CHARACTERISATION OF THE MICROBIAL COMMUNITIES PRESENT IN AN ANAEROBIC BAFFLED REACTOR UTILISING MOLECULAR TECHNIQUES

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THARNIJA LALBAHADUR

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

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THARNIJA LALBAHADUR

I hereby approve the final submission of the following dissertation.

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PROF. FAIZAL BUX

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this _____ day of _____ , 2005, at Durban Institute of Technology

Dedication

This work is dedicated to Dreamers

The journey to realising your dreams begins with a single step. All you have to do is have faith…Faith in God, faith in yourself and faith in your dream

And of course, to Ma, Didz, Dad and Shivarn who walked my journey with me, making the complications encountered so much simpler and for giving me the strength to see my dream through.

ABSTRACT

The provision of safe and sanitary water is a constitutional right and above all, a necessity of life. As a result of the rapid urbanisation and the past policies of apartheid, a large population of South Africa dwell in informal settlements, where there is very little hope of development, as the government does not possess the resources that are necessary for a full-scale sanitation programme. Therefore, on-site treatments have been considered to provide sanitation in these dense peri-urban areas. The anaerobic baffled reactor (ABR) is one such sanitation system. This reactor utilises the phenomenon of anaerobic digestion to degrade substrates.

One of the major disadvantages of any anaerobic treatment processes is the extreme sensitivity of the bacterial communities, thus inducing slow recovery rates following toxic shocks. Therefore, an understanding of these microbial consortia is essential to effectively control, operate and optimise the anaerobic reactor. Fluorescence *in situ* hybridization, 4',6-diamidino-2-phenylindole (DAPI) staining and DNA sequencing techniques were applied to determine the microbial consortium, as well as their reactions to daily operating conditions. With an understanding of these populations and their responses to perturbations within the system, it is possible to construct an anaerobic system that is successful in its treatment of domestic wastewater.

In situ hybridizations were conducted for three operating periods, each characterised by specific flow rates. Results showed Eubacterial population dominance over the Archaeal population throughout both of the operating periods investigated. However, these cells cumulatively consisted of 50% of the total biomass fraction, as determined by DAPI staining. Group-probes utilised revealed a high concentration of fermentative acidogenic bacteria, which lead to a decrease in the pH values. It was noted that the ABR did not separate the acidogenic and methanogenic phases, as expected. Therefore, the decrease in pH further inhibited the proliferation of Archaeal acetoclastic methanogens, which were not present in the second operating period. DNA sequencing results revealed the occurrence of the hydrogenotrophic *Methanobacterium* and *Methanococcus* genera and confirmed the presence of *Methanosarcina.* Sequencing of the bacterial DNA confirmed the presence of the low G+ C Gram Positives (*Streptococcus*), the high G+C Gram Positives (*Propionibacterium*) and the sulfate reducing bacteria (*Desulfovibrio vulgaris*). However, justifications were highly subjective due to a lack of supportive analytical data, such as acetate, volatile fatty acids and methane concentrations. Despite this, findings served to add valuable information, providing details on the specific microbial groups associated with ABR treatment processes.

PREFACE

Some of the material presented in this dissertation has/will be published and presented elsewhere:

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GLOSSARY

Biological Oxygen Demand: refers to the consumption of oxygen in water caused by the activity of bacteria (Koppe *et al*., 1999).

Chemical Oxygen Demand: refers to the concentration of organic compounds in wastewater (oxidation of dichromate with silver ions as a catalyst) (Oerther *et al*., 1999 and Koppe *et al*., 1999).

Chemoorganotrophic heterotrophs: Organisms that use organic compounds as sources of energy, hydrogen, electrons and carbon for biosynthesis (Prescott *et al*., 1999).

Complement: With reference to the Watson-Crick base pairs, Adenine pairs with Thiamine (or Uracil) and Guanine pairs with Cytosine (Stahl and Amann, 1991).

Complementary base pairing rule: Only certain nucleotides can align opposite each other in the two strands of DNA: G pairs with C; A pairs with T (or U in RNA) (Orlica, 1992).

DAPI: DNA intercalating dye, which when exposed to the appropriate wavelength, fluoresces, allowing cells to be detected and enumerated (Porter and Feig, 1980).

Denature: To unfold, to become inactive. (DNA) (Orlica, 1992).

Deoxyribonucleic acid (DNA): DNA is a long, thin, chainlike molecule that is usually found as two complementary chains and is often hundreds to thousands of times longer than the cell in which it resides. The links or subunits of DNA are the four nucleotides called adenylate, cytidylate, thymidylate, and guanylate. The precise arrangement of these four subunits, repeated many times, is used to store all the information necessary for life (Orlica, 1992).

DNA polymerase: the enzyme complex that makes new DNA using the information contained in old DNA (Orlica, 1992).

Facultative anaerobes: Microorganisms that do not require oxygen for growth, but do grow better in its presence (Prescott *et al*., 1999).

Hydrolysis: the addition of water to large molecules that results in their breakdown into smaller molecules (Gray, 1989).

Match: Used to describe complete complementary between the probe and target sequence (Stahl and Amann, 1991).

Mismatch: This occurs at a position when the nucleotide in the probe does not complement the nucleotide at the same position in the target sequence (Stahl and Amann, 1991).

Nucleotide: one of the building blocks of nucleic acids. A nucleotide is com- posed of three parts: a base, a sugar, and a phosphate. The sugar and the phosphate form the backbone of the nucleic acid, while the bases lie flat like steps of a staircase. DNA is composed of deoxyadenylate, deoxythymidylate, deoxyguanylate, and deoxycytidylate, four different kinds of nucleotide represented by the letters A, T, G, and C (Orlica, 1992).

Obligate anaerobes: Organisms that grow only in the presence of oxygen (Prescott *et al*., 1999).

Oligonucleotide: a short piece of DNA or RNA containing 6-50 nucleotides (Stahl and Amann, 1991).

Organism: one or more cells organized in such a way that the unit is capable of reproduction (Orlica, 1992).

Oxidation: the addition of oxygen or the removal of hydrogen (Gray, 1989).

Photolithotrophic autotrophs: Organisms that use light energy, an inorganic electron source (water, hydrogen or hydrogen sulfide) and CO_2 as a carbon source (Prescott *et al*., 1999).

Photoorganotrophic heterotrophs: Microorganisms that use light energy and organic electron donors and employ simple organic molecules, rather than CO_2, as a carbon source (Prescott *et al*., 1999).

Polymerase Chain Reaction: a test tube reaction in which a specific region of DNA is amplified many times by repeated synthesis of DNA using DNA polymerase and specific primers to define the ends of the amplified region (Orlica, 1992).

Probe: a DNA or RNA molecule, usually radioactive, which is used to locate a complementary RNA or DNA by hybridizing to it (Orlica, 1992).

Reduction: the addition of hydrogen or the removal of oxygen (Gray, 1989).

Stringency: Describes the conditions of the hybridization or the hybridization wash step. The greater the stringency of the hybridization or wash step, the fewer the number of mismatches (Stahl and Amann, 1991).

Target: Target sequences for a probe is the complementary sequence to which the probe is designed to hybridize (Stahl and Amann, 1991).

ABBREVIATIONS

ABR	Anaerobic Baffled Reactor
ALF	Oligonucleotide probe specific for the alpha subclass of the *Proteobacteria*
ARC	Oligonucleotide probe specific for the Archaea
BAC	Oligonucleotide probe specific for the *Bacteriodes-Prevotella*
BET	Oligonucleotide probe specific for the beta subclass of the *Proteobacteria*
CF	Oligonucleotide probe specific for the *Cytophaga-Flavobacterium-Bacteriodes*
COD	Chemical Oxygen Demand
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DSB	Oligonucleotide probe specific for the *Desulfobacteriaceace*
DSV	Oligonucleotide probe specific for the *Desulfovibrionaceae*
EUB	Oligonucleotide probe specific for the domain Eubacteria
FISH	Fluorescence in situ hybridization
GAM	Oligonucleotide probe specific for the gamma subclass of the *Proteobacteria*
HGC	Gram-positive bacteria with high G+C DNA content
HRT	Hydraulic retention time
LGC	Gram-positive bacteria with low G+C DNA content
MS	Oligonucleotide probe specific for the *Methanosacina*
MX	Oligonucleotide probe specific for the *Methanosaeta*
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid

SMP	Soluble microbial products
SRB	Sulfate reducing bacteria
TCC	Total cell counts
VFA	Volatile fatty acids

CHAPTER ONE

INTRODUCTION

1.1 SANITATION: THE BASIC HUMAN RIGHT

The Water Services Act No.108 of 1997 states that every South African has the "right of access to basic water supply and basic sanitation necessary to ensure sufficient water and an environment not harmful to health or well being". According to this Act the term basic sanitation refers to "the minimum standard of services necessary for the safe, hygienic and adequate collection, removal, disposal or purification of human excreta, domestic wastewater and sewage from households, including informal households" (South Africa, Water Services Act No.108 of 1997).

Despite this constitutional right it is disturbing to note that approximately 20 million South Africans dwelling in rural and peri-urban areas live without adequately designed sanitation facilities. Partial justification for this occurrence is rooted in the past policies and strategies of the previous government, especially the segregation policy of apartheid, which negated control of sanitation services to certain communities. However, with the advent of a democratic government, change was imminent. Although many such areas still exist within the country, in the province of Kwa-Zulu Natal (K-ZN) certain schemes have been enforced to help overcome the sanitation problems. A non-government organisation, known as The Valley Trust, provided a primary health programme that served the peri-urban population living within the Valley of a Thousand Hills area. This programme entailed the course for the building of Ventilated Improved Pit (VIP) latrines in order to promote awareness for adequate sanitation. A similar scheme was enforced in the Amatikulu region of K-ZN (The Mvula Trust, 1995).

1.2 THE EFFECTS OF INADEQUATE SANITATION: WATERBORNE DISEASES

South Africa is not alone in her struggle for the provision of adequate sanitation and clean water. It is estimated that more than three billion people worldwide lack adequate sanitation and at least three to four million deaths are the result of waterborne diseases annually (Cosgrove and Rijsberman, 2000). Waterborne diseases are a result of inadequate sanitation. The water acts as a conduit for the transportation of microbial pathogens. Generally these pathogens are of intestinal origin and a result of discharge of human and animal faeces into water bodies. Pathogens associated with waterborne diseases include bacteria, viruses and protozoa (Task Force on Water Reuse, 1991).

1.2.1 Bacteria Involved In Waterborne Diseases

Bacteria are located indiscriminately in our environment, existing in water, air and soil, even on and in humans themselves. Not all bacteria are pathogenic but instead help maintain life for larger organisms (Spellman and Drinan, 2000). In humans, the bacterium *Escherichia coli* can be located in the large intestine as natural microflora. However, different strains, when water or foodborne, can lead to severe diarrhoea and vomiting (Nester *et al*., 1998).

There are many pathogenic bacteria that cause waterborne diseases. *Vibrio cholerae* is probably one of the most recognised waterborne pathogen of faecal origin. When ingested, the bacterium adheres to the epithelium of the small intestine and produces an enterotoxin called choleragen. This causes diarrhoea and vomiting in infected individuals and if not adequately treated, leads to death (Nester *et al*., 1998).

Another common waterborne pathogen is *Salmonella*. These bacteria, when introduced into the human system by faecally contaminated water and food, attack the gastro-intestinal tract. Infections are characterised by severe diarrhoea, vomiting and fever. *Shigella* sp. enter the human intestinal tract via the same mechanism as *Salmonella* and cause death of intestinal cells. Symptoms of *Shigella* include fever, diarrhoea, vomiting, pus and blood in faeces, stiff neck and convulsions (Nester *et al*., 1998). Bacteria involved in the transmission of waterborne diseases are tabulated in Table 1.1.

Table 1.1: Waterborne disease-causing bacteria (Spellman and Drinan, 2000).

MICROORGANISM	DISEASE
Salmonella typhi	Typhoid fever
Salmonella sp.	Salmonellosis
Shigella sp.	Shigellosis
Campylobacter jejuni	Campylobacter enteritis
Yersinia enterocolitica	Yersinosis
Escherichia coli	Gastroenteritis

1.2.2 Viruses Involved In Waterborne Diseases

Viruses are the most minuscule living infectious agent. They have no nucleus, cell wall or cell membrane and are unable to multiply in the absence of a living host; however, they are capable of survival within the environment. These viruses have the ability to infect humans through the ingestion of contaminated water. There are approximately a hundred known viruses occurring in human faeces alone, thus increasing the probability of contamination of unsanitized water (Spellman and Drinan, 2000).

The Hepatitis A virus is a common waterborne pathogen and is transmitted via the faecal-oral route. The infection causes damage of the liver but symptoms are mild (Nester *et al*., 1998).

There are many viruses that cause gastroenteritis. These include Rotavirus and Norwalk virus. Both are transmitted via the faecal-oral route and cause diarrhoea, abdominal cramps and vomiting (Nester *et al*., 1998). The viruses involved in the transmission of waterborne diseases are tabulated in Table 1.2.

Table 1.2: Waterborne disease-causing viruses (Task Force on Water Reuse, 1991).

MICROORGANISM	DISEASE/SYMPTOM
Echo virus	Diarrhoea, fever, meningitis
Coxsackie virus	Meningitis, respiratory disease, fever
Hepatitis A virus	Infectious hepatitis
Rotaviruses and Norwalk virus	Gastroenteritis

1.2.3 Protozoa Involved In Waterborne Diseases

Protozoa are eukaryotic organisms that are single-celled. This cell serves as the entire body, within which contains the facilities for performing the bodily functions. Most of these are harmless, however, some are parasitic (Spellman and Drinan, 2000).

Giardia lamblia is a waterborne pathogen that enters the human system following ingestion of faecally contaminated water. They reproduce within the small intestine and cause irreversible

mucosal damage. Symptoms include indigestion, nausea, diarrhoea and weight loss (Nester *et al*., 1998).

Cryptosporidium sp. are transported via unsanitary water and infests in the human body. Symptoms include, diarrhoea and vomiting. Another common parasite is *Entamoeba hystolytica*, which causes severe diarrhoea and abdominal pain (Nester *et al*., 1998). Protozoa involved in the transmission of waterborne diseases are tabulated in Table 1.3.

Table 1.3: Waterborne disease-causing protozoa (Task Force on Water Reuse, 1991).

MICROORGANISM	DISEASE/SYMPTOM
Cryptosporidium	Diarrhoea
Balantidium coli	Diarrhoea, dysentery
Entamoeba histolytica	Dysentery, liver abscess
Giardia lamblia	Diarrhoea

1.2.4 Helminths Involved In Waterborne Diseases

Helminths are intestinal worms that multiply in sewage and wet soil. They are microscopic in size and can be present in even treated water. Ingestion of these worms can lead to a variety of diseases and infections, as detailed in Table 1.4 (Spellman and Drinan, 2000).

Table 1.4: Waterborne disease-causing helminths (Task Force on Water Reuse, 1991).

MICROORGANISM	DISEASE/SYMPTOM
Nematodes (Roundworms)	
Ancylostoma duodenale	Anaemia
Strongyloids stercoralis	Strongyliodiasis (skin inflammation, lung or abdominal disturbances
Trichuris trichuria	Trichuriasis (bloody stool, diarrhoea)
Cestodes (Tapeworms)	
Taenia saginata	Taeniasis (digestive disturbances)
Diphyllobothrium latum	Diphyllobothriasis (anaemia, diarrhoea)
Trematodes (Flukes)	
Chlonorichis sinensis	Chlonorchiasis (diarrhoea, abdominal and liver disturbances)
Paragonimus westermani	Paragonimiasis (blood coughing, cerebral disturbances)

The above diseases may be prevented or at least reduced in incidence if proper sanitation protocols are adhered to. It is the duty of the government to ensure that procedures are arranged to provide the adequate sanitation to its country's people.

1.3 CURRENT SANITATION TECHNOLOGIES

There are systems in place for the treatment of domestic wastewaters arising from rural areas. However, in South Africa, many are still in the design process or are too expensive for the poorer communities to afford.

1.3.1 The Traditional Pit Latrine

This is the most commonly employed sanitation technology in the rural communities of South Africa. It consists of a pit that is dug into the ground and enclosed in a shelter. The disadvantages of this include the occurrence of obnoxious odours, which leads to fly breeding, thus encouraging the progression of disease. The units are rarely kept in a sanitary condition and are regarded as a hazard as the structures are normally unstable (The Mvula Trust, 1995).

1.3.2 The Ventilated Improved Pit Latrine

This improvement on the pit latrine encompasses a strong and stable foundation and a ventilation pipe on the roof of the latrine that is fitted with a fly screen. If properly constructed and utilised, it provides the health benefits of a conventional waterborne sewage system (The Mvula Trust, 1995).

1.3.3 Septic Tanks

This system has been widely used in the peri-urban areas of South Africa (The Mvula Trust, 1995). This system includes a septic tank and a leaching field system, which trap and store solids while letting the effluent flow into a leaching or absorption field, where it seeps into the soil and degrades naturally (Spellman and Drinan, 2000).

The disadvantage of all of the above systems is that the billions of gallons of sewage, which enter the ground annually, are not properly treated, as there is inadequate reticulation for the water borne sewage. These disposal systems are the breeding grounds of faecal bacteria, which contaminate the water supplies and result in the progression of waterborne diseases (Spellman and Drinan, 2000). These disadvantages can be overcome by the use of stabilisation ponds.

1.3.4 Stabilisation Ponds

This system is a high cost sanitation technology. Excreta is flushed in a conventional cistern-toilet system and transported via a network of sewer pipes to a treatment works. For rural areas, the most favourable treatment considered is stabilisation ponds. The advantages of such a plant are that they are robust and easy to operate. However, they do not produce an effluent that conforms to the standard effluent discharge limits (The Mvula Trust, 1995).

1.3.5 Conventional Waterborne Sanitation

This system is widely applied in urban areas and has a great success rate. However, implementation of this system in rural areas is not seen as a possibility. The major disadvantages are that it is very expensive, putting it out of the affordability range of rural settlements, it requires large volumes of water and there are difficulties with operation and maintenance (The Mvula Trust, 1995).

1.4 THE DEVELOPMENT OF AN AFFORDABLE SANITATION TECHNOLOGY FOR APPLICATION IN RURAL AREAS: ANAEROBIC BAFFLED REACTOR

The government of South Africa does not possess the resources that are necessary for a full-scale sanitation programme. Therefore, on-site treatments have been considered to provide sanitation in these dense peri-urban areas (Dama *et al*., 2001).

A Water Research Commission project entitled 'The Anaerobic Baffled Reactor for Sanitation in Dense Peri-Urban Settlements' has been accorded the task of providing an appropriate sanitation system that can be successfully applied in these areas. However, there were some constraints placed on the design of this reactor: -

1. The reactor should be compact and require very little maintenance, as most of peri-urban areas are rocky and steep, therefore, causing difficulties for maintenance teams to navigate there.
2. It should be robust to handle varying hydraulic and organic loads. Since the communities vary in size, its water use patterns will be dependant on the proportion of that community employed and the proximity of this place of employment.
3. It should require the minimum amount of maintenance and have no energy requirements as there is at present inadequate infrastructure to provide such a service and technology.
4. It should have no removable parts in order to decrease the risk of theft.
5. The effluent must meet the South African discharge standards
6. Since there is no pretreatment, the system must be able to handle the possibility of grit build-up and a high solids concentration (Foxon *et al*., 2001).

Therefore, taking into consideration the above, a pilot scale anaerobic baffled reactor (ABR) was developed and investigated by the Pollution Research Group (PRG), University of Kwa-Zulu Natal (Natal campus) (Dama *et al*., 2001). The success of this experimentation has lead to the development of a 3000L ABR that has been operating at Kingsburgh Wastewater Works since 2002.

The ABR meets the requirements for the development of a suitable sanitation technology for peri-urban or rural areas in the following way:

1. It requires very little space and is simple in design.
2. No mechanical mixing is needed; therefore, no power requirements are necessary.
3. The ABR is known to function effectively under a wide range of flow and load conditions.
4. The ABR requires little or no maintenance (Foxon *et al*., 2001).

One of the major limitations of the ABR is that the pathogen removal rate is low and the effluent characteristics do not meet that of the discharge standards (Foxon *et al*., 2001).

1.5 MAIN OBJECTIVES OF THIS STUDY

One of the major disadvantages of any anaerobic treatment processes is the extreme sensitivity of the bacterial communities, thus inducing slow recovery rates following toxic shocks. Therefore, an understanding of these consortia is essential to effectively control and operate the anaerobic reactor (Merkel *et al.*, 1999). This project, therefore, aimed to study the microbial consortia involved in the treatment of wastewater by the ABR with the intention of profiling the significant bacteria, which make up the large community and discerning their reactions to the daily changes

in the reactor. The characterisation was conducted utilising highly accurate novel molecular techniques.

To achieve this aim, the main objectives set out were:

1. To optimise pretreatment of the sludge to achieve accurate quantification of the microorganisms by fluorescent *in situ* hybridization (FISH).
2. To optimise the conditions for the various probes and to carry out FISH.
3. To compare the changes in the microbial population to physical and chemical changes in the ABR.
4. To optimise the extraction of DNA from the microbial consortium.
5. To amplify the DNA using PCR with specific primers.
6. To determine the genetic sequence of the amplified DNA and determine its origin using the GenBank system.
7. To verify FISH results using the sequence analysis obtained from GenBank.

CHAPTER TWO

LITERATURE REVIEW

2.1 WATER: THE UNIVERSAL REQUIREMENT FOR LIFE

"Water is like the blood in our veins" - Levi Eshkol, Israeli Prime Minister, 1962

Water is the basic need of mankind. The arrival of the first life forms, 3.5 billion years ago, began with the formation of water. Ever since, all life forms have been dependant on this resource for sustenance (Cosgrove and Rijsberman, 2000). Water on our planet exists in a closed system referred to as the hydrosphere and the way it circulates through the earth's systems is called the hydrologic cycle (de Villiers, 1999). This cycle (Figure 2.1) is the mechanism by which water, in its three forms (solid, liquid and gas), circulates within our environment. Water is lost from the earth's surface via evaporation (from lakes, oceans and other water bodies) or by transpiration of plants. This lost water forms clouds, which in turn condense to form precipitation, which returns to the earth's surface as rain and snow (Spellman and Drinan, 2000).

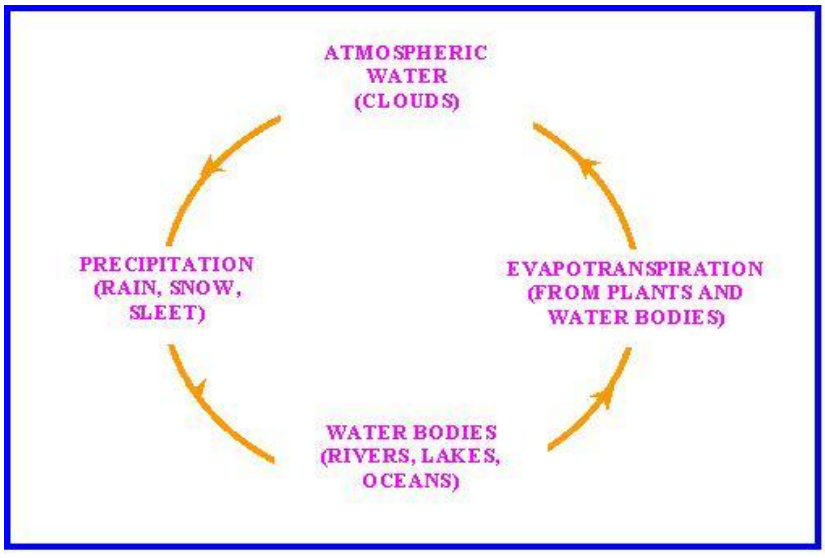

Figure 2.1: The hydrologic cycle (Adapted from Spellman and Drinan, 2000)

Humans, however, have intervened in this natural cycle and this has lead to the formation of an artificial water cycle referred to as the urban water cycle, which is represented in Figure 2.2. Water, from lakes and rivers, is utilised for man's purposes and then treated by various methods. The treated water is then dispersed into receiving water bodies. Thereafter, the natural water cycle commences (Spellman and Drinan, 2000).

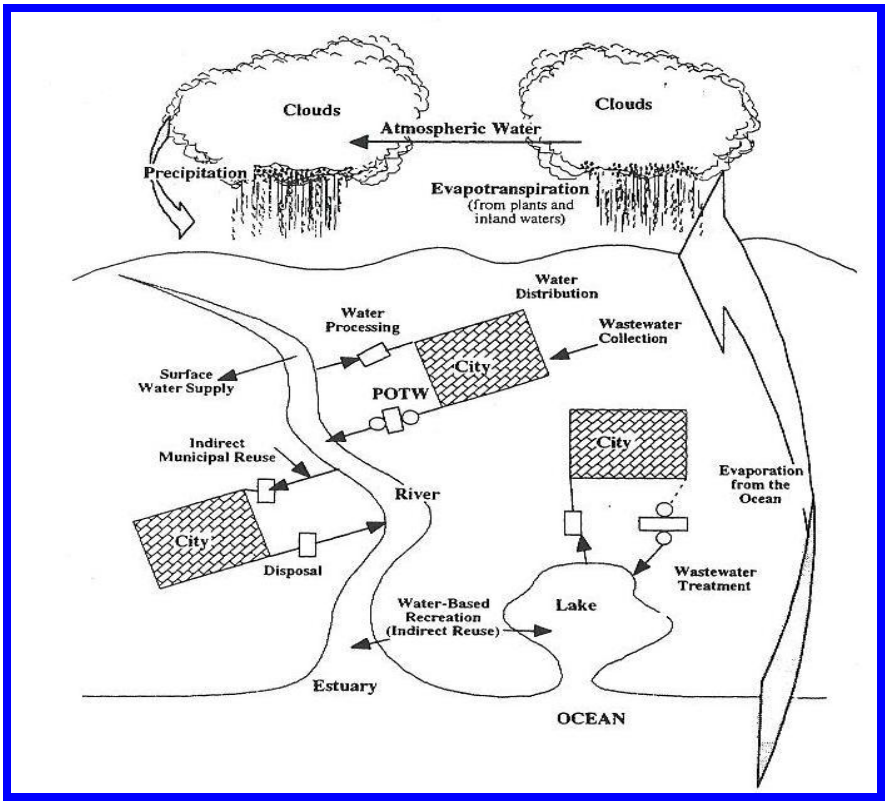

Figure 2.2: The urban water cycle (Spellman and Drinan, 2000).

Despite the recycling of water by these cycles, there is an escalating need for more water. The total amount of water on our planet has not decreased since geological times. Atmospheric moisture, the oceans, the rivers, the lakes, the polar ice caps, saturated soil (wetlands) and groundwater are all reservoirs of water (de Villiers, 1999). It is however, the quality of the freshwater available for human consumption that has diminished. This may be attributed to the

improper management of water, so much so, that millions of people worldwide do not have access to safe and affordable drinking water (Cosgrove and Rijsberman, 2000).

Water is needed for the irrigation of crops, for personal use (drinking, cooking and bathing) and in many industries. This used water, or wastewater, has often been inadequately collected and treated. Not only is this a health hazard for humans, it also leads to pollution of aquatic systems, our fundamental sources of water (Cosgrove and Rijsberman, 2000). This wastewater, when treated and properly recycled, is an alternate water source, which can reduce the needs for fresh water. This also reduces stress on the environment, as treated wastewaters do not pose the infinite health hazards of untreated water and do not cause drastic effects on the aquatic life forms (Task Force on Water Reuse, 1991).

2.2 WASTEWATER CHARACTERISTICS AND LEGISLATIONS

Wastewater may be defined as any water that has been utilised by man and then returned to a water body after being altered physically or chemically (Gallert and Winter, 1999). Generally municipal wastewater is an amalgamation of numerous substances. During dry weather, the majority of the wastewater is of domestic origin, which arises from the flushing of toilets, bathing water, hand washing, cleaning and cooking. The compounds produced from this water include fats, carbohydrates, proteins and urea (Koppe *et al*., 1999). These compounds can be broken down into their elemental levels to produce substances that are harmful to our environment. These elements have been summarised in Table 2.1.

Table 2.1: Components of domestic wastewater (Koppe *et al*., 1999).

COMPOUND	ORIGIN
1) Hydrocarbons	Food, excrement
2) Nitrogen	
Ammonium/Ammonia	Faeces, urine
Nitrite	Pickle salt
Nitrate	Food, drinking water
3) Phosphorus	
Dihydrogen phosphate Hydrogen phosphate Phosphate	} Urea, drinking water, food
Phosphoric acid	Beverages
Magnesium ammonium phosphate	Urea
Calcium phosphate	Faeces
Apatite	Cleaning water, household
4) Sulfur	
Sulfate	Urea, washing agents, drinking water
Sulfites	Created during anaerobic processes
Hydrogen sulfide, sulfide	Reduction of sulfate and proteins under anaerobic conditions
5) Halogens	
Chlorides	Urea, drinking water

Since wastewater is contaminated by the above compounds, purification methods have to be enforced to protect the environment from deterioration (Gallert and Winter, 1999). These methods have to be economical and safe and be able to recycle and recover the valuable

components of wastewater (Gray, 1989). Legislations are enforced to ensure that this aim is achieved. In South Africa, municipal wastewater, according to the Water Act No. 36 of 1998, is not allowed to exceed the following parameters with special limits being set for effluent disposal in catchment areas (Table 2.2).

Table 2.2: Wastewater limit values applicable to discharge of wastewater into a water source according to amended Act 54 of 1956 (South Africa, Water Services Act No.54 of 1956).

SUBSTANCE/PARAMETER	GENERAL LIMIT	SPECIAL LIMIT
Faecal coliforms (per 100 ml)	0	0
Chemical Oxygen Demand (mg/L)	75	30
pH	55-9.5	5.5-7.5
Maximum Temperature	35ºC	25ºC
Chlorine as free Chlorine (mg/L)	0.25	0
Suspended solids (mg/L)	25	10
Orthophosphate as phosphorus (mg/L)	10	1
Fluoride	1	1
Soap, oil or grease (mg/L)	2.5	2.5
The effluent must not contain any substance capable of producing colour, odour or taste.		

2.3 WASTEWATER TREATMENT

Domestic wastewater may be distinguished as a low-strength wastewater. This is characterised by:

1) low COD concentrations

2) high amounts of suspended solids

3) low temperatures and

4) strong fluctuations in hydraulic and organic loading rates (van Lier *et* al., 2001).

In order to meet the requirements for disposal, this wastewater has to first be treated. There are many processes in place to achieve this aim. Conventional wastewater treatments may be divided into three stages, namely, primary, secondary and tertiary treatments.

1. **Primary Treatment:**

This involves the removal of sedimentary material by allowing the passage of the liquid portion of wastewater through a series of screens, while large objects are hampered. Following this removal, the water is allowed to settle for a time period that is conducive for the precipitation of aluminium sulfate and ferrous sulfate. Thereafter, the remaining liquid is treated by the second phase and the sedimentation is either incinerated or treated using digesters (Nester *et al*., 1998).

2. **Secondary Treatment:**

Secondary treatment entails the stabilisation of organic materials present in sewage and a reduction of the biological oxygen demand (BOD). This process is generally carried out aerobically, using a community of microorganisms. However, anaerobic technologies are in place. The organic material is degraded by bacteria and incorporated into their cell material. These bacteria are themselves a food source for ciliates, protozoa and nematodes (Nester *et al*., 1998).

3. **Tertiary Treatment:**

This treatment involves the removal of nitrates and phosphates in order to prevent pollution of the receiving water bodies. Once the levels are at those acceptable for discharge, the treated water is discarded into rivers and lakes (Nester *et al*., 1998)

The most popular form of wastewater treatment is that of activated sludge. This is an aerobic biological strategy that utilises a consortium of mixed microbial communities to degrade the components of wastewater (LaPara *et al*., 2000). However, many of these aerobic treatments are finding disfavour. Aerobic treatments, such as the activated sludge process are too costly to be emplaced in developing regions. Aerobic systems produce large amounts of sludge that have to be later anaerobically degraded. In addition to that, a high-energy input is required for aeration and sometimes nutrient additions are required, all of which is unnecessary for anaerobic treatments. Energy is wasted in aerobic treatment systems, while energy is stored in the form of methane in anaerobic systems (Gijzen, 2001). Therefore, anaerobic systems are being suggested in place of aerobic treatments to help eliminate these problems. Although anaerobic treatments are more expensive to construct, they are less expensive to operate than aerobic treatments (Gallert and Winter, 1999).

Anaerobic treatments have been utilised from as long as 1881. However, following the development of aerobic treatments, anaerobic technologies have been limited to the treatment of aerobic sludge and wastewater treatment in individual households in the form of septic tanks (Garuti *et al*., 1992). This is due to the misconceptions that anaerobic treatments are poor for treatment of low strength wastewaters (<1000 mg COD/l), that they cannot tolerate inhibitory compounds in waste, are inoperable at low temperatures, that is, below 35ºC and have poor removal efficiencies. However, due to the rising cost of aerobic treatments and its disadvantages mentioned above, anaerobic techniques applying the process of anaerobic digestion are finding renewed favour (Langenhoff *et al*., 2000). Anaerobic treatments have many advantages and disadvantages. These have been tabulated in Table 2.3.

Table 2.3: Advantages and disadvantages of anaerobic treatment (Speece, 1996).

ADVANTAGES	DISADVANTAGES
Provision of process stability	Long start-up period
Reduction of waste biomass disposal costs	Insufficient effluent quality
Reduction of N and P supplementation costs	Sulfide and odour generation
Reduction of installation space requirements	Low kinetic rates at low temperatures
Conservation of energy	Nitrification not possible
Minimization of operational attention requirement	

The success of any anaerobic treatment process is dependant on its microbial community. This environment consists of various microorganisms that interact to completely degrade the components of wastewater in the process of anaerobic digestion (McInerney, 1999).

2.4 ANAEROBIC DIGESTION

The process of anaerobic digestion and its formation of methane and carbon dioxide are prevalent throughout nature. This process occurs in a diversity of habitats including the rumen of animals, lower intestinal tract of humans, sewage digesters, landfill sites, rice paddies and the sediments of lakes and rivers (Ferry, 1992). This process has been exploited in the form of anaerobic digesters to aid in the treatment of wastewaters.

2.4.1 The Chemistry Of Anaerobic Digestion

The process of anaerobic digestion involves the systematic conversion of organic compounds to methane via the employment of a consortium of microorganisms (Bitton, 1994). These

microorganisms do not exist in isolation but instead, co-exist to carry out their functions at optimum levels, thus establishing a community, of which bacteria are dominant (Atlas, 1997). These bacteria catalyse the complex conversion of high molecular weight organic compounds to methane utilising the following reaction: **Organic matter** $\longrightarrow$ **$CH_4 + CO_2 + H_2 + NH_3 + H_2S$** Four groups of bacteria within the system carry out the above reaction through synergistic interactions (as seen in Figure 2.3) (Bitton, 1994).

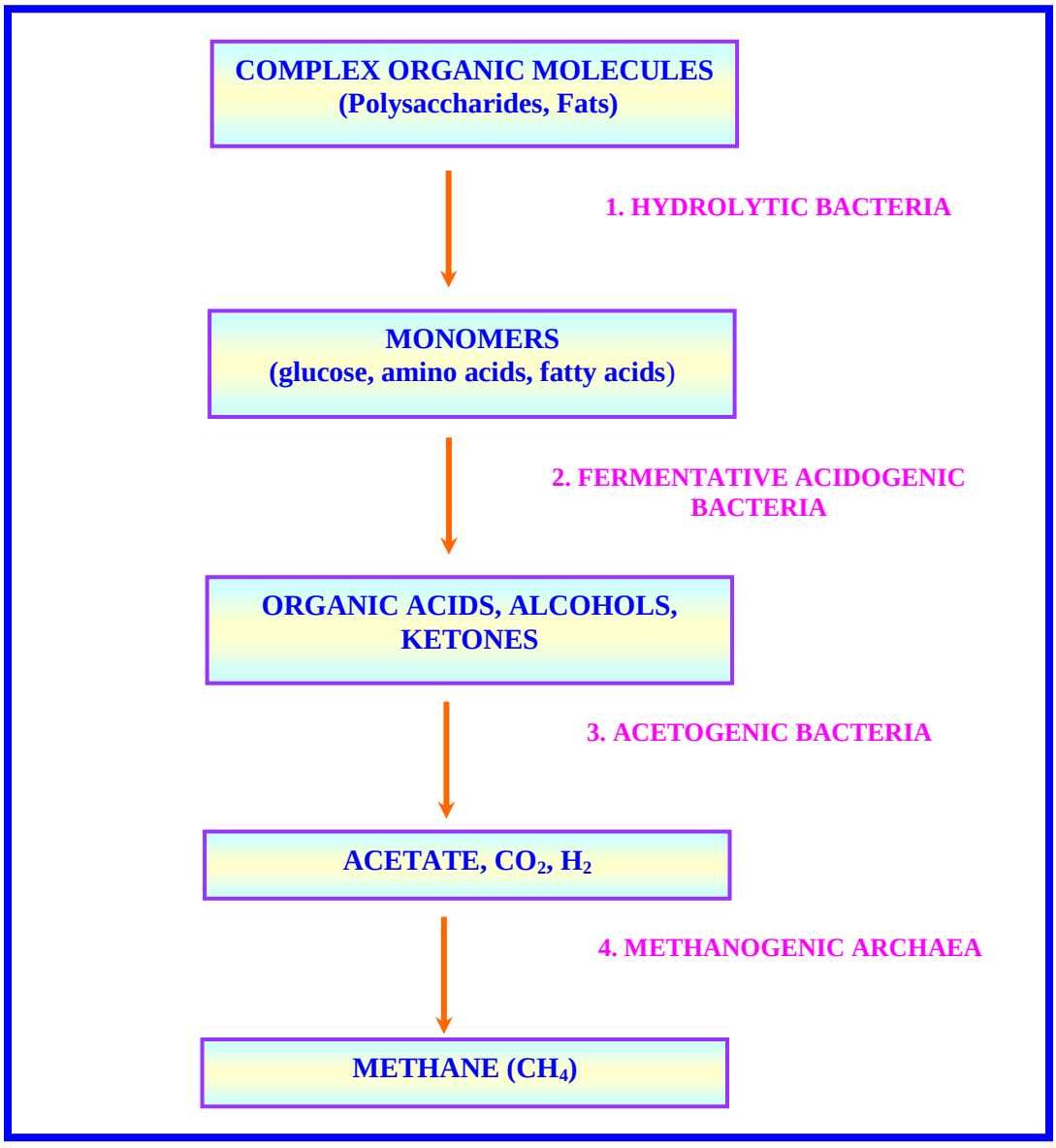

Figure 2.3: Microbial groups involved in the anaerobic digestion of wastes (Bitton, 1994)

With reference to Figure 2.3:

Step 1: The first step involves the hydrolysis of organic polymers by hydrolytic bacteria. These bacteria breakdown the complex organic molecules, such as proteins, cellulose, lignin and lipids into soluble monomers like amino acids, glucose, fatty acids and glycerol. The hydrolysis is catalysed by the enzymes cellulase, protease and lipase (Bitton, 1994 and Guiot *et al*., 1992). This may be the rate-limiting step in the degradation of particles as this process is slower for particles than for soluble organics (Langenhoff *et al*., 2000). This process is generally carried out by the bacteria belonging to the groups *Cytophaga-Firmicutes* (CF) and Low G+C bacteria (LGC) (Prescott *et al*., 1999).

Step 2: The soluble monomers produced in step 1 are now utilized by the fermentative acidogenic bacteria to form organic acids (acetic, propionic, formic and lactic), alcohols, ketones volatile fatty acids (VFAs) carbon dioxide (CO_2) and hydrogen (H_2) (Bitton, 1994 and Guiot *et al*., 1992). Major acid forming bacteria are *Bacillus, Clostridium, Peptostreptococcus, Micrococcus* and *Pseudomonas*. Other bacteria involved in this step belong to the groups *Bacteriodes,* LGC, gamma (*γ)- Proteobacteria and alpha (α)-Proteobacteria* (Gray, 1989 and Prescott *et al*., 1999).

Step 3: Obligate hydrogen-producing acetogens degrade propionate, long chain VFAs and certain aromatic compounds to acetate, CO_2 and H_2. A minor group of hydrogen-consuming acetogens reduce CO_2, CO and methoxyl-groups of aromatic compounds to acetate and sometimes butyrate (Guiot *et al*., 1992). This process requires a low hydrogen tension for the conversion of fatty acids (Bitton, 1994). Common bacteria associated with this process include *Acetobacter* and *Syntrophobacter, Syntrophomonas* and other bacteria belonging to the High G+C (HGC) group (Leach, 1994 and Prescott *et al*., 1999).

Step 4: This is the final step of anaerobic digestion and possibly the most important. The group of microorganisms involved in this process are the Archaeal methanogens. These strictly anaerobic organisms facilitate the conversion of hydrogen, formate and acetate to methane (Ferry, 1992). This process involves the use of both the hydrogenotrophic and acetotrophic methanogens (Bitton, 1994). Methane is not the only end product of anaerobic digestion. Hydrogen sulfide is also produced. This is formed by the utilisation of sulfate by sulfate-reducing bacteria (SRB). This poses a problem as hydrogen sulfide causes environmental pollution and the SRB are competitors of the methanogens for acetate (Barber and Stuckey, 2000).

2.4.2 Factors Influencing Anaerobic Digestion

Many factors have to be taken into consideration for the successful application of anaerobic digestion. These include:

i. Temperature

Temperature is an important factor as it governs the growth and metabolic rate of microorganisms (McInerney, 1999). An increase in temperature increases the rate of reaction and thereby decreasing the retention time requirements. Most systems are therefore operated in the mesophilic temperature range (Muyima *et al*., 1997).

ii. pH

pH stability is of great importance to the anaerobic process. Optimum pH ranges between 6.5 and 7.5. If the pH falls below or close to 6.0, the sensitive methanogenic bacteria are inhibited and start to die (Bitton, 1994 and Muyima *et al*., 1997).

iii. Oxygen

Oxygen is extremely toxic to the obligate anaerobic methanogens and these bacteria are inhibited by even small concentrations (Bitton, 1994 and Muyima *et al*., 1997).

iv. Nutrient requirements

Wastewater being treated must be nutritionally balanced. The major elements required are carbon, hydrogen, nitrogen, phosphorus, magnesium, potassium and calcium. Trace elements are also required by the methanogens. These include iron, zinc, manganese, nickel, molybdenum and cobalt. Many of these elements act as co-factors for enzymes (Leach, 1994).

v. Toxins

There are a wide variety of toxins that adversely affect the digestion process. These include halogenated organics, phenols, ammonia, chlorinated hydrocarbons and formaldehyde (Bitton, 1994 and Leach, 1994).

Sulfide is one of the most formidable inhibitors of anaerobic digestion. Sulfide is more toxic to the methanogens than the acid-forming bacteria and is toxic at concentrations above 150-200mg/L (Bitton, 1994).

Anaerobic digestion may at any time be inhibited by the formation of several of its intermediates that are produced during the process. High concentrations of H_2, and VFAs are toxic and lead to feedback inhibition, inhibiting especially the methanogens (Bitton, 1994 and McInerney, 1999).

The above criteria are important as they help provide optimum conditions for the growth and replication of the microorganisms that make up the anaerobic sludge, which is vital for the successful application of anaerobic digestion.

2.4.3 The Microbiology Of Anaerobic Digestion

The success of any anaerobic digestion process is dependent upon its microbial population. This population is composed of a diverse group of microorganisms that include genera belonging to both the domains of *Bacteria* and *Archaea*.

2.4.3.1 Differentiation between *Bacteria* and *Archaea*

Figure 2.4 represents the universal phylogenetic tree. It is believed that the *Archaea* and *Eubacteria* were the first to develop, followed later by the *Eucarya* (Prescott *et al*., 1999).

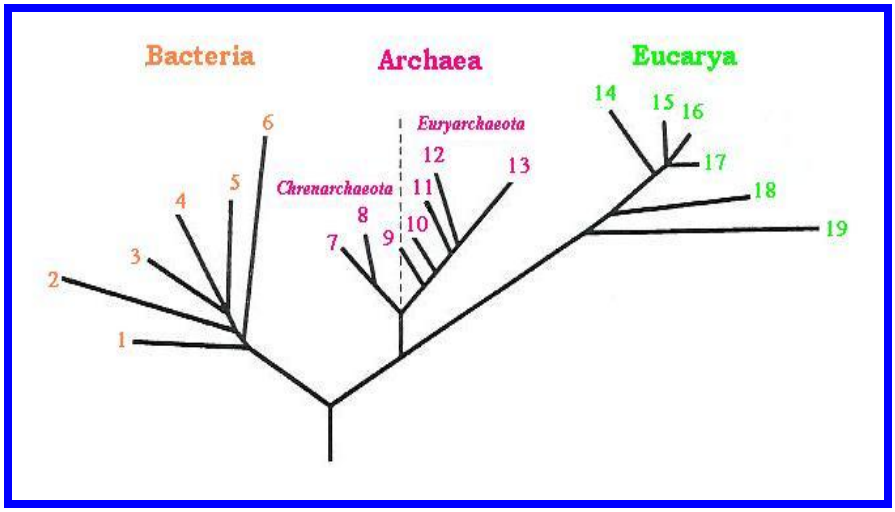

Figure 2.4: Universal phylogenetic tree (Woese *et al*., 1990).

Where:

a. *Bacteria*-

1: Thermotogales

2: The Flavobacteria and Relatives

3: The Cyanobacteria

4: The Proteobacteria (Purple Bacteria)

5: The Gram-positive Bacteria

6: The green non-sulfur bacteria

b. *Archaea*

Kingdom: *Chrenarchaeota*:

7: *Pyrodictium*

8: *Thermoproteus*

Kingdom: *Euryarchaeota*

9: The Thermococcales

10: The Methanococcales

11: The Methanobacteriales

12: The Methanomicrobiales

13: The extreme halophiles

c. The *Eucarya*

14: The animals

15: The ciliates

16: The green plants

17: The fungi

18: The flagellates

19: The microsporidia

The Eubacterial domain consists of prokaryotic cells that contain Eubacterial rRNA and membrane lipids composed primarily of diacyl glycerol diesters. They generally lack extensive complex internal membrane systems and can be found in a variety of shapes together in pairs, clusters and chains. Bacteria are classified according to their gram stain reaction, which is either positive (purple or violet) or negative (red). Gram-positive bacteria are those whose cell walls contain a thick homogeneous layer of peptidoglycan, muramic and teichoic acids. Gram-negative

bacteria, on the other hand, contain a thin layer of peptidoglycan, which is surrounded by a complex outer membrane of lipopolysaccharides (LPS) (Prescott *et al*., 1999).

Archaea resemble bacteria only in their prokaryotic cell structure. They lack muramic acids in their cell walls and possess membrane lipids with ether-linked aliphatic chains. They also contain distinctive RNA polymerase enzymes and have distinctive ribosomes of different composition and shape (Prescott *et al*., 1999). Certain archaeal genomes contain families of repeat sequence DNA, which is common in eukaryotic cells (Woese, 1987). They can occur in a variety of shapes in forms of single cells as well as clusters. Many microorganisms of this domain are extremophiles, existing and proliferating in habitats normally considered too severe for life (Schiraldi *et al*., 2002). However, they cannot readily adapt to aerobic conditions and this domain does not contain any obligate aerobes (Woese, 1987).

2.4.3.2 The domain *Archaea*

A. Methanogens

The most abundant group of *Archaea* involved in anaerobic digestion are the methanogens. These are strict anaerobes that form methane as their major metabolic end product from CO_2, H_2, formate, methanol and acetate (Holland *et al*., 1987). Methanogens construct three different types of cell walls and generally differ from each other in shape, 16S rRNA sequence and other features (Prescott *et al*., 1999). They fluoresce a greenish-blue when viewed under a fluorescent microscope. The methanogens are found in the Kingdom Euryarchaeota and can be divided into three orders, Methanobacteriales, Methanococcales and Methanomicrobiales, which encompass many genera (Holland *et* al, 1987). Selected genera have been described in Table 2.4.

Table 2.4: Characteristics of selected methanogens (König, 1993).

Order I: Methanobacteriales

Family I: Methanobacteriaceae

Optimum Temperature: 37ºC and 70ºC.
Energy Source: H_2 and formate
Gram reaction: Positive

Genus 1: *Methanobrevibacter*

Morphology: short, lancet-shaped rods. Some species have a single flagellum.
Temperature: Mesophilic
DNA Base Composition: 27-32 mol % G +C

Genus 2: *Methanobacterium*

Morphology: straight, long, irregular rods that are non-motile.
Temperature: Mesophilic to thermophilic
DNA Base Composition: 33-61 mol % G +C

Order II: Methanococcales

Family I: Methanococcaceae

Energy Source: H_2/CO_2 and formate
Gram reaction: Negative

Genus 1:*Methanococcus*

Morphology: regular to irregular cocci.
Temperature: Mesophilic to extremely thermophilic
DNA Base Composition: 31-41 mol % G +C

Order III: Methanomicrobiales

Family I: Methanomicrobiaceae

Energy Source: H_2, formate
Gram reaction: Negative

Genus 5: *Methanospirillum*

Morphology: rods forming long filaments. Polar flagellation.
Temperature: Mesophilic
DNA Base Composition: 46-50 mol % G +C

Family II: Methanosarcinaceae

Genus 1: *Methanosarcina*

Energy Source: H_2/CO_2, acetate, methanol
Gram reaction: Positive
Morphology: cocci, singular or in clumps
DNA Base Composition: 40-51 mol % G +C

Genus 5: *Methanosaeta*

Energy Source: Acetate
Morphology: Thick rods, forming long filaments in a protein sheath.
DNA Base Composition: 61 mol % G +C

This diverse group of *Archaea* contain co-enzymes and co-factors that are unique to them. Examples include the co-enzyme M (CoM) is the smallest co-enzyme known and is essential to the enzyme methyl CoM reductase (Holland *et al*., 1987). Factor F_{420} is a biochemical component of methane formation. It fluoresces a bright blue under ultraviolet (UV) light and can be viewed with a UV microscope (Hobson and Wheatley, 1993). The co-factor F_{350} plays a role in the terminal step of methane reduction. This factor fluoresces yellow under UV light (Hobson and Wheatley, 1993 and König, 1993).

The methanogens are most probably the most important group involved in anaerobic digestion. Of the many Methanogenic genera only two are known to perform acetoclastic reactions, that is, the formation of methane from acetate (Rocheleau *et al*., 1999). These are the genus *Methanosarcina* and the genus *Methanosaeta*. Species belonging to the former genus have a higher maximum growth rate than the latter species. *Methanosaeta* species, however, have a lower threshold for acetate than *Methanosarcina* species. This, therefore, suggests that *Methanosaeta* species will proliferate at low acetate conditions (Raskin *et al*., 1994a).

Along with inter-genus competition, methanogens have to compete with the sulfate reducing bacteria (SRB) for common substrates such as hydrogen and acetate. Both these groups catalyse the terminal stage of anaerobic digestion and are dependant upon other microorganisms to convert the complex organics to simpler compounds. In the presence of non-limiting levels of sulfate, the SRB generally outcompete the methanogens. However, in the presence of low-sulfate environments, methanogens are dominant (Raskin *et al*., 1996). In the total absence of sulfate, certain SRB, such as *Desulfovibrio* sp., have been known to grow together with the methanogens to convert ethanol or lactate to acetate (Wu *et al*., 1991).

2.4.3.3 The domain *Bacteria*

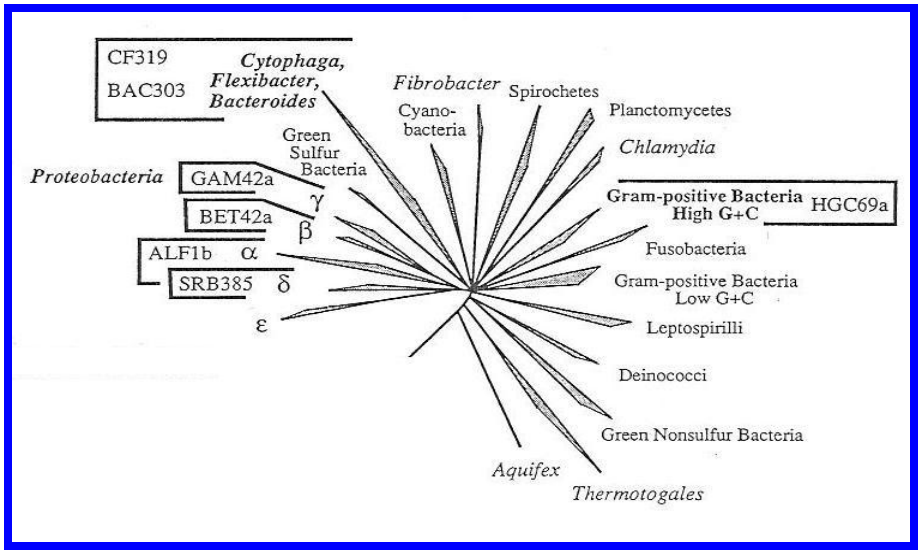

Figure 2.5: The bacterial phylogenetic tree (Amann *et al*., 1995).

Figure 2.5 shows the bacterial phylogenetic tree describing the divisions of the various bacteria, many of which are further described below.

A. Bacteriodes

This group contains species that are obligate anaerobic, non-sporing and motile or non-motile rods. They are chemoheterotrophic, and produce a mixture of organic acids as fermentation end-products (Prescott *et al*., 1999). They generally grow up to 1.0µm in diameter and 2-20µm in length. Most are non-motile with a few containing flagella (Holland *et al*., 1987). They are generally isolated from human faeces (Prescott *et al*., 1999).

B. Cytophaga-Flexibacter

These genera belong to the kingdom *Sphingobacteria* and are non-photosynthetic, nonfruiting, gliding gram-negative eubacteria. Members of the genera *Cytophaga* are slender rods, which have pointed ends. They are a major component of the bacterial population in sewage treatment plants (Prescott *et al*., 1999).

C. The Proteobacteria

The Proteobacteria are the largest and most diverse of the eubacteria. They contain the purple sulfur and non-sulfur bacteria (Logan, 1994). They are gram-negative and do not vary very much in appearance but have diverse metabolisms and lifestyles. They are divided into five subgroups, namely, alpha (α), beta (β), gamma (γ), delta (δ) and epsilon (ε) (Prescott *et al*., 1999).

i. α-*Proteobacteria*:

This group contains those bacteria that are capable of growing at low nutrient levels. One of the genera located within this division are the purple non-sulfur bacteria. They are anaerobic, photoorganoheterotrophs that cannot oxidise elemental sulfur to sulfate (Prescott *et al*., 1999).

ii. β-*Proteobacteria*:

This group generally tends to utilise substances that diffuse form organic composition in the anaerobic zones of habitats. They use H_2, ammonia, methane and VFAs for energy and exhibit a considerable amount of diversity (Prescott *et al*., 1999).

iii. γ-*Proteobacteria*:

This sub-group constitutes the largest group of proteobacteria, with extreme variety of physiological types. Most of the important genera are chemoorganotrophic and facultative anaerobes (Prescott *et al*., 1999).

The purple-sulfur bacteria in this group are strict anaerobes and photolithoautotrophic. They oxidise hydrogen sulfide to sulfur and finally to sulfate (Logan, 1994).

The family *Enterobacteriaceae* contain the gram-negative, motile or non-motile, facultative anaerobic straight rods. They degrade sugars to yield formic acid and certain species can degrade this acid to H_2 and CO_2. They carry out mixed acid fermentations to produce lactate, acetate, succinate and the above-mentioned formate (Prescott *et al*., 1999).

iv. δ-Proteobacteria:

This sub-group is a small one, but with great diversity. These chemoorganotrophs can be found in anaerobic digesters in the form of the sulfate reducing bacteria (SRB). These bacteria generate sulfide from sulfate and sulfur while causing oxidation of organic nutrients (Prescott *et al*., 1999). The SRB can be divided into four groups.

i) Gram-negative mesophilic SRB

ii) Gram-positive spore forming SRB

iii) Thermophilic bacterial SRB and

iv) Thermophilic archaeal SRB

The SRB in the sub-class, δ-*Proteobacteria* belong to the first group. They are further divided into two families, the Desulfovibrionaceae and the Desulfobacteriaceae. Bacteria in these groups

include *Desulfovibrio* and *Desulfobacter* respectively (Castro *et al*., 2000). Selected characteristics of these bacteria have been noted in Table 2.5.

Table 2.5: Characteristics of *Desulfovibrio* and *Desulfobacter* (Castro *et al*., 2000).

CHARACTERISTICS	*Desulfovibrio*	*Desulfobacter*
Shape	Spiral to vibrioid	Oval to rod
Motility	+	+/-
GC content of DNA (%)	49-66	44-46
Oxidation of acetate	Incomplete	Complete
Growth Temperature (ºC)	25-40	20-33

The activity of the SRB can be both related to proton oxidation and reduction (acetate formation) and thereby can grow syntrophically with the methanogens, eliminating the need to reduce sulfate. However, in the presence of sulfate, the SRB compete with the methanogens for common substrates such as hydrogen or formate (Araujo *et al*., 2000).

SRB are known to be ubiquitous and can be found in diverse environments such as, oil production facilities, freshwater lake sediments, marine sediments and wastewater treatment plants (Ito *et al*, 2002).

D. The Low G+C Gram Positives (LGC)

The LGC bacteria are those gram-positive bacteria whose DNA G+C composition is below 50%. The genus *Clostridium* is placed within this group. They are obligate anaerobes that are gram-positive and spore-forming. They are generally chemoorganotrophic and fermentative (Prescott *et al*., 1999).

The *Veillonellaceae* are anaerobic chemoheterotrophic cocci that cause fermentation of carbohydrates, lactate and other organic acids to produce gas and VFAs. They are common parasites of warm-blooded animals (Prescott *et al*., 1999).

The *Lactobacillus* contains bacterial gram-positive rods that are non-sporing, mesophilic and chemohetrotrophic. They produce lactic acid as their final fermentation product (Prescott *et al*., 1999).

Members of the genus *Staphylococcus* are facultative anaerobes that are nonmotile and gram-positive, usually found as cocci in clusters. They ferment glucose anaerobically and are generally found in warm-blooded animals (Prescott *et al*., 1999).

E. The High G+C Gram positives (HGC)

These bacteria are defined as those that contain a DNA base composition above 50% G+C and are gram positive.

The *Propionibacterineae* are found within this group. They are facultative anaerobes, that are non-motile, club-shaped rods. They ferment lactate and sugars to form propionic and acetic acids (Prescott *et al*., 1999). They are generally found in human faeces (Holland *et al*., 1987).

The bacterium *Bifidobacterium* is also located within the HGC kingdom. They are non-motile, non-sporing rods that are anaerobic. They facilitate the fermentation of carbohydrates to produce lactic and acetic acids, without the formation of CO_2 (Prescott *et al*., 1999). Many of these

bacteria are associated with faecal contamination and many are found in sewage (Holland *et al*., 1987).

2.5 THE APPLICATION OF ANAEROBIC DIGESTION FOR THE TREATMENT OF WASTEWATER

The main characteristics that are essential for a biological treatment system include:

- Simplicity and efficiency
- High biomass concentration within a reactor to ensure high cellular retention times
- The formation of a community of microorganisms in a granular or dense sludge form
- Low nutrient requirements with the minimal of sludge formation
- High stability in the presence of normal fluctuations of influent composition and
- The capacity to accommodate high organic loading rates (Foresti, 2002).

2.5.1 Septic Tanks

There are many anaerobic systems equipped to treat wastewater. The most commonly applied system is the septic tank. These are unstirred vessels, which are operated at ambient temperatures. The system consists of mainly a tank and an absorption or leaching field (Figure 2.6). The tank is generally composed from concrete and its function includes the removal of wastewater solids to avoid clogging of the absorption field. The biological degradation of waste occurs in this tank and results in the formation of septage (sludge) and scum (floating layer of solids). The effluent is transported to the absorption field via a system of perforated pipes, which are surrounded by

gravel or crushed stones. This effluent is treated by the soil microflora as it passes down to groundwater. This method of treatment is disadvantageous as it leads to the pollution of groundwater by pathogenic microorganisms, which are unable to be eliminated (Bitton, 1994 and Leach, 1994).

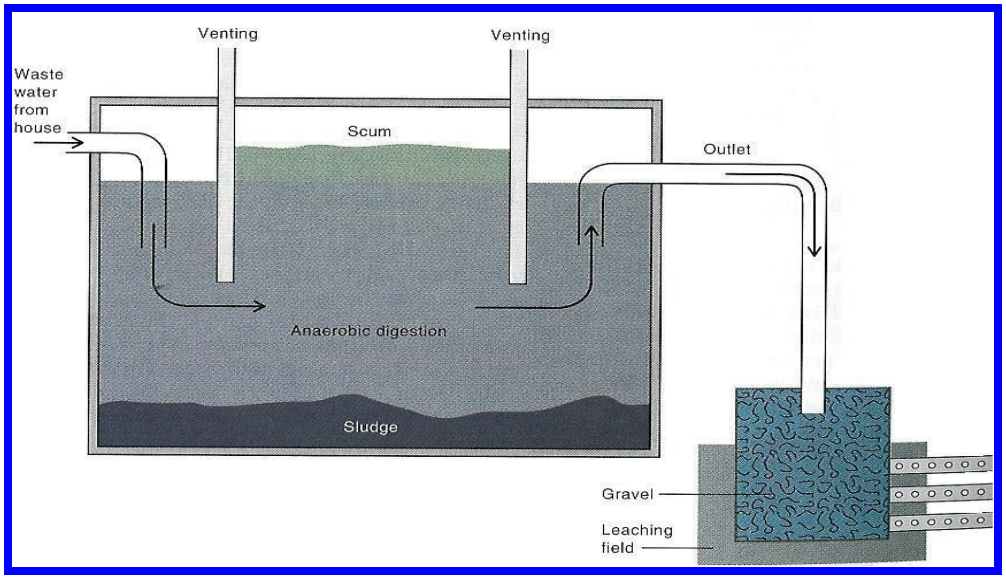

Figure 2.6: Diagrammatic representation of the septic tank (Nester *et al*., 1998).

2.5.2 Conventional Stirred Anaerobic Reactor

This process involves a large holding tank, into which, wastes are continually fed (Figure 2.7). This is a single chamber digester, which is divided into two compartments. The upper compartment acts as a clarifier and the bottom compartment, the digestion chamber. They are separated by a central plate, which allows for the settling of sludge and its descent into the bottom compartment, thus ensuring biomass retention. This process has been successfully applied for the treatment of high strength wastes (McInerney, 1999 and Leach, 1994).

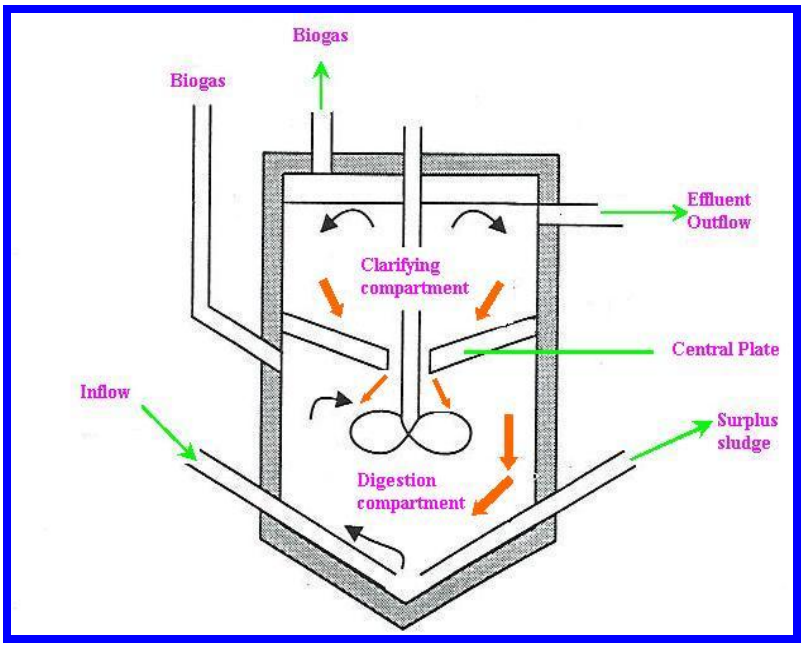

Figure 2.7: Diagrammatic representation of a conventional stirred anaerobic reactor (Leach, 1995).

2.5.3 Anaerobic Filters

These reactors utilise inert support materials on which the anaerobic biomass subsist (McInerney, 1999). Waste flows upwards in the reactor, thus preventing washout of the biomass. They are suitable for wastewaters that are rich in carbohydrates (Bitton, 1994). The disadvantages of this system, however, are that they have a propensity of clogging, they require extremely slow flow rates and are not effective for the treatment of waters with a high organic content (Leach, 1994). A typical anaerobic filter reactor is shown in Figure 2.8.

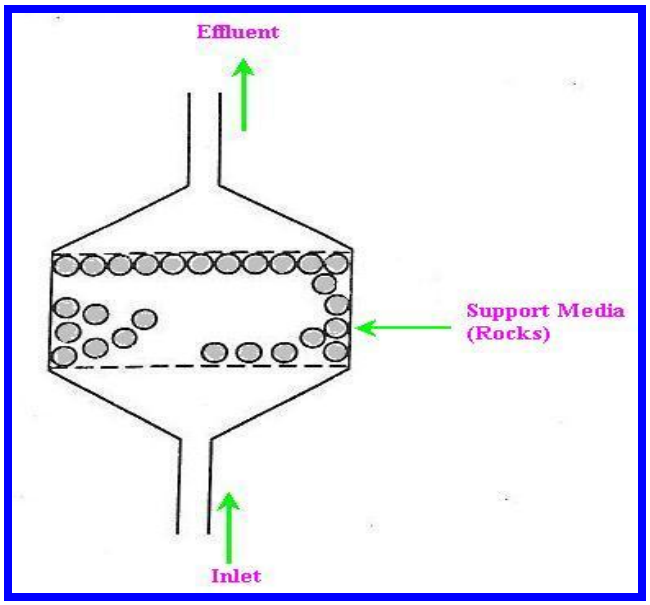

Figure 2.8: Diagrammatic representation of the anaerobic filter (Leach, 1994).

2.5.4 Upflow Anaerobic Sludge Blanket Process (UASB)

The UASB reactor is detailed in Figure 2.9. This reactor consists of a bottom layer of packed sludge, a sludge blanket and an upper liquid layer (Bitton, 1999). Wastewater flows upwards through the sludge, which may be contained in granules. This natural granulation is advantageous, in that, a high population of immobilised bacteria can be achieved without support materials. A number of large-scale systems are in place to treat soluble wastes at low hydraulic retention times (McInerney, 1999).

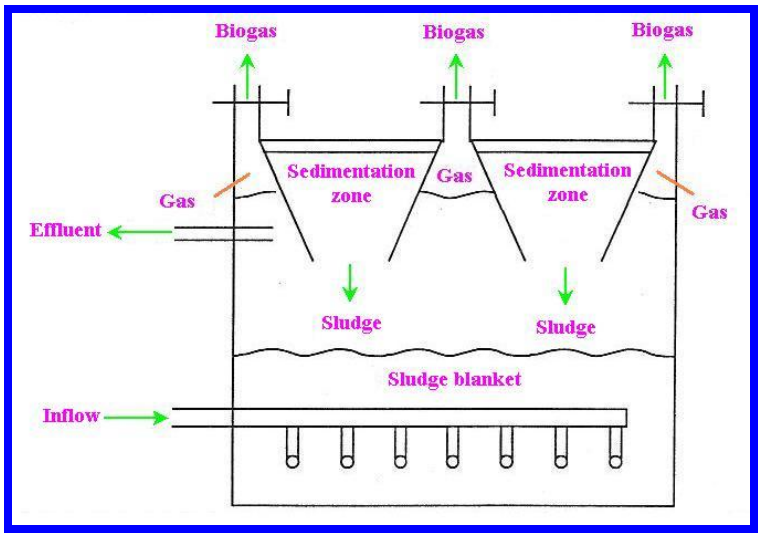

Figure 2.9: Diagrammatic representation of an USAB reactor (Leach, 1994).

2.5.5 The Anaerobic Baffled Reactor

The main drawbacks of many anaerobic systems include a long retention time and instability to hydraulic and organic shocks (Nachaiyasit and Stuckey, 1997a). These shocks are characterised by an increase in volatile fatty acids (VFAs), a decrease in removal efficiency and methane content, an increase in effluent suspended solids and the sludge volume index and an increase or decrease in pH. Such changes lead to upheaval within the reactor, undermining performance (Nachaiyasit and Stuckey, 1997b). These have been overcome by the development of high rate reactors that can separate hydraulic retention times and cell retention times to allow slow-growing anaerobes to remain in the reactor, independently of the wastewater flow (Nachaiyasit and Stuckey, 1997a). The reactors have the added advantage of being a closed and stable ecosystem (Godon *et al*., 1997). An anaerobic baffled reactor (ABR) is one such high rate reactor that also encompasses the necessary characteristics of a biological system.

An ABR, Figure 2.10, is a reactor in which a series of hanging and standing baffles are employed in order to force wastewater to flow upward and under the baffles, through a bed of anaerobic sludge, as it passes from the influent to the effluent pipe. In this way the reactor is divided into separate compartments. Each compartment within the reactor has its own microbial consortium specific for the reactions they carry out (Barber and Stuckey, 1999; Skiadas and Lyberatos, 1998). Therefore, the bacteria move vertically within each segregation without much movement horizontally through the reactor, thus establishing a simple and inexpensive, yet effective method of biomass retention (McInerney, 1999). The biggest advantage of this is that the reactor segregates the individual anaerobic process phases (hydrolysis, acidogenesis, acetogenesis and methanogenesis) so that the different bacterial groups can develop under their optimal conditions. The first compartment of the ABR is able to act as a buffer zone, protecting the latter compartments from toxic and inhibitory compounds in the feed and allowing harmless and balanced influent into the rest of the reactor (Uyanik *et* al., 2002a). The more sensitive methanogens are separated from the front of the reactor, where they might be subjected to toxic shocks (Plumb *et al*., 2001; Barber and Stuckey, 1999 and Hutňan *et al*., 1999).

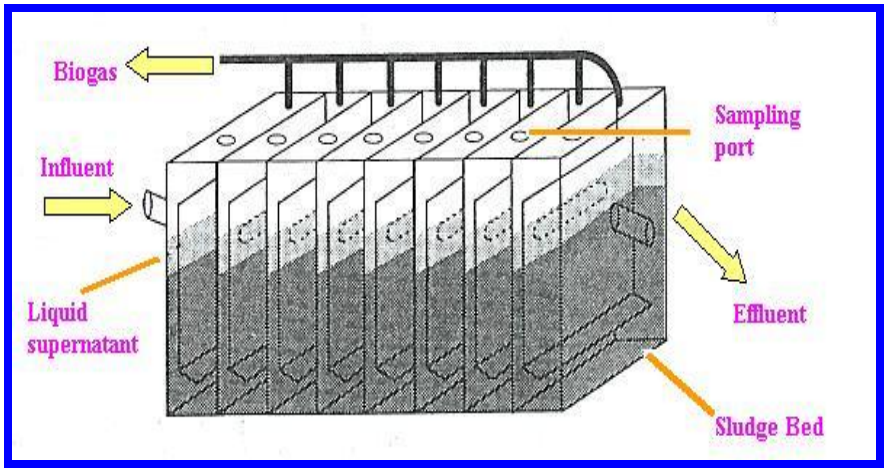

Figure 2.10: Schematic representation of the ABR (Nachaiyasit and Stuckey, 1997b).

The advantages of an ABR include its simple design as it has no moving parts and is inexpensive to construct. It requires no mechanical mixing and provides reduced sludge bed expansion but still prevents clogging. The biomass requires no sludge or gas separation and is retained within the reactor without a fixed medium or a solids settling chamber. It has a low sludge generation and a high solids retention time but low hydraulic retention times (HRT). The communities are extremely stable to hydraulic and organic shock loads. It also provides long operation times without sludge wasting (Barber and Stuckey, 1999). However, problems may be encountered with start-up. Organic loading rates have to be low so that the slow-growing microorganisms do not become overloaded. High rates may lead to an accumulation of volatile fatty acids and a subsequent decrease in pH. These can be overcome by a low organic loading and a dilution of the feed (Uyanik, 2003).

The ABR is an ideal reactor for the treatment of low-strength wastewaters, such as domestic wastewater as was reported by Langenhoff and colleagues (2000). A 91.9% removal of COD was reported by Manariotis and Grigoropoulos (2002). The ABR can also be used to treat wastewater at low temperatures. Most wastewater treatments occur at between 30-35ºC and since influent temperatures are lower than this, heating is required. This increases the cost of treatment. Therefore, it is practical to treat these waters at a lower temperature, if possible. Langenhoff and Stuckey (2000) reported 70% COD removal rate in a low temperature ABR. Manariotis and Grigoropoulos (2002) reported an 87.6% COD removal at 16ºC. Despite this removal efficiency, effluent CODs are still too high to discharge into water bodies due to high levels of soluble residual COD. Therefore, some sort of post-treatment is required. Each effluent is different and should, therefore be characterised fully, before a post-treatment can be chosen. Treatments include solid/liquid separation, aerobic polishing, granular activated carbon, ozonation, chlorination, slow sand filtration, stabilisation ponds, activated sludge and ultra-violet treatment (Barker *et al*., 1999 and Vieira and Garcia Jr, 1992). Modifications to the ABR have also proven

successful. The development of the ANANOX® process by Garuti and co-workers (1992), is one such example. This process involves the treatment of wastewater by an ABR followed by further effluent treatment by an aeration tank containing activated sludge and a final settling tank. Results show a high efficiency removal of COD (Garuti *et al*., 2001).

The ABR is seen as a novel method of treating the wastewaters of rural and peri-urban areas of South Africa. It is estimated that 20 million of South Africans living in these areas do not have access to adequate sanitation. This is not only an infringement of basic human rights but it also leads colossal environmental pollution. The contamination of surface and ground waters results in the dissemination of pathogenic bacteria, which cause waterborne diseases, further exacerbating the problem (The Mvula Trust, 1995). The ABR proposes to be an affordable sanitation system, which can be emplaced in the rural areas of South Africa. Its advantages of simple design and technology, no electricity requirements and its ability of effective functioning under a wide range of flow and load conditions make it an ideal system for the implementation in rural areas (Foxon *et al*., 2001)

In order to ensure optimum running of any microbial treatment reactor, many factors have to be taken into consideration. However, one of the most important is that of the characterisation of the microbial communities within the reactor. The microbial ecology and the interaction of the various populations in wastewater treatment plants remains one of the least understood aspects in anaerobic systems. Developments in anaerobic treatment are dependant on the fundamental relationships between the microbial populations, if efficiency is to be improved (Uyanik *et al*., 2002b). Characterisation of the microflora (bacteria) serves as a basis for improving our understanding of the specific reactions facilitated by the bacteria, and hence helps to optimise the process.

2.6 TECHNIQUES USED TO STUDY ENVIRONMENTAL ANAEROBIC MICROBIAL COMMUNITIES

2.6.1 Conventional Microbiological Techniques

Conventional microbial techniques used to identify microorganisms include the viable plate count and the most-probable number (MPN) technique.

2.6.1.1 Viable plate count technique

This technique involves the growth of the microorganisms on agar plates, using streaking procedures. This technique is simple, sensitive and is widely employed to count microorganisms from environmental samples. However, there is a high probability of incorrect numbers. Low counts result if the cells are not broken up and well dispersed. It is also not possible to be absolutely certain that an individual cell produced one colony. Therefore, results are not accurately quantitative and are expressed as colony-forming-units rather than actual cell numbers (Prescott *et al*., 1999).

2.6.1.2 The MPN technique

This technique involves the growth of microorganisms in varying volumes of water and the subsequent transfer to agar plates. Colonies are counted and multiplied by their dilution factor to provide an indication of actual cell numbers (Prescott *et al*., 1999).

Other techniques that are used to examine and quantify microorganisms in environmental populations included the isolation of pure cultures and the investigation of their physiological and biochemical traits. These conventional microbiological techniques are of limited usefulness, as

most of these bacteria do not grow *in vitro* due to the fact that cultivation media is a poor substitute for natural growth and it does not take into consideration the interdependency of microorganisms. These techniques are also time-consuming, labour-intensive and are inadequate for the determination of reliable cell numbers (Santegoeds *et al*., 1998 and Schramm and Amann, 1999). Difficulties were also encountered with the study of anaerobes due to problems with anaerobic cultivation (Logan, 1994). Therefore, these methods have been replaced by DNA/RNA based techniques that are applied to successfully quantify the communities in different microbial environments.

2.6.2 DNA And RNA Based Techniques

2.6.2.1 Fluorescent in situ hybridization (FISH)

The process of FISH was first described by Delong and colleagues in 1989. This technique involves the identification of bacteria by targeting the ribosomal RNA (rRNA) of bacterial cells. The rRNA molecules are ideal targets for nucleic acid probes for several reasons:

i) They are functionally conserved molecules present in all organisms.

ii) Primary structures of 16S and 23S rRNA molecules are composed of sequence regions of higher and lower evolutionary conservation.

iii) 16S rRNA sequences have already been determined for a large fraction of the bacterial species.

iv) Their natural amplification with high copy numbers per cell greatly increases the sensitivity of the rRNA targeted probing (Amann, 1995; Delong *et al.*, 1989).

FISH involves the detection of rRNA of morphologically intact cells utilizing labelled probes (Amann, *et al*., 1995). Probes are pieces of single stranded DNA, varying in length of 15-25 nucleotides, which are complementary to a site within the rRNA of the bacteria researchers wish to examine. Prior to 1989, these probes were labelled radioactively and were viewed by autoradiography (Amann, *et al*., 1990). The probes are now labelled with fluorescent dyes, which aid in their detection when they bind to their complementary sites (Schramm and Amann, 1999). If no target sequence is found in the ribosomes, the unbound probe does not bind and is removed by the washing step. However, if the complementary sequence is found, the probe will bind. This binding site will be readily detected by epifluorescence microscopy (Hugenholtz *et al*., 2001). This entire process is summarized in Figure 2.11.

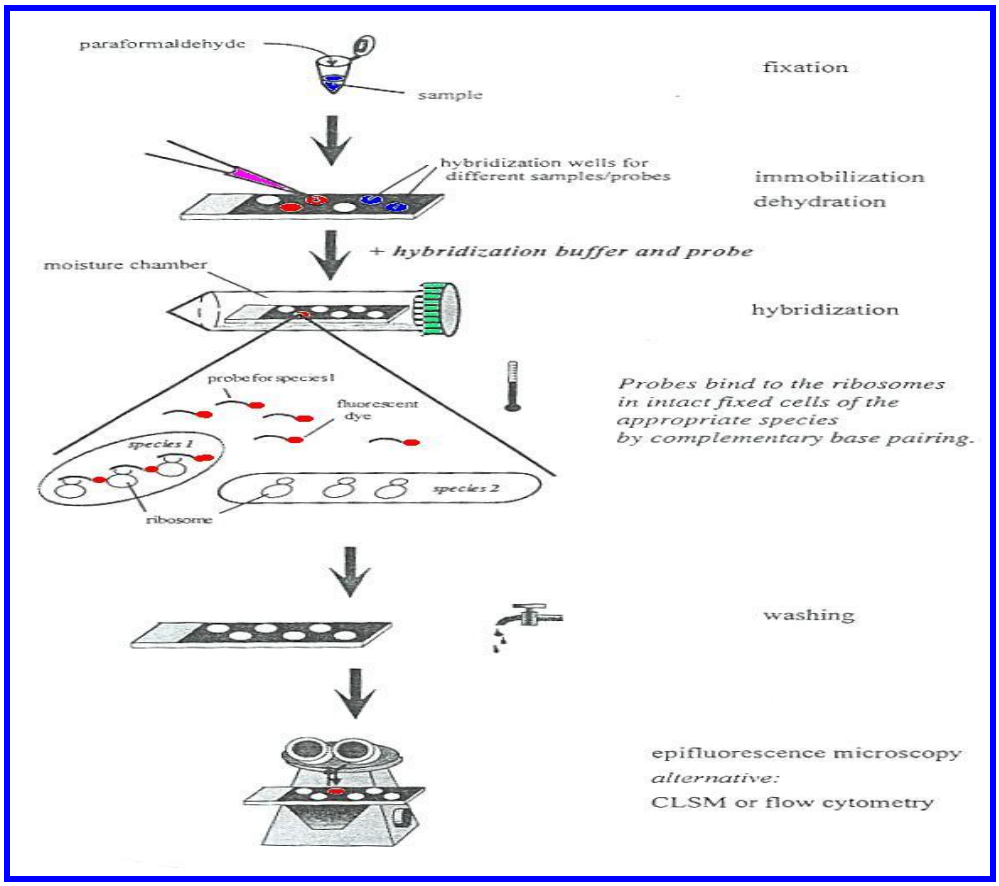

Figure 2.11: The FISH process (Schramm and Amann, 1999)

In order to enhance the FISH process, cells are fixed to maintain their morphological integrity, to make them permeable to the probe and to minimize autofluorescence (Stahl and Amann, 1991). Autofluorescence may originate from sample constituents or from unbound or non-specifically bound probes (Haugland, 1998). Formamide is added to the probes as it lowers the melting point of the double helix, thus increasing the life of the nucleic acid by eliminating degradation (Stahl and Amann, 1991).

The FISH technique is almost always complemented by 4'6-diamidino-2-phenylindole (DAPI) staining. DAPI is a DNA- intercalating dye that, when excited at a wavelength of 365nm, fluoresces bright blue. Unbound DAPI or DAPI that has bound to non-DNA material fluoresces a pale yellow (Porter and Feig, 1980).

Cells stained with DAPI and probe-hybridized were conventionally counted manually. However, the advent of digital analysis tools have virtually replaced this method. The only disadvantage of digital analysis is its limited efficiency. These tools are unable to accurately determine the cell numbers of dense clusters or biofilms. To help reduce this problem, software systems have been developed to differentiate between labelled cells and unlabelled background (Daims *et al*., 2001).

FISH is an extremely valuable technique to detect and study microorganisms in their natural habitat, as it eliminates many of the uncertainties of culture enumeration techniques (Amann *et al*., 1990) However, like many other techniques, it suffers from limitations.

A. Limitations Of FISH

FISH results are accurate but this accuracy is not absolute. Probes may bind non-specifically, or background fluorescence of samples may lead to overestimations of bacterial cells (Amann, 1995). The FISH technique is also limited in accuracy for densely aggregated cells (Daims *et al.*, 2001). Other limitations may also arise from low cellular ribosome content of the target cells. Slow growing or starving cells yield poor signals that may not be able to be detected by FISH. The rRNA of cells may not always be readily accessible. Certain cells walls are difficult for the probe to permeate and even if the probe diffuses into the cell wall, it may have difficulty in accessing the rRNA (Schramm and Amann, 1999). However, there have been improvements to the FISH technique over the past two years, which include the combination of FISH with other techniques to help overcome limitations (Wagner *et al*., 2003).

B. Overcoming The Limitations Of FISH Using Newly Developed Technologies

i. The application of unlabelled oligonucleotides

The accessibility of probe-target sites varies between phylogenetically different organisms. Therefore, it is imperative to verify if the target site of newly designed probes have been successfully applied in FISH of related organisms. The accessibility of the target sites may be increased by the utilisation of unlabelled oligonucleotide probes, which bind adjacent to the probe target site. These helper probes must have the same or greater specificity than the probe itself (Wagner *et al*., 2003).

ii. The use of peptide nucleic acid probes

Peptide nucleic acid (PNA) probes target the 16S rRNA and confer bright signals, even in when the cell contains a low cellular ribosome content. PNA probes have an uncharged polyamide backbone, therefore hybridizations can occur at low salt concentrations and at high temperatures. This is ideal for Gram-positive bacteria and *Cyanobacteria* (Wagner *et al*., 2003).

iii. The use of self-ligating probes

The problem of high background noise is as a result of non-specific binding of probes to the sample material. The use of self-ligating probe pairs helps overcome this. These probes target adjacent regions in the rRNA and are able to self-ligate after hybridization. This self-ligation results in the loss of fluorescence and therefore, only the hybridized and autoligated probe fluoresces (Wagner *et al*., 2003).

iv. The use of polyribonucleic probes

To overcome the limitations of low signal intensity of cells from those that contain a low rRNA content or are slow growing, polyribonucleic probes are utilised. These are RNA transcripts of PCR amplicons of 16S rRNA genes that are fluorescently labelled. They yield higher signal intensities than conventional oligonucleotide probes and are therefore better for the identification of cells with low cellular ribosome. The disadvantage is that they cannot be readily bought and have to be synthesised in the laboratory (Pernthaler *et al*., 2002).

Other molecular techniques may also be used in conjunction with FISH to help verify results. These include the Polymerase chain reaction and DNA sequencing techniques.

2.6.2.2 Polymerase chain reaction

Developed between 1983-1985 by Kary Mullis, the Polymerase Chain Reaction (PCR) technique revolutionised the way large quantities of DNA is synthesised (Prescott *et al*., 1999). PCR may be defined as a method that "uses repeated cycles of oligonucleotide-directed DNA synthesis to carry out *in vitro* replication of target nucleic acid sequences " (Persing, 1991). PCR is a simple, yet fast and efficient method to amplify DNA *in vitro* (Schwab, 1993). The technique involves repeated cycles of high temperature template denaturation (94-97ºC) and primer extension at 72ºC (Zyskind and Bernstein, 1992).

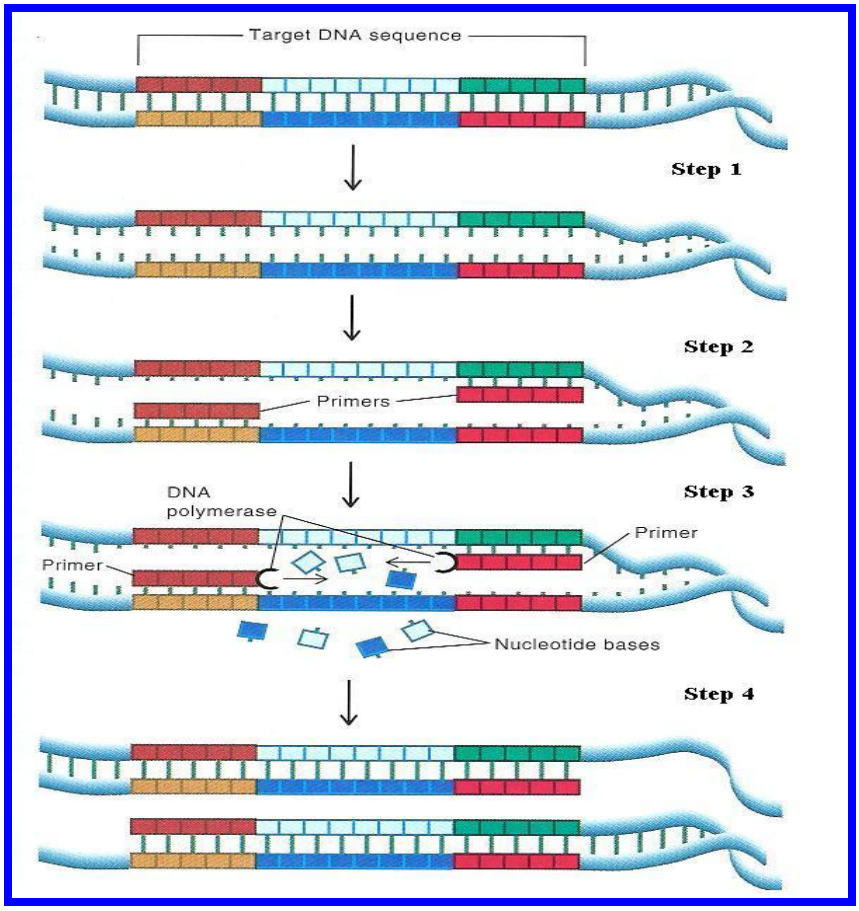

Figure 2.12: The polymerase chain reaction (Nester *et al*., 1998).

With reference to Figure 2.12:

Two oligodeoxynucleotide primers are used. When the DNA template is denatured and cooled, the primers will then each bind to opposite strands of template (Steps 1 and 2). DNA polymerase then extends the primers at the 3' end (Step 3). This process is repeated for many cycles to produce multiple copies of target DNA (Step 4) (Kocher and Wilson, 1991).

A. PCR Reaction Components

In order to carry out PCR, certain components are vital. These include:

i. Deoxynucleotides (DNTP's)

The PCR process employs the use of DNTP's to provide energy and nucleosides for the synthesis of DNA (Taylor, 1993).

ii. Taq Polymerase

This DNA polymerase enzyme used was isolated from the bacterium *Thermus aquaticus*. Taq polymerase is ideal for PCR as it is thermostable; therefore it does not need replenishment after each heating step and is active at high temperatures where annealing of the oligonucleotide primers is more specific and rapid. The disadvantages of Taq include its high processivity at sub-optimal temperatures, which leads to non-specific amplification (Taylor, 1993 and Giovannoni, 1991).

iii. Primers

Primers are hybridization probes that act in concert. They should ideally be 18-24 bases long and without any internal structures, such as hairpin bends. They should not be complementary to each other at the 3' ends and should have a melting temperature that allows annealing at 55-65ºC (Giovannoni, 1991 and Roche Molecular Biochemicals, 1999).

iv. Magnesium Chloride

Taq needs Mg2+ for activity. It also complexes with dNTP's to produce a substrate that the polymerase recognises (Roche Molecular Biochemicals, 1999 and Kocher and Wilson, 1991).

B. Applications Of PCR

PCR is a useful technique that can be applied for the following: -

- Adding useful ends to DNA fragments
- Changing of specific bases in a sequence
- Amplification of cDNA
- Production of single stranded DNA
- Cloning of unknown sequences that are found next to known sequences
- Amplification of RNA
- Diagnosis of diseases
- DNA Fingerprinting (Zyskind and Bernstein, 1992 and Schwab, 1993).

C. Limitations Of PCR

PCR like every other technique has its share of limitations. They include the following.

- Taq polymerase has a high error rate.
- Contamination of the PCR technique can cause the wrong DNA to be amplified, thereby giving false positive results.
- PCR may produce 'shuffle clones' which are recombinant products of two sequences (Kocher and Wilson, 1991).

2.6.2.3 Gel electrophoresis

Following PCR, DNA fragments need to be separated. Early methods included sucrose gradients and paper and cellulose acetate matrices. Modern methods, that is, agarose and polyacrilamide gels produce results that are homogenous and reproducible (Andrews, 1991).

In this process, charged molecules are placed within an electrical field and allowed to migrate to positive and negative poles. Molecules separate as they move at different rates based on their charge and size (Prescott *et al*., 1999). Compact supercoils will migrate faster than the linear DNA, which in turn will migrate faster than the open circular Form II DNA (Zyskind and Bernstein, 1992). Gels can be visualised by ethidium bromide staining, incorporated in the buffer or post-separation staining. Gels are then visualised by UV radiation, as ethidium bromide is a nucleic acid intercalating dye (Andrews, 1991).

2.6.2.4 DNA sequencing

The term DNA sequencing refers to an unravelling of a linear order of nucleotide bases to determine genetic information (Volckaert *et al*., 1993). This technique was exploited by simultaneous developments of the chain termination method by Sanger and co-workers and the chemical method by Maxim and Gilbert (Schwab, 1993).

A. The Chain Termination Method

The procedure involves the use of dideoxynucleotides triphosphates (ddNTPs). These are similar to nucleotides but lack the 3' hydroxyl group. These add to the growing end of a nucleotide chain but terminate the syntheses catalysed by DNA polymerase. Therefore, chains of various lengths are formed. These lengths can be run on agarose gel electrophoresis in four reactions with different fluorescent ddNTPs. The mixture with ddATP produces fragments with an A terminus, ddCTP with a C terminus, and so on. These are run on agarose gels based on size separation. The gels are then read from the bottom up (Prescott *et al*., 1999).

B. The Chemical Degradation Method

This technique involves the preparation of a single or double-stranded DNA fragments that are labelled at one end. These labelled fragments are then divided into four aliquots and treated by four separate chemical reagents that are base specific. Therefore, each aliquot results in a 'nested set' of fragments. Each aliquot is then run on a polyacrilamide gel in adjacent wells. The

sequence is then read by determining the order of the nucleotide residues from the labelled end (Volckaert *et al*, 1993 and Zyskind and Bernstein, 1992).

However, recently automated systems have virtually replaced the manual methods. Reagent kits, computer-assisted handling and analysis have also uncomplicated the process (Schwab, 1993).

C. Automation

An automated system is basically a gel electrophoresis system that detects labelled nested sets that are generated by sequencing reactions. Fluorescent labels that have replaced the radioactive ones, are attached to the primer involved in the reaction. This primer is then incorporated into the nested set. Each ddNTP is labelled with a different coloured dye. Therefore, each base-specific termination reaction is identified by a different coloured dye. The reaction products are electrophoresed on a polyacrilamide gel, past a detector, which excites the fluorescent label. The fluorescence passes through four different filters and a photomultiplier tube and the output that is generated is stored on computer (Volckaert *et al.*, 1993 and Howe and Ward, 1991).

Following the acquisition of the required sequence, this sequence submitted to GenBank, a sequence database, to determine its origins. GenBank is a free public database consisting of numerous nucleotide and protein sequences submitted by researchers and run by the National Centre for Biotechnology Information (NCBI). This database encompasses sequences submitted to Data Libraries in the United Kingdom and Japan and is available online at http://www.ncbi.hlm.nih.gov (Benson *et al*., 2003).

2.7 CONCLUSIONS FROM REVIEW OF LITERATURE

Although anaerobic technologies have in the past been disregarded as a treatment scheme for domestic wastewater, the high costs and other disadvantages associated with aerobic technologies have made researchers rethink this attitude. Many of the disadvantages associated with the application of the anaerobic digestion process in digesters have been overcome by the design of high rate reactors. The anaerobic baffled reactor is an example of such a technology. However, like for every treatment process, the success of its application is dependant on its microbial consortia. Conventional methods used to study these communities were limited in efficiency and therefore, could not provide accurate results. The advent of RNA/DNA based technologies has enabled researchers to determine and quantify these diverse communities. With an understanding of these populations and their responses to perturbations within the system, it is possible to construct an anaerobic system that is successful in all aspects with regards to the treatment of domestic wastewater.

CHAPTER THREE

MICROBIAL COMMUNITY ANALYSIS OF THE ABR

3.1 INTRODUCTION

The characterisation of a microbial community is essential when it relates to wastewater treatment efficiency. Microorganisms are the deciding factor on whether a process is a success or a failure. Therefore, it is imperative that studies on the microbial communities and their response to changes in the ecosystem are undertaken. With regards to anaerobic technologies, conventional microbiological techniques have shown many disadvantages and results do not accurately display the composition of the diverse microbial communities. Therefore, the RNA/DNA based methods, such as FISH and PCR are better suited to these types of analyses. These novel techniques are advantageous in that they are rapid and they eliminate the numerous uncertainties of culture enumeration techniques. Previously, difficult to culture and certain unculturable microorganisms can be detected by these methods (Amann *et al*, 1990). These techniques are especially advantageous for anaerobic communities, which can be studied *in situ*, without the problems encountered by anaerobic culture methods. These techniques were applied to study the microbial communities of a pilot-scale ABR.

3.1.1 Reactor Configuration

An ABR is currently being investigated by the Pollution Research Group (PRG), University of Kwa-Zulu Natal, with the aim of providing an appropriate sanitation system that can be successfully applied in peri-urban areas. The reactor was constructed with mild steel to the dimensions of 3m x 1m x 1.2m. This 3000L pilot scale ABR is divided internally into eight

compartments by alternately hanging and standing baffles. Dimensions of the internal compartments are summarised in Table 3.1.

Table 3.1: Dimensions of each of the eight compartments of the ABR.

Compartment	Number	Height /m	Volume /m^3
1	1	1.10	0.41
2	2	1.07	0.40
3	3	1.04	0.39
4	4	1.01	0.38
5	5	0.99	0.37
6	6	0.96	0.36
7	7	0.93	0.35
8	8	0.90	0.33

Sampling ports are present on the top and on one side of the reactor. Influent feed (100% domestic) was obtained from the raw wastewater channel at the head of Kingsburgh Wastewater Treatment Works by a submersible pump. The flow rate was enforced by a programmable logic controller (PLC). This reactor was initially seeded with sludge from waste activated sludge digesters at Umbilio Wastewater Treatment Works and thereafter allowed to build up its own sludge community. A schematic representation of the ABR is detailed in Figure 3.1.

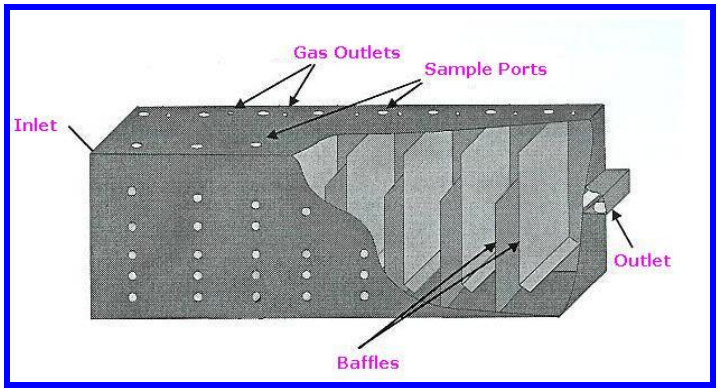

Figure 3.1: Schematic representation of the pilot-scale ABR (Foxon *et al*., 2004).

3.1.2 Operating Conditions

The FISH, PCR and sequencing techniques were applied to study the population changes within the ABR run at Kingsburgh Wastewater Works. Chemical analyses such as COD and pH were provided by the PRG (APPENDIX SEVEN). The reactor was investigated at three different operating periods.

The first operating period was conducted from February-June (2003) period. This operating period was characterised by an average flow rate of 3264L of wastewater per day. This was much higher than the expected flow rate of 2800L per day. During this operating period, a 24-hour campaign was conducted from the 27-29 May 2003 (Day 101). Hourly COD samples were taken for the influent, effluent and for each compartment (APPENDIX SEVEN). However, microbial sampling was only conducted at the end of the campaign.

The second operating period (July-September 2003) was a proposed 15 h HRT, with a flow rate of 4800 L of wastewater per day. However, the actual average flow rate was a much lower 1149 L of wastewater per day. This period was characterised by frequent incidences of biomass washout caused by pump malfunctions, which resulted in a small amount of feed being pushed through the ABR very quickly, thus causing a mixing of microbial populations.

Following the failure of the second operating period, a third (November-December 2003) was implemented, utilising the same flow rate. However, this operating period failed to achieve the required flow rate of 4800L per day and was recorded at an average of 1573.1L/day. Following the similar perturbations of the second operating period, this operating period was halted.

For the second and third operating periods, COD tests were conducted erratically, due to a number of disturbances within the reactor, such as biomass washout as well as pump difficulties.

3.2 MATERIALS AND METHODS

3.2.1 Fluorescent In Situ Hybridization

3.2.1.1 Sampling and cell fixation

Grab samples were obtained from each of the eight compartments of the ABR, on Days 36 (March), 57 (April), 85 (May), 101(May-campaign) and 127 (June) of the first operating period as well as Day 21 (July) of the second operating period and Day 16 (November) of the third operating period. Only one sample was taken for each of the last two operating periods due to the failure of these periods and insufficient chemical data. Cells were fixed using 4% paraformaldehyde (for gram-negative) or ethanol solutions (for gram-positive) for 1½ hours. Thereafter cells were washed and resuspended in 50:50 phosphate buffered saline (PBS): absolute ethanol (Amann, 1995) (APPENDIX ONE).

3.2.1.2 Pretreatment and immobilisation of fixed cells

Sonication parameters were optimised by trial and error, varying wattage and time. Optimum pretreatment conditions proved to be the sonication at 8 watts for 8 minutes. The pretreated sample was diluted with 1 x PBS and Igepal CA-630. Ten microlitre of this mixture was spotted onto pretreated slides and allowed to dry. Once dry, the slides were dehydrated by successive rinses in 60% (v/v), 80% (v/v) and absolute ethanol for 2 minutes each.

3.2.1.3 Total cell counts using membrane filtration DAPI

Pretreated samples were subjected to DAPI staining, using a modified membrane filtration method (Porter and Feig, 1980) (APPENDIX TWO). Total cell counts were calculated using the following equation (Porter and Feig, 1980):

$$\mathbf{TCC = MTCC \times DF \times MF}$$

Where:	**TCC**	=	Total cell count
	MTCC	=	Mean total cell count for x no. of fields
	DF	=	Dilution factor
	MF	=	Total number of microscope fields on a filter

3.2.1.4 Whole cell hybridization with labelled probes

A 50 ml polypropylene centrifuge tube, equilibrated with hybridization buffer was used as the chamber for whole cell hybridization to prevent evaporative loss of the hybridization solution. Thereafter hybridization was carried out according to Amann (1995) (APPENDIX THREE) utilising the probes described in Table 3.1. These probes were selected on the basis of previous investigations by Bell (2002) on anaerobic microbial community analyses treating dye wastewaters. The concentration of probes was varied and the optimum found to be 5ng/ul. Probes were applied at this concentration in a buffer containing 0.9M NaCl, 20mM Tris-HCl (pH 7.4), 0.01% sodium dodecyl sulfate (SDS) and appropriate amounts of formamide (Table 3.2) (Amann, 1995).

Table 3.2: 16S rRNA targeted probes utilised.

Probe	Specificity	Sequence (5'-3')	FA	Ref
EUB338	Bacteria (16S, 338-355)	GCTGCCTCCCGTAGGAGT	20	1
ARC915	Archaea (16S, 915-934)	GTGCTCCCCCGCCAATTCCT	20	1
ALF1b	α-Proteobacteria (16S, 19-35)	CGTTCG(C/T)TCTGAGCCAG	20	2
BET42a	β-*Proteobacteria* (23S, 1027-1043)	GCCTTCCCACTTCGTTT	35	2
GAM42a	γ-*Proteobacteria* (23S, 1027-1043)	GCCTTCCCACATCGTTT	35	2
SRB385	δ-*Proteobacteria* (16S, 385-402)	CGGCGTCGCTGCGTCAGG	35	3
LGC354a	*Firmicutes* low G+C (16S, 354-371)	TGGAAGATTCCCTACTGC	20	4
HGC69a	High G+C (23S, 1901-1918)	TATAGTTACCACCGCCGT	25	5
CF319a	*Cytophaga-Flavobacterium (16S, 354-371)*	TGGTCCGTGTCTCAGTAC	20	1
BAC303	*Baceriodes-Prevotella* (16S, 385-402)	CCAATGTGGGGGACCTT	0	6
DSB985	*Desulfobaceriaceace (16S, 985-1004)*	CACAGGATGTCAAACCCAG	0	6
DSV698	*Desulfovibrionaceae (16S, 698-717)*	GTTCCTCCAGATATCTACGG	20	7
MS821	*Methanosarcina (16S, 821-844)*	CGCCATGCCTGACACCTAGCGAGC	20	8
MX825	*Methanosaeta (16S, 825-847)*	TCGCACCGTGGCCGACACCTAGC	20	8

Where:

1. Snaidr *et al.*, 1997
2. Manz *et al.*, 1992
3. Santegoeds *et al.*, 1998
4. Friedrich *et al.*, 1999
5. Roller *et al.*, 1994
6. Plumb *et al.*, 2001
7. Merkel *et al.*, 1999
8. Raskin *et al.*, 1994b

3.2.1.5 Post hybridization stringent wash

Hybridization was terminated and any unbound probe was washed off using wash solution according to specific parameters relating to each probe's formamide concentration (20mM Tris-HCl (pH 7.4), 0.01% SDS and xNaCl). Thereafter, slides were immersed in 50 ml of wash solution at 48˚C for 20 minutes (Amann, 1995).

3.2.1.6 DAPI staining

Subsequent to washing, slides were removed and air-dried. Hybridized samples were stained with DAPI for 5-10 minutes in the dark. Excess DAPI was washed off using 1 x PBS. A drop of anti-fading agent, Kallestad ™ Mounting Media (Biorad, South Africa), was added to hinder the fading of the DAPI stain. A coverslip was placed over the slide and held in place using clear nail varnish (Hicks *et al*., 1992).

3.2.1.7 Direct counting of bacterial cells

Slides were examined using a Zeis Axiolab microscope attached to a 50 W bulb. Images were saved using a Sony (Germany) CCD camera and image analysis software (Ks 300, Zeis, Germany) was used to determine the area counts of cells bound to DAPI and the probes. Zeis filter set 02 was used for DAPI staining, 09 for fluorescein detection and set 14 for rhodamine. Due to autofluorescence of certain samples, most probe counts were conducted manually. Ten-fifteen fields were saved and evaluated from each hybridized spot under a 1000 fold magnification. The total cell numbers for each probe was determined by using the following equation obtained from Amann (1995):

$$\frac{\mathbf{n(Probe)}}{\mathbf{n(DAPI)}} \times \mathbf{n(MF)}$$

Where:

n(Probe) = average number of cells bearing probe conferred fluorescence.

n(DAPI) = average number of cells bearing DAPI conferred fluorescence.

n(MF) = total cell count as obtained by membrane filtration

3.2.2. Polymerase Chain Reaction

3.2.2.1 Extraction of DNA from whole cells

DNA was extracted from whole cells using the boiling method as described by Jackson and colleagues (1991) (APPENDIX FOUR)

3.2.2.2 Amplification of DNA

Amplification of archaeal 16S ribosomal DNA was performed using *Archaea*-specific primer lAf (forward); (5'- TCY GKT TGA TCC YGS CRG AG-3') and universal reverse primer 1492r (5'-TAC GGY TAC CTT GTT ACG ACT T-3') (Plumb *et al*., 2001).

Amplification of bacterial samples were performed using bacterial specific primers 341f (forward) with G+C clamp (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GG–3') (Kaewpipat and Grady, 2001) and the universal primer 1492 r.

PCR mixtures (Fermentas, Inqaba Biotechnical Industries, South Africa) were made up to a total volume of 100µl. The mixtures were thereafter incubated in a Hybaid PCR Sprint Temperature Cycling System utilizing specific reaction conditions (APPENDIX FIVE)

3.2.3 Agarose Gel Electrophoresis

Amplified fragments were run on agarose gels, using an Omeg Scientific HG/02 Minigel System (Industricord, South Africa) to determine their purity according to a modified protocol developed by Andrews (1991) (APPENDIX SIX). Electrophoresed samples were viewed using a Hoefer® MacroVue UV-20 transilluminator (Pharmacia Biotech AB, South Africa).

3.2.4 DNA Sequencing

DNA sequencing was performed by Inqaba Biotechnical Industries (South Africa) using a Spectrumedix SCE2410 genetic analysis system with 24 capillaries (SpectruMedix LLC, Pennsylvania, USA). For the reactions, the BigDye version 3.1 dye terminator cycle sequencing kit from Applied Biosystems was used. Sequence results were determined using the Chromas Version 2.3 (Technelysium) sequence analysis programme (online version available at www.technelysium.com.au/chromas.html). Similarity searches against database entries were conducted using the online nucleotide BLAST search engine available at the National Centre for Biotechnology Information website (http://www.ncbi.hlm.nih.gov) (Altschul *et al*., 1997). These results were thereafter, submitted to GenBank (http://www.ncbi.hlm.nih.gov) and its bacterial or archaeal origin determined (Benson, *et al*., 2003).

3.3 RESULTS AND DISCUSSION

Latter justifications are highly subjective due to lack of supportive analytical data such as acetate, VFA and methane concentrations.

3.3.1 Results of DNA Sequencing

DNA sequencing of bacteria revealed the presence of the LGC, HGC and δ-*Proteobacterial* classes of bacteria, thereby confirming FISH results (Table 3.3).

Table 3.3: Bacterial populations identified by DNA sequencing.

BACTERIA	GROUP	ALIGNED SEQUENCE	REGION
Streptococcus sp	Low G+C Gram Positive	TGATGATACTAGTAGCAGT	1254-1272
Propionibacterium	High G+C Gram Positive	GTGCTGCTGACTGACGAG	2304718-2304701
Desulfovibrio vulgaris	δ-*Proteobacteria*	CTGCTGTGCTCGCGCTGC	5152-5169

DNA sequencing of Archaea confirmed the presence of *Methanosarcina* as determined by FISH and revealed the presence of *Methanobacterium sp* and *Methanococcus sp*, that had not been probed for (Table 3.4).

Table 3.4: Archaeal populations identified by DNA sequencing.

ARCHAEA	FAMILY	ALIGNED SEQUENCE	REGION
Methanobacterium sp.	Methanobacteriaceae	ACGGACGTAGCGTGCAGG	11473-11456
Methanococcus sp.	Methanococcaceae	ATCAATGTAGTAGTA	317563-317577
Methanosarcina acetivorans	Methanosarcinaceae	ATCATCAAGTAACTC	5377-5363

3.3.2 Operating Period: February-June 2003

This operating period was characterised by an average flow rate of 3264L of wastewater per day.

3.3.2.1 The distribution of microorganisms within the ABR

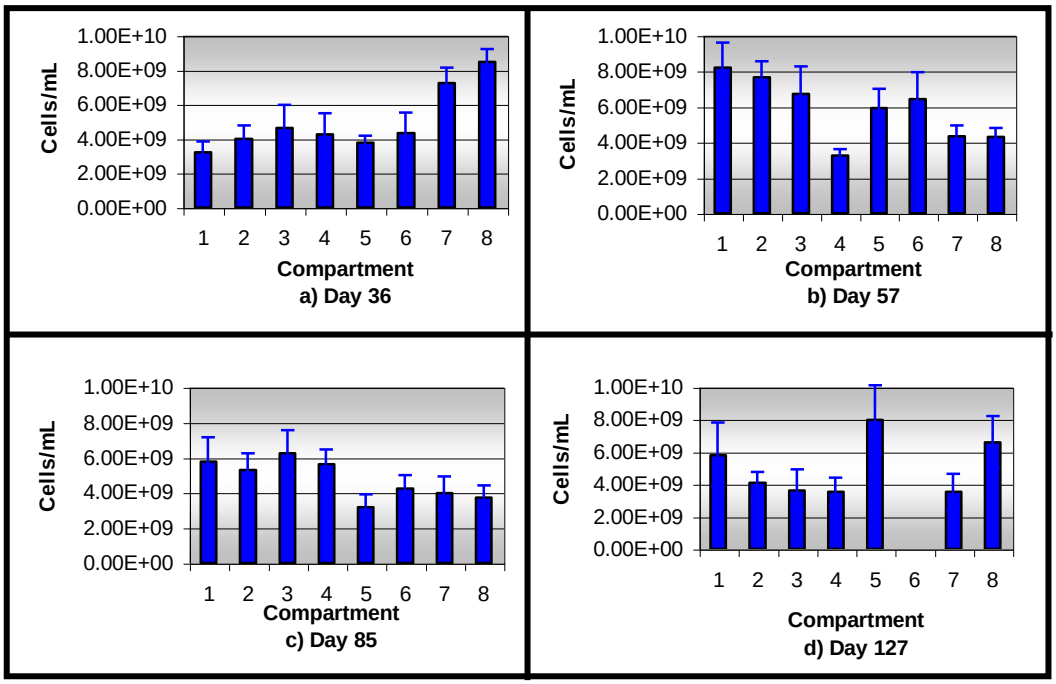

Figure 3.2: Total cell counts (February-June 2003).

Figure 3.2 shows the total cell counts (TCC) as determined by the membrane filtration DAPI method. Cell numbers showed uniformity with numbers between 10^9 and 10^{10} cells/ml. The highest cell numbers on average for the eight compartments was expressed on Day 57.

On Day 36 (Figure 3.2a), total cell counts showed initial low numbers in Compartment 1, thereafter, increasing in Compartments 2 and 3. Compartment 1 is generally regarded as a buffering zone, where most of the inhibitory substances are neutralized (Nachaiyasit and Stuckey, 1997b). Uyanik *et al* (2002a) found that this compartment helps stabilize the feed before it enters the other compartments. Substances inhibitory to anaerobic digestion that are found in domestic effluents may include ammonia from faeces and urine and chlorides from urea (Koppe *et al*., 1999, Bitton, 1994 and Leach, 1994). Following the reduction of these substances, microorganisms are able to multiply and develop better. Slight decreases were noted in Compartments 4 and 5. It is generally thought that the more sensitive organisms are located in the middle of the reactor. It is possible that there were certain inhibitory substances present, or that the COD was depleted, therefore limiting the substrate for the hydrolysing bacteria. These bacteria would then die. When bacteria die, they lyse and release intracellular molecular weight material into their environments (Nester *et al*., 1998 and Barker *et al*., 1999). This molecular weight material is known as soluble microbial products (SMP). Soluble microbial products are the organic molecules formed by biomass. These include cellular compounds that are released during cell lysis, diffused through cell membrane or released by the cell as extracellular material. Soluble microbial products are generally produced in response to environmental stress, extreme temperature changes, nutrient deficiency and the presence of toxic compounds (Aquino and Stuckey, 2004). The high molecular weight component has been identified as biodegradable heteropolysaccharides (Aquino and Stuckey, 2002). This material would provide the necessary organic molecules for the hydrolysing bacteria, which would increase growth in these favorable conditions. Therefore, an increase in the TCC was noted in Compartment 6.

Total cell count results for Day 57 (Figure 3.2b) remained similar throughout, with a stepwise decrease in numbers from Compartments 1 to 3. This differs from Day 36, which shows a stepwise increase in numbers in these Compartments. It is possible that there could have been an inhibitory or toxic substance present in the influent on Day 57, that was lacking on Day 36, or if present was not at high concentrations. There was a sharp decrease in Compartment 4. This could be due to a depletion of nutrients and a subsequent decrease in the hydrolysing bacteria. The SMP released by these lysed bacteria would provide sustenance for the remaining survivors, which begin to grow and replicate as can be seen by the increase in Compartment 6 (Aquino and Stuckey, 2004).

Day 85 (Figure 3.2c) showed TCC that were lower than the previous day's sampling. When looking at the total bacterial and archaeal numbers (to be discussed), it was noted that there was an overall decrease in both these microbial numbers. It can be speculated that there was a lower availability of nutrients (COD) on this day than the previous days. The decrease in Compartment 5 could be as a result of a depletion of nutrients, and the death of sensitive organisms. Other microorganisms would have utilized the SMP released by the lysing cells and replication would have continued, thus increasing the cell numbers in Compartment 6 (Aquino and Stuckey, 2002).

Total cell count results for Day 127 (Figure 3.2d) show that the numbers from Compartments 1-4 generally remain uniform. There is an increase in Compartment 5, which indicates a surge in nutrient availability. No results were available for Compartment 6, as the sample was damaged during the storage period.

DAPI staining for the determination of TCC is advantageous, in that DAPI binds exclusively to any DNA present. Any other material appears a pale yellow colour, as opposed to the bright blue of DNA. This may also be regarded as a disadvantage when determining microbial communities,

as DAPI does not differentiate between the DNA of live, dying or dead organisms (Porter and Feig, 1980). To help give a better indication of the microbial biomass, *in situ* hybridization was performed, using EUB338 and ARC915.

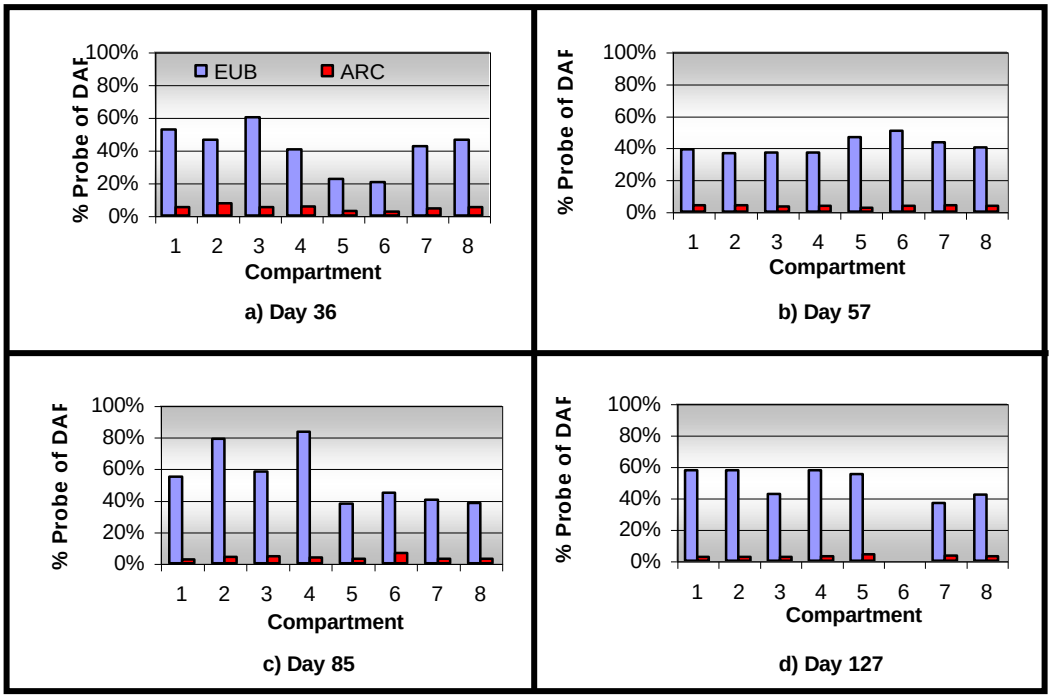

Figure 3.3: Percentage Domain-specific probes of DAPI stained cells (February-June 2003).

The domain-specific probes EUB338 and ARC915 were utilised to determine the active microbial biomass. Hybridizations with these probes showed bacterial dominance over the archaeal community (Figure 3.3). This reiterates that the theory that bacterial populations are generally the dominant species in anaerobic digestion (Bitton, 1994). Eubacterial cells, on average consisted of approximately 47% of the total microbial population. Archaeal cells were detected at approximately 4% of the total microbial population. In total these probes detected 50% of the DAPI stained cells. It is possible that these numbers are lower than the actual values, due to the low amount of cellular ribosomal RNA (rRNA) within intact cells. These microorganisms with low rRNA are unable to be accurately detected by FISH. This low cellular rRNA is characteristic

of slow growing (like the anaerobic microflora) as well as of starving cells (Amann *et al.*, 1990). Bacteria and *Archaea* are not the sole components of an anaerobic community. Fermentative ciliates, protozoa and anaerobic fungi all contribute to this microbial consortium (Bitton, 1994 and Tajima *et al*., 2001). Protozoan associations with two Archaeal orders, *Methanomicrobiales* and *Methanobacteriales* have been noted. Anaerobic ciliates have also been noted to form associations with the delta-*Proteobacteria* (Amann *et al*., 1995). These eukaryotic cells would have been stained by DAPI but not detected by the probes. Díaz *et al.* (2003) noted that in granules from separate batch reactors containing different substrates (formate, acetate, propionate, sucrose, starch and peptone) that less than 10% of the biomass was active prokaryotes that hybridized with Eubacterial or Archaeal specific probes combined. It was noted that most of the microorganisms corresponded to resting forms. This was attributed to low methanogenic activity.

3.3.2.2 Bacterial populations active in the ABR

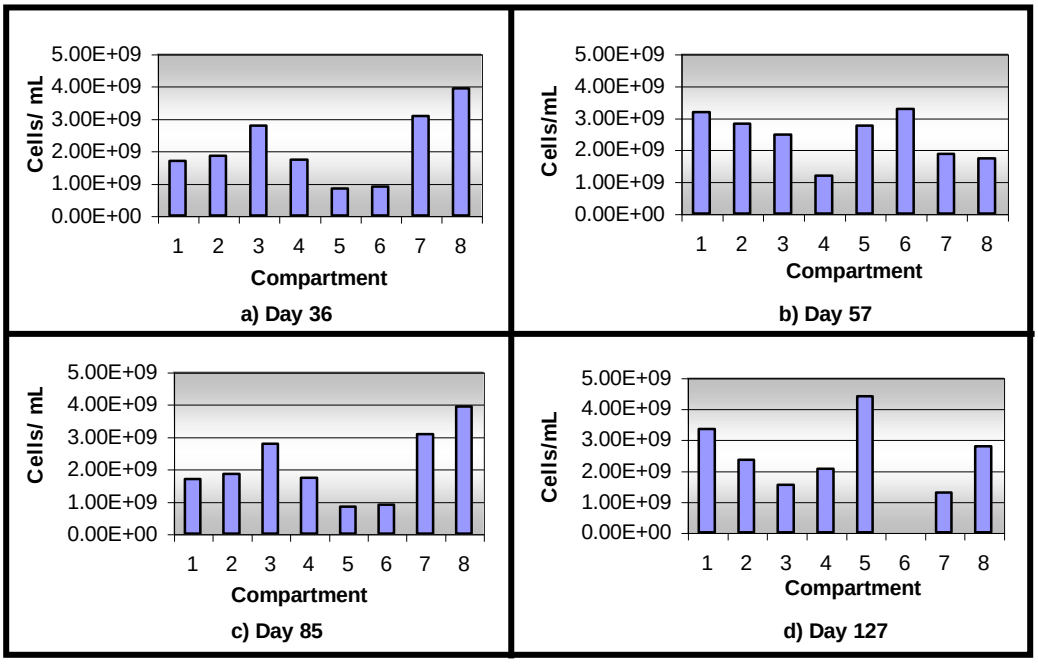

Figure 3.4: Total numbers of Eubacterial cells hybridized by probe EUB338 (February-June 2003).

The probe EUB338 was employed to determine the degree of bacterial population dominance. This probe is complementary to the conserved region of the 16S rRNA of most bacteria (Harmsen, *et al*., 1996). However, recent studies conducted have determined that the probe does not detect all Eubacteria as previously thought. The EUB probe is insufficient for the detection of certain bacteria belonging to the *Cytophaga-Fermicutes-Bacteriodes* phylum, the *Spirochetes* and *Acidobacterium* (Daims *et al*., 1999). Cells that are slow-growing or starving contain low rRNA that cannot be detected accurately by FISH (Wagner *et al*., 2003). This may be why the bacterial population only accounted for 47% of the DAPI-stained cells.

Eubacterial cells detected on days 36 and 85 (Figure 3.4a and c) followed the same trend. Increases were noted in Compartment 3, followed by decreases in Compartments 4-6 and thereafter increases in 7 and 8. The increase in Compartment 3 may be due to the depletion or breakdown of inhibitory compounds, such as ammonia and chloride that enter with the influent (Bitton, 1994 and Leach, 1994). The microbial populations in the buffering zone, Compartment 1 (Figure 3.5 a and c), probably reduce these toxins, thereby numbers providing optimal conditions for the proliferation of the more sensitive bacteria (Nachaiyasit and Stuckey, 1997b and Uyanik *et al*., 2002a). The increase in bacterial numbers indicates a utilisation of nutrients. It is under these conditions that bacteria will compete with one another for common nutrients. Certain bacteria, which are unable to compete with the faster-growing ones, will die. This theory explains the sudden decrease in bacterial numbers in Compartments 4 and 5. When these bacteria die, they release their intracellular material (SMP) (Aquino and Stuckey, 2004), which serves as a nutrient source for the remaining bacteria, thereby increasing the bacterial population, as can be seen in Compartment 6. Another possibility for this decrease and increase could be that bacteria are often inhibited by metabolic products that they produce (Bitton, 1994 and McInerney, 1999). The ideal conditions of Compartment 3 would have lead to the rapid growth of bacteria and an increased production of end products. The growth of bacteria would have then been inhibited by the high

concentration of end products; therefore they would have contained a low rRNA content and would not have been detected by the probe (Amann, *et al*., 1990 and Wagner *et al.*, 1993). Once the concentrations of the products were at a level that was not inhibitory, these bacteria would have continued to grow and replicate (Compartment 6).

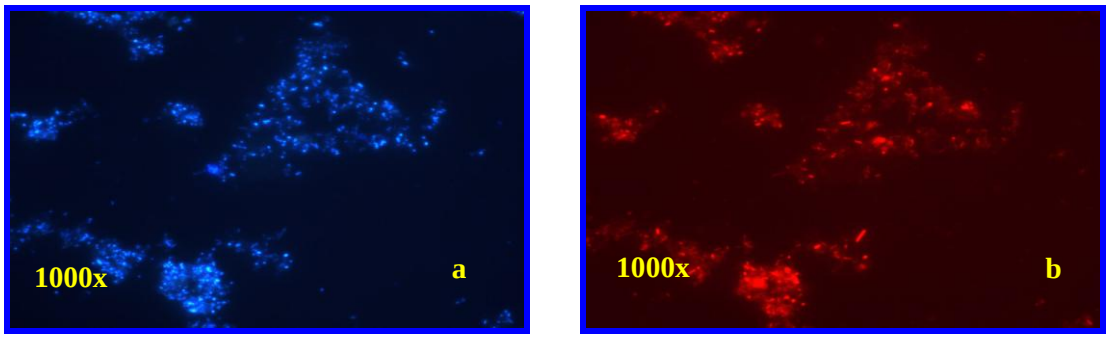

Figure 3.5: Images of the same field showing (a) DAPI stained and (b) EUB338 hybridized cells of Compartment 1 (Day 36).

Days 57 and 127 (Figure 3.4b and d) are similar in that the bacterial populations decreased in the middle of the reactor (Compartments 3 and 4) and increased in Compartments 5 and 6 respectively. Since the more sensitive organisms are located in the middle of the reactor it is sufficient to say that inhibitory substances, such as ammonia and chloride (Bitton, 1994 and Leach, 1994), which may have been degraded slowly throughout the reactor, may have inhibited them. The optimal conditions for proliferation would have been reached only by the fifth of sixth reactor, which is marked by an increase in bacterial numbers. Figure 3.6 below shows EUB 338 hybridized cells of Day 127

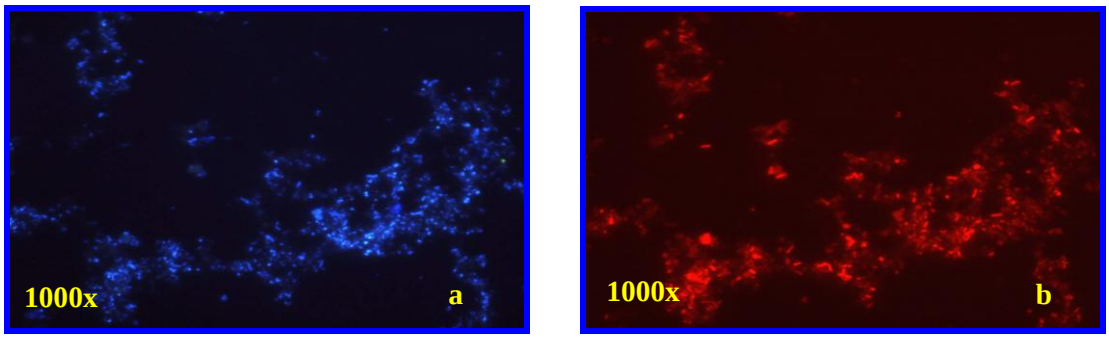

Figure 3.6: Images of the same field showing (a) DAPI stained and (b) EUB338 hybridized cells of Compartment 1 (Day 127).

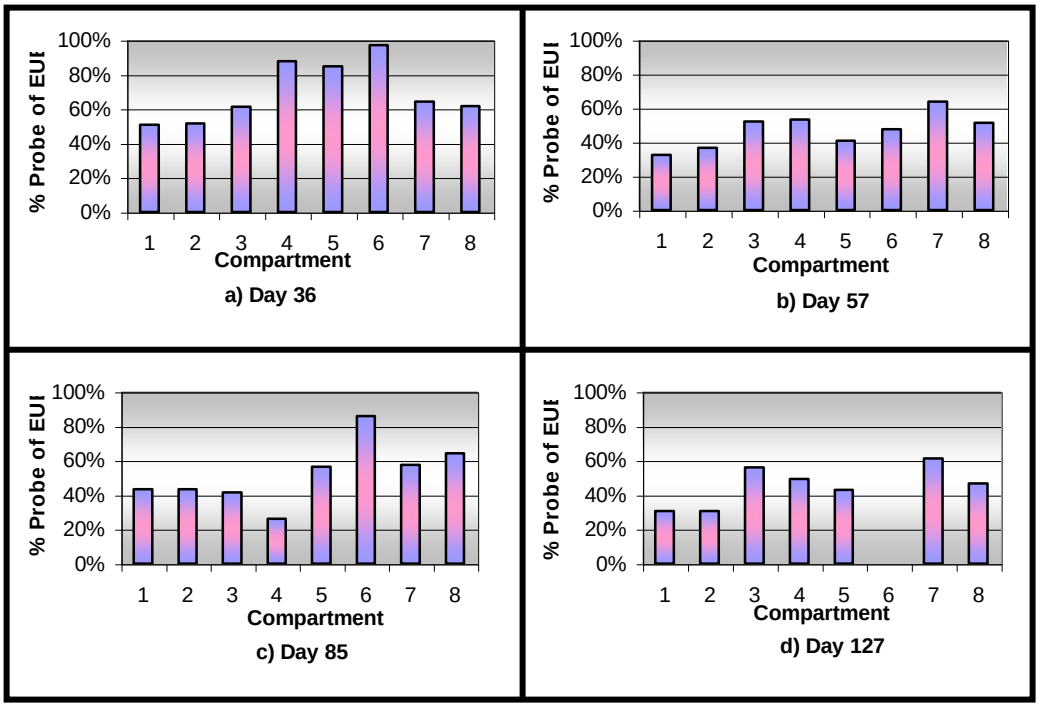

Figure 3.7: Percentage group-specific probes of total Eubacterial count (February-June 2003).

Of the EUB338 detected cells, 51% on average, for the four days sampled, were detected by the other 10 probes (Figure 3.7). Day 36 showed the highest number of Eubacterial cells (average 70% for the eight compartments) being identified by the 10 probes. Day 127, showed the lowest

number of bacterial groups identified (average of 36%). This indicates the presence of bacteria, in the ABR, which have not been accounted for by the probes. Plumb *et* al. (2001), treating dye wastewaters in an 8 compartment ABR, noted that the sum of the group specific probes was less than 100%, thereby signifying the presence of bacteria that were not detected. The epsilon subclass of the *Proteobacteria* was not probed for. This may have been present in the reactor, for example *Arcobacter*. Aerotolerant members of this group have been noted in activated sludge reactors. *Arcobacter*, which is found in humans with enteritis or diarrhoea, may have entered the system with the influent, which is 100% domestic (Snaidr *et al*., 1997). The presence of influent bacteria has been noted by Curtis and Craine (1998). Godon *et al*. (1997), when treating vinases in an anaerobic digester noted the presence of *Spirochetes* and *Planktomyces. Spirochetes* were also detected by Fernández *et al* (1999), when investigating a methanogenic reactor fed on glucose for 605 days. These bacteria may have been present in the ABR, and contributed to the EUB numbers. It is suggested that probes for these bacteria be utilised in further studies pertaining to the ABR.

It is also possible that Eubacterial numbers were inflated by the autofluorescence of the background material (Amann, 1995). Autofluorescence of the background material was reduced slightly by the rinsing of samples in 1 x phosphate buffered saline. However, it was not possible to minimise all of the autofluorescence. The digital analysis programme would have been unable to accurately determine the cell numbers thereby producing overestimated results (Wagner *et al*., 2003). Non-specific binding of probes may also have lead to overestimated results (Amann *et al*., 1995 and Amann, 1990).

A. The Hydrolytic Bacteria

The hydrolytic bacteria consist of bacteria from the groups Low G+C Gram Positives (probe LGC354a) and the *Cytophaga-Fermicutes* (probe CF319a). These bacteria execute the hydrolysis of organic polymers (proteins, cellulose, lignin and lipids) into soluble monomers (amino acids, glucose, fatty acids and glycerol) (Bitton, 1994 and Guiot *et al*., 1992). The presence of the LGC was confirmed by DNA sequencing results, which identified the *Streptococcus* species of this class (Table 3.3). Since the LGC group of bacteria contain different members that each carry out either hydrolysis or acidogenesis, it was assumed that each of these groups consisted of 50% of the total LGC population. These numbers were expressed in Figures 3.8 and 3.10 as a portion of the total hydrolytic and total acidogenic populations respectively.

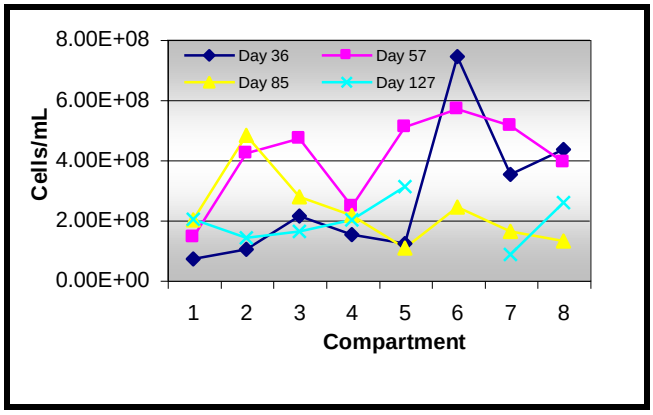

Figure 3.8: Total cell numbers of hydrolytic bacteria (February-June 2003).

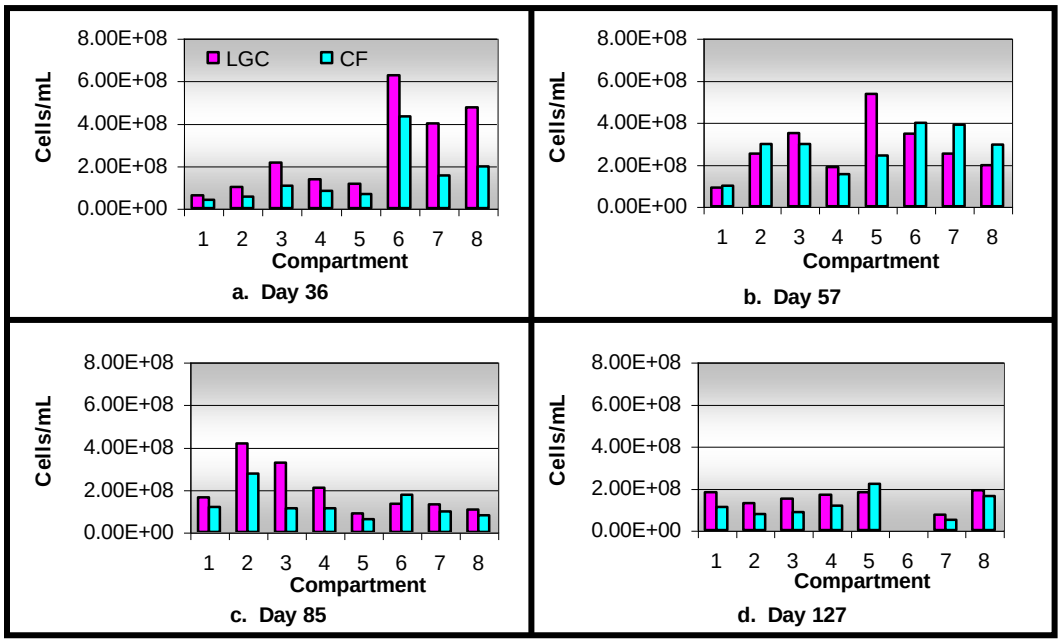

Figure 3.9: Total cell numbers of the hydrolytic Eubacterial cells hybridized by probes LGC354a and CF319a (February-June 2003).

Day 36 (Figure 3.8) exhibited a stepwise increase in the total hydrolytic bacterial population from Compartments 1 to 3. It is noted that both the LGC and the *Cytophaga-Fermicutes* show increases (Figure 3.9a). This increase could be due to the slow breakdown of the toxic and inhibitory substances in Compartment 1 (the buffering zone) (Nachaiyasit and Stuckey, 1997b and Uyanik *et al*., 2002a). The hydrolytic bacteria generally break these compounds down with little difficulty, and therefore the breakdown is step-wise and inversely proportional to growth. There are also enough nutrients for these bacteria to proliferate, as the influent COD was high (792mg COD/L) (Figure A7.1, APPENDIX SEVEN). The decrease in the total population (Figure 3.8) in Compartments 4 and 5 indicates a depletion of nutrients, which would cause inhibition and even death. Death and lysis of cells would have resulted in the production of SMP (Aquino and Stuckey, 2004), which would have provided the nutrients for these bacteria, which show increases in Compartment 6 (Figures 3.8 and 3.9a). The decrease in the population after

Compartment 6, would have been due to a depletion of nutrients, as can be seen by the effluent COD, which was a low 164 mg/L (Figure A7.1, APPENDIX SEVEN).

Day 57 displayed the highest numbers of the total hydrolytic bacteria from all the sampling dates during this operating period. Results 57 showed the similar step-wise increase (Compartments 1 to 3) and the decrease (Compartment 4) as the previous sampling date (Figures 3.8 and 3.9b), following the same trends. However, the hydrolytic bacteria recover quicker than those of Day 36, and show increased growth in Compartment 5. There is an overall increase in hydrolytic bacteria in Compartment 6 (Figure 3.8). When viewing Figure 3.9b, it is noted that there is a decrease in the LGC bacteria, but a steady increase of the *Cytophaga-Fermicutes*. This is probably owing to competition for the same, steadily depleting nutrients (organic molecules). This nutrient source would have been depleted by Compartments 7 and 8, which would have resulted in the death of the organisms (Figure 3.8). This depletion is noted by the low COD, which was recorded at 107.31 mg/L (Figure A7.1, APPENDIX SEVEN).

Day 85 showed the similar trend as the previous sampling dates, differing only in the advanced decrease of the population numbers, which occurred in Compartment 2 (Figure 3.8). There appears to be a larger decrease in the *Cytophaga-Fermicutes* than the LGC (Figure 3.9c). Since the LGC consist of both acidogenic and hydrolytic bacteria, the relatively small decrease could be due to an increasing acidogenic bacterial population, which would have been almost inversely proportional to the decreasing hydrolytic population. The death of these bacteria would have lead to an increase in organic molecules in the total wastewater composition, as dead cells lyse and release intracellular material as SMP (Aquino and Stuckey, 2004). This, then, would have accounted for the increase of COD, and the subsequent utilisation of this material by the hydrolysing bacteria. This is noted by an increase in Compartment 6.

Results of Day 127 depict almost similar hydrolytic bacterial numbers from Compartments 1 to 4 (Figure 3.8). This indicates a slow breakdown (hydrolysis) of the organic polymers. It is possible that conditions were not optimal or there were certain inhibitory substances present in the reactor, which prevented rapid hydrolysis, as was noted during the previous sampling days. However, there is an increase of both bacterial groups in Compartment 5 (Figure 3.9d), indicating the removal of the inhibitory substance. The decrease of the population numbers in Compartment 7 is probably due to a depletion of nutrients.

B. The Fermentative Acidogenic Bacteria

The fermentative acidogenic bacterial group consists of bacteria belonging to the α and γ subclasses of the *Proteobacteria*, the Low G+C Gram Positives and the *Bacteriodes* classes. They utilise the soluble monomers produced by the hydrolytic bacteria and form organic acids (acetic, propionic, formic and lactic), alcohols, ketones VFAs carbon dioxide CO_2 and hydrogen H_2 (Bitton, 1994 and Guiot *et al*., 1992).

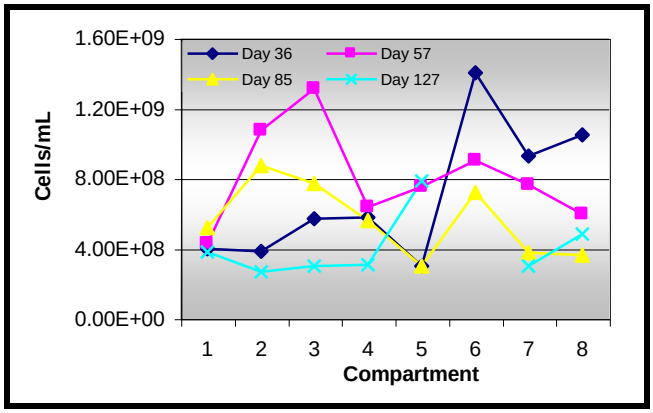

Figure 3.10: Total cell numbers of acidogenic bacteria (February-June 2003).

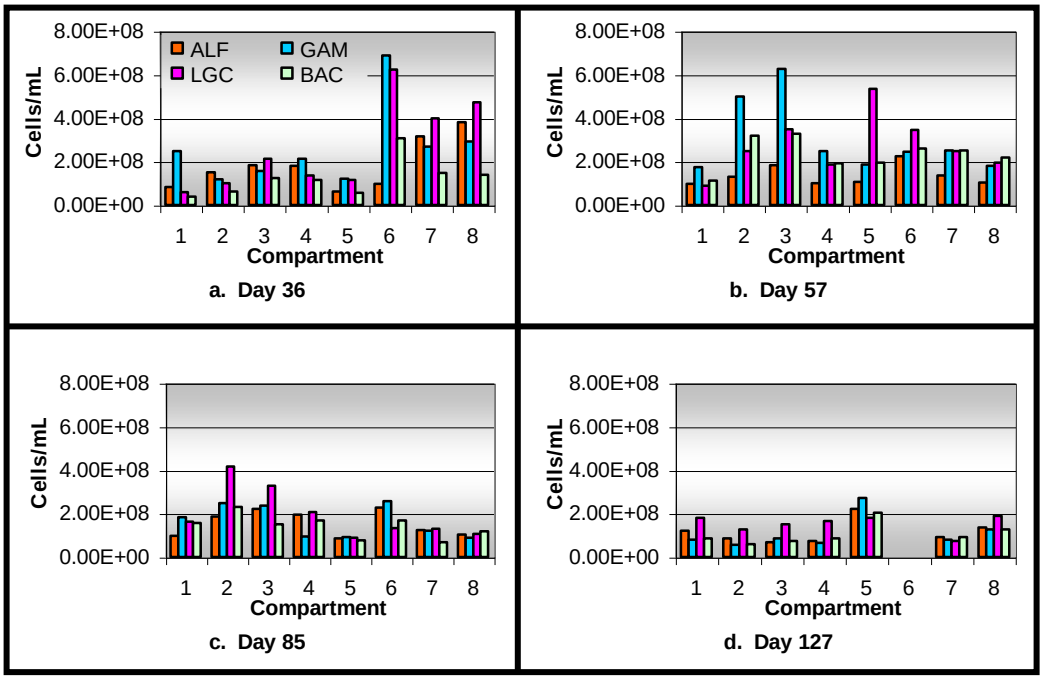

Figure 3.11: Total cell numbers of the hydrolytic Eubacterial cells hybridized by probes ALF1a, GAM1b, LGC354a and BAC303 (February-June 2003).

Results for Day 36 showed increasing acidogenic bacterial numbers from Compartments 1 to 3 (Figure 3.10). It is noted that almost all the individual bacterial populations increase as well (Figure 3.11a). This shows increasing substrate availability for the growth and proliferation of these organisms. The γ-*Proteobacteria* class is the only one that decreases in Compartment 2. It is possible that there was competition from the other bacterial classes, and therefore, the γ-*Proteobacteria* decreased. The total acidogenic bacterial numbers are lowest in Compartment 5 (Figure 3.10). This probably would have been subsequent to utilisation of substrate; therefore the slower-growing bacteria would have decreased first (*Bacteriodes* in Compartment 4) and thereafter followed by the other classes in Compartment 5 (Figure 3.11a). The increase of the acidogenic bacteria in Compartment 6 could be due to the increased substrate concentration that was formed by the utilisation of the organic polymers produced by the hydrolytic bacteria. It is also possible for these bacteria to be inhibited by their end products (Bitton, 1944 and McInerney,

1999), and following the depletion or utilisation of these inhibitory products by the acetogenic bacteria, the numbers increased in Compartment 6.

The acidogenic bacteria show higher numbers on Day 57, as opposed to the previous sampling day (Figure 3.10). All of the bacterial populations show increasing numbers from Compartments 1 to 3. This is in accord with the increasing substrate availability produced by the hydrolysis stage of anaerobic digestion. The decrease in Compartments 4 and 5 indicate a depletion of nutrients, or possible inhibition from the build-up of end products. The LGC are the first to recover, showing increases in Compartment 5 (Figure 3.11b). This increase must be as a result of the increase of the hydrolytic LGC, which increased as a result of the availability of organic polymers from lysing cells (SMP). The formation of the monomers in this step would have provided sustenance for the acidogenic bacteria, which show a large increase in Compartment 6.

Results of Day 85 depict the similar trend of Days 36 and 57, in that; there is an increase in cell numbers from Compartments 1 to 3 (Figure 3.10), possibly due to the increasing availability of substrate from the hydrolysis step. The exception to this increase is the *Bacteriodes* class, which decreases in Compartment 3 (Figure 3.11c). This decrease must be as a result of competition between the acidogenic bacterial groups for the common substrates, which become limiting in Compartment 5. Subsequently, all the bacterial groups decrease in this compartment. These bacteria then increase in Compartment 6, possibly as a result of the formation of organic monomeric substrate by the hydrolytic bacteria (as previously explained).

Day 127 (Figure 3.10) showed the lowest acidogenic population numbers in this operating period. It was previously noted that hydrolysis was slow; therefore, it took an extended time to produce the monomeric substrate required by the acidogens. The substrate present may have been at low concentrations. It was noted that the LGC bacteria remained relatively constant from

Compartments 1 to 4, while the other classes decreased (Figure 3.11d). This could be due to both the acidogenic and hydrolytic populations remaining relatively similar. There was a rapid increase in acidogenic numbers in Compartment 5. This could be due to an increased substrate production by the hydrolytic bacteria, following the removal of the inhibitory substance (as noted previously). Thereafter, the decrease in numbers indicates limiting substrate concentrations.

During this operating period, it was noted that the pH of the effluent was relatively low, between 6.2 and 6.7 (Figure A7.2, APPENDIX SEVEN). This correlates with the acidogenic bacterial numbers, which show high numbers at the end of the reactor (Figure 3.10).

C. The Acetogenic Bacteria

The acetogenic bacteria are those belonging to the classes HGC and δ-*Proteobacteria* (SRB). Obligate hydrogen-producing acetogens degrade propionate; long chain VFAs and aromatic compounds to acetate, CO_2 and H_2. A minor group of hydrogen-consuming acetogens reduce CO_2, CO and methoxyl-groups of aromatic compounds to acetate and sometimes butyrate (Guiot *et al*., 1992). The presence of the HGC and SRB was confirmed by DNA sequencing, of the *Propionibacterium* species and *Desulfovibrio vulgaris* respectively (Table 3.3). It has been noted in literature that the SRB grow syntrophically on lactate, ethanol, propionate, fumarate and that syntrophy with formate utilising methanogens, eradicates the need to reduce sulfate. In this way, the SRB can grow as the proton-inducing acetogenic bacteria. This phenomenon is characteristic of *Desulfovibrio* and *Desulfobacterium* (Raskin *et al*, 1994a and Raskin *et al*., 1995).

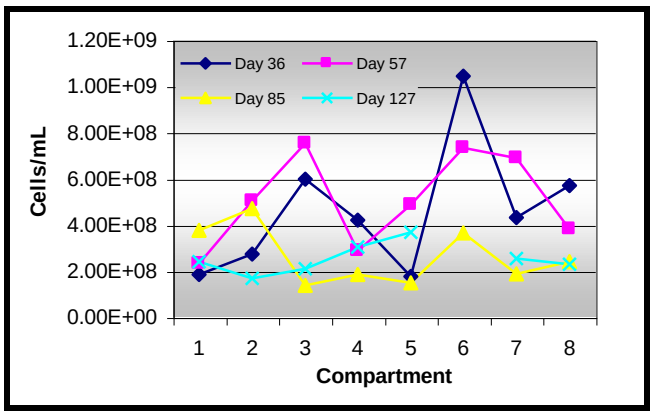

Figure 3.12: Total cell numbers of acetogenic bacteria (February-June 2003).

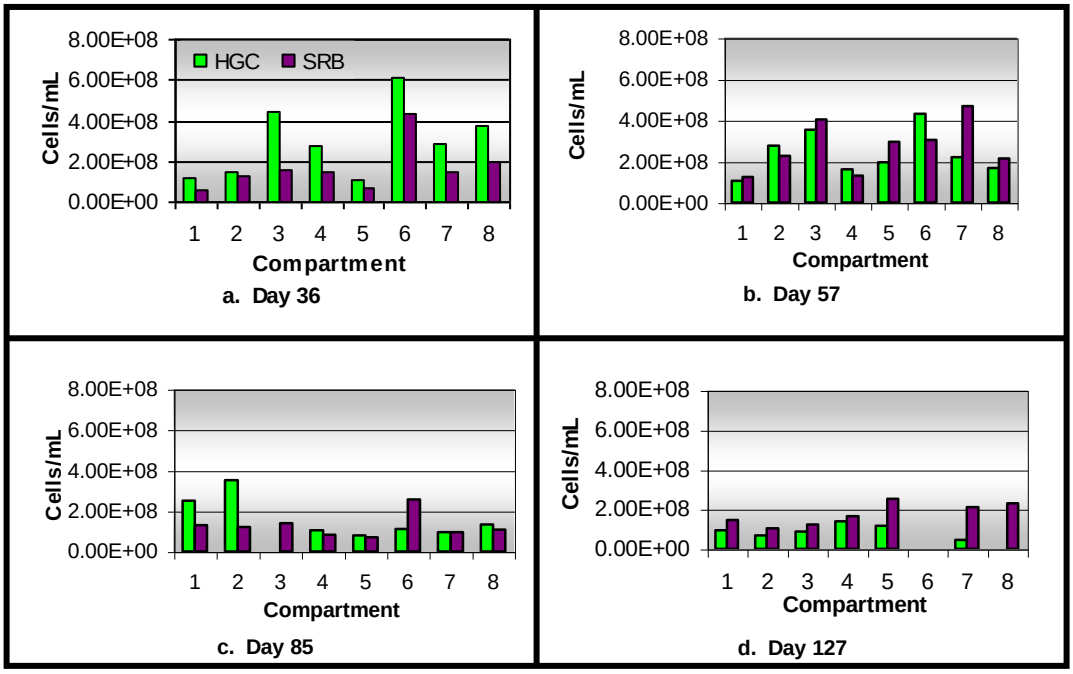

Figure 3.13: Total cell numbers of the acetogenic Eubacterial cells hybridized by probes HGC69a and SRB385 (February-June 2003).

Results of Day 36 depict a step-wise increase of the acetogens from Compartment 1 to 3 (Figure 3.12), which correlates with the increasing substrate availability by the production of acids by the acidogens. Both the acetogenic bacterial classes show increases during this period (Figure 3.13a).

The decrease of the acetogen numbers in Compartment 4 may be attributed to feedback inhibition, where the bacteria are inhibited by their own metabolic end products. It is noted that there is a decrease in the acetoclastic methanogen, *Methanosarcina*, in this compartment (to be discussed in section 3.3.2.3). Therefore, there may not have been sufficient removal of acetate, and the acetogens would have been inhibited. The bacterial numbers increase in Compartment 6 (Figure 3.12), possibly due to the reduction in the acetate concentration and the reversal of inhibition. The decrease in Compartment 7 may be due to an increase in the substrate concentration, produced by the increasing acidogenic population (as discussed previously).

Results of Day 57 show a step-wise increase and decrease of acetogens from Compartments 1 to 4 (Figure 3.12). The decrease in Compartment 4 may due to a decreased production of acids by the acidogenic bacteria, which also decrease. Increases were noted for Compartment 5 and this corresponds to the increase in the acidogenic bacterial numbers. This indicates an increased availability of substrate for the acetogens. The HGC species decrease in Compartment 7, while the SRB population increases (Figure 3.13b). This could be due to the competition between the acetogens for the same substrates.

Results depicted in Figure 3.12, of Day 85, show increasing acetogenic numbers from Compartments 1 to 2. Thereafter, there is a sharp decrease in bacterial numbers in Compartment 3, due to the absence of the HGC (Figure 3.13c). This cannot be attributed to a decrease in substrate as acidogenesis is prevalent and the SRB are still present. Therefore, it must be as a result of the overproduction of acetate, which is not removed, as there are no *Methanosarcina* present in this compartment. The HGC would have been inhibited. It is also noted that the SRB are at almost the same level as the previous compartment. The presence of the HGC in Compartment 5, serves as an indication of the reduction of the acetate concentration. The increase in acetogenic bacterial numbers in Compartment 6 is related to the increased acidogen numbers,

which would have provided an increased substrate concentration for the growth and proliferation of acetogens.

Day 127 results correlate with the other bacterial characterisation analyses, in that the initial numbers are low (Figure 3.12). This could be due to a lower production of substrates as a result of slow hydrolysis. The increase in Compartment 4 is in coordination with the increased production of acidic end products by the acidogens. The HGC numbers however, decrease from Compartment 5 to 7, possibly as a result of competition for substrates between the two classes (Figure 3.13d). The SRB appear to dominate the utilisation of substrates, and depict similar numbers from Compartments 5 to 7, and finally completely out competing the HGC, which are not present in Compartment 8.

The SRB385 probe is often regarded as phylogenetically inconsistent (Santegoeds *et al*., 1998). Therefore, genera of this group were probed using probes DSB985 and DSV698.

Results of Day 36 depicted an increase in the SRB (Figure 3.13a) from Compartments 1 to 2. However, the DSB and DSV results remained relatively similar (Figure 3.14a). This increase might have been due to a slight increase in the SRB, but more so due to an increase in other organisms that this probe detects. Other changes in the reactor, the subsequent increases and decreases of the SRB are mirrored by the DSB and DSV probed bacteria.

Day 57 results (Figure 3.14b) mirrored that of the SRB probed cells (Figure 3.13b), with numbers increasing and decreasing accordingly. The increase in Compartment 7 of the SRB is not uniform with the decrease of the DSB, but this is counteracted by the increase of the DSV.

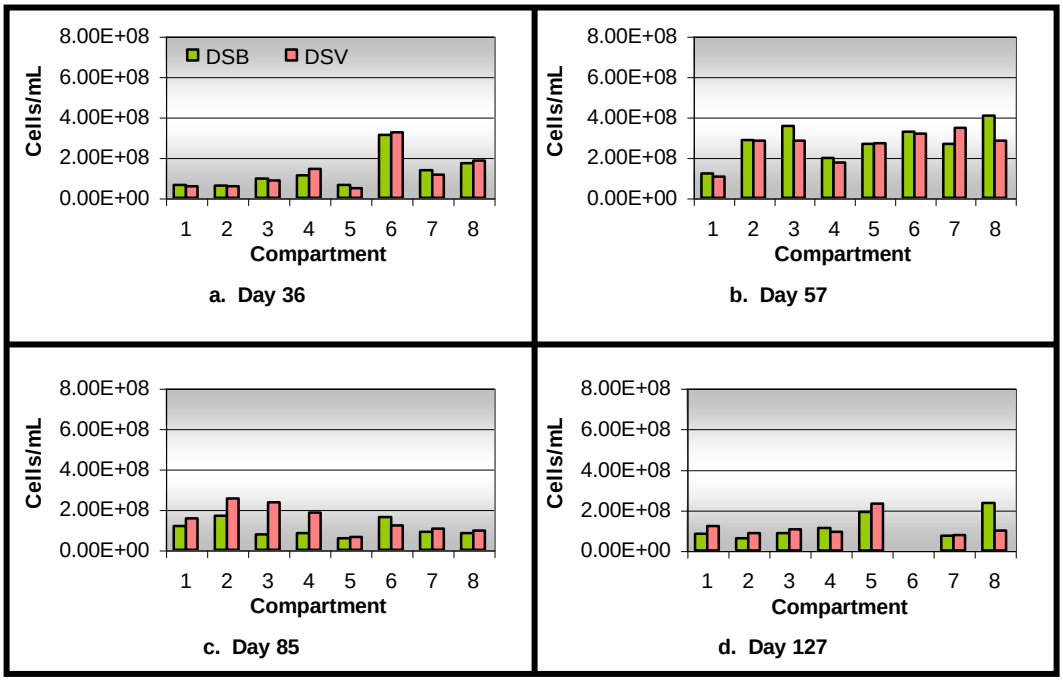

Figure 3.14: Total cell numbers of the sulfate reducing bacteria hybridized by probe DSB985 and DSV698 (February-June 2003).

On Day 85 (Compartment 3), there was a decrease in the DSB numbers (Figure 3.14c), but DSV and SRB numbers remain relatively similar. This could be as a result of an increase of other bacteria that are detected by the SRB probe. The decrease of DSV numbers caused a decrease of the SRB in Compartment 4.

Day 127 results showed the lowest DSB and DSV results for this operating period (Figure 3.14d). The increases and decreases of the SRB where mirrored by the DSB and DSV numbers from Compartments 1 to 7. In Compartment 8, the DSB show a large increase, while the SRB numbers remain relatively similar. This could be due to a decrease in the numbers of the other bacteria that probe SRB385 binds to.

It has been noted that throughout the months that the sum of the DSB and DSV probes were often higher than the total SRB numbers. A possible explanation would be the autofluorescence of the two former probes, which would have lead to an inflation of numbers. Probes may also bind non-specifically and this would have been inaccurately counted by the digital analysis program (Amann, 1995 and Wagner *et al*, 2003).

Probe BET42a was applied to the sludge samples for all of the above days. Signals were only detected on Day 36 (results not shown) and that too only in the first four compartments. When studying this genus further, it was noted that most of these bacteria were aerobic and therefore would not have been able to survive in an anaerobic atmosphere. It is possible that there was a little oxygen present in the entering wastewater and after this was utilised these bacteria died off.

No separation of the phases of anaerobic digestion was noted, with all the microbial communities being present throughout the reactor for all 4 sampling periods. This could be due to the high flow rate, which would have prevented the establishment of a healthy community. Langenhoff and Stuckey (2000) found that no separation of the phases was noted in an 8 chamber ABR, treating low strength synthetic wastewater. Uyanik *et al* (2002b) noted no separation of phases in a 4 compartment ABR, treating synthetic wastewater, as methane production was noted throughout the reactor.

3.3.2.3 Archaeal populations active in the ABR

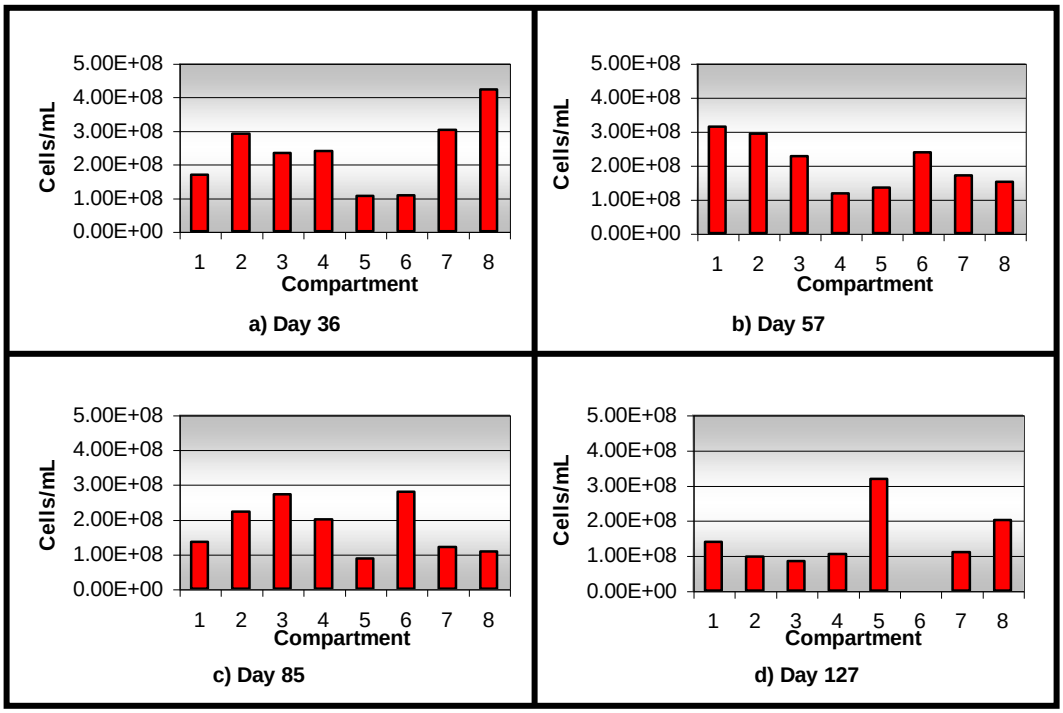

Figure 3.15: Total numbers of Archaeal cells hybridized by probe ARC915 (February-June 2003).

The probe ARC 915 was applied to determine the contribution of Archaea to the community of the ABR. Hybridizations showed total archaeal populations consisting of an average 4% of total DAPI stained cells (Figure 3.3). This number may be underestimated, as it is sometimes difficult for probes to enter Archaeal cell walls. Since the Archaea are slow growing, they would have had a low cellular rRNA content, which would have been unable to detect with the oligonucleotide probes (Wagner *et al*, 2003 and Amann, *et al*., 1990).

Figure 3.15 shows the results of ARC probed cells. On Day 36 (Figure 3.15a), the archaeal cells showed increases from Compartments 1 to 2. The first compartment is regarded as a buffering zone, where toxins are destroyed (Nachaiyasit and Stuckey, 1997b and Uyanik *et al*., 2002a).

Since the Archaea consist of sensitive organisms, these would have had difficulty in growing in the toxic conditions and would only proliferate once these were removed. There is a decrease in cell numbers in Compartments 5 and 6. This may be attributed to a decrease in *Methanosarcina* cells, which are not found after Compartment 4 (Figure 3.16a). There is an increase in Archaeal populations from Compartments 7 and 8, indicating the presence of other methanogens, such as *Methanobacterium* species, which are also found in anaerobic digesters.

Results of Day 57 are depicted in Figure 3.15b. This graph shows the stepwise decrease of Archaeal numbers from Compartments 1 to 4. This decrease is due to the reduction in *Methanosarcina* numbers (Figure 3.16b). The increase in Compartment 5 (Figure 3.15b) would have been due to an increase in methanogens other than *Methanosarcina* as these numbers were decreasing. Compartments 7 and 8 show decreases in the total Archaeal numbers due to the elimination of the *Methanosarcina* cells.

Results for Day 85 (Figure 3.15c) show an increase from Compartments 1 to 3, which would indicate a decrease in toxic substances and thereby supporting the growth of the methanogens. Compartment 4 shows a decrease in Archaeal numbers. This corresponds to the decrease in cell numbers of all the other groups of bacteria due to nutrient depletion (as discussed). The increase in Compartment 6 also correlates with the production of intermediates by the various groups, following hydrolysis of intracellular material by the hydrolytic bacteria.

It was previously noted in Day 127 that a lengthy hydrolysis had occurred. Therefore there was insufficient nutrients/substrate for the methanogens, which showed decreases from Compartment 1 to 4 (Figure 3.15d). The increase in Compartment 5 coincides with the completion of hydrolysis and the release of substrates.

A. The Methanogens

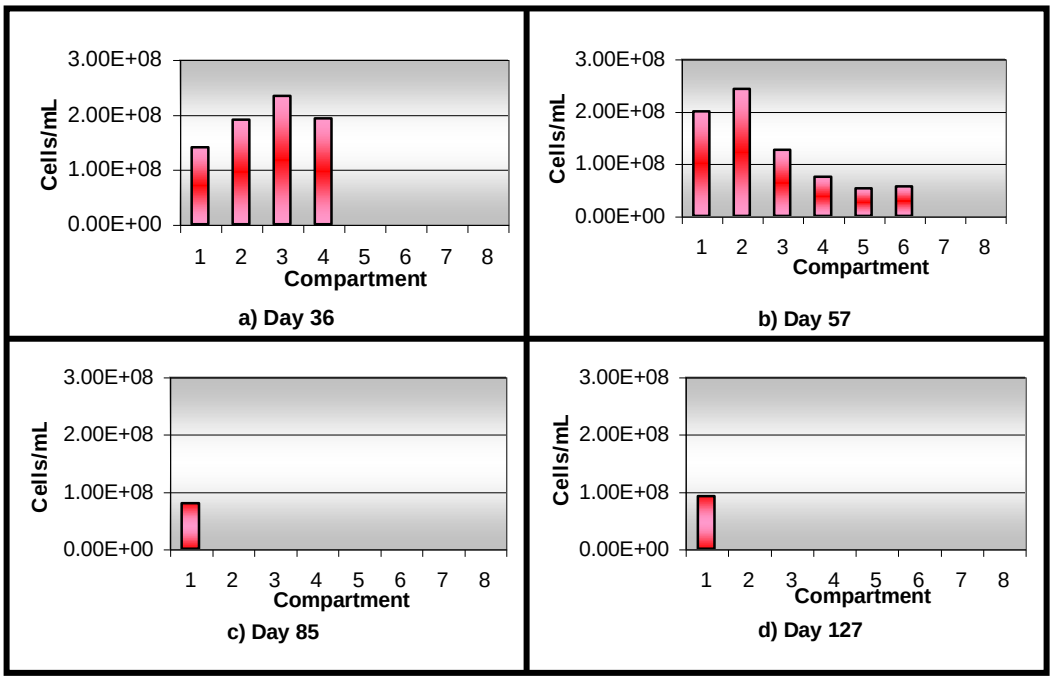

Figure 3.16: Total cell numbers of *Methanosarcina* as determined by probe MS821 (February-June 2003).

Of the total Archaeal population only one genus was identified by *in situ* hybridization. This was the genus *Methanosarcina*. DNA sequencing results confirmed the presence of this methanogen as well as the presence of the genus *Methanobacterium* and the genus *Methanococcus* (Table 3.4).

Methanosarcina is an acetoclastic methanogen that utilise acetate and decrease its concentrations (Rocheleau *et al*., 1999). These cells were found primarily at the beginning of the reactor in their characteristic packet or cluster forms (König, 1993). Tilche and Yang (1987) (as cited in Barber and Stuckey, 1999) noted a large concentration of *Methanosarcina* cells at the beginning of a pilot scale Hybridised ABR treating molasses wastewater. Langenhoff and Stuckey (2000) also

noted a high methanogenic activity at the beginning of an 8-chamber ABR treating low strength synthetic milk substrate. The methanogens compete with the SRB for the same electron donors and generally are out-competed (Araujo *et al.,* 2000). Figure 3.16a shows the decrease of the *Methanosarcina* after Compartment 4. This coincides with an increase of the SRB. The methanogens are slower growers and therefore are out competed by the SRB and therefore decrease.

Results of Day 57 (Figure 3.16b) indicate that the methanogens and SRB utilise acetate equally from Compartments 1 and 2. The stepwise decrease of methanogens is inversely proportional to the increase of the SRB, which out compete the methanogens. The methanogens are eliminated in Compartment 7.

Days 85 and 127 (Figure 3.16c and d) show the presence of *Methanosarcina* only in Compartments 1. The first compartment is generally a buffering zone, where many inhibitory compounds enter with the influent. It is possible that these substances inhibited the methanogens. Acetoclastic methanogens are also very sensitive to changes in pH and generally thrive at a pH between 6.7 and 7.4 (Bitton, 1994). It was noted for this period that the pH values often were below 6.5 (Figure A7.2, APPENDIX SEVEN). Therefore, the acetoclastic methanogens would have been inhibited. Methanogenic bacteria are slow growers and, therefore, require long recovery times following start-up and perturbations within a system (Raskin *et al*., 1994a).

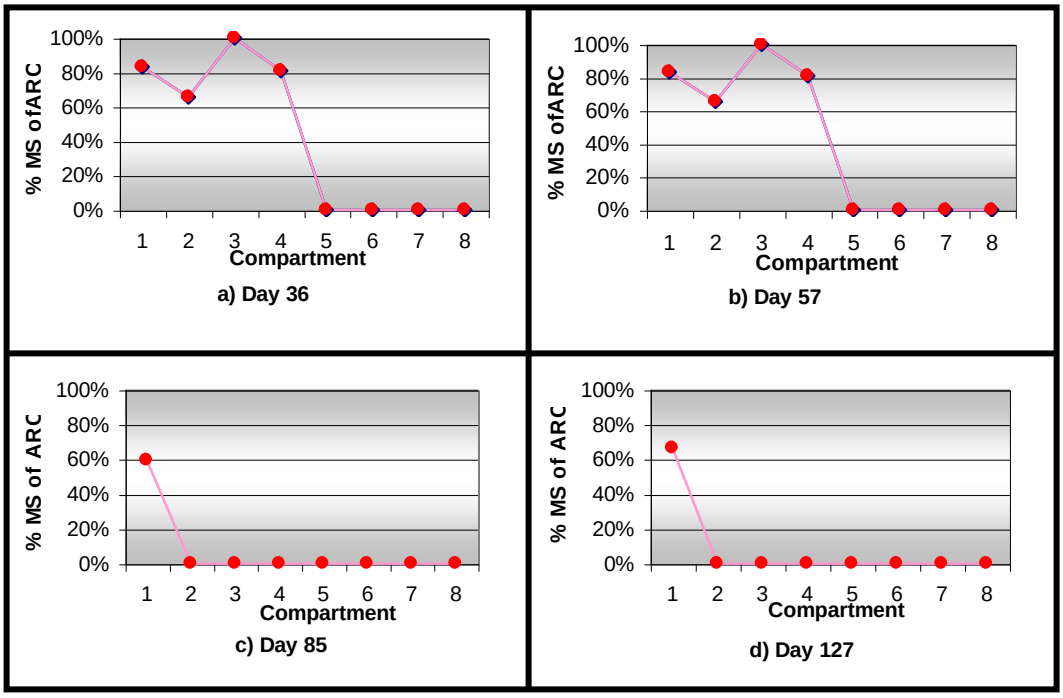

Figure 3.17: Percentage *Methanosarcina* of the total Archaeal population (February-June 2003).

Figure 3.17 depicts the percentage of the *Methanosarcina* population of the total Archaeal population. It was noted that only on Days 36 and 57 (Compartment 3) that the *Methanosarcina* were 100% of the detected Archaea. In most of the instances the *Methanosarcina* do not contribute to the Archaeal community. This indicates the presence of other methanogens, such as *Methanobacterium* and *Methanococcus*, which was identified by DNA sequencing. These methanogens utilised hydrogen and formate to produce methane (König, 1993). It is suggested that probes for the different methanogens be utilised for future studies. *Methanobacterium* sp, in conjunction with *Methanosarcina* sp. have been noted in anaerobic digesters fed with vinases (Godon *et al*., 1997). *Methanobacterium* sp. tend to recover quickly from stresses in a system. It is possible that is why they were able to survive in the ABR. Angenent *et al* (2002) noted that *Methanobacteriaceae* showed increased numbers after feeding resumed following starvation in a 5 compartment anaerobic migrating blanket reactor fed on sucrose, nutrients, bicarbonate, yeast extract and trace elements.

The other methanogenic probe utilised was MX825. This probe was applied to detect *Methanosaeta* cells, however, no signals were detected. These cells are highly filamentous but were not detected even with DAPI-staining. It is noted that scavenging methanogens are limited by low pH (Nachaiyasit and Stuckey, 1997b). *Methanosaeta* cells have a slower growth rate than *Methanosarcina* and are more sensitive to environmental changes like decreased pH. Decreased pH further encourages the growth of acid producing bacteria, which results in a decrease in the methane potential (Barber and Stuckey, 1999).

Raskin *et al* (1994) noted that *Methanosarcina* were the prevalent acetoclastic methanogen in anaerobic chemostats fed with glucose. *Methanosaeta* cells were absent. This proved to be due to the ability of *Methanosarcina* to proliferate at high acetate concentrations. This may have been the reason why this phenomenon was noted in our pilot scale ABR. However, this cannot be verified due to a lack of data for acetate concentrations. The prevalence of *Methanosarcina* was also noted in a continuously stirred tank reactor treating cattle manure (Mladenovska *et al*., 2003).

3.3.3 Twenty-four Hour Campaign (Day 101)

3.3.3.1 The distribution of microorganisms within the ABR

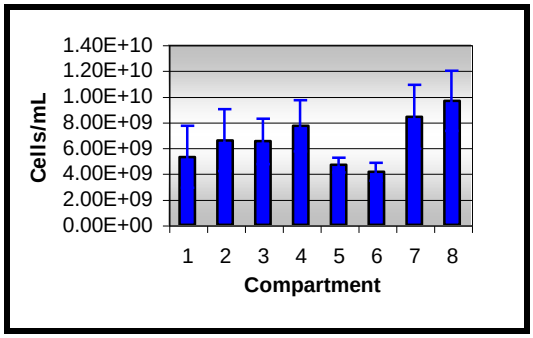

Figure 3.18: Total cell counts (Day 101).

The total cell counts; representing the biomass fraction of the anaerobic sludge was determined for each of the compartments during the 24-hour campaign. The total cell numbers were low in Compartment 1, thereafter, increasing gradually to Compartment 4 (Figure 3.18). When compared with COD results (Figure A7.3, APPENDIX SEVEN)**,** this increase corresponded with an increased use of organics, indicated by the decreasing COD values. Compartment 4 is characterised by a low COD concentration of 70 mg/L. This implies a depletion of nutrients; therefore there are insufficient nutrients for the growth of bacteria in Compartments 5 and 6. When viewing COD results, it is noted that COD values increased in Compartments 5 and 6. This COD could have been as a result of the release of intracellular SMP from lysed cells (Aquino and Stuckey, 2004). The organics released would have been utilised by the microorganisms in Compartments 7 and 8. Due to favourable conditions, these microorganisms would have depicted increased growth (Figure 3.18). Negative removal of COD was also noted by Nachaiyasit and Stuckey (1997a). This, however, was attributed to sampling errors.

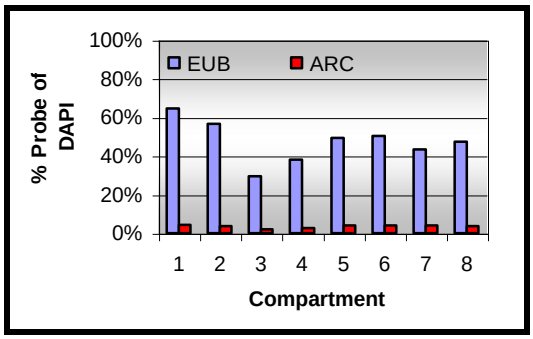

Figure 3.19: Percentage Domain-specific probes of DAPI stained cells (Day 101).

To help accurately determine the active portion of the biomass present, cells were hybridized with the probes EUB338 and ARC 915. EUB populations were dominant; comprising approximately 47% of all DAPI stained cells (Figure 3.19). Archaeal cells were present at approximately 4% of the DAPI stained biomass. Cumulatively, these microorganisms comprised of 51% of the total DAPI biomass, signifying that only half of the DAPI stained cells was active. This could also indicate the presence of microorganisms other than bacteria and Archaea, such as ciliates, protozoa, nematodes and fungi (Bitton, 1994, Tajima *et al*, 2001 and Amann, 1995). It is possible that these numbers are actually lower than the actual values, due to the low amount of cellular rRNA within intact cells, which is difficult for the probes to detect (Schramm and Amann, 1999). DAPI stains all cells, including inactive ones. These cells would have had too low an rRNA content to be detected by the probes (Wagner *et al*., 1993). These results have also been achieved by Díaz *et al* (2003) where less than 10% of their biomass was active prokaryotes that hybridized with Eubacterial or Archaeal specific probes combined.

3.3.3.2 Bacterial populations active in the ABR

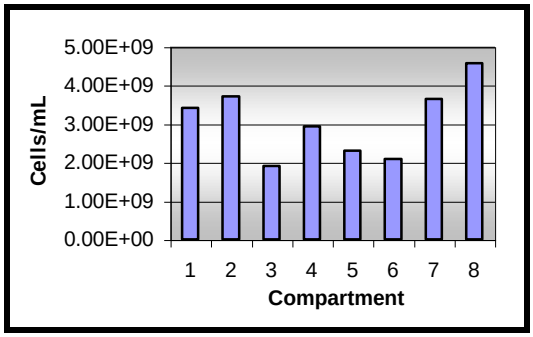

Figure 3.20: Total numbers of Eubacterial cells hybridized by probe EUB338 (Day 101).

Overall bacterial population numbers were determined by EUB338 hybridizations. These numbers showed similarities in Compartments 1 and 2, with a slight decrease in Compartment 3 (Figure 3.20). This decrease is attributed to a decrease in the acidogenic bacteria (Figure 3.23). The increase in Compartment 4 may also be attributed to these bacteria. The decrease of bacteria in Compartments 5 and 6 is a result of a depletion of nutrients as discussed above. The increase in COD, may have been as a result of the production of SMP from dead cells (Aquino and Stuckey, 2002), would have encouraged the growth of the bacteria, which show increases in Compartments 7 and 8.

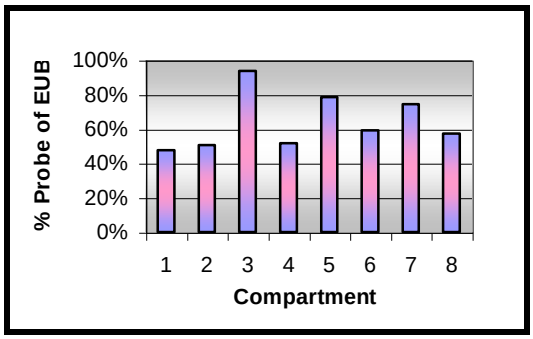

Figure 3.21: Percentage group-specific probes of total Eubacterial count (Day101).

The group specific probes utilised, identified on average 64% of the total bacterial population (Figure 3.21). The highest number of cells identified was in Compartment 3 (94%) and the lowest in the first compartment (48%). This indicates that there are other bacteria present in the reactor that have not been identified. Plumb *et* al. (2001), treating dye wastewaters in an 8 compartment ABR, noted that the sum of the group specific probes was less than 100%, thereby signifying the presence of bacteria that were not detected. These may have included the epsilon subclass of the *Proteobacteria* (Snaidr *et al*., 1997), *Spirochetes* (Fernández *et al.*, 1999) and *Planktomyces.* Godon *et al*., 1997), which have been found in anaerobic digesters. It is possible that EUB numbers were inflated due to autofluorescence of the background material (Amann, 1995) and non-specific binding of the probe (Amann *et al.*, 1995 and Amann, 1990).

No separation of the phases of anaerobic digestion was noted, with the hydrolytic, acidogenic, acetogenic and methanogenic communities being present throughout the reactor. This could be due to the high flow rate, which would have prevented the establishment of a healthy community. Langenhoff and Stuckey (2000) found that no separation of the phases was noted in an 8 chamber ABR, treating low strength synthetic wastewater. Uyanik *et al* (2002b) noted no separation of phases in a 4 compartment ABR, treating synthetic wastewater, as methane production was noted throughout the reactor.

A. The Hydrolytic Bacteria

Certain LGC bacteria also carry out acidogenic reactions. For the purpose of Figures 3.22 and 3.24, it was assumed that each of these bacteria (hydrolytic and acidogenic LGC) comprised of 50% of the total LGC population.

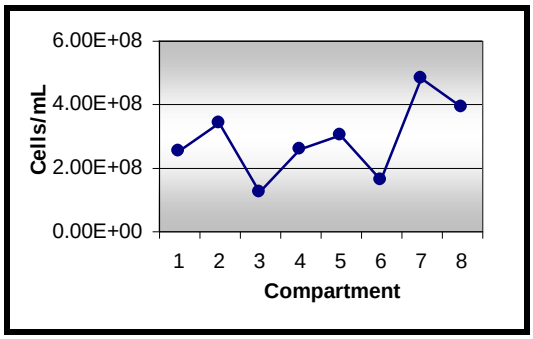

Figure 3.22: Total cell numbers of hydrolytic bacteria (Day 101).

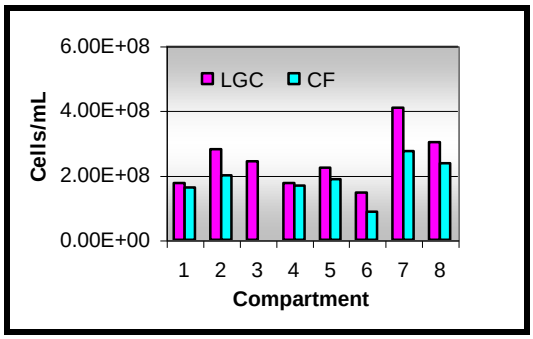

Figure 3.23: Total cell numbers of the hydrolytic Eubacterial cells hybridized by probes LGC354a and CF319a (Day 101).

Increases of the hydrolytic bacteria were noted at the beginning of the reactor (Figure 3.22). This was characterised by an increase in both the LGC and the *Cytophaga-Fermicutes* bacteria. These results correlate with the decrease in COD (Figure A7.3, APPENDIX SEVEN). The increase in numbers may have also been due to the slow removal of toxic compounds, which enter with the influent into the first compartment. It is noted that there is only a slight reduction in COD from Compartments 2 to 3 (Figure A7.3, APPENDIX SEVEN). This can be attributed to the complete disappearance of the *Cytophaga-Fermicutes* (Figure 3.23). This disappearance might have been due to the presence of certain toxins within the reactor. The LGC also decrease slightly, but still carry out hydrolysis. The re-emergence of the *Cytophaga-Fermicutes* in Compartment 4 may

have caused a decrease in the LGC numbers, with the introduction of competition for the limited nutrients, shown by the low COD values (Figure A7.3, APPENDIX SEVEN). The release of intracellular material (SMP) by dead cells would have provided the necessary nutrients needed for these bacteria and therefore, there is an increase in the numbers of hydrolytic bacteria (Figure 3.22). The decrease in numbers in Compartment 6 may have been due to competition between the two groups of bacteria for the nutrients, resulting in the death of certain bacteria. The release of nutrients (SMP) from the lysing cells would have caused an increase in organics in Compartment 6 as is noted by the increased COD (Figure A7.3, APPENDIX SEVEN). This COD is utilised in Compartment 7, where there is an increase in hydrolytic bacterial numbers (Figure 3.22). The decrease in Compartment 8 is again due to a decrease in nutrients.

B. The Fermentative Acidogenic Bacteria

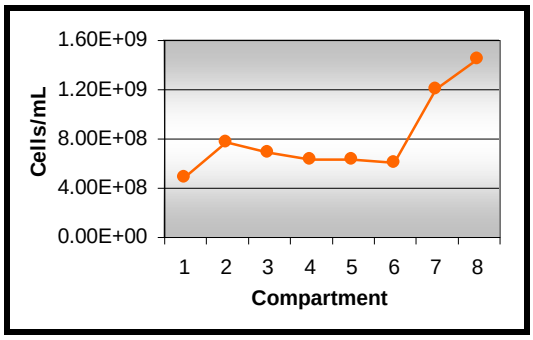

Figure 3.24: Total cell numbers of acidogenic bacteria (Day 101).

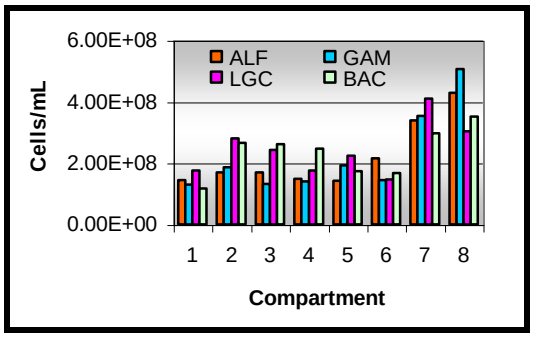

Figure 3.25: Total cell numbers of the hydrolytic Eubacterial cells hybridized by probes ALF1a, GAM1b, LGC354a and BAC303 (Day 101).

The acidogenic bacteria show step-wise increase from Compartments 1 to 2 (Figure 3.24). When viewed in conjunction with the numbers of hydrolytic bacteria it is noted that both follow the same trend. The breakdown of COD by the hydrolytic bacteria yields the substrates required by the acidogenic bacteria. These nutrients, such as, glucose, amino acids and volatile fatty acids, are utilised by the acidogenic bacteria resulting in the formation of acidic end products (Bitton, 1994 and Guiot, *et al*., 1992). The pH is inversely proportional to the acidogenic bacterial growth, and this shows a decrease from Compartments 1 to 2 (Figure A7.4, APPENDIX SEVEN). There is a slight decrease in acidogenic bacterial numbers in Compartment 3 (Figure 3.24). This is owing to a decrease in gamma-Proteobacterial numbers, possibly due to competition from the other acidogens for the limited nutrients, caused by a decrease in hydrolytic bacteria. There is also decrease in LGC numbers but this was attributed to a decrease in the hydrolytic LGC population (mentioned above). The decrease in the acidogen numbers in Compartment 4 is due to a decrease in all the bacterial populations (Figures 3.23 and 3.24). This may be as a result of a low substrate formation by the hydrolytic bacteria, which are affected by the low nutrient availability (as mentioned above). The decrease in acidogens causes an increase in the pH, due to the deficiency in fermentative end products. The rapid increase of bacterial numbers in Compartment 7 and 8

could be due to an improved production of substrates by the hydrolytic bacteria (mentioned above).

It has been noted that acidogenic bacterial numbers were relatively high throughout this campaign period. This is further correlated by the low pH prevalent throughout, indicating the constant production of acids by these bacteria (Figure A7.4, APPENDIX SEVEN). An example of the acidogenic bacteria is depicted in the following FISH image of GAM1b stained cells.

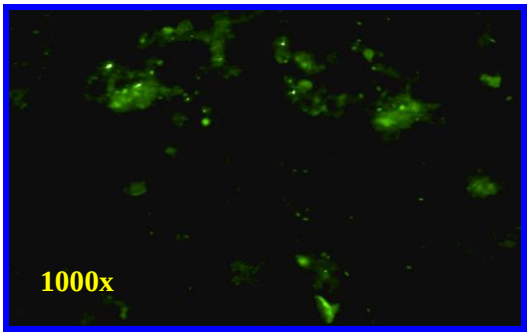

Figure 3.26: FISH image of the gamma-*Proteobacteria* (Day 101)

C. The Acetogenic Bacteria

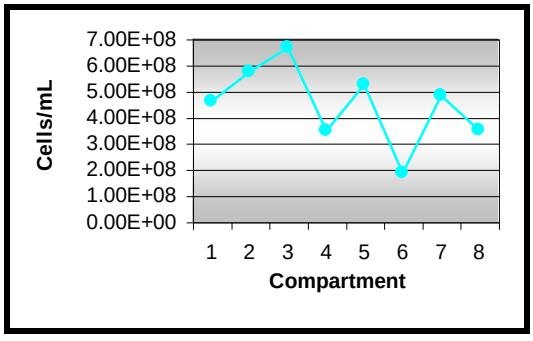

Figure 3.27: Total cell numbers of acetogenic bacteria (Day 101).

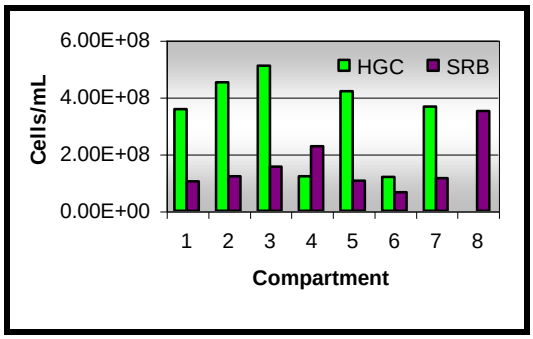

Figure 3.28: Total cell numbers of the acetogenic Eubacterial cells hybridized by probes HGC69a and SRB385 (Day 101).

Bacteria carrying out acetogenic reactions belong to the HGC and SRB groups. They are often in competition with the methanogens for the same electron donors (Raskin *et al*., 1996).

The acetogens show stepwise increase from Compartments 1 to 3, as the first two steps of anaerobic digestion proceed, resulting in the increase of acetate production (Figure 3.27). The SRB numbers are lower than the HGC numbers, possibly due to competition from the methanogens for electron donors (Figure 3.28). There is a decrease in acetogenic numbers in Compartment 4 (Figure 3.27). This decrease is due to a reduction in HGC numbers. This could be due to competition between the two groups of bacteria for the limited substrate as the acidogens, which produce these substrates also decrease in Compartment 4 (as discussed previously). The SRB compete better, since they do not have to compete with the acetoclastic methanogens, which have died off, and therefore increase. The reduction of acetogenic numbers in Compartments 5 and 6 is due to limiting substrate concentrations. It is noted that very little acid is produced as the pH values in these compartments shows an increase (Figure A7.4, APPENDIX SEVEN). The increase of the acidogens in Compartment 7 lead to an increase in substrate production and subsequently, there was an increase in the acetogenic bacteria. The HGC bacteria are not present

in Compartment 8, possibly due to competition with the SRB for the limiting nutrients. Figure 3.29 shows a FISH image of HGC 69a stained cells.

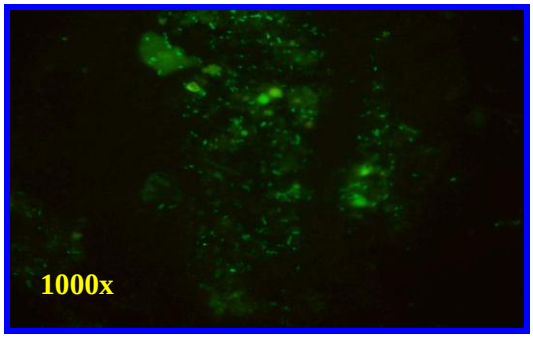

Figure 3.29: FISH image of the HGC (Day 101)

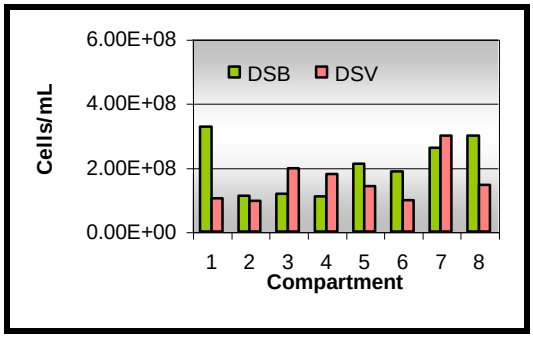

Figure 3.30: Total cell numbers of the sulfate reducing bacteria (Day 101).

Figure 3.30 shows that the DSB is more prevalent than the DSV. The DSV is the acetate producer and follows the trend of the SRB. The DSB show initially higher numbers in Compartment 1, with a sharp decline in numbers in Compartment 2, possibly due to competition with methanogens for electron donors. In Compartments 5 and 6, the SRB numbers decrease (Figure 3.28) but the DSB numbers increase. This could be due to competition between the two SRB, with the DSB proving superior to the DSV, which decrease.

3.3.3.3 Archaeal populations active in the ABR

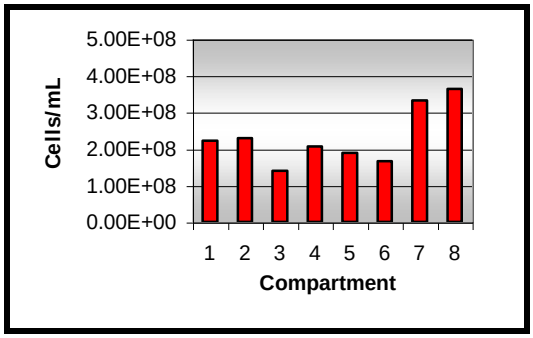

Figure 3.31: Total numbers of Archaeal cells hybridized by probe ARC915 (Day 101).

The probe ARC 915 was applied to determine the contribution of Archaea to the community of the ABR. Hybridizations showed total archaeal populations consisting of an average of 4% of total DAPI stained cells (Figure 3.19). This number may be underestimated, as it is sometimes difficult for probes to permeate Archaeal cell walls. Since the Archaea are slow growing, they would have contained a low cellular rRNA content, which would have been unable to detect with the oligonucleotide probes (Wagner *et al*, 2003 and Amann *et al*., 1990).

The Archaeal numbers remain relatively similar throughout the reactor, with a sharp decline in numbers in Compartment 3 (Figure 3.31). This could be due to a diminished *Methanosarcina* number. The increase in numbers in Compartment 5 and 6 may be due to an increase in numbers of other methanogens, such as *Methanobacterium* and *Methanococcus*, as identified by sequencing (Table 3.4). The decrease in Compartment 6 may be due to a depletion of nutrients (as discussed previously). The increase of numbers in Compartments 7 and 8 could be due to an increased nutrient level, as a result of the lysis of dead cells. This is supplemented by a decrease in COD numbers, which indicates the increased utilisation of substrates (Figure A7.3, APPENDIX SEVEN).

A. The Methanogens

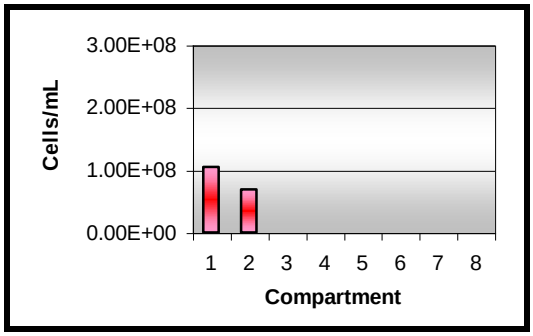

Figure 3.32: Total cell numbers of *Methanosarcina* as determined by probe MS821 (Day 101).

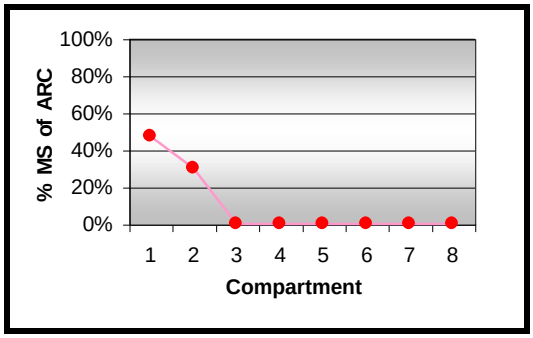

Figure 3.33: Percentage *Methanosarcina* of the total Archaeal population (Day 101).

Of the total Archaeal population only one genus was identified by *in situ* hybridization. This was the genus *Methanosarcina*. DNA sequencing results confirmed the presence of this methanogen as well as the presence of the genus *Methanobacterium* and the genus *Methanococcus* (Table 3.4).

Methanosarcina species were only present in Compartments 1 and 2 (Figure 3.32). It is noted that the pH in Compartment 2 falls below 6.4 (Figure A7.4, APPENDIX SEVEN). This is inhibitory to the acetoclastic methanogens, which generally thrive at a pH between 6.7 and 7.4 (Bitton,

1994). Decreased pH further encourages the growth of acid producing bacteria, which results in a decrease in the methane potential (Barber and Stuckey, 1999). Figure 3.33 reveals *Methanosarcina* comprising less than 50% of the Archaeal population, signifying the presence of other methanogens e.g. *Methanobacterium* and *Methanococcus.*

The other methanogenic probe utilised was MX825. This probe was applied to detect *Methanosaeta* cells, however, no signals were detected. It is noted that scavenging methanogens are limited by low pH (Nachaiyasit and Stuckey, 1997b). *Methanosaeta* cells have a slower growth rate than *Methanosarcina* and are more sensitive to environmental changes like decreased pH and therefore would not have been present in our ABR.

3.3.4 Operating Period: July–September 2003

This operating period was characterised by an average flow rate of 1149L of wastewater per day. However, there were frequent occurrences of biomass washout.

3.3.4.1 The distribution of microorganisms within the ABR

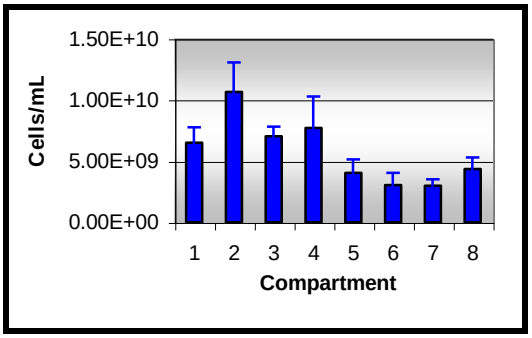

Figure 3.34: Total cell counts (Day 21).

Figure 3.34 shows the total cell counts as determined by the membrane filtration method of Porter and Feig (1980). The cell numbers were high throughout the reactor with the highest being recorded in Compartment 2. This may be due to the diminution of toxic or inhibitory compounds in the buffer zone of Compartment 1, thereby providing ideal conditions for the proliferation of microorganisms (Nachaiyasit and Stuckey, 1997b and Uyanik *et al*., 2002a). The decrease of biomass from Compartments 4 to 7 could be as a result of a consumption of the nutrients available in the influent. The increase in numbers in Compartment 8 may be due to the utilisation of the organic material from lysed cells (SMP), and subsequent growth (Aquino and Stuckey, 2004). It was noted that from Days 18-22, there was a high influent COD (Figure A7.5, APPENDIX SEVEN), which would have provided more nutrients. The effluent values were also high for this period. This was a result of a frequent washout of biomass from the reactor. This

makes it impossible for the biomass in a reactor to degrade a substrate completely (Langenhoff *et al*., 2000)

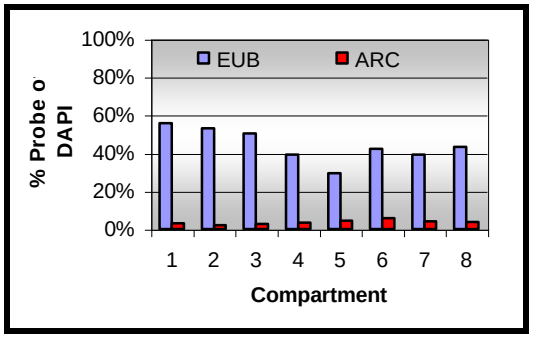

Figure 3.35: Percentage Domain-specific probes of DAPI stained cells (Day 21).

The probes used to identify the active biomass included EUB338 and ARC915. In the vein of the previous operating period, the bacterial portion of the biomass showed dominance over the Archaea (Figure 3.35). Bacterial cells were detected as 44% and Archaea were detected at 4% of the total biomass. This indicates that only 48% of the total DAPI-stained biomass was active. This number may have been underestimated. Slow-growing bacterial and Archaeal cells, which contain a low rRNA content, do not produce bright signals when hybridized and would not have been counted (Schramm and Amann, 1999). DAPI stains all cells, including inactive ones. These cells would have had too low an rRNA content to be detected by the probes (Wagner *et al*., 1993). These results have also been achieved by Díaz *et al* (2003) where less than 10% of their biomass was active prokaryotes that hybridized with Eubacterial or Archaeal specific probes combined. There are other microorganisms besides those probed for that make up an anaerobic community. These include ciliates, fungi and nematodes (Bitton, 1994, Tajima *et al*, 2001 and Amann, 1995).

3.3.4.2 Bacterial populations active in the ABR

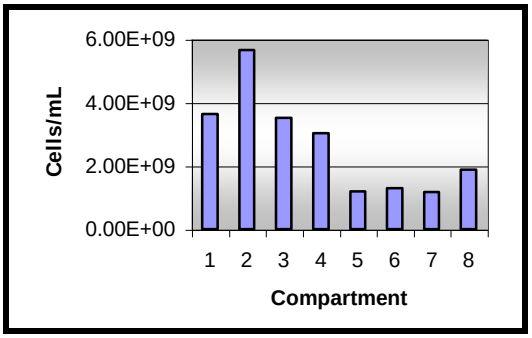

Figure 3.36: Total numbers of Eubacterial cells hybridized by probe EUB338 (Day 21).

Bacterial numbers show increasing values from Compartments 1 to 2 (Figure 3.36). This increase could be as a result of the breakdown of toxins in Compartment 1, which provides optimal conditions for growth in Compartment 2 (Nachaiyasit and Stuckey, 1997b and Uyanik *et al*., 2002a). There is a reduction in bacterial numbers from Compartments 3 to 7, indicating a utilisation of nutrients and a decrease in numbers as the nutrient availability becomes limiting. The increase in Compartment 8 could be due to an escalation of intracellular material released from lysing cells (SMP), which provide the necessary sustenance for the remaining cells. These cells therefore showed improved growth.

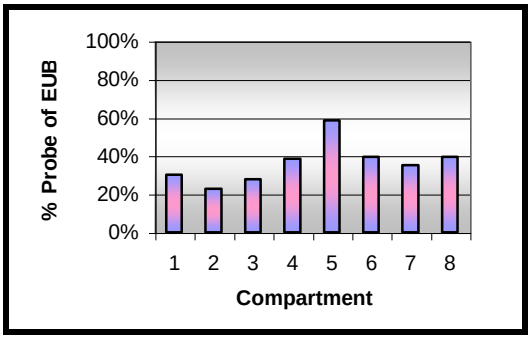

Figure 3.37: Percentage group-specific probes of total Eubacterial count (Day 21).

Of the total bacterial cells identified by EUB338, only 36% were identified by the bacterial probes. The highest number of cells identified was in Compartment 5 (59%) and the lowest in the second compartment (23%). This indicates that there are other bacteria present in the reactor that have not been identified. Plumb *et* al. (2001), treating dye wastewaters in an 8 compartment ABR, noted that the sum of the group specific probes was less than 100%, thereby signifying the presence of bacteria that were not detected. These may have included the epsilon subclass of the *Proteobacteria* (Snaidr *et al*., 1997), *Spirochetes* (Fernández *et al.*, 1999) and *Planktomyces.* Godon *et al*., 1997), which have been found in anaerobic digesters. It is possible that EUB numbers were inflated due to autofluorescence of samples (Amann, 1995) and non-specific binding (Amann *et al.*, 1995 and Amann, 1990).

No separation of the phases of anaerobic digestion was noted, with the same microbial communities being present throughout the reactor. This could be due to frequent occurrences of biomass washout, which would have prevented the establishment of a healthy community. Langenhoff and Stuckey (2000) found that no separation of the phases was noted in an 8 chamber ABR, treating low strength synthetic wastewater. Uyanik *et al* (2002b) noted no separation of phases in a 4 compartment ABR, treating synthetic wastewater, as methane production was noted throughout the reactor.

A. The Hydrolytic Bacteria

Certain LGC bacteria also carry out acidogenic reactions. For the purpose of Figures 3.38 and 3.40, it was assumed that each of these bacteria (hydrolytic and acidogenic LGC) comprised of 50% of the total LGC population.

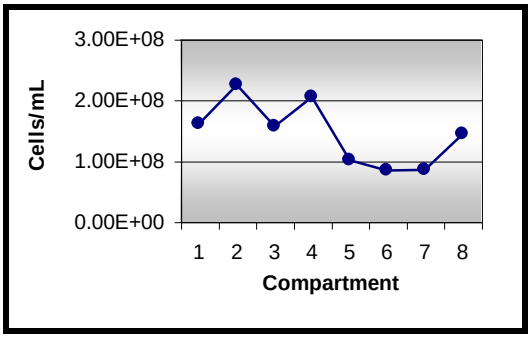

Figure 3.38: Total cell numbers of hydrolytic bacteria (Day 21).

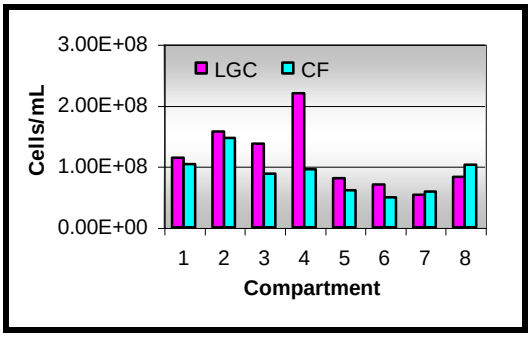

Figure 3.39: Total cell numbers of the hydrolytic Eubacterial cells hybridized by probes LGC354a and CF319a (Day 21).

The hydrolytic bacteria showed increases from Compartments 1 to 2 (Figure 3.38). This increase could be due to a reduction in inhibitory compounds, which are present in the influent. The decrease in acidogen numbers in Compartment 3 could be due to competition between the two groups of bacteria for the same substrates. There is a large increase in the LGC in Compartment 4

(Figure 3.39). This increase may be partially due to an increase in the acidogenic LGC (to be discussed). The decrease in cell numbers from Compartments 5 to 7 (Figure 3.38) may be as a result of a depletion of nutrients. This depletion results in the death of cells. The death of cells is characterised by lysis and the release of SMP (Aquino and Stuckey, 2004). This material would have provided the necessary substrates required by the bacteria for hydrolysis and therefore, their numbers increase in Compartment 8.

B. The Fermentative Acidogenic Bacteria

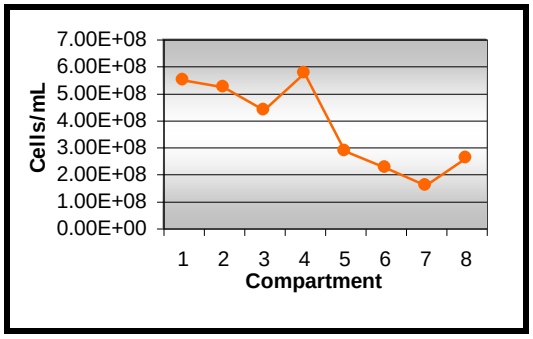

Figure 3.40: Total cell numbers of acidogenic bacteria (Day 21).

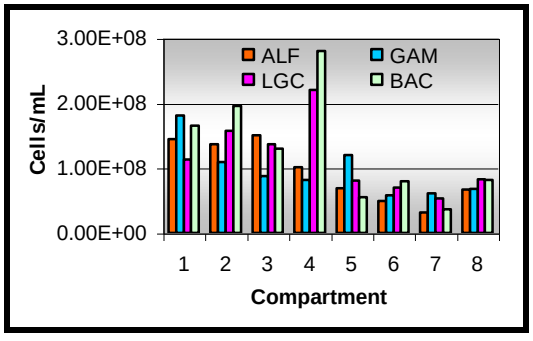

Figure 3.41: Total cell numbers of the hydrolytic Eubacterial cells hybridized by probes ALF1a, GAM1b, LGC354a and BAC303 (Day 101).

The total acidogenic bacterial numbers show a decrease from Compartments 1 to 2 (Figure 3.40). It is possible that these bacteria were inhibited by the presence of inhibitory substances, which enter in the influent. The decrease in numbers in Compartment 3 is due to competition between the groups of bacteria for the limited substrate. There is an increase in numbers in Compartment 4. This increase can be attributed to an increase in the LGC and *Bacteriodes* groups (Figure 3.41). These groups probably compete better for the nutrients and therefore show higher numbers. The other groups decrease as a result of the limited nutrient availability. All the bacterial groups show decreases from Compartments 5 to 7. This corresponds with the death of hydrolytic bacteria, thereby inducing a reduction in the substrates needed by the acidogens. These bacteria die as a result of limiting nutrients. There is an increase in numbers in Compartment 8 and again this coincides with the increase of hydrolytic bacteria, thereby increased substrate is formed for the growth of the acidogens.

C. The Acetogenic Bacteria

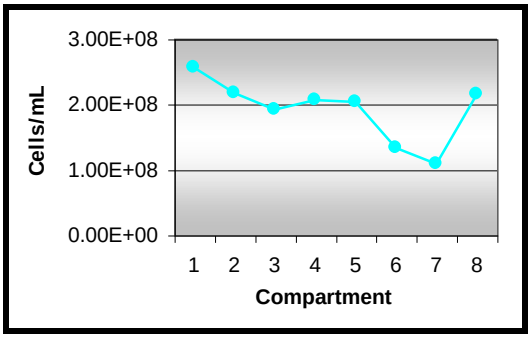

Figure 3.42: Total cell numbers of acetogenic bacteria (Day 21).

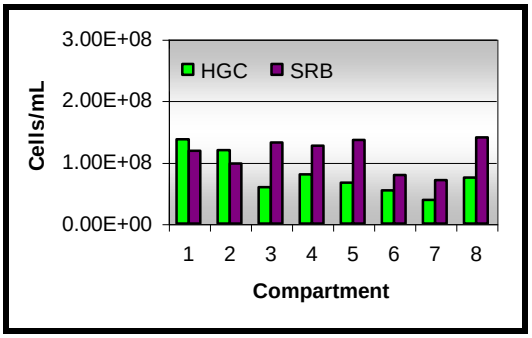

Figure 3.43: Total cell numbers of the acetogenic Eubacterial cells hybridized by probes HGC69a and SRB385 (Day 21).

The total acetogen numbers decrease from Compartments 1 to 2 (Figure 3.42). This could be due to the decrease in the acidogenic bacterial numbers, which would have lead to a decreased formation of acidic substrates, such as propionate and the VFAs. These substrates are required by the acetogens to survive and since this was limiting, they decreased (Gray 1989). In Compartment 3, it is noted that there is an increase in the SRB numbers, but a decrease in the HGC numbers (Figure 3.43). This is due to competition for substrates by the two groups, with the SRB proving dominant. There is a slight increase in numbers in Compartment 4. This concurs with the increase in acidogenic bacteria (as discussed previously), thereby increasing the provision of the necessary substrates required by the acetogens. The decrease in numbers from Compartments 5 to 7 could be a result of a depletion of nutrients caused by a decrease in the acidogenic bacterial numbers. The increase in Compartment 8 is a result of an increasing substrate formation by the acidogenic bacterial numbers.

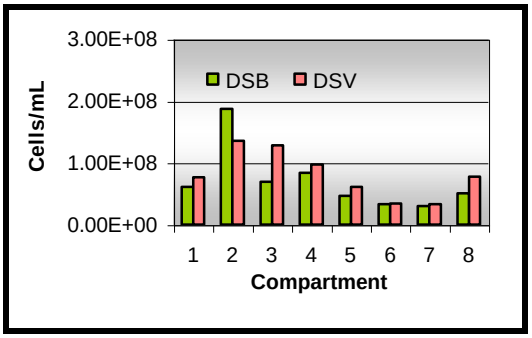

Figure 3.44: Total cell numbers of the sulfate reducing bacteria (Day 21).

The DSV and DSB probes show the same trends as the SRB probe increasing and decreasing accordingly, except in Compartment 2 (Figure 3.44). SRB numbers show a decrease, while the probes show an increase. This may be attributed to a decrease in bacteria other than the SRB, that are detected by the SRB385 probe. The decrease in numbers from Compartments 4-7 concurs with the depletion of nutrients and once nutrients become available, these numbers increase (Compartment 8).

3.3.4.3 Archaeal populations active in the ABR

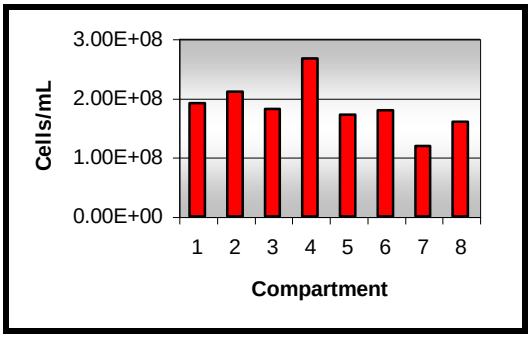

Figure 3.45: Total numbers of Archaeal cells hybridized by probe ARC915 (Day 21).

The probe ARC 915 was applied to determine the contribution of Archaea to the community of the ABR. Hybridizations showed total archaeal populations consisting of 4% of total DAPI stained cells (Figure 3.35).

Archaeal numbers remained similar throughout the reactor (Figure 3.45). Compartments 1 to 2 show a slight increase in numbers, possibly due to a depletion of toxic compounds, creating conditions favourable for growth. The decrease of Archaea from Compartments 4 to 7 could be as a result of a depletion of the nutrients available in the influent. The increase in numbers in Compartment 8 is due to the utilisation of SMP from lysed cells, and subsequent growth.

No *Methanosarcina* or *Methanosaeta* cells were detected. This signifies that there were other methanogens present in the reactor, for example *Methanobacterium, Methanococcus* and *Methanobrevibacter*, which is known to proliferate at the beginning of the reactor (Uyanik *et al*., 2002a). Frequent biomass washout occurrences (such as that experienced during this operating period) leads to the washout of filaments (Alves *et al*., 2000). The high washout rate also disrupts the slow-growing bacteria (like the Archaea), providing them with too short a retention time to establish. An absence of acetoclastic methanogens leads to a low pH, thereby inhibiting them further (Barber and Stuckey, 1999).

Methanogenic bacteria are slow growers and, therefore, require long recovery times following start-up and perturbations within a system (Raskin *et al*., 1994a). It is possible that this is why they were absent during the restart study. *Methanobacterium* sp. tend to recover quickly from stresses in a system. It is possible that is why they were able to grow quickly in the ABR. Angenent *et al* (2002) noted that *Methanobacteriaceae* showed increased numbers after feeding resumed following starvation in a 5 compartment anaerobic migrating blanket reactor fed on sucrose, nutrients, bicarbonate, yeast extract and trace elements.

3.3.5 Operating Period: November-December 2003

This operating period was characterised by an average flow rate of 1573L of wastewater per day. However, there were frequent occurrences of biomass washout.

3.3.5.1 The distribution of microorganisms within the ABR

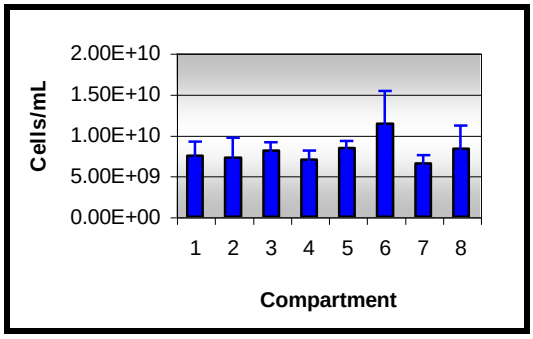

Figure 3.46: Total cell counts (Day 16).

Figure 3.46 shows the total cell counts for the third operating period. Total cell numbers remained between 10^9 and 10^{10} cells/mL. There is an increase in the total cell counts from Compartments 1 to 3. The first compartment serves as a buffering zone, where inhibitory substances are neutralised (Nachaiyasit and Stuckey, 1997b and Uyanik *et al*., 2002a). Therefore, these substances entering with the influent would have been neutralised in the first compartment, thus enabling proliferation of the microbial species. The decrease in Compartment 4 may be as a result of a depletion of nutrients, causing death of the microbial flora. The increase in Compartment 5 may be as a result of organisms other than bacteria and Archaea, as both show low numbers in this compartment (Figures 3.48 and 3.57). Other organisms present may be fungi or ciliates (Tajima *et al*, 2001). Compartment 6 shows a large increase. This is due to an increase in

Archaea. The decrease in Compartment 7 is attributed to a decrease in the archaeal numbers, possibly as a result of competition for nutrients. Compartment 8 shows an increase that can be attributed to fungi and ciliates, as well as to a small degree, Archaea, as bacterial numbers are decreasing. This decrease may be due to predation by the protozoa.

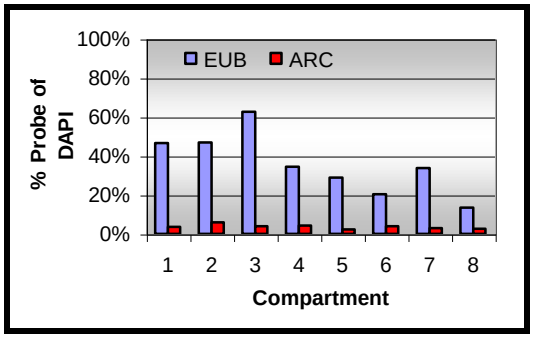

Figure 3.47: Percentage Domain-specific probes of DAPI stained cells (Day 16).

EUB338 and ARC915 probes used to detect the active portion of the biomass revealed bacterial dominance over Archaea (Figure 3.47). Archaeal percentages remained relatively constant throughout at an average of 4% of the total DAPI population. Bacterial percentages were lower during this operating period, detected at an average of 36% of the total DAPI population. Therefore only 40% of the biomass was characterised. It is possible that these cells were acclimatising to the higher COD concentrations than the previous operating periods and were therefore, growing slowly. Slow growing cells contain low amounts of cellular rRNA and therefore, cannot be accurately determined (Wagner *et al*., 2003). Possibly this reason could be why low numbers were detected. These results have also been achieved by Díaz *et al* (2003) where less than 10% of their biomass was active prokaryotes that hybridized with Eubacterial or Archaeal specific probes combined. There are other microorganisms besides those probed for that

make up an anaerobic community. These include ciliates, fungi and nematodes (Bitton, 1994, Tajima *et al*, 2001 and Amann, 1995).

3.3.5.2 Bacterial populations active in the ABR

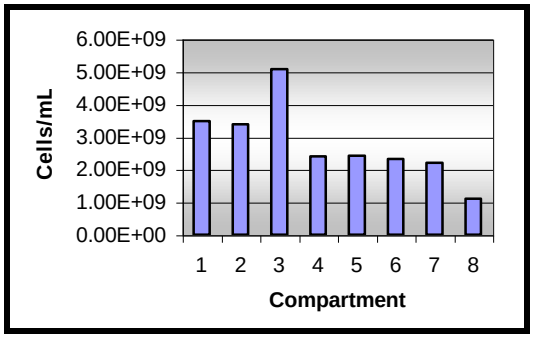

Figure 3.48: Total numbers of Eubacterial cells hybridized by probe EUB338 (Day 16).

Eubacterial numbers determined by hybridization with probe EUB338 showed increasing values from Compartments 1 to 3 (Figure 3.48). This increase can be attributed to an increased nutrient availability as anaerobic digestion progresses. The buffer zone (Compartment 1) would have neutralised the majority of the inhibitory substances present in the influent and this would have lead to a proliferation of the bacterial community (Nachaiyasit and Stuckey, 1997b and Uyanik *et al*., 2002a). Compartments 4 to 8 depict decreasing numbers, possibly due to a depletion of nutrients.

No separation of the phases of anaerobic digestion was noted, with the same microbial communities being present throughout the reactor. This could be due to frequent occurrences of biomass washout, which would have prevented the establishment of a healthy community. Langenhoff and Stuckey (2000) found that no separation of the phases was noted in an 8 chamber

ABR, treating low strength synthetic wastewater. Uyanik *et al* (2002b) noted no separation of phases in a 4 compartment ABR, treating synthetic wastewater, as methane production was noted throughout the reactor.

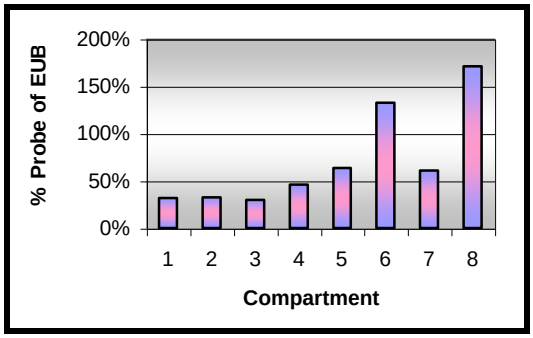

Figure 3.49: Percentage group-specific probes of total Eubacterial count (Day 16).

Group specific probes applied during this operating period identified on average 44% of the total Eubacterial population (Figure 3.49. This suggests the presence of bacteria that have not been accounted for by the probes. These may have included the epsilon subclass of the *Proteobacteria* (Snaidr *et al*., 1997), *Spirochetes* (Fernández *et al.*, 1999) and *Planktomyces.* Godon *et al*., 1997), which have been found in anaerobic digesters. Compartments 6 and 8 show overestimations of the group-specific probes. This could be as a result of non-specific binding and autofluorescence of the background material (Amann *et al*., 1995). Snaidr *et al* (1997) attributed this to a possible under-estimation of DAPI-stained cells.

A. The Hydrolytic Bacteria

Certain LGC bacteria also carry out acidogenic reactions. For the purpose of Figures 3.50 and 3.52, it was assumed that each of these bacteria (hydrolytic and acidogenic LGC) comprised of 50% of the total LGC population.

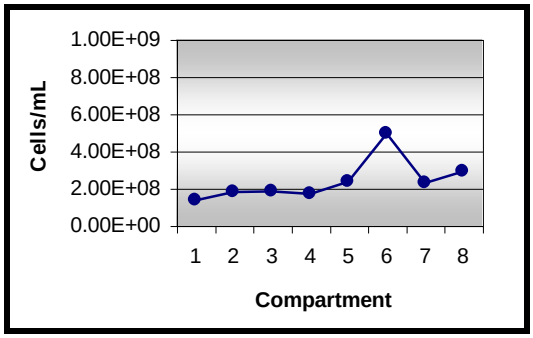

Figure 3.50: Total cell numbers of hydrolytic bacteria (Day 16).

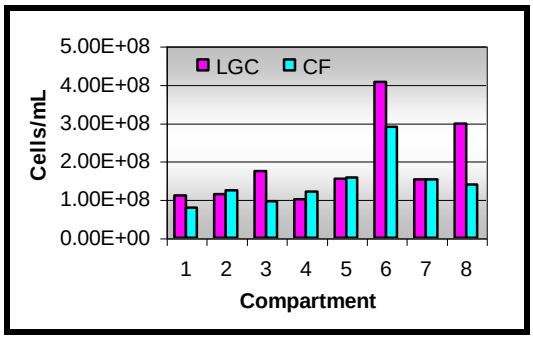

Figure 3.51: Total cell numbers of the hydrolytic Eubacterial cells hybridized by probes LGC354a and CF319a (Day 16).

Figure 3.50 shows low numbers of hydrolytic bacteria from Compartments 1 to 5. Compartments 1to 3 show slight increases, possibly due to neutralisation of inhibitory substances. It is noted that there is a slight decrease in numbers in Compartment 4. When viewing Figure 3.51, it can be seen that although the LGC decrease, the *Cytophaga-Fermicutes* increase. This could be due to

competition between the two groups for substrates. There is a rapid increase in the hydrolytic bacterial numbers from Compartments 5 to 6. This indicates an increased hydrolysis reaction, whereby the COD is degraded into simpler monomeric compounds (Bitton, 1994). It is possible that the numbers in Compartment 6 were overestimated, due to autofluorescence of the background material. The decrease in numbers in Compartment 7 indicates a depletion of nutrients in the reactor, thus leading to death of the bacteria. In Compartment 8, it is noticed that there is a slight increase in hydrolytic bacterial numbers (Figure 3.50). When viewing Figure 3.51, it is seen that the *Cytophaga-Fermicutes* decrease while the LGC increase. It is possible that this increase in LGC numbers is due to an increase in the acidogenic component and not the hydrolytic. It was noted that the hydrolytic bacteria were present at low concentrations throughout this operating period. This therefore signifies that there would not have been sufficient production of substrates for the other bacteria in the anaerobic digestion process. This may be due to a higher COD concentration (Figure A7.6, APPENDIX SEVEN) than that of the previous operating periods, therefore, requiring these bacteria to first acclimatise to these new conditions, before they proliferated.

B. The Fermentative Acidogenic Bacteria

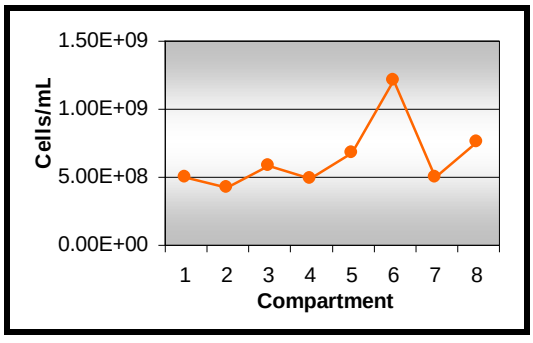

Figure 3.52: Total cell numbers of acidogenic bacteria (Day 16).

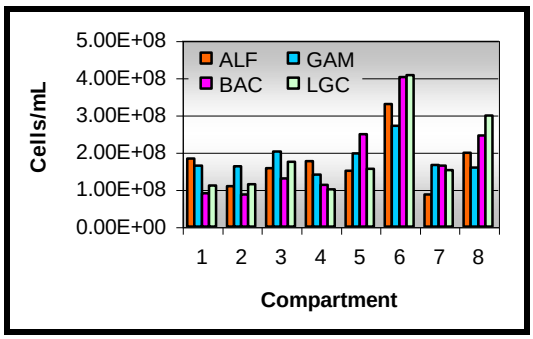

Figure 3.53: Total cell numbers of the hydrolytic Eubacterial cells hybridized by probes ALF1a, GAM1b, LGC354a and BAC303 (Day 16).

The acidogens show decreasing numbers from Compartment 1 to 2 (Figure 3.52). It is possible that inhibitory substances present in Compartment 1 resulted in the decrease of these bacterial numbers (Nachaiyasit and Stuckey, 1997b and Uyanik *et al*., 2002a). The low numbers of the hydrolytic bacteria indicates a low production of the substrates required by the acidogens, thus limiting their growth. The decrease in Compartment 4 of the acidogens is as a result of a decrease in the α-*Proteobacteria* (Figure 3.53). The other bacterial numbers remained constant. This indicates a competition for the limited nutrients (substrate from the hydrolytic bacteria) between the species. The increase in numbers in Compartments 5 to 6 may be as a result of the increased production of substrates from the hydrolytic bacteria, which also show a large increase. The decrease in numbers in Compartment 7 may be due to inhibition by feedback inhibition. Bitton (1994) and McInerney (1999) noted that anaerobic digestion may at any time be inhibited by the formation of several of its intermediates, for example, H_2 and VFAs. The increase in numbers in Compartment 8, indicating that levels of the inhibitory end products were at a lower concentration.

C. The Acetogenic Bacteria

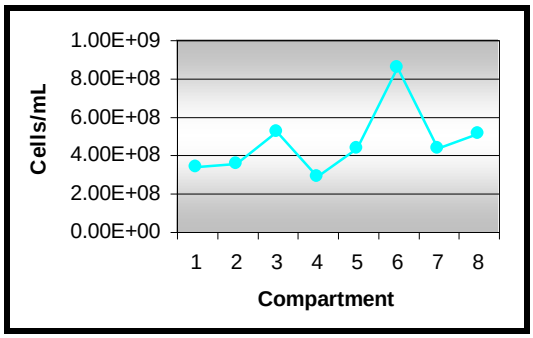

Figure 3.54: Total cell numbers of acetogenic bacteria (Day 16).

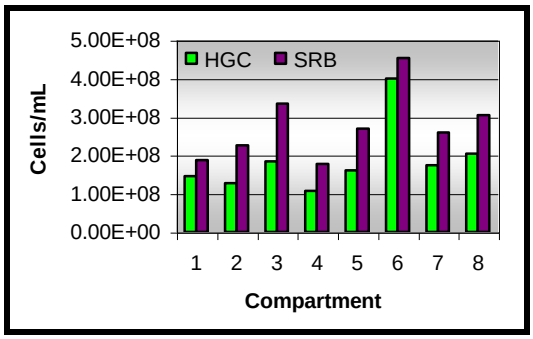

Figure 3.55: Total cell numbers of the acetogenic Eubacterial cells hybridized by probes HGC69a and SRB385 (Day 16).

The acetogenic bacteria (Figure 3.54) show similar numbers from Compartment 1 to 2, with a slight increase in Compartment 3, possibly due to the increased production of substrates from the acidogens. It is noted in Figure 3.55 that there is a decrease in the HGC numbers but an increase in the SRB. This could be due to competition for substrates from the acidogenic reactions, which were limited as the acidogen numbers were low. The large increase of the SRB in Compartment 3 can be attributed to disappearance of the acetoclastic methanogens in Compartment 3 (Figure 3.58), thus eliminating the competition for nutrients. The decrease of the acetogens in Compartment 4 (Figure 3.54) is as a result of a decreased substrate formation by the acidogens,

which also decreased (Figure 3.52). Thereafter, acetogenic numbers showed correlations with the acidogenic bacterial numbers, increasing and decreasing accordingly. This illustrates the dependence of the acetogens to the formation of organic acids by the acidogens.

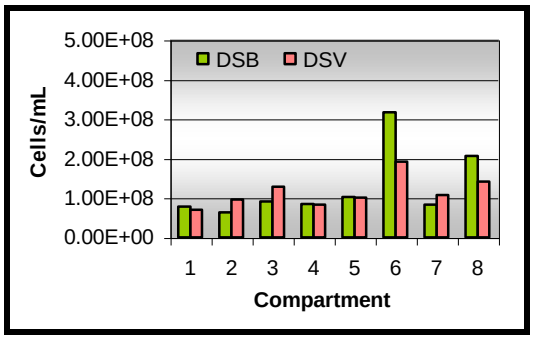

Figure 3.56: Total cell numbers of the sulfate reducing bacteria (Day 16).

The DSB and DSV probes follow the trends of the SRB probe, increasing and decreasing accordingly, except in Compartment 2, where DSB decreases. This may be due to competition with DSV for the limited nutrients as well as the methanogens for electron donors.

3.3.5.3 Archaeal populations active in the ABR

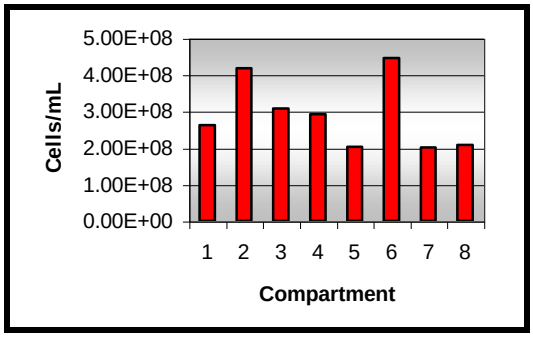

Figure 3.57: Total numbers of Archaeal cells hybridized by probe ARC915 (Day 16).

The probe ARC 915 was applied to determine the contribution of Archaea to the community of the ABR. Hybridizations showed total archaeal populations consisting of 4% of total DAPI stained cells (Figure 3.47). This number may be underestimated, as it is sometimes difficult for probes to permeate Archaeal cell walls (Wagner *et al*., 2003).

Archaeal numbers remained relatively similar throughout the reactor (Figure 3.57). Compartments 1 to 2 show a slight increase in numbers, possibly due to a depletion of toxic compounds, creating conditions favourable for growth. This increase is attributed to the large increase of *Methanosarcina* cells. The decrease of Archaea from Compartments 3 is as a result of a disappearance of the *Methanosarcina*. The decrease of Archaea from Compartments 4 to 5 could be as a result of a depletion of the nutrients available in the influent and a subsequent decrease in the hydrogenotrophic methanogens. The increase in numbers in Compartment 6 could be due to the utilisation of the organic material from lysed cells, and subsequent growth.

A. <u>The Methanogens</u>

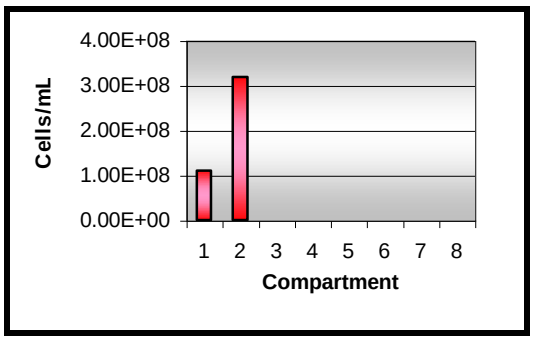

Figure 3.58: Total cell numbers of *Methanosarcina* as determined by probe MS821 (Day 16).

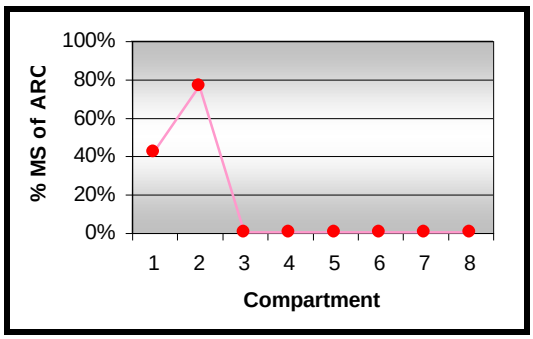

Figure 3.59: Percentage *Methanosarcina* of the total Archaeal population (Day 16).

Methanosarcina cells were detected solely in the first two compartments, with a large increase from Compartments 1 to 2, accounting for 42% and 76% of the total archaeal population respectively (Figure 3.59). The disappearance of the acetoclastic methanogens in Compartment 3 may have been as a result of competition from the SRB for electron donors (Figure 3.58) (Araujo *et al*., 2000). Another rationalisation may be that as there was an increase in the acidogens in this compartment, it would have resulted in a higher production of acidogenic end-products, which would have inhibited the sensitive methanogens (Bitton, 1994). An absence of acetoclastic methanogens leads to a low pH, thereby inhibiting them further (Barber and Stuckey, 1999). The frequent biomass washout occurrences also disrupted the slow-growing bacteria (like the Archaea), providing them with too short a retention time to establish.

3.3.6 Comparison Of The Three Operating Periods

3.3.6.1 The distribution of microorganisms within the ABR

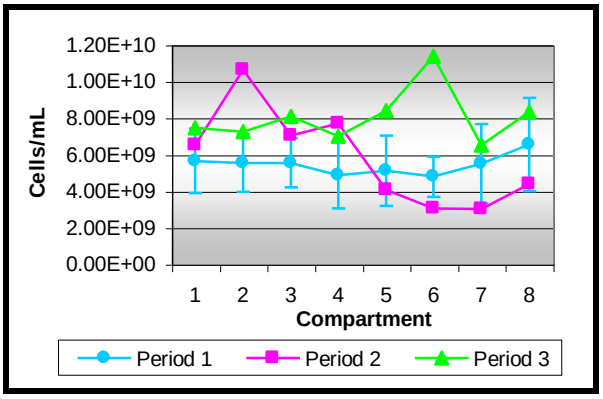

Figure 3.60: Total cell counts for each operating period.

During the first period, total cell counts showed uniformity throughout the reactor, with the only an increase occurring at the end (Figure 3.60). This may have been as a result of an increased nutrient availability from lysed cells (Compartment 6) (SMP), therefore supporting the proliferation of the microbial population.

The second operating period differs in that there is a large increase in total cell numbers from Compartments 1 to 2 (Figure 3.60). This may be due to the diminution of toxic or inhibitory compounds in the buffer zone of Compartment 1, thereby providing ideal conditions for the proliferation of microorganisms in Compartment 2 (Nachaiyasit and Stuckey, 1997b and Uyanik *et al*., 2002a). The second operating period shows the lowest numbers from Compartments 5 to 8, for all the operating periods, possibly as a result of a depletion of nutrients.

Total cell counts showed highest numbers for Operating Period 3 (Figure 3.60), with the highest being present in Compartment 6. It was noted that during this period, influent COD concentrations were higher than either of the previous operating periods. This would have provided a higher nutrient availability for the microbial population (Figure A7.6, APPENDIX 7).

3.3.6.2 Bacterial populations active in the ABR

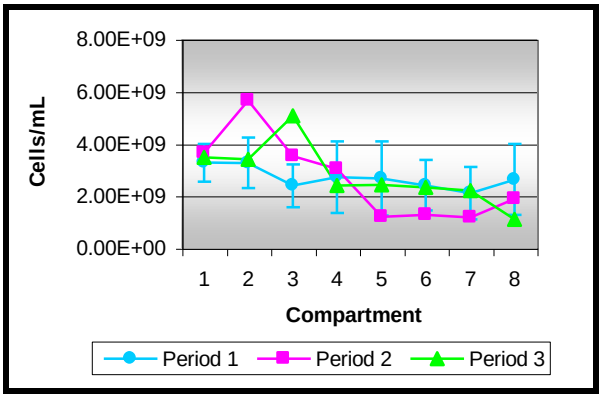

Figure 3.61: Total numbers of Eubacteria for each operating period.

Bacterial numbers depicted in Figure 3.61 shows similar numbers in Compartment 1 for all three operating periods. The first operating period depicts stable numbers throughout the reactor. When viewing flow rate analyses it was noted that the flow rate was higher than expected for this operating period. A high flow rate makes it impossible for the biomass in a reactor to degrade a substrate completely (Langenhoff *et al*., 2000). It also prevents establishment of a healthy community as it being continually mixed.

Operating Period 2 (Figure 3.61) shows the quickest increase in bacterial numbers, which occurs in Compartment 2. This indicates a neutralisation of inhibitory substances quicker than the other operating periods, possibly because these substances were present in much lower concentrations. . Substances inhibitory to anaerobic digestion that are found in domestic effluents may include ammonia from faeces and urine and chlorides from urea (Koppe *et al*., 1999, Bitton, 1994 and Leach, 1994).

Both Operating Periods 2 and 3 (Figure 3.61) show lower numbers at the end of the reactor from Compartment 4 onwards. This indicates a depletion of nutrients in these compartments. It is also possible that a community was unable to establish itself due to frequent biomass washout occurrences that characterised these operating periods.

No separation of the phases of anaerobic digestion was noted, with the same microbial communities being present throughout the reactor for all three operating periods. This could be due to frequent occurrences of biomass washout, which would have prevented the establishment of a healthy community. This was also noted by Langenhoff and Stuckey (2000) and Uyanik *et al* (2002b) in an 8 chamber ABR, treating low strength synthetic wastewater and in a 4 compartment ABR, treating synthetic wastewater, respectively.

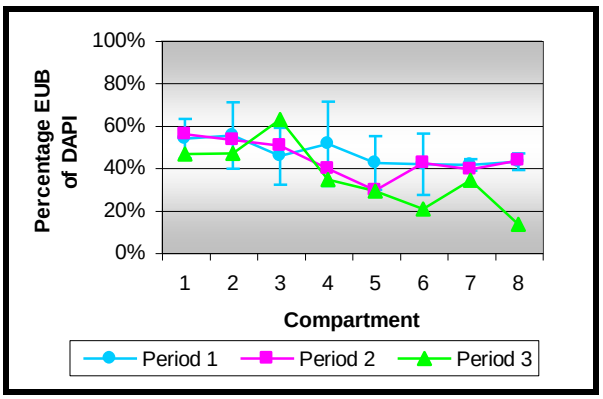

Figure 3.62: Percentage Eubacteria of DAPI for each operating period.

Figure 3.62 depicts the percentage of bacteria of the total DAPI population. Operating Period 1 showed uniformity with bacteria consisting of an average of 47% of the total DAPI stained cells. Operating Period 2 showed decreased bacterial percentages of an average of 44%. The final operating period showed the lowest average percentage of 36%, with decreasing percentages in the latter compartments. The steady decrease in percentages from Operating Period 1 to 3 may have been as a result of the high biomass washout rate, which prevented the establishment of the bacterial community.

It was noted that there was a high diversity of bacteria throughout the operating periods, which followed an unconventional pattern of growth, with no specific trends noted. Fernández *et al* (1999) found, in a functionally stable, glucose-fed methanogenic reactor, that bacterial community structure was highly dynamic; following a chaotic function, suggesting that functional stability did not mean community stability. Zumstein *et al* (2000) (as cited in Kaewpipat and Grady, 2001) noted, in an anaerobic digester that was run for 2 years at constant environmental parameters, that there were rapid shifts in the species composition of the bacterial community and that evolution of this community may have been due to inherent parameters such as phages and predation. This could also explain why the bacterial numbers in the ABR was lower than

expected (Figure 3.61). Bacterial numbers seem to be greatly influenced by the eukaryotic members of the consortium, such as protozoa, which feed on the bacteria. It is possible that a large portion of the DAPI-stained cells may have been protozoa. Since these microorganisms ingest bacteria and therefore, could have reduced the bacterial numbers. Protozoan associations with two Archaeal orders, *Methanomicrobiales* and *Methanobacteriales* have been noted. Anaerobic ciliates have also been noted to form associations with the delta-*Proteobacteria* (Amann *et al*., 1995). These eukaryotic cells would have been stained by DAPI but not detected by the probes. The low EUB/DAPI ratios may have been influenced by the slow growth of cells. These cells would have contained low rRNA thus limiting the target for the oligonucleotide probes (Amann *et al.,* 1990). Delong *et al* (1989) noted that individual *Escherichia coli* cells (growth rate of ± $0.3h^{-1}$) expressed fluorescence intensities only 5 times greater than autofluorescence or non-specific binding. Since anaerobic communities are known to be slow-growing, the low rRNA concentrations would have lead to low fluorescence intensities, which would not have been accurately detected. Díaz *et al.* (2003) noted that in granules from separate batch reactors containing different substrates (formate, acetate, propionate, sucrose, starch and peptone) less than 10% of the biomass was active prokaryotes that hybridized with Eubacterial or Archaeal specific probes combined. It was noted that most of the microorganisms corresponded to resting forms. This was attributed to low methanogenic activity.

Group-specific probes applied in this process, although identifying a number of bacteria, were still less than 100% of the total Eubacterial count. Plumb *et* al. (2001), treating dye wastewaters in an 8 compartment ABR, noted that the sum of the group specific probes was less than 100%, thereby signifying the presence of bacteria that were not detected. Other bacteria present in the ABR may have included the epsilon subclass of the *Proteobacteria*, *Spirochetes* and *Planktomyces*, which have been found in anaerobic digesters. The epsilon subclass of the *Proteobacteria* was not probed for. This may have been present in the reactor, for example

Arcobacter. Aerotolerant members of this group have been noted in activated sludge reactors. *Arcobacter*, which is found in humans with enteritis or diarrhoea, may have entered the system with the influent, which is 100% domestic (Snaidr *et al*., 1997). The presence of influent bacteria has been noted by Curtis and Craine (1998). Godon *et al*. (1997), when treating vinases in an anaerobic digester noted the presence of *Spirochetes* and *Planktomyces*. *Spirochetes* were also detected by Fernández *et al* (1999), when investigating a methanogenic reactor fed on glucose for 605 days. These bacteria may have been present in the ABR, and contributed to the EUB numbers. It is suggested that probes for these bacteria be utilised in further studies pertaining to the ABR.

A. The Hydrolytic Bacteria

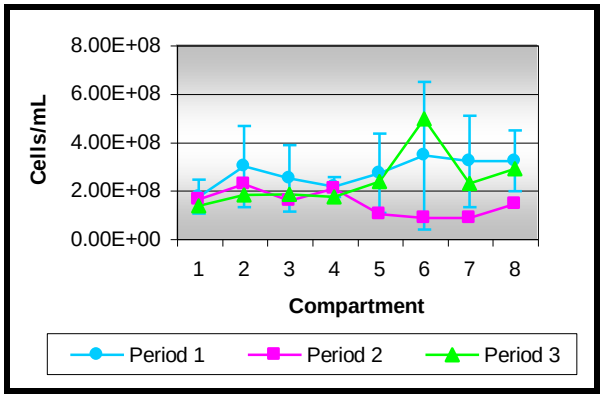

Figure 3.63: Total numbers of hydrolytic bacteria for each operating period.

It was noted that the hydrolytic bacteria were present throughout the reactor for all three operating periods (Figure 3.63). This serves as an indication of the presence of their required substrates throughout the compartments. It was expected that the ABR would facilitate the separation of the phases of anaerobic digestion (Uyanik *et al*, 2002a). However, since the hydrolytic bacteria were

present throughout the reactor, it indicates that hydrolysis occurred slowly, possibly being the main limitation of the anaerobic digestion process. It has been noted that hydrolysis is often the limiting process of anaerobic digestion (Bitton, 1994).

The highest numbers of hydrolytic bacteria were recorded for the first operating period. The other two operating periods were characterised by frequent biomass washouts, thus preventing the establishment of a stable community. This could signify that conditions of the first operating period were relatively more conducive to supporting bacterial growth than when compared to the second and third operating periods.

B. The Fermentative Acidogenic Bacteria

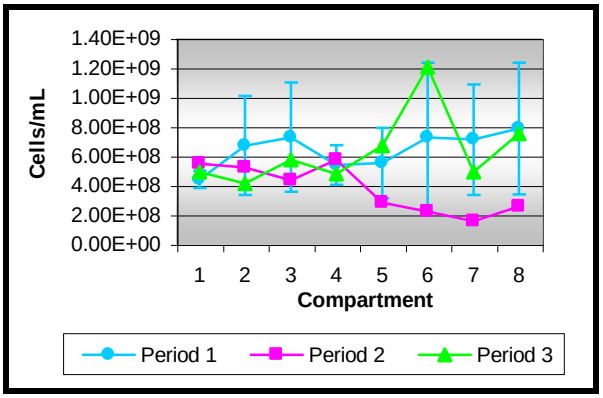

Figure 3.64: Total numbers of acidogenic bacteria for each operating period.

The acidogenic bacteria were present throughout the compartments for all operating periods (Figure 3.64). This group of bacteria displayed the highest numbers comparatively. Many of these bacteria are known to possess extra-cellular enzymes, which can degrade organic polymers to

produce monomers and finally organic acids (Bitton, 1994 and Prescott *et al*., 1999). The presence of these bacteria correlated with that of the low pH noted for the three operating periods. The first operating period shows the highest average acidogens (Figure 3.64). The other two periods were characterised by frequent biomass washout occurrences, which would have prevented the establishment of this community. Inhibitory substances may have been present and the low numbers of hydrolytic bacteria in Operating Periods 2 and 3, would have limited the growth of the acidogens.

C. The Acetogenic Bacteria

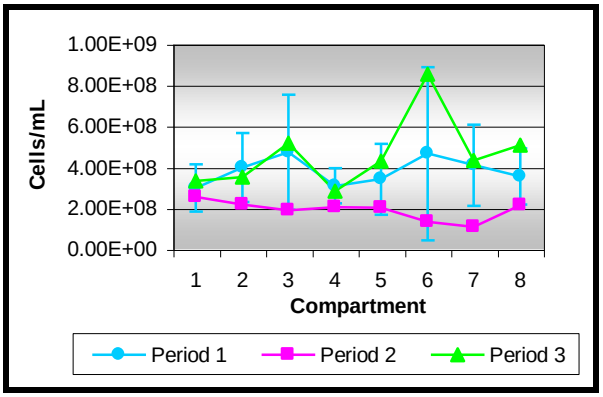

Figure 3.65: Total numbers of acetogenic bacteria for each operating period.

The acetogenic bacteria were present throughout the reactor, with higher numbers at the end of the reactor for Operating Periods 1 and 3 (Figure 3.65). This is probably due to an increased production of acetate by the acidogenic bacteria. Operating Period 2 shows the lowest numbers, possibly due to feed back inhibition of the acetogens by their end products, as no acetoclastic methanogens were present to utilise the acetate produced. Bitton (1994) and McInerney (1999)

noted that anaerobic digestion might at any time be inhibited by the formation of several of its intermediates, for example, H_2 and VFAs.

There appeared to be a large concentration of SRB present, although sulfate concentrations were low. It has been noted in literature that the SRB grow syntrophically on lactate, ethanol, propionate, fumarate and that syntrophy with formate utilising methanogens, eradicates the need to reduce sulfate. In this way, the SRB can grow as the proton-inducing acetogenic bacteria. This phenomenon is characteristic of *Desulfovibrio* and *Desulfobacterium* (Raskin *et al*, 1994a and Raskin *et al*., 1995).

3.3.6.3 Archaeal populations active in the ABR

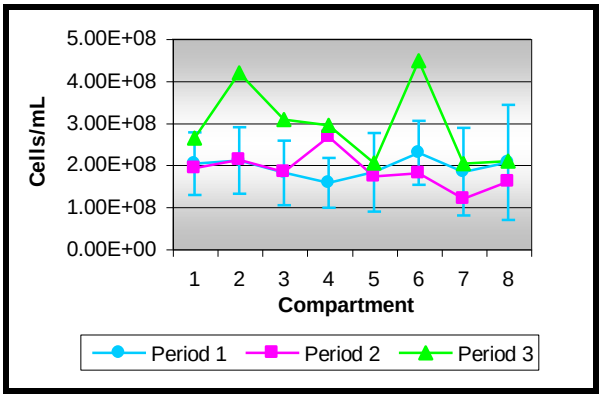

Figure 3.66: Total numbers of Archaea for each operating period.

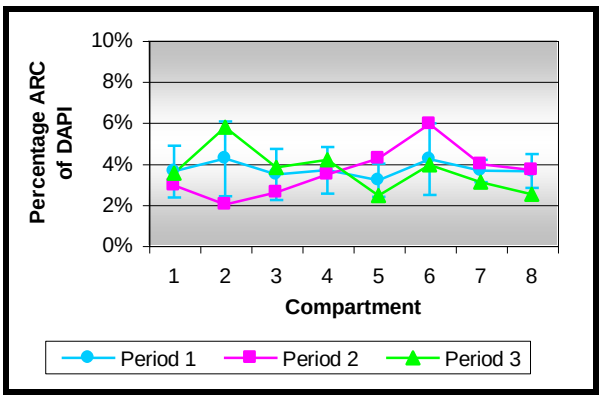

Figure 3.67: Percentage Archaea of DAPI for each operating period.

Figures 3.66 and 3.67 depict the contribution of the Archaea to the total biomass fraction. Archaeal cells were detected throughout the operating periods at an average of 4% of the total DAPI-stained cells. This stability in Archaeal numbers has been found by Fernández *et al* (1999), where Archaea showed less diversity than bacterial numbers. Zumstein *et al* (2000) (as cited in Kaewpipat and Grady, 2001) also noted this stability in Archaeal populations. The percentages, however, were lower than expected as it is thought that Archaea proliferate at the end of an anaerobic reactor. Plumb *et al* (2001) determined, with an ABR treating dye wastewaters, that while Archaeal numbers increased, bacterial numbers decreased, until 90% of all DAPI stained cells being Archaea were detected in Compartments 7 and 8 of the ABR. In this way the ABR facilitates the separation of the acidogenic and methanogenic phases (Uyanik *et al*., 2002a). However, the above results show that since the bacterial population was dominant throughout the compartments, the archaeal community was unable to establish. The presence of the acidogenic bacteria throughout the reactor would have lead to low pH conditions, which is inhibitory to methanogens (Bitton, 1994). The repeated washout occurrences of Operating Periods 2 and 3 prevented the establishment of a healthy methanogenic population.

The Archaea were present at the highest numbers during Operating Period 3 (Figure 3.66). This however, is not representative of the acetoclastic methanogens but of the hydrogenotrophic methanogens, which utilise hydrogen and formate (König, 1993). It was noted that there was a high influent COD for this period, which therefore would have yielded more nutrients for these organisms (Figure A7.6, APPENDIX SEVEN).

Methanosarcina cells were the only acetoclastic methanogen identified by *in situ* hybridizations. These cells were found primarily at the beginning of the reactor in their characteristic packet or cluster forms (König, 1993). Tilche and Yang (1987) (as cited in Barber and Stuckey, 1999) noted a large concentration of *Methanosarcina* cells at the beginning of a pilot scale Hybridised ABR treating molasses wastewater. Langenhoff and Stuckey (2000) also noted a high methanogenic activity at the beginning of an 8-chamber ABR treating low strength synthetic milk substrate. The other acetoclastic methanogen, *Methanosaeta*, was not present. This could be due to the low pH, which inhibits scavenging methanogens (Nachaiyasit and Stuckey, 1997b). The high biomass washout rate also resulted in a washout of filaments (*Methanosaeta*). *Methanosaeta* cells have a slower growth rate than *Methanosarcina* and are more sensitive to environmental changes like decreased pH. Decreased pH further encourages the growth of acid producing bacteria, which results in a decrease in the methane potential (Barber and Stuckey, 1999). Raskin *et al* (1994a) noted that *Methanosarcina* were the prevalent acetoclastic methanogen in anaerobic chemostats fed with glucose. *Methanosaeta* cells were absent. This proved to be due to the ability of *Methanosarcina* to proliferate at high acetate concentrations. This may have been the reason why this phenomenon was noted in the pilot scale ABR. However, this cannot be verified due to a lack of data for acetate concentrations. The prevalence of *Methanosarcina* was also noted in a continuously stirred tank reactor treating cattle manure (Mladenovska *et al*., 2003).

Hydrogenotrophic methanogens, *Methanobacterium* and *Methanococcus*, were identified by DNA sequencing. These methanogens utilised hydrogen and formate to produce methane (König, 1993). It is suggested that probes for the different methanogens be utilised for future studies. *Methanobacterium* sp, in conjunction with *Methanosarcina* sp. have been noted in anaerobic digesters fed with vinases (Godon *et al*., 1997). *Methanobacterium* sp. tend to recover quickly from stresses in a system. It is possible that is why they were able to survive in the ABR. Angenent *et al* (2002) noted that *Methanobacteriaceae* showed increased numbers after feeding resumed following starvation in a 5 compartment anaerobic migrating blanket reactor fed on sucrose, nutrients, bicarbonate, yeast extract and trace elements.

CHAPTER FOUR

CONCLUSIONS AND RECOMMENDATIONS

4.1 GENERAL CONCLUSIONS

It has to be noted that this work has been developmental, with *in situ* analyses of anaerobic communities of an ABR being a relatively young field. Within the Kingsburgh ABR there appeared to be no separation between the phases of anaerobic digestion as expected, with all the bacterial groups being present throughout the compartments. It was noted that hydrolysis was the limiting step, and the possibly slow breakdown of the organic molecules led to the occurrence of these bacteria throughout the reactor. Frequent incidents of biomass washout during Operating Periods 2 and 3 prevented the establishment of a healthy community and resulted in a mixing of the microbial populations. Utilising results obtained from this project, the entire ABR process was re-evaluated and the process parameters altered, in that a lower flow rate was implemented. Thereafter, an improved ABR process, as well as an apparent stability of the microbial populations was noted in 2004.

DAPI staining of the anaerobic sludge from the ABR revealed a high concentration of biomass (10^9-10^{10} cells/mL). However, *in situ* hybridizations revealed only 50% of this biomass as bacteria and Archaea. Previous research has shown that *Eubacteria* and *Archaea* are not the sole living components of anaerobic sludge since fermentative ciliates, protozoa and anaerobic fungi all contribute to this microbial consortium. These cells would have been stained by DAPI but not detected by the probes. It is therefore recommended that DAPI staining for the determination of microbial biomass, should always be accompanied by *in situ* hybridization and vice versa.

Bacterial dominance was noted throughout the operating periods, comprising of between 36 and 46% of the total DAPI-stained cells. This number may have been underestimated as it was previously noted that probe EUB338 does not detect all bacteria. The probe has mismatches in the *Cytophaga-Fermicutes-Bacteriodes* phylum, the *Spirochetes* genera as well as those of the *Acidobacterium* group (Daims *et al*., 1999). Cells that are starving or slow-growing are also unable to be detected by FISH, as they generate low or no signals and therefore cannot be detected.

Of the total bacterial population (determined by the EUB338 probe) only 50% were documented by the group-specific probes utilised. This denotes that there were additional bacteria present that had not been accounted for by the probes, for example, epsilon subclass of the *Proteobacteria*, *Spirochetes* and *Planktomyces*. However, it was not feasible to utilise all probes. It is probable that certain EUB338 numbers would have been inflated, as a result of autofluorescence, which is a limitation of FISH. However, this was overcome by rinsing samples in 1 x phosphate buffered saline. However, it proved impossible to minimise all of the autofluorescence.

The probe SRB385 used for the determination of the sulfate reducing bacteria is known to be phylogenetically inconsistent. Therefore the probes DSB985 and DSV698 were utilised to accurately determine the contribution of the SRB. However, it was noticed that the sum of the latter two probes was often higher than that of the total SRB385. This could be attributable to autofluorescence of background material.

The probe CF319a does not completely detect the members of the *Cytophaga-Fermicutes* phylum. Therefore, there may have been underestimations of this group, which would have lead to lower numbers than that actually present.

Archaeal numbers in the ABR were low, detected at an average of 4% of the total biomass fraction. It is possible that these numbers are lower than the actual values, due to the low amount of cellular ribosomal rRNA within intact cells. These microorganisms with low rRNA are unable to be accurately detected by FISH. This low cellular rRNA is characteristic of slow growing (like the *Archaea*) as well as of starving cells. It is possible that the probes failed to hybridize to these cells and therefore could not be detected.

The total numbers of the acetoclastic methanogens were low throughout the first and third operating periods, with none being found in the second operating period. It was noted that the ABR did not separate the acidogenic and methanogenic phases, as expected. Since the presence of the acidogens leads to a low pH (as previously noted), the acetoclastic methanogens would have been inhibited. The repeated washout occurrences of Operating Periods 2 and 3, prevented the establishment of a healthy methanogenic population.

Methanosarcina clusters were the only archaeal cells detected by *in situ* hybridization. The complete absence of the acetoclastic *Methanosaeta* was due to a high flow rate, which resulted in a washout of filaments. However, the latter could also be due to the low pH, which inhibits scavenging methanogens.

DNA sequencing results proved to be an effective tool in the identification of the microbial population. These results not only verified the FISH analyses, but in fact, provided additional information by identifying the microorganisms according to genus levels. DNA sequencing of the archaeal populations, revealed the presence of both the *Methanobacterium* and *Methanococcus* genera, which were not probed for. It also verified the presence of the *Methanosarcina.* Sequencing of the bacterial DNA revealed the presence of *Streptococcus spp., Propionibacterium spp,* and *Desulfovibrio vulgaris*.

Hybridization and sequencing results are noteworthy techniques of identification of the microbial population of anaerobic treatment processes. Notoriously difficult to culture organisms, such as the methanogens, and their responses have been studied to confer an improved understanding of the communities of the ABR. When applied with further chemical studies, such as VFAs, acetate and methane concentrations, these results will help to optimise the process of anaerobic digestion.

4.2 RECOMMENDATIONS

This research served as a basis for further investigation, which should focus on:

- limiting the disadvantages of probe EUB338. It is suggested that other probes developed for detection of the Eubacterial class be utilised. These include EUB338 II and EUB338 III (Daims *et al.*, 1999).
- reducing autofluorescence. It is suggested that self-ligating probes be utilised. These probes self-ligate following hybridization and therefore only the autoligated probe is recognised (Wagner *et al*., 2003).
- achieving accurate *Cytophaga-Fermicutes* results. It is suggested that probe CFB560 described by O'Sullivan and colleagues (2000) be applied.
- providing accurate numbers of cells with low cellular rRNA. It is suggested that unlabelled oligonucleotides (Wagner *et al*., 2003), multiple probes (Amann, 1990) or polyribonucleic probes be used. Probes targeting the small sub-unit rRNA have also been applied successfully for this purpose, providing more reliable results (Behrens *et al*., 2003).
- determining the contribution of the epsilon subclass of the *Proteobacteria Spirochetes* and *Planktomyces* by *in situ* hybridization, utilising the appropriate probes

- providing accurate Archaeal numbers. It is suggested that probe EURY498, targeting the Euryarchaeota, be applied in place of ARC915 (Burggraf *et al*., 1994).
- understanding the methanogenic population. It is suggested that probes for for both acetoclastic methanogens and hydrogenotrophic methanogens be utilised.
- preventing the washout of biomass. It is suggested that the polymerisation of biomass in granules by chemical aid be attempted. This provides a larger and more compact biomass fraction (Uyanik *et al*., 2002b).
- utilising other effective tools, besides DAPI and FISH for the determination of microbial biomass in wastewater treatment plants. These include the use of polyribonucleic probes, as well as peptide nucleic acid probes.

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APPENDIX ONE

Cell Fixation

1. PREPARATION OF SOLUTIONS

a) 1 x phosphate buffered saline

- 130 mM sodium chloride
- 10 mM sodium phosphate buffer (Na_2HPO_4) (pH 7.2)

b) 3 x phosphate buffered saline

- 390 mM sodium chloride
- 30 mM sodium phosphate buffer (pH 7.2)

c) 4% paraformaldehyde

- Heat 65 ml of ddH_2O to 60°C.
- Add 4 g of paraformaldehyde.
- Add 1 drop of 2M NaOH solution and stir rapidly until the solution has nearly clarified.
- Remove from heat source and add 33 ml of 3 x PBS.
- Adjust pH to 7.2 with HCl.
- Filter solution through a 0.2 µm filter.
- Quickly cool down to 4°C and store in the refrigerator or on ice.

2. PARAFORMALDEHYDE FIXATION OF CELLS *

i. Add 3 volumes of paraformaldehyde fixative to 1 volume of sample and hold for 1-3 hours at 4°C.

ii. Pellet fix cells by centrifugation (3500 rpm, 4 min) and remove fixative.

iii. Wash cells in 1 x PBS and centrifuge.

iv. Resuspend in 1 x PBS to 50% of the original sample volume.

v. Add one volume of ice-cold ethanol (e.g. 0.5 ml) and mix.

vi. Fixed cells may now be spotted on glass slides or may be stored (-20°C) for 2-3 months.

* For ethanol fixation of Gram-positive bacteria use absolute ethanol in place of 4% paraformaldehyde.

APPENDIX TWO

Membrane Filtration DAPI

Procedure:

i. Counter stain cellulose acetate filters (pore size, 0.22 µm, Micron Separations Inc) with Sudan Black solution for 12 hrs.

ii. Add 895 µl of 1 x PBS to 5 µl of activated sludge in a 2 mL micro-test tube.

iii. Add 100 µl of the non-ionic detergent (Igepal CA-30, Sigma Chemicals) and mix.

iv. Add 1 mL of DAPI (0.5 µg/mL) to 1mL of the activated sludge mixture.

v. Allow staining to proceed for 10 min.

vi. Place the stained cellulose acetate filter and a 0.45 µm backing filter at the base of a 15mL filter tower and wet with sterile deionised water.

vii. Quantitatively transfer the stained activated sludge mixture to the filter tower under a slight vacuum.

viii. After filtration, remove excess DAPI stain by washing the filter in the filtering device with sterile deionised water.

ix. Mount the stained cellulose filter on one drop of glycerol/PBS mixture (95:5 v/v) on a glass slide.

x. Add one drop of an anti-fading Kallestad^{TM} Mounting Media (Biorad, South Africa) to the mounted filter surface before placing the coverslip.

xi. DAPI fluorescence is detected with a Zeiss Axiolab microscope (Zeiss, Germany) fitted for epifluorescence microscopy with a 50 W high-pressure mercury bulb and Zeiss filter set 01.

APPENDIX THREE

Whole Cell Hybridization

MATERIALS:

1. 50 ml polypropylene tube
2. Whatman 3MM paper
3. Hybridization buffer

0.9 M sodium chloride	0.01 % sodium dodecylsulfate
20 mM Tris/HCl	x% formamide *

PROCEDURE:

i. Soak a strip of Whatman 3MM paper in hybridization buffer and place in a polypropylene tube.

ii. Allow the chamber to equilibrate for 15 min at 46°C hybridization temperature.

iii. Add 10 µl of the appropriate probe/buffer mixture to each well containing the fixed cells.

iv. Transfer slide to the pre-warmed moisture chamber and hybridize for 2 hours at 46°C.

v. After hybridization, rinse the slide with the pre-warmed (48°C) hybridization buffer and incubate @ 48°C for 20 min.

vi. Rinse the slide with 1 x PBS, shake the excess water and air-dry.

vii. Add 10 µl of DAPI (0.5 µg/ml) and allow staining for 5 minutes in the dark.

viii. Wash the slides with 1 X PBS and air-dry.

ix. Mount the slide in Mounting media and cover with a coverslip.

x. Select random fields for enumeration by image analysis.

* Formamide concentrations for each probe are given in Table 11.

APPENDIX FOUR

DNA Extraction

Procedure:

1. Place 1ml of sample into a centrifuge tube.
2. Centrifuge sample for 10 minutes at 4000rpm.
3. Remove supernatant and resuspend in 1ml of distilled water.
4. Place centrifuge tube in water-bath for 15 minutes at 80ºC.
5. Remove and store in freezer.
6. Use 1µl aliquots of boiled solution for PCR.

APPENDIX FIVE

Polymerase Chain Reaction

PCR mixtures (Fermentas, Inqaba Biotechnical Industries, South Africa), total volume of 100µl contained:

- 10 µl of 10 x PCR buffer (without magnesium)
- 1.5 mM $MgCl_2$
- Deoxynucleoside triphosphate mixture at a concentration of 2mM
- Each primer at a concentration of 50pmol/µl
- 5µl of DNA template for *Archaea* and 10µl of DNA for bacteria
- 5 U of Taq polymerase.
- 5µl of DMSO was added to the Archaeal reactions.

Reaction conditions:

Stage One:	Step 1:	94ºC	4 minutes	x 1
Stage Two:	Step 1:	94ºC	1 minute	
	Step 2:	53ºC	1 minute	x 35
	Step 3:	72ºC	2 minutes	
Stage 3:	Step 1	72ºC	4 minutes	x 1
Hold		4ºC		

APPENDIX SIX

Agarose Gel Electrophoresis

Preparation of Gel

i. Add 50mL of 1x Tris-acetate buffer (0.04M Tris-acetate and 0.001M EDTA) and 0.8g agarose

ii. Bring to boil and ensure that all the agarose has dissolved.

iii. Cool

iv. Add 1µl ethidium bromide

v. Pour into tray with comb and allow to set, 15-20 minutes

vi. Remove gel and transfer to electrophoresis chamber

Sample Loading

i. Load 2µl of loading buffer (0.25 % bromophenol blue and 40% w/v sucrose in water) with 4µl of sample onto each well of the gel.

Electrophoresis settings

- Voltage: 80V Time: 60 minutes

APPENDIX SEVEN

Reactor Performance

A. OPERATING PERIOD FEBRUARY – JUNE 2003

Reactor Performance:

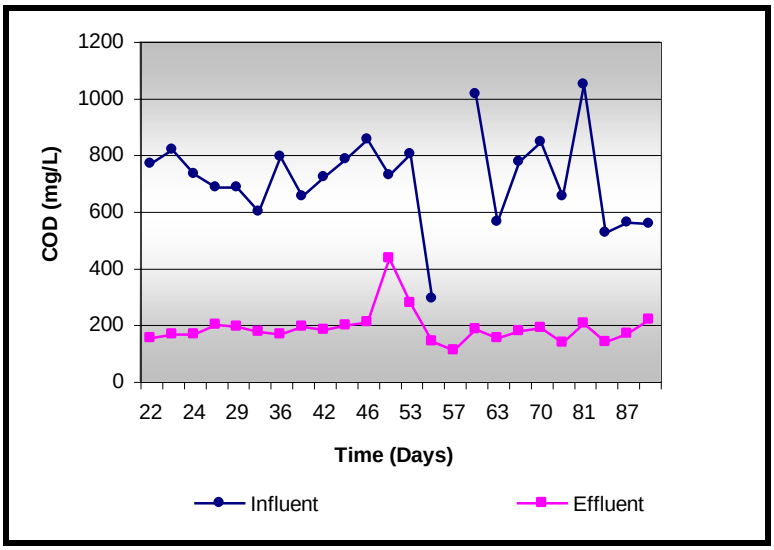

Figure A7.1: Grab influent and effluent COD measurements (February-June 2003).

Figure A7.1 shows COD results for the operating period February to June 2003. Influent COD concentrations were constant throughout this period, with the lowest COD recorded on Day 56 (290 mg/L) and the highest on Day 81 of 1046. Effluent COD concentrations were low, with the highest being over 400 mg/L on Day 50. Average COD influent values were 716 mg/L ±25.74 and average COD effluent values were 192 ± 10.04.

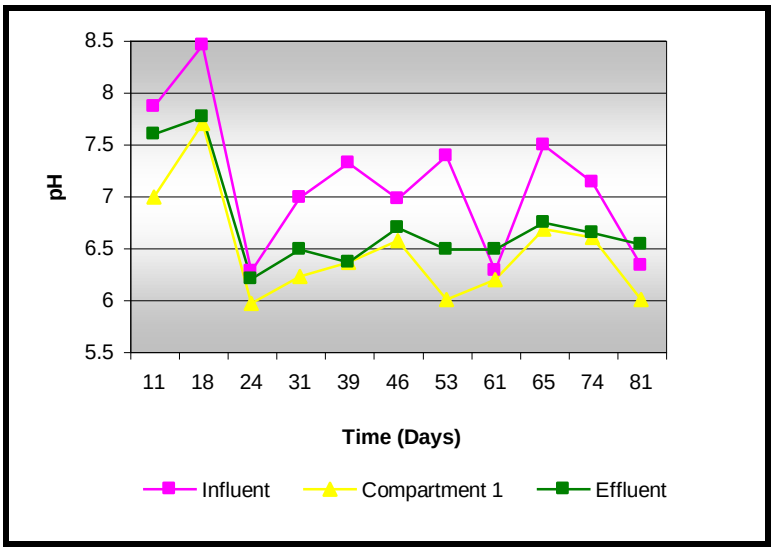

Figure A7.2: Selected pH measurements (February-June 2003).

pH measurements for February-June are detailed in Figure A7.2. pH values remained within the range of 6 and 8. Compartment 1 showed the lowest pH values, not surprisingly, as most of the acid-forming steps take place in this compartment. On Day 18, the highest pH was recorded. It can be seen that the effluent pH did not differ much from the influent, indicating that although acidogenesis took place, it did so on a small scale. This could be due to an inhibition of the acid-forming bacteria.

B. TWENTY-FOUR HOUR CAMPAIGN (DAY 101)

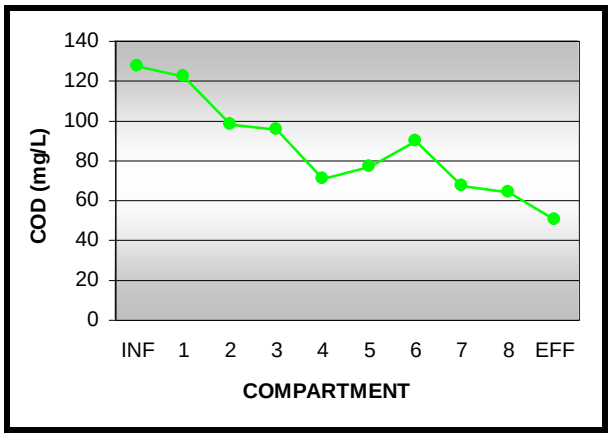

Figure A7.3: Soluble COD measurements for each compartment (Day 101).

COD values for Day 101 are shown in Figure A7.3. On this day an 84% reduction of COD was noted. COD values show a step-wise decrease from compartment to compartment, with the exceptions of 5 and 6. These increases in COD can be attributed to an increase in intracellular material released from dead lysing cells.

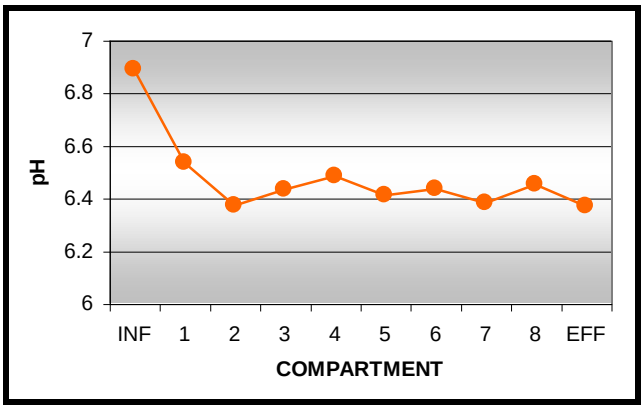

Figure A7.4: pH measurements for each compartment (Day 101).

pH measurements for the 24hr campaign are detailed in Figure A7.4. pH values remained within the range of 6.4 and 6.6., Compartment 2 showed the lowest pH values possibly due to acidogenesis taking place. There seemed very little change in the pH, with only slight increases and decreases throughout the compartments.

C. OPERATING PERIOD: JULY-SEPTEMBER 2003

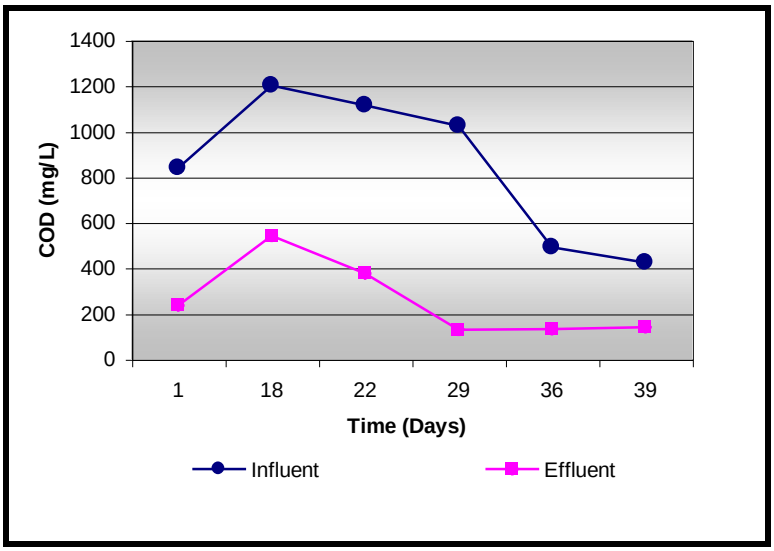

Figure A7.5: Grab influent and effluent COD measurements (July-August 2003).

COD values for the second operating period are shown in Figure A7.5. Influent COD concentrations were fluctuated throughout this period, with the lowest COD recorded on Day 39 (425 mg/L) and the highest on Day 18 of 1200. Effluent COD concentrations were higher than the previous operating period, with the highest being 541 mg/L on Day 50. Average COD influent values were 849 mg/L ± 133.24and average COD effluent values were 259 ± 63.31.

D. OPERATING PERIOD: NOVEMBER-DECEMBER 2003

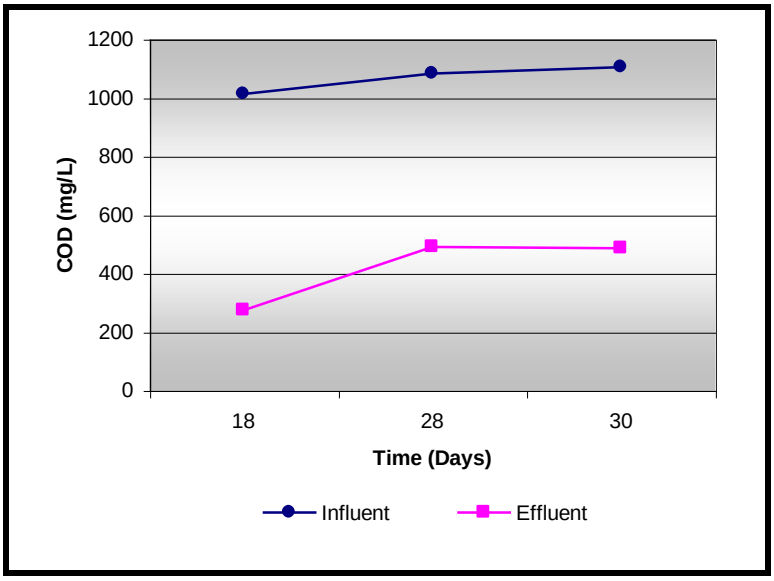

Figure A7.6: Grab influent and effluent COD measurements (November-December 2003).

COD values for the third operating period are shown in Figure A7.6. Samples were obtained for only 3 days, due to pump difficulties and high biomass washout. Influent COD concentrations were high throughout this period, with the lowest COD recorded on Day 18 (1013 mg/L) and the highest on Day 18 of 1104. Effluent COD concentrations were high throughout this operating period, with the highest being 491 mg/L on Day 50. Average COD influent values were 1066mg/L ± 48 and average COD effluent values were 416mg/L ± 124.