ELUCIDATION OF THE MICROBIAL COMMUNITY STRUCTURE WITHIN A LABORATORY SCALE ACTIVATED SLUDGE PROCESS USING MOLECULAR TECHNIQUES

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Dissertation submitted in compliance with the requirements for the Master's degree in Technology in the Department of Biotechnology, Durban University of Technology

2006

DECLARATION

I HEREBY DECLARE THAT THIS DISSERTATION IS MY WORK, UNLESS STATED TO THE CONTRARY IN THE TEXT, AND THAT IT HAS NOT BEEN SUBMITTED IN PART OR IN WHOLE TO ANY OTHER TECHNIKON, UNIVERSITY OR ACADEMIC INSTITUTION

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P.PADAYACHEE

I HEREBY APPROVE THE FINAL SUBMISSION OF THE FOLLOWING DISSERTATION

_____

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This_____Day of _____, 2006, At Durban University of Technology

DEDICATION

To all who has touched my life positively and left their imprint of love for which I will always remember…

ACKNOWLEDGEMENTS

I wish to thank and express my sincere gratitude to the following individuals for their constant support and assistance during the course of my study

- To GOD for making everything in my life possible.
- To my mum and dad for their constant support and encouragement throughout my studies.
- To Rubashini, Shaun and Dharashini, thank you for always assisting and supporting me.
- To Dhiraj for being my support during my studies and for always being there to motivate me. Thank you.
- To my friends Swastika, Dashika, Nishani and Shubnum for their continuous love and encouragement.
- Prof. Faizal Bux, my supervisor for giving me the opportunity to complete my masters in technology degree.
- My colleague, Arshad for always supporting my ideas and for guiding me with my work during the two years of my masters research.
- To my colleagues at the Centre for Water and Wastewater Technology, for all their assistance throughout my studies.
- The NRF for funding my research.

ABSTRACT

The microbial community present in a laboratory-scale modified Ludzack-Ettinger activated sludge system was investigated using a combination of novel molecular techniques. The parent system was investigated for a duration of one year and samples were taken at regular intervals to determine the profile and structure of the microbial community present within the anoxic and aerobic zones of the MLE system. The combination of molecular techniques included fluorescent *in situ* hybridisation (FISH) and denaturing gradient gel electrophoresis (DGGE).

FISH was performed using oligonucleotide probes, which were complementary to conserved regions of the rRNA for the alpha, beta and gamma subclasses of the gram negative family *Proteobacteria* as well as a group-specific HGC oligonucleotide probe as a representative of the gram positive actinomycetes branch. The total eubacteria present was determined using the EUB oligonucleotide probes, EUB388, EUB388-II and EUB388-III.

The DGGE analysis of PCR-amplified 16S rDNA gene segments was used to examine the microbial community profile in the anoxic and aerobic zones. The profile for each of the zones revealed a number of consistent bands throughout the duration of the laboratory-scale process. However, the profiles obtained suggested that a diverse microbial community existed within the aerobic and anoxic zones. The bands also indicated the presence of dominant and less dominant species of bacteria.

Hybridisations obtained from the FISH analyses indicated that the alpha and gamma subclasses were predominant within the anoxic zone and the aerobic zone showed a dominance of the beta subclass of *Proteobacteria*. The steady state behaviour of the MLE system was confirmed with the results obtained from COD, TKN, nitrates and OUR analytical tests. COD and nitrogen mass balances were conducted to confirm the acceptance of the results obtained for each batch as an indication of the system performance for the MLE model. Nitrogen mass balances indicated an upset in the nitrogen levels for batches two and seven.

PREFACE

Aspects of the work covered in this dissertation will be published and presented elsewhere:

Conferences:

P. Padayachee, A. Ismail and F. Bux. *Title*: Elucidation of the microbial community structure within a laboratory scale activated sludge process using molecular techniques. Oral presentation at: WISA Biennial Conference, 21-25 May 2006 (Accepted).

Publications:

P. Padayachee and F. Bux. *Title*: The effects of the microbial community on system performance of a laboratory scale activated sludge system. *In preparation for submission to South African Journal of Science.*

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LIST OF ABBREVIATIONS

A	:	Adenine
a	:	Mixed Liquor Recycle Ratio From Aerobic to the Anoxic Reactor
aq	:	Aqueous
ALF1b	:	Alpha Subclass of *Proteobacteria*
BET42a	:	Beta Subclass of *Proteobacteria*
BOD	:	Biological Oxygen Demand
C	:	Cytosine
CDGE	:	Constant Denaturing Gradient Gel Electrophoresis
CH_4	:	Metane
COD	:	Chemical Oxygen Demand
CO_2	:	Carbon Dioxide
DAPI	:	4,6-Diamidino2-Phenylindole
DGGE	:	Denaturing Gradient Gel Electrophoresis
DNA	:	Deoxyribonucleic Acid
DO	:	Dissolved Oxygen
dATP	:	Deoxyadenine Triphosphate
dCTP	:	Deoxycytosine Triphosphate
dGTP	:	Deoxyguanine Triphosphate
dNTP	:	Deoxynucleoside Triphosphate
dTTP	:	Deoxythymine Triphosphate
EBPR	:	Enhanced Biological Phosphorus Removal
EUB	:	Eubacteria
FISH	:	Fluorescent *In Situ* Hybridisation
f_n	:	Nitrogen Fraction of the MLVSS
f_{cv}	:	COD to VSS ratio of Volatile Sludge Mass
G	:	Guanine
GAM42a	:	Gamma Subclass of *Proteobacteria*
H_2	:	Hydrogen
HGC69a	:	High Guanine Cytosine
KCl	:	Potassium Chloride

MBR	:	Membrane-Separation Bioreactor
MLE	:	Modified Ludzack-Ettinger
MLSS	:	Mixed Liquor Suspended Solids
MLVSS	:	Mixed Liquor Volatile Suspended Solids
N	:	Nitrogen
N_d	:	Nitrate Denitrified
N_{ne}	:	Effluent Nitrate
N_{ng}	:	Nitrate Generated
N_{ti}	:	TKN in Influent
N_{te}	:	TKN in Effluent
N_s	:	Nitrogen Incorporated in VSS per day
N_w	:	TKN in waste sludge
N_2^-	:	Dinitrogen
NO_2^-	:	Nitrite
NO_3^-	:	Nitrate
NH_3	:	Ammonia
O_2	:	Oxygen
O_c	:	Oxygen Required for Carbonaceous degradation
O_d	:	Oxygen Recovered via Denitrification
O_d	:	Oxygen used for Denitrification
O_n	:	Oxygen used for Nitrification
O_{tm}	:	Measured Oxygen per day
OUR	:	Oxygen Utilisation Rate
P	:	Phosphorus
PCR	:	Polymerase Chain Reaction
Q_i	:	Influent Flow Rate
RNA	:	Ribonucleic Acid
rRNA	:	Ribosomal Ribonucleic Acid
s	:	Sludge Recycle from the Clarifier to the First Reactor
spp	:	Species
S_{ti}	:	Influent COD Concentration

S_{te}	:	Effluent COD Concentration
S_{xw}	:	COD in Waste Sludge
T	:	Thymine
Taq	:	*Thermus aquaticus*
TKN	:	Total Kjeldahl Nitrogen
TCA	:	Trichloroacetic Acid
Tris-HCl	:	Tris base Hydrochloride
TSS	:	Total Suspended Soilds
VSS	:	Volatile Suspended Solids
WW	:	Wastewater
X_H	:	Heterotrophic Active Biomass
X_V	:	Mixed Liquor Volatile Suspended Solids in mgVSS

CHAPTER 1

GENERAL INTRODUCTION

1.1 WASTEWATER MANAGEMENT

Water quality management is a social necessity rather than an optional matter, in order for society to operate efficiently. Whilst society increases in numbers, the wastes that are produced and the environmental impact of these wastes become much greater. Water quality management and wastewater treatment has to be effective in maintaining water quality parameters (Schroeder, 1977).

Domestic wastewater represents the most common source of infection because it is contaminated with pathogenic micro-organisms. The most common pathogens found in wastewater are usually associated with the intestinal tract, example *E.coli*. For proper wastewater treatment the wastewater is purified in activated sludge processes, whereby the wastewater enters treatment plants where it is firstly filtered for large particles, thereafter the wastewater enters tanks where specific processes take place. These processes are able to take place because wastewaters contain sufficient carbon, nitrogen, phosphorus and trace nutrients to support the growth of micro-organisms which aid in the removal of organic and inorganic wastes from the wastewaters, thereafter the water is purified by filtration or the biomass is allowed to settle in a settler and further filtration and purification steps with chemicals take place in order for the wastewaters to be discharged into rivers or lakes. (Muyima *et al*., 1997; Madigan *et al*., 2000).

1.2 ACTIVATED SLUDGE MICROBIOLOGY

Ecological study of the activated sludge within the process is important for the determination of which micro-organisms are significant in this process. The relative number and growth of each microbial species present is determined by the availability of oxygen, pH, and the mode of mixing. The predominant bacteria will be those organisms that are capable of utilising the organic wastes which are present. The microbial community of activated sludge consists of bacteria, protozoa, fungi, algae and filamentous organisms (Muyima *et al*., 1997).

Activated sludge is an artificial living ecosystem under the continuous influence of abiotic and biotic factors. For wastewater treatments low effluent concentrations of organic compounds and inorganic nutrients are required for the activated sludge. This eventually leads to a strong competition between individual groups of micro-organisms where only the adapted micro-organisms are able to survive. The surviving micr-organisms that dominate the activated sludge do not stay constant because the influencing factors are not constant during wastewater treatment. Therefore the microbial composition of the activated sludge is not constant but instead reflects the conditions to which the activated sludge system is exposed (Wanner, 1997). Activated sludge systems disperses all substrates present with a suitable mixing system, so micro-organisms can utilise these substrates and may grow as three-dimensional aggregated microbial communities called flocs (Seviour *et al*., 1999).

1.3 MICROBIAL COMMUNITY ANALYSIS

Phylogenetic identification and detection of micro-organisms using the rRNA approach involving *in situ* applications is used to first characterise samples by retrieval of rDNA sequences. Thereafter rRNA sequences are quantified with oligonucleotide probes in a colony using whole-cell hybridisation. It has been established that on various taxonomic levels phylogenetic groups are characterised by rRNA sequences. Oligonucleotide probes are usually designed for the highest taxonomic levels which are the domains such as Archea, Bacteria, Eukarya and for intermediate levels, examples are the alpha, beta and gamma subclasses of *Proteobacteria*. The approach of using domain specific probes followed by probes of a narrower specificity increases the information on the community diversity and the composition which can be obtained by whole-cell hybridisation (Wagner and Amann, 1997). Other molecular techniques such as PCR-DGGE, allows microbial diversity to be studied at a genetic level (Luxmy *et al*., 2000).

Proteobacteria contains most but not all gram negative bacteria. The *Proteobacteria* is further subdivided into four subclasses namely alpha, beta, gamma and delta. The four subclasses are distinguished by two helices within the 16S rRNA with positions of 184 to 193 and 198 to 219 with references to *E.coli* numbers (Woese, 1987). The organisms most common within the subclasses are:

- Alpha: *Caulobacter* spp., *Nitrobacter* spp and *Zoogloea* spp.
- Beta: *Alcaligenes* and *Comamonas, Sphaerotilus* spp., some *Zoogloea* spp and most of the autotrophic nitrifiers.

- Gamma: *Acinetobacter* spp., some *Zooglea* spp, Enterobacteriaceae, fluorescent pseudomoads and *Vibrio* spp.
- Delta: Sulphate reducing bacteria and myxobacteria (Wagner and Amann, 1997).

Significant rRNA homologies have been found between actinomycetes and a group of related bacteria, which are characterised by gram-positive staining and a high G+C DNA content therefore creating a group named actinomycetes branch or gram-positive bacteria with high G+C content of DNA. Monitoring of gram-positive bacteria with a high G+C DNA content in activated sludge is necessary because organisms of the actinomycetes branch play an important role in sewage purification processes (Wagner and Amann, 1997).

The application of the rRNA approach to phylogenetically detecting and analysing the presence of microbial communities in environmental samples is a valuable tool (Wagner and Amann, 1997; Daims *et al*., 2001) used for the treatment of these samples. These molecular biological techniques offer an opportunity to analyse the structure and species composition of the microbial communities present (Muyzer *et al*., 1993).

1.4 TOTAL BIOMASS DETERMINATION IN WASTEWATER TREATMENT

1.4.1 Direct Measurement:

Specific growth rates are often calculated on the basis of total suspended solids (TSS), mixed liquor suspended solids (MLSS) and volatile suspended solids (VSS) of activated

sludge for sufficiently accurate methods to determine the activated sludge biomass (Liebeskind and Dohmann, 1994). The solids in sewage may be either suspended or dissolved. Total suspended solids, is the portion of solids retained by a filter. Total dissolved solids, is the portion that passes through the filter. Total solids, is the term applied to the material residue left in the vessel after evaporation of a sample and its subsequent drying in an oven at a defined temperature. The type of filter holder, the pore size, porosity, area and thickness of the filter, particle size and amount of material deposited on the filter are the principal factors affecting separation of suspended solids from dissolved solids (Liebeskind and Dohmann, 1994).

Volatile solids are solids ignitable at 550^{o}C. The concentration of total volatile solids is considered to be a rough measure of organic content. Suspended volatile solids, is considered to be a measure of the concentration of biological solids such as bacteria and protozoa. Volatile solids may be measured for the total sample and is referred to as total volatile solids, whilst the suspended fraction is referred to as suspended volatile solids or the filtrate-dissolved volatile solids (Bitton, 1999).

The contents of the aeration tank in an activated sludge system, is called mixed liquor. MLSS is the total amount of organic and mineral suspended solids present, including micro-organisms in the mixed liquor. It is determined by filtering an aliquot of mixed liquor and then drying the filter at 105^{o}C, then the weight of the solids from the sample are determined. The organic portion of the mixed liquid suspended solids is represented by MLVSS which comprises of non-microbial organic matter as well as dead and alive

microorganisms including cellular debris. MLVSS is determined after heating of the dried filter samples at 600 to 650^{o}C. This represents approximately 65 to 75% of MLSS (Bitton, 1999).

1.4.2 Quantitative measurement:

A quantitative measurement of biomass-COD concentration could greatly enhance the accuracy and reliability of wastewater treatment models that rely on the component biomass concentration, which is usually expressed in terms of COD (Munch and Pollard, 1997).

COD is used as the standard unit in mathematical models, requiring reliable quantitative estimates of the bacterial biomass. It links the organic substrate and bacterial biomass in a common reaction, which is the reduction of oxygen. Biomass concentrations are sometimes estimated by indirect techniques, which are mostly based on measurements of the metabolic activity. Microscopic methods used to determine bacterial biomass allows a direct and specific measurement of the number of bacteria present (Munch and Pollard, 1997).

1.5 ACTIVE BIOMASS DETERMINATION IN WASTEWATER

Various methods can be used to estimate the amount of active biomass that is present within the MLE process. However, most methods are only applicable to pure culture

studies. The activated sludge of wastewater treatment plants contains not only active biomass, but also a high content of dead organic and inorganic material of unknown composition. Distinguishing between active and non-active biomass is difficult however the best and most reproducible method for biomass estimation is often described as the determination of DNA content (Liebeskind and Dohmann, 1994).

This can be achieved by using epifluorescence microscopy. Epifluorescence microscopy is a technique developed to count viable cells by microscopic examination of samples. This includes total cell counts, which involves counting of samples dried on microscope slides or counts done on samples that are in a liquid form (Cronje *et al*., 2001).

The disadvantage of this method is that living cells could not be distinguished from dead cells. This problem was overcome by staining the organisms with fluorescent dyes specific for living cells. The fluorescent cells can then be counted under an epifluorescent microscope. This overcomes the problem associated with culturing organisms to determine the number of viable cells present (Cronje *et al*., 2001).

In this study a combination of molecular techniques, FISH and PCR-DGGE will be used to monitor the microbial composition and determine the microbial community population shifts within a steady state laboratory-scale parent anoxic and aerobic activated sludge system. Molecular techniques were employed due to the disadvantages of using conventional methods, which according to previous researcher, for example Manz *et al*., (1994), who points out the over-estimation and under-estimation regarding the

quantification of bacteria which occurs using conventional techniques. Previous research has been focused primarily on activated sludge from large-scale wastewater treatment plants. This research allows a comparative study of results obtained from a laboratory scale "controlled" activated sludge system with the large-scale wastewater treatment plants. It is important to establish the microbial population diversity between the anoxic and aerobic zones to be able to empower engineers and to facilitate in wastewater treatment modelling.

1.6 AIM: To apply and optimise molecular techniques (Fluorescent *in situ* Hybridisation, Polymerase Chain Reaction and Denaturing Gradient Gel Electrophoresis) in order to analyse the microbial community, structure and composition present in activated sludge.

OBJECTIVES:

1. To operate and maintain a laboratory scale activated sludge process based on a modified Ludzack-Ettinger (MLE) process.
2. To determine the steady state of the modified Ludzack-Ettinger process.
3. To determine the microbial community structure of the MLE process using fluorescent *in situ* hybridisation (FISH) to target the *Proteobacteria* and high G+C content bacterial families.
4. To confirm the profile of the microbial community present using the denaturing gradient gel electrophoresis (DGGE).

CHAPTER 2

LITERATURE REVIEW

2.1 MAIN BIOLOGICAL PROCESSES TAKING PLACE IN WASTEWATERS

2.1.1 The Nitrogen Process

Nitrogen is an important component necessary for all living organisms. All living cells contain approximately 14% nitrogen, which forms a part of several key cell components which are protein and DNA (Wong *et al*., 2003). Most organisms depend on either the combined source of nitrogen, such as ammonia, nitrate, organic compounds or fixed nitrogen for their nutrition (Muyima *et al*., 1997). Micro-organisms carry out most of the key redox reactions of nitrogen in nature. Nitrogen gas is thermodynamically the most stable form of nitrogen therefore the major reservoir for nitrogen on earth is the atmosphere (Madigan *et al*., 2000).

The breakdown of Nitrogen bonds (N≡N) of molecular nitrogen is the reduction of nitrogen which is an energy demanding process. Few organisms are able to use nitrogen in the process called nitrogen fixation (Madigan *et al*., 2000). The product of nitrogen fixation is ammonia and this in turn is used for the formation of organic nitrogenous compounds such as plant proteins. During the assimilation pathways, nitrogenous compounds of higher oxidation states, such as nitrates and nitrites, can be reduced to organic nitrogen via ammonia (Wong *et al*., 2003).

2.1.1.1 Nitrification and Denitrification

In raw wastewater, nitrogen is present mostly in a reduced form, such as ammonia nitrogen and organic nitrogen (Muyima *et al*., 1997). Nitrogen which is free and saline ammonia is characterised by Total Kjeldahl Nitrogen (TKN) (Lilley *et al*.,

1997). Whilst carbonaceous oxidations of wastewaters occur, many forms of organic nitrogen are converted to ammonia. The reduced form of nitrogen which is ammonia is oxidised by autotrophic nitrifying bacteria converting nitrites to nitrates, through a process known as nitrification. Nitrification requires oxygen and decreases alkalinity in wastewaters. This decrease in alkalinity decreases the pH, however nitrification is possible at a broad range of pH values, but the optimal pH value is between 7.5 and 8.5 (Muyima *et al*., 1997).

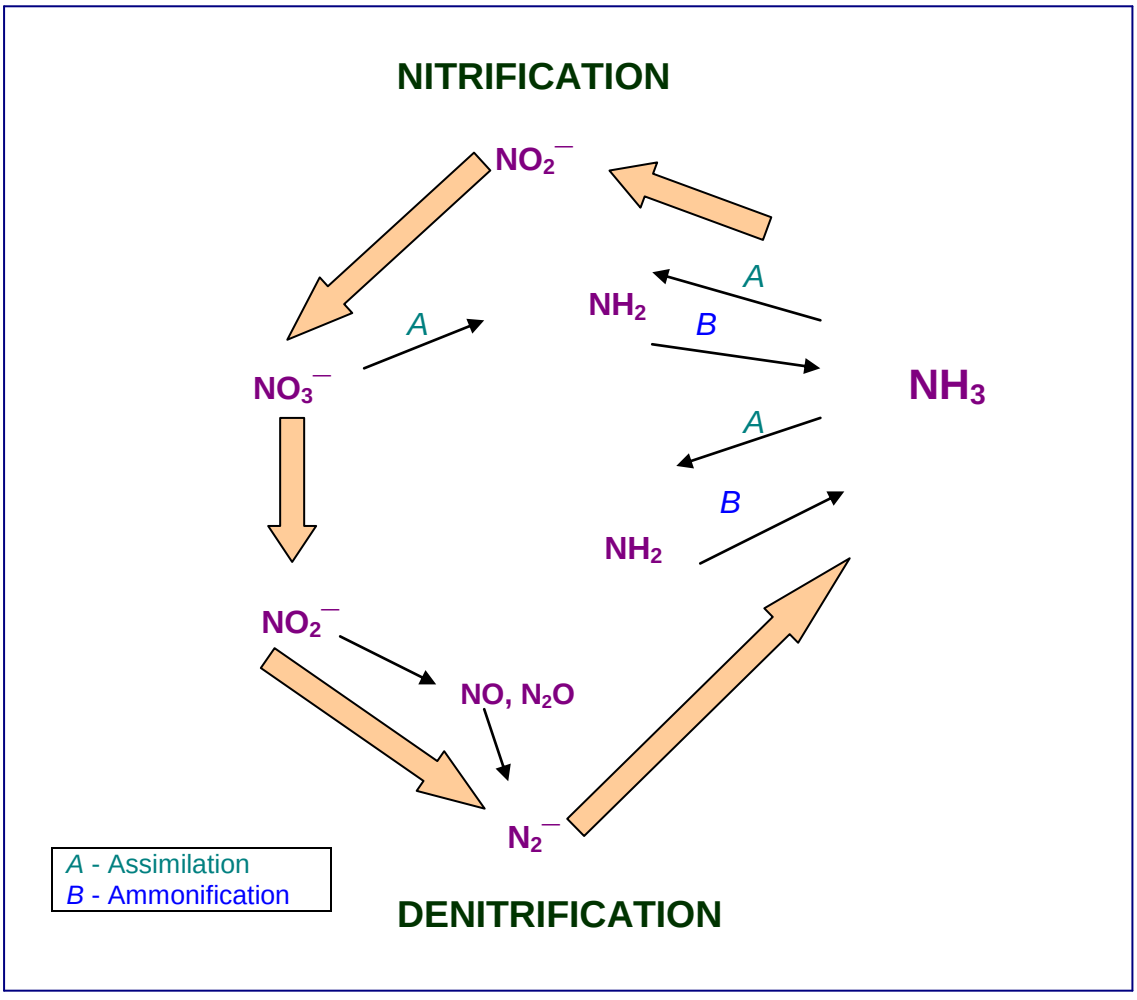

FIGURE 2.1: The Nitrogen Cycle (Madigan *et al*., 2000).

The denitrification process, shown in Figure 2.1, depicts the reduction of oxidised nitrogenous compounds to nitrogen gas (Wong *et al*., 2003). Denitrification is the

main process by which gaseous nitrogen is formed biologically. Micro-organisms prefer the utilisation of nitrates rather than nitrogen gas as a source of nitrogen, therefore denitrification removes mostly fixed nitrogen (Madigan *et al*., 2000). Nitrates are reduced in two stages the first is when nitrate reductase converts nitrate to nitrite and the second stage involves nitrite reductase which converts nitrite to nitrogen gas (Wong *et al*., 2003). Denitrification is beneficial in wastewater treatment where the nitrates are removed from the wastewaters therefore preventing the proliferation of algal growth when treated wastewaters are discharged into lakes and streams (Madigan *et al*., 2000).

2.1.2 The Carbon Process

Carbon is recycled through all of earth's carbon reservoirs, which are lands, oceans, aquatic environments, sediments, rocks and biomass. Carbon and oxygen cycles are combined since carbon dioxide fixation by oxygenic phototrophs releases oxygen and organic matter is oxidised to carbon dioxide by aerobic respiration (Madigan *et al*., 2000).

Concentrations of carbon dioxide in the atmosphere are determined mainly by the processes of photosynthesis and respiration. Carbon dioxide produces bicarbonate and carbonate ions when it is dissolved in slightly alkaline waters. The conversion of organic carbon to carbon dioxide with the reduction of molecular oxygen, shown in Figure 2.2, involves several different types of micro-organisms. Bacteria and fungi which function in an aerobic environment completely oxidises organic substances present. In aerobic conditions the carbon dioxide from aerobic respiration produces carbonate ions which unite with the dissolved calcium ions, this eventually forms

calcium carbonate. In anerobic conditions fermentation decomposes organic compounds and the end products are further oxidised by anaerobic bacteria in the presence of inorganic hydrogen acceptors such as nitrates, sulphates and carbon dioxide (Muyima *et al*., 1997).

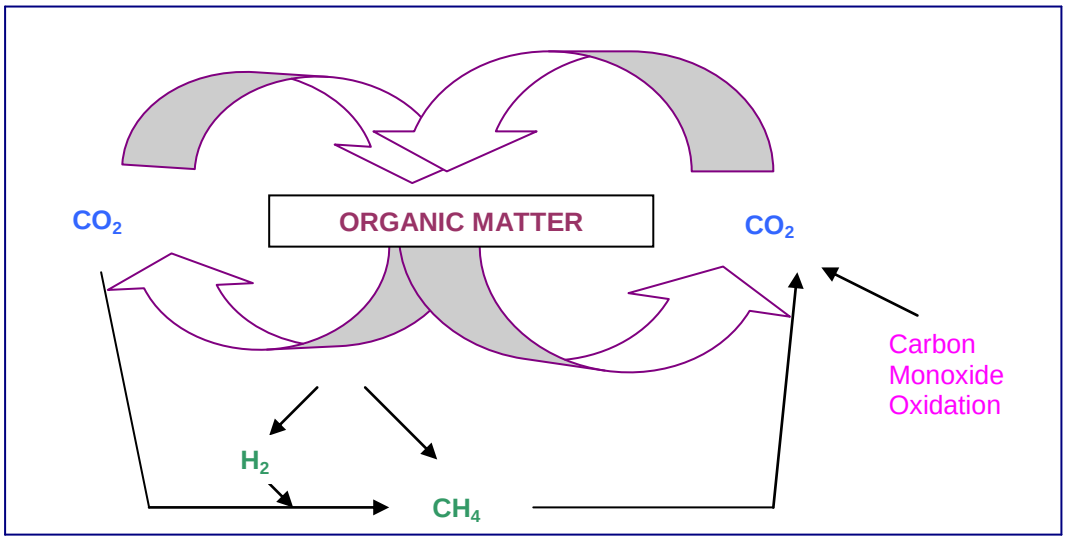

FIGURE 2.2: The Carbon Cycle (Muyima *et al*., 1997).

2.1.3 The Phosphorus Cycle

Phosphorus in wastewaters are found as inorganic, phosphate ion or organic phosphates. The synthesis of organic phosphate occurs within the micro-organism, however when the micro-organism dies the phosphate is released via hydrolysis. Phosphate is limited when involved in the growth of organisms due to the supply of phosphates occurring as insoluble complexes, some of which are calcium, iron and aluminium salts. Figure 2.3 outlines the phosphorus cycle (Muyima *et al*., 1997). Phosphorus is stored and released in sludge by manipulating oxygen concentrations this is known as enhanced nutrient removal processes. *Acinetobacter calcoaceticus* is a genus of bacteria responsible for the removal and release of phosphorus within sludge. These bacteria take up and metabolise compounds contributing to the

Biological Oxygen Demand (BOD) under aerobic conditions. During anaerobic conditions the bacteria cannot metabolise compounds however they can take up volatile fatty acids such as acetate. When the wastewater is aerated aerobic metabolism takes place and the bacteria then breaks down the acetate present to produce energy. This energy is then used to take up phosphorus and to generate polyphosphate. Therefore for the process of phosphorus removal to be achieved anaerobic conditions must prevail (Horan, 2003).

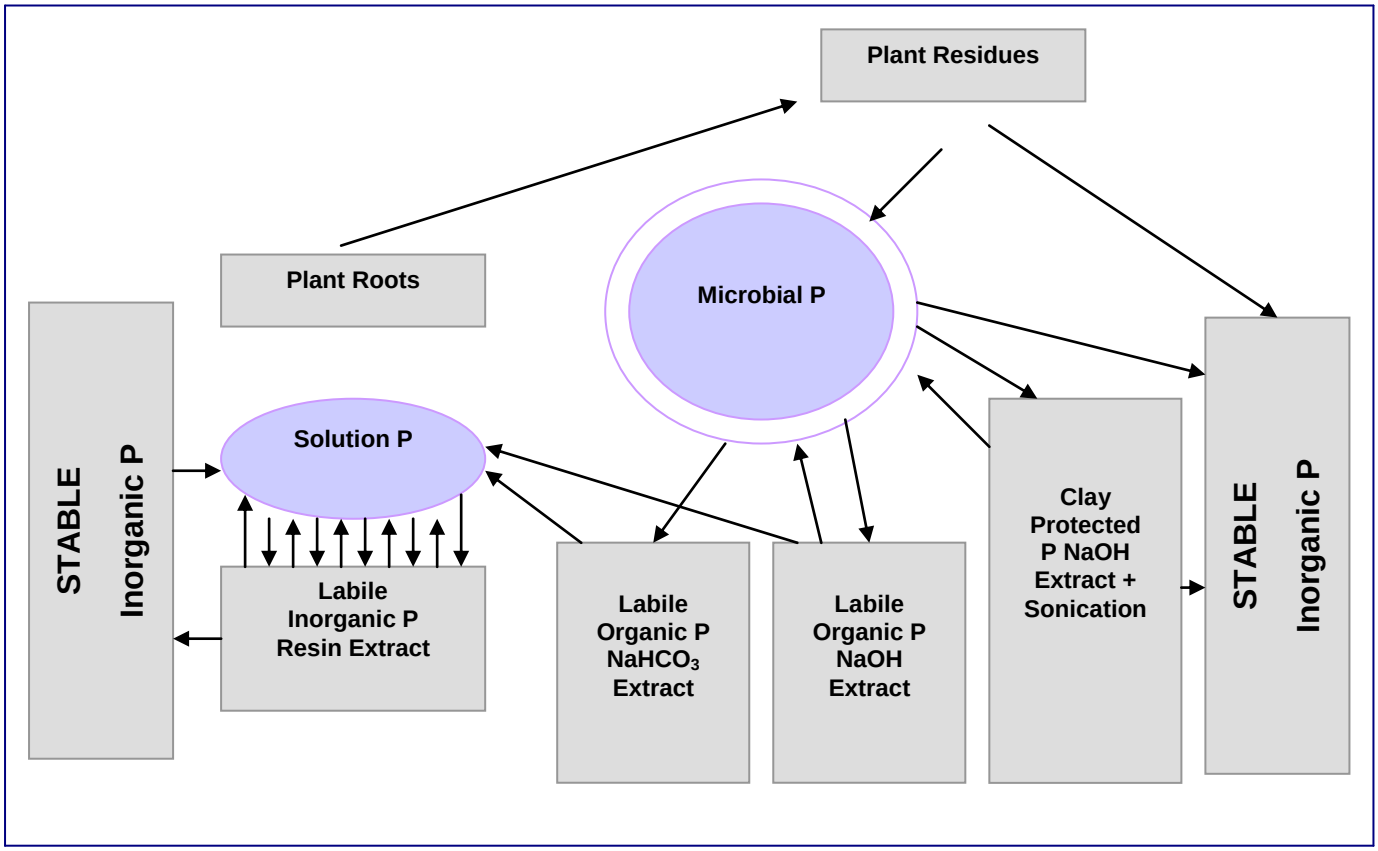

FIGURE 2.3: The Phosphorus Cycle (Muyima *et al*., 1997).

2.2 TREATMENT OF WASTEWATERS

Treatment of activated sludge involves the removal of biodegradable organics, unsettleable suspended solids and other constituents. These biodegradable organic compounds are degraded by bacteria in an aerated reactor and the biomass is allowed

to settle and concentrate in a clarifier (Muyima *et al*., 1997). Domestic wastewater treatment can be classified into primary, secondary and tertiary biological treatment. Primary treatment of wastewaters usually removes approximately 60 percent of suspended solids. Secondary treatment is expected to reduce the balance of the suspended solids concentration by 93 percent (Schroeder, 1977). Tertiary treatment increases the efficiency of the suspended solid removal by 99 percent (Ramalho, 1983).

2.3 THE ACTIVATED SLUDGE PROCESS

Activated sludge processes are responsible for the dense growth of micro-organisms in different reactors (Cloete *et al*., 2003). The system is either a continuous or semi-continuous aerobic method for wastewater treatment involving carbon oxidation and nitrification. With nutrients and oxygen present the microbial population in the wastewater achieves optimal growth and respiration. An activated sludge process has been developed for the removal of carbon, nitrogen and phosphate (Muyima *et al*., 1997). The different stages of nutrient removal within the activated sludge system are achieved in different zones:

- **Anaerobic Zone:** This zone establishes a facultative anaerobic microbial community. Both dissolved oxygen and oxidised nitrogen (nitrates and nitrites) are absent. Removal of phosphates occurs in this zone.
- **Primary Anoxic Zone:** This zone is considered the main denitrification reactor in the system. Oxygen is absent and nitrates and nitrites are present leading to the proliferation of denitrifying bacteria which reduces nitrates and nitrites to molecular nitrogen.

- **Primary Aerobic Zone:** In this zone nitrification takes place whereby ammonia is oxidised to form nitrites and then to nitrates. This provides an environment for the micro-organisms to take up the phosphates present.
- **Secondary Anoxic Zone:** Further denitrification takes place in this zone.
- **Secondary Aerobic Zone and Clarifier:** The secondary aerobic zone increases the levels of dissolved oxygen in the mixed liquor before it can enter the clarifier. It also removes additional phosphates and oxidises residual ammonia. The clarifier allows the activated sludge to settle and recycling of the activated sludge takes place (Cloete *et al*., 2003).

2.3.1 Biological Nutrient removal system configurations

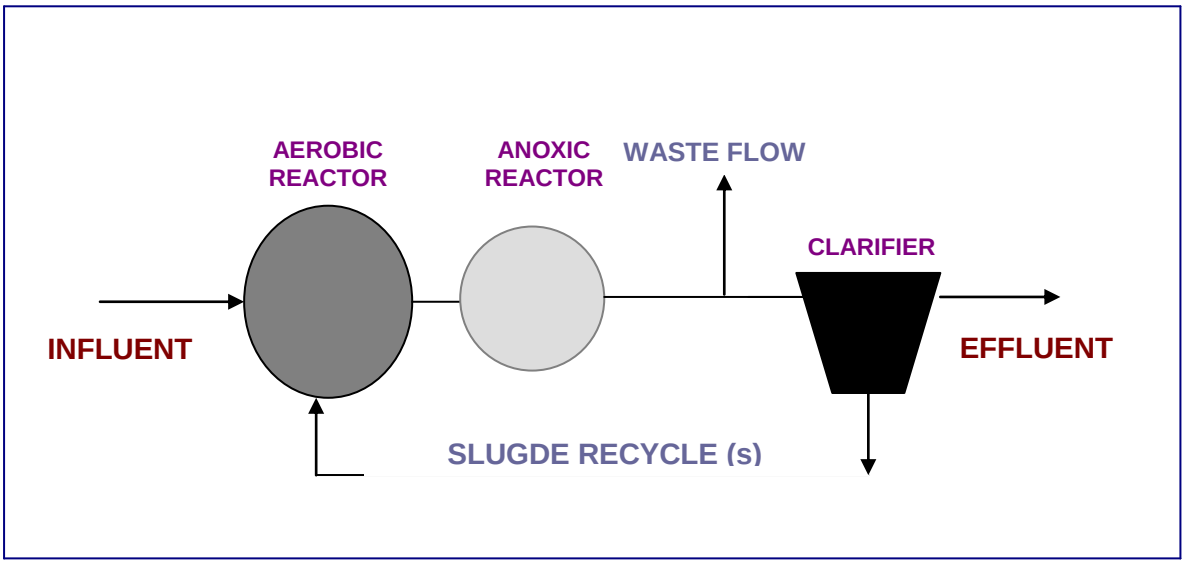

FIGURE 2.4: The Wuhrmann Process (Lilley *et al*., 1997).

The process in Figure 2.4 is a representation of the first nitrogen removal system that was produced. Influent is discharged directly into the aerobic zone, where nitrification takes place. The flow continues to the anoxic zone, where denitrification takes place and then to the clarifier. The sludge is then recycled (s) and returned back to the aerobic zone. This allows for further denitrification to take place (Lilley *et al*., 1997).

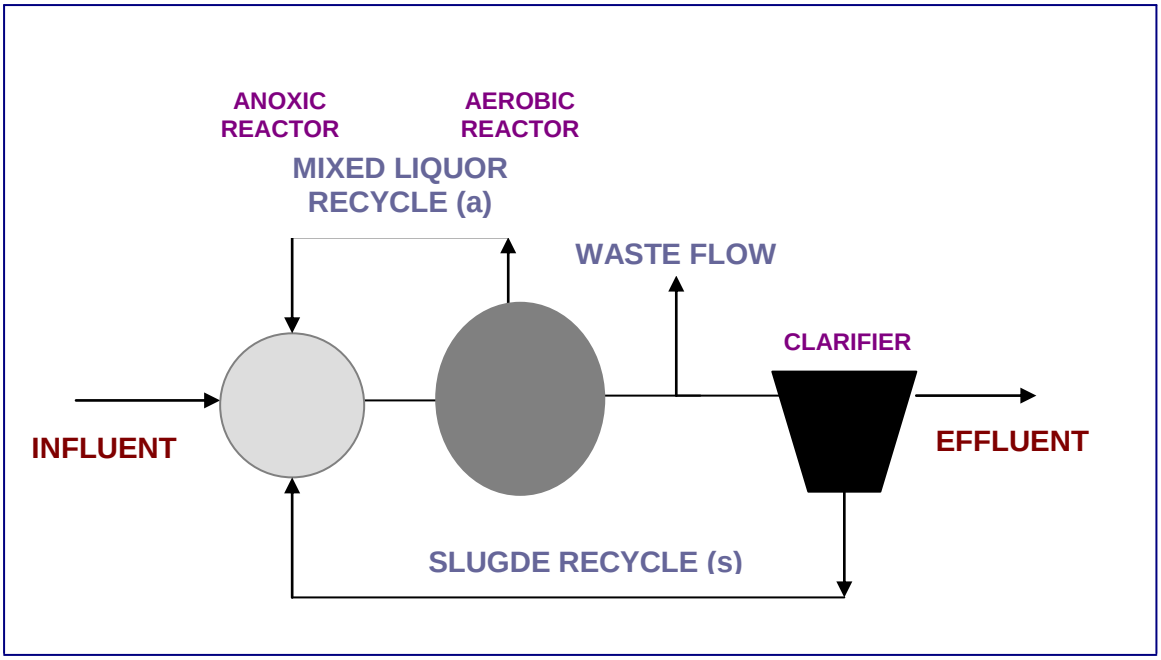

FIGURE 2.5: The Modified Ludzack-Ettinger (MLE) Process (Lilley *et al*., 1997).

The MLE system utilises readily biodegradable organic matter in the influent as an energy source for denitrification. As shown in Figure 2.5 the influent is fed into the first reactor which is the anoxic reactor, where it is stirred without aeration to supply organic matter to micro-organisms, which is used as an energy and carbon source for denitrification. The denitrified wastewaters are then aerated in the second reactor which is the aerobic reactor, where nitrification takes place. Recycling of the settled activated sludge within the clarifier or settler is transferred back to the anoxic reactor, referred to as sludge recycle (s) in Figure 2.5 as well as internal recycling, mixed liquor recycle (a) from the aerobic zone to the anoxic zone (Van Haandel *et al*., 1981).

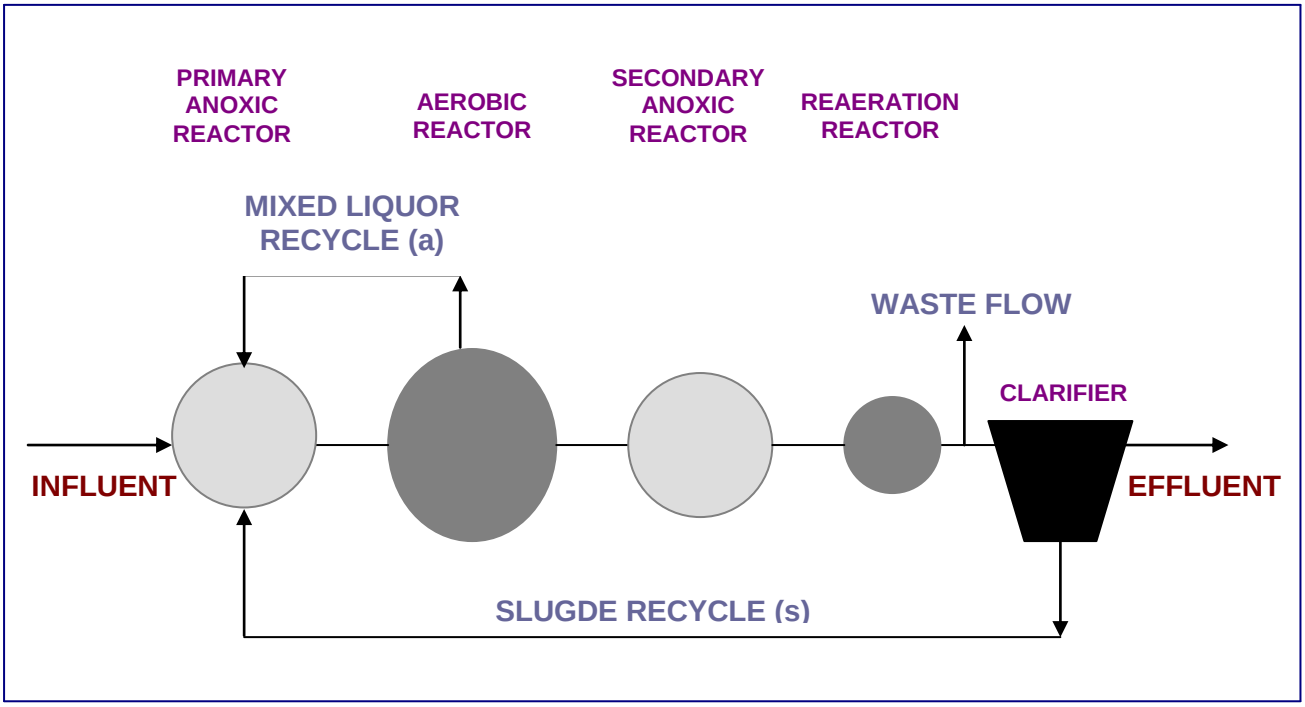

FIGURE 2.6: The Bardenpho Process (Lilley *et al*., 1997).

The Bardenpho system outlined in Figure 2.6 overcomes the problem of incomplete denitrification. The low nitrate concentrations present after leaving the aerobic zone can be denitrified in the secondary anoxic zone, in order to produce a reasonably nitrate free effluent. The reaeration reactor served to remove the nitrogen bubbles from the sludge flow to allow better sedimentation of the sludge and promote nitrification of any ammonium that may be present. The mixed liquor recycle (a) and sludge recycle (s) also assists in the distribution of the nitrates between the primary and secondary anoxic zones (Lilley *et al*., 1997).

2.2 RIBOSOMAL RNA (rRNA)

Ribosomes are large molecular structures that catalyse protein synthesis in every living organism. The ribosome basically consists of RNAs and proteins. It is universal, conserved and play an important role in biological processes (Favaretto *et al*., 2005).

rRNAs are ancient molecules that are universally distributed and moderately conserved in sequences amongst organisms. There are a number of different possible sequences of rRNA, which can indicate phylogenetic relationships between two organisms. Hence the degree of similarity in rRNA sequences among two organisms indicate that they are evolutionary related. Phylogenetic trees indicate the true evolutionary position of organisms and can be constructed from comparative sequence analyses (Madigan *et al*., 2000).

The three rRNA molecules in prokaryotes have sizes of 5S, 16S, and 23S (Madigan *et al*., 2000). The 5S rRNA molecule is approximately made up of 120 nucleotide bases. It is found in most prokaryotic ribosomes. 5S rRNA has been used in sequence analysis and applied in molecular evolution studies. It is ideal as a molecular phylogenetic marker due to its size and ubiquity that enables RNA sequencing using direct methods. The 5S rRNA enhances protein synthesis by stabilising the ribosome structure (Szymanski *et al*., 2002).

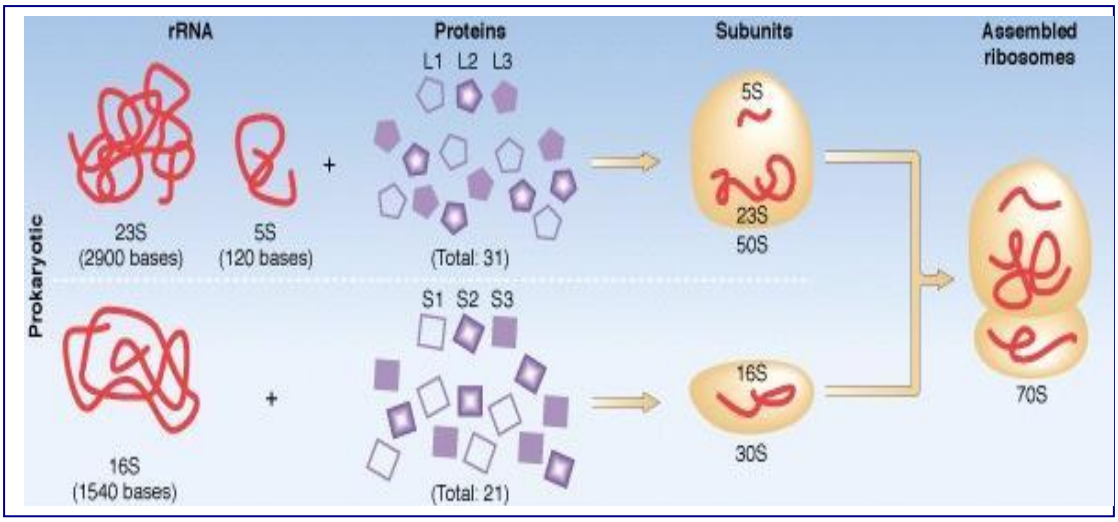

FIGURE 2.7: Representation of the prokaryotic rRNAs, the proteins they respectively produce and the subunits involved (Understanding the Genetic Code, 2005).

The 16S and 23S rRNA molecules have approximately 1500 and 2900 nucleotide bases respectively. These larger bacterial rRNAs contain a number of regions which are highly conserved sequences used for obtaining proper sequence alignments and they serve as phylogenetic chronometers due to their sufficient sequence variability in other regions of the molecule (Madigan *et al*., 2000). The 16S and 23S rRNA prokaryotic rRNAs are represented in Figure 2.7.

2.2.1 rRNA used to Analyse Microbial Communities

Microbial diversity should be analysed in a culture-independent way by direct rRNA sequence retrieval (Daims *et al*., 2001). This can be performed by incorporating molecular techniques such as the polymerase chain reaction (PCR) and fluorescent probing *in situ* (FISH) (Wagner and Amann, 1997). Culture dependent methods for analysing microbial communities have severe limitations:

- They are time consuming,

- The number of colony forming units is usually a minor fraction of cell counts determined by direct microscopic techniques
- The media and conditions used for standard plate counting may be selected for only bacteria that are a small percentage of the actual bacterial population present (Manz *et al*., 1994).

Environmental samples were first characterised by the study of rRNA which took place approximately a decade ago. The 5S rRNA molecules were extracted from mixed samples. The samples under went electrophoresis, allowing the separation of molecules that belong to different communities. The use of the 5S rRNA molecules was limiting and only used for uncomplicated ecosystems due to the fact that the 120 nucleotide base was relatively small. Thereafter the use of the larger rRNA molecules, 16S and 23S rRNA were used for research in microbial ecology. These molecules contain sufficient information enough for phylogenetic analyses (Wagner and Amann, 1997).

2.3 NUCLEIC ACID HYBRIDISATION

2.3.1 Principle of Hybridisation

The genome of every organism has an arrangement of four nucleotides (Adenine, Cytosine, Guanine and Thymine). Genetic make-up of each organism contains a collection of information and the analysis of these genetic messages requires isolation and the characterisation of the DNA sequences of interest. The process of hybridisation is the formation of a double helix from two strands of complementary DNA. Successful nucleic hybridisation is based on the complementarity of the two

target DNAs. After hybridisation the extent of complementarity will determine the stability of the duplex DNA (Bej, 1996) as represented in Figure 2.8.

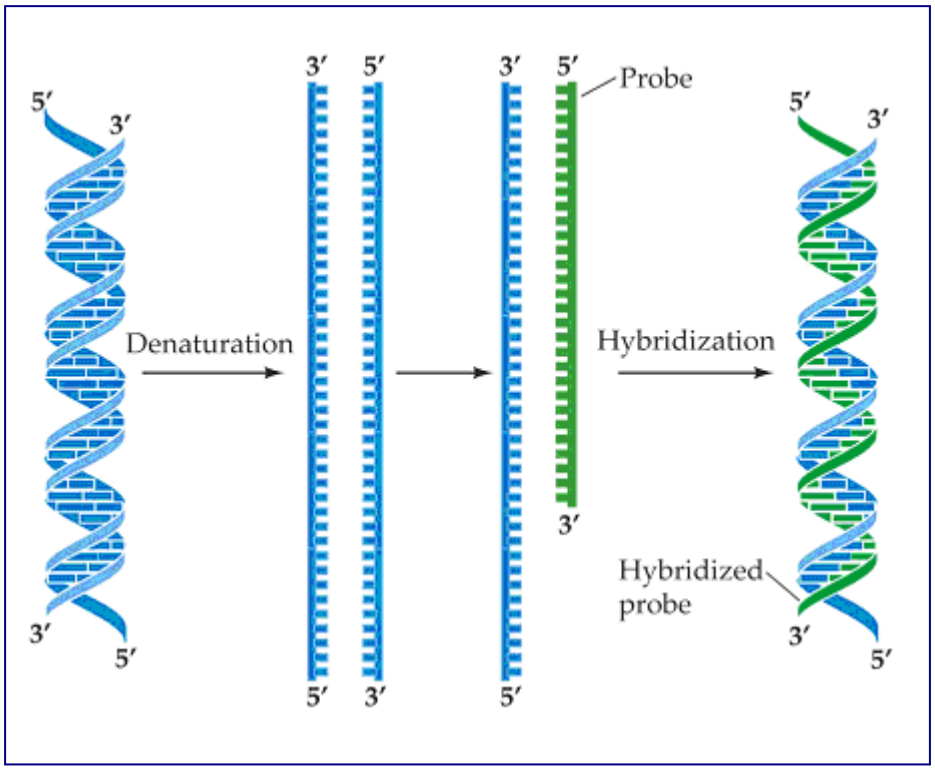

FIGURE 2.8: The process of nucleic acid hybridisation and creating a hybrid strand of DNA or RNA (Molecular and Cellular Biology, 2005).

Hybridisation reactions occur between any two complementary single stranded nucleic acid chain such as DNA and DNA, RNA and RNA or if an RNA transcript is introduced the RNA competes with the coding DNA strand and forms double-stranded DNA and RNA duplexes (Access Excellence, 2005).

2.3.2 Parameters that Influence Hybridisation

Nucleic acid hybridisation is dependent on the reannealing of denatured DNA to complementary strands in an environment that is below their melting point. The melting point is the temperature at which half of the DNA strand is present in a single stranded denatured form. The melting temperature and renaturation are influenced by

tempreature, pH, monovalent cations and formamide concentration (Roche Manual, 2004).

Optimal hybridisation conditions are favoured by additional parameters such as:

- **Probe Length:** Maximum hybridisation rates are obtained with long probes however *in situ* hybridisation requires shorter probes to allow the probes to diffuse into the dense matrix of cells. The length also influences thermal stability.
- **Probe Concentration:** This factor affects the rate at which the first few base pairs are formed. The higher the concentration of the probe the higher the annealing rate.
- **Base Mismatch:** When base pairs are mismatched, hybridisation rates and thermal stability of the duplexes formed are reduced. Mismatching forms the bases of point mutation.
- **Stringency Washes:** Duplexes are formed between matched sequences and sequences that do not match during hybridisation. Imperfectly matched sequences can be limited by varying the stringency of the hybridisation. Removal of non-specific hybridisation occurs with washing the sample with a solution containing a diluted concentration of salt. The lower the salt concentration and a higher wash temperature provide a more stringent wash (Roche Manual, 2004).

2.4 THE POLYMERASE CHAIN REACTION (PCR)

Amplification of specific regions of DNA from target sequences using the polymerase chain reaction is important for many molecular techniques (Cobb and Clarkson, 1994). The PCR process is regarded as a method for rapidly amplifying DNA *in vitro*. This procedure makes use of an enzyme DNA polymerase which copies DNA molecules. The PCR technique requires a template which is a fragment of a nucleotide

sequence of the gene that is going to be amplified. Oligonucleotide primers are also required. These primers are short synthetic fragments of DNA that contain a particular sequence which is complementary to sequences in the target gene which enables the amplification of the correct genes (Madigan *et al*., 2000).

2.4.1 Principal of PCR

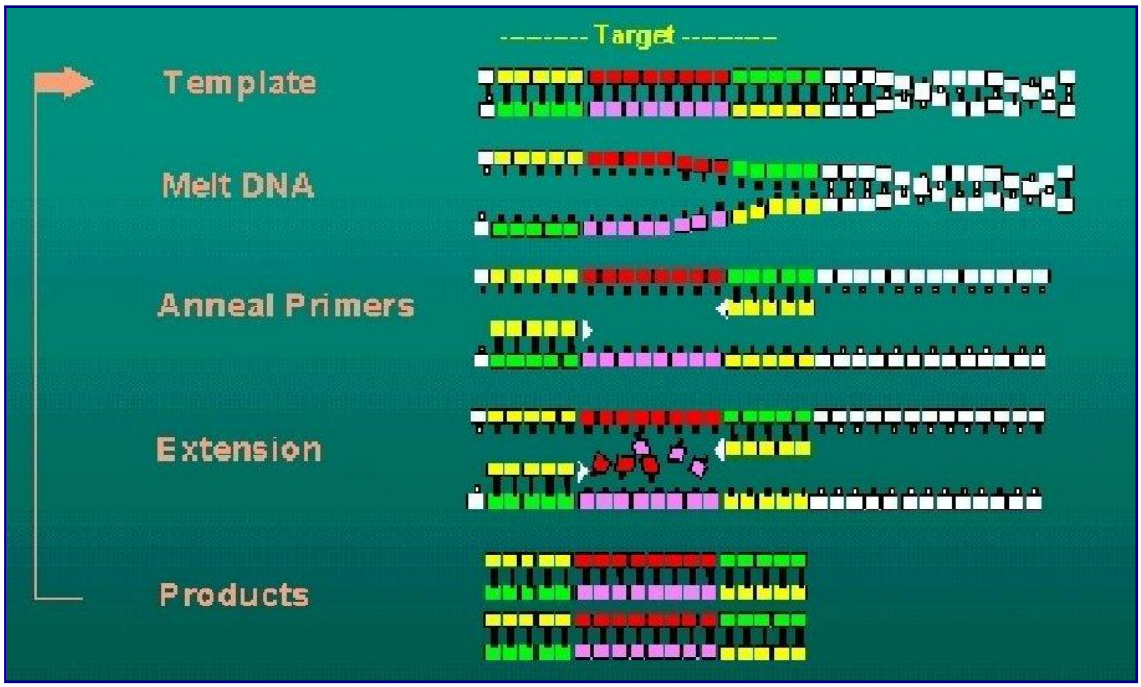

FIGURE 2.9: The PCR Process (Lazo *et al*., 1994).

There are three major steps in PCR illustrated in Figure 2.9 (Principal of the PCR, 2001):

- **Denaturation at 94°C:** During denaturation the double stranded DNA melts to produce two single stranded DNA.
- **Annealing at 54°C:** Oligonucleotide primers move around due to the forces of Brownian motion whilst in motion the primers bind to the DNA template with hydrogen bonds, however if the primer binds to an incorrect sequence then the

bond is broken. When the primer binds to the complementary template correctly, more stable hydrogen bonds are formed. The DNA polymerase then attaches to the primer and template DNA strand that has more stable hydrogen bonds and starts to copy the template. Once a few bases are built to the template, the hydrogen bonds between the primer and template become stronger and do not break.

- **Extension at 72°C:** This is an ideal temperature for the DNA polymerase to function. The force of attraction between the primer and the correct complementary template is stronger than the forces breaking this attraction. However if the primers are attached incorrectly to the template they are removed due to the high temperatures that they are exposed to and this does not produce an extension of the fragment.

2.4.2 Applications of PCR

PCR is a simple, efficient and extremely specific process which requires a few molecules of target DNA to start the reaction. PCR is extremely important for obtaining DNA for cloning and sequencing because the genes of interest can be amplified if the complementary sequences are known. This procedure can also be used to amplify small quantities of DNA. The ability to amplify and analyse DNA of micro-organisms that have not been cultured allows the application of PCR to be involved in diagnostic microbiology. PCR is also used in conjunction with DNA fingerprinting to identify individuals (Madigan *et al*., 2000).

2.4.3 Reaction Components for PCR

- **DNA Polymerase:** The DNA polymerase used commonly is Taq polymerase isolated from *Thermus aquaticus*. It is a heat stable enzyme and its activity is optimum at high temperatures (Taylor, 1991).
- **Deoxynucleoside Triphosphates (dNTPs):** They are stable at -20ºC and can be stored for months. They consist of the following nucleotide bases dATP, dCTP, dGTP and dTTP (Taylor, 1991).
- **Reaction Buffer:** The PCR buffer usually contains Tris-HCl at a concentration of 10 to 50mM, up to 50mM KCl, 1.5mM or higher $MgCl_2$, 50 to 200µM dNTP, gelatine or BSA of 100µg/ml and non-ionic detergents; Tween-20, Nonided P-40 or TritonX-100 (Rybicki, 2001).
- **Primers:** Oligonucleotide primers are synthesised so that they have similar G+C content to the DNA template with minimal secondary structure and low complementary structures to each other in order to optimise the binding capabilities to the complementary DNA template (Taylor, 1991).

2.4.4 Factors Affecting PCR

2.4.4.1 Denaturing Temperature and Time:

Specific complementary binding due to the hydrogen bonding of single stranded nucleic acids is referred to as annealing. However if the nucleic acid is not single stranded it is heated above the melting temperature of the double stranded nucleic acid and thereafter it is cooled rapidly. This ensures that the separated strands do not re-anneal. When nucleic acids are heated in ionic strength buffers lower than 150mM sodium chloride, the melting temperature is less than 100ºC. Therefore PCR is optimum at denaturing temperatures of 91-97ºC. Taq polymerase has a half life of 30

minutes at 95ºC. Therefore approximately 30 amplification cycles can be performed (Rybicki, 2001).

The time taken at each step is the main reason for loss of activity of Taq polymerase. Hence optimum activity of Taq requires the time to be reduced and the number of cycles of the PCR to be increased. Denaturation time is usually 1 minute at 94ºC however it is possible for shorter template sequences to reduce the time to 30 seconds or less. Increasing denaturation temperature and decreasing time can also be used, such as 96ºC for 15 seconds (Rybicki, 2001).

2.4.4.2 Annealing Temperature and Primer Design:

The length and the sequence of the primer are important in designing the parameters of a successful amplification. The annealing temperature used for PCR depends on the length and composition of the primers. The annealing temperature should be 5ºC below the lowest melting temperature of the primers used. When the annealing temperature is too low the primers will anneal to sequences other than the target sequence leading to non-specific amplification. If the annealing temperature is too high the primer will not anneal to the target sequence. Most primers anneal efficiently in 30 seconds at a temperature of about 54ºC (Rybicki, 2001).

2.4.4.3 Primer Length:

Optimum length of the primer depends on the adenine and thymine content as well as the melting temperature. Primers should be complicated in sequence preventing them from easily annealing to the target sequences (Rybicki, 2001).

2.4.4.4 Elongation Temperature and Time:

Elongation is usually set at 70 to 72ºC for 0.5 to 3 minutes. The Taq polymerase has a specific activity at 37ºC which is similar to DNA polymerase I. Elongation occurs from the start of annealing which results in good stability. The optimal temperature for the primer extension which occurs up to 100 bases per second is 70ºC. A longer time is required for longer products. Longer times are also helpful in later cycles when the product concentration exceeds the enzyme concentration and when dNTPs or primers become depleted (Rybicki, 2001).

2.4.4.5 Reaction Buffer:

Reaction buffers that contain potassium chloride or sodium chloride concentrations that is higher than 50mM inhibits the Taq polymerase, however this may be necessary for primer annealing. The concentration of magnesium can affect primer annealing but Taq polymerase requires magnesium so the concentration should be optimally determined. Titrations should be performed with different magnesium concentrations for all new template and primer combinations. The primer concentration should not exceed 1µM unless the primer can easily degenerate. The dNTP nucleotide concentration can be approximately 50µM and below (Rybicki, 2001).

2.4.4.6 Cycle Number:

The starting concentration of the target DNA determines the number of amplification cycles required to produce a visible band on a gel. A 40 to 45 cycle should be used to amplify a 50 nucleotide base target molecule. If 30 cycles does not produce a desired product yield then 1µl of the amplified sample mix can be re-amplified 20 to 30 times in a new reaction mixture. Nested PCR can also be performed to produce a higher

desired product yield. This process involves the reamplification of the PCR product with a second, more specific set of primers (Rybicki, 2001).

2.5 DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE)

2.5.1 Principal of DGGE

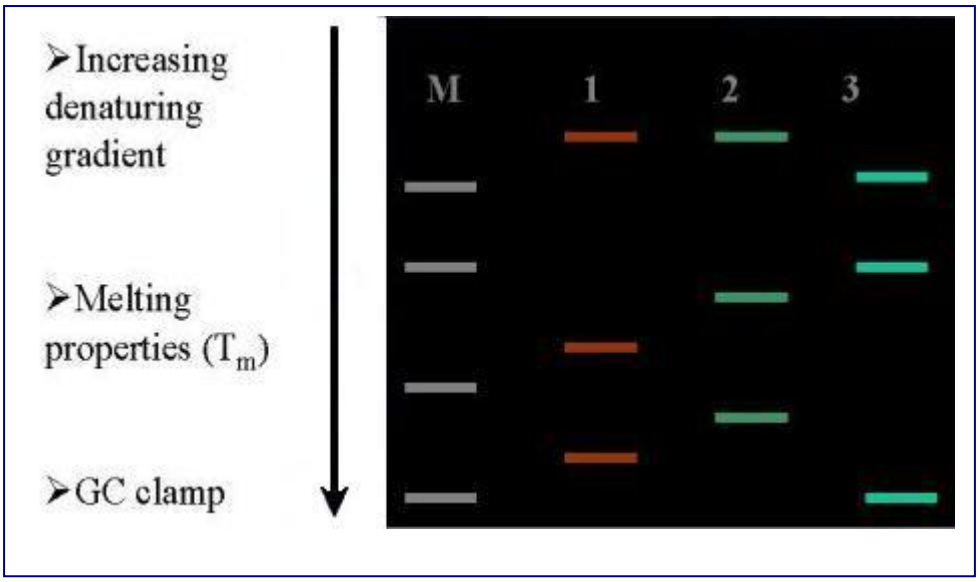

FIGURE 2.10: The Principle of DGGE (Verseveld *et al*., 2001).

DGGE is used to separate DNA fragments of the same length but differ in their base-pair sequences (Kaewpipat and Grady, 2002). The technique, shown in Figure 2.10, is based on the electrophoretic mobility of partially melted DNA molecules in polyacrylamide gels (Muyzer *et al*., 1993). These gels contain an ascending gradient of the DNA denaturants, which consists of urea and formamide (Borresen *et al*., 1988). The melting of the DNA fragments occurs as they migrate down the gel and reach specific melting domains (Muyzer *et al*., 1993). These domains occur due to the difference in concentration of denaturants (Borresen *et al*., 1988).

When two DNA fragments only differing by a single base pair are electrophoresed on a polyacrylamide gel, they will initially move through the gel at a constant rate (Borresen *et al*., 1988). As they move into specific domains, with the appropriate lowest melting temperatures of the DNA fragments, a transition occurs where the helical molecules become partially melted molecules and migration of the molecule ends at that particular point in the gel. When sequences vary within a domain their temperature differs and this causes the sequence variants of fragments to stop migrating at different positions in the gel and thus can be separated effectively (Muyzer *et al*., 1993).

The number of bands theoretically represents the number of species present within the sample analysed. The primers used to amplify a portion of the 16S rRNA genes producers a quantitative relationship between the gene copy number and the PCR-DGGE band intensity. PCR-DGGE is a sensitive and relatively fast technique that can detect most single based variations when a G-C clamp is present and therefore capable of detecting changes within a microbial community or of differences between microbial communities (Kaewpipat and Grady, 2002).

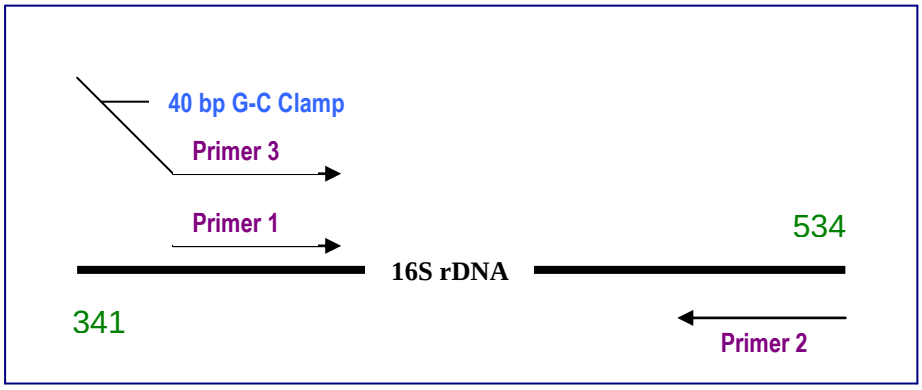

FIGURE 2.11: Representation of the rDNA region amplified by PCR. Primer 1 and primer 2 amplify a specific region in the 16S rDNA. Primer 1 and Primer 3 amplify

the same region however primer 3 is incorporated with a G-C clamp (Muyzer *et al*., 1993).

G-C rich sequences (G-C clamp) can be incorporated into one of the primers used in PCR as illustrated in Figure 2.11. This modifies the melting characteristics of the fragment to which approximately 100 percent of all possible sequence variations can be detected (Muyzer *et al*., 1993). The G-C clamp is present on the 5' end of one of the primers as illustrated in Figure 2.11, imparting melting stability to the PCR products in a DGGE gel. Therefore the individual double stranded DNA molecules denature adjacent to the G-C clamp according to their melting characteristics. This then forms discrete bands in the gel (Ferris *et al*., 1996).

2.5.2 Types of DGGE Gels

2.5.2.1 Perpendicular DGGE

This type of DGGE gel is used to determine the number of melting domains and the optimum denaturing conditions. If the sequence is unknown a 0 to 100% gradient gel should be done, However if the sequence is known the denaturant range should be narrowed in order to improve the resolution between wild-type and mutant sequences (Bio-Rad Laboratories, 1994). The relationship of electrophoretic mobility and denaturant concentration is represented as a sigmoid curve in a gel, showing migration at right angles to the denaturing gradient. The electrophoretic velocity is constant along the path and is proportional to the distance of the curve from the top of the gel. The change in the curve indicates the melting midpoint of the domain. A ratio of low denaturant velocity to the high denaturant plateau indicates the approximate size of the domain (Myers *et al*., 1987).

2.5.2.2 Parallel DGGE

The denaturant concentrations are above and below the melting point of a given domain. When the melting of two or more domains is seen, two different gradient gels can be incorporated to maximise separation. In parallel DGGE gels the denaturation concentrations increases from the top to the bottom of the gel (Muyzer *et al*., 1993). A large number of sequences can be examined and separations are carried out in the gel where the path of the molecule is parallel to the gradient and each molecule can migrate through an ascending gradient of denaturant concentration and reach a domain where migration stops (Myers *et al*., 1987). Therefore in a parallel DGGE gel separation is not time dependent, molecules migrate at a constant velocity until it reaches a domain in the gel where the denaturant concentration causes the molecules to melt and denature. Therefore stopping the migration of the molecules (Bio-Rad Laboratories, 1994). Good resolution of the bands in the gel can be obtained if a narrow segment of the gradient of denaturant concentration is extended over the full height of the gel (Myers *et al*., 1987).

2.5.2.3 Constant Denaturing Gradient Gel (CDGE)

This gel requires one denaturing condition to melt the sequence. The concentration of the denaturant used is determined at the increase in gel retardation as seen in the perpendicular denaturing gradient gel. The constant denaturing gels allow more samples to be electrophoresed under optimal conditions. In CDGE separation of sequences is time dependent due to the wild-type and mutant fragments which partially melts immediately and migrates at a constant rate. Therefore the running time of the gel is dependent on the resolution required (Bio-Rad Laboratories, 1994).

2.5.3 Analyses of Microbial Populations with DGGE

DGGE performed on 16S rRNA genes has been used to produce a genetic fingerprint of mixed microbial communities (Kaewpipat and Grady, 2002). The number of bands that is obtained from DGGE profiles provides an estimate of the different microbial species present. The intensity of each band provides an estimation of the relative abundance of each species (Nasu *et al*., 2000). The primers used to amplify a fragment of the 16S rRNA produces, a quantitative relationship between the gene copy number and the PCR-DGGE band intensity. However many factors can prevent the formation of the number and intensity of the bands in the DGGE gel. Therefore representing the exact number and abundance of species in a microbial community can be difficult (Kaewpipat and Grady, 2002) however DGGE is a sensitive and a rapid technique that detects most singebase variations when a G-C clamp is added to one of the primers in the PCR process. This provides a profile of changes that occur within a microbial community or between microbial communities (Kaewpipat and Grady, 2002). According to Muyzer *et al*., (1993) DGGE as a technique superior to cloning and sequencing of PCR amplified rDNA. When group specific oligonucleotide primers are used for hybridisation with bands derived from DGGE, particular constituents of the microbial populations can be identified. Therefore the identity and relative abundance of the different species can be identified and this produces a profile of the microbial community in both a qualitative and semi-quantitative way. Cloning and sequencing only provides qualitative information about a microbial population (Muyzer *et al*., 1993).

2.6 EPIFLUORESCENCE MICROSCOPY USING 4,6-DIAMIDINO-2-PHENYLINDOLE

DAPI is an intercalating dye that specifically binds to double stranded DNA and exhibits fluorescent characteristics. Binding of DAPI to double-stranded DNA occurs with an appropriate 20-fold fluorescent enhancement, which does not occur with single stranded DNA. A DAPI count is the measure of total bacterial or phage DNA (Tecan, 2001).

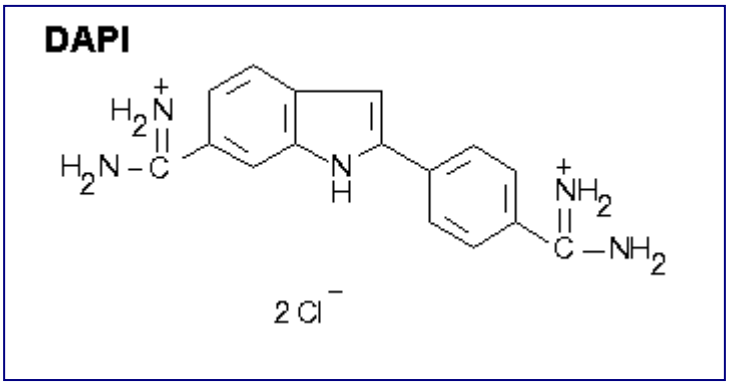

FIGURE 2.12: Chemical structure of the DAPI molecule (Benlot *et al*., 2002).

DAPI is highly specific for DNA under a wide range of conditions. When excited with light at a wavelength of 365nm, the DNA-DAPI complex fluoresces a bright blue, while unbound DAPI and DAPI bound to non-DNA material may fluoresce a weak yellow colour. Micro-organisms can therefore be distinguished from other particulate matter (Porter and Feig, 1980).

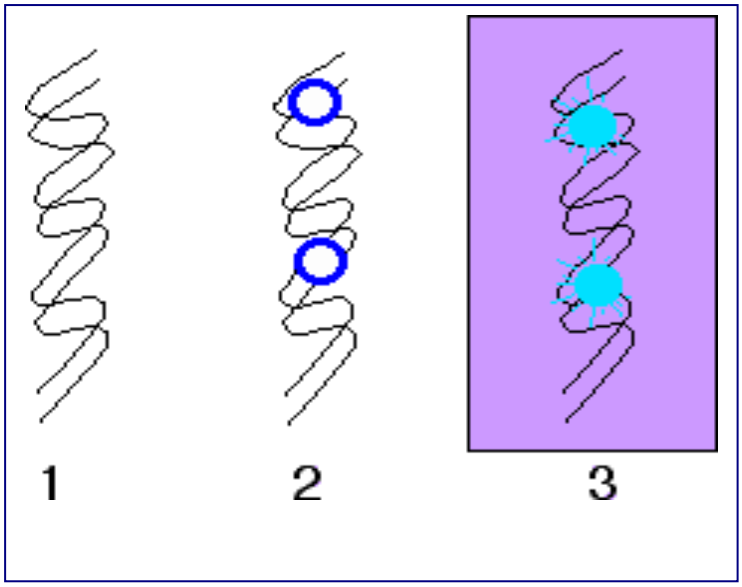

FIGURE 2.13: The process where DAPI binds to DNA. 1: double stranded DNA, 2: DAPI intercalating with the DNA and 3: Under ultra-violet light DAPI fluoresces (Benlot *et al*., 2002).

Polynucleopore filters are better than cellulose filters for the direct counting of bacteria because they have a uniform pore size and a flat surface that retains all of the bacteria on top of the filter. Although cellulose filters retains all the bacteria, many are trapped inside the filter where they cannot be counted. Before use, nucleopore filters must be dyed with Irgalan black to eliminate auto-fluorescence. Criteria for a successful direct enumeration technique are; all bacteria must be retained by the filter; all the bacteria must be visible at the filter surface and the staining and optical conditions must produce high contrasts between the bacteria and the background. The nucleopore filters should be stained before use by soaking for 2 to 24 hours in a solution of 2g Irgalan black in 1 litre of 2% acetic acid (Hobbie *et al*., 1977).

Major factors that affect this method of choice are membrane filter, disaggregation, sample preservation and storage (Daley, 1979). This method allows direct and specific measurement of the active micro-organisms present. The potential deficiency

with regards to application of this method to activated sludge mixed liquor is the yield of inconsistent cell fluorescence. This method is not applicable for organisms which are present in flocs because cells are counted correctly if they are individual cells (Cronje *et al*., 2001).

2.7 FLUORESCENCE *IN SITU* HYBRIDISATION (FISH)

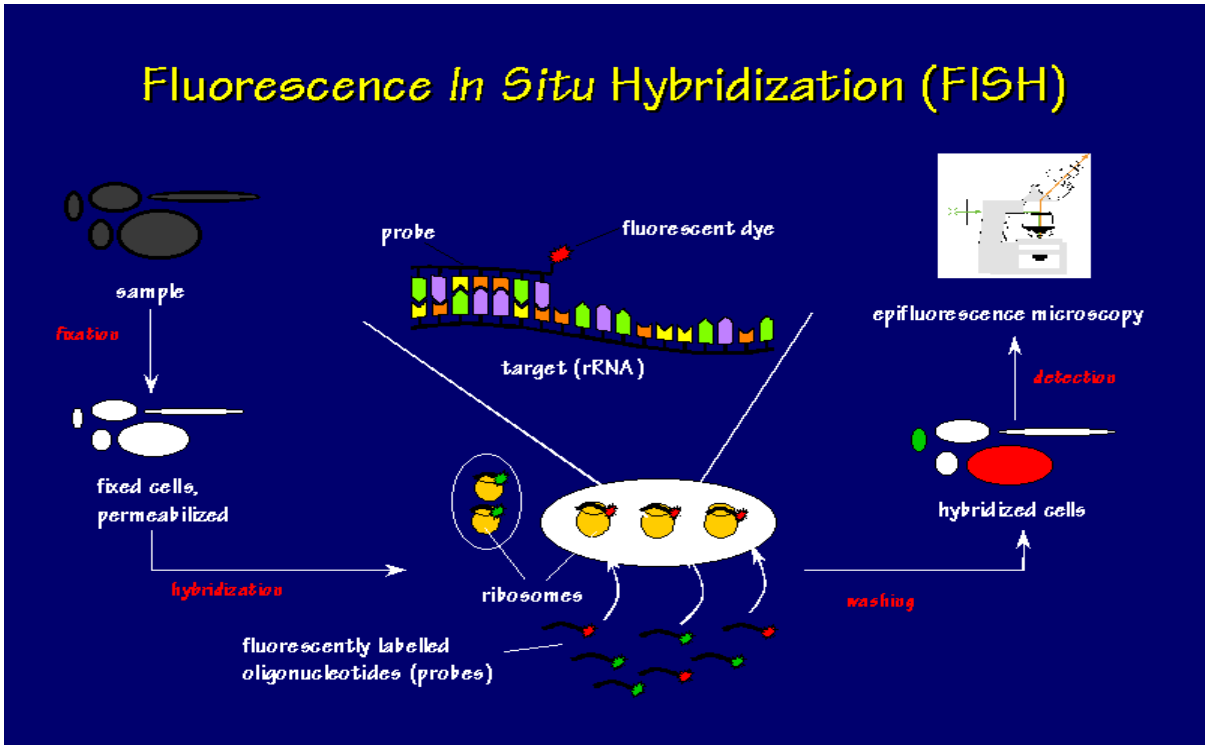

FIGURE 2.14: Flow-Diagram for the procedure of FISH (Baxter, 2005).

FISH requires a labelled probe to be used to detect and localise RNA or DNA sequences. *In situ* hybridisation requires the ability of DNA to re-anneal or hybridise with a complementary strand at a specific temperature. The probe used for the *in situ* hybridisation can be fluorescently labelled and detected by immunocytochemistry. The probe that hybridises to the target sequence can be controlled by the design of the probe, conditions of the hybridisation buffer solution, temperature, pH and salt concentration in the wash buffer. The high stringency conditions allow the probes to

hybridise with similar homology to the target sequence, whilst low stringency conditions allow the probe to bind with a decrease in specificity (Baxter, 2005).

FISH is an invaluable tool for microbial ecology because it allows for the detection and *in situ* identification and quantification of bacteria in several different environmental samples (Yeates *et al*., 2003). Cell numbers of the bacteria can be obtained by enumeration under an epifluorescent microscope. Cell counting procedures involve the use of semi-automated digital image analysis tools, to quantify the fluorescently labelled bacteria in samples (Daims *et al*., 2001).

2.7.1 *In Situ* Hybridisation With rRNA-Targeted Oligonucleotide Probes

Fluorescent *in situ* hybridisation using rRNA targeted probes are frequently applied to quantify the composition of microbial communities. This procedure is based on the comparative analysis of macromolecules, mostly ribosomal RNA molecules and fluorescent derivatives of such probes. These probes have been applied successfully for *in situ* enumeration of defined groups of micro-organisms present in activated sludge (Manz *et al*., 1994).

Fluorescently monolabelled, rRNA targeted oligonucleotide probes show the detection of individual cells, allowing whole-cell hybridisation with rRNA targeted probes to be a suitable tool inferring phylogenetic evolution. The cell morphology of an uncultured microbe and its abundance as well as spatial distributions can be determined *in situ* (Wagner and Amann, 1997).

rRNA molecules are ideal targets for nucleic acid probes because (Amann, 1995):

- They are functionally conserved molecules present in all micro-organisms.

- The primary structures of 16S and 23S rRNA molecules are composed of sequence regions of higher and lower evolutionary conservation.
- 16S rRNA sequences have been determined for a large fraction of the described bacterial species.
- Their natural amplification with high copy numbers per cell greatly increases the sensitivity of rRNA targeted probing, allowing a reliable detection of individual microbial cells by a combination of relatively insensitive fluoresently monolabeled, rRNA-targeted oligonucleotides and epifluorescence microscopy (Amann, 1995).

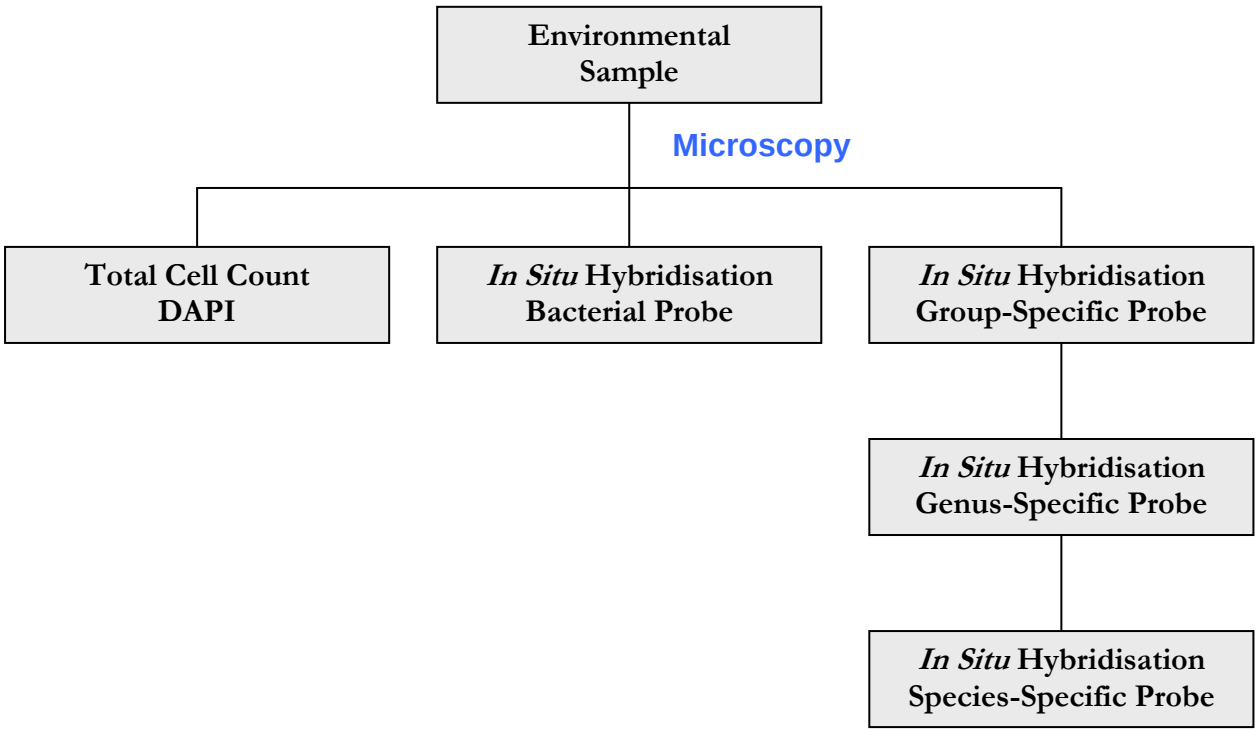

FIGURE 2.15: Flow Chart for an approach to a detailed community analyses (Wagner and Amann, 1997).

As outlined in Figure 2.15, DNA is extracted from environmental samples using standard molecular techniques. Sequence-specific probes are then designed in order to

identify and enumerate whole fixed cells in the sample via *in situ* hybridisation. This method is reliable because sequences are retrieved from cells within the original sample and not from contamination (Wagner and Amann, 1997).

2.7.2 Specificity of Oligonucleotide Probes

A nucleic acid probe is a strand of nucleic acid that is labelled and hybridises to complementary molecules from mixtures of nucleic acids. Probes are either general or specific. Specific probes are those probes that will react or hybridise with cells because of unique sequences in their ribosomal RNA (Madigan *et al*., 2000). Oligonucleotide probes have been designed for the highest taxonomic levels Archaea, Bacteria and Eukarya as well as the intermediate levels. The information gained from higher level probes allows the probe sets to be selected appropriately for the next lower taxonomic level. An example is when the group specific probe shows dominance of bacteria from the gamma subclass of *Proteobacteria* then probes specific for the gamma subclasses will be used (Wagner and Amann, 1997).

Probes are designed based on databases and their specificity is evaluated by hybridisation against culturable reference organisms. This is a disadvantage because the culturable micro-organisms represent a minor part of the true diversity of organisms (Wagner and Amann, 1997).

2.7.3 Optimal Conditions for Hybridisation

Optimal conditions for hybridisations depend on the extent of complementarily of the probe with the nucleic acid needed. High stringent conditions involve the probe and target nucleic acid to have a high degree of complementarity and will allow only well-

matched hybrids to form. However if the probe and target nucleic acid produce a high degree of mismatch the hybridisation reaction is performed in low stringent conditions. Hybridisation takes place in three steps:

- **Prehybridisation:** After transferring the nucleic acids onto the solid support it is prehybridised with blocking agents in order to coat the surfaces and preventing the probe from binding non-specifically to the target nucleic acid.
- **Hybridisation:** The probes are denatured. The ionic strength, temperature and time of the hybridisation are important for a successful hybridisation. Hybridisation can be carried out in an aqueous solution or with formamide.
- **Post-Hybridisation Wash:** This wash removes unhybridised probe from the solid support and dissociates the probe DNA that is loosely bound to the target nucleic acids. The salt concentration, washing temperature and the time of treatment are important for a stringent or nonstringent hybridisation (Bej, 1996).

2.7.4 Labelling Pre-Existing Nucleic Acid Probes

- **Labelling 5' End of DNA:** This method is used for generating labelled DNA fragments which are to be used as gene probes when oligonucleotides are used as the probe. Bacteriophage T4 polynucleotide kinase catalyses the transfer of isotopically labelled phosphate to the 5' terminus of a DNA strand. The attachment of the labelled phosphate group to the 5' end of the probe strand has no impact on the base sequence of the probe.
- **Labelling 3' End of DNA:** 3' end of DNA fragments which have been digested with restriction enzymes produce protruding 5' ends which can be labelled using the Klenow fragment of *E.coli* DNA polymerase I (Debroy and Dangler, 1996).

2.7.5 Purification and Quantification of Probes that are Labelled

The elimination of high background interference involves the removal of unincorporated labelled nucleotides from the reaction mixture before using the newly labelled probe. Purification is achieved by:

- **Ethanol Precipitation:** This is a traditional method for purifying probe molecules from labelling reactions and for concentrating nucleic acid samples. The conditions for salt concentration, incubation temperature, duration and centrifugation specification is dependent on laboratories and their scale of purification.
- **Column Chromatography:** Gel filtration methods are used to separate these labelled probes from unlabelled nucleotides on the basis of size. Sephadex chromatography is used. Separation is dependent on sample molecules entering the pores of the sephadex beads which are held in columns. Smaller nucleotide molecules enter the gel pores and are retained for longer times, whilst the larger probe molecules flow between the beads.
- **Centrifugal Filtration:** unlabelled nucleotides are separated from labelled nucleic acids by ultra-filtration units. Labelled probes due to their larger sizes are retained above a filtration membrane, whilst the unlabelled nucleotides pass through the membrane (Debroy and Dangler, 1996).

Quantification takes place by trichloroacetic acid (TCA) precipitation. The determination of the amount of labelled incorporated probes into nucleic acid molecules is done by precipitating a small aliquot from the mixture containing the labelled probe to ice-cold 5% TCA. The precipitated nucleic acids are collected over a glass fibre filter and counted using a scintillation counter (Debroy and Dangler, 1996).

CHAPTER 3

DETERMINING THE STEADY STATE OF THE MODIFIED LUDZACK-ETTINGER (MLE) PROCESS

3.1 INTRODUCTION

The MLE system, shown in Figure 3.1 is a basic activated sludge process developed for activated sludge biological denitrification. This process was proposed by Ludzack and Ettinger and then later modified by Barnard. It basically comprises of two reactors, anoxic and aerobic reactors (Van Haandel *et al.*, 1981). The anoxic zone is free of oxygen but does contain a substantial amount of nitrates and the aerobic zone is aerated by introducing air or oxygen (Lilley *et al.*, 1997).

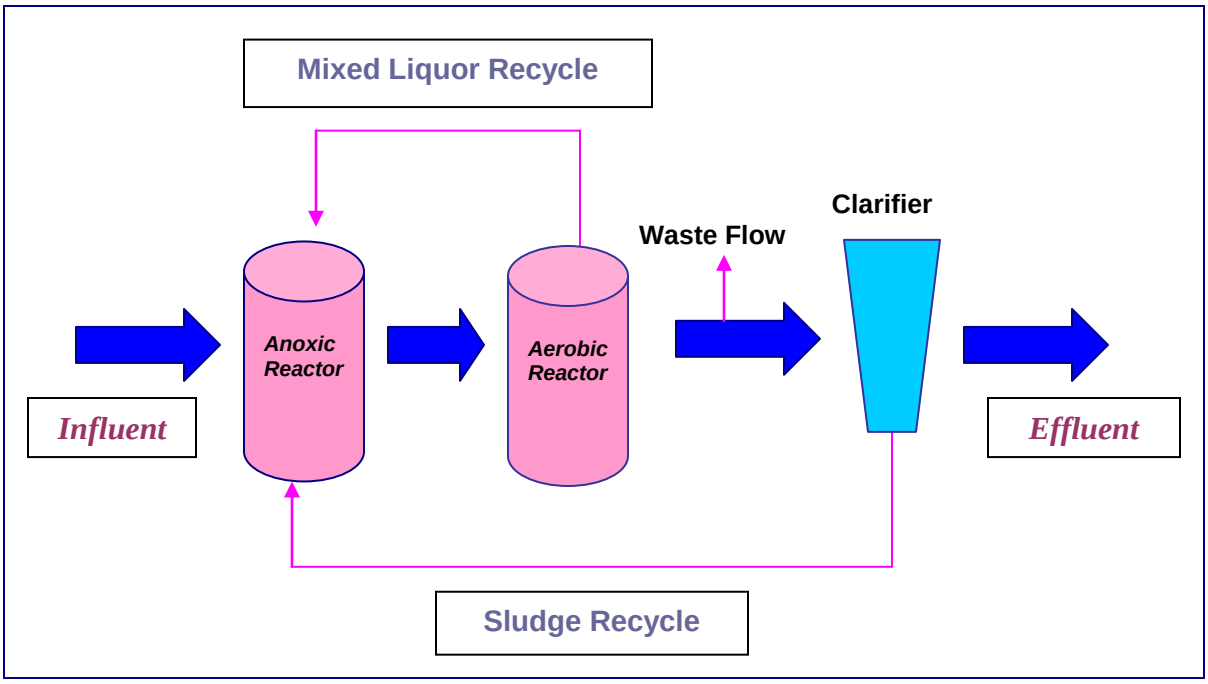

FIGURE 3.1: Representation of a laboratory-scale modified Ludzack-Ettinger process (Lilley *et al.*, 1997).

The culture of organisms present, grow at the expense of the incoming organic material from the influent which is entering the MLE system. The effluent produced is low in carbon and suspended solids. This is due to the processes that occur within the system and by wasting a part of the sludge from the aerobic reactor. The amount of sludge wasted depends on the characteristics of the incoming raw wastewater (influent) and the type of processes that occur within the system (Schroeder, 1977). Microscopic examination of the sludge reveals that it is formed mainly by a heterogeneous population of micro-organisms (Ramalho, 1983). These heterogeneous organisms are usually present as flocculating cells in activated sludge (Lilley *et al.,* 1997).

Flocculating heterotrophic organisms present in aerobic conditions break down carbonaceous materials and a small percentage of energy derived from the breakdown is utilised for synthesis or growth of new cell material, the remaining fraction is used as energy to bring about the synthesis reaction and is eventually lost as heat. The micro-organisms do separate from the sludge and are eventually discharged from the treatment system. This lowers the COD of the sludge before discharging the sludge as effluent (Lilley *et al.,* 1997).

Research of the MLE activated sludge system is important for determining which micro-organisms are predominant and significant in utilising organic wastes (Muyima *et al*., 1997) whilst the system is in steady state. The steady state behaviour of the MLE system is investigated through conducting COD, TKN, nitrate, MLSS and VSS analytical tests. Reliability of the wastewater models with regards to each batch is determined by

conducting COD and nitrogen mass balances which measures the oxygen demand and nitrogen reactions taking place in the influent, anoxic, aerobic and effluent samples.

3.1.1 Mass Balance Equations

3.1.1.1 COD Mass Balance (WRC, 1984).

- M (S_{ti}) = S_{ti} x Q: Mass COD load (M S_{ti}) is calculated by the mean influent COD (S_{ti}) and the mean flow per day (Q).
- M (S_{te}) = S_{te} x Q: Mass of COD effluent (M S_{te}) is calculated by the mean effluent COD and the mean flow per day (Q).
- M (S_{xw}) = f_{cv} x MX_v / Rs: Mass of COD in waste sludge M (S_{xw}) is calculated by COD/VSS ratio of volatile solids (f_{cv}) – 1.48mg COD/mgVSS and MX_v (which is the total volume in litres of the process reactors - V_p) multiplied by X_v (which is the mixed liquor volatile suspended solids in mg VSS) divided by Rs (which is mass of sludge in reactor / mass of sludge wasted per day).
- M (O_c) = M (O_{tm}) + M (O_d) - M (O_n): Mass of the oxygen required for carbonaceous degradation is calculated by [M (O_{tm}) which is measured oxygen per day = OUR x 24 x % aerobic Vp/100 and M (O_n) which is the mass of oxygen used for nitrification - equals to 4.57 M (N_{ng}) Where: N_{ng} = the mass of nitrate that is generated - M(N_{ti}) - M(N_{te}) - M(N_s): Where: M(N_{ti}) is the mass of TKN in the influent and M(N_{te}) is the mass of the TKN in the effluent and M(N_s) which is the mass of nitrogen in the VSS daily

(f_n x M(X_v) / R_s): Where: f_n is the fraction of TKN in VSS ~ 0.1 mg (TKN-N) / mgVSS**]** and M (O_d) is the mass of oxygen recovered due to denitrification = 2.86M (N_d): Where: M (N_d) is the mass of nitrate denitrified which is mass of nitrate generated M (N_{ng}) – mass of nitrate generated daily M (N_{ne}):

Where: M (N_{ne}) = Q x N_{ne})

Mass Balance COD (%) = $\frac{M(Ste) + M(Sxw) + M(Oc)}{M(Sti)}$ x 100

3.1.1.2 Nitrogen (N) Mass Balance (WRC, 1984).

- Mass of influent TKN (M (N_{ti})is calculated by the addition of mass of effluent TKN M (N_{ne}) + mass of effluent NO_3 M (N_{ne})+ mass of N denitrified M (N_d)+ mass of TKN wasted M (N_w).
- Mass of nitrate denitrified M (N_d) = [mass of NO_3 coming in] - [mass of NO_3 going out]
- M (N_d) = [$NO_{3\,(AE)}$ x Qi x a + $NO_{3\,(FE)}$ x Qi x s] – [$NO_{3\,(AN)}$ x Qi x (1+a+s)] :

  Where: Qi is the influent flow rate, a is the a-recycle ratio, s is the s-recycle ratio

  Where: $NO_{3\,(AE)}$ is the concentration of NO_3 in the aerobic reactor

  $NO_{3\,(FE)}$ is the concentration of NO_3 in the filtered effluent

  $NO_{3\,(AN)}$ is the concentration of NO_3 in the anoxic reactor
- M (N_{te}) is the mass of TKN in the effluent, which is N_{te} x Qi
- M (N_{ne}) is the mass of effluent nitrate, which is $NO_{3(FE)}$ x Qi
- M (N_w) is the mass of TKN in waste sludge, which is $\frac{fn \times M(Xy)}{}$:

Rs

Where: fn is the fraction of TKN in VSS ~ 0.1 mg (TKN-N) / mg VSS

- M (N_{ti}) is the mass of TKN in the influent, which is N_{ti} x Qi

Mass Balance Nitrogen (%) = $\frac{M(N_{te}) + M(N_{ne}) + M(N_d) + M(N_w)}{M(N_{ti})} \times 100$

3.2 METHODOLOGY

The laboratory-scale modified Ludzack-Ettinger process (Figure 3.2) was fed daily with 30L of primarily domestic wastewater obtained from Southern Wastewater Works (Durban, South Africa). Raw samples were stored in containers at 4ºC. The MLE system was run on a 10day sludge age process which constituted one batch. After the steady state of the MLE system was achieved, the system was run for a duration of eighteen (18) batches. The wastewater was diluted with tap water to give an influent feed of approximately 500mgCOD/L from the original 800mgCOD/L. The process was maintained by wasting 1.5L/day of the mixed liquor from the aerobic reactor.

Samples from the influent, anoxic, aerobic and effluent was analysed daily for the following: COD (Appendix 1), TKN (Appendix 2) MLSS, VSS (Appendix 3) and nitrate and nitrite analysis (Nitrite tests were not performed because the nitrites were 2% less than nitrates). Samples from the aerobic and anoxic reactors were taken for microbial community analyses (Chapter 4 and 5). The oxygen utilisation rate (OUR) was monitored within the mixed liquor with an online dissolved oxygen (DO) controller (hi-tech microsystem) according to Randall *et al*., (1991). The pH was kept constant at 7.5 and the temperature was kept constant at 20ºC.

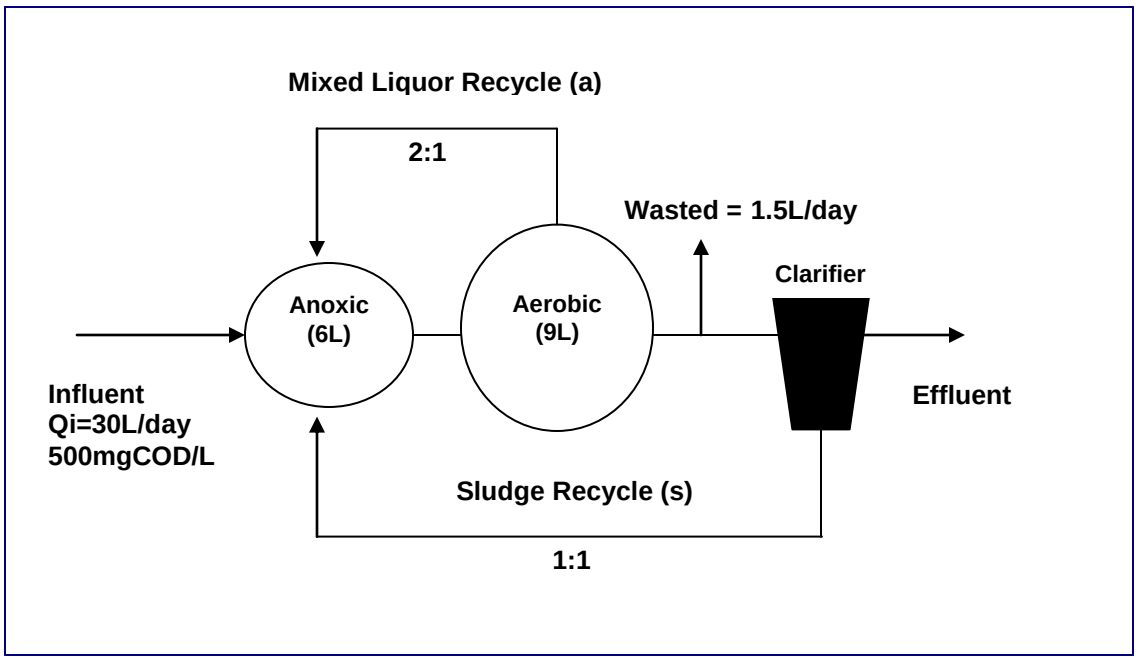

FIGURE 3.2: Design of the modified Ludzack-Ettinger process (Lilley *et al*., 1997).

3.3 RESULTS

TABLE 3.1: Steady state results for the parent laboratory-scale anoxic and aerobic sludge system.

WW Batches	COD(mg/L)		TKN(mg/L)		Nitrate (mgN/L)			OUR	Mixed liquor (mg/L)		
	INF	EFF	INF	EFF	Anoxic	Aerobic	Eff	mgO/L/H	VSS	COD	TKN
1	502.6 (48.8)	46.0 (10.1)	38.8 (6.3)	5.3 (2.1)	0.6 (1.0)	3.9 (1.9)	7.9 (1.5)	30.6 (0.4)	2352.4 (153.1)	3720 (553.2)	234.6 (20.7)
2	492 (52.6)	57.0 (11.6)	48.3 (2.9)	4.3 (0.9)	1.8 (0.4)	7.9 (1.8)	15.4 (0.3)	29.69 (0.460)	1860 (205)	2727.5 (208.2)	229.8 (12.4)
3	504 (10.2)	47.2 (9.5)	42.8 (4.3)	4.4 (0.4)	1.2 (0.7)	8.7 (1.5)	10.1 (0.9)	30.3 (0.1)	1979.6 (273.6)	3188.0 (254.6)	233.8 (5.7)
4	500.2 (43)	47.2 (5.7)	31.2 (2.2)	1.7 (0.8)	0.2 (0.4)	2.9 (1.5)	8.6 (1.6)	31.7 (1.4)	1897.8 (163.2)	2796.5 (159.4)	204.1 (16.8)
5	490.7 (21.8)	39.5 (4.4)	32.2 (2.8)	4.4 (1.2)	0.3 (0.7)	2.7 (1.2)	8.1 (1.6)	30.8 (0.3)	1816.8 (186)	2648.2 (367.4)	129.9 (38.5)
6	512.1 22.5	48.3 7.3	33.6 (1.4)	4.4 (1.2)	0.9 (0.7)	4.3 (2.5)	10.4 (1.5)	31.7 1.5	1860.3 (213.5)	3029.0 344.5	129.9 (38.5)
7	513.4 29.5	53.7 9.2	33.2 (3.5)	2.6 (1.1)	1.2 (1.0)	8.4 (3.6)	12.5 (4.7)	32.0 0.3	1948.3 (141.3)	2906.8 218.2	209.2 (10.4)
8	516.4 (2.5)	48.2 (9.6)	46.4 (9.9)	6.1 (4)	0.8 (1.2)	6.6 (3)	10.1 (2.7)	34.0 (1.2)	2028.7 (189.2)	2962.9 (193.5)	231.4 (55.5)
9	506 (16.1)	46.8 (7.8)	41.9 (3.4)	3.5 (0.9)	1.2 (1.0)	6.9 (1.6)	10.0 (1.3)	26.7 (1.9)	2341.6 (251.9)	3652.8 (426.3)	223.7 (21.9)
10	491.1 (29.6)	41.5 (7.6)	41.8 (6.1)	5.0 (1.9)	2.0 (2.2)	7.0 (3)	9.4 (3.7)	35.3 (1.1)	2076 (232.7)	3043.6 (357.7)	213.3 (24.5)
11	511.7 (23.7)	40.5 (7.4)	50.3 (9.1)	5.1 (1.9)	2.0 (0.9)	9.4 (2.7)	12.2 (1.5)	34.0 (1.2)	2023.7 (256.3)	3112.8 (432.7)	227.8 (31)
12	474.4 (22.3)	43.9 (5.8)	49.3 (5.1)	4.5 (0.5)	2.7 (1.1)	10.4 (1.6)	14.2 (3)	30.4 (0.6)	2151.6 (315.5)	3207 (366.5)	220.8 (13.3)
13	446.8 (45.9)	40.0 (6.0)	62.0 (4.2)	4.6 (0.7)	2.9 (1.3)	11.5 (3.7)	21.4 (4.8)	29.6 (1.4)	2434.5 (338.8)	3578.0 (314.9)	217.1 (10.5)
14	492.3 (32.7)	43.1 (5.3)	45.4 (6.7)	5.7 (1.9)	2.3 (1.7)	8.4 (3.4)	13.5 (3.4)	31.3 (1.6)	2088.2 (137.8)	3145 (227.1)	200.2 32.7
15	489.2 (30.4)	37.0 (6.1)	45.0 (8.9)	6.1 (3.8)	1.3 (1.9)	5.8 (2.6)	10.2 (3.0)	33.5 (0.3)	1933.6 (277.7)	2852.9 (453.2)	231.4 (52.6)
16	479.4 (25.2)	40.2 (4.5)	39.3 (3.7)	4.4 (0.7)	1.5 (0.9)	7.0 (1.9)	10.1 (1.8)	30.3 (0.8)	1985 (239.0)	2949.4 (307.5)	226.5 (24.9)
17	420.0 (39.9)	44.0 (9.5)	34.9 (0.7)	6.6 (1.6)	2.9 (3.3)	7.8 (4.3)	10.9 (4.5)	30.1 (0.4)	1659.4 (446.7)	2254.5 (215.1)	135.5 (35.4)
18	449.2 (40.0)	39.1 (8.2)	42.1 (5.4)	4.4 (0.8)	0.9 (0.5)	7.2 (2.3)	8.9 (2.5)	30.7 (0.7)	1914.5 (239.2)	2903.7 (372.1)	201.3 (49.3)

(Standard deviations appear in brackets)

(The daily results obtained from the parent laboratory scale anoxic/aerobic activated sludge system is shown in Appendix 10).

The COD influent concentration ranged between 420mg/L and 516.4mg/L and the effluent COD concentrations ranged between 37mg/L and 57mg/L. The oxygen demand was calculated to be 443.8mg/L (90.9%). The TKN influent results ranged between 31.2mg/L and 62mg/L whilst the TKN effluent results varied between 1.7mg/L and 6.1mg/L. The nitrogen utilised was calculated to be 37.5mg/L (89.1%). The nitrate concentration present in the anoxic zone ranged between 0.9mgN/L and 2.9mgN/L and the nitrate concentration

for the aerobic zone ranged between 2.7mgN/L and 11.5mgN/L whilst the nitrate concentration for the effluent ranged between 7.9mgN/L and 21.4mgN/L. The OUR rates obtained throughout the 18 batches ranged between 26.7mgO/L/H and 35.3mgO/L/H. Mixed liquor VSS results were found to be between 1659.4mg/L and 2434.5mg/L. The mixed liquor COD levels ranged between 2254.5mg/L and 3720mg/L whilst the TKN mixed liquor results ranged between 129.9mg/L and 234.6mg/L.

TABLE 3.2: COD and nitrogen mass balances for the parent laboratory-scale anoxic and aerobic activated sludge system.

WW Batches	Mass balance (%)	
	COD	Nitrogen
1	105.9	105.6
2	87.3	119.2*
3	95.1	102.6
4	98.6	102.6
5	96.0	100.7
6	97.7	108.4
7	89.5	160.0*
8	94.6	109.0
9	92.4	104.6
10	102.1	102.8
11	95.3	102.7
12	94.8	101.7
13	105.9	102.7
14	97.0	105.3
15	97.3	98.4
16	95.9	106.4
17	98.7	109.1
18	96.9	103.5

* Indicates nitrogen mass balance results which were higher than the acceptable ranges.

The COD mass balances fell in the range of 87.3% and 105.9% and the nitrogen mass balances fell in the range of 98.4% and 119.2%.

3.4 DISCUSSION

The modified Ludzack-Ettinger (MLE) process was setup as a laboratory-scale activated sludge system. The system was run on a 10day sludge age for 18 wastewater batches. Steady state was reached after the fourth sludge age thereafter samples were taken for COD, TKN, MLSS, VSS and microbial population analyses. Table 3.1 represents the steady state results obtained from the anoxic and aerobic parent system for the 18 wastewater batches. The oxygen demand concentration calculated for the MLE system was high with 90.9%. The influent and effluent TKN results obtained verified the high amount of nitrogen (89.1%) that was utilised from the influent which entered the system. The oxygen demand and nitrogen utilisation rates were similar to the findings of Ismail (2003) who found that 87.89% oxygen demand and 82.77% of nitrogen was utilised in a similar MLE system.

The nitrate concentration was analysed within the anoxic, aerobic and effluent wastewaters. The average nitrate concentration for the 18 batches in the anoxic zone was 1.5mgN/L. The results obtained are suggestive that the anoxic zone supported high rates of denitrification. Whereby the nitrogen entering the system in the form of nitrates were converted to nitrites and further converted to molecular nitrogen within the anoxic zone (Lilley *et al*., 1997). The aerobic zone had a higher level of nitrate concentration with an average of 7mgN/L present. The higher concentrations of nitrates present in the aerobic zone indicate an increase in the nitrification and a decrease in the denitrification process. The average nitrate concentration present in the effluent was higher in comparison to the nitrate concentrations found in the anoxic and aerobic zones with a concentration of

11.3mgN/L. The nitrate concentration obtained from the system does signify that a high denitrification potential is prominent within the MLE laboratory-scale process. However complete denitrification is not possible within a laboratory-scale MLE activated sludge system therefore nitrates are discharged with the effluent (Lilley *et al*., 1997) which is evidently shown in the results obtained.

The oxygen utilisation rate (OUR) was analysed in the aerobic zone throughout the 18 batches and the readings ranged between 26.7mgO/L/H and 35.3mgO/L/H which was similar to the findings of Ismail (2003), where it was found that the OUR rates ranged between 29.02mgO/L/H and 32.29mgO/L/H. The mixed liquor from the system was analysed by performing VSS, COD and TKN, the results obtained are represented in Table 3.1. The VSS results obtained from the mixed liquor indicates the presence of a high concentration of volatile suspended solids which consisted of organic matter and dead or live micro-organisms (Bitton, 1999). Therefore it can be noted that a suitably high biomass concentration is present in the mixed liquor.

The COD results of the mixed liquor varied between 2648.2mg/L and 3720mg/L. The average COD results obtained from the mixed liquor in comparison to the influent COD results suggests that a higher COD concentration was present in the aerobic reactor. As confirmed by Munch and Pollard (1997), the amount of organic substrates and bacterial biomass present effects the concentration of oxygen. Therefore a high VSS concentration obtained in the mixed liquor resulted in a high oxygen demand present.

The TKN results from the mixed liquor were higher than the TKN results obtained from the influent due to the high bacterial biomass present in the mixed liquor, which is represented with a high concentration of VSS whereby the bacteria present increases the concentration of nitrogen present. According to WRC (1984) the TKN concentration entering the system with the influent consists of free and saline ammonia and organically bound nitrogen. According to Muyima *et al*., (1997) the primary function of the aerobic zone is to oxidise ammonia to nitrites and then to nitrates. Therefore the zones have nitrifying bacteria which increases the concentration of nitrates present therefore increasing the concentration of the TKN.

COD and nitrogen mass balances from Table 3.2, was calculated in order to validate the reliability of the MLE model throughout the research. According to Wenzel *et al*., (1998) reliable mass balances fall in the range of 90-110%. The COD mass balances fell in the range of 92.4% and 105.9% and the nitrogen mass balances fell in the range of 98.4% and 160.0%. According to Ismail (2003) mass balances calculated for a similar MLE process fell in the range of 77% to 87% for the COD mass balance and 94% to 107% for the nitrogen mass balance. The reliability of the experimental data obtained from the MLE system was checked with the mass balances. However very high nitrogen mass balances were noted in batch 2 with 119.2% and batch 7 with 160.0% these mass balances indicated the unreliability of the MLE model during batches 2 and 7. However the results obtained from the other wastewater 16 batches fell under acceptable ranges according to Clayton *et al*., (1989) and Ubisi *et al*., (1997). The high nitrogen mass balances were contributed to the system performance which was not ideal due to the

spillages that occurred during the second and seventh batches therefore the nitrogen balance in the system was upset.

3.5 CONCLUSION

The MLE configuration outlined in Figure 3.2, illustrated an activated sludge nutrient removal process, which allowed biological processes such as nitrogen removal to prevail within the system. The process indicated a high potential for denitrification within the anoxic zone. However total denitrification cannot be achieved within a laboratory-scale MLE system due to a lack of a secondary anoxic reactor.

The steady state behaviour of the system was shown throughout the 18 wastewater batches ensuring that the mixed liquor reached a consistency throughout the duration of the research. The steady state of the mixed liquor was determined via the measurement of the COD, TKN and nitrate concentrations.

The COD and nitrogen mass balances calculated for each batch determined the reliability of the data obtained for each of the 18 wastewater batches. Nitrogen mass balances calculated for batches 2 and 7 confirmed the unreliability of the results obtained from these two batches. However, these mass balances are calculated only as a representation of the system performance during the research and therefore microbial community analyses were conducted including batches 2 and 7.

CHAPTER 4

MICROBIAL COMMUNITY ANALYSIS OF THE MLE PROCESS USING FLUORESENT *IN SITU* HYBRIDISATION

4.1 INTRODUCTION

The dynamics and diversity of the microbial populations in activated sludge have been analysed by culture-dependent methods, even though many members of the natural bacterial communities are still unculturable (Wagner *et al*., 1994). Hence microscopic identification based on morphological characteristics was researched and developed (Wagner *et al*., 1993) in a culture-independent manner by direct rRNA sequence retrieval, where nucleic acid probes which are complementary to the rRNA are used as tools to monitor population dynamics amongst micro-organisms (Amann, 1995).

Fluorescence *in situ* hybridisation (FISH) using rRNA targeted oligonucleotide probes is often applied to quantify the microbial community compositions in various environmental conditions (Daims *et al*., 2001). The technique of FISH involves the mixing of fixed microbial sample with fluorescently labelled oligonucleotide probes. Phylogenetic group specific probes are oligonucleotide probes that specifically target intracellular rRNAs of many specific groups of organisms. These group-specific probes have been designed in order to target whole bacteria, domains, phyla, subphyla, genera and on a species level (Yeates *et al*., 2003).

Epifluorescent microscopy allows cells to be directly visualised by hybridising them with rRNA targeted oligonucleotides probes that are chemically linked to a fluorochrome molecule. A total cell count is required using a DNA intercalating dye, 4,6-diamidino-2-

phenylindole (DAPI) and thereafter hybridisation takes place with the specific probe required (Cloete *et al*., 2003).

FISH allowed the quantification of the phylum *Proteobacteria* and the high G+C content bacteria present within the anoxic and aerobic zones of the laboratory-scale MLE system. Quantification using FISH allows the steady state behaviour of the MLE system to be analysed with reference to the microbial population dynamics.

4.2 METHODOLOGY

Samples for FISH analyses were taken from the anoxic and aerobic zones of the laboratory-scale MLE activated sludge system. The FISH procedure was done in duplicate to minimise errors and to correctly quantify the bacterial cells according to group specific oligonucleotide probes.

4.2.1 Sample fixation and Sonication:

Samples that were obtained from the anoxic and aerobic zones of the laboratory-scale activated sludge process were immediately fixed (Appendix 4) and pretreated according to Amann (1995). Samples were fixed with 4% paraformaldehyde for gram negative bacteria and with absolute ethanol for gram positive bacteria. The samples were sonicated at 8 watts for 8 minutes with a vibracell sonicator (Virtis), in order to break up the bacterial flocs that were present.

4.2.2 Membrane Filtration with 4,6-diamidino-2-phenylindole (DAPI):

Membrane filtration (Appendix 5) was performed according to (Hicks *et al*., 1992). Fixed samples were sonicated and diluted (dilution factor of 200) with phosphate buffered saline (PBS) and 1% nonidet. Nucleopore filters with pores sizes of 0.2µm and 0.45µm were used. The 0.2µm filters were pretreated with 0.3% sudan black and placed on top of the 0.45µm backing filter. DAPI stain (1.25µg/ml) was used to bind to the DNA that was present. The Zeiss Axiolab microscope (50 watts high-pressure mercury bulb and Zeiss filter set 01) fitted for epifluorescence microscopy was used with the Zeiss image analysis software in order to count the fluorescing cells. Multiple fields (approximately 30) were randomly selected to view and enumerate the fluorescing cells for each slide in order to minimise errors associated with the cells not being evenly distributed on the filter.

4.2.3 Pretreatment and Whole Cell Hybridisation (FISH):

Samples with 4% paraformaldehyde fixation were used for: EUB338 I, II, III; ALF1b; BET42a and GAM42a oligonucleotide probes, and ethanol fixed samples were required for EUB338 I, II, III and HGC69a oligonucleotide probes. The fixed and sonicated samples were immobilised onto pre-treated slides and dehydrated and with 60%, 80% and absolute ethanol to prepare for whole cell hybridisation. Samples were hybridised with appropriate hybridisation buffers and probed *in situ* with the oligonucleotide probes listed in Table 4.1 according to Amann, (1995).

TABLE 4.1: The oligonucleotide probes used for the FISH analysis.

Probe	Specificity	Sequence (5' - 3')	rRNA Target Site (Ecoli numbering)	Formamide (%)	Reference
EUB388 I,II,III	Domain Eubacteria	I: GCTGCCTCCCGTAGGAGT II: GCAGCCACCCGTAGGTGT III: GCTGCCACCCGTAGGTGT	16S, 338 - 355	20	Yeates *et al*., 2003
ALF1b	*Proteobacteria* (Alpha)	CGTTCGYTCTGAGCCAG	16S, 19-35	20	Wagner *et al*., 1993
BET42a	*Proteobacteria* (Beta)	GCCTTCCCACTTCGTTT	23S, 1027 - 1043	35	Wagner *et al*., 1993
GAM42a	*Proteobacteria* (Gamma)	GCCTTCCCACATCGTTT	23S, 1027 - 1043	35	Wagner *et al*., 1993
HGC69a	Gram +ve Bacteria with high G+C content	TATAGTTACCACCGCCGT	16S, 1901 - 1918	25	Onuki *et al*., 2000

4.3 RESULTS

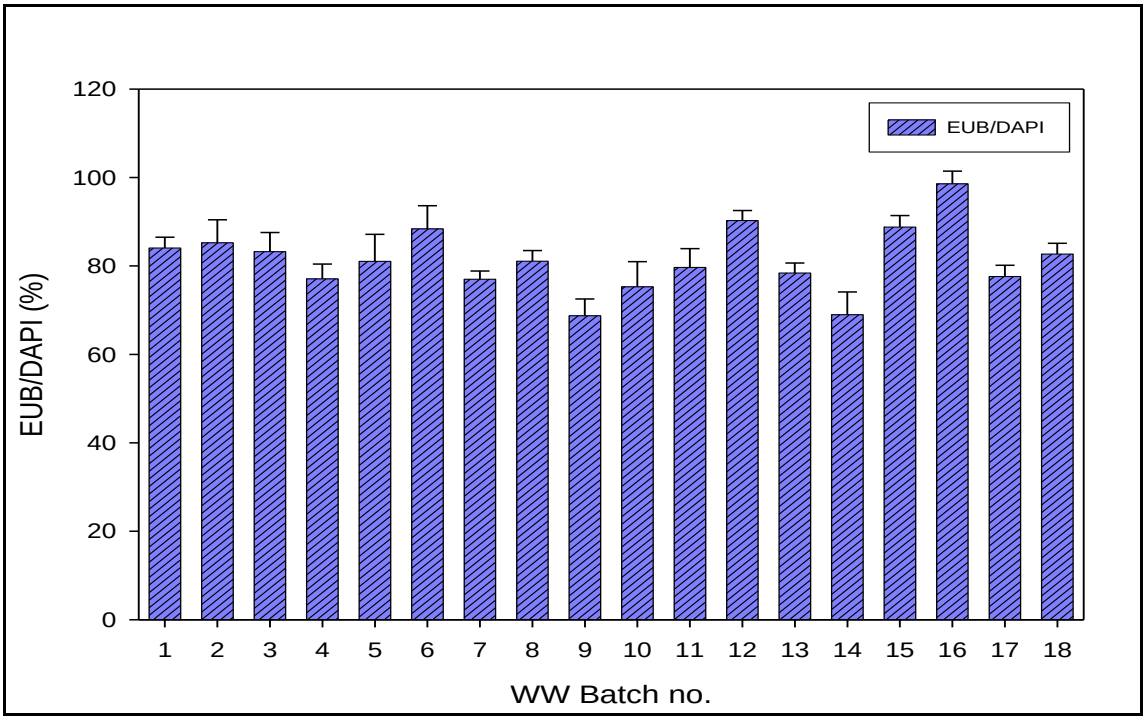

FIGURE 4.1: The EUB as a percentage of the total bacterial cell count (DAPI) for the anoxic zone.

The average EUB/DAPI counts for the 18 batches from the anoxic zones were 81.44% (±3.6%). The highest EUB/DAPI ratio was shown in batch 16 with 98.56% (±2.87%). The lowest EUB/DAPI ratio was found present in batches 9 and 14 with 68.73% (±3.8%) and 68.97% (5.15%) respectively.

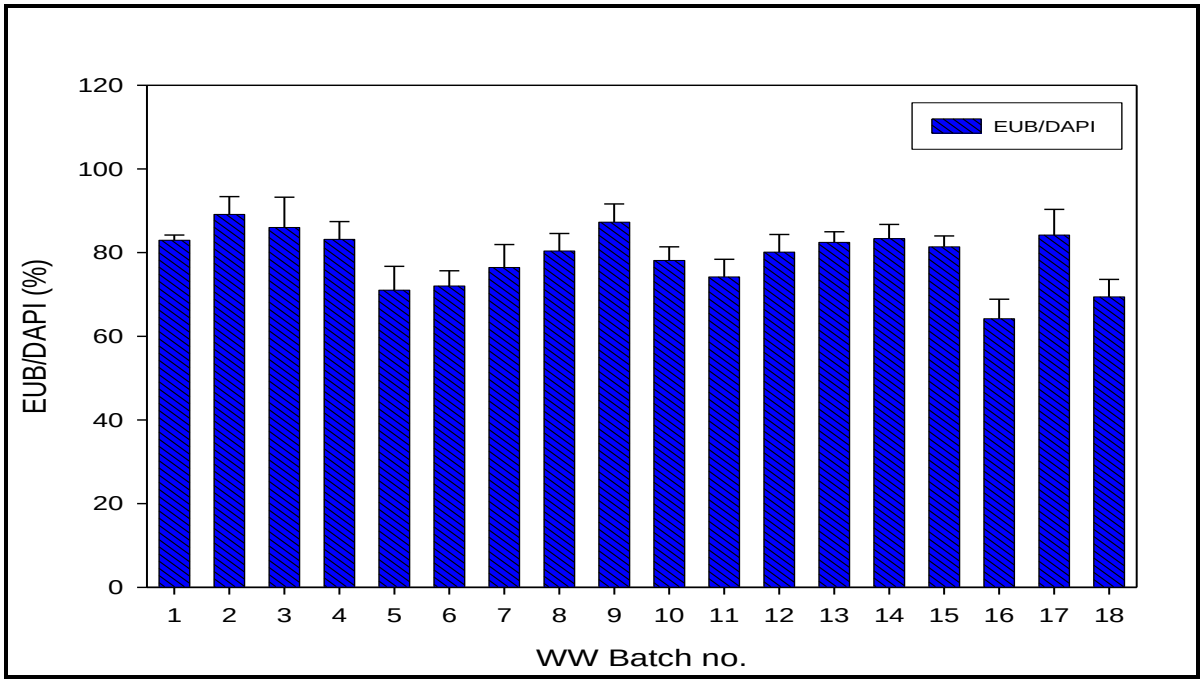

FIGURE 4.2: The EUB as a percentage of the total bacterial cell count (DAPI) for the aerobic zone.

The average EUB/DAPI counts for the 18 batches from the aerobic zones were 79.21% (±4.2%). The highest EUB/DAPI ratio was shown in batch 2 with 89.13% (±4.25%). The lowest EUB/DAPI ratio was found present in batches 16 with 64.19% (±4.67%).

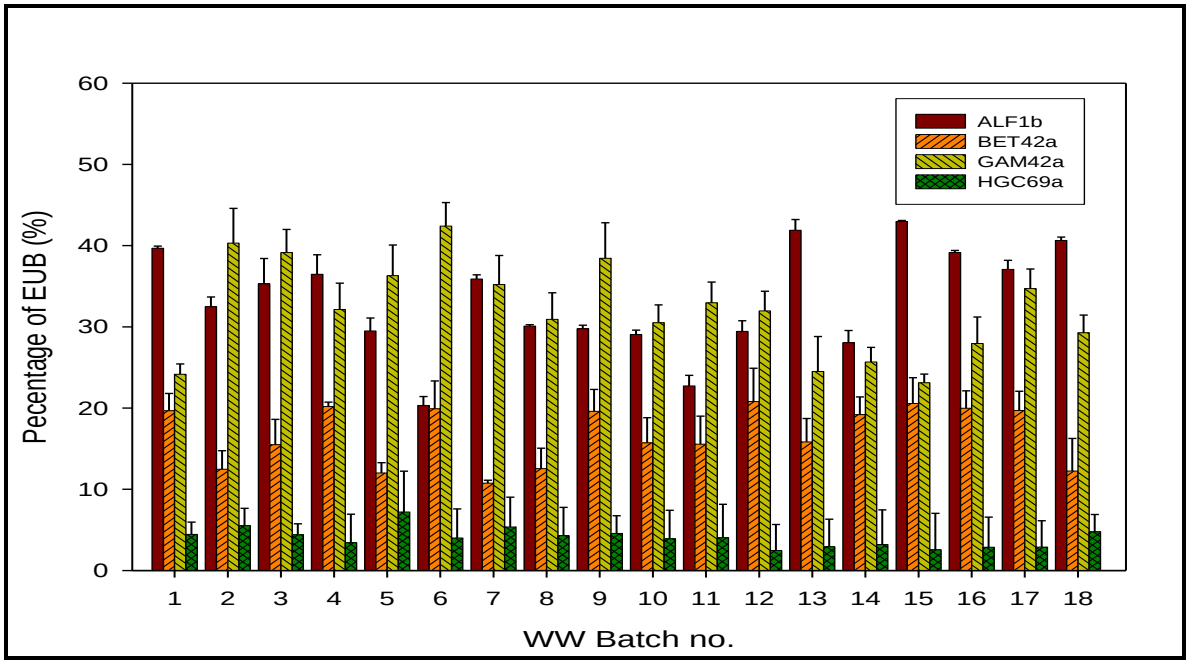

FIGURE 4.3: The total cell counts for the oligonucleotide probes as a percentage of the total eubacterial cells from the anoxic zone.

The anoxic zone showed the dominance of the Alpha subclass, with an average of 33.35% (±1.04%) and Gamma subclass, with an average of 32.20% (±2.87%) of the phylum *Proteobacteria* throughout the 18 wastewater batches. The highest Alpha counts are noted in batches 13 and 15 with 41.88% (±1.32%) and 42.96% (±0.12%) respectively. The highest Gamma bacteria are present in batches 2 and 6 with 40.30% (±4.28%) and 40.40% (±2.89%). An average count of the 18 wastewater batches for the Beta subclass was 16.79% (±2.54%). The high G+C content bacterium represented the least dominant species of bacteria with 4.04% (±3.25%) which is an average for the duration of the 18 wastewater batches.

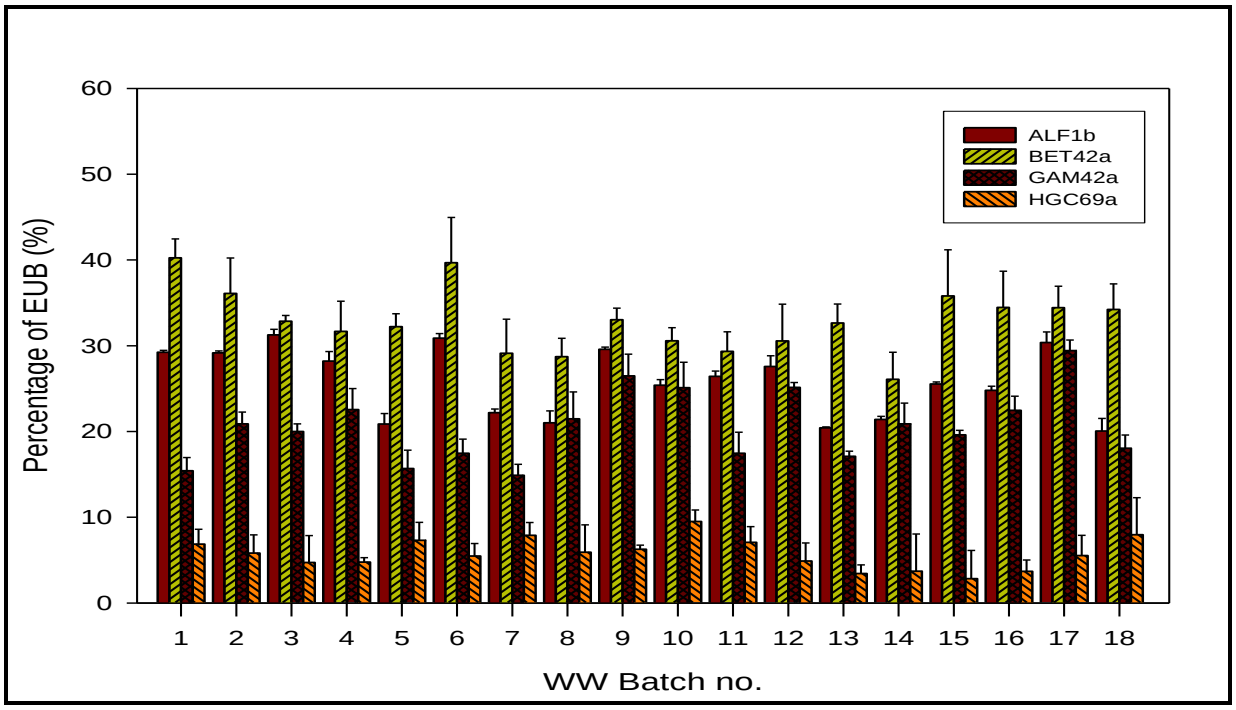

FIGURE 4.4: The total cell counts for the oligonucleotide probes as a percentage of the total eubacterial cells from the aerobic zone.

The aerobic zone showed the dominance of the Beta subclass of *Proteobacteria* throughout the 18 wastewater batches. The highest Beta counts are noted in batches 1 and 6 with 40.24% (±2.21%) and 39.68% (±5.28%) respectively. The Alpha subclass, as an average of the 18 wastewater batches represented 25.80% (±0.69%) of the bacteria present. An average count of the 18 wastewater batches for the Gamma subclass was 20.55% (±1.72%). The high G+C content bacteria showed a presence of 5.76% (±2.12%) as an average of the 18 wastewater batches.

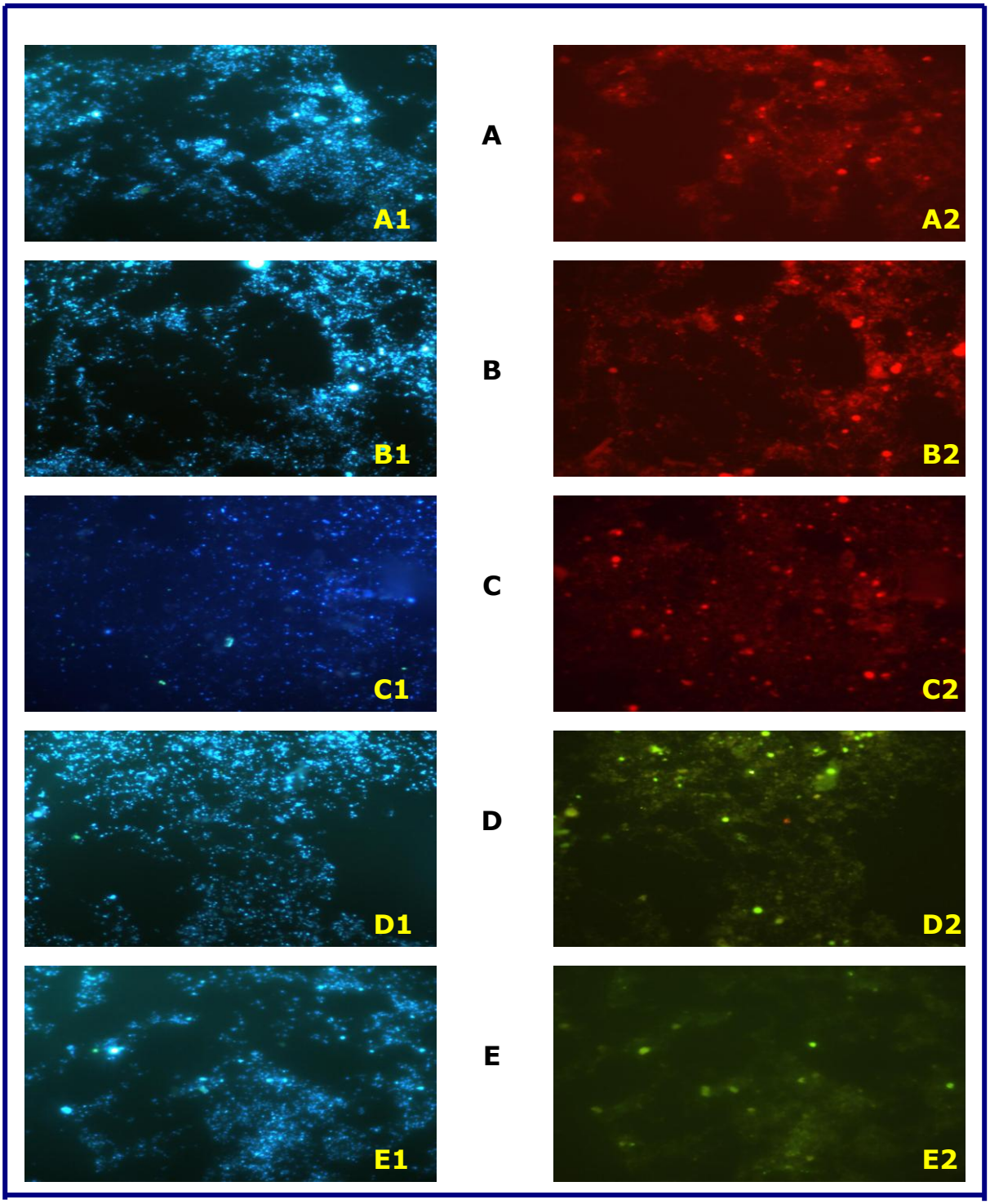

Figure 4.5: *In situ* hybridisation of the anoxic activated sludge samples. (A1 to E1-Left) DAPI stains (A2 to E2-Right) *in situ* hybridisations with probes. Micrographs are shown for identical microscopic fields. A:DAPI stain and corresponding hybridisation with rhodamine labelled EUB338I,II,III, B:DAPI stain and corresponding hybridisation with rhodamine labelled ALF1b, C:DAPI stain and corresponding hybridisation with rhodamine labelled BET42a, D:DAPI stain and corresponding hybridisation with fluorescein labelled GAM42a, E:DAPI stain and corresponding hybridisation with fluorescein labelled HGC69a probe.

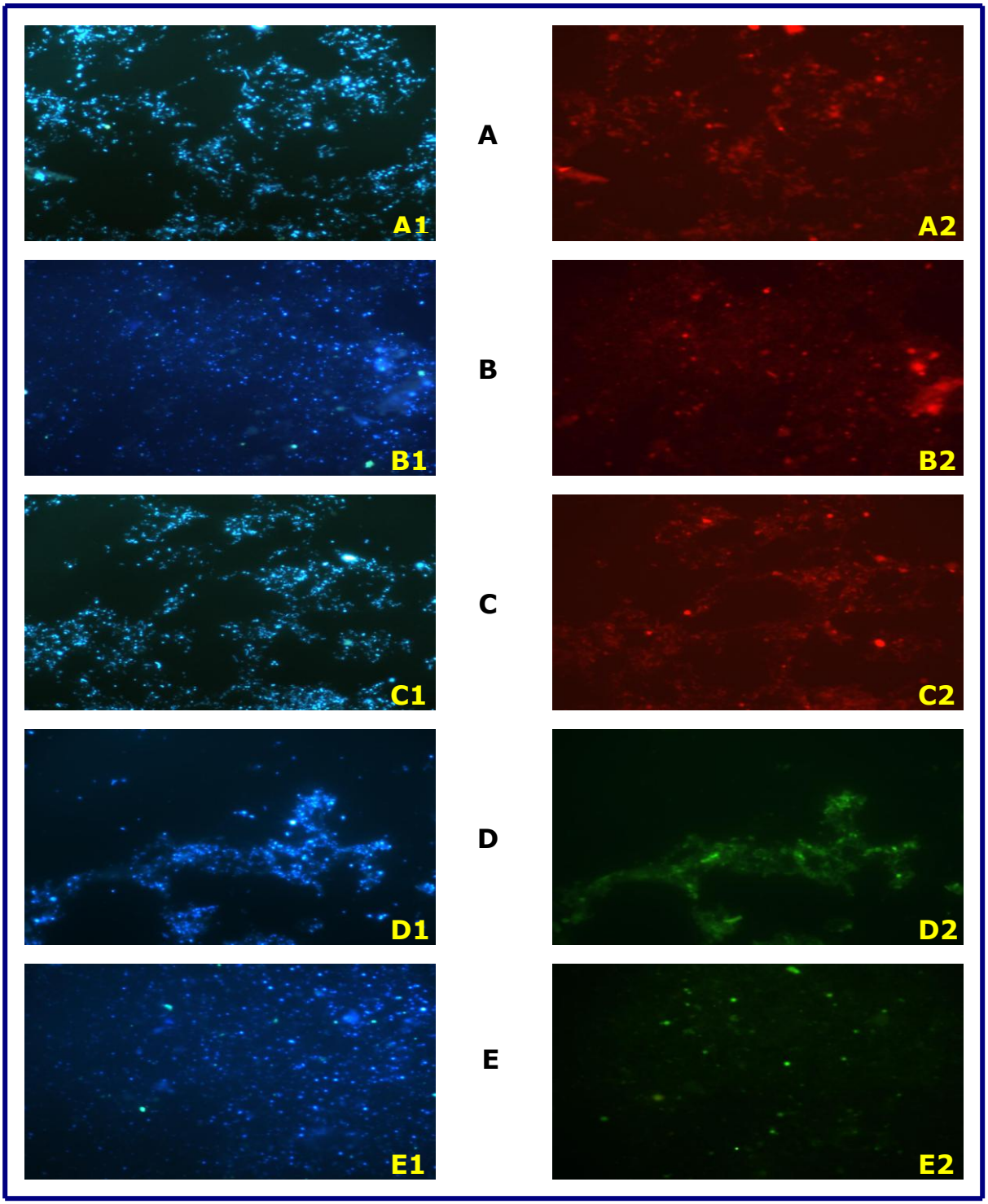

Figure 4.6: *In situ* hybridisation of the aerobic activated sludge samples. (A1 to E1-Left) DAPI stains (A2 to E2-Right) *in situ* hybridisations with probes. Micrographs are shown for identical microscopic fields. A:DAPI stain and corresponding hybridisation with rhodamine labelled EUB338I,II,III, B:DAPI stain and corresponding hybridisation with rhodamine labelled ALF1b, C:DAPI stain and corresponding hybridisation with rhodamine labelled BET42a, D:DAPI stain and corresponding hybridisation with fluorescein labelled GAM42a, E:DAPI stain and corresponding hybridisation with fluorescein labelled HGC69a probe.

4.4 DISCUSSION

Quantification of activated sludge using fluorescence *in situ* hybridisation (FISH) with rRNA targeted oligonucleotide probes provide novel insights with respect to the structure and dynamics of microbial communities (Daims *et al*., 2001). The steady state modified Ludzack-Ettinger process was analysed with group specific oligonucleotide probes such as the alpha, beta, and gamma subclasses of the *Proteobacteria* and bacteria with a high G+C DNA content. The oligonucleotide probes used were EUB 338 I, II, III, ALF1b, BET42a, GAM42a and HGC69a.

The total number of eubacterial cells were determined by hybridising the samples with EUB338 I, II and III. Daims *et al*., (1999) suggested that a more accurate quantification of members from the domain bacteria can be accomplished when incorporating the three probes (EUB 338 I, II and III) for hybridisation. It was shown that important bacterial phyla such as *Planctomycetales* and *Verrucomicrobia* were not hybridising to the probe EUB338 and this brought about the design of the probes EUB338 II and EUB338 III for *in situ* detection of the bacterial phyla that was not detected when using only EUB338 (Daims *et al*., 1999).

The beta and gamma probes were designed to target the 23S rRNA, however these probes only differed by one nucleotide at position 1033, relative to *E.coli*. The BET42a and GAM42a probes have the same sequence, except at position 1033 the BET42a probe has a thymine (T) nucleotide and GAM42a probe has the adenine (A) nucleotide, therefore it was common for GAM42a or BET42a to incorrectly bind to bacteria (Yeates *et al*., 2003). To overcome this problem unlabelled competitor probes were used to differentiate

the relative mismatches at position 1033. The unlabelled competitor probes used in this research were unlabelled BET42a whilst using a labelled GAM42a probe and an unlabelled GAM42a probe was used when hybridising the samples with the BET42a probe.

The microbial community was analysed using FISH within the anoxic and aerobic samples obtained from the MLE systems. The activated sludge samples were first analysed by DAPI staining in order to obtain the total cell counts and then *in situ* hybridisation for specific bacterial probe counts. For the anoxic samples and aerobic samples an average of 81.4% (±3.6%) and 79.2% (±4.2%) (Results shown in Appendix 9) respectively of eubacteria were detected with the bacterial probes EUB338 I, II, III. Figure 4.1 and 4.2 represents the eubacterial counts as a percentage of the total cell counts, these counts are depicted as EUB/DAPI ratios. High eubacterial counts achieved in the anoxic and aerobic zones (81.4% and 79.2% respectively) is, according to Wagner and Amann, (1997) an indication that the bacterial cells present in the activated sludge samples were bacteria that contained sufficient rRNA for detection and were able to be permeabilised for oligonucleotide probes by the fixation procedure used. These high EUB/DAPI ratios also indicate that the bacterial cells in the activated sludge are growing and are metabolically active (Wagner and Amann, 1997).

The samples obtained showed the dominance of the phylum *Proteobacteria* throughout the 18 wastewater batches, from the anoxic and aerobic zones which accounted for 82.3% (±1.3%) and 79.2% (±2.1%) respectively (Results shown in Appendix 9) of the *Proteobacteria* (alpha, beta and gamma) as a percentage of the eubacterial counts. These

findings were similar to that of Wagner *et al*., (1993) who found that 80% of *Proteobacteria* was present in domestic mixed liquor. Figure 4.3 and Figure 4.4 displays the *in situ* distribution among the alpha, beta, gamma (subclasses of *Proteobacteria*) and high G+C content bacteria present in the MLE anoxic and aerobic activated sludge samples.

Figure 4.3 illustrated the average total cell counts of the oligonucleotide probes that hybridised with the anoxic zone samples as a percentage of the total eubacterial cells throughout the 18 wastewater batches. The alpha and gamma subclasses of the *Proteobacteria* were dominant within the anoxic zone, the average percentage of the 18 batches were 33.35% (±1.04%) and 32.20% (±2.87) respectively of eubacteria. The less dominant bacteria found in the aerobic zone was the beta subclass of the *Proteobacteria* whilst the least dominant bacteria present was the high G+C content bacterial cells. According to Onki *et al*., (2000) the beta subclass of *Proteobacteria* was dominant in the aerobic-anaerobic activated sludge system of the enhanced biological phosphorus removal (EBPR), whereby both the nitrification and denitrification processes were prominent within the system. However the results obtained from this current research could have deferred from the results obtained by Onki *et al*., (2000) with reasons taken from Wagner *et al*., (1993) who stated that even though samples are running in similar systems the initial food/micro-organism [F/M] ratios changes the microbial population present, whereby if the sample comes from a system which has an initial low [F/M] ratio then the microbial community present in that sample compared to a sample which is from a system which contains an initial high [F/M] ratio will be different. Kaewpipat and

Grady, (2002) also states that there is a change that occurs between systems and this change is dependent on how much the initial operating conditions varied from each other.

The average total cells counts for the 18 wastewater batches for the *Proteobacteria* subclasses and high G+C content bacterial oligonucleotide probes which hybridised to the aerobic zone samples are displayed in Figure 4.4. Members of the beta subclass of *Proteobacteria* were detected as the most abundant bacteria present in the aerobic mixed liquor comprising of 32.8% (±2.96%) of the total eubacterial cells present as an average of the 18 batches from the aerobic zone. The alpha subclass occupied 25.8% (±0.69%) (average of the 18 batches), whilst the gamma subclass hybridised to 20.5% (±1.72%) (average of the 18 batches), of the cells hybridising with probe EUB 338 I, II, III. The least abundant high G+C content bacteria occupied 5.7% (±2.12%) (average of the 18 batches) of the total eubacterial cell counts as an average of the 18 batches.

According to Wagner *et al*., (1993) aerated activated sludge systems were analysed and the beta subclass of the *Proteobacteria* did predominate the wastewaters with 42% of the cells hybridising to the beta probe with respect to the eubacterial counts. Batch 1 (Figure 4.3) shows the highest dominance of the beta subclass in comparison to the other 17 batches with 40.2% (±2.5%) of cells hybridising with respect to the total eubacterial counts. Luxmy *et al*., (2000), found a dominance in the beta subclass of the *Proteobacteria* with 13.92%, the alpha subclass followed with 12.69%, thereafter the gamma subclass was represented with 7.77% and the high G+C content bacteria was the least dominant bacteria with 3.43% all with respect to the eubacterial counts, these bacteria were found in wastewaters of a membrane-separation bioreactor (MBR). A

similar trend is noticed in the aerobic zone of the MLE system from this current research with reference to the microbial population found in the MBR reactor.

The high G+C content bacteria present in both the anoxic and aerobic zones were the least abundant species present. However higher numbers were noted in the aerobic zone in comparison to the anoxic zone with the highest abundance present in batch 10 with 9.49% (±1.34%). Current findings supported previous research by Wagner and Amann, (1997) who have found only 9% of all bacteria present in activated sludge samples originating from a municipal sewage treatment plant were identified as gram positive high G+C content bacterium. However Wagner *et al*., (2002) found a high abundance of the high G+C content bacteria present in a laboratory scale enhanced biological phosphorus removal (EBPR) with 37% present. This is consistent with the importance of these bacteria for phosphorus removal (Wagner *et al*., 2002). However according to Lilley *et al*., (1997) phosphorus removal usually prevails within the anaerobic zone, and since the MLE system lacks an anaerobic zone, the phosphorus removal is low, hence the decreased numbers of the high G+C content bacterial family.

The sum of the group-specific probes were 86.3% (±4.2%) and 84.9% (±2.4%) (Refer to Appendix 9) for the anoxic and aerobic samples respectively, these counts were higher than the 81.4% (±3.6%) (anoxic zone) and 79.2% (±4.2%) (aerobic zone) (Refer to Appendix 9) which was obtained from the cells hybridising with the bacterial probe EUB338 I, II, III. According to Snaidr *et al*., (1997), this is due to an underestimation of the eubacterial cell counts as determined by the probes EUB338 I, II, III.

The steady state behaviour of the laboratory scale modified Ludzack-Ettinger system throughout the duration of the research was monitored via the analytical tests performed. Table 3.1 in chapter 3, shows the consistent results obtained for COD, TKN, nitrate and mixed liquor analyses. The nitrogen mass balance calculated for the MLE system rejected wastewater batches 2 and 7, with respect to the MLE model therefore theoretically the system performance for those two batches were unreliable. However no distinct changes occurred with the microbial community within the anoxic and aerobic zones during batches 2 and 7. According to Kaewpipat and Grady, (2002) even though COD removal from a reactor is good and the bioreactor is stable the microbial community structures are still dynamic. Changes in the microbial populations are apparent however some microbial communities do remain relatively stable (Kaewpipat and Grady, 2002). As shown in this current research the bacterial families studied remained consistent throughout the 18 wastewater batches for both the anoxic and aerobic zones. This can be a representation of the steady state of the MLE system.

Micrographs represented in Figures 4.5 (anoxic zone) and 4.6 (aerobic zone) visually indicates the relative number of bacteria that were hybridised with either fluorescein or rhodamine labelled oligonucleotide probes. The micrographs depict the cells stained with DAPI and the corresponding *in situ* hybridisation with the appropriate oligonucleotide probe.

4.5 CONCLUSION

FISH used as a tool to quantify bacterial numbers provides precise information for microbial community analysis of activated sludge samples and provides a measure which is independent from cultivation. Wagner and Amann, (1997) confirmed the under-estimation of bacterial numbers when using the cultivation method.

The research focuses on the anoxic and aerobic zones of the steady state MLE system which were analysed according to group specific probes of *Proteobacteria* and high G+C content bacterium which was designed according to conserved regions of *E.coli*. The results conclusively show a defined difference in bacterial consortia present in each of the two zones with the alpha and gamma subclasses dominant in the anoxic zone and the beta subclass was shown to dominate the aerobic zone throughout the 18 wastewater batches.

This evidently confirms the theory that bacteria present within the modified Ludzack-Ettinger system are unique in different zones and the diversity in bacterial consortia are due to the differences in the parameters guiding the aerobic and anoxic zone. The steady state behaviour of the system was validated in Chapter 3, whereby the results obtained for COD, TKN and nitrate analyses were consistent throughout the 18 batches. These consistent results may have influenced the microbial population within the MLE system and hence *in situ* analysis does to an extent validate the stable steady state of the MLE system.

CHAPTER 5

PROFILING THE MICROBIAL COMMUNITY OF THE MLE PROCESS USING DGGE

5.1 INTRODUCTION

Determining the diversity of micro-organisms in an environmental sample is important and the use of microscopy and cultivation of microbes has limited usefulness, since diverse micro-organisms can share similar morphologies and this limits the detection of these organisms that grow exclusively under conditions provided in the culture media (Ferris *et al*., 1996).

Denaturing gradient gel electrophoresis (DGGE) is a powerful tool used for the analyses of sequence diversity in complex natural microbial populations (Kowalchuk *et al*., 1997). Molecular biological techniques offer new opportunities for analysing the structure and species composition of microbial communities. DGGE provides an approach to directly determine the genetic diversity of complex microbial populations. The basis of the procedure relies on the electrophoresis of PCR-amplified 16S rDNA products in polyacrylamide gels, which contains a gradient of denaturant solutions (Muyzer *et al*., 1993).

Changes in melting behaviour of the DNA can be detected with the use of DGGE. DNA fragments that differ by one nucleotide base can be separated by electrophoresis in polyacrylamide gels. Polyacrylamide gels have different concentrations of denaturant solutions at different positions in the gel and as the DNA fragments migrate through the gel they become partially denatured causing an abrupt decrease in mobility. The

fragments melt and become partially melted when they reach specific points in the gel which has a certain concentration of denaturing solutions (Borresen *et al*., 1988).

The microbial community profiles within the anoxic and aerobic zones were determined using the denaturing gradient gel electrophoresis (DGGE). The DGGE pattern obtained analysed the microbial population shifts within each of the zones for the duration of the research.

5.2 METHODOLOGY

Samples were taken from the anoxic and aerobic zones of the laboratory-scale MLE activated sludge reactor for 18 wastewater batches. DGGE denaturation gradients were optimised to obtain profiles that provided the most resolution with respect to the banding pattern.

5.2.1 DNA Extraction:

Samples were taken from the anoxic and aerobic zones of the laboratory-scale MLE activated sludge reactor for the 18 wastewater batches. The phenol-chloroform extraction technique (Appendix 4) produced a high yield of DNA from the 0.2g activated sludge pellet samples (Mayer and Palmer, 1996 and Kuhn *et al*., 2002). The samples were incubated with lysis buffer and then exposed to a cycle of freezing and thawing the cells which was carried out five times. The removal of proteins and RNA contaminants was done by treating the samples with phenol-chloroform (25:24) and 98% chloroform. The

extracted DNA was precipitated with absolute isopropyl alcohol and quantification of the DNA was done using a spectrophotometer according to Maniatis *et al*., (1982).

5.2.2 Polymerase Chain Reaction (PCR):

The extracted DNA from the anoxic and aerobic samples was enzymatically amplified (Appendix 5) with universal primers, targeting the 16S rRNA. The primers used were 341f 5' CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG 3', which had a G-C clamp incorporated into the 5' end of the sequence and 1492r 5' TAC GGC TAC CTT GTT ACG ACT T 3'. Optimisation of the PCR reaction was done using the modified Taguchi method which was outlined by Cobb and Clarkson (1994).

5.2.3 Denaturing Gradient Gel Electrophoresis (DGGE):

Amplified DNA products obtained from the PCR reaction was loaded and run on a 7.5% parallel (wt/vol) acrylamide gel. The gradient was optimised with 30% and 50% denaturation solutions which contained 12ml and 20ml formamide respectively. The parallel DGGE gel was run for 2 hours at 200V at 60ºC. The gel was stained with ethidium bromide (10mg/ml) and viewed on a UV trans-illuminator at a wavelength of 302nm (Bio-Rad Laboratories, 1994) and photographed with a Polaride camera (Hoefer Pharmacia Biotech, USA) (Appendix 6).

5.3 RESULTS

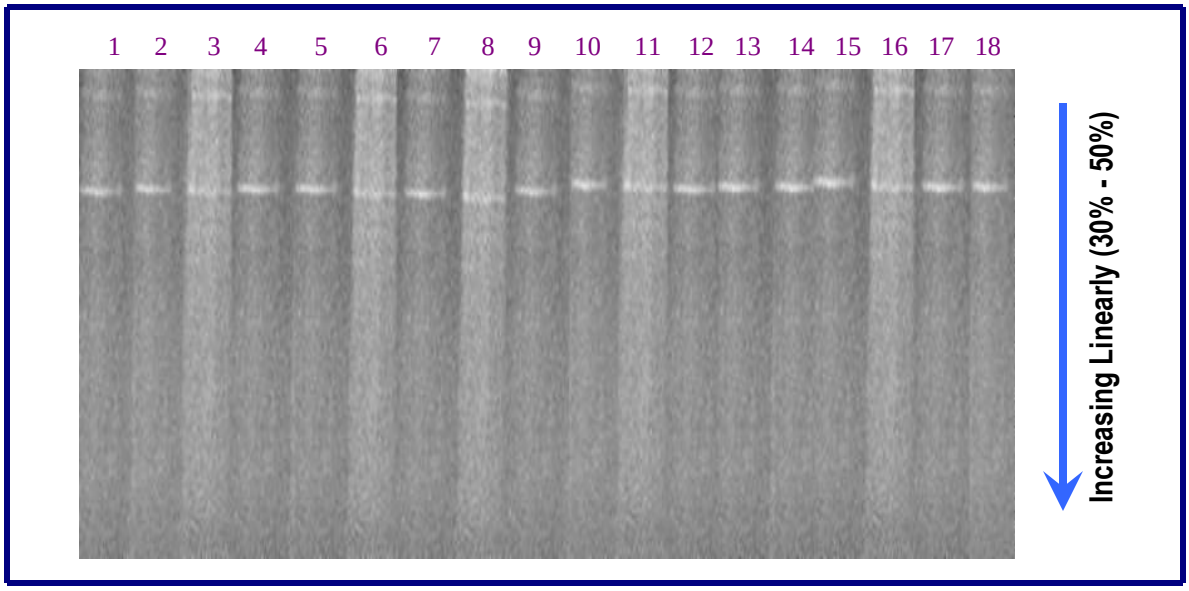

FIGURE 5.1: Image of an ethidium bromide stained DGGE profile of 18 PCR amplified DNA products obtained from the anoxic zone of the laboratory-scale MLE activated sludge process.

The denaturing gradient gel electrophoresis (DGGE) profile of the species present in the anoxic zone for the 18 wastewater batches is shown in Figure 5.1. The profile obtained indicates the presence of two bacterial species. The results show a relative consistency between each batch. There are two bands present; the first band indicates a less dominant species whilst the second species represents a more dominant species that is present throughout the 18 wastewater batches.

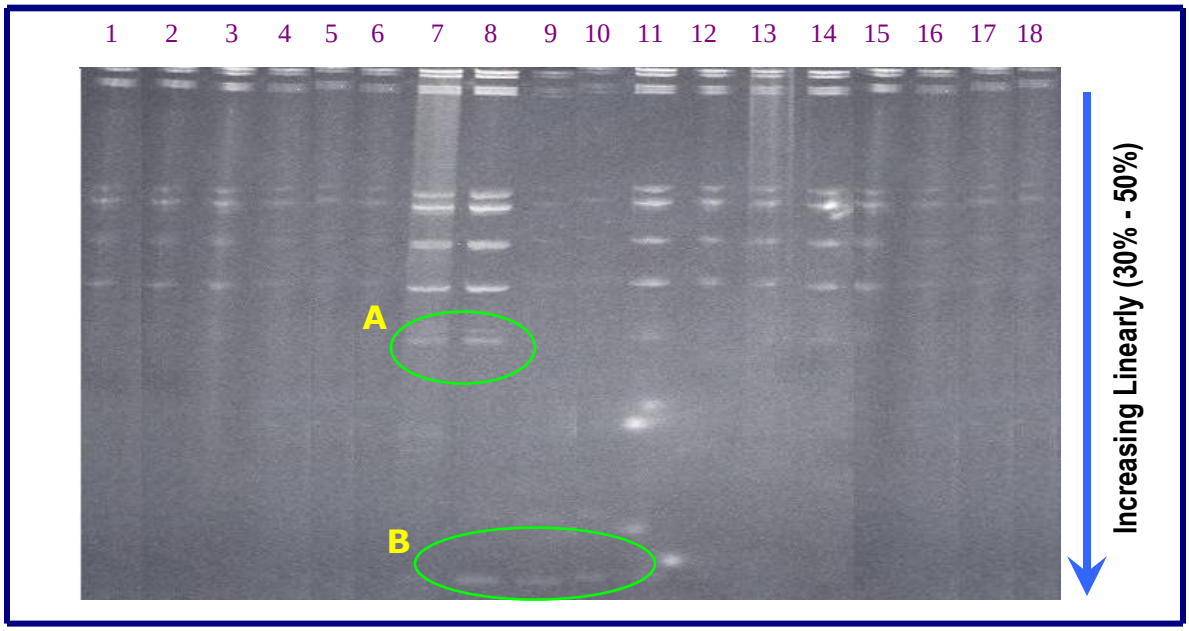

FIGURE 5.2: Image of an ethidium bromide stained DGGE profile of 18 PCR amplified DNA products obtained from the aerobic zone of the laboratory-scale MLE activated sludge process. (Refer to A and B below)

The denaturing gradient gel electrophoresis (DGGE) profile of the species present in the aerobic zone for the 18 wastewater batches is shown in Figure 5.2. The DGGE profile obtained shows the presence of multiple dominant bacterial species present throughout the 18 wastewater batches. Dominant bacterial species are shown in batches 7, 8, 11, 12, 13, 14. Less dominant species are present in batches 1, 2, 3, 4, 5, 6, 15, 16, 17, 18. Batches 9 and 10 have the least dominant bacterial species present. The additional less dominant species present in wastewater batches 7, 8, 9 and 10 is illustrated in the DGGE pattern as A and B.

5.4 DISCUSSION

Denaturing gradient gel electrophoresis (DGGE) performed on enzymatically amplified 16S DNA encoding rRNA is used widely as a molecular approach for analysing microbial communities (Teske *et al*., 1996). Denaturing gradient gel electrophoresis was developed for detecting single base changes and DNA polymorphisms, however it has become a valuable molecular tool for microbial ecology research (Vanhoutte *et al*., 2005). Universal primers incorporated in the PCR enables the DGGE profile to resolve a wide range of bacterial diversity (Teske *et al*., 1996). DGGE conditions were optimised by determining the most appropriate concentrations for the denaturation solutions in order to provide a high resolution banding pattern.

Muyzer *et al*., (1993) suggested that a 40 base pair G-C rich clamp is added to the 5' end of the forward primer involved in amplification of DNA and this provides a better resolution of the PCR amplified DNA fragments. The samples analysed were taken from the anoxic and aerobic zones of the steady state MLE system. Figure 5.1 and Figure 5.2 represents the DGGE banding pattern obtained from the anoxic and aerobic zones respectively. The banding pattern provides a profile of the bacterial species present. The intensity of the band represents the abundance and dominance of bacterial species present within the population (Muyzer *et al*., 1993).

After enzymatic amplifications of nucleic acids from the samples obtained from the anoxic and aerobic zones of the MLE system, the samples were analysed by DGGE. According to Figure 5.1 and Figure 5.2, the profiles obtained from each of the anoxic and

aerobic zones show a relative consistency in the banding pattern within each of the anoxic and aerobic zones. Figure 5.1 indicates the presence of two bacterial species which is present throughout the 18 batches from the anoxic zone. Two bands of different intensities are consistently noted throughout the 18 batches. According to Muyzer *et al*., (1993), the relative intensity of the bands represents the relative abundance of a particular species within the population. Therefore the second band shown in Figure 5.1 shows a higher intensity level, then the first band hence the second band represents the more dominant bacterial species present within the anoxic zone. It is noted that the banding positions varied between the 18 batches. The slight difference in the banding pattern is due to the presence of different species of bacteria therefore slight shifts are noted. As stated by Teske *et al*., (1996) small differences, usually ranging from 1 to 10 nucleotides, in the sequence of amplified 16S rRNA fragments are sufficient to separate PCR products.

Denitrification is the main process that occurs in the anoxic zone and nitrates are present whilst dissolved oxygen is absent (Muyima *et al*., 1997), this zone therefore promotes the growth of denitrifying bacteria. Therefore theoretically it is known that the anoxic zone supports growth of many micro-organisms, however only two bands were obtained from the anoxic zone of the laboratory scale MLE system, but the reason for this is explained by Teske *et al*., (1996), who states that possible overlapping of bands from different bacterial species cannot be excluded and therefore the DGGE profile obtained should be viewed as an approximate representation of the bacterial species and strain diversity. It might be possible to separate identically migrating sequences by using a smaller

denaturation gradient, however the gradient used in this research (30%-50%) was much smaller compared to denaturation gradients used by Muyzer *et al*., (1993) who used a gradient of 15%-55% and Teske *et al*., (1996) who used a gradient of 20%-70%. However according to Jackson *et al*., (2000) the use of smaller denaturation gradients is not advisable when analysing samples which may contain sequences spanning a broad range of melting temperatures.

The profile obtained from the DGGE pattern of the aerobic zone in Figure 5.2 shows the presence of multiple bacterial species present throughout the 18 wastewater batches. According to Kaewpipat and Grady, (2002) micro-organisms which have a low G+C content in their 16S rDNA will stop migrating at lower denaturation concentrations in the gel, therefore the bands closer to the wells represents bacteria with a low G+C content. Banding patterns show similarities between the 18 wastewater batches however according to Muyzer *et al*., (1993) bands that occur at identical positions may not necessarily be from the same bacterial species. Therefore the bands obtained from each of the 18 wastewater batches may represent totally different bacterial species. The profile obtained in Figure 5.2 shows a difference in dominating bacterial species between each of the 18 wastewater batches whereby bands emerge with high intensities in certain batches and then emerges with a lower intensity in other batches. According to Teske *et al*., (1996) the appearance and disappearance of a band from a DGGE profile reflects the increase and/or decrease of the corresponding bacterial species within a population, when a constant PCR amplification is carried out for all samples. Batches 7, 8, 9 and 10 indicates the presence of an additional bacterial species being present as illustrated on the

Figure 5.2 as A and B, however these low intensity bands indicate that the additional bacterial species are not dominant bacterial species or bacterial strains. The proliferating heterotrophic organisms present within the aerobic zone utilises the oxygen and perform nitrification and therefore theoretically the micro-organisms present in this reactor are organisms that are referred to as nitrifying bacteria (Muyima *et al*., 1997).

The DGGE banding pattern from Figure 5.1 and Figure 5.2 confirms the difference in the number of bacterial species present in each of the anoxic and aerobic reactors. The additional bands found in batches 7 to 10 of the aerobic zone is due to the conditions governing the reactor, these conditions could have been most favourable therefore allowing the additional bacterial species to proliferate.

The profiles obtained for the aerobic and anoxic zones show a degree of consistencies within each of the anoxic and aerobic zones. This according to Kaewpipat and Grady, (2002) can be a representation of the system performance. The results obtained in Chapter 3 confirm the steady state of the MLE system and there was a high degree of consistencies obtained from each of the batches with respect to the COD, TKN and nitrate analyses. This therefore could have impacted on the microbial community present within the MLE system.

5.5 CONCLUSION

PCR conditions and primers affect DGGE patterning therefore the PCR technique and conditions must be optimised to be able to produce a valid DGGE pattern. The DGGE banding pattern obtained for the anoxic and aerobic zones of the MLE system consists of a diverse range of bacteria present between each of the two zones. The microbial population present within the anoxic zones was fairly consistent throughout the research even when system performance of the reactor fluctuated however the microbial community present within the aerobic zone indicated the presence of a dynamic community but the population present in the aerobic zone also shared similarities between the 18 wastewater batches.

In conclusion this research established the difference in microbial communities present within the steady state MLE system This information is useful for the monitoring of the activated sludge processes because it was found that unique bacteria proliferates in each of the anoxic and aerobic zones even though there is a continuous established flow rate of the influent that enters the system to the effluent that leaves the system.

CHAPTER 6

GENERAL DISCUSSION

As mentioned previously the modified Ludzack-Ettinger process was basically developed for the biological denitrification of activated sludge, however the MLE process is not capable in performing total denitrification. Optimum nitrogen removal is obtainable with an MLE system when the anoxic zone is loaded to its denitrification potential with efficiency from the a-recycle and s-recycle (Lilley *et al*., 1997) as shown in the MLE configuration in Chapter 3, Figure 3.2. Chapter 3 of this research dealt with the MLE system performance throughout the research. The system performance of the parent laboratory scale anoxic and aerobic sludge system was analysed with respect to the steady state behaviour of the system and the COD and nitrogen mass balances.

The steady state of the MLE system was analysed with the analytical tests performed for COD, TKN, nitrates, VSS and MLSS analysis. Results obtained confirmed the steady state behaviour of the system throughout the 18 wastewater batches researched. Oxygen demand rates of 90.9% and high nitrogen utilisation rates of 89.1% confirmed the ability of the MLE system to perform efficient biological conversions. The nitrate results obtained confirmed a high denitrification potential of the system. COD and nitrogen mass balances calculated as a basis of the MLE model system performance confirmed the reliability of the system for all the wastewater batches except batches 2 and 7 which produced a high nitrogen mass balance confirmed an upset in the nitrogen levels within the system for the two batches.

Microbial community analyses were conducted within the anoxic and aerobic zones of the MLE system. The subclasses of the phylum *Proteobacteria* and the high G+C DNA content bacteria were analysed. FISH analysis was conducted according to Amann, (1995). The eubacterial counts were calculated as a percentage of the total cell counts and thereafter the bacterial probe counts were taken as a percentage of the eubacterial counts. The EUB/DAPI ratios for the anoxic and aerobic zones were 81.44% (±3.6%) and 79.21% (±4.2%) respectively. The EUB/DAPI ratios for the anoxic and aerobic zones were reasonable consistent throughout the 18 wastewater batches. These results are in accordance to the steady state results obtained for the 18 wastewater batches, whereby the results obtained for COD, TKN and nitrate analyses indicates consistencies throughout the research. The bacterial probe counts shown in Chapter 4, Figures 4.3 and 4.4, show a constant trend in bacterial proliferation throughout the 18 wastewater batches for the *Proteobacteria* and high G+C content bacterium within each of the anoxic and aerobic zones. Consistencies in the results were also in accordance to the results obtained from the steady state analyses. Mass balances calculated for nitrogen confirmed a low system performance during batches 2 and 7, however no distinct change in the bacterial community was displayed for both the anoxic and aerobic zones.

FISH allows the group specific microbial community to be determined (Onki *et al*., 2000) from the anoxic and aerobic zones of the MLE activated sludge system. However the *in situ* relationship provided by group specific micro-organisms are not enough to reveal the connection of the microbial community structure and the system performance (Onki *et al*., 2000) however PCR-DGGE helps to characterise the microbial population structure

at a bacterial species and bacterial strain level which is useful for monitoring the community changes over a duration of time. Chapter 5 dealt with the bacterial community DGGE profile obtained at a species level for both the anoxic and aerobic zones of the MLE system. The results obtained showed a definite difference in microbial community structure between each of the two zones. The profiles obtained from the anoxic and aerobic zone shows a good degree of consistency between each batch. DGGE analysis and *in situ* investigations both reflect consistencies in results obtained between each batch of the steady state MLE system. However only two bands were obtained from the anoxic zone and according to the FISH results there was a presence of the alpha, beta, gamma (subclasses of *Proteobacteria*) and high G+C content bacteria, and with reference to system performance denitrification rates were high within the anoxic zone. However according to Teske *et al*., (1996) possible overlapping of bands from different bacterial species cannot be excluded therefore the DGGE profile obtained from each zone must be interpreted as an approximate representation. The profile obtained from the aerobic zone showed a degree of similarity between the batches. Multiple bands were shown representing the different bacterial strains or species present. According to the system performance and the upset in the balance of the nitrogen concentration present in the second and seventh batches no distinct changes in the profiles obtained were evident.

The system was run for a duration of 18 wastewater batches and according to Kaewpipat and Grady, (2002) when analyses of a system is investigated sufficient samples should be analysed over time to give a representative picture of their dynamic character between

zones. Therefore the results obtained are a good representation of the system performance and microbial community present within the system.

CHAPTER 7

7.1 CONCLUSION

Micro-organisms are the primary producers that dominate the biogeochemical cycles on planet earth. These micro-organisms have a vast functional diversity and capability constituting a major genetic resource which is used in order to counteract environmental changes. Biological wastewater treatment processes rely on the interacting capabilities and the metabolisms of micro-organisms. All the organisms present in the treatment of wastewaters participate in various steps of the cyclic conversions (Muyima *et al*., 1997).

Conventional activated sludge processes were not developed for the biological removal of excess phosphates and therefore the process underwent various modifications to be able to treat wastewater more efficiently (Muyima *et al*., 1997). However wastewater treatment is also dependent on the design and operation of the activated sludge process. The engineering aspect related to the design of the process must take into consideration the microbial population present within the system. The efficient and reliable method used to analyse the microbial populations within the process involves novel molecular techniques. These techniques explore the genetic structure of the micro-organisms present and their abilities to perform important biological reactions in which nutrients are either removed or produced.

The molecular techniques used in this research which are fluorescent *in situ* hybridisation (FISH), the polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) gave an insight into the structure and composition of the *Proteobacteria* (Alpha, Beta, Gamma) and high G+C content bacterial populations present within the aerobic and

anoxic zone of a laboratory scale steady state modified Ludzack-Ettinger process. *In situ* application was performed on samples obtained from the aerobic and anoxic zones of the MLE process. The oligonucleotide probes targeted the subclasses of *Proteobacteria* and the high G+C content (actinomycetes branch) families which are the bacterial families present in wastewaters. The gram negative *Proteobacteria* subclasses consisted of the alpha, beta and gamma species of bacteria. The aerobic zone showed the dominance of the beta subclass whilst the anoxic zone was predominant with the alpha and gamma subclasses. The gram positive high G+C content actinomycetes were found in low numbers in both the aerobic and anoxic zones. The distinctive difference in the bacterial communities present in the aerobic and anoxic zone confirms the diversity of organisms that are present. Whole cell hybridisation with the major family level probes validates the results obtained from PCR-DGGE whereby the subclasses of the *Proteobacteria* and high G+C content bacteria with *in situ* investigation showed a difference in bacterial structure between each of the two zones as well as the DGGE profiles showed a distinct difference of bacterial species or strain diversity present between each of the two zones.

PCR-DGGE was used to provide a profile of the population present in the aerobic and anoxic zones and to establish the microbial community shifts that occurred within the MLE system during the 18 batches. The amplified DNA fragments were electrophoresised on a polyacrylamide gel. The difference in microbial populations found in the aerobic and anoxic zone was shown in the two profiles obtained from each of the zones. This information is vital for the design of the activated sludge process, whereby the zones should be treated as different compartments holding different bacterial

communities whilst there is a continuous flow rate of the influent that enters the process to the effluent that leaves the process which is established within the activated sludge treatment process.

System performance was analysed throughout the research with reference to the steady state behaviour of the MLE system and the COD and nitrogen mass balances. However it was noted that the stable steady state of the system impacted on the microbial community within each of the anoxic and aerobic zones, as shown by the *in situ* investigation which showed consistencies in results and the profiles obtained for each of the zones implied that microbial community structure at a species level was fairly consistent. The COD and nitrogen mass balances calculated showed the reliability of the system from an engineering perspective for the wastewater batches however the nitrogen mass balances showed an upset in the nitrogen balance for the system during batch 2 and batch 7. However there were no distinct changes in the microbial population during batches 2 and 7 when investigated *in situ* and with reference to the DGGE profiles obtained.

7.2 RECOMMENDATIONS

Suggestive methodologies that can be applied to this research area is the combination of FISH performed using micro-autoradiography, which involves the introduction of radioactive labelled substrates which cells take up and the developing procedure involves micro-autoradiography. For the microbial population analyses, the bacterial populations can be identified with genetic sequencing and correlations can be made between the microbial structure and the function of the microbial population in its niche. Information

generated from the microbial community analysis using molecular methods should aid in understanding and further optimising activated sludge processes by supporting engineering and process paradigms.

APPENDICES

APPENDIX 1

CHEMICAL OXYGEN DEMAND ANALYSIS (COD)

PROCEDURE: (Standard Methods, 1985)

- Samples are done in duplicate. Erlenmeyer flasks (250ml) are used.
- 0.04g $HgSO_4$ powder is placed into the flasks.
- Add eight to ten glass beads with a diameter of approximately 3mm to each flask.
- Two flasks are used as blanks, 10ml distilled water is added and two flasks are used for sample, 10ml sample is added.
- 5ml $K_2Cr_2O_7$ solution is added to all flasks.
- 15ml H_2SO_4 acid is carefully added to each flask, ensuring that no vapour escapes from the flask. This is done by tilting the flask whilst pouring the acid down the wall of the flask.
- Attach the flask to the condenser.
- Ensure the flasks are leveled onto the heating pad. Contents in the flask is boiled for two hours whilst ensuring that the water flow rate in the condensers is fast enough to condense any vapour arising from the condenser.
- Flasks are cooled to room temperature whilst still attached to the condensers.
- Approximately 80ml of distilled water is added through the top opening of the condensers into sample contents.
- 2 drops of ferroin indicator is added to sample mixture in the flasks when the flasks are removed from the condensers and heating pad and placed on bench top.
- The sample is titrated against Fe $(SO_4)_2$ (NH_4) until the end point is reached. Sample solution end point is reddish brown in colour.

LIST OF REAGENTS:

1. Mercuric Sulphate ($HgSO_4$) Powder

2. Standard Potassium Dichromate ($K_2Cr_2O_7$) Solution – 0.250N:

- ✓ (Pre-treat $K_2Cr_2O_7$ by drying at 103 ºC for three hours and cooled in dessicator) 12.259g $K_2Cr_2O_7$ (standard grade) make up to 1000ml in distilled water.

3. Sulphuric Acid (H_2SO_4) Reagent:

- ✓ 15g silver sulphate (Ag_2SO_4) in 2500ml concentrated (greater than 98%) sulphuric acid (H_2SO_4) – with magnetic stirrer, Dissolving takes one to two days.

4. Ferrion Indicator:

- ✓ 1.485g 1,10-phenanthroline monohydrate is dissolved together with 0.695g $FeSO_4.7H_2O$ in distilled water to 100ml.

5. Standard Ferrous Ammonia Sulphate [$Fe\ (SO_4)_2\ (NH_4)$], 0.05N:

- ✓ 100g $Fe\ (SO_4)_2\ (NH_4).6H_2O$ (analytical grade) dissolved in distilled water. Add 100ml concentrated sulphuric acid (H_2SO_4) make up to 5000ml in volumetric flask.
- ✓ Standardisation: Pipette 5ml standard potassium dichromate ($K_2Cr_2O_7$) solution into a Erlenmeyer flask and dilute with distilled water to 50ml. 15ml Sulphuric Acid (H_2SO_4) Reagent is added and allowed to cool before titrating with $Fe\ (SO_4)_2\ (NH_4)$ titrant using 2 drops ferroin indicator.
- ✓ Normality $Fe\ (SO_4)_2\ (NH_4) = \frac{\text{ml } K_2Cr_2O_7 \text{ x } 0.25}{\text{ml } Fe\ (SO_4)_2\ (NH_4)}$

CALCULATION:

COD (mg/L) = $\frac{(a-b) \times N \times 8000}{\text{ml Sample}}$

Where: a is the average ml Fe $(SO_4)_2$ (NH_4) used to titrate blank of same volume as sample, b is the average ml Fe $(SO_4)_2$ (NH_4) used to titrate sample, N is the normality of Fe $(SO_4)_2$ (NH_4).

APPENDIX 2

TOTAL KJELDAHL NITROGEN TEST (TKN)

PROCEDURE: (Standard Methods, 1985)

- Either 25ml influent or 50ml reactor or effluent sample is pipetted into 250ml Kjeldahl flask. The blank is prepared by adding distilled water in equal volume to that of the sample in a separate flask.
- 10ml digestion mixture is added with three to four glass beads, with a diameter of approximately 3mm to the sample and blank. The same procedure is carried out with the blank as it is for the sample.
- The sample is digested on a heating pad until the solution is clear thereafter heat for twenty minutes in addition.
- The Kjeldahl flask is allowed to cool for thirty minutes when placed on the micro-steam distillation apparatus.
- 50ml of H_3BO_3 solution is pipetted into an Elenmeyer flask (100ml), place on the steam distillation apparatus with the nozzle of the condenser immersed into the solution.
- 100ml distilled water is added to the contents of the Kjeldahl flask in order to dissolve the sediment.
- 10ml NaOH solution is added to the contents of the Kjeldahl flask through the top opening of the steam distiller apparatus.
- Immediately seal the apparatus by placing the seal line in position.
- Steam distillation occurs until the volume of the H_3BO_3 solution is approximately 100ml (Erlenmeyer flask)

- Titrate the sample mixture and blank in the Erlenmeyer flasks with standardised H_2SO_4 (0.02N).

LIST OF REAGENTS:

1. Mercuric Sulphate ($HgSO_4$) Solution:

- ✓ 40g red mercuric oxide HgO is dissolved in 250ml of 1:5 (H_2SO_4 : distilled water), which is 50ml : 250ml respectively. Make up to 1000ml with distilled water.

2. Digestion Mixture (Add Solution 1 and 2 = 5000ml digestion mixture)

- ✓ Solution 1: Sulphuric Acid (H_2SO_4), Mercuric Sulphate ($HgSO_4$), Potassium Sulphate (K_2SO_4) Solution:
  - 333.75g K_2SO_4 is dissolved in 1800ml distilled water, 500ml concentrated (greater than 98%) H_2SO_4 is added and 62.5ml $HgSO_4$ solution is added. Make up to 2500ml.
- ✓ Solution 2: 7N Sulphuric Acid (H_2SO_4):
  - 485ml concentrated (greater than 98%) H_2SO_4 is diluted in distilled water to 2500ml.

3. Mixed Indicator:

- ✓ Two volumes of 0.2% methyl red and 1 volume 0.2% methylene blue in 95% alcohol, (or 200mg in 100ml- methyl red and 100mg in 50ml methylene blue). Solution made up every thirty days.

4. Boric Acid (H_3BO_3):

- ✓ 100g H_3BO_3 is dissolved in distilled water. 100ml of mixed indicator or 40ml blue and 80ml red. Make up to 5000ml.

5. 0.02N Sulphuric Acid (H_2SO_4) Solution:

- ✓ 0.1N H_2SO_4 is prepared by diluting 2.8ml concentrated (greater than 98%) H_2SO_4 to 1000ml. 200ml of the 0.1N H_2SO_4 is diluted to 1000ml with distilled water producing a 0.02N solution.
- ✓ Standardisation:

6. Sodium Hydroxide (NaOH), Sodium Thiosulphate Solution ($Na_2S_2O_3.5H_2O$):

500g NaOH and 25g $Na_2S_2O_3.5H_2O$ is dissolved and made up to 1000ml. This is done in a plastic container (immerse the container in tap water) due to the reaction being extremely exothermic.

CALCULATION:

$$\textbf{TKN (mgN/L)} = \frac{(a-b) \times N \times 14000}{A}$$

Where: a is 0.001N H_2SO_4 for sample in ml, b is 0.001N H_2SO_4 for blank in ml, N is actual normality of 0.001N H_2SO_4 and A is sample in ml.

APPENDIX 3

MIXED LIQUOR SUSPENDED SOLIDS (MLSS) AND MIXED LIQUOR VOLATILE SUSPENDED SOLIDS (MLVSS)

PROCEDURE (MLSS): (Bitton, 1999)

- 50ml activated sludge was placed into a centrifugal tube.
- Centrifuge at 2500 rpm for 5minutes.
- Decant and discard supernatant and quantitatively scoop the sludge into a pre-weighed crucible.
- Crucible and contents are transferred to a drying oven at 105ºC and leave overnight. Remove from oven and cool in dessicator.
- The crucible is re-weighed.

CALCULATION:

$$\textbf{MLSS (g/L)} = \frac{\text{Mass of (Crucible + Sludge)} - \text{Mass of (Crucible) in grams} \times 10}{50\text{ml}}$$

PROCEDURE (MLVSS): (Bitton, 1999)

- The crucible (from MLSS) is then placed in a muffle furnance at 550ºC for 1 hour.
- The crucible is removed and cooled in a dessicator.
- The crucible is re-weighed.

CALCULATION:

$$\textbf{MLVSS (g/L)} = \frac{\text{Mass of (Crucible + Sludge)} - \text{Mass of (Crucible + Ash)} \times 10}{50\text{ml}}$$

APPENDIX 4

DNA EXTRACTION

PROCEDURE: (Mayer and Palmer, 1996 and Kuhn *et al.*, 2002)

- 1ml of raw sample was added to 1.5ml microtubes and centrifuged at 1400 rpm for 5 minutes at 4°C. Samples were done in duplicate.
- The supernatant is discarded and the pellet is resuspended in 500µl 1 x PBS and centrifuged at 5000rpm for 3 minutes.
- The pellet is resuspended in 75µl of Tris-EDTA buffer with 25µl of 10% SDS. Invert tubes 2 to 3 times to mix.
- Samples are incubated for 2 hrs at 65°C in a water-bath.
- 500µl lysis buffer is added to the sample after incubation. Vortex gently.
- The cells are then lysed by the freeze thaw method consisting of dry ice and ethanol slurry for 2 minutes followed by 65°C for 5 minutes. This cycle is repeated 5 times.
- 500µl of Tris-EDTA saturated Phenol was added to the sample and the sample was mixed by inversion.
- The sample is then centrifuged at 5700 rpm for 5 minutes.
- The top layer was removed and added to a clean microtube.
- An equal volume of phenol-chloroform (25:24) was added to the top layer which was removed. Sample was mixed by inversion and centrifuged at 5700 rpm for 5 minutes.
- The top layer was removed and added to a clean microtube and an equal volume of chloroform was added. Sample was mixed by inversion and centrifuged at 5700 rpm for 5 minutes.

- The top layer was removed and added to a clean microtube and an equal volume of 100% isopropyl alcohol was added and the sample was incubated at - 4ºC for 1 hour.
- The DNA was pelleted by centrifugation at 10500 rpm for 30 minutes.
- The pellet is then washed with 70% ethanol centrifuged at 4000 rpm for 5 minutes and then re-suspended in 30µl of sterile distilled water.

CONCENTRATION OF DNA: (Maniatis *et al.*, 1982)

- ✓ To quantify the amount of DNA- readings are taken at 260nm and 280nm.
- ✓ Reading at 260nm calculates the concentration of nucleic acids in the sample.
- ✓ An optical density of 1 corresponds to 50 ug/ml or double stranded DNA, 40 ug/ml for single stranded DNA and 20 ug/ml for oligonucleotides.
- ✓ The ratio between the readings 260nm and 280nm (OD_{260}/OD_{280}) provides an estimate for the purity of the DNA present.
- ✓ Pure preparations of DNA have (OD_{260}/OD_{280}) of 1.8 and 2.0 respectively.
- ✓ Contamination with protein or phenol causes the OD values to decrease significantly.

LIST OF REAGENTS:

1. Tris-EDTA Buffer:

- ✓ 10mM Tris-HCl is added with to 1mM EDTA, pH 8.

2. 10% SDS:

- ✓ 10g SDS is dissolved in 100ml sterile distilled water.

3. Lysis Buffer:

- ✓ 10% SDS is added to 0.1M NaCl and 0.5M Tris-HCl.

4. Equilibrium of Phenol (Tris-EDTA saturated buffer):

- ✓ Liquified phenol is stored at -20ºC therefore the phenol is melted at 68ºC in a water-bath.
- ✓ 50ml phenol is added to a conical flask and 0.1g of 8-Hydroxyquinoline is added.
- ✓ 50ml of Tris-EDTA buffer is added and this solution is stirred on a magnetic stirrer for 15 minutes then turn off the stirrer and allow the two phases present to separate. When the two phases have separated, the top layer is aspirated.
- ✓ The last step is repeated until the pH reaches the pH of the Tris-EDTA buffer.
- ✓ After equilibration of the phenol the final aqueous layer is removed and 100ul Tris-EDTA buffer containing 20ul beta-mercaptoethanol is added.

5. 1 x PBS.

6. Liquidified Phenol.

7. Chloroform

APPENDIX 5

POLYMERASE CHAIN REACTION

PROCEDURE: (Giovannoni, 1991)

- 0.2ml sterile eppendorf tubes are used. The reaction mixture is added in specific quantities to the eppendorf tubes.
- PCR takes place in a Hybaid Thermal Cycler.
- The cycle format used to amplify the template DNA:

TABLE A: Hybaid PCR Sprint Thermal Cycler Program (Hybaid Limited, UK).

STAGES		TEMPEATURE	TIME	CYCLES
STAGE 1	STEP 1	94°C	4 minutes	x 1
STAGE 1	STEP 1	94°C	1 minute	x 35
	STEP 2	53°C	1 minute	
	STEP 3	72°C	2 minutes	
STAGE 1	STEP 1	72°C	4 minutes	x 1
HOLD		4°C		

LIST OF REAGENTS:

1. 10µl of 10 x PCR Buffer without $MgCl_2$: 50mM KCl, 10mM Tris-HCl, pH 8.4.

2. 6µl of 2.5mM $MgCl_2$.

3. 10µl of 10mM dNTP:dATP, dCTP, dGTP, dTTP each at a concentration of 10mM.

4. 10µl of 10ng/µl Genomic DNA Template.

5. 2µl of 50pM Stock Solution of Primers.

6. 1µl of 5units/µl Taq Polymerase

APPENDIX 6

DENATURING GRADIENT GEL ELECTROPHORESIS

PROCEDURE: (Bio-Rad Laboratories, 1994)

- Buffer Tempreature: Preheat running buffer in chamber or tank to 60ºC.
- Assembling the Glass Plates: The acrylamide gel was made between two clean glass plates of different heights. The smaller plate is placed on top of the longer rectangular plate. Spacers are placed between the glass plates and the plates are clamped together. The sandwich clamps are then loosened and an alignment card is inserted. Once a good alignment and seal was achieved, the clamp was tightened.
- The high and low density gel solutions (150µl 10% Ammonium Pesulphate, 15ml Denaturing Solution and 100µl D-GENE dye to only the high density solution) are injected in between the two plates by using the delivery system (Model 475 Gradient Delivery System). A comb to produce wells was added and the gel was allowed to polymerise for 30-45 minutes.
- The samples were loaded into the washed wells (washed with 1 x TAE buffer). 8µl samples were mixed with 2µl D Gene dye solution and loaded into the wells using micropipettes.
- The DGGE apparatus (the sandwiched plates with the loaded gel) was placed in the pre-warmed tank. The voltage was set at 200V for 2 hours.
- After 2 hours the gel was carefully removed from the sandwiched plates and place in a container filled with 250ml of 1 x TAE buffer and 15µl of 10mg/ml ethidium bromide. Staining took 5 minutes and the gel was transferred to another container

which contained only the 250ml 1 x TAE allowing for de-staining (unbound ethidium bromide was washed away from the gel).

- The gel was viewed on a UV transilluminator and photographed with a Polaroid camera (Hoefer Pharmacia Biotech, USA).

LIST OF REAGENTS:

1. 50 x TAE Buffer:

- ✓ 242g of Tris-Base together with 57.1ml glacial acetic acid and 100ml 0.5M EDTA. pH 8. Make to 1000ml. Autoclave.

2. 1 x TAE Buffer:

- ✓ 20ml 50 x TAE buffer is added to 980ml sterile distilled water. Autoclave.

3. Running Buffer:

- ✓ 140ml of 50 x Tae buffer is added to 6860ml distilled water.

4. 40% Acrylamide/Bis-acrylamide:

- ✓ 38g acrylamide and 2g bis-acrylamide are made up to 100ml. Filter through a Whatman No 1.

5. 30% Denaturing Solution:

- ✓ This is made up with 18.8ml 40% Acrylamide/Bis-acrylamide, 2ml 50 x TAE, 12ml Formamide and 12.6g Urea. Make up to 100ml, and degas for 10 to 15 minutes.

6. 50% Denaturing Solution:

- ✓ This is made up with 18.8ml 40% Acrylamide/Bis-acrylamide, 2ml 50 x TAE, 20ml Formamide and 21g Urea. Make up to 100ml, and degas for 10 to 15 minutes.

7. 10% Ammonium Persulphate:

- ✓ 0.1g Ammonium Persulphate is added to 1ml distilled water.

8. D GENE Dye Solution:

- ✓ 0.05g Bromophenol Blue is added to 0.05g Xylene Cyanol and 10ml 1 x TAE buffer.

APPENDIX 7

EPIFLUORESCENCE MICROSCOPY - MEMBRANE FILTRATION

SAMPLE PREPARATION: (Amann, 1995)

- 4% Paraformaldehyde Fixation: 4ml sample was added to 12ml 4% paraformaldehyde (1:3 ratio) in a centrifugal tube and allowed to fixed at 4ºC for 1 to 3 hours and
- 4ml sample was added to 4ml absolute ethanol (1:1 ratio) in another centrifugal tube and allowed to fixed at 4ºC for 1 to 3 hours.
- For each of the centrifugal tubes the samples were centrifuged at 2500 rpm for 5 minutes. Pellet fixed cells and decant the fixatives from each of the centrifuge tubes.
- Wash the fixed cells in 1 x phosphate buffered saline (PBS) three times and re-suspend in 1 x PBS to 50% of the original sample volume (2ml).
- One volume of cold absolute ethanol (2ml) was added and mixed.
- Store at 4ºC

PROCEDURE (MEMBRANE FILTRATION): (Hicks *et al.*, 1992)

- Nucleopore filters (Millipore), with pore size of 0.22µm and 0.45µm are used.
- 0.22µm filter is pretreated by staining with 0.3% sudan black overnight.
- The stained filters are washed with distilled water, to wash-off excess sudan black solution that was not absorbed by the filter.
- A backing filter with a pore size of 0.45µm was placed on a rubber filter which was fitted to the mouth of a filtering conical flask.

- The backing filter was moistened with distilled water.
- The stained 0.22µm filter was then placed on top of the backing filter and a 15ml glass tower was clamped onto the conical flask.
- The sonicated sample, which was diluted to 1000µl, was filtered through the filter paper by using a vacuum pump.
- 1ml of 1.25µg/ml DAPI stain was added to the filter and allowed to filter through by gravitational forces for 5 minutes thereafter a slight vacuum was added to remove the unbound DAPI.
- The glass tower was covered with foil preventing the DAPI from absorbing light and becoming less effective in staining the cells.
- Excess stain that was not absorbed by the cells, were washed 7 times with distilled water.
- The 0.22µm filter was then mounted onto a microscope slide, which contained 1 drop of glycerol/PBS mixture.
- A drop of anti-fading mounting medium, vectashield was added to the surface of the mounted filter paper.
- A coverslip was placed making sure minimum airspaces were formed. The coverslip edges are then laminated onto the slide using clear nail polish.
- The slide is then ready to view using the Zeiss Axiolab microscope (50Watts high pressure mercury bulb and Zeiss filter set 01) fitted for epifluorescence microscopy.

LIST OF REAGENTS:

1. 4% Paraformaldehyde Fixative:

- ✓ 65ml sterile distilled water was heated to 60ºC
- ✓ 4g paraformaldehyde was added and a magnetic stirrer was introduced to the solution
- ✓ 2M sodium hydroxide (NaOH) was added with a Pasteur pipette.
- ✓ Stir until the solution has nearly clarified.
- ✓ Remove from the heat source and add 33ml of 3 x phosphate buffered saline (PBS).
- ✓ pH is adjusted to 7.2 with HCl (hydrochloride acid).
- ✓ The solution is filtered through a 0.45µm filter.
- ✓ Cool down and store in the refrigerator.

2. Phosphate Buffered Saline (PBS):

- ✓ 1 x PBS : 8g sodium chloride (NaCl) is added with 0.2g potassium chloride (KCl), 1.44g sodium hydrogen orthophosphate (Na_2HPO_4) and potassium dihydrogen orthophosphate (KH_2PO_4). Adjust pH to 7.4 and make up to 1000ml. (autoclave and refrigerate)
- ✓ 3 x PBS : 24g sodium chloride (NaCl) is added with 0.6g potassium chloride (KCl), 4.32g sodium hydrogen orthophosphate (Na_2HPO_4) and potassium dihydrogen orthophosphate (KH_2PO_4). Adjust pH to 7.4 and make up to 1000ml. (autoclave and refrigerate)

3. 0.3% Sudan Black:

- ✓ 0.3g sudan black is added to 100ml of 60% ethanol.

4. Glycerol/PBS Mixture:

- ✓ 1ml Glycerol is added to 1ml 1 x PBS.

5. 1.25 μg/ml DAPI

6. Absolute Ethanol.

5. Vectashield.

CALCULATION:

TCC (mg/l) = Mean TCC x Dilution Factor x Total number of Microscope fields

Where: Total number of Microscope fields for the DAPI filter used at 100X magnification is 17594.1.

APPENDIX 8

EPIFLUORESCENCE MICROSCOPY – WHOLE CELL HYBRIDISATION

IMMOBILISATION AND DEHYDRATION OF SAMPLE: (Amann, 1995)

- 20µl of fixed sonicated sample was diluted (dilution factor of 5) with 70µl of 1 x phosphate buffered saline.
- 10µl of this mixture was spotted onto pretreated slides with wells (treated with 1% poly-l-lysine solution for 5 minutes) and allowed to dry at 46ºC for 2 minutes.
- After the slides dried the spotted sample was dehydrated by successive rinses in 60% (v/v), 80% (v/v) and absolute ethanol for 2minutes each.
- Slides were stored in a dessicator until required for hybridization.

Whole Cell Hybridisation with Labeled Probes: (Amann, 1995)

- A strip of Whatman 3MM filter paper is soaked in hybridization buffer and placed in a centrifugal tube.
- The chamber is allowed to equilibrate for 15 minutes at 46ºC (hybridization temperature)
- 10µl of the appropriate probe and buffer mixture was added to each well on the slide.
- The slide was quickly transferred to the prewarmed moisture chamber and hybridise for 2 hours at 46ºC.
- Following hybridization the slide is rinsed with prewarmed 48ºC wash buffer.

- Immerse the washed slide in the wash buffer which is in a centrifugal tube and incubate at 48ºC for 20 minutes.
- The slide was then rinsed with 1 x PBS and air dried.
- 10µl 0.25µg/ml DAPI is added to each well and stained for 5 minutes in an area away from light.
- The slide is rinsed with 1 x PBS and air dried.
- A drop of mounting media (vetashield) is used placed in each well and a coverslip is placed on the slide.
- The edges of the coverslip are laminated with clear nail polish (preventing the escape of vetashield).
- The slide is then ready to view using the Zeiss Axiolab microscope (50Watts high pressure mercury bulb and Zeiss filter set 09 and 15) fitted for epifluorescence microscopy.

LIST OF REAGENTS:

1. Hybridisation buffer:

- ✓ 20% Hybridisation buffer: 20mM Tris-HCl, 5mM EDTA, 0.01% SDS and 20ml Formamide.
- ✓ 25% Hybridisation buffer: 20mM Tris-HCl, 5mM EDTA, 0.01% SDS and 25ml Formamide.
- ✓ 35% Hybridisation buffer: 20mM Tris-HCl, 5mM EDTA, 0.01% SDS and 35ml Formamide.

2. Wash buffer:

- ✓ 20% Hybridisation buffer: 20mM Tris-HCl, 5mM EDTA, 0.01% SDS and 0.19M NaCl.
- ✓ 25% Hybridisation buffer: 20mM Tris-HCl, 5mM EDTA, 0.01% SDS and 0.15M NaCl.
- ✓ 35% Hybridisation buffer: 20mM Tris-HCl, 5mM EDTA, 0.01% SDS and 0.08M NaCl

3. Probe and Hybridisation Buffer Mixture:

- ✓ Probe Dilution: The probe is diluted according to the synthesis report with sterile distilled water.
- ✓ 50ng probe is required per hybridisation. However 10µl spots are used therefore the concentration of probe required is 5ng/µl.
- ✓ Concentration of Probe = $\frac{\text{yield (µg)}}{\text{Dilution volume from the synthesis report}}$
  = ANSWER ug/µl x 1000 (to convert µg to ng)
  = ANSWER ng/µl
- ✓ The hybridisation buffer required to dilute the probe and produce a concentration of 5ng/µl from the stock solution of probes and then calculated with the equation $M_1V_1=M_2V_2$.

4. DAPI.

5. Vectashield.

CALCULATION:

$$\textbf{TCC for Probe} = \frac{n\ (Probe)}{n\ (DAPI)} \times n(MF)$$

Where: n (Probe) is the average number of cells with probe conferred fluorescence, n (DAPI) is the average number of cells with DAPI conferred fluorescence and n (MF) is the total cell count obtained from membrane filtration.

APPENDIX 9

TABLE B: Data obtained from FISH analysis for anoxic zone of the MLE system.

WW BATCHES	EUB/DAPI	ALPHA	BETA	GAMMA	HGC
1	84.05	39.67742 (0.26)	19.67742 (2.12)	24.16129 (1.27)	4.446866 (1.51)
2	85.21	32.47436 (1.2)	12.47436 (2.27)	40.30769 (4.28)	5.512821 (2.14)
3	83.24	35.28302 (3.12)	15.49057 (3.11)	39.15094 (2.84)	4.393531 (1.35)
4	77.05	36.46512 (2.4)	20.18605 (0.54)	32.13953 (3.24)	3.421927 (3.51)
5	81.05	29.48606 (1.61)	12.00398 (1.28)	36.30677 (3.77)	7.211155 (5.02)
6	88.36	20.32813 (1.1)	19.90625 (3.44)	42.40625 (2.89)	4 (3.58)
7	76.98	35.88889 (0.51)	10.75563 (0.37)	35.21003 (3.57)	5.353698 (3.67)
8	81.07	30.09562 (0.17)	12.54183 (2.51)	30.91633 (3.27)	4.302789 (3.47)
9	68.73	29.74713 (0.45)	19.5977 (2.69)	38.44828 (4.37)	4.568966 (2.17)
10	75.26	29.04301 (0.55)	15.70968 (3.1)	30.51254 (2.18)	3.902439 (3.51)
11	79.65	22.72727 (1.29)	15.55556 (3.45)	32.96296 (2.55)	4.037801 (4.12)
12	90.25	29.44044 (1.31)	20.79501 (4.1)	31.96676 (2.41)	2.459834 (3.2)
13	78.39	41.88031 (1.32)	15.83398 (2.87)	24.50965 (4.3)	2.915058 (3.4)
14	68.97	28.03989 (1.51)	19.18803 (2.18)	25.67806 (1.8)	3.190883 (4.27)
15	88.78	42.96912 (0.12)	20.5677 (3.17)	23.11639 (1.06)	2.541568 (4.5)
16	98.56	39.14006 (0.27)	19.97759 (2.15)	27.94678 (3.27)	2.877493 (3.7)
17	77.62	37.07143 (1.12)	19.71429 (2.35)	34.71429 (2.41)	2.889344 (3.24)
18	82.67	40.63946 (0.41)	12.24832 (4.02)	29.27517 (2.18)	4.765101 (2.14)
MEAN	81.44 (3.6)	33.35537 (1.04)	16.79022 (2.54)	32.20721 (2.87)	4.04396 (3.25)
PERCENTAGE OF *PROTEOBACTERIA*		82.3528 (1.3)			
PERCENTAGE OF TOTAL BACTERIA		86.39676 (4.2)			

TABLE C: Data obtained from FISH analysis for the aerobic zone of the MLE system.

WW BATCHES	EUB/DAPI	ALPHA	BETA	GAMMA	HGC
1	82.96 (1.24)	29.23706 (0.23)	40.24751 (2.21)	15.42234 (1.54)	6.870968 (1.72)
2	89.13 (4.25)	29.17219 (0.22)	36.09272 (4.13)	20.89404 (1.36)	5.827815 (2.12)
3	86.02 (7.21)	31.26685 (0.65)	32.84906 (0.68)	20 (0.89)	4.716981 (3.14)
4	83.17 (4.24)	28.21497 (1.1)	31.66987 (3.51)	22.55278 (2.45)	4.779271 (0.51)
5	71.04 (5.68)	20.86379 (1.22)	32.22259 (1.5)	15.68439 (2.13)	7.30897 (2.1)
6	72.00 (3.65)	30.88889 (0.52)	39.68254 (5.28)	17.46032 (1.64)	5.46875 (1.47)
7	76.43 (5.51)	22.1865 (0.44)	29.11897 (3.97)	14.88889 (1.28)	7.888889 (1.5)
8	80.37 (4.21)	21.00348 (1.4)	28.72125 (2.14)	21.46341 (3.14)	5.923345 (3.19)
9	87.27 (4.35)	29.58371 (0.26)	33.03167 (1.35)	26.47059 (2.54)	6.266968 (0.48)
10	78.12 (3.27)	25.40244 (0.64)	30.57317 (1.52)	25.10324 (2.97)	9.498208 (1.34)
11	74.19 (4.21)	26.42612 (0.62)	29.34708 (2.27)	17.45704 (2.46)	7.070707 (1.84)
12	80.09 (4.22)	27.58108 (1.25)	30.55405 (4.29)	25.13514 (0.57)	4.898649 (2.1)
13	82.45 (2.54)	20.42157 (0.11)	32.64706 (2.21)	17.09804 (0.61)	3.431373 (1.02)
14	83.33 (3.41)	21.40921 (0.35)	26.08696 (3.15)	20.89003 (2.42)	3.70844 (4.34)
15	81.37 (2.61)	25.53039 (0.25)	35.80479 (5.38)	19.59669 (0.54)	2.836096 (3.29)
16	64.19 (4.67)	24.80057 (0.47)	34.47293 (4.21)	22.47293 (1.64)	3.697479 (1.32)
17	84.19 (6.12)	30.37705 (1.22)	34.42623 (2.5)	29.42623 (1.24)	5.535714 (2.36)
18	69.40 (4.21)	20.05442 (1.47)	34.22819 (2.98)	18.04762 (1.54)	7.959184 (4.32)
MEAN	75.6 (4.2)	25.80113 (0.69)	32.87648 (2.96)	20.5591 (1.72)	5.760434 (2.12)
PERCENTAGE OF *PROTEOBACTERIA*		79.2367 (2.1)			
PERCENTAGE OF TOTAL BACTERIA		84.99713 (2.4)			

APPENDIX 10

TABLE D: Parent System Daily Results for the Anoxic/Aerobic Activated Sludge System

WW Batches	Date	TKN INF	TKN AER	TKN EFF	NO3 ANO	NO3 AER	NO3 EFF	Nbal %	COD INF	COD ML	COD EFF	OUR	CODbal %	VSS	COD ML	TKN ML
	03-Apr	35.0	230.0	4.5	0.0	1.2	7.5	**101.7**	554	4820	42	30.1	**111.5**	2380	4820	230
	04-Apr	33.0	220.0	2.8	0.2	4.2	8.5	**123.4**	566	4400	66	30.26	**102.9**	2380	4400	220
	05-Apr	40.0	238.0	6.2	0.5	4.2	10.8	**120.7**	505	3790	60	30.62	**109.0**	2036	3790	238
	06-Apr	45.0	288.0	5.6	1.2	5.4	8.2	**101.1**	552	3520	48	30.73	**91.6**	2360	3520	288
	07-Apr	33.0	221.0	6.2	3.2	7.1	6.3	**102.0**	428	3680	34	30.67	**114.2**	2530	3680	221
	08-Apr	39.0	230.0	3.4	0.4	5.6	6.5	**102.8**	480	3460	40	30.7	**100.2**	2544	3460	230
	09-Apr	44.0	246.0	8.4	0.0	3.9	6.7	**100.6**	426	3900	50	30.8	**126.3**	2344	3900	246
	10-Apr	50.0	216.0	8.9	0.0	3.4	8.9	**92.2**	510	3520	40	30.89	**101.6**	2464	3520	216
	11-Apr	39.0	230.0	4.0	0.0	1.2	8.9	**97.0**	490	3130	40	30.8	**106.3**	2196	3130	230
	12-Apr	30.0	227.0	3.4	0.0	2.3	6.3	**114.4**	515	2980	40	30.89	**95.3**	2290	2980	227
1	Mean	38.8	234.6	5.3	0.6	3.9	7.9	**105.6**	503	3720	46	30.65	**105.9**	2352.4	3720	234.6
	Std	6.3	20.7	2.1	1.0	1.9	1.5	10.3	48.8	553.2	10.1	0.263	**10.0**			
	13-Apr															
	14-Apr					NO RESULTS DUE TO MECHANICAL FAILURE OF THE SYSTEM										
	15-Apr															
	16-Apr															
	17-Apr															
	18-Apr															
	19-Apr	50.0	246.0	5.6	2.1	8.2	15.5	**118.3**	464	2430	44	28	**86.4**	1662	2430	246
	20-Apr	50.0	216.0	4.3	1.3	8.2	15.5	**118.6**	434	2740	72	27	**99.5**	1922	2740	216
	21-Apr	44.0	230.0	4.0	1.5	5.4	15.6	**121.6**	520	2890	52	27	**88.4**	1736	2890	230
	22-Apr	49.0	227.0	3.4	2.1	9.8	14.9	**118.5**	550	2850	60	27	**74.8**	2120	2850	227
2	Mean	48.3	229.8	4.3	1.8	7.9	15.4	**119.2**	492	2728	57	27.25	**87.3**	1860	2728	229.8
	Std	2.9	12.4	0.9	0.4	1.8	0.3	1.6	52.6	208.2	11.9	0.5				
	Date															
	23-Apr	45.0	240.0	4.0	1.2	5.2	8.5	**91.4**	515	3450	46	30.14	**96.5**	1686	3450	240
	24-Apr	49.0	230.0	5.0	0.2	6.0	10.1	**102.5**	490	3220	38	30.28	**94.8**	1814	3220	230
	25-Apr	38.0	240.0	4.5	1.5	6.5	7.9	**110.3**	498	2770	40	30.29	**87.2**	1860	2770	240
	26-Apr	42.0	229.0	4.0	2.1	5.6	10.5	**98.9**	512	3190	50	30.48	**96.6**	2224	3190	229
	27-Apr	40.0	230.0	4.5	1.2	5.4	9.8	**109.8**	505	3310	62	30.49	**100.6**	2314	3310	230
	28-Apr															
	29-Apr															
	30-Apr															
	01-May															
3	Mean	42.8	233.8	4.4	1.2	5.7	9.4	**102.6**	504	3188	47.2	30.34	**95.1**	1979.6	3188	233.8
	Std	4.3	5.7	0.4	0.7	0.5	1.1	7.9	10.2	254.6	9.55	0.148	**4.9**			
	02-May	33.6	223.0	2.2	0.2	5.3	5.3	**107.7**	476	2897	54	32.06	**98.9**	1876	2897	223
	03-May	31.0	215.0	2.1	1.3	3.6	5.5	**91.7**	408	2897	42	30.83	**117.0**	1820	2897	215
	04-May	33.6	205.0	1.1	0.2	4.5	6.7	**105.2**	514	2674	51	30.46	**88.1**	1698	2674	205
	05-May	29.1	225.0	1.7	0.0	2.5	5.0	**104.7**	538	2855	45	31.87	**91.1**	1992	2855	225
	06-May	33.6	218.0	2.8	0.3	3.2	6.1	**100.3**	512	2846	45	30.86	**93.1**	1962	2846	218
	07-May	30.3	185.0	2.8	0.1	2.3	6.2	**101.2**	464	2666	46	33.09	**107.1**	2108	2666	185
	08-May	32.0	190.0	1.7	0.0	2.6	7.2	**101.9**	562	2436	55	29.85	**82.0**	1688	2436	190

	09-May	31.0	210.0	1.1	0.1	0.1	7.5	**91.9**	505	2858	50	30.91	**103.8**	2100	2858	210
	10-May	31.0	178.0	1.1	0.2	1.5	9.6	**107.2**	498	2858	48	33.52	**107.3**	1710	2858	178
	11-May	26.9	192.0	0.6	0.2	2.6	6.9	**114.0**	525	2978	36	33.93	**97.8**	2024	2978	192
4		31.2	204.1	1.7	0.2	2.8	6.6	102.6	500	2797	47.2	31.74	**98.6**	1897.8	2797	204.1
		2.2	16.8	0.8	0.4	1.5	1.3	6.9	43	159.4	5.71	1.391				
	Date															
	12-May	34.0	220.0	4.7	2.4	4.6	5.5	**84.7**	482	2763	34	30.49	**94.0**	1858	2763	220
	13-May	34.7	112.0	5.6	0.5	3.6	10.5	**109.0**	485	2433	36	31.5	**94.4**	1810	2433	112
	14-May	31.4	109.2	3.4	0.4	3.4	8.6	**101.6**	517	2804	40	30.77	**91.8**	1990	2804	109.2
	15-May	31.4	120.4	6.7	0.0	1.3	6.8	**94.3**	511	2442	40	30.65	**91.9**	1658	2442	120.4
	16-May	36.0	189.0	5.0	0.0	2.1	5.7	**88.5**	519	3242	43	30.52	**97.7**	1984	3242	189
	17-May	32.5	109.2	3.4	0.0	3.5	9.0	**106.5**	490	2936	38	30.58	**97.1**	1716	2936	109.2
	18-May	28.0	117.6	4.5	0.0	3.7	7.3	**118.6**	500	2836	34	30.75	**92.2**	1689	2836	117.6
	19-May	28.0	120.4	5.0	0.0	1.1	9.7	**119.1**	480	2812	38	30.84	**104.7**	1906	2812	120.4
	20-May	36.0	100.8	2.8	0.0	1.3	8.7	**78.5**	448	2159	46	30.65	**103.3**	1471	2159	100.8
	21-May	30.2	100.8	3.4	0.0	2.6	9.0	**106.2**	475	2055	46	30.83	**93.2**	2086	2442	100.8
5		32.2	129.9	4.4	0.3	2.7	8.1	100.7	491	2648	39.5	30.76	**96.0**	1816.8	2687	129.9
		2.8	38.5	1.2	0.7	1.2	1.6	13.2	21.8	367.4	4.4	0.288				
	22-May	34.0	220.0	4.7	0.6	0.7	10.5	**110.5**	514	3666	46	30.65	**111.6**	2249	3666	220
	23-May	34.7	112.0	5.6	0.2	0.6	9.0	**86.8**	559	3010	48	35.38	**102.2**	1809	3010	112
	24-May	31.4	109.2	3.4	1.5	6.7	8.5	**109.3**	507	3142	36	32.56	**93.4**	2027	3142	109.2
	25-May	33.6	120.4	6.7	1.3	5.4	9.3	**112.4**	501	3107	41	31.32	**96.2**	1776	3107	120.4
	26-May	37.0	189.0	5.0	0.0	5.0	8.4	**116.6**	521	2875	46	31.02	**88.9**	1589	2875	189
	27-May	32.5	109.2	3.4	2.3	7.5	12.8	**126.4**	485	3165	60	32.06	**103.2**	1860	3165	109.2
	28-May	35.0	117.6	4.5	0.8	5.0	9.9	**108.1**	511	3130	51	30.83	**96.3**	1900	3130	117.6
	29-May	34.0	120.4	5.0	0.3	2.3	9.2	**98.9**	478	2382	52	30.46	**98.6**	1611	2382	120.4
	30-May	33.0	100.8	2.8	0.3	2.4	10.3	**98.9**	522	3173	45	31.87	**101.3**	1688	3173	100.8
	31-May	32.0	100.8	3.4	1.5	7.2	9.8	**116.5**	523	2640	58	30.86	**85.1**	2094	2640	100.8
6		33.7	129.9	4.4	0.9	4.3	9.8	108.4	512	3029	48.3	31.7	**97.7**	1860.3	3029	129.9
		1.6	38.5	1.2	0.7	2.5	1.2	10.6	22.5	344.5	7.29	1.459	**7.6**			
	01-Jun	34.0	214.0	1.7	3.5	8.8	7.6	**99.5**	516	2855	53	31.55	**86.7**	2031	2855	214
	02-Jun	42.0	220.0	2.4	0.5	3.5	8.2	**88.3**	488	2778	36	31.61	**97.3**	1973	2778	220
	03-Jun	33.6	218.0	2.8	0.3	9.1	21.0	**222.3**	514	2958	43	31.77	**89.2**	1992	2958	218
	04-Jun	30.3	185.0	2.8	0.1	6.5	7.6	**138.8**	522	3122	65	31.79	**92.5**	1962	3122	185
	05-Jun	33.3	210.0	3.9	1.4	8.5	7.3	**128.9**	565	3268	60	31.94	**83.5**	2056	3268	210
	06-Jun	33.0	211.0	2.8	1.6	6.5	15.7	**161.8**	553	2477	55	32.15	**84.9**	1840	2477	211
	07-Jun	33.0	211.0	2.8	1.6	9.5	14.5	**173.5**	526	2864	52	32.23	**85.7**	1747	2864	211
	08-Jun	31.0	215.0	0.6	0.2	17.0	16.1	**255.6**	485	2898	58	32.27	**74.3**	1798	2898	215
	09-Jun	29.0	210.0	1.7	1.3	8.7	13.9	**188.2**	480	3070	50	32.31	**97.6**	1860	3070	210
	10-Jun	33.2	198.0	4.2	1.4	5.6	13.0	**143.0**	485	2778	65	32.46	**103.7**	2224	2778	198
7		33.2	209.2	2.6	1.2	8.4	12.5	**160.0**	513	2907	53.7	32.01	**89.5**	1948.3	2907	209.2
		3.5	10.4	1.1	1.0	3.6	4.7		29.5	218.2	9.19	0.318	**8.5**			
	Date															
	11-Jun	35.8	205.0	3.5	0.0	3.4	8.6	**111.1**	520	2898	58	35.62	**103.5**	2045	2898	205
	12-Jun	37.0	308.0	2.4	0.0	3.5	9.3	**126.3**	567	3233	60	35.34	**98.6**	2100	3233	308
	13-Jun	47.0	210.0	5.4	0.0	4.4	10.8	**102.5**	507	2989	56	34.96	**104.5**	1900	2989	210
	14-Jun	37.0	143.0	16.0	0.0	3.2	6.5	**116.6**	517	2867	56	34.72	**101.1**	1982	2867	143
	15-Jun	44.0	241.0	2.5	2.3	7.2	8.5	**89.8**	500	2952	38	34.35	**95.8**	2089	2952	241
	16-Jun	35.0	210.0	7.4	0.2	5.8	6.3	**124.1**	479	2707	41	33.91	**95.9**	1956	2707	210
	17-Jun	56.0	188.0	4.2	0.3	10.5	11.5	**104.3**	547	3046	36	33.38	**78.2**	1773	3046	188

	18-Jun	58.0	234.0	5.2	2.1	11.5	14.7	**109.1**	517	2764	36	32.92	**80.2**	1804	2764	234
	19-Jun	58.0	333.0	8.4	3.1	8.6	11.5	**97.2**	512	2855	49	32.54	**90.5**	2367	2855	333
	20-Jun	56.0	242.0	6.2	0.2	7.5	13.1	**109.2**	498	3318	52	32.03	**98.1**	2271	3318	242
8	**Mean**	46.4	231.4	6.1	0.8	6.6	10.1	109.0	516	2963	48.2	33.98	**94.6**	2028.7	2963	231.4
	std	9.9	55.5	4.0	1.2	3.0	2.7	11.4	25	193.5	9.58	1.227	**9.1**			
	21-Jun	39.0	222.0	2.1	0.8	4.4	9.4	**102.4**	532	2858	48	28.69	**86.3**	1727	2858	222
	22-Jun	45.0	238.0	4.5	0.2	4.5	9.4	**101.7**	496	3854	58	27.45	**103.6**	2644	3854	238
	23-Jun	44.0	258.0	3.5	1.4	5.9	9.5	**101.0**	512	3797	34	29.02	**96.3**	2370	3797	258
	24-Jun	48.0	236.0	4.0	0.8	7.5	8.7	**99.0**	485	3375	52	24.15	**85.3**	2400	3375	236
	25-Jun	39.0	224.0	4.5	2.3	6.4	9.9	**106.5**	522	3835	43	29.01	**96.6**	2314	3835	224
	26-Jun	38.0	238.0	3.2	2.4	6.8	7.9	**99.0**	518	3986	41	26.52	**92.6**	2460	3986	238
	27-Jun	39.0	211.0	4.4	0.2	5.6	8.3	**113.5**	506	4004	39	24.56	**91.4**	2534	4004	211
	28-Jun	40.0	213.0	3.2	1.7	8.1	8.8	**108.6**	511	4211	45	24.56	**90.3**	2367	4211	213
	29-Jun	42.0	221.0	3.2	2.5	7.3	12.0	**107.2**	495	3158	56	26.29	**90.1**	2444	3158	221
	30-Jun	45.0	176.0	2.2	0.0	7.2	10.6	**107.5**	483	3450	52	26.35	**91.4**	2156	3450	176
9	**Mean**	41.9	223.7	3.5	1.2	6.4	9.4	104.6	506	3653	46.8	26.66	**92.4**	2341.6	3653	223.7
	Std	3.4	21.9	0.9	1.0	1.3	1.2	4.7	16.1	426.3	7.76	1.862	**5.4**			
	01-Jul	35.0	220.0	10.0	0.8	4.5	6.8	**121.0**	468	3224	48	34.89	**113.0**	2234	3224	220
	02-Jul	42.0	226.0	5.0	0.8	5.3	6.8	**95.1**	481	2942	38	34.91	**102.1**	1780	2942	226
	03-Jul	34.0	248.0	4.5	0.4	4.4	6.3	**115.1**	472	2942	38	34.88	**105.9**	1638	2942	248
	04-Jul	38.0	229.0	3.5	0.0	4.5	7.3	**107.8**	522	2886	38	34.74	**94.5**	2066	2886	229
	05-Jul	46.0	168.0	4.5	2.6	10.5	11.4	**104.7**	455	3271	32	34.58	**102.0**	2312	3271	168
	06-Jul	35.0	182.0	3.5	1.3	6.5	7.7	**108.3**	447	3111	38	34.42	**109.4**	2072	3111	182
	07-Jul	43.0	210.0	4.0	2.3	7.3	9.3	**94.8**	523	3083	36	34.29	**92.9**	2200	3083	210
	08-Jul	50.0	224.0	4.0	2.8	7.6	14.6	**100.5**	512	2970	48	36.57	**102.7**	1908	2970	224
	09-Jul	50.0	196.0	5.0	7.6	13.6	16.9	**94.7**	511	2294	58	36.97	**90.1**	2230	2294	196
	10-Jul	45.0	230.0	5.6	1.4	5.7	6.7	**86.3**	520	3713	41	37.2	**108.2**	2320	3713	230
10	**Mean**	41.8	213.3	5.0	2.0	7.0	9.4	102.8	491	3044	41.5	35.35	**102.1**	2076	3044	213.3
	Std	6.1	24.5	1.9	2.2	3.0	3.7	10.6	29.6	357.7	7.65	1.111	**7.5**			
	Date															
	11-Jul	53.0	230.0	4.5	2.1	5.2	14.0	**90.5**	518	3196	32	35.62	**104.1**	2245	3196	230
	12-Jul	48.0	220.0	2.8	2.9	8.7	12.7	**98.7**	526	2989	34	35.34	**91.9**	1584	2989	220
	13-Jul	64.0	238.0	7.3	1.2	7.9	14.1	**94.6**	489	3250	34	34.96	**102.0**	1892	3250	238
	14-Jul	68.0	288.0	5.6	0.8	8.0	13.1	**91.1**	475	2450	52	34.72	**96.1**	2254	2450	288
	15-Jul	45.0	221.0	6.2	1.2	7.5	10.3	**111.7**	521	3450	39	34.35	**97.0**	2102	3450	221
	16-Jul	47.0	230.0	3.4	1.2	6.9	10.3	**99.7**	523	3835	52	33.91	**104.1**	1680	3835	230
	17-Jul	44.0	246.0	4.4	1.6	8.4	12.4	**123.6**	547	3328	41	33.38	**89.3**	2412	3328	246
	18-Jul	50.0	216.0	8.9	2.5	7.7	11.4	**99.8**	533	3140	47	32.92	**91.8**	2028	3140	216
	19-Jul	39.0	162.0	4.0	3.7	8.2	10.4	**92.7**	505	2390	38	32.54	**85.0**	1980	2390	162
	20-Jul	45.0	227.0	3.4	2.3	10.5	13.5	**124.3**	480	3100	36	32.03	**91.3**	2060	3100	227
11	**Mean**	50.3	227.8	5.1	2.0	7.9	12.2	102.7	512	3113	40.5	33.98	**95.3**	2023.7	3113	227.8
	Std	9.1	31.0	1.9	0.9	1.3	1.5	12.8	23.7	432.7	7.4	1.227	**6.6**			
	21-Jul	48.0	210.0	4.5	3.5	10.8	12.3	**103.0**	490	3280	36	30.36	**88.9**	2250	3280	210
	22-Jul	39.0	244.0	4.0	1.7	5.5	9.5	**107.6**	502	3130	42	30.27	**95.0**	2024	3130	244
	23-Jul	52.0	238.0	4.5	4.5	9.5	14.2	**92.7**	490	2950	50	30.3	**93.1**	2312	2950	238
	24-Jul	52.0	221.0	5.6	2.8	9.5	11.7	**96.7**	438	2420	40	30.12	**89.7**	1934	2420	221
	25-Jul	42.0	217.0	4.2	1.2	8.3	8.4	**109.5**	494	3750	38	30.25	**95.3**	2246	3750	217
	26-Jul	48.0	213.0	5.0	3.2	10.3	11.2	**100.4**	480	3260	48	29.98	**92.3**	2264	3260	213
	27-Jul	54.0	218.0	4.5	3.5	11.5	13.5	**99.5**	475	3420	52	29.95	**94.8**	1386	3420	218

	28-Jul	52.0	224.0	4.5	3.2	10.7	14.2	**105.8**	480	2970	40	29.98	**87.7**	2194	2970	224
	29-Jul	54.0	198.0	4.0	1.3	9.2	11.8	**97.7**	445	3450	42	31.97	**105.6**	2400	3450	198
	30-Jul	52.0	225.0	4.5	2.2	9.5	13.0	**104.4**	450	3440	51	31.02	**105.5**	2506	3440	225
12	**Mean**	49.3	220.8	4.5	2.7	9.5	12.0	101.7	474	3207	43.9	30.42	**94.8**	2151.6	3207	220.8
	Std	5.1	13.3	0.5	1.1	1.7	1.9	5.3	22.3	366.5	5.82	0.626	**6.3**			
	Date															
	31-Jul	58.0	228.0	3.2	1.2	10.5	14.2	**106.2**	528	3490	44	29.63	**83.9**	2274	3490	228
	01-Aug	57.0	210.0	4.2	2.1	11.5	15.9	**110.9**	400	3250	44	29.76	**106.9**	3204	3250	210
	02-Aug	58.0	232.0	4.5	1.2	12.3	17.5	**126.2**	426	3590	38	29.53	**100.8**	2362	3590	232
	03-Aug	62.0	230.0	4.4	3.2	14.6	16.4	**109.0**	440	3100	50	29.68	**89.4**	2102	3100	230
	04-Aug	64.0	210.0	5.6	2.2	13.3	16.5	**107.6**	446	3790	34	29.56	**96.5**	2850	3790	210
	05-Aug	62.0	199.0	5.0	3.2	11.8	14.6	**91.8**	364	3970	36	29.63	**127.5**	2146	3970	199
	06-Aug	66.0	215.0	4.2	4.2	10.3	15.5	**78.4**	440	3830	32	29.66	**109.2**	2267	3830	215
	07-Aug	66.0	218.0	5.6	2.7	2.3	28.0	**101.4**	480	3810	34	29.91	**126.9**	2348	3810	218
	08-Aug	69.0	210.0	4.5	4.1	9.2	28.2	**108.1**	486	3800	44	32.1	**115.4**	2348	3800	210
	09-Aug	58.0	219.0	4.5	5.1	7.5	19.3	**87.0**	458	3150	44	26.21	**102.5**	2444	3150	219
13	**Mean**	62.0	217.1	4.6	2.9	10.3	18.6	102.7	447	3578	40	29.57	**105.9**	2434.5	3578	217.1
	Std	4.2	10.5	0.7	1.3	3.5	5.2	13.7	45.9	314.9	5.96	1.409	**14.5**			
	Date															
	10-Aug	55.0	221.0	5.0	3.2	10.2	15.2	**102.2**	406	3270	46	33.35	**119.4**	2134	3270	221
	11-Aug	54.0	196.0	4.0	1.2	11.2	13.4	**111.5**	505	3300	38	32.15	**86.6**	2202	3300	196
	12-Aug	55.0	210.0	4.5	3.2	10.2	15.5	**101.2**	485	3240	38	29.84	**91.7**	2276	3240	210
	13-Aug	45.0	220.0	4.0	1.2	8.5	9.2	**106.8**	485	3315	36	29.15	**88.8**	2200	3315	220
	14-Aug	40.0	132.0	4.5	1.2	7.1	11.3	**110.4**	500	2780	38	30.28	**86.6**	2118	2780	132
	15-Aug	45.0	232.0	5.2	2.2	8.2	11.6	**110.9**	500	3230	52	30.25	**93.6**	1992	3230	232
	16-Aug	46.0	223.0	10.0	0.3	5.2	7.9	**104.3**	495	2870	48	32.15	**95.6**	2100	2870	223
	17-Aug	37.0	215.0	7.3	3.0	1.6	13.6	**102.4**	522	3090	44	30.16	**104.4**	1842	3090	215
	18-Aug	38.0	143.0	8.0	1.2	3.9	12.1	**113.4**	518	2890	46	32.4	**98.3**	1898	2890	143
	19-Aug	39.0	210.0	4.2	6.8	9.1	13.5	**89.7**	507	3465	45	33.5	**104.7**	2120	3465	210
14	**Mean**	45.4	200.2	5.7	2.3	7.5	12.3	105.3	492	3145	43.1	31.32	**97.0**	2088.2	3145	200.2
	Std	6.7	32.7	1.9	1.7	2.9	2.3	6.6	32.7	227.1	5.3	1.56	**10.2**			
	20-Aug	35.8	205.0	3.5	6.1	5.6	15.0	**90.2**	529	3024	47	33.05	**103.1**	1930	3024	205
	21-Aug	37.0	308.0	2.4	2.2	5.2	8.5	**108.0**	491	2664	31	33.25	**95.5**	2100	2664	308
	22-Aug	47.0	210.0	5.4	0.0	2.9	8.0	**84.4**	457	3042	36	33.31	**111.6**	2268	3042	210
	23-Aug	37.0	143.0	16.0	0.0	1.1	7.5	**109.8**	443	2106	36	33.48	**107.1**	1590	2106	143
	24-Aug	44.0	241.0	2.5	0.0	6.7	5.6	**95.8**	454	2268	36	33.5	**90.1**	1684	2268	241
	25-Aug	35.0	210.0	7.4	0.4	5.3	6.3	**118.7**	517	3186	29	33.66	**93.6**	2308	3186	210
	26-Aug	42.0	188.0	4.2	0.8	4.5	9.0	**93.7**	504	2448	32	33.69	**91.4**	2148	2448	188
	27-Aug	58.0	234.0	5.2	0.2	7.8	9.5	**92.0**	473	3132	38	33.76	**98.4**	1980	3132	234
	28-Aug	58.0	333.0	8.4	0.2	8.9	8.2	**106.8**	515	3472	38	33.79	**91.3**	1522	3472	333
	29-Aug	56.0	242.0	6.2	2.8	10.1	8.5	**84.4**	509	3187	47	33.97	**91.3**	1806	3187	242
15		45.0	231.4	6.1	1.3	5.8	8.6	**98.4**	489	2853	37	33.55	**97.3**	1933.6	2853	231.4
		8.9	52.6	3.8	1.9	2.6	2.4	**11.7**	30.4	453.2	6.06	0.282	**7.5**			
	Date															
	30-Aug	40.0	220.0	4.0	2.3	6.5	10.2	**103.6**	482	3150	41	32.37	**101.7**	2238	3150	220
	31-Aug	46.0	210.0	3.2	1.2	6.0	13.5	**108.4**	463	2898	43	30.37	**100.7**	2056	2898	210
	01-Sep	38.0	260.0	4.5	2.8	2.9	11.0	**96.0**	509	2925	41	29.82	**99.0**	2022	2925	260
	02-Sep	36.0	229.0	4.0	2.7	7.2	8.4	**106.8**	463	3096	32	29.92	**96.1**	1952	3096	229
	03-Sep	40.0	276.0	4.5	0.5	4.1	7.1	**104.2**	522	3258	45	30.6	**94.5**	2052	3258	276
	04-Sep	45.0	220.0	5.3	1.2	7.3	10.3	**108.5**	500	3250	45	30.6	**93.3**	2234	3250	220
	05-Sep	38.0	237.0	5.0	1.2	4.8	8.5	**108.1**	486	3222	38	29.92	**98.5**	2114	3222	237

	06-Sep	36.0	210.0	4.2	0.6	5.5	7.2	**111.0**	472	2340	36	29.92	**85.8**	1402	2340	210
	07-Sep	35.0	198.0	5.3	1.2	5.3	6.9	**105.3**	454	2619	36	29.92	**94.3**	1862	2619	198
	08-Sep	39.0	205.0	3.5	0.8	7.2	8.2	**111.7**	443	2736	45	29.92	**95.1**	1918	2736	205
16	**Mean**	39.3	226.5	4.4	1.5	5.7	9.1	106.4	479	2949	40.2	30.34	**95.9**	1985	2949	226.5
	Std	3.7	24.9	0.7	0.8	1.4	2.1	4.5	25.2	307.5	4.54	0.775	**4.6**			
	09-Sep	33.1	214.0	1.7	0.5	5.2	6.4	**109.0**	369	2241	41	29.87	**109.4**	1538	2241	214
	10-Sep	35.1	220.0	1.9	2.9	6.4	6.0	**81.9**	434	2574	40	29.92	**97.7**	1900	2574	220
	11-Sep	33.6	218.0	2.8	1.2	2.9	7.2	**93.5**	423	2367	40	30	**105.8**	1552	2367	218
	12-Sep	30.3	185.0	2.8	0.8	4.2	6.3	**104.9**	409	2268	41	30	**103.0**	1636	2268	185
	13-Sep	33.3	210.0	3.9	1.2	6.1	6.5	**111.8**	409	2691	49	31	**108.4**	1814	2691	210
	14-Sep	33.0	211.0	2.8	1.2	6.3	10.3	**132.6**	457	2620	40	29.92	**94.0**	1926	2620	211
	15-Sep	33.0	211.0	2.8	1.6	7.2	9.2	**127.5**	360	2750	27	29.92	**114.8**	1744	2750	211
	16-Sep	31.0	215.0	0.6	2.5	6.5	9.1	**113.1**	482	2430	34	29.92	**85.9**	1736	2430	215
	17-Sep	29.0	210.0	1.7	3.7	6.6	7.5	**96.6**	484	2088	49	29.92	**84.7**	1662	2088	210
	18-Sep	33.2	198.0	4.2	2.3	7.2	9.3	**120.2**	493	2400	42	29.92	**83.4**	1576	2400	198
17		32.5	209.2	2.5	1.8	5.9	7.8	**109.1**	432	2443	40.3	30.04	**98.7**	1708.4	2443	209.2
		1.8	10.4	1.1	1.0	1.4	1.5	15.6	44.5	202.4	6.1	0.323	**10.7**			
	Date															
	19-Sep	35.0	178.0	5.0	1.7	4.3	9.8	**105.0**	511	2300	39	29.87	**85.2**	2742	2300	178
	20-Sep	36.0	115.0	8.0	1.2	5.4	8.6	**104.7**	450	2583	43	29.92	**96.9**	1850	2583	115
	21-Sep	34.0	115.0	8.0	4.1	5.1	10.5	**85.9**	427	1845	43	29.92	**98.1**	1164	1845	115
	22-Sep	38.0	218.0	4.0	0.4	5.4	7.8	**110.5**	374	1987	30	29.92	**101.3**	1350	1987	218
	23-Sep	36.0	118.0	8.0	2.3	8.8	7.4	**105.9**	400	2200	52	29.92	**95.5**	1500	2200	118
	24-Sep	34.0	115.0	8.0	2.4	7.5	7.4	**102.6**	382	2178	58	30	**105.7**	1660	2178	115
	25-Sep	35.0	118.0	5.0	2.5	9.9	5.2	**92.9**	409	2313	38	29.92	**87.2**	1814	2313	118
	26-Sep	36.0	118.0	8.0	2.3	7.5	8.5	**104.5**	436	2358	58	31.2	**98.3**	1634	2358	118
	27-Sep	35.0	145.0	4.5	0.3	7.2	9.5	**129.3**	392	2450	34	29.92	**100.2**	1664	2450	145
	28-Sep	35.0	115.0	5.0	0.9	5.1	7.3	**93.7**	419	2331	45	29.92	**100.3**	1216	2331	115
18		35.4	135.5	6.4	1.8	6.6	8.2	**103.5**	420	2255	44	30.05	**96.9**	1659.4	2255	135.5
		1.2	35.4	1.8	1.1	1.8	1.5	11.7	39.9	215.1	9.52	0.405	**6.3**			

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