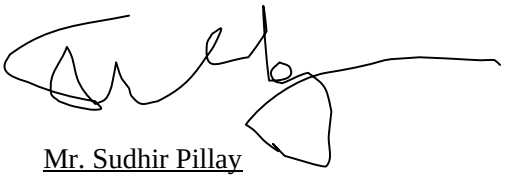
Microbial Studies of an Anaerobic Baffled Reactor (ABR) Treating Domestic Wastewater

by

Sudhir Pillay

PREFACE

I hereby declare that this dissertation is my own work, unless stated to the contrary in the text, and that it has not been submitted in part, or in whole to any other University or Institution. The research was carried out at the University of KwaZulu-Natal (UKZN), Durban in the School of Biological and Conservation Sciences.



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ABSTRACT

Based on previous laboratory studies, the anaerobic baffled reactor (ABR) has been suggested as a waterborne, on-site sanitation option for low-income, peri-urban settlements. This study was part of a larger project, which set out to investigate the performance of a pilot-scale ABR (3 000 L) treating domestic wastewater. For this study, emphasis was placed on the pathogen indicator removal and the microbial population dynamics of the reactor. The reactor was operated at two flow regimes, a hydraulic retention time (HRT) of 22 h (case study 1) and one of between 40-44 h (case study 2), and the various aspects of performance evaluated.

At an average HRT of 22 h, an average chemical oxygen demand (COD) removal efficiency of 72% was achieved, which complied with effluent discharge regulations for agricultural irrigation. Nutrient concentrations remained relatively unaffected by anaerobic digestion. The high level of plant nutrients in the effluent suggests its potential application as a fertiliser. The major re-use concern, however, was the high pathogen indicator counts. Although statistically significant removal efficiencies of *Escherichia coli* (*E. coli*) and total coliforms were observed, a further 3-$\log_{10}$ reduction would be required to produce an effluent which conformed to discharge standards. It was hypothesised that the ABR was capable of improved performance, as several technical difficulties, associated with control and supply apparatus, might have affected the performance of the reactor.

Consequently, the ABR was operated at an average HRT of 40-44 h. Whilst nutrient concentrations remained relatively unaffected, COD removal efficiency increased to 82%, and was again well below agricultural re-use guidelines. There were also improvements in the removal efficiencies of *E. coli* (from 68% to 76%), total coliforms (from 61% to 83%), and total suspended solids (TSS) (from 50% to 68%). It was hypothesised that the improved indicator removal at a HRT of 40-44 h may be due enhanced solids retention and improved reactor stability. Statistically significant reductions of coliphage and *Ascaris* eggs (≥ 94%) were also achieved at a HRT of 40–44 h. However, the pathogen indicator counts in the effluent were still above recommended discharge levels. A small-scale trial, using a microfiltration membrane, was investigated as possible post-treatment option. The limited results indicated that an effluent of appropriate discharge quality could be produced using a microfiltration membrane.

With respect to the microbial population dynamics of the ABR, the hypothesis of the horizontal separation of acidogenic and methanogenic consortia through the ABR was not substantiated by scanning electron microscopy (SEM) observations at a HRT of 22 h. This was thought to be due to the slow rate of hydrolysis of particulate organics within the wastewater. This resulted in scavenging of volatile fatty acids (VFA) by microorganisms resembling methanogens, especially those resembling hydrogenotrophic methanogens (*Methanospirillum*-like microorganisms, *Methanococcus*-like microorganisms, and *Methanobrevibacter*-like microorganisms) in the first few compartments of the reactor. Contrary to literature, microorganisms resembling *Methanosaeta* species were rarely observed. It is suggested that under conditions of 'stress' (high flow and 'washout', low pH), there was a selection for *Methanosarcina*, possibly due to its faster growth and greater tolerance to 'adverse' environmental conditions over *Methanosaeta*.

The results were in marked contrast to the study conducted at an average HRT of 40-44 h, where a partial separation of acidogenic and methanogenic phases was observed. However, this separation was different in form to those described in literature, as methanogenesis occurred predominantly near the front of the reactor. Scavenging by microorganisms resembling hydrogenotrophic methanogens occurred at the front of the reactor. There were also comparatively larger populations of acidogenic and acetogenic bacteria observed at this flow regime. In addition, there were changes in the distribution of microorganisms resembling acetoclastic genera. A few *Methanosarcina*-like populations were observed in compartment 1, whilst larger *Methanosaeta*-like populations were found in subsequent compartments. The latter was found to predominate in compartments 2 and 3, mostly in the form of granular sludge. Although the phenomenon of granulation was observed at both flow rates, they were poorly developed and did not have any significant microbial populations on the surface or within the core when the HRT was 22 h. However, observations made at a HRT of 40-44 h, showed the presence of two-layered granules, consisting of a mixed population on the outer surface and a large central core composed primarily of microorganisms resembling *Methanosaeta* species. The results suggested that *Methanosaeta* was a key-role player in the development of granules, in keeping with other theories of granulation. An additional observation of microorganisms resembling acidogenic bacteria around extracellular polymer (ECP)-bound aggregates of *Methanosaeta*-like cells within the granule core, led to the development of a proposed model of granule biogenesis, which differed from those described to date. These results clearly indicated that the HRT had a major influence on the microbial population dynamics of an ABR treating domestic wastewater.

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GLOSSARY

Acidogens – Bacteria that depolymerise organic polymers, carbohydrates, proteins and lipids and ferment these to organic acids, alcohols, hydrogen and carbon dioxide.

Acetoclastic methanogens – A group of methanogens, that comprises of two main genera, which convert acetate into methane and carbon dioxide.

Acetogens – Bacteria that convert fatty acids (example: propionic acid, butyric acid) and alcohols into acetate, hydrogen, and carbon dioxide.

Aerobes – Microorganisms, which require the presence of air or oxygen for growth.

Anaerobes – Microorganisms that grow in the absence of oxygen, and that do not require molecular oxygen for respiration.

Anaerobic digestion – A microbial fermentation of organic matter to methane and carbon dioxide that occurs in the absence of oxygen.

Basic sanitation - A minimal level of sanitation service, such as access to a septic tank or a ventilated improved pit latrine (VIP).

Biological oxygen demand (BOD) – The amount of dissolved oxygen consumed by microorganisms for the biochemical oxidation of organic and inorganic matter.

Catabolic pathway - A degradative metabolic pathway in which larger molecules are broken into smaller ones.

Chelate – A metal complex formed between an element and metal ion.

Chemical oxygen demand (COD) – The amount of oxygen required to completely oxidise the organic matter in an effluent sample.

Colloidal particles - Finely divided particles, which do not rapidly settle out of suspension or are not readily filtered.

Consortium – An interactive association between microorganisms, that generally results in a combined metabolic activity.

Culture – To encourage the growth of particular organisms under controlled conditions; the growth of particular types of microbes on or within a medium as a result of incoluation and incubation.

Domestic wastewater – A combination of human and animal excreta (faeces and urine), and greywater (washing, bathing and cooking water).

Doubling rates – Time taken for a given population of organisms to become twice as numerous.

Ecosan - A sustainable, "closed-loop" approach to wastewater management, in which, human excreta is contained and sanitised, and the nutrients re-used in agriculture.

Ecosystem – A functional self-supporting system that includes the organisms in a natural community and their environment.

Effluent – The liquid waste discharged from industries or digesters.

Endogenous respiration – The oxidation of cellular mass by aerobic metabolism.

Eutrophication - Process whereby water bodies receive nutrients that result in excessive plant growth. Consequently, the oxygen, which aquatic plants and animals require, is depleted.

Extracellular (Exocellular) enzymes – Enzymes that are secreted outside cells.

Facultative anaerobes – Organisms that make ATP by aerobic respiration if oxygen is present, but switch to fermentation under anaerobic conditions.

Feed – Refers to the substrate supplied to a reactor.

Granules – A mass of microbes cemented together in slime or extracellular polymer produced by microorganisms usually found in waste treatment plants or anaerobic digesters.

Hydrogenotrophic methanogens – A group of methanogens, which convert hydrogen and carbon dioxide into methane.

Influent – Wastewater that flows into a wastewater treatment system.

Liquidification - The enzymatic hydrolysis of complex polymers by the action of extracellular enzymes (see solubilisation).

Metabolism – The total chemical changes and processes in living cells.

Metabolite – Product of a microbial biochemical activity.

Methanogens – Strict anaerobes, belonging to the domain *Archaea*, capable of reducing carbon or low molecular weight fatty acids to methane.

Morbidity – The incidence or prevalence of disease.

Mortality – The number of deaths from a disease.

Obligate anaerobes – Oxygen sensitive organisms, which die when exposed to atmospheric levels of oxygen.

Pathogen - Any organism (virus, bacterium, protozoa, nematode) responsible for the transmission of human disease.

Pollution – An adverse alteration of the environment.

Potable water - Water that is acceptable for consumption.

Scum layer - A floating layer consisting of suspended fats and lipids with entrapped or attached sludge.

Solids - Matter that is suspended or dissolved in water or wastewater.

Solubilisation – The enzymatic hydrolysis of complex polymers by the action of extracellular enzymes (see liquidification).

Souring – Term used to describe an imbalance between acidic and methanogenic fermentation, in which the rate of methane formation falls behind the rate of acid production.

Volatile fatty acid (VFA) – Short-chain organic acid formed by the anaerobic process.

Washout – The removal of solids and associated microorganisms from a reactor.

Water quality - Refers to the chemical, physical and biological characteristics of water in respect to its specific use, and is usually measured in terms of constituent concentrations. The level of water quality is based upon the evaluation of measured quantities and parameters, which then are compared to water quality standards, objectives or criteria.

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ABBREVIATIONS

ABR	Anaerobic baffled reactor
AF	Anaerobic filter reactor
ANOVA	Analysis of variance
APHA	American Public Health Association
AWWA	American Water Works Association
BOD	Biochemical oxygen demand
BPD	Business Partners for Development
CFD	Computational fluid dynamics
CFU	Colony forming units
COD	Chemical oxygen demand
COD_{sol}	Soluble COD
CPD	Critical point drier
DWAF	Department of Water Affairs and Forestry (South Africa)
ECP	Extracellular polymers
FB	Fluidised bed reactor
FISH	Fluorescent *in situ* hybridisation
HPC	Heterotrophic plate count
HRT	Hydraulic retention time
KZN	KwaZulu-Natal
K_s	Substrate affinity
KS	Kolmogorov-Smirnov test
OHPA	Obligate hydrogen-producing acetogens
OLR	Organic loading rate
PCR	Polymerase-chain reaction
RBC	Rotating biological contactor reactor
SEM	Scanning electron microscopy
SRB	Sulphate-reducing bacteria
SPSS	Statistical Package for the Social Sciences software
SS	Suspended solids
PFU	Plaque forming units
PLC	Programmable logic controller

TEM	Transmission electron microscopy
TOC	Total organic carbon
TS	Total solids
TSS	Total suspended solids
UASB	Upflow anaerobic sludge blanket reactor
UKZN	University of KwaZulu-Natal
UNEP	United Nations Environmental Programme
VFA	Volatile fatty acids
VSS	Volatile suspended solids
WEF	Water Environment Federation
WHO	World Health Organisation
WRC	Water Research Commission (South Africa)
WWTP	Wastewater treatment plant

NOTATIONS

	Units
Chemical oxygen demand (COD)	mg COD/L
Colony forming units (CFU)	CFU/100 mL
Flux	$L/m^2.h$
Hydraulic retention time (HRT)	h
Plaque forming units (PFU)	PFU/100 mL
Total solids (TS)	mg TS/L
Total suspended solids (TSS)	mg TSS/L
Volatile suspended solids (VSS)	mg VSS/L

CHAPTER ONE: INTRODUCTION

1.1 Water Situation

South Africa is a semi-arid country, which receives an average rainfall of 500 mm per annum, approximately thirty percent less than the world average of 860 mm (Hoffman and Ashwell, 2001). Rainfall is seasonal and unevenly distributed throughout the country, with approximately twenty-one percent (%) receiving less than 200 mm per annum (Jacobs *et al.*, 1999). Long periods of drought are not uncommon in South Africa, and it is anticipated that by the year 2025, the country will experience chronic water scarcity (Ashton and Seetal, 2002). Population growth and the associated increase in industrial and agricultural activities will increase the demand for water. Furthermore, the water quality of water bodies will become increasingly under threat of pollution as these activities increase, adding further pressure on an already limited resource. Treated and untreated wastewater discharged into water bodies increase the nutrient load therein, resulting in salinity and eutrophication. Microbial pollution as a result of inadequate water and sanitation also represents a major factor contributing to the decline in water quality. Many communities in the country belong to low-income groups and cannot afford basic services and treatment options. Consequently, water bodies, such as rivers, lakes and ponds, are used for a variety of activities such as irrigation, washing and the disposal of human excrement. These highly polluted sources sometimes represent the only source of drinking water, and therefore, greatly increase the risk of the spread of diseases (Tulchin, 1986). Developing countries are especially at risk due to lack of water supply and sanitation systems.

1.2 Health Effects of Poor Water Quality

An estimated 250 million people worldwide are infected with waterborne pathogens, resulting in 10 - 20 million deaths annually (WHO, 2005). Many of the common waterborne pathogens are presented in Table 1.1 Cholera is probably one of the major waterborne diseases in Africa, representing 72% of all cases worldwide (UNEP, 2002). South Africa regularly experiences sporadic outbreaks of cholera, with a peak of 151 182 cases reported occurring from August 2000 to June 2001, representing 58% of total cases reported worldwide during that period (DWAF, 2001). Parasitic infestation also represents a major problem. Nearly 1 billion people are infected worldwide, with children at the greatest risk (WHO, 2005). As a result, they often suffer from nutritional deficiencies and poor growth as a result of such infestations (WHO, 2005). The presence of parasites in water and wastewater is a challenge as they are resistant to various

Table 1.1: Common waterborne transmitted pathogens (Bitton, 1994).

PATHOGEN	DISEASE	MAJOR RESERVOIR
Bacteria		
Salmonella typhi	Typhoid fever	Human faeces
Salmonella paratyphi	Paratyphoid fever	Human faeces
Shigella	Bacillary dysentery	Human faeces
Vibrio cholerae	Cholera	Human faeces
Legionella pneumophilia	Acute respiratory illness	Thermally enriched waters
Yersinia enterocolitica	Gastroenteritis	Human/animal faeces
Campylobacter jejuni	Gastroenteritis	Human/animal faeces
Leptospira	Leptospirosis	Animal faeces and urine
Enteropathogenic *Escherichia coli* (*E. coli*)	Gastroenteritis	Human/animal faeces
Parasites		
Protozoans		
Giardia lamblia	Giardiasis	Human/animal faeces
Entamoeba histolytica	Amoebic dysentery	Human faeces
Acanthamoeba castellani	Amoebic meningoencephalitis	Soil and water
Naeleri gruberi	Amoebic meningoencephalitis	Soil and water
Balantidium coli	Dysentery/intestinal ulcers	Human faeces
Cryptosporidium	Diarrhoea, nausea, low-grade fever	Human/animal faeces
Helminths		
Nematodes (roundworms)		
Ascaris lumbricoides	Ascarisis (intestinal obstruction)	Human/animal faeces
Trichuris trichiura	Whipworm	Human/animal faeces
Necator americanus	Hookworm disease	Soil/faeces
Ancylostoma duodenale	Hookworm disease	Soil/faeces
Cestodes (tapeworm)		
Taenia saginata	Beef tapeworm	Faeces/animals
Taenia solium	Pork tapeworm	Faeces/animals
Trematodes (flukes)		
Schistoma mansomi	Schistosomiasis	Human organs/water (snail/fish)

treatment methods. The cysts of protozoans parasites are able to survive adverse environmental conditions, whilst helminths, such as *Ascaris*, produce eggs that are tolerant to treatment processes, such as chlorination (Bitton, 1994). Contaminated water therefore poses a very serious problem as it can potentially infect large numbers of individuals in a very short period.

Most diseases caused by bacterial pathogens (and to a limited extent, some parasitic pathogens) are characterised by diarrhoea, and other secondary complications. According to Lens *et al*., (2001), the discharge of untreated wastewater is the main cause of diarrhoeal disease worldwide, with an estimated billion infections and 3 million deaths occurring annually. Children are at risk the most, with an estimated 6 000 infant deaths occurring daily as a result of improper sanitation

and hygiene (WEHAB Working Group, 2002). In developing countries, it is the leading infectious cause of infant and child morbidity and mortality (Huttly, 1990; Lonergan and Vansickle, 1991). In South Africa, 43 000 deaths occur annually due to diarrhoeal diseases, and around 1.5 million cases of diarrhoea are reported in children younger than five, annually (DWAF, 2002).

The provision of potable water and sanitation services has been shown to reduce the transmission of waterborne diseases. Esrey *et al.* (1985) noted from studies in various countries that infant morbidity and mortality decreased by up to 80% with improved water supply and sanitation. Similar findings have been reported in other studies (Esrey and Habicht, 1986; Pickering, 1985; Victora *et al*., 1988). Numerous authors have also found a close association between diarrhoea morbidity and mortality, and access to potable water and sanitation. The findings of a study conducted in an urban area in Brazil showed that infants who had access to piped water had 80% less diarrhoeal mortality than those without (Victora *et al*., 1988). Similar studies conducted in Malawi and the Philippines showed that the incidence of diarrhoea was 20% less with infants living in homes with potable water and sanitation facilities (Baltazar *et al*., 1988; Young and Briscoe, 1988). These findings highlight the importance of improving access to potable water and sanitation to reduce waterborne diseases.

1.3 Water and Sanitation Supply in South Africa

As a result of previous apartheid policies, a large proportion of the South Africa's population lack access to basic sanitation and potable water. The South African Department of Water Affairs and Forestry (DWAF) estimates that nearly 18 million people lack basic sanitation, and 12 million have no access to potable water (DWAF, 2002). Furthermore, the level of service is unevenly distributed. Within major cities, municipalities provide potable water of the highest quality and wastewater is taken from homes by waterborne sanitation systems. The total number of people served with potable water and sanitation within urban areas is approximately 80% (Ashton and Seetal, 2002). In residential areas, potable water is also readily available and the sanitary requirements are met either through septic tanks or waterborne sewage systems. In contrast, over half the rural population do not have access to water and sanitation services (Ashton and Seetal, 2002). People living in these areas often use untreated water sources for consumption and faecal waste is discharged into rivers, buried or left in the open.

With a change in political leadership in 1994, the newly elected democratic government recognised the need to address past imbalances in water and sanitation availability. One of the first steps of the Government was to correct previous policies and legislation, which was insufficient and unsuitable for the management of South Africa's water resources, indicative of the deterioration of water quantity and quality experienced at that time (Ashton and Seetal, 2002). With the publishing of the White Paper on Water Supply and Sanitation Policy in 1994, a national strategy was developed to provide drinking water and basic sanitation for all South Africans (DWAF, 2003). The goal was to supply water and basic sanitation to all citizens by 2010. At the moment, the South African government is implementing a programme whereby each household is entitled to 200 litres per day (L/d) of free potable water (DWAF, 2003). The provision is based on the World Health Organisation (WHO) standard, which is considered sufficient to maintain good health and productivity. Similar strategies have produced a dramatic decline in waterborne infections in Europe and North America over the last 130 years (Genthe and Seager, 1996).

The programme has been largely successful with an estimated 9 million people supplied with free basic water from 1994 to 2000 (Kasrils, 2000). The provision of sanitation services, however, is proving to be more difficult. A large sanitation backlog exists with about 1 million households without any means of sanitation and lacking basic hygiene knowledge (DWAF, 2002). This problem is not only confined to housing, but exists in many other sectors, such as education and health. It is estimated that nearly half of all schools use pit latrines, which are often limited in number and are used under unhygienic conditions (DWAF, 2002). More disturbing is the fact that nearly 12% of all schools and 15% of all medical clinics have no sanitation facilities (DWAF, 2002). Reducing the sanitation backlog in these sectors represents a major challenge in itself, but like most other developing countries, the resolution of the problem is complex. One of the major concerns from a public health and environmental pollution aspect has been the effects of increasing urbanisation to cities. This has resulted in the development of high density, informal or peri-urban communities with limited access to potable water and sanitation systems. Providing sanitation to these areas is difficult as conventional sanitation options, such waterborne (flush-and-discharge) and pit systems (drop-and-store), are inappropriate in such congested areas.

1.4 Problems associated with the Delivery of Sanitation in Peri-Urban Settlements

In South Africa, sanitation is usually provided in the form of waterborne sewage system connected to a centralised wastewater treatment or by on-site treatment systems, such as septic

tanks. With respect to waterborne sanitation systems, the surrounding infrastructure near peri-urban settlements is either inadequate or becomes overloaded. The maintenance and future implementation of waterborne sewage systems in these areas is difficult as municipalities have little or no supervision in formalising the housing arrangements and servitudes of rapidly-growing informal settlements. Furthermore, the construction and management costs of these systems are expensive and beyond the financial resources that are available for a developing country. In Nigeria, for example, it is estimated that US$ 9 billion will be required to provide potable water and sanitation facilities to all citizens (Adedipe *et al*., 2000). This method of sanitation is also not sustainable, as it requires large amounts of water for flushing.

As many informal communities are not connected to a sewer system, their sanitation needs are addressed through the use of on-site sanitation systems, such as pit latrines, chemical toilets and septic tanks. The main advantages of theses systems is that they are relatively cheaper and simpler to construct than waterborne sewage systems. However, they show rather low treatment efficiencies, and cannot be used on rocky terrain, where groundwater level is high or in areas that are periodically flooded (Winblad and Simpson-Herbert, 2004). In many instances, failure of these systems has led to groundwater pollution (Stenstrom, 1996). They can also be expensive for municipalities to maintain, as they require emptying at regular intervals, which is often difficult due to the congested nature of the settlements. Furthermore, the available on-site systems are often limited in number, over-utilised and therefore, hygienically unsafe to use.

There exists a considerable need for the development and implementation of treatment systems for use in peri-urban settlements that have high treatment efficiencies at varying loadings, are simple and affordable to construct, have a minimal requirement for land, and low maintenance costs. Among the different treatment processes being considered is anaerobic digestion. Although the process was previously thought of being a less efficient process in terms of performance to aerobic process, research over the last three decades has highlighted advantages over aerobic systems. New reactor types have shown comparable treatment efficiencies, and in some cases, superior performance to conventional aerobic systems (van Haandel and Lettinga, 1994). This technology represents a trend towards sustainable wastewater solutions, and is particularly attractive to developing countries as they offer twin benefits of reducing organic pollutants, and producing energy in the form of combustible methane (Bell, 2000).

1.5 Anaerobic Baffled Reactor (ABR) for Peri-Urban Sanitation

In 1999, the Business Partners for Development (BPD) approved the project entitled "The anaerobic baffled reactor (ABR) for sanitation in dense peri-urban settlements in KwaZulu Natal (KZN)." The motivation for this Water Research Commission (WRC) funded project was to provide on-site sanitation to low-income, informal areas without access to waterborne sanitation systems. Domestic wastewater in these areas is concentrated because of the lack of availability of water, and ambient temperatures are generally high throughout the year (Foxon *et al*., 2005). Under these conditions, the treatment of domestic wastewater by anaerobic degradation is a feasible option. The ABR was proposed as a medium-term, on-site sanitation solution to low-income communities in the KZN province based on results obtained during WRC Project K5/853, in which a laboratory-scale reactor successfully treated soluble, high-strength industrial waste. The versatility and ability of the ABR in removing organic material, as demonstrated in the laboratory-scale project, suggested its application in the treatment of various wastewaters, including domestic wastewater. The reactor also showed several advantages over well-established anaerobic systems, such as the upflow anaerobic sludge blanket (UASB) and the anaerobic filter (AF), including better resilience to hydraulic and organic shock loadings, longer biomass retention times, and lower sludge yields (Barber and Stuckey, 1999). Furthermore, the ABR is easy to construct, has no moving parts, and the requirement for land is minimal.

1.6 Research Aims and Objectives

With a view to addressing the sanitation problem in dense, peri-urban settlements in KZN, the appropriateness of anaerobic treatment on domestic wastewater in an ABR was investigated. The system has the potential to be a cost-effective and efficient system for domestic wastewater treatment. However, most research regarding the ABR has been conducted on laboratory-scale reactors. Very little literature is available for full-scale applications, and even fewer on domestic wastewater treatment. As Anderson *et al*., (2003) aptly stated, "The only real disadvantage that can be levelled against this reactor is that it has not been widely used at full-scale."

The overall objective of this research was to evaluate the performance of a pilot-scale ABR in treating domestic wastewater. Two major objectives chosen to serve as indicators of reactor performance were the determination of:

1. The removal efficiency of selected parameters that serve as indicators of pollution.
2. The microbial population dynamics of the ABR.

The more specific objectives identified were:

- To assess the removal efficiency of pathogen indicators in the ABR;
- To determine the effect of compartmentalisation of pathogen indicator removal;
- To test the hypothesis of spatial separation of acidogenesis and methanogenesis;
- To investigate the changes in microbial community structure and dynamics within the ABR, as well as the treatment efficiency with changing hydraulic retention time (HRT).

1.7 Thesis Organisation

The remainder of the dissertation is divided into six chapters.

Chapter Two presents a review of the literature on anaerobic digestion; the process microbiology and chemistry that occurs in anaerobic systems, the methods available for monitoring the anaerobic digestion process, and the different types of anaerobic reactors used in wastewater treatment, with a detailed focus on the ABR.

Chapter Three deals with the pilot-scale ABR and procedure. It covers the materials and equipment used in the construction and operation of the pilot ABR, choice of test site, periods of operation, and finally, the sampling method used.

Chapter Four details the treatment performance of the pilot ABR, with emphasis on pathogen indicator reduction. The reactor was operated under two flow regimes: a hydraulic retention time (HRT) of 22 h, and one of 40-44 h. The results obtained from two operational periods are presented, followed by a summary of the treatment performance of the pilot ABR. Recommendations for agricultural re-use, based on the results, are also provided.

Chapter Five details the investigation to determine the effect of compartmentalisation on pathogen indicator removal in the ABR. The influence of HRT on indicator removal through the compartments of ABR was also assessed.

Chapter Six details the investigation of the microbial population dynamics in the ABR treating domestic wastewater. Scanning electron microscopy (SEM) was used to describe the microbial communities in each ABR compartment, as well as to observe the changes in the microbial population dynamics with a change in the HRT.

Chapter Seven concludes the study. The main conclusions drawn from the study are presented, and recommendations for future research are made.

CHAPTER TWO: LITERATURE REVIEW

2.1 Introduction to Anaerobic Digestion

Anaerobic digestion is a complex series of reactions, mediated by a consortium of microorganisms, which convert complex organic matter to methane and carbon dioxide in the absence of oxygen (Hawkes *et al*., 1978). The process has been used globally for over a century in the treatment of wastewater (McCarty, 1981; McCarty and Smith, 1986). In spite of its early introduction, which can be traced to the beginnings of wastewater treatment itself, the use of anaerobic systems as the main biological step in wastewater treatment is infrequent (Kassam *et al*., 2003; Seghezzo *et al*., 1998). The reluctance to implement the technology was probably due to concerns over the reliability of the process to treat large, increasing wastewater volumes, especially in industrialised and densely populated areas (Lubberding, 1998). Furthermore, there was a lack of understanding of anaerobic process microbiology and chemistry, as well as experience with the use of the process (Rittmann and McCarty, 2001). Consequently, aerobic systems, such as trickling filters, oxidation ponds, and activated sludge processes, were preferred over anaerobic systems (Rittmann and McCarty, 2001). However, the large increase in energy prices in recent years has increased the operating and maintenance cost of aerobic systems, reducing the attractiveness of the technology (van Haandel and Lettinga, 1994). This has directed research towards alternative, energy-saving treatment systems, such as anaerobic treatment. Through experience, it has been demonstrated that anaerobic systems can offer several advantages over aerobic systems, thereby producing an economical alternative for wastewater treatment (Rittmann and McCarty, 2001).

2.2 Aerobic versus Anaerobic Digestion

A summary of the advantages and disadvantages of anaerobic treatment is presented in Table 2.1. The most obvious advantage of anaerobic digestion is energy production in the form of methane (CH_4). This energy can be used for heating or electrical power, which can then be channelled into the running of a treatment plant. In contrast, the energy requirements to operate aerobic systems are high, as oxygen must be artificially supplied at a cost (van Haandel and Lettinga, 1994). The economic advantage of recovering and utilising methane has been realised by many municipal treatment plants, where often, the energy generated through the anaerobic digestion of sludge from aerobic secondary treatment, satisfies the energy requirements to operate the entire plant, including the aeration tanks (Rittmann and McCarty, 2001). If anaerobic

Table 2.1: Advantages and disadvantages of anaerobic wastewater treatment (adapted from Seghezzo *et al*., 1998).

Advantages	*Simplicity and flexibility*. The construction of anaerobic reactors is relatively simple, and can be applied on either a large or small scale. *Low energy consumption*. The process is generally a net producer of energy in the form of methane. Energy input for aeration is not required as in aerobic systems. *Low decay rate*. Anaerobic microorganisms can remain viable for months, whereas aerobic microorganisms decay within a few weeks. This is important for industries with seasonal activity. *Low sludge production*. Sludge production is much lower in anaerobic systems compared to aerobic systems due to a lower yield co-efficient. *Low nutrient and chemical requirement*. Smaller biomass production occurs in anaerobic systems, and thus the nutrient requirements can be proportionally less. *High organic loading possible*. Anaerobic systems are not limited by the supply of oxygen making it attractive for treating industrial wastewater with high organic loading. *Low space requirement*. The area needed for a reactor is proportionally smaller as it is able to treat wastewater with a high organic loading.
Disadvantages	*Long start-up*. Lower energy yield results in smaller microbial yields and slower growth yields. Due to slow growth rate, start-up time is longer than aerobic systems. *High microbial sensitivity*. Methanogens are sensitive to pH, temperature and assumed to have less resistance towards toxic compounds. *Odour production*. Production of sulphides, especially when there are high concentrations of sulphate in the influent, results in a strong, unpleasant smell. *High buffer requirement for pH control*. The desired pH range for an anaerobic reactor is between 6.5 and 7.6. Chemical addition may be necessary to control pH in wastewaters (mostly industrial wastewaters) with insufficient natural buffering capacity. *Low pathogen and nutrient removal*. Anaerobic digestion results in partial removal of pathogens and incomplete nutrient removal. Post-treatment of anaerobic effluent is required to meet discharge guidelines.

treatment could be used for all or most of the wastewater treatment, the net savings could be substantial (Rittmann and McCarty, 2001).

A second advantage of anaerobic systems is related to the decay rate of microorganisms. Part of the cellular mass of microorganisms is biodegradable and can be used as a source of organic matter for metabolism. The process is called endogenous respiration, and the non-degradable matter that is generated from the process accumulates in the system until it is removed (van Haandel and Lettinga, 1994). When nutrients are limiting or not supplied, microorganisms use

this cellular mass for their energy requirements, and are said to 'decay.' This decay process occurs faster in aerobic organisms than anaerobic ones. As a result, aerobic microorganisms can decay within a week if feed is not supplied (van Haandel and Lettinga, 1994). On the other hand, the slow decay rate of anaerobic microorganisms allows them to remain viable for extended periods without nutrients. This advantage has been realised by many food-processing industries, in which wastewater treatment varies according to seasonal distribution of crops and fruits (Speece, 1996; van Haandel and Lettinga, 1994).

The third advantage of anaerobic systems is also related to bacterial metabolism. Anaerobic digestion of organic matter occurs via fermentative catabolism, in which reduction occurs in the absence of oxygen (Gunnerson and Stuckey, 1986). During this process, organic matter is merely transformed and hence, most of the energy available in the substrate remains unavailable (Gunnerson and Stuckey, 1986). In contrast, metabolism in aerobic systems occurs via oxidative catabolism, in which most of the organic carbon in the substrate is converted to carbon dioxide (Gunnerson and Stuckey; 1986). As a consequence, oxidative reactions yield more free energy than fermentation reactions, which is used for cell growth (Gunnerson and Stuckey, 1986; van Haandel and Lettinga, 1994). Excess cell mass production accumulates in the system, despite the high decay rate, and this must be disposed of. This significantly increases the cost of running the treatment system. According to van Haandel and Lettinga (1994), excess sludge production by aerobic systems, its stabilisation and disposal, can account for 40-60% of the total treatment costs.

Nutrient supplementation also contributes to the treatment costs of both systems. This usually occurs when the feed is deficient in essential macro- and micronutrients, as is the case for some industrial wastewaters. As microbial growth is greater in aerobic systems, the costs of nutrient supplementation are proportionally higher (Rittmann and McCarty, 2001).

Finally, anaerobic systems are able to handle larger organic loading per unit reactor. On the other hand, aerobic systems have typically low loadings, usually less than 1 kg chemical oxygen demand (COD) per day per m^3, because the supply of oxygen limits the system (Rittmann and McCarty, 2001). Anaerobic systems have no oxygen requirement, and can operate at loadings between 5 to 10 kg COD per day per m^3. Consequently, anaerobic digestion is often used in the treatment of concentrated wastewaters with COD of 5 000 mg/L or higher (Rittmann and McCarty, 2001).

It is clear that anaerobic processes offer several advantages over aerobic systems. However, the advantages of the process also have the potential to become the disadvantages. As discussed earlier, anaerobic processes yield less free energy for biological synthesis. Whilst this prevents excessive biomass accumulation in comparison with aerobic systems, anaerobic microorganims, especially the methanogens, typically have slower growth rates (Gunnerson and Stuckey, 1986; Rittmann and McCarty, 2001). Doubling rates of these microorganisms are measured in days as compared to aerobic microorganims, which have doubling rates measured in hours (h) (Rittmann and McCarty, 2001). If the inoculum does not contain a large viable mass of these microorganisms, the start-up of the reactor takes longer. Furthermore, methane-producing microbes are known to be susceptible to changes in environmental conditions, such as pH, temperature, and toxic compounds. If any reactor 'upset' occurs that results in a loss of microbial population, the time for recovery for the reactor is longer because of the slow growth rate of methane producers. Thus, there is "little margin for error" when operating an anaerobic system and this is, perhaps, the major disadvantage of using the process (Rittmann and McCarty, 2001).

Another disadvantage of anaerobic processes is the production of odorous sulphide compounds, particularly gaseous hydrogen sulphide (H_2S), which is toxic and corrosive. These compounds are formed through sulphate reduction and from decomposition of protein-rich wastewaters. Furthermore, it has been shown that sulphate-reducing bacteria (SRB) outcompete methanogens for the same substrates in high-sulphate environments (Kristjansson *et al.*, 1982, Lovley *et al.*, 1982).

The control of pH in anaerobic reactors is critical, especially when treating some industrial wastewaters, which may have a low buffering capacity (this will be discussed in more detail in Chapter 2.4.1). Methanogens have a narrow optimal pH range, between 6.5 and 7.6. Beyond this range will result in reactor 'upset' if the problem persists (Rittmann and McCarty, 2001). Chemical addition or slowing the rate of feeding may be necessary, which increases the running cost and efficiency of the treatment.

The final disadvantage of anaerobic treatment is that the removal efficiency of pathogens is low and nutrient removal incomplete (Seghezzo *et al*., 1998). Post-treatment of the anaerobic effluent is required to meet discharge guidelines and can have a negative impact on processing costs (Seghezzo *et al.*, 1998).

Many ‘substantial developments’ have been made over the years to overcome the problems mentioned above. These include increasing the retention of anaerobic microorganisms through novel reactor design, inoculating or seeding reactors to limit the start-up period, and acclimation of microorganisms to toxic materials.

2.3 The Conversion Process in Anaerobic Systems

The conversion of complex, organic waste into methane requires the mediation of several groups of microorganisms, often linked by their individual substrate and product specificities (Pohland, 1992). A schematic representation of methane fermentation is shown in Figure 2.1. The process involves the degradation of complex organics (polysaccharides, proteins, lipids) by several successive steps into simpler compounds (carbohydrates, amino acids and fatty acids) (Pohland, 1992). The process is not a sequence of independent reactions, but rather, involves indirect and direct symbiotic interactions between different microbial species (Lubberding, 1998).

According to present theory, there are four major metabolic groups that are generally accepted as being present in anaerobic digesters (Anderson *et al.*, 2003; Lubberding, 1998, van Haandel and Lettinga, 1994; Zinder *et al.*, 1984a). Often, the first two or three groups are considered to operate in a group in fermentative reactions, whilst the last is called methanogenic fermentation (Gunnerson and Stuckey, 1986; van Haandel and Lettinga, 1994).

2.3.1 Hydrolysis

In the first step in anaerobic digestion, a consortium of microorganisms degrades complex organic polymers (proteins, cellulose, lignin, lipids) into intermediate organic monomers, such as sugars (mono- and disaccharides), fatty acids, and amino acids (Pohland, 1992; van Haandel and Lettinga, 1994). The reactions are catalysed by a number of extracellular or exocellular enzymes, such as cellulases, proteases, and lipases, which are secreted by relatively fast-growing fermentative bacteria (Bitton, 1994). The function of lipases is to convert lipids to long-chain fatty acids. They are mostly secreted by *Clostridium* species and other micrococci. *Clostridium* species are also responsible for secreting proteases, which degrade proteins into amino acids (Nishio, 1997). Other bacteria also known to secrete proteases include *Bacteroides*, *Butyrivibrio*, *Fusobacterium*, *Selenomas* and *Streptococcus* (Nishio, 1997). Polysaccharides, such as cellulose, starch, and pectin, are degraded by their respective cellulases, amylases, and pectinases (Nishio, 1997).

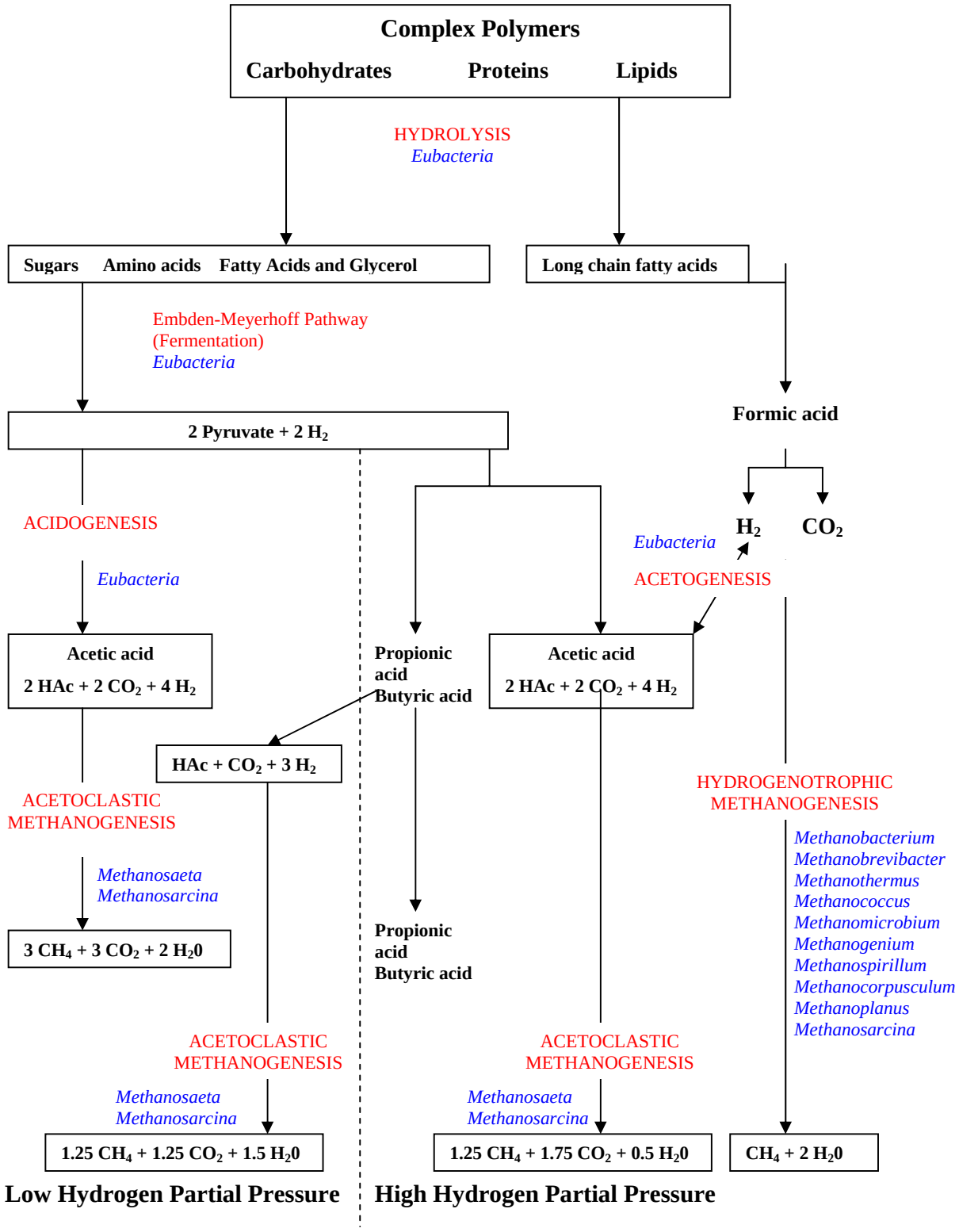


Figure 2.1: Diagram illustrating substrate conversion patterns and the microorganisms involved in the anaerobic digestion of complex macromolecules (from Bell, 2000).

The enzymes facilitate the digestion process by converting complex polymers into soluble monomers. By doing so, substrates, which would otherwise be too large to pass through cell membranes, become available to other metabolic groups (Anderson *et al*., 2003; Bitton, 1994; van Haandel and Lettinga, 1994). Hence, this process is commonly referred to as solubilisation or liquidification (van Haandel and Lettinga, 1994).

Bacteria are not the only microorganisms involved in hydrolysis. Numerous protozoa and fungi have been observed in anaerobic systems. Some protozoa observed in anaerobic reactors include flagellates (*Trepomonas*, *Tetramitus*, *Trichomonas*), amoebae (*Vahlkampfia*, *Hartmanella*) and ciliates (*Metopus*, *Trimyema*, *Saprodinium*). However, they do not occur in large numbers, and are therefore thought to play only a minor role in anaerobic process (Anderson *et al*., 2003). Similarly, a few fungi have been found in low numbers in anaerobic systems. These include Phycomycetes, Ascomycetes and Fungi Imperfecti (Anderson *et al*., 2003).

2.3.2 Acidogenesis

The second step involves the fermentation of soluble products produced by hydrolytic bacteria. Some of the bacteria responsible for this step include: *Clostridium*, *Bacteriodes*, *Ruminococcus*, *Butyribacterium*, *Propionibacterium*, *Eubacterium*, *Lactobacillus*, *Streptococcus*, *Pseudomonas*, *Desulfobacter*, *Micrococcus*, *Bacillus* and *Escherichia* (Anderson *et al*., 2003). Products that are formed by acidogenic bacteria include organic acids (acetic, propionic formic, lactic, butyric, and succinic acids), alcohols and ketones, acetate, carbon dioxide (CO_2), and hydrogen (H_2) (Bitton, 1994). Although obligate anaerobes are responsible for most of the acid fermentation, some of the organic matter is metabolised by facultative bacteria via an oxidative pathway (van Haandel and Lettinga, 1994). Facultative bacteria play a significant role in anaerobic digestion by utilising dissolved oxygen, which would otherwise be toxic to obligate anaerobes (van Haandel and Lettinga, 1994).

2.3.3 Acetogenesis

In this step, acetogenic bacteria produce acetate, CO_2 and H_2 from fatty acids, alcohols and even aromatic compounds. The by-products generated by this step are the only substrates that are efficiently used by methanogens. Two distinct metabolic groups of acetogens have been described in anaerobic systems: obligate hydrogen-producing acetogens (OHPA) and homoacetogens (Anderson *et al*., 2003). To date, only a few OHPA species have been isolated in culture and

identified. These include *Syntrophomonas wolfei* and *Syntrophobacter wolinii*, which degrade butyric and propionic acid, respectively (Anderson *et al.*, 2003).

The OHPA utilise the major fatty acid intermediates (propionic acid, butyric acid) produced by acidogenesis (Anderson *et al.*, 2003; Bitton, 1994). They are also capable of metabolising aromatic compounds as well as higher fatty acids (valeric acid, isovaleric acid, stearic acid, palmitic acid and myristic acid) produced in lipid hydrolysis via β-oxidation (Anderson *et al.*, 2003). Under standard conditions [25^{o} C, pH 7, 1 atmosphere of pressure (atm), 1 M], this fatty acid oxidation is energetically unfavourable due to a high free energy requirement (Δ G > 0) (Lubberding, 1998). Therefore, hydrogen produced as a metabolic by-product by OHPA limits their own growth, and the degradation of fatty acids and other substrates to acetate. This inhibition was demonstrated by McInerney and Bryant (1981), in culture studies with acetogenic bacteria exposed to relatively high hydrogen partial pressures.

In anaerobic environments, this problem is solved by a syntrophic relationship between acetogens and H_2–utilising microorganisms, such as methanogens and SRB. This interaction is a critical requirement for the conversion process and helps prevent an inhibitory feedback effect (Anderson *et al.*, 2003). In other words, the high affinity of methanogens and SRB for hydrogen maintains the low hydrogen partial pressure that is required to make fatty acid oxidation reactions thermodynamically feasible (Lubberding, 1998). However, if this relationship is perturbed in any way, OHPA will be inhibited by high hydrogen partial pressures. The substrates for acetogenesis, namely organic acids, will accumulate in the system, lower the pH, and inhibit pH-sensitive methanogens (Anderson *et al.*, 2003). The growth of acetogens therefore cannot occur without H_2-utilising microorganisms, as demonstrated by the experiments of McInerney *et al.* (1979) and Boone and Bryant (1980). In most anaerobic environments, the hydrogen partial pressure is below 10^{-4} atm, which is sufficiently low enough to prevent inhibition of OHPA and accumulation of fatty acids (Anderson *et al.*, 2003).

The second group of acetogens, known as the homoacetogens, include the genera: *Acetobacterium*, *Acetoanaerobium*, *Acetogenium*, *Buytribacterium*, *Clostridium* and *Pelobacter*. These microorganisms produce acetate from H_2 and CO_2, and in doing so, they maintain the low hydrogen partial pressures required by OHPA. They are found in lower numbers than methanogens in anaerobic systems, and are therefore thought to play a relatively minor role in the conversion process (Anderson *et al.*, 2003).

2.3.4 Methanogenesis

In the last step of anaerobic digestion, a biologically unique group of microbes, known as the methanogens produce methane as a metabolic by-product. The methanogens are critical to the conversion process. Without methanogens, the ultimate degradation of organic matter would not occur (Anderson *et al*., 2003). Methanogenesis is often recognised as the rate-limiting step in anaerobic digestion due to slow growth of methanogens. Furthermore, methanogens have a strictly defined pH optimum (6.5 to 7.6), which means that process will always be under threat of failure due to acid build-up from acidogenesis. Taxonomically, methanogens are distinct from true bacteria (*Eubacteria*) and therefore belong to a separate domain, the *Archaebacteria* (also called *Archaea*) (Lubberding, 1998). The reasons for this separation were due to profound differences in ribosome sequences discovered two decades ago (Anderson *et al*., 2003). Other unique features include:

1. The cell walls of methanogens consist mostly of proteinaceous substances and sugars. Furthermore, their cell walls lack peptidoglycan containing muramic acid, which are a key component of Eubacterial cell walls (Anderson *et al*., 2003).
2. The membranes of methanogens contain modified fatty acids composed of isoprenoids linked to glycerol via ether linkages or other carbohydrates (De Rosa and Gambacorta, 1988; Jones *et al*., 1987; Langworthy, 1985), whereas Eubacterial membranes are composed of glycerol esters of fatty acids (Anderson *et al*., 2003).
3. Methanogens have the following unique co-enzymes: F_{420} and F_{430}. The F_{420} co-enzyme acts as an electron carrier in metabolism and in its oxidised form, absorbs light at 420 nm. This feature is particularly useful for identifying and quantifying methanogens in mixed cultures, or environmental samples by fluorimetric detection (Bitton, 1994).
4. Contain RNA polymerases that have unique subunit structures (Anderson *et al*., 2003).
5. A unique transfer RNA (tRNA) and 5 S ribosomal RNA (rRNA) subunits (Anderson *et al*., 2003).

Within the domain *Archaea*, there are five orders of methanogens as presented in Table 2.2. These orders are represented by diverse morphological variety, consisting of both gram-negative and gram-positive methanogens (Bitton, 1994). Despite this wide diversity, methanogens are able to utilise only a limited number of substrates with acetate, CO_2 and H_2 being the most important (Anderson *et al*., 2003). Two groups of methanogens can be distinguished on their basis of substrate specificity.

Table 2.2: The five orders of the methanogens (Boone *et al*., 1994).

Order	Family	Genus	Species
Methanobacteriales	Methanobacteriaceae	*Methanobacterium*	*M. formicicum, M. bryantii, M. uliginosum, M. alcaliphilum, M. ivanovii, M. thermoaggregans, M. thermoalcaliphilum, M. espanolae, M. thermophilum*
		Methanothermobacter	*M. thermoautotrophicus, M. wolfeii*
		Methanobrevibacter	*M. arboriphilicus, M. ruminantium, M. smithii*
		Methanosphaera	*M. stadtmaniae, M. cuniculi*
	Methanothermaceae	*Methanothermus*	*M. fervidus, M. sociabilis*
Methanococcales	Methanococcaceae	*Methanococcus*	*M. vannielii, M. voltaei, M. maripaludis, "M. aeolicus"*
		Methanothermococcus	*M. thermolithotrophicus*
	Methanocaldococcaceae	*Methanocaldococcus*	*M. jannaschii*
		Methanoignis	*M. igneus*
Methanomicrobiales	Methanomicrobiaceae	*Methanomicrobium*	*M. mobile*
		Methanolacinia	*M. paynteri*
		Methanogenium	*M. cariaci, M. organophilum, M. liminatans*
		Methanoplanus	*M. limicola, M. endosymbiosus*
		Methanoculleus	*M. olentangyi, M. marisnigri, M. thermophilicus*
		Methanofollis	*M. tationis*
	Methanocorpusculaceae	*Methanocorpusculum*	*M. parvum, M. labreanum, M. bavaricum, M. sinense*
	Methanospirillaceae	*Methanospirillum*	*M. hungateii*
Methanosarcinales	Methanosarcinaceae	*Methanosarcina*	*M. barkeri, M. mazeii, M. thermophila, M. acetivorans, M. vacuolata*
		Methanolobus	*M. tindarius, M. siciliae, M. vulcani, M. oregonensis*
		Methanococcoides	*M. methylutens*
		Methanohalophilus	*M. mahii, M. halophilus*
		Methanohalobium	*M. evestigatum*
		Methanosalsus	*M. zhilinaeae*
	Methanosaetaceae	*Methanosaete*	*M. concilii, M. thermophila*
Methanopyrales	Methanopyraceae	*Methanopyrus*	*M. kandleri*

The first group, the acetoclastic methanogens, produce methane from acetate in a pathway known as acetotrophic methanogenesis (Equation 2.1). In anaerobic reactors, approximately two-thirds (65 – 75%) of the methane is derived from this pathway (Anderson *et al.*, 2003; Bitton, 1994; Lubberding, 1998; van Haandel and Lettinga, 1994). Despite this fact, only two genera of methanogens are known to metabolise acetate efficiently: *Methanosarcina* and *Methanosaeta* (formerly *Methanothrix*). Members of the genus *Methanosaeta* can only metabolise acetate, whilst *Methanosarcina* species are more versatile, capable of metabolising methanol, methylamines, and sometimes CO_2 and H_2 to produce methane (Anderson *et al.*, 2003).

$$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^- + \text{energy} \qquad \text{[Equation 2.1]}$$

(Gunnerson and Stuckey, 1986)

The two genera also exhibit different affinities for acetate and thus acetate concentrations have considerable influence over the genera that predominates (Zinder *et al.*, 1984b). At high acetate concentrations, the more versatile *Methanosarcina* is favoured, whilst at low concentrations, *Methanosaeta* dominates. The reason for this shift in predominance is due to differences in specific growth rates and substrate affinity for acetate. *Methanosarcina* has a higher maximum specific growth rate (or shorter doubling time), but a relatively lower substrate affinity for acetate (K_s = 200 – 400 mg/L). *Methanosaeta*, on the other hand, has a high affinity for acetate (K_s = 20 mg/L) but a lower maximum specific growth rate. Thus, *Methanosarcina* is capable of faster growth but only in an environment with a relatively high acetate concentration, whilst *Methansaeta* is a slow-growing, scavenger that grows in environments with relatively low acetate concentrations.

The second group of methanogens, the hydrogenotrophic methanogens, metabolise H_2 and CO_2 to produce methane (Equation 2.2). The pathway is called hydrogenotrophic methanogenesis and accounts for approximately 30% of the methane produced in anaerobic reactors. Although only a relatively small fraction of methane is produced via the hydrogenotrophic pathway, it is critical to the efficiency of the process, as it removes hydrogen produced by hydrolytic and acidogenic bacteria (Gunnerson and Stuckey, 1986). By metabolising hydrogen, these methanogens maintain the low hydrogen partial pressures that are necessary for OHPA to convert fatty acids to acetate. This process, where fermentative microorganisms grow in the presence of hydrogen-utilising microorganisms, is referred to as "interspecies hydrogen transfer" (Pohland, 1992). Without interspecies hydrogen transfer, fermentative microorganisms will shift to produce acids other than acetate (Gunnerson and Stuckey, 1986). This would have serious implications on the rate of methane production. As

discussed earlier, the cleavage of acetate accounts for most of the methane produced. Any decline in acetate production will result in a severe decrease in the rate of methane production. Furthermore, hydrogen accumulation will inhibit the OHPA, and the subsequent accumulation of acids will further inhibit pH-sensitive methanogens.

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O + \text{energy} \quad \text{[Equation 2.2]}$$

(Gunnerson and Stuckey, 1986)

2.4 Environmental Factors Affecting Anaerobic Digestion

There are several factors that affect the rate at which anaerobic digestion occurs. These include pH, temperature, availability of nutrients and, toxic materials and substances.

2.4.1 pH

The control of pH is recognised as one of the most important factors influencing anaerobic digestion. Anaerobic processes generally operate best near neutrality, as the methanogens have a strictly defined pH range (6.5 to 7.6) (Rittmann and McCarty, 2001). The activity of hydrogenotrophic methanogens is reduced when the pH falls to 6.0. This causes imbalances in the conversion process, as acidogenic bacteria are more tolerant to pH change (optimal pH between 5.0 and 6.0) (Hall, 1992). As a result, the rate of acidogenesis proceeds faster than the rate of methanogenesis. Organic acids accumulate in the system, which further reduces the pH, and inhibits the methanogens. Under normal conditions, this acidification is controlled by an aqueous carbonate/bicarbonate buffer system (Bitton, 1994; Gunnerson and Stuckey, 1986; Lubberding, 1998). This buffer system occurs naturally in water systems that are in equilibrium with CO_2 and can be described by the following equations (Rittmann and McCarty, 2001):

$$\begin{aligned}
CO_2\,(aq) &\leftrightarrow CO_2\,(g)\\
CO_2\,(aq) + H_2O &\leftrightarrow H_2CO_3\,(aq)\\
H_2CO_3\,(aq) &\leftrightarrow H^+ + HCO_3^-\\
HCO_3^- &\leftrightarrow H^+ + CO_3^-\\
H_2O &\leftrightarrow H^+ + OH^-
\end{aligned}$$

The bicarbonate buffering system plays an important role in neutralising fatty acid accumulation by acidogenesis and maintains a stable pH; a requirement for pH-sensitive microorganisms such as the methanogens. However, under adverse conditions, the

bicarbonate buffering capacity can be exhausted through excessive acid production (Bitton, 1994). This usually occurs during the start-up of reactors or excessive substrate levels when the growth of methanogens cannot 'match the pace' of faster-growing acidogenic bacteria. Once the buffering capacity of the system is exceeded, pH values will continue to drop and the digester is said to be 'souring' (van Haandel and Lettinga, 1994). When the pH falls below 4.5, which is the maximum limit of the buffering capacity of the system, most methanogenic species will be killed and the digester will fail to operate properly (Lubberding, 1998).

2.4.2 Temperature

As with pH, temperature has a profound influence on the rate of methanogenesis with reaction rates generally increasing with an increase in temperature. Two optimal temperature rates are often cited with respect to the conversion rate of the process: mesophilic (35 - 40^{o} C) and thermophilic (55 – 60^{o} C). Interestingly, decreased rates are observed between the two optimal ranges, that is, between 45 to 48^{o} C. Macki and Bryant (1981) attribute this phenomenon to a lack of adaptation on the part of the methanogens.

Thermophilic digestion is generally more efficient in removing organic matter and pathogens than mesophilic digestion. However, for dilute wastewaters, such as domestic wastewater, thermophilic operation is not an economical option (Rittmann and McCarty, 2001; van Haandel and Lettinga, 1994). The reason for this is that COD values are not high enough to produce enough methane to cause a significant increase in the temperature of the wastewater (Rittmann and McCarty, 2001; van Haandel and Lettinga, 1994). It has been estimated that a maximum 1.5^{o} C increase in temperature is theoretically possible for raw sewage with a COD of 500 mg/L (van Haandel and Lettinga, 1994). This temperature increase, even if utilised at its maximum, which is highly unlikely, will not significantly affect the digestion rates. Hence, anaerobic digestion is more appropriate at ambient temperatures when treating domestic wastewater. For this reason, anaerobic digestion of dilute wastewaters is more attractive for subtropical and tropical countries where temperatures remain over 15^{0} C for most of the year (van Haandel and Lettinga, 1994).

2.4.3 Nutrients

All microorganisms require minerals and nutrients for growth and metabolism. Therefore, the wastewater to be treated must be nutritionally balanced to maintain adequate digestion (Bitton, 1994). Table 2.3 presents the different elemental nutrient requirements for anaerobic reactors. With domestic wastewater, most of the essential nutrients are readily available in the water, and hence, nutrient supplementation is usually unnecessary (Rittmann and McCarty, 2001). The same can be expected from industrial wastewater from food processing, which

Table 2.3: General nutritional requirements of anaerobes (taken from Speece, 1996).

Element	Requirement (mg/g COD)	Desired Excess Concentration (mg/L)
Macronutrients		
Nitrogen	5 – 15	50
Phosphorous	0.8 – 2.5	10
Sulphur	1 – 3	5
Trace metals		
Iron	0.030	10
Cobalt	0.003	0.02
Nickel	0.004	0.02
Copper	0.020	0.02
Manganese	0.004	0.02
Molybdenum	0.004	0.05
Selenium	0.004	0.08
Tungsten	0.004	0.02
Boron	0.004	0.02
Common Cations		
Sodium	-	100 – 200
Potassium	-	200 – 400
Calcium	-	100 – 200
Magnesium	-	75 – 250

also has an adequate supply of these nutrients (Anderson *et al*., 2003; Rittmann and McCarty, 2001).

From all the inorganic nutrients required for anaerobic processes, nitrogen and phosphorus are probably the most important (Hall, 1992; Rittmann and McCarty, 2001). From an operational standpoint, the amount of nitrogen and phosphorus required for anaerobic growth can be determined from estimates of net biological growth using the empirical formula $C_2H_7O_2N$ (Hall, 1992; Rittmann and McCarty, 2001). According to the formula, nitrogen constitutes about 12% of the dry cell mass. As the phosphorous content is approximately a fifth of that of the nitrogen requirement, it can also be estimated (Hall, 1992). By assuming that approximately 10% of the original COD is used for biological synthesis, the amount of nitrogen and phosphorous required by the system can be calculated (refer to Hall, 1992). In anaerobic systems, nitrogen can occur in a variety of inorganic forms such as free ammonia (NH_3), nitrate (NO_3^-), nitrite (NO_2^-) and nitrogen gas (N_2). Nitrogen is usually required in the reduced form as NH_3 or organic amino nitrogen ($NH_4 – N$), as NO_3^- and NO_2^- are likely to be lost through denitrification (Rittmann and McCarty, 2001). Furthermore, it is required in excess of about 50 mg/L to prevent it becoming rate limiting (Rittmann and McCarty, 2001).

As shown in Table 2.3, anaerobic systems also have a nutritional requirement for sulphur. Domestic wastewater generally has sufficient quantities of sulphur in the form of sulphate and therefore supplementation is usually unnecessary (Rittmann and McCarty, 2001). However, if the concentration is too high, reactor performance can be affected, as SRB compete with methanogens for the same substrates. Furthermore, sulphides can be formed from sulphate, which are toxic to methanogens (Lubberding, 1998). Sulphides also form insoluble complexes or chelates with essential trace metals, which then become unavailable for use by methanogens (Rittmann and McCarty, 2001).

According to Speece (1996), the often overlooked influence of trace metals in anaerobic digestion is the reason why anaerobic processes have failed in the past, which subsequently led to a lack of confidence in the process. Trace metals such as iron, nickel, cobalt and zinc are stimulatory to the many reactions and have been implicated in several enzymatic systems of anaerobic microorganisms (Pohland, 1992; Rittmann and McCarty, 2001; Speece, 1996). Nickel, for example, is used by methanogens for growth and synthesis of cofactor F_{430}, which is involved in biogas formation (Diekert *et al.*, 1981), and has been shown to increase the acetate utilisation rate of methanogens by up to five-fold (Speece *et al.*, 1983). Acetogenic bacteria have also shown a similar requirement for nickel for growth (Diekert and Ritter, 1982).

The desired concentration of each trace metal differs considerably, with the requirement for iron being the highest (Table 2.3). Speece (1996) emphasises that the presence of trace metals in the anaerobic reactor does not necessarily indicate that they are readily available to organisms. Although trace metals may be physically present, they can often be in the form of insoluble complexes and this should be kept in mind (Speece, 1996).

Finally, anaerobic systems require a balance of several common cations, such as sodium, potassium, calcium and magnesium (Table 2.3). There is usually a good balance in mixed substrate systems, such as domestic wastewater, but problems occasionally occur when one of the cations is significantly higher compared to the others. When this occurs, it may require supplementation of the other cations to correct the imbalance (Rittmann and McCarty, 2001).

2.4.4 Toxicants

The term ‘toxicity’ is a relative term, which can be applied to numerous chemicals and substances, including those which are stimulatory at low concentrations (Gunnerson and Stuckey, 1986). The effect of toxicants is usually indicated by a reduction in methane production and an accumulation of volatile acids (Bitton, 1994). It is not the purpose of this

section to outline all substances that cause inhibitory effects. Only those relevant in the anaerobic digestion of domestic wastewater will be considered. These include the presence of oxygen and sulphides, and high concentrations of intermediate by-products (H_2, VFA).

Oxygen

Methanogens are notorious for being the strictest of anaerobes and will not grow and produce methane in the presence of oxygen (Zinder, 1994). The presence of oxygen can cause irreversible dissociation of some enzymes and important cofactors, such as F_{420} (Anderson *et al*., 2003). It also has the capacity to increase the standard redox potential in the environment, with methane production being limited at around – 350 mV (Anderson *et al*., 2003). Oxygen generally enters anaerobic reactors through the feeding system as dissolved oxygen. However, this is usually not problematic, as facultative anaerobes metabolise the dissolved oxygen before toxic effects are noticeable downstream.

Sulphides

Sulphides (H_2S, HS^-, S^{2-}) are extremely toxic to most microbes, even at low concentrations (Speece, 1996). They are either introduced with the influent or produced through the reduction of sulphate (Lubberding, 1998). The sensitivity of anaerobic microorganisms to sulphides differs, with acidogens being less sensitive than methanogens. Within the former group, sensitivity also differs, with acetoclastic methanogens less sensitive than hydrogenotrophic methanogens (Rinzena and Lettinga, 1988). For the anaerobic digestion of domestic wastewater, sulphide toxicity is generally not a major problem as concentrations are usually well below the minimum concentration for noticeable toxicity. Problems can occur if the sulphide concentration reaches 50 mg/L, and becomes toxic to methanogens when concentrations over 150 mg/L are reached (Bitton, 1994; Lubberding, 1998). Sulphide inhibition can also arise through competition between SRB and methanogens for common substrates (acetate and hydrogen), and through the precipitation of essential trace elements in the form of insoluble metal complexes (Anderson *et al*., 2003).

Volatile Fatty Acids (VFA)

High concentrations of VFA are generally observed during start-up of an anaerobic system or during organic overload and are usually associated with toxicity and inhibitory effects. Although it is generally believed that VFA inhibition is due to their accumulation and subsequent pH reduction, some VFA are themselves toxic to anaerobic microbes (Anderson *et al*., 2003). Volatile acids, such as acetic and butyric acid, are slightly toxic to methanogens near neutrality, whilst propionic acid can be toxic to both acidogens and methanogens (Bitton, 1994). Long-chain fatty acids (caprylic, capric, lauric, myristic and oleic acids) produced in

hydrolysis have also been shown to inhibit acetoclastic methanogens (Koster and Cramer, 1987).

Ammonia

Ammonia is produced from the degradation of amino acids, proteins, methylamines and other nitrogenous compounds (Anderson and Yang, 1992). It forms part of the bicarbonate buffer system with CO_2 and is stimulatory at low concentrations. However, at high concentrations, process failure can result through ammonia toxicity. This occurs when the feed contains high concentrations of proteins.

Ammonia is present either in the form of ammonium ion (NH_4^+) or free ammonia (NH_3) in anaerobic reactors (Anderson *et al*., 2003). The pH of the system determines which species predominates. At pH values around neutrality, over 99% of NH_3-N is in the form of the less toxic NH_4^+ (Anderson *et al*., 2003). When the pH reaches 8.0, the equilibrium shifts towards the more toxic NH_3. Free ammonia concentrations around 100 mg/L are known to cause inhibition, whilst NH_4^+ concentrations that cause inhibition are much higher, around 3 000 mg/L (Rittmann and McCarty, 2001).

2.5 Evaluating and Monitoring the Performance in Anaerobic Processes

Wastewater contains a number of contaminants, including: biodegradable organics, volatile organic compounds, nutrients, suspended solids (SS), pathogens, and toxic metals (Bitton, 1994). The measurement of these pollutants provides useful insight on the performance of an anaerobic system, as the efficiency of the process is often dictated by their removal. By-products generated from the process (VFA, CH_4) may also be used as performance indicators. Numerous tests are available for measuring these parameters in wastewater samples. Some of these tests have become standardised methods used by various governmental and private laboratories (Bitton, 1994). The section below outlines some of the more routinely measured parameters in assessing the performance of anaerobic reactors.

2.5.1 Physico-chemical analyses

Physico-chemical parameters have been traditionally used to monitor the performance of various wastewater treatment systems. The advantages of these methods are that it allows relatively fast and cheap quantification of process stability and performance.

Organic Content

Domestic wastewater is primarily composed of organic compounds, such as carbohydrates, amino acids, peptides and proteins, and fatty acids and their esters (Bitton, 1994). The measurement of the organic matter in wastewaters is of primary interest, as it describes the metabolism of organic matter by microorganisms present in the system (van Haandel and Lettinga, 1994). As it is impractical to measure all these compounds individually, three main tests are used for determining the organic matter in wastewater. These include the COD, biochemical oxygen demand (BOD), and total organic matter (TOC) tests. The first two tests (COD and BOD) are based on the oxidation of organic matter, a property of all organic compounds, whilst the third (TOC) is based on the determination of the organic carbon concentration (van Haandel and Lettinga, 1994). The COD test is often chosen over the other tests, as they are more accurate and can be carried in a relatively shorter time (van Haandel and Lettinga, 1994).

Gas production

The relative proportions of gases produced during anaerobic digestion provide useful insight to the efficiency of the degradation process. Methane and CO_2 are produced in the largest quantities and are therefore the easiest to determine. Decreases in methane production or sudden increases in the CO_2 of the gas indicate instability (Malina, 1992). Two techniques are employed to measure gas content. These include: gas chromatographic techniques or volumetric methods (APHA-AWWA-WEF, 1998).

pH Value, Alkalinity, and Volatile Fatty Acids (*VFA*)

As mentioned earlier, the values of pH, bicarbonate alkalinity (acid neutralising capacity) and VFA concentration have significant influence on the balance of the anaerobic process. For this reason, they are often measured to provide an indication of the reactor environment and performance. Furthermore, they serve as warning signals of digester failure, as changes in these parameters occur faster than a decrease in gas production (Malina, 1992). The methods used for determining these parameters is relatively simple and inexpensive. The pH value can be obtained from electronic pH meters. Alkalinity can be determined by titrating the sample with a standard sulphuric acid solution to an end point (pH 4.5) (Malina, 1992). The concentration of VFA in the reactor can be measured using gas chromatography techniques, distillation or direct titration methods (APHA-AWWA-WEF, 1998; Malina, 1992).

Nutrients

Although anaerobic digestion does not have any significant effects on the final concentration of nutrients (Lettinga and Hulshoff Pol, 1992; van Haandel and Lettinga, 1994), their

measurement is nevertheless necessary. First, it allows nutrient-deficiency or nutrient-overload conditions to be predicted or established. Secondly, it determines whether post-treatment is required. Concentrations of nitrogen, phosphorus, and to a lesser extent, potassium are routinely measured. There are numerous methods used for the determination of nitrogen in the form of mineral ion (NH_4^+) or oxidised nitrogenous compounds (NO_2^- and NO_3^-), including spectrophotometric and tritration methods, and in some cases, electrode methods (APHA-AWWA-WEF, 1998).

Solids

The presence of solids in wastewater is not particularly desirable as it can adversely affect the anaerobic process in several ways. The anaerobic degradation pathway, especially hydrolysis, can become rate-limited, in reactors treating wastewaters with a high concentration of solids, especially if the solids are poorly degradable. Other disadvantages are that it can lead to formation of a scum layer (floating layer consisting of suspended fats and lipids), accumulate in the reactor and contribute to excess sludge, slow down or inhibit the formation of flocculent or granular sludge by attachment, and contribute to 'washout' (Lettinga and Hulshoff Pol, 1992). For this reason, the determination of solid concentrations is important. Commonly used tests include: total dissolved solids, total suspended solids (TSS), total solids (TS) (total dissolved solids + TSS), volatile suspended solids [(VSS) (provides rough estimation of the amount of organic matter present)] (APHA-AWWA-WEF, 1998).

2.5.2 Pathogen analyses

Pathogens are not routinely measured in anaerobic reactors, except from those that treat various sludges to be re-used in land application. Various methods are available to detect pathogens in water including: concentration in culture media, and molecular tools [monoclonal antibodies, polymerase-chain reaction (PCR) or gene-probing] (Bitton, 1994). As the isolation and identification all pathogens of concern can be costly, laborious, and time-consuming, 'indicators' of faecal contamination are often used instead (Bitton, 1994). Indicator organisms should preferably be non-pathogenic members of the intestinal tracts of warm-blooded animals (Horan, 2003). Other requirements include: suitable for all categories of water, should be present in greater numbers than pathogens; have similar survival rates as pathogens in waters and wastewater treatment processes; able to be detected in low numbers reliably using rapid and inexpensive methods; and should not multiply in the environment (Bitton, 1994; Horan, 2003).

Not one organism has been identified that bears all these essential features (Horan, 2003). Some indicators, however, encompass most of the properties of an ideal indicator and are

standard methods for detecting for microbiological contamination (APHA-AWWA-WEF, 1998). Listed below are some indicators commonly used in water analysis:

- Coliform bacteria – Most widely used indicators in water and wastewater analysis, among which *E. coli* are the most numerous in faecal matter.
- Faecal entercocci – Involves the detection of two genera, *Enterococcus* and *Streptococcus*. Most suitable indicator of faecal pollution as they fulfil most of the requirements for an ideal indicator (Horan, 2003; Larsen *et al*., 1994). However, the method of isolation is not very reliable and needs to be modified (Horan, 2003).
- Bacteriophages – Used as indicators of enteroviral pollution, and include male (F^+) -specific bacteriophages and somatic coliphages.
- Heterotrophic bacteria – Commonly called the heterotrophic plate count (HPC), it is defined as the total number of bacteria that can grow at 35^o C for 48 h. Technique routinely used to determine the water quality in distribution systems.
- Helminth eggs – Routinely used as indicators of parasitic contamination.

2.6 Anaerobic Reactor Configurations

Anaerobic digestion has been traditionally used for the treatment of high-strength wastewaters. However, recent advances in anaerobic reactor design and understanding of the processes that occur in anaerobic digestion have enabled engineers to treat a variety of dilute wastewaters economically. This has been achieved by maintaining a high sludge retention time (SRT) in relation to the hydraulic retention time (HRT) (Anderson *et al*., 2003). This has allowed slow growing anaerobic microorganisms to remain within the reactors independent of wastewater flow, thereby allowing higher volumetric loading rates (Anderson *et al.*, 2003). Increased reaction rates per volume of reactor can be achieved, allowing smaller reactors to be built. To understand why certain high-rate reactors are more appropriate than others in treating domestic wastewater, a thorough understanding of the different reactor designs is required. The following section therefore outlines a brief historical overview of successive anaerobic reactor designs that have been implemented over the years, and their suitability to treat domestic wastewater.

2.6.1 Early developments

The Mouras automatic scavenger was presumably the first anaerobic reactor type developed for sewage treatment (van Haandel and Lettinga, 1994). The reactor was developed in 1881 by the French inventor, M. Louis Mouras, and consisted of an airtight chamber, which was used to liquefy settleable solids from sewage. Around the turn of the century, several new reactor types were developed, including the septic tank and Imhoff tank.

Septic Tank

In 1891, the first septic tank to retain sewage solids was constructed by W.D. Scott in England. It is the simplest and most widely used anaerobic process (Jewel, 1987). The system consists of a buried tank constructed out of concrete, metal, or fibreglass, and a subsurface drainage system, which treats the effluent as it percolates into the soil. Wastewater (blackwater and greywater) enters the reactor, where grease and oil in the wastewater forms a scum layer (Wright, 1999). Often septic tanks contain vertical baffles or grease traps to prevent the scum layer or settable solids from being discharged (Figure 2.2) (McKinney, 1962). These traps are not necessary for residential septic tanks, but rather for those institutions, which generate wastewater with a high fat content (restaurants, hotels) or other foreign materials (hospitals) (Wright, 1999).

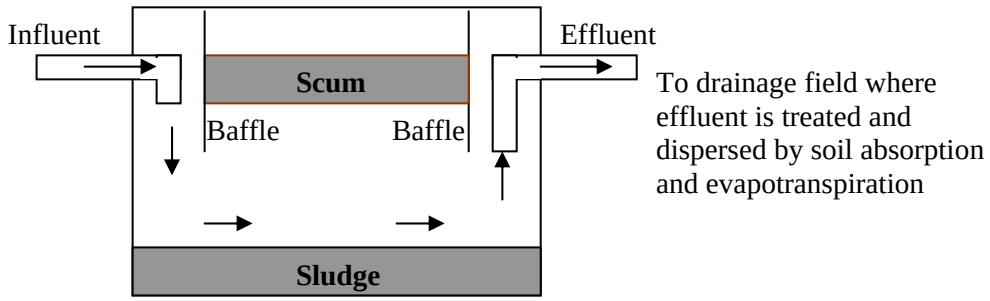


Figure 2.2: Schematic cross-section through a conventional septic tank used for the treatment of domestic sewage (adapted from McKinney, 1962).

The primary function of the tank is to contain settleable solids in the sewage, and treat incoming wastewater by anaerobic digestion. In this way, raw sewage is conditioned to reduce clogging of the drainage system (Wright, 1999). Anaerobic sludge or septage are produced at the bottom of the tank by sedimentation of settleable solids, and eventually becomes compacted by the weight of the liquid and developing layers of sludge. Anaerobic microorganisms in the reactor degrade the organic matter in the sludge. Approximately 1 000 – 2 000 gallons of septage are generated per tank every 2 to 5 years, and has to be disposed of either by land application or introduced with wastewater to be treated by treatment works (Bitton, 1994).

The average retention time of wastewater within the reactor varies between 1 to 4 days (Bitton, 1994; McKinney, 1962). BOD and suspended solid (SS) removals between 65 – 80% and 70 – 90% are achievable, respectively, depending on the ambient temperature. Pathogen

removal, however, is not significant and a large number of viruses, bacteria, protozoa and helminths still persist in the effluent, scum and sludge (Wright, 1999). Another disadvantage of the septic tank is that retention of solids is affected by gas production in the septage layer. Further treatment is therefore necessary and usually takes place in the drainage field.

The effluent enters the drainage field through a series of perforated pipes, and is allowed to percolate towards the groundwater. The drainage field consists of either a soakaway (trench, bed, seepage pit, mound or fill) or an artificially drained system (Wright, 1999). The soil acts as a filter, removing any remaining solids and microbial contaminants in the effluent (Wright, 1999). The drainage field is an integral part of the septic tank system and its functioning is dependent on a number of factors, such as wastewater characteristics, rate of wastewater loading, geology, and soil characteristics (Bitton, 1994).

The risk of groundwater contamination is high, as not all of the remaining contaminants in the effluent are removed by the soil. According to Bitton (1994), septic tank effluents are responsible for a large percentage of disease outbreaks resulting from the consumption of untreated groundwater and are probably the major contributors to of enteric viruses found in subsurface environments. In South Africa, certain municipalities, due to past experiences, do not allow the construction of drainage fields. Instead, municipalities pump out the effluent and combine it with municipal wastewater at the nearest wastewater treatment plant or dispose of it by land application (surface spreading or infiltration in open trenches) (Wright, 1999). This procedure can be costly to the municipality, and as Wright (1999) points out, the danger of this method is that it assumes that the tank has been properly constructed. This is usually not the case as some old tanks have leaks, which are not monitored.

Imhoff tank

The Imhoff tank was developed by Karl Imhoff in Germany to anaerobically treat solids from domestic sewage in the same tank used for settling (McKinney, 1962). The reactor found widespread use in Germany and was used in the construction of a wastewater treatment works in Chicago in 1935, which at the time, was one of the largest sanitary engineering undertakings (van Haandel and Lettinga, 1994). Unlike the septic tank, the Imhoff system consists of two zones: a settling zone and a digestion zone (Figure 2.3). The settleable solids present in the sewage will sediment into a lower compartment containing anaerobic sludge, where it will undergo anaerobic degradation. This division allows gas produced by methanogens in the anaerobic sludge to escape through separate gas vents. Consequently, gas production is prevented from interfering with solid settling, as experienced with septic tanks (van Haandel and Lettinga, 1994). Biological activity is therefore more improved than in

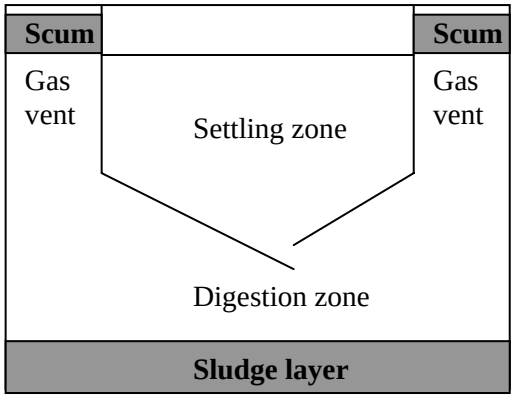


Figure 2.3: Schematic cross-section of a conventional Imhoff tank used for the treatment of domestic sewage (adapted from McKinney, 1962).

septic tanks and there are fewer 'washouts' of methanogens and the substrates that contribute to their growth (McKinney, 1962). Sludge generated by the Imhoff tank must be regularly removed every 6 to 12 months. Other disadvantages include cost of construction, slowness of the digestion process and the accumulation of scum at gas vents (McKinney, 1962).

The major criticism levelled against these earlier treatment systems was the efficiency at which organic matter was removed. These systems are based on the settling and anaerobic digestion of the suspended organic fraction. As only a third to a half of domestic sewage is settable, the maximum biodegradable organic removal that could be achieved was between 30 - 50% (van Haandel and Lettinga, 1994). Most of the organic matter, either in dissolved or hydrolysed form, remains unavailable for use and degradation by anaerobic microorganisms, and remains in the system. The inadequate contact between anaerobic microorganisms and the influent organic matter resulted in poor reactor performance, which led to the belief that anaerobic systems are inferior in performance to aerobic systems (van Haandel and Lettinga, 1994). It is now known that the failures experienced in the past were due to design 'flaws' rather than the process itself.

Designers have subsequently recognised the need to increase the contact between incoming wastewater and maintain a high retention of viable microorganisms in the sludge. This has resulted in a succession of new reactor configurations or modification of previous reactor types, each trying to increase or improve the above-mentioned parameters for greater efficiency. The reactor configurations are displayed in Figure 2.4. Despite the fact that many new reactor designs are available, few have found full-scale application in the treatment of domestic sewage.

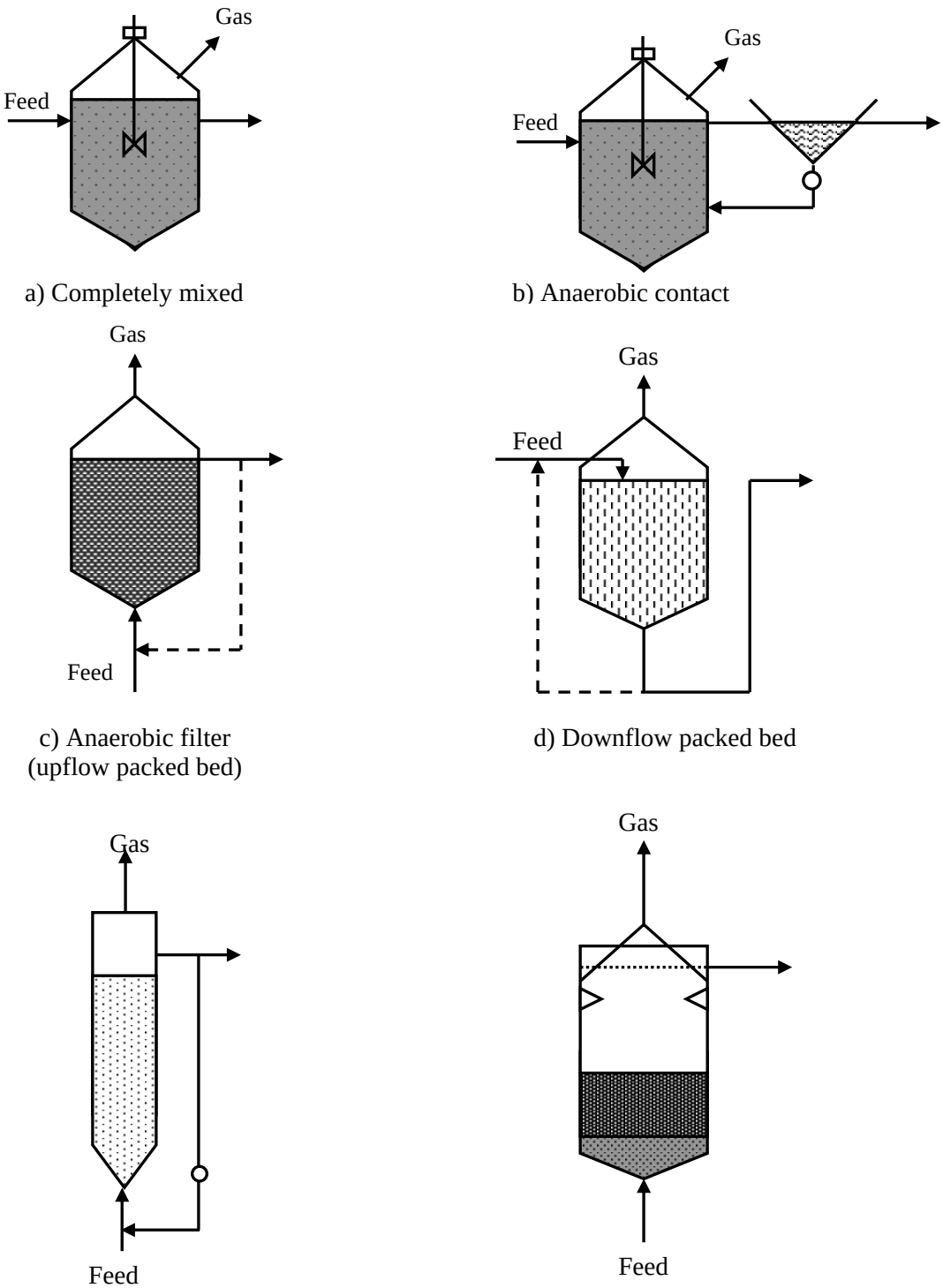


Figure 2.4: Typical anaerobic configurations used in wastewater treatment (Anderson *et al.*, 2003; Rittmann and McCarty, 2001).

2.6.2 Conventional or completely mixed anaerobic digester

The conventional anaerobic process represents the most basic anaerobic treatment system and is commonly used for the treatment of wastewater sludges (Rittmann and McCarty, 2001). It can be operated either in batch or continuous mode, and consists of a single chamber in which solids are mechanically mixed (Figure 2.4a). The mixing increases the contact between the incoming wastewater and anaerobic microorganisms, and prevents the settling of particulate matter, such as sand and grit, which decreases the volume available for treating wastewater (Rittmann and McCarty, 2001). Once the organics are reduced to acceptable levels, the wastewater is discharged from the reactor.

A major disadvantage of the completely mixed system is that the SRT is equal to HRT (Anderson *et al.*, 2003; Hall, 1992). As methanogens have slow doubling times, the HRT and SRT must be at least ten days to prevent 'washout' (loss of methanogens). Consequently, a large reactor is required to accommodate for loss of methanogens (Anderson *et al.*, 2003). Furthermore, as the concentration of the substrate entering a completely mixed system is decreased to its effluent concentration, the reaction rates are decreased accordingly (since pseudo first order kinetics rely on the substrate concentration). Another disadvantage is the poor efficiency at which dilute wastewaters are treated. Treatment of high-strength wastewater (8 000 to 50 000 mg COD/L) is necessary to achieve a high loading per unit volume (Rittmann and McCarty, 2001). Hence, the treatment of dilute wastewaters such as domestic wastewater with a conventional anaerobic digester is neither a practical, nor an economical option.

2.6.3 Anaerobic contact process

The anaerobic contact process is similar in design to the aerobic activated sludge system. The process was developed by Schroepfer and Stander for the treatment of dilute wastewater (1 300 mg COD/L) from house wastes (Rittmann and McCarty, 2001). The reactor overcomes some of the disadvantages of the completely mixed system by recycling effluent suspended solids back to the main tank (Figure 2.4b). This allows for further contact between the biomass and raw waste (Anderson *et al.*, 2003; Hall, 1992). The SRT can then be controlled independently from the HRT, which results in improved removal efficiencies. Furthermore, it allows a smaller tank to be built (Anderson *et al.*, 2003; Hall, 1992).

The process can be applied over a wide range of wastewater concentrations. The digestion process, however, is economically more viable at very high wastewater concentrations (Hall, 1992). A major limitation of this process is a reliance on favourable settling of anaerobic sludge by gravity. This is not often the case, as biosolid loss often occurs, through gas

production by anaerobic flocs and anaerobic microorganisms in the sludge of the settling tank (Anderson *et al*., 2003; Hall, 1992; Rittmann and McCarty, 2001). Biosolid loss is a much bigger problem in anaerobic processes than aerobic processes for the simple reason that the growth rates of methanogens are much longer. This problem has been minimised by a number of approaches, including vacuum degasification, the use of flocculating agents, or inserting inclined baffles in the tank to encourage sedimentation (Anderson *et al*., 2003). These modifications, unfortunately, increase the construction and operating costs of the process.

2.6.4 Upflow and downflow anaerobic packed bed reactors

The initial concept of the upflow anaerobic packed bed or anaerobic filter (AF) was developed by Coulter and co-workers and introduced by Young and McCarty in 1969 (Anderson *et al*., 2003; van Haandel and Lettinga, 1994). The system relies on sludge entrapment by microbial attachment in the form of a biofilm on support material present in the reactor and between the interstices of support material (Lettinga and Hulshoff Pol, 1998). A schematic representation of the reactor is presented in Figure 2.4c. Wastewater is treated as dissolved pollutants are absorbed by the biofilm (Anderson *et al*., 2003). Early systems used rocks as the support material. At present, synthetic materials, such as plastics and reticulated foam particles are used, as they have higher void volumes and greater surface areas (Anderson *et al*., 2003; Rittmann and McCarty, 2001). The cost of the support material, however, can be as high as the construction cost of the reactor itself (Speece, 1996).

A major concern in using the AF for anaerobic treatment is that clogging of bed structure can occur through accumulation of biosolids, influent suspended solid and precipitated minerals (Rittmann and McCarty, 2001). Thus, the use of the AF is therefore not practical for the direct treatment of domestic wastewater (as it contains particulate matter). Consequently, it is commonly used as a secondary treatment from other high-rate anaerobic system, such as the UASB (Anh *et al*., 2003).

This blockage problem can be overcome by operating the packed bed reactors in a downflow mode; hence the name downflow anaerobic packed bed reactors (Figure 2.4d). By operating in a downflow mode, solids accumulate at the top end of the reactor, where there is more substrate available and biological growth is greater (Rittmann and McCarty, 2001). Solid removal is made easier at the top of the reactor through gas re-circulation, and sulphides, which are normally produced in the upper portion of the reactor, are stripped from the liquid before toxicity effects become apparent (Rittmann and McCarty, 2001). The benefit of operating in a downflow mode, however, may be negated through loss of support media, and biosolids to the effluent.

2.6.5 Anaerobic fluidised and expanded bed reactors

The fluidised bed (FB) system was originally developed for removal of nitrate from wastewater but has found application in the anaerobic treatment of wastewaters. Developed by Jeris in 1974 and modified by Jewel *et al.* (1987), the reactor is similar in principle to the anaerobic filter as it relies on the attachment of microbes to support media in the form of sand, plastic and granular activated carbon (Figure 2.4e) (Anderson *et al*., 2003; Rittmann and McCarty, 2001; van Haandel and Letting, 1994). The support media is specifically designed as small particles with high surface: volume ratios. In this way, microbial attachment is maximised, whilst the volume occupied by the media is minimised (Anderson *et al.*, 2003). However, unlike the anaerobic filter, clogging of the sludge bed by fine suspended solids is prevented by operating the reactor at relatively high upflow velocities. The high upflow velocities also encourage mass transfer between particles and dissolved organic matter (Rittmann and McCarty, 2001). Additionally, it determines whether the process is called a FB process or expanded bed process.

Both processes are similar in design with the only differences being the rate of liquid flow and the resulting degree of bed expansion (Anderson *et al*., 2003). Expanded beds are operated at much lower upflow velocities. As a result, bed expansion is much smaller in expanded bed processes (10 - 20%) than FB processes (30 - 90%) (Anderson *et al*., 2003). Possible advantages of using the expanded bed process in comparison to FB process include: higher solids capture and smaller requirement to maintain high recycle rate. Possible disadvantages include: mass transfer not being as great as in FB process, greater likelihood of clogging, and removal of biofilm through abrasion may be greater (Rittmann and McCarty, 2001).

The most undesirable aspect of the application of both systems is the loss of biomass particles from the reactor following sudden changes in particle density, flow rate or gas production (Anderson *et al*., 2003). The control of particle density is very important to the efficiency of the process, yet it is practically impossible to control, as biomass growth is variable. Loss of microorganisms on the outer surface of the support material can often occur through abrasion between particles and fluid shear stress (Rittmann and McCarty, 2001). This is perhaps the major limitation of using the FB and expanded bed systems. Other disadvantages include: lengthy start-up periods, high power requirements for fluidisation or bed expansion, and unsuitability for wastewater with high suspended solids, complex mechanical system design, and high cost of support media (Hall, 1992).

As far as treatment of domestic wastewater is concerned, there have been no full-scale applications used so far, with the process limited to few pilot-scale and laboratory-scale experiments (van Haandel and Lettinga, 1994).

2.6.6 Upflow anaerobic sludge blanket (UASB) reactors

The upflow anaerobic sludge bed (UASB) reactor was developed in the early seventies by Lettinga and colleagues (Lettinga and Hulshoff Pol, 1998; Rittmann and McCarty, 2001; Seghezzo *et al.*, 1998; van Haandel and Lettinga, 1994). The reactor consists of a bottom layer of packed sludge, a sludge blanket, and an upper liquid layer (Figure 2.4f). To date, it has been the most successful and widely-used reactor type, and has been used for the treatment of both industrial and domestic wastewater (Rittmann and McCarty, 2001).

The sludge retention of the UASB reactor is based on the principle that anaerobic sludge has inherently good settling properties, provided that it is not exposed to mechanical agitation (Anderson *et al.*, 2003). Good contact between the wastewater and the sludge is maintained by even flow distribution at the bottom sludge layer of the reactor, coupled with a satisfactory high upflow velocity, as well as, natural mixing through gas production (Lettinga and Hulshoff Pol, 1998). Pollutants within the wastewater are treated as it flows upwards through the sludge bed that consists of a floating layer of active microbial flocs (Bitton, 1994). Eventually, these microbial flocs will grow and form well-defined, compact, spherical granules. The enhanced retention of sludge in the form of granules is the major advantage of the UASB over other anaerobic reactor types (Macleod *et al.*, 1990).

These granules consist of a small ash content (10%) and a mixed population of anaerobic microorganisms (Rittmann and McCarty, 2001). They have superior settling characteristics because of their large size, which allow higher sludge retentions, and consequently, more efficient digestion rates (Anderson *et al.*, 2003). Furthermore, they are extremely stable and can withstand high mixing forces (Lettinga *et al.*, 1980). The stability of the granules is based on the self-immobilisation of various kinds of anaerobic microorganisms associated with methanogenic fermentation. Granules can vary in size and composition depending on wastewater and reactor conditions, but are generally spherical with a diameter of 1-3 mm (Uyanik, 2003). The successful operation of an UASB reactor is very much dependent on their formation. Consequently, various studies have attempted to optimise granulation for the treatment of various wastewaters (Britz *et al.*, 1999; 2002).

The anaerobic treatment of domestic wastewaters in a laboratory-scale, pilot-scale and full-scale UASB reactors has received considerable attention (review by Seghezzo *et al.*, 1998). A

Table 2.4: Summary of published results of UASB reactors treating domestic wastewater (adapted from Seghezzo *et al*., 1998).

Place	Vol. (m^3)	Temp. (^oC)	Influent concentration (mg/L)			Inoculum	HRT (h)	Removal efficiencies (%)			Start-up (months)	Period (months)	Reference
			COD	BOD (COD_{sol})	TSS			COD	BOD (COD_{sol})	TSS			
South Africa	0.008	20	500	(148)	NP	Activated sludge	24	90	(49)	60-65	1	1	Pretorius, 1971
Netherlands	0.030	21	520-590	(73-75)	NP	Digested sewage sludge	9	57-79	(50-60)	30-70	NP	1	Lettinga *et al*., 1983
Netherlands	0.120	18-20	248-581	(163-376)	NP	Granular sludge	12	72	(62)	NP	NP	17	Lettinga *et al*., 1983
Netherlands	0.120	7-18	100-900	53-474	100-700*	Granular sludge	4-14	45-72	(38-59)	50-89	NP	12	de Man *et al*., 1986
Netherlands	6	10-18	100-900	53-474	100-700*	Granular sludge	9-16	46-60	(42-48)	55-75	NP	12	de Man *et al*., 1986
Netherlands	20	11-19	100-900 150-5500	53-474 43-157	100-700* 50-400*	Granular sludge	6.2-18	31-49	(23-46)	NP	NP	12	de Man *et al*., 1986
Colombia	64	25	267	95	NP	Digested cow manure	6-8	75-82	75-93	70-80	6	9	Louwe Kooijmans & van Velsen, 1986; Lettinga *et al*., 1997
Netherlands	0.120	12-20	190-1180	(80-300)	NP	Granular sludge	7-8	30-75	(20-60)	NP	NP	NP	de Man *et al*., 1988
Netherlands	0.116	12-20	150-600	(70-250)	NP	Granular sludge	2-3	NP	(20-60)	NP	NP	NP	de Man *et al*., 1988 (EGSB reactor)
Mexico	0.110	12-18	465	NP	154	Adapted aerobic sludge	12-18	65	NP	73	NP	> 12	Monroy *et al*., 1988
Brazil	0.120	19-28	627	357	376	None	4	74	78	72	4	9	Barbosa & Sant'Anna, 1989
Italy	336	7-27	205-326	55-153	100-250	None	12-42	31-76	40-70†	55-80†	NP	12	Collivignarelli *et al*., 1991; Maaskant *et al*., 1991
India	1200	20-30	563	214	418	None	6	74	75	75	2.5	12	Draaijer *et al*., 1992
Netherlands	120	> 13	391	(291)	-	Granular sludge	2-7	16-34	(20-51)	None	NP	35	van der Last & Lettinga, 1992
Netherlands	205	16-19	391	(291)	-	Self cultivated on sand	1.5-5.8	? 30	(? 40)	None	NP	33	van der Last & Lettinga, 1992 (EGSB reactor)
Colombia	65	NP	NP	NP	NP	NP	5-19	66-72	79-80	69-70	NP	48	Schellinkhout & Collazos, 1992
Netherlands	1.2	13.8	976	454	641*	Digested sewage sludge	44.3	33	50	47*	NP	28	Bogte *et al*., 1993 (UASB-septic tank)
Netherlands	1.2	12.9	821	467	468*	Digested sewage sludge	57.2	3.8	14.5	5.8*	NP	24	Bogte *et al*., 1993 (UASB-septic tank)
Netherlands	1.2	11.7	1716	640	1201*	Granular sludge	202.5	60	50	77.1*	NP	13	Bogte *et al*., 1993 (UASB-septic tank)
Indonesia	086	NP	NP	NP	NP	NP	360	90-93	92-95	93-97	NP	60	Lettinga *et al*., 1993 (UASB-septic tank, blackwater)
Indonesia	0.86	NP	NP	NP	NP	NP	34	67-77	Up to 82	74-81	NP	60	Lettinga *et al*., 1993 (UASB-septic tank, gey + blackwater)
Thailand	0.030	30	450-750	NP	NP	Different sludges	3-12	90	NP	NP	> 2	4	Gnanadipathy & Polprasert, 1993
Brazil	120	18-28	188-459	104-255	67-236	Granular sludge	5-15	60	70	70	> 2	24	Vieira & Garcia, 1992
Columbia	3360	24	380	160	240	None	5	45-60	64-78	? 60	> 6	> 36	Schellinkhout & Osorio, 1994
Brazil	67.5	16-23	402	515	397	Digested sludge	7	74	80	87	NP	14	Vieira *et al*., 1994
Netherlands	0.200	15.8	650	346	217	Digested sludge	3	37-38	26.6	83	None	5	Wang, 1994 (HUSB reactor)
Netherlands	0.120	15.8	397	254	33	Granular sludge	2	27-48	(32-58)	NP	None	3	Wang, 1994 (EGSB)
Puerto Rico	0.059	? 20	782	352	393	Digested sludge	6-24	57.8	NP	76.9	? 4	16	Tang *et al*., 1995
India	12000	18-32	1183	484	1000	NP	8	51-63	53-69	46-64	5	13	Haskoning, 1996a; Tare *et al*., 1997
India	6000	18-32	404	205	362	NP	8	62-72	65-71	70-78	5	11	Haskoning, 1996b; Tare *et al*., 1997
Brazil	477	NP	600	NP	303	Non adapted sludge	13	68	NP	76	2	> 7	Chernicharo & Borges, 1997
Spain	2	20	220-985	63-523	116-336	Mixed mesophilic sludge	5-24	53-85	NP	63-89	NP	> 7	Ruiz *et al*., 1998

NP: not provided; $_{sol}$: soluble; [a]: air temperature; *: expressed as COD; †: obtained at temperatures of 15 – 20^{o}C, HRT = 12 h and V_{up} = 0.58.

summary of the performance of UASB and hybrid variants of the reactor in many countries is presented in Table 2.4. COD removal efficiencies tend to be greater in countries with higher temperatures (> 20^{o} C). In some cases, COD removals exceeding 90% have been achieved (Gnanadipathy and Polprasert, 1993; Lettinga *et al.*, 1993; Pretorius, 1971). The UASB has so far been the most successful anaerobic reactor used in domestic wastewater treatment. However, the process is heavily dependent on the formation and integrity of granules, which may not always be reach desirable levels. Other disadvantages include the: need for electricity to pump wastewater through reactor, and clogging problems occurring when flow is stopped (example: electricity failure).

2.7 Anaerobic Baffled Reactor (ABR)

This section provides a brief review of the development, performance and applications of the anaerobic baffled reactor (ABR) from the literature. The basic design of the reactor consists of a cascade of vertical baffles, where wastewater is forced through compartmental sludge beds at the bottom of the reactor, as it moves from the inlet to outlet (Figure 2.5) (Skiadas *et al.*, 2000). The microorganisms within the reactor tend to rise and settle with gas production, and move horizontally at a relatively slow rate (Nachaiyasit and Stuckey, 1997a; Xing *et al.*, 1991). The wastewater can therefore come into contact with a large amount of active biomass, providing high treatment rates.

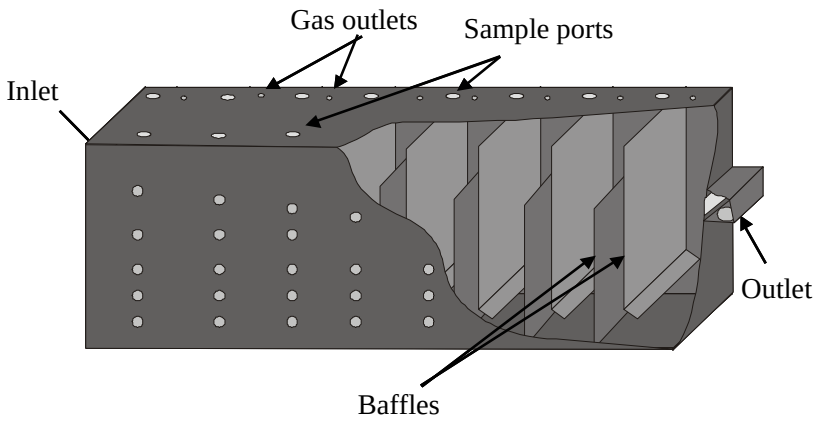


Figure 2. 5: Schematic diagram of the ABR with cut-away, showing hanging and standing baffles (from Foxon *et al.*, 2004).

This compartmentalised design has numerous advantages (Barber and Stuckey, 1999; Bell, 2000). First, the washout of biomass is reduced as solids simply move from one compartment to the next (Polprasert *et al*., 1992). Secondly, it allows spatial separation of anaerobic microbial consortia. This has been shown to confer greater protection against toxic substances and changes to environmental parameters, such as pH and temperature (Barber and Stuckey, 1999, Bell, 2000). Furthermore, it may enhance the hydrolysis of particulate organics in the front of the reactor due to a low pH. The latter point is particularly important as domestic wastewaters contain more particulate organic compounds than soluble wastewaters (Eastman and Ferguson, 1981). Hydrolysis of particulate organics to soluble substrates is usually the rate-limiting step in the degradation process, and therefore, the degradation rate of particles in a reactor is usually slower than that of soluble organics (Eastman and Ferguson, 1981). For this reason, not all reactor types are suitable for treating dilute wastewaters containing particulate matter (Langenhoff *et al*., 2000). Phase separation in an ABR is thought to encourage the hydrolysis of particulate matter at a low pH, without affecting the methanogenesis phase (Langenhoff *et al*., 2000).

Besides these design advantages, the reactor was envisaged as an on-site sanitation solution because of its simple design and an absence of a special gas or sludge separation equipment (Bachmann *et al*., 1983; Polprasert *et al*., 1992; Yu *et al*., 1997). Furthermore, it may be operated without electricity as wastewater could be channelled to the reactor by gravity (Foxon *et al*., 2004). It has also been shown to be cost-effective at full-scale operation. In a study done in Colombia, it was estimated that the construction cost of an ABR was 20% less than that of an UASB, and fives times less than a conventional activated sludge plant serving a population of less than 2 500 people (Orozco, 1997).

2.7.1 Historical overview of the ABR

The anaerobic baffled reactor (ABR) was developed by McCarty and co-workers when they removed the rotating discs from a rotating biological contactor (RBC) (Bachmann *et al*., 1983). Various modifications have been made to the initial design to improve reactor performance, mostly through enhanced solid retention. The main alterations are summarised in Table 2.5. Some modifications, however, have been made in order to treat ‘difficult’ wastewaters, like those with a high solid content (Boopathy and Sievers, 1991) or to reduce costs (Orozco, 1997).

Table 2.5: Historical modifications of the ABR (Barber and Stuckey, 1999).

Modification	Purpose	Reference
Addition of vertical baffles to plug-flow reactor	Solid retention enhanced	Fannin *et al*., 1981
Downflow chambers narrowed	Retention in the upflow region increased	Bachmann *et al*., 1983
Edges on baffles slanted	Mixing is enhanced	Bachmann *et al*., 1983
Settling chamber included after last compartment	Solid retention enhanced	Tilche and Young, 1987
Packing positioned at the top of each chamber	Washout of solids prevented	Tilche and Young, 1987
Separated gas chambers included	Provided enhanced reactor stability by controlling gas measurement	Tilche and Young, 1987
Enlarged first compartment	Improved treatment of wastewaters with a high solid content	Boopathy and Sievers, 1991

2.7.2 Start-up

The start-up period is recognised as the most important step in the operation of an anaerobic system. The purpose of the start-up period is to establish the most appropriate microbial culture, either as a granular particle or floc, for the wastewater to be treated. Once established, the reactor operation is quite stable (Barber and Stuckey, 1999). The initial loadings should be low to prevent washout of slow-growing anaerobic consortia. Gas and liquid upflow velocities should also be low to encourage flocculent and granular growth (Barber and Stuckey, 1999). Initial loadings up to 1.2 kg COD/m^3 d have been cited as the recommended rate for anaerobic systems (Henze and Harremoes, 1983; Speece, 1996). Some success, however, has been achieved at higher loading rates in an ABR (Boopathy *et al*., 1988; Boopathy and Tilche, 1991). Many procedures are available to prevent overload during start-up. These include stimulating the growth of methanogenic microorganisms using methane precursors, such as acetate (Barber and Stuckey, 1999), and adjusting pH in the first compartment (Grobicki, 1989). Barber and Stuckey (1997a) have suggested initially maintaining a long retention time (80 h), and then reducing it in a stepwise fashion, whilst keeping the substrate concentration constant. This procedure was shown to enhance reactor stability and performance in comparison to another 'started-up' using a constant and low retention time (Barber and Stuckey, 1997a).

2.7.3 Low-strength treatment

Several authors have successfully treated various dilute wastewaters in an ABR (Hassouna and Stuckey, 2003; Langenhoff *et al*., 2000; Langenhoff and Stuckey, 2000; Orozco, 1988; Polprasert

et al., 1992; Witthauer and Stuckey, 1982). COD removal efficiencies of more than 90% have been achieved in some cases (Barber and Stuckey, 1999). At lower temperatures, the removal efficiency has been shown to decrease in the ABR (Langenhoff and Stuckey, 2000). However, the decline is not great, as COD removals up to 60% and 70% can be achieved at 10 and 20^{o} C, respectively. Separation of the different phases of anaerobic digestion, as shown by the microbial populations in each compartment, is not substantial when treating dilute wastewaters (Hassouna and Stuckey, 2003). Furthermore, the treatment of dilute wastewaters in an ABR has been shown to favour the dominance of scavenging microorganisms, such as *Methanosaeta* (Polprasert *et al*., 1992).

Low HRT are required, and sometimes necessary, for the treatment of dilute wastewaters in the ABR (Barber and Stuckey, 1999). Orozco (1988) showed that gas production decreased with increasing HRT, suggesting that biomass starvation possible occurs in later compartments at longer retention times. However, low HRT coupled with low loading rates can result in irregular COD removal and low sludge blankets (low active biomass) (Witthauer and Stuckey, 1982). Whitthauer and Stuckey (1982) have suggested starting-up with higher biomass concentrations than used in their study (3 g VSS/L) to overcome this problem.

The majority of studies concerning the application of an ABR in treating dilute wastewater has been performed on soluble wastewaters. In 2000, Langenhoff and co-workers sought to evaluate the performance of an ABR in treating dilute wastewater with a colloidal component (Langenhoff *et al*., 2000). In the study, the performance of identical ABRs in treating synthetic dilute soluble and colloidal wastewater was compared. The colloidal 'feed' was made up of blended dog food and rice with an average particle size of greater than 500 μm, whilst the soluble feed was composed of semi-skimmed milk (500 mg COD/L). The reactors were initially started with the HRT at 80 h, and then gradually reduced to 6 h. COD removal efficiencies were consistently greater than 80% for both 'feeds', at all HRT tested, and a 40% removal was even achieved at a HRT of 1.3 h. Furthermore, the study indicated that the soluble and colloidal feed were degraded to methane at a comparable rate, indicating that hydrolysis was not rate limiting in the degradation of colloidal feed.

2.7.4 'Shock' loadings (organic and hydraulic)

Grobicki and Stuckey (1991) investigated the effect of variation of organic and hydraulic loading rates on mass transfer, and reaction rate limitations in an ABR, and found that the reactors

recovered to their pre-shock levels within 24 h. (Grobicki and Stuckey, 1991). Langenhoff and Stuckey (2000) found that decreasing the HRT from 80 h to 10 h resulted in a temporary rise in COD in the effluent, but the reactor quickly recovered its removal efficiency (> 90%).

Nachaiyasit and Stuckey (1997a and b) conducted two comprehensive, parallel, studies to evaluate the effects of 'shock' loadings on the performance of an ABR. In the first study, they examined the effect of organic 'shock' loads in a laboratory-scale, 8-compartment ABR (Nachaiyasit and Stuckey, 1997a). The reactor was first operated at 20 h HRT, 4 000 mg/L COD at 35^{o} C for 1 month, which resulted in a 98% COD removal. Thereafter, the effect of organic 'shock' loads was examined by increasing the 'feed' concentration to 8 000 mg/L COD and 15 000 mg/L COD for 20 days each. When the 'feed' concentration was doubled, the reactor was stable with COD removal efficiencies remaining relatively unchanged, and the recovery from the 'shock', was rapid. These authors attributed this to a 'buffer' zone, created by the compartmentalised design, which was thought to absorb the overload and prevented most of the biomass from being exposed to low pH values, thereby enhancing reactor stability. In contrast, when the 'feed' concentration was increased to 15 000 mg/L COD, the reactor showed signs of 'overload'. Consequently, VFA were detected in the effluent and the COD removal efficiency decreased to 90%.

In the second study, they examined the effect of transient and stepwise hydraulic loads on ABR performance (Nachaiyasit and Stuckey, 1997b). Two laboratory-scale ABRs were operated at 20 h HRT, 4 000 mg/L COD at 35^{o} C for 1 month and showed a COD removal efficiency of 98%. Hydraulic 'shocks' with an HRT of 1 h, 5 h and 10 h were applied to reactors for 3 h, 3.5 weeks and 2 weeks, respectively. They found that the COD removal efficiency dropped when the HRT was lowered, with some loss of biomass occurring. However, the reactors were generally very stable to large transient 'shocks', and recovered back to its baseline values (98% COD removal) 9 h after the higher 'shock' loadings were ceased.

2.7.5 Effect of temperature

In order to evaluate the effect of low temperature on the performance of the ABR, Nachaiyasit and Stuckey (1997c) undertook a study where two 10 L ABRs were fed with a totally biodegradable feed composed of a synthetic carbohydrate (sucrose) and protein (meat extract) substrate to minimize variations between the reactors. The reactors were first operated at 20 h HRT, 4 000 mg/L COD at 35° C. A COD removal efficiency of 96% was achieved in the

preliminary study. Thereafter, the operating temperature was reduced from 35 to 25^{o} C and it was shown that COD removal efficiency ranged between 93 – 97% between the two reactors. At 15^{o} C, the efficiency of the reactors declined to 73 – 83% over a month operation. Both VFA and COD were detected in the effluent. The authors attributed the presence of VFA in the effluent to the lower rates of metabolism and an increase in the K_s for VFA (at high K_s values, VFA cannot be degraded). The increase in the effluent COD, on the other hand, was attributed to either an enhanced production of soluble microbial products, or a decrease in their metabolism, with these compounds constituting some 10% of the inlet COD (Nachaiyasit and Stuckey, 1997c).

Langenhoff and Stuckey (2000) also conducted a similar study on a 10 L, eight-compartment ABR, treating dilute wastewater (500 mg COD/L). The reactor was initially started with a HRT of 80 h at 35^{o} C, which was then reduced to 10 h. COD removal was greater than 80% in all HRT tested. However, when the temperature was reduced to 20^{o} C and 10^{o} C, the COD removal achieved was 70% and 60%, respectively.

2.7.6 Sulphate treatment

Fox and Venkatasubbiah (1996) investigated the effects of sulphate reduction in the ABR by treating a pharmaceutical wastewater with a COD concentration of 40 000 mg/L and a sulfate concentration of 5 000 mg/L (COD: SO_4 ratio of 8:1). At 40% dilution, COD removal efficiencies greater than 50% were achieved, and the conversion of influent sulfate was greater than 95%, at steady state. Furthermore, it was found that sulphate was completely reduced to sulphide in the first compartment, with increases in sulphide concentration detected along the length of the reactor. The results indicated that sulphate reduction was inhibiting methanogenesis (Barber and Stuckey, 1999).

These authors then varied the COD: SO_4 ratio and found an approximately 45% decrease in sulphate reduction from a COD: SO_4 of 150: 1 to 24: 1. At ratios above 8: 1, both acetoclastic methanogenesis and sulphidogenesis were inhibited by sulphide production. COD removal was reduced to less than 20% and toxic concentrations of sulphide (200 mg/L) were detected in the effluent. The effluent was then recycled back to the reactor, and treated in a trickling filter, which gave greatly improved COD removals and a decrease in effluent sulphide levels.

CHAPTER THREE: MATERIALS

3.1 Introduction

A pilot-scale ABR (3 000 L) was installed at the Kingsburgh wastewater treatment plant (WWTP), south of Durban (KZN Province). The reactor was supplied with domestic wastewater from the surrounding middle-income community, and its performance examined by a number of tests. This chapter presents a brief overview on the construction of the reactor, auxiliary equipment used on the reactor during the operation, duration of experiments and the sampling procedure employed.

3.2 Pilot-Scale ABR for Domestic Wastewater Treatment

3.2.1 Construction of the ABR

The pilot-scale ABR used in this study consisted of alternate hanging and standing baffles, which divided the reactor into eight, identical compartments. A schematic representation of the reactor is shown in Figure 2.5 (Chapter 2.7). The heights of standing baffles were reduced (relative to those used in laboratory-scale reactors) to facilitate fluid flow through the reactor (Foxon *et al.*, 2005). The reactor was constructed with mild steel with a total working volume of 3 000 L (Figure 3.1). Sampling ports were located on the side and top of the reactor. Technical drawings of the reactor can be viewed in Dama *et al.* (2001) and Foxon *et al.* (2005).

3.2.2 Construction of feed box

Raw wastewater from the Kingsburgh WWTP was channelled into a feed splitter box by a submersible pump (Figure 3.1). The splitter box was constructed out of mild steel and was divided into 3 chambers (Figure 3.2). The feed entered the middle chamber, from which 10% entered a feed chamber, and the other 90% entered a return chamber, where it was returned back into the channel of the treatment facility. The feed chamber contained three outlets, one of which was fitted with a butterfly control valve that controlled the feed entering the reactor. When the valve was closed, the level of the wastewater in the feed chamber rose until it entered the feed pipe into the ABR. When the feed rate became too high, the valve opened and the contents of the feed chamber flowed back into the channel of the treatment facility (Foxon *et al.*, 2005).

Figure 3.1: Installation of the pilot-scale ABR (3 000 L) at the Kingsburgh WWTP. The outlet end of the pilot ABR showing the membrane unit (Kubota) attached to the last compartment (left); and a laundry basket housing the submersible pump in a wastewater channel, near the feed end of the ABR (right). Taken from Foxon *et al*. (2005).

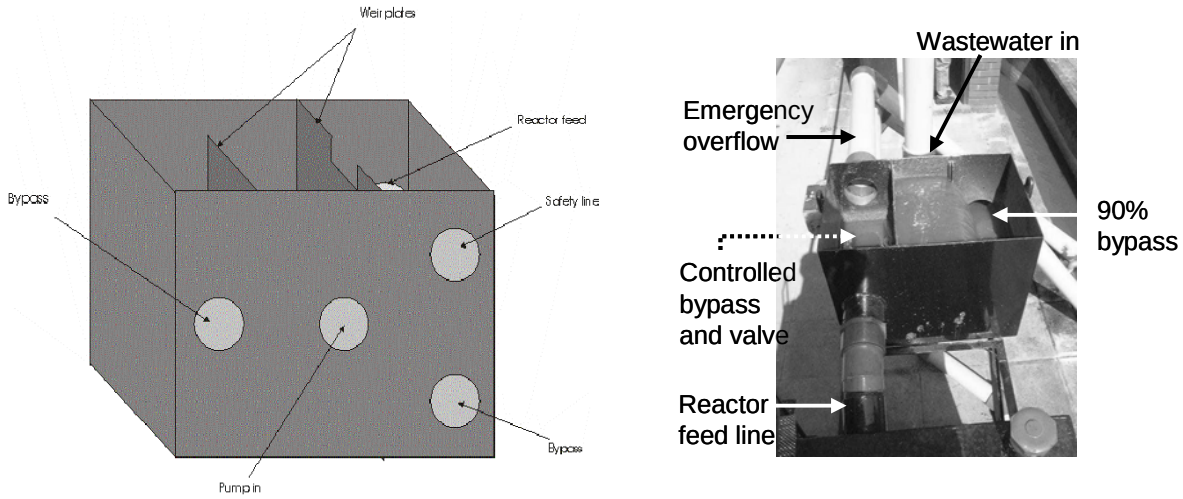


Figure 3.2: Schematic representation of the feed splitter box installed at the inlet of the pilot ABR (left); and photograph of splitter box installed on the ABR (reverse view) (right). Taken from Foxon *et al*. (2005).

3.2.3 Auxiliary equipment

Under field conditions, no additional equipment (no pump or valves), other than that used for screening wastes, would be required for the ABR. For the pilot study, however, additional equipment was required to investigate the performance of the reactor under controlled conditions. Equipment installed with the ABR included (Foxon *et al*., 2005):

- A submersible pump that fed the pilot ABR with raw wastewater entering the treatment plant.
- A pneumatic valve that controlled air supply to the by-pass valve.
- A compressor, which supplied air to the pneumatic valve.
- A magnetic flow meter that was involved in recording effluent flow rate and cumulative flow.
- A programmable logic controller (PLC), which controlled the amount of wastewater entering the ABR and captured flow rate data.
- A timer control switch, which was used to control the by-pass valve when the PLC was off-line.
- A PVC unit, which housed a microfiltration membrane (Figure 3.1), that was attached to the last compartment.

3.2.4 Seeding of ABR

The pilot-scale ABR was seeded with 10 L of anaerobic sludge from an anaerobic digester treating municipal sludge at the Umbilo WWTP.

3.3 Control of Flow

The magnetic flow meter, which was located at the ABR effluent outlet pipe, produced a signal when effluent exited the reactor. The signal was recorded by the PLC, which in turn controlled the flow of wastewater entering the reactor using a number of algorithms that were inputted into the system. The measured flow at the outlet was used to adjust the flow rate at the inlet pipe by controlling the timing of the butterfly control valve. From the numerous control regimes studied, it was found that proportional and integral control (PI) gave the best results. Details of these operational, and other control regimes, can be found in Foxon *et al*. (2005).

3.4 Test Site

The pilot scale reactor was moved from its initial site at Umbilo WWTP to the Kingsburgh plant, south of Durban. The decision to move the reactor to the Kingburgh WWTP was based on the

composition of wastewater generated in the respective areas. The Umbilo WWTP treats both domestic and industrial wastewater, whereas the Kingsburgh WWTP treats only domestic wastewater, generated from a community of around 350 000 people, mostly from middle-income communities. Although the composition of the wastewater may not be the same as those generated from low-income communities, the test site allowed the study to be conducted on wastewater containing no industrial components.

3.5 Operational Periods

This study reports two operational periods. The first was from the 17th February 2003 to 24th June 2003 (127 days). During this period, the reactor was operated at an average HRT of 22 h. This operation was extended from 18 July to 23 September 2003 (63 days), but was characterised by several unplanned technical difficulties, associated with control and supply apparatus. In the second period, the reactor was operated at an average HRT of 40-44 h from the 7th April to the 8th October 2004 (201 days). No significant performance affecting incidents occurred during this operation.

3.6 Sampling

Sampling was conducted on a weekly basis, generally between 10:00 a.m. to 2:00 p.m. Primary influent (raw wastewater) samples were collected from the feed box in glass Schott® (Merck) bottles (1 L), whilst effluent (1 L or 10 L, depending on analyses to be performed) was collected from the outlet pipe that returns ABR effluent back into the WWTP channel. For compartmental samples, a graduated Perspex tube was inserted into sampling ports on top of the reactor. The sampling tube had a 50 mm internal diameter with a built-in rubber bung attached to a steel rod. The rubber bung with steel rod was loosened from the Perspex tube and lowered via the sampling ports into each compartment. The Perspex tube was then closed with the bung and withdrawn from the reactor. The bung was loosened from the tube and the contents placed in a plastic bucket (5 L), mixed and transferred into Schott® bottles (1 L) (Merck). Sludge that was not to be used for sampling was returned to the reactor. In between compartment sampling, the tube and bucket were washed with tap water. Samples were placed on ice whilst in transit to the laboratory.

CHAPTER FOUR: TREATMENT PERFORMANCE WITH EMPHASIS ON PATHOGEN INDICATOR REDUCTION

This chapter presents the results of the pathogen indicator removal in the pilot-scale ABR. The chapter begins by reviewing the literature on the removal of pathogens and indicator organisms by anaerobic digestion (section 4.1), followed by the methods used to measure and analyse experimental variables (section 4.2). The pilot-scale ABR was operated under two flow regimes: an average HRT of 22 h, and one of 40–44 h. The results obtained from two operational periods are presented in sections 4.3 and 4.4, respectively, followed by a summary of the treatment performance of the pilot ABR (section 4.5). Conclusions from the study are presented in section 4.6, followed by recommendations for agricultural re-use (section 4.7).

4.1 Background Literature

Literature regarding pathogen and indicator removal in anaerobic reactors is scarce. The reason for this is that anaerobic reactors are primarily designed for the removal of organic matter (in terms of COD) and suspended matter (van Haandel and Lettinga, 1994). They are generally not used for the disinfection or the removal of biological nutrients. Whilst it is generally accepted that the process has little effect on the final concentration of biological nutrients (van Haandel and Lettinga, 1994), some removal of bacterial and parasitic pathogens can occur in anaerobic systems (Bitton, 1994). This usually occurs "inadvertently as a by-product of the principal design objective" during the removal of organic matter and retention of solids (Curtis, 2003). From the literature, it has been established that a number of parameters can influence the removal of pathogens and microbial indicators in anaerobic systems. These include: temperature; retention time; microbial competition; variations between the survival rates of different pathogens; pH values and chemical interaction (Smith *et al*., 2005). Most of these studies have been conducted on the anaerobic reactors treating waste sludge intended for re-use in agriculture.

4.1.1 Factors affecting pathogen and indicator removal in anaerobic systems

Temperature

Temperature is perhaps the most studied variable, and has great influence on pathogen reduction. Generally, higher temperatures result in greater reduction than lower temperatures (Kearney *et al*., 1993; Kumar *et al*., 1999). Furthermore, it increases deactivation rates (Olsen and Larson, 1987). Cote *et al*. (2005) did, however, have some comparable success at relatively low temperatures (15 – 20^{o} C). Operation within the thermophilic range has been shown to be more

effective in reducing bacterial pathogens and indicator organisms than mesophilic temperatures (Bendixen, 1994; Larsen *et al*., 1994; Watanabe *et al*., 1997). The deactivation at thermophilic temperatures is thought to occur as a result of protein denaturation, whilst at mesophilic temperatures, microbial competition and substrate limitation are thought to be the main parameters influencing removal, with temperature having an indirect effect (Smith *et al*., 2005). Thermophilic operation also results in greater deactivation of parasites than mesophilic operation. Pike *et al*. (1988) observed that almost complete parasite destruction occurred during thermophilic operating conditions at temperatures above 49^{o} C. Mesophilic anaerobic reactors, on the other hand, tend to be less effective in deactivating parasites. Cram (1943) reported that the viability of *Ascaris* eggs is unaffected after 3 months under mesophilic anaerobic conditions. However, significant deactivations (less than 10% viability) were observed when the holding time was increased to 6 months. Exposure time, which is a function of the HRT, is therefore also related to deactivation.

Hydraulic Retention Time (HRT)

Longer HRT generally results in greater pathogen removals (Curtis, 2003). Dahab and Surampalli (2002) reported that the $\log_{10}$ removal of pathogens and indicator bacteria decreased with shorter HRT. Tawfik *et al*. (2003) also found a similar trend in UASBs treating domestic wastewater. This phenomenon is not only applicable to anaerobic reactors. Many waste stabilisation basins are operated at HRTs over 20 days to achieve complete removal of helminth eggs (Feachem *et al*., 1983). Saqqar and Pescod (1991) reported an 88% removal of nematodes in an anaerobic pond operated at a HRT of 8 days, whilst Ayres *et al*. (1993) could only achieve 27% removal in anaerobic pond operated at 1.2 day HRT. However, it must be noted that operating conditions and composition of wastewater between the studies was different.

Variation in Species Survival in Anaerobic Systems

Some literature is available that compares the survival rates of pathogenic organisms in anaerobic processes. Most studies suggest that reduction is species-specific. Moce-Llivina *et al*. (2003) reported that phages were more resistant than enteroviruses in municipal sewage and sludge samples treated at thermophilic temperatures. Kearney *et al*. (1993) found that *Campylobacter jejuni* was the most resistant bacterium in a mesophilic anaerobic digester treating animal waste. *E. coli* and *Yersinia enterocolitica* (*Y. enterocolitica*) showed much lower survival rates, with *E. coli* being more resistant than *Y. enterocolitica*. The differences in survival were attributed to insufficient supply of available nutrients (Kearney *et al*., 1993). Variations in survival rates

between parasitic species have also been reported. Black *et al*. (1982) found that a small percentage of *Ascaris* eggs (less than 25%) were destroyed in a mesophilic anaerobic digester but there was no effect on the viability of eggs of *Trichuris* (whipworms) and *Toxocara* (canine roundworm). Cram (1943), on the other hand, found that *Ascaris* eggs were more resilient to mesophilic digestion than *Ancylostoma* (hookworm) eggs. *Ascaris* eggs remained viable for up to 3 months in the mesophilic anaerobic reactor, whilst hookworm eggs could only survive for up to 40 days under the same conditions (Cram, 1943).

The Effect of pH

This aspect has received little attention. The pH value generally has to be below 6.0 to have any effect on pathogens (Fields, 1979). Whilst this condition may not be achieved (or allowed) in many anaerobic reactors due to the pH-sensitivity of anaerobic processes, it may be applied with a certain degree of success in configurations that separate acid and methanogenic processes. Such configurations are commonly referred to as two-phase reactors. In 2003, Fukushi and co-workers designed a lab-scale experiment to examine the effects of the acid phase on pathogen reduction. They stimulated the acid phase of a reactor treating municipal sludge, and determined the survival of *Salmonella* species. Almost complete destruction of *Salmonella* occurred when pH was adjusted to 5.5 (Fukushi *et al*., 2003). However, it remains unclear how the overall digestion process of a two-phase reactor may be affected when the pH value of the acid phase is that low.

4.1.2 Treatment efficiency of anaerobic systems

Typical reductions of between 1- to 3-log of pathogenic and indicator organisms can be achieved in anaerobic systems (Bitton, 1994). Septic tanks can achieve 50-95% of indicator bacteria with a HRT of 3 days, provided that the tank is well designed and well maintained (Feachem *et al*., 1983). This is usually not the case and consequently, septic tanks cannot be reliably used to contain pathogens, especially if the population is large (Curtis, 2003). The UASB process has also been used with some degree of success. Lettinga *et al*. (1993) observed pathogen reductions greater than 70% operating at very short HRT (5-6 h). Dixo *et al*. (1995) reported a 67% removal of faecal coliforms in an UASB operated at a HRT of 8 h. Nevertheless, post-treatment is required as pathogen and indicator counts in UASB effluents are still very high.

Parasite removal in anaerobic systems is generally much better than bacterial removal (Bitton, 1994), but lower than other treatment processes (Black, 1982; Stott, 2003). The mechanism of removal is thought to be sedimentation and thus, extended HRTs increase the removal of

parasites from the effluent (Stott, 2003). Because of differences in the wastewater treated and reactor design, the removal efficiencies and deactivation of parasitic eggs in anaerobic systems is highly variable. Paulino *et al*. (2001) observed removal efficiencies between 60-93% for helminth eggs and protozoan cysts. Bhaskaran *et al*. (1956) reported a helminth removal of 99.4% in an experimental septic tank with a 3-day HRT. Lloyd and Frederick (2000) also reported similar removal efficiencies (99.5 - 100%) in a two-chambered septic tank operated at a HRT of 2-3 days. UASB reactors have also been reported to produce good parasite reductions. Dixo *et al*. (1995) reported an 89.6% removal of parasite eggs in an UASB operated at a HRT of 7 h. The removal was quite efficient as the raw wastewater contained a high concentration of parasite eggs (1 740 eggs/L to 17 000 eggs/L). Furthermore, the UASB achieved greater removal efficiencies at a higher loading rate than an anaerobic pond (26.6% egg removal) used in the study. In this instance, the high removal efficiencies were not attributed to sedimentation, as the upflow velocities of the reactor were higher than settling velocities of parasitic eggs (Dixo *et al*., 1995).

4.1.3 Discharge standards

All treatment systems need to achieve certain removal efficiencies in order to protect the environment and public health. In South Africa, DWAF (1996) has established discharge guidelines for domestic treated effluents. Depending on the quality of the effluent (COD, N, P, K, TS), it may be released directly into inland surface waters or re-used for agricultural irrigation. The guideline for discharge into surface waters tends to be more stringent than those for agricultural irrigation (DWAF, 1996). The WHO (1989) guidelines are also routinely used as the standard for wastewater discharge for irrigation in many countries. The re-use in agricultural irrigation is seen as more beneficial than discharge, as it offers the option of nutrient recycling. Nutrient-rich effluents could eventually replace costly nitrogen fertilisers, and will play a vital role in balancing water demand and supply in the near future. Furthermore, it could alleviate food shortage within low-income areas and create employment opportunities. Extensive literature exists on the re-use of wastewater for irrigation agriculture worldwide (Bontoux and Courtois, 1996; Haruvy, 1997; Shuval *et al*., 1986; 1997; Tchobanoglous and Angelakis, 1996), but there is very little regarding the re-use of effluent from an ABR. The results presented below are discussed in terms of discharge/re-use according to DWAF (1996) and WHO (1989) guidelines.

4.2 Analytical Methods

Primary influent samples were collected from the feed box in pre-sterilised glass Schott® (Merck) bottles (1 L), and used for all microbiological tests. Effluent samples were collected from the

outlet pipe that returns the effluent back into the WWTP channel. Pre-sterilised glass Schott® (Merck) bottles (1 L) were used to collect effluent samples for both coliform and coliphage analyses, whilst plastic containers (10 L) were used to collect effluent samples for parasitological analyses.

4.2.1 Pathogen indicators

Coliforms

Coliforms were measured using the membrane filtration technique (Standard Method 9222B) (APHA-AWWA-WEF, 1998). Enumerated coliforms included total coliforms (faecal and non-faecal coliforms) and *E. coli.* Primary influent and effluent samples were serially diluted in a sterile saline solution [0.2% NaCl (w/v)] and filtered through gridded 0.45 μm membrane filters (Schleicher and Schüll ME25). Filters were aseptically placed on Chromocult® Coliform Agar (Merck), and incubated at 35^{o} C for 18-24 h. *E. coli* colonies and total coliforms were identified by colour and enumerated as colony forming units (CFU) per 100 mL. A minimum of three plates was prepared for each dilution. All samples were analysed within 12 h after sampling (see Appendix I for a more detailed description of the procedure).

Coliphages

Coliphages are similar to enteric viruses and can be detected easily and rapidly in environmental samples (Bitton, 1994). Furthermore, they occur in higher numbers than enteric viruses in wastewater and other environments (Bitton, 1994; O' Keefe and Green, 1989). Therefore, they are often used as indicators of enteroviral pollution. Coliphages were enumerated according to the double layer technique (eThekwini Waste Water Laboratory Test Method No. MM023) using the host culture *E. coli* (ATCC 13706) (refer to Appendix I). All samples were serially diluted in sterile saline solution [0.2% NaCl (w/v)], and warmed to 48^{o} C. Samples were agitated, an aliquot removed (1 mL), and added to a small volume (10 mL) of semi-solid nutrient medium (6 g agar, 10 g tryptone, 8 g NaCl, 3 g glucose, 1 L sterile distilled water). A culture of host strain (1 mL), which was grown in nutrient broth at 37^{o} C for 18 h, was added to the mixture, and then plated on Petri dishes (90 mm) containing a solid nutrient medium (11 g agar, 13 g tryptone, 8 g NaCl, 1.5 g glucose, 1 L sterile distilled water). The plates were incubated at 37^{o} C for 18 h. Coliphages caused lysis on a lawn *E. coli* host cells, forming visible plaques and were enumerated as plaque forming units (PFU) per 100 mL. A minimum of three plates was prepared for each dilution (a maximum of three different dilutions). All samples were analysed within 12 h after sampling.

Parasite Detection

As coliform indicators are not very good indicators of faecal parasite contamination (Curtis, 2003), a suitable indicator of parasite contamination was required. This was limited to the helminth genus, *Ascaris*. It was chosen as an indicator of parasite contamination as the eggs can readily survive mesophilic digestion of up to 3 months (Cram, 1943). In contrast, almost complete deactivation of protozoans (*Giardia* and *Cryptosprodium*) oocytes can occur after 1 day in a mesophilic anaerobic digester (Gavaghan *et al*, 1993; Stadterman *et al*., 1995) and after 40 days for hookworm eggs (Cram, 1943). *Ascaris* eggs were concentrated and enumerated according the WHO method for parasitological analysis (Ayres and Mara, 1996). Briefly, grab samples of primary influent (1 L) and effluent (10 L) was collected on a weekly basis and allowed to sediment for 18 h. The supernatant of samples was discarded and the remaining sediments were centrifuged at 1 000 g for 15 minutes. The centrifuged supernatant was discarded and the enumeration of parasite eggs realised according to the modified Bailenger method (Ayres and Mara, 1996). Eggs were enumerated as total eggs per litre (eggs/L). Viability of the eggs was not assessed before and after the digestion period due to time constraints. A more detailed description of the method can be viewed in Appendix I.

4.2.2 Physico-chemical analyses

Measurements for chemical oxygen demand (COD), total suspended solids (TSS), free and saline ammonia, and phosphate were obtained using Standard Methods (APHA-AWWA-WEF, 1998). The measurements were performed by the eThekweni Water Services (EWS) laboratory, and by other members of the ABR project team (Foxon *et al*., 2005).

4.2.3 Statistical analysis

Data analysis was carried out using the Statistical Package for the Social Sciences (SPSS) statistical software (version 11.0). First, microbiological data was $\log_{10}$-transformed and tested for homoscedasticity and normal distribution using the Kolmogorov-Smirnov (KS) test. The microbiological data was shown to be normally distributed. The Student's *t*-test was then performed to assess differences between inlet and outlet values. Differences were considered significant at $P \leq 0.05$.

4.3 Case Study 1: HRT of 22 h

The first operating period with the pilot ABR occurred over a period of 127 days (17 February 2003 to 24 June 2003). The pilot ABR was operated at a target HRT of between

20 and 24 h, with an average of 22 h. A total of 353 000 L of raw wastewater from the Kingsburgh WWTP was treated in the pilot ABR. The cumulative flow treated, incidents, and down time during this operation period is presented in Figure 4.1. This operational was extended from 18 July to 23 September 2003 (63 days), but was characterised by several technical difficulties, associated with control and supply apparatus.

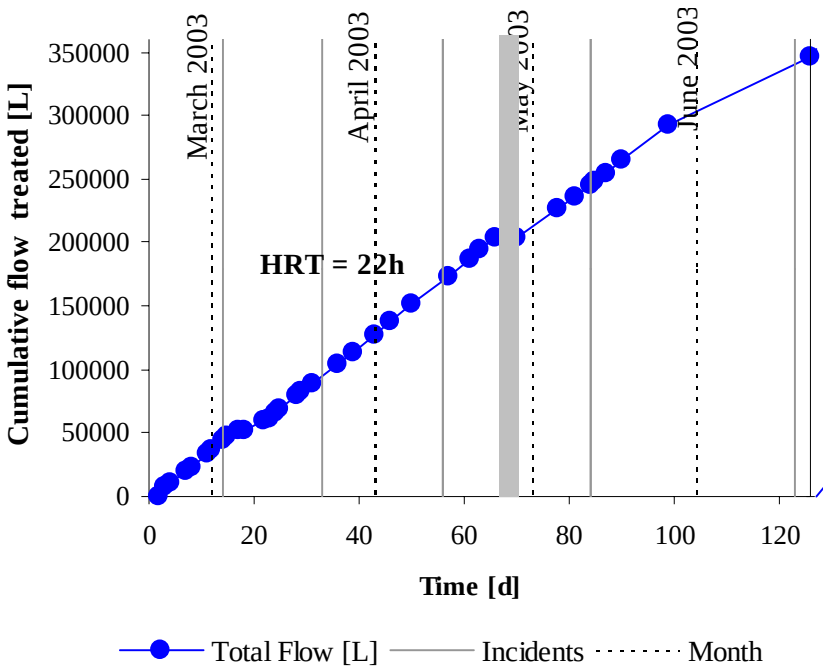


Figure 4.1: Cumulative flow of wastewater treated in the pilot-scale ABR installed at Kingsburgh WWTP from 17 February 2003 to 24 June 2003 (127 days). The reactor was operated at average HRT of 22 h. Solid vertical lines represent possible performance-affecting incidents, and the grey shaded rectangle indicates a reactor down time.

4.3.1 Hypothesis and Objectives

At present, there is no literature regarding the removal efficiency of pathogens and other indicator organisms in any ABR system. The aim of this experiment was to evaluate the pathogen indicator reduction in an ABR treating domestic wastewater. It was hypothesised that anaerobic digestion in the ABR could reduce the number of pathogen indicators.

The objectives of this experiment were to:

1. Determine the extent of pathogen indicator reduction in the ABR.
2. Evaluate the appropriateness of the effluent for re-use based on physico-chemical and pathogen indicator values obtained.

4.3.2 Results and Discussion

Pathogen Indicator Removal at a HRT of 22 h

During the period from February to June 2003, a number of primary influent and effluent samples were obtained and analysed for total coliforms by the eThekweni Water Services (EWS) (Durban) laboratory. The initial results indicated a reduction of microbial indicators (62%) was occurring through the reactor (Table 4.1). A more comprehensive bacteriological examination was conducted from 18 July to 23 September 2003 (63 days) to elucidate the removal efficiency of the ABR. The results from that operating period are presented in Figure 4.2.

Table 4.1: Average physico-chemical and microbiological values for primary influent and effluent samples for an ABR treating domestic wastewater from a middle-income community, for the period February to June 2003, operating at an average HRT of 22 h.

	Units	Influent	Effluent	Discharge Limit[a]	Irrigation Limit[a]
COD	mg COD/L	716 ± 54.4 (32)	192 ± 21.1 (33)	75	400[b]
pH		6.9	6.5	5.5 - 9.5	6 – 9
Ammonia	mg N/L	24.9 ± 4.2 (7)	33.2 ± 2.8 (6)	3	30[c]
Phosphate	mg P/L	4.9 ± 4.1 (4)	5.5 ± 0.5 (5)	10	n/a
TSS	mg TSS/L	480 ± 109 (14)	225 ± 55.2 (14)	25	50
VSS	mg VSS/L	306 ± 60.8 (14)	127 ± 45.9 (14)	No limit	n/a
Total coliforms [d]	CFU/100 mL	1.3×10^8 (6)	5×10^7 (6)	1×10^3	1×10^4 [e]

Abbreviations: (n), values in parentheses are number of samples; na, not applicable (guidelines currently not available); nd, not determined
a: guidelines established by DWAF (1996), unless otherwise stated.
b: for 500 kL/d discharge.
c: tentative guideline, maximum suitable to prevent crop toxicity (DWAF, 1996).
d: presented as geometric mean.
e: limit for unrestricted irrigation (WHO, 1989).

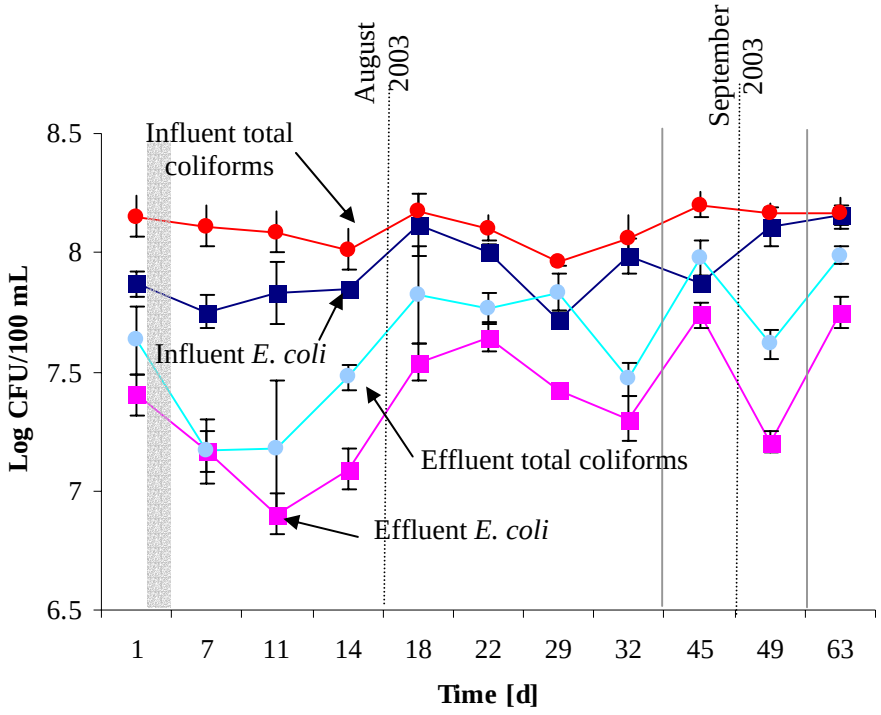


Figure 4.2: Comparison of primary influent and effluent microbial profile of the pilot ABR treating domestic wastewater from 18 July to 23 September 2003 (63 days). Solid grey vertical lines represent possible performance-affecting incidences. Removal from inlet to outlet was statistically significant (Student's *t*-test, $P \leq 0.05$).

Total coliform counts in the primary influent ranged from 9 x 10^7 to 2 x 10^8 CFU/100 mL, with a mean (geometric) of 1.3 x 10^8 CFU/100 mL. Primary influent *E. coli* counts ranged from 5 x 10^7 to 1 x 10^8 CFU/100 mL, with a mean (geometric) of 9 x 10^7 CFU/100 mL. The primary influent coliform counts were relatively consistent throughout the study period, although there were marked variations in coliform removal efficiency (26 to 89%). Effluent *E. coli* and total coliforms counts ranged from 7 x 10^6 to 5 x 10^7 CFU/100 mL and 1 x 10^7 to 1 x 10^8 CFU/100 mL, respectively. This amounted to an average removal of 68% and 61% for *E. coli* and total coliforms, respectively. The indicator removal after anaerobic digestion was shown to be significantly different (Student's *t*-test, $P \leq 0.05$), but still did not comply with discharge guidelines for both surface waters and agricultural irrigation (DWAF, 1996).

Treatment Performance at a HRT of 22 h

The primary influent, effluent and removal of COD, TSS, VSS and other measured parameters in the ABR are presented in Table 4.1. Measurements for COD, TSS and VSS were supplied by Department of Chemical Engineering (University of KwaZulu-Natal) using Standard Methods (APHA-AWWA-WEF, 1998) (Foxon *et al*., 2004). Samples were also analysed for free and saline ammonia, and orthophosphate by the EWS laboratory (Foxon *et al*., 2004; Pillay *et al*., 2004).

At a HRT of 22 h, the effluent COD values ranged between 136 mg COD/L to 433 mg COD/L, with average concentration of 192 ± 21.1 mg COD/L. The ABR was able to achieve an average COD removal of 72 ± 3%. The COD removal was consistent, with effluent concentrations generally below 300 mg COD/L, except for day 50, where a sharp transient increase in the effluent COD (433 mg COD/L) concentration was observed (Foxon *et al*., 2004; Pillay *et al*., 2004).

Ammonia concentrations increased slightly through compartments, whilst no statistical significant difference in orthophosphate profile was observed throughout the compartments, that is, between inlet and outlet values. The average value for orthophosphate in all measurements was 5.5 ± 0.5 mg/L (Foxon *et al*., 2004). Hence, the concentrations of these nutrients remained unaffected by anaerobic digestion, which is in agreement with the literature. TSS and VSS measurements of the primary influent and effluent were obtained for the period February to May 2003 (Table 4.1). Primary influent TSS values ranged between 308 and 965 mg TSS/L, with an average of 480 mg TSS/L. Effluent TSS values ranged between 80 to 390 mg TSS/L (average 225 mg TSS/L), with an average removal of 50% achieved for the period. The primary influent VSS values ranged between 125 to 537 mg VSS/L, with an average of 305 mg VSS/L. An average TSS removal of 56% was achieved, with effluent VSS values ranging between 50 and 200 mg VSS/L (average = 127 mg VSS/L) (Foxon *et al*., 2004).

Indicator removal through the ABR was poor. It was hypothesised that the ABR was capable of improved indicator removal efficiency, but several technical difficulties, associated with control and supply apparatus, might have affected the performance of the reactor during the extended study period (July to September 2003). This was supported by the fact that highest removals were achieved during stable operating period, and lowest following the onset of an incident (Figure 4.2). These installations (pump, valves and compressor) were an essential feature of the

pilot installation, and would not be part of field reactors. Consequently, such difficulties are not expected to occur under field conditions and improved efficiencies are expected. Furthermore, microbial community analysis of the reactor revealed that the ABR was hydraulically overloaded at a HRT of 22 h (Chapter 6.3). This is a common problem with anaerobic reactors treating partially soluble wastewaters, such as domestic wastewater (Lettinga *et al*., 1993). Hydrolysis becomes the rate-limiting step, and a longer HRT is required to entrap and stabilise solids (Lettinga *et al*., 1993). Whilst the ABR is not expected to achieve reductions necessary for re-use, it is hypothesised that a more efficient breakdown of particulate biodegradable matter, and better pathogen indicator removal, will occur at a longer HRT.

4.3.3 Effluent discharge and discharge/re-use options

The results presented above showed that phosphorus concentrations and pH values were the only parameters that complied with guidelines for discharge into water bodies and re-use in agriculture. A substantial removal (72%) of COD was achieved, which compares well with other anaerobic processes treating domestic wastewater. The reduction was not sufficient to allow for discharge into surface water according to the DWAF (1996) guidelines, but was consistently below allowable limits for re-use in irrigation agriculture (Table 4.1).

Effluent ammonia concentrations were at the upper limit for discharge into water resources, but may be used for agricultural re-use (DWAF, 1996). Although there is no strict limitation to ammonia concentration that can be used for irrigation, it is accepted that the risk of eutrophication and groundwater contamination is much higher when ammonia concentrations exceed 30 mg/L (DWAF, 1996). Furthermore, there are restrictions to the type of crop that can be grown in these concentrations (> 30 mg/L). High concentrations have similar effects to excessive fertiliser use on crops, including: excessive vegetative growth and lodging, delayed crop maturity and poor crop quality (DWAF, 1996). Crops that can be irrigated at this level include pepper, tomato, potato and maize (DWAF, 1996). A small-scale potted plant trial was conducted over seven weeks to assess the effects of effluent irrigation on plant growth. The results of the experiment revealed that irrigation with ABR effluent was beneficial to plant growth, and gave comparable results to a hydroponic solution. The results of this trial study are presented in Appendix II.

Hence, the main constraining parameter affecting effluent re-use is the microbial quality of the effluent. The microbial quality of the effluent was poor as *E. coli* and total coliforms counts were high. This usually correlates with high levels of bacterial pathogens, such as *Salmonella*, *Shigella*,

Vibrio cholerae, as well as parasites (DWAF, 1996). The implications of these results are that the ABR effluent may be of potential risk to communities, and may contaminate groundwater supplies. Although coliforms had been significantly reduced through the reactor, the removal did not comply with guidelines for discharge into inland water or unrestricted agricultural irrigation. It may be used for very restricted agricultural purposes according to WHO (1989) guidelines. However, for this to occur, farmers must adhere to very strict limitations. Only certain plants may be grown. These include cereal crops, industrial crops, fodder crops and trees. Additionally, there should be no effluent exposure to farmers and the public. This is a highly unlikely situation given that the ABR is envisaged for high-density communities. The microbial quality also restricts the irrigation technique that can be used. Drip or spray irrigation systems are inappropriate as they are expensive to implement and clogging may occur due to the high bacterial load. Flood or furrow irrigation by bucket would be the most appropriate irrigation technique. The exposure rate with this technique is high, and is often associated with higher risks to Human Norwalk-like virus, diarrhoeal disease and parasitic infestations compared to spray irrigation (Blumenthal *et al.*, 2000). Children in particular, are at most risk (Blumenthal *et al.*, 2000). As the safety of farmers, their families and environment cannot be guaranteed, it is clear that the effluent requires further treatment before any re-use strategy can be considered.

4.4 Case Study 2: HRT of 40-44 h

The results from the second operating period (7 April to 8 October 2004) cover a period of 201 days. The pilot ABR was operated at a target HRT of between 40 and 44 h. A total of 293 000 L of raw wastewater from the Kingsburgh WWTP was treated in the pilot ABR. The cumulative flow treated, incidents, and down time during this operation period is presented in Figure 4.3. In comparison to case study 1, there were less serious performance-affecting incidents. The majority of problems encountered during this study period were associated with pump blockages. Three successful operation periods could be identified from this operation, with mean HRTs of 40.6 h, 44.2 h, and 42.3 h (1.2 L/min; 1.1 L/min and 1.2 L/min average flow rate).

4.4.1 Hypothesis and Objectives

From the previous case study, it was established that there was a significant reduction of pathogen indicators (*E. coli* and total coliforms) through the ABR. However, it was hypothesised that improved removal would occur at a longer HRT. This experiment was designed to test this hypothesis. Furthermore, the survival of species in anaerobic reactors is known to be

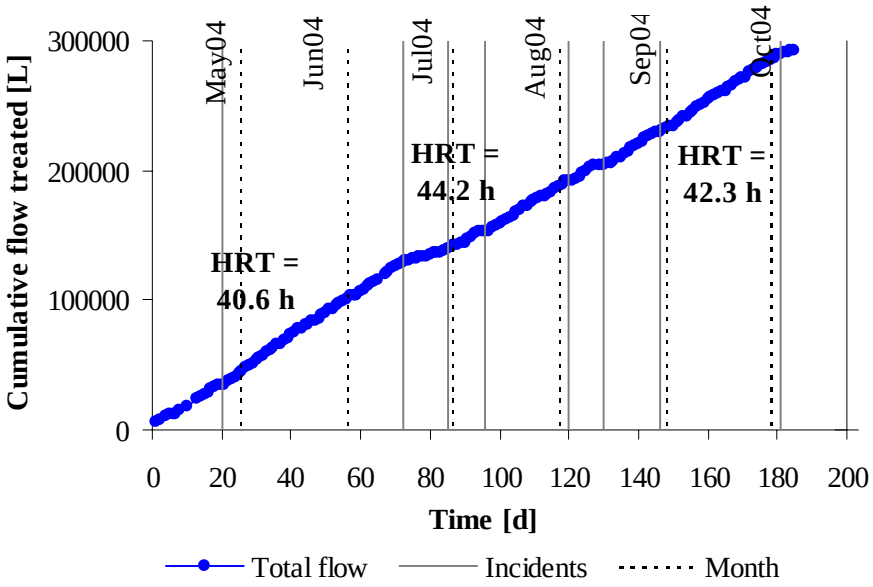


Figure 4.3: Cumulative flow of wastewater treated in the pilot-scale ABR installed at Kingsburgh WWTP from 7 April 2004 to 8 October 2004 (201 days). The reactor was operated at average HRT of 40.6 h, 44.2 h, and 42.3 h, respectively. Solid grey vertical lines represent possible performance-affecting incidents.

species-specific. The experiment was designed to examine the effect of anaerobic digestion in an ABR on other indicators.

The objectives of this experiment were as follows:

1. Evaluate the effect of HRT on the removal of pathogen indicator organisms.
2. Investigate whether there is variation in the removal of indicator organisms with changes in the HRT.
3. Examine the appropriateness of the effluent for re-use based on physico-chemical and microbial indicators results.

4.4.2 Results and Discussion

Pathogen Indicator Removal at a HRT of 40-44 h

There was very little variation in coliform counts entering the reactor. Primary influent *E. coli* counts ranged from 7 x 10^6 to 5 x 10^7 CFU/100 mL, with a mean (geometric) of 2 x 10^7 CFU/100 mL. Total coliform counts in the primary influent ranged from 1 x 10^7 to

2 x 10^8 CFU/100 mL, with a mean (geometric) of 5 x 10^7 CFU/100 mL. Effluent *E. coli* and total coliforms counts ranged from 4 x 10^5 to 1 x 10^7 CFU/100 mL and from 7 x 10^5 to 2 x 10^7 CFU/100 mL. This amounted to an average removal of 76% and 83% for *E. coli* and total coliforms, respectively. Similar patterns of *E. coli* and total coliforms removal were achieved in the ABR (Figure 4.4). Furthermore, the coliform reduction after anaerobic digestion was shown to be significantly different (Student's *t*-test, $P \leq 0.05$). As hypothesised, an increase in the HRT resulted in higher removal efficiencies. However, mean (geometric) counts still did not comply with discharge guidelines for irrigation (DWAF, 1996) (Table 4.2).

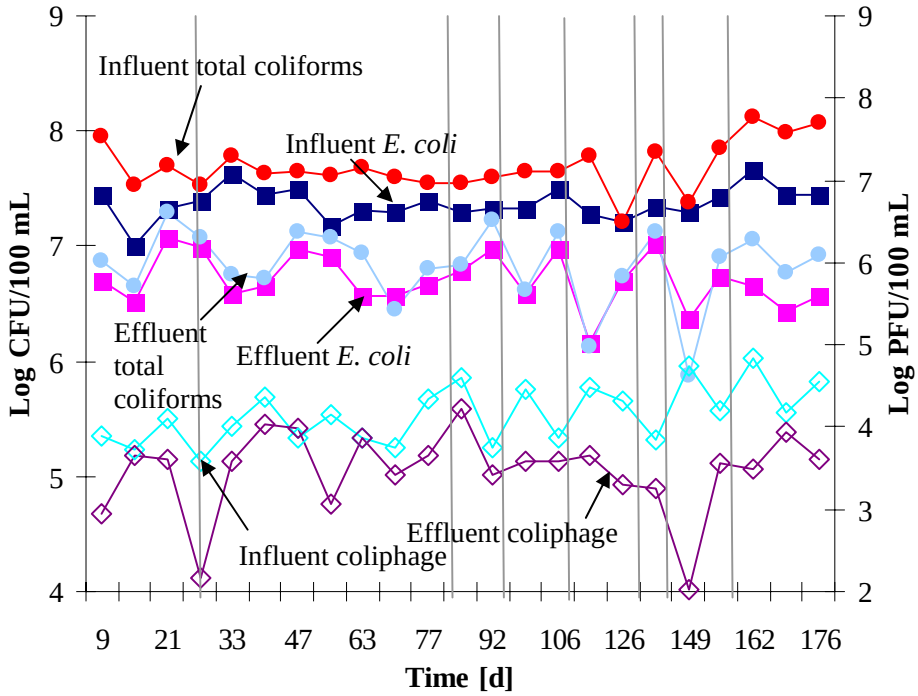


Figure 4.4: Comparison of primary influent and effluent profile of the pilot ABR treating domestic wastewater from April to October 2004. Solid grey vertical lines represent possible performance-affecting incidences. Removal from inlet to outlet was statistically significant (Student's *t*-test, $P \leq 0.05$).

Primary influent coliphage counts ranged from 2 x 10^3 to 8 x 10^4 PFU/100 mL, with a mean (geometric) of 1 x 10^4. Coliphage reduction occurred through the ABR, with the effluent counts ranging from 2 x 10^2 to 1 x 10^4 PFU/100 mL, with an average removal efficiency of 64%. The removal was significant (Student's *t*-test, $P \leq 0.05$), but was not as high as that observed for the

Table 4.2: Average physico-chemical and microbiological values for primary influent and effluent samples for an ABR treating domestic wastewater from a middle-income community, for the period April to October 2004, operating at an average HRT of 40-44 h.

Parameter	Units	Influent	Effluent	Target Limit [a]
COD	mg COD/L	719 ± 0.2 (18)	130 ± 64 (18)	400[b]
pH		6.7 – 7.4	6.2 – 7.4	6 – 9
Ammonia	mg N/L	55 ± 24 (10)	51 ± 23 (10)	30
Phosphate	mg P/L	28 ± 3 (7)	20 ± 6 (7)	Na
Sodium	mg Na/L	150 ± 118 (5)	131 ± 140 (5)	70[c]
Potassium	mg K/L	21 ± 4 (6)	25 ± 5 (6)	Na
TSS	mg TSS/L	416 (1)	135 (1)	50
E. coli	CFU/100 mL	2×10^7 (23)	5×10^6 (23)	1×10^3 [d]
Total coliforms	CFU/100 mL	5×10^7 (23)	8×10^6 (23)	1×10^4
Coliphage	PFU/100mL	1.9×10^4 (23)	4.4×10^3 (23)	20 [e]
Ascaris spp.	(Total eggs/L)	772 (13)	16 (13)	≤ 0.1[f]

Abbreviations: (n), values in parentheses are the number of samples; na, not applicable (guidelines currently not available); nd, not determined
a: agricultural use guidelines established by DWAF (1996), unless otherwise stated.
b: for 500 kL/d discharge.
c: maximum limit to prevent toxicity to the most sodium-sensitive plants (DWAF, 1996).
d: geometric mean, limit for unrestricted irrigation (WHO, 1989).
e: guideline for full and intermediate contact (Venter *et al*., 1996)
f: revised WHO guideline based on the findings of Blumenthal *et al*. (2000).

coliform groups (Figure 4.4). Furthermore, the removal efficiency of coliphages was not closely linked to that of *E. coli* and total coliforms. The results indicate that viruses may have a higher survival rate in an anaerobic reactor.

The number of *Ascaris* eggs in the primary influent was high and varied from 347 to 1 253 eggs/L, with a mean (arithmetic) of 772 eggs/L. The primary influent contained both viable and non-viable eggs (Figure 4.5 A-F). Despite the high load, a statistically significant egg removal (Student's *t*-test, $P \leq 0.05$) of 98% was observed after anaerobic digestion. This equates to a 1-$\log_{10}$ reduction, which is comparable to other anaerobic reactors, such as the septic tank and UASB.

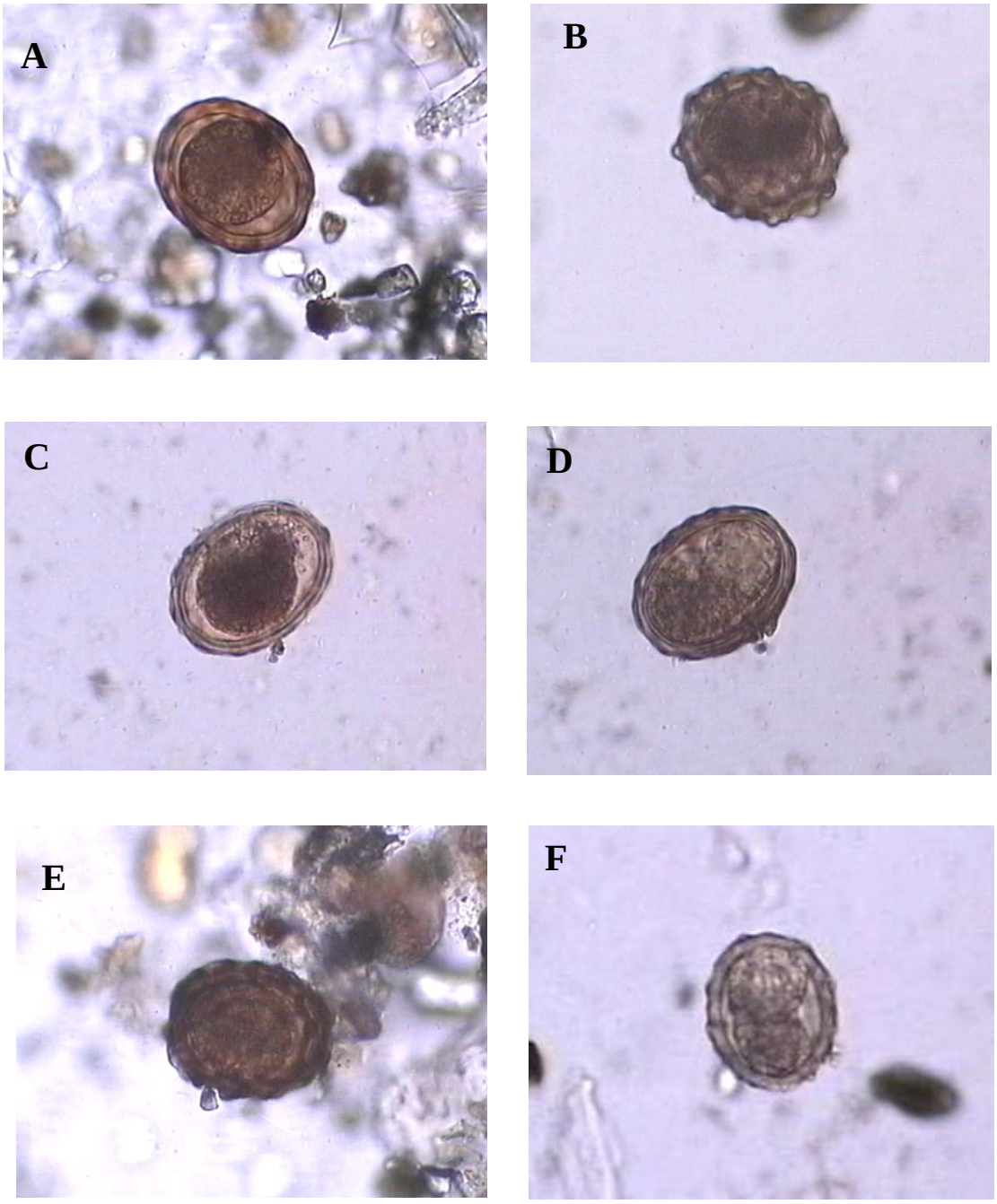


Figure 4.5: Light microscopy of *Ascaris* eggs isolated from influent samples showing that the primary influent contained both viable and non-viable eggs. Photographs A–C represent *Ascaris* eggs in the L1 or non-viable phase. Photographs D-F represent *Ascaris* eggs in the L2 or viable phase (worm development) (magnification x 50).

From all the indicators tested, the greatest reductions were observed with *Ascaris* eggs. The observed results are probably attributed to eggs having a larger mean residence time within the reactor. Helminth eggs are known to sediment (Shuval *et al*., 1986), and the phenomenon is thought to be the main mechanism for helminth removal in anaerobic systems (Stott, 2003). Helminth eggs can also become entrapped in the sludge (von Sperling *et al*., 2001).

Treatment Performance at a HRT of 40-44 h

The primary influent and effluent COD measurements for the pilot-scale ABR over 6 month period are presented in Table 4.2. At a HRT of 40-44 h, the average primary influent COD measured was 719 ± 0.2 mg/L, whilst average effluent COD was 130 ± 64 mg/L, which resulted in an average COD removal of 82%. Effluent COD measurements improved from the previous flow regime (Table 4.1) and were consistently below the guidelines established by DWAF for agricultural re-use (Table 4.2) (DWAF, 1996). Near the end of the study period, an apparently anomalous, sharp transient increase in the primary influent COD was observed at day 181 (Pillay *et al*., 2005). However, COD removal efficiency remained consistent for the study period.

The average values for primary influent and effluent samples for ammonia were 55 ± 24 mg/L and 51 ± 23 mg/L, respectively. No significant reduction in ammonia concentration was observed from the inlet to outlet (Student's *t*-test, $P > 0.05$) (Foxon *et al*., 2005).

Average phosphate concentrations were 28 ± 3 and 20 ± 6 mg/L, whilst average potassium concentrations were 21 ± 4 and 25 ± 5 mg/L for primary influent and effluent samples, respectively.

Sodium levels were highly variable with a mean concentration of 150 ± 118 and 131 ± 140 mg/L being detected for primary influent and effluent samples, respectively (Foxon *et al*., 2005). The nutrient concentrations were higher than those observed for the first operational period (HRT of 22 h), and might reflect a greater extent of treatment at a longer HRT.

The ABR was able to achieve approximately a 1-$\log_{10}$ reduction of coliforms. This is in accordance with most data reported in the literature. The effluent coliform count was still high (10^6) and indicated that the effluent may contain high concentrations of bacterial pathogens. The effect of anaerobic digestion on coliphage reduction was even less marked than coliform bacteria.

The results indicated that viral pathogens might have higher survival rates in the reactor. Mocé-Llivina *et al*. (2003) and Skraber *et al*. (2004) have made similar findings. In both studies, coliphages were shown to be less sensitive than coliforms and other bacterial groups to environmental parameters. With respect to *Ascaris* removal, removal efficiencies ranged between 94 to 99.6% and agreed well with the literature. Although effluent *Ascaris* egg counts ranged between 2 – 56 eggs/L, the removal was relatively efficient given that the reactor received egg loadings up to 1 253 eggs/L, and yet efficiency did not drop below 94% for the days measured.

4.4.3 Effluent discharge and discharge/re-use options

Based on the findings of the previous operation (case study 1), it was established that the effluent quality of ABR might not reach discharge guidelines into surface waters. Restricted agricultural irrigation may be possible provided there is an improvement in the microbial quality of the effluent. Hence, the results presented above are discussed in context to WHO (1989) and DWAF (1996) irrigation re-use guidelines (Table 4.2). In certain instances, revised guidelines have been included to suit the irrigation method to be used and human exposure to effluent (Blumenthal *et al.*, 2000). For example, the WHO (1989) guideline for nematode eggs is 1 egg/L. However, Blumenthal *et al.* (2000) have suggested that when flood or furrow irrigation is used, the guidelines should be more stringent (0.1 egg/L), especially if children are likely to receive any exposure to effluent-irrigated crops. The revision is based on epidemiological studies conducted on farmer workers and their families exposed to various treated and untreated wastewaters (Blumenthal *et al*., 2000). A guideline of 10^3 *E.coli*/100mL has also been included based on the findings of Blumenthal *et al.* (2000). Currently, no limit exists for this parameter, as well as coliphages, in WHO (1989) and DWAF (1996) re-use guidelines.

As was the case in case study 1 (HRT of 22 h), consistent COD treatment efficiencies were observed. Removals for the entire study period were below agricultural irrigation guidelines. The effluent contained high levels of plant nutrients, suggesting its application as a fertiliser replacement. There are concerns, however, that groundwater and surface water contamination may occur as nutrient concentrations exceeded the target limit for irrigation. Furthermore, excessive nutrient supplementation may be detrimental to plant growth, although this was shown not to be the case in a small-scale field study conducted over seven weeks with only three plant varieties (Appendix II). Hence, microbial contamination still remains the major re-use concern, even though improved removal efficiencies were observed during this operation (section 4.5). At its present state, the effluent microbial quality is poor. It may harbour a wide range of bacterial

and viral pathogens, and contains at least one parasitic species (*Ascaris*). It is reasonable to assume that most of eggs will be viable after treatment in the ABR, as parasite deactivation requires very long residence times under mesophilic conditions (Cram, 1943). Therefore, further treatment would be necessary to protect both public health and the environment.

4.5 Summary

The HRT was shown to have a marked influence of the treatment performance, with treatment efficiencies decreasing with reduced HRT. A comparison of the treatment efficiency at the different operating periods is presented in Table 4.3. Although primary influent COD values were relatively similar for both operating periods, removal efficiency increased from 72% to 82%, with an increase in the HRT, indicating a more efficient breakdown of organic matter. Similar patterns were observed for other parameters measured, except nutrient concentration, which remained relatively unaffected by the process (Table 4.3).

Table 4.3: Comparison of ABR treatment performance at two HRT regimes.

	22 h		40-44 h	
Parameter	Mean effluent values	Removal efficiency (%)	Mean effluent values	Removal efficiency (%)
COD (mg/L)	136 – 433	72	63 – 340	82
Ph	6.2 – 7.6	-	6.2 – 7.4	-
Ammonia (mg/L)	28 – 39	-	20 – 90	8
Phosphate (mg/L)	4.7 – 5.9	-	24 – 33	30
TSS (mg/L)	80 – 390	50	134 [a]	68 [a]
E. coli (CFU/100 mL) [b]	$7 \times 10^6 – 5 \times 10^7$	68	4×10^5 - 1×10^7	76
Total coliforms (CFU/100 mL) [b]	$1 \times 10^7 – 1 \times 10^8$	61	7×10^5 - 2×10^7	83

a: lack of range because only one sample measured.
b: presented as geometric means.

TSS removal efficiencies increased from 50% to 68% with an increase in the HRT. Slight improvements in coliform removal efficiencies were also observed. *E. coli* removal increased from 68% to 76% (Table 4.3). However, the reduction efficiency between the two operating periods was shown to be statistically similar (Student's *t*-test, $P > 0.05$). In contrast, the difference in total coliform removal efficiencies was more distinct with a change in HRT. At a

HRT of 22 h, total coliform removal efficiencies were less than *E. coli*. When the HRT was increased (HRT of 40-44 h), total coliform removal efficiencies were higher than *E. coli*. Furthermore, the removal efficiency of total coliforms between the two operating periods was shown to be significantly different (Student's *t*-test, $P \leq 0.05$). Based on these results, it was inferred that removal efficiency of other pathogenic organisms would have also been lower at a HRT of 22 h.

Nutrient concentrations remained relatively unaffected by anaerobic digestion, even with a change in HRT. From an agronomical point of view, this is one of the benefits of using anaerobic digestion to treat wastewaters. The effluent contained high levels of nutrients suggesting its application as a fertiliser replacement. Several countries have realised the potential of nutrient-rich wastewater for irrigation. In Egypt, for example, sludge and biosolids from wastewaters have been successful as an alternative to nitrogen fertilizers, in the growth of wheat, berseem, clover, forage maize and grape vines, and can command a fair price (UNCSD, 1999). It was hoped that the ABR effluent might offer similar benefits by contributing to the alleviation food shortage within low-income areas. The microbial quality of the effluent, however, needs to be improved before this can be realised. Both coliform and viral removal is not adequate to allow for discharge into the environment, and whilst there was a relatively efficient parasite removal (≥ 95%), it was also insufficient. It is recognised that there is a limit to the pathogen reduction that can be achieved by anaerobic digestion. Further treatment in waste stabilization ponds (Bouhoum *et al*., 2000; Lloyd and Frederick, 2000; Shuval *et al*., 1986), membrane filters (Jacobs *et al*., 1999; Odhav, 2004), or constructed wetlands (Bouhoum *et al*., 2002; de Sousa *et al*., 2001; Juwarkar *et al*., 1995) is required to reach the desired microbial quality.

4.6 Conclusion

The results from this study suggest that the ABR would be suitable as a pre-treatment step in dense, peri-urban areas. The ABR can achieve similar treatment rates to that of UASBs, without the use of electricity or a reliance on the granulation process. COD removal is relatively high and consistently met agricultural re-use regulations, even with slight fluctuations in primary influent COD. Sludge production is low compared to conventional on-site treatment systems, such as septic tanks. After 5 years of field operation, the ABR did not require desludging. As this contributes significantly to the operating costs of a septic tank, the comparative cost of operating an ABR is much lower. Another advantage of ABR is that the partial spatial separation of

microbial communities allows it to be more resistant to toxic and shock loadings (Barber and Stuckey, 1999).

As is the case with other anaerobic reactors, only partial removal of pathogens could be achieved. Pathogen treatment efficiencies are comparable to that of septic tanks and high-rate reactors, such as the UASB. The main advantage of the ABR over conventional on-site technologies would thus be higher COD and solid removals. The results are supported by the findings of Wanasen (2003) and Koottatep *et al*. (2004), who found that baffled septic tanks provided higher COD and solid removal than conventional two-chambered septic tanks. This is a vital feature because the area required for any particular post-treatment option can be comparatively smaller. This is particularly important given that space is limited in informal areas. Furthermore, construction costs will be comparatively cheaper. Post-treatment steps can be specifically designed to meet helminthological and bacteriological requirements for unrestricted irrigation, instead of further reduction of solids and COD. A membrane filter, which would fit onto the last compartment of ABR, was considered as a post-treatment option. The results from the trial study are presented in Appendix III.

4.7 Recommendations for Agricultural Irrigation

Although the microbial quality of the effluent suggests its use in restricted irrigation, it is not recommended. Recent evidence has shown that the guideline needs to be revised, as it does not provide adequate protection to farm workers and their families with direct contact with the wastewater (Blumenthal *et al*., 2000). It is recognised that microbial quality of the effluent must be improved in order for it to be discharged. The recommendations presented below are therefore suggested as a tentative guideline:

1. The effluent can be considered as category A wastewater intended for irrigation according to the South African wastewater irrigation guidelines (Rodda *et al*., 1991). This would allow restricted irrigation to non-food crops, such as wood and fibre, nurseries, sportsfields before opening to the public and non-recreational areas of parks (Rodda *et al*., 1991).
2. Agricultural workers wear adequate protective clothing (footwear for farmers and gloves for crop handlers).
3. Proper education on the use and application of ABR effluent, and improving hygiene. For example, (1) hand-washing after picking crops and handling effluent, and (2) washing crops before eating.

4. Provision of adequate potable water to avoid consumption of effluent and for hygiene purposes.
5. The use of low-contaminating irrigation techniques. As mentioned earlier, irrigation by watering can or bucket is seen as the most appropriate and feasible method in light of ABR effluent characteristics, but is known to result in greater incidences of infection (Blumenthal *et al*., 2000). Drip irrigation is known to be a low-contaminating technique (Blumental *et al*., 2000), but it cannot be used, as it requires a substantial capital investment and the likelihood of clogging is high. One option currently being considered is low technology, localised drip irrigation in the form of plastic bottles, which have been punctuated at the base and buried to half its length beside each plant. Effluent can be collected in buckets or watering cans, and be delivered directly into plastic bottles. The advantage of this method is that it can achieve similar protection to conventional drip irrigation systems at a fraction of the cost. A similar strategy has been used in irrigation of commercial crops with greywater (Salukazana *et al*., 2005).
6. Crop requirement for nutrients should be considered. Effluent irrigation should be followed or should only occur during vegetative plant growth stages, where nitrogen requirements are high (DWAF, 1996). Cereals, for example, should have reduced spring applications, to prevent crop lodging before harvesting (DWAF, 1996). Effluent could be regularly used provided it is diluted and another water source is readily available.
7. Although the pH of the ABR effluent is within targets specified by DWAF (1996), nutrient concentrations within the effluent could reduce or increase soil pH, which affects the availability of micro- and macronutrients essential for plant growth and development. The local soil conditions should be considered before irrigating to determine whether the land is suitable for irrigation agriculture with effluent (a soil with a low pH will not meet requirements).

CHAPTER FIVE: THE FATE OF PATHOGEN INDICATORS THROUGH THE ABR

This chapter presents results of the fate of pathogen indicators within individual compartments of the pilot-scale ABR. Section 5.1 provides a brief introduction to the chapter. Section 5.2 presents the methods used to measure and analyse experimental variables during the study period. Experimental data obtained from two operational periods are presented in sections 5.3 and 5.4, respectively. Conclusions from the experiments are presented in section 5.5.

5.1 Introduction

The ABR represents a novel configuration that consists of several compartments. Literature regarding the fate of pathogens and indicators through a multistage anaerobic process, such as the ABR, is scarce. At present, no data exist for an ABR at any scale. The aim of this experiment was to evaluate the fate of pathogen indicators inside the ABR. The results can be used to provide basic information to modify reactor design to improve domestic wastewater treatment.

5.2 Analytical Methods

Primary influent and effluent samples were obtained from the inlet and outlet pipes of the ABR, respectively. Sludge samples (1 L) from each compartment were taken from the sampling points above the reactor. The compartment samples, consisting of both a liquid and solid phase, were representative of the core contents of the upflow side of each compartment. All samples were processed for pathogen indicator quality. The data presented in this chapter represent the mean count over the study period.

5.2.1 Coliforms

E. coli and total coliforms were measured using the membrane filtration technique (Standard Method 9220B) (APHA-AWWA-WEF, 1998) (Appendix I). Primary influent and effluent samples were serially diluted with sterile saline solution [0.2% NaCl (w/v)] and filtered through gridded 0.45 μm membrane filters (Schleicher and Schüll ME25). Compartment samples, consisting of both a sludge and liquid fraction, were also serially diluted with sterile saline solution [0.2% NaCl (w/v)] and filtered. Filters were aseptically placed on Chromocult® Coliform Agar (Merck), and incubated at 35^{o} C for 18-24 h. *E. coli* colonies and total coliforms were

identified by colour and enumerated as colony forming units (CFU) per 100 mL. A minimum of three plates was prepared for each dilution. All samples were analysed within 12 h after sampling.

5.2.2 Coliphages

Coliphages were enumerated according to the double layer technique (eThekwini Waste Water Laboratory Test Method No. MM023) using the host culture *E. coli* (ATCC 13706) (Appendix I). All samples were serially diluted in sterile saline solution [0.2% NaCl, (w/v)], and warmed to 48^{o} C. Samples were agitated, an aliquot removed (1 mL), and added to a small volume (10 mL) of semi-solid nutrient medium (6 g agar, 10 g tryptone, 8 g NaCl, 3 g glucose, 1 L sterile distilled water). A culture of host strain (1 mL), which was grown in nutrient broth at 37^{o} C for 18 h, was added to the mixture, and then plated on Petri dishes (90 mm) containing a solid nutrient medium (11 g agar, 13 g tryptone, 8 g NaCl, 1.5 g glucose, 1 L sterile distilled water). The plates were incubated at 37^{o} C for 18 h. Coliphages caused lysis on a lawn *E. coli* host cells, forming visible plaques and were enumerated as plaque forming units (PFU) per 100 mL. A minimum of three plates was prepared for each dilution (maximum three dilutions). All samples were analysed within 12 h after sampling.

5.2.3 Statistical analysis

Data analysis was carried out using SPSS statistical software (version 11.0). First, microbiological data was $\log_{10}$-transformed for all computations and tests. Log_{10}-transformed data was then tested for homoscedasticity and normal distribution using the Kolmogorov-Smirnov (KS) test. All microbiological data were shown to be normally distributed. Lastly, the Analysis of Variance (ANOVA) test was performed using the post-hoc Scheffe test to determine where the significant differences between compartments lay. Differences were considered significant at $P \leq 0.05$.

5.3 Case Study 1: HRT of 22 h

5.3.1 Hypothesis and Objectives

Barber and Stuckey (1999) have described the ABR as a series of UASB reactors because of its compartmentalisation. Hence, it was hypothesised that each compartment of the ABR may act as an individual treatment process. The objective of this experiment was therefore to determine whether compartmentalisation contributes to pathogen removal.

5.3.2 Results

The longitudinal profiles of mean *E. coli* and total coliform counts through compartments of the ABR are shown Figures 5.1 and 5.2, respectively. Primary influent and effluent counts have also been included. With respect to *E. coli*, there is a general decrease in the coliform count between compartments of the reactor (Figure 5.1). The difference between compartments was shown to be significant (as determined by ANOVA). However, the mean count did not differ significantly from one compartment to the next (Scheffe test, $P > 0.05$), but were observed for a series of compartments (those samples that were furthest away from each other along the reactor).

The following trends were noted for *E. coli* counts between compartments:

1. There was an overlapping of mean *E. coli* counts between compartments.
2. The mean *E. coli* counts of the primary influent was statistically similar to that of compartment 1 (Scheffe test, $P > 0.05$). However, it was significantly higher than latter compartments (Scheffe test, $P \leq 0.05$). The mean *E. coli* counts of compartment 1 were also statistically similar to that of compartments 2 and 3 (Scheffe test, $P > 0.05$), but were significantly higher than later compartments (Scheffe test, $P \leq 0.05$).
3. There were no significant differences in the *E. coli* counts between compartments 2 and the effluent (Scheffe test, $P > 0.05$).
4. There was a slight increase in the *E. coli* counts from compartment 6, although this was not significant (Scheffe test, $P > 0.05$).

With respect to total coliforms, a similar pattern to *E. coli* was observed, with counts generally decreasing between compartments, across the length of the reactor (Figure 5.2). The difference between compartments was shown to be significant (as determined by ANOVA). As with *E. coli*, the mean total coliform counts did not differ from one compartment to the next (as determined by Scheffe test), but occurred between combinations of compartments. The following trends were observed:

1. There were no significant differences in the mean total coliform counts between the primary influent and compartments 1 to 3 ($P > 0.05$, as determined by the Scheffe test).
2. The mean total coliform counts of the primary influent and compartment 1 samples were significantly higher than that from compartments 4 to 8 ($P \leq 0.05$, as determined by the Scheffe test).

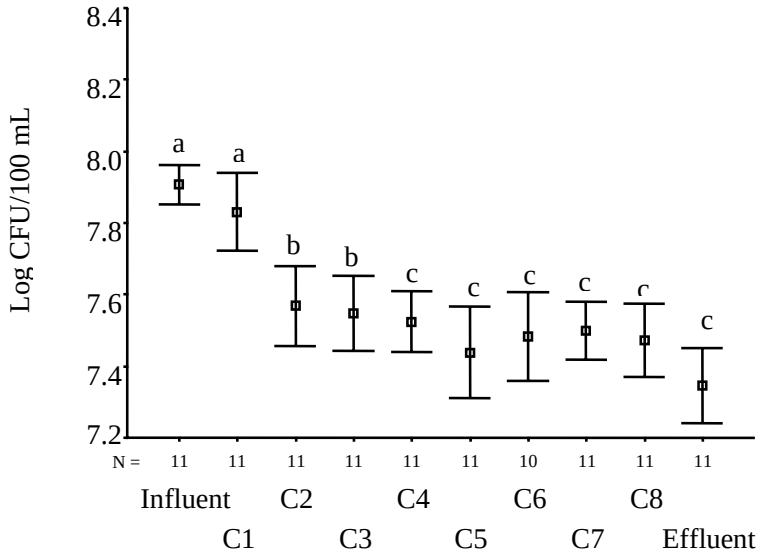


Figure 5.1: Error bar plot of mean *E. coli* counts between compartments (C1 – C8) of the pilot-scale ABR treating domestic wastewater at a HRT of 22 h. The squares represent the mean with vertical lines representing the standard error. Plots with the same letters indicate non-significant means (Scheffe test, $P > 0.05$) (N = number of samples).

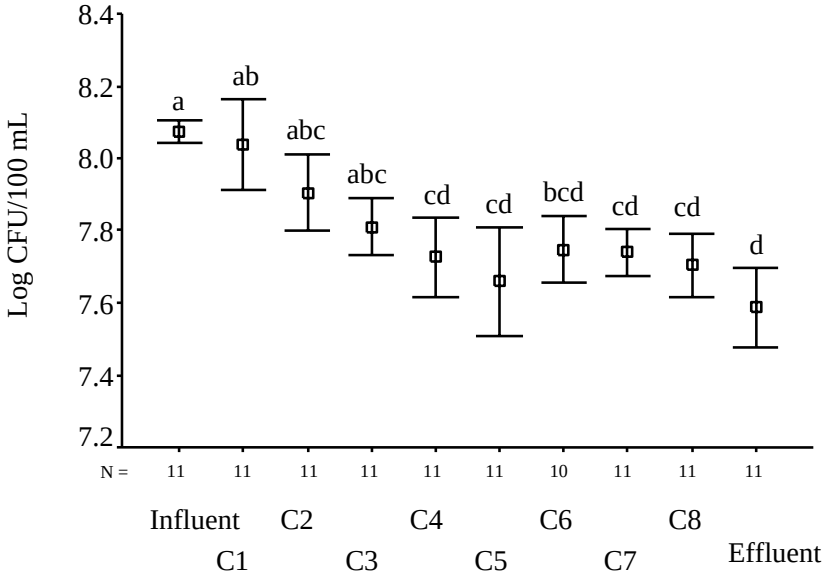


Figure 5.2 Error bar plot of mean total coliform counts between compartments (C1 – C8) of the pilot-scale ABR treating domestic wastewater at a HRT of 22 h. The squares represent the mean with vertical lines representing the standard error. Plots with the same letters indicate non-significant means (Scheffe test, $P > 0.05$) (N = number of samples).

3. There were no significant differences in the mean total coliform counts from compartment 2 to 8, although the effluent count was significantly lower than that of compartment 2 ($P \leq 0.05$, as determined by the Scheffe test).
4. As was the case with *E. coli*, the mean total coliform counts increased in compartment 6. Although the increase was not significantly higher than surrounding compartments, the mean count was statistically similar to that of compartment 1 and 2 ($P > 0.05$, as determined by the Scheffe test).

5.3.3 Discussion

The results presented above suggested that compartmentalisation contributed progressively to coliform removal. However, significant reduction did not occur from one compartment to the next, as originally hypothesised, but occurred over a series of compartments. It is hypothesised that the main mechanism of pathogen removal in an ABR may be due to the retention of solid-associated pathogens and indicators. Although it was not possible to measure the indicator counts between consecutive sections of standing baffles (as only the upflow region of each compartment had a sampling port), the substantially larger difference in the mean coliform count between compartment 8 and the effluent supports this hypothesis. The samples from compartment 8 represent the core contents of that particular compartment. As these samples contain a significant solid fraction, any solid-associated pathogens and indicators will also be included in the count. The effluent, on the other hand, was representative of compartment overflow, and therefore contained a small solid component. As no additional treatment occurred between compartment 8 and the effluent, the difference between the mean coliform counts of the two samples is probably related to differences in sample composition.

Consequently, it was hypothesised that compartments with the largest solids fraction would have the highest coliform count. Contrary to what was expected, coliform counts were not well correlated with the sludge profile through the compartments (data not shown). It is hypothesised that other factors could have influenced the coliform counts within the compartments. These include pH, microbial competition, and substrate limitation (Smith *et al.*, 2005). According to Smith *et al.* (2005), the microbial competition and substrate limitation are the primary factors responsible pathogen reduction under mesophilic anaerobic conditions.

The greatest coliform reductions occurred in the earlier compartments (Figure 5.1 and 5.2). With respect to *E. coli*, the greatest reduction was observed between compartments 1 and 2, although

this was not significant (Scheffe test, $P > 0.05$). Thereafter, a baseline was reached where there was little change in the mean *E. coli* counts. In contrast, there was a gradual 'stepwise' decrease in the total coliform load between compartments 1 to 5, with a baseline being reached at compartment 4. The results suggested that there is no difference in the microbial quality of the effluent compared to compartments 4 to 8.

It is hypothesised that an increase in the HRT may improve pathogen indicator removal through the reactor. As mentioned previously (section 4.3.2), the study period was characterised by several 'washout' incidences, and poor solids degradation. As a major portion of *E. coli* in wastewater is thought to be associated with particles (Gannon *et al*., 1983), it is hypothesised that an increase in the HRT, coupled with a more stable operation, may improve pathogen indicator removal through enhanced solids retention.

5.4 Case Study 2: HRT of 40-44 h

5.4.1 Hypothesis and Objectives

From the previous case study, it was established that compartmentalisation contributed to progressive pathogen indicator removal. However, significant reduction did not occur between adjacent compartments, but over combinations of compartments. It was hypothesised that an increase in the HRT would improve pathogen indicator removal through the ABR.

5.4.2 Results

The longitudinal profiles of the mean *E. coli* and total coliform counts through compartments of the ABR are shown in Figures 5.3 and 5.4, respectively. The coliphage profile is shown in Figure 5.5. Primary influent and effluent counts have also been included. With respect to *E. coli* counts, the mean count increases after the primary influent enters compartment 1, suggesting that there might be a build-up of solid-associated pathogens. Thereafter, the counts decreased in a 'step-wise' manner until the last compartment. The difference between compartments was shown to be significant (as determined by ANOVA). As with the previous operation (HRT = 22 h), the mean *E. coli* counts did not differ from one compartment to the next (as determined by the Scheffe test), but occurred only between sets of compartments. The following trends were observed and are included in Figure 5.3:

1. After an initial increase in the mean *E. coli* count, there was gradual decrease with ranges between successive compartments overlapping.

2. There were no significant differences in the mean *E. coli* counts of the primary influent, compartment 1 and compartment 2 (Scheffe test, $P > 0.05$). However, the mean counts of the other compartments were significantly lower than compartment 1 (Scheffe test, $P \leq 0.05$).
3. There were no significant differences in mean *E. coli* counts of compartments 2 to 4. However, the mean counts of compartment 2 and 3 were significantly different to the latter compartments, whilst for compartment 4, a significant difference was observed after compartment 5 (Scheffe test).
4. There was no significant difference in the mean *E. coli* counts between compartment 6 and the effluent (Scheffe test, $P > 0.05$).

With respect to total coliforms, a similar pattern to that seen for *E. coli* was observed, with mean counts initially increasing after the primary influent enters the reactor, and then steadily decreasing along the length of the reactor (Figure 5.4). The differences between compartments were shown to be significant (as determined by ANOVA). The mean counts did not differ from one compartment to the next (as determined by the Scheffe test). Most of total coliform removal occurred in the first five compartments, after which the mean coliform counts remained relatively unchanged. The following trends were observed and are included in Figure 5.4:

1. After an initial increase in the total coliform counts, there was gradual decrease with ranges between successive compartments overlapping.
2. There were no significant differences in the mean total coliform counts of the primary influent, compartment 1 and compartment 2 (Scheffe test, $P > 0.05$). However, compartment 1 samples were significantly higher than the mean counts in other compartments (Scheffe test, $P \leq 0.05$).
3. There was no significant difference in the mean total coliform count of the primary influent, compartment 2 and compartment 3 (Scheffe test, $P > 0.05$). However, the mean count of compartment 1 was significantly higher than that of the other compartments (Scheffe test, $P \leq 0.05$).
4. There was a significant difference in the mean total coliform counts between compartment 4 and 5 (Scheffe test, $P \leq 0.05$).
5. There were no significant differences in the mean total coliform counts from compartments 5 to the effluent (Scheffe test, $P > 0.05$).

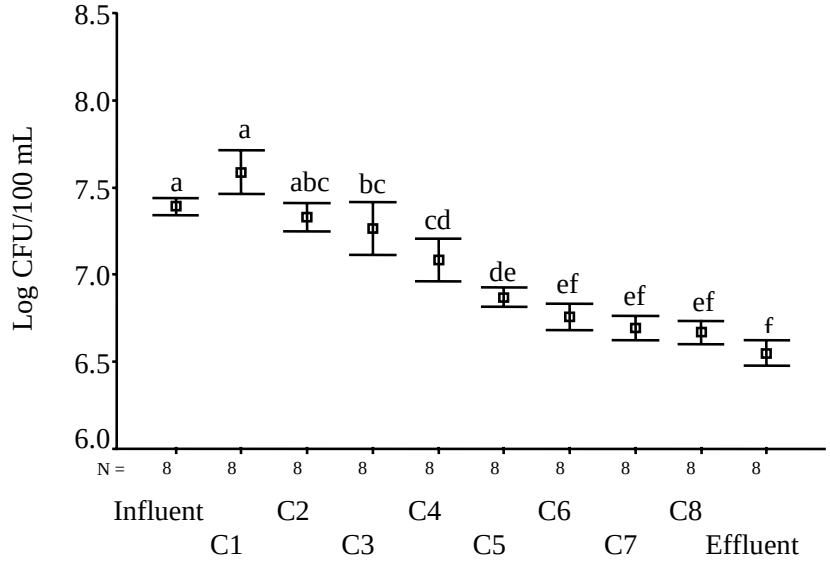


Figure 5.3: Error bar plot of mean *E. coli* counts between compartments (C1 – C8) of the pilot-scale ABR treating domestic wastewater at a HRT of 40-44 h. The squares represent the mean with vertical lines representing the standard error. Plots with the same letters indicate non-significant means (Scheffe test, $P > 0.05$) (N = number of samples).

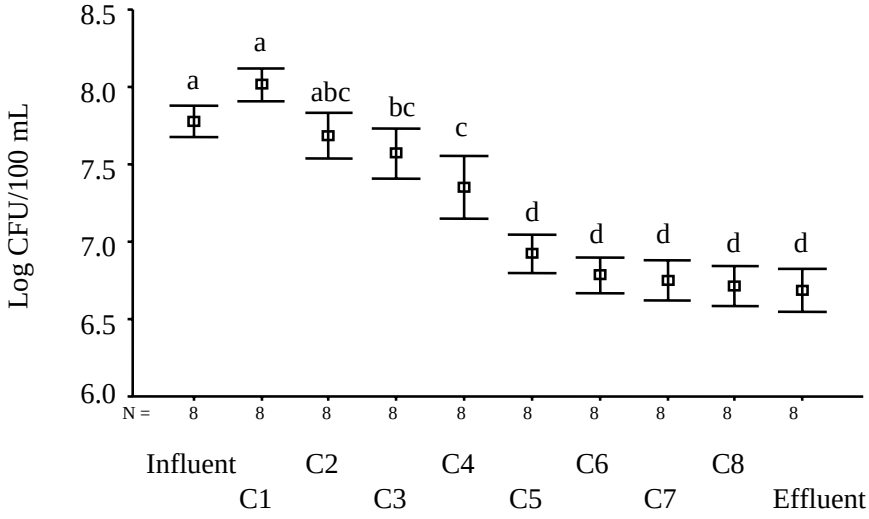


Figure 5.4: Error bar plot of mean total coliform counts between compartments (C1 – C8) of the pilot-scale ABR treating domestic wastewater at a HRT of 40-44 h. The squares represent the mean with vertical lines representing the standard error. Plots with the same letters indicate non-significant means (Scheffe test, $P > 0.05$) (N = number of samples).

With respect to coliphage count per compartment, the longitudinal profile did not resemble the pattern observed for coliform bacteria (Figure 5.5). The difference between compartments was shown to be significant (ANOVA, $P > 0.05$). Post-hoc analysis (Scheffe test) of data revealed the following trends:

1. There was no significant difference in the mean coliphage count between the primary influent and compartment 1(Scheffe test, $P > 0.05$).
2. There was a significant reduction in the mean coliphage count between compartment 1 and 2 (Scheffe test, $P \leq 0.05$), after which, counts remained relatively constant up to the outlet pipe (Scheffe test, $P > 0.05$).
3. There was a significant reduction in the mean coliphage count from compartment 8 to the outlet pipe (Scheffe test, $P \leq 0.05$).

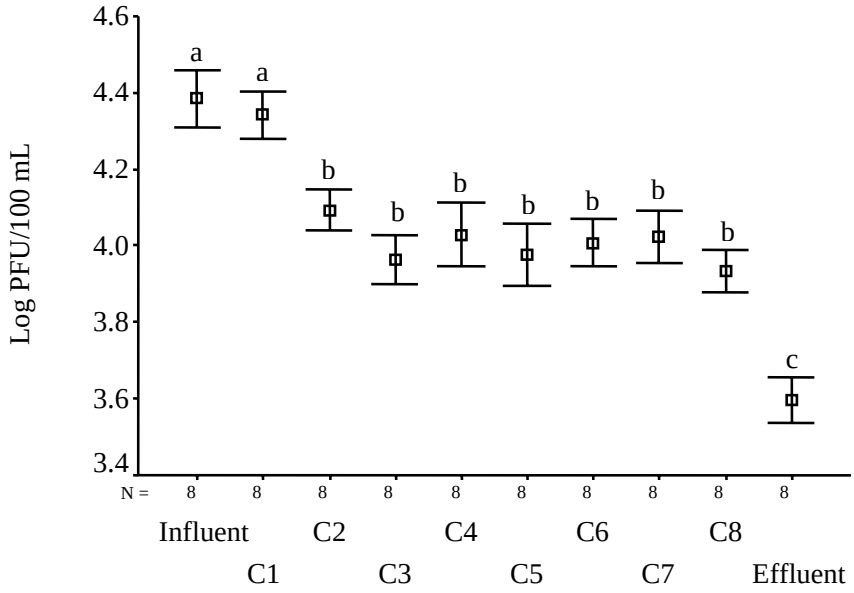


Figure 5.5: Error bar plot of mean coliphage counts between compartments (C1 – C8) of the pilot-scale ABR treating domestic wastewater at a HRT of 40-44 h. The squares represent the mean with vertical lines representing the standard error. Plots with the same letters indicate non-significant means (Scheffe test, $P > 0.05$) (N = number of samples).

5.4.3 Discussion

As with the case study 1, compartmentalisation contributed progressively to coliform removal. Furthermore, there was a 'step-wise' decrease in the mean coliform count from compartment 1 to compartment 6, after which, mean counts remained relatively constant, suggesting that the limit

of coliform removal had been reached in the ABR. Although significant reductions did not occur from one compartment to the next, coliform removal between adjacent compartments were greater than those observed for case study 1 (HRT = 22 h). The improved removal between compartments is thought to be as a result of lower upflow velocities at a longer HRT. The extended HRT allowed for a better containment of solid-associated coliforms between compartments, and hence, better removal efficiencies (see section 4.5). It might be argued that other factors, such as microbial competition and substrate limitation, may also be responsible for coliform removal through the reactor (Smith *et al.*, 2005). However, experimental proof is lacking.

The mean coliphage count profile through the ABR was different to that observed for coliform groups. There were significant decreases between compartment 1 and compartment 2, and between compartment 8 and the effluent. Between compartments 2 to 8, there was very little change in the coliphage plaque count. The data presented suggests that solids retention is also critical to viral removal. It is hypothesised that viruses may be absorbed into the sludge, as wastewater passes through the reactor. This hypothesis is supported by the fact that greatest reduction occurs between compartment 8 and the outlet pipe, and between compartment 1 and compartment 2. As mentioned previously, the effluent samples represented compartment overflow, and therefore contained very little solids as part of their composition. Compartmental samples, on the other hand, represent the core contents of that particular compartment. It therefore contains a significant solids fraction as part of its composition. As there was no additional treatment between compartment 8 and the effluent, it is suggested that the difference in the coliphage plaque count between these samples is probably due to differences in sample composition. This may also explain the large difference in the mean plaque counts between compartment 1 and compartment 2. Compartment 1 had the largest solids fraction in the reactor. This probably explains why higher plaque counts were observed in this compartment compared to others. Based on these results, it is suggested that viral removal in an ABR is associated with solids retention. As the main mechanism of coliphage removal appears to be correlated to solids retention, the results presented here suggest that coliphage removal at a HRT of 40-44 h may have been better than at a HRT of 22 h.

5.5 Conclusion

The longitudinal profiles of pathogen indicators under two operating conditions suggested that the ABR acted a containment unit, and although some measure of pathogen indicator reduction was

seen, the differences were gradual (significant differences did not occur from one compartment to the next, but over a series of compartments). The data presented suggests that the main mechanism of pathogen removal may be due to solids-retention. It might be argued that microbial competition and substrate limitation may also be driving factors, based on the findings of Smith *et al*. (2005). However, experimental proof is lacking. Additionally, longer HRT are thought to improve pathogen removal through better containment of solid-associated pathogens and probably, increased exposure to anaerobic conditions.

In terms of reactor design for dense-peri urban communities, Foxon *et al*. (2005) have suggested a five-compartment ABR, instead of an eight. The modifications to design are based on results obtained from the pilot study (Foxon *et al*., 2005). The results presented above suggest that the pathogen removal capability of the ABR would not be largely affected by this modification. For both operational periods, it was shown that pathogen indicator removal occurred mostly during the first five compartments, after which, mean counts remained largely unchanged. One of the objectives outlined in section 5.1 was achieved, and this suggested that the microbial quality of a five-compartment ABR will be relatively similar to that achieved from an eight-compartment reactor.

CHAPTER SIX: MICROBIAL POPULATION DYNAMICS IN PILOT ABR TREATING DOMESTIC WASTEWATER

This chapter presents the characterisation of the microbial communities in the pilot ABR treating domestic wastewater. Section 6.1 presents a literature review of microbial communities observed within different types of ABRs. Section 6.2 presents the analytical methods used to monitor the population dynamics in the ABR. The results and discussion from the two operational periods are presented in sections 6.3 and 6.4, respectively. The overall performance is summarised in section 6.5.

6.1 Background Literature

The unique design of the ABR promotes the separation of various phases of anaerobic catabolism (Barber and Stuckey, 1999). This results in differences in microbial populations along the length of the reactor. The microbial ecology within each compartment will depend on the type and amount of substrate present and external parameters, such as pH and temperature (Barber and Stuckey, 1997b and 1999). According to the literature, fast-growing microorganisms, capable of growth at high substrate levels and reduced pH, should dominate the first two compartments, whereas slower-growing microorganisms dominate near the end of the reactor at higher pH (Barber and Stuckey, 1999).

Table 6.1 presents a summary of findings observed within ABRs (adapted from Barber and Stuckey, 1999). Various methods have been used to describe the microbial population dynamics in an ABR, from descriptive techniques based on scanning electron microscopy (SEM) and light microscopy, to more quantitative, molecular techniques, such as fluorescent *in situ* hybridisation (FISH). The most common observation is the change in the population of acetoclastic methanogens (Barber and Stuckey, 1999). In treating molasses wastewater in a laboratory-scale ABR, Tilche and Yang (1987), and Yang *et al.* (1988) observed large populations of *Methanosarcina* species at the front of the reactor, whilst *Methanosaeta* species were prevalent towards the end. Similar findings have been observed in the treatment of other types of wastewaters (Barber and Stuckey, 1997b; Bell, 2000; Garuti *et al.*, 1992). These observations suggested that acetate concentrations were highest at the front of the reactor and lowest at the end. According to the literature, *Methanosarcina* species outcompete *Methanosaeta* species at high acetate concentrations, while *Methanosaeta* species outcompete *Methanosarcina* at low

Table 6.1 Summary of observations of genera reported in ABR studies (adapted from Barber and Stuckey, 1999)

Observations	Technique	Reference
Methanosarcina predominant at front of reactor with *Methanosaeta* predominating in the last compartments.	SEM, TEM, LM	Bell, 2000; Boopathy and Tilche, 1991, 1992; Garuti *et al*., 1992; Tilche and Yang, 1987; Yang *et al*., 1988
Active methanogenic fraction within the biomass. Highest at front of reactor and lowest in last compartment.	ATA	Bachmann *et al*., 1985; Orozco, 1988
Microorganisms resembling *Propionibacterium* , *Syntrophobacter* and *Methanobrevibacter* found in close proximity to granules. *Methanosaeta* and colonies of *Syntrophomonas* also observed.	TEM	Grobicki, 1989
Large population of *Methanobacterium* at front of reactor along with *Methanosarcina* granules.	EP	Tilche and Yang, 1987
Biomass activity h ighest in bottom third of each compartment (>85%); first compartment had highest activity (92%).	ATPA	Xing *et al*., 1991
Methanosaeta predominant with some cocci. *Methanosarcina* not observed.	SEM	Polprasert *et al*., 1992
Irregular granules with gas vents covered by single rod -shaped microorganisms.	SEM	Holt *et al*., 1997
Microorganisms resembling *Methanobrevibacter* , *Methanococcus* and *Desulfovibrio* found.	ATPA, SEM, EP	Boopathy and Tilche, 1992
Heterogeneous bacterial population observed at front of reactor.	SEM, TEM	Barber and Stuckey, 1997b; Bell, 2000; Boopathy and Tilche, 1991
Dense bundles of filaments consisting of *Methanosaeta* through all compartments, even at high acetate concentrations. No *Methanosarcina* observed.	LM, SEM, FISH	Angenent *et al*., 2000
Horizontal separation of *Methanospirillum* and *Methanosaeta.*	FISH	Bell, 2000
Methanosaeta predominant with *Methanobacterium* , *Methanospirillum* and *Methanomethylovorans* .	FISH	Bell, 2000
Microorganisms resembling *Methanobr evibacter* and *Methanococcus* found at front of reactor, with *Methanosaeta* and *Methanosarcina* at end.	SEM	Uyanik *et al*., 2002

acetate concentrations. This idea was supported by Polprasert *et al*. (1992) who found that *Methanosaeta* dominated a four-compartment ABR, which had acetate concentrations as low as 20 mg/L.

The distribution of hydrogenotrophic methanogens within the ABR is also determined by the substrate concentration in each compartment. In most cases, they are observed near the front of the reactor, where substrates are the highest (Bell, 2000; Tilche and Yang, 1987; Uyanik *et al*., 2002). In contrast to acetoclastic methanogens, little is known about the hydrogen concentration that will favour one hydrogenotrophic methanogen species over another.

Although not necessary for optimum performance, granulation has been noted in ABRs (Barber and Stuckey, 1999). Xing *et al*. (1991) observed granules in all three compartments of a hybrid ABR (HABR) treating high-strength molasses (molasses alcohol stillage and raw molasses). Granules appeared after 30 days of operation and continued to grow from their initial size (< 0.5 mm). Similarly, Boopathy and Tilche (1992) observed growing granules in all compartments of a HABR treating highly-acidic, molasses wastewater. In contrast, granules formed within 30 days of operation. As with UASBs, granules were predominantly composed of acetoclastic methanogens. In the first compartment, granules were primarily composed of *Methanosarcina* species, whilst in the latter compartments, granules were primarily composed of *Methanosaeta* species. *Methanosarcina*-dominated granules had low densities and were full of 'gas cavities'. As a result, they often floated to the top of the reactor increasing the possibility of biomass 'washout'. When the substrate strength was increased (acidified molasses to raw molasses), granule size steadily decreased along the length of the reactor. At the same time, the sludge weight of compartment 1 increased from less than 600 g to 900 g. Similar findings were observed by Orozco (1988) in an open plug flow baffled reactor treating dilute carbohydrate wastewater.

Uyanik (2003) compared the granulation development in a split-fed and normal fed ABR treating brewery and ice-cream wastewater. Granules were formed from a mixed population of anaerobic microorganisms after 45 days. 'Mature' granules were predominately composed of a *Methanosaeta*-dominated inner core. Contrary to literature reports, the observation of mixed culture in the initial stage of granule formation, lead Uyanik (2003) to believe that *Methanosaeta* was not responsible for granule initiation. It was hypothesised that the predominance of *Methanosaeta* within granules occurs after initiation, when the flow of substrates through the

granule matrix leads to the development of an internal environment difference to that of the surface (Uyanik, 2003).

6.2 Analytical Methods

6.2.1 Scanning electron microscopy

Introduction

Scanning electron microscopy (SEM) was used to elucidate the microbial population dynamics in the ABR. The scanning electron microscope has become an important analytical tool in microbial studies, as it enables resolutions 1 000 times better than a light microscope (Robinson and Gray, 1990). This has allowed scientists to identify microorganisms in environmental samples, based on their size and morphology, which has been previously described from pure cultures or other concentration techniques. More precise tools for examining microbial consortia are available, which yield better and more accurate results. However, these techniques require greater stringency, and therefore can be time-laborious and expensive.

Sampling

Sludge samples, representing the composition of a core of the reactor contents, were collected using a Perspex sampling tube from the upflow side of each compartment. The samples were collected at near steady-state for the two different HRT study periods, and prepared for SEM. Sludge samples were dark grey, except in compartment 1, where samples appeared brown. In addition to collecting core sludge samples from each compartment, individual granules, dispersed within the sludge of compartments 2 and 3, were removed from the sampling bucket and placed in separate, glass screw cap McCartney® (Merck) bottles containing 0.1 M phosphate buffer at pH 7.2. There were subtle differences used in the preparation of the core sludge samples and the granules (refer to Appendix I).

Procedure

Approximately 50 mL of core sludge from each compartment was removed from the collection bottle and centrifuged for 5 minutes to remove the supernatant. The samples were washed three times in 0.1 M phosphate buffer at pH 7.2. Washed samples were decanted and fixed in 10% formaldehyde in 0.1M phosphate buffer. Samples were fixed for 16 h, decanted and washed three times with 0.1M phosphate buffer, and post-fixed with 1% osmium tetroxide for 1 h at room temperature. Fixed samples were then repeatedly rinsed with distilled water to remove excess

fixative, and dehydrated in a graded alcohol series (25, 50, 75 and 100%) for 10 minutes each (Robinson and Gray, 1990).

Granules, on the other hand, were individually removed from McCartney® (Merck) bottles, and gently washed three times in 0.1 M phosphate buffer at pH 7.2 in glass petri dishes. The granules were then transferred to McCartney® (Merck) bottles containing 10% formaldehyde in 0.1M phosphate buffer. Samples were fixed for 16 h, decanted and washed three times with 0.1M phosphate buffer, and post-fixed with 1% osmium tetroxide for 1 h at room temperature. Fixed samples were then repeatedly rinsed with distilled water to remove excess fixative, and dehydrated in a graded alcohol series (25, 50, 75 and 100%) for 20 minutes each (Robinson and Gray, 1990).

Both core sludge and granular sludge samples were collected on nucleopore filters (0.20 μm) (Costar) and further dehydrated in a critical point drier (CPD) (Hitachi HCP-1). Dried samples were then mounted on aluminium stubs, and sputter-coated (Polaron E5100) with gold. At least two stubs were prepared for each core sludge sample. Samples were viewed on a Leo 1450, and images recorded digitally. A detailed description of the procedure used for core sludge and granule sample preparation is presented in Appendix I.

6.2.2 Epi-fluorescence microscopy

Introduction

Epi-fluorescence microscopy was used for the microscopic analysis of ABR granules from case study 1. The method is based on the histological method described by Chui and Fang (1994), which relies on the fluorescence of co-factors F_{350} and F_{420} of methanogens under epi-fluorescent excitations (refer to Appendix I for a detailed description of the method).

Sampling

Sludge was removed from compartments 2 and 3 using the Perspex sampling tube and placed in a bucket. Individual granules were removed from the sampling bucket and placed in separate, glass screw cap McCartney® (Merck) bottles containing 0.1 M phosphate buffer at pH 7.2. The granules were then prepared for epi-fluorescent microscopy.

Procedure

Granules were removed from McCartney® (Merck) bottles and transferred to glass Petri dishes containing 0.1M phosphate buffer at pH 7.2. The granules were washed three times in buffer solution, and then fixed in 10% formaldehyde containing 0.1M phosphate buffer for 16 h. Granules were dehydrated in a graded alcohol series (25, 50, 75, 100%) for 20 minutes each. The alcohol was then replaced with xylene by immersing the granules in a graded xylene series (25, 50, 75, 100%) for 20 minutes each. The xylene-saturated granules were placed in peel-off moulds (Polyscience) containing molten paraffin and left overnight at 60^{o} C – 65^{o} C. The moulds were removed from the incubator, allowed to cool, and sectioned by a microtome (American Optical). The sections were allowed to float on a waterbath and placed on a glass slides (TAAB). The slide was dried in air, placed in an incubator at 70^{o} C for 10 minutes to melt the paraffin, and finally dewaxed with xylene (Chui and Fang, 1994). The slide was then examined by a microscope under epi-fluorescent excitations.

6.3 Case Study 1: HRT of 22 h

6.3.1 Hypothesis and Objectives

A study was undertaken to investigate the hypothesis that spatial separation of anaerobic consortia develop along the length of the reactor. It was hypothesised that fermentative microorganisms, capable of sustained growth at low pH, would predominate in the earlier compartments. A change to slower-growing populations of pH sensitive methanogens was expected in later compartments in keeping with observations in the literature. The objective of this experiment was to determine the distribution of anaerobic populations through the ABR.

6.3.2 Results

Distribution of Anaerobic Microorganisms

During the study period, a variety of morphotypes, resembling methanogens were observed. It is important to note that since many microorganisms have similar morphologies, more precise tools would be required to fully characterise the anaerobic microbial consortia present. However, some methanogenic microorganisms have been well documented and can be distinguished with relative degree of confidence (Fang, 2000). Four morphotypes were frequently observed in sludge samples from each compartment and are presented in detail. The fifth was a bamboo-shaped rod resembling *Methanosaeta* species. This morphotype was infrequently observed within a few granular sludge samples. The most common morphotypes observed within core sludge samples include:

1. Rod-shaped, chain-forming microorganisms resembling *Methanobrevibacter* species (Jianrong *et al*., 1997, Uyanik *et al*., 2002) (Figure 6.1 and 6.2), but could also be *Methanomicrobium* or *Methanobacterium* species. However, the latter two genera are not known to form chains (Boone *et al*., 1994).
2. Coccoidal-shaped microorganisms with cleavage furrows that were observed as individual cells or clumps. The morphology resembles the typical morphology of *Methanosarcina* species (Boone *et al*., 1994, Sprott and Beveridge, 1994) (Figure 6.1).
3. Large cocci (approximately 2 μm in diameter) resembling species of *Methanococcus* or *Methanocorpusculum*. However, the latter is known to be irregular in shape (Boone *et al*., 1994) (Figure 6.1 and 6.3).
4. Small cocci (approximately 0.5 to 1 μm in diameter) resembling *Methanococcus* species, but could also be *Methanosarcina* species (Boone *et al*., 1994) (Figures 6.1 and 6.3).
5. Thin, filamentous microorganisms consisting of 10 or more cells, resembling *Methanospirillum* microorganisms (Boone *et al*., 1994; Jianrong *et al*., 1997) (Figures 6.1 and 6.4). The morphotype could also be filamentous *Methanosaeta*, but it does have typical angular shapes noted for the genus (Boone *et al*., 1994; Cheng *et al*., 1997; Guiot *et al*., 1992; Jianrong *et al*., 1997; Uyanik *et al*. 2002).

The distribution of microorganisms resembling methanogens within the ABR has been summarised in Table 6.2. SEM observations showed that there was a large population of microorganisms resembling methanogens at the front of reactor, which gradually decreased towards the end. The first compartment had the highest numbers and the largest variety of these microorganisms (Figure 6.1). Other morphotypes were also observed, including, slender rods and irregular cocci (not shown). By far, the most dominant morphotype corresponded to that which resembled *Methanobrevibacter* species. However, the number of observations of this morphotype declined drastically in the second compartment. No observations for this morphotype were detected in subsequent compartments. Other microorganisms observed in compartment 1 that resembled hydrogentrophic methanogens included, *Methanospirillum*-like and *Methanococcus*-like microorganisms, with the latter being detected by DNA sequence analysis in a parallel study (Lalbahadur, 2005). In contrast to *Methanobrevibacter*-like microorganisms, these morphotypes were observed in almost all compartments (Table 6.2). There was no discernible variation in number between compartments for *Methanococcus*-like microorganisms, although the number of

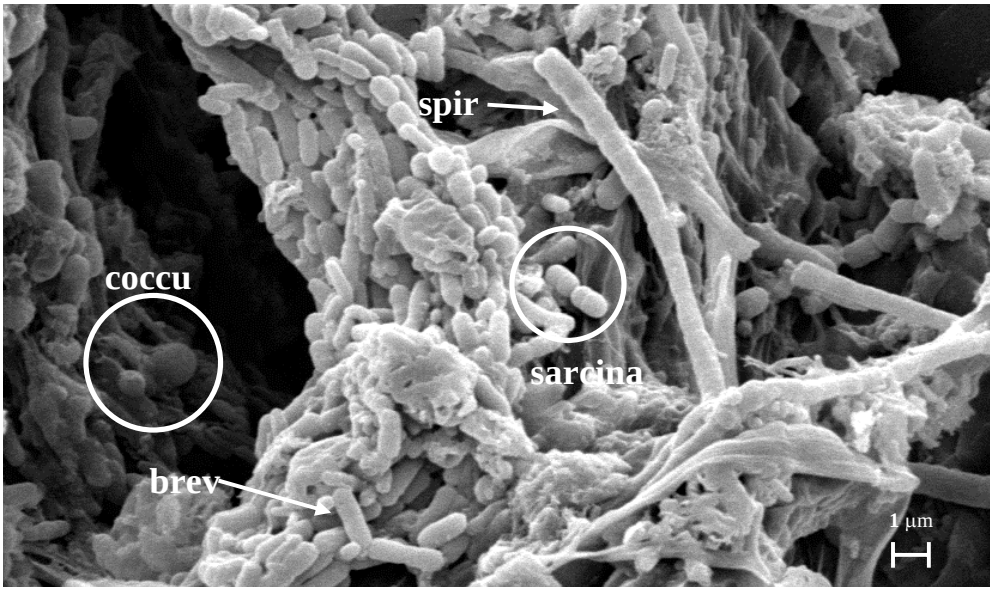


Figure 6.1: SEM micrograph showing the wide range of morphotypes in a core sludge sample from compartment 1, including microorganisms resembling *Methanobrevibacter*-like cells (brevi), *Methanococcus*-like cells (coccus), *Methanosarcina*-like cells (sarcina), and *Methanospirillum*-like cells (spir).

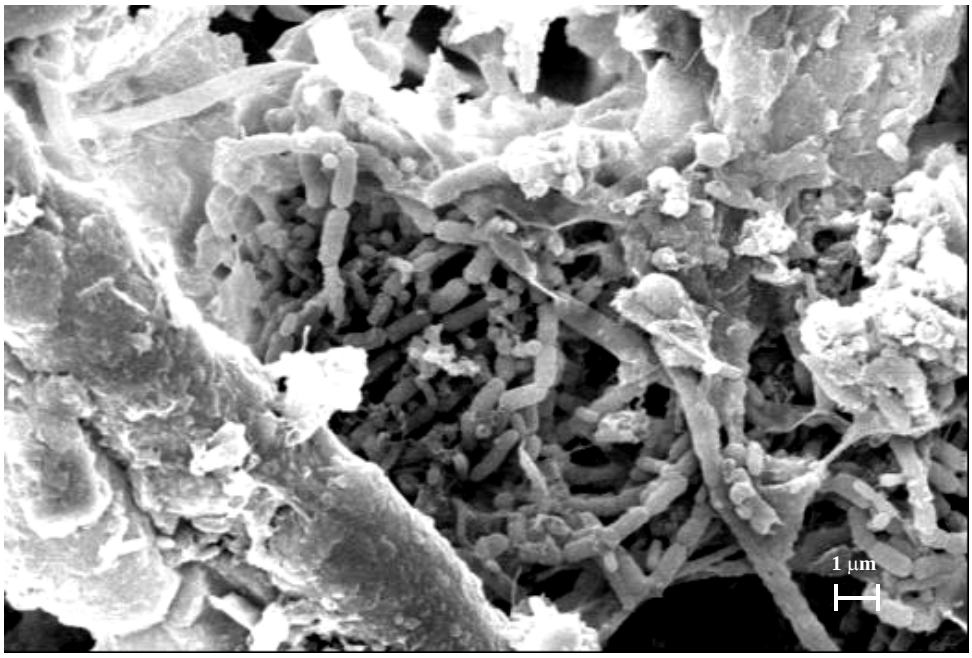


Figure 6.2: SEM micrograph showing a colony of chain-forming *Methanobrevibacter*-like microorganisms in compartment 1. The size and shape also suggest that it may be *Methanomicrobium* or *Methanobacterium* species, although they are not known to form chains (Boone *et al.*, 1994).

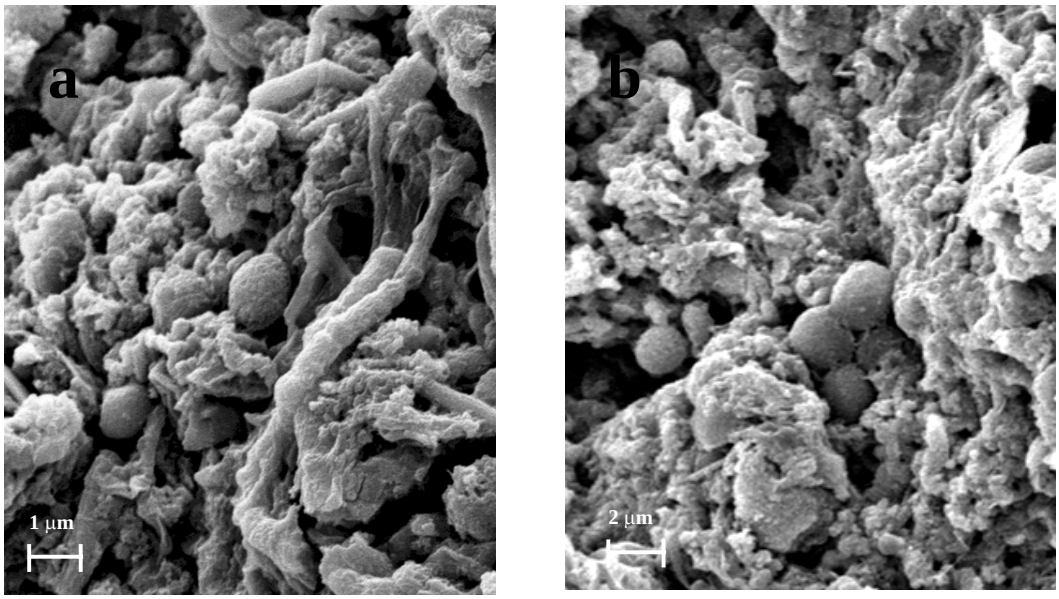


Figure 6.3: SEM micrograph showing: a) *Methanococcus*-like microorganisms in compartment 5; b) *Methanococcus*-like microorganisms in compartment 8.

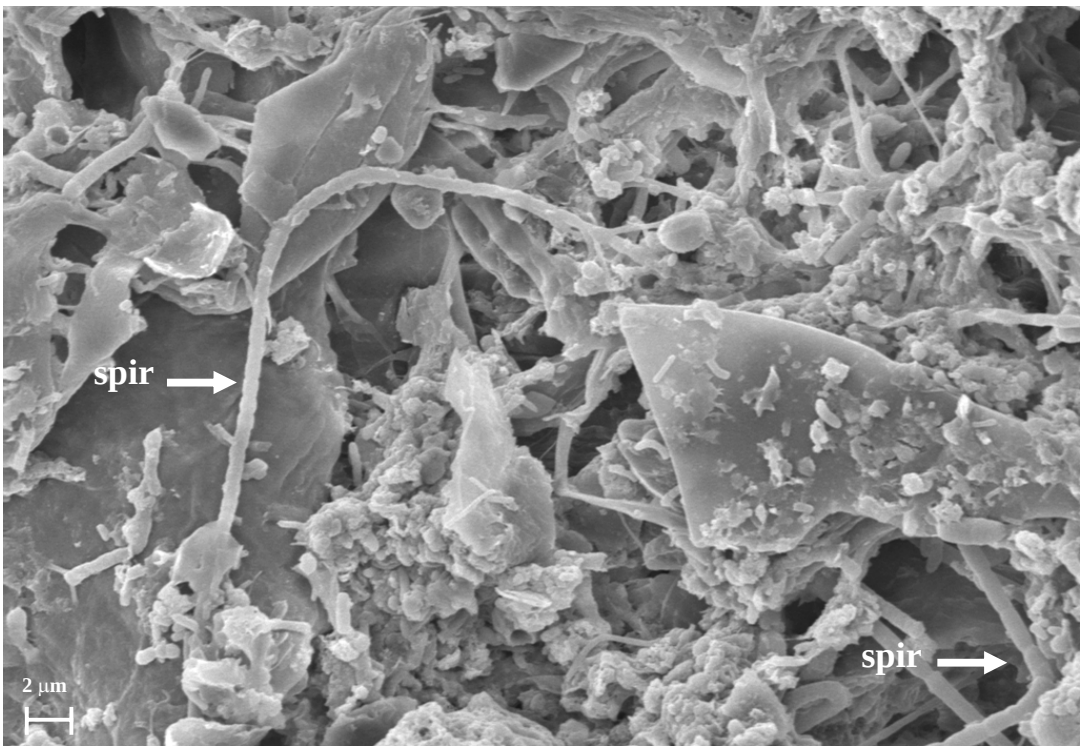


Figure 6.4: SEM micrograph showing filamentous microorganisms resembling *Methanospirillum* cells (spir).

Table 6.2: Location, distribution and tentative identification of microorganisms resembling methanogens in the ABR treating domestic wastewater at a HRT of 22 h.

Shape	Estimated cell size	Possible microorganism	Possible primary substrates [a]	Compartment location
Cell-forming rods	1 μm	*Methanobrevibacter*	Formate, H_2	1,2
		Methanomicrobium	Formate, H_2	
		Methanobacterium[1]	Formate, H_2	
Cocci with cleavage furrows	1 μm	*Methanosarcina*	Trimethlyamine, acetate, methanol, H_2	1
Small cocci	0.5 - 1 μm	*Methanococcus*	Acetate, Formate, H_2	1, 2, 3, 5
		Methanosarcina	Trimethlyamine, acetate, methanol, H_2	
Large cocci	2 - 3 μm	*Methanococcus*	Acetate, formate, H_2	1 – 8
		Methanocorpusculum	Formate, H_2	
Filamentous	1 μm each	*Methanospirillum*	Formate, H_2	1 – 7

[1] *M. formicicum* and *M. espanolae* are the only species that can use formate (Boone *et al*., 1994).
[a] Reference: Boone *et al*. (1994).

observations for *Methanospirillum*-like microorganisms decreased from compartment 5. *Methanosarcina*-like microorganisms were the only acetoclastic methanogens present in large numbers, with the morphotype resembling *Methanosaeta* being observed infrequently within a few granular sludge samples from compartment 3. Furthermore, the *Methanosarcina*-like cells were found almost exclusively in compartment 1 (Table 6.2). The observation was supported using fluorescent probes (FISH) and sequence analyses for the same reactor (Foxon *et al*., 2004 and 2005; Lalbahadur, 2005).

SEM observations also revealed the presence of some precipitates embedded in all sludge samples. The size and shape of precipitates varied, were located throughout the reactor, and were found to be silicon when analysed by energy-dispersive X-ray spectrometry (data not presented) (Figure 6.5). The plate-like nature of the silicon particles suggested that they were not sand particles. It is speculated that the silicon may have derived from microbial activity on the silicone sealant used to seal the reactor before operation began or may be plant-derived wall or cuticular silicates. Evidence for plant-derived components such as xylem vessels was seen (Figure 6.5).

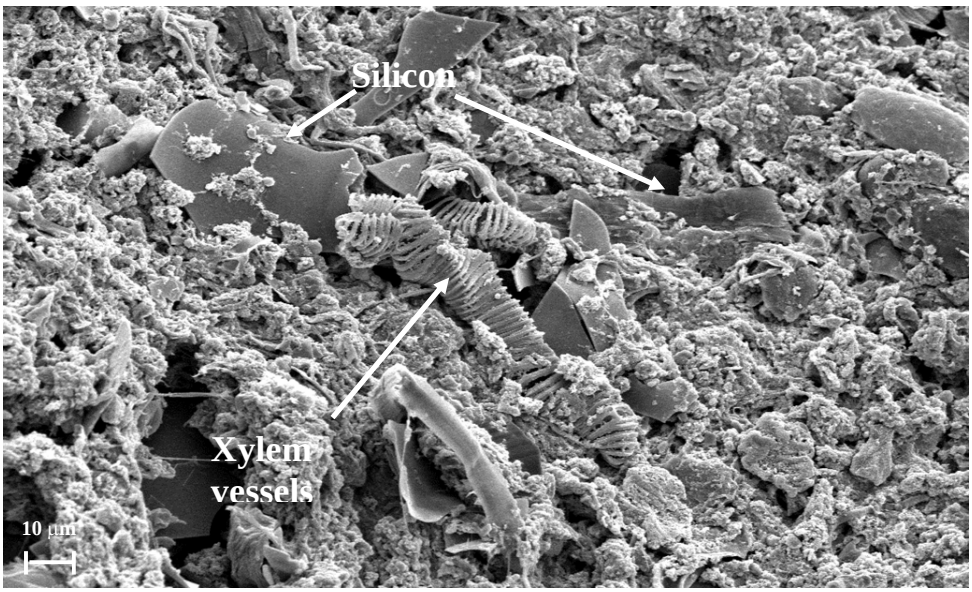


Figure 6.5: SEM micrograph showing possible non-microbial material within the sludge of the ABR. Plate-like precipitates observed in all compartments of the ABR were shown to be silicon by SEM energy-dispersive X-ray microanalysis. Plant material in the form of xylem vessels was also observed.

Granulation

Granulation was shown to occur between the second and third compartments in the ABR. The outer surface of the granules was smooth but irregular in shape (Figure 6.6a). A close examination of granule surface revealed a small microbial diversity; only two morphotypes were observed (not shown). Furthermore, they were not present in large numbers, and observations were infrequent. These included coccoid microorganisms, resembling either *Methanococcus* or *Methanosarcina* cells, and a filamentous microorganism, resembling *Methanospirillum*. It is possible that this morphotype might be *Methanosaeta*, but it does not display the typical characteristics observed for this methanogen. Cleavage of the granule revealed a loosely packed interior, composed of numerous cavities (Figure 6.6b). Granule structure was brittle as they showed a tendency to crumble during sectioning. Microbial diversity within the granule was limited and similar to that of the surface. Interestingly, a third morphotype was observed very infrequently within a few granules. This morphotype was composed of rod-shaped cells with flat ends, bearing the typical characteristics of *Methanosaeta* species (Patel, 1984; Zehnder *et al*., 1980; Zinder *et al*., 1984b) (Figure 6.7). However, they did not occur frequently enough to be included with other prevalent microorganisms resembling methanogens.

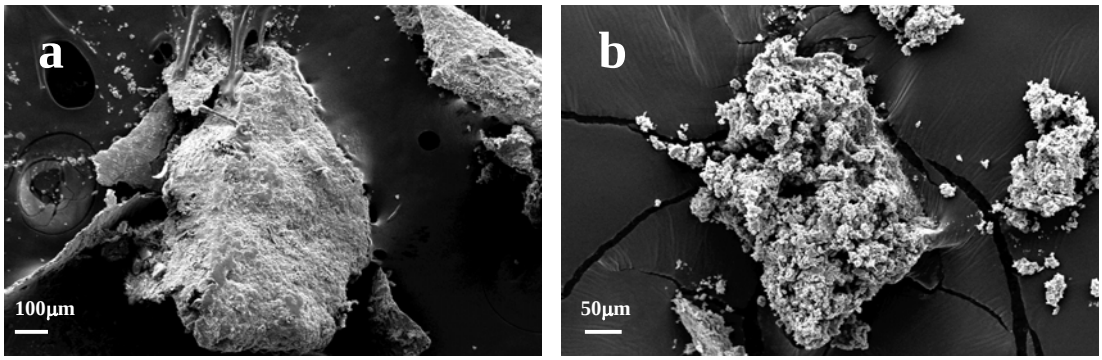


Figure 6.6: SEM micrographs showing: (a) the surface topography of an entire granule; (b) interior structure of granule from ABR treating domestic wastewater (HRT = 22 h).

The limited observations of these acetoclastic methanogens gave rise the hypothesis that archaea of this type may have been present, but bound within granules, and therefore not observed. Hence, a few granules (4) were prepared and examined under epi-fluorescent excitation. The excitation of histological sections of granules at 350 nm and 420 nm revealed only the presence of coccoid microorganisms (Figure 6.8). Contrary to SEM observations, filamentous or rod-shaped microorganisms resembling *Methanosaeta* species were not detected within granules. Similar results were obtained from composite sludge samples using FISH and DNA sequence analysis (Foxon *et al*., 2005; Lalbahadur, 2005). It was hypothesised that the lack of fluorescent signals observed in this study may be due a low metabolic rate, whilst those reported by Lalbahadur (2005) may be due to a lack of probe penetration into the granules.

According to Foxon *et al.* (2005), the lack of *Methanosaeta* may be due to the sampling error. As a core sample of sludge was taken from the upflow region of each compartment, it was hypothesised that *Methanosaeta* might not have been present within the core sludge, but attached to the walls of the reactor. This would explain why it was not detected by the more rigorous method of FISH or DNA sequence analysis used by Lalbahadur (2005). Whilst this hypothesis cannot be confirmed, the results presented here suggest that *Methanosaeta* was not present in substantial numbers, with only a few observations within some granules.

6.3.3 Discussion

According to theory, partial or total separation of acidogenic and methanogenic phases occurs in an ABR. As a result, the reactor has often been described as a two-stage process (Grobicki and Stuckey, 1991), the consequence of which, results in the establishment of different microbial

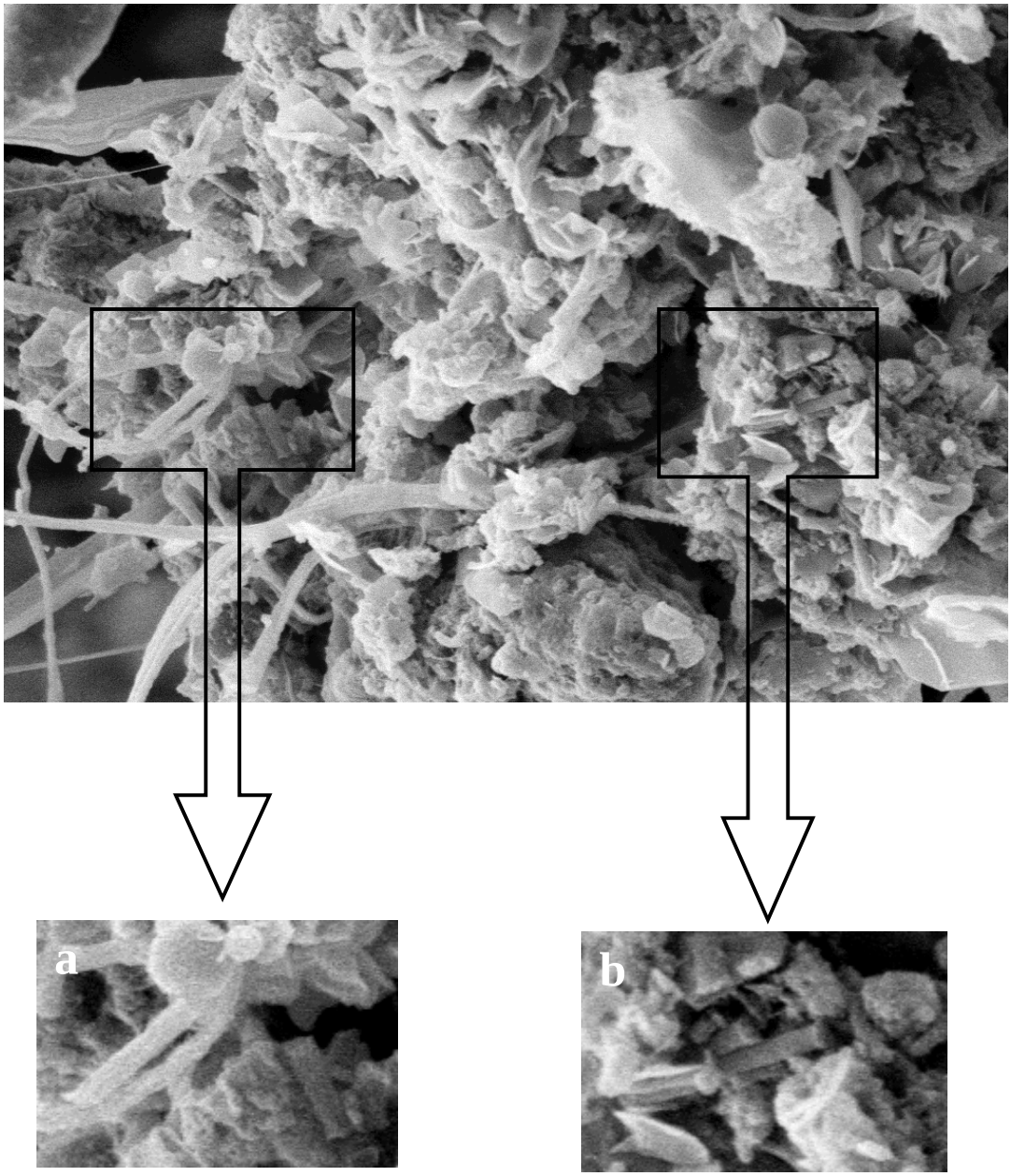


Figure 6.7: SEM micrograph of the interior of a granule showing both coccoid and filamentous microorganisms. The coccoid morphotypes resemble *Methanococcus* or *Methanosarcina* species. The filamentous morphotypes resembles *Methanospirillum* species. Close examination of the micrograph also reveals the presence of third morphotype of bamboo-shaped rods with flat-ends, which closely resemble the typical morphology of *Methanosaeta* species (inserts a and b).

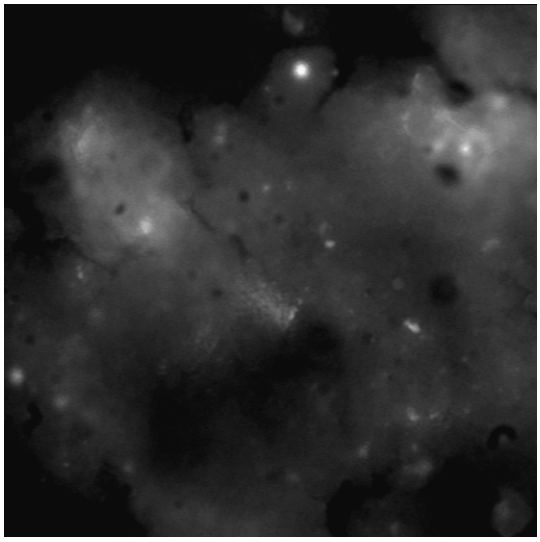


Figure 6.8: Section of typical granule from the ABR treating domestic wastewater under epi-fluorescent microscopy. Only coccoid microorganisms, with relatively low emitted fluorescence, were detected within the granule (mag. X 25).

groups, under more favourable conditions, spatially through the reactor (Akunna and Clark, 2000; Barber and Stuckey, 1999; Holt *et al*. 1997; Uyanik *et al*., 2002). Contrary to these findings, the results presented here show that significant separation did not occur in this ABR, when treating domestic wastewater at a HRT of 22 h. Microorganisms resembling methanogens were observed throughout the reactor with a larger number in the first few compartments. These results were also verified using fluorescent probes to characterise core sludge samples from the same reactor (Lalbahadur, 2005). Langenhoff and Stuckey (2000) observed similar results in treating dilute, synthetic wastewater (semi-skimmed milk diluted with tap water) at various temperatures in an ABR. These authors postulated that phase separation in an ABR does not occur in the treatment of dilute wastewaters due the lack of a physical selection pressure (low COD and low biomass 'washout').

Whilst this may be true for dilute soluble wastewaters, it is hypothesised that the lack of selection of the specific groups in the separate compartments may be due to the slow rate of hydrolysis of particulate organics. The degradation of domestic wastewater is known to be complex, as it contains more particulate organics than soluble ones (Eastman and Ferguson, 1981). As a result, the initial hydrolysis of particulates to soluble substrates may have been the rate-limiting step in

the reactor. The reactor was therefore thought to be hydraulically overloaded at a HRT of 20 h. This hypothesis is supported by FISH studies for the same reactor, which showed the presence of hydrolytic and acidogenic bacteria in all compartments of the reactor (Foxon *et al*., 2005; Lalbahadur, 2005). The results indicate that hydrolysable particles were present in all compartments, implying that the initial breakdown of particulates was the rate-limiting step (Foxon *et al*., 2005). Furthermore, the presence of methanogens at the front of the reactor implies that scavenging of VFA, either produced at low levels by fermentative microorganisms, or present as a soluble fraction entering the reactor, probably occurred. Under such conditions of low substrate availability, it was expected that there would be a larger population of *Methanosaeta* species (Barber and Stuckey, 1999; Polprasert *et al*., 1992; Zinder, 1994). Contrary to findings in the literature, there were very few observations for morphotypes that resemble *Methanosaeta*, with all observations made within a few granules. *Methanosaeta* was not detected by epi-fluorescent excitation in granules, or in any core sludge samples by FISH, indicating a possible low metabolic activity (Uyanik, 2003). As mentioned earlier, the results could also be related to the sampling procedure used. In contrast, there were numerous observations for *Methanosarcina*-like species, especially near the front of the reactor, the results of which were confirmed by FISH (Foxon *et al*., 2005; Lalbahadur, 2005).

It is thought that parameters, other than the acetate concentration, may have influenced the dynamics of the two acetoclastic methanogen populations. This included sludge ‘washout’, poor solids retention, and a low pH (5.95 – 6.85), which were thought to be the effect of reactor instability and poor solids degradation at a HRT of 22 h. Methanosaeta species are known to occur as two forms in anaerobic reactors; a long multicellular, filamentous form and a rod-shaped form in fragments up to five cells (Schmidt and Ahring, 1997). The filamentous form is favoured under conditions with substrate limitation (Hulshoff Pol, 1989; Wiegant and de Man, 1986), such as those observed within this ABR. According to Schmidt and Ahring (1997), this form can lead to bulking of sludge and is more susceptible to ‘washout’. Accidental ‘washout’ through unplanned high flow incidents may have aggravated this situation. This would probably explain why only rod-shaped, Methanosaeta-like cells were observed in the reactor. However, this form (rod-shaped) was observed within poorly developed granules with large cavities, and might have also been susceptible to ‘washout’. According to Yoda and Nishimura (1997), granules with such cavities, caused by substrate limitation, will eventually float out from reactors. In either scenario, it is clear that the flow conditions (sludge ‘washout’ and solids retention characteristics of the reactor) have a major influence on the dynamics of the two acetoclastic populations.

The low pH was also thought to influence the dynamics of the two acetoclastic genera. Hydrolytic and acidogenic bacteria were detected in all compartments of the ABR (Foxon et al., 2005; Lalbahadur, 2005). As a result, the pH values all compartments were generally below the optimum cited for methanogenic organisms. It was postulated that there was a selection for Methanosarcina under such conditions, even if acetate levels were low (not measured). Methanosarcina is considered to be more metabolically versatile than Methanosaeta. They are also known to efficiently produce methane at low pH values (Barber and Stuckey, 1997b), whilst others can lower their minimum thresholds for acetate at a lower pH. Furthermore, they are able to form either clusters or microcysts during unfavourable conditions, a strategy that increases their survival (Sprott and Beveridge, 1994; Zinder, 1994). It is also able to grow on several substrates other than acetate, including trimethylamine, methanol, and H_2/CO_2, using them rapidly to produce higher cell yields (doubling times = 1.5 days) than Methanosaeta (Zinder, 1994). In some instances, Methanosarcina can out-compete hydrogenotrophic methanogens in a medium containing H_2/CO_2 (Daniels et al., 1984). Methanosaeta, on the other hand, are more sensitive than Methanosarcina to environmental conditions, such as reduced pH (Barber and Stuckey, 1997b). Furthermore, they are regarded as specialists that utilise only acetate, and at very low concentrations, and consequently their doubling times are longer (approximately three days) (Zinder, 1994). The predominance of *Methanosaricna* as the main acetoclastic methanogen may therefore be due to their adaptability to grow faster under conditions of high 'washout', use more than one substrate, and a greater tolerance to reduced pH.

With respect to hydrogenotrophic methanogens, large populations were observed towards the front of the reactor, although there was no discernible variation in *Archaeal* numbers observed using FISH (Foxon *et al*., 2005; Lalbahadur, 2005). According to Tilche and Yang (1987), the stimulation of hydrogenotrophic scavenging methanogens at the front of the reactor is due to elevated levels of hydrogen headspace. It is hypothesised that a similar situation may have prevailed in the pilot reactor. Whilst it is uncertain under which conditions one hydrogenotrophic methanogen is favoured over another, it is postulated that the prevalence of *Methanospirillum*-like microorganisms over other hydrogenotrophic methanogens near the end of the reactor may be related to a lower threshold (K_m) for hydrogen (results cited by Zinder, 1994).

With respect to the granulation phenomenon, only a few, poorly developed granules were observed near the front and middle sections of the reactor. Barber and Stuckey (1997a) have also

observed floc development towards the middle of an ABR. These authors hypothesised that it may be due to less shear force and greater gas production.

With respect to granule structure, no evidence of stratification was observed in this study using SEM and fluorescence microscopy. In addition, only two morphotypes were regularly observed within granules. Whilst similar findings have been observed when treating propionate, ethanol, glutamate, sugar refinery wastewaters, and methanol waste in UASBs (Bhatti *et al*., 1993; Fang *et al*., 1995a), the distinct lack of microorganisms on the surface and within the granules, or the predominance of any morphotype, is in stark contrast to the literature regarding granulation. Both *Methanosarcina* and *Methanosaeta* have been identified in anaerobic granules under stable conditions, and their presence is important during the initiation of granulation (de Zeeuw, 1984; Grotenhuis *et al*., 1988; Hulshoff Pol, 1989; Schmidt and Ahring, 1996). The prevalence of one over the other are thought to be related to substrate affinity, specifically, the acetate concentration (Schmidt and Ahring, 1997). Based on the kinetic data for the two methanogens, there should have been a selection for granules dominated by *Methanosaeta* under substrate limitation. The inability of *Methanosaeta* to 'establish' itself, under these conditions, was the thought to be the main reason for poor granule development. Numerous reports have suggested that *Methanosaeta* is the primary initiator of granulation (MacLeod *et al*., 1990; Wiegant, 1987) and play an important role in binding granule components together (MacLeod *et al*., 1990; Morgan *et al*., 1991). As it could not 'establish' itself within the bulk phase of the reactor (for reasons outlined earlier), granule development was probably hindered. Although *Methanosarcina* is also capable of granule formation, its inability to form well-developed granules is probably due to low substrate concentrations, or a high cation concentration in the wastewater (Schmidt and Ahring, 1993).

To conclude, it was suggested that the hydraulic conditions have a major influence on the microbial population dynamics of an ABR treating domestic wastewater.

6.4 Case Study 2: HRT of 40-44h

6.4.1 Hypothesis and Objectives

During the 22 h HRT operation period, no spatial separation of anaerobic consortia was observed along the length of the reactor, as a result of the reactor being hydraulically overloaded. A study was undertaken to investigate the hypothesis that a better spatial separation might occur at a longer HRT. Furthermore, it was hypothesised that a longer HRT may allow *Methanosaeta* to

'establish' themselves through less 'washout' and improved environmental conditions. The objective of this experiment was to determine the effect of increasing the HRT on the composition of microbial populations in the ABR.

6.4.2 Results

Distribution of Anaerobic Microorganisms

A greater mixed population of microorganisms, which were not observed in case study 1, were present at the front of the reactor. These included rod-shaped and coccoid microorganisms of varying size and shape (Figure 6.9). The prominence of these microorganisms at the front of the reactor, especially compartment 1, led to the conclusion that they may have been acid-producing bacteria. Because the absolute identification of these microorganisms could not be made with any degree of confidence, they have been omitted from the descriptive list of morphologies below. Also excluded, were small clusters of two to four spherical cells, with cleavage furrows, which are consistent with the descriptions reported for *Methanosarcina* species (not shown) (Chen and Lun, 1997; Sprott and Beveridge, 1994). These microorganisms were infrequently observed, mostly in compartment 1, and were therefore not included. The most prevalent morphotypes, excluding those that could not be identified confidently, included:

1. Slender rods with a slight curvature, which closely resembled the *Syntrophomonas*-like species described by Harper and Pohland (1997). These authors suggested that the morphotype could also be a slender species of *Desulfovibrio* or *Desulfomonas* (Figure 6.10) (Harper and Pohland, 1997).
2. Rod-shaped, chain-forming microorganisms resembling hydrogenotrophic methanogens *Methanobrevibacter*, *Methanobacterium* or *Methanomicrobium* (Figure 6.11). As previously stated, only *Methanobrevibacter* has been shown to form chains (Boone *et al*., 1994).
3. Long filamentous or chain-forming microorganisms consisting of 10 or more cells resembling the typical morphology of *Methanospirillum* (Figure 6.11 and Figure 6.12).
4. Bamboo-shaped rods with flat-ends resembling the typical morphology of the acetoclastic methanogen, *Methanosaeta* (Patel, 1984; Zehnder *et al*., 1980; Zinder *et al*., 1984b) (Figure 6.12).

The relative abundance of these morphotypes varied between compartments and the results are summarised in Table 6.3. For *Syntrophomonas*-like microorganisms, the number of observations made for this morphotype was highest in compartment 1, after which, numbers steadily declined

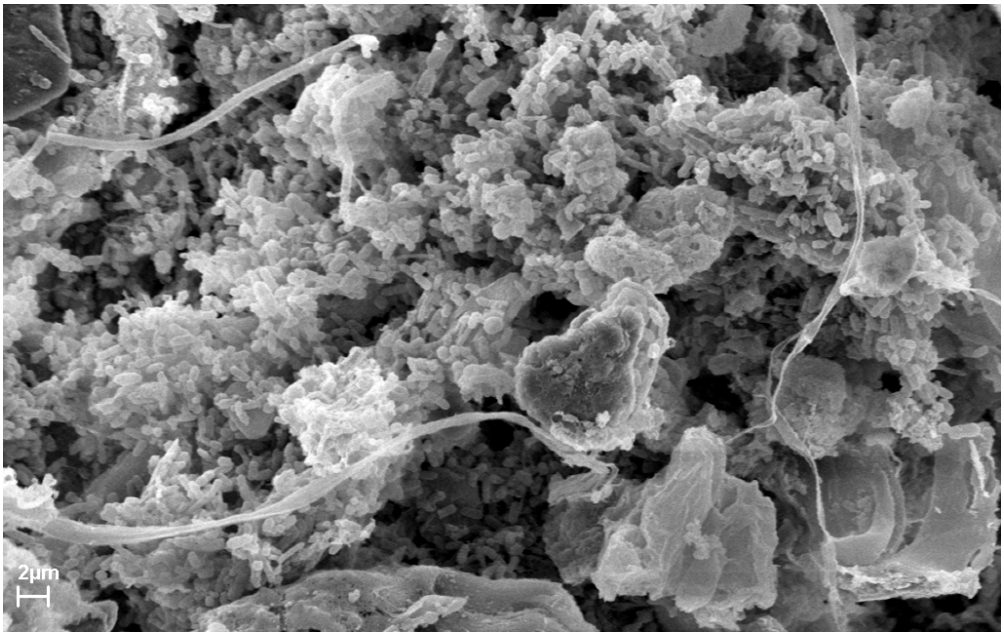


Figure 6.9: SEM micrograph showing a heterogeneous population of morphotypes in compartment 1. It was thought that the majority of the population resembled acidogenic bacteria.

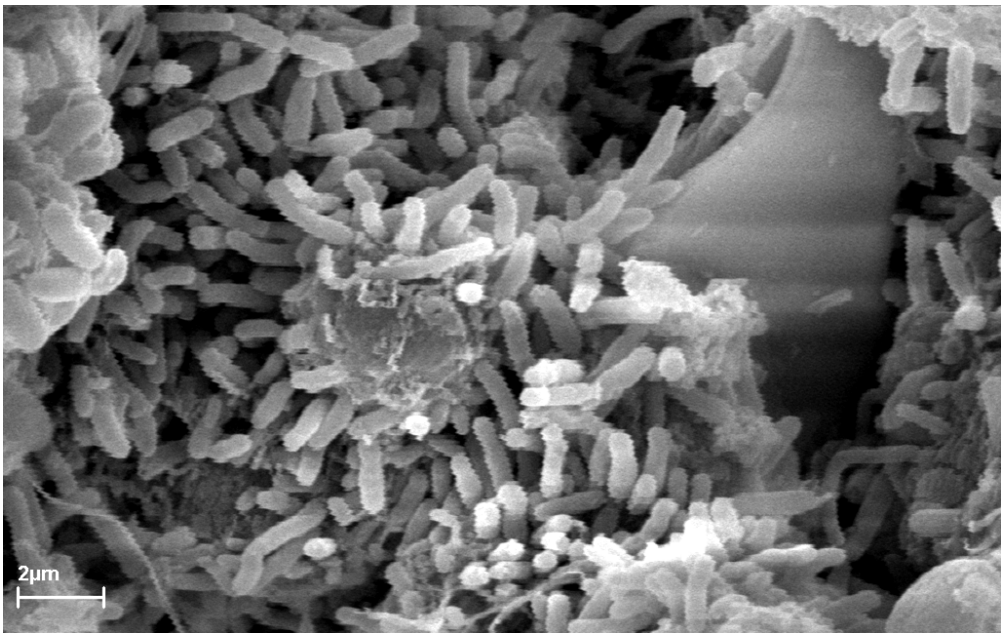


Figure 6.10: SEM micrograph of compartment 1 showing thin, slender rods with a slight curvature that resembles *Syntrophomonas* species, or a slender species of *Desulfovibrios* or *Desulfomonas* (Harper and Pohland, 1997).

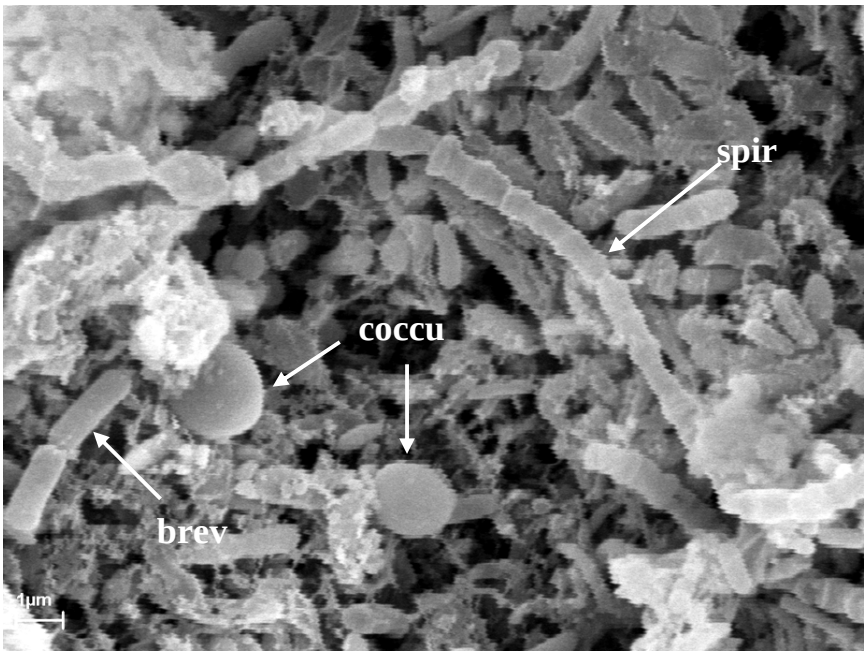


Figure 6.11: SEM micrograph of compartment 1 showing morphotypes resembling *Methanospirillum*-like microorganisms (spir), *Methanoccous*-like microorganisms (coccus) and *Methanobrevibacter*-like microorganisms (brev).

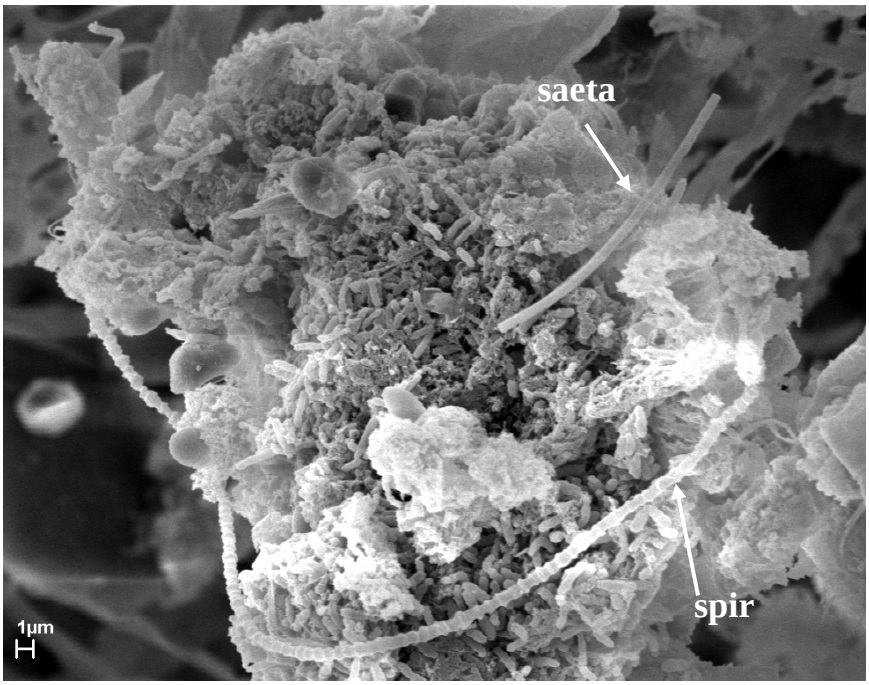


Figure 6.12: SEM micrograph showing a heterogeneous population of microorganisms in compartment 1, with two types of filamentous microorganisms resembling *Methanospirillum* species (spir) and *Methanosaeta* species (saeta).

Table 6.3: Location, distribution and tentative identification of microorganisms observed in the ABR treating domestic wastewater at a HRT of 40-44 h.

Shape	Estimated size	Possible microorganisms	Possible primary substrates [a]	Compartment location
Long rods with slight curvature	1 – 2 μm	*Syntrophomonas*	Butyrate	1
		Desulfovibrio	Acetate, H_2	
		Desulfomonas	Acetate, H_2	
Chain-forming rods	1 μm	Methanobrevibacter	Formate, H_2	1,2
		Methanomicrobium	Formate, H_2	
		Methanobacterium[1]	Formate, H_2	
Small cocci	0.5 - 1 μm	*Methanococcus*	Acetate, Formate, H_2	1, 2, 3, 5
		Methanosarcina	Trimethlyamine, acetate, methanol, H_2	
Large cocci	2 –3 μm	*Methanococcus*	Acetate, formate, H_2	1 – 8
		Methanocorpusculum	Formate, H_2	
Filamentous	1 μm each	Methanospirillum	Formate, H_2	1 – 7
Bamboo-shaped rods and filaments	2 μm each	Methanosaeta	Acetate	2 – 8

[1] *M. formicicum* and *M. espanolae* are the only species that can use formate (Boone *et al*., 1994).
[a] Reference: Boone *et al*. (1994).

up to compartment 3. A similar pattern was observed with respect to morphotype resembling *Methanobrevibacter*-like microorganisms.

For the morphotypes resembling *Methanospirillum*-like microorganisms, the number of observations was relatively similar from compartment 1 to 4, after which, there was a steady decrease.

Two types of cocci were observed within the reactor: large cocci with a diameter of approximately 2 - 3 μm, and smaller cocci with a diameter between 0.5 – 1 μm. Although inconclusive, these cocci resembled *Methanococcus* species. Variations in the relative abundance for the two types of cocci were also evident. The number of observations of smaller cocci was highest in the front of the reactor (compartment 1 to 5), after which, observations became infrequent. In contrast, the larger cocci were found throughout the reactor with increased numbers evident between compartments 5 to 8.

Bamboo-shaped rods were the sixth morphotypes observed, a typical characteristic of the acetoclastic methanogen, *Methanosaeta* (Figure 6.12 and Figure 6.13). This morphotype was present in most compartments, but was most prevalent in compartments 2 and 3, with a decreased occurrence in the later compartments. In compartments 2 and 3, *Methanosaeta*-like microorganisms were present almost exclusively as aggregates or granules, as either a filamentous form or clusters of rod-shaped microorganisms. Within the granules or core sludge samples, *Methanosaeta*-like cells were observed embedded in a complex network of extracellular polymers (ECP) (Figure 6.14). This arrangement led to the formation of internal gas cavities. A more detailed description of the microbial composition of these granules is provided below.

Granulation

The granulation phenomenon was observed in compartments in 2 and 3, and to a lesser extent, compartment 4. Granule shape varied from circular to oval-shaped and were 600 μm to 2000 μm in diameter. Granules were dark-grey to black in colour, with a smooth surface (Figure 6.15). On the surface of larger, well-developed oval granules, apparent 'gas cavities' about 10-20 μm could be seen (Figure 6.16a and b).

Granules had a two-layered structure, with a thin outer surface layer (approximately 2 – 10 μm) and large interior core (Figure 6.17a). A close examination of the granule surface revealed the existence of numerous large and small cocci, resembling *Methanococcus*-like microorganisms, embedded in inert material (Figure 6.17b). Also present to a lesser extent were the morphotypes resembling *Methanospirillum*-like microorganisms and acidogenic bacteria (not shown). Bamboo-shaped rods, resembling *Methanosaeta* species, protrude from the inner layer to the surface of granules (not shown). Similar observations have been made from an upflow sludge bed and filter reactor treating sucrose waste (MacLeod *et al.*, 1990). Cleavage of granules revealed an internal structure different from that seen in surface views. The interior contained a predominance of *Methanosaeta*-like microorganisms, which made up the core of the granule (Figure 6.18). These microorganisms were surrounded by fibrous ECP, and were often embedded within them (Figure 6.19). The structural arrangement of the *Methanosaeta*-like cells may have facilitated the formation of internal 'gas cavities' within granules (Figures 6.20 and 6.21). Morphotypes resembling acidogenic bacteria were shown to surround these cavities (Figure 6.22). They were also shown to occur around mini-aggregates of ECP-bound *Methanosaeta*-like cells (Figures 6.23 and 6.24). The frequent observations of these aggregates within the granule interior seemed to suggest that ABR granules might have developed from them. These mini-aggregates may be

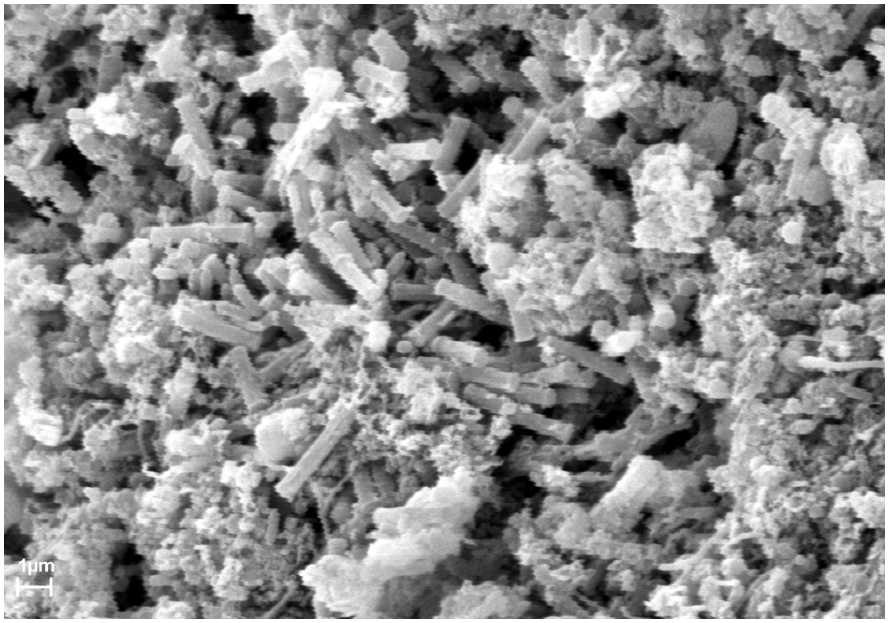


Figure 6.13: Close SEM examination of sludge from compartment 2 showing a cluster of bamboo-shaped cells with flat ends resembling the typical morphology of *Methanosaeta* cells.

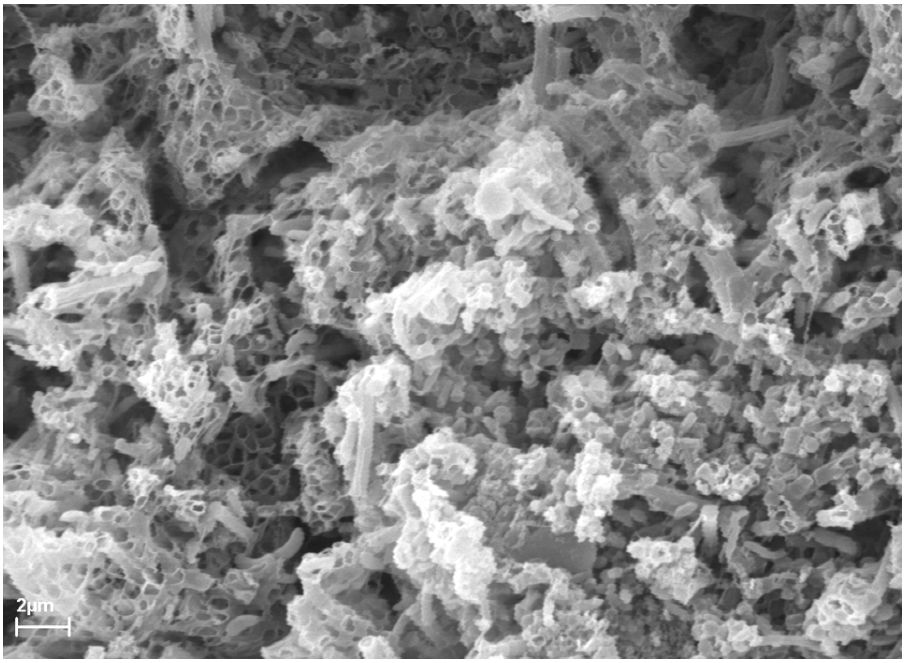


Figure 6.14: SEM micrograph from compartment 2 showing *Methanosaeta*-like cells embedded in ECP, forming a complex aggregate.

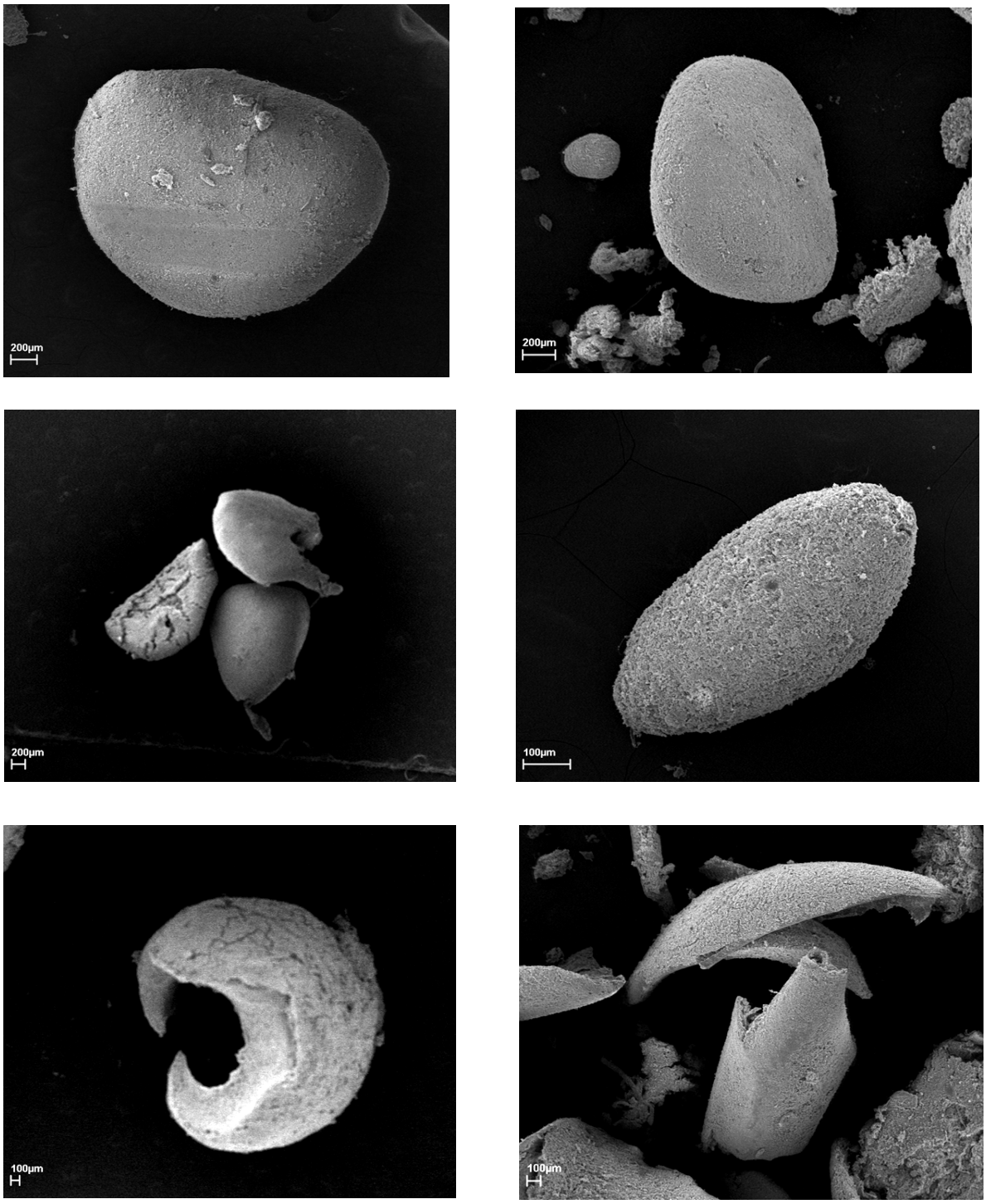


Figure 6.15: SEM micrographs of some selected granules from compartments 2 and 3. Granules varied in size and shape (circular and oval-shaped).

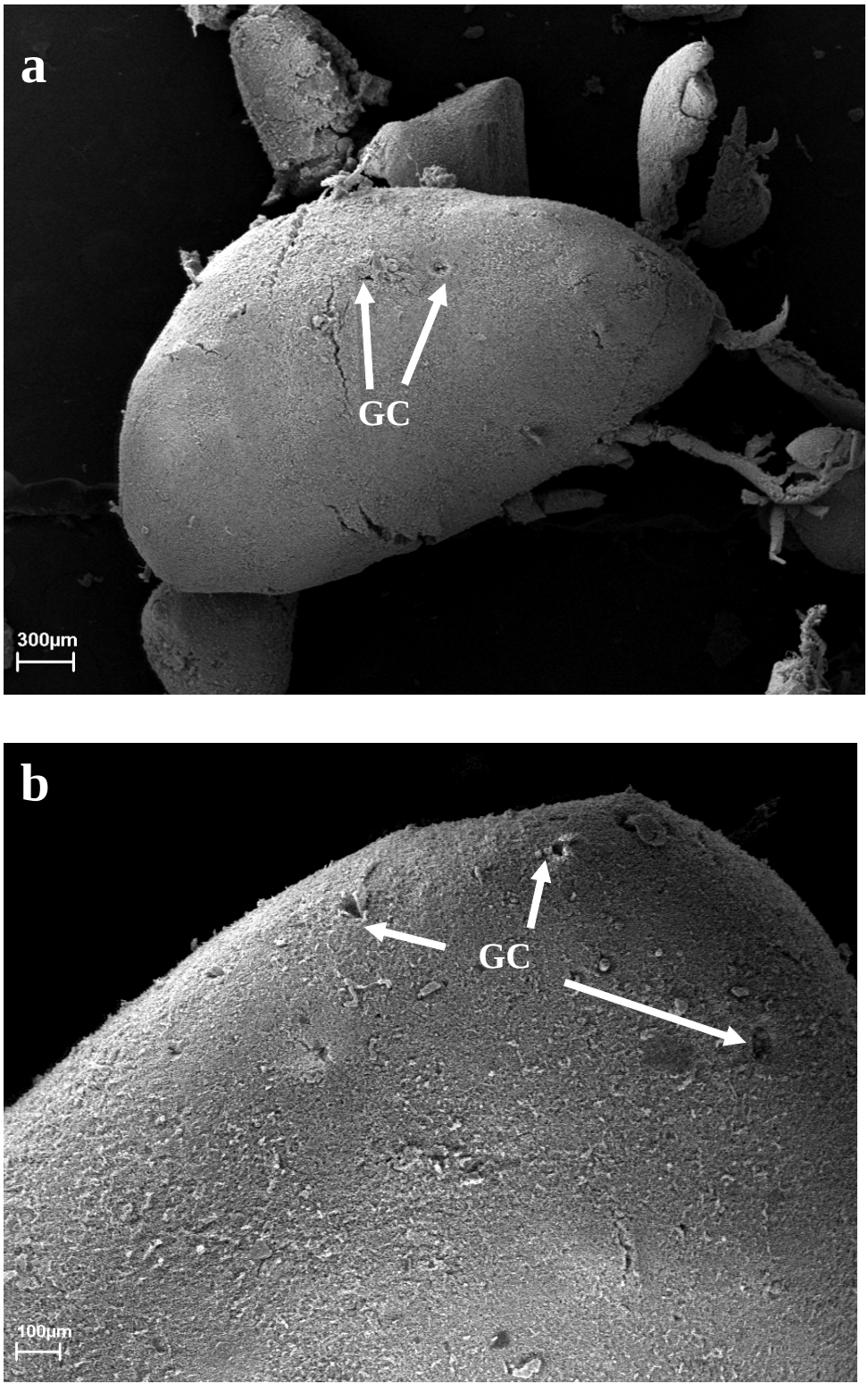


Figure 6.16: SEM micrographs of the surface of granules showing: a) apparent 'gas cavities' (GC, 'gas cavity') on the surface of granule; b) higher magnification of the cavities.

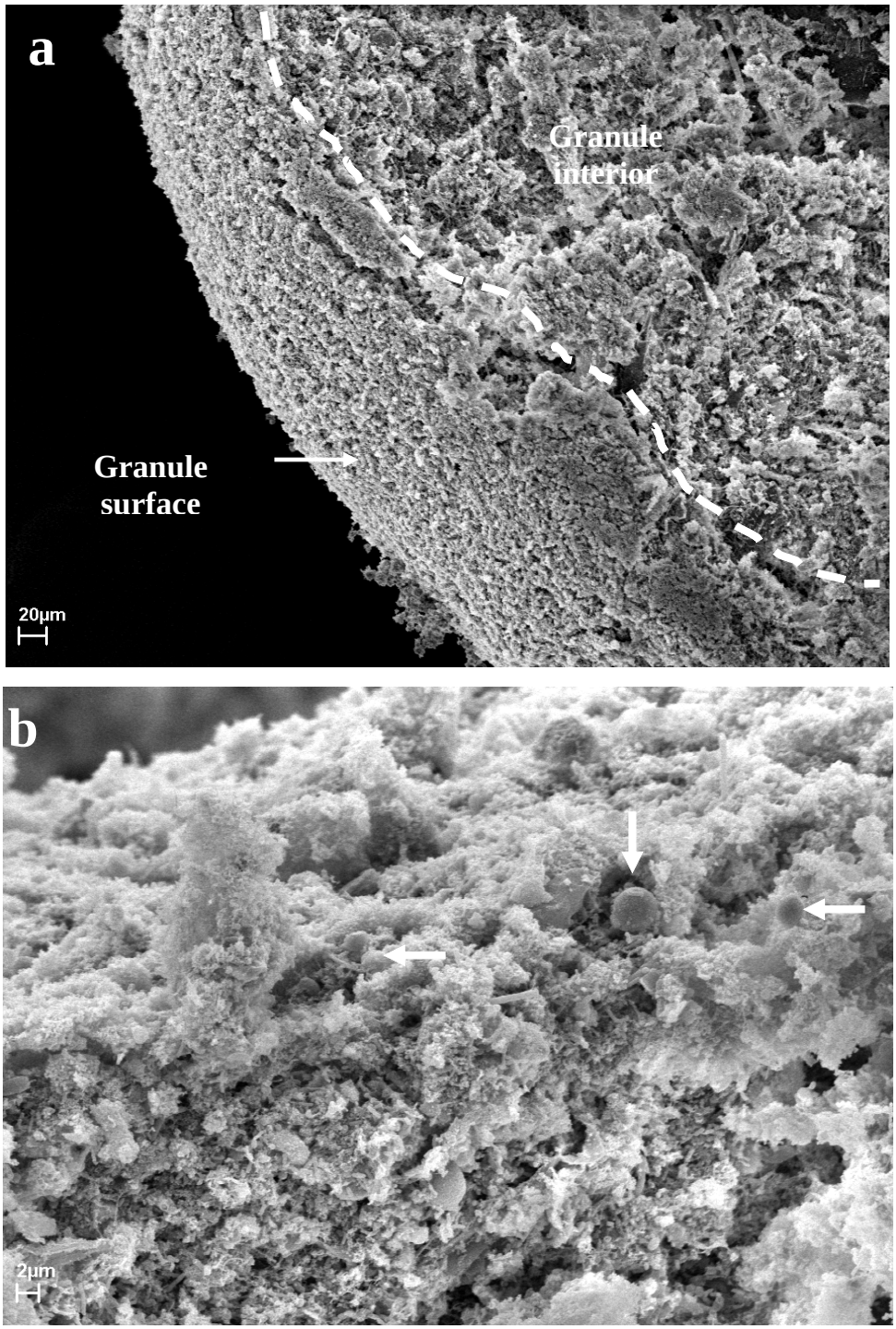


Figure 6.17: SEM micrograph of bisected granule showing: a) internal and external surfaces of granules; b) a heterogeneous microbial population, consisting mostly of coccoid microorganisms of varying size and shape (arrows), at high magnification. Other morphotypes observed on the outer surface included *Methanospirillum*-like microorganisms (not visible) and some irregular rod-shaped microorganisms.

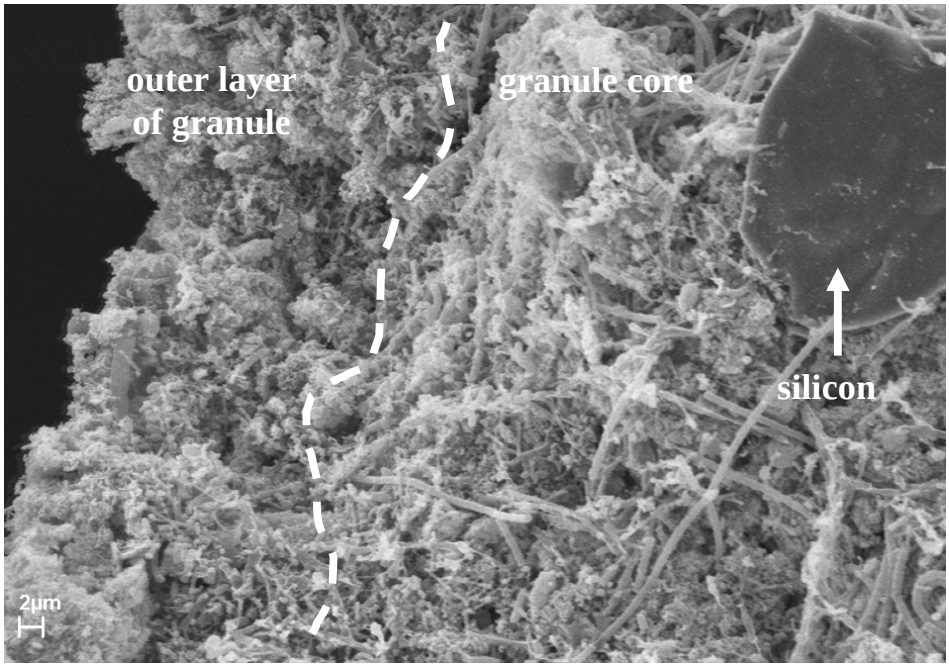


Figure 6.18: SEM micrograph of bisected granule showing a predominant mass of filamentous microorganisms of similar structure to *Methanosaeta* within the granule core. Silicon precipitates are also noted.

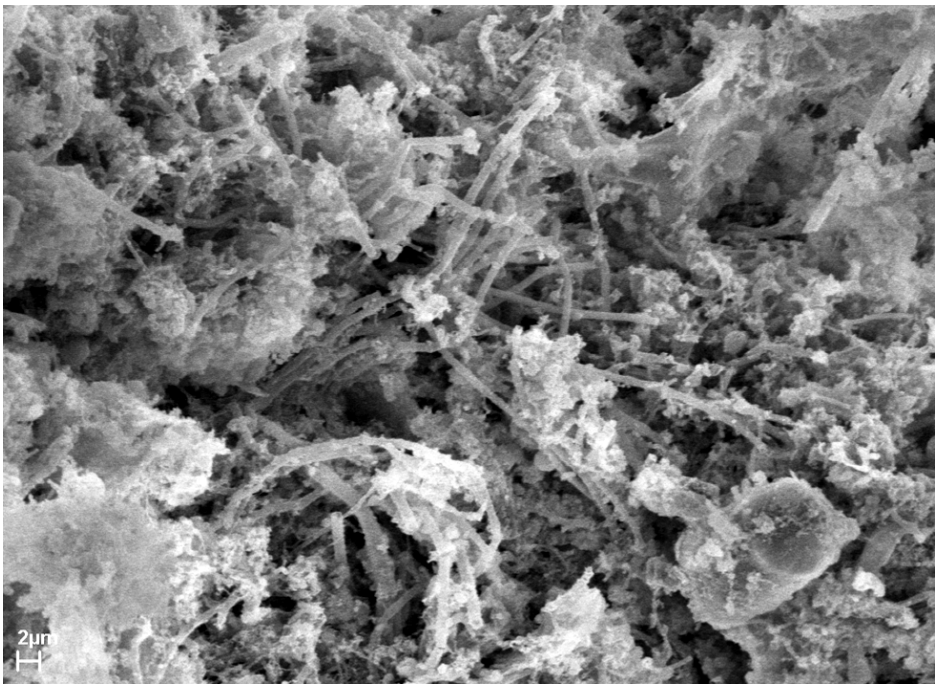


Figure 6.19: High magnification of granule interior showing *Methanosaeta*-like cells surrounded by large amounts of electron-dense ECP matrices.

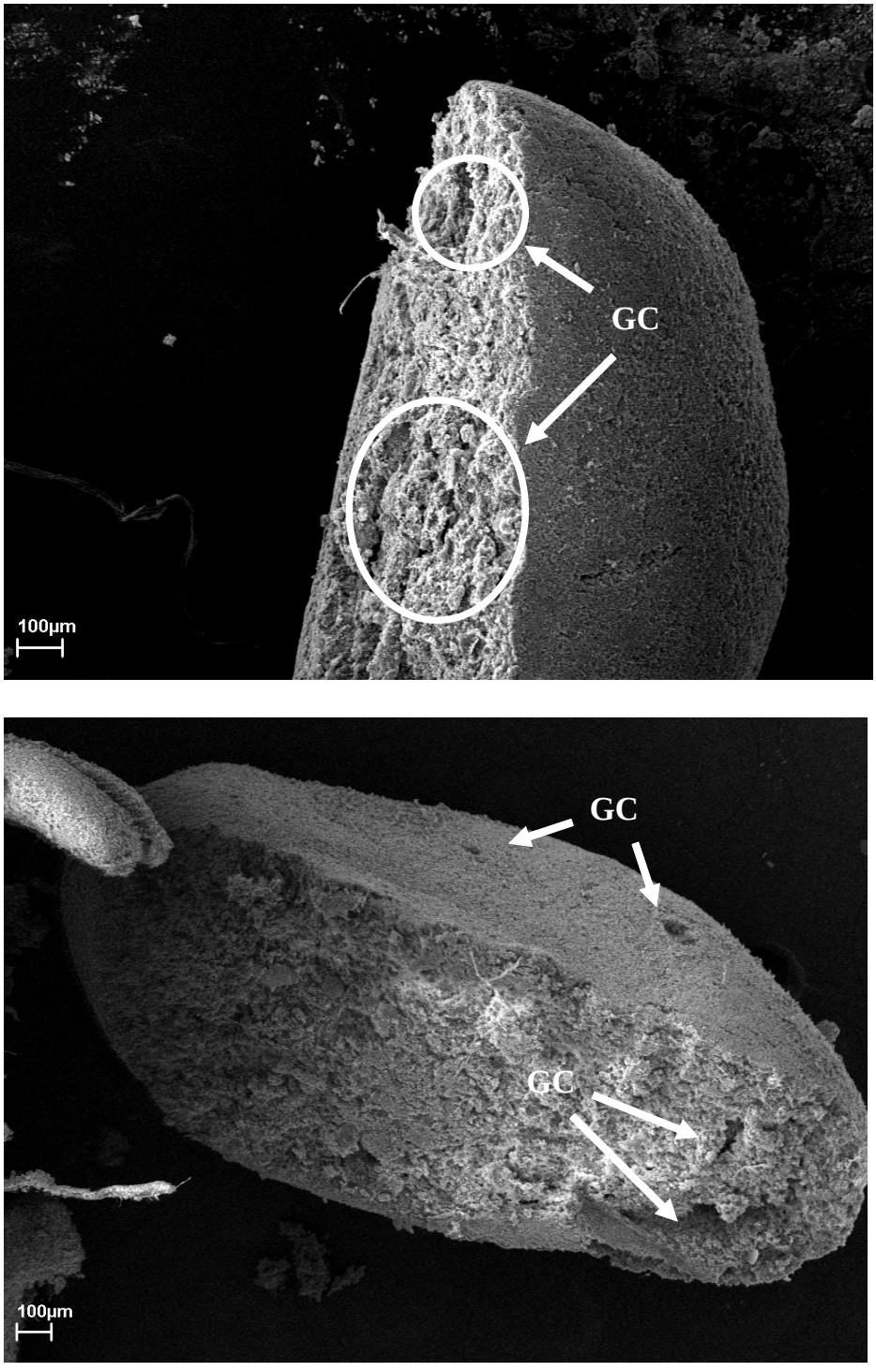


Figure 6.20: SEM micrographs of granules showing the internal surface of granules covered with 'gas cavities' of varying size. Only larger cavities have been highlighted. The bottom micrograph also shows distinct cavities on the outer surface of the granule (GC, 'gas cavity').

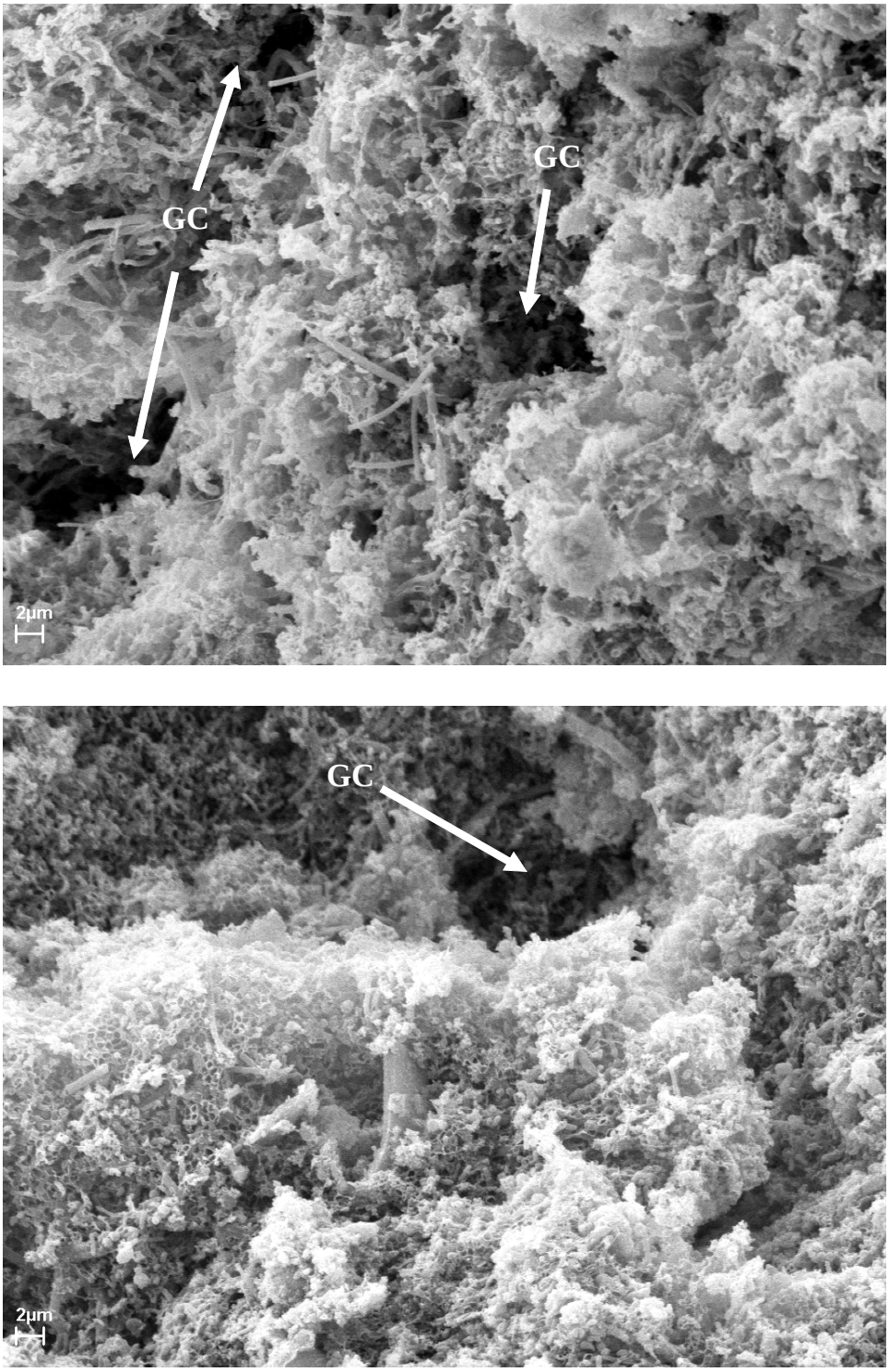


Figure 6.21: High magnification of granule interior showing numerous possible internal 'gas cavities' (GC), surrounded by matrices composed of both rod and filamentous forms of *Methanosaeta*-like microorganisms.

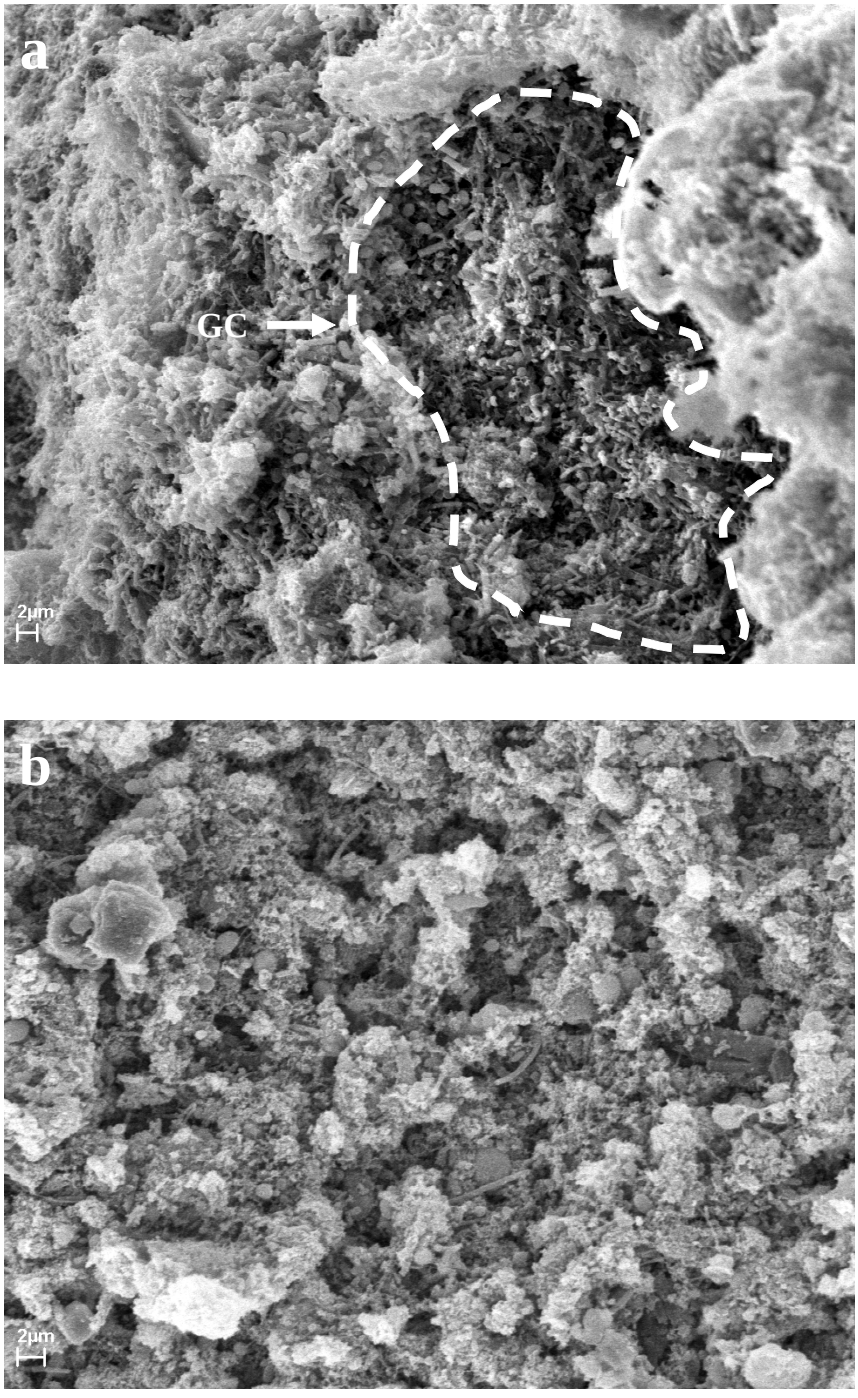


Figure 6.22: SEM micrographs of a ‘gas cavity’ from an ABR granule showing: a) a mixed population, which includes rods and cocci of varying size, covering a large internal ‘gas cavity’ (GC); b) predominance of cocci of various sizes and shapes (0.5 - 2 μm), in association of with other microorganisms, along ‘gas cavities’. The smaller, irregular-shaped cocci resemble acidogenic bacteria.

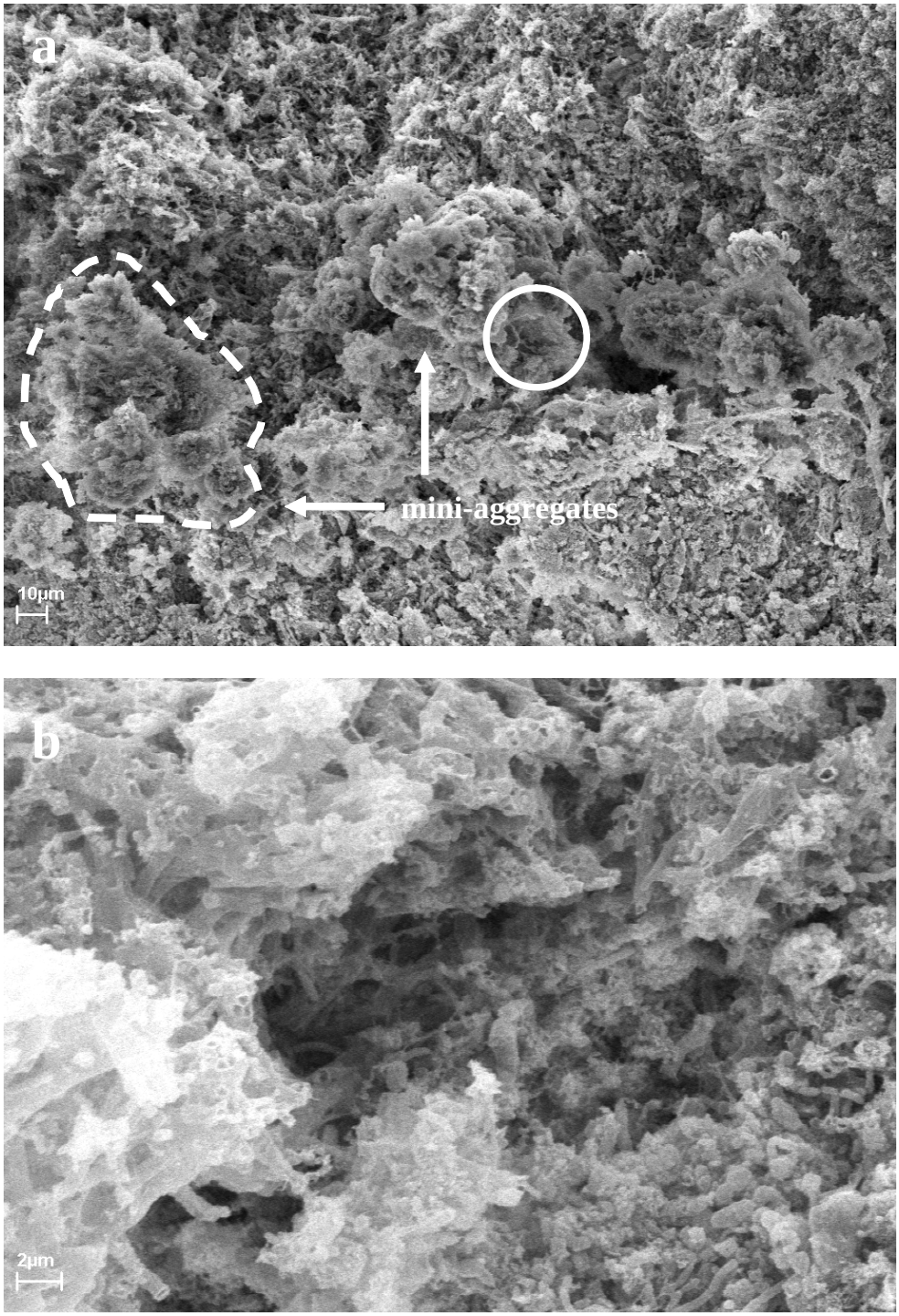


Figure 6.23: SEM micrographs of the granule interior showing: a) mini-aggregates (raised areas on micrograph) of ECP-bound *Methanosaeta*-like cells forming the core of a granule; b) high magnification of circled area in (a) showing microcolonies of acidogenic-like bacteria around ECP aggregates (lower right hand side).

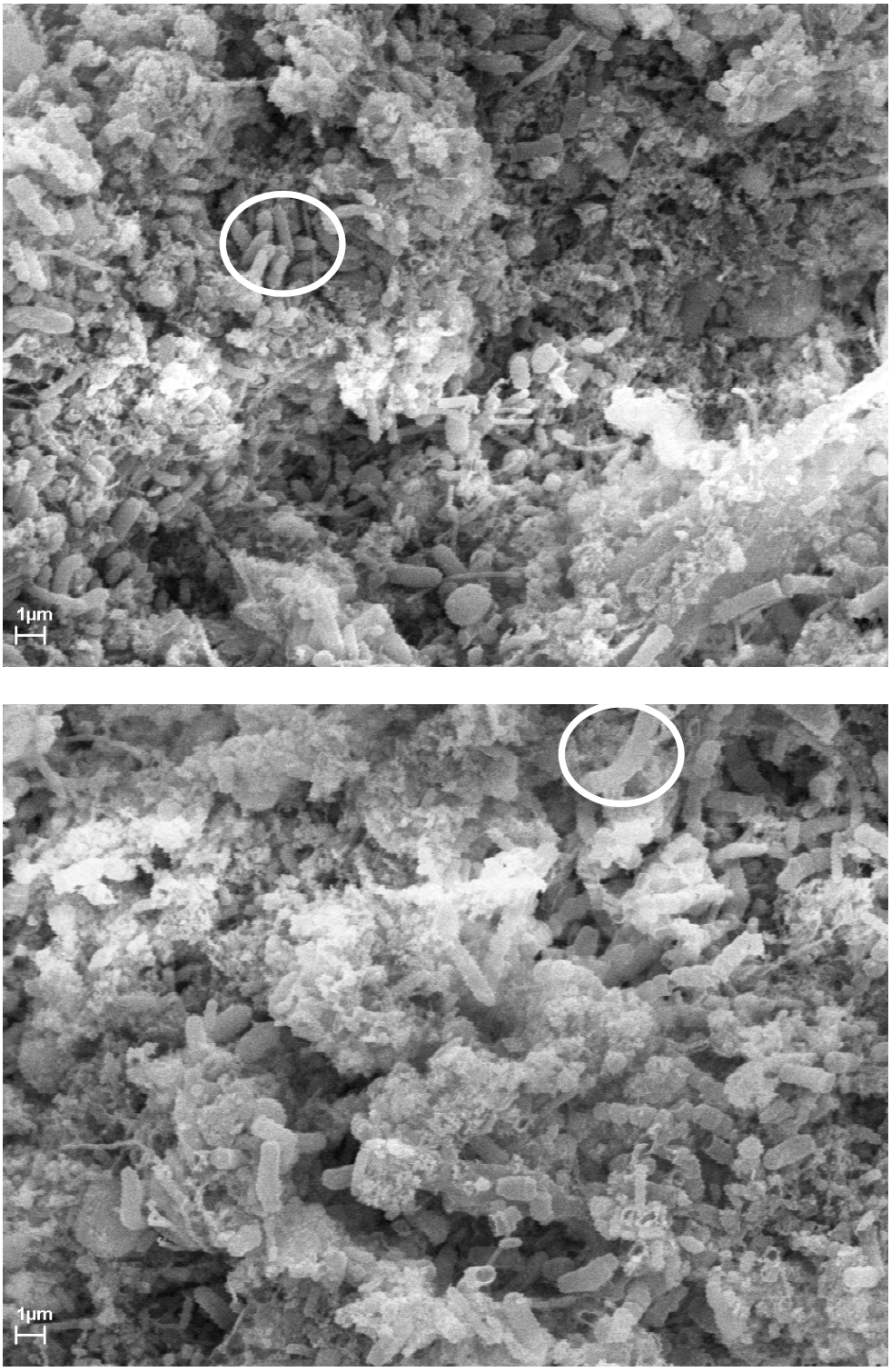


Figure 6.24: SEM micrographs of the surface area around mini-aggregates showing a large microcolony of cocci and rods thought to be acidogenic bacteria. Microorganisms resembling acetogenic bacteria are circled.

joined or held together by ECP secreted by these *Methanosaeta*-like cells as shown in SEM images (Figure 6.25).

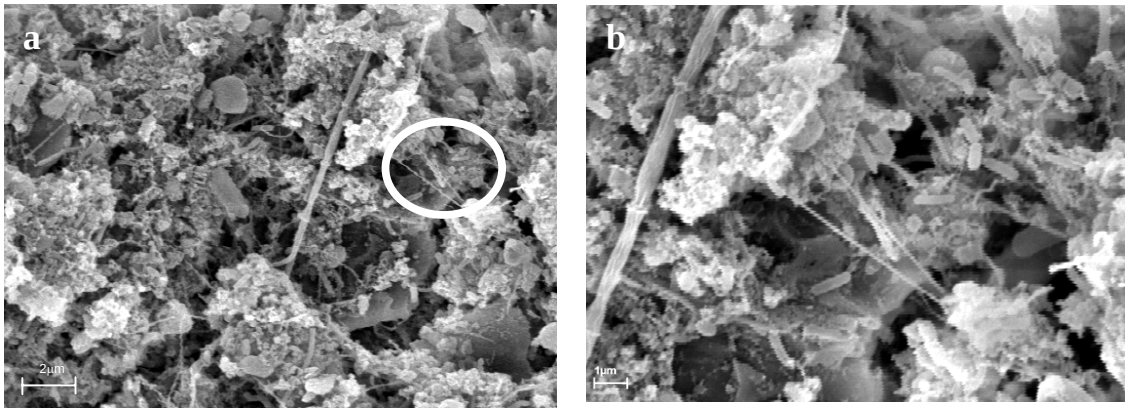


Figure 6.25: SEM micrographs showing: a) bridges composed of ECP between other polymer-bound clumps and inert particulates; b) high magnification of the circled area in (a).

6.4.3 Discussion

Although the results presented are qualitative, micrographs of the core sludge samples suggested that a partial separation of phases occurred in the ABR operated at a HRT of 40-44 h. These findings are consistent with the theory of phase separation in ABRs (Barber and Stuckey, 1999), but different in form, as fermentative steps were mostly confined to compartment 1, whilst methanogenesis occurred near the front and middle sections of the reactor, rather than at the end.

SEM micrographs showed the presence of large numbers of microorganisms resembling acidogens in the first compartment, and to a lesser extent in compartments 2 to 3 as part of granular sludge. This would explain why the VFA concentration in this compartment was higher in comparison to others (Foxon *et al.*, 2005). The VFA produced by this group are probably consumed by the morphotype resembling *Syntrophomonas* species, which degrades butyrate into acetate and hydrogen, forming the precursors of methanogenesis. Hydrogen-generating reactions should be highest at the front of ABR, and probably explains the relatively high proportion of hydrogenotrophic methanogens observed there. Boopathy and Tilche (1992) made similar findings in an ABR treating acidified wastewater. The presence of hydrogen-utilising methanogens in close proximity to acetogenic bacteria is beneficial to the system, as it is thought to keep hydrogen partial pressure low. Several authors have noted the importance for such a syntrophic association, as it allows acetate-generating reactions to be more thermodynamically

possible (Anderson *et al*., 2003; Boone and Bryant, 1980; MacLeod *et al*., 1990; McInerney *et al*., 1979).

As relatively large numbers of microorganisms resembling acetogenic bacteria were observed in compartment 1, it was expected that acetate concentration would be highest in this compartment. The results were confirmed by the grab analyses, which that showed acetate concentrations in compartment 1 were nearly double that of compartments 2 to 4 (Foxon *et al*., 2005). This probably explains the absence of *Methanosarcina*-like microorganisms in the middle of the reactor. These results suggested that the first stages of anaerobic digestion might be limited, as acetate levels were not high enough to maintain a high population of *Methanosarcina*. The results were substantiated by SEM micrographs, which showed the scavenging acetoclastic methanogen, *Methanosaeta*, predominating in the subsequent compartments, indicating likely lower acetate concentrations.

With respect to granulation, the phenomenon occurred predominately in compartments 2 and 3, and not in all compartments, as found by other authors (Boopathy and Tilche, 1992; Orozco, 1988; Uyanik *et al*., 2002). Barber and Stuckey (1997a) did, however, observe an increase in floc size near the middle of the reactor, which they attributed to less hydraulic shear and gas production. The granules had a stratified appearance (different layers), although the extent of this stratification was not great. A stratified appearance has also been reported for other types of granules fed different types of wastewater in UASB reactors (Chui and Fang, 1994; Fang and Chui, 1993; Fang *et al*., 1995b; Guiot *et al*., 1992; MacLeod *et al*., 1990). The sectioning of these granules revealed two distinct layers, with microbes within the core being morphologically different from those in the outer surface layer. The core of was almost entirely composed of bamboo-shaped microorganisms resembling *Methanosaeta* species, whilst the thin outer layer (2 – 10 μm) was comprised of a mixed population, consisting of filaments, rods, and cocci. *Methanosaeta*-like cells dominated granules in two forms; a rod-shaped form embedded in ECP, and a long filamentous form, also associated with ECP. Although there were no discernible differences in observations between the two morphologies, it was thought that there would be a selection for the rod-shaped form over time. This hypothesis is based on the findings of Wiegant (1987), who observed a change in *Methanosaeta* morphology within granules, from filamentous to rod-shaped, as time progressed. This change was attributed to an increase in the density of growth. According to Schmidt and Ahring (1997), the rod-shaped form is favoured over the filamentous form as it is thought to be less susceptible to ‘washout’.

The predominance of *Methanosaeta* within the core of granules has been noted in many other studies (Ahn, 2000; Banik *et al*., 1997; Guiot *et al*., 1992; MacLeod *et al*., 1990; Uyanik, 2003; Wiegant, 1987). The findings presented here suggest that *Methanosaeta* may act as a key structural element in the development of granules. Similar findings have been made in other anaerobic systems, especially the UASB reactor (Banik *et al*., 1997; Dubourgier *et al*., 1987; MacLeod *et al*., 1990; Morgan *et al*., 1991; Wiegant, 1987).

Only two theories contradict these findings. The first is the Cape Town Hypothesis, proposed by Sam-Soon *et al*. (1987), following the analysis of UASB treating a synthetic carbohydrate substrate with adequate macro- and micro-nutrients, but deficient in nitrogen. The second hypothesis is based on SEM observations in an ABR treating ice-cream wastewater (Uyanik, 2003). According to the Cape Town hypothesis, granulation depends on excessive ECP production by *Methanobacterium* strain AZ under conditions of high hydrogen partial pressures, unlimited ammonium, and cysteine deficiency (Sam-Soon *et al*., 1987). The ECP production causes *Methanobacterium* strain AZ to bind with each other and to other microorganisms, forming granules. No mention is made of *Methanosaeta* in the granulation process, although the authors did state that other microorganisms might be capable of similar characteristics (Sam-Soon *et al*., 1987). The major limitation of this hypothesis is that it is unable to explain why granulation occurs in systems that do not yield H_2 in the fermentation process (evidence cited in Hulshoff Pol *et al*., 2004).

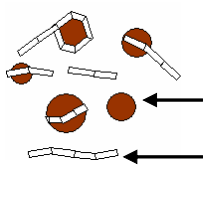
The second hypothesis, developed by Uyanik (2003), suggests that *Methanosaeta* does not initiate nucleation or promote granulation, even though they formed the central core of granules from an ABR. Supporting evidence for this hypothesis were that various microorganisms were observed during the initial stage of granulation. Uyanik (2003) stated these findings were also in contradiction with the idea that a relatively homogeneous microbial population is responsible for initiating granulation (El-Mamouni *et al*., 1997; MacLeod *et al*., 1990; Vanderhaegen *et al*., 1992). Uyanik (2003) believed the predominance of *Methanosaeta* within granules occurred when the flow of substrates through the granule 'matrix' leads to the development of an internal environment significantly different from that of the bulk phase of the reactor.

The results presented for case study 2 (HRT of 40-44h), however, appear to contradict Uyanik's (2003) hypothesis. SEM examination showed that the presence of *Methanosaeta* was essential for promoting granulation and leading to their eventual 'maturation'. These findings are supported by

observations at a HRT of 22 h, where the apparent lack or absence of *Methanosaeta* resulted in poorly developed or 'immature' granules with large cavities. It was thought that these granules probably had very low anaerobic activity (particularly methanogenesis), as there were very few microorganisms associated with them. It was also thought that these 'granules' might actually be particulates that were selected for, in conditions of high 'washout', onto which a few microorganisms have been able to attach. When the HRT was changed to 40-44 h, there was a reduction in the 'washout' rate coupled with a generally more stable operation (Foxon *et al*., 2005), which allowed *Methanosaeta*-like cells to proliferate and 'establish' themselves in the reactor. The granules they formed part of, had a relatively more 'stable' structure (less cavities, higher cell density, greater microbial diversity) than those observed at a HRT of 22 h. The production of ECP by the *Methanosaeta*-like microorganisms was thought to be critical to the granulation process. The ECP formed bridges between *Methanosaeta*-like microorganisms, which were thought to strengthen loosely adhered aggregates to form tight granules (Ross, 1984; Shen *et al*., 1993). It also facilitated the adhesion of other metabolic groups, which probably led to the formation of the surface layer, consisting of a mixed microbial population, and is thought to sustain the mechanical strength between granule layers (Uyanik, 2003).

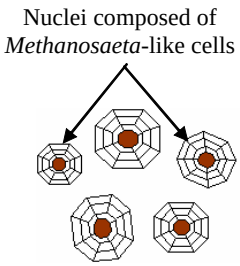
With respect to the internal structure of the granules, the arrangement of *Methanosaeta*-like cells within the granule core led to the formation of numerous cavities. It is thought these cavities represent the site of vigorous gas production (Bochem *et al*., 1982) or could possibly act as channels for substrate diffusion into the granule. The cavities are probably formed by the inactivation and autolysis of acidogenic bacteria through substrate diffusional limitation, as the granule develops (Beeftink and van den Heuval, 1987). These ideas are supported by observations of microorganisms resembling acidogenic bacteria around the external and internal cavities. In addition, these microorganisms were shown to occur around mini-aggregates composed of ECP-bound *Methanosaeta*-like cells within the granule (Figure 6.24). In contradiction to the developmental hypothesis of MacLeod *et al.* (1990), the evidence presented here suggests that granule development may have occurred through a multi-nucleate/multi-aggregate process. A proposed model of this granulation process is presented in Figure 6.26.

The difference in granule formation proposed in this study compared to those described elsewhere (review by Hulshoff Pol *et al*., 2004), is that granules are thought to develop from the aggregation of several nuclei, instead of from a single nucleus or precursor. The proposed model incorporates fundamental elements of many other hypotheses regarding initial stages of



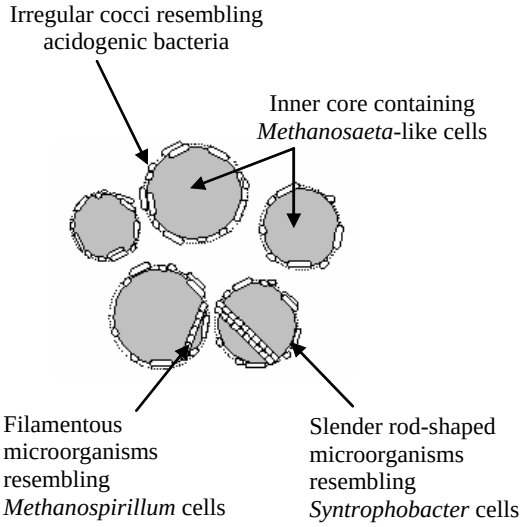
Stage I

Early nucleation sites are formed through the adhesion of *Methanosaeta* cells on particulates or through the aggregation of *Methanosaeta* filaments (Wiegant, 1987). SEM observations from a HRT of 22 h suggest that other microorganisms, such as those resembling *Methanospirillum* and *Methanococcus* species, may also have similar properties.



Stage II

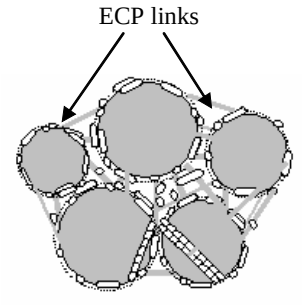
Increase in the number of *Methanosaeta*-like cells. The cells grow on the inside and the outside of particulates. As the *Methanosaeta*-like cells grow, they produce ECP.



Stage III

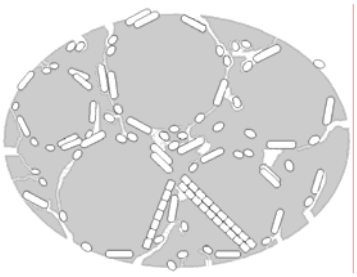
Methanosaeta form the inner core of nuclei, and the ECP they produce entrap non-attached bacteria and possibly other methanogens on the outer surface (Morgan *et al*., 1991; Wiegant, 1987). The ECP may also act as a substrate source for certain metabolic groups. Acetogens are thought to be the first group to colonise the surface of nuclei, followed acidogens and hydrogenotrophs (Ahn, 2000).

(continued on page 118)



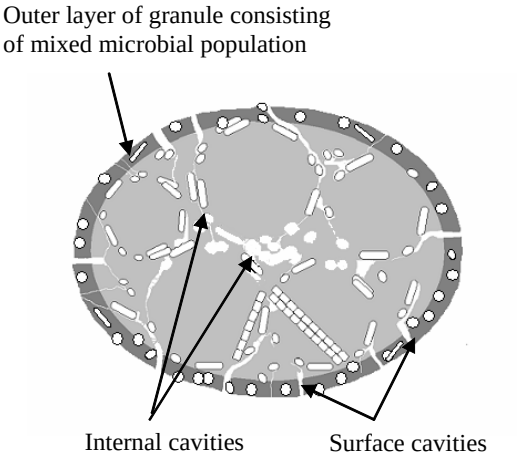
Stage IV

ECP cross-links develop between nuclei, forming a large aggregate, with a loose core (Ahn, 2000). Other microorganisms may also become entrapped between the bridges, as they develop. Cross feeding between the various metabolic groups is thought to result in the growth of the aggregate (MacLeod *et al*., 1990).



Stage V

The cross-links between the nuclei are strengthened by further ECP production. The innermost *Methanosaeta* cells become compacted, due to the increase in cell density, forming a dense core (Ahn, 2000; Wiegant, 1987).



Stage VI

The surface ECP layer accumulates various metabolic groups, mostly those that resemble hydrogenotrophs and acidogens, forming a distinct outer layer. Lysis and digestion of innermost acidogenic bacteria occurs through substrate limitation, resulting in the formation of internal cavities (Beeftink and van den Heuval, 1987). It is hypothesised that these cavities might be site of gas production, or possibly allows for the diffusion of substrate into the granule, and therefore do not close.

Figure 6.26: Proposed model of granulation in an ABR treating domestic wastewater. (Diagrams are not drawn to scale).

granulation (review by Hulshoff Pol *et al.*, 2004). The first step is thought to be either the colonisation of finely dispersed particulates by *Methanosaeta*, or the aggregation of *Methanosaeta* filaments, forming the initial nucleation sites ('Spaghetti theory' by Wiegant, 1987) (Figure 6.26). SEM observations from case study 1 suggested that other microorganisms, possibly hydrogenotrophic methanogens, might also be attached to particulate matter. Individual *Methanosaeta* cells grow and produce ECP, to which non-attached microorganisms adhere. This step is probably the most decisive factor determining granulation, as it is thought to facilitate the attachment of various metabolic groups (Morgan *et al.*, 1991), which leads to the growth of the nuclei (Wiegant, 1987). In addition, it is postulated that the ECP may also represent a source of substrate for other metabolic groups. Some of first microbes that would attach to these nuclei would probably be acetogenic bacteria, which would supply the substrate to *Methanosaeta* cells (Ahn, 2000). SEM examination from this study suggests that *Syntrophobacter* might be the predominant acetogen within the granule (Figure 6.24). The syntrophic relationship between the acetoclastic methanogen and the acetogen results in the growth of the nuclei (Ahn, 2000). The H_2-generating reactions of the former group of bacteria are known to inhibit their own growth, and require a syntrophic relationship with H_2-consuming microorganisms. This probably explains the observations of microorganisms resembling hydrogenotrophic methanogens (*Methanospirillum*- and *Methanococcus*-like microorganisms) on the outer surface of the granule. The two metabolic groups are probably supplied with substrates by microorganisms resembling acidogens, which also become attached to ECP. As suggested by MacLeod *et al.* (1990), the 'cross-feeding' that occurs, as a result of the close physical association between the various metabolic groups, results in the growth of the nuclei.

Whilst findings up to this point have been consistent with the developmental hypothesis cited elsewhere (review by Hulshoff Pol *et al.*, 2004), observations of mini-aggregates surrounded by microorganisms resembling acidogenic bacteria (and *Methanococcus*-like microorganisms) within the granule do not agree with the hypothesis of MacLeod *et al.* (1990) that each granule develops from a single precursor or nucleus. The proposed model, based on SEM observations obtained during this study, instead, suggests that several nuclei, consisting of a core of ECP-bound *Methanosaeta* and a mixed microbial surface population, aggregate together. Such nuclei have been observed within the compartments of the ABR (micrographs not shown). Ahn (2000) and Dubourgier *et al.* (1987) have proposed similar theories on granule formation. As SEM observations suggest, ECP may aid in this aggregation by forming bridges between each aggregate (Figure 6.25). In addition, aggregation may also be encouraged by 'rolling effects' as

seen in UASBs (Ahn, 2000). The aggregation of different nuclei will result in a granule that will probably be less prone to 'washout'. Over time, the growth of individual cells and the subsequent increase in the production of ECP will increase the mechanical strength between aggregates. As the granule grows, the innermost acidogenic bacteria within the granule will become inactivated, probably through limited substrate diffusion, forming internal cavities. The gas production by *Methanosaeta*-like microorganisms probably prevents the cavities from closing. It is also thought that these cavities may allow for diffusion of substrates into the granule. Although unknown at this time, the shape of the granule is probably determined by hydraulic shear forces (Wiegant, 1987).

The differences in granule development observed between MacLeod *et al*. (1990) and the results presented here may be related to the substrate complexity. MacLeod *et al*. (1990) studied granule development in a UASB reactor supplied with a soluble sucrose substrate. Under such conditions, the conversion process would probably be much faster, as would be the growth of the various metabolic groups. As the 'driving force' is higher, granules could have possibly developed from individual nuclei. In addition, the accelerated rate of metabolism may have allowed for the development of a higher level of stratification, as observed by MacLeod *et al*. (1990). In contrast, the treatment of domestic wastewater is more 'problematic' due to the large particulate fraction. The hydrolysis of particulates is often the rate-limiting step, as was shown, resulting in low substrate conditions or a 'low driving force'. This probably limits single nuclei from developing into individual granules, and could possibly limit the size of granules. Furthermore, it may also explain the 'lower level of stratification' observed in 'mature' granules.

6.5 Summary

Although more precise tools are available to document the distribution and shifts in anaerobic consortia over time, SEM can provide a useful description of the microorganisms involved in the ABR. The results are purely qualitative, as they are based on tentative identification of the external morphological characteristics of microorganisms, many of which have similar shapes. Provided the limitations of the technique are recognised, the technique can provide a qualitative understanding of microbial population dynamics that can occur in an ABR.

In contrast to theory, spatial separation was shown not occur in the ABR at a HRT of 22 h (case study 1). It was hypothesised that hydrolysis was the overall rate-limiting step in the ABR treating domestic wastewater, a common occurrence when treating wastewater with particulate

fraction. Hydrolysable organic material, present in all compartments of the reactor, was continuously undergoing hydrolysis (Foxon *et al*., 2005). As a result, scavenging by microorganisms resembling methanogens occurred, particularly near the front of the reactor, where soluble fraction from the influent would be highest. The results were verified using FISH (Foxon *et al*., 2005; Lalbahadur, 2005). Large populations of microorganisms resembling hydrogenotrophic methanogens were observed at the front, including morphotypes resembling *Methanobrevibacter* species, *Methanococcus* species, and *Methanospirillum* species. Only one morphotype resembling an acetoclastic methanogen, namely *Methanosarcina*, was detected in substantial numbers. The other acetoclastic methanogen, *Methanosaeta*, was very infrequently observed in some granules using SEM, but was not detected using FISH or sequence analysis. The absence of *Methanosaeta* as determined by the respective studies, was attributed to sampling method used, lack of penetration of molecular probes, and/or low metabolic rate. Nevertheless, there should have been a large population of scavenging *Methanosaeta* in the bulk of the sludge. The results indicate that the dynamics of acetoclastic methanogen genera are dominated by flow conditions, particularly sludge 'washout' and solids retention. It was hypothesised that under such conditions, there was a selection for the versatile *Methanosarcina* genus, instead of scavenging *Methanosaeta*. In addition, it was hypothesised that the 'failure' of *Methanosaeta* to establish itself within the reactor was reason for poor granule development.

In case study 2 (HRT of 40-44 h), there was a partial separation of phases, but this was different in form to those reported in literature, in that methanogenesis occurred near the front and middle parts of the reactor. As previously seen, scavenging by microorganisms resembling hydrogenotrophic methanogens occurred at the front of the reactor. There were also comparatively larger populations of acidogenic and acetogenic bacteria observed at this flow regime. In addition, there were changes in the distribution of microorganisms resembling acetoclastic genera. A few *Methanosarcina*-like populations were observed in compartment 1, whilst larger *Methanosaeta*-like populations were found in subsequent compartments. The latter was found to dominate in the front and middle sections of the reactor, particularly in compartments 2 and 3, and occurred mostly in the form of granular sludge. The granules were stratified to a certain extent, and composed of primarily of *Methanosaeta*-like cells, with a mixed population on outer surface. Within the granule core, *Methanosaeta*-like cells were observed as ECP-bound aggregates surrounded by microorganisms resembling acidogenic bacteria. In contradiction to the development hypothesis of MacLeod *et al*. (1990), these observations led us to hypothesise that granule development may occur through the aggregation of smaller nuclei

(multi-aggregate hypothesis). The secretion of ECP by *Methanosaeta* is thought to be pivotal in the development of a 'mature' granule. The presence of such granules has several treatment advantages, including: maximal microorganism-to-space ratio; the formation of a stable metabolic arrangement between metabolic groups involved; and excellent settling properties (Guiot *et al*., 1992).

To conclude, it was thought that factors (sludge 'washout', solids retention, low pH), other than acetate concentration, could also influence the predominance of either acetoclastic genus. In the ABR run at a longer HRT, there were lower levels of solids 'washout' and improved hydrolysis of particulate organics. This resulted in a more stable anaerobic sludge that exhibited a degree of adaptation to environmental conditions (Foxon *et al*., 2005).

CHAPTER SEVEN: CONCLUSIONS AND RECOMMENDATIONS

The specific objectives of this study were to examine the pathogen indicator removal and the microbial population dynamics through a pilot-scale ABR of 3 000 L. The reactor was operated at two flow regimes, a HRT of 22 h and a HRT between 40-44 h, and fed with domestic wastewater from a WWTP serving a middle-income population.

7.1 Conclusions

The conclusions have been presented according to the two objectives mentioned above:

7.1.1 Pathogen indicator studies and other treatment performance parameters

In keeping with published results, nutrient and pathogen indicator removal through the ABR was limited. The high nutrient concentration in the ABR effluent raises concern over its re-use in agricultural irrigation. The average ammonia concentration was over 30 mg/L, which increases the risk of groundwater contamination and eutrophication. Furthermore, it restricts the type of crop that can be grown. Using preliminary potted plant trials, it was shown that ABR effluent was not detrimental to plant growth in the short term (Appendix II).

Microbial contamination therefore remained the major re-use concern, even though significant indicator removal was achieved at both flow rates. Consequently, the effluent should be considered a health hazard, and cannot be discharged or re-used without further treatment. A microfiltration membrane, that can be added to the last compartment, was considered as a possible post-treatment option (Appendix III).

As pathogen indicator removal was comparable to that of other anaerobic reactors, the main advantage of an ABR would be enhanced COD removal. COD removal was consistently below irrigation limits for both case studies, with improved removal efficiencies observed at a HRT of 40-44 h. The results also indicated that the treatment performance of an ABR is enhanced at a longer HRT.

The longitudinal indicator profile through the ABR suggested that compartmentalisation contributed progressively to pathogen indicator reduction. However, significant reduction did not occur between adjacent compartments, but over a series of compartments. It was hypothesised

that the main mechanism of pathogen removal in an ABR may be due to the retention of solid-associated pathogens. Other factors, such as pH, microbial competition and substrate limitation, may also be responsible for pathogen reduction, but experimental proof is lacking. In addition, longer HRT are thought to encourage better pathogen removal through enhanced retention of solid-associated microorganisms.

In terms of reactor design for dense-peri urban communities, Foxon *et al*. (2005) have suggested a five-compartment ABR, instead of an eight-compartment one. The modifications to the design are based on results obtained from the pilot study (Foxon *et al*., 2005). The results of this study suggested that the pathogen removal capability of the ABR would not be largely affected by this modification.

7.1.2 Microbial community analysis in the ABR

Although scanning electron microscopy (SEM) was used as a qualitative technique, it enhanced the understanding of the process of anaerobic digestion by providing an indicator of the microbial population dynamics in an ABR. In some instances, it can overcome some of the problems associated with definitive techniques, such as FISH, where low bacterial RNA activity and low probe penetration can be a problem in aggregate-bound organisms. Ideally, both tools should be combined to gain an understanding of anaerobic digestion.

The hypothesis of horizontal separation of acidogenic and methanogenic microorganisms through the ABR was not substantiated by SEM observations at a HRT of 22 h. This was thought to be due to the slow rate of hydrolysis of particulate organics within the wastewater. This resulted in scavenging of VFA by methanogenic-like microorganisms, especially those resembling hydrogenotrophic methanogens, in the first few compartments of the reactor. Contrary to literature, *Methanosarcina* species were the predominant acetoclastic methanogens present. Microorganisms resembling *Methanosaeta* species were rarely observed. These results were verified by FISH and sequence analysis in a parallel study (Lalbahadur, 2000). It was hypothesised that under conditions of high flow, sludge ‘washout’, and low pH, there was a selection for *Methanosarcina*, possibly due to its faster growth and greater tolerance to ‘adverse’ environmental conditions over *Methanosaeta*.

The hypothesis of horizontal separation of acidogenic and methanogenic microorganisms through an ABR was substantiated by SEM observations at a HRT of 40-44 h. This separation, however,

was different in form, in that methanogenesis occurred near the front and middle sections of the reactor, and not the end as reported in other ABR studies. This was possibly due to hydrolysis being rate-limiting. The 'improved' environmental conditions at a HRT of 40-44 h allowed *Methanosaeta*-like microorganisms to predominate under low substrate conditions in all compartments, except compartment 1, where a few *Methanosarcina*-like cells were observed. The results indicated that the HRT has a major influence on the microbial population dynamics of an ABR.

Although not necessary for the reactor performance, the phenomenon of granulation was observed in both operating periods. However, at a HRT of 22 h, granules were not well developed, and did not have any significant microbial population on the surface or within the core when examined by SEM. This was in contrast to literature reports on granulation. It is hypothesised that the absence or lack of *Methanosaeta* under low substrate conditions was probably the reason for poor granule development. This was supported by the observations made at a HRT of 40-44 h, where two-layered granules were observed. The thin outer surface layer was comprised of mixed population, whilst the core consisted predominately of *Methanosaeta*-like cells. These results suggested that *Methanosaeta* was a key role-player in the development of granules, in keeping with other theories of granulation. In addition, the observation of microorganisms resembling acidogens, around ECP-bound aggregates of *Methanosaeta*-like cells within the granule, suggested that the mechanism of granulation in the treatment of low-strength, domestic wastewater differed from those documented to date.

7.1.3 Overall conclusions

The ABR showed 'promise' as a sanitation option for communities who desire waterborne sanitation. It has several biological and hydraulic advantages over septic tanks, including higher solids retention and better COD removal (evidence cited in Foxon *et al*., 2005). The reactor could potentially be used to treat domestic wastewater from a few to a hundred households. However, post-treatment of the effluent would be required to remove nutrients and pathogens.

7.2 Recommendations for Further Study

- The performance of a field-scale ABR treating domestic wastewater from a low-income community should be investigated, and the reduction of other pathogen indicators, such as enterococcus, *Salmonella*, whipworm and canine roundworms,

should be included. In addition, the effect of anaerobic digestion on parasite egg viability should be assessed.

- Further research into post-treatment options, preferably integrated systems (such as submersible membrane filters), is required.
- Once the microbial quality of the effluent achieves agricultural discharge limits, a comprehensive field trial comparing the effects of ABR effluent on plant growth should be undertaken.
- The potential medium and long term effects of metals entering the ABR should be considered, particularly if the community contributing the raw wastewater eats tinned food or uses aluminium pots. This will be an important factor in future irrigation investigations. In addition, the impact of long-term metal accumulation on nitrification and denitrification may be important.
- Microbial community analysis of the second operational period (HRT = 40-44 h) should be verified and quantified using fluorescent *in situ* hybridisation (FISH). The technique can also be used to substantiate the hypothesis of granule formation in anaerobic reactors treating domestic wastewater.

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APPENDIX I: ANALYTICAL METHODS

A 1.1 Membrane Filtration Technique for Coliforms (Standard Method 9222B)

A 1.1.1 Introduction

Coliforms are routinely used as indicators of faecal pollution. The membrane filtration technique (Standard Method 9222B) allows for quick and easy quantification of coliform groups in samples (APHA-AWWA-WEF, 1998).

A 1.1.2 Principle

The technique allows for the simultaneous detection of total coliforms and *E. coli* based on the production of specific colony colours on Chromocult® Coliform agar (Merck). *E. coli* colonies appear dark blue to violet colour whilst total coliforms appear salmon to red colour.

A 1.1.3 Apparatus and Equipment

- Autoclave (Tomy Autoclave, model SD-30N)
- Cold room ($\pm 4^{o}$ C)
- Counting apparatus (Colony Counter, Bel-Art Products)
- Culture tubes (Merck)
- Forceps
- Glass bottles (Schott®, Merck)
- Graduated pipettes (10 mL) (Sarstedt)
- Incubator (37^{0} C)
- Membrane filtration unit
- Microwave for melting media
- Petri dishes (60 mm) (Concorde Plastics)
- pH meter (Hanna pH 211, microprocessor pH meter)
- Water bath (W.A. Sauer)

A 1.1.4 Sample Handling

Heavily contaminated samples will need to be serially diluted.

A 1.1.5 Reagents and Materials

A) Pre-sterilised gridded 0.45 μm membrane filters (Schleicher and Schüll ME25)

B) Detergent solution: 100 mL NaOCl, made up to 1 L with distilled water.

C) Distilled water

D) NaCl (Merck)

E) Chromocult® Coliform agar (Merck): Add 26.5 g of agar to 1 L with distilled water. Heat in a microwave until dissolved. Do not boil solution. Pour agar into Petri dishes, allow it to cool and place in cold room.

F) Sterile saline buffer: 2g of D, made up to 1 000 mL with distilled water. Autoclave at 120^{o} C for 15 minutes.

A 1.1.6 Procedure

Day before Analysis

1. Prepare Chromocult® Coliform agar (Merck) according to manufacturer's directions.
2. Pour agar in Petri dishes (60 mm) and leave in the cold room (± 4^{o} C).

Day of Analysis

1. Heavily contaminated samples will need to be serially diluted in sterile saline solution (1: 10 000 or 1: 100 000 recommended for raw domestic wastewater).
2. Remove sufficient prepared Petri dishes from the cold room and allow the agar to reach room temperature.
3. Remove sterilised filter funnels from autoclave bag (or spray filters with 70% ethanol and boil for 15 minutes before use).
4. Use flamed forceps to transfer filter funnels to boiling water bath.
5. Flame forceps between each operation.
6. Open membrane filter envelope at one end, remove gridded membrane filters using flamed forceps, wet briefly in boiling water and transfer to filter manifold.
7. Turn on vacuum to flatten filter.
8. Turn off vacuum.
9. Using flamed forceps, place filter funnels on manifold.
10. Pour sample into filter
    - if sample is 100 mL, filter as is.
    - if sample is 1 mL or a serial dilution, add sample to approximately 30 mL sterile saline and shake to dispense before pouring into filter.

11. Turn on vacuum until all of sample has passed through membrane filter.
12. Turn off vacuum.
13. Using flamed forceps, remove membrane filter from manifold and roll onto Petri dish with culture medium, grid side up, making sure no air bubbles are trapped beneath filter.
14. Place lid on Petri dish and place dish upside down (lid facing down).
15. Repeat for all samples, making sure that the water trap does not overfill.
16. At the end of analysis, place Petri dishes in incubator at 37^{o} C, lid facing down, to prevent drops of condensate falling on filter and blurring colonies.
17. Spray a little ethanol (70%) through the filters.
18. Empty the water trap down the sluice in wash-up room.
19. Place filter units in boiling water bath for 15 minutes, then place in autoclave bag and autoclave for 15 minutes.
20. Autoclave water trap bottle or wash with detergent.
21. Add a little detergent to any undiluted sample and pour down sluice.
22. Residual serial dilutions can be poured down the sluice without sterilisation.
23. Autoclave all glassware before including in general glassware wash-up.

A 1.2 Double Layer Plaque Assay (eThekwini Test Method No. MM023)

A 1.2.1 Introduction

Coliphages are bacteriophages (bacterial virus) that infect and replicate in coliform bacteria and appear to be present wherever total and faecal coliforms are found (eThekwini Test Method No. MM023). As they are similar to enteric viruses and are relatively easily detected in environmental samples (Bitton, 1994), they often used as indicators of enteric viral contamination.

A 1.2.2 Principle

The sample is mixed with a small volume of semi-solid nutrient medium (top agar), a culture of host strain is added and the mixture is plated onto a solid nutrient medium (base agar).

A 1.2.3 Apparatus and Equipment

- Autoclave (Tomy Autoclave, model SD-30N)
- Counting apparatus (Colony Counter, Bel-Art Products)
- Culture tubes (Merck)
- Graduated pipettes (1 mL and 10 mL) (Sarstedt)
- Glass bottles (Schott®, Merck)

- Incubator (36 ± 2)^{o} C
- McCartney® bottles (Merck)
- Microwave for melting media
- Petri dishes (Concorde Plastics)
- pH meter (Hanna pH 211, microprocessor pH meter)
- Water bath at (45 - 48^{o} C) (W.A. Sauer)

A 1.2.4 Sample Handling

The size of the sample will be dependent on the expected number of phage. Heavily contaminated samples will need to be serially diluted.

A 1.2.5 Reagents and Materials

A) Standard culture *E. coli* (ATCC 13706) (eThekwini Water and Wastewater Laboratory)
B) Distilled water
C) Nutrient broth (Oxoid): Add 13 g to 1 L distilled water. Mix well.
D) Nutrient agar (Merck)
E) Tryptone (Oxoid)
F) NaCl (Merck)
G) Glucose (Oxoid)
H) Phage base agar: Add 11 g of D, 13 g of E, 8 g of F and 1.5 g of G to 1 L distilled water. Autoclave and pour into Petri dishes.
I) Phage top agar: Add 6 g of D, 10 g of E, 8 g of F and 3 g of G to 1 L distilled water. Mix well and dispense 10 mL aliquots into McCartney® bottles. Autoclave at 121 ^{o} C for 15 minutes and then place in water bath at 48 ± 1.

A 1.2.6 Procedure

Day before Analysis

1. Prepare nutrient broth, pour 10 mL aliquots into McCartney® bottles and autoclave at 121 ^{o} C for 15 minutes.
2. Take a loopful of host culture from slant using aseptic techniques.
3. Sub-culture in nutrient broth at 37 ± 1^{o} C for 18 h.

Day of Analysis

1. Pour phage agar base into Petri dishes.

2. Allow to set.
3. Prepare phage top agar and maintain at 48 ± 2^{o} C in water bath.
4. Warm sample in water bath at 48 ± 2^{o} C.
5. Add 1 mL of *E. coli* host culture and 1 mL of sample to 10 mL of top agar (Note: work fast to prevent agar from cooling too fast).
6. Swirl gently to mix, avoiding bubbles.
7. Dispense evenly onto prepared agar base.
8. Allow to set.
9. Incubate at 37 ± 1^{o} C for 18 h.
10. Count plaques.

A 1.3 The Modified Bailenger Method for *Ascaris* Enumeration (Ayres and Mara, 1996)

A 1.3.1 Introduction

The modified Bailenger method (Ayres and Mara, 1996) is a parasitological method endorsed by the WHO for analysis of wastewater to be used in agriculture. The technique can be successfully used to recover a wide range of helminth eggs including: *Ascaris*, *Trichuris*, *Capillaria*, *Enterobius vermicularis*, *Toxocara*, *Taenia* and *Hymenolepis*. However, is not suitable for many of the operculated or trematode eggs (Ayres and Mara, 1996). The analysis was limited to the helminth genus, *Ascaris*, due to time constraints. It was chosen as an indicator of parasite contamination as it shows higher survival rates than other intestinal parasites (Cram, 1943).

A 1.3.2 Principle

The method is based on the separation of fats and other matter in an interphase solution (normally ether or ethyl acetate), while the parasites sediment into a non-miscible buffer below (Ayres and Mara, 1996). In addition, the method relies on centrifugal force to sediment parasite eggs (Ayres and Mara, 1996).

A 1.3.3 Apparatus and Equipment

- Centrifuge capable of generating 1 000 G (Eppendorf 5810R)
- Graduated pipettes (10 mL) (Sarstedt)
- McMaster counting slides (Focal Point)
- Pasteur pipettes (Bilbate) with teats

- Siphon
- Vortex mixer (not essential)

A 1.3.4 Sample handling

The method is efficient for use with raw wastewater (Ayres and Mara, 1996). However, the sample size must be increased to at least 10 L for the efficient recovery of eggs in treated wastewater effluents as egg numbers are lower than in untreated wastewaters (Ayres and Mara, 1996).

A 1.3.5 Reagents and Materials

A) Centrifuge tubes with lids (50 mL) (Sterilin)
B) Detergent solution: 1 ml Tween® 80 (Sigma-Aldrich), made up to 1 litre with tap water.
C) Distilled water
D) Ether (or ethyl acetate) (Merck)
E) Sodium acetate trihydrate (Merck)
F) Glacial acetic acid (Merck)
G) Zinc sulphate (Merck)
H) Acetoacetic buffer: 15 g of E and 3.6 ml of F, made up to 1 litre with distilled water. Adjust pH to 4.5.
I) Zinc sulphate solution (33%, relative density 1.18): 33 g of G, made up to 100 mL with distilled water.

A 1.3.6 Procedure

1. Collect a sample of wastewater of known volume (V litres), usually 1 L for raw or partially treated wastewaters and 10 L for final treated effluents.
2. Allow the sample to sediment for 1-2 h, depending on the size of the container. Ayres and Mara (1996) recommend an open-topped, straight-sided container for sedimentation, as it makes the removal of the supernatant easier and permits thorough rinsing of the container.
3. Remove 90% of the supernatant using a suction pump or siphon.
4. Carefully transfer the sediment to one or more centrifuge tubes. Rinse the container with detergent solution, and add the remaining solution to the sediment. Centrifuge the tubes at 1000 G for 15 minutes.

5. Remove the supernatant. If more than one centrifuge tube has been used in step 4, transfer all the sediments to one tube. Remember to rinse thoroughly with detergent solution to ensure that no sediment is discarded and re-centrifuge at 1 000 G for 15 min.
6. Suspend the pellet in an equal volume of acetoacetic buffer (pH 4.5). If the pellet is less than 2 ml, add buffer up to 4 ml to ensure that, after extraction with ethyl acetate (steps 7 and 8), there is sufficient volume of buffer above the pellet to allow the ethyl acetate layer to be poured off without re-suspension of the pellet.
7. Add two volumes of ethyl acetate or ether, and mix the solution thoroughly in a vortex mixer. The sample can also be shaken by hand if no vortex is available.
8. Centrifuge the sample at 1000 G for 15 minutes. Three distinct phases will form. All the non-fatty, heavier debris, including helminth eggs, larvae and protozoa, will be in the bottom layer. The middle layer should consist of the clear buffer. The fats and other material moves into the ethyl acetate or ether and forms a thick dark plug at the top of the sample.
9. Record the volume of the pellet containing the eggs, and then pour off the rest of the supernatant in one smooth action. It may be necessary to loosen the fatty plug first by running a fine needle around the side of the centrifuge tube.
10. Re-suspend the pellet in five volumes of zinc sulphate solution, (for example: if the volume of the pellet is 1 mL, add 5 mL of $ZnSO_4$). Record the volume of the final product (X ml). Mix the sample thoroughly, preferably using a vortex mixer.
11. Quickly remove an aliquot with a Pasteur pipette and transfer to a McMaster slide for final examination. (Note: a minimum of 1.5 mL is required to fill a two-chambered McMaster slide).
12. Leave the full McMaster slide to stand on a flat surface for 5 minutes before examination. This allows all the eggs to float to the surface.
13. Place the McMaster slide on the microscope stage and examine under 10x or 40x magnification. Count all the eggs seen within the grid in both chambers of the McMaster slide. For greater accuracy, the mean of two slides, or preferably three, should be recorded.
14. Calculate the number of eggs per litre from the equation:

$$N = AX/PV$$

where:

N = number of eggs per litre of sample

A = number of eggs counted in the McMaster slide or the mean of counts from two or three slides

X = volume of the final product (mL)

P = volume of the McMaster slide (0.3 mL)

V = original sample volume (L)

A 1.4 Scanning electron microscopy

A 1.4.1 Introduction

The scanning electron microscopy (SEM) was used to tentatively identify microorganisms in the sludge of the ABR. More precise tools for examining microbial consortia are available, such as FISH and DNA sequence analysis, which yield better and more accurate results. However, these techniques require greater stringency, and therefore can be expensive and time-laborious.

A 1.4.2 Principle

The microscope generates fast electrons that strike the sample, which in turn, emit low energy electrons according the surface or topography of the sample. These low energy electrons are then detected to form an image of the sample (Robinson and Gray, 1990).

A 1.4.3 Apparatus and Equipment

- Aluminium stubs
- Centrifuge (Eppendorf 5810R)
- Critical point dryer (Hitachi Critical Point Dryer)
- Forceps
- Glass collection bottles (Schott®, Merck)
- Glass Petri dishes (90 mm)
- McCartney® glass screw bottles (Merck)
- Pasteur pipettes with teats
- pH meter (Hanna pH 211, microprocessor pH meter)
- Scanning electron microscope (SEM) (Leo 1450)
- Sputter coater (Polaron E5100)

A 1.4.4 Sample handling

Core sludge samples and granules must be collected and prepared separately. Granules tend to soft and can be easily broken. They therefore require greater care during sample preparation (no centrifugation steps).

A 1.4.5 Reagents and Materials

A) Centrifuge tubes with lids (Sterilin)
B) Nucleopore filters (Costar)
C) 0.45 µm filters (Millipore)
D) Alcohol (100%)
E) Distilled water
F) Sodium dihydrogen orthophosphate (Merck)
G) Disodium hydrogen orthophosphate (Merck)
H) Paraformaldehyde (BDH Chemicals)
I) Osmium tetroxide (Sigma-Aldrich)
J) Graded alcohol series: 25, 50 and 75 mL of D, made up to 100 mL with distilled water.
K) Stock solution A: Dissolve 3.12 g of F in 100 mL distilled water.
L) Stock solution B: Dissolve 2.83 g of G in 100 mL of distilled water.
M) 0.1 M phosphate buffer: Add 14 mL of K to 36 mL of L. Make up to 100 mL with distilled water.
N) Stock solution C: Dissolve 9.36 g of F in 100 mL distilled water.
O) Stock solution D: Dissolve 8.49 g of G in 100 mL of distilled water.
P) 0.3 M phosphate buffer: Add 14 mL of N to 36 mL of O. Make up to 100 mL with distilled water.
Q) 10% formaldehyde in 0.1M phosphate buffer: Heat 65 ml of deionised water to 60°C. Add 10 g of H. Add 1 or 2 drops of 2M NaOH solution and stir rapidly until the solution has nearly clarified. Remove from heat source and add 33 ml of solution P. Adjust pH to 7.2. Filter solution through a 0.45 µm filter (Millipore). Cool in a waterbath and store in the refrigerator.
R) 1% osmium tetroxide solution: 0.5 g of I, made up to 50 mL with deionised water in a Schott® bottle. Place Schott® bottle in an airtight container and refrigerate.

1.4.6 Procedure

Core sludge samples

1. Remove approximately 50 mL of sludge from collection bottle.
2. Centrifuge at 2 000 G for 5 minutes and remove the supernatant.
3. Wash samples with 0.1 M phosphate buffer at pH 7.2 for 5 minutes. Repeat washing step three times. (Note: centrifuge and decant supernatant between washing steps).
4. Fix samples with 10% formaldehyde in 0.1M phosphate buffer for 16 h.

5. Wash samples with 0.1 M phosphate buffer at pH 7.2 for 5 minutes. Repeat washing step three times. (Note: centrifuge and decant supernatant between washing steps).
6. Post-fix with 1% osmium tetroxide solution for 1 h at room temperature.
7. Wash samples with distilled water for 5 minutes to remove excess fixative. Repeat washing step three times. (Note: centrifuge and decant supernatant between washing steps).
8. Dehydrate in a graded alcohol series (25, 50, 75 and 100%) for 10 minutes each.
9. Remove sludge from centrifuge tubes and place on 0.2 µm nucleopore filters.
10. Dehydrate samples in a critical point dryer.
11. Place samples on aluminium stubs using double-sided tape.
12. Sputter coat samples.
13. Place samples in SEM and record images.

Granules

1. Gently remove granules from McCartney® bottle using forceps.
2. Gently wash in 0.1 M phosphate buffer at pH 7.2 in glass Petri dishes. Repeat washing step three times.
3. Remove granules from Petri dishes and place in McCartney® bottles containing 10% formaldehyde in 0.1M phosphate buffer for 16 h.
4. Remove fixative with Pasteur pipettes and replace with 0.1 M phosphate buffer at pH 7.2 in glass Petri dishes. Repeat washing step three times.
5. Remove buffer solution and post-fix with 1% osmium tetroxide solution for 1 h.
6. Remove fixative with Pasteur pipettes and replace with distilled water. Repeat washing step three times.
7. Dehydrate in a graded alcohol series (25, 50, 75 and 100%) for 20 minutes each.
8. Remove sludge from centrifuge tubes and place on 0.2 µm nucleopore filters.
9. Dehydrate samples in a critical point dryer.
10. Place samples on aluminium stubs using double-sided tape.
11. Sputter coat samples.
12. Place samples in SEM and record images.

A 1.5 Epi-fluorescence microscopy

A 1.5.1 Introduction

Epi-fluorescence microscopy was used to conduct microscopic analysis of ABR granules from case study 1. The method is based on the histological method described by Chui and Fang (1994).

A 1.5.2 Principle

The method relies on the fluorescence of co-factors F_{350} and F_{420} of methanogens under epi-fluorescent excitations (350 and 420 nm) (Chui and Fang, 1994).

A 1.5.3 Apparatus and Equipment

- Coplin jars (Merck)
- Dessicator (Glaswerx Wertheim)
- Glass Petri dishes
- Glass slides and cover slips (TAAB)
- Microscope equipped with epi-fluorescent attachments (Nikon E-400)
- Microtome (American Optical)
- Incubator (60 – 70^{o} C)

A 1.5.4 Sample handling

Granules tend to soft and can be easily broken. They therefore require greater care than core sludge samples during sample preparation.

A 1.5.5 Reagents and Materials

A) Alcohol (100%)
B) Xylene (Merck)
C) Distilled water
D) Sodium dihydrogen orthophosphate (Merck)
E) Disodium hydrogen orthophosphate (Merck)
F) Paraformaldehyde (BDH Chemicals)
G) Paraffin wax (melting point 45 – 55^{o} C) (Hopkin and Williams)
H) Peel-off moulds (Polyscience)
I) 0.45 µm filters (Millipore)
J) Graded alcohol series: 25, 50 and 75 mL of A, made up to 100 mL with distilled water.

K) Graded xylene series: 25, 50 and 75 mL of B, made up to 100 mL with A.

L) Stock solution A: Dissolve 3.12 g of D in 100 mL distilled water.

M) Stock solution B: Dissolve 2.83 g of E in 100 mL of distilled water.

N) 0.1 M phosphate buffer: Add 14 mL of L to 36 mL of M. Make up to 100 mL with distilled water.

O) Stock solution C: Dissolve 9.36 g of D in 100 mL of distilled water.

P) Stock solution D: Dissolve 8.79 g of E in 100 mL of distilled water.

Q) 0.3 M phosphate buffer: Add 14 mL of O to 36 mL of P. Make up to 100 mL with distilled water.

R) 10% formaldehyde in 0.1M phosphate buffer: Heat 65 ml of deionised water to 60°C. Add 10 g of F. Add 1 or 2 drops of 2M NaOH solution and stir rapidly until the solution has nearly clarified. Remove from heat source and add 33 ml of Q. Adjust pH to 7.2. Filter solution through a 0.45 µm filter (Millipore). Cool in a waterbath and store in the refrigerator.

A 1.5.6 Procedure

1. Gently remove granules from McCartney® bottles using forceps.
2. Gently wash in 0.1 M phosphate buffer at pH 7.2 in glass Petri dishes. Repeat washing three times.
3. Remove granules from Petri dishes and place in McCartney® bottles containing 10% formaldehyde in 0.1M phosphate buffer for 16 h.
4. Gently wash in 0.1 M phosphate buffer at pH 7.2 in glass Petri dishes. Repeat washing step three times.
5. Dehydrate granules in a graded alcohol series (25, 50, 75 and 100%) for 20 minutes each.
6. Replace alcohol with xylene by immersing the granules in a graded alcohol/xylene series (25, 50, 75 and 100%) for 20 minutes each.
7. Heat paraffin wax on a hot tray at 45 – 55^{o} C.
8. Pour the molten wax into peel-off moulds.
9. Carefully transfer xylene-saturated granules into the peel-off moulds and leave overnight in a desiccator at 60 – 65^{o} C.
10. Remove moulds from the incubator and allow samples to cool.
11. Section the samples with a microtome and allow the sections to float on a waterbath.
12. Remove sections from waterbath and allow it to air dry on slides.
13. Place slides in an incubator at 70^{o} C for 10 minutes to melt the paraffin.

14. Carefully dewax with 100% xylene using Coplin jars.
15. Place a coverslip on slide and examine under epi-fluorescent microscope.

APPENDIX II: POTTED PLANT GROWTH TRIAL

A 2.1 Introduction

This part of the project formed part of a third year project, in which, the effect of ABR irrigation on plant growth was assessed. Results from Chapter 4 of this project indicated that ABR effluent was not of suitable microbiological quality for irrigation agriculture. However, the high nutrient levels of the effluent suggested that it might be used as a potential fertilising solution, provided that the microbial quality was improved. A small-scale potted plant irrigation trial was therefore conducted to examine plant growth. A limited microbiological analysis of plants was also included to confirm earlier findings (microbial quality unsuitable for irrigation).

A 2.2 Materials and Methods

A 2.2.1 Experimental design

Potted plant growth trials were conducted at an experimental site near the University greenhouse. Three common vegetable crops were selected for the experiment: peppers, spinach and maize. Spinach and pepper seedlings were obtained from a local nursery (Skye Seedlings), whilst maize plants were grown from seed. The seedlings of each vegetable type were potted in plastic bags containing Berea red sand. Seedlings were given two weeks to adapt to conditions and watered with tap water during this period. Irrigation was carried out using a plastic watering can, in which water was delivered directly onto the soil surface.

Three irrigation treatments were employed. Tap water containing no nutrients served as a negative control, tap water containing commercially available plant nutrient powder (2.2 mg/L) (Chemicult®) served as positive control, and ABR effluent served as the experimental treatment. Plants were watered with 500 mL of the respective treatments on a weekly basis. In addition, all plants were watered with tap water every two days to prevent dehydration damage. A total of 90 plants were used in the experiment, with 10 plants of the same crop type used for each treatment.

A 2.2.2 Plant growth monitoring

Growth was monitored after the first week of planting seedlings, and measured on a weekly basis over a seven-week period. The growth parameters included height, number of leaves, stem diameter, mean leaf length and width. All growth measurements were recorded by third year students as part of a third year project (Badat and Singh, 2003).

A 2.2.3 Microbiological analyses

After a seven-week growth trial, plants were analysed for *E. coli* and total coliforms. Microbiological analyses were performed on the surface of plants by swabbing all leaves of each plant with sterilised gauze swabs. A new swab was used for each plant. Each swab was placed into a sterilised glass bottle containing a saline solution [0.2% NaCl, (w/v)], the contents of which were agitated to suspend microbial contaminants. Samples were serially diluted and coliform analyses performed using the membrane filtration technique (Standard Method 9222B) (APHA-AWWA-WEF, 1998), as described in Appendix I. Samples were analysed with assistance from third year students (Badat and Singh, 2003).

After a 2-month waiting period, soil samples from different treatment bags were tested for microbiological contamination. According to OMEE and OMAFRA (1996), a waiting period of two months is recommended if the coliform count exceeds 10^6 CFU/100 mL. The rationale behind this strategy was to determine whether the waiting period was sufficient to allow significant reduction in coliform numbers. Soil samples (1 g) were collected from the surface, and at a depth of 15 cm below the soil surface (to determine leaching), and transferred into sterilised glass bottles containing a sterile saline solution [0.2% NaCl, (w/v)]. The glass bottles were agitated vigorously to re-suspend microorganisms. Thereafter, samples were serially diluted and coliform analyses performed using the membrane filtration technique (Standard Method 9222B) (APHA-AWWA-WEF, 1998) (see Appendix I).

A 2.2.4 Statistical analysis

Growth measurement data was shown to be normally distributed using the KS test. The assumption of equal variance was then tested using Levene's test, and was rejected. ANOVA was performed using the post-hoc Tamhane's T2 test for data with unequal variances. ANOVA was used to determine whether there were any significant differences between groups of data, whilst Tamhane's T2 test was used to determine where the differences lay. All data were analysed using SPSS for Windows XP.

Microbiological data were not statistically analysed due to a small sample size.

A 2.3 Results

A 2.3.1 Comparison of plant growth

Comparison of plant growth for the different treatments for each crop type is presented in Figures A2.1 to A2.3. Figure A2.1 shows the comparative growth of maize plants for the different treatments and the following growth trends were observed:

a) Length of maize leaves: Crops irrigated with nutrient solution were longest, followed by crops irrigated with ABR effluent. Crops irrigated with tap water only had the shortest leaves. No statistical difference (ANOVA, $P > 0.05$) was observed between the lengths of leaves for the different treatments.
b) Width of leaves: Crops irrigated with nutrient solution had the largest width, whilst those irrigated with tap water only, had the smallest width. Crops irrigated with ABR effluent were similar in size to those watered with nutrient solution, although no statistical difference was seen between any of the treatments (ANOVA, $P > 0.05$).
c) Stem diameter: Crops irrigated with nutrient solution were the largest, whilst those irrigated with tap water were the smallest. The mean diameter of crops irrigated with ABR effluent was intermediate between those of other treatments. No significant difference was observed between the treatments (ANOVA, $P > 0.05$).

Figure A2.2 shows the comparative growth of pepper plants for the different treatments. The following growth trends were observed:

a) Plant height: Pepper plants irrigated with a nutrient solution and ABR effluent had similar heights and both treatments were significantly greater than those irrigated with tap water (Tamhane's T2 test, $P \leq 0.05$).
b) Number of leaves: Crops irrigated with a nutrient solution had a greater number of leaves, followed by crops irrigated with ABR effluent. Pepper plants irrigated with tap water had the smallest number of leaves. No statistical difference was observed between treatments (ANOVA, $P > 0.05$).
c) Stem diameter: Crops irrigated with nutrient solution were the greatest, whilst those irrigated with tap water were the smallest. The mean diameter of crops irrigated with ABR effluent was intermediate between those measured for the other treatments. A significant difference in the mean stem diameter was observed between the nutrient solution and the tap water treatments (Tamhane's T2 test, $P \leq 0.05$), but no significant difference observed between the nutrient solution and ABR effluent, and tap water and ABR effluent.

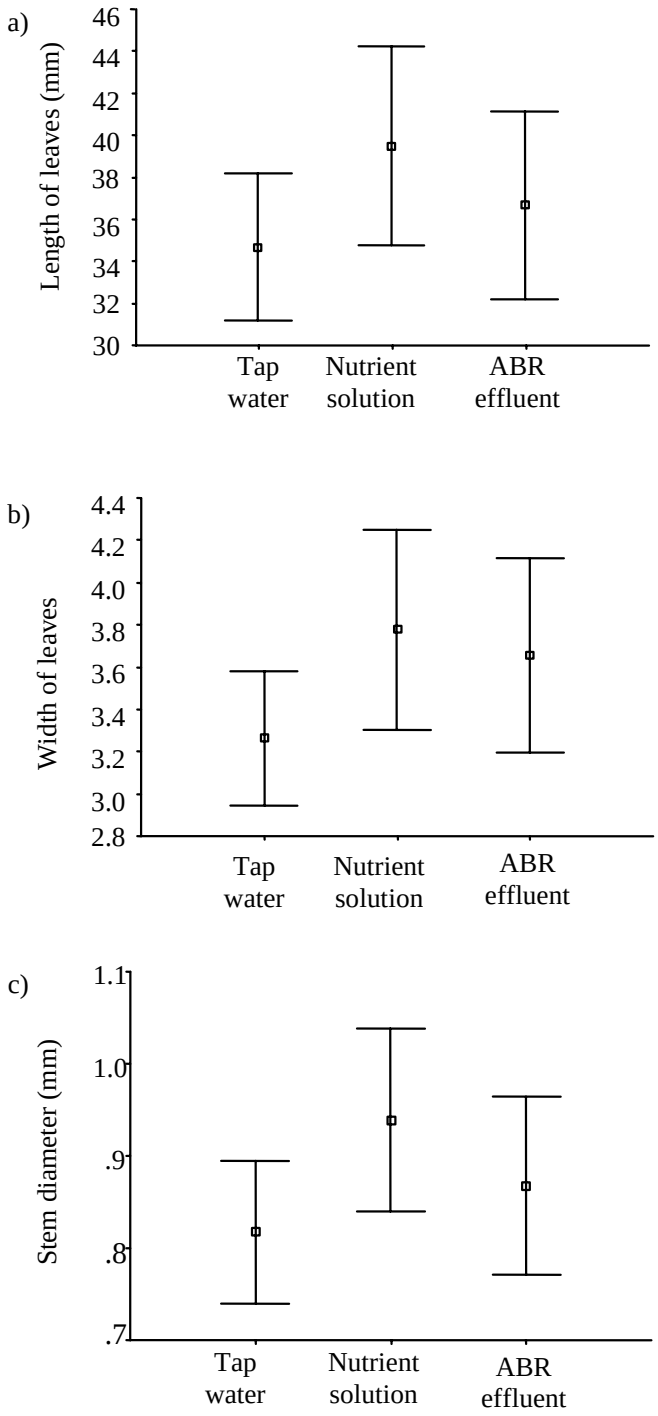


Figure A2.1: Comparison of irrigation treatments of maize for a) length of leaves, b) width of leaves, and c) stem diameter. The squares represent the mean with vertical lines representing the standard error (n = 63).

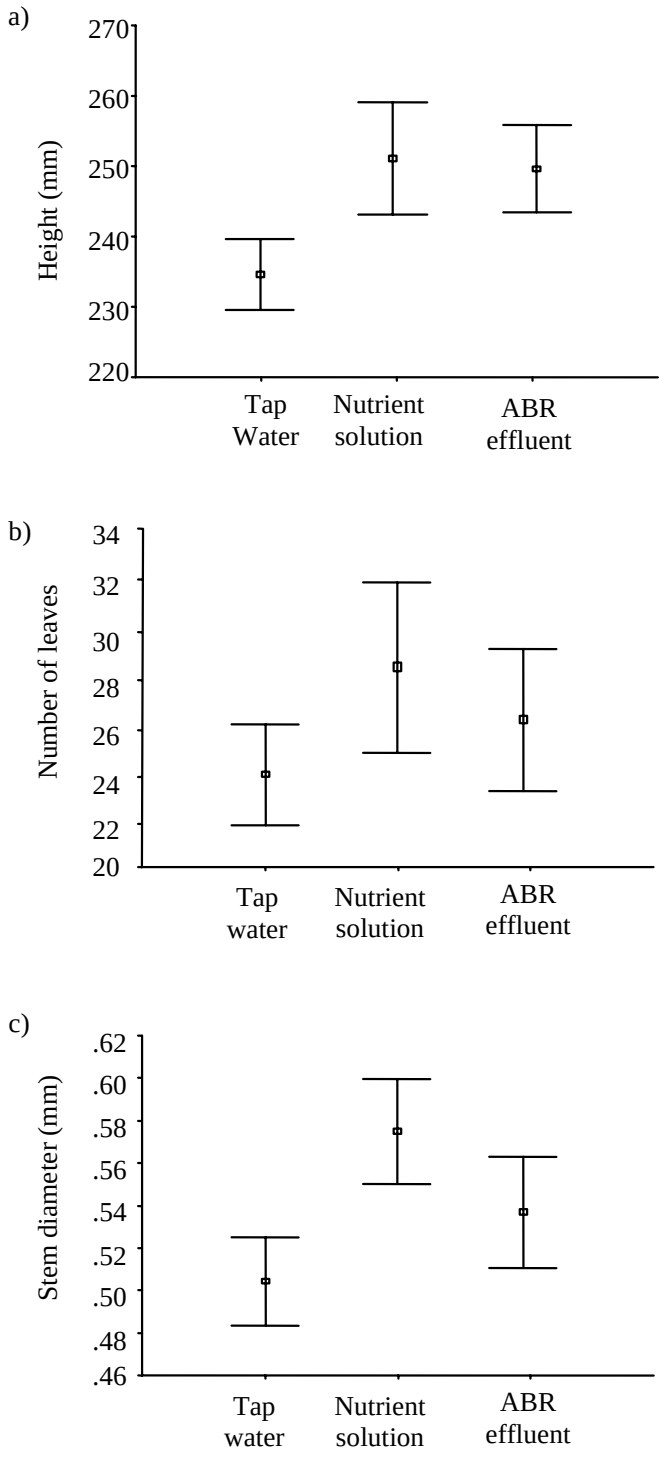


Figure A2.2: Comparison of irrigation treatments of pepper for a) plant height, b) number of leaves, and c) stem diameter. The squares represent the mean with vertical lines representing the standard error (n = 63).

Figure A2.3 shows the comparative growth of spinach plants for the different treatments. The following growth trends were observed:

a) Plant height: Spinach plants irrigated with ABR effluent were the highest, followed by crops irrigated with a nutrient solution, with tap water irrigated crops being the smallest. The height of ABR effluent-irrigated crops were similar to that of irrigated with a nutrient solution, but significantly higher than those irrigated with only tap water (Tamhane's T2 test, $P \leq 0.05$). Crops irrigated with the nutrient solution were also similar in height to tap water treatments.
b) Number of leaves: Crops irrigated with a nutrient solution and ABR effluent had a significantly higher number of leaves than crops irrigated with tap water (Tamhane's T2 test, $P \leq 0.05$).

A 2.3.2 Microbiological contamination

This part of the experiment was aimed at evaluating the potential health risks associated with effluent irrigation. The sampling size was limited as it was pre-determined that the quality of effluent needed to be improved before it could be used in irrigation agriculture. The results presented below are therefore semi-quantitative and were not tested for statistical differences.

As can be seen in Figure A2.4, no consistent trends seen regarding for the surface contamination between the different irrigation treatments. For maize, the *E. coli* count was the highest from plants irrigated with a nutrient solution and lowest with those irrigated with tap water only. On the other hand, the total coliform counts were highest for effluent-irrigated maize crops and lowest for crops irrigated with tap water. The total coliform contamination of nutrient-irrigated maize crops was similar to that of crops irrigated with ABR effluent.

For pepper plants, the *E. coli* count on plant surfaces was relatively similar for all irrigation treatments, with tap water treatments having the highest contamination level. Tap watered pepper crops also had the highest total coliform contamination, but in contrast to *E. coli* counts, the difference between the other treatments was more noticeable.

For spinach plants, the *E. coli* count was the highest from plants irrigated with ABR effluent and lowest with those irrigated with a nutrient solution. In addition, crops irrigated with tap water and a nutrient solution had similar levels of contamination. The total coliform count on the surface of

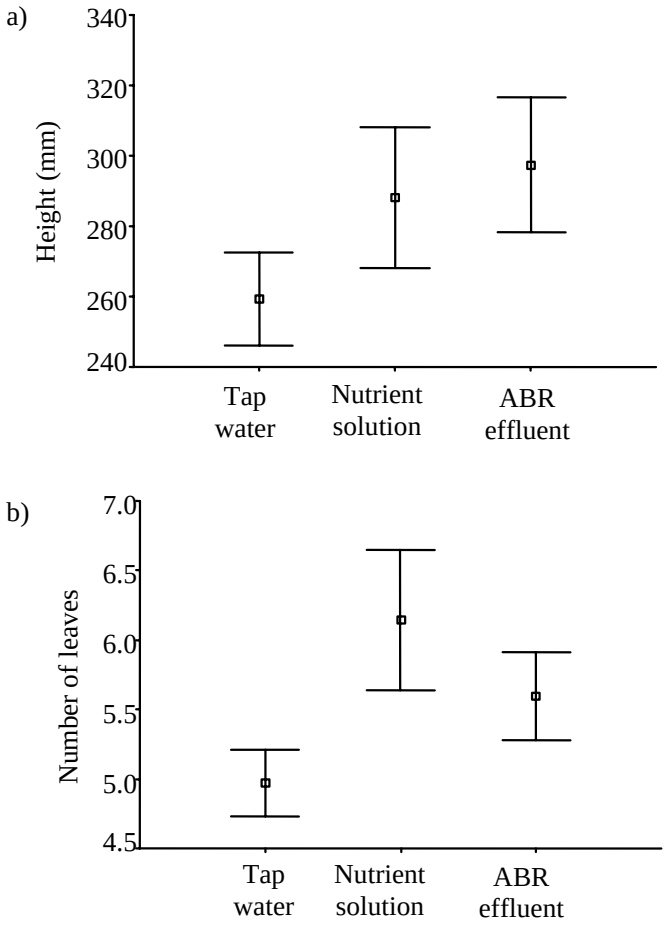
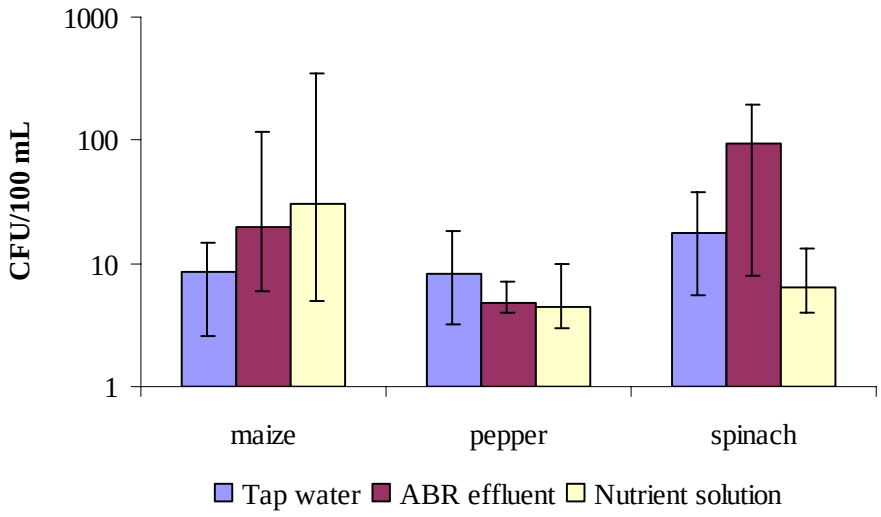


Figure A2.3: Comparison of irrigation treatments of spinach for a) plant height, and b) number of leaves. The squares represent the mean with vertical lines representing the standard error (n = 63).

spinach plants was relatively similar for all treatments, with tap water irrigated crops having the highest contamination, followed by ABR effluent, and finally, nutrient solution.

Considering soil contamination, *E. coli* contamination was generally the highest (from 10^3 to 10^4 CFU/100 mL) in crops irrigated with ABR effluent (Figure A2.5), and clearly posed a health risk. In addition, counts at the soil surface were similar to those at a depth of 15 cm, indicating that leaching of microbial contaminants is likely to occur with this soil type. Unexpectedly, some water and nutrient solution treatments had counts similar, and in one of the cases, a higher count than that of effluent-treated plants. A similar inconsistent pattern was observed with respect to the total coliform count (Figure A2.5).

a) *E. coli*



a) Total coliforms

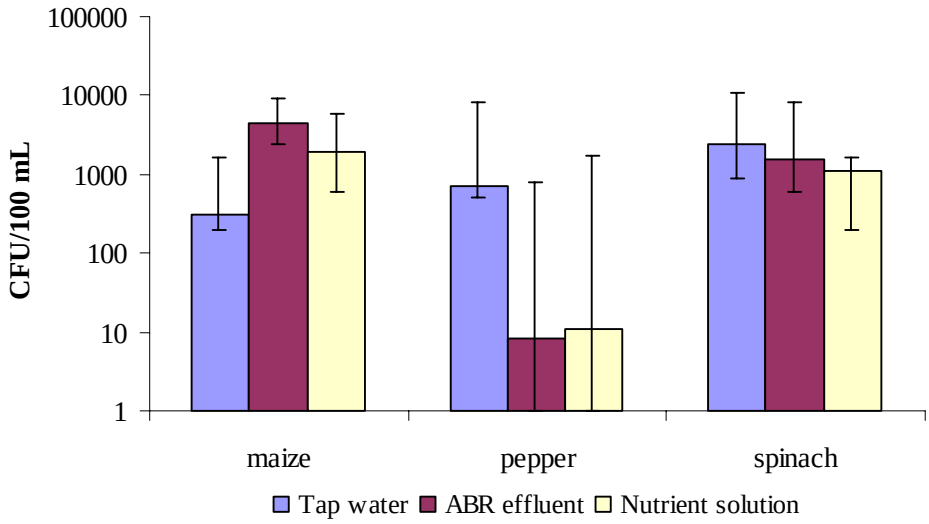
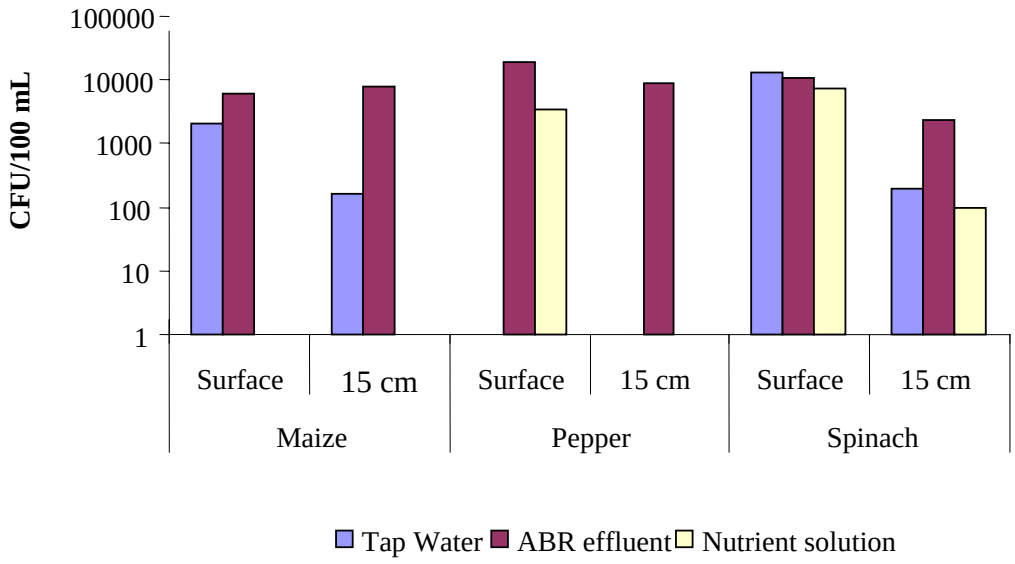


Figure A2.4: Microbial analyses of crops receiving three irrigation treatments. a) *E. coli* counts on the surface of crops; b) total coliform counts on the surface on crops. Results are presented as geometric means (n = 9). Vertical lines represent the standard error.

a) *E. coli*:



b) Total coliforms:

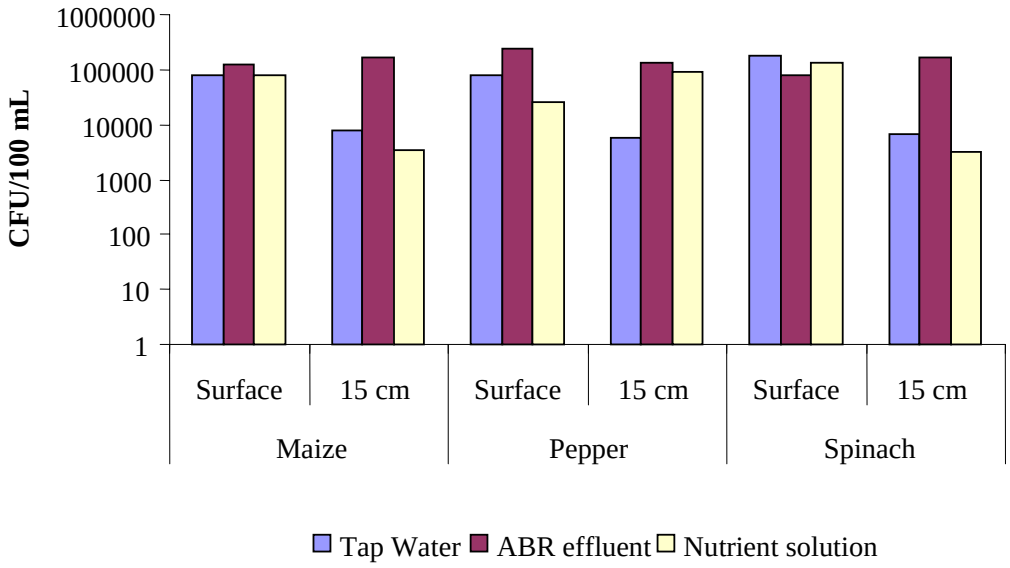


Figure A2.5: Microbial analyses of soil samples from the surface and 15 cm below the soil surface of plastic bags from three different treatments. a) *E. coli* counts from the surface and below the soil of bags; b) total coliform counts from surface and below he soil of bags. Results are presented as geometric means (n = 9).

A 2.4 Discussion and Conclusion

There were few discernible differences in the growth responses of each crop type to the different treatments. Maize plants appeared to have responded the least favourably to ABR effluent or nutrient-supplemented treatments compared to negative treatments (tap water). No statistically significant growth was observed between all treatments for the study period, although tap water treatments (negative control) yielded the poorest growth, whilst the nutrient solution treatments (positive control) yielding the best growth. The growth of maize irrigated with ABR effluent closely resembled that of plants irrigated with nutrient solution, indicating the potential nutrient value of effluent. Pepper plants displayed a similar trend in growth with respect to the different treatments, with a statistically significant difference between the effluent treatment and the tap water treatment, only observed for plant height. In contrast, spinach plants showed the best response when given effluent irrigation. All growth parameters measured showed statistical significance compared to tap water treatments (negative control).

With respect to the microbiological quality of plants, analyses of plant surfaces showed a high variability of counts, with no consistent trends among irrigation treatments, or crop type. Total coliform counts were generally two to three magnitudes higher than that of *E. coli* counts, ranging from 0 CFU/plant and to approximately 10 000 CFU/plant. *E. coli* counts varied between 1 CFU/plant and approximately 100 CFU/plant, with varying geometric means between plants and irrigation treatments.

A similar trend was observed with respect to soil analyses, with coliform counts similar, or in some cases, higher than effluent-treated plants. Whilst this is probably due to a small sample size, there was evidence that *E. coli* contamination was higher in crops irrigated with ABR effluent. Counts were similar to those measured from untreated wastewater, and was never detected below 1 000 CFU/100 mL for the study. In addition, the level of contamination on the surface was similar to that of the bottom of each bag for each crop type indicating that microbial groundwater contamination is likely if the effluent were to be used untreated. Since soil analyses were done after a waiting period, the results indicated that waiting periods have little value as a protective measure, and further re-enforces the need for post-effluent treatment to reduce the pathogen load.

Given the limitations of this study, it can be concluded that ABR effluent was not detrimental to plant growth, and may be even be stimulatory. The re-use of ABR effluent has the potential to alleviate food insecurity and create employment within low-income areas, provided the microbial

quality is improved. The limited microbiological data of plant surface and soil samples suggests that are very slight differences in the contamination levels between irrigation treatments. A more comprehensive experiment will be required in the future, with more frequent sampling and a large size to confirm preliminary findings. However, it is questionable whether resources should be devoted to such studies until the microbial quality of the ABR effluent can be significantly improved.

APPENDIX III: PILOT TRIAL USING MEMBRANE FILTER

A 3.1 Introduction

Microbiological tests were used to evaluate microfiltration membrane filter efficiency as a post-treatment option. The advantage of using a membrane filter as a post-treatment option is that can be included as part of the ABR technology.

Membrane filter technology has several advantages over other post-treatment options, including: superior quality of treated water, which can be consistently achieved in one or two-steps; ability to operate without the use of chemicals; reduced land requirements; and reduced environmental impact (Owen *et al.*, 1995). The major disadvantage of incorporating this technology with the ABR is that it will significantly increase the operating and maintenance costs of the system.

The process involves the separation of components of a fluid mixture by selective permeation though a membrane. The water stream that is retained by the membrane is called the retentate (concentrate), whilst the stream that passes through the membrane is called the permeate stream (Odhav, 2004). The separation of the retentate stream from the permeate stream is brought about by pressure differences (Odhav, 2004). The performances of membranes are based on their selectivity (ability of membrane to retain or reject a specific entity) and permeate flux (permeate rate per unit area of membrane). The former is based on the membrane pore size, whilst the latter is directly proportional to the effective pressure. Higher pressures result in greater efficiency through higher membrane output. However, it also causes greater 'fouling', a phenomenon whereby substances in raw water, such as bacteria, suspended organic and inorganic particles, are absorbed into or 'plug' the membrane (Odhav, 2004). Over time, the deposition of material onto or into the material results in a 'cake' or 'fouling' layer that causes a decrease in flux. This results in altered rejection, reduces treatment efficiency and membrane life, all of which, increase the operating and maintenance costs (Madaeni, 1999). Hence, pre-treatment of wastewater with a particulate content is a prerequisite. To date, 'fouling' is the most important factor that has limited the use of membrane filters to remove microbial contaminants (Jacangelo, 1990; Leiknes *et al.*, 2006).

A 3.2 Materials and Methods

A laboratory experiment was conducted with a submersible plate and frame Kubota® (Copa) microfiltration membrane, with a particle rejection range between 0.1 to 1.0 μm (depending on

'fouling' layer). The filter, which was encased with a plastic housing, was fed with effluent collected in 25 L plastic drums. The membrane enclosure has been illustrated in Figure 3.4. Feeding was carried out for 4 to 4.5 h each day, for a period of three days. The microbial removal capacity was assessed using the membrane filtration technique (Standard Method 9222B) for coliform groups (APHA-AWWA-WEF, 1998) and the double layer plaque assay for coliphages (eThekwini Waste Water Laboratory Test Method No. MM023) (refer to Appendix I).

A 3.3 Results and Discussion

Results indicated that microbial counts were the highest at the start of each 'feeding' period, with a gradual decrease at the time of cessation of 'feeding' (Figure A3.1). There are two possible explanations for the observed trends. First, residual bacteria from the previous feeding could be present downstream of the filter. These would then be dislodged on resumption of flow and result in higher microbial counts. The second explanation is related to the phenomenon of 'caking' on membrane surfaces. As material is deposited on the membrane, a 'fouling' or 'cake layer' develops and the pore size becomes smaller, restricting the passage of microorganisms through it. At the end of day, the filter was removed and immersed in water to prevent dehydration damage. This could have resulted in a partial removal, or cleaning, of the fouling layer, resulting in higher counts at the start of each feeding cycle and lowered counts as the layer re-developed. This explanation is substantiated by flux values, which also follow the same trend as microbial counts over the same period (Figure A3.1).

On day 3 (4:30 h), the experiment was stopped due to observations of bubble formation in the permeate collection pipe. The subsequent decrease in flow indicated that membrane integrity had been compromised. This was borne out by the results obtained on day 4, where permeate microbial counts were shown to increase (Figure A3.1).

The limited microbiological results obtained from the trial suggest that a 'membrane-polishing treatment' may be a suitable post-treatment option for the ABR. The membrane was able to achieve $\log_{10}$ reductions between 1 to 5 and 1 to 2 for coliforms and coliphages, respectively. The coliform counts in permeate samples were generally below the agricultural irrigation limit (Figure A3.1). However, coliphage levels were still high, and represent a health hazard (Table A3.1). A further log reduction is required before the effluent is considered safe. As viruses are generally much smaller than bacteria, it is anticipated that viral rejection (and also bacterial) would increase as the 'fouling' layer develops.

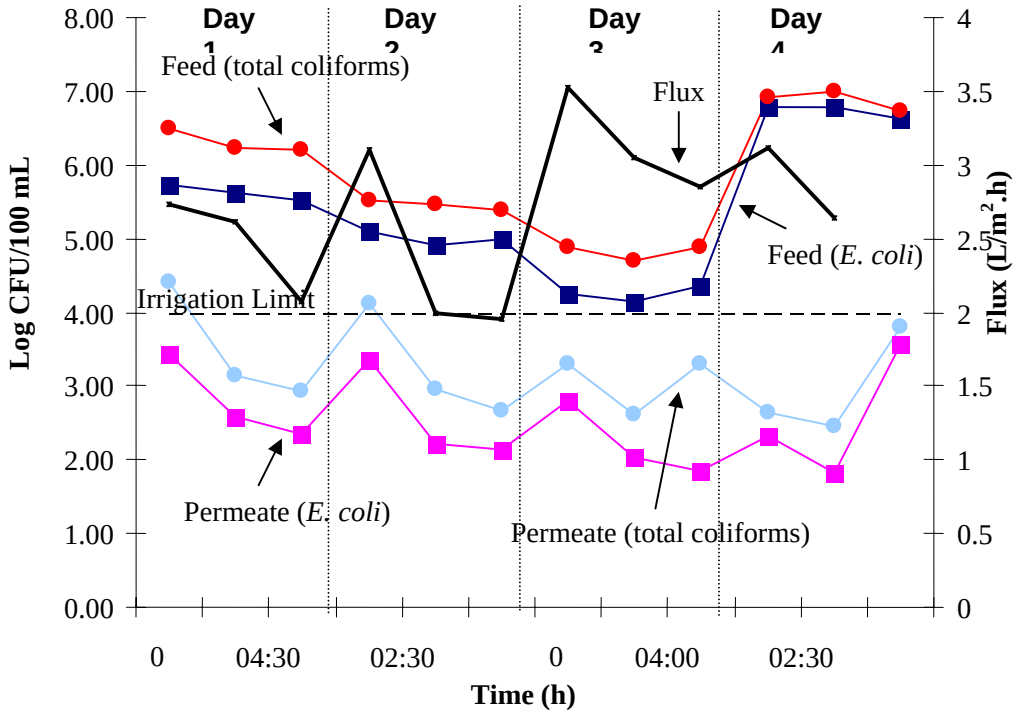


Figure A3.1: Comparison between coliform indicator counts in the feed and permeate during the study period. Flux measurements are also included.

Table A3.1: Average microbiological values from a microfiltration membrane (Kubota®) treating ABR effluent, in relation to discharge standards.

	Units	Feed	Permeate	Discharge Limit
E. coli	CFU/100 mL	7×10^5 (6)	8×10^2 (6)	$1 \times 10^{3\,a}$
Total coliforms	CFU/100 mL	2×10^6 (12)	5×10^3 (12)	$1 \times 10^{4\,b}$
Coliphage	PFU/100mL	1×10^4 (12)	2×10^2 (12)	$20^{\,e}$

Abbreviations: (n), values in parentheses are the number of samples
a: geometric mean, limit for unrestricted irrigation (WHO, 1989).
b: geometric mean for agricultural re-use (DWAF, 1996).
c: guideline for full and intermediate contact (Venter *et al.*, 1996).

A 3.4 Conclusion

It may be concluded from this short study that a microfiltration membrane may be a suitable post-treatment option to reduce the microbial load in the ABR effluent.