THE INDEPENDENT HIGH RATE ALGAL POND AS A UNIT OPERATION IN TERTIARY WASTEWATER TREATMENT

A thesis submitted in fulfilment of the
requirements for the degree of

MASTER OF SCIENCE
(BIOTECHNOLOGY)

of

RHODES UNIVERSITY

by

STEWART JAMES CLARK

November 2001

ABSTRACT

The development of the High Rate Algal Pond (HRAP) as an independent tertiary treatment unit operation for phosphate and nitrate removal is reported. A novel Integrated Algal Ponding System (IAPS) design is proposed for nutrient removal from the effluents of both a conventional domestic sewage treatment plant and from an Advanced Integrated Wastewater Ponding System (AIWPS). The viability of an independently operated HRAP has been identified and termed the Independent High Rate Algal Pond (I-HRAP). A 500 m^2 pilot I-HRAP was operated in such a way as to facilitate the precipitation of calcium phosphate, known to be controlled by pH (greater than 9.4) and resulting in final phosphate levels of less than 1 $mg.L^{-1}$ as PO_4-P. The incorporation of the I-HRAP into a denitrification process was also investigated. Continuously fed column reactors, utilising algal biomass as a carbon source, showed that the heterotrophic bacterial community dominant in the anaerobic algal sludge were denitrifying the nitrate in the feed. It was demonstrated that as the cultures were stressed (using increased nitrate concentrations, anaerobiosis and light starvation) total polysaccharide (TPS) concentrations increased, with a notable increase in the exopolysaccharide (EPS) fraction. These experiments corroborated the hypothesis that harvested microalgal biomass can be manipulated to produce, and release, exopolymeric substances under stress conditions, and which may serve as carbon source for denitrification. In both batch flask studies and in laboratory-scale reactor systems, harvested microalgal biomass from an HRAP was shown to produce exopolymeric substances under stress conditions. Initial high loading-rates of greater than 20 $mg.L^{-1}$ NO_3-N resulted in double the amount of exopolysaccharide production than in flasks with initial low loading-rates (less than 5 $mg.L^{-1}$ NO_3-N). Making use of an upflow anaerobic sludge blanket-type degrading-bed reactor, and an anaerobic, flooded trickle filter (ANTRIC) receiving HRAP effluent, the relationship between denitrification and the changes in polysaccharide content was investigated. This phenomenon has considerable beneficial implications in biological wastewater treatment systems where high nitrate concentration in the final effluent is a potential mitigating factor. Identification of the heterotrophic bacteria active in the denitrification process was attempted. This study presents a first report on the development and operation of the I-HRAP and has been followed by a technical-scale pilot plant evaluation of the process in the tertiary treatment of domestic wastewaters.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

16S rRNA	16S subunit of the ribosomal RNA
907R	reverse primer
AIWPS	Advanced Integrated Wastewater Ponding System
ANTRIC	anaerobic, flooded trickle filter
APS	ammonium persulfate
ARDRA	amplified ribosomal DNA restriction analysis
ASP	Algal Settling Pond
ASP1	Algal Settling Pond receiving HRAP1 overflow
ASP2	Algal Settling Pond receiving HRAP2 overflow
BAT	Best available technology
BF	attached biofilm at bottom section of ANTRIC
BL	loose biological matter at bottom section of ANTRIC
BLAST	Basic local alignment search tool
BNR	Biological nutrient removal
BOD	biological oxygen demand
C/N	carbon nitrogen ratio
Ca	calcium
cd_1-Nir	heme *c*- and heme d_1-containing cytochrome
CNS	central nervous system
COD	chemical oxygen demand
CPS	capsular polysaccharide
CVS	cardio vascular system
dATP	adenine deoxynucleotide triphosphate
dCTP	cytosine deoxynucleotide triphosphate
DEAT	Department of Environment and Tourism
DGGE	denaturing gradient gel electrophoresis
dGTP	guanine deoxynucleotide triphosphate
DH5α	strain of *E.coli* used to make competent cells
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DO	dissolved oxygen
dsDNA	double stranded deoxyribonucleic acid

dTTP	thymine deoxynucleotide triphosphate
EBG	Environmental Biotechnology Group
EBPR	enhanced biological phosphorus removal
EDTA	ethylene diamine tetra-acetic acid
EEC	European Economic Community
EPS	exopolysaccharide
FP	fermentation pit
GC	guanine cytosine
GDWf	Grahamstown Disposal Works final effluent
GM5F	forward primer
HRAP	High Rate Algal Pond
HRAP1	High Rate Algal Pond receiving GDWf
HRAP2	High Rate Algal Pond receiving PFP effluent
HRT	hydraulic retention time
IAPS	Integrated Algal Ponding System
I-HRAP	Independent High Rate Algal Pond
IISD	International Institute for Sustainable Development
MF	attached biofilm at middle section of ANTRIC
Milli-Q H_2O	double distilled water
ML	loose biological matter at middle section of ANTRIC
MPN	most probable number
N	nitrogen
N_2	nitrogen gas
N_2O	nitrous oxide
Nap	periplasmic nitrate reductase
NapA	subunit A of Nap
NapB	subunit B of Nap
nar	gene encoding nitrate reductase
narG	gene encoding α-subunit
NarGHI	major membrane-bound nitrate reductase
narH	gene encoding β-subunit
narI	gene encoding γ-subunit
narY	gene encoding β-subunit in *E. coli*
narZ	gene encoding α-subunit in *E. coli*

NarZYV	*E. coli* membrane-bound nitrate reductase
NH_3	ammonia
NH_4^+	ammonium ion
NH_4-N	nitrogen as ammonia
nir	gene encoding nitrite reductase
*nir*K	gene encoding copper-containing nitrite reductase
*nir*S	gene encoding cd_1 cytochrome-containing nitrite reductase
NO	nitric oxide
NO_2^-	nitrite ion
NO_3^-	nitrate ion
NO_3-N	nitrogen as nitrate
nor	gene encoding nitric oxide reductase
nos	gene encoding nitrous oxide reductase
NO_x-N	sum of nitrate and nitrite as N
o-P	orthophosphate
P	phosphorus
PAO	phosphorus accumulating organism
PCI	phenol : chloroform : isoamyl alcohol
PCR	polymerase chain reaction
PFP Effluent	Primary Facultative Pond overflow
PFP	primary facultative pond
pGEM-T	plasmid
pH	measure of acidity
PHA	polyhydroxyalkanoate
PO_4-P	phosphorus as phosphate
SDS	sodium dodecyl sulfate
SRP	soluble reactive phosphorus
TAE	Tris acetate buffer
TBE	Tris borate buffer
TE	Tris EDTA buffer
TF	attached biofilm at top section of ANTRIC
TGGE	temperature gradient gel electrophoresis
TKN	total Kjeldal Nitrogen
TL	loose biological matter at top section of ANTRIC

TPS	total polysaccharide
UA2SB	Upflow Anaerobic Algal Sludge Blanket
UASB	upflow anaerobic sludge blanket
UV	ultraviolet
VBNC	viable but not culturable
VFA	volatile fatty acid
WCED	World Commission on Environment and Development
WHO	World Health Organization
WRC	Water Research Commission
YT	yeast tryptone

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

I would like to express my appreciation and gratitude to the following people for their input and support during my time at Rhodes University:

- In particular I would like to thank my supervisor, Professor Peter Rose, for his vision, support and guidance throughout this project.
- To my colleague, Len Dekker, for priceless advice and invaluable collaborative work.
- To Dr. Oliver (Doc) Hart, a fountain of wisdom and handyman of note, for his incredible engineering feats both down at the EBG Field Station and in the lab.
- To my labmates in labs 402, 403 and 425, and in particular, Michelle Bowker for introducing me to the wonders of Molecular Microbial Ecology.
- To the postgraduates and staff in the Department of Microbiology, Biochemistry and Biotechnology, Rhodes University, a great bunch of friends I will never forget.
- To my family at His People Christian Church and 1 Bond Street for their unfailing love and encouragement. Guys, you are the best!
- To the Water Research Commission for their generous financial support throughout this project.

CHAPTER ONE

SUSTAINABILITY AND TERTIARY WASTEWATER TREATMENT

1.1 INTRODUCTION

The National State of the Environment Report for South Africa (DEAT, 1999) identified population growth, increased economic activity and intensification of land use practices as factors leading to increased water demand and increasing degradation of freshwater resources. Water resources of the country are under stress and most major rivers have now been dammed to provide water for an increasing population. In many areas wetlands have been converted for other land-use purposes, with more than 50 % of the country's wetlands already lost. Industrial and domestic effluents are polluting the ground- and surface waters, and the resultant changes in habitat have affected the biodiversity of freshwater ecosystems. In addition to the extensive degradation of freshwater resources currently experienced, an estimated overall increase in demand of some 52 % over the next 30 years has been predicted (DEAT, 1999).

The scarcity of water is compounded by pollution of the ground- and surface water resources. The priority pollutants of South Africa's freshwater environment have been identified in the recent White Paper on Integrated Pollution and Waste Management (DEAT, 2000), and include industrial effluents, domestic and commercial sewage, acid mine drainage, agricultural runoff and litter. As many of these sources are spread out across the country, it is difficult to estimate the magnitude of the pollution problem. However rivers in the Western Cape, Eastern Cape, KwaZulu-Natal and the Vaal have major problems with total dissolved solids, and most South African rivers have eutrophication problems (DEAT, 1999).

A growing imbalance between supply and demand of water has focused attention on the potential of water reuse and recycling as a conservation tool, and a means of expanding existing supply. Reuse involves the utilization of an effluent by a user other than the original discharger while recycling is the internal use of such water by the original user. Both processes provide productive ways to reuse wastewater before it is finally dispersed into the environment (Mutembwa, 2000).

In developing countries, particularly those in arid parts of the world, reliable low-cost, low-technology methods are needed for acquiring new water supplies and protecting existing water sources from pollution. The intended water reuse applications dictate the extent of wastewater treatment required, the quality of the finished water and the method of distribution and application. A European Community Commission Directive (91/271/EEC) has declared that "treated wastewater shall be reused whenever appropriate. Disposal routes shall minimize the adverse effects on the environment" (Asano & Levine 1996). It is important however, to recognise that public acceptance of reuse projects is vital to the future of wastewater reclamation, recycling and reuse. The consequences of poor public perception could jeopardize future projects involving the use of reclaimed water (Asano & Levine, 1996).

A Commission on Sustainable Development report by the United Nations Secretary-General to the General Assembly of the Economic and Social Council (UN, 2000) estimated that improper sanitation leads to more than 2 million deaths annually as a result of cholera, diarrhoea and related water-borne diseases. The lack of functional sanitation and proper operation and management of wastewater treatment facilities is a major contributor to contamination of drinking water supplies (Abrams, 1998).

The need for proper management and operation of wastewater treatment plants is thus of paramount importance in developing countries. Wastewater treatment facilities that require little capital outlay, have low-operator costs and are easily repaired must be constructed if global water needs are to be met. Since much of the drinking water available to rural communities is in the form of contaminated river water, simple, low-cost systems are needed for the treatment of abstracted water before use (DEAT, 1999).

1.2 AN ECONOMIC ENVIRONMENT

1.2.1 Cost of Treatment vs Cost of Non-Treatment

By reducing the need for fresh water diversions, water recycling for beneficial uses has the potential to make a substantial contribution to meeting human water needs and to lessen mankind's impact on the environment. However, health and environmental protection measures need to be tailored to suit the local balance between affordability and risk (Anderson *et al.,* 2001). Since economics is the key factor in the choice of treatment philosophy, individual countries have developed different approaches, which vary between high-technology / high-cost / low-risk, and low-technology / low-cost / controlled-risk strategies (Anderson *et al.,* 2001).

Health guidelines for the use of wastewater in agriculture and aquaculture set up by the World Health Organization (WHO, 1989) are intended to allow the introduction of threshold criteria devised from balancing risk and affordability. Development of a single international framework for water recycling guidelines will improve risk management with a better balance between risk and affordability, allow more targeted international research and development, improve public understanding and confidence in water recycling and reduce overall project costs.

These approaches in the formulation of water recycling regulations and guidelines have been developed to provide effective measures to protect against two broad categories of risk : public health risks and environmental risks (DEAT, 2000). Health risks include both microbiological and chemical risks. Provided industrial discharges are properly managed, microbiological risks are usually the dominant risk for non-potable applications of recycled water. While in the past, guidelines have traditionally focused on health protective measures, there has been an increase in recognition of the need to ensure that the impact of the recycled water applications on soils and groundwaters are environmentally sustainable for the long term. There seems to be little value in pursuing reuse of recycled water if the outcome is to render land or groundwater unfit for any further use.

For solutions to be practically implemented they must be both technically feasible and economically viable. Inconsistencies in approach, the absence of a unified scientific approach and increased public concerns about risks sometimes give rise to unnecessarily conservative responses to water recycling projects (Anderson *et al.*, 2001). While doing nothing has high health and environmental costs, activity intended to ensure a very low-risk might be too expensive. This is the situation in most developing countries and in such cases, a controlled-risk approach gives the best balance between risk and cost.

1.3 SUSTAINABILITY AND TECHNOLOGY

Sustainable Development has been defined in the Brundtland Commission Report on Environment and Development, *Our Common Future* (WCED, 1987), as "development that meets the needs of the present without compromising the ability of future generations to meet their own needs." While sustainability mainly concerns environmental issues related to the resilience of ecosystems, rather than any social or economic considerations, sustainable development has a broader context including social and economic objectives (Drummond & Marsden, 1999). Speth (1990) states that technological transformation for environmental sustainability is a process that reduces environmental damage, per unit of added value, fast enough to outpace production increases.

The International Institute for Sustainable Development (IISD) have drawn up a framework identifying four successive generations or stages of "green technology" development (Miller, 1996) :

<u>Stage One</u> : Technology focuses on *remediation*, or cleaning up pollution after it has entered the environment.

<u>Stage Two</u> : Technology focuses on *abatement*, or end-of-pipe cleaning solutions.

<u>Stage Three</u> : Technology focuses on *pollution prevention*, and looks at optimising internal processes to eliminate waste.

<u>Stage Four</u> : *Sustainable technology* seeks to optimise the full life-cycle of a product from raw material to final resting place, not only from an environmental but also from a social and economic perspective.

In the context of sustainable development in the developing world, the World Bank has identified sustainable technology as "technology that lasts" (World Bank, 1992).

1.4 NUTRIENTS IN THE ENVIRONMENT

1.4.1 Eutrophication

Eutrophication is the term applied to increases in the concentration of nutrients (in particular nitrogen and phosphorus) in a water body, and to all the subsequent phenomena: the increase of the primary production by phytoplankton or filamentous algae, the increase of the content of organic matter in water, the marked oscillations of pH values between day and night and the imbalance of the dissolved oxygen values between the surface layers and the deep layers of the water masses, especially in lakes, reservoirs and artificial impoundments. Another effect of eutrophication is the reduction of biodiversity in an ecosystem, due to the ability of some organisms to thrive in these environments, blooming in a short period of time after an input of nutrients into the system (Wu, 1999).

Most of the eutrophication occurring today is closely related to different kinds of human activities. Of note is the generalized use of mineral fertilizers. Extended use of such mineral fertilizers has also produced an increase in the nitrogen and phosphorus in circulation all over the world. Prior to the industrial processes of chemical synthesis and intensive mining, these nutrients were essentially outside of the natural cycles of energy and matter. Now, however, these nutrients have become an active part of these cycles and as such have an impact on the ecosystems into which they are released (Sala & Mujeriego, 2001). The amount of new nitrogen and phosphorus entering the global system each year is equivalent to the mass of these elements contained in the mineral fertilizers to be used in agriculture and landscape gardening. Cultural (or anthropogenic) eutrophication is thus no more than the leakage of nutrients from the predominant production system. Current schemes which usually give priority to primary usage are thus not able to treat the production wastage appropriately and hence a source of pollution goes unchecked. The correct course of action to prevent the spread of this problem all over the world, would be to correct recycling of waste together with a reduction of the consumption of new mineral fertilizers (Anderson *et al.*, 2001).

The present trend in the field of wastewater treatment, enforced by various legislations in different countries as a means of protection for sensitive receiving waters, is to reduce the concentration of nitrogen and phosphorus in the secondary effluents through its elimination in wastewater treatment plants. However, the progressive reduction in the nutrients released at point sources is uncovering another form of pollution which is less apparent and more difficult to combat, namely diffuse pollution or non-point source pollution. Diffuse pollution due to nutrients is mainly caused by rainfall which rinses these nutrient-rich compounds – whether mineral fertilizers or some type of organic waste – which have been applied to the soil. Runoff from rainfall transports these nutrients to the nearest stream, once again disturbing the balance in the natural ecosystem (Sala & Mujeriego, 2001).

1.4.2 Phosphorus in the Environment

Phosphorus plays a critical role in plant nutrition and makes a major contribution to agricultural and industrial development. However, its release to surface waters in agricultural runoff and wastewaters causes serious eutrophication effects and has led to legislation such as the European Union Urban Water Directive (European Community Commission Directive 91/271/EEC), designed to remove phosphorus from domestic and industrial wastewater.

Phosphorus, more than any other nutrient except nitrogen, is apt to be a growth-limiting factor for plants and algae. Phosphate, the anionic form of phosphorus, exists in the soil only in small amounts because it is relatively insoluble and is present only in certain kinds of rocks. Erosion releases phosphate into the environment which is transported by rivers and streams to the ocean where it precipitates. The world's rivers carry 17 million metric tons of phosphorus to the sea every year, half from natural erosion and half from domestic and industrial use and from sewage (Raven & Johnson, 1992).

Anthropogenic activity has increased the availability of phosphate in the environment. Every year, millions of tons of phosphate are added to agricultural lands in fertilisers. In general, four times as much phosphate as the crops require is added each year, usually in the soluble form of superphosphate (calcium dihydrogen phosphate). However, there would seem to be a disproportionate addition of phosphate to crop growth since plants can only take up a certain amount of the phosphorus that is added to the soil. Human sewage represents a potential renewable source of about 1kg of phosphorus per hectare of cultivated land worldwide per year (Raven & Johnson, 1992).

1.4.2 Nitrogen in the Environment

Nitrogen gas constitutes approximately 78 % of the earth's atmosphere. Occurring as paired nitrogen atoms, this form of nitrogen is inert and has very little effect on the ecosystem. Fixed nitrogen in the soil, oceans and biological organisms constitute only about 0.03 % of the above value. Even so, this element has been and continues to be the main limiting nutrient for biomass production on a global scale. The global nitrogen cycle is kept in balance chiefly through two processes : biological nitrogen fixation and denitrification. Figure 1.1 illustrates the main fluxes in the nitrogen cycle. However, through anthropogenic activity, this balance has been radically altered. The predominant reason given for this shift in nitrogen influx and efflux in the biosphere is the invention of the Haber-Bosch process of ammonia synthesis (Gijzen & Mulder, 2001).

Used mainly in the production of nitrogen fertilisers, the Haber-Bosch process has removed a restriction on agricultural productivity. Between 1960 and 2000, the production of nitrogen fertilisers has increased almost tenfold from 1×10^{10} $kg.year^{-1}$ to 9×10^{10} $kg.year^{-1}$ (Gijzen & Mulder, 2001). Current production is equivalent to about 37 % of the total amount of nitrogen input achieved via terrestrial and aquatic biological nitrogen fixation.

Consequences of human impact on the nitrogen cycle include blooms of toxic cyanobacteria (blue-green algae) in freshwater; release of ammonia to the atmosphere through oxidative processes (of eutrophication or biomass degradation) resulting in lowered oxygen levels in affected waterbodies; dramatic decreases in biodiversity; incomplete denitrification resulting in the formation of NO and N_2O, both known to contribute significantly to the global greenhouse effect; and the presence of high levels of nitrate in water which has been associated with methaemoglobinemia and various forms of cancer (Reilly *et al.*, 2000; Shrimali & Singh, 2001).

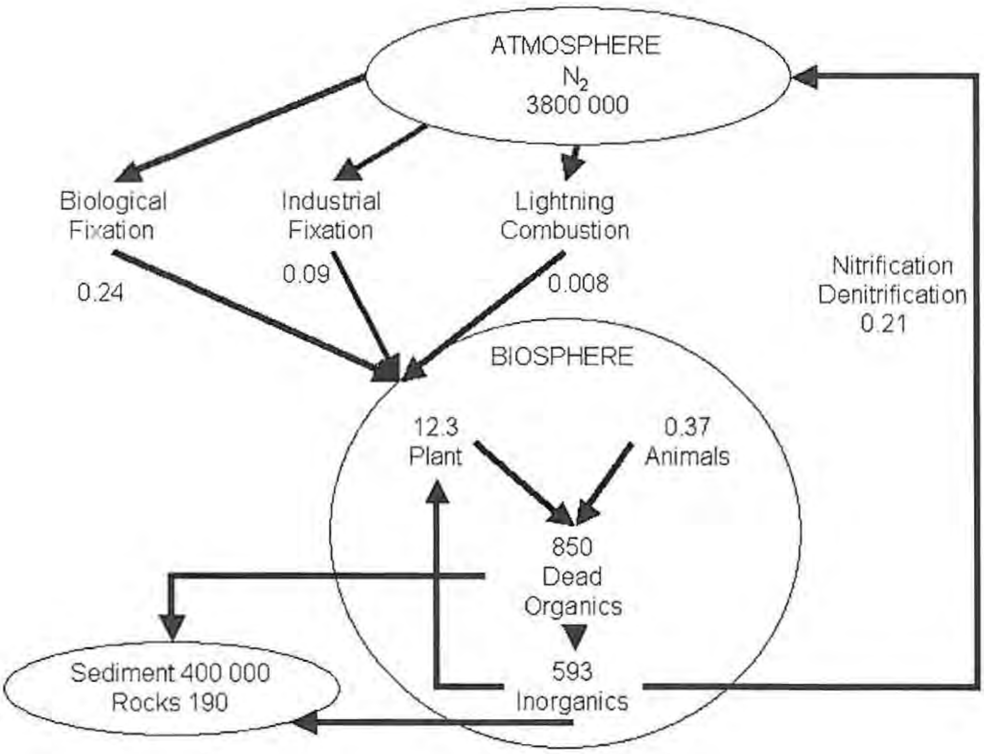


Figure 1.1 **Main fluxes and compartments in the elemental nitrogen cycle. All values x 10^{12} kg.year^{-1} (from Gijzen & Mulder, 2001)**

Nitrate concentrations in drinking water at 10 – 45 mg.L^{-1} or more is considered to be carcinogenic and a causative factor of methaemoglobinemia in infants (blue baby syndrome). The functioning of the central nervous system (CNS) and cardiovascular system (CVS) may also be adversely affected by nitrate-rich water. The World Health Organization (1989) has recommended the limit of 10 mg.L^{-1} NO_3-N for drinking water which has an equivalent value of 45 mg.L^{-1} nitrate.

The concept of the "anthropogenic" nitrogen cycle (Figure 1.2) shows that by a combination of high levels of fertiliser production and inefficient use of fertilisers in man-made plant and animal production systems, the overall result is widespread eutrophication of groundwater, rivers, lakes and coastal waters. Industrial N_2 fixation contributes almost 30 % of the total global nitrogen influx into the biosphere. Only 10 – 15 % of the 9 x 10^{10} kg.year^{-1} of nitrogen fertiliser used in agriculture actually contributes to food protein. The remainder is wasted to air, soil and waterbodies, causing severe health and environmental problems. The human body, in fact, is even less efficient in taking up nitrogen and only 1 – 2 % of food protein is incorporated into the human body while the excess is wasted via domestic sewage. Although many developed nations are now legislating that industry implement tertiary treatment end-of-pipe strategies, the total volume of wastewater that actually receives tertiary treatment on a global scale is probably less than 5 % (Gijzen & Mulder, 2001).

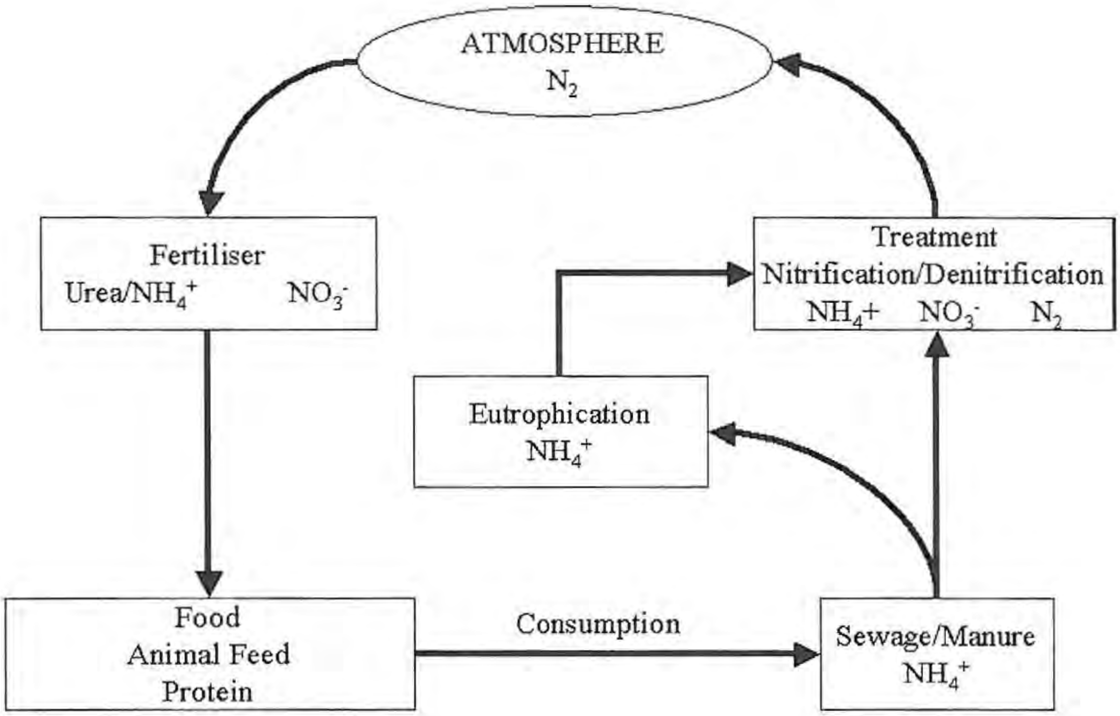


Figure 1.2 The "Anthropogenic" Nitrogen Cycle (from Gijzen & Mulder, 2001)

1.5 TERTIARY WASTEWATER TREATMENT TECHNOLOGIES

Wastewater treatment may be loosely defined as that degree of treatment necessary to restore waste water to the physical, chemical and biological purity of the water originally used to dilute and convey the wastes (Oswald, 1988). Eutrophication and its biological consequences require wastewater engineers to design treatment facilities capable of neutralizing the negative effects of this phenomenon on the environment.

Figure 1.3 shows a generalized overview of wastewater treatment technologies and operations as well as effluent reuse schemes (Mujeriego & Asano, 1999). Based on water quality requirements, any effluent stream can be used as reclaimed water for various beneficial uses.

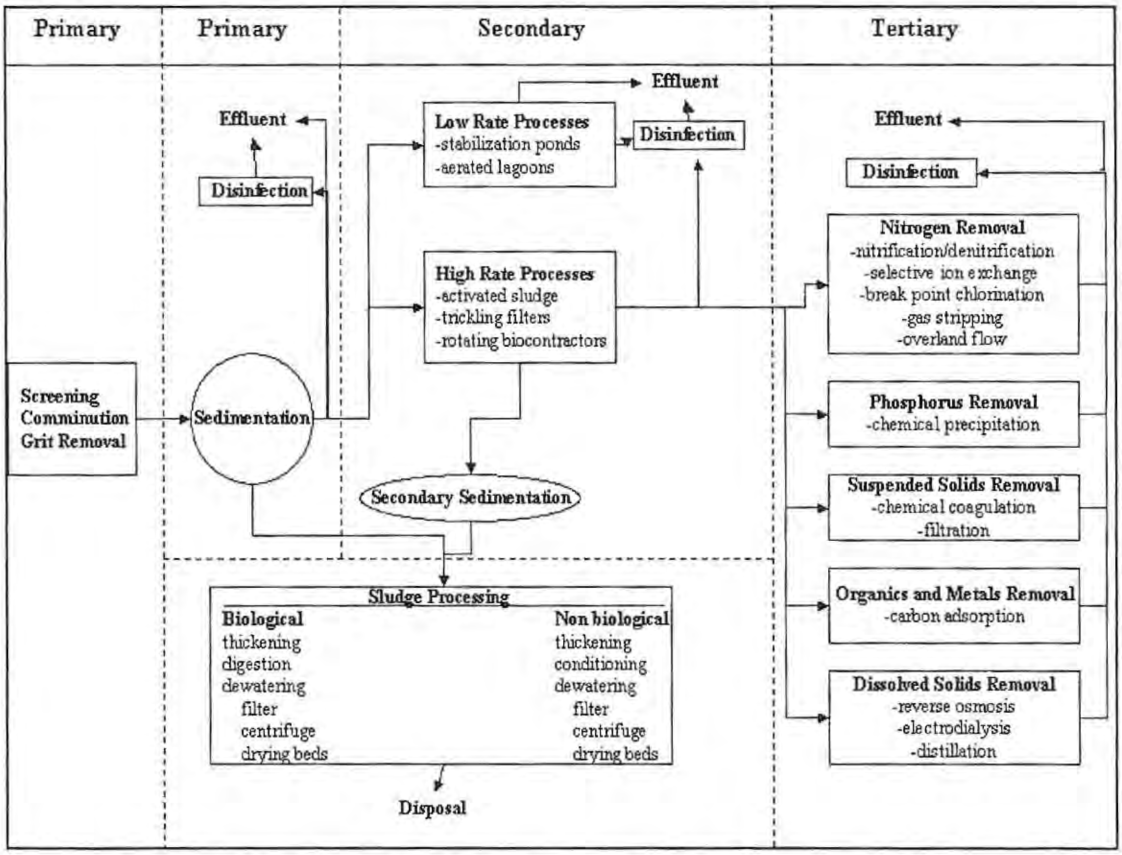


Figure 1.3 **Generalized wastewater treatment processes and operations, and effluent reuse schemes (from Mujeriego & Asano, 1999).**

From Figure 1.3, it can be seen that wastewater treatment can be carried out to the extent required by regulatory agencies for protection of aquatic environments and other beneficial uses. The dashed line represents an increase in treated water quality as required by water reuse. The range of applicable technologies may include: lagoons, wetlands, and natural treatment systems; advanced physical-chemical treatment; advanced biological treatment including biological nutrient removal (BNR); advanced oxidation processes; membrane separation and membrane bioreactors; disinfection technologies; and innovative reactor designs such as sequencing batch reactors and advanced mixing devices (Mujeriego & Asano, 1999).

1.5.1 Phosphorus Removal

In both commercial and industrial wastewaters, phosphorus occurs in two forms: readily digestible orthophosphates and less degradable polyphosphates (Falkentoft *et al.,* 1999). Recent studies on enhanced biological phosphorus removal (EBPR) in activated sludge have shown that the process relies on alternating anaerobic / aerobic cycles (Jenkins and Tandoi, 1991). During the anaerobic stage, phosphate accumulating organisms (PAO) such as *Acinetobacter*, *Pseudomonas* and *Aeromonas* species hydrolyse intracellular polyphosphate to supply energy. In this process, orthophosphate is released into the surrounding aqueous medium. This energy is then used to take up and store short-chain fatty acids or volatile fatty acids (VFA) released from the activated sludge. This carbon is then stored as a polyhydroxyalkanoate (PHA) such as polyhydroxybutyrate and polyhydroxyvalerate (Pereira *et al.,* 1996). In the following aerobic stage, the PAO will degrade the stored organic matter as an energy source while taking up orthophosphate, storing it as polyphosphate. As a result, the nett phosphate concentration decreases during the aerobic stage of the process.

1.5.1.1 Chemical and Biological Precipitation of Phosphate

Chemical precipitation is a very flexible approach to phosphorus removal and can be applied at several stages during wastewater treatment. Primary precipitation involves chemical addition before primary sedimentation and phosphorus is removed in primary sludge. Secondary precipitation involves chemical dosage directly to the aeration tank and phosphate is removed in the secondary sludge. Tertiary treatment seldom occurs and involves the addition of chemicals following secondary treatment and creates an additional, chemical, tertiary sludge. Chemical precipitation typically produces phosphorus bound as a metal salt within the waste sludge, which has the potential for high-value returns as an agricultural additive, although bioavailability studies are as yet inconclusive (Morse *et al.*, 1998). Nevertheless, phosphorus in wastewater and agricultural waste could considerably reduce reliance on non-renewable resources, moving phosphorus towards sustainability (Morse *et al.*, 1998). The disadvantages of phosphorus precipitation in primary settling tanks are high operational costs for chemical addition and increased in sludge quantities of 20 – 25 % above that are usually associated with secondary biological treatment. Estimates for treating 150 million gallons per day for phosphorus removal by precipitation have been as high as US $1,400,000 per annum (Dawson, 1997).

An alternative method for phosphate removal involves pH-controlled chemical precipitation. The pH of water in an HRAP is clearly correlated to photosynthetic activity and as such, the algae indirectly play a central role in the removal of phosphate (Oswald, 1988). In the Advanced Integrated Wastewater Ponding System (AIWPS) configuration, designed and trademarked by Oswald (1991), HRAP containing algal biomass have been shown to remove up to 80 % of the phosphate present in the water. Orthophosphate is the major concern in that it forms the bulk of the phosphate content in wastewater contributing to eutrophication. Soluble orthophosphate will combine with calcium at a pH above 9.5 forming insoluble calcium phosphate precipitate (Moutin *et al.*, 1992). Furthermore, orthophosphate is the only known form of phosphorus used by algae. Assimilation of the o-Phosphate by the algae is however only a secondary phenomenon. Several other authors have also shown that little phosphorus removal occurs in tertiary lagoons where there is little algal growth (Mara and Pearson 1986 ; Maynard *et al.*, 1999). These authors have also found that up to 80 % of phosphorus removal could be accounted for by the precipitation of hydroxyapatite ($Ca_5(PO_4)_3OH$), which is insoluble. The precipitation is known to be controlled by the concentration of H^+ ions over a range of pH 8 – 10 and by the concentration of calcium over a range of 1.25 – 3.75mM and phosphate (Moutin *et al.*, 1992). This method of phosphorus removal is a less expensive method and requires a simpler, low-cost technology. EBPR in activated sludge has often proven ineffective and the costly addition of chemicals has had to be considered.

1.5.2 Nitrogen Removal

Analysis of the total Kjeldal nitrogen (TKN) from conventional wastewater treatment plants has revealed that 85 – 90 % is in the form of ammonia, 10 – 15 % remains in the organic fraction and only a small percentage is present as nitrate (Focht & Chang, 1975). Human wastes contribute nitrogen in significant amounts. Further, fertilizers, animal wastes, municipal and industrial wastes are considered important sources of nitrate contamination in groundwater (Shrimali & Singh, 2001). Focht & Chang (1975) identified the seriousness of the problem of ammonia discharges into receiving waters. They pointed out that the presence of ammonia in surface wastewater must be considered not only in terms of the potential oxygen demand resulting from the conversion to nitrate by nitrifying bacteria, but also of the potential negative impacts of nitrate on soil and the contamination of drinking water.

The primary processes involved in the nitrogen cycle are nitrification and denitrification, both of which are biologically mediated (Figure 1.4).

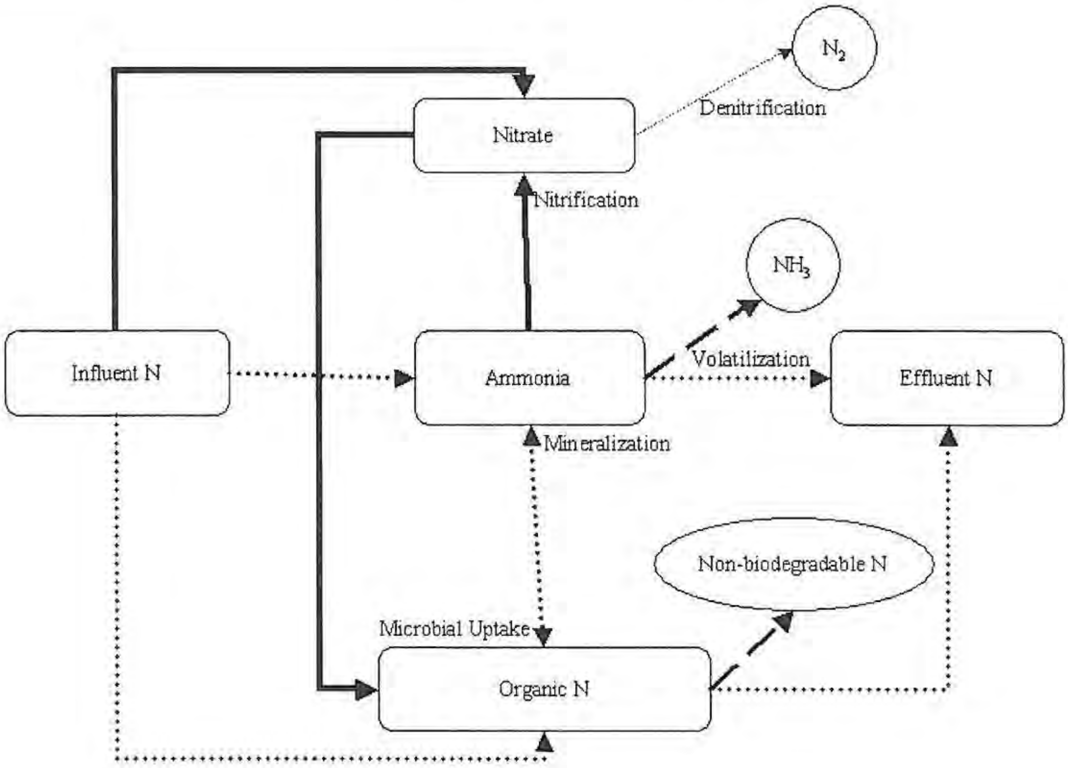


Figure 1.4 The Elemental Nitrogen Cycle

1.5.2.1 Nitrification

Ammonia conversion to nitrates, a process referred to as nitrification, is achieved aerobically in two bacteriologically-mediated steps. In the first step, *Nitrosomonas* converts ammonia to nitrite (Dawson, 1997):

$$55NH_3 + 76O_2 + 109HCO_3^- \rightarrow C_5H_7O_2N + 54NO_2^- + 57H_2O + 104H_2CO_3 \ldots\ldots\ldots\ldots(1)$$

In the second step, *Nitrobacter* converts nitrite to nitrate:

$$400NO_2^- + NH_4^+ + 4H_2CO_3 + HCO_3^- + 195O_2 \rightarrow C_5H_7O_2N + 3H_2O + 400NO_3^- \ldots\ldots(2)$$

1.5.2.2 Denitrification

It is generally agreed that denitrification involves the dissimilatory reduction of one or both of nitrate (NO_3^-) and nitrite (NO_2^-) to the gaseous oxides or molecular nitrogen gas. In this process, the ionic nitrogen oxides act as terminal electron acceptors in the absence of oxygen. The differences between assimilatory nitrate reduction and dissimilatory nitrate reduction are outlined in Table 1.1.

Table 1.1 Comparison of denitrification, assimilatory nitrate reduction and dissimilatory nitrate reduction.

Denitrification	Assimilatory NO_3^- Reduction	Dissimilatory NO_3^- Reduction
Reduction of NO_3^- to gaseous products of nitrogen	Reduction of NO_3^- to NH_4^+ for cellular synthesis	Reduction of NO_3^- to NO_2^-
Coupled with the respiratory electron transport chain	Not coupled with the respiratory electron transport chain	Coupled with the respiratory electron transport chain
Found exclusively among bacteria (usually aerobes)	Common among higher green plants as well as microorganisms	Found to occur among many species of bacteria (mostly facultative anaerobes)

Denitrification is of great potential in waste treatment, since it can permanently remove excess combined nitrogen from local environments by completing the natural nitrogen cycle. In the last decade the role of N_2O in contributing to destruction of the protective ozone layer and in warming of the planet was recognized. Denitrification completes the global nitrogen cycle (Tiedje, 1988).

Denitrification occurs in the presence of nitrogen-free organic compounds such as carbohydrates, cellulose and volatile fatty acids. These substances are oxidized by the oxygen liberated from nitrates, providing energy for the reaction. As such, denitrification can take place in the absence of free oxygen and can be expressed by the following generalized reaction:

$$4KNO_3 + 5C_{org} \rightarrow 2K_2CO_3 + 3CO_2 + 2N_2 \quad \text{....(3)}$$

In environments where organic matter is limiting, autotrophic denitrification can occur with the bacteria utilizing sulphur for energy:

$$10NO_3^- + 11S^0 + 4.1HCO_3^- + 0.5CO_2 + 1.71NH_4^+ + 2.54H_2O \rightarrow$$
$$0.92C_5H_7NO_2 + 11SO_4^{2-} + 5.4N_2 + 9.62H^+ \quad \text{....(4)}$$

Table 1.2 shows a summary of stoichiometric relationships of heterotrophic denitrification with various carbonaceous substrates.

Table 1.2 **The Stoichiometry of heterotrophic denitrification utilising various carbon substrates.**

Substrate	Stoichiometric equation
Ethanol	$5C_2H_5OH + 12NO_3^- \rightarrow 10HCO_3^- + 2OH^- + 9H_20 + 6N_2$
Methanol	$1.08CH_3OH + NO_3^- + H^+ \rightarrow 0.065C_5H_7NO_2 + 0.467N_2 + 0.76CO_2 + 2.44H_2O$
Acetic acid	$5CH_3COOH + 8NO_3^- \rightarrow 8HCO_3^- + 2CO_2 + 6H_2O + N_2$
Cellulose	$5(C_6H_{10}O_5)_n + 24nNO_3^- \rightarrow 6nCO_2 + 13nH_20 + 12nN_2 + 24nHCO_3^-$
Methane	$5CH_4 + 8NO_3^- + 8H^+ \rightarrow 4N_2 + 5CO_2 + 14H_2O$
Glucose	$C_6H_{12}O_6 + 2.8NO_3^- + 0.5NH_4^+ + 2.3H^+ \rightarrow 0.5C_5H_7NO_2 + 1.4N_2 + 3.5CO_2 + 6.4H_2O$

Conventionally, liquid carbon sources like ethanol or acetate are injected into raw water. Most of the published research on wastewater denitrification process indicate the use of methanol, ethanol or acetic acid as the organic carbon sources. Sugar or glucose syrup have also been used as a carbon source for heterotrophic denitrification (Focht & Chang, 1975).

Matějů *et al.*, (1992) examined the stoichiometric relationships of heterotrophic bacterial denitrification using methanol as a carbon source. In the first two steps, the energy reactions may be written as follows:

$6NO_3^- + 2CH_3OH \rightarrow 6NO_2^- + 2CO_2 + 4H_2O$ ....(5)

$6NO_2^- + 3CH_3OH \rightarrow 3N_2 + 5CO_2 + 3H_2O + OH^-$ ....(6)

The above reactions however refer to ideal conditions which are very seldom found in nature. For straightforward nitrate dissimilation, the above reaction dynamics would apply. However, additional methanol is required for deoxygenation (equation 7) and cell synthesis (equation 8) and the following equations have been determined (Matějů *et al.*, 1992):

$O_2 + 0.93CH_3OH + 0.056NO_3^- + 0.056\ H^+ \rightarrow$
$0.056C_5H_7NO_2 + 0.65CO_2 + 1.69H_20$ ....(7)

$14CH_3OH + 3NO_3^- + 4H_2CO_3 \rightarrow 3C_5H_7NO_2 + 20H_2O + 3HCO_3^-$ ....(8)

On the basis of these reactions, the following overall nitrate-removal reaction formula for open systems was developed (Matějů *et al.*, 1992) :

$NO_3^- + 1.08CH_3OH + H^+ \rightarrow 0.065C_5H_7NO_2 + 0.467N_2 + 0.76CO_2 + 2.44H_2O$.......(9)

It has further been determined that the relationship between the amount of denitrified nitrogen and the concentration of the carbon source is linear. With this in mind, the following formula for determining the required concentration of carbon source was developed (Matějů *et al.*, 1992):

For methanol

$C_{met} = 2.47NO_3^--N + 1.53NO_2^--N$..(10)

For ethanol

$C_{et} = 2.00NO_3^--N + 1.28NO_2^--N$..(11)

For acetic acid

$C_{ac} = 3.30NO_3^--N + 2.08NO_2^--N$..(12)

Denitrifiers are essentially facultative anaerobic bacteria which have the alternative capacity to reduce nitrogen oxides when oxygen becomes limiting. These heterotrophic bacteria therefore do not require strict anaerobic conditions and the aerobic respiratory growth of the culture will, after consuming the available oxygen, shift to a denitrifying metabolism. While NO_3^- is the primary electron acceptor in the denitrifying pathway, NO_2^- and N_2O can be substituted, although lower NO_2^- concentration ranges may become necessary as some strains of denitrifiers are inhibited by concentrations as low as 2 to 3 mM. A population of denitrifiers in an ecosystem may be evaluated by enumeration, in media with nitrate as an electron acceptor under anaerobic conditions, and by assessing denitrifying activity. Denitrifying activity is most frequently assayed by the acetylene inhibition method (Stewart *et al.,* 1967). The final step in the denitrification pathway is the reduction of nitrous oxide to dinitrogen. Acetylene used as 10 % of the headspace gas is almost always effective in inhibiting N_2O reduction while not causing toxicity to the growing culture. As a result, the N_2O in the medium can be assayed over time giving a good estimate of denitrification rates. This method has been used to measure denitrification activity in pure cultures, activated sludge and sediments (Etchebehere *et al.,* 2001). Most denitrifiers have the ability to complete the entire denitrification pathway, NO_3^- → N_2. Some strains however lack the ability to reduce NO_3^- → NO_2^- and N_2O → N_2. Only a small number of denitrifiers are known to lack the NO_2^- → N_2O step.

Possible controlling factors observed in denitrification include pH, water temperature, dissolved oxygen, hydraulic retention time, organic substrate and attachment media (Reilly *et al.,* 2000). Denitrification rates are optimal at temperatures between 20 – 25°C, within a pH range of 7.0 - 7.5. Denitrification can be enhanced by the presence of aquatic macrophytes which provide a carbon source as a substrate for bacterial attachment and intermittently anoxic microniches. In natural wetlands, sediment detritus is the main source of organic carbon for denitrification (Reilly *et al.*, 2000).

1.5.3 Conventional Denitrification in Tertiary Wastewater Treatment

Sharma & Ahlert (1977) detail three schemes which can be adopted for conventional nitrogen removal. The first entails separate stages for carbon oxidation (to reduce the carbon-to-nitrogen or C/N ratio for favourable nitrification), oxidation of ammonia to nitrate or nitrite and reduction of nitrate to nitrogen. Such a process is outlined in Figure 1.5.

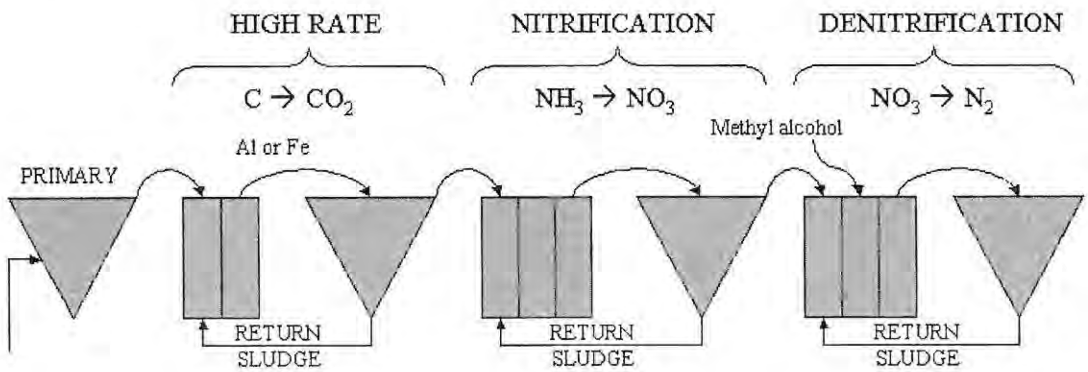


Figure 1.5 Schematic of a three-stage system for nitrogen removal (after Sharma & Ahlert, 1977).

The second scheme involves a combined process for nitrogen removal. In this category, carbon oxidation and nitrification are carried out simultaneously in the same reactor and denitrification in a second reactor. A two-stage system for nitrogen removal is shown in Figure 1.6.

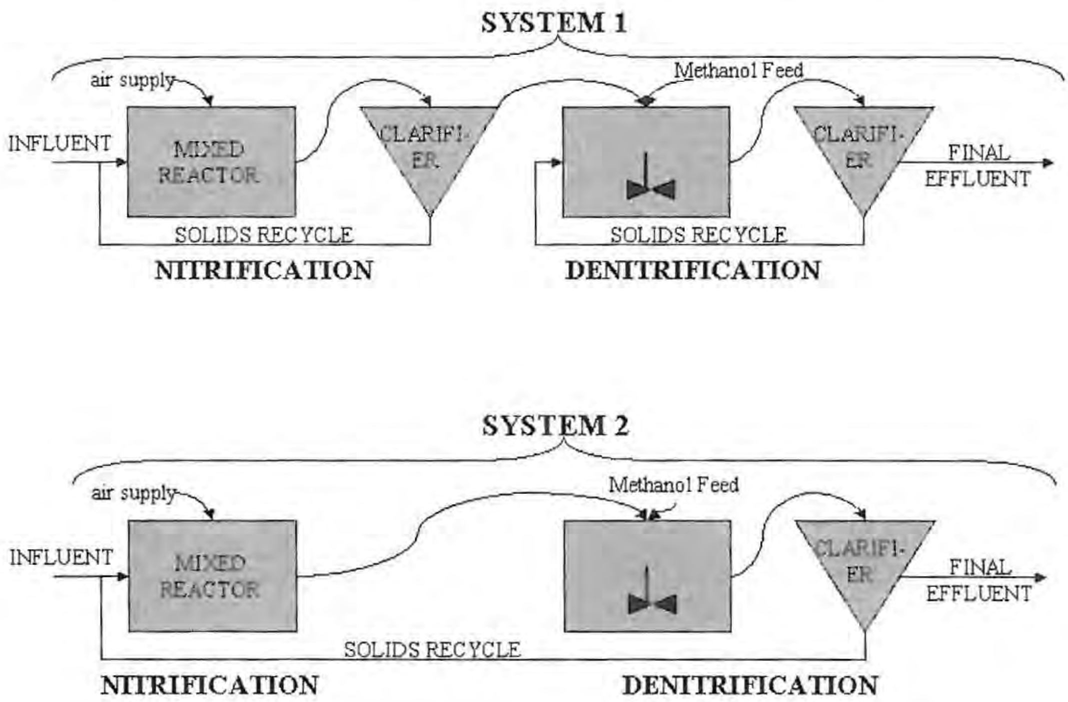


Figure 1.6 **Schematic of a two-stage system for nitrogen removal (after Sharma & Ahlert, 1977).**

The third scheme involves carbon oxidation, nitrification and denitrification in one reactor. This reactor-type has found favour with many researchers and has successfully been implemented in a number of conventional wastewater treatment plants. A recent example of this type of scheme was developed as an intermittently aerated biofilm airlift reactor (van Benthum *et al.,* 1998), as illustrated in Figure 1.7.

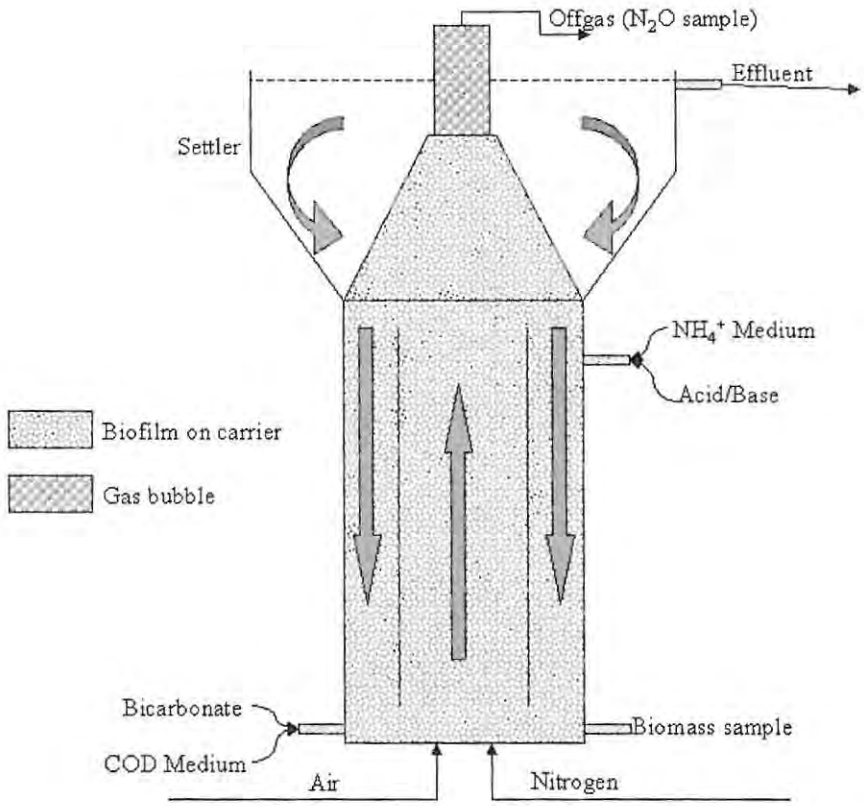


Figure 1.7 Single-stage carbon nitrogen removal scheme (after van Benthum *et al.*, 1998), combining carbon oxidation, nitrification and denitrification in a single reactor.

The conventional processes (coagulation, filtration, chlorination, UV and ozone treatment) applied for water potability are not useful for the elimination of the nitrate ion from wastewater. Techniques capable of nitrate elimination are ion exchange, reverse osmosis and electrodialysis. However, the application of these processes has been limited as they are relatively expensive and merely displace nitrate into a concentrated brine that may pose a disposal problem. Thus, there is considerable interest in developing alternative treatment techniques to remove nitrate from contaminated streams in a cost-effective manner. Biological denitrification reduces nitrate to innocuous nitrogen gas, rather than to ammonia which exerts an adverse impact on drinking water by combining with chlorine to form chloramines and nitrogen trichloride (both agents causing negative organoleptic properties). Furthermore, biological denitrification is the only process that directly targets nitrate and does not shift the concentration of other ions. Biological treatment thus represents a cost-effective technique for removing the nitrate ion from contaminated water (Shrimali & Singh, 2001).

1.6 INTEGRATED ALGAL PONDING SYSTEMS

Integrated Algal Ponding Systems (IAPS) have been shown to be an economical and reliable option in management of wastewaters (Oswald, 1991) and can be considered an appropriate technology in rural communities. The use of algae in wastewater tertiary treatment facilities to eliminate nutrients offers a sustainable alternative to conventional treatment systems and provides several advantages. Martínez *et al.*, (2000) noted some advantages relating to IAPS technology:

- It rests on the principles of natural ecosystems and therefore is not environmentally dangerous;
- causes no secondary pollution if the biomass produced in reused;
- enables the efficient recycling of nutrients contained in the secondary effluents, since the microalgae are highly effective not only at using the inorganic N and P for growth but also at purifying the waste by producing oxygen and removing heavy metals and xenobiotic substances.

The AIWPS, the layout of which is shown in Figure 1.8, consists of a series of ponds, each a unit in itself, in optimal sequence. Developed at the University of California, Berkeley, by Oswald (1991), the system has been tailored for specific use in various countries around the world, preparing wastewater for safe discharge into the environment.

KEY TO NUMBER ON FIGURE BELOW

1. SCREENING GRIT REMOVAL	7. PADDLE WHEEL MIXER	13. ALGAL HARVEST
2. DISTRIBUTER	8. HIGH RATE POND	14. LOW-LEVEL TRANSFER
3. FERMENTATION PITS	9. HIGH-LEVEL TRANSFER	15. MATURATION POND
4. FACULTATIVE POND	10. ALGAE SUBSIDENCE CHAMBERS	16. HIGH-LEVEL TRANSFER
5. OXYGENATED WATER	11. ALGAE SETTLING PONDS	17. WATER REUSE
6. LOW-LEVEL TRANSFER	12. SETTLED ALGAE RETURN	18. SUPPLEMENTARY AERATION

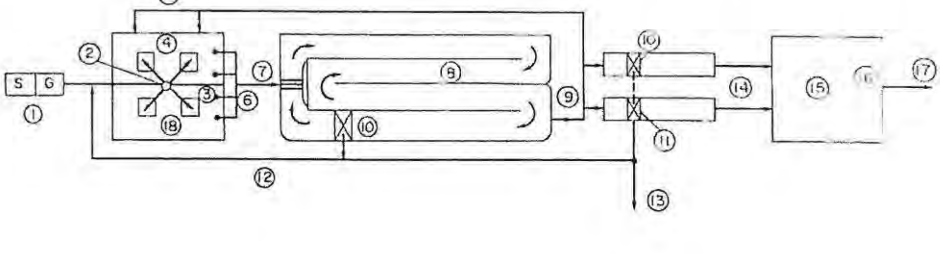


Figure 1.8 **The Advanced Integrated Wastewater Ponding System (Green *et al.*, 1996).**

Several types of ponds exist in the arena of wastewater treatment: deep, unmixed facultative ponds; deep, mechanically mixed and aerated ponds; shallow, wind-mixed ponds; and very shallow high-rate oxidative ponds. Using the AIWPS model, the Environmental Biotechnology Group (EBG) at Rhodes University built an IAPS pilot plant in Grahamstown as part of Water Research Commission (WRC) project K5/651 'Appropriate Low-cost Sewage Treatment Using the Integrated Algal High Rate Oxidation Ponding Process" (Rose *et al.*, 2001c). The plant was officially opened in April 1997 by the then minister of Water Affairs and Forestry, Professor Kader Asmal. This facility consists of a fermentation pit (FP) for early removal of biodegradable matter and removal by settling of parasitic helminth eggs, a primary facultative pond (PFP) for further reduction of chemical oxygen demand (COD), a high rate algal pond (HRAP) for tertiary treatment and finally an algal settling pond (ASP) for removal of excess algal flocs. Figure 1.9 shows some design and operational aspects of the anaerobic components of the IAPS.

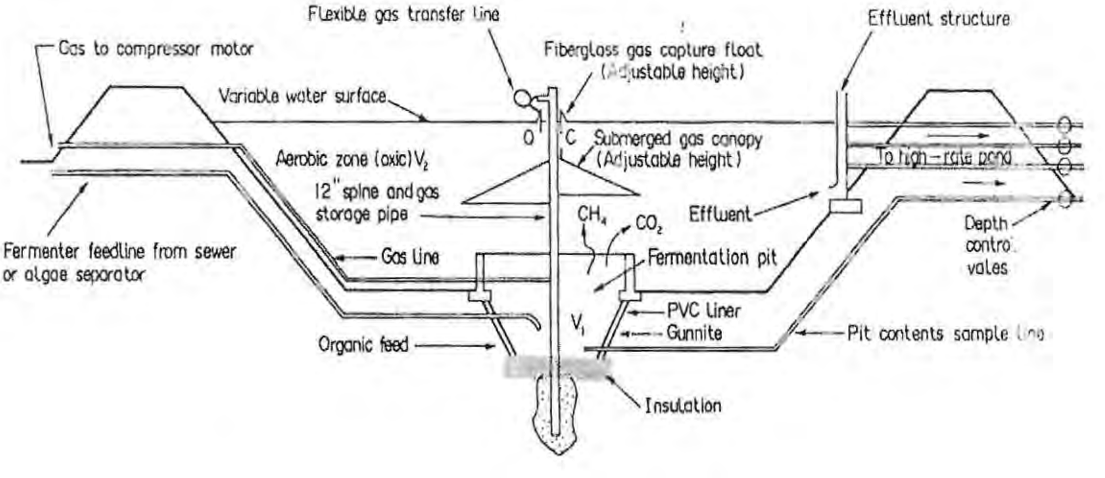


Figure 1.9 **An operating scheme showing the importance of the fermentation pit and facultative pond and their respective functions (Oswald, 1991).**

1.6.1 The Role of Algae in Tertiary Wastewater Treatment

While the IAPS is indeed an integrated system, making use of various low-cost, low-technology components, by far the most crucial component is the HRAP. Microalgae have been noted as being opportunistic phototrophs, exploiting recent anthropogenic alterations of aquatic environments. Nutrient enrichment, termed 'eutrophication', as observed in wastewater treatment systems, provides an ideal niche for micro-algae and cyanobacteria. These and other micro-organisms, including the heterotrophic eubacteria, have been shown to play an active part in the degradation and recycling of the nutrients responsible for eutrophication, namely N and P, into potentially valuable biomass (Oswald, 1988). Nurdogan & Oswald (1995) observed that the role of microalgae in the purification process of an HRAP may be direct and/or indirect. The direct role involves photosynthetic oxygen production for the growth of aerobic bacteria and nutrient uptake while the indirect role involves elevated pH levels of the wastewater, resulting in phosphate and metal precipitation, ammonia stripping and disinfection.

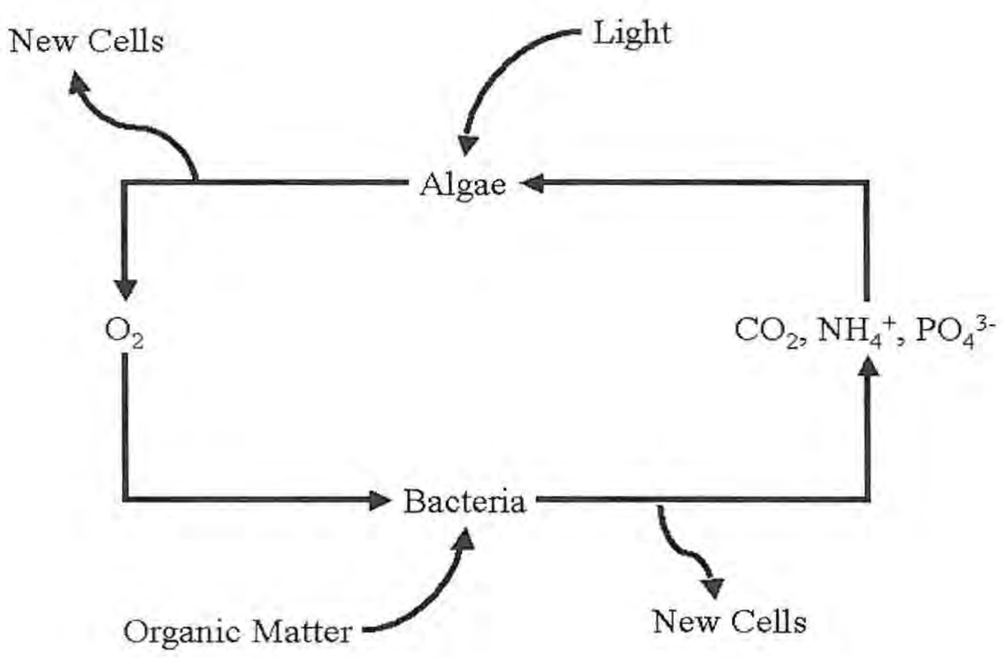


Figure 1.10 **Generalized flow of symbiosis between bacteria and algae.**

During active photosynthesis, the pH of a healthy culture of algae in an HRAP on a typical summer afternoon may be as high as 11 and remains around 9 during winter. Orthophosphate and ammonia can both be removed through precipitation of insoluble complexes such as $CaNH_4PO_4$ and $MgNH_4PO_4$. The solubility of hydroxyapatite ($Ca_5(PO_4)_3OH$), the predominant form of orthophosphate found in HRAP, is so low that even at pH values as low as 9, a large fraction of orthophosphate will be removed provided sufficient calcium ions are available in solution.

In the majority of IAPS, nitrogen removal is facilitated primarily via heterotrophic nitrification and denitrification, bacterial and algal uptake and outgassing of NH_3 at high pH in the HRAP. Oswald (1995) indicated that for algal growth, nitrogen must be available in the form of either ammonium (NH_4^+) or nitrate (NO_3^-). He went on to add that in temperate climates, advanced ponding systems can remove virtually all biologically available nitrogen. Ammonia exists in aqueous solution as either NH_4^+ ion or NH_3, as determined by the pH and temperature of the surrounding medium. Above neutral pH values, the equilibrium is shifted in favour of NH_3 gas. A complete conversion of NH_4^+ ion to NH_3 gas is possible around pH 11. The removal of NH_3 gas by air stripping reaches about 90 % during the summer months at pH 10.5 and ambient temperatures of around 20°C. A design note for HRAP on this issue is that at high pH, the ammonia may be toxic to bacteria. It would therefore be necessary to ensure a pH gradient from surface to bottom in the raceway, keeping the algal-bacterial communities in suspension to prevent bacteria from being exposed to continuously high pH and ammonia concentrations (Nurdogan & Oswald, 1995).

The processes for N and P removal in IAPS have been studied and reported widely in the literature (Oswald, 1991; Green *et al.,* 1996). The mechanisms involved include anoxic denitrification in the facultative pond, algal uptake and ammonia stripping in the HRAP, cell removal in the algal settling pond and final particle separation by flotation and filtration (Green *et al.,* 1996). From the arguments put forward by Green *et al.,* (1996) on denitrification reported to take place in the facultative pond, it is evident that the mechanism remains uncertain.

The use of organic molecules produced in microalgal photosynthesis as a carbon source for a freestanding denitrification unit operation has not been reported. The release of photosynthate by the microalgae has however been the subject of extensive reports.

1.6.2 Photosynthates and Denitrification

The liberation of extracellular polymers by microalgae has been identified as having important ecological effects as early as 1955 (Fogg & Westlake, 1955; Hellenbust, 1965). Algal polysaccharides in particular have been identified as having tremendous commercial application (Myklestad, 1995). As such, research has been done to understand exopolysaccharide (EPS) production and experimentally determine the conditions required for optimum production (Vincenzini *et al.*, 1990; Fulda & Mikkat, 1998). Much of the work has centred around marine algae of the group Rhodophyta, although emphasis is shifting to freshwater microalgae such as *Anabaena*, *Cyanospira* and *Spirulina* (De Philippis *et al.*, 1996).

These polysaccharides most often consist of a glycogen-like polyglucan which acts as an ideal storage compound, easily utilizable as a carbon or energy source. Alternatively, the polysaccharide may be synthesized as an exopolysaccharide and released into the surrounding medium. This can then be incorporated into the cell wall and act as a sheath or capsule for protection or exuded and wasted into the surrounding medium to serve as an available carbon source for heterotrophic bacteria (De Philippis *et al.*, 1996).

1.6.3 Algal Stress Physiology

Studies have been carried out investigating the response of exopolysaccharide-producing algae to changes in their environment. In experiments conducted using the red, unicellular microalgae *Porphyridium* and *Rhodella* to determine the response of these algae to changes in environmental nutrient conditions, Adda *et al.*, (1986) and Arad *et al.*, (1992) clearly showed that, under conditions of nitrate starvation, both cell-wall and internalised polysaccharide content increased. Their findings correlate with previous results obtained using the halophilic microalga *Dunaliella primolecta* (Thomas *et al.*, 1984). Furthermore, Arad *et al.*, (1992) showed that nitrate deficiency in *Rhodella reticulata* caused enhanced production of cell-wall polysaccharide as well as effecting the distribution of the polysaccharides between bound and soluble fractions. They concluded that the response to nitrate depletion can be attributed to the inhibition of protein synthesis. This affects cell division which in turn affects polysaccharide production.

De Philippis *et al.*, (1996) reported the response of an exopolysaccharide-producing, heterocystous cyanobacterium to changes in metabolic carbon flux. In this study, carbon flux was altered by exposing the cultures to nitrogen-deficient media (or media with nitrogen assimilation inhibitors) or by adding glyoxylate (known to stimulate carbon metabolism). The results corresponded to previous studies and they were able to show that the amount of carbon diverted from the synthesis of nitrogenous compounds is preferentially channelled toward formation of reserve compounds which become re-usable once nitrate is reintroduced or inhibition is removed. They were further able to show that the addition of glyoxylate resulted almost exclusively in the overproduction of exopolysaccharide.

Lama *et al.*, (1996) examined the effect of growth conditions on endo- and exopolymer biosynthesis in *Anabaena cyclindrica*, with particular interest in the occurrence and characterization of polyhydroxybutyrate. As an apparent aside, they observed the occurrence of an exopolysaccharide during autotrophic growth and they report that production was influenced by the presence of a nitrogen source. However, unlike previous studies, in media containing nitrate, the EPS content was 7.5-fold higher than in media with the omission of a nitrate source. Interestingly, they further noted that the addition of an outside carbon source (acetate, valerate, glucose or citrate) caused a decrease in EPS concentration.

Enhanced exudation of organic compounds under nutrient limiting conditions may be explained by an "overflow" mechanism. This theory is based on the idea that algae, which grow under N-limitation, exude fixed organic material because they are unable to transform it into biomass. An alternative hypothesis is called the "leakage" hypothesis. According to this theory, a major part of the exudation can be explained as a passive diffusion of low-molecular weight organic compounds through the algal cell membrane (Fulda & Mikkat, 1998). Exudate substances such as vitamins or hormones may be used as nutrients (trophic link) and/or may stimulate heterotrophic growth (Fulda & Mikkat, 1998).

1.7 THE PROPOSAL

The use of IAPS has been studied for wastewater treatment in the tannery, winery, distillery and abattoir industries as well as in domestic sewage facilities by the EBG at Rhodes University (Rose *et al.*, 2001a; Rose *et al.*, 2001b; Rose *et al.*, 2001c). Although nutrient removal in these systems has been reported with some understanding of N and P removal in HRAP, the HRAP as a standalone unit operation in tertiary treatment has not been previously reported. Given the widely identified need for polishing of low-strength raw water and tertiary treatment of domestic sewage outfall water across the developing world, the potential was identified to apply N and P removal in an I-HRAP. The underlying principles supporting such an application formed the substances of the study reported here.

Grahamstown Disposal Works was identified as having a serious problem with regards to effluent quality. The final effluent being released into the Blaaukrantz River was shown to fall outside of water quality guidelines set up by the Department of Water Affairs and Forestry with regard to nutrient status (N and P). The opportunity presented itself at the IAPS pilot plant located at the EBG experimental field station, to examine the possibility of using an I-HRAP as a tertiary treatment step, treating final effluent from the Grahamstown Disposal Works.

Based on previous observations of the IAPS process at the EBG pilot plant, the HRAP has been shown to be able to remove the nutrients phosphorus and nitrogen from domestic wastewater. In addition to polishing wastewater, the HRAP has been observed to yield sufficient amounts of algal biomass, a potential carbon source for subsequent biological reactions. However, no studies have been conducted on the HRAP as a separate unit operation for tertiary treatment of low-strength wastewater. The question thus presented itself : Would an I-HRAP be able to treat low-strength wastewater or river waters? When receiving effluent with low COD, would the algal biomass flourish, effecting pH-mediated nutrient changes and would there be sufficient available carbon to support nitrification, denitrification and phosphorus removal?

1.8 HYPOTHESIS

A freestanding independent high rate algal pond may be manipulated to effect nitrogen and phosphorus removal in tertiary wastewater treatment.

1.9 RESEARCH OBJECTIVES

1. Investigate the use of an I-HRAP as a freestanding unit operation in the tertiary treatment of low-strength final effluent from a conventional sewage treatment facility;
2. Examine the potential for phosphorus removal in an I-HRAP;
3. Examine the potential for nitrogen removal in an I-HRAP;
4. Examine the use of microalgae as a carbon source for nitrogen removal.

CHAPTER TWO

THE INDEPENDENT HIGH RATE ALGAL POND

2.1 INTRODUCTION

High Rate Algal Ponds (HRAP) are low-cost, low-technology oxidation ponds incorporated into the Advanced Integrated Wastewater Ponding System (AIWPS) designed by Oswald (1991). These ponds were based on the concept of algal biotechnology, whereby it may be possible to take advantage of algal metabolism and physiology to meet technological needs.

The opportunity arose at Rhodes University's Environmental Biotechnology Group Experimental Field Station to compare tertiary treatment in final effluent streams from a conventional wastewater treatment plant and from an IAPS plant. In this study, elements of tertiary treatment examined included the reduction of nitrate, ammonia and phosphate concentrations in the wastewater. Algal-based systems have been shown to be successful in controlling pathogen removal (Awuah *et al.,* 2001). Although preliminary data indicated that the HRAP was effective in reducing faecal bacteria and virus particles in the wastewater by elevated pH levels and solar UV irradiation, the results of that study are not included in this report. The stand-alone HRAP was termed the Independent High Rate Algal Pond (I-HRAP) as it was operated apart from the integrated system of the IAPS.

As is the case with all domestic, commercial and industrial wastewater treatment facilities, the composition of the influent to the Grahamstown Disposal Works is affected by climatic, seasonal and anthropogenic activities in the city. For this reason, the chemical makeup of the Primary Facultative Pond (PFP) and Grahamstown Disposal Works final effluents (GDWf) fluctuated widely throughout the study period.

The Grahamstown Disposal Works receives on average 4600 $m^3.day^{-1}$ of raw sewage, with peak flows during specific times of the year reaching 7200 $m^3.day^{-1}$. The typical composition of Grahamstown sewage compares with that of other urban sources worldwide (Table 2.1).

Table 2.1 Composition of typical domestic sewage of some urban areas (Horan, 1990) and for Grahamstown Disposal Works (Mancotywa *pers. comm.*, 1999).

Determinand	Manchester (UK)	Mafraq (Abu Dhabi)	Campina Grande (NE Brazil)	Amman (Jordan)	Nairobi (Kenya)	Grahamstown (SA)
BOD ($mg.O_2\ L^{-1}$)	240	228	240	770	520	N/A
COD ($mg.O_2L^{-1}$)	520	600	570	1830	1120	678
Suspended Solids ($mg.L^{-1}$)	210	198	392	900	520	350
Ammonia as N ($mg.L^{-1}$)	22	35.2	38	100	33	30
pH	7.4	7.6	7.8	N/A	7.0	6.9
Temperature (°C)	14	N/A	26	22	24	20

N/A Data not available

2.2 RESEARCH OBJECTIVES

1. Establish a stable microalgal population and monitor N and P removal in an I-HRAP treating effluent from a conventional wastewater treatment plant (GDWf).
2. Monitor N and P removal in an HRAP treating effluent from and IAPS system.

2.3 MATERIALS AND METHODS

The operational performance of an HRAP acting as an independent unit for tertiary treatment of conventional wastewater was monitored. The Algal Integrated Wastewater Ponding System plant constructed at the Grahamstown Disposal Works, South Africa, is a joint project between the Water Research Commission (WRC), Grahamstown Municipality and Rhodes University. A schematic diagram of the plant is shown in Figure 2.1. Designed as both a demonstration plant and research facility, the plant consisted of the following unit processes: a fermentation pit, a primary facultative pond, an HRAP, an algal settling pond (ASP) and drying beds.

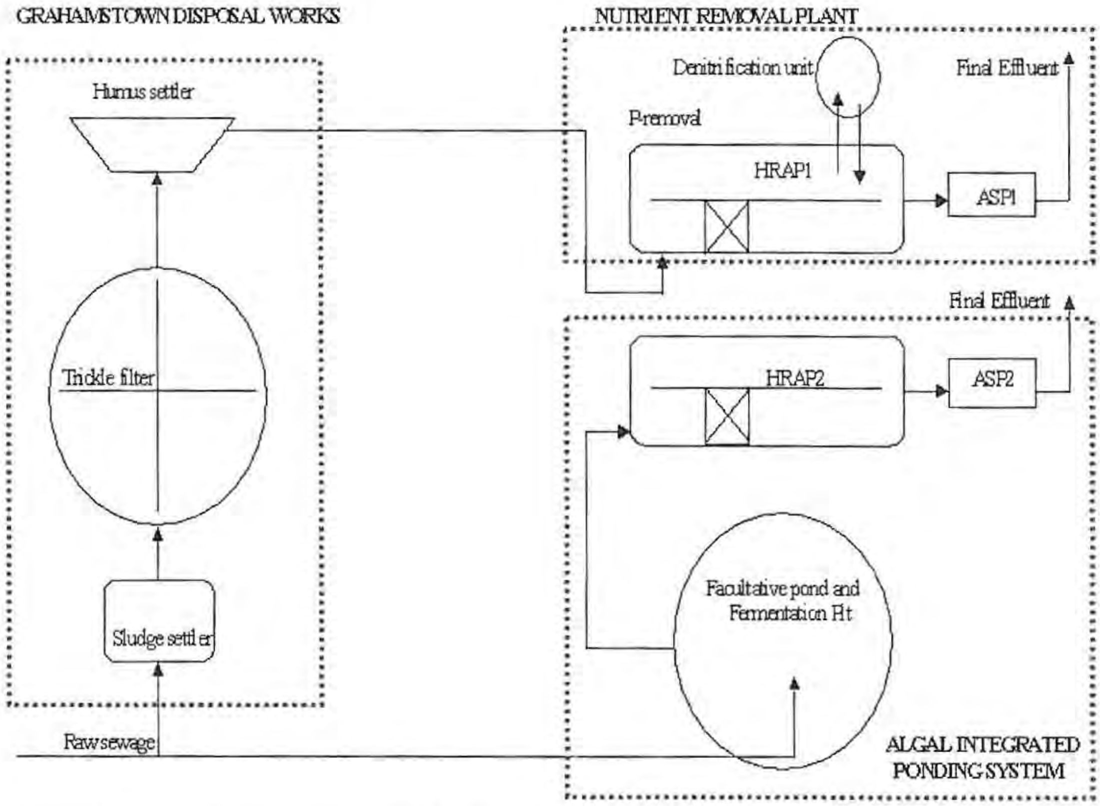


Figure 2.1 Schematic outline of the IAPS pilot plant at the Grahamstown Disposal Works, South Africa. To the left of the diagram is the flow path of the conventional wastewater treatment plant while to the right of the diagram, the integrated algal ponding systems for primary, secondary and tertiary treatment of domestic wastewater is outlined. HRAP1 = high rate algal pond treating conventional effluent, HRAP2 = high rate algal pond treating PFP effluent, ASP1 = algal settling pond receiving treated water from HRAP1 and ASP2 = algal settling pond receiving treated water from HRAP2.

Figure 2.2 The Primary Facultative Pond at the Algal Integrated Wastewater Ponding System pilot plant in Grahamstown, South Africa. The wooden walkway was constructed for experimental access. The Fermentation Pit is submerged below the surface of the PFP at the centre of the pond.

The IAPS pilot plant was designed to treat liquid wastes of 500 person equivalents with an average water consumption and disposal of 150 $L.capita^{-1}.day^{-1}$ giving a total flow rate through the plant of 75 $m^3.day^{-1}$. The ultimate Biological Oxygen Demand daily loading rate was determined to be 40 $kg.day^{-1}$. Design and operating parameters for the fermentation pit and primary facultative pond are indicated below :

Fermentation Pit

Hydraulic Retention Time	:	3 days
Upflow Velocity	:	1.5 $m.day^{-1}$
Depth	:	4.5 m

Primary Facultative Pond

Hydraulic Retention Time	:	20 days
Volume	:	1500 m^3
Surface Area	:	840 m^2

Two identical high rate algal ponds were operated for research purposes. In order to make certain observations for this research project, one HRAP was allowed to receive effluent from the primary facultative pond, while the second HRAP was retrofitted to receive Grahamstown Disposal Works' final effluent, normally discharged into natural water bodies.

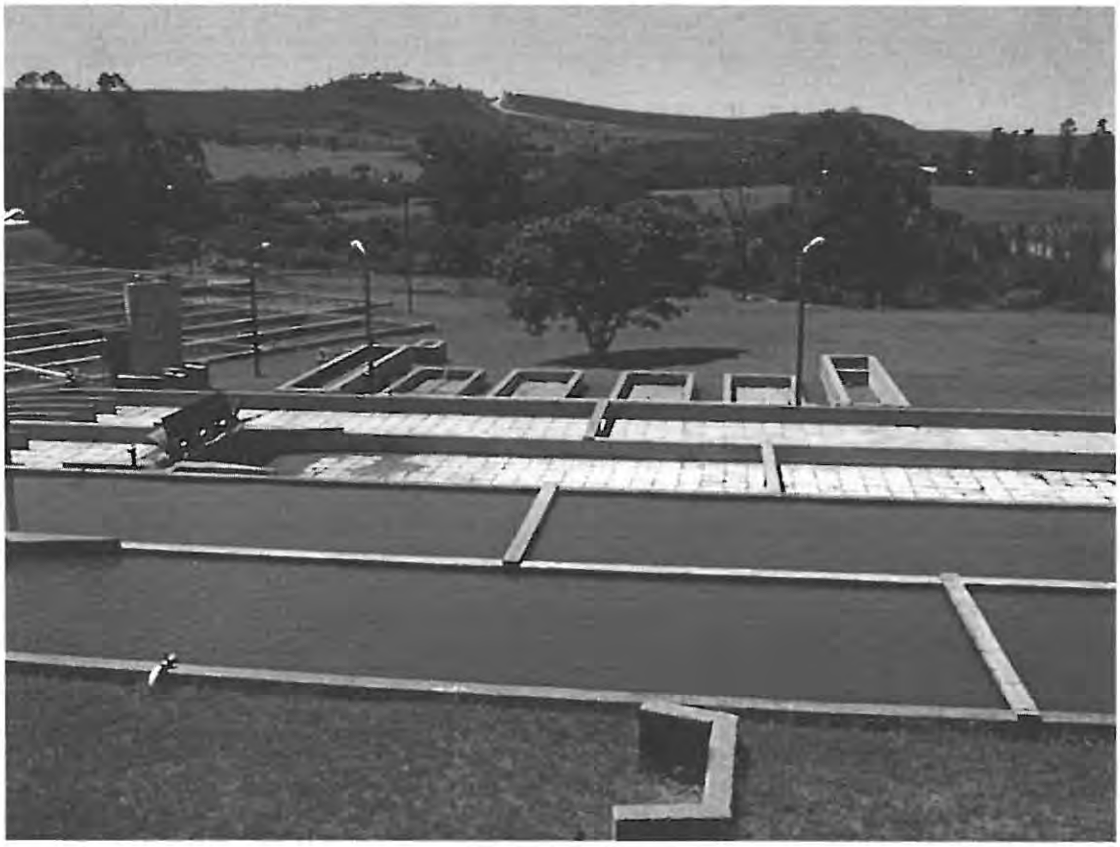


Figure 2.3 A view of the high rate algal ponds, algal settling ponds and algal drying beds at the IAPS pilot plant in Grahamstown, South Africa. The HRAP in the foreground received the PFP effluent while the empty HRAP next to it was retrofitted to receive Grahamstown Disposal Works final effluent. In the background, two longer cement algal settling ponds can be seen flanking four smaller algal drying beds.

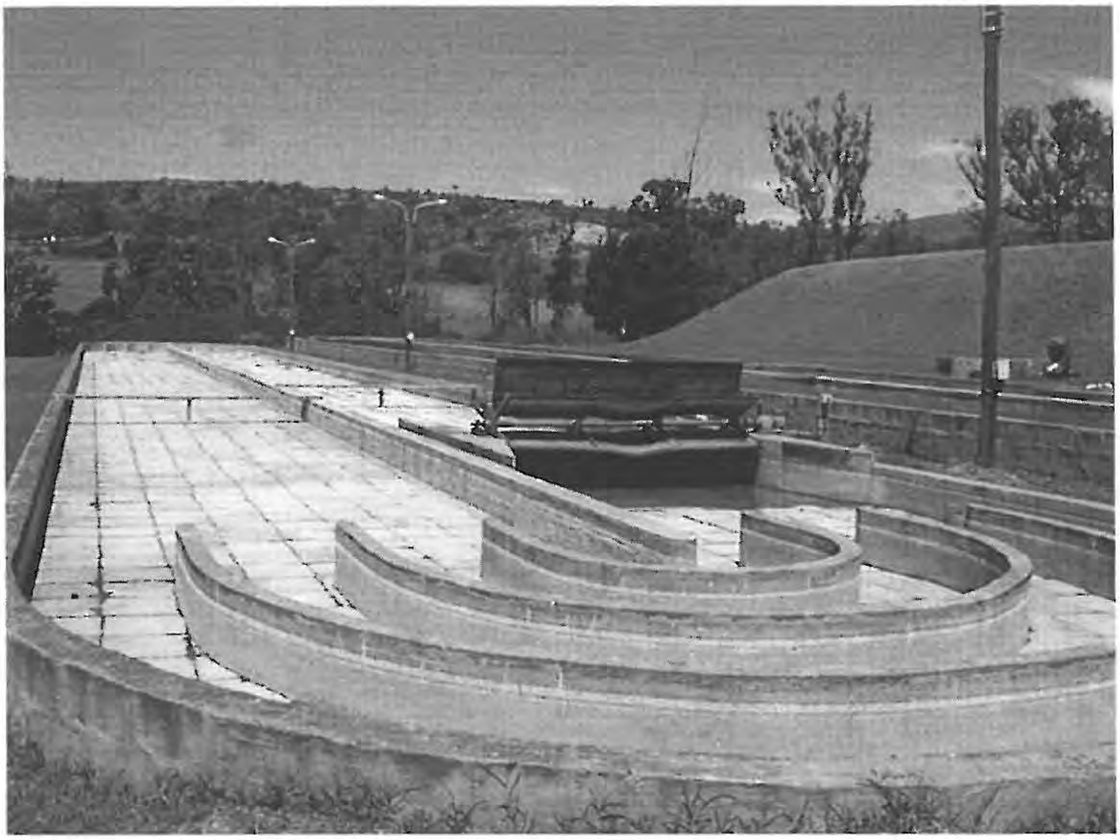


Figure 2.4 An empty high rate algal pond. From the photograph it can be seen that a filled HRAP is not more than 30cm deep. It can also be seen that simple, inexpensive materials can be used in the construction of an HRAP.

Each of the high rate algal ponds have a 500 m^2 surface area with approximately 30 cm water depth, hydraulic retention times of 5 days and linear velocities (achieved by means of mechanical paddle wheels) of 20 – 40 $cm.sec^{-1}$.

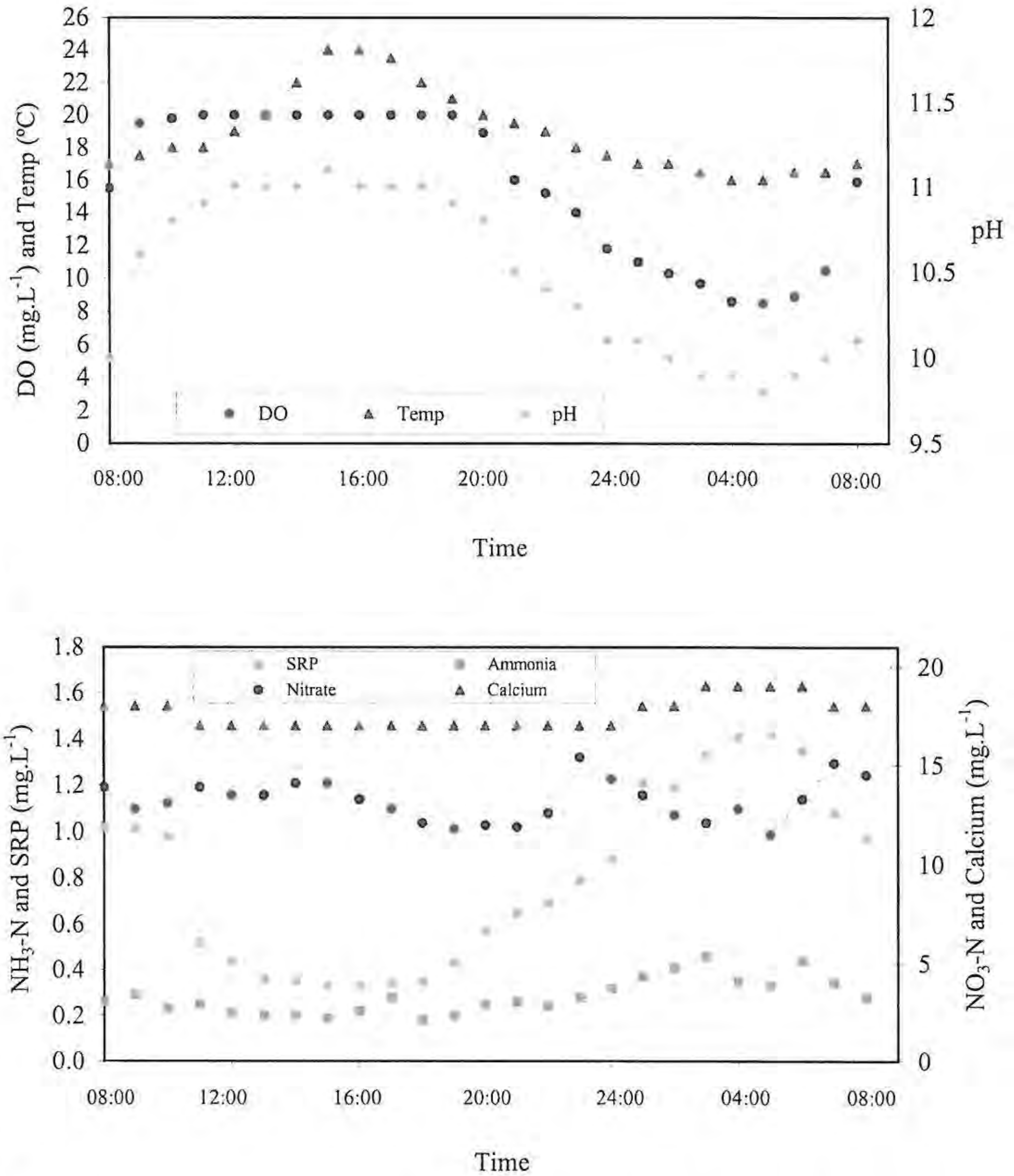
Figure 2.5 A basic algal settling pond as used in the IAPS pilot plant at Grahamstown, South Africa. The insert shows the appearance of dried algae after being pumped from the algal settling pond into the algal drying beds.

Standard nutrient concentrations were measured over a period of time. Calcium (Ca), Nitrate (NO_3-N), phosphate (PO_4-P) and ammonium (NH_4-N) were measured using MERCK Spectroquant® analysis kits. Sampling points were Grahamstown Disposal Works final effluent (GDWf), high rate algal pond receiving GDW-f (HRAP1), algal settling pond effluent from HRAP1 (ASP1), primary facultative pond effluent (PFP Effluent), high rate algal pond receiving PFP Effluent (HRAP2) and algal settling pond effluent from HRAP2 (ASP2). Chlorophyll *a* was determined by the acetone extraction method after Lichtenthaler (1987) as indicated in Appendix B.

2.4 RESULTS AND DISCUSSION

2.4.1 Phosphate Removal by HRAP

In order to determine whether phosphorus removal in the I-HRAP was primarily enhanced biological phosphorus removal (EBPR) or pH induced precipitation due to photosynthetic activity, the performance of the high rate algal pond with respect to physico-chemical properties was monitored over a 24 hour period and the results are displayed in Figure 2.6.



A

B

Figure 2.6 Performance of a high rate algal pond over 24 hours with respect to physico-chemical properties. Figure A shows the dissolved oxygen, temperature and pH profiles in the pond while figure B shows the soluble reactive phosphorus (SRP), ammonia, nitrate and calcium profiles.

From Figure 2.6 A, it can be seen that DO, temperature and pH have similar profiles where DO and pH appear to be directly linked to algal photosynthetic activity (it should be noted that sunrise was around 6am and sunset around 7pm). Oxygen production in the I-HRAP was beyond saturation levels of over 20 $mg.L^{-1}$. However, while DO did seem to change according to photosynthetic activity, the dissolved oxygen concentration in the water during the dark periods (where the DO is at its lowest) was never less than about 8 $mg.L^{-1}$. Thus the HRAP never entered an anoxic state. These findings are in agreement with literature (Awuah *et al.,* 2001).

Also observable in Figure 2.6 B are the phosphorus and calcium profiles which inversely mirror the DO and pH profiles. It is thus evident that during the daytime when the pH in the ponds is high due to increase in hydroxide ions from photosynthesis, the calcium and phosphorus levels are at their lowest and at night, when the algal populations undergo respiration, the pH level drops to its lowest while the calcium and phosphorus concentrations reach their highest levels.

Performance of the I-HRAP over 45 days to remove orthophosphate from IAPS (Figure 2.7) and conventional GDWf (Figure 2.8) is shown. The phosphate levels contained in the final effluent from the conventional treatment system were shown to be approximately 50 % greater than that of the PFP, once again indicating the need for tertiary treatment to be included before conventionally treated effluent is released into the natural environment. Despite this difference between PFP and GDWf phosphate concentrations, the performance of the I-HRAP appeared to be enhanced when treating GDWf compared to PFP effluent. The GDWf phosphate concentration was reduced by approximately 80 % from 8.3±2.9 $mg.L^{-1}$ to 2.0±1.3 $mg.L^{-1}$ PO_4-P while the IAPS phosphate concentration was only reduced by about 30 % from 6.1±2.0 $mg.L^{-1}$ to 4.1±2.2 $mg.L^{-1}$ PO_4-P.

A possible explanation of the poor performance of the HRAP treating PFP effluent was the condition of the algal population in the HRAP. The chlorophyll *a* concentration in the I-HRAP treating the GDWf effluent (HRAP1 was around 0.5 $\mu g.mL^{-1}$) was always greater than in the IAPS HRAP (HRAP2 around 0.3 $\mu g.mL^{-1}$). Owing to the weaker algal population in the IAPS HRAP, the photosynthetic activity was lower and therefore less alkalinity was generated, which is required for precipitation of the orthophosphate. The low phosphate concentrations observed on days 24 and 25 in the PFP effluent (Figure 2.7) were due to heavy rains diluting out the facultative pond wastewater.

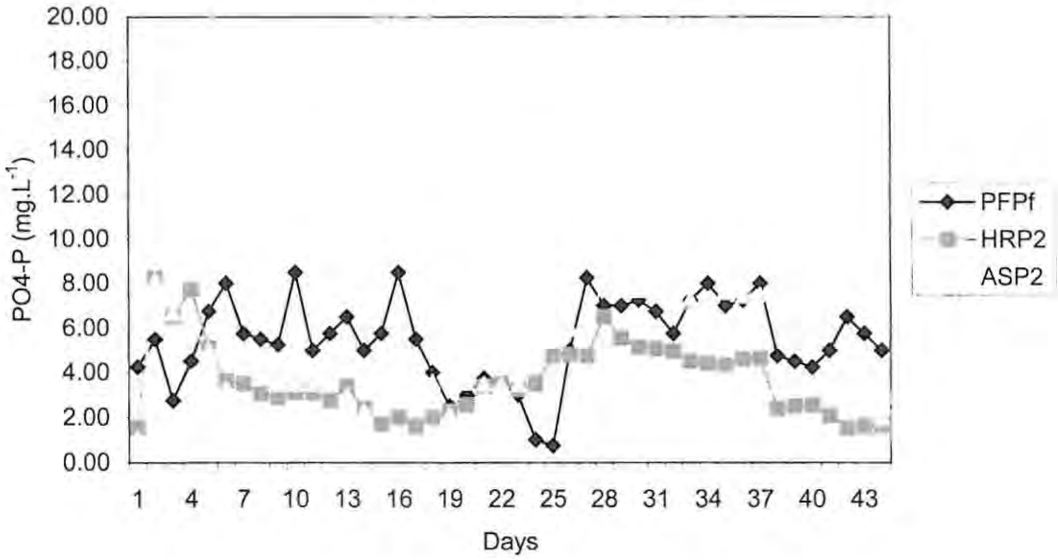


Figure 2.7 The effects of tertiary treatment on phosphate (PO_4-P) levels using an HRAP to treat PFP effluent from the IAPS plant.

It should also be noted that in the case of both streams, the orthophosphate (o-P) levels in the ASP were generally higher than those of the HRAP. This is explained by the nature of the algal settling pond. Since there is no mechanical movement in the ASP, combined with the depth of the pond, settled algal sludge begins to degrade, thus initiating methanogenesis. This process of methanogenesis decreases the pH in the ASP and causes a reverse of the pH-dependent calcium phosphate precipitation. It is thus critical that algal settling bonds are regularly emptied and cleaned, in order to prevent phosphate release by methanogenic algal sludge.

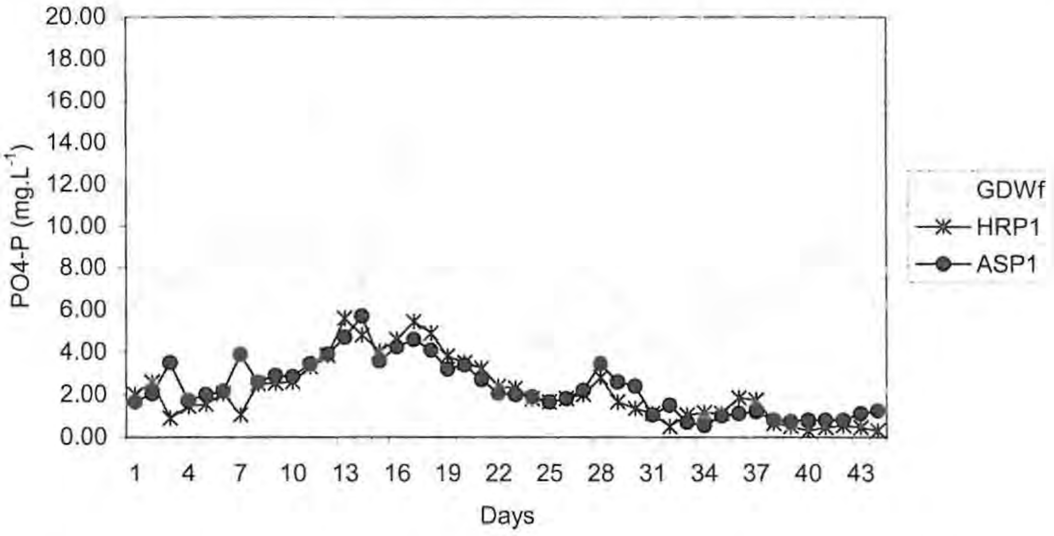
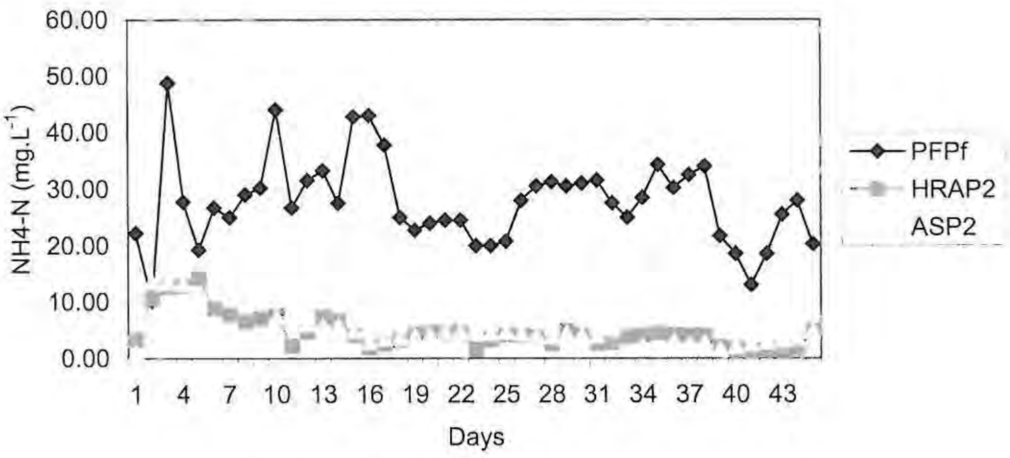


Figure 2.8 **The effects of tertiary treatment on phosphate (PO_4-P) levels using an I-HRAP to treat effluent from Grahamstown Disposal Works.**

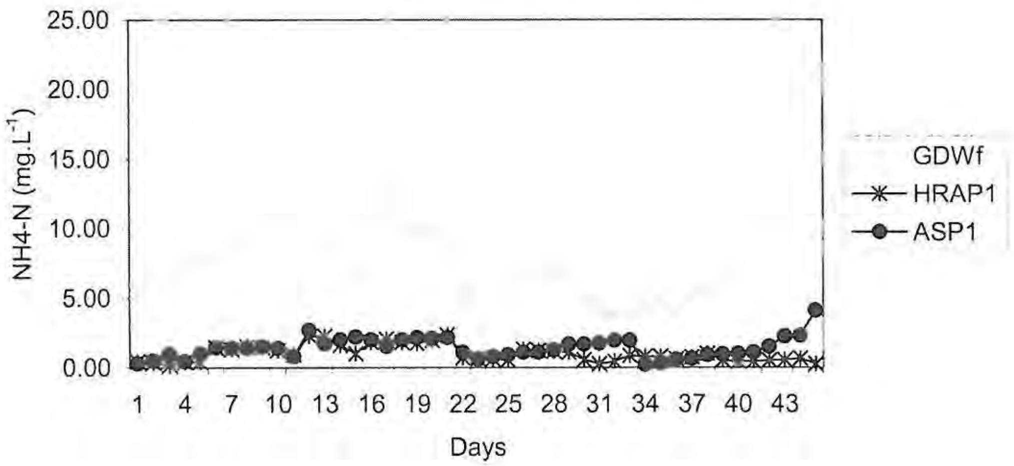
2.4.2 Ammonia Removal by an HRAP

Figure 2.9 illustrates ammonia removal by the HRAP over 45 days. The process of volatilisation is currently held as the most probable cause of ammonia removal from moderately mixed oxidation ponds at elevated pH such as the HRAP in operation at the pilot plant. Ammonia removal was also enhanced by tertiary treatment with the I-HRAP, most notably in the PFP effluent.

It can be proposed that the observed lower ammonia concentration and the corresponding higher nitrate concentration in GDWf effluent over the PFP effluent is a result of the process of nitrification. Since the wastewater from the IAPS stream is not exposed to nitrifying conditions before reaching the HRAP, the predominant form of nitrogen is expected to be ammonia. The majority of ammonia present in the conventional wastewater is nitrified to nitrate during secondary treatment in the trickling filters.



A



B

Figure 2.9 The effects of tertiary treatment on ammonium nitrogen (NH_4-N) removal by (A) an HRAP treating PFP effluent and (B) an I-HRAP treating GDWf.

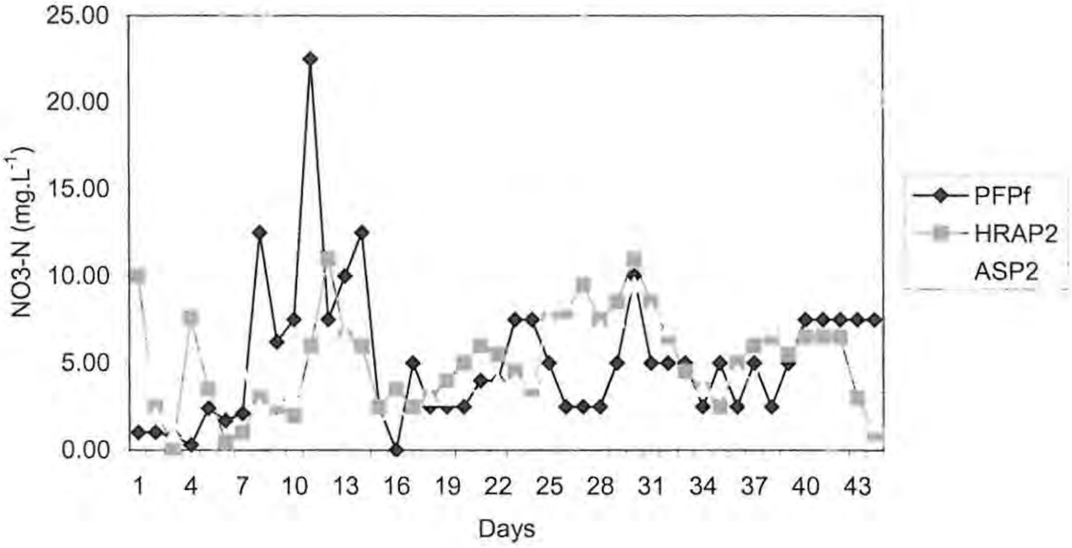
Since the balance between ammonia and nitrate concentrations is affected by environmental conditions, these two forms of nitrogen are expected to show interrelated patterns of appearance and removal.

2.4.3 Nitrate Removal by an HRAP

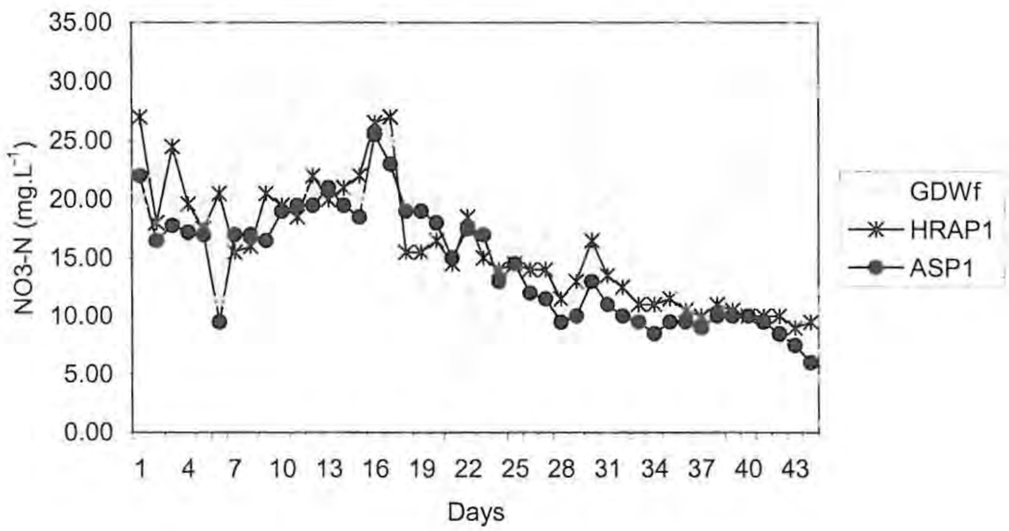
In the IAPS component (Figure 2.10), the nitrate concentration reaching the HRAP2 from the PFP was already low. The highest concentration during the study period was around 20 mg.L^{-1} NO_3-N, although the mean nitrate concentration was 4.14±3.82 mg.L^{-1} NO_3-N. This low level of nitrate is as a result of denitrification inherent in the functioning of the PFP. Due to anaerobic conditions found in the PFP, a large fraction of nitrate in the raw sewage is reduced to nitrogen gas. The current Water Quality

Guidelines in South Africa (Department of Water Affairs & Forestry Water Quality Guidelines) indicate a maximum for NO_x-N of 15 mg.L^{-1}. This large fluctuation would seem to be one of the shortcomings of the entire system (pointed out by Green *et al.*, 1996), since a nitrate concentration of 15 mg.L^{-1} NO_3-N is above effluent quality standard requirements. It is interesting to note that the nitrate levels seem to increase in the oxidation pond, resulting in increased nitrate levels in the algal settling pond. A possible explanation of this is the process of nitrification, the oxidation of ammonia to nitrate under continuous oxidative conditions.

In the Grahamstown Disposal Works component, the nitrate concentration in the effluent is unacceptably high for release into natural water bodies. Occasionally, nitrate concentrations reached as high as 30 mg.L^{-1} NO_3-N in the GDWf effluent which is released into the Blaaukrantz River. For this reason, the tertiary treatment step offered by the I-HRAP is essential in lowering the nitrate concentrations in the effluent to acceptable levels. Nitrate removal in an HRAP occurs predominantly through metabolic uptake by algae and the incorporation of nitrate into cellular products. However, algal metabolism in the HRAP alone is not sufficient to reduce nitrate concentration to desired levels and indeed nitrate levels in the ASP effluent were often still as high as 25 mg.L^{-1} NO_3-N. Furthermore, since the HRAP never enters an anoxic state required for denitrification, the need for a subsequent denitrification step in-line with the I-HRAP was identified, investigated and is reported in Chapter four.



A



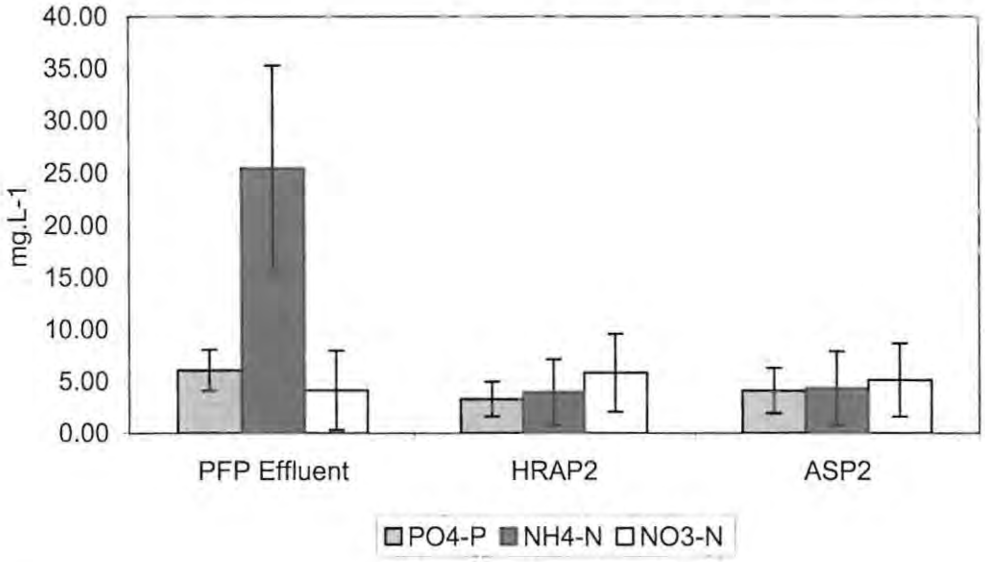
B

Figure 2.10 **The effects of tertiary treatment on nitrate nitrogen (NO_3-N) removal by (A) an HRAP treating PFP effluent and (B) an I-HRAP treating GDWf.**

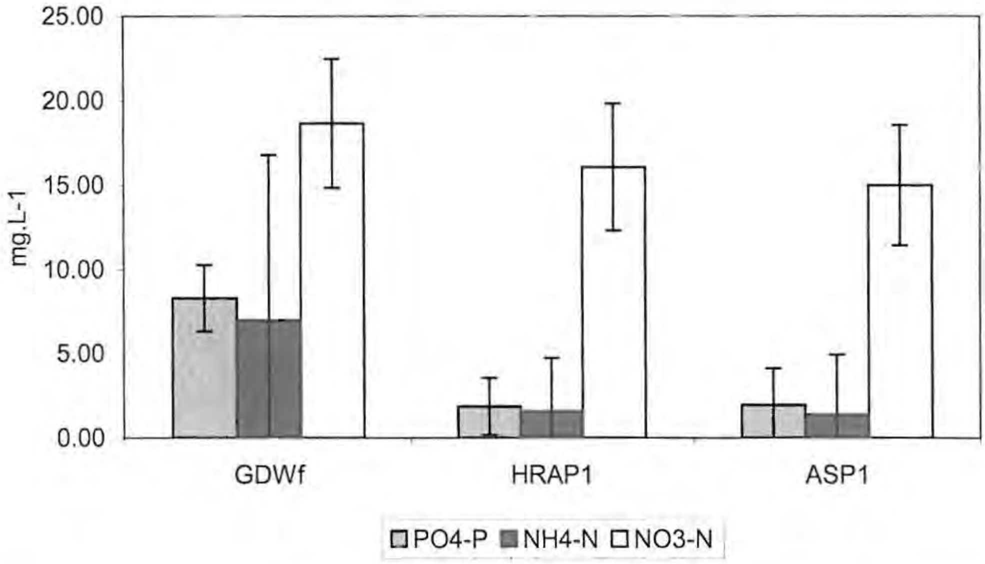
The nitrate concentrations through the ponds treating GDWf do not follow the same increasing trend seen in the ponds treating PFP effluent. This may be confirmation of the fact that the preferred nitrogen source for algae (and indeed many bacteria) is ammonia, and since the ammonia concentrations are higher in the IAPS stream, the algae first utilize the available ammonia before taking up nitrate. In the conventional stream, the ammonia levels are far lower and there may be competition for it, resulting in metabolic diversification allowing some microorganisms to use nitrate as the nitrogen source (McCarthy *et al.*, 1977).

2.4.4 Summary of Tertiary Performance of an HRAP

From the data obtained over a period of 28 weeks, certain trends are clear. For one, phosphate and ammonia removal in the HRAP treating both conventional effluent as well as PFP effluent is evident. Overall percentage removal of total nitrogen (as nitrate and ammonia) from the IAPS stream was 68 % while from the GDWf stream, the I-HRAP was only successful in removing 36 %.



A



B

Figure 2.11 The overall performance of tertiary treatment by (A) an HRAP treating PFP effluent and (B) an I-HRAP treating GDWf over a period of 28 weeks.

The results shown in Figure 2.11 indicate that whether treating conventional effluent or PFP effluent, high rate algal ponds enhance water effluent quality in terms of nutrient composition.

The quality of the water was further measured by perceptual observations. Figure 2.12 shows the differences in algal biomass in each of the two experimental HRAP. It can be seen that the algal cultures were considerably different in each of the two algal ponds. The predominant species found in the HRAP treating IAPS effluent was found to be *Scenedesmus* sp. while the predominant species found in the GDWf I-HRAP was *Microcystis* sp. Furthermore, the physiological state of each of the two algal cultures varied widely. While the *Microcystis* sp. culture appeared healthy, forming distinct flocs, the culture in the IAPS HRAP appeared less healthy, and at times took on a distinct brownish colouration due to an increase in the pigment phaeophytin, a breakdown pigment of chlorophyll, often used as an indicator of algal physiological status (Figure 2.13).

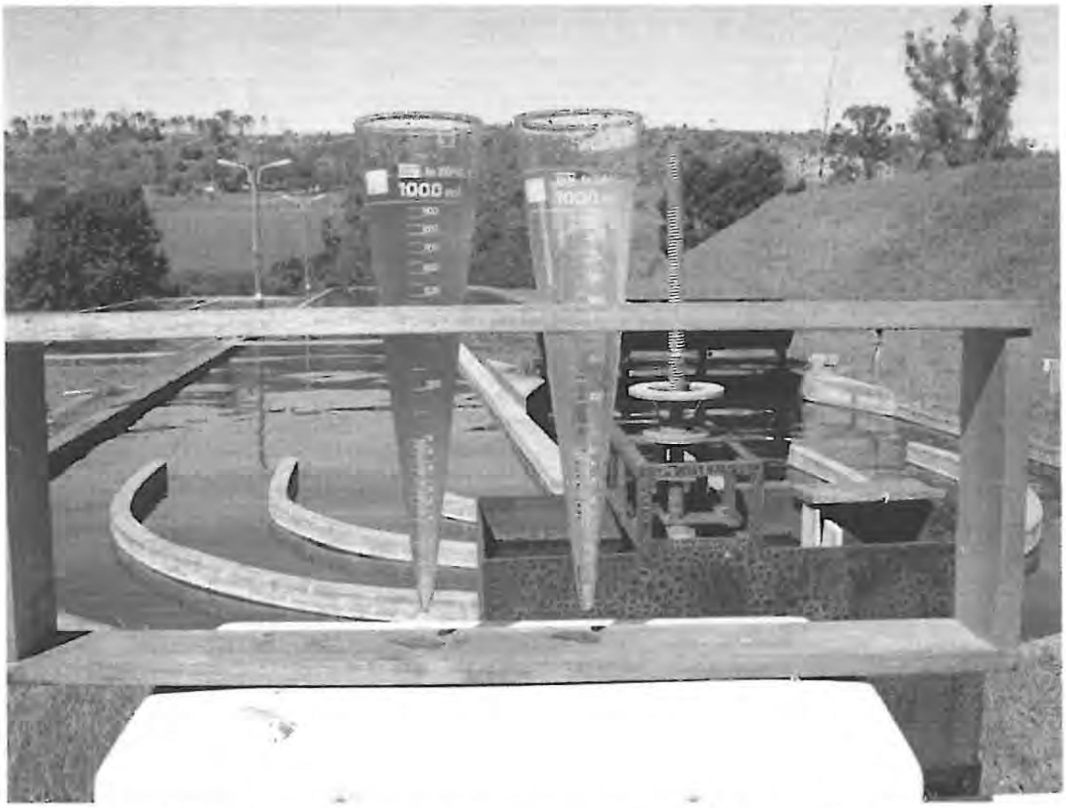


Figure 2.12 Visual observation of the quality of water from the HRAP treating IAPS effluent (left Imhoff Cone) and GDWf (right Imhoff Cone). The darker colouration of the IAPS HRAP is not necessarily indicative of a healthy algal culture, but rather of the increased concentration of phaeophytin, the breakdown pigment of chlorophyll, often released as a result of exposure to extreme stress conditions.

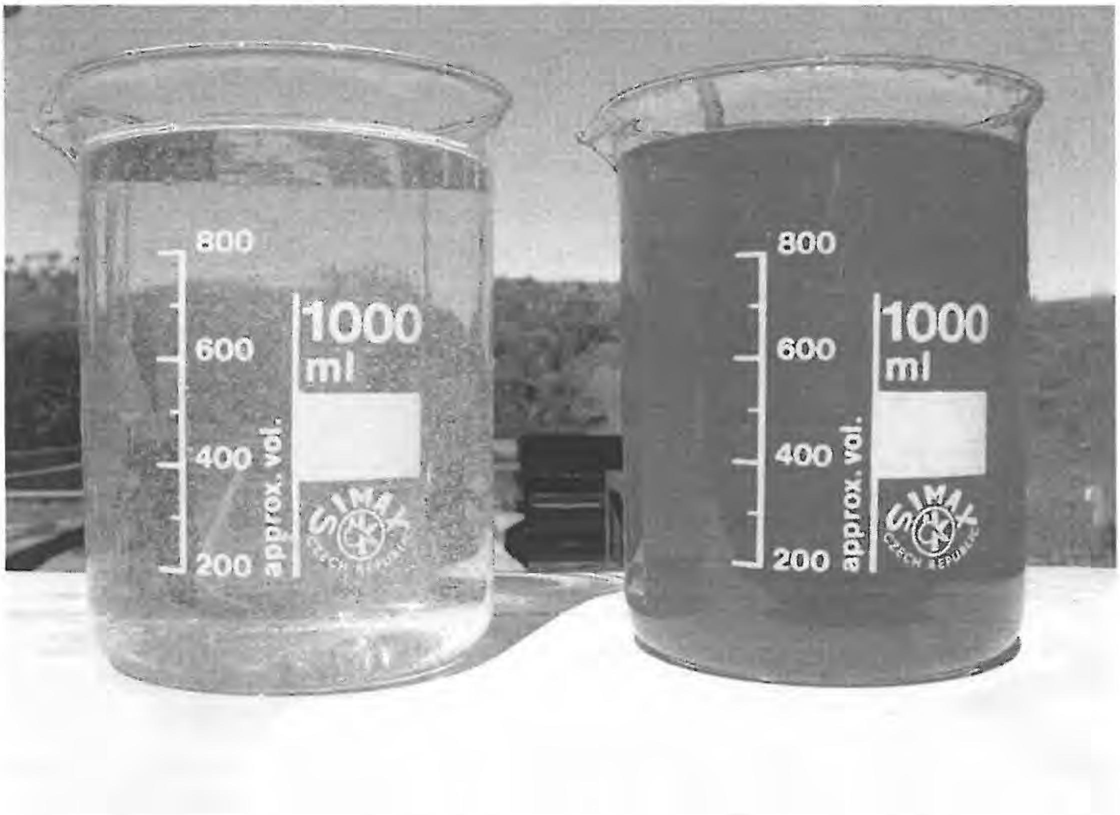


Figure 2.13 Closer visual inspection of the wastewater from the two HRAP's revealed that the algal culture in the I-HRAP treating GDWf (left beaker) consisted predominantly of *Microcystis* sp., forming healthy-looking algal flocs, while the IAPS HRAP culture (right beaker) consisted predominantly of *Scenedesmus* sp. with higher concentrations of phaeophytin.

2.5 CONCLUSIONS

1. The I-HRAP was observed to support good algal growth.
2. The I-HRAP was found to remove phosphorus from effluent treated in conventional wastewater treatment systems primarily by biologically-mediated calcium phosphate precipitation at elevated pH.
3. Nitrogen removal in the I-HRAP was primarily by ammonia removal via volatilisation.
4. Since anoxic conditions are not established in the I-HRAP, even during hours of darkness, nitrate reduction levels are unsatisfactory and a subsequent denitrification step would be needed to effect a more complete nitrogen removal.

CHAPTER THREE

EXTRACELLULAR PRODUCTS OF ALGAE TO SUPPORT DENITRIFICATION

3.1 INTRODUCTION

Polysaccharides may comprise 80 – 90 % of the total extracellular release of organic substances by healthy algae during various phases of growth (Fogg, 1977; Myklestad, 1995). In earlier research, Fogg & Westlake (1955) intimated that even if extracellular products formed only a small part of the total material synthesized by an alga, it is nevertheless possible that the substance concerned may be biologically active at low concentrations, and also that there was evidence to show that their ecological effects of these substances may sometimes be considerable.

High rate algal ponds have been shown to promote good algal growth and are efficient in removing phosphorus by means of pH-mediated precipitation with calcium and ammonia removal by means of nitrification and volatilisation. However, the HRAP is unable to remove excess nitrate from wastewater. Although macrophyte- and algal-based wastewater treatment systems have been shown to remove some nitrate (Awuah *et al.*, 2001), the majority of the nitrate present in the wastewater remains and is disposed of into the environment with the wastewater. Since elevated nitrate levels in natural waterbodies is undesirable, promoting eutrophication and supporting algal growth, a tertiary polishing step is required in wastewater treatment to remove excess nitrate from the effluent.

Combining these two approaches, the proposal was made to investigate the use of HRAP algal growth and associated EPS production to support heterotrophic denitrification. The majority of algal polysaccharides consist of a glycogen-like polyglucan which is an easily utilisable carbon or energy source by heterotrophic bacteria. Stress manipulation of algal physiology was examined in order to determine the conditions required to obtain sufficient polysaccharide to support bacterial denitrification. An alternative to utilising algal EPS as a carbon source for denitrification was the use of degraded algal cells, thereby accessing the full carbon source available from algal production in the HRAP.

3.2 RESEARCH OBJECTIVES

1. Investigate conditions under which algal stress physiology can be manipulated to promote the production and release of photosynthate by microalgae.
2. To establish that EPS, from whole-cell algae, is an available carbon source for heterotrophic denitrification.

3.3 MATERIALS AND METHODS

3.3.1 Analytical Techniques

The pH of the effluent was analysed using a Cyberscan gel electrode pH meter. All COD, NO_3-N, NH_4-N and PO_4-P concentrations were measured using MERCK Spectroquant® Kits and tests were performed according to manufacturers instructions.

Total polysaccharide (TPS), exopolysaccharide (EPS) and capsular polysaccharide (CPS) were assayed by means of a variation of the phenol-sulphuric acid method described by Dubois *et al.*, (1956). One millilitre of 5 % (w/v) phenol was added to 1 mL sample and mixed thoroughly. To this was added 5mL concentrated sulphuric acid. The exothermic reaction was mixed and allowed to develop for 10 minutes at room temperature and then incubated for a further 15 minutes at 25°C. The mixture was then read spectrophotometrically at 495 nm. A zero blank was used by substituting 1 mL of sample with 1 mL of Milli-Q H_2O. Concentration readings were obtained using glucose as a standard (0 – 100 $\mu g.mL^{-1}$).

The three fractions of polysaccharide were separated as follows: total polysaccharide was measured by assaying 5 mL of the entire medium and culture constituents; exopolysaccharide was obtained by centrifuging 5 mL of the culture and medium at 5 000 x g for 15 minutes and analysing the supernatant; capsular polysaccharide was obtained by centrifuging 5mL of the culture and medium at 5 000 x g for 15 minutes, discarding the supernatant, resuspending the pellet in 5 mL Milli-Q H_2O, discarding the supernatant and repeating. Milli-Q H_2O was added to the original level (of 5 mL), incubated at 50°C for 30 minutes, mixing every 10 minutes, centrifuged for 15 minutes at 5 000 x g and the supernatant analysed.

3.3.2 Aerobic Batch Flask Studies – 250 mL

Algal biomass was harvested by collecting 20 L of HRAP effluent and settling for 2 hours. The supernatant was decanted and the remaining biomass was centrifuged for 15 minutes at 15 000 x g in 250mL tubes. The cells were washed up to three times in tap water and finally recombined and resuspended to the original volume. The algal biomass was then evenly distributed, 30 mL into each of 12 x 250 mL flasks. The volumes were then brought up to 200 mL with the appropriate nutrient mix in distilled water. The nutrient regimes tested included (1) a control without nutrients, (2) a high-nitrate (20 $mg.L^{-1}$ NO_3-N final volume) phosphate-free batch, (3) a high-phosphate (20 $mg.L^{-1}$ PO_4-P final volume) nitrate-free batch and (4) a combined high-nitrate high-phosphate (20 $mg.L^{-1}$ each final volume) batch. Typically the final pH of the flasks measured 9.0. The experiment was performed in a dark, aerobic environment. For this, the flasks were covered in aluminium foil to prevent photosynthesis, but not degassed with N_2 gas or sealed with a rubber bung. The flasks were kept on an orbital shaker at 90 rpm at ambient temperature. Experiments were carried out in triplicate. The experiment was carried out over a 24 hour period sampling every 4 hours by removing 20mL of culture and analysing for pH, NO_3-N, PO_4-P, NH_4-N, total polysaccharide (TPS), exopolysaccharide (EPS) and capsular polysaccharide (CPS) as described previously. The mean of triplicate results and the standard deviations were recorded.

3.3.3 Anaerobic Batch Flask Studies – 250 mL

Algal biomass was harvested by collecting 20 L of HRAP effluent and settling for 2 hours. The supernatant was decanted and the remaining biomass was centrifuged for 15 minutes at 15 000 x g in 250 mL tubes. The cells were washed up to three times in tap water and finally recombined and resuspended to the original volume. The algal biomass was then evenly distributed, 30 mL into each of 12 x 250 mL flasks. The volumes were then brought up to 200 mL with the appropriate nutrient mix in distilled water. The nutrient regimes tested included (1) a control without nutrients, (2) a high-nitrate (20 $mg.L^{-1}$ NO_3-N final volume) phosphate-free batch, (3) a high-phosphate (20 $mg.L^{-1}$ PO_4-P final volume) nitrate-free batch and (4) a combined high-nitrate high-phosphate (20 $mg.L^{-1}$ each final volume) batch. Typically the final pH of the flasks measured 9.0. The experiment was performed in a dark, anaerobic environment. The flasks were covered in aluminium foil to prevent photosynthesis, degassed with

N_2 gas for 7 minutes and sealed with a rubber bung. The rubber bung was fitted with three glass tubes : one for sampling, one for flushing the volume with N_2 gas and the third as a pressure outlet. The flasks were kept on an orbital shaker at 90 rpm at ambient temperature. Experiments were carried out in triplicate. The experiment was carried out over a 24 hour period with sampling every 4 hours by removing 20 mL of culture and analysing for pH, NO_3-N, PO_4-P, NH_4-N, total polysaccharide (TPS), exopolysaccharide (EPS) and capsular polysaccharide (CPS) as described previously. Figure 3.1 shows a schematic of the anaerobic batch flask reactor. The mean of triplicate results and the standard deviations were recorded.

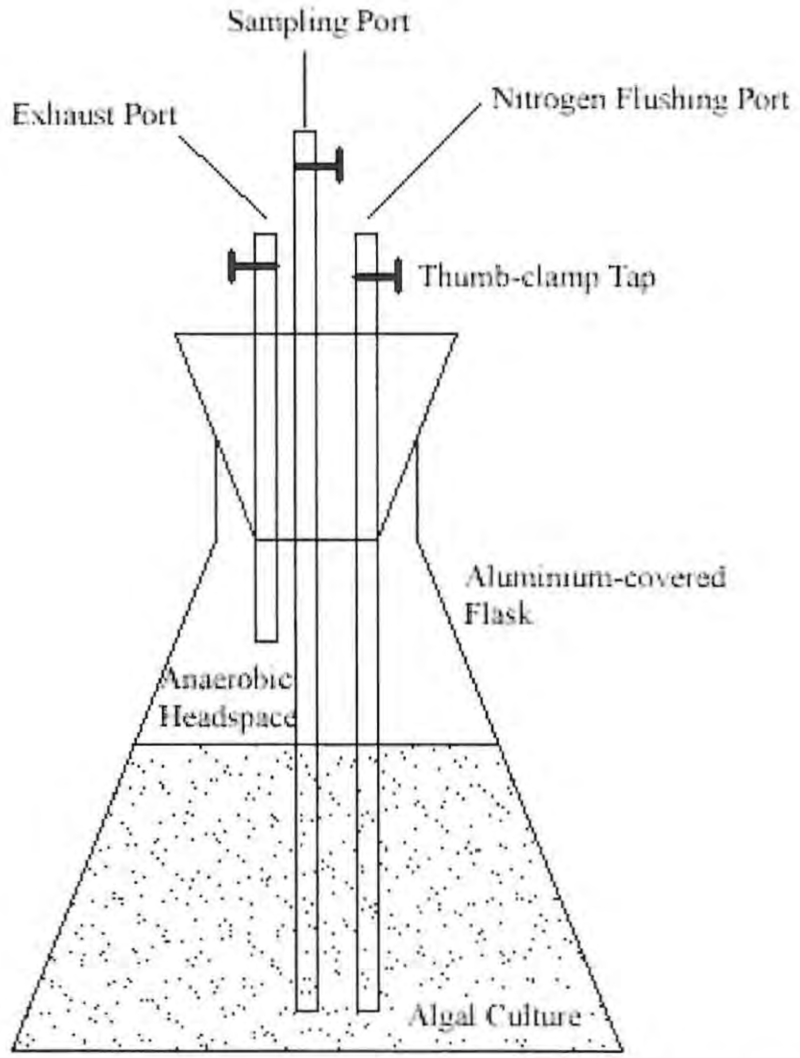


Figure 3.1 Anaerobic Batch Flask Reactor showing sampling, flushing and exhaust valves.

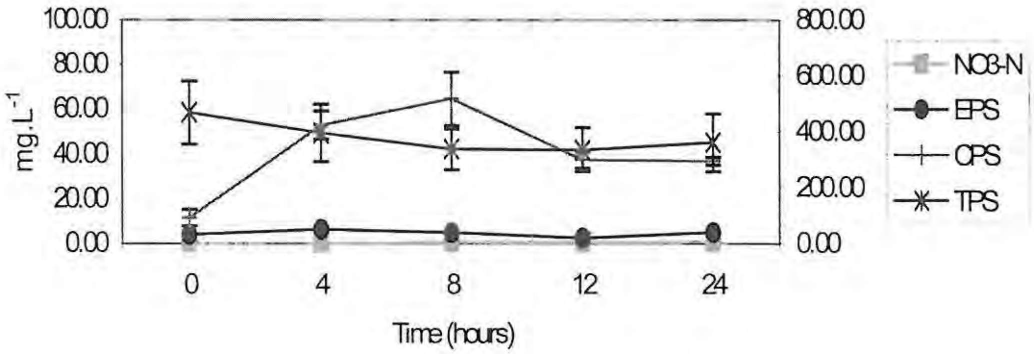
3.3.4 Anaerobic Batch Flask Studies – 1 L

Algal biomass was harvested by collecting 20 L of HRAP effluent and settling for 2 hours. The supernatant was decanted and the remaining biomass was centrifuged for 15 minutes at 15 000 x g in 250 mL tubes. The cells were washed up to three times in tap water and finally recombined and resuspended to the original volume. The algal biomass was then evenly distributed, 100 mL into each of 12 x 1 L flasks. The volumes were then brought up to 500 mL with the appropriate nutrient mix in distilled water. The nutrient regimes tested included (1) a control without nutrient, (2) a high-nitrate (20 $mg.L^{-1}$ NO_3-N final volume) phosphate-free batch, (3) a high-phosphate (20 $mg.L^{-1}$ PO_4-P final volume) nitrate-free batch and (4) a combined high-nitrate high-phosphate (20 $mg.L^{-1}$ each final volume) batch. Typically the final pH of the flasks measured 9.0. The experiment was performed in a dark, anaerobic environment. The flasks were covered in aluminium foil, degassed with N_2 gas for 7 minutes and sealed with a rubber bung. The rubber bung was fitted with three glass tubes : one for sampling, one for flushing the volume with N_2 gas and the third as a pressure outlet. The flasks were kept on an orbital shaker at 90 rpm at ambient temperature. Experiments were carried out in triplicate. The experiment was carried out over a 120 hour period sampling every 24 hours by removing 20 mL of culture and analysing for pH, NO_3-N, PO_4-P, NH_4-N, total polysaccharide (TPS), exopolysaccharide (EPS) and capsular polysaccharide (CPS) as described previously. The mean of triplicate results and the standard deviations were recorded.

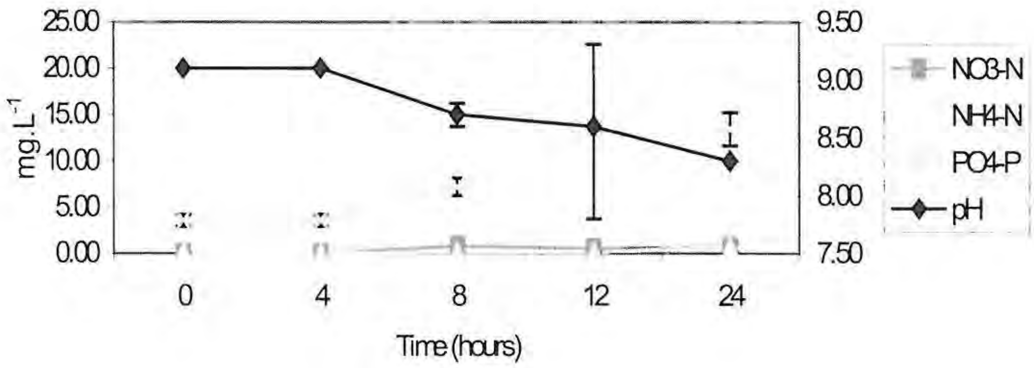
3.4 RESULTS AND DISCUSSION

3.4.1 Aerobic Batch Flask Studies – 250 mL

Batch flask studies were set up to investigate a role for algal biomass in denitrification reactions. The production of polysaccharide by algal biomass was followed through the manipulation of a number of stress conditions. These conditions included dark, aerobic vs dark, anaerobic growth conditions and changing nutrient regimes. Results of the dark, aerobic batch-flask experiment are shown below.



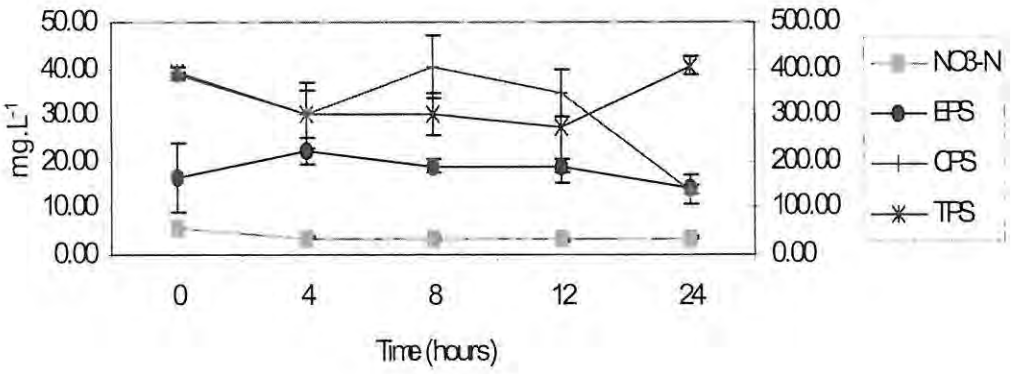
A



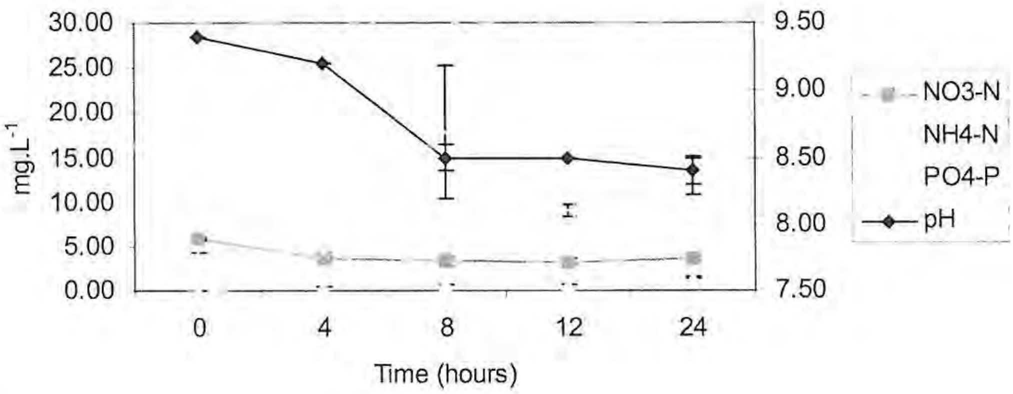
B

Figure 3.2 Performance of batch-flask reactors under nutrient-deficient, dark, aerobic conditions (maximum stress). Graph A represents the nitrate and polysaccharide concentrations while graph B represents the pH, nitrate, ammonium and phosphate concentrations. In Graph A, the Y2 axis is used to indicate the concentration of TPS in $mg.L^{-1}$ while in graph B, the Y2 axis is used to indicate the pH in the flasks.

Figures 3.2 through 3.5 show the results of batch-flask reactor studies performed under dark, aerobic conditions. These studies revealed that very little or no exopolysaccharide is released in nutrient-limiting or nutrient-rich environments by the HRAP microalgae under such conditions. The highest EPS concentration reached under dark, aerobic conditions was around 20 mg.L^{-1} in flasks supplemented with 20 mg.L^{-1} NO_3-N and no phosphate (Figure 3.3A). Total polysaccharide concentrations never rose above 500 mg.L^{-1}.

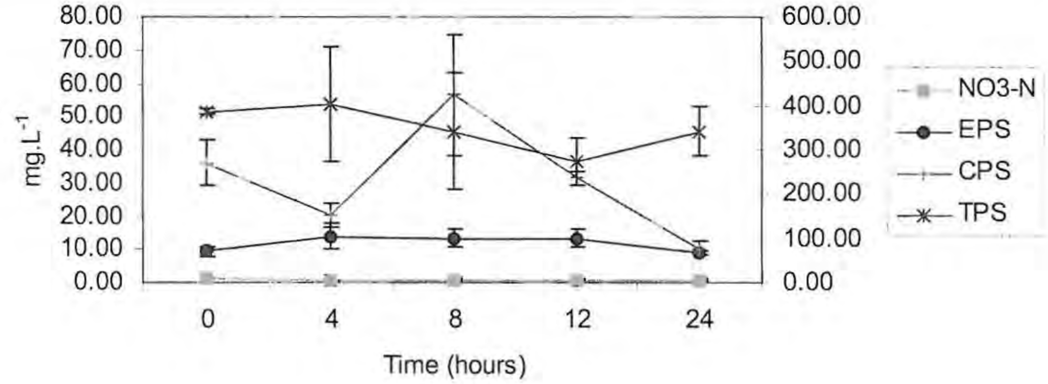


A

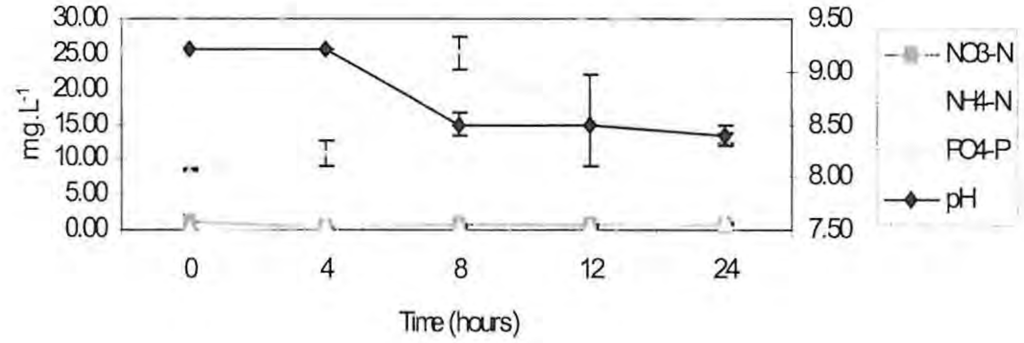


B

Figure 3.3 Performance of batch-flask reactors under dark, aerobic conditions with 20 mg.L^{-1} NO_3-N and no PO_4-P added (reduced N stress). Graph A represents the nitrate and polysaccharide concentrations while graph B represents the pH, nitrate, ammonium and phosphate concentrations. In Graph A, the Y2 axis is used to indicate the concentration of TPS in mg.L^{-1} while in graph B, the Y2 axis is used to indicate the pH in the flasks.

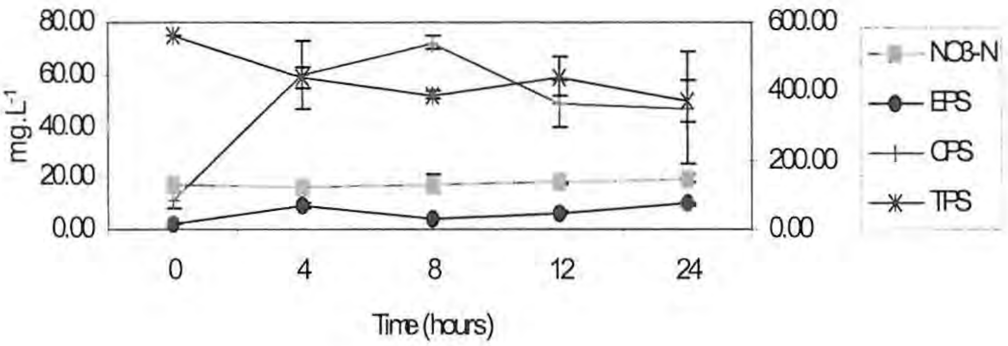


A

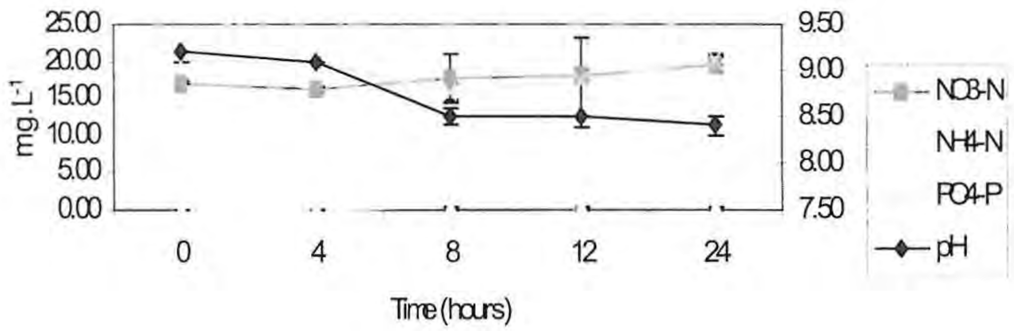


B

Figure 3.4 Performance of batch-flask reactors under dark, aerobic conditions with no NO_3-N and 20 mg.L^{-1} PO_4-P added (reduced P stress). Graph A represents the nitrate and polysaccharide concentrations while graph B represents the pH, nitrate, ammonium and phosphate concentrations. In Graph A, the Y2 axis is used to indicate the concentration of TPS in mg.L^{-1} while in graph B, the Y2 axis is used to indicate the pH in the flasks.



A



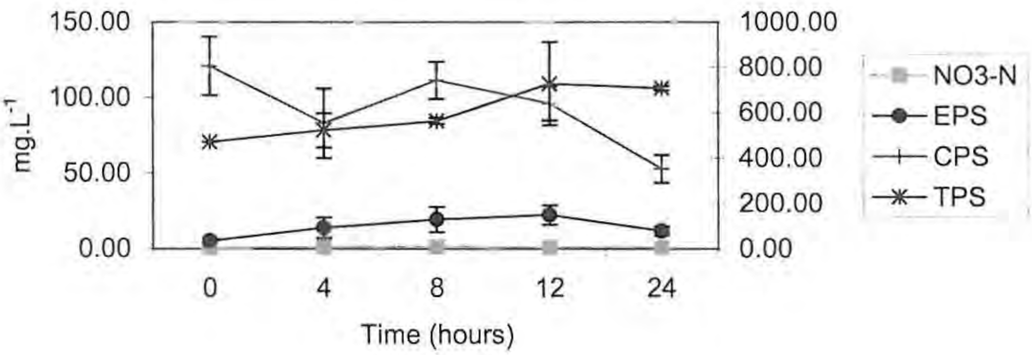
B

Figure 3.5 Performance of batch-flask reactors under dark, aerobic conditions with 20 mg.L^{-1} NO_3-N and 20 mg.L^{-1} PO_4-P added (minimum nutrient stress). Graph A represents the nitrate and polysaccharide concentrations while graph B represents the pH, nitrate, ammonium and phosphate concentrations. In Graph A, the Y2 axis is used to indicate the concentration of TPS in mg.L^{-1} while in graph B, the Y2 axis is used to indicate the pH in the flasks.

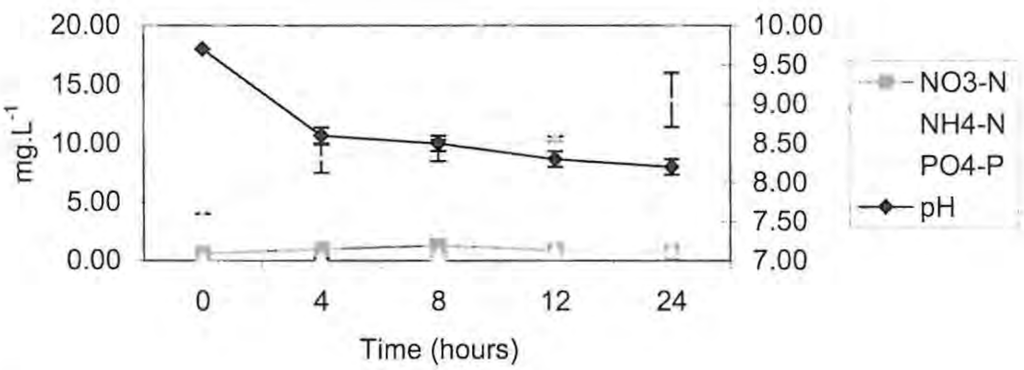
Under all aerobic experimental conditions, CPS seemed to reach a maximum after 8 hours, although total polysaccharide (TPS) concentrations remained unchanged. With regard to the polysaccharides, it would seem that the CPS fraction demonstrated the most dramatic changes. The total polysaccharide and exopolysaccharide concentrations, while fluctuating slightly, showed little change throughout the study period. As stated earlier, there seemed to be little or no change in nitrate or ammonium levels under any of the experimental nutrient conditions. The only observable change was elevation of phosphate concentrations and these can be linked to the slight decrease observed in pH of the cultures during the time trial.

3.4.2 Anaerobic Batch Flask Studies – 250 mL

In the anaerobic experiments, more striking patterns were observed. In all nutrient regimes under anaerobic conditions, an associated decrease in exopolysaccharide concentrations was found to correspond with a decrease in the nitrate concentration, a function attributed to denitrification.



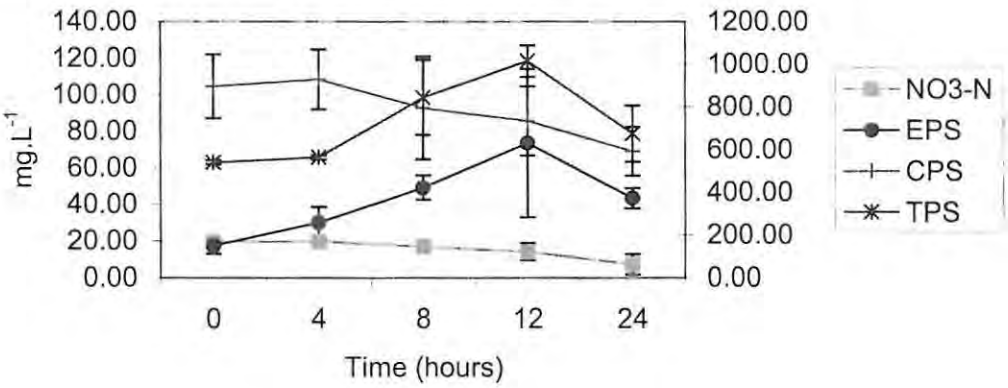
A



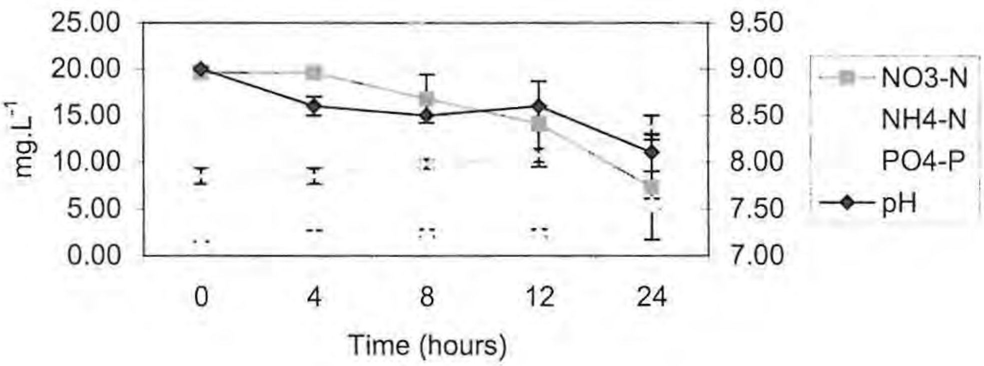
B

Figure 3.6 Performance of 250mL batch-flask reactors under dark, anaerobic conditions with no NO_3-N or PO_4-P added. Graph A represents the nitrate and polysaccharide concentrations while graph B represents the pH, nitrate, ammonium and phosphate concentrations. In Graph A, the Y2 axis is used to indicate the concentration of TPS in $mg.L^{-1}$ while in graph B, the Y2 axis is used to indicate the pH in the flasks.

In Figure 3.6, EPS levels were observed to increase only to a maximum of 20 $mg.L^{-1}$. However, when looking at results from the anaerobic experiments where nitrate is supplemented (Figures 3.8 and 3.10) compared to those flasks which were nitrate-free (Figure 3.6 and 3.8), the EPS concentrations in NO_3-N enriched flasks were two to three times greater than in the flasks where no NO_3-N was added. In NO_3-N enriched flasks, although EPS appears to increase initially, a marked decrease in EPS concentrations can clearly be seen when nitrate levels begin to decrease. In flasks where appreciably little or no nitrate is present for the heterotrophic bacteria to reduce, no noticeable decrease in exopolysaccharide concentration is observed.



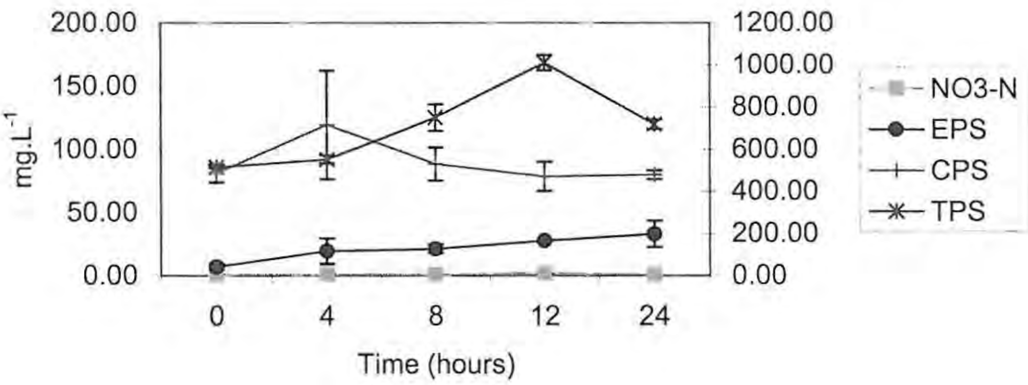
A



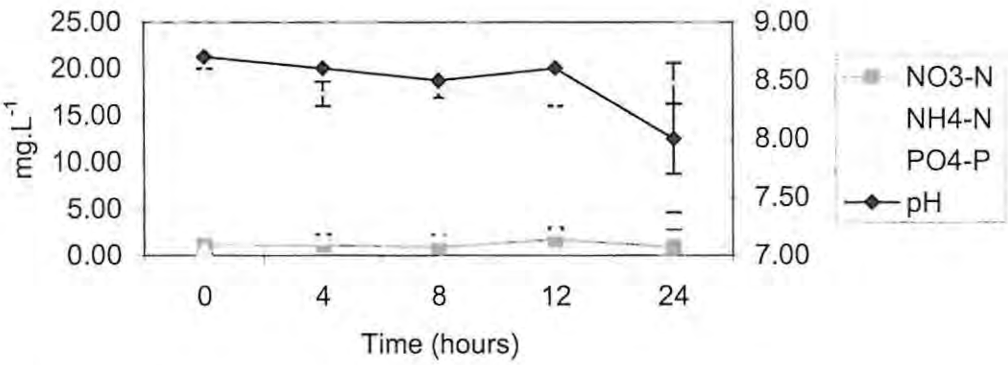
B

Figure 3.7 Performance of 250mL batch-flask reactors under dark, anaerobic conditions with 20 mg.L^{-1} NO_3-N and no PO_4-P added. Graph A represents the nitrate and polysaccharide concentrations while graph B represents the pH, nitrate, ammonium and phosphate concentrations. In Graph A, the Y2 axis is used to indicate the concentration of TPS in mg.L^{-1} while in graph B, the Y2 axis is used to indicate the pH in the flasks.

In Figure 3.7A, the exopolysaccharide concentration peaks at 73.5 ± 40.6 mg.L^{-1} and then drops to 43.3 ± 5.6 mg.L^{-1} at the same time the nitrate concentration decreases from 16.8 ± 2.6 mg.L^{-1} to 7.3 ± 5.6 mg.L^{-1}. The same pattern can be seen in Figure 3.9A where a decrease in EPS concentration is associated with a decrease in nitrate concentration.



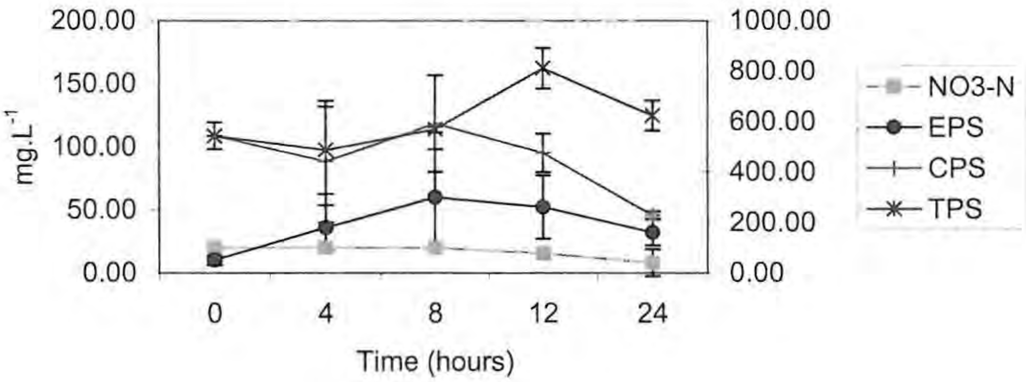
A



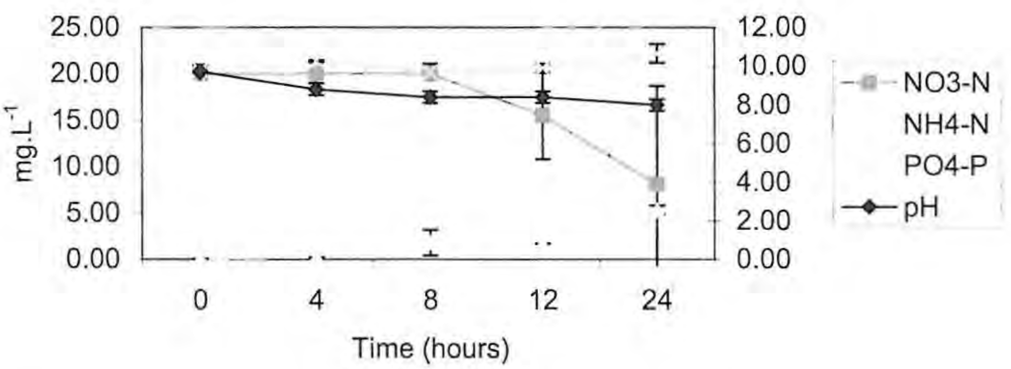
B

Figure 3.8 Performance of 250mL batch-flask reactors under dark, anaerobic conditions with no NO_3-N and 20 mg.L^{-1} PO_4-P added. Graph A represents the nitrate and polysaccharide concentrations while graph B represents the pH, nitrate, ammonium and phosphate concentrations. In Graph A, the Y2 axis is used to indicate the concentration of TPS in mg.L^{-1} while in graph B, the Y2 axis is used to indicate the pH in the flasks.

In Figures 3.6A and 3.8A where appreciably little or no nitrate was present in the system and hence no nitrate reduction was taking place, there was no noticeable decrease in EPS concentration at any stage during the experiment. Furthermore, in flasks supplemented with nitrate to 20 mg.L^{-1}, EPS maxima concentrations were found to be at least two-fold higher than in flasks which were nitrate-free. In Figures 3.6A and 3.8A, EPS reached maximum concentrations of 22.3 $\pm$ 6.4 mg.L^{-1} and 32.9 $\pm$ 10.3 mg.L^{-1} respectively while in Figures 3.7A and 3.9A (results of experiments where nitrate was supplemented), EPS maxima were found to be 73.5 $\pm$ 40.6 mg.L^{-1} and 59.9 $\pm$ 38.1 mg.L^{-1} respectively.



A

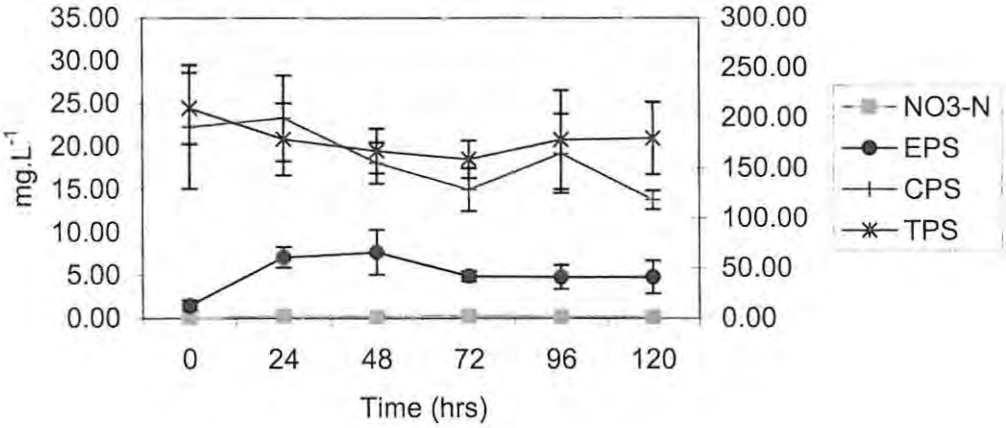


B

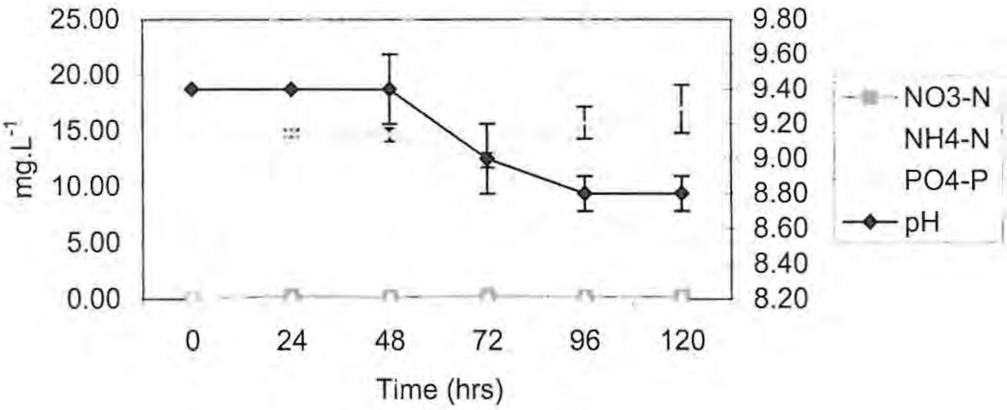
Figure 3.9 Performance of 250mL batch-flask reactors under dark, anaerobic conditions with 20 mg.L^{-1} NO_3-N and 20 mg.L^{-1} PO_4-P added. Graph A represents the nitrate and polysaccharide concentrations while graph B represents the pH, nitrate, ammonium and phosphate concentrations. In Graph A, the Y2 axis is used to indicate the concentration of TPS in mg.L^{-1} while in graph B, the Y2 axis is used to indicate the pH in the flasks.

Comparing overall exopolysaccharide concentrations in the 250 mL batch-flask reactor studies carried out under dark, aerobic conditions to EPS concentrations in the dark, anaerobic 250 mL batch-flask studies, one can see the elevated EPS maxima in the latter. Whereas EPS concentrations seldom reached 20 mg.L^{-1} in the dark, aerobic flask reactors, EPS maxima in the dark, anaerobic reactors reached 70 mg.L^{-1} (those flasks supplemented with 20 mg.L^{-1} NO_3-N as shown in Figure 3.7A). Total polysaccharide showed similar trends, with reactors incubated under dark, anaerobic conditions exhibiting greater TPS maxima (around 1000 mg.L^{-1}), nearly two-fold greater than in aerobic cultures.

3.4.3 Anaerobic Batch Flask Studies – 1 L



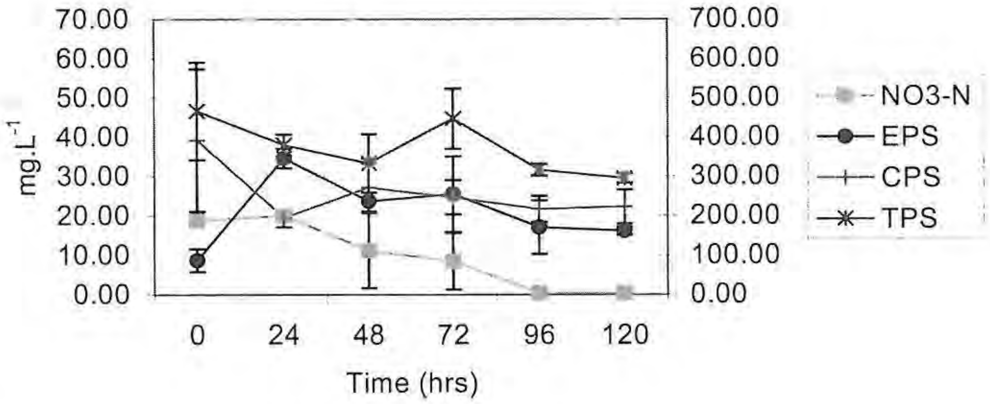
A



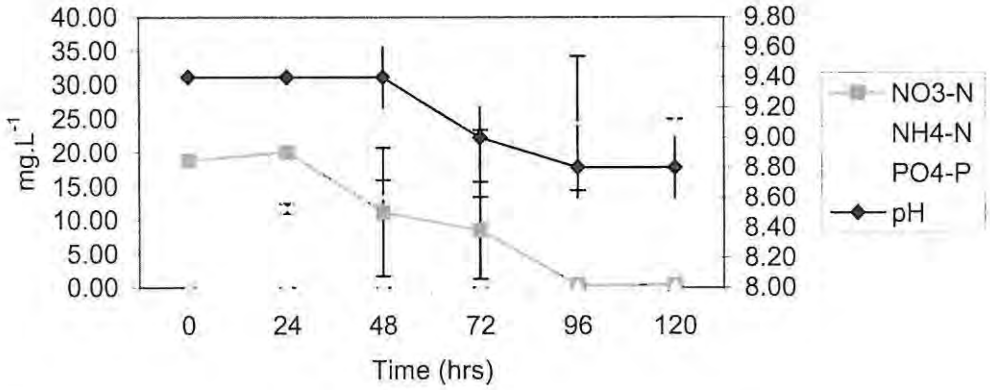
B

Figure 3.10 Performance of 1L batch-flask reactors under dark, anaerobic conditions without NO_3-N or PO_4-P added. Graph A represents the nitrate and polysaccharide concentrations while graph B represents the pH, nitrate, ammonium and phosphate concentrations. In Graph A, the Y2 axis is used to indicate the concentration of TPS in mg.L^{-1} while in graph B, the Y2 axis is used to indicate the pH in the flasks.

In the scaled-up 1 L dark, anaerobic batch flask experiments, a similar pattern was observed to the 250 mL dark, anaerobic batch flask experiments. Once again, the flasks that had been supplemented with nitrate (as seen in Figures 3.11A and 3.13A) showed EPS maxima two-fold greater than in flasks that had not been supplemented with nitrate (as seen in Figures 3.10A and 3.12A) and concomitant nitrate removal in NO_3-N-supplemented flasks with EPS concentrations was once again observed.



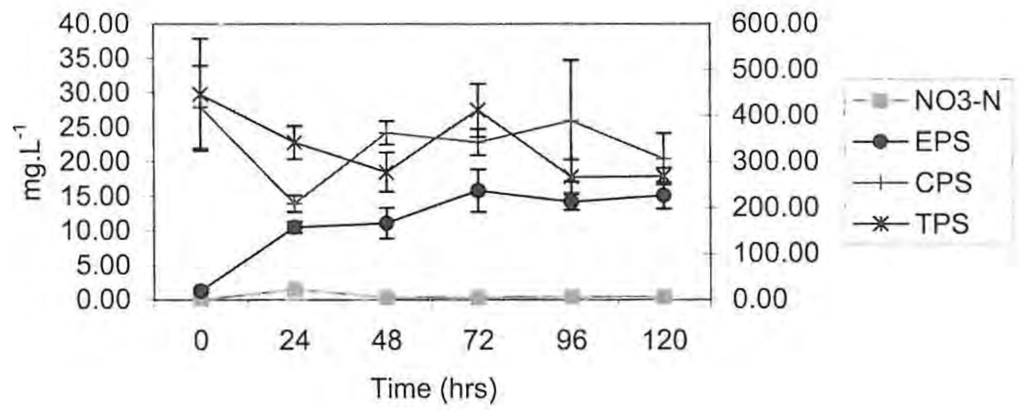
A



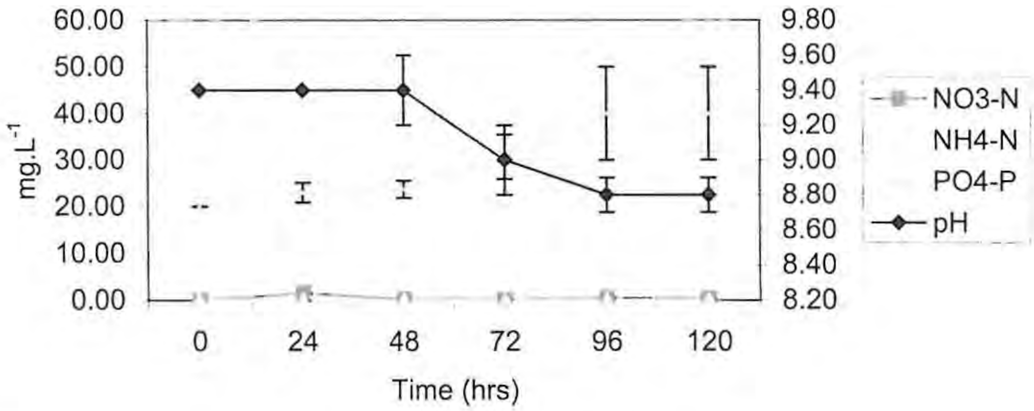
B

Figure 3.11 Performance of 1L batch-flask reactors under dark, anaerobic conditions with 20 mg.L^{-1} NO_3-N and no PO_4-P added. Graph A represents the nitrate and polysaccharide concentrations while graph B represents the pH, nitrate, ammonium and phosphate concentrations. In Graph A, the Y2 axis is used to indicate the concentration of TPS in mg.L^{-1} while in graph B, the Y2 axis is used to indicate the pH in the flasks.

The similar patterns of nitrate and EPS concentrations seen in Figures 3.11 and 3.13 give an indication of the relationship between denitrifying organisms present in the algal mixture and available carbon source for heterotrophic denitrification. It is interesting to note in Figure 3.11A that within the first 24 hour period, the CPS concentration decreased by 20 mg.L^{-1} from 40 mg.L^{-1} while there was a corresponding increase of 20 mg.L^{-1} in EPS concentration. This would suggest that under the conditions present in the culture vessel, some capsular polysaccharide was sloughed from the algal cells and released into the surrounding medium to become available as exopolysaccharide.



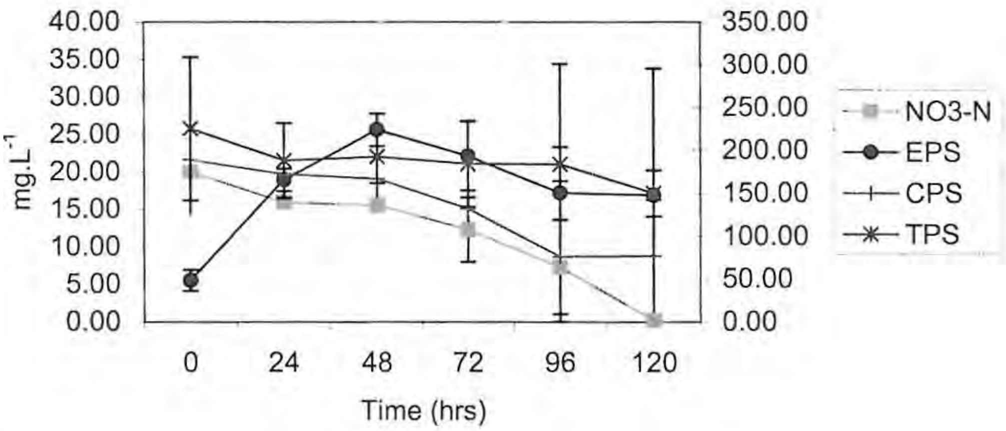
A



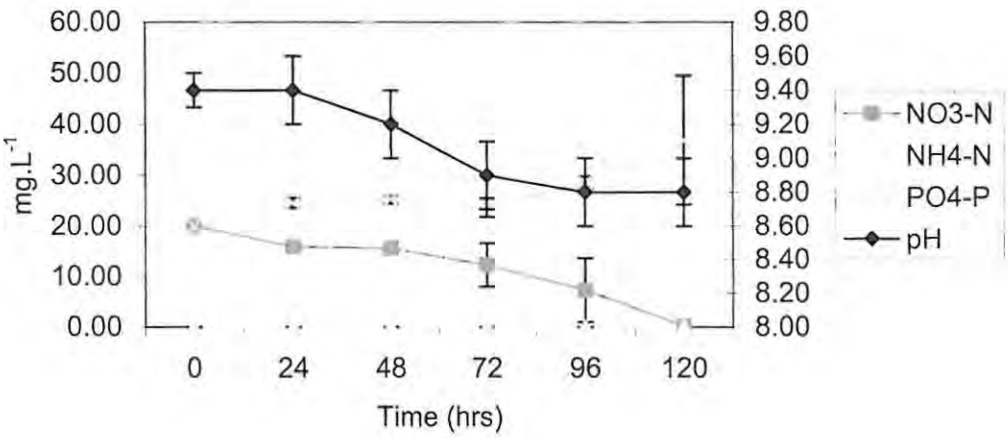
B

Figure 3.12 Performance of 1L batch-flask reactors under dark, anaerobic conditions with no NO_3-N and 20 mg.L^{-1} PO_4-P added. Graph A represents the nitrate and polysaccharide concentrations while graph B represents the pH, nitrate, ammonium and phosphate concentrations. In Graph A, the Y2 axis is used to indicate the concentration of TPS in mg.L^{-1} while in graph B, the Y2 axis is used to indicate the pH in the flasks.

Flask reactors in which no nitrate was present (and hence no denitrification taking place) showed no change in EPS reserves and reached an equilibrium at around 5 mg.L^{-1} EPS where there were no nutrients (Figure 3.10) and 15 mg.L^{-1} where only phosphate was supplemented. This pattern goes further to establish the relationship between denitrification and the availability of a carbon source in the form of EPS for heterotrophic denitrification.



A



B

Figure 3.13 Performance of 1L batch-flask reactors under dark, anaerobic conditions with 20 mg.L^{-1} NO_3-N and 20 mg.L^{-1} PO_4-P added. Graph A represents the nitrate and polysaccharide concentrations while graph B represents the pH, nitrate, ammonium and phosphate concentrations. In Graph A, the Y2 axis is used to indicate the concentration of TPS in mg.L^{-1} while in graph B, the Y2 axis is used to indicate the pH in the flasks.

The sloughing of CPS into the surrounding medium to become part of the EPS fraction of polysaccharide has been discussed. From Figure 3.13 however, it seems as though CPS may also be available directly to some extent as a carbon source for heterotrophic bacteria. The dramatic increase in EPS observed over the first 48 hours in Figure 3.13 may be as a result of CPS dissolution, however the most marked decrease in CPS levels only occurred after 48 hours, once the EPS had already reached its maximum. This points to some aspects of algal physiology that have been observed by other researchers (Vincenzini *et al.*, 1990 ; De Philippis *et al.*, 1996), who noted that nitrogen starvation has been found to stimulate EPS release in both microalgae and cyanobacteria, although they go on to state that this behaviour does not seem to respond to a general rule.

3.5 CONCLUSIONS

1. A correlation between requirements for stress physiology with polysaccharide release and denitrification was identified.
2. Under aerobic conditions, little or no change was observed in ammonia concentrations. Nitrate concentrations also remained predominantly unaffected indicating that little or no denitrification was occurring.
3. Conditions of anoxia and the presence of nitrate promote the production of polysaccharides (with TPS maxima ranging between 800 – 1000 $mg.L^{-1}$) by microalgae and the concomitant reduction of nitrate.
4. Under aerobic conditions, polysaccharide was consumed and TPS maxima rarely exceeded 500 $mg.L^{-1}$, regardless of the nutrient regime.
5. The stress conditions examined (photosynthetic vs anoxic, low nutrients vs high nutrients) have major implications for the design of an I-HRAP integrating N-removal.
6. Since phosphorus is released during N-removal due to decreasing pH, a secondary I-HRAP may be required to follow an anoxic denitrification step.

CHAPTER FOUR

DENITRIFICATION COLUMNS & REACTORS

4.1 INTRODUCTION

4.1.1 Upflow Anaerobic Sludge Blanket (UASB) Reactors

Upflow anaerobic sludge blanket reactors have been successfully implemented in a wide range of wastewater treatment facilities since the late seventies (Lettinga & Hulshoff Pol, 1991). Anaerobic digestion is an important part of wastewater treatment processes, whether they rely on conventional systems or biological systems and, taking into account the increasing requirement for optimum land usage, UASB offer an excellent alternative to conventional anaerobic digesters.

Biological denitrification is a process which by its very nature as an anoxic process lends itself to adaptation to UASB technology. In the early stages of UASB design and implementation, it was recognised that packed bed reactors offer a particular advantage over suspended or fluidised sand bed reactors since in the former, a high sludge concentration can be maintained with little or no washout of particles in the effluent (Klapwijk *et al.,* 1981). Figure 4.1 illustrates a typical UASB design for a denitrification reactor.

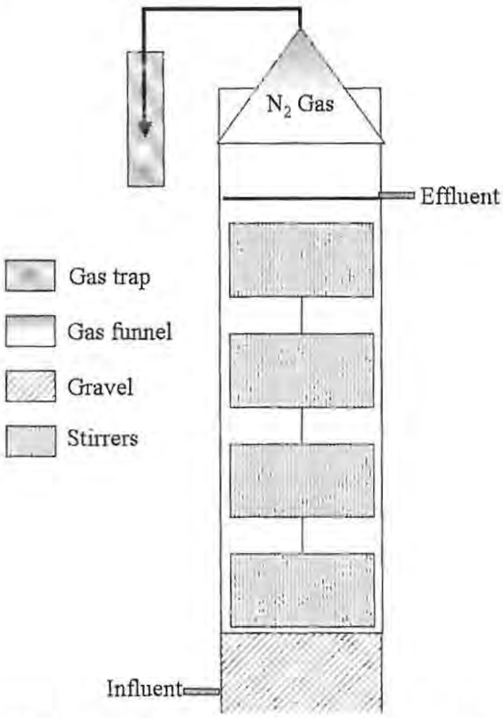


Figure 4.1 A typical UASB design for denitrification (after Klapwijk *et al.*, 1981).

Figure 4.2 illustrates the design of a UASB reactor supporting denitrification. The N_2 gas released during denitrification could be captured and used for industrial processes. In conventional wastewater treatment systems, upflow sludge blanket technology has been limited to utilizing sludge as the packing material and little thought has been given to alternative packing. In the IAPS process, which relies heavily on the oxidizing ability of algae in the high rate algal ponds, the algae itself can act as a sludge packing material through which wastewater can be passed to remove organic nutrients and other unwanted material. This process is a novel design and will be discussed in more detail later.

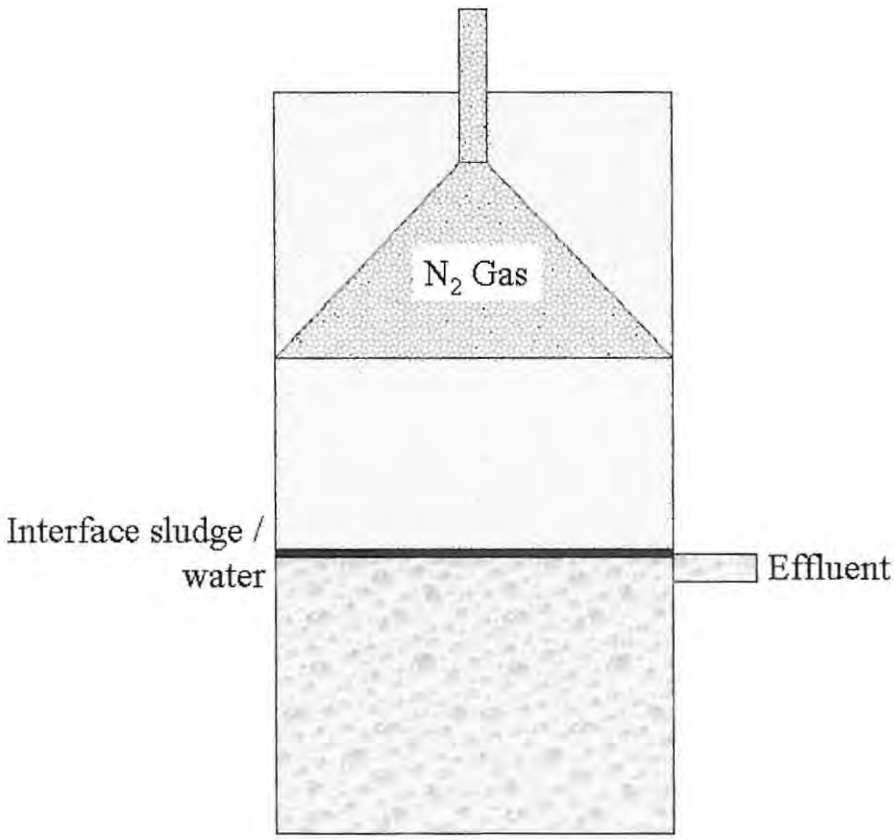


Figure 4.2 **Sludge particles above a sludge bed (after Klapwijk *et al.*, 1981).**

4.2 RESEARCH OBJECTIVES

1) Demonstrate denitrification in a bench-scale reactor utilising algal biomass as a carbon source for heterotrophic bacteria.
2) Scale up the flask reactor studies and examine three reactor configurations to determine optimal conditions for denitrification:

2.1) To examine the use of stress physiology of algae to produce EPS to support heterotrophic denitrification, without destroying the algal cells, and then returning the algae to the HRAP for subsequent phosphorus removal;

2.2) To examine the use of algal biomass as a carbon source for denitrification, where degradation of the algal biomass releases carbon for heterotrophic nitrate reducers;

2.3) To examine a combination of the above configurations.

3. Scale up reactors to pilot scale for processes evaluation.

4.3 MATERIALS AND METHODS

4.3.1 Bench-scale NO_3 Removal

Three UASB-styled denitrification columns (shown in Figure 4.3) were set up in the laboratory as follows. Three glass cylinders with dimensions:

Height : 275 mm

Diameter : 50 mm

Volume : 0.54 L

were used as the vessels. Algal biomass was collected from the HRAP, settled for 2 hours and the supernatant decanted. Each cylinder was inoculated with 450 mL settled algal biomass. The total COD of the algal biomass in each column was 16,9 $g.L^{-1}$. The columns were stoppered with rubber bungs, each bung penetrated by two glass rods. The one rod reached only into the upper layer of the column and the second reached right down to the base of the column. The deep rod acted as the delivery tube for feed while the upper rod served as the sampling port. Before the experiment was started, each of the three columns were subjected to a vigorous 10 minute degassing with N_2 gas to render the environment anaerobic. Each column received a different type of feed. Column A received feed consisting of HRAP supernatant, after having the algal flocs removed by settling, plus the addition of 145 $mg.L^{-1}$ KNO_3 which has an equivalent NO_3-N concentration of 20 $mg.L^{-1}$. Column B received the same feed as column A except that the KNO_3 supplement was omitted. Column C received the same feed as column A except the algal flocs were not removed.

In all three cases, the feed was degassed vigorously with N_2 gas for 7 minutes to remove residual free O_2 and the feed sources were kept covered using aluminium foil with a foil cap sealing the mouth. All three feed sources were kept suspended using magnetic stirrers. All piping, tubes and columns were also covered with aluminium foil to prevent photosynthesis. Movement in the system was achieved using a Watson-Marlow peristaltic pump with an hydraulic retention time of 5 hours in each column, according to the following hydraulic retention time formula:

$$\text{HRT (hours)} = \frac{\text{Volume of Container (mL)}}{\text{Flow Rate (mL.hr}^{-1}\text{)}}$$

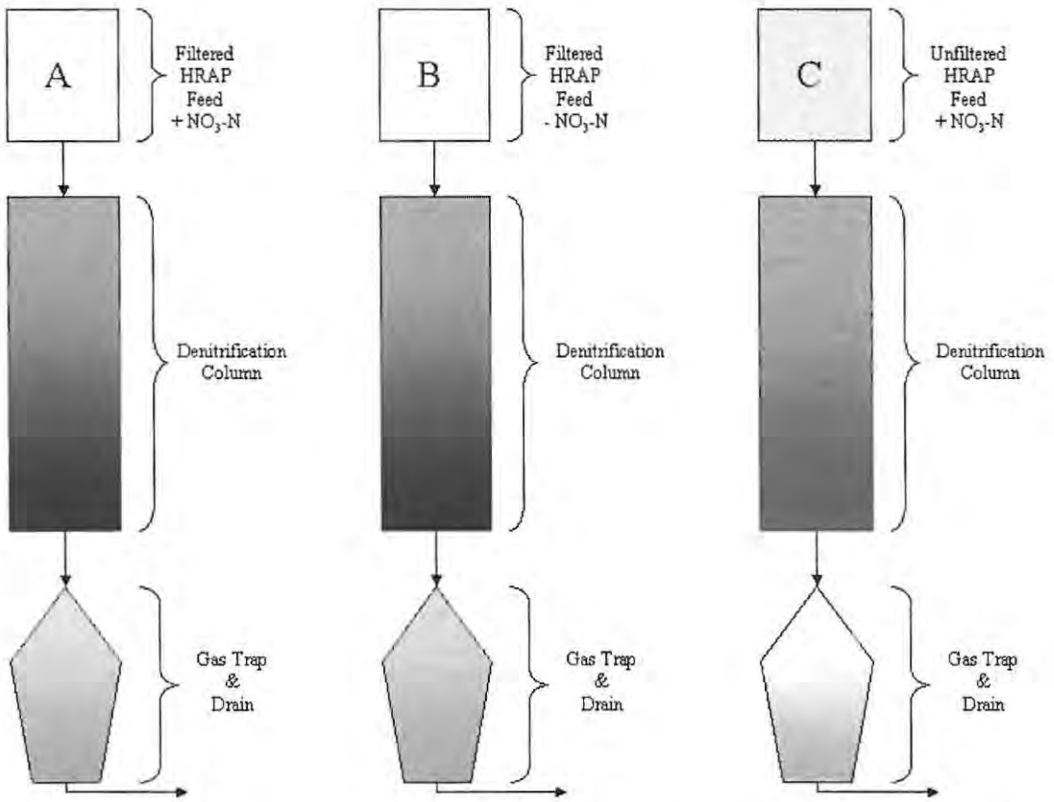


Figure 4.3 **Bench-scale denitrification reactors. Stream A received filtered HRAP with 20 mg.L^{-1} NO_3-N, stream B received filtered HRAP without the addition of NO_3-N and stream C received unfiltered HRAP with the addition of 20 mg.L^{-1} NO_3-N. The columns were packed with settled algal biomass. The gas traps were included in the event that gas samples may be analysed for N_2 gas production.**

The effluent was analysed daily for pH (using a gel electrode pH meter Cyberscan) and total COD, NO_3-N, NH_4-N and PO_4-P (MERCK Spectroquant® Kits – see Appendix A).

4.3.2 Lab-scale Denitrifying Reactors

In order to demonstrate the effectiveness of biological heterotrophic denitrification utilising metabolites from algal stress physiology, two denitrifying reactors were designed, built and operated over a period of 14 weeks.

The first reactor, which was termed the Degrading-bed Reactor, was styled on a typical wastewater UASB reactor as described by Lettinga & Hulshoff Pol (1991). This reactor, with a volume of 23.23 L, was operated with a 5.5 hour hydraulic retention time. On start-up, the reactor was packed with 5 L of algal sludge collected from one of the algal settling ponds at the IAPS plant. The reactor was then fed continuously with effluent from the high rate algal pond receiving GDWf effluent. Owing to the anaerobic nature of denitrification, the reactor was covered with aluminium foil to prevent photosynthesis by the algae.

The second reactor was designed as an anaerobic, flooded trickle filter. This reactor, termed the ANTRIC reactor, consisted of a glass cylinder covered with aluminium foil for reasons explained previously. The cylinder was initially packed with gravel (each with an average diameter of 4 cm) and filled (a liquid volume of 6.4 L) with high rate algal pond water containing a good fraction of algal flocs. This reactor was then connected in series after the Degrading-bed Reactor. The theory behind a flooded trickle filter is as follows: a liquid stream is pumped in at the head of the reactor and allowed to percolate under pressure through the packing material. It is proposed that under such conditions, biofilm development may occur more readily than in an UASB-styled reactor, and as a result of such algal-bacterial biofilm development, denitrification would be enhanced. A supplemental NO_3-N source was combined with the feed (Degrading-bed Reactor effluent) at a concentration of 20 $mg.L^{-1}$ NO_3-N. A schematic diagram of the denitrifying reactor setup is shown in Figure 4.4.

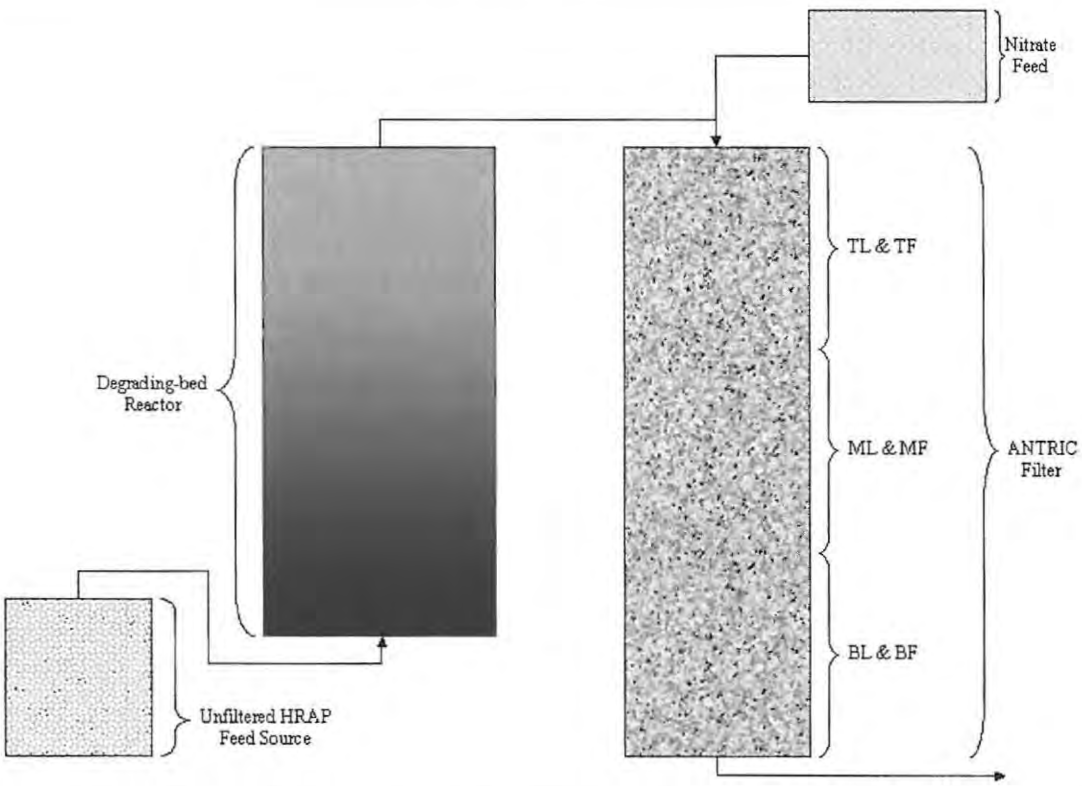


Figure 4.4 **Schematic diagram of the lab-scale denitrification reactor setup. The abbreviations used indicate sampling points FROT = Degrading-bed effluent before nitrate supplementation; AO = ANTRIC effluent; the L and F of TL, TF, ML, MF, BL and BF denote unattached algal sludge and attached algal biofilm respectively while the T, M and B of the same denote top, middle and bottom sections of the reactor respectively.**

4.4 RESULTS AND DISCUSSION

4.4.1 Bench-scale NO_3 Removal

The results of the bench-scale denitrification columns showed some interesting patterns. The columns being fed on high rate algal pond wastewater with the algal flocs removed showed lower nitrate removal from 20 mg.L^{-1} to around 15 mg.L^{-1} NO_3-N while the column receiving feed which included the algal flocs showed nitrate removal from 20 mg.L^{-1} to less than 5 mg.L^{-1} NO_3-N.

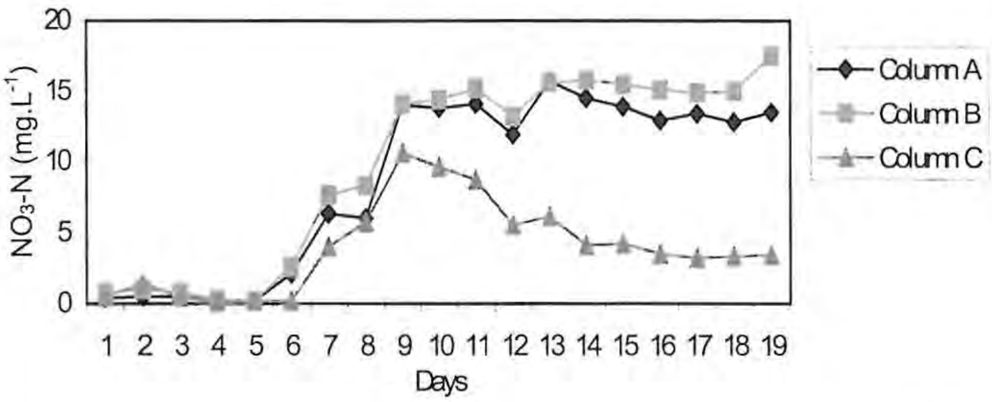


Figure 4.6 Nitrate studies in bench-scale denitrification columns. Column A fed with filtered HRAP wastewater, column B was fed with filtered HRAP wastewater with the addition of 20 mg.L^{-1} NO_3-N and column C was fed with HRAP wastewater including algal flocs and 20 mg.L^{-1} NO_3-N.

Other parameters were monitored occasionally to observe changes in mineral and wastewater characteristics (Figure 4.7). The pH of the HRAP feed was always found to be between 10 and 11 while the pH of the effluent from the columns fluctuated between 9 and 10. This slight decrease could be attributed to anaerobic processes such as methanogenesis which are acidogenic processes. Ammonium concentrations varied only slightly between feed and column effluent within the concentration range of 6.0 – 7.3 mg.L^{-1} NH_4-N. Since the conditions in the columns were by no means oxidative and there was no mixing of the substrate in the columns with the atmosphere, no nitrification or volatilisation of ammonium would have taken place. Orthophosphate concentrations increased slightly from feed to column effluent from a mean value of 0.6 mg.L^{-1} to 2.6 mg.L^{-1} during the experiment. Again, this increase could be due to the acidogenic conditions produced by anaerobic processes, resulting in dissolution of bound orthophosphate from their metal ions (Ca^{2+} in particular).

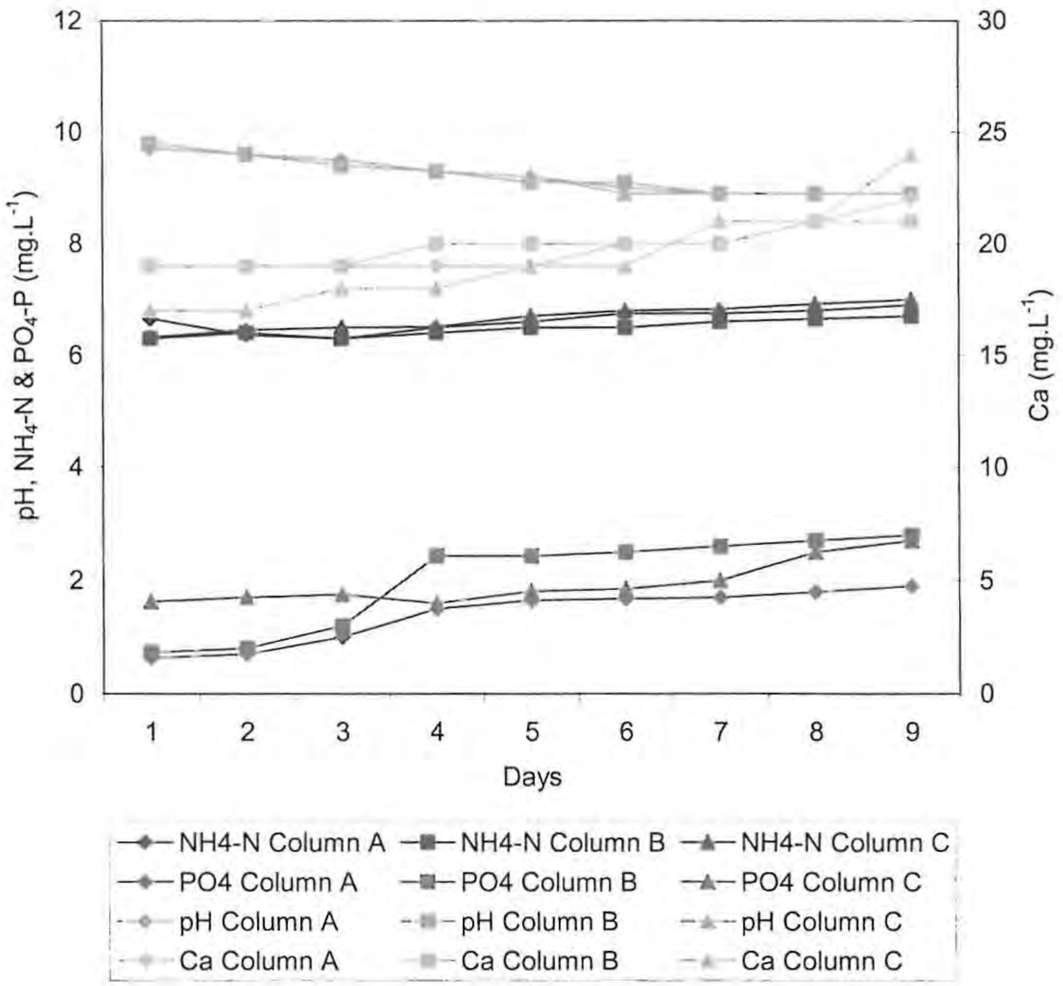


Figure 4.7 **Analysis of conditions in bench-scale denitrification columns.**

4.4.2 Performance of Lab-scale Denitrifying Reactors

The performance of two denitrification reactors are discussed below. These reactors were designed on the assumption that the denitrifying heterotrophic bacteria present in high rate algal pond wastewater are able to utilize algal polysaccharide as a carbon source for the process of denitrification. The first reactor was termed the Degrading-bed Reactor and functioned as a typical UASB reactor packed with algal sludge while the second reactor was termed the ANTRIC Reactor and functioned as an anaerobic, flooded trickle filter. These two reactors were connected in series.

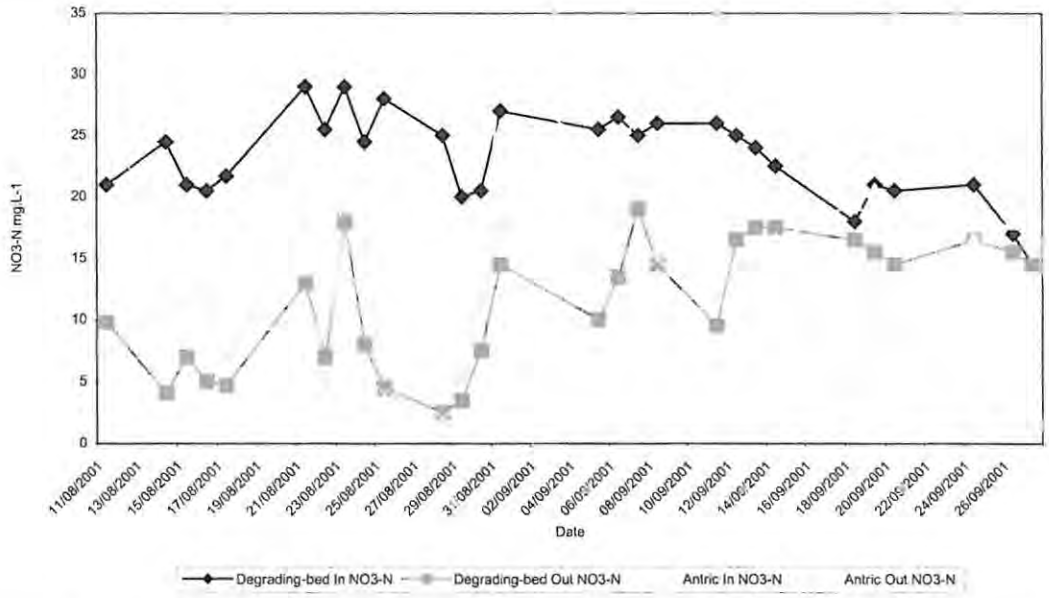


Figure 4.7 **Nitrate removal by the Degrading-bed Reactor and ANTRIC Reactor over a period of 14 weeks. Although the Degrading-bed effluent was fed directly to the ANTRIC filter, the feed was supplemented with a 20 mg.L^{-1} NO_3-N source.**

Figure 4.7 shows the nitrate removal performance of the two denitrifying reactors. Both reactors showed good nitrate removal capabilities, although the ANTRIC filter was able to sustain denitrifying activity more than the Degrading-bed Reactor. While the Degrading-bed initially showed better nitrate removal, the performance seemed to decline toward the seventh week. The state of the packed algal bed possibly reflected this trend. The algal sludge appeared condensed and the organic matter degraded to the extent that the amount of fresh algal biomass entering the bed contributed little or nothing to the available carbon reserves. The heterotrophic denitrifying population in the algal sludge therefore was probably inactivated.

The ANTRIC filter on the other hand continued to show good nitrate removal at the end of the 14 week experimental period. The fact that the two reactors were connected in series would imply that the ANTRIC Reactor could only receive a carbon source from the Degrading-bed Reactor and if that reactor was no longer providing denitrifying conditions, the question remained, where was the carbon source for denitrification in the ANTRIC originating? The answer to this seems to be in the design of the Degrading-bed Reactor. Since the algal sludge bed had become so packed, degradation and subsequent release of available carbon continued to take place but instead of the carbon being made available to the heterotrophic community present in the sludge bed, the majority of it was released into the upflow column and passed directly into the ANTRIC filter where the heterotrophic community present in the biofilm around the packing material were able to utilize it. This would seem to indicate again the benefit of biofilms over sludge beds or free-floating biomass for intense microbial activity.

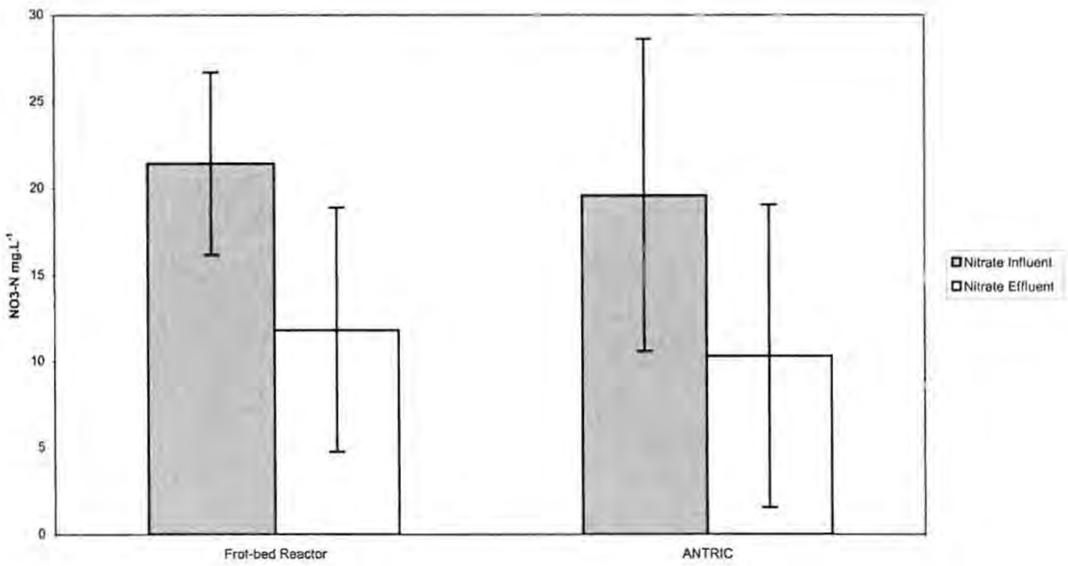


Figure 4.8 **Overall nitrate removal by Degrading-bed Reactor and ANTRIC Reactor. Error bars reflect changes in effluent makeup over a 14 week period.**

4.5 CONCLUSIONS

1) In the bench-scale reactors, denitrification was demonstrated but phosphorus levels were shown to increase due to a decrease in pH. As a result, the need to include the denitrifying reactor in front of the I-HRAP was identified.

2) In the degrading-bed reactor, degradation of algal biomass provides sufficient carbon for heterotrophic denitrification, however a build-up of degraded biomass caused inhibition of denitrification either due to substance toxicity or to a decrease in available carbon.

3) In the ANTRIC reactor, stress manipulation of metabolically active algal cells also supplied sufficient energy to drive denitrification and had the

added benefit of recycling algal biomass to an HRAP for subsequent phosphorus removal.

4) While field trial scale-up reactors were under construction for further detailed analysis, preliminary findings indicated that the denitrification reactors would enhance the nutrient removal capacity of an I-HRAP.

CHAPTER FIVE

MOLECULAR MICROBIAL ECOLOGY

5.1 INTRODUCTION

5.1.1 Development of Molecular Tools for Environmental Biotechnology

The identification of many of the key organisms in both the denitrification process and algal ponding systems has been limited to those microbes that can be cultured on artificial growth medium or directly identified by microscopic techniques. There are however many more fastidious microbes playing vital roles in these processes, but which are considered to be either viable but non-culturable (VBNC) or to have growth requirements which, if implemented in the laboratory, are too expensive to maintain.

As a result of the shortcomings of these traditional techniques, scientists have developed new tools for *in situ* and *ex situ* detection and identification of bacteria. One such novel approach involves the sequence analysis of the highly conserved 16S rRNA gene. In particular, a method termed denaturing gradient gel electrophoresis (DGGE) has been used to profile complex microbial communities based on analysis of the 16S rRNA gene segments or other functional genes (Muyzer *et al.,* 1993; Ferris *et al.,* 1996). Denaturing gradient gel electrophoresis is an experimental method in which a denaturant is used to exploit differences in DNA melting points dependent on GC ratios. If comprehensive sequence scrutiny is desired, careful manipulation of melting domains and use of GC clamps may be necessary. In this method, one of the PCR primers can include a GC clamp on its 5' end which imparts a melting stability to the products in the gel. In general, 45 bp clamps appear to work consistently, whereas shorter clamps are less predictable. The products are then separated by electrophoresis on a polyacrylamide gel containing increasing linear concentration gradient of chemical denaturants such as formamide and urea. Denaturant concentration is specified as percent of an arbitrary standard (100 % denaturant is defined as 40 % (v/v) formamide plus 7.0M urea). An acrylamide concentration of 8 % affords adequate resolution of 0.15 – 1 kb fragments. To obtain conditions where dsDNA is close to melting, the gel is immersed in a heated bath of electrophoresis buffer. During this process, individual double-stranded DNA molecules denature along their length adjacent to the GC clamp according to their melting characteristics.

Mobility is determined by melting behaviour and not fragment length. Denaturation involves the melting domain reaching its melting temperature at a particular position on the gel. At this point, a transition of helical to partially melted molecule occurs (termed helix-to-random coil transition) and migration virtually stops. Sequence-dependent conformational changes are detected as variations in migration distance arising from the effect of partial melting on electrophoretic mobility. Each resulting band may then represent a different microbial population (Myers *et al.*, 1987; Abrams & Stanton, 1992).

5.1.2 Methodological Developments and Limitations

The obvious limitation to these molecular-based methods is the ability to maintain amplification of high-fidelity PCR products. Several methods of ensuring both efficiency and specificity of the amplification process have been developed

PCR amplification of low-copy-number targets is vulnerable to interference by the amplified extension of primer pairs annealed to non-target nucleic acid sequences in the test sample (mis-priming) and by the amplified extension of two primers across one another's sequences without significant intervening sequence (primer dimerisation). Most of the observed mispriming and primer dimerisation arises during the customary and poorly controlled interval (time-scale of minutes) that reactants are mixed at room temperature before starting an amplification. In Hot Start PCR, complete mixture of all reactants is delayed until they have been heated to a temperature which prevents primer annealing to non-specific sequences. This method increases amplification efficiency and specificity to the point that non-probed and nonisotopically probed detection become practical and routine (Chou *et al.*, 1992).

The appearance of spurious smaller bands in the product spectrum of PCR amplification may be due to mis-priming by one or both of the oligonucleotide amplimers internal or external to the target template. Adjustments to the Mg^{2+} concentration or increasing the annealing temperature of the PCR may solve this problem. Such empirical determinations are time-consuming and need to be implemented with optimisation of each sample type. As a simple solution, beginning the amplification cycle at or above the expected annealing temperature and then decreasing the annealing temperature in a stepwise gradient to the "touchdown" annealing temperature, spurious amplifications are avoided (Don *et al.*, 1991).

It is clear that the great phylogenetic diversity of bacteria capable of denitrification makes the classical approach of using 16S rRNA gene sequences to detect and analyse denitrifying bacterial communities in environmental samples unrealistic. That is to say direct analysis of microbial communities based on 16S rRNA alone is not always appropriate to investigate a functional group of the bacterial community consisting of several phylogenetically different genera and species such as denitrifiers.

Amplified ribosomal DNA restriction analysis (ARDRA) can be used in culturable organisms to (i) evaluate for example, the presence of the gene encoding the N_2O reductase and (ii) analyse the physiology of N_2O production and reduction by isolates from a denitrifying community (Chèneby *et al.*, 2000). Alternatively, functional probes (for Southern blotting and hybridisation) and PCR primers based on structural *nir* genes to detect denitrifying bacteria should be considered.

5.1.3 The Molecular Biology of Denitrification

Biological denitrification is a respiratory process involving the reduction of nitrate and nitrite with the evolution of nitric oxide (NO), nitrous oxide (N_2O) and molecular nitrogen (N_2) gases. The reduction pathway is completely catalysed by enzymatic activity and in particular involves the reduction of nitrite by the enzyme nitrite reductase (Nir) (Hochstein & Tomlinson, 1988).

5.1.3.1 Nitrate Reductase

The first step in dissimilatory denitrification is the reduction of nitrate (NO_3^-) to nitrite (NO_2^-). This reaction is controlled by the dissimilatory nitrate reductase gene. On the structural level, there exist three types of nitrate reductases. Two of these are membrane-bound while the third is periplasmic (Bell *et al.*, 1990; Iobbi-Nivol *et al.*, 1990). The major membrane-bound nitrate reductase is termed NarGHI and is composed of three subunits, α, β and γ. The α and β-subunits constitute the cytoplasmic domain and the γ-subunit constitutes the membrane domain required for attachment of the α-and β-subunits to the cytoplasmic side of the inner membrane (Philipot & Højberg, 1999). The γ-subunit is a 19 – 28kDa hydrophobic protein encoded for by the *narI* gene. The larger β-subunit is a 43 – 63kDa globular protein containing iron sulphur clusters and is encoded for by the *narH* gene. The α-subunit (104 – 150kDa) is encoded for by the *narG* gene and it has been reported that this subunit is the active site of nitrate reduction (Philipot & Højberg, 1999).

The second membrane-bound nitrate reductase, termed NarZYV, has only been identified in *E.coli*. There exist many similarities between the two membrane-bound nitrate reductases. Both have the same α β γ-subunit composition with the NarZYV having an α-equivalent subunit of 150kDa encoded for by the *narZ* gene and a β-equivalent subunit of 60kDa encoded for by the *narY* gene (Iobbi-Nivol *et al.,* 1990). Both enzymes catalyse the reduction of nitrate and both are inhibited by azide. Philipot & Højberg (1990) proposed that the presence of NarZYV may assist *E.coli* in the transition from aerobic to anaerobic metabolism.

The third dissimilative nitrate reductase located in the periplasm of various bacteria is termed Nap. This Nap protein is distinct from the membrane-bound nitrate reductases in that it only has two subunits. These subunits are termed NapA (63 – 93kDa) and NapB (13 – 19kDa). It has been proposed that Nap is involved in maintaining the redox balance of the cell during transition from aerobic to anaerobic growth or in aerobic denitrification (Bell *et al.,* 1990).

5.1.3.2 Nitrite Reductase

Two known and distinct types of nitrite reductase enzymes exist, although they are mutually exclusive in any given strain. The first contains heme *c* and heme d_1 proteins of cytochrome type *cd* (cd_1-Nir) and the other is a copper-containing metalloflavoprotein (Cu-Nir). Strains in which cd_1-Nir has been identified include *Alcaligenes faecalis*, *Paracoccus denitrificans*, *Pseudomonas aeruginosa*, *Pseudomonas perfectomarinus* and *Thiobacillus denitrificans*. Although not as abundant in the bacterial genera, the copper reductases exhibit greater variation in molecular weight and immunological reactions. Furthermore, they are present in more taxonomically unrelated strains such as *Achromobacter cycloclastes*, *Pseudomonas denitrificans* and denitrifying strains of *Rhodopseudomonas sphaeroides*.

5.1.3.3 Nitric Oxide Reductase

A few types of nitric oxide reductases have been found in microorganisms. The most common type contains cytochrome *bc*. It would appear that the nitric oxide reductases possess a broader physiological role than simply the reduction of nitric oxide to nitrous oxide. For example, the nitric oxide reductase in *Neisseria gonorrhoeae* has been shown to be required for anaerobic growth (Ye & Thomas, 2001).

5.1.4 Current State of the Field and Advances

All molecular studies on the ecology of denitrifying bacteria are based on functional genes and their products. Genes involved in denitrification (*nar*, *nor*, *nir*S, *nir*K and *nos*) contain highly conserved DNA regions which can be successfully exploited for developing gene probes. The conversion of nitrite to nitric oxide (or nitrous oxide) is the crucial step in the reaction sequence because it leads to gas formation. Therefore, most of the ecological work has been done with probes for both types of nitrite reductases. It is more difficult to design broad-range primers or probes for *nir*S than for *nir*K. A general probe recognizing both *nir*S and *nir*K cannot be developed, since these structurally different enzymes are encoded by genes which do not share sequence homology. While PCR and probing with oligonucleotides or antibodies are primarily used to study the structure of nitrifying and denitrifying communities, studies of their function in natural habitats, which require quantification at the transcriptional level, are currently not possible (Bothe *et al.*, 2000).

5.2 RESEARCH OBJECTIVES

1) To investigate the heterotrophic bacterial populations involved in denitrification using molecular techniques.
2) Develop molecular methods for the analysis of bacterial populations associated with denitrification in the ANTRIC reactor.
3) Determine whether the bacterial populations were primarily associated with the biofilm on the substrate in the reactor or loosely associated with the algal biomass.

5.3 MATERIALS & METHODS

Molecular techniques were employed to analyse the communities involved in the process of denitrification. The purpose of this section of the work was to optimise the procedures needed for biofilm community analysis.

5.3.1 DNA Extraction

Two chromosomal DNA extraction methods were compared. The first and preferred method was the so-called PCI or phenol-chloroform-isoamyl alcohol method (see Appendix C). In this method, bacterial cell walls were ruptured using lysozyme, followed by four freeze-thaw transfers. Remaining cell wall and membrane matter protecting DNA was removed using SDS, proteinase K, CTAB and sodium chloride. Chromosomal DNA was separated and pelletted using phenol:chloroform:isoamyl alcohol in the recommended ratio of 25:24:1. Extracted DNA was precipitated from solution using ice cold rectified ethanol, air dried, resuspended in minimal TE buffer (0.01 M Tris-HCl, 0.001 M EDTA, pH 8.0) and stored at –20°C for further use.

The second method of DNA extraction tested was a modification of the High Pure PCR Template Preparation Kit (Cat. No. 1 796 828 Boehringer Mannheim). This technique of DNA isolation is based on the ability of nucleic acids to bind specifically to the surface of glass fibre fleece in the presence of a chaotropic salt. Since the binding reaction is specific for nucleic acids, impurities such as salts, proteins and other cellular debris may be removed by a washing step. Purified nucleic acid may then be eluted off the glass fibre columns in a pH controlled step using 10 mM Tris, pH 8.5. The modified protocol used involved a combination of the procedures for nucleic acid isolation from whole blood and tissue samples. The sample was pelletted for 30 seconds at 13000 rpm. To the drained pellet, 200 μL tissue lysis buffer and 15 μL lysozyme (10 $mg.L^{-1}$ in Tris-HCl, pH 8.0) was added and the mixture incubated for 30 minutes at 37°C. To this, 40 μL Proteinase K was added and the mixture incubated at 55°C for 1 hour. Next, 200 μL binding buffer was added and the mixture incubated for 10 minutes at 72°C. After incubation, 100 μL isopropanol was added and the mixture pipetted into a High Pure filter column. The column was centrifuged for 1 minute at 8000 rpm, the flowthrough discarded and the collection tube changed. A volume of 500 μL wash buffer was pipetted into the filter tube and cenitrifuged as above. This wash step was repeated and finally centrifuged at 13000 rpm for 30 seconds. The collection tube was discarded and the filter tube fitted into a 1.5 mL reaction tube. The nucleic acids were then eluted off the column matrix using 200 µL prewarmed (70°C) elution buffer, centrifuging at 8000 rpm for 1 minute. The elution step was repeated to increase elution volume. The nucleic acids were stored at -20°C for further use.

5.3.2 Polymerase Chain Reaction Amplification of the 16S rRNA Gene

As mentioned previously, the amplification of high fidelity product is vital for successful manipulation of any system involving molecular biology. For this reason, the bulk of the present study focused on optimising the PCR process for 16S rRNA of denitrifying populations.

Initially *Taq* DNA Polymerase (Cat. No. 1 647 679 Roche Diagnostics GmbH) was used but the research group soon moved over to using the Expand High Fidelity System (Cat. No. 1 732 641 Roche Diagnostics GmbH) owing to the ability of the system to give PCR products with high yield, high fidelity and high specificity from episomal and genomic DNA. A further advantage of Expand System-generated products is the appearance of 3' single A overhangs which are useful for later cloning steps.

The standard protocol used in setting up PCR reactions was as follows (final concentration per reaction) : 200 µM each of dATP, dCTP, dGTP and dTTP (Cat. No. 1 969 064 Roche Diagnostics GmbH), 200 nM each of a forward and reverse primer (GM5F and 907R GC respectively, Integrated DNA Technologies – see Table 5.1), <1 $\mu g.100\mu L^{-1}$ template DNA, PCR reaction buffer with 1.5 mM $MgCl_2$ and 2 units Expand High Fidelity Taq DNA polymerase. All PCR solutions and dilutions were made with sterile Milli-Q water (>18 $M\Omega.cm^{-2}$) and kept on ice until used.

Table 5.1 PCR primer sequences (from Santegoeds *et al.*, 1998).

Primer	Sequence
GM5F	5'-CCTACGGGAGGCAGCAG-3'
907R with GC-clamp at 5' end for DGGE	[a]5'-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG CCGTCAATTCCTTTGAGTTT -3'
907R	5'-CCGTCAATTCCTTTGAGTTT-3'

[a] shaded sequence indicates GC-clamp

In order to improve amplification fidelity, various optimisation changes were made. These changes included varying DNA template concentrations, making use of commercially available nucleic acid purification columns (Zymo Research's DNA Clean & Concentrator™), addition of the detergent Dimethyl Sulfoxide (DMSO), altering $MgCl_2$ concentrations and setting up different temperature programmes during PCR cycling.

From work carried out in the Department of Microbiology, Biochemistry and Biotechnology at Rhodes University, Grahamstown, South Africa, it was shown that substantial dilutions of DNA template from ancient tissue samples (often up to 1/10 000) show marked improvement of amplification product (Professor Greg Blatch *pers. comm.*, 2001). Owing to the sensitivity of the PCR reaction, the presence of inhibitory or other anomalous material may adversely affect amplification. This same property of the PCR reaction also allows for the amplification of minute quantities of DNA. Therefore, dilutions of 1/10, 1/50, 1/100, 1/1000 and 1/1500 were tested in order to determine whether or not amplification was being affected by inhibitors.

High background and non-specific primer-template annealing are often dealt with by changing the concentration of Mg^{2+} in the reaction mixture. Magnesium concentrations of 0.5 mM, 1 mM, 2 mM, 3 mM and 4 mM were evaluated. Furthermore, although it is not usually necessary to add detergents, the addition of up to 2 % DMSO may improve amplification specificity. In the work carried out in this project, addition of 0.25 % DMSO was assessed.

Finally, amplification cycles were addressed. In order to increase product yield and ensure correct primer-template annealing, various protocols have been developed. One such protocol is "hot start" PCR in which the *Taq* polymerase is added at temperatures above the oligonucleotide primer annealing temperature. In so doing, amplification specificity is improved because mis-annealed primers, which occur at lower annealing temperatures, are not extended to generate non-target products (Chou *et al.*, 1992). In this procedure therefore, the PCR reaction mixture was exposed to an initial denaturation step in the Eppendorf PCR MasterCycler of 98°C for five minutes followed by a two minute hold at 80°C at which stage the *Taq* polymerase was added and the cycles continued.

A second approach known as "touchdown" PCR involves setting the annealing temperatures above the theoretical melting temperature of a primer-template pair for the first few cycles and then decreasing the annealing temperature of subsequent cycles in a stepwise fashion to allow correct primer-template pairs to form before spurious priming of non-specific products (Don *et al.*, 1991). The standard thermal cycling conditions used were as follows: 16 cycles of 94°C for 30 seconds; annealing temperature stepdowns every 4 cycles of 2°C (from 68°C to 62°C); 72°C for 3 minutes. The annealing temperature for the final 15 cycles was 60°C with denaturation and extension phases as above. A final extension phase of 72°C for 5 minutes with a 4°C hold ended to amplification cycle. An alternative set of touchdown temperatures was also evaluated: as above but from 62°C to 58°C, with the final 15 cycles run with an annealing temperature of 56°C.

All PCR amplifications were examined on 1 % molecular grade agarose gels in TBE buffer (10.8 $g.L^{-1}$ Tris, 5.5 $g.L^{-1}$ boric acid, 4 $mL.L^{-1}$ 0.5 M EDTA, pH 8.0), run at 80V for 45 minutes. Agarose gels contained ethidium bromide for visualising nucleic acid products under ultraviolet light. The standard nucleic acid marker used was λ DNA, cut using the endonuclease *Pst*I. A standard gel loading buffer (0.25 % bromophenol blue, 0.25 % xylene cyanol FF, 30 % glycerol) was used in all agarose and acrylamide gel work.

5.3.3 Denaturing Gradient Gel Electrophoresis

Products of 16S rRNA PCR amplification were electrophoresed on a denaturing gradient gel, a procedure useful for separating similar products based on their GC content. For this procedure, a 10 x 10 cm Vertical Electrophoresis Unit (Z33,956-3 Sigma-Aldrich) was used. Depending on the denaturing gradient to be used in the experiment, denaturing solutions were made up accordingly. A 50 mL stock of 0 % denaturant consisted of 5mL 10 x TAE (0.4 M Tris-acetate, 0.01 M EDTA, pH 8.0), 7.5 mL 40 % acrylamide and 37.5mL Milli-Q water. A 50mL stock of 100 % denaturant consisted of 5 mL 10 x TAE, 7.5 mL 40 % acrylamide, 20 mL deionised formamide, 21 g molecular grade urea and the volume brought up to 50 mL with Milli-Q water. After 15 minutes of degassing in a vacuum jar, 20 μL 20 % APS and 4μL TEMED were added to 3.7 mL of each of the denaturant solutions. Gradients were poured using a Model 385 Gradient Former (165-2000 BIORAD) and allowed at least an hour at room temperature to set. The gel apparatus was then inserted into the gel tank which was filled with preheated (65°C) 1 x TAE buffer. After the comb was removed, the wells were flushed using TAE buffer from the gel tank to displace excess acrylamide and samples were loaded using long-nosed sterile tips to prevent damage to the gel. The gel was then electrophoresed for 2 hours at 120V. During electrophoresis, the buffer was maintained at a constant 65°C with gentle mixing.

Results were checked first by staining the denaturant gel with ethidium bromide and viewing the gel on a UV transilluminator followed by the more sensitive silver staining procedure, a method adapted from the QIAGEN TGGE manual. Gels were soaked in fixing solution (10 % ethanol, 0.1 % acetic acid v/v in Milli-Q water) for 6 minutes. The buffer was discarded and the gel immersed in 0.17 % w/v silver nitrate in Milli-Q water and stained for 10 minutes. Next the gel was rinsed and excess silver ions removed by washing twice in Milli-Q water. The gel was then soaked in developer (1.5 % w/v sodium hydroxide, 0.01 % w/v sodium borohydride and 0.15 % v/v formaldehyde in Milli-Q water) for no more than 40 minutes (usually 5 – 10 minutes proved sufficient). The reaction was stopped by washing the gel in fixing solution again for 30 seconds. Finally the gel was viewed on a visible light transilluminator.

Visible bands were individually excised from the denaturing gels and the nucleic acid extracted using QIAEX II Polyacrylamide Gel Extraction Protocol (QIAGEN 20021). The extracted DNA was then subjected either to further PCR or cloned into pGEM-T Easy vector (Promega Cat. No. A1360).

5.3.4 Cloning

PCR products were cloned into the pGEM-T Easy vector plasmid (Promega Cat. No. A1360). Ligation reactions were performed as follows (all steps were performed on ice and competent cells pipetted gently): combine 5 µL 2 x ligation buffer, 1 µL pGEM vector (50 ng), 2 µL PCR product, 1 µL T4 DNA ligase (3 $U.\mu L^{-1}$) and Milli-Q H_2O to a final volume of 10 µL. The reaction was incubated for 2 hours at room temp or overnight at 4°C for more transformants. Transformations were done using competent *E.coli* DH5α cells.

To make competent cells, 4 mL of the strain (DH5α) was grown in 2 x YT broth (16 g Bacto-Tryptone, 10 g Bacto-yeast, 5 g NaCl made up to up to 1 L in Milli-Q water and autoclaved) overnight with $100\mu g.mL^{-1}$ ampicillin (final concentration). Overnight culture were diluted 1:200 in 2 x YT broth and grown in containers 10 times the culture volume. Cultures were grow to early log phase (OD_{600} = 0.3 – 0.6). These were then centrifuged at 5000rpm for 5 minutes at 4°C. Cells were kept ice cold from here on. Cells were resuspended in 1 culture volume ice cold 0.1 M $MgCl_2$ and left on ice for about 1 minute. They were then centrifuged at 5000 rpm for 5 minutes at 4°C, resuspended in ½ culture volume ice cold 0.1 M $CaCl_2$ and left on ice for 1 – 2 hours. Cells were collected as before and resuspend in 1/10 volume 0.1 M $CaCl_2$.

Transformation of the template into the cells was achieved by mixing 0.1 mL competent cells with 5 µL DNA and incubating on ice for 20 minutes. Cells were heat shocked at 42°C for 10 minutes. Next, 0.9 mL 2 x YT was added and the cells were left to express antibiotic resistance for 1 hour at 37°C and then plated (200 µL onto MacConkey Agar plates containing 100 µg/mL ampicillin). Plates were incubated overnight at 37°C. White colonies were picked onto a new MacConkey Agar plate with 100 μg.mL^{-1} ampicillin as well as into 2 x YT broth (5 mL) with 100 μg.mL^{-1} ampicillin. These were incubated at 37°C overnight and used for miniprepping.

In order to confirm the correct inserts were transformed into the pGEM-T Easy vector, the cultures were miniprepped as follows. A 1 mL volume of the overnight culture of cells was pelletted by centrifugation at 14000 rpm for 1 minute, the pellet resuspended in 100 μL Solution I (50 mM glucose, 25 mM Tris-Cl, 10 mM EDTA) and the solution left to stand at room temperature for 5 minutes. Next, 200 μL Solution II (0.2 M NaOH, 1 % SDS) was added and the solution left on ice for 5 minutes. After this add 150 μL Solution III (3 M CH_3COOK pH 5 with glacial acetic acid), mix by inversion and leave on ice for 5 minutes. The samples were centrifuged for 10 minutes at 14000 rpm, one volume of isopropanol was added to the supernatant and the mixture at room temperature for 2 minutes. Finally, this was then centrifuged at 14000 rpm for 30 minutes at 4°C, the pellet washed in 200 μL ice cold 70 % ethanol, centrifuged for a further 1 minute, the pellet air dried and resuspended in minimal TE buffer. The plasmid DNA was then stored at –20°C.

Once the plasmid had been purified from the cell, the insert was excised by digesting the pGEM-T Easy plasmid with the restriction enzyme *Eco*R1. This restriction enzyme was chosen since the pGEM-T Easy vector was designed to have *Eco*R1 sites on either side of the cloning site, thus enabling easy purification of the insert DNA.

Once the insert was excised from the vector, it was analysed on a 1 % agarose gel as described previously to confirm that it was the correct insert by virtue of its molecular weight.

5.3.5 Sequencing

Samples to be sequenced using an ABI 3100 Genetic Analyzer were purified using a QIAquick PCR Purification Kit (Cat. No. 28104 QIAGEN). Template DNA was quantified by measuring the absorbance of the sample at 260 nm (one OD_{260nm} unit = 50 ng.μL^{-1} dsDNA) to ensure 5 – 8 ng.μL^{-1} was put forward in the sequencing reaction. The primer chosen in the sequencing reaction was GM5F since 907R is the GC-rich primer used for DGGE. The primer concentration put forward in the sequencing reaction was 0.27 pmol.μL^{-1}. The template and primer mixture was then subjected to labelling using the BigDye Terminator Cycle Sequencing Kit (Cat. No. 4303 Applied Biosystems) as per manufacturer's instructions. The cycling protocol used was as follows : Rapid thermal ramp to 96°C, 96°C for 10 seconds, rapid thermal ramp to 50°C, 50°C for 5 seconds, rapid thermal ramp to 60°C, 60°C for 4 minutes, rapid thermal ramp to 4°C and hold. Labelled template was cleaned using DNA Clean & Concentrator™-5 (Cat. No. D4003 Zymo Research) as per manufacturer's instructions, dried thoroughly using a vacuum centrifuge and kept at -20°C until sequencing.

5.4 RESULTS & DISCUSSION

5.4.1 Molecular Biology Technique Development

Since the initial component of this study involved the development of appropriate techniques, many of the results represented reflect the best of several attempts. Furthermore, many of the electrophoresis gels presented display negative results, showing what techniques did not work in this particular application. Explanations and methodological steps followed to correct the problems have been considered. In all the figures of agarose gels shown below, the DNA marker used was lambda DNA cut using the restriction enzyme *Pst*I, resulting in a DNA ladder called λ*Pst*I). Since the 16S rRNA region amplified using the primer pair GM5F and 907R has been previously described (Santegoeds *et al.,* 1998), correct amplification product size was know to be 589bp and is indicated by the arrow(s) in the figures below.

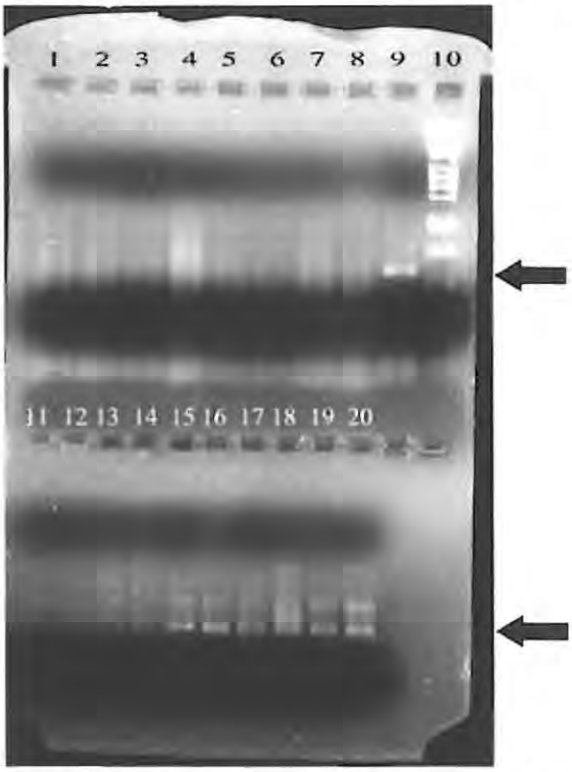


Figure 5.1 **Initial results on a 1 % agarose gel of 16S rRNA PCR amplification run using previously determined cycling conditions. Lane 10 represents the DNA marker λ*Pst*I. Lanes 1 – 2 display the PCR products of TL, 3 – 4 of TF, 5 – 6 of ML, 7 – 8 of MF and 9 of BL. Lanes 11 – 12 of BF, 13 – 16 of AO and 17 – 20 of Frot. Arrows indicate 589bp fragment size as determined by the λ*Pst*I DNA marker.**

Figure 5.1 shows that initial 16S rRNA PCR amplification attempts were generally unsuccessful from most of the sampling points. It was shown that the problem was probably not a lack of template since all total DNA extractions had been successful, yielding average chromosomal DNA concentrations of 0.4 $\mu g.mL^{-1}$, as indicated spectrophotometrically. One exception was the amplification of sample BL (the DNA extract from unattached algal sludge from the base of the ANTRIC filter). This PCR resulted in a good, clean yield of 16S rRNA product. This might be explained by the fact that the total chromosomal yield from the extraction process was substantially lower for this sample than the other samples, yielding only 0.07 $\mu g.mL^{-1}$. Nevertheless, based on the results of the other PCR products, it was suggested that there were possible inhibitory compounds in the DNA extract preventing successful primer binding and resulting template amplification during the PCR process. Since the polymerase chain reaction is such a sensitive procedure, only minute amounts of template are required for successful product amplification. It was therefore decided to make use of dilutions of the chromosomal template in an attempt to overcome possible inhibitors.

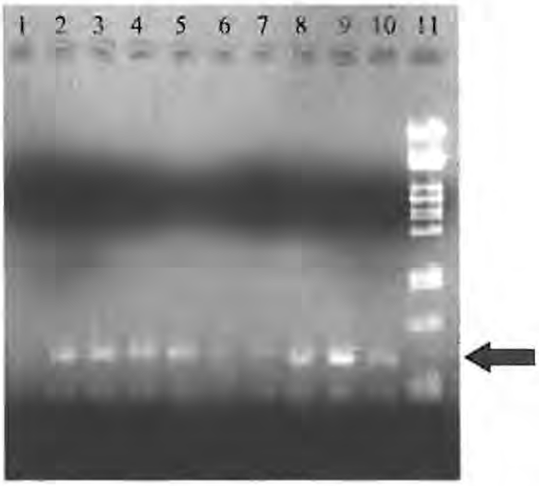


Figure 5.2 A 1 % agarose gel showing an attempt to use template dilution in the PCR mixture to avoid inhibition of amplification by possible interfering salts. Lanes 1 – 5 display results of dilutions performed on TF (1/10, 1/50, 1/100, 1/1000 and 1/1500 respectively). Lanes 6 – 10 display results of dilutions performed on ML (1/10, 1/50, 1/100, 1/1000, 1/1500 respectively). Lane 11 represents the DNA marker λ*Pst*I. Arrow indicates 589bp fragment size as determined by the λ*Pst*I DNA marker.

Figure 5.2 shows the results of the experimental dilutions used in an attempt to overcome amplification inhibition by salts and other such interfering compounds present in the denitrification column samples. It can be seen that the best amplification results came from the PCR reactions performed with 1/100 or 1/1000 template dilutions. It was therefore decided that for PCR amplifications from the original chromosomal DNA extracts, 1/1000 dilutions of template would be used as standard.

Figure 5.3 shows the results of PCR amplification of all original chromosomal DNA extracts using template dilutions of 1/1000. All PCR amplifications yielded reasonable products with the exception of BL and BF, which may have been made too dilute, considering the initial concentrations after extraction. In order to reach a balance between over diluting the template to remove inhibitors and under diluting to ensure the presence of sufficient template, it was decided that a uniform chromosomal DNA template concentration of 0.001 $\mu g.mL^{-1}$ be used in all further PCR amplifications of original chromosomal DNA extract.

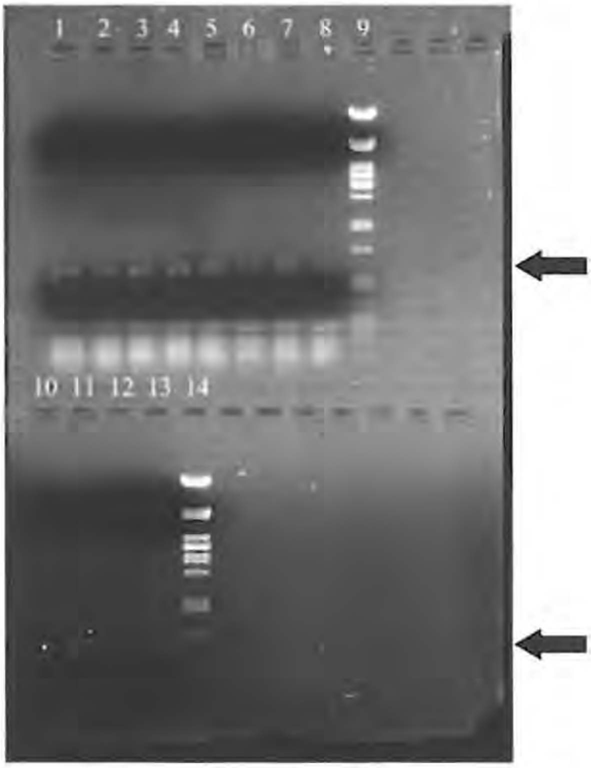


Figure 5.3 **This agarose gel illustrates the results of PCR amplification of 1/1000 dilution product of all sampling zones (TL lanes 1 – 2, TF lanes 3 – 4, ML lanes 5 – 6, MF lanes 7 – 8, BL lanes 10 – 11, BF lanes 12 – 13). Lanes 9 and 14 represent the DNA marker λ*Pst*I. Arrows indicate 589bp fragment size as determined by the λ*Pst*I DNA marker.**

Subsequent PCR amplification using the optimised template concentrations was shown to be inconsistent, not always yielding product. For this reason, other purification and amplification techniques were attempted. One such purification technique involved the use of a commercially available nucleic acid purification column from Zymo Research.

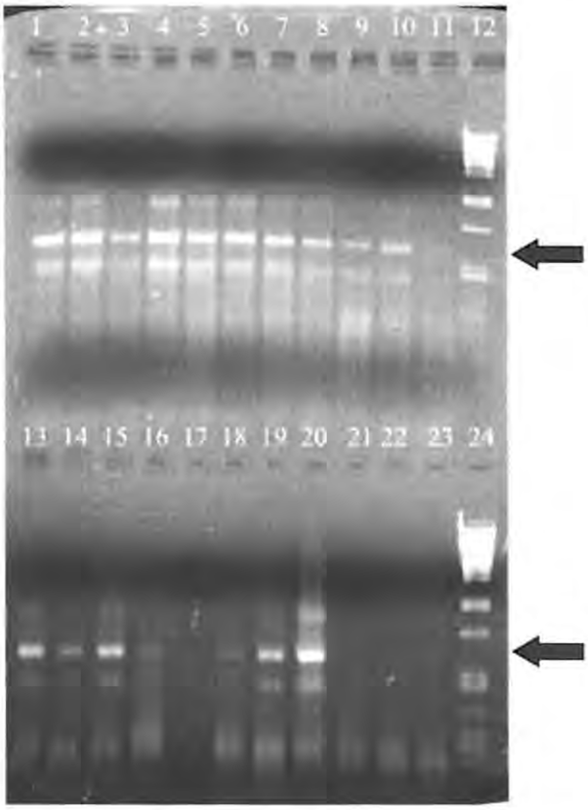


Figure 5.4 **Due to suspected contaminating salts or other such inhibitory substances, the use of Zymo DNA Clean & Concentrator™ columns was attempted to clean PCR template before amplification. PCR products of purified, undiluted template is shown in lanes 1 – 11 and purified, 1/1000 diluted template in lanes 13 – 23. The samples in order are 2xTL, 2xTF, 2xML, 2xMF, 1xBL, 2xBF. Lanes 12 and 24 represent the DNA marker λ*Pst*I. Arrows indicate 589bp fragment size as determined by the λ*Pst*I DNA marker.**

Figure 5.4 shows the effectiveness of removing salts and other inhibitory substances from the PCR mixture to allow for better amplification of product. However, use of these columns may become prohibitively expensive and thus a less expensive alternative was sought. It was suggested that optimising Mg^{2+} concentrations in the PCR mixture and the addition of the detergent DMSO may improve product yield. It was found that the optimum Mg^{2+} concentration in the PCR amplification mixture was 3 mM (double that of the standard 1.5 mM provided with Roche's Expand High Fidelity PCR System). It was also shown that a final concentration of 0.25 % DMSO in the PCR mixture yielded the best results. Figure 5.5 shows the results of PCR amplification using 3 mM Mg^{2+} and 0.25 % DMSO.

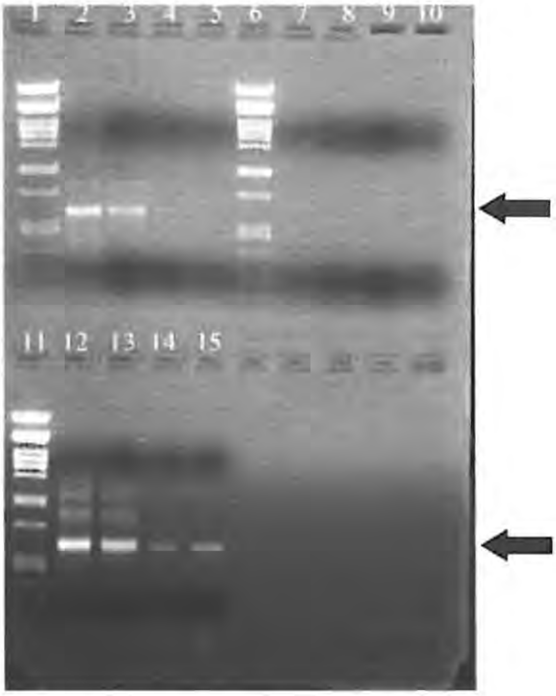


Figure 5.5 **Results of PCR amplification with the addition of 0.25 % DMSO (final concentration per reaction) and optimum Mg^{2+} concentration of 3 mM are shown. Lanes 1, 6 and 11 represent the DNA marker λ*Pst*I. Lanes 2 – 5 represent repeats of TL and TF, 7 – 10 of ML and MF and 12 – 15 of BL and BF. Arrows indicate 589bp fragment size as determined by the λ*Pst*I DNA marker.**

Other efforts to optimise PCR amplification included the use of Hot Start and Touchdown protocols to avoid non-specific primer annealing and the decrease of overall annealing temperature from 62°C to 56°C. Through a combination of these optimisation measures, strong, clean 16S rRNA PCR products (589bp) were obtained. Figure 5.6 shows the results of optimised PCR amplification. Although weak products were obtained from the BL and BF samples, the yield was hoped to be sufficient for further use in denaturing gradient gel electrophoresis. All other yields were deemed sufficient for use in DGGE.

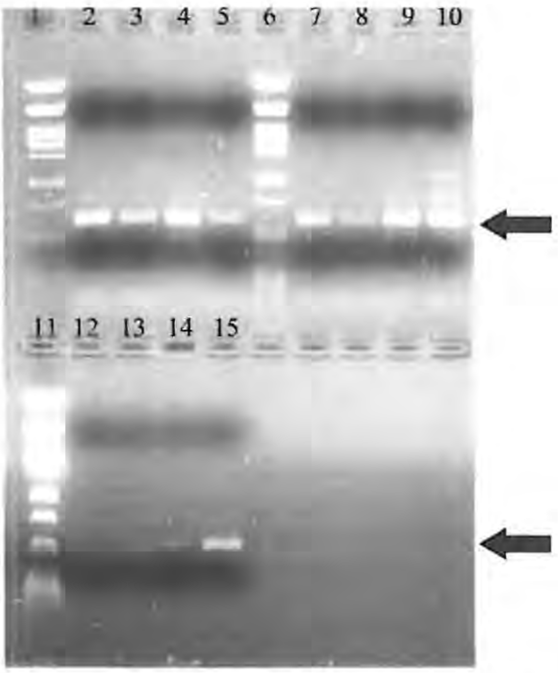


Figure 5.6 Optimum PCR amplification revealed the need to combine techniques. PCR products resulting from a combination of template dilution, the addition of 0.25 % DMSO, amplification using 3 mM Mg^{2+} and a decrease in programmed annealing temperature to 56°C is shown. Lanes 1, 6 and 11 represent the DNA marker λ*PstI*. Lanes 2 – 5 show PCR products of TL and TF, 7 – 10 of ML and MF and 12 – 15 of BL and BF. Arrows indicate 589bp fragment size as determined by the λ*PstI* DNA marker.

Figure 5.7 shows the results of two denaturing gradient acrylamide gels (one 35 – 55 % denaturing gel and one 55 – 70 % denaturing gel). These gels were loaded with PCR product prepared using the above protocols. Each well was loaded with PCR product from one of each of the sampling points from the ANTRIC Filter. Denaturing gradient gels were electrophoresed at constant voltage, constant temperature and the results examined after staining the gels with silver nitrate. Gels were given preliminary examination by staining with ethidium bromide to confirm the presence or absence of bands. However, this stain is not always sensitive enough to detect small quantities of DNA and thus silver nitrate was the preferred method of staining. Although other denaturing gradients were assessed (e.g. 0 – 35 % and 70 – 90 %), no distinct bands were found and it was decided to focus on the 35 – 55 % and 55 – 70 % denaturing range.

Once a gel had been stained with silver nitrate and bands identified, those individual bands were excised using sterile scalpel blades, the DNA extracted, reamplified in the previous manner and rerun on the same denaturing gradient. Confirmed bands (as shown in Figure 5.8) were then cloned into pGEM-T. Sequencing was performed on successfully transformed inserts and sequences submitted to the Basic Local Alignment Search Tool (BLAST) database (Altuschul *et al.*, 1990).

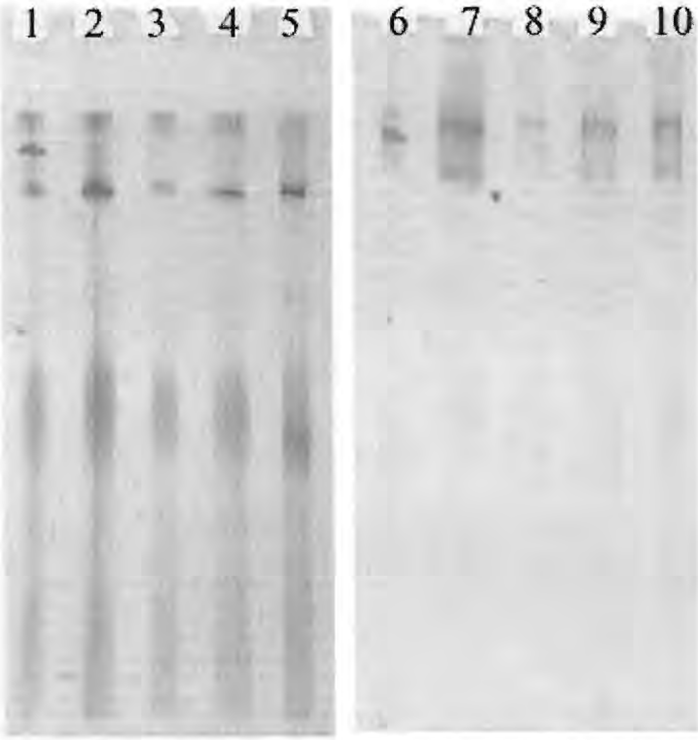


Figure 5.7 **Two acrylamide gels run using the denaturing gradient gel electrophoresis (DGGE) technique are shown. Lanes 1 – 5 show the separation of TL, TF, ML, MF and BF respectively on a 35 – 55 % denaturing gradient while lanes 6 – 10 show the same separation on a 55 – 75 % denaturing gradient.**

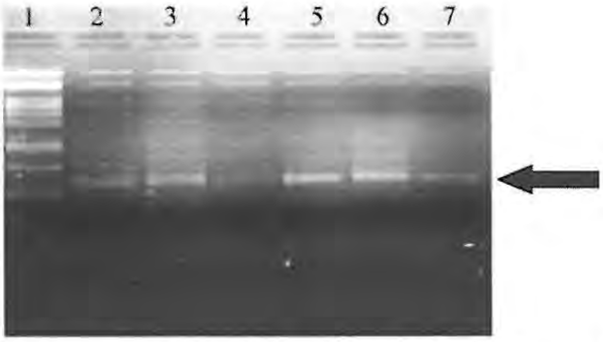


Figure 5.8 **PCR amplified products of excised bands from DGGE. Lane 1 represents the DNA marker λ*Pst*I. Lane 2 – 5 show bands excised from the 35 – 55 % denaturing gradient gel (lane 2 is the product from a band of TL, 3 and 4 are products of separated bands from TF, lane 5 a product from a band of BF). Lane 6 – 7 show bands excised from the 55 – 75 % denaturing gradient gel (lane 6 is the product from a band of TL and lane 7 shows the product from a band of MF). Arrow indicates 589bp fragment size as determined by the λ*Pst*I DNA marker.**

Following restriction digests and analysis of cloned products, it was determined that the cloned inserts were of the desired molecular size of around 589bp. However, on analysis of the sequences returned from the BLAST database, the products were shown to be meaningless. The reason suggested for these results may be that the template used in the PCR reaction was degraded. It was found that the original chromosomal DNA extract had begun to degrade, and since the denitrification columns had been sacrificed during sampling, there was no further biomass for continued investigation.

5.5 CONCLUSIONS

1) Molecular method development for analysis of denitrifying populations from the reactor columns used in this study proved difficult, and after optimizing the entire PCR procedure, extracted chromosomal DNA was found to be degraded, ending further study of the denitrifying bacterial populations at that stage. Further development of methods to enable follow-through to cloning and sequencing is needed for denitrifying systems.
2) Notwithstanding these final negative results, a number of advances were made in understanding molecular analysis of denitrifying systems.
3) The phenol:chloroform:isoamyl alcohol method of DNA extraction proved to be most suitable treatment for this field of application. The High Pure System may not be suitable for DNA isolation from environmental samples.
4) Optimizing PCR conditions is vital in order to ensure a true reflection of environmental populations and integrity of further community analysis. These included:
    - 4.1) Template dilution;
    - 4.2) Varying Mg^{2+} concentrations;
    - 4.3) Use of commercial DNA template clean-up kits;
    - 4.4) Addition of detergents, such as DMSO; and
    - 4.5) Use of novel cycling techniques, such as "hot start" and "touchdown".

5) While the results from the current work were unfavourable, co-workers in similar fields of research have found that DGGE is a suitable method for studying diverse bacterial populations from environmental samples.
6) Cloning of PCR products is necessary to allow high fidelity amplification of required DNA template and to allow confirmation that the desired product is carried forward for DNA sequencing.

CHAPTER SIX

CONCLUSIONS

6.1 IAPS AS AN APPROPRIATE TECHNOLOGY FOR TERTIARY WASTEWATER TREATMENT

The tertiary treatment of wastewater plays a crucial role in both environmental sustainability and sustainable development, and water reuse and recycling have become important strategies in protecting the freshwater resource as it comes under increasing pressure from anthropogenic sources. In developing countries in Africa and Asia the availability of clean, safe drinking water is directly related to the provision of proper sanitation (World Bank 1992; World Bank 1994; WHO 2000). At the same time, high-technology/high-cost/low-risk approaches are impractical in such countries, and low-technology/low-cost/controlled-risk approaches tend to be preferred (Anderson *et al.,* 2001). IAPS technology offers such low-technology/low-cost/controlled-risk advantages and in particular, the control of eutrophication in natural waterbodies, as a result of the release of nutrients in improperly treated wastewater.

According to the European IPPC (Integrated Pollution Prevention and Control) Directive, being implemented under the Pollution and Control Act (1999), pollution control strategies must be based on the concept of Best Available Techniques (BAT). BAT may be defined as "the most effective and advanced stage in the development of activities and their methods of operation which indicate the practical suitability of particular techniques for providing in principle the basis for emissions limit values designed to prevent and, where that is not practicable, generally reduce emissions and the impact on the environment as a whole" (Council Directive 96/61/EC). Simply, BAT may be described as a technology or technique which allows the highest level of preventive measures against pollution to be taken, with regard to what is technically feasible (Lema & Omil, 2001). The HRAP was identified as such an appropriate technology for tertiary wastewater treatment of effluent streams from conventional wastewater treatment systems (Grahamstown Disposal Works) and raw water resources.

6.2 NUTRIENT REMOVAL IN THE I-HRAP

The studies reported here showed the HRAP to be suitable as an independent unit operation, one which can be retrofitted to an existing wastewater treatment plant in order to improve effluent quality. This Independent High Rate Algal Pond was termed the I-HRAP. The I-HRAP was observed to be conducive to support good algal growth with low-strength loading. Due to the photosynthetic nature of algae, certain reactions were observed to be beneficial in its functioning as a tertiary treatment unit. The I-HRAP was shown to be suitable for the removal of phosphorus from both conventional and IAPS effluent primarily by biologically-mediated calcium phosphate precipitation at elevated pH. The elevated pH was shown to be linked to the activity of microalgal photosynthesis. Furthermore, although pH decreased during dark periods due to the nature of microalgal respiration, the DO concentrations in the I-HRAP never decreased to the extent that anoxia was initiated.

The I-HRAP was further shown to facilitate heterotrophic nitrification and ammonia removal via volatilisation in the tertiary treatment of the wastewater. This process too was partly attributed to the photosynthetic activity of the microalgal cultures in the I-HRAP. Ammonia volatilisation is only possible at elevated pH values. Above neutral pH conditions, the equilibrium between NH_4^+ and NH_3 gas is shifted in favour of NH_3 gas which is stripped into the atmosphere. However, ammonia is not the only problematic nitrogenous compound in wastewater environments. Nitrate and to some extent, nitrite, are of particular concern, being associated with toxic algal blooms in eutrophied waterbodies and methaemoglobinemia in infants. The I-HRAP was unable to reduce nitrate levels satisfactorily because anoxia was not reached during periods of darkness, and the need for a subsequent denitrification step was identified.

6.3 ALGAL PHOTOSYNTHATE AND BACTERIAL DENITRIFICATION

A link was demonstrated between organic carbon requirements for denitrification and microalgal stress physiological responses resulting in polysaccharide release to the system. It was shown that the polysaccharides released by the I-HRAP microalgae may be utilised as a carbon source supporting heterotrophic denitrification. Under dark, aerobic experimental conditions little or no change was observed in ammonia concentrations. Nitrate concentrations also remained predominantly unaffected indicating that little or no denitrification was occurring. Under dark, aerobic conditions, polysaccharide was consumed and TPS maxima rarely exceeded 500 $mg.L^{-1}$, regardless of the nutrient stress. Conditions of anoxia and the presence of excess nitrate promoted the production of polysaccharides, with TPS production maxima ranging between 800 – 1000 $mg.L^{-1}$ and the concomitant reduction of nitrate was observed. This supported the idea that microalgal polysaccharide production may be manipulated to provide an available carbon source for heterotrophic denitrification.

The stress conditions including anoxia and high nutrient loads also have an important bearing on the design of an I-HRAP employing a N-removal component. Since phosphorus was observed to be released during the denitrification phase (probably due to decreasing pH, a result of VFA production in the anaerobic reactor), it was noted that P-removal would need to be located in a stage following denitrification. The tertiary wastewater treatment scheme outlined in figure 6.1 was thus proposed.

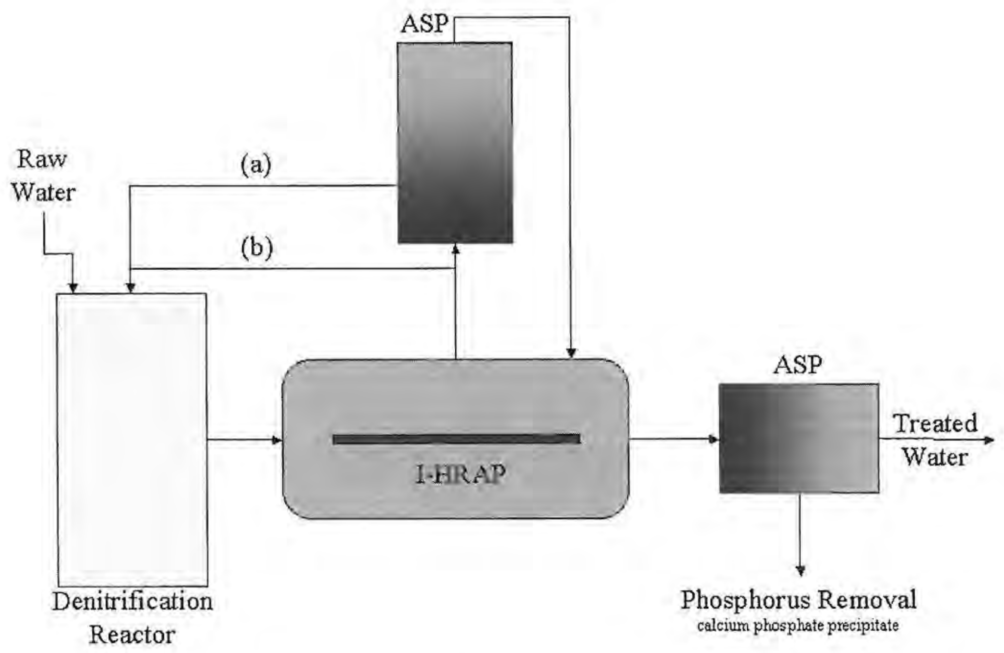


Figure 6.1 Proposed flow diagram for nutrient removal from low-strength, treated wastewater or raw water by an I-HRAP. Stream (a) represents the use of settled algal biomass for a degrading bed denitrification reactor while stream (b) represents the use of live, whole-cell microalgal biomass for carbon production in the denitrification unit. The P-removal ASP is situated post-denitrification due to phosphate release in the reactor.

Two forms of carbon utilisation were studied. In the first form degradation of algal biomass provided sufficient carbon for heterotrophic denitrification, however a build-up of degraded biomass caused inhibition of denitrification either due to substance toxicity or to a decrease in available carbon. In the second form, the ANTRIC Reactor, stress manipulation of metabolically active algal cells resulted in carbon production, supplying sufficient energy to drive denitrification. Algal cells pass through the system and return to the HRAP where solubilised reserves of calcium and phosphate reprecipitate. Some algal biomass remains in the column where cells fracture and become completely degraded. Field trial scale-up reactors are under construction for further detailed analysis and preliminary findings indicate that the denitrification reactors will enhance the nutrient removal capacity of an I-HRAP.

6.4 MOLECULAR MICROBIAL ECOLOGY

A study of the bacterial communities involved in the denitrification process was undertaken using molecular techniques. After optimizing the PCR procedure however, extracted chromosomal DNA was thought to be degraded, ending further examination of the denitrifying bacterial populations. The reason for this could not be determined. The phenol:chloroform:isoamyl alcohol method of DNA extraction proved to be most suitable treatment for this field of application. The High Pure System may not be suitable for DNA isolation from environmental samples. Optimizing PCR conditions is vital in order to ensure a true reflection of environmental populations and integrity of further community analysis. DGGE is a suitable method for studying diverse bacterial populations from environmental samples. Cloning of PCR products is necessary to allow easy amplification of required DNA template and to allow confirmation that the desired product is carried forward for DNA sequencing. Further work will be required to optimise this system for the identification of denitrifying populations in I-HRAP systems.

6.5 FUTURE WORK

1. ^{14}C-labelling tracer studies are needed to follow metabolic pathways of carbon production by microalgae and the utilisation by denitrifying populations of algal photosynthate. This work is necessary in order to

establish kinetic rates for production and consumption reactions involved in the process.

2. Bacterial denitrifying population studies using molecular techniques leading to the study of microbial biofilm dynamics using fluorescent in situ hybridisation.
3. Scale up of the denitrification reactors to compare carbon production by live, whole-cell microalgal manipulation with degrading microalgal biomass.
4. Integrate a unit operation and pilot a full-scale I-HRAP for nutrient removal.

REFERENCES

Abrams, E.S. & Stanton Jnr, V.P. 1992. Use of denaturing gradient gel electrophoresis to study conformational transitions in nucleic acids. *Meth. Enzym.* 212 : 71 – 104.

Abrams, L. 1998. Understanding sustainability of local water services. The African Water Page, http://www.africanwater.org/sustainability.html

Adda, M., Merchuk, J.C. & Arad, S.M. 1986. Effect of nitrate on growth and production of cell-wall polysaccharide by the unicellular red alga *Porphyridium*. *Biomass.* 10 : 131 – 140.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215 (3) : 403 – 410.

Anderson, J., Adin, A., Crook, J., Davis, C., Hultquist, R., Jimenez-Cisneros, B., Kennedy, W., Sheikh, B. & van der Merwe, B. 2001. Climbing the ladder : a step by step approach to international guidelines for water recycling. *Wat. Sci. Tech.* 43 (10) : 1 – 8.

Arad, S.M., Lerental, Y.B. & Dubinsky, O. 1992. Effect of nitrate and sulfate starvation on polysaccharide formation in *Rhodella reticulata*. *Biores. Tech.* 42 : 141 – 148.

Asano, T. & Levine, A.D. 1996. Wastewater reclamation, recycling and reuse : Past, present and future. *Wat. Sci. Tech.* 33 (10 – 11) : 1 – 14.

Awuah, E., Anohene, F., Asante, K., Lubberding, H. & Gijzen, H. 2001. Environmental conditions and pathogen removal in macrophytes- and algal-based domestic wastewater treatment systems. *Wat. Sci. Tech.* 44 (6) : 11 – 18.

Bell, L.C., Richardson, D.J. & Ferguson, S.J. 1990. Periplasmic and membrane-bound respiratory nitrate reductase in *Thiosphaera pantotropha* : the periplasmic enzyme catalyses the first step in aerobic denitrification. *FEBS Lett.* 265 : 85 – 87.

Bothe, H., Jost, G., Schloter, M., Ward, B.B. & Witzel, K-P. 2000. Molecular analysis of ammonia oxidation and denitrification in natural environments. *FEMS Microb. Rev.* 24 : 673 – 690.

Chèneby, D., Philippot, L., Hartmann, A., Hénault, C. & Germon, J-C. 2000. 16S rDNA analysis for characterization of denitrifying bacteria isolated from three agricultural soils. *FEMS Microb. Ecol.* 34 : 121 – 128.

Chou, Q., Russell, M., Birch, D.E., Raymond, J. & Bloch, W. 1992. Prevention of pre-PCR mis-priming and primer dimerisation improves low-copy-number amplifications. *Nucl. Acids Res.* 20 (7) : 1717 – 1723.

Dawson, R.N. 1997. Advances in biological nutrient removal from wastewater. In : Biotechnology in the sustainable environment. G.S. Sayler, J. Sanseverino & K.L. Davis (eds.). Proceedings of a conference on biotechnology in the sustainable environment, Knoxville, Tennessee, USA. 14 – 17 April 1996. Plenum Press, New York.

DEAT (Department of Environmental Affairs and Tourism). 1999. National state of the environment report - South Africa. Department of Environmental Affairs and Tourism, Pretoria, South Africa.

DEAT (Department of Environmental Affairs and Tourism). 2000. White paper on integrated pollution and waste management for South Africa. Department of Environmental Affairs and Tourism, Pretoria, South Africa.

De Philippis, R., Sili, C. & Vincenzini. M. 1996. Response of an exopolysaccharide-producing heterocystous cyanobacterium to changes in metabolic carbon flux. J. *Appl. Phycol.* 8 : 275 – 281.

Don, R.H., Cox, P.T., Wainwright, B.J., Baker, K. & Mattick, J.S. 1991. "Touchdown" PCR to circumvent spurious priming during gene amplification. *Nucl. Acids Res.* 19 (14) : 4008.

Drummond, I. & Marsden, T. 1999. The condition of sustainability. Routledge Research Global Environmental Change Program. Routledge, London.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. & Smith, F. 1956. Colorimetric method for determination of sugars and related substances. *Analyt. Chem.* 28 (3) : 350 – 356.

Etchbehere, C., Errazquin, I., Barrandeguy, E., Dabert, P., Moletta, R. & Muxí, L. 2001. Evaluation of the denitrifying microbiota of anoxic reactors. *FEMS Microb. Ecol.* 35 : 259 – 265.

European Communities Commission Directive. 1991. Council directive regarding the treatment of urban wastewater (91/271/EEC). *Official Journal of the European Communities*. No. L135 of the 91.5.30, 40 – 50.

European Communities Commission Directive. 1996. Council directive concerning integrated pollution prevention and control (96/61/EC). 24 September 1996.

Falkentoft, C.M., Harremoës, P. & Mosbæk, H. 1999. The significance of zonation in a denitrifying, phosphorus removing biofilm. *Wat. Res.* 33 (15) : 3303 – 3310.

Ferris, M.J., Muyzer, G. & Ward, D.M. 1996. Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. *Appl. Environ. Microbiol.* 62 (2) : 340 – 346.

Focht, D.D. & Chang, A.C. 1975. Nitrification and denitrification processes related to waste water treatment. *Adv. Appl. Microbiol.* 19 : 153 – 186.

Fogg, G.E. 1977. Excretion of organic matter by phytoplankton. *Limnol. Oceanogr.* 22 : 576 – 577.

Fogg, G.E. & Westlake, D.F. 1955. The importance of extracellular products of algae in freshwater. *Proc. Internat. Assoc. Theor. Appl. Limnol.* 12 : 219 – 232.

Fulda, S. & Mikkat, S. 1998. The role of cyanobacterial exudates for growth of heterotrophic bacteria. In : Cyanobacterial biotechnology. G. Subramanian, B. Kaushik & G. Venkataraman (eds.). Science Publishers Inc., Enfield, USA.

Gijzen, H.B. & Mulder, A. 2001. The nitrogen cycle out of balance. In : Water21 Magazine of the International Water Association. K. Hayward (ed.). IWA Publishing, London.

Green, F.B., Bernstone, L.S., Lundquist, T.J. & Oswald, W.J. 1996. Advanced integrated wastewater pond systems for nitrogen removal. *Wat. Sci. Tech.* 33 (7) : 207 – 217.

Hellenbust, J.A. 1965. Excretion of some organic compounds by marine phytoplankton. *Limnol. Oceanogr.* 10 : 192 – 206.

Hochstein, L.I. & Tomlinson, G.A. 1988. The enzymes associated with denitrification. *Annu. Rev. Microbiol.* 42 : 231 – 261.

Horan, N.J. 1990. Biological wastewater treatment systems : Theory and operation. John Wiley & Sons Ltd, United Kingdom.

Iobbi-Nivol, C., Santini, C.L. & Giordano, F.B.G. 1990. Purification and further characterization of the second nitrate reductase of *Escherichia coli* K12. *Eur. J. Biochem.* 188 : 679 – 687.

Jenkins, D. & Tandoi, V. 1991. The applied microbiology of enhanced biological phosphate removal – accomplishments and needs. *Wat. Res.* 25 (12) : 1471 – 1478.

Klapwijk, A., van der Hoeven, J.C.M. & Lettinga, G. 1981. Biological denitrification in an upflow sludge blanket reactor. *Wat. Res.* 15 : 1 – 6.

Lama, L., Nicolaus, B., Calandrelli, V., Manca, M.C., Romano, I. & Gambacorta, A. 1996. Effect of growth conditions on endo- and exopolymer biosynthesis in *Anabaena cyclindrica* 10 C. *Phytochemistry* 42 (3) : 655 – 659.

Lema, J.M. & Omil, F. 2001. Anaerobic treatment: a key technology for sustainable management of wastes in Europe. *Wat. Sci. Tech.* 44 (8) : 133 – 140.

Lettinga, G. & Hulshoff Pol, L.W. 1991. UASB-Process design for various types of wastewaters. *Wat. Sci. Tech.* 24 (8) : 87 – 107.

Lichtenthaler, H.K. 1987. Chlorophylls and carotenoids : Pigments of photosynthetic biomembranes. *Met. Enzym.* 148 : 350 – 382.

Mancotywa, A.M. 1999. Analytical advisory service for Grahamstown Municipality. Grahamstown City Engineers Department.

Mara, D.D. & Pearson, H. 1986. Artificial freshwater environment : Waste Stabilization Ponds. In : Biotechnology – A comprehensive treatise 8. H.J. Rehm & G. Red (eds.). Weinheim, Verlagsgesellschaft.

Martínez, M.E., Sánchez, S., Jiménez, J.M., El Yousfi, F. & Muñoz, L. 2000. Nitrogen and phosphorus removal from urban wastewater by the microalga *Scenedesmus obliquus*. *Biores. Technol.* 73 : 263 – 272.

Matějů, V., Čižinská, S., Krejčí, J. & Janoch, T. 1992. Biological water denitrification – A review. *Enzyme Microb. Technol.* 14 : 170 – 183.

Maynard, H.E., Ouki, S.K. & Williams, S.C. 1999. Tertiary lagoons : A review of removal mechanisms and performance. *Wat. Res.* 33 (1) : 1 – 13.

McCarthy, J.J., Taylor, W.R. & Taft, J.L. 1977. Nitrogenous nutrition of the plankton in the Chesapeake Bay I : Nutrient availability and phytoplankton preferences. *Limnol. Oceanogr.* 22 (6) : 996 – 1011.

Miller, D. 1996. Green technology trends. In : Biotechnology in the sustainable environment. G.S. Sayler, J. Sanseverino & K.L. Davis (eds.). Proceedings of a conference on biotechnology in the sustainable environment, Knoxville, Tennessee, USA. 14 – 17 April 1996. Plenum Press, New York.

Morse, G.K., Brett, S.W., Guy, J.A. & Lester, J.N. 1998. Review : Phosphorus removal and recovery technologies. *The Sci. Total Environ.* 212 : 69 – 81.

Moutin, T., Gal, J.Y., El Halouani, H., Picot, B. & Bontoux, J. 1992. Decrease of phosphate concentration in a high rate pond by precipitation of calcium phosphate : Theoretical and experimental results. *Wat. Res.* 26 (11) : 1445 – 1450.

Mujeriego, R. & Asano, T. 1999. The Role of Advanced Treatment in Wastewater Reclamation and Reuse. *Wat. Sci. Tech.* 40 (4 – 5) : 1 – 9.

Mutembwa, A. 2000. Towards a Sustainable Water Management Strategy for Southern Africa. In : The Sustainability Challenge for Southern Africa. J. Whitman (ed.). Macmillan Press LTD, Great Britain.

Muyzer, G., De Waal, E.C. & Uitterlinden, A.G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes encoding for 16S rRNA. *Appl. Environ. Microbiol.* 59 (3) : 695 – 700.

Myers, R.M., Maniatis, T. & Lerman, L.S. 1987. Detection and localization of single base changes by denaturing gradient gel electrophoresis. *Met. Enzym.* 155 : 501 – 527.

Myklestad, S.M. 1995. Release of extracellular products by phytoplankton with special emphasis on polysaccharides. *The Sci. Total Environ.* 165 : 155 – 164.

Nurdogan, Y. & Oswald, W.J. 1995. Enhanced nutrient removal in high-rate ponds. *Wat. Sci. Tech.* 31 (12) : 33 – 43.

Oswald, W.J. 1988. Micro-algae and waste-water treatment. In : Micro-algal Biotechnology. M.A. Borowitzka & L.J. Borowitzka (eds.). Cambridge University Press. United Kingdom.

Oswald, W.J. 1991. Introduction to advanced integrated wastewater ponding systems. *Wat. Sci. Tech.* 24 (5) : 1 – 7.

Oswald, W.J. 1995. Ponds in the twenty-first century. *Wat. Sci. Tech.* 31 (12) : 1 – 8.

Pereira, H., Lemos, P.C., Reis, M.A.M., Crespo, J.P.S.G., Carrondo, M.J.T. & Santos, H. 1996. Model for carbon metabolism in biological phosphorus removal processes based on *in vivo* ^{13}C-NMR labelling experiments. *Wat. Res.* 30 (9) : 2128 – 2138.

Philippot, L. & Højberg, O. 1999. Dissimilatory nitrate reductases in bacteria. *Biochim. Biophys. Acta* 1446 : 1 – 23.

Raven, P.H. & Johnson, G.B. 1992. Biology, Third Edition. Mosby-Year Book, Inc. St. Louis, Missouri, USA.

Reilly, J.F., Horne, A.J. & Miller, C.D. 2000. Nitrate removal from a drinking water supply with large free-surface wetlands prior to groundwater recharge. *Ecol. Eng.* 14 : 33 – 47.

Rose, P.D., Dunn, K.M., Maart, B.A. & Shipin, O. 2001a. Algal biotechnology and sustainable management for saline wastewaters. Meso-saline wastewaters: the *Spirulina* model. Water Research Commission (WRC) Report on Project K5/495.

Rose, P.D., Hart, O.O., Shipin, O. & Müller, J.R. 2001b. Integrated algal ponding systems and the treatment of domestic and industrial wastewaters. Part 2: Abattoir wastewaters. Water Research Commission (WRC) Report on Project K5/658.

Rose, P.D., Shipin, O., Ellis, P. & Hart, O.O. 2001c. Appropriate low-cost sewage treatment using integrated algal high rate oxidation ponding processes. Water Research Commission (WRC) Report on Project K5/651.

Sala, L. & Mujeriego, R. 2001. Cultural eutrophication control through water reuse. *Wat. Sci. Tech.* 43 (10) : 109 – 116.

Santegoeds, C.M., Ferdelman, T.G., Muyzer, G. & De Beer, D. 1998. Structural and functional dynamics of sulfate-reducing populations in bacterial biofilms. *Appl. Environ. Microbiol.* 64 : 3731 – 3739.

Sharma, B. & Ahlert, R.C. 1977. Nitrification and nitrogen removal. *Wat. Res.* 11 : 897 – 925.

Shrimali, M. & Singh, K.P. 2001. New methods of nitrate removal from water. *Environ. Poll.* 112 : 351 – 359.

Speth, J.G. 1990. Needed : An environmental revolution in technology. In : Conferences Proceedings Towards 2000 : Environment, Technology and the New Century, Annapolis, 13 – 15 June 1990.

Stewart, W.D., Fitzgerald, G.P. & Burris, R.H. 1967. In situ studies on N2 fixation using the acetylene reduction techqniue. *Proc. Natl. Acad. Sci. USA* 58 : 2071 – 2078.

Thomas.W.H., Seibert, D.L.R., Alden, M., Neori, A. & Eldridge, P. 1984. Yields photosynthesis efficiencies and proximate composition of dense marine microalgal culture. II. *Dunaliella primolecta* and *Tetraselemis suecica* experiments. *Biomass* 5 : 211 – 215.

Tiedje, J.M. 1998. Ecology of denitrification and dissimilatory nitrate reduction to ammonium. In : Biology of anaerobic microorganisms. A.J.B. Zehnder (ed.). John Wiley & Sons, Inc. USA.

United Nations, Commission on Sustainable Development. 2000. Progress made in providing safe water supply and sanitation for all during the 1990s : Report of the Secretary-General. http://www.un.org/esa/sustdev/csd8/wss4rep.pdf

van Benthum, W.A.J., Garrido, J.M., Mathijssen, J.P.M., Sunde, J., van Loosdrecht, M.C.M. & Heijnen, J.J. 1998. Nitrogen removal in intermittently aerated biofilm airlift reactor. *J. Environ. Eng.* March : 239 – 248.

Vincenzini, M., De Philippis, R, Sili, C. & Materassi, R. 1990. Studies on exopolysaccharide release by diazotrophic batch cultures of *Cyanospira capsulata*. *Appl. Microbiol. Biotechnol.* 34 : 392 – 396.

WCED (World Commission on Environment and Development). 1987. Our common future. Oxford University Press, Oxford.

World Bank. 1992. World development report : Development and the environment, 15th Edition. Oxford University Press, Oxford.

World Bank. 1994. World development report : Infrastructure for development, 17th Edition. Oxford University Press, Oxford.

World Health Organization (WHO). 1989. Health guidelines for the use of wastewater in agriculture and aquaculture. Report of a WHO Scientific Group, Technical Report Series 778, World Health Organization, Geneva.

World Health Organization (WHO). 2000. Global water supply and sanitation assessment 2000 report.
http://www.who.int/water_sanitation_health/Globassessment/GlasspdfTOC.htm

Wu, R.S.S. 1999. Eutrophication, water borne pathogens and xenobiotic compounds: Environmental risks and challenges. Marine Pollution Bulletin 39 (1 – 12) : 11 – 22.

Ye, R.W. & Thomas, S.M. 2001. Microbial nitrogen cycles : physiology, genomics and applications. *Curr. Op. Microb.* 4 : 307 – 312.

APPENDIX A

MERCK SPECTROQUANT® METHODS

AMMONIUM

(Berthelot's Reaction – colourimetry of blue 2.2'-isopropyl-5.5'-methyl-indophenol)

Principle : Following adjustment to pH 13 (with Reagent NH_4-1B), ammonia reacts with hypochlorite (Reagent NH_4-2B) to monochloramine, which in turn forms a blue indophenol dye in a catalysed 2-stage reaction with thymol (Reagent NH_4-3B).

Method : Pipette 5 mL sample into a test tube. Add 0.6 mL Reagent NH_4-1B and mix. Add 1 blue microspoonful of Reagent NH_4-2B and mix. Leave to react for 5 minutes at room temperature. Add 4 drops of Reagent NH_4-3B, mix and react for 5 minutes at room temperature. A blank was prepared in the same way, substituting 3 mL of Milli-Q H_2O for the sample. Transfer the solution to a 10 mm quartz cuvette and read on the MERCK SPECTROQUANT® SQ118 using method 008.

CALCIUM

Principle : The addition of Reagents Ca-1A and Ca-2A produce an alkaline-alcoholic medium. Calcium ions react with Calcospectral (modified glyoxal bis [2-hydroxyanil]) Reagent Ca-3A to give a soluble, red-violet colour complex.

Method : Pipette 0.1 mL of the sample into a test tube. Add 5 mL of Reagent Ca-1A and mix. Add 4 drops of Ca-2A and mix. Add 4 drops of Ca-3A and mix. Allow reaction to proceed for 8 minutes at room temperature. A blank was prepared in the same way, substituting 0.1 mL of Milli-Q H_2O for the sample. Transfer the solution to a 20 mm quartz cuvette and read on the MERCK SPECTROQUANT® SQ118 using method 015.

CHEMICAL OXYGEN DEMAND

Principle : Oxygen, from potassium dichromate in sulphuric acid, reacts with oxidisable substances in the sample, with silver sulphate as catalyst and mercuric sulphate for chloride masking.

Method : Mix 0.3 mL of COD Solution A with 2.3 mL COD Solution B. Carefully pipette 3.0 mL of sample into a reaction cell and tightly screw on the cap. Mix and incubate the reaction cell in a MERCK TR205 heating block for 120 minutes at

148°C. Remove the reaction cell from the heating block and allow to cool to 20 – 20°C. Mix the reaction cell before measurement. A blank was prepared in the same way, substituting 3 mL of Milli-Q H_2O for the sample. Place the reaction cell in the cell compartment and read on the MERCK SPECTROQUANT® SQ118 using method 029.

NITRATE

Principle : Reaction with Nitrospectral in concentrated sulphuric acid to give a dark red-coloured nitro compound.

Method : Place 1 blue microspoonful of Reagent NO_3-1A in a dry, clean test tube. Pipette 5 mL of Reagent NO_3-2A into the test tube and dissolve Reagent NO_3-1A completely. Pipette 1.5 mL sample into mixture and allow exothermic reaction to continue for 10 minutes. A blank was prepared in the same way, substituting 1.5 mL of Milli-Q H_2O for the sample. Transfer the solution to a 10 mm quartz cuvette and read on the MERCK SPECTROQUANT® SQ118 using method 056.

NITRITE

Principle : Solid mixture of sulfanilic acid and N-napthylethylene-diammoniumdichloride brings about a diazo-reaction with subsequent coupling of the amine.

Method : Pipette 10 mL of the sample into a test tube. Add 2 blue microspoonfuls of Reagent NO_2-AN and dissolve. Allow the reaction 10 minutes to complete. A blank was prepared in the same way, substituting 10 mL of Milli-Q H_2O for the sample. Transfer the solution to a 10 mm quartz cuvette and read on the MERCK SPECTROQUANT® SQ118 using method 065.

PHOSPHORUS (ORTHO-PHOSPHATE)

Principle : In a solution of ammonium heptamolybdate and potassium antimony oxide tartrate acidified with sulphuric acid (Reagent P-1A), orthophosphate ions react with molybdate ions to form molybdophosphoric acid. Ascorbic acid (Reagent P-2A) reduces this to phosphomolybdenum blue (PMB), which is then determined photometrically.

Method : Pipette 5 mL of the sample into a test tube. Add 5 drops of Reagent P-1A and mix. Add 1 blue microspoonful of Reagent P-2A and mix / dissolve. Leave to stand for 5 minutes. A blank was prepared in the same way, substituting 5 mL of Milli-Q H_2O for the sample. Transfer the solution to a 10 mm quartz cuvette and read on the MERCK SPECTROQUANT® SQ118 using method 072.

APPENDIX B

CHLOROPHYLL DETERMINATION (AFTER LICHTENTHALER, 1987)

Concentrate the sample by centrifugation (10 mL, 15 minutes, 5000 x g). Pour off the supernatant, rinse with Milli-Q H2O, centrifuge at 5000 x g for 15 minutes and pour off the supernatant. Add 10 mL ice cold 100 % acetone. Flush the headspace with N2 and seal the tube. Cover the tube in tin foil to prevent light degradation and extract the pigment by incubating the tube on an invert-shaker for 6 hours at 4°C. Clarify sample by filtering through a solvent-resistant disposable filter or by centrifugation in a closed tube for 10 minutes at 5000 x g. Read the optical density (OD) of the sample at wavelengths of : 644.8 and 661.6 nm. Calculate the pigment concentrations according to the following formulae ($\mu g.mL^{-1}$) :

$$Chl_a = 11.24(A661.6) - 2.04(A644.8)$$

$$Chl_b = 20.13(A644.8) - 4.19(A661.6)$$

$$Chl_{a+b} = 7.05(A661.6) + 18.09(644.8)$$

APPENDIX C

PHENOL-CHLOROFORM-ISOAMYL ALCOHOL DNA EXTRACTION PROTOCOL

- ✓ Centrifuge cells at 13000 rpm in sterile Eppendorf tubes
- ✓ Pour off supernatant
- ✓ Resuspend in 500 µL TE (10 mM Tris, 1 mM EDTA pH 8.0)
- ✓ Add 6 µL of 50 $mg.mL^{-1}$ lysozyme and incubate for 30 minutes at 37°C, mixing every 10 minutes
- ✓ Boil for 1 minute
- ✓ Perform 4 freeze-thaw cycles (immerse tube in liquid nitrogen followed by 80°C waterbath and repeat)
- ✓ Add 0.5 % SDS (i.e. 50 µL 10 % SDS to 1 mL)
- ✓ Add proteinase K (50 $\mu g.mL^{-1}$) → 2.5 µL, incubate at 37°C for 12 hours
- ✓ Add 100 µL 10 % CTAB, mix
- ✓ Add 200 µL 5 M NaCl, mix
- ✓ Incubate at 55°C for 1 hour
- ✓ Aliquot 500 µL of each into fresh Eppendorf tubes
- ✓ Add 500 µL phenol
- ✓ Vortex and centrifuge at 13000 rpm
- ✓ Remove aqueous upper layer to fresh Epp
- ✓ Add 250 µL chloroform:isoamyl alcohol (24:1) and 250 µL phenol
- ✓ Vortex and centrifuge at 13000 rpm
- ✓ Remove aqueous upper layer to fresh Epp }
- ✓ Add 500 µL chloroform:isoamyl alcohol (24:1) } Repeat until pink
- ✓ Vortex and centrifuge at 13000 rpm } colouration disappears
- ✓ Remove upper aqueous layer to fresh Epp
- ✓ Add 1 mL (2.5 volumes) 96 % rectified ethanol
- ✓ Store at –20°C overnight
- ✓ Centrifuge down DNA to a pellet
- ✓ Pour off 96 % ethanol carefully so as not to lose the pellet
- ✓ Allow to drain and air dry
- ✓ Resuspend pellet in 40 µL TE (10 mM Tris, 1 mM EDTA, pH 8.0)