ELUCIDATION OF MICROBIOLOGICAL-BIOCHEMICAL RELATIONSHIPS IN DENITRIFICATION OCCURRING DURING ACTIVATED SLUDGE TREATMENT

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Dissertation submitted in compliance with the requirements for the Master's Degree in Technology with the Department of Biotechnology, Technikon Natal, Durban.

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GAVIN DAVID DRYSDALE

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.

GAVIN DAVID DRYSDALE

I hereby approve the final submission of the following dissertation.

MR FAIZAL BUX
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this 9 day of MAY, 2001, at Technikon Natal, Durban.

DEDICATION

I would like to dedicate this work to all the people of the world for clean water and life...

ACKNOWLEDGEMENTS

I firstly would like to give special thanks and acknowledgement to Jesus Christ, my Lord and saviour, in whom all of this was made possible for me.

I would also like to sincerely thank the following people:

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- My wife, Jacqueline, for her overwhelming love and support which without I would not be able to succeed in life, let alone this research project.
- My son, Jonathan, who has changed my life irrevocably since his arrival on 1 April 1999.

PREFACE

Of the work presented in this dissertation various aspects have and will be published and presented elsewhere:

Publications

Drysdale, G.D., Mudaly, D., Atkinson, B.W., Kasan, H.C. and Bux, F. (2000). *Investigation of the microbial contribution to nutrient removal in an activated sludge wastewater treatment process*. Report to the Water Research Commission, Project No. K5/822.

Drysdale, G.D., Kasan, H.C. and Bux, F. (2001). Assessment of denitrification by the ordinary heterotrophic organisms in an NDBEPR activated sludge system. *Wat. Sci. Tech.* **43**(1), 147-154.

Conferences

Drysdale, G.D., Bux, F. and Kasan, H.C. (1999). Interactive microbial denitrification in a biological nutrient removal process. African International Environmental Protection Symposium, Anaerobic Digestion Symposium, Imperial Hotel, Pietermaritzburg, 4-8 July.

Drysdale, G.D., Bux, F. and Kasan, H.C. (2000). Assessment of nitrite denitrification behaviour by denitrifying heterotrophic organisms in a NDBEPR activated sludge system. The Water Institute of Southern Africa Biennial Conference and Exhibition, Sun City, 28 May - 1 June.

Drysdale, G.D., Bux, F. and Kasan, H.C. (2000). Assessment of denitrification by the ordinary heterotrophic organisms in a NDBEPR activated sludge system. 1st World Congress of the International Water Association (IWA), Paris, France, 3-7 July.

EXECUTIVE SUMMARY

Up until now extensive work has been done to develop kinetic models and related software that can be used successfully to simulate and design nitrification denitrification (ND) and nitrification denitrification biological excess phosphorus removal (NDBEPR) systems for efficient nitrogen removal. The denitrification kinetics of these systems have primarily been determined and attributed to the ordinary heterotrophic bacteria, now also known as the OHO fraction, otherwise not involved in biological excess phosphorus removal. However, denitrification kinetics determined for ND systems have been found to vary considerably at times when applied to NDBEPR systems because of varying OHO active fraction estimates and the unexplained occurrence of anoxic phosphorus removal and any success achieved to date has been some what fortuitous. Ultimately variations in process performance and kinetics are attributable to inadequate control and lack of understanding of the ecological, physiological and biochemical activities of constituent microorganisms. There is growing concern and movement towards a better understanding of the microbial community within activated sludge in order to gain optimal control of the process.

The aim of this study was, therefore, to isolate and characterise OHOs present in a NDBEPR system in order to gain a better understanding of the organisms involved in denitrification as well as a more holistic and accurate evaluation of the OHO fraction attributable to denitrification in such a system.

Heterotrophic bacteria (OHOs) were isolated, using plating techniques, from the pre- and secondary

anoxic zones of the Darvill NDBEPR process situated on the outskirts of Pietermaritzburg, KwaZulu Natal. Isolates were characterised according to their ability to reduce different concentrations of nitrate and/or nitrite under anoxic conditions and identified to at least generic level.

Results showed that the OHO fraction is more complex than currently accepted and, with respect to denitrification, can be more accurately subdivided into five functional groups, four of which interactively contribute to denitrification occurring in the system and one group that are non-denitrifying. These groups were defined as true denitrifiers (bacteria capable of both nitrate and nitrite reduction), incomplete denitrifiers (bacteria that reduced nitrate to nitrite with no further reduction of the nitrite produced), incomplete-nitrite reducers (bacteria capable of both nitrate and nitrite reduction, however, exhibiting severe inhibition of nitrite reduction by nitrate),exclusive nitrite reducers (bacteria only capable of reducing nitrite) and non-denitrifiers (bacteria not capable of nitrate or nitrite reduction). The non-denitrifiers comprised the largest OHO group isolated (100 isolates) followed by the incomplete denitrifiers (85 isolates), true denitrifiers (56 isolates), incomplete-nitrite reducers (24 isolates) and exclusive nitrite reducers (7 isolates), respectively. The large number of non-denitrifying organisms comprising the OHOs is cause for concern on the accuracy of attributing denitrification behaviour and kinetics to the entire OHO fraction. Of the denitrifying OHOs, most were found capable of nitrate reduction while only approximately half were capable of nitrite reduction. Many of the nitrite reducing OHOs were seen to possibly be regulated by a feedback inhibition mechanism restricting nitrite reduction in the presence of nitrate, therefore, explaining nitrite accumulation observed during denitrification in NDBEPR systems, biofilms and ground water. Identification of denitrifying isolates revealed significant quantities of *Pseudomonas*

spp isolated while *Flavobacterium* spp showed possible predominance amongst exclusive nitrite reducers. *Comamonas testosteroni*, *Escherichia coli 1*, *Pseudomonas aerogenes* and *Seratia marcescens*, which are known incomplete denitrifiers, were found to in fact be true denitrifiers capable of nitrite reduction at lower concentrations of KNO_3 to that currently specified for denitrification screening media. *Aeromonas salmonicida 2*, *Agrobacterium radiobacter*, *Comamonas acidovorans*, *Klebsiella pneumonia* ssp *pneumoniae*, *Pseudomonas cepacia*, *Pseudomonas maltophilia*, *Seratia liquificaens* and *Sphingobacterium multivorum*, which are also known incomplete denitrifiers, were also found to actually be true denitrifiers when grown in more nutrient rich media to that currently being employed for denitrification screening media. Some incomplete denitrifiers were also found to be capable of both nitrate and nitrite reduction by using separate nitrate and nitrite media for reduction screening. These organisms included *Acinetobacter calcoaceticus var lwoffi*, *A. radiobacter*, *Citrobacter freundii*, *E. coli 1*, *Kluyvera* spp, *P. cepacia*, *P. maltophilia* and *Vibrio fluvialis*,which were hence characterised as incomplete-nitrite reducers.

Denitrification is therefore a complex, interactive microbial process which is still not fully understood. More understanding is still required of the different microorganisms involved in denitrification as well as the biochemistry and enzymatic regulation of nitrate and nitrite reduction under the various physiological conditions imposed during activated sludge treatment in ND and NDBEPR systems.

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LIST OF ABBREVIATIONS

1°	primary
2°	secondary
API	analytical profile index
a-recycle	anoxic/aerobic recycle
BNR	biological nutrient removal
BOD	biological oxygen demand
CGY	casitone glycerol yeast autolysate
COD	chemical oxygen demand
d	days
DO	dissolved oxygen
E	enteric
g	grams
h	hour
K_1	primary anoxic denitrification rate for RBCOD (ND systems)
K_2	primary anoxic denitrification rate for SBCOD (ND systems)
K_3	secondary anoxic denitrification rate (ND systems)
K'_2	primary anoxic denitrification rate (M/UCT NDBEPR system)
K'_3	secondary anoxic denitrification rate (M/UCT NDBEPR system)
kL	kiloliters

KNO_2	potasium nitrite
KNO_3	potasium nitrate
L	liters
mL	mililiters
MLE	modified Ludzack Ettinger
min.	minutes
M/UCT	modified University of Cape Town
N	nitrogen
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
ND	nitrification denitrification
NDBEPR	nitrification denitrification biological excess phosphorus removal
NE	non-enteric
No.	number
OHO	ordinary heterotrophic organisms
ortho-P	orthophosphorus
PAO	phosphorus accumulating organism
PCR	polymerase chain reaction
PHA	polyhydroxyalkanoates
RBCOD	readily biodegradable carbon substrate
Ref.	reference
sec.	seconds
SBCOD	slowly biodegradable carbon substrate

spp	species
s-recycle	sludge recycle
ssp	sub-species
VFA	volatile fatty acid

CHAPTER 1

INTRODUCTION

1.1 NITROGEN CONTAMINATION OF WATER

South Africa is a country faced with increasing domestic and industrial activity versus a low rainfall and limited water resources. It is for this reason that a high standard of wastewater treatment is necessary to ensure protection and maximum reuse of available water resources. Long storage times of water in dams is also necessary owing to the limited water resources in the country. In the past, South African dams experienced unexplained eutrophication problems and it was established that this was due to the lack of standards governing the levels of nitrogen and phosphorus in secondary treated water entering these impoundments (Lötter, 1989). Legislation has since prompted further investigation and improvement into the removal of nitrogen and phosphorus in wastewater treatment.

The occurrence of nitrogen in water is primarily due to human and animal excrement (mostly as urea), fertilisers, run off from fertilised lands, decaying vegetative and animal matter, food processing waste, uranium oxide fuel production, wastewater treatment and fixation of atmospheric nitrogen (Bode *et al.*, 1987; Ketchum, 1988; Terblanche, 1991; Otlanabo, 1993). Nitrogen can be present in water sources in various forms all of which can be detrimental to human health and/or the environment. Nitrogen in the form of free ammonia is toxic to fish and other aquatic organisms

(Sedlak, 1991) while also posing serious problems for potable water treatment in that it has a high affinity for chlorine thus reducing the effectiveness of chlorine disinfection of drinking water (American Water Works Association, 1995). Nitrogen in the form of nitrate and nitrite is eutrophic and, when consumed in excess of 6 mgN/L, is responsible for a blood disorder in infants known as methemoglobinemia or blue baby syndrome (Terblanche, 1991; Kempster *et al*, 1997). Nitrate and nitrite have also been implicated in impaired growth and malformation of foetuses, slowing of conditioned motor reflexes of the central nervous system of humans, cardiovascular disorders and hypertension (Terblanche, 1991) and are known to inhibit the removal of phosphorus during waste water treatment (Gruenebaum and Dorgeloh, 1992; Kuba *et al.*, 1996). Nitrite, as with ammonia, also has a high affinity for chlorine and thus also has an adverse effect on potable water treatment (American Water Works Association, 1995). Furthermore, nitrosamines, which are derived from nitrate, are known carcinogens and play a role in the induction of certain gastrointestinal cancers (Terblanche, 1991; Otlanabo, 1993). The removal of nitrogen from waste water streams is therefore of utmost importance. The need therefore exists for further improvement in wastewater treatment to ensure effective safeguarding of remaining available water resources and the environment.

1.2 THE ACTIVATED SLUDGE PROCESS AND NITROGEN REMOVAL

The activated sludge process was first discovered and developed at the turn of the century, whereafter in 1913, Ardern and Lockett took the process to full scale for the first time at the Davyhulme treatment works in Manchester (Gray, 1990). Originally, the process was designed for

the removal of organic matter, especially carbonaceous BOD and COD, from wastewater (Lu and Leslie Grady Jr, 1988 ; Gray, 1990). However, in the early 1970s, significant development and changes occurred further enhancing the biological nutrient removal (BNR) capabilities of the process to progressively include nitrification, denitrification and phosphorus removal (Wentzel *et al.*, 1992). These improvements were induced by changing the process from a single aerobic reactor to multi-reactor processes incorporating anaerobic, anoxic and aerobic reactors/zones with inter-reactor recycles (Wentzel *et al.*, 1992). This was extremely advantageous in that much of the chemical means of wastewater treatment could now be avoided.

Nitrification was the first method of biological nitrogen removal achieved via activated sludge treatment. Under aerobic conditions nitrifying microorganisms convert ammonia, via nitrite, to nitrate in two different oxidative reactions known as nitritation and nitratation (Mauret *et al.*, 1996). The disadvantages, however, of nitrification are that during the oxidation of ammonia, hydrogen ions are released which cause a decrease in the pH and alkalinity of the wastewater (Lilley *et al.*, 1997; Muyima *et al.*, 1997). Low pH in turn results in decreased efficiency in nitrification, poor sludge settleability and corrosive effluent (Muyima *et al.*, 1997). Furthermore, nitrification in activated sludge systems requires long sludge ages and lengthy retention times to achieve complete ammonia and nitrite oxidation.

With the resultant release of nitrate into receiving water bodies via nitrified wastewater, it became necessary to include denitrification into activated sludge systems. Denitrification is the reduction of nitrate, via nitrite, to nitrous oxides and gaseous nitrogen under anoxic conditions (Wu and

Knowles, 1995). The inclusion of denitrification into activated sludge systems is achieved via the addition of unaerated or anoxic zones[1] in the process (Dold and Marais, 1987). Under anoxic conditions ordinary heterotrophic organisms (OHOs) are stimulated into utilising nitrate and nitrite as final electron acceptors for cellular respiration in place of oxygen (Ketchum, 1988; Cappuccino and Sherman, 1992). This results in the production of gaseous nitrogen, which escapes to the atmosphere, accompanied by simultaneous COD removal (Wanner and Grau, 1988). The inclusion of denitrification to nitrifying activated sludge systems has resulted in reduced daily oxygen requirements, reduced effluent nitrate loads, elimination of pH control and alleviation of the stringency of dissolved oxygen (DO) control during wastewater treatment (Dold and Marais, 1987).

Up until now extensive work has been done to develop kinetic models and related software that can be used successfully to simulate and design nitrification denitrification (ND) and nitrification denitrification biological excess phosphorus removal (NDBEPR) systems for efficient nitrogen removal (Dold *et al.*, 1991; Wentzel *et al.*, 1991; Ekama *et al.*, 1992; Gujer and Kappler, 1992; Ekama and Wentzel, 1999). However, denitrification kinetics determined for ND systems have been found to vary considerably at times when applied to NDBEPR systems because of varying OHO active fraction estimates and the unexplained occurrence of anoxic phosphorus removal. Any success achieved to date has therefore been some what fortuitous (Dold *et al.*, 1991; Ekama and Wentzel, 1999). Ultimately, variations in process performance and kinetics are attributable to inadequate control and lack of understanding of the ecological, physiological and biochemical

[1]Anoxic refers to a state of absence of aeration where only nitrate and nitrite are available as electron acceptors for cellular respiration.

activities of the microorganisms within the process (Bux *et al.*, 1994; Hippen *et al.*,1997). Henze (1992) and Kristensen *et al.* (1992) echo concern in that activated sludge models fall short in not taking into consideration the community structure of the biomass present in the process. There is growing concern and movement towards a better understanding of the microbial community within activated sludge in order to gain optimal control of the process (Lu and Leslie Grady Jr, 1988; Davelaar, 1989; Wagner *et al.*, 1993; Bux *et al.*, 1994; Jansen *et al.*, 1994; Hu *et al.*, 1996; Satoh *et al.*, 1996; Ubisi *et al.*, 1997).

1.3 AIMS AND OBJECTIVES OF THE STUDY

The objective of this study was to create a better understanding of the microbial ecology of denitrification occurring during wastewater treatment in a NDBEPR activated sludge system. The project aimed at isolating, identifying and characterising OHOs responsible for, or capable of, nitrate and/or nitrite reduction occurring during denitrification in an existing NDBEPR system in order to establish the interactive nature of microbial denitrification. Improved understanding of the microbiology and biochemistry of nitrate and nitrite reduction will result in a better understanding and characterisation of the OHO fraction, attributable for denitrification, which could lead to improved modelling of denitrification behaviour in BNR activated sludge processes.

CHAPTER 2

REVIEW OF RELATED LITERATURE

2.1 THE NITROGEN CYCLE

Nitrogen constitutes approximately 79% of the earths atmosphere. Although chemically inert, nitrogen is one of the most important elements to living organisms as it forms part of amino acids, purines, pyrimidines and some co-enzymes (Ketchum, 1988; Muyima *et al.*, 1997). Various mechanisms are employed in the assimilation of nitrogen for cellular growth by different organisms and hence the flux of nitrogen in the environment is dependant on numerous factors. Ammonia is released into the environment through excreta, decaying plant and animal matter and nitrogen fixation. Ammonia is assimilated into organic nitrogen by nitrogen fixing bacteria or is oxidised by chemoautotrophic bacteria in the process of nitrification (Ketchum, 1988; Muyima *et al.*, 1997). Nitrate and nitrite can in turn be utilised by various facultative microorganisms for cellular respiration under oxygen limited conditions (Wu and Knowles, 1995). Under such conditions nitrate and nitrite are reduced to nitrous oxides and dinitrogen gas which escape to the atmosphere. Dinitrogen gas can then be converted to ammonia by nitrogen fixing bacteria (Ketchum, 1988; Muyima *et al.*, 1997) or to nitrate by lightning (Otlanabo, 1993). Nitrogen is therefore available in different forms for assimilation by different organisms while concurrently maintaining the natural nitrogen balance in the environment (Ketchum, 1988).

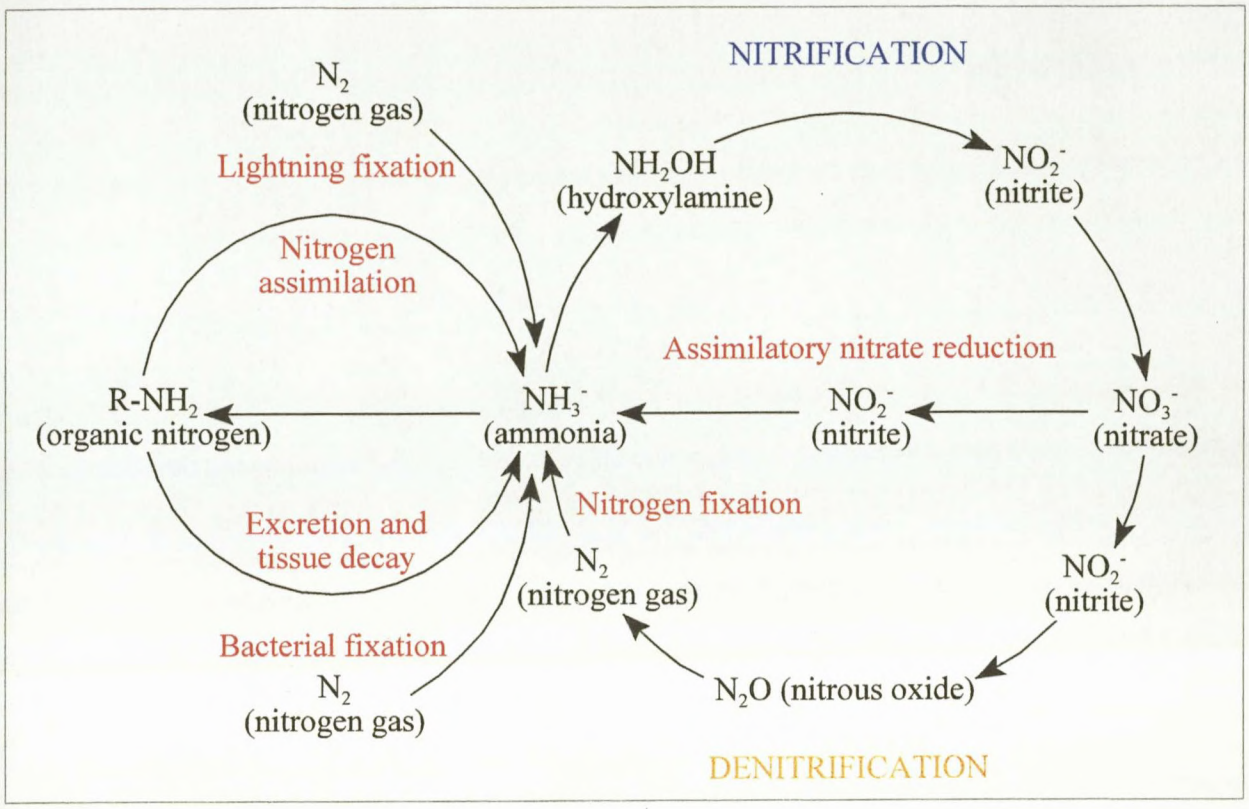

FIGURE 2.1 The nitrogen cycle (Ketchum, 1988; Muyima *et al.*, 1997).

2.2 NITROGEN REMOVAL DURING WASTEWATER TREATMENT

2.2.1 Nitrification

Nitrification was the first method of biological nitrogen removal achieved via activated sludge treatment of wastewaters. In addition to COD removal, single aerobic reactors were found to be capable of ammonia oxidation at sludge ages in excess of 3 days (FIG. 2.2). Under aerobic conditions nitrifying organisms convert ammonia, via nitrite, to nitrate in two different oxidative

reactions known as nitritation and nitratation. In nitritation, ammonia is oxidised to nitrite whereafter nitratation commences with further oxidation of the nitrite to nitrate (Mauret *et al.*, 1996).

Nitritation: $2NH_4^+ + 3O_2 \rightarrow 4H^+ + 2H_2O + 2NO_2^-$

Nitratation: $2NO_2^- + O_2 \rightarrow 2NO_3^-$

However, nitrification does not totally eliminate nitrogen from wastewater but merely converts it into other forms i.e., nitrite and nitrate. In addition, during oxidation of ammonia and nitrite, hydrogen ions are released which cause a decrease in pH and alkalinity of wastewater (Lilley *et al.*, 1997; Muyima *et al.*, 1997). Low pH in turn results in decreased efficiency in nitrification, poor sludge settleability and corrosive effluent (Lilley *et al.*, 1997). Long sludge ages are also required to achieve complete ammonia and nitrite oxidation during nitrification in activated sludge systems.

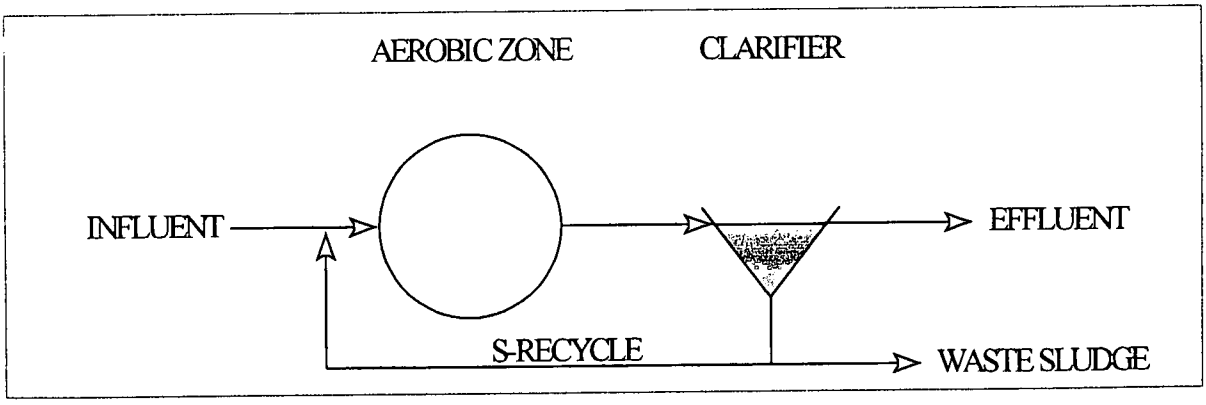

FIGURE 2.2 Single aerobic activated sludge system for COD removal and nitrification (Lilley *et al.*, 1997).

2.2.2 Denitrification

The inclusion of denitrification to wastewater treatment systems has been instrumental in overcoming some of the problems associated with nitrification. Denitrification in activated sludge systems is achieved via the addition of unaerated or anoxic zones in the process (Dold and Marais, 1987). Denitrification is the microbial reduction of nitrate, via nitrite, to nitrous oxides and gaseous nitrogen under anoxic conditions (Wu and Knowles, 1995).

Denitrification: $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$

Under anoxic conditions, denitrifying heterotrophic organisms (OHOs) are stimulated into utilising nitrate and nitrite as final electron acceptors for cellular respiration in place of oxygen with resultant production of gaseous nitrogen accompanied by simultaneous COD removal (Ketchum, 1988; Wanner and Grau, 1988; Cappuccino and Sherman, 1992). The inclusion of denitrification to nitrifying activated sludge systems has resulted in the effective removal of nitrogen from wastewaters, reduced daily oxygen requirements, reduced effluent nitrate loads and alleviation of the stringency of dissolved oxygen (DO) control during wastewater treatment (Dold and Marais, 1987). Denitrification also reduces alkalinity which has resulted in elimination of the need for pH control (Dold and Marais, 1987).

Nitrogen removal processes incorporating denitrification include the Wuhrmann process (FIG. 2.3), modified Ludzack-Ettinger (MLE) process (FIG. 2.4) and Bardenpho process (FIG. 2.5). The

Wuhrmann process was one of the first nitrogen removal processes designed. It consists of an aerobic zone into which the influent is directly discharged for nitrification to occur. Nitrified wastewater is then discharged into an anoxic zone where nitrite and nitrate are reduced into gaseous nitrogen. The underflow from the clarifier (s-recycle) is then returned to the head of the aerobic zone. However, the shortfall of this system is that substrate required for denitrification is obtained only from the death of organisms which results in a slow denitrification rate and subsequent need for a very large anoxic zone for complete denitrification (Lilley *et al.*, 1997). As a large anoxic zone can be inhibitory to nitrification the MLE process incorporates the anoxic zone upstream of the aerobic zone with a recycle (a-recycle) of nitrified wastewater from the aerobic zone to the anoxic zone. This system has proven to facilitate a high rate of denitrification but cannot achieve complete denitrification as part of the nitrified wastewater from the aerobic zone is discharged with the effluent (Lilley *et al.*, 1997). The Bardenpho process was designed with the objective to achieve complete denitrification. The Bardenpho system, as with the MLE process, also consists of an anoxic zone followed by an aerobic zone and an a-recycle, but it also incorporates a secondary anoxic zone and reaeration cycle respectively. The secondary anoxic zone allows for denitrification of nitrite and nitrate which has not been recycled to the primary anoxic zone. This is followed by flash aeration which strips nitrogen bubbles from the biomass and nitrifies any trace quantities of ammonia remaining in the wastewater. While not as efficient as the MLE process the Bardenpho system can obtain lower effluent nitrate loads of below 5 to 7 mg N/L (Lilley *et al.*, 1997).

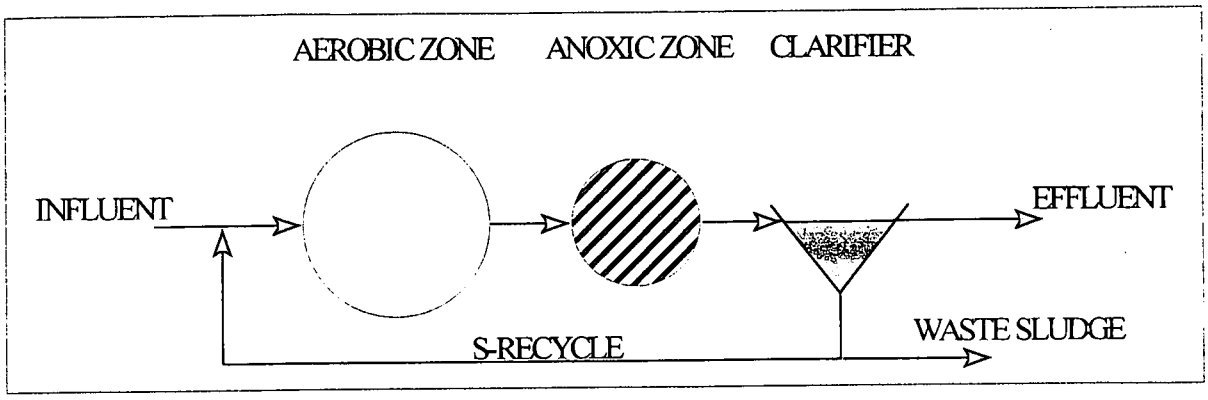

FIGURE 2.3 The Wuhrmann process for nitrogen removal (Lilley *et al.*, 1997).

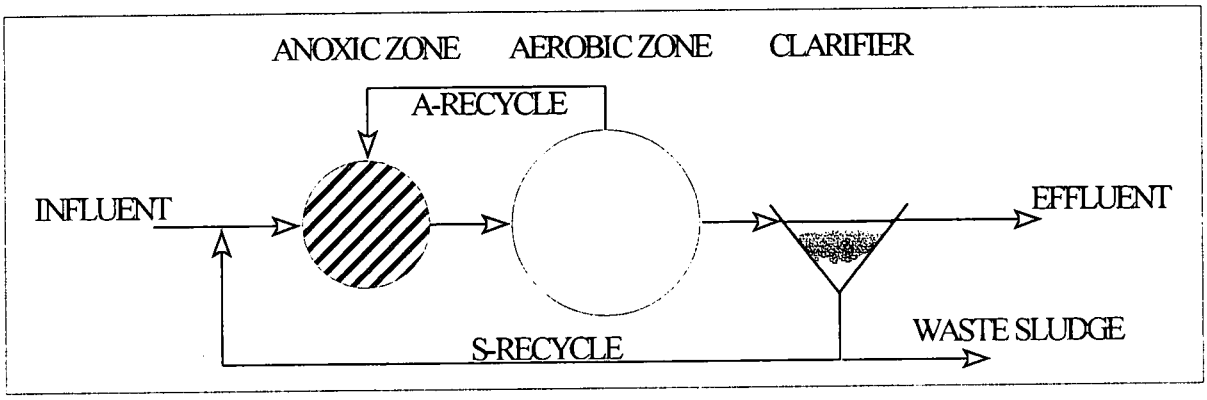

FIGURE 2.4 The modified Ludzack-Ettinger process for nitrogen removal (Lilley *et al.*, 1997).

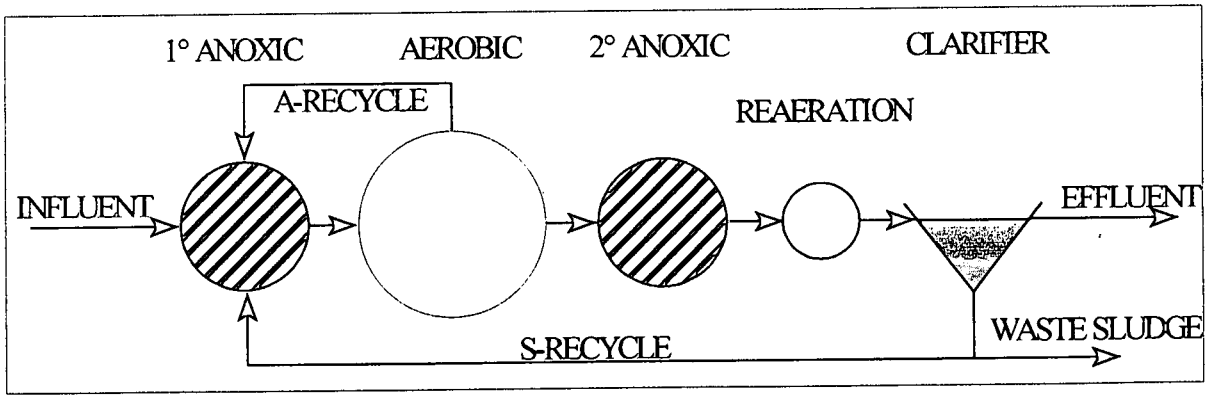

FIGURE 2.5 The Bardenpho process for nitrogen removal (Lilley *et al.*, 1997).

2.3 MICROBIOLOGY OF NITROGEN REMOVAL

2.3.1 Nitrification

2.3.1.1 Autotrophic nitrification

The process of nitrification is generally ascribed to chemoautotrophic bacteria belonging to the family *Nitrobacteraceae* which are Gram negative obligate aerobes (*Bergeys*, 1989; Brock *et al.*, 1991). Although contributing only a small percentage of the total microbial community in wastewater treatment systems, autotrophic nitrifiers are believed to be responsible for the majority of nitrification (Burrell *et al.*, 1998). Nitrifying bacteria have traditionally been classified into two main groups; that of ammonia-oxidisers, which carry out the rate-limiting step of ammonia oxidation to nitrite, and nitrite-oxidisers that oxidise nitrite through to nitrate. Among all the biological reactions responsible for nitrogen removal, ammonia oxidation is generally the rate limiting step because of slow growth and poor yield of ammonia oxidising bacteria (Suwa and Noto, 1998). *Nitrosomanas* spp are the autotrophs most commonly implicated in ammonia oxidation while *Nitrobacter* spp are believed to be the predominant organisms responsible for nitrite oxidation (Van Loosdrecht and Jetten, 1998). The optimal temperature for growth of these organisms is between 25-30°C and a pH of between 7.0-8.4 (Gray, 1990; Underhill, 1990; Bitton, 1994; American Water Works Association, 1995). Any slight variation to one or more of these factors can inhibit growth of these fastidious organisms. The autotrophs also have very slow growth rates with generation times of between 8-24 hours. Thus, the time required for successful isolation and cultivation of these

organisms can be several months (Watson *et al.*, 1981). Secondary autotrophic nitrifiers have also been found to exist in nitrifying ecosystems. These organisms have, in the past, generally been found in lower numbers than *Nitrosomonas* spp and *Nitrobacter* spp which is believed to be due to their narrower range of temperature and pH for growth (Focht and Verstraete, 1977). However, recent studies have revealed that bacteria from the *Nitrospira* phylum are present as the main nitrite-oxidising bacteria in wastewater treatment systems and that *Nitrobacter* spp are often undetectable (Hovanec and DeLong, 1996; Wagner *et al.*, 1996; Burrell *et al.*, 1998). Using polymerase chain reaction (PCR) with *Nitrospira*-specific primers, excellent nitrification activity in full-scale activated sludge plants has been positively correlated with the presence of *Nitrospira* spp whereas their absence is noted in systems experiencing poor nitrification. It has also been demonstrated that *Nitrosomonas* spp are not always detected in natural samples (Hiorns *et al.*, 1995; Hovanec and DeLong., 1996).

2.3.1.2 Heterotrophic nitrification

Numerous studies have also highlighted the possible involvement of heterotrophic bacteria in nitrification (Aleem, 1975; Verhagen and Laanbroek, 1991; Robertson and Kuenen, 1992; Stevens *et al.*, 2000). From studies conducted thus far, heterotrophic nitrification rates have been found to be only one or two orders of magnitude lower than that of autotrophs (Robertson and Kuenen, 1992). Furthermore, most of the heterotrophic nitrifiers studied are far less fastidious and sensitive than their autotrophic counterparts. Taking this into account, as well as their relatively fast growth rates and subsequent dominance in activated sludge, heterotrophs may be contributing significantly to

nitrification in activated sludge systems. However, the role of the heterotrophic nitrifier is not well known, whereas the role of the autotrophic nitrifier is well understood. It is evident that at least two different (organic and inorganic) biochemical pathways exist for nitrification in heterotrophic organisms (Robertson and Kuenen, 1992). These pathways proceed as:

$$RNH_2 \rightarrow RNHOH \rightarrow RNO \rightarrow RNO_2 \rightarrow RNO_3 \text{ (organic pathway)}$$

$$NH_4^+ \rightarrow NH_2OH \rightarrow NOH \rightarrow NO_2 \rightarrow NO_3 \text{ (inorganic pathway)}$$

It is also possible that some organisms use combinations of these pathways (Robertson and Kuenen, 1992). However, as these reactions do not generate energy it is uncertain how heterotrophic nitrification relates to the cytochrome chain (Robertson and Kuenen, 1992). This question, of which pathway of nitrogen oxidation is used, remains a crucial problem to be resolved. Unlike autotrophic nitrification, heterotrophic oxidation of ammonia requires energy that leads to decreased yield coefficients. The problem in assigning a definitive function to heterotrophs in a nitrification system is that the oxidation of nitrogen is not obligatorily linked to growth and, in pure culture, the production of nitrate and nitrite occurs after the logarithmic growth phase (Obaton *et al.*, 1968; Focht and Verstraete, 1977; Tate III, 1980). These bacteria only nitrify if supplied with an external, organic substrate, and it has not yet been demonstrated that they gain any energy from the reaction (Robertson and Kuenen, 1992). The reason for heterotrophic nitrification may be as diverse as the bacteria that do it. For example, heterotrophic nitrification by an *Arthrobacter* species may be related to the production of chelating agents (Robertson and Kuenen, 1992). For other species such as *Thiosphaera pantotropha, Pseudomonas denitrificans* and *Alcaligenes* spp it is thought that the

ammonia oxidation step (which requires NADPH) is utilised to dispose of any excess reducing power, possibly because of a rate limiting step in the electron transport chain to oxygen (Robertson and Kuenen, 1992). In other cases, heterotrophic nitrification may be due to co-metabolism where the reactions are due rather to a "biochemical accident" than a mechanism that is important to the survival of the species (Robertson and Kuenen, 1992). It, however, remains to be shown that the heterotrophic organisms, which can oxidise nitrogen under axenic culture conditions, can do so *in situ*. Heterotrophic nitrification under aerobic conditions can result in production of nitrite and/or nitrate that would subsequently become available for denitrification under anaerobic conditions without any inhibition or competition from autotrophs (Castignetti and Hollocher, 1984). Numerous heterotrophs may, therefore, be predominating in activated sludge systems due their ability to both nitrify and denitrify. Models applied to the nitrifying populations may need to be revised as they have been based on the assumption that *Nitrosomonas* spp and *Nitrobacter* spp are the organisms solely responsible for nitrification occurring during wastewater treatment (Hovanec and DeLong, 1996; Wagner *et al.*, 1996; Yuichi and Noto, 1998). A need exists for a better understanding of the organisms involved in nitrification and their specific mechanisms employed in ammonia and nitrite oxidation in order to further improve nitrogen removal during wastewater treatment.

2.3.2 Denitrification

Denitrification is primarily performed by facultatively anaerobic heterotrophic bacteria. A very wide array of different heterotrophic bacteria are capable of contributing to denitrification (*Bergey's*, 1984,

1986; Otlanabo, 1993; Carter *et al.*, 1995). Organisms most commonly implicated in denitrification during wastewater treatment are *Pseudomonas* spp, *Achromobacter* spp, *Agrobacterium* spp, *Alcaligenes* spp, *Bacillus* spp, *Chromobacterium* spp, *Flavobacterium* spp and *Vibrio* spp (Janda *et al.*, 1988; Gray, 1990; Lazarova *et al.*, 1992; Otlanabo, 1993). However, *Pseudomonas* spp are generally regarded as the predominant organisms involved in denitrification (Janda *et al.*, 1988; Gray, 1990; Lazarova *et al.*, 1992).

Dold *et al.* (1991), Ubisi *et al.* (1997) and Ekama and Wentzel (1999) have offered a simplified community structure for denitrifiers in ascribing denitrification solely to the heterotrophic bacteria (OHO fraction). However, denitrification may not be totally attributable to just the OHOs, or even all the OHOs. Microbial denitrification is being proven to be more complex than the traditional 'black and white' idea of step-wise reduction of nitrate, via nitrite, to gaseous nitrogen by individual bacteria exposed to anoxic conditions (Hippen *et al.*,1997; Van Loosdrecht and Jetten, 1998). Research has shown that numerous different enzymes are required to achieve complete denitrification and these enzymes are often restricted to different bacteria as well as expressed differently, from organism to organism, under varying physiological conditions (Robertson and Kuenen, 1992; Carter *et al.*, 1995; Wu and Knowles, 1995; Martienssen and Schöps, 1999). Denitrifying organisms can be classified into various functional groups according to their enzymatic capacity for nitrate and/or nitrite reduction. True denitrifying heterotrophic bacteria are capable of both nitrate and nitrite reduction owing to the presence of both nitrate and nitrite reductase enzymes within their cytoplasm while many other denitrifying bacteria (incomplete denitrifiers) are restricted (absence of nitrite reductase) to reduction of nitrate to nitrite with no further reduction of the nitrite

produced (Rheinheimer, 1985; Ketchum, 1988; Cappuccino and Sherman, 1992; Robertson and Kuenen, 1992; Glass *et al.*, 1997). In fact, according to Rheinheimer (1985) and Robertson and Kuenen (1992), most of the denitrifying bacteria in aquatic systems are enzymatically restricted to incomplete denitrification with subsequent nitrite accumulation. A third group of denitrifiers existing in the environment are true denitrifiers exhibiting transient accumulation of nitrite prior to nitrite reduction. However, very little is known about the organisms responsible for this type of denitrification as well as the mechanisms responsible for transient nitrite accumulation. In addition, exclusive nitrite reducing bacteria belonging to the *Neisseria* and *Flavobacterium* genera have also been isolated from environmental samples which exhibit an absence of nitrate reductase enzymes (*Bergey's*, 1984). The full impact these organisms have on nitrogen removal during activated sludge treatment has, however, not yet been determined or even properly contemplated. It is uncertain if exclusive nitrite reducers are present and contributing to denitrification behaviour in BNR processes. Numerous denitrifying heterotrophs, including *Escherichia coli*, *Paracoccus denitrificans* (also known as *Thiosphaera pantotropha*) and *Pseudomonas aeruginosa*, have also been found to reduce nitrate under aerobic conditions (Robertson and Kuenen, 1992; Carter *et al.*, 1995; Hentschel and Felbeck, 1995; Helmer and Kunst, 1997; Martienssen and Schöps, 1999). These organisms have been found to contain a periplasmic nitrate reductase which regulates redox balance for the aerobic respiratory chain using nitrate (Carter *et al.*, 1995). Furthermore, many heterotrophic bacteria are enzymatically incapable of denitrification (*Bergey's*, 1984; 1986) and in view of this it is inaccurate to ascribe denitrification in ND and NDBEPR systems to the entire OHO fraction. Microbial denitrification behaviour is complex and requires careful re-evaluation to gain better understanding of the biochemical mechanisms involved and hence improve modelling of BNR systems.

2.4 KINETICS AND MODELLING

Up until now extensive work has been done to develop kinetic models and related software that can be used successfully to simulate and design nitrification - denitrification (ND) and nitrification - denitrification biological excess phosphorus removal (NDBEPR) systems (Dold *et al.*, 1991; Wentzel *et al.*, 1991; Ekama *et al.*, 1992; Gujer and Kappler, 1992; Ekama and Wentzel, 1999). The denitrification kinetics of these systems have primarily been determined and attributed to all the ordinary heterotrophic bacteria, now also known as the OHO fraction, otherwise not involved in BEPR (Dold *et al.*, 1991; Ubisi *et al.*, 1997; Ekama and Wentzel, 1999). However, denitrification kinetics determined for ND systems have been found to vary considerably at times when applied to NDBEPR systems because of varying OHO active fraction estimates and the unexplained occurrence of anoxic phosphorus removal and any success achieved to date has been some what fortuitous (Dold *et al.*, 1991; Ekama and Wentzel, 1999). The OHO active fraction was introduced in 1976 as a hypothetical parameter for defining activated sludge kinetic rates but has never been possible to measure. Applying the OHO parameter to activated sludge kinetics determinations and models has thus encountered problems, particularly when applied to denitrification behaviour of NDBEPR systems (Ekama and Wentzel, 1997). The occurrence of anoxic phosphorus accumulation has largely been responsible for the problems experienced with OHO fraction estimates and denitrification kinetics determinations for NDBEPR systems (Ekama and Wentzel, 1999). Phosphorus removal under anoxic conditions has been observed in a number of BNR systems (Wanner *et al.*, 1992; Kuba *et al.*, 1993; Chuang *et al.*, 1997; Kuba *et al.*, 1997; Barker and Dold, 1996; Ekama and Wentzel, 1999). From a microbiological perspective there is little argument

against denitrifying phosphorus accumulating organisms being able to utilise nitrate and nitrite, instead of oxygen, for oxidation of stored poly-β-hydroxyalkanoates (PHA). However, while *Pseudomonas* spp are thought to possibly be involved due to their widespread capacity for both denitrification and phosphorus removal, much uncertainty exists regarding the exact organisms and mechanisms involved (Janda *et al.*, 1988; Osborn *et al.*, 1989; Gray, 1990; Lazarova *et al.*, 1992; Kavanaugh and Randall, 1994; Jørgenson and Pauli, 1995). Ultimately variations in process performance and kinetics are attributable to inadequate control and lack of understanding of the ecological, physiological and biochemical activities of constituent microorganisms (Bux *et al.*, 1994; Hippen *et al.*,1997). Henze (1992) and Kristensen *et al.* (1992) echo concern in that activated sludge models fall short in not taking into consideration the structure of the biomass present in the process. There is growing concern and movement towards a better understanding of the microbial community within activated sludge in order to gain optimal control of the process (Lu and Leslie Grady Jr, 1988; Davelaar, 1989; Wagner *et al.*, 1993; Bux *et al.*, 1994; Jansen *et al.*, 1994; Hu *et al.*, 1996; Satoh *et al.*, 1996; Ubisi *et al.*, 1997).

Readily biodegradable organics (RBCOD) and slowly biodegradable organics (SBCOD) have also been seen to have an effect on the denitrification kinetics of ND and NDBEPR systems. When compared, ND and NDBEPR systems have been seen to react differently to RBCOD and SBCOD. The denitrification kinetics of ND systems have been found to have an initial rapid denitrification rate (K_1) followed by slower rates (K_2 and K_3) for pre- and post denitrification reactors (FIG. 2.6) while NDBEPR systems show an absence of any initial rapid denitrification rate (FIG.2.7) (Ekama and Wentzel, 1997). The initial rapid K_1 rate seen in ND systems has been thought to be due to

utilisation of RBCOD whereafter remaining SBCOD contributes to K_2 and K_3. In NDBEPR systems RBCOD is utilised under anaerobic conditions for PHA oxidation and phosphorus release leaving only SBCOD available for denitrification (K'_2 and K'_3) and hence there is an absence of any initial rapid denitrification rate. However, the K'_2 and K'_3 rates of NDBEPR systems have been found to be 2.5 and 1.6 times greater respectively than the K_2 and K_3 rates of ND systems (Ekama and Wentzel, 1997). The cause for this difference has not yet been determined and it is possible that this phenomenon is microbially and biochemically mediated and hence a result of different population structures selected for by ND and NDBEPR systems. Andreadakis and Chatjikonstantinou (1994) demonstrated similar denitrification kinetics as discussed above for ND systems (FIG. 2.6) but only used RBCOD for their analyses. Their results, however, demonstrated that the K_1, K_2 and K_3 rates are not entirely dependant on the biodegradability of organics but may be microbially mediated.

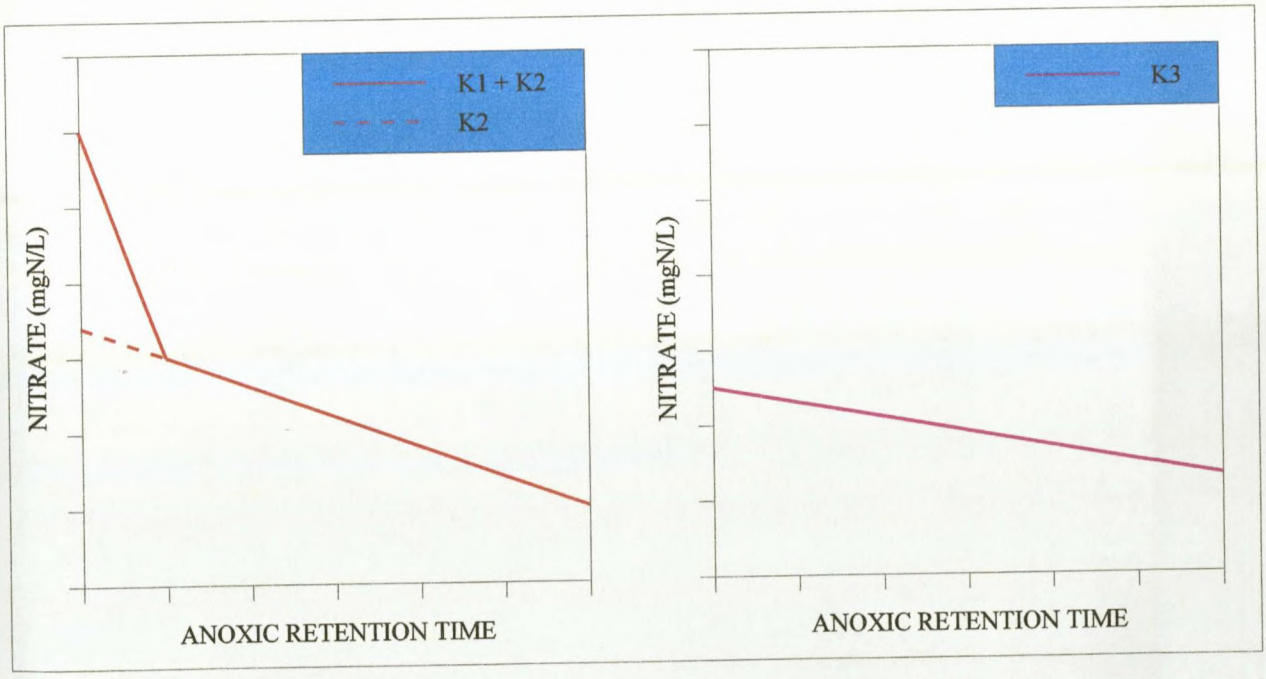

FIGURE 2.6 Schematic representation of the denitrification rates observed in primary and secondary anoxic reactors of ND systems (Ekama and Wentzel, 1997).

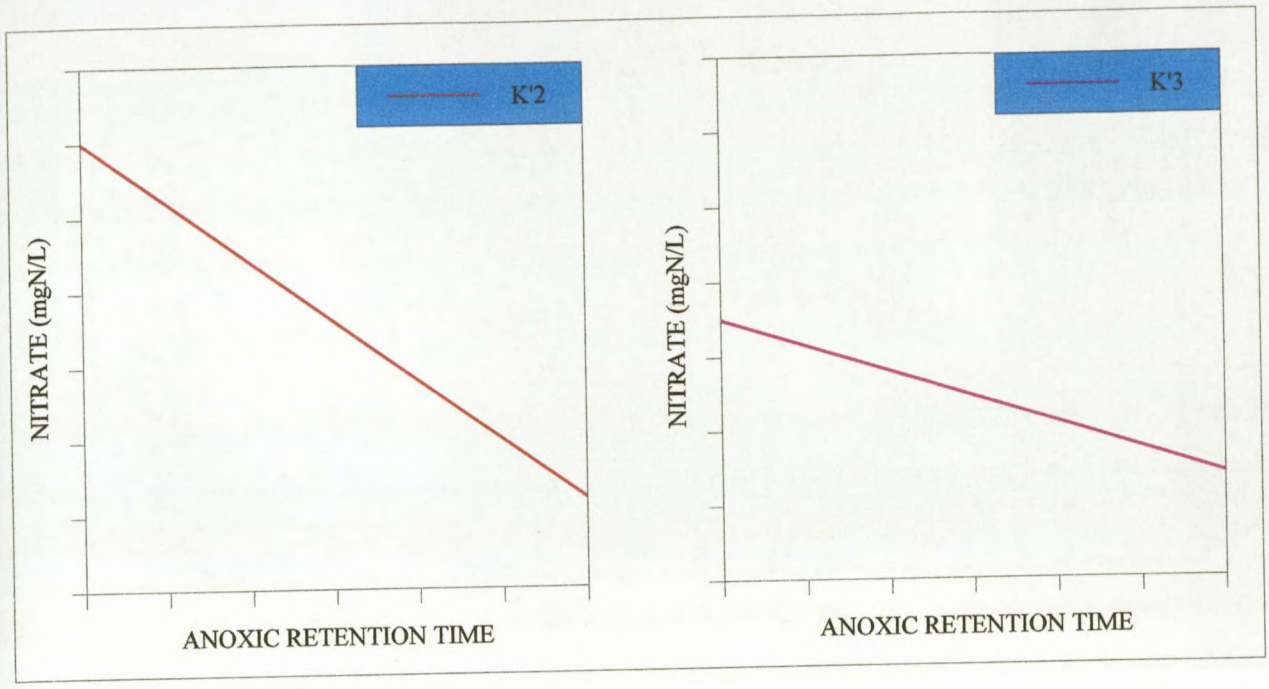

FIGURE 2.7 Schematic representation of the denitrification rates observed in the primary and secondary anoxic reactors of the M/UCT NDBEPR activated sludge system (Ekama and Wentzel, 1997).

The denitrification kinetics of ND and NDBEPR systems also demonstrate differences between nitrate and nitrite reduction rates. Ekama and Wentzel (1999) noted initial nitrite build-up or slow reduction, at approximately 1/10th of the nitrate reduction rate, when determining denitrification kinetics for NDBEPR processes (FIG. 2.8). Significant nitrite reduction would only proceed from initial build-up, or slow reduction, once the nitrate concentration had been reduced to below 1 mgNO_3-N/L (FIG. 2.8). Interestingly, the nitrite denitrification rate was seen to be approximately the same as the nitrate denitrification rate and not faster, as theoretically predicted according to their electron accepting capacities (NO_3:NO_2 = 5:3) (Ekama and Wentzel, 1999). Helmer and Kunst (1997) and Green *et al.*, (1994) noted similar denitrification behaviour (i.e. initial nitrite

accumulation) in biofilms and ground water, respectively. Nitrite accumulation, and subsequent reduction in the absence of nitrate, therefore seems be a general characteristic of denitrifying bio-communities which cannot be said to be restricted to ND and NDBEPR activated sludge systems due to specific process parameters applied.

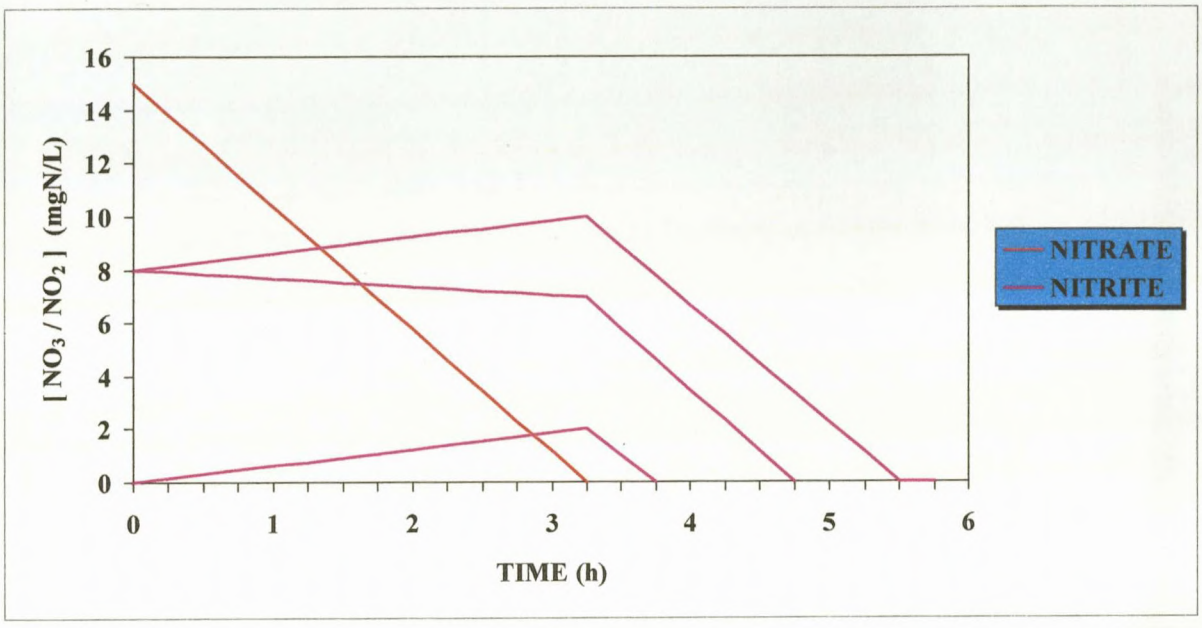

FIGURE 2.8 Schematic representation of nitrate/nitrite denitrification kinetics in primary anoxic reactors of various NDBEPR systems (Ekama and Wentzel, 1999).

2.5 THE DARVILL NDBEPR PROCESS

In order to gain a comprehensive understanding of the results and conclusions drawn in this study, it is important to take into consideration the process configuration and operational parameters of the Darvill BNR process from which all sampling was conducted. The Darvill process is a NDBEPR

system closely configured upon the Johannesburg process (FIG. 2.9). Due to the configuration of the Johannesburg process, the system has the potential to select for twice the concentration of denitrifiers than conventional anoxic zones thus resulting in a high degree of nitrogen removal (Osborn *et al.*, 1989). The Darvill configuration consists of an anaerobic zone (2700 kL) which feeds into an informal anoxic zone at the head of the aeration basin (19 600 kL)[2]. Return sludge is then recycled from the secondary clarifiers, via a pre-anoxic zone (700 kL), back to the anaerobic zone. The inclusion of a pre-anoxic zone is intended to protect the anaerobic zone from nitrate/nitrite overflow from nitrification activity within the aerobic zone (De Haas, 1998, as cited by Atkinson, 1999).

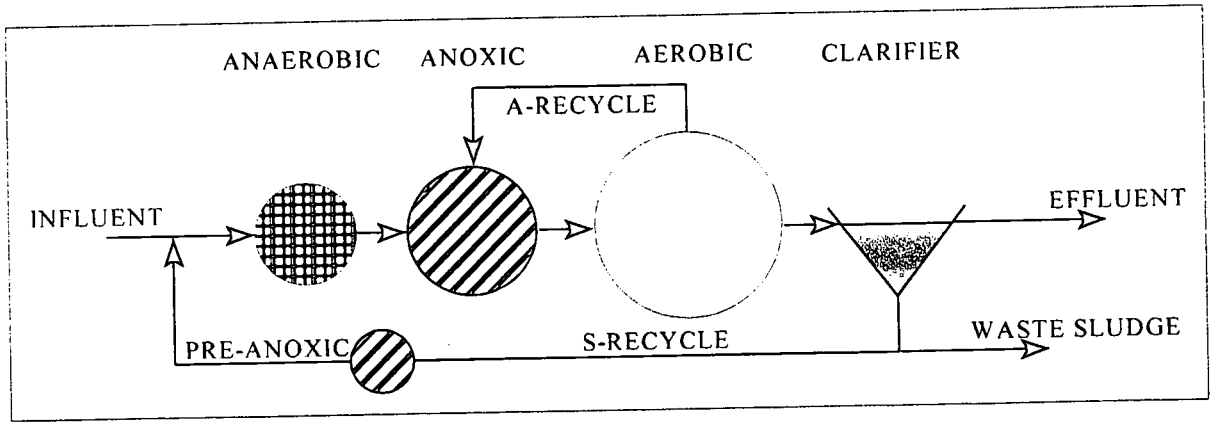

FIGURE 2.9 The Johannesburg activated sludge process configuration (Lilley *et al.*, 1997).

The average settled sewage composition to the Darvill works is shown in TABLE 2.1. Generally

[2]The aeration basin is actually subdivided into 3 basins, each with an informal anoxic zone and underflow recycle (a-recycle), totaling 19 600 kL.

a low COD is typical for the system. However, the phosphorus content of the sewage is prone to large fluctuations due to illegal dumping of vegetable oil effluent to the sewer by cooking oil, margarine and soap manufacturers. As a result, the plant can at times suffer high influent and effluent orthophosphorus (ortho-P) concentrations. The occurrence of high ortho-P concentrations in turn has resulted in the need for chemical precipitation in conjunction with biological treatment. Oily emulsions released by vegetable oil effluent also tend lead to diminished aeration efficiency and a reduction in nitrification (De Haas, 1998, as cited by Atkinson, 1999).

TABLE 2.1 Average settled sewage composition to the Darvill works (January 1995 to February 1997) (De Haas, 1998, as cited by Atkinson, 1999).

Determinant	Mean value
COD	242.9 mg/L
TKN	24.8 mgN/L
NH3	18.2 mgN/L
Alkalinity	141 mg/L as CaCO3
Ortho-P	5.6 mgP/L
TP	8.5 mgP/L

Owing to the low COD of the Darvill influent, settled sewage is fed directly into the anoxic zone of the process while a volatile fatty acid (VFA) containing stream, from the primary sludge fermenters, is fed into the anaerobic zone. VFA production in the primary sludge fermenters encourages phosphorus release in the anaerobic zone for subsequent enhanced phosphorus uptake in the aerobic zone. A minor fraction of the settled sewage influent (*ca.* 4%) is also diverted to the pre-anoxic zone to ensure complete denitrification of the return sludge. Denitrification of the return sludge is

essential to maintain maximum VFA production and phosphorus release in the anaerobic zone (De Haas, 1998, as cited by Atkinson, 1999). Actual operating features for the Darvill system are listed in TABLE 2.2.

TABLE 2.2 Operating features for the Darvill NDBEPR activated sludge system (De Haas, 1998, as cited by Atkinson, 1999).

Operating parameter	Mean value
Design average dry weather inflow	60 ML/d
Design maximum wet weather inflow	160 ML/d
Design MLSS	3 800 mg/L
Mean cell retention time	7.4d

CHAPTER 3

MATERIALS AND METHODS

3.1 ISOLATION OF HETEROTROPHIC BACTERIA

Four mixed liquor samples were taken randomly from the pre- and primary anoxic zones of the Darvill NDBEPR process situated on the outskirts of Pietermaritzburg in KwaZulu Natal. The samples were homogenised using glass beads in order to physically disrupt floc structures and release constituent bacteria for isolation. In order to avoid bias and gain a representative selection of heterotrophic bacteria from the samples, triplicate serial dilutions (10^{-1} to 10^{-6}) were made from each sample and plated, using the spread plate technique, on ten different media all known to be successful isolation media for heterotrophs. The media used were casitone glycerol yeast autolysate (CGY) agar (APPENDIX 1), Heterotrophic Plate Count agar (Difco Laboratories, USA), Blood agar (Biolab, South Africa), Brain Heart Infusion agar (Biolab, South Africa), Chocolate agar, Mueller-Hinton agar (Biolab, South Africa), Nutrient agar (Oxoid, England), Plate Count agar (Biolab, South Africa), Tryptone Glucose Extract agar (Oxoid, England) and Tryptone Soya agar (Oxoid, England) (*Bergey's*, 1984; Osborn *et al.*, 1989; Gray, 1990; Bux *et al.*, 1994; Bridson, 1995). Well defined bacterial colonies were isolated after 5 to 7d of incubation at 20°C (Lötter and Murphy, 1985; Venter *et al.*, 1989) and then maintained and further cultured on CGY agar (Osborn *et al.*, 1989; Gray, 1990; Bux *et al.*, 1994). Pure cultures of the isolates were maintained by storage at 4°C with monthly

subculturing to fresh CGY media.

3.2 NITRATE AND NITRITE REDUCTION SCREENING

To assess the specific nitrate and nitrite reduction capacities of all the OHOs isolated, pure cultures were screened in triplicate for nitrate and nitrite reduction using the colorimetric biochemical reduction test proposed by Cappuccino and Sherman (1992) (APPENDIX 2). Both nitrate and nitrite media were used comprised of CGY broth supplemented with 1.0 g/L potassium nitrate (KNO_3) and potassium nitrite (KNO_2), respectively. However, because high concentrations of nitrite have been reported to be toxic to some microorganisms (Mahne and Tiedje, 1995; Piñar *et al.*, 1997) the concentrations of KNO_3 and KNO_2 used were reduced to 0.5 g/L. Nitrate and nitrite media used were semi-solidified using 0.1% bacteriological agar to impede oxygen diffusion and create anoxic conditions ideal for denitrification (Cappuccino and Sherman, 1992). Incubation was carried out at 20°C for 5d. After incubation, growth of isolates was first assessed where after nitrate and nitrite reduction by each isolate determined using alpha-naphthylamine, sulphanilic acid and zinc powder (Cappuccino and Sherman, 1992). Strength of nitrate and nitrite reduction was postulated on intensity of colorimetric reaction. Based on the results of nitrate/nitrite reduction screening, the isolated OHOs were characterised into specific groups delineating their exact capacity for nitrate and/or nitrite reduction under anoxic conditions. The purpose of these characterisations was to more accurately define the OHO fraction of the Darvill NDBEPR process and so gain a better representation of the actual fraction of OHOs responsible for denitrification as well as their

interactive contribution to nitrate and nitrite reduction occurring in the process.

3.3 IDENTIFICATION OF DENITRIFYING ISOLATES

After denitrification screening, OHOs that could reduce nitrate and/or nitrite under anoxic conditions were identified to at least generic level using combinations of API identification kits, key differential biochemical tests and morphological characteristics (APPENDICES 3-13). Pure cultures were initially screened using the Gram stain technique (Cappuccino and Sherman, 1992) (APPENDIX 3), to establish their cellular morphology. Gram negative rods were then tested for oxidase activity and identified using API 20E and API 20NE identification kits, as described by Bux *et al.* (1994) (APPENDIX 4-5), in conjunction with additional key differential biochemical tests (APPENDICES 5-6). Gram negative cocci were identified using key differential biochemical tests and colonial and cellular morphological characteristics (*Bergey's*, 1984; Cappuccino and Sherman, 1992) (APPENDICES 5, 7-10). Gram positive rods were identified using the spore stain technique in conjunction with key differential biochemical tests and colonial and cellular morphological characteristics (*Bergey's*, 1986; Cappuccino and Sherman, 1992)(APPENDICES 5, 11-12). Gram positive cocci were also identified using key differential biochemical tests and colonial and cellular morphological characteristics (*Bergey's*, 1986; Cappuccino and Sherman, 1992) (APPENDICES 5-6, 9, 13).

CHAPTER 4

RESULTS

The results of this study show that, with respect to denitrification, the OHO fraction within the Darvill NDBEPR process comprises five different functional groups, four of which interactively contribute to denitrification occurring in the system and one group that is non-denitrifying. These groups were characterised according to their ability to reduce nitrate and/or nitrite and classified as true denitrifiers, incomplete denitrifiers, incomplete-nitrite reducers, exclusive nitrite reducers and non-denitrifiers (TABLE 4.1; FIG. 4.1).

TABLE 4.1 Characterisation of the functional groups comprising the Darvill OHO fraction.

OHO Fractions	No. Isolates	Physiology
1. True denitrifiers	56	Reduce both nitrate and nitrite $NO_3 \Rightarrow NO_2 \Rightarrow N_2$
2. Incomplete denitrifiers	85	Reduce nitrate to nitrite with no nitrite reduction $NO_3 \Rightarrow NO_2$
3. Incomplete-nitrite reducers	24	Reduce both nitrate and nitrite, however exhibit inhibition of nitrite reduction by nitrate $NO_3 \Rightarrow NO_2$ // $NO_2 \Rightarrow N_2$
4. Exclusive nitrite reducers	7	Only reduce nitrite $NO_2 \Rightarrow N_2$
5. Non-denitrifiers	100	No nitrate or nitrite reduction

The largest of the five groups isolated proved to be non-denitrifying OHOs (100 isolates) while, of

the four denitrifying OHO groups isolated, incomplete denitrifiers predominated (85 isolates) followed by true denitrifiers (56 isolates), incomplete-nitrite reducers (24 isolates) and exclusive nitrite reducers (7 isolates), respectively (FIG. 4.1 and TABLES 4.1-4.5). It was found that 63.2% (172 isolates) of the total OHO fraction isolated were capable of contributing to denitrification while 36.8% (100 isolates) showed no capacity for nitrate or nitrite reduction under the given experimental conditions (FIG. 4.1). In terms of the nitrate and nitrite reduction capacity of the total OHO fraction isolated (272 isolates), it was seen that 60.7% (165 isolates - true denitrifiers, incomplete denitrifiers and incomplete-nitrite reducers) were able to reduce nitrate, under the given experimental conditions, in contrast to 32.0% (87 isolates - true denitrifiers, incomplete-nitrite reducers and exclusive nitrite reducers) which reduced nitrite (FIG. 4.1).

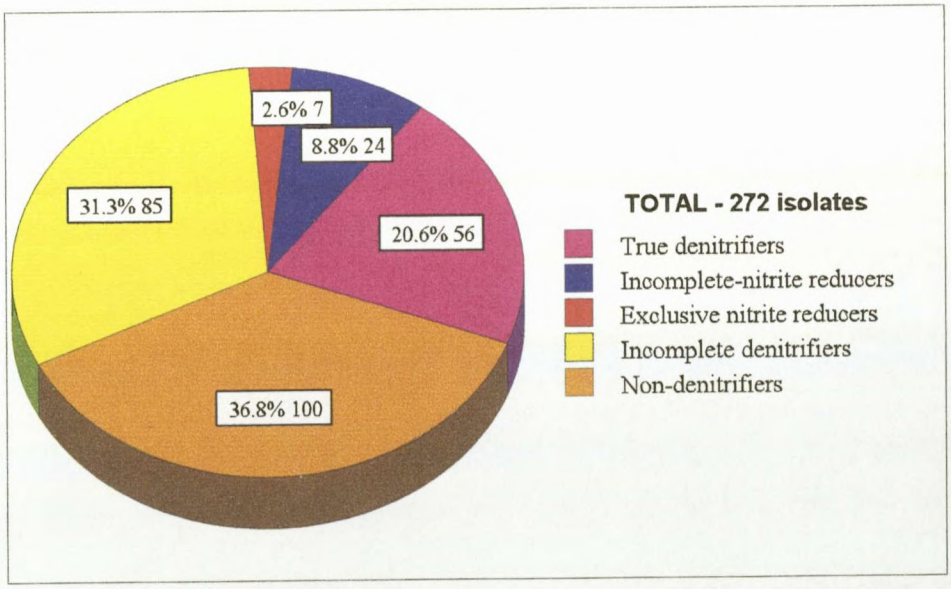

FIGURE 4.1 The OHO community structure of the Darvill NDBEPR anoxic zone using plating techniques.

TABLE 4.2 Identification of true denitrifying heterotrophic bacteria isolated from the Darvill NDBEPR activated sludge system.

Ref. No.	Identification	Cell morphology	Gram reaction
D1	*Aeromonas salmonicida 2*	Rod	negative
D2 - D3	*Agrobacterium radiobacter* - 2 strains	Rods	negative
D4	*Alcaligenes xylosoxidans* ssp *xylosoxidans*	Rod	negative
D5	**Alcaligenes xylosoxidans* ssp *xylosoxidans*	Rod	negative
D6	*Chromobacterium violaceum*	Rod	negative
D7 - D8	*Comamonas acidovorans* - 2 strains	Rods	negative
D9	*Comamonas testosteroni/Pseudomonas alcaligenes*	Rod	negative
D10 - D12	**Comamonas testosteroni/Pseudomonas alcaligenes*	Rods	negative
D13	**Escherichia coli 1*	Rod	negative
D14	*Flavobacterium indologenes*	Rod	negative
D15	**Klebsiella pneumonia* ssp *pneumoniae*	Rod	negative
D16	*Ochrobactrum anthropi*	Rod	negative
D17	*Pasteurella aerogenes*	Rod	negative
D18	*Pseudomonas aeruginosa*	Rod	negative
D19 - D20	*Pseudomonas cepacia* - 2 strains	Rods	negative
D21	**Pseudomonas maltophilia*	Rod	negative
D22 - D23	*Pseudomonas pickettii* - 2 strains	Rods	negative
D24	**Pseudomonas pseudomallei*	Rod	negative
D25 - D30	*Pseudomonas stutzeri* - 6 strains	Rods	negative
D31	**Pseudomonas stutzeri*	Rod	negative
D32 - D33	*Pseudomonas* spp (x 2)	Rods	negative
D34	*Seratia liquificaens*	Rod	negative
D35 - D36	*Seratia marcescens* - 2 strains	Rods	negative
D37	**Sphingobacterium multivorum*	Rod	negative
D38	*Sphingomonas paucimobilis*	Rod	negative
D39 - D40	*Vibrio hollisae* - 2 strains	Rods	negative
D41 - D47	**Unidentified	Rods	negative
D48 - D51	*Neisseria* spp (x 4)	Cocci	negative
D52 - D56	**Unidentified	Rods	positive

*Doubtful identification; **Inconclusive identification

TABLE 4.3 Identification of incomplete denitrifying heterotrophic bacteria isolated from the Darvill NDBEPR activated sludge system.

Ref. No.	Identification	Cell morphology	Gram reaction
D57 - D58	*Aeromonas hydrophila* - 2 strains	Rods	negative
D59	*Aeromonas salmonicida salmonicida*	Rod	negative
D60	*Alcaligenes faecalis*	Rod	negative
D61	*Alcaligenes xylosoxidans* ssp *xylosoxidans*	Rod	negative
D62 - D63	*CDC group IV C-2/CDC group IV E* - 2 strains	Rods	negative
D64	**CDC group IV C-2*	Rod	negative
D65 - D67	*Chryseomonas luteola* - 3 strains	Rods	negative
D68 - D69	*Comamonas acidovorans* - 2 strains	Rods	negative
D70 - D73	*Comamonas testosteroni/Pseudomonas alcaligenes* - 4 strains	Rods	negative
D74 - D75	**Comamonas testosteroni/Pseudomonas alcaligenes* - 2 strains	Rods	negative
D76 - D77	*Enterobacter cloacae* - 2 strains	Rods	negative
D78	**Moraxella* spp	Rod	negative
D79	*Pasteurella aerogenes*	Rod	negative
D80 - D81	*Pasteurella* spp (x 2)	Rods	negative
D82	*Proteus mirabilis*	Rod	negative
D83	*Pseudomonas aeruginosa*	Rod	negative
D84	*Pseudomonas cepacia*	Rod	negative
D85	**Pseudomonas cepacia*	Rod	negative
D86 - D88	*Pseudomonas fluorescens 1* - 3 strains	Rods	negative
D89	*Pseudomonas maltophilia*	Rod	negative
D90 - D91	**Pseudomonas mendocina* - 2 strains	Rods	negative
D92 - D93	*Pseudomonas stutzeri* - 2 strains	Rods	negative
D94 - D95	**Pseudomonas stutzeri* - 2 strains	Rods	negative
D96	*Pseudomonas vesicularis*	Rod	negative
D97 - D99	*Pseudomonas* spp (x 3)	Rods	negative
D100	*Seratia liquefaciens*	Rod	negative
D101 - D103	*Vibrio fluvialis* - 3 strains	Rods	negative
D104 - D112	**Unidentified	Rods	negative

* doubtful identification; ** inconclusive identification

TABLE 4.3 ***Continued***

Ref. No.	Identification	Cell morphology	Gram reaction
D113 - D115	*Bacillus* spp (x 3)	Rods	positive
D116 - D136	**Unidentified	Rods	positive
D137	**Staphylococcus* spp	Coccus	positive
D138 - 139	**Unidentified	Cocci	positive
D140	*Branhamella* spp	Coccus	negative
D141	Unidentified	Coccus	negative

* doubtful identification; ** inconclusive identification

TABLE 4.4 Identification of incomplete-nitrite reducing heterotrophic bacteria isolated from the Darvill NDBEPR activated sludge system.

Ref. No.	Identification	Cell morphology	Gram reaction
D142	*Acinetobacter calcoaceticus var lwoffi*	Rod	negative
D143	*Aeromonas hydrophila*	Rod	negative
D144	**Agrobacterium radiobacter*	Rod	negative
D145	*Citrobacter freundii*	Rod	negative
D146	*Comamonas testosteroni/Pseudomonas alcaligenes*	Rod	negative
D147 - D148	*Escherichia coli 1* - 2 strains	Rods	negative
D149	**Escherichia coli 1*	Rod	negative
D150	*Kluyvera* spp	Rod	negative
D151	*Pasteurella* spp	Rod	negative
D152	*Pseudomonas* cepacia	Rod	negative
D153	*Pseudomonas* maltophilia	Rod	negative
D154	*Shewanella* putrefaciens	Rod	negative
D155	*Vibrio fluvialis*	Rods	negative
D156 - D159	**Unidentified	Rods	negative
D160 - D162	*Neisseria* spp (x 3)	Cocci	negative
D163	**Unidentified	Coccus	negative
D164 - D165	**Unidentified	Rods	positive

* doubtful identification; ** inconclusive identification

TABLE 4.5 Identification of exclusive nitrite reducing heterotrophic bacteria isolated from the Darvill NDBEPR activated sludge system.

Ref. No.	Identification	Cell morphology	Gram reaction
D166	****_Weeksella zoohelcum_ _Flavobacterium odoratum_	Rod	negative
D167	****_Flavobacterium_ spp _Flavobacterium odoratum_ _Pseudomonas diminuta_	Rod	negative
D168	*_Sphingobacterium multivorum_	Rod	negative
D169 - D170	**Unidentified	Rods	negative
D171 - D172	**Unidentified	Cocci	negative

* doubtful identification; ** inconclusive identification; ****low discrimination between two or more organisms

Identification of the denitrifying OHOs isolated showed a very diverse consortia of heterotrophic bacteria (predominantly Gram negative rods) interactively contributing to denitrification in the Darvill NDBEPR system (TABLES 4.2-4.5). Of the true and incomplete denitrifiers identified, *Pseudomonas* spp (including *Comamonas* spp) showed significant involvement comprising 21 (does not include unidentified isolates) of 56 true denitrifying isolates and 25 (does not include unidentified isolates) of 85 incomplete denitrifying isolates (TABLES 4.2-4.3). Of the 24 incomplete-nitrite reducers isolated, both *Pseudomonas* spp (including *Comamonas* spp) and *Neisseria* spp comprised 3 (does not include unidentified isolates) of 24 isolates, respectively (TABLE 4.4). Identification of the exclusive nitrite reducing OHOs isolated, although indefinite, indicated potential predominance of *Flavobacterium* spp (TABLE 4.5).

Nitrate and nitrite concentration was also seen to be a limiting factor for denitrification by numerous isolates. *C. testosteroni*, *E. coli 1*, *P. aerogenes* and *S. marcescens*, which are known incomplete

denitrifiers (*Bergey's*, 1984), were found to be incapable of nitrite reduction when tested in nitrate media comprising 1.0 g/L KNO_3. However, when grown in nitrate media comprising 0.5 g/L KNO_3 these organisms were in fact found capable of both nitrate and nitrite reduction and hence characterised as true denitrifiers (TABLE 4.2). These findings were confirmed with the use of nitrite media at different concentrations of KNO_2. The *Neisseria* spp isolated, as well as three unidentified true denitrifiers (isolates D42, D43 and D52), were also incapable of nitrite reduction at 1.0 g/L KNO_3 and only reduced nitrate and nitrite completely at 0.5 g/L KNO_3. Four of the true denitrifying isolates also demonstrated weak reduction of nitrite when incubated in nitrite media. *C. testosteroni*, *E. coli*, *Pseudomonas* spp (isolate D34) and one unidentified true denitrifier (isolate D42) were not able to reduce nitrite at 1.0 g/L KNO_2 but managed at 0.5 g/L KNO_2. *A. radiobacter*, *P. cepacia* (isolate D16), *P. maltophilia*, *Neisseria* spp (isolate D48) and one unidentified true denitrifier (isolate D43) were, although capable of nitrite reduction in nitrate media, unable to reduce nitrite in nitrite media at either 1.0 or 0.5 g/L KNO_2. Furthermore, *A. salmonicida 2*, *A. radiobacter*, *C. acidovorans*, *K. pneumonia* ssp *pneumoniae*, *P. cepacia*, *P. maltophilia*, *S. liquificaens* and *S. multivorum*,which are known incomplete denitrifiers (*Bergey's*, 1984), reduced both nitrate and nitrite at 1.0 and 0.5 g/L KNO_3 and were also characterised as true denitrifiers (TABLE 4.2). Of the 24 incomplete-nitrite reducers isolated (TABLE 4.4), 13 were found to be incapable of nitrite reduction in nitrite media comprising 1.0 g/L KNO_2 but reduced nitrite effectively at a concentration of 0.5 g/L KNO_2. However, of the organisms characterised as incomplete-nitrite reducers, *A. calcoaceticus var lwoffi*, *A. radiobacter*, *C. freundii*, *E. coli 1*, *Kluyvera* spp, *P. cepacia*, *P. maltophilia* and *V. fluvialis* are known to be incomplete denitrifiers and have not previously been known to reduce any concentration of nitrite (*Bergey's*, 1984) as was demonstrated in this study with

use of nitrite media. Of these, *A. radiobacter*, *C. freundii*, *E. coli 1* (D147), *P. cepacia* and *V. fluvialis* only demonstrated nitrite reduction in nitrite media comprising 0.5 g/L KNO_2 and not at 1.0 g/L KNO_2. *C. testosteroni/P. alcaligenes*, *S. putrefaciens*, the *Nesseria* spp and five unidentified incomplete-nitrite reducers (isolates D156, D157, D163, D164, D165) also exhibited similar behaviour with one demonstrating weak reduction of nitrate at 0.5 g/L KNO_3 with no reduction of nitrate at 1.0 g/L KNO_3 (isolate D163). Overall, it was seen that nitrate and especially nitrite reduction, by heterotrophic bacteria, occurs more readily in media comprising 0.5 g/L KNO_3 and KNO_2, respectively, than at concentrations of 1.0 g/L (TABLE 4.6) as is generally the current specifications for nitrate/nitrite reduction screening media (Atlas, 1995; Food and Drug Administration, 1995; Atlas, 1997).

TABLE 4.6 Numbers of isolated OHOs demonstrating nitrate and nitrite reduction in separate nitrate and nitrite media consisting of different concentrations of KNO_3 and KNO_2 respectively.

OHO fractions	Total isolates	No. isolates showing nitrate reduction		No. isolates showing nitrite reduction			
		Nitrate media - KNO_3		Nitrate media - KNO_3		Nitrite media - KNO_2	
		1.0 g/L	0.5 g/L	1.0 g/L	0.5 g/L	1.0 g/L	0.5 g/L
1. True denitrifiers	56	56	56	45	56	46	50
2. Incomplete denitrifiers	85	85	85	0	0	0	0
3. Incomplete-nitrite reducers	24	23	24	0	0	13	24
4. Exclusive nitrite reducers	7	0	0	0	0	7	7
5. Non-denitrifiers	100	0	0	0	0	0	0
(Total)	-272	-163	-165	-45	-56	-66	-81

CHAPTER 5

DISCUSSION

5.1 TRUE DENITRIFIERS

The true denitrifying OHOs isolated reduced both nitrate as well as nitrite under anoxic conditions. These bacteria all contain nitrate as well as nitrite reductase enzymes enabling them to successfully reduce nitrate, via nitrite, to gaseous nitrogen (Rheinheimer, 1985; Ketchum, 1988; Cappuccino and Sherman, 1992; Robertson and Kuenen, 1992). This group of organisms fit the traditional description and understanding of denitrification occurring in waste water treatment but are not exclusively responsible for denitrification occurring in the Darvill NDBEPR process (FIG. 4.1). The results of the study show true denitrifying OHOs to comprise roughly the second largest functional group of denitrifiers to be found in the Darvill NDBEPR system (FIG 4.1 and TABLES 4.1-4.2). However, the ability to reduce both nitrate and nitrite under anoxic conditions would infer a selective advantage to these denitrifiers in ND and NDBEPR systems thus ensuring a strong presence of these organisms in such processes. It is known that culture based studies can be biased in that, due to the strongly selective nature of culture media, counts of certain microorganisms, or groups thereof, can either be over- or underestimated (Wagner and Amann, 1997). Therefore, although this study focussed on isolation of heterotrophic bacteria and not plate counts, it cannot be taken for granted that the amount of true denitrifying OHOs isolated are a true reflection of the actual size of this

group *in situ* relative to the other groups of denitrifiers present. *In situ* evaluation of the functional groups of denitrifiers, present in ND and NDBEPR systems, is therefore an important criteria necessary for understanding the population dynamics of activated sludge.

Identification of the true denitrifying OHOs revealed *Pseudomonas* spp to comprise a significant amount of the true denitrifiers isolated from the Darvill NDBEPR process (TABLE 4.2) and are therefore important organisms to be considered in denitrification behaviour of such systems. These results substantiate *Pseudomonas* spp to be one of the predominant groups of bacteria involved in denitrification in ND and NDBEPR systems (Janda *et al.*, 1988; Gray, 1990; Lazarova *et al.*, 1992). *Psuedomonas* spp have also been implicated in phosphorus accumulation (Osborn *et al.*, 1989; Kavanaugh and Randall, 1994; Jørgensen and Pauli, 1995) and therefore, with such an overlap in their function, this group of organisms may be predominating in NDBEPR systems. The involvement of *Pseudomonas* spp in activated sludge treatment needs to be properly established in order to fully understand their contribution to nitrogen and phosphorus removal as well as the significance of these organisms as model organisms for activated sludge kinetics. However, it was also seen that many other bacteria are also true denitrifiers capable of interactively contributing to denitrification in the Darvill process (TABLE 4.2). Denitrification is an interactive process involving numerous organisms but it is still uncertain as which of these organisms, or groups thereof, are predominant and most significant to the kinetics of ND and NDBEPR processes.

Numerous isolates were also identified that have not previously been known to reduce nitrite. *E. coli 1*, *C. testosteroni* , *P. aerogenes* and *S. marcescens*, which are known incomplete denitrifiers

(*Bergey's*, 1984), were found to be capable of both nitrate and nitrite reduction when grown in nitrate media comprising 0.5 g/L KNO_3, yet not at 1.0 g/L KNO_3, and hence characterised as true denitrifiers (TABLE 4.2). The *Neisseria* spp isolated, as well as three unidentified true denitrifiers (isolates D42, D43 and D52), were also incapable of nitrite reduction at 1.0 g/L KNO_3 and only reduced nitrate and nitrite completely at 0.5 g/L KNO_3. To date, the specifications for denitrification screening media range from 1.0 g/L to 5.0 g/L KNO_3 addition as the nitrate source (Cappuccino and Sherman, 1992; Atlas, 1995; Food and Drug Administration, 1995; Atlas, 1997). This is substantially higher than 0.5 g/L KNO_3 and therefore it is possible that even more organisms exist that have been incorrectly characterised in terms of their ability to reduce nitrate and/or nitrite. Nitrite has been reported to be toxic at high concentrations (Mahne and Tiedje, 1995; Piñar *et al.*, 1997) and therefore it is possible that nitrate can also be toxic or inhibitory at high concentrations. This would explain the observed true denitrification exhibited by *E. coli 1*, *C. testosteroni*, *P. aerogenes* and *S. marcescens* when incubated at a lower concentration of KNO_3 (i.e. 0.5 g/L). Therefore, it is important that the concentration of nitrate added to nitrate media be reduced to 0.5 g/L KNO_3, or lower, in order to accurately determine the ability of heterotrophic bacteria to reduce nitrate and/or nitrite. In addition, the use of nitrite media may also prove valuable in assessing denitrification. However, it is also apparent that the initial concentration of KNO_2 used needs to be similar to that of KNO_3 in nitrate media. *P. testosteroni*, *E. coli 1*, *Pseudomonas* spp (isolate D34) and one unidentified true denitrifier (isolate D42) were not able to reduce nitrite at 1.0 g/L KNO_2, but managed at 0.5 g/L KNO_2, while *A. radiobacter*, *P. cepacia* (isolate D16), *P. maltophilia*, *Neisseria* spp (isolate D48) and one unidentified true denitrifier (isolate D43) were, although capable of nitrite reduction in nitrate media, unable to reduce nitrite in nitrite media at either 1.0 or 0.5 g/L

KNO_2. Owing to the toxicity of nitrite (Mahne and Tiedje, 1995; Piñar *et al.*, 1997), it is possible that true denitrifiers reduce nitrite as soon as it is produced from nitrate reduction, therefore, never allowing accumulation of the nitrite intermediate. This would explain the inability of *A. radiobacter*, *P. cepacia* (isolate D16), *P. maltophilia*, *Neisseria* spp (isolate D48) and isolate D43 to reduce nitrite in nitrite media, as both 1.0 g/L and 0.5 g/L KNO_2 contain high concentrations of nitrite. However, most of the true denitrifying isolates were found to reduce nitrite efficiently, in nitrite media, at both 1.0 g/L and 0.5 g/L KNO_2 (TABLE 4.6). Therefore, it is uncertain to what degree nitrite toxicity is applicable to all true denitrifiers or if the observed absence of nitrite reduction by some isolates, in nitrite media, is in fact due to nitrite toxicity. It is possible that different enzymes, or feed back mechanisms, exist within different true denitrifiers to optimise energy yield during denitrification and that nitrite may not be as toxic to microorganisms as currently believed.

A. salmonicida 2, *A. radiobacter*, *C. acidovorans*, *K. pneumonia* ssp *pneumoniae*, *P. cepacia*, *P. maltophilia*, *S. liquificaens* and *S. multivorum*, which are known incomplete denitrifiers (*Bergey's*, 1984), had no difficulty in reducing both nitrate and nitrite at 1.0 and 0.5 g/L KNO_3 and were also characterised as true denitrifiers (TABLE 4.2). It is uncertain how nitrite reduction was induced in these isolates as they are not known to be capable of nitrite reduction (*Bergey's*, 1984). Nitrate and nitrite concentration do not explain the observed results as *A. salmonicida 2*, *A. radiobacter*, *C. acidovorans*, *K. pneumonia* ssp *pneumoniae*, *P. cepacia*, *P. maltophilia*, *S. liquificaens* and *S. multivorum* demonstrated nitrite reduction, in nitrate media, at the equivalent concentration of KNO_3 (i.e. 1.0 g/L) to that already specified for nitrate reduction media (Cappuccino and Sherman, 1992; Atlas, 1995; Food and Drug Administration, 1995; Atlas, 1997). Therefore, these organisms would

already have been assessed for nitrate and nitrite reduction at 1.0 g/L KNO_3 and have, to date, been found to only accumulate nitrite in the media used. As far as can be hypothesised two other possibilities exist that may explain the nitrite reduction by these isolates. The first possibility is incorrect identification. Numerous Gram negative rods were unidentifiable or doubtfully identified, even with repeated identification tests, using API 20E and 20NE identification kits (TABLES 4.2-4.5). It is apparent that the consortia of bacteria, and possible presence of mutants, in activated sludge is too diverse for current API databases. It is therefore possible that the organisms identified as *A. salmonicida 2*, *A. radiobacter*, *C. acidovorans*, *K. pneumonia* ssp *pneumoniae*, *P. cepacia*, *P. maltophilia*, *S. liquificaens* and *F. multivorum* are isolates with similar biochemical characteristics to organisms included in the API data bases while themselves being excluded. It is therefore important that identification kits, such as API, be used in conjunction with other biochemical tests and molecular techniques when identifying organisms within environmental samples, including activated sludge. However, as API identification kits have demonstrated a certain level of accuracy and dependability in their use, it must be noted that nitrite reduction by *A. salmonicida 2*, *A. radiobacter*, *C. acidovorans*, *K. pneumonia* ssp *pneumoniae*, *P. acidovorans*, *P. cepacia*, *P. maltophilia*, *S. liquificaens* and *S. multivorum* may possibly have been induced by the nutrient composition of the nitrate media used. Many researchers have reported on the varying affects of different nutrient sources on denitrification (Blaszczyk, 1993; La Cour Jansen *et al.*, 1994; Rosén and Welander, 1994; Watanabe *et al.*, 1994; Martienssen and Schöps, 1999). Clays-Josserand *et al.* (1995) stipulate the importance of testing bacterial cultures for denitrification under non-limiting conditions for proper expression of the genes involved. In this study, CGY broth (APPENDIX 1) supplemented with KNO_3 or KNO_2, was used for nitrate and nitrite reduction screening. Specified

nitrate reduction media (Cappuccino and Sherman, 1992; Atlas, 1995; Food and Drug Administration, 1995; Atlas, 1997) are usually minimal in terms of their nutrient content and lack diversity with the carbon substrate used. CGY is a non-selective, nutrient rich medium known to support the growth of a wide array of organisms and hence is a favoured medium for the isolation of bacteria from environmental, aquatic and sewage samples (Osborn *et al.*, 1989; Gray, 1990; Bux *et al.*, 1994). Owing to the nutritional status of CGY, the isolates tested for nitrate and nitrite reduction under anoxic conditions would not have been limited in terms of available carbon substrate necessary for both nitrate and nitrite reduction. There is therefore need for a comparison of classical nitrate reduction media with more nutrient rich media in order to establish correct parameters for assessing nitrate and nitrite reduction and hence correctly characterise denitrifying heterotrophic bacteria.

5.2 INCOMPLETE DENITRIFIERS

Most of the denitrifying heterotrophic bacteria isolated were incomplete denitrifiers i.e., bacteria that were only capable of reducing nitrate to nitrite with no further reduction of the nitrite produced (FIG. 4.1 and TABLES 4.1 and 4.3). These results substantiate findings by Rheinheimer (1985), Robertson and Kuenen (1992) and Rosén and Welander (1994) who state that denitrifying communities are predominantly comprised of incomplete denitrifiers. According to Rheinheimer (1985) and Robertson and Kuenen (1992), 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. Ekama and Wentzel (1999) noted initial nitrite build-up or slow reduction at approximately 1/10th of the nitrate reduction rate when determining denitrification kinetics for NDBEPR processes (FIG. 2.8). Helmer and Kunst (1997) and Green *et al.*, (1994) noted similar denitrification behaviour in biofilms and ground water, respectively. Results of this study show that this is partly attributable to the large amount of incomplete denitrifiers present in activated sludge. These are therefore a very important group of organisms that need to be considered when modelling denitrification kinetics of NDBEPR processes. Incomplete denitrifiers, comprising the largest of the denitrifying OHO fractions isolated, contribute significantly to nitrate reduction along with true denitrifiers and incomplete-nitrite reducers. However, owing to the absence of nitrite reductase enzymes in incomplete denitrifiers (Robertson and Kuenen, 1992), this group of organisms are unable to contribute to reduction of the nitrite they produce under anoxic conditions. Only true denitrifiers, incomplete-nitrite reducers and exclusive nitrite reducers are able to reduce nitrite under anoxic conditions. However, these three groups of denitrifiers collectively only comprised approximately half of the denitrifying organisms isolated (FIG. 4.1). Therefore, it is clear that more denitrifying organisms are capable of nitrate reduction than nitrite reduction thus explaining temporary nitrite accumulation during denitrification in NDBEPR systems.

Identification of incomplete denitrifiers also revealed a significant quantity of *Pseudomonas* spp isolated (TABLE 4.3). Approximately a third of the incomplete denitrifiers isolated were *Pseudomonas* spp (including *Comamonas* spp), therefore, again substantiating the importance of *Pseudomonas* spp to denitrification. However, numerous other heterotrophic bacteria are also incomplete denitrifiers and, therefore, denitrification behaviour needs to be evaluated on a

community basis with many organisms interactively involved.

Results also showed possible aerobic denitrification by incomplete denitrifiers. These organisms did not grow well under anoxic conditions but preferred the more aerobic conditions at the surface of the nitrate media. It is possible that these organisms can utilise oxygen and nitrate simultaneously for cellular respiration. Rheinheimer (1985), Robertson and Kuenen (1992), Carter *et al.*, (1995), Hentschel and Felbeck (1995) and Martienssen and Schöps (1999) all support claims of denitrifying bacteria being capable of aerobic denitrification. In fact, a denitrifying bacterium has been isolated that is unable to grow under strict anoxic conditions and yet reduces nitrate microaerophilically (Robertson and Kuenen, 1992). Carter *et al.*, (1995) demonstrated numerous incomplete denitrifiers, isolated from soil and sediment, to be capable of aerobic nitrate respiration as compared to only a few true denitrifiers. However, although these results may confirm such findings, it is also possible that biomass production by incomplete denitrifiers is simply restricted under anoxic conditions thus explaining the poor growth observed by these bacteria. The inability to utilise nitrite for cellular respiration will reduce the amount of available electron acceptor under anoxic conditions thus resulting in lower potential growth yield as compared to true denitrifiers and incomplete-nitrite reducers which utilise both nitrate and nitrite. However, exclusive nitrite reducers isolated generally grew well under anoxic conditions (nitrite anoxia) and, although also potentially limited by available electron acceptor, did not grow as poorly under anoxic conditions as did incomplete denitrifiers. A more in depth understanding of nitrate reduction by incomplete denitrifiers is therefore still necessary in order to fully understand their contribution to denitrification.

5.3 INCOMPLETE-NITRITE REDUCERS

The third group of denitrifying heterotrophic bacteria isolated were incomplete-nitrite reducers. These bacteria exhibited typical incomplete denitrification (reduction of nitrate to nitrite with no further reduction of the nitrite produced) when grown in nitrate medium. However, when grown in nitrite media these bacteria proved to reduce nitrite efficiently. It is possible that the nitrite reductase enzymes within these bacteria are subject to a feedback inhibition mechanism restricting expression of the enzymes when nitrate are present. This mechanism may simply be a matter of energetics where nitrate are favoured over nitrite owing to the higher electron accepting capacity of nitrate as compared to nitrite (NO_3:NO_2 = 5:3) (Ekama and Wentzel, 1999). However, the mechanisms responsible for inhibition of nitrite reduction remain uncertain and further research is required to fully understand this phenomenon and its exact impact on denitrification in ND and NDBEPR processes. Ekama and Wentzel (1999) noted that, when determining denitrification kinetics for NDBEPR processes, significant nitrite reduction would only proceed from initial build-up, or slow reduction, once the nitrate concentration had been reduced to below 1 mgNO_3-N/L (FIG. 2.8). Helmer and Kunst (1997) and Green *et al.*, (1994) noted similar nitrite reduction behaviour in biofilms and ground water, respectively. Results of this study show that this can be strongly related to the presence of incomplete-nitrite reducing bacteria in NDBEPR systems. Blaszczyk (1993) identified *P. stutzeri* as an incomplete-nitrite reducer, demonstrating a sequential pattern of nitrate and nitrite reduction whereby nitrate is first completely transformed to nitrite whereafter nitrite reduction commences. Incomplete-nitrite reducers are therefore true denitrifiers which simply express a different pattern of nitrate and nitrite reduction as compared to organisms classically

characterised as true denitrifiers. However, the kinetics of nitrate and nitrite reduction by incomplete-nitrite reducers, or sequential denitrifiers, need to be accurately evaluated. These organisms comprised a substantial fraction of the OHOs isolated (FIG. 4.1 and TABLES 4.1 and 4.4) and therefore their contribution to denitrification in NDBEPR processes needs to be taken into account when modelling denitrification kinetics of such systems.

Pseudomonas spp (including *Comamonas* spp) and *Neisseria* spp were the predominant heterotrophic bacteria identified as incomplete-nitrite reducers. *Neisseria* spp have previously been found to predominantly reduce nitrite over nitrate with some species only being capable of exclusive nitrite reduction (*Bergey's*, 1984). However, of the identified incomplete-nitrite reducers (TABLE 4.4), most are known incomplete denitrifiers previously not characterised as being capable of nitrite reduction. *A. hydrophila*, *A. radiobacter*, *C. freundii*, *C. testosteroni*, *E. coli 1*, *Kluyvera* spp, *Pasteurella* spp, *P. cepacia*, *P. maltophilia* and *V. fluvialis* are all organisms known to be capable of nitrate reduction while being incapable of reducing any of the nitrite produced from nitrate reduction (*Bergey's*, 1984). With the use of both nitrate and nitrite reduction media these organisms were all found to be capable of both nitrate as well as nitrite reduction. Owing to the inability of these organisms to reduce nitrite in the presence of nitrate these organisms can be mistaken as incomplete denitrifiers, when grown in nitrate reduction media, as nitrite accumulation occurs. However, when no nitrate is present (i.e. the use of nitrite reduction media) these organisms do in fact have the enzymatic capacity to utilise nitrite as final electron acceptor for cellular respiration under anoxic conditions. Therefore, it is possible that there are even more organisms, than those identified in this study, that have previously been characterised as incomplete denitrifiers yet are in

fact capable of nitrite reduction. Classical criteria for denitrification screening media do not include the use of nitrite media (Cappuccino and Sherman, 1992; Atlas, 1995; Food and Drug Administration, 1995; Atlas, 1997), as was done in this study, and therefore it is important to evaluate nitrite reduction by heterotrophic bacteria separate from nitrate.

Nitrite concentration was found to be a limiting factor for nitrite reduction by numerous incomplete-nitrite reducers. Approximately half (13 of 24 isolates) of the incomplete-nitrite reducing isolates exhibited inhibition of nitrite reduction at 1.0 g/L KNO_2 but reduced nitrite effectively at 0.5 g/L KNO_2. This is possibly attributable to the toxicity of nitrite at high concentrations (Mahne and Tiedje, 1995; Piñar *et al.*, 1997) but it needs to be noted that nitrite reduction by the true denitrifiers and exclusive nitrite reducers isolated was predominantly strong, even at 1.0 g/L KNO_2, as compared to that of the incomplete-nitrite reducers (TABLE 4.6). The reasons behind this are very uncertain but it is possible that this phenomenon is enzymatic in nature and somehow linked to the observed phenomenon of inhibition of nitrite reduction in the presence of nitrate. The use of lower concentrations of KNO_3 and KNO_2, in nitrate and nitrite reduction media, is therefore important if nitrate and nitrite reduction is to be accurately assessed in heterotrophic bacteria.

5.4 EXCLUSIVE NITRITE REDUCERS

The fourth group of denitrifying heterotrophic bacteria isolated comprised exclusive nitrite reducing bacteria (FIG. 4.1 and TABLES 4.1 and 4.5). These bacteria exhibited no capacity for nitrate

reduction under anoxic conditions but were capable of efficient reduction of nitrite when grown in nitrite media. A few exclusive nitrite reducing bacteria, from the *Neisseria* and *Flavobacterium* genera, have been reported (*Bergey's*, 1984) but little is known about any possible involvement in activated sludge treatment. Although not isolated in great numbers, the contribution to denitrification by exclusive nitrite reducers needs to be more accurately assessed. It is important that more *in situ* understanding is gained in order to assess the exact contribution of exclusive nitrite reducers to denitrification behaviour in NDBEPR systems.

Although attempted identification of the exclusive nitrite reducers isolated proved somewhat indefinite, *Flavobacterium* spp may possibly be significant genera involved (TABLE 4.5). *F. odoratum* has specifically been characterised as being incapable of nitrate reduction yet capable of nitrite reduction (*Bergey's*, 1984) and was also found to possibly be one of the exclusive nitrite reducers isolated in this study (TABLE 4.5). *S. multivorum* and *P. diminuta* were also possible organisms identified as exclusive nitrite reducers (TABLE 4.5). However, these organisms are not known to be capable of nitrate or nitrite reduction (*Bergey's*, 1984). With the use of classical nitrate reduction media these organisms demonstrate no reduction of nitrate and hence show no nitrite accumulation. With no nitrite accumulation it is therefore not possible to determine nitrite reduction by these organisms and consequently it is possible that mere assumptions have been made regarding their inability to reduce nitrite based on the absence of nitrate reduction. However, identification of the exclusive nitrite reducers isolated needs to be substantiated with the use of more biochemical tests and molecular based assays. Identification of exclusive nitrite reducers is important as not much is known about these organisms and how they contribute to the denitrification kinetics of ND

and NDBEPR processes. Nitrite media is not a specified requirement for screening of denitrification capacity of heterotrophic bacteria and, therefore, more non-denitrifying organisms may in fact be capable of exclusive nitrite reduction.

5.5 NON-DENITRIFIERS

The fifth component of OHOs identified in this study were heterotrophic bacteria incapable of any degree of nitrate or nitrite reduction and hence characterised as non-denitrifiers (FIG. 4.1 and TABLE 4.1). Many heterotrophic bacteria are known not to have the enzymatic capacity to denitrify under any given circumstances (*Bergey's*, 1984; 1986). Given this and the fact that the non-denitrifiers comprised such a large fraction of the OHOs isolated (36.8%) it is inaccurate to ascribe denitrification behaviour in NDBEPR processes to the whole OHO fraction when determining the denitrification kinetics of such systems. Although contributing to COD removal under aerobic conditions, and hence forming part of the heterotrophic active biomass or OHO fraction (Ubisi *et al.*, 1997; Ekama and Wentzel, 1999), the non-denitrifiers do not comprise the active denitrifying OHO fraction and should not be included in denitrification kinetics studies under the broad parameter of OHO fraction. Furthermore, it is possible that some or even many of these non-denitrifiers are in fact PAOs as it needs to be remembered that PAOs are also heterotrophic bacteria. Given that so many non-denitrifiers were isolated, the biochemistry of these organisms needs to be evaluated in order to determine wether their survival in a NDBEPR system is due to fermentative capacity or phosphorus accumulation. However, the fraction of fermenters and PAOs comprising

the non-denitrifiers was not determined in this study and therefore it is uncertain as to the exact composition of the non-denitrifier fraction in relation to function under anoxic/anaerobic conditions. It is therefore important that the OHO fraction be more accurately defined in terms of active denitrifiers when applied to kinetic determinations for NDBEPR processes.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

The OHO fraction within the Darvill NDBEPR process comprises five different functional groups which were defined and characterised as true denitrifiers (bacteria capable of both nitrate and nitrite reduction), incomplete denitrifiers (bacteria that reduced nitrate to nitrite with no further reduction of the nitrite produced), incomplete-nitrite reducers (bacteria capable of both nitrate and nitrite reduction, however, exhibiting inhibition of nitrite reduction by nitrate),exclusive nitrite reducers (bacteria only capable of reducing nitrite) and non-denitrifiers (bacteria not capable of nitrate or nitrite reduction). The non-denitrifiers comprised the largest OHO group isolated (100 isolates) followed by the incomplete denitrifiers (85 isolates), true denitrifiers (56 isolates), incomplete-nitrite reducers (24 isolates) and exclusive nitrite reducers (7 isolates), respectively. The large number of non-denitrifying organisms comprising the OHOs is cause for concern on the accuracy of attributing denitrification behaviour and kinetics to the entire OHO fraction. This group therefore needs to be excluded from the active denitrifying OHO fraction and properly defined in terms of their function in NDBEPR systems. Of the denitrifying OHOs, most were found capable of nitrate reduction while only approximately half were capable of nitrite reduction. Many of the nitrite reducing OHOs were also found to be regulated by some sort of feedback inhibition mechanism restricting nitrite reduction

in the presence of nitrate, therefore, explaining nitrite accumulation observed during denitrification in NDBEPR systems, biofilms and ground water. Identification of the denitrifying OHOs revealed a diverse community of OHOs involved of which *Pseudomonas* spp showed the most significance while *Flavobacterium* spp showed possible predominance amongst the exclusive nitrite reducers. *C. testosteroni*, *E. coli 1*, *P. aerogenes* and *S. marcescens*, which are known incomplete denitrifiers, were found to in fact be true denitrifiers capable of nitrite reduction at lower concentrations of KNO_3 to that currently specified for denitrification screening media. *A. salmonicida 2*, *A. radiobacter*, *C. acidovorans*, *K. pneumonia* ssp *pneumoniae*, *P. cepacia*, *P. maltophilia*, *S. liquificaens* and *S. multivorum*, which are known incomplete denitrifiers, were also found to actually be true denitrifiers possibly due to the use of more nutrient rich media to that currently being employed for denitrification screening. Other known incomplete denitrifiers were also found to be capable of both nitrate and nitrite reduction by using both nitrate and nitrite media for reduction screening. These organisms included *A. calcoaceticus var lwoffi*, *A. radiobacter*, *C. freundii*, *E. coli 1*, *Kluyvera* spp, *P. cepacia*, *P. maltophilia* and *V. fluvialis* which were hence characterised as incomplete-nitrite reducers. Denitrification is therefore a complex, interactive microbial process which is still not fully understood. More understanding is still required of the different microorganisms involved in denitrification as well as the biochemistry and enzymatic regulation of nitrate and nitrite reduction under the various physiological conditions imposed during activated sludge treatment in ND and NDBEPR systems.

6.2 RECOMMENDATIONS

The following areas are recommended for future research into denitrification:

- The kinetics of nitrate and nitrite reduction need to be determined for each of the different functional groups of OHOs seen to be involved in denitrification in order to more accurately establish the contribution of each to denitrification behaviour in ND and NDBEPR systems.
- Nitrate and nitrite reductase enzymes, present in the different functional groups, need to be assessed for a more complete understanding of the biochemistry of denitrification and, more particularly, the inhibition of nitrite reduction in the presence of nitrate.
- *In situ* evaluation (i.e. molecular techniques) of the different functional groups is necessary to establish the predominant groups contributing to denitrification as well as possible shifts in denitrifier population structure under different environmental conditions and process parameters.
- The non-denitrifying fraction of the OHOs need to assessed in terms of their specific function in ND and NDBEPR systems.
- Key OHOs need to be identified from each of the different functional groups of denitrifiers as model organisms upon which the kinetics of nitrate and nitrite reduction can be based and further researched.
- Aerobic denitrification needs to properly understood in order to evaluate its possible impact upon nitrate and nitrite reduction during wastewater treatment.
- New parameters need to be established for the media used for nitrate and nitrite reduction

screening in order to correctly assess, characterise and identify microorganisms contributing to denitrification.

- New identification tools are required for accurately assessing and determining population structure and dynamics of activated sludge.

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APPENDIX 1

CASITONE GLYCEROL YEAST AUTOLYSATE AGAR (CGY)

Preparation of CGY agar as follows (Bux et al., 1994; Bridson, 1995):

Measure out:

5g	Bacto casitone or tryptone L42
10ml	Glycerol
1g	Yeast autolysate
16g	Agar

Add to 1 litre distilled water and dissolve by heating. Adjust pH to 7.2. Autoclave at 121°C for 15 min.

APPENDIX 2

COLORIMETRIC BIOCHEMICAL NITRATE REDUCTION TEST

The nitrate reduction test, as described by Cappuccino & Sherman (1992), as follows:

1) Preparation of reagents:

Solution A

8g Sulphanilic acid

1litre Acetic acid, 5N (1 part glacial acetic acid to 2,5 parts distilled water)

Solution B

5g Alpha-naphthylamine

1litre Acetic acid, 5N (1 part glacial acetic acid to 2,5 parts distilled water)

2) Procedure

Inoculate test organism into nitrate media (Nitrate broth supplemented with 0,1%agar).

After incubation add 5 drops of solution A followed by 5 drops of solution B to cultures.

If a red colouration develops then the nitrate has only been reduced to nitrite.

If no red colouration develops then add zinc powder to culture.

If a red colouration develops then no nitrate was reduced at all.

However if no red colouration develops then nitrate was reduced to nitrite which in turn was reduced to either ammonia, nitrogen gas or a less oxidised form of nitrogen.

APPENDIX 3

GRAM STAIN

The Gram stain procedure, as described by Cappuccino & Sherman (1992), as follows:

Prepare a smear of each organism on a clean glass microscope slide.

Allow smear to air dry and then heat fix by passing rapidly through a flame 2-3 times.

Flood smears with crystal violet and leave for approximately 1 min.

Wash excess stain off with tap water.

Flood stain with Grams iodine and leave for approximately 1 min.

Wash excess stain off with tap water.

Drop by drop, decolourise smear using 95% ethyl alcohol.

Wash smear with tap water.

Counter stain smear with safranine and leave for approximately 45 sec.

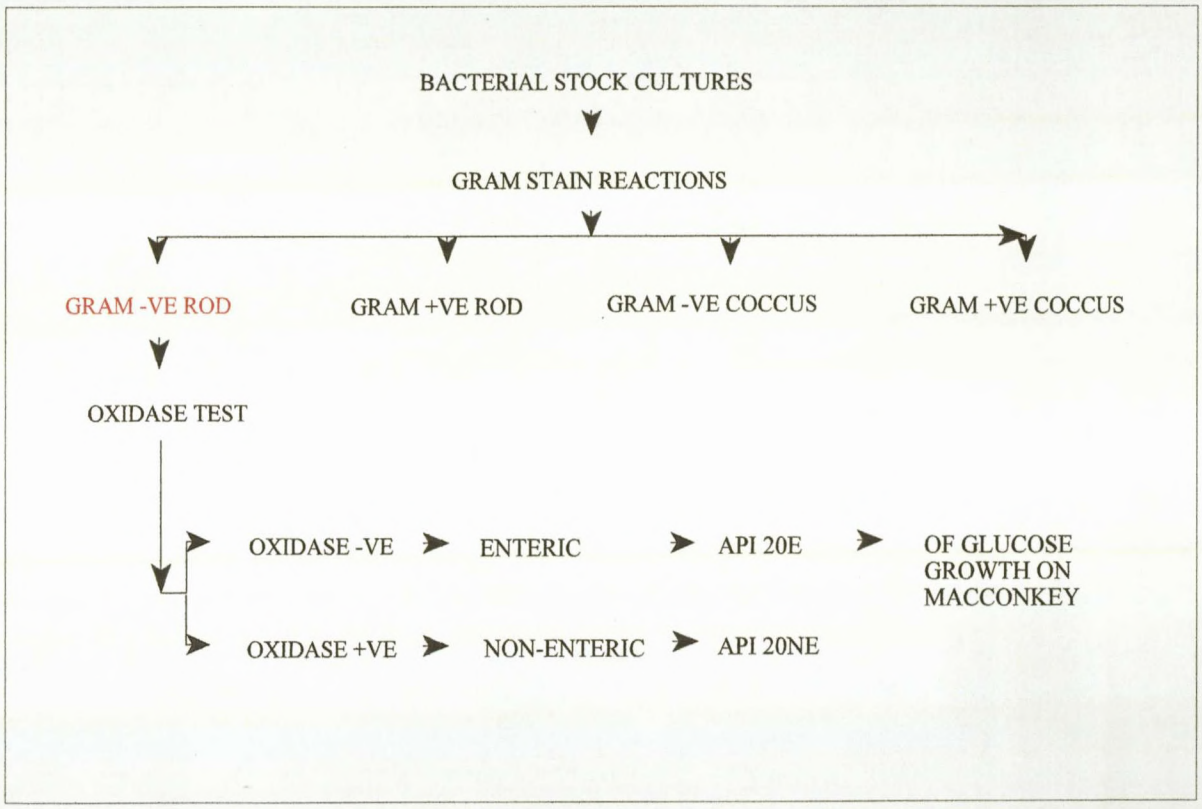
Wash excess stain off with tap water.

Blot slide dry with bibulous paper and view with light microscope under oil immersion.

APPENDIX 4

IDENTIFICATION OF GRAM NEGATIVE RODS

The use of API 20E and API 20NE profile indexes for identification of enteric and non-enteric Gram negative rods, as described by Bux *et al.* (1994), as follows:



APPENDIX 5

OXIDASE TEST

The oxidase test as follows:

1) Reagent

Oxidase reagent OX 70460 (bio Mérieux, France)

2) Procedure

Using an inoculating loop or spatula, smear test organism onto moistened filter paper.

Smear should consist of 1-4 colonies of a 24h old culture.

Add 1 drop of oxidase reagent to smear and allow for maximum reaction time of 1-2 min.

Violet colouration indicates a positive result.

No colouration indicates a negative result.

APPENDIX 6

OF GLUCOSE TEST

The OF Glucose test for determination of oxidative or fermentative metabolism as follows:

Prepare OF basal medium and dispense 10mL allequotes into test tubes.

Sterilise media at 121°C for 15 min.

Allow media to cool to 40-60°C.

Aseptically add filter sterilised glucose solution to media (10% v/v).

Inoculate 2 tubes of media per test organism (24h cultures).

Overlay 1 media with 2-3mL sterile mineral oil for the fermentative test.

The oxidative test must not be overlayed with oil.

Incubate test tubes at 30°C for 24h.

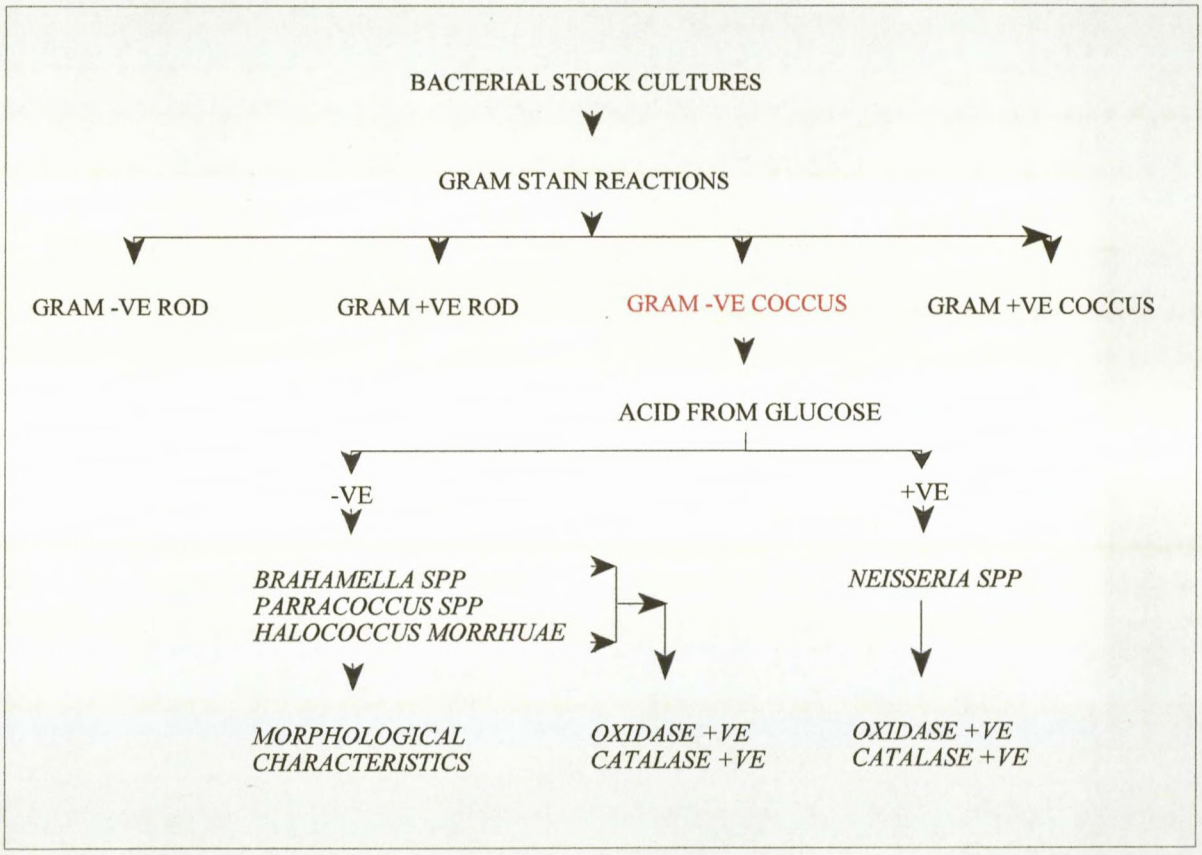
Glucose metabolism is positively indicated by yellow colouration.

Negative results are indicative by no colour change (green) or slight blue/green colouration.

APPENDIX 7

IDENTIFICATION OF GRAM NEGATIVE COCCI

Identification of denitrifying Gram negative cocci, adapted from *Bergeys* (1984) and Cappuccino and Sherman (1992), as follows:



APPENDIX 8

ACID FROM GLUCOSE TEST

Test for acid production from glucose as follows:

Prepare phenol red broth base and dispense 5mL allequotes into bijou bottles.

Sterilise media at 121°C for 15 min.

Assepticlly add 10% glucose solution (v/v) to media.

Inoculate media with test organisms (24h cultures).

Incubate for 24h at 30°C.

Acid production is positively indicated by yellow colouration.

A negative result is indicated by no colour change (red).

APPENDIX 9

CATALASE TEST

The catalase test, according to Cappuccino and Sherman (1992), as follows:

1) Preparation of reagent:

3% Hydrogen peroxide.

2) Procedure

Inoculate test organism onto agar medium and incubate for 24h.

Add 3- 4 drops of 3% hydrogen peroxide to the surface of the culture.

Examine culture for the presence or absence of bubbling or foaming.

Bubbles or foaming is indicative of a positive result while the absence thereof is indicative of a negative result.

APPENDIX 10

COLONIAL MORPHOLOGY ASSESSMENT

Colonial morphology assessment, according to Cappuccino and Sherman (1992), as follows:

Size: Pinpoint, small, moderate, large.

Pigmentation: Colour of colony.

Form: The shape of the colony:

a. Circular: Unbroken peripheral edge.

b. Irregular: Indented peripheral edge.

c. Rhizoid: Rootlike spreading growth.

Margin: The appearance of the outer edge of the colony:

a. Entire: Sharply defined, even.

b. Lobate: Marked indentations.

c. Undulate: Wavy indentations.

d. Serrate: Tooth like appearance.

e. Filamentous: Threadlike, spreading edge.

Elevation: The degree to which colony growth is raised on agar surface:

a. Flat: Elevation not discernable.

b. Raised: Slight elevation.

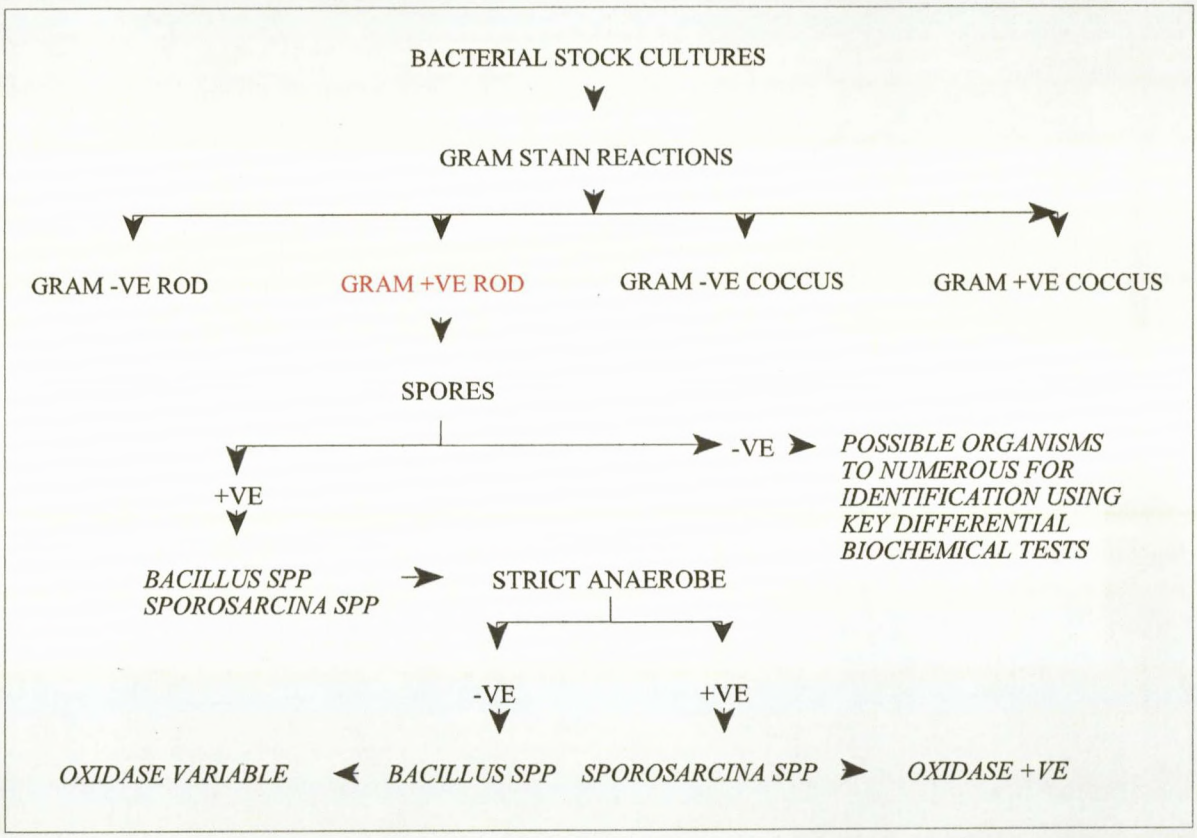
c. Convex: Dome shaped elevation.

d. Umbonate: Raised, with elevated convex central region.

APPENDIX 11

IDENTIFICATION OF GRAM POSITIVE RODS

Identification of denitrifying Gram positive rods, adapted from *Bergeys* (1986) and Cappuccino and Sherman (1992), as follows:



APPENDIX 12

SPORE STAIN (SCHAEFFER-FULTON METHOD)

The spore stain, according to Cappuccino and Sherman (1992), as follows:

Make smears of the test organisms on clean glass slides.

Allow smears to air dry and heat fix.

Flood smears with malachite green and place on a warm hot plate, allowing the preparation to steam for 2-3 min. (do not allow stain to evaporate - replenish if necessary).

Remove slides from hot plate, cool, and wash under running tap water.

Counter stain with safranin for 30 sec.

Wash with tap water.

Blot dry with bibulous paper and examine under oil immersion.

Spores will appear green while vegetative cells will have stained red.

APPENDIX 13

IDENTIFICATION OF GRAM POSITIVE COCCI

Identification of denitrifying Gram positive cocci, adapted from *Bergeys* (1986) and Cappuccino and Sherman (1992), as follows: