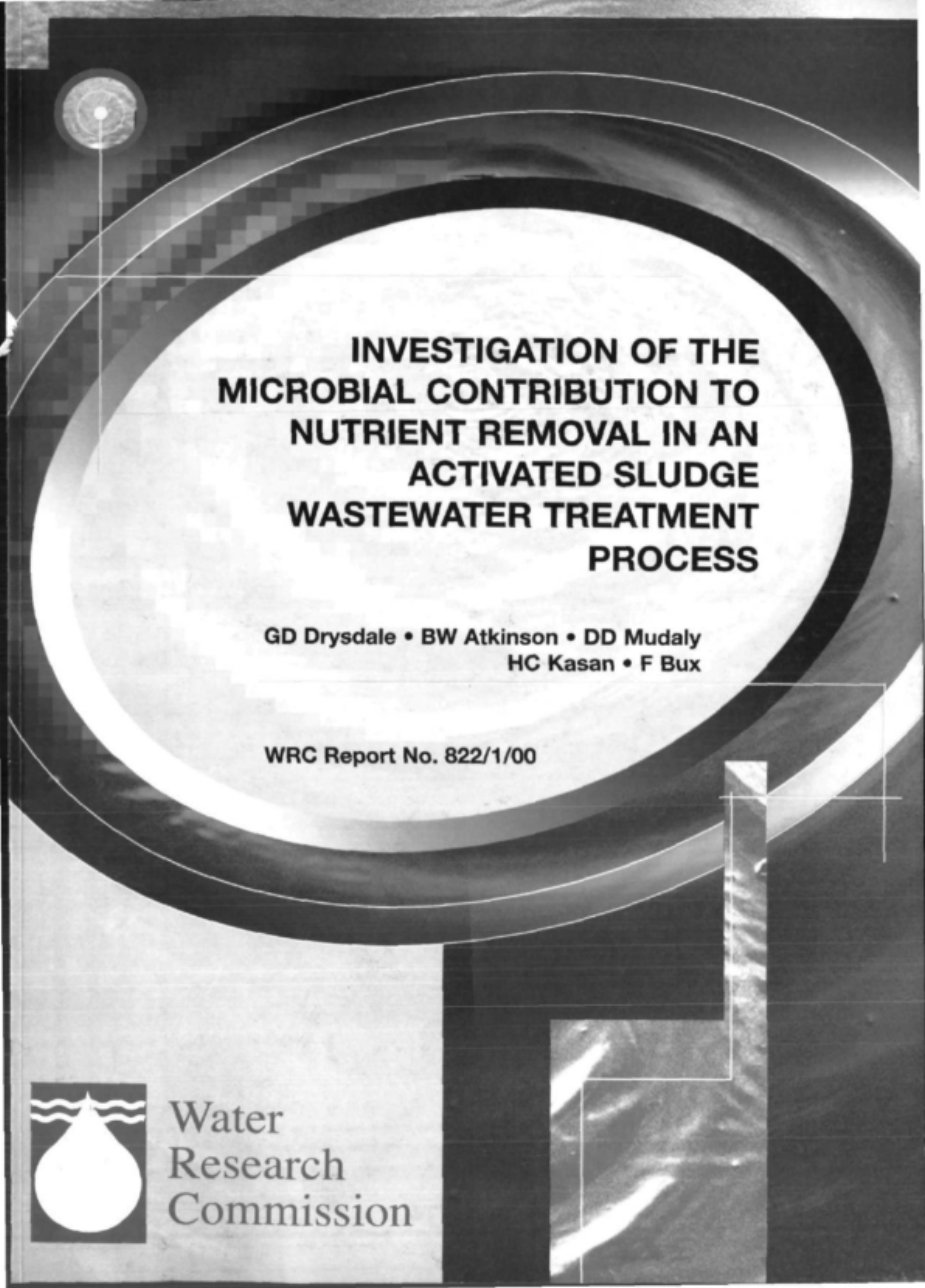

INVESTIGATION OF THE MICROBIAL CONTRIBUTION TO NUTRIENT REMOVAL IN AN ACTIVATED SLUDGE WASTEWATER TREATMENT PROCESS

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WRC Report No. 822/1/00

Water
Research
Commission

Investigation of the microbial contribution to nutrient removal in an activated sludge wastewater treatment process

Final Report to the
Water Research Commission

by

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EXECUTIVE SUMMARY

1. Background and motivation

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 and nitrogen, 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).

Wastewater entering a treatment plant is composed of many different and constantly changing substrates/pollutants which only mixed populations of microorganisms are capable of metabolising. The microbial community in wastewater treatment systems exist in unique and transient ecosystems, dependent upon contaminant type, load and the ambient oxidation reduction potential.

Although Koch's principles for other nutrient removal processes during biological wastewater treatment viz., nitrogen assimilation in biomass, nitrification and denitrification as well as ammonia release from organic nitrogen, have been satisfied, the same cannot be said for biological excess phosphorus removal processes (Jenkins and Tandoi, 1991). Although the process of biological excess phosphorus 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). Since pure cultures which possess complete characteristics of polyphosphate accumulating organisms have not been isolated yet, the biochemical mechanism cannot be definitively described (Mino *et al*., 1998). Without understanding the correlation between the polyphosphate accumulating bacteria community structure within activated sludge mixed liquor (as well as the interactions between autotrophic and heterotrophic bacteria involved in nitrification and denitrifiers involved in simultaneous phosphorus uptake) and the wastewater plant's performance, reliable and efficient nitrification/denitrification biological excess phosphorus removal operations will remain difficult to design. In-depth microbial community analyses and descriptions of the vast interactions occurring amongst the bacteria involved in wastewater remediation are therefore integral to advance the science and efficiency of processes such as biological nutrient removal.

The process engineering and technology (modelling and implementation) and microbiological and biochemical disciplines have traditionally worked independently of one another. A consequence of this is that microbiologists can find it difficult to express information gathered in engineering terms which could otherwise be implemented to address the deficiencies of the existing steady-state design models and kinetic simulation models. Rapid developments in and application of molecular biological techniques have made a considerable contribution to the understanding of biological nutrient removal activated sludge systems. These techniques also provide quantitative data which is a prerequisite for the successful modelling of these systems. Integration of the engineering and microbiological/biochemical disciplines should remain the focus activity area in activated sludge research for the near future. To this end, a new multi-institutional Water Research Commission project has been initiated in which both engineering and microbiological-based research groups are participating.

2. Statement of objectives

The initial research proposal submitted to the Water Research Commission was entitled:

"INVESTIGATION AND COMPARISON OF THE MICROBIAL CONTRIBUTION TO NUTRIENT REMOVAL IN ACTIVATED SLUDGE AND TRICKLING FILTER WASTEWATER TREATMENT PROCESSES"

The aim of the original project was to establish a relationship between the microbial composition of activated sludge and trickling filter processes and the three primary functions of biological wastewater treatment viz., carbon, nitrogen and phosphorus removal.

The objectives of the proposed research programme were as follows:

- microbiological survey at different sites of wastewater treatment at Darvill and Umbilo Wastewater Treatment Plants;
- simultaneous monitoring of plant parameters including chemical and biological oxygen demand, nitrogen, phosphorus, pH etc.;
- establishment of extent of correlation between microbial predominance and nutrient removal viz., carbon, nitrogen and phosphorus;
- comparison of treatment technologies i.e., trickling filter and activated sludge, with respect to the microbial community and nutrient removal; and
- construction of database of above correlations for future use in design and optimization of respective treatment processes.

With the consensus of the Water Research Commission Steering Committee, it was decided to alter the title and objectives of the original proposal slightly. These changes would therefore produce a project of more significance to the activity area of biological nutrient removal and be more in line with national and international trends.

"INVESTIGATION OF THE MICROBIAL CONTRIBUTION TO NUTRIENT REMOVAL IN AN ACTIVATED SLUDGE WASTEWATER TREATMENT PROCESS"

The use of trickling filters for nutrient removal was not investigated as it is not common practice to use this technology in wastewater treatment plants in KwaZulu-Natal any longer. The research project was therefore designed to:

- conduct a microbiological survey of a full-scale nitrification/denitrification biological excess phosphorus removal plant in KwaZulu-Natal (Darvill Wastewater Works, Pietermaritzburg), concentrating on the various redox zones i.e., anaerobic, anoxic and aerobic;

- establish the extent of correlation between microbial dominance in a particular zone and the extent to which this dominance contributes to the removal of specific nutrients;
- increase current knowledge of microbial community structure-functionality;
- assess the removal capacity of specific, isolated bacteria in order to determine their individual contribution to the system as a whole;
- assess molecular techniques (fluorescence *in situ* hybridizations), as opposed to conventional cultivation techniques, to determine the microbial community structure; and
- compare various culture-dependent identification techniques such as the Analytical Profile Index, Microbact and Biolog assays using bacteria isolated from activated sludge.

Within the context of improving the microbiological knowledge of biological nutrient removal, three core areas of activity were defined and selected for in-depth investigation. These included (i) biological excess phosphorus removal; (ii) biological nitrogen removal (nitrification and denitrification processes) and; (iii) the implementation and use of molecular techniques such as fluorescence *in situ* hybridization and dot blot hybridizations to conduct quantitative and qualitative studies of the bacteria involved. Research protocols and results of these three sub-activity areas will be reported and discussed individually for ease of reading.

3. Summary of results

Biological phosphorus removal

The experimental procedure for biological phosphorus removal assays was conducted in two phases: (i) laboratory, where samples of mixed liquor were obtained from Darvill Wastewater Works and, (ii) pilot plant, where an enhanced culture of polyphosphate accumulating organisms was developed. During phase 1 of experimentation, mixed liquor samples were taken from the aerobic zone of Darvill Wastewater Works and cultivated on solid media. Isolates were screened for their ability to accumulate polyphosphate through successive anaerobic/aerobic incubation and only those isolates displaying the desirable phosphorus transformation patterns i.e., anaerobic phosphorus release and aerobic phosphorus uptake, were subsequently identified. Identification of the various isolates was achieved using the Gram stain, various qualitative biochemical tests and the Analytical Profile Index 20NE identification system. Significant findings during biological excess phosphorus removal experimentation on a full-scale wastewater works included:

- cultivation results showed the 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 polyphosphate accumulating bacterial community;
- although *Acinetobacter* spp. accumulated large quantities of polyphosphate from a phosphate enriched liquid medium, low recovery rates of this genus on solid agar media did not warrant its implication as the organism pivotal to phosphate uptake and removal from the activated sludge system in question.

An enhanced culture of polyphosphate accumulating organisms, at pilot-scale, was developed during phase 2 of experimentation by incrementally increasing the acetate fraction whilst concomitantly decreasing the settled sewage fraction to the feed influent of a laboratory unit. The pilot plant, modelled on the 3-stage Phoredox process, was seeded with mixed liquor obtained from a non-nutrient removing (perhaps apart from coincidental nitrification) single aerobic full-scale activated sludge plant (Amanzimtoti Wastewater Works, KwaZulu-Natal South Coast) in order to primarily investigate population dynamics and to monitor changes (if any) in the bacterial community structure when subjected to conditions conducive to the biological excess phosphorus removal mechanism. Community analyses of both activated sludge systems were performed using both conventional cultivation and molecular techniques which involved ribosomal ribose nucleic acid-targeted fluorescently-labeled oligonucleotide probes. Significant findings when conducting cultivation-dependant microbial community analyses on an enhanced biological excess phosphorus removal culture at pilot-scale were:

- *Pseudomonas* spp. appeared to dominate the bacterial community;
- plating resulted in the total dominance of the gamma subclass of Proteobacteria;

- results of this aspect of the study indicated the importance of the Proteobacteria to biological excess phosphorus removal operations. Surprisingly, the dominance of *Acinetobacter* spp. in biological excess phosphorus removing activated sludge was never demonstrated, even when using plating techniques.

In situ probe hybridization investigations

The total bacterial population of both full- and pilot-scale biological excess phosphorus removing activated sludges was found to be underestimated by at least three orders of magnitude by cultivation-dependant methods. In addition, cultivation-dependant techniques have been found to overestimate the gamma Proteobacterial subclass and underestimate the alpha and beta Proteobacteria.

- the alpha and beta Proteobacteria were both shown to be functional groups implicated in full- and pilot-scale biological excess phosphorus removing systems, together comprising more than 60% of the active population;
- however, the overall community profile shows that the biological excess phosphorus removal population is quite diverse and it is unlikely that any one species dominates the process.
- the polyphosphate accumulating population of the total active bacterial community was found to be at percentages of 55% for an enhanced pilot plant and 35% for the full-scale biological excess phosphorus removal system;
- the total number of polyphosphate accumulating organisms required to remove one gram of soluble phosphorus was thereby estimated to be between $1\text{-}2 \times 10^{11}$ cells;
- the quantity of biomass (as metabolically active bacterial cells) to be associated with one gram of volatile suspended solids was determined at 1×10^{13} cells;
- the portion of the volatile suspended solids present as biomass only was estimated to be 15% for the full-scale biological excess phosphorus removal system investigated.

Biological nitrogen removal

Findings confirm the existence of a diverse community of heterotrophic bacteria involved in nitrogen removal during wastewater treatment of which *Pseudomonas* spp. show significant involvement. Numerous heterotrophic bacteria were found to be capable of nitrification.

- heterotrophic isolates demonstrated direct oxidation of ammonium to nitrates with little or no nitrite accumulation;
- different patterns of nitrification behaviour were noted amongst heterotrophic nitrifiers with some displaying potential nitrification/denitrification behaviour;
- *Staphylococcus* and *Micrococcus* spp. showed significant involvement in nitrification along with Streptococcus, *Pseudomonas* and *Bacillus* spp.

With respect to denitrification, the ordinary heterotrophic fraction within the Darvill Wastewater Works mixed liquor was found to comprise five distinct functional groups, four of which interactively contribute to denitrification occurring in the system and one group that are non-denitrifying.

- these groups were defined and characterised as true denitrifiers (bacteria capable of both nitrate and nitrite reduction), incomplete denitrifiers (bacteria that reduced nitrates to nitrites with no further reduction of the nitrites produced), incomplete-nitrite reducers (bacteria capable of both nitrate and nitrite reduction, however, exhibiting severe inhibition of nitrite reduction by nitrates),exclusive nitrite reducers (bacteria only capable of reducing nitrites) and non-denitrifiers (bacteria not capable of nitrate or nitrite reduction).
- 60.7% of the bacteria isolated were capable of nitrate reduction as compared to 32% which showed any capacity for nitrite reduction.

The ordinary heterotrophic fraction is therefore more complex than currently accepted and needs to be amended for more accurate modelling of denitrification kinetics in nitrification/denitrification biological excess phosphorus removal processes.

Numerous heterotrophic bacteria were also found capable of phosphorus uptake under anoxic conditions utilising nitrates instead of oxygen.

- *Serratia* spp. and *Vibrio* spp. were the most efficient anoxic phosphorus accumulators isolated demonstrating 7.10 and 7.29 mgPO_4-P/L removal, respectively;
- weak phosphorus accumulating bacteria were also identified;

- these organisms may not necessarily be weak phosphorus accumulators under aerobic conditions, but, were limited under anoxic conditions due to weak denitrification capacity.
- anoxic phosphorus release was also observed by some denitrifying heterotrophic bacteria;
- *Pasteurella* spp. released a maximum of 5.91 mgPO_4-P/L with concurrent reduction of 6.25 mgNO_3-P/L.

4. Meeting the objectives

It was generally agreed that the research objectives, as formulated and detailed at the beginning of the Executive Summary, were successfully achieved. An in-depth microbiological survey of both a full- and pilot-scale nitrification/denitrification biological excess phosphorus removal activated sludge system was performed. Both cultivation-dependant and molecular *in situ* techniques were applied to obtain an adequate description of the physiologically significant microbial community involved in biological phosphorus removal, heterotrophic nitrification and denitrification. Autotrophic nitrifiers were not investigated during the course of this study due to difficulties in their isolation and excessively long growth rates. Of great significance during this study was the establishment of a molecular biology laboratory and application of the fluorescence *in situ* hybridization technique to obtain both qualitative and quantitative information regarding the microbial community in biological nutrient removal systems. Comparisons between various cultivation-dependant identification techniques were not performed due to the potential excessive costs in the purchase of software and consumables. All isolates were subsequently identified using the Analytical Profile Index.

5. Contribution to state-of-the-art

Upon initiation of this research project, fluorescence *in situ* hybridization technology had not yet been applied to any South African activated sludges. Microbial community analyses appeared to be largely confined to culture-dependant techniques. Although routinely applied at an international level, it appeared after an intensive literature search that fluorescence *in situ* hybridization had not yet been applied to continuous systems designed to stimulate biological excess phosphorus removal either. The majority of these articles seemed to be confined to the use of sequencing batch reactors and/or full-scale systems. Quantitative data provided by molecular based assays can also be used by the engineering and technology disciplines to improve existing activated sludge models and to address the deficiency of the active biomass concept within these models. Based on these points, it was felt that the current project positively contributed to the state-of-the-art in wastewater treatment both locally and abroad.

6. Recommendations for future research

- As microbiologists, efforts to isolate phosphorus accumulating organisms from biological excess phosphorus removal sludges should continue. Although this will require a certain degree of innovation, the presence of viable but non-culturable bacteria in environmental ecosystems should not deter these efforts;
- Although the broad community profile (family and sub-group levels) of an ecosystem can be determined through application of fluorescence *in situ* hybridization technology, techniques involving cloning, sequencing and probe design must also be incorporated to adequately describe the microbial community profile at a generic and species level;
- In order to improve the quantitative potential of molecular techniques when conducting activated sludge studies, methods to allow for maximum floc dispersion should be investigated. Counting of sufficient numbers of microscopic fields will also be required when attempting to provide the raw data required to advance the predictive and descriptive capacity of current mathematical models for nitrification/denitrification biological excess phosphorus removal processes;
- Heterotrophic nitrification needs to be evaluated *in situ* in order to establish the involvement, if any, of heterotrophic bacteria in nitrification occurring during wastewater treatment;
- The kinetics of nitrate and nitrite reduction need to be determined for true denitrifiers,

incomplete denitrifiers, incomplete-nitrite reducers and exclusive nitrite reducers in order to establish the contribution to denitrification by each group;

- A method needs to be established to determine the non-denitrifying and denitrifying fractions of the heterotrophic active biomass for more accurate modelling of denitrification behaviour in biological nutrient removal systems; and,
- The microbiology and biochemistry of anoxic phosphorus accumulation requires further investigation;
- Results of the Analytical Profile Index identification method need to be substantiated using additional biochemical tests and qualitative molecular techniques to allow more conclusive identification of bacterial isolates from environmental samples;
- Microbiologists/biochemists and process engineers must begin to communicate and not work independently of one another. The quantitative information which molecular biologists are now able to generate regarding active cell biomass will prove invaluable in improving the accuracy and predictive ability of the existing or new activated sludge mathematical models.

7. Technology transfer

i) Capacity building

The following students received relevant qualifications as a result of this project:

M. Tech. (Biotechnology) - B Atkinson
- G Drysdale
- D Mudaly

B. Tech. (Biotechnology) - M Sidat
- N Lacko
- M Chetty
- J Moulds
- W Stevens

Drysdale, GD, Kasan, HC and Bux, F (2000) Assessment of denitrification by the ordinary heterotrophic organisms in a NDBEPR activated sludge system. 1st World Congress of the International Water Association, Paris, France, 3-7 July.

Mudaly, DD, Bux, F and Kasan, HC (1998) The application of a fluorescently labeled rRNA targeted oligonucleotide probe for the *in situ* identification of bacteria in activated sludge. The Water Institute of Southern Africa Biennial Conference and Exhibition, Baxter Theater, Cape Town, 4-7 May.

Mudaly, DD, Moulds, J, Bux, F and Kasan, HC (1998) Dual staining of activated sludge with DAPI and a kingdom level 16S rRNA targeted oligonucleotide probe. South African Society for Microbiology 10th Biennial Congress, International Convention Centre, Durban, 5-8 July.

Mudaly, DD, Bux, F and Kasan, HC (1999) Determination of bacteria predominating in a pilot-scale enhanced biological phosphate removal activated sludge process using a cultivation independent approach. African International Environmental Protection Symposium, Imperial Hotel, Pietermaritzburg, 4-8 July.

Mudaly, DD, Atkinson, BW and Bux, F (2000) Combined approach of FISH and dot-blots for a study of bacteria predominating in full- and pilot-scale EBPR activated sludge processes. The COE Symposium on Establishment and Evaluation of Advanced Water Treatment Technology Systems Using Functions of Complex Microbial Communities, University of Tokyo, Tokyo, Japan, 6-8 March.

Mudaly, DD, Atkinson, BW and Bux, F (2000) 16S rRNA *in situ* probing for the determination of the family level community structure implicated in enhanced biological nutrient removal. 1st World Congress of the International Water Association, Paris, France, 3-7 July.

Sidat, M, Bux, F and Kasan, HC (1997) Laboratory scale investigation to improve biological phosphate removal from wastewater. South African Society for Microbiology Conference, University of Natal, 15-17 October.

Stevens, W, Drysdale, GD and Bux, F (1999) An assessment of the nitrifying behaviour of heterotrophic bacteria in a biological nutrient removal system. 12th Annual Symposium of SASM (Natal Branch), ML Sultan Technikon, Durban, 15 October 1999.

Stevens, WE, Drysdale, GD and Bux, F (2000) An assessment of the potential for heterotrophic nitrification in biological nutrient removal systems. BIOY2K Biotech SA 2000 Conference, Rhodes University, Grahamstown, 23-28 January.

Stevens, WE, Drysdale, GD and Bux, F (2000) The potential of heterotrophic bacteria to nitrify in a biological nutrient removal system. WISA 2000, Sun City, 28 May - 1 June.

8. Archiving of data

All raw and processed data collected from this research project will be archived and made available upon request at Technikon Natal.

ACKNOWLEDGEMENTS

The research in this report emanated from a project funded by the Water Research Commission and entitled:

"INVESTIGATION OF THE MICROBIAL CONTRIBUTION TO NUTRIENT REMOVAL IN AN ACTIVATED SLUDGE WASTEWATER TREATMENT PROCESS"

The Steering Committee responsible for this project consisted of the following persons:

Dr N Mjoli	Water Research Commission (Chairman)
Mrs CM Smit	Water Research Commission (Secretary)
Prof CA Buckley	University of Natal (Durban)
Prof TE Cloete	University of Pretoria
Prof GA Ekama	University of Cape Town
Mr P Gaydon	Umgeni Water
Dr HC Kasan	Rand Water (formerly Technikon Natal)
Ms APM Oelofse	Water Research Commission
Mr G Richardson	Durban Metro
Prof E Senior	University of Natal (Pietermaritzburg)

The financing of the project by the Water Research Commission and the contribution of the members of the Steering Committee is gratefully acknowledged. The National Research Foundation is also acknowledged for providing students the financial means to complete their individual projects and the research project in its entirety.

This project was only possible with the co-operation of many individuals and institutions. The authors therefore wish to record their sincere thanks to the following:

Prof H van Versefeld	Vrije University, Netherlands
Dr DW de Haas	Gutterridge, Haskins and Davey, Australia (formerly Umgeni Water)
Dr F Kurisu	University of Tokyo
Mrs M Bekink	Umgeni Water
Miss P Joyce	Umgeni Water
Miss M Smuts	Umgeni Water
Mr S Makhavhu	Umgeni Water
Mr Z Jikazi	Umgeni Water

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AD/TP	adenosine di/triphosphate
AE	aerobic reactor
AMM	acetate mineral media
AN	anaerobic reactor
API	Analytical Profile Index
ATCC	American Type Culture Collection
AX	anoxic reactor
BEPR	biological excess phosphorus removal
BNR	biological nutrient removal
BPR	biological phosphorus removal
C	carbon
CFU	colony forming unit
CGY	casitone glycerol yeast extract agar
COD	chemical oxygen demand
CWWR	Centre for Water and Wastewater Research
DAPI	4',6 diamidino-2-phenylindole
DO	dissolved oxygen
DNA	deoxyribonucleic acid
DPB	denitrifying phosphorus accumulating bacteria
DSVI	dilute sludge volume index
FISH	fluorescent *in situ* hybridization
F/M	food:microorganism ratio
FSA	free and saline ammonia
f_{bs}	biodegradable soluble fraction of total influent COD
f_{ts}	total readily biodegradable COD fraction
HAc	acetate
HRT	hydraulic retention time
MCRT	mean cell retention time
ML	mixed liquor
MLSS	mixed liquor suspended solids
MLVSS	mixed liquor volatile suspended solids
N	nitrogen
N_{ti}	total influent nitrogen
NaAc	sodium acetate
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
ND	nitrification denitrification
NDBEPR	nitrification denitrification biological excess phosphorus removal
O	oxygen
OHO	ordinary heterotrophic organism
ortho-P	orthophosphorus

OUR	oxygen utilization rate
P	phosphorus
P_{ti}	total influent phosphorus
PAO	polyphosphate accumulating organism
PCR	polymerase chain reaction
PHA	poly-β-hydroxyalkonoate
PHB	poly-β-hydroxybutyrate
poly-P	polyphosphate
Q_i	influent flow rate
R_s	sludge age
RBCOD	readily biodegradable COD
rRNA	ribosomal ribonucleic acid
S_{bsi}	influent biodegradable soluble COD
S_{ti}	total influent COD
S_{te}	total effluent COD
SBR	sequencing batch reactor
SCFA	short chain fatty acid
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SRP	soluble reactive phosphorus
SV_{30}	sludge volume after 30 min. settling (mL/L)
TCA	tricarboxylic cycle (Kreb's Cycle)
TKN	total Kjeldahl nitrogen
TP	total phosphorus
TSS	total suspended solids
UCT	University of Cape Town
VFA	volatile fatty acid
VM	vanadate-molybdate
VSS	volatile suspended solids
WWTP	wastewater treatment plant
WWW	wastewater works
X_a	active biomass
X_{AUT}	autotrophic biomass
X_e	endogenous residue
X_H	heterotrophic biomass
X_i	inert particulate material
X_{PAO}	polyphosphate accumulating organism biomass

CHAPTER ONE

INTRODUCTION

1.1 Background and scope of research

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).

Wastewater entering a treatment plant is composed of many different and constantly changing substrates/pollutants which only mixed populations of microorganisms are capable of metabolising. The microbial community in wastewater treatment systems exist in unique and transient ecosystems, dependent upon contaminant type, load and the ambient oxidation reduction potential.

Although Koch's principles for other nutrient removal processes during biological wastewater treatment 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 biological excess phosphorus removal (BEPR) processes (Jenkins and Tandoi, 1991). Although the process of BEPR 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). Since pure cultures which possess complete characteristics of polyphosphate accumulating organisms (PAO's) have not been isolated yet, the biochemical mechanism cannot be definitively described

(Mino *et al*., 1998). Without understanding the correlation between the PAO community structure within activated sludge mixed liquor (as well as the interactions between autotrophic and heterotrophic bacteria involved in nitrification and denitrifiers involved in simultaneous P uptake) and the wastewater plant's performance, reliable and efficient nitrification/denitrification biological excess phosphorus removal (NDBEPR) operations will remain difficult to design. In-depth microbial community analyses and descriptions of the vast interactions occurring amongst the bacteria involved in wastewater remediation are therefore integral to advance the science and efficiency of processes such as biological nutrient removal.

The process engineering and technology (modelling and operation) and microbiological and biochemical disciplines have traditionally worked independently of one another. A consequence of this is that microbiologists can find it difficult to express information gathered in engineering terms which could otherwise be implemented to address the deficiencies of the existing steady-state design models and kinetic simulation models. Rapid developments in and application of molecular biological techniques have made a considerable contribution to the understanding of biological nutrient removal (BNR) activated sludge systems. More significantly, these techniques provide quantitative data which is a prerequisite for the successful modelling of these systems. Integration of the engineering and microbiological/biochemical disciplines should remain the focus activity area in activated sludge research for the near future. To this end, a new multi-national and multi-institutional Water Research Commission project has been initiated in which both engineering and microbiological-based research groups are participating.

1.2 Research objectives

The research project was therefore designed to:

- conduct a microbiological survey of a BNR plant in KwaZulu-Natal (Darvill Wastewater Works) which implements both biological phosphorus and nitrogen removal, concentrating on the various zones ie., anaerobic, anoxic and aerobic;
- establish the extent of correlation between microbial dominance in a particular zone and the extent to which this dominance contributes to the removal of specific nutrients;

- increase current knowledge of microbial community structure-functionality;
- assess the removal capacity of specific, isolated bacteria in order to determine their individual contribution to the system as a whole;
- assess molecular techniques (fluorescent *in situ* hybridization or FISH), as opposed to conventional cultivation techniques, to determine the microbial community structure; and
- compare various culture-dependent identification techniques such as the Analytical Profile Index (API), Microbact and Biolog assays using bacteria isolated from activated sludge.

1.3 Report structure

The report is structured and will be presented as such:

TABLE 1.1 Scope and structure of report.

CHAPTER	SCOPE
1	General introduction and scope of research
2	Literature review
3	Microbiology of BEPR in both full- and pilot-scale treatment plants
4	Microbial community analyses of full- and pilot-scale treatment plants using molecular techniques (FISH and dot blots)
5	Microbiological aspects of denitrification and heterotrophic nitrification in a full-scale nutrient removing plant
6	General conclusions and recommendations

CHAPTER TWO

LITERATURE REVIEW

2.1 Origins of phosphorus and nitrogen in the environment

The weathering and mining of P-containing rocks makes phosphate ions available to plants which accumulate the inorganic ion from the soil. 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 before sedimentation can occur, converting it to the organic form. 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 its 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 activity which increases the quantity of phosphate in the environment includes the mining of phosphate ores for fertiliser production, animal feed supplementation and detergent production.

The occurrence of nitrogen in water is primarily due to human and animal excrement (mostly as urea), fertilisers, run off from fertilised lands, decaying vegetative and animal matter, food processing waste, uranium oxide fuel production and fixation of atmospheric nitrogen (Bode *et al.*, 1987; Terblanche, 1991; Ketchum, 1988; Otlanabo, 1993). Nitrogen can be present in water sources in various forms all of which can be detrimental to human health and/or the environment. Apart from causing various medical disorders in humans and ecological disturbances, nitrites and ammonia pose serious problems for potable water treatment in that they have a high affinity for chlorine thus reducing the effectiveness of chlorine disinfection of drinking water (American Water Works Association, 1995). Nitrates and nitrites also inhibit the removal of phosphorus during waste water treatment which can result in serious eutrophication problems in receiving water bodies (Gruenebaum and Dorgeloh, 1992; Kuba *et al.*, 1996). The removal of nitrogen from waste water streams is therefore of utmost importance to ensure effective safeguarding of remaining available water resources and the environment.

2.2 Technology and microbiology of activated sludge

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. 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; a certain fraction of the settled biomass is recycled to the biological reactor via a clarifier underflow; and dependency of plant performance on the mean cell residence time (MCRT) or sludge age (R_s) of the system (Grady and Lim, 1980).

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 has revealed that the microbial consortium is 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).

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 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:

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

2.3 Principles of biological phosphorus removal

Phosphorus removal from wastewater by biological means is accomplished in two ways: stoichiometric coupling to microbial growth or enhanced storage in the resident biomass as polyphosphate(poly-P) (Mino *et al.*, 1998). Enhanced BPR occurring in activated sludge is a direct result of the ability of 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 BPR 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).

In wastewater technology, anaerobiosis describes an environment in which both dissolved oxygen and oxidised forms of nitrogen i.e., nitrates and nitrites, are absent (Jenkins and Tandoi, 1991; Muyima *et al.*, 1997). The function of the anaerobic zone in BEPR operations is two-fold: (i) the reduced redox potential induces conversion of the influent readily biodegradable COD (RBCOD) to short chain fatty acids (SCFA's or VFA's) via acidogenisis by non-PAO heterotrophs, and (ii) it provides an ideal environment where PAO's are able to take up the VFA's and accumulate them intracellularly as poly-β-hydroxyalkonoates (PHA), the most abundant of which is poly-β-hydroxybutyrate (PHB), if acetate (HAc) is the main constituent in the feed liquor (Ekama and Wentzel, 1997). 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.

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 BPR as a result of weak influent COD and resultant low RBCOD and VFA concentrations.

Two biochemical models currently exist describing the synthesis of PHA and the source of the reducing power required for this anabolic reaction. These are the Comeau-Wentzel model and Mino model. In the Comeau-Wentzel model, the Kreb's cycle operates under anaerobic conditions to partially oxidise HAc to CO_2 and to generate reducing power in the form of NADH. In contrast, the Mino model hypothesises that the anaerobic metabolism of intracellularly stored glycogen to acetyl-CoA as well as its partial oxidation to CO_2 generates reducing power for PHA synthesis (Mino *et al.*, 1987). More precise identification of the correct biochemical pathway is required but it is evident that a pure culture of a PAO will be required in this respect. However, if the biochemical model of Mino's, describing the mode of P release and uptake is accepted, the metabolism of PAO's is well illustrated in TABLE 2.1 (Mino *et al.*, 1998).

The primary function of the aerobic zone is to oxidise organic material in the sewage (Muyima *et al.*, 1997). When a suitable R_s is selected and autotrophic organisms are able to establish themselves in the system, oxidation of ammonia N to nitrite and nitrate i.e., 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.

TABLE 2.1 Metabolism of PAO's in BEPR processes.

Metabolism	Response
Anaerobic phase	
Uptake of external organic substrates	+
Consumption of intracellular glycogen	+
Accumulation of intracellular PHA	+
Consumption of intracellular poly-P and release of ortho-P	+
Aerobic phase	
Recovery of intracellular glycogen	+
Degradation of intracellular PHA	+
Growth	+
Recovery of intracellular poly-P	+

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).

2.4 Biological nitrogen removal processes

Nitrification was the first method of biological nitrogen removal achieved via activated sludge treatment. Under aerobic conditions nitrifying microorganisms convert ammonia, via nitrites, to nitrates in two different oxidative reactions known as nitritation and nitratation. In nitritation, ammonia is oxidised to nitrites whereafter nitratation commences with further oxidation of the nitrites to nitrates (Mauret *et al*., 1996). The disadvantages of nitrification are that during the oxidation of ammonia, hydrogen ions are released which cause a decrease in the pH and alkalinity of the wastewater (Muyima, 1997; Lilley *et al.*, 1997). Low pH, in turn, results in decreased efficiency of nitrification, poor sludge settleability and corrosive effluent (Muyima, 1997). Furthermore, nitrification in activated sludge systems requires a long R_s and lengthy retention times to achieve complete ammonia and nitrite oxidation. Among all the biological reactions responsible for nitrogen removal, ammonia oxidation is generally the rate limiting step because of slow growth and poor yield of chemoautotrophic ammonia oxidising bacteria (Suwa and Noto, 1998). Environmental engineers are still focussed on developing more efficient processes for nitrification.

With the resultant release of nitrates into receiving water bodies via nitrified wastewater, it became necessary to include denitrification into activated sludge systems. Denitrification is the reduction of nitrates, via nitrites, to nitrous oxides and gaseous nitrogen under anoxic conditions (Wu and Knowles, 1995). The inclusion of denitrification into activated sludge systems is achieved via the addition of unaerated or anoxic zones in the process (Dold and Marais, 1987). Under anoxic conditions denitrifying microorganisms are stimulated into utilising nitrates and nitrites as final electron acceptors for cellular respiration with resultant production of gaseous nitrogen accompanied by concurrent COD removal (Ketchum, 1988; Wanner and Grau, 1988; Cappucino and Sherman, 1992). The inclusion of denitrification to nitrifying activated sludge systems has resulted in reduced daily oxygen requirements, reduced effluent nitrate loads, elimination of pH control and alleviation of the stringency of DO control during wastewater treatment (Dold and Marais, 1987). Denitrification kinetics have primarily been determined and attributed to the ordinary heterotrophic bacteria (OHO), otherwise not involved in BEPR (Dold *et al*., 1991; Ubisi *et al*., 1997; Ekama and Wentzel, 1999). However, denitrification kinetics determined for ND systems have been found to vary considerably at times when applied to NDBEPR systems because of varying OHO active fraction estimates and the occurrence of anoxic phosphorus removal (Dold *et al*., 1991; Ekama and Wentzel, 1999). Ultimately, variations in process performance and kinetics are attributable to inadequate control and lack of understanding of the ecological, physiological and biochemical activities of the microorganisms within the process (Bux *et al.*, 1994). Henze (1992) and Kristensen *et al.*, (1992) echo concern in that activated sludge models fall short in not taking into consideration the structure of the biomass present in the process. There is growing concern and movement towards a better understanding of the microbial community within activated sludge in order to gain optimal control of the process (Lu and Leslie-Grady, 1988; Davelaar, 1989; Wagner *et al.*, 1993; Satoh *et al*., 1996; Ubisi *et al*., 1997).

2.5 Microbiology of biological phosphorus and nitrogen removal

Since it is the biomass within the activated sludge process which is responsible for mediating the treatment functions of interest viz. COD removal, nitrogen removal and phosphorus removal, it therefore follows that studies focussed on the microbial composition of the activated sludge mixed liquor and the direct assessment of the metabolic activity of the community would be advantageous towards improving the process description and design. Investigating the biomass in a somewhat detailed manner always presents the danger of neglecting the "process" aspect of the activated sludge system. With such considerations in mind, the microbial investigations conducted in sections of this report have been expressed in terms of some parameters that find common usage amongst process engineers.

A number of heterotrophic organisms from activated sludge have been investigated intensively for their involvement in processes such as BPR. 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 BEPR 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.

Reports from literature, describing the microbial community of BEPR sludges, appear to be unconvincing and haphazard. Bond *et al*., (1997), when applying the clone library approach, have reported that the beta subclass of Proteobacteria appear to have a specific role in the BEPR process and that only a few Actinobacteria (Gram positive bacteria with a high G + C content) were found. Kampfer *et al*., (1996), however, using FISH molecular techniques, found relatively high numbers of Actinobacteria in an BEPR sludge and suggested their significance to the success of BEPR operations. Although it has been conventionally assumed that BEPR would be assigned to one particular dominant species or group of microorganisms, it appears that the microbial community of the BEPR process is diverse.

The process of nitrification is generally ascribed to chemoautotrophic bacteria belonging to the family *Nitrobacteraceae* which are Gram negative obligate aerobes (Watson *et al*., 1981; Brock *et al*., 1991). Although contributing only a small fraction to the total microbial community in wastewater treatment systems, autotrophic nitrifiers are believed to be responsible for the majority of nitrification (Randall *et al*., 1992; Burrell *et al*., 1998). *Nitrosomanas* spp. are the autotrophs most commonly implicated in ammonia oxidation while *Nitrobacter* spp. are believed to be the predominant organisms responsible for nitrite oxidation (van Loosdrecht and Jetten, 1998). The autotrophs have been found to have very slow growth rates, with a generation time of between 8-24 h (Watson *et al*., 1981). Thus, the time required for successful isolation and cultivation of these organisms can be several months. Secondary autotrophic nitrifiers have also been found to exist in nitrifying ecosystems. These organisms have, in the past, generally been found in lower numbers than *Nitrosomonas* spp. and *Nitrobacter* spp. which is believed to be due to their narrower range of temperature and pH for growth (Focht and Verstraete, 1977). However, recent studies have revealed that bacteria from the *Nitrospira* phylum are present as the main nitrite-oxidising bacteria in wastewater treatment systems and that *Nitrobacter* spp. are often undetectable (Hovanec and DeLong, 1996; Wagner *et al*., 1996; Burrell *et al*., 1998).

Numerous studies have highlighted the possible involvement of heterotrophic bacteria in nitrification (Aleem, 1975; Verhagen and Laanbroek, 1991; Robertson and Kuenen, 1992). From studies conducted thus far, heterotrophic nitrification rates have been found to be only one or two orders of magnitude lower than that of autotrophs (Robertson and Kuenen, 1992). Furthermore, most of the heterotrophic nitrifiers studied are less fastidious and sensitive than their autotrophic counterparts. Taking this into account and their fast growth rates, heterotrophs may be contributing significantly to nitrification in activated sludge systems. However, the role of the heterotrophic nitrifier is not well understood.

The pathway of heterotrophic nitrogen oxidation remains a crucial problem yet to be resolved. Unlike autotrophic nitrification, heterotrophic oxidation of ammonia requires energy that leads to decreased yield coefficients. Heterotrophic bacteria only nitrify if supplied with an external, organic substrate and it has not yet been demonstrated that they gain any energy from the reaction (Robertson and Kuenen, 1992). Reasons for heterotrophic nitrification may be as diverse as the bacteria performing it. For example, heterotrophic nitrification by an *Arthrobaceter* species may be related to the production of chelating agents (Robertson and Kuenen, 1992). For other species such as *Thiosphaera pantotropha, Pseudomonas denitrificans* and *Alcaligenes* spp. it is thought that the ammonia oxidation step (which requires NADPH) is utilised to dispose of any excess reducing power, possibly because of a rate limiting step in the electron transport chain to oxygen (Robertson and Kuenen, 1992). It remains to be shown, however, that heterotrophic organisms which can oxidise nitrogen under axenic culture conditions can do so *in situ*. Heterotrophic nitrification under aerobic conditions can result in production of nitrite and/or nitrate that would subsequently become available for denitrication under "anaerobic" conditions (absence of DO; otherwise referred to as anoxic in wastewater technology) without any inhibition or competition from autotrophs (Castignetti and Hollocher, 1984). Patureau *et al.*, (1994) acknowledge that the existence of a mixed ecosystem that can grow rapidly and nitrify, denitrify and consume organic matter in a single stage aerobic system would be an attractive and novel idea in the world of wastewater treatment. Models applied to nitrifying populations may require revision as they have been based on the assumption that *Nirosomonas* spp. and *Nitrobacter* spp. are the organisms solely responsible for nitrification occurring during wastewater treatment (Hovanec and DeLong, 1996; Yuichi and Kazuhiko, 1998; Wagner *et al.*, 1996). The need exists for a better understanding of the organisms involved in nitrification and their specific mechanisms employed in ammonia and nitrite oxidation in order to further improve nitrogen removal during wastewater treatment.

Denitrification is primarily performed by facultative heterotrophic bacteria. Dold *et al.*, (1991), Ubisi *et al.*, (1997) and Ekama and Wentzel (1999) have offered a simplified community structure for denitrifiers in ascribing denitrification solely and entirely to the OHO fraction. However, denitrification may not be totally attributable to just the OHOs, or even all the OHOs. Studies involving denitrification in soils and aquatic ecosystems have indicated strong involvement of many different heterotrophic bacteria (*Bergey's*, 1984;1986; Otlanabo, 1993; Carter *et al.*, 1995). Research has shown that numerous different enzymes are required for true denitrification and these enzymes are often restricted to different bacteria as well as expressed differently, from organism to organism, under varying physiological conditions (Robertson and

Kuenen, 1992; Wu and Knowles, 1995). In fact, according to Rheinheimer (1985) and Robertson and Kuenen (1992), most of the denitrifying bacteria in aquatic systems are enzymatically restricted to incomplete denitrification. The full impact denitrifying organisms have on nitrogen removal during activated sludge treatment has, however, not yet been determined or even properly contemplated. Furthermore, many heterotrophic bacteria are enzymatically incapable of denitrification (*Bergey's*, 1984;1986) and in view of this it is inaccurate to ascribe denitrification in ND and NDBEPR systems to the entire OHO fraction.

2.6 The phylogenetic classification system

The fact that DNA is the expressive machinery through which natural selection, and therefore evolution operates means that it is a valuable tool for mapping evolutionary relationships between microorganisms. Phylogeny thereby seeks to place bacterial taxonomy on a foundation of natural classification. Genotypic information i.e., sequence information, is superior in two respects to phenotypic information for classifying and relating organisms. Sequence information is (i) more readily, reliably and precisely interpreted, and (ii) innately more informative of evolutionary relationships than phenotypic information is. The elements of a genetic sequence are also well defined and are restricted in number. The description of two organisms as being similar is based upon mathematically defined relationships making the determination more objective (Woese, 1987). In addition, sequence data can be readily accumulated creating a "data base" which can be referred to for phylogenetic analysis as new sequence information becomes available (Olsen *et.al.*, 1986).

A molecule whose sequence changes randomly in time can be termed a chronometer. All sequences are not of equal value in determining phylogenetic relationships and ribosomal RNA (rRNA) is presently the most useful molecular chronometer. A good molecular chronometer should have the following characteristics; (i) they should have a random clocklike behaviour, (ii) they should occur in all organisms and different positions in their sequences should change at different rates making their evolutionary range all encompassing and, (iii) they should have a large size made up of many functional domains which are evolutionary separate from one another

(Woese, 1987).

The three primary kingdoms comprising all life (previously termed eubacteria, archaebacteria and eukaryotes) were found to be genealogically very distinct from one another (as revealed by rRNA sequence comparisons. In fact microbiologists can no longer assume that the archaebacteria and eubacteria are related to one another to the exclusion of the eukaryotes. As such it has been recommended that we now refer to these kingdoms as domains (Woese *et al.*, 1990).

The construction of a phylogenetic classification system from rRNA sequence comparisons has revealed how little we knew about the relationships that exist between bacteria. The idea that bacteria can be divided into two categories viz., Gram positive and Gram negative (justified by cell wall composition) is misleading. While the Gram positive bacteria form a coherent phylogenetic group, Gram negative bacteria do not. They are in fact divided into 10 separate and distinct phylogenetic groups. Photosynthetic bacteria do not form a grouping that is phylogenetically distinct from non-photosynthetic bacteria. Autotrophs and heterotrophs are also intimately related to one another within various genealogical phyla (Woese, 1987).

2.7 Cultivation-dependent enumeration methods

Early attempts to characterize and enumerate the microbial consortia in activated sludge employed culture-dependant methods such as the Most Probable Number (MPN) methods and viable plate counts. However, there is a large discrepancy between numbers determined by means of total direct microscopic counts and the percentage of those that could be cultured. For the activated sludge process, this percentage ranges from 1-15% and in most other ecosystems it is usually less than 1% (Wagner *et al.*, 1993). According to Amann *et al.*, (1995), two cell types contribute to the silent but active majority of cells that are not detected: (i) species which are known but for which the applied culture conditions are not suitable, or which have entered a non-culturable state; and (ii) unknown species that have never been cultured before for lack of suitable methods. For activated sludge another factor can be included viz., clumping of cells in the activated sludge floc thereby preventing the quantitative release of bacteria, leading to an underestimation of active cells by viable plate count methods (Wagner and Amann, 1997). 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*., 1993). However, while it is evident that cultivation-dependant methods present a limitation for the enumeration of bacteria in environmental samples, selective cultivation techniques will always remain a valuable tool for the isolation and characterization of microorganisms.

2.8 Background to some popular molecular techniques

Molecular biology presents some alternative approaches to analysing the microbial community comprising activated sludge systems. One is a chemotaxonomic approach using bacterial respiratory quinone profiles. The limitation here however is that given the complexity of the activated sludge community, one cannot translate relative quantities of this biomarker into cell numbers. The immunofluorescent approach provides for the identification and enumeration at the cellular level (Cloete and Steyn, 1987) but problems here include non- specific binding of the antibody and inability of the antibody to penetrate the cell due to the presence of extracellular polymers. One also requires a pure culture of the organism of interest in order to generate sufficient quantities of antibody. Other molecular techniques include protein fingerprinting of activated sludge using SDS-PAGE (Ehlers and Cloete, 1999) and the determination of terminal restriction fragment length polymorphism (T-RFLP) profiles of the 16S rDNA in activated sludge samples (Liu *et al.*, 1997). Here too, quantitative data is lacking or as in the latter case is obtained through an extrapolation that deals with a lumped parameter of rDNA molecules rather than active cell numbers.

Microautoradiography has recently been applied to environmental sample analyses to determine microbial community structures. Typically, a radiolabelled compound appears in the cell through adsorption of a tracer or labelled substrate. The radiolabelled sample is then placed in contact with a radiosensitive emulsion and the emissions from the radioactive sample interact with silver bromide crystals 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. 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*., (1999), 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-functional studies.

Probably the biggest recent contribution to microbial ecology has come from the application of rapid nucleic acid sequencing and recombinant DNA methodologies for the analysis of phylogenetic and quantitative aspects of mixed microbial populations. This analysis is based on nucleotide sequence comparison of rRNA's or their genes extracted from naturally occurring biomass. Because molecules rather than organisms are isolated, the method is not limited to species that are amenable to laboratory cultivation (Olsen *et al.*, 1986). The rRNA sequence collection has provided a powerful determinative framework for the design and application of phylogenetically based nucleic acid hybridization probes. Although rRNA's are highly conserved biopolymers, they exhibit great variation in regional sequence conservation. Some nucleotide positions and locales have remained virtually unchanged since the divergence of all existing life (universal sequences, whereas other regions vary so quickly that they can be used to differentiate between species or subspecies of bacteria). In addition, their high copy number (10^3-10^5 per cell) lends greater sensitivity to directed determinative tests (Stahl and Amann, 1991).

Fluorescently labelled oligonucleotide probes have been used with epifluorescent microscopy to identify single cells of specific phylogenetic groups in the environment (Hicks *et al.*, 1992). This approach targets the rRNA within intact bacterial cells using oligonucleotides that are complementary to conserved signature sequences of phylogenetically defined taxa. By using selected regions within the larger rRNA molecules as hybridization targets, probe specificity can generally be freely adjusted to encompass different phylogenetic groups (Amann *et al*., 1990). Probes have been designed for higher taxonomic levels; domains Archaea, Bacteria and Eukarya

(Delong *et al*., 1989; Amann *et al.*, 1990); for intermediate family levels; alpha, beta and gamma subclasses of the family Proteobacteria (Manz *et al.*, 1992); Actinobacteria subclass within the family Firmicutes (Roller *et al.*, 1994); and lower, genus levels i.e., *Acinetobacter* (Wagner *et al.*,1994b).

2.9 *Acinetobacter* - model polyphosphate accumulating organism?

Among the issues confronting activated sludge researchers are the assessment of the active biomass mediating a particular process or function, the delineation of the key microbial groups comprising the total population, and more specific to BEPR, the quantification of that portion of the population responsible for the release and subsequent uptake of soluble phosphate - the PAO's.

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 centered 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 BEPR (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 BNR systems have emphasised the functional role of *Acinetobacter* in BEPR (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;1989). However, the high incidence and recovery rates of *Acinetobacter* spp. in these systems was more than likely due to the bias imposed upon the gamma Proteobacteria through isolation on solid agar media. The method of identification (API in most cases) must also be viewed in a critical light as its database was not developed for the vast number of species encountered in environmental samples.

Due to the controversy surrounding the role of *Acinetobacter* in P removal operations, microbiologists have, for the past decade, attempted to isolate other bacteria in activated sludge upon which the mechanism can be modelled. Recently, a 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 i.e., 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. Its C and P transformation patterns coincide with those of BEPR sludges and, as such, the bacterium has been considered as a candidate for the model PAO in BEPR processes. *M. phosphovorus* is a Gram positive coccus and nucleotide sequencing shows a high G+C genomic DNA content (65.6 mol%; phylogenetically belongs to the Actinobacteria) (Ubukata and Takii, 1998). However, the bacterium's dominance in BEPR processes has yet to be demonstrated and because it cannot readily be isolated from activated sludge (as opposed to *Acinetobacter* spp.), its application to BPR studies may be limited.

More recently, the application of molecular techniques has provided valuable information regarding those organisms performing the BEPR function. Crocetti *et al.*, (2000), using FISH, various staining procedures and PCR-amplified bacterial 16S rRNA genes prepared in clone liraries, found that the common bacterial group in high performance BEPR sequencing batch reactors (SBR's) belonged to the beta-2 Proteobacteria. The authors were more specific and concluded that the organisms responsible for BEPR were closely related to *Rhodocyclus* and *Propionibacter*. Hesselmann *et al.*, (1999), using techniques similar to those of Crocetti *et al.*, (2000), including dot blot hybridization of extracted nucleic acids, found that the main constituent of a BEPR sludge was a *Rhodocyclus*-like bacteria which they termed *Accumulibacter phosphatis*.

2.10 Microbial community analysis into the future

Molecular based analysis has emerged as a popular tool to facilitate a better understanding of the structure and function of the microbial community comprising nutrient removal activated sludge processes. It is generally believed that this information could assist process retrofitting, design and modelling as these evolve into more complex mathematical descriptions in order to accommodate increasing nutrient loads and to reduce the impact of wastewater on aquatic environments. Increasingly, dynamic models involving BEPR are beginning to include more specific microbiological and biochemical information from lower levels of organization contained within the surrogate biomass (Wentzel and Ekama, 1998). Direct techniques are now the method of choice for quantifying bacteria in a particular ecosystem. Because rRNA can be directly correlated with growth rate (Schaechter *et al.*, 1958; Delong *et al.*, 1989; Wallner *et al.*, 1993, Wagner *et al.*, 1993) probe based hybridizations also provide a basis for determining relative metabolic rates of cells in a particular environment. However they do not give any direct indication of the specific kind of physiology (function) being mediated by a phylogenetic group. Methods proposed to directly assay the physiology of microorganisms in a particular environment include microautoradiography (Nielsen *et al.*, 1999), and the detection of mRNA molecules within whole cells (Wagner *et al.*, 1998). However, these methods are still in their developmental stages and are not as yet amenable to routine application.

Therefore, regarding the present research study and within the context of improving the microbiological knowledge of biological nutrient removal, three core areas of activity were defined and selected for in-depth investigation. These included (i) BEPR; (ii) biological nitrogen removal (nitrification and denitrification processes) and; (iii) the implementation and use of molecular techniques such as FISH to conduct quantitative and qualitative studies of the bacteria involved. Qualitative and quantitative data regarding the bacterial species involved in these processes was obtained by both conventional serial dilution, plating, staining and various biochemical tests as well as the application of FISH technology. Research protocols and results of these three sub-activity areas will be reported and discussed individually for ease of reading.

CHAPTER THREE

BIOLOGICAL PHOSPHORUS REMOVAL

3.1 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.

During the present study, an enhanced culture of PAO's was developed using similar techniques and methods to those employed by Wentzel *et al.*, (1988). The development of an enhanced culture during the present study therefore represents a review of work conducted by Wentzel *et al.*, (1988). Cultures were subsequently isolated and identified using both conventional plating (including API) and molecular techniques (FISH, see CHAPTER 4). 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. It was envisaged that present research findings would offer an intrinsic understanding of the microbial community displaying BEPR.

This chapter represents microbial community analysis work from both a full- and pilot-scale BEPR activated sludge system. Initially, 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 full-scale Darvill BNR plant were therefore applied 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 AIMS AND OBJECTIVES

The main objective of this phase of the research was to determine the incidence of *Acinetobacter* spp. in NDEBPR sludges and to identify other organisms using isolation techniques. The NDEBPR sludges were also used for FISH analysis to determine the microbial community structure. To this end, the aims of work presented in this chapter included:

- development of an enhanced culture of PAO's using a continuous pilot-scale activated sludge process;
- characterization of the unit i.e., detailed description of influent and effluent;
- isolation and identification of bacteria from the sludge samples (full- and pilot-scale) using solid media; and
- determine prevalence of *Acinetobacter* spp. in the sludges.

3.3 MATERIALS AND METHODS

3.3.1 Sampling of activated sludge mixed liquor

Grab samples of mixed liquor were obtained from the aerobic zone of the full-scale activated sludge system at Darvill Wastewater Works (WWW; Pietermaritzburg, KwaZulu-Natal).

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. An operational description of Darvill WWW can be obtained in APPENDIX 1.

3.3.2 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 2) (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.

3.3.3 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.

3.3.4 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. The *A. calcoaceticus* ATCC culture was treated in the same manner to validate the identification procedure.

3.3.5 Development of an enhanced culture of PAO's

3.3.5.1 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, UCT). 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. 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 with settled sewage and rinsed at least once a month. A motorised impellor in the tank ensured that the sewage was continuously mixed.

3.3.5.2 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 schematic design of the pilot plant is given in APPENDIX 3. The reactor configuration consisted of the following: an anaerobic (AN) zone (8 L); an anoxic (AX) zone (4 L); first aerobic (AE1) zone (10 L); and second aerobic (AE2) zone (10 L). The clarifier (2.5 L), downstream of the reactors, was positioned at a 60° angle to the horizontal. 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 using a peristaltic pump (Gilson), was set at a ratio of 1:1 with respect to Q_i. The a-recycle, pumped (Watson-Marlow) from AE2 to the AX zone, was set at a ratio of 3:1.

Batches of settled 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 to reduce biological activity.

Sludge was wasted from the system by daily withdrawal of the appropriate volume of mixed liquor from AE2 and R_s was maintained at 10 d for the duration of experimentation. Oxygen utilization rate (OUR) was measured in AE2 by means of a DO probe and meter (Randall *et al*., 1991). Air supply to the aerated zones was controlled between a specified upper and lower limit such that the aquarium air pumps switched on at a DO concentration of 2.0 mg/L and switched off at a DO concentration of 5.0 mg/L. The meter, operating in OUR mode, recorded OUR over a 24 h period.

3.3.5.3 Acquisition of seed inoculum

Mixed liquor (25 L) was obtained from the return sludge stream of Amanzimtoti Wastewater Treatment Plant (WWTP; South Durban, KwaZulu-Natal). Various operating and wastewater characteristics pertaining to the activated sludge process at Amanzimtoti WWTP are given in APPENDIX 4.

Since the primary objective at this stage of the project was to establish an enhanced culture of PAO's and to identify the resident microflora, it seemed appropriate to inoculate the pilot plant with activated sludge biomass from a non-BNR plant ie., Amanzimtoti WWTP, and monitor changes in the microbial community as the culture became more 'enhanced' i.e., as the culture, through manipulation of certain abiotic parameters, shifted towards accumulating P in excess of normal metabolic requirements.

3.3.5.4 Feed supplementation for enhanced culture development

For the duration of pilot plant operation, a constant influent COD of 500 mg/L was intended to assist in achieving steady-state conditions. During experimental periods A - E this proved difficult since Darvill settled sewage is relatively weak i.e., 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 (NaAc) as the sole organic supplement. Sewage supplementation is tabulised in APPENDIX 5.

Anhydrous NaAc (ACE, South Africa) concentrations to the feed were incrementally increased at regular time intervals (APPENDIX 5). At concentrations of 400 mgNaAc/L as COD (period F), 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) (APPENDIX 6). Ammonium chloride was also added to the feed stock to maintain a TKN value of approximately 25 mgN/L (to maintain a relatively constant TKN/COD ratio).

3.3.5.5 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. 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.

3.3.5.6 Acid dosing

Wentzel *et al*., (1988) found that P uptake in the aerobic reactors results in a marked 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 HAc dosing. Mixed liquor pH in all four reactors was monitored daily using a portable pH probe and meter (Beckman). Dilute hydrochloric acid (20 to 300 mM) was dosed to AE1 at a rate of 500 mL/d. Addition of acid maintained the mixed liquor pH below 7.8.

3.3.5.7 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.

3.3.5.8 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 7.

3.3.5.9 Parameters measured

TABLE 3.1 describes the parameters routinely measured as well as the methods employed to conduct the various analyses.

TABLE 3.1

SAMPLING POSITION AND PARAMETER MEASUREMENT

Test	COD[1]	TP[2]	SRP[3]	TKN[4]	FSA[5]	NO_3[6]	V/MLSS[7]	DSVI[8]	OUR[9]	pH[10]
Influent	○✚	○	✚	○	✚					
AN		○✘								✔
AX		○✘								✔
AE1		○✘								✔
AE2		○✘					✔	✔	✔	✔
Effluent	○✚	○	✚	○	✚	✚				

✔ Measurement taken (filtering not applicable); ○ Unfiltered sample; ✚ Filtered through Millipore 0.45μm glass fibre syringe filter; ✘ filtered through Whatman No. 1 filter paper (or equivalent)

[1] Microwave digestion and potentiometric titration

[2] Sulphuric acid/persulphate digestion at 100°C followed by vanadate-molybdate (VM) colour development for orthophosphate (*Standard Methods*, 1989)

[3] As 2 above, excluding digestion procedure

[4] According to Skalar AutoAnalyser Industrial Method Cat. No. 155-205

[5] According to Skalar AutoAnalyser Industrial Method Cat. No. 155-205

[6] According to Skalar AutoAnalyser Industrial Method Cat. No. A461-S

[7] Separation of solids by centrifugation, drying in crucible at 105°C and incineration at 550°C

[8] According to Lilley *et al.*, (1997)

[9] YSI DO probe (5739) and automated procedure of Randall *et al.*, (1991)

[10] Beckman portable pH meter

Note: S_{bs} concentration was determined according to the physical-chemical separation method of Mamais *et al.*, (1993)

3.4 RESULTS

3.4.1 Identification of presumptive PAO's

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 3.2). This amounted to 41% of the total recovered isolates which were presumed to be PAO's. Ten of the presumptive PAO's were Gram negative (Proteobacteria) whilst six were Gram positive (Firmicutes), relating to 63 and 37% of the total PAO population, respectively (FIG. 3.1). The PAO isolates were subsequently identified and expressed as a percentage of their relevant Gram reactions (FIGS 3.2 and 3.3).

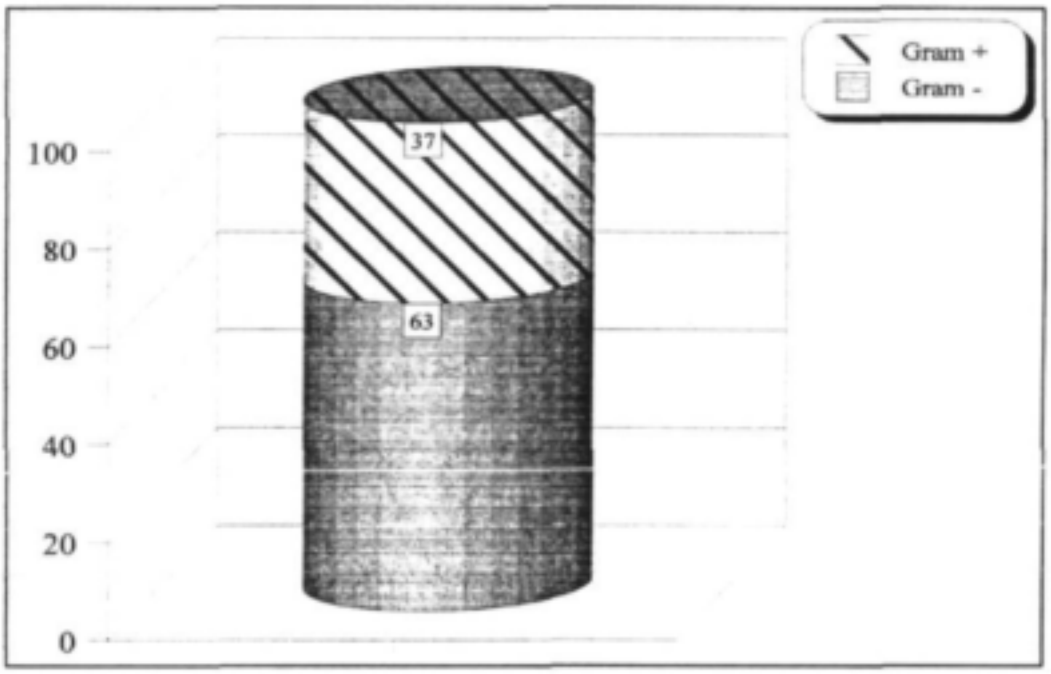

FIGURE 3.1 Percentage PAO bacterial population according to Gram reaction.

It is evident from FIG. 3.2 that either the incidence or recovery rate of *Acinetobacter* spp. in the mixed liquor was very low with respect to the other PAO isolates in the Darvill WWW activated sludge system. In either instance, *Acinetobacter* spp. only amounted to 8% of the Gram negative community. *Pseudomonas* spp. appeared to dominate the Gram negative PAO population (56%) (FIG. 3.2) followed by the Gram positive organisms, *Staphylococcus* spp. and *Streptococcus* spp. (40 and 30%), respectively (FIG. 3.3).

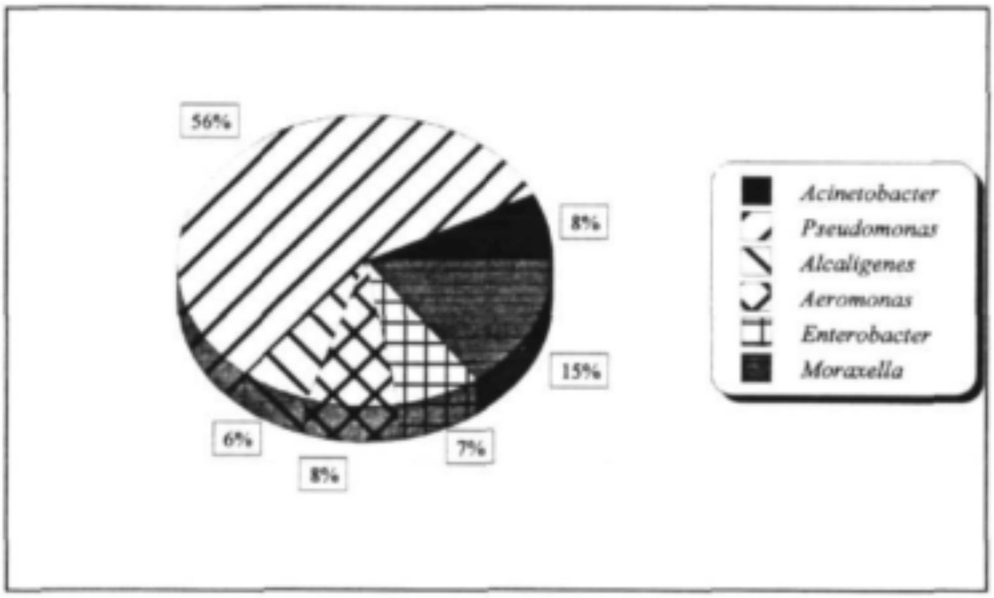

FIGURE 3.2 Percentage heterotrophic Gram negative PAO genera isolated from the aerobic zone of Darvill WWW. Cultures grown and maintained on CGY agar.

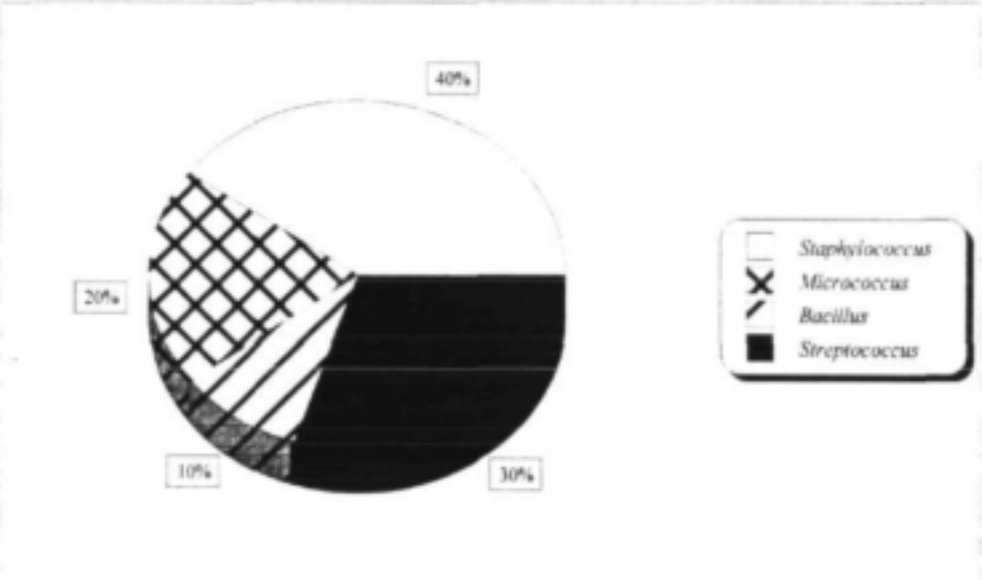

FIGURE 3.3 Percentage heterotrophic Gram positive PAO genera isolated from the aerobic zone of Darvill WWW. Cultures grown and maintained on CGY agar.

3.4.2 Phosphate uptake by isolates

The phosphate accumulating capacity of the various isolates is presented in TABLE 3.2. 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 3.2), 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 i.e., *ca.* 1 to 2 x 10^{-11} mg P per cell. *Bacillus cereus*, from the Bacillus-Clostridium grouping, 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.

3.4.3 Phylogenetic distribution and classification of PAO's

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 3.3 and FIG. 3.4). The most prominent PAO isolates proved to be the gamma subclass members of Proteobacteria (47%), followed by the Bacillus/Clostridium cluster (29%). The beta subclass of Proteobacteria (18%) and the Actinobacteria (6%) constituted the remainder of the PAO community (FIG. 3.4).

TABLE 3.2 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	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)

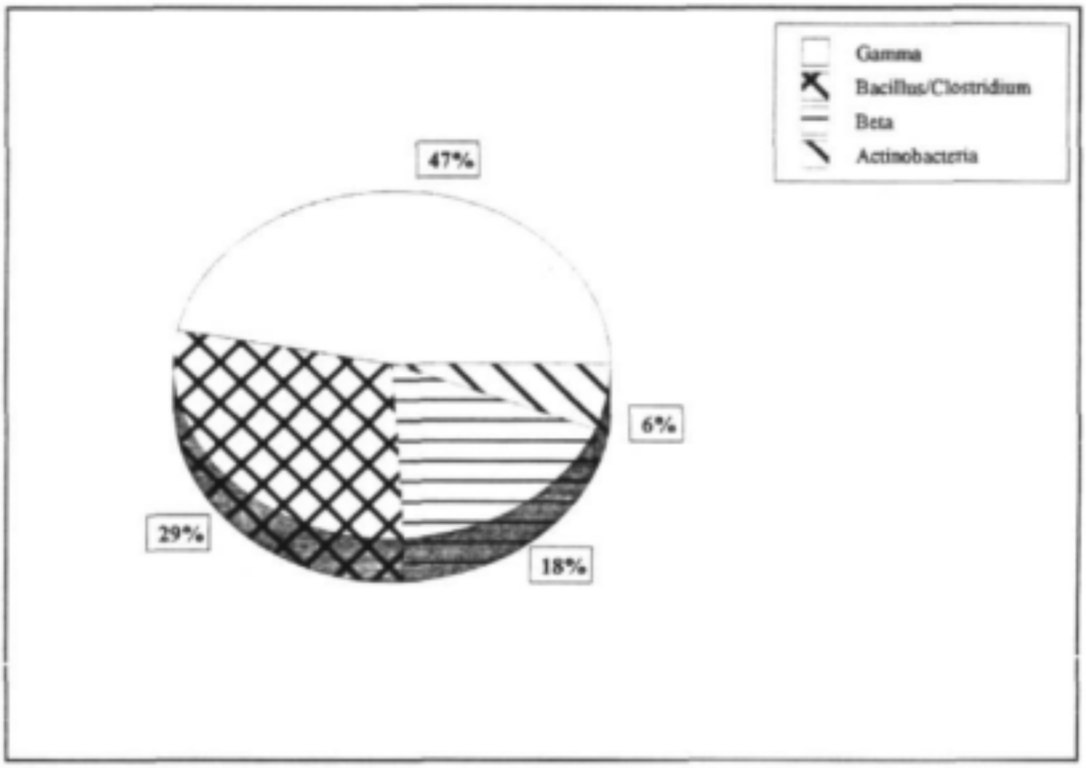

FIGURE 3.4 Graphical representation of isolated PAO's from Darvill WWW mixed liquor at the family level of classification.

TABLE 3.3 Phylogenetic classification and current bacterial names of PAO's cultivated from Darvill WWW.

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	Bacillus/Clostridium group
Micrococcus spp.	NC	Actinobacteria
Moraxella phenylpyruvica	NC	Proteobacteria (γ subdivision)
Staphylococcus epidermidis	NC	Bacillus/Clostridium group
Streptococcus spp.	NC	Bacillus/Clostridium group
Pseudomonas testosteroni	*Comamonas testosteroni*	Proteobacteria (β subdivision)
Staphylococcus aureus	NC	Bacillus/Clostridium group
Pseudomonas acidovorans	*Comamonas acidovorans*	Proteobacteria (β subdivision)
Staphylococcus spp.	NC	Bacillus/Clostridium group
Enterobacter agglomerans	NC	Proteobacteria (γ subdivision)
Alcaligenes denitrificans	*Achromobacter xylosoxidans* subsp. *denitrificans*	Proteobacteria (β subdivision)

NC = no change

3.4.4 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 zones and analysed to obtain a more holistic impression of P release and uptake occurring throughout the system (FIGS 3.5A - 3.5E). It is evident from FIGS 3.5A to 3.5E that the BPR mechanism was functional due to the expected curve representing anaerobic P release and subsequent uptake in the downstream AE reactors. 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 i.e., present in effluent at concentrations > 1 mgP/L. Net P uptake from the system gradually increased as the culture became enhanced. Initial net P uptake during period C was *ca.* 14 mgP/L (FIG. 3.5A) which increased to a maximum of *ca.* 38 mgP/L during period G (FIG. 3.5E). A slight increase in effluent soluble phosphate concentration was noted during periods D (FIG. 3.5B) and F (FIG. 3.5D) which was attributed to prolonged solids retention times in the clarifier which promoted the onset of anaerobiosis.

The transient behaviour of the P removal mechanism is shown in FIG. 3.6, depicting soluble reactive phosphorus (SRP, as P) removal as a function of time. A gradual increase in the BPR mechanism can be noted as the HAc 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 D and E 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.

TABLE 3.4 shows actual influent and effluent SRP concentrations and percentage SRP removal for the experimental periods. During experimentation, soluble P was supplemented to the influent feed (in the form of K_2HPO_4) to ensure P was not limiting to the system. However, during periods D and F the BPR mechanism increased dramatically and effluent ortho-P concentrations decreased below 1 mgP/L (TABLE 3.4). 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 F is as a result of dilution of the settled sewage fraction to the feed. Phosphate supplementation, however, was not increased concomitantly and remained constant at 20 mgP/L. A decrease in P uptake, based on percentage removal, was recorded during period G (86.4% as opposed to 98.8% during period F) but actual P removal continued to increase (TABLE 3.4). Although this doesn't necessarily reflect a decreasing removal mechanism, it may have been an indication that there was no longer sufficient active PAO biomass in the system to cope with increased phosphate loads.

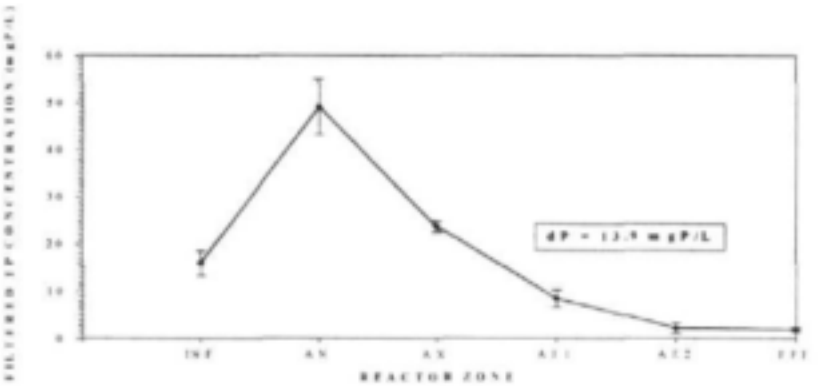

FIGURE 3.5A Average TP concentrations in reactors of pilot plant during enhanced culture development (INF=influent; AN=anaerobic; AX=anoxic; AE1=aerobic 1; AE2=aerobic 2; EFF=effluent) during period C (150 mgCOD/L as NaAc). Standard deviations are indicated at each point.

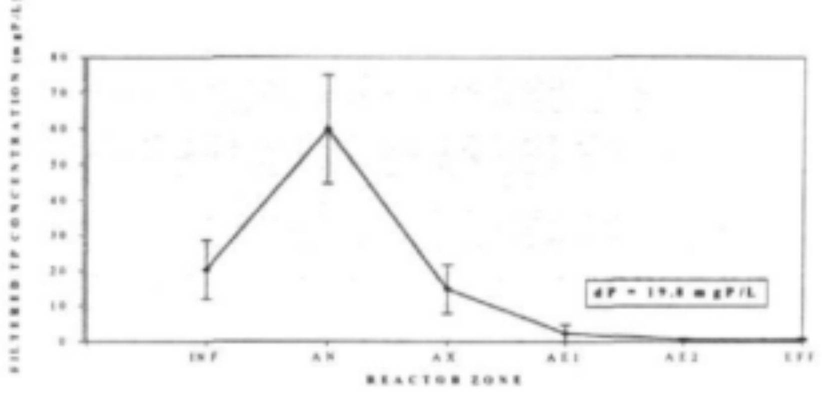

FIGURE 3.5B Average TP concentrations in reactors of pilot plant during enhanced culture development (INF=influent; AN=anaerobic; AX=anoxic; AE1=aerobic 1; AE2=aerobic 2; EFF=effluent) during period D (200 mgCOD/L as NaAc). Standard deviations are indicated at each point.

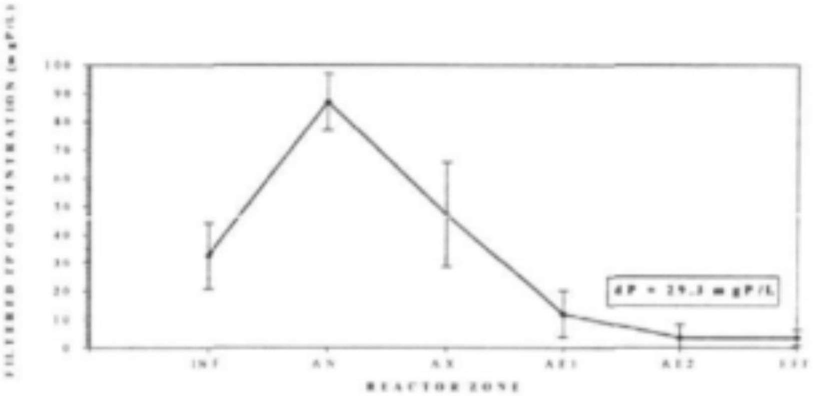

FIGURE 3.5C Average TP concentrations in reactors of pilot plant during enhanced culture development (INF=influent; AN=anaerobic; AX=anoxic; AE1=aerobic 1; AE2=aerobic 2; EFF=effluent) during period E (300 mgCOD/L as NaAc). Standard deviations are indicated at each point.

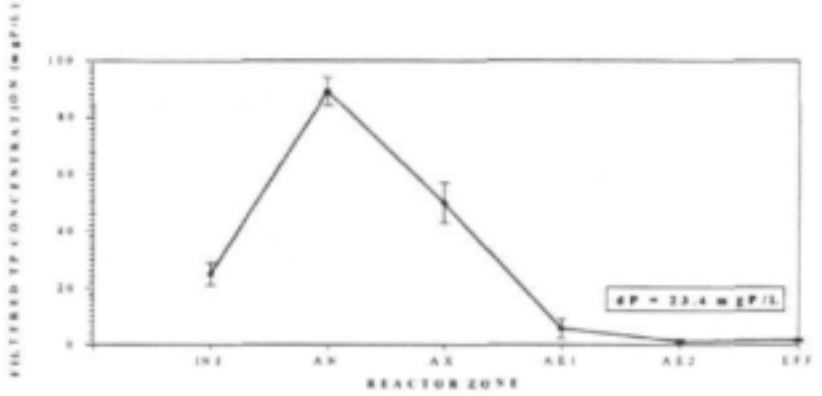

FIGURE 3.5D Average TP concentrations in reactors of pilot plant during enhanced culture development (INF=influent; AN=anaerobic; AX=anoxic; AE1=aerobic 1; AE2=aerobic 2; EFF=effluent) during period F (400 mgCOD/L as NaAc). Standard deviations are indicated at each point.

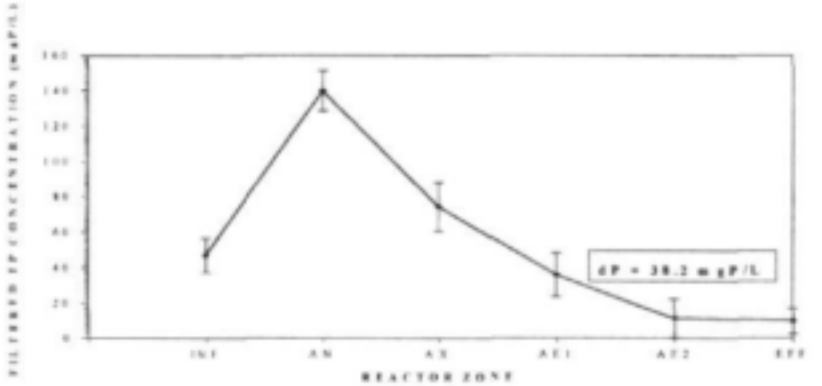

FIGURE 3.5E Average TP concentrations in reactors of pilot plant during enhanced culture development (INF=influent; AN=anaerobic; AX=anoxic; AE1=aerobic 1; AE2=aerobic 2; EFF=effluent) during period G (500 mgCOD/L as NaAc). Standard deviations are indicated at each point.

FIGURE 3.6 Total SRP (as P) removal plotted as a function of time.

TABLE 3.4 Mean influent and effluent SRP concentrations (mgP/L) and percentage removal for periods A to G. Results expressed as mean values with sample standard deviations in parentheses.

Period	Average soluble P concentration (mgP/L)			
	Influent	Effluent	Removal	% P_{sol} removal
A	ND	ND	ND	ND
B	12.2 (1.47)	9.9 (0.91)	2.3	18.9
C	12.7 (0.21)	7.4 (0.39)	5.3	41.7
D	16.7 (6.15)	0.4 (0.20)	16.3	97.6
E	27.0 (3.80)	2.5 (2.53)	24.5	90.7
F	24.1 (2.16)	0.3 (0.35)	23.8	98.8
G	44.2 (7.00)	6.0 (5.60)	38.2	86.4

ND = not determined

3.4.5 Influent and effluent monitoring

3.4.5.1 Influent characterization

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 respectively. However, as mentioned earlier, the influent COD target proved difficult to achieve due to the weak COD load of Darvill settled sewage. Periods prior to E (when HAc supplementation increased to 300 mg/L as COD) were therefore deficient of the target COD load (TABLE 3.5). The contribution of supplemented NaAc (based on stoichiometry) to the various period COD loads was recorded as follows: period B = 3.5 gO_2/d; period C = 5.5 gO_2/d; period D = 7.1 gO_2/d; period E = 11.2 gO_2/d; period F = 14.6 gO_2/d; and period G = 18.1 gO_2/d. At 100 per cent HAc supplementation during period G, theoretical COD (as HAc) addition based on reaction stoichiometry was never achieved which led to lower recorded COD and RBCOD loads for the period (TABLE 3.5).

The sudden increase in COD entering the system during period E (22.7 mgO_2/d) was due to the infiltration of industrial effluent from a local vegetable oil refinery into the sewage system. Total phosphorus and TKN entering the system was also routinely monitored to ensure phosphate was not limiting i.e., always detectable in the clarified effluent, and the denitrification capacity of the anoxic zone was never over-extended. The strength of the BPR removal mechanism became especially evident when one considers that influent TP loads had to be increased from 0.92 g/d during period F to 1.74 g/d during the latter half of period G (TABLE 3.5).

TABLE 3.5 Average influent flow rate (Q_i), hydraulic retention time (HRT) and mass loading rate of the pilot plant for experimental periods A to G. Results expressed as mean values with sample standard deviations in parentheses.

Period	Q_i (L/d)	COD load (gO_2/d)	RBCOD load (gO_2/d)	TP load (gP/d)	TKN load (gN/d)
A	ND	ND	ND	ND	ND
B	34.8 (2.4)	15.14	4.52	0.59	1.04
C	36.3 (4.62)	16.52	5.05	0.59 0.47^*	1.23
D	35.5 (4.08)	15.27	6.39	0.92 0.6^*	1.35
E	37.3 (2.16)	22.68	11.34	1.23 1.0^*	1.38
F	36.6 (2.8)	19.73	12.77	0.92 0.89^*	1.1
G	36.2 (2.75)	17.81	13.61	1.74 1.48^*	0.83

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

3.4.5.2 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 3.6).

TABLE 3.6 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 A to G. Results expressed as mean values with sample standard deviations in parentheses.

Period	S_{ti} (mgO_2/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)
A	ND	ND	ND	ND	ND	ND
B	435 (37.74)	0.3	87.4	0.31	0.03	0.09
C	455 (124.96)	0.31	91.9	0.33	0.03	0.09
D	430 (88.31)	0.42	91	0.28	0.07	0.14
E	608 (171.33)	0.5	91	0.29	0.05	0.09
F	539 (59.26)	0.65	93	0.25	0.05	0.07
G	492 (51.3)	0.76	94.2	0.23	0.08	0.1

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

An unusual observation during period G was that at a theoretical HAc supplementation of 500 mgO_2/L as COD (the only source of organic loading in the influent), the RBCOD fraction (f_{ts}) amounted to only 76% of S_{ti} which itself amounted to 492 mgO_2/L (TABLE 3.6). The mean RBCOD concentration achieved during period G therefore only amounted to 374 mgO_2/L (TABLE 3.6). The same pattern was also recorded during period F, where the mean RBCOD concentration amounted to 350 mgO_2/L as opposed to the target concentration of 400 mgO_2/L. Microwave digestion and potentiometric titration of organic samples are known to render reasonable C recoveries. It may be possible, however, that microwave digestion is unsuitable for samples containing volatile organics and an underestimation will therefore be obtained. Due to the maintenance of the system on HAc alone during period G, the convergence of S_{ti} and S_{bsi}

during the latter stages of system operation was expected. High COD removal was recorded throughout experimentation i.e., 87 to 94% removal (TABLE 3.6).

TABLE 3.6 also summarizes 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 3.6). 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 (*ca.* 5 h) and reasonably limiting conditions as far as organic substrate was concerned i.e., low F/M ratio. These values give an indication of COD utilisation efficiency with regards to phosphate uptake and removal in the aerobic reactor.

3.4.5.3 Phosphorus and nitrogen

Results of mean influent TKN/COD and TP/COD ratios for periods B to G are summarized in TABLE 3.7. The TKN/COD ratio was, as far as possible, maintained at 0.06 mgN/mgCOD. During the latter stages of experimentation (periods F and G), influent required supplementation with ammonium chloride to regulate the TKN/COD ratio. The high TKN/COD ratios, recorded during periods B to D, are mainly due to the reduced COD (<500 mg/L) strength of the influent entering the pilot plant. During period G, 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. The increase in S_{ti} during period E and concomitant decrease in N_{ti} managed to reduce the TKN/COD ratio considerably from 0.09 during period D to 0.06 where the ratio was stabilized through constant N supplementation (as NH_4Cl) to the influent. Generally, nitrification processes were responsible for removing between 90 to 99% of influent ammonia. Effluent nitrates were generally below 2 mgN/L, indicating optimal performance of the anaerobic zone as far as recycled nitrates were concerned (results not shown).

TABLE 3.7 Mean influent TKN/COD (N_{ti}/S_{ti}) and TP/COD (P_{ti}/S_{ti}) ratios for experimental periods B to G.

Period	N_{ti}/S_{ti} (mgN/mgCOD)	P_{ti}/S_{ti} (mgP/mgCOD)
B	0.07	0.04
C	0.07	0.03
D	0.09	0.06
E	0.06	0.05
F	0.06	0.05
G	0.05	0.1

3.4.6 Sludge settleability, system solids and mass fractions

Although system MLSS concentrations increased dynamically (until steady-state was achieved during period E), the active portion or MLVSS of the TSS showed a distinct decline from an initial value of 77% to 53% during period G (TABLE 3.8). The increase in MLSS during period E corresponds directly to the increased COD load during the same period. Data regarding SV_{30} and SVI are also given in TABLE 3.8. The high SVI value recorded during period D 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 E 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 planktonic bacteria (resulting in higher S_{te} values).

During experimental periods D to G, mixed liquor mass fractions were routinely monitored to ensure the anaerobic fraction did not fluctuate significantly with subsequent negative effects (TABLE 3.9). Anaerobic mass fractions of 10% or lower can result in acetate leakage from the reactor i.e., 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 D to G with regards to mass fractions in the respective reactors (TABLE 3.9).

TABLE 3.8 Mean MLSS, MLVSS/MLSS ratios and SV_{30} and DSVI values for experimental periods A to G. Results expressed as mean values with sample standard deviations in parentheses.

Period	MLSS (g/L)	MLVSS/MLSS * (mgVSS/mgTSS)	SV_{30} (mL/L)	DSVI (mL/g)
A	ND	ND	ND	ND
B	1.532 (0.38)	0.77	141.8 (15.80)	92.6 (16.98)
C	1.587 (0.25)	0.71	167.0 (27.00)	105.2 (12.45)
D	1.689 (0.28)	0.69	213.3 (43.87)	126.3 (35.92)
E	2.420 (0.50)	0.61	206.9 (17.10)	85.5 (14.25)
F	2.429 (0.23)	0.61	208.3 (11.69)	85.8 (6.96)
G	2.421 (0.22)	0.53	201.1 (26.12)	83.1 (11.42)

ND = not determined; * calculated in aerobic zone

TABLE 3.9 Mixed liquor mass fractions of individual reactors during experimental periods D to G.

Period	AN	AX	AE*
D	0.24	0.12	0.64
E	0.23	0.13	0.64
F	0.2	0.12	0.68
G	0.23	0.12	0.65

* includes combined AE1 and AE2 reactor zones

3.4.7 Mixed liquor pH

Although alkalinity was continuously fed to the reactor it has been reported that inducing the BPR mechanism can cause the pH of the bulk liquid to increase, potentially resulting in total collapse of the system (Wentzel *et al.*, 1988). Analysis of TABLE 3.10 shows a gradual increase in the pH value of AE1 and AE2 as the BPR 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 E and a decrease in the pH of AX, AE1 and AE2 zones was observed during periods F and G. The anaerobic zone remained within a relatively constant pH range for the duration of experimentation i.e., *ca.* 7.0 - 7.2. Of significance in TABLE 3.10 was that the pH of the aerobic reactors was maintained below pH 7.8 at all times in order to discourage chemical precipitation of phosphate and to observe the biological BEPR mechanism exclusively.

TABLE 3.10 Reactor pH values during experimental periods A to G. Results expressed as mean values with sample standard deviations in parentheses.

Period	AN	AX	AE1	AE2
A	ND	ND	ND	ND
B	6.99 (0.14)	7.09 (0.12)	7.16 (0.13)	7.29 (0.18)
C	7.01 (0.09)	7.15 (0.09)	7.30 (0.03)	7.43 (0.06)
D	7.18 (0.15)	7.42 (0.10)	7.49 (0.04)	7.68 (0.03)
E	7.11 (0.12)	7.30 (0.09)	7.69 (0.08)	7.77 (0.15)
F	7.16 (0.06)	7.26 (0.07)	7.48 (0.26)	7.75 (0.18)
G	7.14 (0.07)	7.23 (0.13)	7.22 (0.15)	7.63 (0.24)

ND = not determined

3.5 DISCUSSION

Many studies have previously been conducted to attempt to identify the heterotrophic PAO population in various activated sludge mixed liquors (Hart and Melmed, 1982; Brodisch and Joyner, 1983; Lötter and Murphy, 1985; 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 focused in attempting to elucidate exactly which organisms are responsible for BEPR. This, however, remains an extremely daunting task due to the vast microbial diversity which exists in activated sludge and various abiotic parameters i.e., influent flow and loads, which affect the BPR mechanism and activated sludge process as a whole.

When applying 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 BEPR using chemifluorescent and API 20E techniques of identification. The major contributing factors to discrepancies between authors when conducting bacterial community analyses of BEPR 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 recovered *Acinetobacter* isolates (8%; FIG. 3.2) 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 (FIG. 3.2) 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 (HAc 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 majority of pseudomonads isolated from Darvill belong to the gamma subclass of Proteobacteria (TABLE 3.3). 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* spp. numbers should have amounted to more than 8% of the Gram negative PAO population (FIG. 3.2). The dominance of the gamma subclass of Proteobacteria is evident in FIG. 3.4 and TABLE 3.3. However, the major contribution to gamma subclass dominance was derived from *Pseudomonas* spp. A possible explanation for this could be the method of identification employed for this study i.e., 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 *A. 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 species types (wild types) of this genus. During this study, only *A. calcoaceticus* was isolated from the mixed liquor. This low diversity range 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 3.2). 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 initial biomass concentrations when 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 *A. 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 seriously disadvantaged the cells physiologically. However, an 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. 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 3.2).

It has been reported that the physiological state of PAO's in the anaerobic zone affects P release (Rustrian *et al*., 1997). The authors found that *Acinetobacter* cells in the stationary phase of growth were able to release more accumulated P (18-58% of intracellular poly-P) than those in exponential growth (5-38%). It is possible that the 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 3.2). 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. 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 subsequently P uptake) would therefore be reduced. Phosphate uptake by the various isolates may therefore be in excess of those values reported in TABLE 2.2. 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 rendered 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 obtained during this study. 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 diversity. 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.

Low *Acinetobacter* cell counts during the present study could be attributable to the organism having a low specific growth rate, therefore been rapidly outgrown by the other isolates. Oerther *et 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 BEPR was successfully reported, 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; Mino *et al*., 1998).

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 characterized 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 (apart from molecular differentiation) of discerning between the two genera i.e., *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 BEPR 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 poly-P accumulation studies.

The low number of isolates recovered and lack of diversity recorded 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.

It is evident from FIGS 3.5A - 3.5E that the BPR 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 BEPR biochemical and kinetic models. A striking feature of the enhanced culture was the magnitude of P release, uptake and removal in the respective reactors. During period G (FIG. 3.5E), anaerobic P release amounted to 93 mgP/L and P uptake amounted to 130 mgP/L resulting in a net 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 and a P uptake of 310 mgP/L, resulting in a net removal of 60 mgP/L for 500 mgCOD/L acetate feed (theoretically the same as period G). Differences in phosphate removal capacities may suggest that a different microbial community was developed during the present study. It is also likely that the activated sludge was not at steady-state when mixed liquor samples were taken and analysed which may have influenced soluble P concentrations.

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 but of further interest is that f_{bs} fractions should exceed 0.20 (Ekama *et al*., 1984). This criterion was maintained throughout experimentation with f_{bs} values ranging from 0.30 through to 0.76 for experimental periods B and G, 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 BEPR response 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. If this was the case, the enhanced culture receiving 500 mgCOD/L as HAc during period G would theoretically only be capable of removing 10 mgP/L. Analysis of FIGS 3.5E and 3.6 and TABLE 3.4 shows that a maximum of *ca.* 38 mgPO$_4$-P/L was removed from the system during period G. A more relevant assumption is 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.

Sewage characteristics have a significant influence on biological nutrient removal. Assuming a sufficient active organism mass constituting the system MLVSS, 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. 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 D) which, according to Ekama *et al*., (1984), is suitable for complete nitrate removal when employing the Phoredox process.

Settling efficiency of activated sludge in the system was measured by means of the DSVI assay (TABLE 3.8). Except for periods C and D, DSVI values indicated that the sludge was settling well. At no stage during culture development did it visually 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 DSVI value of 126 mL/g during period D may have indicated the onset of dispersed growth which became evident during period E, 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 and subsequent poor floc formation in activated sludge (Wanner, 1997). Excessive organic loading seems to be responsible for the high DSVI values during periods C and D as a simultaneous decrease in DSVI to 85.5 mL/g was recorded during period E as the system MLSS increased to 2.420 g/L (TABLE 3.8). The gradual decrease in activated sludge MLVSS from

0.77 (normal range for activated sludge) during period B to 0.53 mgMLVSS/mgMLSS during period G (TABLE 3.8) is indicative of an enhanced culture (Wentzel *et al.*, 1988).

When conducting BEPR pilot-scale studies, Wentzel *et al.*, (1988) suggest two operational procedures which prevent overloading of the PAO's in the anaerobic reactor with HAc (resulting in HAc leakage). These include limiting the HAc load increments and enlarging the anaerobic reactor. They also suggest an anaerobic mass fraction of 0.30 and an anoxic mass fraction of 0.08. During the present study, anaerobic mass fractions fluctuated between 0.20 - 0.24 whilst the anoxic fraction remained reasonably stable at 0.12 (TABLE 3.9). Acetate increments were increased every two to three sludge ages i.e., every 20 to 30 d, to ensure that sufficient time was permitted for maximum HAc sequestration in the anaerobic zone and to allow for significant growth (yield) of PAO's in the system.

3.6 CONCLUSIONS

Acinetobacter spp. were not the dominant PAO's isolated from Darvill WWW activated sludge 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 community.

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 BEPR 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 the phylogenetic group, Firmicutes, were found to be very prominent in the Darvill PAO community, indicating their significance to the BEPR 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 before analysis i.e., adequate floc disruption and dispersion;
- plating bias - will the isolation medium employed recover the maximum number of viable cells and will the incubation parameters selected 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 from environmental samples; 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 i.e., immunofluorescence, quinone profiles and FISH. This is not to say that microbiologists must rebuke the conventional plating and isolation procedure for it remains a powerful tool and our level of understanding would not be where it is today without it.

During the course of this study, it became apparent that a laboratory-scale activated sludge process, displaying a strong BEPR mechanism, had been successfully developed using HAc as the sole supplemented RBCOD source. A primary objective of enhanced culture development was to simulate the system of Wentzel *et al*., (1988) to provide samples for microbial community characterization. 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.

The following chapter includes the FISH molecular technique to identify and quantify organisms in the pilot- and full-scale NDBEPR systems investigated during the course of this chapter.

CHAPTER FOUR

MICROBIAL COMMUNITY ANALYSIS IN BIOLOGICAL NUTRIENT REMOVAL PROCESSES USING RIBOSOMAL RIBONUCLEIC ACID DIRECTED OLIGONUCLEOTIDE PROBES

4.1 INTRODUCTION

The fact that the biomass within the activated sludge process is responsible for mediating the treatment functions of interest viz., COD removal, nitrogen removal and phosphorus removal, substantiates studies focused on the microbial composition of the activated sludge mixed liquor. Current mathematical models describing the behaviour of the activated sludge process are not based on the direct measurement of the different components comprising the biomass (the metabolically active fraction of the MLVSS). Engineers have traditionally used MLVSS as a 'lumped' indication of the active biomass within activated sludge systems. Although the MLVSS parameter has the advantage of fitting directly into mass balance equations, it has the disadvantage of not providing a true indication of the total active biomass (X_a) present. This parameter represents not only X_a but also endogenous residue (dead cellular material; X_e) and inert particulate COD originating from the influent (X_{II}) (Wentzel *et al.*, 1998). Within Activated Sludge Model No.2 (Henze *et al.*, 1995), X_a of NDBEPR sludge encompasses the following components:

- nitrifying organisms (X_{AUT}), responsible for nitrification;
- the 'all-rounder' heterotrophic organisms (X_H), responsible for fermentation in the anaerobic zone, denitrification in the anoxic zone, and chemoheterotrophic activity in the aerobic zone; and
- X_{PAO}, responsible for the accumulation of polyphosphate in the aerobic zone.

Efforts to calibrate and validate the model kinetic and stoichiometric parameters do not currently involve the direct, experimental determination of mixed liquor concentrations of these microbial populations. Through advances in molecular biology, in particular, developments in the field of microbial evolution (Woese, 1987), tools have become available which may prove useful in furthering the descriptive and predictive capacity of current mathematical models for BEPR. One of these improved techniques i.e., FISH, was applied during the present research to allow for quantitative microbiological data to be expressed within the context of process engineering terms.

4.2 AIMS AND OBJECTIVES

- to investigate the major bacterial phylogenetic families implicated in pilot- and full-scale enhanced biological nutrient removal processes;
- to determine the proportion of the biomass that is metabolically active within the sludge; and
- to determine the proportion of the biomass responsible for phosphorus uptake.

4.3 MATERIALS AND METHODS

4.3.1 Sampling and cell fixation

Grab samples of activated sludge mixed liquor were collected from the AN, AX and AE zones of the pilot-scale BEPR process. Representative bacterial strains from each of the classes, sub-classes and genera investigated, used to evaluate probe specificity, are given in TABLE 4.1. Monocultures 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. Activated sludge samples and monocultures were fixed for 2 hours at 4^0C with 3% paraformaldehyde/PBS by the addition of three volumes of fixative to one volume of sample. Cells were then washed in 1 x PBS (130 mM NaCl, 10 mM Sodium phosphate buffer, pH 7.2) and resuspended in PBS/cold absolute ethanol (1:1 v/v). For fixation of Gram positive cells, activated sludge was added to ethanol to a final concentration of 50% (v/v).

TABLE 4.1 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
Corynebacterium glutamicum	SABS	GPBHGC	HGC

SABS - South African Bureau of Standards culture collection; TN - Technikon Natal Microbiology Department; ATCC - American Type Culture Collection

4.3.2 Membrane filtration and staining with DAPI

Membrane filtration was carried out as described by Porter and Feig, (1980). Dual staining of cells with DAPI and fluorescent oligonucleotides was modified from the method of Hicks *et al.*, (1992) so that cells were stained after *in situ* hybridization with DAPI (0.33 µg/mL) for 5 min. PAO's were detected by staining the cells with 50 µg/mL DAPI for 20 seconds.

4.3.3. Oligonucleotide probes

Oligonucleotide probes were synthesized and labelled with either rhodamine (red) or fluorescein (green) by Roche Molecular Biochemicals (Germany). Probes used are given in TABLE 4.2.

TABLE 4.2 Probe sequences and target sites for *in situ* hybridization.

Probe	Sequence	Target site	Fluor	Reference
EUB338	5'- GCTGCCTCCCGTAGGAGT -3'	16S	Rhodamine	Amann *et al.* 1990
ACA23a	5'- ATCCTCTCCCATACTCTA -3'	16S	Rhodamine	Wagner *et al.* 1994b
ALF1b	5'- CGTTCG(C/T)TCTGAGCCAG -3'	16S	Rhodamine	Manz *et al.* 1992
BET42a	5'- GCCTTCCCACTTCGTTT -3'	23S	Rhodamine	Manz *et al.* 1992
GAM42a	5'- GCCTTCCCACATCGTTT -3'	23S	Fluoresceine	Manz *et al.* 1992
HGC	5'- TATAGTTACCACCGCCGT -3'	23S	Rhodamine	Roller *et al.* 1994

4.3.4 FISH hybridization

Aliquots (5 µL) fixed sludge were applied to poly-lysinated slides and allowed to air dry. Hybridization solution (10 µL) (0.9 M NaCl, 20 mM Tris/HCl, pH 7.2, 0.01% SDS, 50 ng probe, X % (v/v) formamide) were applied to each spot and incubated for two hours at 46°C in an isotonically equilibrated humidity chamber. Probe was removed from the slide by rinsing with 2mL prewarmed washing solution (20 mM Tris/HCl, 0.01% SDS, 5 mM EDTA, Y M NaCl; the salt concentration, Y, was adjusted to the formamide concentration, X , in the hybridization buffer according to the formula of Lathe (1985). Slides were rapidly transferred into washing solution and incubated at 48°C for 20 min. Slides were then rinsed briefly with distilled water, air-dried and mounted in vectashield antifading solution for viewing by microscopy.

4.3.5 Microscopy and image analysis

Cells were visualised with a Zeiss (Carl Zeiss, Germany) Axiolab microscope fitted for epifluorescence with a 50W mercury high- pressure bulb and Zeiss filter sets 02, 09 and 15. Images were captured using a Sony (Japan) CCD camera. Image analysis was carried out using the Zeiss KS 300 imaging system.

4.3.6 Nucleic acid extraction and membrane hybridization

Total nucleic acids were extracted from about 0.2 g (wet weight) activated sludge using hot phenol. Briefly, a screw-cap polypropylene vial was filled to one third with 0.1 mm diameter glass beads, 750 µL buffer (50 mM NaOAc, 10 mM EDTA), 50 µL 20% SDS and approximately 600 µL of buffer-saturated phenol. Mechanical cell disruption was carried out by bead beating using a Minibeadbeater (Biospec Products, USA) for 4 min. at 4 200 rpm. The mixture was then heated in a hot block at 60°C for 10-15 min. followed by 2 min. of further beating. The aqueous layer was decanted to a clean microfuge tube and 400 µL of buffer saturated phenol and 100 µL of chloroform-isoamyl alcohol (24:1 v/v) was then added and mixed. Phenol-chloroform extractions were repeated twice. The top layer was removed and total nucleic acids precipitated with 1/10 volume 3 M NaOAc, (pH 5.1) and 2 volumes of ethanol at -20°C overnight (Stahl *et al.*, 1988). Total nucleic acids were resuspended in sterile double distilled water for analysis. Membrane hybridizations (dot blots) were performed as described by Manz *et al.*, (1992).

4.4 RESULTS

TABLE 4.3 Probe specific counts (*in situ*) expressed as a percentage of total cells stained with DAPI.

	Full-scale non-BEPR (%)	Pilot-scale BEPR (%)	Full-scale BEPR (%)	
Probe	FISH	FISH	FISH	Dot Blot
α	11±4	19±3	20±4	30
β	25±4	23±4	30±2	37
γ	14±5	17±3	13±5	14
HGC	13±3	11±2	8±2	12
Total	61	69	79	96
EUB338	73±5	78±8	80±7	100
PAO*	n/d	55±7	25±4	n/d

*as revealed by excess DAPI staining

The percentages listed in TABLE 4.3 represent averages that were calculated by dividing the area of probe specific fluorescence by the area of DAPI fluorescence for at least ten microscopic fields under 400X magnification.

TABLE 4.4 Plate counts and direct cell counts for non-nutrient removal and nutrient removal systems.

	Full-scale non-BEPR	**Pilot-scale BEPR**	**Full-scale BEPR**
Plate counts (CFU/mL)	5.2 x 10^{7}	2.6 x 10^{6}	1.7X10^{6}
DAPI (membrane filtration) (cells/mL)	3.0 x 10^{10}	1.6 x 10^{10}	8x10^{9}
Estimated active biomass (cells/mL)	2.19 x 10^{10}	1.25 x 10^{10}	6.4X10^{9}

TABLE 4.5 Probe specific counts (*in situ*) expressed as a percentage of total cells stained with DAPI compared to culture dependant enumeration.

	Full-scale non-BEPR		Pilot-scale BEPR		Full-scale BEPR	
Probe	Cultivation	FISH	Cultivation	FISH	Cultivation	FISH
α	13	11±4	0	19±3	14	20±4
β	13	25±4	18	25±4	13	30±2
γ	54	14±5	47	17±3	66	13±5
HGC	0	13±3	6	11±2	0	8±2
Total	80	61	71	72	93	71
Acinetobacter	8	5	21	9	24	9
EUB		73±5		78±8		80±7

TABLE 4.6 Percentage endogenous residue (X_e) for enriched pilot-scale plant.

	Average cell no./field
(Light microscopy)	410 ± 53
DAPI count	226 ± 44
X_e(%)	45

TABLE 4.6 shows the percentage contribution of X_e to the VSS for the pilot-scale BEPR plant. This was determined by comparing the number of cells displaying DAPI conferred fluorescence with the total number of cells visible by light microscopy. Since DAPI intercalates the bacterial chromosomal DNA, those cells not showing DAPI fluorescence were assumed to have their DNA degraded and therefore constitute X_e. In order to experimentally determine that fraction of the VSS that exists as active bacterial biomass, the percentage X_e, as determined in TABLE 4.6 must be subtracted from the measured VSS reading. Since at the time of sampling, the pilot-scale BEPR system was fed with a single readily biodegradable substrate source (acetate), the X_u fraction for this system can be assumed to be zero. The fraction of the active biomass as VSS can then be determined (see Eq. 1, results shown in TABLE 4.7). Through multiplying the EUB/DAPI ratio by the total cell count (DAPI, membrane filtration) (from TABLE 4.4), the metabolically active biomass of the sludge in terms of cell numbers can be estimated from Eq. 2 (results shown in TABLE 4.7):

VSS as biomass (g/L) = (100 - %Xe) . measured VSS .... (Eq. 1)
X_a (cells/mL) = (%EUB / DAPI) . DAPI (cells/mL) .... (Eq. 2)

From Eq. 1 and Eq. 2, the mean VSS per cell for the pilot-scale system can be determined by:

Mean VSS per cell (g/cell) = VSS as biomass (g/L) / X_a (cells/L) .... (Eq. 3)

TABLE 4.7 Biomass expressed in terms of key process functions for nutrient removal (pilot- and full- scale) and non-nutrient removal (full-scale) sludges.

Parameter	Full scale non-BEPR (R_s = 20 d)	Pilot-scale BEPR (R_s = 10 d)	Full-scale BEPR (R_s = 8 d)
S_{bi} (mgO_2/L)	800	492	200
Measured VSS (g/L)	3	1.29	2.75
P removal (mgP/L)	n/d	40	5.3
DAPI (cells/mL)	$3x10^{10}$	$1.6x10^{10}$	8×10^9
X_a (EUB/DAPI) (%)	73	78	80
X_a (cells/mL)	$2.19x10^{10}$	$1.25x10^{10}$	6.4×10^9
X_c (%)	n/d	45	n/d
VSS as biomass (g/L)	n/d	0.71	0.36
% measured VSS as X_a	n/d	55	13
Mean VSS (gVSS/cell)	n/d	$5.68x10^{-14}$	n/d
X_{PAO}* (%)	n/d	55	35
X_{PAO}* (cells/mL)	n/d	6.88×10^9	$2.24x10^9$
VSS as PAO's (g/L)	n/d	0.39	0.13
X_{PAO}/mgP removed	n/d	$1.7x10^{11}$	$4.2x10^{11}$

*as revealed by staining in excess with DAPI

By determining the mean VSS per bacterial cell using the pilot-scale system (Eq. 3), the VSS as biomass for the full-scale BEPR system can be calculated by extrapolation from Eq. 4 (results shown in TABLE 4.7):

VSS as biomass (g/L)= Mean VSS / cell (g) . X_a (cells/L) .... (Eq. 4)

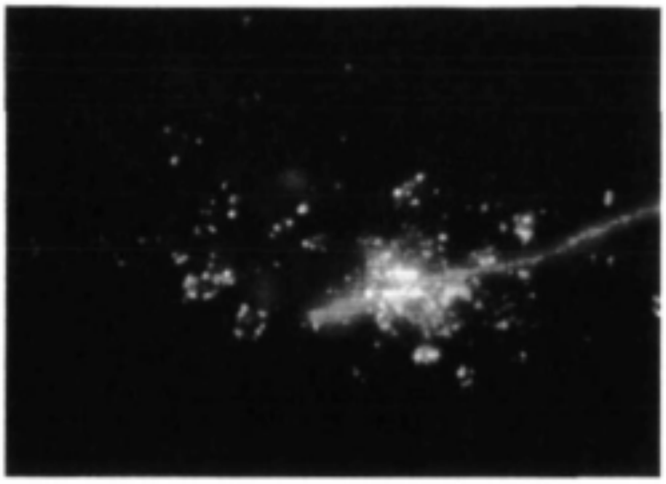

FIGURE 4.1 DAPI stain of activated sludge indicating PAO's stained yellow.

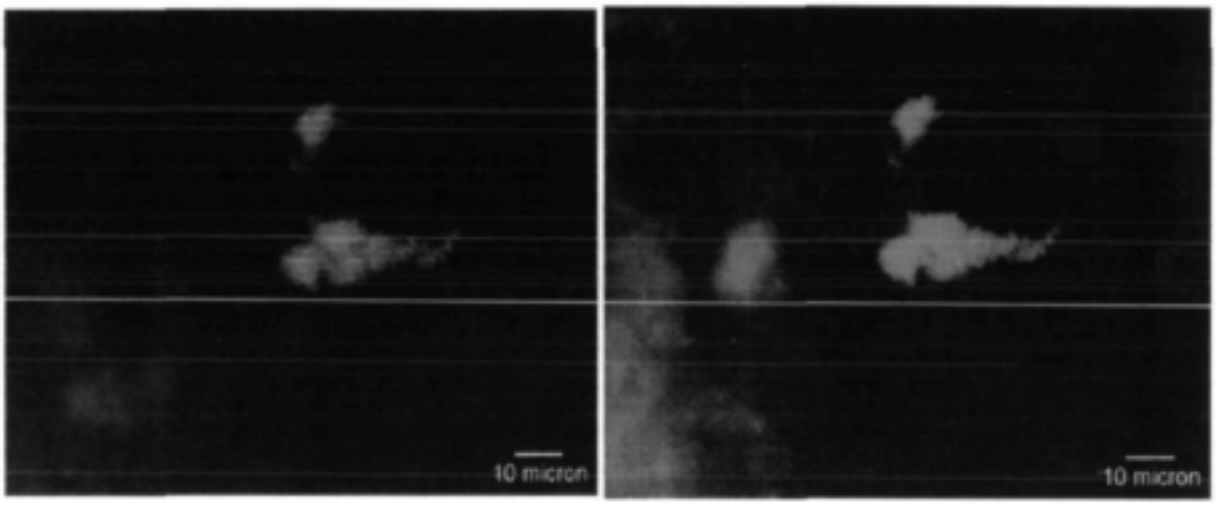

FIGURE 4.2 Application of probes GAM42a (LHS) and ACA23a (RHS) showing cells belonging to the genus *Acinetobacter* for the same microscopic field.

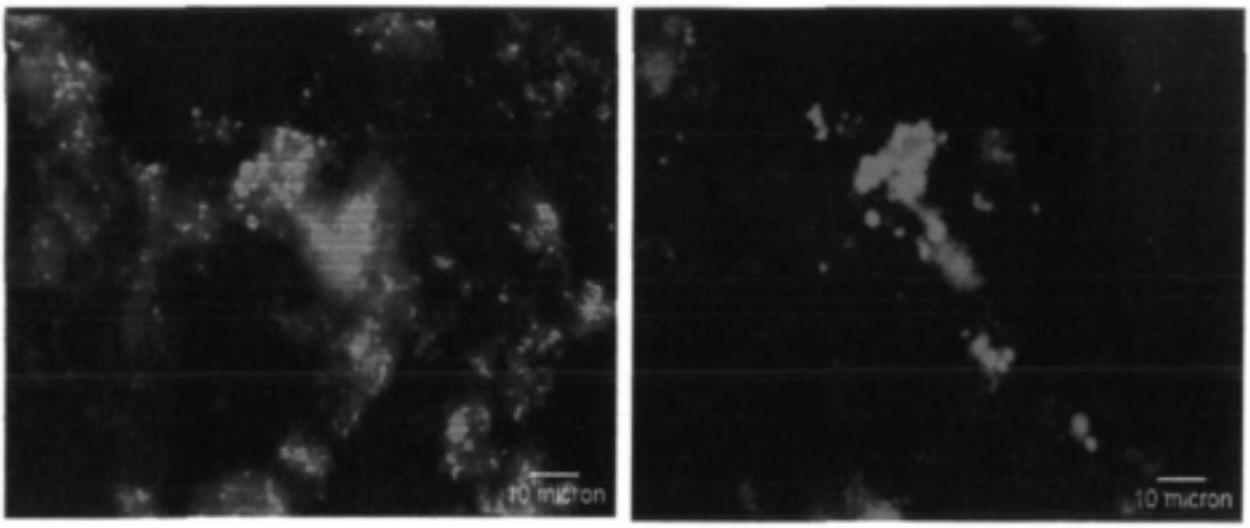

FIGURE 4.3 DAPI stain of activated sludge (LHS) and *in situ* hybridization with rhodamine labeled probe ALF1b (RHS) for the same microscopic field.

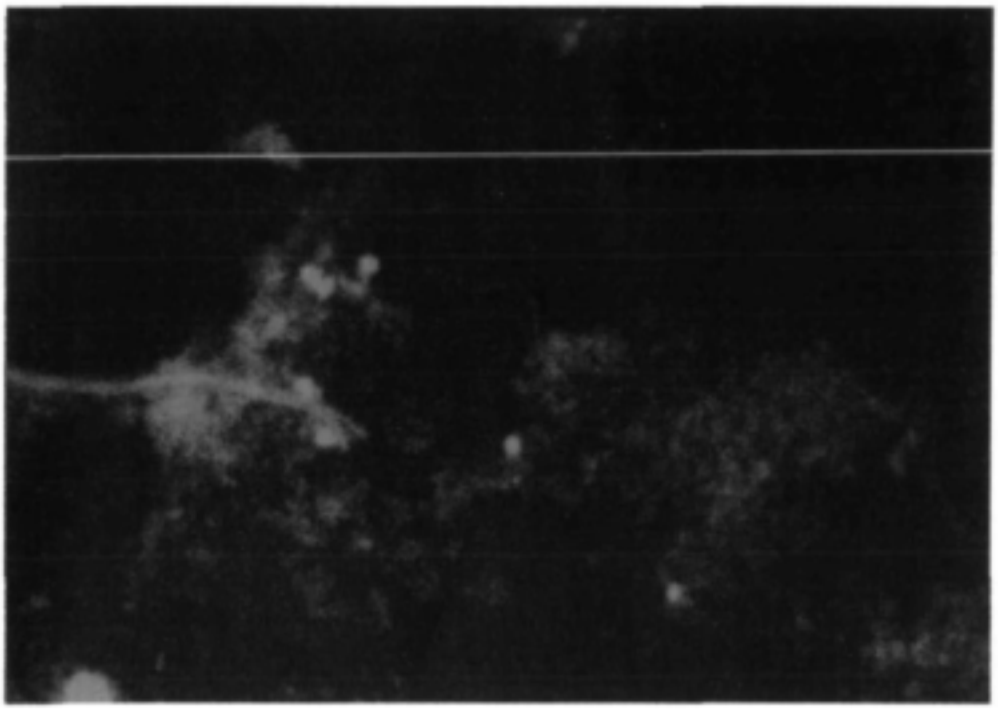

FIGURE 4.4 Activated sludge displaying probe conferred signal after hybridization with ALF1b.

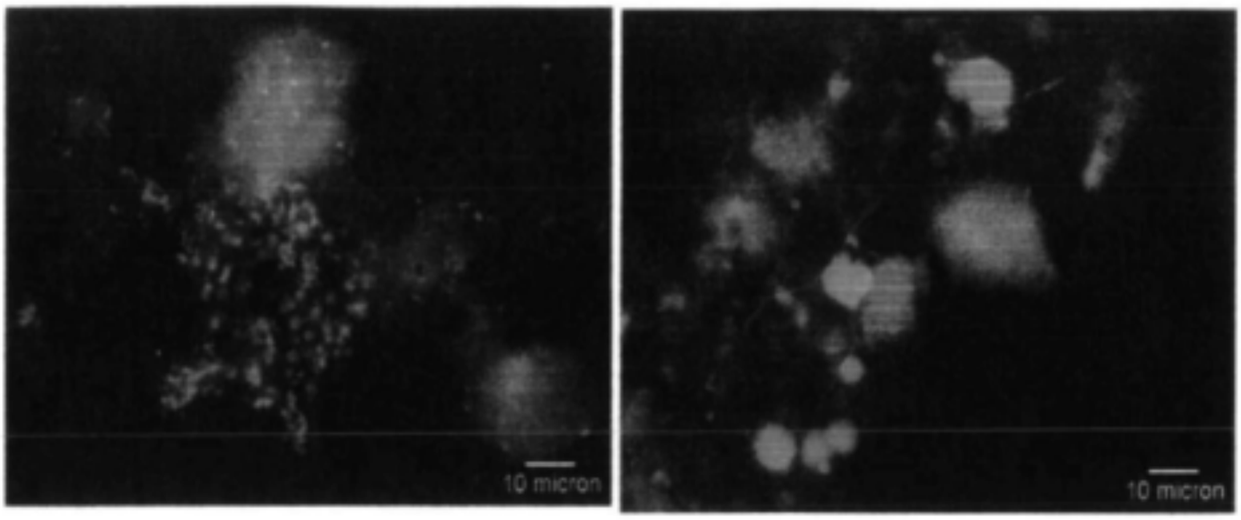

FIGURE 4.5 *In situ* hybridization of activated sludge with rhodamine (red) labelled probe BET42a and fluorescein (green) labelled probe GAM42a showing communities of bacterial cells belonging to beta and gamma Proteobacterial populations.

4.5 DISCUSSION

The microbial community was analysed by measuring the area of cells that displayed probe conferred fluorescence and expressed as the percentage of the area that bound DAPI. This approach was used as opposed to individual cell counts for *in situ* hybridizations due to the fact that most of the activated sludge flocs were very compact. For total cell counts by membrane filtration, mechanical floc disruption was carried out using a mini-beadbeater (Biospec, USA) and 1 mm glass beads. This enabled efficient counting of individual cells without affecting DAPI fluorescence. However, it was found that this treatment led to decreased probe conferred signals during *in situ* hybridization. This was attributed to excessive cell disruption which led to leakage of ribosomes from the cells, decreasing the probe target number. Extracellular polymers (ECP) did not appear to inhibit probe efficacy and cells at the inner and peripheral regions of the flocs showed comparable probe uptake. A high percentage of the activated sludge sampled from both nutrient and non-nutrient removal systems bound the probe EUB338 (TABLE 4.4) indicating that the majority of cells were bacteria which were metabolically active.

4.5.1 Cultivation dependant counts vs FISH

Total plate counts were found to underestimate the metabolically active bacterial population by three to four orders of magnitude (TABLE 4.4). It can be clearly observed from such results that in attempting to quantify the biomass within activated sludge systems plating techniques are presently not useful. Cultivation-dependent plating methods were also found not to give an accurate description of the bacterial families constituting the sludge. The gamma Proteobacteria subclass was overestimated and the contribution of the alpha and beta Proteobacteria and Actinobacteria, to the total biomass, was underestimated (TABLE 4.5).

4.5.2 Determination of the active biomass in BEPR

According to Wentzel and Ekama (1998), the heterotrophic active biomass is a key parameter within the steady-state design and kinetic simulation models for activated sludge systems. This parameter remains hypothetical since it has not been measured directly. The challenge in integrating microbiological data with process modelling is to convert cell numbers to mass units. The calculations as presented here (refer to section 4.4.1 and TABLE 4.7) serve to illustrate an approach that can be taken in order to accomplish this. Using the pilot-scale system, a measure of 5.68×10^{14} bacterial cells can be associated with a VSS reading of 1 g (Eq. 1). It should be noted that this cell number comprises both heterotrophs and autotrophs. This need not be a serious problem since the autotrophs can be inhibited by the addition of allyl-thiourea. This ratio has been applied in determining the true VSS for the Darvill full-scale BEPR system (0.36 g VSS/L, Eq. 4). This would amount to only 13% of the measured VSS value for the system (TABLE 4.7).

4.5.3 Determination of PAO's in BEPR

DAPI staining for the detection of PAO's *in situ* (FIG. 4.1) showed that the PAO population in the enriched culture was much higher than for the full-scale process (TABLE 4.7). It can be hypothesised that fewer fermentative heterotrophs were present in the anaerobic zone of the enhanced culture and a larger proportion of the biomass was dedicated to phosphate release and subsequent uptake in the aerobic zone. In addition, the influent biodegradable COD to the enriched culture is larger by a factor of approximately 2.5 (on a concentration basis). The calculated PAO biomass required to remove 1 mg of phosphorus from both the full- and pilot-scale BEPR plants correlate quite closely at approximately $1.7\text{-}2.75 \times 10^{11}$ PAO's.

4.5.4 Community analysis

The application of the probe specific for *Acinetobacter* spp. enabled the clear visualisation of cocco-bacilli occurring in either chains or clusters (FIG. 4.2). This study supports findings that *Acinetobacter* spp. are not predominant in BEPR sludge in spite of the fact that the gamma subclass is present at a percentage of about 17% of the total community (FIGS 4.2 and 4.5). The presence of *Acinetobacter* can be estimated to be less than 9% of the total population.

While most studies have indicated the predominance of members of the beta Proteobacteria and/or Actinobacteria in BEPR sludges (Kampfer *et al*., 1996; Snaidr *et al*., 1997; Bond *et al*., 1999), the findings presented here indicate that the alpha Proteobacteria are also implicated in BEPR for both the full-scale and enriched cultures (TABLE 4.3). Members of the community belonging to the alpha Proteobacteria were visualised to be of a uniform morphotype, being oval shaped and congregated in compact spherical flocs (FIGS 4.3 and 4.4). A clear shift in this population was observed to occur from the non-BEPR sludge (11%) to the enriched BEPR sludge (19%). This group was also found to be present at a percentage of about 28% of the total bacteria (ALF1b/EUB probe hybridization) for the full scale plant (TABLE 4.3). Members of the alpha Proteobacteria have been implicated by Kawaharasaki *et al*., (1999) as being capable of storing large amounts of polyphosphate, although they were not found to predominate the BEPR sludge. While the FISH method presents a good indication of cell numbers, dot blots represent a good indication of the total rRNA belonging to the group of interest. The measurement of total rRNA is important due to the interest in the relative levels of metabolic activity for the different groups implicated in BEPR. Based on the assumption that rRNA content is directly proportional to metabolic activity over a wide range (Schaechter *et al.*, 1958; Delong *et al.*, 1989; Wallner *et. al.*, 1993; Wagner *et. al.*, 1993), the results presented here using dot blots indicate that the alpha Proteobacteria are indeed metabolically active in BEPR systems (TABLE 4.3).

Bacteria belonging to the Actinobacteria were found in the lowest numbers for all systems studied. In order to eliminate the possibility of bad probe efficacy *in situ*, dot blots were also carried out for this group on the full-scale BEPR sludge. The results obtained supported the FISH determination (TABLE 4.3).

A survey of twenty full-scale activated sludge plants operating in Japan conducted by Mino *et al.*, (1998) showed beta Proteobacteria to be most dominant, followed by the alpha subclass and Actinobacteria, respectively. The BEPR community analysed here is dispersed over a wide phylogenetic range of at least four families but findings suggest that the alpha and beta subclasses of the Proteobacteria predominate the total bacterial biomass present for both full-scale and enriched pilot-scale BEPR systems. Considering that each family comprises numerous genera and species, and noting that the community belonging to the beta and gamma Proteobacteria subclass displayed numerous morphotypes, it may be implied that bacteria capable of excess polyphosphate accumulation probably belong to different phylogenetic groups and cannot be ascribed exclusively to any one genus or species. It is therefore likely that there are a few possible population structures capable of mediating BEPR.

4.6 CONCLUSIONS

- Cultivation-dependant enumerative methods were not found to give an accurate reflection of the predominating bacterial groups or species;
- Community analysis conducted using FISH yields a good quantitative representation of the biomass within BEPR systems, showing that the microbial community displays a high degree of diversity;
- The alpha, beta and gamma Proteobacteria, and the Actinobacteria are implicated in

BEPR, with both the alpha and beta Proteobacteria probably playing a major role;

- For a full-scale BEPR system, approximately 35% of the total bacterial population was found to accumulate phosphorus as polyphosphate granules; and
- The use of rRNA directed oligonucleotide probes can be applied to directly measure the metabolically active biomass fraction within the activated sludge system. This may possibly provide a means for advancing the process description and predictive capacity of the current mathematical models.

CHAPTER FIVE

BIOLOGICAL NITROGEN REMOVAL

5.1 INTRODUCTION

Nitrogen removal during wastewater treatment is achieved via two processes known as nitrification and denitrification. Under aerobic conditions chemoautotrophic bacteria belonging to the family *Nitrobacteraceae* convert ammonia, via nitrites, to nitrates in two different oxidative reactions cumulatively known as nitrification. The resultant nitrates and nitrites produced during this process are subsequently utilised under anoxic conditions as electron acceptors for cellular respiration by denitrifying OHO's resulting in reduction of the nitrates/nitrites with concurrent COD removal (Ketchum, 1988; Wanner and Grau, 1988; Cappucino and Sherman, 1992).

Up until now extensive work has been done to develop kinetic models and related software that can be used successfully to simulate and design ND and NDBEPR systems (Dold *et al.*, 1991; Wentzel *et al.*, 1991; Ekama *et al.*, 1992; Gujer and Kappler, 1992; Ekama and Wentzel, 1999). However, denitrification kinetics determined for ND systems have been found to vary considerably at times when applied to NDBEPR systems because of varying OHO active fraction estimates and the unexplained occurrence of anoxic P removal and any success achieved to date has been some what fortuitous (Dold *et al.*, 1991; Ekama and Wentzel, 1999). Ultimately, variations in process performance and kinetics are attributable to inadequate control and lack of understanding of the ecological, physiological and biochemical activities of the microorganisms within the process (Bux *et al.*, 1994). Furthermore, studies have also begun to highlight the possible involvement of heterotrophic bacteria in nitrification (Aleem, 1975; Verhagen and Laanbroek, 1991; Robertson and Kuenen, 1992). Thus far, heterotrophic nitrification rates have been found to be only one or two orders of magnitude lower than that of autotrophs (Robertson and Kuenen, 1992). Therefore, in addition to denitrification and anoxic phosphorus accumulation, heterotrophs may be contributing significantly to nitrification in activated sludge systems. Models applied to nitrifying populations may require revision as they have been based on the assumption that *Nitrosomonas* spp and *Nitrobacter* spp are the organisms solely responsible for nitrification occurring during wastewater treatment (Hovanec and DeLong, 1996; Yuichi and Kazuhiko, 1998; Wagner *et al*., 1996).

5.2 AIMS AND OBJECTIVES

The objective of this study was to isolate, identify and characterise heterotrophic bacteria (OHO's) responsible for or capable of contributing to:

- nitrification
- denitrification and
- anoxic P accumulation

occurring during wastewater treatment in a full-scale NDBEPR system. It was aimed at identifying predominant organisms, or groups thereof, interactively involved in nitrogen removal as well as gaining a better understanding of the specific mechanisms employed.

5.3 MATERIALS AND METHODS

5.3.1 Enrichment, isolation and cultivation of nitrogen removing bacteria

5.3.1.1 Potential nitrifying bacteria

Pure cultures of *Nitrosomonas europaea* and *Nitrobacter winogradskyi* were obtained from the ATCC as well as from the Vrije University in the Netherlands in order to grow as controls for *in situ* experiments evaluating the presence of autotrophic nitrifiers in activated sludge using FISH. Numerous techniques were employed in attempting to grow these extremely fastidious organisms but with no success (Soriano and Walker, 1968; Schmidt and Belser, 1982; Sandén *et al*., 1994). Attempts to isolate these organisms directly from soil and activated sludge samples have also proved unfruitful. However, due to observed ammonia and nitrite oxidation behaviour in the soil and sludge samples coupled with continuous heterotrophic contamination further research was focussed at nitrification by heterotrophic bacteria. Potential nitrifying heterotrophic bacteria were isolated from the aerobic zones of the NDBEPR process at Darvill WWW using nitrification enrichment media (APPENDICES 9 and 10). Random mixed liquor samples from each of the three aerobic tanks were homogenised using glass beads in order to physically disrupt the floc structures for ease of isolation of constituent bacteria. 10ml aliquots of each sample were inoculated to 3 x 400 mL liquid enrichment media for ammonia oxidisers (APPENDIX 9) and 10 mL of each sample to 3 x 400 mL liquid enrichment media for nitrite oxidisers (APPENDIX 10). The broths were incubated aerobically for 7 d at 25°C to enrich for nitrifying bacteria. Organisms were isolated by serial, ten fold dilutions (10^{-1} to 10^{-6}) using distilled water, and each dilution was plated onto enrichment media, using the spread plate technique, solidified with the addition of 1.5% agar. Due to the lack of an adequate experimental protocol established for heterotrophic nitrifiers, the media used was that known to cultivate autotrophic ammonia and nitrite oxidisers (Atlas, 1993) amended with the addition of organic substrates from a variety of media that have in the past successfully grown heterotrophs isolated from aquatic and environmental samples were supplemented (*Bergey's*, 1984; Bux *et al*., 1994; Bridson, 1995). Monocultures of isolated bacteria were maintained through storage at 4°C and monthly subculturing onto fresh media.

5.3.1.2 Potential denitrifying bacteria

Random mixed liquor samples were taken from the pre- and secondary anoxic zones of the Darvill NDBEPR process. The samples were homogenised using glass beads. In order to gain a representative selection of heterotrophic bacteria from the samples, triplicate serial dilutions (10^{-1} to 10^{-6}) were made from each sample and plated, using the spread plate technique, onto ten different media all known to be successful isolation media for heterotrophs. The media used were CGY agar (APPENDIX 2), Heterotrophic Plate Count agar (Difco Laboratories, USA), Blood agar (Biolab, South Africa), Brain Heart Infusion agar (Biolab, South Africa), Chocolate agar, Mueller-Hinton agar (Biolab, South Africa), Nutrient agar (Oxoid, England), Plate Count agar (Biolab, South Africa), Tryptone Glucose Extract agar (Oxoid, England) and Tryptone Soya agar (Oxoid, England) (Bergey's, 1984; Osborn *et al*., 1989; Gray, 1990; Bux *et al*., 1994;

Bridson, 1995). Heterotrophic bacteria were isolated after 5 to 7 d of incubation at 20°C (Lötter and Murphy, 1985; Venter *et al.*, 1989) and then maintained and further cultured on CGY agar (Osborn *et al.*, 1989; Gray, 1990; Bux *et al.*, 1994). Monocultures of isolated bacteria were maintained by storage at 4°C and monthly subculturing onto fresh media.

5.3.1.3 Potential denitrifying P accumulating heterotrophic bacteria

Two different media were used for the initial enrichment and isolation of potential denitrifying-P accumulating heterotrophic bacteria (DPB's). Acetate mineral media (AMM) (APPENDIX 11) was used to isolate potential P accumulators (Jørgensen and Paulii, 1995) while CGY agar was used for denitrifying bacteria. Random mixed liquor samples were taken from the Darvill NDBEPR process and homogenised using glass beads. Serial dilutions (10^{-1} to 10^{-8}) were made from each sample and plated onto AMM and CGY agar using the spread plate technique. After 5 to 7 d of incubation at 20°C well separated bacterial colonies were isolated and maintained on their respective isolation media at 4°C with monthly subculturing onto fresh media.

5.3.2 Evaluation of nitrification by heterotrophic bacteria

The nitrifying capacity of the isolated heterotrophic bacteria was assessed axenically. Monocultures of isolates obtained from the ammonium and nitrite enrichment media were incubated in liquid ammonium enrichment media (APPENDIX 12), on a Labcon shaker at 140 rpm, at ±25°C for 5 d. Ammonium, nitrite and nitrate concentrations were measured daily using a Merck SQ 118 spectrophotometer while the pH of each broth was measured on the first and last day of incubation in order to monitor possible decrease in pH which would substantiate any observed nitrification behaviour. Uninoculated broths were used as controls for each experiment.

5.3.3 Evaluation of denitrification by heterotrophic bacteria

To assess the specific nitrate and nitrite reduction capacities of all the heterotrophic bacteria isolated, monocultures were screened in triplicate for nitrate and nitrite reduction using the colorimetric biochemical reduction test proposed by Cappuccino and Sherman (1992) (APPENDIX 13). Both nitrate and nitrite media were used comprising of CGYA broths supplemented with 1.0 g/L potassium nitrate (KNO_3) and potassium nitrite (KNO_2), respectively. However, because high concentrations of nitrite have been reported to be toxic to many microorganisms (Piñar *et al.*, 1997) the concentrations of KNO_3 and KNO_2 used were reduced to 0.5 g/L. Furthermore, nitrate and nitrite media used were semi-solidified using 0.1% bacteriological agar to impede oxygen diffusion and create anoxic conditions ideal for denitrification (Cappuccino and Sherman, 1992). Incubation was carried out at 20°C for 5 d whereafter nitrate and nitrite reduction by each of the isolates was determined using alpha-naphthylamine, sulphanilic acid and zinc powder according to the method proposed by Cappuccino and Sherman (1992). Based on the results of the nitrate/nitrite reduction screenings the isolated heterotrophic bacteria were characterised into specific groups delineating their exact capacity for nitrate and/or nitrite reduction under anoxic conditions. The purpose of these characterisations was to more accurately define the OHO fraction of the Darvill NDBEPR process and so gain a better representation of the actual fraction of OHOs responsible for denitrification as well as their interactive contribution to nitrate and nitrite reduction occurring in the process.

5.3.4 Evaluation of anoxic P accumulation

Potential DPB's isolated from AMM and CGY agar were initially screened for denitrification ability using the colorimetric biochemical reduction test proposed by Cappuccino and Sherman (1992). Denitrifying isolates were then grown anaerobically in liquid acetate media (APPENDIX 14) to produce cells devoid of polyphosphate granules (Jørgensen and Paulii, 1995). The presence of polyphosphate granules was monitored using the Neisser staining technique of Jenkins *et al.*, (1984). After 24 h incubation at 30°C on a rotary shaker (120 rpm), the biomass was centrifuged using a Beckman J6 centrifuge (10 min, 8000 rpm, 10°C) and washed with distilled water. Cells were recentrifuged, resuspended in phosphate uptake media (3.27, 4.90 and 16.34 mgPO_4-P/L) (APPENDIX 15) and incubated for a further 6 h. Anoxic conditions were created in the phosphate uptake medium via the addition of 2.26, 11.29 and 13.54 mgNO_3-N/L coupled with helium sparging prior to inoculation (Kuba *et al*., 1993). Nitrate and phosphate concentrations were monitored hourly using a Merck SQ118 photometer. Uninoculated broths were used as controls for the experiments.

5.3.5 Identification of isolates

After screening, heterotrophic bacteria that could nitrify, denitrify or accumulate P under anoxic conditions were identified using Gram stains, API 20E, API 20NE, key differential biochemical tests, and cellular and colonial morphological characteristics (APPENDICES 16-19). All heterotrophic bacteria were identified to at least generic level. Gram negative rods were identified using API 20E and API 20NE, as described by Bux *et al*. (1994), in conjunction with additional key differential biochemical tests while Gram positive rods and Gram negative and positive cocci were identified using key differential biochemical tests and morphological characteristics (*Bergey's*, 1984;1986; Cappuccino and Sherman, 1992). However, numerous Gram negative rods were unidentifiable, even with repeated identification tests using the API bioassay. It is apparent that the consortia of bacteria and possible presence of mutants in activated sludge is too diverse for current API databases. Furthermore, not all Gram positive rods were identified. This was due to difficulty in establishing sufficient key differential biochemical tests to effectively distinguish amongst the wide array of possible denitrifying organisms present in the environment (*Bergey's* 1984;1986). Some Gram negative and positive cocci isolated were also unidentified owing to atypical biochemical characteristics as compared to genera outlined in *Bergey's* (1984;1986) and Cappuccino and Sherman (1992).

5.4 RESULTS AND DISCUSSION

5.4.1 Nitrification by heterotrophic bacteria

Of 60 isolates tested, 27 were found to be capable of nitrification of which *Staphylococcus* spp. and *Micrococcus* spp. showed the most significant involvement followed by *Streptococcus* spp., *Pseudomonas* spp. and *Bacillus* spp. (TABLE 5.1). All the heterotrophic nitrifiers isolated demonstrated a very similar pattern of ammonium utilisation. However, nitrite production was negligible while different patterns were observed in relation to nitrate formation (FIGS 5.1-5.5).

Isolates A1 - A4 and N1 - N4 showed rapid nitrate production from day 1 to day 2 which ceased or slowed drastically onwards (FIG 5.1-5.2). Nitrite accumulation was negligible throughout experimentation and therefore it is apparent that these isolates are capable of direct oxidation of ammonium to nitrate. This nitrification behaviour offers significant differences to nitrification by autotrophic bacterial species which can either oxidise ammonium or nitrites, but not both. Furthermore, the ability to oxidise both ammonium and nitrites would infer selective advantage to these isolates in an activated sludge system. This may be another reason substantiating the predominance of heterotrophs over autotrophs and therefore it is very possible that heterotrophs are contributing significantly to nitrification in ND and NDBEPR systems. Total ammonium and nitrite oxidation, during wastewater treatment, may be incorrectly attributed to autotrophic bacteria and future research needs to focus on determining the combined contribution of heterotrophs and autotrophs to nitrification.

Isolates A5 - A11 demonstrated different nitrification behaviour to isolates A1 - A4 and N1 - N4 in that their initial nitrate production was minimal or absent from day 1 to day 2 where after increasing rapidly to day 3 and then slowing onwards (FIG 5.3). The sudden increase in nitrate production from day 2 onwards was also seen to generally result in higher nitrate concentrations than recorded with any of the other nitrifying isolates (FIGS 5.1-5.5). Most of these isolates, although demonstrating an initial lag phase in nitrate production, were therefore stronger nitrifiers than all the other nitrifying isolates. However, it was also noted that the initial pH of the nitrification media used to test isolates A6 - A11 was very low (5.56) (TABLE 5.2) and therefore it is possible that the initial lag in nitrate production may have been a result of acidic conditions. Nitrification is known to be optimal in neutral to alkaline conditions and can be inhibited under acidic conditions (Gray, 1990; Underhill, 1990; Bitton, 1994). Isolate A5 (*Staphylococcus aureus*), however, was tested at pH 7.00 (TABLE 5.2) and yet demonstrated the same behaviour as isolates A6 - A11 which were tested at pH 5.56. In addition, isolate A6 was found to accumulate the highest concentration of nitrate compared to all the other nitrifying isolates (FIG 5.3). It therefore remains possible that more than one pattern of nitrification behaviour may exist amongst heterotrophic nitrifiers.

Isolates A12 - A17 and N5 - N10 (FIGS 5.4-5.5) exhibited the same nitrification behaviour as isolates A1 -A4 and N1 - N4 (FIGS 5.1-5.2). Rapid nitrate production was also seen from day 1 to day 2 which ceased or slowed drastically onwards (FIGS 5.4-5.5). Nitrite accumulation was again also negligible with these isolates, therefore, further substantiating the ability of heterotrophic bacteria to oxidise both ammonium and nitrite, unlike their autotrophic counterparts. However, isolates A12 -A17 and N5 - N10 demonstrated potential nitrification-denitrification behaviour. The final pH measured for these isolates was found to be near neutral to slightly alkaline which is contrary to the very acidic conditions which prevailed amongst all the other nitrifying isolates (TABLE 5.2). The closest explanation for the higher pH results of these isolates is the concurrent occurrence of denitrification. Many heterotrophic bacteria can denitrify to various extents and therefore it is very possible that nitrifying isolates may also be capable of denitrification. Denitrification is optimal under slightly acidic to neutral conditions and results in the production of alkalinity which in turn results in elevated pH. In addition to the acidic conditions which would have resulted from the nitrification behaviour observed, it is possible that, with increasing biomass generation, oxygen limitation may have occurred due to subsequent increase in competition for available oxygen. Oxygen limitation, coupled with nitrate production, may then have resulted in the creation of anoxic conditions suitable for denitrification to occur. However, aerobic denitrification by heterotrophic bacteria has also been reported previously (Rheinheimer, 1985; Robertson and Kuenen, 1992). It is, therefore, also a possibility that these organisms are in fact capable of aerobic denitrification and hence were capable of reducing the nitrates being produced in the aerobic nitrification broths. Little is known about the occurrence of nitrifying-denitrifying heterotrophic bacteria and need exists for better understanding of the biochemistry and physiology of such organisms as well as their involvement in nitrogen removal in ND and NDBEPR systems.

TABLE 5.1 Ammonium (ref. A) and nitrite (ref. N) enrichment media isolates.

Isolate Ref. No.	Identification	Cellular morphology	Gram reaction
A1	*Micrococcus* spp	Coccus	positive
A2	*Staphylococcus aureus*	Coccus	positive
A3	**Pseudomonas maltophilia*	Rod	negative
A4	**Unidentified	Rod	positive
A5	**Unidentified	Rod	positive
A6	*Staphylococcus aureus*	Coccus	positive
A7	*Staphylococcus epidermidis/saprophyticus*	Coccus	positive
A8	*Pseudomonas fluorescens*	Rod	negative
A9	**Unidentified	-	-
A10	***Unidentified	-	-
A11	***Unidentified	-	-
A12	*Micrococcus* spp	Coccus	positive
A13	*Pseudomonas maltophilia*	Rod	negative
A14	*Staphylococcus aureus*	Coccus	positive
A15	**Unidentified	Rod	positive
A16	**Unidentified	Rod	negative
A17	**Unidentified	Rod	negative
N1	*Bacillus* spp	Rod	positive
N2	*Bacillus* spp	Rod	positive
N3	***Unidentified	-	-
N4	***Unidentified	-	-
N5	**Aeromonas salmonicida 2*	Rod	negative
N6	*Micrococcus* spp	Coccus	positive
N7	*Streptococcus mitis/pneumoniae*	Coccus	positive
N8	*Streptococcus* spp	Coccus	positive
N9	**Unidentified	Rod	positive
N10	**Unidentified	Rod	positive

*Doubtful identification; **Inconclusive identification; ***Organism non-viable for identification

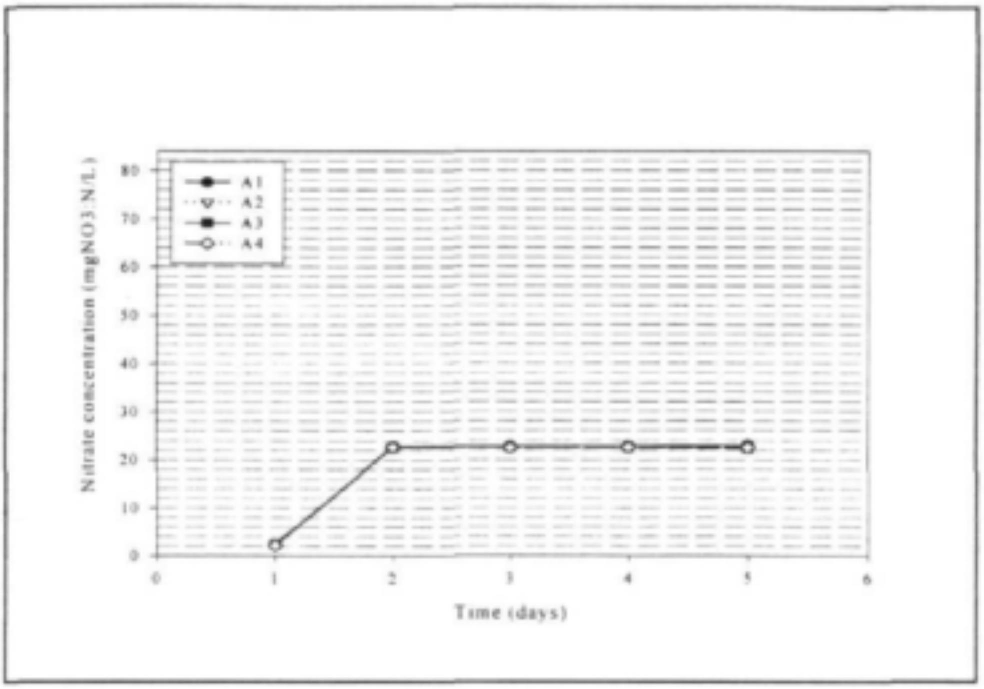

FIGURE 5.1 Nitrate production profiles during nitrification experiments for isolates A1 - A4.

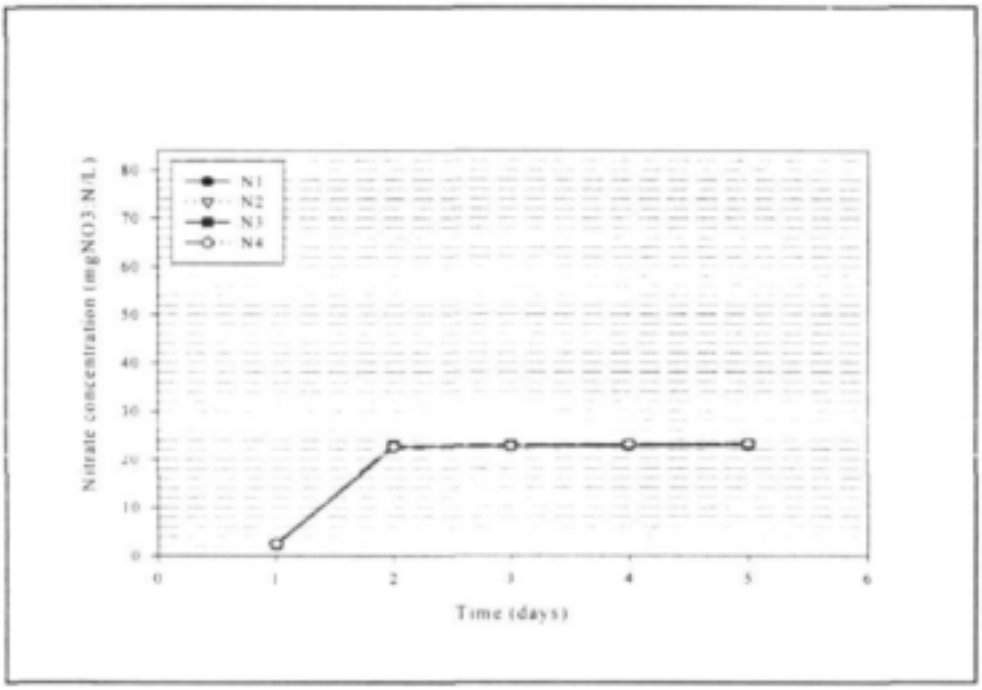

FIGURE 5.2 Nitrate production profiles during nitrification experiments for isolates N1 - N4.

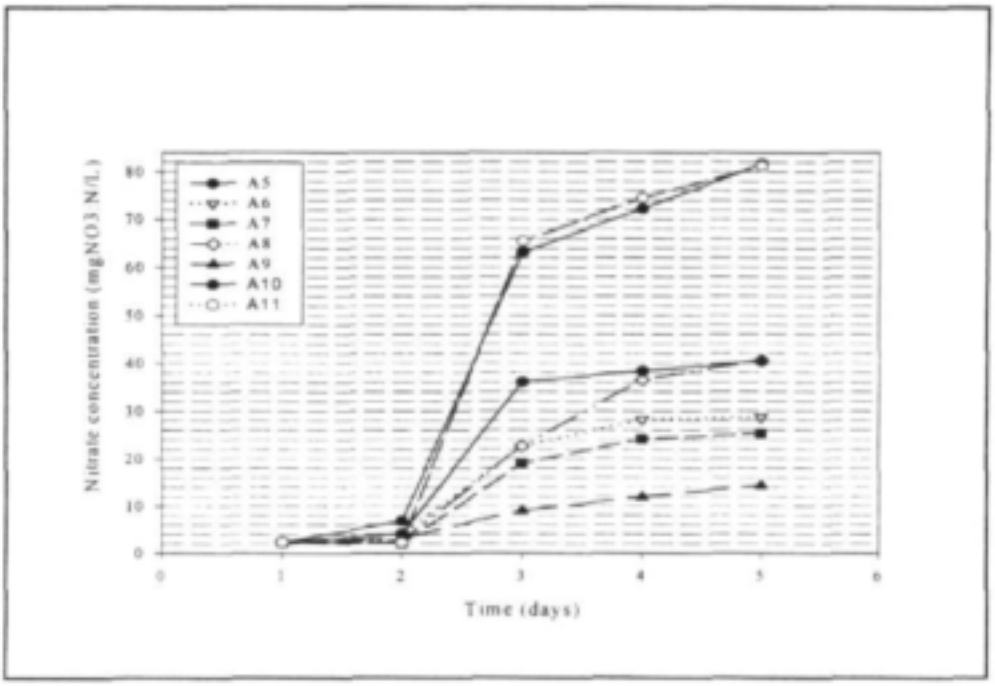

FIGURE 5.3 Nitrate production profiles during nitrification experiments for isolates A5 - A11.

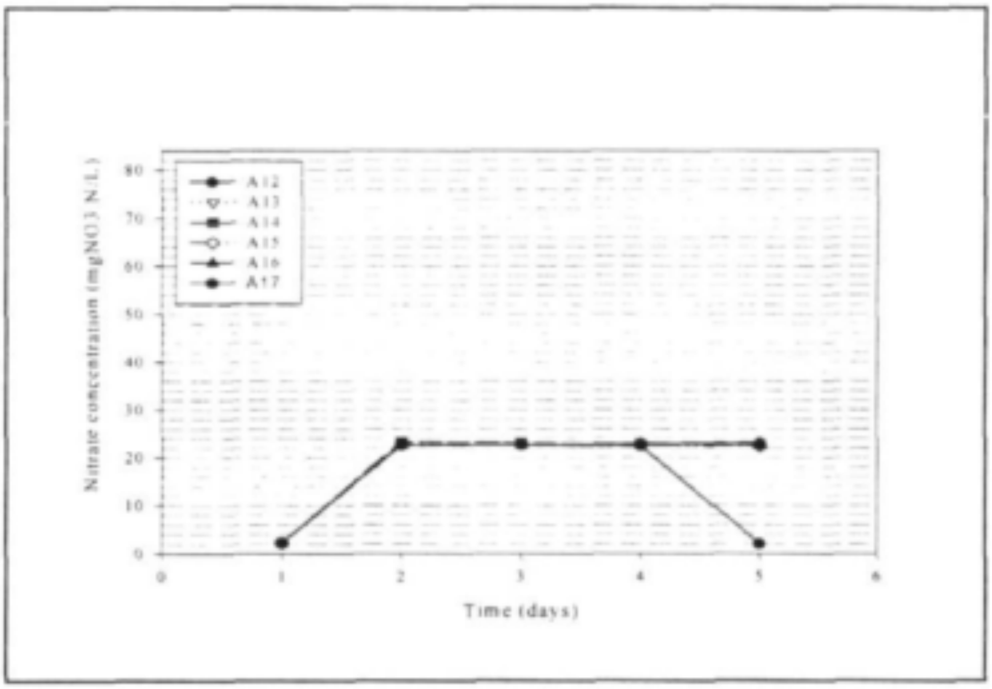

FIGURE 5.4 Nitrate production profiles during nitrification experiments for isolates A12 - A17.

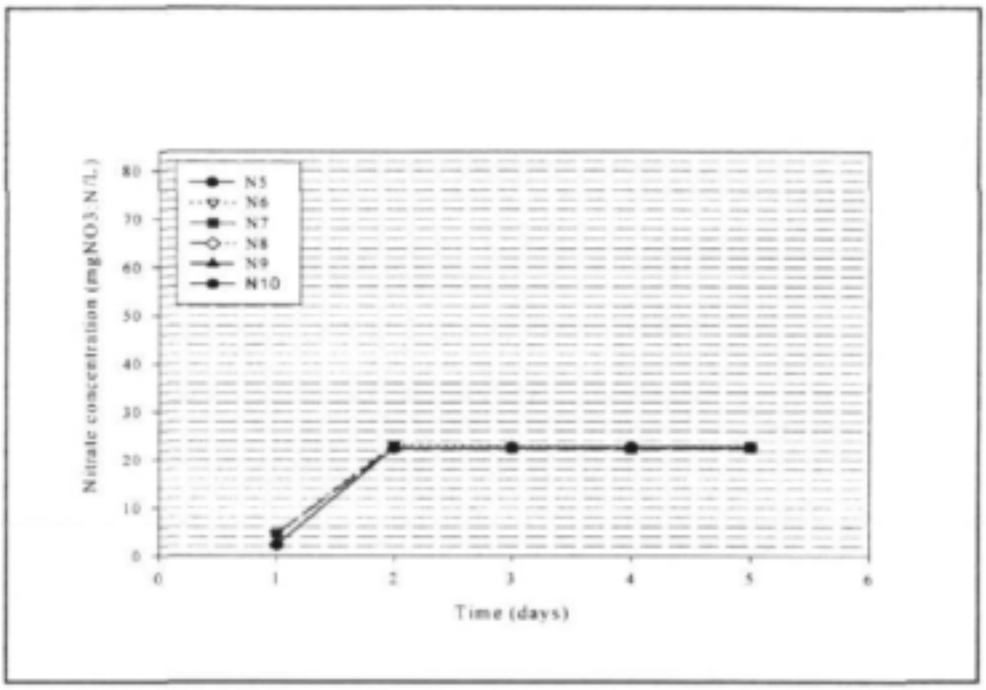

FIGURE 5.5 Nitrate production profiles during nitrification experiments for isolates N5 - N10.

5.4.2 Denitrification by heterotrophic bacteria

The results of this study show that, with respect to denitrification, the OHO fraction within the Darvill NDBEPR process comprises five different functional groups, four of which interactively contribute to denitrification occurring in the system and one group that are non-denitrifying OHO's (FIG. 5.6). These groups were characterised as true denitrifiers (bacteria capable of both nitrate and nitrite reduction), incomplete denitrifiers (bacteria that reduced nitrates to nitrites with no further reduction of the nitrites produced), incomplete-nitrite reducers (bacteria exhibiting severe inhibition of nitrite reduction by nitrates),exclusive nitrite reducers (bacteria only capable of reducing nitrites) and non-denitrifiers (bacteria not capable of nitrate or nitrite reduction). Of the OHO's isolated true denitrifiers comprised 20,6% (56 isolates), incomplete denitrifiers 31,3% (85 isolates), incomplete-nitrite reducers 8,8% (24 isolates), exclusive nitrite reducers 2,6% (7 isolates) and non-denitrifiers 36,8% (100 isolates). In respect to the nitrate and nitrite reduction capacity of the isolated OHO fraction as a whole, 60,7% (165 isolates) were capable of nitrate reduction while only 32,0% (87 isolates) were capable of any degree of nitrite reduction.

Alternatively, it can be interpreted that 95,9% of the denitrifying OHO's isolated were capable of nitrate reduction as compared to only 50,6% capable of contributing to nitrite reduction under anoxic conditions.

TABLE 5.2 Changes measured in pH on day 1 and day 5 of incubation during nitrification studies by heterotrophic bacteria.

Isolate Ref. No.	Ammonium enriched isolates		Isolate Ref. No.	Nitrite enriched isolates	
	Day 1	Day 5		Day 1	Day 5
A1	7.00	3.45	N1	7.00	3.95
A2	7.00	4.26	N2	7.00	3.96
A3	7.00	3.41	N3	7.00	3.03
A4	7.00	3.91	N4	7.00	3.58
A5	7.00	4.02	N5	7.00	6.00
A6	5.56	3.78	N6	7.00	6.88
A7	5.56	3.07	N7	7.00	7.16
A8	5.56	3.23	N8	7.00	6.97
A9	5.56	3.67	N9	7.00	6.08
A10	5.56	4.09	N10	7.00	6.60
A11	5.56	3.98			
A12	7.00	7.03			
A13	7.00	7.39			
A14	7.00	6.52			
A15	7.00	7.10			
A16	7.00	7.41			
A17	7.00	7.15			

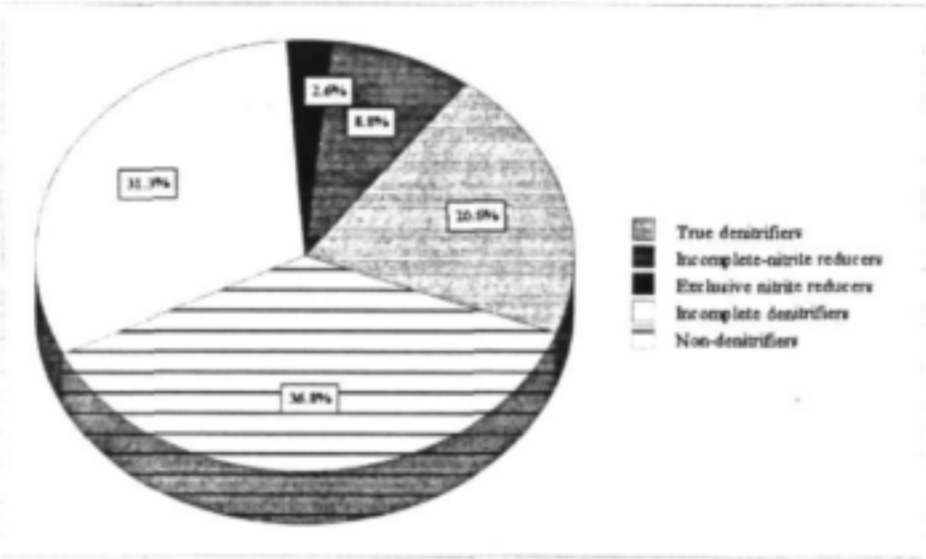

FIGURE 5.6 The heterotrophic bacterial community structure of the Darvill NDBEPR anoxic zone using plating techniques.

5.4.2.1 True denitrifiers

The first of the five groups characterised comprised of true denitrifiers i.e. bacteria that were able to reduce both nitrates as well as nitrites (FIGS 5.6-5.7 and TABLE 5.3). These bacteria all contain nitrate as well as nitrite reductase enzymes enabling them to successfully reduce nitrates, via nitrites, to gaseous nitrogen (Rheinheimer, 1985; Ketchum, 1988; Cappuccino and Sherman, 1992; Robertson and Kuenen, 1992). Of all the true denitrifiers tested, most were capable of efficiently reducing nitrates and nitrites simultaneously with no apparent build up of nitrite as an intermediate. This group of organisms fit the traditional description and understanding of denitrification occurring in waste water treatment but were not isolated in great enough numbers to be exclusively attributable to denitrification occurring in the Darvill NDBEPR process (FIG. 5.7). Results also revealed that a few true denitrifying bacteria cannot reduce nitrates and nitrites as effectively as others with slight nitrite build-up occurring or alternatively demonstrating slower reduction of nitrates. This could possibly be attributed weak nitrate/nitrite reductases or feedback inhibition mechanisms triggered in the microbial cells by nitrate/nitrite concentrations and/or other physiological conditions imposed on the bacteria. However, according to the overall results obtained it is unlikely that this is having profound effect on denitrification and therefore need not be of great concern when characterising the denitrifying OHO fraction. *Pseudomonas* spp comprised a significant amount of the true denitrifiers isolated from the Darvill NDBEPR process (TABLE 5.3) and are therefore important organisms to be considered in denitrification behaviour of such systems.

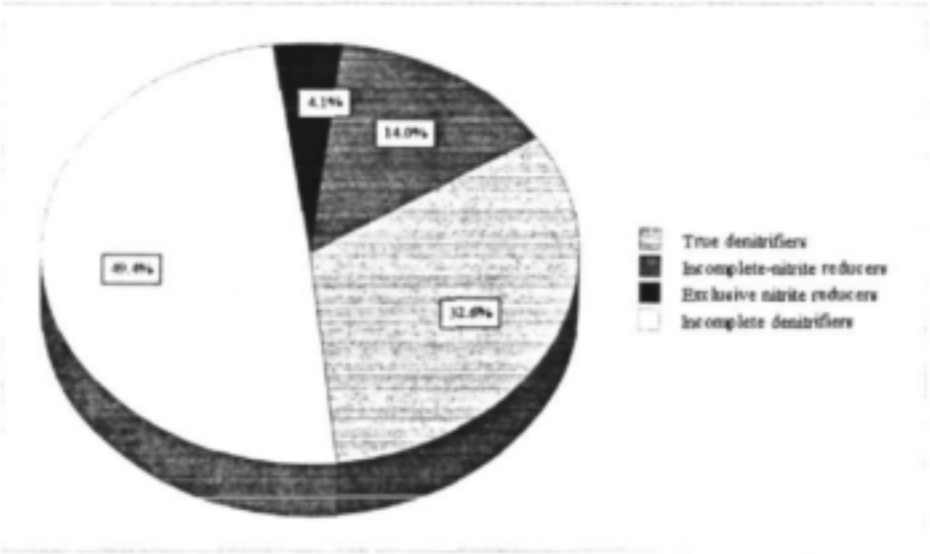

FIGURE 5.7 The denitrifying heterotrophic bacterial community structure of the Darvill NDBEPR anoxic zone using plating techniques.

TABLE 5.3 Identification of true denitrifying heterotrophic bacteria.

Isolate Ref. No.	Identification	Cellular morphology	Gram reaction
D1	*Achromobacter group VD*	Rod	negative
D2	*Achromobacter xylosoxidans*	Rod	negative
D3	**Achromobacter xylosoxidans*	Rod	negative
D4	*Aeromonas salmonicida 2*	Rod	negative
D5 - D6	*Agrobacterium radiobacter* - 2 strains	Rods	negative
D7	*Chromobacterium violaceum*	Rod	negative
D8	**Escherichia coli 1*	Rod	negative
D9	*Flavobacterium indologenes*	Rod	negative
D10	**Flavobacterium multivorum*	Rod	negative
D11	**Klebsiella pneumonia ssp pneumo.*	Rod	negative
D12	*Pasteurella aerogenes*	Rod	negative
D13 - D14	*Pseudomonas acidovorans* - 2 strains	Rods	negative
D15	*Pseudomonas aeruginosa*	Rod	negative
D16 - D17	*Pseudomonas cepacia* - 2 strains	Rods	negative
D18	**Pseudomonas maltophilia*	Rod	negative
D19	*Pseudomonas paucimobilis*	Rod	negative
D20 - D21	*Pseudomonas pickettii* - 2 strains	Rods	negative
D22	**Pseudomonas pseudomallei*	Rod	negative
D23 - D28	*Pseudomonas stutzeri* - 6 strains	Rods	negative
D29	**Pseudomonas stutzeri*	Rod	negative
D30	*Pseudomonas testosteroni alcaligenes*	Rod	negative
D31 - D33	**Pseudomonas testosteroni alcaligenes* - 3 strains	Rods	negative
D34 - D35	*Pseudomonas* spp (x 2)	Rods	negative
D36	*Seratia liquificaens*	Rod	negative
D37 - D38	*Seratia marcescens* - 2 strains	Rods	negative
D39 - D40	*Vibrio hollisae* - 2 strains	Rods	negative
D41 - D47	**Unidentified	Rods	negative
D48 - D51	*Neisseria* spp (x 4)	Cocci	negative
D52 - D56	**Unidentified	Rods	positive

*Doubtful identification; **Inconclusive identification

5.4.2.2 Incomplete denitrifiers

Most of the denitrifying heterotrophic bacteria isolated were incomplete denitrifiers i.e. bacteria that were only capable of reducing nitrates to nitrites with no further reduction of the nitrites produced (FIGS 5.6-5.7 and TABLE 5.4). These results substantiate findings by Rheinheimer (1985), Robertson and Kuenen (1992) and Rosén and Welander (1994). According to Rheinheimer (l985) and Robertson and Kuenen (1992), initial nitrite production by actively denitrifying bacterial communities is usually very high and may, especially if accompanied by high initial nitrate concentrations, result in nitrite build-up. This nitrite build-up is as a result of the predominant presence of incomplete denitrifiers within denitrifying bacterial communities. Robertson and Kuenen (1992) state that these incomplete denitrifying bacteria lack key nitrite reductase enzymes which enable true denitrifiers to reduce nitrites. Ekama and Wentzel (1999) noted initial nitrite build-up or slow reduction, at approximately 1/10th of the nitrate reduction rate, when determining denitrification kinetics for NDBEPR processes. Results of this study show that this is partly attributable to the large amount of incomplete denitrifiers present in activated sludge and therefore this is a very important group of organisms that need to be considered when modelling denitrification kinetics of NDBEPR processes. Incomplete denitrifiers interactively contribute to nitrate reduction along with true denitrifiers and incomplete-nitrite reducers. As these three groups of denitrifiers collectively comprised % of denitrifiers isolated it clear as that more denitrifying organisms are capable of nitrate reduction than nitrite reduction (FIG. 5.7). Very few incomplete denitrifiers exhibited weak reduction of nitrates and therefore overall nitrite production by this group of denitrifying bacteria is substantial enough to be of affect and concern to denitrification behaviour in NDBEPR processes. Results also showed possible aerobic denitrification by incomplete denitrifiers. These organisms did not grow well under anoxic conditions but preferred the more aerobic conditions at the surface of the nitrate media. It is possible that these organisms can utilise oxygen and nitrates simultaneously for cellular respiration. Rheinheimer (1985) and Robertson and Kuenen (1992) support claims of denitrifying bacteria being capable of aerobic denitrification. In fact a denitrifying bacterium has even been found that cannot grow under proper anoxic conditions and reduces nitrates microaerophilically (Robertson and Kuenen, 1992). These results may confirm such findings, although, it is also possible that biomass production by incomplete denitrifiers is simply restricted under anoxic conditions thus explaining the poor growth observed in these bacteria under anoxic conditions. Of the incomplete denitrifiers isolated from the Darvill NDBEPR process *Pseudomonas* spp were again found in appreciable amounts further substantiating the significant involvement of these organisms in denitrification during wastewater treatment (TABLE 5.4).

TABLE 5.4 Identification of incomplete denitrifying heterotrophic bacteria.

Isolate Ref. No.	Identification	Cellular morphology	Gram reaction
D57	*Achromobacter xylosoxidans*	Rod	negative
D58 - D59	*Aeromonas hydrophila* - 2 strains	Rods	negative
D60	*Aeromonas salmonicida*	Rod	negative
D61	*Alcaligenes faecalis*	Rod	negative
D62 - D63	*Bordetella/Alcaligenes* spp - 2 strains	Rods	negative
D64	**CDC group IV C-2*	Rod	negative
D65	*Citrobacter freundii*	Rod	negative
D66 - D67	*Enterobacter cloacae* - 2 strains	Rods	negative
D68	**Moraxella* spp	Rod	negative
D69	*Pasteurella aerogenes*	Rod	negative
D70 - D71	*Pasteurella* spp (x 2)	Rods	negative
D72	*Proteus mirabilis*	Rod	negative
D73 - D74	*Pseudomonas acidovorans* - 2 strains	Rods	negative
D75	*Pseudomonas aeruginosa*	Rod	negative
D76	*Pseudomonas cepacia*	Rod	negative
D77	**Pseudomonas cepacia*	Rod	negative
D78 - D80	*Pseudomonas fluorescens I* - 3 strains	Rods	negative
D81 - D83	*Pseudomonas luteola* - 3 strains	Rods	negative
D84	*Pseudomonas maltophilia*	Rod	negative
D85 - D86	**Pseudomonas mendocina* - 2 strains	Rods	negative
D87 - D88	*Pseudomonas stutzeri* - 2 strains	Rods	negative
D89 - D90	**Pseudomonas stutzeri* - 2 strains	Rods	negative
D91 - D94	*Pseudomonas testosteroni/alcaligenes* - 4 strains	Rods	negative
D95 - D96	**Pseudomonas testosteroni/alcaligenes* - 2 strains	Rods	negative
D97	*Pseudomonas vesicularis*	Rod	negative
D98 - D100	*Pseudomonas* spp (x 3)	Rods	negative
D101	*Seratia liquefaciens*	Rod	negative
D101 - D103	*Vibrio fluvialis* - 3 strains	Rods	negative

TABLE 5.4 *continued*

Isolate Ref. No.	Identification	Cellular morphology	Gram reaction
D104 - D112	**Unidentified	Rods	negative
D113 - D115	*Bacillus* spp (x 3)	Rods	positive
D116 - D136	**Unidentified	Rods	positive
D137	**Staphylococcus* spp	Coccus	positive
D138 - 139	**Unidentified	Cocci	positive
D140	*Branhamella* spp	Coccus	negative
D141	Unidentified	Coccus	negative

*Doubtful identification; **Inconclusive identification

5.4.2.3 Incomplete-nitrite reducers

The third group of denitrifying heterotrophic bacteria isolated were incomplete-nitrite reducers (FIGS 5.6-5.7 and TABLE 5.5). These bacteria exhibited typical incomplete denitrification (reduction of nitrates to nitrites with no further reduction of the nitrites produced) when grown in nitrate medium. However, when grown in exclusive nitrite media these bacteria proved to reduced nitrites efficiently. It is possible that the nitrite reductase enzymes within these bacteria are subject to a severe feedback inhibition mechanism restricting expression of the enzymes when nitrates are present or simply that this is an energy dependant reaction where nitrates are favoured over nitrites owing to the higher electron accepting capacity of nitrates as compared to nitrites (NO_3:NO_2 = 5:3). However, the mechanisms responsible for nitrate inhibition of nitrite reduction remain uncertain and further research is required to fully understand this phenomenon and how to overcome it in the quest to optimise NDBEPR processes. Ekama and Wentzel (1999) noted that, when determining denitrification kinetics for NDBEPR processes, significant nitrite reduction would only proceed from the initial build-up or slow reduction once the nitrate concentration had been reduced to below 1 mgNO_3-N/L. Results of this study show that this can

TABLE 5.5 Identification of incomplete-nitrite reducing heterotrophic bacteria.

Isolate Ref. No.	Identification	Cellular morphology	Gram reaction
D142	*Aeromonas hydrophila*	Rod	negative
D143	**Agrobacterium radiobacter*	Rod	negative
D144	*Citrobacter freundii*	Rod	negative
D145 - D146	*Escherichia coli 1* - 2 strains	Rods	negative
D147	**Escherichia coli 1*	Rod	negative
D148	*Kluyvera* spp	Rod	negative
D149	*Pasteurella* spp	Rod	negative
D150	*Pseudomonas* cepacia	Rod	negative
D151	*Pseudomonas* maltophilia	Rod	negative
D152	*Pseudomonas* putrefaciens	Rod	negative
D153	*Pseudomonas tostesteroni/alcaligenes*	Rod	negative
D154 - D155	*Vibrio fluvialis* - 2 strains	Rods	negative
D156 - D159	**Unidentified	Rods	negative
D160 - D162	*Neisseria* spp (x 3)	Cocci	negative
D163	**Unidentified	Coccus	negative
D164 - D165	**Unidentified	Rods	positive

*Doubtful identification; **Inconclusive identification

be strongly related to the presence of incomplete-nitrite reducing bacteria in NDBEPR systems. This group of bacteria comprised a substantial fraction of the OHO's isolated (FIG. 5.7) and therefore their contribution to denitrification in NDBEPR processes needs to be carefully considered and taken into account when modelling denitrification kinetics of such systems. Furthermore, nitrite concentration was found to be a limiting factor for nitrite reduction by incomplete-nitrite reducing heterotrophic bacteria. Approximately half of the incomplete-nitrite reducing isolates exhibited inhibition of nitrite reduction at 1.0 g/L KNO_2 but reduced nitrite effectively at 0.5 g/L KNO_2. This is possibly attributable to the toxicity of nitrites at high concentrations (Piñar *et al.*, 1997) but it needs to be noted that nitrite reduction by the true denitrifiers and exclusive nitrite reducers isolated was very strong, even at 1.0 g/L KNO_2. The reasons behind this are very uncertain but it is possible that this phenomenon is enzymatic in nature and somehow linked to the phenomenon of nitrate inhibition of nitrite reduction. *Pseudomonas* spp and *Neisseria* spp were the predominant heterotrophic bacteria identified as incomplete-nitrite reducers (TABLE 5.5). *Neisseria* spp have previously been found to predominantly reduce nitrites over nitrates with some species only being capable of exclusive nitrite reduction (*Bergey's*, 1989). Denitrification behavior observed in this study, by *Neisseria* spp, indicate that nitrate and nitrite reduction by these organisms is even more complex than currently accepted and need exists for better understanding of the mechanisms involved.

5.4.2.4 Exclusive nitrite reducers

The fourth group of denitrifying heterotrophic bacteria isolated comprised exclusive nitrite reducing bacteria (FIGS 5.6-5.7 and TABLE 5.6). These bacteria exhibited no capacity for nitrate reduction under anoxic conditions but were capable of efficient reduction of nitrites when grown in an exclusive nitrite media. Exclusive nitrite reducing bacteria have been isolated and characterised from environmental samples (*Bergey's*, 1984) but little is known about their involvement in activated sludge treatment. Exclusive nitrite reducers lack nitrate reductase enzymes and are, therefore, incapable of contributing to nitrate reduction. Furthermore, of the exclusive nitrite reducers isolated none were found that exhibited weak reduction of nitrites even in the presence of higher concentrations of nitrite which were inhibitory to many of the incomplete-nitrite reducing bacteria. Although not isolated in great numbers (FIGS 5.6-5.7), the contribution to denitrification by exclusive nitrite reducers needs to be more accurately assessed considering the efficiency of reduction of high concentrations of nitrite seen in this study. It is important that more understanding is gained in order to assess their exact contribution to denitrification behaviour in NDBEPR systems. *Flavobacterium* spp. seem to be significant genera involved in exclusive nitrite reduction (TABLE 5.6). However, this needs to be further substantiated as exclusive nitrite reducers, isolated from the Darvill NDBEPR process, were difficult to conclusively identify demonstrating atypical biochemical traits to that of organisms included in the API 20E and 20NE databases.

TABLE 5.6 Identification of exclusive nitrite reducing heterotrophic bacteria.

Isolate Ref. No.	Identification	Cellular morphology	Gram reaction
D166	*****CDC group II J* *Flavobacterium odoratum*	Rod	negative
D167	**Flavobacterium multivorum*	Rod	negative
D168	*****Flavobacterium* spp *Pseudomonas diminuta* *Flavobacterium odoratum*	Rod	negative
D169 - D170	**Unidentified	Rods	negative
D171 - D172	**Unidentified	Cocci	negative

*Doubtful identification; **Inconclusive identification; ****Low discrimination between two or more organisms

5.4.2.5 Non-denitrifiers

The fifth component of OHOs identified in this study were heterotrophic bacteria incapable of any degree of nitrate or nitrite reduction and hence characterised as non-denitrifiers (FIG. 5.6). Many heterotrophic bacteria are known not to have the enzymatic capacity to denitrify under any given circumstances (*Bergey's*, 1984;1986). Given this and the fact that the non-denitrifiers comprised such a large fraction of the OHOs isolated (FIG. 5.6) it is inaccurate to ascribe denitrification behaviour in NDBEPR processes to the whole OHO fraction when determining the denitrification kinetics of such systems, as is currently being done (Dold *et al*., 1991; Ubisi *et al*., 1997; Ekama and Wentzel, 1999). Although contributing to COD removal under aerobic conditions, and hence forming part of the heterotrophic active biomass or OHO fraction (Ubisi *et al*., 1997; Ekama and Wentzel, 1999), the non-denitrifiers do not comprise the active denitrifying OHO fraction and should not be included in denitrification kinetics studies under the broad parameter of OHO fraction. Furthermore, it is possible that some or even many of these non-denitrifiers are in fact PAOs as it needs to be remembered that PAOs are also heterotrophic bacteria. However, the fraction of PAOs comprising the non-denitrifiers was not determined in this study and therefore it is uncertain as to the exact composition of the non-denitrifier fraction in relation to PAOs. It is therefore important that the OHO fraction be more accurately defined in terms of active denitrifiers when applied to kinetic determinations for NDBEPR processes.

5.4.3 Anoxic P accumulation by denitrifying heterotrophic bacteria

Anoxic P accumulation was observed in ten heterotrophic bacterial isolates of which *Pseudomonas* spp. predominated (TABLE 5.7). Of these, six isolates proved capable of good P accumulation under anoxic conditions (TABLE 5.8 and FIG 5.8A-B) while four were only capable of accumulating P in small amounts at low initial phosphate an nitrate concentrations (FIG 5.9A-B and TABLE 5.8). Of the six strong DPB's, *Serratia* spp. and *Vibrio* spp. were identified as the best anoxic P accumulators with 7.10 mg/L and 7.29 mgPO$_4$-P/L removal, respectively, at an initial nitrate concentration of 13.54 mgNO$_3$-N/L and P concentration of 16.34 mgPO$_4$-P/L. Nitrate reduction observed by the strong DPB's confirms that some denitrifying bacteria are capable of P removal using nitrates instead of oxygen. It is most probable that these organisms are also capable of aerobic P accumulation but it is uncertain as to how their P removal capacities will vary between anoxic and aerobic conditions. Although apparent that anoxic P accumulation tends to be less than that possible under aerobic conditions (Kerrn-Jespersen and Henze, 1993) it uncertain as to how this corresponds with the individual organisms capable of anoxic P removal. Kerrn-Jespersen and Henze (1993) demonstrated that anoxic P accumulation was dependent on the ratio of DPB's to aerobic P accumulators. Anoxic P accumulation gives an added survival strategy to those organisms capable and therefore NDBEPR systems should select for and allow predominance of such organisms. This may offer some explanation to difficulties experienced in modelling NDBEPR systems upon the kinetics of ND systems as the physiological conditions imposed by ND systems may not neccessarily select for the same organisms as that of NDBEPR systems. Furthermore, COD requirements of DPB's and normal denitrifiers will vary owing to ability of DPB's to utilise internally stored PHB thus adding further challenges to modelling NDBEPR processes. The weak DPB's identified were only capable of accumulating small amounts of P at an initial P concentration of 4.90 mgPO$_4$-P/L in the presence of 2.26 mgNO$_3$-N/L (FIG 5.9A-B and TABLE 5.8). Their removal capacities varied between 1.83 mg/L and 1.05 mgPO$_4$-P/L removal with 1.25 mg/L to 0.95 mgNO$_3$-N/L reduction. It is possible that the weak anoxic P accumulation observed by these organisms is due to weak denitrification capacity demonstrated. These organisms may, therefore, not necessarily be weak P accumulators under aerobic conditions but are limited, due to weak denitrification capacity, under anoxic conditions.

TABLE 5.7 Identification of DPB's.

Isolate Ref. No.	Identification	Cellular morphology	Gram reaction
DP1	*Vibrio* spp	Rod	negative
DP2	*Serratia* spp	Rod	negative
DP3	*Aeromonas* spp	Rod	negative
DP4	*Pseudomonas* spp	Rod	negative
DP5	*Pseudomonas* spp	Rod	negative
DP6	*Pseudomonas* spp	Rod	negative
DP7	*Pseudomonas* spp	Rod	negative
DP8	*Pseudomonas* spp	Rod	negative
DP9	*Pseudomonas cepacia*	Rod	negative
DP10	*Klebsiella* spp	Rod	negative

TABLE 5.8 P and nitrate removal efficiencies of DPB's under anoxic conditions.

Isolate Ref. No.	Identification	Initial P (mgPO_4-P/L)	P removal (mgPO_4-P/L)	Initial N (mgNO_3-N/L)	N removal (mgNO_3-N/L)
DP1	*Vibrio* spp	16.34	7.29	13.54	2.92
DP2	*Serratia* spp	16.34	7.10	13.54	6.22
DP3	*Aeromonas* spp	16.34	3.86	13.54	3.27
DP4	*Pseudomonas* spp	16.34	3.29	13.54	3.65
DP5	*Pseudomonas* spp	16.34	2.05	13.54	3.57
DP6	*Pseudomonas* spp	16.34	3.43	13.54	4.37
DP7	*Pseudomonas* spp	4.90	4.63	3.27	1.10
DP8	*Pseudomonas* spp	4.90	1.51	3.27	0.95
DP9	*Pseudomonas cepacia*	4.90	1.13	3.27	1.25
DP10	*Klebsiella* spp	4.90	1.05	3.27	1.14

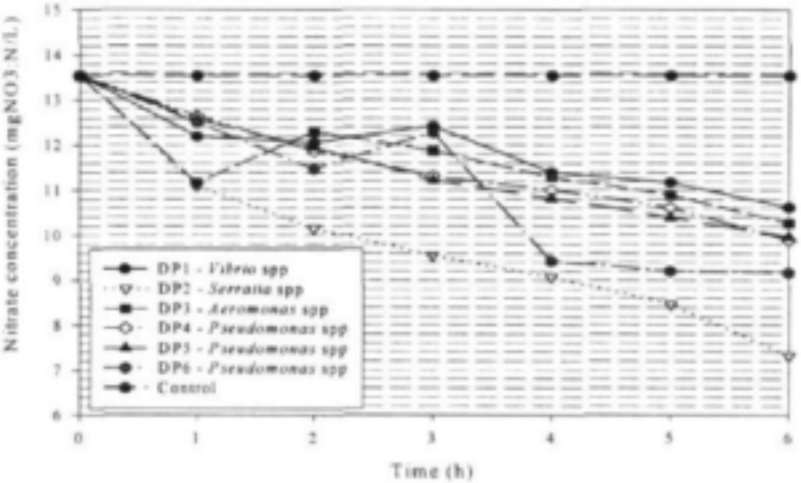

FIGURE 5.8A Nitrogen (as nitrate) reduction profiles by isolates DP1-DP6 when cultivated in phosphate uptake media under anoxic conditions.

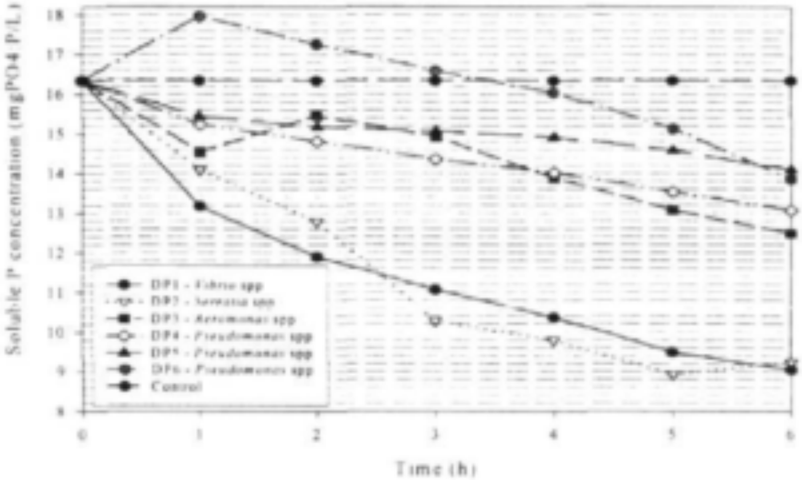

FIGURE 5.8B P removal profiles by isolates DP1-DP6 when cultivated in phosphate uptake media under anoxic conditions.

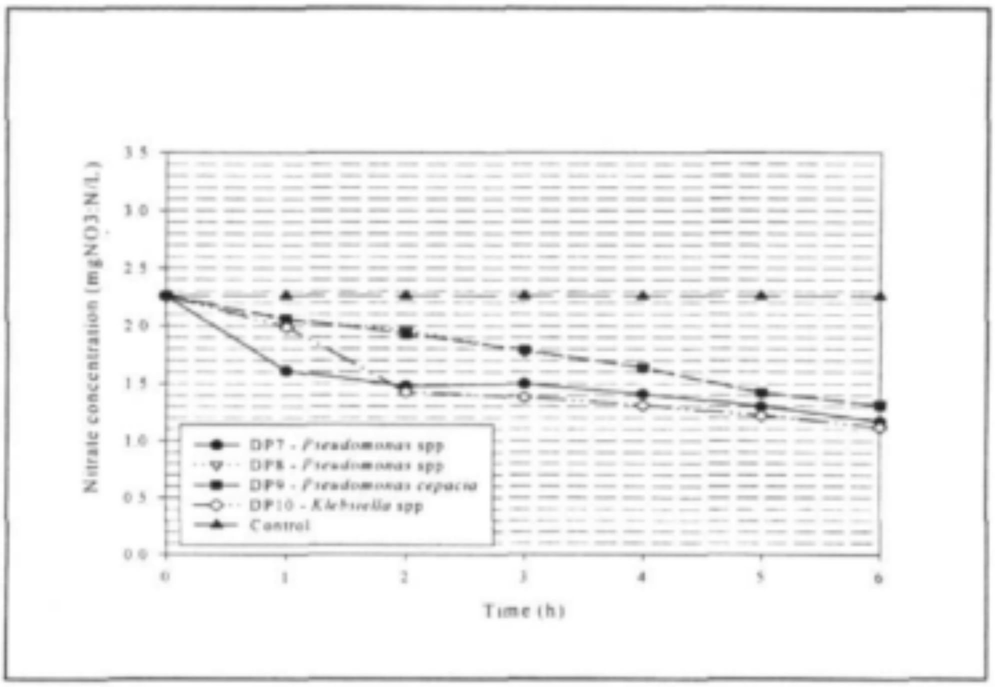

FIGURE 5.9A Nitrogen (as nitrate) reduction profiles by isolates DP7-DP10 when cultivated in phosphate uptake media under anoxic conditions.

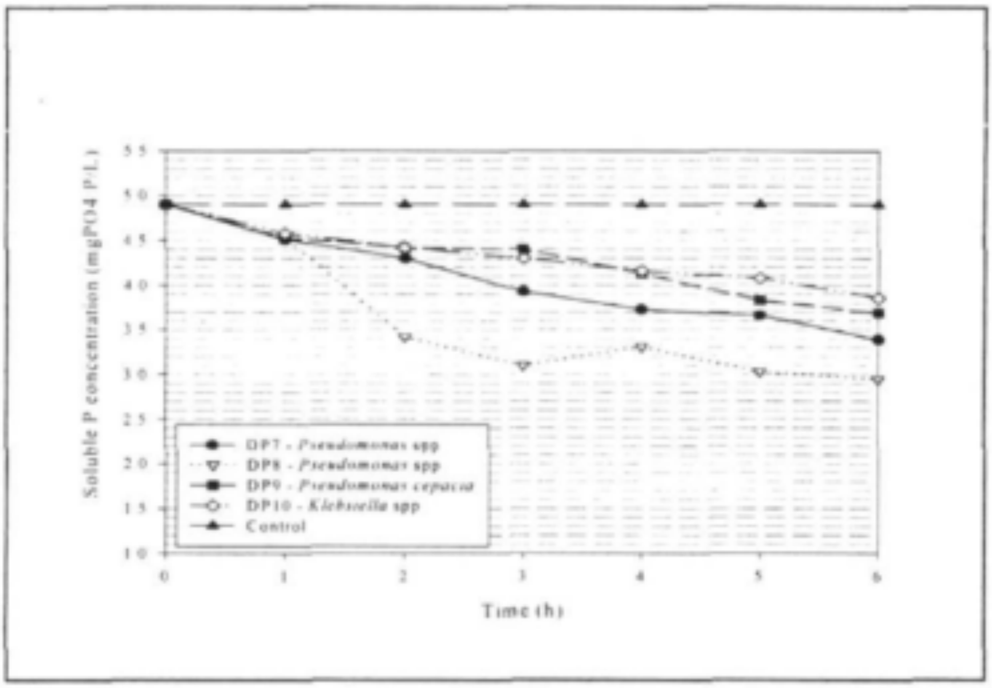

FIGURE 5.9B P removal profiles by isolates DP7-DP10 when cultivated in phosphate uptake media under anoxic conditions.

Anoxic P release was also observed by seven isolated denitrifying bacteria (FIGS 5.10A-5.11B and TABLES 5.9-5.10). Of these *Pasteurella* spp. released as much as 5.91 mgPO_4-P/L with concurrent reduction of 6.25 mgNO_3-N/L. Although four of the isolates demonstrated slight P uptake after initial P release (FIGS 5.10A-B) these organisms cannot be positively linked to BEPR under anoxic conditions. It is interesting to note that P release occurred simultaneously to nitrate reduction as the presence of nitrates is known to inhibit P release under anaerobic conditions (Gruenebaum and Dorgeloh, 1992; Kuba *et al.*, 1996). However, as acetate was present in the phosphate uptake media it is possible that P release was induced by the acetate even though nitrate was available as electron acceptor. Researchers have shown that P release is directly dependant on the presence of acetate and not necessarily anaerobic conditions which only affect P release indirectly by stimulating fermentation of RBCOD (Muyima *et al.*, 1997). In the absence of an external carbon source these organisms may be capable of anoxic P accumulation as their denitrification capacity was good (FIG 5.10A). Furthermore, the slight P uptake observed after initial release (FIG 5.10B) may be a result of decline in acetate concentration due to denitrification and intracellular PHA production. As a result acetate would be non limiting allowing for P accumulation to proceed. However, the P uptake experiments were not conducted for longer than six hours to verify this possibility. Alternatively, these organisms may be PAO's incapable of anoxic P accumulation but able to denitrify under anoxic conditions. This would suggest alternative biochemical pathways and enzymes and indicate possible overlap in the function of some PAO's in NDBEPR systems. This, however, remains uncertain and more detailed understanding of these organisms is required to fully understand the mechanisms of BEPR and denitrification occurring in NDBEPR systems.

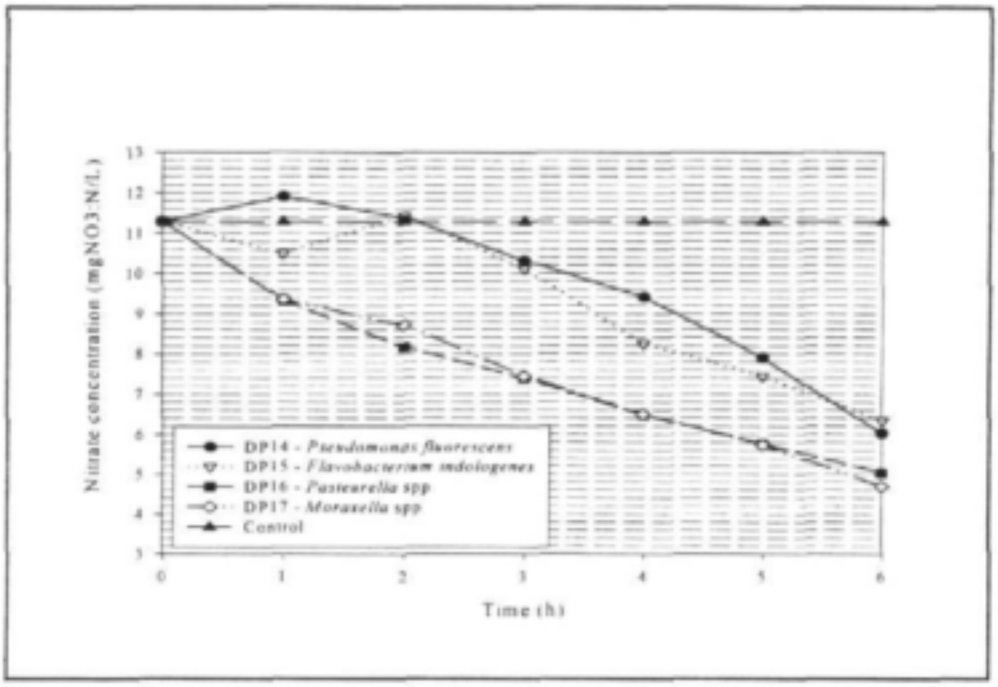

FIGURE 5.10A Nitrogen (as nitrate) reduction profiles by isolates DP11-DP14 when cultivated in phosphate uptake media under anoxic conditions.

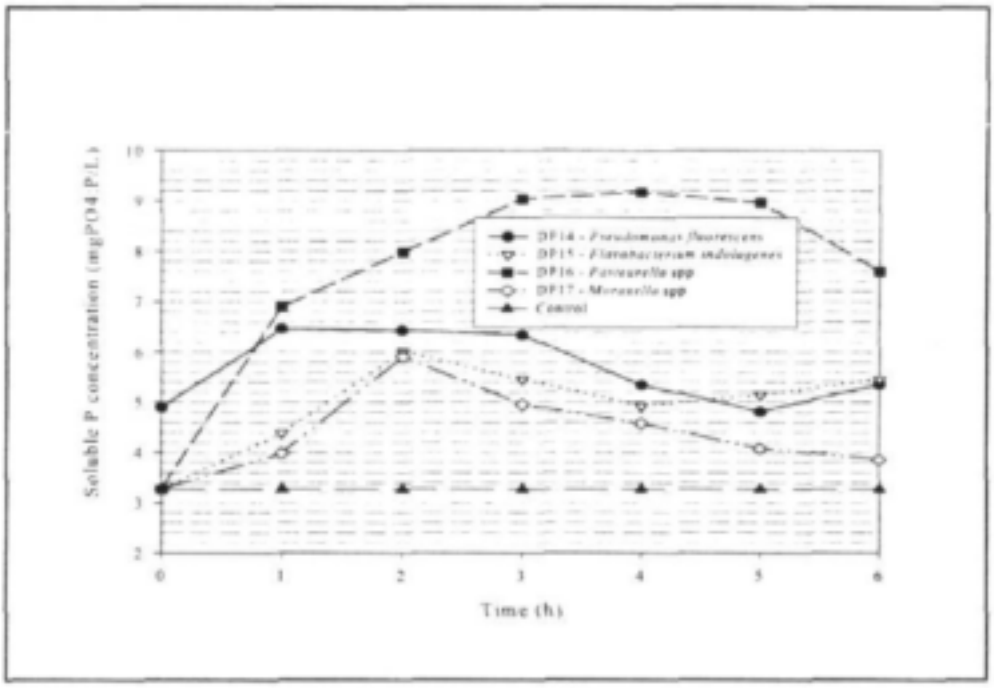

FIGURE 5.10B P release profiles by isolates DP11-DP14 when cultivated in phosphate uptake media under anoxic conditions.

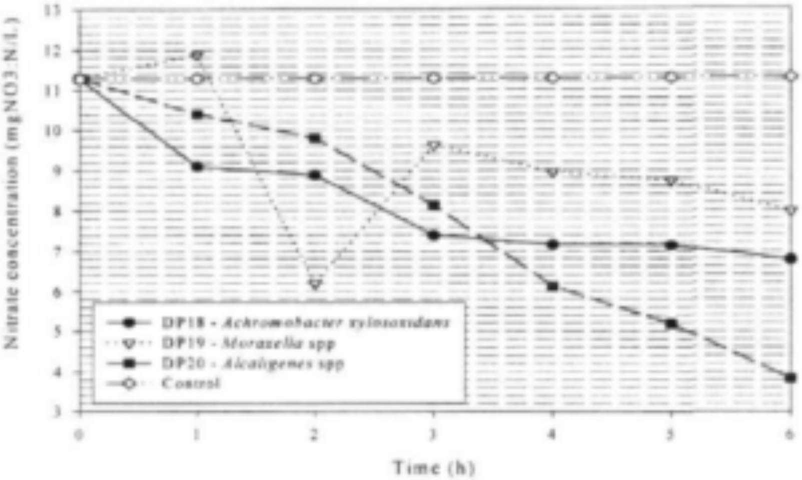

FIGURE 5.11A Nitrogen (as nitrate) reduction profiles by isolates DP15-DP17 when cultivated in phosphate uptake media under anoxic conditions.

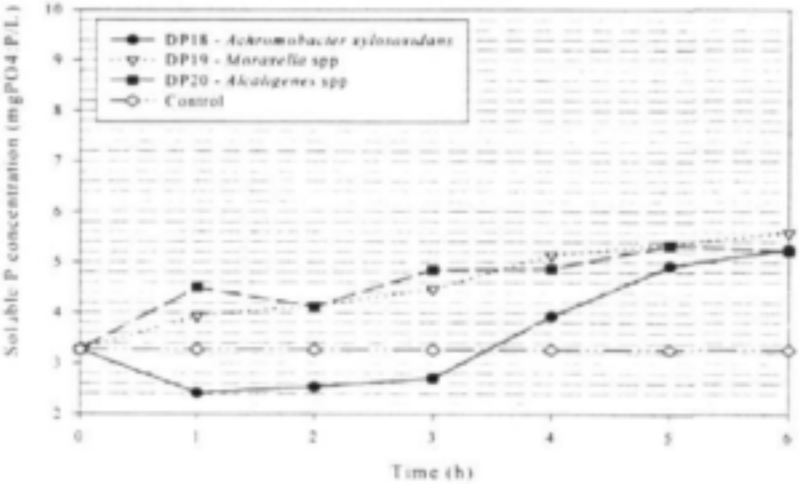

FIGURE 5.11B P release profiles by isolates DP15-DP17 when cultivated in phosphate uptake media under anoxic conditions.

TABLE 5.9 Identification of anoxic P releasing heterotrophic bacteria.

Isolate Ref. No.	Identification	Cellular morphology	Gram reaction
DP11	*Pseudomonas fluorescens*	Rod	negative
DP12	*Flavobacterium indologenes*	Rod	negative
DP13	*Pasteurella* spp	Rod	negative
DP14	*Moraxella* spp	Rod	negative
DP15	*Achromobacter xylosoxidans*	Rod	negative
DP16	*Moraxella* spp	Rod	negative
DP17	*Alcaligenes* spp	Rod	negative

TABLE 5.10 Maximum P release observed by denitrifying heterotrophic bacteria under anoxic conditions.

Isolate Ref. No.	Identification	Initial P ($mgPO_4$-P/L)	Max. P released ($mgPO_4$-P/L)	Initial N ($mgNO_3$-N/L)	N removal ($mgNO_3$-N/L)
DP14	*Pseudomonas fluorescens*	4.90	1.56	11.29	5.26
DP15	*Flavobacterium indologenes*	3.27	2.75	11.29	4.94
DP16	*Pasteurella* spp	3.27	5.91	11.29	6.25
DP17	*Moraxella* spp	3.27	2.32	11.29	3.31
DP18	*Achromobacter xylosoxidans*	3.27	1.98	11.29	4.51
DP19	*Moraxella* spp	3.27	2.64	11.29	6.59
DP20	*Alcaligenes* spp	3.27	2.06	11.29	7.46

Achromobacter xylosoxidans showed initial uptake of P under anoxic conditions but later released 3.17 $mgPO_4$-P/L (FIG 11B). This organism however only accumulated 2.11 $mgPO_4$-P/L prior to releasing P and therefore is not likely to be involved in anoxic P accumulation to any appreciable extent. It is possible that the declining nitrate concentration during incubation may have created sufficient anaerobiosis to induce secondary P release. However, the exact mechanisms responsible for the observed behaviour are unclear and further research into the biochemistry of this organism is necessary.

5.5 CONCLUSIONS

The findings of this study confirm the existence of a diverse community of heterotrophic bacteria involved in nitrogen removal during wastewater treatment of which *Pseudomonas* spp show significant involvement. Numerous heterotrophic bacteria were found to be capable of nitrification. These isolates demonstrated direct oxidation of ammonium to nitrates with little or no nitrite accumulation which is contrary to nitrification behaviour observed in autotrophic bacteria which can only oxidise ammonium or nitrite and not both. Different patterns of nitrification behaviour were also noted amongst heterotrophic nitrifiers with some even displaying potential nitrification-denitrification behaviour. *Staphylococcus* spp. and *Micrococcus* spp. showed significant involvement in nitrification followed by *Streptococcus* spp., *Pseudomonas* spp. and *Bacillus* spp. However, although heterotrophic bacteria are capable of contributing to nitrification little is still known about the exact mechanisms involved and interactive contribution heterotrophs make, if any, to nitrification in BNR systems. There is a need for more insight into the exact role of the heterotrophic bacteria in nitrification in BNR systems in order to optimise modelling of such systems. In respect to denitrification, the OHO fraction within the Darvill NDBEPR process comprises five different functional groups, four of which interactively contribute to denitrification occurring in the system and one group that are non-denitrifying. These groups were defined and characterised as true denitrifiers (bacteria capable of both nitrate and nitrite reduction), incomplete denitrifiers (bacteria that reduced nitrates to nitrites with no further reduction of the nitrites produced), incomplete-nitrite reducers (bacteria capable of both nitrate and nitrite reduction, however, exhibiting severe inhibition of nitrite reduction by nitrates),exclusive nitrite reducers (bacteria only capable of reducing nitrites) and non-denitrifiers (bacteria not capable of nitrate or nitrite reduction). The OHO fraction is therefore more complex than currently accepted and needs to be amended for more accurate modelling of denitrification kinetics in NDBEPR processes. The large amount of non-denitrifying organisms comprising the OHOs is cause for concern on the accuracy of attributing denitrification behaviour and kinetics to the entire OHO fraction, as is currently the practice, and therefore this group needs to be excluded from the active denitrifying OHO fraction and properly defined in terms of their function in NDBEPR systems. Various denitrifying heterotrophic bacteria also demonstrated the capacity for anoxic P uptake utilising nitrates instead of oxygen. *Serratia* spp. and *Vibrio* spp. were the most efficient anoxic P accumulators isolated. Weaker P accumulating bacteria were also identified which were only capable of accumulating small amounts of P at low initial P and nitrate concentrations. However, these organisms may not necessarily be weak P accumulators under aerobic conditions, but, were limited under anoxic conditions due to weak denitrification capacity. Anoxic P release was also observed by some denitrifying heterotrophic bacteria. Although P release may be optimal under anaerobic conditions, inhibition of P release under anoxic conditions is not true to all denitrifying heterotrophic bacteria. The mechanisms of anoxic P removal need to be understood better in order to model such behaviour in NDBEPR systems.

CHAPTER SIX

GENERAL CONCLUSIONS AND RECOMMENDATIONS

6.1 GENERAL CONCLUSIONS

Mixed liquor samples were taken from the aerobic zone of Darvill WWW and cultivated on solid media. Isolates were screened for their ability to accumulate poly-P through successive anaerobic/aerobic incubation and only those isolates displaying the desirable P transformation patterns i.e., anaerobic P release and aerobic P uptake, were subsequently identified. Results showed unequivocal dominance of the gamma subclass of the Proteobacteria cluster. 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 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.

Development of enhanced PAO cultures at pilot-scale and subsequent community analysis using cultivation-dependant techniques, once again showed the dominance of *Pseudomonas* spp. in the system. Plating also resulted in the total dominance of the gamma subclass of Proteobacteria. The apparent dominance of *Pseudomonas* spp. and the gamma Proteobacteria in BEPR operations was attributed to the bias imposed by the applied isolation and identification techniques. However, results of this aspect of the study indicated the importance of the Proteobacteria to BEPR operations. Surprisingly, the dominance of *Acinetobacter* spp. in BEPR activated sludge was never demonstrated when using plating techniques.

Using *in situ* molecular techniques (FISH), the total bacterial population of both full- and pilot-scale BEPR activated sludges was found to be underestimated by at least three orders of magnitude by cultivation-dependent methods. The alpha and beta Proteobacteria were both shown to be functional groups implicated in full- and pilot-scale BEPR systems, together comprising more than 60% of the active population. However, the overall community profile shows that the BEPR population is quite diverse and it is unlikely that any one species dominates the process.

As well as qualitative data, molecular techniques also yield reliable quantitative information regarding biological systems. The PAO population of the total active biomass was found to be at percentages of 55% for the enhanced pilot plant culture and 35% for the full-scale NDBEPR system. The total number of PAO's required to remove 1 g of soluble phosphorus was calculated to be between 1-2 x 10^{11} cells. In attempting to determine the quantity of biomass (as metabolically active bacterial cells) to be associated with 1g of VSS, the pilot-scale system proved useful and yielded a number of 1×10^{13} cells. From this calculation, that portion of VSS present as biomass only was estimated to be only 15% of the total for the full-scale BEPR system.

Findings confirm the existence of a diverse community of heterotrophic bacteria involved in nitrogen removal during wastewater treatment of which *Pseudomonas* spp. show significant involvement. Numerous heterotrophic bacteria were found to be capable of nitrification with most demonstrating direct oxidation of ammonium to nitrates with little or no nitrite accumulation. Different patterns of nitrification behaviour were also noted amongst heterotrophic nitrifiers with some even displaying potential nitrification-denitrification behaviour. *Staphylococcus* and *Micrococcus* spp. showed significant involvement in nitrification along with *Streptococcus*, *Pseudomonas* and *Bacillus* spp.

With respect to denitrification, the OHO fraction within the Darvill NDBEPR process was comprised of five different functional groups, four of which interactively contribute to denitrification occurring in the system and one group that are non-denitrifying. These groups were defined and characterised as true denitrifiers (bacteria capable of both nitrate and nitrite reduction), incomplete denitrifiers (bacteria that reduced nitrates to nitrites with no further reduction of the nitrites produced), incomplete-nitrite reducers (bacteria capable of both nitrate and nitrite reduction, however, exhibiting severe inhibition of nitrite reduction by nitrates), exclusive nitrite reducers (bacteria only capable of reducing nitrites) and non-denitrifiers (bacteria not capable of nitrate or nitrite reduction). The OHO fraction is therefore more complex than currently accepted and needs to be amended for more accurate modelling of denitrification kinetics in NDBEPR processes.

Numerous heterotrophic bacteria were also found capable of phosphorus uptake under anoxic conditions *in vitro*. *Serratia* spp. and *Vibrio* spp. were the most efficient anoxic phosphorus accumulators isolated demonstrating 7.10 and 7.29 mgPO_4-P/L removal, respectively. Weaker phosphorus accumulating bacteria were also identified. However, these organisms may not necessarily be weak phosphorus accumulators under aerobic conditions but were limited under anoxic conditions due to weak denitrification capacity. Anoxic phosphorus release was also observed by some denitrifying heterotrophic bacteria. *Pasteurella* spp. released as much as 5.90 mgPO_4-P/L with concurrent reduction of 6.25 mgNO_3-N/L. Although phosphorus release may be optimal under anaerobic conditions, inhibition of phosphorus release under anoxic conditions is not true to all denitrifying heterotrophic bacteria.

It was generally agreed that the research objectives, as formulated and detailed at the beginning of the Executive Summary, were successfully achieved. An in-depth microbiological survey of both a full- and pilot-scale nitrification/denitrification biological excess phosphorus removal activated sludge system was performed. Both cultivation-dependant and molecular *in situ* techniques were applied to obtain an adequate description of the physiologically significant microbial community involved in biological phosphorus removal, heterotrophic nitrification and denitrification. Autotrophic nitrifiers were not investigated during the course of this study due to difficulties in their isolation and excessively long growth rates. Of great significance during this study was the establishment of a molecular biology laboratory and application of the fluorescence *in situ* hybridization technique to obtain both qualitative and quantitative information regarding the microbial community in biological nutrient removal systems. Comparisons between various cultivation-dependant identification techniques were not performed due to the potential excessive costs in the purchase of software and consumables. All isolates were subsequently identified using the Analytical Profile Index.

Upon initiation of this research project, fluorescence *in situ* hybridization technology had not yet been applied to any South African activated sludges. Microbial community analyses appeared to be largely confined to culture-dependant techniques. Although routinely applied at an international level, it appeared after an intensive literature search that fluorescence *in situ* hybridization had not yet been applied to continuous systems designed to stimulate biological excess phosphorus removal either. The majority of these articles seemed to be confined to the use of sequencing batch reactors and/or full-scale systems. Quantitative data provided by molecular based assays can also be used by the engineering and technology disciplines to improve existing activated sludge models and to address the deficiency of the active biomass concept. Based on these points, it was felt that the current project positively contributed to the state-of-the-art in wastewater treatment both locally and abroad.

6.2 RECOMMENDATIONS

- As microbiologists, efforts to isolate PAO's from BEPR sludges should continue. Although this will require a certain degree of innovation, the presence of viable but non-culturable bacteria in environmental ecosystems should not deter these efforts;
- Although the broad community profile (family and sub-group levels) of an ecosystem can be determined through application of FISH technology, techniques involving cloning, sequencing and probe design must also be incorporated to adequately describe the microbial community profile at a generic and species level;
- In order to improve the quantitative potential of molecular techniques when conducting activated sludge studies, methods to allow for maximum floc dispersion should be investigated. Counting of sufficient numbers of microscopic fields will also be required when attempting to provide the raw data required to advance the predictive and descriptive capacity of current mathematical models for NDBEPR processes;
- Heterotrophic nitrification needs to be evaluated *in situ* in order to establish the involvement, if any, of heterotrophic bacteria in nitrification occurring during wastewater treatment;
- The kinetics and biochemistry of nitrate and nitrite reduction need to be determined for

true denitrifiers, incomplete denitrifiers, incomplete-nitrite reducers and exclusive nitrite reducers in order to establish the contribution to denitrification by each group;

- A method needs to be established to determine the non-denitrifying and denitrifying fractions of the heterotrophic active biomass for more accurate modelling of denitrification behaviour in BNR systems.
- The microbiology and biochemistry of anoxic P accumulation requires further investigation;
- Results of API identification need to be substantiated using additional biochemical tests and qualitative molecular techniques to allow more conclusive identification of bacterial isolates from environmental samples;
- Microbiologists/biochemists and process engineers must begin to communicate and not work independently of one another. The quantitative information which molecular biologists are now able to generate regarding active cell biomass will prove invaluable in improving the accuracy and predictive ability of the existing or new activated sludge mathematical models.

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8. APPENDICES

APPENDIX 1

Configuration and operational parameters of Darvill WWW

When Darvill 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. A1).

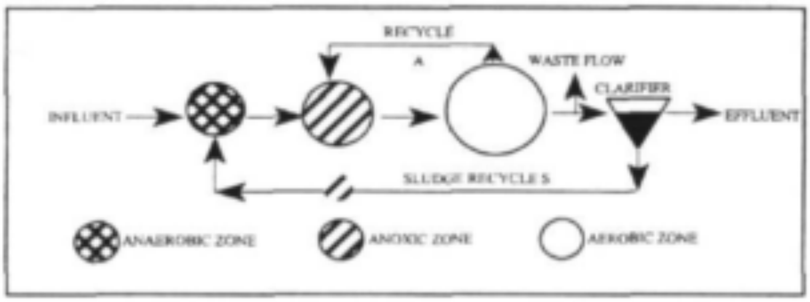


FIGURE A1 The Johannesburg system incorporating NDBEPR features.

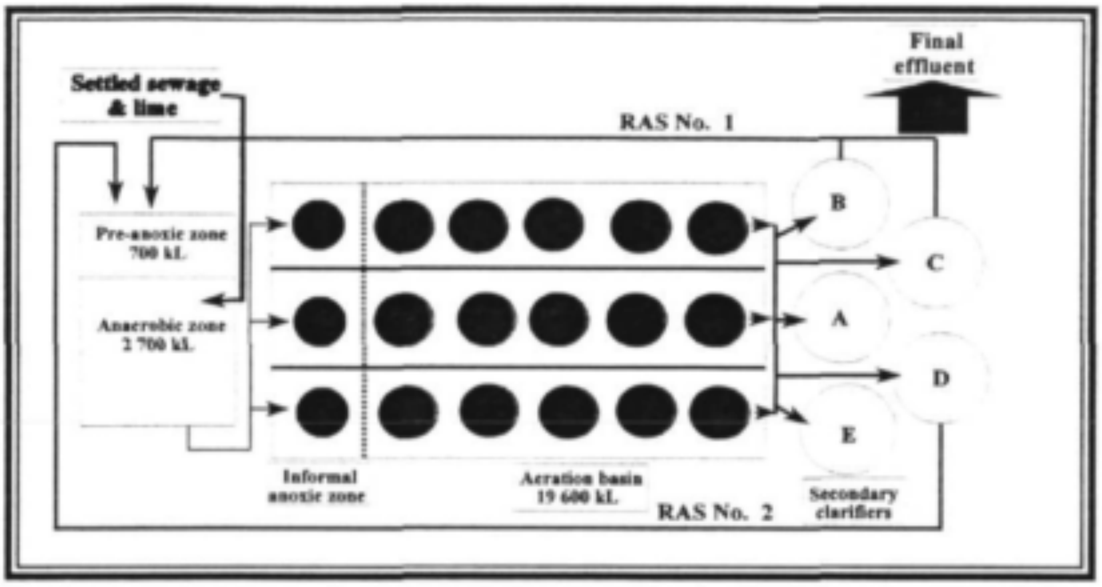


FIGURE A2 Schematic representation of Darvill activated sludge plant modified for BPR.

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. A2 for a diagrammatic description of the Darvill WWW activated sludge plant.

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 HRT for mixed liquor passing through the anaerobic zone (De Haas, 1998). TABLE A1 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 A2. 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 A1 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 A2 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
FSA	18.2 mgN/L
Alkalinity	141 mg/L as CaCO3
Ortho-P	5.6 mgP/L
TP	8.5 mgP/L

APPENDIX 2

Casitone glycerol yeast autolysate agar (CGY)

Preparation of CGY agar as follows:

Measure out:

5 g Bacto casitone; 10 ml glycerol; 1 g yeast autolysate; 16 g agar

Add to 1 litre distilled water and dissolve by heating. Adjust pH to 7.2. Autoclave at 121°C for 15 min.

APPENDIX 3

Layout of activated sludge EBPR pilot plant

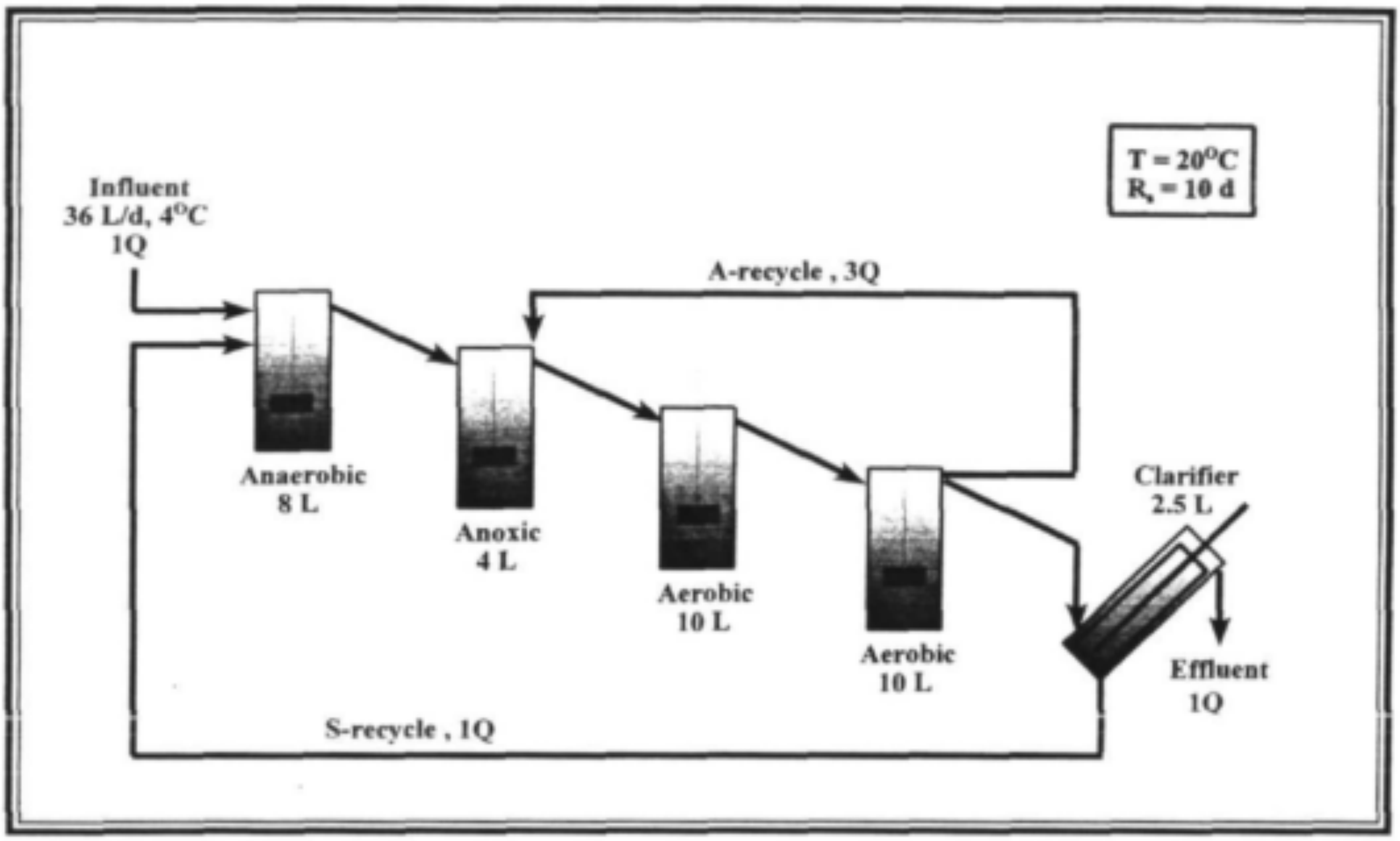


FIGURE A3 Layout of three-stage Phoredox pilot plant.

APPENDIX 4

Operating parameters of the single aerobic activated sludge system at Amanzimtoti WWTP

TABLE A3 Operating parameters and average feed composition to Amanzimtoti WWTP activated sludge process.

Total reactor volume - east plant	4 680 m^3
west plant	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
MCRT	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 $CaCO_3$

APPENDIX 5

Sewage supplementation to the BEPR activated sludge pilot plant

TABLE A4 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 $CaCO_3$
A 19/6/98 - 25/6/98	7	0	0	0	100
B 26/6/98 - 03/8/98	39	100	5	8.5	100
C 04/8/98 - 22/8/98	19	150	5	11.3	100
D 23/8/98 - 11/9 98	20	200	23/8 - 02/9 = 5	11.3	100
			03/9 - 09/9 = 10	11.3	
			10/9 - 11/9 = 20	11.3	
E 12/9/98 - 02/10/98	21	300	20	25.5	100
F 03/10/98 - 22/10/98	20	400	20	25.5	100
G 23/10/98 - 21/11/98	30	500	23/10-28/10=30	25.5	100
			29/10-31/10=40	25.5	
			01/10-04/11=50	25.5	
			05/11-21/11=50	42.5	

APPENDIX 6

Macro- and micronutrient supplementation to pilot plant influent

TABLE A5 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

APPENDIX 7

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. Sampling bottles were 250 mL plastic honey jars. The following preservation techniques were used for the duration of the project:

TABLE A6 Sample preservation techniques.

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
TKN	H_2SO_4 addition to pH<2; refrigerate	24 h
TP	H_2SO_4 addition to pH<2; refrigerate	24 h
SRP	immediate 0.45 μm filtration; refrigerate	24 h
Solids	refrigerate	6 h

APPENDIX 8

Determination of influent readily biodegradable soluble COD (S_{bsl}) 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). The method involves removal of colloidal material 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:

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

where:

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

APPENDIX 9

Ammonium enrichment medium

Preparation of ammonium enrichment medium as follows:

Measure out:

0.5 g KH_2PO_4; 0.5 g K_2HPO_4; 10 g $(NH_4)_2SO_4$; 0.2 g $MgSO_4.7H_2O$; 0.2 g NaCl; 0.01 g $CaCl_2.2H_2O$; 0.01 g $FeSO_4.7H_2O$; 5.0 g tryptone; 1.0 g yeast extract; 10 ml glycerol

Add to 1 litre of distilled water. Dissolve and distribute into flasks. Autoclave at 121°C for 15 min.

APPENDIX 10

Nitrite enrichment medium

Preparation of nitrite enrichment medium as follows:

Measure out:

0.5 g KH_2PO_4; 0.5 g K_2HPO_4; 0.6 g KNO_2; 0.2 g $MgSO_4.7H_2O$; 0.2 g NaCl; 0.01 g $CaCl_2.2H_2O$; 0.01 g $FeSO_4.7H_2O$; 5.0 g tryptone; 1.0 g yeast extract; 10 ml glycerol

Add to 1 litre of distilled water. Dissolve and distribute into flasks. Autoclave at 121°C for 15 min.

APPENDIX 11

Acetate mineral medium (AMM)

Preparation of AMM as follows:

Measure out:

3.68 g $CH_3COOHa.3H_2O$; 28.73 mg $Na_2HPO_4.2H_2O$; 57.27 mg NH_4Cl; 131.82 mg $MgSO_4.7H_2O$; 26.74 mg K_2SO_4; 17.2 mg $CaCl_2.2H_2O$; 12 g HEPES buffer; 15 g agar; 2 ml trace mineral solution

Trace Mineral Solution:

50 g EDTA; 5 g $FeSO_4.7H_2O$; 1.6 g $CuSO_4.5H_2O$; 5 g $MnC12._4H_2O$; 1.1 g $(NH_4)_6Mo_7O_{24}.4H_2O$; 50 mg H_3BO_3; 10 mg KI; 50 mg $CoCl_2.6H_2O$; 1 L distilled water

Add to 1 litre distilled water. Adjust pH to 7.0 with 1 M NaOH. Autoclave at 121°C for 15 min.

APPENDIX 12

Ammonium nitrification medium

Preparation of ammonium nitrification medium, as adapted from Schmidt and Belser (1982), as follows:

Measure out:

10 ml $(NH_4)_2SO_4$ (5 g/100 ml distilled water); 1 ml $CaCl_2.H_2O$ (1.34 g/100 ml distilled water); 1 ml $MgSO_4.7H_2O$ (4 g/100 ml distilled water); 7.5 ml KH_2PO_4 (0.2 M); 1 ml chelated iron

Chelated Iron Solution:

0.246 g $FeSO_4.7H_2O$; 0.331 g EDTA disodium; 100 ml distilled water; 1 ml trace element solution

Trace Element Solution:

0.02 g $MnCl_2$; 0.02 g $ZnSO_4.7H_2O$; 0.002 g $CuSO_4.5H_2O$; 0.0002 g $CoCl_2.6H_2O$; 0.01 g $NaMoO_4.2H_2O$; 100 ml distilled water; 10 ml glycerol

Adjust pH to 7.0 using 0.1N NaOH. Autoclave at 121°C for 15 min.

APPENDIX 13

Colorimetric biochemical nitrate reduction test

The nitrate reduction test, as described by Cappuccino & Sherman (1992), is as follows:

1) Preparation of reagents:

Solution A

8 g sulphanilic acid; 1 litre acetic acid, 5N (1 part glacial acetic acid to 2.5 parts distilled water)

Solution B

5 g alpha-naphthylamine; 1 litre acetic acid, 5N (1 part glacial acetic acid to 2.5 parts distilled water)

2) Procedure and interpretation of results

Inoculate test organism into nitrate media (Nitrate broth supplemented with 0,1%agar); incubate cultures; after incubation add 5 drops solution A followed by 5 drops solution B to cultures; if a red colour develops, nitrate has only been reduced to nitrite; if no red colour develops, add zinc powder to culture; if a red colour develops, nitrate was not reduced at all; if no red colour develops, nitrate was reduced to nitrite which, in turn, was reduced to either ammonia, nitrogen gas or a less oxidised form of nitrogen.

APPENDIX 14

Phosphate limiting liquid acetate medium

Preparation of phosphate limiting liquid acetate medium as follows:

Measure out:

3.23 g $CH_3COONa.3H_2O$; 22.98 mg $Na_2HPO_4.2H_2O$; 152.76 mg NH_4Cl; 81.12 mg $MgSO_4.7H_20$; 17.83 mg K_2SO_4; 11.00 mg $CaCl_{2.}2H_2O$; 7 g HEPES buffer; 2 ml trace mineral solution

Trace Mineral Solution:

50 g EDTA; 5 g $FeSO_4.7H_2O$; 1.6 g $CuSO_4.5H_2O$; 5 g $MnCl_{2.4}H_2O$; 1.1 g $(NH_4)_6Mo_7O_{24}.4H_2O$; 50 mg H_3BO_3; 10 mg KI; 50 mg $CoCl_{2.}6H_2O$; 1 litre distilled water

Add to 1 litre distilled water. Adjust pH to 7 with 1M NaOH. Autoclave at 121°C for 15 min.

APPENDIX 15

Phosphate uptake medium

Preparation of phosphate uptake medium as follows:

Measure out:

3.23 g $CH_3COONa.3H_2O$; 35.11 mg K_2PO_4; 305.52 mg NH_4Cl; 91.26 mg $MgSO_4.7H_2O$; 25.68 mg $CaCl.2H_2O$; 8.5 g PIPPES buffer; 2 ml trace mineral solution

Trace Mineral Solution:

50 g EDTA; 5 g $FeSO_4.7H_2O$; 1.6 g $CuSO_4.5H_2O$; 5 g $MnCl_2._4H_2O$; 1.1 g $(NH_4)_6Mo_7O_{24}.4H_2O$; 50 mg H_3BO_3; 10 mg KI; 50 mg $CoCl_2.6H_2O$; 1litre distilled water

Add to 1 litre distilled water. Adjust pH to 6.8 with 1 M NaOH. Autoclave at 121°C for 15 min.

APPENDIX 16

Identification of Gram negative rods

The use of API 20E and API 20NE profile indexes for identification of enteric and non-enteric Gram negative rods, as described by Bux *et al.* (1994), was conducted as follows:

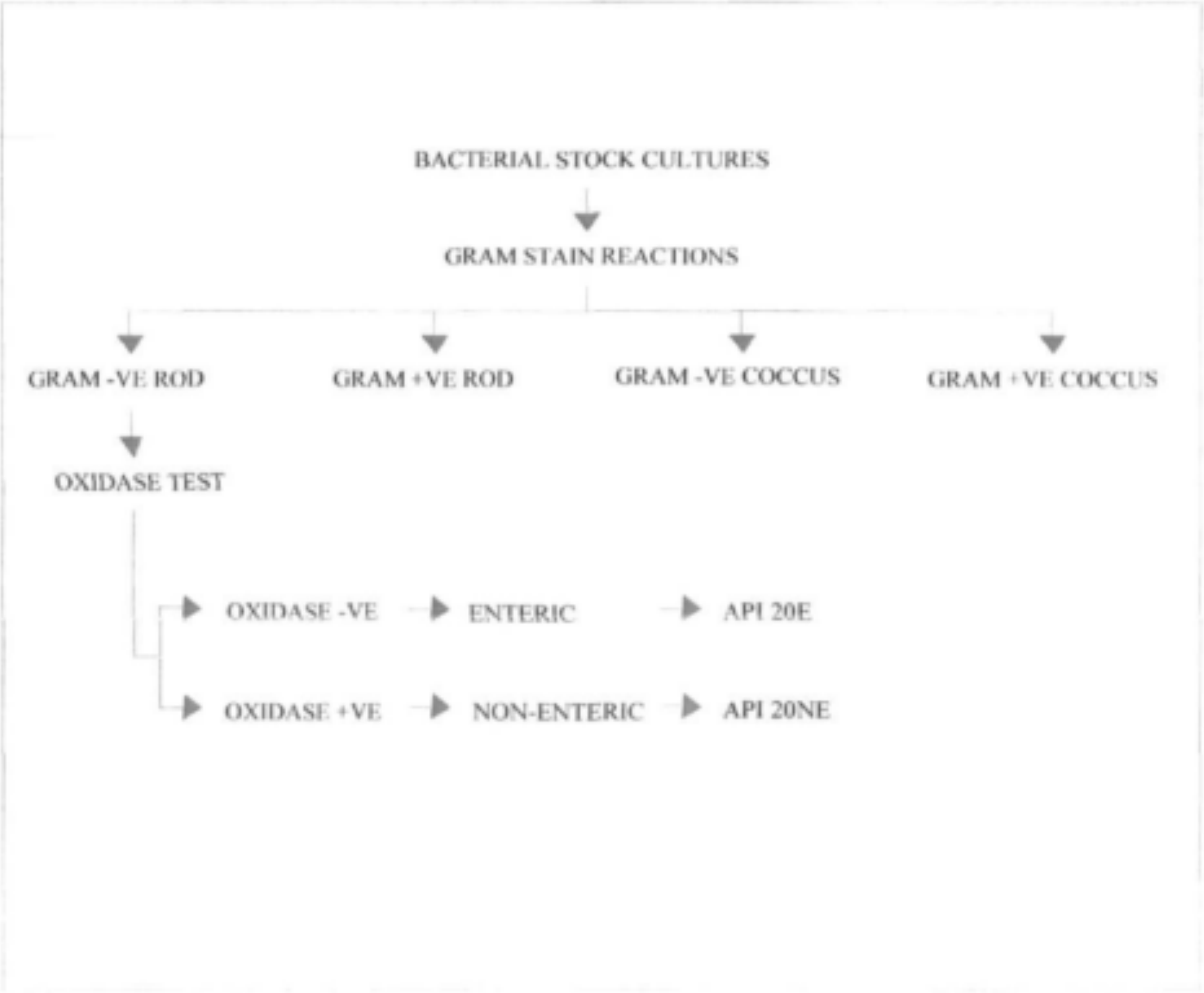


FIGURE A4 Experimental protocol to isolate and identify Gram negative rods.

APPENDIX 17

Identification of Gram negative cocci

Identification of Gram negative cocci, according to *Bergey's* (1984) and Cappuccino and Sherman (1992), was conducted as follows:

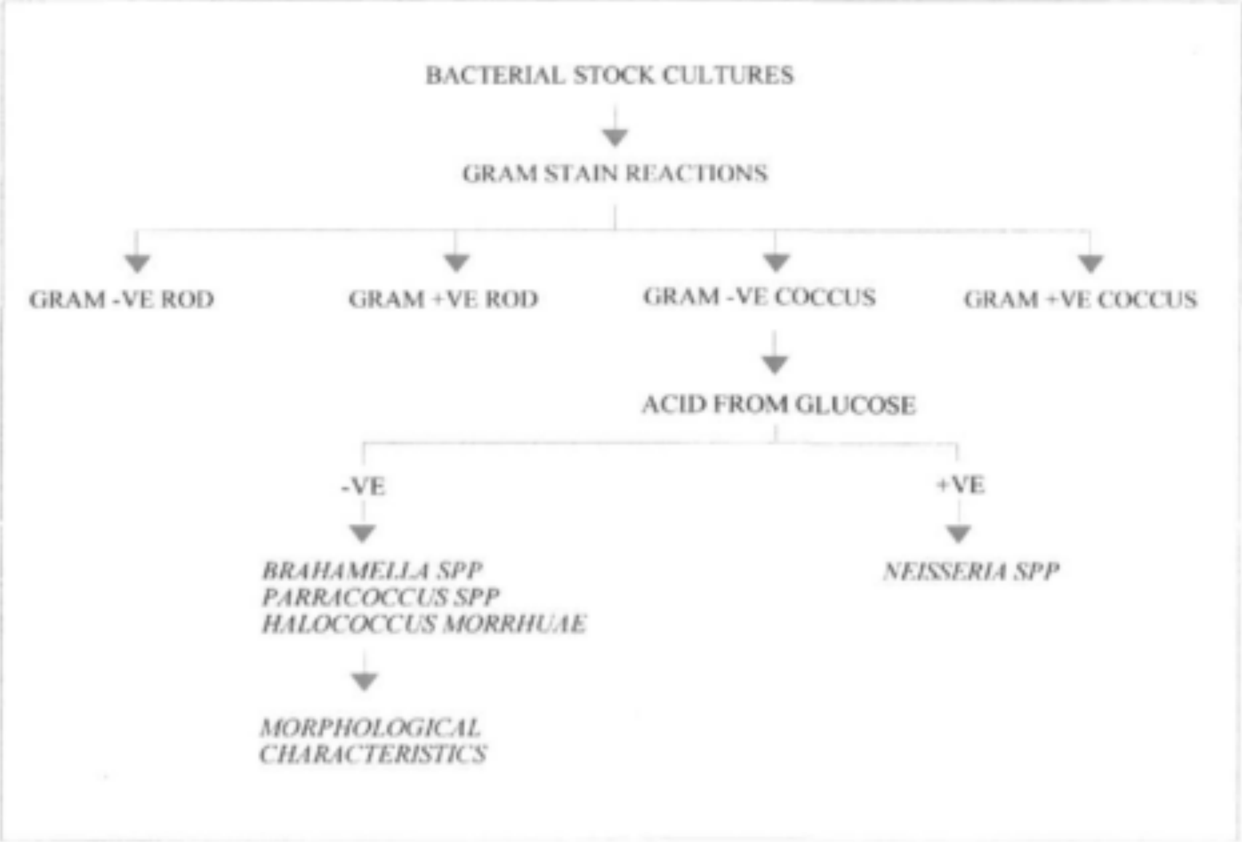


FIGURE A5 Experimental protocol to isolate and identify Gram negative cocci.

APPENDIX 18

Identification of Gram positive rods

Identification of Gram positive rods, according to *Bergey's* (1984;1986) and Cappuccino and Sherman (1992), was conducted as follows:

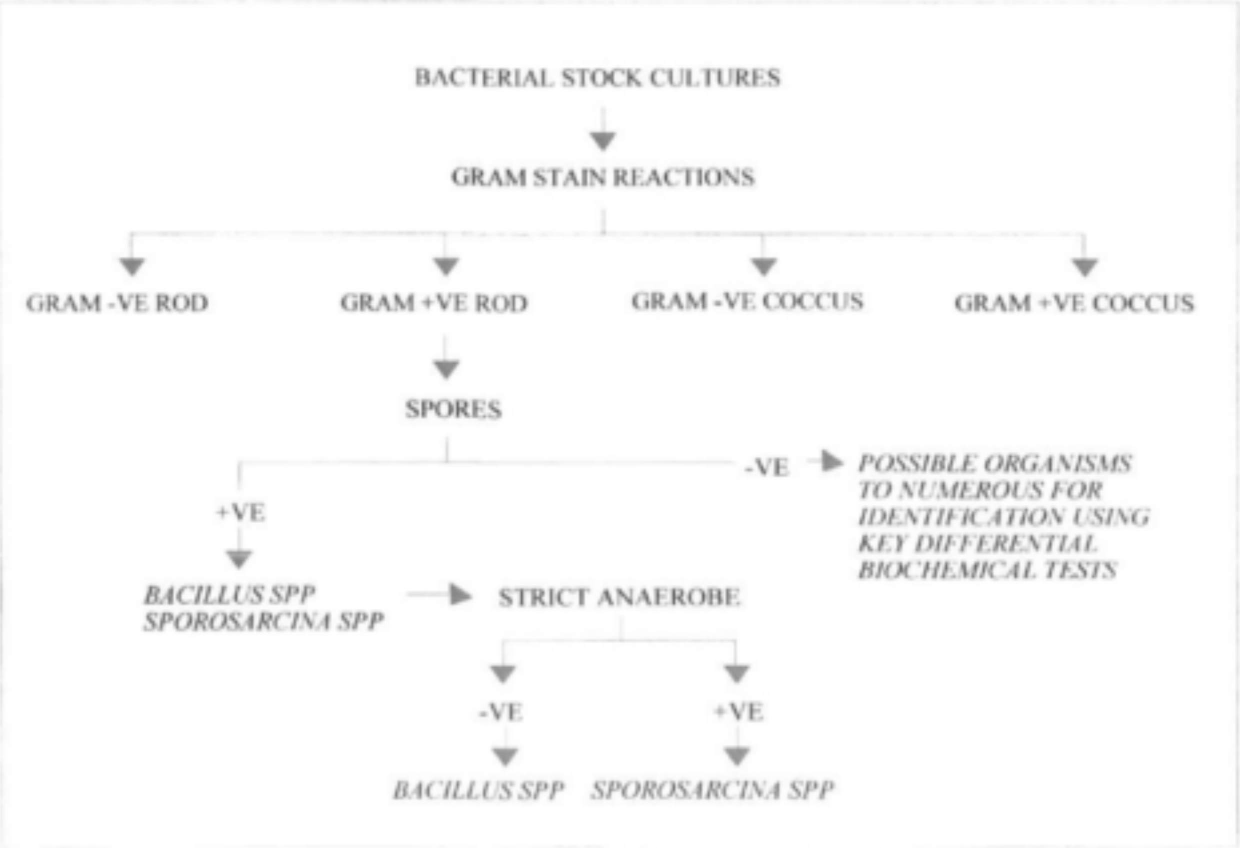


FIGURE A6 Experimental protocol to isolate and identify Gram positive rods.

APPENDIX 19

Identification of Gram positive cocci

Identification of Gram positive cocci, according to *Bergey's* (1984; 1986) and Cappuccino and Sherman (1992), was conducted as follows:

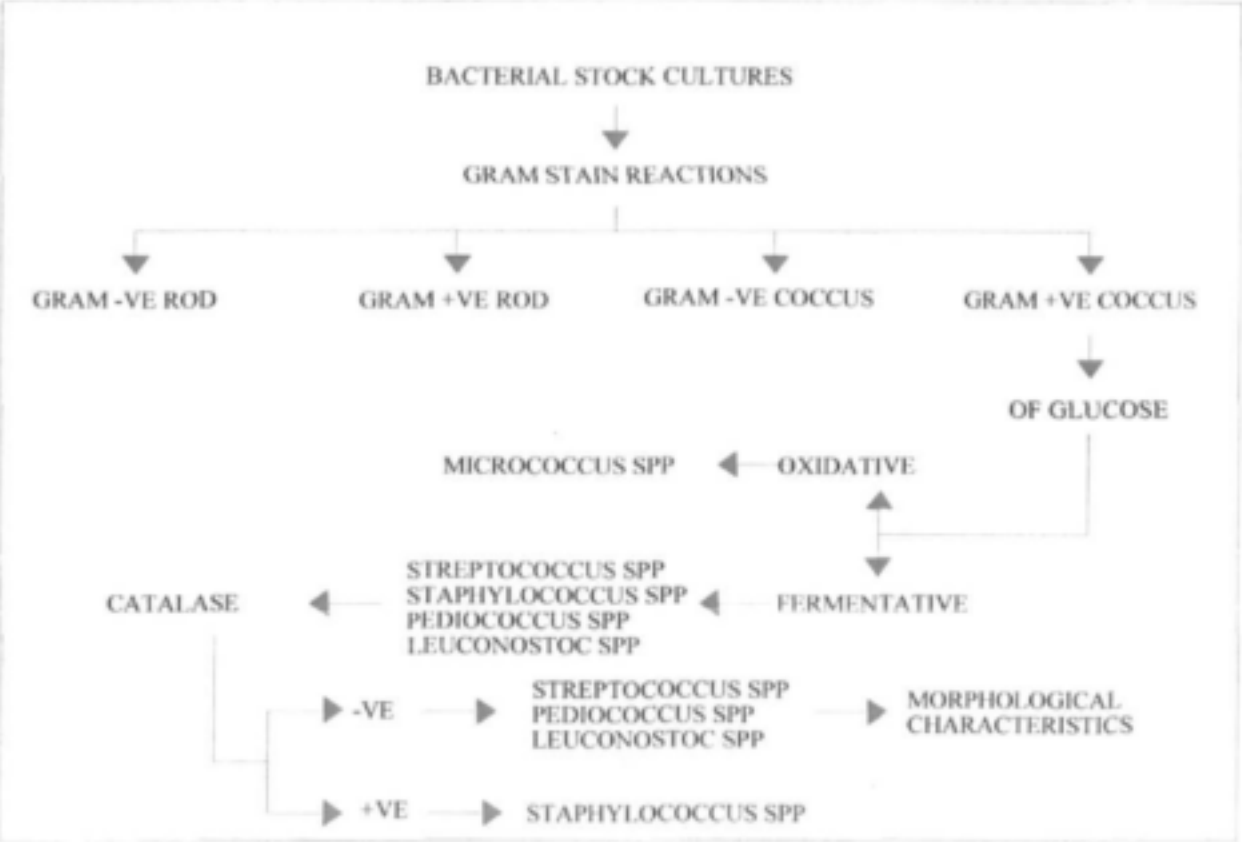
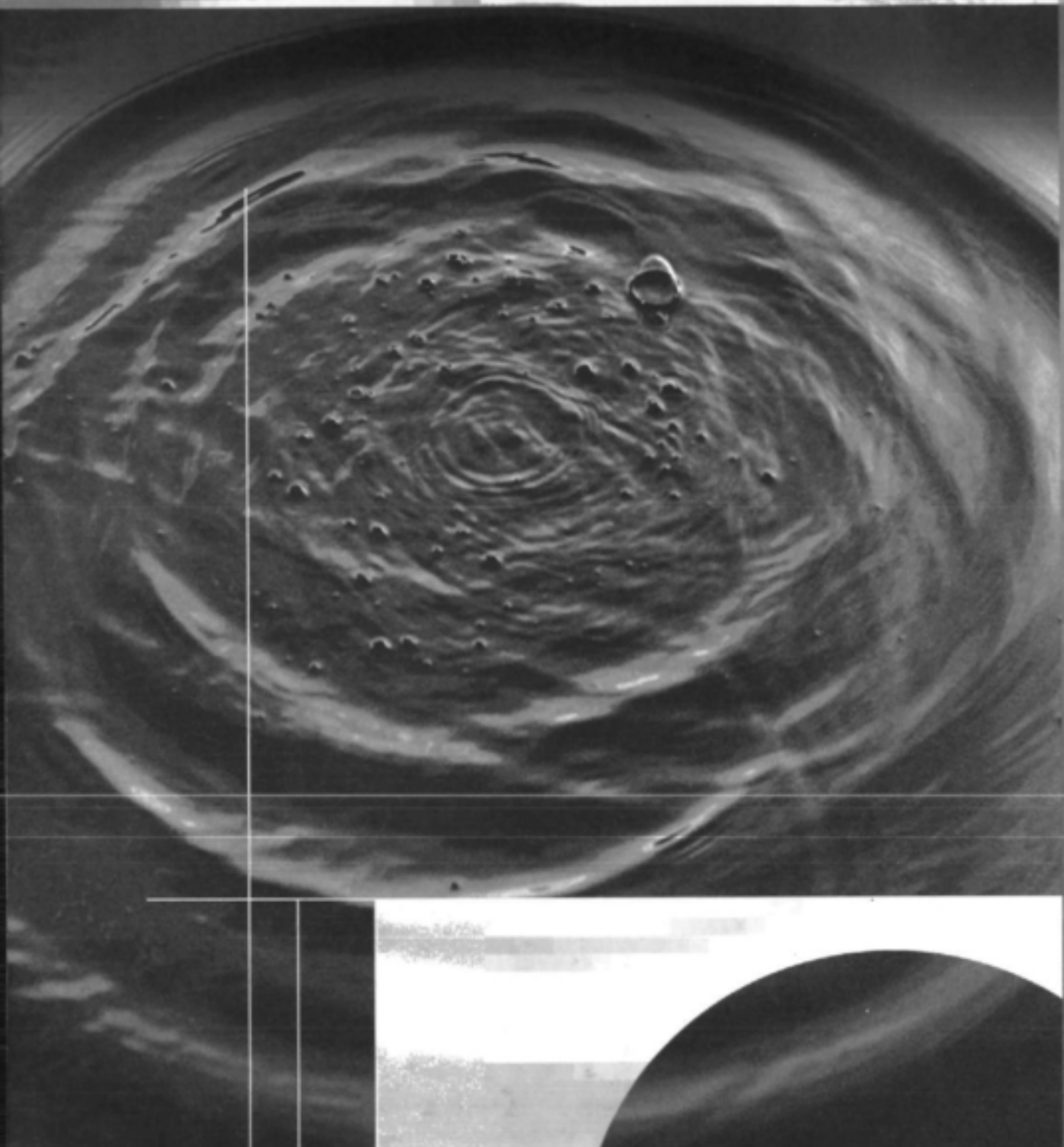


FIGURE A7 Experimental protocol to isolate and identify Gram positive cocci.



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