Determination of heterotrophic active bacteria in activated sludge using novel molecular techniques

Final report to the
Water Research Commission

by

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EXECUTIVE SUMMARY

1. Background and motivation

In the current steady state design and kinetic simulation models for activated sludge, the heterotrophic active biomass is a key parameter. However, this parameter remains hypothetical within the structure of the models; it has not been measured directly, primarily due to the lack of "suitable simple experimental techniques" (Ubisi *et al.,* 1997a). The heterotrophic active biomass component of activated sludge mediates the biodegredation processes of COD removal and denitrification and the rates of these processes are therefore directly related to the fraction present in the mixed liquor. To ensure that both new and existing wastewater treatment installations are designed/modified in such a way that they will adequately satisfy the need for sustainability of potable water supplies, it is essential that this fraction of the activated sludge biomass be accurately quantified. Investigations conducted by the Centre for Water and Wastewater Technology (CWWT, DIT, Durban; WRC Report K5/1178) has indicated that direct methods of quantification may be possible. To this end, the CWWT, through collaboration with the Civil Engineering Department (University of Cape Town), proposed an in-depth investigation to evaluate the applicability of molecular techniques to quantify the heterotrophic fraction present in activated sludge mixed liquors.

Process engineers and wastewater treatment plant operators alike have traditionally relied upon mixed liquor suspended solids (MLSS) and in particular, mixed liquor volatile suspended solids (MLVSS) to indicate the concentration of biological mass (biomass) in the activated sludge system. However, these measurements do not take cognizance of the fact that a certain fraction of the reading consists of unbiodegradable particulate and inert organic matter and either dead or metabolically inactive organisms. It is well established that a large majority of the bacteria in activated sludge remain viable yet non-culturable so that conventional cultivation techniques are not sufficient for total community recovery (only 5-15% of total bacteria can usually be recovered when using optimized media) (Wagner *et al.,* 1993). Reliable measurement of the metabolic active bacterial community (in particular, heterotrophic) and accurate calibration of the models describing the process have therefore remained elusive.

With the advancement and refinement of various molecular techniques, it has now become possible to measure the active fraction of activated sludge mixed liquors directly or *in situ*. Due to its high copy number (10^4–10^5 per cell in rapidly growing bacteria), rRNA hybridizations and nucleic acid extractions are beginning to play an integral part in microbial analyses, making it possible to correlate community structure with function. Intracellular ribonucleic acid (RNA) concentration is directly proportional to metabolic activity over a wide range (DeLong *et al.*, 1989) i.e., the more active a bacterial cell, the higher the RNA content. Once a bacterial cell enters a state of endogenous respiration the RNA is rapidly degraded. It is therefore possible to correlate visualized fluorescence, originating from specific binding of fluorescently labeled oligonucleotide probes, with metabolic activity for a particular organism. We proposed to express RNA content in an activated sludge sample as a function of total nucleic acid (TNA), the index (RNA/TNA) of which should indicate the active biomass fraction. Similarly the EUB/DAPI (FISH) index would give an indication of the ratio of total number of metabolically active bacterial cells to the total cell number. Both parameters should therefore give a reliable indication of heterotrophic activity in activated sludge.

The deficiency in accurately quantifying the heterotrophic active biomass fraction of mixed liquor has recently caste some measure of doubt on the entire framework within which the steady state design and kinetic simulation models have been developed. Although theoretical measurements derived from the simulation models and oxygen utilization measurements derived from batch test procedures support one another (Ubisi *et al.,* 1997a), there is an indication that more confidence in the procedure will be promoted if the biomass fraction can be measured directly. Molecular techniques offer such an alternative, the results of which have the potential to be incorporated directly into the current models.

2. Statement of objectives

The objectives of the research project are summarized as:

- To optimize nucleic acid extraction from activated sludge;
- To determine the relationship between rRNA content and metabolic activity for activated sludge biomass;

- To determine a coefficient (F_{VB}) for converting cell numbers to VSS units;
- To set up and operate a Lab-scale process; and
- To directly detect the active heterotrophic organisms of all systems that have a steady-state model and/or respirometric batch-test value for the parameter Z_{BH}, to correlate the two types of measurement as well as compare to modeling theory.

3. Summary of results

Relationship between rRNA and metabolic activity

The relationship between rRNA quantity per cell and metabolic activity has been established in pure culture studies and in several natural mixed populations in a variety of environments. The relationship between rRNA content and metabolic activity was investigated by making use of a batch test, in order to monitor the metabolic activity of the aerobic biomass component via oxygen utilization rate (OUR) measurement. The respirogram of the oxygen utilization rate and RNA/cell versus time for the batch experiment indicated a pattern. As soon as there is a decrease in OUR the cellular RNA levels also begins to drop, thus indicating that the RNA/cell drops appreciably after the cessation of metabolic activity. This analysis confirmed the usefulness of the rRNA molecule as a target for the detection of active biomass.

Community analysis

A study was done to determine the phylogenetic affiliation of the microbial community comprising a full-scale process in order to investigate to what extent the community profile correlates with communities found in an acetate-enriched pilot scale. Both FISH, dot blots and cultivation techniques were used. Community analysis of the activated sludge using FISH and Dot blots resulted in a good representation of the biomass, displaying the high degree of diversity of the microbial community, whereas the cultivation dependant enumerative methods were not found to give an accurate reflection of the bacterial species present. These results explained the “gamma-shift” caused by plating of activated sludge bacteria on nutrient rich agar as observed in previous studies.

Conversion factor

To compare the FISH results with model outputs, conversion of cell numbers to mass units are required. Since size of bacterial cells in nature varies between species and with growth rate any conversion factor for the total biomass will only be an estimate. A variety of monocultures would broaden the survey and allow for more reasonable assessment of the possible deviation associated with this value. In this study 12 monocultures were included. With mathematical calculation using VSS and cell numbers, a conversion factor of 8.49×10^{-11} was determined.

Comparison of microbiological measurements of heterotrophic active biomass with engineering measurements.

With this conversion factor available it was possible to compare the $Z_{BH(0)}$ from the modified batch test with the $Z_{BH(0)}$ measured by molecular probing. This comparison indicated a close correlation between the two values. The slight differences in measured Z_{BH} values could be attributed to sensitivity in regression analysis of Ln OUR data (Cronje *et al.*, 2002). With these results it was concluded that molecular biology shows powerful advantage over conventional microbiological and biochemical techniques as a tool for the direct determination and separation of mixed liquor components.

4. Recommendations for future research

Confidence in the molecular probing of heterotrophic active biomass was established with this study. It is recommended that an extensive study be launched to determining the autotrophic biomass fraction in activated sludge systems, as preliminary studies indicated a much higher amount present than that predicted by analytical procedures. A cross-link between the engineering and microbiological paradigms has been developed with the *in situ* measurement of heterotrophic biomass. The *in situ* measurement of autotrophic biomass is expected to be of great importance to the engineers in order to improve the activated sludge process.

This project is related to two other concurrent WRC projects, viz:

- Microbial Characterization of activated sludge mixed liquor suspended solids (Cloete and Thantsha, 2003, WRC Report No. 1191/1/03).

- Activity of heterotrophic and autotrophic biomass in BNR activated sludge (Cronje, Beeharry, Lakay, Wentzel and Ekama, WRC Report No. 1179/1/2002).

5. Archiving of data

All raw and process data collected from this research project will be archived and made available upon request at Durban Institute of Technology.

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

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ACA23a	oligonucleotide probe specific for Acinetobacter spp.
ADP	adenosine diphosphate
AE	aerobic reactor
ALF1b	oligonucleotide probe specific for the alpha-subclass of *Proteobacteria*
AN	anaerobic reactor
API	analytical profile index
AS	activated sludge
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
AX	anoxic reactor
EBPR	excess biological phosphorous removal
BET42a	oligonucleotide probe specific for the beta-subclass of *Proteobacteria*
BNR	biological nutrient removal
BOD	biochemical oxygen demand
CFU	colony forming units
CGYA	casitone glycerol yeast autolysate agar
COD	chemical oxygen demand
RBCOD	readily biodegradable COD
SBCOD	slowly biodegradable COD
COD_{FIL}	filtered COD
CWWT	Centre for Water and Wastewater Technology
DAPI	4',6-diamidino-2-phenylindole
DGGE	denaturing gradient gel electrophoresis
DIG	digoxigenin
DNA	deoxyribonucleic acid
DO	dissolved oxygen
EPS	exopolymeric substance
EUB338	oligonucleotide probe specific for most members of the kingdom Bacteria
f_{CV}	COD/VSS ratio

f_{VB}	VSS/biomass ratio
FISH	fluorescent *in situ* hybridization
GAM42a	oligonucleotide probe specific for the gamma-subclass of Proteobacteria
GPBHGC	Gram-positive bacteria with high G+C DNA content
HGC69a	oligonucleotide probe specific for *Actinobacter*
INF	influent
MLSS	mixed liquor suspended solids
MLVSS (VSS)	mixed liquor volatile suspended solids (volatile suspended solids)
NDEBPR	nitrification denitrification excess biological phosphorus removal
Ortho-P	ortho phosphorus (PO_4^{3-})
OHO	ordinary heterotrophic organisms
OUR	oxygen utilization rate
P	phosphorus
PAO	polyphosphate accumulating organism
PCR	polymerase chain reaction
PHA	polyhydroxyalkanoates
PSS	protein synthesizing system
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
mRNA	messenger RNA
rRNA	ribosomal ribonucleic acid
tRNA	transfer RNA
SBR	sequenced batch reactor
SCFA	short chain fatty acid
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
S_0/X_0	initial substrate (S_0) to initial biomass (X_0) ratio
TCA	tricarboxylic acid
TKN	total Kjedahl nitrogen
TNA	total nucleic acid
TP	total phosphorus
UCT	University of Cape Town

V	volume
VFA	volatile fatty acid
VSS	volatile suspended solids
WRC	Water Research Commission
WWW	wastewater works
X_{AUT}	autotrophic organism
X_B	biomass
Z_{BH}	heterotrophic organisms
X_{PAO}	polyphosphate

CHAPTER ONE

INTRODUCTION

1.1 Background and scope of research

Mathematical models for biological carbon and nitrogen removal from domestic and industrial wastewaters are essential for the design and operation of biological wastewater treatment plants. To aid the design and operation of the single sludge activated sludge system, a suite of steady state design models (e.g. WRC, 1984; Wentzel *et al*., 1990; Maurer and Gujer, 1994) have been developed. In the development of these models, it was recognized that it would not be possible to incorporate the behavior of specific microorganisms species, the mixed liquor in the activated sludge system contains a wide diversity of different microorganisms species, and only recently identification techniques have become available to study these microorganisms.

In terms of models, in the non-nitrifying aerobic COD removal activated sludge system, the mixed liquor organic suspended solids (MLOSS) is made up of three components; (1) ordinary heterotrophic organism (OHO) active biomass, (2) endogenous residue and (3) inert material. With the inclusion of nitrification in the system, a fourth component is included; (4) autotrophic organisms (AO) active biomass. The activated sludge process is a biological process used to treat both domestic and industrial wastewater. In current steady state design and kinetic simulation models for activated sludge, the OHO active biomass is a key parameter since the entire activated sludge process depends upon the physiology of the heterotrophic organisms. To ensure that the engineers develop and incorporate adequate mathematical models for the treatment processes it is important to quantify this portion of the activated sludge accurately. The direct determination of OHO active biomass is not common practice due to a lack of suitable techniques. Historically the MLOSS have been measured as a lumped parameter, via the VSS or COD test (Standard Methods, 1985). Specific rates for these biological processes (denitrification; oxygen utilization) are often expressed in terms of this lumped parameter, but only part of the MLOSS, the OHO active biomass is responsible for mediating the biological processes for COD removal and denitrification (Wentzel *et al*., 1998).

The batch test procedure developed by Kappeler and Gujer, (1992) presented a means of quantifying the OHO active biomass concentration through monitoring the organisms OUR response with time in a batch reactor. This procedure was extended by Wentzel *et al*., (1995), Ubisi *et al*., (1997a; 1997b) and Cronje *et al*., (2002) to quantify the OHO active biomass concentration drawn from aerobic and anoxic/aerobic activated sludge systems. Cronje *et al*., (2002) compared the results for the OHO active biomass obtained from the modified batch test with theoretical values for the OHO active biomass concentration from the steady state design model (WRC, 1984). Mixed liquor samples were drawn from a parent anoxic/aerobic activated sludge system. From this comparison, they concluded that the results obtained showed good agreement between the measured and theoretical values. Parallel to the developments in the engineering and technology of the activated sludge system, significant advances have been made in the microbiological and biochemical areas. Fluorescent *in situ* hybridization (FISH) with rRNA-targeted nucleic acid probes is a new molecular tool for rapid, reliable and cultivation-independent monitoring of phylogenetically defined bacterial populations in activated sludge samples (Amann *et al*., 1995).

Fluorescent *in situ* hybridization (FISH) makes use of a fluorescently labelled probe specific for a region on the 16S and 23S ribosomal RNA of a bacterial subclass or group. In order to make use of this technique it was first necessary to determine the relationship between the metabolic activity and rRNA content, since only active cells needs to be counted (CHAPTER 3). Secondly, in order for this cell numbers, determined by FISH, to be meaning full to the engineers, a conversion factor is needed to convert the cell numbers to mass units (CHAPTER 5). And lastly this cell numbers and conversion factor will be used to determine the OHO active biomass. This will then be compared to the values obtained from the modified batch tests and theoretical values (CHAPTER 6).

1.2 Research objectives

The research project was therefore designed to:

- Determine the relationship between rRNA content and metabolic activity for activated sludge biomass;

- Determine a coefficient (F_{VB}) for converting cell numbers to VSS units and use this value to;
- Directly detect the active heterotrophic organisms of all systems that have a steady-state model and/or respirometric batch-test value for the parameter Z_{BH}, to correlate the two types of measurement as well as compare to modeling theory.

1.3 Report Structure

The report is structured and will be presented as such:

TABLE 1.1 Scope and structure of report.

CHAPTER	SCOPE
1	General introduction and scope of research
2	Literature review
3	The relationship between rRNA and metabolic activity
4	FISH and dot blots using group-specific oligonucleotide probes
5	Biomass determination for BNR mixed liquor
6	Comparison of microbiological measurements of heterotrophic active biomass with engineering measurements
7	General conclusions and recommendations

CHAPTER TWO

LITERATURE REVIEW

2.1 Wastewater composition

In this day and age, water and wastewater management is considered a necessity for the formation and maintenance of a modern society. The modernization of society has resulted in the production of larger volumes of wastewater that are increasingly becoming more concentrated and hence more harmful to the environment (Johnson Smith Consulting LtD, 2001). The composition of wastewater does not remain constant from place to place. Wastewater or sewage consists of over 95% water. The remaining 5% determines the nature of the mixed liquor (Gray, 1989). Wastewater mainly consists of domestic waste, industrial waste and storm sewage.

2.2 Domestic sewage

Fresh domestic sewage is a grey relatively turbid liquid with a characteristic odor. It usually has a neutral pH and a temperature of 15 to 25°C (Bolton and Klein, 1971). As the sewage ages, its characteristics change due to biodegradation. Biological degradation changes the chemical composition and produces gases such as hydrogen sulphide. The design parameter for domestic sewage treatment is based on the population:pollution load ratio (Qasim, 1994). The pollution load refers to the COD and biochemical oxygen demand (BOD) and oxygen absorbed (OA) (Qasim, 1994).

TABLE 2.1. Composition of typical domestic sewage. (McGhee, 1991)

Parameter measured	**Range (mg/L)**
Oxygen absorbed	70 – 80
Biochemical Oxygen Demand	350 – 400
Chemical Oxygen Demand	700 – 800
Settleable Solids	10 – 12
Suspended Solids	250 – 350
Ammonia	40 – 45
Phosphate	10 – 13

2.3 Bacterial community analysis

When working with activated sludge or any biological wastewater treatment process, it is important to have a good understanding of the microbial community involved in the process as this would assist in improving system design and performance and in calculating the biodegrading capacity of the system. Biological wastewater systems depend on the metabolic interactions of microorganisms to degrade chemicals and other pollutants to less harmful products (Cloete and Muyima, 1997).

In a study done in 1984, Boyd found that the composition of the microbial community is determined by the wastewater. Bacteria make up about 95% of the microbial community in activated sludge (Wisconsin Department of Natural Resources, 2000). They make up such a large percentage in activated sludge because they are the most versatile of all organisms, in terms of their growth rates and catabolic capabilities.

The dominant bacteria in wastewater are the aerobic heterotrophic Gram-negative bacilli, these heterotrophs obtain their energy from the oxidation of organic matter (Gray, 1989). The organic matter also serves as a source of carbon (Bitton, 1999). When the organic compounds are oxidized the resultant products are carbon dioxide and water (Gray, 1989).

2.4 The morphology and size of prokaryotes

It can be expected that small, relatively simple organisms like bacteria would be similar in terms of shape and size. Notwithstanding the fact that many bacteria are similar in terms of morphology, there are many significant differences. Most of the commonly occurring bacteria have one of three basic cell shapes, namely: cocci, bacilli or spirals. Cocci are spherical cells. These cells can exist individually, but are very often organized into groups. One of the other main forms of bacteria are those which are rod shaped and are often referred to as bacilli. Bacilli differ significantly with regards to their length and width ratio. The shape of the rod may vary amongst different species and may be round, shaped, flat or forked. Many bacteria occur in the form of long rods and spirals. They are called spirilla if they are rigid and spirochetes if flexible. Bacteria differ with regards to their shape as much as they differ with regards to their size. The smallest bacteria, such as some members of the Mycoplasma genus, are approximately 100 to 200 nm in diameter. Some bacteria are relatively big. Some spirochetes may reach a length of up to 500 nm (Cloete, 1998). Therefore this difference may have an effect on the measurement of the cells.

2.5 Microbial growth under steady-state conditions

An almost constant ratio of DNA/cell, RNA/cell and protein/cell and also a constant cell density and minimum cell size is indicative of steady state conditions of growth (Gray, 1989). As wastewater contains most of the nutrients required for bacterial growth, provided that environmental conditions are favorable, a diverse group of organisms will develop (Gray, 1989). Under steady state conditions the nutrient concentration remains constant, thus of the groups of microorganisms capable of responding to these conditions the fastest tend to predominate at these times (Marais and Ekama, 1976). Heterotrophs require two categories of nutrients:

1. Those needed for energy production, growth and metabolism.
2. The chemical elements required for biosynthesis (Gray, 1989).

The glucose synthesized by the autotrophs forms the basic source of energy for the heterotrophic cells to synthesize the more complex molecules that constitute the cell mass, including proteins

(Marais and Ekama, 1976). As conditions change in terms of nutrient breakdown, other groups begin to respond to availability of nutrients and energy (Gray, 1989). The F/M ratio maintained in the aeration tank in an activated sludge system controls the rate of biological oxidation as well as the volume of microbial biomass produced. The activated sludge ecosystem can be completely balanced so that nearly all the available organic matter is utilized by the primary heterotrophic activity, which in turn is used by the grazers so that there is no excess microbial biomass, composed mainly of heterotrophic bacteria, which is surplus to that required to maintain the microbial population density in the aeration tank via the returned sludge. Therefore, the excess microbial biomass must be disposed of separately as unwanted sludge. Under steady-state conditions the growth rate of the microorganisms will be equal to the specific sludge wastage rate (Gray, 1989). Under steady-state conditions the predominance of various bacterial populations at different intervals during the process is not fully understood. Factors such as nutrient and oxygen availability as well as the ability of bacteria to break down and utilize complex organic matter play a vital role. The ratios of Proteobacterial species in relation to each other as well as the entire bacterial population will be evaluated.

2.6 Measurement of microbial growth

There are many ways to measure microbial growth to determine growth rates and generation times. Either population or number may be used because growth leads to increase in both. The most obvious way to determine microbial numbers is through direct counting. The use of a counting chamber is easy, inexpensive and relatively quick. It also gives information about the size and morphology of the microorganisms. The disadvantage of this technique is that the microbial population must be fairly large for accuracy because such a small volume is sampled. It is also difficult to distinguish between living and dead cells and the cells tend to clump together making it difficult to view. Increases in the total cell mass as well as in cell numbers accompany population growth. Therefore, techniques for assuming changes in cell mass can be used to determine growth. The most direct approach is the determination of microbial dry weight. Cells growing in liquid medium are collected by centrifugation, washed, dried in an oven and weighed. It is time consuming however and not very sensitive. Because bacteria weigh so little it may be necessary to centrifuge several hundred millilitres of culture to collect a

significant quantity. More rapid sensitive techniques are based upon the fact that microbial cells scatter light striking them. Because microbial cells in a pure culture are at roughly constant size, the amount of scattering is proportional to the concentration of cells present (Cloete, 1998).

2.7 Factors affecting growth

Domestic sewage and wastewater from industries, mainly food-processing industries, are generally rich in organic and inorganic nutrients. Thus, wastewaters provide an ideal ground for the growth of bacteria, provided that environmental conditions are suitable.

Heterotrophic bacteria require two types of nutrients:

- Nutrients for growth and metabolism
- Chemical nutrients for biosynthesis of enzymes and co-factors.

(Gray, 1989; Schroeder, 1977)

Bacteria readily utilize soluble compounds such as sugars, organic acids and amino acids. Secondary metabolites may only be utilized as secondary substrates (Wisconsin Department of Natural Resources, 2000). Carbon is required in relatively large amounts as this is the main source of energy. The basic nutrients of abundance in normal raw sewage are carbon (C), nitrogen (N) and phosphorous (P); with the ratio of C:N:P being about 100:10:1 (Wisconsin Department of Natural resources, 2000).

Some bacteria require growth factors such as vitamins, which serve as co-enzymes or co-factors. Wastewaters generally contain sufficient amounts of growth factors such as biotin and riboflavin (Brock, 1979). Many bacterial species are capable of synthesizing their own growth factors. A large number of other elements are required, but the concentrations that are required for optimum growth depends on the species.

2.7.1 Dissolved Oxygen

Heterotrophic aerobes utilize dissolved oxygen as electron acceptors. The growth of aerobic bacteria increases as the concentration of dissolved oxygen increases, until the bacteria reach a maximum where, faster growth cannot take place under the present conditions. A dissolved oxygen concentration of 1-2 mg/L is adequate to support the growth of heterotrophic active species (Bitton, 1999).

Dissolved oxygen is usually supplied by mechanical means in activated sludge systems. This involves the use of large stirrers, which continuously mix the suspension. This ensures that a high concentration of dissolved oxygen is always present. Activated sludge systems require higher concentrations of dissolved oxygen because of the presence of floc structures (Schroeder, 1977).

2.7.2 Temperature

Temperature needs to be kept relatively constant to ensure good bacterial growth. Temperature controls the rate of reactions within the bacterial cell. Changes in temperature results in significant changes to the bacterial community structure. Activated sludge systems operate in the mesophilic temperature range with the optimum temperature being between 14°C and 25°C (Schroeder, 1977).

Raised temperatures increase microbial activity and metabolism and hence, increases substrate utilization. Due to increased metabolic rates, there will be a need for increased oxygen supply. A decrease in growth occurs once the temperature rises above 25°C (Schroeder, 1977).

2.7.3 pH

Bacterial monocultures grow well within a pH range of 6.5–8.1 (Gray, 1989). In biological treatment processes, the pH is maintained within this range to ensure efficient removal of the waste matter. Domestic sewage usually falls within this range, depending on the season and

climate (Schroeder, 1977). If the wastewater is characterized by acidic or very alkaline conditions, then it must be modified. The pH is modified by diluting the wastewater with another effluent to neutralize pH (Gary, 1989).

2.7.4 Inhibitors of Growth

Many heavy metals and organic compounds have been found to be toxic to aerobic bacteria. Bacterial monocultures are affected by relatively small concentrations of toxins. Activated sludge requires larger concentrations of toxins to inhibit the growing cells. This is due to the large population of bacterial cells (Brock, 1979).

Metals such as copper and mercury are considered to be the most toxic. These metals form complexes with the bacterial enzymes and other growth agents, rendering them inactive (Gray, 1989). High concentrations of salts and nitrogen compounds severely inhibit bacterial growth. Salt concentrations of about 3000 mg/L lead to increased osmotic pressure within the cell. This build up of pressure may eventually cause the cell to burst (Brock, 1979).

Bacterial cells that utilize ammonia as a sole source of nitrogen are inhibited by nitrogen concentrations greater that 2000 mg/L (Brock, 1979). Anionic detergents that are normally present in domestic sewage have been found to inhibit a wide range of bacteria.

2.8 Bacterial growth kinetics

In an activated sludge system, the rate at which organic matter is utilized depends upon the rate of bacterial growth. Bacteria reproduce mainly by binary fission, each cell gives rise to two daughter cells (Bitton, 1999). The rate of cell division varies with species. Some species have division times that are less than thirty minutes while, others many hours (Bitton, 1999).
When bacteria are inoculated they follow a particular growth pattern:

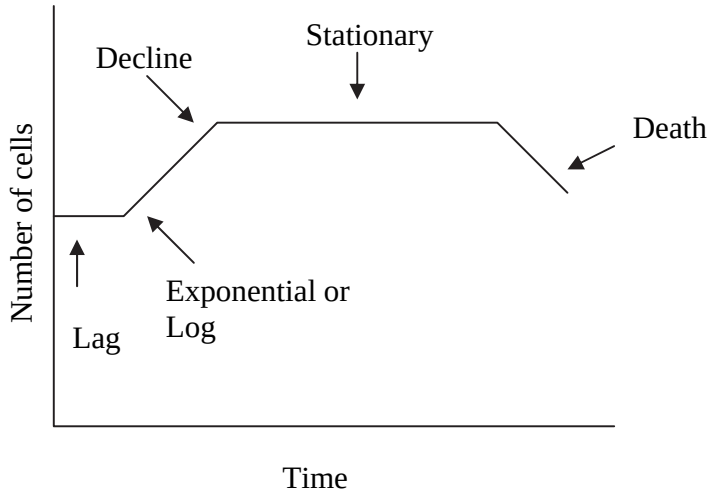

FIGURE 2.1 Typical bacterial growth curve (Bitton, 1999).

The five phases of growth (Bitton, 1999) are:

2.8.1 The Lag phase

This is the period of acclimatization of the bacterial cells to their new environment. No growth occurs during this stage. The cells take in nutrients only for the biosynthesis of enzymes and other cell components. The duration of the phase is determined by the degree of adaptation of the cells to the environment.

2.8.2 The Exponential or Log phase

During this phase the generation time decreases and there is a constant increase in the growth rate. This results in an increase in mass and the number of cells. The growth rate is at its maximum. Cells in this phase of growth are more sensitive to physical and chemical damage. This phase continues until the nutrients become limited.

2.8.3 The Decline phase

Bacterial growth begins to slow down due to the limited nutrient supply. Toxic metabolites begin to accumulate because of decreased metabolic rates. Chemical conditions in the medium start to decline. The growth curve gradually begins to flatten out.

2.8.4 The Stationary phase

This phase follows immediately after the decline phase. At this stage most of the nutrients have been utilized. The cells start producing secondary metabolites. Some cells undergo reproduction but the number of cells that are dying balances this. Thus, the bacterial population remains fairly constant.

2.8.5 The Death phase

At this phase of growth, the nutrients have been completely utilized and there is a high concentration of toxins. These conditions are unfavorable for the survival of bacteria cells. The cell death rate increases.

The bacterial growth curve is an indication of the response of cells to environmental conditions within a closed system (Schroeder, 1977). Biological treatment processes are continuous systems where bacterial populations need to be maintained at a particular growth phase. In activated sludge systems the bacterial population is maintained at the mid-log phase, when the cells are the most metabolically active (Cloete and Muyima, 1997).

2.9 Molecular methods versus culture dependant methods

The use of the activated sludge process in the treatment of wastewater is still one of the most important biotechnological processes. Although our understanding of process engineering has matured, our current knowledge on the structures and dynamics of the involved microbial communities, and consequently our understanding of the microbiology of the activated sludge

process is still very limited (Wagner and Amann, 1997). Culture dependent methods such as the viable plate count for the most part have been used for the analysis of these microbial communities. However, due to its selectivity towards certain organisms, inadequacies with these methods were observed. Standard plate counts reflect not so much the actual microbial community structure of the activated sludge but rather the selectivity of the growth media for certain bacteria. It has been found that in aquatic habitats, direct microscopic counts can exceed viable cell counts by several (2-4) orders of magnitude (Wagner and Amann, 1997). It is now recognized that in most cases most of the microscopically visible cells are viable, but do not form visible colonies on plates. In activated sludge two effects cause this mainly:

1. Clumping of the cells in the activated sludge flocs prevents a quantitative release of individual bacteria and consequently leads to an underestimation of the number of active cells determined by viable plate count techniques.
2. The cultivation conditions are not suitable for all bacteria.

The direct identification and enumeration of bacteria in the activated sludge requires different techniques that analyze at a cellular level. One of the methods used has been immunofluorescence (Wagner and Amann, 1997). This method has been used in the monitoring of bacteria in complex environments. However, this technique using fluorescent antibodies can be disturbed by extracellular substances that are thought to hinder antibody penetration. Non–specific binding of the antibodies to detritus particles and fungal spores can result in high levels of background fluorescence. In addition the production of specific antibodies requires a pure culture of the organism of interest. As most bacteria in the activated sludge are still uncultured, methods for the identification of bacteria in situ are necessary that are completely independent of cultivation methods (Wagner and Amann, 1997). Delong *et al*., (1989) have developed a staining method that provides phylogenetic information on single microbial cells and requires no previous knowledge of the organisms detected. This approach was based on oligodeoxynucleotide hybridization probes complementary to ribosomal RNA (rRNA) sequences that are diagnostic for selected phylogenetic groups. When these probes are labeled with fluorescent dyes, they can be used for the detection and phylogenetic characterization of organisms with the microscope (Delong *et al.*, 1989).

In situ nucleic acid hybridization with isotopically or fluorescently labeled probes is widely used for the intracellular localization and quantification of RNA and genes. Isotopically labeled oligodeoxynucleotides bind to the rRNA of intact, fixed cells and together with autoradiography, can be used for the phylogenic identification of organisms. The abundance of cellular ribosomes 10^4-10^5 per cell in rapidly growing bacteria, suggest that the binding of phylogenetic group–specific probes for the rRNA might be viewed directly in the fluorescent microscope (Delong *et al.*, 1989).

2.10 Investigating Proteobacteria in activated sludge

In probing COD removing activated sludge with oligonucleotides specific for Proteobacteria, Wagner *et al.*, (1993) found that the Proteobacteria together comprises 60-75% of the microbial cells visualized by DAPI. Between aeration basins that differed in F/M ratios they also observed a shift in the population profile within this division. In samples taken from the high load first stage basin (B1, F/M ratio=1.8), alpha and beta counts (37% each) dominated over gamma counts (7%). These results indicate that the substrate to microorganism ratio could be critical in determining the microbial community structure of the environment being analyzed.

2.11 Proteobacteria

The Proteobacteria contain most, but not all, of the traditional Gram-negative bacteria. However the arrangement of classically defined families, genera and even species within this phylum is a jumbled one (Woese, 1987); Photosynthetic species group with non-photosynthetic ones, heterotrophs are paired with chemolithotrophs; anaerobes are paired with aerobes. Because the purple photosynthetic phenotype is distributed throughout the group and because photosynthesis is complex enough that it is unlikely that it has arisen more than once, the ancestral phenotype of the group is undoubtedly photosynthetic (Woese, 1987). However, the Proteobacteria form one of the few groups that cannot be defined by a simple signature. This class falls into four subclasses that have been designated alpha, beta, gamma and delta-Proteobacteria (Manz *et al*., 1992). This class makes up one of a few that cannot be defined by a simple signature. These subdivisions can be defined and distinguished by two helices in the 16S rRNA. Within a given

subclass the structure of the helices remains relatively constant, while varying remarkably between the subclass. The beta and gamma subclass are closely related to each other. The alpha-Proteobacteria seems predominately photosynthetic. Photosynthesis is seen in the two main subgroups of the beta-subclass; while in the gamma subclass photosynthesis appears to be confined to one of its 3 main subgroups (Woese, 1987).

2.11.1 Alpha-Proteobacteria

The intimate juxtaposition of photosynthetic species in the alpha subdivision suggests a more or less continual evolution of the latter from the former. Aerobic metabolisms appear to have arisen a few times in this subdivision. Reduction and oxidation of nitrogenous compounds is common among these species. The close association of species reducing and oxidizing nitrogen compounds, e.g., *Pseudomonas palustris* and *Nitrobacter winogradskii* suggests some sort of evolutionary connection between the two metabolisms (Woese *et al.,* 1984). Sequence difference in the 16S rRNA among members of these clusters is less than 7% (Woese, 1987).

2.11.2 Beta-Proteobacteria

Most of the beta-Proteobacterial species fall into two main subgroups, beta1 and beta2. This subdivision is a mixture of different genera, some of which are not even phylogenetically coherent within the subdivision (Manz *et al.,* 1992). The beta-photosynthetic species reclassified in the genus Rhodocyclus are quite distinct from other purple non-sulphur bacteria i.e., those residing in the α-subdivision. These species differ in rRNA sequences as well as in type of cytochrome C. Beta-cytochromes are of the small sub-unit type, while those from the alpha subdivision are larger. Photoreaction centers are structurally different between these two subgroups (Woese, 1987). The view that the gamma and beta subclass of Proteobacteria as separated from one another has to be revised in that the beta subclass is a clearly defined subgroup of the gamma subclass (Hugenholtz *et al*., 1998). Representative examples of this subclass include Thiobacillus, Alcaligenes, Spirillum and Nitrosovibrio. The genera Burkholderia, Comamonas, Ralstonis and Telluria are now the home of all former pseudomonads within the beta-subclass (Hugenholtz *et al*., 1998).

2.11.3 Gamma-Proteobacteria

The gamma-Proteobacteria is the most extensively characterized of the sub-divisions of the Proteobacteria (Wagner *et al*., 1994). The beta and gamma subdivisions seem to be more closely related to each other than to any of the other proteobacterial subdivisions (Woese, 1987; Hugenholtz *et al*., 1998). The subdivision is a mixture of phenotypes, e.g. Photosynthetic with non-photosynthetic, aerobic with anaerobic. Oligonucleotide catalogue analysis divides the gamma-Proteobacteria into three main subgroups; one containing mainly photosynthetic species of the purple sulphur type e.g. *Chromatium*; a second known to contain only species associated with Legionnaires disease and a third that is a mixture of non-photosynthetic genera from the enteric, vibrio's oceanospirilla, the fluorescent pseudomonads and relatives (Woese *et al*., 1985). The genus *Pseudomonas* is now restricted to species related to *Pseudomonas aeruginosa*, the type species. Besides *Pseudomonas*, *Stenotrophomonas* and *Deleya* are genera harboring former pseudomonads within the gamma subclass of Proteobacteria (Hugenholtz *et al*., 1998).

2.11.4 Gram-positive Eubacteria

Cell wall type distinguishes the Gram-positive Eubacteria from other species. The accessibility of intracellular nucleic acids is dependant on the permeability of the cell periphery (e.g. cell walls, membranes, capsules) to the probe molecules, and the availability of the probe-binding site for hybridization (Roller *et al.*, 1994). This phylum appears to consist of 4 subdivisions, only 2 of which are well characterized. These 2 are easily differentiated on the basis of DNA composition. The first includes species whose DNA contain more than 55% guanine and cytosine (G+C), the other is made up of species whose DNA contains less than 50% G+C. Species in the high G+C Gram-positive subdivision conform to a general Actinomycete phenotype: They tend to be pleomorphic; form branched filaments. Most are aerobic with the exception of the deeper branches, e.g. the Bifidobacteria (Woese, 1987).

2.11.5 The Actinobacteria (Gram-positive bacteria with high G+C DNA)

Cell wall type distinguishes the Gram-positive bacteria from other divisions (Fox *et al.,* 1980). However, for this phylum, the cell wall phenotype does not link all the genotypically defined members; both Heliobacterium and the mycoplasmas are members of the Firmicutes (Gram-positive division) that lack the characteristic Gram-positive cell wall (Gest and Flavinger, 1983; Stackebrandt *et al.,* 1985). The phylum appears to consist of 4 subdivisions. Two of these are well characterized and are readily distinguishable based on their DNA composition. The one (Actinobacteria) includes species whose DNA contains greater than 55% guanine plus cytosine (G+C) residues; the other is made up of species whose DNA contains less than 50% G+C (Bacillus/Clostridium group), and includes the mycoplasmas (Fox *et al.,* 1980). The phototroph *Heliobacterium chlorum* is the only characterized representative of the third subdivision (Gest and Flavinger, 1983) and the genera Megashaera, Selenomonas and Sporomusa constitude the fourth division (Stackebrandt *et al.,* 1985) having a Gram-negative cell wall type. Species belonging to the Actinobacteria conform to the general actinomycete phenotype; they tend to be pleomorphic, form branched filaments and are mostly aerobic with the exception of the deeper branches e.g. the bifidobacteria (Woese, 1987).

2.12 Measuring of evolutionary relationships

Highly complex characteristics (phenotypic patterns) are unlikely to have changed more than once and thus are good indicators of relationships (Woese, 1987). Measuring of evolutionary relationships is now well established by the sequencing of proteins and nucleic acids. Classification and relating these organisms have been based on phenotypic information, however it has been found that genotypic (Sequence) information is superior in two major ways:

1. It can be more readily, reliably and precisely interpreted.
2. It is more informative of evolutionary relationships (Woese, 1987).

Unlike these 3-dimensional phenotypic patterns, sequence patterns are one-dimensional and can be measured in simple ways, in terms of simple relationships. This is because the elements of a

sequence, i.e. the nucleotides or amino acids are limited in number and well defined (Woese, 1987).

2.13 Ribosomal ribonucleic acid

There is no more fundamental and straightforward way to classify and relate organisms than by appropriate nucleic acid sequence comparisons (Olsen *et al.*, 1986). Since rRNA is present in all organisms and different positions in their sequences change at very different rates, this allows for most phylogenetic relationships to be measured (Woese, 1987). The 5S and 16S rRNA has been used most for rRNA–based phylogenetic characterizations (Olsen *et al*., 1986). The average bacterial 16S rRNA molecule has a length of 1500 nucleotides. Thus it contains more than sufficient information for reliable phylogenetic analysis (Wagner and Amann, 1997). Because substantial portions of the 16S rRNA sequences are similar among all known organisms, low-stringency hybridization permits the identification of rRNA genes from unknown organisms. Other portions are unique to certain organisms or related groups of organisms and thus can be used as targets for hybridization probes with various specificities (Wagner and Amann, 1997). The ubiquity of the rRNA ensures that probes can be designed to identify virtually any organisms or group of related organisms. The application of these techniques allows for the detection of actively growing microorganisms with relatively high cellular rRNA content.

2.14 Oligonucleotide Probes

The method of oligonucleotide cataloging has been used to determine partial rRNA sequences (Woese, 1987). An oligonucleotide is a short single strand of DNA (oligodeoxyribonucleotide) usually 6-50 nucleotides in length (Stahl and Amann, 1991). A collection of these sequence fragments from a given rRNA constitutes an oligonucleotide catalog, i.e. a detailed complex pattern characteristic of a given bacterial species. Comparisons among these catalogs permit phylogenetic groupings to be identified at various taxonomic levels (Woese, 1987). Under appropriate conditions of stringency, oligonucleotide probes often discriminate between targets that differ in a single nucleotide (Stahl and Amann, 1991). The specificity of a probe is determined by its length and the complexity of the target sequence. A minimum probe size of

about 9 bases (with 4 Guanine-Cytosine pairs) is required for a stable hybridization (Stahl and Amann, 1991). The oligonucleotide hybridization probes complementary to rRNA sequences can be used as diagnostic tools for selected phylogenetic groups. When these probes are labeled with fluorescent dyes, they can be used for the detection and phylogenetic characterization of organisms with the aid of a fluorescent microscope (Delong *et al.*, 1989). Group specific rRNA-targeted oligonucleotide hybridization probes have the potential to facilitate the characterization of microorganisms (Manz *et al.*, 1992). Phylogenetic groups are defined by sequence similarities often reflected in so called signatures (Manz *et al*., 1992). This term is used to describe individual rRNA sequence positions, combinations of positions, or higher order structural elements characteristics for all, or most, members of one group and not occurring in the rRNA of most other groups. Short oligodeoxynucleotides complimentary to these signature regions are used as nucleic acid probes for the differentiation of major subclass of Proteobacteria (Woese, 1987).

2.15 Probe design for the major classes of Proteobacteria

Based on comparative sequence analysis of 16S and 23S rRNA, Manz *et al*., (1992) located sites specific for the alpha, beta and gamma subclasses of Proteobacteria. Alpha subclass specific sites were identified close to the end 5' end of the 16S rRNA (ALF1b) and at helix 73 of the 23S rRNA (ALF73a). Signature sites for the beta and gamma subclasses were found located at helix 42 of the 23S rRNA. Site ALF73a was found to be unsuitable for probe design due to some members of the alpha subclass possessing at least two mismatches at the binding site. The ALF1b binding site was subsequently chosen for probe design. However, position 29 within the ALF1b binding site is not perfectly conserved for the alpha subclass of Proteobacteria, most species within this subclass have an adenine (A) residue but some have a guanidine (G). Thus for detection of all members of the alpha subclass, probe ALF1b was devised a 1:1 mixture of two oligonucleotides. Some members of the alpha subclass have only one or no mismatches. Also most of 16S rRNA molecules from the phylum "Spirochetes and relatives" have the ALF1b binding site as do *Flexistipes sinusarabicus*. Consequently probe ALF1b should not be regarded as being specific for only alpha subclass of Proteobacteria (Manz *et al*., 1992).

The probe designed for the beta and gamma subclasses (BET42a and GAM42a respectively) are directed against one homologous site (position 1027-1043, E. coli 23S rRNA numbering; Brosius *et al.*, 1981) and are identical except for one nucleotide. All hitherto sequenced 23S rRNA genes of beta subclass organisms have a thymidine (T) residue at position 1037, whereas all sequenced 23S rRNA molecules of gamma subclass organisms have an adenine (A) at this position. All other available 23S rRNA sequences have at least two differences within this target site (Manz *et al.*, 1992).

2.15.1 Probes specific for alpha, beta and gamma species of Bacteria

2.15.1.1 Alpha subclass of Proteobacteria

Alpha subclass specific sites were found close to the 5' end of the 16S rRNA (Alf1b), and at helix 73 of the 23S rRNA (Alf 73a). Probe ALF73a is capable of hybridization with most of the alpha subclass sequences available. It has been found however, that two sequences on the alpha subclass molecule (*Bradyhisobium zaponicum* and *Rhodopseudomonas palustris*) have two mismatches within the ALF73a binding site. Thus Alf 73a only hybridizes with part of the alpha subclass of Proteobacteria (Manz *et al.*, 1992).

2.15.1.2 Beta and gamma subclass of Proteobacteria

Signature sites for the beta and gamma subclass are situated at helix 42 of the 23S rRNA. The probes for the beta (BET42a) and gamma (GAM42a) subclass are directed against one homologous site and are identical except for one nucleotide. All sequenced 23S rRNA genes of beta subclass organisms have a Thymidine (T) residue at position 1037, whereas all sequenced 23S rRNA molecules of the gamma subclass organisms have an adenine (A) at this position. All other available 23S rRNA sequences have at least 2 differences within this target site. Members of the beta and gamma subclass of Proteobacteria have usually 2 (or more) differences located centrally in the target site (Manz *et al.*, 1992).

2.15.1.3 Gram-positive bacteria with a high Guanine-Cytosine content

23S rRNA sequences of Gram-positive bacteria with a high guanine-cytosine content (GPHGC) showed a potential target site for a group specific oligonucleotide probe within the stem of helix 69 (Roller *et al.,* 1994). Most bacterial species contain a CC in position 1908-1909 paired with GG 1912-1913. However, all 23S rRNA sequences determined for GPHGC have an AG/CU (1908-1909/1912-1913) in place of CC/GC. Thus probe HGC was designed as a 18mer complementary to positions 1901-1918 (Woese, 1987).

2.15.1.4 Probe design for the Actinobacteria

Roller *et al.,* (1994) by comparative sequence analysis of 14 complete 23S rRNA's of representatives Actinobacteria strains, found potential target sites at helix 69 of the 23S rRNA (domain IV) for a group-specific probe, and a hyper variable insertion between helix 54 and 55 in the 23S rRNA (domain III) that could serve as a target site for more specific probes. Within the stem of helix 69 most bacterial sequences contain CC in positions 1908-1909; presumably paired with GG (1912-1913). However, all 23S rRNA sequences so far determined for GPBHGC have AG/CU (1908-1909/1912/1913) in place of CC/GG. Probe HGC was therefore constructed as a 18mer complementary to positions 1901-1918.

2.15.2 Probes for nitrifying bacteria

Wagner *et al.,* (1995) by comparative analysis of 16S rRNA sequences of *Nitrosomonas europaea* and *Nitrosomonas eutropha* with 3000 other (complete or almost complete) 16S rRNA sequences, designed probe NEU complementary to part of helix 23a (position 653-670, *Escherichia coli* numbering; Brosius *et al*., 1981) of these two ammonia-oxidizing species. Database checks showed that all non-target sequences had at least one mismatch with probe NEU. Using optimized conditions (40% v/v formamide, 46°C in the hybridization buffer) the specificity of the NEU probe was further evaluated by whole cell hybridization with 24 reference strains, including 13 described genospecies of the genus *Nitrosomonas*. It was found that *N. cryotolerans*, *N. marina*, *N. halophilla,* N. *aestuarii* and *Nitrosococcus mobilis* were also

detected with probe NEU. Bacteria of the genus *Nirtobacter* belong to the alpha-2 branch of the Proteobacteria. They have been considered to be the 'model' organism for the conversion of NH_4^+ to NO_2^- (Dawes, 1986). Wagner and co-workers (1996) by comparative analysis of sequenced Nitrobacter strains designed probes NIT1, NIT2 and NIT3 specific for *Nitrobacter* spp. and applied this probe set for in situ analysis of 9 sewage treatment plants. The presence of Nitrobacter species in nitrifying sludge was observed only by NIT1 in 3 of the sewage treatment plants investigated. Mobarry *et al.,* (1996) designed a set of five 16S rRNA targeted probes for phylogenetically defined groups of autotrophic ammonia-and nitrite-oxidizing bacteria. This includes a comprehensive probe set of both Nso190 and Nso1225 encompassing all sequenced ammonia-oxidizers of the beta subclass of Proteobacteria (Manz *et al.*, 1992). Site 19-35 for probe Alf1b was selected for use. Positions 29 within this Alf1b target site is not perfectly conserved within the alpha subclass of Proteobacteria: most species of this subclass have an A (adenine) but some have a G (guanine). Thus to detect all members of this class, the probe Alf1b is produced by a 1:1 mixture of 2 oligonucleotides (Manz *et al.*, 1992).

2.15.3 Basic steps in probe design and applications for *in situ* identification

2.15.3.1 Synthesis of fluorescent oligonucleotides

Oligonucleotides with an average length of 15-25 nucleotides are chemically synthesized. An aminoethylphosphate-linker is attached to the 5' end of the oligonucleotide. A range of activated labels e.g. biotin, enzymes, fluorescein dyes can be coupled to the primary amino group rhodamine and tetramethylrhodamine. The biotin and fluorescent dyes are available in an activated form (isothiocyanate and N-hydroxysuccinimide derivatives, Stahl and Amann, 1991).

2.15.3.2 Purification of fluorescent oligonucleotides

The oligonucleotide–dye conjugate is separated from the bulk of unreacted dye by passing the reaction mixture two times through spin columns equilibrated with water (Stahl and Amann, 1991).

2.15.3.3 Cell Fixation

Cell fixation or maintenance of morphological integrity is necessary as the sample is exposed to high temperature, detergents and osmotic gradients. Fixation allows for accessibility of the probe molecules to target nucleic acids, by increasing the permeability of cell periphery (Stahl and Amann, 1991).

2.15.3.4 Hybridization

In an isotonically equilibrated humidity chamber the binding of the probe to its target intracellular nucleic acid is allowed to take place. Stringent conditions have to be maintained to prevent non-specific binding (Stahl and Amann, 1991).

2.15.3.5 Viewing of hybrids

The hybrids are separated from any unreacted probe by rinsing with wash solution and briefly with distilled water. Fluorescence from hybridized cells is detected by epifluorescent microscopy.

2.16 Optimization of hybridization conditions for Proteobacteria-specific probes

Optimization of hybridization conditions entails balancing probe sensitivity (i.e. ensuring that all target sequences are detected) with hybridization stringency (i.e. ensuring that the probe binds only to the specific target sequence). Whole cell hybridization requires a minimization of thermic stress to maintain cell integrity. Manz and co-workers (1992) therefore sought to increase stringency by incrementally increasing the formamide concentration (5% intervals) in the hybridization buffer as apposed to increasing the temperature. Using six reference strains for dot blot analysis the following optimal hybridization conditions for the alpha, beta and gamma-Proteobacteria probes were obtained:

- Probe ALF1b; 20% formamide (0.9 M NaCl, 20 mM Tris/HCl pH 7.2, 0.01% SDS) at 46°C

- Probes BET42a and GAM42a; 45% formamide (0.9 M NaCl, 20 mM Tris/HCl pH 7.2, 0.01% SDS) at 46°C.

However whole cell hybridizations required the formamide concentration to be decreased to 35% for both probes BET42a and GAM42a in order to enhance probe sensitivity.

2.17 Enhancing probe specificity with competitor oligonucleotides

A competitor probe is usually an unlabeled (or differently labeled) oligonucleotide added to the hybridization mixture together with the labeled probe to block off sequences with one or more mismatches compared to the specific target site. This competitor bears a sequence that is complementary to the potential mismatched sequence. Manz and co-workers (1992) found that addition of competitor oligonucleotide in 10 fold excess of the labeled probe in dot blot analyses, resulted in a reduction of non-specific probe binding from 31%-2% for the GAM42a binding site with BET42a functioning as the unlabeled competitor. As a result conventional whole cell probe hybridizations for detecting members of the beta and gamma-Proteobacteria subclasses are carried out by adding both labeled probes simultaneously to the hybridization mixture.

2.18 Fixation of Gram-positive cells and optimization of whole cell hybridization

Experimental evidence suggests that fixation of Actinobacteria with paraformaldehyde is detrimental to whole cell hybridization with labeled oligonucleotides (Braun-Howland *et al.*, 1992; Kawaharasaki *et al.*, 1999). Roller and co-workers (1994) also found that whole cell hybridization of selected actinobacterial monocultures after parafomaldehyde fixation did not result in reliable visualization of these cells. Fixation in 50% ethanol showed homogenous, strong hybridization with fluorescently labeled oligonucleotide probes. The desired balance between stringency and sensitivity was obtained by gradually increasing the formamide concentration in the hybridization buffer. It was found that at formamide concentrations of 25% (v/v) the HGC probe could distinguish clearly between target and non-target cells (Roller *et al.*, 1994).

2.19 Factors affecting the hybridization

2.19.1 Melting point

The melting point is an important feature defining a given double helix structure. The break-down of the helix occurs at a specific melting temperature. The double-helix structure does not dissociate completely at a specific temperature. This occurs over a temperature range. The unzipping of the helix is influenced by nucleotide composition in the area of base pair formation or denaturation (Stahl and Amann, 1991).

2.19.2 Percentage of mismatched base pairs

A 1% base-pairing mismatch corresponds to about a 1-1.5°C in melting temperature (Tm). The rate of DNA reassociation is approximately halved (at the optimum temperature for reassociation) for every 10°C reduction (Stahl and Amann, 1991).

2.19.3 Influence of formamide

The melting point of double helix is lowered by addition of formamide. For every 1% increase in the concentration of formamide the Tm is reduced by about 0.7%. Thus by the addition of formamide to the hybridization solution, the hybridization can be carried out at a lower temperature without loss of high stringency. This avoids the loss of membrane bound nucleic acid at high temperatures, as well as increasing the life of nucleic acid by preventing degradation (Stahl and Amann, 1991) loss of membrane bound nucleic acids at high temperatures, as well as increasing the life of nucleic acid by preventing degradation (Stahl and Amann, 1991).

2.19.4 Probe length

The specificity of a probe is determined by its length and the complexity of the target sequence. A minimum probe size of about 9 oligonucleotides is required for a stable hybridization (Stahl and Amann, 1991).

2.20 DAPI: a simple but useful stain for BNR

The fluorescing stain, 4'6-diamidino-2-phenylindole (DAPI) was evaluated by Porter and Feig (1980) for identifying and enumerating aquatic microflora. DAPI is a highly specific stain for DNA under a wide range of conditions. When excited with light at a wavelength of 365 nm, the DNA-DAPI complex fluoresces a bright blue, while unbound DAPI and DAPI bound to non-DNA material may fluoresce a weak yellow. In order to provide a dark background for fluorescing bacteria Porter and Feig (1980) stained nucleopore filters with Irgalan Black for 12 hr prior to filtration. They found that the minimum period required to expose the sample to DAPI for effective visualization and counting of lake microflora was 5 min. DAPI was applied at a final concentration of 0.01 µg/mL. Hicks *et al.*, (1992) developed a method of dual staining samples with DAPI and fluorescently labeled oligonucleotide probes to allow for the effective quantification of bacterioplankton obtained from pond samples. Hicks and co-workers (1992) found that DAPI stained cells could be subsequently probed with fluorescently labeled oligonucleotides without affecting probe hybridization. Using this approach they were able to estimate the percentage of total bacterioplankton that hybridized with the fluorescent oligonucleotide probe.

2.20.1 Probe EUB, EUB/DAPI Ratio

Fluorescent oligonucleotide probing to the activated sludge samples can be tested with probe EUB, which is specific for the domain of bacteria. The amount of cells that hybridizes with the bacterial probe is indicative of the percentage of bacteria present. This also indicates that the cells have sufficient rRNA for detection, which was permeabilised for oligonucleotide probes by standard fixation (Wagner *et al.*, 1994). A high EUB/DAPI ratio shows that most bacteria are growing and are metabolically active. Bacteria stained with DAPI that do not hybridize with the probe EUB are either metabolically inactive or not permeable for oligonucleotide probes by standard fixation (Wagner and Amann, 1997).

2.21 Assessment of biomass

In the design and operation of activated sludge systems, it is important to have a relatively accurate measure of the mixed liquor suspended solids (MLSS). The MLSS is the dry weight of solids in mg/L of mixed liquor in an activated sludge system (Hantpols Technical Service Department, 2001). The MLSS represents the total amount of organic and inorganic solids and microorganisms. If the MLSS is too high, there is a problem of limited oxygen supply, owing to the high concentration of biomass (Gray, 1989). Most activated sludge plants operate with an average MLSS concentration of about 2500-3000 mg/L (Gray, 1989).

The organic portion of the MLSS is represented by the volatile suspended solids (VSS). The VSS has traditionally been used as a lumped indication of the active biomass present in the system. VSS comprises viable and non-viable microorganisms, cellular debris and unbiodegradable organic matter. VSS measurements have proven advantageous when doing mass balances. Mass balances are basically accounting for everything that goes into the system (influent + biomass), the changes that occur within the system during treatment and everything that comes out of the system (effluent). Clearly, the VSS is a poor reflection of the active biomass. Direct cell counts are usually performed to determine exactly what fraction of the VSS is active biomass. Liebeskind and Dohmann, (1994) applied the method of DNA extraction for biomass determination of activated sludge mixed liquor. They followed the procedure of using acid extraction of DNA, quantitative determination of the deoxyribose sugar by a color reaction with standard DNA and mathematical conversion of the measured DNA into biomass. They found that the conversional DNA method was strongly affected by unknown sludge constituents and in particular iron. They also found that treating the sludge with EDTA first improved DNA extraction; but concluded that they were not sure as to whether all of the DNA was successfully extracted.

The development of molecular probes enables the ability to elucidate the structure of complex microbial ecosystems. By directly targeting the cells genetic code bacteria can be identified and enumerated (Rittmann *et al.*, 1999). At present these methods are more suitable for bacteria identification and the study of particular organism species or groups, than for quantification of

the active biomass. However the methods appear to hold promise to provide quantitative data on active biomass after further investigation (Cronje *et al.,* 2002). To overcome the problems associated with other methods to determine viable bacterial numbers, techniques have been developed to count viable cells by microscopic examination of samples, like total cell count which involves counting of samples dried on the slides or on samples in liquid. The principle deficiency identified with this method was that it could not distinguish living cells from dead cells but this problem was overcome by staining the organisms with fluorescent dyes specific for living cells, and the fluorescent cells can then be counted under a microscope. The method is called epifluorescent microscopy and is combined with a membrane filter technique. Fluorescent dyes such as acradine orange (AO) and 4'6-diamidno-2-phenylindole (DAPI) are used as they help in microscopic counting. As an alternative to cell counts, biomass volume can be determined. Both the cell count and biomass volume can be converted to biomass using the conversion factor such as the weight of carbon per cell or cell volume (Cronje *et al.,* 2001).

Epifluorescence microscopy has been used for three types of measurements on bacteria: the total numbers of bacteria, the total carbon biomass and the living biomass (or numbers). The basic principle of this method is to dilute the sample with phosphate buffer to give a suitable density for counting, stain with fluorochrome, and collect bacterial cells on membrane filters. The filters are subsequently illuminated through en epifluorescence condenser and the bacteria in a known volume are counted. From knowledge of the sample volume, dilution factor, and appropriate control counts, the number of bacteria per unit volume or area can be calculated. Although simple in principle, DAPI technique is subjective to systematic and random errors that can reduce analytical precision significantly. Major factors that affect this method are choice of membrane filter, desegregation, sample preservation and storage (Daley, 1979). The advantages of this method are that it allows direct and specific measurement of the number of bacteria, is not affected by the presence of extracellular materials and is independent of this method to activated sludge mixed liquor. The disadvantages are (i) yielding of inconsistent cell fluorescence, (ii) the method is complex, expensive and tedious, (iii) with the organisms in the activated sludge mixed liquor binding in flocs, counting of individual cells is difficult as the dispersion of the cells is a problem (Cronje *et al.,* 2001).

The DAPI technique was used by Porter and Feig (1980) for identification and enumeration of aquatic micro-flora. DAPI is a cell permanent stain, specifically binding to double-stranded DNA occurs with an approximately 20-folds fluorescence enhancement, which does not occur with single-stranded DNA. The DNA-DAPI complex fluoresces at a wavelength of 365 nm or at greater than 390 nm to produce bright blue when excited with light, while the unbound DAPI and DAPI bound to DAPI non DNA material may fluoresce a weak yellow. This enables the detection of cells and counting of less than 1µm bacteria and blue-green algae in seston-rich samples, and the sample storage is increased to at least 24 weeks (Porter and Feig, 1980).

2.21.1 Batch test method

A simple batch test method to quantify heterotrophic active biomass in activated sludge mixed liquor were described by Kappelar and Gujer (1992), where a small quantity of mixed liquor is mixed with centrifuged wastewater and the oxygen utilization rate (OUR) response is monitored with time. From the observed exponential increase in OUR, the initial OUR in the batch test can be determined, this can be used to derive an estimate for the heterotrophic active biomass concentration. This method was extended and modified in 1995 (Wentzel *et al.*, 1995 and Mbewe *et al.,* 1995) for application to the characterization of municipal wastewaters. This batch test was conducted on unsettled municipal wastewater without the addition of activated sludge mixed liquor. From the OUR-time response and floculated filtered COD measurement at the end of the test, the wastewater heterotrophic active biomass, readily biodegradable COD (RBCOD) and unbiodegradable soluble COD (USCOD) could be determined. A close correlation between the RBCOD and USCOD measured in the batch test and those measured via conventional methods were found by Mbewe *et al.,* (1995). However, they were not able to evaluate the results for wastewater heterotrophic active biomass, since no conventional tests was available. This simple batch test method was extended by Ubisi *et al.,* (1997a; 1997b) to quantify the OHO active biomass concentration in an activated sludge system. A small sample of mixed liquor is drawn from the activated sludge system and mixed with raw wastewater in a batch reactor where the oxygen utilization rate (OUR) and nitrate and nitrite concentrations are monitored with time. In parallel, a similar batch test is conducted on the raw wastewater without mixed liquor addition. From analysis of the OUR and nitrate and nitrite responses of the two parallel tests, the

mixed liquor OHO active biomass concentration can be quantified. This batch test was evaluated by drawing mixed liquor samples from well defined laboratory-scale anoxic/aerobic sludge systems operated at 12 and 20 d sludge age (Wentzel *et al.*, 1998). A comparison between the results from the batch tests and theoretical values for OHO active biomass concentrations from steady state design (WRC, 1984) and kinetic simulation models (Dold *et al.*, 1991) was made and they concluded that the batch test method may prove to be a valuable tool that can be used.

2.21.1.1 Parent system theoretical heterotrophic active biomass.

The theoretical heterotrophic active biomass of the mixed liquor drawn from the parent system and added to the batch tests was calculated by Ubisi *et al.*, 1997a. This can be achieved by one of two approaches. Either the steady state design or the kinetic simulation models could be used.
Steady state model:
From WRC (1984) the heterotrophic active biomass fraction of the mixed liquor volatile suspended solids (VSS) (f_{av}) can be determined from:

$$f_{av} = MX_{BH}/MX_V$$
$$= MX_{BH}/(MX_{BH} + MX_E + MX_I + MX_{BA}) \quad (1.1)$$

Where;

MX_{BH} = mass of heterotrophic active biomass, VSS units (mgVSS)
$= V \cdot X_{BH}$
MX_E = mass of endogenous material, VSS units (mg VSS)
$= V \cdot X_E$
MX_I = mass of inert material, VSS units (mgVSS)
$= V \cdot X_{BA}$
V = system volume (L)
X_{BH} = heterotrophic active biomass concentration, VSS units (mgVSS/L)
X_E = endogenous material concentration, VSS units (mgVSS/L)
X_I = inert material concentration, VSS units (mg VSS/L)

X_{BA} = autotrophic active biomass concentration, VSS units (mgVSS/L)

MX_V = mass of volatile suspended solids, VSS units (mgVSS)

= $V \cdot X_V$

X_V = volatile suspended solids concentration, VSS units (mg VSS/L)

The autotrophic active biomass (M) components of the mixed liquor organic suspended solids is very small compared to the other three components for activated sludge systems receiving normal municipal wastewaters, in eq (1.1). Therefore the autotrophic active biomass can be neglected when calculating the mixed liquor VSS, with very little error. Accordingly, from WRC (1984), substituting in eq (1.1) for MX_{BH} and $MX_V = (MX_{BH} + MX_E + MX_I)$:

$$\frac{1}{f_{av}} = 1 + f_E{*}b_{HT}{*}R_S + \frac{f_{S,up}\,(1 + b_{HT}{*}R_S)}{f_{cv}\,Y_H{*}(1 - f_{S,us} - f_{S,up})} \qquad (1.2)$$

Where;

f_E* = fraction of heterotrophic active biomass that is endogenous residue

= 0.2 (endogenous respiration theory, Dold *et al.* 1980)

b_{HT}* = specific endogenous mass loss rate at temperature T (/d)

= b_{H2O}* 1.029

b_{H2O}* = specific endogenous mass loss rate at 20 ºC

= 0.24/d @ 20ºC (endogenous respiration theory, Dold *et al.* 1980)

R_S = system sludge age (d)

= 12 d

$f_{S,up}$ = fraction of influent substrate that is unbiodegradable particulate

$f_{S,us}$ = fraction of influent substrate that is unbiodegradable soluble

f_{CV} = COD to VSS ratio of mixed liquor organic suspended solids (mgCOD/mgVSS)

Y_H* = heterotrophic active biomass yield, VSS units (mgVSS/mgCOD)

= 0.45 mgVSS/mgCOD (WRC, 1984)

The kinetic simulation models include autotrophic active biomass and non-utilized slowly biodegradable COD in the mixed liquor organic suspended solids (MLOSS), whereas these are ignored in the steady state design models. The f_{av} from either the steady state or the kinetic simulation models could be used, due to their near equality.
Knowing f_{av} and the concentration of the mixed liquor VSS that was drawn from the parent system to be added to the batch tests, the theoretical heterotrophic active biomass concentration in the batch reactor due to the added mixed liquor is given by:

$$Z_{BH}\,(theo)_{BT} = [X_V\,(PS) \cdot f_{av} \cdot f_{CV} \cdot V_{ML}]/(V_{ML} + V_{WW}) \qquad (1.3)$$

Where;

$Z_{BH}\,(theo)_{BT}$ = theoretical heterotrophic active biomass concentration in batch test reactor due to added mixed liquor, COD units (mgCOD/L batch reactor)
$X_V\,(PS)$ = mixed liquor VSS concentration measured in parent system, (mgVSS/L)
V_{ML} = volume of mixed liquor from parent system added to batch test, (L)
V_{WW} = volume of wastewater added to batch test, (L)

In eq (1.3) the parent system mixed liquor organic suspended solids are expressed in VSS units, whereas the heterotrophic active biomass is expressed in COD units. This is because the conventional measurement of the mixed liquor suspended solids in activated sludge systems is done via the VSS test, whereas the kinetic models used to develop the batch test are in terms of the COD parameter. The two units are however directly related through the COD/VSS ratio of the mixed liquor organic suspended solids (f_{CV}). If a value for f_{CV} is not available from measurement, the standard of 1.48 mgCOD/mgVSS (WRC, 1984) can be accepted.

2.21.1.2 Batch test

Wentzel *et al.,* (1995) applied the UCT model (Dold *et al.* 1991) to the waste water only batch test and noted that the model could be simplified if it is recognized that specific conditions prevail in the batch test, namely:

- Aerobic conditions-denitrification processes need not be included.
- No nitrification, nitrification processes need not be included. Excess ammonia present.
- Nitrate as an N-source for growth need not be considered.
- Transformations from organic to ammonia N need not be included.

Accepting these conditions a simplified UCT model can be developed that is applicable to the batch test (Beehary *et al.*, 2001).

From the simplified UCT model, the rate of growth of heterotrophic biomass (dZ_{BH}/dt) is given by:

dZ_{BH}/dt = growth on RBCOD + growth on SBCOD - death

$$\frac{dZ_{BH}}{dt} = \mu_H \frac{S_{bs}}{K_{SH} + S_{bs}} + K_{MP} \frac{S_{ads}/Z_{BH}}{K_{SP} + S_{ads}/Z_{BH}} Z_{BH} - b_H Z_{BH}$$

where

Z_{BH} = heterotrophic active biomass concentration (mgCOD/L)

S_{bs} = RBCOD concentration (mgCOD/L)

K_{SH} = half saturation constant for RBCOD
= 5 mgCOD/L (Dold *et al.* 1991)

S_{ads} = absorbed SBCOD concentration (mgCOD/L)

K_{SP} = half saturation constant for SBCOD
= 0.027 mgCOD/mgCOD (Dold *et al.* 1991)

It can be accepted that during the initial stages of the batch test $S_{bs} >> K_{SH}$ and $S_{ads} / Z_{BH} >> K_{SP}$, and therefore,

$$\frac{dZ_{BH}}{dt} = (\mu_H + K_{MP} - b_H) \cdot Z_{BH} \qquad (1.4)$$

Integrating eq (1.4) and solving yields the active organism concentration at time t [$Z_{BH(t)}$, mgCOD/L] in terms of the initial active organism concentration [$Z_{BH(o)}$, mgCOD/L], time (t in hr) and the net specific growth rate ($\mu_H + K_{MP} - b_H$) viz;

$$OUR_{(t)} = \frac{1 - Y_{ZH}}{Y_{ZH}} (\mu_H + K_{MP}) Z_{BH(t)}/24 \qquad (1.5)$$

Substituting eq (1.3) for $Z_{BH(t)}$ in eq (1.5) and taking natural logs yields

$$\ln OUR_{(t)} = \frac{1 - Y_{ZH}}{Y_{ZH}} (\mu_H + K_{MP}) Z_{BH(0)}/24 + (\mu_H + K_{MP} - b_H)\, t/24$$

which is a straight line with,

$$\text{slope} = (\mu_H + K_{MP} - b_H)/24$$

$$\text{y-intercept} = \ln OUR_{(t=0)} = \ln \frac{1 - Y_{ZH}}{Y_{ZH}} (\mu_H + K_{MP}) Z_{BH(0)}/24$$

To determine heterotrophic active biomass at the start of the batch test ($Z_{BH(0)}$), the OUR values for the data up to the precipitous drop in OUR are plotted Ln OUR versus time (hr), and linear regression applied to determine the Y-intercept, slope and correlation coefficient. From the slopes y-intercepts, $Z_{BH(0)}$ can be determined (Wentzel *et al.* 1995).

$$Z_{BH(0)} = \frac{e^{\text{y-intercept}} \cdot 24}{\frac{1 - Y_{ZH}}{Y_{ZH}} \cdot (\text{slope} \cdot 24 + b_{HT})}$$

Where;

$Z_{BH(0)}$ = heterotrophic active biomass concentration at the start of the batch test (mgCOD/L batch reactor)

Y_{ZH} = heterotrophic active biomass yield, COD units (mgCOD/mgCOD)

b_{HT} = heterotrophic specific death rate at temperature T (d)

= b_{H2O} $1.029^{(T-20)}$

b_{H2O} = heterotrophic specific death rate at 20ºC

= 0.62/d (death/regeneration theory, Dold *et al.* 1980; Wentzel *et al.* 1995).

2.21.2 Measured versus theoretical heterotrophic active biomass concentrations

The values of the measured and theoretical mixed liquor $Z_{BH(0)}$ (ML) are plotted against each other to compare. Ubisi *et al.,* (1997a; 1997b) observed good agreement between the measured and theoretical values for a 12 d sludge age but for the 20 d sludge age system the values compared poorly, with the theoretical values being approximately twice those measured. These contrasting results raised uncertainty about the reliability of these results obtained from the batch test. Cronje *et al.,* (2000) suggested a modification in the batch test method. These include the removal of the heterotrophic active biomass from the wastewater through pre-flocculation and filtration.

2.22 Background on MLE process

The MLE process is a basic activated sludge process developed for single sludge biological denitrification, proposed by Ludzack-Ettinger and later modified by Barnard (Lilly *et al.,* 1997). It utilizes readily biodegradable organic matter in the influent as an energy source for denitrification. The process consists of two reactors (anoxic and aerobic) in series, completely separated from each other. The influent is fed to the first reactor, which is maintained in an anoxic state by stirring without aeration, to supply organic matter used as energy and carbon source for dinitrification. The denitrified effluent is aerated in the second reactor where nitrification takes place. The settled activated sludge from the settler is recycled to the anoxic reactor and there is an additional internal recycle of mixed liquor from the aerobic zone to the anoxic zone (Van Haandel *et al.,* 1981). The system layout of the MLE process is shown in FIG 2.2.

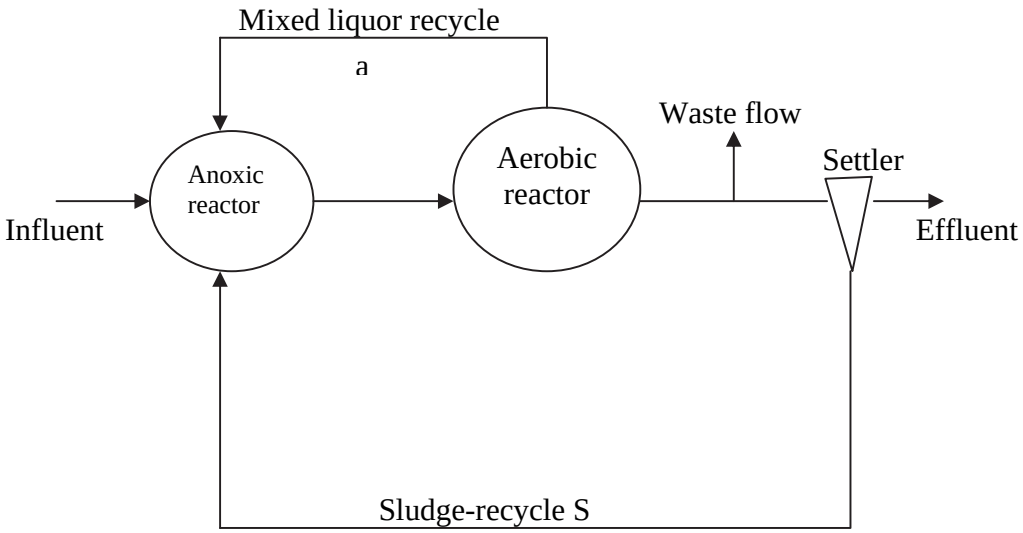

FIGURE 2.2 Schematic layout of laboratory-scale MLE process.

2.23 Background of Johannesburg process

The Johannesburg system was developed to overcome the major disadvantage of the Phoredox system in that nitrate present in the underflow will be discharged to the anaerobic zone, thus reducing the efficiency of the zone. The S-recycle passes through a small anoxic zone in the underflow. Any nitrate present in the underflow will be denitrified before being discharged to the anaerobic zone (Lilly *et al.*, 1997; FIG 2.3).

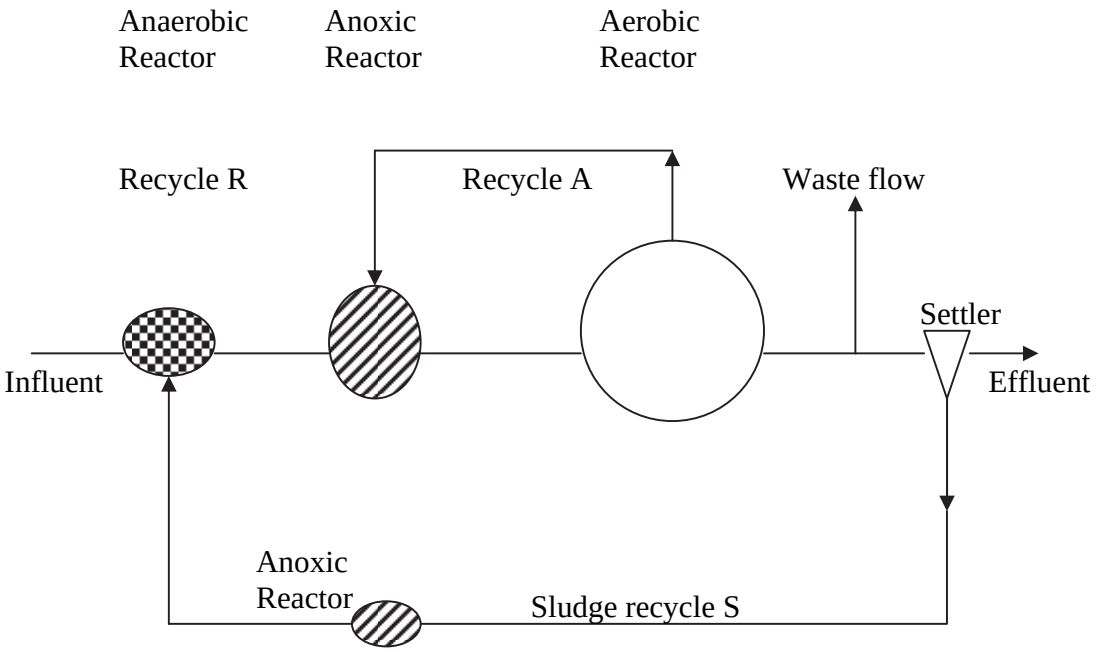

FIGURE 2.3 Schematic layout of laboratory-scale Johannesburg process.

CHAPTER THREE

THE RELATIONSHIP BETWEEN rRNA AND METABOLIC ACTIVITY

3.1 INTRODUCTION

The advance of molecular microbiology is making the direct determination of active biomass fractions in activated sludge systems a tractable problem (Oether *et al.,* 1999; Rittmann *et al.,* 1999). However, the application of fluorescently labeled oligonucleotide probes targeting the rRNA in cell-free or intact bacterial cells assumes that the detected cells have been metabolically active up to the point of sampling. The correlation between cellular RNA content (the majority of which comprises rRNA) and growth rate has been shown for monocultures of *Salmonella typhimurium* from as early as 1958 (Shaechter *et al.,* 1958). Delong *et al.,* (1989), have also shown that the RNA:DNA ratio of *Escherichia coli* monocultures are linearly correlated with the growth rate over a range of μ=0.3-1.6 h^{-1}. Also, for starving *E. coli* cells ribosome degradation has been shown to precede a loss of viability (Davis *et al.,* 1986). However, there is evidence to suggest that such trends are not universal; bacteria with low specific growth rates, growing in nutrient limited environments, like *Nitrosomonas* spp. and marine strains of *Vibrio* spp. have been found to maintain their rRNA intact, even after the cessation of metabolic activity (Wagner *et al.,* 1995; Flärdh *et al.,* 1992). Since the activated sludge process is generally a nutrient-limited environment, and in EBPR particularly a key biomass component the PAO's have low specific growth rates; it is necessary to evaluate the correlation between RNA content and metabolic activity prior to the application of rRNA directed FISH studies for biomass determination in BEPR systems.

In order to investigate the relationship between rRNA content and metabolic activity a batch test was devised to monitor the metabolic activity of the aerobic biomass component of a full-scale EBPR process via oxygen utilization rate (OUR) measurements. The biomass was sampled at various time intervals and total cell counts and RNA extractions were performed. The OUR profile was compared to the average RNA content per cell to investigate the relationship between the two.

3.2 MATERIALS AND METHODS

3.2.1 Batch test set-up

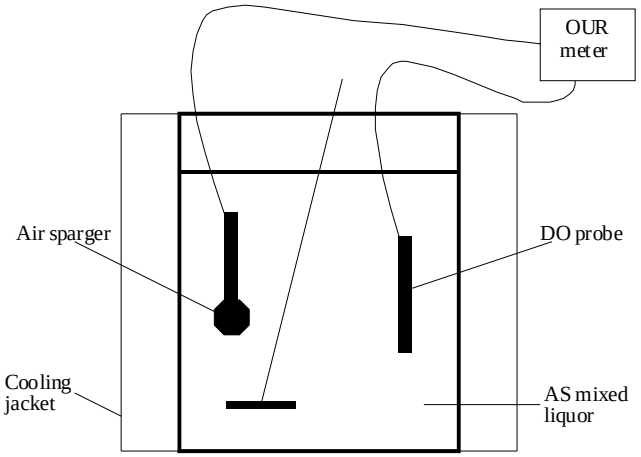

FIGURE 3.1 A schematic representation of the batch experiment to investigate the relationship between rRNA and heterotrophic/autotrophic activity.

A batch test was conducted using activated sludge from the anaerobic zone of the Darvill Wastewater Works as a seed inoculum. The wastewater (containing SCFA's) from the pre-fermenter feeding the anaerobic zone of the plant was used as a readily biodegradable substrate source. Air supply to the reactor was controlled by an oxygen utilization rate (OUR) meter (University of Cape Town) such that the aquarium air pumps switched on at a DO concentration of 2.0 mg/L and switched off at DO concentrations of 4.0 mg/L. The surface of the activated sludge mixed liquor was covered by small molded polystyrene pieces to prevent oxygen diffusion between the liquid-air interface (Wentzel *et al*., 1998). The pH was maintained at 7.4 (± 0.2) and temperature at 20^{o}C (± 2^{o}C). Samples of 20 mL activated sludge mixed liquor was taken every 30 min over a period of 12 hr for total cell counts (APPENDIX 5) and RNA extractions (APPENDIX 8). Mass balances were not performed across the reactor. Refer to FIG 3.1 for a schematic representation of the batch experiment.

3.2.2 Measurement of COD, MLVSS and S_o/X_o

In order for the biomass in the batch test to be representative of the original treatment environment Chudoba *et al.*, (1991) and Chudoba *et al.*, (1992) recommended a S_o/X_o ratio (on a COD basis) of <2. The MLVSS was measured and adjusted to approximately 2800 mg/L (APPENDIX 1) and the filtered COD (0.45 μm pore size, APPENDIX 1) was adjusted to approximately 500 mg/L. Taking f_{CV} = 1.42 mgCOD/ mgVSS (WRC, 1984), S_o/X_o (on a COD basis) was calculated as follows:

COD (S_o) = 500 mg/L
MLVSS = 2800 mg/L;

X_o = 2800 mgVSS/L x 1.42 mg COD/mgVSS
= 3976 mg COD/L
S_o/X_o = 0.13

3.3 RESULTS

3.3.1 Observed changes in COD and VSS

TABLE 3.1 Substrate and VSS values measured at t=0 hr and t=12 hr for the batch experiment.

Parameter	Time Initial (0 h)	Time Final (12 h)
Filtered COD (mg/L)	521	108
MLSS (mg/L)	3610	3746
VSS (mg/L)	2780	3190

The measured COD has been termed filtered COD and not soluble COD since it has been shown that particulate colloidal matter can pass through a filter having a pore size of 0.45 μm (Marais *et*

al., 1993; Dold *et al.*, 1980). From TABLE 3.1 it is clear that approximately 80% of the original filtered COD has been consumed by the biomass with a concomitant increase in VSS from 2780-3190 mg/L after the completion of the batch test.

3.3.2 OUR and changes in cell numbers

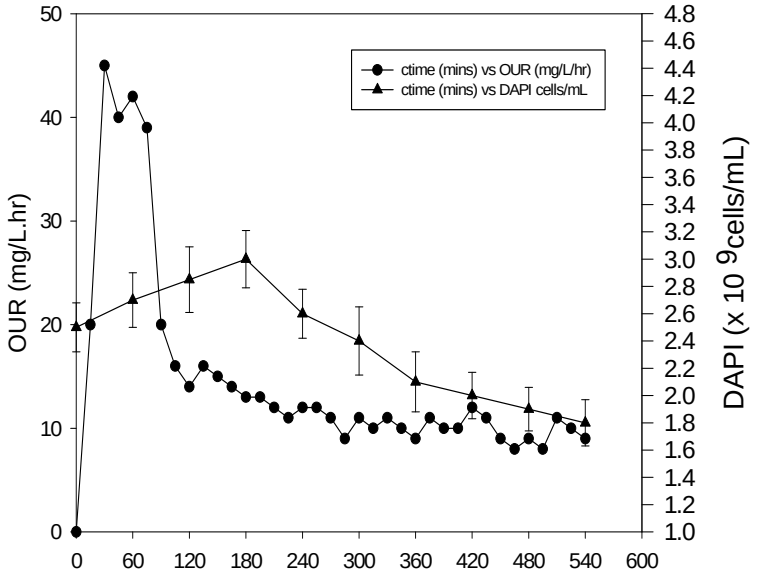

FIGURE 3.2 Oxygen Utilization Rate and DAPI cell counts versus time for the batch experiment.

The respirogram in FIG 3.2 shows the characteristic profile of an initial peak phase due to the rapid consumption of readily biodegradable COD followed by a tail phase due to metabolism of exogenous slowly biodegradable COD (Wentzel *et al.*, 1995) or internal storage compounds like polyhydroxyalkanoates (PHA) or glycogen (Dircks *et al.*, 1999). From an original total cell count of approximately 2.5 x 10^9 cells/mL the cell numbers increase to a maximum of approximately 3.0x10^9 cells/mL over a period of 3 hr (t=0 to t=180 min). Cell numbers then drop steadily to a value of 1.8x10^9cells/mL at t=12 hr.

3.3.3 OUR and changes in cell-free RNA

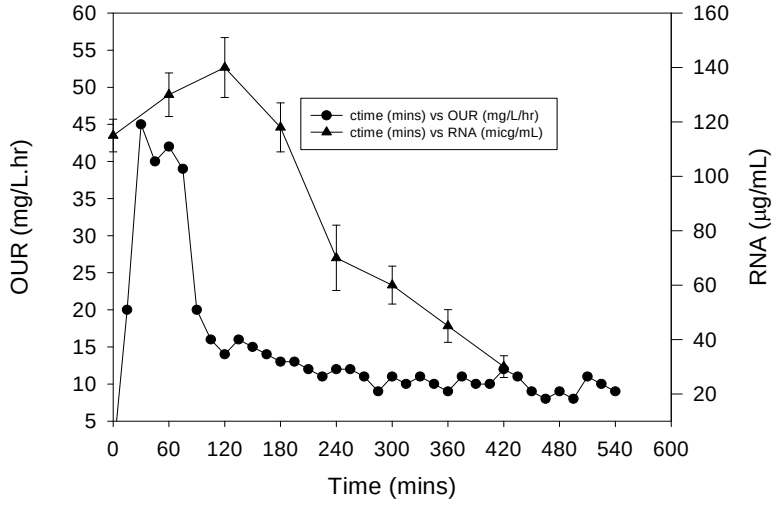

FIGURE 3.3 Oxygen utilisation rate and cell free RNA versus time for the batch experiment.

FIG 3.3 indicates an initial increase in total RNA from 115 to 140 µg/mL of mixed liquor synthesized by biomass in the system over the first 2 hr of the batch test. The RNA then proceeds to be degraded at a relatively rapid rate over the next 2 hr to approximately 70 µg/mL. This is then followed by a period where the system RNA is degraded at a slower rate over the following three hours to approximately 30 µg/mL. From data presented in FIG 3.2 and 3.3 it is possible to estimate the average amount of RNA per cell for the biomass active in the system. This is given in FIG 3.4.

3.3.4 OUR and changes in RNA per cell

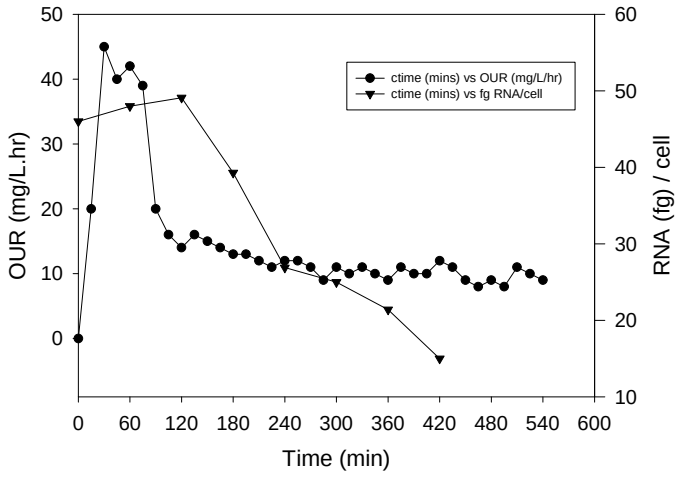

FIGURE 3.4 Oxygen utilization rate and RNA/cell versus time for the batch experiment.

FIG 3.4 indicates a very slight increase in the average amount of RNA per cell from 46 mg/cell to 49 mg/cell over the first 2 hr of the batch test. There is then a sudden drop in the RNA per cell over the next 2 hr to approximately 27 mg/cell, which is followed by a period of RNA degradation at a slower rate over the following three hours to a final concentration of 15 mg/cell.

3.4 DISCUSSION

3.4.1 Changes in filtrate COD and VSS

The 20% of initial total filtered COD remaining at the end of the batch test (TABLE 3.1; t =12 hr) represents non-biodegradable COD which could be inert soluble and (colloidal) particulate COD either originating from the influent (WRC, 1984) or residue from endogenous respiration (cell lysis) processes occurring in the biomass (Dold et al, 1980) during the batch test. The biodegradable fraction from the filtered COD (TABLE 3.1) has been utilized by the biomass for generating energy and for incorporation into new cell material; either by cell multiplication or for

metabolic processes of maintenance and storage (Chudoba *et al.,* 1992). The VSS of the system increased from 2880 mg/L to 3290 mg/L. This increase in VSS reflects the conversion of the biodegradable COD into biomass by their metabolic activity of utilizing oxygen to generate free energy (WRC, 1984). The initial high OUR observed (FIGS 3.2, 3.3, 3.4) are related only to the initial energy generated from oxidation of RBCOD substrates. The OUR does not indicate as to how the cell utilizes the energy from substrate oxidation (Pollard *et al.,* 1998). Also, the VSS measurement by itself can give no indication as to whether cell division or a storage response has occurred in the conversion of substrate from the bulk liquid to the particulate (cellular) phase of the system. The nature of the biomass response can only be determined by the direct determination of changes occurring at a cellular level in the system. A So/Xo ratio of 0.13 has proven suitable for representing the biomass of the original treatment environment since negligible cell multiplication has occurred.

3.4.2 Changes in cell numbers

According to Chudoba *et al*., (1991; 1992), at an So/Xo ratio <2 the cell multiplication response is negligible due to limiting substrate availability for the biomass and therefore, there is insufficient energy available for growth purposes. The increase in cell numbers from 2.5×10^9 to 3.0×10^9 observed in FIG 3.2 (t=0 to t=180 min) can be described as negligible, and may represent cell multiplication by those species possessing relatively higher specific growth rates (Poulsen *et al.,* 1993; Grady *et al,* 1996). Alternatively, the presence of PAO's which have accumulated PHA's in the anaerobic zone could have enabled them to supersede a phase of exclusive storage and accumulation and also utilize the RBCOD for growth (multiplication) purposes under aerobic conditions. Thus, in spite of their relatively lower specific growth rates (Mino *et al.,* 1998a; van Loosdrecht and Heijnen, 1997) the PAO's may have gained a competitive advantage over the other organisms due to the prior exposure to anaerobic conditions. It is also likely that the storage response of the PAO's has been one of glycogen replenishment (Mino *et al.,* 1998a). Such interpretations of the data remain hypothetical, since the biomass has been collectively monitored as a 'surrogate' organism (Wentzel and Ekama, 1997); individual biomass fractions have not been targeted, nor have internal storage polymers been analyzed. Most importantly however, it can be said that the batch experiment retains its

validity as being representative of the biomass in the original BEPR treatment environment since the population could not have shifted significantly with the negligible degree of growth observed (FIG 3.2).

Energy generated from the oxidation of exogenous RBCOD by the biomass can be allocated into a complex array of cellular functions. These energy resources may be allocated to cell maintenance and new cell synthesis (Grady *et al.,* 1996; Chudoba *et al.,* 1991; Chudoba *et al.,* 1992; Speece *et al.,* 1973) the accumulation of endogenous polymers (that can again be oxidized) (Mino *et al.,* 1998a; Smolders *et al.,* 1994 a; Smolders *et al.,* 1994b; Wentzel *et al.,* 1986) and secreted exopolymers (Jorand *et al.,* 1998; Bura *et al.,* 1998).

3.4.3 Change in system RNA composition

The slight increase in cell-free RNA for the system (FIG 3.3) and the RNA/cell (FIG 3.4) are probably related to only a small increase in the VSS due to activation of previously inactive ribosomal units without the need for novel synthesis of rRNA (Koch, 1971). This is because the So/Xo ratio remains low resulting in *de novo* protein synthesis and enzyme induction being minimized (Grady *et al.,* 1996). Shortly after the precipitous decrease in OUR, cellular RNA levels (FIG 3.4) also begin to drop sharply. During this first phase of RNA degradation the biomass undergoes a shift in metabolism to more slowly biodegradable COD that has adsorbed on to the cell surface (Burke *et al,* 1986) or could represent respiration on internal storage compounds such as PHA (Dircks *et al.,* 1999) and is substantiated by an OUR profile during this phase (t=120 min to t=240 min) which is characteristic of metabolism on SBCOD (Wentzel *et al.,* 1995). The final phase of slower RNA degradation represented at t=240 min to t=420 min in FIG 3.3 may indicate that even though the biomass has exhausted most of the available biodegradable COD by this period, the cells maintain a reserve pool of rRNA to initiate *de novo* protein synthesis in the event of favorable conditions returning (Wagner *et al.,* 1995; Flärdh *et al.,* 1992; Koch, 1971). The overall effect however, as seen in FIG 3.4 from t=120 min to t=240 min is that the RNA content per cell drops appreciably after the cessation of metabolic activity (Dawes, 1986, Davis *et al.,* 1986).

3.5 CONCLUSIONS

i. The results confirm the usefulness of the rRNA molecule as a target for the detection of biomass activity.

ii. The direct monitoring of cell numbers gives more information of the metabolic response of the activated sludge population employed in the batch assay.

iii. FISH is a therefore a usable tool to target active bacteria in the activated sludge system.

CHAPTER FOUR

FISH AND DOT BLOTS IN EBPR USING GROUP SPECIFIC OLIGONUCLEOTIDE PROBES

4.1 INTRODUCTION

Molecular based analysis involving suitably labeled oligonucleotide probes has emerged as a popular tool to facilitate a better understanding of the structure and function of the microbial community comprising both natural (Stahl *et al.*, 1988) and engineered ecosystems (Kawaharasaki *et al.,* 1998). Increasingly, dynamic models involving enhanced biological phosphate removal (EBPR) are beginning to include more specific microbiological and biochemical information from lower levels of organization contained within the surrogate biomass (Wentzel *et al*., 1998). The direct determination and quantification of that portion of the population responsible for the release and subsequent uptake of polyphosphate the polyphosphate accumulating organisms (PAO's) has been particularly elusive (Tandoi, 2000; Mino *et al.,* 1998a).

Research undertaken to elucidate the PAO population implicated in EBPR identifies particular bacterial phylogenetic families as being predominant in either SBR operated in aerobic/anaerobic cyclic conditions or continuous anaerobic/anoxic/aerobic conditions to stimulate EBPR. These studies indicate the predominance of the beta-Proteobacteria and Gram-positive bacteria with a high DNA (G+C) content. (Blackall *et al.,* 1998; Bond *et al.,* 1999). Sudiana *et al.,* (1998) found that the beta-Proteobacteria group predominated in activated sludge communities which had been acclimatized with acetate as the major carbon source under phosphorus limited, or rich conditions. Kawaharasaki *et al.,* (1999) found that although the beta-Proteobacteria were predominant in the EBPR sludge (64%) fed with acetate, they did not seem to accumulate large amounts of polyphosphate as revealed by staining with DAPI at a polyphosphate staining concentration. Instead up to 85% of the alpha-Proteobacteria which was only present at 7% of the total population was inferred to accumulate large amounts of polyphosphate.

The studies reviewed above were conducted using sequencing batch reactor-type processes. In South Africa most full-scale EBPR processes (e.g., the Darvill Wastewater Works) are of a continuous type. The influence of the process-type on the structure of the microbial community comprising the system has been extensively researched (Pollard *et al.*, 1998; Grady *et al.*, 1996; Chudoba *et al.*, 1992) and findings suggest that population shifts are likely to occur between the microbial communities found in each process type. Mudaly *et al.*, (2000) have also found a high degree of microbial diversity in a continuous type process designed to stimulate the EBPR mechanism within the PAO biomass. Their findings suggest that the alpha-Proteobacteria might also be implicated in EBPR.

This study therefore involves the determination of the phylogenetic affiliation (at a family level) of the microbial community comprising a full-scale continuous EBPR process, viz., the Darvill Wastewater Works, in order to investigate to what extent the community profile correlates with communities found in acetate-enriched pilot scale systems.

4.2 METHODS AND MATERIALS

4.2.1 Sampling of mixed liquor and cell fixation

Grab samples of activated sludge mixed liquor were collected from the anaerobic, anoxic, and aerobic zones of the Darvill Wastewater Works. Samples were also collected from the Amanzimtoti Wastewater Works (Durban, South Africa), where secondary biological treatment consists of two parallel activated sludge units. Each unit is divided into six aeration bands consisting of four surface aeration units per band (Pillay, 1998). Representative bacterial strains used to evaluate probe specificity, are given in TABLE 4.1. Monocultures were grown aerobically at 30^{o}C in nutrient broth and harvested at mid-logarithmic phase in order to ensure a high cellular rRNA content. For *in situ* hybridization activated sludge samples and monocultures were fixed for 2 hr with 3% paraformaldehyde/PBS (APPENDIX 3). For fixation of Gram-positive cells activated sludge was added to ethanol to a final concentration of 50% (v/v) (Roller *et al.*, 1994).

TABLE 4.1 Bacterial reference strains used to assess probe specificity.

Organism	Source	Phylogenetic affiliation
Acetobacter acetii	SABS	alpha (α)-Proteobacteria
Alcaligenes faecalis	ATCC	beta (β)-Proteobacteria
Acinetobacter calcoaceticus	ATCC	gamma (γ)-Proteobacteria,
Corynebacterium glutamicum	SABS	Gram-positive high G+C (GPBHGC)

SABS - South African Bureau of Standards culture collection

ATCC - American Type Culture Collection

4.2.2 Membrane filtration and staining with DAPI

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

4.2.3 Cultivation and plate counts

Serial dilutions (10^{-2}-10^{-6}), using 9 mL PBS, were performed on homogenized (1 mm glass beads) mixed liquor samples. 0.1 mL aliquots of each dilution were spread in duplicate on individual CGY agar plates (APPENDIX 2) and incubated at 20^{o}C for 5 d. Plates containing between 30-100 CFU's were scored and retained for further study. Well developed, individual colonies were re-streaked on solid isolation medium (CGY agar) and incubated for a further 5 d at 20^{o}C. Isolates were then identified according to the API 20NE identification system (BioMérieux, France). Isolates corresponding to family and species level groups of interest were quantified and expressed as fractions of total plate counts.

4.2.4 Nucleic acid extraction and membrane hybridization

Total nucleic acids were extracted from about 2 mL of activated sludge mixed liquor using the hot phenol method (APPENDIX 8). Total nucleic acids were resuspended in sterile ddH_2O for analysis. Extracted nucleic acids were spotted onto positively charged nylon membranes (Boehringer Mannheim, Germany) in 2x dilution series using a dot blotting device. Membrane hybridizations were carried out as detailed in APPENDIX 9. Hybridization stringencies (% formamide) for the probes used were as follows; 20% formamide for probes ALF1b and EUB338, 45% formamide for probes BET42a and GAM42a (Manz *et al.,* 1992); 25% for probe GPBHGC (Roller *et al.,* 1994).

4.2.5 Oligonucleotide probes

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

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

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

4.2.6 FISH hybridization

Aliquots of 5 µL fixed sludge were applied to poly-L-lysinated slides and allowed to air dry. Hybridization was carried out as detailed in APPENDIX 7. The formamide stringencies for the different probes were as follows; EUB338; 20%, ALF1b; 25%, BET42a; 35%, GAM42a; 35%, GPHGC; 25%, ACA23a; 35%.

4.2.7 Microscopy and image analysis

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

4.3 RESULTS

4.3.1 Community profiles

TABLE 4.3 Probe specific cell counts compared to culture dependant enumeration.

	Full-scale Non-EBPR		**Full-scale EBPR**		
	Cultivation	**FISH[a]**	**Cultivation**	**FISH[a]**	**DOT BLOT[b]**
α	13	12±4	12±3	28±5	25
β	13	26±4	11±2	30±4	30
γ	54	17±3	70±5	13±3	20
HGC	0	13±3	0	8±2	10
Aeromonas	13	< 3	12±2	< 4	N/d
Pseudomonas	20	< 4	30±5	< 4	N/d
Acinetobacter	9	< 5	28±5	< 9	N/d
EUB		79±6	N/d	92±8	100

[a] = percentage of DAPI

[b] = percentage of EUB

Note that the genus level counts (*Pseudomonas* spp., *Aeromonas* spp., and *Acinetobacter* spp.) listed in TABLE 4.3 are given as average percentages for those selected fields (<10 fields) observed bearing probe signals and do not represent randomly selected average counts. The fact that their sum totals approach the total for the gamma-Proteobacteria probe in the P and NP sludge should therefore not be assumed to imply that the gamma-Proteobacteria have been accounted for by these three genera. Results are not given for individual aerobic, anoxic and anaerobic zones (in the case of the P sludge) since community composition was found to be stable across the reactors.

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

	Amanzimtoti WWTP non-EBPR Full-scale	**Darvill WWW[a] EBPR Full-scale**
Plate counts (CFU/mL)	$5.2x10^7$	$1.7x10^6$
DAPI (membrane filtration) (cells/mL)	$8.0x10^{10}$	$5.2x10^9$
Estimated active biomass (cells/mL)	$5.8x10^{10}$	$4.16x10^9$

[a] = aerobic zone

The active bacterial biomass in TABLE 4.4 was estimated by multiplying the EUB/DAPI ratio and the total cell count as obtained by membrane filtration (TABLE 4.3). Results are shown for the aerobic zone only.

4.3.2 Nucleic acid extractions

TABLE 4.5 RNA yields for different zones of Darvill Wastewater Works.

	Aerobic	**Anaerobic**	**Anoxic**
RNA (mgRNA/gVSS)	47±6	33±4	36±4

The 260/280 absorbance ratio for all samples were in the range of 1.8-2.0 which is appropriate for pure RNA (Lane, 1996).

4.4 DISCUSSION

Extracellular polymers (ECP) did not appear to inhibit probe efficacy since cells at the inner and peripheral regions of the flocs showed comparable probe uptake. Since rRNA content has been shown to be directly proportional to growth rates (Schaechter *et al.,* 1958; Delong *et al.,* 1989; Wallner *et al.,* 1993; Grouse *et al.,* 1996) and based on the findings of CHAPTER 2 of this work, it is assumed that all cells bearing probe conferred fluorescence are metabolically active. A high percentage of the activated sludge sampled from both nutrient and non-nutrient removal systems bound the probe EUB338 (TABLE 4.4) indicating that the majority of cells were metabolically active bacteria. The informal name "bacteria" is frequently used loosely to refer to all prokaryotes, but care should be taken to interpret its meaning in a particular context. Because this study employs oligonucleotide probes that are phylogenetically based, one should note that the domain Bacteria (previously referred to as kingdom Eubacteria) are considered phylogenetically distinct from the domain Archae (previously referred to as kingdom Archaebacteria) and in all foregoing discussion reference to bacteria excludes members of the domain Archae (Woese *et al.,* 1990).

4.4.1 Cultivation dependant counts versus FISH

Total plate counts were found to underestimate the metabolically active bacterial population by 3–4 orders of magnitude (TABLE 4.3). Cultivation-dependent plating methods were also found not to give an accurate description of the bacterial families constituting the sludge. The gamma-Proteobacteria subclass was overestimated and the alpha, beta-Proteobacteria, and Actinobacteria, were underestimated (TABLE 4.3). The overestimation of bacteria belonging to the γ-Proteobacteria could be specifically observed for the three genera *Pseudomonas*, *Aeromonas* and *Acinetobacter* and explains the major part of the "gamma-shift" caused by plating of activated sludge bacteria on nutrient rich agar media (Cloete and Muyima, 1997).

4.4.2 Community analysis

The application of the probe specific for *Acinetobacter* spp. enabled the clear visualization of cocco-bacilli occurring in either chains or clusters. While the gamma-subclass is present at a percentage of about 17% of the total community, the presence of *Acinetobacter* can be estimated to be approximately 9% of the total population, which comes to approximately 53% of the gamma-subclass population. Considering the observed diversity of the EBPR community it may be necessary to revisit the concept and definition of the term "dominance" for the EBPR process at the species level since a single geno-species present at 9% of the total DAPI count may indeed be a "predominating" population.

While most studies have indicated the predominance of members of the beta-Proteobacteria and/or GPBHGC in EBPR sludge (Kampfer *et. al*., 1996; Snaidr *et al*. 1997; Bond *et al.*, 1999) the findings presented here indicate that the α-Proteobacteria may also be implicated in EBPR. A clear shift in this particular population was observed to occur when comparing the NP sludge (11%) to the P sludge (28%) (ALF1b/DAPI, TABLE 4.4). It is possible that the reactor configuration (inclusion of an anoxic zone) and/or the type of system used (continuous process) may have resulted in this population coming into predominance. This might explain the discrepancies between the results obtained in the current study and work conducted using SBR's, operated in only aerobic/anaerobic modes, where the contribution of the alpha-Proteobacteria to the total active sludge biomass was found to be minimal. As such, a few possibilities arise as to the functional groups present within this family; viz., they may be PAO's capable of successfully competing in EBPR at low S_o/X_o ratio's and/or they may also be alpha-Proteobacteria representatives which are efficient denitrifiers at low S_o/X_o ratios (Grady *et al.,* 1996; Chudoba *et al.,* 1991 and Chudoba *et al.,* 1992). Members of the community belonging to the α-Proteobacteria were visualized to be of a uniform morphotype, being oval shaped and congregated in compact spherical flocs. It should be noted that most 16S rRNA from the phylum "spirochetes and relatives" and *Flexistipes sinusarabicus* (Manz *et al.,* 1992) also have the ALF1b binding site. Members of the α-Proteobacteria have been implicated by Kawaharasaki *et al.,* (1999) as being capable of storing large amounts of polyphosphate, although they were not found to predominate the EBPR sludge. Studies performed on a pilot scale continuous acetate

enriched system by Mudaly *et al.*, (2000) also showed the alpha-Proteobacteria to be present at levels of approximately 20% (ALF1b/DAPI).

While the FISH method presents a good indication of cell numbers, dot blots represent a good indication of the total rRNA belonging to the group of interest. The measurement of total rRNA is important since rRNA gives an indication of the relative levels of metabolic activity for the different groups implicated in EBPR based on the assumption that rRNA content is directly proportional to metabolic activity over a wide range (Schaechter *et al.*, 1958; Delong *et al.*, 1989; Walner *et al.*, 1993; Wagner *et al.*, 1993). The results presented here using dot blots indicate that the alpha-Proteobacteria are indeed metabolically active in EBPR systems (TABLE 4.4).

Bacteria belonging to the Actinobacteria were found in the lowest numbers (8%) at the Darvill Wastewater Works. This represents another discrepancy as far as the identification of the possible family level-affiliation of PAO's are concerned, since the Actinobacteria are also believed to include PAO's (Kampfer *et al.*, 1996; Bond *et al.*, 1999 and Crocetti *et al.*, 2000). There have been reports of problems with probe penetration of the thicker peptidoglycan cell wall layer found in this group (Kamfer *et al.*, 1996; Braun-Howland *et al.*, 1992; Roller *et al.*, 1994; Kawaharasaki *et al.*, 1999). In order to eliminate the possibility of bad probe efficacy *in situ*, dot blots were also carried out for this group on full-scale EBPR sludge. The results by dot blots also support the FISH determination for this group (TABLE 4.4).

The EBPR community is clearly dispersed over a wide phylogenetic range of at least four families but the findings shown in TABLE 4.3 suggest that the alpha and beta subclasses of the Proteobacteria predominate the total bacterial biomass present in the Darvill Wastewater Works, being present at 28% and 30% respectively. It is also noteworthy that there is a correlation between the community profile observed for the continuous pilot scale process investigated by Mudaly *et al.*, (2000) and the continuous full-scale system studied here since the alpha-Proteobacteria can be implicated as playing an important role in both systems. For the full-scale system however the gamma-Proteobacteria does not seem to be as predominant when compared to pilot-scale studies on EBPR (Mudaly *et al.*, 2000). The overall observed community profile by

FISH gains substantial support from the results of the dot blot investigations, all membrane hybridizations have also been performed at their maximal stringencies thus eliminating the possibility of non-specific hybridization. For the alpha-Proteobacteria the results of dot-blot investigations have been particularly revealing since even at hybridizations conducted with formamide concentrations ranging in 5% to 10% (v/v) excess of normal stringency, there was no appreciable decrease in signal intensity.

Considering that each family comprises numerous genera and species, and noting that the community belonging to the beta and gamma-Proteobacteria subclass displayed numerous morphotypes, it may be implied that bacteria capable of excess polyphosphate accumulation probably belong to different phylogenetic groups and cannot be ascribed exclusively to any one genus or species. Liu (1995) has reported at least three dominant morphologically distinguishable microorganisms being present in sludge with a high phosphate removal. A survey of twenty full-scale activated sludge plants operating in Japan conducted by Mino *et al.,* (1998b) showed beta-Proteobacteria to be most dominant, followed by the alpha subclass and Actinobacteria, respectively. They also concluded that the PAO population is quite diverse. It is therefore likely that there are a few possible population structures capable of mediating EBPR.

rRNA-based *in situ* studies have expanded perspectives with respect to the bacteria implicated in EBPR (Crocetti *et al.,* 2000; Mudaly *et al.,* 2000), and have revealed the disadvantage of cultivation-based determinations. However the isolation of PAO's remains to be fulfilled in order to confirm the physiological mechanisms hypothesized by biochemists (Mino *et al.*, 1998a; Smolders *et al.*, 1994a; 1994b; Wentzel *et al.,* 1986; 1991). The results shown in this study reveals substantial phylogenetic diversity with little or no representation among organisms previously studied in isolation. If phylogenetic differences between the bacterial divisions are reflected in substantial physiological differences (Hugenholtz *et al.,* 1998) then the results shown here may indicate why the determination and isolation of a 'model PAO organism' has proven to be so challenging (Tandoi, 2000; Kawaharasaki *et al.,* 1998; Nakamura *et al* 1991; Nakamura *et al.*, 1995) for microbiologists. The possibility of the lateral transfer of biochemical properties for polyphosphate accumulation between the divisions, as hypothesized for the photosynthetic complexes also cannot be ruled out (Blankenship, 1992).

4.5 CONCLUSIONS

i. Cultivation-dependant enumerative methods were found not to give an accurate reflection of the predominating bacterial groups or species;

ii. Community analysis conducted using FISH yields a good quantitative representation of the biomass within EBPR systems, showing that the microbial community displays a high degree of diversity;

iii. The gamma-shift observed in previous studies are explained by the over estimation of cultivation-dependant plating methods;

iv. The alpha, beta and gamma-Proteobacteria, and the Actinobacteria are implicated in EBPR, with both the alpha and beta-Proteobacteria probably playing a major role in full-scale continuous type processes.

CHAPTER FIVE

BIOMASS DETERMINATION FOR EBPR MIXED LIQUOR

5.1 INTRODUCTION

Engineers have traditionally used mixed liquor volatile suspended solids (MLVSS) as a 'lumped' indication of the active biomass within activated sludge systems. Although the MLVSS parameter has the advantage of fitting directly into mass balance equations, it has the disadvantage of not providing a true indication of the active biomass (X_B) present. This parameter represents not only X_B but also endogenous residue (dead cellular material; X_e) and inert particulate COD (originating from the influent; X_{ii}) present in the sludge. (Wentzel *et al.*, 1998.)

Within Activated Sludge Model No.2 (Henze *et al.*, 1995), X_B of nitrification/denitrification biological excess phosphorus removal (NDBEPR) sludge encompasses the following components;

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

Efforts to calibrate and validate the model kinetic and stoichiometric parameters do not currently involve the direct, experimental determination of mixed liquor concentrations of these microbial populations. Through advances in molecular biology, in particular, developments in the field of microbial evolution (Woese, 1987), tools have become available which may prove useful in furthering the descriptive and predictive capacity of current mathematical models for BEPR. In this study probe results for the determination of the total active bacterial biomass (X_B) for the full scale system are converted to mass units in order to determine what fraction of the measured VSS is metabolically active bacterial biomass.

5.2 METHODS AND MATERIALS

5.2.1 Growth of monocultures

In order to make a conversion from cell numbers to mass units a conversion factor is required. This conversion factor will have the units mg VSS/cell. Since the size of bacterial cells in nature varies between species (Bergey's, 1984) and with the growth rate (Dow and Wittenbury, 1980) any conversion factor for the total biomass will only be an estimate. In this study in addition to conversion factors determined by Mudaly *et al.,* (2001) and Munch and Pollard, (1997) overnight nutrient broth cultures of *Acetobacter acetii*, *Alcaligenes eutrophus*, *Corynebacterium glutamicum*, *Micrococcus luteus*, *Acinetobacter calcoaceticus* and *Pseudomonas aeruginosa* were grown in sterile, filtered activated sludge mixed liquor (Amanzimtoti Wastewater Works) (500 mg COD/L; 90 mL mixed liquor + 10 mL inoculums) and harvested in the mid-log growth phase for cell enumeration by DAPI (APPENDIX 5) and VSS determination (APPENDIX 1). Average operational conditions of VSS, S_{bi} for the Amanzimtoti Wastewater works were obtained from Pillay (1998), and specific operational conditions of VSS, S_{bi} and P-removal for the particular period of analysis at the Darvill Wastewater Works and the pilot-scale activated sludge plants were obtained from Singh (2000); and Mudaly *et al.,* (2000), respectively.

5.3 RESULTS AND DATA INTERPRETATION

5.3.1 Determination of VSS per unit cell

TABLE 5.1 VSS per unit cell for monocultures and activated sludge biomass

Biomass	DAPI cell counts (cells/mL)	VSS (mg/mL)	VSS/cell (mg)	Reference
Acetobacter acetii	$5x10^{9}$	0.290	$5.8x10^{-11}$	this study
Acetobacter acetii	$2.06x10^{9}$	0119	$5.77x10^{-11}$	this study
Alcaligenes eutrophus	$1.95x10^{9}$	0.110	$5.6x10^{-11}$	this study

Corynebacterium glutamicum	$2.45x10^{9}$	0.200	$8.16x10^{-11}$	this study
Corynebacterium glutamicum	$3.6\ x10^{9}$	0.198	$5.48x10^{-11}$	this study
Acinetobacter calcoaceticus	$2.21x10^{9}$	0.290	$13.12x10^{-11}$	this study
Alcaligenes faecalis	$4.28x10^{9}$	0.186	$4.35x10^{-11}$	this study
Micrococcus luteus	$7.48x10^{9}$	0.852	$11.4x10^{-11}$	this study
Pseudomonas aeruginosa	$4.65x10^{9}$	0.310	$6.66x10^{-11}$	this study
Pseudomonas aeruginosa	$3.38x10^{9}$	0.290	$8.58x10^{-11}$	this study
Gram positive cocci	$2.82x10^{9}$	0.458	$16.2x10^{-11}$	this study
Gram positive rods	$3.34x10^{9}$	0.250	$7.48x10^{-11}$	this study
Nitrobacter sp.	n/d	n/d	$9x10^{-11}$	Sanden *et al.*, 1996
Full-scale BNR	n/d	n/d	$14x10^{-11}$	Munch and Pollard, 1997
Pilot -scale BNR	$1.25x10^{10}$ **a**	0.71 **b**	$5.68x10^{-11}$	Mudaly *et al.*, (2001)

a = EUB cell count

b = VSS as biomass

From TABLE 5.1 the average VSS/cell (f_{VB}) can be calculated to be $8.49x10^{-11}$ mgVSS/cell.

TABLE 5.2 Biomass expressed in terms of key process functions for nutrient removal (pilot- and full- scale) and non-nutrient removal (full-scale) sludge.

Parameter measured	**Full-scale Non BEPR (20 d sludge age)**	**Pilot-scale Enriched BEPR (10 d sludge age)**	**Full-scale BEPR (8 d sludge age)**
S_{bi} (CODmg/L)	800	500	200
Measured VSS (g/L)	3	1.29	2.75
P removal (mgP/L)	n/d	40	5.89

DAPI (cells/mL)	$3x10^{10}$	$1.6x10^{10}$	$8x10^{9}$
X_a (EUB/DAPI) (%)	73	78	80
X_a (cells/mL)	$2.19x10^{10}$	$1.25x10^{10}$	$6.4x10^{9}$
X_e (%)	n/d	45	n/d
VSS as biomass (g/L)	n/d	1.0	0.54
% measured VSS as X_a	n/d	55	20
X_{PAO}**(%) of X_B	n/d	55	20
X_{PAO}** (cells/mL)	n/d	$6.88x10^{9}$	$1.28x10^{9}$
VSS as PAO's (g/L)	n/d	0.59	0.11
X_{PAO}/mgP removed	n/d	$1.7x10^{11}$	$2.17x10^{11}$

**as revealed by staining in excess with DAPI

The biodegradable COD fractions for the Amanzimtoti and Darvill Wastewater Works was determined by taking 80% of the total measured COD (WRC, 1984) as obtained from Pillay, (1998) and Singh (2000).

By determining the mean VSS per bacterial cell from TABLE 5.1, the VSS as biomass for the full-scale BEPR system can be from the following equation (results shown in TABLE 5.2):

$$\text{VSS as biomass (g/L)} = \text{Mean VSS/cell (g)} \times X_B \text{ (cells/L)}$$

The active bacterial biomass X_B (TABLE 5.2) was estimated by multiplying the EUB/DAPI ratio and the total cell count as obtained by membrane filtration.

From TABLE 5.2: VSS as biomass (g/L) for full scale BEPR = $8.49x10^{-11} \times (0.80 \times 8 \times 10^{9})$

$= 0.54$ g/L

If this is compared to measured VSS (TABLE 5.2) = 2.75 g/L, the true VSS amount to only 20%.

5.3.2 The determination of biomass in EBPR

The challenge in integrating microbiological data with process modelling is to convert cell numbers to mass units. The calculations as presented here serve to illustrate an approach that can be taken in order to accomplish this. Using the data given in TABLE 5.1, a conversion factor (f_{VB}) of $8.49x10^{-11}$ mgVSS/cell has been applied in determining the true VSS for the Darvill Full-scale EBPR system. This would amount to only 20% of the measured active biomass for the system (TABLE 5.2). This implies that 80% of the measured VSS for the full-scale system is present as inert material and endogenous residue that has become enmeshed in the sludge mass. DAPI staining for the detection of PAO's *in situ* showed that the PAO population in the pilot-scale enriched culture (Mudaly *et al.*, 2001) was much higher than for the full-scale process (TABLE 5.2). This is expected as the P-removed performance of the pilot-scale system is about 7.5 times higher than that observed in the full-scale plant. The calculated PAO biomass required to remove 1 mg of phosphorus from the EBPR for both systems compare reasonably at approximately $1.7x10^{11}$ PAO's for the pilot plant and $2.17x10^{11}$ PAO's for the full-scale plant.
Using such an approach model outputs for pilot-scale studies in the research environment can be compared to directly determined values of biomass fractions for the purposes of model calibration prior to full-scale plant design. In the case of batch assays such a technique can be used supplement the respirometric test for the determination of the heterotrophic active biomass component in AS mixed liquor (Wentzel *et al.*, 1995; 1998).

5.3.3 Implications for the So/Xo ratio

The possibility of converting active cell numbers to VSS mass units also has advantages for batch experiments where the S_o/X_o ratio is a key parameter in the assay (Grady *et al.*, 1996; Chudoba *et al.*, 1991; 1992). Here, the value for X_o can be converted to a true active biomass reading, i.e; VSS consisting of biomass only. For example taking the batch assay performed in section 3.2.2 of this study,

Given: S_o = 500 mgCOD/L

X_o = 2800 mgVSS/L

DAPI cell count = $2.5x10^9$ cells/mL

f_{cv} = 1.42 mgCOD/mgVSS

f_{VB} = $8.49x10^{-11}$mgVSS/cell

Apparent S_o/X_o ratio:

X_o = 2800 mgVSS/L x 1.42 mg COD/mgVSS

= 3976 mg COD/L

S_o/X_o = 0.13

True S_o/X_o ratio:

X_o = DAPI cell count x f_{VB} x f_{cv}

= $2.5x10^{12}$ cells/L x $8.49x10^{-11}$mgVSS/cell x 1.42 mgCOD/mgVSS

= 301.395 mgCOD/L

S_o/X_o = 500/301

= 1.66

The influence of parameters related to the history of the inoculums such as sludge age (Grady *et al.,* 1996) can be eliminated from the So/Xo ratio. This will therefore give the S_o/X_o ratio a more universal significance for studies performed using seed sludge from different parent plants and results can therefore be more realistically compared.

5. 4 CONCLUSIONS

i. A conversion factor (fVB) for the conversion of cell numbers to VSS mass units has been determined as $8.49x10^{-11}$mgVSS/cell.

ii Only 20% of the total measured VSS was found to represent metabolically active biomass for the Darvill Wastewater Works.

iii The PAO biomass required for the removal of 1 mg of phosphorus was determined to be $2.17x10^{11}$ PAO's for the full-scale plant.

iv The use of f_{VB} to normalize the value of X_o during batch assays can allow for more reliable comparison of data obtained using seed innocula with different culture histories.

CHAPTER SIX

COMPARISON OF MICROBIOLOGICAL MEASUREMENTS OF HETEROTROPHIC ACTIVE BIOMASS WITH ENGENEERING MEASUREMENTS.

6.1 INTRODUCTION

The fact that biomass within the activated sludge process is responsible for mediating the treatment functions of interest viz. COD removal, nitrogen removal and phosphorus removal, substantiates studies focused on the microbial composition of the activated sludge mixed liquor. Current mathematical models describing the behavior of the activated sludge process are not based on the direct measurement of the different components comprising the biomass. Current techniques incorporating aspects of molecular biology and microbiology are making it possible to quantify the key microbial groups active in the process, with a view to improving the process description and design.

Historically the mixed liquor organic suspended solids (MLOSS) have been measured as a lumped parameter, via the VSS or COD test (Standard Methods, 1985). Specific rates for these biological processes (denitrification; oxygen utilization) are often expressed in terms of this lumped parameter, but only part of the MLOSS, the heterotrophic active biomass (Z_{BH}), is responsible for mediating the biological processes for COD removal and denitrification (Wentzel *et al*., 1998).

The batch test procedure developed by Kappeler and Gujer, (1992) presented a means of quantifying the Z_{BH} concentration through monitoring the organisms OUR response with time in a batch reactor. This procedure was extended by Wentzel *et al*., (1995), Ubisi *et al*., (1997a; 1997b) and Cronje *et al*., (2002) to quantify the heterotrophic active biomass concentration drawn from aerobic and anoxic/aerobic activated sludge systems. In the research of Ubisi *et al*., (1997a; 1997b) and Wentzel *et al*., (1998), the measured and theoretical Z_{BH} concentrations of mixed liquor from the 12 day sludge age parent system showed a close 1:1 correlation while those for mixed liquor from the 20 day sludge age parent system showed poor correlation. Ubisi

et al., (1997a; 1997b) and Wentzel *et al.*, (1998) did not provide any explanation for this inconsistency. In the investigation of Cronje *et al.*, (2002) on mixed liquor drawn from a 10 day sludge age parent system, a reasonably close 1:1 correlation was found. In contrast, in the investigation of Beeharry *et al.*, (2001) on mixed liquor from 10 day sludge age parent system, the correspondence line between measured and theoretical Z_{BH} fell parallel to the 1:1 correspondence. Lee *et al.*, (2003) investigation on mixed liquors from 10 and 20 day sludge age parent systems found similar correspondence to that of Beeharry *et al.*, (2001). To explain these observations, Novak *et al.*, (1994) proposed that the batch conditions might favour growth of sections of Z_{BH} population (e.g. fast growing Z_{BH}), causing the community structure to change with time in the batch test. This change in community structure will effect a change in the observed response, which is not taken into account in the batch test analysis.

Parallel to the developments in the engineering and technology of the activated sludge system, significant advances have been made in the microbiological and biochemical areas. Fluorescent *in situ* hybridization (FISH) with rRNA-targeted nucleic acid probes is a new molecular tool for rapid, reliable and cultivation-independent monitoring and quantification of phylogenetically defined bacterial populations in activated sludge samples (Amann *et al*., 1995). The delectability of bacteria by such oligonucleotide probes is not only dependant on the penetration of the labeled probes into cells but also on the ribosomes contents and consequently on the growth rates of cells (De Long *et al*., 1989). Because substantial portions of the 16S rRNA sequences are similar among all known organisms, low stringency hybridization permits the identification of rRNA genes from unknown organisms and thus can be used as targets for hybridization probes with various specificities. Thus, the ubiquity of the rRNA ensures that the probes can be designed to identify virtually any organism or group related organisms. The application of this technique allows for the detection and quantification of actively growing microorganisms with relatively high cellular rRNA content.

It remains for the results that these techniques provide to be integrated with the design and kinetic design modeling theory. The consequence of this is that the engineering and technology paradigm (modeling) has largely worked independently of the molecular biology paradigm. To facilitate links and overlap between the two paradigm sets, the new developments in the

molecular biology analytical techniques (FISH) can be implemented to address the deficiency in the engineering and technology paradigm of the active biomass concept. This should prove possible because, in contrast to the more traditional analytical techniques, the new techniques provide quantitative information, a prerequisite for modeling. Some initial integration between modeling and these techniques has been initiated, but this is still in its infancy.

In this study 16S rRNA- targeted oligonucleotide probes for phylogenetically defined groups of autotrophic ammonia and nitrite oxidizing bacteria were used. These probes were coupled with a universal probe targeting all active bacteria for the quantification of the heterotrophic active biomass in an activated sludge system. The modified batch test method is used to correlate heterotrophic active biomass concentrations to results comparable to molecular probing.

6.2 MATERIALS AND METHODS

6.2.1 D.I.T parent system

The parent laboratory-scale system layout and operational details are shown in FIG 6.1. The system was fed 24 L/d raw (unsettled) municipal wastewater obtained from Southern Works (Durban, South Africa). This wastewater is primarily domestic with no industrial contribution. The wastewater was collected in batches, stored in 25 L containers at 4°C and served as feed for both the parent system and batch tests. For the parent system, daily wastewater was drawn from the storage containers after thorough mixing and diluted with tap water to approximately 500 mgCOD/L Daily monitoring included influent COD, TKN; all reactors nitrate and nitrite; aerobic reactor VSS, COD and TKN, OUR; effluent COD, TKN, nitrate and nitrite (Standard Methods, 1985). To ensure steady state, the parent system was run for more than five sludge ages before mixed liquor was harvested for the batch test.

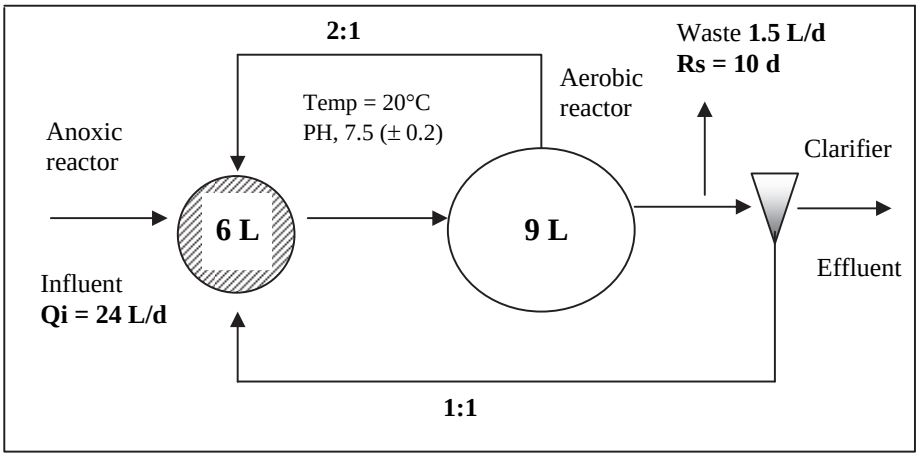

FIGURE 6.1 Schematic layout and operational data for DIT parent laboratory-scale Modified-Ludzack-Ettinger (MLE) anoxic/aerobic activated sludge system

6.2.1.1 UCT operated parent system

The parent laboratory-scale system layout and operational details for 10 and 20 day sludge age systems are shown in FIG 6.2. The system was fed 10 L/d raw (unsettled) wastewater from Mitchell's Plain Treatment Plant in Cape Town (South Africa). This wastewater is primarily domestic with a small industrial component. The wastewater batch was brought to the laboratory and stored in 400 L stainless steel tanks in a cold room at 4°C. The total COD concentration which served as feed to the parent system was targeted at 750 ± 50 mgCOD/L for both the 10 days and 20 day sludge age systems. Daily monitoring included influent COD, TKN; all reactors nitrate and nitrite; aerobic reactor VSS, COD and TKN, OUR; effluent COD, TKN, nitrate and nitrite (Standard Methods, 1985).

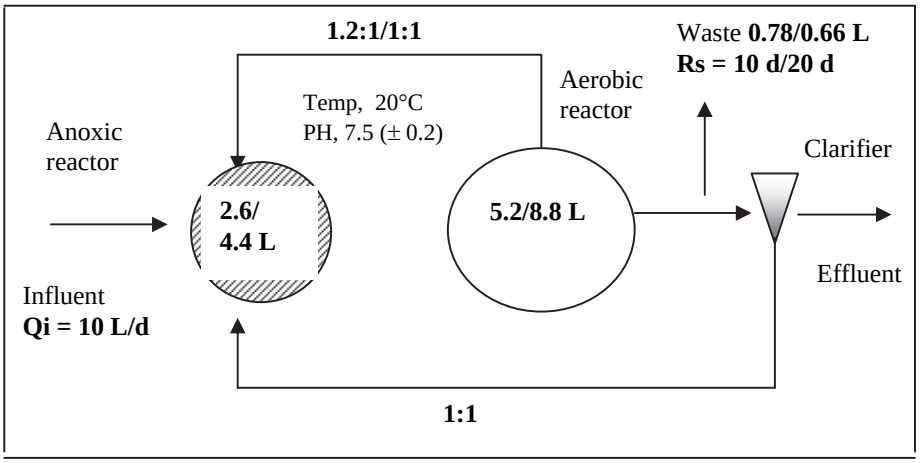

FIGURE 6.2 Schematic layout and operational data for UCT operated parent laboratory-scale Modified Ludzack Ettinger (MLE) anoxic/aerobic activated sludge system

6.2.2 Modified batch test

Flocculation/settling. A volume of 10 ml of stock $AL(SO_4).15H_2O$ (50 g/L) were added per 1 L of wastewater, the mixture was stirred rapidly (~200 rpm) for 2 minutes and then slowly (~1 rpm) for 30 min. The mixture was allowed to settle for a further 30 min.

Filtration. The clear supernatant that developed in the settling cylinders was drawn off and filtered through a 0.45 µm glass fibre filter (Whatman's GF/C). The flocculated-filtered wastewater was stored overnight in a cold room at 4ºC.

Filtered wastewater and mixed liquor batch test. The required volume of flocculated-filtered wastewater was measured, preheated to 20ºC in a warm water bath and placed in a continually stirred batch reactor maintained at a constant temperature of 20ºC. The pH of the flocculated-filtered wastewater was raised to 7.5 by adding sodium bicarbonate ($NaHCO_3$) prior to the commencement of the batch test. The required volume of mixed liquor was harvested from the aerobic reactor of the parent system and added to flocculated-filtered wastewater, giving a

combined volume of 5 L for the mixture in the batch reactor. Immediately after the mixed liquor was added to the flocculated-filtered wastewater, a sample was drawn to obtain the initial total COD concentration (Standard Methods, 1985). The oxygen supply and OUR response in the batch test were measured using an automated technique (Randall *et al*., 1991). At regular intervals, samples were drawn from the batch reactor, immediately filtered through 0.45 µm filter paper, 2-3 drops of $HgCl_2$ were added to the filtrate which was stored for subsequent nitrate and nitrite analysis. At the end of the batch tests, the contents of the batch reactor were homogenized in a liquidizer, a sample drawn and the final COD concentration measured. The OUR results were downloaded from the DO meter to a PC.

6.2.3 Sampling and cell fixation

A grab sample of mixed liquor was collected at the start of each batch test and fixed for 1.5 hr at 4ºC with 4% paraformaldehyde. Cells were fixed by the addition of three volumes of fixative to one volume of sample. Cells are then washed in 1 x PBS (130 mM NaCl; 10 mM sodium phosphate buffer, pH 7.2) and then resuspended in PBS/cold absolute ethanol (1:1).

6.2.4 Membrane filtration and staining with DAPI

Membrane filtration was carried out as described by Porter and Feig, (1980) with the following modification. Cellulose acetate (Millipore, 0.22 µm pore size, 25 mm diameter) filters were stained for 24 hr in Sudan Black (0.3% w/v in 60% ethanol). Dual staining of cells with DAPI and fluorescent oligonucleotides was modified from the method of Hicks *et al*., (1992) so that cells were stained after in situ hybridization with DAPI (0.33 µg/ml) for 5 min.

6.2.5 Oligonucleotide probes and hybridization

Oligonucleotides probes used during the present study are given in TABLE 5.1. 40 µl of the pre-treated sample was mixed with 60 µl of 1 x PBS and 10 µl Nonidet. 10 µl of this mixture was spotted onto pre-treated slides and allowed to dry. Spotted cells were then dehydrated by serial immersions through 60%, 80% and 96% (v/v) ethanol (3 min each). Samples of 10 µl

hybridizations solution (0.9 M NaCl, 20 mM Tris/HCl, pH 7.2, 0.01% SDS, 50 ng probe, X% (v/v) formamide) were applied to each spot and incubated for 2 hr at 46ºC in an isotonically equilibrated humidity chamber. Probe was removed from the slide by rinsing in 2 ml pre-warmed washing solution (20 mM Tris/HCl, 0.01% SDS, 5 mM EDTA, Y M NaCl). The salt concentration and formamide concentration was adjusted according to the formula of Lathe, (1985). Slides were rapidly transferred into washing solution and incubated at 48ºC for 20 min. Slides were then rinsed briefly with distilled water, air-dried and mounted in Mounting media anti-fading solution (Bio-Rad, USA) for viewing by microscopy.

6.2.6 Microscopy and image analysis

Cells were visualized with a Zeiss Axiolab microscope (Carl Zeiss, Germany) fitted for epifluorescence with a 50W mercury high-pressure bulb and Zeiss filter sets 02, 09 and15. Images were captured using a Hamamatsu (Japan) CCD camera. 30 microscopic fields under the 100X objective were randomly selected for enumeration.

TABLE 6.1 Probe sequences and formamide percentages for *in situ* hybridization.

Probe	Sequence	% F[1]	Fluor-	Reference
EUB	5'- GCT GCC TCC CGT AGG AGT -3'	20	Rhodamine	Amann *et al*., 1990
NIT 3	5'- CCT GTG CTC CAT GCT CCG - 3'	40	Rhodamine	Wagner *et al*., 1996
NEU	5'- CCC CTC TGC TGC ACT CTA - 3'	40	Rhodamine	Wagner *et al*., 1995
Nso 190	5'- CGA TCC CCT GCT TTT CTC C - 3'	55	Rhodamine	Mobarry *et al*., 1996

[1] percentage formamide (v/v) in the hybridisation buffer

6.3 RESULTS AND DATA INTERPRETAION

6.3.1 DIT Parent system

For each of wastewater batches fed to the parent system (9 batches), the daily results were averaged and sample standard deviations calculated (APPENDIX 11; TABLE 11.1). Batch tests were conducted during 7 wastewater batches. Following Ekama *et al*., (1986) the following were determined: the influent wastewater unbiodegradable soluble and particulate COD fractions (fs,us and fs,up respectively); system COD and N mass balances; the COD and TKN to VSS ratios of the mixed liquor (f_{CV} and f_N respectively) (APPENDIX 11, TABLE 11.2). N and COD mass balances were in the acceptable range, ranging from 85-106% and 80-89% respectively. From the steady state data, the theoretical Z_{BH} of the mixed liquor drawn from the parent system and added to the batch tests needs to be calculated (Ubisi *et al*., 1997a; 1997b). To do this the steady state design model was used. For the steady state design model from WRC, (1984) and the data in APPENDIX 11, TABLE 11.2, the Z_{BH} as fractions of the MLOSS (f_{av}) were calculated and are listed in APPENDIX 11, TABLE 11.2. Knowing f_{av}, the concentration of mixed liquor VSS or COD drawn from the parent system (APPENDIX 11, TABLE 11.1), and the volume of mixed liquor added to the batch tests, the theoretical Z_{BH} concentration in the batch reactor due to the added mixed liquor could readily be calculated (Wentzel *et al*., 1998; Ubisi *et al*., 1997a;1997b).

6.3.2 UCT operated system

Daily results for the parent activated sludge systems at 10 day and 20 day sludge ages are listed in APPENDIX 10, TABLE 10.1 and TABLE 10.2 respectively. Each sewage batch was accepted as a steady-state period. For each sewage batch the daily data was average and the sample standard deviations calculated. The influent wastewater unbiodegradable soluble and particulate COD fractions (fs,us and fs,up respectively); system COD and N mass balances; the COD and TKN to VSS ratios of the mixed liquor (f_{CV} and f_N respectively) for 10 and 20 day sludge systems are listed in APPENDIX 10, TABLE 10.2 and TABLE 10.4 respectively. From this data the theoretical Z_{BH} concentration in the batch reactor due to the added mixed liquor could

readily be calculated (Wentzel *et al*., 1998; Ubisi *et al*., 1997a; 1997b). N and COD mass balance were consistent and in the range of 92-105% and 92-101% respectively for the 10 day sludge age system. For the 20 day sludge age system COD range from 91-107%. Sewage batches that gave mass balances falling outside this range were 21/02, 22/03 and 23/02 (APPENDIX 10 and TABLE 10.4). Examining the data more closely showed that for these sewage batches, the errors for COD mass balance did not lie in VSS, COD, or TKN but probably lies in the measurement of the OUR.

6.3.3 Batch Test

TABLE 6.2. COD recovery, regression data from ln (OUR_H) versus time plot and heterotrophic active biomass at the start of the batch test ($Z_{BH\,(0)}$).

Sewage batch no	Batch test no	Volume (L)		COD Recov. (%)	Regression			$Z_{BH(0)}$ (mgCOD/L)			
								Measured		Theoretical	
		WW	ML		Y-int	Slope	R^2	Batch test	ML	Batch test	ML
4	B1	4.5	0.5	97	1.64	0.129	0.919	64.65	646.5	124.57	1245
4	B2	4.6	0.4	97.02	1.69	0.124	0.918	70.26	878.25	99.65	1245
5	B3	4.6	0.4	95.08	1.67	0.118	0.902	72.53	906.62	127.16	1589.5
5	B4	4.6	0.4	98	1.47	0.075	0.943	84.16	1052	127.16	1589.5
6	B5	4.65	0.35	107.21	1.73	0.12	0.917	75.05	1072	86.25	1232
6	B6	4.6	0.4	91.41	1.28	0.112	0.925	48.42	605.25	98.57	1232
7	B7	4.5	0.5	93.84	1.27	0.113	0.904	49	490	121.12	1211.2
8	B8	4.45	0.45	90.42	1.39	0.154	0.925	43	478	104.13	1157
8	B9	4.5	0.5	93.48	1.28	0.104	0.995	53.49	534.9	116.59	1165.9
8	B10	4.5	0.5	92.45	1.11	0.138	0.904	35.78	357.8	116.59	1165.9
9	B11	4.5	0.5	90.73	1.06	0.134	0.962	35.02	350.2	100.93	1009.3

Eleven batch tests were conducted on mixtures of various quantities of mixed liquor and wastewater. The OUR due to nitrification ($OUR_{N(t)}$) was taken in to account in deriving estimates for % COD recovery and $X_{H(o)}$, since both parameters are determined from the OUR for heterotrophs only ($OUR_{H(t)}$). The $OUR_{N(t)}$ was determined from the nitrate concentration time

profile (Ubisi *et al*., 1997a; 1997b). Then from each measured OUR value ($OUR_{M(t)}$), the nitrification OUR ($OUR_{N(t)}$) was subtracted to give the OUR due to the heterotrophs only ($OUR_{H(t)}$). The %COD recoveries ranged from 90-107% (TABLE 5.4). To determine Z_{BH} present at the start of the test, $Z_{BH\ (0)}$, the $OUR_{H(t)}$ up to the precipitous drop in OUR were plotted Ln $OUR_{H(t)}$ versus time. Linear regression was used to determine the Y-intercept, slope and correlation coefficients (R^2) (TABLE 6.4). From the Y-intercept and slope, values for $Z_{BH\ (0)}$ were calculated (Wentzel *et al*., 1995; Ubisi *et al*., 1997a; 1997b). These values represent the heterotrophic active biomass concentrations in the batch test due to that added with the mixed liquor. The $Z_{BH\ (0)}$ (ML) values represent the measured mixed liquor heterotrophic active biomass concentration in the total batch test volume (TABLE 6.4). To illustrate the comparison, the measured versus theoretical mixed liquor OHO active biomass data for all batch tests are shown in FIG 6.3.

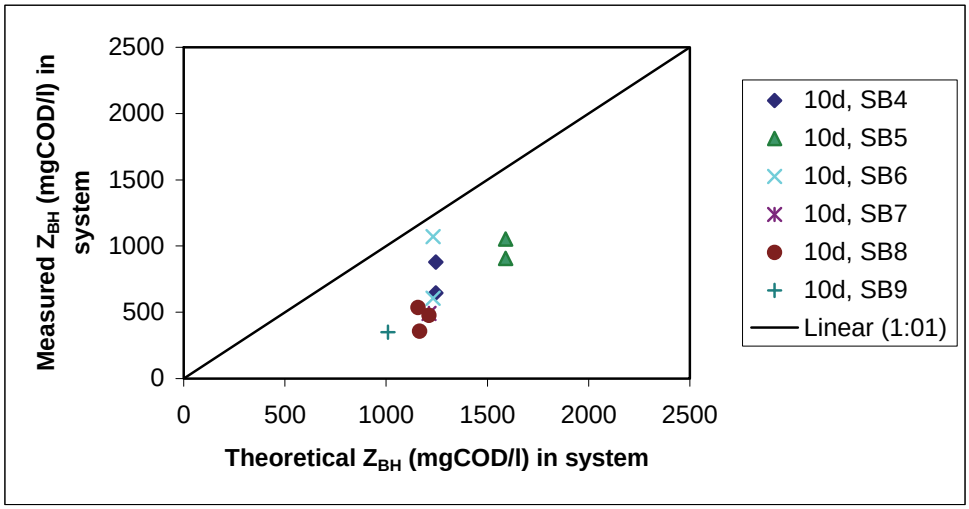

FIGURE 6.3 Modified batch test results; graph of measured versus theoretical OHO active biomass concentration at the start of the test ($Z_{BH(0)}$) for various sewage batches (SB) for the 10 day DIT MLE activated sludge system; Scale 2500 x 2500 mgCOD/L.

6.3.4 Fluorescent *in situ* hybridization

The oligonucleotide probes used were targeted to cells 16S rRNA, which provides a phylogenetic identification based directly on cell genetic code (Stahl *et al.*, 1998). The following nitrifying probes were used, NEU complemenary to a signature region of most halophilic and halotolerant ammonia oxidizers (Wagner *et al*., 1995), Nso190 specific for the ammonia oxidizers in the beta subclass of *Proteobacteria* (Mobarry *et al*., 1996) and NIT3 complementary to a region of all previously sequenced *Nitrobacter* species (Wagner *et al*., 1996). EUB338 was used to target all eubacteria (Amann *et al*., 1990), which includes the nitrifiers. Cell counts for the determination of the active biomass (DAPI) for each batch test is converted to mass units in order to determine what fraction of the measured VSS is metabolically active. In order to make a conversion from cell numbers to mass units a conversion factor (F_{VB}) of 8.49×10^{-11} (Mudaly *et al*., 2001) is applied in the following equation:

$$CODmg/L = \frac{cell\ count \times F_{VB} \times F_{CV} \times original\ vol\ of\ sample(mL)}{Measured\ vol\ of\ sample(mL) \times 1000 \times DF}$$

TABLE 6.3 Probe cell counts and heterotrophic active biomass concentration at the start of the test ($Z_{BH\ (0),}$ DIT system).

Batch test No.	Eub cell count (cells/ml)	Nitrifiers cell count (Neu, Nit, Nso 190) (cells/ml)	Probe Measured $Z_{BH(0)}$ (mgCOD/L)	Measured $Z_{BH(0)}$ (mgCOD/L) ML
B1	2.03×10^9	3.33×10^8	504.78	656.5
B2	2.80×10^9	5.68×10^8	665.34	878.25
B3	2.03×10^9	3.72×10^8	521.39	906.62
B4	2.61×10^9	4.73×10^8	672.14	1052
B5	2.30×10^9	3.60×10^8	557.08	1072
B6	1.94×10^9	4.08×10^8	441.30	605.25
B7	2.15×10^9	5.96×10^8	468.90	490
B8	1.95×10^9	4.23×10^8	451.21	478
B9	1.78×10^9	2.70×10^8	444.77	534.9
B10	1.56×10^9	5.62×10^8	293.56	357.8
B11	1.60×10^9	5.93×10^8	302.53	350.2

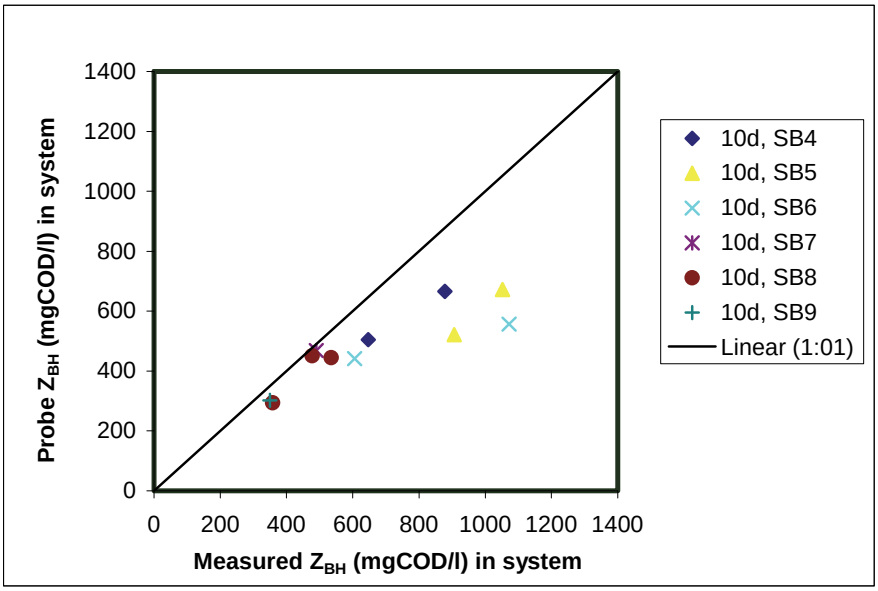

FIGURE 6.4 Measured versus probe OHO active biomass concentration at the start of the batch test ($Z_{BH(0)}$) for the various sewage batches for 10 day DIT parent system.

TABLE 6.4 Measured and theoretical biomass present at the start of the test ($Z_{BH(0)}$, UCT operated system) for 10 and 20 day sludge age parent MLE activated sludge system.

	Sewage batch no.	Date	Vol of ML added to BT	$Z_{BH(0)}$ (mgCOD/l)			
				Theoretical		Measured	
				Batch Test	ML	Batch Test	ML
10d	21/02	24/11	300	158.60	1586.00	143.20	1432.00
		27/11	200	105.70	1585.50	31.30	469.50
		2/12	100	52.90	1587.00	7.10	213.00
	22/02	10/12	300	132.30	1323.00	119.00	1190.00
		17/12	300	132.50	1325.00	108.60	1086.00
	23/02	22/12	300	133.80	1338.00	53.00	530.00
	01/03	7/1	300	144.60	1446.00	112.00	1120.00
		14/1	200	96.40	1446.00	50.50	757.50
	02/03	24/1	300	145.80	1458.00	139.60	1396.00
		1/2	200	97.20	1458.00	89.20	1338
20d	21/02	24/11	400	165.30	1239.75	55.60	417.00
		27/11	270	111.60	1240.00	28.50	316.67
		2/12	150	62.00	1240.00	5.70	114.00
	22/02	10/12	400	100.10	750.75	39.11	293.33
		17/12	300	75.10	751.00	22.50	225.00
	23/02	22/12	400	73.20	549.00	20.43	153.23
	01/03	7/1	400	138.00	1035.00	83.90	629.25
		14/1	300	103.50	1035.00	87.60	876.00
	02/03	24/1	400	140.50	1053.75	157.70	1182.75
		1/2	300	105.40	1054.00	52.10	521.00

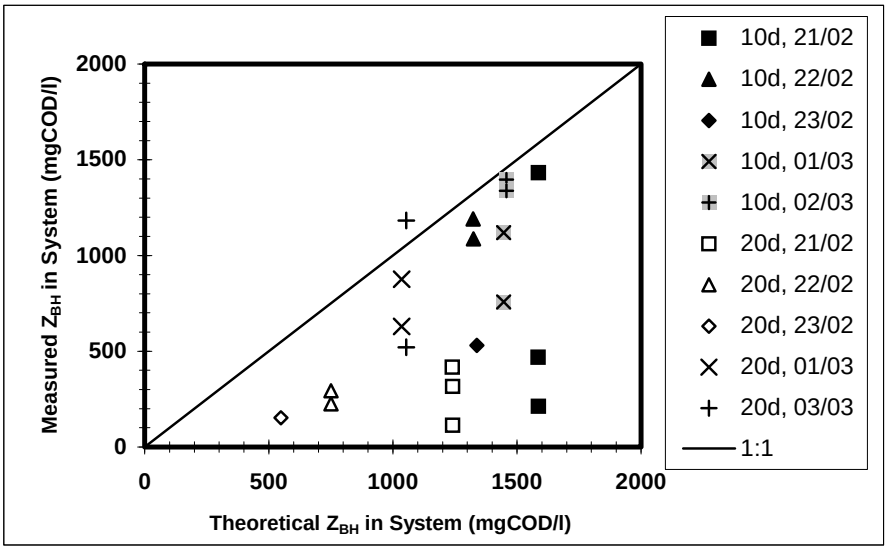

FIGURE 6.5 Measured versus theoretical OHO active biomass concentration at the start of the test ($Z_{BH(0)}$) for various sewage batches for 10 and 20 day UCT operated parent system.

TABLE 6.5 Probe cell counts and heterotrophic active biomass concentration at the start of the test ($Z_{BH(0)}$, UCT operated system).

	Sewage Batch No.	Date	Eub cell count (cells/ml)	Nitrifiers cell count (cells/ml)	Vol of sample (ml)	Fcv	Probe Measured values $Z_{BH(0)}$ (mgCOD/l)	Theoretical values $Z_{BH(0)}$ (mgCOD/l)
10d	21/02	25/11	3.460E+09	2.95E+08	18	1.47	959.60	1599.13
		3/12	2.961E+09	1.38E+08	18	1.47	821.20	1599.13
	22/02	10/12	2.936E+09	3.64E+08	18	1.38	764.42	1336.32
		16/12	3.560E+09	3.31E+08	18	1.38	926.88	1336.32
	23/02	24/12	3.669E+09	7.24E+08	18	1.46	1010.64	1351.78
	01/03	6/01	2.980E+09	3.09E+08	18	1.43	803.98	1465.90
		13/01	2.915E+09	1.00E+09	18	1.43	786.45	1465.90
	02/03	23/01	1.593E+09	4.44E+08	18	1.54	462.84	1481.63
		31/01	3.180E+09	6.07E+08	18	1.54	923.94	1481.63
20d	21/02	25/11	3.664E+09	6.97E+08	18	1.51	1043.82	1257.3
		3/12	2.058E+09	1.37E+08	18	1.51	586.30	1257.3
	22/02	10/12	3.234E+09	7.28E+08	18	1.50	915.22	765.63
		16/12	2.822E+09	6.71E+08	18	1.50	798.63	765.63
	23/02	24/12	2.740E+09	7.56E+08	18	1.37	708.22	518.331
	01/03	6/01	4.618E+09	4.60E+08	18	1.43	1245.91	1061.76
		13/01	4.403E+09	3.89E+08	18	1.43	1187.90	1061.76
	02/03	23/01	2.829E+09	4.04E+08	18	1.50	800.61	1078.85
		31/01	3.931E+09	7.39E+08	18	1.50	1112.47	1078.85

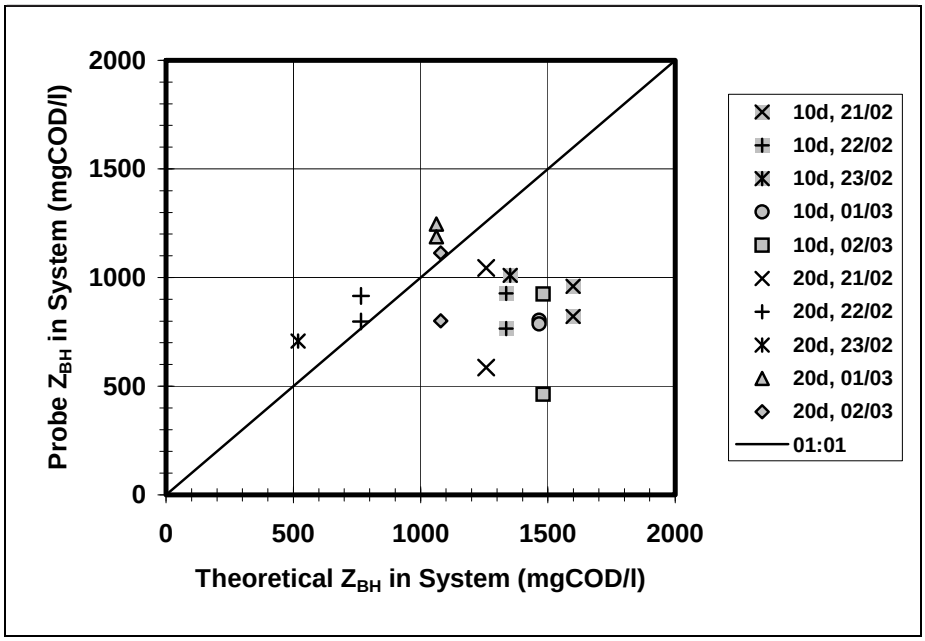

FIGURE 6.6 Measured probe versus theoretical OHO active biomass concentration at the start of the test ($Z_{BH(0)}$) for various sewage batches for 10 and 20 day UCT operated parent system.

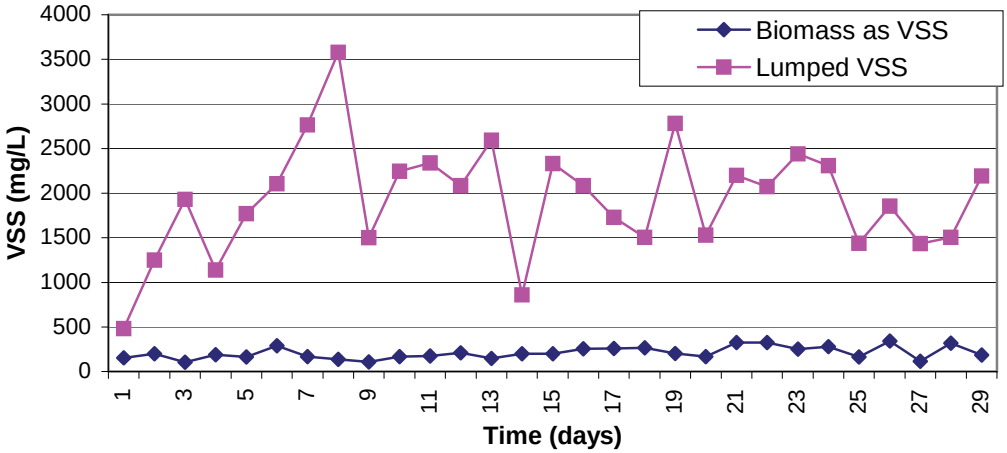

FIGURE 6.7 VSS concentration in the MLE aerobic zone.

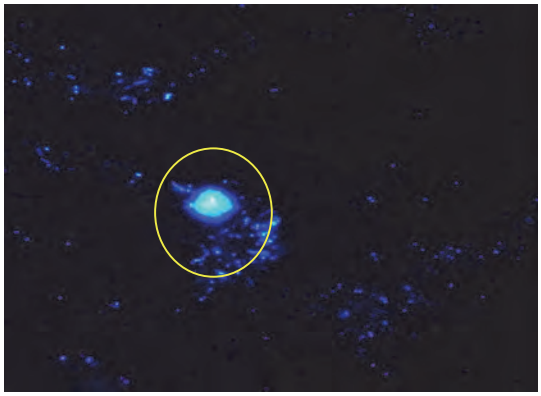

FIGURE 6.8 (a): Epifluorescent micrograph of nitrifying bacteria stained with DAPI.

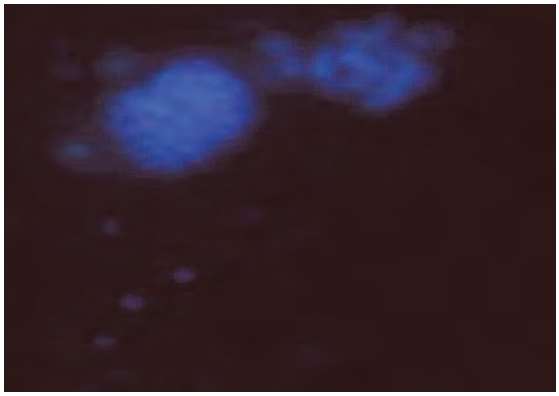

FIGURE 6.8(b): Epifluorescent micrograph of nitrifiers stained with DAPI.

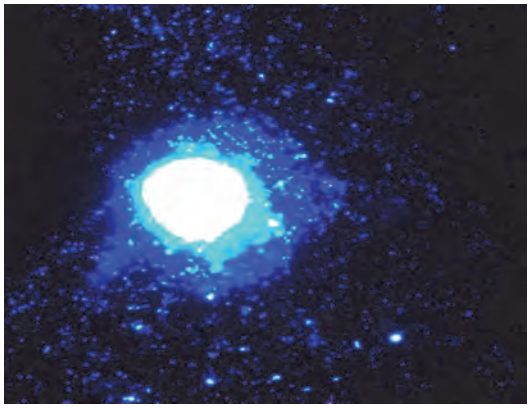

FIGURE 6.9 (a): Epifluorescent micrograph of cells stained with DAPI.

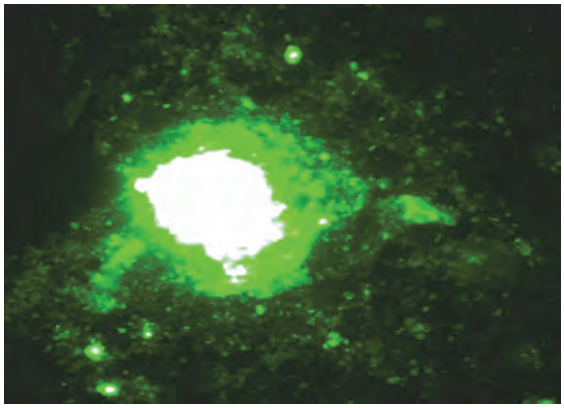

FIGURE 6.9 (b): Epifluorescent micrograph of bacterial cells bearing the probe EUB.

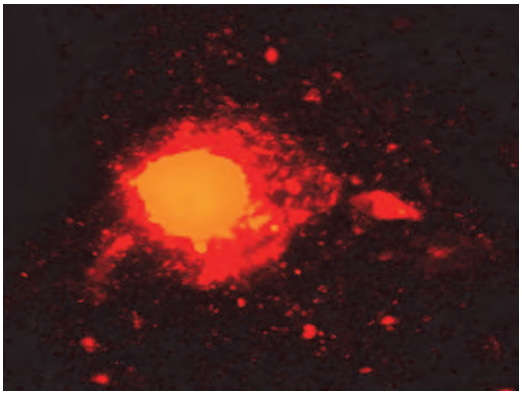

FIGURE 6.9 (c): Epifluorescent micrograph of the Nitrifying bacteria, bearing the probes NIT3, NEU and Nso190

6.4 DISCUSSION

6.4.1 DIT results

6.4.1.1 Comparison between measured and theoretical OHO active biomass for 10 day sludge age MLE sludge system

In TABLE 6.4, the measured OHO active biomass concentration at the start of each batch test [$Z_{BH(0)}$(meas.)] is compared to the theoretical OHO active biomass concentration at the start of the test due to mixed liquor sample which was drawn from the parent system and added to the batch test [$Z_{BH(0)}$(theor.)]; theoretical values were predicted via the steady state design model. To illustrate the comparison, the measured versus theoretical mixed liquor OHO active biomass data for all the batch tests are shown in FIG 6.3. From FIG 6.3 there is a correspondence between measured and theoretical $Z_{BH(0)}$, but the values plot parallel to and below the 1:1 correspondence line. Lee *et al*., (2003) identified three trend regions when examining the correspondence between measured and theoretical $Z_{BH(0)}$. The first, in the region of theoretical $Z_{BH(0)}$ from 0 to about 30 mgCOD/L as the theoretical $Z_{BH(0)}$ increases, the measured values decrease, to approach near zero. The second, in the region from 30 to 150 mgCOD/L theoretical $Z_{BH(0)}$, as theoretical increases, the measured values increase parallel to 1:1 correspondence line between the

measured values and the theoretical values. The third, above 150 mgCOD/L theoretical $Z_{BH(0)}$, as the theoretical $Z_{BH(0)}$ increases further the measured $Z_{BH(0)}$ values increase sharply. Thus, it is evident that the trends observed in this investigation are consistent with those of Lee *et al.*, (2003).

6.4.1.2 Comparison between measured and probe OHO active biomass for 10 day sludge age MLE sludge system

In TABLE 6.5, the measured OHO active biomass concentration at the start of each batch test [$Z_{BH(0)}$ (meas.)] is compared to the probe OHO active biomass concentration at the start of the test [$Z_{BH(0)}$ (probe)]. The modified batch test translated OUR response into parameters that reflect the $Z_{BH(0)}$ and are directly comparable to the $Z_{BH(0)}$ measures from the molecular probes. The $Z_{BH(0)}$ values obtained my molecular probing correlated closely with measured $Z_{BH(0)}$ (ML) values obtained from the modified batch tests (FIG 6.3). However molecular probing provides a more accurate quantification of the Z_{BH} since rRNA-targeted oligonucleotide probes are used for the direct enumeration of individual cells. The slight differences in measured Z_{BH} values could be attributed to sensitivity in regression analysis of Ln OUR data (Cronje *et al*., 2002). FIG 6.2 allows a comparison between the measured VSS and the VSS as biomass from the system. These results were calculated from total cell counts, it seems like the active biomass represents only a very small fraction (10%-15%) of the measured VSS.

6.4.2 UCT results

6.4.2.1 Comparison between measured and theoretical OHO active biomass for 10 and 20 day sludge age MLE sludge system

In TABLE 6.6, the measured OHO active biomass concentration at the start of each batch test is compared with the theoretical OHO active biomass concentration at the start of the batch test due to the mixed liquor sample drawn from the parent system and added to the batch test; theoretical values predicted via steady state design model. To illustrate the comparison, the measured versus theoretical mixed liquor OHO active biomass data for all the batch test are shown plotted in FIG.

6.4. From FIG. 6.4 there is a correspondence between measured and theoretical $Z_{BH(0)}$, at a 10 and 20 day sludge age, but the values plot parallel to and below the 1:1 correspondence line. From FIG. 6.4 the data for the batch tests on the 20 day sludge age mixed liquor are very similar to that for the batch tests on the 10 day sludge age mixed liquor. The data examined in more detail reveals three trend regions as mentioned in 6.4.1.1, which corresponds closely. Further, as noted above, there is a close similarity between DIT results and UCT results.

6.4.4.2 Comparison between probe and theoretical OHO active biomass for 10 and 20 day sludge age MLE sludge system

In TABLE 6.6, the probe OHO active biomass concentration at the start of each batch test [$Z_{BH(0)}$ (probe.)] is compared to the theoretical OHO active biomass concentration at the start of the test due to mixed liquor sample which was drawn from the parent system and added to the batch test [$Z_{BH(0)}$(theor.)]; theoretical values were predicted via the steady state design model. To illustrate the comparison, the probe versus theoretical mixed liquor OHO active biomass data for all batch tests are shown plotted in FIG 6.4. From FIG. 6.4 there is a close correspondence between probe and theoretical $Z_{BH(0)}$, for 10 and 20 day sludge age but the values plot parallel to the 1:1 correspondence line. However data for the comparison between probe and measured $Z_{BH(0)}$ were not collected.

What was noted, were the large numbers of nitrifiers observed (TABLE 6.5 and 6.6) during this analysis. In analytical procedures and batch-tests, the nitrifiers [FIGs 6.3; 6.4(c)] are considered to be present in negligible quantities (Wentzel *et al*., 1998).

However, it is our belief that there are significant numbers present and a need has arisen for the investigation of autotroph numbers.

6.5 CONCLUSION

i. In this study a combination of DAPI staining and group specific oligonucleotide probing was applied to the rapid in situ characterization of the heterotrophic active biomass in batch tests. Values obtained by molecular probing corresponded closely with $Z_{BH(0)}$(ML) values obtained from the modified batch test. This good correspondence of molecular

probing values with the modified batch test [measured $Z_{BH(0)}$ (ML)] give confidence that FISH analysis can be used to address the active biomass concept. By the application of this technique, which targets individual cells, we are able to quantify the portion of active biomass (Z_{BH}) responsible for COD removal and denitrification.

ii. Active biomasses (heterotrophic and autotrophic biomass) are invariably included in the current design and simulation models, but they remain hypothetical as they have not been directly measured and compared to theoretical values. A cross-link between the engineering and microbiological paradigms has been developed with the *in situ* measurement of heterotrophic biomass and this project could establish a firm collaboration, with the result being incorporated into current models.

iii. The main deduction from this study is that molecular biology shows its powerful advantage over conventional microbiological and biochemical techniques as a tool for the direct determination and separation of mixed liquor components.

CHAPTER SEVEN

GENERAL CONCLUSIONS AND RECOMMENDATIONS

For the analysis done thus far a rapid degradation (70% of the RNA per cell degraded over 2 hr) of a large proportion of the rRNA for the bacterial community involved in EBPR was observed after a "downshift" in metabolic activity. This indicates metabolic activity or recent (an estimated window period is two hours) metabolic activity for the activated sludge cells bearing probe conferred fluorescence after hybridization.

By examining the total RNA of the system, the relationship between RNA and metabolic activity was investigated. Certain groups within the total biomass may also behave differently, therefore the RNA of these specific groups should be quantified (e.g. in dot blot assays using DIG-labeled probes specific for the groups of interest) and compared with cell numbers of the group (e.g. FISH-based cell counts) under suitable experimental conditions. In order for the results to truly reflect the treatment environment, such experiment should be performed prior to the application of rRNA based probes. This is especially the case if rRNA based assays are to be compared with model outputs of different biomass fractions. These precautions are essential in rRNA based assays as ignorance can lead to an over estimation of the active biomass in the system, leading to larger discrepancies between model outputs and directly determined results.

To facilitate comparison to model outputs, which are expressed in COD or VSS mass units, a conversion factor (f_{VB}) of 8.49×10^{-11} mgVSS/cell has been derived for application in direct biomass determinations. It is recommended that his conversion factor be more exhaustively evaluated, using bacterial monocultures which vary in dimension over a wide range and pilot scale experiments conducted with a readily biodegradable substrate source and a low sludge age (in order to minimize the contribution of inert material to the VSS reading).

The modified batch test translated OUR response into parameters that reflect the Z_{BH} and are directly comparable to the Z_{BH} measures from the molecular probes. Molecular probing provides a more accurate quantification of the Z_{BH} since rRNA-targeted oligonucleotide probes are used

for the direct enumeration of individual cells. The Z_{BH} values obtained by molecular probing correlated closely with measured $Z_{BH(0)}$ (ML) values obtained from the modified batch tests. Probing the nitrifiers revealed a surprisingly high amount of cells, it is therefore recommended that a more extensive study be done on the autotrophs present in activated sludge systems with the prospect of incorporating the results into current models.

8. REFERENCES

AMANN, R.I., BINDER, B.J., OLSEN, R.J., CHRISHOLM, S.W., DEVEREUX, R. AND STAHL, D.A. (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Applied Environmental Microbiology* **56** (6): 1919-1925.

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

BEEHARRY, A.O., WENTZEL, M.C., AND EKAMA, G.A. (2001) Evaluation of batch test for measurement of active biomass in activated sludge mixed liquor. Res. Dept. W112, Department of Civil Engineering, University of Cape Town, Rondebosch 7700, South Africa.

Bergey's manual of Systematic bacteriology (1984) Volume 1. Williams and Wilkins, Baltimore, USA.

BITTON, G. 1999. *Wastewater Microbiology* (2nd ed.). John Wiley and Sons: USA.

BLACKALL, L.L. and BURRELL, P.C. (1998) The microbiology of nitrogen removal in activated sludge systems. *In*: SEVIOUR, R. J. and BLACKALL, L. L. (ed). *The microbiology of activated sludge*. Chapman & Hall: London. p 203-226.

BLACKALL, L.L., BOND, P.L., CROCETTI, G.C. AND CHRISTENSON, M. (1998) The microbial diversity of complex consortia in waste treatment processes: correlation between process performance and microbial composition. *Microbial community and functions in wastewater treatment processes:* proceedings of the second International Symposium of the Center for Excellence, University of Tokyo. p 94-101.

BLAKENSHIP, R.E. (1992) Origin and early evolution of photosynthesis. *Photosynthetic Research.* **33**: 91-111.

BOLTON, R.I. AND KLEIN, L. (1971) *Sewage Treatment* (2nd ed.) Butterworth and Co. Limited: London.

BOND, P.L., KELLER, J. AND BLACKALL, L.L. (1999) Bio-P and non-bio-P bacteria identification by a novel microbial approach. *Water Science and Technology*. **39** (1): 13-20.

BRAUN-HOWLAND, E.B., DANIELSEN, S.A. and NIERZWICKI-BAUER, S.A. (1992) Development of a rapid method for detecting bacterial cells *in situ* using 16S rRNA-targeted probes. *Biotechniques* **13**(6): 928-933.

BROCK, T.D. (1979) *Biology of microorganisms*. (3rd ed) Prentice Hall, Englewood Cliff. New Jersey.

BROSIUS, J., DULL, T.L., SLEETER, D.D. and NOLLER, H.F. 1981. Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*. *Journal of Molecular Biology* **148**: 107-127.

BURKE, R.A., DOLD, P.L. AND MARIAS G.V.R (1986) Biological excess phosphorus removal in short sludge age activated sludge system. *Research Report W58*, Department of Civil Engineering, University of Cape Town, Rondebosch 7700, South Africa.

BURA, R., CHEUNG, M., LIAO, B., FINLAYSON, J., LEE, B.C., DROPPO, I.G., LEPPARD, G.G. AND LISS, S.N. (1998) Composition of extracellular polymeric substances in the activated sludge floc matrix. *Journal of Environmental Engineering* **37** (4-5): 325-333.

CHUDOBA, P., CAPDEVILLE, B. AND CHUDOBA, J. (1992) Explanation of the biological meaning of the So/Xo ratio in batch cultivation. *Water Science and Technology* **26** (3-4): 743-751.

CHUDOBA, P., CHANG, J. AND CAPEDEVILLE, B. (1991) Synchronised division of activated sludge microorganisms. *Water Research* **25** (7): 817-822.

CLOETE, T.E. (1998) *Introduction to Microbiology*

CLOETE, T.E. and MUYIMA, N.Y.O. (eds.). 1997. *Microbial Community Analysis: The Key to the Design of Biological Wastewater Treatment Systems*. Perfect Page Publishing Services: London.

CLOETE, T.E. AND THANTSHA, M. (2003) Microbial characterization of activated sludge mixed liquor suspended solids. *Water Research Commision Report no.*1191/1/03.

CROCETTI, G.R., HUGENHOLTZ, P., BOND, P.L., SCHULER, A., KELLER, J., JENKINS, D. AND BLACKALL, L. (2000) Identification of polyphosphate accumulating organisms and design of 16S rRNA-directed probes for their detection and quantitation. *Applied Environmental Microbiology* **66**: 1175-1182.

CRONJE, G.L., BEEHARY, A.O., WENTZEL, M.C. and EKAMA, G.A. (2002) Active biomass in activated sludge mixed liquor. *Water Research* **36**: 439-444.

DALEY, R.J. (1979) Direct epifluorescence of the native bacteria: Uses, limitations and comparative accuracy. In: Costerton, J.W. and Colwell, R.R. *Native aquatic bacteria Enumeration, activity and ecology*. ASTM STP 695. Am. Soc. Test. Mater.

DAVIS, B.D., LUGER, S.M. AND TAI, P.C. (1986) Role of ribosome degradation in the death of starved *Esterichia coli* cells. *Journal of Bacteriology* **166** (2): 439-445.
DAWES, E.A. (1986) Microbial energetics. Blackie and Sons Ltd., Glasgow.

DELONG, E.F., WICKHAM, G.S. and PACE, N.R. (1989) Phylogenetic stains: ribosomal RNA-based probes for the identification of single microbial cells. *Science* **243**: 1360-1363.

DIRCKS, K., PIND, P.F., MOSBAEK AND HENZE, M. (1999) Yield determination by respirometry- The possible influence of storage under aerobic conditions in activated sludge. *Water SA* **25** (1): 69-74.

DOLD, P.L., EKAMA, G.A. AND MARAIS, G.V.R. (1980) A general model for the activated sludge process. *Prog. Water Technology* **12** (60): 47-77.

DOLD, P.L., WENTZEL, M.C., BILLING, A.E., EKAMA, G.A. AND MARAIS, G.V.R. (1991) Activated sludge simulation programs. Water Research Commision, PO Box 824, Pretoria 0001, South Africa.

DOW, C.S. AND WITTENBURY, R. (1980) Prokaryote form and function. In: Ellwood, D.C., Hedger, M.J., Latham, M.J., Lynch, J.M. and Slater, J.H. (eds): *Contemporary microbial ecology*. Academic press: London. p 391-417.

EKAMA, G.A., DOLD, P.L. and MARAIS, G.V.R. 1986. Procedures for determining influent COD fractions and the maximum specific growth rate of heterotrophs in activated sludge systems. *Water Science and Technology* **18**: 91-114.

FLäRDH, K.P.S., COHEN, S. AND KJELLEBERG (1992) Ribosomes exist in large excess over the apparent demand for protein synthesis during carbon starvation in marine *Vibrio sp.* Strain CCUG 15956. *Journal of Bacteriology* **174**: 6780-6788.

FOX, G.E., STACKEBRANDT, E., HESPELL, R.B., GIBSON, J., MANILOFF, J., DYER, A., WOLFE, R.S., BALCH, W.E., TANNER, R., MAGRUM, L., ZABLEN, L.B., BLAKEMORE, R., GUPTA, R., BONEN, L., LEWIS, B.J., STAHL, D.A., LUEHRSEN, K.R., CHEN, K.N. and WOESE, C.R. (1980) The phylogeny of prokaryotes. *Science* **209**: 457-463.

GEST, H. and FAVINGER, J.L. (1983) *Heliobacterium chlorum*, an anoxygenic brownish-green photosynthetic bacterium containing a ‘new’ form of bacteriochlorophyll. *Arch. Microbiology* **136**: 11-16.

GRADY, J., SMELTS, B.F. AND BARBEAU, D.S. (1996) Variability in kenetic parameter estimates: a review of possible causes and a proposed terminology. *Water Research* **30** (3): 742-748.

GRAY, N.F. (1989) *Biology of Wastewater*. Bookcraft Ltd. Great Britain.

GROUSE, R.L., GAAL, T., BACTLETT, M.S., APPLEMAN, J.A. AND ROSS, W. (1996) rRNA transcription and growth rate dependant regulation of ribosome synthesis in *Escherichia coli. Annual Review of Microbiology* **50**: 645-677.

HANTPOL'S TECHNICAL SERVICE DEPARTMENT. (2001) Glossary of terms [online]. Available from: http://www.hantpol.com.htm

HENZE, M., GUJER, W., MINO, T., WENTZEL, M.C. AND MARAIS, G.V.R. (1995) *Activated Sludge Model No. 2*. IAWQ Scientific and Technical Report No. 3. IAWQ: London.

HICKS, R.E., AMANN, R.I. and STAHL, D.A. (1992) Dual staining of natural bacterioplankton with 4', 6-diamidino-2-phenylindole and fluorescent oligonucleotide probes targeting kingdom level 16S rRNA sequences. *Applied and Environmental Microbiology*, **58**: 2153-2163.

HUGENHOLTZ, P., GOEBEL, B.M. and PACE, N. 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *Journal of Bacteriology* **180** (18): 4765-4774.

JOHNSTON SMITH CONSULTING LTD. (2001) Treatment of Domestic Sewage [online]. Available from: http://www.johnstonsmith.co.uk/fact12.html

JORAND, F., BOUE-BIGNE, F., BLOCK, J.C. AND URBAIN, V. (1998) Hydrophobic/hydrophilic properties of activated sludge exopolymeric substances. *Water Science and Technology* **37** (4-5): 307-315.

KAMPFER, P., ERHART, R., BEIMFOHR, C., BOHRINGER, J., WAGNER, M. AND AMANN, R. (1996) Characterization of bacterial communities from activated sludge: culture-dependant numerical identification versus *in situ* identification using group- and genus-specific rRNA- targeted oligonucleotide probes. *Microbial Ecology* **32**: 101-121.

KAPPELER, J., AND GUJER, W. (1992). Estimation of kinetic parameters of heterotrophic biomass under aerobic conditions and characterization wastewater for activated sludge modeling. *Water Science Technology*. **25** (6): 125-140.

KAWAHARASAKI, M., KANAGAWA, T., TANAKA, K. AND NAKAMURA, K. (1998) Development and application of 16S rRNA-targeted oligonucleotide probe for detection of the phosphate accumulating bacterium *Microlunatus phosphovorus* in an enhanced biological phosphorous removal process. *Water Science and Technology* **37** (4-5): 481-484.

KAWAHARASAKI, M., TANAKA, H., KANAGAWA, T. AND NAKAMURA, K. (1999) *In situ* identification of polyphosphate accumulating bacteria in activated sludge by dual staining with rRNA-targeted oligonucleotide probes and 4', 6-diamidino-2-phenylindol (DAPI) at a polyphosphate-probing concentration. *Water Research* **33**: 257-265.

KOCH, A.L. (1971) The adaptive response of *Escherichia coli* to feast and famine existence. *Advance Microbiology and Physiology* **6**: 147-217.

LANE, D.J. (1996) 16S/23S rRNA sequencing. In: Stackebrandt, E. and Goodfellow, M. (eds.), *Nucleic acid techniques in bacterial systematics.* John Wiley and Sons: New York. pp. 115-148.

LATHE, R. (1985) Synthetic oligonucleotide probes deduced from amino acid sequence data. Theoretical and practical considerations. *Journal of Molecular Biology* **183**: 1-12.

LEE, B.J., WENTZEL M.C. AND EKAMA G.A. (2003) Batch test for measurement of ordinary heterotroph organism active mass in activated sludge mixed liquor. Res. Dept. W118, Department of Civil Engineering, University of Cape Town, Rondebosch 7700, South Africa.

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

LILLEY, I.D., PYBUS, P.J. AND POWER, S.P.B. (1997) Operating manual for biological nutrient removal wastewater treatment works. *Water Research Commision Report No.* TT 83/97.

MAMAIS, D., JENKINS, D. AND PITT, P. (1993) A rapid physical-chemical method for the determination of readily biodegradable soluble COD in municipal wastewater. *Water Research* **27** (1): 195-197.

MANZ, W., AMANN, R.I., LUDWIG, W. and SCHLEIFER, K-H. (1992) Phylogenetic olygonucleotide probes for the major subclasses of proteobacteria: problems and solutions. *Systematic and Applied Microbiology* **15**: 593-600.

MARAIS, G.V.R. and EKAMA, G.A. (1976) The activated sludge process: Part 1 – Steady state behavior. *Water SA* **2** (4): 163-200.

MAURER M. AND GUJER W. (1994) Prediction of the performance of enhanced biological phosphorus removal plants. *Water Science and Technology* **30** (6) 333-334.

MBEWE, A., WENTZEL, M.C. AND EKAMA, G.A. (1995) Characterisation of Municipal Wastewaters, UCT Report No. W 84. Dept. Civil Eng., Univ. of Cape Town, Rondebosch 7701, South Africa.

MCGHEE, T.J. (1991) *Water Supply and Sewerage* (6^{th} ed.). McGraw-Hill Inc.: Singapore.

MINO, T., VAN LOOSDRECHT, M.C.M. AND HEIJNEN, J.J. (1998a) Microbiology and biochemistry of the enhanced biological phosphate removal processes. *Water Research* **32** (11): 3193-3207.

MINO, T., SATOH, H. AND MATSUO, T. (1998b) New aspects of population dynamics of enhanced biological phophate removal processes. *Microbial community and functions in wastewater treatment processes:* proceedings of the second International Symposium of the Centre for Excellence, University of Tokyo. pp 102-114.

MOBARRY, B., WAGNER, M., URBAIN, V., RITTMANN, B. and STAHL, D.A. (1996) Phylogenetic probes for analysing abundance and spatial organisation of nitrifying bacteria. *Applied and Environmental Microbiology*, **62**(6): 2156-2162.

MUDALY, D.D., ATKINSON, B.W. AND BUX, F. (2000) Microbial community profile of a biological excess phosphorous removal activated sludge system using a cultivation-independent approach. *Water SA* **26** (3): 343-352.

MUDALLY, D.D., ATKINSON, B.W. AND BUX, F. (2001) FISHing for biomass in activated sludge mixed liquor: the slippery VSS fraction. In: *Advances in Water and Wastewater Treatment Technology in - Molecular Technology, nutrient removal, sludge reduction and environmental health*, Matsuo T, Hanaki K, Takizawa S and Satoh H (eds), Elsevier Science, Amsterdam: Netherlands.

MUNCH, E. AND POLLARD, P.C. (1997) Measuring Bacterial Biomass-COD in Wastewater Containing Particulate Matter. *Water Research* **31**(10): 2550-2556.

NAKAMURU, K., HIRAISHI, A., YOSHIMA, Y., KAWAHARASAKI, M., MASUDA, K. AND KAMAGATA, Y. (1995) Microlunatus phosphovorus gen. Nov. sp. Nov., a new Gram-positive polyphosphate accumulating bacterium isolated from activated sludge. *International Journal of Systematic Bacteriology* **45**: 17-22.

NAKAMURU, K., MASUDA, K. AND MIKAMI, E. (1991) Isolation of a new type of phosphate accumulating bacterium and its phosphate removing characteristics. *Journal of Fermentation and Bioengineering* **71**: 258-263.

NOVAK, L., LARREA, L. AND WANNER, J. (1994) Estimation of maximum specific growth rate of heterotrophic and autotrophic biomass: a combined technique of mathematic modeling and batch cultivations. *Water Science and Technology* **30** (11): 171-180.

OETHER, D.B., DE LOS REYES, F.L. AND RASKIN, L. (1999) Interfacing phylogenetic oligonucloetide probe hybridizations with representations of microbial populations and specific growth rates in mathematical models of activated sludge processes. *Water Science and Technology* **39** (1): 11-20.

OLSEN, G.J., LANE, D.J. GIOVANNONI, S.J., PACE, N.R., and STAHL, D.A. (1986) Microbial ecology and evolution: a ribosomal RNA approach. *Annual Review of Microbiology* **40**: 337-365.

PILLAY, S. (1998) Personal communication. Works operator, Amanzimtoti Wastewater Works.

POLLARD, P.C., STEFFENS, M.A., BIGGS, C.A. AND LANT, P.A. (1998) Bacterial growth dynamics in activated sludge batch assays. *Water Research* **32** (3) 587-596.

PORTER, K.G. and FEIG, Y. 1980. The use of DAPI for identifying and counting aquatic microflora. *Limnology and Oceanography* **25**: 943-948.

POULSEN, L.K., BALLARD, G. AND STAHL, D.A. (1993) Use of rRNA fluorescence in situ hybridization for measuring the activity of single cells in young and established biofilms. *Applied and Environmental Microbiology* **59** (5): 1354-1369.

QASIM, S.R. (1994) *Wasterwater Trearment Plants, Planning, Design and Operation.* Technomic Publishing Company Inc. Lancaster: USA.

RANDALL, E.W., WILKINSON, A. AND EKAMA, G.A. (1991). An instrumentation for the direct determination of oxygen utilization rate. *Water SA.,* **17** (1), 11-18.

RITTMANN, B.E., LASPIDOU, C.S., FLAX, J., STAHL, D.A., URBAIN, V., HARDUIN, H., VAN DER WAARDE, J.J., GEURKINK, B., HENSSEN, M.J.C., BROUWER, H., KLAPWIJK, A. and WITTERAUW, M. (1999) Molecular and modeling analysis of the structure and function of nitrifying activated sludge. *Water Science and Technology* **39**: 51-60.

RITTMANN, B.E., LASPIDOU, C.S., FLAX, J., STAHL, D.A., URBAIN, V., HARDUIN, H., VAN DER WAARDE, J.J., GEURKINK, B., HENSSEN, M.J.C., BRAOUWER, H., KLAPWIJK, A. AND WETTERAUW, M. (1999) Molecular and modeling analysis of the structure and function of nitrifying activated sludge. *Water Science and Technology* **39** (1): 51-60.

ROLLER, C., WAGNER, M., AMANN, R., LUDWIG, W. and SCHLEIFER, K-H. (1994) *In situ* probing of Gram-positive bacteria with high DNA G+C content using 23S rRNA-targeted oligonucleotides. *Microbiology* **140**: 2849-2858.

SANDEN, B., BJORLENIUS, B., GRUNDITZ, C. AND DALHAMMAR, G. (1996) Nitrifying bacteria in the influent of a wastewater treatment plant- influence and importance on nitrifying capacity. *Water Science and Technology* **34** (1-2): 75-82.

SCHAECHTER, M.O., MAALOE, O. AND KJELDGAARD (1958) Dependency on medium and temperature of cell size and chemical composition during balanced growth of *Salmonella typhimurium*. *Journal of General Microbiology* **19**: 592-606.

SCHROEDER, E.D. (1977) Water and Wastewater Treatment. McGraw-Hill Inc.: USA.

SINGH, N. (1998) Personal communication. Works operator, Darvill Wastewater Works.

SMOLDERS, G.J.F., VAN DER MEIJ, J., VAN LOOSDRECHT, M.C.M. AND HEIJNEN, J.J. (1994a) Stoichiometric model of the aerobic metabolism of the biological phosphorous removal process. *Biotechnology and Bioengineering.* **44**: 837-848.

SMOLDERS, G.J.F., VAN DER MEIJ, J., VAN LOODSRECHT, M.C.M. and HEIJNEN, J.J. (1994b) Model of the anaerobic metabolism of the biological phosphorous removal process: stoichiometry and pH influence. *Biotechnology and Bioengineering* **43**: 461-470.

SNAIDR, J., AMANN, R., HUBER, I., LUDWIG, W. AND SCHLEIFER, K-H. (1997) Phylogenetic analysis and *in situ* identification of bacteria in activated sludge. *Applied Environmental Microbiology* **63**: 2884-2896.

SPEECE, R.E., ENGELBRECHT, R.S. AND AUKAMP, D.R. (1973) Cell replication and biomass in the activated sludge process. *Water Research* **7**: 361-374.

STACKEBRANDT, E., POHLA, H., KROPPENSTEDT, R., HIPPE, H. AND WOESE, C.R. (1985) 16S rRNA analysis of *Sporomusa, Selenomonas* and *Megasphaera*: on the phylogenetic origin of Gram-positive eubacteria. *Arch. Microbiology* **143**: 270-276.

STAHL, D.A. and AMANN, R.I. (1991) Development and application of nucleic acid probes. *In*: STACKELBRANT, E. and GOODFELLOW, M.. *Nucleic acid techniques in bacterial systematics*. John Wiley & Sons Ltd: England. pp 205-248.

STAHL, D.A., FLESHER, B., MANSFIELD, H.R. AND MONTGOMERY, L. (1988) Use of phylogenetically based hybridization probes for studies in ruminal microbial ecology. *Applied Environmental Microbiology* **54**: 1079-1084.

STANDARD METHODS. (1985) *Standard methods for the Examination of Water and Wastewater* (16th edn.). American Public Health Assoc., 1015 15th Str. NW, Washington DC 200005, USA.

SUDIANA, I.M., MINO, T., SATOH, H. AND MATSUO, T. (1998) Morphology, in situ characterization with rRNA targeted probes and respiratory quinone profiles of enhanced biological removal sludge. *Water Science and Technology* **38** (8-9): 69-76.

TANDOI, V. (2000) The identity of poly-P bacteria in the third millennium. *Newsletter of the IWA specialist group on activated sludge population dynamics.* Vol. 12, No. 1. pp. 13-14.

UBISI, M.F., JOOD, T.W. WENTZEL, MC. AND EKAMA GA. (1997a) Activated sludge mixed liquor heterotrophic active biomass. *Water SA* **23** (3), 239-248.

UBISI, M.F., WENTZEL, MC. AND EKAMA, GA. (1997b) Organic and inorganic components of activated sludge mixed liquor. Res. Rept. W94, Dept. Civil Eng., Univer. of Cape, Ronderbosch 7700, South Africa.

VAN HAANDEL, A.P.C, EKAMA, G.A. AND MARAIS G.v.R. (1981) The activated sludge process 3-single sludge denitrification. *Water Research.,* **15**: 1135-1152.

VAN LOOSDRECHT, M.C.M. AND HEIJNEN, J.J (1997) Importance of bacterial storage polymers in bioprocesses. *Water Science and Technology* **35** (1): 41-47.

WAGNER, M, RATH, G, KOOPS, H-P, FLOOD J and AMANN, R (1996) *In situ* analysis of nitrifying bacteria in sewage treatment plants. *Water Quality International '96 18th IAWQ Biennial International Conference and Exhibition*. pp 207-214.

WAGNER, M. and AMANN, R. (1997) Molecular techniques for determining microbial community structures in activated sludge. In: Cloete, T.E. and Muyima, N.Y.O. (eds.) *Microbial Community Analysis: The key to the design of Biological Wastewater Treatment Systems.* International Society on Water Quality. Scientific report no. 5..

WAGNER, M., AMANN, R.I., LEMMER, H. and SCHLEIFER, K-H. (1993) Probing activated sludge with oligonucleotides specific for Proteobacteria: inadequacy of culture-dependant methods for describing microbial community structure. *Applied and Environmental Microbiology* **59**: 1520-1525.

WAGNER, M., ERHART, R., MANZ, W., AMANN, R., LEMMER, H., WEDI, D. and SCHLEIFER, K. (1994a) Development of an rRNA – targeted oligonucleotide probe specific for the genus Acinetobacter and its application for in situ monitoring in activated sludge. *Applied and Environmental Microbiology*, **60** (3): 792-800.

WAGNER, M., RATH, G., AMANN, R., KOOPS, H-P. and SCHLEIFER, K-H. (1995) *In situ* identification of ammonia-oxidizing bacteria. *Systematic and Appied Microbiology*. **18**: 251-264.

WAGNER, M., RATH, G., KOOPS, H-P. and SCHLEIFER, K-H. (1996) *In situ* analysis of nitrifying bacteria in sewage treatment plants. *Water Science and Technology* **3**: 237-244.

WALLNER, G., AMANN, R. AND BEISKER, W. (1993) Optimizing fluorescent *in situ* hybridization of suspended cells with rRNA-targeted oligonucleotide probes for the flow cytometric identification of microorganisms. *Cytometry* **14**: 136-143.

WENTZEL, M.C., DOLD P.L., EKAMA G.A. AND MARAIS G.V.R. (1990) Biological excess phosporus removal – Steady state process design. *Water SA* **16** (1): 29-48.

WENTZEL, M.C. AND EKAMA, G.A. (1997) Principles in the modeling of biological wastewater treatment plants. In: Cloete, T.E. and Muyima, N.Y.O. (eds.) *Microbial community analysis: The key to the design of biological wastewater treatment systems.* I.A.W.Q. Scientific and Technical Report no. 5. Cambridge University Press: London pp. 73-82.

WENTZEL, M.C., LOTTER, L.H., LOEWENTHAL, R.E. AND MARAIS, G.V.R. (1986) Metabolic behaviour of *Acinetobacter spp.* in enhanced biological phosphorous removal- a biochemical model. *Water SA* **12** (4): 209-224.

WENTZEL, M.C., LOTTER, L.H., LOEWENTHAL, R.E. AND MARAIS, G.V.R. (1991) Evaluation of biochemical models for biological excess phosphorous removal. *Water Science and Technology* **23**: 567-576.

WENTZEL, M.C., MBEWE, A. AND EKAMA, G.A. (1995) Batch test for the measurement of readily biodegradable COD and active organism concentrations in municipal wastewaters. *Water SA*. **21** (2): 117-124.

WENTZEL, M.C., UBISI, M.F. AND EKAMA, G.A. (1998) Heterotrophic active biomass component of activated sludge mixed liquor. *Water Science and Technology* **37** (4-5), 79-87.

WISCONSIN DEPARTMENT OF NATURAL RESOURCES. (2000) The Microbiology of Activated Sludge [online]. Available from: http://www.dnr.state.wi.html

WOESE, C.R. (1987) Bacterial evolution. *Microbiology Review* **51** (2): 221-271.

WOESE, C.R., KANDLER, O. AND WHEELIS, M.L. (1990) Towards a natural system of organisms: proposal for the domains Archaea, Bacteria and Eukarya. *Proclamations of the National Academy of Science USA* **87**: 4576-4579.

WOESE, C.R., STACKEBRANDT, E., WEISBURG, W.G., PASTER, B.J., MADIGAN, M.T., FOWLER, V.J., HAHN, C.M., BLANZ, P., GUPTA, R., NEALSON, K.H. and FOX, G.E. (1984a) The phylogeny of purple bacteria: the alpha subdivision. *Systematic and Applied Microbiology* **5**: 315-326.

WOESE, C.R., WEISBURG, W.G., HAHN, C.M. PASTER, B.J., ZABLEN, L.B., LEWIS, B.J., MACKE, T.J., LUWIG, W and STACKEBRANDT, E. (1985) The phylogeny of purple bacteria: the gamma subdivision. *Systematic and Applied Microbiology* **6**: 25-33.

WRC (1984) Theory, Design and operation of nutrient removal Activated Sludge processes. Water Research Commission, PO Box 824, Pretoria 0001, South Africa.

9. APPENDICES

APPENDIX 1

Determination of filtrate COD, MLSS and MLVSS

a) Determination of filtrate COD (Standard methods, 1985)

Reagents:

1.1 Standard potassium dichromate ($K_2Cr_2O_7$) solution, 0.250 N

Dissolve 12.259 g. $K_2Cr_2O_7$ (primary standard grade) previously dried at 103ºC for 3 hours and then cooled in a desiccator, in distilled water and make up to 1000 mL.

1.2 Sulphuric acid (H_2SO_4) reagent

Dissolve 15 g of silver sulphate (Ag_2SO_4) in 2500 mL of concentrated (<98%) H_2SO_4 using a magnetic stirrer. Dissolution takes place between 1 and 2 days.

1.3 Standard ferrous ammonia sulphate [Fe $(NH_4)_2(SO_4)_2$] 0.05 N

Dissolve 100g analytical grade Fe $(NH_4)_2(SO_4)_2$. $6H_2O$ in distilled water using a 5 L volumetric flask. Add 100 mL of concentrated H_2SO_4 and make up to 5000 mL.

Standarization: Pipette 5 mL of standard $K_2Cr_2O_7$ (1.1) solution into an Erlenmeyer flask and dilute to 50 mL with distilled water. Add. 15 mL of the H_2SO_4 (1.2) and allow to cool before titrating with Fe $(NH_4)_2(SO_4)_2$ titrant, using 2 drops of ferroin indicator (1.4)

$$\text{Normality Fe } (NH_4)_2(SO_4)_2 = \frac{\text{mL } K_2Cr_2O_7 * 0.25}{\text{mL Fe } (NH_4)_2(SO_4)_2}$$

1.4 Ferroin indicator

Dissolve 1.485 g 1,10-phenanthroline monohydrate, together with 0.695 g $FeSO_4.7H_2O$ in distilled water and dilute to 100 mL.

1.5 Mercuric sulphate ($HgSO_4$) powder

Procedure:

1. Collect the correct number of clean, dry 250 mL Erlenmeyer flasks with the ground-glass 24/40 necks. Two flasks are used for each test. Include 2 extra flasks if influent COD is being tested and 2 extra flasks if reactor or effluent COD is being tested.
2. Place approximately 0.04 g $HgSO_2$ powder in each flask. This is the e quivalent to the volume of 2 match heads.
3. Place 8 - 10 glass beads (± 3mm diameter) in each flask.
4. For each influent sample pipette 10 mL of unfiltered influent into each of the two flasks. Pipette 10 mL of distilled water into each of the two flasks. (these are the blanks)
5. Add 5 mL of $K_2Cr_2O_7$ solution to each of the flasks.
6. In turn, carefully add 15 mL of the H_2SO_4 COD acid (1.2) to each flask, ensuring that no vapor escapes from the flask. This is most easily done by pouring the acid down the wall of the flask while the flask is tilted. Immediately attach the flask to the jacket condenser.
7. Once acid has been added to each flask ensure that the flasks are level on the heating pad. Allow the contents of the flask to boil for 2 hours. Ensure that the water flow rate in the condensers is swift enough to condense all vapors rising up the condensers.
8. Allow the flasks to cool to room temperature with the condensers still in position.

9. Pour approximately 80mL of distilled water through the top opening of each of the condensers into the sample mixture, ensuring that the side walls of the condensers are well washed.
10. Remove the flasks from the condensers and the heating pad and add 2 drops of ferroin indicator to each flask.
11. Titrate with Fe $(SO_4)_2$ (NH_4) solutions until one drop of titrant changes the solution color to reddish brown.

Calculation:

$$\text{mg/L COD} = \frac{(a-b)* N * 8000}{\text{mL sample}}$$

Where;

a = average mL Fe $(SO_4)_2$ (NH_4) used to titrate blank of same volume as sample
b = average mL Fe $(SO_4)_2$ (NH_4) used to titrate sample
N = normality of Fe $(SO_4)_2$ (NH_4)

b) Determination of MLSS:

1. Place 100 mL of activated sludge mixed liquor in a centrifuge tube.
2. Centrifuge at 4000xg for 8 min.
3. Discard the supernatant and quantitatively scoop the sludge into a pre-weighed crucible.
4. Place the crucible into a drying oven at 105°C and leave overnight. Remove from oven and allow cooling in a dessicator.
5. Re-weigh crucible.

6. Determine the MLSS according to the following calculation.

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

c) Determination of MLVSS:

1. Place the pre-weighed crucible (from the MLSS determination) into a muffle furnace.
2. Ash at 550°C for 1h. Remove the crucible and allow cooling in dessicator.
3. Re-weigh the crucible
4. Determine the MLVSS according to the following equation:

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

APPENDIX 2

Preparation of Casitone glycerol yeast autolysate agar

Ingredients:

Bacto® casitone	5g
Glycerol	5g
Yeast autolysate	1g
Bacteriological agar	16g
Distilled water	1000 mL

Preparation:

With the exception of the glycerol, heat all ingredients and continuously stir until clarified. Remove from heat, add glycerol and continue stirring until dissolved. Adjust pH to 7.2 and autoclave at 121ºC for 20 min.

APPENDIX 3

Cell fixation with paraformaldehyde

a) Preparation of 4% Paraformaldehyde in phosphate buffered saline (Amann *et al*, 1990):

1. 65 mL of dd H_2O was heated to 60°C
2. 4 g of paraformaldehyde (Sigma, St. Louis, MO, USA) was weighed out using a top balance (Scaltec SPB 52)
3. One drop of 2 M NaOH solution was added and rapidly stirred until the solution is clear.
4. The solution was removed from the heater/magnetic stirrer (Agimatic – N), upon which 33 mL of 3x PBS is added.
5. The solution was than be filtered through a 0.22 µL filter.
6. pH was adjusted to 7.2 with HCl
7. The solution was allowed to cool at 4°C before use.

b) Fixation of cells (Amann *et al*, 1990a; Roller *et al*, 1994):

1. One volume of cells was mixed with three volumes of fixation buffer.
2. Incubate for 1.5 hours.
3. Centrifuge at 4000xg for 8 min and pellet cells.
4. Wash pellet with PBS.
5. Centrifuge at 3000xg for 5 min.
6. Resuspend cells in PBS.
7. Add an equal volume of absolute ethanol.

APPENDIX 4

Sonication of activated sludge for dispersion

The activated sludge floc structure can sometimes be highly compact. In order to satisfactorily conduct cell counts it is often necessary to disperse the cells by sonication (Virsonic 100, Virtis, USA) at low frequency, after fixation.

Procedure:

1. Place 1mL of fixed activated sludge in a 2mL micro test tube.
2. Immerse the sonication probe into the mixed liquor to at least ¾ of the total volume.
3. Cover the top of the tube surrounding the probe with parafilm paper.
4. Sonicate at 5-10 watts for 8-12 min for an MLSS of 3000-3500 mg/L.

APPENDIX 5

Total cell counts by membrane filtration and staining with DAPI

Membrane filtration and DAPI staining (Porter and Feig, 1980)

Counter stain cellulose acetate filters (pore size, 0.22 µm, Micron Separations Inc.) with Sudan Black solution for 12 h. Add 900 µl of PBS (1x) to 10 µl activated sludge in a 2 ml micro-test tube. Add 100 µl of the non-anionic detergent (Igepal CA-30, Sigma Chemicals) and mix. Add 1ml of DAPI (0.5 µg/ml) to 1 ml of the activated sludge mixture. Allow staining to proceed for ten minutes. Place the stained cellulose acetate filter and a 0.45µm backing filter at the base of a 15ml filter tower and wet with sterile double distilled water. Quantitively transfer the stained activated sludge mixture to the filter tower under a slight vacuum. After filtration, remove excess DAPI stain by washing the filter in the filtering device with sterile double distilled water. Mount the stained cellulose filter on one drop of a glycerol/PBS mixture (95:5 v/v) on a glass slide. Add one drop of a antifading mounting medium (VECTASHIELD, Vector Laboratories, California) to the mounted filter surface before placing the cover slip. DAPI fluorescence was detected with a Zeiss Axiolab microscope (Zeiss, Germany) fitted for epifluorescence microscopy with a 50 W high-pressure mercury bulb and Zeiss filter set 01.

Determination of total cell counts

View the slide with the amount filter under an epifluorescence (1000x magnification) microscope using an appropriate filter set for DAPI fluorescence (e.g. Zeiss filter set 01). Select twenty random fields for cell counts using the image analysis software (MACRO).Determine the mean total cell count for the twenty fields. Determine the total cell count using the following equation:

$$TCC = MTCC \times DF \times MF$$

Where:

TCC = total cell count

MTCC = mean total cell count for twenty fields

DF = dilution factor

MF = total number of microscope fields on filter (55703 under 1000 x magnification)

APPENDIX 6

Immobilization of fixed cells on microscope slides

Note: Slides on which a hydrophobic coating separating eight glass surface windows (Merc, Germany) are routinely used.

a) Pretreatment of microscope slides (Sigma diagnostics, procedure no. P 8920):

1. Clean the slide surface by soaking in warm detergent solution for 1 h, rinse thoroughly and air dry.
2. Place clean slides in diluted (1:10) Poly-L-Lysine solution (Sigma diagnostics, USA) for 5 min at room temperature (18-26°C).
3. Drain slides and dry in 60°C oven for 1 h or at room temperature overnight.

b) Immobilization of fixed cells on pre-treated slides (Amann, 1995a):

1. Spread approximately 3 to 5 µl of fixed cell suspension on the Poly-L-lysinated slide over an area of about 5 mm in diameter.
2. Allow the smear to air dry.
3. Dehydrate the cells by successive passages through 50, 80 and 98% ethanol washes (3 min each).
4. Slides can be stored dry at room temperature indefinitely.

APPENDIX 7

Whole cell hybridization

Hybridization should be carried out in a properly sealed moisture chamber to prevent evaporative concentration of the hybridization solution which might result in nonspecific binding of the probe to the cells. A 50ml polypropylene screw top tube (Corning Glass works, USA) serves as a convenient and portable hybridization chamber.

Materials:

50ml polypropylene tube

Whatman 3MM paper

Hybridization buffer

0.9 M sodium chloride

0.01% sodium dodecyl sulphate

20 mM Tris/HCl

X % formamide

pH 7.2

Procedure:

1. Soak a strip of Whatman 3MM paper in hybridization buffer and place in polypropylene tube.
2. Allow the chamber to equilibrate for 15min at 46°C hybridization temperature.
3. For each spot to be hybridized mix 9 µl hybridization buffer (prewarmed to 46°C) with 1 µl of fluorescent probe (50 ng/µl).
4. Spread 10µL of hybridization buffer/probe mix on each spot of fixed cells.
5. Quickly transfer slide to the prewarmed moisture chamber and hybridize for 2 h at 46°C.

6. After hybridization remove the slide from the moisture chamber and immediately stop hybridization by rinsing the probe from the slide with hybridization buffer prewarmed to a wash temperature of 48°C.
7. Transfer the slide to a polypropylene tube filled with 50 µl hybridization buffer and incubate for 20 min at 48°C.
8. Dip the slides in ddH2O, shake away excess water and air dry.
9. Mount the slides in VECTASHIELD and view with an epifluorescence microscope. Select twenty fields randomly for enumeration by image analysis.
10. The cell count for each probe is determined by using the following equation:

$$\frac{n\,(probe)}{n\,(DAPI)} \times n\,(MF)$$

Where:

n (probe) = average number of cells bearing probe conferred fluorescence.
n (DAPI) = average number of cells bearing DAPI conferred fluorescence.
n (MF) = total cell count as obtained by membrane filtration.

APPENDIX 8

RNA extraction, quantification and purity determination

a) Extractionof RNA:

Reagents

Bead beating buffer: 50 mM NaOAc, 10mM disodium EDTA, pH 5.1
Phenol equilibrated with bead beating buffer (Sambrook *et al.*, 1989)
Chloroform-isoamyl alcohol (24:1, v/v)
20% SDS
3 M NaOAc, pH 5.1

Acid washed glass beads. Soak glass beads in 6 M HCl for 10min. Rinse with distilled water, checking with pH paper until the pH is equivalent to that of fresh distilled water. Bake the rinsed beads at 180°C until completely dry.

Procedure:

Fill the bead beating vial (Biospec products cat. No. 1083-2) one third with acid washed, baked glass beads (0.1 - 0.15 mm beads, Sigma chemical Co.). Centrifuge 1 ml of activated sludge mixed liquor (3000xg, 5 min, 4°C) and wash twice in ice cold bead beating buffer. Resuspend the pellet in 750µl bead beating buffer and transfer to the bead beating vial. Add 20 µl of 20 % SDS and fill the remaining portion of the vial with buffer saturated phenol. Beat for 4 min in a bead beating machine at 5000 rpm (Biospec products, Mini-Beadbeater). Transfer the vial to a heating block for 15 min at 60°C. Beat for a further 2 min. Spin the vial in a micro centrifuge at 1000 x g for 10 min to separate the organic and aqueous phases. Remove the aqueous (top) phase to a clean microfuge tube. Add 400µl of buffer saturated phenol and vortex. Add 100µl of chloroform-isoamyl alcohol and vortex. Spin mixture in a microfuge (4°C, 3000xg) for 5 min. Remove the top layer and repeat the phenol-

chloroform extraction two times. Remove the top layer, add 1/10 volume of 3 M NaOAc (pH 5.1), and precipitate with two volumes of ethanol at -20 C for 12 hr. Air dry the pellet and resuspend in 500 µl of TE buffer. (pH 7.4). Take at least 30 µl of RNA for quantification and purity determination.

b) Quantification and purity determination of RNA

Procedure:

1. Pipette 30 µl of sample RNA extract into a quartz cuvette.
2. Add 2970 µl of TE buffer. Mix gently using a 1ml pipette.
3. To a separate cuvette add 3 ml of TE buffer.
4. Place both cuvettes in the spectrophotometer.
5. Select for the reference cuvette and set reference absorbance at 260 nm to 0.
6. Select for the sample RNA cuvette and measure the absorbance of RNA at 260 nm.
7. Select for the reference cuvette and set the reference absorbance at 280 nm to 0.
8. Select for the sample RNA cuvette and measure the absorbance at 280 nm.
9. Determine OD 260/ OD 280 ratio.
10. Determine RNA [ ] using the following:

RNA (µg/mL) = AB 260 x OD 260 EQ (RNA) x DF x CF

Where: AB260 = measured absorbance at 260nm

OD 260 EQ (RNA) = OD 260 equivalence for RNA where 1 OD unit is equivalent to 40 µg/ ml

DF = dilution factor = 3000/30

CF = concentration actor = 500/1000

The dilution factor is determined by dividing the total liquid volume in the cuvette (3000 µL) by the volume of extracted nucleic acid taken for quantification during absorbance determination.

The concentration factor takes into account that nucleic acid extracted from 1000 µl of activated sludge mixed liquor was made up to 500 µl.

APPENDIX 9

Dot blot Hybridization

Materials:

50ml screw cap polypropelene tube

RNA denaturing solution:

- formamide/formaldehyde(37%)/3-(N-morpholino)-propanesulfonic acid (5:1. 62:1)

Prehybridization buffer:

- 5x Saline sodium citrate (SSC) (Sambrook *et al*., 1989)
- 2% blocking reagent (Roche Molecular Biochemicals, Germany)
- 0.1 % N-lauroylsarcosine
- 0.02 % SDS

Hybridization buffer:

- 4% blocking agent
- 0.1 % N-laoroylsarcosine
- 0.01% SDS
- 0.9 M NaCl
- X % formaldehyde

Wash buffer:

- 20 mM Tris
- 0.01% SDS
- X % formaldehyde

Procedure:

1. Denature RNA by adding 3 volumes of denaturing solution. Incubate at 60°C for 10 min.

2. Transfer samples to a positively charged nylon membrane using a dot blotting device.
3. Immobilize nucleic acids by baking at 120°C in an oven for 30 min.
4. Prehybridize the filters for 1.5h at 46°C with 10ml of prehybridization solution.
5. After pre-hybridization, transfer the filters to hybridization solution containing 5 pm/µl of DIG-labeled probe and hybridize at 46°C for at least 12h
6. After hybridization wash the filters twice for 15 min in 10 mL washing buffer at 48°C.
7. Detect probe conferred DIG-molecules using anti-DIG antibodies coupled with alkaline phosphatase and the colorimetric substrate reagents NBT/BCIP according to the manufacturers instructions. (Boehringer Mannheim, 1995)

APPENDIX 10

Steady state data for the 10 and 20 days UCT operated parent laboratory-scale system

TABLE 10.1 Steady state results for 10 day parent lab-scale anoxic/aerobic activated sludge system. For each sewage batch tested, the daily results have been averaged and the averages are listed with standard deviations.

		COD (mg/l)			TKN (mgN/l)			Nitrate (mgN/l)			OUR	Mixed liquor (mg/l)		
Sewage Batch		Inf	Effl (unfilt)	Effl (filt)	Infl	Effl (unfilt)	Effl (filt)	Anoxic	Aerobic	Effl	(mgO/l/h)	VSS	COD	TKN
21/02	Average	755	51	38	68.4	2.6	3	3.8	18.8	20.2	43	2320	3439	149
(21/11 -	SSD	41	14	14	4.5	0.6	0.3	0.6	1.5	1.8	4.3	272	351	16
05/12/2002)	No of tests	10	11	11	11	9	9	10	10	9	7	9	11	11
22/02	Average	714	41	33	49.9	1.8	2	0.25	10.4	10.6	40.8	2442	3409	164
(06/12 -	SSD	35	24	14	4.4	0.7	0.4	0.13	1.3	1.4	2.1	159	190	27
20/12/2002)	No of tests	7	7	7	8	8	7	6	6	6	7	7	8	8
23/02	Average	688	50	33	50.4	2.4	3.4	0.35	11.6	12.3	40.3	2308	3405	147
(21/12 -	SSD	-	-	-	-	-	-	-	-	-	-	-	-	-
05/01/2003)	No of tests	1	1	1	1	1	1	1	1	1	1	1	1	1
01/03	Average	741	71	41	64	2.8	2.9	1.6	14.6	15	37.8	2229	3236	174
(06/01 -	SSD	49	42	23	3.6	0.6	0.6	0.6	1.9	2.3	2.4	122	292	31
17/01/2003)	No of tests	10	10	9	10	9	10	9	9	9	9	9	10	9
02/03	Average	684	55	51	74	4.1	3.6	6	22.7	22.6	41.4	2066	3235	174
(18/01 -	SSD	84	21	21	10	1.9	1.3	3.1	2.5	3.3	3.4	389	562	15
04/02/2003)	No of tests	12	11	11	12	11	11	11	11	11	8	11	11	11
03/03	Average	654	74	40	51.9	5.2	4.3	0.9	9.8	10.4	35.7	2198	3024	196
(05/02 -	SSD	47	33	15	3.7	2.2	1.8	0.6	1.8	1.2	7	163	304	22
20/02/2003	No of tests	8	8	8	8	7	7	6	8	7	7	8	8	8

TABLE 10.2 Steady state COD and N mass balances, wastewater fractions and mixed liquor parameters for 10 day parent anoxic/aerobic activated sludge system.

ww	Mass balance (%)		Wastewater COD fractions		Mixed liquor		
Batch No.	COD	N	Unbiod. Soluble (fs,us)	Unbio. Particulate (fs,up)	COD/VSS ratio (mgCOD/mgVSS) (fCV)	TKN/VSS ratio (mgN/mgVSS) (fN)	Active Fraction (fav)
21/02	96	95	0.051	0.110	1.47	0.065	0.465
22/03	102	95	0.046	0.16	1.38	0.068	0.392
23/02	105	101	0.048	0.159	1.46	0.063	0.397
01/03	92	92	0.056	0.12	1.43	0.079	0.453
02/03	97	95	0.074	0.12	1.54	0.086	0.458

TABLE 10.3. Steady state results for 20 day parent lab-scale anoxic/aerobic activated sludge system. For each wastewater batch (ww) tested, the daily results have been averaged and the averages are listed with standard deviations.

		COD (mg/l)			TKN (mgN/l)			Nitrate (mgN/l)			OUR	Mixed Liquor (mg/l)		
Sewage Batch		Inf	Effl (unfilt)	Effl (filt)	Infl	Effl (unfilt)	Effl (filt)	Anoxic	Aerobic	Effl	(mgO/l/h)	VSS	COD	TKN
21/02	Average	753	37	29	68.4	3	3.1	4.2	20.5	21.5	22.7	1659	2540	123
(21/11 -	SSD	43	11	13	4.5	0.8	0.6	0.5	1.2	1.2	3.1	208	300	21
05/12/2002)	No of tests	9	8	9	11	10	11	10	10	10	8	9	7	9
22/02	Average	714	42	34	49.9	1.9	2	4.8	24.5	19.9	17.8	1183	1810	95
(06/12 -	SSD	35	8	8	4.4	0.8	0.5	1	2	1.9	0.9	138	240	21
20/12/2002)	No of tests	7	7	8	8	7	7	6	6	5	8	8	7	8
23/02	Average	688	29	25	50.4	2.7	2.8	1.35	11.9	14.8	17.3	890	1243	116
(21/12 -	SSD	--	-	-	-	-	-	-	-	-	-	-	-	-
05/01/2003)	No of tests	1	1	1	1	1	1	1	1	1	1	1	1	1
01/03	Average	741	153	132	64	21.4	21.9	13	26	26	27.5	1560	2370	142
(06/01 -	SSD	49	71	69	3.6	22.7	23.9	0	0	0	13.6	123	236	26
17/01/2003)	No of tests	10	10	10	10	8	8	4	4	4	7	10	10	9
02/03	Average	684	79	61	72	12.7	12.6	10.8	28.7	30.4	25.6	1679	2581	158
(18/01 -	SSD	84	30	27	6.2	15.3	15.5	0.9	3.3	5.6	2.8	153	385	20
04/02/2003)	No of tests	12	12	12	11	12	12	5	5	4	8	12	12	11
03/03	Average	654	49	36	51.9	5.5	4.6	1.15	10.9	13.1	25.9	1815	2719	162
(05/02 -	SSD	47	13	7	3.7	1.5	1.3	0.32	1.1	2.8	1.4	119	328	12
20/02/2003	No of tests	8	8	8	8	8	8	8	8	8	6	8	8	8

TABLE 10.4 Steady state COD and N mass balances, wastewater fractions and mixed liquor parameters for 20 day parent anoxic/aerobic activated sludge system.

ww	Mass balance (%)		Wastewater COD fractions		Mixed liquor		
Batch No.	COD	N	Unbiod. Soluble (fs,us)	Unbio. Particulate (fs,up)	COD/VSS ratio (mgCOD/mgVSS) (fCV)	TKN/VSS ratio (mgN/mgVSS) (fN)	Active Fraction (fav)
21/02	72	90	0.039	0.006	1.51	0.076	0.495
22/03	59	91	0.048	-0.07	1.50	0.082	0.726
23/02	58	94	0.036	-0.089	1.37	0.133	0.861
01/03	100	107	0.178	0.024	1.43	0.105	0.448
02/03	91	110	0.089	0.044	1.50	0.102	0.418

APPENDIX 11

Steady state data for the 10 days DIT parent laboratory-scale system

TABLE 11.1 Steady state results for parent lab-scale anoxic/aerobic activated sludge system. For each wastewater batch (ww) tested, the daily results have been averaged and the averages are listed with standard deviations in brackets.

ww	COD (mg/l)		TKN (mgN/l)		Nitrate + Nitrite (mgN/l)			OUR mgO/l/h	Mixed liquor (mg/l)		
	Inf	Eff	Inf	Eff	Anoxic	Aerobic	eff		VSS	COD	TKN
1	500 (14.09)	32.00 (8.47)	38.08 (2.35)	3.36 (0.25)	1.31 (2.23)	5.04 (5.02)	9.14 (6.02)	30.98 (1.87)	2057 (49) 1864	2935 (318)	140 (7.95)
2	515 (10.44)	33.84 (2.96)	33.18 (3.17)	3.73 (0.89)	2.39 (3.04)	4.38 (3.71)	9.44 (4.90)	29.23 (2.75)	(300)	2736 (220)	122.25 (16.44)
3	499.33 (23.43)	34.79 (8.17)	31.85 (2.30	3.64 (1.44)	0.62 (0.84)	3.23 (2.07)	5.67 (4.49)	27.00 (1.59)	2002 (198)	3184 (179)	137.86 (7.62)
4	498.2 (29.68)	37.92 (12.6)	31.36 (3.25)	1.91 (0.77)	0.187 (0.17)	2.245 (3.7)	6.77 (7.03)	28.28 (5.71)	1940 (88)	2836 (139)	148.96 (9.66)
5	494.7 (16.37)	29.30 (10.10	25.45 (4.23)	1.24 (0.67)	0.25 (0.21)	0.129 (0.05)	6.50 (5.83)	28.18 (4.73)	1656 (76)	2554 (133)	157.91 (10.21)
6	507.07 (20.10)	28.5 (8.7)	31.21 (2.15)	1.62 (0.76)	0.17 (0.01)	0.154 (0.097)	14.00 (8.2)	27.40 (373)	2028 (102)	2850 (151)	137.75 (3.05)
7	507.07 (20.10	31.62 (11.9)	30.52 (1.64)	1.96 (0.89)	0.52 (0.21)	1.88 (0.93)	7.25 (0.75)	29.10 (0.73)	2146 (207)	3174 (336)	92.62 (7.85)
8	483.62 (36.02)	28.25 (7.12)	27.65 (4.40)	2.49 (1.79)	0.32 (0.03)	1.28 (0.81)	6.30 (1.40)	26.96 (4.53)	2114 (79)	3061 (326)	95.5 (16.50)
9	496 (26.24)	28.66 (1.15)	34.5 (2.12)	6.8 (0.28)	0.51 (0.29	0.95 (0.16	5.14 (.58)	27.54 (1.43)	2004 (56)	2766 (380)	122.5 (4.94)

TABLE 11.2 Steady state COD and N mass balances, wastewater fractions and mixed liquor parameters for parent anoxic/aerobic activated sludge system (FIG.1) using steady state design model (19).

ww Batch No.	Mass balance (%)		Wastewater COD fractions		Mixed liquor		
	COD	N	Unbiod. Soluble (fs,us)	Unbio. Particulate (fs,up)	COD/VSS ratio (mgCOD/mgVSS) (fCV)	TKN/VSS ratio (mgN/mgVSS) (fN)	Active Fraction (fav)
1	87.52	103.30	0.064	0.145	1.43	0.080	0.408
2	80.00	100.86	0.066	0.085	1.47	0.066	0.501
3	84.22	98.81	0.070	0.135	1.59	0.068	0.437
4	84.90	99.87	0.076	0.123	1.46	0.077	0.434
5	80.00	93.72	0.059	0.049	1.54	0.095	0.578
6	81.88	86.20	0.056	0.128	1.41	0.068	0.430
7	89.70	103.38	0.064	0.170	1.48	0.060	0.382
8	88.50	106.98	0.058	0.175	1.45	0.0922	0.374
9	84.93	85.41	0.058	0.134	1.48	0.0611	0.378

APPENDIX 12

Technology transfer

Capacity building

M.Tech	Delon Donnovan Mudaly 2001 Microbial community analysis of the activated sludge process using ribosomal ribonucleic acid directed oligonucleotides.
M. Tech	Arshad Abdool Hak Ismail 2003 Optimization of Food to Microorganism ratios during activated sludge respirometric batch assays.
D. Tech	Arshad Abdool Hak Ismail (Current) Determination of the heterotrophic and autotrophic active biomass during activated sludge respirometric batch assays using molecular techniques.

5 Btech students

4 Experiential Trainees

Publications and conferences

Mudaly, D.D., Atkinson, B.W. and Bux, F. (2000) Microbial community profile of a biological excess phosphorus removal activated sludge system using a cultivation-independent approach. *Water SA* **26**(3): 343-352

Mudaly, D.D., Atkinson, B.W. and Bux, F. (2001) FISHing for biomass in activated sludge mixed liquor: the slippery VSS fraction. In: Matsuo, T., Hanaki, K., Takizawa, S. and Satoh, H. (eds.) Advances in Water and Wastewater Treatment Technology in 2000-

Molecular technology, nutrient removal, sludge reduction, and environmental health. Elsivier Science, Amsterdam: Netherlands.

Mudaly, D.D., Atkinson, B.W. and Bux, F. (2001) 16S rRNA *in situ* probing for the determination of the family level community structure implicated in EBPR. *Water Science and Technology* **43**(1): 91-98.

Mudaly, D.D., Degenaar, A.P., Manganyi, A. and Bux, F. (2001) An investigation of volatile suspended solids as a measure of activated sludge biomass. South African Journal of Science

The COE Symposium on Establishment and evaluation of advanced water treatment technology systems using functions of complex microbial community, 6-8 March 2000, Univ. of Tokyo, Japan 1 oral presentation entitled "The combined approach of FISH and dot blots for the study of bacteria predominating in a full scale EBPR activated sludge process."

The Water Institute of Southern Africa, Biennial Conference and Exhibition, Sun City, 28 May- 1 June 2000. Poster presentation entitled "An evaluation of volatile suspended solids as a true measure of metabolic activity in activated sludge".

Paris 2000: 1st World Congress of the International Water Association, IAWQ, 3-7 July 2000, Paris Conference Centre, Porte Maillot, Paris Oral presentation entitled "16S rRNA in situ probing for the determination of the family level community structure implicated in enhanced biological nutrient removal"