

FUNCTIONAL CHARACTERISATION OF HETEROTROPHIC DENITRIFYING BACTERIA IN WASTEWATER TREATMENT SYSTEMS

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I hereby declare that the dissertation represents my own work. It has not been submitted before for any diploma/degree or examination at any other Technikon/University.

NISHANI RAMDHANI

I hereby approve the final submission of the following dissertation.

PROF. FAIZAL BUX

This _____ day of _____, 2005, at Durban Institute of Technology.

DEDICATION

*This dissertation is dedicated to the loving memory of my dad....
....this one's for you!*

ABSTRACT

Atmospheric nitrogen pollution is on the increase and human activities are directly or indirectly responsible for the generation of the various nitrogen polluting compounds. This can lead to the two major problems of eutrophication and groundwater pollution. Therefore, the removal of nutrients such as nitrogen and phosphorus from wastewater is important. Nitrogen removal from wastewater is achieved by a combination of nitrification and denitrification.

Thus, there is a need to identify and characterise heterotrophic denitrifying bacteria involved in denitrification in wastewater treatment systems. The aim of this study, therefore, was to characterise heterotrophic denitrifying bacteria through detailed biochemical and molecular analysis, to facilitate the understanding of their functional role in wastewater treatment systems. Drysdale (2001) isolated heterotrophic denitrifiers to obtain a culture collection of 179 isolates. This culture collection was used to screen for nitrate and nitrite reduction using the colorimetric biochemical nitrate reduction test. The isolates were thereafter Gram stained to assess their gram reaction, cellular and colonial morphology. Based on these results identical isolates were discarded and a culture collection of approximately 129 isolates remained.

The genetic diversity of the culture collection was investigated by the analysis of polymerase chain reaction (PCR)-amplified 16S ribosomal DNA (rDNA) fragments on polyacrylamide gels using denaturing gradient gel electrophoresis (DGGE). Thus DNA fragments of the same length but different nucleotide sequences were effectively separated and microbial community profiles of eight predominant isolates were created. Batch experiments were conducted on these eight isolates, the results of which ultimately confirmed their characterisation and placed them into

-v-

their four functional groups i.e. 3 isolates were incomplete denitrifiers, 2 isolates were true denitrifiers, 2 isolates were sequential denitrifiers and 1 isolate was an exclusive nitrite reducer.

PREFACE

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

Conferences:

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TABLE OF CONTENTS

TITLE PAGE.....	i
DECLARATION.....	ii
DEDICATION.....	iii
ABSTRACT.....	iv
PREFACE.....	vi
ACKNOWLEDGEMENTS.....	vii
TABLE OF CONTENTS.....	viii
LIST OF TABLES.....	xiii
LIST OF FIGURES.....	xiv
LIST OF ABBREVIATIONS.....	xvi

CHAPTER ONE

GENERAL INTRODUCTION.....	1
MAIN OBJECTIVES OF THE STUDY.....	5

CHAPTER TWO

LITERATURE REVIEW.....	7
2.1 WATER POLLUTION AND CONTROL.....	7
2.2 WATER QUALITY AND HEALTH HAZARDS.....	8

2.3 WATER MANAGEMENT AND DEMAND.....	9
2.3.1 Domestic Water Demand.....	10
2.3.2 Industrial Water Demand.....	10
2.4 WASTEWATER.....	11
2.5 WATER TREATMENT PROCESSES.....	12
2.5.1 Methods of Treatment.....	12
2.5.2 Disinfection.....	13
2.6 THE ACTIVATED SLUDGE PROCESS.....	13
2.7 BIOLOGICAL NUTRIENT REMOVAL.....	16
2.8 THE NITROGEN CYCLE.....	17
2.8.1 Nitrogen Fixation.....	19
2.8.2 Nitrogen Assimilation.....	19
2.8.3 Nitrogen Mineralization.....	20
2.8.4 Nitrification.....	20
2.8.5 Denitrification.....	20
2.9 DENITRIFICATION.....	21
2.9.1 Classification of Denitrifying Bacteria.....	22

2.9.2 Enzymes Present in Denitrifying Isolates.....	23
2.10 THE ROLE OF MOLECULAR TECHNIQUES.....	25
2.11 EXTRACTION OF NUCLEIC ACIDS.....	26
2.11.1 Methods of DNA Extraction.....	26
2.12 POLYMERASE CHAIN REACTION (PCR).....	27
2.12.1 Detection and Analysis of the Reaction Product.....	30
2.13 AGAROSE GEL ELECTROPHORESIS.....	31
2.13.1 Applications of Gel Electrophoresis.....	32
2.14 DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE).....	33
2.14.1 Advantages of DGGE.....	34
2.14.2 Disadvantages of DGGE.....	34
 CHAPTER THREE	
MATERIALS AND METHODS.....	36
3.1 ISOLATION & MAINTENANCE OF HETEROTROPHIC DENITRIFIERS.....	36
3.2 ANALYSIS OF CULTURE PURITY, ELIMINATION OF IDENTICAL ISOLATES.....	36
3.3 DENITRIFICATION SCREENING AND SUBSEQUENT CLASSIFICATION.....	37
3.4 EXTRACTION OF DNA FROM ISOLATES.....	38

3.5 AMPLIFICATION OF EXTRACTED DNA.....	38
3.6 DENATURING GRADIENT GEL ELECTROPHORESIS OF AMPLIFIED FRAGMENTS.....	39
3.7 BATCH EXPERIMENTS.....	40

CHAPTER FOUR

RESULTS AND DISCUSSION.....	42
4.1 ANALYSIS OF CULTURE PURITY / ELIMINATION OF IDENTICAL ISOLATES.....	42
4.2 DENITRIFICATION SCREENING AND SUBSEQUENT CLASSIFICATION.....	43
Incomplete Denitrifiers.....	47
True Denitrifiers.....	48
Sequential Denitrifiers.....	49
Exclusive Nitrite Reducers.....	50
4.3 MOLECULAR ANALYSIS OF DENITRIFYING ISOLATES.....	52
4.4 NITRATE AND/OR NITRITE REDUCTION BY PREDOMINANT ISOLATES.....	55

CHAPTER FIVE

GENERAL CONCLUSIONS AND RECOMMENDATIONS.....	64
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5.1 CONCLUSIONS.....	64
5.2 RECOMMENDATIONS.....	65
REFERENCES.....	67
APPENDICES.....	73
APPENDIX 1	:CASITONE GLYCEROL YEAST AUTOLYSATE AGAR (CGYA)
APPENDIX 2	:GRAM STAIN
APPENDIX 3	:COLONIAL MORPHOLOGY ASSESSMENT
APPENDIX 4	:COLORIMETRIC BIOCHEMICAL NITRATE REDUCTION TEST
APPENDIX 5	:AGAROSE GEL ELECTROPHORESIS
APPENDIX 6	:DENATURING GRADIENT GEL ELECTROPHORESIS
APPENDIX 7	:STANDARDIZATION OF AUTOANALYZER II
APPENDIX 8	:GRAM REACTION, CELLULAR MORPHOLOGY OF DENITRIFIERS

LIST OF TABLES

Table 4.1	Comparison of the classification of bacterial isolates into their functional groups based on denitrification capacity.....	46
Table 4.2	Comparison of the classification of the denitrifying isolates after biochemical and molecular analysis.....	54

LIST OF FIGURES

Figure 1.1	The hydrological cycle (Tebbutt, 1998).....	3
Figure 2.1	Adaptation of a conventional activated sludge system (Bitton, 1994).....	13
Figure 2.2	Schematic representation of the nitrogen cycle (adapted from Prescott <i>et al.</i>, 1996).....	17
Figure 2.3	Different steps in PCR (Vierstraete, 1999).....	28
Figure 2.4	Verification of the PCR product on gel (Vierstraete, 1999).....	29
Figure 2.5	Example of DGGE according to the melting properties of DNA (Verseveld <i>et al.</i>, 2001).....	33
Figure 4.1	Picture illustrating the results of the nitrate reduction test.....	44
Figure 4.2	Picture illustrating the results of the test after the addition of zinc.....	45
Figure 4.3	PCR profile of DNA-extracted samples.....	53
Figure 4.4	DGGE of 15 PCR-amplified fragments.....	53
Figure 4.5	Graph showing the amount of nitrate reduced by Organism 7.....	57
Figure 4.6	Graph showing the amount of nitrates reduced by Organism 12.....	57

Figure 4.7	Graph showing the amount of nitrates reduced by Organism 56.....	58
Figure 4.8	Graph showing the amount of nitrates and nitrites reduced by Organism 87.....	59
Figure 4.9	Graph showing the amount of nitrates and nitrites reduced by Organism 88.....	60
Figure 4.10	Graph showing the amount of nitrates and nitrites reduced by Organism 144.....	61
Figure 4.11	Graph showing the amount of nitrates and nitrites reduced by Organism 147.....	62
Figure 4.12	Graph showing the amount of nitrites reduced by Organism 176.....	63

LIST OF ABBREVIATIONS

BNR	:	biological nutrient removal
CGYA	:	casitone glycerol yeast autolysate
°C	:	degrees celcius
DGGE	:	denaturing gradient gel electrophoresis
g	:	grams
g/L	:	grams per litre
KNO ₃	:	potassium nitrate
KNO ₂	:	potassium nitrite
ml	:	millilitres
min	:	minutes
mg/L	:	milligrams per litre
N	:	nitrogen
NO	:	nitric oxide
NO ₃ ⁻	:	nitrate
NO ₂ ⁻	:	nitrite
N ₂	:	nitrogen gas
N ₂ O	:	nitrous oxide
PCR	:	polymerase chain reaction
dNTP	:	deoxy-nucleoside triphosphate
spp	:	species
µl	:	microlitres

CHAPTER ONE

GENERAL INTRODUCTION

Freedom from hunger and malnutrition, good health and a safe and stable environment are essential for human development. The growing population of the hungry and malnourished and the re-emergence of infectious diseases are all indicators that progress achieved thus far regarding food and health security may be wasted unless effective development policies are formulated and implemented globally. It is undoubtedly clear that the solution to these problems is the management and conservation of our natural resources (Maksimović, 1996).

Among these resources that are indispensable for human welfare and development, water ranks as number one. Water is the most important natural resource in the world since without it life ceases to exist and most industries cannot operate. However, scarcity and misuse of this life-supporting resource poses serious threats to food security, human health and the environment. The advantages and disadvantages that have resulted from the wise use and misuse of water has been witnessed globally (Kandiah, 1999).

An uncontaminated water supply is compulsory to establish and sustain a healthy society since it serves as a source of potable water, supports the growth of marine life and also functions to supply important food supplements. Therefore, attention should be paid to the management and control of these water resources regarding the pollutants that enter them (Robertson and Kuenen, 1992).

Water plays a very significant role in supporting all forms of life, however if polluted, it has great potential for transmitting a variety of diseases and illnesses. In developing countries around the

world water-related diseases are unusual since they have efficient water supplies and wastewater disposal systems. However in parts of the world of developing countries a huge fraction of the population are without a safe water supply and ample hygiene. Therefore the toll of water-related diseases has been taken to alarming extents (Tebbutt, 1998).

The aims of wastewater treatment are: to convert the waste matter present into stable oxidized end-products which can be safely discharged to inland or coastal waters without any adverse ecological effects to protect public health; to ensure the effective disposal of wastewater on a regular and reliable basis without offence; to provide an economical method of disposal and recover valuable components of wastewater (Gray, 1989).

Waters and wastewaters have highly complex compositions and adjustments to the composition are usually essential to suit a particular use or avoid degradation of the environment. Therefore a variety of treatment processes are necessary to treat the range of contaminants (Pearson, 1999).

Most of the water on earth is found in the sea and whilst this may appear to be abundant, most of this water is unavailable for most uses (Sanders, Dunn and Yevjevich, 1996). Fresh water is a renewable resource by virtue of the hydrological cycle (Figure 1.1). This cycle is a continuous process by which water is transported from the ocean to the atmosphere to the land and back to the sea (Kandiah, 1999).

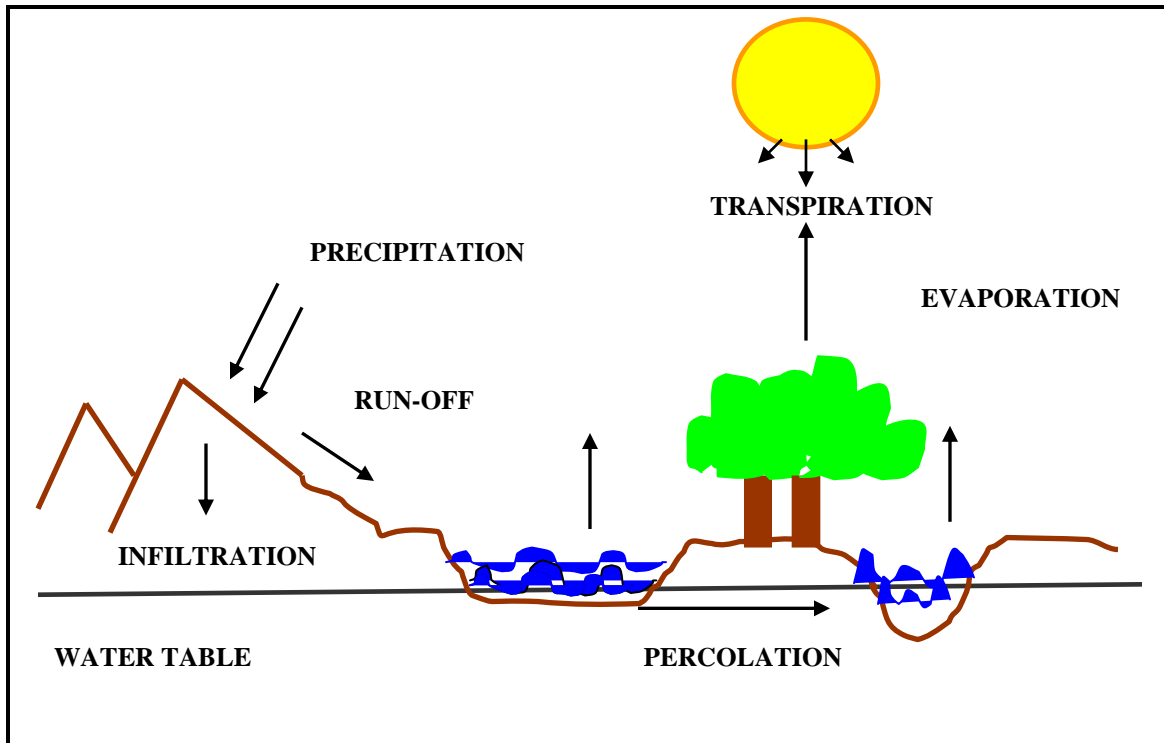


Figure 1.1 The hydrological cycle (Tebbutt, 1998).

Contamination of water bodies with nitrogen compounds is associated with atmospheric deposition with run-off from agricultural land and leaching of nitrate and nitrite from soil (Robertson and Kuenen, 1992). Interest is being shown in the use of biological systems to remove nitrogen. These systems are commonly referred to as biological nutrient removal (BNR) systems (Daigger, 1998).

In a BNR process denitrification is achieved in the anoxic zone of the process. This is a biological environment that is deficient in molecular oxygen, but may contain bound oxygen, such as nitrates and nitrites. Under these conditions certain heterotrophic bacteria are stimulated into using nitrates and nitrites as final electron acceptors for respiration, instead of oxygen. This results in oxidation of organic matter as well as the reduction of nitrates and nitrites into nitrous oxides and nitrogen gas (Wanner and Grau, 1988 as cited in Drysdale *et al.*, 1999).

The discharge of ammonia and nitrogen in receiving streams results in increased oxygen demand and depressed dissolved oxygen concentrations. Ammonia-nitrogen can be toxic to aquatic organisms, as well as nitrite-nitrogen. Nitrogen discharges enrich receiving water bodies, which result in the undesirable growth of algae and other aquatic plants. This process is known as eutrophication (Daigger, 1998).

Nitrogen removal from wastewater is achieved by a combination of nitrification and denitrification (Kandiah, 1999). In activated sludge systems an aerobic zone may exist above the anoxic zone thus allowing for simultaneous nitrification and denitrification (Blackall and Burrell, 1999). Under aerobic conditions, denitrifying bacteria use oxygen as a final electron acceptor in preference to nitrate, during respiration. Therefore in order for denitrification to occur anoxic conditions must be provided (Bitton, 1999). Under anoxic conditions, heterotrophic denitrifiers require nitrate as an electron acceptor, which indicates that in wastewater treatment nitrification should occur before denitrification, in order to provide the denitrifiers with the nitrate that they require (Haandel *et al.*, 1981).

Nitrogen in municipal wastewater is the result of human excreta, ground garbage and industrial wastes. The presence of nitrogen in wastewater discharges can be undesirable, as free ammonia it is toxic to fish and other aquatic organisms; as the ammonium ion or ammonia it is an oxygen

consuming compound, which will deplete the dissolved oxygen in receiving water; and as the nitrate ion it is a potential public health hazard in water consumed by infants. Nitrogen is removed microbiologically from wastewater by the sequential processes of nitrification followed by denitrification (Kornaros *et al.*, 1996).

Nitrate must be removed from water bodies, especially if the receiving water body serves as a source of drinking water. Assimilatory nitrate reduction and dissimilatory nitrate reduction are the two main mechanisms of biological nitrate reduction (Robertson and Kuenen, 1992).

Thus, there was a need to identify and characterise heterotrophic denitrifying bacteria. Therefore the aim of this study was to functionally characterise heterotrophic denitrifying bacteria, previously isolated by Drysdale (2001), through detailed biochemical and molecular analysis, in order to understand their functional role in wastewater treatment systems.

The objectives were:

- a) To conduct colorimetric biochemical nitrate reduction tests on all isolates of the culture collection, as proposed by Drysdale (2001) and Thomson (2002).
- b) To verify the functional characterisation of the culture collection.
- c) To identify and group all identical isolates by performing Gram stains to determine their gram reaction as well as cellular and colonial morphology in order to discard all identical isolates.
- d) To extract DNA from all the isolates in order to amplify the DNA using PCR.
- e) To conduct DGGE on all PCR-amplified samples to create a microbial community profile of all the isolates.

- f) To conduct batch experiments on all the remaining isolates to test the efficiency of the colorimetric biochemical test for the characterisation of the isolates into their functional groups.

CHAPTER TWO

LITERATURE REVIEW

2.1 WATER POLLUTION AND CONTROL

A clean water supply is necessary to establish and maintain a healthy community. Not only does it serve as a source of potable water, but also functions to provide valuable food supplements by means of supporting the growth of aquatic life and also serves important functions for irrigation in agriculture. Yet, despite its importance, all forms of watercourses worldwide are, generally, inadequately treated. Therefore, attention should be paid to the management and control of these water resources with regard to the pollutants that enter them, to ensure that the above-mentioned problems are minimized or avoided. (Robertson and Kuenen, 1992).

All natural waters contain a variety of contaminants that arise from corrosion, leaching and weathering processes. To this natural contamination is added on that which arises from domestic and industrial wastewaters which may be disposed off in several ways e.g., into the sea, onto land, into underground strata or surface waters (Sanders and Yevjevich, 1996).

Any water body is capable of accumulating a certain amount of pollution without any serious consequences due to the dilution and self-purification factors that are present. If additional pollution occurs then the nature of the receiving water will be altered and its suitability for several purposes might be affected (Tebbutt, 1998).

Most countries have pollution control to maintain and hopefully improve water quality due to the need to reconcile various demands on the aquatic environment and on water resources. In order

for a discharge to be considered polluting there must be evidence of actual harm or damage (Pearson, 1999).

Microorganisms have several important functions in pollution control. Natural waters possess the capacity of self-purification due to the microbial content of aquatic systems in which microorganisms respond to organic pollution by increased growth and metabolism. It is necessary to apply this same process in biological treatment systems to treat wastewater. Wastewater also contains microorganisms themselves and by providing a controlled environment for optimum microbial activity in a treatment unit or reactor, nearly all the organic matter present can be degraded (Sanders and Yevjevich, 1996).

The microorganisms utilize the organic matter for energy production by cellular respiration as well as protein synthesis for the manufacture of new cells. Waterborne microorganisms, a large number of which are pathogenic to humans, cause many diseases. The transmission of these diseases through wastewater is a constant public threat. Therefore the use of microorganisms as indicators to assess the microbial quality of water as well as in the assessment of wastewater treatment efficiency, is an important tool in pollution control e.g., *Escherichia coli* (Gray, 1989).

2.2 WATER QUALITY AND HEALTH HAZARDS

Water quality describes the physical, chemical and biological constituents of water. Water is regarded as of high quality when it is safe to drink i.e., when its constituents are below a level at which they can be harmful. Physical and chemical monitoring are particularly required when a known contaminant is being targeted, however knowledge of the behaviour of the contaminant is required to plan an effective programme for their elimination (Neves, 1996). Plants and animals have different tolerances and requirements and can prove to be very good indicators of environmental conditions e.g., plants, birds and fish. Water plays a very important role in

supporting all forms of life, however if contaminated, it has great potential for transmitting a variety of diseases and illnesses (Pearson, 1999).

In developing countries around the world water-related diseases are rare since they have efficient water supplies and wastewater disposal systems. However in parts of the world of developing countries a large portion of the population are without a safe water supply and adequate sanitation. Therefore the toll of water-related diseases has been taken to frightening extents (Tebbutt, 1998).

Millions of people die every year as a consequence of unsafe water or inadequate sanitation. In developing countries, however, millions of people are without safe water supply and do not have adequate sanitation. In the developed world there are major concerns about the long-term health hazards that may arise due to the presence of trace amounts of impurities in drinking water, especially carcinogenic compounds. There are also other chemical contaminants that are naturally occurring or man-made which are known to affect the health of consumers (Kandiah, 1999).

2.3 WATER MANAGEMENT AND DEMAND

Increasing public awareness of the water environment has caused more emphasis to be placed on water pollution control activities and the employment of the best available technology and treatment processes, particularly for industrial wastewaters. As technologies improve it may be possible to achieve higher effluent qualities. As effluent standards become more demanding, financial implications for industries and the public may reach the point where the economic solution may be to cease the discharge. This is impractical for domestic wastewater discharges but could be an option for industry that results in change of the process or closure of the production facility (Neves, 1996).

As a persons standard of living increases, so too does their consumption of water in the home, although the water for these purposes is not normally ingested. Most of the water taken into a home is returned as wastewater and many industries discharge volumes of wastewater that are similar to their consumption of water. Agricultural uses of water place the largest demands on water resources, particularly in developing countries (Veltri, 1996).

2.3.1 Domestic Water Demand

There are many domestic uses of water that are highly important, aside from the fact that water is essential for survival e.g., personal hygiene, cooking, washing, etc. The amount of water used depends on the lifestyle of the community as well as the availability of water that is a measure of both quantity and cost. The demand of water increases with developing lifestyles (Veltri, 1996).

In developed countries the provision of multiple taps, flush toilets, washing machines, swimming pools, etc increase domestic water demands greatly. The flush toilet accounts for a great portion of domestic water consumption and although it displays hygienic attractions, it carries away large quantities of water that carry human wastes to treatment works where the polluting matter is concentrated at a considerable expense (Tebbutt, 1998).

2.3.2 Industrial Water Demand

Many industrial operations consume an amount of water in addition to that required by workers and in manufacturing areas the demand for industrial water may equal or exceed the domestic demand. Most industrial uses of water do not necessarily require a supply of potable quality and there is increasing usage of lower grade supplies e.g., raw river water and sewage effluents (Veltri, 1996).

Although water remains a relatively cheap raw material, the rising cost of water supplies and the treatment of wastewaters have motivated many industries to reduce and limit their water intake. Water consumption is now being increasingly recognized by industry as expenditures that can be controlled. Clean technology concepts are giving rise to reductions in water consumption and water production that benefits both industry and the environment (Tebbutt, 1998).

2.4 WASTEWATER

Wastewater is defined as domestic (sanitary) or industrial. Domestic wastewater comes specifically from residences, commercial places and institutions e.g., schools, while industrial wastewater comes from manufacturing plants. A wastewater treatment plant is a combination of separate treatment processes designed to produce an effluent of specific quality from wastewater of known composition. Unit treatment processes can be classified into five stages:

- a) Preliminary treatment: removal and digestion of solids and grit and the separation of storm-water. Oil and grease are also removed if present in large amounts.
- b) Primary (sedimentation) treatment: involves the removal of settleable solids that are separated as sludge.
- c) Secondary (biological) treatment: dissolved organics are oxidized in the presence of microorganisms.
- d) Tertiary treatment: further treatment of a biologically treated effluent to remove bacteria, suspended solids, specific toxic compounds or nutrients to enable the final effluent to comply with more stringent standards before discharge.
- e) Sludge treatment: the dewatering, stabilization and disposal of sludge (Pearson, 1999).

Large towns and cities have mixtures of both domestic and industrial wastewaters. This is commonly called municipal wastewater and usually includes effluents from service industries

e.g., laundries. It is uncommon for modern municipal treatment plants to accept wastewater from major industries e.g., meat processing unless the treatment plant is specifically designed to do so. The usual practice is for water authorities to charge industry for the treatment and disposal of their wastewater (Gray, 1989).

2.5 WATER TREATMENT PROCESSES

The aims of wastewater treatment are: to convert the waste matter present into stable oxidized end-products which can be safely discharged to inland or coastal waters without any adverse ecological effects to protect public health; to ensure the effective disposal of wastewater on a regular and reliable basis without offence; to provide an economical method of disposal and recover valuable components of wastewater (Gray, 1989).

Waters and wastewaters have highly complex compositions and adjustments to the composition are usually necessary to suit a particular use or prevent degradation of the environment. Therefore a variety of treatment processes are necessary to treat the range of contaminants (Pearson, 1999).

In certain cases it may be necessary to add substances to the water or wastewater to improve its characteristics, e.g., chlorine for disinfection of water, oxygen for the biological stabilization of organic matter (Tebbutt, 1998).

2.5.1 Methods of Treatment

There are three main types of treatment processes:

1. Physical processes: this depends on physical properties of the impurity, e.g., particle size, gravity, viscosity, etc. Typical examples of this type of process include screening, sedimentation, filtration and gas transfer.

2. Chemical processes: this depends on the chemical properties of an impurity or which use the chemical properties of added reagents. Examples of this process include coagulation, precipitation and ion exchange.
3. Biological processes: this depends on the biochemical reactions to remove soluble or colloidal impurities, usually organics. Aerobic biological processes include biological filtration and activated sludge. Anaerobic oxidation processes are used to stabilize organic sludge and highly concentrated organic wastes (Tebbutt, 1998).

In certain situations a single treatment process may be sufficient to provide the desired change in composition, however in most cases it is necessary to utilize several combinations of processes (Tebbutt, 1998).

2.5.2 Disinfection

Microorganisms are small in size and their complete removal from water by processes like coagulation and filtration cannot provide any degree of surety. With regard to ground waters there may be no need for treatment, but there is always a possibility of bacteria and viruses. Due to the public health importance of waterborne organisms, it is necessary to ensure the removal of potentially harmful microorganisms from potable water by means of suitable disinfection processes (Vidić, 1996).

2.6 THE ACTIVATED SLUDGE PROCESS

Activated sludge refers to the brown flocculent culture developed in an aeration tank under controlled conditions. This is a secondary treatment process in which sewage is aerated with a biologically active sludge, which causes the microorganisms to remove pollutants from the sewage (Bitton, 1994).

This process was developed in England and is probably the most versatile of all biological treatment processes since it has found wide applicability with regard to wastewater treatment processes. Treatment of wastes utilizing the activated sludge process represents a portion of the largest biotechnology industry in the world (Seviour and Blackall, 1999).

Bacteria are the most important group of microorganisms found in this biological contact process, since they are responsible for the structural and functional activity of the activated sludge floc. Predominant bacteria are determined by the nature of organic substances in the wastewater, the mode of operation of the plant and the environmental conditions that are present for the organisms in the process. Fungi are relatively rare in activated sludge, but, when present, tend to be of the filamentous form (Seviour *et al.*, 1999).

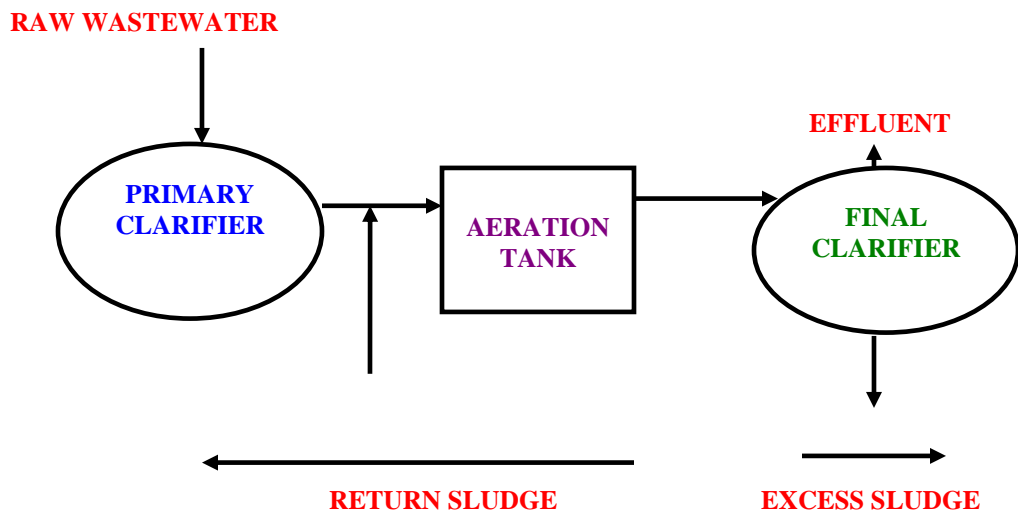


Figure 2.1 Adaptation of a conventional activated sludge system (Bitton, 1994).

Raw wastewater (wastewater before it receives any treatment), as shown in Figure 2.1, is passed into the primary clarifier, where the settleable pollutants are removed by physical means (usually gravity sedimentation), which results in an effluent. This effluent is thereafter passed into an

aeration tank where it is kept for the required length of time. Aeration serves at least 3 important functions:

- 1) Mixing the returned sludge with effluent from primary treatment,
- 2) Keeping the activated sludge in suspension, and
- 3) Supplying oxygen to the biochemical reactions necessary for the stabilization of the wastewater (Bitton, 1994).

The air requirements for good treatment can be satisfied either by a diffused air system or mechanical aerators. Substantially shorter periods are used in some of the modifications of the conventional process. These shorter aeration periods generally result in a lowering of the quality of plant effluents. The effluent is thereafter passed into the final clarifier where the activated sludge solids are separated from the effluent. Following primary clarification, the wastewater contains some suspended and colloidal solids and when agitated in the presence of air, suspended solids form nuclei on which biological life develop and gradually build up to larger solids, which are known as activated sludge (Seviour *et al.*, 1999).

The generation of activated sludge or floc in wastewater is a slow process and the amount so formed from any volume of wastewater during its period of treatment is small and inadequate for the rapid and effective treatment of the wastewater, which requires large concentrations of activated sludge. Collecting the sludge produced from each volume of wastewater treated and re-using it in the treatment of subsequent wastewater flows build up such concentrations. The sludge that is re-used is known as returned sludge. The purpose of this sludge is to maintain a concentration of activated sludge in the aeration tank sufficient for the desired degree of treatment (Seviour and Blackall, 1999).

Excess activated sludge should be wasted as required to maintain the desired solids concentration in the aeration tank. Either withdrawing mixed liquor directly from the aeration tank or to waste from the sludge return line can achieve this. The wasted mixed liquor can then be discharged to a thickening tank or to the primary tanks where the sludge settles and mixes with the raw primary sludge. The waste sludge, usually from the sludge return line, is further thickened by final sedimentation, centrifugation, or flotation thickening and then treated by biological or chemical means. The effluent refers to the water leaving the treatment unit (Bitton, 1994; Seviour and Blackall, 1999).

2.7 BIOLOGICAL NUTRIENT REMOVAL (BNR)

Contamination of water bodies with nitrogen compounds is associated with atmospheric deposition with run-off from agricultural land and leaching of nitrate and nitrite from soil (Robertson and Kuenen, 1992). Interest is being shown in the use of biological systems to remove nitrogen. These systems are commonly referred to as Biological Nutrient removal (BNR) systems (Daigger, 1998).

In a biological nutrient removal (BNR) process denitrification is achieved in the anoxic zone of the process. This is a biological environment that is deficient in molecular oxygen, but may contain bound oxygen, such as nitrates and nitrites. Under these conditions certain heterotrophic bacteria are stimulated into using nitrates and nitrites as final electron acceptor for respiration, instead of oxygen. This results in oxidation of organic matter as well as the reduction of nitrates and nitrites into nitrous oxides and nitrogen gas (Wanner and Grau, 1988 as cited in Drysdale *et al.*, 1999).

The discharge of ammonia and nitrogen in receiving streams results in increased oxygen demand and depressed dissolved oxygen concentrations. Ammonia-nitrogen can be toxic to aquatic organisms, as well as nitrite-nitrogen. Nitrogen discharges enrich receiving water bodies, which result in the undesirable growth of algae and other aquatic plants. This process is known as eutrophication (Daigger, 1998).

In activated sludge systems an aerobic zone may exist above the anoxic zone thus allowing for simultaneous nitrification and denitrification (Blackall and Burrell, 1999). Under aerobic conditions, denitrifying bacteria use oxygen as a final electron acceptor in preference to nitrate, during respiration. This is due to glucose oxidation, which in the presence of oxygen yields more energy than in the presence of nitrate (Delwiche, 1970 as cited in Bitton, 1999). Therefore in order for denitrification to occur anoxic conditions must be provided (Bitton, 1999). Under anoxic conditions, heterotrophic denitrifiers require nitrate as an electron acceptor, which indicates that in wastewater treatment nitrification should occur before denitrification, in order to provide the denitrifiers with the nitrate that they require (Haandel *et al.*, 1981).

2.8 THE NITROGEN CYCLE

Nitrogen in municipal wastewater is the result of human excreta, ground garbage and industrial wastes. The presence of nitrogen in wastewater discharges can be undesirable, as free ammonia it is toxic to fish and other aquatic organisms; as the ammonium ion or ammonia it is an oxygen-consuming compound, which will deplete the dissolved oxygen in receiving water; and as the nitrate ion it is a potential public health hazard in water consumed by infants. Nitrogen is removed microbiologically from wastewater by the sequential processes of nitrification followed by denitrification (Kornaros *et al.*, 1996). Depending on the circumstances, removal of all forms of nitrogen or just ammonium may be required (Sedlak, 1991).

The biological nitrogen cycle is an important part of global interconversion of nitrogen compounds. Nitrogen is essential to life and is a component of proteins and nucleic acids in microbial, animal and plant cells. Ironically, nitrogen is the most abundant gas (79% of the earth's atmosphere), yet it is a limiting nutrient in aquatic environments and agricultural lands (Bitton, 1994).

Most organisms cannot use nitrogen unless it is first converted to ammonia since nitrogen is a stable molecule and will only undergo changes under extreme conditions e.g. high temperatures. Nitrogen atoms are constantly moving in a giant circle from the air, through the soil, into the bodies of plants and animals, and eventually back to the air. This process is called the nitrogen cycle, as depicted in Figure 2.2. Microorganisms play an important role in the nitrogen cycle, which are determined by five processes: nitrogen fixation, assimilation, mineralization, nitrification and denitrification (Bitton, 1994).

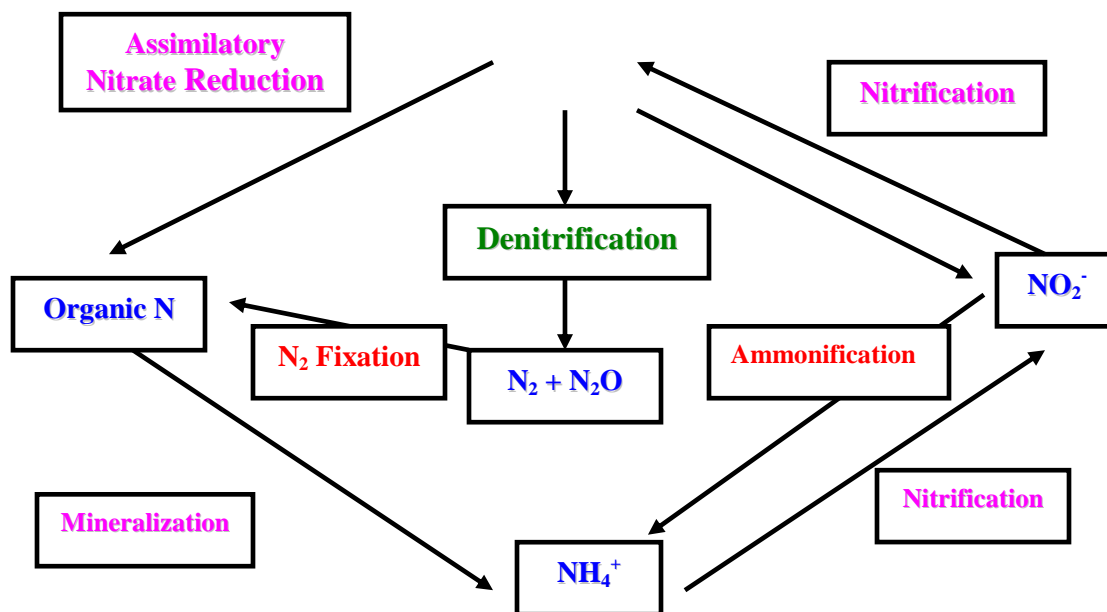


Figure 2.2 Schematic representation of the nitrogen cycle (adapted from Prescott *et al.*, 1996).

2.8.1 Nitrogen fixation – chemical reduction of nitrogen is expensive and requires large amounts of energy. Only a few species of bacteria and cyanobacteria are capable of carrying out nitrogen fixation, which ultimately results in ammonia. This process is driven by the enzyme nitrogenase, which has the ability to reduce triple-bonded molecules e.g., nitrogen (Bitton, 1994).

2.8.2 Nitrogen assimilation – heterotrophic and autotrophic microorganisms take up and assimilate ammonia and nitrate after reduction to ammonia. Plant and algal cells take up nitrogen preferably in the form of ammonia. Cells convert nitrate or ammonia to proteins and grow until nitrogen becomes limiting (Bitton, 1994).

2.8.3 Nitrogen mineralization (Ammonification) – transformation of organic nitrogenous compounds to inorganic forms (Bitton, 1994).

2.8.4 Nitrification – this process entails the conversion of ammonium to nitrate by microbial action (primarily by aerobic chemoautotrophs - nitrifiers). This is carried out by two categories of organisms:

- Ammonia oxidizers - conversion of ammonia to nitrite - known as ammonium oxidation. The organisms involved are called ammonia oxidizers. There are four recognized genera: *Nitrosomonas*, *Nitrospira*, *Nitrosococcus* and *Nitrosolobus*.
- Nitrite oxidizing bacteria - conversion of nitrite to nitrate – known as nitrite oxidation e.g., *Nitrobacter* (Corbin, 1998).

The microbes that perform nitrification are inefficient since most of them are autotrophs, which use energy gained from oxidizing ammonia to fix carbon. Therefore these bacteria have a dual role: they are involved in recycling nitrogen and fixing carbon into organics, but this is not efficient. Microbes that perform nitrification are fragile and acid-sensitive, even though they produce acid. Nitrification is a nuisance to the agricultural industry since the conversion of ammonia to nitrate is very rapid under optimal conditions (Burrell *et al.*, 1998).

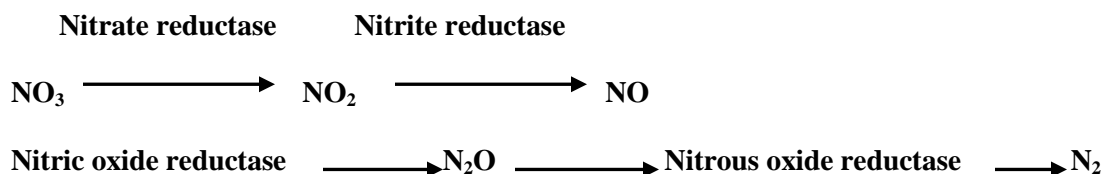
2.8.5 Denitrification – this is the term used to describe the biological reduction of nitrate and nitrite to nitrous oxide and nitrogen. This is one of the key processes in the nitrogen cycle (Blackall and Burrell, 1999).

2.9 DENITRIFICATION

Nitrate must be removed from water bodies, especially if the receiving water body serves as a source of drinking water. Assimilatory nitrate reduction and dissimilatory nitrate reduction are the two main mechanisms of biological nitrate reduction (Robertson and Kuenen, 1992).

- Assimilatory nitrate reduction – a process whereby nitrate is taken up and converted to nitrite and then to ammonium by plants and microorganisms. Several enzymes are involved especially a wide range of assimilatory nitrate reductases, the activity of which is unaffected by oxygen. Some microorganisms possess both an assimilatory nitrate reductase and a dissimilatory nitrate reductase, which is sensitive to oxygen (Blackall and Burrell, 1999).
- Dissimilatory nitrate reduction – a process of anaerobic respiration where nitrate (NO_3^-) is reduced to nitrous oxide (N_2O) and nitrogen gas (N_2). N_2 is liberated but has low water solubility and tends to escape as rising bubbles, which may interfere with the settling of the sludge in a sedimentation tank. Microorganisms involved in denitrification are aerobic autotrophic or heterotrophic, that can switch to anaerobic growth when nitrate is used as an electron acceptor (Ward, 1998; Blackall and Burrell, 1999).

Denitrification is carried out in the following sequence (Sedlak, 1991):



N₂O may be produced during denitrification in wastewater, which leads to the incomplete removal of nitrate. Up to 8 % of nitrate is converted to N₂O under certain conditions (Szekeres *et al.*, 2001).

2.9.1 Classification of Denitrifying Bacteria

According to the denitrification process, denitrifying bacteria comprise of four different functional groups (Drysdale *et al.*, 2000). Based on their ability to reduce nitrate or nitrite, heterotrophic denitrifiers are characterized into specific groups depicting their capacity for nitrate and/or nitrite reduction under anoxic conditions. These groups are the incomplete denitrifiers, true denitrifiers, sequential denitrifiers and exclusive nitrite reducers (Drysdale, 2001).

- Incomplete denitrifiers – capable of reducing nitrates to nitrite but incapable of further reduction of the nitrite. These organisms predominate broth surfaces that indicate their preference for oxygen as an electron acceptor. They lack nitrite reductase enzymes. These bacteria prefer oxygen but are capable of adapting to oxygen-free environments, where nitrate is used as an alternative electron acceptor (Drysdale *et al.*, 2000; Thomson *et al.*, 2003).
- True denitrifiers – these bacteria are capable of reducing both nitrates and nitrites simultaneously since they contain both nitrate reductase and nitrite reductase enzymes. The utilization of both nitrate and nitrite as electron acceptors results in more abundant growth throughout test broths.
- Additionally, nitrite reduction to nitrogen gas results in the production of insoluble gas bubbles, which rise to the surface of test broths. This is indicative of complete denitrification (Drysdale *et al.*, 2000; Thomson *et al.*, 2003).

- Sequential denitrifiers – this group is also capable of both nitrate and nitrite reduction (like the true denitrifiers) except that nitrite reduction is inhibited in the presence of nitrate, which results in nitrite accumulation during nitrate reduction. These bacteria are regulated by a feedback inhibition mechanism that regulates nitrite reduction for the absence of nitrate (Drysdale *et al.*, 2000; Thomson *et al.*, 2003).
- Exclusive nitrite reducers – exhibit non-denitrification characteristics in nitrate media but are capable of efficient nitrite reduction in nitrite media due to the absence of nitrate reductase enzymes (Drysdale *et al.*, 2000).

2.9.2 Enzymes Present in Denitrifying Isolates

The enzymes found in the four different categories of denitrifying bacteria i.e., true denitrifiers, incomplete denitrifiers, sequential denitrifiers and exclusive nitrite reducers are essential to the biological denitrification process. These enzymes are involved in the stepwise reduction of nitrous oxides associated with electron transport phosphorylation and evolution of the gases nitric oxide (NO), nitrous oxide (N₂O) and molecular nitrogen (N₂) (Hallin and Lindgren, 1999).

The nitrogen oxides of the denitrification pathway, which comprise of the more reduced species i.e. nitrite, nitric oxide and nitrous oxide, are referred to as the intermediates of the denitrification pathway and dinitrogen as the end product of denitrification. The transfer of electrons along the pathway accompanies the reduction of one-nitrogen oxide intermediate to another to the specific nitrogen oxide. A specific enzyme catalyses reduction of each specific nitrogen oxide; these enzymes belong to a group which are generally referred to as the nitrogen oxide reductases. These are known specifically as the nitrate reductases, nitrite reductases, nitric oxide reductases and nitrous oxide reductases (Casey *et al.*, 1999).

Denitrification involves a suite of reductase enzymes all of which require metal co-factors, known as metalloenzymes. Nitrate reductases (Nar) which contain molybdenum and iron, are usually membrane bound and oxygen sensitive. Two major forms of nitrate reductases are found in denitrifying bacteria, one containing iron (heme d1) and one containing copper (type I and type II) (The Princeton Environmental Institute, 2000).

There are two known and distinct types of nitrite reductase enzymes (Nir), one with heme c and heme d1 and the other containing copper (Hallin and Lindgren, 1999). Nitric oxide reductase enzymes (Nor) are membrane-bound and catalyze the reduction of two molecules of nitric oxide to one molecule of nitrous oxide and one molecule of water. The metalloenzyme contained in nitrous oxide reductase enzymes is copper (Suharti *et al.*, 2001).

The function of these enzymes may be plasmid-related. Plasmids are independent, circular, self-replicating DNA molecules that carry only a few genes. Plasmids exist as extra-chromosomal genomes in cells, although they can be inserted into a bacterial chromosome, where they become a permanent part of the bacterial genome (Casey *et al.*, 1999).

The ability of these isolates to reduce nitrate or nitrite could be the result of the increase or reduction in plasmid copy numbers (number of plasmids contained in a specific cell). Metabolic plasmids carry genes coding for enzymes that degrade specific substances e.g., nitrite, which has been reported to be toxic (Prescott *et al.*, 1996).

Different bacteria have different tolerance levels for nitrite and this could be due to different bacterial species having different plasmid copy numbers. Those with high copy numbers are possibly able to tolerate higher concentrations of nitrite, whereas those bacteria with low copy

numbers may not produce sufficient amounts of enzyme and are thus incapable of reducing larger quantities of nitrite (Thomson, 2002).

2.10 THE ROLE OF MOLECULAR TECHNIQUES

The progress of methodologies for the analysis of microorganisms and microbial ecology at molecular level has shown extraordinary improvement in recent years. Each methodology has specific advantages and disadvantages, or complications. However, the advances in PCR and sequencing have enabled techniques exploiting nucleic acids to be extensively utilised for microbial analysis (Moore *et al.*, 2004).

Current advances in microbial ecology have been relying on the exploitation of modern tools of molecular biology. The purpose of molecular techniques to study microorganisms in their natural environments not only led to the identification of new and uncultured species but it also improved our understanding of the interactions in complex microbial communities (Eichler *et al.*, 2004).

Although molecular techniques have been successful in analyzing microbial communities, classical isolation techniques for bacteria are still essential (Blackall, 1994). However, nucleic acid-based techniques can support classical techniques by providing the tools for identification, characterization, and screening of bacteria (Muyzer *et al.*, 1993). Molecular methods are more rapid and more reliable, since they are unaffected by culture media and growth conditions, as compared with microbiological and biochemical methods (Schramm and Amann, 1999; Muyzer *et al.*, 2004).

New molecular techniques such as DGGE of PCR-amplified DNA fragments can be used to determine the ecological importance of denitrifiers as well as detect and identify new denitrifying bacteria (Hallin and Lindgren, 1999).

2.11 EXTRACTION OF NUCLEIC ACIDS

The extraction of nucleic acids from biological material requires cell lysis, inactivation of cellular nucleases, and separation of the desired nucleic acid from cellular debris. Conventional methods usually employ a lysis procedure that is rigorous enough to fragment the complex starting material and inactivate nucleases, yet gentle enough to preserve the desired nucleic acid (Burtscher and Wuertz, 2003).

Good quality DNA or RNA can be easily extracted, however nucleic acids from archival material tends to be impure and therefore needs to be prepared such that it will be uncontaminated before analysis. When extracting DNA for use as PCR template it is useful to be made aware of the concentration and quality of the DNA in the sample. Samples may be quantitatively assayed by spectrophotometry or fluorometry by measurement of the fluorescence enhancement of a dye as it binds to DNA (Jackson *et al.*, 1991).

Qualitative analysis involves the assaying of DNA by running an agarose electrophoresis ‘mini-gel’. A molecular weight marker is run alongside the samples to give a ladder of fragments of various sizes such that the molecular weight of the DNA can be estimated. This type of information allows the success of the extraction technique to be determined so that in event of any negative PCR’s, it might be explained by a failure of the DNA extraction procedure (Burtscher and Wuertz, 2003).

2.11.1 Methods of DNA extraction

- a) **Proteinase K incubation:** this procedure involves incubation of fresh tissue or cells with the proteolytic enzyme proteinase K for up to 24 hours. This is followed by the purification of the DNA by several organic extraction steps using phenol and chloroform.

The DNA is thereafter re-dissolved in a buffer and stored at 4°C. This technique has

several advantages: it is very efficient and can produce large amounts of very high molecular weight DNA from as little as a single fresh frozen tissue section; the DNA can be stored for several months; fresh tissue needs to only be incubated with proteinase K for 1 hour to produce as good yields of high molecular weight DNA as much longer incubations therefore reducing the time required for this procedure; this method can be applied to blocks of fresh tissue e.g., cultured cells, cervical smears, etc; and, the DNA produced by this method is of a suitable quality for use with standard molecular biological techniques as well as PCR (Kuhn *et al.*, 2002).

- b) DNA suitable for PCR amplification can also be produced from fresh tissue by simple boiling in distilled water. The main advantage of this technique is its speed since it takes only a matter of minutes. Prolonged boiling of tissues reduces the yield of released DNA so the optimum boiling time is only 15 minutes. Once again, a small amount of tissue will produce sufficient DNA for several amplification reactions. DNA extracted by this method can be stored at 4°C or frozen, Boiling tissue produces DNA sample of sufficient concentration and quality to allow the amplification of a single copy gene, however this might not always be efficient enough for the amplification and detection of lower copy number sequences e.g. persistent viral infections (Jackson *et al.*, 1991).

2.12 POLYMERASE CHAIN REACTION (PCR)

Who would have thought a bacterium found in a hot spring in Yellowstone National Park would spark a revolutionary new laboratory technique?

The PCR relies on the ability of DNA-copying enzymes to remain stable at high temperatures. This was achieved by *Thermus aquaticus*, a bacterium that now helps scientists to produce millions of copies of a single DNA segment in a matter of hours (Jackson *et al.*, 1991).

PCR is basically a primer extension reaction for amplifying specific nucleic acids *in vitro*. This method was devised and named by Mullis and colleagues at the Cetus Corporation (Sinclair, 2002). The use of PCR was limited until heat stable DNA polymerase became widely available. The PCR is a major development in the analysis of DNA and RNA because it has both simple and existing technology and enabled the rapid development of new techniques (Burtscher and Wuertz, 2003).

In nature, most organisms copy their DNA in the same manner and PCR mimics this process, the only difference being that it is performed in a test tube. When any cell divides, enzymes called polymerases make a copy of the entire DNA in each chromosome. The first step, as shown in figure 2.3, in this process is to “unzip” the two DNA chains of the double helix. As the two strands separate, DNA polymerase makes a copy using each strand as a template (Sinclair, 2002).

The four nucleotide bases, the building blocks of every piece of DNA, are represented by the letters A, C, G, and T, which represent their chemical names: adenine, cytosine, guanine and thymine, respectively. The A on one strand always pairs with the T on the other, whereas C always pairs with G. the two strands are said to be complementary to each other (Sinclair, 2002).

To copy DNA, polymerase requires two other components, a supply of the four-nucleotide bases and a primer. DNA polymerases cannot copy a chain of DNA without a short sequence of nucleotides to “prime” the process, or get it started. So the cell has another enzyme called a primase that actually makes the first few nucleotides of the copy. This stretch of DNA is called a primer and once this is made the polymerase can take over making the rest of the new chain (Taylor, 1991).

A PCR vial contains all the necessary components for DNA duplication: a piece of DNA, large quantities of the four nucleotides, large quantities of the primer sequence, and DNA polymerase. The polymerase is the *Taq* polymerase, named for *Thermus aquaticus*, from which it was isolated. The three parts of the PCR reaction are carried out in the same vial, but at different temperatures (Sinclair, 2002).

The first part of the process separates the two DNA chains in the double helix, as shown in Figure 2.3, which can be done by simply heating the vial to 94-96°C for 30 seconds. The primers cannot bind to the DNA strands at such a high temperature, so the vial is cooled to 50-65°C. At this temperature the primers bind or “anneal” to the ends of the DNA strands. This takes about 20 seconds. The final step of the reaction is to make a complete copy of the templates (Taylor, 1991).

Since the *Taq* polymerase works best at around 72-75°C (the temperature of the hot springs where the bacterium was discovered), the temperature of the vial is raised. The *Taq* polymerase begins adding nucleotides to the primer and eventually makes a complementary copy of the template. If the template contains an A nucleotide, the enzyme adds on a T nucleotide to the primer. If the template contains a G, it adds a C to the new chain, and so on to the end of the DNA strand. This completes one PCR cycle (Taylor, 1991).

At the end of a cycle, each piece of DNA in the vial has been duplicated. The cycle however can be completed 30 or more times. Each newly synthesized piece of DNA can act as a new template, so after 30 cycles, 1 billion copies of a single piece of DNA can be produced (Jackson *et al.*, 1991).

PCR has rapidly become one of the most widely used techniques in molecular biology since: it is a rapid, inexpensive and simple means of producing relatively large numbers of copies of DNA molecules from minute quantities of source DNA material even when the source DNA is of relatively poor quality (Taylor, 1991).

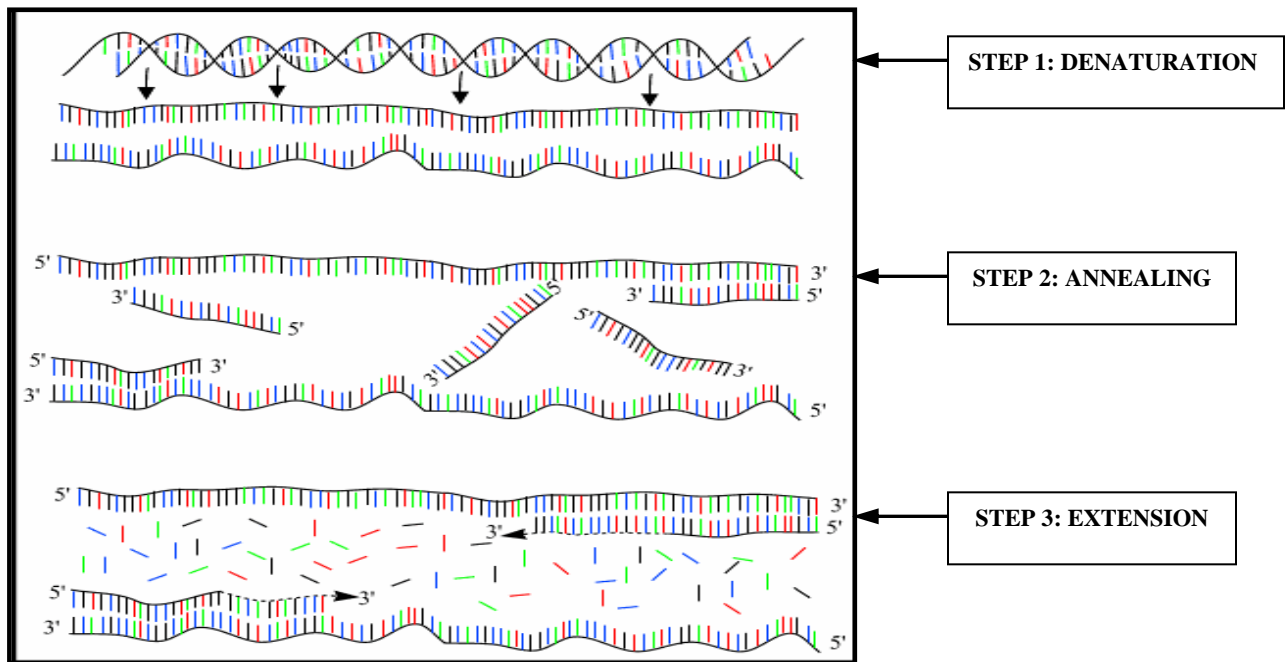


Figure 2.3 Different steps in PCR (Vierstraete, 1999).

2.12.1 Detection and analysis of the reaction product

The product of a PCR should be a fragment or fragments of DNA of defined length. The simplest way to check this is to load a fraction of the reaction product and appropriate molecular weight markers onto an agarose gel of 0.8-4 % containing ethidium bromide, as shown in figure 2.4. The product should be readily visible under ultraviolet transillumination (Ledbetter and Nelson, 1991). By comparing product bands with bands from the known molecular-weight markers, one should be able to identify any product fragments that are of the appropriate molecular weight (Taylor, 1991).

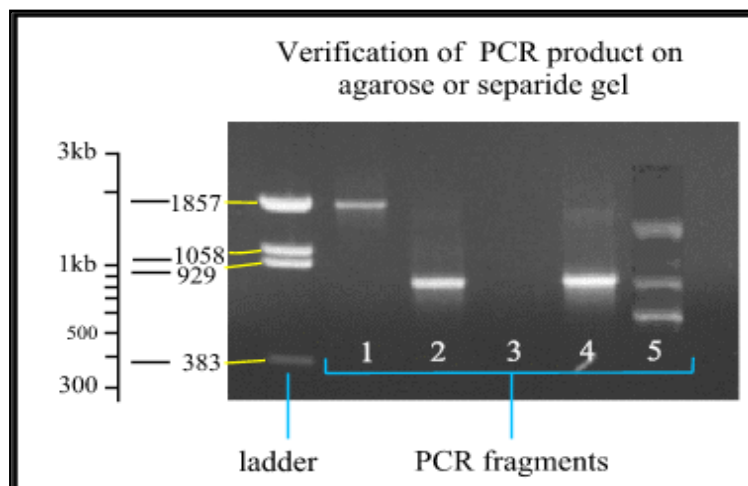


Figure 2.4 Verification of the PCR product on gel (Vierstraete, 1999).

2.13 AGAROSE GEL ELECTROPHORESIS

Gel electrophoresis is a method that separates macromolecules-either nucleic acids or proteins-on the basis of size, electric charge, and other physical properties. DNA is a negatively charged molecule and is moved by electric current through a matrix of agarose (Vierstraete, 1999).

A gel is a colloid in a solid form. Electrophoresis describes the migration of charged particles under the influence of an electric field. *Electro* refers to the energy of electricity. *Phoresis*, from the Greek verb *phoros*, means, “to carry across.” Thus gel electrophoresis refers to the technique in which molecules are forced across a span of gel, motivated by an electrical current. Activated electrodes at either end of the gel provide the driving force. A molecule’s properties determine how rapidly an electric field can move the molecule through a gelatinous medium (Knight and Monroe, 1996).

Many important biological molecules such as amino acids, peptides, proteins, nucleotides and nucleic acids possess ionisable groups and therefore at any given pH exist in solution as

electrically charged species either as cations (+) or anions (-). Depending on the nature of the net charge, the charged particles will migrate either to the cathode or to the anode. The frictional force of the gel material acts as a “molecular sieve,” separating the molecules by size. After staining, the separated molecules in each lane can be seen in a series of bands spread from the top of the gel to the bottom (Vierstraete, 1999).

There are two basic types of materials used to make gels: agarose and polyacrilamide. Agarose is a natural colloid extracted from seaweed. It is very fragile and is easily destroyed by handling. Agarose gels have very large “pore” sizes and are used primarily to separate very large molecules but their resolution is inferior to polyacrilamide gels. Polyacrilamide is the same material that is used for skin electrodes and in soft contact lenses. This type of gel offers greater flexibility and more sharply defined banding than agarose gels (Taylor, 1991).

2.13.1 Applications of Gel Electrophoresis

- a) It is one of the staple tools in molecular biology and is of critical value in many aspects of genetic manipulation and study.
- b) It is used in the identification of particular DNA molecules by the band patterns they yield after being cut with various restriction enzymes.
- c) Viral DNA, plasmid DNA and particular segments of chromosomal DNA can all be identified in this way.
- d) It is also used in the isolation and purification of individual fragments containing interesting genes, which can be recovered from the gel with full biological activity.
- e) It is possible to determine the genetic difference and evolutionary relationship among species of plants and animals.
- f) It is possible to separate and identify protein molecules that differ by as little as a single amino acid.

- g) Can be used for DNA fingerprinting.

2.14 DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE)

DGGE is a powerful, sensitive tool for analyzing the sequence diversity of complex natural microbial populations. It provides information about sequence variation in a mixture of PCR fragments of identical length based on the different mobility in an acrylamide gel matrix of increasing denaturant concentration. The separation principle of DGGE is based on the melting properties (denaturation) of DNA in solution. It is a convenient method of inferring differences in the composition of microbial communities and requires the use of GC clamps in one of the primers for each PCR product (Kowalchuk *et al.*, 1997).

It has previously been shown that minor differences, such as single base pair substitutions, between otherwise identical DNA fragments can result in altered melting behaviour detectable by DGGE. The ability to detect single-base changes in human DNA is of importance in the diagnosis of genetic diseases and the detection of both induced and spontaneous mutations that may underly-developing malignancies. DNA fragments differing by as little as one single-base substitution can be separated from each other by electrophoresis in polyacrylamide gels containing an ascending gradient of the DNA denaturants urea and formamide (Körkkö, 1998).

Two identical DNA fragments that differ by only a single base pair will initially move through the polyacrylamide gel at a constant rate. As they migrate into a critical concentration of denaturant the lowest melting domain within the fragments melt to produce partially denatured DNA. The melting of a domain is accompanied by an abrupt decrease in mobility (Muyzer *et al.*., 1993).

The position in the gel at which decrease in mobility is noticed corresponds to the melting temperature of that domain. Since single-base substitutions within a melting domain results in a melting temperature difference and altered stability characteristics of the double helix, partial denaturation of the two DNA fragments will occur at different positions in the gel. Thus DNA molecules can be separated on the basis of very small differences in the melting temperature as shown in figure 2.5 (Børresen, *et al.*, 1988).

2.14.1 Advantages of DGGE

- a) High detection rate and sensitivity.
- b) The methodology is simple and a non-radioactive detection method is used.
- c) PCR fragments may be isolated from the gel and used in sequencing reactions (Körkkö, 1998).

2.14.2 Disadvantages of DGGE

- a) Computer analysis and preliminary experiments are essential when setting up DGGE from scratch.
- b) Primers are more expensive because of the 40 bases of GC clamp. Additional primers may be required for sequencing.
- c) Analysis of PCR fragments over 400bp is less successful.
- d) Method involves the use of formamide (Körkkö, 1998).

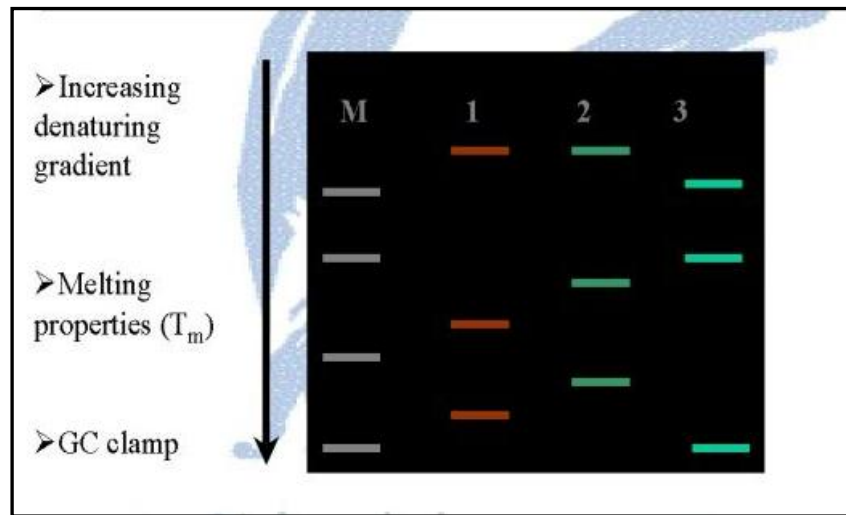


Figure 2.5 Example of DGGE according to the melting properties of DNA (Verseveld *et al*, 2001).

CHAPTER THREE

MATERIALS AND METHODS

3.1 ISOLATION AND MAINTENANCE OF HETEROTROPHIC DENITRIFIERS

Drysdale (2001) obtained mixed liquor activated sludge samples from the anoxic zones of the Darvill Wastewater Treatment Works situated in Pietermaritzburg, Kwa-Zulu Natal. Heterotrophic denitrifiers were isolated from these samples and plated onto Casitone Glycerol Yeast Autolysate (CGYA) agar medium (Appendix 1) to obtain a culture collection (179 isolates). CGYA was used to maintain the cultures as it is one of the most widely and effectively used media for growing and isolating heterotrophic bacteria from activated sludge (Osborn *et al.*, 1989; Gray, 1990; Bux *et al.*, 1994; Drysdale *et al.*, 1999; 2000). This culture collection previously identified and screened for denitrification capacities by Drysdale (2001), was used as the basis for this project. Monthly sub-culturing and storage at 4°C on fresh CGYA slants was used to maintain the culture collection, which was supplemented with potassium nitrate (KNO₃) and potassium nitrite (KNO₂).

3.2 ANALYSIS OF CULTURE PURITY AND ELIMINATION OF IDENTICAL ISOLATES

The purity of the isolates were determined by performing Gram stains (Appendix 2) to analyse their colonial and cellular morphology as proposed by Cappuccino and Sherman (1992). Cultures were inoculated onto CGYA agar plates, using the streak plate technique, and incubated at room temperature. Gram stains were performed within the first 24 hours of growth, since staining of bacterial cultures older than one day may produce variable results. A further four-day incubation period of the inoculated CGYA agar plates produced well-defined colonies for the analysis of colonial morphology. Only cultures that exhibited uniform morphology with regard to size, pigmentation, form, margin and elevation as well as cultures that exhibited single gram

reactions, when examined under oil immersion, were regarded as pure (Appendix 3). Cultures were considered to be identical if they exhibited the same nitrogen conversion characteristics as well as cellular and colonial morphology. With regard to identical isolates, only one culture was retained so as to prevent the repeated testing of organisms.

3.3 DENITRIFICATION SCREENING AND SUBSEQUENT CLASSIFICATION

Pure cultures from the culture collection were screened in triplicate to determine their ability to reduce nitrate and/or nitrite. These were subsequently classified into the following functional groups incomplete denitrifiers, true denitrifiers, sequential denitrifiers (previously termed incomplete nitrite reducers) and exclusive nitrite reducers.

This was achieved by inoculating the bacteria onto nitrate and nitrite media, incubating the bacteria at 20°C for four days. The colorimetric biochemical nitrate reduction test proposed by Cappuccino and Sherman (1992) and Drysdale (2001), was used for screening the denitrification capacities of the isolates (Appendix 4).

The nitrate and nitrite media was prepared by supplementing CGYA broths with KNO_3 and KNO_2 respectively, as proposed by Drysdale (2001). Bacteriological agar, at a concentration of 0.1%, instead of 1.5%, was incorporated into the media that semi-solidified it, thus preventing oxygen from diffusing into the media. This enabled the achievement of anoxic conditions that is essential for most denitrification processes (Cappuccino and Sherman, 1992).

Additionally, the denitrification capacity of the isolates were analysed at lower test concentrations to determine if the bacterial isolates would display a different mode of denitrification. The three concentrations of KNO_3 and KNO_2 tested were 1.0 g/L, 0.5 g/L and 0.2 g/L, respectively.

3.4 EXTRACTION OF DNA FROM ISOLATES

DNA was extracted from whole cells using an adaptation of the boiling method as described by Jackson *et al* (1991). About 3-4 loopfuls of pure culture was dispensed into 1 ml of sterile distilled water and vortexed. Sample vials were placed in a water bath and suspensions were boiled at 48°C for 15 minutes. Thereafter, samples were stored at -4°C until further use.

3.5 AMPLIFICATION OF EXTRACTED DNA

Amplification of bacterial 16S rDNA was performed using bacterial-specific primers. Reaction mixtures (total volume, 100µl) included: 10 µl 10 X PCR buffer excluding MgCl₂, 6 µl 1.5 mM MgCl₂, 10 µl Genomic DNA, 10 µl 2 mM dNTP, forward and reverse primers at a concentration of 50 µM (2 µl of each), 59 µl sterile distilled water and 1 µl Taq polymerase (Giovannoni, 1991).

The following universal primers were used:

~P 341 - 358 forward (with 40 bp GC-clamp): 5' CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG 3' (Kaewpipat and Grady, 2001).

~P 1492 - 1512 reverse: 5' TAC GGC TAC CTT GTT ACG ACT T 3' (Blackall, 1994).

The 40 bp GC-rich sequence at the 5' end of the 341 forward primer brings about a transition for each DNA molecule, from double-stranded (ds DNA), helical to a partially single-stranded (ss DNA) secondary structure. This occurs at a certain position in the gel and when DNA is analysed on a polyacrilamide gel containing an increasing gradient of DNA denaturants. This GC-rich sequence will stop or strongly slow down the migration of the respective gene fragment. The GC region acts as a “clamp” to prevent the formation of ss DNA (Schramm and Amann, 1999).

The reaction mixture was vortexed and thermal cycling was performed with a hot start at 94°C for 4 minutes to inactivate any contaminating proteases. Thereafter, Taq DNA polymerase was added to the reaction mixture to give a final volume of 100 µl. This process was followed by 35 cycles of 94°C for 1 minute, 53°C for 1 minute, 72°C for 2 minutes and a final extension at 72°C for 4 minutes in a thermal cycler (Hybaid PCR Sprint Temperature Cycling System). PCR-amplified products were visualized on 2.5% agarose gel electrophoresis (Appendix 5) (Brown, 1991).

3.6 DENATURING GRADIENT GEL ELECTROPHORESIS OF AMPLIFIED FRAGMENTS

DGGE of PCR amplified DNA fragments were carried out using the BIO-RAD D Gene™ Denaturing Gel Electrophoresis System and performed in accordance with the BIO-RAD D Gene instruction manual and applications guide. A 7.5%, 16 x 16-cm gel was used. Optimum concentrations of the denaturing solutions were experimentally determined by varying the concentrations of urea and formamide. Good separation and resolution of DNA into clear bands on the gel demonstrated optimum concentrations. A 30% low density denaturing solution and a 50% high density denaturing solution was used (Appendix 6).

DGGE settings:	Buffer temperature	60°C
	Maximum buffer temperature	65°C
	Voltage	200V
	Time	80 minutes

Staining and photographing of gel:

After the specified run time, the gel was removed and stained with 1 X TAE buffer and 10 µl Ethidium Bromide (10 mg/ml) for 1-2 minutes. The gel was then transferred to another container containing 1 X TAE buffer where it was de-stained for 5-20 minutes. The gel was then viewed under a Hoefer® MacroVue UV transilluminator. The gel was photographed and allowed to develop for 30 seconds.

All identical isolates were discarded after running DGGE.

3.7 BATCH EXPERIMENTS

The colorimetric biochemical nitrate reduction test proposed by Drysdale (2001) and Thomson (2002) had limitations with regard to the characterization of the denitrifying isolates into its four functional groups. Therefore quantitative work was conducted which was a much more sensitive method of characterisation. CGYA broth was prepared and supplemented with 0.1 g/L of potassium nitrate and 0.05 g/L of potassium nitrite. Aliquots of 195 ml of CGYA broth were placed into schott bottles and inoculated with 5 ml of 24-hour culture suspensions. This was carried out for each of the remaining isolates from the culture collection. A positive control was also used for each organism. All experiments were conducted in duplicate.

The schott bottles were then incubated at 20°C for 6 hours with regular sampling intervals of 30 minutes. Anoxic conditions were maintained using airtight bottles and flushing with nitrogen gas (Suharti *et al.*, 2001). Naidoo and Buckley (WRC Report 820/1/00) also used during batch experiments to maintain anoxic conditions during batch experiments.

The broth samples were thereafter analysed for nitrate and/or nitrite concentrations using the Technicon Autoanalyzer II equipped with a cadmium reduction column (Appendix 7). The results were obtained as total nitrogen in (mg/L) and as true nitrites (mg/L). For true nitrate results

(mg/L) the difference between the total nitrogen and true nitrite readings were used. Previous work conducted by Naidoo and Buckley (WRC Report 820/1/00) also made use of a cadmium reduction method in an automatic continuous flow system. This method served to ultimately differentiate between the four functional groups of the denitrifying isolates.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 ANALYSIS OF CULTURE PURITY / ELIMINATION OF IDENTICAL ISOLATES

Approximately 50 isolates were lost during the sub-culturing process from Thomson (2002) up until this study, and thus the gram stain was performed on only the remaining isolates of the culture collection.

This could be due to one of several serious disadvantages of routine sub-culturing: after prolonged sub-culturing the risk of selection and adaptation to the cultivation medium arise and changes or losses of important properties could occur; the selective and synthetic nature of the media and incubation times with regard to the organisms' resident environmental conditions could play an important role; laboratory conditions can, in extreme cases, lead to the loss of important morphological features and physiological qualities; the possibility of contamination; the possibility of mislabeling or other handling mistakes; routine sub-culturing is always a labour-and-consumable-intensive process and it is therefore difficult to maintain large numbers of strains in a culture collection (Malik, 1984).

The methods used in the maintenance of stock cultures of microorganisms usually involve serial sub-culturing or other simple methods of preservation, which are time consuming and could also result in genetic instability. Future work will involve preservation methods such as freeze-drying with liquid nitrogen using cryo-protective agents i.e., glycerol. Bacteria preserved in liquid nitrogen usually show high rates of survival and good strain stability during long-term storage (Dumont, 2004). Even though these are the usual standard procedures for the preservation of

cultures, these techniques need to be optimised or modified to suit the requirements of the organisms in the culture collection, in order to prevent their deterioration and degradation.

Isolates from the culture collection were analysed for their gram reaction, colonial and cellular morphology, as shown in Appendix 8. Results of this study show that there were no gram-positive cocci present, although gram-negative heterotrophic bacteria predominated the culture collection. A study conducted by Wagner *et al.*, (1994) showed, with molecular analysis, that although gram-negative heterotrophic bacteria are predominant in activated sludge, gram-positive heterotrophic bacteria may be playing a greater role than previously believed. All the isolates were rods except isolates 48 – 51, 137 – 141 and 160 – 163, which were gram-negative cocci.

4.2 DENITRIFICATION SCREENING AND SUBSEQUENT CLASSIFICATION

Analysis of the denitrification capacities of the isolates, using the colorimetric nitrate reduction test, revealed that denitrifying bacteria in activated sludge can be classified into four functional groups based on their abilities to reduce nitrate and/or nitrite.

The nitrate reduction test involved the addition of Solution A (sulphanilic acid) and Solution B (alpha-naphthylamine) to the cultured bacteria (Appendix 4). A cherry red colour was indicative of nitrate reduction to nitrite. If however there was no colour change after the addition of solutions A and B, then a small quantity of zinc powder was added to the medium to reduce any nitrate to nitrite. If the addition of zinc did not produce a colour change this indicated that there was no more nitrate left in the medium to be reduced by the zinc, since it had already been reduced by the bacterium to nitrite, which was further reduced to nitrogen gas.

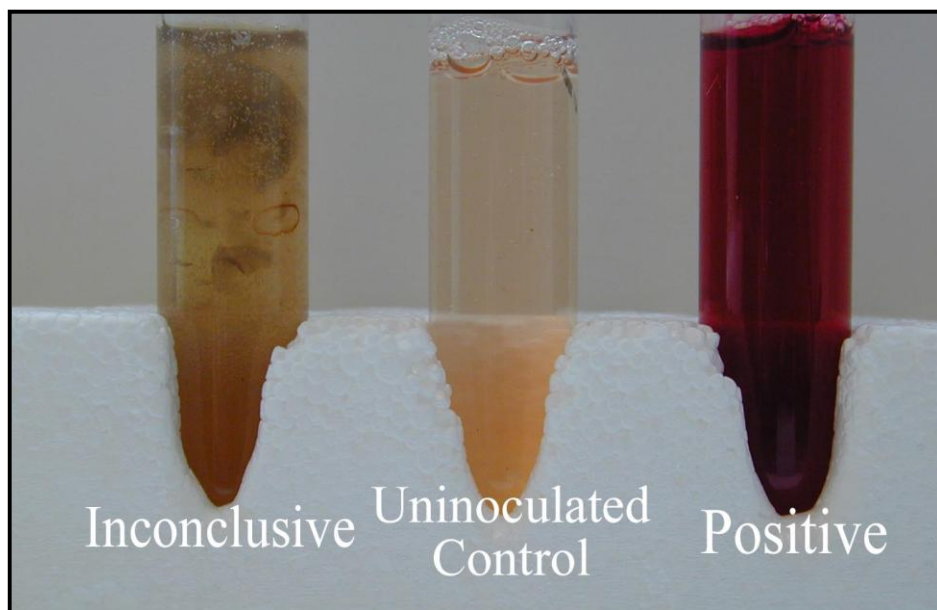


Figure 4.1 Picture illustrating the results of the nitrate reduction test.

Figure 4.1 depicts the principle of the nitrate reduction test. These tubes were treated with dimethyl 1-naphthylamine and sulphanilic acid. The tube on the left illustrated no change, thus it was inconclusive. The tube in the centre (uninoculated control) also illustrated no change. This was expected. The tube on the right indicated that the organism present reduced nitrate to nitrite (positive). To further determine a positive/negative reduction of nitrate, zinc powder was added to detect the presence of nitrate.

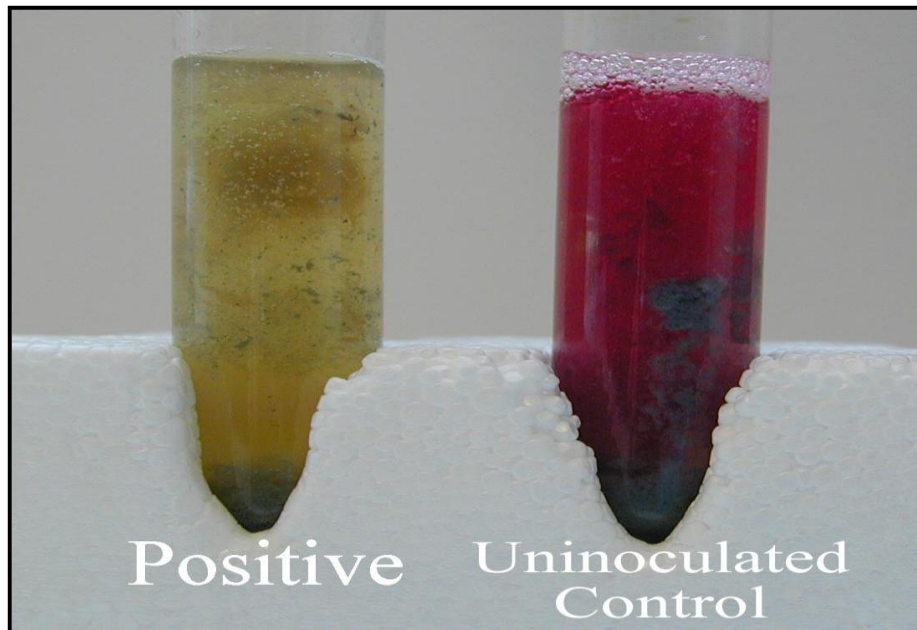


Figure 4.2 Picture illustrating the results of the test after the addition of zinc.

Figure 4.2 illustrates that a tube will turn a deep red colour on the addition of zinc if nitrate is present. The inconclusive tube (figure 4.1) did not turn red since the organism present reduced nitrate to something besides nitrite, which is indicated by the tube labelled 'positive' (figure 4.2). The uninoculated control turned red which indicated that nitrate was still present in the tube. This was expected.

Table 4.1 Comparison of the classification of bacterial isolates into their functional groups based on denitrification capacity.

CULTURE NUMBER	DRYSDALE (2001)	THOMSON (2002)	RAMDHANI (2003)
1 - 26	Incomplete	Incomplete	Incomplete
27 - 30	Sequential	Incomplete	Sequential
31	Sequential	Incomplete	Sequential
32 - 38	Incomplete	Incomplete	Incomplete
39	Not Analyzed	Incomplete	Sequential
40 - 59	Incomplete	Incomplete	Incomplete
60	Sequential	Incomplete	Sequential
61	Incomplete	Incomplete	Incomplete
62 - 64	Not Analyzed	Incomplete	Sequential
65	Sequential	Incomplete	Incomplete
66 - 82	Incomplete	Incomplete	Incomplete
83 - 107	True	True	True
108	Not Analyzed	True	True
109 - 123	True	True	True
124	Inconclusive results	True	True
125 - 129	True	True	True
130 - 133	Sequential	Sequential	Sequential
134 - 136	Incomplete	Sequential	Sequential
137	True	Sequential	Sequential
138	Sequential	Sequential	Sequential
139	Incomplete	Sequential	Sequential
140 - 142	Sequential	Sequential	Sequential
143	Incomplete	Sequential	Sequential
144 - 145	Sequential	Sequential	Sequential
146	True	Sequential	Sequential
147 - 148	Sequential	Sequential	Sequential
149	Incomplete	Sequential	Sequential
150	Sequential	Sequential	Sequential
151	True	Sequential	Sequential
152	Sequential	Sequential	Sequential
153 - 154	Incomplete	Sequential	Sequential
155	Sequential	Sequential	Sequential
156	True	Sequential	Sequential
157 - 161	Not analyzed	Sequential	Sequential
162 - 166	Sequential	Sequential	Sequential
167	True	Sequential	Sequential
168 - 174	Incomplete	Sequential	Sequential
175	Sequential	Exclusive nitrite reducer	Exclusive nitrite reducer
176 - 179	Exclusive nitrite reducer	Exclusive nitrite reducer	Exclusive nitrite reducer

Results of this study confirmed those of the studies conducted by Drysdale (2001) and Thomson (2002), in that denitrifying bacteria can be classified into four functional groups, namely incomplete denitrifiers, true denitrifiers, sequential denitrifiers and exclusive nitrite reducers. The denitrification capacity of the isolates was assessed using the colorimetric nitrate reduction test proposed by Cappuccino and Sherman (1992) and Drysdale (2001). The nitrate reduction test results are displayed in Table 4.1.

Incomplete Denitrifiers

The formation of a red colour after the addition of Solutions A and B indicated the reduction of nitrates to nitrites and bacteria that displayed this reaction were classified as incomplete denitrifiers (Cappuccino and Sherman, 1992). The inability of incomplete denitrifiers to further utilize nitrite restricts their growth under anoxic conditions and limits cellular reproduction. The incomplete denitrifiers were the predominant group of denitrifiers screened, their constituent numbers in a wastewater treatment system needs to be verified *in situ* (Thomson, 2003).

Incomplete denitrifiers have been reported to be predominant in various denitrifying ecosystems (Rheinheimer, 1985; Robertson and Kuenen, 1992; Rosén and Welandar, 1994), however, this was preceding the discovery of great numbers of sequential denitrifiers reported by Drysdale (2001), Thomson (2002) and in this study.

Results of this study show the presence of incomplete denitrifying gram-positive rods as well as gram-negative cocci in the Darvill activated sludge (Appendix 8). The presence of gram-positive heterotrophic bacteria offers confirmation to findings by Wagner *et al.*, (1994) who claimed that gram-positive bacteria might be playing a greater role in activated sludge treatment than previously believed.

True Denitrifiers

If however, there was no colour change after the addition of solutions A and B, then a small quantity of zinc powder was added to the medium to reduce any nitrates to nitrites. If the addition of zinc did not produce colour change then this indicated that there were no more nitrates left in the medium since it had already been reduced by the bacteria to nitrites, which was then further reduced to N_2 . Cultures that exhibited this type of reaction were classified as true denitrifiers (Cappuccino and Sherman, 1992).

Previous studies, based on the classification of the heterotrophic denitrifying bacteria, by Drysdale *et al.*, (1999), showed that *Pseudomonas* spp. was predominant amongst the true denitrifiers. This was the same culture collection used during this study.

According to Robertson and Kuenen (1992) nitrate and nitrite reduction by heterotrophic bacteria is controlled by nitrate and nitrite reductases present in the cytoplasm of the bacteria. These nitrate and nitrite reductases enable denitrifying bacteria to reduce both nitrates and nitrites simultaneously when both the enzymes are present in the cells (Robertson and Kuenen (1992). Robertson and Kuenen (1992) further state that these enzymes are usually induced when the bacterial cells are exposed to oxygen-limited conditions, or even when merely placed in contact with nitrates thus explaining the reason why denitrification occurs predominantly under anoxic conditions.

In this study isolates 83 – 108 (Table 4.1), which were classified as class I true denitrifiers by Thomson (2002), were capable of true denitrification at all three KNO_3 and KNO_2 concentrations tested. Isolates 109 – 129 (Table 4.1), which were classified as class II true denitrifiers by Thomson (2002), displayed true denitrification in the nitrate broths at all test concentrations. However, in nitrite media, the organisms were either incapable of nitrite reduction or capable of nitrite reduction only at low KNO_2 concentrations. This inability to reduce nitrite at high

concentrations may be as a result of the toxic nature of nitrite (Piñar *et al.*, 1997 and Presott *et al.*, 1996).

Piñar *et al.*, (1997) stated that nitrite is toxic to many organisms, is mutagenic in its acid form and at high concentrations, removes proteins from the cell membrane. This would explain why nitrite reduction occurs simultaneous to nitrate reduction so as to avoid toxic build-up of nitrite. However, it is unclear from the results of this study why high concentrations of nitrite do not negatively affect all denitrifiers. The class I true denitrifiers, sequential denitrifiers and exclusive nitrite reducers were capable of nitrite build-up reduction and were not negatively influenced like the class II true denitrifiers.

Sequential Denitrifiers

Cultures that displayed incomplete denitrification on the nitrate medium (red colour after the addition of solutions A and B) and nitrite reduction on the nitrite medium (no colour change after the addition of solutions A and B) were classified as sequential denitrifiers. Additionally, cultures that displayed incomplete denitrification at high nitrate concentrations but displayed true denitrification at low nitrate concentrations were also classified as sequential denitrifiers (Drysdale, 2001).

Current research was conducted with the intention of verifying the denitrification capacities of the isolates based on the nitrate reduction test. Various differences in functional classifications were noted between this study and that of Thomson (2002), based principally on the results using lower concentrations (0.2g/L) of KNO_3 and KNO_2 (Table 4.1). Thus a minor number of isolates were classified as incomplete denitrifiers as compared to the findings of Drysdale (2001) due to nitrite reduction occurring at lower nitrate and nitrite concentrations. These isolates were therefore classified as sequential denitrifiers, as shown by Thomson (2002) and this study.

True denitrifiers reduce nitrate and nitrite simultaneously as compared to the sequential denitrifiers, where nitrite reduction is inhibited by the presence of nitrate, leading to nitrite accumulation during nitrate reduction (Drysedale, 2001). Sequential denitrifiers are regulated by a feedback inhibition mechanism regulating nitrite reduction. It is very likely that nitrate is the regulatory compound and the feedback inhibition is a mechanism to conserve energy due to different electron accepting capacities of nitrate and nitrite. However, more research is required to understand sequential denitrification, with regard to the enzymes involved in denitrification.

Nitrite accumulation is often observed in denitrifying ecosystems (Blaszczyk, 1993; Green *et al.*, 1994; Martienssen *et al.*, 1998). Ekama and Wentzel (1999) suggested the possibility that nitrate is the preferred electron acceptor in cellular respiration since nitrate can accept five electrons in comparison to nitrite that can only accept three. Blaszczyk (1993) stated that the reasons for nitrite accumulation could be due to (i) unbalanced reduction reactions for nitrate and nitrite, or (ii) delayed induction of nitrite reductase in comparison with nitrate reductase. The results of this study show that nitrite accumulation could be partly due to the large amount of sequential denitrifiers present in activated sludge.

Sequential denitrifiers are therefore very important and need to be considered when modeling denitrification kinetics of wastewater treatment processes.

Exclusive Nitrite Reducers

Cultures that produced a red colour after the addition of zinc (when grown on nitrate medium) indicated that no nitrate reduction took place at all. However, if these isolates displayed nitrite reduction (no colour change after the addition of solutions A and B) when grown on nitrite medium, they were classified as exclusive nitrite reducers (Drysedale, 2001). The intensity of the colour reactions indicated the extent to which nitrate and nitrite was reduced. Current findings substantiated previous research by Drysdale (2001) and Thomson (2002) whereby only a few

exclusive nitrite reducers were detected (Table 4.1). Therefore, *in situ* verification of this functional group is essential to establish their precise contribution to denitrification in full-scale processes and other environments. The loss of nitrate reduction capacity by this group of denitrifiers requires further investigation. While it is possible that nitrate reductase genes can be orientated by plasmids, further research is needed to understand the occurrence of this phenomenon (Lötter, 1989).

Therefore, inferred nitrate reduction may be as a result of transfer of genetic material under conditions of continual anoxic exposure, and being plasmid mediated, can be lost under conditions of low plasmid copy numbers and repeated sub-culturing.

Of all the remaining isolates analysed, five were classified into a different functional group as compared with the results observed by Drysdale (2001) and Thomson (2002) (Table 4.1). It must be noted that five of the 179 isolates were not completely analysed by Drysdale (2001), therefore for these organisms, comparisons could only be made with the results observed by Thomson (2002). Cultures investigated in this study had undergone lengthy storage and sub-culturing on CGYA media (Drysdale, 2003).

The observed inability of certain cultures to reduce nitrite, due to lengthy maintenance periods, is possibly attributed to plasmid loss. The presence of metabolic plasmids in denitrifying bacteria is still in need of investigation. However, the presence of two megapasmids in the symbiotic N₂-fixing bacterium *Sinorhizobium meliloti*, was reported by Finan *et al* (2001). The genes involved in N₂-fixation are located on these plasmids as well as several genes involved in nitrate/nitrite reduction. Therefore, it is possible that plasmids do play a role in nitrogen conversion in certain environments.

4.3 MOLECULAR ANALYSIS OF DENITRIFYING ISOLATES

Although molecular techniques have been successful in analyzing microbial communities, classical isolation techniques for bacteria are still essential (Blackall, 1994). However, nucleic acid-based techniques can support classical techniques by providing the tools for identification, characterization, and screening of bacteria (Muyzer *et al.*, 1993). Molecular methods are more rapid and more reliable, since they are unaffected by culture media and growth conditions, as compared with microbiological and biochemical methods (Schramm and Amann, 1999; Muyzer *et al.*, 2004).

New molecular techniques such as DGGE of PCR-amplified DNA fragments can be used to determine the ecological importance of denitrifiers as well as detect and identify new denitrifying bacteria (Hallin and Lindgren, 1999).

In order to unravel the microbial ecology of natural ecosystems it is no longer sufficient to make diversity assessments using only traditional culture based methods. The molecular approach of extracting total nucleic acids and analysing the sequence of PCR-amplified 16S rDNA genes offers a more effective approach for assessing microbial communities without the need for cultivation (Moore *et al.*, 2004).

Figure 4.3 represents an example of an agarose gel electrophoresis that was carried out for all the isolates to amplify 16S rRNA gene segments from the bacterial DNA, which was extracted from the denitrifying isolates. Most PCR applications have used gel electrophoresis to demonstrate the presence of the amplified DNA. This method provides maximum sensitivity and specificity (Muyzer *et al.*, 1993). The fluorescent bands (lanes 2-10) represented a positive result for the presence of DNA in the samples. A molecular weight marker (lane 1) was also run to determine the molecular weight of each sample of DNA.

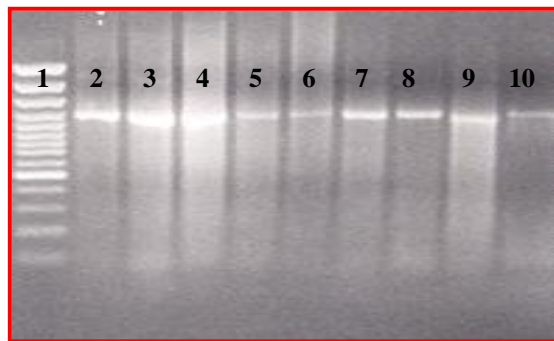


Figure 4.3 PCR profile of DNA-extracted samples; Where, 1 = molecular weight marker & 2-10 = samples.

DGGE was thereafter conducted on all PCR-amplified 16S rDNA samples to aid in the process of discarding all the identical isolates of the culture collection, since DGGE is very effective in differentiating DNA fragments of the same length but different nucleotide sequences. This procedure allows for the direct identification of the presence of different species and thus to profile them in both a qualitative and a semi-quantitative way (Muyzer, 1993). The result of DGGE analysis of PCR products of a microbial community is a band pattern, and the number of bands provides an estimate for the microbial diversity of a system (Schramm and Amann, 1999).

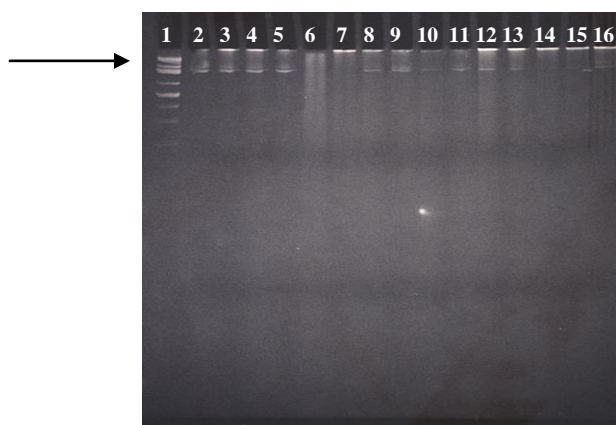


Figure 4.4 DGGE of 15 PCR-amplified fragments

Figure 4.4 shows an example of a typical DGGE gel. Bands that had the same horizontal alignment (indicated by the arrow) in each lane of the gel, was indicative of organisms with DNA fragments of the same length but different nucleotide sequences (lanes 2-5 and 8-16). A molecular weight marker was also run in the DGGE gels as a comparison to determine the molecular weight of all the isolates (lane 1).

Similar banding patterns in the DGGE gels were observed for all samples. The presence of similar bands in different samples is indicative of community similarity and the number of bands present in a sample was used to deduce the richness of species present (Jackson *et al.*, 2001). Clear lanes (6 and 7) in the gel indicated the absence of DNA.

Like many molecular techniques, DGGE focuses on the primary members of a community and will unlikely detect organisms that account for less than 1 % of the community. A band in a DGGE gel is representative of a fraction of the community, not the total abundance of a given population (Jackson *et al.*, 2001).

Table 4.2 Comparison of the classification of the denitrifying isolates after biochemical and molecular analysis.

CLASSIFICATION	NUMBER OF ISOLATES	
	BIOCHEMICAL ANALYSIS (NO ₃ ⁻ REDUCTION TEST)	MOLECULAR ANALYSIS (DGGE)
Incomplete Denitrifier	73	3
True Denitrifier	47	2
Sequential Denitrifier	54	2
Exclusive Nitrite Reducer	5	1

Using molecular analysis, the findings clearly show that those isolates that were regarded as different based on gram reaction, colonial and cellular morphology, were actually the same organism. e.g., 73 morphologically different incomplete denitrifiers were reduced to just 3 distinguishable isolates, as shown in Table 4.2 which displays the results of the comparison in the classification of the isolates after analysis utilizing biochemical tests and molecular techniques.

The classification of the denitrifying isolates based on biochemical analysis (Appendix 8) was performed as a verification of previous research performed by Drysdale (2001). Of all the isolates analysed after biochemical analysis, 47 were true denitrifiers, 54 isolates were sequential denitrifiers and 5 isolates were exclusive nitrite reducers. Whereas, molecular analysis resulted in 2 predominant true denitrifiers, 2 predominant sequential denitrifiers and only 1 predominant exclusive nitrite reducer.

4.4 NITRATE AND/OR NITRITE REDUCTION BY PREDOMINANT ISOLATES

Batch experiments were conducted under anoxic conditions on the remaining 8 predominant isolates since no characterization of the isolates could be made on the basis of the nitrate reduction test alone. These batch experiments were performed since the nitrate reduction test may give false negative or false positive results if the medium is not prepared accurately or the test is not performed accurately. Denitrifying bacteria are usually characterized by their ability to use nitrate and nitrite as electron acceptors when producing gaseous nitrogen.

Most denitrifiers are aerobic i.e. oxygen takes priority to be used as an electron acceptor as compared to nitrate. Therefore it is known that oxygen inhibits the denitrification process (Otani *et al.*, 2004). However, reports have been made that certain cultural species can undergo denitrification even under aerobic conditions, since these bacteria have nitrogen reductases that are oxygen-tolerant (Bell *et al.*, 1991; Gupta, 1997).

The capacities of the remaining isolates to reduce nitrate and/or nitrite were determined experimentally by measuring the quantity of nitrates utilised and the quantity of nitrites remaining or converted to N₂ after the batch experiment. This was achieved by means of a Technicon Autoanalyser II (Appendix 7).

Of all the isolates biochemically profiled, molecular characterization showed the microbial community to comprise of only 3 incomplete denitrifiers, 2 true denitrifiers, 2 sequential denitrifiers and 1 exclusive nitrite reducer. The incomplete denitrifiers were represented by isolates 7, 12 and 56; the true denitrifiers were represented by isolates 87 and 88; the sequential denitrifiers were represented by isolates 144 and 147; and the exclusive nitrite reducers were represented by isolate 176.

Incomplete Denitrifiers:

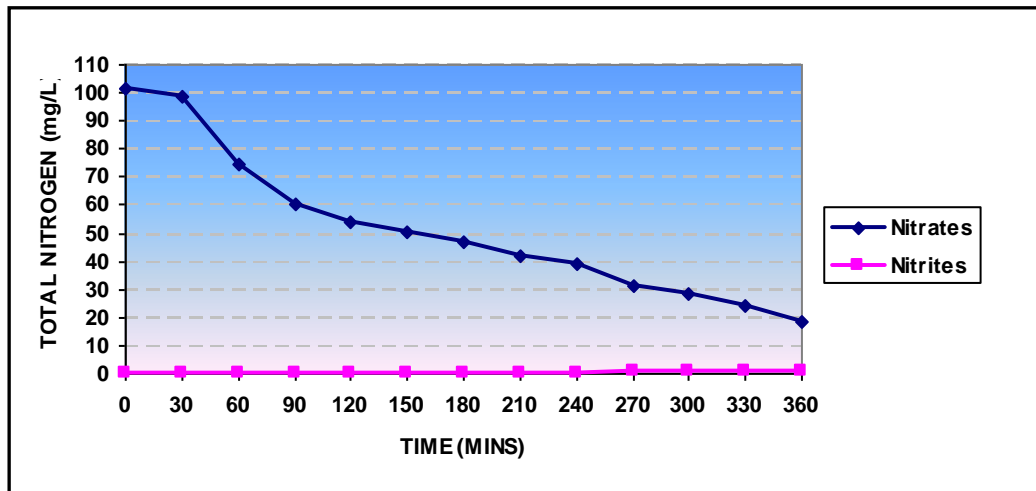


Figure 4.5 Graph showing the amount of nitrates and/or nitrites reduced by Organism 7.

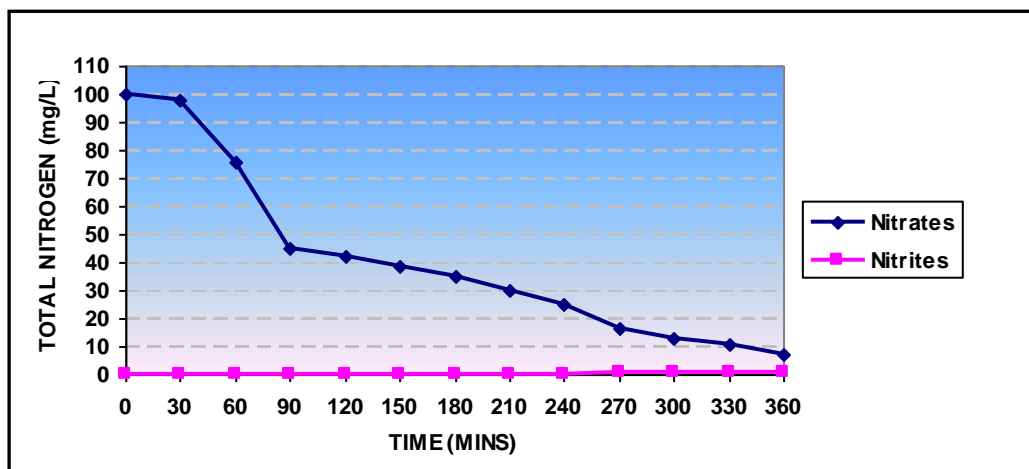


Figure 4.6 Graph showing the amount of nitrates and/or nitrites reduced by Organism 12.

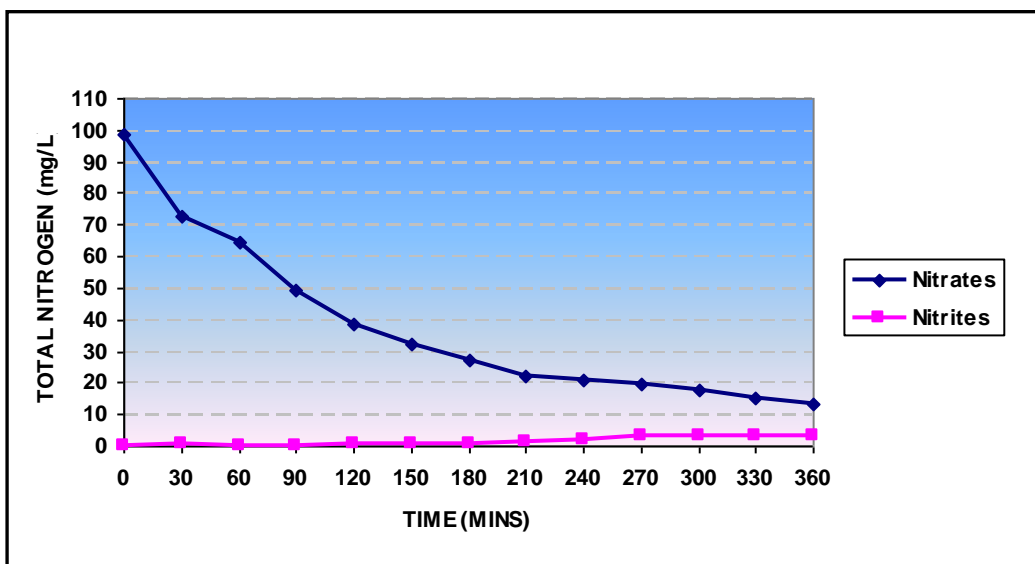


Figure 4.7 Graph showing the amount of nitrates and/or nitrites reduced by Organism 56.

Figures 4.5, 4.6 and 4.7 all depict the capacities of organisms 7, 12 and 56 respectively, to reduce nitrates and /or nitrites. Incomplete denitrifiers are capable of reducing nitrates to nitrite, but are incapable of further reduction of the nitrite. There is an increase in nitrite concentrations, as time increases, for these three figures that is very gradual, thus it cannot clearly be seen. The initial nitrite concentrations for figures 4.5, 4.6 and 4.7 are 0.01, 0.01 and 0.3 mg/L respectively, whilst their final nitrite concentrations are 0.74, 0.70 and 3.0 mg/L respectively. Thus it can be seen that for these organisms the amount of nitrates decreases as the amount of nitrite increases accordingly, at any given time interval.

These results substantiate findings by Rheinheimer (1985), Robertson and Kuenen (1992) and Rosén and Welander (1994), who found that initial nitrite production by actively denitrifying

bacterial communities is usually very high and may, especially if accompanied by high initial nitrate concentrations, result in nitrite build-up. This build-up is as a result of the predominant presence of incomplete denitrifiers within denitrifying bacterial communities. Robertson and Kuenen (1992) state that these incomplete denitrifying bacteria lack key nitrite reductase enzymes that enable the true denitrifiers to reduce nitrites.

True Denitrifiers:

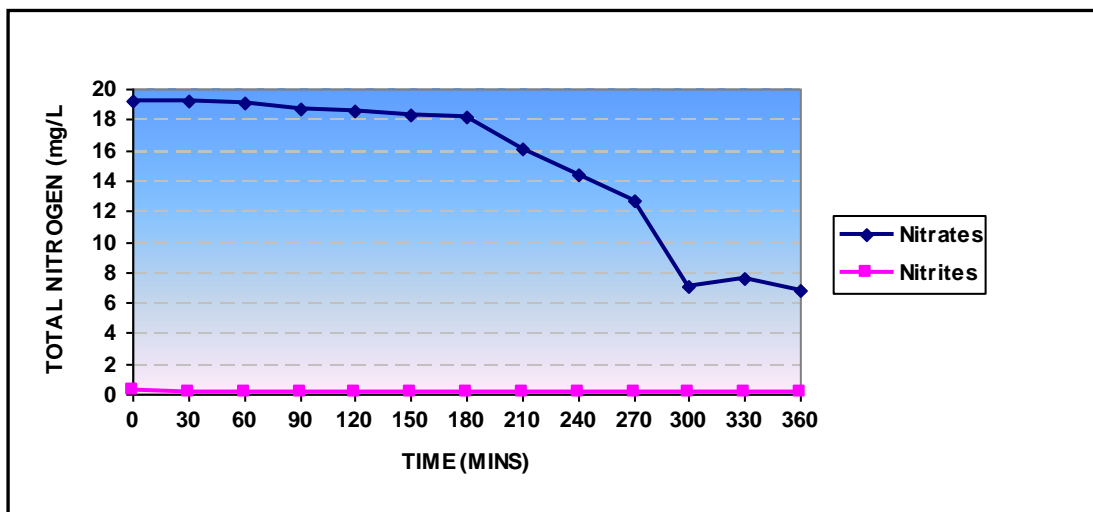


Figure 4.8 Graph showing the amount of nitrates and/or nitrites reduced by Organism 87.

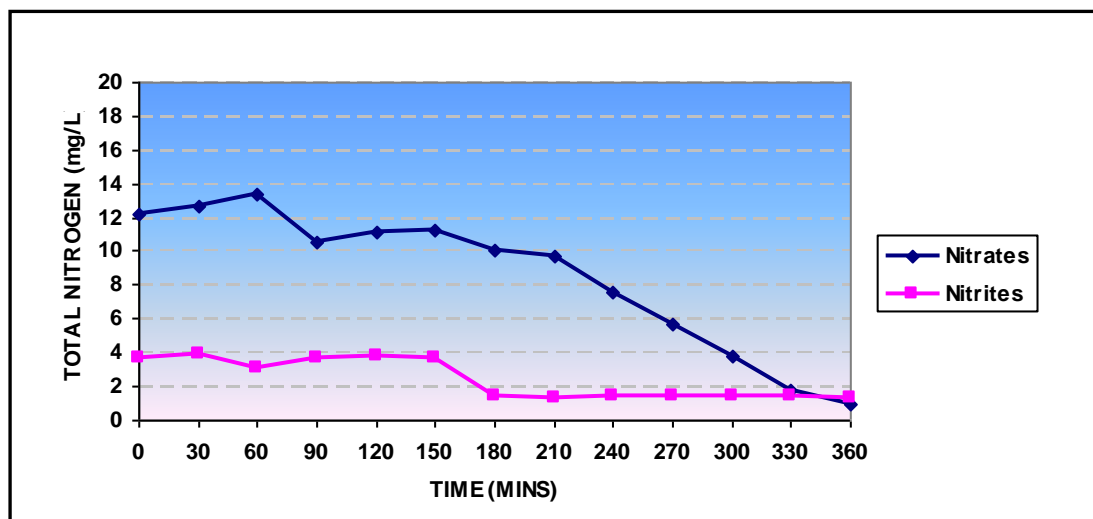


Figure 4.9 Graph showing the amount of nitrates and/or nitrites reduced by Organism 88.

Figures 4.8 and 4.9 depict the capacities of organisms 87 and 88 respectively, to reduce nitrates and /or nitrites. True denitrifiers are capable of reducing both nitrates and nitrites simultaneously. Thus, for these organisms, the amount of nitrates decreases proportionally as the amount of nitrite also decreases at any given time interval. However, it can be noted that for figure 4.8, the rate at which the nitrite concentration increases, is very gradual and cannot clearly be seen. Thus, initial nitrite concentrations for figures 4.8 and 4.9 are 0.3 and 3.615 mg/L respectively, whilst their final nitrite concentrations are 0.14 and 1.301 mg/L respectively.

These bacteria all contain nitrate and nitrite reductase enzymes enabling them to successfully reduce nitrates, via nitrites, to gaseous nitrogen (Rheinheimer, 1985; Ketchum, 1988; Cappuccino and Sherman, 1992; Robertson and Kuenen, 1992). In this study, two classes of true denitrifiers (Thomson, 2002) were analysed. The class II true denitrifiers displayed true denitrification in all

three test concentrations of KNO_2 and KNO_3 , however, in nitrite media, were either incapable of nitrite reduction or could only reduce nitrites at low KNO_2 concentrations.

This inability to reduce nitrites at high concentrations may be due to the toxicity of nitrites (Piñar *et al.*, 1997). Piñar *et al.*, (1997) also stated that nitrite is toxic to many organisms at high concentrations since it removes protein from cell membranes. This would explain why nitrite reduction occurs simultaneous to nitrate reduction so as to prevent the toxic build-up of nitrite.

Sequential Denitrifiers:

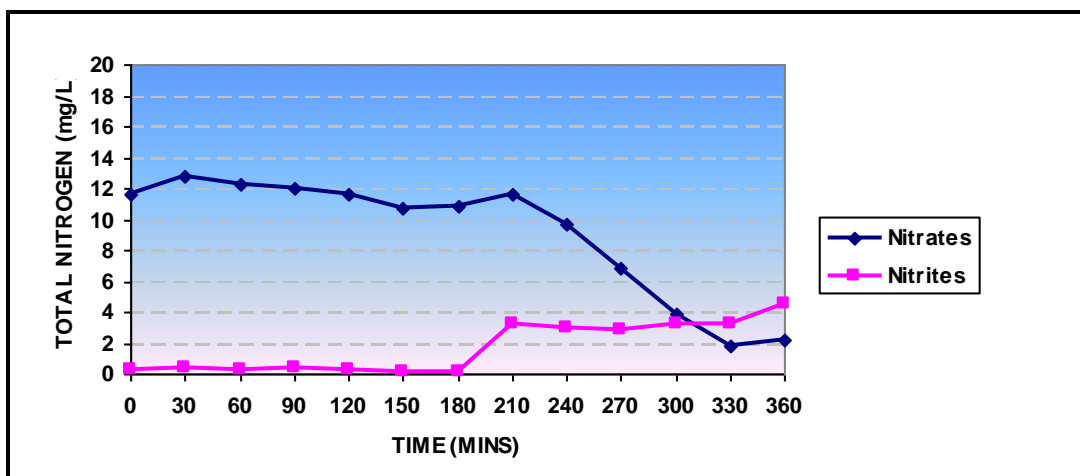


Figure 4.10 Graph showing the amount of nitrates and/or nitrites reduced by Organism 144.

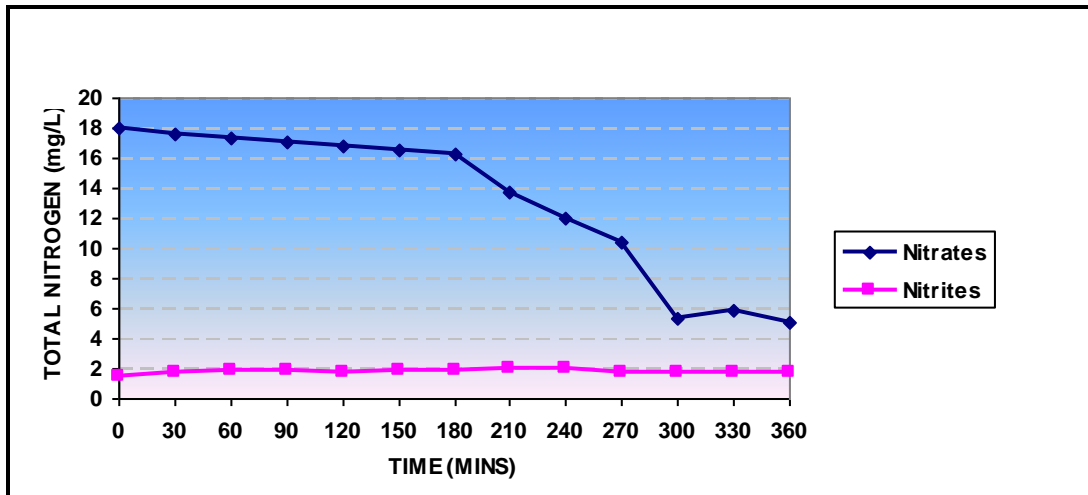


Figure 4.11 Graph showing the amount of nitrates and/or nitrites reduced by Organism 147.

Figures 4.10 and 4.11 depict the capacities of organisms 144 and 147 respectively, to reduce nitrates and /or nitrites. Sequential denitrifiers are capable of reducing both nitrates and nitrites simultaneously. However, nitrite reduction is inhibited in the presence of nitrate, which results in nitrite accumulation during nitrate reduction at any given time interval (Drysdale, 2001). The initial nitrite concentrations for figures 4.10 and 4.11 are 0.276 and 1.483 mg/L respectively, whilst their final nitrite concentrations are 4.491 and 1.777 mg/L respectively.

Ekama and Wentzel (1999) suggested that the possibility for this type of phenomenon is that nitrate is the preferred electron acceptor in cellular respiration since it is capable of accepting five electrons in comparison to nitrite which can only accept three. The reasons for nitrite accumulation were stated by Błaszczyk (1993):

- a) unbalanced reduction reactions for nitrate and nitrite, or
- b) delayed induction of nitrite reductase as compared to nitrate reductase.

Exclusive Nitrite Reducer:

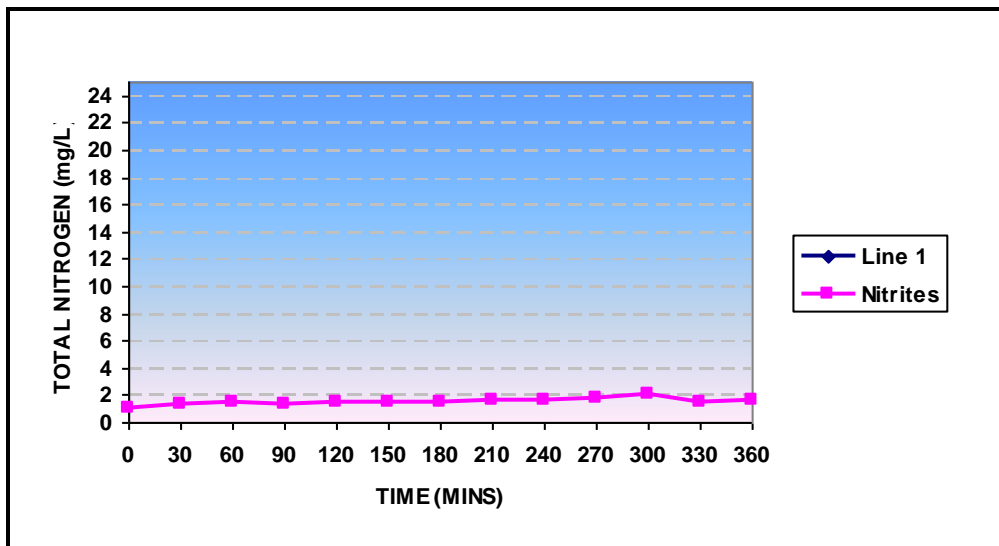


Figure 4.12 Graph showing the amount of nitrites reduced by Organism 176.

Figure 4.12 displays the capacity of organism 176, to reduce nitrites. Exclusive nitrite reducers exhibit non-denitrification characteristics in nitrate media but are capable of efficient nitrite reduction in nitrite media (Drysdale, 2001). The initial nitrite concentration for figure 4.12 is 1.05 mg/L and the final is 1.6 mg/L.

This group of organisms have been isolated and characterised from environmental samples (Roberson and Kuenen, 1992) but little is known with regard to their role in activated sludge treatment. These organisms lack nitrate reductase enzymes and are therefore incapable of contributing to nitrate reduction. Although these organisms have not been isolated in great amounts, their contribution to denitrification requires a much more accurate assessment, considering their efficiency at the reduction of high concentrations of nitrite as seen in this study.

CHAPTER FIVE

GENERAL CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

The findings of this study confirm the existence of a diverse community of heterotrophic bacteria involved in nitrogen removal during wastewater treatment of which incomplete denitrifying bacteria show significant involvement. The results showed that only five isolates from the culture collection differed with regard to their classification into four functional groups. This can be clearly seen by the results shown by Thomson (2002) as compared to the results shown by this study (Refer to table 4.2). For certain isolates, the results obtained by Drysdale (2001) were inconclusive or were not analysed, thus no comparisons could be made for these particular isolates.

The heterotrophic community within the Darvill Wastewater Works process comprises of four different functional groups. These were characterised as incomplete denitrifiers (bacteria capable of reducing nitrate to nitrite with no further reduction of the nitrite produced), true denitrifiers (bacteria capable of both nitrate and nitrite reduction) (Glass *et al.*, 1997), sequential denitrifiers (bacteria which are capable of both nitrate and nitrite reduction, however, exhibiting inhibition of nitrite reduction by nitrate) and exclusive nitrite reducers (bacteria capable of only reducing nitrite).

Based on molecular analysis of the heterotrophic community analysed, it was found that there were a large number of incomplete denitrifiers (73 isolates), 54 sequential denitrifiers, 47 true denitrifiers, and only 5 exclusive nitrite reducers, respectively.

However, DGGE principles revealed that the culture collection could be identified up until species level (Muyzer *et al.*, 1993). This was achieved by considering all DNA fragments having the same molecular weights as being the same species of organism. Thus DGGE profiling revealed 8 predominant isolates which consisted of 3 incomplete denitrifiers, 2 true denitrifiers, 2 sequential denitrifiers and 1 exclusive nitrite reducer. Although all the isolates were initially classified into their respective functional groups, this classification cannot be regarded as conclusive.

Further research will focus on the excision of prominent bands from the DGGE gels for nucleotide sequencing.

More research is still required of the different microorganisms involved in denitrification, since it is a complex, interactive microbial process that is still not fully understood.

5.2 RECOMMENDATIONS

The following areas are recommended for future research into denitrification:

- ❖ Nitrate and nitrite reduction kinetics need to be ascertained for each of the different functional groups of denitrifiers to accurately establish their contribution to nitrification and denitrification systems.
- ❖ Nitrate and nitrite reductase enzymes present in the denitrifying bacteria need to be assessed for a greater understanding of the biochemistry of denitrification.
- ❖ Important ordinary heterotrophic organisms need to be evaluated from each of the different functional groups, which can be used as model organisms from which nitrate and nitrite reduction kinetics can be based.

- ❖ Aerobic denitrification requires proper understanding in order to evaluate its possible impact on nitrate and nitrite reduction during wastewater treatment.
- ❖ Sequencing of the genes coding for nitrate and nitrite reductase enzymes is beneficial to the development of molecular probes for the identification of each of the four functional groups *in situ*, which will result in the indication of the predominant functional groups present in activated sludge.
- ❖ The possibility of metabolic plasmids containing genes coding for nitrite reductase enzymes requires investigation. If such plasmids do exist, the extent of nitrite reduction in the presence and absence of these plasmids needs to be determined.

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APPENDICES

APPENDIX 1

CASITONE GLYCEROL YEAST AUTOLYSATE AGAR (CGYA)

Preparation of CGYA is as follows (Bux *et al.*, 1994):

5g Bacto casitone or Tryptone L42

10mL Glycerol

1g Yeast autolysate

16g Agar

0.1g Potassium nitrate

0.05g Potassium nitrite

Weigh out all the ingredients and add all except the glycerol to 1 litre of distilled water and bring to the boil. When the ingredients are properly dissolved add the glycerol and heat for a further 2-3 minutes only. Thereafter, adjust pH to 7.2 and autoclave at 121°C for 15 minutes.

APPENDIX 2

GRAM STAIN

The procedure, as proposed by Cappuccino and Sherman (1992), is as follows:

- a) Smears of each isolate need to be prepared on a clean glass microscope slide.
- b) Smears need to air dry and then be heat fixed by rapidly passing the slide through a flame 2-3 times.
- c) Smears need to be flooded with crystal violet and left on for 1 minute.
- d) Slides need to be flooded with tap water to wash off the excess stain.
- e) Stains need to be flooded with Grams iodine and left on for 1 minute.
- f) Excess stain needs to be washed off with tap water.
- g) Smears should be decolourised by adding 95% ethyl alcohol, drop by drop.
- h) Smears need to be washed with tap water.
- i) Smears should be counter stained with Safranin and left on for 45 seconds.
- j) Excess stain should be washed off with tap water.
- k) Slides need to be blotted dry with paper towel and viewed with a light microscope under oil immersion.

APPENDIX 3

COLONIAL MORPHOLOGY ASSESSMENT

Assessment of the colonial morphology of isolate according to Cappuccino and Sherman (1992):

- a) **Size:** Pinpoint, small, moderate or large
- b) **Pigmentation:** Colour of colonies
- c) **Form:** Shape of colonies:
 - Circular: unbroken peripheral edge
 - Irregular: indented peripheral edge
 - Rhizoid: root-like spreading growth
- d) **Margin:** The appearance of the outer edge of the colony:
 - Entire: sharply defined, even
 - Lobate: marked indentations
 - Undulate: wavy indentations
 - Serrate: tooth-like appearance
 - Filamentous: thread-like spreading edge
- e) **Elevation:** The degree to which colony growth is raised on the agar surface:
 - Flat: elevation not discernable
 - Raised: slight elevation
 - Convex: dome-shaped elevation
 - Umbonate: raised, with elevated convex central region

APPENDIX 4

COLORIMETRIC BIOCHEMICAL NITRATE REDUCTION TEST

The nitrate reduction test as proposed by Cappuccino and Sherman (1992) is as follows:

1) Preparation of reagents

Solution A

8g Sulphanilic acid

1L Acetic acid, 5N (1 part glacial acetic acid : 2.5 parts distilled water)

Solution B

5g Alpha-naphthylamine

1L Acetic acid, 5N (1 part glacial acetic acid : 2.5 parts distilled water)

2) Procedure

The test organism needs to be inoculated into CGYA broth supplemented with 0.1% agar. This is incubated at room temperature for 5 days. After incubation, 5 drops of solution A followed by 5 drops of solution B needs to be added to the cultures. Development of a red colour indicates that the nitrate is only reduced to nitrite. If no red colour develops then a small quantity of zinc powder should be added. Development of a red colour after the addition of zinc indicates that no nitrate has been reduced at all. However, if there is no development of a red colour, then this indicates that nitrate is reduced to nitrite, which is in turn reduced to either ammonia, nitrogen gas or a less oxidised form of nitrogen.

APPENDIX 5

AGAROSE GEL ELECTROPHORESIS

50 mL TAE buffer

0.8g Agarose

1 μ l Ethidium bromide

Microwave the above ingredients, except the Ethidium bromide, together for 60 seconds. Cool the solution and add the Ethidium bromide. Pour this solution into a gel tray and allow to set for 15-20 minutes.

Loading of samples

2 μ l of Loading buffer + 2 μ l of sample

Running buffer

25 mL of gel buffer

250 mL Distilled water

Electrophoresis settings

Voltage ~ 80 V

Time ~ 60 minutes

APPENDIX 6

DENATURING GRADIENT GEL ELECTROPHORESIS

Solutions and Reagents

40% Bis-Acrylamide

38 g Acrylamide

2 g Bis-Acrylamide

Mix the two ingredients together with 100 mL Distilled water. Filter the solution through Whatman Filter paper No.1. Store the solution at 4°C.

Denaturing Solution (DNS)

18.8 mL 40% Bis-Acrylamide

2.0 mL 50 X TAE Buffer

40.0 mL Formamide*

42.0 g Urea*

Mix all the ingredients together to 100 mL Distilled water.

* The concentrations of Formamide and Urea are varied according to the required concentration of the denaturing solutions.

10% Ammonium Persulphate

0.1g Ammonium Persulphate

1.0 mL Distilled water

Mix the ingredients together. This solution should be freshly made up for every use.

D-Gene Dye Solution

0.05 g Bromophenol Blue

0.05 g Xylene Cyanol

10.0 mL 1 X TAE Buffer

Mix all the ingredients together and store at room temperature.

Denaturing Gel: High Density DNS (50%) + Low Density DNS (30%)

15 mL DNS

150 µl Ammonium Persulphate

15 µl Temed

300 µl Dye

Running Buffer

140 mL 50 X TAE Buffer

6860 mL Distilled water

Fill upper buffer chamber with 350 mL 1 X TAE Buffer.

APPENDIX 7

STANDARDIZATION OF AUTOANALYZER II

REAGENTS

Colour Reagent

Add 50 mL concentrated phosphoric acid to 400 mL deionized water. Dissolve 20 g sulphanilimide and 1 g N - (1-naphthyl) ethylenediamine dihydrochloride in this solution and dilute to 500 mL with deionized water. Store in a dark glass bottle. This solution is stable for one week.

Ammonium Chloride Buffer Solution

Dissolve 30 g ammonium chloride and 0.2 g disodium EDTA in 800 mL deionized water. Add 0.5 mL Brij-35 wetting agent and dilute to one litre. Adjust the pH to 6.6 with diluted NH_3 solution (10 mL concentrated NH_3 solution diluted to 100 mL with deionized water).

Nitrate Stock Solution

Dissolve 0.7218 g potassium nitrate (dried for 16 h at 60°C) and dilute to one litre with deionized water.

Nitrate Standard Solutions

Standard solutions must be prepared at 0.4, 1.0 and 1.4 mg/L respectively, in one-litre volumetric flasks by appropriate quantitative dilution of the nitrate stock solution.

Nitrite Stock Solution

Dissolve 0.6076 g potassium nitrite (dried for 16 h at 60°C) and dilute to one litre with deionized water.

Nitrite Standard Solutions

Standard solutions must be prepared at 0.4, 0.6 and 0.8 mg/L respectively, in one-litre volumetric flasks by appropriate quantitative dilution of the nitrite stock solution.

It must be noted that the standard solutions for both nitrates and nitrites need to be preserved by adding HgCl_2 solution to give a final concentration of 10 mg/L Hg (II) and thereafter be stored in polyethylene containers at 4°C.

APPENDIX 8

GRAM REACTION & CELLULAR MORPHOLOGY OF DENITRIFIERS

CULURE NUMBER	GRAM REACTION	CELLULAR MORPHOLOGY
1	Negative	Long rod
2	Negative	Short rod
3	Negative	Long rod
4	Negative	Short rod
5	Negative	Long rod
6	Negative	Short rod
7	Negative	Long rod
8	Negative	Short rod
11	Negative	Short rod
12	Negative	Long rod
13	Negative	Long rod
15	Negative	Long rod
18	Negative	Short rod
19	Negative	Short rod
22	Negative	Short rod
24	Negative	Short rod
25	Negative	Short rod
26	Negative	Short rod
27	Negative	Short rod
28	Negative	Short rod
29	Negative	Long rod
30	Negative	Long rod
32	Negative	Long rod
33	Negative	Short rod
34	Negative	Short rod
35	Negative	Short rod
37	Negative	Short rod
38	Negative	Short rod
39	Negative	Short rod
40	Negative	Long rod
41	Negative	Long rod
43	Negative	Short rod

CULURE NUMBER	GRAM REACTION	CELLULAR MORPHOLOGY
44	Negative	Long rod
45	Negative	Short rod
46	Negative	Long rod
47	Negative	Short rod
48	Negative	Cocci
49	Negative	Cocci
50	Negative	Cocci
51	Negative	Cocci
52	Positive	Long rod
53	Positive	Long rod
56	Positive	Long rod
57	Negative	Long rod
58	Negative	Short rod
60	Negative	Long rod
61	Negative	Short rod
62	Negative	Short rod
63	Negative	Short rod
64	Negative	Long rod
66	Negative	Short rod
67	Negative	Short rod
68	Negative	Long rod
70	Negative	Short rod
71	Negative	Short rod
72	Negative	Long rod
73	Negative	Short rod
74	Negative	Short rod
76	Negative	Short rod
77	Negative	Long rod
78	Negative	Short rod
79	Negative	Long rod
80	Negative	Short rod
82	Negative	Long rod
84	Negative	Short rod
94	Negative	Long rod
97	Negative	Short rod

CULURE NUMBER	GRAM REACTION	CELLULAR MORPHOLOGY
98	Negative	Long rod
99	Negative	Short rod
100	Negative	Long rod
101	Negative	Long rod
102	Negative	Long rod
103	Negative	Short rod
105	Negative	Short rod
106	Negative	Short rod
109	Negative	Long rod
110	Negative	Short rod
111	Negative	Short rod
113	Positive	Short rod
116	Positive	Short rod
117	Positive	Long rod
119	Positive	Short rod
120	Positive	Short rod
123	Positive	Short rod
124	Positive	Long rod
125	Positive	Long rod
126	Positive	Short rod
127	Positive	Short rod
128	Positive	Long rod
130	Positive	Short rod
131	Positive	Long rod
132	Positive	Short rod
133	Positive	Short rod
134	Positive	Long rod
135	Positive	Short rod
136	Negative	Short rod
137	Negative	Cocci
138	Negative	Cocci
139	Negative	Cocci
140	Negative	Cocci
141	Negative	Cocci
142	Negative	Short rod

CULURE NUMBER	GRAM REACTION	CELLULAR MORPHOLOGY
145	Negative	Short rod
146	Negative	Short rod
147	Negative	Short rod
149	Negative	Short rod
150	Negative	Short rod
151	Negative	Short rod
152	Negative	Long rod
153	Negative	Long rod
154	Negative	Long rod
155	Negative	Short rod
156	Negative	Long rod
157	Negative	Short rod
158	Negative	Short rod
159	Negative	Short rod
160	Negative	Cocci
162	Negative	Cocci
163	Negative	Cocci
164	Positive	Short rod
168	Negative	Long rod
169	Negative	Short rod
170	Negative	Long rod
171	Negative	Short rod
172	Negative	Short rod
173	Negative	Short rod
174	Negative	Long rod
175	Negative	Long rod
176	Negative	Short rod
177	Negative	Long rod
178	Negative	Short rod
179	Negative	Short rod