THE ROLE OF ACTIVATED SLUDGE EXTRACELLULAR POLYMERS AND AEROBIC BIOMASS IN THE REMOVAL OF PHOSPHORUS FROM WASTEWATER

by

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Submitted in partial fufillment of the requirements for the degree

M.Sc Microbiology

In the Faculty of Natural and Agricultural Science

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January 2001

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or part been submitted at any university for a degree.

Signature: _____

Date: 16 July 2001

"Science has 'explained' nothing; the more we know
the more fantastic the world becomes and the
profounder the surrounding darkness."

— Aldous Huxley (1894-1964), British novelist.

"An expert is a man who has made all the mistakes which
can be made in a very narrow field."

— Niels Bohr (1885-1962)

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SUMMARY

Research has indicated the relationship between biomass and phosphorus removal in activated sludge. Mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) are often used as indicators of biomass, and used as such in the mathematical modelling of biological phosphorus removal. Not all phosphorus removed in activated sludge systems can be accounted for by polyphosphate accumulating organisms (PAO). The objectives of this study were to determine the relationship between the MLSS and MLVSS fractions and phosphorus removal in activated sludge, to compare these fractions, together with total plate count (TPC) and adenosine triphosphate (ATP) bacterial counts as measures of viable biomass and to investigate the role of extracellular polysaccharides (EPS) in the removal of phosphorus from wastewater. The hypothesis of the study was that the same amount of MLSS and, specifically MLVSS, of different activated sludges should show similar orthophosphate uptake abilities if these were to be indicative of biomass. To this end two experiments were conducted. In experiment 1, sterile mixed liquor growth medium was inoculated with equal amounts (40 grams) of wet sludge pellets from five different 3-stage Bardenpho activated sludge systems with similar sludge ages. In experiment 2, the MLSS and

orthophosphate concentrations in the same plants used in experiment 1 were simulated. Orthophosphate removal was determined hourly and differed amongst systems. Different orthophosphate removal capacities were attributed to differences in the MLSS active biomass fraction of the different activated sludges. Although MLSS and MLVSS showed the same trend in orthophosphate removal, initial concentrations of these fractions could not be directly linked to differences in orthophosphate uptake abilities of different sludges, indicating the unsuitability of MLSS and MLVSS as indicators of viable biomass in activated sludge. However, orthophosphate removal was consistently higher in the sludges with higher ATP and TPC values, indicating a relationship between the active biomass fraction of the MLSS and orthophosphate removal.

A method for the qualitative and quantitative *in situ* characterization of PAO cell clusters and closely associated EPS is described. X-ray microanalysis was performed on samples from four activated sludge plants. Analyses were done by means of scanning electron microscopy (SEM) combined with energy dispersive spectrometry (EDS). On average, cell clusters with associated EPS contained between 57 and 59 % phosphorus, while EPS alone contained between 23 and 30 % phosphorus. Results suggest that phosphorus removal in activated sludge might be due not only to PAO, but also by EPS acting as a phosphorus reservoir. Extraction of EPS from two different activated sludge plants yielded similar amounts of EPS. Comparison of EDS results before and after EPS extraction, indicated possible intracellular leakage during homogenization, while phosphorus may be complexed in localized iron and aluminium precipitates in wastewater treatment plants employing chemical treatment to attain effluent standards. These precipitates were probably removed by filtration during the extraction procedure employed.

UNIVERSITEIT VAN PRETORIA
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DIE ROL VAN GEAKTIVEERDE SLYK EKSTRASELLULêRE POLIMERE EN AëROBE BIOMASSA IN DIE VERWYDERING VAN FOSFOR UIT AFVALWATER

deur

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OPSOMMING

Navorsing het die verband tussen biomassa en fosfor verwydering in geaktiveerde slyk aangetoon. Mengvloeistof gesuspendeerde soliede materiaal (MGSM) en mengvloeistof vlugtige gesuspendeerde soliede materiaal (MVGSM) word dikwels gebruik as indikators van biomassa en gebruik in die wiskundige modellering van biologiese fosfor verwydering. Nie alle fosfor verwyder in geaktiveerde slyk sisteme kan toegeskryf word aan poli-fosfaat akkumulerende organismes (PAO) nie. Die doelwitte van hierdie studie was om die verwantskap tussen die MGSM en MVGSM fraksies en fosfor verwydering in geaktiveerde slyk te bepaal, om hierdie fraksies, tesame met die totale plaat telling (TPT) en adenosien trifosfaat (ATF) bakteriese tellings as indikators van lewensvatbare biomassa te vergelyk en die rol van ekstrasellulêre polisakkariede (EPS) in die verwydering van fosfor uit afvalwater te ondersoek. Die hipotese van die studie was dat dieselfde hoeveelheid MGSM, en spesifiek, MVGSM van verskillende geaktiveerde slyke dieselfde ortofosfaat opname behoort te toon as hierdie fraksies biomassa sou aandui. Om die hipotese te toets is twee verskillende eksperimente uitgevoer. In eksperiment 1 is steriele mengvloeistof groeimedium geïnokuleer met gelyke hoeveelhede (40 g) nat slyk pille verkry uit vyf 3-fase Bardenpho geaktiveerde slyk

aanlegte met ooreenkomstige slykouderdomme. In eksperiment 2 is dieselfde geaktiveerde slyk aanlegte as in eksperiment 1 gebruik en is die MVGSM en ortofosfaat konsentrasies in die aanlegte nageboots. Ortofosfaat verwydering was uurliks bepaal en het verskil tussen die sisteme. Die verskillende ortofosfaat verwyderings kapasiteite wat waargeneem is was toegereken aan verskille in die MGSM aktiewe biomassa fraksie van die verskillende slyke. Al het MGSM en MVGSM dieselfde patroon in ortofosfaat verwydering getoon, kon aanvanklike konsentrasies van hierdie fraksies nie direk gekoppel word aan verskille in die ortofosfaat opname vermoë van die verskillende slyke nie, wat die onaanvaarbaarheid van MGSM en MVGSM as indikators van lewensvatbare biomassa in geaktiveerde slyk aandui. Ortofosfaat verwydering was egter konsekwent hoër in die slyke met hoër ATF en TPT waardes, wat die verwantskap tussen die lewensvatbare biomassa fraksie van die MGSM en ortofosfaat verwydering aantoon.

'n Metode vir die kwalitatiewe en kwantitatiewe *in situ* karakterisering van PAO sel-trosse en naby-geassosieerde EPS word beskryf. X-straal mikroanalise was uitgevoer op monsters uit vier geaktiveerde slyk aanlegte. Analises was uitvevoer deur middel van skandeer elektron mikroskopie (SEM) gekombineer met energie verstrooïende spektrometrie (EVS). Gemiddeld, het sel-trosse met geassosieerde EPS tusen 57 en 59 % fosfor bevat, terwyl EPS alleen tussen 23 en 30 % fosfor bevat het. Resultate het voorgestel dat fosfor verwydering in geaktiveerde slyk nie net toegeskryf kan word aan PAO nie, maar ook aan EPS wat optree as a fosfor reservoir. Ekstraksie van EPS vanuit twee verskillende geaktiveerde slyk aanlegte het eenderse hoeveelhede EPS opgelewer. Vergeyking van EVS resultate voor en na EPS ekstraksie het moontlike intrasellulêre lekkasie gedurende homogenisasie aangedui, terwyl fosfor moontlik gekomplekseerd met gelokaliseerde yster en aluminium presipitate mag voorkom in afvalwater behandelings aanlegte wat chemiese beandeling gebruik om uitvloei-standaarde te handhaaf. Hierdie presipitate is moontlik verwyder deur filtrasie gedurende die ekstraksie prosedure wat gebruik is.

ACKNOWLEDGEMENTS

I would like to thank the following persons and organizations without whose help this thesis would probably not have been possible:

- Prof. T.E. Cloete, Head of Department, Department of Microbiology and Plant Pathology, for his invaluable leadership, support and patience as promoter during my years of study;

- The Water Research Commission whose financial support made this study possible, as well as the members of the steering committee for valuable input and positive criticism;

- My parents and brother Braam for their confidence in me, support (physical and moral) during my years of study;

- Mr. T. Nevondo, Mr. V. Molepo, Ms. B. Ntsudisane, Ms. R. van Zyl and Mr. M. van Heerden for technical assistance;

- Prof. J. Coetzee and Mr. A. Botha from the Unit for Microscopy and Microanalysis, University of Pretoria for EDS analyses and valuable insight;

- The managers and operators of the Daspoort, Centurion, Zeekoegat, Baviaanspoort and Rooiwal wastewater treatment plants for their trust and willingness to assist me in this study;

- Almighty God Who gave me the wisdom and insight and, above all, the opportunity to use my talents to His glory.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

<:	Smaller than
>:	Larger than
%:	Percent
µ:	Micron
µg:	Microgram
Acetoacetyl-CoA:	Acetoacetyl coenzyme A
Acetyl-CoA:	Acetyl coenzyme A
ADP:	Adenosine diphosphate
Al^{3+}:	Aluminium ion
AMP:	Adenosine monophosphate
AO:	Acridine Orange
API:	Analytical profile index
ATP:	Adenosine triphosphate
BEPR:	Biological excess phosphorus removal
Bio-P:	Biological phoshorus
BNR:	Biological nutrient removal
BOD5:	5 day biological oxygen demand test
BPR:	Biological phosphorus removal
°C:	Degrees Celcius
^{13}C:	Carbon-13 isotope
^{14}C:	Radio-labelled carbon-14 isotope
Ca^{2+}:	Calcium ion
$CaCO_3$:	Calcium carbonate
cal:	Calories
cap:	Capita
cells.ml^{-1}:	Cells per millilitre
cfu:	Colony forming units
CGY:	Casitone glycerol yeast autolysate

$^{14}CO_2$:	Radio-labelled carbon dioxide
CO_2:	Carbon dioxide
COD:	Chemical Oxygen Demand
CLSM:	Confocal laser scanning microscopy
d:	Day/s
DAPI:	4'6-diamidino-2-phenylindole
DNA:	Deoxyribonucleic acid
DO:	Dissolved oxygen
EBPR:	Enhanced biological phosphate removal
EDS:	Energy dispersive spectroscopy
EDTA:	Ethylenediaminetetraacetic acid
Eh:	Redox potential
EM:	Electron microscopy
EPS:	Extracellular polysaccharides/extracellular polymeric substances
ESEM:	Environmental scanning electron microscope
EUB:	Eubacterial probe
FDA:	Fluorescein diacetate
Fe^{3+}:	Ferric iron ion
FISH:	Fluorescent *in situ* hybridization
FITC:	Fluorescein isothiocyanate
FT-IR:	Infrared spectroscopy
G6PDH:	Glucose–6-phosphate dehydrogenase
g:	Gram
GAO:	Glycogen accumulating organisms
GC:	Gas chromatography
GC-MS:	Gas chromatography – mass spectroscopy
$g.g^{-1}$:	Gram per gram
^1H:	Hydrogen-1 isotope
H^+:	Hydrogen ion
H_2O:	Water

H_2S:	Hydrogen sulfide
H_2SO_4	Sulphuric acid
h:	Hour
HPLC:	High performance liquid chromatography
HPO_4^{2-}:	Hydrogen phosphate ion
$H_2PO_4^-$:	Dihydrogen phosphate ion
H_3PO_4:	Phosphoric acid
K^+:	Potassium ion
kg:	Kilogram
kcal:	kilocalories
kJ:	Kilojoule
KH_2PO_4:	Potassium dihydrogen phosphate
KNO_3:	Potassium nitrate
l:	Liter
M:	Molar
MCRT:	Mean cell residence time
Mg^{2+}:	Magnesium ion
$MgSO_4.7H_2O$:	Magnesium sulphate heptahydrate
mg:	Milligram
$mg.g^{-1}$:	Milligram per gram
$mg.l^{-1}$:	Milligram per litre
min:	Minutes
ml:	Millilitre
MLOSS:	Mixed liquor organic suspended solids
MLSS:	Mixed liquor suspended solids
MLVSS:	Mixed Liquor Volatile Suspended Solids
MPN:	Most Probable Number
MRNA:	Messenger RNA
N:	Nitrogen
N_2:	Nitrogen gas

N_2O:	Nitrous oxide
NA:	Nutrient Agar
$NaC_2H_3O_2$:	Sodium acetate
NaCl:	Sodium chloride
NAD:	Nicotinamide adenine dinucleotide
NaOH:	Sodium hydroxide
Na_2SO_4:	Sodium sulphate
NaS_2O_3:	Sodium thiosulphate
ND:	Not determined
NH_3:	Ammonia
NH_4^+:	Ammonium ion
NH_3-N:	Ammonia nitrogen
nm:	Nanometer
NMR:	Nuclear magnetic resonance spectroscopy
NO_2^-:	Nitrite ion
NO_3^-:	Nitrate ion
NO_3-N:	Nitrate nitrogen
NO_x:	Nitrogen oxides
O_2:	Oxygen
OH^-:	Hydroxide ion
OUR:	Oxygen utilization rate
P:	Phosphorus
PAO:	Polyphosphate accumulating organisms
PCR:	Polymerase chain reaction
pH:	Hydrogen ion concentration
PHA:	Poly-ß-hydroxyalkanoates
PHB:	Poly-ß-hydroxybutyrate
PO_4^{3-}:	Orthophosphate ion
Poly-P:	Polyphosphate
R:	Reactive group/substituent

RBCOD:	Readily biodegradable chemical oxygen demand
rpm:	Revolutions per minute
RNA:	Ribonucleic acid
rRNA:	Ribosomal ribonucleic acid
S^0:	Elemental sulphur
S_2^-:	Sulphide ion
SCFA:	Short chain fatty acids
SDS-PAGE:	Sodium dodecyl sulphate polyacrylamide electrophoresis
SEM:	Scanning electron microscopy
SO_4^{2-}:	Sulphate ion
SRB:	Sulphate reducing bacteria
SS:	Suspended solids
TCA:	Tricarboxylic acid
TEM:	Transmission electron microscopy
TKN:	Total Kjelldahl nitrogen
tRNA:	Transfer RNA
TOC:	Total organic carbon
TP:	Total phosphorus
TPC:	Total plate counts
T_x:	Time x
UCT:	University of Cape Town
VFA:	Volatile fatty acids
VSS:	Volatile suspended solids
WTP:	Wastewater treatment plant/s
Yr:	Year

Chapter 1

INTRODUCTION

Eutrophication of South Africa's natural waters is greatly accelerated by human activities, resulting in the discharge of the nutrients nitrogen (N) and phosphorus (P). Nutrients are introduced into the water from point sources, e.g. wastewater treatment works, and diffuse sources, e.g. from fertilizers or the excreta of animals and birds (Lilley *et al.*, 1997). Phosphate is recognised as one of the major nutrients contributing to the eutrophication of aquatic environments (Momba and Cloete, 1996a,b). Gross eutrophication is marked by large visible blooms of algae which make water treatment difficult (Atlas and Bartha, 1993; Lilley *et al.*, 1997). Degradation in the water quality of lakes is noticed by an offensive odour, appearance, taste, as well as depletion of oxygen from the lower water, resulting in extensive fish kills.

Many algae have the ability to fix nitrogen in water and therefore phosphorus is the element that should be restricted in order to minimize eutrophication (Lilley *et al.*, 1997). Sources of phosphorus include fertilizers, synthetic detergent and excreta. In the United States of America the concentration of phosphate in raw municipal wastewaters has been significantly reduced by the implementation of detergent "phosphate bans" (Martin and Wilson, 1994).

Phosphate is a major component of nucleic acids and therefore no organism can reproduce without phosphate. Phosphate is however a limiting nutrient, and therefore the removal of phosphate from effluents should limit one of the basic causes of eutrophication or algal blooms (Momba, 1995).

Both chemical and biological nutrient removal may be used in limiting eutrophication of water (Lilley *et al.*, 1997). Phosphate removal by chemical precipitation always remains an option,

although its high cost relative to the biological route reduces its appeal (Lötter and Murphy, 1988).

Activated sludge processes are currently the most widely used biological wastewater treatment process in the developed world treating both domestic and industrial wastewaters (Gray, 1989). Activated sludge systems, modified for enhanced P removal during effluent treatment are now operative in at least nine countries spanning the globe: Australia, Brazil, Canada, France, New Zealand, South Africa, United States, Zimbabwe and Namibia (Toerien *et al.*, 1990).

The activated sludge process relies on a dense microbial population being mixed in suspension with the wastewater under aerobic conditions (Gray, 1989). Kuba *et al.* (1997) indicated that biological P removal is achieved by recirculation of activated sludge through anaerobic and aerobic or anoxic conditions. There is consensus that enhanced P removal is a result of microbial action (Toerien *et al.*, 1990) and the ability of certain organisms (for e.g. *Acinetobacter* spp.) to accumulate large quantities of polyphosphates has been well elucidated by Fuhs and Chen (1975). However it has been illustrated that no single species of bacteria is capable of removing all the phosphate from wastewater.

Biological phosphate removal plants in South Africa have not always given reliable and satisfactory performance (Osborn *et al.*, 1986). In 1983, an extensive review of 11 of the South African Bardenpho plants was conducted and it was found that only one was achieving consistently good phosphorus removal, while a further 3 plants achieved good removal only after improving operational and control procedures (Oldham *et al.*, 1994). The reasons are not well elucidated, but a significant feature is dilution of substrate in sewage influents by continuous wet weather and storm periods. Other microorganisms can compete with polyphosphate accumulating organisms (PAO) in anaerobic-aerobic activated sludge systems, leading to system failure.

Sludge, in which PAO dominate, consumes carbohydrates while taking up short-chain fatty acids in the anaerobic phase and accumulates poly-ß-hydroxy-alkanoates (PHA). Significant

release of phosphate was not observed because polyphosphate was not utilized. Energy was rather obtained from the utilization of glycogen (Erasmus, 1997).

One of the factors contributing to poor performance of biological treatment plants (especially for P removal) is the so-called nitrate feedback whereby the anaerobic conditions required for phosphate removal are lost (Lilley *et al.*, 1997). In South African activated sludge systems it has been indicated that high concentrations of sulphide also has a detrimental effect on enhanced biological P removal. Apart from being toxic, excessive sulphide concentrations may reduce the concentrations of essential trace elements to sub-critical concentrations, thus interfering directly with biochemical reactions occurring during P release (Toerien *et al.*, 1990).

It has been indicated that no population differences have been observed between P removing and non-P removing activated sludge systems and neither does the community differ from one zone to another (Ehlers, 1997). Bosch (1992), Momba (1995) and Muyima (1995) indicated that biomass was related to phosphorus removal. Preliminary research has indicated that biomass quantity is more important than the microbial community of the biomass (Bosch, 1992). The higher the biomass the better the P-removal. This suggested that the main difference between P removing and non-P removing systems was biomass related and not due to the microbial community structure.

The secret to designing and running an activated sludge plant for successful P removal, therefore, lies in creating conditions in the plant which favour propagation and growth of biomass capable of removing P (Lilley *et al.*, 1997).

To this end simple, fast and effective measures of determining, not only total biomass, but also viable biomass should be developed, which could assist in the operation and management of wastewater treatment plants.

Although research effort has been directed towards improving the understanding of biological excess phosphorus removal (BEPR), designs of activated sludge systems to accomplish BEPR

are still based on experience and semi-empirical methods. Current research in wastewater treatment has been directed towards mathematical modelling of basic design and operational procedures. Quantification, as well as kinetic models of biomass in activated sludge are routinely used in design of wastewater treatment plants, in spite of the limitations which are currently encountered in biomass determination. One important parameter in such models has been the amount of viable biomass, therefore attempts have been made to find simple, fast and reliable methods of determining biomass in wastewater and activated sludge. The simplest and most often used method is to measure suspended solids (SS) or volatile suspended solids (VSS). These methods, however, do not distinguish between living cells and debris of either organic or inorganic origin. One key component of the mixed liquor suspended solids (MLSS) is the heterotrophic active biomass, mediating the biodegradation processes of chemical oxygen demand (COD) removal and denitrification. However, this parameter has only been hypothetical within the structure of models, and has not been measured directly, primarily due to the lack of simple, suitable measurement techniques (Ubisi *et al.*, 1997). In literature, principally microbiological techniques have been proposed for biomass estimation (Ubisi *et al.*, 1997), including pour plate or other culturing techniques, DNA analysis, the use of fluorescent probes for rRNA and sequencing of rDNA. These techniques, however, have not yet been adequately integrated with design and kinetic modelling, while culturing techniques have been widely criticized for their unreliability (Cloete and Steyn, 1988). With the use of traditional plate count techniques, an underestimation of biomass is done due to the selectivity of the media employed (Jørgensen *et al.*, 1992), as well as the concept of flocs acting as colony forming units (cfu), which may consist of thousands of cells. Although good progress has been made with the RNA and two DNA approaches, these methods are still in their infancy. All the methods, with exception of culturing techniques, also require sophisticated equipment and experimental techniques not widely available (Ubisi *et al.*, 1997).

Microbial activity has also been used as a parameter to determine the microbial potential. Methods include respirometry, ATP content and different enzyme assays. However, there have been very few attempts to relate activity to biomass. ATP is a fundamental component of living matter, involved in metabolic activities and disappearing immediately after cell death (Atlas, 1982). Specific proportions of ATP in relation to total cellular carbon are constant

with variations not exceeding 17 % (Atlas, 1982). ATP has been found to reflect viable biomass (Patterson *et al.*, 1970; Kucknerowicz and Verstraete, 1979; Jenkinson and Ladd, 1981; Jørgensen *et al.*, 1992).

Extracellular polymeric substances (EPS), also referred to as extracellular polysaccharides are macromolecules formed by polymerization of similar or identical building blocks. Building blocks include monosaccharides, uronic acids and amino sugars (containing various substituents), proteins (for example glycoproteins and lipoproteins), nucleic acids, phospholipids and humic substances (Wingender *et al.*, 1999). Among activated sludge EPS, proteins dominate, which, on the basis of their relatively high content of negatively charged amino acids, are supposed to be more involved than sugars in electrostatic bonds with multivalent cations, underlining their key role in floc structure (Wingender *et al.*, 1999). Functions attributed to EPS include sorption of exogenous organic compounds and inorganic ions. Adsorptive properties of exopolymers have been well documented especially in terms of biosorption of pollutants and toxics (Beech and Cheung, 1995; Loaëc *et al.*, 1997).

Although the macro-environmental conditions of activated sludge have been well described, very little is known about the micro-environment in activated sludge flocs, including diffusion gradients and the role of EPS in EBPR. However, the role of EPS in biological phosphorus removal has not been well studied. In previous studies, Buchan (1980) used EDS to determine the location of phosphorus volutin granules in activated sludge, without attention to the phosphorus content of EPS. As far as the author could establish, no attempts have been made as yet to elucidate the phosphorus content of activated sludge EPS by means of *in situ* methods. Results could be included in modelling of activated sludge systems to streamline the process and to attain constant and reproducible effluents conforming to standards set by Government.

EDS entails the bombardment of a sample with electrons, producing, along with various secondary signals, X-rays. The release of X-rays is produced by ionization of atoms in the sample. This ionization creates a vacancy in one of the energy levels of the atoms, which is almost immediately filled by an electron from a higher energy level. As these electrons

transfer to lower energy levels, the excess energy is emitted as X-rays (Buchan, 1983). Each element in the periodic table contains electrons in orbits with particular discrete energy levels, and it is on the basis of the differences between these specific energies that each element can be identified (Buchan, 1983). The spectrometer plots energies of different X-rays against counts of each specific energy, giving both qualitative and quantitative information about the elemental composition of a sample. EDS was therefore chosen as a technique to study the role that EPS plays in phosphate removal from wastewater in the activated sludge process.

The objectives of the study were therefore as follows:

* to investigate ATP, TPC, MLSS and MLVSS of activated sludge, as measures of viable biomass, and its relation to phosphorus removal;
* to investigate the *in situ* phosphorus content of EPS by means of EDS of air-dried sludge and bulk EDS analysis of extracted EPS.

REFERENCES

Atlas R.M. (1982). Enumeration and estimation of microbial biomass. In: *Experimental Microbial Ecology*. Blackwell Scientific, Oxford. Pp. 84-101.

Atlas R.M. and Bartha R. (1993). Microbial Ecology-Fundamentals and Applications. Third Edition. The Benjamin/Cummings Publishing Company, Inc., Redwood City, California, U.S.A.

Beech I.B. and Cheung C.W.S. (1995) Interactions of exopolymers produced by sulphate-reducing bacteria with metal ions. *International Biodeterioration & Biodegradation* **35**: 59.

Bosch M. (1992). P uptake kinetics of *Acinetobacter* in activated mixed liquor. M.Sc. Thesis, University of Pretoria, Pretoria, South Africa.

Kuba T., Van Loosdrecht M.C.M., Murnleitner E. and Heijnen J.J. (1997). Kinetics and stoichiometry in the biological P removal process with short cycle times. *Water Research* **31**(4): 918-928.

Kucknerowicz F. and Verstraete W. (1979). Direct measurement of microbial ATP in activated sludge samples. *Journal of Chemical Technology and Biotechnology* **29** :707-712.

Lilley I.D., Pybus P.J. and Power S.P.B. (1997). Operating manual for biological nutrient removal wastewater treatment works. Water Research Commission of South Africa. WRC Report No. TT 83/97.

Loaëc M., Olier, R. and Guezennec, J. (1997) Uptake of lead, cadmium and zinc by a novel bacterial exopolysaccharide. *Water Research* **31**: 1171-1179.

Lötter L.H. and Murphy M. (1988). Microscopic evaluation of carbon and P accumulation in nutrient removal activated sludge plants. *Water Science and Technology* **20**: 37-49.

Martin R.F. and Wilson T.E. (1994). Design and operation of nutrient removal facilities in the U.S. In: *Nutrient Removal From Wastewaters*. N.G. Horan, P. Lowe and E. Stentiford (eds.). Technomic Publishing Company, Inc., Lancaster, Pennsylvania, U.S.A. Pp. 173-186.

Momba M.N.B. (1995). Phosphate removal in activated sludge and its relationship to biomass. M.Sc. Thesis, University of Pretoria, Pretoria, South Africa.

Momba M.N.B. and Cloete T.E. (1996). Biomass relationship to growth and phosphate uptake of *Pseudomonas fluorescens*, *Escherichia coli* and *Acinetobacter radioresistans* in mixed liquor medium. *Journal of Industrial Microbiology* **16**: 364-369.

Muyima N.Y.O. (1995). Phosphate uptake by immobilized activated sludge in activated sludge mixed liquor. Ph.D. Thesis, University of Pretoria, Pretoria, South Africa.

Osborn D.W., Lötter L.H., Pitman A.R. and Nicholls H.A. (1986). Enhancement of Biological Phosphate Removal by Altering Process Feed Composition. Report to the Water Research Commission of South Africa by the City Health and City Engineers Departments of Johannesburg City Council. WRC Report no. 137/1/86.

Oldham W., Abraham K., Dawson R.N. and McGeachie G. (1994). Primary sludge fermentation design and optimization for biological nutrient removal plants. In: *Nutrient Removal from Wastewaters*. N.G. Horan, P. Lowe and E. Stintford (eds.). Technomic Publishing Company, Inc., Lancaster, Pennsylvania, U.S.A. Pp. 187-198.

Patterson J.W., Brezonik P.L. and Putnam H.U. (1970). Measurement and significance of adenosine triphosphate in activated sludge. *Environmental Science & Technology* **4**: 569-575.

Toerien D.F., Gerber A., Lötter L.H. and Cloete T.E. (1990). Enhanced biological P removal in activated sludge systems. *Advances in Microbial Ecology* **1**: 173-230.

Ubisi M.F., Jood T.W., Wentzel M.C. and Ekama G.A. (1997). Activated sludge mixed liquor heterotrophic active biomass. *Water SA* **23**(3): 239-248.

Wingender J., Neu T.R and Flemming H-C. (1999). What are bacterial extracellular polymeric substances? In: *Microbial Extracellular Polymeric Substances – Characterization, Structure and Function*. Wingender J., Ney T.R and Flemming H-C. (eds.). Springer-Verlag, Berlin, Germany. Pp. 1-19.

Chapter 2

LITERATURE REVIEW

2.1 Introduction

General removal of phosphorus (P) from wastewater was introduced in Scandinavia in the late 1960's (Atkinson, 1999). At the time it was believed that P alone was limiting to algal growth and that only P removal would solve the problem of eutrophication. It is now known that both nitrogen (N) and P contribute to this phenomenon, although P is still regarded as the limiting growth factor of algae (Lilley *et al.*, 1997). Much research regarding both the chemical and biological removal of these nutrients from wastewaters have been conducted (Atkinson, 1999). Enhanced biological P removal (EBPR), which can be described as biological accumulation of soluble P as polyphosphate (poly-P) from the bulk liquid in excess of normal metabolic requirements, tends to be sensitive to many external parameters and, thus, is subject to fluctuations (Atkinson, 1999). This creates difficulties for wastewater treatment plants (WTP) to achieve and maintain full compliance with discharge regulations (Atkinson, 1999). A more comprehensive understanding of the microbial communities within mixed liquors of wastewater treatment systems is therefore required, which will ultimately assist in the improvement of system design and performance (Atkinson, 1999).

Chemical and civil engineers only consider the biological or chemical processes taking place in reactors when designing biological wastewater treatment systems, with little or no regard for individual microbial species or the entire microbial community involved (Atkinson, 1999). Process design seems to be tackled empirically, with biological reactions or processes occurring within a system (such as wastewater treatment) all lumped together and attributed to a single organism, of which the response accounts for the total system response (Atkinson, 1999). If one were to consider every microbial species present in a highly organized

community such as activated sludge, process models, designed to make quantitative and qualitative predictions as to the effluent quality from a particular design, would become increasingly complex and superfluous (Atkinson, 1999). It is evident from accomplishments to date that engineers have succeeded, to a certain degree, in modelling wastewater treatment systems, especially if one considers the tremendous successes achieved with biological P (bio-P) removal and nitrification denitrification processes at full-scale (Atkinson, 1999). However, there are limitations to this empirical approach and EBPR processes occasionally deteriorate in P removal efficiency (Atkinson, 1999), or even routinely fails (Bond *et al.*, 1995). In order to further optimize biological processes (for example oxidation of organics, bio-P removal, nitrification and denitrification), biological community analyses will have to play a more significant role in design (Atkinson, 1999). The better the microbial community structure and function of a system is known, the better the control and management of the system will be (Atkinson, 1999). Also the precise role and optimum concentration of biomass in systems should be determined to attain effluent standards and in broader terms make systems more efficient. The precise role of extracellular polysaccharides (EPS), beyond aggregation properties for cells in activated sludge has also not yet been determined and/or included in modelling. Elucidation of these factors will lead to an better-understood and optimized EBPR process.

2.2 Water

Water is the essence of life on earth. However, despite being the precious commodity it is, this resource is grossly ignored, only being considered under severe droughts and/or other crises. Water resources are simply too precious to be exploited. Umgeni Water (1996) states that 97.5 % of the earth's water is present as salt water, followed by frozen water and fresh water with 1.7 % and 0.8 %, respectively. Of the 0.8 % fresh water, two-thirds are lost due to evapo-transpiration, leaving approximately 0.3 % remaining fresh water. Some 20 % of this amount of potentially potable water is located in areas too remote for human access, and three-quarters of the remaining 80 % is not effectively captured for use as it arrives in the form

of monsoons and floods. Therefore, we only get to use less than 0.05 % of the total amount of water on the planet (Serageldin, 1998).

Available fresh water for human consumption is rapidly becoming scarcer due to massive amounts of pollutants being discharged directly into waterways. Subsequently, these pollutants contaminate ground water supplies and aquifers, diminishing reserves even further (Atkinson, 1999). The number of countries in the world today confronted by a water deficit total 26, equivalent to a total population of 300 million. However, this figure will increase drastically to approximately two-thirds of the world's population by the year 2050 if pro-active steps to curb the trend are not implemented (Abu-Zeid, 1998). It has been estimated that South Africa alone already utilizes approximately 60 % of its useable water supplies, a demand which will increase as the country strives for first-world status (Asmal, 1998). It is evident that water resource demands have escalated dramatically with population explosions, urbanization and the subsequent growth of mega-cities. Thus, the concept of sustainability of our natural resources has now become a matter of urgency and should no longer be viewed as an ideological philosophy (Atkinson, 1999).

N and P are the two key nutrients determining the productivity (trophy) of lakes and other water impoundments. Greater human activities in catchment areas (whether by urban development or agriculture) will lead to greater annual loads of N and P entering an impoundment. P is usually the limiting nutrient, since the planet's reservoir of P is generally much lower than that of N. P compounds also tend to be less soluble than N compounds (Atkinson, 1999).

2.3 Eutrophication

Eutrophication is a natural ageing process which usually occurs in lakes and other quiescent bodies of water through introduction of plant nutrients (P and N) to the impoundment (Atkinson, 1999). The process occurs naturally over hundreds of years, but the process has

been greatly accelerated by human activities in sensitive areas (Atkinson, 1999), including the use of detergents and fertilizers. Eutrophication of natural and man-made water impoundments is a problem encountered in many countries, including South Africa (Atkinson, 1999). However, problems experienced in South Africa which promulgate eutrophication are long storage times of dams and reservoirs, high summer temperatures and long daylight hours (Bolitho, 1976).

A water body can be classified according to its trophic status, describing the rate at which organic material is supplied to or by the relevant impoundment per unit of time (Wetzel, 1983). Thus, the term refers to the "productivity" of a water body, and therefore the amount of new organic biomass formed per unit time within the aquatic ecosystem (Atkinson, 1999). The trophic status of water is directly influenced by the concentration of P and N in solution (Atkinson, 1999).

In contrast to other nutritional components in the hydrosphere, P is the least abundant and usually limits biological productivity (Atkinson, 1999). The most significant form of P in lake systems is inorganic P (orthophosphate or PO_4^{3-}), of which the uptake satisfies metabolic requirements for living organisms (Muyima *et al*, 1997). Organic P is then synthesized intracellularly. The sum of inorganic and organic forms of P in a body of water (in particulate or solubilized form) is known as total P (TP). Release of phosphate from aquatic sediments is primarily accomplished by bacterial metabolism of organic matter at the bottom of the lake (Atkinson, 1999). This metabolism usually creates conditions conducive to the resolubilization of P by a decrease in ambient pH through the production of acids (Wetzel, 1983). Low trophic states in an impoundment can exist despite high P loadings, provided that water residence times are low (Thornton, 1980).

A classic example of eutrophication in South Africa is the deterioration of the Hartbeespoort Dam. Constructed 66 years ago, the dam became so overgrown with water hyacinth (60% of surface area) during the mid 1970's, that the Department of Water Affairs was compelled to spend R200 000 on various rehabilitation programmes (Rudd, 1979). Constant clearing of water weeds from the canal system amounted to R40 000 per annum. Hypereutrophic

conditions intensified during the 1980's, resulting in algal hyperscums (crusts of algal biomass) which were concentrated up to a metre thick at the dam wall (Haarhoff *et al.*, 1992). The dam now enjoys oligotrophic status, mainly by greater understanding of the prevailing conditions and implementation of intensive remedial programmes (Atkinson, 1999). Speculators, however, agree that the improvement might only be temporary (Atkinson, 1999).

Enrichment of water in an impoundment results in undesirable effects, the primary being the establishment of profuse algal blooms and excessive growth of nuisance causing aquatic plants (Atkinson, 1999). These include several species of blue-green (*Cyanobacteria*) and green (*Chlorophyta*) algae, the diatoms and flagellates, water grasses, rooted broad leaf plants as well as floating water plants (hyacinth)(Rudd, 1979). Joska and Bolton (1994) found *Cladophora glomerata* to be a major problem algal weed in South Africa. The algal population which blooms as a result of the lake or pond becoming rich in nutrients, eventually crashes as a result of exhaustion of micro-nutrients or accumulation of toxic by-products. The dead algal biomass is decomposed by heterotrophic microbes, which exhaust the dissolved oxygen in water, resulting in extensive fish kills and septic conditions (Atlas and Bartha, 1993). Secondary effects (a direct result of weed and algal growth) include (Rudd, 1979):

* rapid oxygen uptake from the water causing lower layers of water to become anaerobic;
* stratification with respect to oxygen and temperature of the water occurs due to the exclusion of sunlight and heat from the lower waters;
* production of methane and sulphides from anaerobic digestion of dead plants which sink to the bottom of the water body;
* these anaerobic acid conditions which occur cause the release of bound phosphates, iron and manganese from sediments;
* production of sulphides results in fish kills (especially in winter) and a negative aesthetic appeal of the dam due to overturning of the water and the appearance that the water is black;
* sulphides also cause a strong rotten egg smell to the water; the water can no longer be used for potable consumption purposes for livestock or humans as certain species of the algae are toxic.

Costs of purification of such "over-fertilized" waters escalate dramatically and the chemical quality of these waters become altered, resulting in the need for alternative treatment methods and higher chlorine demands (Atkinson, 1999). Many limnological studies have been conducted concerning eutrophication, concerned primarily with its causes and effects (Walmsley and Thornton, 1984; Chutter, 1990; Dillon and Molot, 1996). And results have conclusively indicated that eutrophication is promoted if P and N is released into a reservoir or a catchment area. These studies have also shown that eutrophication can be effectively controlled and curtailed if the P load to receiving waters is controlled. Gross eutrophication becomes marked when the inorganic soluble N and P concentrations in waters are in excess of 0.3 $mg.l^{-1}$ and 0.015 $mg.l^{-1}$, respectively (Lilley *et al.*, 1997). It is virtually impossible to control eutrophication by limiting N, due to the ability of algae to fix and assimilate atmospheric N, which is the made available to other aquatic life forms when these cells die and the assimilated N is released (Atkinson, 1999). The increased awareness that P is the limiting nutrient has led to the introduction of more stringent legislation governing the discharge of P to receiving water bodies (Atkinson, 1999).

It is not necessary to enforce nutrient limitation when effluents are to be used directly for irrigation purposes, industrial use or to be discharged directly to sea (Atkinson, 1999). Only if the effluent is to be discharged to a lake or impoundment where eutrophication and its effects are undesirable, for example sensitive catchment areas, is nutrient removal strictly enforced (Atkinson, 1999). In 1980, the Department of Water Affairs introduced legislation limiting the dissolved orthophosphate content of point source discharges to seven sensitive catchment areas to 1 $mg.l^{-1}$ (Government Gazette, 1984) and this became known as the Special Phosphate Standard. A five-year grace period was, however, permitted before legislation enforcement in order to encourage P removal technology development and implementation. Excessive chemical precipitation was not encouraged due to its mineralising effect on water. The mechanism of biological P removal was studied intensely due to its attractive features and, especially low operating costs. During this period many existing WTP in South Africa were modified or new plants constructed to incorporate EBPR (Atkinson, 1999). Although initial capital outlay is high, the cost of EBPR operations in terms of operation and maintenance is significantly lower than chemical dosing plants, purely because chemical precipitants cannot be

recovered and are expensive (Atkinson, 1999). However, although the biological P removal mechanism is understood to a certain extent, it is still difficult to achieve full compliance with the Special Phosphate Standard and the biological process often has to be enhanced by simultaneous chemical dosing (Atkinson, 1999).

Problems caused by eutrophication are extensive in South Africa, being a country with limited water resources, and cannot be ignored due to the eminent threat on her limited water resources. To this end legislation have been made (Water Act, 1956). Continual failure of some activated sludge systems to remove limiting nutrients in their effluents continues to threaten our limited water resources. It is therefore empirical that effluents from wastewater treatment plants comply with the stipulated regulations of the Water Act of 1956. On the one hand it is also necessary that extensive research be carried out as to comprehend the phosphate removal mechanisms in order to assist in process design of wastewater plants which will optimally remove phosphate and other eutrophication-causing nutrients. The prevention of eutrophication can be achieved by removing phosphate from wastewater by chemical as well as by biological or by combination both methods (Momba and Cloete, 1996a,b). The biological methods due to their low maintenance expenses and by virtue of their environmental friendliness have become most popular of recent.

2.4 Algae

Algae are an essential component of the aquatic environment and play a significant role in eutrophication. Algae are defined as an assemblage of eukaryotic organisms that contain chlorophyll and carry out oxygenic photosynthesis. Most algae are of microscopic size and hence are clearly microbes. There are, however, a number of forms, which are macroscopic, for example species of seaweed, which can grow over 100 m in length.

Algae are important autochthonous members of freshwater ecosystem. In large, deep lakes, phytoplankton contributes most of the organic carbon, which supports the growth of the heterotrophic organisms in freshwater ecosystems (Atlas and Bartha, 1993).

Algae are largely, if not exclusively photoautotrophic, their growth inevitably depending upon intercepting sufficient light energy to sustain photosynthetic carbon fixation in excess of immediate respiratory needs. Radiant energy of suitable wavelengths (photosynthetically-active radiation) is neither universally nor uniformly available in water but it is attenuated hyperbolically with depth, through absorbency by the water and scattering by particulate matter there is less available photosynthetically-active radiation with increasing depth. For any given autotroph in a given water mass, there is likely to be a critical depth (the light compensation point) below which nett accumulation of photosynthetate is impossible. It is therefore implicit that the long-term survival of the algae depends upon its ability to enter or remain in the upper illuminated part of the water mass, for at least parts of its life.

2.5 Wastewater

Wastewater consists of materials derived from domestic sewage or industrial processes, which for reasons of public health and for recreational, economic and aesthetic considerations cannot be disposed of merely by discarding them untreated into lakes or streams. Rather, the undesirable and toxic materials in water must either be removed or rendered harmless. Gray (1989) defines wastewater as consisting of domestic (sanitary) or industrial (trade) effluents. Domestic wastewater comes exclusively from residences, commercial buildings and institutions such as schools and hospitals, while industrial wastewater comes from manufacturing plants. Large towns and cities have a mixture of both domestic and industrial wastewater which is commonly referred to as municipal wastewater and normally include effluents from the service industries such as dairies, laundries and bakeries, as well as a variety of small factories (Gray, 1989). Sewage is a complex mixture of natural inorganic and organic materials with a small proportion of man-made substances.

The main source of pollution in sewage is human excreta with smaller contributions from food preparation, personal washing laundry and surface drainage. The chemical and physical nature of wastewater can be further complicated by the inclusion of industrial wastes which are composed of strong spent liquors from main industrial processes and comparatively weak wastewater from rinsing, washing and condensation (Gray, 1989).

Sewage composition is normally measured in terms of BOD5 (the five-day biochemical oxygen demand test), COD (chemical oxygen demand), suspended solids (SS), ammonia (NH_4^+) and the P content (Gray, 1989). Enrichment of waters with nutrients, notably nitrogen and P in conjunction with carbon dioxide results in the prolific growth of algae (algal blooms), a process previously defined as eutrophication (Lilley *et al.*, 1997).

2.5.1 Inorganic properties of sewage

Inorganic components of sewage are sodium, calcium, potassium, magnesium, chlorine, sulphur (as sulphates and other forms), phosphate, bicarbonates and ammonia. Traces of heavy metals are also found. Domestic wastewater contains a very wide range of inorganic salts and trace elements, including all those necessary for biological growth and activity. Among the major ions in wastewater are chloride, nitrogen and P (Gray, 1989).

N and P are both essential nutrients for plant growth. N is generally necessary for the synthesis of protein and biological growth. In fresh wastewater, N is primarily present as proteinaceous matter and urea. This organic N is rapidly decomposed by bacterial action in the case of proteins, or by hydrolysis in the case of urea to ammonia, the concentration of which, in wastewater, is indicative to some extend of its age (Gray, 1989).

Ammonia exists in aqueous solution as either the ammonium (NH_4^+) or as ammonia (NH_3) depending on the pH of the wastewater. At pH values of >7 the equilibrium of the reaction $NH_3 + H_2O \rightleftharpoons NH_4^+ + OH^-$ is displaced to the left so that ammonia predominates and at pH

values <7 equilibrium moves to the right and ammonium predominates. The oxidized forms of ammonia, nitrite and nitrate are normally absent from fresh sewage as they are products of the biological oxidation process within the treatment plant.

P is present in sewage in three distinct forms, i.e. orthophosphate, polyphosphate and organic phosphate. Organic P is a minor constituent of sewage and like the polyphosphates requires further decomposition to the more assimilable orthophosphate form, which is normally fairly slow. Sources of phosphate in sewerage are the alkyl-benzene-sulphonate detergents and surfactants (Gray, 1989).

2.5.2 Organic properties of sewage

Organic matter comprises of carbon, hydrogen, and oxygen with N frequently present. Carbohydrates comprise the largest group in sewage followed by non-volatile and volatile acids. Urea is a major component of urine but is hydrolized so rapidly to ammonia that it is only found in very fresh sewage. Fats are the major organic constituents in the suspended solids fraction, together with carbohydrates and proteins account for 60-80% of the organic carbon present (Gray, 1989).

2.6 Wastewater treatment

Effective removal of pollutants from wastewater prior to discharge is of prime importance and the production of high quality effluents can have an economic advantages by supplementing existing water resources (Slim, 1987).

Wastewater treatment is necessary before disposal of effluent into rivers, lakes or ponds so as to avoid excessive eutrophication. If the wastewater was recycled, additional costs would be incurred by water purification works using this water due to problems associated with tastes and odours, filter and screen clogging, slime accumulation in pipes and toxicity caused by

certain algae. In addition to these treatment problems, the appeal of the water for recreational purposes is also reduced (Lilley *et al.*, 1997).

Wastewater treatment is a process achieved by physical, chemical and biological nutrient removal referred to as Biological Nutrient Removal (BNR). This process is divided into three levels (Alas and Bartha, 1993):

* primary treatment
* secondary treatment
* tertiary treatment.

In wastewater treatment, the objective of BNR is to remove the primary nutrients which cause eutrophication namely carbon, N and P (Lilley *et al.*, 1997).

Some of the floc material is then returned to the aerator to serve as inoculum, while the rest is sent to the sludge digester. The residence time in an activated sludge system is generally 5-10 h. The main process occurring during this time is adsorption of soluble organic matter to the floc and incorporation of some of the soluble material into microbial cell material. The BOD of the liquid is considerably reduced by this process (75-90%) but the overall BOD liquid plus solids is only slightly reduced, because most of the absorbed organic matter still resides in the floc. The main process of BOD reduction thus occurs in the sludge digester, to which the floc is transferred (Atlas and Bartha, 1993).

2.6.1 Conventional activated sludge systems

Conventional activated sludge systems have been used to purify domestic wastewater for many decades (Toerien *et al.*, 1990). Conventional activated sludge systems consist primarily of an aeration basin and a settling basin. Microbes are retained in the system as an activated sludge. It should be noted that the conventional activated sludge process is not designed or operated to

achieve biological excess P removal (BEPR). The suspended bacteria of this system use P in quantities which satisfy only their basic metabolic requirements (Pitman, 1984).

Activated sludge systems have become an integral part of municipal wastewater treatment. The process relies upon the dense growth of microbes in a reactor where air is continuously supplied to allow for carbonaceous oxidation (Atkinson, 1999). The term "activated sludge" refers to an aerobic slurry of microbes which can be removed from the process by sedimentation and returned in quantifiable amounts to the wastewater stream (Grady and Lim, 1980). All activated sludge systems operate with the following characteristics in common (Grady and Lim, 1980):

* utilization of a flocculent slurry of microbes to remove organic matter from the surrounding wastewater;
* prior to effluent discharge from the plant, microbes are removed by sedimentation, thereby reducing outgoing solids loads;
* settled microbes are recycled to the biological reactor via a clarifier underflow;
* dependency of plant performance on the mean cell residence time (MCRT or sludge age) in the system.

In the presence of both oxygen and nutrients, high rates of microbial growth are achieved. Microbial metabolism of the organic matter present results in the production of oxidised end-products such as carbon dioxide, nitrates, sulphates and phosphates, as well as the biosynthesis of new microbial biomass (Gray, 1989; Horan, 1990; Bitton, 1994; Muyima *et al.*, 1997, Atkinson, 1999). In conventional activated sludge systems, the aspects of the process that can be varied independently to achieve different responses, are the process layout, the loading rate and the method of aeration (Atkinson, 1999).

The basic ecological unit of activated sludge is the floc. Microbial floc formation is essential to the success of activated sludge processes, as it allows for rapid and efficient separation of sludge from treated wastewater in the sedimentation tank. The exact mechanism of formation of flocs is not well understood, but seems to be almost entirely bacterially mediated (Muyima

et al., 1997). The model proposed by Forster and Dallas-Newton (1980), explaining the structure of the floc, has gained acceptance and forms the basis of our perception of floc arrangement. According to this model, filamentous microbes form the matrix or backbone of the structure to which zoogleal (floc-forming) microbes attach (Bitton, 1994). Attachment is thought to be brought about by exopolysaccharides present in the form of a capsule or discrete slime layer (Horan, 1990). These polymers are composed of sugars, amino sugars, uronic acids and amino acids, are produced during the endogenous growth phase and form the three-dimensional matrix (Bitton, 1994). As extracellular polysaccharide production gradualy continues, other microbes and colloidal material become entrapped in the matrix and the floc diameter increases. Surface charges on the microbial cells, as well as bridge formation by polyvalent cations, also contribute to flocculation (Gray, 1989). The very rigid structure of the floc has impeded the quantitative analysis of the activated sludge community structure, as complete dispersion of the floc is extremely difficult. Clumping of cells in the floc leads to an underestimation of the number of active cells present in the mixed liquor when using viable plate count techniques (Atkinson, 1999). Problems attributable to poor floc formation can be one of two causes: non-filamentous bulking and filamentous bulking. These characteristics are undesirable and will have negative economic consequences to the plant in question (Atkinson, 1999).

The activated sludge process has been developed primarily for carbon, N and P removal (Momba, 1995). The activated sludge process is capable of producing an effluent of high quality at reasonable cost (Saayman, 1995). Activated sludge reactors are relatively resistant to shock loads and can achieve acceptable effluent in spite of dynamic inputs (Grady and Lim, 1980). The activated sludge system is efficient and flexible and is able to withstand considerable variations in sewage flow rate and concentration (Atlas and Bartha, 1993). The potential of activated sludge in controlling eutrophication is well known so is its capability to prevent salinity as a result of discharging in the receiving water body. Increasing industrial and population demands on water supplies have ensured that the activated sludge process has had to undergo various modifications in order to cope with the increase in carbonaceous and nutrient loadings (Atkinson, 1999).

Single aerobic systems are no longer sufficient to treat domestic and industrial wastes and changes incorporated into the process relate to size, number and configuration of the reactors, flow direction and mixed liquor recycle and flow regime within the reactors (Wentzel *et al.*, 1992). P and N removal from wastewater is essential to ensure the sustainablity of potable water supplies. However, in conventional systems, phosphate is only taken up in quantities, which satisfy basic bacterial metabolic requirements. Thus, nutrient loads to receiving waters would subesquently remain excessive (Atkinson, 1999). Therefore, in order to encourage resident microflora in the system to accumulate P in excess of basic metabolic needs, modifications and extensions to conventional reactor configurations have been implemented.

Nitrification denitrification systems were developed in 1974 by Barnard, who recognized the importance of an anaerobic zone at the head of the biological reactor to encourage excess biological P uptake (Wentzel *et al.*, 1992). He realized that biological P removal was induced if the organism mass was stressed by subjecting it to conditions of anaerobiosis, thereby releasing P to the bulk liquid (Ekama *et al.*, 1984). This particular system was termed the Bardenpho system, and, since its inception, a number of further modifications have taken place to enhance biologically mediated nutrient removal (Atkinson, 1999).

2.6.2 Biological P removal

Biological treatment of wastewater can be accomplished in a number of ways, the determining factors depending upon efficiency and economics of wastewater contact with the microbes concerned (Atkinson, 1999). Some technologies depend upon immobilization techniques (such as biofilm systems), suspended growth systems (such as activated sludge) and lagoon systems (such as anaerobic and aerobic lagoons, maturation ponds and high-rate algal ponds)(Muyima *et al.*, 1997). However, in this section only the activated sludge process will be discussed.

Although P can be removed from wastewater by chemical precipitation (precipitants including ferrous and ferric ions, aluminium salts, lime and polyelectrolytes), biological P removal has

several advantages over chemical precipitation. These include low sludge production, the fertilizer value of the sludge, less expensive operation costs and the avoidance of anion enrichment of the treated water (Wentzel, 1992; Lilley *et al.*, 1997; Romanski *et al.*, 1997). Although biological P removal (BPR) can effectively compete with chemical removal in terms of cost, the characteristics of the influent wastewater will dictate the performance of the removal mechanism (Atkinson, 1999).

However, a distinction must be made between P removal by additional chemical dosing by the plant operator in addition to biological action and P removal by means of chemical changes of the wastewater as a result of biological action like changes in alkalinity, acidity and pH (Atkinson, 1999). It can be assumed that when treating municipal wastewater by an appropriately designed activated sludge process, excess P removal is principally mediated by the biological mechanisms of adsorption and absorption (Ekama *et al.*, 1984).

There exists within the activated sludge mixed liquor a microbial population capable of accumulating soluble P as poly-P granules (volutins). These microbes are termed P accumulating organisms (PAO)(Wentzel *et al.*, 1988) and are able to take up P in excess of normal metabolic requirements. The process is, therefore, termed biological excess P removal (BEPR). These organisms will, however, only proliferate in the system, and the mechanism of poly-P accumulation will only be induced if certain structural alterations are made to the aeration basin (for example an anaerobic zone at the head of the basin). Research has shown that the anaerobic zone leads to the enrichment of fermentative organisms such as *Enterobacter*, *Klebsiella*, *Citrobacter*, *Pasteurella*, *Proteus* and *Aeromonas* (Lötter and Murphy, 1985). The PAO are then able to accumulate the fermentation products (including acetic, lactic, succinic, propionic, butyric acids) and store them intracelularly as carbon and energy reserves, for example poly-ß-hydroxyalkonoates (PHA's), of which poly-ß-hydroxybutyrate (PHB) is an example (Satoh *et al.*, 1992; Lilley *et al.*, 1997). As a result of internal carbohydrate accumulation, the PAO release P back into solution and the orthophosphate concentration in the anaerobic zone increases. In the subsequent aerobic reactor, the PAO utilize the internally stored carbon for growth, which increases their biomass in the sludge. PHB is also used as an energy source in the aerobic reactor to take up P from

the bulk solution and to re-synthesize the poly-P degraded in the anaerobic reactor. P uptake, together with an increase in the quantity of PAO in the system, leads to a nett removal of P from the wastewater (Wentzel, 1992). Soluble P, accumulated as biological poly-P in the solid phase, is then removed from the system with the waste sludge.

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

Although the process of EBPR is well understood from a technical aspect, it remains difficult to achieve consistent and reproducible removal rates at the full-scale due to a lack of understanding of the process from a biochemical and microbiological point of view (Satoh *et al.*, 1996; Wang and Park, 1998). Without understanding the correlation between the PAO community structure within activated sludge mixed liquor and the wastewater plant performance, reliable and efficient biological P removal operations will remain difficult to design (Atkinson, 1999).

2.6.3 Modified activated sludge systems

Various modifications have been made on the activated sludge process in order to meet most wastewater treatment needs (Toerien *et al.*, 1990). The need for nutrient removal from wastewater has been stated and thus modifications of the activated sludge processes have been developed to achieve maximum potential in terms of nutrient removal. Barnard (1975) reviewed the available technology for biological nitrification and denitrification and proposed the Bardenpho process (Figure 2.1). The Bardenpho process consists of four stages i.e.: primary anoxic, primary aerated, secondary anoxic and secondary aerated followed by a

clarifier. Carbon removal and nitrification takes place in the main aeration basin. Nitrified mixed liquor is recycled from this basin to the primary anoxic basin where, in the absence of free dissolved oxygen, denitrification occurs, using organic compounds as the carbon source. Mixed liquor not recycled to the primary anoxic basin passes on to the secondary anoxic basin, where additional denitrification takes place at a slow rate under conditions of endogenous respiration. Before entering the secondary clarifier, the mixed liquor from the secondary anoxic basin passes through a small re-aeration basin, the function of which is to ensure that:

* NH_3-N formed during endogenous respiration in the secondary anoxic basin is converted to NO_3-N;
* aerobic conditions exists in the secondary clarifier, as any denitrification that occurs under anoxic conditions would produce nitrogen gas that could cause rising sludge;
* aerobic conditions exists in the secondary clarifier to prevent P release from the sludge into the effluent (Toerien *et al.*, 1990).

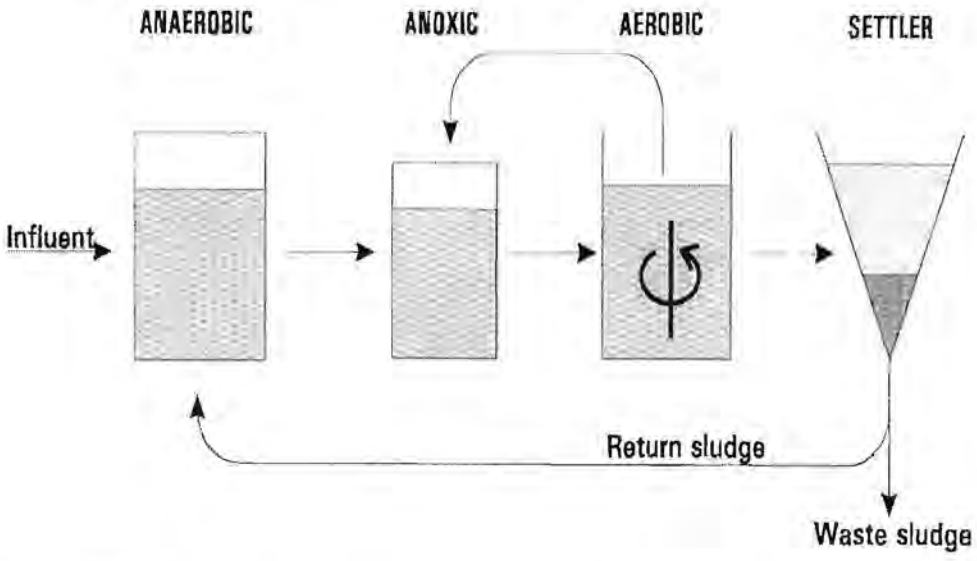

Figure 2.1: The three-stage Phoredox system (modified Bardenpho)(Erasmus, 1997).

2.6.3.1 Anaerobic zone

The term "anaerobic zone" (in the activated sludge process) means that the contents of the zone are kept, as far as possible, deficient of nitrate and dissolved oxygen and the input of nitrate and oxygen to this zone is severely restricted (Buchan, 1984; Lilley *et al.*, 1997). The anaerobic zone is characterized by an oxidation-reduction potential that excludes any oxidative processes. The most important reactions are anaerobic fermentation (especially acido- and aceto-genesis), poly-P depolymerization and desulphation. Readily degradable organic substrate from wastewater is converted to low-molecular organic compounds, which are then stored by PAO in the form of PHB (Wanner and Grau, 1988). The anaerobic zone is essential for phosphate removal, as the bacteria in the activated sludge passing through this zone are preconditioned to take up excess phosphate under aerobic conditions (Momba, 1995).

The presence of nitrate in an anaerobic zone has been reported as a handicap to the phosphate removing potential of the activated sludge systems and there is need to exclude nitrate in the anaerobic zone. In the anaerobic zone, sludge from the clarifier jointly flows with the influent wastewater. Influent nitrate levels has to be kept low in order to ensure that nitrates returned with the underflow of the final clarifier does not negatively affect the performance of the initial oxygen limiting zone (Momba, 1995).

2.6.3.2 The anoxic zone

The anoxic zone refers to the presence of nitrates and the absence of dissolved oxygen (Buchan, 1984; Pitman, 1984; Streichan *et al.*, 1990). Substrate is oxidised anoxically in the anaerobic zone (i.e. electrons from organic compounds are transferred to nitrate- and/or nitrite-nitrogen which is reduced to elementary N) (Wanner and Grau, 1988).

The anoxic zone is the main denitrification reactor in the activated sludge process. It is fed by the effluent from the anaerobic zone and the mixed liquor recycled from the aerobic zone.

This zone is fundamental to the biological removal of N and due to the absence of oxygen (O_2). Non-poly-P organisms utilize nitrate as electron acceptors, reducing it to N gas, thus carrying out denitrification of the mixed liquor and allowing the elemental N formed to escape as a gas (Lilley *et al.*, 1997).

2.6.3.3 Primary aerobic zone

The primary aerobic zone within the activated sludge plant, is the zone which is aerated by introducing either air or oxygen (Lilley *et al.*, 1997). Various mechanical devices are used for aerating this zone (Nogaj, 1980). The main function of this zone is to oxidize organic material in the sewage, oxidize ammonia to nitrite and to nitrate, and provide an environment in which the biomass can take up the phosphate released in the anaerobic zone, plus the phosphate which enters the process in the feed sewage. (Momba, 1995; Wanner and Grau, 1988). The aeration rate seems to be the principal operational determinant of the efficiency of phosphate removal (Pitman 1984).

Ammonia is oxidised in the aerobic zone. Chemoautotrophs are responsible for this oxidation. Ammonia is oxidized to nitrate by *Nitrosomonas*, *Nitrospira* and *Nitrosolobus* spp., whereas nitrite is oxidized to nitrate by *Nitrobacter*, *Nitrospira* and *Nitrococcus* spp. (Momba, 1995). The nutrifying organisms are sensitive to the pH and alkalinity of the wastewater. The growth rate of these organisms is severely inhibited outside the pH range of 7 to 8,5. During the conversion of ammonia to nitrate, hydrogen ions are released resulting in a decrease in the alkalinity of the wastewater. If the alkalinity of wastewater decreases to below 40 $mg.l^{-1}$ (as $CaCO_3$), the pH becomes unstable resulting in a sharp decrease in nitrification efficiency due to the retarded growth rate of the autotrophs (Lilley *et al.*, 1997).

2.6.3.4 The clarifier

The clarifier is used for producing a clear effluent free of suspended solids and a thickened sludge for recycling to the inlet of the process. The quality of the underflow sludge should be such that nitrate is not recycled to the anaerobic zone (Ekama *et al.*, 1984). The operation of a clarrifier can greatly affect its performance. However, sludge settling properties have an overriding effect with the clarifier operations having to be adapted to the sludge settling characteristics. The latter are largely influenced by the operating conditions in the biological reactor (Pitman, 1984).

2.6.4 Preconditioning of the poly-P organisms for EBPR

Under aerobic conditions the PAO are not able to compete with non-PAO for substrate (food sources) such as glucose or other saccharides. Under anaerobic conditions (no nitrate or oxygen present) and in the presence of short chain fatty acids (SCFA or VFA)(Osborn *et al.*, 1986) the poly-P-organisms break down and hydrolyze stored poly-P. This process releases orthophosphate to the surrounding liquid and leads to the phenomenon known as P release in the anaerobic zone. The bound energy released in the hydrolyzing poly-P is utilised by the poly-P organisms to absorb, process and store the SCFA within the organisms, thereby reserving substrate for their exclusive use when they enter an environment which contains external electron acceptors such as nitrate or oxygen. In this way they do not have to compete with the non-poly-P-organisms which are unable to utilize SCFA under the anaerobic conditions because of lack of a suitable electron acceptor (Alexander *et al.*, 1994, Lilley *et al.*, 1997; Osborn *et al.*, 1986).

Upon re-entering the aerobic environment the poly-P organisms utilise the reserved SCFA both for growth and to replenish their poly-P pool by abstracting orthophosphate from the surrounding medium. This gives rise to the phenomenon known as excess P uptake which occurs in aerobic environments (Alexander *et al.*, 1994; Lilley *et al.*, 1997).

Alexander *et al.* (1997) postulated that in order to promote the growth of poly-P organisms the following conditions are required:

* an anaerobic environment which receives or generates an adequate supply of SCFA, the mass of released P being proportional to the mass of SCFA utilised by the poly-P system; followed by
* an aerobic environment for P uptake by the poly-P organisms.

2.7 Biogeochemical cycles related to wastewater treatment

Pathways by which nutrients are circulated within ecosystems are referred to as biogeochemical cycles. For every element the cycling process involves (Mader, 1998):

* a reservoir where the source normally remains unavailable to the surrounding biota (for example rocks and sediments); assimilable forms of the element are only released by various geological components;
* an exchange pool, represented by an elemental source from which organismsare able to accumulate nutritional components (for example hydrosphere and atmosphere);
* a biotic community through which elements move along either simple or elaborate food chains.

N is involved in a gaseous cycle where the element is assimilated from and returned to the atmosphere. P, on the other hand, is sedimentary, where the element is absorbed from the soil by plant roots, exchanged to heterotrophs and returned to the soil by decomposers (Mader, 1998). Both P and N are interchangeable with reference to ecosystems they inhabit, as both elements are capable of moving between terrestrial and aquatic systems (Atkinson, 1999).

Microbes like all biological systems respond to their environment. Microbes have diverse metabolic capabilities and high enzymatic activity rates, and thus they play an important role in

biogeochemical cycling. Microbes are vital for the functioning and maintenance of the earth's ecosystems (Ehlers, 1995). Biogeochemical cycling describes the movement and conversion of materials by biochemical activities within the ecosphere. Biogeochemical cycles include physical transformation (e.g. dissolution, precipitation, volatilization and fixation), chemical transformation (e.g. biosynthesis, biodegradation, and oxido-reductive biotransformations) and various other combinations of physical and chemical changes (Atlas and Bartha, 1993). Microbes play a major role in biogeochemical cycling because of their ubiquity, diverse metabolic capabilities and high enzymatic activity rates (Jørgensen 1989; Pomeroy, 1984). Wastewater treatment by biological nutrient removal or activated sludge processes fulfills the biogeochemical cycles of the nutrients involved in the process. In wastewater treatment the objective of BNR is to remove the primary nutrients which cause eutrophication namely carbon, N and P from wastewaters (Lilley *et al.*, 1997).

2.7.1 The carbon cycle

Carbon is one of the important nutrients contributing to the growth of algae and other macrophytes in aquatic environments and thus is directly associated with eutrophication. Wastewater treatment processes have to minimise the discharge of carbon to water bodies in order to minimize eutrophication. Carbon in wastewater streams occurs as organic and inorganic compounds. Organic compounds can be utilised by heterotrophs, while inorganic compounds are utilised by autotrophs.

Boths forms of carbon are removed from wastewater through a series of redox reactions, oxidising the carbon source to carbon dioxide and water (Lilley *et al.*, 1997). It is empirical that an understanding of the carbon cycle be established in order to maximize knowledge on carbon removal in wastewater treatment.

In the carbon cycle, carbon moves from reservoirs in the atmosphere and oceans through living organisms back to these reservoirs. Carbon is introduced into the atmosphere by aerobic

respiration, fossil fuel burning and volcanic eruptions which release carbon from rocks deep in the earth's crust (Starr and Taggart, 1981). In the atmosphere carbon exists as bicarbonates or carbonate ions (Purves, *et al.*, 1995).

Carbon undergoes oxidation and reduction during the carbon cycle. Carbon is assimilated by autotrophs in gaseous form (CO_2). The involvement of microbes in the carbon cycling can be best discussed within the context of the food web. Autotrophic organisms carry out the nett fixation of carbon dioxide to form organic compounds, this includes both the photosynthetic and chemolitotrophic microbes. The most important groups of microbes, in terms of their ability to convert carbon dioxide to organic matter are the algae, the cyanobacteria, and the green and purple photosynthetic bacteria. Chemolithotrophic microbes contribute to a lesser extend (Atlas and Bartha, 1993). Photosynthetic microbes fix CO_2 via the pentose-phosphate or Calvin cycle.

Microbes are capable of incorporating CO_2 through the phospho-enol pyruvate carboxylase system. In the case of heterotrophic microbes, exchange, but no nett CO_2 fixation occurs, but some chemolitotrophic microbes use this system instead of, or in addition to the pentose cycle for nett CO_2 fixation (Atlas and Bartha, 1993.

Methanogenic archaebacteria play an important role in the anaerobic reduction of carbon dioxide. The resulting methane can be utilised only by a limited number of microbes (Gottschalk, 1979); these methylotrophs are important in carbon cycling, however, especially as it relates to atmospheric carbon transfers.

2.7.2 The nitrogen cycle

Matter required for maintaining and reproducing life is obtained from five major nutrient sources, carbon, hydrogen, oxygen, N and P are more often the limiting nutrients, although N can be readily obtained from the air. The availability of N and P (and sometimes carbon)

therefore often limits the growth of algae and plant life. This in turn limits the growth of the heterotrophs (Lilley *et al.*, 1997).

N is essential to life and is a component of proteins and nucleic acids in microbial, animal, and plant cells. In fresh wastewater, N is primarily present as proteinaceous matter and urea. This organic N is rapidly decomposed by bacterial action in the case of proteins, or hydrolysis in the case of urea to ammonia, the concentration of which in wastewater is indicative to some extend of its age (Gray, 1989). N is the most abundant gas in the atmosphere. Most organisms are, however, incapable of utilizing N gas unless it is first converted to ammonia. This is because N gas is a very stable molecule that can undergo changes only under extreme conditions (for example electrical discharge, high temperatures and pressures).

2.7.2.1 Microbiology of the nitrogen cycle

Atlas and Bartha, 1987 and Grady and Lim, 1980 agree that microbiologically the N cycle consists of several steps which include:

* N fixation
* N assimilation
* mineralization
* nitrification
* denitrification

2.7.2.1.1 Nitrogen fixation

N fixation is a process only a few species of bacteria and cyanobacteria are capable of carrying out. N fixation results with the production of ammonia. Organisms capable of N fixation are classified into two categories:

* nonsymbiotic N-fixing microbes. Included in this category are members of *Azotobacter* (for example *A. Agilis*, *A. chrooccum*, *A. vinelandii*), gram-negative bacteria that form cysts and fix N in soils and other environments. Other N-fixing microbes are *Klebsiella, Clostridium* (anaerobic, spore-forming bacteria active in sediments) and cyanobacteria (for example *Anabaena* and *Nostoc*). The latter fix N in natural waters and soils and their fixation rate is said to be ten times higher than free N-fixing microbes in soils (Bitton, 1994).

* Symbiotic N-fixing microbes. These organisms enter into symbiotic relationships with higher plants. A popular example is that of legumes and Rhizobia. *Rhizobium* infects the roots of legumes and fix N (Atlas and Bartha, 1993). N fixation in this symbiotic relationship is of great importance both in global N cycling and in agriculture.

2.7.2.1.2 Nitrogen assimilation

The term assimilation refers to the incorporation of nutrients into the biomass of an organism (Atlas and Bartha, 1993). Both heterotrophic and autotrophic microbes take up and assimilate NO_2^- and NO_3^- after reduction to NH_4^+. Bitton (1994) states that assimilation is the process responsible for some N removal in wastewater treatment plants. Cells convert NO_3^- or NH_4^+ to proteins and grow until N becomes limiting. The proportion of carbon to N (C:N) assimilation is 100:10.

2.7.2.1.3 Nitrogen mineralization (ammonification)

The mineralization of N is also known as ammonification. Bitton (1994) defines ammonification as the transformation of organic nitrogenous compounds to inorganic forms. Ammonification is a process driven by a variety of microbes (e.g. acinomycetes and fungi).

Proteins are mineralized to ammonium ions during ammonification in accordance with the following sequence:

proteins → amino acids → deamination to NH_4^+

For example in the transformation of urea to ammonia:

$(NH_2)_2CO + H_2O +$ urease enzyme$\rightarrow 2NH_3 + CO_2$

Ammonification usually involves deamination as one of the intermediate steps, prior to the formation of NH_3 or NH_4^+. Deamination of ammonia takes place either oxidatively or by reduction depending on the availability of oxygen and other important extracellular proteolytic enzymes.

Oxidative deamination:

$R\text{-}CH\text{-}NH_2\text{-}COOH + \frac{1}{2}O_2 \rightarrow R\text{-}CO\text{-}COOH + NH_3$

Reductive deamination:

$R\text{-}CH\text{-}NH_2\text{-}COOH + 2H^+ \rightarrow R\text{-}CH_2\text{-}COOH + NH_3$

2.7.2.1.4 Nitrification

Nitrification is defined as the process in which ammonia is oxidized to nitrite and nitrite to nitrate (Atlas and Bartha, 1993; Bitton 1994). Nitrification is the first step in N removal in wastewater treament processes (Lilley *et al.*, 1997). It is a process primarily carried out by aerobic, chemolithotrophic bacteria of the family *Nitrobacteraceae* (Atlas and Bartha,1993).

Organisms responsible for nitrification may be divided into two groups:

* organisms responsible for the conversion of NH_4^+ to NO_2^- which include *Nitrosomonas* species (for example *N. europaea* and *N. oligocarbogenes*). The conversion of ammonia to nitrite takes place via hydroxylamine. Included also amongst ammonium oxidizers are *Nitrosospira, Nitrosococcus and Nitrosolobus* (Bitton 1994).

$$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + 2H^+ + H_2O + 275\ kJ$$

* organisms responsible for conversion of nitrite to nitrate (NO_2^- to NO_3^-) are the *Nitrobacter* species (for example *N. agilis* and *N. winogradsky*) as well as *Nitrospora* and *Nitrococcus*.

$$NO_2^- + 1/2O_2 \rightarrow NO_3^- + 75\ kJ$$

The oxidation of NH_4^+ to NO_2^- is an exogonic exothermic process. The generated energy is utilised by microbes to assimilate CO_2. Carbon dioxide, bicarbonate and carbonate satisfy the carbon requirements of nitrifiers. Nitrification is favored by the presence of oxygen and sufficient alkalinity to neutralize the hydrogen ions produced during the oxidation process. Although autotrophic nitrifiers are predominant in nature, nitrification may also be carried out by heterotrophic bacteria (for example *Arthrobacter*) and fungi (for example *Aspergillus*). Nitrification occurs in the aeration tank (4-6 hr retention time), and sludge containing high numbers of nitrifiers is recycled to maintain nitrifier activity. Factors such as ammonia/nitrite concentration, oxygen concentration, pH, temperature, BOD_5:TKN ratio and the presence of toxic chemicals all control nitrification in wastewater treatment plants (Bitton, 1994).

2.7.2.1.5 Denitrification

Atlas and Bartha (1993) defined denitrification as the formation of gaseous N or gaseous N oxides from nitrate or nitrite by microbes. Denitrification is the second stage in N removal culminating in the production of N gas, which eventually escape from the anaerobic zone

reactor in the activated sludge process. The two most important mechanisms for denitrification are accomplished by assimilatory and dissimilatory nitrate reduction (Bitton, 1994).

Assimilatory nitrate reduction:

During assimilatory nitrate reduction, nitrate is taken up and converted to nitrite and then to ammonium by microbes. This process involves several enzymes that convert NO_3^- to NH_3, which is subsequently incorporated into proteins and nucleic acids.

Dissimilatory nitrate reduction:

Dissimilatory nitrate reduction is an anaerobic respiration by which NO_3^- serves as an electron acceptor. NO_3^- is reduced to nitrous oxide (N_2O) and N gas (N_2). N gas liberation is the predominant output of denitrification. N_2, however, has low water solubility and thus tends to escape as rising bubbles. The microbes involved in denitrification are aerobic autotrophic or heterotrophic microbes that can switch to anaerobic growth when nitrate is used as the electron acceptor. The denitrification process is carried out according to the following sequence mediated by the nitrate reducatase, nitrite reductase, nitric oxide reductase and nitrous oxide reductase enzymes, respectively:

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

Denitrifiers belong to several physiological (organotrophs, lithotrophs, and phototrophs) and taxonomic groups and they can use various energy sources (organic or inorganic chemicals or light). Microbes that are capable of denitrification belong to the following genera: *Pseudomonas, Bacillus, Spirillum, Hyphomicrobium, Agrobacterium, Acinetobacter, Propionobacterium, Rhizobium, Cytophaga, Thiobacillus and Alcaligenes*. The most widely spread genus is probably *Pseudomonas* (*Ps. fluorescens, Ps. aeruginosa, Ps. denitrificans*).

The benefits of denitrification in wastewater treament are the following:

* it reduces the concentration of oxidized N in the water, not only as nitrate but also nitrite which may be present in high concentrations in certain industrial wastewater if *Nitrobacter* is inhibited;
* it utilizes some of the organic matter during denitrification;
* it releases oxygen into solution which can be used by heterotrophs thereby offsetting the extra aeration cost for nitrification (Gray, 1989).

2.7.3 The phoshorus cycle

P is a macronutrient that is necessary to all living cells. It is an important component of adenosine triphosphate (ATP), nucleic acids (DNA and RNA), and phospholipids in cell membranes. P can be stored in intracellular volutin granules as poly-P in both prokaryotes and eukaryotes. It is a limiting nutrient with regard to algal growth in lakes and thus contributes to eutrophication. The average concentration of TP (inorganic and organic forms) in wastewater is in the range of 10-20 $mg.l^{-1}$ (Bitton, 1994) and requirements for its removal calls for maximal concentration lower than 1 $mg.l^{-1}$ (Water Act 1954).

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 up the orthophosphate ion 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 environemt once they die and are decomposed (Mader, 1998). Phosphate is referred to as a limiting nutrient because many of the sources consist of insoluble complexes such as calcium, iron and aluminium salts (Muyima *et al.*, 1997). At any given time, freshwater supplies usually only contain trace amounts of the phosphate ion.

However, human intervention increases the quantity of phosphate in the environment and includes the mining of phosphate ores for fertilizer production, animal feed supplementation and detergent production (Atkinson, 1999).

In this section, only the sources of P contamination will be discussed. The primary sources of P pollution entering the environment can be categorised as either diffuse or point. Basically, diffuse sources originate from rural and urban areas and agricultural fertilizers contribute the greatest amount to these sources (Atkinson, 1999). As the name implies, this pollution problem cannot be targeted to a specific area and treatment to remove the P content from diffuse sources is not usually economically feasible. However, quantification of the P load to an impoundment can be achieved (Atkinson, 1999). Atmospheric precipitation and dry fall-out are also classified as diffuse pollution (Rudd, 1979).

On the other hand, point source pollution is due to industrial and domestic effluents and includes effluents emanating from wastewater treatment plants (Atkinson, 1999). Point source pollution contributes the highest P load to the environment, although methods to treat the water do exist (Wentzel, 1992).

The difficulty in consistently achieving the mandatory effluent P standard in wastewater treatment has led to alternatives being considered, one option being to eliminate P from detergents. It has been calculated that P in detergents only constitutes approximately 40 % of the total P load in domestic wastes (Wentzel, 1992). In-depth feasilbilty studies rendered little economic incentive to remove P from detergents. The decision was therefore taken (in the South African context) not to introduce a P ban, but rather to investigate methods of improving existing P removal technologies or to develop new technologies (Atkinson, 1999).

The main sources of P in sewage are human waste (30-35 %), and phosphate builders in detergents (50-70 %). In the industrial wastewater field, the P content presents a problem in food processing and canning effluents, effluents containing poly-P used to prevent corrosion and scale formations, effluents from some fertilizer plants, etc. Human excretion contains between 226 to 1004 g P/cap/yr, and phosphate load in detergent builders is estimated at 1004

g P/cap/yr. (Ganczarczyk, 1983). P is present in sewage in three distinct forms: orthophosphate, poly-P, and organic phosphate. Organic phosphate is a minor constituent of sewage and like the poly-P requires further decomposition to the more assimilable orthophosphate form, which is normally fairly slow. About 25 % of the total P in settled sewage is present as orthophosphate in forms such as PO_4^{3-}, HPO_4^{2-}, $H_2PO_4^-$ and H_3PO_4, which are available for immediate biological metabolism. Therefore, in terms of utilization, both in the treatment plant and subsequently in receiving waters, it is the inorganic phosphate concentration that is important rather than the total P concentration. After secondary treatment, about 80 % of the total P in a final effluent is in the orthophosphate form (Gray, 1989).

The major steps in the P cycle are:

* mineralization
* assimilation
* precipitation

Microbes play an active role in the P cycle.

2.7.3.1 Mineralization

Mineralization of organic P compounds is carried out by a variety of microbes, which include bacteria (e.g. *Baccilus subtilis*, *Arthrobacter*), actinomycetes (e.g., *Streptomyces*) and fungi (e.g. *Aspergillus, Penicillium*). Organic P compounds such as phytin, inositol phosphates, nucleic acids and phospholipids are mineralized to orthophosphate. The enzymes responsible for the degradation of P compounds are the phosphatases (Bitton, 1994).

2.7.3.2 Assimilation

Microbes assimilate or absorb P, which enters into the composition of several macromolecules within the cell. P may be stored by some microbes as poly-P granules. The poly-P serves as both an energy and P source in microbes (Bitton, 1994). The natural ability of microbes to assimilate P has been exploited in terms of explaining the removal of P from activated sludge systems. To this end various theories have been formulated to explain the mechanism of P uptake by activated sludge microbes. P enters bacterial cells either by diffusion or biochemically by an active transport mechanism such as ATP. In the activated sludge process, where the concentration of P is normally low in comparison to the cell, active transport is more important. Gray (1989) showed that there was a constant movement of P into cells by active transport, but also out of cells by diffusion, with an overall gain in the P concentration in the cell due to the active transport mechanism.

In the activated sludge process, the mixed liquor sludge normally contains 1.0 - 2.5 % of P. However, under certain environmental conditions this can increase to 5.0 % or even more due to enhanced P accumulation by the microbial biomass. Some bacteria, when deprived temporarily of adequate concentrations of P for metabolism, rapidly accumulate the element once they are exposed to it again and store it as poly-P within their cells a process is known as luxury uptake.

Luxury uptake occurs when a bacterial cell takes up P more than for its normal metabolic requirements (Toerien *et al.*, 1990). Excess P is taken up and stored in the cell as long as sufficient energy is available (Carberry and Tenney, 1973). Enhanced P uptake from wastewater can be observed when obligate aerobic bacteria are exposed to the stress of an anaerobic environment. These cells take up P rapidly by luxury uptake reaction immediately on entry into the aerobic zone where the stress is released and there is an abundant supply of both energy and P.

A number of studies have indicated that P removing microbes proliferate in the aerobic zone as a result of the accumulation of fermentation breakdown products during anaerobiosis. The fermentation breakdown products consist of acetate and butyrate which are short chain carbohydrates (fatty acids), suitable for P removing bacteria. A model organism of this kind is *Acinetobacter* spp. (Buchan 1983; Lötter, 1985), which have been shown to accumulate phosphates under aerobic conditions of up to 25 % of their cell mass. Enhanced biological P removal plants have since been either designed or redesigned to employ luxury uptake of P (Atkinson, 1999). Vaker *et al.* (1967) reported EBPR in a full-scale plant and this was attributed to the phenomenon of luxury uptake of P by activated sludge bacteria.

2.7.3.3 Precipitation

The solubility of orthophosphate is controlled by the pH of the aquatic environment and the presence of Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+}. Precipitation results with the formation of insoluble hydroxy-apatite compounds such as $Ca_{10}(PO_4)_6(OH)_2$, $Fe_3(PO_4)_2.8H_2O$ or $AlPO_4.2H_2O$ (Lilley *et al.*, 1997)

Insoluble forms of P are solubilised by microbes through their metabolic activities. Mechanisms of solubilization used by microbes include: the use of enzymes, production of organic and inorganic acids (for example succinic acid, oxalic acid, nitric and sulphuric acid), production of CO_2 (which lowers pH), production of H_2S (which may react with iron phosphate and liberate orthophosphate), and the production of chelators that can complex calcium, iron or aluminium (Bitton, 1994). Chemical precipitation as a method for phosphate removal is gaining less popularity due to the high costs that are incurred for buying lime (Lilley *et al.*, 1997) and added to this, are the lime dumps that become characteristic of plants using chemical precipitation.

2.7.4 The sulphur cycle

The South African experience in terms of EBPR (in the activated sludge process) indicates that high concentrations of sulphide are detrimental to the process. Apart from being toxic, excessive sulphide may reduce the concentration of essential trace elements to subcritical levels, thus interfering directly with biochemical reactions occurring during P release. Once sulphate reducing bacteria (SRB), such as *Desulfovibrio desulphuricans* develop, the nett availability of SCFA declines because these bacteria utilize such compounds as carbon sources for growth. Furthermore, sulphide contributes to the phenomenon of bulking sludge due to the growth of filamentous organisms such as *Thiothrix* and is thus best avoided. It has, however been claimed that such organisms contribute to the process of enhanced P removal (Toerien *et al.*, 1990).

Sources of sulphur in wastewaters are organic sulphur found in excreta and sulphate, which is the most prevalent anion in natural waters.

2.7.4.1 Microbiology of the sulphur cycle

Organic sulphur compounds are mineralized by several types of microbes through aerobic and anaerobic pathways (Bitton, 1994)

Sulphatase enzymes are involved in the degradation of sulphate esters to SO_4^{2-} under aerobic conditions:

$$R\text{-}O\text{-}SO_3^- + H_2O + \text{sulphatase enzyme} \rightarrow R\text{-}OH + H^+ + SO_4^{2-}$$

Sulphur containing amino acids (e.g. cysteine, cystine and methionine) are degraded to inorganic sulphur compounds or mercaptans, which are odorous sulphur compounds under anaerobic conditions (Bitton, 1994; Gray, 1989). Most anaerobic bacteria are able to produce

sulphide from protein, e.g. *Proteus* and *Bacteroides* spp., and some *Clostridium* spp. Although all can grow anaerobically, only *Bacteroides* spp., which can be present in feces at concentrations up to 10^{10} per gram, are obligate species (Gray 1989).

2.7.4.1.1 Assimilation

Microbes assimilate oxidized as well as reduced forms of sulphur. Anaerobic microbes assimilate reduced forms such as H_2S, whereas aerobes utilize more oxidized forms.

2.7.4.1.1.1 Oxidation reactions

Several groups of microbes are involved in sulphur oxidation (Bitton, 1994). H_2S is oxidized to elemental sulphur under aerobic and anaerobic conditions. Under anaerobic conditions, *Thiobaccillus thioparus* oxidizes S^{2-} to S^0:

$$S^{2-} + \frac{1}{2}O_2 + 2H^+ \rightarrow S^0 + H_2O$$

Under anaerobic conditions, oxidation is carried out by photoautotrophs and chemoautotrophs, such as *Thiobacillus denitrificans*. Photosynthetic bacteria use H_2S as an electron donor and oxidize H_2S to S^0, which it stores within the cells of *Chromatiaceae* (purple sulphur bacteria) or outside the cells of *Chlorobiaceae* (green sulphur bacteria). Filamentous sulphur bacteria (e.g. *Beggiatoa*, *Thiotrix*) also carry out H_2S oxidation to elemental S, which is deposited in sulphur granules (Bitton, 1994).

Oxidation of elemental sulphur:

These reactions are carried out mainly by the aerobic, Gram negative, non-spore-forming thiobacilli (for example *Thiobacillus thioxidans*) which grow at very low pH values.

$2S + 3O_2 + 2H_2O \rightarrow 2H_2SO_4$

$NaS_2O_3 + 2O_2 + H_2O \rightarrow Na_2SO_4 + H_2SO_4$

Sulphur oxidation by heterotrophs:

Heterotrophs (for example *Arthrobacter*, *Micrococcus*, *Bacillus*, *Pseudomonas*) can also be responsible for sulphur oxidation in neutral and alkaline soils.

2.7.4.1.2 Sulphate reduction

Sulphides are produced by assimilatory and dissimilatory sulphate reduction.

Assimilatory sulphate reduction:

H_2S may result from the anaerobic decomposition by proteolytic bacteria (for example Clostridia, *Vellionella*) of organic matter containing sulphur amino acids such as methionine, cysteine and cystine.

Dissimilatory sulphate reduction:

Sulphate reduction is the most important source of H_2S in wastewater. Sulphur reducing bacteria which are strict anaerobes, reduce sulphate:

SO_4^{2-} + organic compounds $\rightarrow S^{2-} + H_2O + CO_2$

$S^{2-} + 2H^+ \rightarrow H_2S$

H_2S is toxic to animals and plants including fish, as well as wastewater treatment operators (Bitton, 1994).

Sulphate reducing bacteria belonging to the following genera have been isolated from environmental samples (anaerobic sludge digestors, aquatic sediments and gastro-intestinal tracts): *Desulfovibrio*, *Desulfotomaculum*, *Desulfobulbus*, *Desulfobacter*, *Desulfococcus*, *Desulfonema*, *Desulfosarcina*, *Desulfobacterium* and *Thermodesulfobacterium*.

2.8 Pasteur effect and enhanced phosphate uptake

The Pasteur effect is a phenomenon associated mostly with the change in the metabolic rate of an organism when shifted from anaerobic to aerobic metabolism. For example, when yeast cells are transferred from anaerobic to aerobic conditions, they increase the rate of glucose breakdown by a factor of 3 to 4. This change is accompanied by a reduction of the fermentation rate and a stoppage of alcohol formation. The Pasteur effect is apparently the result of differences in the cell's energy charge under aerobic and anaerobic conditions. In the presence of oxygen, the respiratory chain and the sites of substrate-level phosphorylation in the glycolytic pathway compete for ADP. The activity of phosphofructokinase is known to be regulated by the level of ATP and citrate. Under anaerobic conditions the activity of phosphofructokinase increases because it is activated by ADP and AMP. Also, more ADP is available for enzymes catalyzing substrate-level phosphorylation reactions. The Pasteur effect could possibly be used to explain the logic behind the ability of activated sludge bacteria phosphate uptake phenomenon subsequent to stressful conditions of the anaerobic zone (Gottschalk, 1979).

2.9 Mechanisms of biological P removal

EBPR by bacteria present in the mixed liquor of activated sludge was first observed by Vaker *et al.* (1967). Since then this phenomenon has gained world-wide support and it utilized both in new and existing wastewater treatment plants, which are either constructed or upgraded to accommodate biological nutrient removal (Atkinson, 1999). The tendency to opt for biological

P removal is due to the low sludge production of the system, the fertilizer value of the waste sludge and the use of wastewater components like influent COD and its various fractions as process chemicals to control the mechanism (Henze, 1996).

Biological P removal is a direct result of the ability of certain microbes (PAO) to accumulate large quantities of poly-P intracellularly. The terms "enhanced" and "excess" are often incorporated to emphasize the ability of these microbes to accumulate poly-P in excess of their normal metabolic requirements. In order to encourage the growth and proliferation of these microbes, as well as to induce the biological P mechanism, two conditions are essential, namely sequential anaerobic and aerobic reactors, as well as the presence of VFA in the anaerobic reactor (Wentzel *et al.*, 1990).

2.9.1 Anaerobic zone

In wastewater technology, the term anaerobiosis describes an environment free both of dissolved oxygen and oxidized forms of nitrogen like nitrates and nitrites (Jenkins and Tandoi, 1991; Muyima *et al.*, 1997). The function of the anaerobic zone is two-fold:

* the reduced redox potential induces the conversion of influent readily degradable COD (RBCOD) to SCFA via acidogenesis by non-PAO heterotrophs;
* it provides an ideal environment where PAO are able to take up the SCFA and accumulate the intracellularly as PHA (the most common form being PHB)(Ekama and Wentzel, 1997).

The function of the anaerobic zone was originally thought to be one of stimulation of P release. However, it has now been realized that the zone is solely responsible for the production of suitable substrates (VFA) through fermentation, which allows for the proliferation of PAO in the system due to a lack of substrate competition from non-PAO (Fuhs and Chen, 1975; Muyima *et al.*, 1997). Due to the absence of terminal electron acceptors, PAO are able to

utilize the VFA exclusively through the energy generated from poly-P hydrolysis (Atkinson, 1999). This zone is essential for the nett removal of P from the wastewater, as it preconditions the PAO to take up excess P under aerobic conditions, resulting in diminished intracellular poly-P storage granules (volutin), which must be replenished in the following aerobic zone. It is essential that neither nitrates nor dissolved oxygen are recycled to, or enter the anaerobic zone, as the effects on P removal are adverse. This may be due to (Henze *et al.*, 1995):

* competition for VFA between PAO and other normal heterotrophs;
* reduction in PAO activity due to reduced fermentation of RBCOD leading to a reduction in VFA;
* some species of PAO are able to denitrify (in the case of nitrate infiltration) and all are aerobic (in the case of dissolved oxygen), so the organisms will preferably utilize these terminal electron acceptors to obtain energy, rather than switching their metabolism to poly-P hydrolysis (which directly affects P accumulation in the subsequent aerobic zone).

2.9.1.1 Volatile fatty acid synthesis and sequestration

In the anaerobic zone, facultative organisms (non-PAO) are able to derive a small amount of energy (sufficient only for survival) through the generation of intracellular electron acceptors. Through the Embden-Meyerhof pathway, they are able to degrade readily biodegradable organics such as glucose to fatty acids, including acetate, lactate, butyrate, succinate and fumarate. However, under anaerobic conditions, these fatty acids cannot enter the Krebs cycle and are subsequently released into the bulk liquid (Ekama *et al.*, 1984). PAO have a distinct advantage over other normal heterotrophs as they are able to utilize accumulated poly-P stores to supply the energy required to accumulate these fatty acids (Satoh *et al.*, 1992). Wentzel *et al.* (1990) found that the rate of acetate sequestration by PAO is zero order with respect to soluble acetate and occurs rapidly. Although VFA are known to be a primary carbon source

accumulated by PAO in the anaerobic zone, other short chain organic compounds are also known to be taken up and accumulated as PHA (Satoh *et al.*, 1997).

VFA concentrations 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 wastwater and can either be included as an in-line or side-stream facility (Banister and Pretorius, 1998). Primary sludge fermentation is therefore a practical solution for plants experiencing erratic biological P removal as a result of weak influent COD and the resultant low RBCOD and VFA concentrations. Seeding the fermenters with partially digested sludge (1-3 d digestion) has been shown to improve VFA production efficiency with maximum VFA yields of 10 % of the total influent COD being recorded after 6 d (Banister and Pretorius, 1998).

2.9.1.2 Poly-ß-hydroxybutyrate synthesis

A consequence of the high organic carbon concentration in the anaerobic zone is that, provided both an electron and energy source is available, foe example NADH/NAD and ATP/ADP, carbon can be taken up intracellularly and accumulated as PHB. Due to the inhibition of the tricarboxylic acid (TCA) cycle in the anaerobic zone due to the high NADH/NAD ratio, the synthesis of PHB acts as an electron sink, decreasing this ratio (Wentzel *et al.*, 1986). This promotes the TCA cycle, which generates more electrons. The interaction between the TCA cycle and PHB synthesis, mediated by the NADH/NAD ratio, ensures that all VFA taken up is stored as PHB. Reducing equivalents such as $NAD(P)H_2$ are also essential for PHB synthesis. Stoichiometrically, one $NAD(P)H_2$ molecule is required for the conversion of 2 mol acetate to PHB (Appeldoorn *et al.*, 1992). Before acetate can be stored as PHB, the molecule needs to be activated by acetyl-CoA. This is done at the expense of 1 mol ATP per mol acetate if acetate kinase is invloved, or 2 mol ATP per mol acetate if the acetyl-CoA synthetase enzyme catalyzes the reaction (Appeldoorn *et al.*, 1992).

2.9.1.3 Orthophosphate release

A high external concentration of small organic molecules such as acetate allows for passive diffusion into the cell, without expenditure of energy. Activation of sequestered acetate to acetyl-CoA by coupled ATP hydrolysis has the effect of decreasing the ATP/ADP ratio to such an degree that ATP synthesis is stimulated via poly-P degradation (Wentzel *et al.*, 1986). On a molar basis, 1 mol intracellular P is released per mol VFA sequestered (Wentzel *et al.*, 1990). Therefore, if acetate is used as substrate, approximately 0.5 mg P is released per mg acetate (as COD) sequestered (Atkinson, 1999). The mass of P released will always be proportional to the VFA feedstock in solution, in other words, the greater the VFA concentration, the greater the mass of P released. A minimum prerequisite for P release in the anaerobic reactor is a biodegradable COD concentration of 60 mg $COD.l^{-1}$ (Punrattanasin and Randall, 1998) or a RBCOD fraction of 25 mg $COD.l^{-1}$ surrounding the organism (Ekama et al., 1984). Compounds such as acetate, formate and propionate, are capable of inducing P release from phosphate-laden sludge under anaerobic, anoxic and aerobic conditions. This has compelled the belief that the release of P is primarily dependent on the nature of the substrate interacting with the biomass and not the prevailing anaerobic conditions (Gerber *et al.*, 1987).

2.9.2 Anoxic zone

Anoxia refers to an environment in which nitrates and nitrites are present, although dissolved oxygen is absent (Atkinson, 1999). This zone is responsible for denitrification in the activated sludge system and reduces the level of nitrates recycled to the anaerobic zone. The anoxic zone is fed by the effluent from the anaerobic zone and by the mixed liquor recycle from the aerobic zone. The variable nature of this recycle ensures that no dissolved oxygen enters the anoxic zone, resulting in the reduction and removal of NO_x from the system (Atkinson, 1999). It has been found that the PAO community can be divided into two fractions, those only capable of aerobic respiration with oxygen as an electron acceptor, and those capable of utilizing both oxygen and nitrate as electron acceptor (Kerrn-Jespersen and Henze, 1993). P

will therefore be taken up in the anoxic zone if no VFA or other low molecular organic compound is available for assimilation. Thus, if acetate leaks from the anaerobic zone into the anoxic zone, P release and PHB accumulation will continue (Artan *et al.*, 1997). Although considerable, anoxic P uptake is quantitatively lower lower than aerobic P uptake and is a first order reaction with respect to the size of anaerobically accumulated PHB stores (as is aerobic P uptake)(Kerrn-Jespersen and Henze, 1993; Artan *et al.*, 1997). Kerrn-Jespersen and Henze (1993) found a linear relationship between accumulated PHB concentration, denitrification rates and P uptake rates under anoxic conditions. Anoxic P uptake has the potential to reduce costs of wastewater treatment considerably due to simultaneous P removal and denitrification occurring in one zone (Atkinson, 1999).

2.9.3 Aerobic zone

The primary function of the aerobic zone is to oxidize organic material in the sewage. When a suitable sludge age is selected and the autotrophic organisms are able to establish themselves in the system, oxidation of ammonia N to nitrite and nitrate (nitrification) occurs simultaneously. Aerobiosis also provides an environment where the PAO are able to take up the P released in the anaerobic zone, as well as the P entering the system through the feed sewage (Atkinson, 1999).

2.9.3.1 Poly-ß-hydroxybutyrate degradation

The degradation of PHB proceeds via its hydrolysis to free ß-hydroxybutyrate, the oxidation of the acid to acetoacetate and, finally, activation of acetoacetate to acetoacetyl-CoA. Two molecules of acetyl-CoA are then formed by the activated cleavage of acetoacetyl-CoA and these end products enter the TCA cycle (Wentzel *et al.*, 1986). This degradation pathway is regulated by high concentrations of pyruvate and/or a high NADH/NAD ratio and it can, therefore, be concluded that the pathway will only be functional in an environment where

concentrations of extracellular organic substrate is low and a terminal electron acceptor is present, for example in the aerobic zone (Wentzel et al., 1986). Degradation of PHB to acetate provides a carbon and energy source for cell metabolism and function (Atkinson, 1999).

2.9.3.2 Phosphorus uptake and poly-P synthesis

Translocation of extracellular soluble phosphate ions occur via hydroxyl mediated antiport. The cations required for neutralizing the charge imbalance and stabilizing the phosphoryl bonds, for example Mg^{2+}, Ca^{2+} and K^{+} are taken up by the cell via 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 ration and an increase in the ATP/ADP ratio. A high and non-limiting ATP/ADP ration stimulates poly-P synthesis (Atkinson, 1999). The principal mechanism of poly-P synthesis is via the phosphorylation of accumulated phosphate by ATP:

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

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).

2.10 Mixed liquor suspended solids (MLSS)

The concentration of suspended solids in the system, which to a large extent constitutes the resident biomass, is referred to as the MLSS. This value offers the system operator a crude measure of the biomass contained within the process (Atkinson, 1999). With the advent of

steady state and kinetic design modelling, the accurate determination of biomass in activated sludge systems has become a significant criterion (Wentzel *et al.*, 1990). Historically, sludge biomass and specific growth rates were calculated on the basis of MLSS, volatile suspended solids (VSS) or COD of the activated sludge (Liebeskind and Dohmann, 1994). In nitrifying denitrifying EBPR systems the mixed liquor organic suspended solids (MLOSS or mixed liquor VSS) is made up of four components: heterotrophic active biomass, endogenous residue, inert material and autotrophic active biomass (Ubisi *et al.*, 1997; Wentzel *et al.*, 1998). The heterotrophic active biomass component arises from the synthesis of heterotrophic organisms on influent biodegradable COD and is lost via endogenous respiration or death processes. The autotrophic active biomass arises from the synthesis of autotrophic organisms in the nitrification of ammonia and is also lost via endogenous respiration processes. Endogenous residue is generated as a result of the unbiodegradable fraction from heterotrophic and autotrophic biomass death processes, while inert material represents the unbiodegradable particulate COD fraction entering the system via the influent (Ubisi *et al.*, 1997). All four components are returned to the anaerobic reactor (in biological P removal systems) via the clarifier recycle and are removed via the waste flow. Ubisi *et al.* (1997) and Wentzel *et al.* (1998) described a simple batch test procedure for heterotrophic biomass determination in activated sludge mixed liquors. Liebeskind and Dohmann (1994) formulated an improved method of DNA extraction from activated sludge for quantitative biomass determination. Although the proposed method showed close correlation to the kinetic models when applied to pure cultures and high-rate aeration sludges, the full DNA complement could not be extracted from sludges containing precipitating agents such as ferric iron. Unfortunately, the procedures are unable to distinguish between heterotrophic PAO and non-PAO in mixed liquor samples originating from EBPR operations. The requirement for a simple method to determine the active PAO fraction therefore still exists and should be actively investigated.

2.11 Nutrients in sewage for microbes

In activated sludge, the relative number of each species is determined by its growth rate, availability of a suitable food source and predation. Furthermore, physical conditions prevailing in the plant have a different effect on the rate of proliferation of various species (Buchan, 1984).

Domestic sewage, and to a lesser extent industrial wastewater, contains a rich variety of organic and inorganic compounds, including important trace elements and organic growth factors. All the nutritional requirements for bacterial and fungal growth are present in the wastewater. Thus, provided the environmental factors are favourable, a wide range of heterotrophic microbes will develop within the biological reactor of a wastewater treatment plant. The microbes present are not only those species which can metabolize the raw constituents of the wastewater, but can also utilize the breakdown products of other microbes and species which prey upon other microbes (Gray, 1989).

Microbes require certain basic nutrient elements. Carbon is required as a source of energy and for the synthesis of various macromolecules. Important nutrients required by microbes include N, P, sulphur, potassium, calcium and magnesium. Trace elements such as iron, copper, zinc and cobalt are required by many species and more fastidious organisms require growth factors such as vitamins.

Carbon, N and P is required by microbes in balanced amounts. The ratio is COD:N:P of 100:6:1.5. Buchan (1984) states that only a fraction of the incoming constituents are involved in bacterial synthesis.

Large quantities of inert or non-biodegradable material can be present, which are removed by processes other than metabolism (such as adsorption onto floc particles). N is only considered fully available when present as ammonia and P as soluble phosphate, although other forms of these elements can be converted to ammonia and phosphate and thus become available.

Domestic sewage usually provides a nutritionally balanced food with the necessary elements and vitamins for bacterial activity. However, for the processes required for denitrification and enhanced P removal, the organic carbon source is often not available (Buchan, 1984). The most important nutrients for poly-P organisms involved in enhanced biological P uptake are the SCFA.

2.12 Microbial ecology of activated sludge systems

The activated sludge system, like all biological treatment processes, relies on a mixed culture of bacteria (in order to carry out basic oxidation of the substrate present) with higher grazing microbes also present, forming a complete ecosystem which has various trophic levels. The activated sludge aeration tank is a truly aquatic environment, although the high level of nutrients and high level of bacterial activity makes it unlike any natural aquatic habitat. Gray (1989), however, views the activated sludge aeration tank as an extreme environment or habitat for most aquatic species, due to the constant aeration and recirculation.

Aquatic species which find the activated sludge aeration tank being inhospitable or inhabitable include organisms which are larger than the smaller mesofauna, such as rotifers and nematodes, or those with long life cycles (Gray, 1989). Ganczarczyk (1983) views activated sludge as a representation of a complicated mixture of viruses, bacteria, protozoa and other organisms, found either singly or clumped together, often enmeshed in a fabric of organic debris, dead cells and other waste products.

Microbes which play an important role in the activated sludge system include groups of bacteria, fungi, protozoa, rotifers and nematodes (Gray, 1989). Activated sludge is a complex ecological system made up of species forming several trophic levels, which compete for food resources, with predator-pray and/or parasite-host relationships clearly discernible. Other species interactions, which effect species diversity and species dominance, such as antibiotic production and phage activity are also important (Gray, 1989). The response of this ecological

system to the environment is often difficult to predict as individual organisms have varying sensitivities to nutrient conditions, waste composition, and other stresses imposed upon the system (for example inorganic salt concentrations, turbulence, pH, temperature and the presence of competing microbes). These influences are poorly understood from a theoretical perspective and their control in any treatment unit may not be as meaningfully desired. This factor may be due to the fact that many of the characteristics indigenous to biological wastewater treatment systems, especially those related to floc formation, remain largely outside any direct control (Ganczarcyk, 1983). The origin of wastewater may limit the type of bacterial species present in the activated sludge system, for example wastewater from industry (Ganczarcyk, 1983).

The physical characteristics of the treatment system can also affect activated sludge population diversity by a washout of slow-growers, elimination of species more sensitive to turbulence, etc. (Ganczarcyk, 1983). Activated sludge as a habitat has its set of selctive parameters which are characteristic of each zone in the process, hence the difference in terms of those organisms which constitute the bulk of the biomass and/or those that make the largest contribution to the total metabolic activity (Buchan, 1984). Some of the known parameters include pH, temperature, nutrients and growth stage of the microbes (Momba, 1995; Toerien *et al.*, 1990).

2.12.1 Bacteria

Bacteria constitute the major component of activated sludge flocs and are the most active organisms (Bitton, 1994; Ganczarcyk, 1983). Bacteria in the activated sludge system is the predominant group (Ganczarcyk, 1983) and more than 300 strains of bacteria are known to thrive in activated sludge (Bitton, 1994). Both heterotropic and chemolithotrophic organisms are found in activated sludge systems (Ganczarcyk, 1993). Heterotrophic bacteria utilise organic material as a source of both carbon and energy, while chemolitotrophic bacteria generally depend on the oxidation of mineral compounds for energy requirements and utilize carbon dioxide as a carbon source (for example nitrifying bacteria).

The major genera found in the flocks are *Zooglea, Pseudomonas, Flavobacterium, Alcaligenes, Bacillus, Achromobacter, Corynebacterium, Commomonas, Brevibacterium* and *Acinetobacter* as well as filamentous microbes. Some examples of filamentous microbes are the sheathed bacteria (for example *Sphaerotilus*) and gliding bacteria (for example *Beggiatoa, Virtreoscilla*) which are responsible for sludge bulking. *Caulobacter,* as well as *Zooglea,* has been isolated from wastewater treatment plants in general and activated sludge in particular. Activated sludge flocs also harbor autotrophic bacteria such as nitrifiers (*Nitrosomonas, Nitrobacter*) which convert ammonium to nitrate and phototrophic bacteria such as the purple non-sulphur bacteria (*Rhodospirillaceae*), which are detected at concentrations of approximately 10 cells per ml. The purple and green sulphur bacteria are found at much lower levels (Bitton, 1994).

In the anaerobic zone microbes of the species *Aeromonas, Citrobacter, Klebsiella, Pasteurella, Proteus* and *Serratia* are capable of fermentation and thus accumulate and produce organic compounds such as lactic acid, succinic acid, propionic acid, butyric acid, acetic acid and ethanol during fermentation. These organic compounds serve as electron donors and acceptors. However, these are not utilised under anaerobic conditions instead they are only consumed in the anoxic and aerobic zone. It appears thus that the anaerobic zone provides substances for the proliferation of aerobic phosphate-accumulating bacteria (Buchan, 1984; Fuhs and Chen, 1975).

Activated sludge represents a stable community where both the number of species and cell numbers of the representative species are high (Atkinson, 1999). These types of communities are best suited to overcome environmental stress like toxic shock. Wentzel *et al.* (1988) identified organisms from an enhanced PAO culture originating from laboratory-scale UCT and 3-stage Bardenpho/Phoredox systems by means of culture dependent methods of isolation and the Analytical Profile Index (API) system of identification, and discovered that >90 % of the bacterial community consisted of the genus *Acinetobacter*. On the other hand, Bond *et al.* (1997), by means of *in situ* molecular techniques, while operating a sequencing batch reactor employing BPR, detected very low numbers of *Acinetobacter* and concluded that bacteria other than *Acinetobacter* are responsible for EBPR.

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 particular attention and has subsequently practically become optimized. Yet, systems based on nitrifying denitrifying EBPR principles still regularly fail to achieve the desired end result (Atkinson, 1999). 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 characterized from the following viewpoints:

* characterization and quantification of microbial constituents according to metabolic activities;
* identification and classification of microbes;
* activated sludge quality, for example settleability and dewaterability.

Ecological studies of activated sludge are a integral part of 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, algae, protozoa, fungi and filamentous organisms, although species 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 heterotrophic bacteria will dominate the community structure (Kämpfer *et al.*, 1996). Probing of activated sludge with fluorescently labeled oligonucleotide probes specific for the alpha, beta and gamma subclasses of the *Proteobacteria* has revealed that the microbial consortia are dominated by these bacteria (approximately 80 %), a phylum containing the majority of the traditional Gram negative bacteria, the majority of which are heterotrophic (Wagner *et al.*, 1993). A number of heterotrophic organisms have been investigated intensively for their involvement in processes such as BPR. These organisms include *Acinetobacter*, *Moraxella*, *Pseudomonas*, *Microlunatus*, *Achromobacter*, *Aeromonas*, *Vibrio*, *Citrobacter*, *Pasteurella*, *Enterobacter*,

Proteobacter, *Klebsiella*, *Bacillus* and coliforms like *Escherichia coli* and *Escherichia intermedium* (various authors as cited by Atkinson, 1999).

Many of these studies have implicated *Acinetobacter* as the principle agent responsible for BPR in activated sludge. Although Koch's principles for other nutrient removal processes, namely, N assimilation in biomass, nitrification and denitrification, as well as ammonia release from organic N, have been satisfied, the same cannot be said for EBPR processes (Jenkins and Tandoi, 1991). Due to *Acinetobacter*'s inability to fulfill the Koch-Henle postulates adapted to microbial ecology, the exactrole of the organism in BPR has become dubious (Cloete *et al.*, 1985; Steyn and Cloete, 1989). These revised postulates state that (Steyn and Cloete, 1989):

* the microbe in question must be associated with a certain phenomenon (BPR) under all circumstances;
* the organisms must be isolated and studied in pure culture;
* pure culture studies must render similar results to those found in the natural habitat;
* the microbe must be present in sufficiently high numbers to warrant its association with the particular function.

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 (Atkinson, 1999).

The effects of growth phase and initial cell concentration of *Acinetobacter* spp. on P release and uptake have been investigated by Momba and Cloete (1996a,b) and Rustrian *et al.* (1997). Momba and Cloete (1996b) found a linear relationship between initial biomass and phosphate uptake and that at low cell densities (10^2-10^5 per ml) there was a nett release of P rather than uptake. The physiological state of the cells also determines the response of the biomass to P accumulation. Cloete and Bosch (1994) have observed that P is accumulated at the end of log and during stationary phase once active growth has ceased and concluded that a maximum number of cells in the stationary phase of growth should be accumulated in the aerobic zone to optimize biological P removal. Rustrian *et al.* (1997) found that *Acinetobacter* released more

P under anaerobic conditions when in the stationary phase of growth. Yet, P uptake was equally efficient with cells in the log and stationary phase. These results can have a direct implication on EBPR operations as growth conditions for the PAO community can be better optimized to improve and promote the mechanism (Atkinson, 1999).

2.12.2 Fungi

Activated sludge does not usually favour growth of fungi, although some fungal filaments are occasionally observed in activated sludge flocs. Fungi may grow abundantly under specific conditions of low pH, toxicity and nitrogen deficient wastes. The predominant genera found in activated sludge are *Geotrichum, Penicillium, Cephalosporium* and *Alternaria* (Bitton, 1994; Gray, 1989).

Gray (1989) found both endoparasitic and predatory nematophagous fungi from a number of activated sludge plants in Ireland, where they had a significant role both in floc formation and in the regulation of the nematode population density.

2.12.3 Protozoa

Protozoa are common components in activated sludge with population densities reaching up to 50 000 ml^{-1} (Gray, 1989). Three classes are important in activated sludge: the *Rhizopodia*, *Mastigophora* and the *Ciliophora*. The ciliophora in activated sludge are mostly phagotrophic and are divided into free-swimming types (for example *Paramecium*), crawling types (for example *Aspidisca*) and the stalked types (for example *Vorticella*) (Buchan, 1984).

2.13 The "G" bacteria

When assessing EBPR efficiency, a group of normal inhabitants found in activated sludge mixed liquor must be kept in mind: the "G" bacteria (Atkinson, 1999). There is a microbial community in activated sludge capable of organic substrate uptake and assimilation in the anaerobic zone with subsequent metabolism of these storage granules in the aerobic zone. This community is composed of two distinct groups of organisms with distinctly different modes of substrate uptake and synthesis of storage granules. The first group are the PAO, the metabolism of which has been previously described. The second group are the "G" or glycogen accumulating organisms (GAO). In conventional systems, GAO are involved in normal organic oxidation processes, but in selection systems like BPR, their presence and impact to system efficiency can become more prominent (Atkinson, 1999). These Gram negative cocci grow as tetrads and are able to out-compete PAO in anaerobic/aerobic systems by accumulating polysaccharide and not polyphosphate in the aerobic zone (Cech and Hartman, 1993; Cech *et al.*, 1994; Maszenan *et al.*, 1998). The GAO are thought to be able to effectively compete with fermentative organisms for RBCOD and PAO for VFA in the anaerobic zone as they are able to obtain the necessary reducing power and energy required for uptake through glycolysis (the Embden-Meyerhof pathway). Their proliferation in an EBPR system will eventually lead to a decline in phosphate removal (Atkinson, 1999). Cech and Hartman (1993) found that GAO were able to dominate in an anaerobic-oxic system when the influent consisted of an acetate and glucose mixture. Yet, when acetate alone was used as a substrate, the PAO were able to dominate their competitors. Influent P/COD ratios also affect the microbial community structure of EBPR activated sludge. Liu *et al.* (1997) found that a low P/COD ration enriches for GAO, while high P/COD ratios promote the growth of PAO, while suppressing the growth of GAO. Competing with the non-PAO (fermentative bacteria) for RBCOD in the anaerobic zone, reduces VFA production which ultimately influences P release by the PAO (Atkinson, 1999). Molecular phylogenetic classification of GAO isolates in the *Proteobacteria* phylum of the domain *Bacteria* shows their relatedness to many of the PAO, indicating that some relationship between the two competing bacteria does exist. This

implies that an identical or similar mode of metabolism between the two bacterial types must have existed at some stage of their evolution (Atkinson, 1999).

2.14 Failure of enhanced biological phosphate removal systems

The successful operation of nutrient removing activated sludge plants is dependent on good operation, coupled with good design and the maintenance of the correct biomass. Biological phosphate removal plants in South Africa have not always given reliable and satisfactory performance (Osborn *et al.*, 1986). EBPR fail either as a result of toxic shock due to the eccumulation of H_2S or the nitrate feedback into the anaerobic zone (Bitton, 1994). Operational optimization of the EBPR process is dependent on meeting the oxygen demand in the aerobic zone and providing the correct substrate in the anaerobic zone (for example SCFA). Other organisms can compete with poly-P bacteria in anaerobic-aerobic activated sludge systems, leading to system failure (Erasmus, 1997). The failure of EBPR process has attracted various methods to remedy this problem, for instance, augmenting of activated sludge by addition of readily biodegradable COD to promote the growth of phosphate removing bacteria has been applied by Osborn *et al.* (1986).

2.15 Bioremediation and bioaugmentation

The objective of wastewater treatment is to reduce or prevent the accumulation of pollutants in the environment, thus transforming them into harmless products such as biomass, carbon dioxide, methane and nitrate. This objective is achieved by the use of microbes which biodegrade these pollutants. From one perspective, activated sludge processes may be viewed as bioremedation of wastewater.

Atlas and Bartha (1993) define bioremedation as the use biological agents to reclaim soils and waters polluted by substances hazardous to human health, and/or the environment. It is an

extension of biological treatment processes that have traditionally been used to treat wastes in which microbes are typically used to biodegrade environmental pollutants. Bioremedation techniques are diverse and may involve the design of special reactors, as well as the use of specialized microbes, a process referred to as bioaugmentation.

Oellerman and Pearce (1995) explained that bioaugmentation involves the use of genetically manipulated or specially adapted microbes to enhance the biodegradation of specific toxic, hazardous and recalcitrant compounds which are difficult to remove from the environment by conventional treatment processes. Microbial strains for enhancing biodegradation of specific chemicals are generally isolated from environmental samples (wastewater, sludge, compost or soil) and selected by conventional enrichment techniques. They are grown in nutrient media that contain a specific organic chemical as the sole source of carbon and energy or as a sole source of nitrogen. Furthermore, strains that can handle relatively high concentrations of the target chemical are selected. Some of the microbial strains may be subsequently irradiated to obtain a desirable mutation (Bitton, 1994).

Exposure of wastewater microbial communities to toxic xenobiotics results in the selection of resistant microbes that have the appropriate enzymes to use the xenobiotic as the sole source of carbon and energy. This process is called acclimation or adaptation. Most microbes need acclimation prior to the onset of metabolism. Prior exposure to the xenobiotic helps reduce the acclimation period (Bitton, 1994).

In wider sense, augmentation also infers the stimulation of microbes indigenous to the contaminated environment and wastewater to enhance and improve the treatment performance. The aim is to attack preferential substrates which are marginally available or difficult to degrade as pollutants. Bioaugmentation technologies simply attempt to optimize the natural capacity of microbes to degrade and recycle such compounds by controlling pH, oxygen supply or anaerobicity and temperature, and supplying essential limiting nutrients to minimize the stress on such systems.

Bioaugmentation may involve the addition of selected bacteria to a bioreactor to maintain or enhance the biodegradation potential in the reactor. Although the technology has been known for decades, unfortunately, information on the formulation of the mixtures of the microbial cultures is scanty because of trade secrets (Bitton, 1994).

Biodegration techniques are versatile and can be utilized at various stages of treatment. Applications include removal of contaminants from raw materials prior to precessing, treatment of waste before discharge, treatment of effluent streams and decontamination of soils, sediments, surface water and groundwater (Oellermann and Pearce, 1995).

Enhanced biological phosphate removal (EBPR) is usually not optimized and routinely fails (Bond *et al.*, 1995). Under favourable conditions, phosphate-removing sludge has been observed to take several sludge ages to develop. This suggests that the phosphate-removing community might need to be established and may not occur merely as a result of conditioning of the existing population (Bond *et al.*, 1995). Therefore, it is of prime importance that a fixed biomass concentration be maintained if there is a need to obtain high reactor performance in every biological wastewater treatment process (Nicolella *et al.*, 1997). Another limitation of bioaugmentation is that there is usually a requirement by microbes used, for an acclimation period prior to the onset of the process. Short survival or lack of growth of microbial inocula in the seeded bioreactors is another well-known problem (Bitton, 1994). A sludge age ranging from 15 to 20 days is required for the removal of phosphate in activated sludge systems (Toerien *et al.*, 1990) and, thus, subsequent to toxic shock or the development of unfavorable conditions for phosphate uptake, effluents with high phosphate concentration may be discharged into waterways. The use of activated sludge biomass for augmentation may assist the wastewater plant operators to avoid discharging limiting nutrients into the rivers, lakes and ponds whilst the natural biomass of the plant is resuscitated.

Bioaugmentation could be used in this regard to rectify the depleted activated sludge microbial that fails to optimally remove phosphate.

2.16 Use of bioaugmentation in wastewater treatment

Some of the applications of bioaugmentation are the following (Grubbs, 1984; Rittmann *et al.*, 1990):

* increased BOD removal. Microbial strains may be used to increase BOD removal in wastewater treatment plants;

* reduction of sludge volume. Production of large amounts of sludge is a serious problem associated with aerobic waste treatment, and thus reduction of sludge volumes is highly desirable. The reduction is the result of increased organic removal following addition of a mixed culture of selected microbes. Reductions in 17 % to nearly 30 % have been documented;

* use of mixed cultures in sludge digestion. In aerobic digesters, the use of mixed cultures has led to significant savings in energy requirements. In anaerobic digesters, bioaugmentation has resulted in enhanced methane production;

* biotreatment/bioremedation of hazardous wastes. The use of added microbes for treating hazardous wastes (for example phenols, ethylene glycol, formaldehyde) has been attempted and has a promising future. Bioaugmentation with parachlorophenol-degrading bacteria achieved a 96% removal in 9 h, as compared with a control that exhibited 57 % removal after 58 h (Kennedy *et al.*, 1990). *Candida tropicalis* cells also have been used to remove high concentrations of phenol in wastewater (Kumaran and Shivaraman, 1988). *Desulfomonile tiedjei*, when added to a methanogenic upflow anaerobic granular-sludge blanket, increased the ability of the bioreactor to dechlorinate 3-chlorobenzoate (Arhing *et al.*, 1992). Oellermann and Pearce (1995) achieved significant results on phenol bioremedation;

Bioaugmentation by means of biomass could be used to rectify wastewater treatment plants in South Africa especially those plants which fail to remove phosphate.

2.17 General aspects of biomass

Atlas and Bartha (1993) define biomass as the dry weight, volume, or other quantitative estimation of organisms, thus, the total mass of living organisms in an ecosystem. The various types of organisms in activated sludge system thus constitute the biomass of that particular system. The biomass parameter measures the extent of growth and it enters into certain important derived parameters such as growth yields, metabolic quotients (Pirt, 1975), the quantity of energy being stored in a particular segment of the biological community, as well as the transfer of energy between trophic levels within an ecosystem (Atlas and Bartha, 1993). Biomass can be expressed in units of weight (g) that can be converted to units of energy (cal).

The methods used to measure biomass are based on eight types of measurements: mass, volume or linear extent, mass of a biomass component, mass of substrate consumed or product formed, metabolic rates, light scattering, cell and organelle counts and staining methods (Pirt, 1975).

Choice of the method to be used for measuring biomass is a crucial decision to make and often limitations of the methods make the decision difficult. The factors which influence the choice are (Pirt, 1975):

* properties of the biomass;
* properties of the culture medium;
* accuracy required;
* sensitivity required; and
* required speed of measurement.

Properties of the biomass which affect the choice of the method to use are, whether it is filamentous or particulate, the ease at which separation from the medium can be obtained during quantification and the age of the biomass or its growth rate (Pirt, 1975).

The rate of phosphate removal in activated sludge process is dependent on the biomass concentration and cell sizes (Momba, 1995). Excess uptake of phosphate has been related to the increase in biomass (Bosch, 1992; Streichan *et al.*, 1990) and the rate of removal is dependent on concentration of the sludge (Srinath *et al.*, 1959). Momba and Cloete (1996a,b) demonstrated, using *Acinetobacter junii* that the concentration of phosphate uptake is related to the biomass concentration and nutrient gradients which have to be defined and, which in full-scale plants, are separated and governs P release and uptake.

Momba and Cloete (1996a,b) noted that:

* there was a rapid phosphate release when low initial cell biomass concentration were used in mixed liquor medium;
* the most favourable nett phosphate removal from mixed liquor medium was obtained by the use of a high initial biomass concentrations.

Biomass of 10^7 cells.ml^{-1} released phosphate during the logarithmic growth phase, whereas biomass of 10^8 cells.ml^{-1} (*Acinetobacter junii* and *Pseudomonas fluorescens*) removed phosphate during the lag phase, logarithmic growth phase and up to the stationary phase. Enhanced uptake of phosphate occurred when cells reached the stationary growth phase for *A. junii* and logarithmic growth phase for *Ps. fluorescens*.

Previous research has indicated that phosphate uptake was related to an increase in biomass concentration (Streichan *et al.*, 1990; Bosch, 1992). Momba and Cloete (1996a,b) reported that high initial cell concentrations of *Acinetobacter junii* (a PAO) removed more phosphate than low cell concentrations, and that phosphate uptake was therefore directly related to biomass concentration and high nutrient availability. Bosch (1992) stipulated that biomass was a more significant factor than the type of organism/s present with reference to biological

phoshorus removal. Sidat *et al.* (1999) also reported on findings suggesting a direct relationship between the biomass concentration and phosphate removal capacity. Optimal phosphate removal was achieved at a biomass concentration of 1900 $mg.l^{-1}$.

2.18 Limitations to maximum biomass determination

The maximum density of biomass of any kind that can be reached in a given medium is determined by using one of the following four conditions (Pirt, 1975):

* the amount of growth-limiting substrate supplied;
* maximum packing density of the biomass;
* the accumulation of an inhibitory producr;
* cell death.

Techniques for direct determination of biomass, such as filtration and dry weight or by centrifugation and packed cell volume, are rarely applicable to environmental samples. These techniques tend to measure mineral and detritus particles and fail to discriminate between trophic levels, that is, between producers and consumers. Consequently, determination of biomass by these methods is imprecise (Atlas and Bartha, 1993).

Current research in wastewater treatment has been directed towards mathematical modelling of basic design and operational procedures. One important parameter in such models is the amount of viable biomass. For this reason attempts have been made to find simple and reliable methods to determine the biomass in wastewater and activated sludge. The simplest and most often method is to measure SS or VSS. Such methods, however, do not distinguish between living cells and debris of either organic or inorganic origin. Using traditional plate techniques, the problem is normally an underestimation of the biomass due to selectivity of the media (Jørgensen *et al.*, 1992). Microbial activity has been used as a parameter to determine the microbial potential. Methods include respirometry, ATP content and different enzyme assays.

Very few attempts however have been done to relate activity to biomass. Roe and Bhagat (1982) estimated the ATP to SS ratio at maximum viability in activated sludge, and they found that viability varied significantly which mean cell residence time. Henze (1986) reported comparative studies of denitrification and oxygen utilization rates of raw wastewater and used conversion factors to relate activity to biomass. Kucknerowicz and Verstraete (1979) found a linear relationship between oxygen utilization rate (OUR) and ATP content suggesting that ATP reflected viable biomass. In a study to relate biomass activity with ATP content, oxygen respiration and fluorescein diacetate (FDA) hydrolysis, Jørgensen *et al.* (1992) reported that FDA hydrolysis fail to show any correlation.

Attempts at understanding microbial ecology are limited by the lack of suitable methods and it is thought that bacteria from aquatic and soil environments cannot be accurately enumerated. (Atlas and Bartha, 1987). The non-critical use of so-called standard methods may yield data of questionable accuracy as microbes live in micro-environments having physico-chemical properties, which may be distinctly different from those of the surrounding environment. Bacterial culture media used in laboratories are mostly designed for the cultivation of human pathogens or other fastidious bacteria and consists mainly of proteinaceous substrates such as peptones, meat and yeast extract and hence this may not necessarily satisfy the nutritional requirements of microbes isolated from the environment. Planktonic bacteria in water systems have different nutrient requirements to the fastidious bacteria (Brözel and Cloete, 1992).

The viable (cultivable) bacteria in activated sludge constitute only a small fraction (< 10 %) of the total number of cells present (Cloete and Steyn, 1988). Cloete (1984) has warned against the acceptance of organisms that can be cultivated as a true reflection of the entire population of the activated sludge. Cloete (1984) compared the total microscopic counts of two activated sludge samples that had been centrifuged onto density gradients with the number of *Acinetobacter* estimated with fluorescent antibodies and the numbers of volutin-containing bacteria revealed by metachromatic staining. *Acinetobacter* and volutin-containing bacteria occurred only in 3 out of 10 fractions and constituted only 2.4 -20.5 % of the total number of bacteria applied to the density gradients. The fluorescent antibody number of *Acinetobacter* corresponded closely to the colony forming units (cfu) of *Acinetobacter* of activated sludge

samples, thus giving reasonable justification that the bacteria which were non-cultivable were not *Acinetobacter*. The *Acinetobacter* (or cultivable bacteria) could therefore not represent the population structure of those bacterial groups that could not be cultivated. Bond *et al.* (1995) argued that culturing techniques have provided a misleading picture of bacterial community structure in activated sludge and, that in general, the role of *Acinetobacter* spp. in activated sludge processes has been overrated. For instance, non-culture-dependent methods such as quinone profiles and flourescent in situ hybridization (FISH) probes have indicated that *Acinetobacter* spp. are present in small proportions in activated sludge (*ca.* 3 to 6 %). A promising method for the quantification of uncultured microbes in the environment has been suggested by Polz and Cavanaugh (1997). This method is based on generating nucleic acid standards for the quantification of uncultured microbes. rRNA transcribed from cloned templates has been shown to afford a quantitative estimate of a species when added at different concentrations to an artificially assembled community. This replaces the need for nucleic acids standards obtained from pure cultures of the organisms to be quantified.

Although considerable research effort has been directed towards improving understanding of the BEPR phenomenon, designs of activated sludge systems to accomplish BEPR are still based on experience and semi-empirical methods. Clearly, the need exists for design procedures based on more fundamental behavioural patterns and kinetics (Wentzel *et al.*, 1990). Quantification, as well as kinetic models of biomass in activated sludge are routinely used in design of wastewater treatment plants, in spite of the limitations which are currently encountered in biomass determination. For instance, in the current steady state design and kinetic simulation models for activated sludge, the mixed liquor suspended solids is made up of a number of components. One key component is the heterotrophic active biomass, as this component mediates the biodegration processes of COD removal and denitrification. Thus, the rates for these processes are directly related to the heterotrophic active biomass present, and the specific rates should be expressed in terms of this parameter to allow a meaningful comparison of the rates measured in different systems. However, the heterotrophic active biomass parameter has been only hypothetical within the structure of these models; it has not been measured directly, primarily due to the lack of suitable simple measurement techniques. This deficiency has cast some measure of doubt on the entire framework within which the

steady state design and kinetic simulation models have been developed, in the past. However, Ubisi *et al.* (1997) reported close correlation between measured heterotrophic active biomass concentration with those calculated theoretically, thus promoting confidence in the application of the models for design, operation and control of activated sludge systems.

In the literature, principally microbiological techniques for biomass estimation has been proposed (Ubisi *et al.*, 1997), including pour plate or other culturing techniques (Gaudy and Gaudy, 1980), ATP analysis (Nelson and Lawrence, 1980), DNA analysis (Liebeskind and Dohmann, 1994), the use of fluorescent probes for rRNA (Wagner *et al.*, 1994c) and sequencing or ribosomal DNA (Blackall, 1994). However, these techniques have not yet been adequately integrated with the design and kinetic modelling theory, while culturing techniques have been widely criticised for their unreliability (Cloete and Steyn, 1988). The RNA and the two DNA methods are still in their infancy. The last four named methods also require sophisticated equipment and experimental techniques that are not widely available (Ubisi *et al.*, 1997).

2.19 Estimation of microbial numbers

A range of direct counting techniques has been developed to count dead and living cells separately. All these techniques are based on the indication of metabolic activity of the living cells (Roszak and Colwell, 1987).

2.19.1 Direct count procedures

A means to overcome the quantitative and qualitative bias imposed by cultivation-dependent methods is the use of chemotaxonomic and molecular techniques for *in situ* analyses of communities. The term *in situ* indicates that all microbial cells in an environmental sample are not isolated prior to investigation, but are rather studied intact, within the original sample

(Atkinson, 1999). Techniques employed include application of biomarker approaches, immunofluorescence and sequence-based molecular methods (otherwise referred to as FISH). These methods have proven to be powerful tools in their ability to quantitatively enumerate specific species in diverse microbial communities and many perceptions (like *Acinetobacter* as the model poly-P organism have been shattered due to their accurate and reliable identification abilities (Atkinson, 1999).

2.19.1.1 Light microscopy

Microbes can be counted by direct microscopic observation. These procedures yield the highest estimations of numbers of microbes, although there are several major drawbacks to direct observational methods, including (Coetzee, 1999):

* dead and alive microbes are counted;
* different types of microbes, especially bacteria, cannot be distinguished on the basis of morphology;
* the sample cannot be used for further experimentation.

2.19.1.2 Epifluorescence microscopy

Epifluorescence microscopy, with stains such as acridine orange (AO), 4,6-diamidino-2-phenylindole (DAPI) and fluorescein isothiocyanate (FITC), is widely used for direct counting of bacteria (Jacobs *et al.*, 1996). When AO is used as a stain, bacteria and other microbes fluoresce orange and green. DAPI, which stains the DNA of bacterial cells and produces an intense blue fluorescence, has been found to be superior to AO for visualizing small bacterial cells (Wolfaardt *et al.*, 1991).

Counts by direct epifluorescent microscopy are typically two orders of magnitude higher than counts obtained by cultural techniques (Coetzee, 1999). The value of the direct count epifluorescent microscopy approach to enumeration is that it is applicable to a variety of habitats without the bias inherent to viable count procedures (Wolfaaardt et al., 1991).

2.19.1.3 Scanning electron microscopy (SEM)

SEM is performed by scanning a focussed electron probe across the surface of the sample to be studied. Secondary electrons emitted from the sample are tyoically detected by a photomultiplier system, the uotput of which is used to modulate the brightness of a TV monitor that is rastered in synchronization with the electron beam scan. The more electrons a particular region emits, the brighter the image at that point. SEM images typically contain a good deal of topographical detail, in other words a very good depth of field. Electron microscopy (EM), instead of light microscopy, can be used for direct counting of microbes, although the technique is impractical for routine use (Caldwell *et al.*, 1997).

2.19.1.4 Confocal laser microscopy

This is a new, non-destructive technique with major advantages, bridging the gap between traditional light microscopy and SEM (Coetzee, 1999). It gives higher resolution and thinner non-invasive optical sections or planar views than those obtained by classical bright-field or dark-field microscopy and increased contrast is another advantage (Rochow and Tucker, 1994). Integrating a light microscope, a scanning laser and a computer, confocal microscopy allows the generation of three-dimensional images of biological cells and tissues, or successive profiles of multi-layer structures (Coetzee, 1999).

Confocal configuration of the microscope optics, along with the capability of the laser to scan the specimen in a point-by-point fashion to produce sharp, high contrast and high resolution images of very thin, well defined optical sections (Kuehn *et al.*, 1998).

In an image-processing system, a hundred or more very thin optical sections can be stored and combined into a composite three-dimensional image. These image views can resemble those from SEM, but the specimen need not be in a vacuum (Rochow and Tucker, 1994).

2.19.1.5 Fluorescent *in situ* hybridization (FISH)

Of the three categories of RNA in prokaryotes (mRNA, tRNA and rRNA), only rRNA can be used for probing purposes because of its central role in cell survival, maintenance and reproduction. In addition, rRNA and their genes are almost universally present in cellular life forms, are functionally constrained and therefore evolutionally conserved, which makes them valuable indicators of identity and relatedness (Atkinson, 1999). Perhaps their greatest quality as far as spatial distribution and identification in activated sludge or other environmental samples is concerned, is their natural amplification and high copy number per cell (usually > 10 000 per cell)(Atkinson, 1999). An intact rRNA molecule is comprised of three sub-unit molecules: 5S, 16S and 23S. An average bacterial 16S rRNA molecule is approximately 1 500 nucleotides long (Amann *et al.*, 1995). There is therefore sufficient information contained within this molecule to establish reliable phylogenetic analyses, even if they are not fully sequenced, although > 1000 nucleotides should be determined for accuracy and confidence (Amann *et al.*, 1995).

Each rRNA-targeted oligonucleotide is chemically linked to a fluorochrome which allows cells hybridized with the labelled oligonucleotide to be directly visualized using epifluorescence microscopy or scanning confocal laser microscopy (Amann *et al.*, 1995). The choice of fluorochrome for immunofluorescence or probe hybridization is dependent on availability in a stable, purified form, the ease with which it can be coupled to a carrier molecule (antibody or

oligonucleotide probe) without influencing biological activity, as well as the availability of optical apparatus that enable detection of the emitted fluorescent light (Bosch and Cloete, 1993).

The general procedure of bacterial cell quantification from an environmental sample using FISH, usually incorporates a total cell count using the DNA intercalating dye DAPI, followed by hybridization with an universal bacterial probe (Atkinson, 1999). AO can also be used to microscopically visualize DNA molecules, although there are a number of difficulties inherent to its application (Porter and Feig, 1980). DNA-DAPI complexes fluoresce bright blue when visulaized at wavelenghts of > 390 nm, while unbound DAPI and non-DNA-DAPI complexes fluoresce a weak yellow, which enhances the visibility of DAPI fluorescence (Porter and Feig, 1980). The eubacterial to DAPI ratio gives an indication as to the bacterial composition of the sample (Atkinson, 1999). Dual EUB/DAPI staining of activated sludge samples has revealed that approximately 80 % of the microbial cells in the samples were metabolically active bacteria, of which only 3-19 % could be cultivated on optimized media (Wagner and Amann, 1997). The specificity of oligoprobes is freely adjustable, dependent on the requirements of the user. Different phylogenetic levels ranging from kingdom, subclass, genus, species and subspecies can be probed (Wagner *et al.*, 1994a).

In situ identification of the organisms in a batch-type EBPR sludge has revealed that the four major bacterial groups present were the alpha and beta subclasses of the *Proteobacteria*, Gram positive bacteria with a high G+C content and bacteria belonging to the *Cytophaga-Flavobacterium* cluster of the *Cytophaga-Flavobacterium-Bacteroides* phylum (Kawaharasaki *et al.*, 1999). However, Wagner *et al.* (1994a), when characterizing the Proteobacterial microbial consortia in municipal mixed liquor, found that the beta subclass dominated over the alpha and gamma subclasses. Simultaneous plating on nutrient rich media showed dominance of the gamma subclass, emphasizing the bias introduced with cultivation techniques. FISH of mixed liquor samples at the family level of organization of the anaerobic and aerobic zones of the same plant indicated no markable population shift between zones in the EBPR process when characterized at the family level.

Direct *Acinetobacter* FISH counts of an enhanced pilot-scale culture based on the 3-stage Phoredox system by Atkinson (1999) revealed that the genus only constituted approximately 3 % of the total bacterial community. The alpha and gamma subclasses of the *Proteobacteria* appeared to dominate the system with approximate total DAPI counts of 23 and 21 %, respectively, followed by the beta subclass with approximately 16 %. The *Cytophaga-Flavobacterium* group and the high GC Gram positive bacteria only constituted 5 and 8 % of the bacterial community, respectively. Results indicate the definite involvement of the *Proteobacteria* in EBPR operations, although which specific genera still needs to be clarified. The author found that cell counts using plating techniques were generally 1 000-fold lower than counts obtained with membrane filtration and DAPI staining. The microbial population profile using FISH did not seem to alter throughout the system, with counts of the various classes of bacteria remaining fairly constant within the various reactor zones. These results compare well to those of a study done by Ehlers *et al.* (1998), who found little community variation by comparing protein profiles using SDS-PAGE of different plants and different zones in a particular plant. The results of the study by Atkinson (1999) seem to indicate that due to their diversity of metabolism, *Pseudomonas* spp. are able to achieve dominance in BNR activated sludge plants. Although they are not able to accumulate poly-P as efficiently as other PAO like *Acinetobacter*, their high cell counts in mixed liquors will enable them to remove high quantities of P from the system, which may account for the majority of the observed P removal.

Staining activated sludge samples with DAPI at elevated concentrations of those used for DNA staining, referred to as the polyphosphate probing concentration, results in the fluorescence of intracellular volutin and lipid inclusions (Nakamura *et al.*, 1998; Kawaharasaki *et al.*, 1999). Bacteria, which accumulate large quantities of poly-P, are easily distinguished by colour and intensity of fluorescence due to the following DAPI stain characteristics (Kawaharasaki *et al.*, 1999):

* DNA-DAPI fluorescence is blue-white;
* polyphosphate-DAPI fluorescence is bright yellow;
* lipid-DAPI fluorescence is weak yellow and fades rapidly (within seconds).

Dual staining of samples with DAPI at elevated concentrations and EUB will result in the determination of the PAO population in EBPR sludges by means of the PAO/EUB ratio. *In situ* identification of those bacteria exhibiting strong poly-P accumulation (identified through DAPI staining) can also be achieved through dual staining with class and subclass oligonucleotide probes which totally negates culture-dependent methods of isolation and/or Neisser (poly-P) staining procedures (Atkinson, 1999).

For all its promise and potential, there are still technical problems inherent to FISH when applying the technology to microbial systems such as activated sludge. Qualitatively, the protocol of hybridization and detection has been optimized at all levels of organization. However, due to the nature of activated sludge, quantitative results are often difficult and limited because of incomplete dispersion of flocs, a technical problem which limits the application (Atkinson, 1999). Other problems include DNA retrieval for sequence determination, PCR bias when amplifying the sequence of interest and an imposed selection of the retrieved or target sequences (Hiraishi *et al.*, 1998). One of the possible solutions directed towards these problems is the combination of molecular and biomarker methods (Atkinson, 1999).

2.19.1.6 Immunofluorescence

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

technique also permits aut-ecological studies (studies of individual microbes in their natural environment). This technique has been applied to studies on selected microbial species in their natural habitats, including ecologically important organisms (Cloete and Steyn, 1988).

2.19.1.7 Quinone profiles

Respiratory or isoprenoid quinones are a class of lipids, which are constituents of bacterial plasma membranes and play important roles in electron transport, oxidative phosphorylation and active transport across the plasmamembrane (Collins and Jones, 1981). The numerical analysis of lipoquinone profiles has offered an effective method for monitoring population shifts and for classifying bacterial communities in wastewater sludges (Hiraishi *et al.*, 1991). Quinones are usually extracted from an environmental sample using an organic solvent. After evaporation and re-extraction, the concentrated quinone is applied to column chromatography to separate menaquinone and ubiquinone fractions (Hiraishi *et al.*, 1998). Municipal sludges are usually characterized according to these fractions (Hiraishi *et al.*, 1989, Hiraishi *et al.*, 1998). Quinone components are then identified and quantified using spectrochromatography and mass spectrometry. Numerical analyses of quinone profiles can enhance the information regarding bacterial community dynamics in wastewater ecosystems (Atkinson, 1999). The strength of the technique lies not only in its ability to assess taxonomic structure of bacterial communities, but also that variations in bacterial population structure over space and time can be quantified (Hiraishi *et al.*, 1991).

2.19.1.8 Microautoradiography

Autoradiography has classically been used in the medical field, but has recently been introduced to environmental sample analysis to determine microbial community structures (Atkinson, 1999). Typically, a radiolabelled compound appears in the cell or biological structure of interest through either adsorption of a tracer or labelled substrate uptake. The

radiolabelled ample 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 (Nielsen *et al.*, 1998; Nielsen *et al*, 1999). Although autoradiography can be applied successfully to study the *in situ* physiology of various microbes, it is limited by its lack of proper identification of the organisms in question. However, Nielsen *et al.* (1998), through simultaneous use of autoradiography and FISH, were able to correlate function/activity with identification, which is a tremendous breakthrough for activated sludge identification/diversity/functional studies (Atkinson, 1999).

2.19.2 Viable count procedures

A revolution is occurring in the field of microbiology. Previously, microbiologists concerned with analysis of various microbial communities and population dynamics within a specific eco-system have relied heavily upon conventional plating techniques for isolation purposes (Atkinson, 1999). Although the determination of bacterial numbers in a given sample is a basic prerequisite for any microbiologist, attempts at understanding microbial diversity have been severely limited due to a lack of suitable isolation and identification techniques (Brözel and Cloete, 1992). The main concern amongst wastewater scientists is that many of the microbial constituents of the activated sludge community are viable but non-culturable (Wagner *et al.*, 1994a,b; Amann *et al.*, 1995), which inevitably results in biased assumptions regarding biodiversity. When plating on solid media, the number of cfu represents only a fraction (< 1 to 10 %) of the viable cell counts made by direct microscopic techniques (De Haas, 1998).

The activated sludge process is a habitat that consists of a complex mixture of generalist and specialist microbes. In an attempt to obtain a fundamental understanding and to optimize the biological component of key processes such as EBPR occurring within sludge, isolation and identification techniques have progressed to the molecular level of organization. The

conventional method of dilution and spread plating is the main reason why *Acinetobacter* has traditionally been implicated as the primary active agent in BPR operations (Atkinson, 1999).

A wide range of bacteria can be isolated from activated sludge mixed liquors using conventional microbiological techniques such as sample dilution and spread plate inoculation (Atkinson, 1999). A number of methods such as the Most Probable Number (MPN) technique, pour plate method, surface plate method and membrane filtration are available for estimating numbers of selected metabolic groups in sludge (Schade and Lemmer, 1994). However, these methods (either by the composition of the media or the incubation protocol) have been shown to be extremely selective as to which organisms are allowed to grow and form visible colonies (Atkinson, 1999). The two different types of cells which remain viable but non-culturable in environmental samples can either be (Amann *et al.*, 1995):

* known species for which the cultivation conditions are not suitable for growth or which have entered a non-culturable state;
* unknown species that have not yet been cultured for lack of suitable techniques.

Sufficient documentation of the true community structure is therefore not made available (Snaidr *et al.*, 1997). This becomes evident when one considers EBPR. The most significant effect of cultivation of activated sludge mixed liquor on nutrient rich solid media is the underestimation of bacteria belonging to the beta subclass of the *Proteobacteria* and a gross overestimation of members of the gamma subclass of the *Proteobacteria* (Wagner *et al.*, 1994b). Standard plate counts do not reveal the bacterial community structure of activated sludge, but rather reflect the selectivity of the growth media for certain bacteria (Wagner and Amann, 1997). In effect, typical isolation media and conditions give rise to typical bacteria, whilst the unculturable fraction of the population, which may also be functionally active, are overlooked (Atkinson, 1999).

As far as solid media are concerned, Casitone Glycerol Yeast Autolysate Agar (CGY) has been shown to yield the highest plate counts for activated sludge samples (Pike *et al.*, 1972; Bux *et al.*, 1994). However, due to its high nutrient content, the suitability of the medium to isolate

viable bacteria from activated sludge is questionable. Solid cultivation media should, as far as possible, imitate the environment from which the bacteria of interest originate. For example, bacteria from oligotrophic systems are best enumerated with low nutrient agars like R2A and R3A agars, while bacteria from eutrophic systems are best enumerated with high nutrient agars like CGY, trypticase soy broth, nutrient and plate count agar (Osborne *et al*, 1989; Brözel and Cloete, 1992; Kämpfer et al., 1996). Nutrient rich media, when applied to population community analyses of activated sludge, will render erroneous results as it will support the growth of faster growing bacteria, which may not actually play a major role in the system (Osborn *et al.*, 1989). When isolating bacteria from an EBPR activated sludge system, Kämpfer *et al.* (1996) found that recovery rates using R2A agar were usually approximately one order of magnitude higher than the nutrient rich trypticase soy agar. Osborn *et al.* (1989) found that diluted CGY agar yielded the highest recoveries from activated sludge when compared with dilute Fuhs and Chen media, balance tank effluent and settled sewage. Yet, not one of the media was able to isolate the total amount of bacteria present in the mixed liquor samples. Different media, however, result in different population compositions, so direct comparisons of the various culture-dependent methods available to evaluate isolation efficiency are not beneficial (Atkinson, 1999). Although no medium can be expected to recover all viable cells from wastewater samples, it seems appropriate to use either chemically defined or custom manufactured media designed to yield highest counts (Reasoner and Geldreich, 1985).

The agar plate count is based on the assumption that a viable bacterial cell is capable of multiplying to form two progeny, and so on, under "optimal" conditions for the cell concerned (Postgate, 1969). Continued growth under such conditions on agar will result in a visible colony (Hattori, 1988). The count therefore reflects the number of cells capable of dividing under the given conditions, and not necessarily the total viable number present in the original sample.

There are two basic approaches to viable count procedures: the plate count technique and the MPN technique (Postgate, 1969).

2.19.2.1 Plate count methods

Plate count procedures employ various media and incubation techniques. The number of colonies formed in the standard plate count is normally counted after 48 or 72 h (Gibbs and Hayes, 1988). Yet, water contains many slow-growing bacteria which take longer to form a visible colony (Hattori, 1988). Dilution of samples can be spread onto the surface of the agar (surface spread method), or the sample suspension can be mixed with the agar just before the plates are poured (pour plate method)(Coetzee, 1999). One must consider whether the microbes can survive the plating procedure. Some microbes are killed upon exposure to air in the spread plate method, while others cannot tolerate the temperatures (45°C) needed to maintain melted agar in the pour plate method (Postgate, 1969).

The plates are incubated under specific conditions for a period of time to allow the bacteria to multiply and form macroscopic colonies, after which these colonies are counted. It is assumed that each colony originated from a single bacterial cell. Plates with too many colonies (> 300) cannot be accurately counted because one overlapping colony may represent more than one original bacterium. Plates with too few colonies (< 30) also must be discarded from the counting procedure for statistical reasons (Postgate, 1969).

2.19.2.2 Most probable number

The MPN method is an alternative to the plate count method for determination of viable organisms and uses statistical analysis and successive dilutions of the sample to reach a point of extinction. Replicate dilutions, usually three to ten per dilution, are scored as positive or negative and the pattern of scores are used in connection with appropriate statistical tables to obtain the nost probable number of viable microbes. The MPN procedure gives a statistically based estimate of the number of microbes in the sample and when relatively few replicate tubes are used, the confidence intervals are quite large. The MPN method has the advantage of permitting the use of liquid culture, avoiding the need to add a solidifying agent such as agar

with its possible contaminants. However, the method is more laborious than the plate count (Postgate, 1969).

As in the plate count procedure, media and incubation conditions can be adjusted in the MPN procedure to select for particular groups of microbes or to differentiate microbes with desired characteristics. Each procedure must be carefully selected and tested to permit the correct interpretation of results (Postgate, 1969).

2.19.3 Biochemical assays for estimation of bacterial numbers

Common techniques for estimation of the biomass activity consist of biochemical tests to measure either certain specific enzymes or specific products of bacterial metabolism. The sensitivity, reproducibility and compatibility of a number of biochemical assays for bacterial activity estimations are discussed below.

2.19.3.1 Adenosine triphosphate (ATP)

ATP is present in all microbes and can be measured with great sensitivity (Coetzee, 1999). Because ATP is rapidly lost following the death of cells, measuring ATP concentrations can be used to estimate living biomass (Holm-Hansen and Booth, 1966). ATP can be detected by the luciferin-luciferase assay in which reduced luciferin reacts with oxygen to form oxidized luciferin in the presence of the luciferase enzyme, magnesium ions and ATP. Light is emitted in this reaction, the amount of emitted light being directly proportional to the ATP concentration. There are some difficulties, however, in the accuracy of estimating microbial biomass based upon ATP measurements. Some microbes alter their ATP concentration radically when nutritional or physiological conditions change. Also, in some ecosystems such as soil, sediment and nearshore aquatic areas, ATP may be adsorbed on particles (Holm-Hansen and Booth, 1966).

2.19.3.2 Cell wall components

Almost all bacteria contain muramic acid in their cell walls and the specific relationship between murein and bacteria makes quantitation of this cell wall component useful for estimating bacterial biomass (Millar and Casida, 1970). There is a gradient of concentrations of muramic acid in Gram positive and Gram negative bacteria. To accurately use this method, it is necessary to estimate the proportion of Gram negative and Gram positive bacteria in the sample. Erroneous estimates of these proportions will yield inaccurate estimates of biomass (Millar and Casida, 1970).

2.19.3.3 Chlorophyll measurements

Chlorophyll a, the dominant photosynthetic pigment in algae and cyanobacteria, is a useful measure of the biomass of these photosynthetic microbes, even though there may not be a constant relationship between biomass and chlorophyll content (Bance, 1977). Estimation of the biomass of photosynthetic microbes based upon chlorophyll determinations has been found to correlate well with estimates such as those based upon ATP determination (Paerl *et al.*, 1976).

2.19.3.4 DNA concentration

Concentrations of DNA are maintained in relatively constant proportions within microbes and determination of DNA can be used as a measure of microbial biomass. Because DNA is synthesized in growing cells at a rate proportional to biomass, the rate of DNA synthesis reflects the growth rate of microbes. Microbial growth rates have been determined in environmental samples by incubating samples with tritiated thymidine, using autoradiography of samples to determine rates of nucleotide incorporation (Cloete and Flemming, 1997).

2.19.3.5 Photosynthesis

Both heterotrophic and autotrophic assimilation of carbon dioxide can be measured using radiolabelled bicarbonate by incubating a sample containing the indigenous microbial community with the radiolabelled substrate and then determining the amount of ^{14}C assimilated into the cellular organic matter by filtering the cells and counting the ^{14}C trapped on the filters. Washing the filters eliminates any unincorporated ^{14}C radiolabelled bicarbonate. The residual ^{14}C-containing organic compounds can be oxidized with acid dichromate and the released $^{14}CO_2$ trapped and quantitated (Cloete and Flemming, 1997).

2.19.3.6 Respiration

The release of $^{14}CO_2$ from labelled substrates can also be used to determine decomposition rates for specific substrates and, hence, microbial activity. The complete degradation of a compound to its mineral components, in which the organic carbon of the compound is converted to carbon dioxide by respiration, is called mineralization.

2.19.3.7 Specific enzyme assays

A variety of enzyme assays can be used for measuring the metabolic activities of microbes. Enzymes involved in the biogeochemical cycling of sulphur for example are of interest to microbial ecologists studying microbiologically induced corrosion. It is important that the microbial community not be altered during the assay procedure if the measurement of the enzymatic activity is to reflect *in situ* activities accurately. Caution must be taken in maintenance of *in situ* conditions, particularly with reference to temperature, moisture content and redox potential. Also, incubation periods must be short enough to preclude changes in numbers of microbes, which could alter the levels of enzymes present in the sample (Cloete and Flemming, 1997).

From the above it is clear that numerous techniques exist for the quantification of microbes, some of which represent greater ease and accuracy than others. However, direct microscopy and plate count procedures remain to be the most commonly used in industry (Coetzee, 1999).

2.19.4 Spectrophotometric measurements (turbidity and absorbance)

Light scattering is the most widely used, conveneient and least complex method for estimating total microbiological material in a liquid medium (Mallette, 1969). When light passes through matter, it is scattered apart from its original path by inhomogeneities present. If these inhomogeneities of interest are particles considerably larger than small molecules, scattering becomes relatively intense (Mallette, 1969). Light scattering is dependent upon the concentration, size and shape of the particles, the relative refractive indices of particle and medium and the wavelenght of the incident light (Mallette, 1969). Bacteria scatter light primarily in the forward direction and the amount of scattering is proportional to the mass of cells present (Kheshgi and Saunders, 1959). Scattering is measured by passing a beam of light through the culture in an instrument containing a photocell that registers the amount of light that is scattered at a 90°-angle (turbidity meter) or the amount of light that passes through without being scattered (photometer)(Sebata, 1998). The absorbancy measurement routinely employed in microbiology is more related to total bacterial mass than to bacterial numbers (Mallette, 1969). Most spectrophotometers have wavelenghts of between 350 and 800 nm and this flexibility is important as different substances absorb light at different wavelenghts (bacteria for example absorb most of the light at 540-550 nm)(Sebata, 1998). Spectrophotometers, even the simplest types, are convenient in this respect and have become the most widely used class of instruments in microbiological turbidimetry. However, several problems may arise, including light absorption, the need for calibration curves, collection of light in low scattering angles and the unwanted changes in the biological material (for example aggregation of *Pseudomonas aeruginosa* cells at high levels of phosphate) may introduce large uncertainties (Mallette, 1969).

2.20 Adenosine triphosphate (ATP)

All living things, including plants, animals and bacteria, require a continual supply of energy in order to function. This energy is used for all cellular processes which keep the organism alive. Some of these processes occur continually, such as the metabolism of food, the synthesis of large, biologically important molecules like proteins and DNA and the transport of molecules and ions throughout the organism. Other processes occur only at certain times, such as cellular movement. However, before the energy can be used, it must first be transformed into a form that the organism can easily handle. This special carrier of energy, is the ATP molecule (Brock, 1979).

The ATP molecule is composed of three components. At the center is a sugar molecule (ribose – the same molecule that forms the basis of DNA). Attached to one side of this sugar group is a base (a group consisting of linked rings of carbon and nitrogen atoms). In this case, the base is adenine. The other side of the sugar is attached to a string of phosphate groups, which are the key to the activity of ATP (Brock, 1979).

ATP is and endergonic molecule, requiring energy to be formed. Energy is stored in the covalent bonds between each phosphate group making up the tail of the molecule (Lee *et al.*, 1971). The last phosphate bond holds the most energy (approximately 7 $kcal.mol^{-1}$) and is called the pyrophosphate bond. In order to release its energy, ATP breaks down to form ADP (adenosine diphosphate) and an inorganic phosphate group, while releasing energy from the pyrophosphate bond. ADP is an exergonic molecule, yielding energy when formed. When ADP reacts and comes in contact with enough energy and an inorganic phosphate ion, it becomes ATP and stores energy yet again. ADP also needs the energy from the third phosphate group from respiration processes to become ATP (Lundin and Thore, 1976). More ATP is produced from aerobic respiration than from anaerobic respiration because there is more energy involved (Lundin and Thore, 1976).

$$ATP \rightleftharpoons ADP + \text{inorganic phosphate} + \text{energy}$$

2.20.1 Luciferin-luciferase reaction

Luciferase is an enzyme, which reacts with a small molecule called luciferin in the presence of oxygen and ATP. The resulting high-energy compound releases its energy in the form of visible light in a fraction of a second. The emitted light is "cold" and has practically no waste heat (Lundin and Thore, 1976). Luciferins vary in chemical structure. For example, the luciferin in luminescent bacteria is completely different from that of fireflies. For each type of luciferin, there is a specific luciferase. One of the advantages of using luciferase as a reporter of biomass is the convenience and the speed of performing the assay (Stanley, 1989). Using luciferase assay reagents that support maximal luciferase activity is critical because the luminescent intensity of the luciferase-mediated reaction directly impacts on the detection sensitivity of the reporter assay (Stanley, 1989).

2.20.2 The application of ATP for monitoring microbial biomass

Attempts have been made to find simple and reliable methods to determine the biomass in wastewater and activated sludge (Jørgensen *et al.*, 1992). The simplest and most often used method is to measure SS or VSS (Ali *et al.*, 1985). These methods, however, do not distinguish between living cells and debris of organic or inorganic origin.

Using the traditional total plate count technique, an underestimation of the biomass is done due to the selectivity of the media employed (Jørgensen *et al.*, 1992).

Jørgensen *et al.* (1992) determined biomass of activated sludge growth cultures in terms of dry weight and compared the data with ATP content, the OUR and FDA hydrolysis data. ATP content showed the best correlation with biomass. A conversion factor of 3 mg ATP per g dry weight was calculated. With the same methods applied to 4 full-scale systems, ATP results indicated a relationship of 67 mg dry weight per gram SS.

Roe and Bhagat (1982) estimated the ATP to suspended solids ratio at maximum viability in activated sludge, and they found that viability varied significantly with mean cell residence time. Kucknerowicz and Verstraete (1979) found a linear relationship between OUR and ATP, suggesting that ATP reflected viable biomass. However, a requirement that must be met to obtain reliable biomass estimations, is that the activity/biomass ratio remains fairly constant during the measurement period (Jørgensen *et al.*, 1992). According to Jenkinson and Ladd (1981), however, the ATP concentration of a resting soil population did not differ much from ATP in pure cultures of actively growing microbes. This was in agreement with results by Jørgensen *et al.* (1992) which showed constant ATP content to biomass ratios, independent of growth phase. Van de Werf and Verstraete (1984) however, showed extensive variation in ATP to biomass ratios during different metabolic conditions.

It can be assumed that culture is at maximum activity when it is in the exponential phase of growth. In the study by Jørgensen *et al.* (1992), the amount of viable biomass was estimated on the basis of maximum activity measurements and the conversion factor of 3 mg per g dry weight found, was well in agreement with values reported by Patterson *et al.* (1970) and Nelson and Lawrence (1980). The percentage of viable biomass in the experiments by Jørgensen *et al.* (1992) also correlated well with values in literature (Patterson *et al.*, 1970). Roe and Bhagat (1982) measured ATP levels in activated sludge from a lab-scale sludge plant, and indicated that the fraction of SS made up by viable biomass depended on sludge age, so that the highest viable biomass was obtained at the lowest sludge age.

Few studies have dealt with the estimation of viable biomass in wastewater (Jørgensen *et al.* (1992). By means of OUR measurements, Henze (1986) found that viable biomass constitutes 6 to 78% of VSS, depending on treatment and the type of wastewater.

Patterson *et al.* (1970) developed the method for ATP measurement, using the reaction between luciferin, luciferase and ATP. The finalized procedure was highly sensitive and reliable. The authors reported relative standard deviations of less than 2 % for activated sludge replicates and nearly 100 % recovery of added ATP from activated sludge. Also, the

authors claimed ATP levels in activated sludge to be relatively constant under endogenous conditions, indicating the potential of ATP as an estimate of viable biomass.

Operational control of biological waste treatment has long been dependent on estimates of *in situ* biomass in the waste stabilization process (Patterson *et al.*, 1970). A more appropriate and desirable parameter would evaluate the metabolic activity of those organisms responsible for the treatment (Patterson *et al.*, 1970). The standard parameter of biomass in activated sludge is mixed liquor suspended solids (MLVSS), although it is recognized as an indirect and incomplete measure of the viable sludge floc (Fair and Geyer, 1954; Patterson and Brezonik, 1969, Patterson *et al.*, 1970). Other biomass parameters have been suggested, including particulate organic nitrogen and protein, but these are also unsatisfactory because of the variable concentrations of nonviable particulate organic material present in sewage (Patterson *et al.*, 1970). Furthermore, rapid changes in biological activity are only slowly reflected by changes in any of these parameters (Patterson *et al.*, 1970).

A suitable parameter must fulfill certain criteria to be a useful and appropriate estimate of biomass. For example, the measured quantity should be proportional to some cellular entity (Patterson *et al.*, 1970), such as total organic carbon or dry weight. Also, the substance should have a short survival time after cell death, otherwise it would not be specific for viable biomass. There should also be a sensitive and accurate analytical procedure available to measure the parameter. The authors investigated the occurrence of ATP in activated sludge for the purpose of utilizing this parameter as a measure of metabolic activity and/or biomass. The ATP pool measured, approximated 2 µg per mg MLVSS.

To relate ATP concentration to microbial biomass, it is necessary to know the approximate ATP concentration per cell of the microbial species present (Patterson *et al.*, 1970). If ATP is also related to metabolic activity, the physiological state of the culture must be determined (Patterson *et al.*, 1970). Since it is impossible to make a taxonomic analysis of the microbiota present in activated sludge, the accuracy of biomass estimations would depend upon the constancy of the ATP pool among species (Patterson *et al.*, 1970). D'Eustachio and Levin (1967) reported a constant pool of ATP for *Escherichia coli*, *Pseudomonas fluorescens* and

Bacillus subtilis, which was also constant during all growth phases. In a later study, D'Eustachio and Johnson (1968) investigated the endogenous ATP pool of 13 species of Gram positive and Gram negative aerobic bacteria and found a mean ATP pool of 2.1 μg per mg dry cell material. Also, a linear correlation existed between the endogenous ATP pool and standard plate count for the species involved.

It was uncertain, in the study of Patterson *et al.* (1970), as to the response of the ATP pool to changes in metabolic activity. If there was no change, or only erratic variation, then ATP could not be used as an activity parameter in studies on activated sludge. Thus, an experiment was designed to this extent. Results indicated that the ATP pool is affected by the metabolic activity of an activated sludge culture and may be expected to respond rapidly and decisively to an increase in substrate loading, while only being gradually reduced as the organisms enter an endogenous phase.

Results by Patterson *et al.* (1970) indicated that a significant portion of the MLVSS is non-viable organic material not associated with the oxidative degradation of the substrate. Assuming a mean endogenous ATP pool of 2 μg per mg, dry cell material would result in an estimate that only 40% of the laboratory unit MLVSS was actually viable cell material. In a separate experiment carried out on a contact stabilization plant indicated that only 15 to 20 % of the MLVSS may be active biomass under actual operating conditions.

Upadhyaya and Eckenfelder (1975) found, in a laboratory-scale activated sludge setup, that in general, the viable fraction, as measured by ATP analysis, was found to be higher in experiments with low MLVSS because of less accumulation of non-active mass at lower MLVSS levels. Also, ATP per mass of MLVSS decreased with increases in the cell detention period. The authors also found that the ATP per plate count colony was fairly stable, substantiating the claim that ATP is a measure of viable biomass.

Levin *et al.* (1975) conducted tests at two full-scale municipal treatment plants where ATP was used to control the return sludge flow rate. BOD decreased, VSS remained constant and ATP increased for progression through a plug-flow aeration basin. The result seemed to indicate

that ATP will measure increased biomass formation by oxidation and incorporation of the BOD, but VSS will not. However, as with the investigation of Upadhyaya and Eckenfelder (1975), the ATP content of the return sludge fluctuated substantially, possibly by environmental stress in the form of low dissolved oxygen levels (Roe and Bhagat, 1982).

Specific proportions of ATP in relation to total cellular carbon was found to be constant by Atlas (1982), with variations not succeeding 17 %.

2.21 Energy Dispersive Spectrometry (EDS)

EM combined with energy dispersive spectrometric X-ray microanalysis (EDS) is a method of elemental analysis within well-defined regions of a specimen (Buchan, 1980). However, there are many problems associated with this technique when applied to biological material, as the standard specimen preparation techniques for EM can displace, transform and dissolve many elements within biological samples, which are therefore not representative of the *in vivo* situation (Buchan, 1980). Morgan (1983) discusses the chemical artifacts that tend to be introduced by the exposure of biological material to aqueous sample preparation media. Artifacts discussed include:

* element loss;
* phase transformations in mineralized tissues;
* redistribution of elements within the specimen;
* introduction of extraneous elements.

The only preparation method known which would give a reasonable representation of the *in vivo* situation would be rapid freezing, freeze sectioning and X-ray analysis in the frozen state (Buchan, 1980; Zierold and Schäfer, 1988). However, this is a complex technique requiring special instrumentation (Buchan, 1980).

In EDS, high velocity incident electrons cause ionization of the atoms in the specimen. This ionization creates a vacancy in one of the energy levels of the atoms, which is almost immediately filled by an electron, which was at a higher energy level. As these electrons transfer to the lower energy levels, the excess energy is emitted in the form of X-ray photons. If the vacancy was created in the K-shell of the atom, it is called a K X-ray. Since an electron from one of several higher energy levels could fill the vacancy, a photon corresponding to one of these transitions is emitted. These are called $K\alpha_1$, $K\alpha_2$, $K\beta$ etc. X-ray photons.

Each element in the periodic table contains electrons in orbits with particular discrete energy levels and it is on the basis of the differences between these specific energies that each element can be identified (Buchan, 1980).

In thin biological specimens, the amount of characteristic photons emitted by a particular element in a specimen is proportional to the number of atoms of that element present in the volume of material excited by the primary electron beam (Buchan, 1980).

In addition to the characteristic X-ray radiation emitted by each element in a specimen, a background radiation is also emitted over the whole energy spectrum up to the energy of the incident electrons. This radiation is known as background radiation or "bremsstrahlung" and arises from the deceleration of the electrons due to the interaction with the nuclei of all thew atoms in a specimen (Buchan, 1980).

In the case of thin biological specimens, the amount of bremsstrahlung is proportional to the mass of the specimen through which the electrons pass. The ratio of the characteristic elemental X-rays to the total bremsstrahlung X-rays generated (or the peak to background ratio) is thus proportional to the ratio of the elemental mass to the total mass in the excited volume. An example of an excitation volume can be seen in Figure 2.2. This relationship is the basis of quantitative analysis in biological specimens. The bremsstrahlung forms the background underlying the characteristic peaks as shown in an energy dispersive spectrum (Figure 2.3).

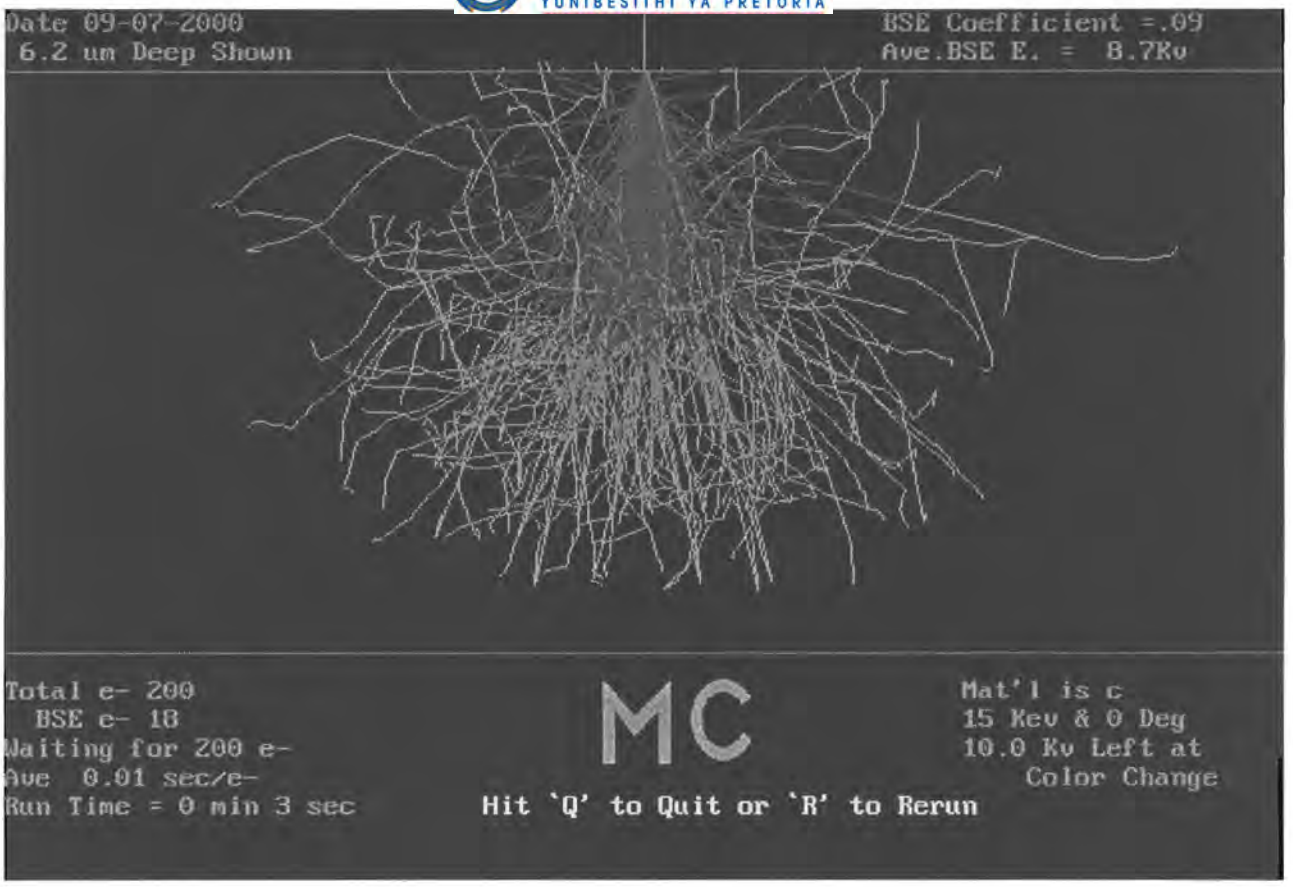

Figure 2.2: Example of the excitation volume created by EDS in a particular specimen (Monte Carlo electron flight simulator).

For quantitative analysis of biological samples, sections are considered to be thin if (Buchan, 1980):

* X-rays emitted by the elements in a specimen are not absorbed significantly by their passage through the specimen;
* the generation of additional X-rays by the process of fluorescence is also negligible.

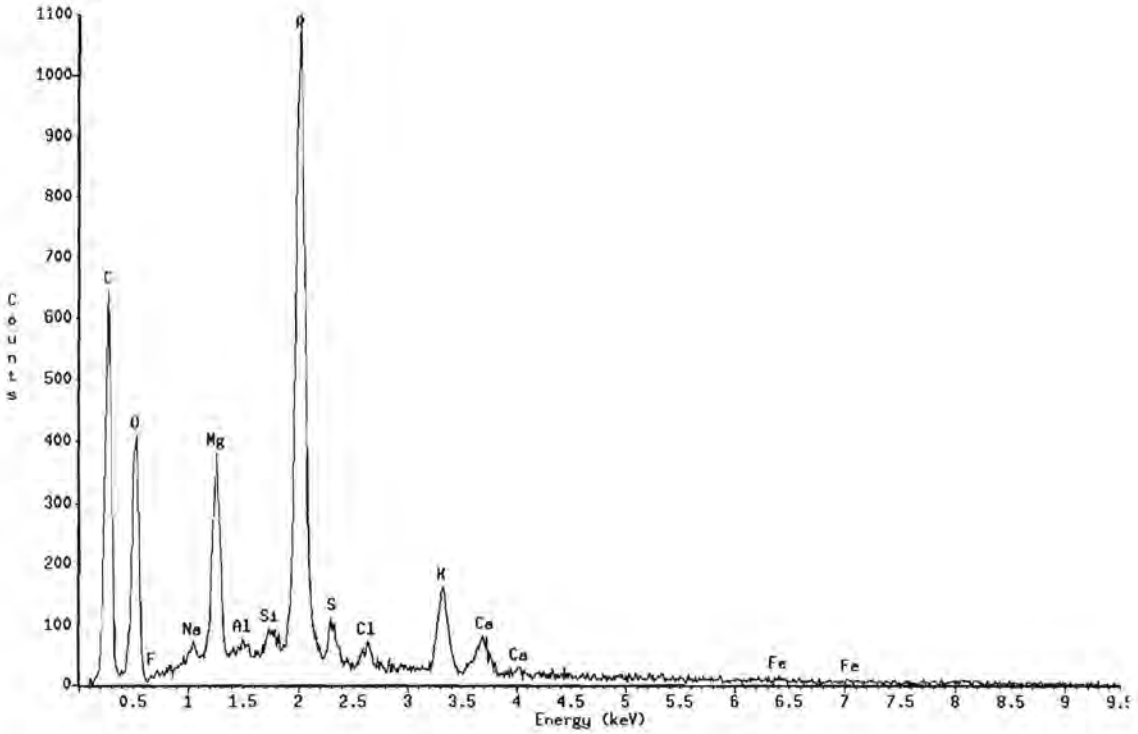

Figure 2.3: Typical EDS energy spectrum. Notice background bremsstrahlung between the Cl and K, as well as the Ca and Fe peaks.

Russ (1978) concluded that regardless of the details of the procedure, the general accuracy of the method is in the order of 10 %, and that in the analysis of biological thin sections, the greatest, and therefore, limiting errors arise in counting statistics, specimen preparation variables and in the difficulty in determining the density and thickness of specimens.

Buchan (1981) used electron microscopy in combination with EDS to examine the nature of accumulated P in seven activated sludge plants exhibiting EBPR. Large P accumulations were located in identical structures in the sludges examined. The P was located in large electron-dense bodies within large bacterial cells, characteristically grouped in clusters and later identified as *Acinetobacter* spp. (Buchan, 1983). The calcium to P ratio of these bodies precluded them from being any form of calcium phosphate precipitate. Quantitative analysis

indicated that the electron-dense bodies contained in excess of 30 % P. Extracellular P containing precipitates were not located.

2.22 Extracellular polymeric substances (EPS)

2.22.1 Definition of EPS

The production of extracellular polymeric substances (EPS) is a general property of microbes in natural environments and has been shown to occur both in prokaryotic and eukaryotic microbes. Microbial EPS are biosynthetic polymers (biopolymers). EPS were defined by Geesey (1982) as extracellular polymeric substances of biological origin that participate in the formation of microbial aggregates. The abbreviation EPS has been used for extracellular polysaccharides, exopolysaccharides, exopolymers and extracellular polymeric substances (Wingender *et al.*, 1999). Polysaccharides have often been assumed to be the most abundant components of EPS in early biofilm research (Costerton et al., 1981). However, proteins and nucleic acids (Frølund *et al.*, 1996; Nielsen *et al.*, 1997), as well as amphiphilic compounds including phospholipids (Neu, 1996; Takeda *et al.*, 1998) have been shown to appear in significant amounts or even predominate in EPS preparations from activated sludges, biofilms and pure cultures of bacteria. In addition, some researchers described humic substances as components of EPS matrices (Nielsen *et al.*, 1997, Jahn and Nielsen, 1998).

2.22.2 Composition, secretion and spatial arrangement of EPS

EPS are organic macromolecules that are formed by polymerization of similar or identical building blocks, which may be arranged as repeating units within the polymer molecules (Wingender *et al.*, 1999). Building blocks of polysaccharides include: monosaccharides, uronic acids and amino sugars bound by glycocidic bonds, and which may have the organic substituents O-acetyl, N-acetyl, succinyl and pyruvyl groups and the inorganic substituent

groups, sulphate and phosphate. Building blocks of proteins are amino acids linked by peptide bonds and my have the substituent groups oligosaccharides (in the case of glycoproteins) or fatty acids (for lipoproteins). Nucleic acids are made up of nucleotides, while phospholipids consist of fatty acids, glycerol, phosphate, ethanolamine, serine, choline and sugars, linked by ester bonds. Humic substances are made up of phenolic compounds, simple sugars and amino acids, linked by ether bonds, C-C bonds and peptide bonds. As described above, EPS may contain nonpolymeric substituents of low molecular weight, which greatly alter their structure and physicochemical properties (Wingender *et al.*, 1999).

By definition, EPS are located at or outside the cell surface, independent of their origin (Wingender *et al.*, 1999). The extracellular localization of EPS and their composition may be the result of different processes such as active secretion, shedding of cell surface material, cell lysis and adsorption from the enviroment. In all cases, EPS form the outermost surface layers of bacteria as boundary structures mediating contact and exchange processes with their biotic and abiotic environments (Wingender *et al.*, 1999).

Although EPS are not essential structures of bacteria in laboratory cultures (as loss of EPS does not impair growth and viability of cells), under natural conditions, EPS production seems to be an important survival feature, with most bacteria occur in microbial aggregates such as flocs, whose structural and functional integrity is based upon the presence of an EPS matrix (Wingender *et al.*, 1999).

2.22.3 Functions of EPS

Among activated sludge extracellular polymers, proteins predominate and, on the basis of their relatively high content of negatively charged amino acids, are supposed to be more involved than sugars in electrostatic bonds with multivalent cations, underlining their key role in the floc structure (Wingender *et al.*, 1999). In addition, proteins have also been suggested to be involved in hydrophobic bonds within the EPS matrix (Dignac *et al.*, 1998).

General functions of EPS (some of which are relevant in activated sludge) include (Wingender *et al.*, 1999):

* adhesion to surfaces;
* aggregation of bacterial cells and formation of flocs and biofilms;
* cell-cell recognition, including symbiotic relationships with plants and animals and initiation of pathogenic processes;
* structural elements of biofilms including mechanical stability and shape of EPS structures;
* protective barrier, including resistance to host defenses and resistance to biocides, disinfectants and antibiotics;
* retention of water, preventing desiccation;
* sorption of exogenous organic compounds, including scavenging and accumulation of nutrients from the environment and sorption of xenobiotics;
* sorption of inorganic ions, including accumulation of toxic metal ions (detoxification), promotion of polysaccharide gel formation and mineral formation;
* enzymatic activities, including digestion of exogenous macromolecules for nutrient acquisition and release of biofilm cells by degradation of EPS structure;
* interaction of polysaccharides with enzymes for accumulation/retention and stabilization of secreted enzymes.

2.22.4 Analysis of EPS

Historically, microbial polysaccharides were studied for three reasons (Neu and Lawrence, 1999):

* they represent a structural feature of the microbial cell, therefore investigated for pure and basic research interests;

* polysaccharides were recognized as antigen determinants of the microbial cell surface, the knowledge of their structure being of great importance in medical microbiology;
* they were recognized as a source of polymers with unique properties.

These applied aspects of polysaccharides were a reason to study their structure, properties and production on the pilot- and industrial-scale. Generally due to the pure culture philosophy in microbiology, the production, isolation and preparation of EPS were done with pure cultures only. Nowadays, more microbiologists are starting to work on defined mixed or even fully complex cultures derived from natural or technical habitats (Neu and Lawrence, 1999). This is mainly because of the fact that a study of a natural system does not involve a pure culture, but a very complex microbial community (Caldwell *et al.*, 1996), and as these complex communities produce complex mixtures of EPS, we are confronted with the challenge of analyzing these complex EPS matrices (Neu and Lawrence, 1999).

2.22.4.1 Destructive analysis of EPS

2.22.4.1.1 Physical and chemical techniques

Apart from a high water content of up to 99 %, biofilms consist mainly of EPS and cells (Neu and Lawrence, 1999). The traditional approach to investigate polysaccharides is based on the isolation of the polysaccharides from the complex cell/EPS matrix. However, these techniques disrupt the original structure of the cells, aggregates or biofilm matrix (Neu and Lawrence, 1999). Several methods have been suggested to isolate polysaccharides from biofilm systems and most are concerned with the extraction of polysaccharides from activated sludge (Nielsen and Jahn, 1999). In early publications, a variety of methods were used for extraction, although recently, employment of cation exchange resins seems to be the method of choice (Neu and Lawrence, 1999).

In typical scheme of sampling, handling and analysis of bound EPS in bioaggregates involves (Nielsen and Jahn, 1999):

* sampling and pretreatment (including washing steps, homogenization or storage);
* extraction by a suitable method;
* purification before analysis;
* analysis for macromolecular composition or other characteristics.

For optimal extraction, samples should be well homogenized without cell disruption. Microscopic checks of particle size during and after homogenization is recommended.

Washing before extraction is often conducted to remove soluble EPS (Nielsen and Jahn, 1999). It is important to select a washing buffer carrying an ionic strength and composition not too different from the sample preventing some bound EPS from desorption and being washed away from the EPS matrix (Nielsen and Jahn, 1999). The mixing of a sample with deionized water can, for example, be regarded as a simple form of extraction (Gaudy and Wolfe, 1962).

Physical methods of extraction include centrifugation, mixing or shaking, sonication or heat treatment (Nielsen and Jahn, 1999). In general, methods employing only physical methods, give lower yields than those employing both physical and chemical techniques. Heat treatment may cause significant lysis and disruption of macromolecules.

Chemical methods of extraction include addition of various chemicals to the bacterial sample that can break different linkages in the EPS matrix and facilitating a release of EPS into the water. Treatments include (Nielsen and Jahn, 1999):

* alkaline treatment by addition of NAOH, causing ionization of charged groups, like carboxylic groups, in proteins and polysaccharides, causing repulsion within the EPS matrix;

* exchange of divalent cations (for example Ca^{2+} and Mg^{2+}), responsible for cross-linking of charged compounds in the EPS matrix, with monovalent ions; the divalent ions can be removed by means of a complexing agent like EDTA;
* cation exchange by a high concentration of NaCl;
* enzymatic digestion.

The more hydrophobic part of the EPS is usually not extracted by the above methods, but may be extracted from pure cultures by the use of detergents (Nielsen and Jahn, 1999).

In many extraction studies, the accumulation of protein and nucleic acids in the crude extract has been taken as an indication of cell lysis (Nielsen and Jahn, 1999). However, it has been found that the EPS matrix usually contains large amounts of protein, nucleic acids and probably also glycoprotein (Frølund *et al.*, 1996), so that the presence of these compounds in the extract is very difficult to use as an indicator for cell lysis. Instead, other methods must be applied, for example substances that are truly intracellular and do not accumulate in the EPS matrix (Nielsen and Jahn, 1999). ATP has been used in addition to DNA as intracellular marker, but difficulty is experienced in the accuracy of the ATP measurements (Grotenhuis *et al.*, 1991). Another promising method is the appearance of intracellular enzymes like glucose-6-phosphate dehydrogenase (G6PDH) in the extract, indicating the extent of lysis (Platt *et al.*, 1985). Cell counts can only be used to see whether cells are destroyed, but not to assess whether leakage of intracellular material occurs (Frølund *et al.*, 1996).

Despite problems with the analysis of cell lysis, boiling and addition of NaOH seem to cause severe cell lysis in activated sludge (Karapanagiotis *et al.*, 1989). The cation exchange method with Dowex resin did not show strong lysis if short extraction times (less than 2 h) were conducted (Frølund *et al.*, 1996). It can be argued that some lysis does not make much of a difference on EPS composition during extraction (Frølund *et al.*, 1996), since in activated sludge for example, the cell biomass represents only 10-20 % of the total organic matter (Wanner, 1994; Frølund *et al.*, 1996; Jahn and Nielsen, 1998; Münch and Pollard, 1997).

Disruption of macromolecules during extraction may take place. It is critical if the chemical structure or some macromolecular properties are to be investigated, but not if only the total amount of EPS is determined (Nielsen and Jahn, 1999). Boiling and alkaline treatments have been reported to cause disruption of macromolecules (Karapanagiotis *et al.*, 1989). Deacylation of acylated alginates may take place, resulting in increased solubility and decreased hydrophobicity. High pH also breaks disulphide bindings in glycoproteins and uronic acids are degraded (Nielsen and Jahn, 1999). Possible disruption has not been investigated in detail for extraction by heating or sonication (Nielsen and Jahn, 1999), although no disruption is caused by the Dowex cation exchange method (Karapanagiotis *et al.*, 1989; Frølund *et al.*, 1996).

Extraction efficiency can be defined in one of two ways (Nielsen and Jahn, 1999):

* the total amount of EPS extracted from all the organic matter in a certain sample;
* the total amount of EPS extracted from the total EPS pool in a certain sample.

The second definition is the most correct definition, but since the total amount of EPS is usually unknown, this definition is rarely used (Nielsen and Jahn, 1999). The second definition does not reveal anything about the total amount of EPS, but only how much EPS a certain method can extract of the total organic matter. Comparative extraction studies using different extraction and analysis methods reflect large variation. In order to compare extraction yields, it is important to refer to a common measurement, for example the amount of organic matter (volatile matter)(Nielsen and Jahn, 1999).

It is still uncertain which part of the exopolymers is extracted by the various methods (Nielsen and Jahn, 1999). Many of the hydrophobic compounds, together with some polysaccharides, are not extracted by commonly used methods. This might explain why some tightly bound exopolymers associated with cell clusters are not extracted from activated sludge (Frølund *et al.*, 1996). It is important to realize that use of even well-standardized extraction procedures is still qualitative in nature, and perhaps only a minor part of the EPS is extracted (Nielsen and Jahn, 1999).

In many studies, it is important to know how much of the EPS is extracted (Nielsen and Jahn, 1999). Because no direct quantitative methods for separation of cell biomass and EPS are available, indirect methods must be used. A possibility is to measure the total biovolume by measuring the number and sizes of bacteria present and using conversion factors from biovolume to total organic carbon (TOC), or protein, to calculate the total contribution rom the cellular biomass fraction (Nielsen and Jahn, 1999). Cellular molecules like DNA or polysaccharide cannot be used, since the cellular content varies. Considering the usually very low extraction yields from the total organic matter recorded in many studies (3-27 %), the extraction yields in respect of the total EPS amount are in general very small (Nielsen and Jahn, 1999).

In many studies of EPS, no further purification of the crude extract is conducted before analysis of the EPS composition and amount (Nielsen and Jahn, 1999). This is common in investigations of activated sludge, granular sludge and biofilms in technical systems (Karapanagiotis *et al.*, 1989; Frølund *et al.*, 1996). In some studies, however, purification before any further analysis takes place. Methods include heat extraction, macromolecular precipitation in alcohol/acetone overnight and rinsing and dehydration in acetone/petroleum ether before analysis of components (Nielsen and Jahn, 1999). This purification step will remove various macromolecules from the EPS matrix. Further purification steps include precipitation of polysaccharides in cold alcohols, removal of protein by protease treatment or phenol extraction, or the use of gel filtration chromatography (Domenico *et al.*, 1989).

After extraction, chemical analyses include sugar analyses, linkage analysis, sequencing and determination of the anomeric configuration. The destructive analysis of polysaccharides includes (Neu and Lawrence, 1999):

* isolation by precipitation from the culture supernatant or extraction from the cell surface;
* purification by precipitation and size fractionation by gel chromatography;
* release of single constituents by various types of hydrolyses;
* determination of charged compounds by high voltage electrophoresis;

* analysis of polysaccharide constituents (sugar and non-sugar) by high performance liquid chromatography (HPLC) or gas chromatography (GC).

Additional investigation may include methylation analysis to determine the linkage pattern of the carbohydrates, analysis of the products by gas chromatography-mass spectroscopy (GC-MS), specific degradation and isolation of di- or oligosaccharides to confirm the structure (Lindberg *et al.*, 1975). ^{1}H NMR and ^{13}C NMR spectroscopy may also be used to follow derivatization procedures of the polysaccharide molecule (Perlin and Casu, 1982). Finally the anomeric configuration (D or L form) of the carbohydrates has to be determined and if the polysaccharide is available in a pure form, the three-dimensional structure may be investigated by X-ray crystallography (Rees *et al.*, 1982).

2.22.4.1.2 Electron microscopy

Electron microscopy is another destructive analysis method for EPS and the presence and significance of EPS in microbial adhesion and biofilm development has been demonstrated by different electron microscope techniques (Neu and Lawrence, 1999). SEM includes fixation and dehydration techniques and results in thin dried strands between organisms, representing the remains of the original EPS (Costerton *et al.*, 1986; Richards and Turner, 1984). Newer SEM techniques, using a cryo-transfer chamber and cold stage, were more gentle, but revealed similar strands of polymers (Richards and Turner, 1984). A technique claiming to allow examination of fully hydrated environmental samples, is the environmental SEM (ESEM)(Danilatos, 1991). However, in reality the process starts with fully hydrated samples, moves to partially hydrated samples and ends with dehydrated samples. Highest resolution is obtained in the latter stage, the result being similar to cryo-SEM techniques.

Transmission electron microscopy (TEM) also requires fixation and dehydration, as well as embedding and sectioning of the specimen. Thereby, high-resolution images of the dried EPS fibrils became visible (Costerton *et al.*, 1986).

The major disadvantage of all electron microscopic techniques lies in the creation and interpretation of artifacts caused by the various preparation steps (Neu and Lawrence, 1999).

2.22.4.1.3 Scanning probe techniques

These techniques include scanning tunneling electron microscopy, atomic force microscopy, scanning ion-conductance microscopy and scanning tunneling microscopy. In these techniques, a scanning probe provides a surface view of the material, providing resolution at the atomic level (Neu and Lawrence, 1999).

2.22.4.2 Non-destructive analysis of EPS

Methods in this section include infrared spectroscopy or FT-IR (with information given being a signal of chemical groups from conditioning films, coatings, EPS and early biofilm events), nuclear magnetic resonance spectroscopy (NMR) (giving information about biomass, flow velocity, oxygen tension and cell distribution) and confocal laser scanning microscopy or CLSM (with a wide range of probes for polysaccharides, proteins and nucleic acids) (Neu and Lawrence, 1999).

2.23 Summary

It is evident from accomplishments to date that engineers have succeeded, to a certain degree, in modelling wastewater treatment systems, especially if one considers the tremendous successes achieved with biological P removal and nitrification denitrification processes at full-scale (Atkinson, 1999). However, there are limitations to this empirical approach and EBPR processes occasionally deteriorate in P removal efficiency (Atkinson, 1999), or even routinely fails (Bond *et al.*, 1995).

The successful operation of nutrient removing activated sludge plants is dependent on good operation, coupled with good design and the maintenance of correct biomass levels. Biological phosphate removal plants in South Africa have not always given reliable and satisfactory performance (Osborn *et al.*, 1986).

Although considerable research effort has been directed towards improving understanding of the EBPR phenomenon, designs of activated sludge systems to accomplish EBPR are still based on experience and semi-empirical methods. Clearly, the need exists for design procedures based on more fundamental behavioural patterns and kinetics (Wentzel *et al.*, 1990). Quantification, as well as kinetic models of biomass in activated sludge are routinely used in design of wastewater treatment plants, in spite of the limitations which are currently encountered in biomass determination. For instance, in the current steady state design and kinetic simulation models for activated sludge, the mixed liquor suspended solids is made up of a number of components. One key component is the heterotrophic active biomass, as this component mediates the biodegration processes of COD removal and denitrification. However, the heterotrophic active biomass parameter has been only hypothetical within the structure of these models; it has not been measured directly, primarily due to the lack of suitable simple measurement techniques.

In the literature, principally microbiological techniques for biomass estimation has been proposed (Ubisi *et al.*, 1997), including pour plate or other culturing techniques (Gaudy and Gaudy, 1980), ATP analysis (Nelson and Lawrence, 1980), DNA analysis (Liebeskind and Dohmann, 1994), the use of fluorescent probes for rRNA (Wagner *et al.*, 1994c) and sequencing or ribosomal DNA (Blackall, 1994). However, these techniques have not yet been adequately integrated with the design and kinetic modelling theory, while culturing techniques have been widely criticised for their unreliability (Cloete and Steyn, 1988). The RNA and the two DNA methods are still in their infancy. The last four named methods also require sophisticated equipment and experimental techniques that are not widely available (Ubisi *et al.*, 1997).

Although the macro-environmental conditions of activated sludge have been well described,

very little is known about the micro-environment in activated sludge flocs, including diffusion gradients and the role of EPS in EBPR. Also, data on the P content of EPS has not been included in modelling of activated sludge systems and/or processes. In previous studies, Buchan (1980) used EDS to determine the location of phosphorus volutin granules in activated sludge, without attention to the phosphorus content of EPS.

The traditional approach to investigate polysaccharides is based on the isolation of the polysaccharides from the complex cell/EPS matrix. However, these techniques disrupt the original structure of the cells, aggregates or biofilm matrix (Neu and Lawrence, 1999). Several methods have been suggested to isolate polysaccharides from biofilm systems and most are concerned with the extraction of polysaccharides from activated sludge (Nielsen and Jahn, 1999).

Physical methods of extraction include centrifugation, mixing or shaking, sonication or heat treatment (Nielsen and Jahn, 1999). In general, methods employing only physical methods, give lower yields than those employing both physical and chemical techniques. Heat treatment may cause significant lysis and disruption of macromolecules.

In many extraction studies, the accumulation of protein and nucleic acids in the crude extract has been taken as an indication of cell lysis (Nielsen and Jahn, 1999).

The need exists to study the role of EPS in P removal *in situ*, generating data that can be directly incorporated into activated sludge models.

2.24 References

Abu-Zeid M.A. (1998). Water and sustainable development: the vision for world water, life and the environment. *Water Policy* **1**(1): 9-19.

Alexander W.V., Kolbe F.F., Pybus P.J., Louw A. and Terblanche A. (1994). Theoretical and case study between side stream and main stream biological removal systems. In: *Nutrient Removal from Wastewaters*, Horan J.N., Lowe P., Stentiford E. (eds.). Technomic Publishing Company, Inc., Lancaster, Pennsylvania, U.S.A. Pp. 215-226.

Ali I., Khararjian H. and Ahmed M. (1985). Viability of microbial mass in compartmentalised single activated sludge process. *Water Research* **19**: 927-932.

Alleman J.E. and Prakasam T.B.S. (1983). Reflections of activated sludge history. *Journal Water Pollution Control Federation* **55**: 436-443.

Amann R.I., Ludwig W. and Schleifer K-H. (1995). Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiological Reviews* **59**(1): 143-169.

Anderson J.P.E. (1989). Principles of and assay systems for biodegradation. In: *Biotechnology and Biodegradation*. Kamely D., Chakrabarty A. and Omenn G.S. (eds.). Gulf Publishing Co., Houston, Texas. Pp. 129-145.

Andrews J.F. (1992). Dynamics and control of the activated sludge process. Technomic Publishing Company, Inc., Lancaster, Pennsylvania, U.S.A.

Appeldoorn K.J., Kortstee G.J.J. and Zehnder A.J.B. (1992). Biological phosphate removal by activated sludge under defined conditions. *Water Research* **26**(4): 453-460.

Arhing B.K., Christiansen N., Mathrani L., Hendriksen H.V., Macario A.J.L. and De Macario E.V. (1992). Introduction of *de novo* bioremediation ability, aryl reductive dechloronation, into anaerobic granular sludge by inoculation of sludge with *Desulfomonile tiedjei*. *Applied and Environmental Microbiology* **58**: 3677-3682.

Artan N., Taşli R., Özgür N and Orhon D. (1997). The fate of P under anoxic conditions in biological nutrient removal activated sludge systems. Proceedings of the *Second International Conference on Microbes in Activated Sludge and Biofilm Processes*. IAWQ, 21-23 July, 1997, Berkeley, California.

Asmal K. (1998). Water as a metaphor for governance: issues in the management of water resources in Africa. *Water Policy* **1**(1): 95-101.

Atkinson B.W. (1999). Identification of polyphosphate accumulating bacteria from pilot- and fulll-scale nutrient removal activated sludges. M.Tech. Dissertation, Technikon Natal, Durban, South Africa.

Atlas R.M. (1982). Enumeration and estimation of microbial biomass. In: *Experimental Microbial Ecology*. Blackwell Scientific, Oxford. Pp. 84-101.

Atlas R.M. and Bartha R. (1987). Measurement of numbers, biomass, and activities in microbial ecosystems. In: *Microbial Ecology - Fundamentals and Applications*. Second edition. Cummings, Menlo Park, California, U.S.A. Pp. 195-232.

Atlas R.M. and Bartha R. (1993). Microbial Ecology-Fundamentals and Applications. Third Edition. The Benjamin/Cummings Publishing Company, Inc., Redwood City, California, U.S.A.

Bance K. (1977). Determining the carbon to chlorophyll ratio of natural phytoplankton. *Marine Biology* **41**: 199-212.

Banister S.S and Pretorius W.A. (1998). Optimisation of primary sludge acidogenic fermentation for biological nutrient removal. *Water SA* **24**(1): 35-41.

Barnard J.L. (1975). Biological nutrient removal without the addition of chemicals. *Water Research* **9**: 485.

Beacham A.M., Seviour R.J., Lindrea K.C. and Livingston I. (1990). Genospecies diversity of *Acinetobacter* isolates from a biological nutrient removal pilot plant of a modified UCT configuration. *Water Research* **24**(1): 23-29.

Bitton G. (1994). *Wastewater Microbiology*. John Wiley & Sons, New York, U.S.A.

Blackall L.L. (1994). Molecular identification of activated sludge foaming bacteria. *Water Science and Technology* **29**(7): 35-42.

Bolitho V.N. (1976). Controlling the access of nutrients from point and diffused sources with special reference to the Pretoria/Witwatersrand/Vereeniging region. *Water SA* **2**(4): 145-149.

Bond P.L., Hugenholtz P., Keller J. and Blackall L.L. (1995). Bacterial community structures of phosphate and non-phosphate-removing activated sludges from sequencing batch reactors. *Applied and Environmental Microbiology* 61(5): 1910-1916.

Bond P.L., Keller J. and Blackall L. (1997). Characterisation of enhanced biological P removal activated sludges with dissimilar P removal performance. Proceeding of the *IAWQ Second International Conference on Microbes in Activated Sludge and Biofilm Processes*. 21-23 July, 1997, Berkeley, California.

Bosch M. (1992). P uptake kinetics of *Acinetobacter* in activated mixed liquor. M.Sc. Thesis, University of Pretoria, Pretoria, South Africa.

Bosch M. and Cloete T.E. (1993). Research on biological phosphate removal in activated sludge. Water Research Commission of South Africa. WRC Report No. 314/1/93.

Brock T.D. (1979). Biology of Microbes. 3rd edition. Prentice Hall, Inc., New Jersey, U.S.A. P. 677.

Brözel V.S. and Cloete T.E. (1992). Evaluation of nutrient agars for the enumeration of viable aerobic heterotrophics in cooling water. *Water Research* **26**(8): 1111-1117.

Buchan L. (1980). The location and nature of accumulated P in activated sludge. D.Sc. Dissertation, University of Pretoria, Pretoria, South Africa.

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

Buchan L. (1983). Possible biological mechanism of P removal. *Water Science and Technology* **15**: 87-103.

Buchan L. (1984). Microbiological aspects. In: *Theory, design and operation of nutrient removal in activated sludge processes*. Wiechers H.N.S., Ekama G.A., Gerber A., Keay G.F.P., Malan W., Marais G.V., Osborn D.W., Pitman A.R., Potgieter D.J.J. and Pretorius W.A. (eds.). Water Research Commission of South Africa, Pretoria, South Africa. Pp. 9.1-9.6.

Bux F., Swalaha F.M. and Kasan H.C. (1994). Microbiological transformation of metal contaminated effluents. Water Research Commission of South Africa. WRC Report No. 357/1/94.

Caldwell D.E., Wolfaardt G.M., Korber D.R. and Lawrence J.R. (1996). Cultivation of microbial consortia and communities. In: *Manual for Environmental Microbiology*. Hurst C.J., Knudson G.R., McInerney M.J., Stetzenbach L.D. and Walker M.V. (eds.). American Society for Microbiology. Pp. 79-90.

Caldwell N.H.M, Breton B.C. and Holburn D.M. (1997). Towards the intelligent SEM. *Electron microscopy and Analysis* (Institute of Physics Conference Series No. 153). Institute of Physics Publishing. Pp. 53-56.

Carberry J.B. and Tenney M.W. (1973). Luxury uptake of phosphate by activated sludge. *Journal Water Pollution Control Federation* **45**: 2444-62.

Cech J.S. and Hartman P. (1993). Competition between polyphosphate and polysaccharide accumulating bacteria in enhanced biological phosphate removal systems. *Water Research* **27**(7): 1219-1225.

Cech J.S., Hartman P. and Macek M. (1994). Bacteria and protozoa population dynamics in biological phosphate removal systems. *Water Science and Technology* **29**(7): 109-117.

Chuang S-H. and Ouyang C-F. (2000). The biomass fractions of heterotrophs and phosphate-accumulating organisms in a nitrogen and P removal system. *Water Research* **34**(8): 2283-2290.

Chutter F.M. (1990). Evaluation of the impact of the 1 mgP/L phosphate P standard on the water quality and trophic status of Hartbeespoort Dam. *Water Sewage Effluent* **10**(1): 29-33.

Cloete T.E. (1984). The detection of *Acinetobacter* in activated sludge and its possible role in biological phosphorus removal. D.Sc. Thesis, University of Pretoria, Pretoria, South Africa.

Cloete T.E. (1994). Introduction to Microbiology. Microbios, Brooklyn, Pretoria, South Africa.

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

Cloete T.E. and Bosch M. (1994). *Acinetobacter* cell biomass, growth stage and P uptake from activated sludge mixed liquor. *Water Science and Technology* **30**: 219-230.

Cloete T.E. and Flemming H-C. (1997). Monitoring microbial populations in industrial water systems. In: Proceedings of *Biofouling and its Control in Industrial Water Systems – A One-day Management Workshop*. University of Pretoria, South Africa. Pp. 39-47.

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

Cloete T.E., Bosch and Mienie N.J.J. (1994). Organisms other than *Acinetobacter* capable of P removal from activated sludge mixed liquor. In: *Nutrient Removal from Wastewaters*. Horan N.J., Lowe P. and Stentiford E. (eds.). Technomic Publishing Co., Inc., Lancaster, Pennsylvania, U.S.A. Pp. 263-266.

Coetzee F.J. (1999). Biofilm biomass quantification and efficiency in a biofilm reactor for treating municipal wastewater. M.Sc. Thesis, University of Pretoria, Pretoria, South Africa.

Collins M.D. and Jones D. (1981). Distribution of isoprenoid quinone structural types in bacteria and their taxonomic implications. *Microbiological Reviews* **45**(2): 316-354.

Comeau Y., Hall K.J., Hancock R.E.W. and Oldham K.W. (1986). Biochemical model for enhanced biological P removal. *Water Research* **20**(12): 1511-1521.

Costerton J.W., Irvin R.T. and Cheng K-J. (1981). The bacterial glycocalyx in nature and disease. *Annual Review of Microbiology* **35**: 299-324.

Costerton J.W., Nickel J.C. and Ladd T.I. (1986). Suitable methods for the comparative study of free-living and surface-associated bacterial populations. In: *Bacteria in Nature*. Pointdexter J.S. and Leadbetter E.R. (eds.). Plenum Press, New York, U.S.A. Pp. 49-84.

D'Eustachio A.J. and Levin G.V. (1967). Levels of adenosine triphosphate during bacterial growth. *Bacteriological Proceedings, American Society for Microbiology, Microbial Physiology Division* **20**: 121.

D'Eustachio A.J. and Johnson D.R. (1968). Adenosine triphosphate content of bacteria. *Federation Proceedings, American Society for Experimental Biology*. **27**: 761.

Danilatos G.D. (1991). Review and outline of environmental SEM at present. *Journal of Microscopy* **162**: 391-402.

De Haas D.W. (1998). The use of simultaneous chemical precipitation in modified activated sludge systems exhibiting enhanced biological phosphate removal. Ph.D. Thesis, University of Cape Town, Cape Town, South Africa.

Dignac M-F., Urbain V., Rybacki D., Bruchet A., Snidaro D. and Scribe P. (1998). Chemical description of extracellular polymers: implication on activated sludge floc structure. *Water Science and Technology* **38**: 45-53.

Dillon P.J. and Molot L.A. (1996). Long-term P budgets and an examination of the steady-state mass balance model for central Ontario lakes. *Water Research* **20**(10): 2273-2280.

Domenico P., Diesrich D.L. and Cunha B.A. (1989). Quantitative extraction and purification of exopolysaccharides from Klebsiella pneumoniae. *Journal of Microbiological Methods* **9**: 211-219.

Eckenfelder W.W. Jr., Patockza J. and Watkin P. (1985). Wastewater treatment. *Chemical Engineering Journal* **90**: 60-74.

Ekama G.A. and Wentzel M.C. (1997). Denitrification kinetics in biolgical N & P removal activated sludge systems treating municipal wastwaters. *Journèes Internationales d'Etude du Cebedeau*, Liège, Palais des Congrès, 22-23 May, 1997.

Ekama G.A., Marais G.V.R. and Siebritz I.P. (1984). Biological excess phosphate removal. In: *Theory, Design and Operation of Nutrient Removal Activated Sludge Processes*. Wiechers H.N.S., Ekama G.A., Gerber A., Keay G.F.P., Malan W., Marais G.V., Osborn D.W., Pitman A.R., Potgieter D.J.J. and Pretorius W.A. (eds.). Water Research Commission of South Africa, Pretoria, South Africa. Pp. 7.1-7.32.

Ehlers M.M. (1995). The sensitivity of DNA reannealing kinetics for bacterial diversity determination. M.Sc. (Agric) Thesis, University of Pretoria, Pretoria, South Africa.

Ehlers M.M, Erasmus A. and Cloete T.E. (1998). Fingerprinting of activated sludge systems using PAGE analysis of total protein extractions for the optimization of biological P removal. Water Research Commission of South Africa. WRC Report No. 776/1/98.

Erasmus A.S. (1997). Immunochemical investigation of enhanced phosphate removal by activated sludge. M.Sc. (Agric) Thesis, University of Pretoria, Pretoria, South Africa.

Fair G.M. and Geyer J.C. (1954). *Water Supply and Waste Water Disposal*. Wiley, New York, USA. P. 721.

Forster C.F. and Dallas-Newton J. (1980). Activated sludge settlement – some suppositions and suggestions. *Water Pollution Control* **79**: 338-351.

Frølund B, Palmgren R., Keiding K and Nielsen P.H. (1996). Extraction of extracellular polymers from activated sludge using a cation exchange resin. *Water Research* **30**:1749-1758.

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

Ganczarcyk J.J. (1983). Activated Sludge Process: Theory and Practice (Pollution Engineering and Technology 23). Marcell Dekker Inc.

Gaudy A.F. and Gaudy E.T. (1980). *Microbiology for Environmental Scientists and Engineers*. McGraw-Hill Book Co., New York, U.S.A.

Gaudy E. and Wolfe R.S. (1962). Composition of an extracellular polysaccharide produced by *Sphaerotilus natans*. *Applied Microbiology* **10**: 200-205.

Geesey G.G. (1982). Microbial exopolymers: ecological and economic considerations. *ASM News* **48**: 9-14.

Gerber A., Mostert E.S., Winter C.T. and De Villiers R.H. (1987). Interactions between phosphate, nitrate and organic substrate in biological nutrient removal processes. *Water Science and Technology* **19**: 183-194.

Gibbs R.A. and Hayes C.R. (1988). The use of R2A medium and the spread plate method for the enumeration of heterotrophic bacteria in drinking water. *Letters in Applied Microbiology* **6**: 19-21.

Grotenhuis J.T.C., Smit M., Van Lammeren A.A.M., Stams A.J.M and Zehnder A.J.B. (1991). Localization and quantification of extracellular polymers in methanogenic granular sludge. *Applied Microbiology and Biotechnology* **36**: 115-119.

Gottschalk G. (1979). *Bacterial Metabolism*. Springer-Verlag, Inc., New York, U.S.A.

Government Gazette (1984). Requirements for the purification of waste water or effluent. *Government Gazette* **227**(991): 12-17.

Grady C.P.L. Jr and Lim H.C. (1980). Activated sludge. In: *Biological Wastewater Treatment Theory and Applications*. Cheremisinoff P.N. (ed.). Marcel Dekker Inc., New York. Pp. 619-681.

Gray N.F. (1989). *Biology of Wastewater Treatment*. Oxford University Press, Oxford.

Grubbs R.B. (1984). Panel discussion: Emerging Industrial Applications. In*: Genetic Control of Environmental Pollutants*. Omenn G.S. and Hollander A. (eds.). Plenum, New York, U.S.A. Pp. 331-349.

Haarhoff J., Langenegger O. and Van der Merwe P.J. (1992). Practical aspects of water treatment plant design for a hypertrophic impoundment. *Water SA* **18**(1): 27-36.

Hattori T. (1988). *The viable count*. Springer Verlag, Berlin.

Helmer C. and Kunst S. (1998). Low temperature effects on P release and uptake by microbes in EBPR plants. *Water Science and Technology* **37**: 531-539.

Henze M. (1986). Nitrate vs. oxygen utilization rates in wastewater and activated sludge systems. *Water Science and Technology* **18**: 115-122.

Henze M., Gujer W., Mino T., Matsuo T., Wentzel M.C. and Marais G.v.R. (1995). *Activated sludge model No. 2*. IAWQ Scientific and Technical Report No. 3. IAWQ, London.

Heukelekian H. (1951). Factors affecting the quantity of sludge production in the activated sludge process. *Sewage and Industrial Wastes* **23**: 945.

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

Hiraishi A., Morishima Y. and Takeuchi J-I. (1991). Numerical analysis of lipoquinone patterns in monitoring bacterial community dynamics in wastewater treatment systems. *Journal of General and Applied Microbiology* **37**: 57-70.

Hiraishi A., Ueda Y. and Ishihara J. (1998). Biomarker and molecular approaches to the microbial community analysis of activated sludge. *Proceedings of Microbial Community and Functions in Wastewater Treatment Processes*. The International Symposium of the Centre of Excellence, Department of Urban Engineering, School of Engineering, University of Tokyo, 10-11 March, 1998.

Holm-Hansen O. and Booth C.R. (1966). The measurement of adenosine triphosphate in the ocean and its ecological significance. *Limnology and Oceanography* **11**: 510-519.

Horan N.J. (1990). *Biological Wastewater Treatment Systems: Theory and Operations*. John Wiley & Sons, Chichester, England.

Howgrave-Graham A.R. (1986). Detection of filamentous bacteria in activated sludge. M.Sc. Thesis, University of Pretoria, Pretoria, South Africa.

Jacobs L., De Bruyn E.E. and Cloete T.E. (1996). Spectrophotometric monitoring of biofouling. *Water Science and Technology* **34**: 533-540.

Jahn A. and Nielsen P.H. (1998). Cell biomass and exopolymer composition in sewer biofilms. *Water Science and Technology* **37**: 17-24.

Jenkins D. and Tandoi V. (1991). The applied microbiology of enhanced biological P removal – accomplishments and needs. *Water Research* **25**(12): 1471-1478.

Jenkinson D.S. and Ladd J.N. (1981). Microbial biomass in soil: measurement and turnover. In: *Soil Biochemistry*. Paul E.A. and Ladd J.N. (eds.). Vol. 5. Dekker, New York, U.S.A. Pp. 415-471.

Jørgensen B.B. (1989). Biogeochemistry of chemoautotrophic bacteria. In: *Autotrophic Bacteria*. H.G. Schlegel and B. Bowien (eds.). Springer-Verlag, Berlin. Pp. 117-146.

Jørgensen P.E., Eriksen T. and Jensen B.K. (1992). Estimation of viable biomass in wastewater and activated sludge by determination of ATP, oxygen utilisation rate and FDA hydrolysis. *Water Research* **26**:1495-1501.

Joska M.A. and Bolton J. (1994). Preliminary investigation into algal weeds in inland waters. Water Research Commission of South Africa. WRC Report No. 426/1/94.

Kämpfer P., Erhart R., Beimfohr C., Böhringer J., Wagner M. and Amann R. (1996). Characterisation of bacterial communities from activated sludge: culture-dependent numerical identification versus *in situ* identification using group- and genus-specific rRNA-targeted oligonucleotide probes. *Microbial Ecology* **32**: 101-121.

Karapanagiotis N.K., Rudd T., Sterritt R.M. and Lester J.N. (1989). Extraction and characterisation of extracellular polymers in digested sewage sludge. *Journal of Chemical Technology and Biotechnology* **44**: 107-120.

Kavanaugh R.G. and Randall C.W. (1994). Bacterial populations in a biological nutrient removal plant. *Water Science and Technology* **7**: 25-34.

Kawaharasaki M., Tanaka H., Kanagawa T. and Nakamura K. (1999). *In situ* identification of polyphosphate-accumulating bacteria in activated sludge by dual staining with rRNA-targeted oligonucleotide probes and 4'6-diamidino-2-phenylindole (DAPI) at a polyphosphate-probing concentration. *Water Research* **33**(1): 257-265.

Kennedy M.S., Grammas J. and Arbuckle W.B. (1990). Parachlorophenol degradation using bioaugmentation. *Journal Water Pollution Control Federation* **62**: 227-233.

Kerrn-Jespersenn J.P. and Henze M. (1993). Biological P uptake under anoxic and aerobic conditions. *Water Research* **27**(4): 617-624.

Kheshgi S. and Saunders R. (1959). Microbiological evaluation of peptones. *Journal of Biochemical and Microbiological Technology and Engineering* **1**:115-127

Knight G.C., McDonnell S.A., Seviour R.J. and Soddell J.A. (1993). Identification of *Acinetobacter* isolates using the Biolog identification system. *Letters in Applied Microbiology* **16**: 261-264.

Kuba T., Van Loosdrecht M.C.M., Murnleitner E. and Heijnen J.J. (1997). Kinetics and stoichiometry in the biological P removal process with short cycle times. *Water Research* **31**(4): 918-928.

Kucknerowicz F. and Verstraete W. (1979). Direct measurement of microbial ATP in activated sludge samples. *Journal of Chemical Technology and Biotechnology* **29** :707-712.

Kuehn M., Hausner M., Bungartz H-J., Wagner M., Wilderer P.A. and Wuertz S. (1998). Automated confocal laser scanning microscopy and semiautomated image processing for analysis of biofilms. *Applied and Environmental Microbiology* **64**(11): 4115-4127.

Kumaran P. and Shivaraman N. (1988). Biological treatment of toxic industrial waste. In: *Biotreatment Systems*, Vol. 1. D.L. Wise (ed.). CRC Press, Boca Raton, Florida, U.S.A. Pp. 227-283.

Lee C.C., Harris K.F., Syers J.K. and Armstrong D.E. (1971). Adenosine triphosphate content of Selenastrum capricornutum. *Applied Microbiology* **21**(5): 957-958.

Liebeskind M. and Dohmann M. (1994). Improved method of activated sludge biomass determination. *Water Science and Technology* **29**(7): 7-13.

Lilley I.D., Pybus P.J. and Power S.P.B. (1997). Operating manual for biological nutrient removal wastewater treatment works. Water Research Commission of South Africa. WRC Report No. TT 83/97.

Lindberg B., Lönngren J. and Svensson S. (1975). Specific degradation of polysaccharides. *Advances in Carbohydrate Chemistry and Biochemistry* **31**: 185-240.

Liu W-T., Marsh T.L. and Forney L.J. (1997). Determination of the microbial diversity of anaerobic-aerobic activated sludge by a novel molecular biological technique. Proceedings of the *IAWQ Second International Conference on Microbes in Activated Sludge and Biofilm Processes*. 21-23 July, 1997, Berkeley, California.

Lötter L.H. (1985). The role of bacterial phosphate metabolism in enhanced P removal from the activated sludge process. *Water Science and Technology* **17**: 127-138.

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

Lötter L.H. and Murphy M. (1988). Microscopic evaluation of carbon and P accumulation in nutrient removal activated sludge plants. *Water Science and Technology* **20**: 37-49.

Lundin A. and Thore A. (1976). Comparison of methods for extraction of bacterial adenosine triphosphate determined by firefly assay. *Applied Microbiology* **30**(5): 713-721.

Mader S.S. (1998). *Biology*. Sixth edition. McGraw-Hill Companies, U.S.A.

Mallettte M.F. (1969). Evaluation of growth by physical and chemical means. In: *Methods in Microbiology*. Norris J.R. and Ribbons D.W. (eds.). Vol. 1. Pp. 522-560.

Marais G.v.R and Ekama G.A. (1984). Fundamentals of biological behaviour. In: *Theory, Design and Operation of Nutrient Removal Activated Sludge Processes*. Wiechers H.N.S., Ekama G.A., Gerber G.F.P., Keay G.F.P., Malan W., Marais G.v.R., Osborn D.W., Pitman A.R., Potgieter D.J.J. and Pretorius W.A. (eds.). Water Research Commission of South Africa, Pretoria, South Africa. Pp. 1.1-1.8.

Martin R.F. and Wilson T.E. (1994). Design and operation of nutrient removal facilities in the U.S. In: *Nutrient Removal From Wastewaters*. N.G. Horan, P. Lowe and E. Stentiford (eds.). Technomic Publishing Company, Inc., Lancaster, Pennsylvania, U.S.A. Pp. 173-186.

Maszenan A.M., Seviour R.J., Patel B.K.C., Rees G.N. and McDougall B. (1998). The hunt for the G-bacteria in activated sludge biomass. *Water Science and Technology* **37**(4): 65-69.

Millar W.N. and Casida L.E. (1970). Evidence for muramic acid in soil. *Canadian Journal of Microbiology* **16**: 299-304.

Momba M.N.B. (1995). Phosphate removal in activated sludge and its relationship to biomass. M.Sc. Thesis, University of Pretoria, Pretoria, South Africa.

Momba M.N.B. and Cloete T.E. (1996a). The relationship of biomass to phosphate uptake by *Acinetobacter junii* in activated sludge mixed liquor. *Water Research* **30**(2): 364-370.

Momba M.N.B. and Cloete T.E. (1996b). Biomass relationship to growth and phosphate uptake of *Pseudomonas fluorescens*, *Escherichia coli* and *Acinetobacter radioresistans* in mixed liquor medium. *Journal of Industrial Microbiology* **16**: 364-369.

Morgan A.J. (1983). Non-freezing techniques of preparing biological specimens for electron microprobe X-ray microanalysis. In: *Basic Methods in Biological X-ray Microanalysis*. Compiled by G.M. Roomans and J.D. Shelburne. Published by Scanning Electron Microscopy, Inc. Pp. 67-80.

Münch E. and Pollard P.C. (1997). Measuring bacterial biomass-COD in wastewater containing particulate matter. *Water Research* **31**: 2550-2556.

Muyima N.Y.O. (1995). Phosphate uptake by immobilized activated sludge in activated sludge mixed liquor. Ph.D. Thesis, University of Pretoria, Pretoria, South Africa.

Muyima N.Y.O., Momba M.N.B. and Cloete T.E. (1997). Biological methods for the treatment of wastewaters. In: *Microbial Community Analysis: The Key to the Design of Biological Wastewater Treatment Systems*. Cloete T.E. and Muyima N.Y.O. (eds.). IAWQ Scientific and Technical Report No. 5. IAWQ, London. Pp. 1-24.

Nakamura K., Kawaharasaki M., Hanada, S., Kamagata Y. and Kanagawa T. (1998). Characterization of bacterial community constructing anaerobic/aerobic activated sludge by in situ identification and cloning of 16S rDNA's. Proceedings of *Microbial Community and Functions in Wastewater Treatment Processes*. The International Symposium of the Centre of Excellence, Department of Urban Engineering, School of Engineering, University of Tokyo, 10-11 March, 1998.

Nelson P.O. and Lawrence A. (1980). Microbial viability measurements and activated sludge kinetics. *Water Research* **14**: 217-225.

Neu T.R. (1996). Significance of bacterial surface-active compounds in interaction of bacteria with interfaces. *Microbiological Reviews* **60**: 151-166.

Neu T.R. and Lawrence J.R. (1999). In situ characterization of extracellular polymeric substances (EPS) in biofilm systems. In: *Microbial Extracellular Polymeric Substances – Characterization, Structure and Function*. Wingender J., Ney T.R and Flemming H-C. (eds.). Springer-Verlag, Berlin, Germany. Pp. 21-47.

Nicholls H.A. and Osborn D.W. (1979). Bacterial stress: prerequisite for biological removal of P. *Journal Water Pollution Control Federation* **51**(3): 557-569.

Nicolella C., Di Felice R. and Rovatti M. (1997). Biomass concentration in fluidized bed biological reactors. *Water Research* **31**(4): 936-940.

Nielsen P.H. and Jahn A. (1999). Extraction of EPS. In: *Microbial Extracellular Polymeric Substances – Characterization, Structure and Function*. Wingender J., Ney T.R and Flemming H-C. (eds.). Springer-Verlag, Berlin, Germany. Pp. 49-72.

Nielsen P.H., Jahn A. and Palmgren R. (1997). Conceptual model for production and composition of exopolymers in biofilms. *Water Science and Technology* **36**: 11-19.

Nielsen P.H., Andreasen K., Lee N., Wagner M. and Nielsen J.L. (1998). Autoradiography for *in situ* analysis of microbial community structure in wastewater processes. *Proceedings of Microbial Community and Functions in Wastewater Treatment Processes*. The International Symposium of the Centre of Excellence, Department of Urban Engineering, School of Engineering, University of Tokyo, 10-11 March, 1998.

Nielsen P.H, Andreasen K., Lee N. and Wagner M. (1999). Use of microautoradiography and fluorescent *in situ* hybridization for characterization of microbial activity in activated sludge. *Water Science and Technology* **39**(1): 1-9.

Nogaj R.J. (1980). Selecting wastewater aeration equipment. In: *Industrial Wastewater and Solid Waste Engineering*. Cavaseno V. and the Staff of Chemical Engineering (eds.). McGraw-Hill Publications Co., New York, U.S.A. Pp. 144-152.

Oellermann R.A. and Pearce K. (1995). Bioaugmentation Technology for Wastewater Treatment in South Africa. Report to the Water Research Commission of South Africa by the Division of Water technology, CSIR. WRC Report no. 429/1/95.

Osborn D.W., Lötter L.H., Pitman A.R. and Nicholls H.A. (1986). Enhancement of Biological Phosphate Removal by Altering Process Feed Composition. Report to the Water Research Commission of South Africa by the City Health and City Engineers Departments of Johannesburg City Council. WRC Report no. 137/1/86.

Oldham W., Abraham K., Dawson R.N. and McGeachie G. (1994). Primary sludge fermentation design and optimization for biological nutrient removal plants. In: *Nutrient Removal from Wastewaters*. N.G. Horan, P. Lowe and E. Stintford (eds.). Technomic Publishing Company, Inc., Lancaster, Pennsylvania, U.S.A. Pp. 187-198.

Osborn D.W., Lötter L.H., Pitman A.R. and Nicholls H.A. (1989). Two-year study on the enhancement of biological phosphate removal by altering process feed composition (plant and laboratory studies). Water Research Commission of South Africa. WRC Report No. 137/2/89.

Owen W.F., Stuckey D.C., Healy J.B., Young L.Y. Jr. and McCarthy P.L. (1979). Bioassay for monitoring biochemical potential and anaerobic toxicity. *Water Research* **13**: 485 492.

Pirt S.J. (1975). *Microbe and Cell Cultivation*. Halsted Press, New York, U.S.A.

Paerl H.W., Tilzer M.M. and Goldman C.R. (1976). Chlorophyll a versus adenosine triphosphate as algae biomass indicators in lakes. *Journal of Phycology* **12**: 242-246.

Patterson J.W. and Brezonik P.L. (1969). *Journal of the Sanitary Engineering Division*. Printed by the American Society of Civil Engineering. **95**: 775.

Patterson J.W., Brezonik P.L. and Putnam H.U. (1970). Measurement and significance of adenosine triphosphate in activated sludge. *Environmental Science and Technology* **4**: 569-575.

Perlin A.S. and Casu B. (1982). Spectroscopic methods. In: *The Polysaccharides*. Aspinall G.O. (ed.). Vol. 1. Academic Press, New York, U.S.A. Pp. 133-196.

Pike E.B., Carrington E.G. and Ashburner P.A. (1972). An evaluation of procedures for enumerating bacteria in activated sludge. *Journal of Applied Bacteriology* **35**: 309-321.

Pitman A.R. (1984). Operation of biological nutrient removal plants. In: *Theory, design and operation of nutrient removal activated sludge processes*. A collaborative information document prepared for the Water Research Commission by the University of Cape Town, City Council of Johannesburg and the National Institute for Water Research of the CSIR, Pretoria, South Africa. Pp. 11-1 to 11-6.

Platt R.M., Geesey G.G., Davis J.D. and White D.C. (1985). Isolation and partial chemical analysis of firmly bound exopolysaccharide from adherent cells of a freshwater sediment bacterium. *Canadian Journal of Microbiology* **31**: 675-680.

Polz M.F. and Cavanaugh M.C. (1977). A simple method for quantification of uncultured microbes in the environment based on *in vitro* transcription of 16S rRNA. *Applied and Environmental Microbiology* **63**(3): 1028-1033.

Pomeroy, L.R. (1984). Significance of microbes in carbon and energy flow in marine ecosystems. In: *Current Perspectives in Microbial Ecology*. M.J. Klug and C.A. Reddy (eds.). American Society for Microbiology, Washington, D.C., U.S.A. Pp. 405-411.

Porter K.G. and Feig Y. (1980). The use of DAPI for identifying and counting aquatic microflora. *Limnology and Oceanography* **25**(5): 943-948.

Postgate J.R. (1969). Viable counts and viability. In: *Methods in Microbiology*. Vol. 1. Norris J.R. and Gibbons D.W. (eds.). Academic Press, London. Pp. 611-628.

Punrattanasin W. and Randall C.W. (1998). The effect of the influent COD/TP ratio upon the performance of biological nutrient removal processes. Proceedings (Book 1) of the *19th IAWQ Biennial Water Quality International Conference*. 21-26 June 1998, Vancouver, Canada.

Purves W.K., Orians G.H., Heller C.H. (1995). *Life - The Science of Biology*. Fourth Edition. Sinauer Associates, Inc.

Reasoner D.J. and Geldreich E.E. (1985). A new medium for the enumeration and subculture of bacteria from potable water. *Applied and Environmental Microbiology* **49**: 1-7.

Rees D.A., Morris E.R., Thom D. and Madden J.K. (1982). Shapes and interaction of carbohydrate chains. In: *The Polysaccharides*. Aspinall G.O. (ed.). Vol. 1. Academic Press, New York, U.S.A. Pp. 185-290.

Richards S.R. and Turner R.J. (1984). A comparative study of techniques for the examination of biofilms by scanning electron microscopy. *Water Research* **18**: 767-773.

Rittmann B.E., Smets B.F., and Stahl D.A., (1990). The role of genes in biological treatment processes. *Environmental Science and Technology* **24**: 23-29.

Rochow T.G. and Tucker P.A. (1994). *Introduction to Microscopy by means of Light, Electrons, X-rays or Acoustics*. 2nd edition. Plenum Press, New York, U.S.A.

Roe P. and Bhagat S.K. (1982). Adenosine triphosphate as a control parameter to activated sludge processes. *Journal Water Pollution Control Federation* **54**: 244-254.

Romanski J., Heider M. and Wiesmann U. (1997). Kinetics of anaerobic orthophosphate release and substrate uptake in enhanced biological P removal from synthetic wastewater. *Water Research* **31**(12): 3137-3145.

Roszak D.B. and Colwell R.R. (1987). Survival strategies of bacteria in the natural environment. *Microbiological Reviews* **51**: 365-379.

Rudd R.T. (1979). The necessity for the promulgation of standards for the limitation of nutrients in effluents in sensitive areas in South Africa. In: *Nutrient Removal from Municipal Effluents*. Technology Transfer Seminar, Pretoria, 17 May 1979.

Russ J.C. (1978). Electron probe X-ray microanalysis – principles. In: *Electron Probe Microanalysis in Biology*. Erasmus D.A. (ed.). Chapman and Hall, London. Pp. 5-36.

Rustrian E., Delgenes J.P. and Moletta R. (1997). P release by pure cultures of *Acinetobacter* sp.: effect of the growth stage with cells cultivated on various carbon sources. *Letters in Applied Microbiology* **24**: 144-148.

Saayman G.B. (1995) Chemical control of sludge bulking in a full-scale biological nutrient removal activated sludge plant. M.Sc. Thesis, University of Pretoria, Pretoria, South Africa.

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

Satoh H., Ramey W.D., Koch F.A., Oldham W.K., Mino T. and Matsuo T. (1996). Anaerobic substrate uptake by the enhanced biological P removal activated sludge treating real sewage. Proceedings of *Water Quality International '96*. 18th IAWQ Biennial International Conference and Exhibition, 23-28 June, Singapore.

Satoh H., Mino T. and Matsuo T. (1997). Anaerobic uptake of glutamate and aspartate by the enhanced biological P removal activated sludge. Proceedings of the *Second International Conference on Microbes in Activated Sludge and Biofilm Processes*. IAWQ, 21-23 July 1997, Berkeley, California.

Schade M. and Lemmer H. (1994). Counting bacteria of selected metabolic groups in activated sludge – an assessment of methods. *Water Science and Technology* **29**(7): 75-79.

Sebata S.L. (1998). The development of peptone and yeast extract as microbiological culture media components. MSc Thesis, University of Pretoria, Pretoria, South Africa.

Serageldin I. (1998). Water in the 21st century: some issues. *Water Policy* **1**(1): 123-127.

Shelton D.R. and Tiedje J.M. (1984). General method for determining anaerobic biodegradation potential. *Applied and Environmental Microbiology* **47**: 850-857.

Sidat M., Kasan H.C. and Bux F. (1999). Laboratory-scale investigation of biological phosphate removal from municipal wastewater. *Water SA* **25**(4): 459-462.

Slim J.A. (1987). Some developments in the water industry in South Africa. *Water Pollution Control* **86**(2): 262-271.

Snaidr J., Amann R., Huber I., Ludwig W. and Schleifer K-H. (1997). Phylogenetic analysis and *in situ* identification of bacteria in activated sludge. *Applied and Environmental Microbiology* **63**(7): 2884-2896.

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

Stanley P.E. (1989). A concise beginner's guide to rapid microbiology using adenosine triphosphate (ATP) and luminescence. In: *ATP Luminescence, Rapid Methods in Microbiology, Technical Series No. 26*. Stanley P.E., McCarthy B.J. and Smither R. (eds.). Blackwell Scientific Publications. Pp. 1-10.

Starr C. and Taggart R. (1981). *Biology - The Utility and Diversity of Life*. Fifth edition. Wadsworth Inc.

Steyn P.L. and Cloete T.E. (1989). The adaptation of Koch's postulates to microbial ecology. *South African Journal of Science* **85**: 360-361.

Streichan M., Golecki J.R. and Schon G. (1990). Polyphosphate accumulating bacteria from sewage plants with different processes for biological removal. *FEMS Microbiology Ecology* **73**:113-124.

Takeda M., Nakano F., Nagase T., Iohara K. and Koizumi J-I. (1998). Isolation and chemical composition of the sheath of *Sphaerotilus natans*. *Bioscience, Biotechnology and Biochemistry* **62**: 1138-1143.

Thornton J.A. (1980). A comparison of the summer P loadings to three Zimbabwean water-supply reservoirs of varying trophic states. *Water SA* **6**(4): 163-170.

Toerien D.F., Gerber A., Lötter L.H. and Cloete T.E. (1990). Enhanced biological P removal in activated sludge systems. *Advances in Microbial Ecology* **1**: 173-230.

Ubisi M.F., Jood T.W., Wentzel M.C. and Ekama G.A. (1997). Activated sludge mixed liquor heterotrophic active biomass. *Water SA* **23**(3): 239-248.

Umgeni Water (1996). Make Every Drop Count. *Natal Witness Supplement*, 15 July, 1996.

Vaker D., Connell C.H. and Wells W.N. (1967). Phosphate removal through municipality wastewater treatment at San Antonio, Texas. *Journal Water Pollution Control Federation* **39**: 750-771.

Van de Werf H. and Verstraete W. (1984). ATP measurement by bioluminescence: environmental applications. In: *International Symposium on Analytical Applications of Bioluminescence and Chemiluminescence*. Kricka L.J. *et al.* (eds.). Academic Press, London.

Venter S.N, Lötter L.H., De Haas D.W. and MacDonald L. (1989). The use of the analytical profile index in the identification of activated sludge bacteria: problems and solutions. *Water SA* **15**(4): 265-267.

Wagner M. and Amann R. (1997). Molecular techniques for determining microbial community structures in activated sludge. In: *Microbial Community Analysis: the Key to the Design of Biological Wastewater Treatment Systems*. Cloete T.E. and Muyima N.Y.O. (eds.). IAWQ Scientific and Technical Report No. 5. IAWQ, London. Pp. 61-72.

Wagner M., Amann R., Lemmer H., Manz W. and Schleifer K-H. (1994a). Probing activated sludge with fluorescently labelled rRNA targeted oligonucleotides. *Water Science and Technology* **29**(7): 15-23.

Wagner M., Erhart R., Manz W., Amann R., Lemmer H., Wedi D. and Schleifer K-H. (1994b). Development of an rRNA-targeted oligonucleotide probe specific for the genus *Acinetobacter* and its application for *in situ* monitoring in activated sludge. *Applied and Environmental Microbiology* **60**(3): 792-800.

Wagner M., Amann R.I., Kampfer P., Assmus B., Hartmann A., Hutzler P., Springer N. and Schleifer K-H. (1994c). Identification and *in situ* detection of Gram-negative filamentous bacteria in activated sludge. *Systematic and Applied Microbiology* **17**: 405-417.

Walmsley R.D. and Thornton J.A. (1984). Evaluation of OECD-type P eutrophication models for predicting the trophic status of southern African man-made lakes. *South African Journal of Science* **80**: 257-259.

Wang J.C. and Park J.K. (1998). Effect of wastewater composition on microbial populations in biological P removal processes. Proceeedings of *Water Quality International 1998*. 19th IAWQ Biennial International Conference, 21-26 June, Vancouver, Canada.

Wanner J. (1994). *Activated Sludge Bulking and Foaming Control*. Technomic Publishing.

Wanner J. (1997). Microbial population dynamics in biological wastewater treatment plants. In: *Microbial Community Analysis: The Key to the Design of Biological Wastewater Treatment Systems*. Cloete T.E. and Muyima N.Y.O. (eds.). IAWQ Scientific and Technical Report No. 5. IAWQ, London. Pp. 35-59.

Wanner J. and Grau P. (1988). Filamentous bulking in nutrient removal activated sludge systems. *Water Science and Technology* **20**: 1-8.

Wentzel M.C. (1992). P removal from sewage in activated sludge systems. *First IAWQ Technical Tour Nutrient Removal and Anaerobic Digestion in South Africa*. Volume One – Nutrient Removal. 11-23 October, 1992.

Wentzel M.C., Lötter L.H., Loewenthal R.E. and Marais G.v.R. (1986). Metabolic behaviour of Acinetobacter spp. in enhanced biological P removal – a biochemical model. *Water SA* **12**(4): 209-224.

Wentzel M.C., Loewenthal R.E., Ekama G.A. and Marais G.v.R. (1988). Enhanced polyphosphate organism cultures in activated sludge systems – Part 1: Enhanced culture development. *Water SA* **14**(2): 81-92.

Wentzel M.C., Ekama G.A., Dold P.L. and Marais G.v.R. (1990). Biological excess P removal – steady state process design. *Water SA* **16**(1): 29-48.

Wentzel M.C., Ekama G.A. and Marais G.v.R. (1992). Processes and modelling of nitrification denitrification biological excess P removal systems – a review. *First IAWQ Technical Tour Nutrient Removal and Anaerobic Digestion is South Africa*. Volume 1 – Nutrient Removal. 11-23 October, 1992.

Wentzel M.C., Ubisi M.F. and Ekama G.A. (1998). Heterotrophic active biomass component of activated sludge mixed liquor. *Water Science and Technology* **37**: 79-87.

Wetzel R.G. (1983). *Limnology*. Second edition. CBS College Publishing, New York, U.S.A.

Wingender J., Neu T.R and Flemming H-C. (1999). What are bacterial extracellular polymeric substances? In: *Microbial Extracellular Polymeric Substances – Characterization, Structure and Function*. Wingender J., Ney T.R and Flemming H-C. (eds.). Springer-Verlag, Berlin, Germany. Pp. 1-19.

Wolfaaardt G.M., Archibald R.E.M. and Cloete T.E. (1991). The use of DAPI in the quantification of sessile bacteria on submerged surfaces. *Biofouling* **4**: 265-274.

Zierold K. and Shäfer D. (1988). Preparation of cultured and isolated cells for X-ray microanalysis. *Scanning Microscopy* **2**(3): 1775-1790.

Chapter 3

SEM-EDS FOR DETERMINING THE PHOSPHORUS CONTENT IN ACTIVATED SLUDGE EPS

Accepted for publication in Water Science and Technology.
The editorial style of Water Science and Technology was followed in this chapter.

3.1 ABSTRACT

Not all phosphorus removed in activated sludge systems can be accounted for by polyphosphate accumulating organisms (PAO). A method for the qualitative and quantitative *in situ* characterization of PAO cell clusters and closely associated extracellular polysaccharides (EPS) is described. X-ray microanalysis was performed on samples from four activated sludge plants situated in Pretoria, South Africa. Analyses were done by means of Scanning Electron Microscopy (SEM) combined with Energy Dispersive Spectrometry (EDS). Cell clusters with associated EPS on average contained between 57 and 59 % phosphorus, while EPS alone contained on average between 23 and 30 % phosphorus. Results suggest that phosphorus removal in activated sludge might be due not only to PAO, but also by EPS acting as a phosphorus reservoir. Extraction of EPS from two different activated sludge plants yielded similar amounts of EPS. Comparison of EDS results of EPS before and after extraction, indicated possible intracellular leakage during homogenization by means of sonication, while phosphorus may be complexed with iron and aluminium in localized precipitates in WTPs employing chemical treatment to attain effluent standards. These precipitates were probably removed by filtration during the extraction procedure employed.

3.2 INTRODUCTION

The activated sludge system for wastewater treatment is one of today's most important biotechnological processes (Wagner *et al*, 1993). Although a considerable amount of work has

been done on system design and process engineering, most systems designed for enhanced biological phosphorus removal (EBPR) routinely fail (Bond *et al.*, 1995), necessitating chemical precipitation to meet effluent standards. This has been attributed to limited knowledge and understanding of the community structure-function and, consequently, the microbiology behind the activated sludge process (Ehlers *et al.*, 1998). Understanding the microbial community of biological wastewater treatment systems would assist in improving design and performance (Bond *et al.*, 1995; Cloete and Muyima, 1997). Studies on the microbial ecology of activated sludge in order to optimize the process have received much attention. A combination of classic culture-dependent and molecular techniques have contributed to a better, but as yet incomplete understanding of the EBPR process and can be attributed to the intrinsic limitations of techniques used to study microbial population dynamics in natural habitats. Microbial diversity and, more importantly, the function of populations in a specific community have not been elucidated to date and, therefore, EBPR cannot be attributed as a function of a specific microbial population in activated sludge. Until now, it has not been possible to isolate a pure culture of bacteria that could be responsible for biological phosphorus removal (Ehlers *et al.*, 1998). Also, very little is known about the microenvironment in activated sludge flocs, including the role of extracellular polysaccharides (EPS) in EBPR. Therefore methods other than classic culture dependent techniques need to be developed to differentiate between phosphorus-removing and non-phosphorus-removing systems and to study the mechanisms involved.

A large part of the floc structure in activated sludge is composed of EPS. These polymers are composed of sugars, amino acids and uronic acids (Bitton, 1994). However, the role of EPS in biological phosphorus removal has not been well studied. In previous studies, Buchan (1980) used energy dispersive spectrometry (EDS) to determine the location of phosphorus volutin granules in activated sludge, although little attention was paid to phosphorus located in the EPS outside these cell clusters.

EDS is a process whereby qualitative and quantitative analysis of a sample may be done following excitation of atoms in such sample. Excitation of atoms lead to the production of X-rays, the energies of which differ for different elements. The spectrometer plots energies of

these X-rays against specific counts of each specific energy, giving a compositional spectrum of the sample (Buchan, 1983). For this study, a combination of scanning electron microscopy (SEM) and EDS was chosen as a technique to study the role that EPS plays in phosphate removal from wastewater in the activated sludge process. Also, an investigation was done regarding amounts of EPS in different activated sludge plants by means of centrifugation extraction (Zhang *et al.*, 1999) following homogenization with a sonic probe. Results, in combination with SEM-EDS, may account for different uptake abilities of different sludges.

3.3 MATERIALS AND METHODS

Sampling:

Four EBPR wastewater treatment plants (WTP) situated in Pretoria, South Africa were sampled. Two of these (Rooiwal and Daspoort WTP) were functioning exclusively biological. The Baviaanspoort WTP employed ferric chloride, while the Centurion WTP used aluminium oxide as post-treatment for orthophosphate precipitation to meet effluent standards. These plants were sampled at the end of the aerobic zones before chemical addition. To standardize the experiments, 500 ml of sludge at the end of the anaerobic or aerobic zones were drawn into sterile Schott bottles and transported on ice to the laboratory for processing.

Orthophosphate analyses:

Orthophosphate analyses were done for the influent wastewater, as well as at the end of the aerobic zone directly before secondary settling. These analyses were done immediately after sampling for the Daspoort WTP and 45 min after sampling for the Rooiwal WTP. For the Baviaanspoort and Centurion WTPs, orthophosphate analyses were done on-site. The orthophosphate analyses were done using a SQ118 spectrophotometer (Merck) and the relevant test kit P(VM) after filtration of 10 ml of sludge through Whatman no. 1 filter paper.

Sample preparation:

1 ml of sludge from each sample was transferred to sterile 1.5 ml Eppendorf tubes and washed three times with sterile double distilled water by centrifugation at 10 000 rpm for 5 min in a BHG Hermle 360K centrifuge. The biomass pellet was finally resuspended in 1 ml of sterile double distilled water. The samples were then diluted 1:10 in sterile double distilled water and 10 µl spotted on high purity carbon stubs. These were left to air-dry at 37°C before being coated under vacuum with approximately 25 nm of high purity carbon. Two controls consisting of only a carbon stub without sample and a carbon stub containing only 10 µl of sterile double distilled water were included to check the purities of both the carbon and double distilled water.

EDS Analysis:

Samples were analysed by means of EDS. The SEM used was a Jeol model JSM-5800LV using a backscatter detector for better compositional contrast and depth resolution than secondary electron detectors. Samples were analysed by a pre-standardized Noran Voyager system at 15 keV for a livetime of 100 s. Ten analyses of each sample were done. Digital images were captured by means of an Orion frame-grabber.

EPS extraction:

For the EPS extraction, the regular centrifugation method of Zhang *et al.* (1999) was used, but instead of homogenization by means of blending, homogenization in this case was done with a Cole-Palmer (Series 4710) ultrasonic homogenizer probe with an output of 55 %. This method was preferred to other methods as reported by Zhang *et al.* (1999) as it was reported to have the least contamination by protein and DNA. Five Eppendorff tubes were filled with 750 µl of sludge for each of the Baviaanspoort and Centurion WTP. Samples were centrifuged at 3500

rpm for 10 min and the supernatant removed. The tubes were again filled with 750 µl double distilled water and homogenized with three 15 s bursts. Samples were kept on ice during homogenization steps. After homogenization, the previously removed fractions were added to the tubes and the samples centrifuged for 30 min at 12000 rpm. After centrifugation, the supernatant was pooled and filtered through 0.22 µ MILLEX®-GS syringe filters (Millipore) into McCartney bottles and freeze-dried overnight. Preparation of freeze-dried EPS for EDS was done as described earlier, although, in this case, the dried EPS was attached to double-sided carbon tape on an aluminium stage before being coated with carbon. EDS analysis was performed as described earlier.

3.4 RESULTS AND DISCUSSION

At the time of sampling all the WTP produced effluents which conformed to the special standard of 1 $mg.l^{-1}$ orthophosphate discharged to water sources as stipulated in the Republic of South Africa Water Act of 1956, as amended in 1980 (Slim, 1987).

There are many problems associated with EDS of biological samples, as normal preparation techniques for electron microscopy can displace, transform and dissolve many elements within biological samples, and is therefore not representative of the *in vivo* situation (Buchan, 1980). In this study we avoided normal SEM preparation to minimize abovementioned problems.

SEM indicated that the Daspoort and Rooiwal EBPR sludges seemed to consist of clusters of cells (Figures 3.1 and 3.2). The Baviaanspoort and Centurion sludges contained more filamentous organisms than Rooiwal and Daspoort (Figures 3.3 and 3.4).

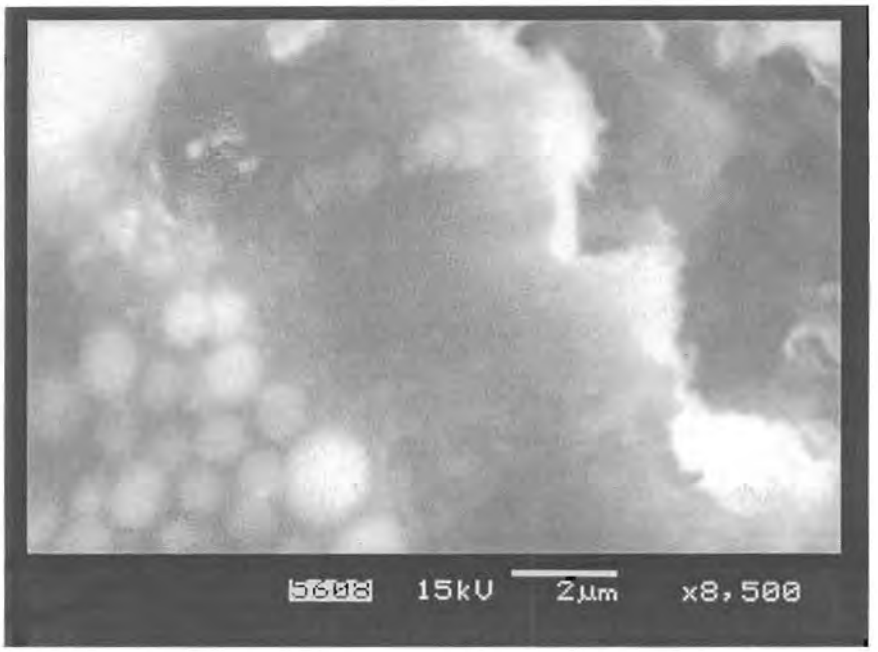

Figure 3.1. Backscatter electron micrograph indicating the spatial distribution of cell clusters and EPS in a Daspoort sludge sample. Note compositional contrast.

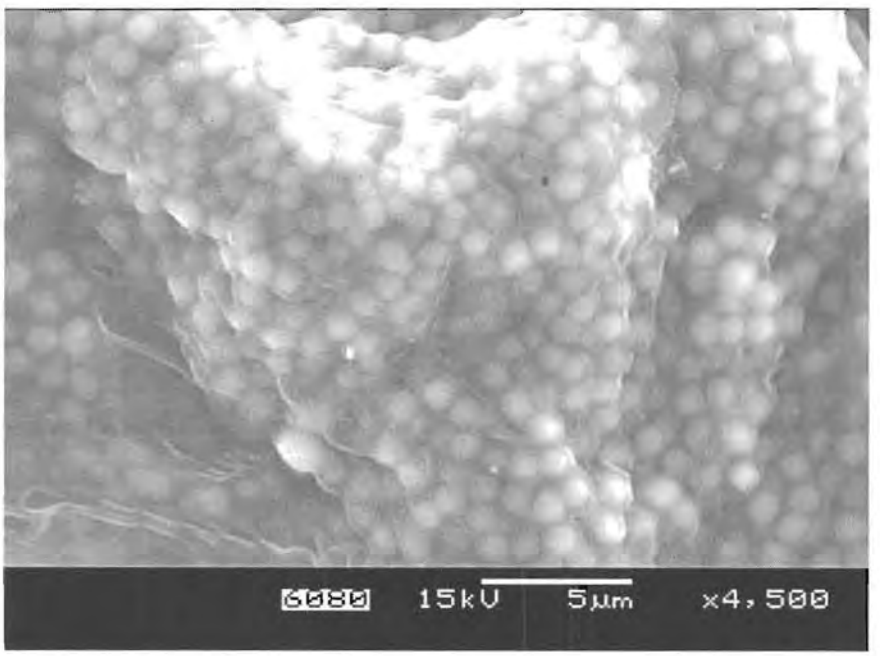

Figure 3.2. Backscatter electron micrograph indicating the spatial distribution of cell clusters and EPS in a Rooiwal sludge sample. Note compositional contrast.

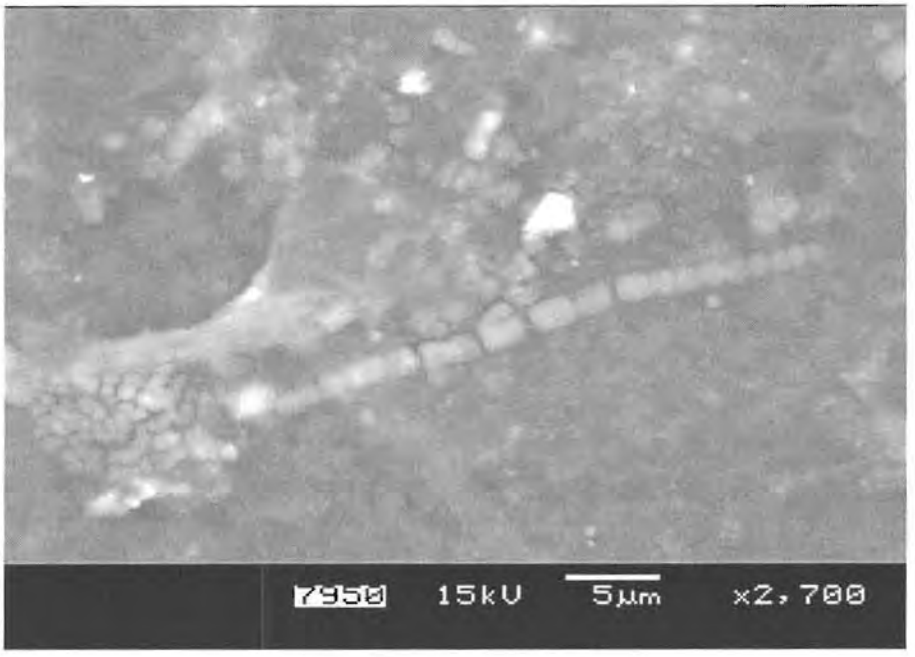

Figure 3.3. Backscatter electron micrograph indicating the spatial distribution of EPS and cell clusters in Baviaanspoort sludge. Note the filamentous organism and compositional contrast.

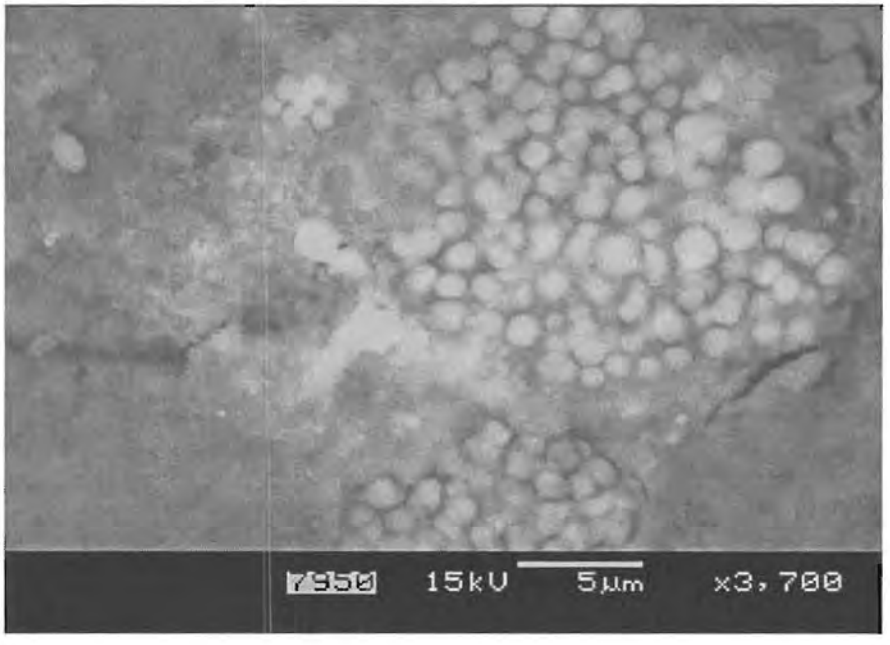

Figure 3.4. Backscatter electron micrograph indicating the spatial distribution of EPS and cell clusters in Centurion sludge. Note compositional contrast.

On average, cell clusters with associated EPS from the four EBPR sludges contained between 57 and 59 % phosphorus (Table 3.1, Figure 3.5), while EPS alone contained between 23 and 30 % phosphorus (Table 3.1, Figure 3.6). These values also showed little variation amongst different plants and different zones in the same plant (Daspoort)(Table 3.1).

Table 3.1. EDS analysis of cell clusters and EPS of different activated sludge plants and zones of the same plant.

Description	**Range (%)**	**Average (%)**	**SD* (%)**
Daspoort – cell clusters (anaerobic zone)	55-60	58	2
Daspoort – cell clusters (aerobic zone)	51-59	57	2
Rooiwal – cell clusters (anaerobic zone)	56-64	59	3
Baviaanspoort - cell clusters (aerobic zone)	53-60	57	2
Centurion - cell clusters (aerobic zone)	56-60	57	1
Daspoort – EPS (anaerobic zone)	23-37	30	8
Daspoort – EPS (aerobic zone)	19-33	27	4
Rooiwal – EPS (anaerobic zone)	16-35	29	8
Baviaanspoort - EPS (aerobic zone)	20-40	30	1
Centurion – EPS (aerobic zone)	17-30	23	2

*SD – Standard deviation.

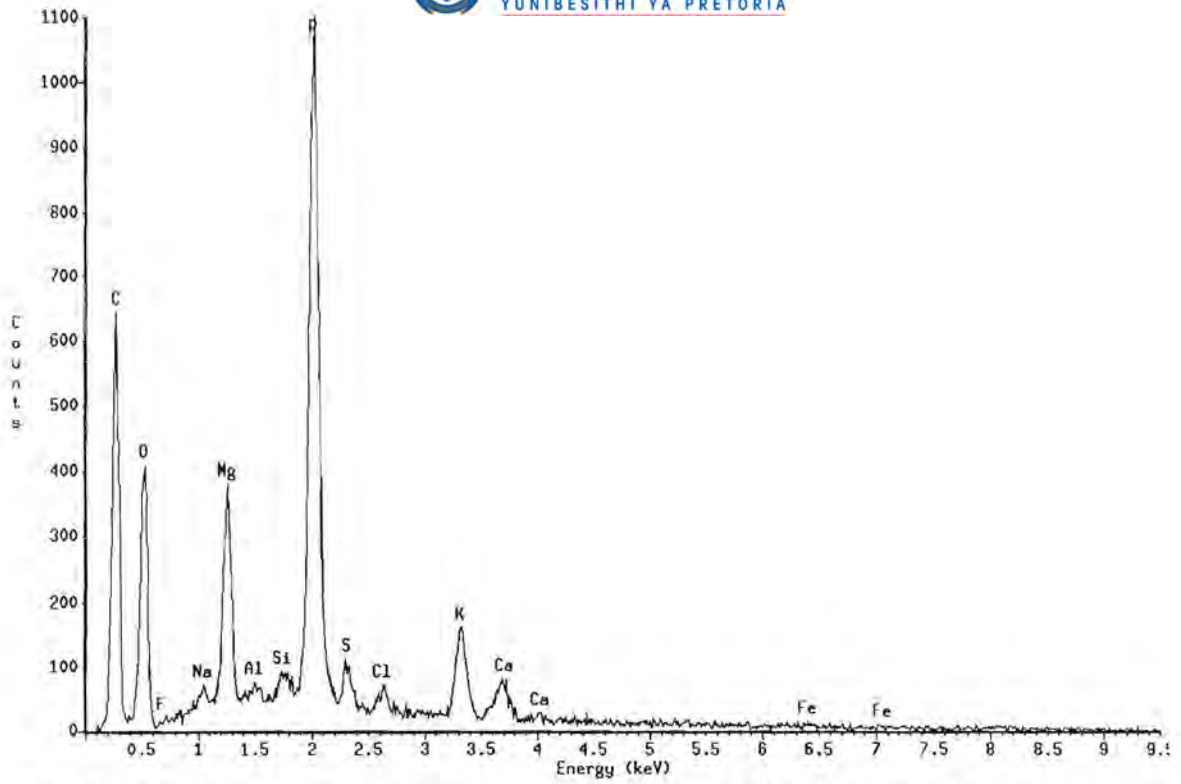

Figure 3.5. Analytical X-ray spectrum of Daspoort anaerobic sludge cell clusters.

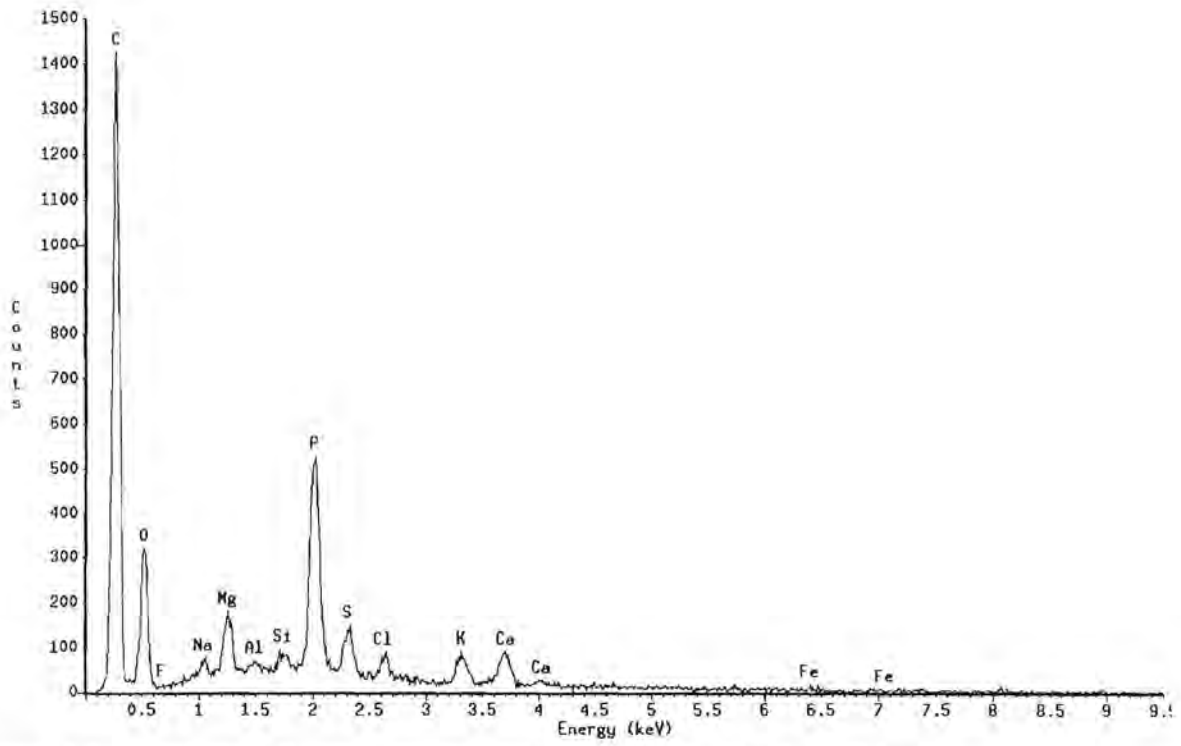

Figure 3.6. Analytical X-ray spectrum of Daspoort anaerobic sludge EPS.

As counter-ions to phosphorus, magnesium, potassium and calcium were dominant in cell clusters, while, in addition to these ions, sulphur, and silicon ions were also found as counter-ions in EPS (Tables 3.2, 3.3 and 3.4). Baviaanspoort cell clusters and EPS was found to be rich in iron, probably due to recycling of sludge after chemical treatment (Table 3.4). The EPS of Centurion sludge was rich in iron and aluminium. The aluminium was probably present due to recycling of sludge after chemical treatment and the iron due to the influent metal concentration (Table 3.3). Copper was also found in the Centurion EPS (data not shown).

Table 3.2. Typical data set for analysis of cell clusters and EPS (Rooiwal – anaerobic zone)(the K following every element indicates the K-shell of the specific atom).

Cell clusters			EPS		
Element	**Weight %**	**SD (%)**	**Element**	**Weight %**	**SD (%)**
Na-K	0.89	0.60	Na-K	3.59	0.59
Mg-K	17.83	0.91	Mg-K	9.28	0.41
Al-K	0.08	0.55	Al-K	5.90	0.63
Si-K	0.46	0.50	Si-K	8.71	0.37
P-K	59.92	1.48	P-K	35.45	1.00
S-K	0.96	0.77	S-K	12.35	0.55
Cl-K	1.07	0.72	Cl-K	1.79	0.41
K-K	18.79	1.73	K-K	12.41	0.52
Ca-K	ND*	ND	Ca-K	6.82	0.49
Fe-K	ND	ND	Fe-K	3.72	1.00

*ND – Not detected

Table 3.3. Typical data set for analysis of cell clusters and EPS (Centurion – aerobic zone)(the K following every element indicates the K-shell of the specific atom).

Cell clusters			EPS		
Element	**Weight %**	**SD (%)**	**Element**	**Weight %**	**SD (%)**
Na-K	ND	ND	Na-K	0.68	1.11
Mg-K	15.91	0.82	Mg-K	1.59	0.93
Al-K	1.31	0.51	Al-K	25.55	1.00
Si-K	0.15	0.48	Si-K	5.76	1.04
P-K	57.12	1.36	P-K	23.29	1.20
S-K	1.33	0.73	S-K	5.69	1.19
Cl-K	ND	ND	Cl-K	3.23	1.12
K-K	16.68	0.80	K-K	0.48	1.03
Ca-K	6.37	0.78	Ca-K	14.95	1.27
Fe-K	1.14	1.52	Fe-K	18.77	3.27

It was easy to distinguish between bacterial cells and EPS for the Daspoort and Baviaanspoort sludges, making EDS analysis easy (Figures 3.1 and 3.3). However, due to the very close encapsulation of cells by the EPS in the Rooiwal and Centurion sludges (Figures 3.2 and 3.4), EDS analysis was more difficult. Cell clusters of these sludges may also have contributed to X-rays produced from EPS being analyzed, due to overlap of EPS and cells in the large excitation volume (approximately 2 μ x 2 μ) created.

EDS analyses of phosphorus in cell clusters in this study [60 % (cells and EPS) minus 30 % (EPS alone) equaling 30 %] are in agreement with those of Buchan (1980), who indicated a 18 % phosphorus content in *Acinetobacter* cells.

Table 3.4. Typical data set for analysis of cell clusters and EPS (Baviaanspoort – aerobic zone)(the K following every element indicates the K-shell of the specific atom).

Cell clusters			EPS		
Element	**Weight %**	**SD (%)**	**Element**	**Weight %**	**SD (%)**
Na-K	ND	ND	Na-K	1.34	0.49
Mg-K	14.98	1.42	Mg-K	3.93	0.38
Al-K	1.36	0.91	Al-K	2.38	0.35
Si-K	2.18	0.83	Si-K	3.09	0.35
P-K	53.73	2.10	P-K	26.27	0.90
S-K	1.82	1.29	S-K	9.63	0.94
Cl-K	ND	ND	Cl-K	0.99	0.42
K-K	8.05	1.11	K-K	1.92	0.42
Ca-K	10.05	1.27	Ca-K	19.44	1.14
Fe-K	7.84	2.74	Fe-K	31.01	1.78

Jenkins *et al.* (1971) reviewed various aspects of chemical precipitation of phosphorus. According to their theory, phosphorus uptake in excess of the normal metabolic requirements of organisms in activated sludge is caused by chemical precipitation of phosphates by metals. These precipitates are then entrapped in the sludge matrix. Compounds such as monetite ($CaHPO_4$), hydroxyapatite [$Ca_5(PO_4)_3OH$], tricalcium phosphate [$Ca_3(PO_4)_2$] and octacalcium phosphate [$Ca_4H(PO_4)_3$] have been considered to be precipitated. Morgan and Fruh (1972) also considered magnesium and iron precipitates. According to the theory of Kerdachi and Roberts (1980), colloidal calcium phosphate crystals are precipitated through mediation of bacterial extracellular enzymes. These crystals are then supposedly chemically bound to an extracellular polymer matrix of microbial origin. Adsorptive properties of exopolymers have been well documented, especially in terms of biosorption of pollutants and toxics (Beech and Cheung, 1995; Loaëc *et al.*, 1997). Results of EDS analyses of EPS for the Baviaanspoort and

Centurion WTP in this study tend to support abovementioned theories to a certain extent, as chemical precipitation of orthophosphate with iron and aluminium follows the stochiometry of 1:1 (Bitton, 1994)(Tables 3.3 and 3.4). EPS extraction from the Baviaanspoort and Centurion WTP led to similar yields of freeze-dried EPS (0.8 $mg.ml^{-1}$) from similar volumes (3.75 ml) of sludge (Figure 3.7). A summary of results for phosphorus in these samples can be seen in Table 3.5, while typical results for the Baviaanspoort and Centurion freeze-dried EPS are given in Tables 3.6 and 3.7. Unfortunately, there is no way of knowing to what extent orthophosphate contamination of the EPS fractions occurred during homogenization due to cell breakage or lysis and further steps to prevent this phenomenon should be investigated.

Figure 3.7. Comparison of different EPS yields following extraction after homogenization with a sonic probe. Samples are from left to right Baviaanspoort and Centurion.

Table 3.5. Summary of results for phosphorus content of extracted and freeze-dried EPS from the Baviaanspoort and Centurion WTP by means of SEM-EDS.

Description	**Range (%)**	**Average (%)**	**SD (%)**
Baviaanspoort (aerobic zone)	13-24	15	5
Centurion (aerobic zone)	17-23	20	2

Table 3.6. Typical SEM-EDS dataset for extracted EPS from the Baviaanspoort WTP (the K following every element indicates the K-shell of the specific atom).

Extracellular polysaccharides		
Element	**Weight %**	**SD (%)**
Na-K	17.25	0.39
Mg-K	6.52	0.22
Al-K	0.68	0.16
Si-K	2.47	0.16
P-K	14.73	0.44
S-K	6.58	0.26
Cl-K	15.75	0.57
K-K	19.10	0.64
Ca-K	16.03	0.41
Fe-K	0.89	0.54

Table 3.7. Typical SEM-EDS dataset for extracted EPS from the Centurion WTP (the K following every element indicates the K-shell of the specific atom).

Extracellular polysaccharides		
Element	**Weight %**	**SD (%)**
Na-K	13.09	0.42
Mg-K	8.20	0.26
Al-K	1.36	0.19
Si-K	4.26	0.21
P-K	18.59	0.51
S-K	5.56	0.27
Cl-K	16.6	0.36
K-K	13.09	0.36
Ca-K	18.01	0.44
Fe-K	1.23	0.55

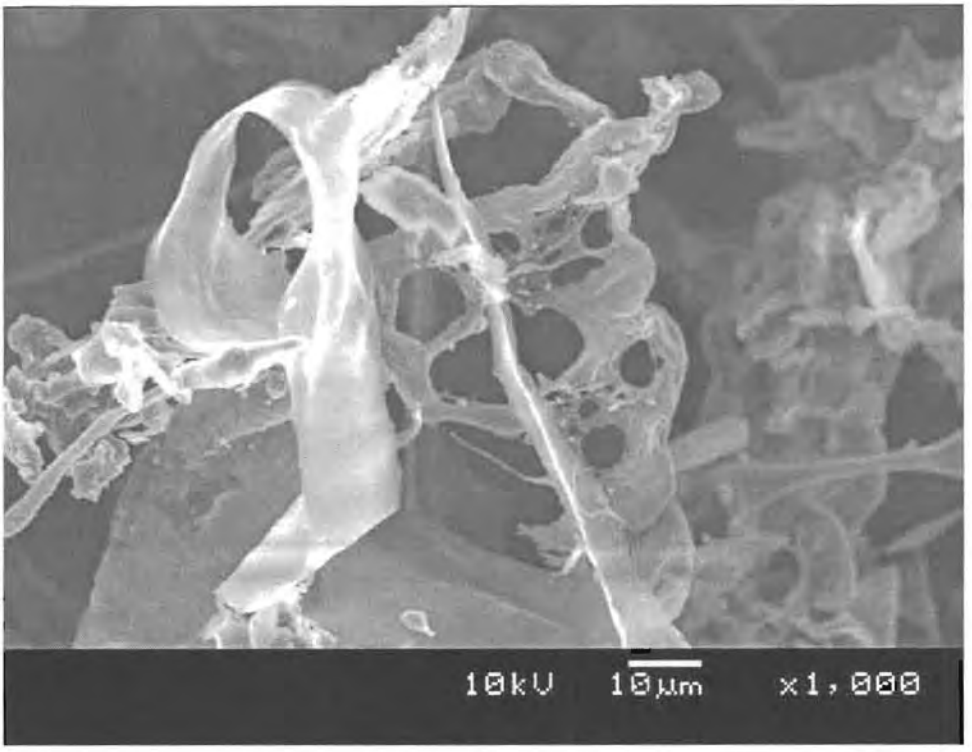

Figure 3.8. Backscatter electron micrograph of extracted and freeze-dried Centurion EPS.

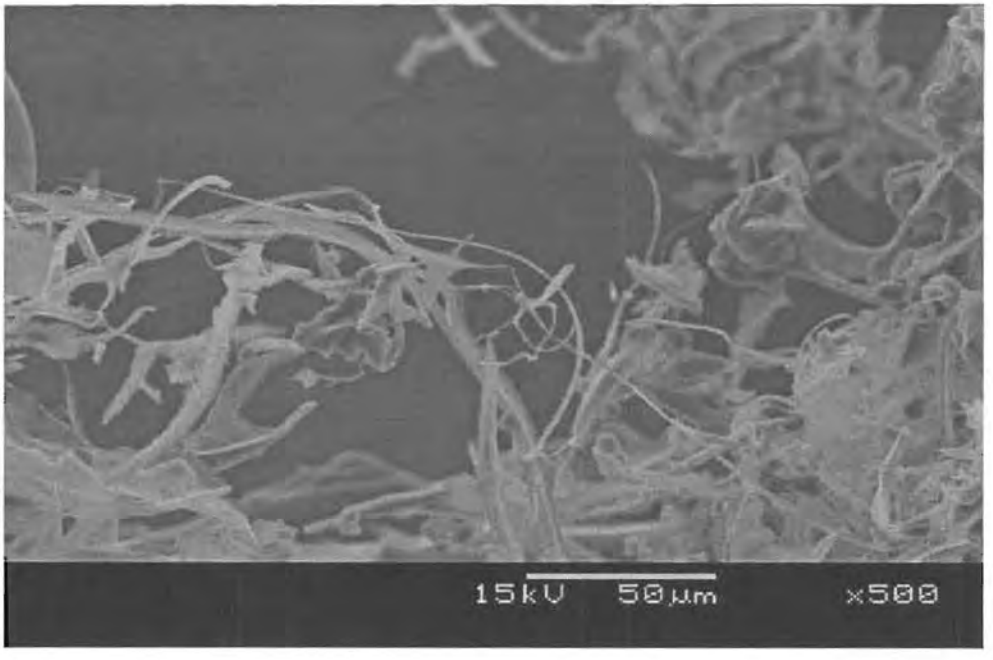

Figure 3.9. Backscatter electron micrograph of extracted and freeze dried Baviaanspoort EPS.

For the Baviaanspoort WTP, on average, the phosphorus content dropped from 30 % in whole-sludge EDS results (Table 3.1) to 15 % for EPS extracts (Table 3.5). Whole-sludge EDS of EPS for this plant indicated high levels of iron, not associated with cell clusters (Table 3.4). Levels of iron and aluminium, however, dropped after EPS extraction, indicating insoluble iron and aluminium precipitates of phosphorus, probably removed by filtration. The iron in the EPS originates from the recycling of sludge treated with ferric chloride to attain phosphorus effluent standards, while the aluminium probably originates from the influent wastewater. It is not certain to what extent the remaining phosphorus in the extracted EPS reflects the true *in situ* situation of the sludge, as leakage of intracellular material might have occurred during sonication. This phenomenon can be seen by increases in the levels of Na, Mg, S, Cl, K and, to a certain, extent Ca in the EPS following extraction (Tables 3.4 and 3.6).

On average, phosphorus levels in the Centurion WTP EPS was somewhat (3 %) lower after EPS extraction (Tables 3.1 and 3.5), indicating some removal by filtering out of iron and aluminium precipitates. The plant treats effluent with aluminium oxide to attain its phosphorus effluent standard, and recycling of this metal takes place, as can be seen in the high levels of this metal in EPS (Table 3.3). Once again, the aluminium was not associated with the cell clusters (Table 3.3). On the other hand, some EPS parts analyzed contained no aluminium (data not shown), indicating localization of this metal in the EPS. The iron in the EPS probably originated from the influent being treated (Table 3.3). If all of the aluminium and iron was removed by means of filtering out of phosphorus precipitates, the leakage of intracellular phosphorus during sonication must have been great to result in an average percentage of phosphorus of 20 % (Table 3.5) after extraction. Leakage of DNA, for example, caused by cell lysis during sonication, may have led to the high levels of phosphorus after removal of phosphorus precipitates with aluminium and iron. Once again, possible leakage of intracellular compounds after extraction can be seen by increases in the levels of Na, Mg, S, Cl, K and Ca in the EPS after extraction (Tables 3.3 and 3.7).

In general, a difference could be observed in crude EPS extracts before and after filtration. Before filtration, the crude extracts were straw-coloured, while they were colourless after filtration. This could be due to the filtering out of specific metal-phosphorus precipitates.

The type and amount of intracellular leakage during extraction should be investigated further and steps should be taken to minimize this phenomenon. Also, the effect of EPS extraction on WTP employing only biological treatment (that is, without addition of chemicals to attain effluent standards) could shed light on the leakage of intracellular leakage during homogenization steps. In addition, more gentle extraction methods for EPS could be employed. For example, addition of deionized water to a sample may be regarded as a simple form of extraction (Gaudy and Wolfe, 1962). However, gentle extractions will probably only extract loosely bound EPS (Nielsen and Jahn, 1999).

Further attempts to optimize the EDS analysis of activated sludge should be investigated. This could include Transmission Electron Microscopy (TEM) in combination with EDS to avoid large excitation volumes, rapid freezing and cryo-sectioning, as well as freeze-substitution to avoid displacement or migration of ions inside the samples. In the author's opinion, these methods will give better results than extraction procedures, which have more inherent disadvantages like intracellular leakage.

3.5 CONCLUSIONS

SEM-EDS indicated that cell clusters with associated EPS of different activated sludge plants, on average, contained between 57 and 59 % phosphorus, while EPS alone contained on average between 23 and 30 % phosphorus. Results suggest that phosphorus removal in activated sludge might be due not only to PAO, but also by EPS acting as a phosphorus reservoir. Results of EDS for EPS extracts from two WTP employing chemical treatment, indicated possible localized iron and aluminium precipitates of phosphorus which were seemingly removed by filtration (part of the extraction procedure). Although intracellular leakage during extraction was indicated, EPS could play an important role in removal of phosphorus, even in these plants.

3.6 ACKNOWLEDGEMENTS

The authors would like to thank the Unit for Microscopy and Microanalysis at the University of Pretoria for analyses as well as their valuable contributions to this research. This study was funded by the Water Research Commission of South Africa.

3.7 REFERENCES

Beech I.B. and Cheung C.W.S. (1995). Interactions of exopolymers produced by sulphate-reducing bacteria with metal ions. *Int. Biodeterior. Biodegrad.* **35**, 59.

Bitton G. (1994). *Wastewater Microbiology*. John Wiley & Sons, New York.

Bond P.L., Hugenholtz J.K., Keller J. and Blackall L.L. (1995). Bacterial community structure of phosphate- removing and non-phosphate-removing activated sludge from sequencing batch reactors. *Appl. Environ. Microbiol.* **61**, 1910-1916.

Buchan L. (1980). *The location and nature of accumulated phosphorus in activated sludge*. DSc (Agric) thesis, University of Pretoria, Pretoria, South Africa.

Buchan L. (1983). Possible biological mechanism of phosphorus removal. *Wat. Sci. Tech.* **15**, 87-103.

Cloete T.E. and Muyima N.Y.U. (1997). *Microbial Community Analysis: The Key to the Design of Biological Wastewater Treatment Systems*. International Association on Water Quality, Cambridge, Great Britain.

Ehlers M.M., Erasmus A. and Cloete T.E. (1998). *Fingerprinting of Activated Sludge Systems Using PAGE Analysis of Total Protein Extractions for the Optimization of Biological Phosphorus Removal*, Water Research Commission Report No. 776/1/98, Water Research Commission, Pretoria, South Africa.

Gaudy E. and Wolfe R.S. (1962). Composition of an extracellular polysaccharide produced by Sphaerotilus natans. *Appl. Microbiol.* **10,** 200-205.

Jenkins D., Menar A.B. and Ferguson J.F. (1971). Chemical processes for phosphate removal. *Wat. Res.* **5**, 369-389.

Kerdachi D.A. and Roberts M.R. (1980). *The ability of the extended aeration activated sludge process to remove phosphorus consistently to less than 0,1mg P/l in a simple surface aerated reactor*. Presented to the S.A. Branch of the Institute of Water Pollution Control Conference, Pretoria, June 1980.

Loaëc M., Olier, R. and Guezennec, J. (1997). Uptake of lead, cadmium and zinc by a novel bacterial exopolysaccharide. *Wat. Res.* **31**, 1171-1179.

Morgan W.E. and Fruh E.G. (1972). *An investigation of phosphorus removal mechanisms in activated sludge systems*, Environmental Protection Technology Series, EPA-R2-72-031, Office of Research and Monitoring, U.S. Environmental Protection Agency, Washington D.C.

Nielsen P.H. and Jahn A. (1999). Extraction of EPS. In: *Microbial Extracellular Polymeric Substances – Characterization, Structure and Function*. Wingender J., Neu T.R. and Flemming H-C. (eds.). Springer-Verlag, Berlin, Germany. Pp. 49-72.

Slim J.A. (1987). Some developments in the water industry in South Africa. *Wat. Pollut. Control.* **86**, 262-271.

Wagner M., Amann R., Lemmer H. and Schleifer K. C(1993). Probing activated sludge with oligonucleotides specific for proteobacteria: Inadequacy of culture-dependent methods for describing microbial community structure. *Appl. Environ. Microbiol.* **59**, 1520-1525.

Zhang X., Bishop P.L. and Kinkle B.K. (1999). Comparison of extraction methods for quantifying extracellular polymers in biofilms. *Wat. Sci. Tech.* **39**(7), 211-218.

Chapter 4

PHOSPHORUS REMOVAL CAPACITY OF MLSS AND MLVSS FRACTIONS OF FIVE ACTIVATED SLUDGE PLANTS

4.1 ABSTRACT

Research has indicated the relationship between biomass and phosphorus removal in activated sludge. Mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) are often used as indicators of biomass, and used as such in the mathematical modelling of biological phosphorus removal. The objectives of this study was therefore to determine the relationship between the MLSS and MLVSS fractions and phosphorus removal in activated sludge and to compare these fractions, together with the total plate count (TPC) and adenosine triphosphate (ATP) bacterial counts as measures of viable biomass. The hypothesis of the study was that the same amount of MLSS and, specifically MLVSS, of different activated sludges should show similar orthophosphate uptake abilities. To this end two experiments were conducted. In experiment 1, 600 ml quantities of sterile mixed liquor containing 219 $mg.l^{-1}$ orthophosphate was inoculated with equal amounts (40 grams) of wet sludge pellets from five different 3-stage Bardenpho activated sludge systems with similar sludge ages. In experiment 2, conditions in the same plants used in experiment 1, were simulated by centrifuging 900 ml of each sludge, dividing the pellets equally and resuspending the pellets in duplicate in 450 ml quantities of sterile mixed liquor growth medium containing 28 $mg.l^{-1}$ orthophosphate. Orthophosphate removal was determined hourly over an eight hour experimental period for experiment 1 and a seven hour period for experiment 2. Orthophosphate removal differed amongst systems where the same quantity (66.67 $mg.ml^{-1}$) of wet sludge was used, while little orthophosphate removal was observed in

the control flasks containing no sludge. In experiment 1, the Centurion Wastewater Treatment Plant (WTP) showed, on average, the highest orthophosphate removal capacity (30.79 mg $P.g^{-1}$ initial MLSS), followed by the Baviaanspoort, Zeekoegat, Rooiwal and Daspoort WTPs with average orthophosphate removal capacities of 23.78, 20.17, 15.40 and 14.88 mg $P.g^{-1}$ initial MLSS, respectively. For experiment 2, the Centurion WTP performed best, with an orthophosphate removal of 9.19 mg $P.g^{-1}$ initial MLSS, followed by the Baviaanspoort, Daspoort, Rooiwal and Zeekoegat WTPs with orthophosphate removals of 7.97, 4.60, 4.55 and 2.82 mg $P.g^{-1}$ initial MLSS, respectively. In terms of initial MLVSS, the same pattern was observed as that for initial MLSS. The different orthophosphate removal capacities observed were attributed to differences in the MLSS active biomass fraction of the different activated sludges. Results indicated that the MLSS and MLVSS fractions of activated sludge *per se*, were not good indicators of biomass in activated sludge. ATP proved to be a more reliable method for indicating the viable biomass concentration than TPC. Although MLSS and MLVSS showed the same trend in orthophosphate removal, initial concentrations of these fractions could not be directly linked to differences in orthophosphate uptake abilities of different sludges, indicating the unsuitability of MLSS and MLVSS as indicators of viable biomass in activated sludge. However, orthophosphate removal was consistently higher in the sludges with higher ATP and TPC values, indicating a relationship between the active biomass fraction of the MLSS and orthophosphate removal.

4.2 INTRODUCTION:

Water is a limited resource in South Africa. Rainfall is erratic and infrequent and droughts are a constant threat, making it essential to protect aquatic environments from pollution (Bosch, 1992). This necessitated the building of man-made dams in major river systems. However, slow flow-through rates, high summer temperatures and long daylight hours combined with inflow of domestic and industrial wastewaters rich in nutrients like nitrogen and phosphorus due to accelerated human activities lead to the process of eutrophication in these water bodies (Atkinson, 1999; Bolitho, 1976). Nitrogen can be fixed by algae in the water and therefore,

phosphorus is considered the limiting nutrient and, therefore, the element that should be eliminated from wastewater to minimise eutrophication (Lilley *et al.*, 1997). Phosphorus removal from wastewater is seen as one of the best mechanisms for eutrophication control (Gleisberg, 1992, Toerien *et al.* 1975), having many advantages over chemical precipitation (Lilley *et al.*, 1997).

The activated sludge process was developed in 1914 in England and was so named because it consists of a mixed slurry of microbes which have the ability to stabilize wastes aerobically (Grady and Lim, 1980; Metcalf & Eddy, 1991). It has been known since 1959 that phosphorus can be removed during the activated sludge wastewater treatment process (Srinath *et al.*, 1959) and excess biological phosphate removal by bacteria in the mixed liquor of activated sludge was first observed by Vaker *et al.* (1967). Since then, this phenomenon has gained world-wide support and is utilized in both new and existing wastewater treatment plants which are either constructed or upgraded to accommodate biological nutrient removal (Atkinson, 1999).

In 1976 Barnard proposed the simultaneous removal process of phosphorus and nitrogen known as the Phoredox process in South Africa and the Bardenpho or modified Bardenpho process in the United States.

The activated sludge process has undergone various modifications and is able to meet most wastewater treatment needs (Toerien *et al.*, 1990). There is interest to better understand the enhanced biological phosphorus removal process, since system performance is often erratic (Bond *et al.*, 1995), necessitating additional chemical treatment to attain effluent standards. This failure has mainly been attributed to the lack of knowledge of the roles of microbes and optimum conditions for their growth in order to facilitate biological phosphorus removal. Phosphorus removal fails either as a result of biomass depletion following toxic shock due to sulphide accumulation or toxic influents, or nitrate feedback into the anaerobic zone (Bitton, 1994). Insufficient aeration in the aerobic zone and insufficient nutrients in the anaerobic zone (short chain fatty acids) is also thought to lead to system failure. In addition, organisms other than polyphosphate accumulating organisms can compete for phosphorus in anaerobic-aerobic systems, leading to process failure (Erasmus, 1997).

Ubisi *et al.* (1997) states that in the bioreactor of the non-nitrifying aerobic activated sludge system, the mixed liquor organic suspended solids is made up of three components: heterotrophic active biomass, endogenous residue and inert material. In the nitrifying aerobic and anoxic/aerobic activated sludge systems, a fourth component is included: autotrophic active biomass. Currently, the heterotrophic active biomass still exists only as a hypothetical parameter within the structure of the design procedures and kinetic models and has not been directly measured experimentally and compared to theoretical values (Ubisi *et al.*, 1997). The main problem in its measurement has been the lack of suitable experimental techniques and although microbiological techniques have principally been proposed, these techniques have not yet been adequately integrated with the design and kinetic modelling theory (Ubisi *et al.*, 1997). Ubisi *et al.* (1997) used a batch test procedure to quantify the heterotrophic active biomass from a well-defined laboratory-scale anoxic/aerobic activated sludge system. Results were in close agreement with those calculated theoretically using steady state design and kinetic models. This data is however not suited for activated sludge systems incorporating anaerobic reactors to mediate phosphorus removal, as organisms mediating this removal will contribute to the mixed liquor organic suspended solids.

Traditional methods for determination of active biomass concentrations, such as volatile suspended solids or particulate COD, are often inappropriate because particulate substrate is present in most wastewater treatment systems (Münch and Pollard, 1997). Determining the biomass concentration in such wastewaters is generally regarded as very difficult, if not impossible (Jørgensen *et al.*, 1992). Modellers of activated sludge rarely quantify the biomass concentration in their systems. Instead, they estimate biomass concentrations by indirect techniques, which are mostly based on measurements of metabolic activity (Münch and Pollard, 1997), for example, in measuring the rate of oxygen or nitrate uptake in batch tests. These rates are then used to estimate the biomass concentration in the system (Münch and Pollard, 1997). However, such measurements are tedious and generally cannot be applied to anaerobic systems (Münch and Pollard, 1997).

Attempts have been made to find simple and reliable methods to determine the biomass in wastewater and activated sludge (Jørgensen *et al.*, 1992). The simplest and most often used

method is to measure suspended solids (SS) or volatile suspended solids (VSS) (Ali *et al.*, 1985). These methods, however, do not distinguish between living cells and debris of organic or inorganic origin. The standard parameter of biomass in activated sludge is MLVSS, although it is recognized as an indirect and incomplete measure of the viable sludge floc (Fair and Geyer, 1954; Patterson and Brezonik, 1969, Patterson *et al.*, 1970). Other biomass parameters have been suggested, including particulate organic nitrogen and protein, but these are also unsatisfactory because of the variable concentrations of nonviable particulate organic material present in sewage (Patterson *et al.*, 1970). Furthermore, rapid changes in biological activity are only slowly reflected by changes in any of these parameters (Patterson *et al.*, 1970).

Currently, the heterotrophic active biomass exists only as a hypothetical parameter within the structure of the design procedures and kinetic models (Ubisi *et al.*, 1997).

In literature, principally microbiological techniques, including pour-plate and other culturing techniques, DNA analysis, fluorescent probes for ribosomal RNA, as well as sequencing of ribosomal DNA have been proposed to measure the heterotrophic active biomass parameter (Ubisi *et al.*, 1997). These methods are, however, mostly still in their infancy and still need to be optimized. For example, using the traditional total plate count technique, a underestimation of the biomass is done due to the selectivity of the media employed (Jørgensen *et al.*, 1992) and flocs which may contain thousands of cells, but resembles one colony forming unit. It has been proved that newer molecular techniques like fluorescent *in situ* hybridization (FISH) gives a totally different picture of the microbial community in activated sludge (Atkinson, 1999). For example, total plate counts of activated sludge samples indicate high levels (> 90 %) of *Acinetobacter* spp. On the other hand, FISH has indicated minute quantities of these species (3 to 6 %). However, even with homogenization techniques, counting of individual cells within these structures are difficult, due to difficulty experienced in breaking up these structures. This also leads to concern about entry of dyes and probes. Most of these methods also require sophisticated equipment and experimental techniques not widely available (Ubisi *et al.*, 1997).

A suitable biomass measurement parameter must fulfill certain criteria to be a useful and appropriate estimate of biomass. For example, the measured quantity should be proportional to some cellular entity (Patterson *et al.*, 1970), such as total organic carbon or dry weight. Also, the substance should have a short survival time after cell death, otherwise it would not be specific for viable biomass. There should also be a sensitive and accurate analytical procedure available to measure the parameter. A simple and rapid method of biomass determination in activated sludge might be the use of ATP. Patterson *et al.* (1970) developed the method for ATP measurement, using the reaction between luciferin, luciferase and ATP. The finalized procedure was highly sensitive and reliable. The authors reported relative standard deviations of less than 2% for activated sludge replicates and nearly 100% recovery of added ATP from activated sludge. Also, the authors claimed ATP levels in activated sludge to be relatively constant under endogenous conditions, indicating the potential of ATP as an estimate of viable biomass. The authors investigated the occurrence of ATP in activated sludge for the purpose of utilizing this parameter as a measure of metabolic activity and/or biomass. The ATP pool measured, approximated 2 µg per mg MLVSS.

Jørgensen *et al.* (1992) determined biomass of activated sludge growth cultures in terms of dry weight and compared the data with ATP content, the oxygen utilization rate (OUR) and fluorescein diacetate (FDA) hydrolysis data. ATP content showed the best correlation with biomass. A conversion factor of 3 mg ATP per g dry weight was calculated. With the same methods applied to 4 full-scale systems, ATP results indicated a relationship of 67 mg dry weight per gram suspended solids.

Roe and Bhagat (1982) estimated the ATP to suspended solids ration at maximum viability in activated sludge, and they found that viability varied significantly with mean cell residence time. Kucknerowicz and Verstraete (1979) found a linear relationship between OUR and ATP, suggesting that ATP reflected viable biomass. However, a requirement that must be met to obtain reliable biomass estimations, is that the activity/biomass ratio remains fairly constant during the measurement period (Jørgensen *et al.*, 1992). According to Jenkinson and Ladd (1981), however, the ATP concentration of a resting soil population did not differ much from ATP in pure cultures of actively growing microbes. This was in agreement with

results by Jørgensen *et al.* (1992) which showed constant ATP content to biomass ratios, independent of growth phase. Van de Werf and Verstraete (1984) however, showed extensive variation in ATP to biomass ratios during different metabolic conditions.

It can be assumed that culture is at maximum activity when it is in the exponential phase of growth. In the study by Jørgensen *et al.* (1992), the amount of viable biomass was estimated on the basis of maximum activity measurements and the conversion factor of 3 mg per g dry weight found, was well in agreement with values reported by Patterson *et al.* (1970) and Nelson and Lawrence (1980). The percentage of viable biomass in the experiments by Jørgensen *et al.* (1992) also correlated well with values in literature (Patterson *et al.*, 1970). Roe and Bhagat (1982) measured ATP levels in activated sludge from a lab-scale sludge plant, and indicated that the fraction of SS made up by viable biomass depended on sludge age, so that the highest viable biomass was obtained at the lowest sludge age.

To relate ATP concentration to microbial biomass, it is necessary to know the approximate ATP concentration per cell of the microbial species present (Patterson *et al.*, 1970). If ATP is also related to metabolic activity, the physiological state of the culture must be determined (Patterson *et al.*, 1970). Since it is impossible to make a taxonomic analysis of the microbiota present in activated sludge, the accuracy of biomass estimations would depend upon the constancy of the ATP pool among species (Patterson *et al.*, 1970). D'Eustachio and Levin (1967) reported a constant pool of ATP for *Escherichia coli*, *Pseudomonas fluorescens* and *Bacillus subtilis*, which was also constant during all growth phases. In a later study, D'Eustachio and Johnson (1968) investigated the endogenous ATP pool of 13 species of Gram positive and Gram negative aerobic bacteria and found a mean ATP pool of 2.1 μg per mg dry cell material. Also, a linear correlation existed between the endogenous ATP pool and standard plate count for the species involved.

In the study of Patterson *et al.* (1970), it was not certain as to the response of the ATP pool to changes in metabolic activity. If there was no change, or only erratic variation, then ATP could not be used as an activity parameter in studies on activated sludge. Thus, an experiment was designed to this extent. Results indicated that the ATP pool is affected by the

metabolic activity of an activated sludge culture and may be expected to respond rapidly and decisively to an increase in substrate loading, while only being gradually reduced as the organisms enter an endogenous phase.

Results by Patterson *et al.* (1970) indicated that a significant portion of the MLVSS is non-viable organic material not associated with the oxidative degradation of the substrate. Assuming a mean endogenous ATP pool of 2 µg per mg, dry cell material would result in an estimate that only 40% of the laboratory unit MLVSS was actually viable cell material. In a separate experiment carried out on a contact stabilization plant indicated that only 15 to 20% of the MLVSS may be active biomass under actual operating conditions.

Kucknerowicz and Verstraete (1979) found a linear relationship between the oxygen utilization rate (OUR) and ATP content in activated sludge, suggesting that ATP reflected the viable biomass. This is in agreement with results obtained in this study, which indicated larger cell counts by means of ATP analysis, as well as smaller standard deviations when compared to TPC.

The objective of this study was to investigate ATP, TPC, MLSS and MLVSS of activated sludge, as measures of viable biomass, and its relation to phosphorus removal. The hypothesis was that P-removal is directly correlated to biomass in activated sludge. The rationale of the study was, therefore, that equal quantities of MLSS and/or MLVSS from different activated sludge plants with similar configurations and inflow characteristics should remove the same quantity of orthophosphate in standardized batch culture experiments if MLSS and/or MLVSS were good indicators of biomass.

4.3 MATERIALS AND METHODS:

Experimental design

Two experiments were conducted. In the first (experiment 1), 40g of wet sludge pellets from five activated sludge plants were used to evaluate orthophosphate uptake from sterile mixed liquor growth medium containing 219 $mg.l^{-1}$ orthophosphate. Average orthophosphate removal was expressed as mg P removed per g wet sludge, as well as mg P removed per g of initial MLSS.

For the second experiment (experiment 2), the same systems as in experiment 1 were used. Original sludge MLSS (as present at the time of sampling) and orthophosphate concentrations were simulated by centrifuging 900 ml quantities of sludge, dividing the pellets equally and resuspending the pellets in duplicate in 450 ml sterile mixed liquor growth medium containing 28 $mg.l^{-1}$ orthophosphate. The average orthophosphate removal was expressed as mg P removed per g of initial MLSS and MLVSS of the activated sludges.

Sample collection

For experiment 1, grab samples (7 l) were taken from the aerobic zones of five activated sludge systems in and around Pretoria (i.e. Daspoort, Centurion, Baviaanspoort, Zeekoegat and Rooiwal)(Table 1). For experiment 2, grab samples (2 l) were taken from the same activated sludge plants. All samples were collected at the end of the aerobic zones of the WTPs.

Samples were taken in sterile Schott bottles, transported on ice and initial analyses performed immediately upon return to the laboratory. All samples were analysed within 8 h of sampling and all analyses were performed in triplicate. Average results of sludge analyses before inoculum preparation for experiments 1 and 2 are given in Tables 4.1 and 4.2 respectively.

Table 4.1: Characteristics of the activated sludge collected from five different plants used in experiment 1. Standard deviations are shown in brackets.

Analysis	Daspoort	Centurion	Zeekoegat	Rooiwal	Baviaanspoort
ATP (cells.ml^{-1})					
(on-site)	$4.17x10^7$ ($9.02x10^6$)	$3.95x10^7$ ($6.35x10^6$)	$4.30x10^7$ ($7.21x10^6$)	$5.13x10^7$ ($1.05x10^7$)	$3.27x10^7$ ($4.04x10^6$)
(laboratory)	$5.90x10^7$ ($2.18x10^7$)	$5.36x10^7$ ($2.34x10^7$)	$7.23x10^7$ ($3.06x10^6$)	$5.13x10^7$ ($9.81x10^6$)	$5.33x10^7$ ($4.16x10^6$)
MLSS (mg.l^{-1})	4700 (420)	4600 (0)	4740 (20)	4600 (170)	2800 (120)
COD (mg.l^{-1})	63 (29.70)	52 (4.24)	76 (12.02)	60 (30.41)	6 (0.00)
TPC (cfu.ml^{-1})	$4.37x10^6$ ($1.58x10^6$)	$7.32x10^6$ ($3.69x10^6$)	$3.30x10^6$ ($3.96x10^6$)	$3.46x10^6$ $9.09x10^5$)	$2.42x10^6$ ($8.24x10^5$)
PH	6.92 (-)	7.10 (-)	6.95 (-)	7.00 (-)	7.23 (-)
PO_4^{-3} (mg.l^{-1})	11.00 (0.00)	8.00 (0.00)	7.33 (0.58)	2.00 (0.00)	6.67 (0.58)
NO_3^- (mg.l^{-1})	5.50 (3.54)	9.00 (5.66)	6.03 (1.21)	11.40 (2.71)	34.40 (1.25)
SO_4^{2-} (mg.l^{-1})	45.67 (3.21)	104.33 (4.62)	50.00 (1.73)	54.00 (3.61)	41.00 (3.46)
NH_4^+ (mg.l^{-1})	10.32 (0.36)	7.53 (0.04)	5.07 (0.15)	0.08 (0.02)	9.95 (1.00)

Table 4.2: Characteristics of the activated sludge collected from five different plants used in experiment 2. Standard deviations are shown in brackets.

Analysis	Daspoort	Centurion	Zeekoegat	Rooiwal	Baviaanspoort
PH	7.21 (0.00)	7.43 (0.00)	7.07 (0.00)	7.30 (0.00)	7.34 (0.00)
NO_3^- (mg.l^{-1})	12.00 (2.83)	5.50 (2.12)	16.50 (0.71)	11.00 (11.30)	6.00 (0.00)
NH_4^+ (mg.l^{-1})	1.55 (0.07)	0.15 (0.07)	1.40 (0.14)	0.45 (0.07)	0.10 (0.00)
COD (mg.l^{-1})	2388 (395)	3554 (346)	3147 (489)	3651 (1318)	4292 (678)
Al (mg.l^{-1})	ND*	0.18 (0.23)	ND	0.01 (0.01)	0.05 (0.04)
Fe(mg.l^{-1})	0.32 (0.01)	0.95 (0.92)	0.30 (0.00)	0.50 (0.14)	1.65 (1.48)
TPC (cfu.ml^{-1})	$1.04x10^6$ ($2.12x10^4$)	$4.55x10^6$ ($1.34x10^6$)	$1.45x10^6$ ($4.67x10^5$)	$1.29x10^6$ ($2.33x10^5$)	$8.45x10^6$ ($7.07x10^4$)
MLSS (mg.l^{-1})	2830 (14.14)	4280 (0.00)	4090 (98.99)	2965 (49.50)	6025 (21.21)
MLVSS (mg.l^{-1})	2070 (0.00)	2810 (0.00)	3020 (70.71)	2365 (49.50)	4095 (7.07)

*ND – Not detected

Microbiological analyses:

1) ATP

ATP was measured on-site as well as in the lab by means of the ATP Bioprobe system (Hughes Whitlock).

2) Total plate counts

Total plate counts were done using the spread-plate technique on Nutrient Agar (NA) after 48 h incubation at room temperature.

Physico-chemical analyses:

3) MLSS

MLSS was determined by filtering 100ml of sample through a glass fibre filter (Whatman), after which the filter paper was dried for one hour at 105°C (Standard Methods, 1995) and the dry weight was determined (for experiment 1 Whatman no. 1 filter paper was used and for experiment 2 Whatman no. 44 (ashless) filter papers were used);

4) pH

pH was measured with a Beckman Φ6 pH meter and a relevant probe;

5) Chemical analyses

Chemical analyses (NO_3^-, PO_4^{3-}, SO_4^{2-} and NH_4^+) were done on the filtrate from the MLSS determination by means of the Spectroquant (SQ118) spectrophotometer (Merck) and the relevant test kits. Initial sludge analyses for experiment 2 included aluminium and iron and were also conducted by means of the SQ118 system and relevant test kits.

6) COD

Initial COD analysis on the sampled sludges before the experiments were conducted by means of the Spectroquant spectrophotometer (Merck) and was done on soluble COD for experiment 1

(done on the filtrate of the MLSS determination), while total COD analyses were done on 1 ml aliquots diluted 1:10 with distilled water in experiment 2.

Table 4.3: Characteristics of the activated sludge plants used in the phosphorus removal study.

	Daspoort	**Baviaanspoort**	**Zeekoegat**	**Centurion**	**Rooiwal**
Sludge age	12 days	13 days	13 days	12 days	12 days
Plant configuration	3-stage Bardenpho	3-stage Bardenpho	3-stage Bardenpho	3-stage Bardenpho	3-stage Bardenpho
Mean daily flow	45 megalitres	35-40 megalitres	35 megalitres	36 megalitres	120 megalitres
Inflow characteristics	Domestic and industrial	Domestic and industrial (85:15)	Domestic and industrial (60:40)	Domestic and industrial (80:20)	Domestic and industrial (70:30)
Type of treatment process	Biological	Biological and chemical*	Biological and chemical*	Biological and chemical**	Biological

* chemical treatment with ferric chloride

** chemical treatment with aluminium oxide

Preparation of wet sludge inoculum

For experiment 1, wet sludge pellets were prepared by centrifugation of the settled sludges in a Beckman J6 ultracentrifuge at 3000 rpm for 20 min. Forty g of wet sludge from each system was weighed off in triplicate and aseptically transferred to sterile 1 l Erlenmeyer flasks.

For experiment 2, wet sludge pellets were prepared by centrifuging 900 ml quantities of unsettled (shaken-up) sludges in a Beckman J6 ultracentrifuge at 3000 rpm for 20 min. The resulting wet sludge pellets were weighed and distributed in equal quantities in duplicate into sterile 1 l Erlenmeyer flasks.

To induce phosphate uptake, the pellets were stored at 4°C overnight to allow for anaerobic/microaerophilic conditions to develop.

Preparation of sterile mixed liquor growth medium

Mixed liquor from Daspoort was used as a nutrient media for both experiments (Table 4.4). Grab samples (25 l) from the anaerobic zone (already containing orthophosphate after anaerobic release from polyphosphate and influent wastewater) were taken. After settling (1 h), the top clear mixed liquor was filtered through Whatman No. 1 filter papers using a vacuum pump (Edwards E.B.3).

The mixed liquor obtained was sterilized by autoclaving in 5 l Schott bottles for 60 min. After cooling, the pH of the liquor was determined and adjusted to 6.89 for both experiments with concentrated sulphuric acid. The phosphate concentration was measured and adjusted to 219 $mg.l^{-1}$ for experiment 1 and 28 $mg.l^{-1}$ for experiment 2 with sterile 2M KH_2PO_4 (made up in mixed liquor and autoclaved).

Table 4.4: Characteristics of sterile mixed liquor from Daspoort used in experiments 1 and 2. Standard Deviations are shown in brackets.

Analysis	Experiment 1	Experiment 2
ATP cells.ml^{-1}	2.15x10^{3} (1.77x10^{3})	ND*
MLSS mg.l^{-1}	400.00 (140.00)	0.00 (0.00)
MLVSS mg.l^{-1}	ND	0.00 (0.00)
COD mg.l^{-1}	87.50 (7.78)	61.00 (2.83)
TPC cfu.ml^{-1}	< 10 (0)	< 10 (0)
pH (original) **pH** (adjusted)	8.65 (0.00) 6.89 (0.00)	8.55 (0.00) 6.89 (0.11)
PO_4^{3-} (original) mg.l^{-1} **PO_4^{3-}** (adjusted) mg.l^{-1}	13.60 (0.00) 219.50 (0.00)	12.90 (0.00) 28.00 (0.00)
NO_3^- mg.l^{-1}	< 5.00 (0.00)	5.50 (2.12)
SO_4^{2-} mg.l^{-1}	165.50 (4.95)	ND
NH_4^+ mg.l^{-1}	16.59 (0.14)	13.50 (0.21)

* ND = Not determined

Experimental protocol

At time zero (T_0), before the onset of the experiments, 600 ml (experiment 1) and 450 ml (experiment 2) of the sterile mixed liquor growth medium was added to every flask containing the wet sludge pellets, as well as to two sterile flasks containing no sludge. These two flasks without sludge would act as controls. One millilitre of the sludge suspensions was drawn from each flask for total plate counts (experiments 1 and 2) and ATP analysis (experiment 1 only). A 100 ml aliquot was also extracted from each flask for the determination of MLSS. The filtrate from the MLSS determination was used for all chemical analyses. The dissolved oxygen concentration was determined at T_0 (before the onset of the experiment) by means of a Yellow

Springs Industries dissolved oxygen meter and an oxygen probe. The dissolved oxygen concentration of each flask was also measured 1, 3, 5, 7 and 8 h into experiment 1 and 1, 4 and 7 h into experiment 2. Experiment 1 was terminated 8 h after the experiment started, while experiment 2 was terminated after 7 h. For both experiments, the flasks were put on a Labotec reciprocal platform shaker and the flasks stoppered with cotton wool. The shaker was operated at a speed of 130 cycles.min^{-1}. The flasks were also aerated by means of an Elite air pump (Elite 802) and a specially made 15-point glass adapter to which plastic hoses were attached.

Phosphate concentrations were determined hourly from T_0 through T_8 for experiment 1 and T_0 through T_7 for experiment 2 after filtration of 10 ml aliquots through Whatman no.1 filter papers. All physico-chemical and microbiological tests were again performed at T_8 for experiment 1 and T_7 for experiment 2.

The experimental protocol for experiments 1 and 2 is summarized in Figure 4.1, while actual averaged results with standard deviations (for experiment 1 only) are given in Appendix 1 (Tables 4.5 and 4.6).

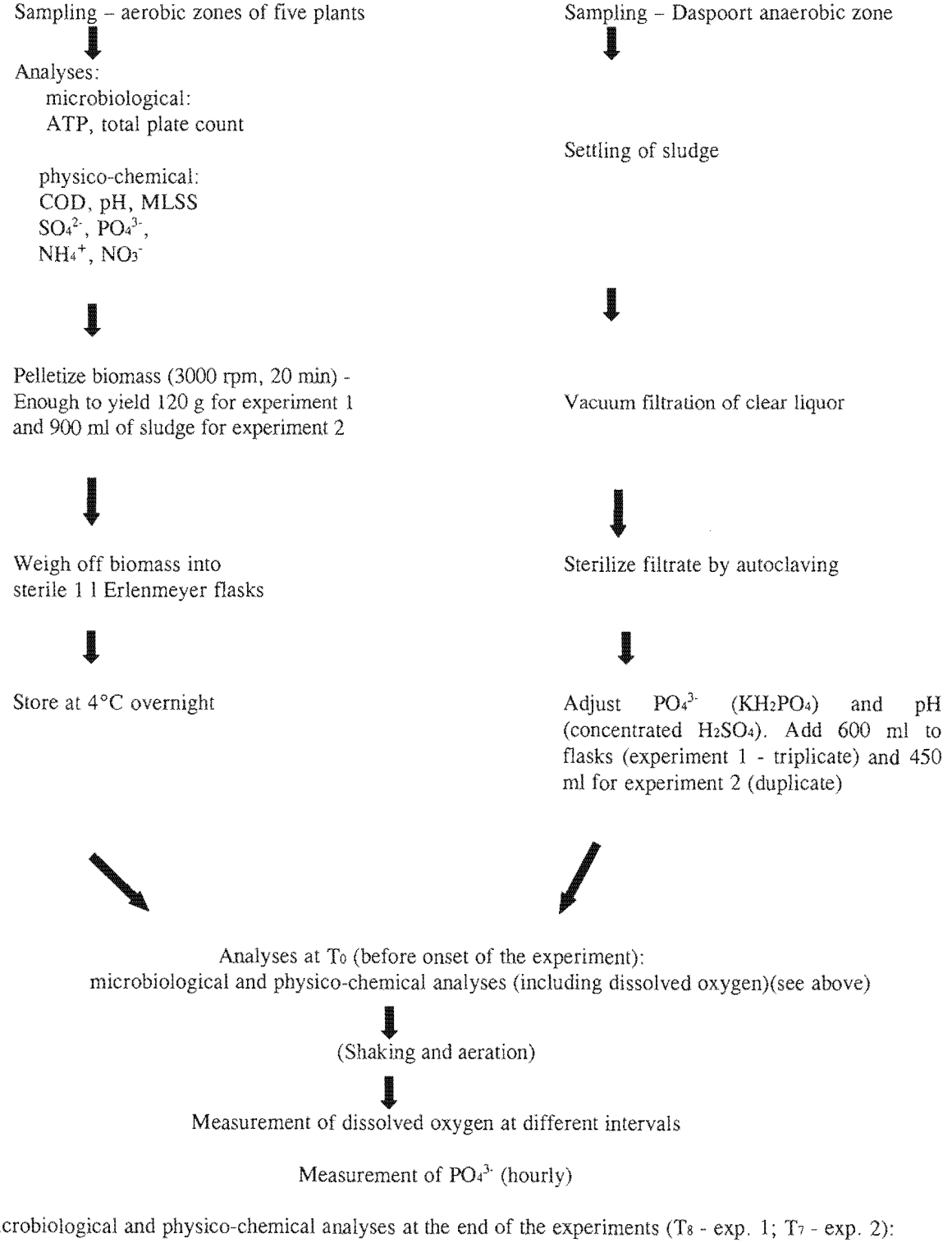

Figure 4.1: Graphic representation of the experimental protocol used in experiments 1 and 2.

4.4 RESULTS AND DISCUSSION:

4.4.1 Experiment 1

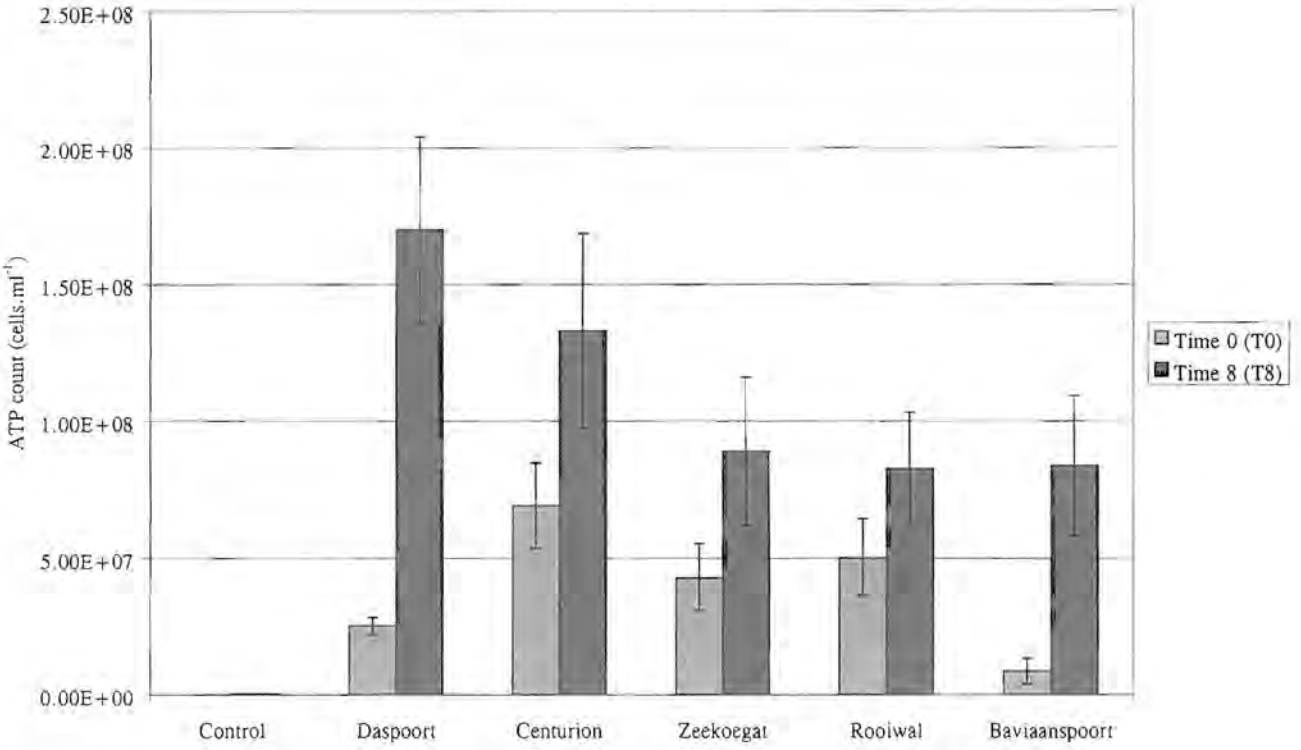

Figure 4.2: Average ATP results at Time 0 and Time 8.

The ATP values indicated that the initial active biomass fraction in the MLSS from the different systems varied. Centurion had the highest ATP concentration, followed by Rooiwal, Zeekoegat, Daspoort and Baviaanspoort (Figure 4.2). After 8 h, Daspoort had the highest ATP concentration, followed by Centurion, Zeekoegat, Baviaanspoort and Rooiwal. Daspoort showed the largest increase in ATP concentration, followed by Baviaanspoort, Centurion, Zeekoegat and Rooiwal. The increase in MLSS values during the experimental period was attributed to the increase in bacterial numbers as indicated by the ATP concentrations (Figure 4.7). The increase in ATP concentrations indicated that bacterial growth took place during the experimental period.

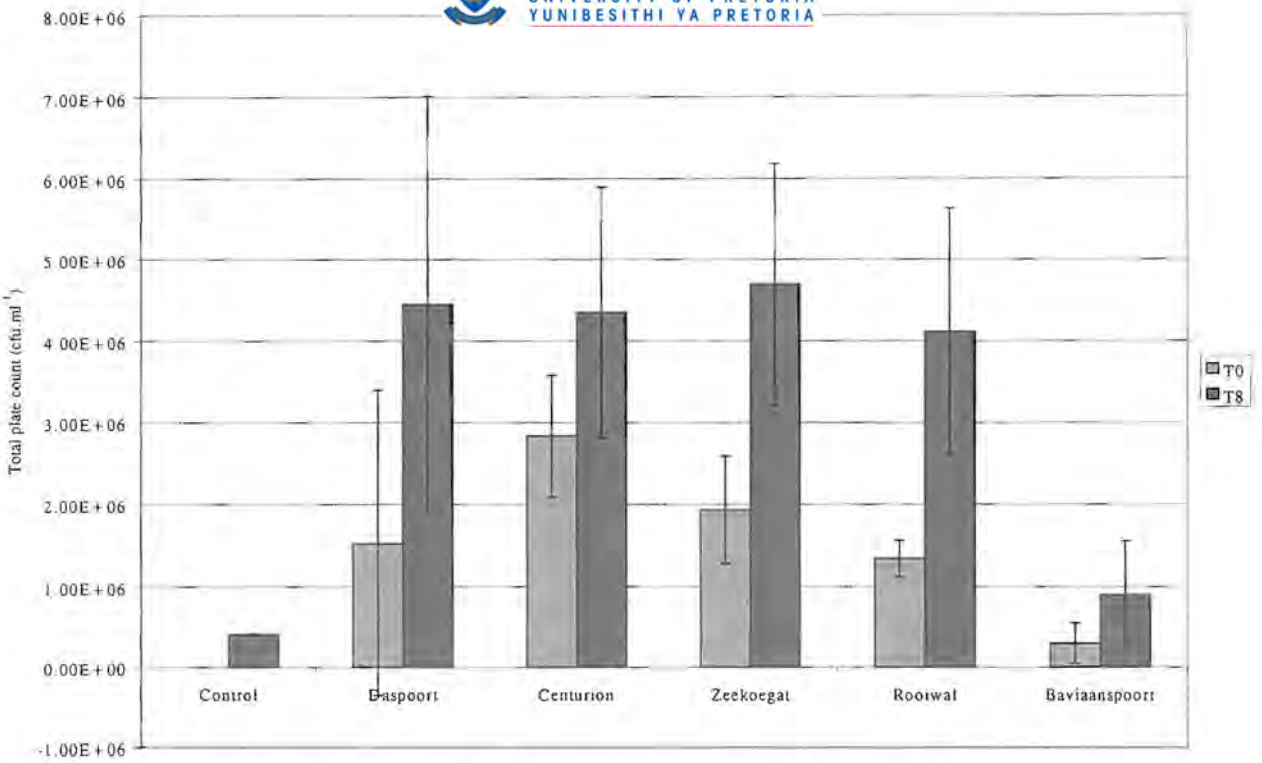

Figure 4.3: Average total plate count (TPC) results at Time 0 and Time 8.

On average, the TPC was the highest for the Centurion sludge, followed by Zeekoegat, Daspoort, Rooiwal and Baviaanspoort (Figure 4.3). The TPC indicated an increase in cell numbers during the experimental period. This was in agreement with the ATP concentrations (Figure 4.2). The standard deviation for the TPC was larger than the standard deviations for ATP analysis (Figure 4.2). The larger variation in the TPC data was ascribed to the method, which relies on colony formation. The colony forming unit in activated sludge would be the floc, which may contain any number of individual bacteria. Since the floc size and distribution in a sample will vary, one would expect a greater variance in the result, as was observed in this study (Figures 4.4 and 4.5). On the other hand, ATP analysis relies on an extraction method, which is not reliant on floc size or distribution, hence the smaller variation in the results. This is furthermore substantiated by the higher ATP cell number values compared to the TPC (on average a one log difference). This also confirms previous data indicating that less than 10% of the viable organisms in activated sludge are culturable (Cloete and Steyn, 1988). These results indicated that ATP was the better method for determining the biomass concentration in activated sludge. This is in agreement with results in previous studies (Jørgensen *et al.*, 1992; Roe and Bhagat, 1982).

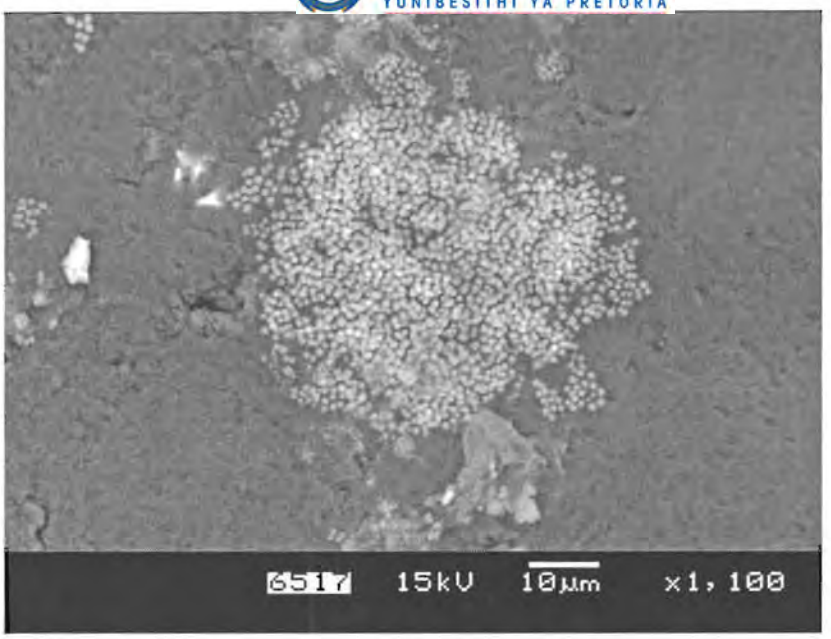

Figure 4.4: Backscatter electron micrograph of activated sludge indicating the floc structure and concept of the colony forming unit.

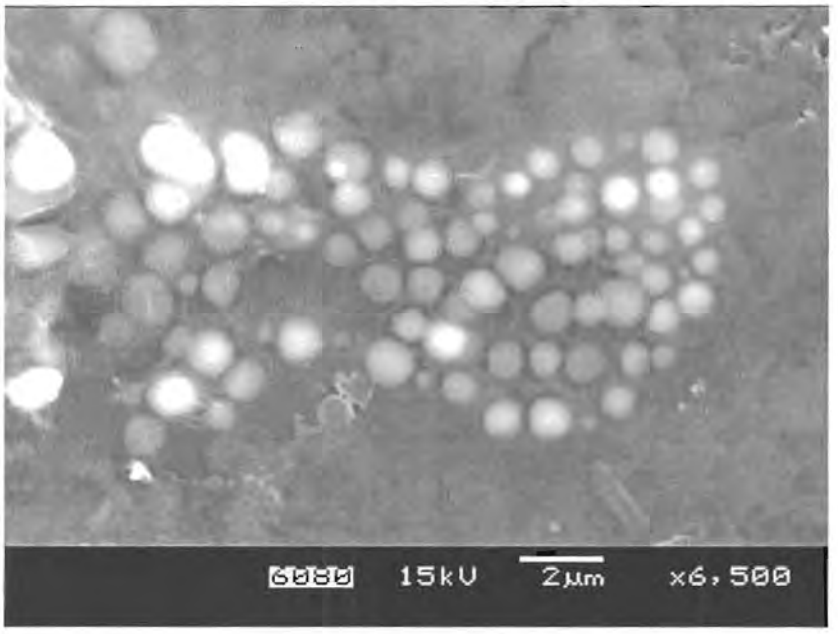

Figure 4.5 Backscatter electron micrograph of activated sludge indicating the floc structure and concept of the colony forming unit.

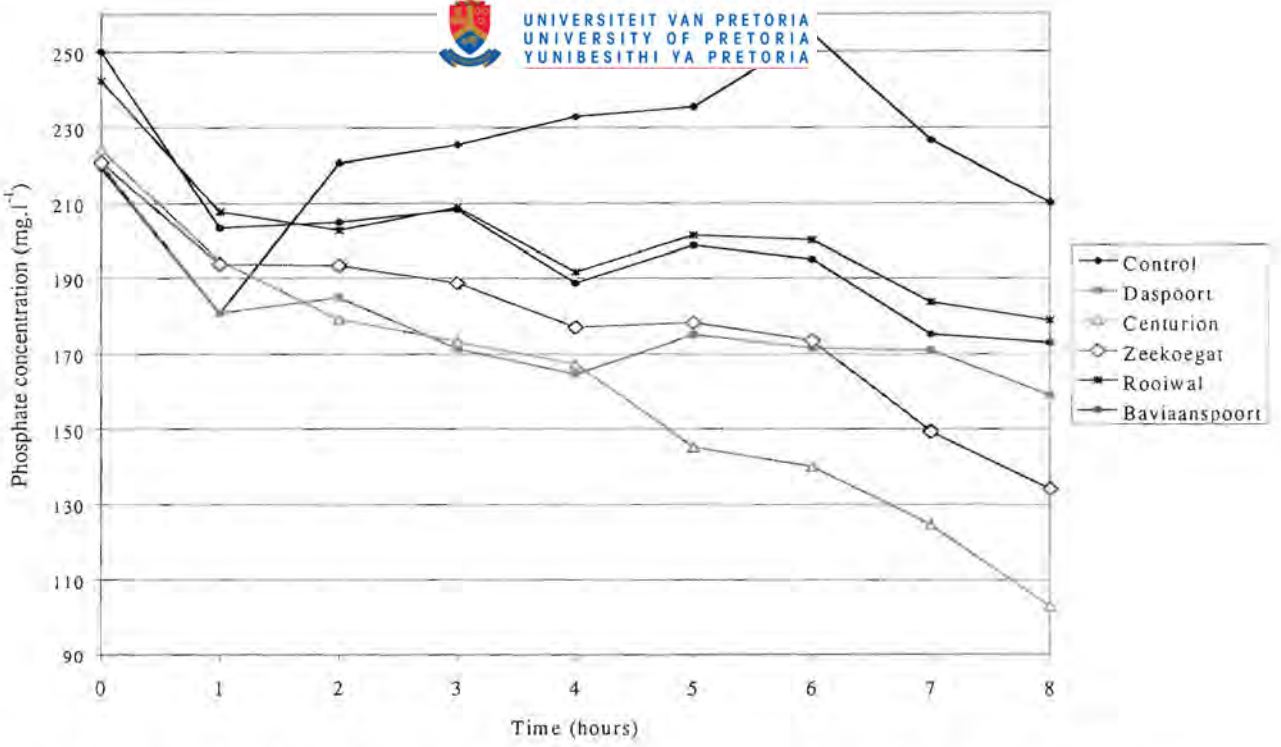

Figure 4.6: Average orthophosphate uptake from different activated sludge systems for wet sludge (66.67 mg.ml^{-1}).

When calculated in terms of the average quantity of orthophosphate removed per gram wet mass, the Centurion WTP performed best, removing 3.03 mg P.g^{-1}, followed by the Zeekoegat, Baviaanspoort, Rooiwal and Daspoort WTPs with uptakes of 2.17, 1.93, 1.59 and 1.54 mg P.g^{-1}, respectively (Figure 4.6). These values were smaller than those calculated in terms of initial MLSS (Figure 4.8). For example, the Centurion WTP showed a removal of 30.79 mg P.g^{-1} initial MLSS, compared to 3.03 mg P.g^{-1} wet sludge. This was not surprising, since the MLSS does not include the hydrated fraction and, hence, the higher removal rates in terms of phosphorus expressed as mg P.g^{-1} MLSS. On average, the Centurion WTP performed best at orthophosphate removal, removing a total of 121 mg P.l^{-1} orthophosphate, followed by the Zeekoegat, Baviaanspoort, Rooiwal and Daspoort with changes in orthophosphate concentration of 86, 78, 64 and 62 mg P.l^{-1}, respectively.

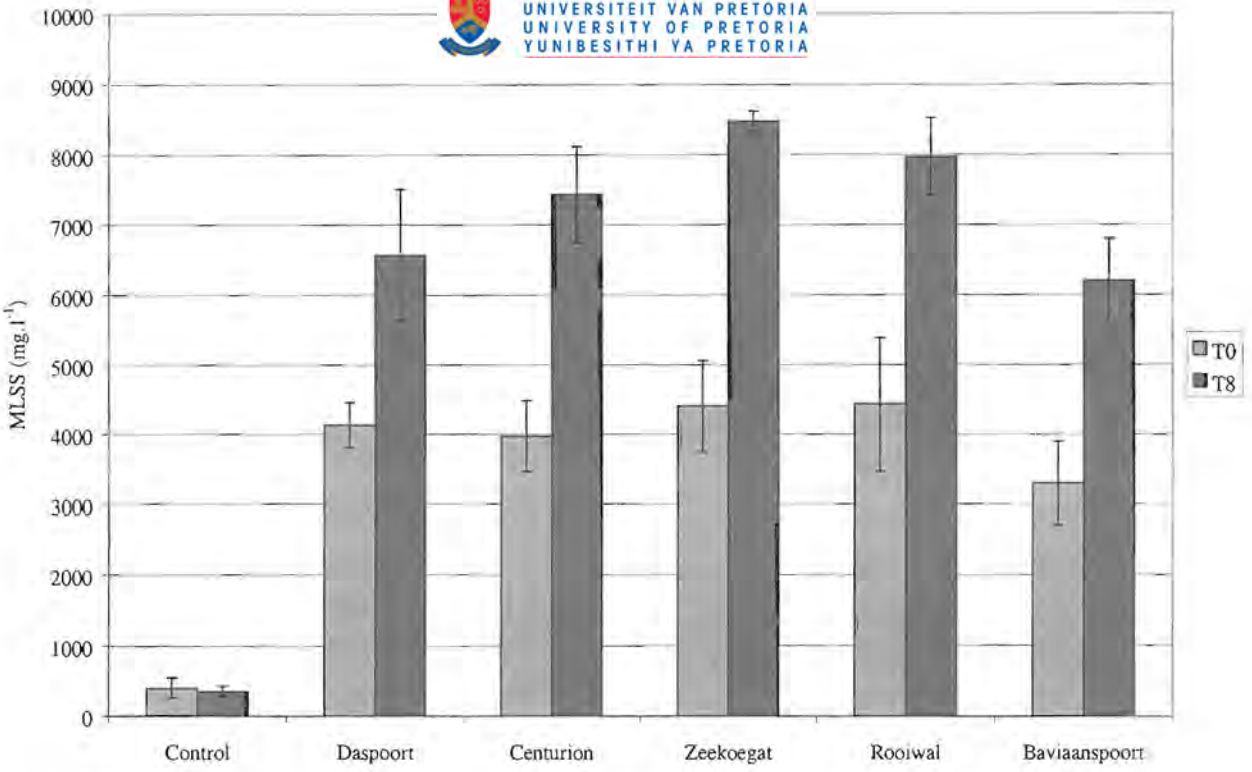

Figure 4.7: Average MLSS values at Time 0 and Time 8 during experiment 1.

MLSS values at time 0 h were similar for all the systems. Values increased for all the systems during the 8 h experiment. MLSS values increased by 4070, 3540, 3370, 2900 and 2440 $mg.l^{-1}$ for the Zeekoegat, Rooiwal, Centurion, Baviaanspoort and Daspoort WTPs, respectively. The increase in MLSS values was attributed to an increase in viable bacterial cell numbers as indicated by total plate counts and ATP analyses (Figures 4.2 and 4.3). The good orthophosphate removal by the Centurion system can, however, not only be attributed to the increase in MLSS, as it did not show the greatest increase in MLSS. It is therefore clear that the MLSS composition, especially in terms of viable cells might play an important role in different orthophosphate uptake abilities of different sludges, as observed in better orthophosphate removal in sludges containing higher initial TPC and ATP counts (Figures 4.2 and 4.3). No pattern could be observed for the rest of the WTPs, although the Daspoort WTP showed the smallest increase in MLSS (2440 $mg.l^{-1}$), while removing the least amount of orthophosphate (62 mg $P.l^{-1}$)(Figure 4.6). The control flasks showed an initial MLSS value of 400 $mg.l^{-1}$ at time 0 h that can be attributed to residue not filtered out in the preparation of the sterile growth medium.

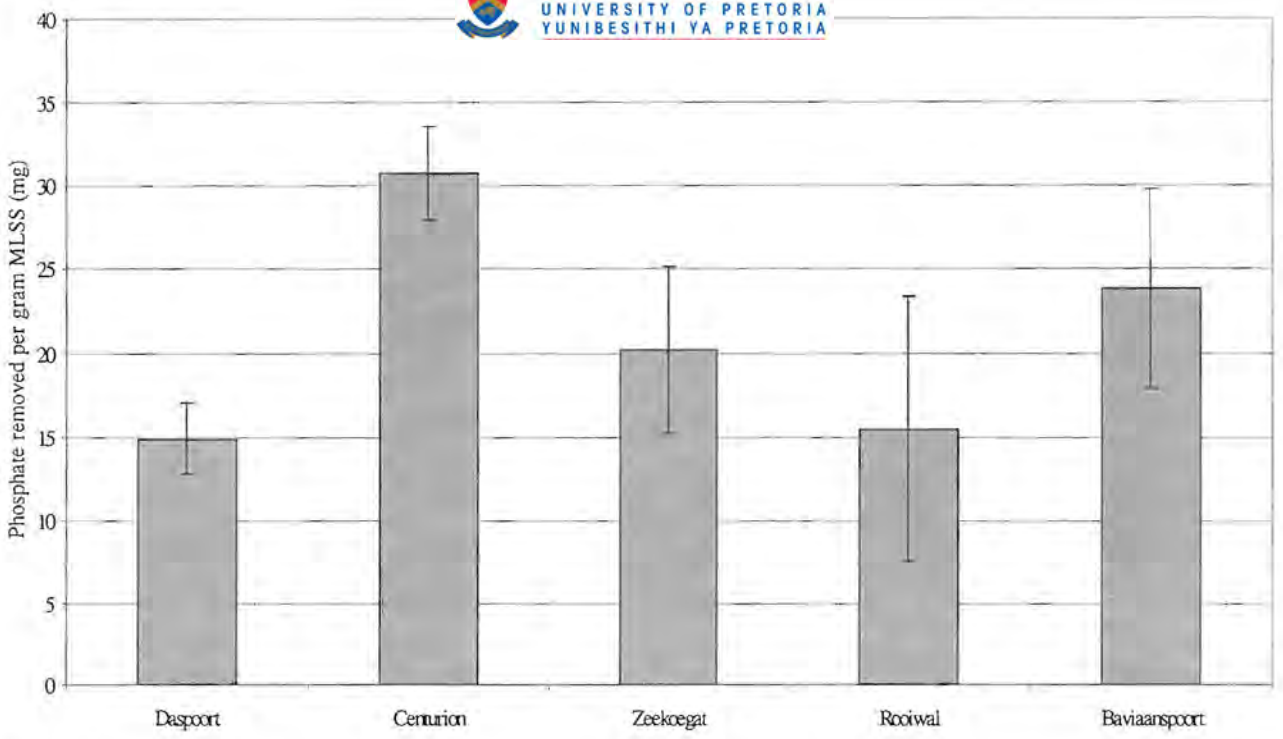

Figure 4.8: Average orthophosphate uptake (mg) per gram of initial MLSS.

With the orthophosphate removed calculated in terms of initial MLSS, Centurion performed the best (30.79 mg $P.g^{-1}$ removal) followed by Baviaanspoort, Zeekoegat, Rooiwal and Daspoort with values of 23.78, 20.17, 15.40 and 14.88 mg $P.g^{-1}$ MLSS, respectively (Figure 4.8). Although the initial MLSS values were similar for all the systems, the quantities of orthophosphate removed over the experimental period were different. Judging from the standard deviations, it was concluded that these differences were, however, not significant for Baviaanspoort, Daspoort, Zeekoegat and Rooiwal. However, the orthophosphate removal was significantly higher in the Centurion WTP (Figure 4.8).

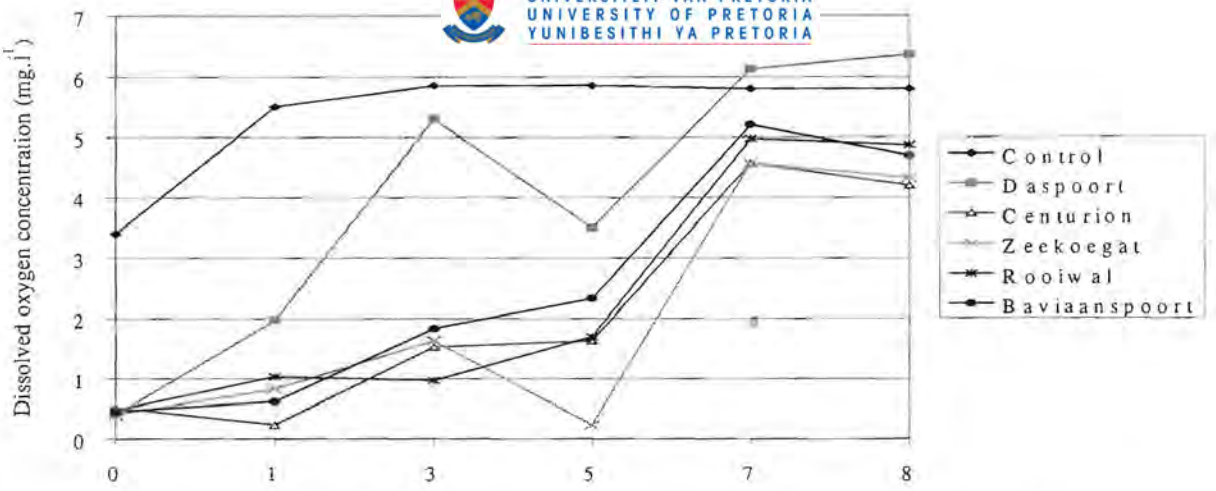

Figure 4.9: Average dissolved oxygen concentration during the eight hour experimental period.

It is desirable to have dissolved oxygen concentration of more than 1 $mg.l^{-1}$ for optimum phosphorus removal in full-scale systems (Lilley *et al.*, 1997). The dissolved oxygen concentration during this experiment was constantly above 0.23 $mg.l^{-1}$, and above 1.5 $mg.l^{-1}$ from time 3 h onwards (Figure 4.9). This indicated that aeration conditions in this batch test were optimal for orthophosphate uptake.

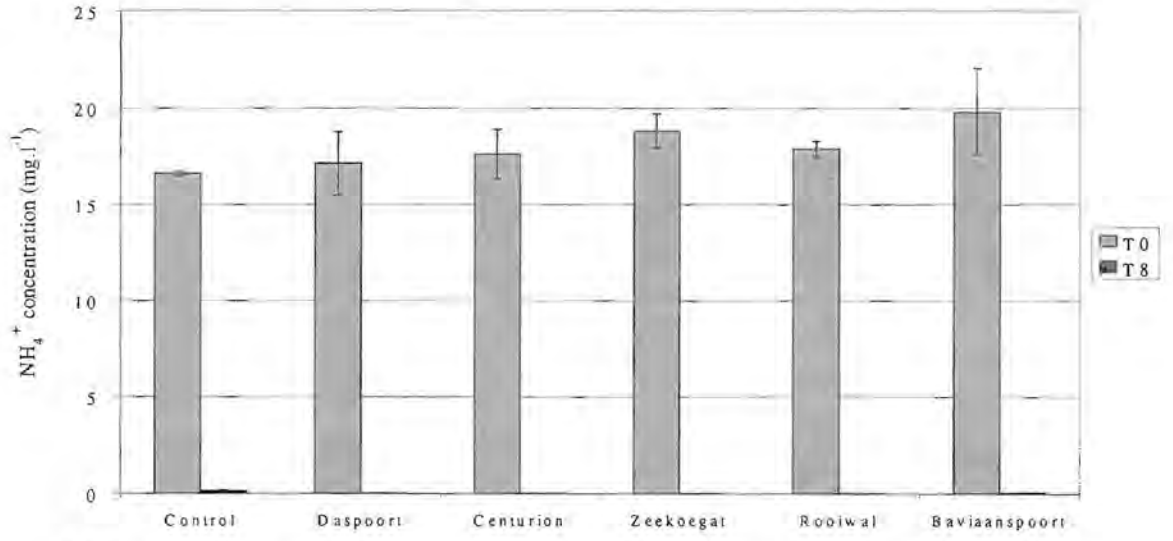

Figure 4.10: Average ammonium concentration at Time 0 and Time 8.

Almost complete nitrification was observed in all the systems (Figure 4.10). This was expected due to the prevailing aerobic conditions, stimulating nitrification, which is an aerobic process.

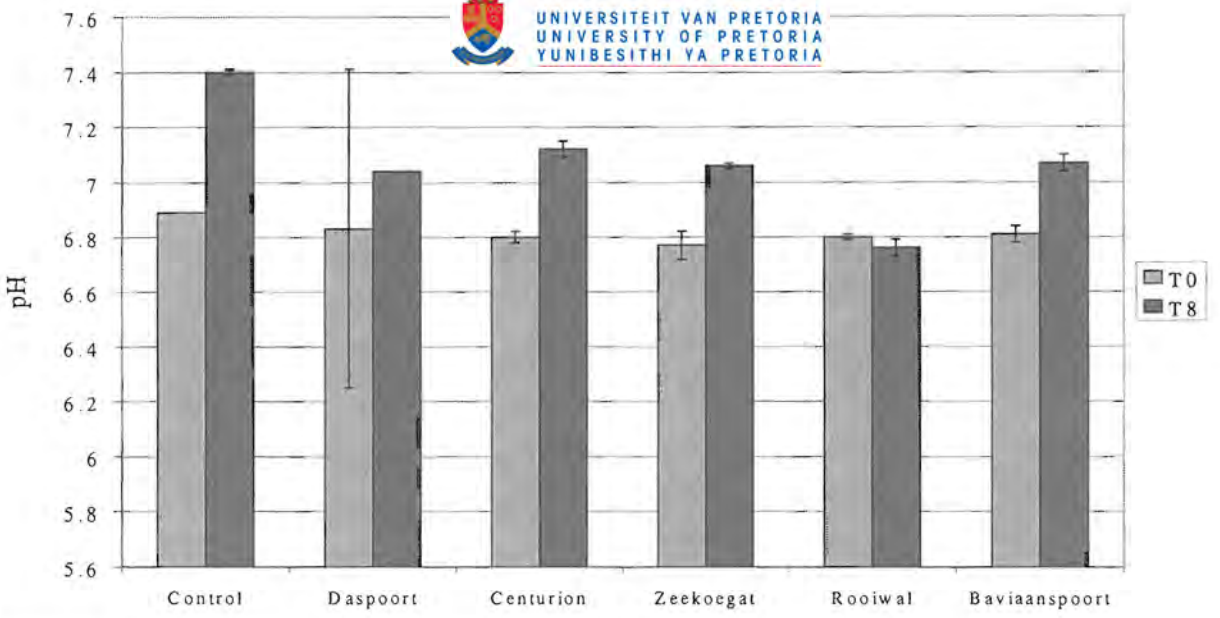

Figure 4.11: Average pH values at Time 0 and Time 8.

Values for pH for time 0 h and time 8 h were constant during the experimental period (Figure 4.11) and within the optimal range for orthophosphate removal as reported by Fuhs and Chen (1975).

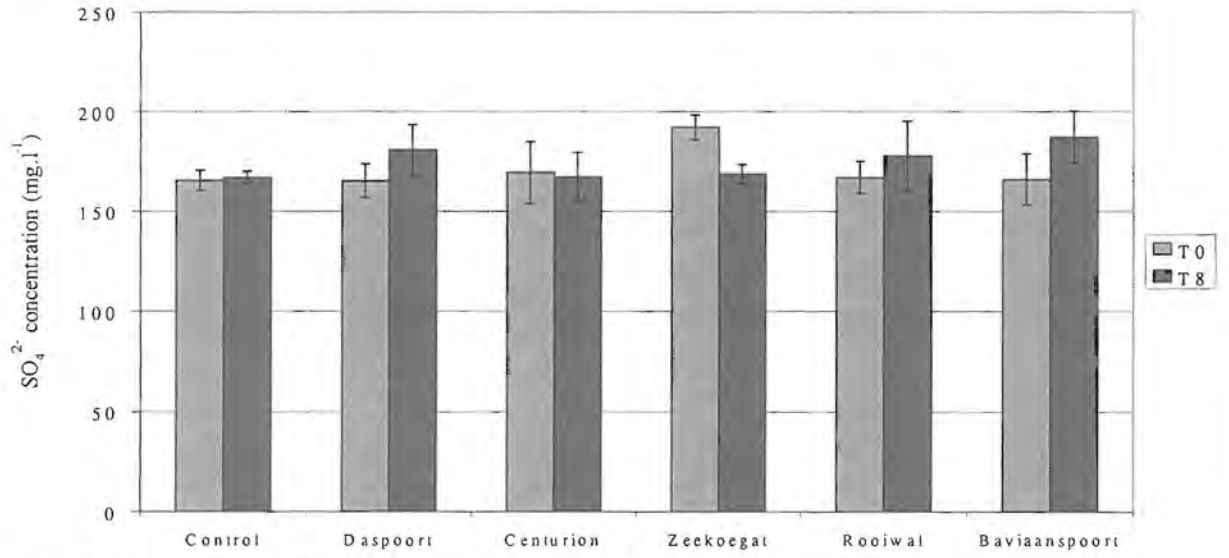

Figure 4.12: Average sulphate concentration at Time 0 and Time 8.

Sulphate results were similar at time 0 and time 8 for all the systems (Figure 4.12). This was expected, since aerobic conditions prevailed and, hence, no sulphate reduction would occur.

4.4.2 Experiment 2

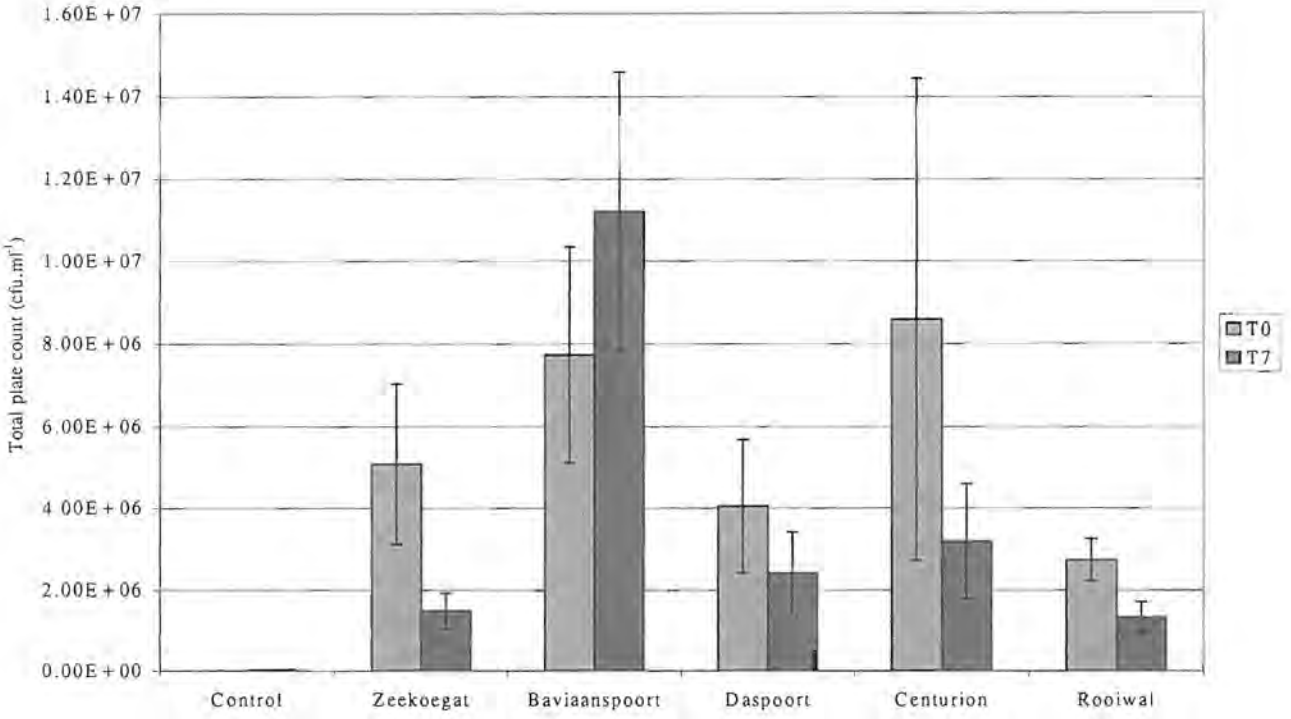

Figure 4.13: Average total plate count results at Time 0 and Time 7.

On average, for all the systems except Baviaanspoort, a decrease in cell numbers was observed (Figure 4.13). The Centurion and Baviaanspoort systems, on average, contained most colony forming units (cfu) at time 0 h, although large standard deviations (as also seen in experiment 1) were observed. Centurion removed the most orthophosphate (33 mg $P.l^{-1}$, followed by Baviaanspoort with a removal of 29 mg $P.l^{-1}$ (Figure 4.14). However, over the experimental period, the Baviaanspoort WTP showed an increase in cell numbers ($3.47x10^6$ $cfu.ml^{-1}$)(Figure 4.13), while Centurion showed the largest decrease in cell numbers ($5.38x10^6$ $cfu.ml^{-1}$). Clearly different mechanisms for the different orthophosphate removal patterns for these two plants exist. Although the large initial cell counts might be responsible the good orthophosphate uptake ability of these two sludges (Figure 4.14), either their bacterial composition could be different or, in the case of Centurion, the bacteria were not adapted to nutritional stress, as no additional nutrients were added to the sterile growth medium. This behaviour is typical of r-strategists (Andrews and Harris, 1985; Atlas and Bartha, 1993).

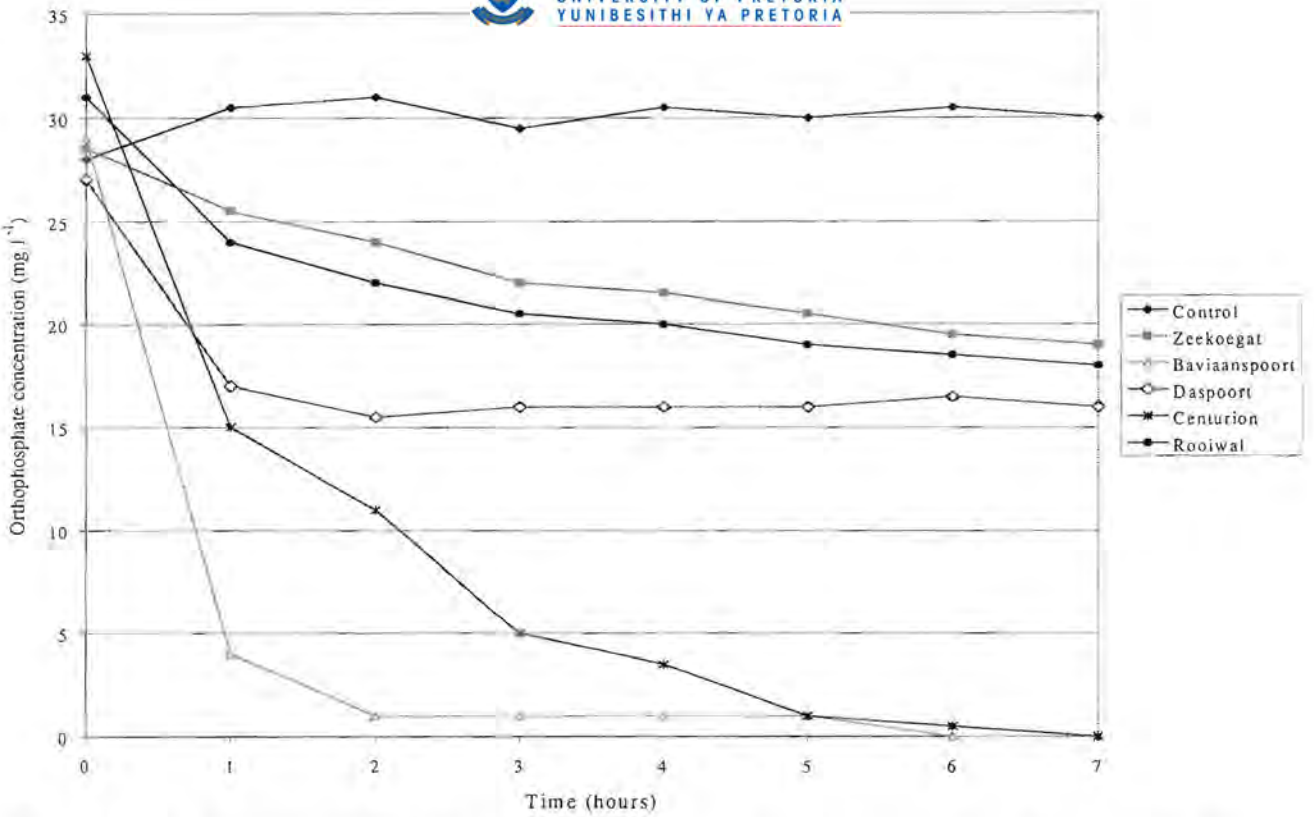

Figure 4.14: Average orthophosphate uptake from different activated sludge systems for sludge re-suspended to the original MLSS concentration.

Centurion removed the most orthophosphate (33 mg $P.l^{-1}$), followed by the Baviaanspoort, Rooiwal, Daspoort and Zeekoegat plants with uptakes of 29, 13, 11 and 10 mg $P.l^{-1}$, respectively (Figure 4.14). However, Baviaanspoort removed the orthophosphate quicker (within 2 h all the orthophosphate was removed). Levels of aluminium in the original sludge sampled from the Centurion system were higher (0.18 mg.l^{-1}) compared to the other systems (<0.05 mg.l^{-1})(Table 4.2). The fast removal of orthophosphate in the Centurion sludge was, however, not attributed to the amount of aluminium present, mainly because of the 1:1 stochiometry requirement of aluminium precipitation of orthophosphates (Lilley *et al.*, 1997). The large orthophosphate removal capacities of the Centurion and Baviaanspoort systems was attributed to the large initial bacterial numbers in these plants as indicated by the total plate count (Figure 4.13) and initial MLSS and MLVSS values (Figures 4.15 and 4.16, respectively).

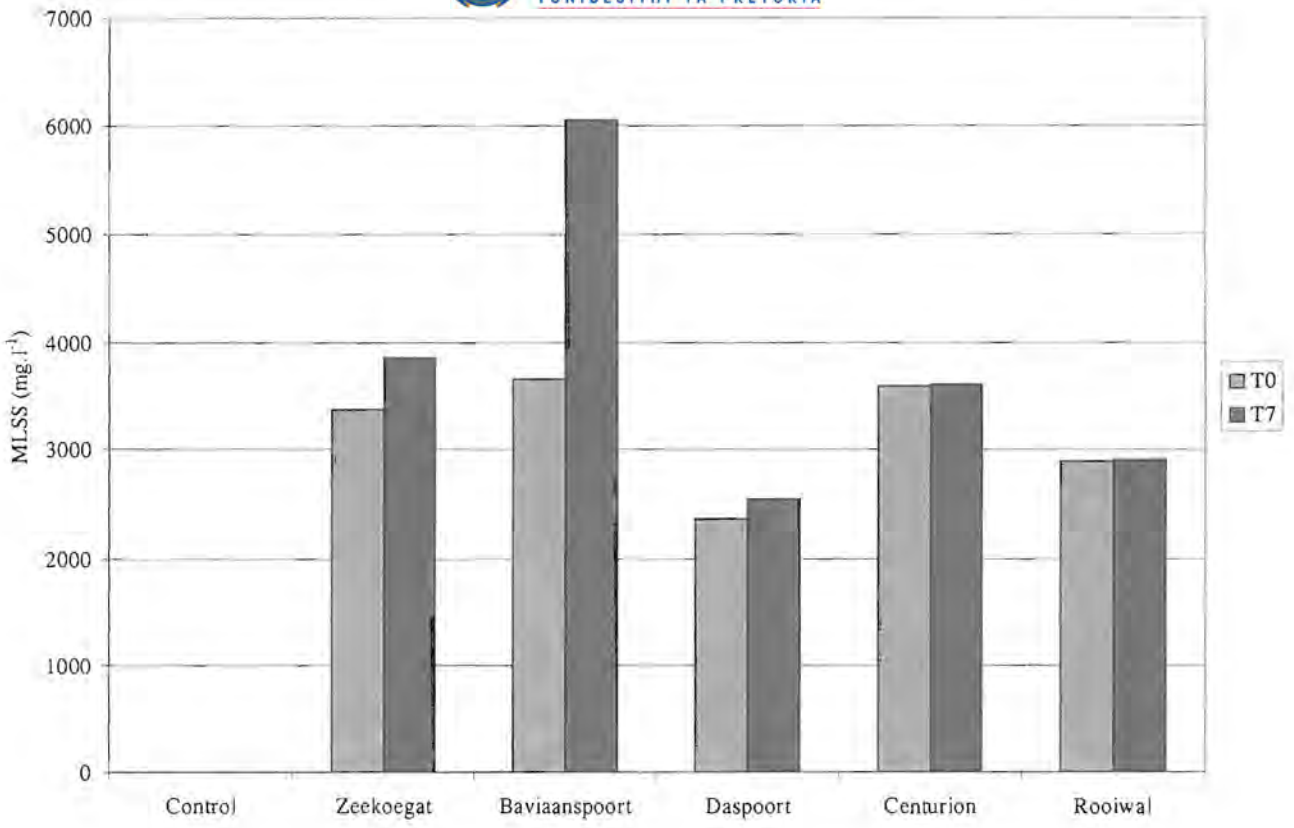

Figure 4.15: Average MLSS values at time 0 and time 7.

In the Baviaanspoort sludge, an average increase in MLSS of 2390 $mg.l^{-1}$ was observed (Figure 4.15). This was followed by the Zeekoegat and Daspoort plants, which showed average increases of 480 and 190 $mg.l^{-1}$, respectively, while both the Centurion and Rooiwal plants showed an increase of 15 $mg.l^{-1}$. The increase in MLSS in the Baviaanspoort sludge was attributed to an increase in bacterial cell numbers (Figure 4.13). These results, furthermore, indicate that little microbial growth that could contribute to the MLSS occurred in the other systems over the 7 h experimental period. The small increase (15 $mg.l^{-1}$) in MLSS in the Centurion plant (which removed the most orthophosphate) and the large increase in MLSS for the Baviaanspoort plant (which removed nearly the same amount of orthophosphate, only faster) indicated differences in the viable biomass fraction of these sludges. The results indicated that MLSS was not a good indicator of viable biomass and microbial activity in activated sludge.

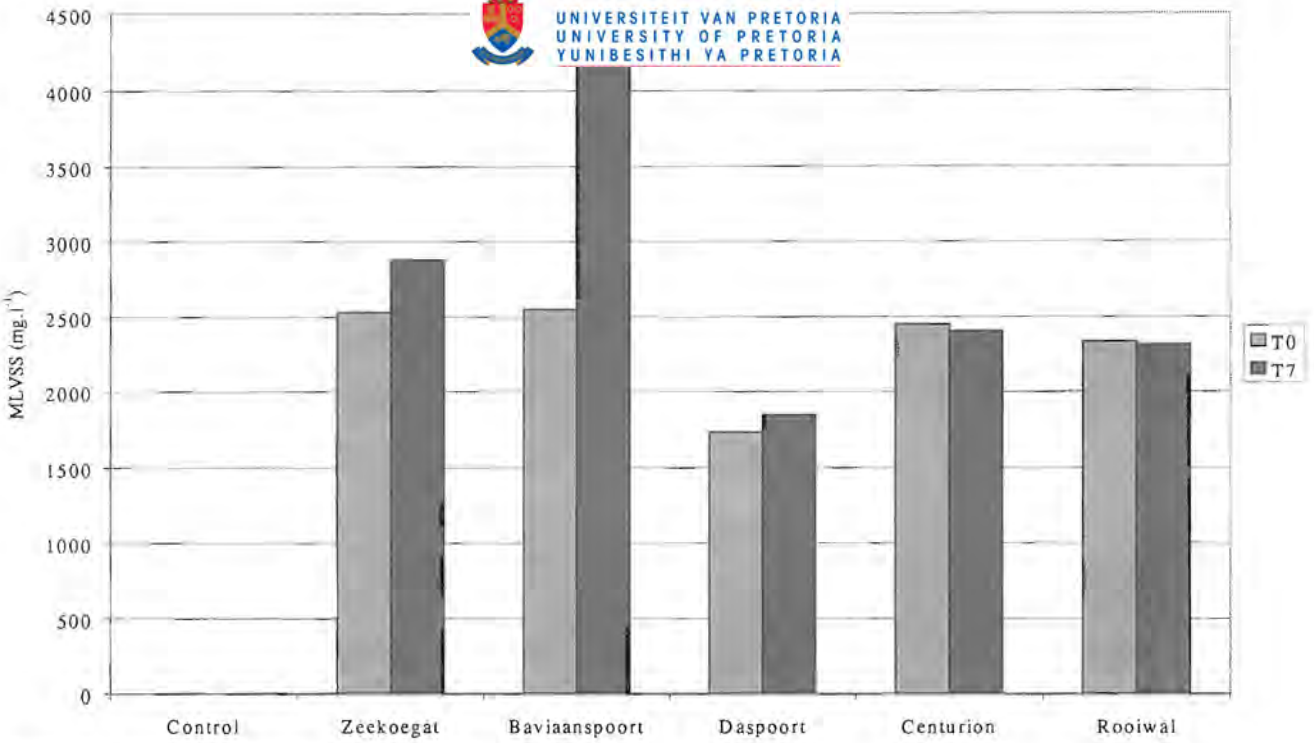

Figure 4.16: Average MLVSS values at Time 0 and Time 7.

The MLVSS values of all the systems remained rather constant during the experiment, except for Baviaanspoort where an increase of 1650 $mg.l^{-1}$ was observed (Figure 4.16). This was followed by the Zeekoegat and Daspoort plants, which showed increases of 350 and 110 $mg.l^{-1}$, respectively. The Rooiwal and Centurion plants showed decreases in MLVSS of 20 and 45 $mg.l^{-1}$, respectively. These decreases could have indicated the inability of these sludges to perform well at orthophosphate removal under nutritional stress, also causing dectreases in cell numbers by means of TPC (Figure 4.13). This phenomenon is reminiscent of r-strategists. The results were similar to results obtained for MLSS (Figure 15). Again, the increase in MLVSS for Baviaanspoort was attributed to an increase in bacterial cell numbers and, hence, viable biomass. However, the discrepancy between MLSS and MLVSS values for Baviaanspoort and Centurion, indicated that MLVSS was a more sensitive method for indicating microbial biomass, but not a good indicator of viable biomass and microbial activity in activated sludge. The MLVSS fraction of the MLSS was 75, 70, 73, 68 and 80 %, respectively for the Zeekoegat, Baviaanspoort, Daspoort, Centurion and Rooiwal WTPs and this fraction did not change over the 7 h experimental period. These values also compared well with values at the time of sampling, indicating success at simulating these fractions of activated sludge in this experiment (Table 4.2).

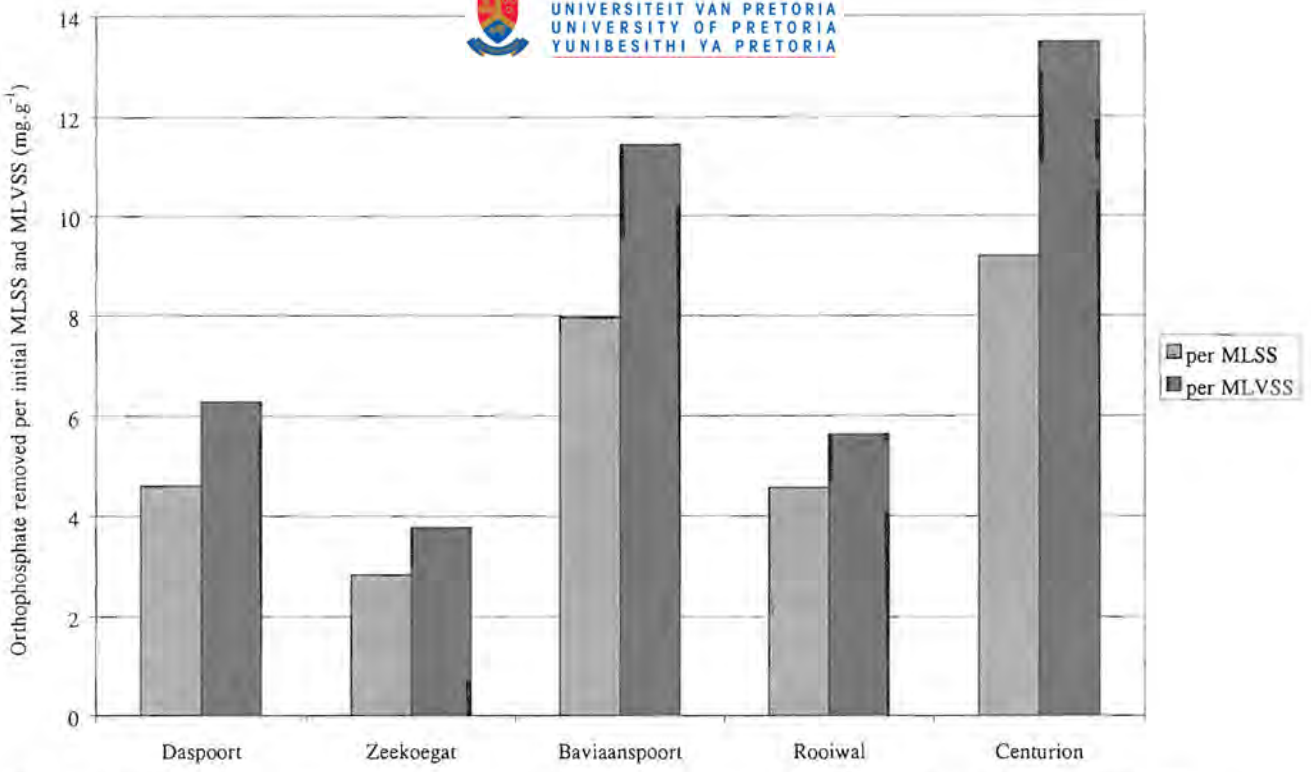

Figure 4.17: Average orthophosphate uptake (mg) per gram of initial MLSS and MLVSS.

MLVSS values were, as expected, always lower than the MLSS, since it is the volatile fraction of the MLSS (Figures 4.15 and 4.16). Nevertheless, the MLVSS followed the same orthophosphate uptake trend as the MLSS for all the sludges (Figure 4.17). Average orthophosphate removed calculated in terms of initial MLSS indicated that Centurion performed best (9.19 mg $P.g^{-1}$) followed by Baviaanspoort, Daspoort, Rooiwal and Zeekoegat with removal of 7.97, 4.60, 4.55 and 2.82 mg $P.g^{-1}$, respectively. In terms of initial MLVSS, the same pattern was observed with removal of 13.48, 11.43, 6.28, 5.63 and 3.76 mg $P.g^{-1}$, respectively. The Baviaanspoort and Centurion systems performed better than the other systems in terms of orthophosphate uptake, both in terms of initial MLSS and MLVSS. In both cases this was attributed to the larger initial bacterial numbers (Figure 4.13), resulting in the higher initial MLSS and MLVSS values (Figures 4.15 and 4.16). The Baviaanspoort and Centurion systems removed all the orthophosphate within 2 h and 5 h, respectively (Figure 4.14). The true potential for orthophosphate uptake ability in these systems, in terms of MLSS, was therefore probably an underestimation, since no residual orthophosphate was left in the flasks.

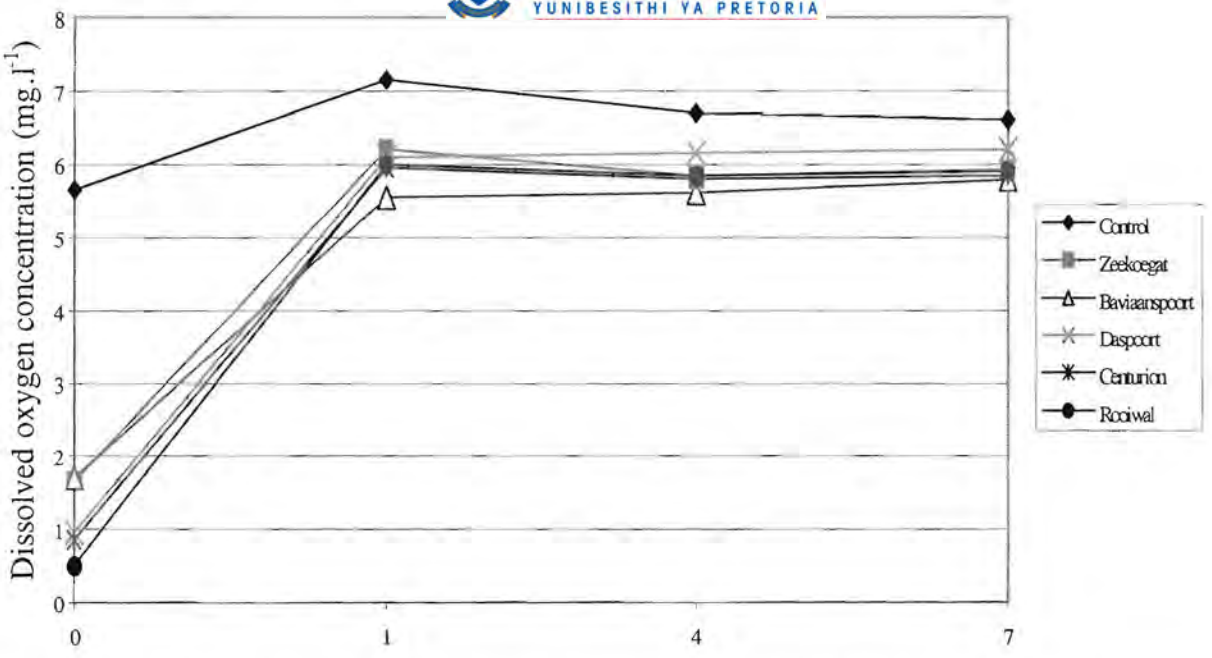

Figure 4.18: Average dissolved oxygen concentrations during the 7 h experimental period.

The dissolved oxygen concentrations were above 1 $mg.l^{-1}$ from time 1 h onwards, indicating optimal conditions for orthophosphate uptake (Figure 4.18).

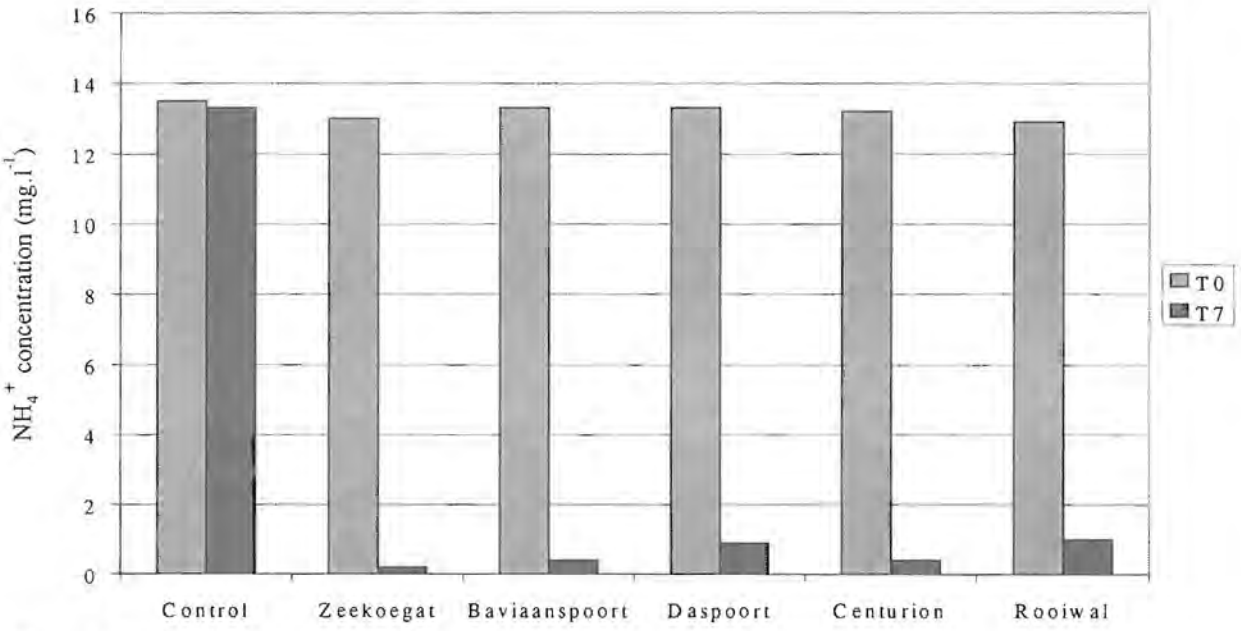

Figure 4.19: Average ammonium concentration at Time 0 and Time 7.

As with experiment 1, almost complete nitrification was observed in all the systems (Figure 4.19). No nitrification was observed in the control flasks, due to the absence of biomass.

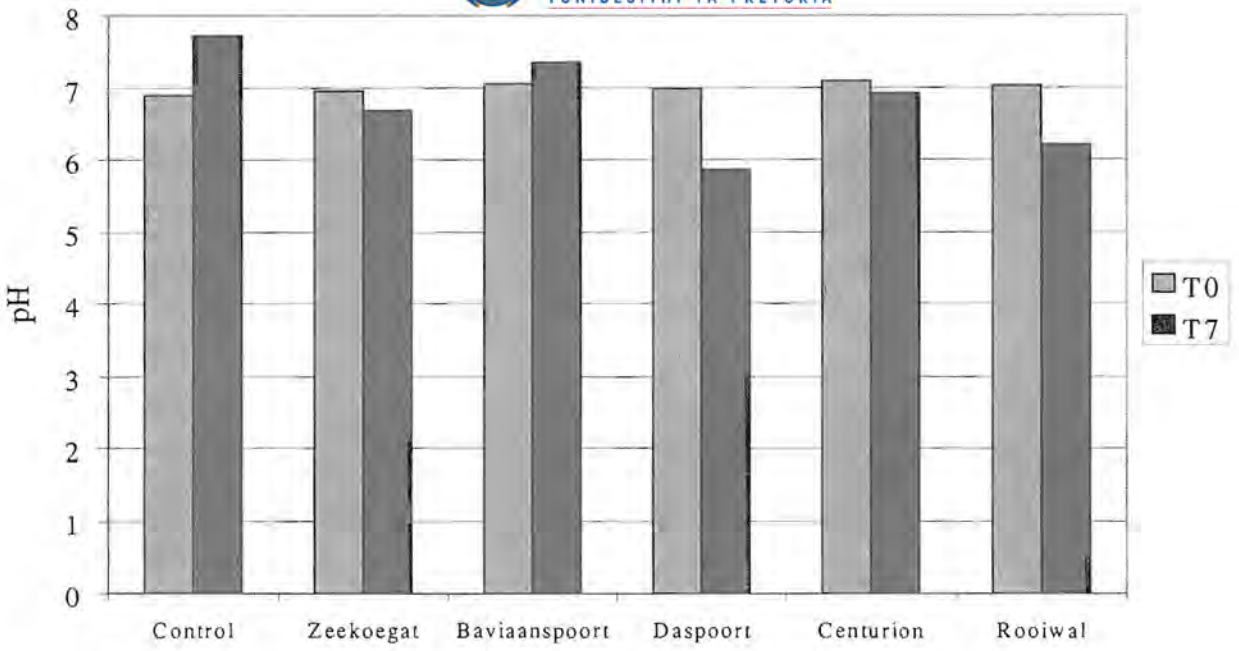

Figure 4.20: Average pH values at Time 0 and Time 7.

As with experiment 1, pH values for the duration of the experiment were in the optimal range for orthophosphate uptake (Fuhs and Chen, 1975)(Figure 4.20).

4.5 GENERAL DISCUSSION:

Historically, the mixed liquor organic suspended solids have been measured as a lumped parameter via the VSS test, or more recently, the COD test. However, as stated above, only a part of the mixed liquor organic suspended solids is heterotrophic active biomass, the active part of activated sludge, and only this part mediates the biological processes of COD removal and denitrification. Currently, the heterotrophic active biomass exists only as a hypothetical parameter within the structure of the design procedures and kinetic models. Although indirect evidence provides support for this parameter (by consistency between observations and predictions over a wide range of conditions) it has not been directly measured experimentally and compared to theoretical values.

The problem in measurement of this parameter has been the lack of simple, suitable experimental techniques. In the literature, principally microbiological techniques like pour-plate or other culturing techniques, ATP analysis, DNA analysis, using fluorescent probes for ribosomal RNA and sequencing of ribosomal DNA. However, these techniques have not yet been adequately integrated with the design and kinetic modelling theory. The culturing techniques have been widely criticized for their unreliability, while the RNA and the two DNA methods are still in their infancy. The last-named four techniques also require sophisticated equipment and experimental techniques that are not widely available (Ubisi et al., 1997).

Quantification, experience, semi-empirical methods, as well as kinetic models of biomass in activated sludge are still routinely used in design of wastewater treatment plants, in spite of the limitations currently experienced in biomass determination. For example, Chuang and Ouyang (2000) estimated the the biomass fractions of heterotrophs and phosphate accumulating organisms (PAO) by experimental results and theoretical calculation on the mass balance of organic matter, nitrogen and phosphorus in a pilot-scale biological nutrient removal process. The results showed that heterotrophs comprised the majority (48%) of the community and that only 12% of PAO were present in the nitrogen and phosphorus removal in the laboratory scale system studied for municipal wastewater treatment.

The active biomass is probably the most important process parameter and currently no adequate method exists to measure it. In order to accurately measure the active biomass concentration in activated sludge, MLSS is of critical importance for:

* monitoring the performance of a system
* troubleshooting
* verifying theoretical models
* optimization of biomass concentration for maximum phosphate removal.

Further biomass studies should include the physical, chemical and biological characterization of MLSS for different activated sludge systems.

Methods to elucidate the physical composition of, and to characterize activated sludge MLSS should include a polyphasic approach, combining culture-dependent microbiological methods with non-culture-dependent techniques like ATP analysis and counting procedures like DAPI, acridine orange (AO) and nalidixic acid staining. Molecular techniques, including 16S rRNA, fluorescent *in situ* hybridization (FISH), DNA extraction, PCR, cloning, sequencing and analysis should also be included.

In terms of design procedures and kinetic models, the bioreactor of the non-nitrifying aerobic activated sludge system is composed of three components: heterotrophic active biomass, endogenous residue and inert material (Ubisi *et al.*, 1997). The inert material arises from the influent wastewater, containing unbiodegradable particulate organics, which on entry into the bioreactor, are enmeshed in the mixed liquor organic suspended solids (Ubisi *et al.*, 1997).

The relationship between biomass and phosphate removal in activated sludge has been indicated by previous research that indicated that phosphate uptake was related to an increase in biomass concentration (Streichan *et al.*, 1990; Bosch, 1992). The higher the biomass, the higher the phosphate removal. Momba and Cloete (1996) reported that high initial cell concentrations of *Acinetobacter junii* (a PAO) removed more phosphate than low cell concentrations, and that phosphate uptake was therefore directly related to biomass concentration and high nutrient availability. Bosch (1992) stipulated that biomass was a more significant factor than the type of organism/s present with reference to biological phoshorus removal. Sidat *et al.* (1999) also reported on findings suggesting a direct relationship between the biomass concentration and phosphate removal capacity. Optimal phosphate removal was achieved at a biomass concentration of 1900 $mg.l^{-1}$. Current research, however, indicated that not only the amount of biomass is important in orthophosphate removal, but also initial bacterial numbers (as determined by means of ATP and TPC) constituting these fractions. It would seem that larger viable numbers could lead to better orthophosphate removal. Also, results would seem to indicate differences in the microbial composition of the active biomass fraction of activated sludge. For example, the Centurion system performed best at orthophosphate removal, although a decrease in cell numbers, MLSS and MLVSS occurred. On the other hand Baviaanspoort performed just as good at orthophosphate removal, although

large increases in TPC, MLSS and MLVSS were observed. This phenomenon would warrant further research, as Ehlers (1997) indicated by use of sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PGE) of protein extracts that the community structure of different activated sludges do not differ much from each other.

In the current study, orthophosphate removal was consistently high with higher biomass concentrations as measured by TPC and ATP. This supports the notion that the viable biomass fraction of the MLSS is the key to orthophosphate removal by activated sludge. However, maintenance of large fractions of viable biomass in activated sludge will select for smaller flocs, causing poor settling Roe and Bhagat, 1982). It is thus important to find a situation of equilibrium between viable biomass and settling performance to optimize the activated sludge process.

Optimization of the ATP measurement procedure is also important. Although most authors claim ATP measurement to be an accurate measure of viable biomass (Weddle and Jenkins, 1970; Roe and Bhagat, 1982), there is still concern as to its constancy under different metabolic conditions. In the experiments by Weddle and Jenkins (1970) and Upadhyaya and Eckenfelder (1975) the ATP per plate count colony was fairly stable, substantiating the claim that ATP is a measure of viable biomass. However in the current study, at time 0 h (before the start of experiment 1) the ATP cell count ranged between 17 and 37 times the TPC cell count, while at the end of the experiment (time 8 h) with controlled aeration and conditions, this range differed even more, ATP counts being between 19 and 93 times the TPC cell count. ATP measurements have shown variation in response to environmental stresses like anoxic conditions in return sludge (Roe and Bhagat, 1982). However, the method has been shown in the current study to be superior to the traditionally used methods of TPC, MLSS and MLVSS for biomass determination. MLSS and MLVSS did not resemble the viable population as measured by ATP or TPC. Also, a seemingly impossible result indicated that the Centurion plant (which had the best orthophosphate removal) had the lowest MLVSS fraction of the MLSS (that is, 68 % compared with up to 80 % for the Rooiwal plant) of all the plants used in this study. ATP was also found to be a better biomass estimator than TPC due to higher (at least one log unit) bacterial counts and smaller standard deviations. It is a

cheap, simple and fast method, not requiring special training for laboratory personnel and with a small capital input for a portable luminometer, giving on-the-spot results. It is clear, however, that further research is necessary to dismiss any disagreement on the suitability of the method as a measure of viable biomass. This should include studies on the proper homogenization of activated sludge to break up flocs, ensuring that the extractant enters the floc effectively for extraction of ATP. Also, different extraction procedures and chemicals should be compared to ensure optimal extraction. Decent calibration curves are also a necessity, while studies on ATP levels under different environmental stresses, conditions and growth phases should be conducted.

4.6 CONCLUSIONS:

- ATP proved to be a more reliable method for indicating the biomass concentration than TPC, due to the higher yield and a smaller standard deviation.
- Orthophosphate removal was consistently higher in the sludges with higher initial ATP and TPC values, indicating a relationship between viable biomass and orthophosphate removal.
- The MLVSS showed the same trend in orthophosphate removal as the MLSS, although always somewhat lower, due to it being the volatile fraction of the MLSS.
- Neither initial MLSS, initial MLVSS nor changes in the concentrations of these fractions could be directly linked to different orthophosphate uptake abilities of different sludges, indicating the unsuitability of MLSS and MLVSS to indicate viable biomass and/or differences in the viable biomass fraction in activated sludge.

4.7 ACKNOWLEDGEMENTS:

The author would like to thank V. Molepo, B. Ntshudisane and T.S. Nevondo for technical assistance, the operators and managers of the different activated sludge plants for their valuable input, as well as the Water Research Commission for funding of this research.

4.8 REFERENCES:

Ali I., Kharajian H. and Ahmed M. (1985). Viability of microbial mass in compartmentalised single activated sludge process. *Water Research* **19**: 927-932.

Andrews J.H. and Harris R.F. (1985). r- and K-selection and microbial ecology. *Advances in Microbial Ecology* **9**: 99-148.

Atkinson B.W. (1999). Identification of polyphosphate accumulating bacteria from pilot- and full-scale nutrient removal activated sludges. M. Tech. Thesis, Technikon Natal, Durban, South Africa.

Atlas R.M. and Bartha R. (1993). *Microbial Ecology: Fundamentals and Applications*. Third edition. The Benjamin/Cummings Publishing Company, Menlo Park, California, U.S.A.

Bitton G. (1994). *Wastewater Microbiology*. John Wiley & Sons, Inc., New York.

Bolitho V.N. (1976). Controlling the access of nutrients from point and diffused sources with special reference to the Pretoria/Witwatersrand/Vereeeniging region. *Water SA* **2**(4): 145-149.

Bond P.L., Hugenholtz J.K., Keller J. and Blackall L.L. (1995). Bacterial community structure of phosphate-removing and non-phosphate-removing activated sludge from sequencing batch reactors. *Applied and Environmental Microbiology* **61**: 1910-1916.

Bond, P.L., Keller J. and Blackall L.L. (1999). Anaerobic phosphate release from activated sludge with enhanced biological phosphorus removal. A possible mechanism of intracellular pH control. *Biotechnology and Bioengineering* **63**(5): 507-515.

Bosch M. (1992). Phosphorus uptake kinetics of *Acinetobacter* in activated sludge mixed liquor. M.Sc. thesis. University of Pretoria, Pretoria, South Africa.

Chuang S-H. and Ouyang C-F. (2000). The biomass fractions of heterotrophs and phosphate-accumulating organisms in a nitrogen and phosphorus removal system. *Water Research* **34**(8): 2283-2290.

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

D'Eustachio A.J. and Levin G.V. (1967). Levels of adenosine triphosphate during bacterial growth. *Bacteriological Proceedings, American Society for Microbiology, Microbial Physiology Division* **20**: 121.

D'Eustachio A.J. and Johnson D.R. (1968). Adenosine triphosphate content of bacteria. *Federation Proceedings, American Society for Experimental Biology*. **27**: 761.

De Haas D.W. (1999). Investigation into a biosupplement for possible reduction of activated sludge production in a system with excess biological phosphorus removal. *Water SA* **25**: 75-83.

Ehlers M.M. (1997). Bacterial community structures of activated sludge determined with SDS-PAGE. PhD. Thesis. University of Pretoria, Pretoria, South Africa.

Erasmus A.S. (1997). Immunochemical investigation of enhanced phosphate removal by activated sludge. M.Sc. (Agric) thesis. University of Pretoria, Pretoria, South Africa.

Fair G.M. and Geyer J.C. (1954). *Water Supply and Waste Water Disposal*. Wiley, New York, USA. P. 721.

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

Gleisberg D. (1992). Phosphate. In: N.T. de Oude (ed.), *Detergents*. Springer-Verlag, Berlin. Pp. 179-203.

Grady C.P.L. (Jr) and Lim H.C. (1980). Activated sludge. In: P.N. Cheremisinoff (ed.). *Biological wastewater treatment theory and applications*. Marcel Dekker (Inc.), New York. Pp. 619-681.

Jenkinson D.S. and Ladd J.N. (1981). Microbial biomass in soil: measurement and turnover. In: *Soil Biochemistry*. Paul E.A. and Ladd J.N. (eds.). Vol. 5. Dekker, New York, U.S.A. Pp. 415-471.

Jørgensen P.E., Eriksen T. and Jensen B.K. (1992). Estimation of viable biomass in wastewater and activated sludge by determination of ATP, oxygen utilisation rate and FDA hydrolysis. *Water Research* **26**: 1495-1501.

Kucknerowicz F. and Verstraete W. (1979). Direct measurement of microbial ATP in activated sludge samples. *Journal of Chemical Technology and Biotechnology* **29**: 707-712.

Lilley I.D., Pybus P.J. and Power S.P.B. (1997). *Operating manual for biological nutrient removal wastewater treatment works*. Water Research Commission report no. TT 83/97. Beria Printers, South Africa.

Metcalf & Eddy, Inc. (1991) *Wastewater engineering – Treatment, disposal and reuse. 3rd edition*. p. 379. (Revised by Tchobanoglous G. and Burton F.L). McGraw-Hill, Inc., Singapore.

Momba M.N.B. (1995). Phosphate removal in activated sludge and its relationship to biomass. MSc thesis. University of Pretoria, Pretoria, South Africa.

Momba M.N.B. and Cloete T.E. (1996). The relationship between *Acinetobacter junii*, biomass and phosphate uptake in activated sludge mixed liquor. *Water Research* **30**: 364-370.

Münch E.v-. and Pollard P.C. (1997). Measuring bacterial biomass-COD in wastewater containing particulate matter. *Water Research* **31**(10): 2550-2556.

Nelson P.O. and Lawrence A. (1980). Microbial viability measurements and activated sludge kinetics. *Water Research* **14**: 217-225.

Oellermann R.A. and Pearce K. (1995). Bioaugmentation technology for wastewater treatment in South Africa. Water Research Commission Report No. 429/1/95. Pretoria, South Africa.

Patterson J.W. and Brezonik P.L. (1969). *Journal of the Sanitary Engineering Division.* Printed by the American Society of Civil Engineering. **95**: 775.

Patterson J.W., Brezonik P.L. and Putnam H.U. (1970). Measurement and significance of adenosine triphosphate in activated sludge. *Environmental Science and Technology* **4**: 569-575.

Roe P. and Bhagat S.K. (1982). Adenosine triphosphate as a control parameter to activated sludge processes. *Journal Water Pollution Control Federation* **54**: 244-254.

Schön G., Geywitz S. and Mertens F. (1993). Influence of dissolved oxygen and oxidation-reduction potential on phosphate release and uptake by activated sludge from sewage plants with enhanced biological phosphorus removal. *Water Research* **27**(3): 349-354.

Sidat M., Kasan H.C. and Bux F. (1999). Laboratory-scale investigation of biological phosphate removal from municipal wastewater. *Water SA* **25**(4): 459-462.

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

Standard Methods for the Examination of Water and Wastewater (1995). Published by American Public Health Association, American Water Works Association and Water Environment Federation. Eaton A.D., Clesceri L.S. and Greenberg, A.E. (eds.) 19th edition. United Book Press, Inc., Baltimore, Maryland, United States. Pp 2-53 – 2-58.

Streichan M., Golecki J.R. and Schon G. (1990). Polyphosphate accumulating bacteria from sewage plants with different processes for biological removal. *FEMS Microbiology Ecology* **73**: 113-124.

Toerien D.F., Hyman K.L. and Bruwer M.J. (1975). A preliminary trophic status classification of some South African impoundments. *Water SA* **1**(1): 15-23.

Toerien D.F., Gerber A., Lötter L.H. and Cloete T.E. (1990). Enhanced biological phosphorus removal in activated sludge systems. *Advances in Microbial Ecology* **11**: 173-230.

Ubisi M.F., Jood T.W., Wentzel M.C. and Ekama G.A. (1997). Activated sludge mixed liquor heterotrophic active biomass. *Water SA* **23**(3): 239-248.

Upadhyaya A.K. and Eckenfelder W.W. (Jr.). 1975. Biodegradable fraction as an activity parameter of activated sludge. *Water Research* **9**: 691.

Vaker D., Connel C.H. and Wells W.N. (1967). Phosphate removal through municipality wastewater treatment at San Antonio, Texas. *Journal Water Pollution Control Federation* **39**: 750-771.

Van de Werf H. and Verstraete W. (1984). ATP measurement by bioluminescence: environmental applications. In: *International Symposium on Analytical Applications of Bioluminescence and Chemiluminescence*. Kricka L.J. *et al.* (eds.). Academic Press, London.

Weddle C.L. and Jenkins D. (1970). The viability and activity of activated sludge. *Water Research* **5**: 621.

Chapter 5

CONCLUSIONS

- ATP proved to be a more reliable method for indicating the biomass concentration than TPC, due to the higher yield and a smaller standard deviation.

- Orthophosphate removal was consistently higher in the sludges with higher initial ATP and TPC values, indicating a relationship between viable biomass and orthophosphate removal.

- The MLVSS showed the same trend in orthophosphate removal as the MLSS, although always somewhat lower, due to it being the volatile fraction of the MLSS.

- Neither initial MLSS, initial MLVSS nor changes in the concentrations of these fractions could be directly linked to different orthophosphate uptake abilities of different sludges, indicating the unsuitability of MLSS and MLVSS to indicate viable biomass and/or differences in the viable biomass fraction in activated sludge.

- SEM-EDS indicated that cell clusters with associated EPS of different activated sludge plants, on average, contained between 57 and 59 % phosphorus, while EPS alone contained on average between 23 and 30 % phosphorus. Results suggest that phosphorus removal in activated sludge might be due, not only to PAO, but also by EPS acting as a phosphorus reservoir.

- Results of EDS for EPS extracts from two WTPs employing chemical treatment, indicated possible localized iron and aluminium precipitates of phosphorus which were seemingly removed by filtration (part of the extraction procedure). Although intracellular leakage

during extraction was indicated, EPS could play an important role in removal of phosphorus, even in these plants.

Appendix 1 (pertaining chapter 4):

Table 4.5: Average experimental values for microbiological and physico-chemical analyses performed in the 8 h orthophosphate uptake experiment (experiment 1). Standard deviations are indicated in brackets.

Analysis	Control	Daspoort	Centurion	Zeekoegat	Rooiwal	Baviaanspoort
ATP (cells.ml^{-1})						
(T_0)	$2.15x10^3$ ($1.77x10^3$)	$2.53x10^7$ ($3.06x10^6$)	$6.91x10^7$ ($1.57x10^7$)	$4.30x10^7$ ($1.22x10^7$)	$5.02x10^7$ ($1.41x10^7$)	$8.83x10^6$ ($4.86x10^6$)
(T_8)	$4.31x10^5$ ($9.69x10^4$)	$1.70x10^8$ ($3.40x10^7$)	$1.33x10^8$ ($3.58x10^7$)	$8.88x10^7$ ($2.71x10^7$)	$8.27x10^7$ ($2.05x10^7$)	$8.38x10^7$ ($2.55x10^7$)
MLSS (mg.l^{-1})						
(T_0)	400 (140)	4130 (320)	3970 (510)	4400 (660)	4430 (960)	3300 (600)
(T_8)	350 (70)	6570 (930)	7430 (680)	8470 (150)	7970 (550)	6200 (600)
TPC (cfu.ml^{-1})						
(T_0)	<10 (0)	$1.52x10^6$ ($1.88x10^6$)	$2.83x10^6$ ($7.42x10^5$)	$1.93x10^6$ ($6.53x10^5$)	$1.34x10^6$ ($2.24x10^5$)	$2.88x10^5$ ($2.53x10^5$)
(T_8)	$4.00x10^5$ ($1.13x10^4$)	$4.45x10^6$ ($2.56x10^6$)	$4.35x10^6$ ($1.54x10^6$)	$4.69x10^6$ ($1.49x10^6$)	$4.11x10^6$ ($1.52x10^6$)	$8.98x10^5$ ($6.53x10^5$)
pH (T_0)	6.89 (0.00)	6.83 (0.58)	6.80 (0.02)	6.77 (0.05)	6.80 (0.01)	6.81 (0.03)
(T_8)	7.40 (0.01)	7.04 (0.00)	7.12 (0.03)	7.06 (0.01)	6.76 (0.03)	7.07 (0.03)
PO_4^{-3} (mg.l^{-1})						
(T_0)	219.50 (0.00)	220.33 (10.90)	224.30 (6.69)	220.93 (6.40)	242.47 (4.90)	250.13 (1.16)
(T_1)	180.70 (29.42)	180.80 (9.46)	194.68 (5.90)	193.73 (7.72)	207.73 (8.92)	203.37 (6.95)
(T_2)	220.75 (1.48)	184.87 (18.18)	179.07 (15.88)	193.40 (6.16)	202.80 (5.59)	204.90 (1.96)
(T_3)	225.50 (17.39)	171.43 (10.50)	173.27 (12.53)	188.70 (8.03)	208.93 (4.47)	208.27 (2.90)
(T_4)	232.95 (20.15)	164.63 (13.18)	167.30 (8.51)	177.03(11.01)	191.70 (3.48)	188.73 (5.88)
(T_5)	235.55 (4.31)	174.97 (16.38)	145.30 (27.70)	178.17 (17.12)	201.50 (15.31)	198.67 (9.73)
(T_6)	254.80 (5.94)	171.53 (7.98)	140.10 (5.92)	173.47 (14.69)	200.17 (6.82)	195.00 (9.28)
(T_7)	226.70 (15.84)	171.00 (10.53)	124.60 (3.50)	149.43 (10.42)	183.63 (9.31)	175.10 (16.22)
(T_8)	210.15 (9.40)	158.90 (18.26)	103.03 (9.30)	134.17 (10.86)	178.87 (13.36)	172.97 (16.63)

Table 2 (continued):

Analysis	Control	Daspoort	Centurion	Zeekoegat	Rooiwal	Baviaanspoort
NO_3^- ($mg.l^{-1}$)						
(T_0)	<5 (0)	10.07 (7.09)	6.72 (4.74)	13.30 (8.84)	7.57 (1.79)	9.15 (0.64)
(T_8)	13.55 (3.00)	32.16 (10.55)	6.09 (5.06)	7.01 (2.42)	12.50 (14.02)	7.02 (5.20)
SO_4^{2-} ($mg.l^{-1}$)						
(T_0)	165.50 (4.95)	165.33 (8.33)	169.33 (15.50)	192.00 (6.24)	167.00 (7.81)	166.00 (12.73)
(T_8)	167.00 (2.83)	180.67 (12.74)	167.33 (12.34)	168.67 (4.62)	177.67 (17.50)	187.00 (13.11)
NH_4^+ ($mg.l^{-1}$)						
(T_0)	16.59 (0.14)	17.13 (1.64)	17.59 (1.31)	18.81 (0.89)	17.86 (0.41)	19.79 (2.21)
(T_8)	0.13 (0.05)	0.02 (0.02)	0.02 (0.01)	0.01 (0.01)	0.02 (0.01)	0.05 (0.01)
Dissolved Oxygen ($mg.l^{-1}$)						
(T_0)	3.40 (0.42)	0.40 (0.10)	0.52 (0.03)	0.40 (0.10)	0.47 (0.15)	0.45 (0.05)
(T_1)	5.50 (0.14)	1.97 (1.59)	0.23 (0.11)	0.83 (0.59)	1.03 (0.50)	0.62 (0.42)
(T_3)	5.85 (0.35)	5.30 (0.26)	1.53 (1.22)	1.63 (1.30)	0.97 (0.98)	1.83 (1.95)
(T_5)	5.85 (0.21)	3.50 (1.51)	1.63 (0.49)	0.23 (0.06)	1.70 (1.76)	2.33 (1.33)
(T_7)	5.80 (0.28)	6.13 (0.06)	4.57 (0.61)	4.57 (0.15)	4.97 (0.97)	5.20 (0.44)
(T_8)	5.80 (0.42)	6.37 (0.06)	4.20 (1.13)	4.33 (0.23)	4.87 (1.40)	4.70 (0.61)

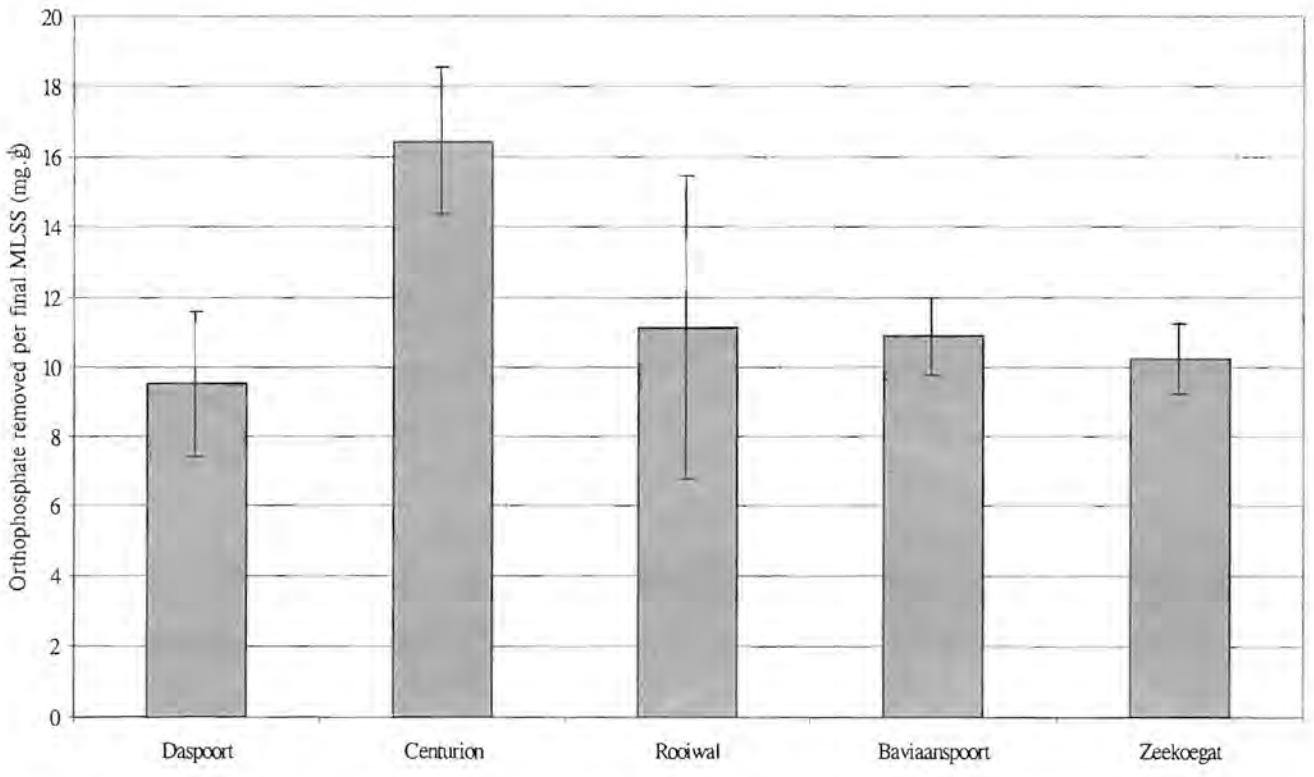

Figure 4.21: Average orthophosphate uptake (mg) per gram final MLSS.

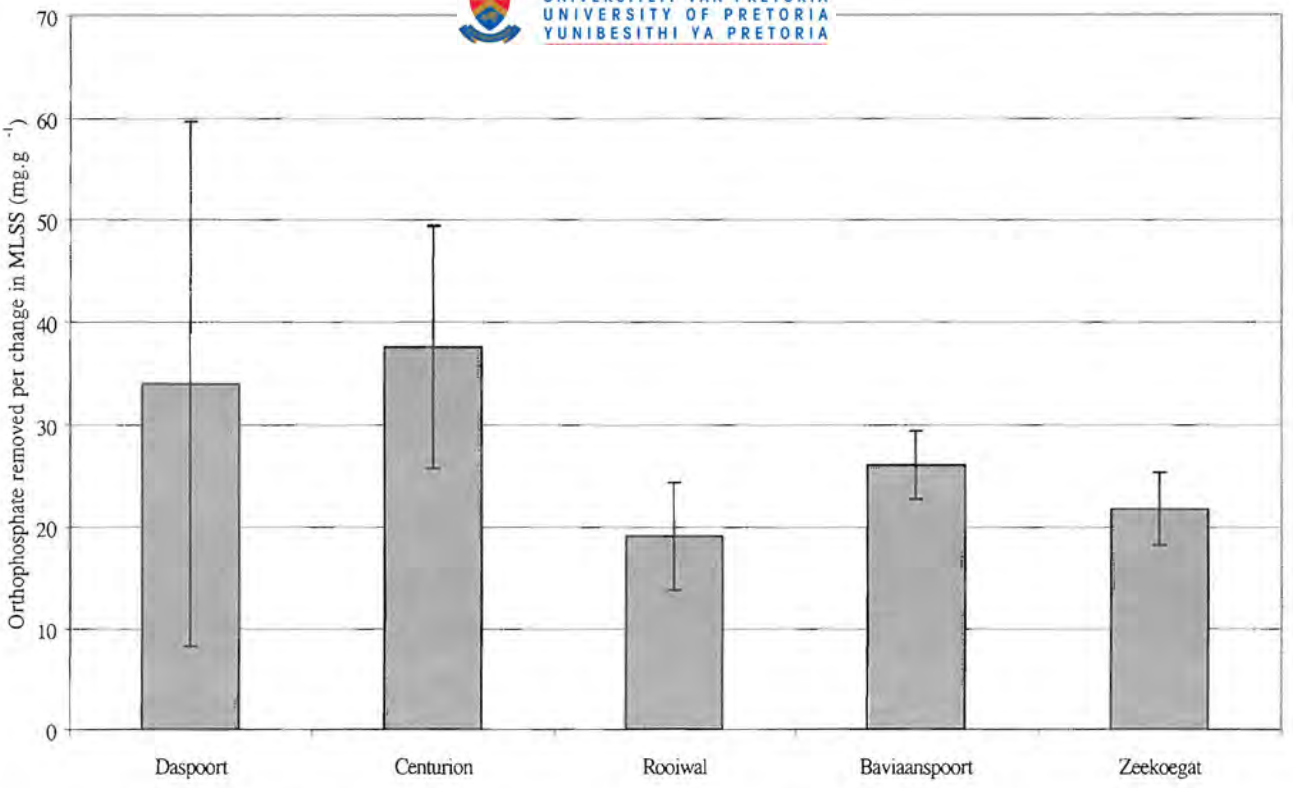

Figure 4.22: Average orthophosphate uptake (mg) per gram change in MLSS.

Table 4.6: Average experimental values for microbiological and physico-chemical analyses performed in the 7 h phosphate uptake experiment (experiment 2). Standard deviations are indicated in brackets.

Analysis	Control	Zeekoegat	Baviaanspoort	Daspoort	Centurion	Rooiwal
PO_4^{3-} (mg.l^{-1})						
T_0	28.00	28.50	29.00	27.00	33.00	31.00
T_1	30.50	25.50	4.00	17.00	15.00	24.00
T_2	31.00	24.00	1.00	15.50	11.00	22.00
T_3	29.50	22.00	1.00	16.00	5.00	20.50
T_4	30.50	21.50	1.00	16.00	3.50	20.00
T_5	30.00	20.50	1.00	16.00	1.00	19.00
T_6	30.50	19.50	0.00	16.50	0.50	18.50
T_7	30.00	19.00	0.00	16.00	0.00	18.00
pH						
T_0	6.89	6.95	7.05	6.98	7.09	7.02
T_7	7.72	6.69	7.34	5.85	6.91	6.20
NO_3^- (mg.l^{-1})						
T_0	5.50	5.50	4.50	5.50	10.50	9.00
T_7	11.00	3.50	4.00	24.50	11.50	15.50
NH_4^+ (mg.l^{-1})						
T_0	13.50	13.00	13.30	13.30	13.20	12.90
T_7	13.30	0.20	0.40	0.90	0.40	1.00
TPC (cfu.ml^{-1})						
T_0	<10 (0)	5.07×10^6 (1.95×10^6)	7.73×10^6 (2.62×10^6)	4.05×10^6 (1.64×10^6)	8.57×10^6 (5.87×10^6)	2.73×10^6 (5.19×10^5)
T_7	1.39×10^4 (4.32×10^3)	1.49×10^6 (4.37×10^5)	1.12×10^7 (3.39×10^6)	2.40×10^6 (1.01×10^6)	3.19×10^6 (1.40×10^6)	1.33×10^6 (3.90×10^5)
MLSS (mg.l^{-1})						
T_0	0	3375	3655	2365	3590	2900
T_7	0	3855	6045	2555	3605	2915
MLVSS (mg.l^{-1})						
T_0	0	2530	2550	1735	2450	2340
T_7	0	2880	4200	1845	2405	2320
Dissolved oxygen (mg.l^{-1})						
T_0	5.65	1.65	1.70	0.95	0.85	0.50
T_1	7.15	6.20	5.55	6.10	5.95	6.00
T_4	6.70	5.85	5.60	6.15	5.80	5.85
T_7	6.60	5.93	5.78	6.20	5.85	5.90

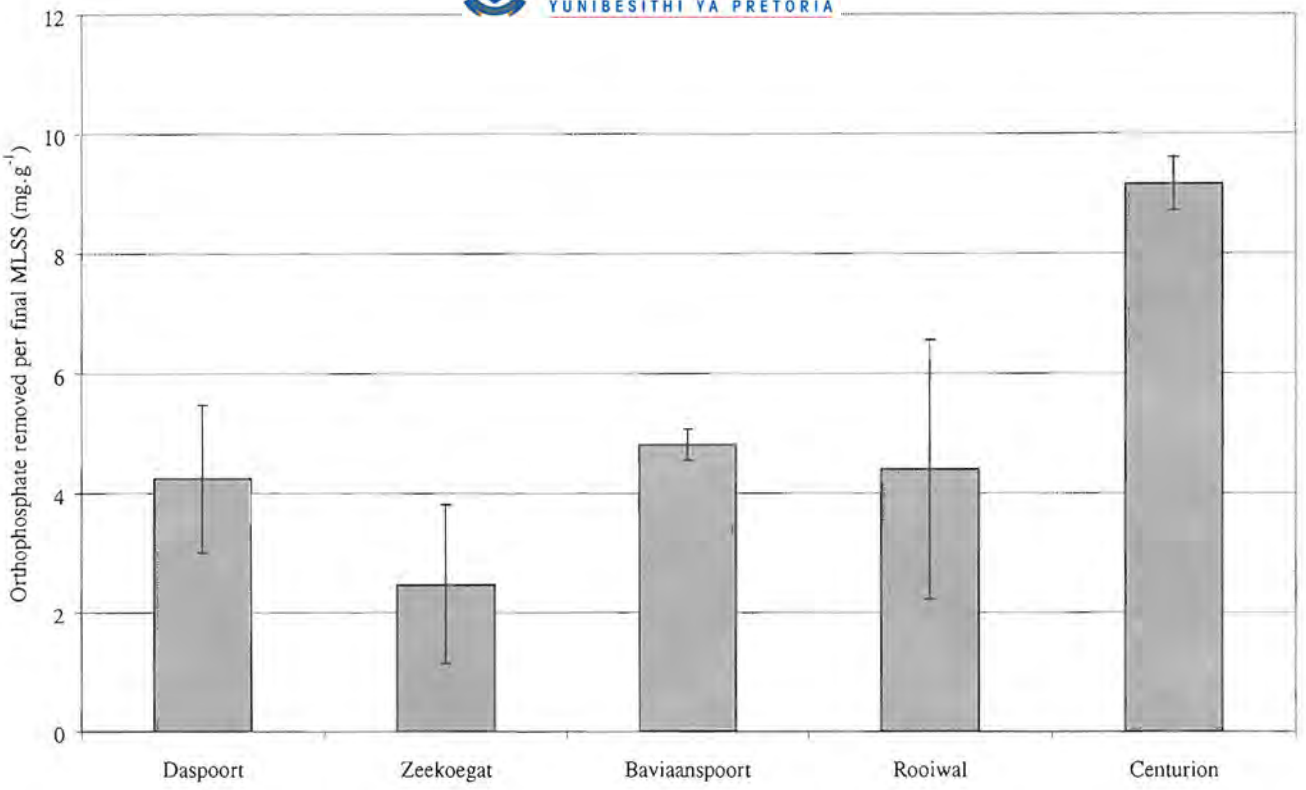

Figure 4.23: Orthophosphate removed (mg) removed per gram final MLSS.

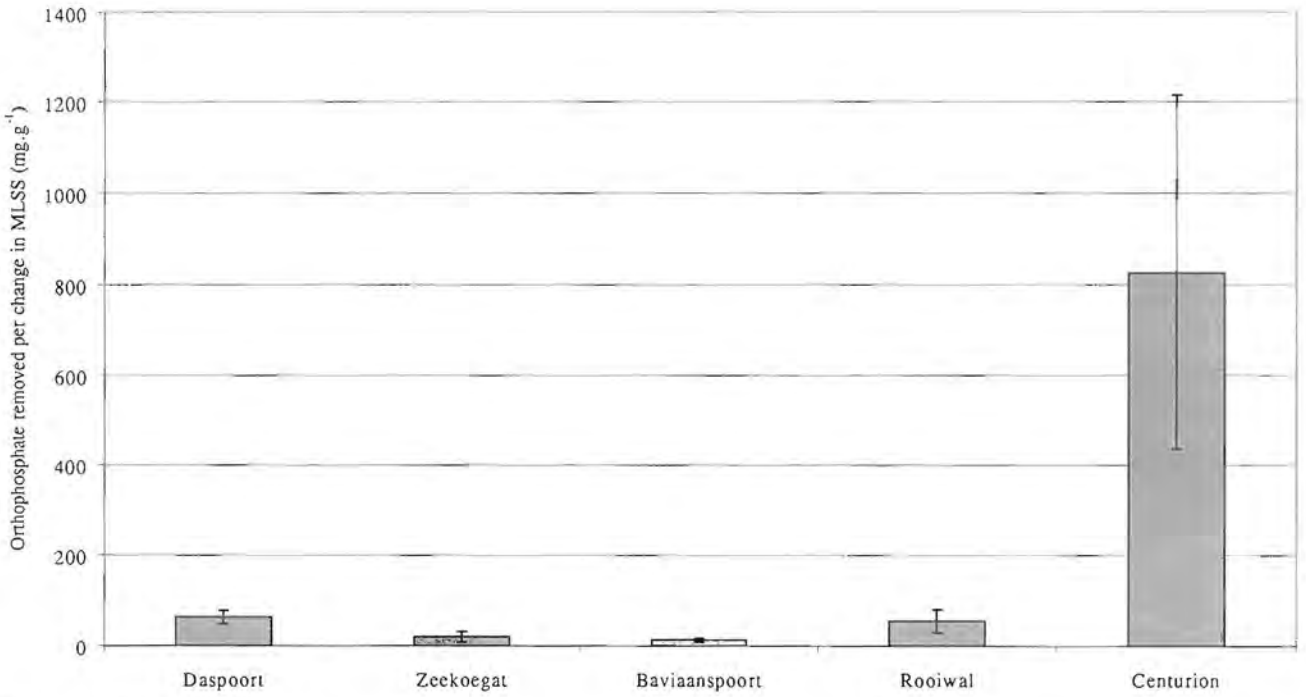

Figure 4.24: Orthophosphate removed (mg) per gram change in MLSS.

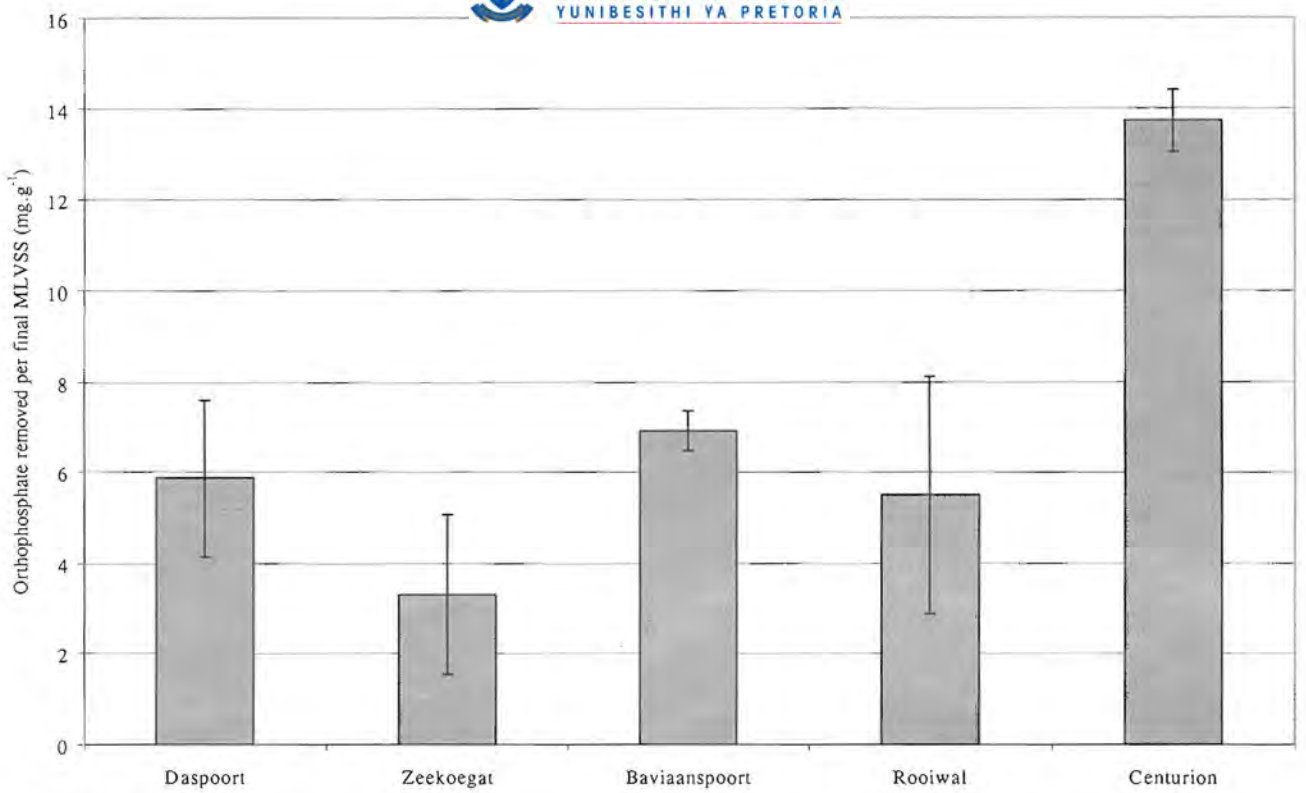

Figure 4.25: Orthophosphate removed (mg) per gram final MLVSS.

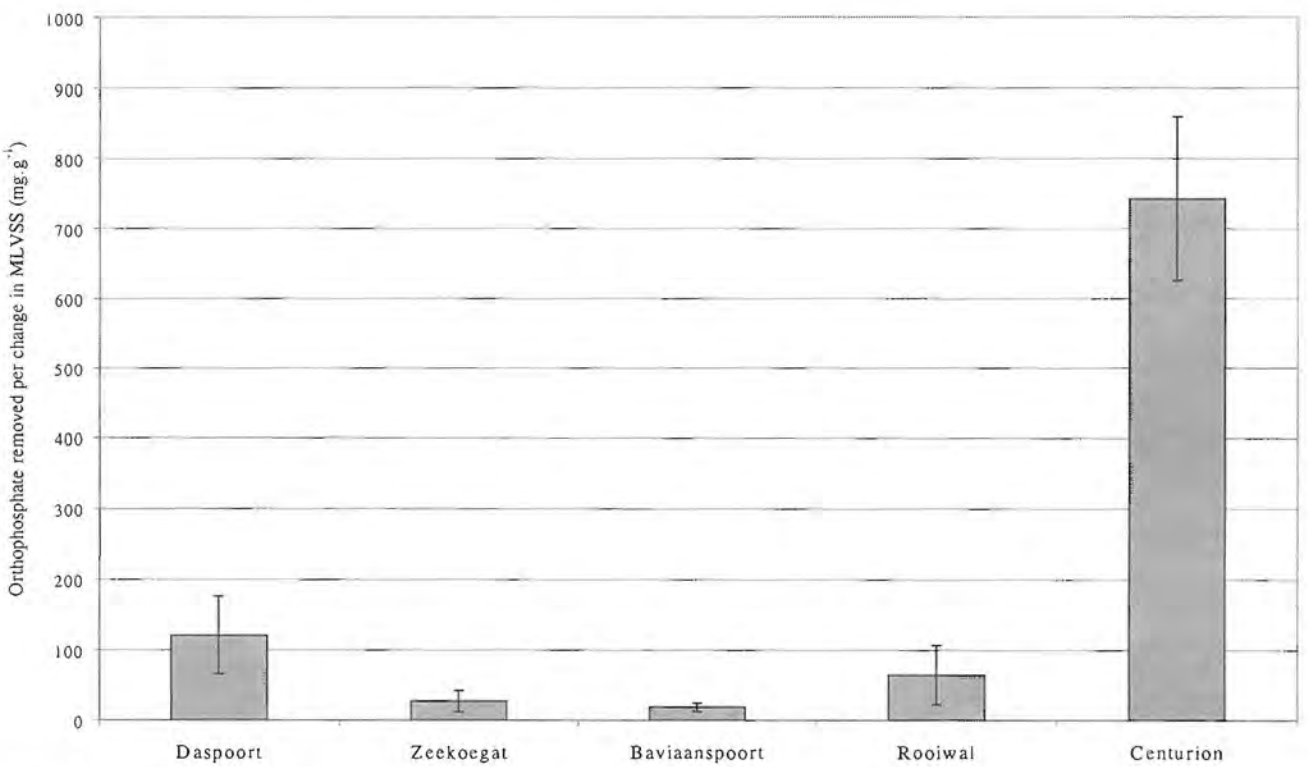

Figure 4.26: Orthophosphate removed (mg) per gram change in MLVSS.