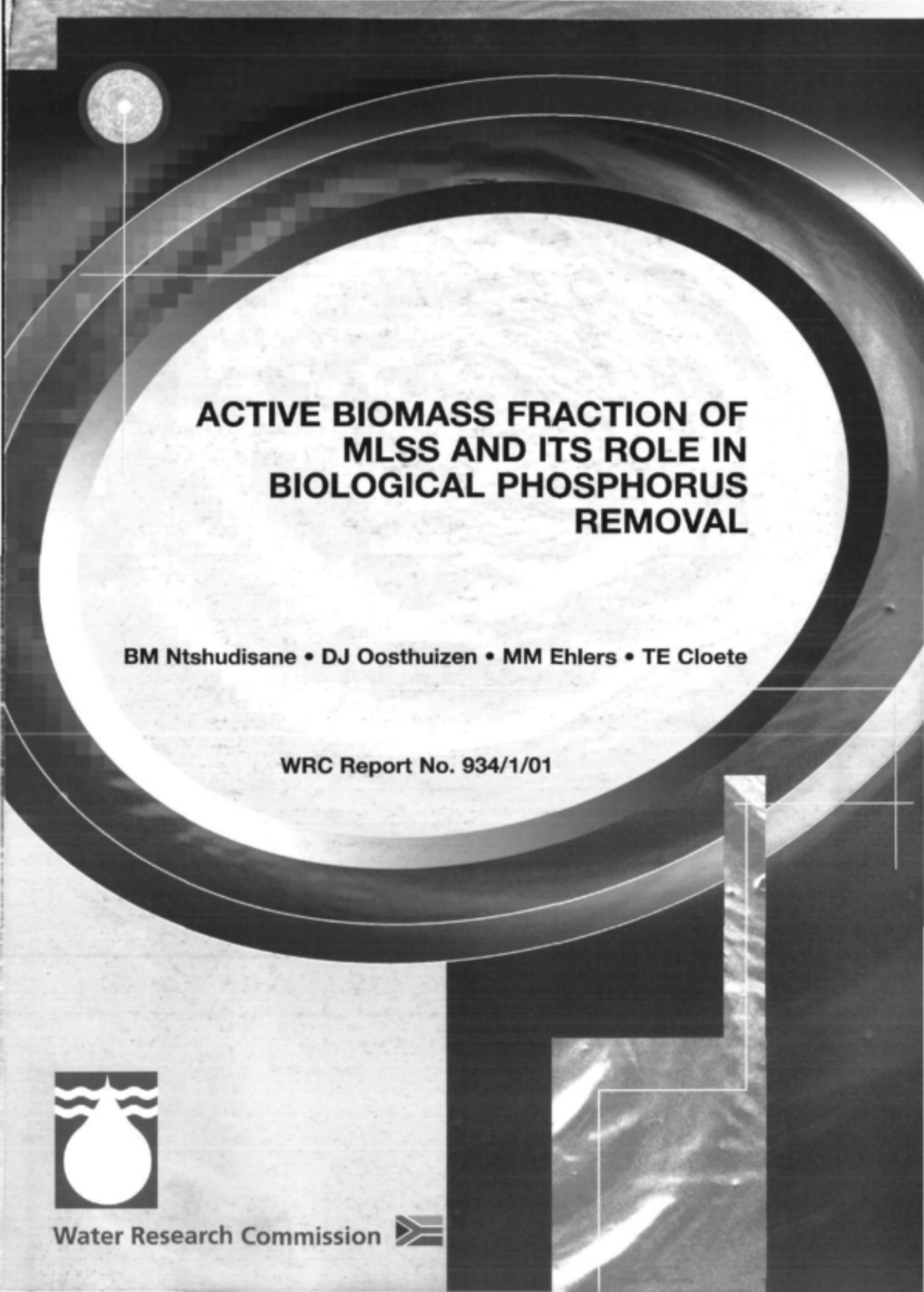

ACTIVE BIOMASS FRACTION OF MLSS AND ITS ROLE IN BIOLOGICAL PHOSPHORUS REMOVAL

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WRC Report No. 934/1/01

Water Research Commission

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Final Report

to the Water Research Commission

by

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EXECUTIVE SUMMARY

BACKGROUND:

Eutrophication of South Africa's natural waters is accelerated by human activities which result in the discharge of the nutrients nitrogen (N) and phosphorus (P) (Lilley *et al.*, 1997). Eutrophication is marked by large visible blooms of algae, which makes water treatment difficult. Nitrogen can be fixed by algae in the water and therefore, phosphorus is the element that should be considered to minimise eutrophication (Lilley *et al.*, 1997).

The prevention of eutrophication can be achieved by removing phosphate from wastewater by chemical or biological methods (Lilley *et al.*, 1997). Biological phosphate removal has gained support since chemical removal is very expensive and increases the salt and mineral concentrations of the effluent (Slim, 1987). The activated sludge process has been designed and operated for excess phosphate removal to reach effluent concentrations of 0.5 to 1.0 mg phosphate per litre (Barnard, 1975; Fuhs and Chen, 1975). However, many activated sludge systems fail, necessitating chemical addition. This has mainly been attributed to the lack of knowledge of the role of microorganisms and optimum conditions for their growth in order to facilitate biological phosphorus removal.

Research has indicated that there were no differences amongst the bacterial community structures of different activated sludge zones or amongst different activated sludge systems (Momba, 1995; Ehlers 1997). Hence, phosphorus removal cannot be attributed to the activity of a single population, but rather to the combined activity of all the populations in the microbial community. Momba and Cloete (1996) indicated that an increase in biomass resulted in an increase in phosphorus removal. This raised the question whether wastewater treatment systems could be bioaugmented in order to increase biomass.

Bioaugmentation involves the use of specially selected and adapted microorganisms for the biodegradation of wastewater (Oellermann and Pearce, 1995). The objective is not to replace the existing biomass, but to supplement it for improved efficiency. Its use could enhance the degradative potential of indigenous microbial populations to avoid predation, nutrient competition and biomass inactivation.

The application of bioaugmentation in wastewater treatment was originally the result of efforts to solve operational problems, such as shock loads in treatment plants (Oellerman and Pearce, 1995). According to De Haas (1999), results from the commercial application of bioaugmentation products, particularly in wastewater treatment systems facing operational problems, have tended to be positive. However, laboratory research investigations have contradicted these results. A number of bioaugmentation products do not perform as claimed by the suppliers (Oellermann and Pearce, 1995). Cases were reported with little or no advantage of bioaugmentation on the improvement of treatment works (Yu and Hung, 1992). It was concluded that no significant improvement in process performance can be achieved with bioaugmentation (De Haas, 1999). As an alternative, the biomass could be increased in a number of different ways.

An alternative to the application of commercial biosupplements would be to use total biomass from activated sludge systems, which optimally remove P. The use of such biomass would be inexpensive and the acclimation period shorter than that for commercially available biosupplements. Biomass could be augmented subsequent to system failure as a result of either toxic shock or biomass depletion.

OBJECTIVES:

The objectives of this study were:

1) To determine the effect of bioaugmentation on phosphorus removal in laboratory experiments by adding commercially available bioaugmentation products.

2) To determine the relationship between biomass and phosphate removal in different activated sludge systems.

3) To evaluate the following:
    i) Anaerobic sludge biomass as supplement
    ii) Return sludge biomass as supplement
    iii) Aerobic sludge biomass as supplement
    iv) Bioaugmentation biomass as supplement

4) To determine the P removal capacity of a system based on biomass.

5) To determine the effect of bioaugmentation on phosphorus removal in a conventional activated sludge system by adding biosupplements and/or anaerobic sludge etc. in order to increase the biomass.

6) To compare different methods for the determination of biomass in activated sludge systems.

7) To investigate the locality and quantity of phosphorus associated with extracellular polysaccharides (EPS).

SUMMARY OF RESULTS:

For determining the effect of bioaugmentation on phosphorus removal in laboratory experiments different concentrations of bioaugmentation product A (SA Biotech) (2 g.l^{-1}, 6 g .l^{-1}, 8 g.l $^{-1}$, 10 g.l^{-1}, 20 g.l^{-1}, 40 g.l^{-1}, 80 g.l^{-1}, 100 g.l^{-1}, 160 g.l^{-1}, 200 g.l^{-1}) were used as inocula in sterile anaerobic mixed liquor medium. There was an initial relationship between phosphate uptake and biomass, which thereafter was negatively affected by the phosphate concentration of the bioaugmentation product. At concentrations exceeding 80 g.l^{-1} of mixed liquor, no phosphorus was removed, instead the phosphate concentration increased as a result of the phosphorus content of the bioaugmentation product. The product obtained from SA Biotech had a high phosphate content, making it unsuitable for bioaugmentation.

For determining the possibility of culturing a bioaugmentation product in a separate fermentation unit for addition to activated sludge, the growth of bioaugmentation product B (Amitek) and anaerobic activated sludge in sterile mixed liquor was compared. The hypothesis was to use an inexpensive substrate to grow these products in separate tanks to have a reserve of biomass which can then serve as biomass inoculum to increase the already existing biomass in the system. Therefore, a commercially available bioaugmentation product (Product B obtained from Amitek) as well as anaerobic sludge were evaluated for this purpose.

Different volumes (2.5 ml and 10 ml) of the bioaugmentation product and anaerobic sludge were inoculated in sterile mixed liquor medium (250 ml and 90 ml), Nutrient broth (90 ml) and sterile mixed liquor added with nutrients (sodium acetate, magnesium sulphate and potassium nitrate) respectively. The experiments were conducted under aerobic and anaerobic conditions at room temperature (±21°C). No growth of the microorganisms occurred when sterile mixed liquor medium was used, even when nutrients were added. The microorganisms were able to grow in the Nutrient Broth.

For determining the P removal capacity of a system based on biomass, aerobic sludge was used as biomass in sterile mixed anaerobic mixed liquor.

The increase in P removal with an increase in biomass indicated a direct relationship between biomass and P removal from mixed liquor. Our results are in agreement with that of Lemos *et al.* (1997) and others (Momba and Cloete, 1996; Muyima, 1995) who stated that a phosphorus free effluent could be obtained when there are enough phosphorus accumulating bacteria in a wastewater process. This suggested that the failure of EBPR under certain conditions could be due to insufficient biomass.

An investigation was done into the relationship between the MLSS and MLVSS fractions of activated sludge, as measures of total biomass, and phosphorus removal. In this study,

by means of two independent experiments, differences in orthophosphate uptake ability of different activated sludges treated in exactly the same way were observed. 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. Orthophosphate uptake differed amongst systems in terms of wet sludge mass. In experiment 1 (constant wet mass of 40 g), the Centurion Wastewater Treatment Plant (WTP) showed, on average, the highest orthophosphate removal capacity (30.79 $mg.g^{-1}$ 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 $mg.g^{-1}$ initial MLSS, respectively. For experiment 2 (simulated MLSS values), for average orthophosphate uptake in terms of initial MLSS, the Centurion WTP performed best (9.19 $mg.g^{-1}$), followed by the Baviaanspoort, Daspoort, Rooiwal and Zeekoegat WTPs with uptakes of 7.97, 4.60, 4.55 and 2.82 $mg.g^{-1}$, respectively. For average orthophosphate uptake calculated in terms of initial MLVSS, the same pattern was observed as that for initial MLSS and followed the same order of systems 13.48, 11.43, 6.28, 5.63 and 3.76 $mg.g^{-1}$, respectively for Centurion, Baviaanspoort, Daspoort, Rooiwal and Zeekoegat. The different 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*, are not good indicators of biomass in activated sludge. ATP proved to be a more reliable method for indicating biomass concentration than TPC. Although MLSS and MLVSS showed the same trend in orthophosphate removal, initial concentrations of these fractions did not correlate with ATP or TPC, indicating the unsuitability of these fractions as indicators of biomass in activated sludge. In addition, orthophosphate removal was consistently higher in the sludges with higher ATP and TPC values, indicating a relationship between biomass and orthophosphate removal.

Analysis of extracellular polysaccharides (EPS) of activated sludge was 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 27 and 30%

phosphorus. Results suggest that phosphorus removal in activated sludge might be due not only to polyphosphate accumulating organisms (PAO), but also by EPS acting as a phosphorus reservoir.

CONCLUSIONS

Bioaugmentation product A caused an increase in the phosphate concentration of the mixed liquor in all our experiments and could therefore not be considered for increasing the biomass in activated sludge. Bioaugmentation product B also contained a high P concentration making it unsuitable for P-removal studies. Bioaugmentation product B and anaerobic sludge did not grow in the substrate used, except in Nutrient Broth.

When aerobic sludge was used as augmentation, an increase in biomass led to an increase in P removal. When calculating the quantity of P removal per g of wet sludge no significant difference was observed indicating that there was a direct relationship between P removal and MLSS for a specific system.

ATP proved to be a more reliable method for indicating the biomass concentration in activated sludge than TPC, due to the higher yield and a smaller standard deviation. 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, nor MLVSS concentrations correlated with ATP and/or TPC, indicating that MLSS and MLVSS were not good indicators of biomass in activated sludge. Orthophosphate removal was consistently higher in the sludges with higher ATP and TPC values, indicating a relationship between biomass and orthophosphate removal.

Results of EDS suggest that phosphorus removal in activated sludge might be due not only to PAO, but also by EPS acting as a phosphorus reservoir.

RECOMMENDATIONS FOR FURTHER RESEARCH:

Further biomass studies should include the physical, chemical and biological characterization of MLSS for different activated sludge systems.

LIST OF PRODUCTS:

Degrees:

MSc Microbiology – B.M Ntshudisane
MSc Microbiology - D.J. Oosthuizen

Presentations:

Molepo V., Ehlers M.M. and Cloete T.E., 1999. The relationship of activated sludge mass to phosphate removal in sterile mixed liquor - Oral presentation. BioY2K Combined Millenium Meeting, 23-28 January, 2000. Grahamstown, South Africa.

Oosthuizen D.J., Ehlers, M.M. and Cloete, T.E., 2000. Phosphorus removal using equal amounts of biomass from five different activated sludge systems - Poster presentation. BioY2K Combined Millenium Meeting, 23-28 January, 2000. Grahamstown, South Africa.

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Dr G. Offringa:	Water Research Commission (Chairman)
Mr G.N. Steenveld:	Water Research Commission
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LIST OF ABBREVIATIONS

$<$:	Smaller than
$>$:	Larger than
%:	Percent
µg:	Microgram
ADP:	Adenosine diphosphate
AO:	Acridine Orange
ATP:	Adenosine triphosphate
BOD:	Biological Oxygen Demand
BEPR:	Biological excess phosphorus removal
°C:	Degrees Celcius
^{14}C:	Radio-labelled carbon-14 isotope
$^{14}CO_2$:	Radio-labelled carbon dioxide
ca:	*circa*
$cells.ml^{-1}$:	Cells per millilitre
cfu:	Colony forming units
CO_2:	Carbon dioxide
COD:	Chemical Oxygen Demand
DAPI:	4'6-diamidino-2-phenylindole
DNA:	Deoxyribonucleic acid
DO:	Dissolved oxygen
EBPR:	Enhanced biological phosphate removal
e.g.:	For example
Eh:	Redox potential
FDA:	Fluorescein diacetate
FISH:	Fluorescent *in situ* hybridization
FITC:	Fluorescein isothiocyanate
g:	Gram
$g.g^{-1}$:	Gram per gram

H_2S:	Hydrogen sulfide
H_2SO_4	Sulphuric acid
h:	Hour
kg:	Kilogram
kcal:	kilocalories
KH_2PO_4:	Potassium dihydrogen phosphate
KNO_3:	Potassium nitrate
l:	Litre
M:	Molar
MCRT:	Mean cell residence time
$MgSO_4.7H_2O$:	Magnesium sulphate heptahydrate
mg:	Milligram
$mg.g^{-1}$:	Milligram per gram
$mg.l^{-1}$:	Milligram per litre
min:	Minutes
ml:	Millilitre
MLSS:	Mixed liquor suspended solids
MLVSS:	Mixed Liquor Volatile Suspended Solids
MPN:	Most Probable Number
N:	Nitrogen
NA:	Nutrient Agar
$NaC_2H_3O_2$:	Sodium acetate
ND:	Not Determined
NH_4^+:	Ammonium
nm:	Nanometer
NO_3^-:	Nitrate
O_2:	Oxygen
OUR:	Oxygen utilization rate
P:	Phosphorus

ΔP:	Change in phosphate concentration
PAO:	Polyphosphate accumulating organisms
PCR:	Polymerase chain reaction
pH:	Hydrogen ion concentration
PO_4^{3-}:	Phosphate
Poly-P:	Polyphosphate
rpm:	Revolutions per minute
RNA:	Ribonucleic acid
rRNA:	Ribosomal ribonucleic acid
SEM:	Scanning electron microscopy
SO_4^{2-}:	Sulphate
SRB:	Sulphate reducing bacteria
SS:	Suspended solids
Tra+:	Transfer positive
TPC:	Total plate counts
T_x	Time x
UCT:	University of Cape Town
VSS:	Volatile suspended solids
WTP:	Wastewater treatment plant

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CHAPTER 1

INTRODUCTION

The increase in the amount of effluent being disposed to natural water bodies is due to rapid industrialisation. Major contaminants found in wastewater include biodegradable, volatile and recalcitrant organic compounds, toxic metals, suspended solids, nutrients (nitrogen and phosphorus), microbial pathogens and parasites (Bitton, 1994). The discharge of nitrogen (as nitrates) and phosphorus (as phosphates) to natural waters causes eutrophication (Lilley *et al.*, 1997). This is marked by large visible blooms of algae, which makes water treatment difficult. Since nitrogen (N) can be fixed by algae, phosphorus (P) is the element that should be considered to minimise eutrophication (Lilley *et al.*, 1997). Phosphorus removal from wastewater is therefore seen as one of the best methods for eutrophication control (Toerien *et al.*, 1990). According to the Water Act (Act 54 of 1956) the concentration of orthophosphate in purified wastewater is limited to 1 $mg.l^{-1}$.

Phosphate can be removed from wastewater by chemical or biological methods. Chemical method are expensive since it involves the addition of lime, aluminium and ferric chloride (Momba, 1995). Unfortunately these chemicals become part of the sludge and through additional binding with water, contribute to significant volume increases of the sludge (Grubbs, 1979). Hence, biological phosphate removal has gained support. The activated sludge process was therefore designed and operated for excess biological phosphate removal (Barnard, 1976). However, many activated sludge systems fail necessitating chemical addition. One of the most important reasons for systems failure, is the lack of knowledge of the role of microorganisms in these systems.

Research has indicated that there were no differences amongst the bacterial community structures of different activated sludge zones or amongst different activated sludge systems (Momba, 1995; Ehlers, 1997). Hence, phosphorus removal cannot be attributed to the activity of a single population, but rather to the combined activity of all the populations in the microbial community.

Momba and Cloete (1996) indicated that an increase in biomass resulted in an increase in phosphorus removal. This necessitates the development and/or evaluation of appropriate methods to determine biomass concentration in activated sludge.

Different methods exist to determine biomass (Holm-Hansen and Booth, 1966; Kucnerowicz and Verstraete, 1979; Herbert, 1990; Jϕrgensen *et al.*, 1992; Liebeskind and Dohmann, 1994):

i) direct counting procedures (light microscopy, epifluorescence microscopy, fluorescent antibody techniques, electron microscopy and confocal scanning light microscopy),
ii) viable count procedures (plate count methods, MPN),
iii) biochemical assays for the estimation of bacterial numbers (ATP, cell wall components, chlorophyll measurements, DNA concentration, photosynthesis, respiration, specific enzyme assays), MLSS.

Bioaugmentation presents an opportunity to manipulate the biomass in activated sludge. Bioaugmentation is the practice of enhancing the performance of indigenous bacterial populations of wastewater treatment systems through the addition of bacterial cultures with specific degradative abilities (www.sybronchemicals.com/biochem/waste). It does not replace an existing bacterial population, but augments its ability to respond to certain situations or its ability to degrade components of the waste stream, resulting in improved treatment.

The availability and use of bioaugmentation products in wastewater treatment systems has increased significantly in recent years. The effectiveness of adding either live cultures of microorganisms produced commercial to systems which naturally develop mixed and complex populations of many different microorganisms may be questioned. Biosupplements have been marketed and used in wastewater treatment processes for a number of years with varying degrees of success (De Haas, 1999). According to Koe and Ang (1992), results from the commercial application of such biosupplements, particularly in wastewater treatment systems facing operational problems, have tended to be positive. However, laboratory research investigations have contradicted these results and many researchers have concluded that no significant improvement in process performance can be achieved with biosupplementation (De Haas, 1999).

The objectives for this study were therefore:

1) To determine the effect of bioaugmentation on phosphorus removal in laboratory experiments by adding commercially available bioaugmentation products.
2) To determine the relationship between biomass and phosphate removal in different activated sludge systems.
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5) To determine the effect of bioaugmentation on phosphorus removal in a conventional activated sludge system by adding biosupplements and/or anaerobic sludge etc. in order to increase the biomass.
6) To compare different methods for the determination of biomass in activated sludge systems.

CHAPTER 2

LITERATURE REVIEW

2.1 Basic principles of the activated sludge process

The activated sludge process refers to the production of an activated mass of microorganisms capable of stabilizing a waste aerobically (Tchobanoglous and Burton, 1991). The process relies on dense microbial populations being mixed in suspension with wastewater under aerobic conditions. In the presence of adequate nutrients and oxygen a high rate of microbial growth and respiration is achieved (Muyima *et al.*, 1997).

The basic principle of the process is that wastewater is brought into contact with a mixed bacterial microbial population in the form of flocculent suspension in an aerated and agitated system (Gray, 1989). Suspended and colloidal material is removed from the wastewater by adsorption and agglomeration on to the microbial flocs (Wrinkler, 1981). The materials and the dissolved nutrients are then broken down slowly by microbial metabolism. Part of the nutrient material is oxidized to simple substances such as carbon dioxide and part is converted into new microbial cell material (Gray, 1989). When the desired degree of treatment is achieved, the sludge is separated from the treated wastewater by settling. The supernatant from the separation stage should be free of sludge and it is thus the treated wastewater (Gray, 1989). Part of the settled sludge is drawn off as waste and the rest is recycled to the aeration basin in order to maintain a high concentration of bacteria and serve as microbial inoculum (Eckenfelder *et al.*, 1985).

The activated sludge process has undergone various modifications to meet wastewater treatment needs (Toerien *et al.*, 1990). These changes relate to the flow regime in the reactor, the size, number and configuration of the reactors, recycled flow, influent flow and others incorporated either emphatically or present inadvertently or unavoidably (Muyima *et al.*, 1997).

The Phoredox activated sludge process is a five staged process designed to remove nitrogen and phosphorus by biological means. The system consists of a sequence of primary anaerobic, primary anoxic, primary aerated, secondary anoxic and an aerated basin followed by a clarifier (Toerien *et al.*, 1990). In the Phoredox activated sludge process, the incorporation of an anaerobic zone allows the release of phosphate and after it has been released from the biomass in the anaerobic zone, is reincorporated in the biomass during aerobiosis together with part or all of the influent phosphate (Gerber *et al.*, 1986). The anaerobic zone is required to effect the phosphorus removal (Toerien *et al.*, 1990). It has been indicated that the release of phosphate is a basic requirement for successful phosphate uptake (Cloete, 1984). The anaerobic stage receives the influent sewage feed and the sludge recycle from the clarifier underflow (Toerien *et al.*, 1990).

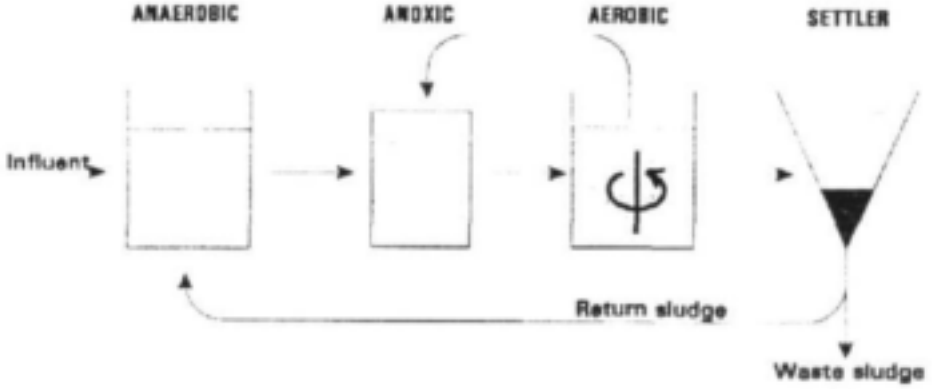

Figure 2.1: The three-stage Phoredox system (modified Bardenpho)(Erasmus, 1997).

The outflow from the anaerobic stage is passed into the primary anoxic stage, which receives the mixed liquor recycle (Toerien *et al.*, 1990). The mixed liquor recycle is drawn either from a point within the primary aeration stage or from the outflow from this stage. The remainder of the outflow enters a secondary anoxic stage, which is followed by a secondary aeration stage and finally a clarifier, from which the supernatant liquor is discharged and the settled solids are returned to the head of the process (Toerien *et al.*, 1990).

The application of this process in South Africa has led to the removal of phosphate in the final effluent of activated sludge plants to levels between 0.2 and 0.8 $mg.l^{-1}$ together with the removal of between 80 % - 90 % of the nitrogen (Barnard, 1976).

2.2 The role of biomass in phosphorus removal

It was indicated that biomass was related to phosphorus removal (Bosch, 1992; Momba, 1995; Muyima, 1995). 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.

Atlas and Bartha (1993) defined biomass as the dry weight, volume, or other quantitative estimation of organisms; the total mass of living organisms in an ecosystem. Thus the various types of organisms in activated sludge system constitutes 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, 1974). 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, 1993). Biomass can be expressed in units of weight (grams) that can be converted to units of energy (calories).

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 of measuring biomass is a crucial decision to make in the approach to a culture problem and often limitations of the methods make the decision difficult. The factors which influence the choice are: i) the properties of the biomass, ii) the properties of the culture medium, iii) the accuracy required, iv) the sensitivity required, and v) the 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 maximum density of biomass of any kind which can be reached in a given medium is determined by using one of the following four conditions: i) the amount of growth-limiting substrate supplied, ii) maximum packing density of the biomass, iii) the accumulation of an inhibitory product, iv) cell death (Pirt, 1975). 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, the determination of biomass 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 suspended solids or volatile suspended solids. 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. Attempts have been made to relate activity to biomass. Henze (1986) reported comparative studies of denitrification and oxygen utilization rates of raw wastewater and used conversion factors to relate activity to biomass. Kucnerowicz and Verstraete (1979) found a linear relationship between oxygen utilization rate 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.

Bond *et al.* (1995) argues that culturing techniques have provided a misleading picture of bacterial community structure in activated sludge and that in general the role of *Acinetobacte*r spp in activated sludge processes has been overrated. For instance non-culture-dependent methods such as quinone profiles and fluorescent in situ hybridization 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 microorganisms 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 microorganisms, 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 biological excess phosphorus (P) removal phenomenon, designs of activated sludge systems to accomplish biological excess P removal still are based on experience and semi-empirical methods. Clearly, the need exists for design procedures based on fundamental behavioral 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 facilities, regardless 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 (MLSS) which is made up of a number of variable components. One key component of MLSS is the heterotrophic active biomass. This component mediates the biodegradation 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. Ubisi *et al.* (1997) has however 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.

2.3 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 load. Biological phosphate removal plants in South Africa have not always given reliable and satisfactory performance (Osborn *et al.*, 1986). Enhanced biological phosphorus removal fail either as a result of toxic shock due to the accumulation 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 i.e. short chain fatty acids. Other organisms can compete with poly-P bacteria in anaerobic-aerobic activated sludge systems, leading to systems failure (Cech and Hartman, 1993; Mino *et al.*, 1994).

Failure of the 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 (Osborn *et al.*, 1986). Bioaugmentation or bioremediation has also been recommended as an approach to redress system failure (Bitton, 1994; Oellermann and Pearce, 1995).

The availability and use of biosupplements (bioaugmentation products) in wastewater treatment system has increased significantly in recent years (De Haas, 1999). The effectiveness of adding live cultures of microorganisms produced commercially to systems, which naturally develop mixed and complex populations of many different microorganisms, may be questioned.

Biosupplements have been marketed and used in wastewater treatment processes for a number of years with varying degrees of success (De Haas, 1999). According to Koe and Ang (1992), results from the commercial application of such biosupplements, particularly in wastewater treatment systems facing operational problems, have tended to be positive. However, laboratory research investigations have contradicted these results and many researchers have concluded that no significant improvement in process performance can be achieved with biosupplementation (De Haas, 1999).

2.4 Use of bioaugmentation in wastewater treatment

Grubbs (1984) and Rittmann *et al.* (1990) reported that bioaugmentation technology is currently applied in activated sludge for the following purposes:

Increased BOD removal whereby microbial strains are 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 microorganisms. Reductions in 17% to nearly 30% have been documented.

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 or Bioremediation of hazardous wastes. The use of added microorganisms for treating hazardous wastes (e.g. 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 bioremediation.

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

2.5 Current features and future methods for bioaugmentation in activated sludge

Bioaugmentation of activated sludge systems with specialised bacterial strains could be a powerful tool to improve several aspects in wastewater treatment processes, such as improved flocculation and degradation of recalcitrant compounds (Van Limbergen *et al.*, 1998). The addition of strains to activated sludge, either through activity of inoculated strains or after transfer of degradative plasmids to activated bacteria, could enhance the biodegradation of recalcitrant compounds.

The construction of new genetically modified organisms with the potential for enhanced breakdown of organic compounds can be a promising method for bioaugmentation of activated sludge (McClure *et al.*, 1989; McClure *et al.*, 1990; McClure *et al.*, 1991). There are indications that horizontal transfer of plasmids between bacteria has played a significant role in the adaptation of bacteria to the presence of recalcitrant compounds (Stotzky and Babich, 1986; Sayler *et al.*, 1990). The methods of bioaugmentation that will be discussed can lead to a more efficient bioaugmentation of activated sludge in the future.

2.5.1 Bioaugmentation in activated sludge and other wastewater treating bioreactors

The introduction of degradative bacteria and mobile catabolic genes into activated sludge in order to enhance biodegradation of xenobiotics was evaluated (Van Limbergen *et al.*, 1998). McClure *et al.* (1991) showed that the transfer of genes into indigenous bacteria can result in enhanced biodegradation. McClure *et al.* (1991) also indicated that the use of strains that are well adapted to the activated sludge conditions could be very important. The studies indicate that the introduction and dissemination of catabolic plasmids in natural or reactor communities could be a promising bioaugmentation strategy (Van Limbergen *et al.*, 1998).

2.5.2 Flocculation in activated sludge processes and its influence on bioaugmentation

Aerobic wastewater treatment relies on the ability of microorganisms to aggregate, allowing a separation of the formed microbial biomass and the effluent in the final settling tank (Van Limbergen *et al.*, 1998). Aerobic wastewater treatment plants that do not face major shocks or toxic pulses do not attain the discharge standards (Berthouex and Fan, 1986). Hence, even under normal operation conditions, activated sludge microbial communities are quite unstable (Van Limbergen *et al.*, 1998).

Techniques have been developed to stimulate the formation of a good settling sludge. Before the start of bioaugmentation of activated sludge, a good settling sludge should be available (Van Limbergen *et al.*, 1998). The inoculated strains should be integrated in the flocs and they should not negatively influence the sedimentation process.

The identification and characterisation of the genes and plasmids involved in the flocculation-promoting processes can help to construct bacteria that incorporate more efficiently into activated sludge flocs (Van Limbergen *et al.*, 1998). The construction of these bacteria can improve bioaugmentation.

2.5.3 Inoculation of strains in activated sludge

The nature of xenobiotics, the physicochemical conditions and the metabolic potential of the microbiota determines the efficiency, efficacy and cost of bioaugmentation (Van Limbergen *et al.*, 1998). Mineralization in a natural environment is preceded by an acclimation period (time between the inoculation and the onset of degradation of the pollutant).

Research has been conducted whereby the strains have been inoculated into the activated sludge bacteria. It was described that *Pseudomonas putida*, containing the recombinant plasmid, survived for more than 8 weeks in the activated sludge unit but did not degrade the target substrate (McClure *et al.*, 1991). The reason for not degrading the target substrate could be the preferential use of alternative substrates. The introduced strain may face intense competition, predation or parasitism in sewage. The conversion of pollutants by several members of an indigenous microbial community can also have negative effects (Van Limbergen *et al.*, 1998).

The genetic optimization of microorganisms, able to degrade target compounds and the design of novel pathways for the catabolism of recalcitrant xenobiotics, are promising strategies to enhance bioremediation processes (Van Limbergen *et al.*, 1998).

2.5.4 Plasmids and their conjugative transfer in activated sludge

Plasmids and their ability to transfer between different bacteria seem to play an important role in the adaptation of bacteria to xenobiotics through the acquisition of new genetic traits (Mergeay *et al.*, 1990). Plasmids genes can be disseminated into bacteria through conjugation, transformation and transduction. Transformation and transduction provide limited opportunities for genetic exchange (Hirsch, 1990). Plasmid transfer via conjugation play an important role in the environment and has been studied more intensively (Van Limbergen *et al.*, 1998). Conjugation is limited by plasmid incompatibility and modification systems (Frank *et al.*, 1996).

Recombinant plasmids lacking mobilisation and transfer genes have a low probability of dissemination. However, transfer of genes located on these plasmids after recombination with or transposition into Tra+ plasmids is possible and has been demonstrated (Top *et al.*, 1990). Cases of transfer of catabolic plasmids in activated sludge plants clearly indicate that genes from inoculated strains can be disseminated into activated sludge bacteria (Van Limbergen *et al.*, 1998).

2.6 Methods to determine biomass

A range of direct counting techniques have been developed to count dead and living cells separately. All the techniques are based on the indication of metabolic activity of the living cells (Herbert, 1990).

2.6.1 Direct counting procedures

The folowing direct counting procedures exist:

Method	Reference
Light microscopy	Atlas and Bartha (1993)
Epifluorescence microscopy	Wolfaardt *et al.* (1991)
Fluorescent antibody techniques	Cloete and Steyn (1988)
Confocal scanning laser microscopy	Rochow and Tucker (1994)

2.6.2 Viable count procedures

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 conditions that are optimal for the cell concerned (Atlas and Bartha, 1993). Continued growth under such conditions on agar will result in a visible colony. 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 Herbert, 1990) and the Most Probable Number (MPN) technique (Atlas and Bartha, 1993).

2.6.3 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 the bacterial metabolism. The sensitivity, reproducibility and compatibility of a number of biochemical assays for bacterial activity estimations are discussed below:

Adenosine triphosphate (ATP)

ATP is present in all microbes and can be measured with great sensitivity. Because ATP is lost rapidly following death of cells, measuring ATP concentrations can be used to estimate living biomass (Holm-Hansen *et al.*, 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 and the amount of emitted light is 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. In some ecosystems, such as soil, sediment and near shore areas, ATP maybe absorbed on particles (Holm-Hansen *et al.*, 1966).

All living things, plants, animals, and bacteria require a continual supply of energy in order to function. This energy is used for all the processes which keep the organism alive. Some of these processes occur continually such as the metabolism of foods, 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 movements. However, before the energy can be used, it must first be transformed into a form which the organism can easily handle. This special carrier of energy is the molecule adenosine triphosphate (ATP) (Brock, 1979).

The ATP molecule is composed of three components. At the centre is the sugar molecule ribose (the same sugar that forms the basis of DNA). Attached to one side of the sugar 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. These phosphates are the key to the activity of ATP (Brock, 1979).

ATP is an endergonic molecule, meaning it requires energy to be formed. Energy is stored in the covalent bonds between each phosphate group, which make up the tail of the molecule. The last phosphate bond holds the most energy (approximately 7 $kcal.mole^{-1}$). It is called the pyrophosphate bond. In order to release its energy to the organism, ATP breaks down into ADP (adenosine diphosphate) and an inorganic phosphate group and releases energy from the pyrophosphate bond. ADP is an exergonic molecule, which means that it yields energy when formed. When ADP reacts and comes into contact with enough energy and an inorganic phosphate group (PO_4^{3-}), it becomes ATP and stores energy yet again. To become ATP, ADP gets its energy and its third phosphate from respiration. More ATP forms from aerobic respiration than from anaerobic respiration (fermentation), because there is more energy involved.

Cell wall components

Almost all bacteria contain muramic acid in their cell walls and the specific relationship between murein and bacteria makes quantification of this cell-wall component useful for estimating bacterial biomass. 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 (Atlas and Bartha, 1993).

Chlorophyll measurements

Chlorophyll a, the dominant photosynthetic pigments 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. Estimation of the biomass of photosynthetic microbes based on chlorophyll determinations has been found to correlate well with such estimates based upon ATP determinations (Atlas and Bartha, 1993; Herbert, 1990).

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 (Herbert, 1990).

Photosynthesis

Both heterotrophic and autotrophic assimilation of carbon dioxide can be measured by using radiolabelled bicarbonate by incubating a sample containing the indigenous microbial community with the radio-labelled 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 radio-labelled bicarbonate. The residual ^{14}C-containing organic compounds can be oxidized with acid dichromate and the released $^{14}CO_2$ trapped and quantified.

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 CO_2 by respiration is called mineralization. Measurements of $^{14}CO_2$ release from synthetic organic chemicals, such as pesticides, are essential for evaluating the environmental safety of such compounds because, unless mineralized by microbes, they will accumulate in the environment.

Specific enzyme assays

A variety of enzyme assays can also be used for measuring the metabolic activities of microbes. Enzymes involved in the biogeochemical cycling of sulphur are of interest to microbial ecologists studying microbially 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 given to maintenance of *in situ* conditions, particularly with reference to temperature, moisture content, and Eh and incubation periods must be short enough to preclude changes in numbers of microbes, which could alter the levels of enzymes present in the sample.

It is clear from the above that numerous techniques exist for quantifying microbes, some of which represent greater ease and accuracy than others. Direct microscopy and plate count procedures remain to be the most commonly used in industry.

Bacteria within biofilms can break down contaminants in water. Through the use of the Biochemical Oxygen Demand (BOD) test one could determine the amount of oxygen the microbes in a biofilm consume and in result determine the decrease in organic concentration of the waste water (Gray, 1989).

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 (Mailette, 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 (Mailette, 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 (Mailette, 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 (Mailette, 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 (Mailette, 1969).

2.6.4 Mixed liquor suspended solids

One parameter which is used in activated sludge to measure biomass is the mixed liquor suspended material. The mixed liquor organic suspended solids are made of three components:

heterotrophic active biomass; endogenous residue and inert material. In the nitrifying aerobic and anoxic/aerobic activated sludge systems, a fourth mixed liquor organic suspended solids components included: autotrophic active biomass. The heterotrophic active biomass arises from synthesis of living heterotrophic organisms on biodegradable organic substrates and is "lost" via endogenous respiration/death processes; in the activated sludge system this mixed liquor component performs the biodegradation process of COD removal and denitrification (Ubisi *et al.*, 1997).

The autotrophic active biomass arises from synthesis of autotrophic organisms in the nitrification of ammonia to nitrate under aerobic conditions and is "lost" via endogenous respiration/death processes. The non-biodegradable portion of the heterotrophic and autotrophic active biomass that are lost in the endogenous respiration/death process. The inert material arises from the influent wastewater non-biodegradable particulate organics which, on entry into the bioreactor, are enmeshed in the mixed liquor organic suspended solids. All four mixed liquor suspended solids settle out in the secondary settling tank and are returned to the bioreactor via underflow recycle; these components leave the activated sludge system via the waste flow (Ubisi *et al.*, 1997).

Historically the mixed liquor organic suspended solids have been measured as a lump parameter, via the VSS test (Standard Methods, 1985), or more recently, the COD test. The problem in measurement of this parameter has been the lack of suitable experimental techniques. In the literature, principally microbiological techniques have been proposed; for example, pour plate or other culturing techniques (e.g Gaudy and Gaudy, 1980), ATP analysis (Nelson and Lawrence, 1980; Osborn *et al.*, 1986), DNA analysis (Liebeskind and Dohmann, 1994) using fluorescent probes for ribosomal RNA (Wagner *et al.*, 1994) and sequencing of ribosomal sequencing of ribosomal DNA (Blackall, 1994). However, these techniques have not yet been adequately integrated with the design and kinetic modelling theory; the culturing techniques have been widely criticised for their unreliability (Cloete and Steyn, 1988); the RNA and two DNA methods are still in their infancy; and the last-named four methods require sophisticated equipment and experimental techniques that are not widely available (Ubisi *et al.*, 1997).

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CHAPTER 3

BIOAUGMENTATION PRODUCTS FOR THE OPTIMIZATION OF PHOSPHORUS REMOVAL IN ACTIVATED SLUDGE

3.1 Abstract

Biological phosphorus removal during the activated sludge wastewater treatment process is an acknowledged phenomenon having gained worldwide support. However, the phenomenon of enhanced phosphate removal by activated sludge systems is not yet fully understood. Research has indicated that an increase in biomass resulted in an increase in phosphorus removal in activated sludge. The aims of the study were to determine the effect of bioaugmentation on phosphorus removal in activated sludge by adding a commercial bioaugmentation product in order to increase biomass and to determine the possibility of culturing a bioaugmentation product in a separate fermentation unit for addition to activated sludge. Two bioaugmentation products were used. Different concentrations of bioaugmentation product A were used as inocula in sterile anaerobic mixed liquor medium. Our results indicated that 18.86 mg.l^{-1} of phosphate was removed per gram of the bioaugmentation product A added when using 10 g of inoculum in 1000 ml of the mixed liquor. At concentrations exceeding 80 g per 1000 ml of mixed liquor, no phosphorus was removed, instead the phosphate concentration increased as a result of the phosphorus content of the bioaugmentation product. Bioaugmentation product A had a high phosphate content, making it unsuitable for bioaugmentation. For determining the possibility of culturing a bioaugmentation product in a separate fermentation unit for addition to activated sludge, the growth of bioaugmentation product B and anaerobic sludge was compared. Different concentrations (2.5 ml and 10 ml) of the bioaugmentation product and anaerobic sludge were inoculated into sterile anaerobic mixed liquor, Nutrient broth and sterile anaerobic mixed liquor with added nutrients (sodium acetate, magnesium sulphate and potassium nitrate), respectively. The experiments were conducted under aerobic and anaerobic conditions at room temperature. No growth was observed in the sterile mixed liquor. Nevertheless growth did occur in Nutrient broth. It was concluded that the products tested would be impractical and too expensive to use for bioagmentation in activated sludge.

3.2 Objectives

The objectives of the study were to determine the effect of bioaugmentation on phosphorus removal in activated sludge by adding a commercially available bioaugmentation product in order to increase biomass and to determine the possibility of culturing a bioaugmentation product in a separate fermentation unit for addition to activated sludge. The hypothesis of culturing a bioaugmentation product in a separate fermentation unit, was to use an inexpensive substrate to grow biomass in a separate tank as a reserve of biomass which could then serve as inoculum to increase the already existing biomass in the system, as and when necessary.

3.3 Materials and Methods

Rationale of experiments 1 and 2

The purpose of these experiments was to determine the relationship between biomass and phosphate removal and not to determine whether bioaugmentation will be of any practical value.

3.3.1 Bioaugmentation products

Commercially available bioaugmentation products were obtained from SA Biotech and Amitek.

3.3.2 Total plate count

One ml of each bioaugmentation product was serially diluted in sterile Ringer's solution. The Ringer's solution was sterilized by autoclaving at 121^0C for 15 min. The bioaugmentation products were then plated on Nutrient agar plates and incubated at room temperature (± 21^0C) for 48 h, respectively.

3.3.3 Chemical analysis of the bioaugmentation products

The phosphate (PO_4^{3-}) concentration of the bioaugmentation products was determined on samples filtered through Whatman no. 1 filter papers. The sulphate (SO_4^{2-}), nitrate (NO_3^-) and Chemical Oxygen Demand (COD) concentrations in the bioaugmentation product were determined on unfiltered samples. All the chemical analyses were determined using relevant test kits and an SQ 118 photometer (Merck).

3.3.4 Evaluation of bioaugmentation product A

3.3.4.1 Preparation of the mixed liquor medium

Grab mixed liquor samples (15 l) were collected from the anaerobic zone of the Daspoort wastewater care plant, Pretoria, South Africa. The samples were filtered using Whatman no.1 filter papers and then the supernatant was autoclaved at 121 ^{0}C for 1h. After autoclaving, the pH was adjusted to between 6 and 7 with concentrated H_2SO_4. The phosphate concentration of the supernatant was determined and adjusted to 32 mg.l^{-1} with KH_2PO_4.

3.3.4.2 Experimental setup

Sterile anaerobic mixed liquor (500 ml) was suspended in 1 l Erlenmeyer flasks. Each flask was inoculated with different quantities (1 g, 3 g, 4 g, 5 g, 10 g, 20 g, 40 g, 50 g, 80 g and 100 g per 500 ml of mixed liquor) of bioaugmentation product. A control, which was not inoculated, was included. The flasks were stirred using a 6 plate magnetic stirrer (Instrulab) and aerated using aquarium air pumps. Experiments were performed in triplicates at room temperature (22 to 25^0 C). The same experiment was repeated with different concentrations (1 g, 3 g, 4 g and 5 g) of sterile bioaugmentation product inoculated into sterile anaerobic mixed liquor, respectively. Bioaugmentation product was sterilized by autoclaving at 121^{0}C for 15 min.

3.3.4.3 Phosphate analysis

The phosphate content of the mixed liquor medium was analyzed hourly by taking 10 ml samples from the flasks with a micro-pipette. Samples were filtered through Whatman no.1 filter papers. Phosphate concentration was determined using the P (VM) 14842 test kit and SQ 118 photometer (Merck).

3.3.5 Evaluation of bioaugmentation product B

3.3.5.1 Determination of the growth curve

The following experiments were conducted in order to determine the possibility of growth of the bioaugmentation product in sterile anaerobic mixed liquor medium.

3.3.5.1.1 Experiment 1

Different quantities of bioaugmentation product (2.5 ml and 10 ml) and anaerobic sludge (2.5 ml and 10 ml) were inoculated in side-armed flasks containing sterile anaerobic mixed liquor medium (250 ml and 90 ml) and Nutrient broth (90 ml), respectively. A control, which was not inoculated was also included. One flask from each set was incubated aerobically and the other anaerobically. The aerobic conditions were created by shaking the flasks on a rotary shaker incubator at 130 rpm. The anaerobic conditions were created by putting the flasks on the bench, without shaking. The flasks were incubated at room temperature ($\pm 30^0$C). The experiments were conducted in duplicate. The absorbency was measured every 30 min at the wavelength of 590 nm using a spectrophotometer.

3.3.5.1.2 Experiment 2

Sterile anaerobic mixed liquor (90 ml) was suspended in each of the 5 side-armed flasks. The solutions to be added in the sterile mixed were prepared separately. For the first solution, 1.25 g Na-acetate was added into 250 ml mixed liquor, in a Schott bottle. The second solution contained 2.5 g sodium-acetate in 250 ml mixed liquor. The third solution contained 1.25 g Na-acetate, 0.125 g $MgSO_4.7H_2O$ and 0.045 g KNO_3 in 250 ml mixed liquor and the fourth solution was prepared by adding 2.5 g Na-acetate, 0.25 g $MgSO_4.7H_2O$ and 0.09 g KNO_3 g into 250 ml mixed liquor. All solutions were prepared in 500 ml Schott bottles respectively and sterilized by autoclaving at 121^0C for 15 min. The bioaugmentation product (10 ml) was inoculated into each of the 4 side-armed flasks and the last side-armed flask was not inoculated and served as a control. The experiment was done in duplicate. The flasks were shaken on a rotary shake incubator at the speed of 130 rpm at room temperature. Other flasks were incubated anaerobically. The absorbency was measured every 30 min at a wavelength of 590 nm using a spectrophotometer.

3.4 Results

3.4.1 Chemical and microbiological analyses

Table 3.1: Chemical and microbiological analyses of the bioaugmentation products.

Bioaugmentation product	COD ($mg.l^{-1}$)	Phosphate ($mg.l^{-1}$)	Sulphate ($mg.l^{-1}$)	Nitrate ($mg.l^{-1}$)	TPC ($cfu.ml^{-1}$)
A	14 542	107.6	39	50.7	1.22×10^6
B	*ND	87.5	ND	43	9.0×10^6

*ND - Not Determined

The high COD, phosphate, sulphate and nitrate concentrations served as nutrients for sustaining the microorganisms in the product.

3.4.2 Evaluation of bioaugmentation product A

When using 2 g of the bioaugmentation product per 1 000 ml of the mixed liquor, the phosphate concentration increased from 30.00 $mg.l^{-1}$ to 39.40 $mg.l^{-1}$ after the addition of the bioaugmentation product (time 0 h). The phosphate concentration decreased to 37.20 $mg.l^{-1}$ after 9 h (Figure 3.1). The amount of phosphate removed per gram of bioaugmentation product added was 2.20 $mg.l^{-1}$ (Table 3.2).

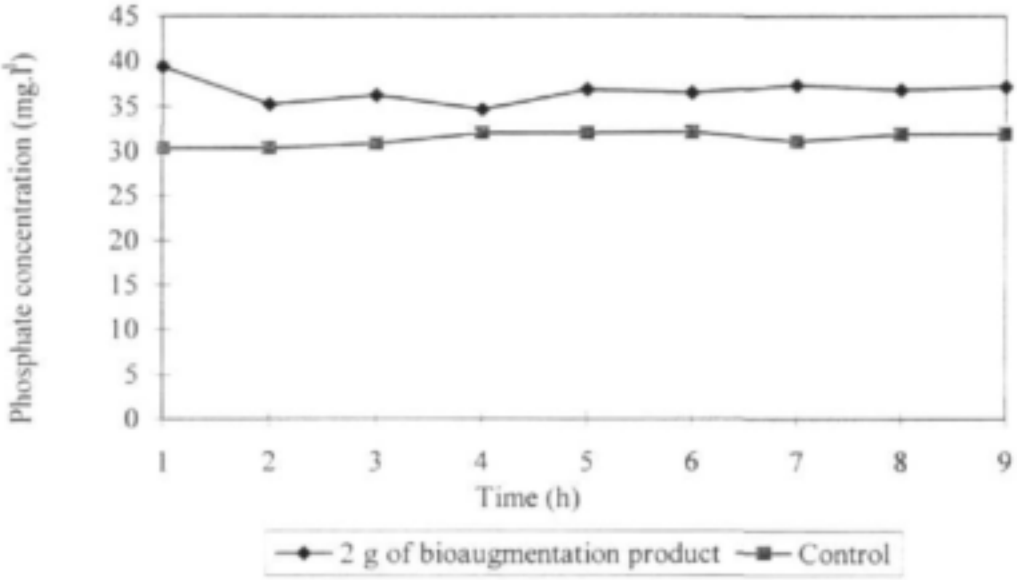

Figure 3.1: **Phosphate removal when 2 g of bioaugmentation product per 1 000 ml of mixed liquor was used.**

When using 6 g of the bioaugmentation product per 1 000 ml of mixed liquor, the phosphate concentration increased from 30.00 mg.l^{-1} to 82.40 mg.l^{-1} after the addition of the bioaugmentation product (time 0 h). The phosphate concentration decreased to 56.50 mg.l^{-1} after 9 h (Figure 3.2). The amount of phosphate removed per gram of bioaugmentation product added was 8.60 mg.l^{-1} (Table 3.2).

When using 8 g of the bioaugmentation product per 1 000 ml of mixed liquor, the phosphate concentration increased from 30.00 mg.l^{-1} to 111.50 mg.l^{-1} after the addition of the bioaugmentation product (time 0 h). The phosphate concentration decreased to 39.90 mg.l^{-1} after 9 h (Figure 3.2). The amount of phosphate removed per gram of bioaugmentation product added was 17.90 mg.l^{-1} (Table 3.2).

Table 3.2: Phosphate removed per gram of the bioaugmentation product added.

Bioaugmentation product added per 1 000 ml of mixed liquor	**Phosphate (P) concentration** (mg.l^{-1}) T = 0 h	**Phosphate (P) concentration** (mg.l^{-1}) T = 9 h	**Change in phosphate concentration, ΔP*** (mg.l^{-1})	**Phosphate removed per gram of bioaugmentation product added***** (mg.l^{-1})
Control	30.00	30.30	0.00	-
2 g.l^{-1}	39.40	37.20	2.20	2.20
6 g.l^{-1}	82.40	56.50	25.90	8.60
8 g.l^{-1}	111.50	39.90	71.60	17.90
10 g.l^{-1}	156.10	61.80	94.30	18.86
20 g.l^{-1}	183.34	49.42	133.92	13.40
40 g.l^{-1}	283.17	95.72	187.45	9.370
80 g.l^{-1}	186.65	338.00	-151.35**	-3.78**
100 g.l^{-1}	306.92	751.50	-444.58**	-8.89**
160 g.l^{-1}	949.90	1129.90	-180.00**	-2.25**
200 g.l^{-1}	946.60	834.15	112.45	1.124

*$\Delta P = (\text{P concentration})_{T9} - (\text{P concentration})_{T0}$, **Phosphate release

***ΔP/gram(s) of the bioaugmentation product added

When using 10 g of the bioaugmentation product per 1000 ml mixed liquor, the phosphate concentration increased from 30.00 mg.l^{-1} to 156.10 mg.l^{-1} after the addition of the bioaugmentation product (time 0 h). The phosphate concentration decreased to 61.80 mg.l^{-1} after 9 h (Figure 3.2). The amount of phosphate removed per gram of the bioaugmentation product added was 18.86 mg.l^{-1} (Table 3.2).

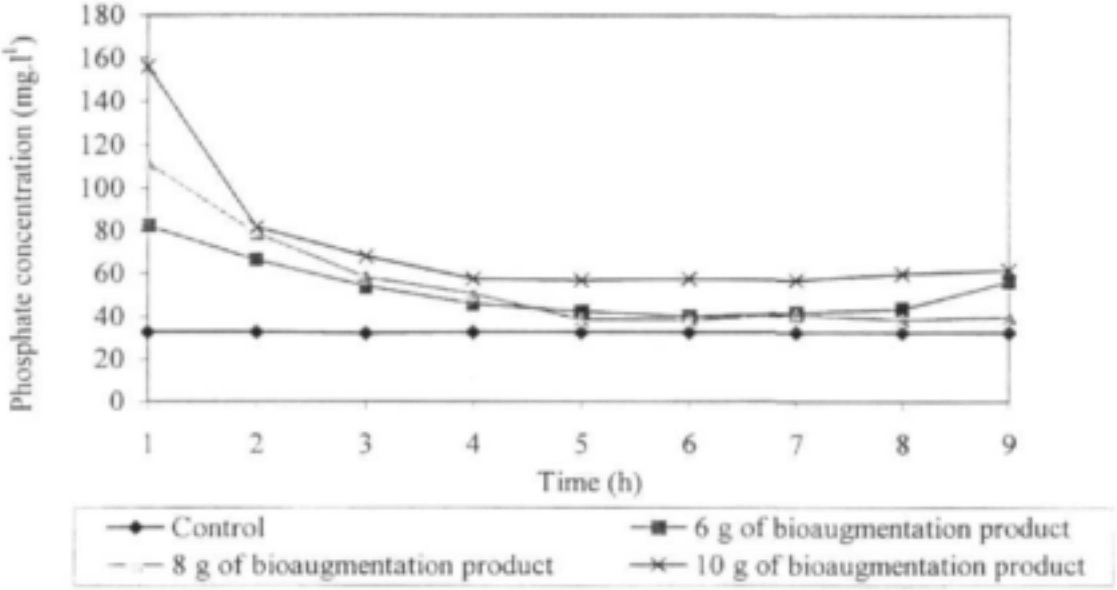

Figure 3.2: Phosphate removal when 6 g, 8 g and 10 g of bioaugmentation product per 1000 ml of mixed liquor were used.

When using 20 g of the bioaugmentation product per 1000 ml of mixed liquor, the phosphate concentration increased from 30.00 mg.l^{-1} to 183.34 mg.l^{-1} after the addition of the bioaugmentation product (time 0 h). The phosphate concentration decreased to 49.42 mg.l^{-1} after 9 h (Figure 3.3). The amount of phosphate removed per gram of the bioaugmentation product added was 13.41mg.l^{-1} (Table 3.2).

When using 40 g of the bioaugmentation product per 1000 ml of mixed liquor, the phosphate concentration increased from 30.00 mg.l^{-1} to 283.17 mg.l^{-1} after the addition of the bioaugmentation product (time 0 h). The phosphate concentration decreased to 95.72 mg.l^{-1} after 9 h (Figure 3.3). The amount of phosphate removed per gram of the bioaugmentation product added was 9.46 mg.l^{-1} (Table 3.2).

When using 80 g of the bioaugmentation product per 1000 ml of mixed liquor, the phosphate concentration increased from 30.00 mg.l^{-1} to 186.65 mg.l^{-1} after the addition of the bioaugmentation product (time 0 h). The phosphate concentration increased to 338.00 mg.l^{-1} after 9 h (Figure 3.4).

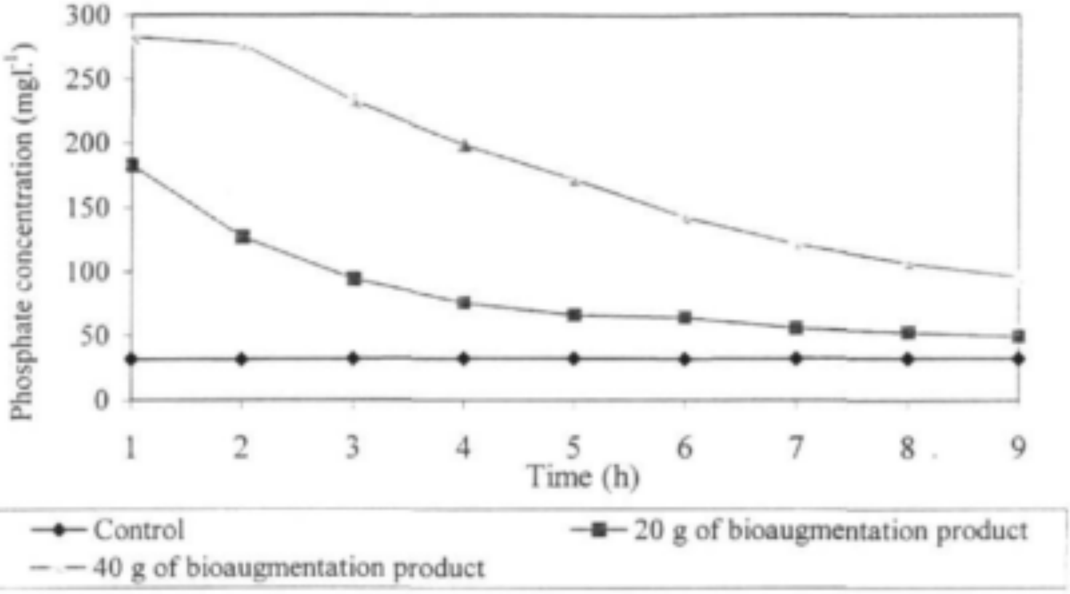

Figure 3.3: **Phosphate removal when 20 g and 40 g of bioaugmentation product per 1000 ml of mixed liquor were used.**

When using 100 g of the bioaugmentation product per 1000 ml of mixed liquor, the phosphate concentration increased from 30.00 mg.l^{-1} to 306.92 mg.l^{-1} after the addition of the bioaugmentation product (time 0 h). The phosphate concentration increased to 751.5 mg.l^{-1} after 9 h (Figure 3.4).

When using 160 g of the bioaugmentation product per 1000 ml of mixed liquor, the phosphate concentration increased from 30.00 mg.l^{-1} to 949.90 mg.l^{-1} after the addition of the bioaugmentation product (time 0 h). The phosphate concentration increased to 1129.90 mg.l^{-1} after 9 h (Figure 3.4).

When using 200 g of the biosupplement per 1000 ml of mixed liquor, the phosphate concentration increased from 30.00 mg.l^{-1} to 946.60 mg.l^{-1} after the addition of the bioaugmentation product (time 0 h). The phosphate concentration decreased to 834.15 mg.l^{-1} after 9 h (Figure 3.4). The amount of phosphate removed per gram of the bioaugmentation product added was 1.124 mg.l^{-1} (Table 3.2).

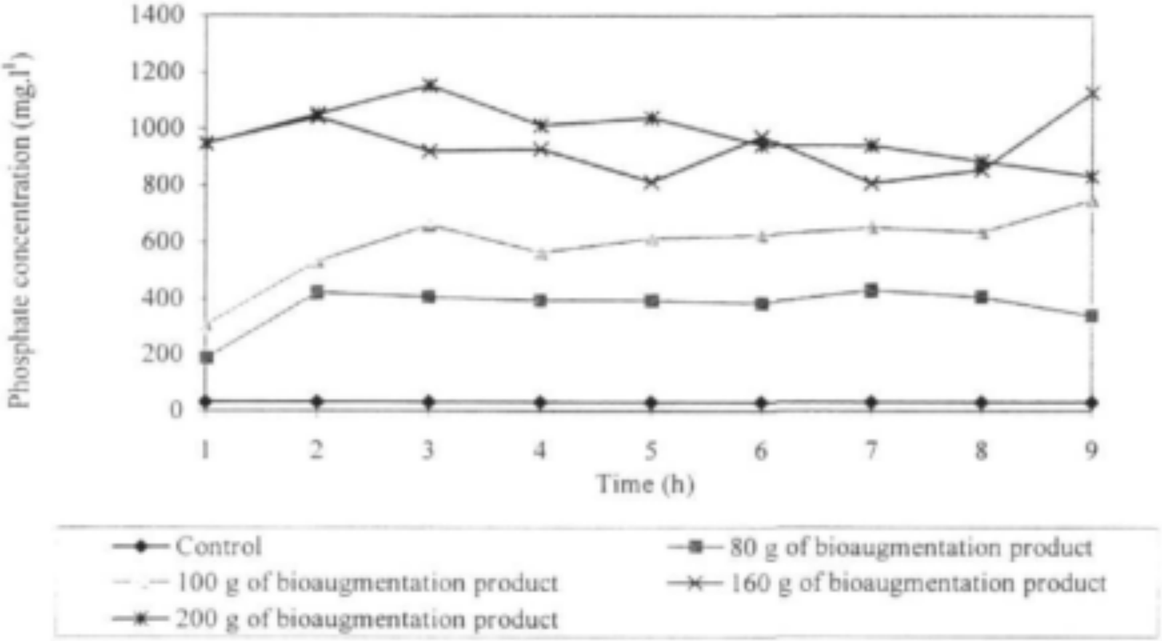

Figure 3.4: Phosphate removal when 80 g, 100 g, 160 g and 200 g of bioaugmentation product per 1000 ml of mixed liquor were used.

When sterile bioaugmentation product was used as inoculum, for 2 g per 1000 ml, initially, the phosphate concentration was 32 mg.l^{-1} and after 9 h it remained 32 mg.l^{-1} (Figure 3.5). When using 6 g of the sterile biosupplement per 1000 ml of mixed liquor, phosphate concentration increased from 32 mg.l^{-1} to 35.2 mg.l^{-1} after the addition of the product (time 0 h). The phosphate concentration increased to 43.3 mg.l^{-1} after 9 h (Figure 3.5). The amount of phosphate released per gram of the bioaugmentation product added was 8.1 mg.l^{-1} (Table 3.3).

When using 8 g and 10 g of the sterile bioaugmentation product per 1000 ml of the mixed liquor, phosphate concentration increased from 32 mg.l^{-1} to 35.6 mg.l^{-1} and 37.6 mg.l^{-1} after the addition of the bioaugmentation product, respectively. The phosphate concentration increased to 40.3 mg.l^{-1} and 43.3 mg.l^{-1}, respectively, after 9 h (Figure 3.5). The amount of phosphate released per gram of the bioaugmentation product added was 4.7 mg.l^{-1} and 5.7 mg.l^{-1}, respectively (Table 3.3).

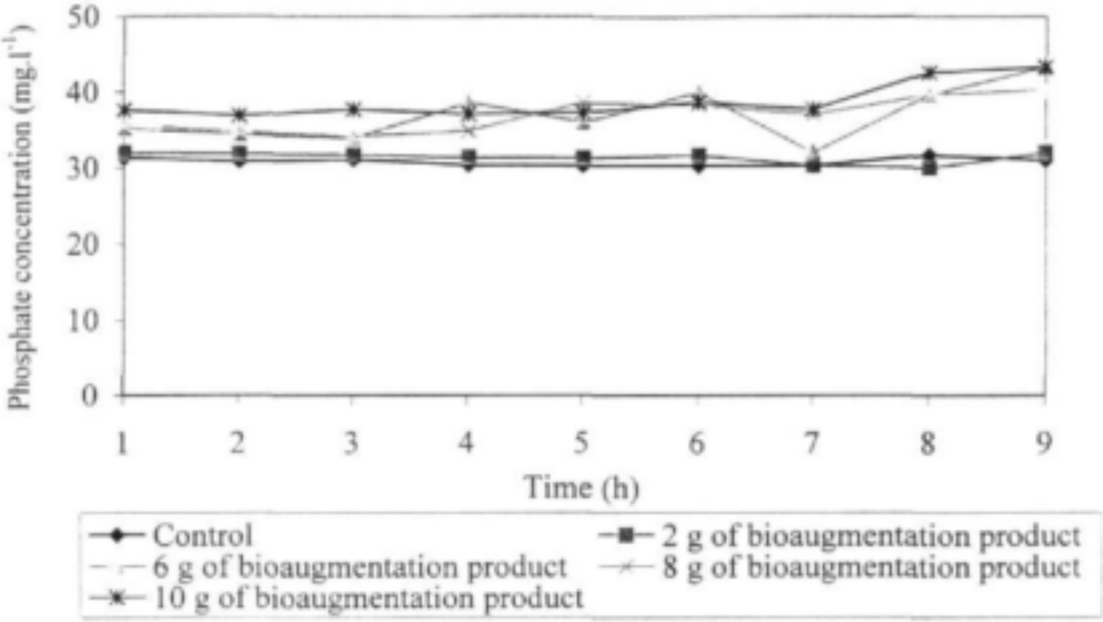

Figure 3.5: Phosphate removal when 2 g, 6 g, 8 g and 10 g of sterile bioaugmentation product per 1000 ml of mixed liquor were used.

Table 3.3: Phosphate removed per gram of the sterile bioaugmentation product added.

Bioaugmentation product added per 1000 ml of mixed liquor	**Phosphate (P) concentration** (mg.l^{-1}) T = 0 h	**Phosphate (P) concentration** (mg.l^{-1}) T = 9 h	**Change in phosphate concentration,** **ΔP*** (mg.l^{-1})	**Phosphate removed per gram of bioaugmentation product added***** (mg.l^{-1})
2g.l^{-1}	32.00	32.00	0.00	0.00
6g.l^{-1}	35.20	43.30	8.10	2.70
8g.l^{-1}	35.60	40.30	4.70	1.175
10g.l^{-1}	37.60	43.30	5.70	1.14
Control	31.30	31.00	0.30	-

*ΔP = (P concentration)$_{T9}$ – (P concentration)$_{T0}$, ***ΔP/gram(s) of the bioaugmentation product added

The sterile bioaugmentation product did not remove phosphate.

3.4.3 Evaluation of bioaugmentation product B

When 2.5 ml of bioaugmentation product and anaerobic sludge were inoculated in 250 ml mixed liquor, to determine the growth curve of the product, there was no increase in the absorbance readings. The absorbance reading of the medium under aerobic conditions of the bioaugmentation product was initially 0.20 and decreased to 0.03 and that under anaerobic conditions was 0.20 and decreased to 0.02. The absorbance reading of the medium under aerobic conditions inoculated with anaerobic sludge was initially 0.04 and increased to 0.06. The medium under anaerobic conditions indicated the absorbance reading of 0.14 and decreased to 0.12 (Figure 3.6).

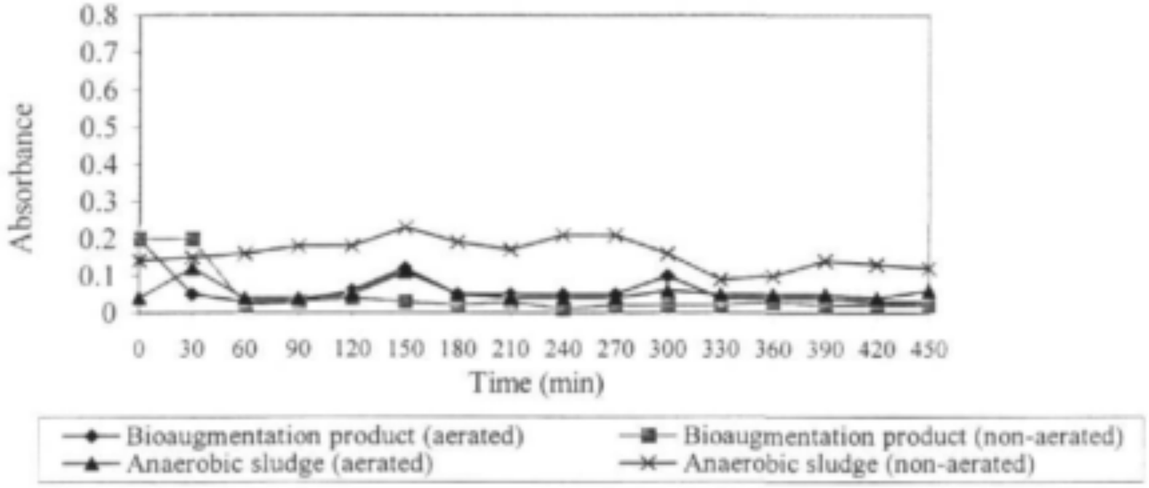

Figure 3.6: The growth of bioaugmentation product (2.5 ml) and anaerobic sludge (2.5 ml) inoculated in 250 ml sterile mixed liquor, during aerobic and anaerobic conditions.

When using 10 ml of the bioaugmentation product and anaerobic sludge per 90 ml of mixed liquor, there was no increase in the absorbance readings, that is, the readings remained the same. The absorbance reading of the medium under aerobic conditions, inoculated with the bioaugmentation product, was initially 0.12 and increased to 0.16 after 24 h and under anaerobic conditions the initial absorbance was 0.06 and decreased to 0.05 after 24 h. In the medium inoculated with anaerobic sludge, for aerobic conditions, the absorbance reading was initially 0.12 and decreased to 0.00 after 24 h and for anaerobic conditions, the absorbance reading was 0.17 and decreased to 0.02 after 24 h (Figure 3.7).

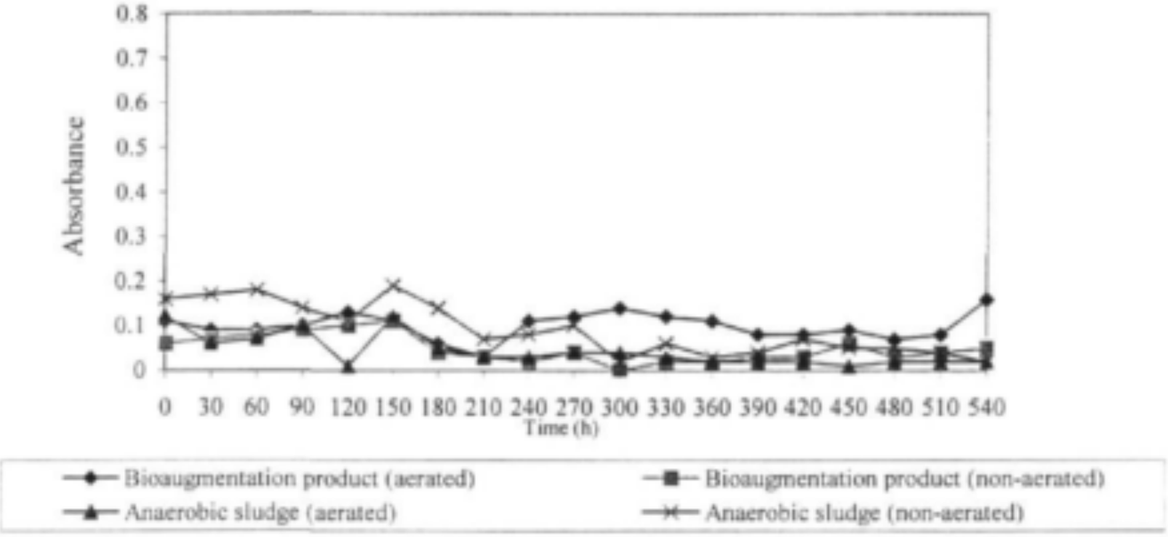

Figure 3.7: The growth of bioaugmentation product (10 ml) and anaerobic sludge (10 ml) inoculated in 90 ml of sterile mixed liquor, during aerobic and anaerobic conditions.

The absorbance readings of the inoculation of the bioaugmentation product and the anaerobic sludge in 90 ml of Nutrient broth showed an increase. The absorbance readings of the medium incubated under aerobic conditions, inoculated with the bioaugmentation product, were initially 0.04 and increased to 2.00 after 24 h and for anaerobic conditions medium, the absorbance reading was initially 0.03 and increased to 0.76 after 24 h. The absorbance reading for the medium inoculated with the anaerobic sludge, incubated under aerobic conditions, was 0.56 and it increased to 2.00 after 24 h. During anaerobic conditions, the absorbance was 0.55 and it increased to 1.35 after 24 h (Figure 3.8).

There was no growth of the microorganisms in the medium when using 10 ml of the bioaugmentation product per 90 ml of the mixed liquor with added nutrients (see materials and methods).

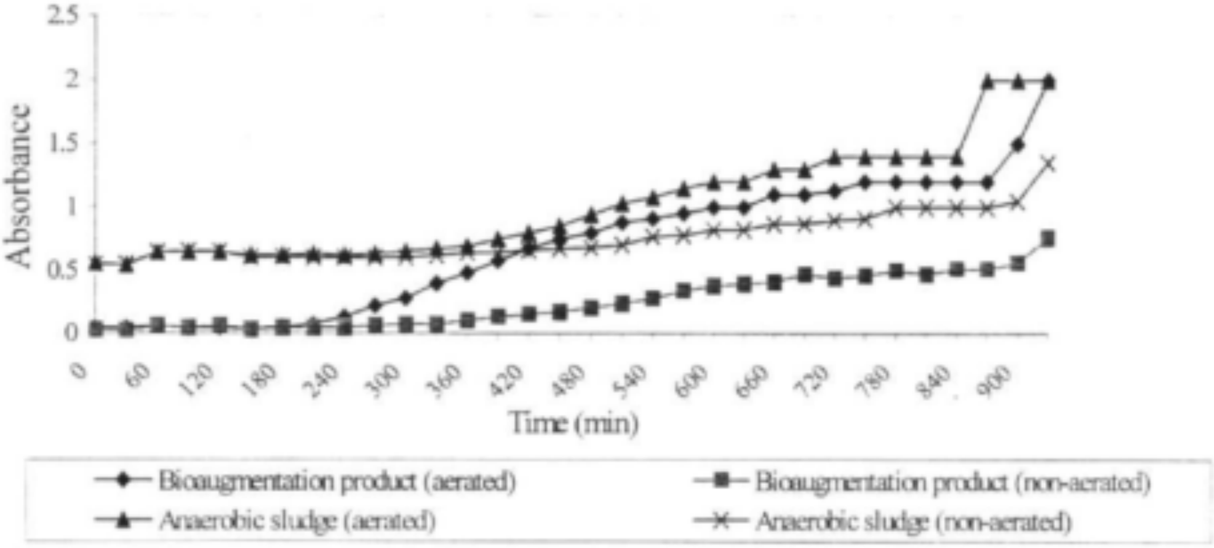

Figure 3.8: **The growth of bioaugmentation product (10 ml) and anaerobic sludge (10 ml) inoculated in 90 ml Nutrient broth, during aerobic and anaerobic conditions.**

3.5 Discussion and Conclusion

Bioaugmentation products A and B contained phosphate. This increased the phosphate concentration of the mixed liquor in all the experiments upon addition. Initially, the phosphate removal increased proportional to the quantity of bioaugmentation product added. Eight g and ten g of bioaugmentation product per 1000 ml of mixed liquor resulted in 17.90 $mg.l^{-1}$ and 18.86 $mg.l^{-1}$ phosphate removal per g of bioaugmentation product added, respectively. Hereafter, the rate of phosphate removal per g of bioaugmentation product decreased. This could be ascribed to a number of factors. The first factor could be the presence of organic matter which was introduced by the bioaugmentation product which contained 14 542 $mg.l^{-1}$ COD. Previous studies have indicated that the presence of organic carbon matter would inhibit phosphate removal (Bosch and Cloete, 1993). The second limiting factor could be the presence of nitrate, also introduced during bioaugmentation. The bioaugmentation product A contained 39 $mg.l^{-1}$ nitrate. It is known that nitrate limits phosphate uptake in activated sludge (Barnard, 1976). Oxygen limitations could also have occurred limiting phosphate uptake in the experiments, due to the elevated organic carbon content as previously discussed. From our results, it became clear that there was a relationship between phosphate uptake and the quantity of biougmentation product added up to a point where other factors became limiting.

Since 8 g and 10 g per 1000 ml of mixed liquor removed phosphorus, full-scale applications will need between 8 kg and 10 kg of bioaugmentation product per 1000 liters to remove phosphorus from the activated sludge. Even at these concentrations, the limit of 1 mg.l^{-1} phosphate was never reached. Full-scale applications of the bioaugmentation product would be impractical and too expensive. It was therefore concluded that the bioaugmentation product evaluated was unsuitable for use in activated sludge systems.

When determining the possibility of culturing a bioaugmentation product B and anaerobic sludge in sterile mixed liquor medium, as reserve biomass, no growth of the microorganisms was observed in the sterile mixed liquor and sterile mixed liquor with added nutrients. Growth only occurred in Nutrient broth. The hypothesis was to use an inexpensive substrate to grow this product in separate tanks to have a reserve of biomass, which could then serve as biomass inoculum to increase the already existing biomass in the system when necessary. The results indicated that there were not enough nutrients in the sterile mixed liquor to support the growth of the microorganisms of the bioaugmentation product, or it was not suitable for their growth. In order for this approach to be successful, alternative inexpensive substrates will have to be evaluated in order to increase biomass.

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CHAPTER 4

PHOSPHATE REMOVAL CAPACITY OF AEROBIC, ANAEROBIC AND RETURN SLUDGE MLSS

4.1 Abstract

It has been reported that biological phosphorus removal (BPR) often fails. Various reasons for the failure of activated sludge systems have been reported. Biomass depletion has been suggested as one of the reasons for system failure in BPR. The role of biomass in biological excess phosphate removal from activated sludge is widely accepted, though its role is not yet clearly understood. To better understand how activated sludge systems remove phosphate five sets of experiments were conducted, using 32 $mg.l^{-1}$, 100 $mg.l^{-1}$ and 200 $mg.l^{-1}$ phosphate in sterile mixed liquor. In the first experiment (40 $g.l^{-1}$, 80 $g.l^{-1}$, 120 $g.l^{-1}$ and 160 $g.l^{-1}$) and (33 $g.l^{-1}$, 66 $g.l^{-1}$, 100 $g.l^{-1}$ and 133 $g.l^{-1}$) aerobic sludge mass was used as inocula in sterile mixed liquor containing 32 $mg.l^{-1}$ and 100 $mg.l^{-1}PO_4^{3-}$ respectively. Also 100 $g.l^{-1}$ and 133 $g.l^{-1}$ aerobic sludge mass was used in sterile mixed liquor containing 200 $mg.l^{-1}$ PO_4^{3-}. In the second experiment, 33 $g.l^{-1}$, 66 $g.l^{-1}$, 100 $g.l^{-1}$ and 133 $g.l^{-1}$ of anaerobic sludge mass was used in sterile mixed liquor containing 200 $mg.l^{-1}$ PO_4^{3-}. In the third experiment, 33 $g.l^{-1}$, 66 $g.l^{-1}$, 100 $g.l^{-1}$ and 133 $g.l^{-1}$ of return sludge mass was used in sterile mixed liquor containing 200 $mg.l^{-1}$ PO_4^{3-}. The fourth experiment was done simulating the actual MLSS of the aerobic, anaerobic and return sludge. When using aerobic sludge mass in mixed liquor at a concentration of 32 $mg.l^{-1}$ PO_4^{3-}, all the phosphate was removed within 6 h for all the concentrations. This indicated that the sludge to PO_4^{3-} ratio was too high to determine the relationship between the sludge PO_4^{3-} removal. When using aerobic sludge mass from the same system at different concentrations of mixed liquor media containing 100 $mg.l^{-1}$ PO_4^{3-} and 200 $mg.l^{-1}$ PO_4^{3-}, sludge concentration was linearly related to PO_4^{3-} removal. However, there was no difference in the removal of phosphate by different quantities of anaerobic and return sludge mass from the same system. When simulating the actual MLSS concentration in a

specific plant, in the first experiment, no difference could be detected between the phosphate removal capacity of aerobic and anaerobic sludge although the use of return sludge resulted in a higher phosphate removal when compared to aerobic and anaerobic sludge. In the second simulation experiment, the anaerobic sludge indicated variable results. However, the return sludge performed consistently well at phosphate removal. The return and the anaerobic sludges were more effective than aerobic sludge. It was concluded that an increase in sludge mass resulted in a proportional increase in phosphate removal.

4.2 Objectives

The objectives of the study were to evaluate the anaerobic sludge, return sludge and aerobic sludge as supplements and to determine the phosphate removal capacity of a system based on sludge mass and also MLSS concentrations similar to that found in full-scale plants.

4.3 Materials and Methods

The purpose of the experiments was to determine the relationship between biomass and phosphate removal using aerobic, anaerobic and return sludge mass as supplements.

4.3.1 Samples

Grab mixed liquor samples of aerobic (10 l), anaerobic (20 l) and return (10 l) sludge were collected from the Daspoort wastewater treatment plant in Pretoria, South Africa. The total plate count, mixed liquor suspended solid (MLSS), chemical oxygen demand (COD), mixed liquor volatile suspended solids (MLVSS), phosphate, nitrate and ammonium concentration and pH were performed immediately after collection, for experiment 4. The samples were stored at 4 ^{0}C overnight for further use.

4.3.2 Preparation of the mixed liquor medium

The same procedure for preparation of the growth medium was followed as described at section 3.3.4.1. However, the following changes were made:

The filtrate was adjusted to 32 mg.l^{-1}, 100 mg.l^{-1} and 200 mg.l^{-1} for experiment 1 (aerobic sludge) and 200 mg.l^{-1} for experiment 2 (anaerobic sludge) and for experiment 3 (return sludge) by the addition of KH_2PO_4, respectively.

4.3.3 Inoculum

Grab mixed liquor samples of aerobic sludge (for experiment 1), anaerobic sludge (for experiment 2), return sludge (for experiment 3) and the experiment simulating the actual MLSS of the plant (experiment 4 and 5) were centrifuged at 3000 rpm for 20 min using a Beckman Model J 6 Centrifuge, respectively. For the wet sludge experiments, specific quantities of concentrated sludge were weighed. For the experiment simulating the actual MLSS of the system, the sludge was concentrated as follows: for half the sludge concentration, 500 ml of the aerobic sludge was used. For the standard sludge concentration, 1 l was concentrated and for double the sludge concentration, 2 l of the sludge was concentrated. The aerobic, anaerobic and return pellets were then put in 1 l glass beakers, respectively. To induce phosphate uptake, the pellets were stored at 4 ^{0}C overnight for anaerobic/microaerophilic conditions to develop.

4.3.4 Experimental setup and running

4.3.4.1 Experiments with sludge mass

The prepared sludge pellets, which were stored overnight were weighed off into specific concentrations into sterile 1 l Erlenmeyer flasks for the sludge mass experiments. For experiment 1, the sterile mixed liquor medium (500 ml) containing 32 mg.l^{-1} phosphate concentration was dispensed into 5 1 litre flasks containing 20 g, 40 g, 60 g and 80 g of aerobic sludge pellets. A control, which was not inoculated, was also included. The same experimental procedure was repeated using aerobic sludge pellets (20 g, 40 g, 60 g and 80 g) and (60 g and 80 g) per 600 ml of sterile mixed liquor containing 100 mg.l^{-1} and 200 mg.l^{-1} phosphate concentration, respectively. The experiments were conducted in triplicate. For experiment 2 (anaerobic sludge), the sterile mixed liquor (600 ml) containing 200 mg.l^{-1} of phosphate was dispensed into 5 1 l Erlenmeyer flasks containing 20 g, 40 g, 60 g and 80 g of sludge mass. A control, which was not inoculated, was included. The experiment was done in triplicate. For experiment 3 (return sludge), the sterile mixed liquor medium (600 ml) containing 200 $mg.l^{-1}$ of phosphate was dispensed into 5 1 l Erlenmeyer flasks containing 20 g, 40 g, 60 g and 80g of sludge mass. A control was also included. The flasks were constantly shaken on a Labotec shaker at room temperature, respectively. In experiment 1, where aerobic sludge was used per 500 ml of mixed liquor medium containing 32 $mg.l^{-1}$ phosphate, the mixed liquor was aerated by means of aquarium pump (Elite 802). Samples (6 flasks) were constantly shaken by the use of a six-plate magnetic stirrer device (Instrulab). In the experiment where aerobic sludge per 600 ml of mixed liquor medium containing 100 $mg.l^{-1}$ and 200 $mg.l^{-1}$, mixed liquor was aerated by means of a compressor-vacuum pump (Edwards E.B.3). In other experiments, the mixed liquor media were not aerated but shaken on a Labotec shaker (Figure 4.1).

4.3.4.2 Experiment with MLSS concentrations simulating full-scale MLSS *concentrations.*

In the experiment simulating the actual MLSS of the plant, the half, standard and double mass of the aerobic, anaerobic and return sludge was inoculated in 3 1 l Erlenmeyer flasks containing sterile mixed liquor (600 ml) containing 32 $mg.l^{-1}$ phosphate concentration, respectively. A control was also included. The experiment was done in duplicate. The flasks were constantly shaken on a Labotec shaker at room temperature, respectively (Figure 4.2).

4.3.5 Total Bacterial Count

Immediately after inoculation, 0.1 ml of sample was extracted from each flask. The total bacterial count was determined on the original sample, at time 0 h and again at time 8 h as described in section 3.3.2. The bacterial count was also determined using ATP bioprobe (Merck) for aerobic sludge.

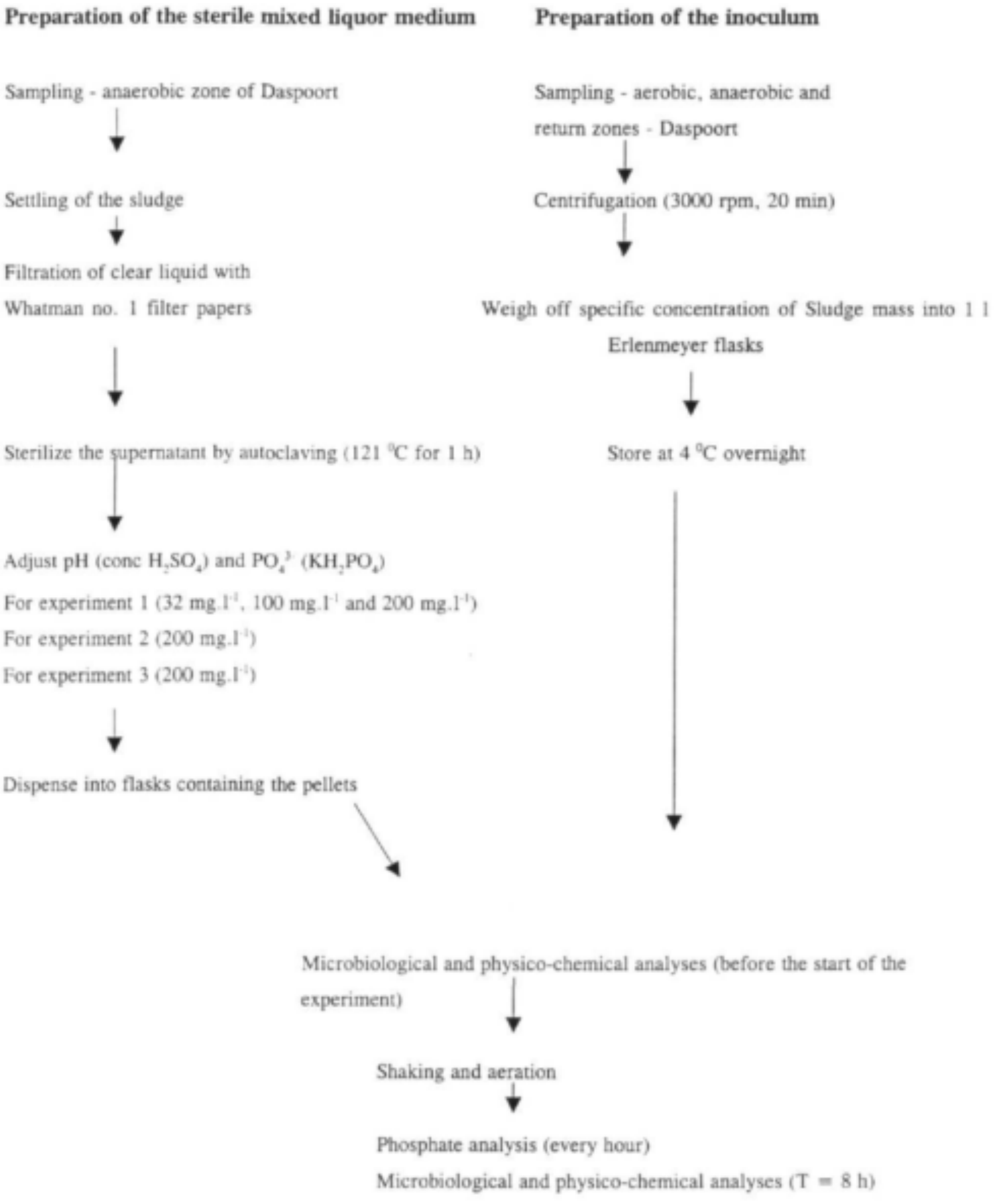

Figure 4.1: **Schematic representation of the experimental protocol used in the constant biomass experiments.**

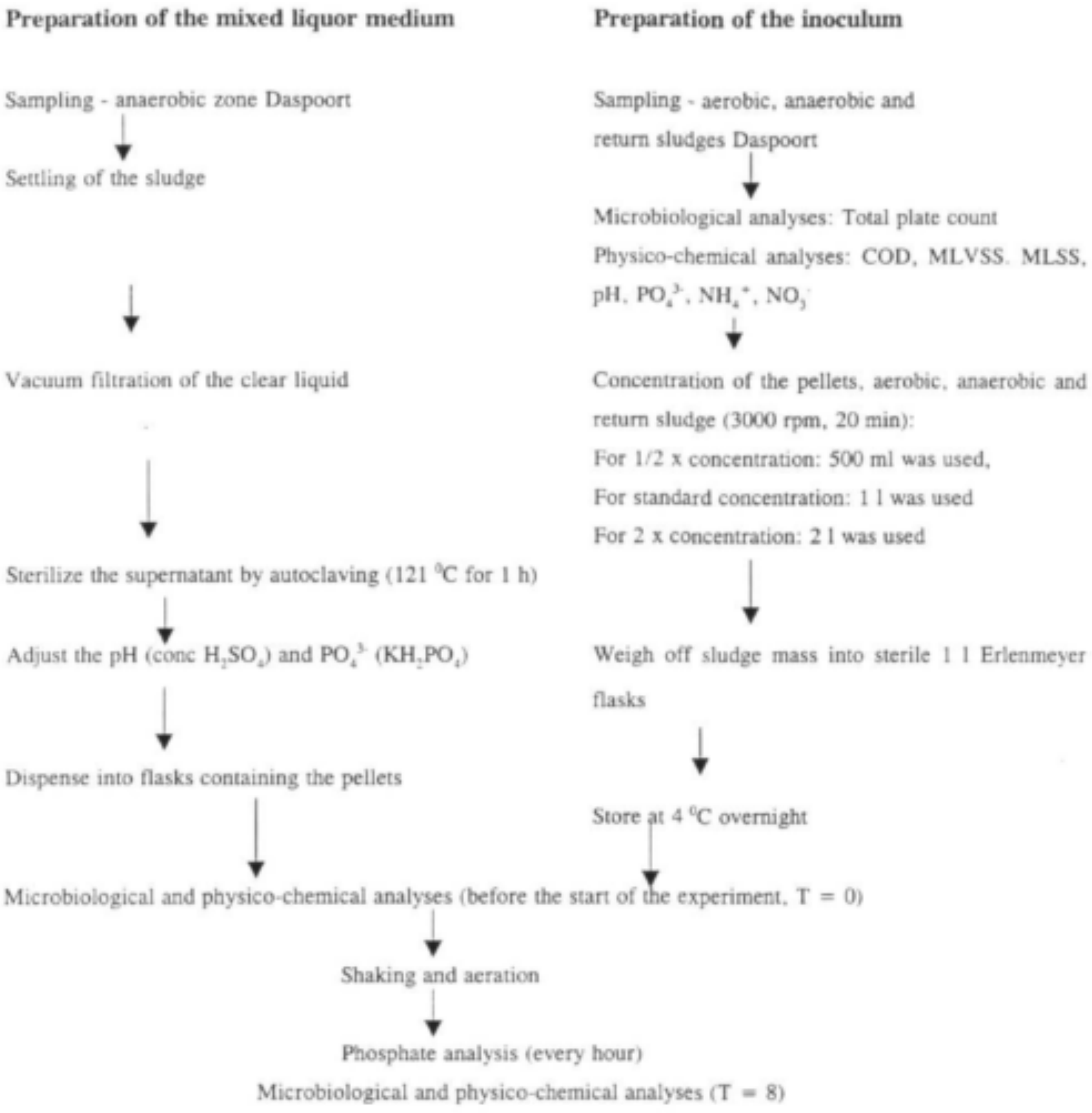

Figure 4.2: **Schematic representation of the experimental protocol used in the biomass simulation experiments.**

4.3.6 Mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS)

Mixed liquor and volatile suspended solids were determined using standard methods (Standard Methods, 1995). This was conducted on the original sample, at time 0 h and time 8 h.

4.3.7 Oxygen concentration

Oxygen concentration was determined at time 0 h and time 8 h of the experiment using an oxygen probe (Oxygen meter YSI Model 54).

4.3.8 Phosphate removal studies

The same procedure was followed as described in section 3.3.4.3. The first samples were drawn at time 0 h (i.e. prior to aeration and shaking) and also at time 8 h (before termination of the experiment) as well as hourly during the experiment. The phosphate concentration was also determined on the original sample prior preparation of the experiment.

4.3.9 Chemical analysis

The same procedure was followed as described in section 3.3.3. The chemical analyses were determined on the original sample, at time 0 h and time 8 h.

4.3.10 pH

pH of the mixed liquor media was determined on the original sample, at time 0 h and time 8 h. The pH was determined by a Beckman Φ34 pH meter and a relevant probe.

4.4 Results and Discussion

4.4.1 Experiments with sludge mass

4.4.1.1Experiment 1 (Aerobic sludge)

In experiment 1, where an average of 32 mg.l^{-1} PO_4^{3-} was used in the mixed liquor medium, the inoculum led to the removal of all the PO_4^{3-} within relatively short period of time (± 3 h). In some cases (80 g.l^{-1}, 120 g.l^{-1} and 160 g.l^{-1} of sludge mass), PO_4^{3-} removal was in less than 1 hour (Figure 4.3). No difference could be detected between 40 g.l^{-1}, 80 g.l^{-1}, 120 g.l^{-1}, and 160 g.l^{-1} sludge mass after 3 h. Hence, no quantitative conclusion could be made with regards to sludge mass and its relationship to PO_4^{3-} removal excepting that the PO_4^{3-} concentration was too low in relation to the sludge mass. Hence the PO_4^{3-} concentration was increased in subsequent experiments.

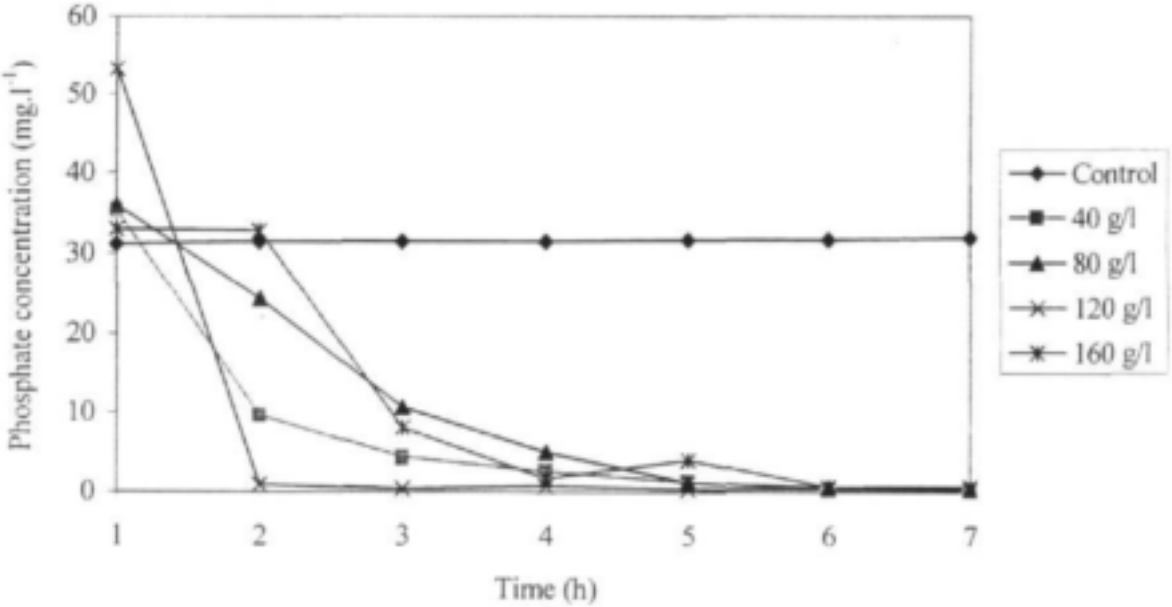

Figure 4.3: Phosphate removal by 40 g.l^{-1}, 80 g.l^{-1}, 120 g.l^{-1} and 160 g.l^{-1} of aerobic sludge mass in sterile mixed liquor containing 32 mg.l^{-1} P.

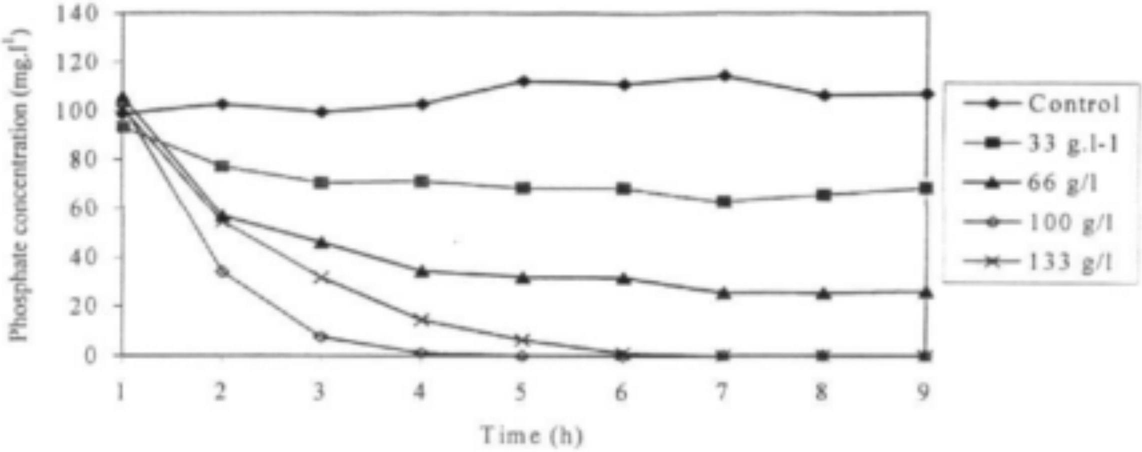

Figure 4.4: **Phosphate removal by 33 g.l^{-1}, 66 g.l^{-1}, 100 g.l^{-1} and 133 g.l^{-1} of aerobic sludge mass in sterile mixed liquor containing 100 mg.l^{-1} P.**

When different quantities (33 g.l^{-1}, 66 g.l^{-1}, 100 g.l^{-1} and 133 g.l^{-1}) of aerobic sludge mass in sterile mixed liquor containing 100 mg.l^{-1} of phosphate was used, the results indicated that 100 g.l^{-1} and 133 g.l^{-1} of sludge mass removed all the phosphate within 4 h and 6 h respectively. There was also an increase in phosphate removal as the sludge mass increased (Figure 4.4). It was decided to repeat the experiment for the 100 g.l^{-1} and 133 g.l^{-1} of sludge mass, using a higher phosphate concentration (200 mg.l^{-1}).

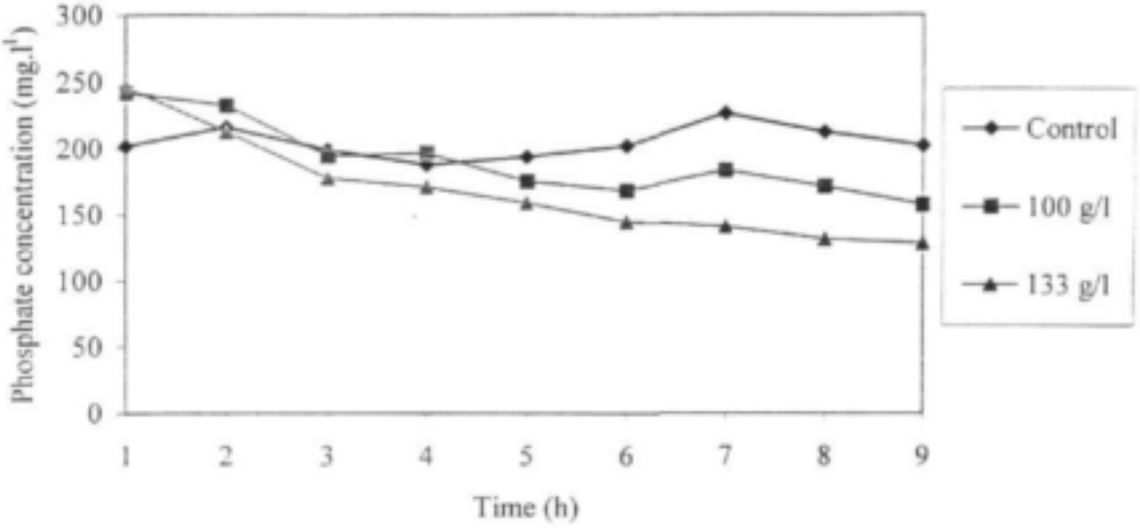

Figure 4.5: Phosphate removal by 100 g.l^{-1} and 133 g.l^{-1} of aerobic sludge mass in sterile mixed liquor containing 200 mg.l^{-1} P.

The 133 g.l^{-1} of aerobic sludge mass removed more phosphate than the 100 g.l^{-1} (Figure 4.5). These results also indicated that an increase in sludge mass resulted in increase in phosphate removal.

The increase in PO_4^{3-} removal with an increase in aerobic sludge mass indicated a direct relationship between sludge mass and PO_4^{3-} removal from mixed liquor (Figures 4.4 and 4.5). In terms of phosphate removed per gram of sludge mass, values were in the range of 1.25 mg $PO_4^{3-}.g^{-1}$ of sludge to 1.99 mg $PO_4^{3-}.g^{-1}$ of sludge (Table 4.1). However, in terms of PO_4^{3-} removed per gram of initial MLSS, a larger range of values was observed (10.69 mg $PO_4^{3-}.g^{-1}$ sludge to 19.91 mg $PO_4^{3-}.g^{-1}$sludge). In the experiment with 100 mg.l^{-1} phosphate, the optimum sludge concentration of 66 g.l^{-1} was indicated (Table 4.1). For experiment pertaining 200 mg.l^{-1} phosphate, an increase in phosphate removal was observed for an increase in sludge mass (Table 4.1). Although the optimum sludge concentrations could not be determined.

Table 4.1: Phosphate removal by 40 g.l^{-1}, 80 g.l^{-1}, 120 g.l^{-1} and 160 g.l^{-1} of aerobic sludge mass in sterile mixed liquor containing 100 and 200 mg.l^{-1} phosphate concentration.

[PO_4^{3-}] (mg.l^{-1})	Sludge mass (g.l^{-1})	PO_4^{3-} (mg.l^{-1})		PO_4^{3-} removed (mg.l^{-1})	* PO_4^{3-} removed per g of sludge (mg.g^{-1})	PO_4^{3-} Removed per initial MLSS (mg.g^{-1})
		T = 0 h	T = 8 h			
100	Control	98.66	107.00	-8.34	-	-83.4
	33	93.33	68.33	25.00	1.25	11.26
	66	105.66	26.00	79.66	1.99	19.91
	100	102.33	0.00	102.33	1.70	18.05
	133	101.33	0.00	101.33	1.27	13.84
200	Control	200.00	202.00	-2.00	-	0.57
	100	242.00	157.67	84.33	1.40	10.96
	133	246.67	128.67	118.00	1.48	13.28

* P removed per gram of sludge = P removed/Amount of sludge mass used.

We assumed that an increase in sludge mass would also result in a proportional increase in the biomass since the sludge was taken from the same system. Therefore, we concluded that phosphate removal was proportional to the biomass. Our results are in agreement with that of Lemos *et al*. (1997) and others (Momba and Cloete, 1996; Muyima, 1995) who stated that a phosphorus free effluent could be obtained when there was enough biomass in a wastewater process. This suggested that the failure of EBPR under certain conditions could be due to insufficient biomass.

Table 4.2: ATP, Total Plate Count and MLSS of aerobic sludge mass in sterile mixed liquor containing 100 mg.l^{-1} and 200 mg.l^{-1} phosphate concentration.

[PO_4^{-3}] (mg.l^{-1})	Sludge mass (g.l^{-1})	ATP		Total Plate Count (cfu.ml^{-1})		MLSS (mg.l^{-1})	
		T= 0 h	T = 8 h	T = 0 h	T = 8 h	T = 0 h	T = 8 h
100	Control	1.23×10^4	4.51×10^6	0	*ND	100	200
	40	3.34×10^6	4.93×10^7	1.69×10^6	1.44×10^6	2220	3260
	80	5.41×10^6	5.36×10^7	5.34×10^6	6.17×10^7	4001	6080
	120	5.81×10^6	2.93×10^7	8.71×10^6	1.49×10^7	5670	7570
	160	8.92×10^6	1.60×10^7	6.50×10^6	1.93×10^7	7320	10240
200	Control	7.18×10^2	1.60×10^4	0.0	1.17×10^5	3486	3508
	120	2.13×10^7	3.75×10^7	6.43×10^6	1.01×10^7	7691	12101
	160	3.11×10^7	4.78×10^7	5.26×10^6	1.13×10^7	8886	21811

*ND - Not determined

The ATP results indicated the biomass fraction in the MLSS of aerobic sludge mass. The ATP values increased during the experimental period (Table 4.2). At time 0 h, when using 100 mg.l^{-1} of phosphate in sterile mixed liquor, the 160 g.l^{-1} of sludge mass concentration had the highest ATP value. After 8 h, the 80 g.l^{-1} of sludge concentration had the highest value of ATP followed by 40 g.l^{-1}, 120 g.l^{-1} and 160 g.l^{-1}, when 100 mg.l^{-1} phosphate was used. The increase in ATP concentrations in all the experiments indicated that bacterial growth occurred during the experimental period. The increase in MLSS values was attributed to the increase in ATP values, reflecting bacterial growth (Table 4.2). The total plate count of aerobic sludge in all the experiments also increased during the experimental period (Table 4.2). The 120 g.l^{-1} of sludge mass had the highest initial TPC values for both the 100 mg.l^{-1} and the 200 mg.l^{-1} experiments at time 0 h. After 8 h, the 80 g.l^{-1} sludge concentration had the highest TPC value for 100 mg.l^{-1} phosphate followed by 160 g.l^{-1}, 120 g.l^{-1} and 40 g.l^{-1} of sludge mass. For mixed liquor containing 200 mg.l^{-1} phosphate, the 160 g.l^{-1} sludge concentration had the highest value of TPC followed by the 120 g.l^{-1} sludge mass. These results are in agreement with the ATP values indicating bacterial growth and thus increasing biomass.

4.4.1.2Experiment 2 (Anaerobic sludge)

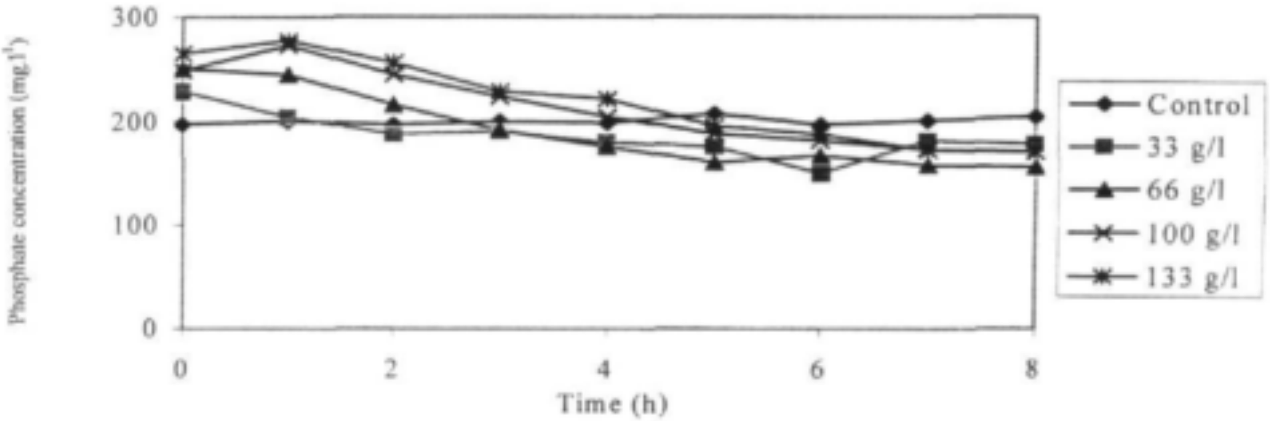

Figure 4.6: Phosphate removal by 33 g.l^{-1}, 66 g.l^{-1}, 100 g.l^{-1} and 133 g.l^{-1} of anaerobic sludge mass in sterile mixed liquor containing 200 mg.l^{-1} P.

The 66 g.l^{-1}of sludge mass removed phosphate (2.08 mg.g^{-1}) better than the 33 g.l^{-1}, (1.62 mg.g^{-1}), 100 g.l^{-1} (0.89 mg.g^{-1}) and the 133 g.l^{-1}(1.07 mg.g^{-1}) (Figure 4.6 and Table 4.3). Similar phosphate removal capacities were observed (as in the aerobic sludge experiment).

Table 4.3: Phosphate removal by 40 g.l^{-1}, 80 g.l^{-1}, 120 g.l^{-1} and 160 g.l^{-1} of anaerobic sludge mass in sterile mixed liquor containing 200 mg.l^{-1} phosphate concentration.

[PO_4^{3-}] (mg.l^{-1})	Sludge mass (g.l^{-1})	PO_4^{3-} (mg.l^{-1})		**PO_4^{3-} removed** (mg.l^{-1})	**PO_4^{3-} removed per g of sludge** (mg.g^{-1})	**PO_4^{3-} removed per initial MLSS** (mg.g^{-1})
		T = 0 h	T = 8 h			
200	Control	197.28	217.8	-20.52	-	1.17
	33	229.00	196.67	32.33	1.62	12.13
	66	250.76	167.33	83.43	2.08	17.50
	100	244.00	190.10	53.90	0.89	8.69
	133	265.27	179.00	86.27	1.07	14.10

Table 4.4: ATP, Total Plate Count and MLSS of anaerobic sludge mass in sterile mixed liquor containing 200 mg.l^{-1} phosphate concentration.

PO_4^{3-} concentration (mg.l^{-1})	Sludge mass (g.l^{-1})	ATP		Total Plate Count (cfu.ml^{-1})		MLSS (mg.l^{-1})	
		T = 0 h	T = 8 h	T = 0 h	T = 8 h	T = 0 h	T = 8 h
200	Control	9.40×10^2	1.00×10^5	0.00	1.74×10^4	1750	2000
	40	2.06×10^7	5.30×10^7	4.90×10^6	3.77×10^6	2666	2866
	80	2.26×10^7	7.33×10^7	1.06×10^6	5.80×10^6	4766	5166
	120	1.60×10^7	6.50×10^7	4.10×10^6	7.73×10^6	6200	7933
	160	1.39×10^7	6.20×10^7	1.99×10^6	2.21×10^7	6116	10133

The ATP and the TPC values of the anaerobic sludge mass indicated an increase in cell numbers during the experimental period (Table 4.4). This indicated an increase in the biomass fraction of the anaerobic sludge mass during the experimental period. The 80 g.l^{-1} of anaerobic sludge mass had the highest value of ATP, followed by the 40 g.l^{-1}, 120 g.l^{-1} and 160 g.l^{-1} concentrations at time 0 h. After 8 h, the 80 g.l^{-1} concentration still had the highest value followed by the 120 g.l^{-1}, 160 g.l^{-1} and 40 g.l^{-1} concentrations. The increase in MLSS was attributed to an increase in ATP and TPC of the anaerobic sludge.

Higher ATP values were observed then those for TPC. This was ascribed to the fact that TPC relies on colony formation. The activated sludge bacterial suspension occurs in flocs, which may contain thousands of individual bacteria. The floc size and distribution in an activated sludge sample will vary and one would expect a variance in the results. On the other hand, ATP relies on chemical extraction and is hence not influenced by floc size or distribution. These results indicated that ATP is a better method than TPC for determining the bacterial numbers in activated sludge.

Jorgensen *et al.*, (1992) determined the biomass of activated sludge growth cultures in terms of dry weight and compared to with ATP content, the oxygen utilization rate and fluorescein hydrolysis data. ATP content showed the best correlation with biomass. ATP was also estimated to suspended solids ratio at maximum viability in activated sludge, it was found that viability varied with mean cell residence time. This showed that ATP is the best and reliable method to estimate bacterial numbers in activated sludge.

4.4.1.3 Experiment 3 (Return sludge)

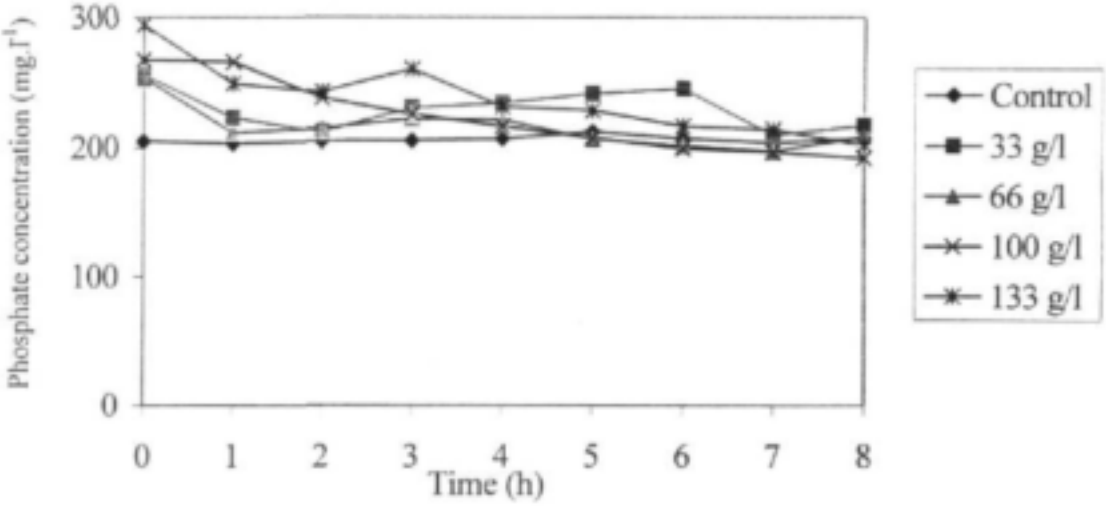

Figure 4.7: **Phosphate removal by 33 g.l^{-1}, 66 g.l^{-1}, 100 g.l^{-1} and 133 g.l^{-1} of return sludge mass in sterile mixed liquor containing 200 mg.l^{-1} P.**

When different quantities (33 g.l^{-1}, 66 g.l^{-1}, 100 g.l^{-1} and 133 g.l^{-1}) of return sludge mass was used, very little phosphate removal was detected (Figure 4.7). No quantitative conclusion could be made by phosphate removal of this sludge mass, hence the actual MLSS of the plant was used in subsequent experiments.

4.4.2 Experiment with MLSS concentrations simulating full-scale MLSS concentrations (experiment 4).

The original samples were chemically and microbiologically analyzed immediately after sampling (Table 4.5). The results indicated low concentrations of phosphate and ammonium. The nitrate concentration was below the lower limit (5 mg.l^{-1}) of the spectrophotometric method. The results also indicated a total plate count of 1.25 x 10^{6} cfu.ml^{-1} (return sludge), 1.89 x 10^6 cfu.ml^{-1} (anaerobic sludge) and 1.45 x 10^6 cfu.ml^{-1} (aerobic sludge). The return sludge had higher MLSS (6447 mg.l^{-1}) and COD (6012 mg.l^{-1}) than the aerobic and anaerobic sludge. The MLVSS of the return sludge was also higher (8117 mg.l^{-1}) than that of the aerobic and anaerobic sludge.

Table 4.5: Chemical and microbiological analyses of the original sludge from Daspoort used in experiment 4.

Sample	[PO_4^{3-}] (mg.l^{-1})	pH	COD (mg.l^{-1})	NH_4^+ (mg.l^{-1})	Total plate count (cfu/ml)	MLSS (mg.l^{-1})	MLVSS (mg.l^{-1})
Influent	6.46	*ND	ND	ND	ND	ND	ND
Effluent	1.33	ND	ND	ND	ND	ND	ND
Return sludge	3.00	7.16	6012	0.60	1.25 x 10^6	6447	8117
Anaerobic sludge	6.90	7.39	2298	9.61	1.89 x 10^6	3053	5307
Aerobic sludge	ND	7.27	2848	0.34	1.45 x 10^6	3073	2263

*ND = Not Determined

Experiment with Aerobic sludge

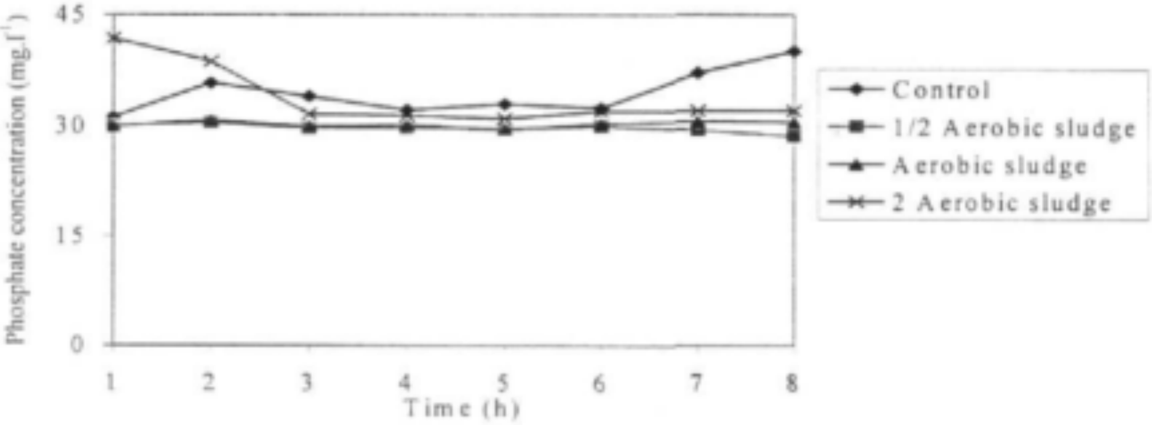

Figure 4.8: Phosphate removal by ½ x aerobic (1045 mg.l^{-1} MLSS), standard aerobic (2190 mg.l^{-1} MLSS) and 2 x aerobic (4430 mg.l^{-1} MLSS) sludge in sterile mixed liquor containing 32 mg.l^{-1} P.

The 2 x aerobic sludge (4430 $mg.l^{-1}$) removed phosphate (0.22 mg $PO_4^{3-}.g^{-1}$ MLSS) better than the ½ x aerobic (0.12 mg PO_4^{-3} g^{-1} MLSS) and the standard aerobic sludge (0.02 mg $PO_4^{3-}.g^{-1}$ MLSS) (Figure 4.8 and Table 4.6).

Experiment with anaerobic sludge

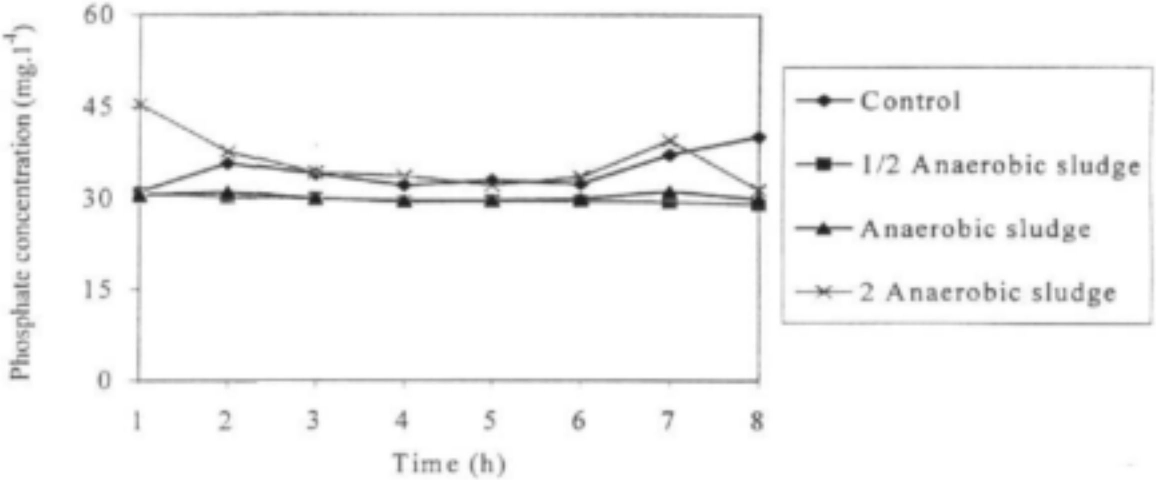

Figure 4.9: Phosphate removal by ½ x anaerobic (1095 $mg.l^{-1}$ MLSS), standard anaerobic (2095 $mg.l^{-1}$ MLSS) and 2 x anaerobic (4240 $mg.l^{-1}$ MLSS) sludge in sterile mixed liquor containing 32 $mg.l^{-1}$ P.

When the actual MLSS of the plant for anaerobic sludge was used, the 2 x anaerobic sludge (4240 $mg.l^{-1}$) removed higher concentration of phosphate (0.33 mg $PO_4^{3-}.g^{-1}$ MLSS) compared to ½ x anaerobic (0.14 mg $PO_4^{3-}.g^{-1}$ MLSS) and standard anaerobic sludge (0.02 mg $PO_4^{3-}.g^{-1}$ MLSS) (Figure 4.9 and Table 4.6).

Experiment with return sludge

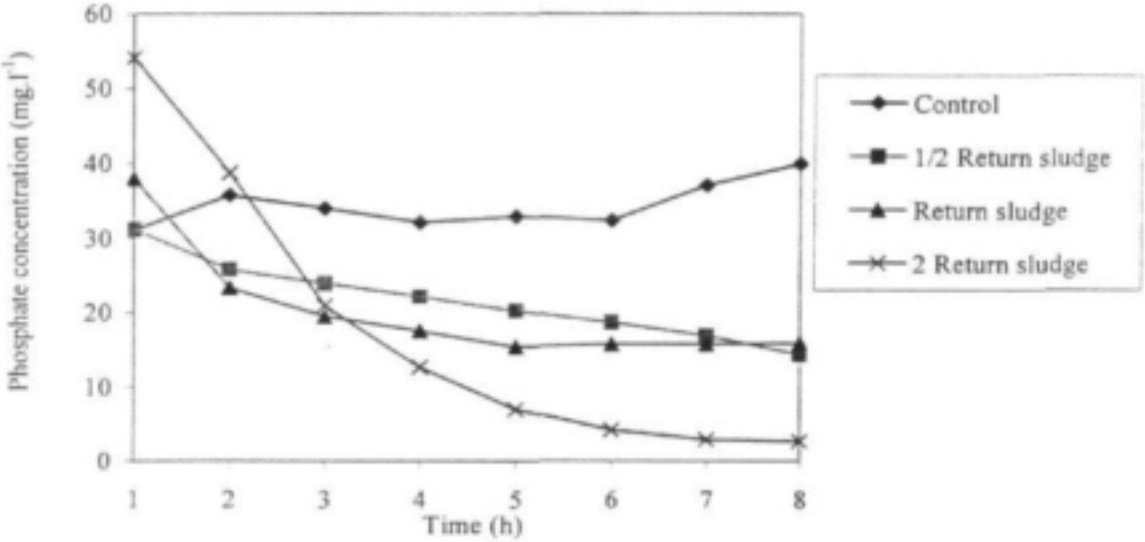

Figure 4.10: Phosphate removal by ½ x return (2590 mg.l^{-1} MLSS), standard return (4790 mg.l^{-1} MLSS) and 2 x return (7840 mg.l^{-1} MLSS) sludge in sterile mixed liquor containing 32 mg.l^{-1} P.

The phosphate removal by the actual MLSS of the plant for the return sludge indicated that the ½ x return (2590 mg.l^{-1}) and the 2 x return (7840 mg.l^{-1}) sludge removed phosphate better than the standard return sludge (Figure 4.10 and Table 4.6). The ½ x return, the standard return and the 2 x return sludge removed 0.69 mg PO_4^{3-}.g^{-1} MLSS, 0.51 mg PO_4^{3-}.g^{-1} MLSS and 0.67 mg PO_4^{3-}.g^{-1} MLSS, respectively.

Table 4.6: Phosphate removed by ½ the actual, the actual and double the actual MLSS of the Daspoort plant inoculated into sterile mixed liquor containing 32 mg.l^{-1} phosphate concentration.

Sample	Mass (g.l^{-1})	[PO$_4^{3-}$] (mg.l^{-1})		PO$_4^{3-}$ removed (mg.l^{-1})	PO$_4^{3-}$ removed per gram sludge (mg.l^{-1})	PO$_4^{3-}$ removed per initial MLVSS (mg.g^{-1})	PO$_4^{3-}$ removed per initial MLSS (mg.l^{-1})
		T = 0 h	T = 7 h				
Control	-	31.10	40.05	-8.95	-	-447.5	0.00
½ x Aerobic sludge	20.00	30.05	28.60	1.45	0.12	1.946	1.387
Standard Aerobic sludge	39.58	29.90	30.45	-0.55	0.02	-0.346	-0.251
2 x Aerobic sludge	75.16	41.75	32.00	9.75	0.22	2.607	2.200
½Anaerobic sludge	21.50	30.80	29.00	1.8	0.14	1.895	1.644
Standard Anaerobic sludge	37.91	30.50	29.85	0.65	0.02	0.445	0.310
2 x Anaerobic sludge	70.33	45.40	31.40	14.00	0.33	4.409	3.302
½ Return sludge	40.33	31.20	14.40	16.8	0.69	8.865	6.486
Standard Return sludge	72.00	38.0	15.95	22.05	0.51	6.602	4.603
2 x Return sludge	128.50	54.15	2.70	51.45	0.67	8.809	6.562

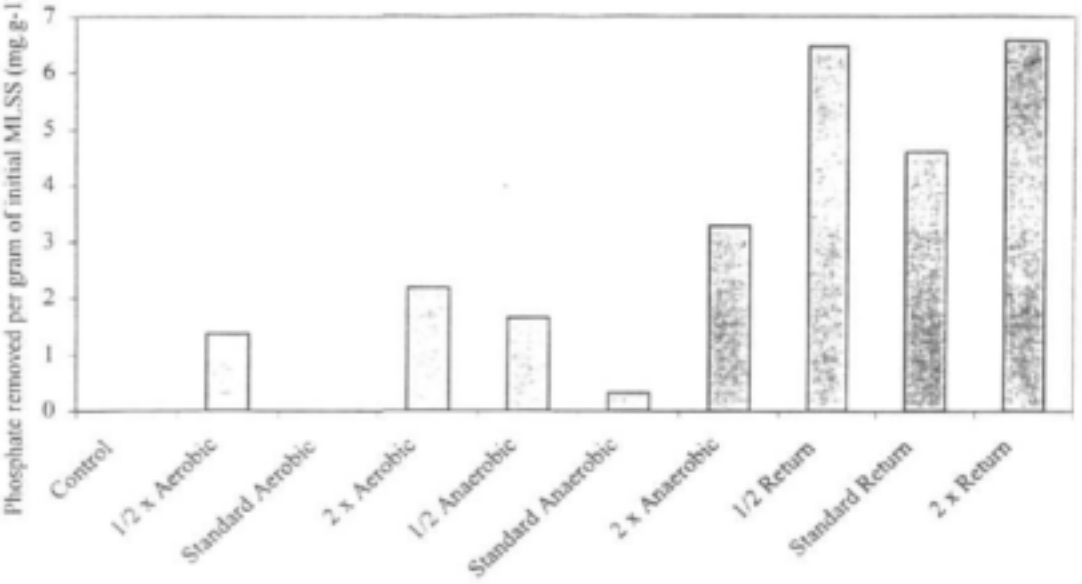

Figure 4.11: Phosphate removed per gram of initial MLSS of aerobic, anaerobic and return sludge in sterile mixed liquor containing 32 mg.l^{-1} P (experiment 4). A negative value of 0.251 mg.g^{-1} was obtained for standard aerobic sludge concentrations (not indicated).

The mixed liquor suspended solids increased with increasing inoculum size and time (Figure 4.11 and Tables 4.2, 4.4 and 4.7) in all the experiments. The return sludge removed the highest concentration of phosphate (6.48 mg $PO_4^{3-}.g^{-1}$for ½ x return, 4.60 mg $PO_4^{3-}.g^{-1}$ for standard return and 6.56 mg $PO_4^{3-}.g^{-1}$ of MLSS for 2 x return sludge) (Table 4.6). The results suggested that the return sludge had a higher biomass than aerobic and anaerobic sludge. These results also indicated that the return sludge removed phosphate better than aerobic and anaerobic sludge.

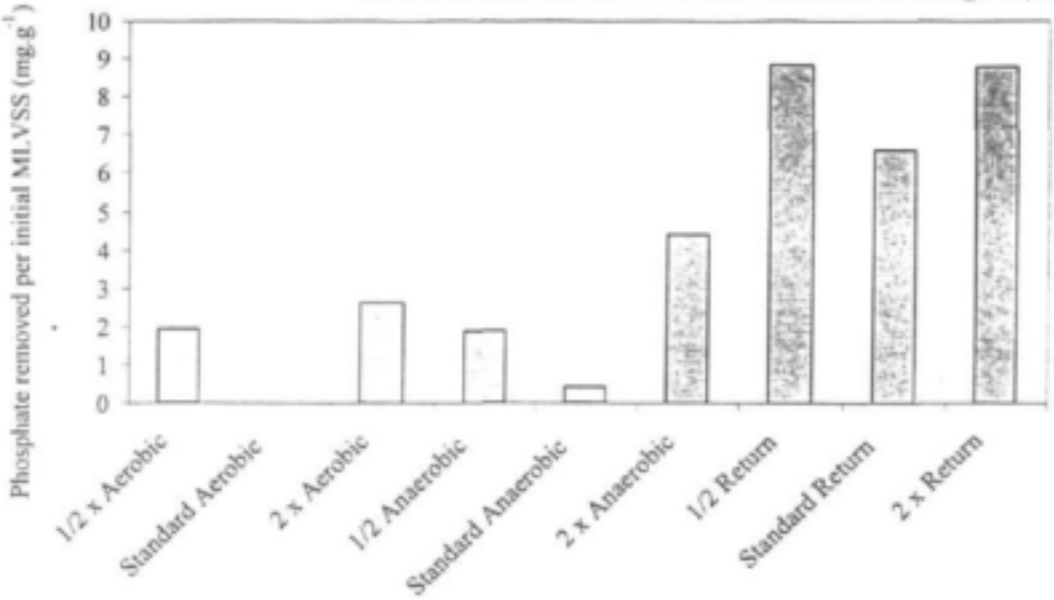

Figure 4.12: Phosphate removed per gram of initial MLVSS of aerobic, anaerobic and return sludge in sterile mixed liquor containing 32 mg.l^{-1} P (experiment 4). A negative value of 0.346 mg.g^{-1} was obtained for standard aerobic sludge concentration (not indicated).

The MLVSS followed the same phosphate removal trend as the MLSS for all the sludges (Figure 4.12). Average phosphate removal calculated in terms of MLVSS indicated that the return sludge removed the highest concentration of phosphate. The ½ x return sludge removed 8.865 mg PO_4^{3-}.g^{-1} of MLSS, standard return removed 6.602 mg PO_4^{3-}.g^{-1} MLSS and the 2 x return removed 8.809 mg PO_4^{3-}.g^{-1} of MLSS (Table 4.6). The return sludge performed better than the aerobic and anaerobic sludges. This was attributed to the larger initial bacterial numbers resulting in higher MLVSS values.

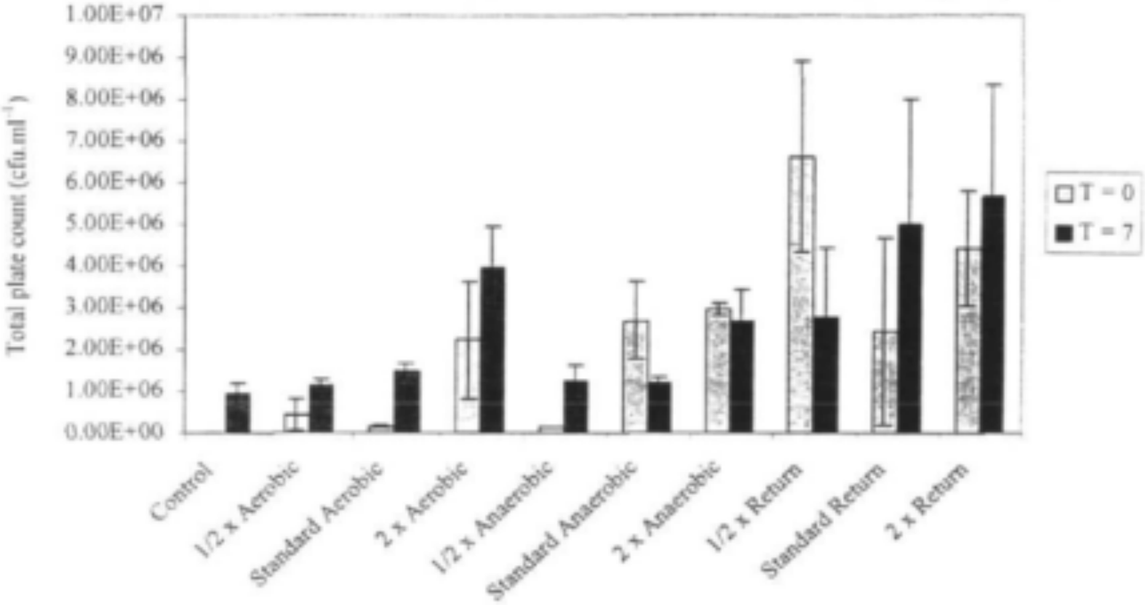

Figure 4.13: **The total plate count of ½ x aerobic (1045 mg.l^{-1} MLSS), standard aerobic (2190 mg.l^{-1} MLSS), 2 x aerobic (4430 mg.l^{-1} MLSS), ½ x anaerobic (1095 mg.l^{-1} MLSS), standard anaerobic (2095 mg.l^{-1} MLSS), 2 x anaerobic (4240 mg.l^{-1} MLSS), ½ x return (2590 mg.l^{-1} MLSS), standard return (4790 mg.l^{-1} MLSS) and 2 x return (7840 mg.l^{-1} MLSS) sludge.**

The TPC of the actual MLSS of the plant increased during the experimental period (Figure 4.13). On average, the return sludge had the highest bacterial numbers followed by the anaerobic and then the aerobic sludge (Table 4.7). The ½ x return sludge had high TPC value (6.60 x 10^6 cfu.ml^{-1}) followed by 2 x return sludge (4.42 x 10^6 cfu.ml^{-1}), 2 x anaerobic sludge (2.9 x 10^6 cfu.ml^{-1}) and the standard anaerobic sludge (2.69 x 10^6 cfu.ml^{-1}) for time 0 h. After 7 h, the ½ x return sludge had a high TPC value followed by 2 x return (5.67 x 10^6 cfu.ml^{-1}), 2 x aerobic (3.97 x 10^6 cfu.ml^{-1}), standard return (5.00 x 10^6 cfu.ml^{-1}) and the 2 x anaerobic (2.65 x 10^6 cfu.ml^{-1}) sludge.

Table 4.7: Total Plate Count, MLSS and MLVSS of aerobic, anaerobic and return sludge inoculated in sterile mixed liquor containing 32 mg.l^{-1} phosphate concentration (experiment 4).

Sample	Biomass (g.l^{-1})	Total Plate Count (cfu.ml^{-1})		MLSS (mg.l^{-1})		MLVSS (mg.l^{-1})	
		T = 0 h	T = 7 h	T = 0 h	T = 7 h	T = 0 h	T=8 h
Control	-	< 10	0.93 x 10^{6}	0.00	-10.00	20	-1590
½ x Aerobic sludge	20.00	4.25 x 10^{5}	1.16 x 10^{6}	1045	1060	745	755
Standard Aerobic sludge	39.58	1.58 x 10^{5}	1.47 x 10^{6}	2190	2095	1590	1620
2 x Aerobic sludge	75.16	2.22 x 10^{6}	3.97 x 10^{6}	4430	4440	3740	3220
½Anaerobic sludge	21.50	1.43 x 10^{5}	1.22 x 10^{6}	1095	1070	950	800
Standard Anaerobic sludge	37.91	2.69 x 10^{6}	1.20 x 10^{6}	2095	2080	1460	1550
2 x Anaerobic sludge	70.33	2.95 x 10^{6}	2.65 x 10^{6}	4240	4415	3175	3085
½ Return sludge	40.33	6.60 x 10^{6}	2.77 x 10^{6}	2590	2745	1895	1860
Standard Return sludge	72.00	2.42 x 10^{6}	5.00 x 10^{6}	4790	5030	3340	3430
2 x Return sludge	128.50	442 x 10^{6}	5.67 x 10^{6}	7840	8540	5840	5575

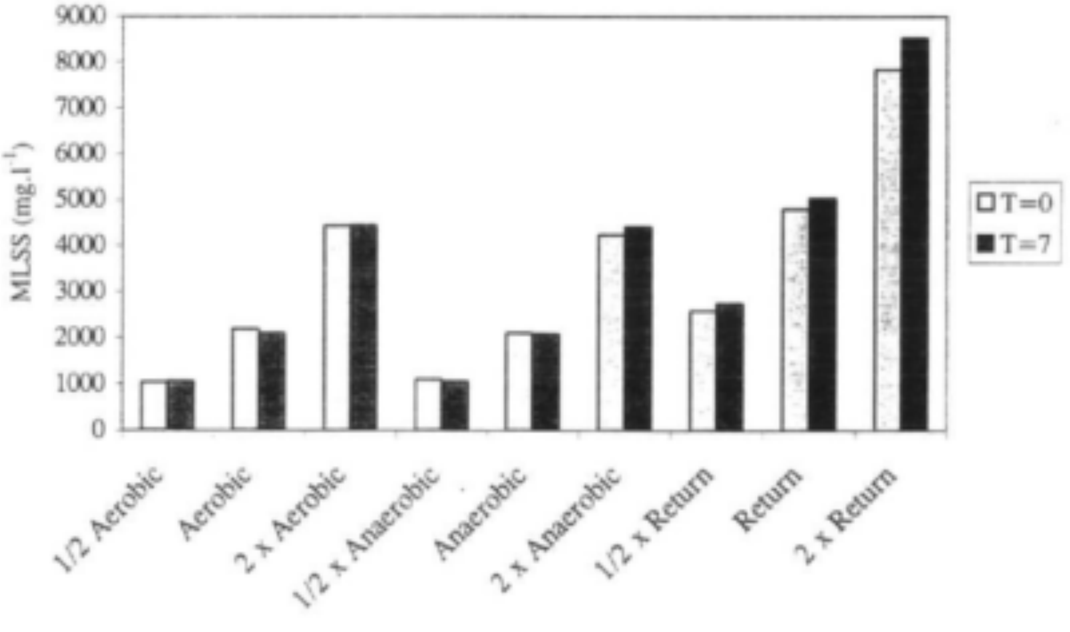

Figure 4.14: Average MLSS values at time 0 h and time 7 h.

The MLSS increased in all the samples except in the standard aerobic sludge and ½ x aerobic sludge (Figure 4.14 and Table 4.7). The 2 x return sludge mass indicated the highest MLSS values (7840 mg.l^{-1} and 8540 mg.l^{-1}) for both time 0 h and time 7 h and similar MLSS values for standard return sludge, 2 x aerobic and the 2 x anaerobic sludge were observed for both time 0 h and 7 h. The increase in MLSS was attributed to an increase in bacterial cell numbers indicated by TPC. The results also indicated that microbial growth occurred during the experimental period.

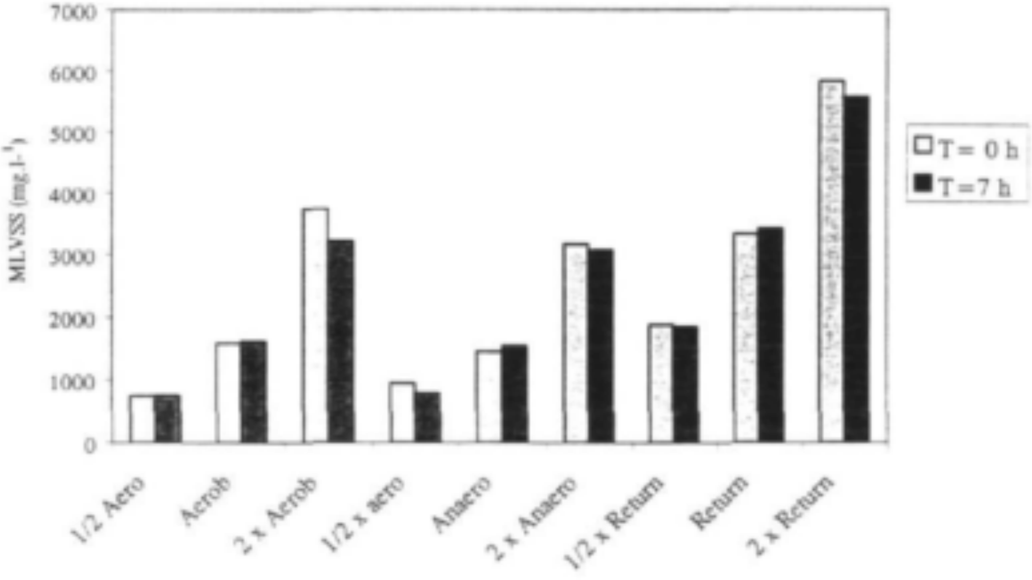

Figure 4.15: Average MLVSS values at time 0 h and time 7 h.

The 2 x return sludge concentration had highest MLVSS values at time 0 h and time 7 h followed by 2 x aerobic and standard return sludge concentrations (Figure 4.15). The results of the MLVSS were lower than those for MLSS (Figure 4.14 and Table 4.7) since it represents the volatile fraction of the MLSS. The increase in MLVSS of the standard aerobic, standard anaerobic and standard return sludge concentrations was attributed to an increase in bacterial cell numbers indicated by the TPC analyses. The MLVSS is a standard parameter of biomass in activated sludge, although it is recognized as an indirect and incomplete measure of the viable sludge floc (Patterson *et al.*, 1970).

Experiment with MLSS concentrations simulating full-scale MLSS concentrations (Experiment 5)

To check the constancy of the MLSS and MLVSS simulation results, a second experiment using the same experimental layout was done three months after the first experiment.

Experiment with aerobic sludge

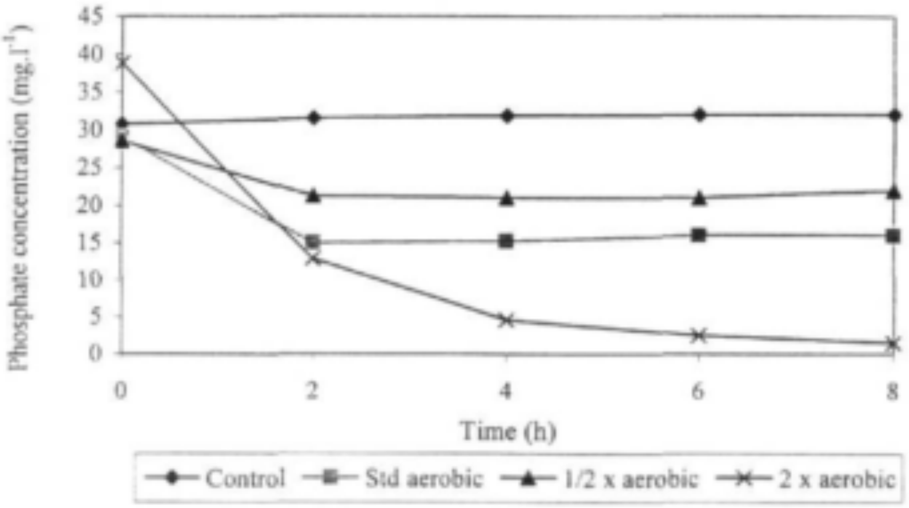

Figure 4.16: Phosphate removal by ½ x aerobic (1140 mg.l^{-1} MLSS), standard aerobic (3230 mg.l^{-1} MLSS) and 2 x aerobic (4030 mg.l^{-1} MLSS) sludge in sterile mixed liquor containing 32 mg.l^{-1} P.

The phosphate removal by the actual MLSS of the plant for the aerobic sludge indicated that 2 x aerobic sludge removed phosphate better (0.40 mg PO_4^{3-}.g^{-1} MLSS) than the standard aerobic (0.21 mg PO_4^{3-}.g^{-1} MLSS) and the ½ x aerobic (0.28 mg PO_4^{3-}.g^{-1} MLSS) sludge (Figure 4.16 and Table 4.8). The 2 x aerobic sludge was most effective after 8 h whereby it removed almost the phosphate in the mixed liquor.

Experiment with anaerobic sludge

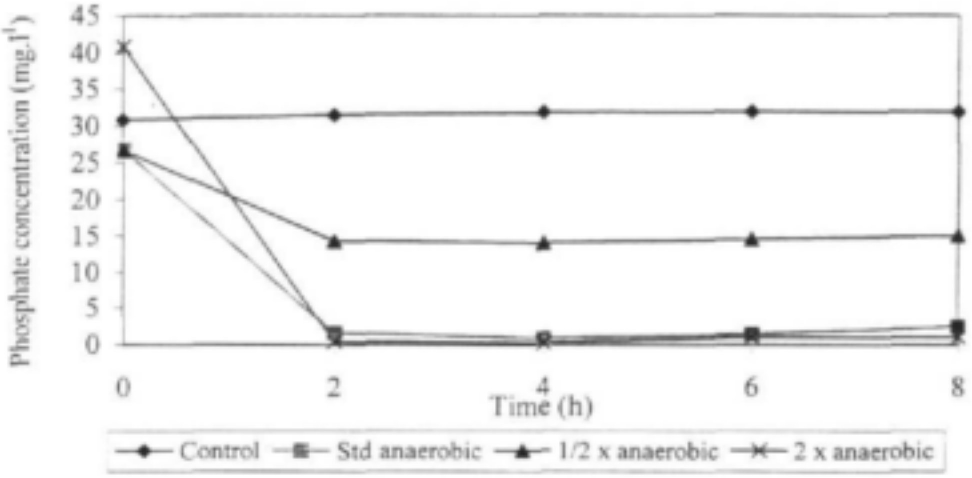

Figure 4.17: Phosphate removal by ½ x anaerobic (1650 mg.l^{-1} MLSS), standard anaerobic (3320 mg.l^{-1} MLSS) and 2 x anaerobic (5600 mg.l^{-1} MLSS) sludge in sterile mixed liquor containing 32 mg.l^{-1} P.

When the actual MLSS of the plant for anaerobic sludge was used, the standard anaerobic and the 2 x anaerobic sludge removed almost all the phosphate in the mixed liquor after 2 h of the experiment, at the same rate (Figure 4.17 and Table 4.8). Both removed 0.41 mg PO_4^{3-}.g^{-1} MLSS compared to the ½ x anaerobic sludge which removed 0.39 mg PO_4^{3-}.g^{-1} MLSS.

Experiment with return sludge

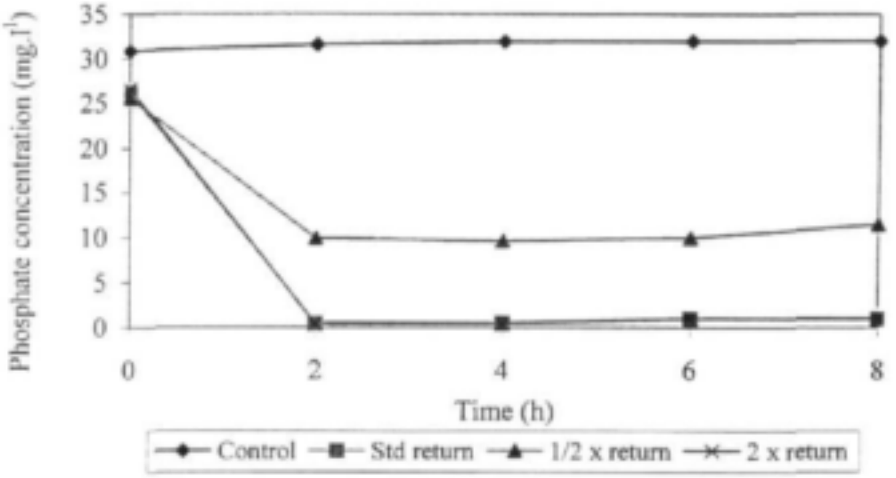

Figure 4.18: Phosphate removal by ½ x return (2430 mg.l^{-1} MLSS), standard return (4010 mg.l^{-1} MLSS) and 2 x return (4800 mg.l^{-1} MLSS) sludge in sterile mixed liquor containing 32 mg.l^{-1} P.

The phosphate removal by the actual MLSS of the plant for the return sludge indicated the same pattern as the anaerobic sludge (Figure 4.18 and Table 4.8). The standard return and the 2 x return sludge removed all the phosphate (0.30 mg PO_4^{3-}.g^{-1} MLSS and 0.20 mg PO_4^{3-}.g^{-1} MLSS) in the mixed liquor after 2 h of the experiment, respectively. The ½ x return sludge removed the least phosphate.

Table 4.8: Phosphate removed by ½ the actual, the actual and double the actual MLSS of the Daspoort plant inoculated into sterile mixed liquor containing 32 mg.l^{-1} P.

Sample	Sludge Mass (g.l^{-1})	[PO$_4^{3-}$] (mg.l^{-1}) T = 0 h	T = 8 h	PO$_4^{3-}$ Removed (mg.l^{-1})	PO$_4^{3-}$ removed per gram of sludge (mg.l^{-1})	PO$_4^{3-}$ removed per initial MLVSS (mg.g^{-1})	PO$_4^{3-}$ removed per initial MLSS (mg.g^{-1})
Control	-	30.80	32.00	-1.2		-12.12	0.00
½ x aerobic sludge	23.50	28.55	22.00	6.55	0.28	5.776	5.771
Std aerobic sludge	60.00	29.10	16.00	13.1	0.21	4.057	4.056
2 x aerobic sludge	92.70	38.85	1.50	37.35	0.40	11.202	11.199
½ x anaerobic sludge	29.50	26.70	15.00	11.7	0.39	7.073	7.069
Std anaerobic sludge	58.70	26.80	2.50	24.3	0.41	7.321	7.319
2 x anaerobic sludge	96.00	40.80	1.00	39.8	0.41	7.112	7.100
½ x return sludge	45.90	25.50	11.50	14.00	0.30	5.756	5.773
Std return sludge	83.70	26.15	1.00	25.15	0.30	6.275	6.271
2 x return sludge	131.50	26.60	1.00	25.6	0.20	5.340	5.344

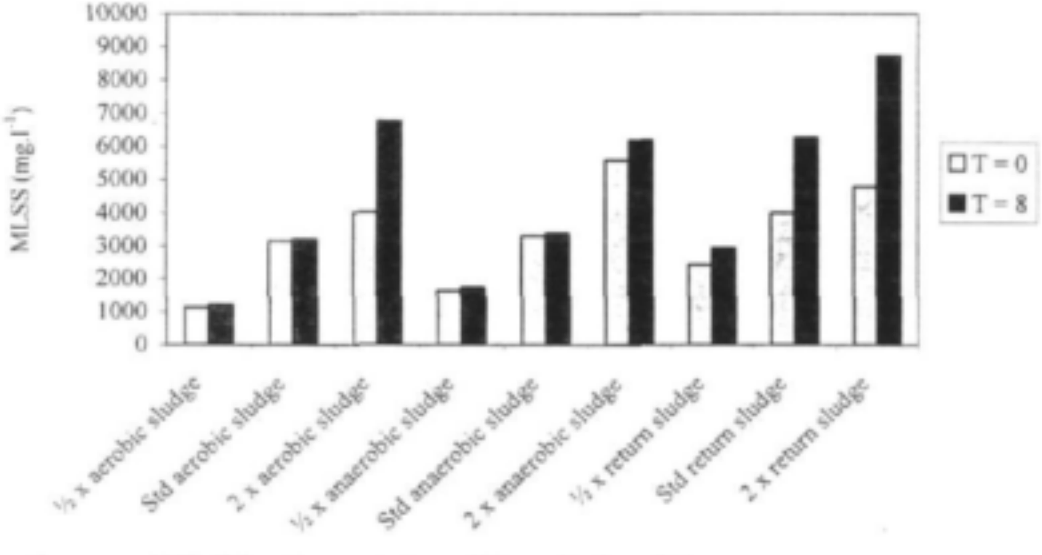

Figure 4.19: Average MLSS values at time 0 h and time 8 h.

The MLSS increased with the increasing inoculum and size in all the sludges (Figure 4.19 and Table 4.9). The 2 x anaerobic sludge indicated the highest MLSS value (5600 mg.l^{-1}) at time 0 h followed by 2 x return (4800 mg.l^{-1}) and standard return (4010 mg.l^{-1}) sludge. After time 8 h, the highest MLSS value was indicated by the 2 x return sludge (8730 mg.l^{-1}) followed by the 2 x aerobic (6760 mg.l^{-1}), standard return (6270 mg.l^{-1}) and 2 x anaerobic (6190 mg.l^{-1}) sludge. The results indicated that microbial growth occurred during the experiment.

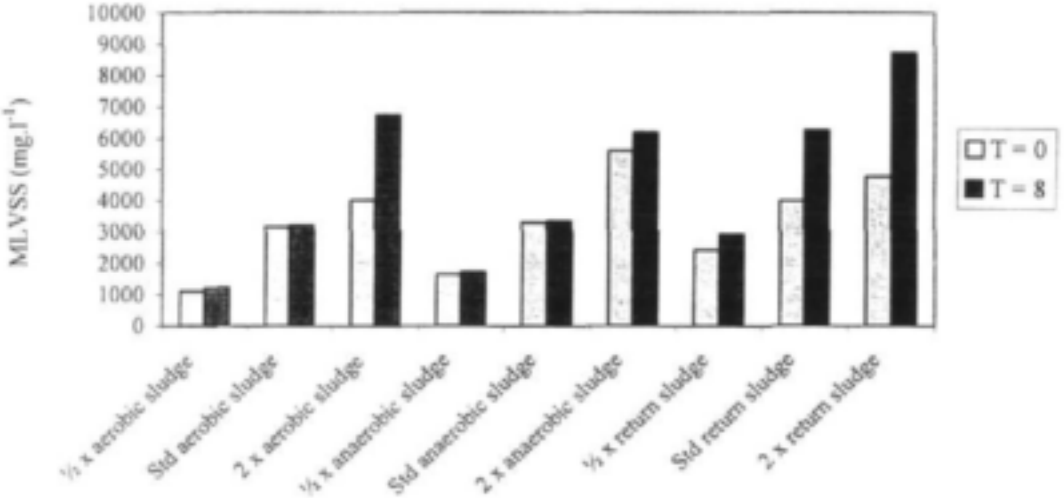

Figure 4.20: Average MLVSS values at time 0 h and time 8 h.

The MLVSS followed the same trend as the MLSS for all the sludges (Figure 4.20). The 2 x anaerobic sludge indicated the highest MLVSS value (5596 mg.l^{-1}) at time 0 h followed by 2 x return (4794 mg.l^{-1}) and standard return (4008 mg.l^{-1}) sludge. After time 8 h, the highest MLVSS value was indicated by the 2 x return sludge (8722 mg.l^{-1}) followed by the 2 x aerobic (6753 mg.l^{-1}), standard return (6263 mg.l^{-1}) and 2 x anaerobic (6188 mg.l^{-1}) sludge. The MLVSS results were lower than those for MLSS (Table 4.9) since it represents the volatile fraction of the MLSS.

Table 4.9: MLSS and MLVSS of aerobic, anaerobic and return sludge inoculated in sterile mixed liquor medium containing 32 mg.l^{-1} phosphate concentration.

Sample	Sludge mass (g.l^{-1})	MLSS (mg.l^{-1})		MLVSS (mg.l^{-1})	
		T = 0 h	T = 8 h	T = 0 h	T = 8 h
Control	-	0.000	20.00	-99	-199
½ x aerobic sludge	23.50	1140	1230	1134	1229
Std aerobic sludge	60.00	3170	3230	3169	3229
2 x aerobic sludge	92.70	4030	6760	4034	6753
½ x anaerobic sludge	29.50	1650	1740	1654	1739
Std anaerobic sludge	58.70	3320	3390	3319	3384
2 x anaerobic sludge	96.00	5600	6190	5596	6188
½ x return sludge	45.90	2430	2950	2432	2949
Std return sludge	83.70	4010	6270	4008	6263
2 x return sludge	131.50	4800	8730	4794	8722

Current research in wastewater treatment has been directed towards mathematical modeling of basic design and operational procedures (Jorgensen *et al.*, 1992). One important parameter in such models is the amount of viable biomass. For this reason, attempts have been made to find methods to determine the biomass in wastewater and activated sludge. The choice of a method is a crucial decision to make. One parameter, which is used to measure biomass in activated sludge is the MLSS. The MLSS is made of heterotrophic active biomass, endegenous residue and inert material. Historically, the MLSS has been measured as a lump parameter via the VSS test (Standard methods, 1985).

The viable counting methods such as the total plate count, can also be used to differentiate between living and dead cells, by assessing their ability to grow either in liquid or solid media (Herbert 1990). The viable counting methods substantially underestimate the true microbial populations presence, since there is no universal growth medium on which microorganisms will grow.

Momba and Cloete (1996) indicated that an increase in biomass of pure cultures resulted in increase in phosphate removal. They proved that the high initial cell concentration of the phosphate accumulating organisms removed more phosphate than low cell concentrations and phosphate uptake was directly related to biomass and high nutrient availability.

4.5 Conclusion

- ATP was a more reliable method for biomass determination in activated sludge than TPC, due to higher bacterial counts.
- An increase in sludge mass resulted in a proportional increase in phosphate removal.
- In the first simulation experiment, the return sludge MLSS removed phosphate better than the aerobic MLSS and anaerobic sludge MLSS.
- In the second simulation experiment, the anaerobic and the return sludges indicated the same trend in phosphate removal and were more effective than the aerobic sludge.
- The MLSS and the MLVSS indicated the same trend in phosphate removal, although the MLVSS was lower since it represents the volatile fraction of the MLSS.

4.6 References

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CHAPTER 5

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

5.1 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 objective of this study was therefore to determine the relationship between the MLSS and MLVSS fractions and phosphorus removal in activated sludge. In experiment 1, 600 ml quantities of sterile mixed liquor containing 219 mg.l^{-1} orthophosphate was inoculated with equal amounts (40 grams) of sludge from five different 3-stage Bardenpho activated sludge systems with similar sludge ages. In experiment 2, 450 ml amounts of sterile mixed liquor growth medium containing 28 mg.l^{-1} orthophosphate was inoculated with sludge amounting to original sludge amounts. Orthophosphate removal was determined hourly over an eight hour experimental period for experiment 1 and a seven hour period for experiment 2. Orthophosphate removal capacities differed amongst systems, while little orthophosphate removal was observed in the control flasks containing no sludge. Orthophosphate uptake differed amongst systems. In experiment 1, the Centurion Wastewater Treatment Plant (WTP) showed, on average, the highest orthophosphate removal capacity (30.79 mg.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.g^{-1} initial MLSS, respectively. For experiment 2, for average orthophosphate uptake in terms of initial MLSS, the Centurion WTP performed best (9.19 mg.g^{-1}), followed by the Baviaanspoort, Daspoort, Rooiwal and Zeekoegat WTPs with uptakes of 7.97, 4.60, 4.55 and 2.82 mg.g^{-1}, respectively. For average orthophosphate uptake calculated in terms of initial MLVSS, the same pattern was observed as that for initial MLSS and followed the same order of systems 13.48, 11.43, 6.28, 5.63 and 3.76 mg.g^{-1}, respectively for Centurion, Baviaanspoort, Daspoort, Rooiwal and Zeekoegat. The different 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*, are not good indicators of biomass in activated sludge. ATP proved to be a more reliable method for indicating biomass concentration than TPC. Although MLSS and MLVSS showed the same trend in orthophosphate removal, initial concentrations of these fractions did not correlate with ATP or TPC, indicating the unsuitability of these fractions as indicators of biomass in activated sludge. In addition, orthophosphate removal was consistently higher in the sludges with higher ATP and TPC values, indicating a relationship between biomass and orthophosphate removal.

5.2 Objectives:

The purpose of these experiments was to use concentrations of MLSS from five similar activated sludge plants and to determine the relationship between MLSS/MLSS and P-removal.

5.3 Materials and methods:

Rationale of experiments 1 and 2

The hypothesis was that P-removal is directly correlated to biomass in activated sludge. The rationale in these experiments was, therefore, that equal quantities of MLSS and/or MLVSS should remove the same quantity of phosphorus.

5.3.1 Experimental design

Two experiments were conducted. In the first (experiment 1), 40g of sludge 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 removed per g of initial MLSS.

In the second experiment (experiment 2), the original sludge MLSS contents and orthophosphate concentrations of the same systems were simulated by inoculating similar sludge concentrations as present in the systems at the time of sampling into sterile mixed liquor growth medium containing 28 $mg.l^{-1}$ orthophosphate. The average orthophosphate removal was expressed as mg removed per g of initial MLSS and MLVSS of the activated sludges.

5.3.2 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. To standardize the experiment, 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 (appendix) and all analyses were performed in triplicate.

5.3.3 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 as described in section 3.3.2.

5.3.4 Physico-chemical analyses:

3) MLSS and MLVSS

MLSS and MLVSS determinations were done as described in section 4.3.6.

4) pH

pH was measured as described in section 4.3.10.

5) Chemical analyses

Chemical analyses (NO_3^-, PO_4^{3-}, SO_4^{2-} and NH_4^+) were done as described in section 3.3.3.

6) COD

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

Table 5.1: Characteristics of the activated sludge plants used in this 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

5.3.5 Preparation of inoculum

For experiment 1, sludges were prepared by centrifugation of the settled sludges as described in section 4.3.3 Forty g sludge from each system was weighed off in triplicate and aseptically transferred to sterile 1 l Erlenmeyer flasks.

For experiment 2, sludges were prepared by centrifuging 900 ml quantities of unsettled (shaken-up) sludges as described in section 4.3.3. The resulting sludge mass was 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.

5.3.6 Preparation of sterile mixed liquor growth medium

Mixed liquor from Daspoort was used as a nutrient media for both experiments (Tables 2 and 3) and was prepared as described in section 3.3.4.1. 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).

5.3.7 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. Soluble COD analyses were done on the filtrate obtained from the MLSS determinations for experiment 1, while 1 ml aliquots were taken from every flask and diluted 1:10 with distilled water for total COD determinations in experiment 2. 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.

Table 5.2: 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

5.4 Results and Discussion:

Experiment 1

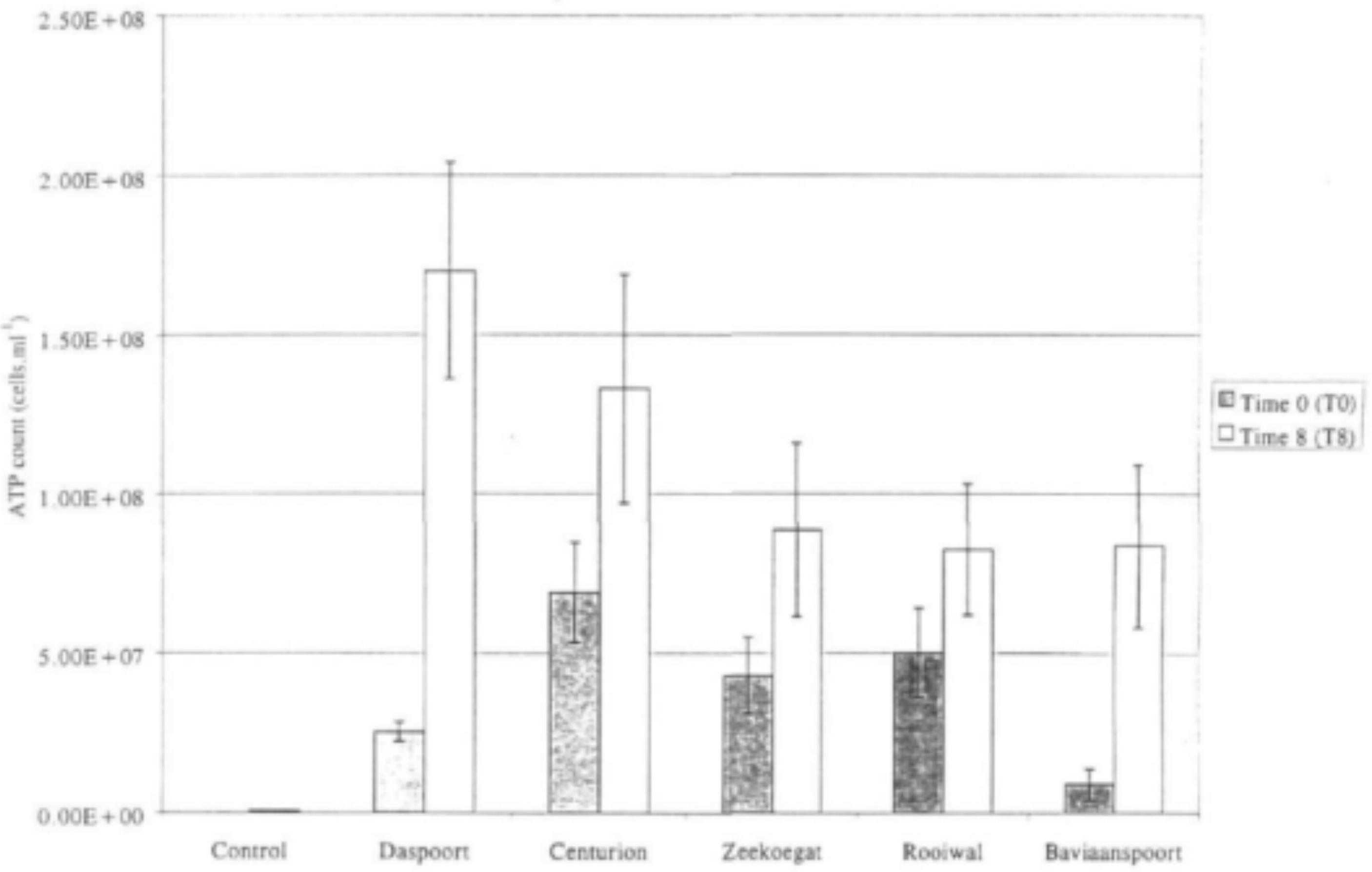

Figure 5.1: Average ATP results at Time 0 and Time 8.

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 5.1). 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 (Figure 5.4) was attributed to the increase in bacterial numbers as indicated by the ATP concentrations. The increase in ATP concentrations indicated that bacterial growth took place during the experimental period.

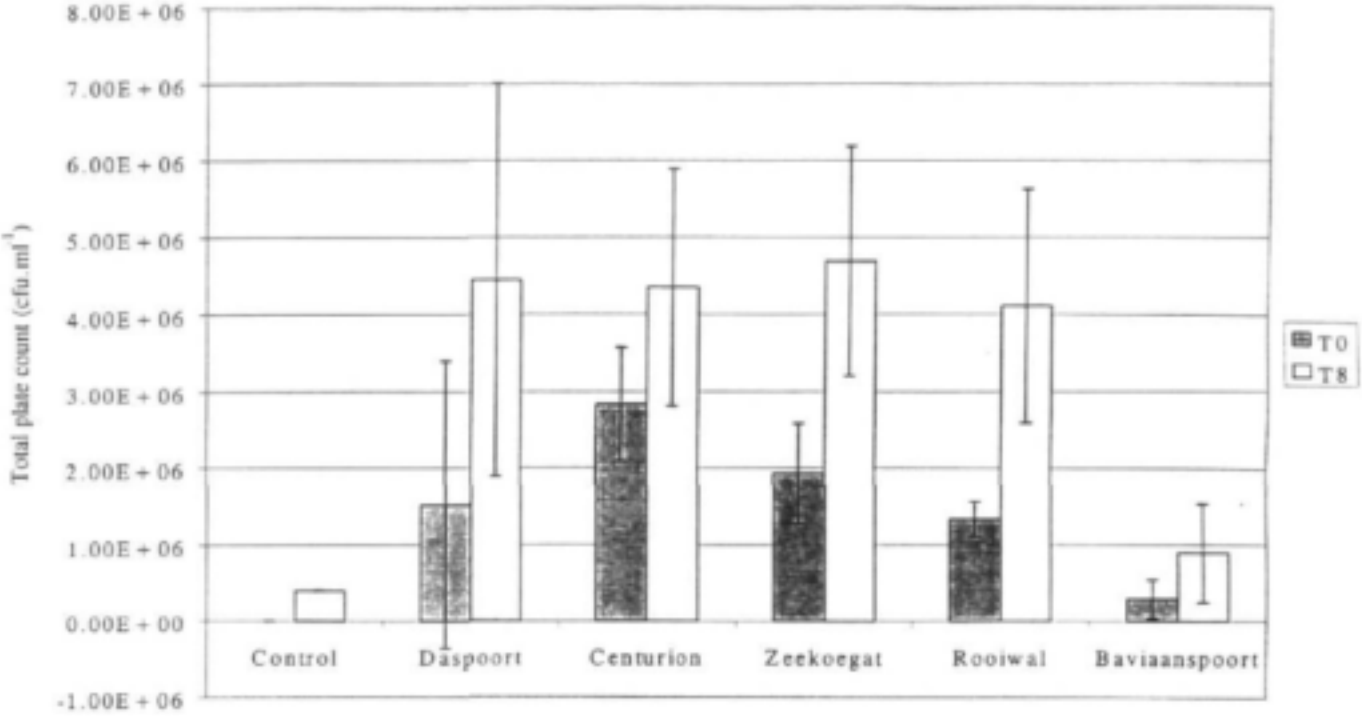

Figure 5.2: Average total plate count (TPC) results at Time 0 and Time 8.

On average, the TPC was the highest for the Centurion sludge, followed by Zeekoegat, Daspoort, Rooiwal and Baviaanspoort (Figure 5.2). The TPC indicated an increase in cell numbers during the experimental period. This was in agreement with the ATP concentrations (Figure 5.1). The standard deviation for the TPC was larger than the standard deviations for ATP analysis (Figure 5.1). 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. 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).

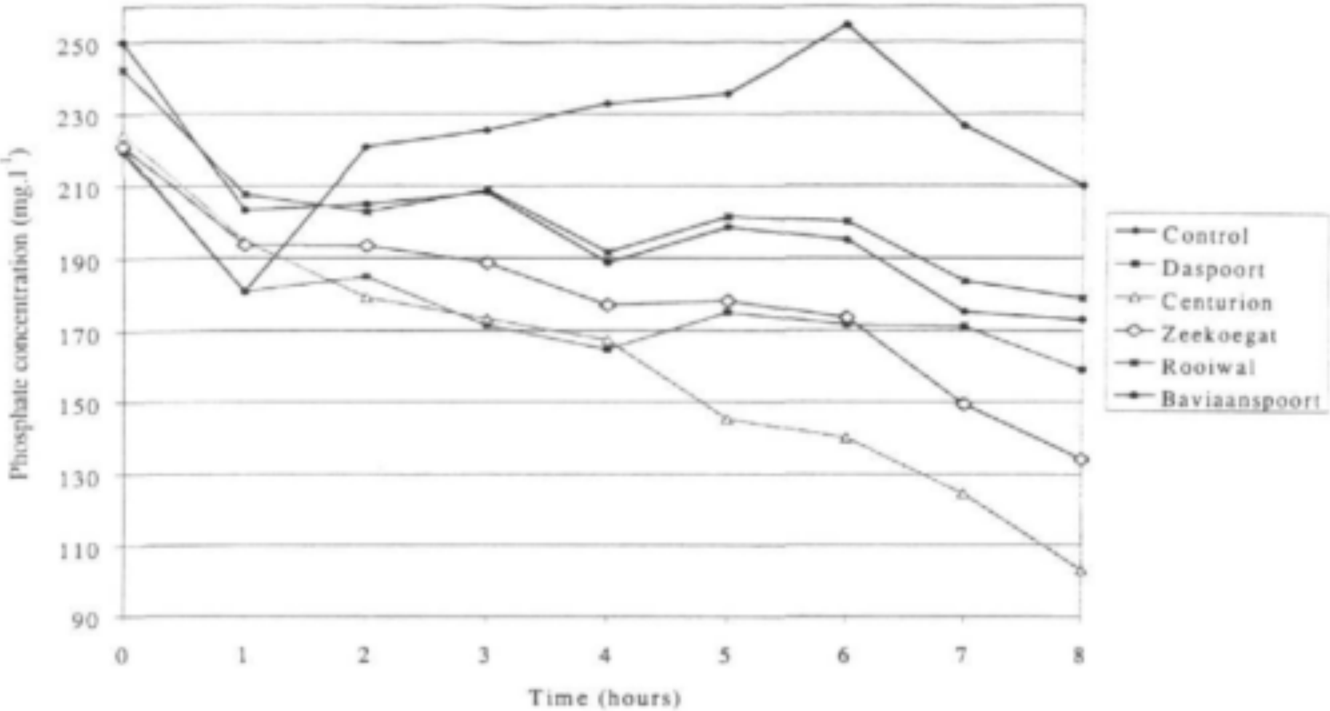

Figure 5.3: **Average orthophosphate uptake from different activated sludge systems for wet sludge (40 g/600 ml).**

When calculated in terms of average orthophosphate removed per gram wet mass, Centurion performed best (3.03 mg.g^{-1}), followed by Zeekoegat, Baviaanspoort, Rooiwal and Daspoort with uptakes of 2.17, 1.93, 1.59 and 1.54 mg.g^{-1}, respectively (Figure 5.3). These values were smaller than those calculated in terms of initial MLSS (Figure 5.5). For example, Centurion had a removal of 30.79 mg.g^{-1} initial MLSS, compared to 3.03 mg.g^{-1} wet sludge. The MLSS mass would be lower than the wet biomass, since it does not include the hydrated fraction and, hence, the higher removal rates in terms of phosphorus expressed as mg.g^{-1} MLSS.

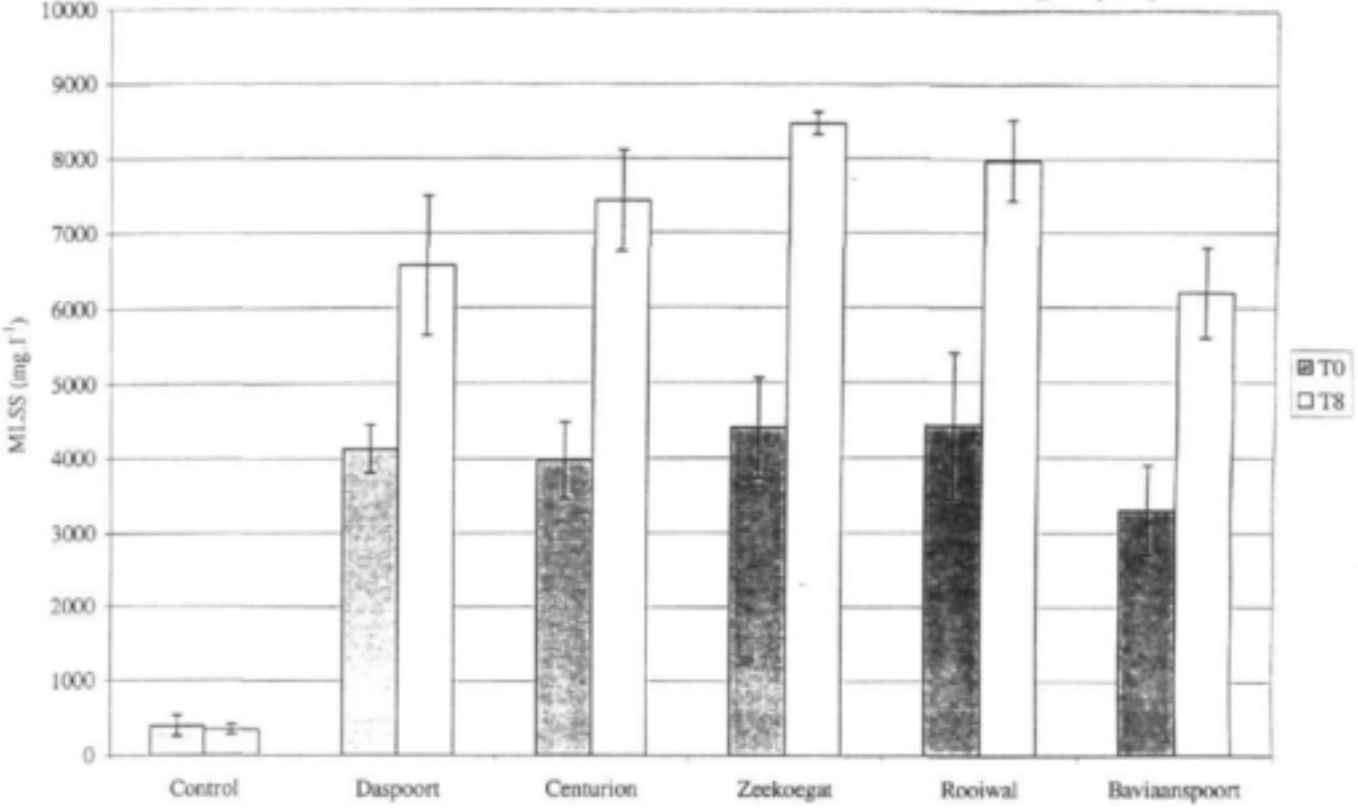

Figure 5.4: Average MLSS values at Time 0 and Time 8 during the phosphate uptake experiment.

MLSS values at time 0 h were similar for all the systems. Values increased for all the systems during the 8 h experiment. The increase in MLSS values was attributed to an increase in viable bacterial cell numbers as indicated by total plate counts and ATP analyses (appendix, Figures 5.1 and 5.2). The control flasks showed an initial MLSS value of 400 mg.l^{-1} at time 0 h.

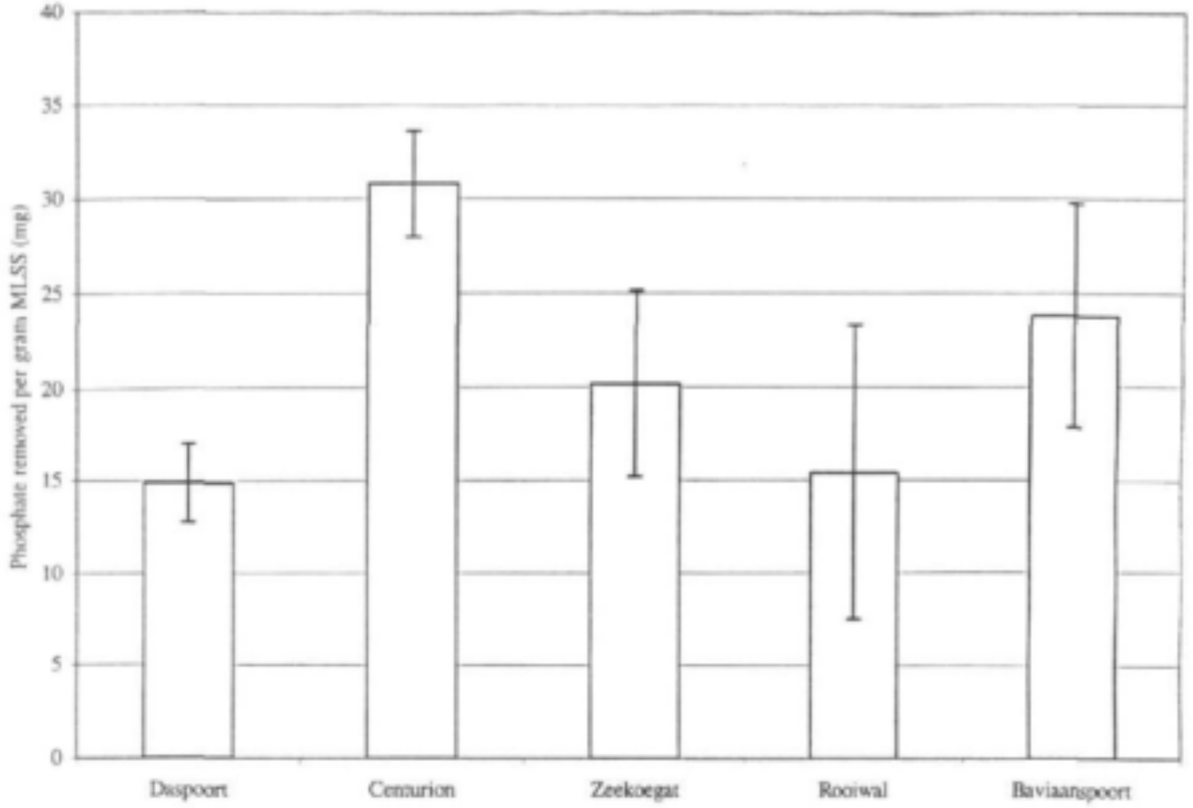

Figure 5.5: **Average orthophosphate uptake (mg) per gram of initial MLSS.**

With the orthophosphate removed calculated in terms of initial MLSS, Centurion performed the best (30.79 $mg.g^{-1}$ initial MLSS) followed by Baviaanspoort, Zeekoegat, Rooiwal and Daspoort with values of 23.78, 20.17, 15.40 and 14.88 $mg.g^{-1}$ initial MLSS, respectively (Figure 5.5). Although the initial MLSS values were similar for all the systems, the quantities of orthophosphate removed 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 different when comparing these systems with Centurion.

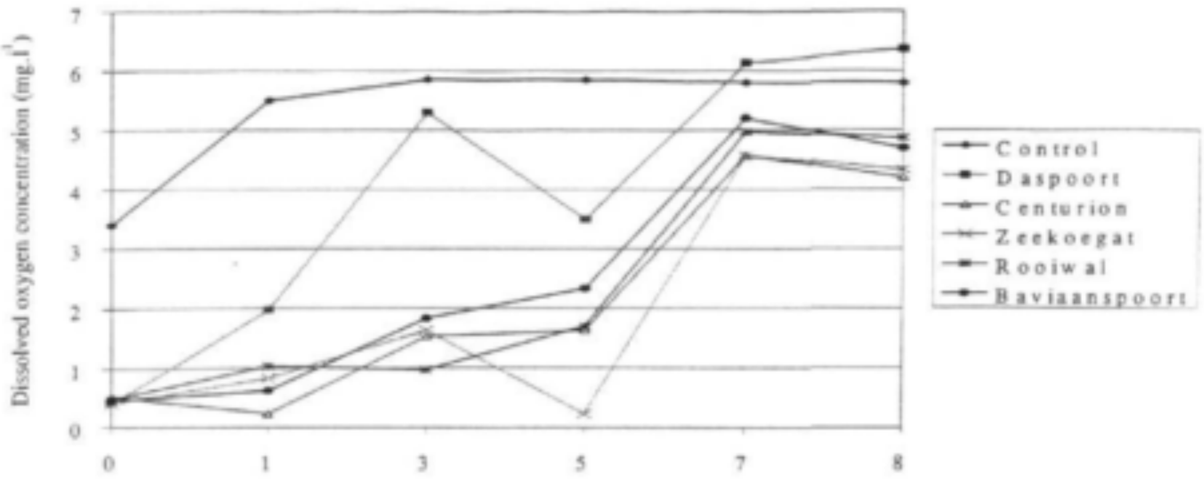

Figure 5.6: Average dissolved oxygen concentration during the eight hour experimental period.

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 5.6). This indicated that aeration conditions in this batch test were optimal for orthophosphate uptake.

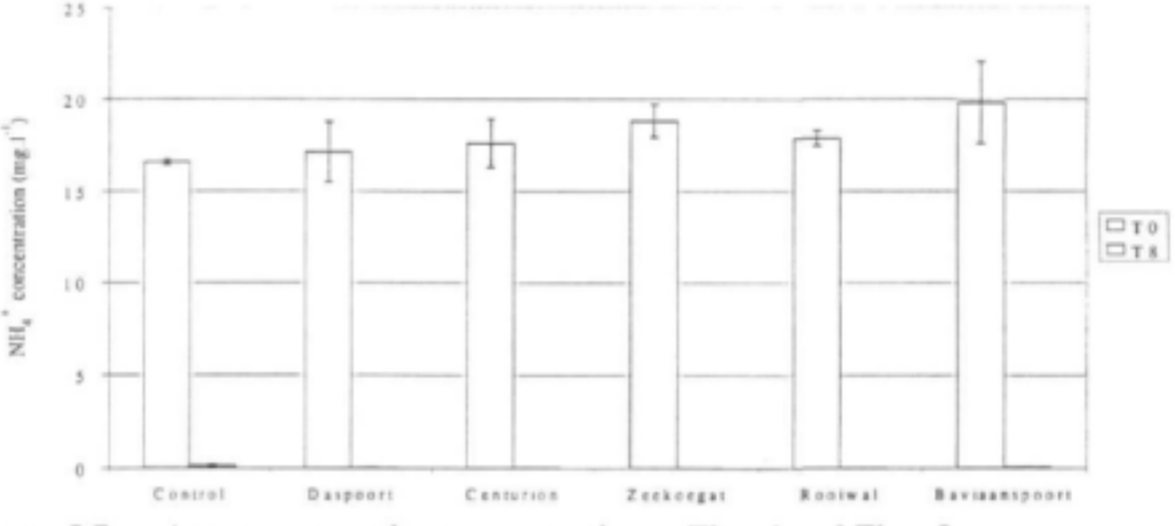

Figure 5.7: Average ammonium concentration at Time 0 and Time 8.

Almost complete nitrification was observed in all the systems (Figure 5.7). This was expected due to the prevailing aerobic conditions, stimulating nitrification, which is an aerobic process.

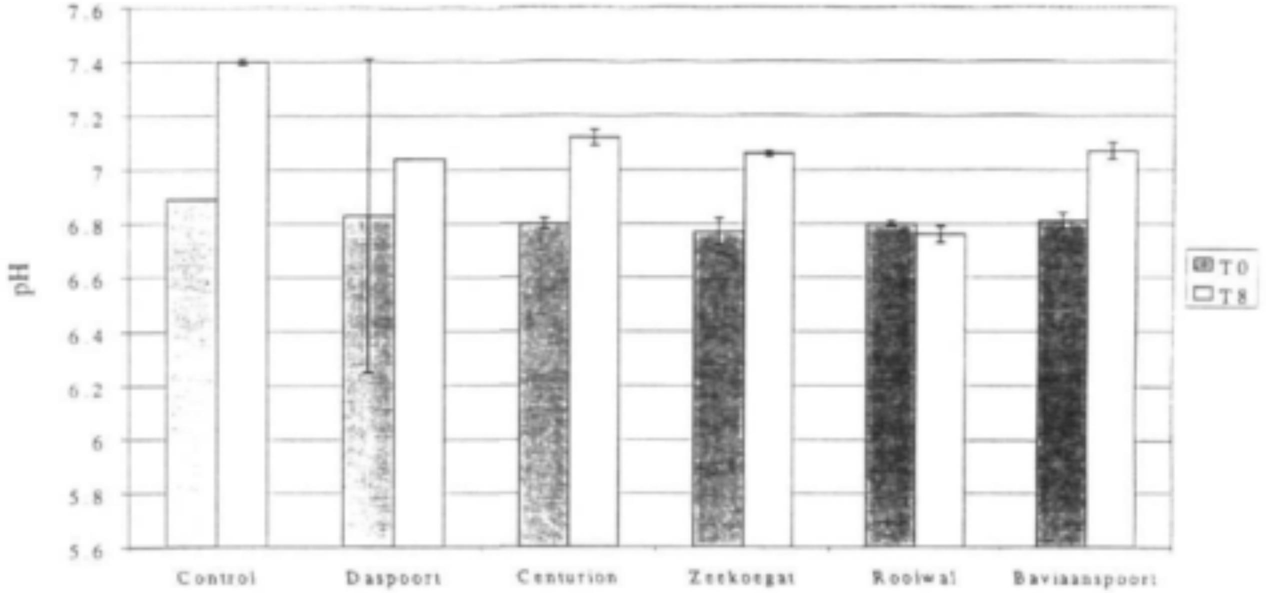

Figure 5.8: **Average pH values at Time 0 and Time 8.**

Values for pH for time 0 h and time 8 h were constant during the experimental period (Figure 5.8) and within the optimal range for orthophosphate removal as reported by Fuhs and Chen (1975).

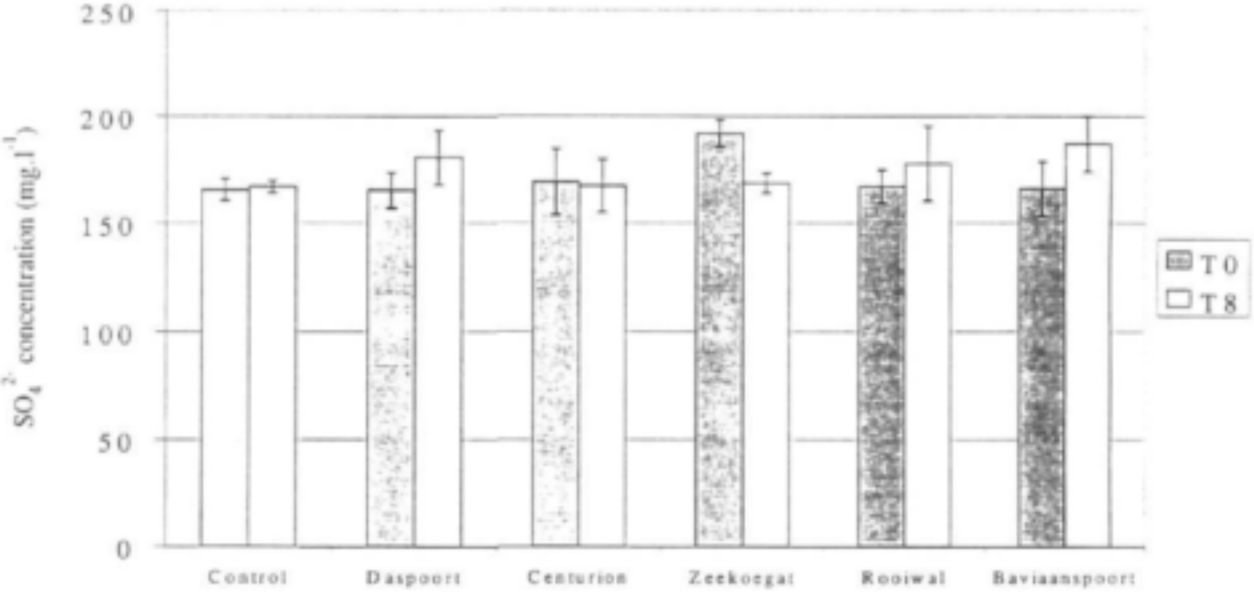

Figure 5.9: **Average sulphate concentration at Time 0 and Time 8.**

Sulphate results were similar at time 0 and time 8 for all the systems (Figure 5.9). This was expected, since aerobic conditions prevailed and, hence, no sulphate reduction would be expected to occur.

Experiment 2

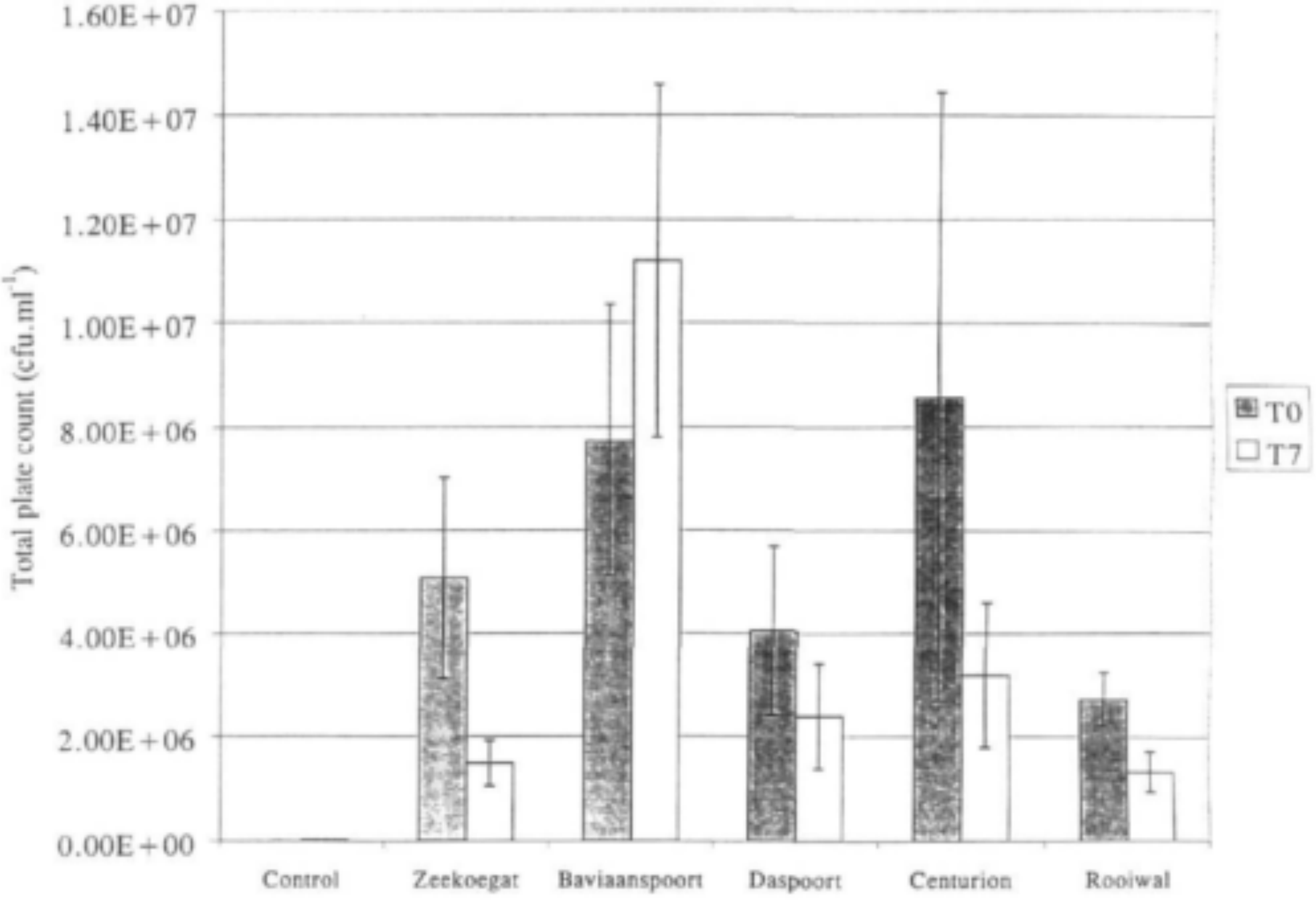

Figure 5.10: **Average total plate count results at Time 0 and Time 7.**

On average, for all the systems except Baviaanspoort, a decrease in cell numbers were observed as determined by total plate counts (Figure 5.10). 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. The large initial cell count might be responsible the good orthophosphate uptake ability of these two sludges (Figure 5.11).

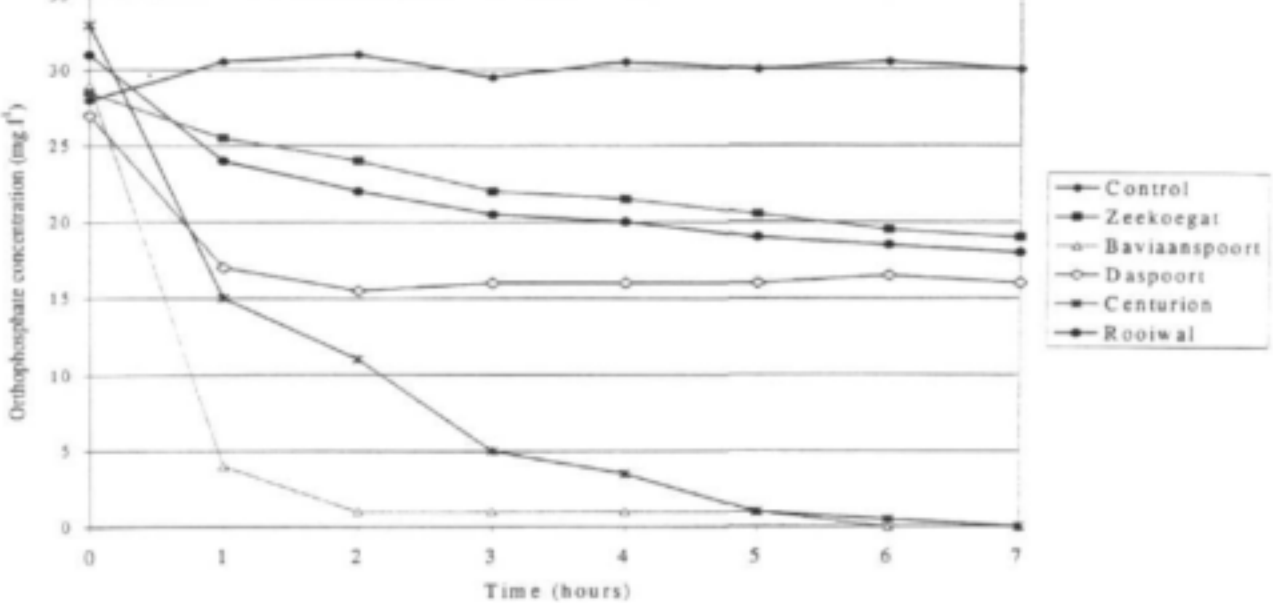

Figure 5.11: Average orthophosphate uptake from different activated sludge systems for sludge re-suspended to the original MLSS concentration.

Baviaanspoort removed all the orthophosphate within 2 h, followed by Centurion, Rooiwal, Daspoort and Zeekoegat (Figure 5.11). 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})(appendix). 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 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 bacterial numbers in these plants as indicated by total plate counts (Figure 5.10) and initial MLSS and MLVSS values (Figures 5.12 and 5.13, respectively).

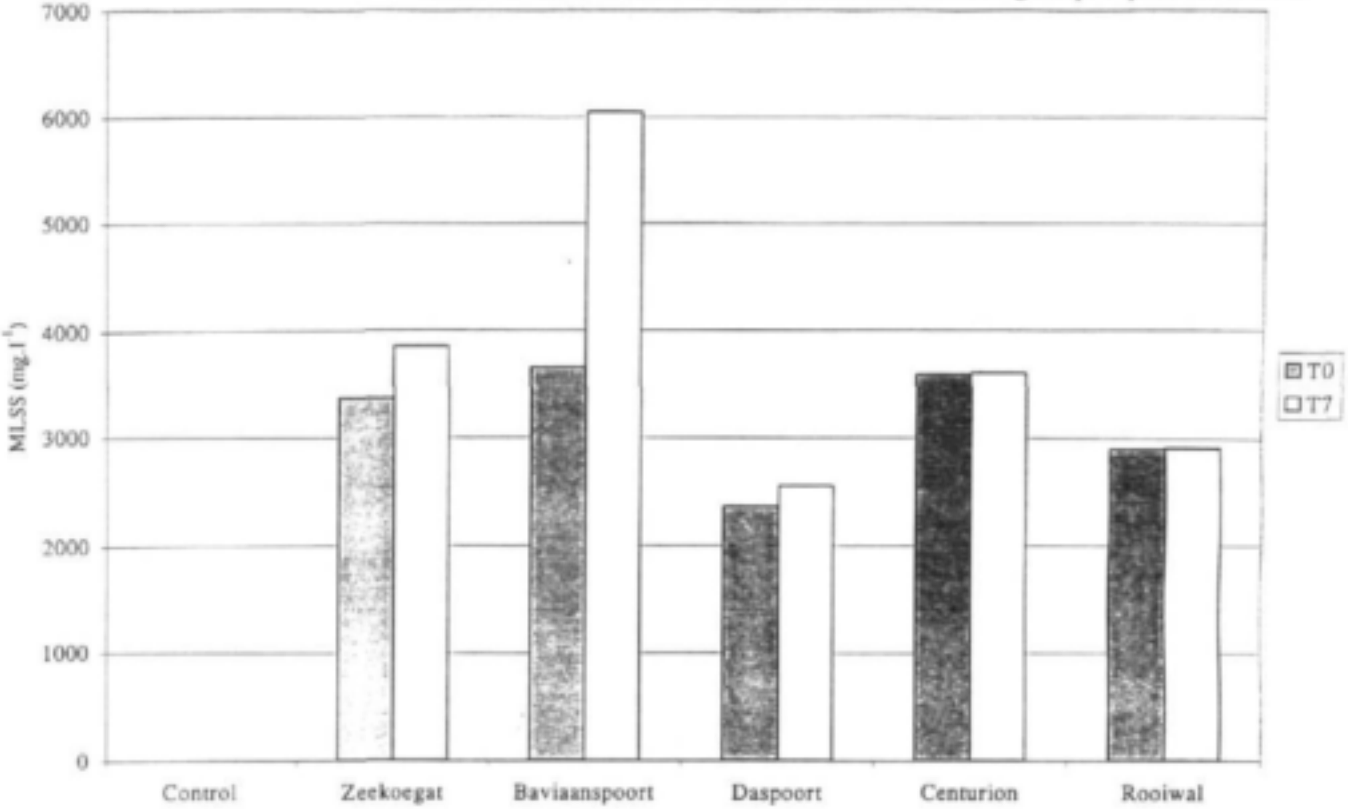

Figure 5.12: Average MLSS values at Time 0 and Time 7.

In the Baviaanspoort sludge, an average increase in MLSS of 2390 mg.l^{-1} was observed (Figure 5.12). This was followed by the Zeekoegat and Daspoort plants, which showed average increases of 480 and 190 mg.l^{-1}, respectively and for both the Centurion and Rooiwal plants an increase of 15 mg.l^{-1} was measured. The increase in MLSS in the Baviaanspoort sludge was attributed to an increase in bacterial cell numbers (Figure 5.10). These results, furthermore, indicate that no microbial growth occurred in the other systems over the 7 h experimental period.

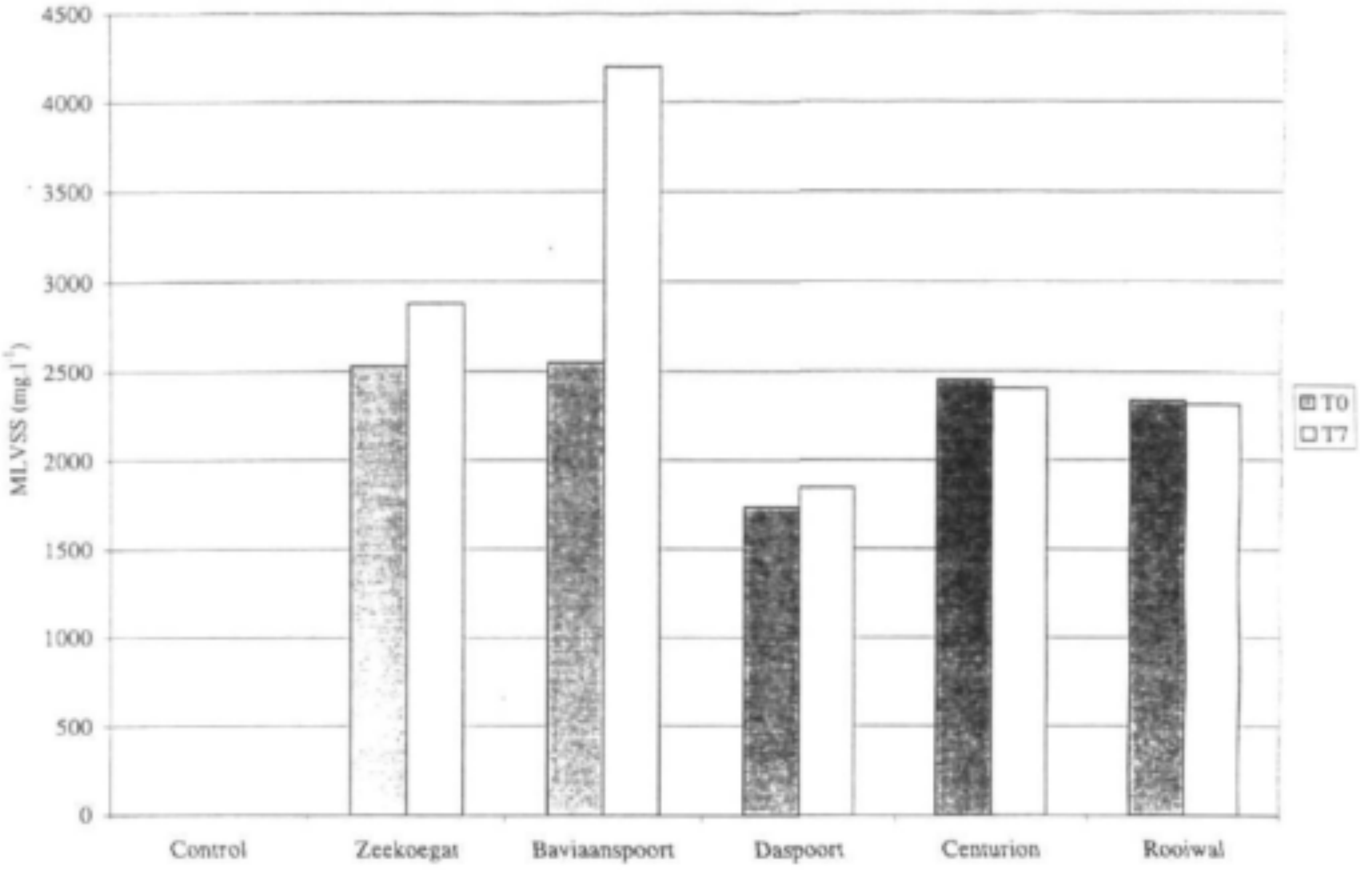

Figure 5.13: Average MLVSS values at Time 0 and Time 7.

The MLVSS values of all the systems remained constant during the experiment, except for Baviaanspoort where an increase of 1650 mg.l^{-1} was observed (Figure 5.13). 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. The results were similar to results obtained for MLSS (Figure 5.12). Again, the increase in MLVSS for Baviaanspoort was attributed to an increase in bacterial cell numbers and, hence, biomass.

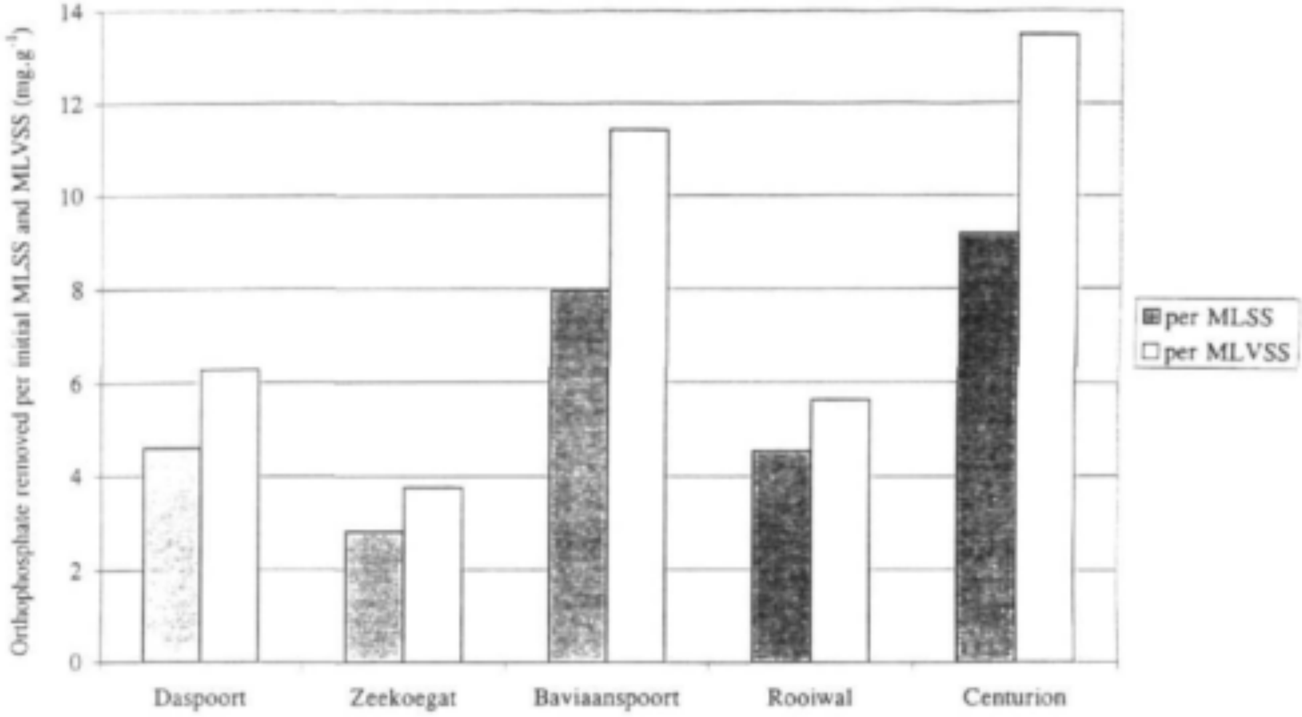

Figure 5.14: Average orthophosphate uptake (mg) per gram of initial MLSS and MLVSS.

MLVSS values were, as expected, always lower than the MLSS, since it is the volatile fraction of the MLSS (Figures 5.12 and 5.13). Nevertheless, the MLVSS followed the same orthophosphate uptake trend as the MLSS for all the sludges (Figure 5.14). Average orthophosphate removed calculated in terms of initial MLSS indicated that Centurion performed best (9.19 $mg.g^{-1}$) followed by Baviaanspoort, Daspoort, Rooiwal and Zeekoegat with removal of 7.97, 4.60, 4.55 and 2.82 $mg.g^{-1}$, 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 $mg.g^{-1}$, 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, resulting in the higher initial MLSS and MLVSS values (Figures 5.12 and 5.13). The Baviaanspoort and Centurion systems removed all the orthophosphate within 2 h and 5h, respectively (Figure 5.11). 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.

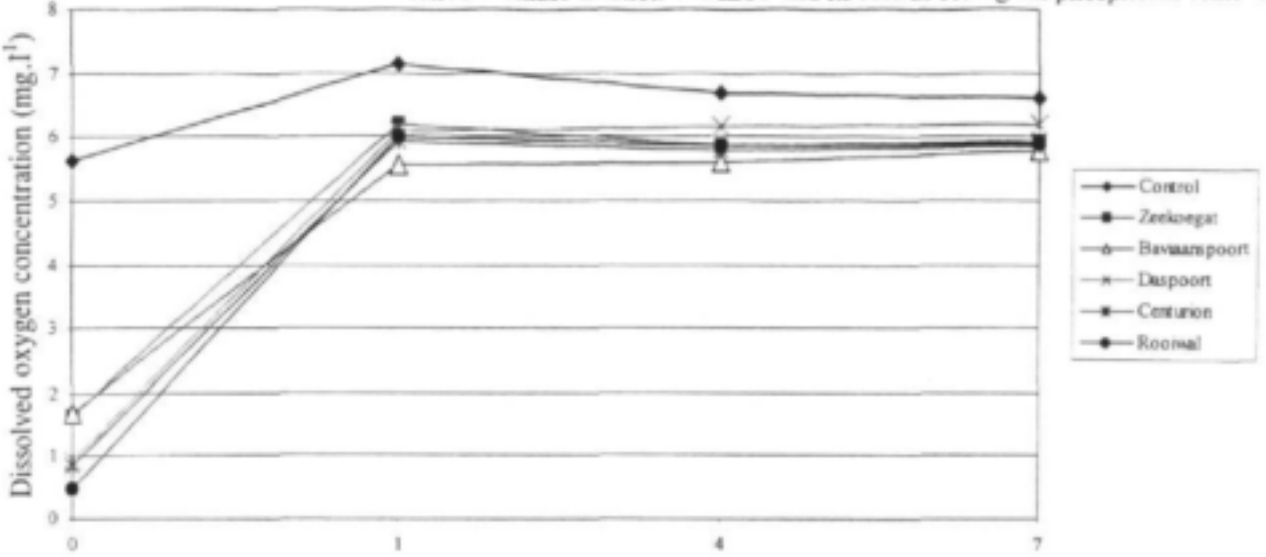

Figure 5.15: Average dissolved oxygen concentrations during the 7 h experimental period.

The dissolved oxygen concentrations were above 1 mg.l^{-1} from time 1 h onwards, indicating optimal conditions for orthophosphate uptake (Figure 5.15).

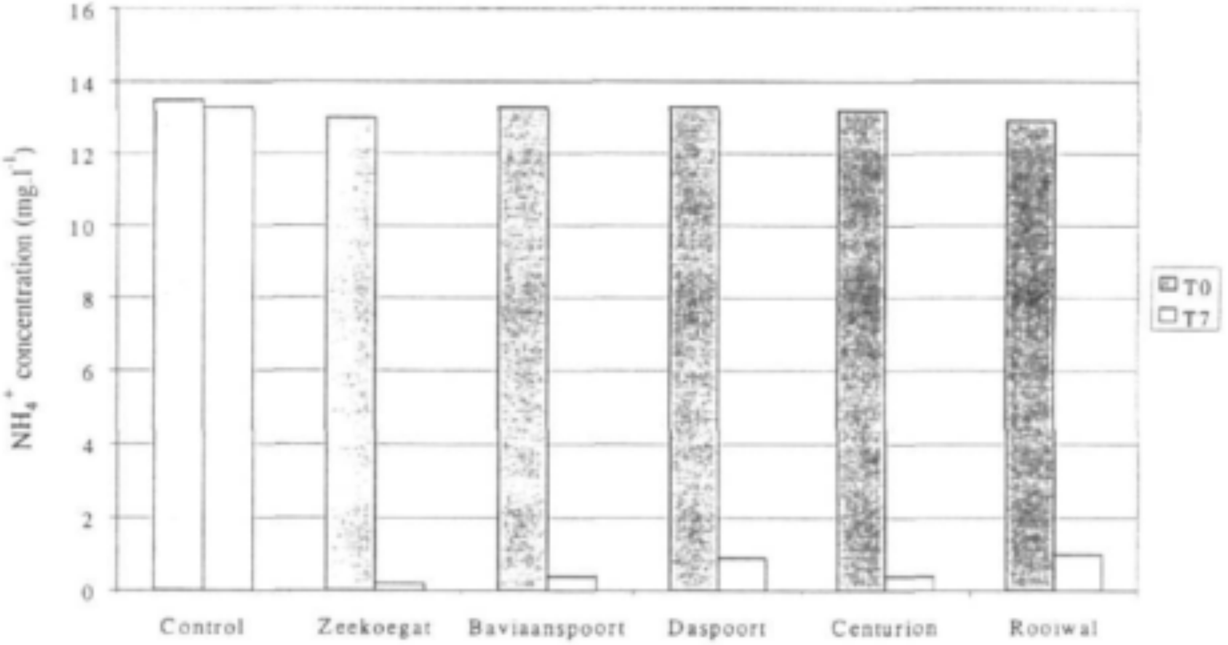

Figure 5.16: Average ammonium concentration at Time 0 and Time 7.

As with experiment 1, almost complete nitrification was observed in all the systems (Figure 5.16).

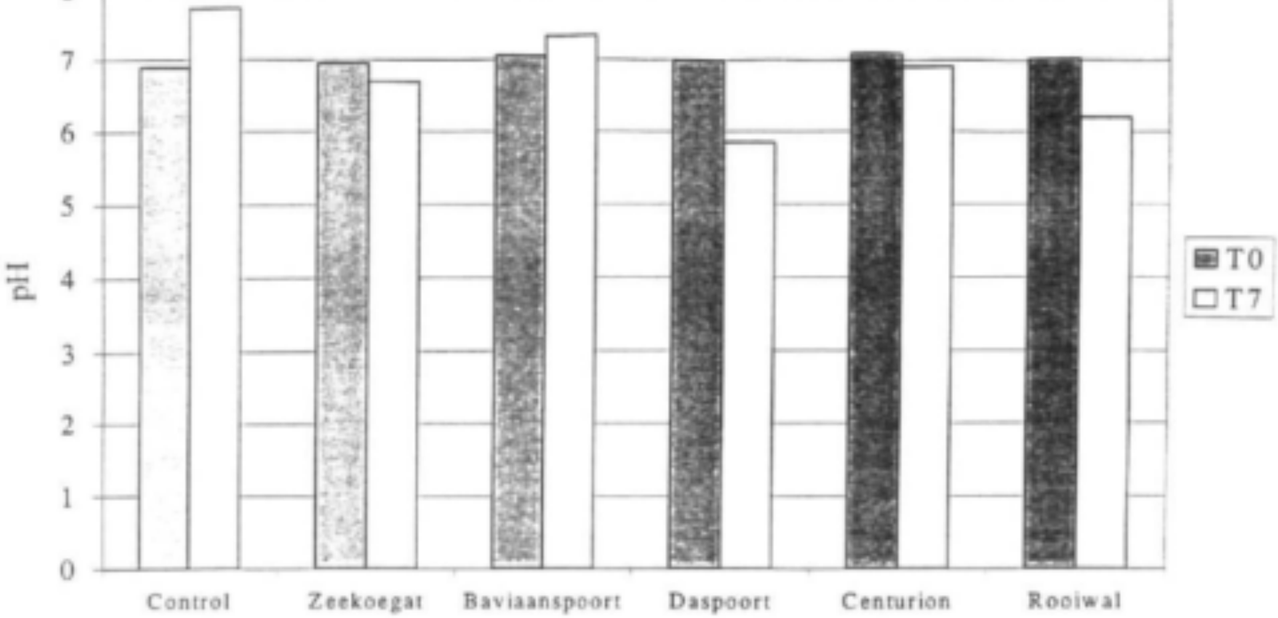

Figure 5.17: Average pH values at Time 0 and Time 7.

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 5.17).

5.5 General discussion

Biomass determination

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 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.

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.

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, ATP analysis, 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).

Chuang and Ouyang (2000) estimated 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 laboratory scale system studied for municipal wastewater treatment. In this system, 47% of the phosphorus was removed as polyphosphate.

In terms of design procedures and kinetic models, the bioreactor of non-nitrifying aerobic activated sludge systems 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 non-biodegradable particulate organics, which on entry into the bioreactor, are enmeshed in the mixed liquor organic suspended solids (Ubisi *et al.*, 1997).

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.

Previous research has indicated that phosphate uptake was related to an increase in biomass concentration (Streichan *et al.*, 1990; Bosch, 1992). 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}. These findings were supported by the current research, although indicating that not only the amount of MLSS (or the MLVSS fraction thereof) is important in orthophosphate removal, but also initial bacterial numbers (as determined by means of ATP and TPC) constituting these fractions.

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).

Theoretically, a colony is formed by a single bacterial cell. However, bacteria do not occur as single bacteria in nature, but as clusters, chains or micro-colonies (Figure 5.18). Hence, when doing a total plate count on agar, these micro-colonies, which may contain numerous bacteria, will give rise to a macro-colony, referred to as a colony-forming unit. A colony forming unit (cfu) does therefore not represent a single bacterial cell, as in theory, but thousands of cells occuring in clusters and hence, the underestimation of true bacterial numbers, when using culture-based techniques. On the other hand, ATP relies on chemical extraction and is, hence, not influenced by cluster formation.

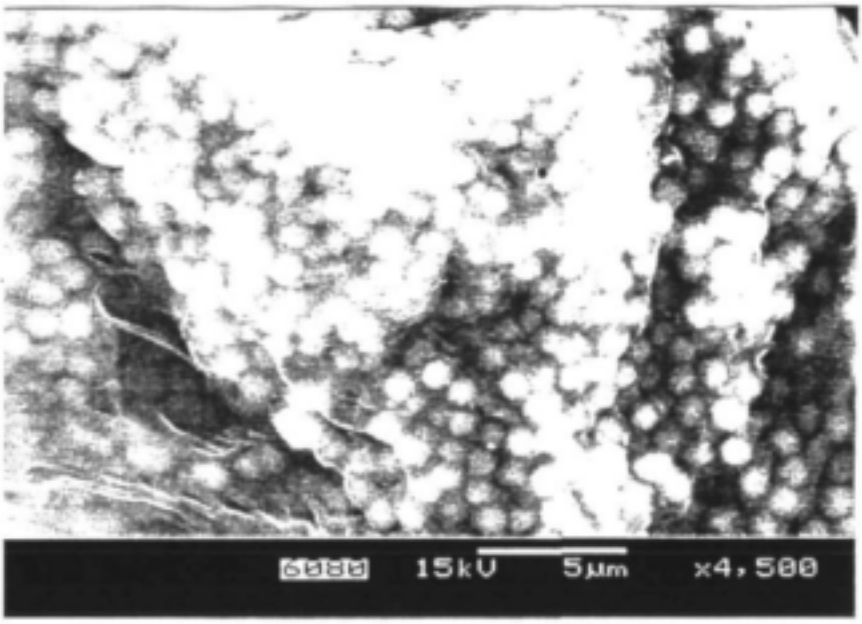

Figure 5.18: Electron micrograph indicating the spatial distribution of cell clusters (colony forming units) in activated sludge.

Biomass determination in activated sludge by means 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.

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 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.

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.

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.

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). Studies have indicated a constant pool of ATP for *Escherichia coli*, *Pseudomonas fluorescens* and *Bacillus subtilis*, which was also constant during all growth phases (Patterson *et al.*, 1970). Another study indicated a mean endogenous ATP pool of 2.1 μg per mg dry cell material for 13 species of Gram positive and Gram negative aerobic bacteria (Patterson *et al.*, 1970). 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, it was indicated that only 15 to 20% of the MLVSS may be active biomass under actual operating conditions.

In this study, by means of two independent experiments, differences in orthophosphate uptake ability of different activated sludges treated in exactly the same way were observed.

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.

5.6 Conclusions:

- Despite similar operating conditions and design of the five activated sludge systems used in this study, in terms of both experiments, differences in orthophosphate removal could be observed as expressed per unit MLSS and MLVSS.
- 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.
- 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, nor MLVSS concentrations correlated with ATP and/or TPC, indicating that MLSS and MLVSS were not good indicators of biomass in activated sludge.
- Orthophosphate removal was consistently higher in the sludges with higher ATP and TPC values, indicating a relationship between biomass and orthophosphate removal.

5.7 Acknowledgements:

The authors would like to thank 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.

5.8 References:

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CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

6.1.1 DETERMINATION OF THE EFFECT OF BIOAUGMENTATION ON PHOSPHORUS REMOVAL IN LABORATORY EXPERIMENTS BY ADDING A COMMERCIALLY AVAILABLE BIOAUGMENTATION PRODUCT

The bioaugmentation products contained a high initial phosphate concentration which caused an increase in the phosphate concentration of the mixed liquor in all the experiments. Four grams and five grams of bioaugmentation product per 500 ml of mixed liquor resulted in 17.90 $mg.l^{-1}$ and 18.86 $mg.l^{-1}$ phosphate removal per gram of bioaugmentation product added, respectively. At inoculum quantities exceeding 40 g per 500 ml of mixed liquor, the phosphorus concentration increased during the experimental period.

From an economical point of view, bioaugmentation products will be too expensive to increase the biomass of a full scale activated sludge plant, because our results indicated that between 8 kg - 10 kg of product per 1 000 litre will be required.

When trying to culture bioaugmentation product B and anaerobic sludge as reserve biomass, no growth occurred using sterile mixed liquor and sterile mixed liquor with added nutrients. The results indicated that there were not enough nutrients in the sterile mixed liquor to support the growth of the microorganisms of the bioaugmentation product and anaerobic sludge, or it was not suitable for their growth. In order for this approach to be successful, alternative inexpensive substrates have to be evaluated in order to increase the biomass.

6.1.2 DETERMINATION OF THE RELATIONSHIP BETWEEN BIOMASS AND PHOSPHATE REMOVAL IN DIFFERENT ACTIVATED SLUDGE SYSTEMS

For all sludge mass experiments employing activated sludge from a single system, an increase in sludge mass led to an increase in P removal. When calculating the quantity of P removal per g of sludge mass, no significant difference was observed. The same trend was also observed for P removal in terms of MLSS, indicating that there was a direct relationship between P removal and MLSS for a specific system.

In one experiment simulating the actual MLSS and MLVSS concentrations of a single plant, the anaerobic and aerobic sludges removed similar amounts of phosphorus when calculated in terms of initial MLSS and MLVSS. In another experiment conducted three months later, the anaerobic sludge performed better at phosphorus removal than aerobic sludge. The return sludge mass performed consistently good at phosphorus removal.

For the experiments employing sludges from different systems, differences in P removal capacities were observed. Larger P removal capacities were attributed to larger initial MLSS and TPC values

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. 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, nor MLVSS concentrations correlated with ATP and/or TPC, indicating that MLSS and MLVSS were not good indicators of biomass in activated sludge.

6.2 RECOMMENDATIONS

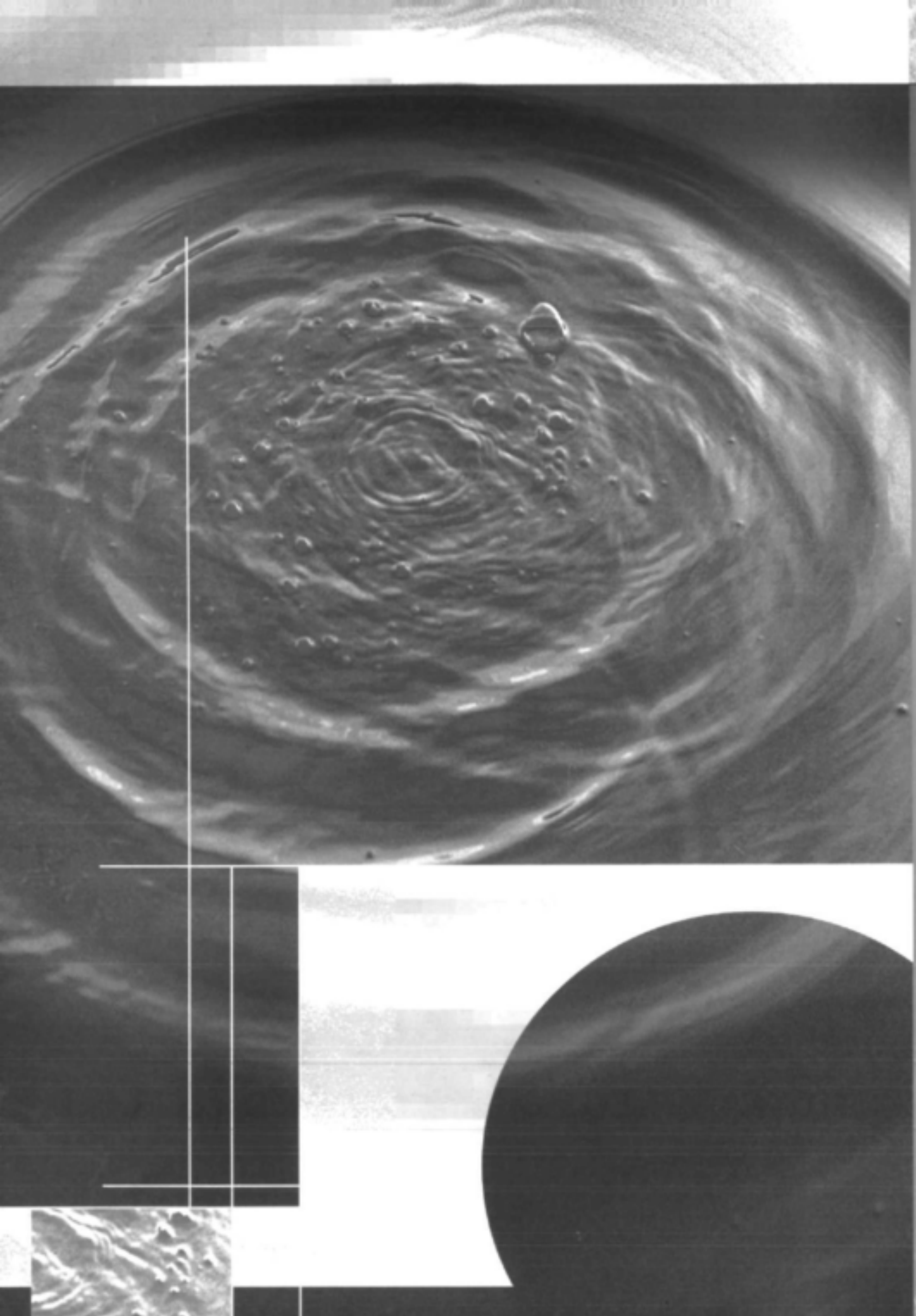
The relationship between biomass and phosphate removal in activated sludge has been indicated. The higher the biomass, the higher the phosphate removal. To optimize the design and operation of activated sludge systems, a number of steady state design models have been developed to progressively include aerobic COD removal and nitrification, anoxic denitrification and anaerobic/anoxic/aerobic BEPR (biological excess phosphorus removal).

In terms of these design procedures and kinetic models, in the bioreactor of the non-nitrifying aerobic activated sludge system, the mixed liquor organic suspended solids are made up of three components: heterotrophic active biomass; endogenous residue and inert material. In nitrifying aerobic and anoxic/aerobic systems, a fourth component is included: autotrophic active biomass.

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 suitable experimental techniques. In the literature, principally microbiological techniques have been proposed e.g. 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.

In contrast, recently a simple batch test procedure has been developed to quantify heterotrophic active biomass.



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