OPTIMISATION OF FOOD TO MICROORGANISM RATIOS DURING ACTIVATED SLUDGE RESPIROMETRIC BATCH ASSAYS

ARSHAD ABDOOL HAK ISMAIL

2003

OPTIMISATION OF FOOD TO MICROOGANISM RATIOS DURING ACTIVATED SLUDGE RESPIROMETRIC BATCH ASSAYS

ARSHAD ABDOOL HAK ISMAIL

Dissertation submitted in compliance with the requirements of the Master's Degree in Technology in the Department of Biotechnology, Durban Institute of Technology

Approved for final submission

7-05-03

Supervisor:
Dr. Faizal Bux
(D. Tech: Biotechnology, D.I.T.)

Date

2003

DECLARATION

I hereby declare that the dissertation is my own 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.

A.A.H. Ismail

APRIL, 2003

DEDICATION

To my Parents... for their constant support and encouragement

ABSTRACT

The measured kinetics of a bacterial culture degrading a single organic compound as a sole carbon source in a batch reactor depends on the history of the culture, the identifiability of the parameters, and the manner in which the experiment to measure them is run. The initial substrate to biomass ratio (So/Xo) used in the experiment is particularly important because it influences both parameter identifiability and the expression of the culture history.

In this study five batch tests were run at So/Xo ratios of 0.8, 1.5, 4, 8 and 11. Mixed liquor harvested for each batch test was drawn from a well-defined parent anoxic/aerobic activated sludge system. Contemporary molecular techniques were applied to investigate the microbial community profile of batch test at defined So/Xo ratios and how the microbial community structure is influenced by these ratios.

Denaturing gradient gel electrophoresis (DGGE) analysis of PCR-amplified 16S rDNA gene segments was used to examine microbial population shifts in respirometric batch test at defined So/Xo ratios. Biomass growth and substrate removal curves for batch test with So/Xo ratios of 0.8 and 1.5 were typical for a system without cell multiplication. This was confirmed by the consistencies of two predominant common DGGE bands evident throughout the duration of the batch tests, suggesting no shift in microbial community structure away from its original treatment environment. At So/Xo ratios of 4, 8 and 11 biomass growth and substrate removal curves were typical for a system with cell multiplication. The DGGE banding patterns evident at these ratios were suggestive of a large qualitative shift in microbial community structure, this was indicative by the absence of predominant DGGE bands (one or two bands) present in its original microbial culture.

PREFACE

Aspects of the work covered in this dissertation can be found in the following publication.

Ismail A.A.H (2003) Denaturing Gradient Gel Electrophoresis of Microbial Community in Activated Sludge Respirometric Batch Assays at Defined So/Xo Ratios. *Water Research* (Submitted)

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude and appreciation to the following people for their assistance in the completion of this dissertation.

- Mr. Faizal Bux for giving me the opportunity, supporting my ideas and understanding my financial position.

- Mr. Michael de Villiers and Miss Francisca Holden for their interest, valuable insight and assistance during the course of my work.

- My colleagues at the Centre for Water and Wastewater Research: Adrian Degenaar, Serusha Inarman and Pamela Padayachee for their constant involvement, technical assistance and understanding.

- My siblings: Riyad, Surfaraz and Isma for their love, support, encouragement and patience through the years.

TABLE OF CONTENTS

CHAPTER 2

Literature Review

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

a	=	mixed liquor recycle ratio from aerobic to the anoxic reactor
A	=	adenine
ADP	=	adenosine diphosphate
ATP	=	adenosine triphosphate
aq	=	aqueous
BNR	=	biological nutrient removal
BOD	=	biological oxygen demand
bp	=	base pair
C	=	cytosine
CDGE	=	constant denaturing gradient gel electrophoresis
CO_2	=	carbon dioxide
CoA	=	coenzyme A
COD	=	chemical oxygen demand
DGGE	=	denaturing gradient gel electrophoresis
DNA	=	deoxyribonucleic acid
rDNA	=	ribosomal DNA
DO	=	dissolved oxygen
ETP	=	electron transport pathway
f_n	=	nitrogen fraction of the MLVSS (0.1mgN/mgVSS)
f_{cv}	=	COD to VSS ratio of volatile sludge mass (1.48mgCOD/mgVSS)
FAD	=	flavin adenine dinucleotide – oxidized
$FADH_2$	=	flavin adenine dinucleotide – reduced
F/M	=	food to microorganism ratio
FMN	=	flavin mononucleotide – oxidized
$FMNH_2$	=	flavin mononucleotide – reduced
fp	=	flavoprotein
g	=	gaseous
G	=	guanine
GTP	=	guanosine triphosphate

MLE	=	modified Ludzack-Ettinger
MLOSS	=	mixed liquor organic 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 the influent
N_{te}	=	TKN in the effluent
N_s	=	nitrogen incorporated in VSS per day
N_w	=	TKN in waste sludge
NAD^+	=	nicotinamide adenine dinucleotide – oxidized
NADH	=	nicotinamide adenine dinucleotide – reduced
NaR	=	nitrate reductase
NH_3	=	ammonia
NiR	=	nitrite reductase
NOR	=	nitric oxide reductase
N_2OR	=	nitrous oxide reductase
NO	=	nitric oxide
NO_2^-	=	nitrite
NO_3^-	=	nitrate
N_2	=	dinitrogen
N_2O	=	nitrous oxide
O_2	=	oxygen
O_c	=	oxygen required for carbonaceous degradation
O_d	=	oxygen recovered via denitrification
O_n	=	oxygen used for nitrification
O_{tm}	=	measured oxygen per day
OUR	=	oxygen utilization rate
P	=	phosphorous
PCR	=	polymerase chain reaction

PSS	=	protein synthesizing system
Q	=	ubiquinone
QH_2	=	ubiquinol
Qi	=	influent flow rate
RNA	=	ribonucleic acid
R_s	=	sludge age
s	=	sludge recycle ratio from the settler to the anoxic reactor
So/Xo	=	initial substrate to initial biomass ratio
S_{ti}	=	influent COD concentration
S_{te}	=	effluent COD concentration
S_{xw}	=	COD in waste sludge
T	=	Thymidine
Taq	=	*Thermus aquaticus*
TCA	=	Tricarboxylic acid (cycle)
TKN	=	total Kjeldahl nitrogen
UCT	=	University of Cape Town
VSS	=	volatile suspended solids
WW	=	wastewater
X_H	=	heterotrophic active biomass
X_v	=	mixed liquor volatile suspended solids

CHAPTER 1

General Introduction

1.1 Nutrient Overload: Unbalancing the Global Nitrogen Cycle

As a basic building block of plant and animal proteins, nitrogen is a nutrient essential to all forms of life (Fig 1.1). Recent studies have shown that excess nitrogen from human activities such as agriculture, energy production, and transport has begun to overwhelm the natural nitrogen cycle with a range of ill effects from diminished soil fertility to toxic algal blooms (Vitousek, 1997). Until recently, the supply of nitrogen available to plants and animal has become limiting. Although it is the most abundant element in the atmosphere, plants cannot use nitrogen from the air until it is chemically transformed, or fixed, into ammonium or nitrate compounds that plants can metabolize. In nature, only certain bacteria and algae (and, to a lesser extent, lightning) have this ability to fix atmospheric nitrogen, and the amount that they make available to plants is comparatively small. Other bacteria break down nitrogen compounds in dead matter and release it to the atmosphere again. As a consequence, nitrogen is a precious commodity, a limiting nutrient, in most undisturbed natural systems (Bitton, 1995).

Driven by a massive increase in the use of fertilizer, the burning of fossil fuels, and an upsurge in land clearing and deforestation, the amount of nitrogen available for uptake at any given time has more than doubled since the 1940s. In other words, human activities now contribute more to the global supply of fixed nitrogen each year than natural processes do, with human-generated nitrogen totaling about 210 million metric tons per year, while natural processes contribute about 140 million metric ton (Table 1.1) (Vitousek, 1997).

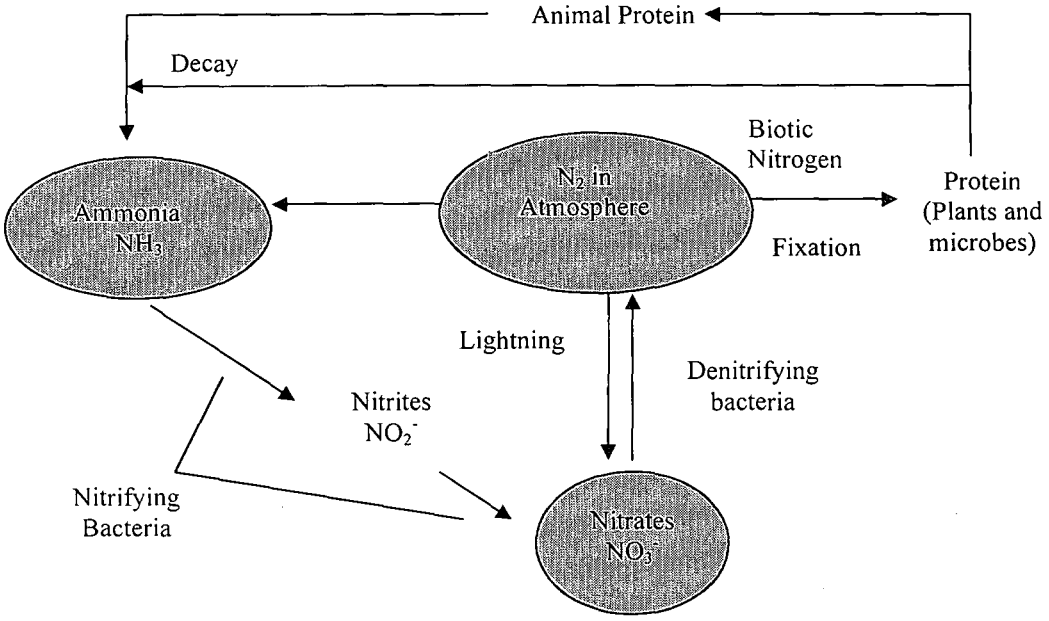


Figure 1.1: Schematic representation of the nitrogen cycle (Stryer, 1981)

Table 1.1: Global sources of biologically available (fixed) nitrogen (Vitousek, 1997).

A Global Glut of Nitrogen	
ANTHROPOGENIC SOURCES	**ANNUAL RELEASE OF FIXED NITROGEN (teragrams)**
Fertilizer	80
Legumes and other plants	40
Fossil fuels	20
Biomass burning	40
Wetland draining	10
Land clearing	20
Total from human sources	**210**
NATURAL SOURCES	
Soil bacteria, algae, lightning, etc.	140

Although terrestrial ecosystems are vulnerable to the global nitrogen glut, aquatic ecosystems in lakes, rivers, and coastal estuaries have probably suffered the most so far. They are the ultimate receptacle of much of the nutrient overload, which tends to accumulate in runoff or to be delivered directly in the form of raw or treated sewage. (Sewage is very high in nitrogen from protein in the human diet.) In these aquatic systems, excess nitrogen often greatly stimulates the growth of algae and other aquatic plants. When this extra plant matter dies and decays, it can deprive the water of its dissolved oxygen, suffocating many aquatic organisms (Vitousek, 1997).

This overfertilization process, called eutrophication (Steyn *et al.,* 1975), is one of the most serious threats to aquatic environments today, particularly in coastal estuaries and inshore waters where most commercial fish and shellfish species breed .One of the more troubling aspects of this nutrient assault on aquatic systems has been a steady rise in toxic algal blooms, which can take a heavy toll on fish, seabirds, and marine mammals (Vitousek, 1997).

Limitation of nutrient discharges into waters from point sources (sewers) is usually achieved by biological means, either by way of biological trickling filters or activated sludge systems. Both these systems utilize naturally occurring bacteria to reduce the nutrient concentrations entering waters. Artificial conditions favourable for the controlled growth of these bacteria are created within these systems and results in concentrations of at least a million times that found in the natural environment. The bacteria in these systems utilize the nutrients for growth and in this way the nutrients pass from the liquid phase into a solid phase and are concentrated in the biological culture (Lilley *et al.*, 1997)

1.2 Biological Treatment Processes

Municipal wastewaters commonly contain sufficient concentrations of carbon, nitrogen, phosphorus, and trace nutrients to support the growth of microbial culture. Average domestic wastewater exhibits a surplus of nitrogen and phosphorus with a BOD/N/P ratio of about 100/17/5. If municipal waste contains a large volume of nutrient-deficient industrial waste, supplemental nitrogen is generally supplied by the addition of anhydrous ammonia (NH_3) or phosphoric acid (H_3PO_4) as is needed (Hammer, 1977).

Biological processing is the most efficient way of removing organic matter from municipal wastewaters. These living systems rely on mixed microbial cultures to decompose, and to remove colloidal and dissolved organic substances from solution. The treatment chamber holding the microoganisms provides a controlled environment; for example, activated sludge is supplied with sufficient oxygen to maintain an aerobic condition. Wastewater contains the biological food, growth nutrients, and innoculum of microoganisms. The most important factors affecting biological growth are temperature, availability of nutrients, oxygen supply and pH. The rate of biological activity doubles for every 10 to 15°C temperature rise with the range of 5 to 35°C. This higher temperature range is not used in waste treatment, since it is difficult to maintain such a high operating temperature, and because thermophilic bacteria are more sensitive to small temperature changes (Needham and Needham, 1962).

Diffused and mechanical aeration basins must supply sufficient air to maintain dissolved oxygen for the biota to use in metabolizing the waste organics. Rate of microbial activity is independent of dissolved oxygen concentration above a minimum critical value, below which the rate is reduced by the limitation of oxygen required for respiration. The exact minimum depends on the type of activated sludge process and the characteristics of the wastewater being treated (Hammer, 1977).

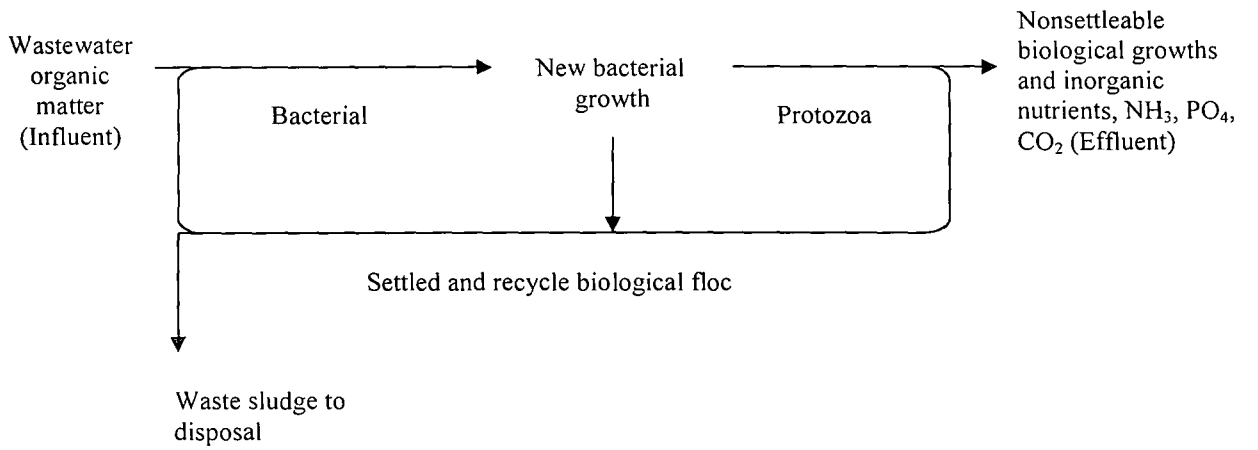
Hydrogen ion concentration has a direct influence on the biological treatment systems which operate best in a neutral environment. The general range of operation of aeration

systems is between pH 6.5 and 8.5. Above this range microbial activity is inhibited, and below pH 6.5 fungi are favoured over bacteria in the competition for metabolizing the waste organics. Normally the bicarbonate buffer capacity of wastewater is sufficient to prevent acidity and reduced pH; while carbon dioxide production by the microorganisms tends to control the alkalinity of high pH wastewaters (McKinney, 1962).

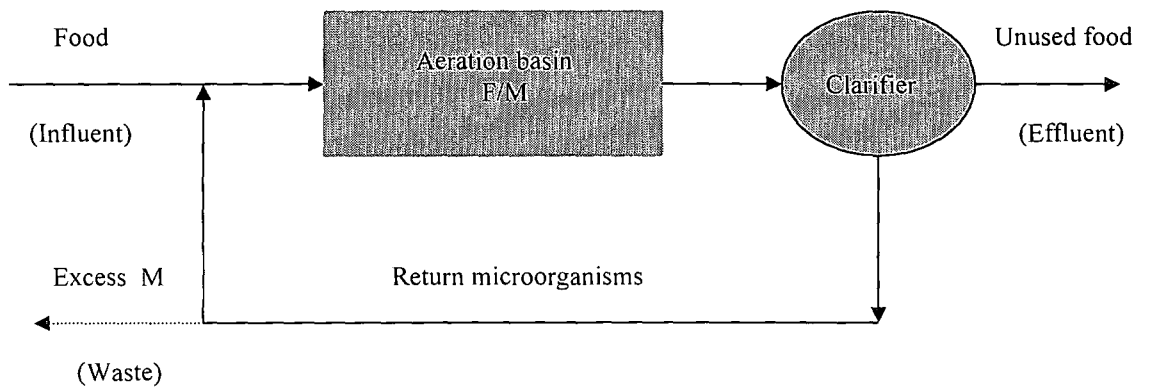
1.2.1 Population Dynamics

Three of the major factors in population dynamics are: competition for the same food, predator-prey relationship, and symbiotic association. When organic matter is fed to a mixed population of microoganisms, competition arises for this food, and primary feeders that are most competitive become dominant. Under normal operating conditions, bacteria are primary feeders in both aerobic and anaerobic operations. Protozoa consuming bacteria is the common predator-prey relationship in activated sludge (Clark *et al.*, 1977).

In an activated sludge process, waste organics serve as food for the bacteria and the small population of fungi that might be present. Some of the bacteria die and lyse, releasing their contents which are resynthesized by other bacteria. The secondary feeders (protozoa) consume several thousand bacteria for a single reproduction. The benefit of this predator-prey action is twofold, firstly removal of bacteria stimulates further bacterial growth, accelerating metabolisms of organic matter, and secondly settling characteristics of the biological floc is improved by reducing the number of free bacteria in solution. (Fig. 1.2) (Hammer, 1977).



(a)



(b)

Figure 1.2: Generalized biological population dynamics in the activated sludge wastewater treatment process. (a) Predator-prey relationship between protozoa and bacteria in the activated sludge process. (b) Sedimentation and recirculation maintains the desired food to microorganism (F/M) ratio in the aeration basin (Hammer, 1977).

Control of microbial populations is essential for the efficient aerobic treatment. If wastewater were simply aerated, the liquid detention times would be intolerably long, requiring a time period of about five days at 20°C for 70 percent reduction. However, extraction of organic matter is possible within a few hours of aeration provided that a large number of microorganisms are mixed with the wastewater. In practice this is achieved by settling the microorganisms out of solution in a final clarifier and returning them to the aeration tank to metabolize additional waste organics. The effluent from the process consists of nonsettleable organic matter and dissolved organic salts (Fig. 1.2) (Hammer, 1977).

Good settling characteristics occur when activated sludge is held in the endogenous (starvation) phase. Furthermore, a large population of under fed biota removes BOD very rapidly from solution. Excess microorganisms are wasted from the process to maintain proper balance between food supply and biological mass in the aeration tank. This balance is referred to as food-to-microorganism ratio (F/M) which is normally expressed in units of grams of BOD applied per day per gram of MLSS in the aeration basin. Operation at a high F/M ratio results in incomplete metabolism of the organic matter, poor settling characteristics of the biological floc and consequently poor BOD removal efficiency. At low F/M ratios, the mass of organisms are in near starvation condition that results in a high degree of organic matter removal, good settleability of the activated sludge and efficient BOD removal (Hawkes, 1963; Hammer, 1977).

1.3 Activated Sludge Process

Since its development by Ardern and Lockett in 1914, the activated sludge system has gained increasing importance in the treatment of municipal wastewaters. This is a consequence of its adaptability to variation in wastewater composition, high rates of removal of organic material and ability to remove nutrients nitrogen (N) and phosphorus (P) to low levels without chemical addition (Casey *et al.*, 1995).

Its initial development as an aerobic process was a consequence of its greater economy and surety of effluent quality than the trickling filter, especially with regard to nitrification. Barnard introduced significant developments in the activated sludge system in 1973 and 1975. By incorporating anoxic and anaerobic zones he demonstrated that a high percentage of the influent N and P could be removed by biological mechanisms in the system without the aid of chemical addition (Wentzel *et al.*, 1992). It was later demonstrated that through the imposition of specific environmental conditions and substrate supply, the growth of certain species could be promoted so as to fulfill some desired function. Examples are:

- With a sufficiently long aerobic sludge age, nitrifying organisms develop and produce an effluent low in ammonia
- With the appropriate sludge age, and a sequence of in-series reactors operating in anaerobic, anoxic and aerobic states, with inter-reactor recycles, different species of microorganisms develop that
    - Nitrifiy;
    - Denitrify; and
    - Store phosphorus in excess of their metabolic requirements (WRC,1984; Casey *et al.*, 1995)

By these means the eutrophic elements, N and P, are removed from the wastewater biologically.

1.3.1 Biological Nutrient Removal System Configurations

It is not always possible to achieve complete biological nitrogen and phosphorus removal. However, to achieve biological nutrient removal (BNR) of nitrogen and phosphorus, the process must incorporate an anaerobic zone, an anoxic zone and an aerobic zone (Lilly *et al.*, 1997).

A number of mainstream removal streams have evolved in South Africa over the years. For the purpose of this investigation three common wastewater treatment processes for nitrogen removal will be discussed. These include Wuhrmann process (Fig. 1.3), modified Ludzack-Ettinger process (detailed in chapter 3) and Bardenpho process (Fig. 1.4). However other systems such as the phoredox process, 3-stage phoredox process, UCT process, modified UCT process and Johannesburg process were designed to incorporate both P and N removal. These systems, however, are beyond the scope of this study and will not be discussed further (Lilley *et al.*, 1997).

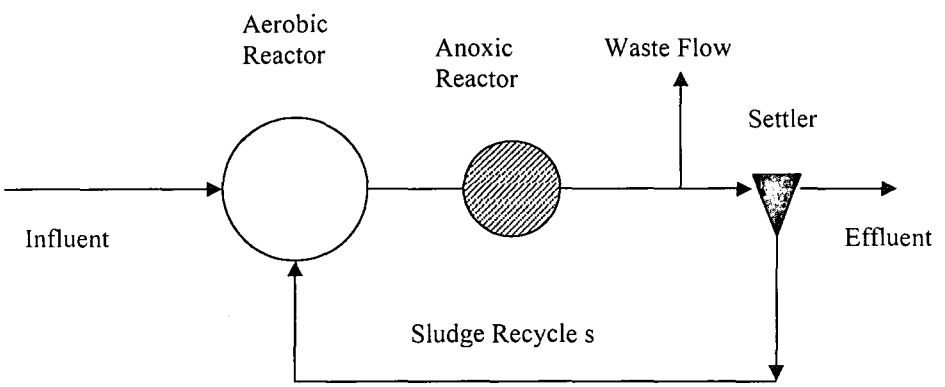


Figure 1.3: The Wuhrmann process for nitrogen removal (Lilley *et al.*, 1997).

The Wuhrmann process comprises an aerobic zone followed by an anoxic zone. The influent is discharged directly into the aerobic zone in which nitrification takes place. The flow is then discharged to the anoxic zone. The underflow from the clarifier (s-recycle) is returned to the aerobic zone. The substrate source for denitrification in the anoxic is obtained from death of organisms. However, the release rate of substrate is very low. This unfortunately leads to large anoxic zones and small aerobic zones. Theoretically, complete denitrification could be achieved. However, this is not practically possible because the anoxic zone would have to be very large due to the slow denitrification rate which may lead to a loss of nitrification (WRC, 1984; Lilley *et al.*, 1997).

The Bardenpho system (Fig. 1.4) was developed to overcome incomplete denitrification of the modified Ludzack-Ettinger (MLE) system. Barnard (1973) considered that low nitrate concentration discharged from the aeration zone could be denitrified in a secondary anoxic zone placed after the aerobic zone, to give a relatively nitrate-free effluent. Prior to discharge to the clarifier a flash aeration basin was introduced after the second anoxic zone to strip the nitrogen bubbles from the sludge. This assisted with sedimentation of the sludge. The flash aeration also served to nitrify any ammonia release within the anoxic zone.

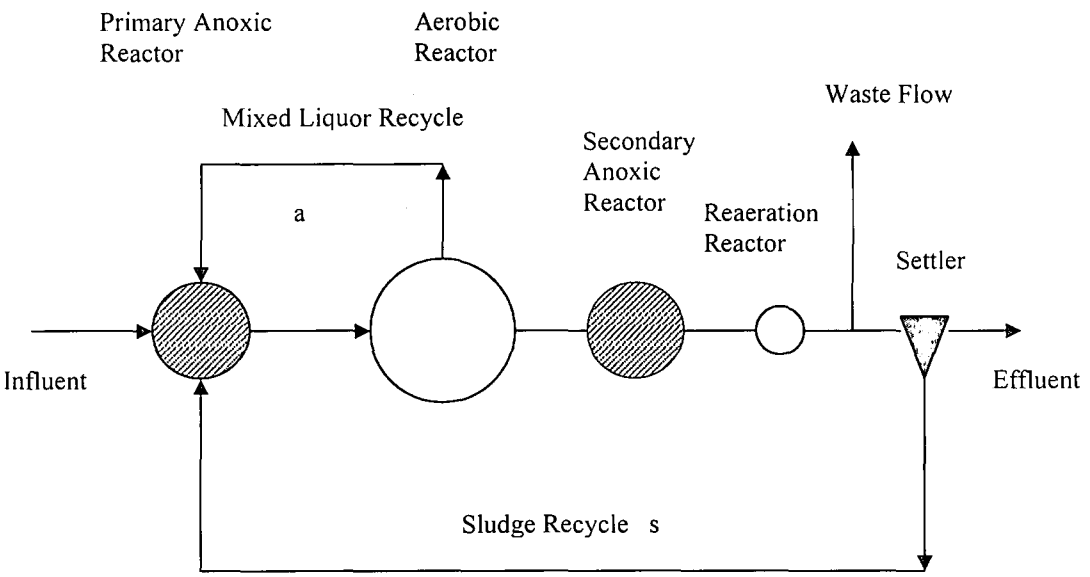


Figure 1.4: The Bardenpho process for nitrogen removal (Lilley *et al.*, 1997).

For a fixed underflow s-recycle ratio, the mixed liquor a-recycle governs the distribution of nitrate between the primary and secondary anoxic zones. The best denitrification performance will be obtained when a- and s-recycle values are chosen such that the primary anoxic zone is just loaded to its denitrification potential (maximum amount of nitrate the reactor could remove) and the nitrate concentration in the flow leaving this zone will thus be zero (WRC, 1984; Lilley *et al.*, 1997).

The balance of nitrate generated in the aerobic zone (and not recycled to the primary anoxic zone) flows to the secondary anoxic zone. If this load of nitrate to the secondary anoxic zone is less than the denitrification potential of this zone then complete denitrification will be achieved. In practice the Bardenpho system for nitrogen removal is appropriate if the calculated effluent nitrate concentration is greater than 5 to 7 mgN/L (which is usually the case for $^{TKN}/_{COD}$ ratios > 0.1 mg N/mg COD) then the MLE process is better suited for higher nitrogen removal efficiency (Table 1.2) (Lilley *et al.*, 1997).

Table 1.2: Advantages and disadvantages of nitrogen removal processes (Lilley *et al.*, 1997).

Process	Advantages	Disadvantages
Wuhrmann	Theoretically possible to remove all nitrate	Large anoxic mass fraction required which may inhibit nitrification Due to organisms die-off, ammonia and organic nitrogen are discharged with the effluent Low denitrification rate
Modified Ludzack-Ettinger	High rate of denitrification Simple configuration Higher N-removal than the Bardenpho system for $^{TKN}/_{COD}$ ratios > 0.1	Complete denitrification cannot be achieved Effluent nitrate concentration will be greater than 5mgN/L a-recycle limiting
Bardenpho	Theoretically possible to remove all the nitrate, but not possible in practice Higher N-removal than the MLE system for $^{TKN}/_{COD}$ ratios < 0.1	Complex configuration a-recycle limiting Mainly used for treating raw wastewaters where $^{TKN}/_{COD}$ ratios < 0.1.

1.4 Active Biomass

In wastewater treatment, it is the bacteria that are primarily responsible for the oxidation of organic matter. However, fungi, algae, protozoans (collectively known as Protista), and higher organisms all have important secondary roles in the transformation of soluble and colloidal organic matter into biomass. In order to function properly the microorganisms involved in wastewater treatment require a source of energy and carbon for the synthesis of new cells as well as other nutrients and trace elements. The microorganisms are classified as either heterotrophic or autotrophic according to their source of nutrients. Heterotrophs require organic matter both for energy and as a carbon source for the synthesis of new microoganisms, whereas autotrophs do not utilize organic matter but oxidize inorganic compounds for energy and use carbon dioxide as a carbon source (Hammer, 1977).

In a bioreactor of a non-nitrifying aerobic activated sludge system the mixed liquor organic suspended solids (MLOSS) is made up of three components;

- Heterotrophic active biomass
- Endogenous residue and
- Inert material (Wentzel *et al.*, 1998)

In the nitrifying aerobic and anoxic/aerobic activated sludge systems, a fourth component is included;

- Autotrophic active biomass

All four MLOSS components settle out in the secondary settling tank and are returned to the bioreactor via the underflow recycle and leave the system via the wasteflow (Wentzel *et al.*, 1998).

Historically the MLOSS has been measured as a lumped parameter using the VSS and COD tests (Standard Methods, 1985). Specific rates for the biological processes (e.g denitrification; oxygen utilization) often are expressed in terms of this lumped parameter. Only a part of the MLOSS is heterotrophic active biomass (X_H), the active fraction, and only this part mediates the biological process of COD removal and denitrification.

Accordingly, the specific rates for these biological processes should be expressed in terms of X_H to allow meaningful comparison of rates measured in different systems. The proliferation of kinetic simulation computer programs that invariable includes X_H as a parameter has become widely accepted. However X_H exist only hypothetically within the structure of the design procedures and kinetic models (Wentzel *et al.*, 1998).

Wentzel *et al.*, (1998) used a simple batch test procedure to quantify the heterotrophic active biomass concentration of mixed liquor drawn from a well-defined parent anoxic/aerobic activated sludge system operated at 12 day and 20 day sludge ages. With the parent system at 12 day sludge age there was a close agreement between the measured and theoretical values. However with the parent system at 20 day sludge age the agreement was poor, with the theoretical values been about 2 times those measured. No explanation could be found for this inconsistency.

Novel microbiological and molecular techniques have been proposed such as ATP analysis (Nelson and Lawrence, 1980), DNA analysis (Muyzer *et al.*, 1993; Liebeskind and Dohmann, 1994) and using florescent probes for ribosomal RNA (Wagner *et al.*, 1997). With the development and the integration of these techniques to the design and kinetic modeling theory, it makes it possible to directly quantify the key microbial groups active within the BNR process with a view to improving the process description and design (Rittmann *et al.*, 1999).

1.5 Microbial Community Analysis

The activated sludge treatment process uses undefined populations of bacteria to treat a variety of wastes. The conditions in a reactor are manipulated to promote the presence and activity of desirable organisms, such as ammonia oxidizers, and to discourage the growth of undesirable organisms such as the bacteria associated with foaming. The design and maintenance of such systems is, in effect, the engineering the bacterial population and its activities (Curtis and Craine, 1998)

Unfortunately, it is difficult to engineer something that cannot be measured. Consequently, the explicit engineering of bacterial populations has been impeded by the crude, labour intensive and inadequate nature of the tools available to quantify and identify bacteria: suspended solids measurements (MLOSS), morphological examination and culture (Bitton, 1995).

However, progress has been made recently with the advent of 16S RNA based technology, in particular: whole cell hybridization (Amman *et al.*, 1995), the polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) (Muyzer *et al.*, 1993). These techniques exploit differences between the ribosomal gene sequence of different organisms. In whole cell hybridization, individual bacteria can be identified and enumerated using fluorescent probes targeted at specific signature sequences in ribosomal RNA.

DGGE allows away of comparing rDNA sequences by separating them into discrete bands based on their sequence. In DGGE the double stranded DNA products undergo electrophoresis through a polyacrylamide gel containing an increasing linear gradient of denaturants. Strand separation occurs when a sufficiently high denaturant concentration is reached. The sequence of PCR product determines the denaturant concentration at which this occurs (Muyzer *et al.*, 1993; Myers *et al.*, 1987). A ladder of bands, each corresponding to the individual PCR products of a specific sequence is thus produced from analysis of a complex microbial population. The electrophoresis gels can be probed with diagnostic oligonucleotides to identify particular sequences (Muyzer *et al.*, 1995) or bands can be excised, reamplified and sequenced (Ferris *et al.*, 1996). To enhance the resolution of the process a GC rich sequence can be incorporated into one of the primers to modify the melting behavior of the fragment and allow the majority of sequence variation to be detected in the denaturing gel (Muyzer *et al.*, 1993).

Ferris and Ward (1997) used DGGE analysis to evaluate seasonal distributions of Octopus Spring microbial mat populations defined by 16S rRNA sequences along a

thermal gradient. No shift in the thermal gradient was detected, and populations at spatially or temperature-defined sites exhibited only slight changes over the annual sampling period.

Curtis and Craine (1998) used DGGE analysis to compare spatial and temporal differences within activated sludge plants and thus defined a sampling strategy for 16S RNA based comparisons of diversity. This strategy was used to compare a variety of activated sludge plants.

1.6 Aims and Objectives

- Operation of parent laboratory scale Modified Ludzack Ettinger (MLE) anoxic/aerobic activated sludge system

- To obtain initial and final profiles of microbial community using DGGE at defined So/Xo ratios during activated sludge respirometric batch assays

- Comparison of initial and final DGGE profiles to determine a So/Xo ratio where change in the composition of microbial community is minimal

CHAPTER 2

Literature Review

2.1 Biochemistry of Heterotrophic Respiratory Metabolism

Metabolism can be broadly described as the manner by which facultative heterotrophic organisms derive energy and matter for growth. It consists of two processes:

- The enzymatic biosynthesis of the complex molecular components of the organism itself (anabolism)
- The enzymatic bio-reactions which generate energy to perform this biosynthesis (catabolism)

The process of catabolism is referred to as respiratory metabolism. In catabolism, complex organic compounds (substrate) are enzymatically degraded such that some of its electrons originally present in the organic compound (called the electron donor) are removed (oxidation) and transferred through a sequence of controlled biochemical reactions to a final compound (called a terminal electron acceptor). In this sequence of biochemical reactions there is a release of chemical energy, some of which is conserved through the formation of energy-rich molecule ATP (Casey *et al.*, 1999)

The principle objective of the catabolic process is the formation of ATP and without electron donors and acceptors this is not possible. The ATP produced by the catabolic process is utilized by the organism in the anabolic process for the synthesis of new cell material and in maintenance of cell function and structure. Facultative heterotrophic organisms could more appropriately be named chemo-heterotrophs as a consequence of their derivation of energy (catabolism) from the break down of organic compounds and their derivation of the principal source of carbon for cell synthesis (anabolism) from the same organic compounds (heterotrophs). The term facultative refers to their ability to switch between available terminal electron acceptors in response to their environmental conditions; oxygen (O_2) under aerobic conditions, and nitrate (NO_3) and/or nitrite (NO_2)

under anoxic conditions (Casey *et al.*, 1999). This study is limited to facultative heterotrophic organisms since these are the organisms likely to be present under the cyclic anoxic-aerobic conditions found in a parent laboratory scale anoxic/aerobic activated sludge system.

2.1.1 Stages of Respiratory Metabolism

The process of respiratory metabolism by heterotrophic organisms wherein organic substrates, such as carbohydrate, protein and lipids, are oxidized to end products of H_2O and CO_2 can be divided into four stages and conceptualized as illustrated in Fig. 2.1. In Stage I, large complex organic molecules are hydrolysed to simpler ones; carbohydrates to hexoses and pentoses, proteins to amino acids, and lipids to fatty acids and glycerol.

In Stage II, the endproducts of Stage I are degraded further, resulting in the formation of acetyl-coenzyme A (acetyl-CoA) and carbon dioxide. For the degradation of each group of Stage I endproducts, i.e. amino acids, hexoses/pentoses, and fatty acids/glycerol, different biochemical pathways are employed.

In Stage III, the endproduct from Stage II, acetyl-CoA, enters the TCA cycle, a cyclic sequence of reactions catalysed by a series of enzymes. During the cycle, the acetyl group of acetyl-CoA is oxidized to form two molecules of CO_2, eight protons (H^+), and four pairs of electrons (e^-) and one molecule of GTP which is energetically equivalent to one ATP; Coenzyme A is recovered. The TCA cycle is the final catabolic pathway common to all aerobic and facultative organisms. It can be conceptualized as the mechanism by which all the foregoing substrates (in the form of acetyl-CoA) are converted to common products, i.e. electrons and protons, in the form of reduced NADH and reduced $FADH_2$.

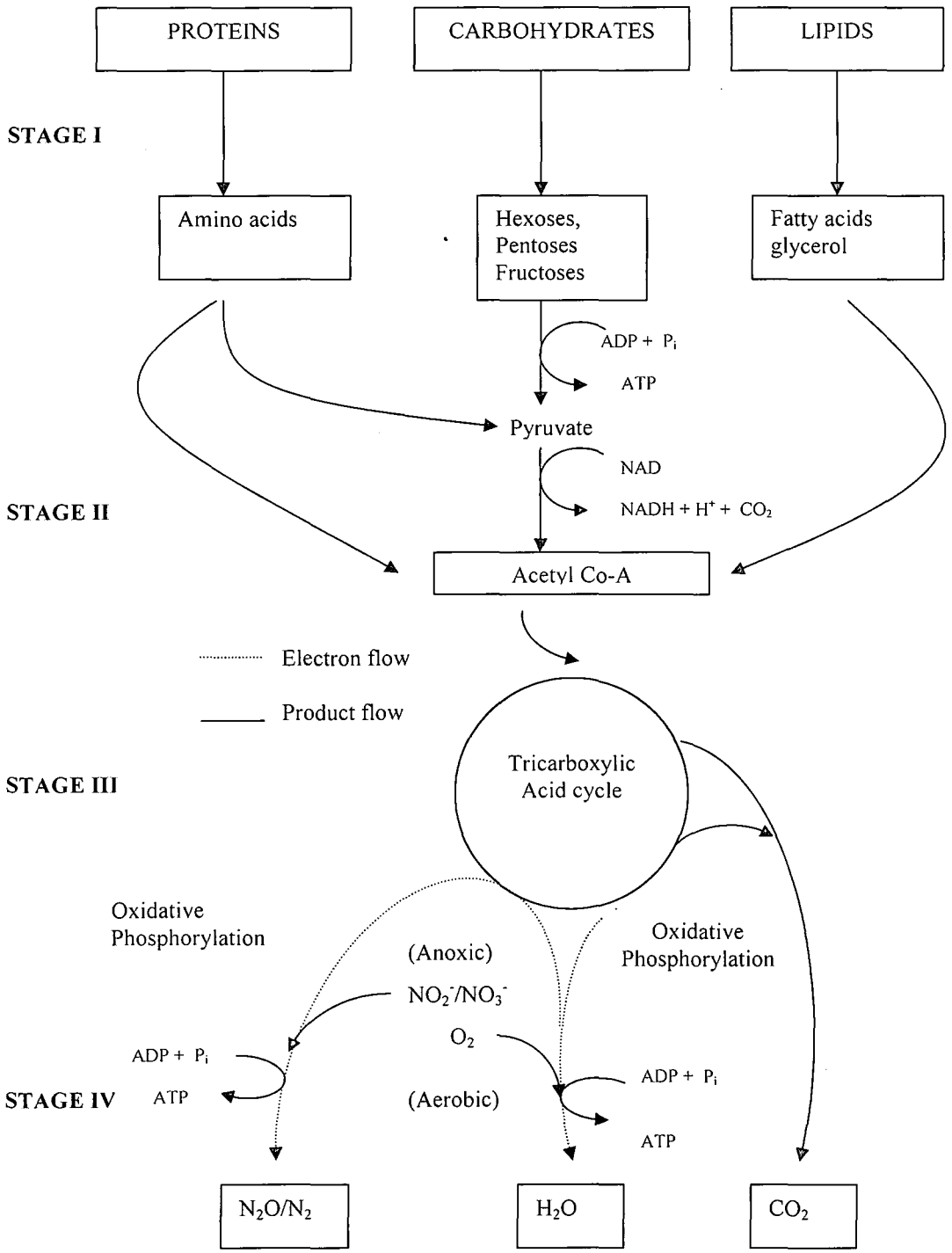


Figure 2.1: Stagewise division of substrate breakdown by facultative heterotrophic organisms indicating the major metabolic pathways (Lehninger, 1975)

In Stage IV, the electrons and protons associated with NADH and $FADH_2$ are removed and transferred along a pathway of electron and proton carrier enzymes of successively lower energy (Electron Transport Pathway) to a final (terminal) electron acceptor. During the process, much of the free energy of the electrons is conserved in the form of the energy-rich molecule ATP in a process termed oxidative phosphorylation. Under aerobic conditions, the electrons and protons are transferred to the final electron acceptor O_2 (with the formation of H_2O) and under anoxic conditions; electrons and protons are transferred to nitrate (NO_3^-) and/or nitrite (NO_2^-) (with the formation of H_2O and N_2). With NO_3^- or NO_2^- as external terminal electron acceptors, the gaseous products nitric oxide (NO) and nitrous oxide (N_2O) are produced intracellularly; these gaseous nitrogen oxides act as intermediate electron acceptors during the formation of dinitrogen (N_2) (Casey *et al.*, 1999; Lehninger, 1975)

2.1.2 Aerobic Respiration

The respiratory enzyme complexes involved in the transfer of electrons and protons from NADH and $FADH_2$ to the terminal electron acceptor oxygen are shown in Fig. 2.2. The complexes shown in Fig. 2.2 are the complexes synthesized under aerobic conditions. These are, NADH dehydrogenase, ubiquinone (Q), cytochrome bc_1, cytochrome *c* and the two oxidases, cytochromes aa_3 and *o*. These complexes are considered as proton pumping energy conserving sites, i.e. sites at which protons are pumped from the cytoplasmic (inner) to the periplasmic (outer) side of the membrane, this being the first step of the mechanism which links the processes of respiration (transfer of electrons to oxygen) and oxidative phosphorylation (the production of the high energy molecule, ATP, from low energy molecule, ADP). Usually the Electron Transport Pathways (ETPs) to cytochrome *o* and cytochrome aa_3. Cytochrome aa_3 and cytochrome *o* are referred to as the primary and alternative oxidases, respectively (Casey *et al.*, 1999; Lehninger, 1975).

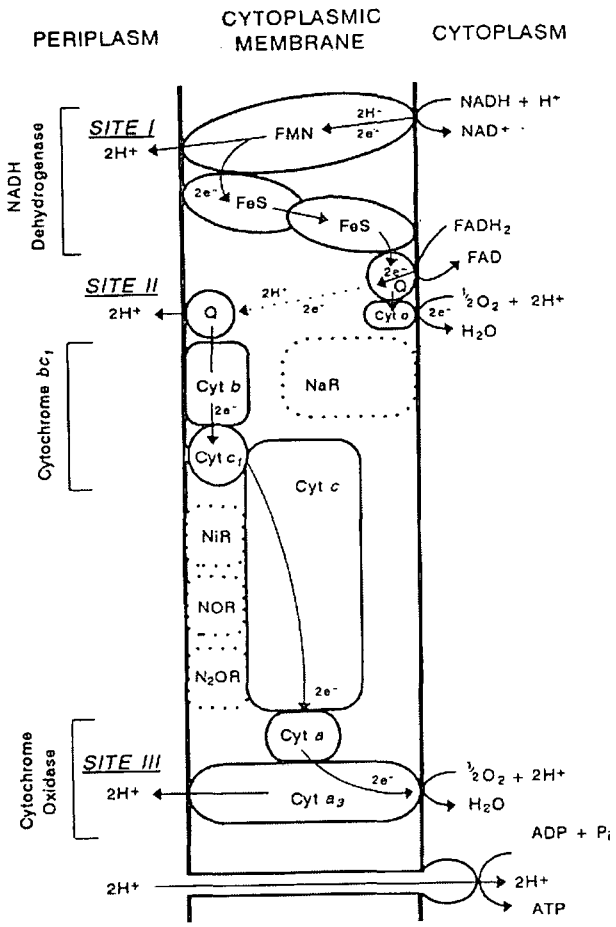


Figure 2.2: Electron Transport Pathway (ETP) for a typical facultative aerobic heterotrophic organism grown under aerobic conditions (Casey *et al.*, 1999)

2.1.2.1 NADH dehydrogenase enzyme complex

The NADH dehydrogenase enzyme complex consists of polypeptide chains collectively referred to as flavoprotein (Fp) and a series of iron-sulphur protein complexes (FeS). Flavoprotein contains a prosthetic group called flavin mononucleotide (FMN) or flavin which acts as the electron and proton transferring site of complex. The NADH dehydrogenase complex catalyses the transfer of two protons and a pair of electrons from NADH to ubiquinone in three steps.

In the first step, the prosthetic group, FMN of the flavoprotein (fp) gains protons and electrons to give a reduced form $FMNH_2$ as follows:

$$NADH + H^+ + FMN \longrightarrow FMNH_2 + NAD^+$$

In the second step, a pair of electrons are transferred from $FMNH_2$ to a series of iron-sulphur protein complexes (FeS) on the periplasmic side of the membrane at which point two protons are released to the periplasm. The reaction is as follows:

$$FMNH_2 + 2Fe^{3+} \longrightarrow 2Fe^{2+} + FMN + 2\,H^+$$

In the third step, a pair of electrons are transferred between the FeS complexes, and each reduced FeS complex donates one electron to the next carrier in the ETP, ubiquinone (Q) (Casey *et al.*, 1999; Lehninger, 1975).

2.1.2.2 Ubiquinone

Ubiquinone is a mobile molecule which transports electrons and protons from the cytoplasmic to the periplasmic side of the membrane. Either NADH or $FADH_2$ can act as the initial electron donor. For NADH as the initial donor, the transfer of electrons and protons is as follows:

$$Q\ (\text{ubiquinone}) + 2Fe^{2+} + 2\,H^+ \longrightarrow QH_2\ (\text{ubiquinol}) + 2Fe^{3+}$$

For $FADH_2$ as the donor, the transfer of electrons and protons is as follows:

$$Q + FADH_2 \longrightarrow QH_2 + FAD$$

Ubiquinone transports electrons and protons to the periplasmic side of the membrane where it extrudes two protons to the periplasm and transfers a pair of electrons to

cytochrome *b* contained in the second of the main respiratory complexes, the cytochrome bc_1 complex is as follows (Casey *et al.*, 1999; Lehninger, 1975):

$$QH_2 + 2 \text{ cyt } b_{ox} (Fe^{3+}) \longrightarrow 2 \text{ cyt } b_{red} (Fe^{2+}) + 2H^+ + Q$$

2.1.2.3 Cytochrome bc_1 complex

An important characteristic of cytochromes in general is that they are able to transfer electrons only, unlike NADH, NADH dehydrogenase, $FADH_2$ and ubiquinone, which transport both electrons and protons. The cytochrome bc_1 complex contains two sequential electron transporting proteins, cytochrome *b* and cytochrome c_1. The cytochromes of the cyt bc_1 complex are intermediates in the transfer of electrons from ubiquinol to cytochrome *c*, the next complex in the pathway. Cytochromes are electron transporting proteins which contain iron in a prosthetic group and are referred to as haem proteins (Casey *et al.*, 1999)

2.1.2.4 Cytochrome c complex

The cytochrome c complex accepts electrons from cytochrome c_1 of the cytochrome bc_1 complex and transfers them to the terminal electron transferring complex for aerobic respiration, the cytochrome oxidase complex, cytochrome aa_3 (Casey *et al.*, 1999)

2.1.2.5 Cytochrome oxidase complex

The terminal cytochrome complex in the aerobic ETP, the cytochrome oxidase complexes (cytochrome aa_3 and *o*), transfer electrons to the terminal electron acceptor, oxygen. For obligate aerobic heterotrophic organisms, the aerobic cytochromes in the ETP to oxygen are contained in the cytochrome aa_3 complex (Poole, 1982). For facultative denitrifying organisms, an additional cytochrome oxidase complex, cytochrome *o* is present (Willison and John, 1979). It has been found that for facultative organisms maintained under anoxic conditions, three to four times more cytochrome *o* is

synthesized than when the same facultative organisms are maintained under aerobic conditions (Sapshead and Wimpenny, 1972).

The cytochrome aa_3 complex contains four electron transferring metal centres; two iron and two copper. Iron atoms are contained in each of cytochrome *a* and a_3 and alternate between a reduced ferrous (+2) state and an oxidized ferric (+3) state during electron transport. Copper atoms are also contained in each of cytochromes *a* and a_3 and also mediate electron transport and alternate between a reduced (+1) state and an oxidized (+2) state (Poole, 1982). The Cytochrome aa_3 complex is as follows:

cyt *a* (Fe^{2+}, Cu^{+}) + cyt a_3 (Fe^{2+}, Cu^{+}) + O_2 + $4H^{+}$ $\longrightarrow$ $2H_2O$ + cyt *a* (Fe^{3+}, Cu^{2+}) + cyt a_3 (Fe^{3+}, Cu^{2+})

For cytochrome *o* as oxidase, 4 protons from the cytoplasm combine with 1 oxygen molecule and 2 pairs of electrons (transferred from ubiquinol to cytochrome *o*) to form water as follows (Poole, 1982):

2cyt *o* (Fe^{2+}, Cu^{+}) + O_2 + $4H^{+}$ $\longrightarrow$ $2H_2O$ + 2cyt *o* (Fe^{3+}, Cu^{2+})

2.1.3 Anoxic respiration

When dissolved oxygen becomes limiting, facultative heterotrophs switch from oxygen to nitrate/nitrite as terminal electron acceptor and respire anoxically. For nitrate/nitrite as electron acceptor, the first three stages illustrated in Fig. 2.1 and the greater part of the fourth stage of the ETP continue functioning unchanged; a difference in electron transport between aerobic and anoxic respiration becomes apparent only after the ubiquinone complex. Figure 2.3 illustrates the respiratory complexes present in a typical facultative aerobic organism grown under anoxic conditions (Casey *et al.*, 1999).

Organisms can utilise nitrate through two processes, assimilatory nitrogen removal and dissimilatory nitrogen removal. Assimilatory nitrogen removal is the reduction of nitrate

to ammonium (NH_4^+), the ammonium being used for synthesis of cellular material. This is an anabolic process, is energy consuming, is not associated with the respiratory ETP and can occur under both aerobic and anoxic conditions.

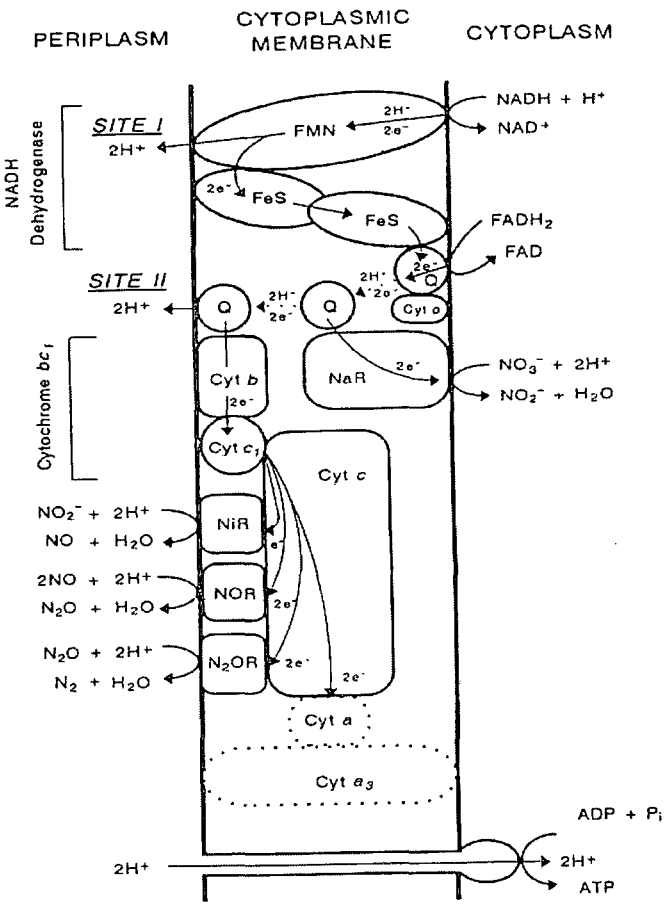


Figure 2.3: Electron transport pathway (ETP) for a typical facultative aerobic heterotrophic organism grown under anoxic conditions (Casey *et al.*, 1999)

Dissimilatory nitrogen reduction is the reduction of nitrate to nitrite, or nitrite to one of the more reduced gaseous nitrogen oxide compounds, nitric oxide (NO), nitrous oxide (N_2O) or dinitrogen (N_2). The process whereby nitrate and nitrite act as terminal electron acceptors during utilization of organic substrate are referred to as nitrate and nitrite respiration respectively. The term nitrate reduction applies to the reduction of nitrate to nitrite only. The term denitrification is applied to the reduction of one of the ionic

nitrogen oxides (nitrate or nitrite), to one of the gaseous nitrogen compounds, nitric oxide, nitrous oxide, or dinitrogen. The pathway for denitrification is composed of a number of sequential steps by which electrons are passed via the ETP to one of the nitrogen oxides, to produce another more reduced nitrogen oxide (Casey *et al.*, 1999). The sequential production of the intermediates as indicated by Payne (1973) are as follows:

$$\text{Nitrate } (NO_3^-{}_{aq})^{(+5)} \longrightarrow \text{Nitrite } (NO_2^-{}_{aq})^{(+3)} \longrightarrow \text{Nitric Oxide } (NO_g)^{(+2)} \longrightarrow$$
$$\text{Nitrous Oxide } (N_2O_g)^{(+1)} \longrightarrow \text{Dinitrogen } (N_{2g})^{(0)}$$

2.1.3.1 Nitrate reductase

Nitrate is reduced to nitrite by nitrate reductase, the catalytic site of which is situated on the cytoplasmic side of the membrane (Boogerd *et al.*, 1983). In the transfer of electrons from NADH to nitrate reductase, electrons pass sequentially through the complexes of NADH dehydrogenase to ubiquinone as described for aerobic respiration. In the process, the first of the energy conserving (proton-pumping) sites (Site I) is passed. The transfer of electrons through the NADH dehydrogenase complex is common to reduction of each of the nitrogen oxide reductases. The electrons then pass from ubiquinol to nitrate reductase. At the catalytic site of nitrate reductase, 2 electrons are passed to nitrate which in process takes up 2 protons from the periplasm to form nitrite and water as follows (Casey *et al.*, 1999):

$$2H^+ + 2e^- + NO_3^- \longrightarrow NO_2^- + H_2O$$

2.1.3.2 Nitrite reductase

The function of nitrite reductase is to reduce nitrite to nitric oxide. Nitrite reductase situated on the periplasmic side of the membrane accepts one electron from cytochrome *c* and at the catalytic site of the reductase the electron is passed to nitrite, which in the

process also takes up 2 protons from the periplasm to form nitric oxide (NO) and water as follows (Casey *et al.*, 1999):

$$2H^+ + e^- + NO_2^- \longrightarrow NO + H_2O$$

2.1.3.3 Nitric oxide reductase

The function of nitric oxide reductase is to reduce nitric oxide to nitrous oxide. A feature which distinguishes nitric oxide reductase is to reduce nitric oxide to nitrous oxide. A feature which distinguishes nitric oxide reductase from the other nitrogen oxide reductases is that it is the site at which the N-N bond of N_2O is formed and two molecules of NO are required for the formation of 1 molecule of N_2O. Nitric oxide reductase receives a pair of electrons from cytochrome *c* and at the catalytic site of the reductase the electrons are passed to 2 molecules of nitric oxide which in the process take up 2 protons to form one molecule each of nitrous oxide and water as follows (Casey *et al.*, 1999):

$$2H^+ + 2e^- + 2NO \longrightarrow N_2O + H_2O$$

2.1.3.4 Nitrous oxide reductase

The function of nitrous oxide reductase is to reduce nitrous oxide to dinitrogen. Nitrous oxide reductase situated on the periplasmic side of the membrane receives a pair of electrons from cytochrome c and at the catalytic site of the reductase the electrons are passed to nitrous oxide which in the process takes up 2 protons from the periplasm as follows (Casey *et al.*, 1999):

$$2H^+ + 2e^- + N_2O \longrightarrow N_2 + H_2O$$

2.2 The Development of Molecular Biology

Understanding that DNA contains the blueprint for all life (Avery *et al.*, 1944) led to the discovery of the structure of the DNA molecule ca. 1953 by Watson and Crick in 1953 and the "cracking of the genetic code" about a decade later (Jacob and Monod, 1961; Nirenburg and leder, 1964; Khorana, 1966). Manipulative techniques showing that nucleic acid molecules can be cut into smaller fragments and copied (Smith, 1970; Southern, 1975; Grunstein and Hogness, 1975) and that its informational content can be interpreted (Maxam and Gilbert, 1977; Sanger *et al.*, 1977) and controlled (Tacon *et al.*, 1980; Russel and Bennet, 1981; Amann *et al.*, 1983) has given birth to the field of study we now call 'recombinant DNA technology'

For most of the history of microbiology, microbiologists have struggled with simplicity of bacterial morphology and phenotypic characters in an attempt to construct an evolutionarily valid taxonomy for the prokaryotes (Stackebrandt and Woese, 1981). Zuckerkandl and Pauling (1965) were among the first to note that molecular sequences were the best source of evolutionary information. With the advent of nucleic acid sequencing and phylogenetic inference methods, the way from phenotypic to genotypic characteristics for evolutionary inference was clear. Ribosomes within biological cells are the sites of protein synthesis. They are composed of a mixture of nucleic acids (ribosomal Ribonucleic acids- rRNA) and proteins, and have an average size of 70S in bacteria. Because of their central role in cell survival, maintenance and reproduction, rRNAs and their genes are almost universally present in cellular life forms, are functionally constrained and therefore are described as being evolutionally conserved (Olsen and Woese, 1993). Within the Bacteria, the small subunit rRNA is the 16S rRNA and the genes that code for this molecule are 16S rDNAs. In most cases, the 16S rDNA is exactly transcribed to form the 16S rRNA i.e. the primary nucleic acid sequences of these two molecules are the same. Additionally, ribosomes of Bacteria contain the larger 23S rRNA (genes = 23S rDNAs) and the sequence information from 23S rDNAs is also used to address evolutionary relationships between different Bacteria (Blackall *et al.*, 1998)

2.2.1 The rRNA revolution

16S rRNA was the first molecule examined for studying prokaryotic phylogenies, and has played a seminal role in evolutionary studies. Between 1965 and the mid-90s, 16S rRNA has had influence at all levels of bacterial systematics, and was used to demonstrate the phylogenetic relationship between the eubacteria, eukaryotes and the archaebacteria. It was demonstrated that eubacterial sequences are far closer to each other than they are to archaebacteria and also pointed towards a theoretical organism from which all life has evolved (Woese, 1987). The use of 16S rRNA sequence information thereby led to a proposal by Woese *et al.* (1990), stating that there should be a new taxon called a domain established above the level of kingdom. All life on this planet is therefore divided into the three domains Bacteria, Archaea and Eucarya (Woese *et al.*, 1990).

Information from rRNA sequences demonstrated that very little was known about bacterial phylogeny, and what was known was often wrong. Before rRNA sequencing, it was believed that bacteria fell into one of two groups – Gram-positive and Gram-negative. This was an almost truth, where Gram-positive turned out to be a coherent grouping, but the Gram-negative group consisted of 10 distinct groups each as distinguishable as the Gram-positive group. Many other groupings previously based on morphological or otherwise 'behavioural' attributes were also changed. This included the mycoplasmas, which in lacking a cell wall were segregated, however rRNA sequencing indicates that they are merely degenerate clostridia. Similarly, the segregation of autotrophs and heterotrophs, were shown not to be phylogenetically separate groupings but intermixed with numerous other bacterial phyla. 16S rRNA is particularly useful for the resolution of the bacteria phylum branching orders, therefore showing the progression of bacterial evolution (Woese, 1987).

In addition to these major taxonomic resolutions, there are various examples of how 16S rRNA sequence information has assisted in the resolution of bacterial genera. One of the best examples of how this information has been applied to such a problem is with the bacterial order actinomycetes (Blackall *et al.*, 1994).

2.2.2 Analysis of rRNA molecules

The first attempts to characterize environmental samples by studying rRNA began about more than a decade ago. In these studies, 5S rRNA molecules were directly extracted from mixed samples, the molecules belonging to the different community members were electrophoretically separated, and a comparative sequence analysis yielded phylogenetic placements (Lane *et al.*, 1985; Stahl *et al.*, 1985; Amann *et al.*, 1995). These pioneering studies yielded interesting insights. However, the information content in the approximately 120-nucleotide 5S rRNA is relatively small, and the requirement for electrophoretic separation of the different 5S rRNA molecules limited this approach to less complex ecosystems. Consequently, the use of larger rRNA molecules for studies in microbial ecology were implemented (Olsen *et al.*, 1986)

An average bacterial 16S rRNA molecule has a length of 1,500 nucleotides, and 23S rRNA molecules are around 3,000 nucleotides. When fully or almost fully analyzed (>1,000 nucleotides should be determined), both molecules contain sufficient information for reliable phylogenetic analysis (Amann *et al.*, 1995).
The principle steps of the proposed procedure were:

- Extraction of total community DNA
- Preparation of shot-gun DNA library in bacteriophage lamda
- Screening by hybridization with a 16S rRNA-specific probe
- Sequence determination from clones containing 16S rRNA genes and
- Comparative analysis of the retrieved sequence

The first thorough application of this approach was the characterization of a marine picoplankton sample (Schmidt *et al.*, 1991). Numerous unknown sequences could be identified in the library. Among those were 15 unique bacterial sequences related to the cyanobacteria and proteobacteria and one eucaryotic sequence.

With the advent of PCR (Saiki *et al.*, 1988), a method became available to speed up this quite laborious procedure. By using PCR, 16S rRNA gene fragments can be selectively

amplified from mixed DNA. Gene libraries derived from mixed amplification products should contain only defined fragments that can be rapidly sequenced from known priming sites. Giovannoni *et al.*, (1990) was the first to apply this approach in an analysis of Sargasso Sea picoplankton. Results indicated again the presence of defined clusters of proteobacterial and cyanobacterial origin.

Finally, there is yet another route to the molecular characterization of natural microbial communities. With techniques such as denaturant gradient gel electrophoresis, it is possible to physically separate PCR products originating from the amplification of rDNA fragments from environmental samples. Besides obtaining a characteristic fingerprint for the examined community, the possibility of isolating less common fragments should allow their specific retrieval (Muyzer *et al.*, 1995).

Kowalchuk *et al.*, (1997) used denaturing gradient gel electrophoresis of specifically amplified 16S rRNA gene fragments to analyze the β-subdivision ammonia-oxidizing populations from coastal sand dune samples. This approach coupled with sequence analysis allowed the detection of specific groups of β-subdivision ammonia oxidizers and permitted their presence to be correlated with specific environmental factors.

2.3 Polymerase Chain Reaction

The polymerase chain reaction (PCR) for gene amplification was first introduced by Saiki and colleagues in 1985. PCR quickly and efficiently produces many copies of specific DNA regions. Standard DNA cloning methods achieve similar results with viral or plasmid vectors, but with much greater effort. Thus, it is evident that PCR will replace or modify many applications of cloning technology. The main limitations of PCR are the size of the region that can be amplified, and the requirement for some knowledge of the sequences flanking the 'target' DNA. (Giovannoni, 1991).

The PCR reaction mixture consists of template DNA, deoxynucleotide triphosphates (dNTPs), primers and DNA polymerase (Taq polymerase). PCR reactions can be broken down into three steps (Fig. 2.4), which are repeated in cycles:

- The melting of duplex sample DNA (94°C)
- The annealing of two primers to opposite DNA strands (53°C)
- The extension of primers by enzymatic nucleotide additions to produce a copy of the gene (72°C)

The specificity of the PCR reaction is governed by the oligonucleotide primers which hybridize to opposite DNA strands at opposite ends of the DNA target, and direct the replication of the intervening region. Thus, the mechanism of PCR is simple primer-directed DNA synthesis, with the added dimensions that the process is repetitive, and the number of copies produced increases exponentially (Giovannoni, 1991).

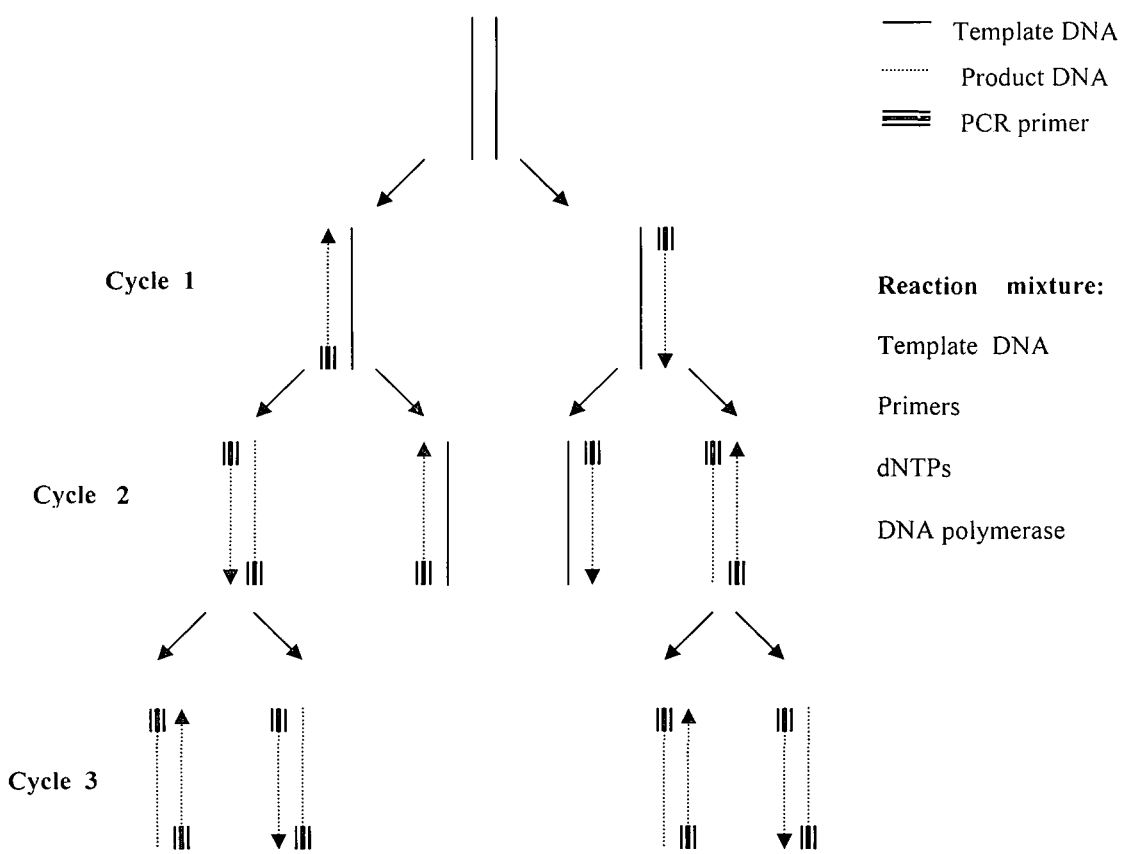


Figure 2.4: A schematic outline of the polymerase chain reaction (Giovannoni, 1991).

In theory, with each replication cycle, the number of copies of the DNA target doubles. Copies of the target DNA are produced according to the equation:

$$N = N_0 I^x$$

Where x is the number of cycles, N_0 is the starting number of copies, and I is the efficiency of the reaction expressed as the number of complete target molecules produced per cycle from each template molecule. In theory, the maximum value for I is 2. An example will serve to illustrate the remarkable outcome of this process. Starting with a single copy of 1-Kilobase piece of DNA, after 36 complete cycles of synthesis, 10^{11} copies, or 100ng, of DNA would be produced (Giovannoni, 1991).

2.3.1 *Thermus aquaticus* DNA Polymerase

The first PCR experiments used the Klenow fragment of *Escherichia coli* DNA polymerase 1 to catalyze the extension of the annealed primers (Saiki *et al.*, 1985). The Klenow fragment is irreversibly denatured at 94°C. After each denaturing step fresh enzyme was added to the reaction. The process was tedious and prone to errors. The substitution of the thermostable DNA polymerase isolated from *Thermus aquaticus* (Taq polymerase; Chen *et al.*, 1976) circumvented these problems (Saiki *et al.*, 1988). In addition, the primer elongation step of the reaction could be run at higher temperatures, improving specificity. The result was a greatly simplified procedure that increased the yield, sensitivity, and the length of products that could be amplified.

Thermus aquaticus is a thermophilic eubacterium that can routinely be isolated from hotsprings and household water heaters. Innis and colleagues (1988) studied the enzymology of the *T. aquaticus* DNA polymerase. Taq DNA polymerase exhibits an unusually high optimal processing activity, 8000 bases per minute at 75°C, and significant extension rates at much lower temperatures: 90 bases per a minute at 37°C

and 15 bases per a minute at 22°C. Taq polymerase does not possess a 3' exonuclease activity, which may in part explain the high processing rate of the enzyme (Giovannoni, 1991).

2.3.2 The Design of Amplification Primers

PCR amplification primers are simply pairs of hybridization probes that must act in concert. Each probe must be relatively specific for its binding site. If one or both of the primers fails to hybridize, there will be no PCR products. If the primers hybridize non-specifically, which is often the case with degenerate primers when used at conditions of low hybridization stringency, then non-specific amplifications will occur (Giovannoni, 1991).

Usually PCR primers are synthetic DNA molecules 20-30 bases in length. In theory, any specific 20-base-long DNA sequence will occur in random DNA segments of 3.4×10^6 bases (the size of the *E. coli* genome) with a frequency of $(1/4)^{20} \times 3.4 \times 10^6 = 3.09 \times 10^{-6}$ (Giovannoni, 1991).

With highly conserved molecules, such as rRNAs, it is possible to design amplification primers that will universally amplify all genes. In this study the variable V3 region of 16S rDNA was enzymatically amplified in the PCR with primers to conserved regions of the 16S rRNA genes. Primers 1 and 2 (chapter 5) was used to amplify the 16S rDNA regions in different bacterial species which correspond to position 341 to 534 in *E. coli* (Fig. 2.5) (Muyzer *et al.*, 1993; Giovannoni, 1991)

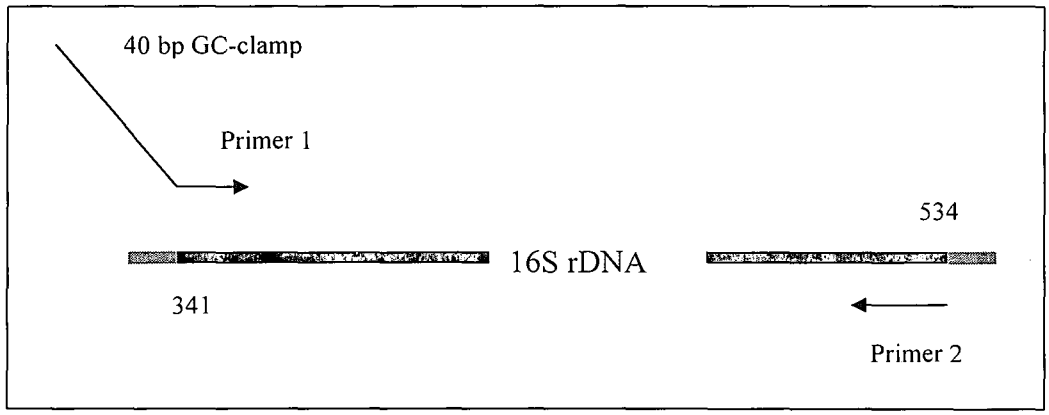


Figure 2.5: Schematic diagram of the rDNA region amplified by PCR. Primers 1 and 2 amplify a fragment, which corresponds to position 341 and 534 in the 16S rDNA of *E. coli*. The fragment is incorporated with a 40-bp GC clamp at its 5' end at position 341 (Muyzer *et al.*, 1993).

2.3.3 Sources of Error using PCR

2.3.3.1 Taq Polymerase Reading Errors

Replication errors brought about by the misincorporation of bases are a potential source of concern when PCR products are cloned and sequenced. Innis *et al.*, data (1988) reported an error rate of one mistake in 4000 to 5000 base pairs after 35 cycles of PCR. Dunning *et al.*, (1988) reported 22 probable mistakes in 8000 nucleotides sequenced, an error rate of one mistake per 364 nucleotides (0.3%). Innis *et al.*, suggest that misincorporations promote chain terminations, thus attenuating the amplification of defective molecules.

In the study of microbial systematics, the highest error rates reported (0.0025) are negligibly small when compared to the similarities observed between microbial species. Thus, it is highly unlikely that a mistake in phylogenetic inference would result from Taq polymerase base misincorporation errors (Giovannoni, 1991).

2.3.3.2 Chimeric Gene Products

One consideration in the use of PCR for the amplification of genes from mixed populations of homologous genes (such as rRNA genes in mixed microbial population DNA) is the concern that chimeric amplification products might be created. The formation of chimeric products can occur by a mechanism in which some templates are not copied completely during a period of primer extension. During ensuing cycles the partial copies could re-anneal to related homologues, to be further extended on a second template. The result would be the creation of chimeric genes that contain regions copied from different homologues (Dunning *et al.*, 1988; Giovannoni, 1991)

Innis *et al.*, (1988) reported that Taq polymerase is highly processive. In the presence of sufficient dNTP substrates, premature terminations are rare events. This suggests that chimeric genes, like misincorporated nucleotides, may be the result of low dNTP substrate concentrations in late cycles. Accordingly, Giovannoni (1991) suggest limiting the number of amplification cycles so that no more than 25% of the initially present substrate nucleotides are incorporated.

2.4 Denaturing gradient gel electrophoresis

There are three types of DGGE gels that can be run to detect mutations in DNA. The first gel type is a perpendicular gradient gel, in which the gradient is perpendicular to the electric field and uses a broad denaturing gradient range, such as 0-100% or 20-70% (Fisher and Lerman, 1983). From this gel one can determine the concentration of denaturants in which the wild-type and mutant fragments can be separated. The other types of gels are parallel DGGE and constant denaturing gradient gel electrophoresis (CDGE). In parallel DGGE, the denaturing gradient is parallel to the electric field, and the range of denaturants is narrowed to allow better separation of fragments (Myers *et al.*,

1987). In CDGE there is no gradient. Instead the optimum denaturing concentration which allowed separation in perpendicular DGGE is used (BRL, 1994).

When running a denaturing gel, both the mutant and wild-type DNA fragments are run on the same gel. This way one can detect a mutation by seeing a band shift on the gel. In some cases, it is possible to detect a mutation by running just homoduplex DNA, for example, if the mutation is a base change from A to G (G-C pairing has higher melting temperature than A-T pairing) (Myers *et al.*, 1987). The method of heteroduplex analysis helps in resolving wild-type and mutated fragments when it is not possible to detect a mutation by running homoduplex fragments, for example if the mutation is a base change from A to T. Heteroduplexes can be formed by adding the wild type and mutant template sequence in the same PCR reaction or by adding separate PCR products together, then denaturing and allowing them to re-anneal. A heteroduplex has a mismatch in the double strand causing a distortion in its usual shape; this has a destabilizing effect and causes the DNA strands to separate at a lower concentration of denaturant (Fig. 2.6). The heteroduplex bands always migrate more slowly than the corresponding homoduplex bands (Myers *et al.*, 1987; BRL, 1994)

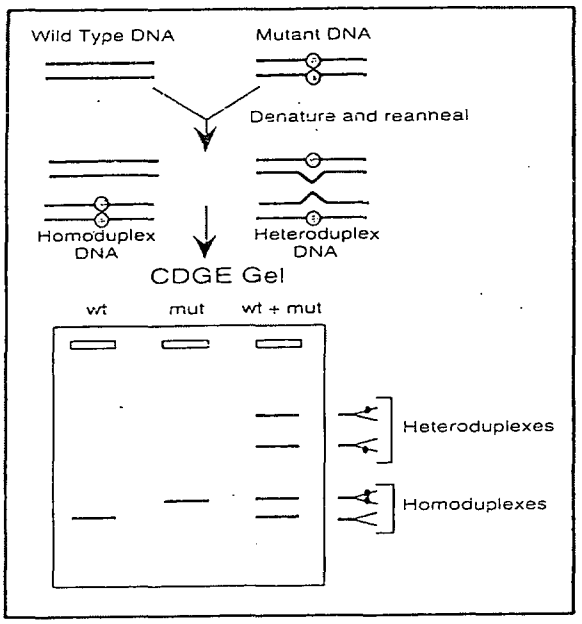


Figure 2.6: An example of wild-type and mutant DNA fragments that were denatured and re-annealed to generate four fragments, two Heteroduplexes and two homoduplexes (adapted from BRL, 1994).

2.4.1 Principle of the Method

DNA molecules are separated in denaturing gradient gels according to differences in the stringency of conditions required to melt 50-200 bp of the helix (Lerman *et al.*, 1984). Melting is regarded as an equilibrium between two well-defined states for each base pair, that of the double helix and that of a more nearly random chain in which bases are neither paired nor stacked on adjacent bases in an orderly way. The change to the latter state is effected by a combination of temperature and the composition of solvent in the gel (Myers *et al.*, 1987). A simplified description of denaturing conditions is possible since the effect of the denaturants used in the system, urea and formamide, appears to be fully equivalent to further elevation of the temperature, and sequence dependent differences in melting appear to be the same, regardless of which agent effects the change (Lerman *et al.*, 1981; Myers *et al.*, 1987).

The portion of a sequence in which a GC pair replaces an AT will melt at a slightly higher temperature, or equivalently, at a slightly higher concentration of urea and formamide in the gel (Fisher and Lerman, 1983). The structural difference caused by the presence of opposing bases that do not constitute a Watson-Crick pair in otherwise complementary strands substantially lowers the melting temperature of that portion of the molecule or equivalently, lowers the denaturant concentration at which melting takes place (Myers *et al.*, 1987).

The electrophoretic mobility of a fully helical DNA molecule in a gel appears to be insensitive to the composition or sequence of the helix, except with respect to minor effects where certain sequence patterns introduce a persistent curvature of the helix. Under conditions that maintain complete melting and strand dissociation, the separated strands exhibit an electrophoretic mobility in the gel considerable lower than that of a double helical molecule (Myers *et al.*, 1987).

The progression of melting of a DNA molecule as the temperature is gradually raised occurs as a succession of more or less well-defined steps, where each step represents the simultaneous change in state of a considerable number, 50-300, of contiguous base pairs. All of the pairs comprising a segment of this kind melt within a relatively narrow temperature interval. Each such block of base pairs is conventionally termed a domain; each domain is usually clearly demarcated from the adjacent domains on either side. Small changes in the properties of a molecule, such as the replacement of a single base pair by a different base pair, raises or lowers the melting temperature of the domain that includes the substitution without significant effects beyond its boundaries (Myers *et al.*, 1987). The positions along the base sequence that correspond to domain boundaries can be identified by examining the effects of a series of closely spaced single base substitutions and by computation using sequence-specific melting theory, which shows a close agreement with the experimental results (Myers *et al.*, 1985a; Myers *et al.*, 1985b).

More substantial changes in the molecule, such as sequence alteration involving many base pairs or the lack of perfect Watson-Crick complementarity in the molecule, can alter domain boundaries, establish new domains, or result in the merger of adjacent domains that were otherwise slightly different (Myers *et al.*, 1987).

The typical length of domains is roughly 50-300 bp under the conditions prevailing in denaturing gradient gels. The effective persistence length of 35-40 bases characterizes the flexibility of melted strands of DNA. Consequently only the melting of the first or the first few domains gives a melted section of at least 91 bp, the length required to reduce the electrophoretic mobility of a partly melted DNA molecule to 20% of its mobility when fully helical. The use of the denaturing gradient gels to examine changes that occur after further mobility reductions is not feasible. Further melting usually results in the complete dissociation of the two strands in molecules shorter than roughly 1 kb. Thus, only those changes in sequence that occur in the lowest temperature domains can be detected by denaturing gradient gel electrophoresis (Myers *et al.*, 1985b).

Composite molecules can be constructed in which nearly any sequence can constitute the lowest temperature domains by attachment to a very high-melting portion. The high melting portion is described as a "GC clamp" (Fisher and Lerman, 1983; Shefield *et al.*, 1989).

Shefield *et al.*, (1989) cloned a high-temperature melting G+C-rich sequence, designated a GC clamp, into a plasmid vector next to the mouse β^{major} globin promoter. This cloned GC-clamp increased the number of mutations in the promoter detectable by DGGE from 40% of all possible single-base changes to close to 100%.

In the experimental configuration that is most useful for comparing a large number of similar molecules and for preparative process, the electrophoretic field drives the molecule into an ascending gradient of the denaturant mixture. The limits of denaturant concentration at the top and bottom of the gel and the ambient temperature are selected such that melting transition in the domain of interest takes place somewhere in the middle

region of the gel. Each molecule begins its migration at the top, moving rapidly as a more or less complete double helix, then drops to a much lower mobility as the first domain melts, or drops stepwise as the first few domains melt. The run is terminated when all molecules of interest have dropped to low mobility. The drop in mobility not only renders the final position of the molecule in the gel relatively insensitive to the duration of the electrophoretic run, but also results in the substantial sharpening of the band (Myers *et al.*, 1987).

2.4.2 Perpendicular DGGE

Perpendicular DGGE a useful step in the analysis of any DNA fragment. This technique is used to determine the number of melting domains and the optimum denaturing conditions. If the sequence is not known, it is useful and advisable to examine the relationship between electrophoretic mobility and denaturant concentration. This relation is displayed as a sigmoid curve in a gel where migration is at right angles to the denaturing gradient and the sample is applied as a continuous line across the top of the gel, such that each molecule travels in a path of constant denaturant concentration (Fisher and Lerman, 1979). Thus the electrophoretic velocity is constant along any part and is approximately proportional to the distance of the curve from the top of the gel. This configuration is designated a perpendicular gradient gel (Fig. 2.7). The inflection in the curve directly indicates the melting midpoint of the relevant domain. The ratio of the low denaturant velocity to that of the final, high denaturant plateau indicates roughly the size of the domain (Myers *et al.*, 1987; BRL, 1994)

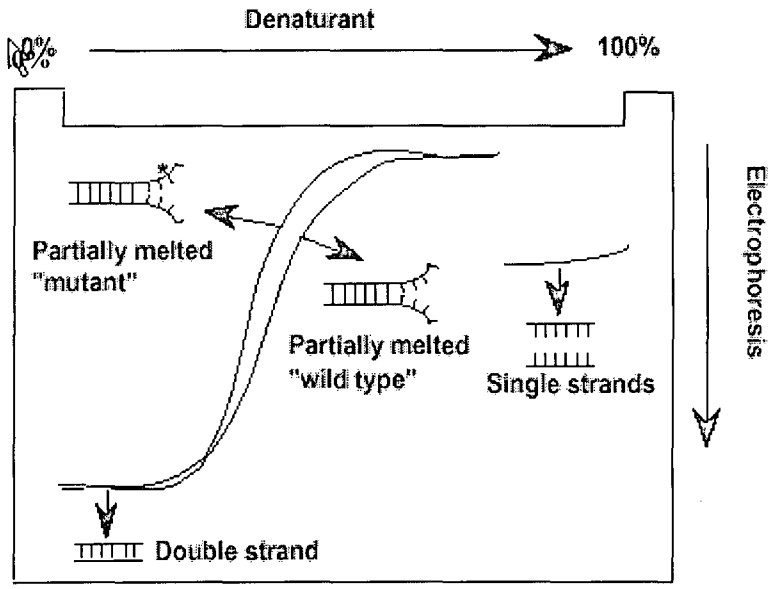


Figure 2.7: An example of DNA melting properties in a perpendicular denaturing gradient gel (adapted from BRL, 1994)

2.4.3 Parallel DGGE

Examination of a large number of fragments and preparative separations are more conveniently carried out in the gel configuration where the path of the molecule is parallel to the gradient, such that each molecule travels through an ascending denaturant concentration and reaches a gradient level where its continued migration would be exceedingly slow. Samples are loaded into adjacent lanes and appear as simple, short bands at characteristic positions along the lane (Myers *et al.*, 1987).

2.4.3.1 Analysis of Natural Microbial Population

Recently, denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA gene segments has been used to profile complex microbial communities (Muyzer *et al.,* 1993) and to infer the phylogenetic affiliation of the community members (Muyzer *et al.*, 1994; Muyzer *et al.*, 1995). Ferris *et al.*, (1996) used DGGE to profile microbial populations inhabiting different temperature regions in the microbial mat community. Two known cyanobacterial populations and one known green nonsulphur bacterium-like population were detected by DGGE. A new cyanobacterial 16S rRNA sequence type was detected at temperatures from 63 to 75°C and a new green nonsulphur bacterium-like sequence type was also detected at temperatures from 53 to 62°C (Ferris *et al.*, 1997).

The use of DGGE for the analysis of the microbial community of activated sludge provides a powerful means for detecting perturbations in community structure. Marsh *et al.*, (1998) applied DGGE to an activated sludge community maintained in a laboratory-scale bioreactor. Over the course of five weeks, analysis with DGGE revealed dramatic changes in the eukaryotic community based on differences in the denaturation profiles. Curtis and Craine, (1998) used DGGE to compare the bacterial diversity of activated sludge plants and to determine the optimal sample regime for comparative studies.

DGGE process allows rapid comparison of bacterial diversity in activated sludge plants. DGGE approach can help answer important questions about which bacteria undertake which functions in activated sludge. Answering these questions will be important step towards the explicit engineering of bacterial populations in activated sludge and other bacterial treatment processes (Curtis and Craine, 1998).

2.5 Respirometric Batch assay

2.5.1 Growth rate in batch reactors

Some biological reactors, e.g. anaerobic digesters, are operated as batch reactors, where the waste containing some microorganisms is place in the reactor and mixed at a constant temperature. After a given time the reactor contents are emptied and disposed of and the reactor is recharged. Since there is no flow of the substrate or bacteria into or out of the reactor in batch processes, the concentrations of substrate and bacteria vary with time. If a small number of innoculum bacteria are placed into a substrate and nutrient rich liquid, the mass of bacteria as well as the substrate remaining within the liquid will vary according to Figure 2.7. Initially the bacterial population remains constant during a lag phase during which time the cells become accustomed to the new media. After the lag phase of up to several hours, the organisms begin to grow and multiply during the acceleration phase. During this second phase, the many intermediates involved in the metabolic reaction chain build up to steady levels. Once the organisms have become accustomed to the media, they multiply very rapidly according to the first order reaction rate (Schroeder, 1977),

$$\frac{dX}{dt} = kX \qquad (2.1)$$

where

X = weight of dry cells/volume

K = specific growth rate, time^{-1}

Since the integral of Eq. (2.1) is a logarithmic expression, this growth is called a log phase. During this phase, there is a high food to microorganism (So/Xo) with a very high fraction of cells being viable; cell deaths are not important. Eventually the food is consumed to a point where there are too many organisms and not enough food left to sustain the rapid growth rate during the log phase.

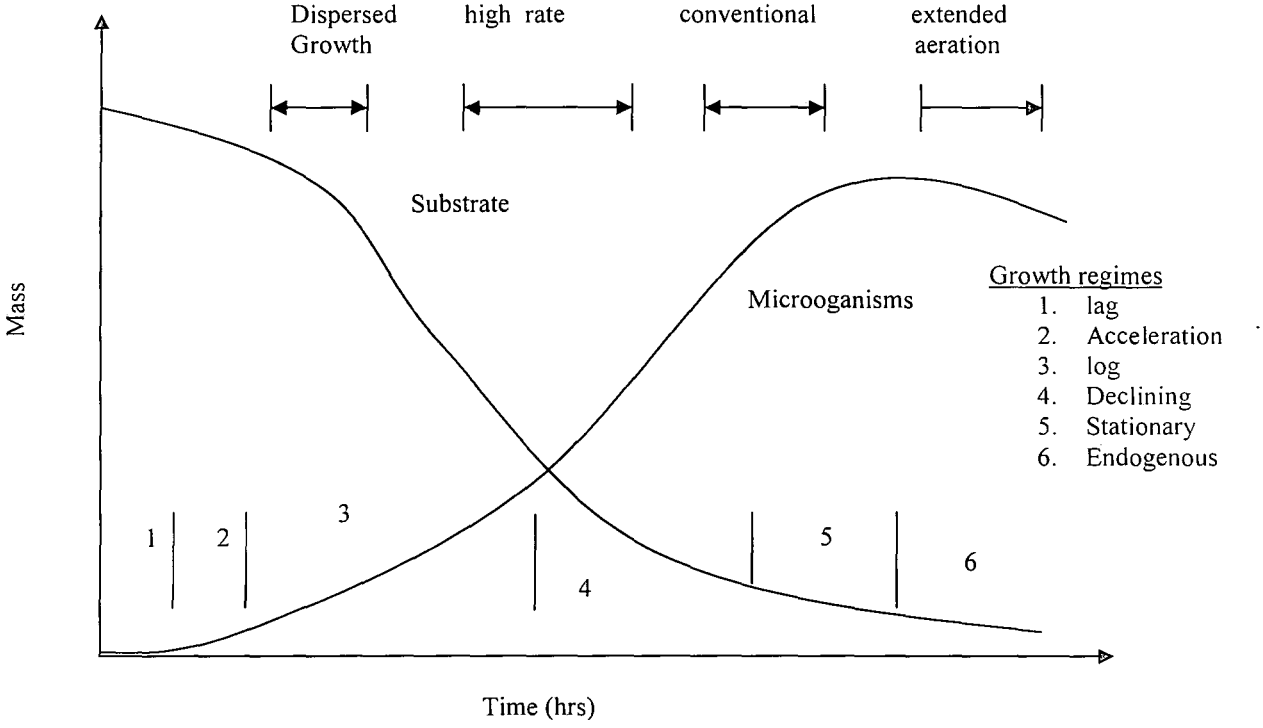


Figure 2.8: Typical growth curves for a batch reactor (Schroeder, 1977)

Essential nutrients are also depleted and toxins, e.g. acids or alcohols, accumulate during this declining growth phase. As the food concentration becomes limiting, the death rate of the organisms increases until the stationary phase is reached where the death rate is nearly equal to the rate of cell synthesis. Eventually the food supply will become low enough to cause the death rate to be greater than the synthesis rate and a decrease in the viable cell concentration occurs. During this endogenous phase the cells use the stored ATP energy for respiration and motion until the ATP is depleted and the cells die. The cell wall then ruptures, releasing carbon-containing compounds as food for the remaining viable bacteria (Bitton, 1995; Schroeder, 1977).

2.5.2 The So/Xo ratio

The variations in relationship between biomass growth and substrate removal rate, during batch experiments have been studied by numerous authors (Fujimoto, 1963; Rao and Gaudy, 1966; Gaudy and Ramanathan, 1967). However the various experimental data found in the literature cannot be compared because of different operational conditions used. In order to make them suitable for more correct interpretation, the data obtained from batch experiments have to be related to a universal parameter. According to Pitter and Chudoba (1990), such a parameter is the ratio of initial substrate concentration (So) to initial biomass concentration (Xo). This ratio is one of the most important parameters in batch cultivations, determining whether or not cell multiplication will take place during exogenous substrate removal (Chudoba *et al.*, 1992).

Microbial growth reflects an increase in biomass with (multiplication) or without (storage or accumulation) the increase in cell number (Pitter and Chudoba, 1990). The increase in biomass due to cell multiplication results from the oxidation of extracellular substrates, to obtain energy and also to synthesize all components of the biomass (Daigger and Grady, 1982). The storage response involves only the transport of substrates, the oxidation of a smaller portion, and the synthesis of storage polymers like carbohydrate or lipid.

Storage or accumulation processes have been observed most usually during batch cultivation when microbial growth is limited by low amount of utilizable carbon and energy source, with respect to the innoculum concentration. Thus, at low So/Xo ratio, a relatively high amount of biomass is supplied with a low quantity of substrate. The initial energy level is then low as well, and the increase in cell mass reflects only the increase in molecular polymer content in the biomass. Consequently, weight changes do not necessarily reflect similar changes in cell number. Due to the fact that the cell replication is a process energetically enough demanding, no, or only negligible cell multiplication takes place at low So/Xo ratios during exogenous substrate removal (Speece *et al.*, 1973; Pitter and Chudoba, 1990; Chudoba *et al.*, 1992)

At high So/Xo ratio, a relatively low amount of biomass is supplied with a higher quantity of substrate. The initial energy level is higher, and thus sufficient for different synthetic reactions which take place during cell replication cycle, like enzyme, protein and nucleic acids syntheses. Consequently, the number of microorganisms increases during the exogenous substrate removal, which is indicated by increasing rates of substrate removal and biomass growth (Chudoba *et al.*, 1992).

2.5.2.1 Considerations for Determining the So/Xo ratio

Mixed culture of microoganisms (e.g. activated sludge) contains different bacterial species and predators (protozoa, rotifers etc.) which differ in their growth constants. They can be divided into two groups; slow growers and fast growers (Chudoba *et al.*, 1992). Under high So/Xo ratios substantial cell multiplication occurs in a batch cultivation which changes the proportion between slow-growers and fast growers and the measured kinetics will reflect the characteristics of the fastest growing species, resulting in a shift in bacterial community structure away from its original mixed culture. Therefore the measured growth parameters would not be representative of the original treatment environment (Chudoba *et al.*, 1992).

Chudoba *et al.*, (1992) in recognizing the culture history and the nature of the assay procedure, argued in favour of low So/Xo ratios (to limit cell multiplication) during batch tests since they mimic the most common condition of the biomass present in typical full scale, completely mixed, continuous activated sludge process.
This is evident from the following calculation for such a system:

Given:

Influent substrate concentration (S_i)	=	500 mgCOD/L
Effluent substrate concentration (S_e)	=	20 mgCOD/L
Volatile suspended solids	=	2000 mgVSS/L
F_{cv}	=	1.48 mgCOD/mgVSS (WRC, 1984)

Therefore: Xo = VSS x F_{cv}

= 2000 x 1.48

= 2960 mgCOD/L

In such a system the available substrate concentration (S_o) is approximately the same as that in the effluent (S_e)

Therefore;

So/Xo = 20/2960

= 0.0067

This So/Xo ratio (0.0067) would therefore represent the condition of the biomass found in most BNR treatment plants (Chudoba, 1992).

CHAPTER 3

The Modified Ludzack-Ettinger Process for Nitrogen Removal

3.1 Introduction

Ludzack and Ettinger in 1962 were the first to propose a single sludge nitrification-denitrification process utilizing the biodegradable material in the influent as an energy source for denitrification. In 1973, Barnard proposed an improvement of the Ludzack-Ettinger process, by completely separating the anoxic and aerobic reactors, recycling the underflow from the settler to the anoxic reactor, and providing an additional recycle from the aerobic to the anoxic reactor (Fig. 3.1) (WRC, 1984).

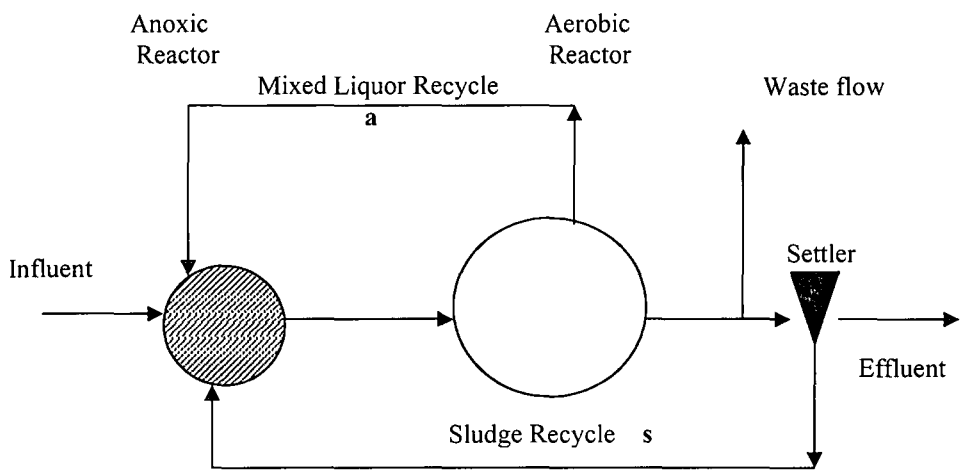


Figure 3.1: The modified Ludzack Ettinger process for nitrogen removal (WRC,1984)

In this process, the influent is discharged directly into the anoxic zone. The anoxic zone is virtually free of oxygen but contains nitrite and nitrate or has a substantial input of nitrate. This zone is fundamental to the biological removal of nitrogen because the absence of oxygen allows non-poly-P organisms to utilize nitrate as electron acceptors, reducing it to nitrogen gas, thus carrying out denitrification of mixed liquor and allowing the elemental nitrogen formed to escape to the atmosphere as a gas. The aerobic zone is aerated by introducing either air or oxygen. In this environment the utilization of biodegradable organic matter is virtually completed while ammonium nitrogen is converted to nitrate by the nitrifiers present in the population of microorganisms A recycle (A-recycle) from the aerobic zone recycles nitrite and nitrate back to the anoxic zone. The underflow recycle (S-recycle) from the clarifiers also recycles nitrite, nitrate and mixed liquor to the anoxic basin. As the influent contains substrates or COD that can be used rapidly, a high rate of denitrification in the anoxic zone is observed. Complete denitrification cannot be achieved because part of the total flow from the aerobic reactor (containing nitrite and nitrate) is discharged directly with the effluent and is not recycled back to the anoxic zone. (Lilly *et al.*, 1997)

The characterization of carbonaceous material in the influent is done via the chemical Oxygen Demand (COD) test (Standard Methods, 1985). The COD of the influent is divided into three main fractions, viz. unbiodegradable, biodegradable and heterotroph active biomass (Figure 3.2). The unbiodegradable COD has two subfractions, the unbiodegradable particulate and unbiodegradable soluble. The biodegradable COD also has two subfractions, slowly biodegradable (SBCOD) and readily biodegradable (RBCOD). Thus for a complete characterization of municipal wastewater, the five COD fractions need to be quantified (Wentzel *et al.*, 1995).

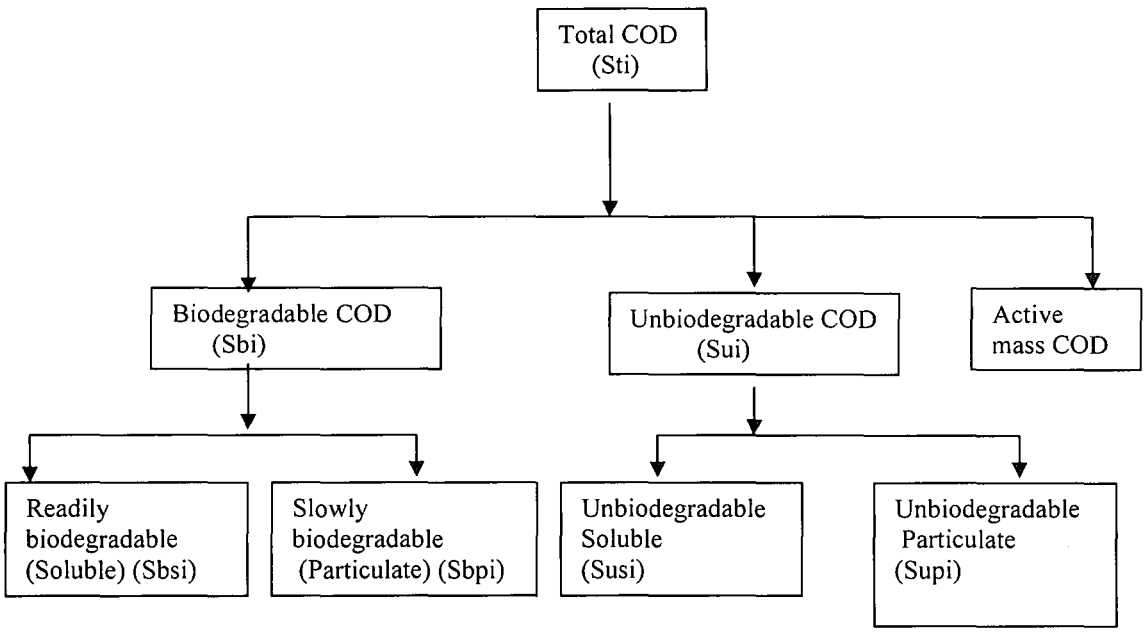


Figure 3.2: Division of influent COD into its constituent fractions (Dold *et al.*, 1991)

Characterization of the nitrogenous material in the influent is with the Total Kjeldahl Nitrogen (TKN) and Kjeldahl free and saline ammonia test (Standard Methods, 1985). As for the carbonaceous material, the nitrogenous material is also subdivided into different fractions. The subdivision is shown in figure 3.3.

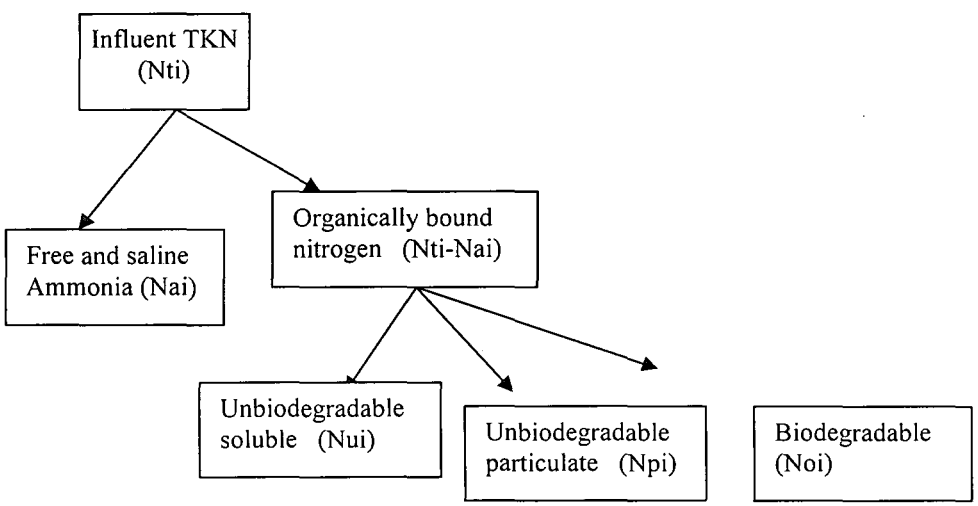


Figure 3.3: Division of the total influent TKN into its constituent fractions (WRC, 1984)

The free and saline ammonia is immediately available for the incorporation into the bacterial protoplasm and for the conversion to nitrite or nitrate. The proteinaceous fraction consists of a number of subfractions, unbiodegradable soluble and unbiodegradable particulate organic nitrogen fractions, Nui and Npi respectively, and a biodegradable organic nitrogen fraction, Noi. The unbiodegradable soluble fraction passes unaffected through the system and is discharged in the effluent similar to the soluble unbiodegradable COD. The biodegradable organic nitrogen is broken down by the heterotrophs to free and saline ammonia which with its influent counterpart participates in any further biologically mediated reactions (WRC, 1984).

3.1.1 Mass balance equations

In the design of activated sludge processes the characteristics of wastewater are of prime importance. These characteristics govern both the selection of the process and the removal of nitrogen attainable in the process. Wastewater characteristics of importance are:

- Mean influent COD and TKN concentrations (S_{ti} and N_{ti} respectively) and mean daily flow (Q)
- Mean effluent COD and TKN concentrations (S_{te} and N_{te} respectively)
- Influent readily biodegradable COD fraction (f_{bs})
- Influent TKN/COD concentration ratio (N_{ti}/ S_{ti})
- Maximum specific growth rate of the nitrifiers at reference temperature of 20°C (μ_{nm20})
- Average minimum and maximum temperatures (T_{min} and T_{max}) in the process

3.1.1.1 COD mass balance

The mean influent COD concentration (S_{ti}) and the mean flow per a day (Q) define the mass COD load day on the process, M (S_{ti}), (WRC, 1984) by

$$M(S_{ti}) = S_{ti} . Q$$

Mass of COD in the effluent is defined by,

$$M(S_{te}) = Q . S_{te}$$

Mass of COD in the waste sludge

$$M(S_{xw}) = f_{cv} . MX_v/Rs$$

Where

X_v = mixed liquor volatile suspended solids (mgVSS)

f_{cv} = COD/VSS ratio of volatile solids (1.48mgCOD/mgVSS)

V_p = total volume of the process reactors (L)

R_s = mass of sludge in reactor / mass of sludge wasted per day

$MX_v = V_p . X_v$

Mass of oxygen required daily for carbonaceous degradation

$$M(O_c) = M(O_{tm}) + M(O_d) - M(O_n)$$

Where

$M(O_{tm})$ = measured oxygen per day

= OUR . 24. % aerobic $V_p/100$

$M(O_n)$ = mass of oxygen used for nitrification

$= 4.57\ M(N_{ng})$

$M(N_{ng})$ = mass of nitrate generated

$= M(N_{ti}) - M(N_{te}) - M(N_s)$

$M(N_{ti})$ = mass of TKN in the influent

$= Q \cdot N_{ti}$

$M(N_{te})$ = mass of TKN in the effluent

$= Q \cdot N_{te}$

$M(N_s)$ = mass of nitrogen incorporated in the VSS per day

$$= \frac{f_n \cdot M(X_v)}{R_s}$$

Where

f_n = fraction of TKN in VSS ~ 0.1 mg (TKN-N)/mgVSS

$M(O_d)$ = mass of oxygen recovered via denitrification

$= 2.86\ M(N_d)$

$M(N_d)$ = mass of nitrate denitrified

$= M(N_{ng}) - M(N_{ne})$

$M(N_{ne})$ = mass of nitrate generated per day

$= Q \cdot N_{ne}$

COD mass balance(%) = $$\frac{M(S_{te}) + M(S_{xw}) + M(O_c)}{M(S_{ti})} \times 100$$

3.1.1.2 Nitrogen (N) mass balance

In the N mass balance, the influent TKN mass must be accounted for by the sum of the mass of TKN and nitrate in the effluent, mass of nitrate denitrified to nitrogen gas in the anoxic reactor and the mass of TKN abstracted through the waste sludge(WRC, 1984):

Mass of Influent TKN = mass of effluent TKN + mass of effluent NO_3 + mass of N denitrified + mass of TKN wasted

$$M(N_{ti}) = M(N_{te}) + M(N_{ne}) + M(N_d) + M(N_w)$$

$M(N_d)$ = mass of nitrate denitrified

= NO_3 mass in - NO_3 mass out

$$M(N_d) = [NO_{3(AE)} \cdot Qi \cdot a + NO_{3(FE)} \cdot Qi \cdot s] - [NO_{3(AN)} \cdot Qi \cdot (1 + a + s)]$$

Where

Qi = influent flow rate

a = a- recylce ratio

s = s- recycle ratio

$NO_{3(AE)}$ = NO_3 concentration in the aerobic reactor

$NO_{3(FE)}$ = NO_3 concentration of the filtered effluent

$NO_{3(AN)}$ = NO_3 concentration of the anoxic reactor

$M(N_{te})$ = mass of TKN in the effluent

= $N_{te} \cdot Qi$

$M(N_{ne})$ = mass of effluent nitrate

= $NO_{3(FE)} \cdot Qi$

$M(N_w)$ = mass of TKN in waste sludge

$$= \frac{f_n \cdot M(X_v)}{R_s}$$

Where

f_n = fraction of TKN in VSS ~ 0.1 mg (TKN-N)/mgVSS

$M(N_{ti})$ = mass of TKN in the influent

$$= N_{ti} \cdot Qi$$

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

3.2 Methodology

3.2.1 Parent System

The parent laboratory-scale system layout and operational details are shown in figure 3.4. The system was fed 30L/d raw (unsettled) municipal wastewater obtained from Southern Works (Durban, South Africa). This wastewater is primarily domestic with no industrial contribution. The wastewater was collected in batches, stored in 25L containers at 4°C and served as feed for both the parent system and batch tests. For the parent system, daily wastewater was drawn from the storage containers after thorough mixing and diluted with tap water from its raw COD of approximately 800 mgCOD/L to give a total influent feed of approximately 500 mgCOD/L.

3.2.2 System performance monitoring and analytical methods

To monitor the system performance, samples were drawn daily for analysis from each of the reactors, anoxic, aerobic and final effluent. Daily monitoring included influent COD (APPENDIX 1), TKN (APPENDIX 2); all reactors nitrate and nitrite; aerobic reactor VSS (APPENDIX 3), COD and TKN, OUR; effluent COD, TKN, nitrate and nitrite. (Nitrite was less than 2% of nitrate and therefore neglected). Oxygen utilization rate (OUR) was monitored continuously online with the DO controller, OUR meter developed by Randall *et al.*, (1991). In addition, aerobic batch tests (Chapter 4) were conducted on sludge drawn from the system to investigate microbial population shifts at defined So/Xo ratios. To ensure steady state, the parent system was run for more than five sludge ages before mixed liquor was harvested for the batch test.

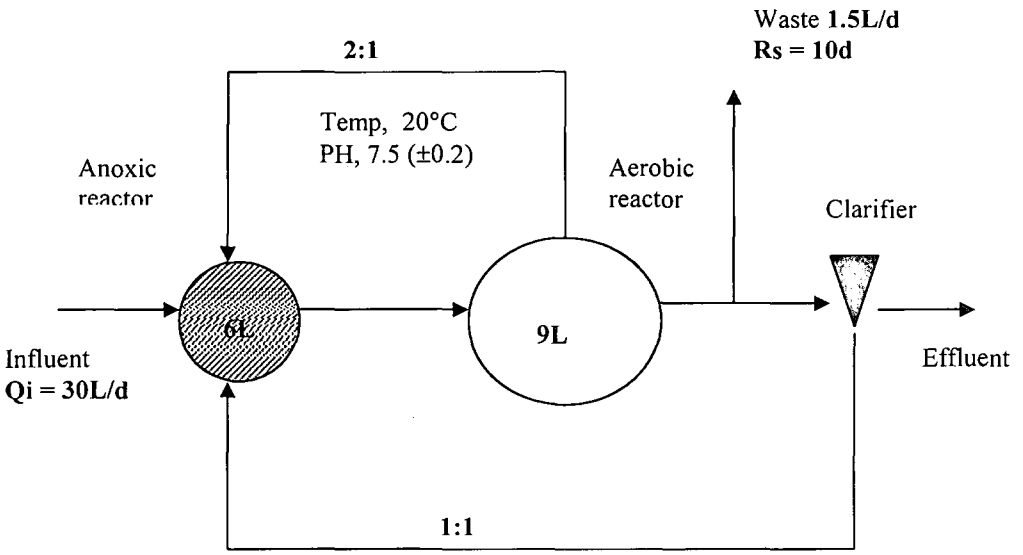


Figure 3.4 : Schematic layout and operational data for parent laboratory-scale Modified . Ludzack Ettinger (MLE) anoxic/aerobic activated sludge system.

3.3 Results

Daily results for the parent activated sludge system are listed in Appendix 7, Table A7. Each sewage batch was accepted as a steady state period. For each of the batches of wastewater fed to the parent system (12 batches), the daily results were averaged and the sample standard deviations calculated (Table 3.1).

Table 3.1: Steady state results for parent laboratory-scale anoxic/aerobic activated sludge

Anoxic/Aerobic steady state system									
WW batch	**COD (mg/l)**		**TKN (mg/l)**		**Nitrates (mgN/l)**			**Aerobic OUR (mgO/l/h)**	**Mixed liquor (mg/l)**
	Inf	Eff	Inf	Eff	Anoxic	Aerobic	Eff		VSS
1	470.93 (25.52)	46.13 (8.82)	29.50 (4.33)	3.07 (0.60)	0.48 (0.50)	1.76 (0.65)	7.58 (3.17)	30.33 (0.31)	2069 (119.7)
2	491.49 (39.34)	48.39 (12.76)	27.84 (5.08)	6.48 (3.25)	0.32 (0.03)	1.28 (0.81)	3.78 (1.21)	29.57 (0.40)	2610 (135.7)
3	481.92 (33.89)	48.42 (11.94)	29.96 (2.32)	4.87 (0.57)	0.29 (0.11)	1.89 (0.45)	4.98 (1.50)	30.57 (0.29)	2195.14 (101.6)
4	497.52 (44.18)	60.08 (16.01)	32.15 (2.94)	4.51 (3.72)	0.1 (0.02)	1.30 (0.45)	6.21 (1.64)	29.64 (0.16)	2363 (112.9)
5	475.4 (28.01)	68.6 (11.89)	34.24 (5.25)	8.04 (4.87)	0.1 (0.01)	1.26 (0.86)	6.01 (1.87)	29.02 (0.24)	2301.71 (85.40)
6	482.82 (31.49)	67.32 (9.00)	31.04 (3.92)	4.86 (1.03)	0.52 (0.21)	1.88 (0.93)	7.25 (0.75)	31.01 (0.86)	2038.4 (187.3)
7	473.892 (25.16)	68.46 (8.02)	32.01 (1.88)	10.08 (1.90)	0.51 (0.29)	1.18 (0.87)	5.14 (1.58)	29.29 (0.24)	1904 (187.1)
8	485.7 (46.75)	58.29 (12.26)	28.21 (2.53)	3.24 (0.71)	0.285 (0.18)	0.98 (0.52)	8.03 (0.85)	32.29 (0.52)	1814.5 (155.9)
9	484.14 (31.35)	51.21 (10.43)	27.27 (5.26)	4.58 (0.91)	0.1 (0.03)	0.39 (0.16)	6.58 (0.95)	30.13 (0.47)	2258 (132.1)
10	473.39 (36.66)	62.8 (20.20)	28.45 (3.88)	3.35 (0.71)	0.1 (0.02)	0.26 (0.51)	7.30 (2.04)	30.54 (0.34)	1912.4 (154.5)
11	489.59 (33.40)	59.19 (11.94)	30.63 (2.99)	4.29 (1.20)	0.1 (0.01)	0.12 (0.06)	7.26 (1.84)	31.23 (0.63)	2237 (131.70)
12	485.16 (35.27)	62.34 (16.03)	27.44 (5.72)	4.38 (0.92)	0.1 (0.004)	0.1 (0.07)	6.30 (1.40)	30.54 (0.73)	2045 (92.26)

It can be seen in Table 3.1 that the average influent COD concentrations varied between 470 and 497 mgCOD/L and average effluent COD concentrations ranged from 46 to 68 mgCOD/L. This was suggestive of the high rate of substrate consumption. Average influent TKN concentrations range from 27 to 34 mg/L and effluent TKN concentrations range from 3 to 10 mg/L. Nitrate concentrations in the anoxic reactor ranged from 0.1 to 0.48 mgN/L, in the aerobic reactor ranged from 0.1 to 1.88 mgN/L and in the effluent ranged from 3.78 to 8.03 mgN/L. Average MLVSS concentrations ranged from 1814 to 2610 mgVSS/L. Average OUR concentrations ranged from 29.02 to 32.29 mgO/h/L.

The system operating and sample analytical procedures and the accuracy of the experimental data were checked by means of COD and N mass balances calculated from the sewage batch averages of measured parameters (Table 3.2).

Table 3.2: Steady state COD and N mass balances for parent anoxic/aerobic activated sludge system.

Anoxic/aerobic steady state system		
WW batch	Mass balance (%)	
	COD	**N**
1	78.43	102.28
2	87.37	101.90
3	81.46	94.89
4	81.10	96.25
5	85.30	98.38
6	81.47	100.61
7	81.27	95.91
8	77.19	103.48
9	81.99	107.84
10	79.58	97.11
11	82.53	97.40
12	81.13	98.41

The COD mass balances fall in the range of 77 to 87% and the nitrogen mass balances fall in the range of 94 to 107%. Acceptable N and COD mass balances were obtained for all 12 wastewater batches.

3.4 Discussion

The parent laboratory-scale activated sludge system was run for more than five sludge ages to ensure steady state. Once steady state was acquired, the mixed liquor was harvested for the batch tests (chapter 4). Steady state data obtained for 12 wastewater batches are represented in Table 3.1. COD entering the system was approximately 500mg/L and in the range of 50-70 mgCOD/L leaving the system. TKN leaving the system was in the range 3-10 mgN/L. $^{TKN}/_{COD}$ ratios > 0.1 were suggestive of a high rate of the nitrogen removal efficiency in the system (WRC, 1984), however this resulted in high effluent nitrate concentrations of > 5mgN/L. The low concentrations of NO_3 in anoxic reactor were evident of the high rates of denitrification in the system. The proportion of COD utilized with nitrate also indicates that the denitrification potential of the system is very high.

COD and N mass balances verified the reliability of the experimental data (Table 3.2). In the COD balance, the COD mass leaving the system via the final effluent flow; oxygen utilized; sludge wasted; nitrate denitrified; and COD utilized in the system is reconciled with the COD mass entering the system with the influent flow (3.1.1.1). In the N balance, the N mass leaving the system via the final effluent flow; sludge wasted; nitrate denitrified; and the nitrogen removed in the system is reconciled with the TKN mass entering the system with the influent flow. The N nitrified and denitrified is calculated from a nitrate mass balance around the reactors (3.1.1.2) (Zhi-rong Hu *et al.*, 2000). The reliability of the experimental data is directly proportional to the mass balance deviation from the target value of 100%, i.e. the closer the mass balances are to 100%, the more reliable the data. The N mass balance falls in the range of 90-110%.

Although the COD and N mass balances of some of batches were lower than 100%, these are similar to COD and N mass balances observed in other investigations with nutrient removal systems, viz. Clayton *et al.*, (1989), 92% and 91%; Ubisi *et al.*, (1997), 84% and 102% and Wentzel *et al.*, (1998), 81% and 92%.

3.5 Conclusion

A biological nutrient removal activated sludge system with MLE configuration for nitrogen removal was proposed. The laboratory-scale evaluation of the system indicates that it holds considerable potential for denitrification. From the 120d laboratory scale evaluation of 10d sludge age acceptable nitrogen and COD mass balances were obtained.

The importance of steady state behavior in this study is to ensure that the mixed liquor has acclimatized to the existing environment, which was observed by the consistencies of the measured parameters. In order to analyze the bacterial community in activated sludge respirometric batch assays and to obtain kinetic constants the mixed liquor has to be representative of the original mixed culture (Chudoba *et al.*, 1992).

CHAPTER 4

The Relationship between Biomass Growth and Substrate Removal

4.1 Introduction

During a batch experiment, an initial substrate concentration (So) is put into contact with an initial quantity of microbial culture (Xo). The parameter So represents the carbon and energy source for biosynthesis, while Xo represents a source of carbon and energy consumption. After being put into contact with the substrate, microorganisms of mixed culture start to remove the substrate, which is demonstrated by changes in substrate removal and biomass growth curves or lines. Therefore the most important parameter in batch cultivation of mixed cultures is the ratio of initial substrate concentration to initial biomass concentration (So/Xo as COD/Biomass) (Chudoba *et al.*, 1992)

At high substrate to biomass ratios the batch assay will measure the maximum rate of substrate oxidation, but it does not represent the growth rate and physiological state of bacteria in the original treatment environment. The greater the batch reactor So/Xo ratio, the greater the change in the bacterial community away from that of the original wastewater environment, and the more likely it is that the measured kinetics will reflect the characteristics of the fastest growing bacteria, or changes in the physiological state of the bacterial community. Hence, lower So/Xo ratios (<2) are preferred in batch assays. Under these conditions, bacterial cell multiplication rate is assumed not to be altered from that of the original treatment environment (Chudoba *et al.*, 1991, 1992). In order to investigate the relationship between biomass growth and substrate removal batch test were set up with various So/Xo ratios. The substrate and biomass were sampled at regular intervals and OUR profiles were determined.

4.2 Methodology

4.2.1 Batch Test Experiment

The mixed liquor (bacterial biomass, Xo) was sampled from the parent system aerobic reactor and the raw sewage (substrate, So) was taken from the influent that had been diluted to 500mgCOD/L. Both were added into a 5L stirred aerobic batch reactor (figure 4.1) and run as described by Ekama *et al.* (1986). To inhibit autotrophic nitrifying bacterial growth, thiourea was added to the mixed liquor to a final concentration of 20mg/L. The OUR was monitored continually using an automated technique (Randall *et al.*, 1991). The dissolved oxygen (DO) concentration was logged continuously with a DO probe linked to a computer. The DO was allowed to decrease from 5mg/L to 2mg/L and the mixture aerated until the DO again reached 5mg/L. OUR was represented by the negative slope of the DO concentration profile during the non-aerated periods. The pH was maintained at 7.4 (±0.2) and the temperature at 20°C (±2°C). Because of the low OUR values, the walls of the reactor was thoroughly brushed during aeration cycles to prevent particulate matter adhering to them. Samples of 50mL mixed liquor were taken every 1-hour over a period of 16 hours for COD (APPENDIX 1), MLVSS (APPENDIX 3) and DNA extractions (APPENDIX 4).

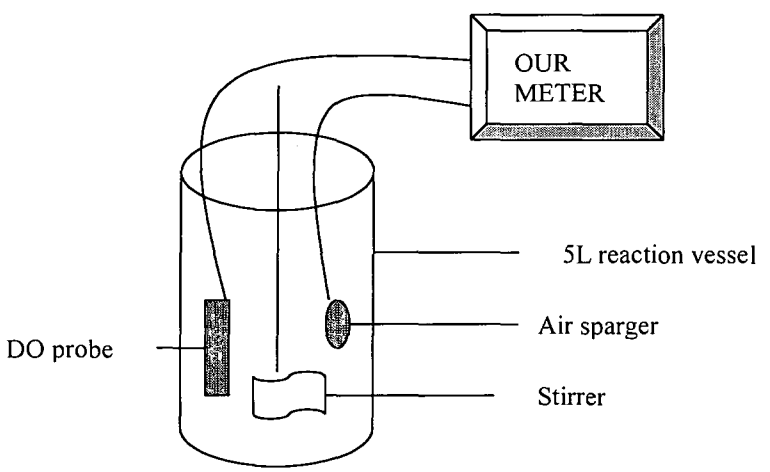


Figure 4.1: A schematic representation of stirred aerated Batch reactor.

4.2.2 Measurement of COD, MLVSS and determination of So/Xo ratio

In order to investigate biomass growth and substrate removal five batch tests were conducted at defined So/Xo ratios (0.8, 1.5, 4.0, 8.0 and 11). Before mixing, the COD (APPENDIX 1) and MLVSS (APPENDIX 3) concentrations of the sewage feed and mixed liquor were measured. The initial substrate to biomass ratio (So/Xo) was determined by dividing COD (mg/L) measurements of raw sewage by those of the mixed liquor. The COD was adjusted to approximately 500mg/L and the mixed liquor volatile suspended solids was measured and adjusted according to the So/Xo ratio. Taking f_{cv}= 1.48mgCOD/mgVSS (WRC, 1984), So/Xo (on a COD basis) was calculated as follows:

Batch test 1 :

COD (So) = 500 mg/L
MLVSS = 417 mgVSS/L

Xo = 417 mgVSS/L x 1.48 mgCOD/mgVSS
= 617 mgVSS/L
So/Xo = 0.8

Batch test 2 :

COD (So) = 500 mg/L
MLVSS = 225 mgVSS/L

Xo = 225 mgVSS/L x 1.48 mgCOD/mgVSS
= 333 mgVSS/L
So/Xo = 1.5

Batch test 3 :

COD (So) = 500 mg/L
MLVSS = 84 mgVSS/L

Xo = 84 mgVSS/L x 1.48 mgCOD/mgVSS
= 124 mgVSS/L
So/Xo = 4.0

Batch test 4 :

COD (So) = 500 mg/L
MLVSS = 42 mgVSS/L

Xo = 42 mgVSS/L x 1.48 mgCOD/mgVSS
= 62 mgVSS/L
So/Xo = 8

Batch test 5 :

COD (So) = 500 mg/L
MLVSS = 30 mgVSS/L

Xo = 30 mgVSS/L x 1.48 mgCOD/mgVSS
= 45.45 mgVSS/L
So/Xo = 11

4.3 Results

Batch test 1

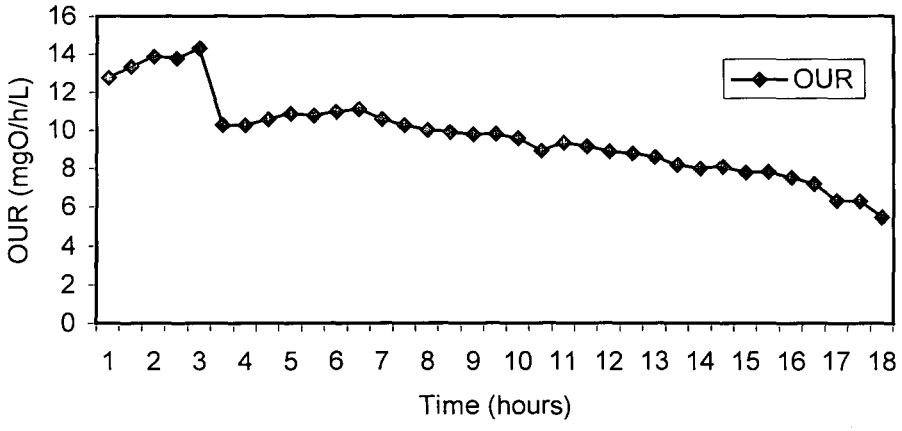


Figure 4.2 : OUR response with time for aerobic batch test with So/Xo ratio of 0.8.

At first the OUR measurements gives a very fast response (approximately 14mgO/h/L) and then after a short period, the respiration rate drops to approximately 10mgO/h/L. Thereafter decreasing at a steady rate to approximately 5mgO/h/L.

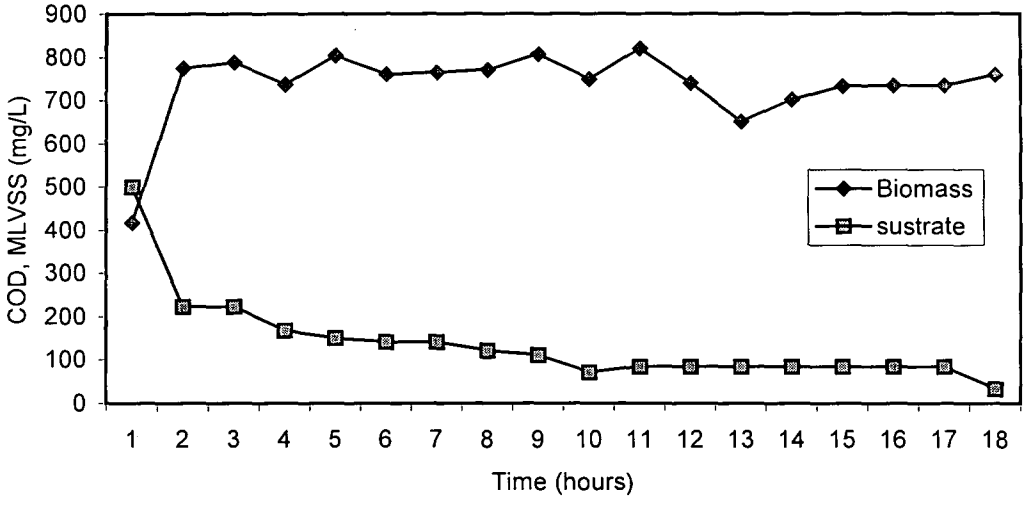


Figure 4.3 : Substrate removal and biomass growth curve for aerobic batch test with So/Xo ratio of 0.8

Figure 4.3 represents substrate removal and biomass growth curves, obtained in a batch system with mixed culture (activated sludge) fed with a substrate concentration of 500 mgCOD/L. Initially there is a rapid increase in biomass from 417 mgVSS/L to 774 mgVSS/L in the system over the first two hours of the batch test. The substrate is consumed at a rapid rate, first decreasing to approximately 200 mgCOD/L for first two hours thereafter decreasing at a slower rate over the next 16 hours to approximately 30 mgCOD/L.

Batch test 2

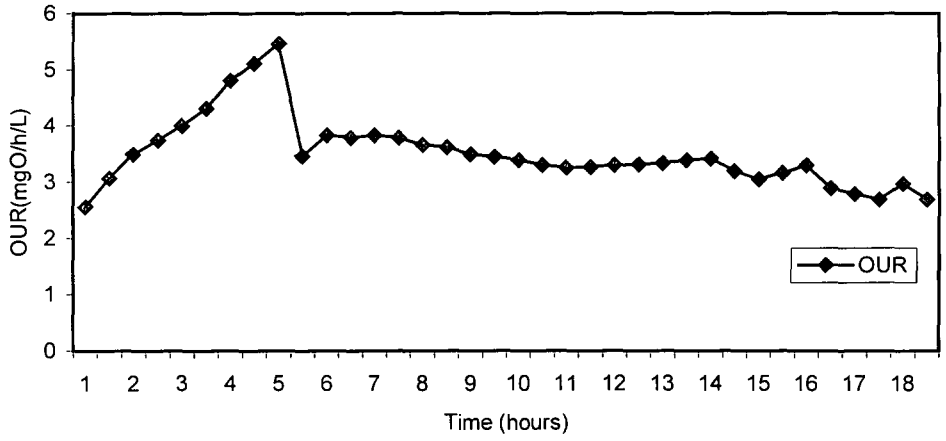


Figure 4.4 : OUR response with time for aerobic batch test with So/Xo ratio of 1.5.

The graph shows a characteristic profile of an initial peak OUR (approximately 6mgO/h/L) due to the rapid consumption of readily biodegradable COD, after a short period the OUR drops to approximately 3mgO/h/L, thereafter a slowly levelling out until endogenous respiration is reached.

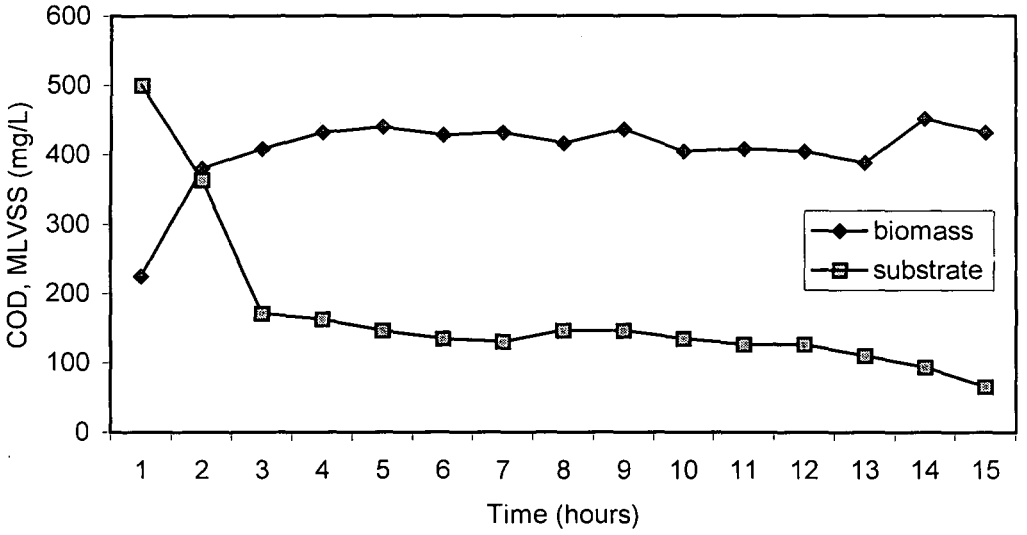


Figure 4.5 : Substrate removal and biomass growth curve for aerobic batch test with So/Xo ratio of 1.5.

Figure 4.5 represents substrate removal and biomass growth curves, obtained in a batch system with mixed culture (activated sludge) fed with a substrate concentration of 500 mgCOD/L Initially there is a rapid increase in biomass from 225 mgVSS/L to 380 mgVSS/L in the system over the first two hours of the batch test. The substrate is consumed at a rapid rate, first decreasing to approximately 200 mgCOD/L for first two hours thereafter decreasing at a slower rate over the next 13 hours to approximately 65 mgCOD/L.

Batch test 3

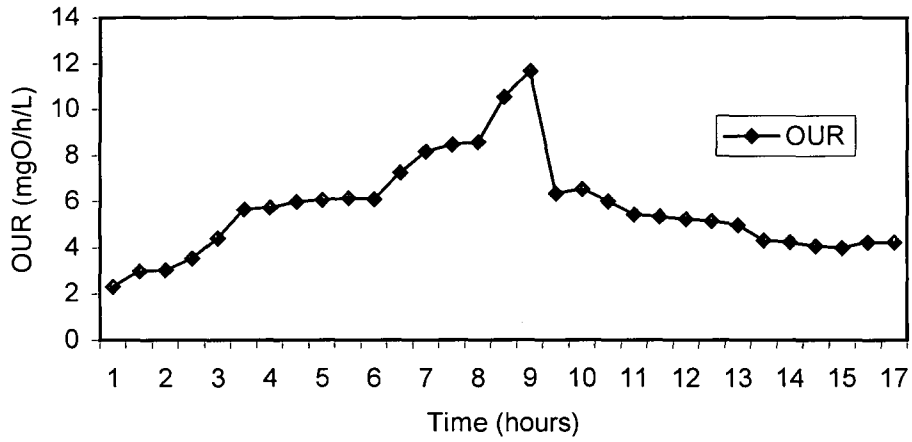


Figure 4.6 : OUR response with time for aerobic batch test with So/Xo ratio of 4

The graph shows an initial slow increase in OUR which peaks at approximately 12mgO/h/L. This is then followed by a rapid decrease to approximately 6mgO/h/L, thereafter slowly levelling out until endogenous respiration is reached.

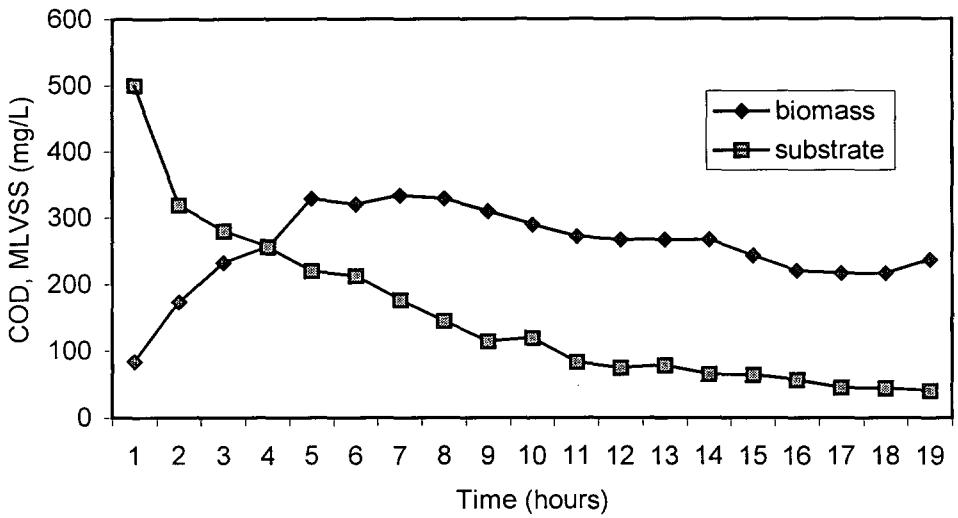


Figure 4.7 : Substrate removal and biomass growth curve for aerobic batch test with So/Xo ratio of 4.

Figure 4.7 represents substrate removal and biomass growth curves, obtained in a batch system with mixed culture (activated sludge) fed with a substrate concentration of 500 mgCOD/L. Initially there is a gradual increase in biomass from 84 mgVSS/L to 329 mgVSS/L in the system over a period of five hours. The substrate is consumed at a rapid rate, first decreasing to approximately 319 mgCOD/L for first two hours thereafter decreasing at a steady rate over the next 17 hours to approximately 40 mgCOD/L.

Batch test 4

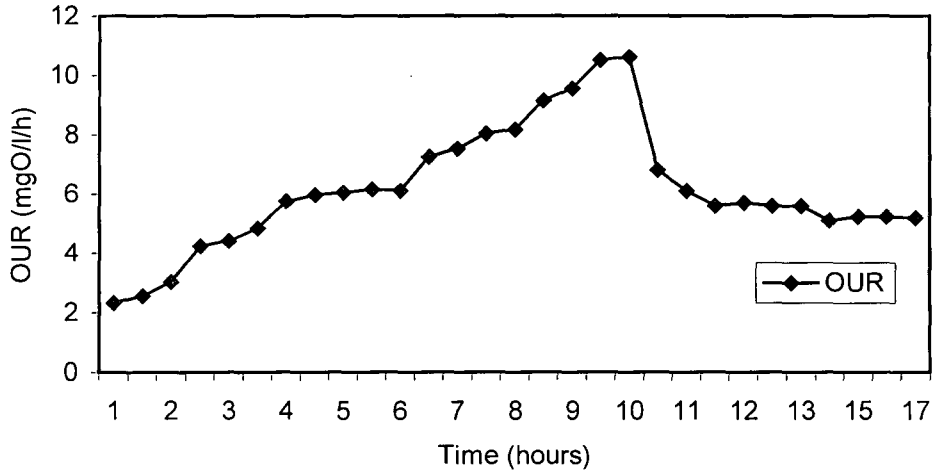


Figure 4.8 : OUR response with time for aerobic batch test with So/Xo ratio of 8.

The OUR response for Figure 4.8 has an almost linear increase which results in an OUR peak phase of approximately 11mgO/h/L. After a long period there is a sudden drop to approximately 6mgO/h/L followed by a slow levelling out until the endogenous respiration is reached.

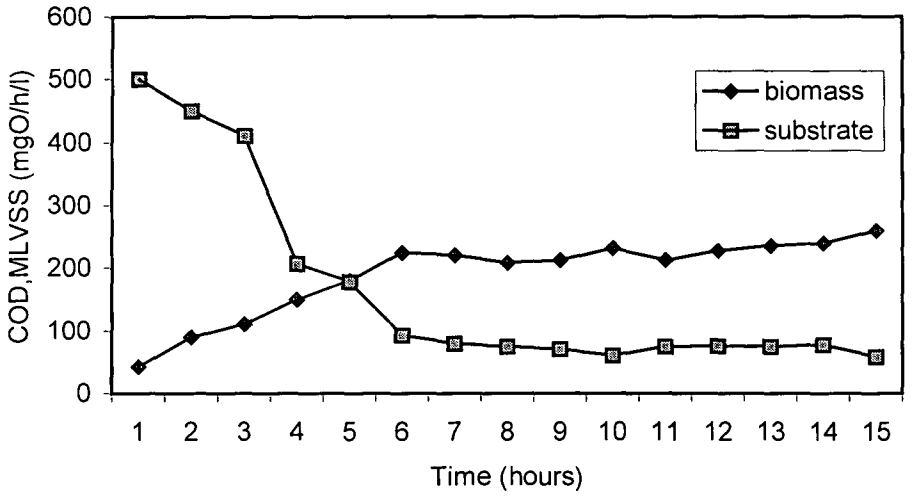


Figure 4.9 : Substrate removal and biomass growth curve for aerobic batch test with So/Xo ratio of 8.

Figure 4.9 represents substrate removal and biomass growth curves, obtained in a batch system with mixed culture (activated sludge) fed with a substrate concentration of 500 mgCOD/L. There is a gradual increase in biomass from 42 mgVSS/L to 225 mgVSS/L in the system over a period of six hours. The substrate is consumed at a gradual rate, decreasing to approximately 411 mgCOD/L after the first three hours thereafter decreasing at a steady rate over the next 12 hours to approximately 58 mgCOD/L.

Batch test 5

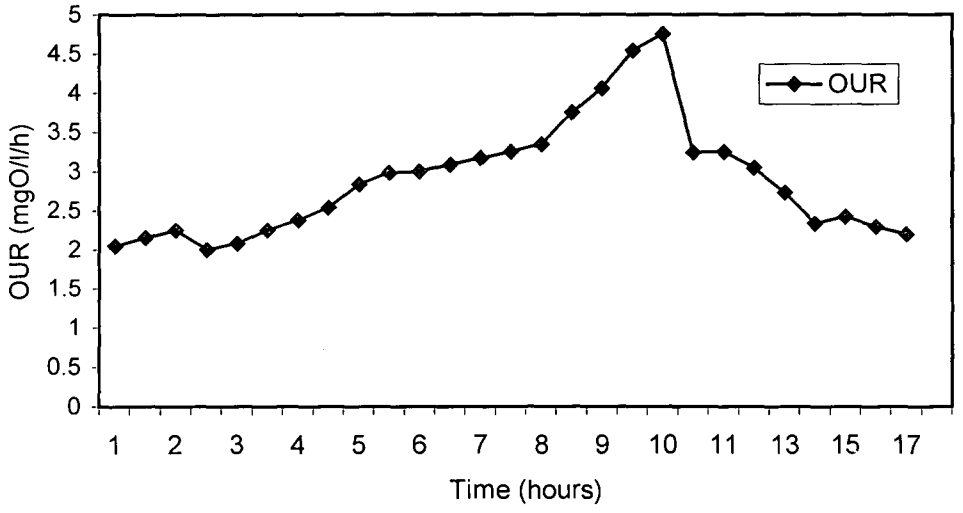


Figure 4.10 : OUR response with time for aerobic batch test with So/Xo ratio of 11.

The OUR response in figure 4.10 has similar look as the OUR response in figure 4.8. First a slow increase to approximately 5 mgO/h/L then a rapid decrease to approximately 3 mgO/h/L and after this a slow levelling out until the endogenous respiration is reached.

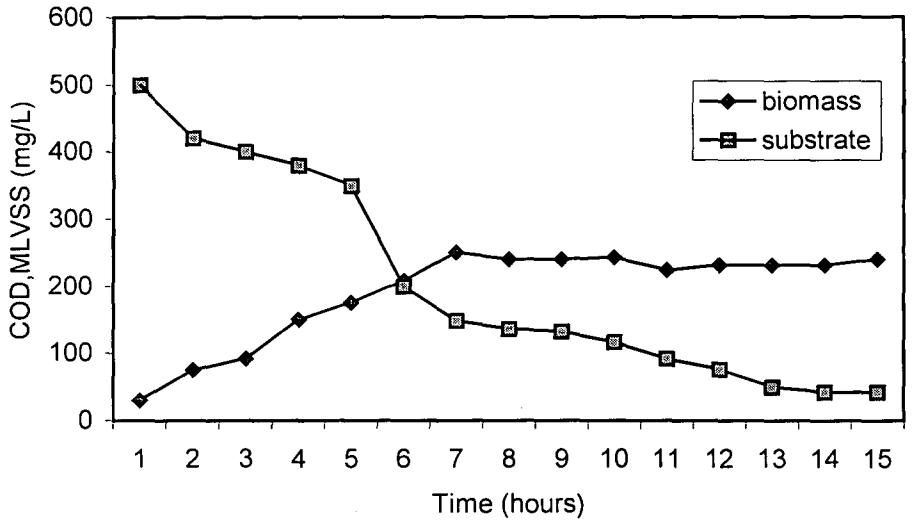


Figure 4.11 : Substrate removal and biomass growth curve for aerobic batch test with So/Xo ratio of 11.

Figure 4.11 represents substrate removal and biomass growth curves, obtained in a batch system with mixed culture (activated sludge) fed with a substrate concentration of 500 mgCOD/L. Initially there is a gradual increase in biomass from 30 mgVSS/L to 250 mgVSS/L in the system over a period of seven hours. The substrate is consumed at a gradual rate, decreasing to approximately 200 mgCOD/L after the first five hours thereafter decreasing at a steady rate over the next 10 hours to approximately 42 mgCOD/L.

4.4 Discussion

Storage or accumulation processes have been observed most usually during batch cultivation when microbial growth is limited by low amount of utilizable carbon and energy source, with respect to the innoculum concentration. Both Figure 4.3 and 4.5 show substrate removal and biomass growth curves with decreasing rates, typical for a system without cell multiplication during exogenous substrate removal (Chudoba *et al.*, 1992). Thus at low So/Xo ratio (Fig. 4.3 and 4.5) a relatively high amount of biomass is supplied with a low quantity of substrate. The initial energy level is then low as well, and the increase in cell mass reflects only the increase in molecular polymer content in the biomass. Consequently, weight changes do not necessarily reflect similar changes in cell number. Due to the fact that the cell replication is a process energetically enough demanding, no, or only negligible cell multiplication takes place at low So/Xo ratios during exogenous substrate removal (Speece *et al.*, 1973; Pitter and Chudoba, 1990; Chudoba, 1991).

The first increase of the OUR curve (Fig 4.2 and 4.4) can be explained by the adaptation of the organisms to this sudden much higher concentration of organic substrate or it could be caused by growth of the biomass. Then the curve exhibits a sudden drop, indicating that the added substrate has been removed from the wastewater. The sudden drop of the OUR curve is then followed by a constantly decreasing respiration which is probable due to the transformation of a secondary substrate (storage polymer) or it could be due to more difficult access to the remaining primary substrate in the flocs (Dircks *et al.*, 1999).

Under high So/Xo (Fig. 4.7, 4.9, and 4.11) the growth and substrate consumption curves have sigmoidal shapes. The rates of biomass growth and substrate removal increase in the initial stage of the batch experiment and after reaching a maximum, decrease (Chudoba *et al.*, 1992). The increase in cell number (cell multiplication) is indicated by an acceleration of both biomass growth and substrate removal rates (Speece *et al.*, 1973)

Thus at high So/Xo ratios a relatively low amount of biomass is supplied with a higher quantity of substrate. The initial energy level is higher, thus sufficient for different synthetic reactions which take place during cell replication cycle, like enzyme, protein and nucleic acids syntheses. Consequently, the number of microorganisms increases during the exogenous substrate removal, which is indicated by increasing rates of substrate removal and biomass growth. It is obvious that the portion of substrate consumed for energy production by catabolic processes will be higher as well, because of higher energetic demand of cell multiplication and maintenance processes (Chudoba *et al.*, 1992).

The OUR curves for aerobic batch test with high So/Xo ratios (Fig 4.6, 4.8 and 4.10) exhibits an initial almost linear increase, this is probably due to the organisms adapting to a very high concentration of substrate or it could be caused by the increase in biomass (Dircks *et al.*, 1999). Then the curve exhibits a sudden drop, indicating that the added substrate has been removed from the wastewater. The sudden drop of the OUR curve is followed by a tail phase due to the metabolism of exogenous slowly biodegradable COD (Wentzel *et al.*, 1995) or internal storage compounds (Dircks *et al.*, 1999).

By only monitoring OUR it is not possible to tell how the cell has allocated its resources. Small variations in cell maintenance have been shown to have a great influence on cell growth (Stouthamer and van Versevld, 1987). Characteristically, adding nutrients to batch cultures stimulates bacterial growth and alters the physiology of the bacteria (Dawes and Sutherland, 1992). Pollard *et al.*, (1998) have suggested that when excess substrate was added to the batch reactor, bacteria progressively channelled more energy into new cell synthesis and less into cell maintenance. The rapid increase in bacterial growth was a normal response of bacteria that have been exposed to a sudden increase in the nutrients. These changes were due to either bacteria adapting to the new nutrient environment (Dawes and Sutherland, 1992), or the selection of sub-populations of bacteria that were more able to efficiently use the higher concentration of nutrients (Grady *et al.*, 1996).

Energy generated from the oxidation of substrate by the bacterial cell in the batch reactor environment is allocated into a complex array of cellular functions. Oxygen utilization rates (OUR) are related only to the initial energy generated from substrate oxidation. On its own, the OUR does not indicate how the cell used the energy from substrate oxidation. The energy resource may be allocated to cell maintenance, new cell synthesis, endogenous polymers (that can again be oxidized) and secreted exopolymers (Jorand *et al.*, 1995)

More recently, others (Grady *et al.*, 1996; Ellis *et al.*, 1996) similarly explained that So/Xo ratios must be low for kinetic growth parameters to represent those of the original treatment environment. The purpose of low ratios is to ensure minimal changes of the bacterial physiological state and community structure away from those of the original treatment process.

4.5 Conclusion

It has been shown that So/Xo ratio is one of the most important parameters in batch cultivation. It determines whether or not cell multiplication will take place during exogenous substrate removal. According to the value of this ratio, it is possible to estimate which processes will take place during batch experiment, accumulation of storage polymers under low So/Xo ratios and cell multiplication under high So/Xo ratios (Chudoba *et al.*, 1992)

An So/Xo ratio of 0.8 and 1.5 has proven suitable for representing the biomass of the original treatment environment since negligible cell multiplication has occurred. It can therefore be expected that the deviation from the community present in the original treatment environment will be negligible and that the batch test conducted at these ratios is representative of the biomass active in the full scale plant.

CHAPTER 5

Determination of Microbial Population profiles at defined So/Xo ratios

5.1 Introduction

The activated sludge process comprises a complex enrichment culture of a mixture of microorganisms. A wide range of bacteria can be isolated using conventional microbiological techniques of sample dilution and spread plate inoculation. However, these techniques are known to be bias the types of microorganisms that are obtained in pure culture and many well known activated sludge bacteria such as *Microthrix parvicella* cannot be isolated using them (Blackall *et al.*, 1994). For most habitats the vast majority of microorganisms are not cultivatable using standard techniques, leading Amann *et al.* (1995) to suggest that these techniques created the "Great Plate Count Anomaly".

Denaturing gradient gel electrophoresis provides a new approach for directly determining the genetic diversity of complex microbial populations. The procedure is based on electrophoresis of PCR-amplified 16S rDNA fragments in polyacrylamide gels containing a linearly increasing gradient of denaturants. In DGGE, DNA fragments of the same length but with different base-pair sequences can be separated (Fisher and Lerman, 1979).

Separation in DGGE is based on the electrophoretic mobility of a partially melted DNA molecule in polyacrylamide gels, which is decreased compared with that of the completely helical form of the molecule. The melting of fragments proceeds in discrete so-called melting domains: stretches of base pairs with an identical melting temperature. Once the melting domain with the lowest melting temperature reaches its melting temperature at a particular position in the DGGE gel, a transition of helical to partially

melted molecules occurs, the migration of the molecule will practically halt. Sequence variation within such domains causes their melting temperatures to differ. Sequence variants of particular fragments will therefore stop migrating at different positions in the denaturing gradient and hence can be separated effectively by DGGE (Lerman *et al.*, 1984).

This technique has been successfully applied to identifying sequence variations in a number of genes from several different organisms (Muyzer *et al.*, 1993). DGGE can be used for direct analysis of genomic DNA from organisms with genomes of millions of base pairs. This is done by transferring separation patterns to hybridization membranes by capillary blotting with modified gel media (Borresen *et al.*, 1988) or by electroblotting (Lerman *et al.*, 1984) followed by analysis with DNA probes. Alternatively, PCR can be used to selectively amplify the sequence of interest before DGGE is used (Cariello *et al.*, 1988). In modification of the latter method, GC-rich sequences can be incorporated into one of the primers to modify the melting behavior of the fragment of interest to the extent to which all possible sequence variations can be detected (Myers *et al.*, 1985b; Muyzer *et al.*, 1993).

Denaturing gradient gel electrophoresis of enzymatically amplified 16S DNA encoding rRNA (rDNA) was used as a molecular approach for the analysis of microbial population shifts in five different batch tests with defined So/Xo ratios (previously discussed in Chapter 4). Bacterial DNA was extracted at regular intervals, amplified by PCR and microbial population profiles of each batch test were determined by DGGE.

5.2 Methodology

5.2.1 DNA extractions

Samples of 25mL were taken in one-hour intervals from each of the five-batch test previously discussed in chapter four. DNA was extracted using phenol chloroform and precipitated with absolute ethanol (APPENDIX 4). The phenol chloroform extraction was repeated four times until the interface after centrifugation appeared clear. These DNA preparations were used as template DNAs in the PCR.

5.2.2 PCR

The variable V3 region of 16S rDNA was enzymatically amplified in the PCR with primers to conserved regions of the 16S rRNA genes. The nucleotide sequences of the primers are as follows: primer 1, 5'-CGCCCGCCGCGCCGGCGGGCGGGGCGGG GGCACGGGGGGCCTACGGGAGGCAGCAG-3'primer 2, 5'-ATTACCGCGGCTG CTGG-3' corresponding respectively to positions (in *E. coli*) 341-358 and 534-517. Primer 1 has at its 5' end a 40-nucleotide GC-rich sequence (GC clamp). A combination of primers 1 and 2 was used to amplify the 16S rDNA regions (Muyzer *et al.*, 1993). PCR amplification was carried out on a Hybaid Thermal Cycler (Hybaid limited, Ashford, UK) in 0.2ml reaction volumes. The reaction mixture and PCR procedure is detailed in APPENDIX 5.

5.2.3 DGGE

All DGGE gels were prepared using a D-gene denaturing gel electrophoresis system (Bio-Rad, Hercules, Ca, USA). PCR products were loaded onto a 7.5% (wt/vol) acrylamide gel (37.5:1, acrylamide: N,N'-methylene-bis-acrylamide) in 1 x TAE buffer with gradients which were formed with 7.5% (wt/vol) acrylamide stock solutions. An optimum denaturant range of 20% to 50% was determined. Parallel DGGE gels

(APPENDIX 6) containing a linear gradient of 20% to 50% parallel to the direction of electrophoresis were run with PCR products obtained from batch test samples. Gels were run for 3 hours at a constant 200V at 60°C by which time the bands had stabilized. Gels were stained with ethidium bromide (1mg/ml) and photographed with UV transillumination (302nm) (Hoefer Pharmacia Biotech Inc, USA). DGGE profiles of batch tests at defined So/Xo ratios were determined.

5.3 Results

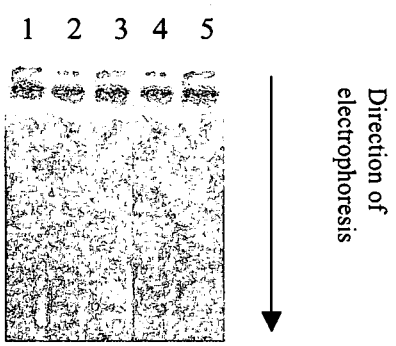


Figure 5.1: Negative image of an ethidium bromide-stained DGGE pattern of PCR products obtained from the aerobic reactor mixed liquor before being harvested for the batch test. The PCR products were eletrophoresed on a 20-50% denaturing, 7.5% polyacrylamide gel. Lanes 1-5 represents the original mixed culture of each of the batch test respectively.

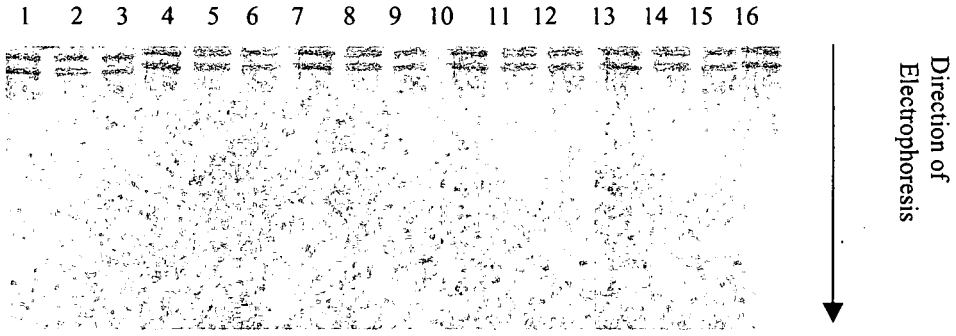


Figure 5.2: Negative image of an ethidium bromide-stained DGGE pattern of PCR products obtain from a batch test with So/Xo ratio of 0.8. The batch test was sampled every hour for a period of 16 hours. The PCR products were eletrophoresed on a 20-50% denaturing, 7.5% polyacrylamide gel. Lanes 1-16 represents the hourly intervals at which the samples were taken.

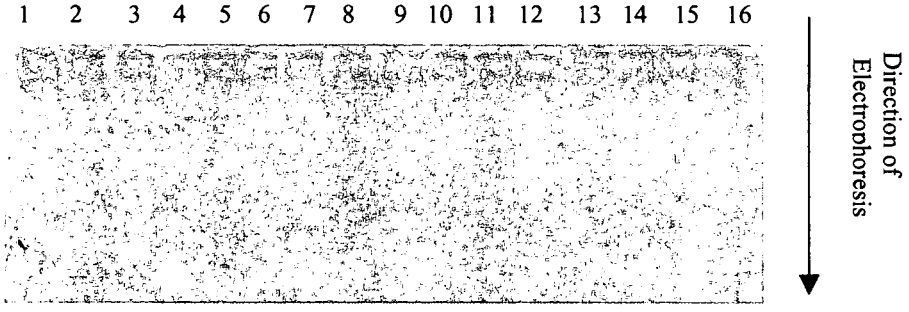


Figure 5.3: Negative image of an ethidium bromide-stained DGGE pattern of PCR products obtain from a batch test with So/Xo ratio of 1.5. The batch test was sampled every hour for a period of 16 hours. The PCR products were eletrophoresed on a 20-50% denaturing, 7.5% polyacrylamide gel. Lanes 1-16 represents the hourly intervals at which the samples were taken.

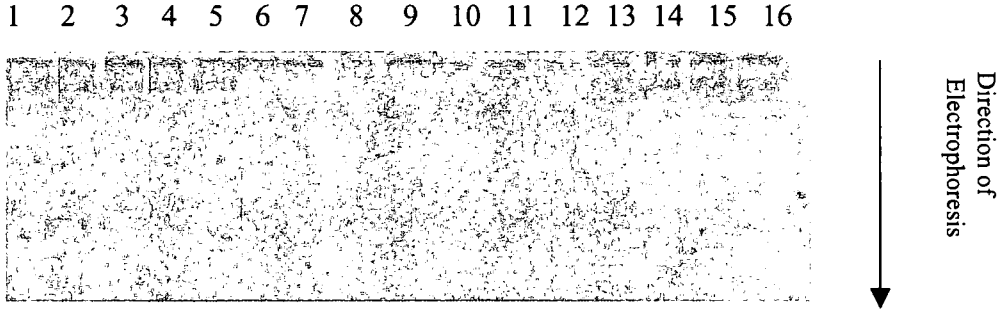


Figure 5.4: Negative image of an ethidium bromide-stained DGGE pattern of PCR products obtain from a batch test with So/Xo ratio of 4. The batch test was sampled every hour for a period of 16 hours. The PCR products were eletrophoresed on a 20-50% denaturing, 7.5% polyacrylamide gel. Lanes 1-16 represents the hourly intervals at which the samples were taken.

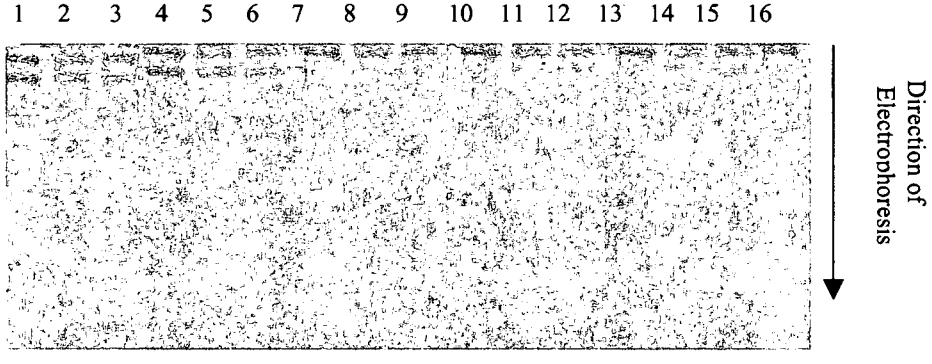


Figure 5.5: Negative image of an ethidium bromide-stained DGGE pattern of PCR products obtain from a batch test with So/Xo ratio of 8. The batch test was sampled every hour for a period of 16 hours. The PCR products were eletrophoresed on a 20-50% denaturing, 7.5% polyacrylamide gel. Lanes 1-16 represents the hourly intervals at which the samples were taken.

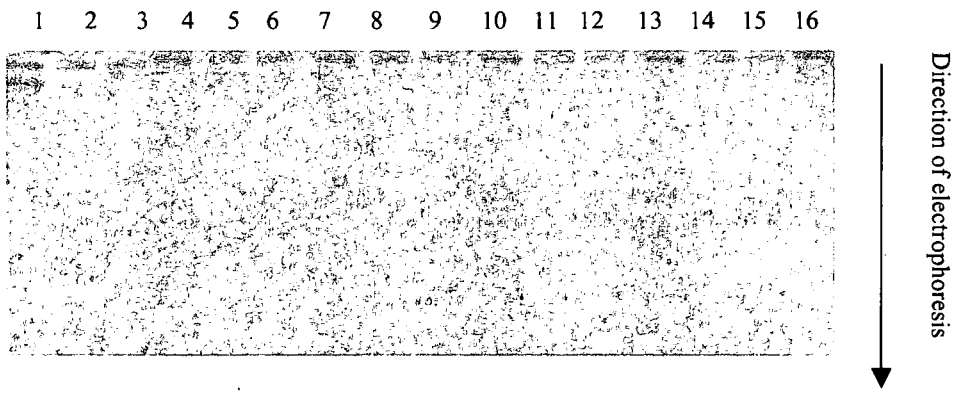


Figure 5.6: Negative image of an ethidium bromide-stained DGGE pattern of PCR products obtain from a batch test with So/Xo ratio of 11. The batch test was sampled every hour for a period of 16 hours. The PCR products were eletrophoresed on a 20-50% denaturing, 7.5% polyacrylamide gel. Lanes 1-16 represents the hourly intervals at which the samples were taken.

The 16S rDNA gene fragments amplified from mixed liquor batch test samples were resolved using DGGE. DGGE analysis of the microbial communities present the original treatment environment (Fig. 5.1) demonstrated the presence of three distinguishable bands in the separation pattern. Based on the band intensities two prominent bands were observed in lanes 1-5.

Figure 5.2 represents DGGE analysis of amplified 16S rDNA fragments of mixed liquor sampled at regular intervals from a batch test with So/Xo ratio of 0.8. Based on the band intensities two prominent bands were observed throughout the batch test (Lanes1-16). The electrophoretic mobility and the intensities of the bands at So/Xo of 0.8 were similar at So/Xo ratio of 1.5 (Fig. 5.3), suggesting that the predominant populations of these microbial communities were not detectably different. DGGE band patterns of both figure 5.2 and 5.3 share these predominant common bands evident in figure 5.1, suggesting that

the microbial communities present in batch test 1 (So/Xo =0.8) and batch tests 2 (So/Xo= 1.5) are representative of the original treatment environment (Fig. 5.1).

Figure 5.4 represents the DGGE pattern of 16S rDNA fragments of mixed liquor sampled at regular intervals from a batch test with So/Xo ratio of 4. The DGGE analysis of batch test 3 (So/Xo= 4) showed 2 common bands in lanes 1-5, after 5 hours a single band was observed for the next seven hours (Lanes 6-12) thereafter 2 bands were observed for the remaining of the batch test (Lanes 13-16). Similar banding patterns were found in batch test 4 at So/Xo of 8 (Fig. 5.5), mixed liquor sampled for the first six hours showed two common bands (Lanes1-6) thereafter a single band was observed throughout the batch test (Lanes 7-16). Figure 5.6 (So/Xo =11) show 2 bands in the first hour (Lane 1) after the first hour a single band was observed throughout the duration of the batch test (Lanes 2-16).

Figures 5.4, 5.5 and 5.6 show DGGE patterns of 16S rDNA fragments which are not representative of the original treatment environment (Fig. 5.1). This is indicated by the inconsistencies of the 2 prominent bands found in the original microbial population.

5.4 Discussion

The novel approach presented here is based on DGGE analysis of 16S rDNA sequences obtained after enzymatic amplification of genomic DNA isolated from five different batch tests at defined So/Xo ratios. This system was optimized for the analysis of microbial populations by designing PCR primers and DGGE conditions that result in high resolution banding patterns. For optimal DGGE separation, incorporation of 40-bp GC-rich clamp in the 5' primer proved necessary for optimal resolution of the fragments in the denaturing gradient. The banding pattern provided a profile of the populations in that the relative intensity of each band and its position most likely represented the relative abundance of a particular species in the population (Muyzer *et al.,* 1993).

The sensitivity of the detection of 16S rDNA sequence variants by this approach was demonstrated by different band intensities and profiles observed at various So/Xo ratios in a batch test. Each band on a gel represents a species of distinctive duplex stability (Curtis and Craine, 1998). A DGGE analysis of the microbial community in each of the batch test clearly identified a number of species. Figure 5.1 represents the presence of 3 species, 2 of which are abundant (indicated by the intensities of the bands) in its original environment. Figure 5.2 and 5.3 (So/Xo ratio of 0.8 and 1.5 respectively) represents an ethidium bromide stained DGGE gel of the microbial community sampled at hourly intervals for 16 hours. Two prominent bands are present throughout the batch test suggesting that there is no qualitative shift in microbial community from the original mixed culture (Fig. 5.1) at low So/Xo ratios. However Figure 5.4, 5.5 and 5.6 (So/Xo ratio of 4, 8 and 11 respectively) lack the consistency of the two prominent bands suggesting that a qualitative shift in the microbial community has occurred over the course of the experiment.

The amount of substrate provided to cells in a batch tests will determine the extent to which the physiological state of the cells will change during the assay. When the provided substrate is very small relative to the amount of biomass present limited changes in the protein synthesizing system (PSS) and synthesis of the new enzymes can

take place before the substrate is depleted and changes in the physiological state will be minimal. This is evident in Figure 5.2 and 5.3 where there is no microbial community shift at low So/Xo ratios. Conversely, when So/Xo ratio is sufficiently high (Fig. 5.5 and 5.6) to allow several cell divisions, synthesis of new PSS components can be significant and a change in physiological state will occur. The kinetics measured under such conditions will be more representative of the cultures ultimate capabilities than of its capabilities prior to the test. Finally for all So/Xo values in between these two extremes (Fig. 5.4), bacteria will achieve only a partial change of physiological state, with the extent of the change depending on the ratio (Grady *et al.*, 1996).

All the conclusions above have to be viewed in the context of the limitations of the process of DNA extraction, PCR and DGGE which could lead to either over or under estimation of the diversity. Underestimates could be caused by an inability to detect organisms present in relatively low numbers or problems with cell lysis or primer binding. Curtis and Craine, (1998) found that diversity assessments were sensitive to the method of DNA extraction employed, this is probably because some organisms are easier to lyse than others. Although bead beating was the best method available, it is possible that some bacteria remained undetected because they were difficult to lyse.

Overestimates of diversity could be caused by a number of factors. Some bacteria may have more than one rDNA sequence which could lead to one bacterium producing two bands. Fortunately, this phenomenon is thought to be relatively rare in bacteria (Curtis and Craine, 1998). The corruption of the DNA in the PCR process leading to the formation of chimeric sequences (when the sequences of more than one bacteria is incorporated into one PCR product) and heteroduplex fragments (where two similar but different strands join together). The sequencing of DGGE products in a hot spring study found evidence for heteroduplex formation (Ferris and Ward, 1997) in a minority of bands; there was no evidence of chimera formation. Another study (Kowalachuk *et al.*, 1997) also found no evidence of chimeras and no true heteroduplex formation (the degenerate primers caused multiple bands from a single template). Muyzer et al., (1993) used DGGE analysis for detecting sequence differences in uncharacterized microbial

populations and excluded the possibility of the creation of chimeric genes in the course of PCR.

5.5 Conclusion

The use of contemporary molecular techniques for the analysis of the microbial community of activated sludge provides a powerful means for detecting perturbations in community structure. DGGE as a technique for studying microbial diversity is superior to cloning and subsequent sequencing of PCR-amplified rDNA or ribosomal copy DNA fragments. First, it provides an immediate display of the constituents of a population in both a qualitative and semiquantitative way, and second it is less time consuming and laborious (Marsh *et al.*, 1998)

This study focuses on the microbial community using primers previously designed for the PCR-amplification of 16S rDNA (Muyzer *et al.,* 1993). DGGE reveals two prominent bands which are evident throughout batch test at low So/Xo ratios (0.8 and 1.5), while at high So/Xo ratios (4,8 and11) lack the consistency of the two prominent bands indicative of a shift in microbial community structure. Therefore it can be concluded that suitable So/Xo ratios of 0.8 and 1.5 can be used for performing batch experiments as the microbial community structure was maintained throughout the batch test, this implies that biomass has utilized energy in storage and accumulation processes and the microbial community under these conditions was representative of the original treatment environment.

CHAPTER 6

General Conclusion and Recommendations

The activated sludge process is the most widely used biological process for wastewater treatment. Patented in 1914 by Ardern and Lockett, then industrialized in 1920 by John and Atwood as a continuous process (Lilley *et al.*, 1997). The activated sludge process design and operation is mainly based on engineering and biological considerations. However, the integration of novel molecular techniques to the activated sludge process will enable improved design and optimization.

The novel approach that is presented here is based on DGGE analyses of 16S rDNA sequences obtained after enzymatic amplification of genomic DNA extracted from microbial populations. In this study DGGE was used to determine microbial community profiles in batch tests at defined So/Xo ratios. At low So/Xo predominant common DGGE bands were observed through the batch test. Therefore suggesting no shift in microbial community structure away from its original environment. At high So/Xo ratios there was a large qualitative shift in microbial community structure, this was indicative by the absence of predominant DGGE bands (one or two bands) present in its original microbial culture.

It has been shown that So/Xo ratio is one of the most important parameters in batch cultivation. It determines whether or not a shift in microbial community structure will take place during exogenous substrate removal. So/Xo ratios of 0.8 and 1.5 have proven to be suitable when conducting a respirometric batch test. This parameter ensures that the microbial community structure will be representative of the original treatment environment.

In this study we have established defined technological parameters in which a batch test could be conducted and have determined microbial community profiles apparent at low and high So/Xo ratios. One step further in the analysis of microbial populations will be to identify these populations and determine correlations between community structure and the function of the participating microbial consortia. The molecular techniques suggestive in facilitating this approach are:

- DNA extractions
- PCR
- DGGE
- Direct rDNA sequencing
- rDNA database
- Phylogenetic identification

REFERENCES

Avery OT, Macleod CM, and Macarty M (1944) Studies on the chemical nature of the substance inducing transformation of pneumococcal types. *Journ. Exper. Med.* **79**: 137-158

Amann E, Brosius J and Ptashne M (1983) Vectors bearing a hybrid trp-lac promoter useful for regulated expression of cloned genes in *E. coli*. *Gene* **25**: 167-178

Amann RI, Ludwig W and Schleifer K-H (1995) Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev.* **59**: 143-169

Bitton G (1995) *Wastewater Microbiology*. John Wiley and sons, New York

Blackall LL, Burell PC, Gwilliam H, Bradford D, Bond PL and Hugenholtz P (1998) The use of 16S rDNA clone libraries to describe the microbial diversity of activated sludge communities. *Wat. Sci. Tech.* **37** (4-5): 451-454

Blackall LL, Seviour EM, Cunningham MA, Seviour RJ and Hugenholtz P (1994) *Microthrix parvicella* is a novel, deep branching member of the actinomycetes subphylum. *System. Appl. Microbiol.* **17**: 513-518

Boogerd FC, Appeldoorn KJ and Stouthamer AH (1983) Effects of electron transport inhibitors and uncouplers on denitrification in *Paracoccus denitrificans*. *FEMS Microbiol. Lett.* **20**: 455-460

Borresen AL, Hovig E and Brogger A (1988) Detection of base mutations in genomic DNA using denaturing gradient gel electrophoresis (DGGE) followed by transfer and hybridization with gene specific probes. *Mutat. Res.* **202**: 77-83

BRL (1994) Instruction manual to the D Gene system. *Bio-rad Laboratories*. Hercules, CA, USA.

Cariello NF, Scott JK, Kat AG, and Thilly WG (1988) Resolution of a missense mutant in human genomic DNA by denaturing gradient gel electrophoresis and direct sequencing using *in vitro* DNA amplification: $HPRT_{Munich}$. *Am. J. Hum. Genet.* **42**: 726-734.

Casey TG, Ekama GA, Wentzel MC and Marais GvR (1995) Filamentous organism bulking in nutrient removal activated sludge systems Paper 1: a historical overview of causes and control. *Wat. SA* **21** (3): 231-237.

Casey TG, Wentzel MC and Ekama GA (1999) Filamentous organism bulking in nutrient removal activated sludge systems Paper 9: Review of biochemistry of heterotrophic respiratory metabolism. *Wat. SA* **25** (5): 409- 424

Chen A, Edgar DB and Trela JM (1976) Deoxyribonucleic acid polymerase from the extreme thermophile *Thermus aquaticus. J. Bacteriol* **127**: 1550-1557

Chudoba P, Capdeville B and Chudoba J (1992) Explanation of the biological meaning of the So/Xo ratio in batch cultivation. *Wat. Sci. Tech.* **26** (3-4): 743-751

Chudoba P, Chang J and Capdeville B (1991) Synchronised division of activated sludge microoganisms. *Wat. Res.* **25** (7): 817-822

Clark JW, Viessman WJr and Hammer MJ (1977) *Biological-Treatment Process, Water Supply and Pollution Control.* IEP, Dun-Donnelley Publisher, New York

Clayton JA, Ekama GA, Wentzel MC and Marais GvR (1989) Denitrification kinetics in biological nitrogen and phosphorus removal systems treating municipal wastewaters. *Res. Rep. W95*, Dept. of civil Eng., Univ. of Cape Town, Rondebosch, 7701, RSA

Curtis TP and Craine NG (1998) The comparison of the diversity of activated sludge plants. *Wat. Sci. Tech.* **37** (4-5): 71-78

Daigger GT and Grady CPL (1982) The dynamics of microbial growth on soluble substrates. A unifying theory. *Wat. Res.* **16**: 365-382

Dawes IW and Sutherland IW (1992) *Microbial Physiology.* Blackwell Scientific Publications, Oxford.

Dircks K, Pind PF, Mosbæk and Henze M (1999) Yield determination by respirometric-The possible influence of storage under aerobic conditions in activated sludge. *Wat SA.* **25** (1): 69-74.

Dunning AM, Talmud P and Humphries S (1988) Errors in the Polymerase Chain reaction. *Nucleic acid Res.* **16**: 10393

Ekama GA, Dold PL and Marais GVR (1986) Procedure for determining influent COD fractions and the maximum specific growth rate of heterotrophs in activated sludge. *Wat. Sci. Tech.* **18**: 91-114.

Ellis TG, Barbeau DS, Smets BF and Grady CPLJ (1996) Respirometric technique for determination of extant kinetic parameters describing biodegradation. *Water Environ. Res.* **68** (5): 917-926.

Ferris MJ, Muyzer G and Ward DM (1996) Denaturing gradient gel electrophoresis profiles of 16s RNA-defined populations inhabiting a hot spring microbial mat community. *Appl. Env. Microbiol.* **62**: 340-346

Ferris MJ and Ward DM (1997) Seasonal distributions of dominant 16s rRNA defined populations in a hot spring microbial mat examined by denaturing gradient gel electrophoresis. *Appl. Env. Microbiol.* **63**: 1375-1381.

Fisher SG, and Lerman LS (1979) Length-independent separation of DNA restriction fragments in two dimensional gel electrophoresis. *Cell* **16**: 191-200

Fisher SG and Lerman LS (1983) DNA fragments differing by single-base pair substitutions are separated in denaturing gradient gels: Correspondence with melting theory. *Proc. Natl. Acad. Sci. USA* **80**: 1579-1583

Fujimoto Y (1963) Kinetics of microbial growth and substrate consumption. *J. Theoret. Biol.* **5**: 171-191

Gaudy AF jr, Ramanathan M and Rao BS (1967) Kinetic behavior of heterogeneous populations in completely mixed reactors. *Biotechnol. & Bioeng.* **9**: 387-411

Giovannoni, S.J. (1991). The polymerase chain reaction, *In* E. Stackerbrandt and M.Goodfellow (ed.), *Nucleic acid techniques in bacterial systematics*. John Wiley & Sons, Chichester, England.

Giovannoni SJ, Britschgi TB, Moyer CL and Field KG (1990) Genetic diversity in Sargasso Sea Bacterioplankton. *Nature* **345**: 60-63

Grady CPLJ, Smets BF and Baebeau DS (1996) Variability in kinetic parameter estimates: a review of possible causes and proposed terminology. *Wat. Res.* **30** (3): 742-748.

Grunstein M and Hogness DS (1975) Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. *Proc. Natl. Acad. Sci. USA* **72**: 3961-3965

Hammer MJ (1977) *Water and Wastewater Technology*. John Wiley and Sons, Inc, Canada.

Hawkes HA (1963) *The Ecology of Wastewater Treatment*. Pergamon Press, London, England.

Innis MA, Myambo KB, Gelfand DH and Brow MAD (1988) DNA sequencing with *Thermus aquaticus* DNA polymerase, and direct sequencing of PCR amplified DNA. *Proc. Natl. Acad. Sci. USA* **85**: 9436-9440

Jacob F and Monod J (1961) Genetic regulatory mechanisms in the synthesis of proteins. *Journ. Mol. Biol.* **3**: 318-356

Jorand F, Zartarian F, Thomas F, Block JC, Bottero JY, Villemin G, Urbain V and Manem J (1995) Chemical and structural (2D) linkage between bacteria within activated sludge flocs. *Wat. Res.* **29** (7): 1639-1647.

Khorana HG (1966) Polynucleotide synthesis and the genetic code. *Harvey lectures 1966-1967*. Series 62. Academic press: New York, USA pp. 79-105

Kowalachuk GA, Stephen JR, Boer W de, Prosser JI, Embley TM and Woldendorp JW (1997) Analysis of ammonia oxidizing bacteria of the ß subdivision of the class Proteobacteria in coastal sand dunes by denaturing gadient gel electrophoresis and sequencing of PCR-amplified 16s ribosomal DNA fragments. *Appl. Env. Microbiol.* **63**: 1489-1497

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

Lane DJ, Stahl DA, Olsen GJ, Heller DJ and Pace NR (1985) Phylogenic analysis of the genera Thiobacillus and Thiomicrospira by 5S rRNA sequences. *J. Bacteriol.* **163**: 75-81

Lehninger AL (1975) *Biochemistry (2nd edn.)*. Worth Publishers Inc., New York, USA.

Lerman LS, Fisher SG, Bregman DB, and Silverstein KJ (1981) *In* RH Sarma (ed), *Biomolecular Steriodynamics*. Adenine Press, Albany, New York, USA.

Lerman LS, Fisher SG, Hurley I, Silverstein K and Lumelsky N (1984) Sequence-determined DNA separations. *Annu. Rev. Biophys. Bioeng.* **13**: 399-423

Liebeskind M and Dohmann M (1994) Improved method of activated sludge biomass determination. *Wat. Sci. Tech.* **29** (7): 7-13.

Lilley ID, Pybus PJ and Power SPB (1997) Operating manual for biological nutrient removal wastewater treatment works. *Water Research Commision Report No.* TT 83/97

Marsh TL, Liu W-T, Forney LJ and Cheng H (1998) Beginning a molecular analysis of the eukaryal community in activated sludge. *Wat. Sci. Tech.* **37** (4-5): 455-460.

Maxam AM and Gilbert W (1977) A new method for sequencing DNA. *Proc. Natl. Acad. Sci. USA* **74**: 560-564

McKinney RE (1962) *Microbiology for Sanitary Engineers*. McGraw-Hill Book Co., New York, USA.

Muyzer G, Teske A, Wirson CO and Jannasch HW (1995) Phylogenetic relationship of Thiomicrospira species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16s rDNA. *Appl. Env. Microbiol.* **59**: 695-700

Muyzer G and Waal EC (1994) Determination of the genetic diversity of microbial communities using DGGE analysis of PCR-amplified 16S rDNA. In LJ Stal and P Caumette (ed), *Microbial mats: structure, development and environmental significance*. Springer-Verlag, Heidelberg, Germany.

Muyzer G, Waal EC and Uitterlinden AG (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16s rRNA. *Appl. Env. Microbiol.* **59**: 695-700

Myers RM, Fisher SG, Lerman LS and Maniatis T (1985a) Nearly all single base substitutions in DNA fragments joined to a GC-clamp can be detected by denaturing gradient gel electrophoresis. *Nucleic Acids Res.* **13**: 3131-3145

Myers RM, Fisher SG, Maniatis T and Lerman LS (1985b) Modification of the melting properties of duplex DNA by attachment of a GC-rich DNA sequence as determined by denaturing gradient gel electrophoresis. *Nucleic Acids Res.* **13**: 3111-3129

Myers RM, Maniatus T and Lerman LS (1987) Detection and localization of single base changes by denaturing gradient gel electrophoresis. *Methods Enzymol.* **155**: 501-527

Needham JR and Needham PR (1962) *A Guide to the Study of Fresh-water Biology*, Holden-Day, Inc., San Francisco.

Nelson PO and Lawrence AW (1980) Microbial viability measurements and activated sludge kinetics. *Wat. Res.* **14**: 217-225

Nirenburg M and Leder P (1964) RNA codewords and protein synthesis: the effect of trinucleotides upon the binding sRNA to ribosomes. *Science* **145**: 1399-1407

Olsen GJ, Lane DJ, Giovannoni SJ, Pace NR and Stahl DA (1986) Microbial ecology and evolution: a ribosomal RNA approach. *Annu. Rev. Microbiol.* **40**: 337-365

Olsen GJ and Woese CR (1993) Ribosomal RNA: a key to phylogeny. *FASEB* **7**: 113-123

Payne WJ (1973) Reduction of nitrogenous oxides by microorganisms. *Bacteriol. Rev.* **37** 409- 452

Pitter P and Chudoba J (1990) *Biodegradablity of organic substances in the aquatic environment.* CRC Press, Boca Raton, USA

Pollard PC, Steffens MA, Biggs CA and Lant PA (1998) Bacterial growth dynamics in activated sludge batch assays. *Wat. Res.* **32** (3): 587-596.

Poole RK (1982) The oxygen reactions of bacterial cytochrome oxidases. *TIBS* (January) 32-34

Rao BS and Gaudy AF jr (1966) Effect of sludge concentration on various aspects of biological activity in activated sludge. *J. Wat. Pollut. Control. Fed.* **38** (5): 794- 812

Randall EW, Wilkinson A and Ekama GA (1991) An instrumentation for the direct determination of oxygen utilization rate. *Wat. SA* **17** (1): 11-18

Rittmann BE, laspidou CS, Flax J, Stahl DA, Urbain V, Harduin H, van der Waarde JJ, Geurkink B, Henssen MJC, Brouwer H, Klapwijk A and Wetterauw M (1999) Molecular and modeling analysis of the structure and function of nitrifying activated sludge. *Wat. Sci. Tech.* **39** (1): 51-60.

Russel DR and Bennet GN (1981) Characterization of the B-lactamase promoter of pBR322. *Nucleic acids Res.* **9**: 2517-2533

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, and Ehrlich HA (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**: 487-491.

Saiki RK, Scharf SJ, Faloona F, Mullis KB, Horn GT, Erlich HA, and Arnheim N (1985) Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230**: 1350-1354

Sanger F, Nicklen S and Coulsen AR (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**: 5463-5467

Sapshead LM and Wimpenny JWT (1972) The influence of oxygen and nitrate on the formation of the cytochrome pigments of the aerobic and anaerobic respiratory chain of *Micrococcus denitrificans*. *Biochim. Biophys. Acta.* **267**: 388- 397

Schmidt TM, Delong EF and Pace NR (1991) Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing. *J. Bacteriol.* **173**: 4371-4378

Sheffield VC, David RC, Lerman LS, and Myers RM (1989) Attachment of a 40-base-pair G+C-rich sequence (GC-clamp) to Genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. *Proc. Natl. Acad. Sci. USA* **86**: 232-236

Schroeder ED (1977) *Water and Wastewater Treatment*. McGraw-Hall Inc. USA

Smith HO, (1970) Nucleotide sequence specificity of restriction endonucleases. *Science* **205**: 455-462

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**: 503-517

Speece RE, Engelbrecht RS and Aukamp DR (1973) Cell replication and biomass in the activated sludge process. *Wat. Res.* **7**: 361-374

Stackebrandt E and Woese CR (1981) The evolution of prokaryotes. In: Molecular and Cellular Aspects of Microbial Evolution. Charlie MJ, Collins JF and Moseley BEB (eds). Vol. 32, Cambridge University Press, London. pp 1-31

Stahl DA, Lane DJ, Olsen GJ, and Pace NR (1985) Characterization of a Yellowstone hot spring microbial community by 5S rRNA sequences. *Appl. Environ. Microbial.* **49**: 1379-1384.

Standard Methods (1985) *Standard Methods for the Examination of Water and Wastewater* (16th edn) American Public Health Assoc., 1015 15th Str. NW, Washington DC 20005, USA.

Steyn DJ, Toerien DF and Visser JH (1975) Eutrophication levels of some SA impoundments II Hartbeesport Dam. *Water SA* **1** (3): 93-101

Stouthamer AH and van Verseveld HW (1987) Microbial energetics should be considered in manipulation metabolism for biotechnological purposes. *Trends Biotechnol.* **5**: 149-155

Stryer L (1981) Biochemistry (2nd edn.). WH Freeman & Co, San Francisco.

Tacon W, Cary N and Emtage S (1980) The construction and characterization of plasmid vectors suitable for the expression of all DNA phases under the control of the *E. coli* tryptophan promoter. *Molec. Gen. Genet.* **177**: 427-438

Ubisi MF, Jood TW, Wentzel MC, and Ekama GA (1997) Activated sludge mixed liquor heterotrophic active biomass. *Water SA* **23** (3): 239-248

Vitousek PM (1997) Human Alteration of the Global Nitrogen Cycle: Causes and Consequences. *Issues in Ecology* **1**: 4-6.

Wagner M and Amann R (1997) Molecular techniques for determining microbial community structures in activated sludge. In: Cleote TE and Muyima NYO (eds.) *Microbial community analysis: The key design of biological wastewater treatment systems.* I.A.W.Q scientific and technical report no.5. Cambridge University Press: London. pp. 61-72.

Wentzel MC, Ekama GA and Marais GvR (1992) Processes and modeling of nitrification denitrification biological excess phosphorus removal systems- a review. *First IAWQ Technical Tour Nutrient Removal and Anaerobic Digestion in South Africa.* Volume one-Nutrient Removal, 11-13 October, 1992

Wentzel MC, Mbewe A and Ekama GA (1995) Batch test for the measurements of readily biodegradable COD and active organisms concentrations in municipal wastewaters. *Wat. SA* **21** (2): 117-124

Wentzel MC, Ubisi MF and Ekama GA (1998) Heterotrophic active biomass component of activated sludge mixed liquor. *Wat. Sci. Tech.* **37** (4-5): 79-87

Willison JC and John P (1979) Mutants of *Paracoccus denitrificans* deficient in c-type cytochromes. *J. Gen. Microbiol.* **115**: 443- 450

Woese, CR. (1987) Bacterial evolution. *Microbiol. Rev.* **51**: 221-271

Woese CR, Kandler O, and Wheelis ML (1990) Towards a natural system of organisms: Proposal for the domains archaea, bacteria, and eucarya. *Proc. Natl. Acad. Sci. USA* **87**: 4576- 4579.

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

Zhi-rong Hu, Wentzel MC and Ekama GA (2000) External nitrification in biological nutrient removal activated sludge systems. *Water SA* **26** (2): 225-237

Zuckerkandl E and Pauling L (1965) Molecules as documents of evolutionary history. *J. Theor. Biol.* **8**: 357-366

APPENDIX 1

Chemical Oxygen Demand (COD) Test
(Standard methods, 1985)

Reagents

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

Dissolve 12,259g, $K_2Cr_2O_7$ (primary standard grade) previously dried at 103°C for 3 hours and then cooled in a desiccator, in distilled water and make up to 1000mL

1.2 Sulphuric acid (H_2SO_4) reagent

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

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

Dissolve 100g analytical grade Fe $(NH_4)_2(SO_4)_2$. $6H_2O$ in distilled water using a 5L volumetric flask. Add 100mL of concentrated H_2SO_4 and make up to 5000mL. Standardization: Pipette 5mL of standard $K_2Cr_2O_7$ (1.1) solution into a Erlenmeyer flask, and dilute to 50mL with distilled water. Add 15mL of the H_2SO_4 (1.2) and allow to cool before titrating with Fe $(NH_4)_2(SO_4)_2$ titrant, using 2 drops of ferroin indicator (1.4)

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

1.4 Ferrion indicator

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

1.5 Mercuric sulphate ($HgSO_4$) powder

Procedure

1. Collect the correct number of clean, dry 250mL Erlenmeyer flasks with the ground-glass 24/40 necks. Two flasks are used for each test. Include 2 extra flasks if influent COD is being tested and 2 extra flasks if reactor or effluent COD is being tested.
2. place approximately 0.04g $HgSO_2$ powder in each flask. This is the equivalent to the volume of 2 match heads.
3. place 8-10 glass beads (± 3mm diameter) in each flask.
4. for each influent sample pipette 10mL of unfiltered influent into each of the two flasks. Pipette 10mL of distilled water into each of the two flasks. (these are the blanks)
5. add 5mL of $K_2Cr_2O_7$ solution to each of the flasks.
6. in turn, carefully add 15mL of the H_2SO_4 COD acid (1.2) to each flask, ensuring that no vapour escapes from the flask. This is most easily done by pouring the acid down the wall of the flask while the flask is tilted. Immediately attach the flask to the jacket condenser.
7. once acid has been added to each flask ensure that the flasks are level on the heating pad. Allow the contents of the flask to boil for 2 hours. Ensure that the water flow rate in the condensers is swift enough to condense all vapour rising up the condensers.
8. allow the flasks to cool to room temperature with the condensers still in position
9. pour approximately 80mL of distilled water through the top opening of each of the condensers into the sample mixture, ensuring that the side walls of the condensers are well washed.
10. remove the flasks from the condensers and the heating pad and add 2 drops of ferroin indicator to each flask.

11. titrate with Fe $(SO_4)_2$ (NH_4) solution until one drop of titrant changes the solution colour to reddish brown.

Calculation:

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

where

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

b = average mL Fe $(SO_4)_2$ (NH_4) used to titrate sample

N = normality of Fe $(SO_4)_2$ (NH_4)

APPENDIX 2

Total Kjeldahl Nitrogen (TKN) Test
(Standard methods, 1985)

Reagents

2.1 Mercuric sulphate ($HgSO_4$) solution

Dissolve 40g red mercuric oxide (HgO) in 250mL of 1 H_2SO_4: 5 water (50mL : 250mL) and dilute to 800mL with distilled water.

2.2 Sulphuric acid (H_2SO_4)- mercuric sulphate ($HgSO_4$) - potassium sulphate (K_2SO_4) solution

Dissolve 333.75g K_2SO_4 in 1800mL distilled water and add 500mL concentrated H_2SO_4. Add 62.5mL $HgSO_4$ solution and dilute to 2500mL. This reagent crystallizes at temperature lower than 14°C.

2.3 7N Sulphuric acid (H_2SO_4)

Dilute 485mL concentrated H_2SO_4 (>98%) in distilled water and make up to 2500mL with distilled water.

2.4 Digestion mixture

Mix the two solutions made up in 2.2 and 2.3 above to give 5000mL of digestion mixture

2.5 Mixed indicator

Mix two volumes of 0.2% methyl red in 95% alcohol with 1 volume of 0.2% methylene blue in 95% alcohol. This solution must be made fresh every 30 days.

2.6 Boric acid (H_3BO_3)

Dissolve 100g H_3BO_3 in distilled water. Add 100mL of mixed indicator (or 40mL blue and 80mL red), and dilute to 5000mL.

2.7 Sulphuric acid H_2SO_4 solution 0.02N

Prepare a stock solution of 0.1N H_2SO_4 by diluting 2.8mL concentrated H_2SO_4 to 1000mL. Dilute 200mL of the 0.1N H_2SO_4 stock solution to 1000mL with distilled water to give a 0.02N stock solution. Standardize this solution as outlined in section 102.3 (c) – Alkalinity of "Standard Methods", p.54

2.8 Standard Sulphuric acid (H_2SO_4) solution 0.001N

Dilute 100mL of the 0.02N H_2SO_4 stock solution to 2000mL to give an approximately 0.001N H_2SO_4 solution.

2.9 Sodium Hydroxide (NaOH)- sodium thiosulphate solution ($Na_2S_2O_3.5H_2O$)

Dissolve 500g NaOH and 25g $Na_2S_2O_3.5H_2O$ in distilled water and make up to 1000mL. This procedure is done in a plastic container as the reaction is highly exothermic.

Procedure

1. Pipette 25mL influent sample or a 50mL reactor or effluent sample into a 250mL Kjeldahl flask. Add 10mL digestion mixture (2.4) and 3 glass beads (approximately 3mm diameter). Prepare a blank by placing a volume of distilled water equal in volume to that of the sample with digestion mixture and beads, in a second kjeldahl flask. Follow the same procedure for the sample and the blank.
2. digest the sample mixture on a heating pad until the sample is clear, and heat for another 20 minutes.
3. Transfer the Kjeldahl flask to the micro steam distillation apparatus and allow to cool for approximately 30 min.

4. pipette 25mL of H_3BO_3 solution (2.6) into a 100mL Erlenmeyer flask and place in position on the steam distillation apparatus with nozzle of the condenser immersed in the solution.
5. add about 100mL of distilled water to the contents of the Kjeldahl flask to dissolve the sediment.
6. add 10mL of the NaOH solution (2.9) to the contents of the Kjeldahl flask via the top opening of the steam distillation apparatus, and immediately seal the apparatus by placing the steam line in position.
7. Steam distil until the volume of the H_3BO_3 solution in the Erlenmeyer flask is approximately 100mL.
8. Titrate the contents of the Erlenmeyer flask for the sample and the blank with standardized 0.02N H_2SO_4 (2.7).

Calculation

$$\text{TKN mgN/L} = \frac{(a-b)^* N^* 14000}{A}$$

Where

a = mL 0.001N H_2SO_4 for sample

b = mL 0.001N H_2SO_4 for blank

N = actual normality of 0.001N H_2SO_4

A = mL sample

APPENDIX 3

Determination of MLSS and MLVSS

Determination of MLSS

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

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

Determination of MLVSS

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

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

APPENDIX 4

DNA Extraction (Lane, 1996)

Reagents

DNA extraction buffer: 0.2M Tris-HCl pH 8.5, 0.25M NaCl, 25mM EDTA

Phenol equilibrated with DNA extraction buffer (Sambrook *et al.*, 1989)

Chloroform-isoamyl alcohol (24:1, v/v)

20% SDS

3M NaOAc, pH 5.1

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

Procedure:

Fill the bead beating vial (Biospec products cat. no. 1083-2) one third with acid washed, baked glass beads (0.1-0.15mm beads, Sigma chemical Co.). Add approximately 0.2g of cell pellet (wet weight). Add 750µL of DNA extraction buffer and 50µL of 20% SDS. Fill the remaining portion of the vial with buffer saturated phenol. Beat for 4 min in a bead-beating machine at 4600rpm (biospec products, Mini-Beadbeater). Transfer the vial to a heating block for 15 min at 60°C. Beat for a further 2 min. Spin in a microfuge at 4000rpm for 10 min to separate the organic and aqueous phases. Remove the aqueous (top) phase to a clean microfuge tube. Add 400µL of buffer saturated phenol and vortex. Add 100µL of chloroform-isoamyl alcohol and vortex. Spin in a microfuge at 4000rpm for 10 min. Remove the top layer and repeat the phenol chloroform extraction three times. Remove the top layer, add 1/10 volume of 3M NaOAc (pH 5.1), and precipitate with two volumes of ethanol at -20°C overnight. Air dry the pellet and resuspend in 500µL of TE buffer (pH 7.4). Store at -20°C until further use.

APPENDIX 5

Polymerase Chain Reaction (Giovannoni, 1991)

Reagents:

10 X PCR reaction buffer: 50mM KCl, 10mM Tris-HCl pH 8.4, 2.5mM $MgCl_2$, 100µg/ml Gelatin, 0.05% (v/v) Nonidet-40.

50µM stock solution of primers

10ng/µl of DNA template

2.0mM of nucleotide triphosphate solution (concentration of each nucleotide)

1 unit/µl of Taq polymerase

Reaction Mixture:

The reaction mixture is prepared in a 0.2ml microfuge tube by mixing 10ng/µl of DNA template; 10µL of 10X PCR buffer; 65µl distilled water; 10µl of 2.0mM nucleotide triphosphates solution; 2µl of 50µM stock solution of each probe. The reaction mixture is then incubated at 94°C for 4 min to inactivate any contaminating proteases, followed by the addition of 1µl of Taq polymerase (1unit/µl). The final reaction volume is 100µl.

Hybaid PCR Sprint Thermal Cycler program (Hybaid limited, Ashford, UK)

Stage 1	Step 1	94°C	4min	x 1
Stage 2	Step 1	94°C	1min	
	Step 2	53°C	1min	x 35
	Step 3	72°C	2min	
Stage 3	Step 1	72°C	4min	x 1
	Hold	4°C		

APPENDIX 6

DENATURING GRADIENT GEL ELECTROPHORESIS PROTOCOL

D GENE System (Bio-Rad Laboratories, Hercules Ca, USA)

Reagent preparation

40% Acrylamide/Bis (37.5:1)

Acrylamide	38 g
Bis-acrylamide	2 g

Add dH_2O to 100ml. Filter through a Whatmann No. 1 and store at 4

Preparation of 50 x DGGE/TAE buffer solution

Tris-Base:	242 g
Acetic acid, glacial	57.1 ml
0.5M EDTA, pH 8.0	100 ml

Mix and add dH_2O to 1 liter. Autoclave for 20-30 mins. Store at room temperature

0% Denaturing Solution (100ml)

40% Acrylamide/bis (37.5:1)		18.8 ml
50 x TAE buffer		2 ml
dH_2O	to	100 ml

100% Denaturing solution (100ml)

40% Acrylamide/Bis (37.5:1)		18.8 ml
50 x TAE buffer		2 ml
Formamide (deionized)		40 ml
Urea		42 g
dH_2O	to	100 ml

Denaturing Solution

	10%	20%	30%	40%	50%	60%	70%	80%	90%
Formamide (ml)	4	8	12	16	20	24	28	32	36
Urea (g)	4.2	8.4	12.6	16.8	21	25.2	29.4	33.6	37.8

10% Ammonium Persulfate (1ml)

Ammonium Persulfate 0.1 g

dH_2O 1.0 ml

Mix and store at -20°C for about a month

D Gene Dye Solution (10ml)

Bromophenol blue 0.05 g

Xylene cyanol 0.05 g

1 x TAE buffer 10.0 ml

Store at room temperature.

1 x TAE Running Buffer (7 L)

50 x TAE buffer 140.0 ml

dH_2O 6860.0 ml

Procedure

6.1 Assembling the Glass plate Sandwiches

Assemble the gel sandwich on a clean surface. Lay the long rectangular plate down first, and then place the left and right spacers of equal thickness along the short edges of the rectangular plate. For parallel gradient gels place the spacers so that the holes in the spacers are at the top of the plate, facing inward and the grooved side of the spacer facing upward or against the short glass plate. Place each clamp by the appropriate side of the glass plate. Place the sandwich assembly in the alignment slot of the casting stand with the short glass plate forward. Loosen the sandwich clamps and insert a D GENE alignment card to keep the spacers upright.

6.2 Pouring gradients

Position the DGGE apparatus below the gradient maker (Model 475 Gradient delivery system) about 30 cm in order to generate enough of a gravity-gradient. Prepare the high and low-density gel solution by pipeting the desired amounts into two disposable culture tubes. Add 1/100 of the total volume of 10% ammonium Persulfate and 1/1000 of the total gel volume of TEMED to the tubes containing the gel solutions. Withdraw the high and low-density gel solutions using a syringe. Place the high density and low-density syringes into the respective syringe holders (high density side for top filling). Rotate the cam wheel slowly and steadily to deliver the gel solution. It is important to cast the gel solutions at a steady pace to avoid mixing of the gradients within the gel sandwich. Let the gel polymerize for about 30-45 minutes.

6.3 Electrophoresis

When the running buffer has reached the correct temperature in the D GENE chamber, the samples are ready to be loaded on the gel. Load samples into the wells using a pipette and a sequencing-loading tip. Do not pierce the gel during sample delivery. Use about 8 µl of PCR product with 2µl of gel loading dye. Place the DGGE apparatus into the prewarmed (60°C) tank. Run at 200V for 3 hours.

6.4 Staining and photographing the gel

Remove the gel from the glass plate by gently grasping two corners and lifting off. Place the gel into a dish containing 250ml of 1 x TAE buffer and 100µl of 1mg/ml ethidium bromide. Stain for about 1-2 minutes. After staining, carefully transfer the gel into a dish containing 250ml of 1 x TAE buffer. Destain for 5-20 minutes. Photograph the gel on a UV transilluminator with a Polaroid camera (Hoefer Pharmacia Biotech, USA).

APPENDIX 7

TABLE A7: Daily results obtained from parent laboratory scale anoxic/aerobic activated sludge system.

Date	WW	COD(mg/l)		VSS(mg/l)	TKN (mg/l)		Nitrates (mgN/l)			OUR
	Batch	influent	effluent		influent	effluent	anoxic	aerobic	effluent	(mgO/l/h)
01-Mar		449.28	43.68	2059	40.24	2.6	0.5	3.1	13	30.1
02-Mar		497.12	43.68	1950	32.56	3.12	0.5	1.61	13	30.03
03-Mar		436.8	45.6	2200	27.65	3.53	0.3	1.9	6.85	30.7
04-Mar		436.8	45.44	1900	30.24	2.35	1.2	2.4	7.5	30.45
05-Mar		499.36	49.92	2300	25.67	2.8	0.3	1.2	8.15	30.11
06-Mar		451.36	37.44	2100	25.34	3.17	0.1	1.8	7.56	29.99
07-Mar		486.72	41.6	1980	27.56	2.6	0.2	1.9	5.67	30.55
08-Mar		499.2	62.4	2000	28.98	3.34	1.5	0.8	4.98	30.15
09-Mar		482.56	33.28	2100	27.45	2.76	0.1	1.2	5.67	30.33
10-Mar	1	470.08	58.24	2100	29.34	4.43	0.1	1.7	3.45	30.89
11-Mar		557.44	68.64	2500	41.44	3.92	0.3	1.2	5.65	30.1
12-Mar		517	50.24	2700	25.76	4.52	0.3	0.1	4.34	29.95
13-Mar		534.56	74.72	2576	25	5.88	0.35	1.3	2.3	29.57
14-Mar		517.3	43.46	2430	28	4.48	0.3	2.8	3.48	29.33
15-Mar		482.36	41.12	2766	30.24	4.92	0.3	1.8	2.4	29.14
16-Mar		498.36	43.46	2856	27.35	10.64	0.4	2	3	29.05
17-Mar		453.76	41.6	2589	25.56	4.62	0.3	0.14	3.4	29.78
18-Mar		455.52	41.6	2578	24.54	5.98	0.3	1.3	3.23	29.94
19-Mar		449.28	41.6	2455	25	5.88	0.35	1.2	4.3	29.8
20-Mar	2	449.28	37.44	2650	25.55	14	0.3	0.95	5.7	29.05
21-Mar		504.88	41.6	2084	28.9	4.56	0.4	1.89	4.8	29.8
22-Mar		499.36	49.92	2426	27.89	5.04	0.4	1.32	5.7	30.52
23-Mar		551.2	72.08	2200	32.09	5.03	0.4	1.52	5.54	30.74
24-Mar		478.4	34.5	2200	33.56	4.07	0.4	2.3	3.45	30.83
25-Mar		459.68	60.32	2195	32.48	4.89	0.35	2.43	2.2	30.65

Table A7- continued

26-Mar		449.44	55.12	2200	31.3	5.2	0.25	1.77	6.5	30.55
27-Mar		463.22	34.98	2084	28.79	5.6	0.2	1.5	7.56	30.59
28-Mar		507.76	46.64	2097	27.34	4.56	0.2	1.8	4.54	30.64
29-Mar		449.44	38.16	2198	30.34	5.7	0.2	2.7	4.95	30.79
30-Mar	**3**	455.8	50.88	2267	26.9	4	0.1	1.7	4.56	30.59
31-Mar		447.32	57.24	2540	32.6	n/s	0.1	1.5	8.1	29.69
01-Apr		486.92	50.52	2340	30.56	2.8	0.1	1.2	6.65	29.61
02-Apr		536.38	51.94	2250	29.4	4.7	0.1	1.24	7.5	29.74
03-Apr		478.06	49.82	2378	30.24	3.36	0.1	1.4	8.15	29.51
04-Apr		510.56	93.28	2460	29.4	6.44	0.1	1.45	7.56	29.5
05-Apr		416	44	2515	36	0	0.05	1.25	5.67	29.66
06-Apr		480	52	2213	35.84	8.68	0.1	1.56	4.98	29.48
07-Apr		556	76	2340	33.09	n/s	0.1	1.7	5.67	29.5
08-Apr		524	50	2348	35.9	10.08	0.15	0.1	3.45	29.98
09-Apr	**4**	540	76	2245	28.5	n/s	0.1	1.6	4.35	29.73
10-Apr		460	60	2260	41.4	n/s	0.14	1.5	7.3	29.4
11-Apr		450	74	2320	40.32	5.04	0.1	1.23	5.67	29.41
12-Apr		452	64	2140	33.6	8.4	0.1	0.7	7.6	29.01
13-Apr		436	48	2204	29.68	7.56	0.1	1.3	5.1	28.9
14-Apr		456	80	2300	34.16	8.4	0.1	1.5	8.2	28.9
15-Apr		494	66	2200	30.5	n/s	0.1	1.2	8.5	28.7
16-Apr		480	84	2392	29.9	n/s	0.1	0.9	5.4	28.7
17-Apr		508	84	2598	28.9	n/s	0.1	0.81	3.2	29.12
18-Apr		512	68	2204	42.56	15.68	0.1	3.4	3.4	29.02
19-Apr	**5**	506	58	2400	31.36	11.2	0.1	0.1	5.7	29.06
20-Apr		511	60	2100	28.9	4.04	0.35	1.2	6.5	29.94
21-Apr		489	60	1918	27.67	4.06	0.3	0.36	7.56	30.73
22-Apr		452	61	2280	30.78	5.45	0.45	1.4	8	31.1

Table A7- continued

23-Apr		450.84	65	2298	30.56	4.67	0.5	3.1	7.6	31.78
24-Apr		446.76	71.4	1700	29.3	3.07	0.95	2.5	6.78	31.56
25-Apr		491.64	77.52	2034	30.78	5.45	0.4	3.2	7.9	31.67
26-Apr		448.8	61.2	1880	32.04	4.2	0.8	2.6	5.8	31.78
27-Apr		536.52	75.48	1987	40.98	5.34	0.56	1.2	8.1	31.9
28-Apr		495.72	83.64	1980	32.48	5.67	0.35	1.8	7.5	29.8
29-Apr	**6**	516.12	69.56	2200	26.88	6.6	0.5	1.4	6.8	29.88
30-Apr		448.8	53.04	1790	29.87	9.78	0.5	0.2	7.7	29.5
01-May		490	74	1789	30.2	9	0.1	1.2	5.1	29.35
02-May		446	76	1800	30.67	11.45	0.5	0.96	6.2	29.5
03-May		490	62	1828	32.56	8.4	0.5	1.18	3.2	29.23
04-May		504	76	1828	30.45	7.56	0.55	0.2	3.6	29.09
05-May		470	72	1689	32.98	8.4	0.35	1.3	3.6	29.07
06-May		462	70	2100	32.56	10.45	0.96	2.8	5.3	29.09
07-May		468	74	2175	34.56	12.34	0.1	1.8	3.9	29.29
08-May		444	58	2222	30.98	13.45	0.65	2	5.6	29
09-May	**7**	544	56	1820	35.28	9.95	0.89	0.14	7.2	29.77
10-May		488	48.96	1689	28.9	2.6	0.28	0.9	8.5	31.75
11-May		472	52	1790	31.75	3.12	0.15	1.2	7.56	31.23
12-May		480	52	1678	27.89	3.53	0.2	0.7	8	32.25
13-May		454	36	1900	26.78	2.35	0.25	0.35	7.6	32.07
14-May		440	64	1700	32.04	2.8	0.1	0.88	7.25	32.29
15-May		450	60	1600	25.45	3.17	0.4	1.2	7.9	32.32
16-May		582	74	1899	30.1	3.6	0.56	0.16	8.8	32.45
17-May		506	78	1987	28.5	3.34	0.21	0.97	8.1	32.78
18-May		441	62	1800	25.3	3	0.6	1.89	9.8	32.78
19-May	**8**	500	62.7	2098	25.4	4.9	0.1	1.56	6.8	32.98
20-May		450	24.4	2389	25	4.04	0.1	0.1	6.54	31.33

Table A7- continued

21-May		510	51.4	2350	29.89	4.06	0.025	0.36	7.6	30.43
22-May		515	57	2156	31.752	5.45	0.1	0.24	7.2	29.91
23-May		444	53.46	2145	14.112	4.67	0.1	0.4	6.2	29.94
24-May		444	53.45	2250	27.89	3.07	0.1	0.5	6.7	30.13
25-May		519	57.56	2299	31.164	5.45	0.1	0.23	5.8	29.73
26-May		502	45.23	2278	30.85	3.4	0.175	0.56	8.1	30.2
27-May		501	53.46	2250	25.5	5.34	0.1	0.39	6.56	29.95
28-May		456.43	53.46	2220	26	5.67	0.1	0.49	4.7	29.73
29-May	**9**	528	74	2243	30.576	4.67	0.1	0.6	6.4	29.98
30-May		416	76	1850	22.344	4.5	0.1	0.1	5.9	29.8
31-May		496	80	1775	28.9	3.12	0.07	0.1	9.8	30.42
1-Jun		499	24	1876	27.89	3.53	0.1	0.1	6.8	30.23
02-Jun		474	40	1900	25.78	2.35	0.1	1.7	4.1	30.8
03-Jun		447	48	1987	30.45	2.8	0.1	0.1	5.6	30.33
04-Jun		551.2	66	1536	31.752	3.17	0.13	0.1	5.4	30.74
05-Jun		478.4	84	1879	23.52	2.6	0.1	0.1	7.7	30.76
06-Jun		459.68	84	1920	33.45	3.34	0.1	0.1	8.6	30.73
07-Jun		449.44	68	2100	27.048	3.7	0.1	0.1	9.3	30.87
08-Jun	**10**	463.22	58	2300	33.4	4.43	0.1	0.1	9.8	30.73
09-Jan		507.76	60	2224	34.104	2.34	0.1	0.1	4.9	31.33
10-Jun		449.44	60	2400	28.224	2.8	0.1	0.1	5.8	30.34
11-Jun		455.8	61	2345	36.456	4.7	0.082	0.3	5.1	30.91
12-Jun		447.32	65	2428	29.4	3.36	0.1	0.1	6.4	30.9
13-Jun		486.92	71.4	2250	31.752	6.44	0.118	0.1	7.3	31.89
14-Jun		536.38	77.52	2318	28.9	4.34	0.1	0.1	7.8	31.98
15-Jun		478.06	60.32	2135	26.3	4.4	0.1	0.1	9.8	31.87
16-Jun		534.56	55.12	2084	31.752	5.34	0.1	0.1	9	30.17
17-Jun		517.3	34.98	2089	28.9	4.35	0.1	0.1	9.3	31.38

Table A7- continued

18-Jun	**11**	482.36	46.64	2100	30.56	4.8	0.1	0.1	8.1	31.54
19-Jun		498.36	54	2128	25	4.04	0.1	0.1	6.65	30.09
20-Jun		453.76	55.55	2189	29.89	4.06	0.1	0.1	7.5	30.28
21-Jun		455.52	65.54	2044	31.752	5.45	0.092	0.07	8.15	30.73
22-Jun		449.28	50.52	2100	14.112	4.67	0.11	0.1	7.56	30.85
23-Jun		449.28	51.94	2089	27.89	3.07	0.102	0.3	5.67	30.71
24-Jun		504.88	62.36	2020	31.164	3.4	0.1	0.09	4.98	31.58
25-Jun		504.88	93.28	1987	30.85	3.45	0.1	0.08	5.67	31.82
26-Jun		499.36	62.34	1900	27.2	5.34	0.1	0.1	6.15	29.8
27-Jun		551.2	62.34	1950	26	5.67	0.1	0.1	4.35	29.8
28-Jun	**12**	478.4	65.54	2043	30.576	4.67	0.1	0.1	4.5	29.77