TREATMENT OF DYE WASTEWATERS IN THE ANAEROBIC BAFFLED REACTOR AND CHARACTERISATION OF THE ASSOCIATED MICROBIAL POPULATIONS

Joanne Bell

MScEng (Chemical)

Submitted in fulfilment of the academic requirements for the degree of

PhD in Engineering

in the

School of Chemical Engineering

University of Natal, Durban

January 2002

Abstract

There is potential for the anaerobic baffled reactor (ABR) to be implemented on-site for pre-treatment of coloured wastewaters. The implementation of waste minimisation and cleaner production strategies in industry will result in the production of smaller volumes of concentrated wastewaters. With implementation of the ABR, the concentrated waste stream could be pre-treated, with an acclimated biomass, which should facilitate sufficient degradation such that the effluent could be discharged to sewer for further treatment.

The ABR is a high-rate compartmentalised anaerobic bioreactor, the design of which promotes the spatial separation of microorganisms. The use of molecular techniques to characterise the microbial populations and the dynamics of these populations with time and/or changing operating conditions will add to the current understanding of the process, which is based on the biochemical pathways and chemical analyses. This knowledge will allow for optimisation of the design of the ABR.

The hypothesis of the horizontal separation of acidogenesis and methanogenesis through the ABR was proven. Changes in the HRT affected the operation of the reactor, however, recovery from these upsets was almost immediate and operation of the reactor was stable.

Two synthetic dye waste streams, one food dye (tartrazine) and one textile dye (CI Reactive Red 141), and a real industrial dye wastewater, were treated in separate laboratory-scale ABRs. These investigations showed that successful treatment of a highly coloured wastewater is possible in the ABR. The design of the ABR facilitates efficient treatment of concentrated dye wastewaters by protecting the sensitive methanogens from the inhibitory dye molecules and promoting efficient colour and COD reduction.

The molecular-based method, fluorescent *in situ* hybridisation, allowed the direct identification and enumeration of microbial populations active in the ABR. In all of the reported investigations, there was a definite shift in the microbial populations through the ABR, with a predominance of eubacteria in the first compartments (acidogenesis) and archaea (methanogenesis) in the later compartments. The number of compartments involved in each depended on the strength of the substrate (organic loading rate - OLR). A combination of FISH probing, and the analysis of 98 archaeal 16S rDNA clone inserts provided useful descriptions of the methanogens actively involved within each compartment. These showed a predominance of the *Methanosaeta* spp., particularly in the last compartments of the reactor. Methanogens present in the first four compartments consisted of species of *Methanobacterium* and *Methanospirillum,* a relatively unstudied methanogen *Methanomethylovorans hollandica,* and an unidentified short filamentous species.

I, Joanne Bell, declare that unless indicated, this dissertation is my own work and has not been submitted for a degree at another University or Institution.

_____

Joanne Bell

January, 2002

As the candidate's supervisor I have approved this thesis for submission.

_____

Prof CA Buckley

January, 2002

Acknowledgements

Several years have passed in my pursuit of this degree. During this time, my life has been touched and influenced by so many wonderful people.

Firstly to my supervisor, Prof. Chris Buckley, my deepest gratitude for all your guidance and support.

I am grateful for the opportunity that this project gave me to spend a year at Imperial College, in London, and I gratefully acknowledge the advice and guidance of Prof. David Stuckey. To my fellow post-grads at Imperial: Lucy, William, Ravi, Mai, Darryl and Duncan, as well as post-docs, Jason and Alette, for teaching me the ropes and for being such good mates! A special thanks to Jason Plumb for re-introducing me to the exciting world of molecular biology and for the clone library work.

My fellow post-grads and staff in the School of Chemical Engineering, at the University of Natal, in particular Priyal, Zama and Kuvarshan for their help with the reactors. Also, thanks to Kelly, Ken, Mike and Les for their help with the construction of the reactors and so many other little things over the years.

My gratitude to the research team at the Centre for Water and Wastewater Research, at Technikon Natal, for the use of their labs and equipment for the FISH work, especially Adrian and Delon for their help and conversation.

I gratefully acknowledge the South African Water Research Commission for funding the project, as well as the National Research Foundation (NRF) and the British Council for financial assistance.

Thanks to the members of the WRC Steering Committee for their valuable contributions.

I am so fortunate to have had so many close friends who have offered such valuable emotional support that will never be forgotten. Thank you especially to Karen, Tina, Cary, Debbie, Steve, Susan and Astrid.

And finally, thank you

To Mom and Dad, I could never have achieved this without your support.

To my beautiful Ethan, who filled my life with such purpose and gave me the inspiration to strive to reach this goal.

To Connel, your confidence in me has remained unwavering throughout the years. My work, my struggles, and my dreams are blessed because our paths are joined.

Glossary

Acclimation	The adaptation of a microbial community to degrade a previously recalcitrant compound, through prior exposure to that compound.
Acetogenesis	The reaction that degrades short chain fatty acids such as propionic acid, butyric acid, or longer chain fatty acids, as well as other intermediates such as ethanol, to acetic acid and hydrogen.
Acid dye	An anionic dye characterised by substantivity for protein fibres and often applied as an acidic dye solution.
Acidogenesis	The process in which long chain soluble monomers or dimers, such as carbohydrates and amino acids, are reduced to short chain volatile fatty acids, such as acetic acid, propionic acid, butyric acid, lactic acid and ethanol, or longer chain fatty acids.
Adsorption (dye)	Binding of dye compounds to surfaces such as microbial cells or activated carbon, usually through electrostatic interaction between the dye and the charged cell.
Aerobic	The condition of living or acting in the presence of molecular oxygen.
Anaerobic digestion	The microbial degradation of an organic compound in the absence of oxygen.
Anionic dye	A dye that dissociates in aqueous solution to give a negatively charged coloured ion.
Anthraquinone dye	Dye based on the structure of 9,10-anthraquinine, with powerful electron donor groups in one or more of the four alpha positions.
Auxiliary	A chemical or formulated product which enables a processing operation in preparation, dyeing, printing or finishing to be carried out more effectively or which is essential if a given effect is to be obtained.

Azo dye	Dye which contains at least one azo group (-N=N-) and can contain up to 4 azo bonds.
Basic dye	A cationic dye characterised by its substantivity for the acidic types of acrylic fibre and for tannin mordanted cotton.
Batchwise processing	Processing of materials as lots of batches in which the whole of each batch is subjected to one stage of the process at a time.
Biodegradation	The microbial degradation of organic compounds to inorganic molecules.
Carcinogenic	Cancer-causing
Chemical Oxygen Demand (COD)	A measure of the total amount of organic material in the waste stream.
Chromophore	A chemical group which, when present in a compound, is responsible for the appearance of colour.
Colour Index	An authoritative, descriptive catalogue of natural and synthetic colourants and intermediates in terms of generic name.
Colourant	Organic chemical used for colouring fabrics or food products and includes dyes and pigments.
Decolourisation	The removal of colour from solution by destruction of the chromophore.
Electron transport chain	A chain of carrier molecules with fixed orientation in the cell membrane, through which electrons are transported and ATP generated.
Enrichment	Selection of microorganisms with certain characteristics, from a mixed culture, through manipulation of culture conditions.
Exhaustion	The proportion of dye or other substance taken up by a substrate at any stage of a process, to the amount that was originally available.
Facultative anaerobe	An organism capable of either aerobic or anaerobic respiration.
Fermentation	Amino acids and sugars are degraded to propionic acid and other intermediary products, acetic acid and hydrogen by

	fermentative or acidogenic bacteria.
Headspace	The volume in a sealed vessel not occupied by the liquid phase.
Hydrolysis	Breakdown of complex long-chain macromolecules (carbohydrates, lipids and proteins), via the Embden-Meyerhof pathway, to short-chain compounds (sugars, fatty acids and glycerol, and amino acids, respectively). First phase of the anaerobic digestion process.
Inhibition	An impairment of bacterial function.
Intermediates (dye)	The compounds used to synthesise dyes.
Labile	Readily degradable.
Medium	Mixture of nutrient substances required by cells for growth and metabolism.
Metabolism	The physiochemical transformations through foodstuffs are synthesised into complex elements, complex substances are rendered into simple ones and energy is made available for use by the organism.
Metabolites	Intermediate compounds formed during dye catabolism.
Methanogenesis	The process by whereby low molecular weight substrates are degraded to form methane.
Mineralisation	Microbial decomposition of an organic compound into inorganic constituents such as carbon dioxide, methane and water.
Mixed culture	Culture consisting of two or more types of microorganisms.
Pollution	An adverse alteration of the environment.
Reactive dyes	Coloured components capable of forming a covalent bond between the dye molecule and the fibre.
Recalcitrant	Resistant to microbial degradation.
Residence time distribution	The distribution of ages of liquid elements in a vessel.

Textile finishing	A collection of processes in which raw cloth/yarn is cleaned and prepared for dyeing and printing.
Volatile fatty acid (VFA)	Short-chain organic acid formed by the anaerobic digestion process.
Xenobiotic	A compound not found in nature.

Table of Contents

List of Figures

List of Tables

Abbreviations

ABR	Anaerobic Baffled Reactor
ADMI	American Dye Manufacturers Institute
APHA	American Public Health Association
CI	Colour Index
COD	Chemical Oxygen Demand
CFD	Computational Fluid Dynamics
CSTR	Continuously Stirred Tank Reactor
EDTA	Ethylenediamine Tetra-Acetic Acid
ETAD	Ecological and Toxicological Association of the Dyestuffs Manufacturing Industry
FISH	Fluorescent *in situ* hybridisation
GC	Gas Chromatograph
HPLC	High Performance Liquid Chromatography
HRT	Hydraulic Retention Time
IC	Inorganic Carbon
MW	Molecular Weight
NMR	Proton Nuclear Magnetic Resonance Spectroscopy
OD	Optical density
OLR	Organic Loading Rate
OTU	Operational Taxonomic Unit
rRNA	Ribosomal Ribonucleic Acid
RTD	Residence Time Distribution
SMP	Soluble Microbial Products
SRB	Sulphate Reducing Bacteria
SRT	Solids Retention Time
SVI	Sludge Volume Index
TC	Total Carbon
TOC	Total Organic Carbon
TS	Total solids
TSS	Total Suspended Solids
UASB	Upflow Anaerobic Sludge Blanket Reactor
UV-VIS	Ultraviolet-visible
VFA	Volatile Fatty Acids
VS	Volatile solids
VSS	Volatile Suspended Solids

Notation

		UNITS
COD	Chemical Oxygen Demand	mg/L
HRT	Hydraulic Retention Time	h
IC	Inorganic Carbon	mg/L
IC_{50}	Concentration which is inhibitory to 50 % of the test population	mg/L
LD_{50}	Concentration which is lethal to 50 % of the test population	mg/kg
MW	Molecular weight	g/mol
OLR	Organic Loading Rate	g COD/L.d
SRT	Solids Retention Time	h
TC	Total Carbon	mg/L
TOC	Total Organic Carbon	mg/L
TS	Total solids	mg/L
TSS	Total Suspended Solids	mg/L
VFA-COD	Contribution of VFAs to total soluble COD measured	mg/L
VS	Volatile solids	mg/L
VSS	Volatile Suspended Solids	mg/L

Chapter One

Introduction

In the constitution of the Republic of South Africa (Act 108 of 1996) (Constitutional Assembly, 1996), it states in Chapter 2, Section 24 that *….everyone has the right to an environment that is not harmful to their health or well-being and have the environment protected, for the benefit of present and future generations, through responsible legislative and other measures that prevent pollution and ecological degradation, promote conservation, and secure ecologically sustainable development and use of natural resources while promoting justifiable economic and social development. Everyone has the right to have access to water....*

South Africa is a water scarce country with an average annual rainfall of < 60 % of the world average (Gravelet-Blondin *et al*., 1997). Water is a public commodity and the actions of users and polluters affect others. The monitoring and management of water quality in rivers is thus vital for the adequate long-term protection of South Africa's water resources. In coastal areas, such as KwaZulu-Natal, the biggest problem with industrial water users is the amount of fresh water lost via effluent pipelines to sea; the water should be treated and returned to the rivers for reuse. It is, therefore, important to encourage industries to minimise water consumption, and recycle and re-use water and effluent where possible. Cleaner production is defined as *the continuous application of an integrated environmental strategy, applied to processes, products and services to increase eco-efficiency and to reduce risks for humans and the environment*. Implementation of cleaner production and waste minimisation practices, at the effluent source, will lead to the production of more concentrated effluents. Anaerobic digestion has the potential to treat these effluents. Agro-industries characteristically produce effluents of a xenobiotic nature and/or of high organic strength. Industries such as the food, leather and textile industries also utilise synthetic colourants in their processes, resulting in coloured effluents.

Increasing awareness in recent years of the cumulative effects of pollution has led to growing public concern and increasingly strict legislation relating to the discharge of industrial wastes. Anaerobic digestion is a naturally occurring process which has been harnessed for its twin benefits of reducing organic pollutants in waste streams and producing energy in the form of a methane-rich biogas. The challenge in advancing anaerobic digestion for high-strength or toxic organic waste streams lies largely in enhancing the bacterial activity taking place per unit of reactor volume and, in the case of xenobiotics, in the acclimation of the biomass to the compound. Taking into consideration the slow growth rate of many anaerobic microorganisms, particularly methanogens, the main objectives of efficient reactor design must be high retention time of bacterial cells within the reactor, together with good mixing to ensure contact between cells and their substrate (Grobicki, 1989). The anaerobic baffled reactor (ABR) achieves both objectives by means of a design that is both simple and inexpensive to construct. High rates of hydraulic

throughput are possible with very little loss of bacteria from the reactor; the reactor is extremely stable during operation and achieves high rates of removal (Barber, 1999).

The results of previous research (Sacks, 1997) identified a number of industries, in the KwaZulu-Natal region, that produced effluent streams that would be amenable to treatment by anaerobic digestion. The organic content of these effluents (based on COD) was too high to permit conventional treatment at a wastewater treatment works. In the short term, these effluents could be treated in the under-utilised anaerobic digestion capacity in the region. However, with the projected increase in the load on wastewater treatment works, this available capacity will ultimately be needed for sewage treatment.

1.1 WATER QUALITY IN SOUTH AFRICA

The South African Department of Water Affairs and Forestry is responsible for the management of water resources in South Africa, thereby ensuring the provision of adequate water supplies of acceptable quality for all recognised users (Gravelet-Blondin *et al*., 1997). Adequate long term protection of South Africa's water resources is of vital importance for sustained economic growth and development (Department of Water Affairs and Forestry, 1993). South Africa is a semi-arid country in which rainfall and water bodies are unevenly distributed, both temporally and spatially. The rapidly increasing population and demographic changes have resulted in an expansion in demand which could lead to water becoming increasingly scarce in many parts of South Africa. In addition, greater pollution loads and reduced flows in the country's rivers will place further pressure on the limited resources (Department of Water Affairs and Forestry, 1993).

1.1.1 The South African Water Act

The strict effluent discharge regulations promulgated in terms of the Water Act of 1956 resulted in the construction of wastewater purification plants. Before the advent of the Water Act of 1956, there was no statutory provision for State control over the purification and disposal of effluent, except that the discharge of sewage into public streams was prohibited (Department of Water Affairs, 1986). The Water Act, in anticipation of water shortages, made provision for the compulsory purification of effluent by the user to specified standards and its subsequent disposal in a manner that would make it available for reuse. The Act provided for control over the use of water for industrial purposes as well as for control over and the prevention of water pollution.

The Department of Water Affairs and Forestry completed an intensive review of the 1956 Water Law in 1997. The review was motivated by the need for preparation for new legislation that would reflect democratic principles and equitable access to the resource by all; symbolised by the slogan *some for all, forever* (Department of Water Affairs and Forestry, 1997). With the adoption of the White Paper, in 1997, participation from communities, water users, academic institutions, scientific councils and Government at national, provincial and local levels, was encouraged, for the development of the new National Water Bill. At its meeting on 21 January 1998 Cabinet approved the presentation of the National Water Bill, 1998.

The new Water Act (1998) has integrated resource-directed measures for protection, such as resource quality objectives, with source-directed measures, such as effluent standards. The source-directed measures include the use of discharge or impact standards. These standards should be stringent enough to protect the specific water resource affected.

1.2 THE SOUTH AFRICAN TEXTILE INDUSTRY

South Africa is in a unique position, having the relatively low cost labour of a developing country, but with sophisticated infrastructure. The highest concentration of textile factories, in South Africa, is in KwaZulu-Natal, with 20 located in the Durban Metropolitan area (Gilfillan, 1997). New restrictions on wastewater discharges are forcing dyestuff manufacturers and textile wet processors to reuse process water and chemicals. This has prompted research into new advanced treatment technologies e.g. polishing treatments such as filtration, chemical oxidation and specialised flocculation techniques and pre-treatment steps including anaerobic digestion, fixed-film bioreactors, Fenton's reagent oxidation, electrolysis or foam flotation (Vandevivere *et al*., 1998).

1.2.1 The Problem of Colour

The South African textile industry is the sixth largest employer in the manufacturing sector, with 68 700 people employed directly, an additional 200 000 indirectly in dependent industries, and supports 80 000 cotton workers. It has local annual sales of R 9.8 billion and is the eleventh largest exporter of manufactured goods. In addition, it is the second largest user of electricity and the second largest payer of rates and taxes in towns and cities across South Africa (Anon, 2000). Currently, in addition to international trade pressure, the industry is also facing increasing pressure from local authorities to reduce their environmental impact due to the limited water resources within South Africa (Gravelet-Blondin *et al*., 1997).

The textile industry is a water-intensive industry; the specific water intake varies from 95 to 400 L/kg fabric depending on the type of processes and water efficiency (Steffen Robertson and Kirsten, 1993). Dyehouse effluents are complex, containing a wide and varied range of dyes and other products. In general, the effluent is highly coloured, high in biochemical oxygen demand (BOD) and chemical oxygen demand (COD), has a high conductivity and is alkaline in nature (Gravelet-Blondin *et al*., 1997). Of these, colour is perceived to be the most problematic as it is visual pollution and gives rise to public complaints. Colour is noticeable at concentrations of 1 mg/L (Bell, 1998).

The majority of textile industries in South Africa discharge to sewer and their effluents must, therefore, comply with limits set by the local authorities, who in turn, must comply with the requirements set by the Department of Water Affairs and Forestry. Due to the variable and complex nature of textile effluents, conventional sewage treatment processes often do not sufficiently treat the effluents with the result that colour and other pollutants enter the receiving water bodies. The discharge of textile effluent into receiving water bodies without adequate treatment can impact on the lives of the people/communities living alongside the water body (Bell, 1998). Colour not only elicits highly emotive responses from the

public, because it is a visible source of pollution, but may also impact on aquatic life by preventing the penetration of light, thereby interfering with photosynthesis.

1.2.2 Legislation for the Discharge of Coloured Effluents

In general, the Department of Water Affairs and Forestry follows the precautionary principle and the *polluter pays* principle, whereby polluters are increasingly required to treat their effluents, and undertake and fund monitoring programmes and ecological impact studies to assess the environmental effects of their discharge (Department of Water Affairs and Forestry, 1993). The Water Act makes provision for the prosecution of offenders that do not comply with its conditions and regulations.

Legislation in South Africa states that discharged effluent must adhere to a general standard of zero colour, however, in practice the measurement of colour is complicated by inadequate analytical methods as well as natural colouration and suspended solids in receiving water bodies. In addition, when colour is undesirable for aesthetic reasons it is difficult to correlate analytical colour measurements with colour perception by the human eye (Bell, 1998). Therefore, in practice, the zero colour standard may be modified so that the impact of the coloured effluent on the receiving water body is such that the total colour in the water is acceptable to all existing and potential downstream users. With regards colour, the Department of Water Affairs and Forestry issued a statement, in 1994, that *the final effluent colour (from a waste water treatment works) should be such that it does not give rise to public complaints and effluent leaving dyehouses should be such that its contribution to the wastewater treatment works does not cause the colour of the final effluent to be displeasing* (Gravelet-Blondin *et al*., 1997).

1.3 MICROBIAL POPULATION DYNAMICS

The fundamental aspects of the anaerobic digestion process have been investigated, yet there is still the need for more basic information on the biological aspects of the anaerobic ecosystem (Godon *et al*., 1997). The complete identification and quantification of all contributing populations is necessary to establish the link between microbial structure and function. This lack of knowledge is due to the limitations of traditional identification and enumeration techniques, such as selective enrichment, pure culture isolation and most probable number estimates.

Molecular-based methods, such as fluorescent *in situ* hybridisation (FISH) with ribosomal RNA (rRNA) oligonucleotide probes, allow the direct identification and enumeration of microbial populations in complex environments (Griffin *et al*., 1998). These techniques can provide a clearer insight into the interactions, concentrations and growth rates of the various trophic groups involved in anaerobic digestion. Molecular techniques by themselves can provide useful qualitative and quantitative information on the microbial populations present in wastewater treatment plants. However, in isolation, they are simply a tool that can complement other methods and approaches for analysing wastewater treatment systems. The combined use of traditional culture-based and microscopic techniques, chemical analyses, and molecular techniques should serve to better link microbial structure and function.

1.4 PROJECT OBJECTIVES

There is potential for the ABR to be implemented on-site for pre-treatment of coloured wastewaters. The overall objective is the implementation of waste minimisation and cleaner production strategies in factories. However, wherever there is industrial activity, some waste is unavoidable; but the effluent produced is of much smaller volume and hence more concentrated. With implementation of the ABR, the concentrated waste stream could be pre-treated, with a biomass acclimated to the particular effluent, which should facilitate sufficient degradation such that the effluent could be discharged to sewer for treatment.

The ABR is a high-rate anaerobic bioreactor, the design of which promotes the spatial separation of microorganisms. The use of molecular techniques to characterise the microbial populations and the dynamics of these populations with time and/or changing operating conditions would add to the current understanding of the process, which is based on the biochemical pathways and chemical analyses. This knowledge should allow for optimisation of the design of the ABR.

Hence, the overall objective of this project was to investigate the operation of the ABR, on a laboratory-scale, and to assess whether the application of fluorescent *in situ* hybridisation (FISH) could enhance the operation of the reactor by relating the active microorganisms to the biochemical pathways and chemical analyses.

The more specific objectives were:

1. To assess the feasibility of the ABR as an on-site pre-treatment mechanism for coloured wastewaters.

2. To test the hypothesis of spatial separation of acidogenesis and methanogenesis through the reactor.

3. To assess the inherent anaerobic toxicity and the ultimate anaerobic biodegradability of a range of food and textile dyes using batch serum tests, and to assess the applicability of these batch tests to operation of the ABR.

4. To investigate the changes in structure and dynamics of the microbial communities, within the ABR, with changing hydraulic retention time.

5. To investigate the efficiency of the ABR in the treatment of coloured wastewaters, with concurrent monitoring of the changes in the microbial populations, using molecular methods.

1.5 THESIS ORGANISATION

The thesis begins (**Chapter 2**) with a review of the literature on the anaerobic digestion and microbiology; the anaerobic baffled reactor; and the physical, chemical, microbiological and molecular techniques available for monitoring anaerobic digestion processes, with a detailed focus on fluorescent *in situ* hybridisation (FISH) with oligonucleotide probes.

Food dyes are the focus of **Chapter 3**. A synthetic pure tartrazine waste stream was treated in a laboratory-scale ABR and the results are presented. The inherent anaerobic toxicity and the ultimate anaerobic biodegradability of a range of food dyes was assessed using batch serum bottle tests. A real coloured waste water was collected from a food dyestuff manufacturer and fed to a second laboratory-scale ABR. These results, together with the microbial community characterisation, are presented.

Chapter 4 details the operation and analytical results of a laboratory-scale ABR, to investigate the effect of changes in the hydraulic retention time, with a defined sugar/peptone feed. FISH was used to monitor the consequential changes in the microbial communities, within each ABR compartment.

Chapter 5 details the investigation of textile reactive dyes. Batch serum bottle tests were used to determine the anaerobic toxicity and biodegradability of a range of reactive textile dyes. CI Reactive Red 141 was fed to a laboratory-scale ABR and the operational results and characterisation of the microbial communities, with increasing dye concentration, are presented. The details of the effect of a short-term dye shock load on the operational efficiency of the reactor are given.

The thesis is concluded with **Chapter 6**. The main conclusions drawn during the study are presented and recommendations for future research are made.

Chapter Two

Literature Review

Anaerobic digestion (**Section 2.1**) is a biological process in which organic matter is catabolised to methane and carbon dioxide. This literature review describes the mechanism, biochemistry and microbiology of anaerobic digestion (**Section 2.2**), with a detailed review of the classification and microbiology of the methanogens. The anaerobic baffled reactor (ABR) is reviewed in **Section 2.3**. It is crucial that the performance of an anaerobic digester be monitored to prevent digester failure and to assess the efficiency of the digestion process. The physical and chemical analyses are described in **Section 2.4**, together with a description of batch screening tests to assess the anaerobic biodegradability and inherent toxicity of a particular waste stream. Molecular-based methods, such as ribosomal RNA (rRNA) probe hybridisation (**Section 2.5**) allow the direct identification and enumeration of microbial populations in complex environments. The combined use of traditional culture-based and microscopic techniques, chemical analyses, and molecular techniques should serve to better link microbial structure and function

2.1 ANAEROBIC DIGESTION

Anaerobic digestion is a biological process in which organic matter is catabolised to methane and carbon dioxide. It has the potential to break down complex refractory organic compounds so that they may be further degraded aerobically or to completely mineralise them (Tracey *et al*., 1989). In aerobic respiration, molecular oxygen serves as an external electron acceptor, accepting electrons from electron carriers such as NADH by way of an electron transport chain (Brock and Madigan, 1991). In the absence of oxygen, carbon atoms associated with the organic substrate become electron acceptors and are reduced, while other compounds are oxidised to carbon dioxide (Pohland, 1992).

The anaerobic degradation process has several advantages over aerobic treatment. In aerobic treatment the microorganisms use oxygen in the air to metabolise a portion of the organic waste to carbon dioxide and water. The microorganisms obtain energy from this oxidation, thus their growth is rapid and a large portion of the organic waste is converted to new cells (Speece, 1996). The portion converted to biomass is not actually stabilised but is simply biotransformed. Although these cells can be removed from the waste stream, the biological sludge they produce still presents a significant disposal problem. In contrast, the anaerobic conversion to methane gas provides relatively little energy to the microorganisms, resulting in a slow growth rate and only a small portion of the waste being converted to new biomass. Conversion to methane represents waste stabilisation since methane is poorly soluble and escapes from the waste stream where it can be collected. As much as 80 to 90 % of the degradable organic portion of a waste can be stabilised in anaerobic treatment, even in highly loaded systems. This is in contrast to aerobic systems where only ca. 50 % of the waste is actually stabilised, even with conventional loadings (McCarty, 1964).

Another advantage of anaerobic digestion is, since only a small portion of waste is converted to cells, the problem of disposal of excess sludge is greatly reduced. The absolute quantity as mass of organic matter is low and is readily de-watered. Since anaerobic treatment does not require oxygen, oxygen transfer does not limit the treatment rates and the power requirements are reduced. In contrast, the methane gas produced is a source of energy.

2.2 ANAEROBIC MICROBIOLOGY

Anaerobic digestion is a complex, multi-phased microbial process involving a number of strongly interacting groups of microorganisms. The biochemical pathways are illustrated in **Figure 2.1**.

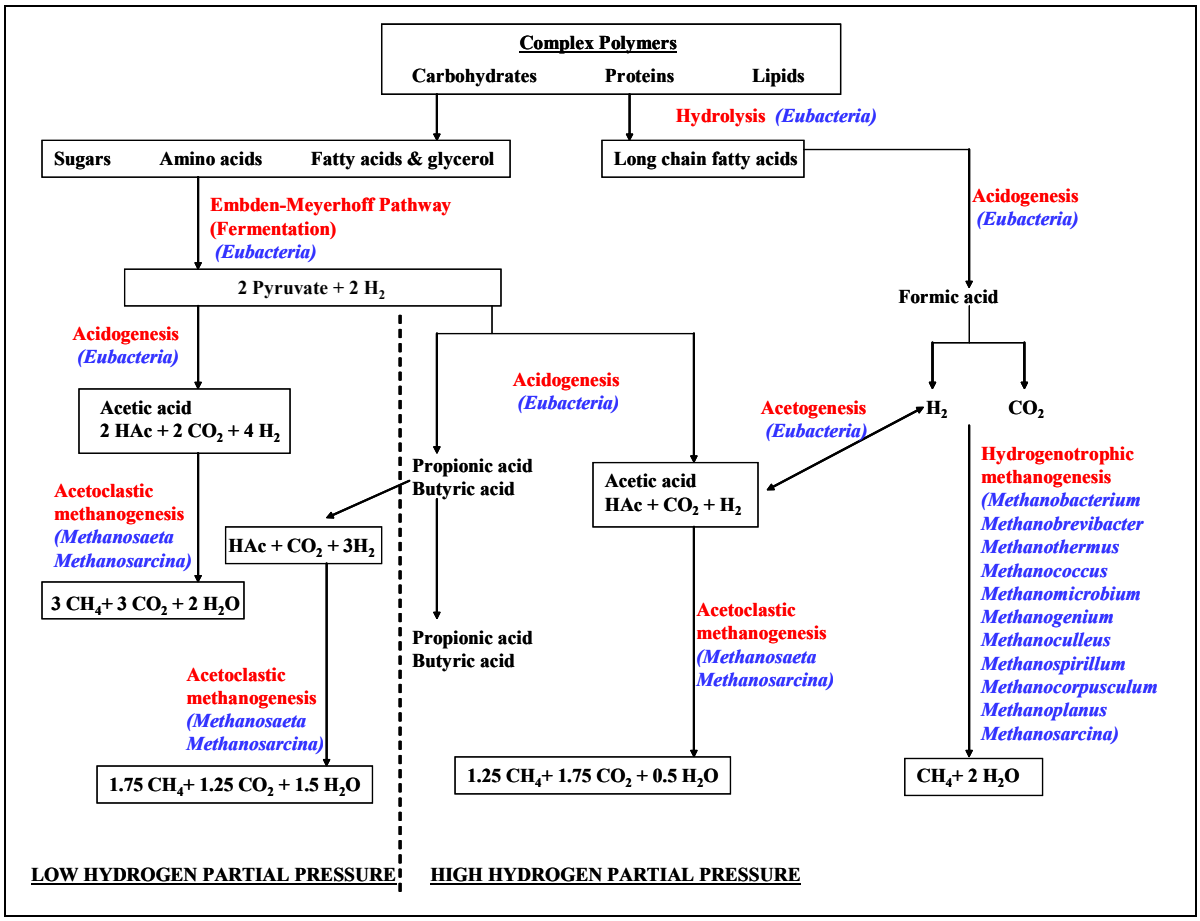


FIGURE 2.1 : Diagram showing the biochemical pathways involved in anaerobic digestion, detailing the microorganisms responsible for each process.

During the first phase, hydrolysis, complex long-chain macromolecules (carbohydrates, lipids and proteins) are hydrolysed extracellularly to short-chain compounds (sugars, fatty acids and glycerol, and amino acids, respectively). Hydrolysis can be a slow process and can be the rate-limiting step in fermentation particularly if the influent contains particulate or large complex molecules in significant quantities. The resulting monomers are fermented to various volatile fatty acid (VFA) intermediates, primarily acetate, propionate and butyrate, with the production of carbon dioxide and hydrogen. The biochemical pathways and end products for this phase depend upon the substrate and the hydrogen partial

pressure. The propionate and butyrate cannot be used directly for methanogenesis and are converted to acetic acid, carbon dioxide and hydrogen, only under conditions of low hydrogen partial pressures (Sam-Soon *et al*., 1991). Acetic acid is the substrate for a group of strictly anaerobic methanogenic bacteria which ferment the acetic acid to methane and carbon dioxide. Bacteria that reduce carbon dioxide, using hydrogen gas or formate also produce methane. The rate at which various substrates fed to a reactor are converted by the various groups of bacteria to biogas is controlled by the kinetics of each reaction.

The presence of other terminal electron acceptors will result in the production of alternative products e.g. sulphate and elementary sulphur will be converted to sulphide by sulphate reducing bacteria (SRB) and nitrate will be reduced to molecular nitrogen and nitrogen dioxide by denitrifying bacteria.

2.2.1 Methanogens

All living things are classified as either eukaryotes (cells contain a true nucleus) or prokaryotes (cells do not contain a nucleus), as illustrated in **Figure 2.2**. All microorganisms are classified as prokaryotes. Nucleotide sequence analysis of rRNA led to the discovery of one group of bacteria, so different from all other groups that a very clear division of the prokaryotes into two branches was assumed. This group is the archaea (formerly archaebacteria) and all other groups are collectively designated as the eubacteria.

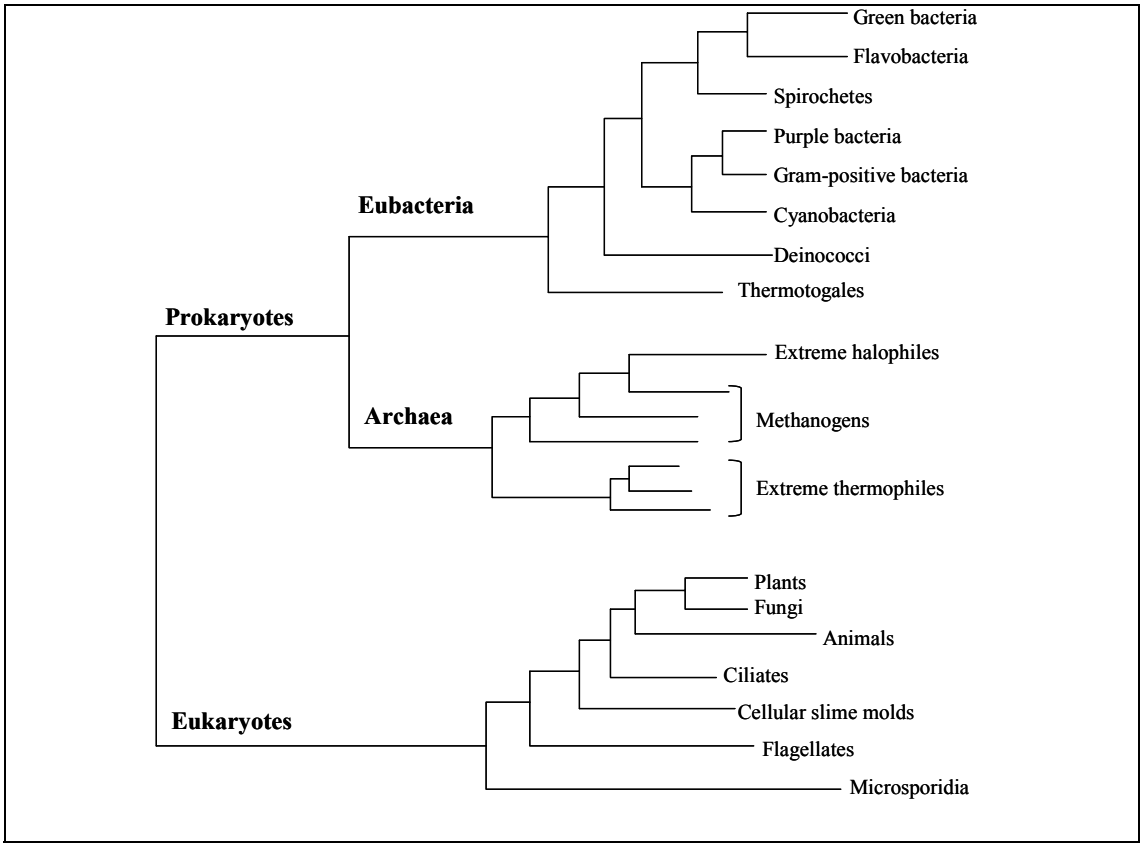


FIGURE 2.2 : Classification of living things.

The archaea differ in that the cell wall does not contain a peptidoglycan skeleton, only proteins and polysaccharides are present. The RNA polymerases and some of the co-enzymes of the archaea differ from those of the eubacteria. Methanogens are classified within the archaea domain. Even though great

advances in our understanding of the role of methanogens in anaerobic processes have been made, much needs to be learnt about microbial interactions in anaerobic systems (Raskin *et al*., 1994). The classification of the methanogens is given in **Table 2.1**.

It is generally accepted that two-thirds or more of the methane produced in an anaerobic bioreactor is derived from acetate. Of the many methanogenic genera, only two, *Methanosaeta* and *Methanosarcina,* are known to grow by an acetoclastic reaction, producing methane from acetate.

$$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^- \qquad [\Delta G^o = -31kJ/mol] \qquad [2.1]$$

Methanosaeta spp. are solely acetoclastic. *Methanosarcina* spp., however, are metabolically the most versatile of all the mesophilic methanogens since they can form methane from hydrogen and carbon dioxide (hydrogenotrophs), from methanol and methylamines (methylotrophs, and from acetate (acetoclasts) (Rocheleau *et al*., 1999).

The two genera have very different morphologies and growth kinetics. *Methanosaeta* spp. are sheathed rods, sometimes growing as long filaments; they are slow growing with minimum doubling times of 4 d under mesophilic conditions. Their survival is due to their high affinity for acetate (K_s of 30 mg/L at pH 7); these microorganisms are termed scavengers. In contrast, *Methanosarcina* spp. grow faster (minimum doubling time of 1.5 d) but have a poor affinity for acetate with a K_s of 400 mg/L at pH 7.

Figure 2.3 shows the growth kinetics of these two genera. It demonstrates that the *Methanosaeta* spp. would out-compete the *Methanosarcina* spp. at low substrate concentrations. However, with increasing substrate concentration, *Methanosarcina spp*. would dominate.

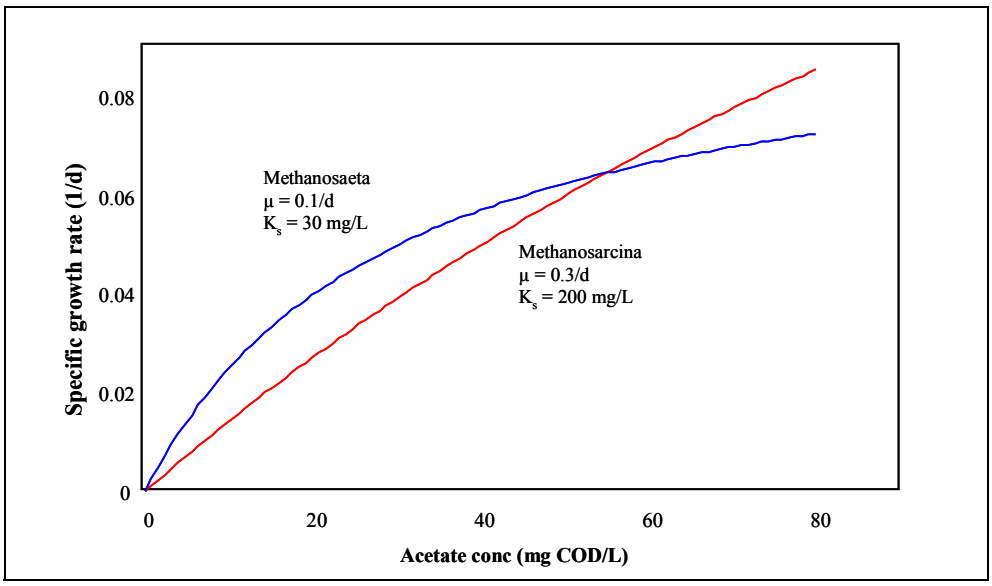


FIGURE 2.3 : Growth kinetics of the two acetoclastic genera, *Methanosaeta* and *Methanosarcina.*

When acetate levels are below 70 mg/L, *Methanosaeta* spp. will have a distinct competitive advantage over *Methanosarcina* spp. The higher activity of *Methanosarcina* spp. provides resistance to shock loadings, whilst the scavenging capacity of *Methanosaeta* spp. will result in an effluent low in COD and

relatively free of acid intermediates. However, in the absence of trace metals and nutrients, the filamentous growth of *Methanosaeta* will predominate even at acetate concentrations as high as 1 000 mg/L (Speece, 1996).

TABLE 2.1 : Classification of methanogens (Raskin *et al*., 1994).

Classification	Morphology	Substrates for methanogenesis
ORDER I: METHANOBACTERIALES		
Family I: Methanobacteriaceae		
Genus I: *Methanobacterium*	Long rods	$H_2 + CO_2$, formate
Genus II: *Methanobrevibacter*	Short rods	$H_2 + CO_2$, formate
Genus III: *Methanosphaera*	Cocci	Methanol + H_2 (both needed)
Family II: Methanothermaceae		
Genus I: *Methanothermus*	Rods	$H_2 + CO_2$
ORDER II: METHANOCOCCALES		
Family I: Methanococcaceae		
Genus I: *Methanococcus*	Irregular cocci	$H_2 + CO_2$, formate
ORDER III: METHANOMICROBIALES		
Family I: Methanomicrobiaceae (*MG1200*)		
Genus I: *Methanomicrobium*	Short rods	$H_2 + CO_2$, formate
Genus II: *Methanogenium*	Irregular cocci	$H_2 + CO_2$, formate
Genus III: *Methanoculleus*	Irregular cocci	$H_2 + CO_2$, alcohols, formate
Genus IV: *Methanospirillum*	Spirilla	$H_2 + CO_2$, formate
Family II: Methanocorpusculaceae (*MG1200*)		
Genus I: *Methanocorpusculum*	Irregular cocci	$H_2 + CO_2$, formate, alcohols
Family III: Methanoplanaceae (*MG1200*)		
Genus I: *Methanoplanus*	Plate-shaped cells	$H_2 + CO_2$, formate
Family IV: *Methanosarcinac*eae		
Genus I: *Methanosarcina* **(*M2821* and *MB4*)**	Large irregular cocci in packets	$H_2 + CO_2$, formate, methanol, methylamines, acetate
Genus II*: Methanolobus*	Irregular cocci in aggregates	Methanol, methylamines
Genus III: *Methanococcoides*	Irregular cocci	Methanol, methylamines
Genus IV: *Methanohalophilus*	Irregular cocci	Methanol, methylamines, methyl sulphides
Genus V: *Methanosaeta* **(*MX825* and *MS5*)**	Long rods to filaments	Acetate

The specific oligonucleotide probes used in this study are written, in brackets, alongside the target organisms. All of the other methanogens hybridised with the universal archaea probe, ARC915.

2.3 THE ANAEROBIC BAFFLED REACTOR

The successful application of anaerobic technology to the treatment of industrial wastewaters is dependent on the development of high rate anaerobic bioreactors. These reactors separate the hydraulic retention time (HRT) from the solids retention time (SRT), which allows the slowly growing microorganisms to remain within the reactor independent of the wastewater flow, thereby allowing application of significantly higher volumetric loading rates (Iza *et al*., 1991; Weiland and Rozzi, 1991). This is achieved by the incorporation of various techniques to immobilise the biomass within the reactor resulting in an increased reaction rate per unit volume of reactor, and subsequent reduction in reactor size. There is improved contact between the biomass and the wastewater; and biomass activity is enhanced due to adaptation (Iza *et al*., 1991).

2.3.1 Design of the Anaerobic Baffled Reactor

The ABR is similar in design and application to the up-flow anaerobic sludge blanket (UASB) but requires no special granule formation for its operation. Bachmann *et al*. (1983) developed the ABR, however, baffled reactor units had previously been used to generate methane-rich biogas as an energy source (Chynoweth *et al*., 1980). As reviewed by Barber and Stuckey (1999), the design of the ABR has undergone many modifications, as summarised in **Table 2.2**.

Table 2.2 : History of the modifications made to the ABR (Barber and Stuckey, 1999).

	Modification	Purpose	Reference
1	Addition of vertical baffles to a plug-flow reactor	Enhanced solids retention to facilitate better substrate accessibility to methanogens	(Fannin *et al*., 1981)
2	Down-flow chambers narrowed	Encouraged cell retention in up-flow chambers	(Bachmann *et al*., 1983)
3	Baffle edges slanted (45 °)	Encouraged mixing by routing the flow towards the centre of the compartment	(Bachmann *et al*., 1983)
4	Included a solids settling chamber after the final compartment	Enhanced solids retention and recycle of washed out solids	(Tilche and Yang, 1987)
5	Positioned packing at the top of each compartment	Prevented washout of solids	(Tilche and Yang, 1987)
6	Included separated gas chambers	Ease and control of gas measurement provided enhanced reactor stability	(Tilche and Yang, 1987)
7	Enlarged first compartment	Improved treatment of high solids wastewaters	(Boopathy and Sievers, 1991)

The laboratory-scale ABRs used in this study had alternately hanging and standing baffles, which divided the reactor into eight compartments (**Figure 2.4**). The reactors were made of Perspex, each with a volume

of 10 L (7.68 L working volume i.e. 0.96 L/compartment). The reactor dimensions were: length of 500 mm (366 mm working) x 118 mm width (98 mm working) x 275 mm height (25.5 mm working). Technical drawings of the reactor are presented in Barber (1999). The lower edges of the hanging baffles were slanted (45 °) to route the flow of liquid and to reduce channelling. The internal baffles were 5 mm thick. The reactor lid and sides were removable. Sample ports on the lid allowed for the removal of gas and liquid samples. There was a gas outlet from each compartment, while the liquid flowed from one compartment to the next through 20 mm high windows cut into the baffles below the level of the liquid. The liquid flow was alternately upward and downward between the partitions. The down-flow chamber was narrower than the up-flow chamber to prevent accumulation of biomass. On its upward passage, the waste flowed through the anaerobic biomass, hence, the waste was in contact with the active biomass but, because of the design, most of the biomass was retained within the reactor. In principle, all phases of the anaerobic degradation process can proceed simultaneously. The sludge in each compartment would differ depending on the specific environmental conditions prevailing and the compounds or intermediates to be degraded (Nachaiyasit and Stuckey, 1997a). A staged reactor can provide higher treatment efficiency since non-labile substrates will be in an environment conducive to degradation. Process stability is good.

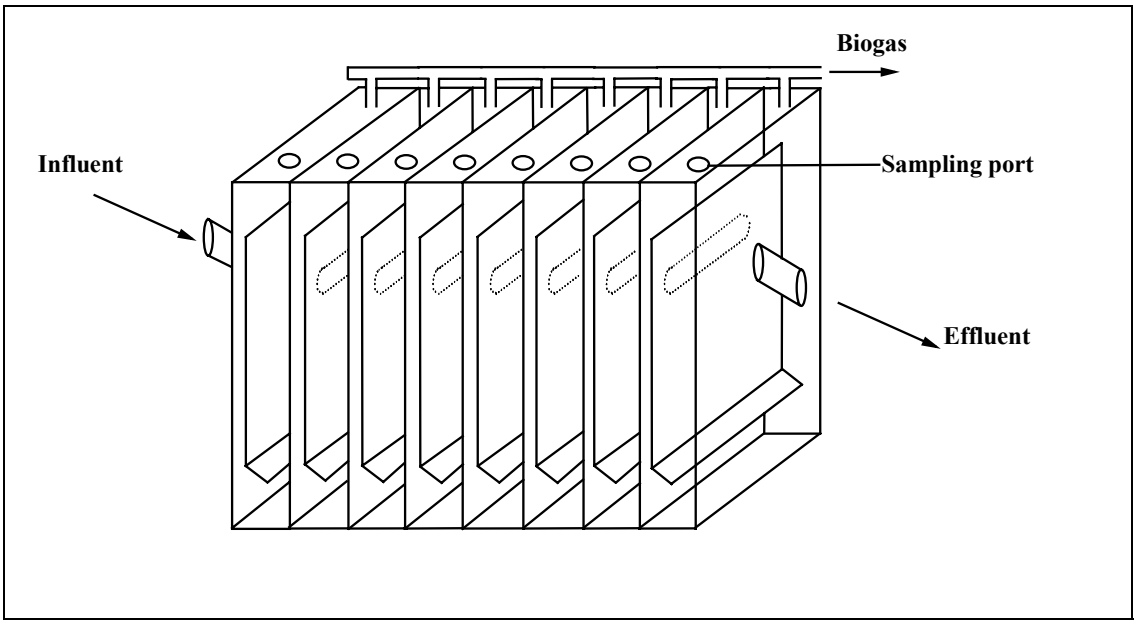


FIGURE 2.4 : Schematic diagram of the anaerobic baffled reactor.

The hydrodynamics and degree of mixing that occur within a reactor of this design strongly influence the extent of contact between substrate and microorganisms, thus controlling mass transfer and potential reactor performance. Microorganisms within the reactor gently rise and settle due to the flow characteristics and gas production, however, their rate of movement down the reactor is slow. The main driving force behind reactor design has been to enhance the solids retention capacity.

2.3.2 Advantages and Disadvantages of the Anaerobic Baffled Reactor

The ABR has several advantages over other high-rate reactor systems. Probably the most significant advantage is its ability to separate acidogenesis and methanogenesis longitudinally, allowing the reactor to behave as a two-phase system without the associated control problems and high costs.

The reactor design is simple, with no moving parts or mechanical mixing, making it relatively inexpensive to construct. There is no requirement for biomass with unusual settling properties. Sludge generation is low and the SRT is high; this is achieved without the need for biomass to be fixed to media particles or a solid-settling chamber. Gas separation is not required.

Since the HRT and SRT are separate, increased volumes of wastewater can be treated, relative to a continuously stirred tank reactor (CSTR) where the HRT equals the SRT. Intermittent operation is possible, which would facilitate treatment of seasonal wastewaters. The ABR has been found to be stable to hydraulic and organic shock loads and the reactor configuration provides protection of the biomass to toxic compounds in the influent (Barber and Stuckey, 1999).

The concentrated, variable and intermittent nature of industrial effluents make them intrinsically unsuitable for treatment in a completely mixed system. The ABR is well suited to intermittent high organic or hydraulic loads; due mainly to the high efficiency with which the active biomass is retained within the reactor. The high rates and efficiency with which the substrates are degraded is due to the compartmentalisation of different microbial associations that have been acclimated to the range of effluent constituents. The series of microbial associations allow acclimated bacteria to degrade the effluent stepwise producing degradation products that may be toxic or inhibitory to a mixed culture. These acclimated bacteria are retained within the reactor due to the high SRT.

One possible disadvantage of the baffled reactor design, at pilot or full-scale, is the requirement to build shallow reactors to maintain acceptable liquid and gas upflow (Barber and Stuckey, 1998).

2.3.3 Literature Review of the Anaerobic Baffled Reactor

One of the major problems associated with anaerobic treatment systems is the start-up procedure. The reduction of the duration of start-up and the improvement of process control are important factors in order to increase the competitiveness of an anaerobic high-rate reactor (Weiland and Rozzi, 1991). The overall objective of start-up is the development of the most appropriate microbial culture for the waste stream to be treated. Initial loading rates should be low so that the slow growing microorganisms are not over-loaded and both gas and liquid up-flow velocities should be low to facilitate flocculent and granular sludge growth. The recommended initial loading rate is ca. 1.2 kg COD/m^3.d (Speece, 1996; Henze *et al*., 1997). Barber and Stuckey (1997) showed that by starting with a long HRT (80 h) and gradually reducing it, in a stepwise fashion, whilst keeping the substrate concentration constant, greater reactor stability was maintained, with superior performance in comparison to a reactor started up with a constant and low HRT coupled to a stepwise increase in substrate concentration. This assessment was based on improved

solids accumulation, promotion of methanogenic populations and faster recovery to hydraulic shocks (Barber and Stuckey, 1997).

A recent modification of the ABR has been the development of the split fed ABR (Uyanik *et al*., 2001). To prevent the accumulation of VFAs during start-up, the feed can be split between the compartments which would result in a longer HRT, longer SRT in the initial compartments and greater availability of substrate for the microorganisms in the final compartments.

An advantage of the ABR is that it does not require granulation of the sludge (Barber and Stuckey, 1999). However, a study by Freese and Stuckey (2000) showed that when granular sludge was used to seed the ABR, there was a dramatic reduction in the overall start-up time of the reactor. Tilche and Yang (1987) first observed the granulation capacity of the ABR. Boopathy and Tilche (1992) also observed pelletisation of the biomass in a hybrid ABR treating a molasses wastewater. The long term stability of granules in an ABR has been shown to be strongly linked to the availability of nutrients (Freese and Stuckey, 2000).

The anaerobic digestion process can be simplified into two distinct phases, namely, acidogenesis and methanogenesis. The literature states that physical separation and operation of these two cultures should result in improved process efficiency. Phase separation can be achieved by selective inhibition, dialysis separation and kinetic control, by adjustment of dilution rates and cell mass recycling (Ghosh and Klass, 1978). The ABR has been described as a two stage process (Grobicki and Stuckey, 1991) where the design of the reactor results in the separation of acidogenesis and methanogenesis, through the compartments, and this has a direct effect on the reactor start-up. Two phase operation can increase both acidogenic and methanogenic activity by a factor of four (Cohen *et al*., 1982) and different microbial groups can develop under more favourable conditions. Advantages of staged operation include faster start-up, increased stability, enhanced efficiency and better conversion of suspended solids (Weiland and Rozzi, 1991; Alexiou and Anderson, 2001).

Several authors have treated low-strength wastewaters effectively in the ABR (Barber and Stuckey, 1999; Langenhoff and Stuckey, 2000). Dilute wastewaters inherently provide a low mass transfer driving force between the biomass and substrate, subsequently reducing biomass activities according to Monod kinetics. As a result, treatment of low-strength wastewaters has been found to encourage the dominance of scavenging microorganisms, such as *Methanosaeta* spp. (Polpraset *et al*., 1992). Biomass retention is enhanced significantly due to lower gas production rates, suggesting that low HRTs are feasible during low-strength treatment.

Whereas low retention times are possible for dilute wastewaters, the opposite applies when treating concentrated wastes. This is due to the high gas mixing caused by improved mass transfer between the biomass and substrate. The reactor configuration and the improved settling ability of the biomass have reduced solids loss caused by the increased gas production. Boopathy and Tilche (1991) investigated the treatment of a concentrated raw molasses wastewater in a hybrid ABR. A 77 % reduction in soluble COD was attained at an organic loading rate (OLR) of 20 kg COD/m^3.d. According to kinetic considerations,

high substrate concentrations will encourage both fast growing microorganisms and microorganisms with high K_s values. Methane production will be derived mainly from acetate decarboxylation by *Methanosarcina* spp. and hydrogen scavenging methanogens, such as *Methanobrevibacter* and *Methanobacterium*. Subsequently *Methanosarcina* spp. were observed as the dominant microbial species formed during high-strength treatment (Boopathy and Tilche, 1991). Treatment of other concentrated wastestreams, such as a slaughterhouse (Polpraset *et al*., 1992), swine (Sievers, 1988), and molasses (Xing *et al*., 1991) wastewaters has been investigated.

Recycling the ABR effluent stream tends to reduce removal efficiency because the reactor approaches a completely mixed system and therefore, the mass transfer driving force for substrate removal decreases despite a small increase in the loading rate. Mixing caused by recycle has also been found to cause a return to single phase digestion, therefore, the benefits arising from the separation of the acidogenic and methanogenic phases are lost (Barber and Stuckey, 1999). However, the addition of a recycle stream can alleviate the problems of low pH caused by high levels of volatile acids in the first compartments of the reactor (Chynoweth *et al*., 1980); and has the benefit of dilution of toxicants and reduction of substrate inhibition in the influent.

The ABR has been shown to tolerate hydraulic and organic shock loads. To a steady-state reactor, with an HRT of 20 h and an organic loading rate of 4.8 kg COD/m³.d, Grobicki (1989) introduced a hydraulic shock by decreasing the HRT to 1 h, for a period of 3 h. The reactor returned to its previous COD removal efficiency of > 95 % within 24 h of resuming normal operating conditions. Less than 15 % of the active biomass was lost. In a similar experiment, the organic loading rate was increased to 20 kg COD/m³.d and, under these conditions, a COD removal efficiency of 72 % was still achieved (Grobicki, 1989).

Nachaiyasit and Stuckey (1997) studied the operation of the ABR at low temperatures. Generally, biochemical reactions double in relative activity for every 10 C ° increase in temperature, however, these authors found no significant reduction in overall COD removal efficiency when the temperature of an ABR was dropped from 35 °C to 25 °C. Further reduction in temperature, to 15 °C, resulted in a 20 % decrease in COD removal. Changes in performance were gradual which is advantageous since this slow response would inherently provide improved protection to shocks, in comparison to other reactor systems. It was found that the production of refractory material increased substantially at the lower temperatures. Langenhoff and Stuckey (2000) operated a laboratory-scale ABR at an HRT of 10 h and feed concentration of 500 mg/L. The reactor temperature was gradually reduced from 35 °C, to 20 °C, and finally to 10 °C. The COD removal efficiency decreased with each reduction in temperature; from 95 % removal at 35 °C, to 70 % at 20 °C, and 60 % at 10 °C.

One of the main constituents of the effluent from a wastewater treatment plant is soluble microbial products (SMPs), which can be defined as compounds of microbial origin which result from substrate metabolism and biomass decay (Schiener *et al*., 1998; Aquino and Stuckey, 2001). In their investigation of SMP production in the ABR, Schiener *et al*. found that a decrease in HRT and an increase in the OLR both resulted in increased effluent SMPs. Quantitatively, between 26 and 48 % of the incoming feed

COD was converted to SMPs in the first compartment of the ABR. SMPs are categorised as (i) those that are produced at a rate proportional to the rate of substrate utilisation (utilisation-associated products, UAP); and (ii) those formed at a rate proportional to the concentration of active biomass (biomass-associated products, BAP) (Rittman *et al*., 1987).

Fox and Venkatasubbiah (1996) investigated the effects of sulphate reduction in the ABR by treating a sulphate containing pharmaceutical wastewater up to a final strength of 20 g COD/L with a COD:SO_4 ratio of 8:1. At steady-state, 50 % COD removal and 95 % sulphate reduction was possible with an HRT of 1 d. Reactor profiles showed that sulphate was almost completely reduced to sulphide within the first compartment and a concomitant increase in sulphide levels down the reactor indicated that sulphate redirected electron equivalents to hydrogen sulphide in preference to methane. After altering the COD:SO_4 ratio by adding glucose, isopropanol and sulphate, the authors noted a fall in potential sulphate reduction from > 95 % at COD:SO_4 = 150:1 to < 50 % at COD:SO_4 = 24:1. Increasing sulphate concentrations showed inhibition pf sulphate reduction due to elevated sulphide concentrations (Fox and Venkatasubbiah, 1996). Barber (1999) investigated two different COD:SO_4 ratios and found that a variation in the ratio altered the production and utilisation of intermediate products, ultimately affecting the reactor performance (Barber, 1999).

Barber (1999) showed that an aerobic polishing step can be inserted within an ABR, in the last or last two compartments, with no detrimental effect on reactor performance. This is due to the fact that the methanogenic archaea are well shielded from oxygen by the outer layers of facultative anaerobes, in immobilised aggregates. Therefore, processes which inherently require both anaerobic and aerobic treatment, e.g. dye wastewaters, could be treated within a single reactor unit.

In terms of modelling, several attempts have been made to predict reactor performance in the ABR and these have all been based on acetoclastic methanogenesis being the rate-limiting step for anaerobic degradation. Early work by Bachmann's group (1985) used a planar biofilm approach, which expanded the work of Williamson and McCarty (1976) by introducing the concept of a variable order coefficient first described by Rittman and McCarty (1978). After comparing two methods (deep film diffusion and completely mixed tanks in series) the authors concluded that treatment efficiency dropped with decreasing influent substrate concentrations, increase in organic loading with constant inlet concentration and an increase in recycle ratio at constant HRT. Performance improved with reductions in substrate concentration at constant HRT. However, at high loading rates the model performed badly and the authors hypothesised that this was due to the assumption of a constant diffusion depth, which in reality is reduced at high loading due to gas production.

Some of these findings were verified in the work of Xing *et al*. (1991) on the modelling of a hybrid baffled reactor. The model was based on results of ATP tests which showed that most of the active biomass was located at the bottom of each compartment, hence, biomass weight and not concentration was used in this model. These authors found that COD removal efficiency improved with increasing biomass weight until a certain concentration was reached, above which, reactor performance became independent of biomass concentration (Xing *et al*., 1991). A spherical model was derived by Nachaiyasit

(1995) and adequately described reactor performance at high loading rates. However, the model overestimated efficiency in the first compartments with low to medium loading rates.

To date, application of the ABR, on a full- or pilot-scale has been limited to the treatment of domestic wastewaters (Barber, 1999). The ABR shows promise for industrial wastewater treatment, on a full-scale, since it can withstand severe hydraulic and organic shocks, intermittent feeding, temperature changes, and tolerate certain toxic materials due to its inherent two-phase behaviour. Despite comparable performance with other well-established technologies, its future use will depend on exploiting its structure in order to treat wastewaters, which cannot be readily treated.

2.4 ASSESSMENT OF THE ANAEROBIC DIGESTION PROCESS

It is crucial that the performance of an anaerobic digester be monitored to prevent digester failure and to assess the efficiency of the digestion process. The anaerobic biodegradability and inherent toxicity of a particular waste stream should be assessed prior to loading to an anaerobic system.

2.4.1 Physical and Chemical Analyses

Physical and chemical analyses are used to monitor the performance of an anaerobic digestion process. The organic load to a digester has a significant effect on the process efficiency since if the organic content is too high, it may result in a shock load to the digester with a concomitant reduction in degradation efficiency or even digester failure (Sacks, 1997).

The efficiency of the degradation process can be assessed in terms of biogas production with an ideal system producing 1 m^3 biogas/ kg COD destroyed (Ross *et al.*, 1992). The gas composition is an important indicator of the state of the process. The ratio of carbon dioxide to methane should be in the region of 35 % to 65 %. A change in this ratio is indicative of stress in the system.

The COD represents the organic portion of the feed, thus the reduction in COD gives an indication of the degradability of the organic molecules in the substrate. A reduction of 50 to 70 % of the COD is expected in a properly functioning system.

A change in temperature can affect the metabolic rates.

Monitoring and control of the reactor pH, alkalinity and VFA concentrations is important to predict and prevent reactor failure, due to a build-up of VFAs in the system.

The mixing efficiency of a digester can be assessed by the temperature and total solids profiles throughout the digester. Efficient mixing is represented by a uniform distribution of temperature and solids.

2.4.2 Batch Screening Tests

Toxicity and inhibition refer to adverse effects on microbial metabolism (Speece, 1996). It is generally accepted that toxicity refers to irreversible damage to the biomass. Inhibition, however, is a more transient detrimental effect, from which the biomass recovers upon removal of the toxicant. These definitions will be adhered to throughout this thesis. For simplicity, the term toxicant has been used to refer to a substance causing either an inhibitory or toxic effect.

Toxicity monitoring is useful for determining, in advance, potential toxic or inhibitory effects of an industrial effluent. Source identification and control of toxicants is the most effective management strategy (Willetts, 1999). Anaerobic toxicity assays can be used to determine the IC_{50} value and thus quantitatively describe the inhibitory effect of a given compound on the anaerobic biomass. The method followed in this study was that described by Owen *et al*. (1979). Bioassay techniques for measuring the presence or absence of inhibitory substances are effective since they are simple to set up, several substances can be tested simultaneously, they are inexpensive, and do not require knowledge of specific inhibitory substances (Owen *et al*., 1979). The methanogenic activity is stimulated, at the start of the test, by the addition of an acetate-propionate solution. The methanogenic metabolism of this solution is monitored by total gas production, in the controls. Inhibition due to substrate addition is determined as a decreased rate of gas production, relative to the controls. The first data points are critical as these reflect the true, unadapted response of the microorganisms.

Laboratory-scale models attempt to simulate the conditions prevailing in the whole or part of the natural environment under study (Atlas and Bartha, 1993). Batch biodegradability assays can function as preliminary screening tests to assess the anaerobic degradability of a particular substrate. It is critical that these tests are conducted prior to operation of a continuous reactor in order to evaluate the efficiency of the degradation process and to assess volumes and concentrations of the substrate that can be treated effectively, i.e. without causing reactor failure.

2.5 MICROBIAL POPULATION DYNAMICS

The fundamental aspects of the anaerobic digestion process have been investigated, yet there is still the need for more basic information on the biological aspects of the anaerobic ecosystem (Godon *et al*., 1997). The complete identification and quantification of all contributing populations is necessary to establish the link between microbial structure and function. This lack of knowledge is due to the limitations of traditional identification and enumeration techniques, such as selective enrichment, pure culture isolation and most probable number estimates. It has frequently been reported that direct microscopic counts exceed viable cell counts by several orders of magnitude; the majority of microscopically visualised cells are viable but do not form visible colonies on plates. Two different types of cells contribute to this: (i) known species for which the applied cultivation conditions are not suitable or which have entered a non-culturable state and (ii) unknown species that had not been cultured before for lack of suitable methods (Amann *et al*., 1995a). It has been estimated that > 99 % of microorganisms observable in nature typically are not cultivated by standard techniques (Hugenholtz *et al*., 1998). These

problems are exacerbated in studies of fastidious anaerobes; because of their low growth rates and obligate anaerobiosis, methanogens are among the microorganisms that are most difficult to study by culture-based techniques.

Molecular-based methods, such as ribosomal RNA (rRNA) probe hybridisation, allow the direct identification and enumeration of microbial populations in complex environments (Griffin *et al*., 1998). These techniques can provide a clearer insight into the interactions, concentrations and growth rates of the various trophic groups involved in anaerobic digestion. Molecular techniques by themselves can provide useful qualitative and quantitative information on the microbial populations present in wastewater treatment plants. However, in isolation, they are simply a tool that can complement other methods and approaches for analysing wastewater treatment systems. The combined use of traditional culture-based and microscopic techniques, chemical analyses, and molecular techniques should serve to better link microbial structure and function.

2.5.1 Ribosomal RNA

An actively growing microbial cell contains up to 10^4 ribosomes, the sites of protein synthesis (Schlegel, 1992). The number of ribosomes within a cell and hence the number of rRNA molecules, is proportional to the growth rate of the cell. Bacterial ribosomes are comprised of a mixture of nucleic acids (rRNA) and proteins and have an average size of 70S (Svedburg units – based on settling). The small subunit rRNA is the 16S rRNA and the genes that code for this are 16S rDNAs. Additionally, ribosomes contain the larger 23S rRNA. An average bacterial 16S rRNA molecule has a length of 1 500 nucleotides, and 23S rRNA molecules are approximately 3 000 nucleotides (Amann *et al*., 1995a).

Ribosomal RNA remains relatively constant in structure because its essential function dictates that it must be resistant to evolutionary change. It comprises a mosaic of regions of highly conserved and highly variable regions of sequence. These properties make rRNA molecules useful for inferring phylogenetic relationships. Essential rRNA sequence domains are conserved across all phylogenetic lineages thus universal tracts of sequence can be identified. Species and subspecies specific sequences have also been identified (Griffin *et al*., 1998).

2.5.2 Fluorescent *in situ* Hybridisation (FISH)

Whole cell hybridisation is the technique that allows for the *in situ* identification and enumeration of microorganisms containing a certain rRNA sequence. It is termed fluorescent *in situ* hybridisation (FISH) when fluorescent-labelled oligonucleotide probes, targeted at specific signature sequences in rRNA, are used (Rocheleau *et al*., 1999). This allows cells to be detected in their natural microhabitat. The cell morphology of an uncultured microorganism and its abundance can be determined as well as the spatial distributions *in situ*. Quantification of the signal conferred by the probes can estimate the *in situ* growth rates of individual cells (Amann *et al*., 1995a).

Oligonucleotides (short strands of nucleic acids; usually 15-30 nucleotides in length), complementary to the 16S rRNA sequence regions with an intermediate degree of conservation and characteristic for phylogenetic entities like genera, families and subclasses, have been successfully used for the rapid identification of microorganisms. The oligonucleotides, or probes, are able to enter fixed bacterial cells and once inside the cells, they may form stable associations (hybrids via hydrogen bonding between complementary nucleotides) with the 16S rRNA in the ribosomes. The microorganisms are made permeable by fixation with paraformaldehyde. Fixation is essential for maintaining the morphological integrity of the cell; the paraformaldehyde cross-links the proteins and nucleic acids, thereby immobilising the cellular structures. If the complementary sequence for the probe is not present in the 16S rRNA in the ribosome, hybridisation does not occur and the probe is washed from the cell. In order to observe when hybridisation occurs, the probes are labelled with a fluorescent marker. Currently the standard probes are labelled with a single fluorescent dye marker attached to the 5' end via a linker molecule. The most frequently used dyes are fluoroscein (excitation wavelength, 490 nm; emission wavelength, 520 nm) and tetramethylrhodamine (550 nm, 575 nm). Probe specificities and sensitivities are strongly dependent on the exact hybridisation conditions. Parameters such as hybridisation and washing temperatures, concentrations of monovalent cations and denaturing agents e.g. formamide, have to be optimised (Amann, 1995b).

Cells, in which the fluorescently labelled oligonucleotide has hybridised, with the 16S rRNA in the ribosome, can be visualised directly by epifluorescent microscopy.

2.5.3 Epifluorescence Microscopy

Fluorochromes, such as fluoroscein and rhodamine, are excited by particular wavelengths of light and generate secondary emitted wavelengths, which are detected as an image of a fluorescing object. The excitation process generally requires light of short wavelengths in the near-UV or blue range. The lamp to generate such light is a high-pressure mercury vapour arc lamp. The lenses are made of quartz. The light source and the arrangement of specific filters in the light path are important. The filters vary depending on the type of fluorochrome being detected. In epifluorescence, the objective acts as both the objective and the condenser. There is no direct light beam from the mercury vapour arc lamp to the eye of the operator. Instead, the excitor beam is reflected to the objective from a rear port by a beam-splitting mirror that reflects the exciting wavelength but transmits visible light back from the objective, through the eyepieces. The filters are between the lamp and the specimen. The filters pass wavelengths of light required for excitation and adsorb most other wavelengths. The confocal laser microscope can be used to evaluate the spatial configuration of microbial granules or flocs.

The use of whole cell hybridisation provides a basis to estimate the *in situ* growth rates of species in natural populations, since the cellular ribosome content and, consequently, the rRNA concentration vary with the growth rate (this would be detected by changes in the strength of the fluorescent signal). Thus, an advantage of using FISH is the detection of metabolically active cells, so that descriptions of the physiologically important population members can be obtained.

FISH is an ideal technique for screening *in situ* samples. Probes can be applied to parallel sub-samples in an ordered top-to-bottom approach, initially using universal and domain-specific probes followed by probes of narrower specificity, such that increasingly refined information on the community diversity and composition can be obtained very rapidly. In this approach, the information gained from the higher-level probes can be used to select the probe sets tailored for the next lower taxonomic levels. Apart from initial screening, FISH can also be used to follow dynamic changes in populations, in response to changes in conditions. Thus, coupled with standard chemical and biochemical analyses, this strategy should enable the identification of the most sensitive members of the communities following initial responses and subsequent acclimation.

Genetic analysis of 16S rRNA gene sequences extracted directly from microbial ecosystems (cloning) provides the means to accurately identify population members for which specific nucleic acid probes do not exist, and provides additional information for the development of new probes.

Chapter 3

Food Dyes in the ABR

This chapter reviews the literature on dye wastewaters and dye chemistry (**Section 3.1**). The focus of the chapter is food dyes, particularly the ability of the ABR to decolourise and degrade food dye wastewaters, together with investigation of the associated microbial populations, using molecular techniques. A laboratory-scale ABR was operated with a synthetic tartrazine feed (**Section 3.2**). Chemical analyses of the operation of this reactor indicated efficient treatment of the wastewater, however, the FISH experiments were unsuccessful due to interference of the tartrazine with the fluorescent probes. The concept of batch screening tests to assess the anaerobic toxicity and biodegradability of each of the dye compounds is introduced in **Section 3.3** and the applicability of these bioassays to the ABR is discussed. **Section 3.4** describes the experiment in which a real wastewater, sampled from a food dye manufacturer, was treated in a laboratory-scale ABR. The chemical analyses together with the successful characterisation of the changing microbial communities, using molecular techniques, provided a detailed description of the processes occurring within the reactor, however, the results were still difficult to interpret because of the complexity and variability of the dye wastewater.

3.1 DYE WASTEWATERS

Dye wastewaters enter the environment from dye manufacturers and dye consumers e.g. textile, leather and food industries (Cooper, 1995). The majority of dyes are xenobiotic chemicals, substances having structural features that are not normally encountered in nature. With modern methods of manufacture and use, dyestuffs do not enter the environment in major quantities, but losses are inevitable and it is important to consider to what extent such products may persist (Brown and Laboureur, 1983). It has been stated that treatments that minimise water and energy consumption will be required, for the economic survival of dyeing and finishing companies (Holme, 1997).

Dyes and pigments are usually released into the environment in the form of a dispersion or a true solution in the industrial effluent (Seshadri *et al*., 1994). The presence of very small amounts of dyes in water (< 1 mg/L) is highly visible and aesthetically unpleasant. The predicted environmental dye concentration (PEC) averages ca. 1 mg/L, but can be higher since batch dyeing is common practice. The PEC is calculated from the daily usage of the dye; the degree of fixation; the degree of removal in the effluent treatment plant; and the dilution factor in the receiving water (Cooper, 1995).

3.1.1 General Dye Chemistry

Dyes are generally small molecules comprising two key components: the chromophore, responsible for the colour, and the functional groups that bond the dye to the fibre (Correia *et al*., 1994). The vast

number of dyes available commercially required a form of classification, based on their chemical structure or in terms of their application to the fibre type. The Colour Index (Society of Dyers and Colourists and American Association of Textile Chemists and Colourists) is the internationally accepted classification system for dyes.

Azo dyes are water-soluble, synthetic organic colourants possessing the characteristic -N=N- (azo) bond and showing great structural diversity. Approximately one-half of all known dyes are azo dyes, making them the largest group of synthetic colourants (Bumpus, 1995). Generally azo dyes contain between one and three azo linkages, linking phenyl and/or naphthyl rings that are usually substituted with some combination of functional groups including: amino, chloro, hydroxy, methyl, nitro and sulphonate groups (Razo-Flores *et al*., 1997). Azo dyes can be used to colour many different substrates, such as synthetic and natural textile fibres, plastics, leather, paper, mineral oils, waxes, foodstuffs and cosmetics.

The attachment of an **acid dye** to a substrate is dependent on the presence of one or more acidic groups. **Basic dyes** form ionic bonds with acid or anionic groups on the substrate. They have a basic amino group which becomes protonated under acidic conditions. **Direct dyes** have sulphuric acid groups but are not acid dyes because these groups are not used for attachment. The dye molecules are large, flat, linear molecules and NaCl is used to enhance the dyeing process by the sodium ions promoting equilibrium between the dye and the substrate. The dyeing process is reversible and used because it is economical and easy to apply. **Sulphur dyes** are insoluble dyes, which need to be reduced with sodium sulphide, to transform them to a soluble form, which adsorbs to the substrate. Upon exposure to air, the dyes become oxidised to form the original insoluble form, which is now present inside the substrate and, therefore, resistant to removal by washing (Maynard, 1983). **Reactive dyes** form covalent bonds with hydroxyl or amino groups on the substrate. They have excellent fastness because the dye becomes a part of the substrate. **Disperse dyes** are non-ionic dyes with a low water solubility. Colouring is due to the formation of a solid solution in the substrate.

3.1.2 Discharge Standards and Treatment Options

Public perception of water quality is influenced by colour; unnatural colour is associated with contamination. Strong colours can reduce light penetration, thus affecting the growth of plants and the aquatic ecosystem. The current environmental concern with azo dyes revolves around the potential carcinogenic health risk that they, or their intermediate biodegradation products, present when exposed to microflora in the human digestive tract (Seshadri *et al*., 1994). Azo dyes are intentionally designed to be recalcitrant under typical usage conditions (Laing, 1991), and it is this property, allied with their toxicity to microorganisms, that makes biological treatment difficult. There is the potential for these dyes to build up in the environment since many of them pass through wastewater treatment plants virtually untreated.

In the setting of discharge standards, dye concentration is not used as a measuring parameter since different dyes have different intensities and hues. Colour standards are, therefore, based on absorbance. Samples are filtered (0.45 μm) and the absorbance read between 400 and 700 nm. In the United Kingdom, the following colour standards are set for discharge to sewer (**Table 3.1**):

TABLE 3.1: United Kingdom colour standards for effluent discharge to sewer (Cooper, 1995).

Wavelength (nm)	Absorbance
400	0.060
450	0.040
500	0.035
550	0.025
600	0.025
650	0.015

In South Africa, the General Standard requires that the effluent be free of colour.

Several chemical and physical decolourisation methods are available, such as coagulation/flocculation and precipitation; oxidation treatment with chlorine (sodium hypochlorite), chlorine dioxide, hydrogen peroxide (Fenton's oxidation) or ozone; adsorption with activated carbon (GAC or PAC), clays or bioadsorbants; electrolysis; and membrane extraction (Grau, 1991; Buckley, 1992; Cooper, 1995; Banat *et al*., 1996; Dubrow *et al*., 1996; Liakou *et al*., 1997) However, these methods are costly, so the move has been toward biological decolourisation of wastewaters. It must be noted that, generally, no one specific treatment process can completely decolourise an industrial wastewater. In this study anaerobic pre-treatment in the ABR, followed by conventional wastewater treatment, is suggested.

Azo dyes are used in a wide variety of products and can be found in the effluent of most sewage treatment facilities. Once discharged into the environment, the fate of these dyes is uncertain and dependent on many unknown factors. Azo dyes are resistant to aerobic degradation (Chung and Stevens, 1993). The strong electron withdrawing character of the azo group stabilises these aromatic pollutants against conversion by oxygenases (Razo-Flores *et al*., 1997). Seshadri *et al*. (1994) described an experiment in which the partitioning of 18 azo dyes in an activated sludge process showed that most of the dyes passed through the system largely unchanged, four dyes were significantly adsorbed onto the mixed liquor solids (bioelimination), and three dyes were apparently degraded. Oxygen is often an inhibitor of azo reduction. A possible explanation for the effect of oxygen on azo reduction is rate competition in the oxidation of reduced electron carriers by either oxygen or azo compounds as oxidants. In the absence of oxygen, azo compounds will be utilised as terminal electron acceptors in microbial respiration, and are reduced and decolourised concurrently with re-oxidation of reduced flavin nucleotides. In the presence of oxygen, however, the reduced flavin nucleotides will be competitively oxidized and the rate of azo reduction concomitantly slowed (Chung and Stevens, 1993).

The initial step in the degradation of azo dyes involves the cleavage of the azo bond. This has been achieved under anaerobic conditions (Brown and Hamburger, 1987):

$$\left(R_1 - N = N - R_2\right) + 4e + 4H^+ \rightarrow R_1 - NH_2 + R_2 - NH_2 \qquad [3.1]$$

where R_1 and R_2 are various phenyl and naphthol residues. The anaerobic azo reductases are non-specific (Bumpus, 1995). The resultant aromatic amines are generally more basic than the azo compounds, thus the pH of the reactor may increase after cleavage of the azo linkages (Knapp and Newby, 1995). Azo compounds are not decolourised until all nitrite and nitrate has been denitrified (Chung and Stevens, 1993). Further degradation of the intermediates, which are recalcitrant under anaerobic conditions, is readily achieved under aerobic conditions; thus suggesting sequenced anaerobic/aerobic treatment system for the total degradation of azo dyes (Glässer *et al*., 1992; FitzGerald and Bishop, 1995; Field *et al*., 1995; Dubrow *et al*., 1996). Tan (2001) evaluated the mineralization of three azo dyes (Mordant Orange 1, 4-Phenylazophenol, and Mordant Yellow 10) under integrated and sequential anaerobic/aerobic conditions. Increasing oxygen concentrations showed decreasing azo dye reduction with ethanol as the co-substrate. These rates were higher when acetate was used as a co-substrate. All aromatic amines were removed if sufficient oxygen was present; oxygen was primarily used to oxidise the co-substrate and, if sufficient oxygen was available, the formed aromatic amines were further degraded. Thus, it was concluded that degradation of azo dyes is possible under integrated anaerobic/aerobic conditions of co-substrate and oxygen are in balance.

The potential accumulation of azo dyes in the environment was viewed with concern and resulted in research sponsored by the American Dye Manufacturers Institute (ADMI) and the Ecological and Toxicological Association of the Dyestuffs Manufacturing Industry (ETAD) (Brown and Laboureur, 1983). Several of these studies showed that the toxicity of commercial dyes to fish and mammals was minimal. Bumpus (1995) reviewed these tests: only ca. 1 % of all the dyes tested (4 461 dyes of all classes) had an $LD_{50} < 250$ mg/kg while 7 % had an LD_{50} between 250 and 2 000 mg/kg, 9.7 % had an LD_{50} between 2 000 and 5 000 mg/kg and 82.2 % had an $LD_{50} > 5\,000$ mg/kg. It was concluded that commercial dyes, with the exception of benzidine dyes and the triphenylmethane type of cationic dyes, were generally not hazardous chemicals (Chung and Stevens, 1993). However, azo reduction of a large number of azo dyes results in the formation of benzidine and benzidine derivatives. Ingestion of these derivatives has been shown to cause bladder cancers and reduced fertility in rodents (Bumpus, 1995).

Investigations have shown that the addition of an additional carbon source, such as glucose or a VFA mixture enhances anaerobic decolourisation (Carliell *et al*., 1995). The carbon source functions as a donor of reduction equivalents, and thus enhances the cleavage of the azo linkage. The addition also results in more actively respiring cells which deplete any oxygen present and thereby enhance the azo reductase activity (Haug *et al*., 1991). However, Razo-Flores *et al*. (1997) found that a pharmaceutical azo dye, azodisalicylate, was completely decolourised and mineralised to methane without an additional carbon source, at dye loading rates of up to 225 mg/L. These results indicated that some azo dyes can be degraded in anaerobic environments, in contrast to the common assumption that they are only biotransformed to mutagenic and carcinogenic aromatic amines.

The primary rate-limiting step in the degradation of azo dyes is the rate of permeation of the dye through the cell membrane since azo reduction must occur intracellularly (Haug *et al*., 1991; Carliell *et al*., 1995). Dye permeability has been shown to be a function of the adsorption-desorption equilibrium of the dye at

the cell membrane and the food:microorganism ratio. Older cells with a reduced supplemental nutrient supply have been shown to have better degradative capabilities. There is increased azo reductase efficiency with damaged cells. Sulphonic acid substitution of the azo dye structure has been shown to block effective dye permeation (Chung and Stevens, 1993).

3.1.3 Food Dyes

Azo dyes are widely used as colourants in foods such as sweets, soft drinks, hot dogs, ice cream and cereals (Chung *et al*., 1978). The extent of use is related to the degree of industrialisation of the society. Since intestinal cancer is more common in highly industrialised societies, a possible connection between these tumours and the use of azo dyes has been investigated (Chung *et al*., 1978). Several carcinogenic aromatic amines have been identified in food dyes (Prival *et al*., 1993). There is increasing legislative control of the dyes used in foodstuffs. Use is restricted to colourants that have not shown any harmful effects when subjected to rigorous examination.

Freedom from toxicity is the first consideration in the choice of colourants for foods (Society of Dyers and Colourists and American Association of Textile Chemists and Colourists). After that, the properties commonly required are high solubility in water, alcoholic solvents, edible oils etc.; freedom from reaction with other components of the foodstuff e.g. flavourings and preservatives; freedom from attack by bacteria; stability to light and heat; and an aesthetically acceptable hue.

3.2 TREATMENT OF CI FOOD YELLOW 4 IN THE ANAEROBIC BAFFLED REACTOR

CI Food Yellow 4, or Tartrazine, is a monoazo synthetic organic colourant (**Figure 3.1**) with a molecular mass of 534.38 g/mole. Maximum absorption of the dye is at a wavelength of 430 nm. Tartrazine is a universally used lemon yellow colourant with excellent stability for all foodstuffs. In 1979, because of allergic reactions produced in humans, the identification of tartrazine, if contained in food and drugs, was required by name on the label. In 1987 tartrazine was certified by the FDA for use in food and beverages. At this time the dye was the third most commonly used food colourant (Collins *et al*., 1990).

The anaerobic degradation of tartrazine by intestinal microbes, particularly *Proteus vulgaris* has been studied extensively (Chung *et al*., 1978). It has been found that tartrazine is not readily degraded under anaerobic conditions (Haug *et al*., 1991). According to toxicological data, tartrazine is mildly toxic by ingestion, with a death concentration of 14 µg/kg. Ingested in high concentrations, it may cause peripheral nervous system effects and musculo-skeletal effects.

Tartrazine was chosen for investigation in this study because it accounted for ca. 50 % of the production of a food dye manufacturing company. It was, therefore, assumed that tartrazine dye and its precursors would constitute a significant proportion of the final trade effluent. From discussions with the plant managers, it was ascertained that the tartrazine waste streams could be segregated on site, therefore, there

was the potential for anaerobic treatment of the tartrazine waste alone, if treatment of the total effluent did not prove efficient.

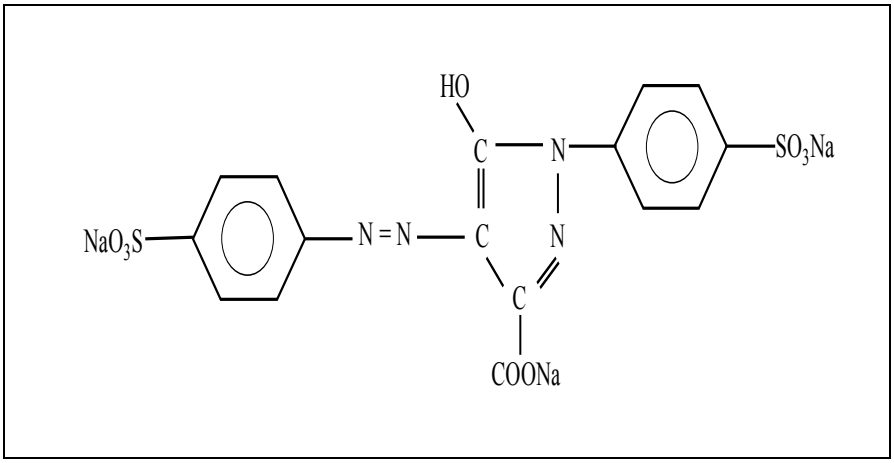


FIGURE 3.1: Chemical structure of Tartrazine (CI Food Yellow 4).

Results of batch anaerobic toxicity assays (**Appendix 3**) showed that tartrazine was not inhibitory to the methanogenic biomass, with an IC_{50} concentration of 14.3 g/L. In the biodegradability assays (**Appendix 3**) methanogenic utilisation of the dye was observed (0.8 %). Biogas production was greater than in the controls, suggesting metabolism of the dye by other anaerobic microbial populations, also resulting in the reduction of COD and colour.

These results suggest the potential for anaerobic degradation of the tartrazine molecule. This may require acclimation of the biomass, or selection for particular populations, since tartrazine is not readily degraded. Problems may also be encountered with colour reduction and colour change.

3.2.1 Hypothesis and Objectives

It was hypothesised that anaerobic digestion could reduce the COD and colour of the tartrazine waste stream; and that the design and structure of the ABR would prevent inhibition of the anaerobic biomass (by the xenobiotic nature of the waste stream, the variable flow and load), and allow for more efficient degradation and decolourisation at a low (20 h) HRT.

It was also hypothesised that fluorescent *in situ* hybridisation of the microbial communities that develop in the ABR compartments, during treatment of the tartrazine waste stream, would provide improved knowledge of the biochemical pathways and the microorganisms involved in the decolourisation.

The specific objectives of this investigation were to:

1. Determine whether adsorption to the anaerobic biomass played a significant role in the decolourisation of the waste stream.

2. Assess the feasibility of the ABR for treatment of a tartrazine waste stream; including reduction of COD and decolourisation.

3. Determine whether the anaerobic biomass became acclimated to the dye, thereby improving degradation and decolourisation, with time.

4. Use 16S rRNA oligonucleotide probes to characterise the microbial populations within each compartment, and the dynamics of these populations during treatment of the tartrazine waste stream.

3.2.2 Physical Decolourisation

Dye permeability through the cell membrane has been shown to be a rate-limiting factor in the biological treatment of dyes. Decolourisation of a wastewater, in a biological treatment system, may be attributed to adsorption of the dye to the anaerobic biomass, and not entirely to degradation or breakdown of the dye molecules. Dyes can be adsorbed by the biomass to the extent of 40 to 80 % (Laing, 1991). Structural factors influence adsorption. It is assumed that a saturation point would be reached where the dye could no longer adsorb to the biomass. A test was conducted to determine the extent of adsorption of tartrazine to the digester sludge since this could contribute to the decolourisation potential in the ABR.

Anaerobic digester sludge was inactivated by autoclaving at 110 °C for 80 min. Once the sludge had cooled it was aliquoted into a series of serum bottles. The TS of the inoculum sludge was measured (26.3 mg/L). Five serum bottles were set up with 10, 20, 50, 75 and 90 mL autoclaved sludge, respectively. The working volume was 100 mL, in each bottle. The tartrazine dye stock solution was diluted to the required volume. The same dye concentration (250 mg/L) was added to each bottle. A control was set up for each bottle, containing the same amount of sludge, with no dye. The function of the controls was to evaluate the background absorbance of the sludge. The bottles were sealed and incubated in a waterbath, at a constant temperature of 35 °C. The absorbance for each was measured (430 nm) at the start of the test and then periodically thereafter, for a period of 6 days.

The bottle contents were well mixed, prior to sampling. Samples (1.5 mL) were withdrawn by syringe, through the rubber septa. The samples were sealed in Eppendorf tubes, centrifuged (13 000 rpm) for 5 min and the supernatant liquor filtered through PVDF filters (0.45 μm). The supernatant was withdrawn (1 mL) and diluted 1 in 10 with distilled water. The absorbance was read at 430 nm. The background absorbance (control) was subtracted from the absorbance measured in the dye samples to give the absorbance of the dye alone. The results were plotted (**Figure 3.2**).

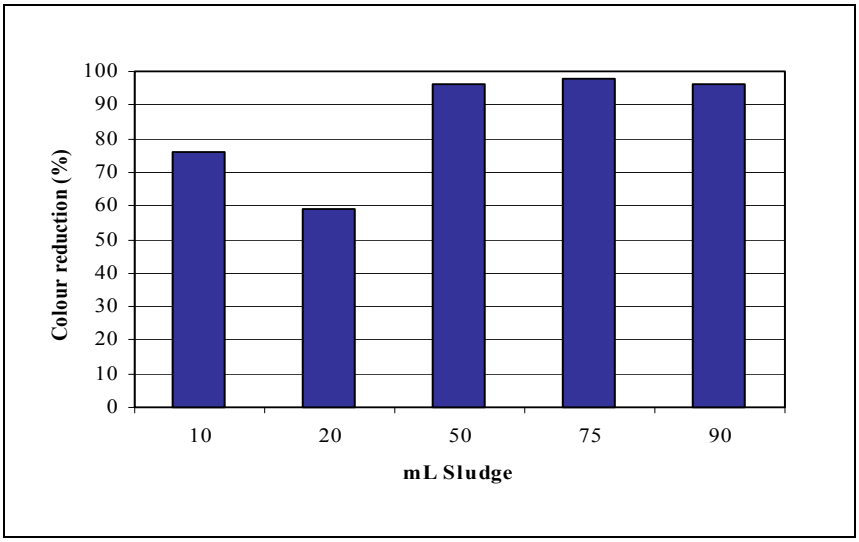


FIGURE 3.2: Tartrazine decolourisation due to adsorption to increasing volumes of inactivated anaerobic sludge.

The biomass was autoclaved so as to inactivate the cells, thus, any observed decolourisation was due to physical and not biological mechanisms. The bottles were inoculated with increasing volumes of sludge to test for increased decolourisation with increased sludge volume, and therefore, increased surface area for adsorption.

The results did not follow the trend expected, i.e. increased decolourisation with increased sludge volume. Colour reduction between 90 and 100 % was achieved in the bottles containing 50, 75 and 90 mL of sludge (**Figure 3.2**). Colour reduction was lower in the bottles with 20 mL sludge than in those with 10 mL sludge. The reason for this is unknown. The decolourisation in these bottles was due to adsorption to the biomass but the results may not be completely representative since the autoclaving may have increased the surface area available for adsorption by rupturing the cells. It is also unknown whether all of the biomass was inactivated by the autoclaving, therefore, some of the decolourisation may have been due to degradation or breakdown of the dye.

The initial dye concentration added to each bottle was 250 mg/L. The tartrazine concentration in each serum bottle, at the end of the test period, was calculated from the final measured absorbance and the tartrazine calibration curve (**Appendix 2**). A plot of dye removal as a function of TS is shown.

From **Figure 3.3** it can be seen that the amount of decolourisation due to adsorption to biomass was a logarithmic (saturation) response. From the equation of the line it can be estimated that 22 mg tartrazine in solution is adsorbed per mg of total solids. From this experiment it can be concluded that adsorption does play a role in the decolourisation of the dye.

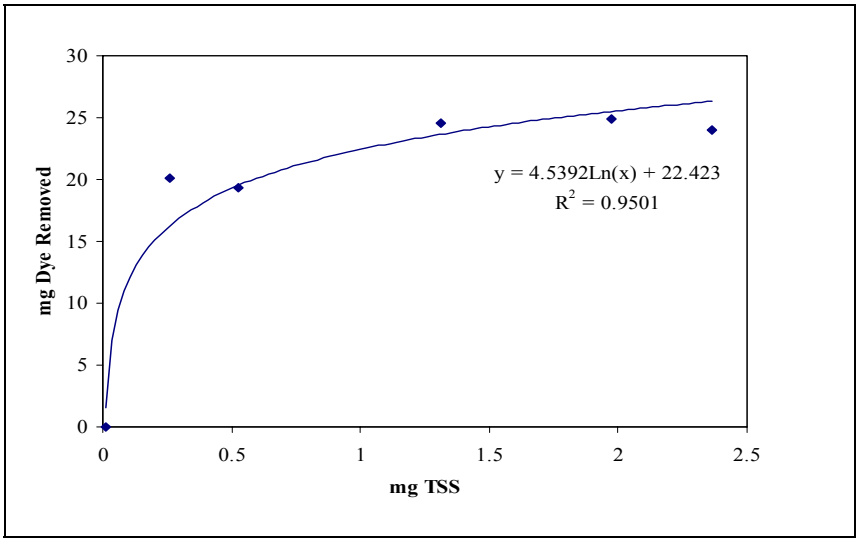


FIGURE 3.3: Plot of Tartrazine adsorption on inactivated biomass.

To verify these results and because of the uncertainty of the inactivation of the autoclaved sludge, a separate test was run, in which 800 mL of anaerobic sludge (TS = 15.4 g/L, VS = 10 g/L) was added to each of two 1 L glass bottles. To one bottle, sodium azide, which is an inhibitor of metabolic activity, was added to give a final concentration of 100 mM. Both bottles were sealed with an anaerobic headspace gas, were mixed thoroughly, and left to equilibrate for 1 h at 35 °C. After equilibration, 200 mL of 500 mg/L tartrazine was added to each bottle, to give a final tartrazine concentration of 100 mg/L. The bottles were shaken thoroughly and sampled (2 mL). Samples were collected from each bottle after 1; 2; 3; 4; 20 and 24 h, for analysis. Immediately after their collection, the samples were centrifuged (13 000 rpm) for 5 min and the supernatants filtered through PVDF filters (0.45 µm). The resulting samples were diluted 1 in 5 with distilled water and then analysed using a spectrophotometer at 430 nm to determine tartrazine concentration. As a control experiment, for examining any potential interaction between sodium azide and tartrazine, these two compounds were mixed at the same concentrations as above, without any biomass, and sampled accordingly.

The results are shown in **Figure 3.4**. In the control experiment, where sodium azide and tartrazine were mixed in the absence of biomass, no decrease in the concentration of tartrazine, compared with the starting solution, was observed after 20 h. Gas production in each bottle, measure qualitatively over time, showed that the biomass amended with sodium azide produced a negligible amount of gas compared with the live biomass bottle, indicating inhibition of metabolic activity.

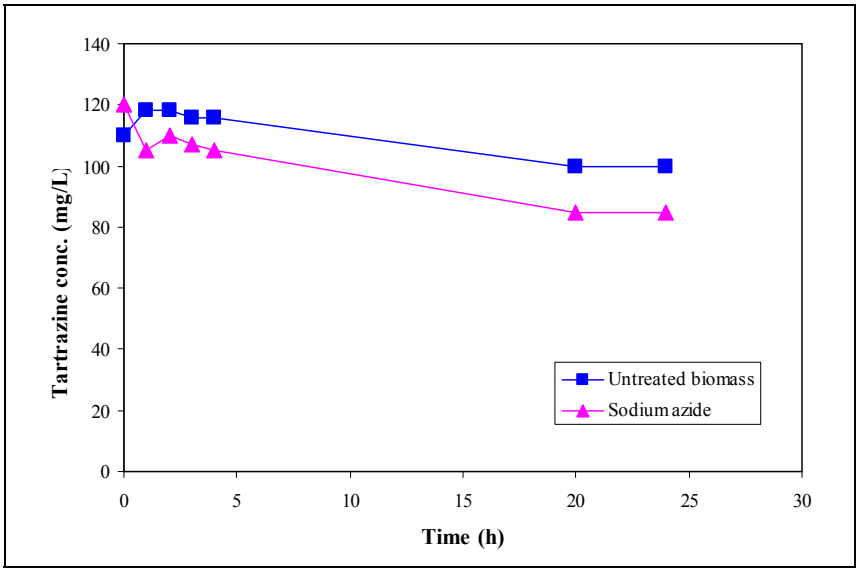


FIGURE 3.4: Plot showing tartrazine concentration measured over time for the live biomass assay and the sodium azide inactivated biomass assay.

The data in **Figure 3.4** shows a slight decrease in tartrazine concentration over time. Assuming that the background absorbance contributed by the biomass was the same for each, and is ignored, the concentration of tartrazine decreased from 119.5 to 98 mg/L and 117.5 to 87 mg/L for the live and inactivated biomass treatments, respectively. Over the first 4 h of the experiment, however, the concentration of tartrazine did not change appreciably compared to the measurements taken after 20 h. Based on this observation, it would appear that adsorption of tartrazine to the biomass did not occur to a great degree in a short period of time. When compared with residence times in an ABR, operating at 20 h HRT, there would be potential for adsorption of tartrazine to the biomass, however, given that reactor measurements (**Section 3.2.9**) showed that most of the colour was removed in the first three compartments, it would appear that adsorption did not occur at a rate even closely approximating the observed removal. Also, the biomass growth in the ABR is low, thus the biomass would rapidly become saturated with the dye.

A tartrazine solution was fed to a laboratory-scale (10 L) ABR to assess the efficiency of the reactor, and its configuration and separation of microbial populations, in the decolourisation of the dye.

3.2.3 Experimental Design

The design and configuration of the ABR is discussed in **Section 2.3.1** and illustrated in **Figure 2.4**. A laboratory-scale reactor was set up in a waterbath, which was maintained at a constant temperature of 35 °C. The reactor was seeded with 7.68 L (0.96 L/compartment) of screened digester sludge taken from Mogden Sewage Works. The inoculum sludge consisted of 18 g/L TS, of which 12 g/L were VS. The sludge was allowed to settle for one week before feeding began. The feed connections for tartrazine degradation were set up as illustrated in **Figure 3.5**. Gas production was not measured.

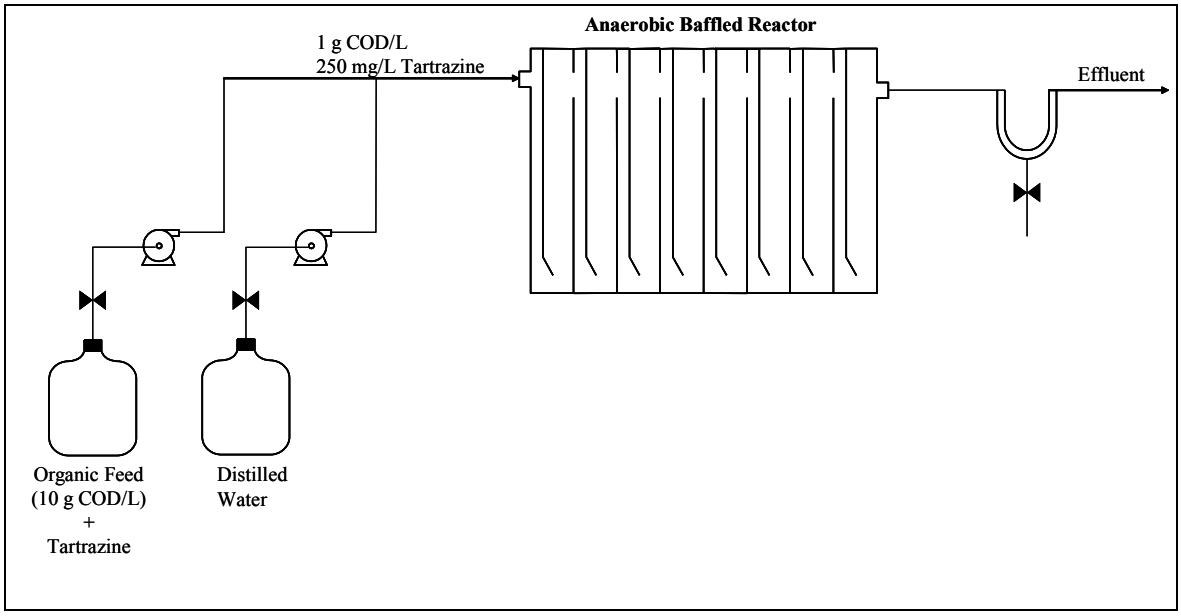


FIGURE 3.5: Schematic diagram showing the experimental layout of the laboratory-scale ABR treating a synthetic tartrazine stream (not to scale).

The feed solution was continuously pumped, by a variable speed Watson-Marlow peristaltic pump (model 101U/R), and diluted with distilled water pumped by a variable-speed peristaltic pump (model 505s). The two streams combined to form a single feed stream just before the inlet to the reactor. The treated effluent passed through a glass U-tube for level control and a biomass trap before running to the effluent reservoir. Effluent samples were taken from the bottom of the U-tube.

A standard sucrose/protein feed solution (Barber, 1999) was made up, as described in **Appendix 1**. This feed was used throughout the project. The feed was completely soluble and biodegradable, and has been used by other researchers to run anaerobic reactors continuously for long periods of time (Grobicki and Stuckey, 1991). Feeding began with the standard feed solution at an organic loading rate of 1 g COD/L and an HRT of 40 h. The flowrate was gradually changed with a stepwise decrease in the HRT from 40 h to 35, 30, 25 and then 20 h. The HRT was maintained at 20 h and the reactor was operated for ca. 60 d with the sugar/protein feed, at an organic load of 1 g COD/L.

Once the reactor had reached steady state, tartrazine dye was added to the feed solution (on day 68). The dye powder (15 g) was diluted in 6 L of the sterilised feed solution (concentration of 10 g COD/L). The feed was diluted 10x with distilled water, such that the feed to the reactor contained a dye concentration of 250 mg/L. The organic load to the reactor was maintained at 1 g COD/L and the HRT at 20 h.

3.2.4 Analytical Methods

Once or twice a week, samples were taken from the ABR and analysed. Each compartment was sampled (10 mL), through the sampling ports in the lid. Sampling began at the U-tube (effluent), and worked through the reactor, finishing at compartment 1. The samples were drawn through a long stainless steel needle and syringe, which were first used to mix the compartment contents by repeatedly plunging the

syringe. The samples were taken on the assumption that the individual compartments were perfectly mixed, i.e., samples were not taken at different levels within a compartment. The samples were immediately sealed in centrifuge tubes. The pH of each was measured. The samples were then centrifuged at 10 000 rpm, for 15 min at 20 °C. The supernatants were filtered through 0.45 µm filters into plastic vials and sealed. The liquid was used to analyse COD and VFAs. The analytical techniques are detailed in **Appendix 1**. The remaining sample supernatant liquor was acidified and stored at – 20 °C, for future reference.

Biogas samples (0.2 mL) were taken from the headspace of each compartment and injected into a GC (GowMac 350), for composition analysis (**Appendix 1**).

3.2.5 16S rRNA Probing

Periodically, samples were taken from each compartment of the ABR and probed to identify the microbial populations, the predominant populations and microbial changes, or shifts in population, with time. The samples were fixed and probed according to the method outlined in **Appendix 1**. A range of 16S rRNA oligonucleotide probes were used. The oligonucleotide probes and their target groups are listed in **Table 3.2**.

TABLE 3.2: Sequences, target sites and specificities of rRNA-targeted oligonucleotide probes used for whole-cell hybridisation.

Probe	Specificity	Reference
ARC915	Archaea	(Stahl and Amann, 1991)
EUB338	Bacteria	(Stahl *et al*., 1989)
ALF1b	Proteobacteria (alpha, delta)	(Manz *et al*., 1992)
BET42a	Proteobacteria (beta)	(Manz *et al*., 1992)
GAM42a	Proteobacteria (gamma)	(Manz *et al*., 1992)
SRB385	Proteobacteria (delta)	(Amann *et al*., 1990)
CF319a	Cytophaga-flavobacteria	(Manz *et al*., 1992)
BAC303	Bacteroides (CFB phylum)	(Manz *et al*., 1992)
HGC69a	High mol%G+C gram-pos.	(Roller *et al*., 1994)
LGC354a	Low mol%G+C gram-pos.	(Meier, 1998)
LGC354b		
LGC354c		
DSV698	*Desulfovibrionaceae*	(Plumb *et al*., 2001)
DSB985	*Desulfobacteriaceae*	(Plumb *et al*., 2001)
MX825	*Methanosaeta*	(Rocheleau *et al*., 1999)
MS821	*Methanosarcina*	(Rocheleau *et al*., 1999)

* Probes specific for gram-positive bacteria LGC (not Clostridia and mycoplasma). Made up an equimolar mixture of the three probes.

The experimental results are presented in two parts: results of reactor analyses, i.e., assessment of the tartrazine degradation; and results of the FISH experiments, describing the observed population dynamics.

3.2.6 Reactor pH

Tartrazine was added to the feed after 68 d of operation of the reactor (indicated by the dotted line on the graph).

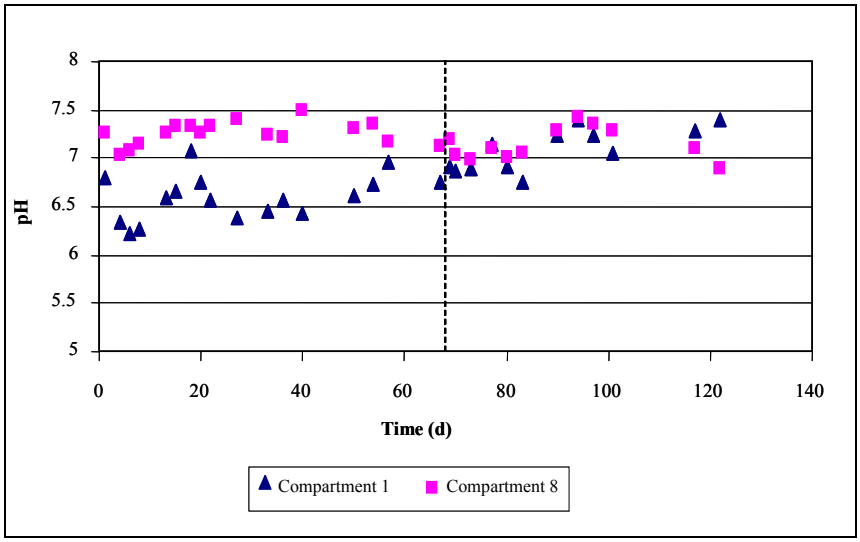


FIGURE 3.6: Plot of the pH profiles in the laboratory-scale ABR treating a tartrazine stream.

The pH of compartment 1 was observed to increase slightly after the addition of the tartrazine, such that there was not much of a distinction between the pH in compartment 1 and compartment 8. This indicated a diversion from the horizontal separation of acidogenesis and methanogenesis, i.e. addition of the dye waste resulted in increased methanogenic activity in the first compartments. This was verified by the biogas composition results (**Section 3.2.11**). The convergence in pH values could have been due to a more active mixed population in compartment 1.

3.2.7 Reactor Solids

A decrease in the solids, measured in compartments 4 and 8, after addition of the tartrazine (day 68) indicated washout of the biomass. Biomass activity remained high in compartment 1. The reason for the washout could have been increased gas production in compartment 1 as suggested by the pH and biogas data, indicating increased methanogenic activity in the first compartments.

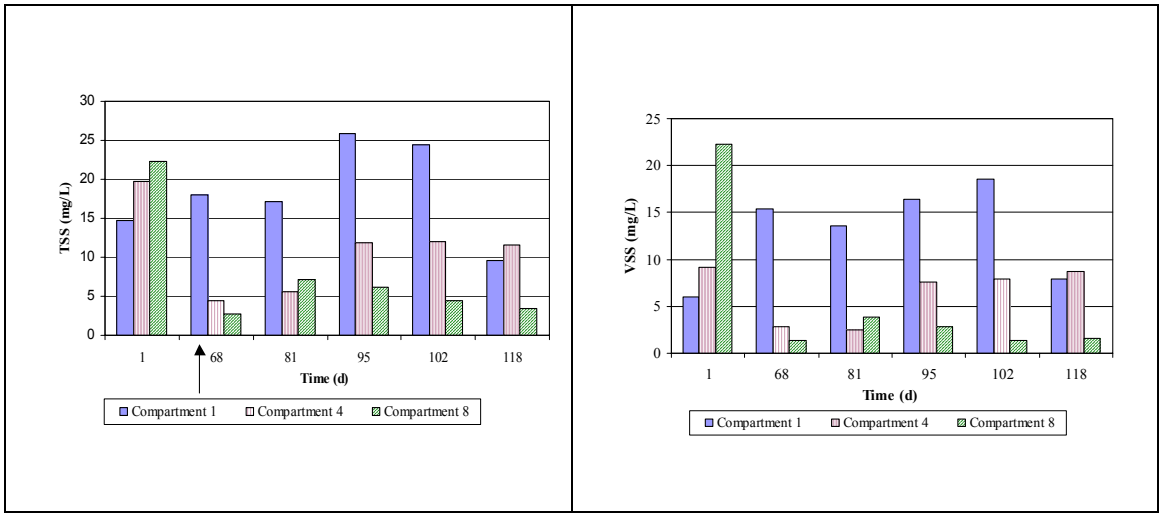


FIGURE 3.7: Plots of the total solids and volatile solids measured in compartments 1, 4 and 8 of the laboratory-scale ABR treating a tartrazine stream, at different sampling times.

3.2.8 Reactor Chemical Oxygen Demand (COD)

Figure 3.8 depicts the soluble COD removed from the reactor over time. The initial fluctuations were during the start-up of the reactor and can be attributed to technical problems with the peristaltic pump, resulting in inaccurate flow rates.

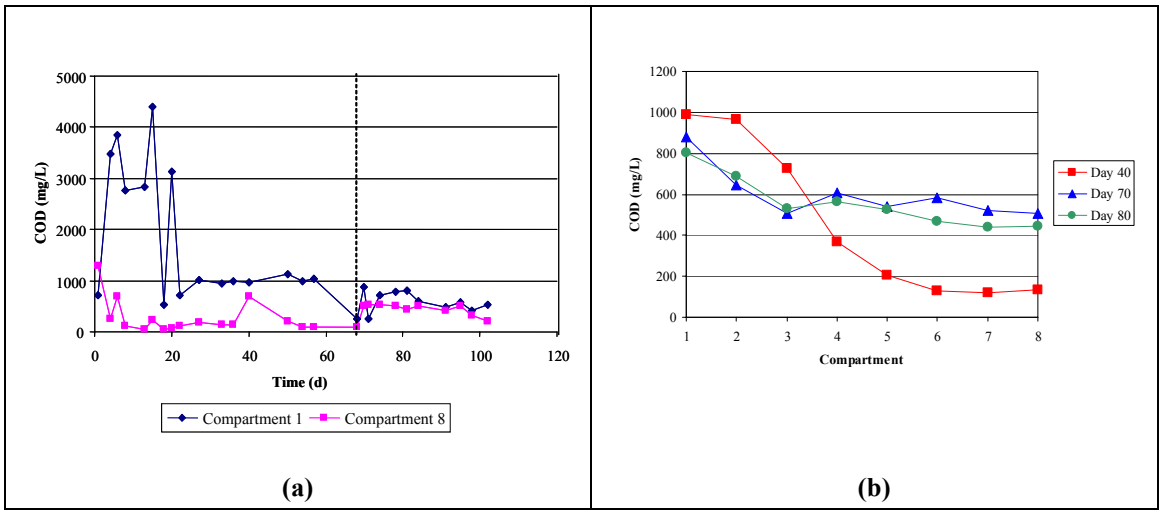


FIGURE 3.8: Plots showing (a) the CODs in compartments 1 and 8 and (b) the COD profiles through the laboratory-scale reactor, treating a tartrazine stream, at different times during the experimental period.

The efficiency of the COD reduction decreased after addition of the tartrazine. The flowrates were corrected and the inlet COD stabilised at 1 000 mg/L on ca. day 25. At steady state, COD reduction was between 80 and 95 %. **Figure 3.8 (b)** shows the COD profiles through the reactor, at different time periods during the experiment. The profile for day 40 illustrates the efficient COD reduction (effluent COD ca. 100 mg/L), before addition of the tartrazine. After addition of the tartrazine (day 68), the COD profiles fluctuated. The COD in the effluent increased (50 to 60 % reduction of the influent COD). These

results suggest that the addition of the tartrazine had an inhibitory or negative effect on the microorganisms, resulting in less efficient degradation of the waste. COD reduction did improve with time as can be seen in the comparison profiles for days 70 and 80. It is possible that the microorganisms required a period to acclimate to the dye.

3.2.9 Reactor Colour

Difficulty was encountered in accurately measuring the colour in the reactor. Although the solution in the reactor was visibly yellow, during the sampling procedure and preparation of the samples for absorbance measurements, the samples tended to change to a maroon/brown colour. The reason for this could be that the degradation products became oxidised, resulting in a colour change. Another explanation could be that degradation products were binding to form another dye structure. Other authors have experienced problems with auto-oxidation during sample preparation (Chung *et al*., 1978; Knapp and Newby, 1995). Haug *et al*. (1991) overcame this problem by using gas-tight cuvettes for colour measurement. The method was improved and accurate colour measurements obtained. For this reason, colour results are only given from day 95. A tartrazine calibration curve was plotted (**Appendix 2**), thus the tartrazine concentration in samples could be calculated from the measured absorbance. These results show that colour removal increased with time.

Over the long term there was no breakthrough of colour in the effluent, so while the colour may have been adsorbed initially, it must have eventually been metabolised.

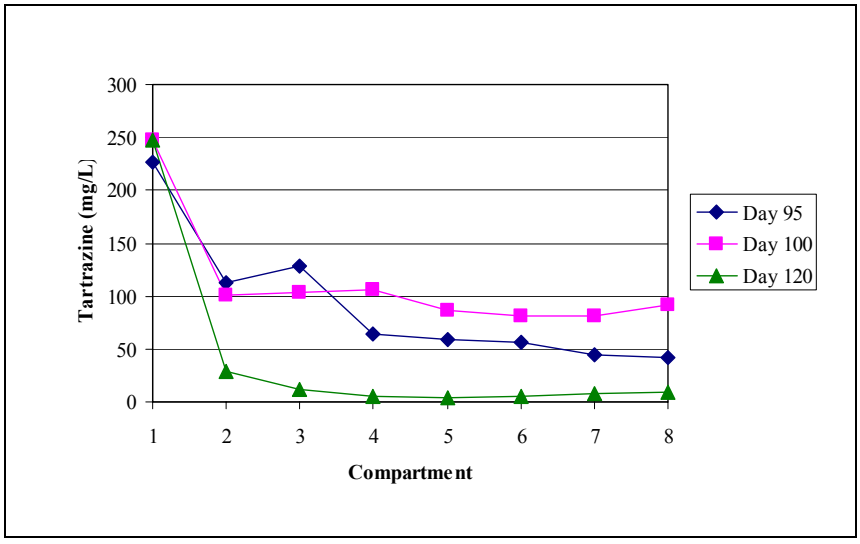


FIGURE 3.9: Plot showing the colour reduction profiles in the laboratory-scale ABR treating a tartrazine stream.

Figure 3.9 shows the colour profiles in the reactor, on three different days. The plot shows increased colour removal with time, suggesting acclimation of the biomass to decolourisation of the tartrazine. By day 120, the tartrazine concentration in the effluent was 12 mg/L. However, the effluent was still highly coloured because even a small amount of dye in solution results in visible colour. According to the results, most of the colour reduction was achieved in the first compartment of the ABR. Barber (1999)

showed that an aerobic polishing step can be inserted within an ABR with no detrimental effect on reactor performance. This is due to the fact that the methanogenic archeae are well shielded from oxygen by the outer layers of facultative anaerobes, in immobilised aggregates. Therefore, processes which inherently require both anaerobic and aerobic treatment, e.g. dye wastewaters, could be treated within a single reactor unit (Barber, 1999). However, FitzGerald and Bishop (1995) found for three investigated azo dyes, that ca. 90 % of the colour and 85 % of the COD was removed in the anaerobic stage with very little additional removal in the secondary aerobic stage. They concluded that a two stage system was not necessary, except as a polishing step. According to Field *et al*., (1995), anaerobic and aerobic metabolic activities are a prerequisite for the complete biodegradation of recalcitrant aromatic pollutants which contain electron withdrawing substituents.

3.2.10 Reactor Volatile Fatty Acids

Figure 3.10 is a plot of the total VFAs measured in the ABR effluent, over time. The dotted line illustrates the addition of tartrazine to the feed stream, on day 68.

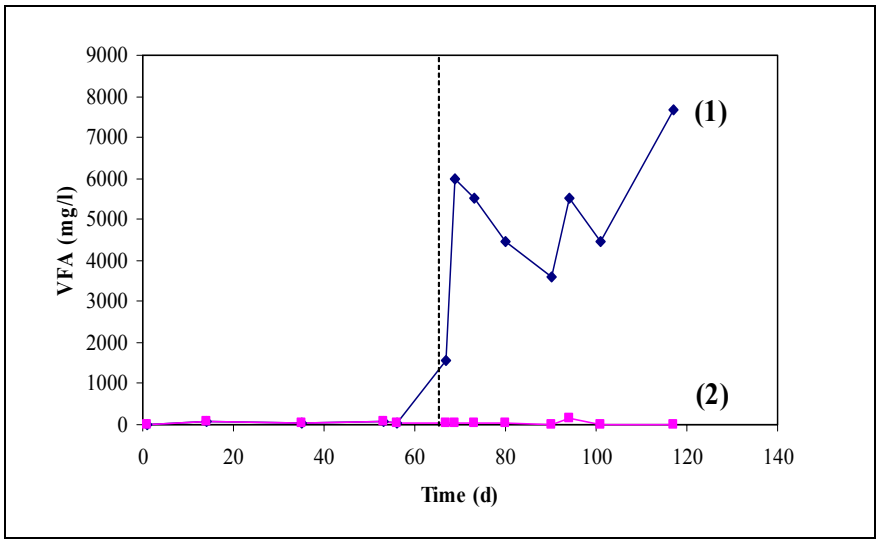


FIGURE 3.10: Plot of the total volatile fatty acids in the effluent of the laboratory-scale ABR treating a tartrazine stream, as a function of time, showing (1) the high concentration as propionate and (2) the high concentration as a dye degradation product.

There was a sharp increase in the measured VFAs, specifically high propionate concentrations, after the addition of the tartrazine to the feed stream. However, since other measured parameters did not indicate VFA accumulation, i.e. the reactor pH did not decrease and reactor performance did not change significantly, it was thought that the tartrazine or its degradation products, which were obviously accumulating in the reactor due to adsorption and slow degradation, was being detected at a retention time similar to that at which propionate was usually detected. **Figure 3.10** illustrates the two scenarios. The first is where the high concentration is plotted as propionate and contributes to the total effluent VFA concentration. This results in VFA concentrations > 5 000 mg/L, which would not satisfy a COD balance since only 1 000 mg/L COD was being added in the feed. The second scenario plotted shows the total

VFA in the effluent, without the measured propionate concentrations. Here, the VFA concentration remains below 150 mg/L and is thought to be more representative of the reactor conditions.

Acetate concentrations in the reactor effluent were below 50 mg/L, thus indicating efficient conversion to methane and carbon dioxide. The propionate profiles showed significant increase in propionate concentrations after the addition of the tartrazine. These levels remained relatively constant over the reactor and increased with time. If these concentrations were representative of tartrazine it would account for the increasing concentrations since the tartrazine was not readily degraded and towards the end of the test when the tartrazine was degraded, these values may be representative of degradation products or accumulated dye in the biomass. *i*-Butyrate was not often detected in the reactor and remained at concentrations < 10 mg/L. The formate profiles showed relatively constant levels throughout the reactor, however, these concentrations were always < 30 mg/L. Previous authors have concluded that formate plays an important role during process stability and shock loading (Grobicki and Stuckey, 1992).

3.2.11 Reactor Biogas

The biogas composition was monitored throughout the operation of the reactor, particularly for detection of methanogenic activity. The results are illustrated in **Figure 3.11**.

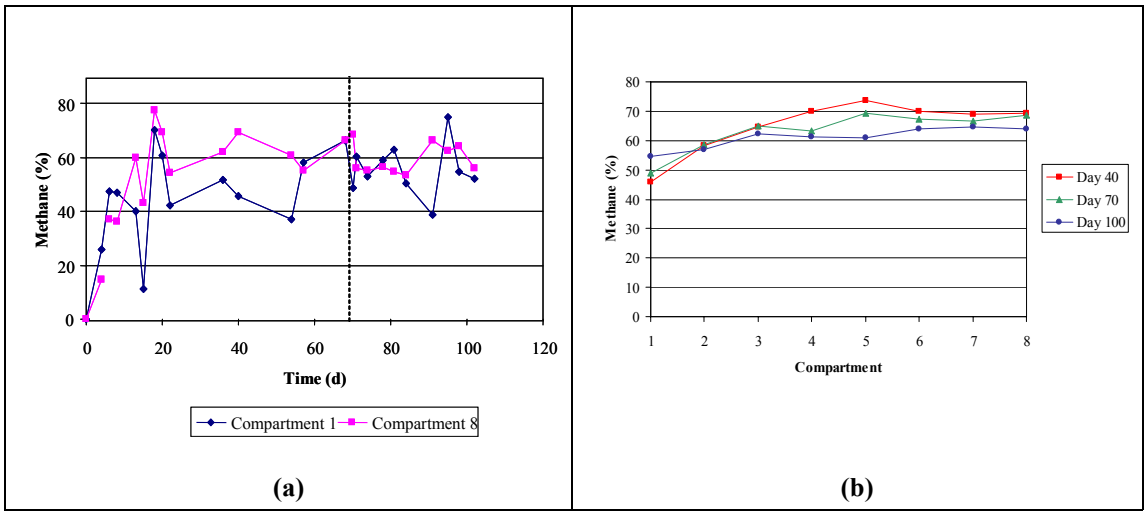


FIGURE 3.11: Plots showing (a) the methane content of the biogas in compartments 1 and 8 and (b) methane profiles through the laboratory-scale ABR treating a tartrazine stream.

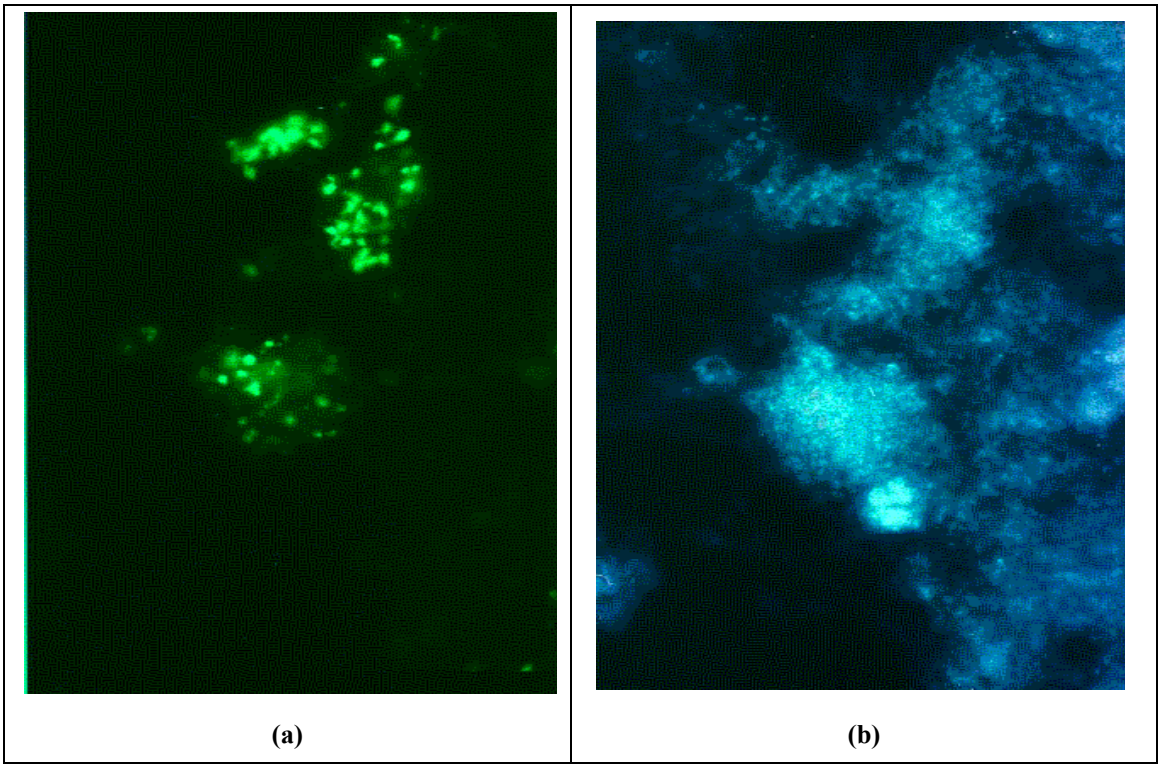
Figure 3.11(a) shows that the methane composition in compartment 8 was higher than in compartment 1, but not significantly so after the tartrazine addition. The profile plot (**Figure 3.11 (b)**) shows the increase in methane production through the reactor. On day 40, the methane content in compartment 1 was 40 %, this increased, with increased methanogenic activity, to ca. 70 % in compartment 4 and remained relatively stable at this value through the remainder of the reactor. After the addition of the tartrazine, the methanogenic activity increased in the first compartments of the ABR.

From these results, it can be concluded that tartrazine was not readily degraded by anaerobic digestion. In batch experiments, Haug *et al*. (1991) assessed the degradation of a number of different azo dyes. For tartrazine, 94 % of the initial dye remained after 3 d of anaerobic incubation, whereas the majority of the other azo dyes had been decolourised. Glucose was added, resulting in complete metabolism of several of the azo dyes but only 16 % of the tartrazine was degraded. These results verify that tartrazine is not readily biodegradable. The increased reduction in COD and colour do, however, suggest some acclimation of the biomass to the dye and indicate the potential for more efficient treatment.

The ultimate objective of treating a coloured wastewater cannot be removal of the colour alone since many of the aromatic amines, which could be present in the effluent as degradation products, are extremely toxic. Benzidine is a carcinogenic aromatic amine present in tartrazine. The concentration in tartrazine is limited, by the FDA, to 1 ng/g (Prival *et al*., 1993). Benzidine contained in tartrazine most commonly originates as an impurity in the sulfanilic acid used for the synthesis of the dye. Aniline is a precursor in the synthesis of sulfanilic acid, and benzidine is a possible oxidation product of aniline. Any benzidine present in the sulfanilic acid could diazotise and couple during manufacture of tartrazine. The reduction in colour, in the ABR, proves that there is degradation of the tartrazine, however, degradation products in the effluent were not identified in this study.

3.2.12 Population Characterisation

Reactor samples were fixed and hybridised with 16S rRNA oligonucleotide probes. Probing of samples from this ABR resulted in brightly fluorescing objects, making it impossible to focus on, and count, surrounding cells. Initially, it was thought that the bright areas were clumps of tartrazine and because the fluorescent probes were detected at a similar wavelength to the tartrazine, that the presence of the dye was affecting the visibility. To prove this hypothesis and to try and view the cells associated with the dye clusters, samples were DAPI stained but not probed. It was hoped that the dye would still be visible but that the cells associated with them could be detected and give some indication of the cell morphology and numbers. However, no bright dye regions were observed. It was therefore, deduced that the bright regions were formed during probing. It is possible that the probe, or the amino linker in the probe label attached to the dye, had a greater affinity for the dye than for hybridisation to the microbial rRNA and actually probed the dye, resulting in the brightly fluorescing regions. This hypothesis is substantiated in **Figure 3.12** where **(a)** is a sample probed with EUB338, and **(b)** is the same field, stained with DAPI. In figure **(a)** only a few, very bright regions are visible and no definite cells can be seen, relative to the numbers of cells that are actually present, shown in **(b)**. It was concluded that the tartrazine dye, associated with the biomass interfered with the probe hybridisation resulting in the probes binding to the dye and not to the associated biomass. This prevented counting and characterisation of microbial populations in the reactor.



(a) **(b)**

FIGURE 3.12: FISH images of the same field of a sample taken from compartment 1 of the laboratory-scale ABR treating a tartrazine stream, (a) probed with the universal eubacterial probe, EUB338 and (b) stained with DAPI.

Identifying suitable end-of-pipe treatment process is made difficult by the combining of effluent streams from individual operations, resulting in large diurnal variations in effluent chemical composition. Clearly, waste treatment techniques need to be dedicated to individual process effluents, rather than the combined discharge, in order to be reliable and effective. This investigation has shown that the ABR, with an acclimated biomass, would be effective at treating a segregated tartrazine waste stream, on site. However, correlation of the active microorganisms with the biochemical pathways and chemical analyses was not possible due to the interference of the tartrazine with the fluorescent oligonucleotide probes.

3.2.13 Conclusions

1. Adsorption to anaerobic biomass did not play a significant role in the decolourisation of the dye.

2. Colour removal increased with time, suggesting acclimation of the biomass. After ca. 60 d, the tartrazine concentration in the effluent was 12 mg/L (95 % reduction).

3. Most of the colour reduction was achieved in the first compartment of the reactor.

4. Methanogenic activity decreased with addition of the tartrazine.

5. Tartrazine was not readily degraded by anaerobic digestion, however, degradation may be improved with acclimation of the biomass.

6. The tartrazine dye, associated with the biomass interfered with probe hybridisation resulting in the 16S rRNA oligonucleotide probes binding to the dye and not to the biomass. Thus, this experiment showed that there are problems associated with FISH and that it may not be applicable in all situations.

3.3 BATCH SCREENING TESTS

Dye compounds and their degradation products can be toxic to humans, animals and microorganisms. It was hypothesised that bioassay techniques for measuring the presence or absence of inhibitory substances are an effective indication of the effect that these substances would have on an anaerobic system.

The objective of this phase of the study was to assess the toxicity of a range of food dyes to the methanogens in anaerobic digester sludge. A food dye manufacturer provided samples of 15 food dyes, of varying chemical classes. The dyes are listed in **Table A3.1**, with both the commercial and Colour Index names. Anaerobic toxicity assays were set up in serum bottles, according to the method of Owen *et al*., 1979 (details in **Appendix 3**). Although these assays provided valuable information in that the concentration at which each dye became inhibitory to the anaerobic biomass was determined, these results could not be directly applied to the ABR. The reason for this is that the serum bottle test approximated a CSTR with a mixed anaerobic sludge, whereas in the ABR, the biomass within each compartment differs as does the substrate entering each compartment.

Since these results were not directly relevant to the operation of the ABR and the focus of the thesis as a whole, the results and discussion have been included in **Appendix 3**.

The results of the anaerobic toxicity assays were used to guide the set-up of the biodegradability assays. Biodegradability of the dyes was determined by monitoring the cumulative biogas production during anaerobic incubation, according to the method of Owen *et al*. (1979). Similarly, the details of these experiments, the results and discussion are presented in **Appendix 3**.

3.4 TREATMENT OF AN INDUSTRIAL DYE WASTEWATER IN THE ANAEROBIC BAFFLED REACTOR

The industrial partner in this investigation was a manufacturer of food dyestuffs, based in Northumberland, England. The factory operated continuously, i.e. 24 h per day, 7 days per week, producing an average volume of 310 m³ of effluent per day. The main organic components of the effluent were azo dyes, sub-dyes and unchanged raw materials (dye precursors). The pH range of the effluent was 7.9 to 8.1. The average salt content was 3 % NaCl, sulphate concentrations averaged 1 040 mg/L and COD, 620 mg/L. As much as 50 % of the production of the factory was dedicated to tartrazine production, resulting in high concentrations of this dye, and its precursors, in the final effluent.

At the time of these investigations, the effluent was chemically treated with sodium dithionite, which forms a precipitate, thus removing a significant amount of the colour from the wastewater. The purpose

of this treatment was to achieve compliance with the discharge optical consent levels, for discharge to sewer and treatment at a local wastewater treatment works. Chemical treatment was, however, not favoured because of the cost associated with the chemicals, the problem of disposal of the precipitate that was formed, and because the company was still charged high tariffs for effluent discharge, based on volume, organic content and settleable solids. The trade effluent discharge standards, set by Northumbrian Water Limited, under the Environmental Protection Act, 1990, the Water Industry Act, 1991 and the Environment Act, 1995, required colour to be less than 5 optical density (OD) units at any point between 480 and 700 nm; volume < 432 m^3/d; pH > 6 and < 10; COD < 6 000 mg/L and sulphate < 5 000 mg/L.

Due to the wide range of dyestuffs produced at the factory, the composition of the effluent varied considerably depending on which dyes were being synthesised and the amount of washing and cleaning of machinery and pipes. A pre-treatment system, such as an ABR, on-site, has the potential of reducing running costs for the company as it would alleviate the need for chemicals, for chemical treatment, and reduce the discharge tariffs since the wastewater would be more stabilised.

3.4.1 Hypothesis and Objectives

It was hypothesised that anaerobic digestion could decolourise the trade effluent and reduce the COD of the waste stream, thereby reducing costs to the company. Again, the design and structure of the ABR would prevent inhibition of the anaerobic biomass and allow for more efficient degradation and decolourisation at a low (20 h) HRT.

It was also hypothesised that fluorescent *in situ* hybridisation of the microbial communities that develop in the ABR compartments, during treatment of the dye waste stream, would provide improved knowledge of the biochemical pathways and the microorganisms involved in the decolourisation.

The specific objectives of the investigation were to:

1. Assess the feasibility of the ABR for treatment of a real food dye waste stream; including reduction of COD and decolourisation.

2. Determine whether the anaerobic biomass became acclimated, thereby improving degradation and decolourisation, with time.

3. Use 16S rRNA oligonucleotide probes to characterise the microbial populations within each compartment, and the dynamics of these populations during treatment of the waste stream.

3.4.2 Experimental Design

A second laboratory-scale ABR was dedicated to the treatment of the food dye trade effluent. The reactor set-up and starting conditions were the same as those described for the tartrazine ABR. The inoculum

sludge had a total solids content of 27 g/L, of which 19 g/L were volatile solids. The experimental set-up is illustrated in **Figure 3.13**.

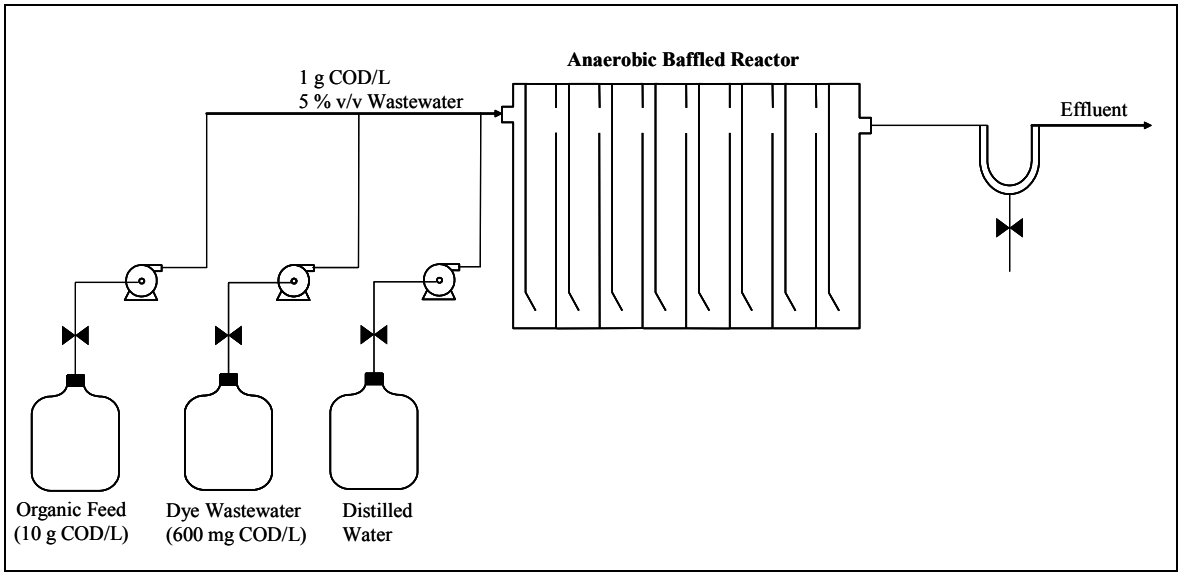


FIGURE 3.13 : Schematic diagram of the experimental layout of the laboratory-scale ABR treating a food dye wastewater.

Once the reactor had reached steady state (day 68), the wastewater was added to the sugar/peptone feed stream. The anaerobic toxicity assays (**Appendix 3**) showed the wastewater to be relatively inhibitory, therefore, feeding commenced with a wastewater concentration of 5 % (v/v). A batch of the wastewater were collected from the factory every 2 to 3 weeks, and stored at 4 °C until use. The sterilised feed solution (10 g COD/L) was diluted with water, such that the organic load to the reactor was 1 g COD/L, with an HRT of 20 h. On day 95, the wastewater feed concentration was increased to 10 % (v/v). Gas production was not measured.

3.4.3 Analytical Methods

The sampling technique and analytical methods were the same as those described in **Section 3.2.4**, except for the COD measurement. The high salt content in the wastewater reacted with the COD reagents resulting in the formation of precipitates in the COD tubes and inaccurate optical COD data. To overcome this problem, the total organic carbon was measured (**Appendix 1**) to provide an indication of the reduction of organic content in the reactor and the reduction profile, through the compartments.

The maximum absorbance wavelength for the wastewater was 500 nm.

3.4.4 Reactor pH

The wastewater (5 % v/v) was added to the feed after 68 d of operation. On day 95, the concentration was increased to 10 % (v/v). No significant effects were observed in the pH of compartment 1 or the reactor effluent (**Figure 3.14)**, even when the wastewater concentration was increased. This suggests that the

reactor was not stressed due to the addition of the wastewater. The pH was maintained within the range required for anaerobic digestion.

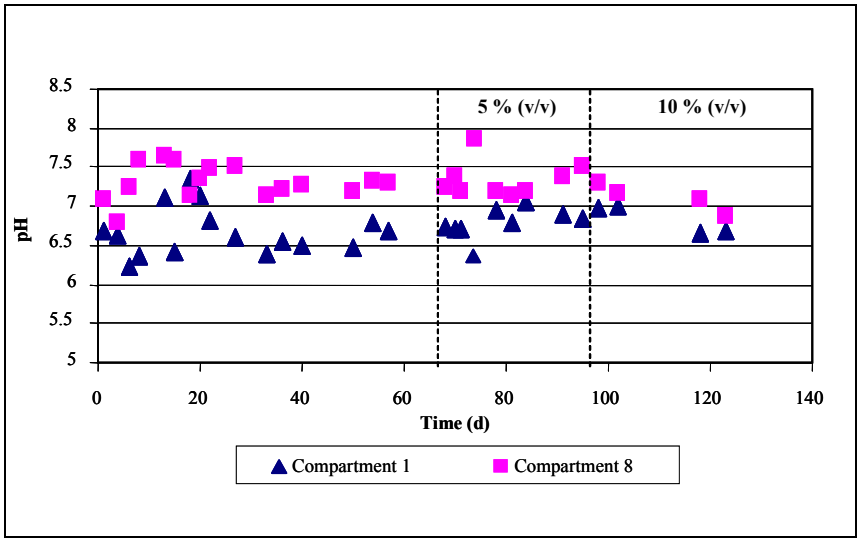


FIGURE 3.14: Plot of the pH profiles in the laboratory-scale ABR treating a food dye wastewater.

3.4.5 Reactor Solids

The total solids and volatile solids were measured periodically during the test period (**Figure 3.15**). The plots show that the solids, in compartment 1, decreased after the addition of the wastewater. There was also a reduction in solids after day 95, when the wastewater concentration was increased to 10 % (v/v).

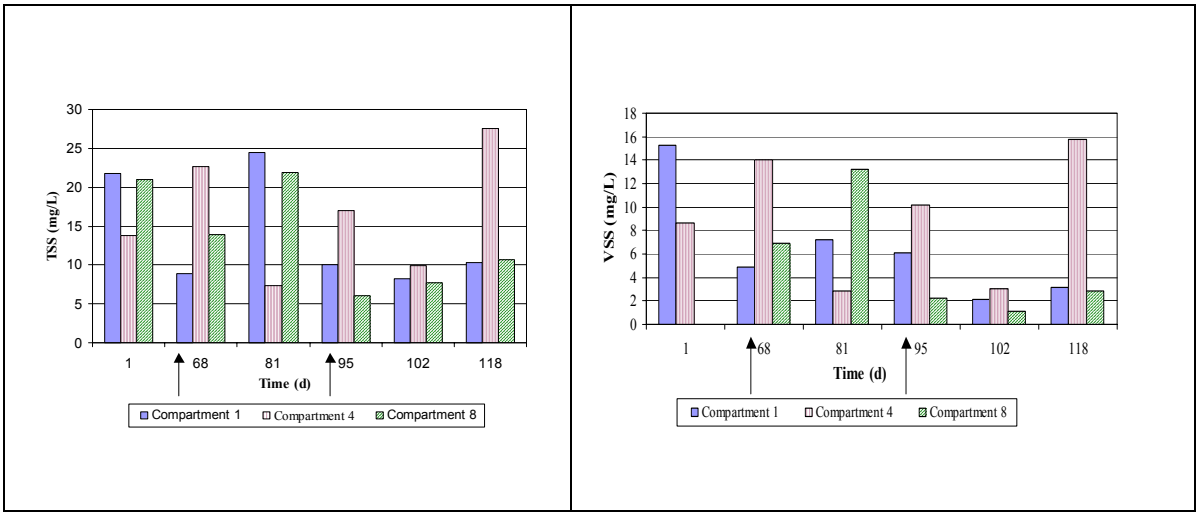


FIGURE 3.15: Plots of the total solids and volatile solids measured in compartments 1, 4 and 8 of the laboratory-scale ABR treating a food dye wastewater.

This reduction in solids after the addition of the wastewater indicates that there was a degree of washout of the biomass. It is common during the start-up of a reactor to experience some biomass loss. This is usually due to the increased activity of the microorganisms, resulting in increased biogas production and

subsequent floating and loss of biomass. Foaming was observed in the first 3 compartments, when the wastewater was initially added and then again when the wastewater concentration was increased to 10 % (v/v). This activity settled after approximately 2 d.

3.4.6 Reactor Chemical Oxygen Demand (COD)

The soluble COD within each compartment and the effluent COD was monitored and the results are shown in **Figure 3.16**. The initial fluctuations were during the start-up of the reactor and can be attributed to technical problems with the peristaltic pump, resulting in inaccurate flow rates.

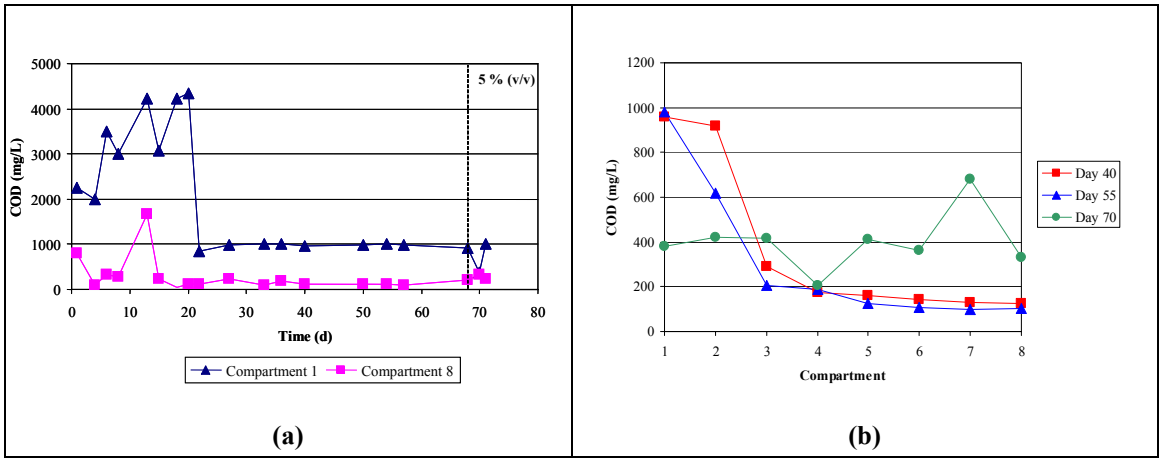


FIGURE 3.16: Plots showing (a) the CODs in compartments 1 and 8 and (b) the COD profiles through the laboratory-scale reactor, treating a tartrazine stream, at different times during the experimental period.

The reduction in COD was measured until the addition of the wastewater to the feed stream. The inlet and effluent COD fluctuated during the first 20 d of start-up (**Figure 3.16 (a)**). The levels then stabilised and the reactor reached steady state, with a consistent COD reduction of ca. 90 %. The COD profiles through the reactor (**Figure 3.16 (b)**) show that before the addition of the wastewater, the majority of the COD was removed in the first three compartments. The plot includes a profile for day 70, which was 2 d after the addition of the 5 % (v/v) wastewater concentration. The fluctuation of this profile indicates the inaccuracy of the COD measurements caused by the high salt concentration in the wastewater. The salt was thought to react with the COD reagents, resulting in precipitates forming. The organic content was then determined by measurement of the total organic carbon (TOC) in the reactor samples (**Appendix 1**). The reduction in TOC was consistent between 70 and 80 %.

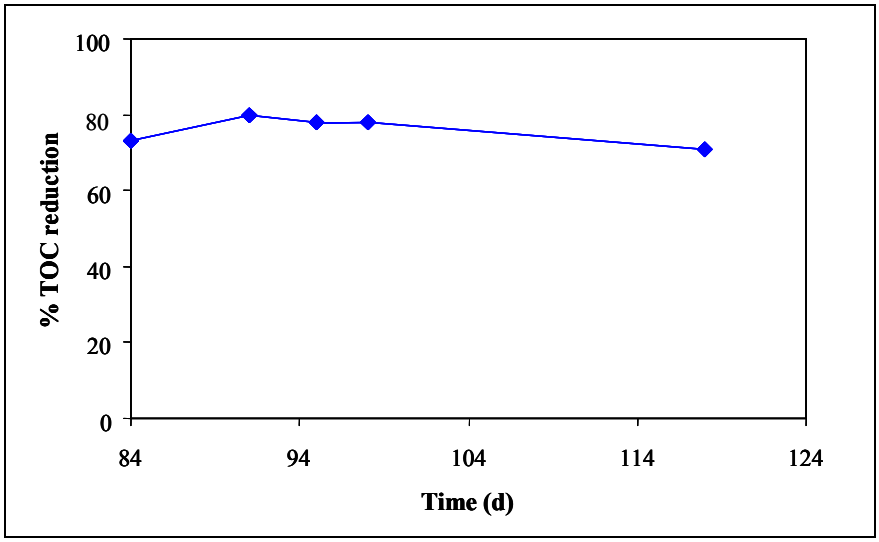


FIGURE 3.17: Plot of the total organic carbon (TOC) measured in compartment 8 of the laboratory-scale ABR treating a food dye wastewater.

3.4.7 Reactor Colour

As with the colour measurement in the tartrazine reactor, it took some time to develop an accurate technique for the measurement of colour in the ABR effluent. The same technique was used and the absorbance was read at 500 nm (**Appendix 1**). A colour calibration curve was plotted for the wastewater (**Appendix 2**), however, due to the variability in the composition of the wastewater, it was found that this curve was not always an accurate estimation of the wastewater concentration, thus, colour reduction was plotted as a reduction in absorbance and not as a concentration. The results are shown in **Figure 3.18**. These results show significant colour reduction.

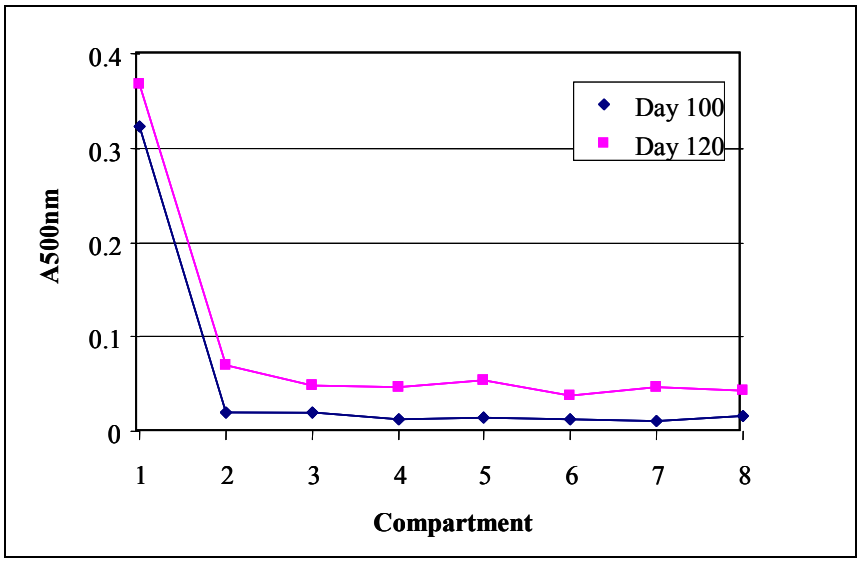


FIGURE 3.18: Plot showing the colour reduction profiles in the laboratory-scale ABR treating a food dye wastewater.

The results in **Figure 3.18** show that the colour reduction in the ABR was significant and that most of the reduction occurred in the first compartment. The profile on day 120 indicates an increase in the effluent colour. The reason for this was that the particular wastewater sample, received from the factory, had a distinct green colour caused by a high concentration of a particular dye. The green hue was evident in the reactor effluent, indicating that the dye was recalcitrant. This illustrates the difficulty in treating real industrial wastewaters, because of the variability. The biomass would need time to acclimate to the specific dye. However, it is expected that acclimation to a real wastewater containing a continuously changing dye composition, would be difficult. If acclimation to certain types of dye structures were to occur, it could be hoped that this acclimation process would also increase resistance to toxic effects caused by other dye compounds. The literature does not support such a hypothesis. Instead, Fu *et al*. (1994) showed that acclimation of a biofilm reactor to Acid Red 14 did not reduce the toxic effect of Acid Orange 7. In addition, the joint inhibitory effect of a mixture of compounds is in general additive of the individual effects (Speece, 1996).

3.4.8 Reactor Volatile Fatty Acids

The total volatile fatty acids measured in the effluent are plotted in **Figure 3.19**. As in the results for the tartrazine ABR, the plot labelled (1) represents the VFA levels incorporating the high *propionate* concentrations. Plot (2) assumes that the detected VFA is not propionate, but tartrazine or one of its degradation products. With this assumption, effluent VFA levels remained below 200 mg/L.

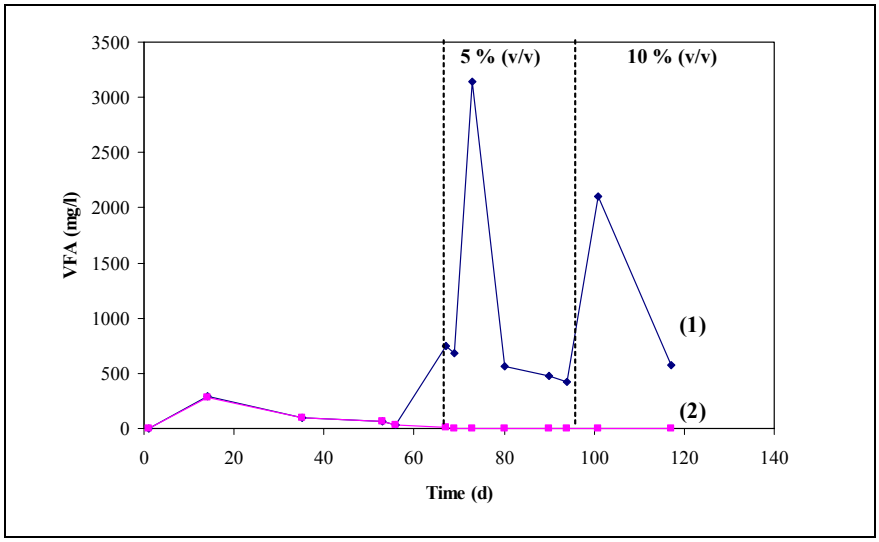


FIGURE 3.19: Plot of the total volatile fatty acids in the effluent of the laboratory-scale ABR treating a food dye wastewater, as a function of time, showing (1) the high concentration as propionate and (2) the high concentration as a dye degradation product.

Since tartrazine was a major component of the industrial effluent, the same explanation was given for the high VFA concentrations measured in these samples. The apparent propionate concentrations increased with the addition of the wastewater to the reactor. Again, other operating parameters did not support the accumulation of VFAs in the reactor, thus, it was thought that the tartrazine, or a dye degradation

product, had been detected on the HPLC column at a similar residence time to that at which propionate was detected. The total VFA concentrations were much lower, ca. 3 000 mg/L, than those in the tartrazine ABR which can be explained by the fact that less tartrazine was being fed to the reactor because it was diluted by the other effluent components. Analysis of VFAs in compartment 1, however, showed an increase in the level of acetate from < 100 mg/L to ca. 800 mg/L upon addition of the dye waste to the ABR before returning to ca. 100 mg/L after several days, and another slightly smaller increase in acetate when the loading of the dye waste was increased to 10 % (v/v) (Bell *et al.*, 2000). It would appear that at first the dye waste may have slightly inhibited the acetotrophic methanogens before these populations acclimated to the changed environment and resumed more complete acetate utilisation.

3.4.9 Reactor Biogas

Biogas composition was monitored throughout the operation of the reactor, particularly for detection of methanogenic activity. The plots in **Figure 3.20** show that the methanogens were present throughout the reactor and that the relative methanogenic activity increased with increasing concentrations of the wastewater.

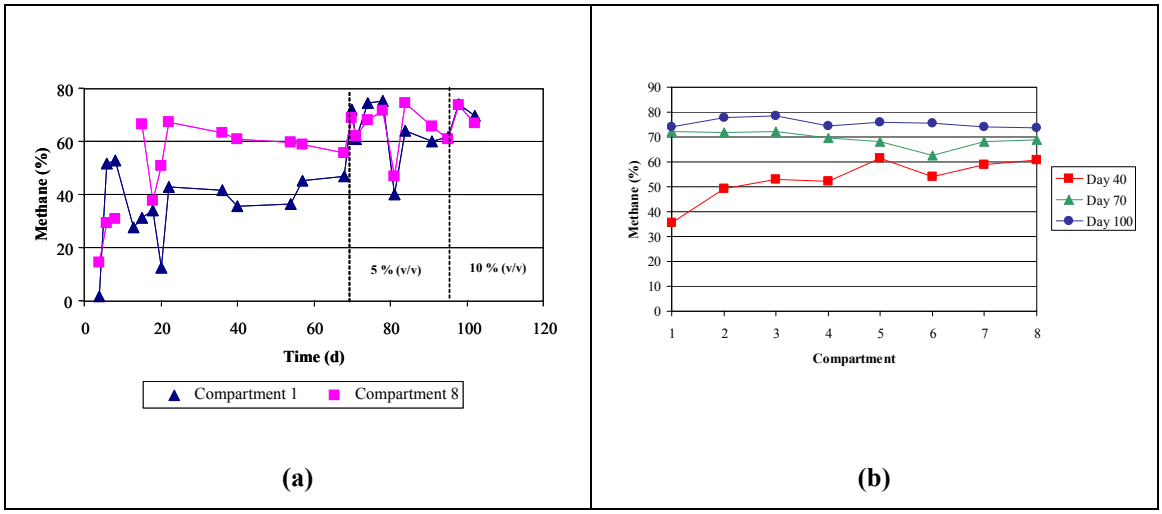


FIGURE 3.20: Plots showing (a) the methane content of the biogas in compartments 1 and 8 and (b) methane profiles through the laboratory-scale ABR treating a food dye wastewater.

Evidence of methanogenic activity is critical in an anaerobic reactor because it proves that the reactor is running efficiently, that there is no accumulation of acids and that the organics being fed to the reactor are being mineralised to methane and carbon dioxide. The results plotted in **Figure 3.20 (a)** show that the methanogenic activity increased in compartment 1 when the wastewater was added to the feed. This suggests that the wastewater contained readily biodegradable components which were converted to acid intermediates in compartment 1, thereby promoting methanogenic activity. The presence of methanogens in each compartment was verified by the 16S rRNA probing experiments (**Section 3.4.10**). The profiles of methane production, in each compartment (**Figure 3.20 (b)**), show that before the addition of the wastewater (day 40), the methane composition in compartment 1 was ca. 35 %, which increased to 60 % by compartment 5 and remained relatively constant throughout the remainder of the reactor. The day 70

profile, however, shows the methane content within every compartment to be ca. 70 %. The methane activity in the first compartments was maintained and the methane content increased (ca. 75 %) after the wastewater concentration in the feed was increased to 10 % (v/v).

The results of this experiment indicate that anaerobic digestion, in the ABR, has potential as an efficient treatment option for the food dye wastewater, contrary to the results of the anaerobic toxicity assays. Methanogenic activity was high in the reactor, the organic content of the influent was reduced by ca. 70 % and colour was reduced by almost 90 %. A potential problem is the variability of the wastewater, which could result in less efficient degradation and variations in the effluent quality. This could be alleviated with an on-site reactor, where the sludge would become acclimated to the dedicated waste stream.

3.4.10 Population Characterisation

Reactor samples were taken, from each compartment, on days 60, 80 and 100 of operation. The samples were hybridised (**Appendix 1**) with the fluorescent-labelled oligonucleotide probes listed in **Table 3.2** to identify the microbial populations, the predominant populations and microbial changes, or shifts in population, with time.

Initial hybridisations with the universal probes for eubacteria (EUB338) and archaea (ARC915) revealed an abundance of members of both these phyla in the first three compartments, at each sampling date. This correlated with the analytical data from the reactor operation, where it was evident that there was methanogenic activity in the first three compartments. Since the majority of the COD and colour was removed in the first three compartments, it was assumed that these were the most active and crucial for the treatment process. It was decided to focus the molecular studies on the first three compartments of the reactor, thereby reducing the number of samples to be analysed so that a more thorough investigation could be completed.

Comparisons with DAPI staining, in order to quantify the relative proportions of the eubacteria and archaea were attempted, however, due to the extensive filamentous or tightly clustered microcolonies of the archaea present, accurate counting of individual cells by visual means was not possible. However, it was estimated that at least 80 % of all DAPI stained cells hybridised with either the universal bacterial (EUB338) or archaeal (ARC915) probes in compartments 1, 2 and 3. Signal intensity of cells in the first 3 compartments when hybridised to the bacterial or archaeal probes was relatively strong compared with cells present in compartments 4 to 8, indicating decreased metabolic activity in these latter compartments. The metabolic activity profile between compartments correlated with the measured COD/TOC removal profiles throughout the ABR (**Figures 3.16 and 3.17**). Estimations of the relative ratio of eubacterial and archaeal members in compartments 1 to 3 ranged from ca. 70:30 in compartment 1 to 50:50 in compartment 3 for each sampling date. Analysis of samples taken from compartment 4 through to 8 revealed a decline in the bacterial populations relative to archaeal cells, with an estimated 90 % of DAPI-stained cells detected using ARC915 by compartments 7 and 8. The characteristic morphology of

Methanosaeta (long sheathed filaments) was visualised using ARC915, and confirmation of the identity of these filaments using the genus specific probe MX825 was obtained. Other morphotypes observed hybridising to ARC915 included *Methanospirillum*-like shorter filaments, single rods and sarcina-like clusters of irregular cocci often found in microcolonies of up to 50 or more cells. On the basis of the intense probe-conferred fluorescence, these cocci appeared very active. However, these cocci, either in sarcina-like arrangement or as microcolonies did not hybridise with the *Methanosarcina*-specific probe MS821.

Further probing of the samples using group-specific probes targeting various eubacterial phyla (**Table 3.2**) revealed the presence of considerable diversity. Cell counts versus EUB338 from the first 3 compartments for each of the sampling dates are presented in **Figure 3.21**. The sum of the counts obtained for each group-specific probe was < 100 % for each of the samples analysed, indicating the presence of bacteria not detected using these probes. The bacterial populations present prior to the addition of the dye waste (Day 60), were dominated by cells detected with the GAM42a probe (between 20 and 25 % in each compartment). The presence of these bacteria, in particular those which are fermentative organisms, was expected in the first compartments of the reactor because of their ability to rapidly metabolise the synthetic feed components.

Significant numbers of cells (usually >5 %) were detected with probes BAC303, SRB385 and also the LGC probe set in the Day 60 samples. For each of probes CF319a, ALF1b, BET42a and HGC69a, cells were detected at ca. 5 % or less in each compartment, except for the compartment 3 sample in which no cells were detected with the HGC69a probe. In general, the proportion of cells detected with each group-specific probe remained fairly constant from compartments 1 to 3 except for the aforementioned HGC69a with which no cells were detected in compartment 3, and BAC303 where levels varied from 18 % in compartment 1 to 4 % in compartment 3.

In the Day 80 samples (after addition of the dye waste), cells detected with the LGC probe set were numerically dominant in compartments 1 and 2 (ca. 29 % and 19 % respectively). Also, cells detected with the BAC303 probe comprised > 10 % of the eubacteria in compartments 1 and 2. With probes SRB385, DSV698 and GAM42a, between 5 and 10 % of bacterial cells were detected in all 3 compartments. The relative decline in the gamma proteobacteria was a noticeable change in population structure, as was the increase in the Gram positive bacteria detected with the LGC probe set. It is likely that the introduction of the dye waste into the ABR favoured growth of the bacterial populations detected with the LGC probe set rather than the gamma proteobacteria, although this is not conclusive. For probes CF319a and ALF1b, between 3 and 5 % of all bacteria were detected in each compartment. Of the other probes, only in compartment 1 were cells detected with either HGC69a and BET42a, and probe DSB985 detected < 1 % of bacterial cells in each compartment.

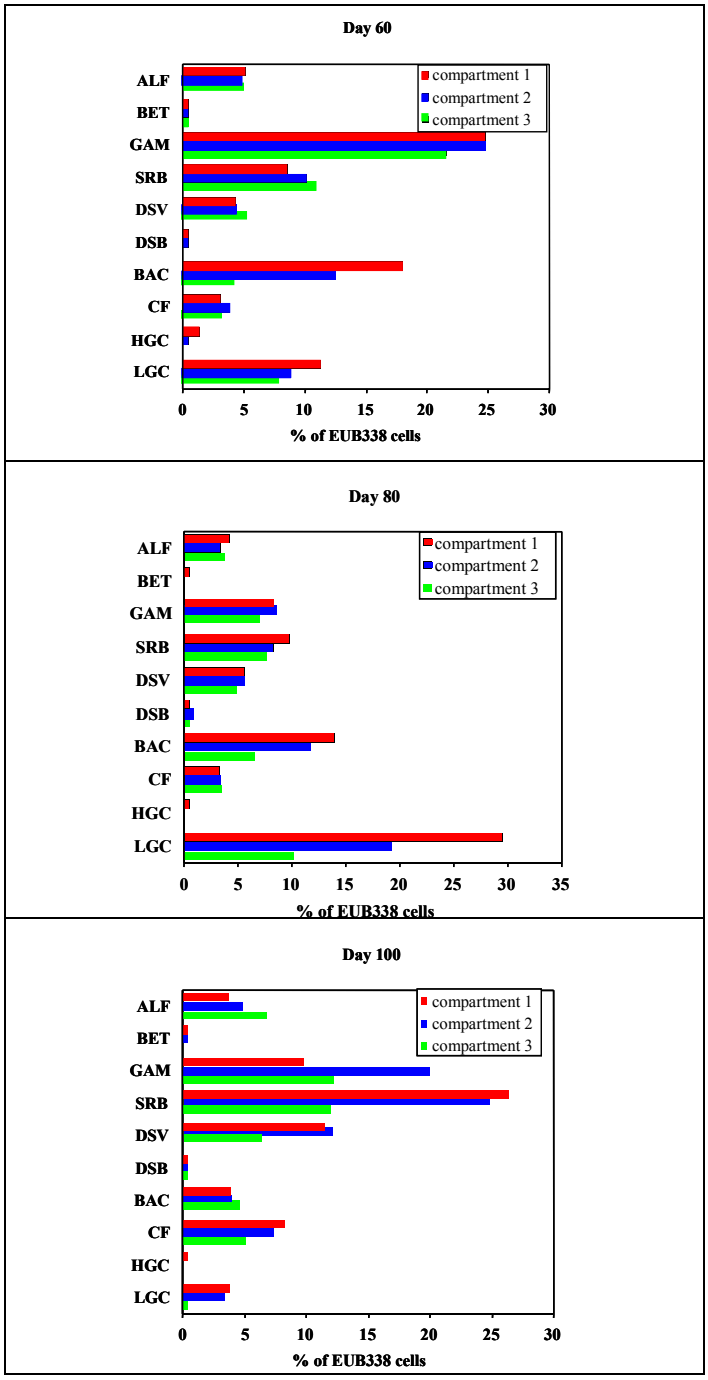
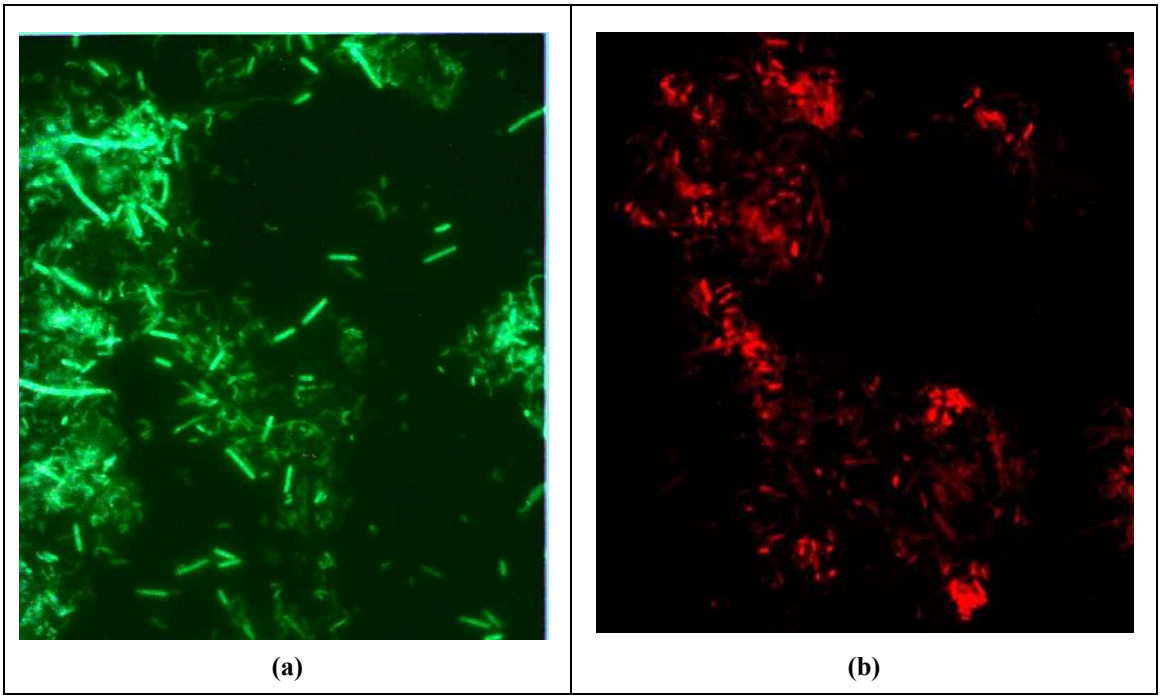


FIGURE 3.21: Bacterial community analysis of ABR compartments 1, 2 and 3 sampled at Days 60, 80 and 100 showing counts obtained using 10 different group-specific probes expressed as a percentage of total bacterial counts achieved using probe EUB338.

Analysis of the Day 100 samples revealed a different population structure. Cells detected with the SRB385 and GAM42a probes were present at greater levels in each compartment (10 % or more) with ca. 25 % of all bacterial cells detected with EUB338 in compartments 1 and 2 also detected with SRB385. In the first two compartments, counts for probe DSV698 were ca. 12 % of bacterial cells. Probe CF319a detected between 5 and 10 % of bacterial cells in each compartment, whereas counts for

BAC303 and the LGC probe set were < 5 %. Counts obtained with ALF1b varied from < 5% in compartments 1 and 2 to almost 7 % in compartment 3. Cells were detected in low numbers with each of probes HGC69a and BET42a in some compartments and low numbers of cells in each compartment were detected using probe DSB985.



(a) **(b)**

FIGURE 3.22 : Whole cell hybridisation of a sample taken from compartment 1 of the laboratory-scale ABR treating a food dye wastewater, on day 80, showing the same field probed with EUB338 (a) and GAM42a (b).

Members of the *Cytophaga-Flexibacter-Bacteroides* (CFB) phylum (cells detected using probes CF319a and BAC303) comprised a considerable proportion (at least 10 %) of the bacterial community in each of the samples analysed. Members of the *Cytophaga-Flexibacter* group within the phylum are ubiquitous microorganisms and have diverse physiology. The *Bacteroides* group are obligate anaerobes which commonly comprise a considerable proportion of intestinal or ruminal microflora (Plumb *et al*., 2001). The hypothesis that intestinal microflora were involved in the degradation of ingested food dyes has long been considered, and experimental evidence has shown that predominant intestinal microorganisms are capable of azo dye reduction (Chung *et al*., 1978).

Throughout the operation of the ABR, especially after the addition of the dye waste, considerable blackening of the inside of the first compartment and the influent tube due to the production of sulphide was observed. Two probes, DSV698 and DSB985 were used in hybridisations to provide further descriptions of sulphate reducing bacteria. Cell counts for the DSV698 probe were between 4 and 6 % in each compartment, whereas counts for probe DSB985 were very low in compartments 1 and 2 and none in compartment 3. As a means of rationalising the counts obtained using probes SRB385 and DSV698, simultaneous hybridisations using these probes with the Day 100 compartment 1 sample revealed that 44.1 % of cells detected using SRB385 also hybridised with DSV698. Cells with vibrio morphology

comprised the majority of cells detected with DSV698 compared with the mixed cell morphotypes possessed by cells detected using SRB385.

The increase of the dye waste from 5% to 10% (v/v), on day 95, may have favoured the growth of other microorganisms such as gamma proteobacteria and cells detected with the SRB385 probe. The specificity of probe SRB385 is not phylogenetically consistent, as the probe target sequence is not only present in members of the delta proteobacteria (some SRB), but also present in some *Actinobacteria* e.g. *Frankia* species, some clostridia, at least one species of *Nitrospira* and many other phylogenetically diverse organisms. At times, SRB385 has been used as a general probe for detecting SRB, however, Manz *et al*. (1992) highlighted the limitations of this probe for studying sulphate reducers and designed and tested several other more specific probes for these organisms. The use of two of these probes in this study, namely DSV698 and DSB985 (**Table 3.2**) provided more accurate enumeration of the SRB. The counts obtained using DSV698 show that species of *Desulfovibrio* comprised a considerable proportion of the community in each of the compartments at Day 100. The results of the simultaneous hybridisations performed using SRB385 and DSV698 demonstrated the relatively broad specificity of the SRB385 probe. Further examination of this diversity by PCR amplifying community 16S rDNA using SRB385 as a primer followed by cloning and analysis of clone inserts could be used to characterise this diversity.

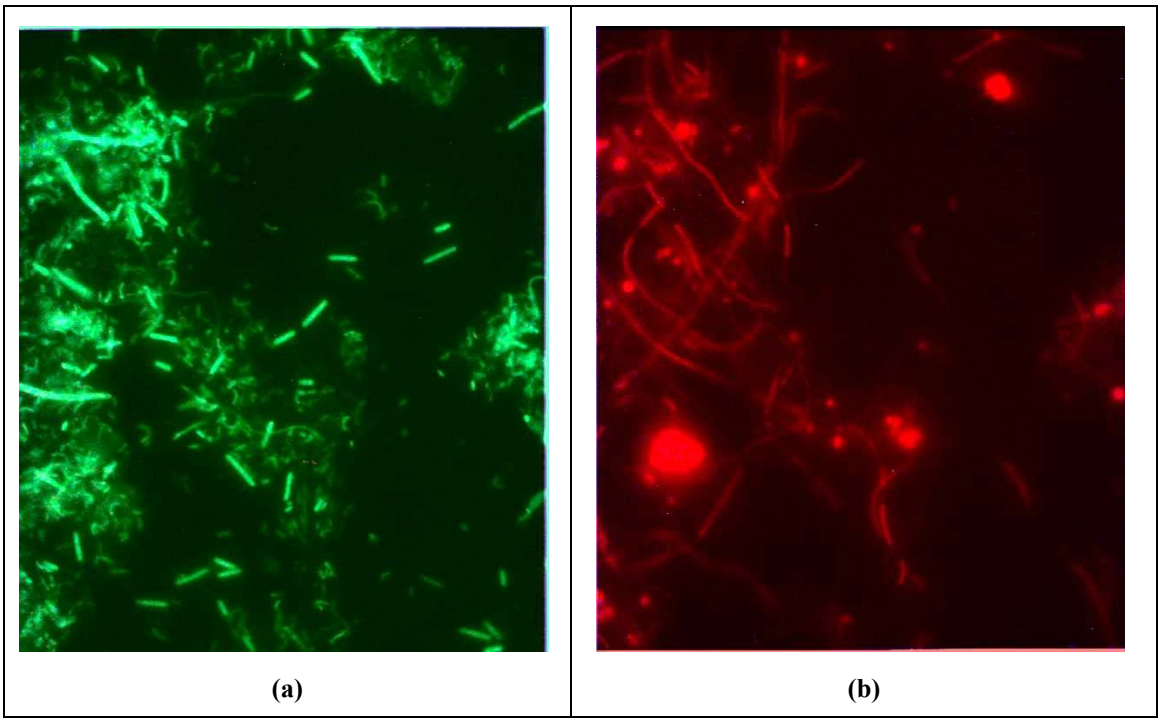
The precise role of SRB in the samples is not clearly defined although as mentioned earlier, sulphide is capable of chemically reducing azo dyes. SRB are commonly detected in anaerobic reactors and their abundance has been shown to vary relative to sulphate levels (Godon *et al*., 1997; Merkel *et al*., 1999). The sources of sulphate for use as a terminal electron acceptor in the ABR are likely to be compounds in the synthetic feed (ca. 50 mg/L) and also the dye waste (up to 1 770 mg/L). The 5 %, then 10 % dilution of the dye waste in the influent would therefore result in a combined influent sulphate level of less than 250 mg/L. Although this concentration is above 100 mg/L, a limiting concentration for sulphate reduction (Overmeire *et al*., 1994), it seems unlikely that this sulphate concentration was sufficiently high to explain the observed sulphide production in the ABR, and the two and a half times increase in SRB. Cell counts in compartments 1 and 2 rose from < 5 % at day 60 to ca. 12 % at Day 100 for probe DSV698, and cell counts for the less specific probe SRB385 showed a similar increase. Based on these increases, it appeared that favourable conditions for sulphidogenesis existed in the first two compartments of the ABR.

The range of compounds known to be used as terminal electron acceptors by sulphidogenic bacteria has been extended. Other than sulphate and sulphite, other inorganic ions such as nitrate, nitrite, and chromate together with organic molecules such as fumarate and the sulphonic acid taurine, can serve as terminal electron acceptors (Barber, 1999). All but one of the dyes typically found in the waste stream were sulphonated dyes. The dye waste, therefore, provided a considerable source of sulphonate for use as a terminal electron acceptor. Members of the genus *Desulfovibrio* and also *Bilophila wadsworthia* are among the growing list of bacteria shown to reduce sulphonates (Plumb *et al*., 2001). Although metabolism of sulphonate under anaerobic conditions is not completely understood, it appears that SRB

may both directly (sulphonate reduction) and indirectly (sulphide production) aid in the overall degradation of the dye waste.

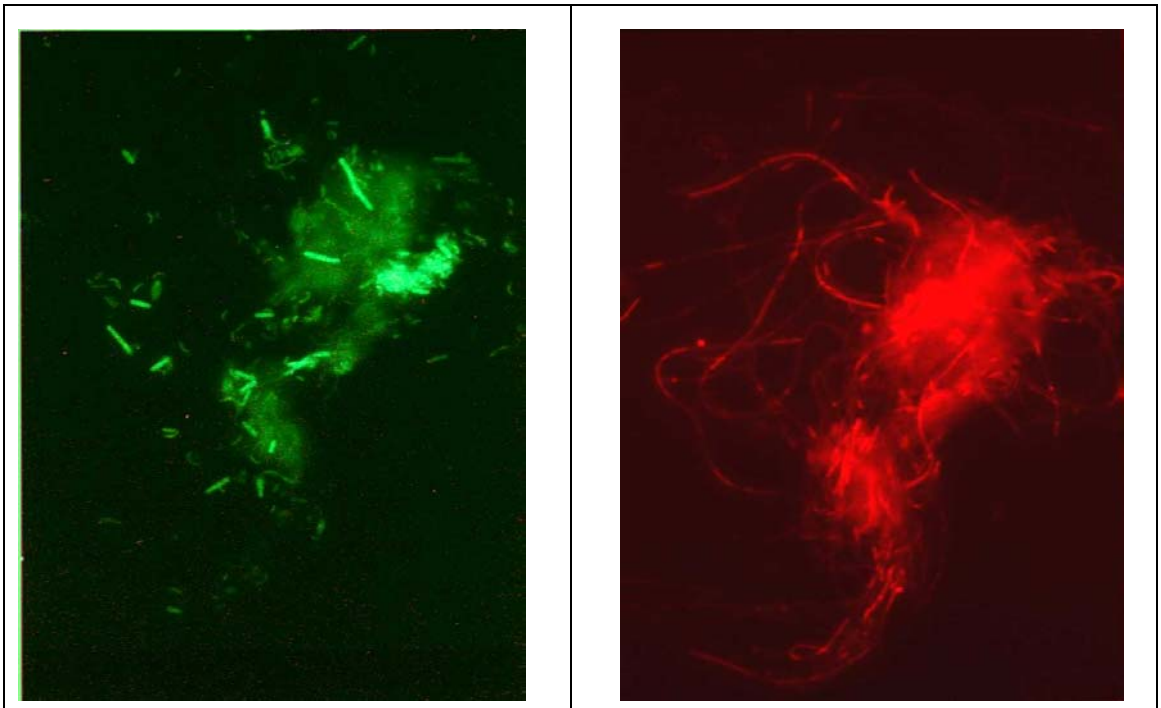
A noticeable trend with respect to the archaeal population members in the compartments sampled was the increase in the relative proportion of sarcina or tightly clustered microcolonies of cells detected using the universal archaea (ARC915) probe. Although some sarcina were present in the day 60 sample, numbers appeared to increase by Day 80 and were numerous in the day 100 sample, which correlated with the observed increased methanogenic activity, based on the biogas composition (**Section 3.4.9**). The sarcina and tight clusters of irregular cocci detected with ARC915 were abundant in compartment 1 (**Figure 3.23 (b)**) and virtually non-existent in compartment 2 onwards, with archaeal morphotypes dominated by filaments typical of *Methanosaeta* (**Figure 3.24 (b)**) and to a lesser degree *Methanospirillum*-like morphotypes with other single rods.

From the FISH results and the rRNA clone library constructed from day 100 compartment 1 sample material (see below), methanogenic species of archaea were shown to be present in compartment 1. These organisms were considered metabolically active, based on the strength of the fluorescent signals, thus it was deduced that potential toxicity of the dye compounds towards the methanogens was negligible at the dye loading rate investigated. From this study, it was not possible to discern any inhibition of methanogenesis in the reactor compartments.



(a) **(b)**

FIGURE 3.23: Whole cell hybridisation of a sample taken from compartment 1 of the laboratory-scale ABR treating a food dye wastewater, on day 80, showing the same field probed with EUB338 (a) and ARC915 (b).



(a) **(b)**

FIGURE 3.24: Whole cell hybridisation of a sample taken from compartment 3 of the laboratory-scale ABR treating a food dye wastewater, on day 100, showing the same field probed with EUB338 (a) and ARC915 (b).

Whole cell hybridisation with fluorescent 16S rRNA oligonucleotide probes showed the separation of microbial trophic groups through the ABR compartments. The universal bacterial (EUB338) and archaeal (ARC915) probes showed an aspect of this separation, with a higher proportion of bacterial population members relative to archaea in the front of the reactor contrasting with compartments 7 and 8 in which comparatively few bacteria were detected.

3.4.11 Construction of an Archaeal 16S rDNA Clone Library

An effort to identify the archaea observed using FISH with ARC915, in particular the clusters of irregular colonies present in the first compartment (which did not hybridise with the available oligonucleotide probes), was made using 16S rDNA clone library construction (Plumb *et al*., 2001). To obtain a clone library, to retrieve rDNA sequence information and to perform a comparative analysis of the retrieved sequences yields information on the identity or relatedness of new sequences in comparison with the available databases and provides a minimal estimate of the genetic diversity. However, this is not proof that the retrieved sequences were from cells thriving in this habitat.

The DNA from a sludge sample, collected from compartment 1 on day 100, was extracted and purified by the method described in **Appendix 1** (Plumb *et al*., 2001). The extracted DNA was amplified by the polymerase chain reaction (PCR), cloned into competent *E. coli* cells and the full-sized-insert clones were screened using the two restriction endonucleases *Hha*I and *Hae*III and visualised using 2.5 % agarose gel

electrophoresis. Clones possessing different restriction profiles were selected for sequence analysis. Automated DNA sequencing was performed on ABI Model 377 sequencer (Applied Biosystems) (**Appendix 1**).

Total community DNA was extracted from a Day 100 compartment 1 sample. A total of 98 clones, with full-sized inserts were obtained for analysis after PCR amplification of archaeal 16S rDNA. Screening of these 98 clones using the restriction endonucleases *Hha*I and *Hae*III, grouped the clones into 10 operational taxonomic units (OTUs). OTUs 1 and 2 were the largest groupings, containing 42 and 41 clones respectively. OTU 3 contained 6 clones, OTUs 4 and 5 contained 2 clones, with the remaining OTUs containing 1 clone each. Where possible, at least 2 representatives of each OTU group were sequenced. Phylogenetic analysis of partial sequence data (ca. 650 nucleotides) from clones representing OTU groupings was used to determine the phylogenetic position of each clone. According to these analyses, all but one of the clones was affiliated with the domain *Archaea*. Sequence data from each of OTUs 1 and 2 representative clones grouped closely with sequence data from *Methanosaeta concilii*. Together with clones from OTUs 5, 6, 7 and 9, also grouping with the representatives of OTUs 1 and 2, and assuming that unsequenced members of OTUs 1 and 2 possessed similar sequences, then ca. 90 % of all the clones obtained were affiliated with the *Methanosaeta* genus. Clones grouped into OTU 3 were closely related to *Methanobacterium formicicum*, and comprised ca. 6 % of the library. The only two clones in OTU 4 grouped closely with sequence data from *Methanospirillum hungatei*. The OTU 10 single clone (AC72) was closely affiliated with sequence data from *Methanomethylovorans hollandica*, a member of the *Methanosarcinaceae*.

The dominance of *Methanosaeta* phylotypes in the clone library was not unexpected as the numerous *Methanosaeta*-like sheathed filaments that hybridised to the ARC915 probe also hybridised to the *Methanosaeta*-specific probe MX825. Species of *Methanosaeta concilii* are known to be important members of anaerobic methanogenic communities due to their ability to metabolise acetate into carbon dioxide and methane, and their numerical dominance compared to other methanogens in anaerobic reactors has been previously reported (Merkel *et al*., 1999; Sekiguchi *et al*., 1999; Domingues *et al*., 2001; Leclerc *et al*., 2001). The presence of phylotypes closely related to other well studied methanogenic species, *Methanobacterium formicicum* and *Methanospirillum hungatei* correlated with microscopic observations. These two species utilise hydrogen and formate for methanogenesis and are commonly found in anaerobic reactors.

The observation of many tightly clustered sarcina-like irregular cocci detected with the ARC915 probe suggested that *Methanosarcina* species would be represented in the clone library, although these cells did not hybridise with the *Methanosarcina* specific probe MS821. This was not the case. One phylotype, very similar (99 % sequence similarity) to sequence data from the newly described *Methanomethylovorans hollandica* (Lomans *et al*., 1999), was the only other representative of the *Methanosarcinaceae* in the clone library. Unlike *Methanosarcina* species, this organism does not utilise hydrogen, carbon dioxide or acetate, and was isolated from freshwater sediment using dimethyl sulphide as a sole source of carbon and energy. The morphology of this species has been described as being between that of *Methanosarcina*

cell clusters and the irregular cocci typical of *Methanolobus* and *Methanococcoides* species (Lomans *et al*., 1999). This accurately describes the morphotypes seen in this study, and the aggregation of small clusters into larger microcolonies has also been observed for this species. As an obligate methylotroph, *Methanomethylovorans hollandica* has been claimed to be a key consumer of dimethyl sulphide and methanethiol in anaerobic environments, although it is also able to utilise methanol and methylamines. It seems possible that compounds such as methanethiol are formed during the reduction of sulphonate from the dye compounds. This methanethiol could then be utilised by *Methanomethylovorans hollandica* for methanogenesis. Alternatively, it has been shown that methanethiol and dimethyl sulphide formation readily occurs under anaerobic conditions where high sulphide concentrations exist (Lomans *et al*., 1999). It seems likely that conditions in the front of the ABR approximated this, resulting in production of these two key substrates for the growth of *Methanomethylovorans hollandica*. Although not as numerous as *Methanosaeta*, these methanogenic cocci comprised a significant proportion of the archaeal biomass, possibly as much as 15 %, and appeared active on the basis of the intense fluorescence observed using FISH. Together with sulphidogenic bacteria, *Methanomethylovorans hollandica* appears to play an important role in the overall degradation of the dye waste.

The use of molecular approaches in this study provided useful descriptions of the microorganisms actively involved in the degradation and decolourisation of the industrial dye waste.

3.4.12 Conclusions

1. Anaerobic degradation and decolourisation of the dye wastewater, in the ABR, was achieved. The methanogenic activity was high throughout the reactor, the organic content of the influent was reduced by ca. 70 % and colour was reduced by ca. 90 %.

2. Most of the colour reduction was achieved in compartment 1.

3. Efficient degradation may be dependent on the composition of the wastewater, which is variable and may upset the degradation process. This could be alleviated with a dedicated, on-site reactor.

4. Application of molecular methods to describe the microbial populations showed considerable diversity in both the eubacteria and archaea involved in the treatment process.

5. FISH enumerations showed that the gamma proteobacteria, and bacteria within the *Cytophaga-Flexibacter-Bacteroides* phylum together with sulphate reducing bacteria were prominent members of a mixed bacterial population. It is suggested that sulphate reducers may contribute to the treatment process through their metabolism of dye-associated sulphonate groups.

6. A combination of FISH probing, and the analysis of 98 archaeal 16S rDNA clone inserts, revealed that together with the bacterial population, a methanogenic population dominated by *Methanosaeta*, together with species of *Methanobacterium* and *Methanospirillum*, and a relatively unstudied methanogen *Methanomethylovorans hollandica*, contributed to the successful anaerobic treatment of the industrial waste.

7. FISH was successful in characterising the microbial populations present in the reactor compartments and those cells which did not hybridise to the oligonucleotide probes were successfully sequenced and identified through the construction of the clone library. Thus, molecular techniques added to the understanding of this process. The results were still difficult to interpret, however, because of the complexity and variability of the dye wastewater. Since new batches of the wastewater were collected every few weeks, and the composition of these was variable, it is believed that the system was too complex and dynamic to allow for complete evolution of the microbial species in the reactor compartments.

Chapter 4

Sucrose in the ABR

Investigation of the food dye wastewater, in **Chapter 3**, showed that the wastewater was effectively decolourised and the COD degraded, in the ABR. The application of molecular techniques provided an indication of the microorganisms actively involved in the metabolism. However, the complexity and variability of the dye wastewater made the results difficult to interpret. Thus, in this next phase of experimentation it was decided to use a well defined, labile sucrose/protein feed to investigate the changes in the microbial communities, with changes in the HRT. The results of the physical and chemical analyses are presented and discussed in **Section 4.2**. The characterisation of the microbial communities and the evolution of these communities, with changing HRT, are presented in **Section 4.3**.

4.1 INTRODUCTION

The unique design of the ABR promotes the development of various profiles of microbial communities within each compartment. The microbial ecology within each reactor compartment will depend on the type and amount of substrate present as well as parameters such as pH and temperature (Barber and Stuckey, 1999). In principle, this facilitates a fundamental analysis of the effects of various components in the inlet stream on the population dynamics and microbial interactions.

4.1.1 Hypotheses and Objectives

A study was undertaken to investigate the hypothesis that, due to the unique design of the ABR, various profiles of microbial communities develop within each compartment and that in the acidification zone of the ABR (first compartments) fast growing bacteria capable of growth at high substrate levels and reduced pH will dominate. A shift to slower growing scavenging bacteria that grow better at higher pH is expected to occur towards the end of the reactor. Experiments were conducted with the following specific aims:

1. To determine the effect of changing hydraulic retention time (HRT) on the operation of the reactor.

2. To determine the effect of changing HRT on the composition and activity of the microbial populations.

3. To quantify the ratios of eubacteria to archaea and to determine the distribution of these groups through the ABR.

4. To identify (genus level) the acetoclastic and hydrogen-utilising methanogens active in the ABR, thus relating the microbiology to function.

5. To correlate the microbial data with the chemical data.

4.1.2 Experimental Design

A laboratory-scale ABR was set up in a temperature-controlled (35 °C) room. The reactor was seeded with 7.68 L (0.96 L/compartment) of screened digester sludge taken from the Umbilo Sewage Works. This sludge had a total solids content of 28 g/L, of which 20 g/L were volatile solids. This gave an inoculum of 19.2 g biomass per compartment, or 153.6 g biomass in the reactor. The sludge was allowed to settle for one week before feeding began. The feed connections for the experiment were set up as illustrated in **Figure 4.1**. Gas production was measured by water displacement, however, this was found to be inaccurate as gas was seen to escape through the effluent tube, and thus a COD balance was not done over this reactor.

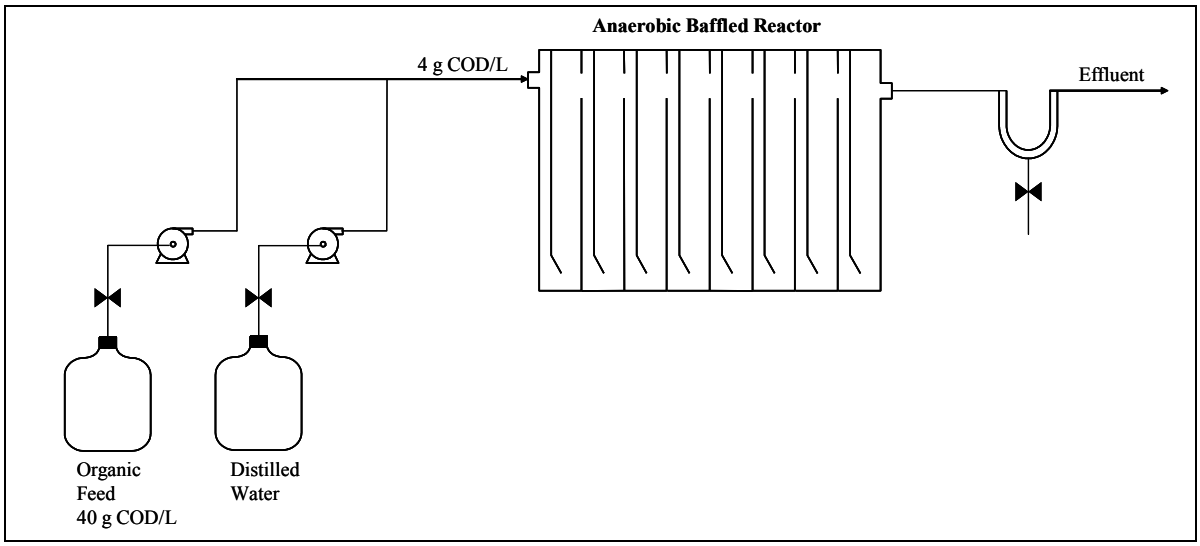


FIGURE 4.1: Schematic diagram of the experimental set-up of the laboratory-scale ABR used to assess the microbial population dynamics with changes in the hydraulic retention time (not to scale).

The feed solution was continuously pumped, by a variable speed Watson-Marlow peristaltic pump (model 101U/R), and diluted by distilled water pumped by a Watson-Marlow variable-speed peristaltic pump (model 503S). The two streams combined to form a single feed stream just before the inlet to the reactor. The treated effluent passed through a glass U-tube for level control and a biomass trap before running to the effluent reservoir. Effluent samples were taken from the bottom of the U-tube.

The standard sucrose/protein feed solution (Barber, 1999) was made up, as described in **Appendix 1**. For the purposes of simplifying stoichiometric calculations, the molecular formula was taken to be that of sucrose ($C_{12}H_{22}O_{11}$), although sucrose made up only 75 % of the total COD of the feed solution. The feed was strongly buffered with bicarbonate because sucrose degrades very quickly to acetic acid. The feed and nutrients were autoclaved for 40 min at 110 °C (HLMC HL-340 autoclave). Feeding began with an HRT of 80 h and an ORL of 1.2 g COD/L.d. The flow rates were gradually changed with a stepwise

decrease in the HRT to 45 h, 20, 18, 12 and then back to 20 h (**Table 4.1**). Steady state was assumed to be reached when operating parameters and removal efficiencies remained constant over three HRTs. However, given the very slow adaptation of mixed anaerobic cultures to change, this is only a quasi-steady state (Grobicki and Stuckey, 1991).

TABLE 4.1 : Summary of the operating conditions.

Day	HRT	Organic Loading Rate	Organic Loading Rate
0	**80**	**1.20**	**0.060**
41	**45**	**2.14**	**0.107**
76	**20**	**4.80**	**0.240**
145	**18**	**5.34**	**0.267**
227	**12**	**8.00**	**0.400**
306	**20**	**4.80**	**0.240**

4.1.3 Analytical Methods

Experimental data were obtained from grab samples, taken from the ABR, once or twice a week. The reactor was sampled and analysed as described in **Section 3.2.4**. The VFAs were analysed by HPLC (**Appendix 1**).

To assess the changes in the microbial populations, sludge samples were taken from each compartment, and fixed with 4 % paraformaldehyde. These samples were taken when the reactor had reached steady-state, at a particular HRT, and prior to a change in the HRT. The method for sample fixation, probe hybridisation and analysis is detailed in **Appendix 1**. The cells were dual stained with DAPI (4',6-diamidino-2-phenylindole – DNA stain) and the fluorescent 16S rRNA-targeted oligonucleotide probes (**Table 4.2)**. The methanogens were the focus of this investigation. The various groups of the eubacteria were not probed. The oligonucleotides comprised domain-specific probes for the eubacteria (EUB338) and archaea (ARC915) and order-, family-, and genus-specific probes for several phylogenetic groups of methanogens. The general archaeal probe, ARC915 also encompasses most non-methanogenic archaea, however, since methanogens are the only presently known archaea that are not restricted to extreme environments, the advantages of probing can be realised by using the general archaea probe in combination with methanogenic probes (Raskin *et al*., 1994). The hybridisations were viewed with a Zeiss Axiolab epifluorescence microscope, using Zeiss filter sets 01 (DAPI), 09 (Fluoroscein) and 14 (Rhodamine).

The probe sequences and hybridisation conditions are given in **Appendix 1**.

TABLE 4.2: Specificities of the rRNA-targeted oligonucleotide probes used for whole-cell hybridisation in samples taken from the laboratory-scale ABR.

Probe	Specificity	Reference
ARC915	Universal Archaea	(Stahl and Amann, 1991)
EUB338	Universal Eubacteria	(Stahl *et al*., 1989)
MX825	Order III: Methanomicrobiales Family IV: *Methanosarcinaceae* Genus V: *Methanosaeta*	(Rocheleau *et al*., 1999)
MS5	Order III: Methanomicrobiales Family IV: *Methanosarcinaceae* Genus V: *Methanosaeta* Species: *concilli*	(Rocheleau *et al*., 1999)
MS821	Order III: Methanomicrobiales Family IV: *Methanosarcinaceae* Genus I: *Methanosarcina*	(Rocheleau *et al*., 1999)
MB4	Order III: Methanomicrobiales Family IV: *Methanosarcinaceae* Genus I: *Methanosarcina* Species: *barkeri*	(Rocheleau *et al*., 1999)
MG1200	Order III: Methanomicrobiales Family I: *Methanomicrobiaceae* Family II: *Methanocorpusculaceae* Family III: *Methanoplanaceae*	(Sekiguchi *et al*., 1999)

4.2 OPERATION OF THE ANAEROBIC BAFFLED REACTOR

The laboratory-scale reactor was operated for a period of 350 d. The results of these analyses are presented and discussed.

4.2.1 Reactor pH

The measured pH values of compartments 1 and 8 are shown, since with the theoretical horizontal separation of acidogenesis and methanogenesis through the reactor, the pH in compartment 1 should be lower than in compartment 8, due to the production of VFAs. The results were analysed to determine any effects of changing the HRT on the measured pH.

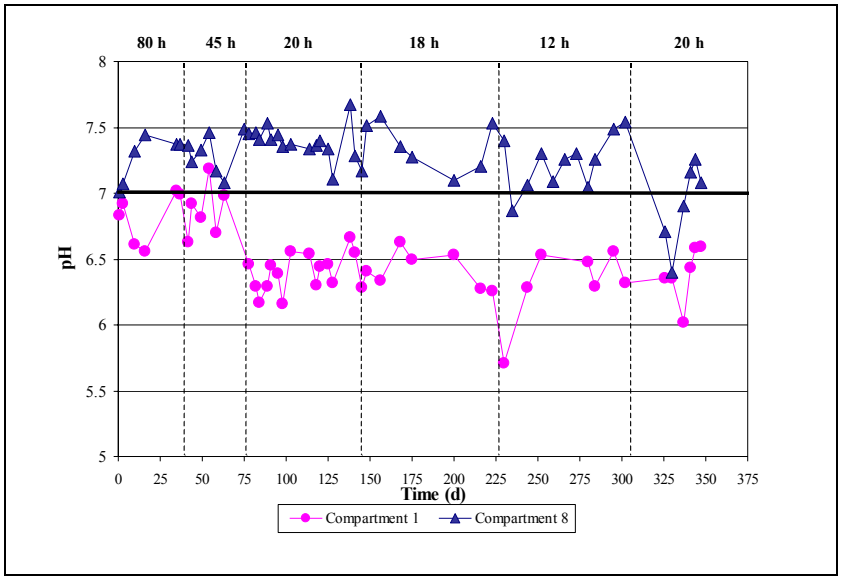


FIGURE 4.2 : Plot of the pH profiles in the laboratory-scale ABR.

Analysis of the data indicates that changes in the HRT did have an effect on the reactor pH. The results support the hypothesis that the pH in compartment 1 is lower than the pH in compartment 8 due to the separation of acidogenesis and methanogenesis. In the plot, the bold line indicates pH 7. Generally, the pH in compartment 1 was below pH 7 and above pH 7 in compartment 8. The changes in HRT are indicated as dotted lines on **Figure 4.2**.

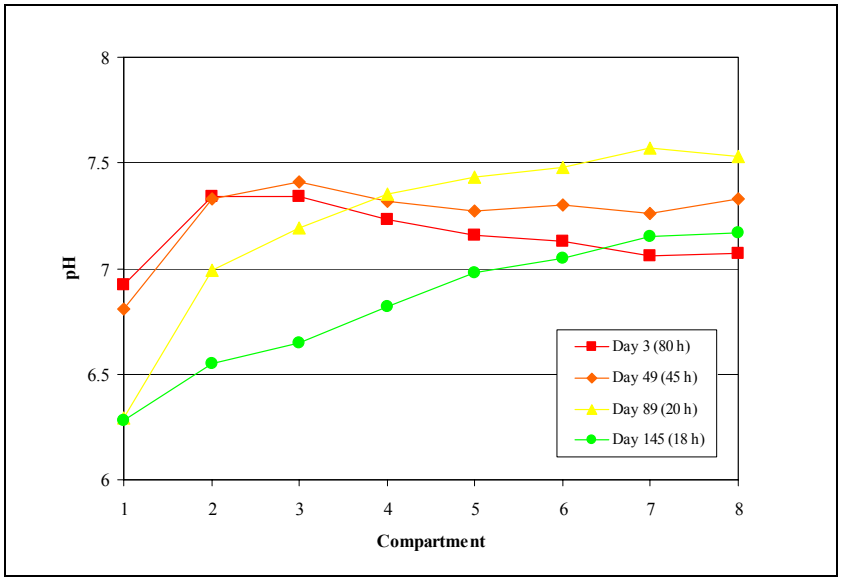


FIGURE 4.3 : Plot of the pH profiles through the laboratory-scale ABR at different times during the experimental period.

The pH values in each compartment (**Figure 4.3**) showed that, for the 80 h and 45 h HRTs, the pH was lowest in compartment 1 and increased to a maximum in compartment 2; this suggests that these were the two most metabolically active compartments of the reactor. When the HRT was 20 h (day 89), the pH in compartment 1 was lower and the pH increased in each compartment through the reactor, with the

maximum in compartment 8. A similar trend was observed during the 18 h HRT (day 145), however, the pH values were much lower than with the 20 h HRT, indicating increased acidogenic activity due to the increased OLR.

When the HRT was 80 h and 45 h, the pH in compartment 1 was relatively high, between 6.5 and 7. This decreased to pH 6.49 when the HRT was changed to 20 h, which indicates that the increased OLR resulted in increased acidogenic activity, and a subsequent reduction of the pH in compartment 1. The pH in compartment 1 remained relatively constant, around pH 6.4, except for a drop to pH 5.71 when the HRT was changed to 12 h, on day 227. At this point the pH in compartment 8 also decreased, to 6.87. This suggests that increasing the organic load to 8 g COD/L.d was a temporary shock load to the reactor, resulting in an over-production of VFAs, reduced methanogenic activity and a subsequent reduction in pH throughout the reactor. However, the pH had recovered to pH 6.3, in compartment 1, and pH 7.06 in compartment 8, by day 244. The results show another pH decrease in both compartments 1 and 8, when the HRT was returned to 20 h. Again, the recovery of the pH was almost immediate.

From these pH data it can be concluded that different metabolic processes were occurring in compartments 1 and 8 of the reactor and, therefore, that they were populated by different types of microorganisms.

4.2.2 Reactor Solids

The measure of volatile solids was taken as an indication of the biomass concentration. **Figure 4.4** is a plot of the cumulative total solids washed out of the reactor. These results show that the majority of the biomass washout, ca. 91 %, occurred during the first 100 d of operation of the reactor. The solids washout was almost entirely volatile solids during the 80 h and 45 h HRTs. The most significant amount of washout was observed with the HRT change from 45 h to 20 h. This could have been due to the increased gas production due to the higher OLR. After this period, there was very little biomass washout, even with the changes in HRT. The solids washout, with the HRT change to 18h and 12h, was low, which suggests stabilisation of the biomass.

These results show a significant loss of solids from the reactor. The difference in mass between the total and volatile solids can be attributed to ash or tightly hydrated water. The ash content of the feed was high, with the sodium bicarbonate alone contributing 24 g/d of ash, or 8.64 kg ash over 360 d.

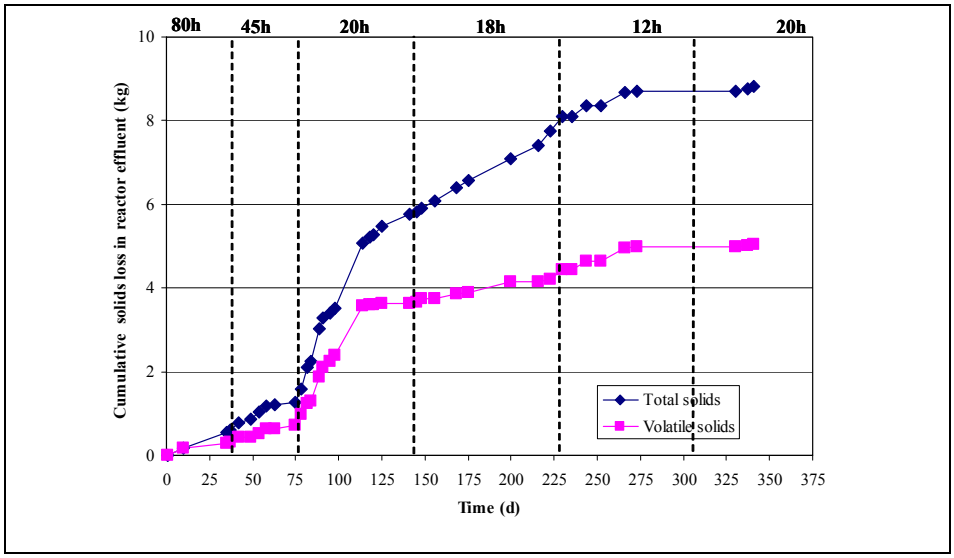


FIGURE 4.4 : Plot of the cumulative solids lost from the laboratory-scale ABR.

The level of the sludge bed in each compartment was measured with every set of analyses. **Figure 4.5** represents a typical profile through the reactor. The sludge level was lower in compartment 1 than in compartments 2 and 3; this could have been due to increased gas production in compartment 1 and resultant carry-over of sludge into compartments 2 and 3. There were variations in the sludge levels with changes in the HRT, however, none of the level changes were significant. The sludge level was always higher in the first compartments than in the later compartments.

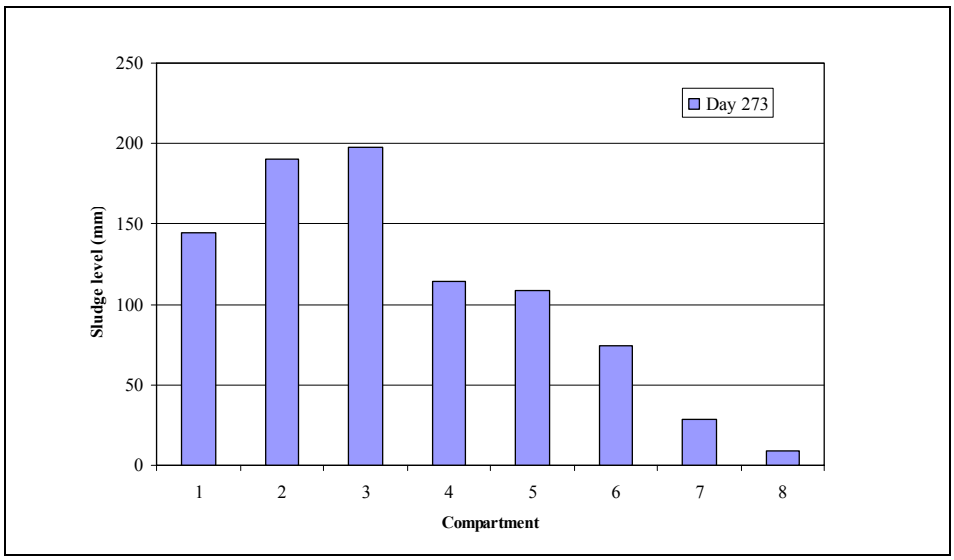


FIGURE 4.5 : Plot of the sludge level in each compartment of the ABR.

4.2.3 Reactor Chemical Oxygen Demand (COD)

Figures 4.6 and **4.7** depict the soluble COD removal in the reactor over time. Soluble COD was measured since the feed was completely soluble. Previous research has shown a correlation between low

hydraulic retention times, high hydraulic dead space and increased channelling (Grobicki and Stuckey, 1992). These factors may control the amount of biomass that is in direct contact with the substrate at any time. A low contact time between the substrate and biomass favoured the acidogens, which have faster growth kinetics and adapt better to reduced pH than the methanogens. The result is a low substrate flux into the biomass flocs, and subsequently the VFAs are washed through the reactor, largely unmetabolised, resulting in low COD removal and low pH (Nachaiyasit and Stuckey, 1997a; Nachaiyasit and Stuckey, 1997b). When the HRT was long, there was an elevated substrate flux into the flocs, resulting in high COD removal. High substrate levels and low pH values in the front compartments resulted in a selective pressure on the mixed microbial population present (McCarty and Mosey, 1991). The experimental results were analysed and compared with these findings.

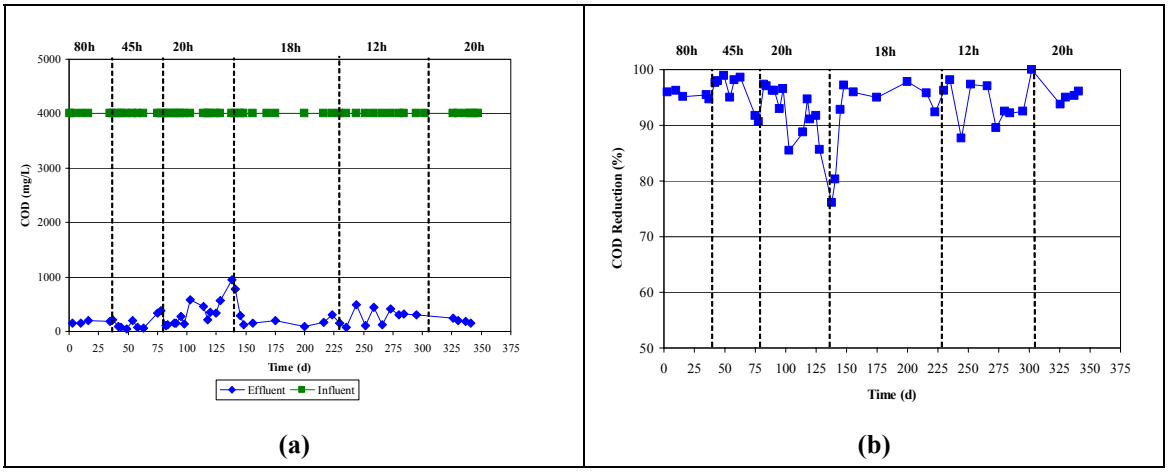


FIGURE 4.6 : Plots showing (a) the influent and effluent CODs and (b) the COD reduction in the laboratory-scale ABR.

Throughout the experiment, the soluble COD in the effluent was low, with an average COD reduction of 94 %. The effluent COD averaged 179 mg/L (standard deviation, $\sigma = 0.56$ mg/L) at the 80 h HRT, and 125 mg/L ($\sigma = 2.4$ mg/L) at the 45 h HRT. The COD removal became less efficient and more variable with the HRT reduction to 20 h; the mean effluent COD was 372 mg/L ($\sigma = 6.2$ mg/L). It then stabilised around 450 mg/L for a period of 16.8 HRTs. At this time, day 145, the HRT was reduced to 18 h, which resulted in a sharp increase in the effluent COD, to 955 mg/L. This increase in the OLR and the reduced contact time between the substrate and the biomass resulted in a lower pH in all of the compartments (**Section 4.2.1**) and thus reduced methanogenic activity such that the VFAs were not well metabolised, resulting in the increased effluent COD. However, within three HRTs (i.e. day 141) the effluent COD had reduced to 785 mg/L and to 145 mg/L by day 148. The reactor was held at the 18 h HRT for a period of 70 d and during this time, the effluent COD averaged a stable 189 mg/L ($\sigma = 1.9$mg/L). The reduction of the HRT to 12 h resulted in less efficient degradation of the soluble COD (297 mg/L) and greater variability ($\sigma = 6.4$ mg/L). This suggests that increasing the organic load to 8 g COD/L.d was a temporary shock to the reactor, resulting in reduced methanogenic activity and a subsequent decrease in COD reduction throughout the reactor. The reactor then seemed to stabilise, with the effluent COD averaging 305 mg/L for a period of 18 HRTs. The HRT was then returned to 20 h and the COD reduction

was efficient with an average value of 191 mg/L, or 95.2 % reduction (σ = 0.83 mg/L). This was stable for a period of 35 d, or 42 HRTs.

These COD data show that although the microorganisms in the reactor were responsive to changes in the HRT, the feed was efficiently degraded and recovery of operation, after a change in the HRT, was quick.

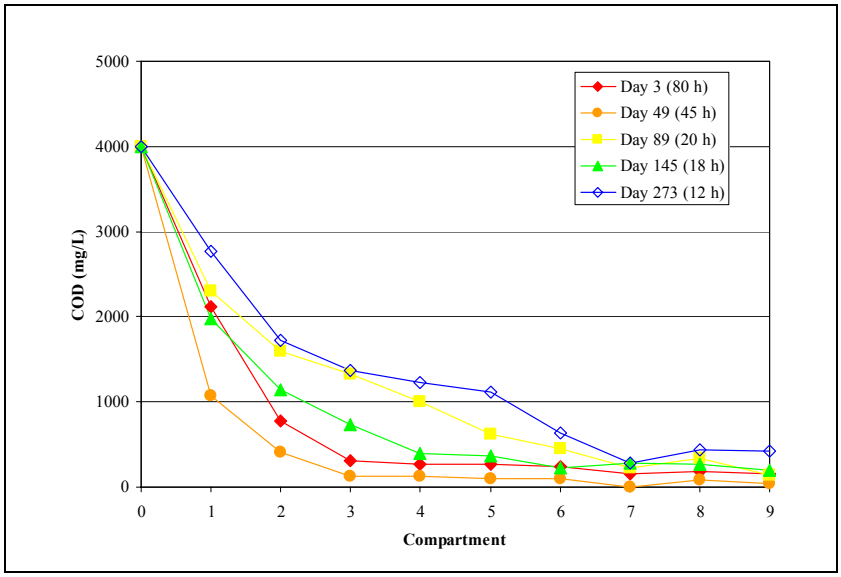


FIGURE 4.7 : Plot showing the soluble COD profiles through the laboratory-scale reactor at different times during the experimental period.

Figure 4.7 shows the COD profiles through the reactor, at different time periods during the experiment. On the plot, compartment 0 represents the reactor feed and compartment 9 represents the reactor effluent. What is immediately evident is that, in all cases, > 50 % of the COD was reduced in the first two compartments of the reactor. The horizontal separation of acidogenesis and methanogenesis was supported by the pH data, therefore it was assumed that the majority of the organic molecules in the feed were hydrolysed and converted to VFAs in the first compartments, with concurrent reduction in COD. The VFAs were then degraded by the methanogens, through the remaining compartments. This assumption can be verified by the VFA-COD data described below. The COD profiles indicate that changing the HRT did have an effect on the COD reduction, however, in all cases the COD was below 300 mg/L by compartment 7 which suggests that the compartmentalised design of the reactor prevents severe upset of the entire reactor contents due to step changes in the feed flow rate.

4.2.4 Reactor Volatile Fatty Acids

Figure 4.8 shows the total VFA concentration in the reactor effluent over 350 d of operation. The initial long HRTs (80 h and 45 h) and subsequent increased substrate/biomass contact time and enhanced methanogenic activity, resulted in efficient metabolism of the VFAs and, thus low concentrations of VFAs in the effluent. The effluent VFA concentration increased when the HRT was changed to 20 h (day 76) and remained variable (σ = 70 mg/L) during this time, which corresponded to the COD data.

From the plot it can be seen that the effluent VFA concentration increased with each HRT change, however, for all HRTs subsequent to the initial 20 h, the VFA levels soon decreased to < 10 mg/L. Stable reactor performance is indicated by an effluent total VFA concentration below 500 mg/L (Willetts, 1999), thus, it can be concluded that the reactor operation was stable since the effluent VFA was < 300 mg/L throughout the test period.

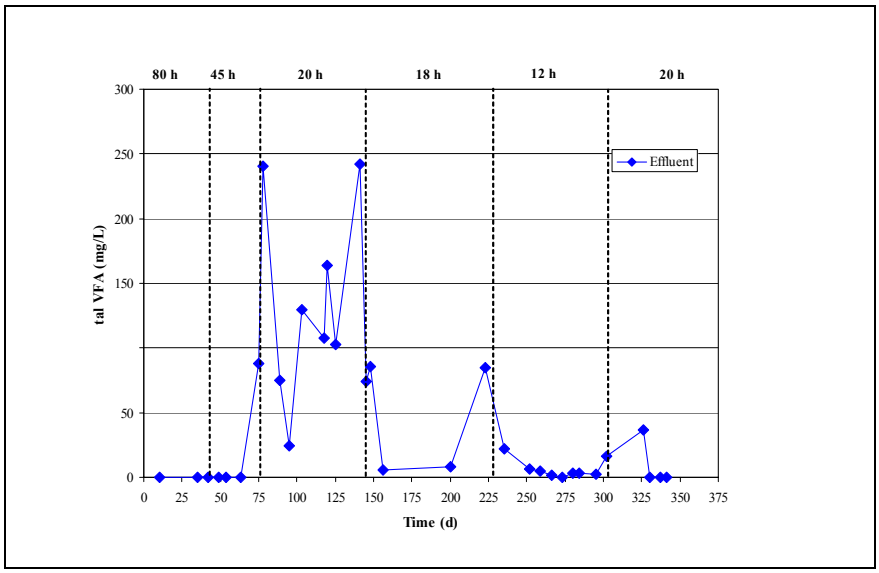


FIGURE 4.8 : Plot of the total VFAs in the laboratory-scale ABR effluent.

The VFA data in **Figure 4.8** correspond to the measured pH data in **Section 4.2.1**. The concentrations of each of the individual acids in compartment 1 and the reactor effluent are illustrated in **Figure 4.9**. Consistent with the hypothesis of acidogenesis occurring in the first compartments, **Figure 4.9 (a)** shows relatively high concentrations of acetic acid in compartment 1 of the reactor and low, or almost negligible, concentrations in the effluent. Similarly, for the other acids, the concentrations were higher in compartment 1 than in the effluent; most were not detected in the effluent. The concentration of propionic acid (**Figure 4.9 (b)**) in the reactor was low except for a peak in concentration with the HRT change to 20 h (day 76). The concentration then stabilised but became variable during the 12 h retention time and remained quite variable when the HRT was changed back to 20 h, suggesting that the methanogenic activity had not fully recovered from the 8 g COD/L.d OLR.

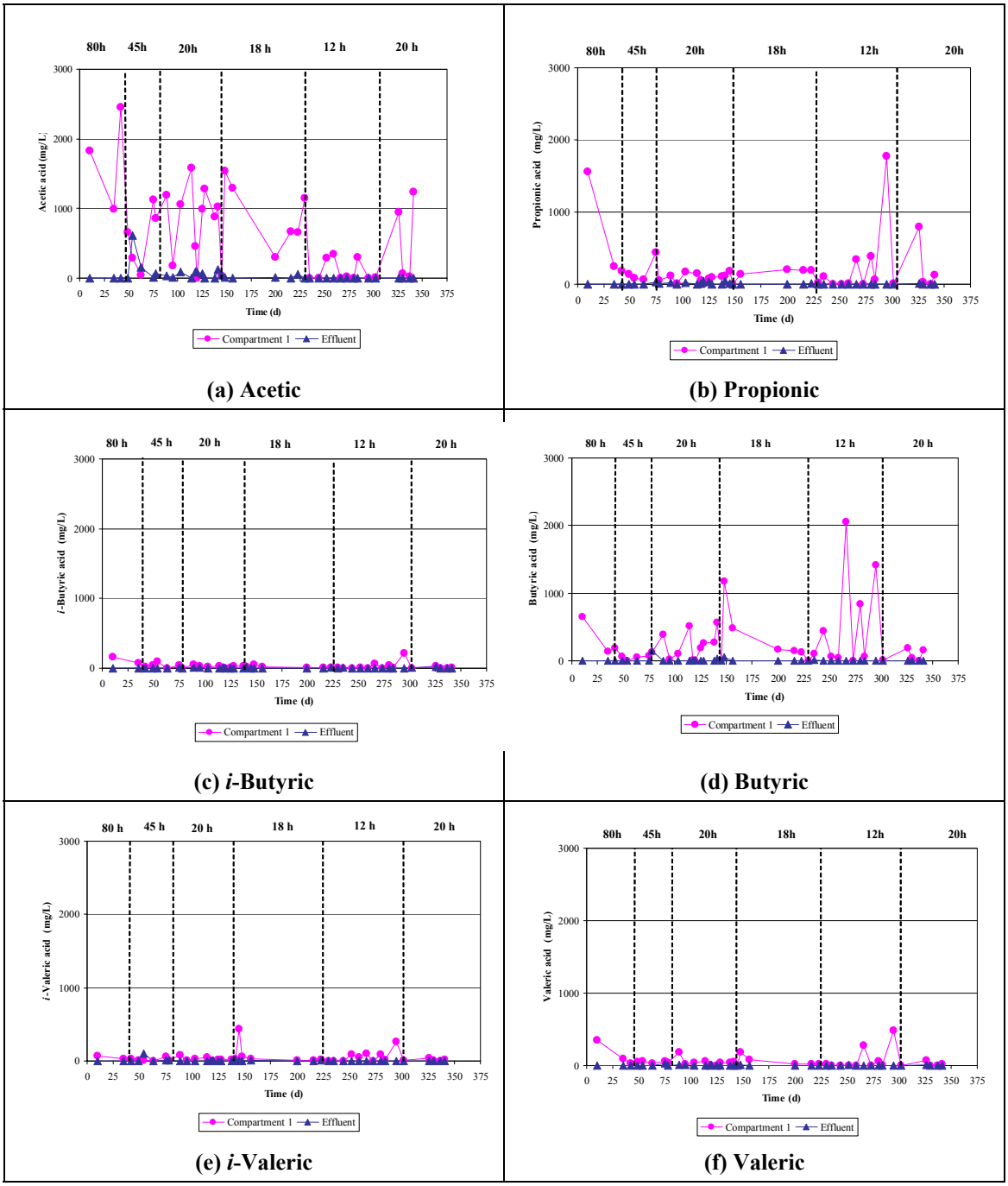


FIGURE 4.9 : Plots of each of the individual volatile fatty acids in compartment 1 and the effluent of the laboratory-scale ABR.

The *i*-butyric acid concentrations were negligible in the reactor, except for two small concentration peaks, in compartment 1, during start-up of the reactor (166 mg/L on day 10) and during the 12 h HRT, with a concentration peak of 218 mg/L on day 295. Relatively high concentrations of butyric acid (**Figure 4.9 (d)**) were detected in compartment 1, especially during start-up when a concentration of 651 mg/L was detected on day 10. The concentration was quite variable during the 20 h and 12 h HRTs. There was an increase in the acid concentration when the HRT was changed to 18 h but this stabilised quickly. Butyrate plays a significant role in the reactor dynamics as it is a store for acetate when acetate

levels are high (Grobicki and Stuckey, 1991). Since butyrate is not a feed stock for methanogens, it is converted to acetate when the acetate concentrations drop; this conversion can only occur under conditions of low hydrogen partial pressures (Sam-Soon *et al*., 1991). A shock increase in the OLR to an anaerobic system results in an increase in the hydrogen partial pressure, causing certain reactions to become non-spontaneous. This results in a build-up of VFAs, which decreases the pH of the system (Ristow, 1999). The increased OLR with the change to the 12 h HRT resulted in increased acidogenic activity. The acetate levels increased immediately and the butyrate levels were highest in the latter part of the 12 h HRT test period. The reason for this was the increased acidogenic activity, resulting in a reduction of the reactor pH and, thus a high hydrogen partial pressure. The butyrate could not be converted to acetate and, therefore, accumulated in the reactor. Comparison of **Figures 4.9 (a)** and **(d)** show that when the HRT was returned to 20 h, i.e., a lower OLR, with reduced acidogenic activity and increased hydrogen partial pressure, the acetate levels increased and the butyrate levels decreased; it was deduced that the butyrate was converted to acetate.

Negligible concentrations of *i*-valeric acid were detected in the reactor, except for two concentration peaks in compartment 1, on day 145 when the HRT was changed to 18 h and during the 12 h HRT when a concentration of 263 mg/L was detected. The same trend was observed with valeric acid, with small concentration peaks (ca. 200 mg/L) detected in compartment 1, with each HRT change. This was favourable since high concentrations of valeric acid are associated with cell lysis (Grobicki and Stuckey, 1991).

Formic acid was not detected in these analyses because with an FID gas chromatograph, formic acid is difficult to detect (Grobicki and Stuckey, 1989).

Profiles through the reactor (**Figure 4.10**) all showed a tail-off from maximum concentrations in the first compartments to very little in the later compartments, except for periods of increased VFA concentration and decreased methanogenic activity, due to an HRT change. **Figure 4.10 (a)** shows the VFA profiles in the reactor of day 10, i.e., during start-up. The acetate and propionate levels were high throughout the reactor, indicating that the methanogenic activity was low. By day 75 (45 h HRT), the reactor was more stable and the acid concentrations were lower, indicating methanogenic utilisation of the acids. The acetate levels were high in compartments 1 and 2 and then tailed off. This shows that the acetate was formed in compartment 1 and started to be utilised in compartment 2 and in subsequent compartments, by the methanogens. Propionic acid and valeric acid were both detected in compartment 1. **Figure 4.10 (c)** shows the VFA profiles in the reactor on day 145, i.e., with the HRT change to 18 h. Consistent with the pH and COD data, there was a sharp increase in the acetate concentration in the reactor due to the increased acidogenic activity. The acetate concentration did not decrease exponentially through the compartments because the methanogenic activity was low, due to the reduced pH in the reactor. By day 230 (12 h HRT) the acetate concentrations in the reactor had stabilised and the profile supported the hypothesis of horizontal separation of acidogenesis and methanogenesis. These results indicate that the anaerobic digestion process was occurring efficiently.

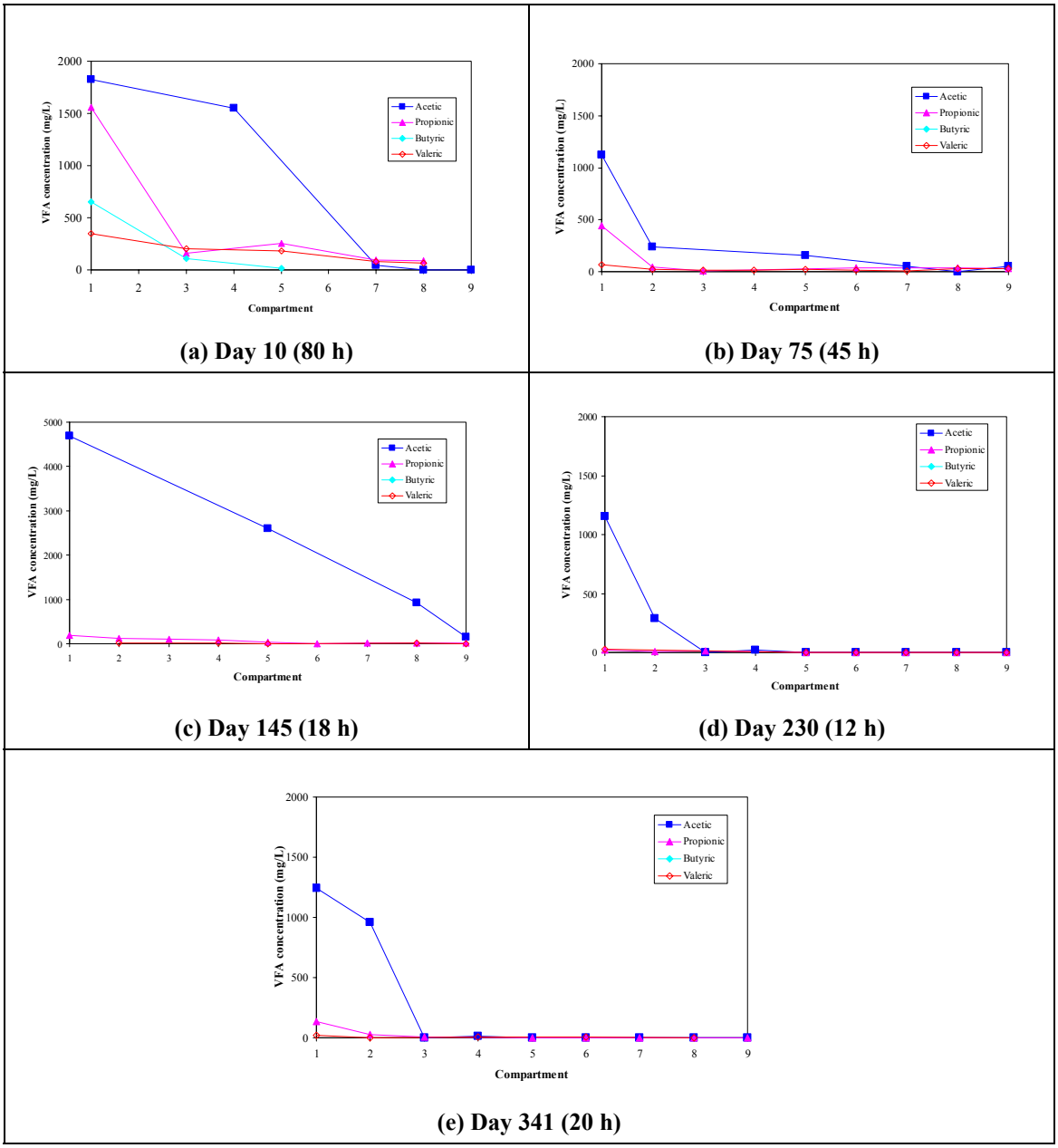


FIGURE 4.10 : Plots of the VFA profiles through the reactor, with time.

Despite high levels of VFAs detected in the acidification zone of the reactor, the longer start-up retention times and high substrate flux into the microbial flocs appeared to allow efficient metabolism to occur which resulted in an effluent that contained < 300 mg VFA /L. In principle, the concentrations of individual volatile acids, especially acetic, propionic and butyric, can be considered as the best control parameters in the liquid phase, as they give indications about the metabolic state of the obligate hydrogen producing acetogens and the acetoclastic methanogens (Weiland and Rozzi, 1991).

To summarise the information regarding the relative importance of the VFAs, the concentration is shown as a proportion of the total VFA contribution to COD. The COD equivalent of each VFA is given in **Table 4.3**.

TABLE 4.3 : COD equivalents of the volatile fatty acids.	
Volatile Fatty Acid	**g COD/g VFA**
Formic	0.348
Acetic	1.066
Propionic	1.512
Butyric	1.816
Valeric	2.037

The COD equivalent of each VFA, detected in the reactor effluent, was calculated and the total is presented in **Figure 4.11** with the measured effluent COD. These results show that in the first 150 d of operation, unmetabolised VFAs accounted for the majority of the effluent COD; the remainder would have been made up of SMPs. However, the VFA concentrations were low during the 18 h and 12 h HRTs. The reason for this is unclear as it was expected that reduced substrate/biomass contact time, enhanced acidogenic activity and reduced methanogenic activity would have resulted in less efficient metabolism of the VFAs and thus high effluent VFAs.

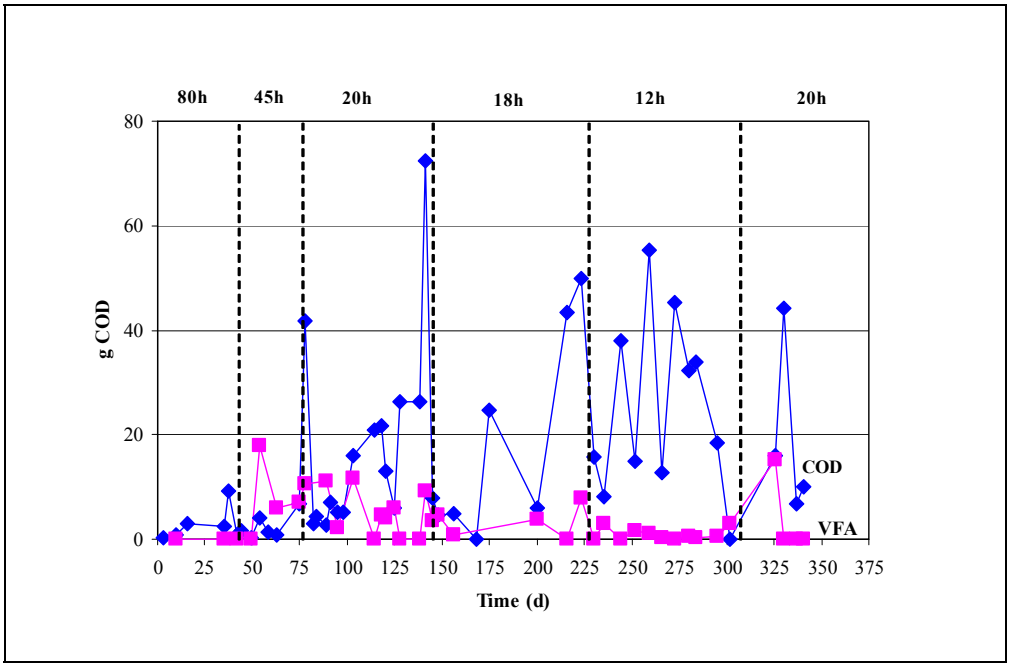


FIGURE 4.11 : Plot showing the measured COD and the calculated VFA-COD in the reactor effluent.

4.2.5 Reactor Biogas

The biogas composition was analysed regularly, by gas chromatography (**Appendix 1**) and the results are given as a percentage of each gas component, of the total gas. Due to problems with the water displacement method, biogas production could not be measured accurately.

The methane composition of the biogas in compartments 1 and 8 is shown in **Figure 4.12 (a)** and the methane profiles through the reactor, at different times during the test period, in **Figure 4.12 (b)**.

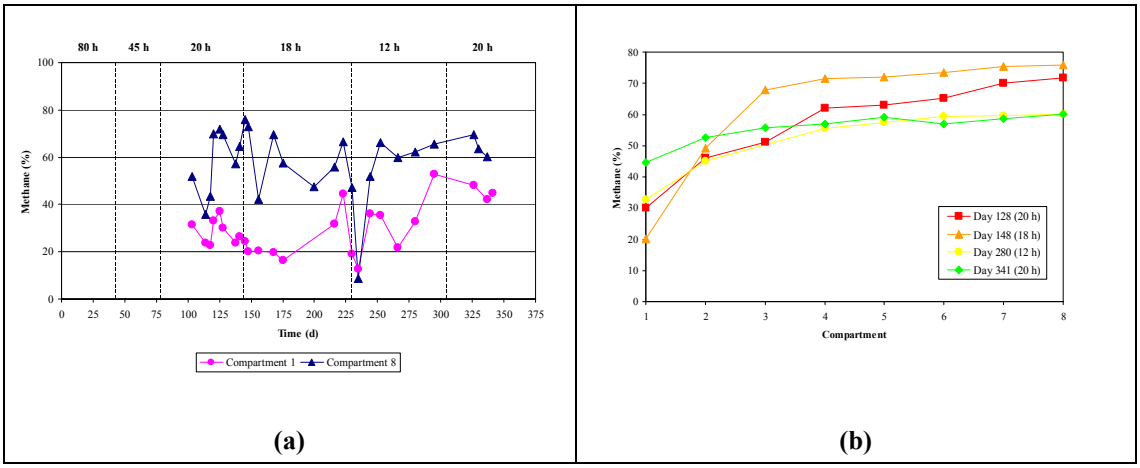


FIGURE 4.12 : Plots showing (a) the methane content of the biogas in compartments 1 and 8 and (b) methane profiles through the laboratory-scale ABR.

These results show that the methane composition in compartment 8 was always higher than in compartment 1, which supports the hypothesis of horizontal separation of acidogenesis and methanogenesis. The methane content of the biogas was relatively constant and averaged ca. 60 % in compartment 8, except for a point when the HRT was changed to 12 h and the methane content dropped to 8.7 % which suggests that the methanogens were inhibited by the organic shock load. However, recovery was almost immediate. There was a slight decrease in the methane content with each HRT change.

The profile plot (**Figure 4.12 (b)**) shows the increase in methane production through the reactor. On day 148, the methane content in compartment 1 was 20 %, this increased, with increased methanogenic activity, to 68 % in compartment 3 and then stabilised at ca. 75 % through the remainder of the reactor. With increasing OLRs, the acidogenic activity was enhanced and methanogenic activity reduced, resulting in lower methane compositions through the reactor. These changes in the biogas composition can be related to the changes in the microbial populations due to the HRT changes.

4.3 MICROBIAL POPULATION CHARACTERISATION

Research into anaerobic digestion using rRNA-based molecular techniques has provided detailed descriptions of the complex bacterial and archaeal populations present, obviating the need for anaerobic culturing techniques (Raskin *et al*., 1994; Godon *et al*., 1997; Merkel *et al*., 1999; Plumb *et al*., 2001). An obvious advantage of using FISH with rRNA-targeted nucleic acid probes is the detection of metabolically active cells, so that descriptions of the physiologically important population members can be obtained. The purpose of this investigation was to describe the microbial populations in the ABR in order to further understand the treatment process. It was hoped that a more detailed understanding of the microbiology within the ABR will provide useful knowledge for the optimisation of it use.

4.3.1 Ratios of Eubacteria to Archaea

Initial hybridisations using universal bacterial (EUB338) and archaeal (ARC915) probes revealed the distribution of both these phyla through the reactor, at each sampling date. DAPI is a blue fluorescent stain that associates with the minor groove of double stranded DNA, preferentially binding to AT clusters (Haugland, 1998). Both prokaryotic and eukaryotic cells containing intact nucleic acids fluoresce with the DAPI stain; i.e. all cells (active and inactive) containing DNA are stained. Comparisons with DAPI staining in order to quantify the relative proportions of each of these population groups were estimated, however, due to the extensive filamentous or tightly clustered microcolonies of the archaea present, accurate counting of individual cells by visual means was not always possible. The relative ratio of eubacteria to archaea in each compartment, at each HRT, was determined and the results are presented in **Figure 4.13**.

The results of day 1 indicate the composition of the reactor seed sludge. Since the seed sludge was taken from an operating CSTR at the Umbilo Sewage Works, it was expected that the sludge would consist of a mixed anaerobic culture. This is evident in the EUB:ARC ratios, which were generally 50:50 in composition. The reactor had been in operation for one day when these samples were taken, therefore, there had not been selection for particular microorganisms based on the substrate available. The ratios in compartments 6, 7 and 8 showed a predominance of the archaea, however, which suggests that the low acetate concentrations available in these compartments, at the low OLR (1.2 g COD/L.D), was being utilised by the scavenging *Methanosaeta* spp. In all compartments there were more cells visible with the DAPI stain than had hybridised with the oligonucleotide probes, i.e., the activity of the sludge was low after sampling, storage and the settling period of the sludge in the reactor.

By day 63 (45 h HRT) separation of acidogenesis and methanogenesis and selection of the appropriate microbial cultures was evident. The ratios in **Figure 4.13** show a predominance of eubacteria in compartments 1 (85 %) and 2 (68 %). Acidogenesis did not spread further than the first two compartments because of the relatively low OLR (2.14 g COD/L.d). The archaea were dominant from compartment 3, through the rest of the reactor. Although the data for compartments 7 and 8 indicate that the population was composed almost entirely of archaea, there were a lot of cells that were DAPI stained but not hybridised with the oligonucleotide probes. This suggests that the populations in these two compartments were relatively inactive and the archaea that did hybridise were probably scavenging *Methanosaeta* spp., capable of survival at very low acetate concentrations.

The cells hybridised in compartment 1, on day 145 (20 h HRT) were entirely eubacteria which, due to the higher OLR (4.8 g COD/L.d) were dominant in the first three compartments of the reactor. The population in compartment 4 was composed of almost equal numbers of eubacteria and archaea. The archaea dominated from compartment 5 through to 8 and the EUB338 hybridisations were faint, indicating low metabolic activity of the eubacteria in these compartments. The results from these samples show that the increased OLR, due to the HRT change to 20 h, resulted in increased archaeal activity within compartments 7 and 8.

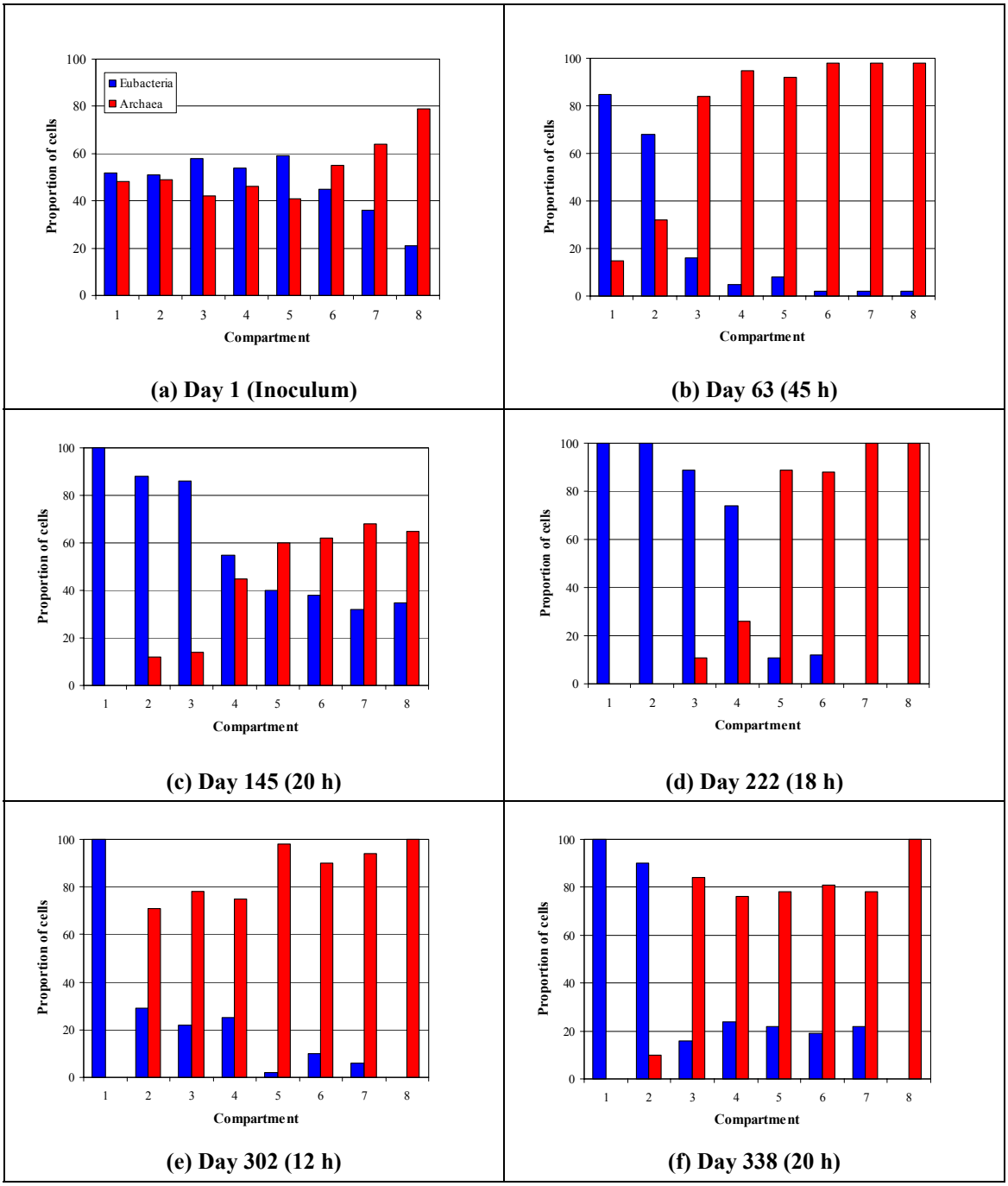
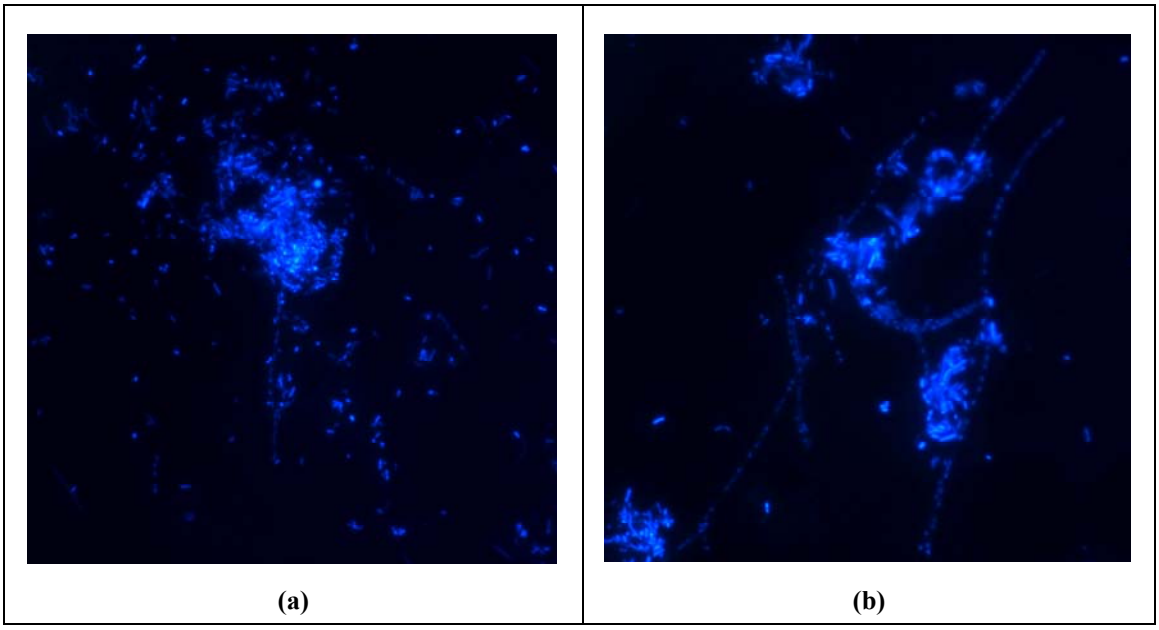


FIGURE 4.13 : Ratios of Eubacteria (EUB338-hybridised) to Archaea (ARC915-hybridised) in each compartment of the ABR, for each investigated HRT.

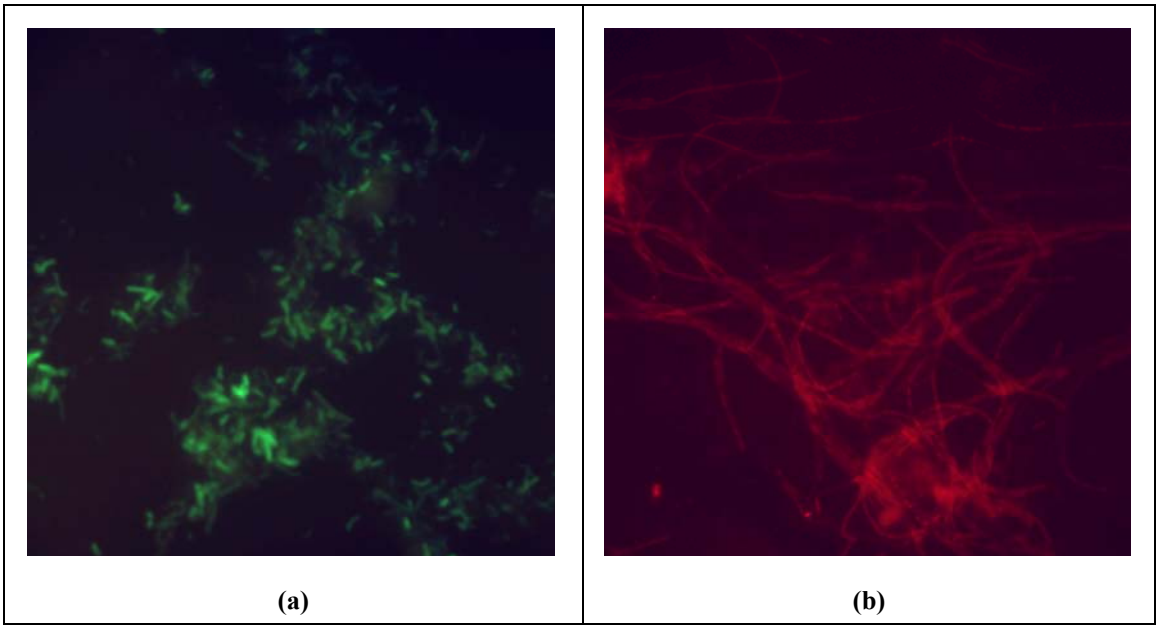
These results show a definite population shift through the reactor, with the predominance of eubacteria in the first three compartments, equal composition in the fourth compartment and predominance of the archaea, or methanogens, in compartments 5 to 8. The pH data (**Figure 4.2**) showed a definite decrease of the pH in compartment 1, with the HRT change to 20 h. It was deduced from these data that the acidogens were dominant in the first compartments and this was verified by the FISH results, as were the biogas composition data (**Figure 4.12 (b)**).



(a) (b)

FIGURE 4.14 : DAPI-stained images of the day 145 (20 h) samples showing (a) the predominance of eubacteria in compartment 2 and (b) the archaea filaments in compartment 8.

Further reduction of the HRT to 18 h and the subsequent increase in the OLR to 5.34 g COD/L.d resulted in the selection of eubacteria in the first 4 compartments of the reactor, ranging from 100 % of hybridised cells in compartment 1, to 74 % in compartment 4. The EUB338-hybridised cells accounted for ca. 70 % of the DAPI stained cells in these compartments, i.e., there were other inactive cells present. From compartment 5 onwards, the populations shifted and were dominated by the archaea. These distributions explain the pH, VFA and biogas data.



(a) (b)

FIGURE 4.15 : FISH images of the day 222 (18 h) samples showing (a) the EUB338-hybridised eubacteria in compartment 2 and (b) theARC915-hybridised archaeal filaments in compartment 8.

The day 302 data represent the microbial populations developed during the 12 h HRT. As expected, with the high OLR (8 g COD/L.d), there was a predominance of eubacteria in compartment 1. However, unexpectedly, the archaea dominated the fields counted from compartment 2 through to compartment 8. These results are in contrast to the VFA and biogas results described above. It is reported in the literature that some problems may be encountered with *in situ* hybridisation. Whole cell hybridisation can identify an individual cell, however, cells often need to be concentrated to bring a particular cell into the examined microscope fields (Amann *et al*., 1995a). Low signal intensity is another frequently encountered problem. The correlation between growth rate and cellular rRNA content means that slowly growing cells are difficult to detect because of their low cellular rRNA content. Fluorescence detection sensitivity can be severely compromised by background signals, which may originate from endogenous sample constituents (referred to as autofluorescence) or from unbound or non-specifically bound probes (referred to as background fluorescence). Autofluorescence is minimised by fixation in fresh (< 24 h) formaldehyde solution (Amann, 1995b).

A possible reason for the high archaeal counts is that, although the sludge samples and the fields selected for counting were dominated by the archaea, they were not necessarily representative of the actual sludge compostion, since only a small volume (3 μL) is applied to the slide. Although the eubacteria numbers were smaller than those of the archaea, the fluorescent signals were far brighter, indicating that the eubacteria were more active than the archaea even though the counts were lower. The archaeal hybridisations became brighter in the later compartments of the reactor.

The counts of the day 338 samples (20 h HRT) demonstrated the horizontal separation of acidogenesis and methanogenesis, with the eubacteria dominant in the first two compartments and the archaea dominant in the remaining six compartments of the reactor.

Examination of the population shifts within a compartment, over the entire test period, shows that the changes in HRT had an effect on the microorganisms. From these results, it can be concluded that there was a definite shift in the microbial populations through the ABR, with a predominance of eubacteria in the first compartments (acidogenesis) and archaea (methanogenesis) in the later compartments. The number of compartments involved in each depended on the strength of the substrate (OLR).

4.3.2 Methanogenic Activity

To gain insight into the methanogens making up the archaeal populations, the samples were hybridised with family- and genus-specific rRNA oligonucleotide probes. In a study by Raskin *et al*. (1994) it was concluded that MS821 was the preferred probe to detect *Methanosarcina* spp. and MX825 for *Methanosaeta* spp. (Sekiguchi *et al*., 1999), although MX825 was found not to hybridise to a thermophilic *Methanosaeta* spp. Rocheleau *et al*. (1999) developed two new oligonucleotide probes, MS5 and MB4. These probes were specific for *Methanosaeta concilli* and *Methanosarcina barkeri*, respectively. They found that all *Methanosaeta*-specific oligonucleotide probes (MS1, MS2, MS5 and MX825) had 100 % homology to *M. concilli* and *M. soehngenii* 16S ribosomal gene sequences, which indicated that these probes might show equivalent specificities. The *Methanosarcina*-specific

oligonucleotide probe MB4 was 100 % homologous to *M. barkeri*. The other *Methanosarcina*-specific probes (MB1, MB3 and MS821) had 100 % homology to other *Methanosarcina* spp. and MS821 had 100 % homology to two non-*Methanosarcina* spp. Probe MG1200 hybridised to the 16S rRNAs of the target organisms and did not hybridise to non-target sequences (Raskin *et al*., 1994).

Based on these findings, the probes used in this investigation are listed in **Table 4.2**. The *Methanosaeta* spp. were detected with the probes MX825 and MS5, however, better results were obtained with MX825. The *Methanosarcina* spp. were detected with MS821, which was also found to give more consistent results than MB4. The probe MG1200 is a family-specific probe, for the *Methanomicrobiaceae*, *Methanocorpusculaceae* and *Methanoplanaceae*, all of which utilise hydrogen and carbon dioxide, as well as formate for methane production. The changes in these populations were determined for each HRT change, and the results are presented in **Figure 4.16**, with detailed descriptions below.

The characteristic morphology of *Methanosaeta* (long sheathed filaments) was visualised using the universal archaeal (ARC915) probe, and confirmation of the identity of these filaments using the genus specific probe MX825 was obtained. Other morphotypes observed hybridising to ARC915 included *Methanospirillum*-like shorter filaments, single rods and sarcina-like clusters of irregular cocci often found in microcolonies of up to 50 or more cells. On the basis of the intense probe-conferred fluorescence, these cocci appeared very active.

The observation of the tightly clustered sarcina-like irregular cocci in compartment 1 (93 %) and compartment 2 (46 %), of the day 63 (45 h HRT) samples suggested the presence of *Methanosarcina* spp. However, they did not hybridise with MS821 and the sum of the counts obtained from each specific probe was < 100 %, indicating the presence of archaea not detected using these probes. Identification of these sarcina-like spp. would require isolation, amplification and sequencing of the rRNA (**Chapter 3**). They are referred to as *sarcina-like* in **Figure 4.16**, and were only detected in compartments 1 and 2.

The MX825 hybridisations in compartments 2 and 3 emitted very faint signals, indicating low metabolic activity. The majority of the archaea in compartments 3, 4 and 5 hybridised with MG1200 and were observed as shorter *Methanospirillum*-like filaments. The archaea in compartments 6, 7 and 8 were composed entirely of *Methanosaeta* spp. (MX825 hybridisations). The fluorescent signals observed in compartments 7 and 8 were weaker, verifying the reduced metabolic activity, due to the low organic load, described above. Species of *Methanosaeta concilii* are known to be important members of anaerobic methanogenic communities due to their ability to metabolise acetate into carbon dioxide and methane, and their numerical dominance compared to other methanogens in anaerobic reactors has been previously reported (Merkel *et al*., 1999; Sekiguchi, *et al*., 1999).

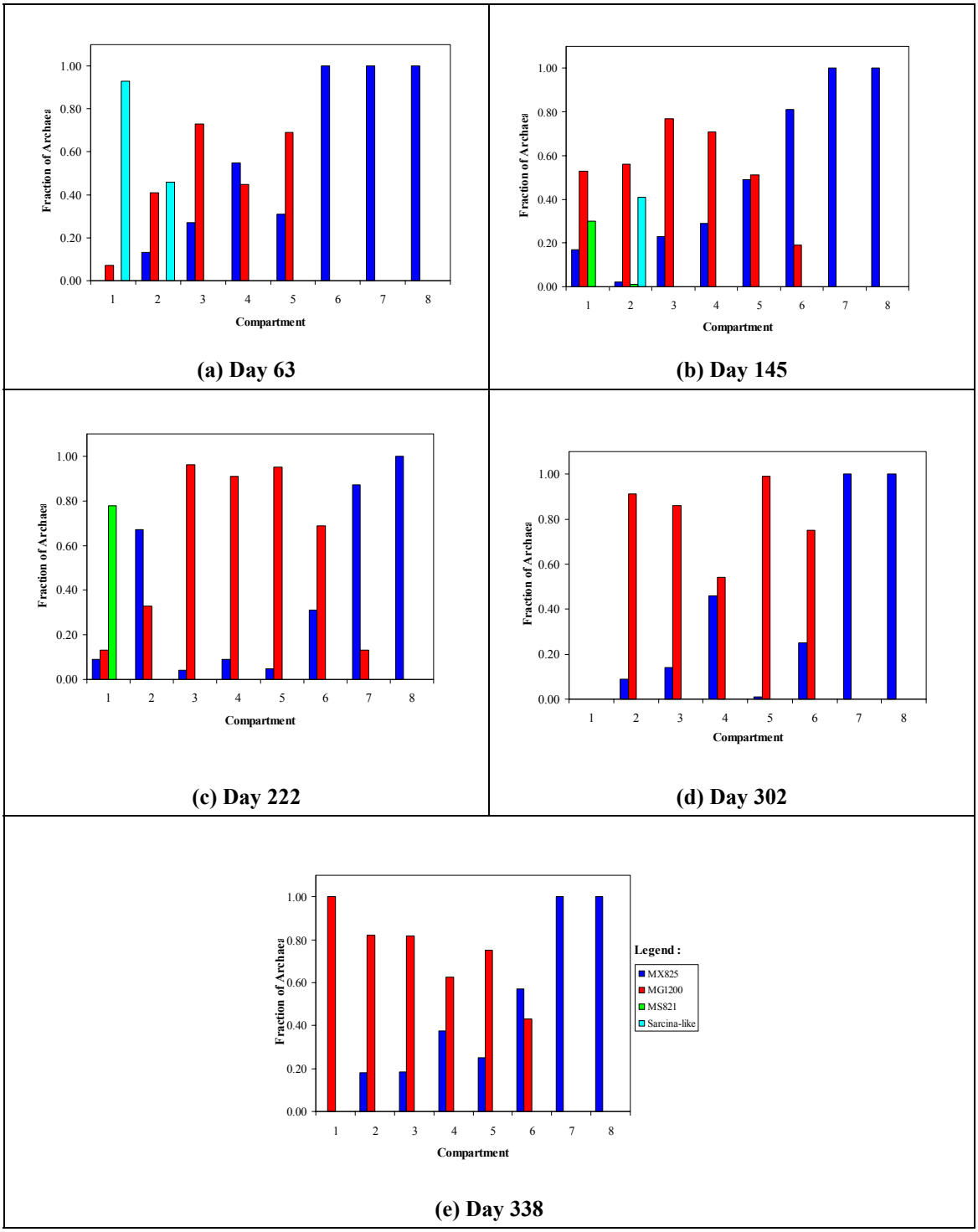


FIGURE 4.16 : Archaeal community analysis of ABR compartments 1to 8, sampled at each investigated HRT, showing counts obtained using family- and genus-specific probes expressed as a fraction of total archaeal counts achieved using probe ARC915.

The cyst-like structure illustrated in **Figure 4.17** was observed in a compartment 2 sample, hybridised with the probe MB4. The identity and purpose of this structure is unknown. The rod-shaped cells within the cyst-like structure appear to have endospores, with emitted a brighter signal than the remainder of the

cells, suggesting a concentration of nucleic acids. It is believed that these cells were in an inactive state and were hybridised non-specifically by the MB4 probe.

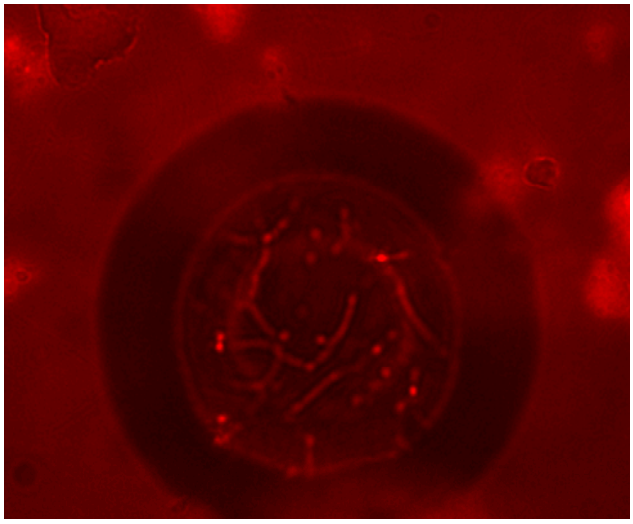
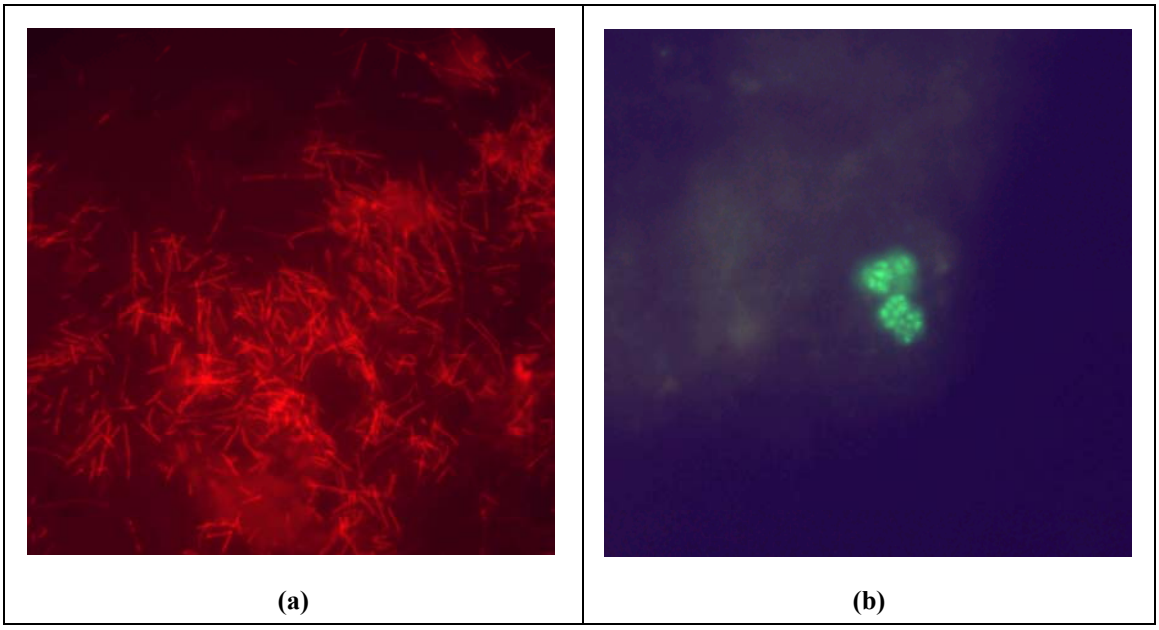


FIGURE 4.17 : Cyst-like structure observed in a sample taken on day 63 (45 h HRT) from compartment 2, and hybridised with the *Methanosarcina*-specific probe MB4.

The samples taken on day 145 (20 h HRT) showed a predominance of the shorter *Methanospirillum*-like filaments, hybridised with MG1200 (**Figure 4.18 (a)**), in compartments 1 through to 5. These methanogens utilise hydrogen and carbon dioxide, as well as formate, for methane production. It is possible that formate was present in the first one or two compartments, due to the increased OLR (Grobicki, 1989; Grobicki and Stuckey, 1992), however, as stated previously, the concentrations could not be measured accurately and it is unlikely that the concentrations would have persisted through to compartment 5. Therefore, it is believed that hydrogen and carbon dioxide were the methane precursors for these microorganisms; the increased organic load resulted in increased acidogenic activity, a reduced pH and an abundance of hydrogen ions and carbon dioxide. These organisms were active at the lower pH, whereas the *Methanosaeta* spp. were inactive (very faint fluorescent signals). Therefore, whilst it was expected that there would be horizontal separation of *Methanosarcina* and *Methanosaeta* spp. through the reactor, these results have shown far fewer sarcina cells (**Figure 4.18 (b)**) than expected, although in accordance with the growth kinetics, those detected were all within the first two compartments. Instead, a horizontal separation of the shorter filamentous Me*thanospirillum*-like spp. and the long sheathed filamentous *Methanosaeta* spp was observed.



(a) **(b)**

FIGURE 4.18 : FISH images of the day 145 (20 h) samples showing (a) the MG1200-hybridised *Methanospirillum*-like filaments in compartment 3 and (b) the MS821-hybridised *Methanosarcina* clusters in compartment 1.

The MX825-hybridised *Methanosaeta* spp. were predominant in the last three compartments of the reactor, in accordance with their growth kinetics and ability to survive at low acetate concentrations. The fluorescent signals observed with these hybridisations were bright, indicating high metabolic activity (**Figure 4.19**).

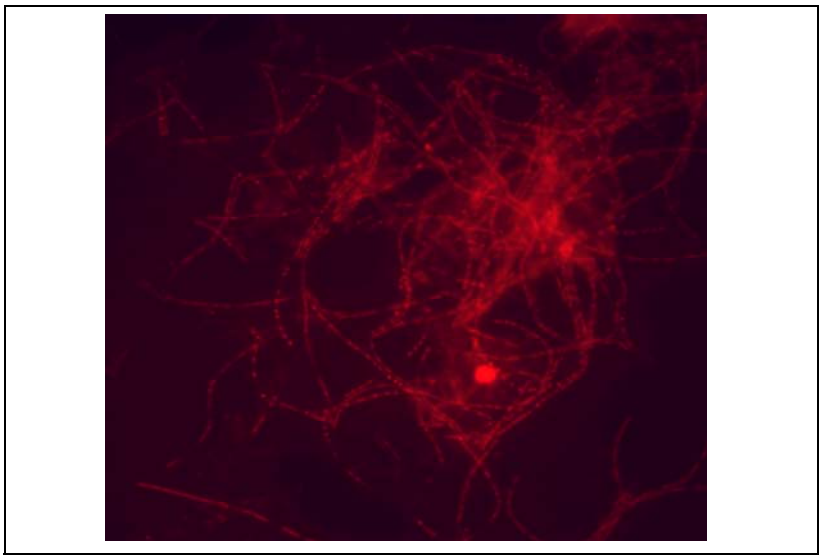


FIGURE 4.19 : FISH image of the filamentous *Methanosaeta* spp. hybridised with the oligonucleotide probeMX825.

The predominance of eubacteria in the first compartments, by day 222 (18 h HRT) resulted in only 23 archaeal cells being hybridised and counted in compartment 1. The majority (78 %) of these were made up by a *Methanosarcina* cluster, which hybridised with probe MS821. The MX825 and MG1200

hybridisations observed in the first five compartments emitted faint signals indicating low metabolic activity, due to the predominance of the acidogenic eubacteria in these compartments. Analysis of the ratios of MG1200 hybridisations to MX825 hybridisations again showed a horizontal separation through the reactor. Similar results were observed with the day 302 (12 h HRT) and day 338 (20 h HRT) samples.

Tilche and Yang (1987) and Yang *et al*. (1988) compared the performance and microbial populations of an anaerobic filter and a hybrid baffled reactor, treating molasses wastewater, with maximum loading rates of 10.5 and 5.5 kg COD/m^3.d for the anaerobic filter and ABR, respectively. They found a large concentration of *Methanosarcina* spp. in the first compartments of the baffled reactor, which shifted to *Methanosaeta* spp. towards the later compartments, compared with a predominance of *Methanosaeta* spp. in the filter reactor. Explanations offered to explain these results were that the acetate loading in the first compartment of the baffled reactor was high (1 000 mg/L) and thus favoured the growth of *Methanosarcina* spp. In contrast, the acetate levels were 10 times lower in the filter reactor, thus *Methanosaeta* had a kinetic advantage and dominated in the reactor. These results were supported by Polpraset *et al*. (1992) where acetate concentrations as low as 20 mg/L facilitated the predominance of *Methanosaeta* spp. in a 4-compartment reactor.

Although granulation is not necessary in the ABR for optimum performance, various reports have noted the appearance of granules in the reactor (Boopathy and Tilche, 1992; Freese and Stuckey, 2000; Boopathy and Tilche, 1991). It is well documented that the filamentous *Methanosaeta* spp. play an important role in the formation of granules by forming a network to stabilise the overall structure (Chang *et al*., 1995). *Methanosarcina* clumps have been found entrapped in pellets formed by the filamentous *Methanosaeta* (Tilche and Yang, 1987; Boopathy and Tilche, 1992). However, when *Methanosarcina* spp. are the predominant acetate-utilising methanogens in a sludge consortium, there is greater susceptibility to biomass washout and process failure, in comparison to a sludge predominated by *Methanosaeta* spp. (Uemura and Harada, 1993). The reason for this is that granules composed of *Methanosarcina* clusters are of low density and full of gas cavities and therefore, tend to lift to the surface of the reactor as a result of high gas and liquid velocities during high loading (Boopathy and Tilche, 1991). Sekiguchi (2000) investigated the microbial community structure of mesophilic methanogenic granular sludge, which had been treating sucrose, propionate and acetate-based artificial wastewaters. The most dominant methanogens in the investigated granules were members of the genus *Methanosaeta*.

From the literature, it is known that *Methanosaeta* cells are predominant in anaerobic sludges (Sekiguchi *et al*., 1999; Domingues *et al*., 2001; Leclerc *et al*., 2001). This investigation showed an unexpected result, namely the abundance of the short filamentous *Methanospirillum*-like spp. and the horizontal separation of these two groups of methanogens through the reactor. Whilst it was expected that the *Methanosarcina* numbers would be greater and that these species would predominate in the first compartments, the observed number of sarcina cells was low and some of these were not even *Methanosarcina* spp. since they did not hybridise with the MS821 probe.

4.4 CONCLUSIONS

1. The hypothesis of the horizontal separation of acidogenesis and methanogenesis through the reactor was supported by the measured pH profiles, the VFA profiles and the biogas composition within each compartment of the reactor.

2. Changes in the HRT did affect the operation of the reactor; an increase in acidogenic activity with an increased OLR resulted in lower pH values within the compartments; reduced methanogenic activity; increased COD in the effluent; and increased VFAs in the effluent. However, it was noted that recovery from these upsets was almost immediate.

3. The reactor HRT was returned to 20 h on day 306. Comparison of operation at this time, with operation at the 20 h HRT from days 76 to 145, showed improved digestion efficiency. The COD removal was comparable but was more stable from day 306. The effluent VFA concentration was much lower than with the first 20 h HRT period and the FISH experiments showed increased methanogenic populations in compartments 3 to 8.

4. Operation of the reactor was stable, based on the analytical data.

5. The molecular-based method, fluorescent *in situ* hybridisation, allowed the direct identification and enumeration of microbial populations active in the ABR. This study, together with the investigations in Chapters 3 and 5, is the first use of rRNA-based molecular approaches to study the population dynamics of an ABR.

6. There was a definite shift in the microbial populations through the ABR, with a predominance of eubacteria in the first compartments (acidogenesis) and archaea (methanogenesis) in the later compartments. The number of compartments involved in each depended on the strength of the substrate (OLR).

7. The use of molecular approaches provided useful descriptions of the methanogens actively involved within each compartment. These results showed far fewer *Methanosarcina* cells than expected. Instead, a horizontal separation of the shorter filamentous Me*thanospirillum*-like spp. and the long sheathed filamentous *Methanosaeta* spp was observed.

Chapter 5

Textile Dyes in the ABR

The base study of the labile sucrose feed, in **Chapter 4**, showed the applicability of fluorescent *in situ* hybridisation for characterisation of the evolving microbial populations in the ABR compartments. In this next phase of the investigation, a well-defined and previously researched reactive textile dye, CI Reactive Red 141, was added to the sugar/protein feed to a laboratory-scale reactor. The literature on textile dyes, particularly reactive dyes is reviewed in **Section 5.1**. Batch screening tests were devised to be applicable to the operation of the ABR; these are detailed in **Sections 5.2 and 5.3.** The chemical and molecular results from the laboratory-scale ABR treating the synthetic CI Reactive Red 141 waste stream, are presented and discussed in **Section 5.4**

5.1 TEXTILE WASTEWATERS

Textile industries consume substantial volumes of water and chemicals for the wet processing of textiles. Over 100 000 commercially available dyes exist with more than 7×10^5 metric tons of dyestuff produced annually (Banat *et al*., 1996).

Wastewater from textile industries is highly coloured and of a complex and variable nature. The adsorption and retention of a dye inside a fibre can be chemical, physical or both, depending on the textile fibre and dye. The adsorptive strength is controlled by several factors including time, temperature, pH, and auxiliary chemicals, such as salts, acids, bases, buffers, dispersing and surface active agents. Thus, a large range of substances, other than dyes, can be found in a dyeing effluent at any one time (Correia *et al*., 1994). In recent years, new and/or tighter regulations coupled with increased enforcement concerning wastewater discharges have been established in many countries (Vandevivere *et al*., 1998). This new legislation, in conjunction with international trade pressure, such as increasing competition and the introduction of ecolabels for textile products, has threatened the survival of the textile industry in many industrialised countries. Appropriate technology is required for the treatment of textile wastewaters, especially in developing regions. As yet, potential solutions are prohibitively expensive, unsustainable or inadequate in terms of their removal of the coloured dye compounds, particularly reactive dyes (Willetts, 1999).

Dyes represent the most problematic component of textile wastewaters (Banat *et al*., 1996; Holme, 1997). Azo dyes account for 60 to 70 % of all textile dyestuffs produced and are the most common chromophore of reactive textile dyes (Carliell *et al*., 1995). Colouration of textile effluents can usually be linked to the presence of water-soluble (reactive) azo dyes. In 1997 reactive dyes comprised ca. 32 % of global dye consumption (Holme, 1997). The use of reactive dyes has increased rapidly due to their properties,

growing consumption of cellulosic fibres, and decrease in the production of other classes of dyes because of cost and health hazards (Gatewood, 1996).

Water insoluble dyes, e.g. disperse and vat dyes, generally exhibit good exhaustion properties, i.e. most of the dye bonds to the fibre and can be removed from the effluent by physical means, such as flocculation (Carliell *et al*., 1996). When this effluent is discharged, to a conventional wastewater treatment works, most of the colour is removed by adsorption to the biomass. However, ca. 10 to 40 % of a water-soluble reactive dye is lost during the dyeing process. Reactive dyes either combine with the fibre or react with water, the reaction being competitive between the two (Dolby, 1980). These dyes pass through a conventional wastewater treatment works and give rise to colouration of the receiving water body.

Dyes are normally present in dyehouse effluent at concentrations of 10 to 50 mg/L, colour being noticeable at concentrations $\geq$ 1 mg/L (Laing, 1991). General characteristics of a dyeing and finishing wastewater are given in **Table 5.1**. (Correia *et al*., 1994).

Table 5.1 : Properties of a typical dyeing and finishing wastewater.

Parameter	
BOD_5	250 mg/L
TSS	75 mg/L
COD	800 mg/L
Oil and grease	0 mg/L
Total chrome	0.27 mg/L
Phenol	0.12 mg/l
Sulphide	0.09 mg/L
Colour	600 ADMI
pH	11
Temperature	38 °C
Water usage	150 L/kg

The many waste streams that originate in a textile factory vary greatly in their nature and the concentration of the different constituents (Correia *et al*., 1994). Segregation of these streams is possible. It would be most effective to treat smaller, concentrated streams of a particular nature in the most appropriate manner (Willetts, 1999).

Textile dye wastewater treatment remains an unsolved problem in many countries. A variety of different systems have been employed to assess the anaerobic reduction of dye wastewater with varying degrees of success. The majority of laboratory-based studies have focused on attached growth systems (Seshadri *et al*., 1994; FitzGerald and Bishop, 1995; Nigam *et al*., 1996) and UASB systems (Donlon *et al*., 1997; Willetts, 1999). The potential of adapting reuse systems to reactive dyes has been investigated but with

limited success (Gatewood, 1996).The first order kinetics which describe anaerobic dye reduction imply that a preferable reactor set-up would be plug-flow since the reduction rate is dependent on dye concentration; in a CSTR, the dye is immediately diluted to a low bulk concentration by the mixing thus the dye reduction rate in a CSTR would be expected to be much lower than in batch experiments or a plug-flow reactor (Willetts, 1999).

The purpose of this investigation was to assess the feasibility of the ABR as an on-site pre-treatment mechanism, of a concentrated dye waste stream. The study was limited to reactive azo dyes used for dyeing cotton, as these present the largest and most problematic group of dyes for treatment purposes (Bumpus, 1995; Carliell *et al*., 1995; Vandevivere *et al*., 1998).

A series of serum bottle anaerobic toxicity tests were run. The details, results and discussion are presented in **Appendix 4**. Although these results gave an indication of the anaerobic toxicity of each of the investigated dyes, they could not be directly applied to the ABR because of the differences in the biomass composition. The following screening tests were devised to provide more valuable information for the ABR.

5.2 ACIDOGENIC TOXICITY ASSAYS

It is known that the reduction of the azo bond requires an anaerobic or reducing environment. In the context of the ABR, and the horizontal separation of acidogenesis and methanogenesis, reduction of the azo bond, with concurrent decolourisation, of a dye molecule in the feed would occur in the first compartments of the reactor. This has been observed in previous experiments (**Sections 3.2.9** and **3.4.7**). Therefore, it would be the acidogenic populations, in the first compartments that would be exposed to the dye compounds, and the methanogenic populations in the later compartments that would be exposed to the dye degradation products, or intermediates. Thus, in the context of treatment of dye wastewaters in the ABR, the toxicity of the dye compounds to the acidogens and the toxicity of the dye degradation products to the methanogens should be determined.

5.2.1 Hypotheses and Objectives

It was hypothesised that the reactive dye compounds, present in textile dye wastewaters, could exert an inhibitory effect on the acidogenic microorganisms in the first compartments of the ABR, thereby adversely affecting the entire anaerobic degradation process.

The objective of this investigation was to assess the toxicity of four textile reactive dyes to the acidogens in anaerobic digester sludge.

5.2.2 Materials and Methods

After consultation with water authorities and textile manufacturers in the Hammarsdale and Pinetown regions, four reactive dyes were chosen for investigation. These are listed, with both the commercial and Colour Index names, in **Table 5.2**. The selected dyes were all azo reactive dyes that gave rise to aesthetic

problems at low concentrations, were known to be problematic with respect to both loading and treatability, and were high consumption dyes, used throughout the year (Hansa, 1999).

TABLE 5.2 : List of the textile reactive dyes investigated.

Commercial Dye	Colour Index Classification	Dye Class
Evercion Red HE7B	CI Reactive Red 141	Disazo
Evercion Navy HER	CI Reactive Blue 171	Disazo
Evercion Green HE4BD	CI Reactive Green 19	Disazo
Evercion Yellow HE4R	CI Reactive Yellow 84	Monoazo

A stock solution (10 % w/v) of each dye was made up. The experiments were performed in 160 mL glass serum bottles, which were sealed with butyl rubber septa and aluminium crimp seals. A defined nutrient medium containing trace elements, minerals and vitamins was prepared according to Owen *et al*. (1979) (**Appendix 1).**

The bottles were prepared in the same manner as for the food dye anaerobic toxicity assays (**Section A3.1**), with 100 mL working volumes. The bottles were seeded with anaerobic digester sludge collected from the Umbilo Sewage Works (TS = 30.67 g/L; VS = 13.67 g/L). Approximately 50 % of the feed to the Umbilo Sewage Works is industrial (Carliell *et al*., 1994; Sacks, 1997). The dye concentrations investigated, for each dye, were: 50 mg/L; 250 mg/L; 1 g/L; 5 g/L; 10 g/L and 20 g/L. The anaerobic toxicity assays were also run with a mixture of the four dyes. Each concentration was repeated in triplicate. The activity of the acidogens and acetogens was stimulated by the addition of a glucose solution, , added to each bottle after equilibration for 1 h, at the incubation temperature of 35 °C.

The controls, or blanks, contained only the inoculum sludge, the anaerobic nutrient medium and the glucose solution. The methanogenic metabolism of the glucose solution was monitored by total gas production, in the controls. Inhibition due to the addition of a dye was determined as a decreased rate of gas production, relative to the controls.

5.2.3 Results

Figure 5.1 shows the cumulative biogas production for each concentration of the investigated textile reactive dyes, relative to the gas production measured in the controls.

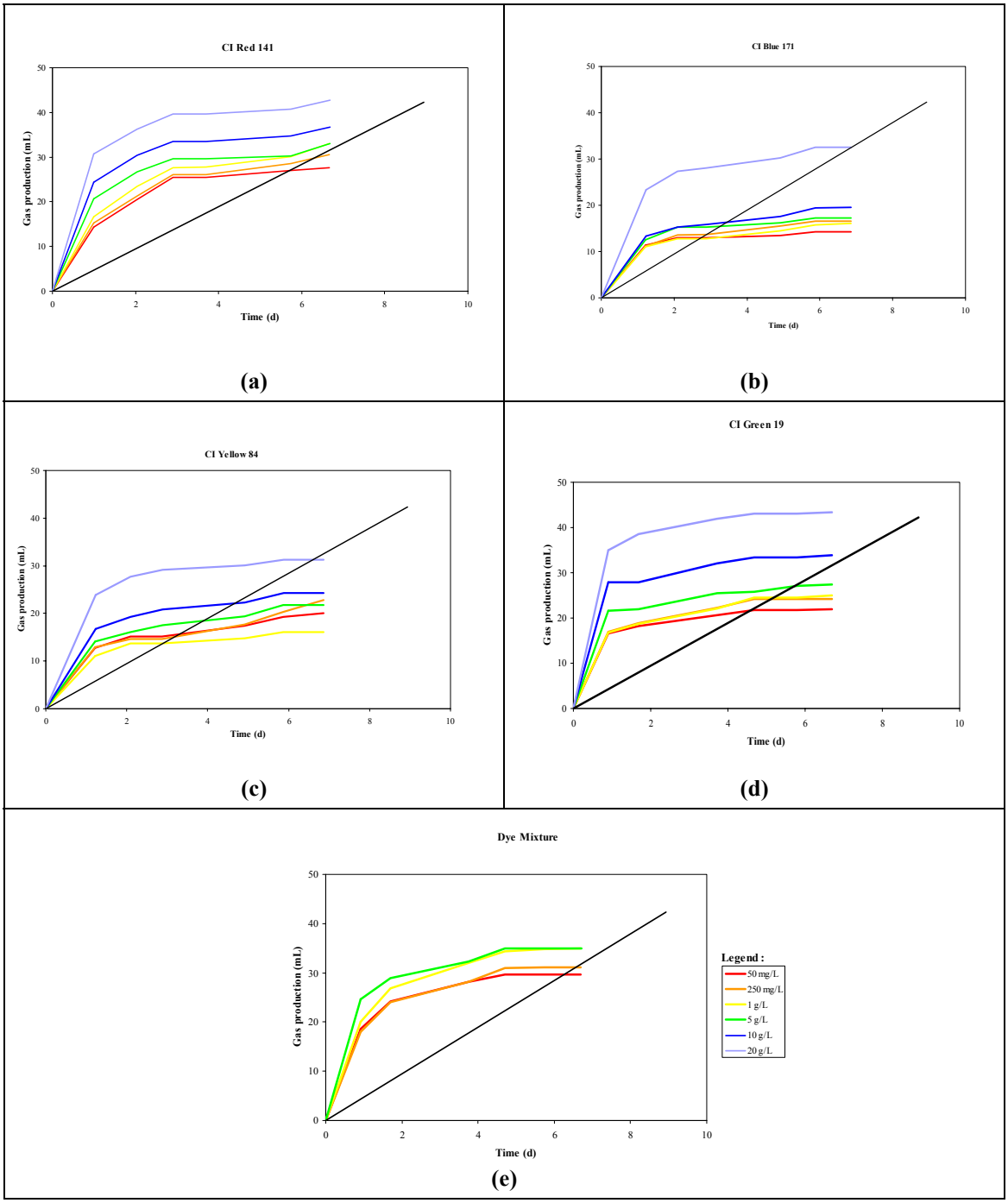


FIGURE 5.1 : Gas production plots for the acidogenic toxicity assays, showing the cumulative biogas production for each concentration of the investigated textile reactive dyes, relative to the gas production measured in the controls (black line).

5.2.4 Discussion

From the biogas production plots in **Figure 5.1** it is evident that the reactive dyes were not inhibitory to the acidogenic populations. In all of the samples, the biogas production was greater than in the controls and the volume of biogas produced increased with increasing dye concentration. This suggests that the dyes were being actively metabolised by the microorganisms. If the dyes were not inhibitory and only the

azo bond was being reduced, i.e. there was no metabolism of the dye, the biogas production would have been similar or equalled that in the controls. However, the biogas production was greater than in the control which suggests that the dye molecules were being utilised as substrate.

The results of these tests indicate that the addition of these dyes to an ABR feed would not adversely affect the microbial activity in the first acidogenic compartments.

5.2.5 Conclusions

1. The reactive dyes were not inhibitory to the acidogenic populations.

2. The biogas production increased with increasing dye concentration which suggests that the dyes were being actively metabolised by the acidogens.

3. Reactive dye compounds, present in textile dye wastewaters, did not adversely affect the anaerobic degradation process.

5.3 BIODEGRADABILITY OF DYE DEGRADATION PRODUCTS

In the treatment of coloured wastewaters, decolourisation cannot be the sole objective since several of the aromatic amines, resulting from the reduction of the dye molecules, have been shown to be toxic or inhibitory, both to microorganisms and higher organisms (Chung *et al*., 1978; Prival *et al*., 1993).

As stated above, in an ABR, the acidogenic populations in the first compartments would be exposed to the dye compounds, and the methanogenic populations in the later compartments would be exposed to the dye degradation products, or intermediates. Thus, in the context of treatment of dye wastewaters in the ABR, the toxicity of the dye degradation products to the methanogens should be determined.

5.3.1 Hypotheses and Objectives

It was hypothesised that the reactive dye degradation products, or intermediates, could exert an inhibitory effect on the methanogenic microorganisms in the later compartments of the ABR, thereby adversely affecting the efficiency of the anaerobic degradation process.

The objective of this investigation was to assess the ability of the methanogens, in anaerobic digester sludge, to degrade the reactive dye degradation products.

5.3.2 Materials and Methods

After termination of the biodegradability assays (**Section A4.2**), the serum bottles were over-gassed and re-sealed. The methanogenic activity was stimulated by the addition of a sodium acetate solution (2 mL) to each bottle to give a concentration of 0.5 g/L in the 44.5 mL working volume. The bottles were incubated at 35 °C and gas production monitored by the syringe method. After 30 d incubation, the gas composition was determined by gas chromatography, and the colour and COD were measured.

5.3.3 Results

The results are shown in **Appendix 4**. In these plots, the gas production curve up to day 64, i.e. left of the dotted line, shows the initial results of the biodegradability assays (**Section A4.2**), the results of which were not directly applicable to operation of the ABR. From day 64 (right of the dotted line) the plots represent the results of the assays investigating the degradability of the dye degradation products. The results are summarised in **Table 5.3**.

TABLE 5.3 : Results of the dye degradation products batch toxicity assays.

Dye	Methanogenic Activity (mL CH_4/g VS)	COD Reduction (%)	Colour Reduction (%)
CI Reactive Red 141 (with nutrient medium)	32.2	0	0
CI Reactive Red 141 (without nutrient medium)	36.7	0	0
CI Reactive Blue 171	34.0	0	0
CI Reactive Green 19	33.4	0	0
CI Reactive Yellow 84	25.8	0	0
Dye Mixture	22.4	0	0

5.3.4 Discussion

The ability of the methanogens to further degrade the dye degradation products was monitored by biogas production relative to the controls. The VFA analyses, after 60 d incubation, showed negligible concentrations of VFAs which was expected since no additional carbon source was added to the assay bottles. It was deduced that the samples contained aromatic amines from the reduction of the azo bonds by the acidogenic bacteria, however these could not be identified since methods were not developed during this project.

In all of the investigated dyes, except the CI Reactive Red 141 without nutrient medium, the biogas production was lower than in the controls which indicated that the methanogens were inhibited by the dye degradation products.

Microbial inhibition was thought to be caused by intercalation of dye compounds between DNA base pairs, so preventing enzymatic activity and cell replication (Carliell *et al*., 1995). However, this requires that the dye pass through the cell membranes of the microorganisms and become inserted between the base pairs of DNA. CI Reactive Red 141 is a large, highly sulphonated compound (MW = 1 634 g/mole); therefore, permeation of the dye through the microbial cell membranes and subsequent intercalation of the dye between DNA base pairs is thought to be unlikely as a mechanism of inhibition. Instead, the degradation products are smaller than the dye compound and may be capable of penetrating the cell and inhibiting the microorganisms either by intercalation or some other intracellular mechanism (Carliell *et al*., 1995).

There was no further reduction in COD or colour in any of the assay samples, thus it was deduced that the methanogens did not further degrade the reactive dye degradation products. Although methanogenic activity was measured in the serum bottles, it was lower than in the controls which indicated that the activity was not due to the degradation of the dye intermediates but utilisation of the added sodium acetate solution and endogenous respiration.

Donlon *et al*. (1997) found that, in batch toxicity assays, azo dye compounds were many times more toxic than their cleavage products (aromatic amines) towards methanogenic activity. These bioassays showed inhibition of methanogenic activity by the degradation products.

In the context of treatment of dye wastewaters in the ABR, these results suggest that the compartments dominated by methanogenesis would not contribute to further degradation of the wastewater and that the inhibited methanogenic activity could reduce the efficiency of the entire degradation process. However, if the coloured wastewater was fed to the ABR together with a high-COD wastewater e.g. a desize wastewater, it is believed that these compartments would contribute to the further reduction in COD.

5.3.5 Conclusions

1. Methanogenic activity was inhibited by the dye degradation products.

2. There was no further reduction in COD or colour.

5.4 TREATMENT OF CI REACTIVE RED 141 IN THE ANAEROBIC BAFFLED REACTOR

CI Reactive Red 141, or Evercion Red HE7B, is an azo reactive dye (**Figure 5.2**) with a molecular mass of 1 634 g/mole. Maximum absorption of the dye was measured at a wavelength of 545 nm (**Appendix 2**). The dye was chosen for investigation as it was representative of a dye class known to be problematic with respect to treatability in a conventional wastewater treatment system; reactive dyes are hydrophilic and, therefore, have little affinity to adsorb to biomass and generally pass through activated sludge systems (Bell, 1998). The red hue is known to give rise to aesthetic problems at relatively low concentrations. Previous investigations have determined the reaction kinetics of CI Reactive Red 141 (Carliell, 1993; Carliell *et al*., 1994; Carliell *et al*., 1995; Carliell *et al*., 1996; Bell, 1998).

A batch control run (data not shown) investigated the difference in degradation potential of both un-hydrolysed and hydrolysed dyes. The four dyes (**Table 5.2**) were hydrolysed, to imitate their form in a wastewater stream, by raising the pH to 11 with 0.2 M NaOH and heating at 80 °C for 2 h. There was a negligible difference in the results, thus un-hydrolysed dyes were used for the remainder of the study. Fontenot *et al*. (2001) also found no significant difference between the decolourisation reaction of a hydrolysed and unhydrolysed reactive dye.

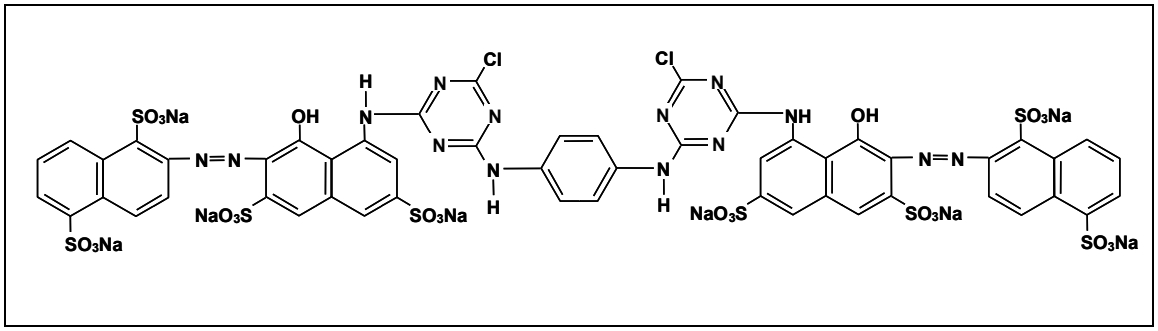


FIGURE 5.2: Chemical structure of CI Reactive Red 141.

Investigations by Carliell *et al*. (1995), using column chromatography, thin layer chromatography and NMR, positively identified 2-aminonaphthalene-1,5-disulphonic acid as a degradation product of CI Reactive Red 141(**Figure 5.3**). This confirmed that azo reduction was responsible for decolourisation of the azo dye.

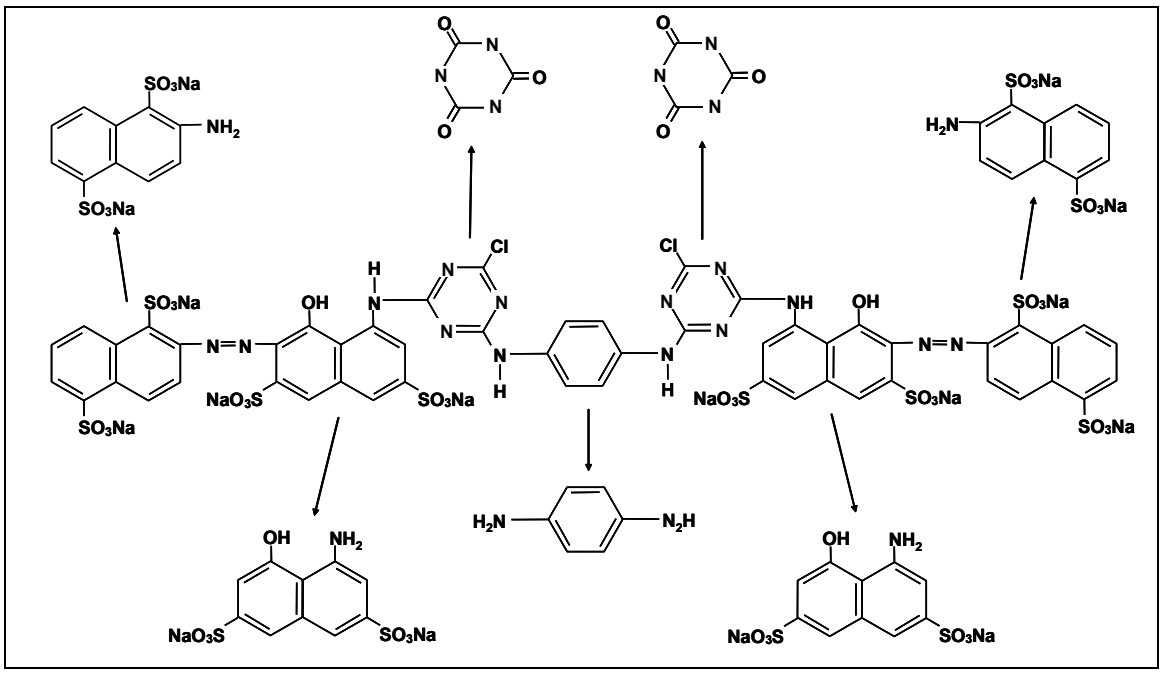


FIGURE 5.3 : Proposed degradation of CI Reactive Red 141 in an anaerobic system (Carliell *et al*., 1995).

Results of the anaerobic toxicity assays (**Section A4.1**) showed that CI Reactive Red 141 was inhibitory to the methanogenic biomass, with an IC_{50} concentration of 2.46 g/L. The acidogenic microorganisms were not inhibited by the dye (**Section 5.2**). In the biodegradability assays, methanogenic utilisation of the dye was not observed, however, the biogas production suggested metabolism of the dye by other anaerobic microbial populations, caused a reduction of COD and colour.

Because of the variable nature of industrial wastes, reactor stability to organic shock loads is one of the most important aspects of reactor design. These shock loads can manifest themselves in two ways: either

as a short-term transient which only lasts a few hours, or as a longer-term step change of days to weeks before reversion back to the original operating conditions. In the first few hours to days, the microbial response to both these shock loads are identical; however, the longer-term shock leads to a new steady state which may or may not be identical to the original operating conditions in terms of COD removal and other parameters. In high SRT reactors such as the ABR, in contrast to CSTRs, the time required to establish a new steady state after a step change is considerably longer than the accepted norm of three HRTs and little is known about the dynamic changes the microbial populations undergo in the transition from one steady state to another.

Due to the partial separation of trophic groups in the ABR, shock loads may also provide insights into microbial responses and interactions under various environmental conditions. McCarty and Mosey (1991) suggested that the relative ratios of reduced end-products produced by the catabolism of carbohydrates were controlled more by population dynamics as a result of competition between bacteria producing propionic and butyric acid under varying substrate concentrations and pH, rather than through the concentration of hydrogen in the system controlling the kinetics and thermodynamics of catabolism. This hypothesis is relevant to the ABR, since the ratio of VFAs produced in the first compartment is controlled by the feed COD and HRT (via pH and substrate concentration), and therefore, this compartment determines the feed composition to the rest of the reactor, and possibly its response to shock loads.

The objective of this experiment was to assess the efficiency of the ABR for the degradation, or treatment, of a CI Reactive Red 141 waste stream; and to determine whether acclimation of the biomass to the dye resulted in increased methanogenic activity.

5.4.1 Hypotheses and Objectives

It was hypothesised that anaerobic digestion, in the ABR, could reduce the COD and colour of a CI Reactive Red 141 waste stream at a low (20 h) HRT. The compartmentalised design of the ABR would prevent inhibition of the anaerobic biomass and methanogenic activity would increase with acclimation of the biomass to the dye.

It was also hypothesised that fluorescent *in situ* hybridisation of the microbial communities that develop in the ABR compartments, during treatment of the CI Reactive Red 141 waste stream, would provide improved knowledge of the biochemical pathways and the microorganisms involved in the decolourisation.

The objectives of this investigation were to:

1. Determine whether adsorption to the anaerobic biomass played a significant role in the decolourisation of the waste stream.

2. Assess the feasibility of the ABR for treatment of a CI Reactive Red 141 waste stream; including reduction of COD and decolourisation.

3. Evaluate any impact on reactor performance with increasing dye concentrations.

4. Determine whether the anaerobic biomass became acclimated to the dye, thereby improving degradation and decolourisation, with time.

5. Determine the effects of a dye shock load on the reactor performance and the ability of the reactor to return to stable operation after the shock load.

6. Use 16S rRNA oligonucleotide probes to characterise the microbial populations within each compartment, and the dynamics of these populations during treatment of the CI Reactive Red 141 waste stream.

5.4.2 Physical Decolourisation

As stated previously, decolourisation of a wastewater in a biological treatment system may be attributed to adsorption of the dye to the anaerobic biomass, and not entirely to degradation or breakdown of the dye molecules. Two tests were conducted to determine the extent of adsorption of CI Reactive Red 141 to the digester sludge since this could contribute to the decolourisation potential in the ABR.

The test conditions are outlined in **Table 5.4**.

TABLE 5.4 : Test conditions to assess the extent of physical decolourisation of CI Reactive Red 141.

Test	Sludge	Dye	Chemical addition
Test 1	Non-acclimated Autoclaved (80 min at 121 °C)	2 g/L CI Red 141	None
Test 2	Non-acclimated Un-autoclaved	2 g/L CI Red 141	0.05 g/L sodium azide

In test 1, the Umbilo Sewage Works mixed anaerobic digester sludge was inactivated by autoclaving at 121 °C for 80 min. Once the sludge had cooled it was aliquoted into a series of serum bottles. The CI Reactive Red 141 dye stock solution was diluted to the required concentration (2 g/L).

In test 2, sodium azide, which is an inhibitor of metabolic activity, was added to give a final concentration of 0.05 g/L in the serum bottle. For each test, a control was set up for each bottle, containing the same amount of sludge, with no dye. The function of the controls was to evaluate the background absorbance of the sludge. The bottles were sealed and incubated in a constant temperature room, at 35 °C. Samples were periodically taken from the bottles, over 7 d. Immediately after their collection, the samples were centrifuged (10 000 rpm) for 5 min and the supernatants filtered through glass fibre filters (0.45 μm). The samples were diluted 1 in 5 with distilled water and then analysed using a spectrophotometer at 545 nm (**Appendix 2**) to determine the dye concentration. The results of each test are presented and discussed below.

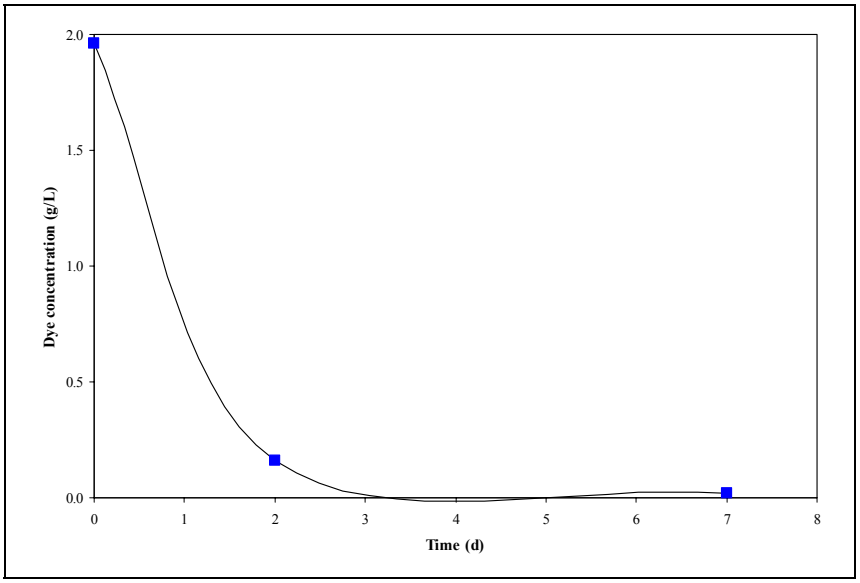


FIGURE 5.4 : Plot showing the CI Reactive Red 141 concentration measured over time for the adsorption test (1), with autoclaved biomass.

The results of test 1 (**Figure 5.4**) showed almost complete decolourisation. The decolourisation in these bottles was due to adsorption to the biomass but the results may not be completely representative since the autoclaving may have increased the surface area available for adsorption by rupturing the cells. It is also unknown whether all of the biomass was inactivated by autoclaving, therefore, some of the decolourisation may have been due to degradation or breakdown of the dye although this is unlikely since no biogas was produced in any of the assay bottles. The decolourisation could have occurred due to the reducing conditions of the liquid associated with the biomass.

The results of test 2 (**Figure 5.5**) exhibited linear decolourisation. There was some biogas production in these assay bottles which suggests that the biomass was not completely inactivated by the sodium azide (the concentration may have been too low) and that some of the decolourisation may have been due to metabolic degradation or associated reduced conditions.

The results of these tests suggest significant decolourisation due to adsorption to the biomass, however, it is also possible that the dyes were reduced due to the anaerobic, or reducing environment within the serum bottles.

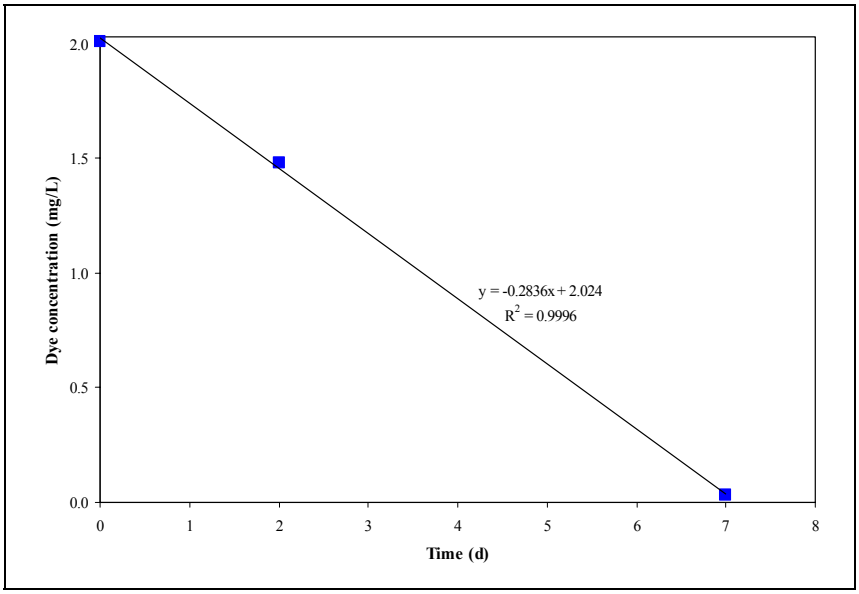


FIGURE 5.5 : Plot showing the CI Reactive Red 141 concentration measured over time for the adsorption test (2), with sodium azide inactivated biomass.

A CI Reactive Red 141 solution was fed to a laboratory-scale (10 L) ABR to assess the efficiency of the reactor, and its configuration and separation of microbial populations, in the degradation of the dye.

5.4.3 Experimental Design

A laboratory-scale ABR was set up in a constant temperature room at 35 °C. The reactor was seeded with 7.68 L (0.96 L/compartment) of screened digester sludge taken from Umbilo Sewage Works (TS = 28 g/L; VS = 20 g/L). This gave an inoculum of 19.2 g biomass per compartment, or 153.6 g biomass in the reactor. The sludge was allowed to settle for one week before feeding began. The feed connections for CI Reactive Red 141 degradation were set up as illustrated in **Figure 5.6**.

The feed solution was continuously pumped, by a variable speed Watson-Marlow peristaltic pump (model 101U/R), and diluted with distilled water pumped by a variable-speed peristaltic pump (model 505s). The two streams combined to form a single feed stream just before the inlet to the reactor. The treated effluent passed through a glass U-tube for level control and a biomass trap before running to the effluent reservoir. Effluent samples were taken from the bottom of the U-tube.

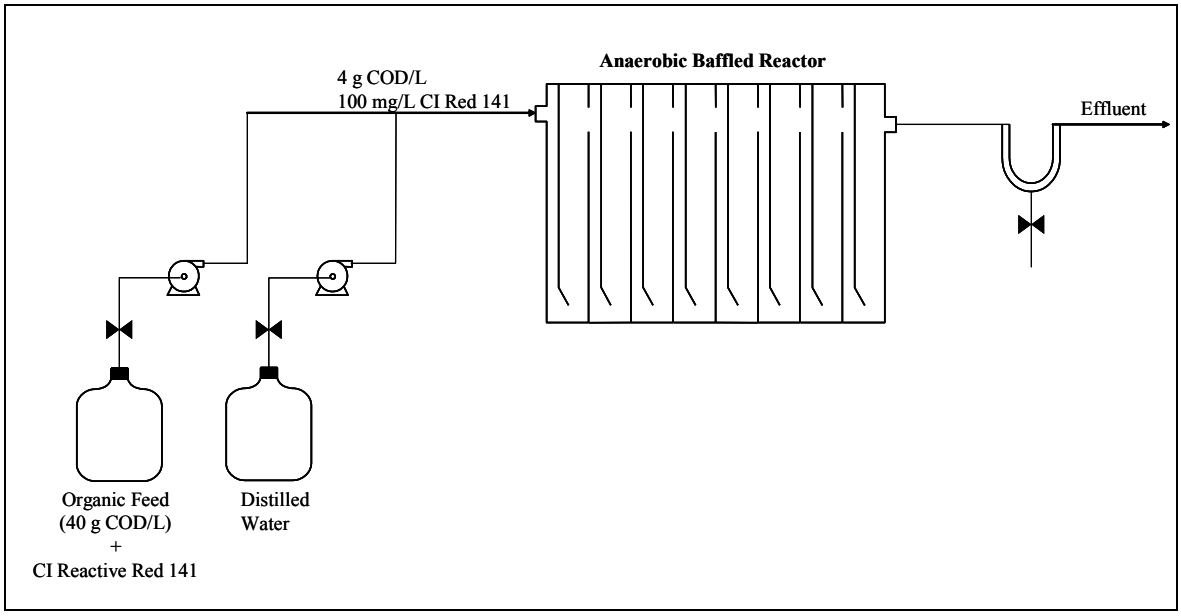


FIGURE 5.6 : Schematic diagram showing the experimental layout of the laboratory-scale ABR treating a synthetic CI Reactive Red 141 stream (not to scale).

The standard sucrose/protein feed solution (**Appendix 1**) was used. The operating conditions are outlined in **Table 5.5**. Once the reactor had reached steady state, at a 20 h HRT, the CI Reactive Red 141 dye was added to the feed solution. The dye powder (2 g) was diluted in 2 L of the sterilised feed solution (concentration of 40 g COD/L). The feed was diluted 10x with distilled water, such that the feed to the reactor contained a dye concentration of 100 mg/L. The COD concentration to the reactor was maintained at 4 g COD/L. To achieve acclimation, the concentration of CI Reactive Red 141 was increased stepwise from 100 mg/L (0.12 g/L.d), to 250 mg/L (0.3 g/L.d) on day 96, to 500 mg/L (0.6 g/L.d) on day 127. Throughout the experimental period, the reactor was supplied with a constant COD loading of 4.8 g COD/L.d of the synthetic feed co-substrate. On day 155, a dye shock load (1 g/L) was fed to the reactor for one HRT. The feed dye concentration was then reduced to 100 mg/L to determine the ability of the reactor to return to stable operation after the shock load.

TABLE 5.5 : Summary of the operating conditions.

Day	HRT (h)	Organic Loading Rate (g COD/L.d)	CI Reactive Red 141 (mg/L)
1	80	1.2	0
16	60	1.6	0
30	30	3.2	0
50	20	4.8	0
65	20	4.8	100
96	20	4.8	250
127	20	4.8	500
155/156	20	4.8	1 000
156	20	4.8	100

5.4.4 Analytical Methods

Experimental data were obtained from snap samples, taken from the ABR, once or twice week. The reactor was sampled and analysed as described in **Section 3.2.4**. The VFAs were analysed by gas chromatography (**Appendix 1**). Colour removal was monitored by the method described in **Appendix 1**. The absorbance was measured (Pharmacia Biotech Ultrospec 2000 UV/VIS) at a wavelength of 545 nm and the dye concentration was determined from the calibration curves (**Appendix 2**).

To assess the changes in the microbial populations, sludge samples were taken from each compartment, and fixed with 4 % paraformaldehyde. These samples were taken when the reactor had reached steady-state, at a particular dye concentration, and prior to a change in the dye concentration. The method for sample fixation, probe hybridisation and analysis is detailed in **Appendix 1**. The cells were dual stained with DAPI and the fluorescent 16S rRNA-targeted oligonucleotide probes (**Table 4.2)**. They comprised domain-specific probes for the eubacteria and archaea and order-, family-, and genus-specific probes for several phylogenetic groups of methanogens.

5.4.5 Reactor pH

The measured pH values of compartments 1 and 8 are shown, to assess the effect of the increasing dye concentration on the reactor pH.

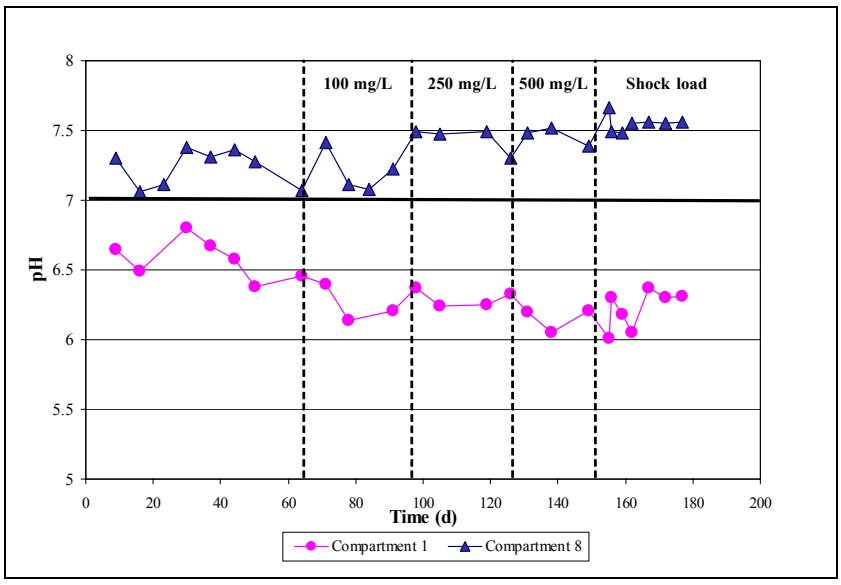


FIGURE 5.7 : Plot of the pH profiles in the CI Reactive Red 141 ABR.

Analysis of the data indicates that changes in the dye concentration had a slight effect on the reactor pH. These results also illustrate the horizontal separation of acidogenesis and methanogenesis through the ABR. In **Figure 5.7** the bold line indicates pH 7 and the changes in dye concentration are indicated as dotted lines.

The pH was quite variable during the start-up of the reactor but stabilised to an average pH of 6.33 in compartment 1 and 7.36 in compartment 8. When the dye was first introduced to the reactor, at a

concentration of 100 mg/L on day 65, the pH in compartment 1 dropped to 6.14 and the pH in compartment 8 was variable over 4 data points. The pH in compartment 1 dropped again when the dye concentration was increased to 500 mg/L, however, the pH in compartment 8 was not affected which illustrates the ability of the compartmentalised reactor to protect the more sensitive methanogenic species, in the later compartments, from inhibitory components or concentrations in the feed stream. During the dye shock load, when the dye concentration was increased to 1 g/L for a period of 1 HRT, the pH in compartment 1 was variable but it did not drop below pH 6. The pH in compartment 8 was not affected by the shock load. The dye concentration was reduced to 100 mg/L on day 156 and by day 167 the pH in compartment 1 was stable at ca. pH 6.3. The fact that the pH did not drop below pH 6 with the shock load indicated that the acidogenic process would not have been inhibited and, therefore, the anaerobic digestion process in the ABR would not have been adversely affected by the dye shock load.

5.4.6 Reactor Solids

Figure 5.8 is a plot of the cumulative solids washed out of the reactor. The measure of volatile solids was taken as an indication of the biomass concentration. These results show that there was biomass washout with each change in dye concentration and that the total amount of solids lost was relatively high (3.37 kg of which 1.47 kg were volatile solids). Only ca. 50% of the solids washed out of the reactor were volatile solids.

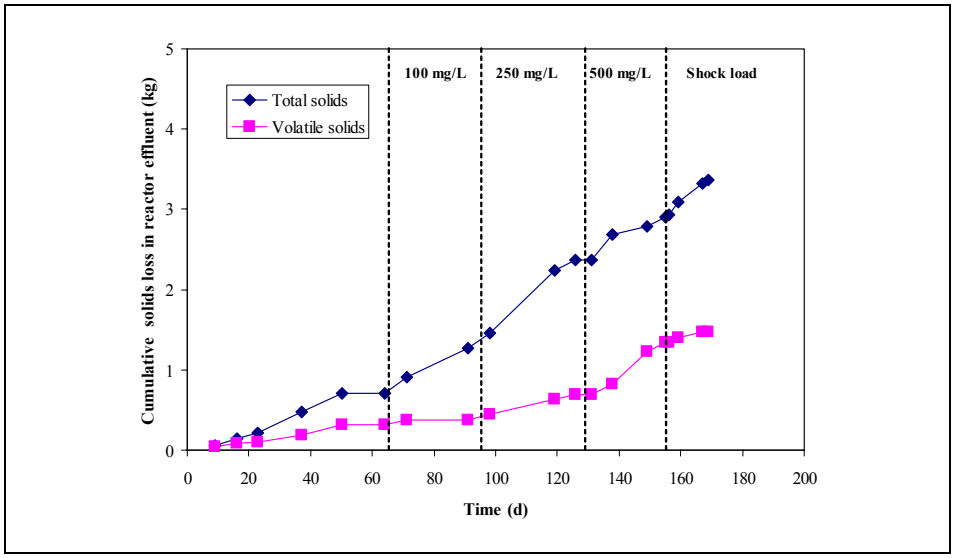


FIGURE 5.8 : Plot of the cumulative solids lost from the CI Reactive Red 141 ABR.

5.4.7 Reactor Chemical Oxygen Demand (COD)

Figures 5.9 and **5.10** depict the soluble COD removed by the reactor over time. The COD removal during start-up, or before the addition of the dye to the feed stream, averaged 95 %, or an effluent COD of 189 mg/L. A COD removal efficiency of > 80 % is considered acceptable to conclude the start-up period (Willetts, 1999).

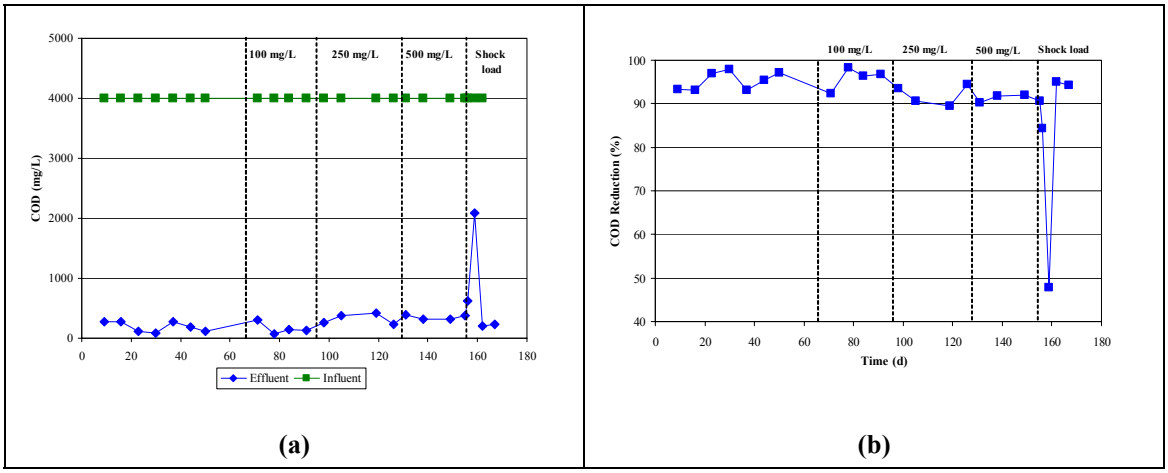


FIGURE 5.9 : Plots showing (a) the influent and effluent CODs and (b) the COD reduction in the laboratory-scale ABR.

CI Reactive Red 141 was added to the feed stream at a concentration of 100 mg/L, on day 65. This resulted in a slight decrease in the COD reduction with an effluent COD of 303 mg/L. The COD reduction stabilised within 3 HRTs to give an effluent COD concentration of 163 mg/L. The average COD reduction for the 250 mg/L dye concentration was 92 % and 91 % for the 500 mg/L concentration. Thus, there was a slight decrease in the COD removal efficiency with each increase in the dye concentration. The dye shock load (1 g/L CI Reactive Red 141) resulted in a sharp decrease in the COD removal, to 47.8 %. It is thought that the high dye concentration caused a temporary inhibition of microbial metabolism, resulting in the substrate and intermediate acids not being completely metabolised. These results correlate with the VFA results (**Section 5.4.9**). The biomass recovery was almost immediate with an effluent COD of 198 mg/L attained within 3 HRTs of the shock load. Thus, these results indicate that addition of the dye to the ABR feed stream did not have a long-term adverse or inhibitory effect on the anaerobic degradation process, except that the shock load caused a temporary inhibition of the microbial metabolism.

Figure 5.10 shows the COD profiles through the reactor, at different time periods during the experiment. On the plot, compartment 0 represents the reactor feed and compartment 9 represents the reactor effluent. The profiles show that the majority of the COD was reduced in the first three compartments of the reactor, due to the horizontal separation of acidogenesis and methanogenesis. The COD profiles indicate that the COD reduction decreased with each increase in dye concentration. This could be attributed to reduced metabolic activity of the methanogens (degrading the intermediates from the sucrose in the feed) since they were shown to be inhibited by CI Reactive Red 141 and its degradation products (**Sections A4.1** and **5.3**). The profiles also show very little COD reduction in the last three compartments of the reactor; this was substantiated by the biogas results and the population characterisation experiments, which showed low metabolic activity in these compartments.

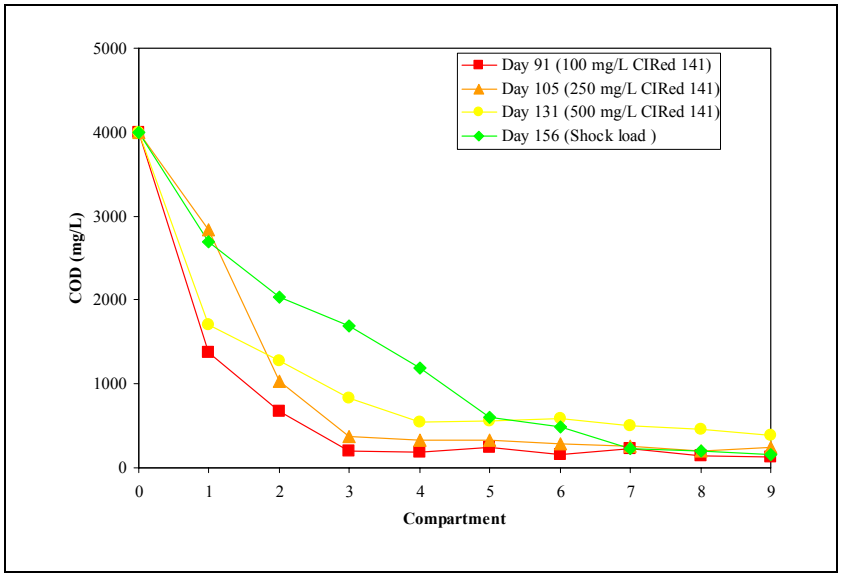


FIGURE 5.10 : Plot showing the COD profiles through the laboratory-scale reactor at different times during the experimental period.

The general response of anaerobic processes to a shock load can be characterised by an increase in VFAs, a decrease in removal efficiency, a decrease in methane content and higher effluent suspended solids. Consequently, the highest concentration shock load causes the lengthiest deterioration of effluent quality in terms of peak soluble COD and VFA concentrations (Nachaiyasit and Stuckey, 1997a). This increase in soluble COD in the effluent will consist of both unmetabolised VFAs and SMPs. The degree of deterioration in performance depends on the duration and magnitude of the shock and the rate of adaptability of the microorganisms; hence, the function of higher biomass concentrations in anaerobic reactors is usually to enhance their stability rather than improve COD removal (Nachaiyasit and Stuckey, 1997a).

The theoretical volume of methane produced was calculated from the organic loading rates and the known conversion of 1 g COD being equal to 0.395 L CH_4 at 35 °C (Speece, 1996).

TABLE 5.6 : Theoretical methane production values calculated from the organic loading rate.

Organic Loading Rate (g COD/L/d)	Theoretical methane production (L/d)
1.2	3.64
1.6	4.85
3.2	9.71
4.8	14.56

Thus, the theoretical total methane production, over the 162 day test period, was **1 951 L**. The total measured methane production was **248.03 L**. Unfortunately this large discrepancy was not identified until

analysis of the experimental data, after termination of the test. Possible reasons for the large biogas loss could be (i) that the diameter of the gas measurement U-tube (**Appendix 1**) was too wide to support sufficient level changes; (ii) there was less resistance to the biogas to follow the liquid route out the effluent tube, than up through the gas port; (iii) there was inefficient gas separation between adjacent compartments and (iv) some biogas could have been lost during sampling. No gas leaks were found.

These poor gas production measurements are reflected in the COD balance. The balance was calculated for each time step and the results are presented in **Appendix 4.** The COD balance over the whole test period was 18.2 %. The poor balance indicates that the gas measurement set-up was also inefficient. The reduction in COD from the inlet feed to the outlet stream was not reflected in the amount of methane produced by each compartment i.e. the measured methane concentrations were too low, suggesting that the COD was not conserved. Any COD which is not converted to methane and is not present in the effluent may be assumed to be consumed in cell maintenance and synthesis.

5.4.8 Reactor Colour

The dye concentration in each compartment and in the final effluent was measured by absorbance (545 nm). The concentration of CI Reactive Red 141 in the influent was increased periodically after at least 20 hydraulic retention times (HRT of 20 h) and when more than 75 % removal of the dye and the co-substrate (sucrose in the synthetic feed) COD had been achieved. Sugar, in the form of glucose or sucrose, is deemed a model wastewater substrate necessary for providing the electrons for the reduction of azo compounds (Donlon, Razo-Flores *et al*., 1997). Sucrose was added to the synthetic feed (**Appendix 1**) and acted as a co-substrate for the azo dye reduction. **Figure 5.11** shows the colour reduction with time. The dotted lines indicate the changes in dye concentration in the feed.

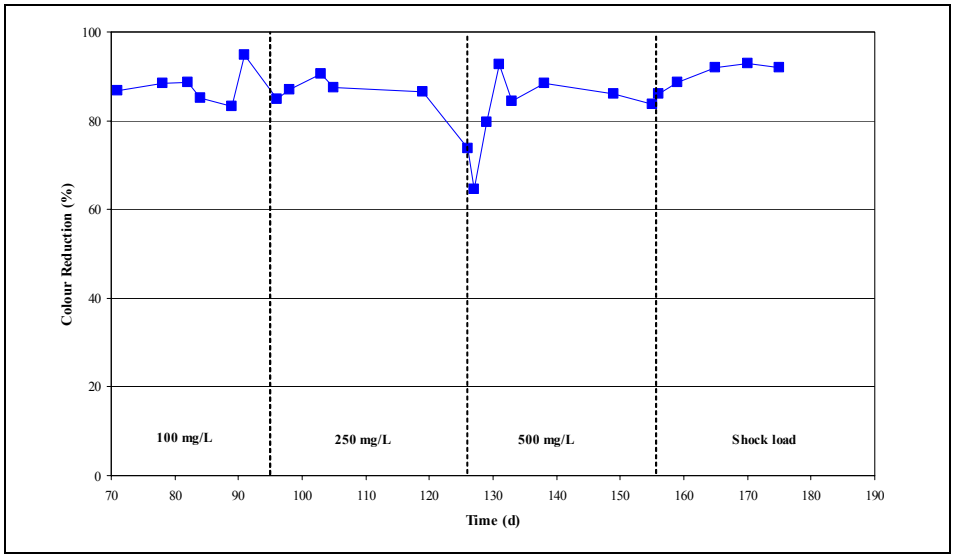


FIGURE 5.11: Plot showing the colour reduction achieved in the laboratory-scale ABR treating a synthetic CI Reactive Red 141 waste stream.

These results show efficient decolourisation of the CI Reactive Red 141 waste stream. The colour reduction averaged 87 % with a 100 mg/L dye concentration in the feed and 85 % for the 250 mg/L concentration. The colour removal dropped to 65 % when the dye concentration was increased to 500 mg/L (day 127). This was the lowest colour removal achieved throughout the duration of the test, and had increased to 80 % within one HRT. The dye shock load of 1 g/L did not inhibit the anaerobic process and a colour reduction of 85 % was achieved during the shock load. When the dye concentration was reduced back to 100 mg/L, to assess the ability of the reactor to return to stable operation after the shock load, colour reduction stabilised at ca. 90 %. Thus, these results show that colour removal was efficient with an average colour removal of 86 % over the whole test period. The minimum dye concentration achieved in the effluent was 5 mg/L, on day 91, however, this concentration is still significant since colour is visible at concentrations ≥ 1 mg/L. The effluent would require further treatment before discharge to a water source. If this colour reduction was achieved by pre-treatment at the factory, further aerobic reduction of the aromatic amines could be achieved by conventional treatment at a wastewater treatment works.

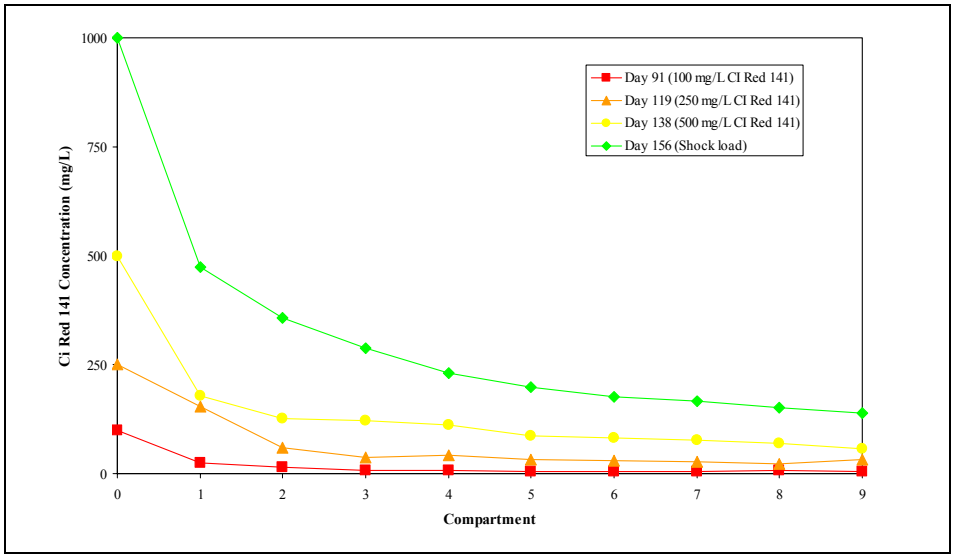


FIGURE 5.12 : Plot showing the colour reduction profiles in the laboratory-scale ABR treating a synthetic CI Reactive Red 141 waste stream.

The colour reduction profiles (**Figure 5.12**) show that, for all investigated dye concentrations, at least 50 % of the colour was removed in the first two compartments of the reactor. This verifies the theory proposed in **Section 5.2** that in the context of the ABR, and the horizontal separation of acidogenesis and methanogenesis, reduction of the azo bond, with concurrent decolourisation, would occur in the first compartments of the reactor. Therefore, it would be the acidogenic populations that would be exposed to the dye compounds, and the methanogenic populations in the later compartments that would be exposed to the dye degradation products, or intermediates. The toxicity assays in **Section 5.2** showed that the acidogens were not inhibited by CI Reactive Red 141 and this was verified by the significant colour removal achieved in the reactor. The final effluent concentration was < 150 mg/L for all investigated dye concentrations.

Carliell *et al*. (1995) found a first-order relationship of CI Reactive Red 141 decolourisation vs. time with respect to dye concentration. It was noted, however, that increasing the initial dye concentration in the anaerobic test system resulted in decreasing reaction rates for decolourisation.

The results of the adsorption assays (**Section 5.4.2**) indicated that adsorption would contribute significantly to the decolourisation of the CI Reactive Red 141 waste stream. If decolourisation was only achieved by adsorption, i.e. there was no reduction of the dye molecules, then the biomass would have reached a saturation point where no more dye molecules could be adsorbed and a break-through of dye would have been observed in the reactor effluent. This was not observed, therefore, it was concluded that there was also metabolic reduction of the dye molecules contributing to the decolourisation of the dye waste.

The literature shows that azo reduction exhibits different reduction potentials in different environments. Many redox reactions occur simultaneously in a biological system and the reduction potentials vary for each reaction. Therefore, the reduction potential of the azo bond in a biological system will be the apparent reduction potential of the bulk solution. Many authors have reported non-specific decolourisation of single dyes, mixtures of dyes or real textile effluent, in the presence of anaerobic or facultative mixed cultures acquired from varying sources (Brown and Laboureur, 1983; Banat *et al*., 1996). In contrast to pure culture studies, the reduction of dyes by mixed cultures appears independent of the structure of the dye molecule involved. Hence, the bulk of decolourisation would appear to occur extracellularly and be dependent on the redox potential of both the bulk phase and the dye. Carliell *et al*., (1995) noted that the presence of other electron acceptors in the bulk phase, such as nitrate, caused a lag phase before dye reduction, during which time the nitrate was reduced. This preference was simply explained by the lower redox potential required for reduction of azo dyes as compared with nitrate (Carliell *et al*., 1995; Wisjnuprapto *et al*., 2001). The reduction potential was not measured in this investigation. Carliell *et al*. (1995) showed that the redox potential of an anaerobic system decreased from ca. -375 mV (addition of CI Reactive Red 141) to ca. -475 mV by the end of a 5 h decolourisation period (Carliell *et al*., 1995). Wisjnuprapto *et al*. (2001) also concluded that, in order to achieve colour removal, a redox potential of – 375 mV was required. Although the precise redox potential for optimum decolourisation is not known, it can be concluded that strictly anaerobic conditions are conducive to decolourisation. Bell (1998) found that the rate and extent of decolourisation of CI Reactive Red 141 was affected by the presence of oxygen in the anaerobic system. It was concluded that the presence of oxygen increased the bulk oxidation reduction potential (ORP) of the solution thus inhibiting the reduction of the dye molecule (Bell, 1998).

5.4.9 Reactor Volatile Fatty Acids

Figure 5.13 shows the total VFAs found in the reactor effluent over time. There was an initial VFA peak in the effluent, during start-up, after which the measured VFA concentration in the effluent was constant at 0 mg/L. When the reactor was exposed to the dye shock load, the VFA concentration in the effluent increased to 735 mg/L. It is thought that the high dye concentration caused a temporary inhibition of microbial metabolism, resulting in the VFAs being present in the effluent. However, recovery was almost

immediate with the effluent VFA concentration returning to < 100 mg/L. It was concluded that the reactor operation was stable since the effluent VFA was < 200 mg/L throughout the test period, except for the temporary response to the dye shock load, and stable reactor performance is indicated by an effluent total VFA concentration below 500 mg/L (Willetts, 1999).

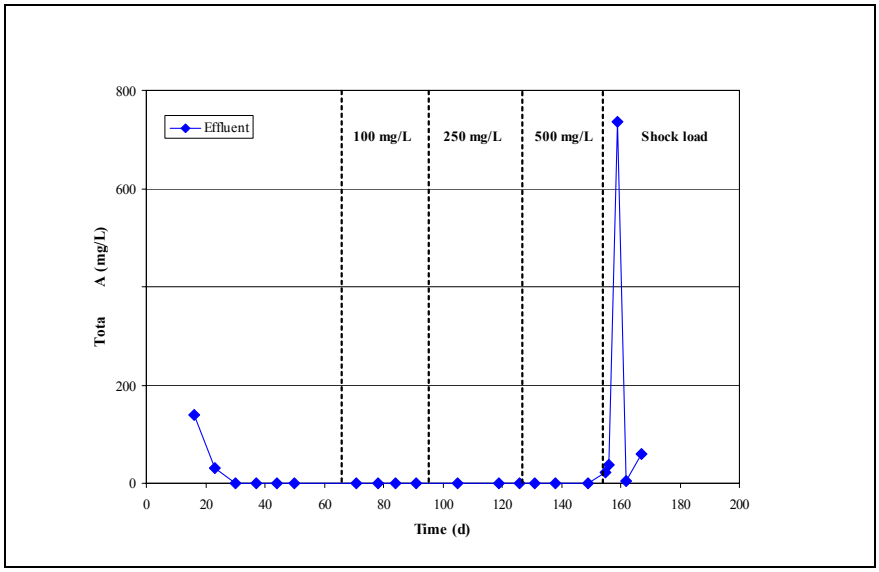


FIGURE 5.13 : Plot of the total VFAs in the laboratory-scale ABR effluent.

The concentrations of each of the individual acids in compartment 1 and the reactor effluent are illustrated in **Figure 5.14**. Consistent with the hypothesis of acidogenesis occurring in the first compartments, **Figure 5.14 (a)** shows relatively high concentrations of acetic acid in compartment 1 of the reactor and low, or almost negligible, concentrations in the effluent. Similarly, for the other acids, the concentrations were higher in compartment 1 than in the effluent; most were not detected in the effluent. There was, however, a peak in the effluent concentration as a result of the dye shock load. The concentration of propionic acid (**Figure 5. 14 (b))** in the reactor was low except for an initial peak during start-up (438 mg/L on day 23), a small increase in concentration (141 mg/L) when the dye was first added to the reactor (day 65) and then another peak (259 mg/L) in response to the dye shock load.

i-Butyric acid concentrations were negligible in the reactor, except for two small concentration peaks, in compartment 1, during start-up of the reactor (613 mg/L on day 16) and in response to the dye shock load, with a concentration peak of 318 mg/L on day 155. During start-up, a high concentration (1 349 mg/L) of butyric acid (**Figure 5.14 (d))** was detected in compartment 1. Within 5 HRTs this concentration had reduced to < 10 mg/L and remained constant with a small peak concentration (63 mg/L) in response to the dye shock load. Since butyrate concentrations did not increase during the duration of the test, it was deduced that the OLR was not high enough to necessitate accumulation of these acetate stores. Negligible concentrations of *i*-valeric and valeric acids were detected in the reactor.

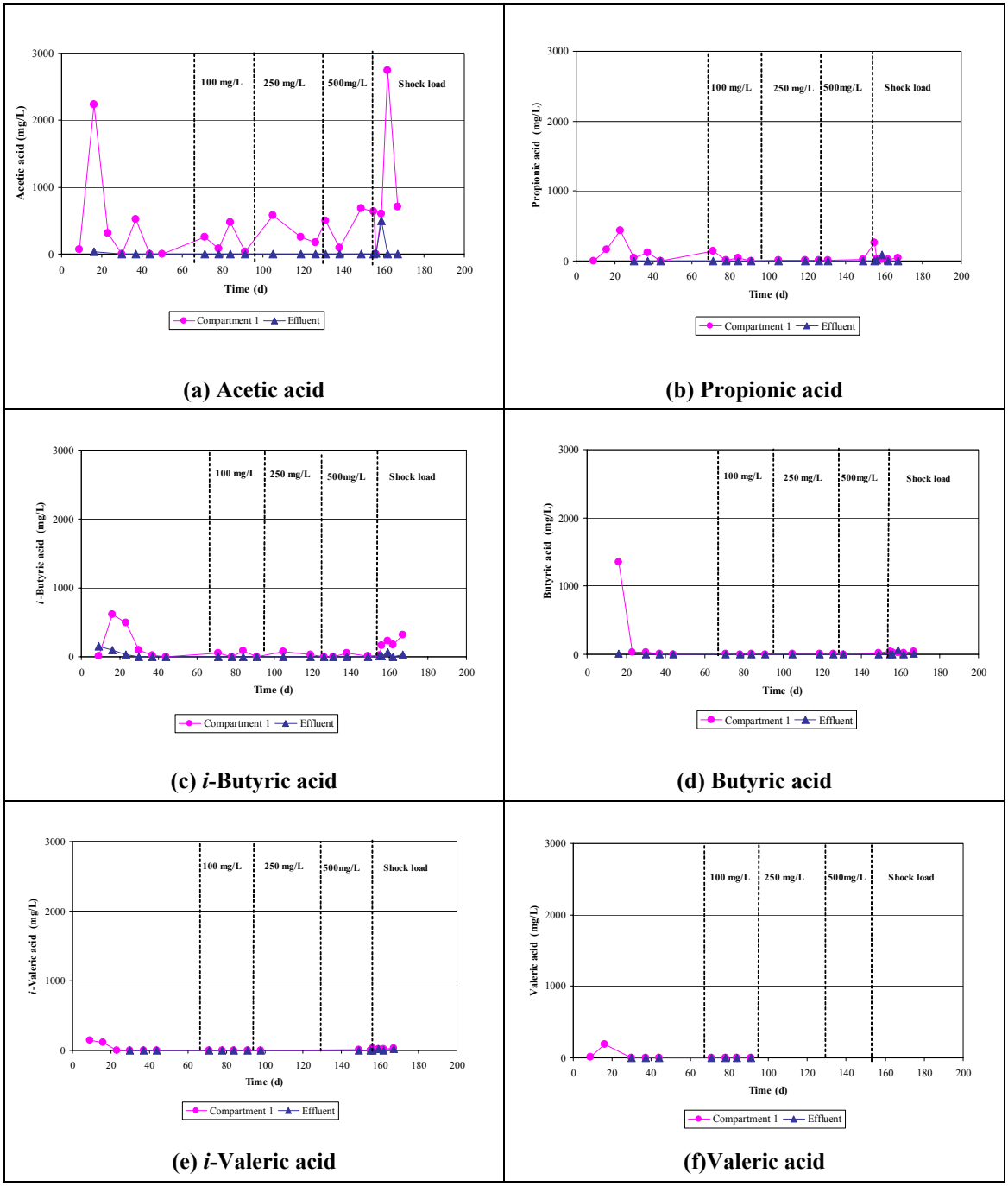


FIGURE 5.14 : Plots of each of the individual volatile fatty acids in compartment 1 and the effluent of the laboratory-scale ABR.

Profiles through the reactor (**Figure 5.15**) all showed a tail-off from maximum concentrations in the first compartments to very little in the later compartments. The reactor was stable and the acid concentrations were low, indicating methanogenic utilisation of the acids formed from the sucrose in the reactor feed. The VFA concentrations were low until the dye shock load, where the acetate concentration increased to 2 741 mg/L and remained relatively high throughout the remainder of the reactor, only dropping to < 100 mg/L in the reactor effluent.

These results indicate that the addition of CI Reactive Red 141 to the ABR feed stream did not have an adverse effect of the anaerobic digestion process. The only observed response to the dye was when the concentration was increased to 1 g/L, as a dye shock load to the reactor. The results show that the response to this was temporary inhibition but recovery to stable operation within 5 HRTs.

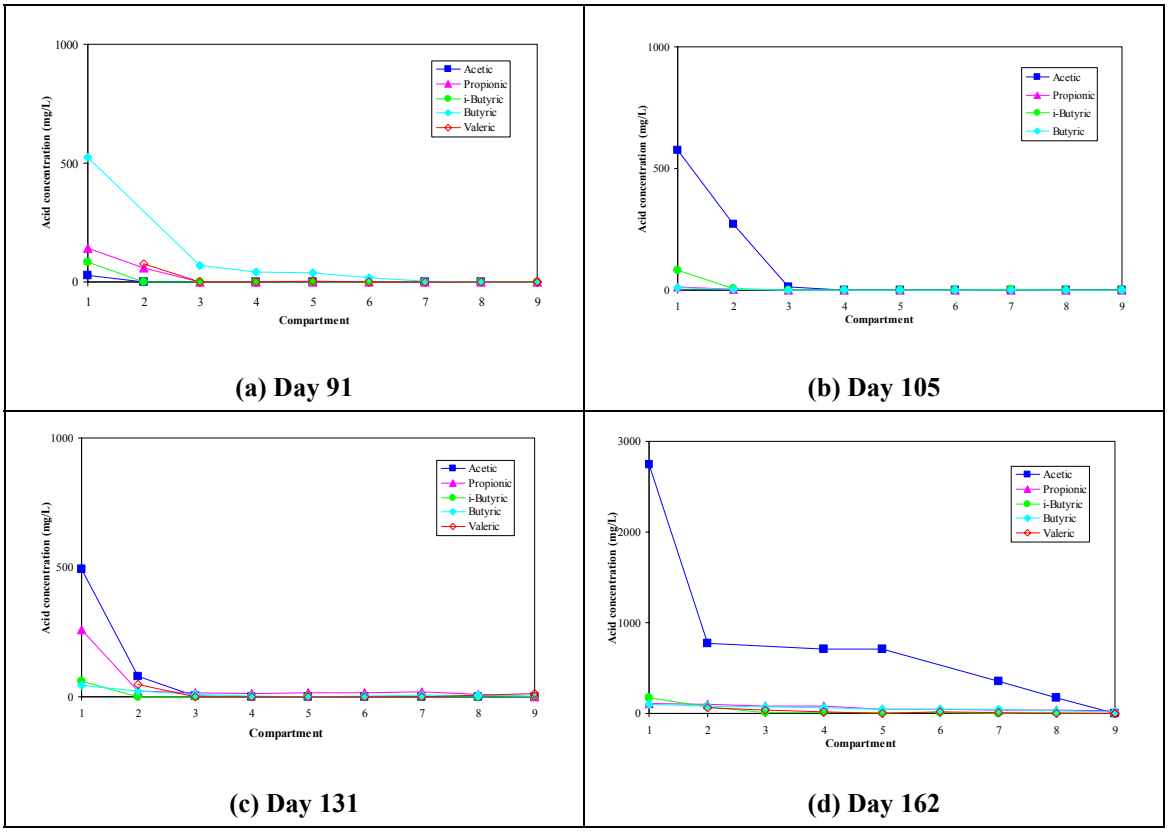


FIGURE 5.15 : Plots of the VFA profiles through the reactor, with time.

To summarise the information regarding the relative importance of the VFAs, the concentration is shown as a proportion of the total VFA contribution to COD. The COD equivalent of each VFA is given in **Table 4.3**.

The COD equivalent of each VFA, detected in the reactor effluent, was calculated and the total is presented in **Figure 5.16** with the measured effluent COD. These results show the high VFA-COD associated with the dye shock load, indicating that the unmetabolised VFAs, due to inhibition of the microorganisms by the high dye concentration, made up the majority of the effluent COD. During the remainder of the test, the VFA-COD was very low, thus the effluent COD must have been composed of the dye degradation products and SMPs.

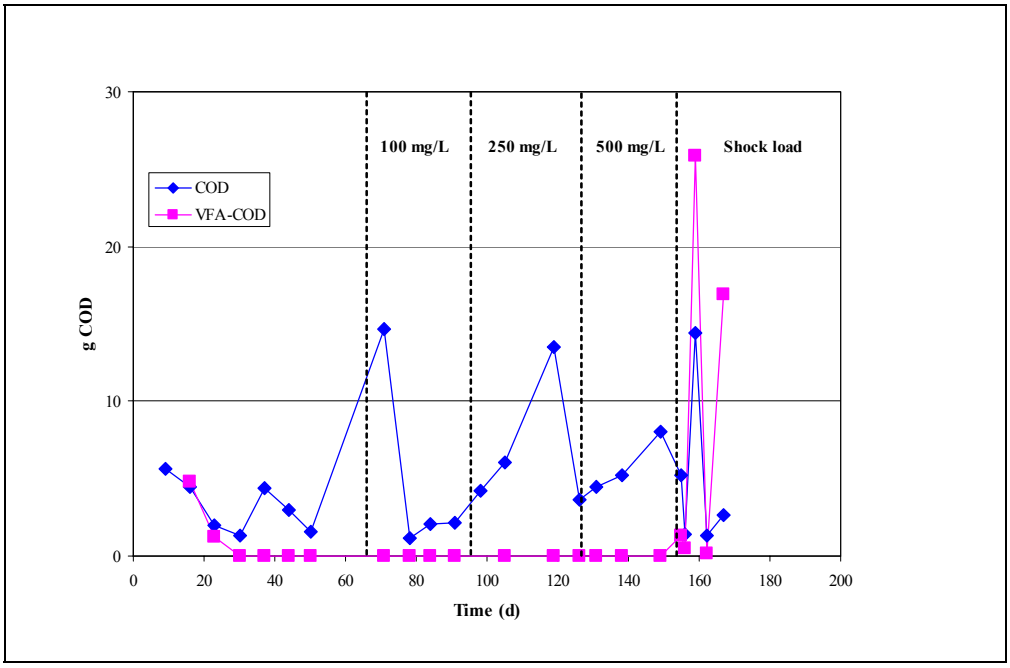


FIGURE 5.16 : Plot showing the measured COD and the calculated VFA-COD in the reactor effluent.

5.4.10 Reactor Biogas

The volume of biogas produced in each compartment was measured by the level sensor system described above and the biogas composition was analysed by gas chromatography (**Appendix 1**). The methane composition is given as a mole fraction of the total biogas (**Figure 5.17**).

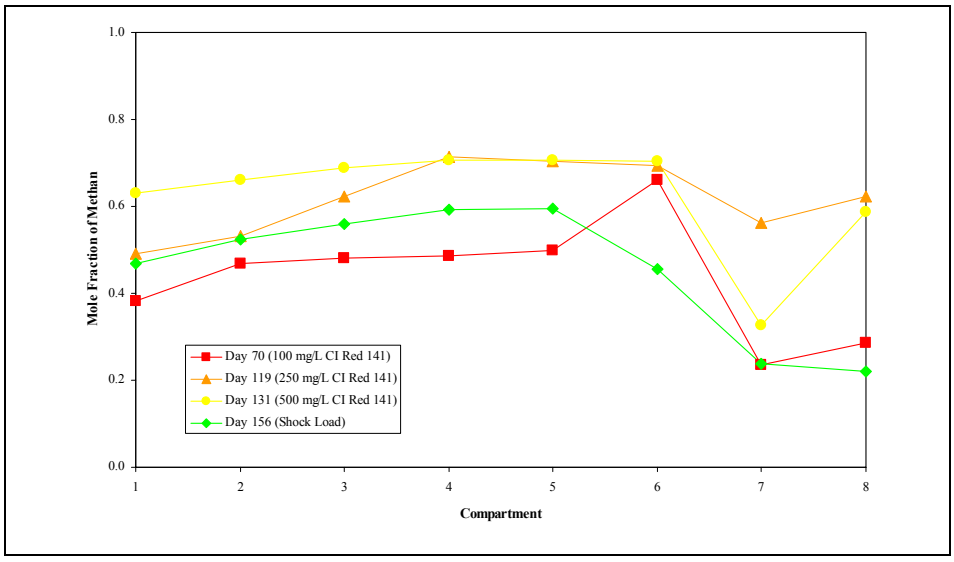


FIGURE 5.17 : Plot showing the methane concentration profiles through the laboratory-scale ABR treating a synthetic CI Reactive Red 141 waste stream.

The profile plot shows that for all of the investigated dye concentrations, the methane composition of the biogas increased in each compartment, from compartment 1 through to a maximum methane composition

in either compartment 5 or 6 (ca. 70%). In all cases the methanogenic activity was low in compartments 7 and 8 which could indicate that the methanogens in these compartments (predominantly the scavenging *Methanosaeta* spp.) were inhibited by the dye degradation products, or that the organic load to the reactor was such that the metabolic activity in these compartments was low. This correlates with the COD results (**Section 5.4.7**).

These results show that the methane composition in the first compartment was relatively high, ranging from ca. 40 % with the 100 mg/L dye concentration, to 63 % with the 500 mg/L concentration. Tilche and Yang (1987) found that 70 % of all methane produced in a hybrid ABR was produced in the first compartment, despite having only 10 % of the VS present within the reactor (Tilche and Yang, 1987).

The methane composition in the compartments increased with increasing dye concentration. This was unexpected since the anaerobic toxicity assays showed the methanogens to be inhibited by CI Reactive Red 141. The dye shock load resulted in a decrease in methanogenic activity, especially in compartments 6, 7 and 8, which suggests that the methanogens were inhibited by the dye degradation products.

These changes in the biogas composition can be related to the changes in the microbial populations due to the dye concentration changes (**Section 5.4.11**).

Willetts (1999) stated that successful treatment of highly concentrated dye wastewater was unlikely. This investigation has shown that successful treatment of a highly coloured wastewater is possible in the ABR. Although there was some inhibition of the methanogens with each increase in the dye concentration (up to 0.5 g/L), the COD and colour removal were efficient. The dye shock load (1 g/L) resulted in a temporary inhibition of the biomass, however, stable operation was resumed within 3 HRTs. The effect of a longer shock load on the reactor operation should be investigated prior to full-scale design and operation. Thus, it can be deduced that the design of the ABR facilitates efficient treatment of concentrated textile dye wastewaters by protecting the sensitive methanogens from the inhibitory dye molecules and degradation products, and promoting efficient colour and COD reduction, primarily in the first compartments. The results of this experiment showed very low metabolic activity in the final three compartments of the reactor, therefore, in a pilot- or full-scale design, these compartments could be removed. If the dye wastewater was to be treated with a high strength COD wastewater, the metabolic activity in the last three compartments would be higher and facilitate further COD reduction. The final three compartments could also be aerated for further reduction of the aromatic amines.

5.4.11 Population Characterisation

Reactor samples were taken, from each compartment, on days 64, 96, 126 and 150 of operation. The samples were hybridised (**Appendix 1**) with the fluorescent-labelled oligonucleotide probes listed in **Table 4.2** (except MS5 and MB4) to identify the microbial populations, the predominant populations and microbial changes, or shifts in population, with time and in response to changes in the dye concentration.

Initial hybridisations with the universal eubacteria (EUB338) and universal archaea (ARC915) probes revealed an abundance of members of both in the first compartment, at each sampling date. This correlated with the analytical data from the reactor operation, where it was evident that there was methanogenic activity in the first compartments and that the methanogenic activity increased with each increase in dye concentration. The relative ratio of eubacteria to archaea in each compartment, at each dye concentration, was determined and the results are presented in **Figure 5.18**.

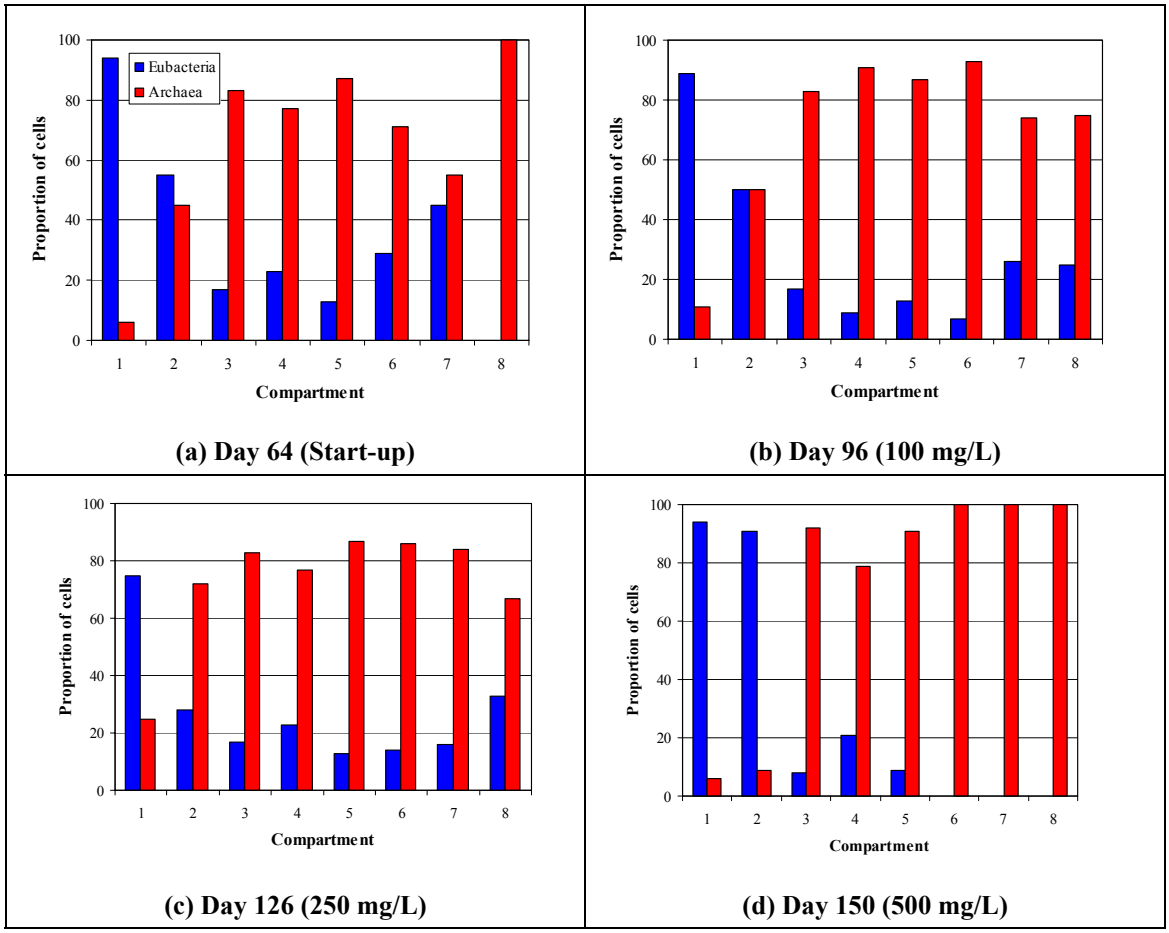


FIGURE 5.18: Ratios of Eubacteria (EUB338-hybridised) to Archaea (ARC915-hybridised) in each compartment of the ABR, for each investigated CI Reactive Red 141 concentration.

Analysis of samples taken from compartment 4 onwards revealed a decline in the ratio of bacterial cells to archaeal cells. The characteristic morphology of *Methanosaeta* (long sheathed filaments) was visualised using ARC915, and confirmation of the identity of these filaments using the genus specific probe MX825 was obtained. Another morphotype observed hybridising to ARC915 included *Methanospirillum*-like shorter filaments, which dominated the archaeal populations in the first four compartments, but did not hybridise with either the MX825 or MG1200 probes. Detailed results for each sample set are given below.

Day 64 (Start-Up) : The samples taken on day 64 were representative of the reactor biomass during start-up. The biomass had been exposed to a stepwise decrease in the HRT, from 80 h to 60, 30, then 20 h, with the synthetic sugar/protein feed (**Appendix 1**). The components of the feed were readily biodegradable, thus there was a predominance of the eubacteria (**Figure 5.19**) in compartment 1 (94 %) and equal populations of eubacteria and archaea in compartment 2. The archaea were the dominant microorganisms making up the microbial populations from compartment 3, through the rest of the reactor.

Figure 5.18 shows a predominance of archaea in compartments 7 and 8, however, the fluorescent signal emitted by these hybridisations was very faint, indicating low metabolic activity. This correlates with the biogas and COD data. All of the archaea in these compartments hybridised with the MX825 probe, i.e. scavenging *Methanosaeta* spp., which are able to survive at low acetate concentrations.

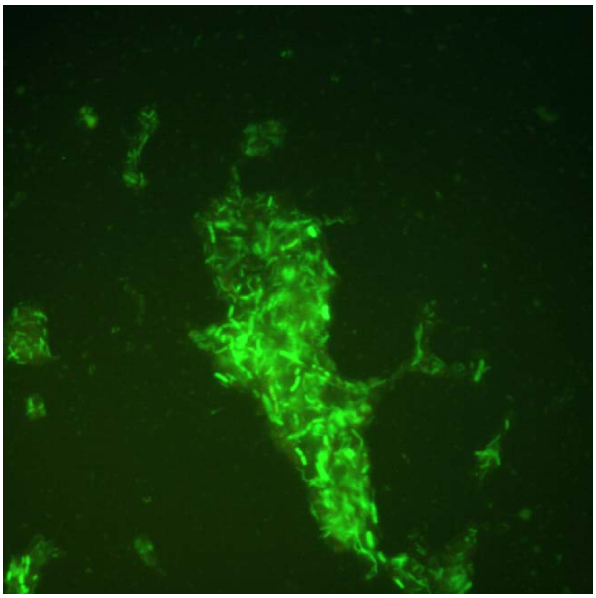
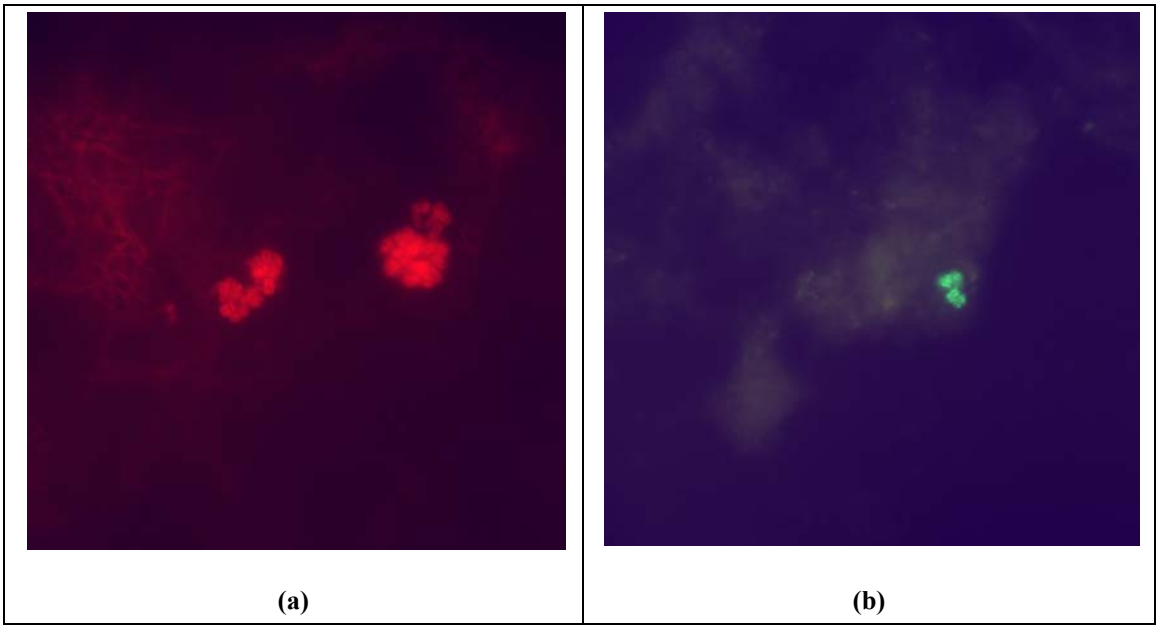


FIGURE 5.19 : FISH image of bacterial cells in compartment 1 of the CI Reactive Red 141 ABR, hybridised with EUB338.

Figure 5.21 shows the plots of the archaeal community analysis of the ABR compartments 1 to 8, sampled at each investigated dye concentration, showing counts obtained using family- and genus-specific probes expressed as a fraction of the total archaeal counts achieved using probe ARC915. On day 64, 32 % of the archaeal population in compartment 1 was made up a short filamentous species. *Methanosarcina* cells were observed in compartment 2; they made up 28 % of the archaea and hybridised with the MS821 probe (**Figure 5.20**).



(a) **(b)**

FIGURE 5.20 : FISH images of *Methanosarcina* cells, from compartment 2 of the CI Reactive Red 141 ABR, hybridised with (a) ARC915 and (b) MS821.

The remainder of the compartments, i.e. compartment 3 onwards, were dominated by the MX825-hybridised *Methanosaeta* spp. No MG1200 hybridisations were observed in these samples.

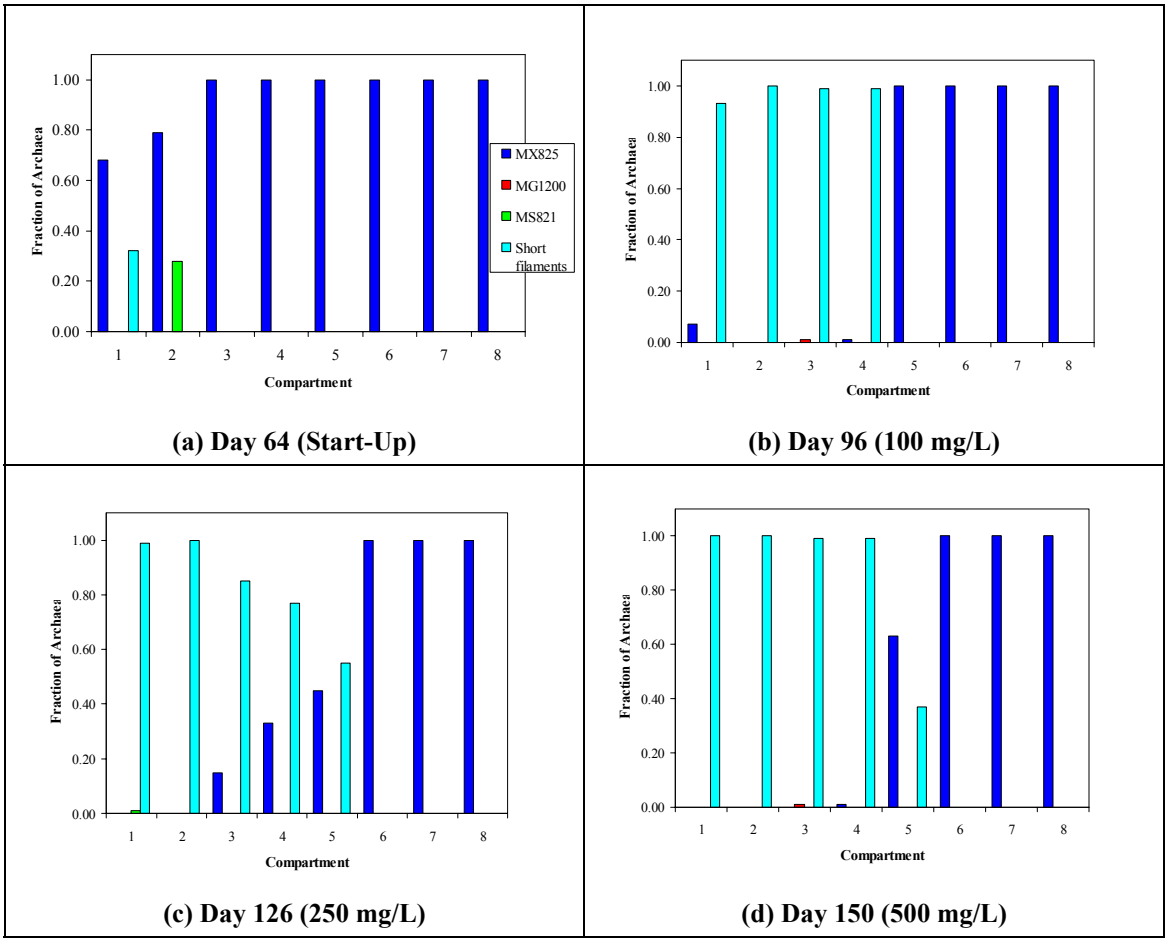
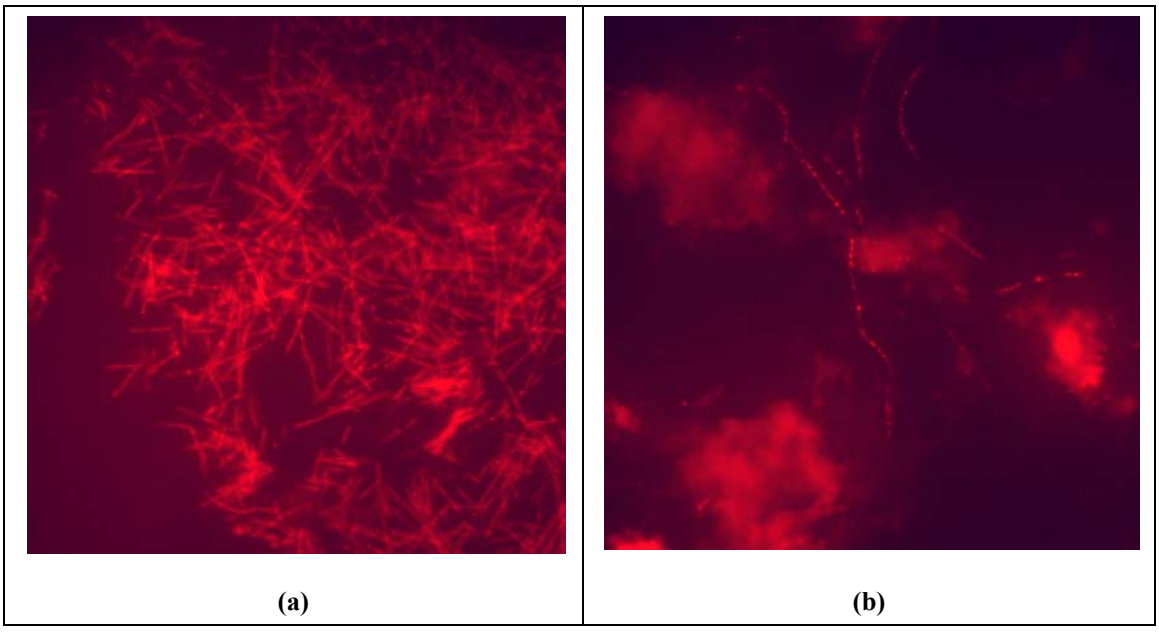


FIGURE 5.21 : Archaeal community analysis of ABR compartments 1 to 8, sampled at each investigated dye concentration, showing counts obtained using family- and genus-specific probes expressed as a fraction of total archaeal counts achieved using probe ARC915.

Day 96 (100 mg/L CI Reactive Red 141) : The biomass samples taken from the reactor on day 96 represented the sludge which had been treating a CI Reactive Red 141 concentration of 100 mg/L for 31 d. **Figure 5.18** shows that 89 % of the microbial population in compartment 1 was made up of eubacteria; the hybridisations emitted bright fluorescent signals indicating high metabolic activity. These organisms were responsible for the majority of the COD and colour reductions (**Sections 5.4.7** and **5.4.8**). The relatively low OLR did not require an extended acidogenic phase, thus the microbial population in compartment 2 was composed of ca. 50:50 eubacteria to archaea. The archaea dominated the populations from compartment 3 onwards. Compartments 7 and 8 contained very few, undefined cells, as observed by the microscope. The fluorescent signals emitted by the hybridisations in these compartments were very faint, indicating low metabolic activity, which correlates with the biogas and COD results.

Figure 5.21 (b) illustrates the composition of the archaeal population on day 96. Compartments 1 to 4 were dominated by a short filamentous species (**Figure 5.22 (a)**). Although these cells were similar in morphology to those observed in **Chapter 4**, they did not hybridise with the MG1200 probe. It was thought that they were *Methanosaeta* spp., however, they did not hybridise with the MX825 probe either. It was evident that these cells had proliferated due to the addition of the dye to the reactor. They were not inhibited by the dye or the dye degradation products since the bright fluorescent signals indicated high metabolic activity. These microorganisms would not have been present in the anaerobic toxicity assay sludge; the results suggest that selection occurred due to the addition of the dye to the ABR feed stream. Further identification of these microorganisms would require extraction and sequencing of the DNA.



(a) **(b)**

FIGURE 5.22 : FISH images of (a) the short archaeal filaments from compartment 2, hybridised with ARC915 and (b) the long *Methanosaeta* filaments from compartment 6, hybridised with MX825.

A portion (7 %) of the archaeal community in compartment 3 hybridised to the MG1200 probe. The *Methanosaeta* spp. dominated the archaeal populations from compartment 5 through the remainder of the

reactor. These organisms hybridised with the MX825 probe (**Figure 5.22 (b)**). The metabolic activity of the *Methanosaeta* spp. was relatively low, as can be seen by the faint fluorescent signal emitted in **Figure 5.22 (b)**. This corresponded with the reactor biogas and COD data which showed low methanogenic activity in the final reactor compartments.

Day 126 (250 mg/L CI Reactive Red 141) : The biomass samples taken from the reactor on day 126 represented the sludge which had been treating a CI Reactive Red 141 concentration of 250 mg/L for 30 d. The EUB:ARC ratios in **Figure 5.18** show that although the microbial population in compartment 1 was dominated by the eubacteria (75 %), there was a larger fraction of archaea in this compartment, compared to previous samples. This was unexpected since the anaerobic toxicity assay showed inhibition of the methanogens by the dye, thus it was expected that increasing the dye concentration would result in lowering the methanogenic activity. However, the biogas results showed increased methanogenic activity in the first compartments of the reactor, with increased dye concentration. The FISH experiments verified this with hybridisation of the short filamentous archaeal microorganism which showed high metabolic activity in compartments 1 to 4. The archaea dominated the communities from compartment 2, through the remainder of the reactor (**Figure 5.21 (c)**). *Methanosarcina* spp., hybridised with MS821, comprised ca. 1 % of the archaeal community in compartment 1.

Day 150 (500 mg/l CI Reactive Red 141) : The biomass samples taken from the reactor on day 150 represented the sludge which had been treating a CI Reactive Red 141 concentration of 500 mg/L for 23 d. The eubacteria dominated in compartment 1 (94 %) and compartment 2 (91 %). The reason for the increased eubacterial activity could be that the methanogens were inhibited by the higher dye concentration, although the biogas results (**Figure 5.17**) showed an increase in the methanogenic activity with the dye concentration increase to 500 mg/L. The results of the FISH experiments (**Figure 5.21 (d)**) verified these showing that the short filamentous archaeal species was predominant and active in the first four compartments. The EUB338 hybridisations emitted a bright fluorescent signal, indicating that these organisms were metabolically active and obviously involved in the colour reduction of the dye and COD reduction of the waste stream. The main component of the archaeal community, from compartment 5 onwards was the MX825-hybridised *Methanosaeta* spp. Very few cells were present in compartments 7 and 8 and the MX825 hybridisations in these compartments emitted very faint fluorescent signals, indicating low metabolic activity.

This investigation showed an unexpected result, namely the abundance of the short filamentous archaeal microorganism, which did not hybridise with either the MG1200 or MX825 probes. This species became metabolically active after the addition of the dye to the ABR feed stream, thus, it was deduced that there was selection for the organism. The bright fluorescent signals, together with the measured increased methane production suggest that these organisms were actively involved in the colour reduction of the dye and the COD reduction of the waste stream, in the first four compartments of the reactor. There was a horizontal separation through the reactor of this short filamentous species and the MX825-hybridised *Methanosaeta* spp. Metabolic activity was low in compartments 7 and 8.

5.4.12 Conclusions

1. The results of the physical decolourisation tests suggested significant decolourisation due to adsorption to the biomass, however, it is possible that the dye chromophores were reduced due to the low redox potential environment within the test bottles. No dye break-through, due to adsorption saturation, was observed during operation of the reactor.

2. COD reduction was consistently > 90 %, except for the period during the dye shock load. Colour reduction averaged 86 %.

3. The biomass showed acclimation to the dye, with increased methanogenic activity with each increase in dye concentration.

4. The inaccurate biogas measurement resulted in the poor COD balance of 18.2 %.

5. The reactor operation was stable, even with increases in the dye concentration. The only observed response to the dye was when the concentration was increased to 1 g/L (shock load), resulting in temporary inhibition but recovery to stable operation within 5 HRTs.

6. This investigation has shown that successful treatment of a highly coloured wastewater is possible in the ABR. The design of the ABR facilitates efficient treatment of concentrated textile dye wastewaters by protecting the sensitive methanogens from the inhibitory dye molecules and promoting efficient colour and COD reduction, primarily by the acidogens in the first compartments.

7. Metabolic activity was low in the final three compartments of the reactor.

8. There was a definite shift in the microbial populations through the ABR, with a predominance of eubacteria in the first compartments (acidogenesis) and archaea (methanogenesis) in the later compartments.

9. The use of molecular approaches provided useful descriptions of the methanogens actively involved within each compartment. These results showed a horizontal separation of a short filamentous archaeal microorganism and the long sheathed filamentous *Methanosaeta* spp.

10. The short filamentous archaeal microorganism proliferated after addition of the dye to the reactor and was metabolically active in the first four compartments of the reactor. Identification of the microorganism will require DNA extraction and sequencing.

11. The application of molecular techniques to the ABR process improved the understanding of the metabolic processes occurring within each compartments and the microorganisms involved in these reactions.

Chapter 6

Conclusions and Recommendations

From this investigation on the treatment of dye wastewaters in the anaerobic baffled reactor and the characterisation of the associated microbial populations, the following can be concluded:

1. There is potential for the ABR to be implemented on-site for pre-treatment of coloured wastewaters. The overall objective is the implementation of waste minimisation and cleaner production strategies in factories. However, wherever there is industrial activity, some waste is unavoidable; but the effluent produced is of much smaller volume and hence more concentrated. With implementation of the ABR, the concentrated waste stream could be pre-treated, with a biomass acclimated to the particular effluent, which should facilitate sufficient degradation such that the effluent could be discharged to sewer for further treatment.

2. Molecular methods can enhance the understanding of an anaerobic digestion process by providing information on the dynamics and evolution of the microbial communities. This information, together with the chemical and biochemical data will facilitate improved reactor design and optimisation of processes.

3. Batch toxicity and biodegradability assays are important screening tests, however, the results cannot be directly applied to the ABR since they use a mixed anaerobic sludge, whereas specialised sludges evolve in each compartment of the ABR. Screening tests can be devised to provide useful toxicity and biodegradability data for application to the ABR.

4. The hypothesis of the horizontal separation of acidogenesis and methanogenesis through the ABR was proven in the experiments reported in this thesis. Changes in the HRT affected the operation of the reactor, however, recovery from these upsets was almost immediate and operation of the reactor was stable.

5. Tartrazine was not readily degraded by anaerobic digestion, however, degradation may be improved with acclimation of the biomass. A COD reduction of 50 to 60 % was achieved in the laboratory-scale ABR and colour removal increased with time, suggesting acclimation of the biomass. After ca. 60 d, the tartrazine concentration in the effluent was 12 mg/L (95 % reduction). Most of the colour reduction was achieved in the first compartment of the reactor. The tartrazine dye, associated with the biomass interfered with probe hybridisation resulting in the 16S rRNA oligonucleotide probes binding to the dye and not to the biomass.

6. For the industrial dye wastewater, anaerobic degradation of a 10 % dilution was efficient. Methanogenic activity was high, the organic content of the influent was reduced by approximately

70 % and colour was reduced by almost 90 %. Degradation is affected by the complexity and variability of a real industrial wastewater, however the long SRT in the ABR can reduce these effects.

7. Anaerobic treatment of the textile reactive dye, CI Reactive Red 141, was successful with the COD reduction consistently > 90 %, except for the period during the dye shock load. Colour reduction averaged 86 %.The biomass showed acclimation to the dye, with increased methanogenic activity with each increase in dye concentration. The reactor operation was stable, even with increases in the dye concentration. The only observed response to the dye was when the concentration was increased to 1 g/L (shock load), resulting in temporary inhibition but recovery to stable operation within 5 HRTs.

8. The design of the ABR facilitates efficient treatment of concentrated dye wastewaters by protecting the sensitive methanogens from the inhibitory dye molecules and promoting efficient colour and COD reduction, primarily in the first compartments.

9. The molecular-based method, fluorescent *in situ* hybridisation (FISH), allowed the direct identification and enumeration of microbial populations active in the ABR. In all of the reported investigations, there was a definite shift in the microbial populations through the ABR, with a predominance of eubacteria in the first compartments (acidogenesis) and archaea (methanogenesis) in the later compartments. The number of compartments involved in each depended on the strength of the substrate (OLR).

10. The use of molecular approaches provided useful descriptions of the methanogens actively involved within each compartment. These results showed far fewer *Methanosarcina* cells than expected. In the laboratory-scale ABR, investigating the effect of changes in HRT on the microbial populations, a horizontal separation of the shorter filamentous Me*thanospirillum*-like spp. and the long sheathed filamentous *Methanosaeta* spp was observed.

11. A combination of FISH probing, and the analysis of 98 archaeal 16S rDNA clone inserts, revealed that together with the bacterial population, a methanogenic population dominated by *Methanosaeta*, together with species of *Methanobacterium* and *Methanospirillum*, and a relatively unstudied methanogen *Methanomethylovorans hollandica*, contributed to the successful anaerobic treatment of the industrial food dye waste stream.

12. Fluorescent *in situ* hybridisation (FISH), with 16S rRNA oligonucleotide probes, showed a horizontal separation of a short filamentous archaeal microorganism and the long sheathed filamentous *Methanosaeta* spp. in the laboratory-scale ABR treating the CI Reactive Red 141 stream. The short filamentous archaeal microorganism proliferated after addition of the dye to the reactor and was metabolically active in the first four compartments of the reactor. Identification of the microorganism will require DNA extraction and sequencing.

Based on the above conclusions, the following work is recommended:

1. Hydrodynamics studies to investigate the biomass concentration, the effects of gas mixing, changes in the viscosity of the bulk liquid and the degree of granulation of the biomass (particle size distribution). Knowledge of these would further enhance understanding of the flow and mixing patterns within the ABR, for optimisation of the design.

2. Develop batch test specifically for the ABR; this may involve sampling of biomass from each compartment of an operating ABR.

3. Further batch tests could be used to evaluate the half lives of dyes, under varying conditions such as inoculum source, dye class, sulphide concentrations and the presence of redox mediators.

4. An in-depth study on the acclimation of biomass to a dye, with concurrent molecular characterisation of the microbial population dynamics.

5. An accurate gas measurement system should be designed such that the operational efficiency of the reactor can be simultaneously monitored by an accurate mass balance.

6. Investigate complete treatment of a coloured wastewater in the ABR by aerating the final compartment for aerobic post-treatment to remove the residual colour.

7. The effect of a longer dye shock load on the reactor operation should be investigated prior to full-scale design and operation.

8. Develop HPLC methods to identify dye degradation products in the batch serum bottle tests and in the ABR effluent.

9. Trial investigations at Imperial College assessed the feasibility of using adsorption as a secondary treatment, to remove the remaining colour from the tartrazine and dye wastewater ABR effluents. Of the tested adsorbants (activated carbon in powder; synthetic clay; granular activated carbon; diatomaceous earth; Montmorilonite K10; cationic exchange resin; anionic exchange resin; rice; charcoal; and bentonite), the greatest colour removal was obtained with granular activated carbon. Total colour removal was obtained for the tartrazine effluent and 85.94 % for the dye wastewater effluent. An in-depth study on the cost-effectiveness of this post-treatment and the efficiency on a pilot- or full-scale would be valuable.

10. Construct and commission a pilot-scale ABR for pre-treatment of an industrial wastewater.

References

Alexiou, I.E. and Anderson, G.K. (2001). *Acidifcation Reactors for the Pre-Treatment of High Strength Agro-Industrial Wastwaters*. Anaerobic Digestion 2001. Antwerp, Belgium.

Amann, R., Binder, B.J., Olsen, R.J., Chrisholm, S.W., Devereux, R. and Stahl, D.A. (1990). Cited in:. *Amann et al., (1995)* .

Amann, R., Ludwig, W. and Schleifer, K.-H. (1995a). Phylogenetic Identification and *in Situ* Detection of Individual Microbial Cells without Cultivation. *Microbiological Reviews,* **59**(1): pp. 143-169.

Amann, R.I. (1995b). *In Situ* Identification of Micro-Organisms by Whole Cell Hybridization with rRNA-Targeted Nucleic Acid Probes. *Molecular Microbial Ecology Manual,* **3.3.6:** pp. 1-15.

American Public Health Association, Ed. (1989). *Standard Methods for the Examination of Water and Wastewater*. 16th Edition. Washington.

Anderson, G.K., Campos, C.M.M., Chernicharo, C.A.L. and Smith, L.C. (1991). Evaluation of the Inhibitory Effects of Lithium When Used as a Tracer for Anaerobic Digesters. *Water Research,* **25**(7): pp. 755-760.

Anon. (2000). *Textile Statistics and Economic Review***.** The Textile Federation. Doornfontein, South Africa.

Aquino, S.F. and Stuckey, D.C. (2001). *Characterization of Soluble Microbial Products (SMP) in Effluents from Anaerobic Reactors*. Anaerobic Digestion 2001. Antwerp, Belgium.

Athanasopoulos, N. (1991). Biodegradation of Textile Wastewaters. In: *Biological Degradation of Wastes*. A.M. Martin (ed.). Elsevier Science Publishers Ltd, London.

Atlas, R.M. and Bartha, R. (1993). *Microbial Ecology: Fundamentals and Applications.* 3rd edition. Benjamin/Cummings, Redwood City.

Bachmann, A., Beard, V.L. and McCarty, P.L. (1983). Comparison of Fixed Film Reactors with a Modified Sludge Blanket Reactor. In: *Fixed Film Biological Processes for Wastewater Treatment*. Y.C. Wu and E.D. Smith (eds.). Noyes Data Corp., NJ.

Bachmann, A., Beard, V.L. and McCarty, P.L. (1985). Performance Characteristics of the Anaerobic Baffled Reactor. *Water Research,* **19**(1): pp. 99-106.

Banat, I.M., Nigam, P., Singh, D. and Marchant, R. (1996). Microbial Decolourisation of Textile-Dye-Containing Effluents: A Review. *Bioresource Technology,* **58:** pp. 217-227.

Barber, W. and Stuckey, D. (1999). The Use of the Anaerobic Baffled Reactor (ABR) for Wastewater Treatment: A Review. *Water Research,* **33**(7): pp. 1559-1578.

Barber, W.P. (1999). *Effect of Nitrate and Sulphate on the Performance of an Anaerobic Baffled Reactor*. PhD Thesis. Imperial College, London.

Barber, W.P. and Stuckey, D.C. (1997). *Start-Up Strategies for Anaerobic Baffled Reactors Treating a Synthetic Sucrose Feed*. The 8th International Conference on Anaerobic Digestion. Sendai, Japan.

Bell, C.B. (1998). *Biological Decolourisation of Textile Effluent in a Nutrient Removal System.* MScEng Thesis. University of Natal, Durban.

Bell, J., Plumb, J.J., Buckey, C.A. and Stuckey, D.C. (2000). Treatment and Decolourisation of Dyes in an Anaerobic Baffled Reactor. *Journal of Environmental Engineering,* **126:** pp. 1026-1032.

Boopathy, R. and Sievers, D.M. (1991). Performance of a Modified Anaerobic Baffled Reactor to Treat Swine Waste. *Transactions of the ASAE,* **34**(6): pp. 2573-2578.

Boopathy, R. and Tilche, A. (1991). Anaerobic digestion of high strength molasses wastewater using hybrid anaerobic baffled reactor. *Water Research,* **25**(7): pp. 785-790.

Boopathy, R. and Tilche, A. (1992). Pelletization of Biomass in a Hybrid Anaerobic Baffled Reactor (HABR) Treating Acidified Wastewater. *Bioresource Technology,* **40:** pp. 101-107.

Brock, T.D. and Madigan, M.T. (1991). *Biology of Microorganisms.* 6th edition. Prentice-Hall International, Inc., USA.

Brown, D. and Laboureur, P. (1983). The Degradation of Dyestuffs: Part I - Primary Biodegradation Under Anaerobic Conditions. *Chemosphere,* **12**(3): pp. 379-404.

Brown, D. and Hamburger, B. (1987). The Degradation of Dyestuffs: Part III - Investigations of their Ultimate Degradability. *Chemosphere,* **16**(7): pp. 1539-1553.

Buckley, C.A. (1992). Membrane Technology for the Treatment of Dyehouse Effluents. *Water Science and Technology,* **25**(10): pp. 230-209.

Bumpus, J.A. (1995). Microbial Degradation of Azo Dyes. In: *Biotransformations: Microbial Degradation of Health-Risk Compounds*. V.P. Singh (ed.). Elsevier Science B.V., Amsterdam.

Carliell, C., Barclay, S.J. and Buckley, C.A. (1996). Treatment of Exhausted Dyebath Effluent using Anaerobic Digestion: Laboratory and Full-Scale Trials. *Water SA,* **22**(3): pp. 225-235.

Carliell, C.M. (1993). *Biological Degradation of Azo Dyes in an Anaerobic System.* MScEng Thesis. University of Natal, Durban.

Carliell, C.M., Barclay, S.J., Naidoo, N., Buckley, C.A., Mulholland, D.A. and Senior, E. (1994). Anaerobic Decolourisation of Reactive Dyes in Conventional Sewage Treatment Processes. *Water SA,* **20**(4): pp. 341-344.

Carliell, C.M., Barclay, S.J., Naidoo, N., Buckley, C.A., Mulholland, D.A. and Senior, E. (1995). Microbial Decolourisation of a Reactive Azo Dye under Anaerobic Conditions. *Water SA,* **21**(1): pp. 61-69.

Casey, T.J. (1993). *Unit Treatment Processes in Water and Wastewater Engineering.* John Wiley & Sons, Chichester.

Chang, Y.-J., Nishio, N. and Nagai, S. (1995). Characteristics of Granular Methanogenic Sludge Grown on Phenol Synthetic Medium and Methanogenic Fermentation of Phenolic Wastewater in a UASB Reactor. *Journal of Fermentation and Bioengineering,* **79**(4): pp. 348-353.

Chung, K.T. and Stevens, S.E. (1993). Degradation of Azo Dyes by Environmental Microorganisms and Helminths. *Environmental Toxicology and Chemistry,* **12:** pp. 2121-2132.

Chung, K.-T., Fulk, G.E. and Egan, M. (1978). Reduction of Azo Dyes by Intestinal Anaerobes. *Applied and Environmental Microbiology,* **35**(3): pp. 558-562.

Chynoweth, D.P., Srivastava, V.J. and Conrad, J.R. (1980). *Research Study to Determine the Feasibility of Producing Methane Gas from Sea Kelp***.** Institute of Gas Technology, IIT Center, Chicago, Illinois.

Cohen, A., Breure, A.M., Van Andel, J.G. and Van Deursen, A. (1982). Influence of Phase Separation on the Anaerobic Digestion of Glucose - II : Stability and Kinetic Responses to Shock Loadings. *Water Research,* **16:** pp. 449-455.

Collins, T.F.X., Black, T.N., Brown, L.H. and Bulhack, P. (1990). Study of the Taratogenic Potential of FD and C Yellow No. 5 when given to Rats. *Food and Chemical Toxicology,* **28**(12): pp. 812-827.

Constitutional Assembly (1996). *The Constitution of the Republic of South Africa. (http://www.polity.org.za/govdocs/constitution/saconst.htmI).*

Cooper, P. (1995). *Colour in Dyehouse Effluent.* Society of Dyers and Colourists. Alden Press, London.

Correia, V.M., Stephenson, T. and Judd, S.J. (1994). Characterisation of Textile Wastewaters - A Review. *Environmental Technology,* **15**: pp. 917-929.

Department of Water Affairs (1986). *Management of the Water Resources of the Republic of South Africa.* CTP Book Printers, Cape Town.

Department of Water Affairs and Forestry (1993). *South African Water Quality Guidelines. Volume 3 : Industrial Use.*

Department of Water Affairs and Forestry (1997). White Paper on Water Policy. *Internet URL http://www.polity.org.za/govt/white_papers/water.html.*

Dolby, P.J. (1980). Dyeing with Reactive Dyes. *Textile Chemist and Colorist,* **12**(9): pp. 231-233.

Domingues, M.R., Araujo, J.C., Varesche, M.B.A. and Vazoller, R.F. (2001). *Evaluation of the Microbial Composition of Thermophilic Anaerobic Biofilms and Suspension Cultures Grown in Acetate plus Sulfate using Fluorescence In Situ Hybridization (FISH) and Scanning Electron Microscopy (SEM)*. Anaerobic Digestion 2001. Antwerp, Belgium.

Donlon, B., Razo-Flores, E., Luijten, M., Swarts, H., Lettinga, G. and Field, J. (1997). Detoxification and Partial Mineralization of the Azo Dye Mordant Orange 1 in a Continuous Upflow Anaerobic Sludge-Blanket Reactor. *Appl Microbiol Biotechnol,* **47:** pp. 83-90.

Dubrow, S.F., Boardman, G.D. and Michelsen, D.L. (1996). Chemical Pretreatment and Aerobic-Anaerobic Degradation of Textile Dye Wastewater. In: *Environmental Chemistry of Dyes and Pigments*. A. Reife and H.S. Freeman (eds.). John Wiley & Sons, Inc., New York.

Fannin, K.F., Srivastra, V.J., Conrad, J.R. and Chynoweth, D.P. (1981). *Marine Biomass Program: Anaerobic Digester System Development***.** Annual Report for General Electric Company, Institute of Gas Technology, Chicago, Illinois.

Field, J.A., Stams, A.J.M., Kato, M. and Schraa, G. (1995). Enhanced Biodegradation of Aromatic Pollutants in Cocultures of Anaerobic and Aerobic Bacterial Consortia. *Antonie van Leeuwenhoek,* **67:** pp. 47-77.

FitzGerald, S.W. and Bishop, P.L. (1995). Two Stage Anaerobic/Aerobic Treatment of Sulfonated Azo Dyes. *J. Environ. Sci. Health,* **A30**(6): pp. 1251-1276.

Fontenot, E.J., Beydilli, M.I., Lee, Y.H. and Pavlostathis, S.G. (2001). *Kinetics and Inhibition During the Decolorization of Reactive Anthraquinone Dyes Under Methanogenic Conditions*. Anaerobic Digestion 2001. Antwerp, Belgium.

Fox, P. and Venkatasubbiah, V. (1996). Coupled Anaerobic/Aerobic Treatment of High-Sulphate Wastewater with Sulphate Reduction and Biological Sulphide Oxidation. *Water Science and Technology,* **34:** pp. 359-366.

Freese, L.H. and Stuckey, D.C. (2000). Influence of Seed Inoculum on the Start-Up of an Anaerobic Baffled Reactor. *Environmental Technology,* **21**: pp. 909-918.

Fu, Y., Jiang, H. and Bishop, P. (1994). An Inhibition Study of the Effect of Azo Dyes on Bioactivity of Biofilms. *Water Science and Technology,* **29**(7): pp. 365-372.

Garuti, G., Dohanyos, M. and Tilche, A. (1992a). Anaerobic-Aerobic Wastewater Treatment System Suitable for Variable Population in Coastal Areas: The ANANOX® Process. *Wat. Sci. Tech.,* **25**(12): pp. 185-195.

Garuti, G., Dohanyos, M. and Tilche, A. (1992b). Anaerobic-Aerobic Combined Process for the Treatment of Sewage with Nutrient Removal: The ANANOX® Process. *Water Science and Technology,* **25**(7): pp. 383-394.

Gatewood, B.M. (1996). Evaluation of Aftertreatments for Reusing Reactive Dyes. *Textile Chemist and Colorist,* **28**(1): pp. 38-42.

Ghosh, S. and Klass, D.L. (April, 1978). Two-Phase Anaerobic Digestion. *Process Biochemistry.* pp. 15-24.

Gilfillan, C.M. (1997). *Water and Effluent Management in the South African Textile Industry.* MScEng Thesis. University of Natal, Durban.

Glässer, A., Liebelt, U. and Hempel, D.C. (1992). *Design of a Two-Stage Process for Total Degradation of Azo Dyes*. DECHEMA Biotechnology Conference 5. Verlagsgesellschaft.

Godon, J.-J., Zumstein, E., Dabert, P., Habouzit, F. and Moletta, R. (1997). Molecular Microbial Diversity of an Anaerobic Digester as Determined by Small-Subunit rDNA Sequence Analysis. *Applied and Environmental Microbiology,* **63**(7): pp. 2802-2813.

Grau, P. (1991). Textile Industry Wastewaters Treatment. *Water Science and Technology,* **24**(1): pp. 97-103.

Gravelet-Blondin, L.R., Barclay, S.J., Carliell, C.M. and Buckley, C.A. (1997). Management of Water Resources in South Africa with respect to the Textile Industry. *Water Science and Technology,* **36**(2-3): pp. 303-310.

Griffin, M.E., McMahon, K.D., Mackie, R.I. and Raskin, L. (1998). Methanogenic Population Dynamics During Start-Up of Anaerobic Digesters Treating Municipal Solid Waste and Biosolids. *Biotechnology and Bioengineering,* **57**(3): pp. 342-355.

Grobicki, A. (1989). *Hydrodynamic Characteristics and Performance of the Anaerobic Baffled Reactor.* Ph.D. Thesis. Imperial College, London,

Grobicki, A. and Stuckey, D. (1991). Performance of the anaerobic baffled reactor under steady state and shock loading conditions. *Biotechnology and Bioengineering,* **37:** pp. 344-355.

Grobicki, A. and Stuckey, D.C. (1989). The Role of Formate in the Anaerobic Baffled Reactor. *Water Research,* **23**(12): pp. 1599-1602.

Grobicki, A. and Stuckey, D.C. (1992). Hydrodynamic characteristics of the anaerobic baffled reactor. *Water Research,* **26**(3): pp. 371-378.

Hansa, A. (1999). *The Development of Techniques for the Analysis of Reactive Dyes in Textile Dyeing Wastewater.* Master of Technology Thesis. ML Sultan Technikon, Durban.

Haug, W., Schmidt, A., Nörtemann, B., Hempel, D.C., Stolz, A. and Knackmuss, H.J. (1991). Mineralization of the Sulphonated Azo Dye Mordant Yellow 3 by a 6-Aminonaphthalene-2-sulphonate Degrading Bacterial Consortium. *Applied and Environmental Microbiology,* **57**(11): pp. 3144-3149.

Haugland, R.P. (1998). *Handbook of Fluorescent Probes and Research Chemicals***.** .

Henze, M., Harremoës, P., la Cour Jansen, J. and Arvin, E. (1997). *Wastewater Treatment.* 2nd edition. Springer-Verlag, Germany.

Holme, I. (March, 1997). Cotton Dyeing and Finishing to 2000 and Beyond. *International Dyer.* pp. 32-41.

Hugenholtz, P., Goebel, B.M. and Pace, N.R. (1998). Impact of Culture-Independent Studies on the Emerging Phylogenetic View of Bacterial Diversity. *Journal of Bacteriology,* **180**(18): pp. 4765-4774.

Iza, J., Colleran, E., Paris, J.M. and Wu, W. (1991). International Workshop in Anaerobic Treatment for Municipal and Industrial Wastewaters: Summary Paper. *Water Science and Technology,* **24**(8): pp. 1-16.

Knapp, J.S. and Newby, P.S. (1995). The Microbiological Decolourisation of an Industrial Effluent Containing a Diazo-linked Chromophore. *Water Research,* **29:** pp. 1807-1809.

Kugelman, I.J. and Chin, K.K., Eds. (1971). *Toxicity, Synergism, and Antagonism in Anaerobic Waste Treatment Processes*. Anaerobic Biological Treatment Processes. Washington, D.C., American Chemical Society.

Laing, I.G. (1991). The Impact of Effluent Regulations on the Dyeing Industry. *Rev. Prog. Coloration,* **21:** pp. 56-71.

Langenhoff, A.A.M. and Stuckey, D.C. (2000). Treatment of Dilute Wastewater Using an Anaerobic Baffled Reactor: Effect of Low Temperature. *Water Research,* **34**(15): pp. 3867-3875.

Lawrence, A.W. and McCarty, P.L. (1969). Kinetics of methane fermentation in anaerobic treatment. *Journal of the Water Pollution Control Federation.,* **41**(2): pp. R1-R17.

Leclerc, M., Godon, J.J. and Moletta, R. (2001). *Diversity of Archaea in Anaerobic Digesters*. Anaerobic Digestion 2001. Antwerp, Belgium.

Liakou, S., Pavlou, S. and Lyberatos, G. (1997). Ozonation of Azo Dyes. *Water Sceince and Technology,* **35**(4): pp. 279-286.

Lomans, B.P., Maas, R., Luderer, R., Op den Camp, H.J.M., Pol, A., Van Der Drift, C. and Vogels, G.D. (1999). Isolation and characterization of *Methanomethylovorans hollandica* gen. nov., sp. nov., isolated from freshwater sediment, a methylotrophic methanogen able to grow on dimethyl sulfide and methanethiol. *Applied and Enviornmental Microbiology,* **65:** pp. 3641-3650.

Maidak, B.L., Cole, J.R., Parker, C.T., Garrity, G.M., Larsen, N., Li, B., Lilburn, T.G., McCaughey, M.J., Olsen, G.J., Overbeek, R. (1999). A new version of the RDP (Ribosomal Database Project). *Nucleic Acids Research,* **27:** pp. 171-173.

Manz, W., Amann, R., Ludwig, W., Wagner, M. and Schleifer, K.H. (1992). Cited in: *Amann et al., (1995).*

Maynard, C.W. (1983). Dye Application, Manufacture of Dye Intermediates and Dyes. In: *Riegles Handbook of Industrial Chemistry*. J.A. Kent (ed.). Van Nostrand Reinheld, New York.

McCarty, P.L. (1964). Anaerobic waste treatment fundamentals. *Public Works,* **95:** pp. 107-112.

McCarty, P.L. and Mosey, F.E. (1991). Modelling of anaerobic digestion processes (A discussion of concepts). *Water, Science and Technology,* **24**(8): pp. 17-33.

Meier, H. (1998) Personal Communication.

Merkel, W., Manz, W., Szewzyk, U. and Krauth, K. (1999). Population dynamics in anaerobic wastewater reactors: modelling and *in situ* characterization. *Water Research,* **33:** pp. 2392-2402.

Munson, M.A., Nedwell, D.B. and Embley, T.M. (1997). Phylogenetic analysis of *Archaea* in sediment samples from a coastal salt marsh. *Applied and Environmental Microbiology,* **63:** pp. 4729-4733.

Nachaiyasit, S. (1995). *The Effect of Process Parameters on Reactor Performance in an Anaerobic Baffled Reactor.* Ph.D. Thesis. Imperial College, London.

Nachaiyasit, S. and Stuckey, D.C. (1997a). The Effect of Shock Loads on an Anaerobic Baffled Reactor (ABR), 1. Step Changes in Feed Concentration at Constant Retention Time. *Water Research,* **31:** pp. 2737-3747.

Nachaiyasit, S. and Stuckey, D.C. (1997b). The Effect of Shock Loads on an Anaerobic Baffled Reactor (ABR), 2. Step and Transient Hydraulic Shocks at Constant Feed Strength. *Water Research,* **31:** pp. 2747-2755.

Nigam, P., Banat, I.M., Singh, D. and Marchant, R. (1996). Microbial Process for the Decolorization of Textile Effluent Containing Azo, Diazo and Reactive Dyes. *Process Biochemistry,* **31**(5): pp. 435-442.

Orozco, A. (1997). *Pilot and Full-scale Anaerobic Treatment of Low-Strength Wastewaters at Sub-Optimal Temperature (15 °C) with a Hybrid Plug Flow Reactor*. The 8th International Conference on Anaerobic Digestion. Sendai, Japan.

Overmeire, A., Lens, P. and Verstraete, W. (1994). MassTransfer Limitation of Sulphate in Methanogenic Aggregates. *Biotechnology and Bioengineering,* **44:** pp. 387-391.

Owen, W.F., Stuckey, D.C., Healy Jr, J.B., Young, L.Y. and McCarty, P.L. (1979). Bioassay for monitoring biochemical methane potential and anaerobic toxicity. *Water Research,* **13:** pp. 485-492.

Plumb, J.J., Bell, J. and Stuckey, D.C. (2001). Microbial Populations Associated with Treatment of an Industrial Dye Effluent in an Anaerobic Baffled Reactor. *Applied and Environmental Microbiology,* **67**(7): pp. 3226-3235.

Pohland, F.G., Ed. (1992). *Design of Anaerobic Processes for the Treatment of Industrial and Municipal Wastes.* Water Quality Management Library. Technomic Publishing Company, Inc., Pennsylvania.

Polpraset, C., Kemmadamrong, P. and Tran, F.T. (1992). Anaerobic Baffle Reactor (ABR) Process for Treating a Slaughterhouse Wastewater. *Environmental Technology,* **13:** pp. 857-865.

Prival, M.J., Peiperl, M.D. and Bell, S.J. (1993). Determination of Combined Benzidine in FD & C Yellow No.5 (Tartrazine), Using a Highly Sensitive Analytical Method. *Food and Chemical Toxicology,* **31**(10): pp. 751-758.

Raskin, L., Stromley, J.M., Rittmann, B.E. and Stahl, D.A. (1994). Group-Specific 16S rRNA Hybridization Probes to Describe Natural Communities of Methanogens. *Applied and Environmental Microbiology,* **60**(4): pp. 1232-1240.

Razo-Flores, E., Donlon, B.A., Luijten, M., Lettinga, G. and Field, J.A. (1997). Biotransformation and Biodegradation of Azo Dyes by Anaerobic Granular Sludge Bed Reactors. *Applied Microbiology and Biotechnology,* **47:** pp. 83-90.

Ristow, N.E. (1999). *The Modelling of a Falling Sludge Bed Reactor using AQUASIM.* MSc Thesis. University of Cape Town.

Rittman, B.E., Bae, W., Namkung, E. and Lu, C.J. (1987). A Critical Evaluation of Microbial Product Formation in Biological Processes. *Water Science and Technology,* **19**(Rio): pp. 517-528.

Rittmann, B.E. and McCarty, P.L. (1978). Variable-Order Model of Bacterial-Film Kinetics. *Journal of the Environmental Engineering Division,* **104**(EE5): pp. 889-900.

Rocheleau, S., Greer, C.W., Lawrence, J.R., Cantin, C., Laramée, L. and Guiot, S.R. (1999). Differentiation of *Methanosaeta concilii* and *Methanosarcina barkeri* in Anaerobic Mesophilic Granular Sludge by Fluorescent In Situ Hybridization and Confocal Scanning Laser Microscopy. *Applied and Environmental Microbiology,* **65**(5): pp. 2222-2229.

Roller, C., Wagner, M., Amann, R., Ludwig, W. and Schleifer, K.H. (1994). Cited in: *Amann et al., (1995).*

Ross, W.R., Novella, P.H., Pitt, A.J., Lund, P., Thomson, B.A., King, P.B. and Fawcett, K.S. (1992). *Anaerobic Digestion of Waste-Water Sludge : Operating Guide***.** Water Research Commission, South Africa.

Sacks, J. (1997). *Anaerobic Digestion of High-Strength or Toxic Organic Effluents.* MScEng Thesis. University of Natal, Durban.

Sam-Soon, P.A.L.N.S., Wentzel, M.C., Dold, P.L., Loewenthal, R.E. and Marais, G. (1991). Mathematical modelling of upflow anaerobic sludge bed (UASB) systems treating carbohydrate waste waters. *Water SA,* **17**(2): pp. 91-106.

Schiener, P., Nachaiyasit, S. and Stuckey, D.C. (1998). Production of Soluble Microbial Products (SMP) in an Anaerobic Baffled Reactor: Composition, Biodegradability, and the Effect of Process Parameters. *Environmental Technology,* **19:** pp. 391-400.

Schlegel, H.G. (1992). *General Microbiology.* 6th Edition. Cambridge University Press.

Sekiguchi, Y. (2000). *Biodiversity and Ecology of Microorganisms in Anaerobic Wastewater Treatment Processes: Polyphasic Approach for Revealing Microbial Community in UASB Granular Sludges.* PhD Thesis. Nagaoka University of Technology, Japan.

Sekiguchi, Y., Kamagata, Y., Nakamura, K., Ohashi, A. and Harada, H. (1999). Fluorescence In Situ Hybridization Using 16S rRNA-Targeted Oligonucleotides Reveals Localization of Methanogens and Selected Uncultured Bacteria in Mesophilic and Thermophilic Sludge Granules. *Applied and Environmental Microbiology,* **65**(3): pp. 1280-1288.

Seshadri, S., Bishop, P.L. and Agha, A.M. (1994). Anaerobic/Aerobic Treatment of Selected Azo Dyes in Wastewater. *Waste Management,* **14**(2): pp. 127-137.

Sievers, D.M. (1988). *Particle Trapping in an Anaerobic Baffle Reactor*. International Winter Meeting of the American Society of Agricultural Engineers,Chicago.

Society of Dyers and Colourists and American Association of Textile Chemists and Colourists *Colour Index.* 3rd Edition.

Speece, R.E. (1983). Anaerobic biotechnology for industrial wastewater treatment. *Environment, Science and Technology,* **17**(9): pp. 416A-427A.

Speece, R.E. (1996). *Anaerobic Biotechnology.* Archae Press, Nashville Tennessee.

Spencer, P. (1999) Personal Communication.

Stahl, D.A. and Amann, R. (1991). Cited in: *Amann et al., (1995)*.

Stahl, D.D., Devereux, R., Amann, R., Flesher, B., Lin, C. and Stromley, J. (1989). Cited in: *Amann et al., (1995).*.

Steffen Robertson and Kirsten (1993). *Natsurv 13: Water and Waste-Water Management in the Textile Industry***.** Water Research Commission, South Africa.

Tan, N. (2001). *Integrated and Sequential Anaerobic/Aerobic Biodegradation of Azo Dyes.* PhD Thesis. Wageningen University.

Tarvin, D. and Buswell, M. (1934). The methane fermentation of organic acids and carbohydrates. *Journal of the American Chemical Society,* **56:** pp. 1751-1755.

Tilche, A. and Yang, X. (1987). *Light and Scanning Electron Microscope Observations in the Granular Biomass of Experimental SBAF and HABR reactors*. Gasmat Workshop,The Netherlands.

Tracey, R.P., Spangenberg, G.J. and Britz, T.J. (1989). *Isolation and characterization of aerobic, facultative and anaerobic non-methanogenic acetate-utilizing bacteria from anaerobic digesters.* Second Anaerobic Digestion Symposium. Bloemfontein, South Africa.

Uemura, S. and Harada, H. (1993). Microbial Characteristics of Methanogenic Sludge Consortia Developed in the Thermophilic UASB Reactors. *Applied Microbiology and Biotechnology,* **39:** pp. 654-660.

Uyanik, S., Sallis, P.J. and Anderson, G.K. (2001). *Development of Split Fed Anaerobic Baffled Reactor*. Anaerobic Digestion 2001.Antwerp, Belgium.

Vandevivere, P.C., Bianchi, R. and Verstraete, W. (1998). Treatment and Reuse of Wastewater from the Textile Wet-Processing Industry: Review of Emerging Technologies. *J. Chem. Technol. Biotechnol.,* **72:** pp. 289-302.

Weiland, P. and Rozzi, A. (1991). The Start-Up, Operation and Monitoring of High-Rate Anaerobic Treatment Systems: Discusser's Report. *Water Science and Technology,* **24**(8): pp. 257-277.

Willetts, J.R.M. (1999). *Thermophilic Decolourisation of Textile Dye Wastewater*. PhD Thesis. University of New South Wales.

Williamson, K. and McCarty, P.L. (1976). A Model of Substrate Utilization by Bacterial Films. *Journal of the Water Pollution Control Federation,* **48**(1): pp. 9-24.

Wisjnuprapto, Lufti Firdaus, M. and Kardena, E. (2001). *Oxido Redox Potential in the Co-Metabolism of Synthetic Dyes Color Index Reactive Orange 16 and Colour Index Reactive Red 3*. The International Water Association Conference on Water and Wastewater Management for Developing Countries. Kuala Lumpur, Malaysia.

Xing, J., Boopathy, R. and Tilche, A. (1991). Model Evaluation of Hybrid Anaerobic Baffled Reactor Treating Molasses Wastewater. *Biomass and Bioenergy,* **1**(5): pp. 267-274.

Zinder, S.H., Anguish, T. and Cardwell, S.C. (1984). Effects of temperature on methanogenesis in a thermophilic (58°C) anaerobic digestor. *Applied and Environmental Microbiology,* **47**(4): pp. 808-813.