated with ethanol:formaldehyde (90:10, v/v) for 1 min, rinsed briefly in distilled water and air dried (Braun-Howland *et al.*, 1992).

5.2.5 Membrane filtration and DAPI staining for total cell counts

Single strength PBS (895 μL) was added to 5 μL activated sludge in an Eppendorf test tube. Glass beads (1 mm diameter) were added to the tube to occupy approximately one-third of the volume and the contents bead-beaten for 10 min at 2 400 rpm in a Mini-Beadbeater™ (Biospec Products). Igepal CA-630 was added (100 μL) and the contents beaten for a further 2 min. Cellulose acetate filters (pore size, 0.22 μm; Micron Separations Inc.) were counterstained with Sudan Black for 12 h. Activated sludge was stained with 4', 6-diamidino-2-phenylindole

(DAPI; Sigma) by adding 1 mL DAPI (0.50 μg/mL) to 1 mL dispersed activated sludge mixture (containing PBS and Igepal CA-630) in a filtering tunnel. The stained cellulose acetate filter, as well as a 0.45 μm backing filter were placed at the base of the filter tunnel and the contents allowed to stain for 10 min in the dark (cover tunnel with aluminium foil) before vacuum filtration. After filtration, excess stain was removed by washing with double distilled water in the filtering device. The stained cellulose filter was mounted on one drop of a glycerol/PBS mixture (95:5, v/v) on glass slides pre-treated with poly-L-lysine (Sigma). One drop of the antifadent VECTASHIELD® mounting medium (Vector Laboratories, California) was added to the mounted filter surface before placing and sealing a coverslip. DAPI fluorescence was detected with a Zeiss Axiolab microscope (Zeiss, Germany) fitted for epifluorescence microscopy with a 50 W mercury high-pressure bulb and Zeiss filter set 01 and total cell counts were determined (APPENDIX 14).

5.2.6 *In situ* hybridization with oligonucleotide probes

Hybridizations of fixed activated sludge samples (immobilised on glass slides) were done as described by Amann (1995) (APPENDIX 15). Probes used and hybridization stringency required after addition of formamide to the hybridization buffer are given in TABLE 5.1. Due to a single mismatch discrimination in the probe sequences and to prevent non-specific binding, probes BET42a and GAM42a were added to the slides simultaneously as competitors in equal molar concentration (TABLE 5.1). In the washing buffer containing no formamide, the sodium chloride solution was reduced accordingly (Manz *et al.*, 1992; APPENDIX 15). After probing, slides were examined with a Zeiss Axiolab epifluorescence microscope (Zeiss, Germany) using Zeiss filter sets 09 (fluorescein) and 14 (rhodamine).

Representative bacterial strains from each of the classes, sub-classes and genera investigated, used to evaluate probe specificity, are given in TABLE 5.2. All cells were grown aerobically at 30°C in nutrient broth and harvested at mid-logarithmic phase (OD = 0.6 to 0.8 at A_{600}) in order to ensure a high cellular rRNA content. Cells were then fixed in either 4% paraformaldehyde, 50% absolute ethanol or ethanol:formaldehyde (90:10, v/v) for 1 min and centrifuged (5 000 *g*, 10 min) at 4°C. Cells were washed in PBS and pelleted. Pellets were then resuspended in

PBS and cold absolute ethanol (1:1, v/v). Fixed bacterial cells were stored at -20°C.

TABLE 5.1 Probe sequences, target sites and formamide concentration in the hybridization buffer required for specific *in situ* hybridization.

Probe	Sequence	Target site	% Formamide	Fluor
EUB338	5'- GCTGCCTCCCGTAGGAGT -3'	16S	20	5' Rhodamine
ACA23a	5'- ATCCTCTCCCATACTCTA -3'	16S	30	5' Rhodamine
ALF1b	5'- CGTTCG(C/T)TCTGAGCCAG -3'	16S	20	5' Rhodamine
BET42a	5'- GCCTTCCCACTTCGTTT -3'	23S	30	5' Rhodamine
GAM42a	5'- GCCTTCCCACATCGTTT -3'	23S	30	5' Fluorescein
CF	5'- TGGTCCGTGTCTCAGTAC -3'	16S	25	5' Fluorescein
HGC	5'- TATAGTTACCACCGCCGT -3'	23S	25	5' Rhodamine

TABLE 5.2 Bacterial reference strains used to assess probe specificity.

Organism	Source	Probe specificity	Synonym
Acetobacter acetii	SABS	*Proteobacteria* - alpha	ALF
Alcaligenes faecalis	TN	*Proteobacteria* - beta	BET
Acinetobacter calcoaceticus	ATCC	*Proteobacteria* - gamma *Acinetobacter* spp.	GAM ACA
Flavobacterium spp.	UCT	*Cytophaga-Flavobacterium*	CF
Corynebacterium glutamicum	SABS	GPBHGC	HGC

SABS - South African Bureau of Standards culture collection
TN - Technikon Natal Microbiology Department
ATCC - American Type Culture Collection
UCT - University of Cape Town Microbiology Department
GPBHGC - Gram Positive Bacteria with High G + C DNA content

5.2.7 Image analysis and probe specific counts

A Zeiss Axiolab fluorescent microscope (Zeiss, Germany) equipped with a 50 W high pressure mercury bulb, Sony

DXC-930P 3CCD colour video camera (Sony, Japan) modified by AVT-HORN (Aalen, Germany) to be cooled to -25°C and KS300 image analysing software (Zeiss, Germany) was used for epifluorescent microscopic observation and image analysis. Threshold intensities were always determined by comparing the microscopic observation and the acquired images. Cell quantification could not be done using simple statistical evaluation due to a non-homogenous distribution of the bacteria (aggregates). Population analyses (for each probe) were therefore carried out by creating computer macros and measuring the area of each signal (ten fields per slide) in the image analysing system.

5.3 RESULTS

The diversity of bacteria existing in the mixed liquor of both the enhanced culture and Amanzimtoti WWW, using both cultivation and FISH techniques, is shown in FIGS 5.1 to 5.4 and TABLES 5.3 to 5.10. A marked feature of FIGS 5.1 to 5.4 and TABLES 5.3 to 5.7 is the extent to which nucleic acid sequencing has modified and compelled change to the classical system of bacterial classification, especially of the genus *Pseudomonas*. Name changes for the various genera were obtained using the Taxonomy Browser from the National Centre for Biotechnology Information (NCBI, 1999) web page (URL: http://www.ncbi.nlm.nih.gov/Taxonomy/). This indicates that previously employed phenotypic and morphotypic characteristics of microorganisms are no longer sufficient for accurate and reliable classification of bacteria due to the ambiguity intrinsic to these techniques. It is now generally accepted that genotypic characteristics must essentially be applied in order to confirm the relatedness existing between both new and existing bacterial genera/species.

5.3.1 Biodiversity of the enhanced culture using cultivation techniques

Results of bacteria isolated from the enhanced PAO culture are shown in FIGS 5.1 to 5.3 and TABLES 5.3 to 5.6. Bacteria isolated aerobically from the anaerobic reactor are given in FIG. 5.1 and TABLE 5.3; the anoxic reactor in FIG. 5.2 and TABLE 5.4; and the aerobic reactor in FIG. 5.3 and TABLE 5.5. The total community profile in each reactor, based on phylogenetic classification, is given in TABLE 5.6.

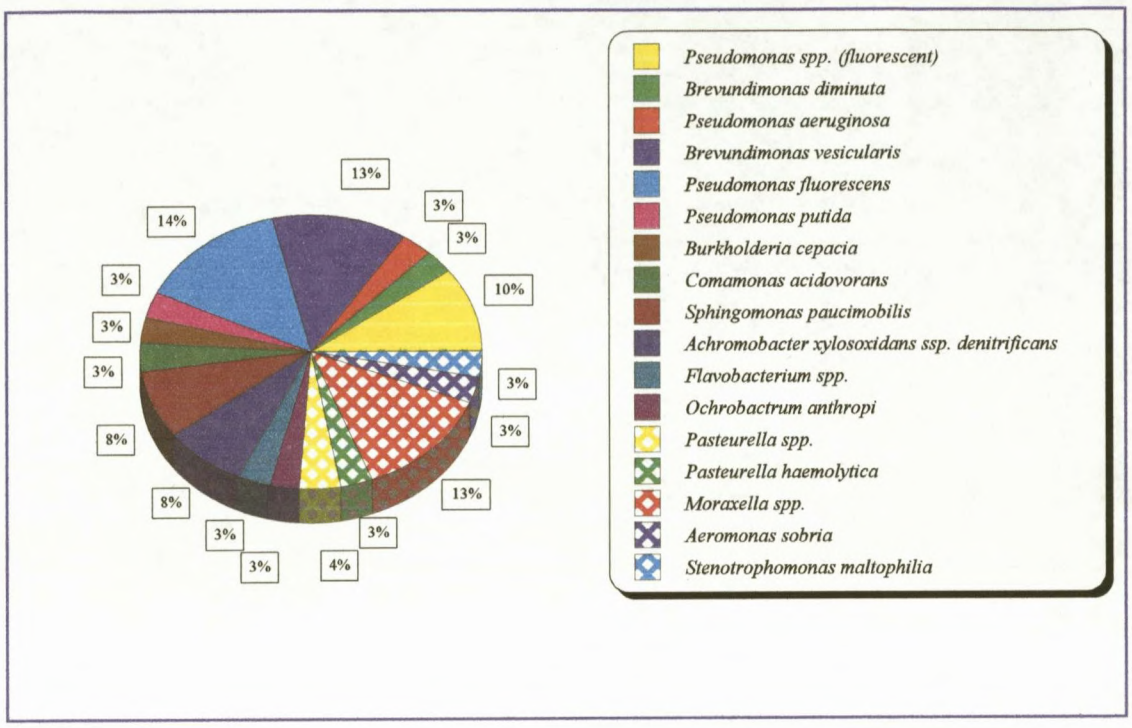

FIGURE 5.1 Bacterial monocultures isolated on CGY agar from the anaerobic zone of the enhanced EBPR culture and identified using the API 20NE system.

FIGURE 5.1 and TABLE 5.3 indicate aerobic bacteria isolated from the anaerobic zone of the enhanced culture after aerobic cultivation. Of particular significance is the apparent dominance of the Pseudomonads in the anaerobic mixed liquor. Dominance of this genus in the mixed liquors of both the anoxic and aerobic reactors was also recorded (FIGS 5.2 and 5.3). *Pseudomonas* spp. numbers in the anaerobic zone amounted to 30% of the total number of isolates recovered, of which *Pseudomonas fluorescens* constituted 14% of the total bacterial community (FIG. 5.1). The complete absence of *Acinetobacter* spp. in the anaerobic reactor was very prominent. No Gram positive bacteria with high G+C (GPBHGC) DNA content in the anaerobic zone were isolated either. *Acinetobacter* spp. became more prominent in the anoxic and aerobic reactors, the sum of which amounted to 16% and 21% of the total isolated bacterial community, respectively (FIGS 5.2 and 5.3). Only one representative of the *Cytophaga-Flavobacterium* phylum, *Flavobacterium* spp., was recorded from the anaerobic reactor (TABLE 5.3). The absolute dominance of the *Proteobacteria*, in particular those of the gamma subclass, in the entire system is evident

(TABLES 5.3 to 5.6). Total plate counts from the various reactors are also shown in TABLES 5.3 to 5.5. Although counts from the anoxic and aerobic zones are very comparable, those from the anaerobic zone are approximately one order of magnitude greater than the two previously mentioned zones (1 x 10^7 CFU/mL).

TABLE 5.3 Phylogenetic classification, synonyms and total plate counts of isolates recovered from the anaerobic zone of the enhanced pilot plant culture.

Organism	Synonym	Phylogenetic classification
Pseudomonas spp. (fluorescent)	NC	*Proteobacteria* (γ)
Brevundimonas diminuta	*Pseudomonas diminuta*	*Proteobacteria* (α)
Pseudomonas aeruginosa	NC	*Proteobacteria* (γ)
Brevundimonas vesicularis	*Pseudomonas vesicularis*	*Proteobacteria* (α)
Pseudomonas fluorescens	NC	*Proteobacteria* (γ)
Pseudomonas putida	NC	*Proteobacteria* (γ)
Burkholderia cepacia	*Pseudomonas cepacia*	*Proteobacteria* (β)
Comamonas acidovorans	*Pseudomonas acidovorans*	*Proteobacteria* (β)
Sphingomonas paucimobilis	*Pseudomonas paucimobilis*	*Proteobacteria* (α)
Achromobacter xylosoxidans ssp. denitrificans	*Alcaligenes denitrificans*	*Proteobacteria* (β)
Flavobacterium spp.	NC	*Cytophaga-Flavobacterium*
Ochrobactrum anthropi	NC	*Proteobacteria* (α)
Pasteurella spp.	NC	*Proteobacteria* (γ)
Pasteurella haemolytica	NC	*Proteobacteria* (γ)
Moraxella spp.	NC	*Proteobacteria* (γ)
Aeromonas sobria	NC	*Proteobacteria* (γ)
Stenotrophomonas maltophilia	*Pseudomonas maltophilia*	*Proteobacteria* (γ)
TOTAL CELL COUNT	**1 x 10^7 CFU/mL**	

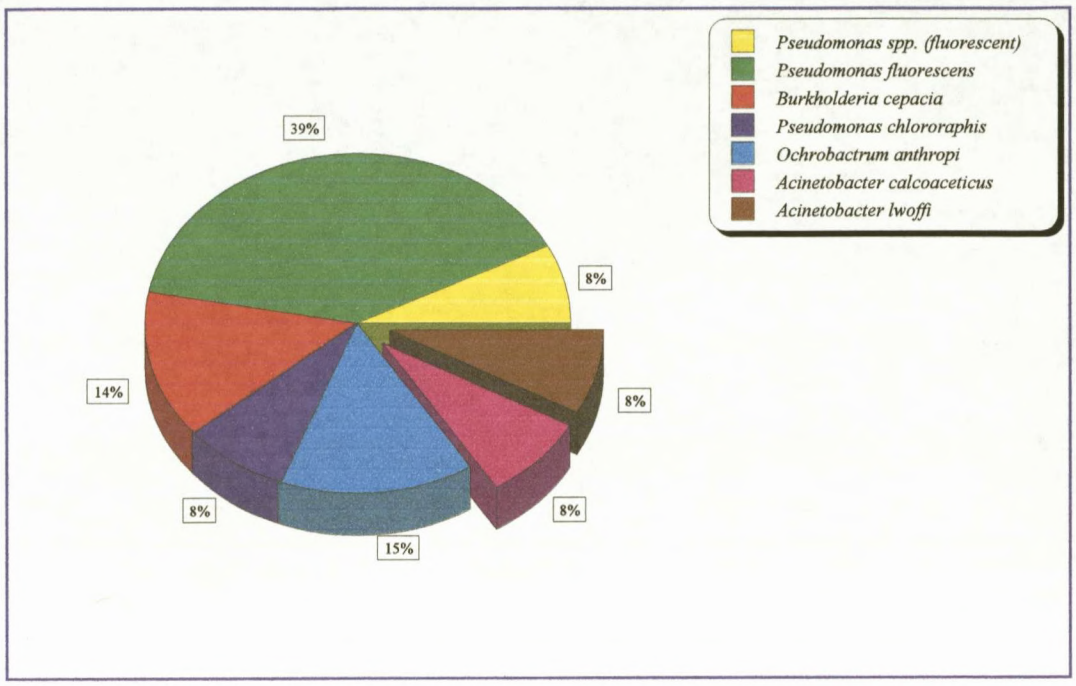

FIGURE 5.2 **Bacterial monocultures isolated on CGY agar from the anoxic zone of the enhanced EBPR culture and identified using the API 20NE system.**

TABLE 5.4 **Phylogenetic classification, synonyms and total plate counts of isolates recovered from the anoxic zone of the enhanced pilot plant culture.**

Organism	Synonym	Phylogenetic classification
Pseudomonas spp. (fluorescent)	NC	*Proteobacteria* (γ)
Pseudomonas fluorescens	NC	*Proteobacteria* (γ)
Burkholderia cepacia	*Pseudomonas cepacia*	*Proteobacteria* (β)
Pseudomonas chlororaphis	*Pseudomonas fluorescens bv. D*	*Proteobacteria* (γ)
Ochrobactrum anthropi	NC	*Proteobacteria* (α)
Acinetobacter calcoaceticus	NC	*Proteobacteria* (γ)
Acinetobacter lwoffi	NC	*Proteobacteria* (γ)
TOTAL CELL COUNT	**2.6×10^6 CFU/mL**	

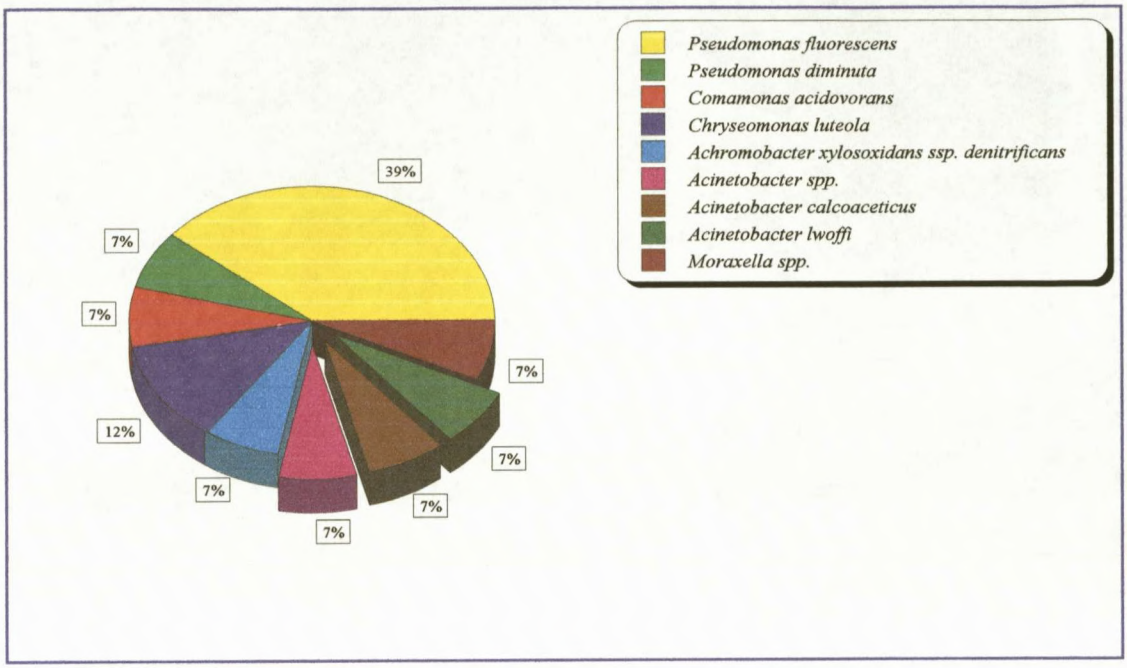

FIGURE 5.3 **Bacterial monocultures isolated on CGY agar from the aerobic zone of the enhanced EBPR culture and identified using the API 20NE system.**

TABLE 5.5 **Phylogenetic classification, synonyms and total plate counts of isolates recovered from the aerobic zone of the enhanced pilot plant culture.**

Organism	Synonym	Phylogenetic classification
Pseudomonas fluorescens	NC	*Proteobacteria* (γ)
Pseudomonas diminuta	NC	*Proteobacteria* (α)
Comamonas acidovorans	*Pseudomonas acidovorans*	*Proteobacteria* (β)
Chryseomonas luteola	*Pseudomonas luteola*	*Proteobacteria* (γ)
Achromobacter xylosoxidans ssp. denitrificans	*Alcaligenes denitrificans*	*Proteobacteria* (β)
Acinetobacter spp.	NC	*Proteobacteria* (γ)
Acinetobacter calcoaceticus	NC	*Proteobacteria* (γ)
Acinetobacter lwoffi	NC	*Proteobacteria* (γ)
Moraxella spp.	NC	*Proteobacteria* (γ)
TOTAL CELL COUNT	**2×10^6 CFU/mL**	

As mentioned previously, the complete dominance of the gamma subclass of *Proteobacteria* throughout the entire pilot plant system became very evident using conventional cultivation techniques (TABLE 5.6). Numbers of gamma subclass representatives increased and appeared to equilibriate in the anoxic and aerobic reactors, respectively. Higher numbers of alpha and beta representatives from the *Proteobacteria*, as well as a representative of the *Cytophaga-Flavobacterium* phylum, were recorded in the anaerobic zone. No members of the GPBHGC or GPBLGC were recovered from the enhanced culture at all.

TABLE 5.6 Distribution of cultured isolates from the enhanced culture using the API 20NE identification system.

Reactor	Phylogenetic classification				
	ALPHA	BETA	GAMMA	CF	HGC
Anaerobic	23%	18%	53%	6%	-
Anoxic	14%	14%	72%	-	-
Aerobic	13%	12%	75%	-	-

5.3.2 Biodiversity of Amanzimtoti WWW mixed liquor using cultivation techniques

The initial seed inoculum to the pilot plant during enhanced culture development ie., Amanzimtoti WWW mixed liquor, appeared to be more diverse with respect to the bacterial species present. This can be observed by the total absence of *Pseudomonas* spp. in the activated sludge sample (FIG. 5.4 and TABLE 5.7) and a wide distribution of bacterial genera. The isolation of *Acinetobacter* spp. ie., *A. lwoffi* (4%) in the mixed liquor can be expected from an aerobic activated sludge system, numbers of which indicate that the genus is not necessarily dominant in a process based primarily on organics removal and nitrification. Organisms recovered in the highest counts proved to be *Sphingomonas paucimobilis* and *Weeksella virosa* (15% respectively), followed by *Flavobacterium* spp. and *Aeromonas salmonicida* (11% respectively). All other isolates were below 10% recovery (TABLE 5.4). Representatives of *Cytophaga-Flavobacterium* (*Flavobacterium* spp. and *W. virosa*) amounted to 35% of the total bacterial population isolated, the *Proteobacteria* constituting the balance (65%; FIG. 5.4).

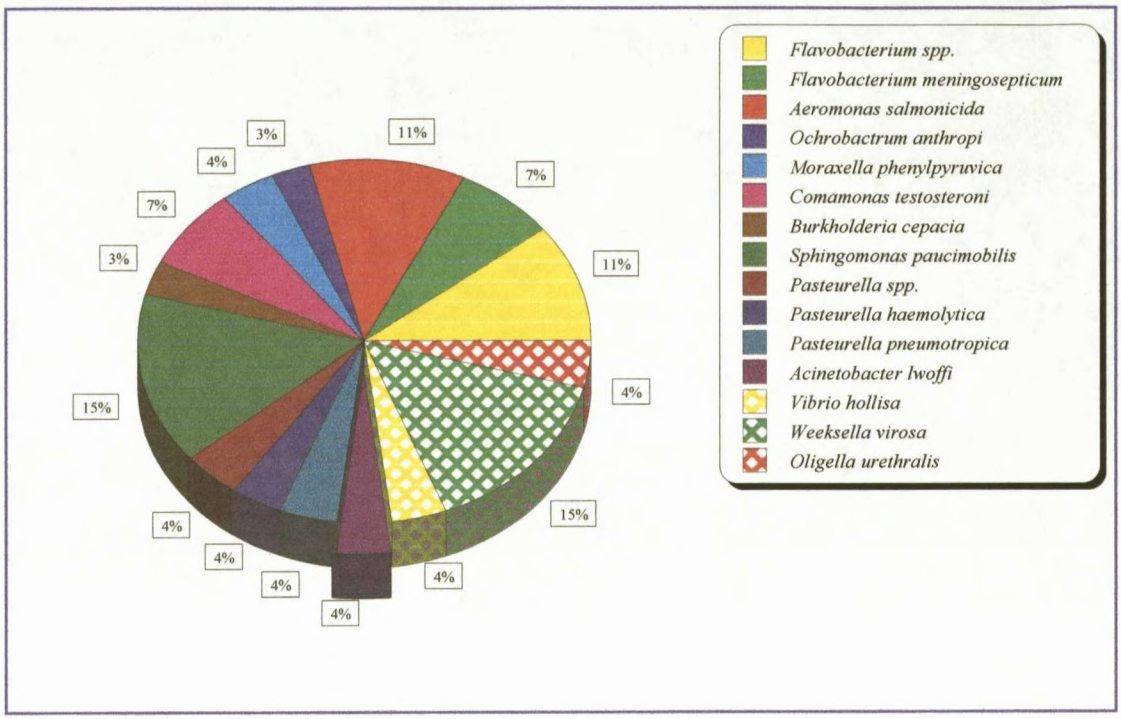

FIGURE 5.4 Bacterial monocultures isolated on CGY agar from Amanzimtoti mixed liquor and identified using the API 20NE system.

The dominance of the *Proteobacteria* in the Amanzimtoti mixed liquor is evident in TABLES 5.7 and 5.8. Total plate counts amounted to 4.6 x 10^6 CFU/mL (TABLE 5.7) which correlates with plate counts obtained for the enhanced culture aerobic zone (2 x 10^6 CFU/mL; TABLE 5.5). When expressed as a percentage of the total phyla isolated, the *Proteobacteria* amounted to 80%; subclass division constituted the following: alpha = 13%; beta = 13%; and gamma = 54% (TABLE 5.8). The *Cytophaga-Flavobacterium* cluster constituted 20% of the bacterial community whilst no GPBHGC or GPBLGC were isolated.

TABLE 5.7 Phylogenetic classification and synonyms of isolates recovered from the Amanzimtoti activated sludge mixed liquor.

Organism	Synonym	Phylogenetic classification
Flavobacterium spp.	NC	*Cytophaga-Flavobacterium*
Flavobacterium meningosepticum	NC	*Cytophaga-Flavobacterium*
Aeromonas salmonicida	NC	*Proteobacteria* (γ)
Ochrobactrum anthropi	NC	*Proteobacteria* (α)
Moraxella phenylpyruvica	NC	*Proteobacteria* (γ)
Comamonas testosteroni	*Pseudomonas testosteroni*	*Proteobacteria* (β)
Burkholderia cepacia	*Pseudomonas cepacia*	*Proteobacteria* (β)
Sphingomonas paucimobilis	*Pseudomonas paucimobilis*	*Proteobacteria* (α)
Pasteurella spp.	NC	*Proteobacteria* (γ)
Pasteurella haemolytica	NC	*Proteobacteria* (γ)
Pasteurella pneumotropica	NC	*Proteobacteria* (γ)
Acinetobacter lwoffi	NC	*Proteobacteria* (γ)
Vibrio hollisae	NC	*Proteobacteria* (γ)
Weeksella virosa	*Flavobacterium genitale*	*Cytophaga-Flavobacterium*
Oligella urethralis	*Moraxella urethralis*	*Proteobacteria* (γ)
TOTAL CELL COUNT	**4.6×10^6 CFU/mL**	

TABLE 5.8 Distribution of cultured isolates from Amanzimtoti WWW using the API 20NE identification system.

Reactor	Phylogenetic classification				
	ALPHA	BETA	GAMMA	CF	HGC
Aerobic	13%	13%	54%	20%	-

Data regarding the bacterial community obtained from cultivation of Darvill WWW mixed liquor is presented in TABLE 5.9 for direct comparison between the two other activated sludge systems investigated. Although the

dominance of the gamma subclass of *Proteobacteria* is common amongst all the systems under investigation, the presence of GPBLGC in relatively significant numbers and absence of the alpha subclass of *Proteobacteria* and *Cyttophaga-Flavobacterium* are amongst the more atypical features of the bacterial community in the Darvill mixed liquor activated sludge when TABLE 5.9 is compared to TABLE 5.6 (both EBPR processes).

TABLE 5.9 Distribution of cultured PAO's from the aerobic zone of Darvill WWW using the API 20NE identification system.

Detailed results can be obtained in CHAPTER 3; FIG. 3.7

Reactor	Phylogenetic classification					
	ALPHA	BETA	GAMMA	CF	HGC	LGC
Aerobic	-	18%	47%	-	6%	29%

5.3.3 Staining of intracellular granules for light microscopy visualisation

Amanzimtoti WWW (return sludge) and enhanced culture (AN, AX and AE) mixed liquor samples were stained to view intracellular polyphosphate and PHB granules (FIGS 5.5 to 5.8). PHB staining of the anaerobic mixed liquor from the enhanced culture showed large aggregates of blue-black granules, representing either individual cells or small, dispersed flocs, indicating large quantities of stored PHB (FIG. 5.5). From the micrograph supplied, it appears that the PHB granules occupied a large fraction of cellular content and that a large number of bacterial cells were actively metabolising VFA's and accumulating PHB, indicative of an operative, efficient EBPR mechanism. Subsequent Neisser staining of the enhanced culture anoxic and aerobic mixed liquor (FIGS 5.6 and 5.7) and Amanzimtoti return sludge (FIG. 5.8) was done to view metachromatic granules, indicative of polyphosphate inclusions. Aerobic uptake of P and accumulation of poly-P was prolific, the majority of the microscopic fields observed been occupied by purple stained cells (FIG. 5.6). It appeared that the contribution of the filaments to P uptake was minimal. Anoxic phosphate uptake and accumulation was confirmed although the frequency of stained cells observed was not comparable to that of the aerobic reactor (FIG. 5.7). Amanzimtoti mixed liquor samples, as expected, showed very little poly-P accumulation (FIG. 5.8).

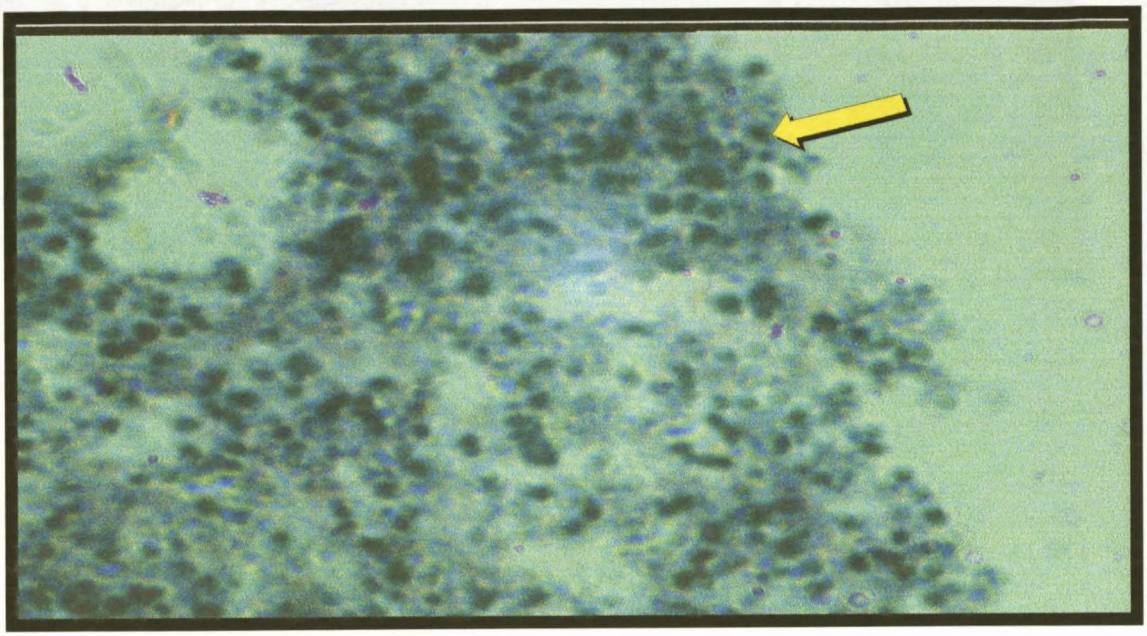

FIGURE 5.5 **Micrograph of polyhydroxybutyrate (PHB) stain from the anaerobic reactor of the enhanced pilot plant culture (X 1000 magnification).**

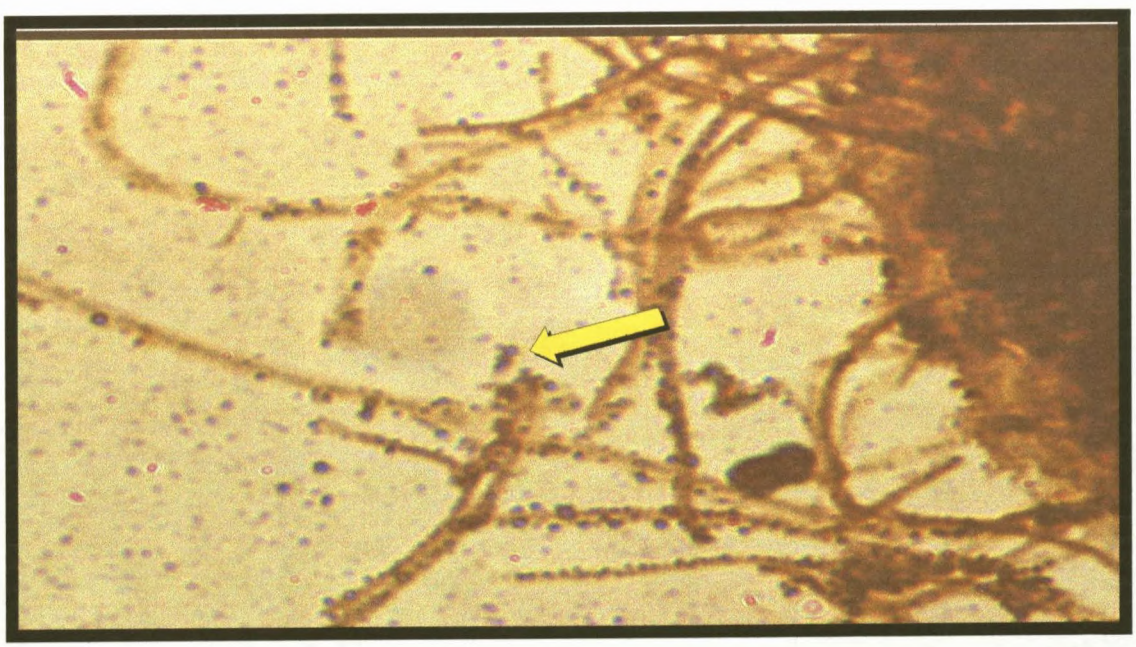

FIGURE 5.6 **Micrograph of Neisser stain (showing intracellular poly-P granules) from the aerobic reactor of the enhanced pilot plant culture (X 1000 magnification).**

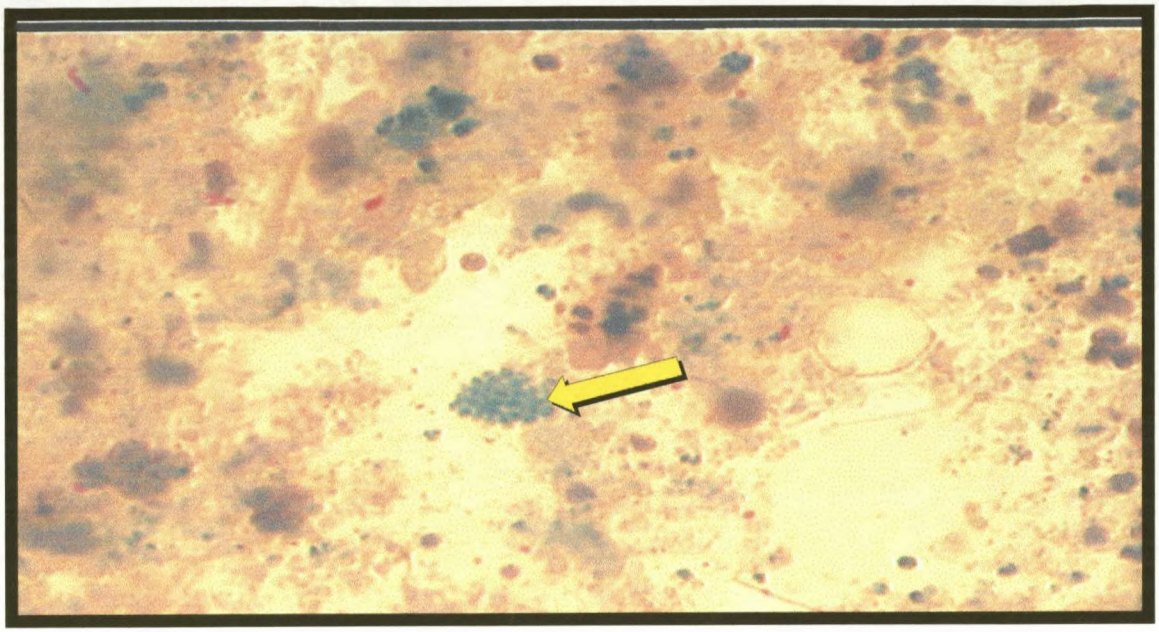

FIGURE 5.7 **Micrograph of Neisser stain (showing intracellular poly-P granules) from the anoxic reactor of the enhanced pilot plant culture (X 400 magnification).**

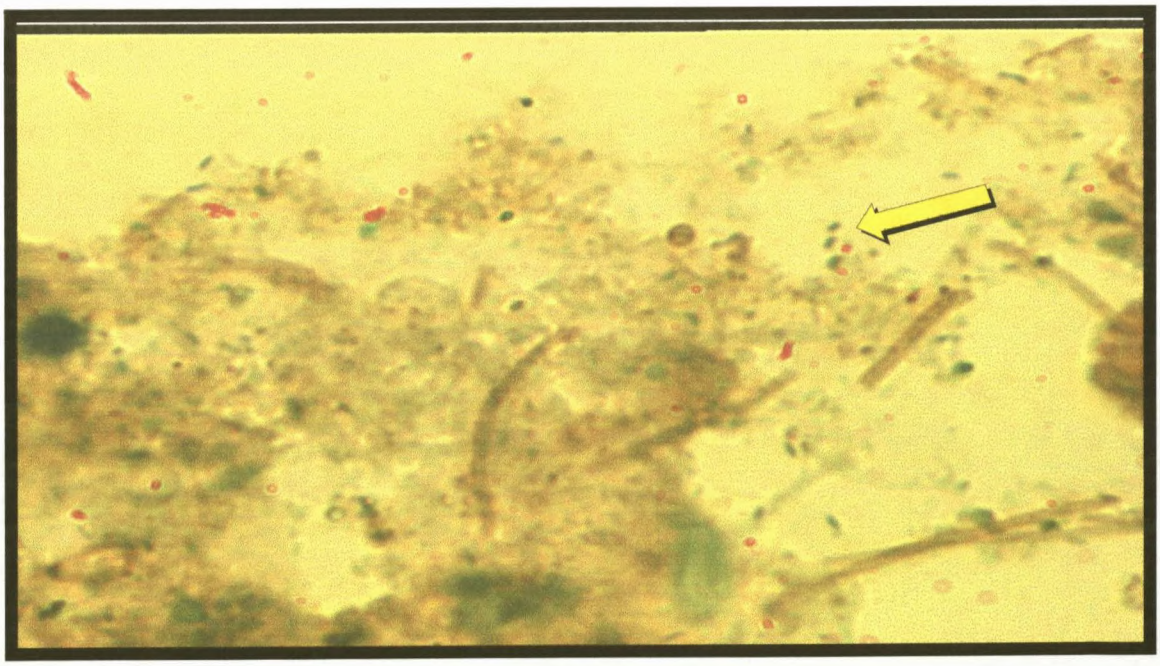

FIGURE 5.8 **Micrograph of Neisser stain (showing intracellular poly-P granules) from the activated sludge mixed liquor at Amanzimtoti Wastewater Treatment Works (X 1000 magnification).**

5.3.4 Biodiversity of enhanced culture and Amanzimtoti WWW using FISH

The composition of the microbial (bacterial) population in the seed inoculum (Amanzimtoti WWW) and the AN, AX and AE zones of the enhanced culture, as determined by FISH, is shown in TABLE 5.10. Community structure profiles of the enhanced culture are indicative of the system at day 150 of operation when phosphate (as P) removal averaged approximately 37 mgP/L. For both activated sludge types, 72-81% of the cells visualised by DAPI were detected by the bacterial probe EUB (TABLE 5.10). These results indicate that the majority of fixed cells were from the domain *Bacteria*. Hybridization with probes for the alpha, beta and gamma subclasses of *Proteobacteria*, CF, HGC and ACA was also performed but the proportions of these groups were not readily quantified due to inadequate floc disruption in the samples and weak fluorescence, possibly due to prolonged bead-beating treatment. Results are therefore expressed as a percentage of area occupied by fluorescence in relation to the area occupied by DAPI and not individual cell counts (results are expressed as an average of 10 microscopic fields). It is therefore recommended that direct interpretation of these results be guarded. Problems with probe CF became evident when hybridization with activated sludge was recorded yet no fluorescence after hybridization with the monoculture control was detected. Total cell counts (DAPI) of the two activated sludge systems and the various zones within the enhanced culture are also given in TABLE 5.10. Colony counts on agar media were approximately 1 000-fold lower than those recorded using DAPI staining (TABLE 5.3 to 5.5 and TABLE 5.7).

Cells belonging to the beta subclass of the *Proteobacteria* were dominant in samples from Amanzimtoti WWW (22%), gradually decreasing to occupy 12-16% of the enhanced culture population (TABLE 5.10). Results of probe CF also showed a slight decline in numbers (9 to *ca.* 5%) due to amplification of the EBPR mechanism, possibly indicating that the growth conditions imposed ie., anaerobic/aerobic sequenced reactors and acetate supplementation, did not favour these organisms. Hybridization with probes ALF and GAM showed an increase in representatives of these phylogenetic groups during enhanced culture development. No fluctuation in numbers of *Acinetobacter* (ACA) and GPBHGC (HGC) was recorded. Results of TABLE 5.10 indicate that the *Proteobacteria* were dominant in all the reactors (AN, AX and AE) of the pilot plant with no tremendous shifts in the population structure between zones recorded.

TABLE 5.10 Comparison of community compositions in activated sludge samples of Amanzimtoti WWW and the enhanced culture pilot plant as determined with oligonucleotide probing.

Probe	Amanzimtoti (%)	Enhanced (Anaerobic) (%)	Enhanced (Anoxic) (%)	Enhanced (Aerobic) (%)
DAPI	100	100	100	100
EUB	72	79	79	81
ALF	9	22	21	24
BET	22	16	15	16
GAM	12	20	21	21
CF	9	5	5	6
HGC	10	9	8	8
ACA	4	2	2	3
CELL COUNT (per mL) *	**1.66×10^9**	**1.2×10^9**	**1.35×10^9**	**1.6×10^9**

* as determined by membrane filtration

Micrographs representing DAPI staining and *in situ* hybridization of activated sludge from the enhanced culture are given in FIGS 5.9A to 5.12B. The genus-specific, rhodamine-labelled 16S rRNA-targeted oligonucleotide ACA (*Acinetobacter* spp.) probe (FIG. 5.9B) showed that the bacterium was present in the sludge in very low numbers, constituting approximately 2-3% of the visualised DAPI stain (TABLE 5.10 and FIGS 5.9A and B). Total cell counts obtained with DAPI staining and membrane filtration are more representative of activated sludge than those obtained with cultivation-dependent methods (TABLE 5.10). Typical morphology of visualised *Acinetobacter* cells was rod to coccus shaped cells, usually arranged in chains (FIG. 5.9B). *Acinetobacter* cells were nearly always closely associated with the sludge floc, emphasising the requirement to effectively disrupt the floc structure to release free cells and increase cell counts. Visualisation of the actual floc under epifluorescence resulted in a large degree of background noise, often resulting in problems with the detection of probe-conferred, specific fluourescence. Bead-beating of the fixed activated sludge samples negatively affected fluorescence, presumably due to structural damage of the cell walls and possible leakage of cytoplasm containing rRNA (results not shown).

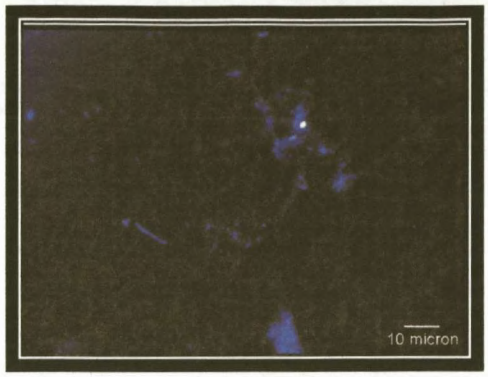

FIGURE 5.9A DAPI stain of enhanced culture activated sludge (aerobic zone).

FIGURE 5.9B *In situ* hybridization of identical microscopic field with tetramethylrhodamine-labelled probe ACA23a.

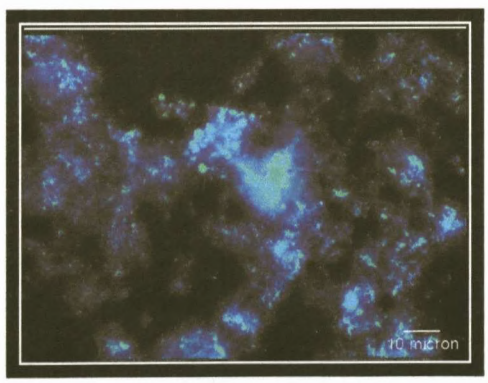

FIGURE 5.10A DAPI stain of enhanced culture activated sludge (aerobic zone).

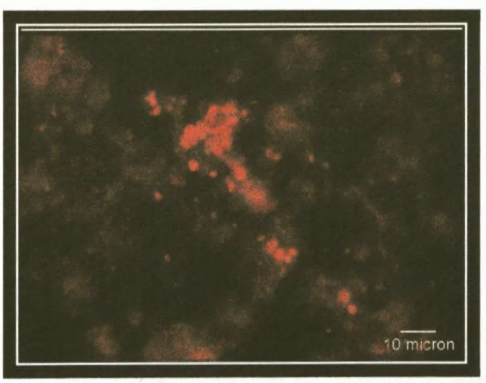

FIGURE 5.10B *In situ* hybridization of identical microscopic field with tetramethylrhodamine-labelled probe ALF1b.

FIGURE 5.11A DAPI stain of enhanced culture activated sludge (aerobic zone).

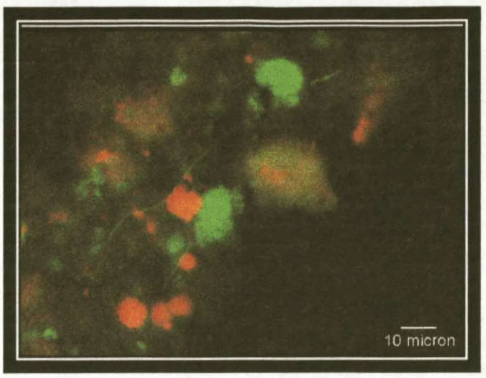

FIGURE 5.11B Simultaneous *in situ* hybridization of identical microscopic field with tetramethylrhodamine-labelled probe BET42a and fluorescein-labelled probe GAM42a.

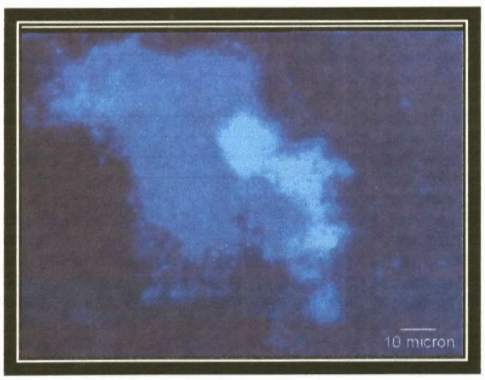

FIGURE 5.12A DAPI stain of enhanced culture activated sludge (aerobic zone).

FIGURE 5.12B *In situ* hybridization of identical microscopic field with tetramethylrhodamine-labelled probe HGC.

DAPI staining and hybridization of the same microscopic field with rhodamine-labelled (red) probe ALF1b are shown in FIGS 5.10A and 5.10B. Hybridization of rhodamine-labelled probes BET42a and HGC and fluorescein-labelled (green) GAM42a (as well as the corresponding DAPI stains) are shown in FIGS 5.11A-5.12B. Due to the single mismatch in the probe sequences, probes BET42a and GAM42a were hybridized simultaneously to the activated sludge samples to prevent non-specific binding. Occasional 'bright' fluorescence when visualising the DAPI stain is evident in FIGS 5.10A and 5.12A. These bright patches of fluorescence seem to be always be associated with the tetramethylrhodamine fluorochrome, possibly indicating that a certain degree of rhodamine excitation at blue wavelengths of visualisation occurs. A more likely explanation is that these patches may also represent aggregates (with high DNA content) of metabolically active cells therefore allowing for greater interchelation and greater reported detection of the DAPI stain.

5.4 DISCUSSION

5.4.1 Cultivation-dependent techniques

It is evident from FIGS 5.1 to 5.3 and TABLES 5.3 to 5.6 that the gamma subclass of *Proteobacteria* and *Pseudomonas* spp. in particular dominated the enhanced culture of PAO's when counting CFU's on CGY enrichment agar (this is not to say that all the isolates were necessarily PAO's). Although these results are in agreement with Wentzel *et al.*, (1988) in as far as both studies found the predominance of the gamma subclass of the *Proteobacteria* in enhanced cultures, they differ in that Wentzel *et al.*, (1988) found the population structure to be very specific; more than 90% of the organisms cultured aerobically were identified as *Acinetobacter* spp. using the API procedure. A striking feature of the enhanced culture community profile in the present study was the diversity of bacteria isolated aerobically, immediately rendering direct comparisons between the two cultures based on FISH as implausible. Although not indicated, it is possible that Wentzel *et al.*, (1988) operated their pilot plant at a different temperature than that used in the present experiment (T=20°C). It has been shown using dry denaturing gradient gel electrophoresis (DDGGE) that temperature, especially long-term elevated temperature (>30°C), has a strong effect on bacterial population structures in activated sludge (Brdjanovic *et al.*, 1998).

According to Bergey's Manual (1984), most *Acinetobacter* strains have a temperature optima of 33-35°C. Temperatures nearing these values will obviously favour growth of the particular organism allowing it to achieve dominance in a system if other growth constraints (such as nutrient and energy supplementation) are satisfied.

Brodisch and Joyner (1983), when isolating bacteria from a laboratory-scale unit comprised of anaerobic, anoxic and aerobic zones and displaying EBPR (effluent PO_4-P = 0.8 mg/L), found a wide distribution of organisms with Gram positive bacteria and *Pseudomonas* spp. dominating the system and *Acinetobacter* spp. only constituting approximately 5% of the total bacteria isolated. They concluded that, due to its aerobic metabolism, recovery of *Acinetobacter* spp. is indirectly proportional to anaerobic hydraulic retention time. It appears that Wentzel *et al.*, (1988) operated their system at an actual anaerobic hydraulic retention time of *ca.* 2 h as opposed to 5 h during this study (TABLE 4.6; page 98). The dual role of *Pseuodomonas* spp. in both denitrification and phosphate removal may account for its dominance in the enhanced culture (Kavanaugh and Randall, 1994).

The complete absence of GPBHGC from the enhanced culture (TABLE 5.6) when cultivating on solid agar media may be due to their absence in the seed Amanzimtoti mixed liquor (TABLES 5.7 and 5.8). It is unlikely, however, that Gram positive bacteria were totally excluded from either of the mixed liquors. Wagner *et al.*, (1994b) showed that cultivation on nutrient rich medium selected against the growth of GPBHGC. Two other possibilities which may account for this group of bacteria's apparent absence may be due to (1) low initial cell numbers resulting in poor competition and recovery on solid media and (2) bacteria from this group, entering the unit through the settled sewage fraction of the influent feed (during early stages of pilot plant operation), may have been unable to grow and become established members of the bacterial community before been washed out/diluted from the system (due to environmental stress from entering the system through the anaerobic reactor).

At the family and subclass level of bacterial classification using the API system, no significant difference between the Amanzimtoti and enhanced culture community structure was recorded (TABLES 5.6 and 5.8). In general, the same groups of bacteria were found in the full-scale process and laboratory unit. A distinct deviation existing between both communities was the low numbers of *Cytophaga-Flavobacterium* recorded in the enhanced culture

(6% as compared to 20% in the initial seed inoculum). The higher numbers of alpha and beta *Proteobacteria* in the anaerobic zone of the enhanced culture (TABLE 5.6) may possibly be due to influent entering the system through this reactor, indicating that these organisms were able to efficiently utilise the influent COD under anaerobiosis ie., possibly PHB accumulation or fermentative organisms. No actual growth (increased yield) should be recorded in the anaerobic zone by aerobic organisms as any assimilated organic material is used primarily for maintenance purposes. Although individual bacterial species cultivated from the various zones of the enhanced culture varied considerably, the microbial profile at the class and subclass level of classification generally remained stable. SDS-PAGE protein profiles of activated sludge from a BNR process have indicated that the microbial community remains relatively stable throughout the various zones of the system (Ehlers *et al.*, 1998). The increase in gamma *Proteobacteria* in the enhanced culture anoxic and aerobic zones (TABLE 5.6) indicates that these organisms are able to efficiently metabolise and synthesise energy (ATP) from intracellular storage granules when a terminal electron acceptor is present in the liquid medium. It appears therefore that the alpha and beta *Proteobacteria* are r-strategists[2] whilst the gamma *Proteobacteria* are K-strategists[3], capable of dominance in the resource limited aerobic reactor (Andrews and Harris, 1985).

5.4.2 Incidence of *Acinetobacter* spp. using cultivation-dependent techniques

A remarkable feature of the current study was the lack of dominance of *Acinetobacter* spp., using direct cultivation methods, was never shown in any of the three sludges investigated (although, at times, its numbers were relatively high; FIGS 5.1 to 5.4). Lötter and Murphy (1985) found *Acinetobacter* spp. to be dominant in aerobic cultures of mixed liquor originating from both the anaerobic and aerobic zones of a full-scale BNR plant (CGY agar plates were also used during their study). In a later study, Osborn *et al.*, (1989) were unable to detect *Acinetobacter* spp. in high numbers from a similar activated sludge process using CGY agar. Kavanaugh and Randall (1994), using various carbon (VFA including acetate) based agar media, could not demonstrate the dominance of *Acinetobacter* spp. in a BNR activated sludge process either. The low fraction of *Acinetobacter* spp. in the Amanzimtoti sludge

[2] The r-strategist microorganism is one that through rapid growth rates, dominates environments in which resources are temporarily abundant; in uncrowded environments r-strategists can attain maximum specific growth rates and are generally regarded as specialists.
[3] K-strategists generally have a lower growth rate than the r-strategists and tend to be successful in resource-limited environments through physiological adaptations to the carrying capacity of the environment; these microorganisms are generally referred to as generalists.

(FIG. 5.4 and TABLE 5.7) was surprising as high numbers of this genus have previously been reported in completely mixed aerobic single reactor systems (Lötter *et al.*, 1986b). These discrepancies in results regarding the incidence of *Acinetobacter* spp. in activated sludge mixed liquors show how misrepresentative cultivation-dependent methods are. According to Ehlers *et al.*, (1998), analysing the protein profile of activated sludge mixed liquors using the culture independent method of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), system design, seasonal changes and characteristics of the waste liquid been treated do not adversely affect protein profiles, indicating little variation in microbial community between plants. One would therefore expect a stronger correlation in the results of plating than those reported, to date, in the literature. However, according to the results reported above, *Acinetobacter* spp. were not prominent in either the single aerobic Amanzimtoti WWW or the enhanced EBPR mixed liquors, constituting 4-21% of the total bacteria recovered on solid agar media (FIGS 5.1 to 5.4 and TABLES 5.3 to 5.5; TABLE 5.7).

5.4.3 *In situ* hybridizations

The bias imposed regarding the species or groups of organisms which cultivation techniques allow to grow is well documented (Wagner *et al.*, 1993; Wagner *et al.*, 1994a,b; Amann *et al.*, 1995; Kämpfer *et al.*, 1996). Cultivation of all three sludge types under investigation ie., Darvill and Amanzimtoti WWW and the enhanced culture, in the present study on CGY agar showed a distinct pattern of cell recovery - complete dominance of the gamma subclass of *Proteobacteria* in the mixed liquor (TABLES 5.6, 5.8 and 5.9). Subsequent hybridization with specific probes of two of the sludge types ie., Amanzimtoti WWW and the enhanced culture, revealed that the beta (in the Amanzimtoti ML) and alpha (in the enhanced culture ML) subclasses of the *Proteobacteria* were dominant (TABLE 5.10; micrographs of the various specific hybridized probes for the enhanced culture are given in FIGS 5.9A to 5.12B). In other similar studies, direct detection methods have not demonstrated the dominance of the alpha *Proteobacteria* for reasons which may be partially ascribed to different process configurations used to develop enhanced EBPR cultures (Nakamura *et al.*, 1998; Blackall *et al.*, 1998b). Kawaharasaki *et al.*, (1999), however, when staining EBPR activated sludge with DAPI at poly-P probing concentrations revealed that both the GPBHGC and the alpha subclass of *Proteobacteria* fluoresce bright yellow, indicating that these groups of bacteria accumulate

large amounts of poly-P. The authors found that although the alpha *Proteobacteria* did not constitute a major proportion of the microbial community (7%), 85% of bacteria which hybridized with probe ALF1b accumulated poly-P, indicating the significance of this subclass to phosphate removal operations. Nakamura *et al.*, 1998 and Blackall *et al.*, 1998b, operating sequencing batch reactors (SBR's) with no anoxic phases, established enhanced cultures of PAO's. Both these studies showed the dominance of the beta subclass of the *Proteobacteria* and the GPBHGC when strong EBPR mechanisms were reported. In fact, Blackall *et al.*, (1998b) found that the SBR operating under poor phosphate removal conditions was dominated by the alpha *Proteobacteria* (42%) and GPBHGC (40%) which, according to the author, were presumably different strains of Gram positive bacteria to those normally affiliated with EBPR systems. A clone library of a second SBR and continuous reactor (both showing comparably strong EBPR mechanisms) was prepared and the clones sequenced and phylogenetically analysed (Blackall *et al.*, 1998b). They reported the dominance of the *Cytophaga-Flavobacterium* cluster (51%) and beta *Proteobacteria* (17%) in the SBR whilst the GPBHGC (37%) and alpha *Proteobacteria* (18%) dominated the continuous culture, indicating that it may be process operation (batch versus continuous) which determines activated sludge diversity and species predominance at laboratory-scale. Care should therefore be exercised when comparing results to those of the literature, ensuring that similar process configurations (with respect to feeding regime and design) are used by the authors before drawing any significant conclusions.

In a recent study, the specificity of the gamma subclass probe (GAM42a) was cast into doubt (even at high formamide concentrations [45%] in the hybridization buffer), indicating that gamma *Proteobacteria* counts in natural and engineered systems still remain over-estimated during whole-cell rRNA hybridizations (Nielsen *et al.*, 1999b). The possibility that gamma *Proteobacteria* counts during the present study may even be lower than those reported in TABLE 5.10 cannot be ignored. It seems, however, that the presence of an anoxic zone in the activated sludge process increases the number of gamma *Proteobacteria* significantly (the majority of *Pseudomonas* spp. are grouped in this subclass). A diverse bacterial community was still shown to exist using FISH even though numbers of the gamma *Proteobacteria* were presumably exaggerated (TABLE 5.10). It is evident that PAO's are not confined to any specific group of organisms and that bacterial diversity is maintained even under EBPR selection. Indeed, one would expect greater diversity under EBPR conditions (as compared to single aerobic processes) due

to the presence of various redox zones which permit the establishment of a number of bacteria in the system with distinctly different metabolic processes. Conversely, many bacteria are known to have a dual function in activated sludge ie., denitrification and phosphate uptake, which may reduce species diversity considerably (Kavanaugh and Randall, 1994).

The contribution of GPBHGC to EBPR seems unclear at the moment. This group of bacteria has previously been implicated in the removal of phosphate from anaerobic-aerobic activated sludge processes, often dominating the system entirely when using *in situ* hybridization techniques and clone libraries (Kämpfer *et al.*, 1996; Christensson *et al.*, 1998). However, Hiraishi *et al.*, (1989) found that the respiratory quinone extracted from activated sludge with a strong EBPR mechanism was composed primarily of ubiquinone, a major component of Gram negative bacteria (*Proteobacteria*), rather than menaquinone, the major component of Gram positive bacteria. Liu *et al.*, (1997) also found that GPBHGC did not constitute a major fraction of activated sludge biomass based on PCR-amplified bacterial 16S rDNA. These discrepancies may be attributable to the different techniques applied to analyse the bacterial community structures and implies that some form of standardisation needs to be applied when conducting ecological studies. It may be possible that a number of molecular techniques be applied to environmental samples simultaneously in the hope that they compliment one another and offer more representative results.

The addition of acetic acid to the anaerobic zone has been shown to enhance the growth of GPBHGC and of bacteria belonging to the beta subclass of the *Proteobacteria* (Wagner *et al.*, 1994b). Although the numbers of beta bacteria decreased slightly during enhanced culture development, a distinct increase in the numbers of GPBHGC was recorded using *in situ* hybridization (TABLE 5.10). Paraformaldehyde fixation of the activated sludge samples initially resulted in low visualised fluorescence of the HGC probe (results not shown). However, after treatment with ethanol:formaldehyde (90:10, v/v) fixative (Braun-Howland *et al.*, 1992), probe-conferred fluorescence increased significantly to those values reported in TABLE 5.10. Reference can also be made to Roller *et al.*, (1994) for HGC probe design and specificity and sample preparation for visualisation of GPB in activated sludge. This clearly shows the importance of adequate sample preparation for quantitative monitoring of this functionally

significant (when considering EBPR operations) group by *in situ* monitoring.

The increase in the number of viable bacteria in the enhanced culture, detected using probe EUB338 (72% in the Amanzimtoti sludge as opposed to *ca.* 80% in the enhanced culture), can be made attributable to the lower operational R_s ie., 10 d as opposed to 20-25 d at full-scale, and the supplementation of a RBCOD source to the influent feed (TABLE 5.10). At the time of mixed liquor sampling for *in situ* hybridizations, the pilot plant was maintained for approximately two sludge ages solely on HAc as an organic source. In NDBEPR processes, MLVSS values consist of heterotrophic and autotrophic viable biomass, endogenous residue and inert particulate material which is absorbed to the surface of individual cells and flocs (Ubisi *et al.*, 1997). In the present enhanced culture, one could assume that the endogenous residue and inert particulate fractions were negligible due to the relatively low sludge age and supplementation of an RBCOD source (as S_{ti}), respectively. Therefore, the average MLVSS value of 1.283 g/L during period 4.7 (TABLE 4.10; page 129) should theoretically consist primarily of active heterotrophic and autotrophic bacteria. Once the autotrophic fraction is determined (Sandén *et al.*, 1994) or even negated using sludge ages of <3 d, the heterotrophic active biomass, responsible for phosphate uptake, can be quantified using FISH (it has been previously shown that the rRNA content of microorganisms is proportional to growth rate over a wide range; DeLong *et al.*, 1989). This indicates the potential of molecular based techniques to be integrated in process engineering and activated sludge modelling. This falls beyond the scope of the present research, however, and will not be discussed further suffice to say that it seems that a major portion of the culture MLVSS consisted of metabolically active bacteria (of which the *Proteobacteria* constituted a major fraction).

5.4.4 Incidence of *Acinetobacter* spp. using *in situ* hybridizations

Direct *in situ* detection of *Acinetobacter* spp. in Darvill WWW and the EBPR pilot plant mixed liquors showed the presence of the genus in very low numbers viz., *ca.* 2-4% of DAPI total area counts (FIG. 5.9A,B and TABLE 5.10). The finding of *Acinetobacter* spp. in the sludge of the EBPR pilot plant in low numbers implies that its role in BPR operations has been grossly overestimated and is in agreement with other reports (Oerther *et al.*, 1997; Christensson *et al.*, 1998). Oligonucleotide hybridization of activated sludge from full-scale BNR processes have

indicated that organisms other than *Acinetobacter* spp. are responsible and contribute to EBPR; numbers of detected *Acinetobacter* cells seem to be below 10% (Wagner *et al.*, 1994a,b) and 1 to 2% (Kämpfer *et al.*, 1996). Less than 1% of the total bacterial population were detected by probe ACA in a laboratory-scale SBR (Bond *et al.*, 1997). Other various non-culture dependent methods for community analysis have also shown that *Acinetobacter* is not primarily responsible for EBPR (Cloete and Steyn, 1987; Hiraishi and Morishima, 1990). The evidence seems clear that *Acinetobacter* spp. are not the model PAO's in activated sludge yet until a substitute bacterium, with carbon and phosphorus transformations conducive to EBPR, is isolated and identified, the biochemical and kinetic models for activated sludge EBPR processes will remain based on this organism.

5.5 CONCLUSIONS

Although organisms found in the enhanced culture of PAO's were not assessed for their individual carbon and phosphorus transformation patterns, it is assumed that those bacteria (genus or phylogenetic class) which were isolated/detected in the system were the bacteria principally responsible for the EBPR mechanism. It may be that many of the bacteria were not directly involved in phosphate removal, *per sè*, but their presence indicates that they may have been involved in other important aspects such as fermentation of RBCOD to VFA and/or denitrification-nitrification processes, all of which are integral to the success of the EBPR process as a whole. Unfortunately, the results of this study do not conclusively indicate which bacteria, isolated or detected (using FISH) in the enhanced culture, were actually PAO's.

The microbial community structure of the pilot plant EBPR sludge was defined and it was shown that, when using FISH, no specific class of organism predominates even in specially developed cultures of activated sludge. Cultivation methods resulted in the total dominance of the gamma subclass of the *Proteobacteria* in all three sludge types investigated during the course of this study. Subsequent hybridization of two of the sludge types ie., Amanzimtoti WWW and the enhanced PAO culture, with specific oligonucleotide probes showed that the cultivated gamma subclass counts were drastically overestimated. It appears that activated sludge maintains its bacterial species diversity when operated as either a single aerobic reactor or when EBPR ie., presence of an anaerobic zone,

is selected.

Acinetobacter spp., when applying both cultivation and *in situ* methods of identification and enumeration, did not show any dominance in the activated sludge samples presently under investigation. *Acinetobacter* spp. do not appear to be the primary organisms responsible for EBPR; this function seems to be the activity of a number of organisms from various clusters of the phylogenetic tree (*Proteobacteria*, *Cytophaga-Flavobacterium* and *Actinobacteria* [GPBHGC]). It appears that isolation and recovery of bacteria on solid agar media results in the dominance of the gamma subclass of *Proteobacteria*, especially members of the genus *Pseudomonas*. When using FISH, the alpha *Proteobacteria* were shown to be dominant in the enhanced culture of PAO's which is thought to be primarily due to the presence of an anoxic zone in the process which places additional stress and restructures the microbial community in the system. The marked shift in alpha and gamma Proteobacterial counts from a non-EBPR sludge (9 and 12%, respectively) to one exhibiting a strong bio-P removal mechanism (*ca.* 22 and 21%, respectively) also indicates that these organisms are able to flourish under EBPR selective pressures are therefore the primary mediators of BPR.

CHAPTER SIX

GENERAL CONCLUSIONS AND RECOMMENDATIONS

6.1 GENERAL CONCLUSIONS

The results of this study seem to unquestionably indicate that *Acinetobacter* spp. are not present in EBPR sludges in any appreciable amount and that its role in phosphate removal has previously been exaggerated. The fact that *Acinetobacter* is unable to satisfy one of the Koch-Henle postulates ie., the microorganism must be present in sufficiently large numbers to warrant its association with a particular function, supports the notion that *Acinetobacter* is not the model PAO.

Cultivation-dependent techniques and sample manipulation was used to isolate and identify PAO's from mixed liquor originating from a full-scale BNR activated sludge plant (Darvill WWW). Isolates were tested to determine their individual phosphate uptake capacities. Although *Acinetobacter* isolates took up the highest quantity of soluble P in axenic cultures, the number of *Acinetobacter* isolates from the mixed liquor (*ca.* 4% of the total isolated PAO population) did not warrant their sole implication in the reported P removal capacity of the system. It became evident that other bacteria were present in the biological sludge which were responsible for the EBPR mechanism. Of the 39 isolates initially subjected to P uptake screening tests, 16 showed propensity to accumulate P in any appreciable amount. Subsequent identification of the 16 isolates revealed dominance of *Pseudomonas* spp. (58%) and the gamma subclass of *Proteobacteria* (47%). Although the quantity of phosphate taken up by Pseudomonad spp. proved to be less than that of *Acinetobacter* spp. in axenic cultures, their high cell counts when isolated on solid media may account for the majority of P removal from the Darvill activated sludge process. The contribution of this genus to EBPR operations must not be ignored. Other isolates displaying the ability to accumulate poly-P included members from the GPBHGC (*Micrococcus*) and GPBLGC (*Bacillus*, *Staphylococcus* and *Streptococcus*) although these two Gram positive clusters only constituted 6 and 29% respectively of the isolated PAO community. Improved methods of PAO isolation from activated sludge mixed liquors should be investigated so that the time-

consuming poly-P screening assay of isolates can be omitted.

An enhanced culture of PAO's was successfully developed and operated for a period of five months using a laboratory-scale activated sludge process based on the 3-stage Phoredox configuration. The unit was initially fed with settled municipal sewage, the fraction of which was incrementally decreased whilst the supplemented acetate fraction was increased. The system was eventually supported with acetate as the sole carbon source at a maximum of 500 mgCOD/L. A high P/COD ratio (0.05-0.1 mgP/mgCOD) was selected in an attempt to enrich the microbial community with PAO's and to suppress the growth of 'G' bacteria or glycogen accumulating bacteria (GAO's). Influent to the unit was routinely characterised and defined with respect to S_{ti} (including S_{bsi}), TKN (including a separate saline ammonia assay), TP and SRP. Effluent was also routinely monitored to evaluate unit efficiency. Steady-state conditions, with respect to S_{ti}, were as far as possible maintained. An increase in the phosphate uptake capacity of sludge was recorded as the acetate fraction to the anaerobic zone of the system was increased. A maximum of *ca.* 40 $mgPO_4$-P/L was removed from the system which was attributed solely to the biological mechanism due to maintenance of mixed liquor pH below 7.8 (which should negate chemical P precipitation). Other significant features of the pilot plant were P/VSS (mgP/mgVSS) values of approximately 0.27, VSS/TSS (mgVSS/mgTSS) of 0.53 and continuous phosphate uptake in the anoxic reactor indicating the presence of denitrifying PAO's in the system. Simultaneous anoxic and aerobic phosphate uptake in the enhanced system seems to indicate that the PAO population consists of both those organisms which are able to denitrify and those which are only capable of aerobic respiration. It seems that a certain soluble acetate fraction must be present in the anaerobic zone before the EBPR mechanism is activated. It is possible that recycled nitrates to the anaerobic zone (generally < 2 $mgNO_3$-N/L) required denitrification (a COD consuming process) before true anaerobiosis was established and phosphate release occurred. In this study, however, an acetate concentration of 200 mg/L was required before excess phosphate uptake was achieved. The physical-chemical protocol for S_{bsi} determination does not appear to be pH sensitive and flocculation does occur between a pH range of 10-11 (not only at pH 10.5).

Direct *Acinetobacter* counts of the enhanced culture mixed liquor using FISH techniques revealed that the genus only constituted approximately 3% of the total bacterial community. The alpha and gamma subclass of the

Proteobacteria appeared to dominate the system (*ca.* 23 and 21% of the total DAPI count, respectively), followed by the beta subclass of the *Proteobacteria* (*ca.* 16%). The *Cytophaga-Flavobacterium* and GPBHGC clusters only constituted 5 and 8% of the bacterial community, respectively. These results indicate the definite involvement of the *Proteobacteria* in EBPR operations although exactly which genera are implicated remains to be clarified. The large biodiversity existing in even enhanced cultures of PAO's makes it impossible to ascribe the mechanism to any one class of organism, never mind only a few genera of bacteria. The bio-P removal mechanism does not appear to be class, genus or species specific but seems to be a function of a number of organisms in which the required metabolic transformation pattern remains latent until activated through the introduction of an anaerobic zone to the head of the activated sludge plant. Cultivation of the bacteria from the enhanced culture on solid media and subsequent identification of the isolates using the API 20NE profile resulted in high counts of *Pseudomonas* spp. and the gamma *Proteobacteria*. The bias imposed upon the gamma *Proteobacteria* using cultivation-dependent methods has been previously reported and discussed in this report. The high numbers of *Pseudomonas* spp. using this technique cannot therefore be accepted although it was surprising that *Acinetobacter* counts remained low even though cultivation conditions conducive to their proliferation were provided. Total cell counts using plating techniques were generally 1 000-fold lower than counts obtained with membrane filtration and DAPI staining. These results indicate how unsuitable plating methods are for isolating and identifying the bacterial community from activated sludge. The microbial population profile using FISH did not seem to alter throughout the system with counts of the various classes of bacteria remaining fairly constant within the various reactor zones. This implies that when conducting microbial community analyses of activated sludge from full-scale plants, point of sampling should not affect results drastically.

Exposing a single aerobic reactor activated sludge (Amanzimtoti WWW) to EBPR selection appeared to allow for the proliferation of the alpha and gamma subclasses of the *Proteobacteria*. Although beta *Proteobacteria* counts decreased slightly, high cell counts indicated that this group of bacteria is still active and functional in EBPR operations. Greater bacterial metabolic activity in the enhanced culture also seemed to occur, evident from higher EUB hybridizations.

The results of this study seem to indicate that due to the diversity of their metabolism, *Pseudomonas* spp. are able to achieve dominance in BNR activated sludge plants (as indicated by cultivation of Darvill WWW and enhanced culture mixed liquors on CGY agar). Although they are unable to accumulate poly-P as efficiently as other known PAO's ie., *Acinetobacter* spp., their high cell counts in mixed liquors will enable them to remove high quantities of P from the system which may account for the majority of the observed phosphate removal.

6.2 RECOMMENDATIONS

- the API system of identification (or other identification systems such as BIOLOG™ or Microbact) can be used as pre-cursory methods to presumptively identify bacteria from environmental samples prior to the application of molecular techniques such as FISH. Although not conclusive, identification will generally assist the molecular biologist as to which probes to purchase/synthesise and which to apply to the sample (if other techniques such as denaturing gradient gel electrophoresis (DGGE) are not available).
- it appears from literature that more qualitative data regarding the presence of intracellular lipophilic and metachromatic granules can be gathered using the dual staining technique of Rees *et al.*, (1992) (using Nile blue A and Neisser's methyleneblue) rather than the techniques suggested by Jenkins *et al.*, (1984). It seems that recently there is much confusion concerning the quality of the conventional Neisser staining procedure in that its ability to conclusively differentiate between intracellular poly-P and PHB granules is questionable.
- the protocol suggested by Ubukata and Takii (1998) for the isolation and identification of PAO's from activated sludge should be investigated further. The authors state that the procedure requires the use of a minimum amount of sterile equipment, a reduced number of experimental steps and reduced labour time.
- there is potential to use molecular techniques (EUB/DAPI ratios and nucleic acid extractions) to extrapolate the heterotrophic active biomass fraction of calculated MLVSS values, essential to the validation of existing kinetic and stoichiometric activated sludge models (Ubisi *et al.*, 1997).
- more rigorous application of molecular analyses of EBPR sludge is required to conclusively identify PAO's. One approach may be to use probes that are more discriminating than the group specific probes

which were used in this study.

- it appears that a thorough molecular based investigation has not yet been conducted where a non-EBPR sludge is gradually influenced to exhibit EBPR and population dynamics monitored. Results of this type of study (with probes applied at the species level of organisation) should indicate exactly which bacteria are implicated in phosphate uptake and removal.

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APPENDICES

APPENDIX 1

PREPARATION OF CASITONE GLYCEROL YEAST AUTOLYSATE AGAR

Ingredients

Bacto® casitone	5 g
Glycerol	5 g
Yeast autolysate	1 g
Bacteriological agar	16 g
Distilled water	1 000 mL

Preparation

All ingredients, except for the glycerol, are heated and continuously stirred until clarified. Remove from heat, add glycerol and continue stirring until dissolved. Adjust pH to 7.2 and autoclave at 121°C for 20 mins.

APPENDIX 2

PREPARATION OF NEISSER STAIN

Preparation

Solution 1:

Solution 1 consists of two separate solutions, A and B which must be prepared and stored separately.

A		B	
Methylene Blue	0.1 g	Crystal Violet (10% w/v in 95% ethanol)	3.3 mL
Ethanol (95%)	5 mL	Ethanol (95%)	6.7 mL
Glacial acetic acid	5 mL	Distilled water	100 mL
Distilled water	100 mL		

Mix 2 volume parts of A with 1 volume part of B and prepare fresh monthly.

Solution 2:

Bismark Brown Y (1% w/v in distilled water)	33.3 mL
Distilled water	66.7 mL

Procedure

- Thin smears of the culture are prepared on the microscope slide and air dried (no heat fixation).
- Stain for 30 sec with Solution 1 and rinse for 1 sec with water.
- Stain for 1 min with Solution 2, rinse well and blot dry.
- Examine under oil immersion at 1 000X magnification using light microscopy.

Interpretation of results

- Positive = blue-violet (either entire cell or intracellular granules)
- Negative = yellow-brown

APPENDIX 3

PREPARATION OF POLYHYDROXYBUTYRATE (PHB) STAIN

Preparation

Separately prepare the following solutions:

Solution 1	**Solution 2**
Sudan Black B (IV) (0.3% w/v in 60% ethanol)	Safranin O (0.5% w/v in distilled water)

Procedure

- Thin smears of the culture are prepared on the microscope slide and air dried (no heat fixation).
- Stain 10 min with Solution 1.
- Rinse 1 sec with water.
- Stain 10 sec with Solution 2, rinse well with water and blot dry.
- Examine under oil immersion at 1 000X magnification with light microscopy.

Interpretation of results

Positive = PHB granules appear as intracellular blue-black granules whilst cytoplasm will either be pink or clear.

APPENDIX 4

COLLECTION AND PRESERVATION OF SAMPLES

Samples were, as far as possible, collected and preserved according to Standard Methods (1989). As stated in Standard Methods, the primary objective of sampling is that relative proportions and concentrations of all pertinent components within the sample will be representative of the material been sampled and that no significant changes in composition occur before analysis.

During operation of the pilot-plant, daily grab samples were considered adequate for analysis due to the fairly constant load entering the plant. All sampling bottles were triple rinsed with the water of interest ie., influent, effluent or mixed liquor, prior to collection. The sampling bottles were 250 mL plastic honey jars. The following preservation techniques were used for the duration of the project:

Determinand	Preservation technique	Maximum storage
Total Plate Count	none	immediate
COD	H_2SO_4 addition to pH<2; refrigerate	24 h
Ammonia	H_2SO_4 addition to pH<2; refrigerate	24 h
Nitrate	H_2SO_4 addition to pH<2; refrigerate	24 h
Kjeldahl nitrogen	H_2SO_4 addition to pH<2; refrigerate	24 h
Total phosphorus	H_2SO_4 addition to pH<2; refrigerate	24 h
Soluble reactive phosphorus	immediate 0.45 μm filtration; refrigerate	24 h
Solids	refrigerate	6 h

APPENDIX 5

DETERMINATION OF CHEMICAL OXYGEN DEMAND (CLOSED REFLUX MICROWAVE DIGESTION)

Overview

The chemical oxygen demand (COD) of a wastewater is a measure of the oxygen equivalent of the organic matter content which can be oxidised by a strong chemical oxidant ie., potassium dichromate. Samples are heated by microwave radiation in strongly acidic solutions with a known excess of potassium dichromate. After digestion, the remaining unreduced potassium dichromate is titrated with standard ferrous ammonium sulphate (FAS) and COD is expressed in units of mgO_2/L.

Reagents

A. Sulphuric acid/silver sulphate solution

Add 25 g silver sulphate to 2.5 L conc. H_2SO_4. Allow to stand for 2 d before use. Prepare every 2 weeks.

B. Ferroin indicator

Weigh 1.485 g 1,10 phenanthroline monohydrate and 0.695 g ferrous sulphate and dissolve in ~ 20 mL distilled water. Dilute to 100 mL. Prepare every month.

C. Mercuric sulphate

Analytical procedure

Add ~ 0.4 g mercuric sulphate to each 120 mL teflon PFA pressure digestion vessel and add 20 mL homogenised sample. Add 30 mL sulphuric acid/silver sulphate reagent and mix. Add 10 mL 0.25 N potassium dichromate solution. Samples are microwaved according to procedure. After digestion, vessels are cooled to room temperature in a cooling bath. Digestion contents are then quantitatively transferred to titration vessels and the residual dichromate titrated against FAS. Titration end points are either indicated potentiometrically or by a colour change

from green to reddish-brown after the addition of 3-4 drops ferroin indicator.

Preparation of standards

Ferrous ammonium sulphate working solution (~0.13 N)

Weigh 52 g FAS and dissolve in ~700 mL distilled water. Add 26 mL conc. H_2SO_4. Allow to cool and dilute to 1 L with distilled water. Prepare solution every 2 weeks.

Potassium dichromate working solution (0.25 N)

Weigh 12.2588 g potassium dichromate (dried to constant weight at 105°C) and dissolve in distilled water (dilute to 1 L). Prepare solution every 2 weeks.

Calculation of results

Standardisation of potassium dichromate and the calculation of COD can be calculated using the following equations:

$$\textbf{Concentration of FAS (N)} = \frac{\textbf{0.25 (N) x 10 (mL)}}{\textbf{Titre}_{std}\ \textbf{(mL)}}$$

where: $Titre_{std}$ = volume (mL) of FAS used in titration against 10 mL standard potassium dichromate

$$\textbf{COD (mgO}_2\textbf{/L)} = \frac{\textbf{(Titre}_{blank}\textbf{ - Titre}_{sample}\textbf{)mL x conc. FAS (N) x 8000}}{\textbf{sample volume (mL)}}$$

where: $Titre_{sample}$ = volume (mL) FAS used in titration against sample

$Titre_{blank}$ = volume (mL) FAS used in blank titration)

APPENDIX 6

DETERMINATION OF INFLUENT READILY BIODEGRADABLE SOLUBLE COD (S_{bsi}) IN WASTEWATER

Overview

Influent total biodegradable soluble COD (COD_{sol}) can be divided into two sub-fractions ie., influent readily biodegradable soluble COD (S_s) and influent non-readily biodegradable soluble COD (S_i). However, in systems where the wastewater of interest is treated biologically at a MCRT of > 3 d, influent S_i can be considered equal to the truly soluble effluent COD ie., influent soluble unbiodegradable COD. S_s consists of simple organic molecules such as volatile fatty acids and low molecular weight saccharides that can pass directly through the cell membrane and be metabolised directly or accumulated intracellularly within minutes. The method applied to calculate S_s during this study was based on the physical-chemical method devised by Mamais *et al.*, (1993) (see Chapter 2, section 2.3.2.1 for details). The method involves removal of colloidal matter that normally passes through 0.45 μm membrane filters by flocculation and precipitation prior to filtration.

Method

Influent and effluent samples (100 mL) were flocculated by adding 1 mL of a 100 g/L zinc sulphate solution ($ZnSO_4.7H_2O$, ACE Chemicals) to each and mixed vigorously with a magnetic stirrer for approximately 1 min. Mixed sample pH was adjusted to approximately 10.5 with 6 M sodium hydroxide solution and allowed to settle quiescently for 5 min. Clear supernatant aliquots (20 - 30 mL) were withdrawn with a disposable syringe and passed through a 0.45 μm membrane filter (Millipore). The COD of both samples was determined and influent soluble biodegradable COD was determined according to the following equation:

$$S_s = COD_{sol} - S_i$$

where:

S_s	=	influent truly soluble biodegradable COD
COD_{sol}	=	influent total truly soluble COD
S_i	=	effluent truly soluble COD

APPENDIX 7

TOTAL PHOSPHATE DETERMINATION

Overview

Total phosphorus (TP) determination of a wastewater sample includes all orthophosphate and polyphosphate, both dissolved and particulate, organic and inorganic. To release organically bound phosphorus, a digestion and oxidation procedure is necessary. Samples are digested using a persulphate digestion technique and an autoclave. All forms of phosphorus are then converted to orthophosphate, the concentration of which is determined colorimetrically.

Reagents

A. Vanadomolybdate solution

Dissolve 20 g ammonium molybdate ($(NH_4)_6Mo_7O_{24}.4H_2O$) and 1 g ammonium meta-vanadate (NH_4VO_3) in ~ 500 mL distilled water. Add 140 mL conc. nitric acid (HNO_3, 55%) and mix well. Allow to cool and make up to 1 L with distilled water. Store reagent in the dark and prepare weekly. Discard if precipitate or growth occurs.

B. Digestion reagent - 7.1% (w/v) potassium persulphate

Dissolve 7.1 g potassium persulphate ($K_2O_8S_2$) in ~ 80 mL distilled water and make up to the 100 mL mark. Dissolve with gentle stirring and heating. Prepare a fresh solution for every digestion.

C. Digestion reagent - 0.6 M sulphuric acid

Add 33 mL conc. sulphuric acid (97%) to ~ 800 mL distilled water. Allow to cool and make up to 1 L.

Preparation of standards

Stock solution 100 mg/L

Dissolve precisely 0.8794 g potassium dihydrogen orthophosphate (KH_2PO_4) in ~ 1 500 mL distilled water. Fill up to 2 L with distilled water and mix well. Store the solution in a fridge at 4°C and prepare every 3 months.

Working standards

0	mgP/L:	distilled water
5	mgP/L:	dilute 50 mL stock solution to 1 000 mL with distilled water
10	mgP/L:	dilute 100 mL stock solution to 1 000 mL with distilled water
15	mgP/L:	dilute 150 mL stock solution to 1 000 mL with distilled water
25	mgP/L:	dilute 250 mL stock solution to 1 000 mL with distilled water
50	mgP/L:	dilute 500 mL stock solution to 1 000 mL with distilled water

Add 1 mL conc. HNO_3 to each standard (preservative).

Analytical procedure

Transfer 20 mL sample, standard and AQC to 50 mL borosilicate tubes. Add 5 mL 0.6 M H_2SO_4 and 5 mL 7.1% (w/v) $K_2O_8S_2$) to each tube and mix well. Cover tubes with aluminium foil and digest for 1 hour in a pressure cooker at 100 kPa. Allow tubes to cool to room temperature and mix contents by inverting. Turbid samples are filtered into clean tubes (standards must not be filtered). Transfer 5 mL aliquots of each sample, standard and AQC into 20 mL test tubes. Add 5 mL vanadomolybdate solution to each tube and allow to stand for 30 mins for full colour development before reading absorbancy at 470 nm. Use deionised water as a reference.

Calculation of results

All results were entered onto a computer using Lotus 1-2-3 software and a standard curve plotted. Concentration (mgP/L) was calculated from absorbance using the equation for a linear curve:

$$y = mx + c$$

where y = concentration (mgP/L)
m = gradient of curve
x = absorbance (nm)
c = y-intercept

Values for m, x and c can be determined using data analysis software.

APPENDIX 8

SOLUBLE REACTIVE PHOSPHATE DETERMINATION

Overview

Phosphates that respond to colorimetric tests without preliminary hydrolysis or oxidative digestion of the sample are termed 'reactive phosphorus'. Filtration through 0.45 μm membrane filters is a replicable method used to separate suspended and soluble forms of reactive phosphorus. Soluble reactive phosphorus is routinely monitored in wastewater treatment plants due to its availability to the resident microflora of receiving water bodies.

Reagents

A. Vanadomolybdate solution

As for Appendix 7

Analytical procedure

Samples were immediately filtered through 0.45 μm membrane filters. Filtered sample (5 mL) was pipetted into a test tube. All samples were done in duplicate to allow for blanks. Deionised water (5 mL) and vanadomolybdate solution (5 mL) were added to each blank and sample, respectively. Contents of the tubes were mixed well and allowed to stand for 30 mins for full colour development. The absorbance of samples and blanks was measured at 470 nm using deionised water as a reference.

Calculation of results

SRP concentration of wastewater samples (mgP/L) was calculated as follows:

SRP = (Abs of sample) - (Abs of blank) x 110.46

It must be noted that this was a rapid formula for SRP determination proposed by de Haas (1998). Although the formula was initially adapted for this project, standard curves were also plotted due to discrepancies in the results.

APPENDIX 9

DETERMINATION OF SOLIDS (MIXED LIQUOR SUSPENDED SOLIDS AND VOLATILE SUSPENDED SOLIDS)

Overview

Mixed liquor suspended solids (MLSS) is defined as the total amount of organic and mineral suspended solids contained in the mixed liquor of the activated sludge reactor. The procedure of MLSS determination is relatively simple to perform and offers system operators a rapid indication of sludge biomass concentration. The organic portion of MLSS is represented by mixed liquor volatile suspended solids (also referred to as mixed liquor organic suspended solids) which is comprised of non-microbial organic matter as well as dead and live microorganisms and cellular debris. MLVSS values are usually reported as a percentage of the MLSS

Determination of MLSS

Pipette 100 mL of sample into a centrifuge tube. Centrifuge at 3 000 rpm for 6 min. Discard supernatant and quantitatively scoop sludge pellet into a pre-weighed crucible. Place crucible into a drying oven at 105°C and leave overnight. Remove from oven and allow to cool in desiccator. Reweigh crucible. MLSS is determined according to the following calculation:

$$\text{MLSS (g/L)} = \frac{\text{mass of (crucible + sludge) - mass of (crucible)}}{\text{100 mL}} \times 10$$

Determination of VSS

Place pre-weighed crucible containing sludge (from MLSS determination) into a muffle furnace. Ash at 550°C for 1 h. Remove crucible, allow to cool in desiccator and re-weigh. It is common practice to express VSS as a percentage of the MLSS according to the following equation:

$$\text{VSS (\%)} = \frac{\text{mass of (crucible + sludge) - mass of (crucible + ash)}}{\text{mass of (crucible + sludge) - mass of (crucible)}} \times 100$$

APPENDIX 10

DETERMINATION OF SLUDGE VOLUME INDEX (SVI) AND DILUTED SVI (DSVI)

Overview

The significance of calculating SVI and DSVI is to monitor the settling characteristics of the mixed liquor and to alert the system operator if the sludge is displaying bulking characteristics. The test involves settling a sludge sample (SVI) or diluted sludge sample (DSVI) in a measuring cylinder for a specific time period and recording the volume it occupies, in conjunction with a MLSS test. It is common practice to perform a DSVI test when SVI sedimentation exceeds 200 mL/L.

Procedure

The procedure for SVI determination will be explained in conjunction with DSVI as both methods require the same protocol. Four 1 000 mL measuring cylinders are required. Fill a container with mixed liquor from the outlet of the aeration basin and agitate ie., do not allow the sludge to settle. Fill each of the measuring cylinders as follows:

- cylinder 1 - fill to the 1 000 mL mark;
- cylinder 2 - fill to the 500 mL mark;
- cylinder 3 - fill to the 250 mL mark; and
- cylinder 4 - fill to the 125 mL mark.

Cylinders 2, 3 and 4 are then filled to the 1 000 mL mark using clarified effluent. All four cylinders are then inverted three times (using your hand over the top of the cylinder) to homogenise the sludge mixture. The cylinders are then placed on a flat surface and allowed to settle quiescently for 30 mins. After 30 mins of settling, the volume occupied by the sludge in each cylinder is recorded.

Determination of SVI/DSVI

Cylinder 1 ie., cylinder containing only mixed liquor is used to calculate SVI, defined by the following equation:

$$\textbf{SVI (mL/g)} = V_{30} / X$$

where: V_{30} = volume of settled sludge after 30 mins sedimentation
X = concentration of activated sludge (g/L)

The DSVI is calculated from the cylinder (either 2, 3 or 4) which has a sludge volume of less than 200 mL after settling and is defined by the following equation:

DSVI = SV30 / adjusted X

where: V30 = volume of settled sludge after 30 mins sedimentation
adjusted X = factor by which to divide the MLSS (see table below)

Cylinder number	Dilution	Factor to divide MLSS for adjusted MLSS calculation
1	Nil	1
2	50%	2
3	25%	4
4	12.5%	8

APPENDIX 11

CALCULATION OF SLUDGE MASS IN REACTOR COMPARTMENTS

Overview

The mass fraction of a particular zone ie., unaerated or aerated, in an activated sludge reactor is defined as the mass of sludge in the particular compartment divided by the total mass of sludge in the reactor. It is important to constantly monitor mass fractions for even in completely mixed reactors the concentration of sludge in each zone will not be constant. Mass fractions are significant to the success of any nutrient removal operation in that they influence:

- the minimum sludge age for nitrification;
- the extent to which the plant will denitrify; and
- the phosphorus removal ability.

Mass fraction determination

Calculate the MLSS in each zone (see APPENDIX 9). Multiply the measured MLSS values by the volume of the respective reactor. The total mass of sludge in the system is then determined and used to fractionate the sludge in the various zones.

An example of how to calculate mass fractions for a 3-stage Bardenpho configuration follows:

Compartment	Volume (m^3)	Measured [MLSS] (g/L)	Mass of sludge (kg) (V x MLSS)	Fraction (%)
Anaerobic	4	3	12	24
Anoxic	3	2	6	12
Aerobic	8	4	32	64
TOTAL	**15**		**50**	**100**

APPENDIX 12

FIXATION OF BACTERIAL CELLS FOR FISH ANALYSIS

Overview

Due to the unfavourable conditions which microbial cells are exposed to during cell-probe hybridization ie., elevated temperatures, detergents and osmotic gradients, fixation of the environmental sample is essential to maintain the morphological integrity of the cells. Although gluteraldehyde can be used to fix cells, it often results in excessive autofluorescence which interferes with interpretation of the microscopic field. Fixation with paraformaldehyde solutions reduces this excessive background 'noise' and assists with cell counting. However, probe permeability of paraformaldehyde fixed Gram positive cells is limited although probe penetration into these cells can be enhanced by fixation in an ethanol series or by short time fixation in an alcohol/formaldehyde mixture.

Procedure

(1) Prepare the following solutions:

(a) 1 x phosphate buffered saline

- 130 mM sodium chloride
- 10 mM sodium phosphate buffer (Na_2HPO_4); correct solution pH to 7.2.

(b) 3 x phosphate buffered saline

- 390 mM sodium chloride
- 30 mM sodium phosphate buffer (Na_2HPO_4); correct solution pH to 7.2.

(c) 4% paraformaldehyde in PBS (not older than 24 h)

- heat 65 mL deionised water to 60°C and add 4 g paraformaldehyde;
- add one drop 2 M NaOH solution and stir rapidly until solution has nearly clarified;
- remove from heat, add 33 mL 3 x PBS and adjust pH to 7.2 with Hcl;
- cool rapidly to 4°C and store in refrigerator.

(d) absolute ethanol

(2) Paraformaldehyde fixation of microbial cell suspensions

(i) add three volumes paraformaldehyde fixative to one volume sample and hold for 1 h at 4°C;

(ii) pellet fixed cells by centrifugation (5 000 x g) for 4 mins and remove fixative;

(iii) wash cells in 1 x PBS and resuspend in 100 μL 1 x PBS;

(iv) add one volume ice-cold absolute ethanol and mix;

(v) fixed cells may be hybridized immediately or stored in the freezer (-20°C) for several months.

APPENDIX 13

IMMOBILIZATION OF FIXED CELLS ON MICROSCOPE SLIDES

1. Pretreatment of microscope slides

Slides are cleaned by soaking in a warm detergent solution (10% w/v potassium hydroxide in absolute ethanol) for two hours followed by a thorough rinse with distilled water. Slides are allowed to air dry. Poly-L-lysine solution (Sigma; 0.1%, w/v) is diluted 1:10 with deionised water. Clean slides are placed in diluted poly-L-lysine (room temperature) for 5 min. Slides are drained and dried in an oven at 60°C for 1 h or at room temperature overnight.

2. Immobilization of fixed microbial cells on poly-L-lysinated slides

Three microlitres (μL) of the fixed cell suspension is applied to the poly-L-lysinated slide over a surface area of approximately 5 mm diameter and allowed to air dry. A hydrophobic coating can be applied to the slide thereby separating several spots on the same slide. The hydrophobic coating prevents mixing of probes applied to adjacent spots on the slide. Immobilised cells are then dehydrated through successive passaging through an ethanol series (60, 80 and 96% ethanol of 3 min each).

APPENDIX 14

DETERMINATION OF TOTAL CELL COUNTS

View the slide with the mounted filter under epiflourescence (400 X) and select ten random, well dispersed fields for cell counts. Determine the mean total cell count for the ten fields. Multiply the mean by the dilution factor (see below) and the number of possible microscopic fields under 400 X magnification (22 281). The result is expressed as the total number of cells per mL of mixed liquor.

Activated sludge dilution: 5 μL/1000 μL = 1/200; therefore multiply by 200.

$$\mathbf{TCC = MTCC \times DF \times MF}$$

where:	TCC	=	total cell count
	MTCC	=	mean total cell count for ten microscopic fields
	DF	=	dilution factor (200)
	MF	=	microscopic field permutations (22 281 under 400 X magnification)

APPENDIX 15

WHOLE CELL HYBRIDIZATION

Materials required

- 50 mL polypropylene screw top tube
- Whatman 3MM paper or equivalent
- Hybridization buffer

  0.9 M sodium chloride

  0.01% sodium dodecylsulphate (SDS)

  20 mM Tris/HCl

  X% formamide (depending on the probe and stringency required) [1]

  pH 7.2

Procedure

1. Soak a slip of Whatman 3MM paper in hybridization buffer and place in tube. Hybridization must be conducted in a properly sealed moisture chamber to prevent evaporative concentration of the hybridization solution which might result in nonspecific binding of the probe to cells.
2. Allow humidity chamber to equilibrate at the hybridization temperature of 46°C.
3. For each spot to be hybridised, mix 9 μL of hybridization buffer with 1 μL fluorescent probe.
4. Spread 10 μL hybridization buffer/probe mix on each spot of fixed cells.
5. Transfer slide to pre-warmed moisture chamber and hybridise for 2 h.
6. Remove slide from chamber and immediately stop hybridization by rinsing the probe from the slides with 2 mL washing buffer [2] (containing no formamide) pre-warmed to 48°C.
7. Transfer the slide in polypropylene screw top tube filled with 50 mL washing buffer to a water bath/incubator and incubate for 20 min at 48°C.
8. Remove salts by quickly dipping slide in double distilled water, shake vigorously to remove excess water and air dry in the dark.

9. Spot 10 μL DAPI (0.5 μg/mL) to the air dried, hybridised smears and leave in the dark for 5 min.

10. Wash with double-distilled water, air dry and mount coverslip with VECTASHIELD®.

11. View slide with an epifluorescent microscope.

Note

① formamide concentration stringency required for hybridization of probe during the present study is given in TABLE 5.1;

② the stringency of the washing step can be increased by decreasing the sodium chloride concentration; for probes EUB338 and ALF1b washing solution contains 20 mM Tris, 0.01% SDS, 180 mM NaCl, 5 mM EDTA; for probes CF and HGC washing solution contains 20 mM Tris, 0.01% SDS, 150 mM NaCl, 5 mM EDTA; and for probes ACA23a, BET42a and GAM42a washing solution contains 20 mM Tris, 0.01% SDS, 120 mM NaCl, 5 mM EDTA.