THE MOLECULAR MICROBIAL ECOLOGY OF SULFATE REDUCTION IN THE RHODES BioSURE PROCESS

by

Chesa Gift Chauke

Submitted in fulfilment of the requirements for the degree of

Master of Science

Of

Rhodes University, Grahamstown

January 2002

TABLE OF CONTENTS

CHAPTER 2
DEVELOPMENT AND PERFORMANCE OF A LAB-SCALE BAFFLE REACTOR

CHAPTER 3
ISOLATION AND MORPHOLOGICAL CHARACTERISATION OF BACTERIAL STRAINS IN A SULFATE REDUCING SLUDGE POPULATION

CHAPTER 4
IDENTIFICATION OF SRB STRAINS FROM PURE CULTURE ISOLATES USING 16S rRNA ANALYSIS

CHAPTER 5
PHYLOGENETIC DIVERSITY OF A SULFATE REDUCING SLUDGE POPULATION DETERMINED BY ANALYSIS OF 16S rRNA GENE EXTRACTED DIRECTLY FROM SLUDGE

ABSTRACT

The research reported here investigated the use of a Baffle Reactor in order to study aspects of the biological sulfur cycle, where a floating sulfur biofilm formation occurs and where complex organic compounds provide electron donor sources. The development of a laboratory-scale Baffle Reactor model system satisfied the requirements for sulfate reducing bacterial biomass growth and sulfur biofilm formation. Since relatively little is known about the microbial ecology of floating sulfur biofilm systems, this study was undertaken to describe the sulfate reducing sludge population of the system together with its performance.

A combination of culture- and molecular- based techniques were applied in this study in order to investigate the microbial ecology of the sulfate-reducing bacteria component of the system. These techniques enabled the identification and the analysis of the distribution of different sulfate reducing bacterial strains found within the sludge bioreactors. Strains isolated from the sludge were characterised based on culture appearance, gram staining and scanning electron microscopy morphology. Molecular methods based on the PCR-amplified 16S rRNA including denaturing gradient gel electrophoresis were employed in order to characterise sulfate-reducing bacteria within the reactors. Three novel Gram negative sulfate-reducing bacteria strains were isolated from the sludge population. Strains isolated were tentatively named *Desulfomonas rhodensis*, *Desulfomonas makanaiensis*, and *Clostridium sulforhodensis*. Results obtained from the Baffle Reactor showed that three dominant species were isolated from

the DNA extracted from the whole bacterial population by PCR. Three of these were similar to those mentioned above. The presence of these three novel unidentified species suggest that there are a range of other novel organisms involved in sulfate reduction processes.

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

A	adenine
AFM	atomic force microscopy
AMD	acid mine drainage
Amp	ampicillin
APS	adenosine phosphosulfate
ATP	adenosine triphosphate
bp	base pair(s)
BR	baffled reactor
C	cytosine
°C	degrees Celsius
CLSM	confocal laser scanning microscopy
COD	chemical oxygen demand
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphates
ds	double EDTA ethylenediaminetetraactetic acid
EBG	environmental biotechnology group
EPS	extracellular polymeric substance
EtBr	ethidium bromide
FISH	fluorescent *in situ* hybridisation
FSBR	falling sludge bed reactor
G	guanine
G+C	glutamine:cytosine ratio

HMB	hydrogenotrophic methanogenic bacteria
HPLC	high pressure liquid chromatography
IPTG	isopropyl-β-D-galactosidase
Kb	kilobase pair(s)
L	litre
LA	Luria agar
LB	Luria broth
M	molar
MB	methanogenic bacteria
mg	milligram
min	minutes
mL	millilitres
mM	millimolar
MPN	most probable number
PCR	polymerase chain reaction
PMF	protein motive force
PSS	primary sewage sludge
rDNA	ribosomal deoxyribonucleic acid
RDP	ribosomal database project
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulfate/ sodium lauryl sulfate
SEM	scanning electron microscopy
SOB	sulfide oxidising bacteria

SPB	sulfide producing bacteria
SRB	sulphate reducing bacteria
SSC	sodium citrate buffer
T	thymine
TAE	Tris-acetate-EDTA buffer
TBE	Tris-borate-EDTA buffer
TE	Tris-HCI buffer
TEM	transmission electron microscopy
UASB	Upflow anaerobic sludge bed reactor
µg	microgram
µL	microlitre
µM	micromolar
µm	micrometer
X-Gal	5-bromo-4-chloro-3-indolyl-β-galactosidase

ACKNOWLEDGEMENTS

The author gratefully acknowledges the following people:

Professor Peter Rose, Department of Microbiology, Rhodes University, for his guidance, support, encouragement and financial generosity throughout the course of this project.

Dr. R.A. Dorrington for her interest and co-supervision especially with the molecular studies.

Professor R. Kirby for his input, comments and constructive criticisms.

Staff and post-graduate students in the Department of Biochemistry and Microbiology, Rhodes University Grahamstown especially: Anna Clarke and Michelle Bowker, for their helpful suggestions, comments and criticisms during the course of this project.

Joan Gilfillan and Ilse Brünning for their help in developing the reactor and optimising the DGGE techniques.

Robin Cross, Shirley Pinchuck and Marvin Randall of the Rhodes Electron Microscopy Unit, for their help with scanning, photographing and for their assistance with the use of the Adobe Photohouse software package.

The Water Research Commission for the financial support that made this project possible.

Thandiwe Notshe for her friendship, encouragement and her unending emotional support.

Finally, my family, for their support and encouragement.

Chapter 1

MICROBIAL ECOLOGY OF SULFATE REDUCING SYSTEMS

1.1 ACID MINE DRAINAGE WASTEWATERS

Pollution of surface waters with acid mine drainage (AMD) follows geochemical trauma induced by mining operations (Rose *et al.*, 1998). The main sources of AMD in abandoned mine areas are usually old waste rock dumps and rock walls in tunnels and shafts. Open pits and underground workings will often be filled partly or completely with polluted water after the closure of a mine, and overflow from such systems may in some cases contribute significantly to the total transport of pollutants out of the area (Christensen *et.al.*, 1999).

All the active gold mines in the major mining basins of South Africa are dewatered in order to remain operational (Scott, 1995). Water leaving the mine may be characterised by one or more of the following: low pH, high Total Dissolved Solids (TDS), high sulfates (SO_4^{2-}), and/or high levels of heavy metals especially Iron (Fe), Manganese (Mn), Nickel (Ni), and/or Cobalt (Co) (Scott, 1995).

Mineral build-up caused by acidic mine drainage presents a formidable problem in South Africa, particularly in view of the fact that it imposes a severe restriction on the beneficial use of water. The effects of this are compounded by the fact that the majority of the gold and coal mines are located in the most highly industrialised and, therefore, the most intensely populated areas in the country where the demands on the fresh water supplies for domestic and industrial use are the highest. The discharge of acid mine drainage, whether from

underground workings or as seepage from slimes and waste dumps into the natural drainage system in these areas, has a profound effect on the critical balance between demand and supply. It is, therefore, advisable to consider the acid mine drainage problem in the context of the critical balance existing between the present and future demands for and availability of water (Henzen and Pieterse 1978).

Although mines only use 4.2 per cent of the total amount of water used in the Republic, the pollution loads carried in mine effluents can impose limitations on the usefulness of the fresh water resources. It has been estimated that when mine pumping operations finally cease, filling of the voids would occur within about a decade, and then to be followed by long term surface flows of AMD (Scott, 1995).

Traditionally these waters have been treated by the addition of an alkaline agent, usually lime, to increase the pH and induce the formation and precipitation of metal hydroxides. Lime treatment is simple and produces a predictable water quality that has been proven by decades of use. However, lime treatment cannot meet new, more restrictive, metal discharge standards and produces a voluminous, mixed metal-hydroxide sludge that is difficult to dewater and dispose (De Vegt *et al.*, 1996).

1.2 PROCESSES FOR THE TREATMENT OF AMD WASTEWATER

Treatment of the AMD problem has been investigated from the perspectives of both physicochemical and biological processes.

1.2.1 Chemical Treatment Processes

A range of chemical processes have been applied to the treatment of AMD. These include precipitation with barium salts, neutralisation with limestone and slurry precipitation recycle reverse osmosis (SPARRO).

1.2.1.1 Precipitation with Barium Salts

Barium has been extensively used in the treatment of boiler feed water for the removal of sulfates. Barium sulfate is a very insoluble compound, and one way of removing sulfate from water is its precipitation as the barium sulfate salt. The source of barium is usually barium carbonate. The removal of sulfate using barium salts consist of two stage barium carbonate and four stage barium sulfide processes (Maree *et al.,* 1989). The removal of sulfate and calcium from water by means of barium carbonate (BaCO3) and barium sulfide (BaS2-) can be presented by the following reactions respectively:

$$Ca^{2+} + SO_4^{2-} + BaCO_3\,(s) \rightarrow BaSO_4\,(s) + CaCO_3 \qquad (1)$$

$$Ca^{2+} + SO_4^{2-} + BaS^{2-} \rightarrow BaSO_4\,(s) + CaS^{2-} \qquad (2)$$

In both processes, sulfate is precipitated as barium sulfate, calcium as calcium carbonate, magnesium as magnesium hydroxide where **M** represents heavy metals such as iron,zinc or copper, and heavy metals as either metal hydroxides or metal sulfides as show in equation 4 below.

$$BaCO_3 + MgSO_4 + Ca(OH)_2 \rightarrow BaSO_4(s) + Mg(OH)_2 + CaCO_3 \qquad (3)$$

$$\mathbf{MS}(OH)^{2+} + H_2O \rightarrow \mathbf{MS} + 2H_2O \qquad (4)$$

1.2.1.2 Neutralisation with limestone

Limestone ($CaCO_3$) can be used as an alternative to lime for the neutralisation of AMD as it is cheaper and occurs naturally in a pure state as limestone, and with magnesium as dolomitic limestone. Maree *et al.,* (1989) investigated the practicality of using cheaper limestone instead of lime for the treatment of acidic effluents. From this study, it was determined that in case of lime treatment, the rate of neutralisation is fast when stiochiometric dosages of lime are applied.

1.2.1.3 Slurry Precipitation Recycle Reverse Osmosis (SPARRO)

Two of the main advantages of this process are that it produces a high quality solid gypsum by-product which could be sold, and can operate at very high recovery ratios, which reduces the quantity for brine disposal.

1.2.1.4 Disadvantages of Chemical Treatment Processes

Chemical treatment has the disadvantages of producing large volumes of unstable metal hydroxides mixed with gypsum which are costly to dispose of, especially when toxic metals content classifies it as hazardous waste (Hulshoff Pol *et al.*, 1998). The sludges may also precipitate in the reactor, which may cause problems of plugging, abrasion and toxicity. Schemes for recovery and reuse of the chemicals would be the key for not only developing a satisfactory solution to the problem of ultimate sludge disposal, but also would lead to the development of a process wherein superior economic advantages could be demonstrated.

The need for an economically viable alternative treatment of AMD has led to the investigation of many processes to determine the most feasible one. Thus, the biological anaerobic

treatment of industrial wastewater and acid mine water in particular, utilising sulfate reduction by sulfate-reducing bacteria (SRB) has become a major topic of interest.

1.2.2 Biological Treatment Processes

Biological sulfate reduction has been identified as a potentially available process for removing sulfates and heavy metals from industrial effluents (Maree *et al.*, 1987). The use of sulfate reducing bacteria permits the selective recovery of copper and zinc, and the separation of sludges containing hazardous elements from those containing only innocuous elements. The application of the process has been demonstrated in active treatment systems for acid mine drainage (AMD) remediation at laboratory and pilot-scale tests in various reactor designs, including packed bed anaerobic reactors (Maree *et al.*, 1987), fluidised bed systems, anaerobic filters, and the Baffle Reactor (Grobiki & Stuckey, 1991). Laboratory and pilot-scale tests in bioreactors and artificial wetlands have proven that sulfate-reduction is effective in raising pH and removing metals and sulfate from mine waters (Hammack and Edenborn, 1992; Dvorak *et al.*, 1992).

Sulfate reduction is carried out by SRB as shown in the following equations, where CH_2O represents a carbohydrate (Herlihy *et al.*, 1987)

$$2CH_2O + SO_4^{2-} \rightarrow S^{2-} + 2CO_2 + 2H_2O \qquad (5)$$

$$S^{2-} + 2CO_2 + 2H_2O \rightarrow H_2S + 2HCO_3^- \qquad (6)$$

The quality of sulfate reduced is directly coupled to the SRB growth, which is related to the energy available in the carbonaceous substrate. Therefore, sulfate conversion can be controlled by the quantity of carbon substrate supplied. Acetate, often a metabolite of SRB growth, is only slowly degraded. However, methanogens readily degrade this compound.

Application of biological approaches in AMD treatment, and related process developments, have concentrated on active and passive treatment systems, both of which rely on microbial activity related to the biological sulfur cycle, including high SRB growth rates and the associated precipitation of metals as sulfide salts (Hulshoff Pol *et al.*, 1998). Since numerous SRB reactor design studies have been reported, bioreactor design, cost of bioreactor construction and the cost of carbon source and electron donor for the biological sulfate reduction process are the singular factors constraining process development. The evaluation, in the active AMD treatment application, of a wide range of reactor designs and carbon sources has been reported (Rose e*t al.*,1998), with most successful full-scale developments focussed on UASB type reactors, and using refined carbon sources, such as ethanol and methanol, and also producer gas as the electron donor for sulfate reduction (Maree JP & Hill, 1989). These have proved to be effective but relatively higher cost options.

The Rhodes BioSURE Process was developed from studies conducted on the use of complex organic compounds derived from waste streams as the carbon source for sulfate reduction. This integrated resource management approach linking AMD treatment and sewage sludge disposal has been driven primarily by a requirement for low-cost and long-term sustainability. The studies of hydrolysis, sulfate reduction and use of the sulfide product to precipitate metals in AMD waste water were incorporated into features that formed the basis of the Rhodes 'BioSURE' pilot plant constructed at Grootvlei Gold Mine.

Based on the circumstances relating to the dewatering of the Grootvlei Gold Mine, a competitive technology evaluation exercise was undertaken by the Government to determine the long-term course of action by which mine drainage wastewaters would be treated. The Rhodes University Environmental Biotechnology Group (EBG) was requested to participate

in this exercise and Figure 1.1 illustrates the site on which the 'BioSURE' Process pilot plant was constructed at No. 3 Shaft, Grootvlei Mine, early in 1998.

Figure 1.1: The Rhodes 'BioSURE' Process pilot plant at Grootvlei Gold Mine, Gauteng

The results of studies reported by Whittington-Jones (2000) and Corbett (2001) have demonstrated the development of active biological treatment of sulfate saline wastewater based on complex organic carbon utilisation as the electron donor source in bacterial sulfate reduction. Given the volume of the treatment requirement at Grootvlei Mine it was decided to concentrate on the use of sewage sludge as the most freely available carbon source.

1.3 THE BIOLOGICAL SULFUR CYCLE

Sulfur (S) is an important element biochemically and geochemically. It constitutes 1% of the dry mass of organisms, in which it serves many structural and enzymatic functions. Sulfur

also acts as a significant electron donor and acceptor during many bacterial metabolic activities (Hulshoff Pol *et al.*, 1998). Sulfur can be found in a range of valence states from highly reduced sulfide (-2) to the most oxidised form in sulfate (SO_4^{2-}) (+6). There are several intermidiate valence forms of sulfur that can act as both electron donor and electron acceptor, depending on environmental conditions, the most notable being elemental sulfur (S^0) and thiosulfate ($S_2O_3^{2-}$) (Voordouw *et al.*, 1995).

1.3.1 The microbial sulfur cycle

Microbial sulfur transformations are closely linked with the carbon cycle in which sulfur reduction coupled with organic matter utilisation is a major mineralisation pathway in anaerobic habitats, while sulfur oxidations, some of which are autotrophic and/or phototrophic (Jorgensen, 1988; Voordouw, 1995), can occur aerobically and anaerobically. The reactions of the sulfur cycle alter the chemical, physical, and biological status of sulfur and its compounds so that sulfur cycling can occur. Many of the reactions of the sulfur cycle are mediated by microorgansms. Many sulfur compounds are highly reactive, and microorganisms often must compete with abiotic reactions. The transformation reactions involved represent a continuous flow of sulfur containing compounds among Earth's various compartments (soil, water, air, and biomass). Disrupting of the sulfur cycle can lead to several serious environmental problems. On the other hand, sulfur biotransformations are the basis of a whole set of environmental bioremediation technologies (Hulshoff Pol *et al.*, 1998).

The behaviour of sulfur compounds in the environment is highly influenced by the activity of living organisms, particularly microbes. In Figure 1.2, the biological sulfur cycle with different oxidation and reduction status is given. Since the 1980s the ecology of bacteria with a role in the sulphur cycle has received considerable attention from different scientific fields

(e.g., microbial mats and sediments, wastewater treatment biofilms, and corrosion (Hulshoff Pol *et al.*, 1998).

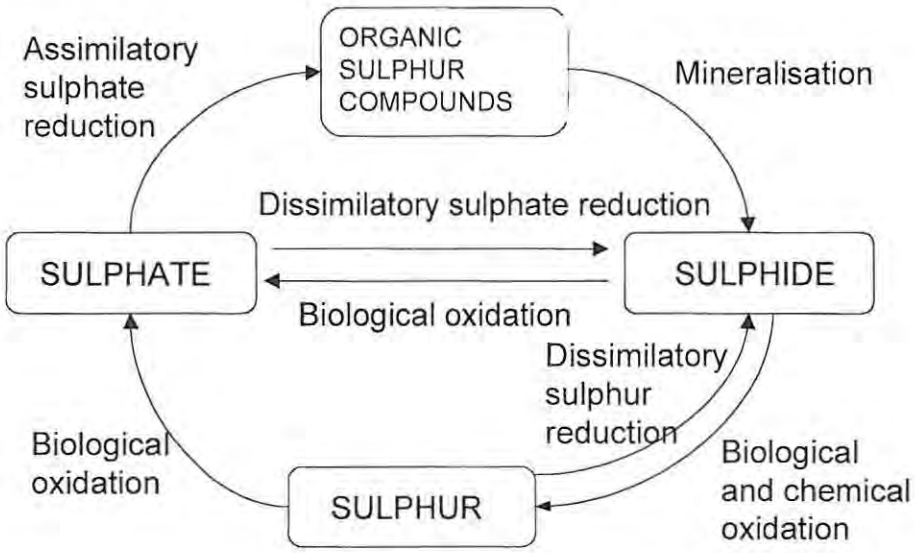


Figure1. 2: The Biological Sulphur Cycle adapted from Janssen *et al.*, (1998)

1.3.2 Microbial Sulfate Reduction

Sulfate salts are the major stock of mobile sulphur compounds. In the microbial sulfur cycle, sulfate is converted to sulfide by sulfate reducing bacteria (SRB) via dissimilatory sulfate reduction. This process of bacterial respiration occurs under strictly anaerobic conditions and use sulfate as terminal electron acceptor. Electron donors are usually organic compounds, eventually, hydrogen:

$$8H_2 + 2SO_4^{2-} \longrightarrow H_2S + HS^- + 5H_2O + 3OH^- \quad (7)$$

1.3.2.1 Metabolic Properties of SRB

SRB include the traditional sulfate reducing genera *Desulfovibrio* and *Desulfotomaculum* in addition to the morphologically and physiologically different genera *Desulfobacter, Desulfobulbus, Desulfococcus, Desulfonema* and *Desulfosarcina* (Widdel, 1988). In the presence of sulfate, SRB are able to use several intermediates of the anaerobic mineralisation process. Besides the direct methanogenic substrates molecular hydrogen (H_2), formate, acetate, and methanol, they can also use propionate, butyrate, higher and branched fatty acids, lactate, ethanol, and higher alcohols, fumarate, succinate, malate, and aromatic compounds. In sulfidogenic breakdown of volatile fatty acids, two oxidation patterns can be distinguished. Some SRB are able to completely oxidise volatile fatty acids to CO_2 and sulfide as end products. Other SRB lack the tricarboxylic acid cycle and carry out an incomplete oxidation of volatile fatty acids, with acetate and sulfide as end products (Hulshoff Pol *et al.,* 1998).

In addtition to the reduction of sulfate, reduction of sulfite and thiosulphate is also common among SRB. *Desulfovibrio* strains have been reported to be able to reduce di, tri, and tetrathionate. Some SRB were found to be able to respire oxygen, despite being classified as strictly anaerobic bacteria. Thus far, however, aerobic growth of pure cultures of SRB has not been demonstrated. The ability of SRB to carry out sulfate reduction under aerobic conditions, nevertheless, remains intriguing and could be of significance for micro-scale sulfur cycles (Hulshoff Pol *et al.,* 1998).

In the absence of an electron acceptor, SRB are able to grow through a fermentative or acetogenic reaction. Pyruvate, lactate, and ethanol are easily fermented by many SRB (Widdel, 1988; Dolfing, 1988). An interesting feature of SRB is their ability to perform acetogenic oxidation in syntrophy with hydrogenotrophic methanogenic bacteria (HMB), as

described for cocultures of HMB with *Desulfovibrio* sp. using lactate and ethanol (Widdel, 1988) or with *Desulfobalbus*-like bacteria using propionate (Janssen *et al.,* 1998). In the presence of sulfate, however, these bacteria behave as true SRB and metabolise propionate as electron donor for the reduction of sulfate.

Wastewater treatment bioreactors are complex ecosystems that contain many bacterial species. In such mixed cultures, SRB will compete in the presence of sulfate with methanogenic bacteria (MB) and obligatory hydrogen-producing acedogenic bacteria (AB) for the substrates available. The importance of this competition increases with a decrease in the COD/sulfate ratio of the wastewater. The outcome of this competition will determine to what extent sulfide and methane, the end products of the anaerobic mineralisation processes, is produced (Hulshoff Pol *et al.,* 1998).

1.3.3 Microbial Sulfur Oxidation

Different bacteria can oxidise various reduced sulfur compounds, for example, sulfide, elemental sulfur, or thiosulfate. Oxidation of sulfide to elemental sulfur is performed by autotrophic bacteria. Equation 8 gives the stoichiometry of the chemoautotrophic process, which proceeds aerobically or microaerobically. In addition, photoautotrophic sulfide oxidation can also occur under anaerobic conditions (Hulshoff Pol *et al.,* 2000). Photosynthetic sulfur bacteria are capable of photoreducing CO_2 while oxidising H_2S to S^0 (Eq. 5).

$$2H_2S + O_2 \longrightarrow 2S^0 + 2H_2O \quad (8)$$

$$2H_2S + CO_2 \longrightarrow 2S^0 + [CH_2O] + H_2O \quad (9)$$

Sulfide can also be completely oxidised to sulfate. Equation (10) gives a formula for the chemoautotrophic process, although photoautotrophic oxidation of sulfide to sulfate can occur.

$$H_2S + 2O_2 \longrightarrow SO_4^{2-} + 2H^+ \qquad (10)$$

This oxidation reaction, catalysed by, for example, *Thiobacillus*, involves a series of intermediates, including sulfide, elemental sulfur, thiosulfate, tetrathionate, and sulfate (Kelly *et al.*, 1997):

$$SH^- \longrightarrow S^0 \longrightarrow S_2O_3^{2-} \longrightarrow S_4O_6^{2-} \longrightarrow SO_4^{2-} \qquad (11)$$

Further studies on sulfide oxidation process and floating sulfur biofilms has been undertaken by other members of the EBG.

1.4 MICROBIAL ECOLOGY OF SULFATE REDUCING BACTERIAL SYSTEMS

The scientific study of microorganisms can be considered under three main headings; molecular biology, cell physiology and population ecology. Clearly there is a hierarchical relationship among these sub-disciplines, both with regard to the increasing structural complexity they represent, and in respect of the functional dependence of ecological relationships on the physiology of the individual organisms, also of physiology on the molecular genetic make up of the particular species. Progress in the overall understanding of the discipline is largely dependent upon problems in one area being illuminated by techniques and insights developed in another. The current widespread application of molecular methods to microbial ecosystems is revolutionising our understanding, both of present day ecological relationships and of past evolutionary development.

This study seeks to expand this interactive view of microbiology in general, and of the SRB in particular, through the consideration of three separate topics:

- The deterministic influence of the cell physiology on population ecology;
- Techniques for elucidating the true complexity of microbial consortia;
- Some technological consequences of the activities of microbial ecosystems.

1.4.1 Cell physiology of the SRB, and ecological concequences

Although the extent of the diversity within the group has only recently been fully recognised (Rabus *et al.*, 1996), the SRB have generally been regarded as a collection of organisms, largely unrelated in the convential taxonomic sense, but clearly forming a broad physiological/ecological grouping. This latter property derives directly from their mode of energy-generating metabolism. The SRB are obligately anaerobic, employing a respiratory mechanism with sulfate as terminal electron acceptor and consequently giving rise to sulfide as the major metabolic end product. The anaerobiosis and requirement for sulfate determine the environments in which they are active, while the sulfide produced underlies their environmental and technological impact (Hamilton, 1998).

Sulfate reducing bacteria (SRB) are a group of anaerobic bacteria which, as a part of their normal activities, generate hydrogen sulfide (H_2S). This product can cause a number of significant problems in water. These range from "rotten egg" odours, through to the blackening of equipment, waters and slime formations, and the initiation of corrosive processes. Detection of these microorganisms is made more challenging because they are anaerobic and tend to grow deep down within biofilms (slimes) as a part of a microbial community. Detection of the SRB is therefore made difficult because these microorganisms may not be present in the free-flowing water over the site of the fouling (Widdel *et al.*, 1988).

The sulfate reducing bacteria are an unusual group of bacteria in that they utilise hydrogen rather than oxygen as the basic driver for many of the metabolic activities. As a result of this, the SRB are anaerobic and are inhibited by the presence of oxygen. Sulfate reduction appears to be coupled to the formation of ATP (a major energy driver in metabolism) by a proton motive force (PMF) derived from electron transport. The bottom line is that the sulfate is reduced in a step-wise fashion to H_2S while releasing energy for growth. It is the H_2S which creates the problems through electrolytic corrosion, "rotten" egg smells, bad taste problems and the formation of black slimes.

Most of the sulfate-reducing bacteria can also use other oxyanions of sulfur as terminal electron acceptors, eg. sulfite and thiosulfate. Additionally, there exists another, quite separate, mixed group of archaea and bacteria that couple their energy metabolism to the reduction of sulfur (Widdel 1988; Davey *et al.*, 1993). Like the more familiar sulfate-reducing bacteria, these sulfur-reducing organisms are characterised by their sulfide production. As this common property is of such defining practical importance, and since the two groups are often found together in a range of natural environments, it is often appropriate to use the more all-embracing generic terms sulfide-producing bacteria (SPB), or sulfidogens to cover both groups of organisms (Hamilton, 1998).

Sulfate, however, is unique in that it is only capable of acting as terminal electron acceptor after its metabolic conversion to adenosine phosphosulfate (APS). The generation of APS involves reaction with ATP, with a net energy cost of two 'high energy bonds' (Hamilton, 1998).

The first genera described, *Desulfotomaculum* and *Desulfovibrio,* comprised mesophilic or moderately thermophilic endospore-forming and nonspore forming bacteria, respectively. These organisms are nutritionally similar; both incompletely oxidise a number of simple organic compounds (e.g., lactate to acetate). Diagnostically, *Desulfovibrio* species test positive (with some exceptions) for the sulfite reductase desulfoviridin and contain the unique tetraheme cytochrome c_3. These observations have contributed to the notion that although the sulfate-reducing bacteria are ecologically very significant, they comprise a small and nutritionally limited group. However, recent isolations and descriptions of new genera have dispelled this view and revealed much greater morphological and nutritional diversity among these bacteria, including several species capable of completely oxidising acetate or other organic compounds. At present, more than 17 genera of sulfate-reducing eubacteria (one of which is affiliated with gram-positive bacteria) and an extremely thermophilic sulfate-reducing archaebacterium have been described (Devereux *et al.,* 1989).

While the nutritional versatility and phylogenetic diversity of the sulfate-reducing bacteria have been established, questions of relatedness among species remain unanswered. 16S rRNA gene sequence similarity is now a generally accepted measure of phylogenetic relationships among bacterial groups.

1.4.2 Species diversity of microbial consortia

Whereas there has been a developing appreciation over the last ten to fifteen years that meaningful data on microbial consortia can only be obtained where due recognition is given to the complex nature of these entities, the true extent of that complexity is only now becoming widely recognised. This increase in our knowledge stems very largely from the addition of molecular methods, such as 16S rRNA gene sequencing, to the armory of

techniques used to examine the species diversity of natural consortia and of enrichment cultures derived therefrom. With regard to SRB, the application of molecular techniques to biofilm systems has been relatively limited (Amann *et al.,* 1992), but recent studies of the nature of subsurface microbial communities, and their putative role in the souring of petroleum reservoirs, have brought to light much information both on newly identified species, and on the scale of the species diversity within such natural consortia (Hamilton, 1998).

1.4.3 Sulfate reduction in microbial ecosystems

Many biotechnological processes of sulfate reduction have been investigated throughout the world. In the past decades, the basic objectives of these investigations was often to provide sulphur from gypsum or sulfate wastes when the sulphur supplies were running low (Butlin *et al.,* 1956; Burgess & Wood 1961, Sadana & Moley 1962; Maree 1987; Maree *et al.,* 1989). More recently, the objective of the study of the biological sulfate reduction was the protection of the environment through the removal of heavy metals (Cork & Cusanovitch 1979; Barnes *et al.,* 1991; Dvorak *et al.,* 1992) and/or sulfate (Hiligsmann *et al.,* 1998) from waste water or solid wastes.

The fundamental approach of most of these investigations was to optimise the sulfate reduction by comparing different kinds of reactor design, biomass carrier, organic substrate, electron donor or other nutrients and physical conditions without paying real attention to the strain selection (Hiligsmann *et al.,* 1998). The biotechnological process recently investigated (Hiligsmann *et al.,* 1996) involved dissimilatory sulfate-reducing bacteria (SRB) in order to produce sulfide from gypsum wastes and oxidise a cheap residual organic substrate as an electron donor. In this study, several strains of lactate-oxidising SRB were isolated using

method described by Postgate (1984), from sludge samples obtained from the Falling Sludge Bed Reactor (FSBR), and were studied in a bench-scale bioreactor. The results clearly showed the feasibility of the biotechnological process which took advantage of the isolated SRB strains in order to oxidise completely the organic substrate used as an electron donor.

Sulfate-rich wastewater is generated by many industrial processes that use sulphuric acid or sulfate-rich feed stock (e.g., fermentation or sea food processing industry) Also the use of reduced sulphur compounds in industrial processes, i.e. sulfide (tanneries, Kraft pulping), sulfite (sulfite pulping), thiosulphate (fixing of photographs) or dithionite (pulp bleaching) contaminated wastewater with sulfate (Hulshoff-Pol *et al.,* 1998). Sulfate emissions are not a direct threat for the environment as sulfate is a chemically inert, non volatile and non toxic compound (Dvorak *et al.,* 1992). However, high sulfate concentrations can cause an imbalance in the natural sulphur cycle. Under anaerobic conditions, dissimilatory sulfate reducing bacteria (SRB) use sulfate as a terminal electron acceptor for the degradation of organic compounds and hydrogen (Okabe *et al.,* 1999). Therefore, the major problem associated with the anaerobic treatment of sulfate-rich wastewater is the production of sulfide.

1.4.4 Electron donors and carbon sources

Numerous substrates have been reported as electron donors for SRB, some of which are recorded in Table 1. SRB do not degrade polysaccharides, proteins or lipids but depend on the acidogenic bacteria for the supply of electron donors from these compounds. The SRB are unique in their ability to grow with reduced organic compounds that cannot be utilise by other bacterial groups. These compounds include propionate, butyrate, higher fatty acids or phenyl substituted organic acids. By using sulfate as an external electron acceptor the SRB can utilise reduced compounds as energy sources (Widdel, 1988). The exception is when carbon

monoxide or carbon dioxide is used as the carbon source. An additional electron donor, usually hydrogen, is then required.

Table 1.1 The morphology, carbon and energy source, growth pH and temperature ranges of SRB genera (Widdel, 1988).

Groups	Morphology and Size	Carbon and Energy Source	pH		Temp (^{0}C)
			Range	Optimum	Range
Desulfobacter	curved (1-2μm)	acetate	6.2 – 8.5	7.3	28 – 32
Desulfobulbus	tapered spheres (1-1.3μm)	propionate, lactate, pyruvate, ethanol, propanol	6.0 - 8.6	7.2	28 – 39
Desulfococcus	sphere, in clusters (1.5-2.2μm)	formate, acetate, lactate, butyrate, pyruvate			30 - 36
Desulfotomaculum	straight	lactate, pyruvate, acetate, ethanol, hydrogen			
Desulfomonas			6.5 – 8.5	7.2	30
Deaulfonema	long filaments	acetate, malate, benzoate, pyruvate			
Desulfosarcina	clusters of rod shaped Cell (1-1.5μm)	formate, acetate, propionate, butyrate	6.9 – 7.0	7.4	33 - 384
Desulfovibrio	curved (2.5-10μm)	lactate		7.5	25 - 35

1.5 THE RHODES 'BIOSURE' PROCESS DEVELOPMENT

For the past five to ten years, the work done by the Environmental Biotechnology Group (EBG) at Rhodes University had focussed on the role of complex carbon substrates as electron donors for biological sulfate reduction. Investigations to assess the degradation of complex organics were undertaken by Molepane (MSc, 1999) and Whittington-Jones (2000). Solubilisation of complex carbon substrates provides the primary reaction in the 'BioSURE' Process, and is effected in the Falling Sludge Bed Reactor (FSBR). The scale-up development of the FSBR formed the basis of the 'BioSURE' pilot plant constructed at Grootvlei Gold Mine and the results of these studies have been reported by Whittington-Jones (2000) and Corbett (MSc, 2001).

In the FSBR utilising primary sewage sludge, particulates are drawn down and then recycled to the inlet, large particulates are hydrolysed, while consumption of small organic compounds is inhibited within an increasing sulfide and alkalinity concentration gradient. On recycle, the hydrolysed compounds in solution pass forward and become available to sulfate reduction in a subsequent operation. Residual particulates settle through a further cycle of hydrolysis.

Based on the widespread availability of primary sewage sludge (PSS) in comparison with other complex carbon sources, Corbett (2001) followed-up studies done by Whittington-Jones (2000) on laboratory-scale FSBR and constructed a full-scale FSBR to test findings that conversion of PSS to a hydrolysate would make complex carbon available to SRB in a simple and more utilisable form. Preliminary studies of sludge solubilisation in the FSBR have shown the activity of sulfide and alkalinity, as physico-chemical effects enhancing enzymatic hydrolysis processes, and accelerating the breakdown of protein, carbohydrate and lignocellulose components in sewage sludge. Follow-up studies on lignocellulositic

hydrolysis in sulfate reducing conditions is currently being conducted by some members of the EBG.

Given the central importance of the solubilisation and hydrolysis reactions as the initial steps in which complex organic carbon structures are made available to biological processes, in this study, sulfate reduction was subjected to more detailed investigation in a 8.5L bench-scale Falling Sludge bed Reactor (FSBR) illustrated in Figure 1.3. Since the performance of the reactor was coupled with the SRB activity, the microbial ecology of sulfate-reducing bacterial system became apparent. Therefore, this study focussed on the characterisation and identification of strains of SRB distributed within different sections of the reactor. In order to relate the microbial spatial distribution of SRB directly to their activity profiles, there is a need to provide comprehensive information to understand a complex biological sulfur cycle in the wastewater biofilms.

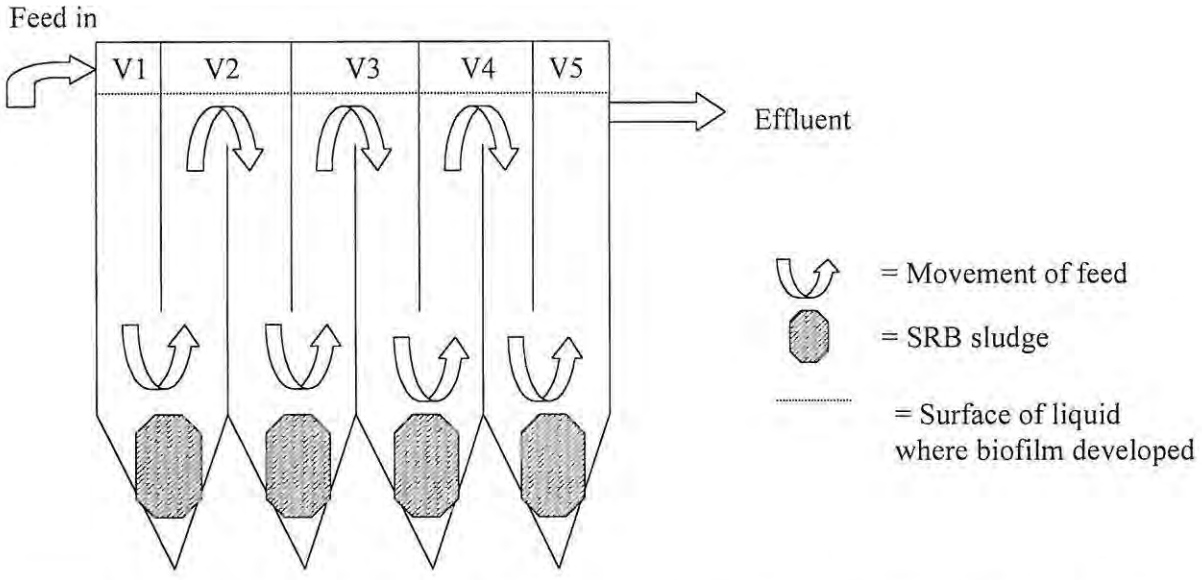


Figure 1.3: Schematic diagram of the baffled reactor used to cultivate sludge and sulphur biofilm. The dimensions of the reactor were 140 mm x 180 mm x 170 mm, with a volume of 8.5L (Janssen *et al.*, 1998).

1.6 METHODS TO DETERMINE SPECIES DIVERSITY

1.6.1 Classical sludge characterisation methods

Classical sludge characterisation methods are often based on selective growth media. The Most Probable Number (MPN) method is a technique in which serial sludge dilutions are inoculated in selective liquid media or on solidified agar-media. This method can give very useful information on the number of microorganisms that are able to grow on artificial media. However, it should be kept in mind that often the real numbers can be much higher than those detected by the MPN method. Direct microscopic analyses are useful as well in the characterisation of sludge. A major drawback of most microscope techniques is the fact that the identification of microbes is usually based on morphology only, which for most bacteria is not very distinctive. Recently, Surman *et al.*, (1996) have compared the applicability of several light, fluorescence, and electron microscopy techniques such as scanning and transmission electron microscopy (SEM and TEM), atomic force microscopy (AFM), and confocal laser scanning microscopy (CLSM) for the examination of microbial biofilms. All techniques have their special advantages and disadvantages, but a combined microscopic approach can give a good picture of the bacterial composition of the sludge (Hulshoff Pol *et al.*, 1998).

1.6.2 Molecular-based sludge characterisation methods

Microbiological transformations of geochemicals are largely community-level processes. Yet, despite perceived species richness, there is little explicit data defining the underlying phylogenetic and physiological diversity of the communities that carry out transformations (Devereux *et al.*, 1996). In the past, studies of microbial diversity have been limited, as most of the bacteria that are observed microscopically in an environmental sample cannot be isolated or readily identified. As recently detailed by Amann *et al.*, (1995), the traditional

limitations of pure culture techniques can be circumvented through molecular biological methods, particularly those that employ rRNA sequence-based approaches.

Comparisons of 16S rRNA gene sequences have provided the major outlines of microbial phylogenetic relationships. There are now well over 3,000 16S rRNA sequences available that serve to relate sequences recovered directly from environment (molecular isolates) with pure culture collections. To some extent, this also permits inference into the physiology of the community member from which the sequence was derived (Amann *et al.*, 1995).

The phylogeny has also served as the basis for the design of phylogenetic probes. It is generally possible to identify nucleotide tracts within a 16S rRNA sequence that are conserved within phylogenetic groups. These tracts can serve as hybridisation sites for DNA probes or PCR primers of phylogenetically defined specificity. Ribosomal RNA-targeted hybridisation probes have been used for measurements of the relative abundance of specific rRNAs in a community and for determinative studies (e.g., to identify culture isolates). The PCR primers have been used to amplify and clone rRNA gene segments of defined groups within a sample for subsequent sequence determination and phylogenetic placement. This provides the basis for a phylogenetic overview of community structure (Devereux *et al.*, 1996).

Without doubt rRNA based detection and identification methods have become important in the unravelling of the microbial composition of anaerobic sludge. Several rRNA based methods have been developed to identify and quantify microorganisms in complex environments. They constitute the cloning of ribosomal copy DNA or polymerase chain reaction (PCR)-amplified ribosomal DNA (rDNA) followed by sequence analysis of the

resulting clones. Some of these methods which can be used for the analysis of the microbial sludge composition are depicted in Figure 1.4.

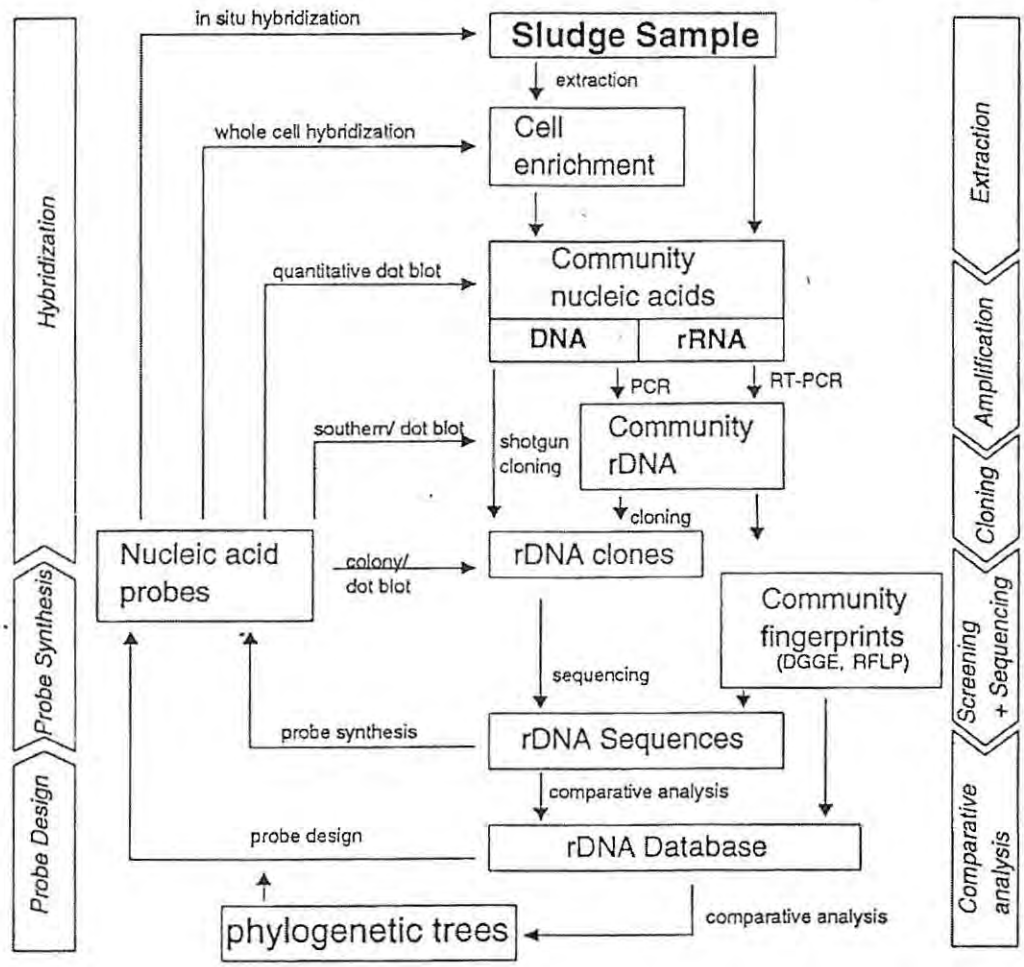


Figure 1.4: Strategies based on rRNA sequences for the characterisation of sludge microbial communities, adapted from Amann *et al.*, (1995).

1.6.2.1. Polymerase chain reaction

Polymerase chain reaction (PCR) has become one of the most widely used techniques in molecular biology and for good reason: it is a rapid, inexpensive and simple means of producing relatively large numbers of copies of DNA molecules from minute quantities of source DNA material-even when the source DNA is of relatively poor quality.

PCR involves preparation of the sample, the master mix and the primers, followed by detection and analysis of the reaction products. PCR is versatile, and many types of samples can be analysed for nucleic acids. By following a few basic rules, problems can be avoided in the preparation of DNA for the PCR. The essential criteria for any DNA sample are that it contain at least one intact DNA strand encompassing the region to be amplified and that any impurities are sufficiently diluted so as not to inhibit the polymerisation step of the PCR reaction (Welsh and McClelland, 1990).

PCR is a technique that involves the amplification of specific sequences of DNA from minute quantities of starting material. It is also an *in vitro* technique for replicating a target i.e. a defined DNA sequence, so that its amount is increased exponentially. Whereas previously only small amounts of a specific gene could be obtained from a cell, now even a single cell can be used to amplified a gene yielding a million copies within a few hours using PCR (Collier et al., 1996).

Before its inception, DNA sequences had to be amplified by time consuming cloning. This method would take days, whereas PCR can be set up easily and completed in a few hours. The PCR technique is based on enzymatic amplification of DNA sequences using oligonucleotide primers that flank the region of interest in the target DNA.

The principle involves a repetitive series of cycles consisting of template denaturation, primer annealing and extension of the annealed primers by a DNA polymerase to create the exponential accumulation of a specific fragment whose ends are determined by the 5' ends of the primers. PCR is so named because it involves a polymerase and the products synthesised in each cycle can serve as templates in the next cycle, thereby almost doubling the number of DNA copies at every cycle, creating a chain reaction similar to the principles in a nuclear reactor (Kwok *et al.*, 1989).

PCR consists of repetitive cycles of DNA denaturation through melting at elevated temperature to convert double-stranded DNA to single-stranded DNA, annealing of oligonucleotide primers to the target DNA, and extension of the DNA nucleotide addition from the primers by the action of DNA polymerase. The target region for amplification is defined by unique oligonucleotide primers that flank a DNA segment. The oligonucleotide primers are designed to hybridise to regions of DNA that flank a desired target sequence, annealing to complementary strands of the target sequence. The primers are then extended across the target sequence by using a heat-stable DNA polymerase (*Taq* DNA polymerase, a thermostable and thermoactive enzyme from *Thermus aquaticus)* in the presence of free deoxynucleoside triphosphates (dNTPs), resulting in a doubled replication of the starting target material. By repeating the three stage process many times, an exponential increase in the amount of target DNA results. The product of each PCR cycle is complementary to and capable of binding to the primers, and so the amount of DNA is potentially doubled in each successive cycle (Eckert *et al.*, 1991).

1.6.2.1.1 PCR amplification of the 16S rRNA genes

With the polymerase chain reaction (PCR) amplification method a few target rRNA genes can be amplified to make them detectable and quantifiable (Giovannoni 1990). The selection of the PCR primers determines which rRNA genes and which part of the rRNA gene will be amplified. By combining universal PCR primers with cloning and sequence analysis techniques, it is possible to get information about the microbial sludge composition.

1.6.3 Denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA genes

To get a general impression of the heterogeneity of the sludge community it is imperative to separate the PCR products and this may be accomplished using denaturing gradient gel electrophoresis (DGGE). This technique is based on electrophoresis of PCR-amplified 16S rDNA fragments in polyacrilamide gels containing a linearly increasing gradient of denaturants. In denaturing gradient gel electrophoresis (DGGE), DNA fragments of the same length but with different base-pair sequences can be separated. Separation in DGGE is based on the electrophoretic mobility of a partially melted DNA molecule in polyacrylamide gels, which is decreased compared with that of the completely helical form of the molecule. The melting of fragments proceeds in discrete so-called melting domains: stretches of base pairs with an identical melting temperature. Once the melting domain with the lowest melting temperature reaches its melting temperature at a particular position in the DGGE gel, a transition of helical to partially melted molecules occurs, and migration of the molecule will practically halt. Sequence variation within such domains causes their melting temperatures to differ. Sequence variants of particular fragments will therefore stop migrating at different positions in the denaturing gradient and hence can be separated effectively by DGGE (Muyzer *et al.,* 1993). PCR is used to selectively amplify the sequence of interest before DGGE is used. In a modification of the latter method, GC-rich sequences can be incorporated into one

of the primers to modify the melting behaviour of the fragment of interest to the extent to which close to 100% of all possible sequence variations can be detected.

In this study, the application of DGGE to the analysis of fragments derived from the variable region of 16S rRNA was described. These fragments were obtained after amplification of 16S rDNA genes from genomic DNA. The results demonstrate the presence of different rDNA fragments in microbial populations. By subsequent hybridisation analysis with group-specific oligonucleotide probes, particular constituents of the population could be identified. The procedure allows one for the first time to directly identify the presence and relative abundance of different species and thus, to profile microbial populations in both a qualitative and a semiquantitative way (Muyzer *et al.*, 1993).

1.6.4 Southern Blotting and DNA Hybridisation

Southern blotting, the capillary transfer of DNA fragments from gels to various types of filter material, revolutionised the study of genomes. The ability to detect rare sequences in complex population of restriction fragments paved the way for the cloning of eukaryotic genes, reverse genetics, and modern molecular biology. Modification of the basic Southern blot concept have led to major developments in hybridisation technology e.g., northern and western blotting (McPherson *et al.*, 1993).

1.6.5 Fluorescent in situ hybridisation

Ribosomal RNA based detection and identification methods have become most important in the unravelling of the microbial composition of anaerobic sludge. Several rRNA based methods have been developed to identify and quantify microorganisms in complex environments. Some of these methods which can be used for the analysis of the microbial

sludge composition are depicted in Figure 3. One of these methods is hybridisation with rRNA-based oligonucleotide probes (Amann *et al.*, 1995).

Detection of microorganisms by using fluorescent in situ hybridisation (FISH) with rRNA-targeted nucleic acid probes is a very useful molecular tool for rapid, reliable and cultivation-independent monitoring of phylogenetically defined populations in environmental samples (Amann *et al.*, 1996). In combination with confocal laser scanning microscopy, FISH allows us to study the spatial arrangement of microbial probe-target populations within biofilms and activated sludge flocs (Wagner *et al.*, 1994). The localisation of certain microorganisms in the sludge can give very valuable information for reactor operation conditions.

Like any other method, FISH also has limitations. Dormant or metabolically inactive cells contain insufficient ribosomes to show fluorescent signals after hybridisation. Moreover, quantification of the number of microorganisms can be a problem when cells have irregular shapes or when they form chains or compact micro-colonies. Insufficient permeability of the cells can be another limitation. Although Gram negative bacteria usually are readily permeabilised with routine chemicals, Gram positive bacteria need additional enzymatic treatment. Finally, the detection limit is determined by the use of microscope techniques, i.e. at least 10,000 cells/ml are needed to detect one cell (Ramsing, 1998).

1.7 RESEARCH OBJECTIVES

The characterisation and quantification of microorganisms in anaerobic bioreactors is difficult, but essential for optimising the bioreactor process. Good results have been obtained by combining classical microbiological and modern molecular-based sludge characterisation methods (Ramsing, 1998). Especially rRNA based techniques have offered new opportunities for the analysis of species and the spatial distribution of microorganisms in bioreactor sludge. Applying these techniques makes it possible to relate the microbial spatial distribution of SRB directly to their activity profiles, which provides comprehensive information to understand the complexity of the sulfur cycle in wastewater biofilms.

The results from work done by Whittington-Jones (2000) and Corbett (MSc, 2001) focussed on the role of complex carbon substrates as electron donors for biological sulfate reduction. This led to the scale-up development and construction of the Rhodes 'BioSURE' Process Pilot Plant at Grootvlei in Gauteng province whereby the process design was incorporated in a dual-stage sulfate reduction operation. The observations of effective degradation of organic carbon occurring within these reactor systems appeared to indicate the operation of a full biological sulfur-cycle or sulfuretum.

With the advantage of a fully optimised bioprocess based on the operation of an artificial sulfuretum, the opportunity existed to study the microbial ecology of SRB under controlled conditions not easily available in natural systems. The broad focus of this study was therefore, to investigate the spatial distribution of SRB species within the sulfate reducing compartments of the artificial sulfuretum.

The specific objectives of the study included the following:

1. To construct and operate a Baffle Reactor as a model system for the study of complete sulfur-biocycle sulfureta;
2. To compare the application of classical colony isolation and whole genome extract molecular techniques in studying the microbial ecology of laboratory-generated sulfureta;
3. To compare the SRB populations in laboratory-scale and pilot-scale Baffle Reactors using sewage sludge as innoculum;
4. To describe the microbial ecology and spatial distribution of SRB in sulfureta.

1.7.1 Research hypothesis

The study was based on the investigation of the principal hypothesis that classical and molecular techniques would provide different interpretation of population dynamics in the bioreactors studied, and that these would show that different species predominate in the compartments providing different metabolic environments.

Chapter 2

DEVELOPMENT AND PERFORMANCE OF A LAB-SCALE BAFFLE REACTOR

2.1 INTRODUCTION

Work done by the EBG at Rhodes University had focussed on the role of complex carbon substrates as electron donors for biological sulfate reduction. Based on the widespread availability of PSS in comparison to other complex carbon sources, investigations were undertaken by Whittington-Jones, (2000) and Corbett, (2001); to assess the degradation of complex organics, and this resulted in the scale up development and construction of the Rhodes 'BioSURE' Process at Grootvlei Gold Mine No. 3 Shaft.

In this chapter, Whittington-Jones and Corbett's prototype FSBR was subjected to more rigorous examination at lab-scale in a Baffle Reactor configuration. The development of a Baffle Reactor model system which satisfied the growth requirements of a sulfur biofilm layer as well as a sulfate reducing bacterial (SRB) sludge bed is described in some detail. The Baffle Reactor design allowed a comparative study of the biofilm where sulfide and COD gradients occurred across the various baffles of the reactor, while operating conditions and the inoculum remained identical through the reactor. This study was undertaken in order to describe the microbial ecology of the system together with its performance.

2.2 MATERIALS AND METHODS

2.2.1 Development of sulfate reduction model system

The prototype Baffle Reactor was constructed as an 8.5L bench-scale reactor and sulfate-enriched PSS was used as the innoculum (COD = 4000 $mg.L^{-1}$; SO_4^{2-} = 2000 $mg.L^{-1}$. A schematic diagram of the laboratory-scale Baffle Reactor used in this study is shown in Figure 1.3 and Figure 2.1. The reactor was fed at a flow rate of 1.2 L/day (80 mL/hr) and the hydraulic retention time was approximately 7 days. The reactor was made up of four valleys with their sides having a slope of 45^0. Each valley was partially separated from adjacent valleys by perspex baffles that extended to 5 cm below the water surface. Each of the four valleys was further divided into two equal valleys by a central baffle extending from above the water level to 5 cm above the base of each valley, thus allowing the maintenance of a continuous flow of nutrient medium throughout the reactor without disrupting or causing washout of the biofilm. The bottom of the valleys were seeded with SRB-enriched sludge obtained from the Rhodes BioSURE Process pilot plant at the Grahamstown Disposal Works.

The SRB growing in the reactor sludge produced sulfide from sulfate in the feed, and the resulting continuous high production of sulfide, was then utilised by the sulfide oxidising bacteria to produce a floating sulphur biofilm on the surface of the reactor. Sampling was made from a number of points in the reactor and the positions are indicated in Figure 1.3. Five parameters (COD, sulfide, sulfate, pH and sulfur) were measured each day to monitor the performance of the reactor.

2.2.1.1 Determination of sulphur compounds

Sulfide in the reactor was monitored each day using the methylene blue method (Truper and Schlegel, 1964) as follows:

A 100 µL sample was added to 100 µL of 15% zinc acetate and 4800 µL of dH_2O. 500 µL ferric chloride (16 g/L in 6M HCL) and 500 µL amine-sulfuric acid stock solution (4 g/L N-N dimethyl-p-pheylenediamine dihydrochloride in 6M HCL) was then added to the solution and left to stand for 1 hour before reading the absorbance at 670 nm.

The sulfate concentration was determined using an HPLC method and this was carried out by filtering 1 ml of sample through a 0.45 µ nylon filter. Reverse phase C_{18} columns (Machery-Nagel, Germany) were then used to pre clean the supernatant before injection into the HPLC column (Hemilton PRP-X 100 anion exchange columns, 150 x 4.1 mm). The columns were first rinsed with 1 ml of water, 1 ml of methanol, then 1 ml of water again before filtering the sample.

Sulfur was also determined using an HPLC method. A 1 ml sample was microfuged at 13 000 rpm for 10 minutes. The pellet was resuspended in 1 ml of acetone, then filtered through a 0.45 µ nylon filter. The sample was then injected into the sample chamber of the HPLC (Beckman Instruments, USA). The mobile phase consisted of 97% methanol in $dddH_2O$ with a flow rate of 1 ml per minute and a UV spectroscopy was used for detection at 230 nm.

2.2.1.2 Chemical oxygen demand (COD)

The Spectroquant COD kit (Merck) was used in the determination of COD concentration, according to the manufacturer's instructions.

2.3 RESULTS AND DISCUSSION

2.3.1 Monitoring the surface layer and the activities taking place within the reactor

Since wastewater sludges are complex multispecies systems, considerable heterogeneity with respect to both the microorganisms present and their physicochemical microenvironments are displayed. Moreover, multiple electron donors and electron acceptors are present in the wastewaters. Therefore, successive vertical zonations of predominant respiratory processes occuring simultaneously in close proximity have been found in aerobic wastewater biofilms with a typical thickness of only a few millimeters (de Beer *et al.,* 1999; Kuhl and Jorgensen, 1992; Ramsing *et al.,* 1993 and Santegoeds *et al.,* 1998).

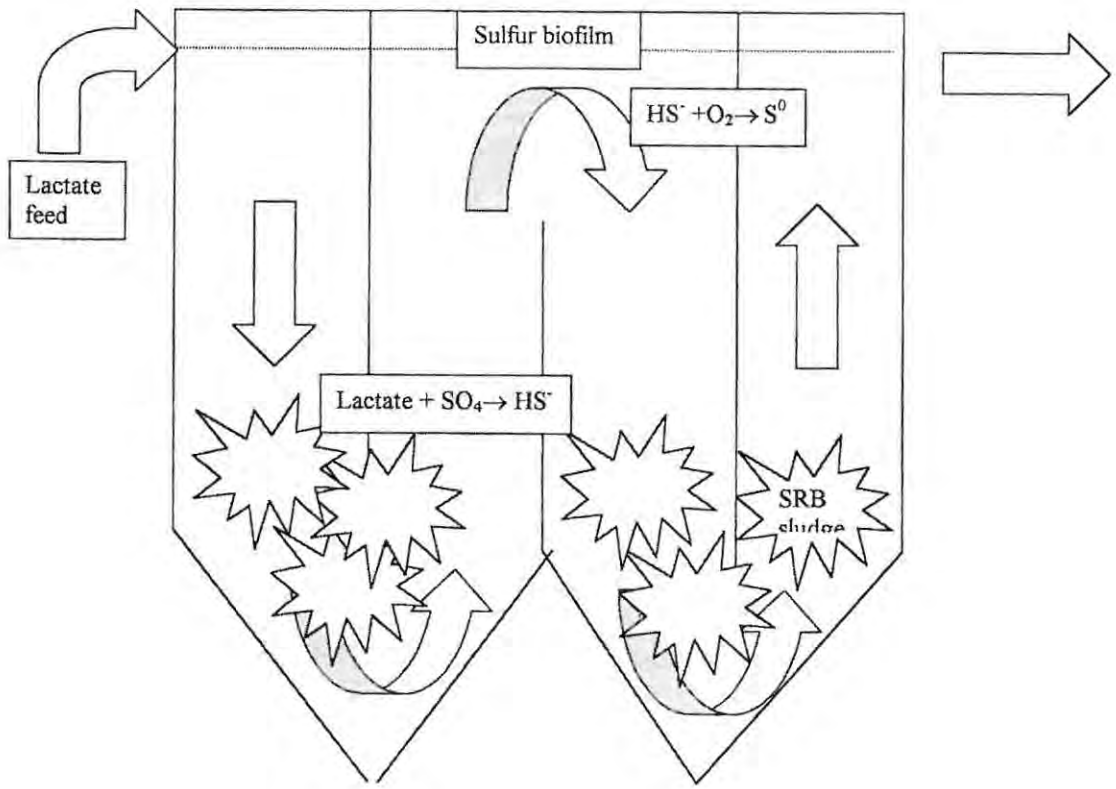


Figure 2.1: Schematic diagram of Baffle Reactor illustrating the position of the sulfate reduction reaction and sulphur biofilm (Janssen *et al.*, 1998).

Since bacterial growth was studied using the laboratory-scale Baffle Reactor, the SRB present in the sludge bed in the valleys of the reactor produced sulfide as a by-product of sulfate reduction, as described by equation 1 in the first chapter. The sulfide was utilised by the sulfide oxidising bacteria (SOB) on the surface of the reactor. Depending on the status of the redox potential at the liquid-air interface the SOB would form a sulfur biofilm on the surface of the liquid and this is illustrated in Figure 2.2. Grobicki and

Stuckey (1991) have reported the effective separation of microorganisms within each zone of a Baffle Reactor, which proved useful in this study.

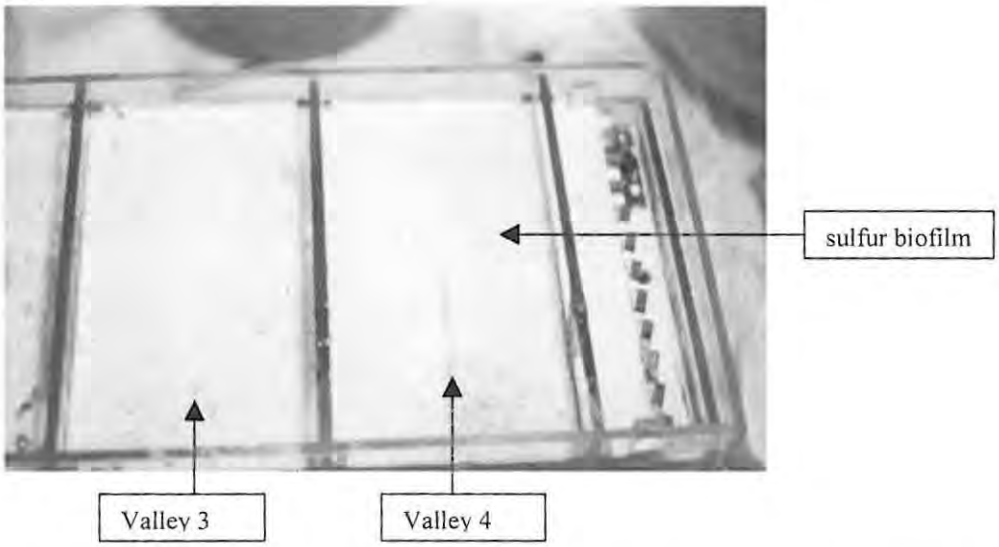


Figure 2.2: Lab-scale Baffle Reactor illustrating the surface of sulfur biofilm production.

2.3.1.1 Sulfate concentrations in the Baffle Reactor

The results for sulfate removal over a period of a month are shown in Figure 2.3 and 2.8. During the start-up and the adaptation period, the sulfate concentration decreased from 750 mg/L to 350 mg/L representing sufficient removal efficiency. Looking into the three valleys, sulfate reduction occurred more in valley 3 than in valley 1 and 2. This profile may be linked to the availability of hydrolysis products after the up-welling of the feed from valley 2.

When the system was subjected to stress at day 29 (Figure 2.3), sulfate concentration increased since the SRB metabolic activity was stressed. This led to an elevation of COD

concentration with low sulfide production and less sulfur being produced since the sulfide was directly converted to sulfate. Immediately the lactate feed was circulated throughout the reactor, the system operated as before.

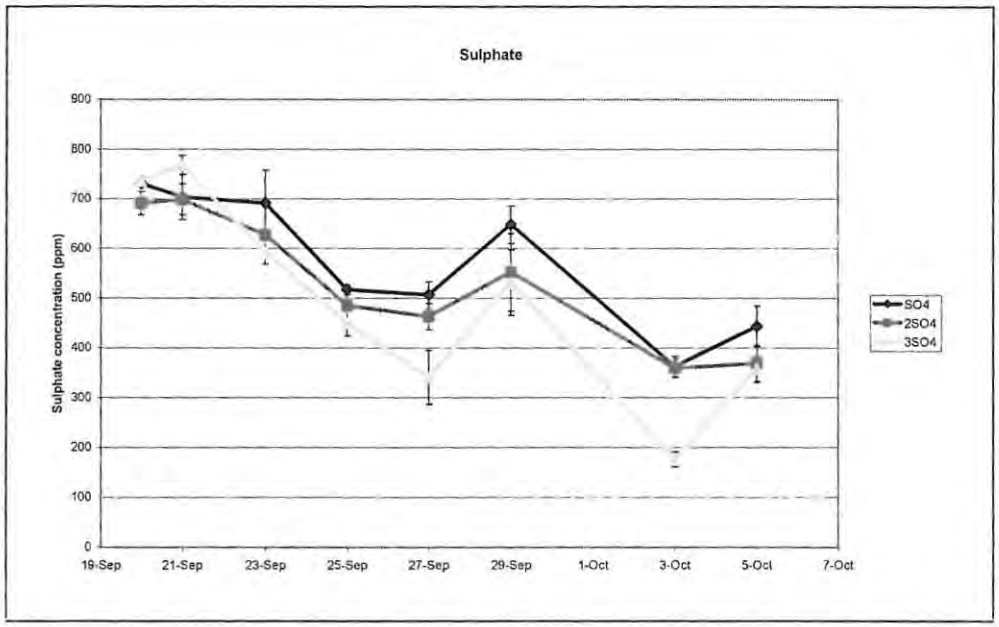


Figure 2.3: Sulfate concentration reported for the Baffle Reactor over the period of a month for valleys 1 = ● ; 2 = ■ and 3 = ▲.

2.3.1.2 Sulfide concentrations in the Baffle Reactor

The sulfide concentration in the valleys of the Baffle Reactor over a period of a month is illustrated in Figure 2.4, where the sulfide concentration increased from one valley to another. Figure 2.2 shows the formation of the sulfur biofilm and this may be related to the increase in sulfide concentration across the valleys, whereby the conversion to elemental sulfur by sulfide oxidising bacteria takes place.

The SRB sludge produced sulfide which moved along the reactor with the feed, resulting in higher concentrations of sulfide in valley 2 than in valley 1. The sulfur biofilm grown from valley 2 was seen to be more brittle and yellow in colour with full coverage of the biofilm occurring. In every second day the biofilm was removed and some would sink when the biofilm become more brittle, however, the high sulfide concentrations resulted in rapid re-formation of the biofilm on the surface of the reactor.

The reactor was subjected to stressful conditions by stopping the feed on the last week of its operation. This incident had an immediate and profound effect on destabilising the Baffle Reactor, affecting COD (Figure 2.6) and sulfate concentrations (Figure 2.3). However, once the source of the disturbance had been removed, the system quickly returned to steady state operation. The system was allowed to run without the lactate feed for several days before the feed was resumed. The sulfide concentration began to decrease immediately the system was starved as illustrated in Figure 2.4. Immediately the feed was resumed, the system picked up and operated as before. The reactor proved to be highly resilient, and was able to withstand stressful events without prolonged loss of performance. The performance of the Baffle Reactor during the stressful conditions provided further evidence for a possible link between biological sulfate reduction and enhanced solubilisation of PSS.

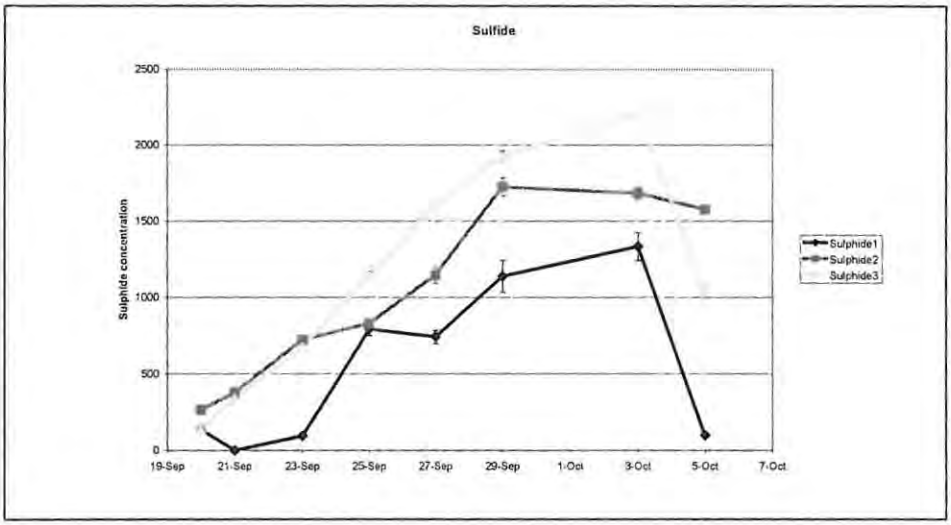


Figure 2.4: Sulfide concentrations reported for the Baffle Reactor over the period of a month for valleys 1 = ● ; 2 = ■ and 3 = ▲.

2.3.1.3 Sulfur concentration in the Baffle Reactor

The high sulfide concentrations present in the reactor resulted in the rapid formation of a more pronounced sulfur biofilm. This may have been due to sulfide oxidising bacteria (SOB) being forced to convert sulfide to elemental sulfur rather than sulfate. Sulfate formation is then preferred for SOB under optimal conditions because of the higher Gibbs free energy available to the cells: -772,43 kJ/mol for sulfate formation compared to –129.50 kJ/mol for sulfur formation (Janssen *et al.,* 1998). The sulfate production rate can be suppressed by controlling the oxygen concentration. The sulfur formation reaction is, however, favoured under oxygen limiting and high sulfide conditions because sulfur is formed faster than sulfate. Sulfur is an intermediate in the sulfate formation reaction, and under more favourable conditions where oxygen is available and sulfide loading values

are not as high, the reaction will be completed with sulfur being converted to sulfate by the bacteria (Gommers *et al.*, 1988; Boogerd *et al.*, 1991).

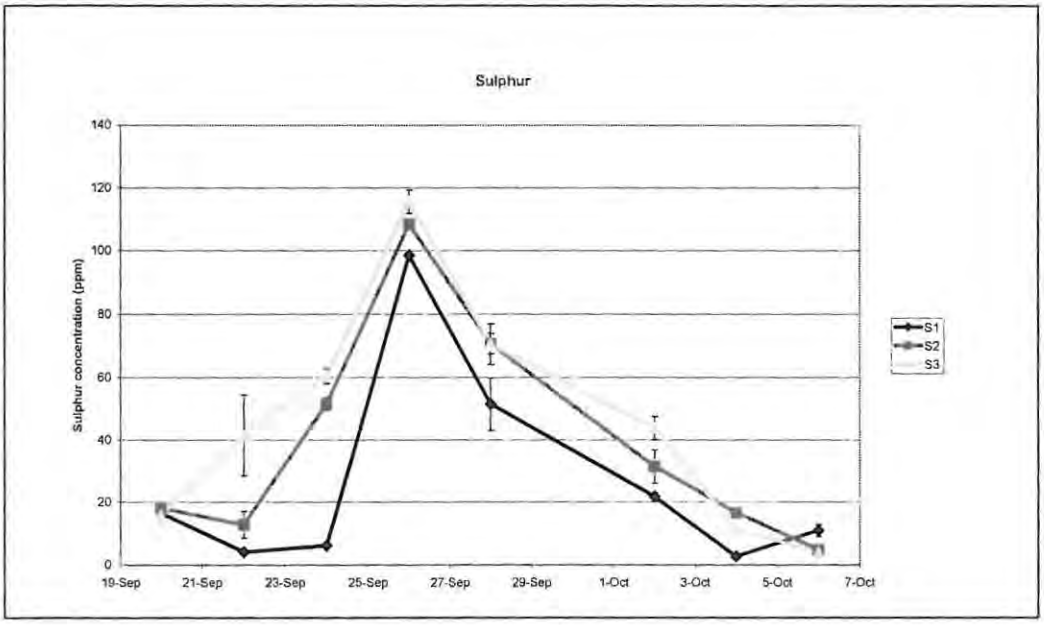


Figure 2.5: Sulfur concentration reported for the Baffle Reactor over the period of a month for valleys 1 = ● ; 2 = ■ and 3 = ▲.

Figure 2.5 illustrates the sulfur concentration of the system whereby sulfur formation increased from valley 1 to valley 3. These results can be directly related to the higher sulfide concentrations found in valleys 2 and 3 (Figure 2.4). Although it is more likely that the metabolic activity of all the sludge beds were similar, high production of sulfide in these valleys may have been due to more metabolically active SRB sludge. During the start-up period, sulfur concentration increased along with sulfide concentration as illustrated in Figure 2.5, 2.4 and 2.8. After the lactate feed was stopped, the sulfur concentration began to decrease and the same results were observed for sulfide

concentration. When the lactate feed resumed, the sulfur and sulfide concentrations began to increase, whereas, sulfate concentration decreased. This suggests that sulfide oxidising bacteria in the biofilm may have adapted to high sulfide concentrations and could withstand levels which are toxic to most organisms.

2.3.1.4 COD

During the performance of the system, complex organic substrates from sewage sludge supplied the energy requirement for SRB to create carbonaceous elements suitable for new cell synthesis. The net effect of these reactions was a decrease in biodegradable chemical oxygen demand (COD). COD removal is reported in Figure 2.6 with the reactor showing an improved performance over time. At steady state operation, the COD concentration was reduced from 4500 to 1000 mg/L. During this period, the initial SO_4:COD ratio was 750:4500 mg/L or 1:6 and was reduced to 350 mg/L SO_4 and 1000 mg/L COD or 1:3.

The COD concentration, which was a measure of the amount of organic material present, was higher where the feed entered the system compared to the effluent because lactate was consumed by the SRB population as an energy and carbon source in order to reduce sulfate to sulfide. When the system was subjected to a stressful conditions on the 2nd of October, the COD concentration increased since there was no lactate feed for the SRB metabolic activities. However, the COD seemed to decrease once the lactate feed was resumed.

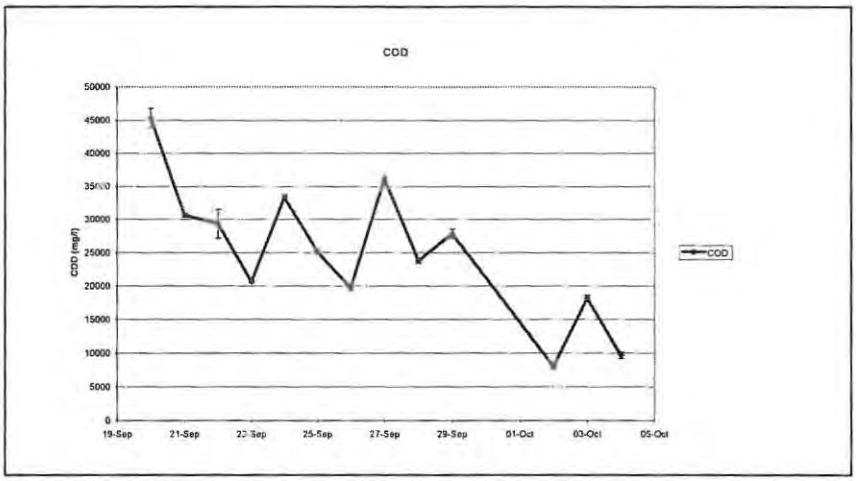


Figure 2.6: COD concentrations reported for the Baffle Reactor over the period of a month, measured as lactate entered the reactor .

2.3.1.5 pH

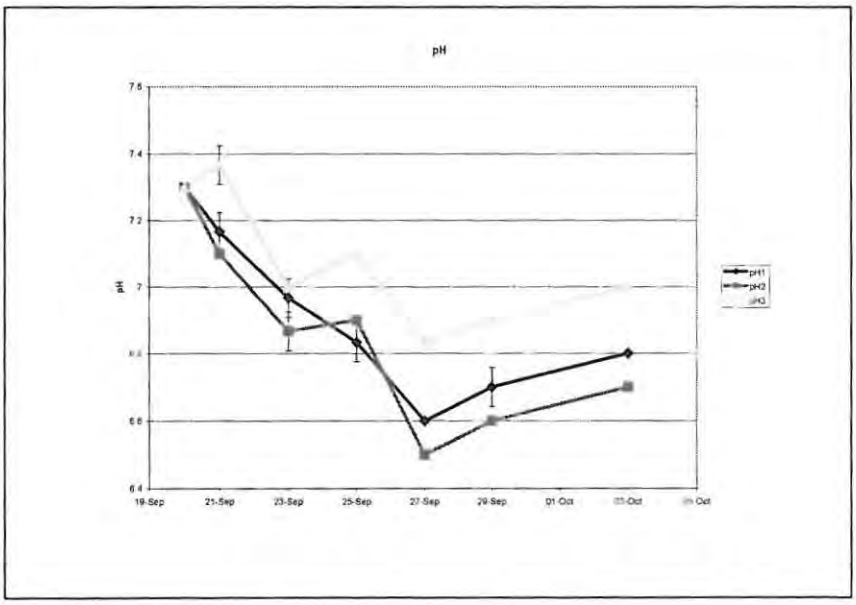


Figure 2.7: The pH profile of the Baffle Reactor monitored over the period of a month for valleys 1 = ● ; 2 = ■ and 3 = ▲ .

The pH of the medium in the Baffle Reactor directly beneath the biofilm was monitored daily over a period of a month. The pH ranged from pH 6.5 – 7.4, with the highest pH values measured in valley 2 and 3, corresponding to the trend found with sulfide concentrations. The increase in pH from the second to the third valley was probably due to the constant removal of soluble products from the lower region during the recycling of sludge.

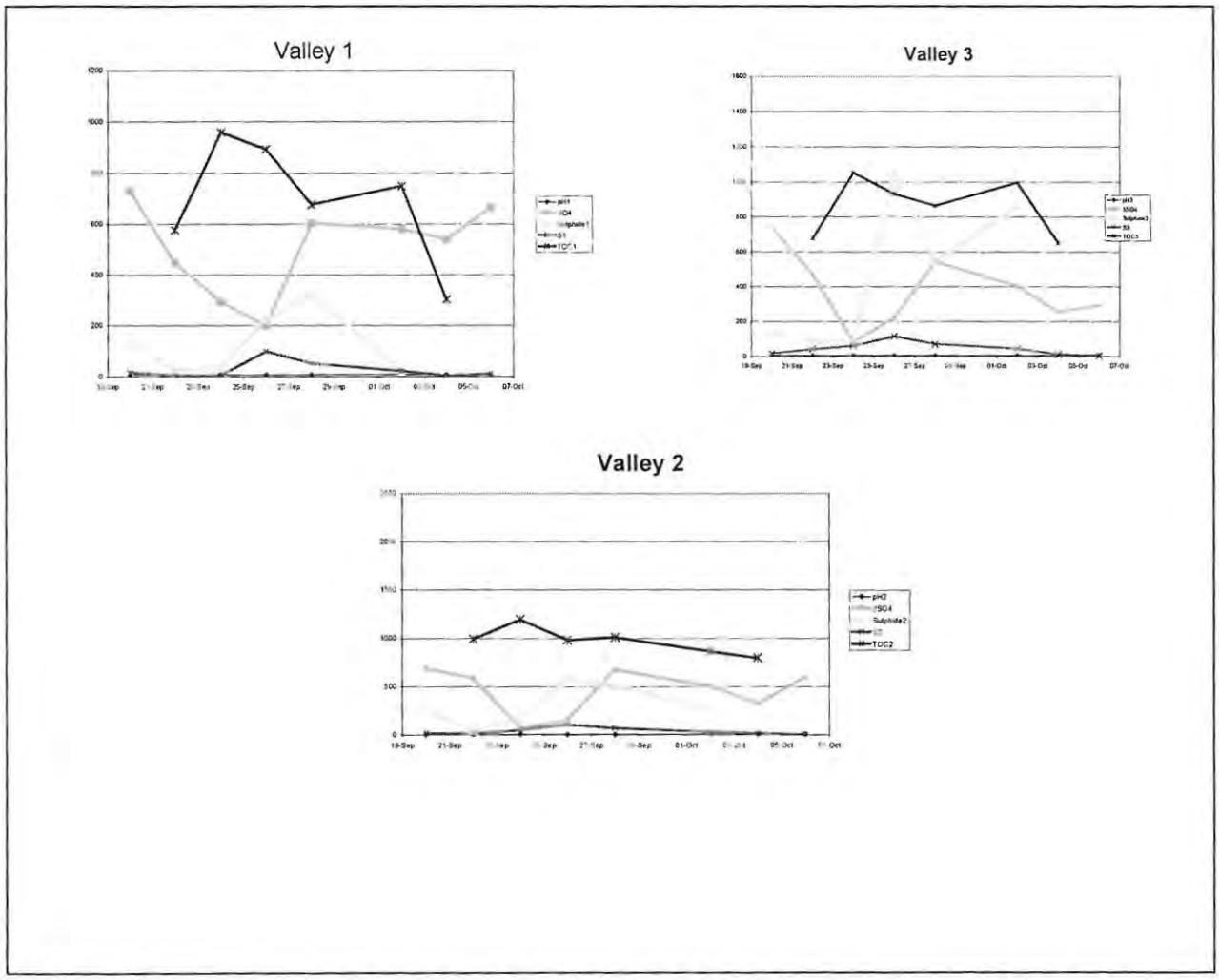


Figure 2.8: A comparison of sulfate, sulfide,sulfur,COD concentrations and pH in the Baffle Reactor for valleys 1, 2 and 3.

2.4 CONCLUSION

The laboratory-scale Baffle Reactor results showed that sewage sludge may be used as an innoculum for bacterial sulfate reduction. Lactate medium was used as a continuous feed for the operation of the reactor. The reactor provided a continuous flow of nutrients to the biofilm without disruption of the biofilm, while the SRB sludge supplied a constant high concentration of sulfide. The Baffle Reactor also produced a gradient of sulfate, sulfide and COD across the reactor, separated by the baffles, which contained varying amounts of sulfide and COD.

The development of this experimental system in which a gradient of increased sulfide concentration and decreased COD concentration was achieved, resulted in the formation of morphological and molecular studies. Figure 3.8 illustrates the overall results of all parameters in those three wells where samples were taken. Since each well depicted different performance, the microbial ecology of such system needed to be observed in detail. Chapter 6 deals with the population diversity of sulfate-reducing bacteria sampled in different wells and at different times to assess if the performance achieved in all the wells are controlled by the bacterial population present.

During the course of the study, the system was exposed to a perturbation that demonstrated the robustness of the process under shock load conditions. The reactor proved to be highly resilient, and was able to withstand perturbation events without prolonged loss of performance. The performance of the baffled reactor during the

perturbation events provided further evidence for a possible link between biological sulfate reduction and enhanced solubilisation of primary sewage sludge.

Chapter 3

ISOLATION AND MORPHOLOGICAL CHARACTERISATION OF BACTERIAL STRAINS IN A SULFATE REDUCING SLUDGE POPULATION

3.1 INTRODUCTION

An understanding of the biology of sludge microbial populations depends in part on a characterisation of the microbial community members occurring in these systems. Identifying microbial types occurring in the sludge population is the first step in determining their individual performance, and also what their combined role in the sludge biomass would be. A broad range of procedures are available for studying the components of mixed microbial communities. These include traditional isolation techniques, where microorganisms are grown and separated on solid media to obtain pure cultures of the species present in the system.

The structure, physiology and microbiology of sulfate reducing biofilms has been studied extensively using light and electron microscopy, micro-electrodes, nucleic acid probes and fluorescent in-situ hybridisation (FISH) techniques (Kuhl and Jorgensen, 1992; De Beer *et al.,* 1995; Kolmert *et al.,* 1997; Okabe *et al.,* 1998; Yu and Bishop, 1998; Okabe *et al.,* 1999).

Scanning electron microscopy (SEM) was used in this study to observe sulfate reducing sludge population morphology and structure. SEM has a large depth of field which allows a considerable amount of the sample to be in focus at a time and a high resolving power, making this type of microscopy useful in structural studies. The fixation and dehydration steps in sample preparation can, however, alter the morphology of the sludge population, and lead to shrinkage and loss of the polysaccharide matrices (Stewart *et al.,* 1995). While techniques needed to be developed for this application, SEM provided good morphological and structural detail on the biofilm components.

Traditional plate isolation techniques were used in this study as part of the overall approach followed to identify the microbial components of the suspended sulfate reducing biofilm growing on sewage sludge flocs in the Baffle Reactor system. The procedures for identification of bacterial species present in the biofilm included strain isolation on solid media, followed by their morphological and metabolic characterisation. Identification of these strains was attempted using Bergey's Manual (Krieg *et al.*, 1989), after which the molecular techniques of ribosomal DNA extraction and PCR amplification of DNA fragments corresponding to parts of the 16S rRNA genes were investigated The sequences of these PCR fragments were then determined and compared with reported sequences in the Ribosomal Database. This work is described in more detail in the next chapter. The results were then compared with whole genome DNA extraction studies and this work is reported in chapter 5.

3.2 MATERIALS AND METHODS

3.2.1 Culture-dependent techniques

3.2.1.1 Isolation of bacterial cultures

Bioreactor Sludge samples were collected and SRB strains were isolated by streaking plates in an anaerobic growth cabinet (Forma Scientific). Several selective media were evaluated. Modified Postgate's Medium B was chosen as the standard medium since it gave best growth after 5 days of incubation period. The medium was composed of: KH_2PO_4 (0.3g), $FeSO_4.7H_2O$ (0.5g), $MgSO_4.7H_2O$ (0.5g), $CaSO_4.2H_2O$ (1.0g), NH_4Cl (0.5g), yeast extract (1.0g), ascorbic acid (0.2g), sodium thioglycolate (0.1g), EDTA (15 μM), resazurin (0.001g), sodium lactate (3.5 ml) and agar (15g). HCl and NaOH were used to adjust the pH to 8.5 before the media was autoclaved (Postgate, 1984). Individual colonies were picked and restreaked at least three times to obtain pure cultures.

3.2.1.2 Morphological characterisation

(SEM) and gram staining techniques were used in order to characterise the SRB strains by cell morphology. Both methods illustrated the different morphological characteristics of the selected single colonies. Cells observed included spherical, ovoid, rod-shaped, and spiral forms. Different colonies were selected on the basis of their apparent physical characteristics, i.e. size, shape and colour.

3.2.1.3. Gram staining

Samples were stained with crystal violet (Saarchem) for 30 seconds then rinsed with dH_2O for 5 seconds. The samples were stained with Gram iodine (Saarchem) for 45 sec. The samples were treated with 4 drops of 95% ethanol before rinsing with dH_2O for 5 seconds. Safrinin was then used as the last stain for 30 seconds, and rinsed again with water for 10 seconds.

3.2.1.4 Scanning Electron Microscopy

The samples were fixed in a 2.5% gluteraldehyde solution in 0.1M phosphate buffer (pH7.0) overnight. The next day the samples were washed twice in 0.1M phosphate buffer for 10 minutes. The samples were then passed through an alcohol dehydration series; 30%, 50%, 70%, 80%, 90% and 100% ethanol for 10 minutes, followed by two 100% ethanol washes for 10 minutes. Following the alcohol dehydration, samples were critical point dried using liquid CO_2 in a Polaron E3000 Critical Drying Apparatus, and sputter coated in gold with Polaron E5100 Sputter Coating Unit. The samples were then observed in a JEOL JSM 840 Scanning Electron Microscope.

3.3 RESULTS AND DISCUSSION

3.3.1 Isolation and culture of strains from sulfate reducing sludge

Sludge samples were collected from the bioreactor at Grootvlei Gold Mine, previously noted, and strains of SRB were isolated anaerobically. Single colonies were selected based on colony shape and colour, cell shape and size and gram stain reactions. Eight morphologically distinct colonies were chosen which represented the potential diversity at a morphological level and were designated as SRB 10, SRB 12, SRB 24, SRB 26, SRB 29, SRB 30, SRB 31 and SRB 35 (Figures 4.1 a-h). The colonies were re-streaked onto Modified Postgate Medium B to select for sulfate-reducing bacteria and to ensure that pure cultures were retained. Growth was observed one week after inoculation.

Table 3.1: Comparison of the nine strains isolated from a sulfate reducing sludge population

Isolates	Colour and shape of colony (Figures 4.1a-h)	Cell shape (Figures 4.3a-h)	Gram stain (Figures 4.2a-h)	Cell size (length)
SRB 10	concave, shiny black with brown edges	Ovoid	Positive	1 µm
SRB 12	concave, whitish rough edges and irregular shaped colonies	Short rods	Negative	1 µm (both)
SRB 24	flat, black and rough edged colonies	Long rods	Negative	1 µm
SRB 26	concave, brown, smooth with slightly rough edged colonies	Short rods	Negative	1 µm
SRB 29	small, round, concave and greyish colonies	Rods	Negative	1 µm
SRB 30	flat, white smooth edged colonies	Spiral	Negative	1 µm
SRB 31	black, orange rough edged colonies	Short rods	Negative	1 µm
SRB 35	concave, metallic shine, round colonies	Rods	Negative	1 µm

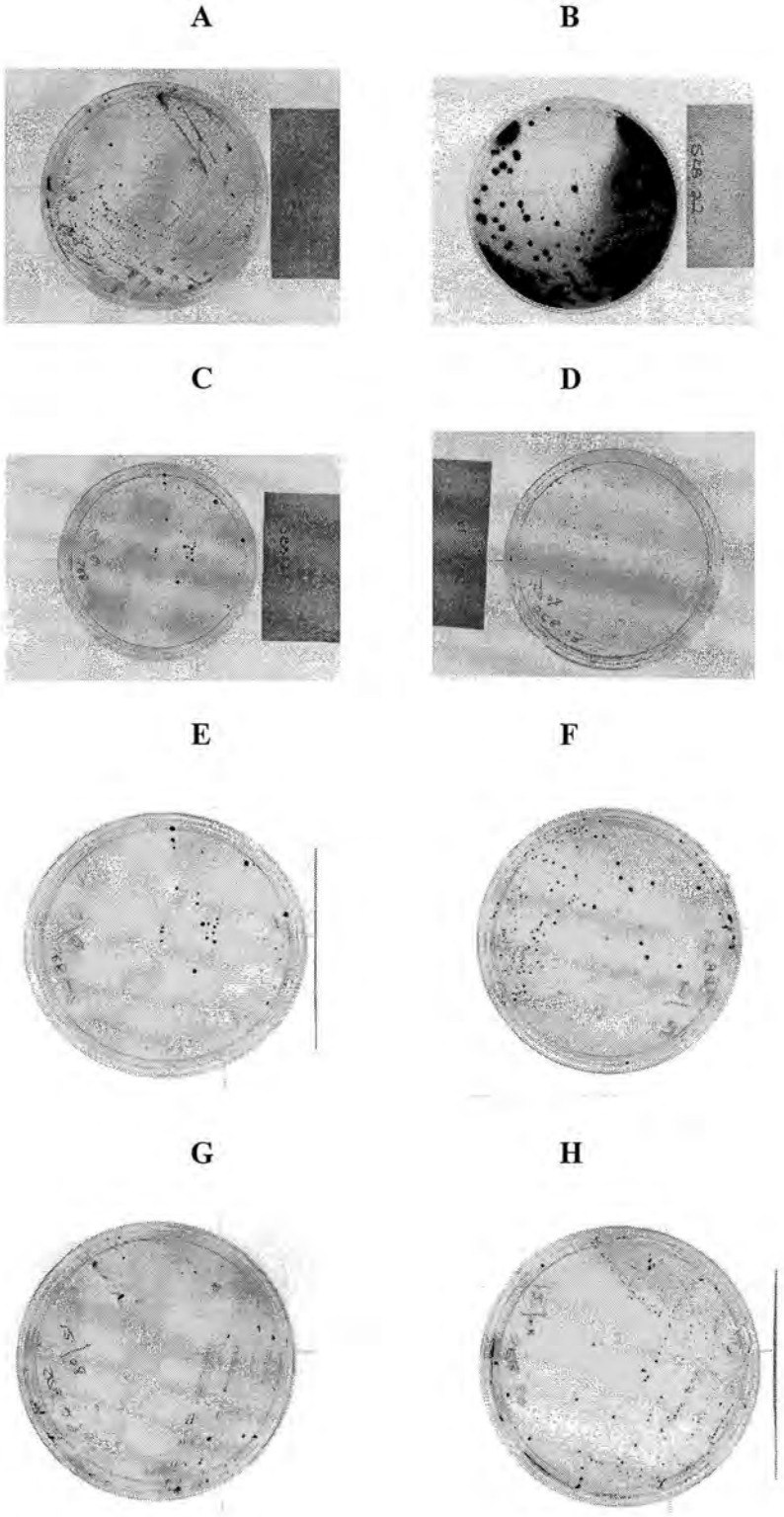


Figure 3.1: Colonies of isolates grown on Modified Postgate Medium B. A: SRB 24, B: SRB 26, C: SRB 29, D: SRB 12, E: SRB 10, F: SRB 30, G: SRB 31, H: SRB 35.

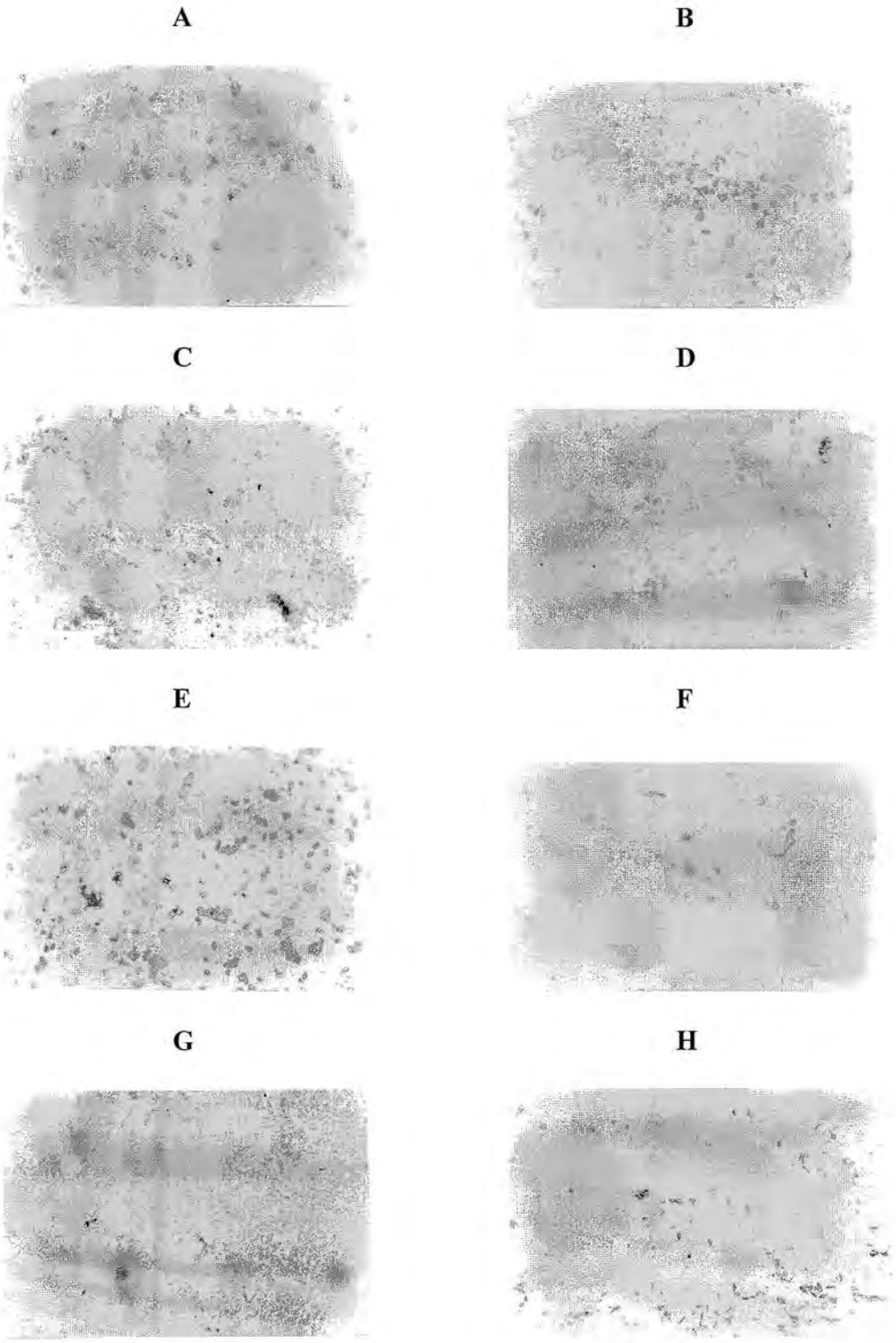


Figure 3.2: Gram staining of the eight isolates. A: SRB 10, B: SRB 24, C: SRB 12, D: SRB 26, E: SRB 29, F: SRB 30, G: SRB 31, H: SRB 35.

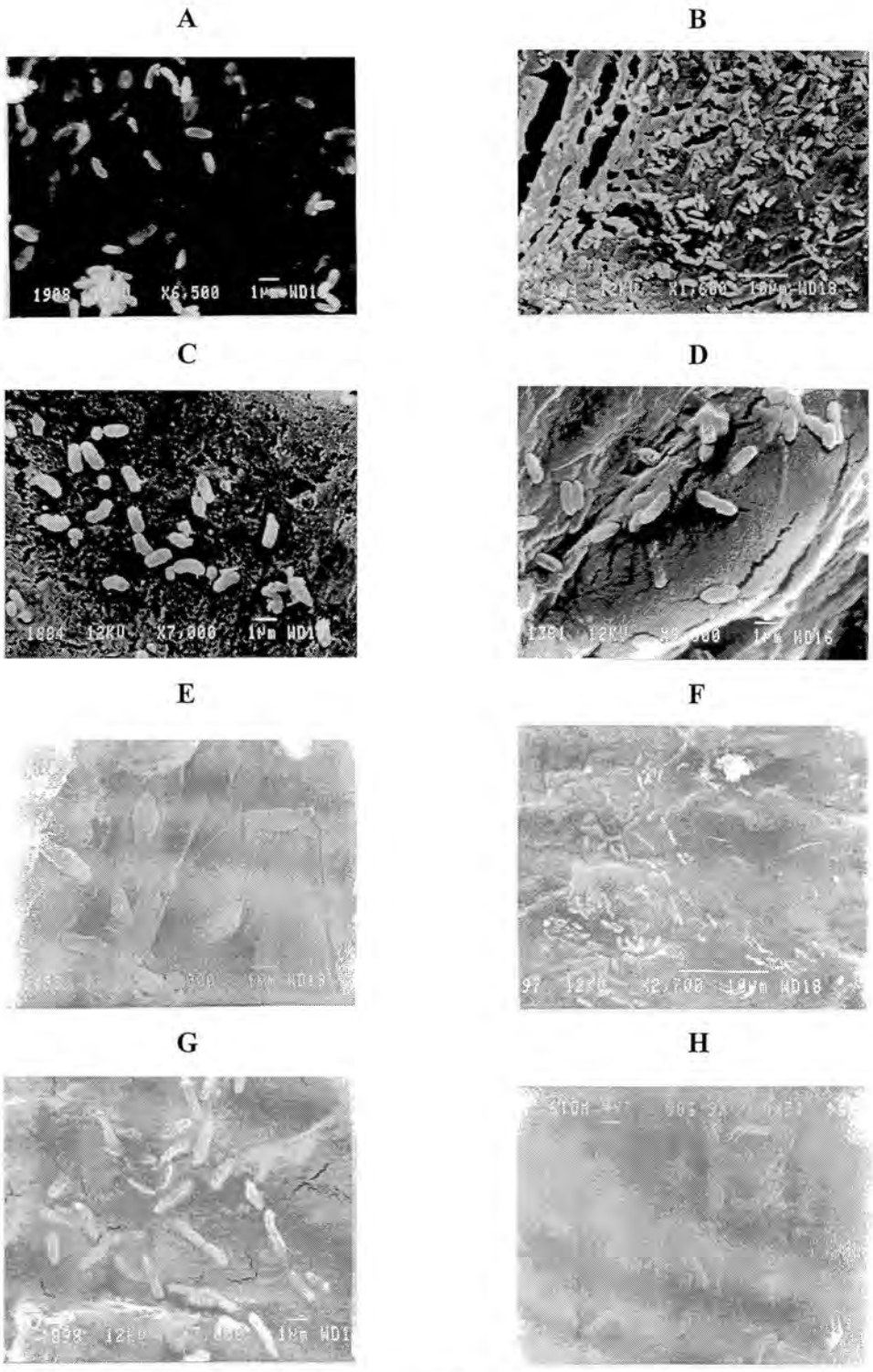


Figure 3.3: Scanning electron micrographs of the eight isolates, illustrating cellular morphology. A: SRB 31, B: SRB 24, C: SRB 12, D: SRB 26, E: SRB 29, F: SRB 30, G: SRB 10, H: SRB 35. Scale bar A-H = 1 μm.

The SEM micrographs reveal that isolate C is composed of two morphologically distinct cell types (Figure 3.3b). Isolate C consists of short rods over 1 μm long and coccoid cells. Although the colonies of C were restreaked numerous times in order to establish pure colonies, the mixed cultures persisted. This is visible in gram stain of Isolate C, where the cells consist of distinct red and blue areas (Figure 3.2b). The species in Isolate C could be co-dependent, and therefore are unable to survive alone.

3.4 CONCLUSION

Traditional plate culture techniques were used in this study to isolate eight morphologically distinct strains from the sulfate reducing sludge population based on their growth on Postgate Medium B and due to the formation of black colonies, they were provisionally identified as SRB-type organisms. These isolates were characterised morphologically and differentiated using gram stain and SEM. Traditional isolation techniques are, however, problematic in microbial ecology because the exact conditions of the original environment are not duplicated in the isolation medium, leading to recovery patterns on solid media which might not reflect the actual situation. Also, pure culture performance may be different compared to mixed cultures growing in proximity with each other in their natural environment. Kalmbach *et al.,* (1997) have estimated that less than 10% of microscopically visible bacteria are culturable on standard media, which leads to a huge discrepancy between direct counts and plate counts. These factors have made traditional plate isolation techniques less widely used in microbial ecology studies.

Due to known limitations of the traditional isolation techniques used in this study, it was necessary to identify components of the microbial community of the sludge population using molecular techniques. The next chapter describes in detail all the various molecular techniques employed. Clone library of the 16S rDNA fragments amplified directly from the biofilm DNA was constructed and the fragments were then sequenced. This would present a more reliable picture of the population present in the sludge and could be used to confirm the results obtained using traditional techniques.

Chapter 4

IDENTIFICATION OF SRB STRAINS FROM CULTURED ISOLATES USING 16S rRNA ANALYSIS

4.1 INTRODUCTION

Past studies of microbial communities responsible for geochemical transformations have been limited by an inability to representatively cultivate, and then identify, the constituent members (Kalmbach *et al.,* 1997). Ribosomal RNA sequences, particularly 16S-like rRNAs, provide a measure of phylogenetic relationship that can be now used to examine the structure and diversity of microbial communities. Because the Gram-negative mesophilic SRB comprise a phylogenetically coherent assemblage, their communities are well suited to explorations through rRNA sequence-based methodologies (Devereux *et al.,* 1996). In this chapter molecular biology methods were employed to determine the presence and distribution of individual bacterial species of SRB in the sulfate reducing sludge population using the pure culture isolates described in chapter 3.

Analysis of rRNA sequences is favoured by microbial ecologists because rRNA is found to in all organisms and has both conserved and variable regions, allowing the construction of general and specific probes (Muyzer and Ramsig, 1995). Using this approach, single cellular organisms may be divided into Archaea (Archaebacteria), Bacteria (Eubacteria) and Eucaryote (Eucarya). The rRNA gene is preferred over other

genes as genetic markers because there are large public rRNA databases with known sequences, and rRNA genes are highly conserved and can therefore be used to examine phylogenetic relationships between species (Britschgi and Giovannoni, 1991). In order to identify a species, the rRNA gene is sequenced, and the sequence is compared to known sequences in the public databases to match the unknown sequence to the closest sequence available.

PCR amplification of the rRNA genes is one of the RNA-based species detection and identification methods used in this study. Once the sequence of an organism has been determined, primers specific to the conserved region of that species can be designed. A positive PCR reaction using those specific primers would therefore indicate the presence of that particular organism.

Denaturing gradient gel electrophoresis (DGGE) is one of the techniques which is frequently used in molecular ecology to compare the structures of complex microbial communities, and is a powerful tool when used in combination with DNA sequencing. The rRNA sequences from environmental samples can be compared using sequence similarities detected by DGGE to elucidate phylogenetic information, as it is assumed that fragments denaturing in the same position have a high probability of being the same, or closely related species. The distribution of SRBs in a water column have been studied using this method (Muyzer and Ramsig, 1995).

Eichner *et al.*, (1999) noted that each DGGE band in simple communities such as hot springs can be assigned to cultured organisms or retrieved ribosomal sequences, whereas in complex environments such as sludges, DGGE becomes limited because the banding patterns becomes too complicated. The number, position and relative abundance of the bands allow comparison of number and relative abundance of dominant rDNA types between communities. However, the number and intensity of the bands do not equal the number and abundance of species within the community because of an inherent bias of PCR amplification from complex template mixtures when one organism can produce more than one band because of multiple, heterogenous rRNA operons (Eichner *et al.*, 1999). Because of the possibility that one band might contain more than one sequence, especially when analysing complex communities, DGGE should be used in conjuction with cloning (Felske *et al.*, 1998).

The molecular techniques described above are useful not only for species identification, but also for the information that can be inferred from the species identification. In this study, the genomic DNA from a sludge biofilm was extracted and amplified to yield a 586 bp product with a GC-clamp attached to the 5' end, corresponding to a portion of the 16S rRNA gene. These fragments were used to construct a clone library from which the excised fragments were analysed using DGGE, and assigned to groups, from which representatives were selected and sequenced. The sequence results were used to determine taxonomic identities of the bacterial types within the sulfate reducing sludge population.

4.2 MATERIALS AND METHODS

4.2.1 Genomic DNA extraction from sludge sample

Bacterial genomic DNA was obtained from the sludge samples as follows: 0.5 g of the sludge was collected and resuspended in 500 µL of TE buffer (10mM Tris-HCL, 1mM EDTA, pH8). After addition of 0.3 mg lysozyme, the samples were incubated at 37^0C for 30 minutes and mixed every 10 minutes by vortexing for three seconds. The tubes were then boiled for 1 minute, followed by four freeze-thaw cycles performed at -196^0C, in liquid nitrogen, and at 80^0C. The homogenate was mixed with 10 mL chilled extraction buffer (0.01M Tris-HCL pH8.0, 5mM EDTA pH 8.0 and 0.5% SDS). Proteinase K (50µg/mL), was added and the suspension was incubated for 12 hours at 37^0C, after which 1%(v/v) CTAB and 1M NaCl final concentration were added and the suspension was incubated at 55^0C for 1hour. An initial phenol-chloroform-isoamyl alcohol (1:24:1 v/v) extraction was performed, followed by chloroform-isoamyl alcohol (24:1 v/v) extractions to remove proteins until a clear inter-phase was obtained. This was followed by a final chloroform extraction (without isoamyl-alcohol). The DNA was precipitated by adding 2.25 volumes of ethanol (96%) and incubated at -20^0C for 12 hours. The precipitate was collected by centrifugation (Beckman JA 20 rotor) at 10 000 rpm for 30 minutes at 4^0C, washed with 70% ethanol, dried and resuspended in 500 µL TE buffer (10mM Tris-HCL, 1mM EDTA, pH8. A Rnase digestion (40 µg/mL Dnase free Rnase, Roche) was performed at 37^0C for 90 minutes to remove RNA. DNA concentrations between 100-300 ng/µL were routinely extracted from the valleys of the lab-scale Baffle Reactor described above.

4.2.2 PCR amplification of the 16S rRNA genes

Primers GM5F (5'-CCT ACG GGA GGC AGC AG-3') and 907R (5'-CC GTC AAT TCC TTT RAG TTT-3'), which correspond to positions 341-358, and 927-997 on the 16S rRNA gene of *E. coli* respectively, were used to amplify a 586 bp fragment from chromosomal rDNA isolated from sludge samples (Muyzer *et al.*, 1995). A GC-clamp (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3') attached to primer 907R was used. This was necessary for use in DGGE to minimise complete strand separation during electrophoresis through the denaturing gel. The PCR reactions contained 250 ng chromosomal DNA, 50 mol of each primer and 1U *Taq* DNA polymerase (Roche) in a final volume of 100 µL. A hot-start touchdown program was run for a total of 28 cycles with a final annealing temperature of 60^0C (Table 4.1). The presence of the amplified fragments were detected by electrophoresing 5-10 µL of the PCR reaction on a 1% agarose gel, as described in Appendix C.

Table 4.1: Touchdown PCR program used for primers GM5F and 907R

STEP	*TEMP*	*TIME*	*No of Cycles*
Initial Denaturation	98^0C	5 min	1
1. Denaturation	94^0C	30 s	
2. Annealing	80^0C 68^0C 66^0C 64^0C 62^0C	2 min 45 s 45 s 45 s 45 s	28 cycles of steps 1-6
3. Extension	72^0C	3 min	
Final Extension	72^0C	5 min	1

4.2.3 Cloning of the amplified fragments

The pGEM-T Easy Vector System (Promega) was used for cloning studies (Figure 4.1). The PCR fragments were cloned into the vector using the manufacturers protocol. 200ng of target DNA was ligated into 50ng plasmid and half shots were prepared for the ligation reaction. The mixture was then incubated overnight at 4^0C to ensure maximum ligation efficiency. The recombinant plasmids were transformed into *E. coli* DH5α using the heat shock technique. Each transformation culture was plated onto LB plates supplemented with ampicillin, IPTG and X-Gal; and incubated overnight at 37^0C.

The pGEM-T Easy vector carries the Beta-lactamase gene, which confers ampicillin resistance, allowing any DH5α*E. coli* cells with the plasmid to grow on the Luria agar plates containing ampicillin. The plasmid also has a multiple cloning region within the K-peptide coding region of 2-galactosidase. When the fragment is inserted into the coding region, the K-peptide is inactivated, allowing colour screening of recombinant plasmids. In the presence of X-Gal and IPTG, the K-peptide of the 2-galactosidase peptide produces a blue product, resulting in blue colonies where there is no insert in the plasmid. If the fragment has been inserted into the multiple cloning region, reulting in a recombinant plasmid, the K-peptide is inactivated, producing white colonies.

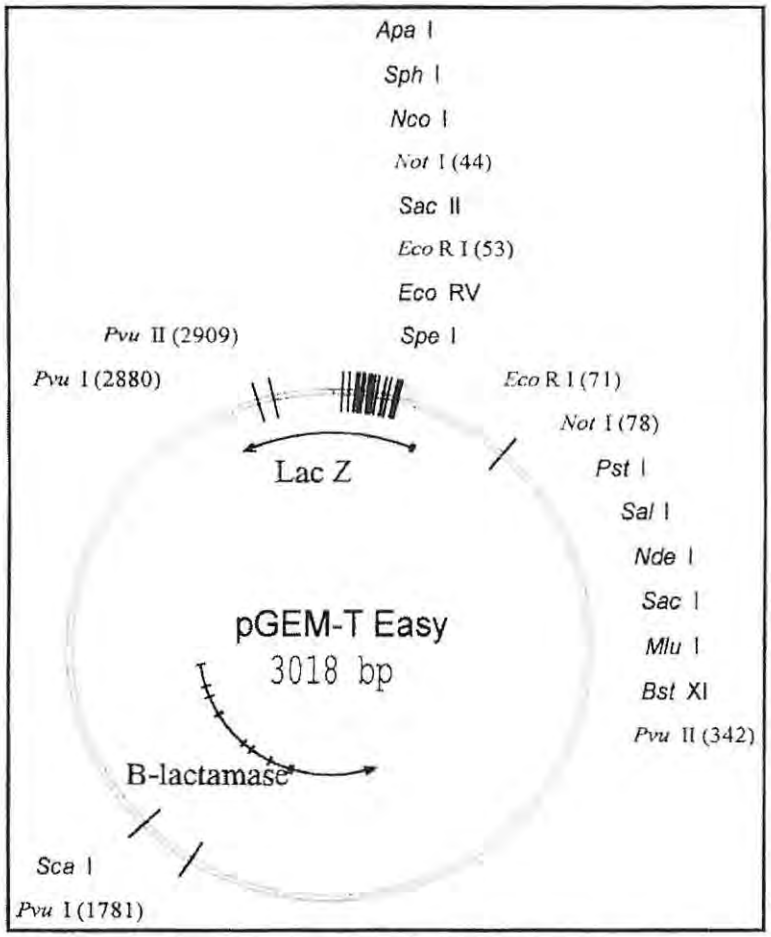


Figure 4.1 Restriction map of pGEM-T Easy Vector

4.2.4 Denaturing Gradient Gel Electrophoresis analysis of the 586 bp fragments

DGGE analysis was performed using a Bio-Rad Protean II system, as previously described by Muyzer *et al.*, (1993; 1995). The 586 bp rDNA fragments were loaded directly onto 6% (w/v) polyacrylamide gels in a 0.5 X TAE (0.4M Tri-acetate, 0.01M EDTA, pH8.3). Gradients were formed with 6% acrylamide solutions (acrylamide-bisacrylamide 37:1 w/w) that contained variations of between 35-80% denaturants. 10 μL of PCR products were loaded onto the gel with 5 μL tracking dye. Electrophoresis was

performed at a constant temperature of 60^0C and at a constant voltage of 100V for 17 hours. After electrophoresis, the gels were stained in 1X TAE buffer with ethidium bromide (0.5 mg/l) for 10 minutes. Fluorescence of the dye bound to DNA was excited by UV irradiation from a UV transilluminator, and was then photographed with a digital image gel documentation system (Kodac ds Electrophoresis Documentation and Analysis System 120 camera) and analysed using Kodac digital Science 1D version 2.0.3 software.

4.2.5 Nucleotide sequencing

Sequencing reactions were performed using half reactions. The Thermosequenase fluorescent labelled primer cycle sequencing kit (Amersham Pharmacia Biotech) was used, and analysed on an ALFexpress automated DNA sequencer (Pharmacia Biotech) with assistance from Mrs Di James (Department of Microbiology, UCT). The DNA fragments were sequenced in both directions. The 16S rDNA sequences with the two primer sequences removed were submitted to the Ribosomal Database Project (RDP) for comparison with other 16S rDNA sequences. Sequence alignments were performed using ClustalW version 1.5 software.

4.3 RESULTS AND DISCUSSION

4.3.1 Analysis and classification of the 16S rRNA gene fragments

Genomic DNA was isolated from all the pure cultures described in chapter 3, and equal volumes (10µl) of the isolated nucleic acids were analysed by DGGE. The resulting pattern of bands was visualised by ethidium bromide staining (Figures 4.2 and 4.3). In Figure 4.4, a mixture of template DNA of the different bacteria isolated from the BR was also used in the PCR amplification, and the resulting products were analysed on the same

gel with those PCR products from pure cultures to compare if any of the pure culture constituents were present in the mixed population of the reactor. DGGE analysis of these fragments demonstrated the presence of distinguishable bands in the separation pattern. Although not clearly visible in Figure 4.2, similar banding patterns were found illustrating that some of the pure cultures were of the same strain.

Screening of the same recombinant plasmids obtained from cloning of the amplified 586 bp rDNA fragments originating from pure cultures was carried out using restriction enzyme analysis and DGGE. The recombinant plasmids were digested with *Eco R1* restriction enzyme, and it was found that some of the fragments contained an internal *Eco R1* recognition site, while other fragments did not. This information was used to classify the inserts into two groups and only the group containing the *Eco R1* site was reported in this study.

After duplicate inserts were eliminated using DGGE analysis, eight clones were chosen. Figure 4.3 illustrates a DGGE gel on which 586 bp DNA fragments of the clones have separated, giving information on their G+C contents. Clone representatives designated SRB 12 and SRB 29, in Figure 4.4 (lane E and F), would be expected to have a much lower G+C content than the other representatives, which was corroborated by analysing the fragments sequence. Therefore, all the fragments migrated to positions in the denaturing gel which corresponded with their G+C contents. It may be concluded that the DGGE system can be a reliable tool to the extent that it separates DNA sequences on G+C % in a logical and reliable manner.

Figure 4.2: Negative image of ethidium bromide stained 6% acrylamide gel with a denaturant gradient of 35-70%. Lane 1: SRB 10; lane 2: SRB 15; lane 3: SRB 12; lane 4: SRB 24; lane 5: SRB 22; lane 6: SRB 29; lane 7: SRB 19; lane 8: SRB 28; lane 9: SRB 26; lane 10: SRB 30; lane 11: SRB 31; lane 12: SRB 35.

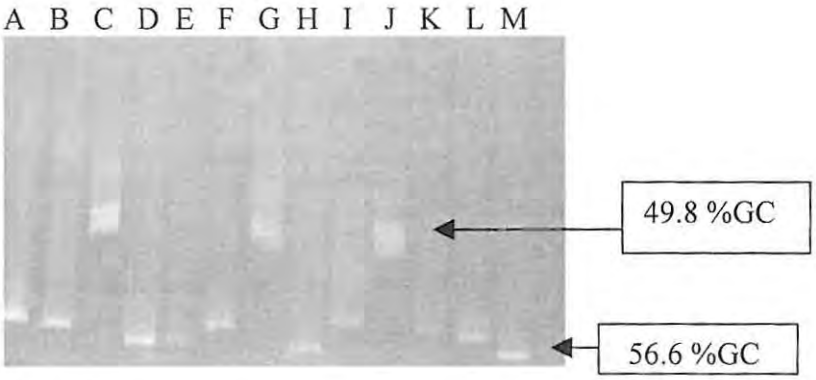


Figure 4.3 Negative image of ethidium bromide stained 6% acrylamide gel with a denaturant gradient of 50-80%. Lane A: SRB 9; lane B: SRB 28; lane C: SRB 29; lane D: SRB 22; lane E: SRB 15; lane F: SRB 24; lane G: SRB 19; lane H: SRB 30; lane I: SRB 10; lane J: SRB 12; lane K: SRB 35; lane L: SRB 26; lane M: SRB 31.

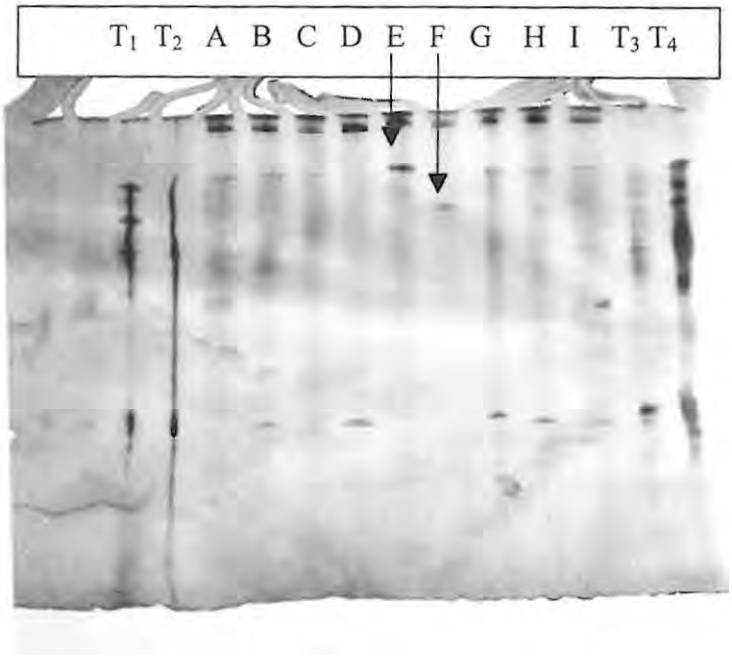


Figure 4.4: Negative image of ethidium bromide stained 6% acrylamide gel with a denaturant gradient of 50-80%. Lane T1, T2, T3 and T4: total DNA isolated from FSBR on different days; lane A: SRB 28; lane B: SRB 10; lane C: SRB 26; lane D: SRB 35; lane E: SRB 29; lane F: SRB 12; lane G: SRB 24; lane H: SRB 30; lane I: SRB 31.

4.3.2 DNA sequence determination

Two DNA libraries were obtained; these libraries were designated libraries I and II. Library I had Eco R1 site while library II did not, and only library I was analysed as mentioned above. Twenty clones were picked from library I and only eight different bands were produced on DGGE gels. The eight clones which were chosen based on their different band separation in a DGGE gel were sequenced after DGGE analysis. Only one sequence could be identified from the DGGE fragments of culture SRB 12, the mixed population. The reason for this is unknown. The sequence results for these clones of the 16S rRNA from various SRB isolates were aligned using Clustal X (Thompson et al.,

1997) with their closest 16S neighbour identified using Blast (Higgins *et al.*, 1996). The results are shown in Appendix G-1 to G-5.

SRB 10, SRB 26, SRB 30 and SRB 35 were all different to each other and different to SRB 24, SRB 29 and SRB 31. In contrast, SRB 24, SRB 29 and SRB 31 all were quite similar to each other. This suggests that there is at least five distinct bacterial species involved in the process that is being studied here.

4.4 CONCLUSION

The sulfate reducing sludge population studied in this project is probably composed of many uncultured or unclassified sulfate-reducing microorganisms. '*Desulfovibrio*', which is a gram-negative bacteria might be expected to be the dominant genus in such a population. DGGE analysis proved successful in separating the isolates based on their G+C contents, and the separation was confirmed based on a combination of morphological criteria.

The grouping of SRB 24, SRB 29 and SRB 31 as similar strains baed on their 16S sequence is not supported to any large extent by their morphological characteristics. However, morphology is not necessarily a good criteria for classification in a system such as that being studied here because the strains are unknown. The remaining SRBs, by their 16S sequence, are distinct species and thus, there is a minimum of five distinct organisms in the reactor system and perhaps up to eight.

Chapter 5

PHYLOGENETIC DIVERSITY OF A SULFATE REDUCING SLUDGE POPULATION DETERMINED BY ANALYSIS OF 16S rRNA GENE EXTRACTED DIRECTLY FROM SLUDGE

5.1 INTRODUCTION

In most natural and engineered bioprocess systems offering a sufficient nutrient supply, microorganisms grow as spatially organised, matrix-enclosed, multispecies communities in biofilms, aggregates, or flocs rather than as single planktonic cells (Costerton *et al.*, 1995). When studying these systems, microbial ecologists attempt to determine population structure and dynamics, as well as the spatial distribution of species and the function of species within communities which are characterised both by various cell-cell interactions (Ward, 1990), and by pronounced architectural and chemical heterogeneity (Costerton *et al.*, 1994 and Massol-Deya *et al.*, 1995). Since a rigorous biomass disaggregation step is a common feature of all traditional cultivation-based microbiological approaches used for analysis of complex microbial communities, spatial information is lost. In addition, biases introduced by conventional cultivation methods result in pronounced population shifts in the community structure and lead to a failure to detect dominant bacterial community members (Wagner *et al.*, 1993 and 1994; Ward *et al.*, 1990). Determining the physiological and biochemical properties of a single species in a laboratory pure culture may also bias the phenotype since gene expression is strongly

influenced by environmental constraints and the growth mode of the cells (Costerton *et al.,* 1994). Since isolated strains are known to adapt genetically to the environmental conditions which they encounter, they may also not be genetically representative of their environmental counterparts (Ward, 1990).

With the advent of rRNA based techniques in microbial ecology, the dependence on isolation of pure cultures as a means of studying the diversity and structure of microbial communities has been reduced. In this chapter, a study was performed with a laboratory-scale Baffle Reactor supplemented with a lactate feed. The goal of this study was to combine the techniques of selective PCR amplification, denaturing gradient gel electrophoresis (DGGE), comparative sequencing, and Southern blot hybridisation to selectively identify sulfate-reducing bacteria in the reactor. One of the open and unanswerable questions was whether the lactate feed indeed selects for those bacteria that are responsible for sulfate reduction in the reactor.

The main objectives for this chapter were to compare laboratory-scale and Pilot Plant Baffle Reactors using sewage sludge innocula and also to determine whether a correlation could be found between conventional plate isolation and molecular techniques. In the course of the study, molecular data were then compared to those obtained by traditional cultivation-dependent techniques.

5.2 MATERIALS AND METHODS

5.2.1 Sludge sampling

The laboratory-scaled Baffle Reactor described in Chapter 2 was used to grow a sulfur biofilm. Once the biofilm had developed on the surface of the reactor, sludge samples were taken daily from three of the five valleys in the Baffle Reactor. Samples were also drawn from the Pilot-scale Baffle Reactor.

5.2.2 Scanning electron microscopy

A 1 cm^2 section of the sludge sample was lifted onto a 0.22 micron supported nylon membrane (Micron Separations Inc), with another membrane placed on top of the sample. The two membranes were stapled together, and the sample fixed in 2.5% gluteraldehyde in 0.1M phosphate buffer (pH 7.0) overnight, before being processed through the alcohol dehydration series described in Appendix B. After critical point drying, cross-sections were cut through the membranes using sharp scissors and placed between two copper "bookends". The copper structures were anchored to a sample stub using colloidal graphite, with the cross section of the sludge sample facing upwards. The sample was sputter coated with gold in this position.

5.2.3 PCR reactions of the 16S rRNA genes

Primers GM5F (5'-CCT ACG GGA GGC AGC AG-3') and 907R (5'-CC GTC AAT TCC TTT RAG TTT-3') in Appendix E were used to amplify a 586 bp fragment from chromosomal rDNA isolated from sludge samples. The PCR reactions contained 250 ng chromosomal DNA, 50 pmol of each primer and 1U taq DNA polymerase (Roche) in a

final volume of 100 μL. A touchdown program (Table 4.1) was used for a total of 28 cycles with a final annealing temperature pf 58^0C.

5.2.4 Denaturing Gradient Gel Electrophoresis analysis

DGGE analysis was performed as described in Chapter 4. The 586 bp DNA were loaded directly onto 6% (w/v) polyacrylamide gels in a 0.5 X TAE (0.4M Tri-acetate, 0.01M EDTA, pH8.3). Gradients were formed with 6% acrylamide solutions (acrylamide-bisacrylamide 37:1 w/w) that contained variations of between 35-80% denaturants. 10 μL of PCR products were loaded onto the gel with 5 μL tracking dye. The electrophoresed gels were stained in 1X TAE buffer with ethidium bromide (0.5 mg/l) for 10 minutes. Fluorescence of the dye bound to DNA was excited by UV irradiation from a UV transilluminator, and was then photographed with a digital image gel documentation system (Kodac ds Electrophoresis Documentation and Analysis System 120 camera) and analysed using Kodac digital Science 1D version 2.0.3 software.

5.3 RESULTS AND DISCUSSION

5.3.1 Morphological characterisation of sludge sample in a baffled reactor

Chapter 2 dealt with the reactor performance which was emphasised in the first two valleys of the reactor. Five parameters of the reactor were evaluated closely and the results obtained showed a general trend of reactor performance with different levels of microbial activity taking place in different wells over time. It was proposed that the reactor might harbour different types of microorganisms as it matures. Therefore,

morphological characterisation of the bacteria in the sludge was conducted from start-up of the reactor until the last day. Figure 5.1a illustrates different gram negative bacterial cells obtained by gram staining while Figure 5.1b-e reveals the presence of several morphologically distinct bacteria in the sludge, including long rods, short rods, coccoid cells, ovoid and spiral cells obtained by scanning electron microscopy (SEM).

In order to investigate and identify dominant participants within the bacterial population of the reactor, molecular techniques were employed.

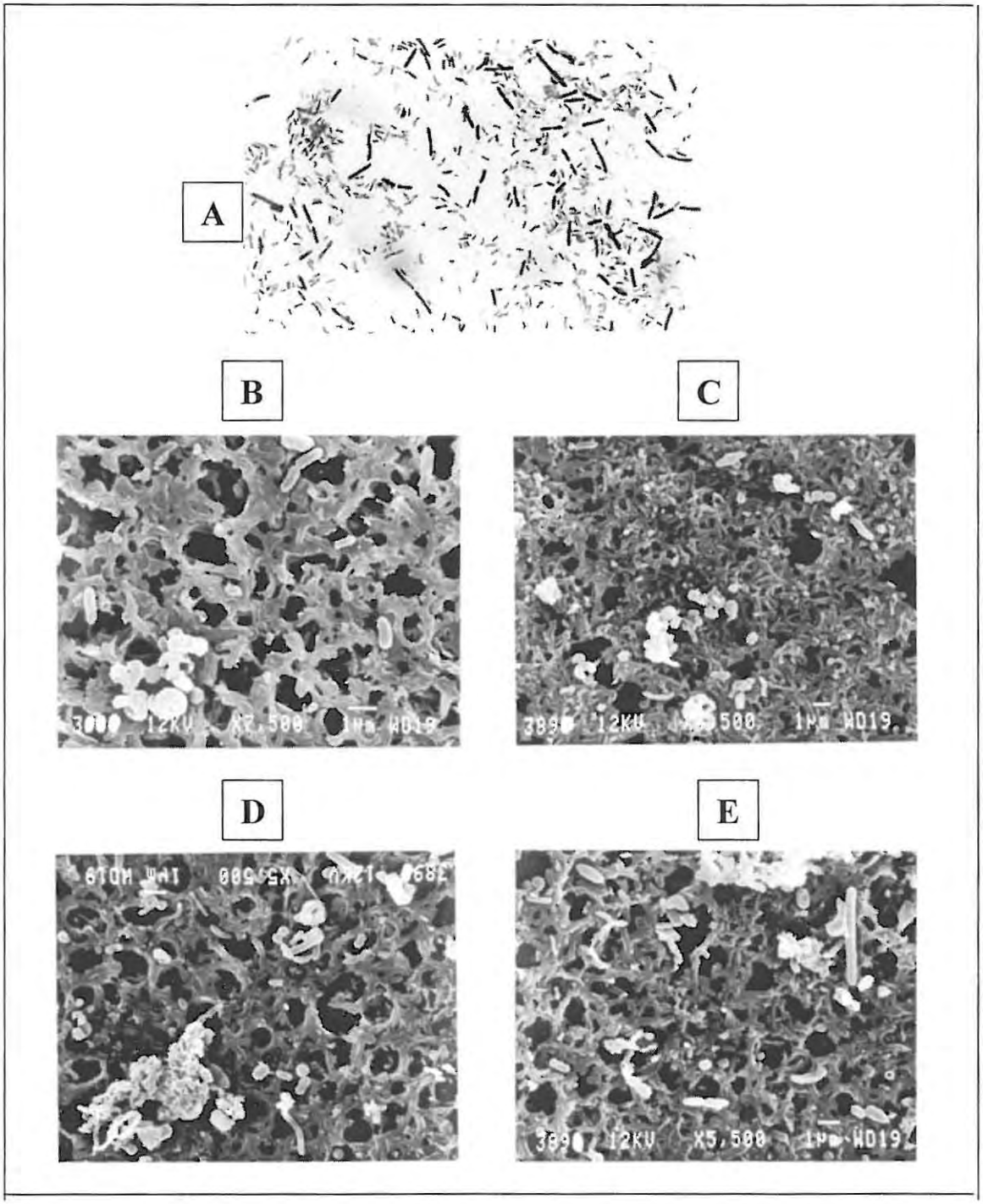


Figure 5.1: Morphological characteristics of Baffle Reactor sludge sample: A: gram staining; B-E: SEM of sludge. Scale bar A-E = 1μm.

5.3.2 Analysis of microbial diversity

5.3.2.1 Denaturing Gradient Gel Electrophoresis analysis

The microbial diversity of the Baffle Reactor system was analysed by DGGE of PCR-amplified 16S rRNA (Figure 5.3 and 5.4). Electrophoresis for 2 to 17 hours was used to optimise the separation of the amplified 16S rRNA genes. Running time of 17 hours gave the best resolution of the 16S rRNA, with up to 8 bands or sequence types observed (Figure 5.4). The migration of several bands was observed even after extended running times, emphasising the importance of proper selection of running conditions. Although only a few dominant morphotypes were observed, a complex microbial diversity with at least 8 diferent sequence types was documented in the DGGE analysis of amplified rRNA gene sequences.

The three most dominant fragments were isolated and reamplified by PCR (Figure 5.4). Before DNA sequencing, the recovered DGGE bands were run on a DGGE gel to confirm their positions relative to the original sample. This step was repeated at least twice to obtain a pure DNA product for sequencing, since reamplification of a (presumably) single fragment often resulted in the formation of multiple amplicons from the adjacent bands.

In chapter 2 sulfur cycle illustrating sulfate reduction and elemental sulfur production was demonstrated. The bacteria that are involved i.e. SRB and SOB were examined closely by isolating both the sludge and sulfur biofilms. DNA was extracted from the two biofilms and enzymatically amplified by PCR and 586 bp 16S rRNA gene fragments

were obtained (Figure 5.2). Scanning electron microscopy was also performed in order to identify morphologically different types of bacterial cells from both biofilms as illustrated in Figure 5.1b-e. PCR-amplified 16S rRNA was analysed by DGGE and this is illustrated in Figure 5.3 whereby different types of bands were depicted illustrating that the bacteria in both the biofilms were different and probably performed different activities within the Baffle Reactor.

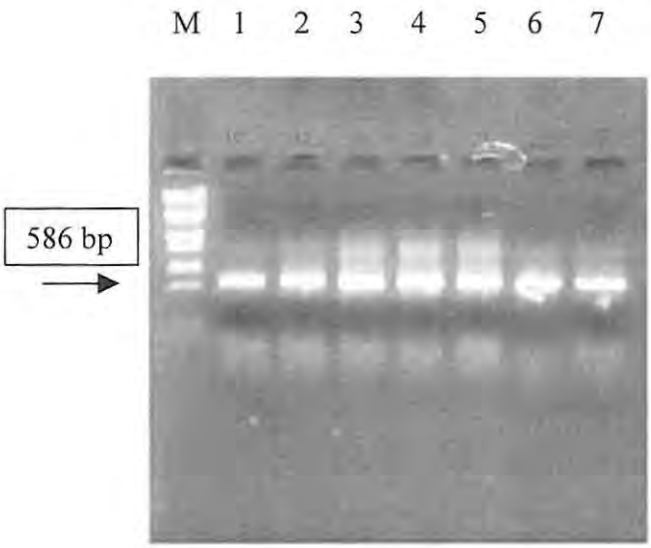


Figure 5.2: PCR amplification of the 16S rRNA gene. Lane 1 to 7: total DNA extracts from Baffle Reactor on different days.

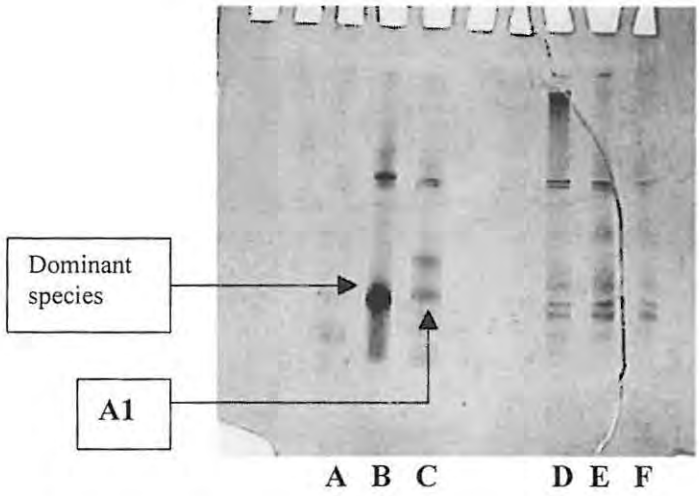


Figure 5.3: Negative image of ethidium bromide stained 6% acrylamide gel with a denaturant gradient of 50-80%. Lane A, B and C: total DNA extracts from laboratory-scale Baffle Reactor sludge biofilm; Lane C, Dand E: total DNA extracts from laboratory-scale Baffle Reactor sulfur biofilm.

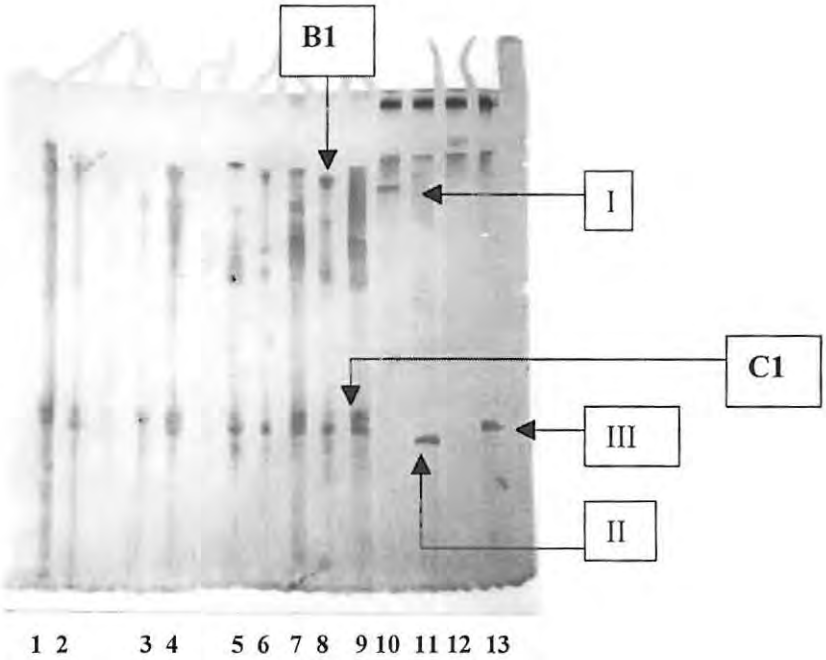


Figure 5.4: Negative image of ethidium bromide stained 6% acrylamide gel with a denaturant gradient of 50-80%. Lane 1-9: total DNA isolated from Pilot Plant Baffle Reactor on different days; lane 10: I, lane 11: II, lane 13: III. Lane 10 and 13: DNA extracted from band B1 and C1, Lane 11:DNA extracted from A1 in the laboratory-scale Baffle Reactor.

5.3.3 DNA sequence determination and phylogenetic analysis

The three dominant DGGE bands were purified and their DNA cloned into pGEM-T. One clone from each band was then sequenced to give about 590 nucleotides. These sequences were compared with the sequences available in GenBank. It was previously shown that relatively short sequences, such as those obtained from DGGE analysis, are sufficient for an approximate phylogenetic identification (Schmidt *et al.*, 1991; Schuppler *et al.*, 1995 and Ward *et al.*, 1990).

The sequences obtained were aligned with their neighbour as obtained by Blast (Higgins *et al.*, 1996) in a similar manner to the isolates sequence in the previous chapter and the results are shown in Appendix G-1 to G-5. Included in this analysis were the SRB strains and Appendix G-1 to G-5 also show the relationship of 16S rRNA sequences of the DGGE bands to the SRB isolates.

DGGE band I is very similar to SRB 10. DGGE band II is very similar to SRB 30. DGGE band III is very similar to SRB 35. These groups were then analysed by the Neighbour-Joining method to produce phylogenetic trees for the five major groupings obtained from both DGGE and strain isolation. The results are shown in Appendix H-1 to H-5

5.3.4 Structure of the Bioreactor microbial population

Analysis of the phylogenetic trees in Appendix H-1 to H-5 show that there seem to be five major groupings of organism present in the bioreactor. No information is available

on the quantitative distribution of these organisms, but it is possible to place a taxonomic grouping on four of the five types.

SRB 10 and DGGE I both would seem to be Desulfomonas species and can be tentatively named *Desulfomonas rhodensis* nov sp. based on their distinct separation from other Desulfomonas species. SRB 30 and DGGE II both resemble a Clostridium species. Clostridium species are strictly anaerobic microorganisms and are most likely to be found in sewage. Literature states that 9 out of 11 strains of Clostridium are capable of producing H_2S (Bergeys Manual, 1989), therefore the strain represented by band II could be involved in sulfate reduction within the reactor.

The 16S RNA sequence is distinct from all known Clostridium and it is suggested that the species be named *Clostridium sulforhodensis* nov sp. SRB 35 and DGGE III also would seem to be Desulfomonas species. In order to analyse the relationship between SRB 10/DGGE I and SRB 35/DGGE III, they were analysed together as shown in Appendix H-6. Although both branch close to each other, they are distinct and it is suggested that SRB 35/DGGE III is a distinct species from SRB 10/DGGE I and should be tentatively named *Desulphomonas makanaiensis* nov sp. SRB 26 was not identified in the DGGE analysis but would seem to be a Desulfomicrobium species and is tentatively named *Desulfomicrobium rhodensis* nov sp.

5.4 CONCLUSION

The study of complex microbial communities such as activated sludge with independent molecular techniques has shown that the microbial diversity is very high and that only a very low number of the microbes have been successfully isolated and characterised (Amann *et al.*,1995). In this chapter, an application of molecular techniques was discussed for cultivation independent analysis of the microbial population structure and its functions. Applying these techniques, the identity and the specific activity of the microorganisms was simultaneously examined in the sludge in order to establish an understanding of the population dynamics in the Baffle Reactor.

Several morphological, biochemical, and genetic characteristics have been used to identify constituents in complex populations of microorganisms. The16S rDNA sequence divergence of different bacterial species has been exploited as an indicator of diversity. To assess this diversity in this study, PCR amplification of 16S rDNA and sequence analysis have been applied. The novel approach that is presented here is based on DGGE analysis of 16S rRNA sequences obtained after PCR amplification of genomic DNA isolated from complex microbial populations in both the laboratory-scale and Pilot Plant Baffle Reactor. The system was obtimised for the analysis of microbial population by using PCR primers and DGGE conditions that resulted in high-resolution banding patterns. For optimal DGGE separation, incorporation of a 40-bp GC-rich clamp in the 5' primer proved necessary for optimal resolution of the fragments in the denaturing gradient. The banding pattern provided a profile of the populations in that the relative intensity of each band and its position most likely represented the relative abundance of a

particular species in the population. The sensitivity of the detection of 16S rDNA sequence variants by this approach was demonstrated by the number and different intensities of the bands observed in the DGGE profile (Figure 5.3). The DGGE profiling approach described in this chapter was to address the biological problem of genetic diversity of microbial populations and to assess the presence of sulfate-reducing bacteria in the bacterial biofilm of the two reactors.

Same sludge innoculum was used for laboratory-scale and pilot-scale reactors and different population distribution patterns were observed in both reactors (Figure 5.3 and Figure 5.4). Dominant species were identified by the intensity of the bands observed in the DGGE profiles of laboratory-scale reactor and the bands (A1, B1 and C1 in Figures 5.3 and 5.4) were excised, purified, cloned and sequenced. The SRB isolates from which 16S sequences was obtained came from the Pilot plant while the 16S sequences from the DGGE bands were amplified from DNA isolated from laboratory-scale plant. The close correlation between the DGGE results and SRB 10, SRB 30 and SRB 35 show that the processes involved in both plants are similar. Thus it can be concluded that Desulfomonas and Clostridium predominate in the SRB process.

The presence of Desulfomicrobium in the Pilot plant does not in any way suggest that it is not present in the laboratory-scale plant because further work would be necessary to identify the full diversity of strains present. The presence of three novel unidentified species SRB 24, SRB 29 and SRB 31 all closely related to each other in the Pilot plant suggest that there are a range of other novel organisms involved in the Pilot plant process.

Chapter 6

GENERAL DISCUSSION AND CONCLUSIONS

6.1 DISCUSSION

It has been noted that most sulfate reducing bacteria (SRB) studies have focussed mainly on the use of refined carbon as electron donor sources for sulfate reduction than complex carbon sources. Whittington-Jones, (2000) and Corbett, (2001) from the EBG at Rhodes University have examined and evaluated the usage of complex carbon sources as electron donors for biological sulfate reduction. This resulted in the scale up development and construction of the Rhodes 'BioSURE' Process at Grootvlei Gold Mine No. 3 Shaft. The results they reported showed a full biological sulfur cycle whereby SRB were involved in sulfate reduction and sulfide oxidising bacteria (SOB) oxidised sulfide to sulfur. However, these processes were obtained using different reactors and they called this a dual-stage sulfate reduction operation. These findings suggested a model system which was undertaken in this study, and the system could also be used to study the various components of the sulfur cycle.

Since relatively little is known about the microbial ecology of these systems, an experimental bench scale Baffle Reactor was constructed and operated for this study. Lactate carbon source was used as the substrate to establish controlled feed conditions and its performance was evaluated.

Sulfate-reducing bacteria (SRB) present in the sludge bed in the valleys of the reactor reduced sulfate to sulfide, which was converted in turn by the sulfide oxidising bacteria (SOB) in the sulfur biofilm to elemental sulphur at the correct redox potential. The sulfur biofilm developed on the surface of the reactor as sulfide oxidising bacteria require a carefully poised redox balance between oxygen and sulfide concentrations. The presence of a too high oxygen concentration resulted in high rates of chemical sulfide oxidation to sulfate.

Since the study mainly focused on the suspended sludge biofilm, techniques such as gram staining and scanning electron microscopy were used to examine the biofilm. The examination by SEM revealed the complex differentiated biofilm structure, where morphologically distinct cells were found to occur as the reactor matures. Bacilli, cocci and spiral forms were the dominant bacterial morphologies found in the sulfate reducing sludge population.

The performance and morphology of the sludge population was carefully assessed and the culture of SRB was shown to reduce sulfate to sulphide. Having described the performance, an attempt was made to identify the sulfate reducing bacterial species present in the biofilm and to achieve that, strains were isolated from the sludge population using traditional plate culture techniques. Eight strains, chosen on the basis of colony morphology, were characterised in an attempt to identify them. These studies revealed that the isolates grown on modified Postgate's Medium B were not comparable

with the dominant species isolated from the laboratory-scale and pilot-scale Baffle Reactors. Thereafter amplified rRNA gene from each isolate were sequenced for identification purposes. Two Desulfomonas species, one Clostridium species and one Desulfomicrobium species were identified in the Pilot plant system and this agrees with the expected type of bacterial population. Three related unidentified species were also found and their importance is as yet unknown.

Results obtained from the laboratory and pilot-scale Baffle Reactors showed that three dominant species were isolated from the whole genome extraction. Their sequences were compared with the sequences available in GenBank and it was clear when analysed phylogenetically that all three DGGE clones were related to the isolated organisms from the Pilot plant.

The more recent comparative 16S rRNA sequencing studies by Giovannoni *et al.*, (1990) and Ward *et al.*, (1990) pointedly illustrated the limitations of pure-culture isolation in surveys of natural microbial diversity. Community members directly identified by molecular criteria were absent from pure-culture isolation representations of the communities. Thus, less biased molecular measures are increasingly used in studies of microbial systematics and ecology. Comparative rRNA sequencing has added a phylogenetic framework to molecular characterisation.

6.2 CONCLUSIONS

The following objectives were achieved in this study:

1. The construction and operation of the Baffle Reactor as a model system to study aspects of the biological sulfur-cycle;
2. The study of SRB sludge populations from bioreactors treating high sulfate wastewaters;
3. The comparison of populations by identifying and comparing the bacterial species present in laboratory-scale and pilot-scale reactors using sewage sludge innocula.

The combined use of selective PCR amplification and comparative sequencing in this study offered the basis for a systematic dissection of sludge biofilm microbial community architecture. The characterisation and quantification of microorganisms in anaerobic reactors is difficult, but essential for optimising the reactor process. Characterisation of sulfidogenic sludge population has been known to be difficult due to the presence of many unidentified species in the sludge and the lack of available hybridisation probes for those microorganisms which have already been isolated. In this study classical microbiological and modern molecular-based sludge characterisation methods have been compared and it has been shown that different species identification results were obtained.

This study was a component of a wider investigation of the Baffle Reactor as a model system for studying integrated sulfur cycle biology under controlled laboratory

conditions. Future work will involve the investigation of interactions between sulfate reducing and sulfide oxidising microbial populations in these systems. The results here indicate that the relatively easy and inexpensive conventional methods of population analysis have little role to play in this investigation. Also that considerable care needs to be exercised in the extrapolation of model laboratory systems to larger-scale environmental applications. Nevertheless, the model laboratory systems seems to provide a useful mechanisms to study sulfureta and the performance of sulfur cycle interactions. Supporting this, the predominant species by DGGE in the laboratory-scale plant correlate closely with three of the dominant species in the Pilot plant.

The classical and molecular approaches used in this study show that a correlation to a significant degree can be obtained between the two methods. However, both also provide unique insights in different aspects of the SRB process.

7. REFERENCES

Abram, J.W. and Nedwell, D.B. (1978) **Inhibition of Methanogenesis by Sulphate Reducing Bacteria Competing for Transferred Hydrogen** Arch. Microbiol. **117**: 89-92

Amann, R.I.; Binder, B.J.; Olson, R.J.; Chisholm, S.W.; Devereux, R. and Stahl, D.A. (1990) **Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analysing mixed microbial populations** Appl. Environ. Microbiol. **56:** 1919-1925

Amann, R.I.; Krumholz, L. and Stahl, D.A. (1990) **Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology** J. Bacteriol. **172:** 762-770

Amann, R.I.; Stromley, J.; Devereux, R.; Key, R. and Stahl, D.A. (1992) **Molecular and Microscopic Identification of Sulfate-Reducing Bacteria in Multispecies Biofilms** Appl. Env. Microbiol. **58**: 614-623

Amann, R.I.; Ludwig, W. and Schleifer, K.H. (1995) **Phylogenetic identification and in situ detection of individual microbial cells without cultivation** Microbial Reviews **59:** 143-169

Amann, R.I.; Snaidr, M.; Ludwig, W. and Schleifer, K.H. (1996) **In situ visualisation of high genetic diversity in a natural microbial community** J. Bacteriol. **178:**3496-3500

Ausubel *et al.,* (1989) **Current Protocols in molecular biology** John Wisley & Sons, New York

Barnes, L.J.; Janssen, F.J.; Sherren, J.; Versteegh, J.H.; Koch, R.O. and Scheeren, P.J.H. (1991) **A new process for the microbial removal of sulphate and heavy metals from contaminated waters extracted by a geohydrological control system** Trans IchemE **69**: 184-188

Bishop, P.L. (1997) **Biofilm structure and kinetics** Wat. Sci. Tech. **36:** 287-294

Boogerd, F.C.; van den Beemd, C.; Stoelwinder, T.; Bos, P. and Kuenen, J.G. (1991) **Relative contributions of biological and chemical reactions to the overall rate of pyrite oxidation at temperatures between 30^0C and 70^0C** Biotechnol. Bioeng. **38:** 109-115

Boshoff, G.; Duncan, J.R. and Rose, P.D. (1996) **An algal-bacterial integrated ponding system for the treatment of mine drainage waters** J. Appl. Phycol. **8:**442

Britschgi, T.B. and Giovannoni, S.J. (1991) **Phylogenetic analysis of a natural marine bacterioplankton population by rRNA gene cloning and sequencing** Appl. Environ. Microbiol. **57:** 1707-1713

Campbell, R. (1983) **Microbial Ecology, Vol 5** Blackwell Scientific, Oxford

Christensen, H.; Hansen, M. and Sørensen, J. (1996) **Counting and size classification of active soil bacteria by fluorescence in situ hybridisation with an rRNA oligonucleotide probe** Appl. Environ. Microbiol. **65:** 1753-1761

Collier *et al.*, (1996) **Comparison of PCR-based approaches to molecular epidemiologic analysis of *Clostridium difficile*** J. Clin. Microbiol. **34 :** 1153-1157

Cooney, M.J.; Roschi, E.; Marison, I.W.; Comninellis, Ch. And von Stockar U. (1996) **Physiologic studies with the sulfate-reducing bacterium *Desulfovibrio desulfuricans*: Evaluation for use in a biofuel cell** Enzyme Microbial Technol. 18: 358-365

Corbett, C.J. (2001) **Development of the Rhodes 'BioSURE' Process in the treatment of acid mine drainage wastewaters** MSc Thesis, Rhodes University.

Cork, D.J. and Cusanovich, M.A. (1979) **Continuous disposal of sulphate by a bacterial mutualism** Dev. Ind. Microbiol. **20:** 591-602

Costerton, J.W. (1995) **Overview of microbial biofilms** J. Industrial Microbiol. **15:** 137-140

Costerton, J.W.; Lewandowski, Z.; De Beer, D.; Caldwell, D; Korber, D. and James, G. (1994) **Biofilms, the customised microniche** J. Bacteriol. **176:** 2137-2142

Davey, M.E.; Wood, W.A.; Key, R.; Nakamura, K. and Stahl, D.A. (1993) **Isolation of three species of *Geotoga* and *Petrotoga:* two new genera, representing a new lineage in the bacterial line of descent distantly related to the 'Thermotogales'** System. Appl. Microbiol. **16:** 191-200

De Beer, D. and Muyzer, G. (1995) **Multispecies biofilms: report from the discussion session** Wat. Sci. Tech. **32:** 269-270

De Beer, D. and Schramm, A. (1999) **Micro-environments and mass transfer phenomena in biofilms studied with microsensors** Wat. Sci. Tech. **39:** 173-178

De Bruyn, E.E. and Cloete, T.E. (1993) **Media for the detection of sulphide-producing bacteria in industrial water systems** J. Microbiol. Methods **17**: 261-271

De Vegt, A.; Krol, J.P. and Buisman, C.J. (1996) **Biological sulfate removal and metal recovery from mine waters** Biotech.Bioeng. **35:** 1-12

Devereux, R.; Delaney, M.; Widdel, F. and Stahl, D.A. (1989) **Natural relationships among sulfate-reducing eubacteria** J. Bacteriol. **171:** 6689-6695

Devereux, R. and Mundfrom, G.W. (1994) **A phylogenetic tree of 16S rRNA sequences from sulfate-reducing bacteria in a sandy marine sediment** Appl. Environ. Microbiol. **60:** 3437-3439

Devereux, R.; Hines, M.E. and Stahl, D.A. (1996) **S Cycling: Characterisation of Natural Communities of Sulfate-Reducing Bacteria by 16S rRNA Sequence Comparisons** Microb. Ecol. **32**: 283-292

Devereux, R. and Mundfrom, G.W. (1994) **A Phylogenetic Tree of 16S rRNA Sequences from Sulfate-Reducing Bacteria in a Sandy Marine Sediment** Appl. Env. Microbiol. **60**: 3437-3439

Dvorak, D.H.; Hedin, R.S.; Edenborn, H.M. and McIntire P.E. (1992) **Treatment of metal contaminated water using bacterial sulfate reduction: results from pilot scale reactors** Biotech. Bioeng. **40:** 609-616

Eckert, K.A.and Kunkel, T.A. (1991) **The fidelity of DNA polymerase and the polymerases used in the PCR.** In: M. J. McPherson, P. Quirke, G. R. Taylor (eds.), Polymerase Chain Reaction I: A Practical Approach. IRL Press, Oxford,

Eichner, C.A.; Erb, R.W.; Timmis, K.N. and Wagner-Döbler, I. (1999) **Thermal gradient gel electrophoresis analysis of bioprotection from pollutant shocks in the activated sludge microbial community** Appl. Environ. Microbiol. **65:** 102-109

Felske, A.; Wolterink, A.; Van Lis, R. and Akkermans, A.D.L. (1998) **Phylogeny of the main bacterial 16S rRNA sequences in Drestse A grassland soils (The Netherlands)** Appl. Environ. Microbiol. **64:** 871-879

Gilfillan J. (2000) **The structure and microbiology of floating sulphide oxidising biofilms.** MSc Thesis, **Rhodes** University.

Gillan, D.C.; Speksnijder, A.G.C.L.; Zwart, G. and de Ridder, C. (1998) **Genetic diversity of the biofilm covering *Montacuta ferruginosa* (Mollusca, Bivalvia) as evaluated by denaturing gradient gel elactrophoresis analysis and cloning of PCR-amplified gene fragments coding for 16S rRNA** Appl. Environ. Microbiol. **64:** 3464-3472

Giovannoni, S.J.; Britschgi, T.; Moyer, C.L. and Field, K.G. (1990) **Genetic diversity in Sargasso Sea bacterioplankton** Nature **345:** 60-63

Godon, J.; Zumstein, E.; Dabert, P.; Habouzit, F. and Moletta, R. (1997) **Molecular Microbial diversity of an Anaerobic digestor as determined by small-subunit rDNA sequence analysis** Appl. Environ. Microbiol. **63:** 2802-2813

Gommers, P.J.F.; Bijleveld, W. and Kuenen, J.G. (1988) **Simultaneous sulfide and acetate oxidation in a denitrifying fluidised bed reactor – start-up and reactor performance** Wat. Res. **22:** 1075-1083

Grobicki, A. and Stuckey, D.C. (1991) **Performance of the anaerobic baffled reactor under steady state and shock loading conditions** Biotech. Bioeng. **37:** 344-350

Hamilton, W.A. (1998) **Bioenergetics of sulfate-reducing bacteria in relation to their environmental impact** Biodegradation **9**: 201-212

Hammack, R.W. and Edenborn, H.M. (1992) **The removal of nickel from mine water using bacterial sulfate reduction** Appl. Microbiol. Biotechnol. **37:** 674-678

Henzen, M.R. and Pieterse M.J. (1978) **Acidic mine drainage in the republic of South Africa** Progress in Water Technology **9:** 981-1000

Herlihy, A.T.; Mills, A.L.; Horneberger, G.M. and Bruckner, A.E. (1987) **The importance of sediment sulfate reduction to the sulfate budget of an impoundment receiving acid mine drainage** Water Resources Research **23:** 287-292

Higgins, D.G., Thompson, J.D. and Gibson, T.J. (1996) **Using CLUSTAL for multiple sequence alignments** Methods Enzymol. **266:** 383-402

Hiligsmann, S., Jacques, P. and Thonart, P. (1998) **Isolation of highly performant sulfate reducers from sulfate-rich environments** Biodegradation **9**: 285-292

Hulshoff Pol, L.W.; Lens, P.N.L.; Stams, A.J.M. and Lettinga, G. (1998) **Anaerobic treatment of sulfate-rich wastewaters** Biodegradation **9**: 213-224

Janssen, A.J.H.; Sleyster, R.; van der Kaa, C.; Jochemsen, A.; Bontsema, J. and Lettinga, G. (1995) **Biological sulphide oxidation in a fed-batch reactor** Biotech. And Bioeng. **47:** 327-333

Janssen, A.J.H.; Ma, S.C.; Lens, P. and Lettinga, G. (1997) **Performance of a sulfide-oxidising expanded-bed reactor supplied with dissolved oxygen** Biotech. And Bioeng. **53:** 32-40

Janssen, A.J.H.; Meijer, S. Bontsema, J. and Lettinga, G. (1998) **Application of the redox potential for controlling a sulfide oxidation bioreactor** Biotech. Bioeng. **60:** 147-155

Janssen, A.J.H.; Lettinga, G. and de Kaizer, A (1999) **Removal of hydrogen sulphide from wastewater and waste gases by biological conversion to elemental sulphur** Physicochemical and engineering aspects **151:** 389-397

Kalmbach, S.; Manz, W. and Szewzyk, U. (1997) **Isolation of new bacterial species from drinking water biofilms and proof of their in situ dominance with highly specific 16S rRNA probes** Appl. Environ. Microbiol. **63:** 4164-4170

Kämpfer, P.; Erhart, R.; Beimfohr, C.; Bohringer, J. and Wagner, M. (1996) **Characterisation of Bacterial Communities from Activated Sludge: Culture-Dependent Numerical Identification Versus In Situ Identification Using Group- and Genus-Specific rRNA-Targeted Oligonucleotide Probes** Microb. Ecol. **32**: 101-121

Kane, M.D.; Poulsen, L.K. and Stahl, D.A. (1993) **Monitoring the Enrichment and Isolation of Sulfate-Reducing Bacteria by Using Oligonucleotide Hybridisation Probes Designed from Environmentally Derived 16S rRNA Sequences** Appl. Env. Microbiol. **59**: 682-686

Kelly, D.P. (1985) **Physiology of the thiobacilli: elucidating the sulphur oxidation pathway** Microbiological Sciences **2:** 105-109

Kwok, S. and Higuchi, R. (1989) **Avoiding false positives with PCR**. Nature **339:** 237

Kolmert, A.; Henrysson, T.; Hallberg, R. and Mattiasson, B. (1997) **Optimisation of sulphide production in an anaerobic continuous biofilm process with sulphate reducing bacteria** Biotechnol. Lett. **19:** 971-975

Krieg, N.R. and Holt, J.G. (Eds) (1989) **Bergey's Manual of systematics bacteriology, Volumes 1 and 3** Williams and Walkins, Baltimore.

Kühl, M. and Jorgensen, B.B. (1992) **Microsensors measurements of sulfate reduction and sulfide oxidation in compact microbial communities of aerobic biofilms** Appl. Environ. Microbiol. **58:** 1164-1174

Lee, N.; Nielsen, P.H.; Andreasen, K.H.; Juretschko, S.; Nielsen, J.L.; Schleifer, K.H. and Wagner, M. (1999) **Combination of fluorescent in situ hybridisation and microautoradiography – a new tool for structure-function analyses in microbial ecology** Appl. Environ. Microbiol. **65:** 1289-1297

Maniatis, T.; Fritsch, F. and Sambrook, J. (1989) **Molecular cloning: a laboratory manual.** Cold Spring Harbour Laboratory, Cold Spring Harbour, New York.

Maree, J.P.; Gerber, A. and Hill, E. (1987) **An integrated process for biological treatment of sulphate-containing industrial effluent** JWPCF **59:** 1069-1074

Maree, J.P. and Hill, E. (1989) **An integrated process for biological treatment of sulfate-containing industrial effluent** J. Water Pollution Control Federation **59:** 1069-1074

Massol-Deya, A.A.; Whallon, J.; Hickey, R.F. and Tiedje, J.M. (1995) **Channel structures in aerobic biofilms of fixed-film reactors treating contaminated groundwater** Appl. Environ. Microbiol. **61:** 769-777

Mau, M. and Timmis, K.H. (1998) **Use of subtractive hybridisation to design habitat-based oligonucleotide probes for investigation of natural bacterial communities** Appl. Environ. Microbiol. **64:** 185-191

McPherson, M.J., *et al.*, Eds. **PCR: A Practical Approach**. Oxford: Oirl Press. 1993

Molipane, N.P. (1998) **Sulphate reduction utilising hydrolysis of complex carbon sources** MSc Thesis, **Rhodes** University.

Muyzer, G.; De Waal, E.C. and Uitterlinden, A.G. (1993) **Profiling of Complex Microbial Populations by Denaturing Gradient Gel Electrophoresis Analysis of Polymerase Chain Reaction-Amplified Genes Coding for 16S rRNA** Appl. Env. Microbiol. **59**: 695-700

Muyzer, G. and Ramsig, N.B. (1995) **Molecular methodes to study the organisation of microbial communities** Wat. Sci. Tech. **32:** 1-9

Muyzer, G.; Teske, A.; Wirsen, C.O. and Jannasch, H.W. (1995) **Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea**

hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments Arch Microbiol. **164**: 165-172

Nealson, K.H. (1997) **Sediment bacteria: Who's there, what are they doing, and what's new?** Annu. Rev. Earth Planet. Sci. **25:** 403-434

Nielsen, P.H.; Andreasen, K.; Lee, N. and Wagner, M. (1999) **Use of Microautoradiography and Fluorescent *In Situ* Hybridisation for Characterisation of Microbial Activity in Activated Sludge** Wat. Sci. Tech. **39**: 1-9

Nielsen, A.T.; Liu, W-T.; Filipe, C.; Grady, L.; Molin S. and Stahl, D.A. (1999) **Identification of a Novel Group of Bacteria in Sludge from a Deteriorated Biological Phosphorus Removal Reactor** Appl. Env. Microbiol. **65**: 1251-1258

Okabe, S.; Matsuda, T.; Satoh, H; Itoh, T. and Watanabe, Y. (1998) **Sulfate reduction and sulfide oxidation in aerobic mixed population biofilms** Wat. Sci. Tech. **37:** 131-138

Okabe, S.; Satoh, H.; Itoh T. and Watanabe, Y. (1999) **Microbial Ecology of Sulfate-Reducing Bacteria in Wastewater Biofilms Analysed by Microelectrodes and Fish (Fluorescent *In Situ* Hybridisation) Technique** Wat. Sci. Tech. **39**: 41-47

Okabe, S.; Satoh, H.; Itoh T.; Satoh, H. and Watanabe, Y. (1999) **Analyses of spatial distridutions of sulfate-reducing bacteria and their activity in aerobic wastewater biofilms** Appl. Environ, Microbiol. **65:** 5107-5116

Postgate, J.R. (1984) **The sulphate-reducing bacteria.** Cambridge University Press, Cambridge.

Power, M.E.; Araujo, J.C.; van der Meer, J.R.; Harms, H. and Wanner, O. (1999) **Monitoring Sulfate-Reducing Bacteria in Heterotrophic Biofilms** Wat.Sci.Tech. **39:** 49-56

Pulles, W.; Howie, D.; Otto, D. and Easton, J. (1995) **A manual on mine water treatment and management practices in South Africa** WRC report no. TT 80/96. Water Research Commision. South Africa

Rabus, R.; Fukui, M.; Wilkes, H. and Widdel, F. (1996) **Degradative Capacities and 16S rRNA-targeted whole-cell hybridisation of sulfate-reducing bacteria in an anaerobic enrichment culture utilising alkylbenzenes from crude oil** Appl. Environ. Microbiol. **62:** 3605-3613

Ramsig, N. (1998) **Molecular biological tools to study population dynamics of sulphur cycle in natural environments and bioreactors (16S rRNA molecular probes, PCR).** TMR Summer School Programme, Wageningen, Netherlands.

Ravenschlag, K.; Sahm, K.; Knoblauch, C.; Jorgensen, B.B. and Amann, R. (2000) **Community structure, cellular rRNA content, and activity of sulfate-reducing bacteria in marine arctic sediments** Appl. Environ. Microbiol. **66:** 3592-3602

Rose, P.D. (1999) Pers. Comm.

Rose, P.D. and Corbett, C. (1999) Pers. Comm.

Rose, P.D.; Maart, B.A.; Dunn, K.M.; Roswell, R.A. and Britz, P. (1996) **High rate algal oxidation ponding for the treatment of tannery effluent** Wat. Sci. Tech. **33:** 219-227

Rose, P.D.; Boschoff, G.A.; van Hille, R.P.; Wallace, L.C.M.; Dunn, K.M. and Duncan, J.R. (1998) **An integrated algal sulphate reducing high rate ponding process for the treatment of acid mine drainage wastewaters.** Biodegradation **9:** 247-257

Sahm, K.; MacGregor, B.J.; Jorgensen, B.B. and Stahl, D.A. (1999) **Sulphate reduction and vertical distribution of sulphate-reducing bacteria quantified by rRNA slot-blot hybridisation in a coastal marine sediment** Environ. Microbiol. **1:** 65-74

Santegoeds, C.M.; Damgaard, L.R.; Hesselink, G.; Zopfi, J.; Lens, P.; Muyzer, G. and de Beer, D. 1999 **Distribution of Sulfate-Reducing and Methanogenic Bacteria in**

Anaerobic Aggregates Determined by Microsensor and Molecular Analyses Appl. Env. Microbiol. **65**: 4618-4629

Santegoeds, C.M.; Ferdelman, T.G.; Muyzer, G. and De Beer, D. (1998) **Structural and Functional Dynamics of Sulfate-reducing Populations in Bacterial Biofilms** Appl. Env. Microbiol. **64**: 3731-3739

Sayler, G.S.; Shields, M.S.; Breen, A.; Tedford, E.T.; Hooper, S.; Sirotkin, K.M. and Davis, J.W. (1985) **Application of DNA-DNA colony hybridisation to the detection of catabolic genotypes in environmental samples** Appl. Environ. Microbiol. **49:** 1295-1303

Schmidt, T.M.; DeLong, E.F. and Pace, N.R. (1991) **Analysis of a picoplankton community by 16S rRNA gene cloning and sequencing** J. Bacteriol. **173:** 4371-4378

Schuppler, M.; Mertens, F.; Schön, G. and Göbel, U.B. (1995) **Moecular characterisation of nocardioform actinomycetes in activated sludge by 16S rRNA analysis** Microbiology **141:** 513-521

Scott, J.R. (1993) **Catchment water quality deterioration as a result of water level recovery in abandoned gold mines on the Eastern and central Witwatersrand** Provisional, Interim report Covering the far East Rand Investigation Area. Volume 1. Institute for Groundwater Studies, University of the Orange Free State, Bloemfontein.

Scott, J.R. (1995) **Flooding of Central East-Rand Gold Mines** Water research commission report 486/1/95. Pretoria

Sekiguchi, Y.; Kamagata, Y.; Nakamura, K.; Ohashi, A. and Harada, H. (1999) **Fluorescence in situ hybridisation using 16S rRNA-targeted oligonucleotides reveals localisation of methanogens and selected uncultured bacteria in mesophilic sludge granules** Appl. Environ. Microbiol. **65:** 1280-1288

Snaidr, J.; Amann, R.; Huber, I.; Ludwig, W. and Schleifer, K-H. (1997) **Phylogenetic analysis and in Situ identification of bacteria in activated sludge** Appl. Environ. Microbiol. **63:** 2884-2896

Stewart, P.S.; Murga, R.; Srinivasan, R. and De Beer, D. (1995) **Biofilm structural heterogeneity visualised by three microscopic methods** Wat. Res. **29:** 2006-2009

Stoffels, M.; Amann, R.; Ludwig, W.; Hekmat, D. and Schleifer, K-H. (1998) **Bacterial community dynamics during start-up of a trickle-bed bioreactor degrading aromatic compounds** Appl. Environ. Microbiol. **64:** 930-939

Teske, A.; Sigalevish, P.; Cohen, Y. and Muyzer, G. (1996) **Molecular Identification of Bacteria from a Coculture by Denaturing Gradient Gel Electrophoresis of 16S**

Ribosomal DNA Fragments as a Tool for Isolation in Pure Cultures Appl. Env. Microbiol. **62**: 4210-4215

Teske, A.; Wawer, C.; Muyzer, G. and Ramsing N.B. (1996) **Distribution of Sulfate_Reducing Bacteria in a Stratified Fjord (Mariager Fjord, Denmark) as Evaluated by Most-Probable-Number Counts and Denaturing Gradient Gel Electrophoresis of PCR-Amplified Ribosomal DNA Fragments** Appl. Env. Microbiol. **62**: 1405-1415

Thompson, J.D.; Gibson , T.J.; Plewniak, F.; Jeanmangin, F. and Higgins D.G. (1997) **The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools** Nucleic Acid Research **24:** 4876-4882

Vetriani, C.; Jannasch, W.H.; MacGregor, B.J.; Stahl, D.A. and Reysenbach, A-L. (1999) **Population structure and phylogenetic characterisation of marine benthic archaea in deep-sea sediments** Appl. Environ. Microbiol. **65:** 4375-4384

Voordouw, G.; Armstrong, S.M.; Reimer, M.F.; Fouts, B.; Telang, A.J.; Shen, Y. and Gevertz, D. (1995) **Characterisation of 16S rRNA genes from oil field microbial communities indicates the presence of a variety of sulfate-reducing, fermantative and sulfide-oxidising bacteria** Appl.Environ.Microbiol. **62:** 1623-1629

Wagner, M.; Amann, R.; Lemmer, H.; Schleifer, K.H. (1993) **Probing activated sludge with oligonucleotides specific for proteobacteria: inadequacy of culture-dependent methods for describing microbial community structure** Appl. Environ. Microbiol. **59:** 1520-1525

Wagner, M.; Erhart, R; Manz, W.; Amann, R. Lemmer, H.; Wedi, D. and Schleifer, K.H. (1994) **Development of an rRNA-targeted oligonucleotide probe specific for the genus *Acinetobacter* and its application for in situ monitoring in activated sludge** Appl. Environ. Microbiol. **60:** 792-800

Ward, D.M.; Weller, R. and Bateson, M.M. (1990) **16S rRNA sequences reveal numerous uncultured microorganisms in a natural community** Nature **345:** 63-65

Welsh, J. and McClelland (1990) **Fingerpriting genome using PCR with arbitrary primers** Nucleic Acids Research **18:** 7213-7218

Widdel, F. (1988) **Microbiology and ecology of sufate and sulfur reducing bacteria**, p. 469-585. In: Zehnder, A.J.B. (Ed.) Biology of anaerobic microorganisms. Wiley. New York.

Widdel, F. and Bak, F. (1992) **Gram-negative mesophilic sulfate-reducing bacteria, p. 3352-3378. In A. Balows, H.G. Trüper, M. Dworkin, W.Harder, and K.H. Schleifer (ed),** The prokaryotes, 2nd ed. Vol IV. Springer-Verlag, New York.

Whittington-Jones, K. (2000) Unpublished PhD thesis. **Sulphide-enhanced hydrolysis of primary sewage sludge: Implications for the bioremediation of AMD.** Goldfields Biotechnology Centre, Rhodes University, Grahamstown, South Africa.

Yu, T. and Bishop, P.L. (1998) **Stratification of microbial metabolic processes and redox potential change in an anaerobic biofilm studied using microelectrodes** Wat. Sci. Tech. **37:** 195-198

APPENDIX A: GROWTH MEDIA

1. MODIFIED POSTGATE'S MEDIUM B

0.3 g	KH_2PO
0.5 g	$FeSO_4.7H_2O$
0.5 g	$MgSO_4.7H_2O$
1 g	$CaSO_4.2H_2O$
0.5 g	NH_4Cl
1 g	Yeast extract
0.2 g	Ascorbic acid
0.1 g	Sodium thioglycolate
15 μM	EDTA
0.001 g	Resazurin
3.5 ml	Sodium lactate

HCl and NaOH were used to adjust the pH in media B to 8.5 before being autoclaved. After sterilisation, pH is near 7.1.

2. LACTATE SRB MEDIUM

Modified from: Atlas (1993)

3.5 g	70% sodium lactate solution
2 g	$MgSO_4.7H_2O$
1 g	NH_4Cl

1 g	Na_2SO_4
1 g	Yeast extract
0.5 g	K_2HPO_4
0.1 g	CaCl.2H

Combine components and make up to 1L with ddH2O. The medium was then autoclaved.

3. LURIA BROTH

Luria broth with ampicillin (per L):

10 g Tryptone

5 g Yeast extract

5 g NaCl

Adjust to pH 7.0 with NaOH

Autoclave and cool to 50°C

Add ampicillin to a final concentration of 100 mg/L

Luria agar (LA) with ampicillin

Add 15 g agar to 1L LB

Autoclave and cool to 50°C before adding ampicillin to a final concentration of 100 mg/L

Luria agar (LA) with ampicillin, IPTG and X-Gal

Make LA with ampicillin plates as described above. When agar is set, spread 10 µL of 100 mg/mL X-Gal (100 mg 5-bromo-4-chloro-3-indolyl-β-D-galactosidase dissolved in 1 mL N,N-dimethylformamide, stored at -20°C) and 12 µL of 200 mg/mL IPTG solution (200 mg IPTG

dissolved in 1 mL ddH_2O) over the plates half an hour before the transformed cells are spread onto the plates.

APPENDIX B: MORPHOLOGY STUDY

1. SAMPLE PREPARATION FOR SCANNING ELECTRON MICROSCOPY

The sample preparation protocol was modified from Cross (1999). The process started with sample fixation in a 2.5% gluteraldehyde solution in 0.1 M phosphate buffer (pH7.0) overnight. The next day the samples were washed twice in 0.1 M phosphate buffer for ten minutes. The samples were then sent through an alcohol dehydration series; 30%, 50%, 70%, 80%, 90% and 100% ethanol for 10 minutes each, followed by two 100% ethanol washes for 10 minutes. Following the alcohol dehydration, samples were critical point dried from liquid CO_2 in a Polaron E3000 Critical Drying Apparatus, and sputter coated in gold with a Polaron E 5100 Sputter Coating Unit, after which the samples were ready for observation in the JEOL JSM 840 Scanning Electron Microscope.

2. GRAM STAIN (Voet and Voet, 1990)

Table 6.1: Protocol followed for Gram stains

Chemical	Time
Grams crystal violet (Saarchem)	30 sec
dH_2O	rinse for 5 sec
Grams iodine (Saarchem)	45 sec
95% ethanol	approximately 4 drops
dH_2O	rinse for 5 sec
Safrinin (Saarchem)	30 sec
dH_2O	rinse for 10 sec

APPENDIX C: Recombinant DNA techniques

1. SPECTROPHOTOMETRIC QUANTIFICATION OF NUCLEIC ACIDS (Maniatis, 1989)

DNA quantification was performed at the wavelengths of 260 nm and 280 nm. The reading at 260nm allows calculation of the concentration of nucleic acid in the sample, where an OD of 1 corresponds to approximately 50u g/mL for double-stranded DNA. The ratio between the readings at 260nm and 280 nm (OD_{260}/OD_{280}) provides an estimate of the purity of the nucleic acid, with pure preparations of DNA giving OD_{260}/OD_{280} values of 1.8. Accurate quantification of the amount of nucleic acid is only possible if there is no protein or phenol contamination of the sample.

2. RESTRICTION DIGESTS:

The reaction consisted of 1μg DNA, 2U enzyme, 2 μL 10X buffer for the enzyme and ddH_2O to bring the volume to 20 μL. The mixture was incubated at 37°C for three hours.

3. PCR AMPLIFICATION OF A FRAGMENT FROM THE 16S RRNA GENE

DNA extracted from the sulfate reducing sludge population was used as target DNA in the polymerase chain reaction to amplify the 16S rRNA gene. Two primers were used for cloning and DGGE analysis. GM5F and 907R (Muyzer *et al* 1995) were used to amplify a 586-bp rDNA fragment, corresponding to positions 341-927 on the 16S rDNA of *E. coli*, with a 40bp GC-clamp attached to the 5'-end of the 907R primer. Sequences and exact positions of primers are given in Appendix E.

PCR amplifications were performed as follows: 100-250 ng of purified genomic DNA, 50 pmol of each of the appropriate primers, 20nmol of each deoxyribonucleoside triphosphate, and 10 ul of 10X PCR buffer + $MgCl_2$ were added to a 0.2 mL tube, where the volume was adjusted to 50 ul using $dddH_2O$. The tubes were then transferred to the Hybaid PCR Sprint Thermal Cycler.

A touchdown program was used in order to increase specificity and prevent the amplification of non-target sequences during the amplification process. Samples were first incubated for 5 min at 98°C for the hot start where the template is denatured completely. The cycle then cooled to 80°C, at which point 1 U of *Taq* DNA polymerase (Roche) was added. The temperature was then lowered to 8°C above the expected annealing temperature (68°C for GM5F/907R) and decreased by 2°C every third cycle until the desired annealing temperature of 60°C (GM5F/907R), where 16

additional cycles were carried out. Denaturing was performed at 94°C for 30 seconds, primer extension at 72°C for 3 minutes and a final extension was carried out at 72°C for 5 minutes. The total number of cycles was 30.

4. CLONING

Cloning was done into the p GEM-T Easy Vector System (Promega). The 586bp fragment was cloned into the vector using the manufacturers protocol, although half the recommended quantities of reagent were used. 150 ng of target DNA was ligated into 50 ng plasmid. The ligations were incubated at 4°C overnight to ensure maximum number of transformants.

The controls included:* Positive control, using the control DNA insert given with the kit. More than 60% of the cells should be white when competent cells with 1 x 10^8 cfu/μ g DNA are transformed.

* Back ground control, with no insert to ensure the plasmid does not re-circularise

* Transformation efficiency, where an insert was added with 2 pg pUC18 vector and no pGEM-T vector. The transformation efficiency should be at least 1 x 10^8 cfu/μ g DNA.

* Negative control, where water is added to the *E. coli* cells with no ligation reaction, and no cells should grow.

The recombinant plasmids were transformed into competent *E. coli* DH5α cells. 2.5 μL of the ligation mix was added to a 50 μL aliquot of competent *E. coli* DH5α cells, which was then heated for 45-50 seconds at 42°C before being cooled in ice for 2 minutes. 950 μL of cold LB medium

was pipetted into the eppendorf and mixed with the cells then incubated at 37°C for 2 hours. 100 µL of the cells in broth were plated onto a LB agar plate with 10 µL of 100 mg/mL 5-bromo-4-chloro-3-indolyl-β-galactosidase (X- Gal) and 12 µL of 200 mg/mL isopropyl-β-D-galactosidase (IPTG) and incubated overnight at 37°C.

5. PREPARATION OF COMPENENT DH5α *E. COLI* CELLS

A 100 µL aliquot of a 5 mL overnight culture of DH5α *E. coli* cells in Luria broth was plated out onto Luria agar. The next morning, the lawn of cells was scraped off the agar and added to the Luria broth. Four 100 mL Erlenmyer flasks containing Luria broth were inoculated with 1.5, 1.0, 0.7 and 0.3 mL of the pre-inoculum and incubated for 3 hours at 37°C on an orbital shaker set at 100 rpm. Once the absorbance (600nm) of the flask inoculated with 1.5 mL of pre-inoculum had reached 0.6-0.8, the flasks were cooled for 5-10 minutes in an ice bath and were then processed separately until the final step. After centrifugation at 5 000 rpm (Beckman centrifuge with JA 14 rotor) for 10 minutes, the supernatant was discarded and the pellet was resuspended in 50 mL RF1 (100 mM KCl, 50 mM $MnCl_2$, 30 mM CH_3COOH; 10 mM $CaCl_2$, 15% m/v glycerol pH 5.8), followed by a 20 minute incubation on ice. The cells were pelleted by a 10 minute centrifugation at 5 000 rpm (Beckman centrifuge with JA 14 rotor). The supernatant was discarded and each of the four pellets was resuspended in 4 mL RF2 (10 mM MOPS, 10 mM KCl, 75 mM $CaCl_2$, 15% m/v glycerol pH 6.8) and the flask content were pooled. Aliquots of 50 µL were pipetted into sterile Eppendorf tubes and stored at -70°C for further use.

6. EASYPREP PLASMID EXTRACTION (Berghammer and Auer, 1993)

1.5 mL overnight culture was spun for 1 minute in a microfuge at 13 000 rpm, and repeated, before resuspending the pellet in 75 µL lysis buffer (10mM Tris-HCl pH8, 1mM EDTA, 15% (w/v) sucrose, 2 mg/mL lysozyme, 0,2 mg/mL pancreatic RNase, 0.1 mg/mL BSA. Stored at -20°C) . The cells were incubated at 37°C for 10-15 min before boiling for 90 seconds then chilling on ice for 60 seconds. The mix was then microfuged at 13 000 rpm for 15 min, after which the supernatant was transferred to a clean eppendorf and stored at -20°C for further work.

7. SEQUENCING REACTIONS

Sequencing was performed using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer). Approximately 200 ng DNA was used per reaction, and half shots were used (Table 6.2)

Table 6.2: Sequencing reactions, as described in the Perkin-Elmer ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit manual (1998)

Reagent	Quantity
Terminator Ready Reaction mix (dye terminators, dUTP*, dCTP, dATP, dITP**, AmpliTaq DNA polymerase, r*Tth* pyrophoshatase, magnesium chloride, buffer)	8 μL
DNA template	100 - 200 ng
-21 M13 Primer (forward)	1.6 pmol
$dddH_2O$	x
Total volume	10 μL

* dITP is used in place of dGTP to minimise band compressions

** dUTP is used in place of dTTP as it results in a better T patterns because dUTP improves incorporation of T terminators.

The reagents were vortexed then spun briefly before the PCR sequencing reaction was started (Table 6.3)

Table 6.3: Sequencing reaction using a Hybaid PCR Sprint Thermal Cycler

	Temperature	Time
Denaturing	96°C	10 seconds
Annealing	50°C	5 seconds
Extension	60°C	4 minutes
Repeat this sequence for 25 cycles, then store at 4°C before purification		

Table 6.4: Precipitation protocol

Reagent	Quantity	Time
3 M Sodium acetate (pH 4.6)	2 μL	
96% Rectified ethanol	50 μL	
PCR product	10 μL	Vortex and keep at room temperature for 1 hour, before spinning down at 13 000rpm for 20 minutes and discarding the supernatant
70% Ethanol	200 μL	Spin for 5 min and discard supernatant

The resultant pellet was air dried overnight before sending off for sequencing.

APPENDIX D: GEL ELECTROPHORESIS

1. AGAROSE GEL ELECTROPHORESIS

Agarose gels were made by dissolving the appropriate amount of agarose in 1X TBE buffer (12.1 g Tris, 0.37 g EDTA and 5.14 g Boric acid made up to 1L and adjusted to pH 8.4 with 1 M HCl) for 0.8 - 1.5 % gels, depending on the fragment size loaded onto the gel. Genomic DNA was run on 0.8% gels, whereas 1.5% gels were used with fragment sizes of 190 bp and 550 bp. The agarose gels were electrophoresed in TBE buffer at a voltage range between 80 - 120 V for approximately 1 hour. In the case of restriction digests, 2% MetaPhor agarose was used as it gives better resolution of smaller fragments. Samples were loaded into the wells with 10% tracking dye. 0.5 μg/mL ethidium bromide added to the gels to allow visualisation of DNA when it was placed on a UV transilluminator, which caused any DNA bound to ethidium bromide to fluoresce.

Tracking Dye III (Maniatis *et al*, 1993)

0.25% bromophenol blue

0.25% xylene cyanol FF

30% glycerol in ddH_2O

Store at 4°C

Markers

The markers were made using Lambda DNA cut either with *Eco*RI and *Hin*d III (resulting in a fragment size range from 0.56 to 24.76 Kb), or with *Pst* (resulting in a fragment size range from

0.3 to 20 Kb). 25 μg λ DNA (Roche) was digested with either 40 U *Pst* or a mixture of 20 U each of *Eco* RI and *Hin*d III in a final volume of 50 μL, using the buffers specified by the maufacturers. The mixture was incubated for 3 hours at 37°C, after which the reaction was stopped by adding 5 μL 0.5 M EDTA and 5 μL tracking dye.

2. DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE)

Reagents for DGGE analysis (Adapted from Myers *et al* 1987)

- 10x TAE electrophoresis buffer

  [0.4 M Tris acetate, 0.01 M EDTA, pH 8-8.3). For 1 litre: 48.45 g Tris base, 3.72 g Na_2EDTA and water to 1 litre. Adjust pH to 8-8.3 with glacial acetic acid (11.5 mL).

- Acrylamide stock solution:

  40% acrylamide (37.5:1 acrylamide: bisacrylamide). For 250 mL, dissolve 100 g electrophoresis- grade acrylamide and 2.7 g bisacrylamide in water to 250 mL.

- Denaturant stock solutions:

  0% denaturant: 6 or 8% acrylamide (depending on fragment size) in TAE buffer

  100% denaturant: 6 or 8% acrylamide, 7M urea, and 40% (v/v) formamide in TAE buffer

  - To deionize formamide: Gently mix 250 mL formamide with approximately 12.5 g Dowex mixed-bed Resin AG 501X8 for 1 hour at room temperature. Filter to remove resin.

- Ammonium persulfate stock (20%)

  For 50 mL: 20 g ammonium persulphate to 50 mL with water.

- TEMED (*N,N,N',N'*-tetramethylethylenediamine)

Preparation of polyacrylamide gels and Polymerization (Adapted from Myers *et al* 1987)

The Bio-Rad Protean II system was assembled as described by the manufacturers, with ethanol and acetone cleaned glass plates. Two solutions of equal volume (12 mL each, which was the volume that filled the void between the glass plates) were prepared from the stock solutions of denaturants and acrylamide to give the desired denaturant concentration range. The chilled solutions were degassed under vacuum for 10 min, before adding 100 μL 20% ammonium persulphate and 10 μL TEMED to each denaturant and gel solution. The solution of higher denaturant concentration was poured into the chamber of the gradient maker which was the first to exit the gradient maker and enter the plate cavity. The solution of lower denaturant concentration was poured into the remaining chamber of the gradient maker. A syringe needle was placed on the end of the tubing and inserted between the glass plates. The needle was kept approximately 10mm above the level of the gel as it was being pumped between the plates by a peristaltic pump. The solution with the highest denaturant concentration was stirred during pouring by a magnetic stirrer, allowing effective mixing of the two denaturant solutions in the chamber. The entire gel was poured in less than 10 minutes, to ensure that polymerization did not occur in the tubing or in the chambers.

After completion of polymerization, the gel was prepared for electrophoresis by removing the comb and heating it to 60°C. The buffer (1X TAE) level was adjusted to be slightly above the level of the wells in the gel. The buffer was pumped by a peristaltic pump, allowing the buffer to circulate from the lower buffer chamber (anode) to the upper buffer chamber (cathode), thereby avoiding increases in buffer pH during electrophoresis.

The wells were flushed with buffer using a syringe in order to remove non-polymerized acrylamide before pre-running the gel for approximately 15-20 min at 120V. The wells were then flushed again before loading the samples, and running the gel at the determined voltage.

Table 6.5: Components of 6% acrylamide DGGE gels in 1X TAE buffer used for separating amplified DNA fragments from the sulfate-reducing sludge population

% Gel	Quantity of reagent	
	Formamide	Urea
0	4%	0.7 M
20	8%	1.4 M
30	12%	2.1 M
35	14%	2.45 M
60	24%	4.2 M
70	28%	4.9 M
80	32%	5.6 M
100	40%	7 M

APPENDIX E: DNA SEQUENCES

1. PRIMER SEQUENCES

GM5F: 5'-CCT ACG GGA GGC AGC AG-3' (position: 341-357; annealing temp = 60°C) (Muyzer *et al*, 1995)

907R: 5'-**CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GCC** GTC AAT TCC TTT RAG TTT-3' position: 907-927; annealing temp = 60°C (Muyzer *et al*, 1995) CG clamp in indicated in bold font.

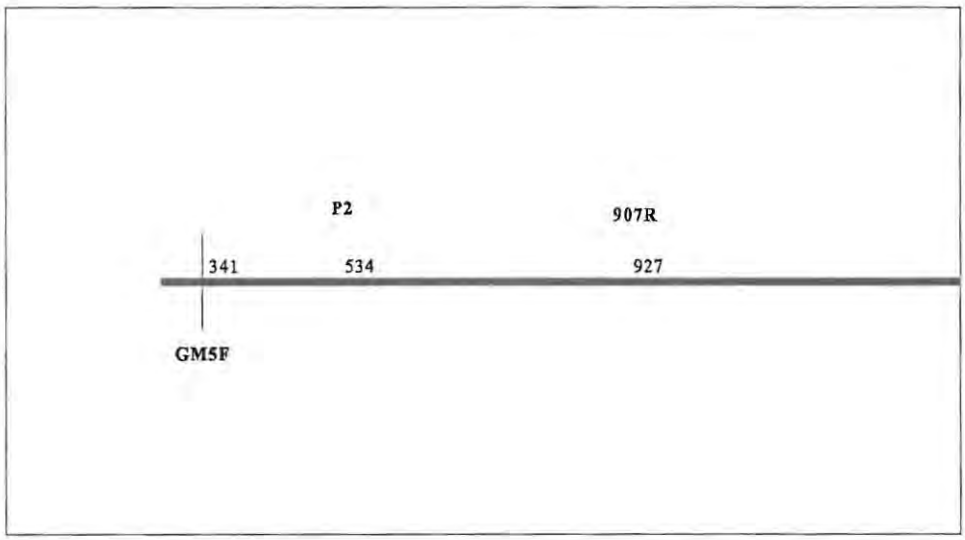


Figure 6.1: Relative positions of primer sets on the 10s rRNA gene from 5' to the 3' end

The primer used in sequencing reactions was the forward primer - **21 M13:** 5' -TGT AAA ACG ACG GCC AGT -3'.

APPENDIX F: STANDARD CURVES

1. SULPHIDE DETERMINATION

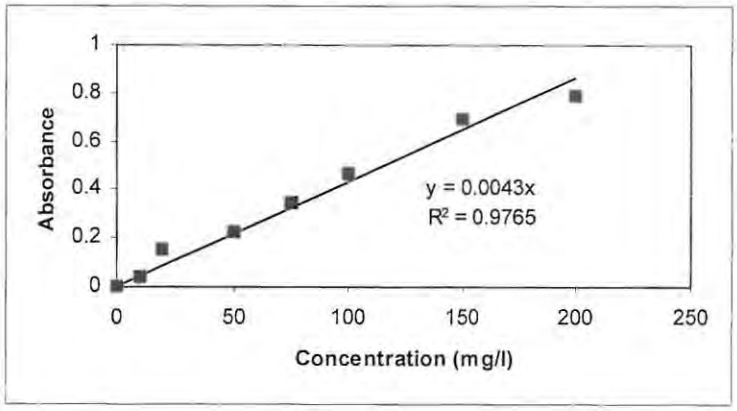


Figure 6.2: Standard curve for sulfide determination, with a dilution factor of 50.

2. SULFATE DETERMINATION

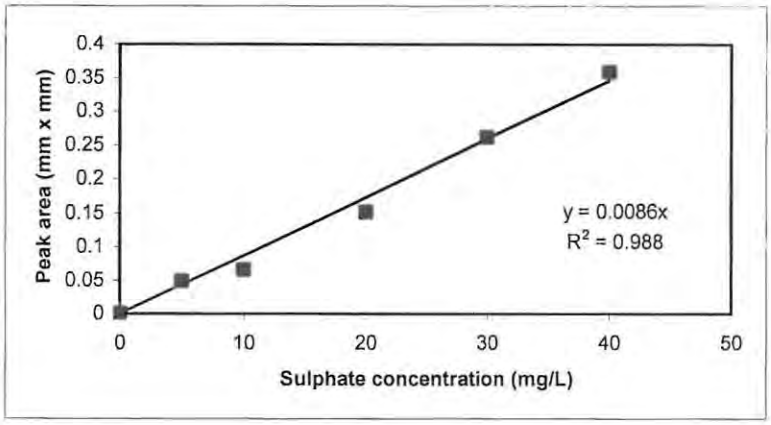


Figure 6.3: Standard curve for sulfate determination

3. SULFUR DETERMINATION

The sulphur standard curve was constructed using standard sulphur flower solutions extracted in acetone overnight and processed as in the protocol described in chapter 3

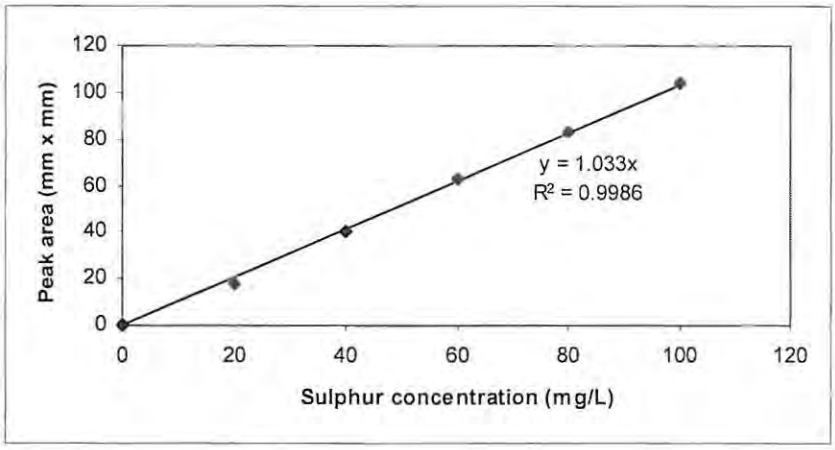


Figure 6.4: Standard curve for sulphur determination.

APPENDIX G: CLUSTAL ALIGNMENT

G-1: Group 1

```
gi_4239810_sulfate-reducing_ba      --------------------------------AATGA-ACGCTGGCGGCGTG
gi_829103_D.amnigenus               ----------------TGATTATGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_1816616_unidentified_sulfat      --------------------------------------------------
gi_18034276_Desulfococcus_mult      ----------AGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_11558175_Desulfococcus_biac      ------------------TCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_6707498_DesulfosarcinaCE105      --------------------------------------------------
gi_3820900deltaproteobacterium      ----------------------------AGAATGA-ACGCTGGCGGCGTG
gi_4581231_Desulfobacterium_ce      --------------------CCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_174347_D.variabilis              -NTTNATTGGAGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_11528067_deltaproteobacteri      ------------------------------------------GGCGGCGTG
gi_18034278_Desulfobacter_curv      ----------AGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_1816610_Desulfobacter_BG23       --------------TTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_1816607_Desulfobacter_BG8        ----------AGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_2388781_Desulfobacter_halot      ------------------------------------------------TG
gi_2501751_Desulfobacter_vibri      ----------------------TGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_1816614_Desulfobacter_BG72       ----------AGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_18034283_Desulfobacter_post      ----------AGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_1816609_DesulfobacteriumBG1      ----------AGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_1816612_Desulfobacterium_BG      ----------AGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_4826627_Desulfobacterium_BS      ---------TAGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_1293665_Desulfobacterium_ni      ------------------------------------------GGCGGCGTG
gi_18034281_Desulfobacterium_v      ------------AGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
```

```
gi_18034280_Desulfobacterium_a  ----------AGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_6453696_eubacterium_BSV76    ------------------------------------------------
gi_174130_Clostridium_litusebu  TTTTTATTTGAGAGTTTGATCCTGGCTCAGGATGA-ACGCTGGCGGCGTG
gi_437756_C.irregularis         ---NNNNNNGAGAGTTTGATCCTGGCTCAGGATGA-ACGCTGGCGGCGTG
gi_15418707_Clostridium_glycol  ---------------AGTATCCTGGCTCAGGATGA-ACGCTGGCGGCGTG
gi_572644C.glycolinum_DSM1288   ----------------------------------A-ACGCTGGCGGCGTG
gi_15072629_Clostridium_glycol  ------------------------------------------------
gi4377977_Clostridium_difficil  ----------AGTTTGATCCTGGCTCAGGATGA-ACGCTGGCGGCGTG
gi_174162_Clostridium_mayombei  --------TGAGAGTTTGATCCTGGCTCAGGATNN-NCGCTGGCGGCGTG
gi_643670_Pyrococcus_furiosus   --------ATTCCGGTTGATCCTGCCGGAGGCCACTGCTATGGGGGTCCG
gi_1235787_T.hydrothermalis     ----------TCCGGTTGATCCTGCCGGAGGCCACTGCTATGGGGGTCCG
gi_11558174_Desulfomicrobium_e  ------------------------------------------------
gi_1490243_DesulfomicrobiumSTL  ----------------------------AATGA-ACGCTGGCGGCGTG
gi_4838529_Desulfomicrobium_hy  ------------------------------------------------
gi_1217950_isolate_Aspo1        ----------------------------AATGA-ACGCTGGCGGCGTG
gi_174332_D.baculatus           -----ACTGGAGAGTTTGATTCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_14625041_DesulfomicrobiumCM  ----------------------------AATGA-AVGCTGGCGGCGTG
gi_1619915_Desulfomicrobium_ap  ----------AGAGTTTGATCCTGNCTCAGAATGA-ACGCTGNCGGCGTG
gi_4581232_Desulfobacterium_ma  ------------------TCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_11558179_Desulfomicrobium_n  ------------------CCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_5114195_Desulfomicrobium'Sc  ------------------------------------------------
gi_5114197_Desulfomicrobium'Sc  ----------------------------AATGA-ACGCTGGCGGCGTG
gi_9650687_Desulfomicrobium_or  ----------------------------AATGA-ACGCTGGCGGCGTG
gi_14422328_Desulfomonas_ovile  ------------------------------------------------
gi_6979515_Desulfomonas_pigra   ----------AGAGTTTGATCCTGGCTCAGATTGA-ACGCTGGCGGCGTG
gi_14269273_Uncultured_bacteri  ------------------------------------------------
gi_13625869_deltaproteobacteri  ----------------------------------AA-ACGCTGGCGGCGTG
gi_2808554_Desulfocapsa_sulfoe  ----------------------------AACGA-ACGTTGGCGGCGTG
I                               ------------------------------------------------
SRB10                           ------------------------------------------------
```

G-2: Group 2

```
gi_6453696_eubacterium_BSV76    ------------------------------------------------
gi_174130_Clostridium_litusebu  TTTTTATTTGAGAGTTTGATCCTGGCTCAGGATGA-ACGCTGGCGGCGTG
gi_437756_C.irregularis         ---NNNNNNGAGAGTTTGATCCTGGCTCAGGATGA-ACGCTGGCGGCGTG
gi_15418707_Clostridium_glycol  ---------------AGTATCCTGGCTCAGGATGA-ACGCTGGCGGCGTG
gi_572644C.glycolinum_DSM1288   ----------------------------------A-ACGCTGGCGGCGTG
gi_15072629_Clostridium_glycol  ------------------------------------------------
gi4377977_Clostridium_difficil  ----------AGTTTGATCCTGGCTCAGGATGA-ACGCTGGCGGCGTG
gi_174162_Clostridium_mayombei  --------TGAGAGTTTGATCCTGGCTCAGGATNN-NCGCTGGCGGCGTG
gi_11558174_Desulfomicrobium_e  ------------------------------------------------
gi_1490243_DesulfomicrobiumSTL  ----------------------------AATGA-ACGCTGGCGGCGTG
gi_4838529_Desulfomicrobium_hy  ------------------------------------------------
gi_1217950_isolate_Aspo1        ----------------------------AATGA-ACGCTGGCGGCGTG
gi_174332_D.baculatus           -----ACTGGAGAGTTTGATTCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_14625041_DesulfomicrobiumCM  ----------------------------AATGA-AVGCTGGCGGCGTG
gi_1619915_Desulfomicrobium_ap  ----------AGAGTTTGATCCTGNCTCAGAATGA-ACGCTGNCGGCGTG
gi_4581232_Desulfobacterium_ma  ------------------TCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_11558179_Desulfomicrobium_n  ------------------CCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_5114195_Desulfomicrobium'Sc  ------------------------------------------------
gi_5114197_Desulfomicrobium'Sc  ----------------------------AATGA-ACGCTGGCGGCGTG
gi_9650687_Desulfomicrobium_or  ----------------------------AATGA-ACGCTGGCGGCGTG
gi_1816616_unidentified_sulfat  ------------------------------------------------
gi_18034276_Desulfococcus_mult  ----------AGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_11558175_Desulfococcus_biac  ------------------TCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_6707498_DesulfosarcinaCE105  ------------------------------------------------
gi_3820900deltaproteobacterium  ----------------------------AGAATGA-ACGCTGGCGGCGTG
gi_4581231_Desulfobacterium_ce  ------------------CCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_174347_D.variabilis          -NTTNATTGGAGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
```

```
gi_11528067_deltaproteobacteri      ----------------------------------------GGCGGCGTG
gi_18034278_Desulfobacter_curv      ----------AGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_1816610_Desulfobacter_BG23       ------------TTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_1816607_Desulfobacter_BG8        ----------AGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_2388781_Desulfobacter_halot      ------------------------------------------------TG
gi_2501751_Desulfobacter_vibri      ----------------------TGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_1816614_Desulfobacter_BG72       ----------AGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_18034283_Desulfobacter_post      ----------AGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_1816609_DesulfobacteriumBG1      ----------AGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_1816612_Desulfobacterium_BG      ----------AGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_4826627_Desulfobacterium_BS      ---------TAGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_1293665_Desulfobacterium_ni      ----------------------------------------GGCGGCGTG
gi_18034281_Desulfobacterium_v      ------------AGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_18034280_Desulfobacterium_a      ------------AGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_4239810_sulfate-reducing_ba      ----------------------------AATGA-ACGCTGGCGGCGTG
gi_829103_D.amnigenus               ----------------TGATTATGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_2808554_Desulfocapsa_sulfoe      ----------------------------AACGA-ACGTTGGCGGCGTG
gi_14269273_Uncultured_bacteri      ------------------------------------------------CAGG
gi_13625869_deltaproteobacteri      ---------------------------------AA-ACGCTGGCGGCGTG
gi_14422328_Desulfomonas_ovile      --------------------------------------------------
gi_6979515_Desulfomonas_pigra       ----------AGAGTTTGATCCTGGCTCAGATTGA-ACGCTGGCGGCGTG
gi_643670_Pyrococcus_furiosus       --------ATTCCGGTTGATCCTGCCGGAGGCCACTGCTATGGGGGTCCG
gi_1235787_T.hydrothermalis         ----------TCCGGTTGATCCTGCCGGAGGCCACTGCTATGGGGGTCCG
II                                  --------------------------------------------------
SRB30                               --------------------------------------------------
```

G-3: Group 3

```
gi_6453696_eubacterium_BSV76        --------------------------------------------------
gi_174130_Clostridium_litusebu      TTTTTATTTGAGAGTTTGATCCTGGCTCAGGATGA-ACGCTGGCGGCGTG
gi_437756_C.irregularis             ---NNNNNNGAGAGTTTGATCCTGGCTCAGGATGA-ACGCTGGCGGCGTG
gi_15418707_Clostridium_glycol      --------------AGTATCCTGGCTCAGGATGA-ACGCTGGCGGCGTG
gi_572644C.glycolinum_DSM1288       ---------------------------------A-ACGCTGGCGGCGTG
gi_15072629_Clostridium_glycol      --------------------------------------------------
gi4377977_Clostridium_difficil      ------------AGTTTGATCCTGGCTCAGGATGA-ACGCTGGCGGCGTG
gi_174162_Clostridium_mayombei      --------TGAGAGTTTGATCCTGGCTCAGGATNN-NCGCTGGCGGCGTG
gi_643670_Pyrococcus_furiosus       --------ATTCCGGTTGATCCTGCCGGAGGCCACTGCTATGGGGGTCCG
gi_1235787_T.hydrothermalis         ----------TCCGGTTGATCCTGCCGGAGGCCACTGCTATGGGGGTCCG
gi_5114195_Desulfomicrobium'Sc      --------------------------------------------------
gi_5114197_Desulfomicrobium'Sc      ----------------------------AATGA-ACGCTGGCGGCGTG
gi_11558174_Desulfomicrobium_e      --------------------------------------------------
gi_1490243_DesulfomicrobiumSTL      ----------------------------AATGA-ACGCTGGCGGCGTG
gi_4838529_Desulfomicrobium_hy      --------------------------------------------------
gi_1217950_isolate_Aspo1            ----------------------------AATGA-ACGCTGGCGGCGTG
gi_11558179_Desulfomicrobium_n      --------------------CCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_4581232_Desulfobacterium_ma      --------------------TCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_14625041_DesulfomicrobiumCM      ----------------------------AATGA-AVGCTGGCGGCGTG
gi_1619915_Desulfomicrobium_ap      ----------AGAGTTTGATCCTGNCTCAGAATGA-ACGCTGNCGGCGTG
gi_9650687_Desulfomicrobium_or      ----------------------------AATGA-ACGCTGGCGGCGTG
gi_174332_D.baculatus               -----ACTGGAGAGTTTGATTCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_14422328_Desulfomonas_ovile      --------------------------------------------------
gi_6979515_Desulfomonas_pigra       ----------AGAGTTTGATCCTGGCTCAGATTGA-ACGCTGGCGGCGTG
gi_14269273_Uncultured_bacteri      ------------------------------------------------CAGG
gi_1816616_unidentified_sulfat      --------------------------------------------------
gi_18034276_Desulfococcus_mult      ----------AGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_11558175_Desulfococcus_biac      --------------------TCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_6707498_DesulfosarcinaCE105      --------------------------------------------------
gi_3820900deltaproteobacterium      ----------------------------AGAATGA-ACGCTGGCGGCGTG
gi_4581231_Desulfobacterium_ce      --------------------CCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_174347_D.variabilis              -NTTNATTGGAGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_11528067_deltaproteobacteri      ----------------------------------------GGCGGCGTG
gi_18034278_Desulfobacter_curv      ----------AGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
```

```
gi_1816610_Desulfobacter_BG23      -------------TTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_1816607_Desulfobacter_BG8       ----------AGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_2388781_Desulfobacter_halot     ---------------------------------------------TG
gi_2501751_Desulfobacter_vibri     --------------------TGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_1816614_Desulfobacter_BG72      ----------AGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_18034283_Desulfobacter_post     ----------AGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_1816609_DesulfobacteriumBG1     ----------AGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_1816612_Desulfobacterium_BG     ----------AGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_4826627_Desulfobacterium_BS     ---------TAGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_1293665_Desulfobacterium_ni     ------------------------------------GGCGGCGTG
gi_18034281_Desulfobacterium_v     -----------AGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_18034280_Desulfobacterium_a     -----------AGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_13625869_deltaproteobacteri     ---------------------------------AA-ACGCTGGCGGCGTG
gi_4239810_sulfate-reducing_ba     ------------------------------AATGA-ACGCTGGCGGCGTG
gi_829103_D.amnigenus              ---------------TGATTATGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_2808554_Desulfocapsa_sulfoe     ------------------------------AACGA-ACGTTGGCGGCGTG
SRB35                              ------------------------------------------------
III                                ------------------------------------------------
```

G-4: Group 4

```
gi_643670_Pyrococcus_furiosus      --------ATTCCGGTTGATCCTGCCGGAGGCCACTGCTATGGGGGTCCG
gi_1235787_T.hydrothermalis        ----------TCCGGTTGATCCTGCCGGAGGCCACTGCTATGGGGGTCCG
gi_6453696_eubacterium_BSV76       ------------------------------------------------
gi_174130_Clostridium_litusebu     TTTTTATTTGAGAGTTTGATCCTGGCTCAGGATGA-ACGCTGGCGGCGTG
gi_437756_C.irregularis            ---NNNNNNGAGAGTTTGATCCTGGCTCAGGATGA-ACGCTGGCGGCGTG
gi_15418707_Clostridium_glycol     --------------AGTATCCTGGCTCAGGATGA-ACGCTGGCGGCGTG
gi_572644C.glycolinum_DSM1288      ---------------------------------A-ACGCTGGCGGCGTG
gi_15072629_Clostridium_glycol     ------------------------------------------------
gi4377977_Clostridium_difficil     -----------AGTTTGATCCTGGCTCAGGATGA-ACGCTGGCGGCGTG
gi_174162_Clostridium_mayombei     --------TGAGAGTTTGATCCTGGCTCAGGATNN-NCGCTGGCGGCGTG
gi_5114195_Desulfomicrobium'Sc     ------------------------------------------------
gi_5114197_Desulfomicrobium'Sc     ------------------------------AATGA-ACGCTGGCGGCGTG
gi_11558174_Desulfomicrobium_e     ------------------------------------------------
gi_1490243_DesulfomicrobiumSTL     ------------------------------AATGA-ACGCTGGCGGCGTG
gi_4838529_Desulfomicrobium_hy     ------------------------------------------------
gi_1217950_isolate_Aspo1           ------------------------------AATGA-ACGCTGGCGGCGTG
gi_11558179_Desulfomicrobium_n     --------------------CCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_4581232_Desulfobacterium_ma     ------------------TCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_14625041_DesulfomicrobiumCM     ------------------------------AATGA-AVGCTGGCGGCGTG
gi_1619915_Desulfomicrobium_ap     ----------AGAGTTTGATCCTGNCTCAGAATGA-ACGCTGNCGGCGTG
gi_9650687_Desulfomicrobium_or     ------------------------------AATGA-ACGCTGGCGGCGTG
gi_174332_D.baculatus              -----ACTGGAGAGTTTGATTCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_14422328_Desulfomonas_ovile     ------------------------------------------------
gi_6979515_Desulfomonas_pigra      ----------AGAGTTTGATCCTGGCTCAGATTGA-ACGCTGGCGGCGTG
gi_14269273_Uncultured_bacteri     ------------------------------------------------CAGG
gi_1816616_unidentified_sulfat     ------------------------------------------------
gi_18034276_Desulfococcus_mult     ----------AGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_11558175_Desulfococcus_biac     --------------------TCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_6707498_DesulfosarcinaCE105     ------------------------------------------------
gi_3820900deltaproteobacterium     ------------------------------AGAATGA-ACGCTGGCGGCGTG
gi_4581231_Desulfobacterium_ce     --------------------CCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_174347_D.variabilis             -NTTNATTGGAGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_11528067_deltaproteobacteri     ---------------------------------------GGCGGCGTG
gi_18034278_Desulfobacter_curv     ----------AGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_1816610_Desulfobacter_BG23      -------------TTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_1816607_Desulfobacter_BG8       ----------AGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_2388781_Desulfobacter_halot     ---------------------------------------------TG
gi_2501751_Desulfobacter_vibri     --------------------TGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_1816614_Desulfobacter_BG72      ----------AGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
```

```
gi_18034283_Desulfobacter_post      ----------AGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_1816609_DesulfobacteriumBG1      ----------AGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_1816612_Desulfobacterium_BG      ----------AGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_4826627_Desulfobacterium_BS      ---------TAGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_1293665_Desulfobacterium_ni      ------------------------------------------GGCGGCGTG
gi_18034281_Desulfobacterium_v      ------------AGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_18034280_Desulfobacterium_a      ------------AGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_13625869_deltaproteobacteri      ---------------------------------AA-ACGCTGGCGGCGTG
gi_4239810_sulfate-reducing_ba      ------------------------------AATGA-ACGCTGGCGGCGTG
gi_829103_D.amnigenus               ----------------TGATTATGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_2808554_Desulfocapsa_sulfoe      ------------------------------AACGA-ACGTTGGCGGCGTG
SRB31                               ------------------------------------------------
SRB29                               ------------------------------------------------
SRB24                               ------------------------------------------------
```

G-5: Group 5

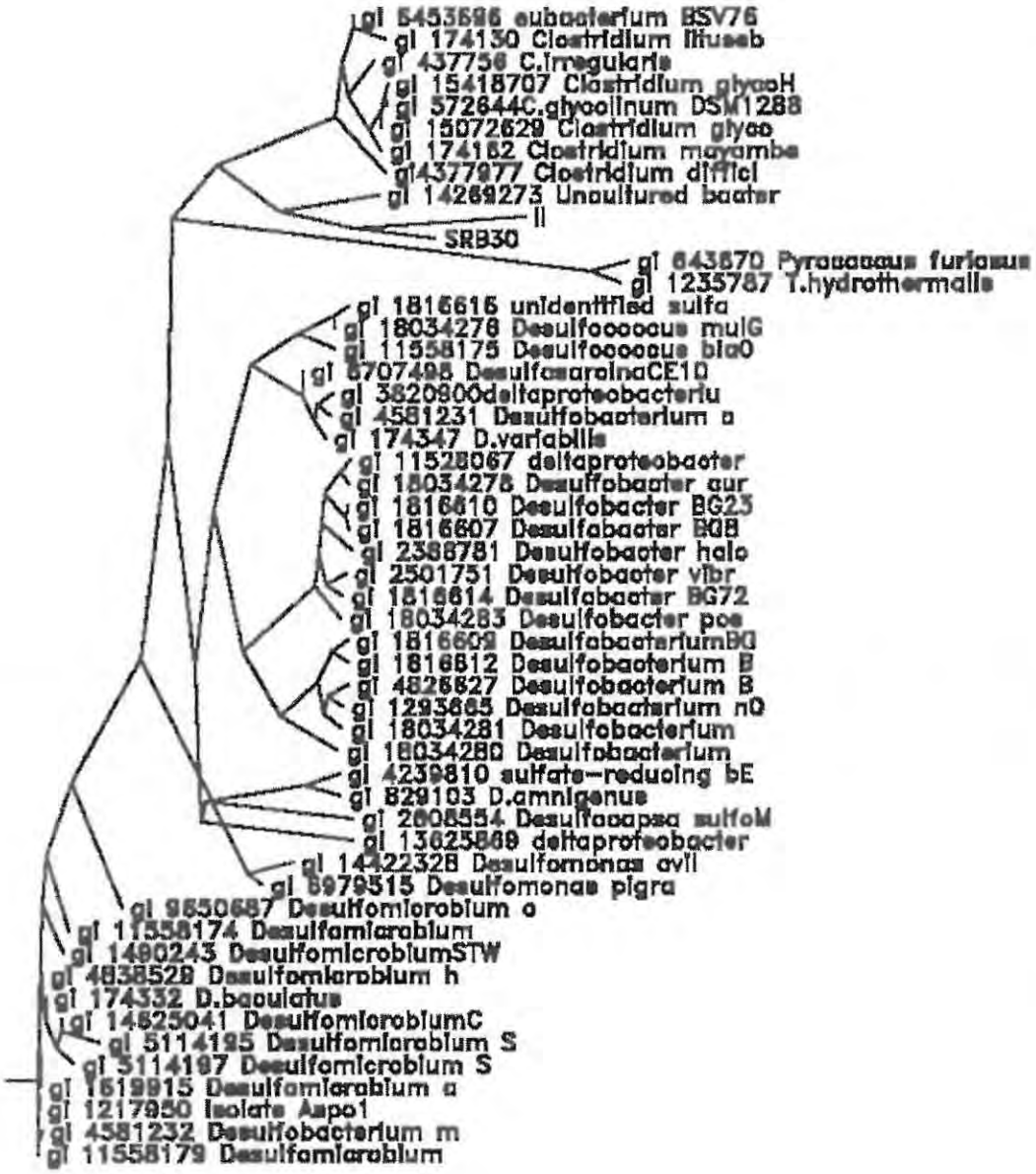
```
gi_6453696_eubacterium_BSV76        ------------------------------------------------
gi_174130_Clostridium_litusebu      TTTTTATTTGAGAGTTTGATCCTGGCTCAGGATGA-ACGCTGGCGGCGTG
gi_437756_C.irregularis             ---NNNNNNGAGAGTTTGATCCTGGCTCAGGATGA-ACGCTGGCGGCGTG
gi_15418707_Clostridium_glycol      --------------AGTATCCTGGCTCAGGATGA-ACGCTGGCGGCGTG
gi_572644C.glycolinum_DSM1288       ---------------------------------A-ACGCTGGCGGCGTG
gi_15072629_Clostridium_glycol      ------------------------------------------------
gi4377977_Clostridium_difficil      ------------AGTTTGATCCTGGCTCAGGATGA-ACGCTGGCGGCGTG
gi_174162_Clostridium_mayombei      --------TGAGAGTTTGATCCTGGCTCAGGATNN-NCGCTGGCGGCGTG
gi_11558174_Desulfomicrobium_e      ------------------------------------------------
gi_4838529_Desulfomicrobium_hy      ------------------------------------------------
gi_1490243_DesulfomicrobiumSTL      ------------------------------AATGA-ACGCTGGCGGCGTG
gi_1217950_isolate_Aspol            ------------------------------AATGA-ACGCTGGCGGCGTG
gi_11558179_Desulfomicrobium_n      --------------------CCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_14625041_DesulfomicrobiumCM      ------------------------------AATGA-AVGCTGGCGGCGTG
gi_4581232_Desulfobacterium_ma      -------------------TCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_5114197_Desulfomicrobium'Sc      ------------------------------AATGA-ACGCTGGCGGCGTG
gi_5114195_Desulfomicrobium'Sc      ------------------------------------------------
SRB26                               ------------------------------------------------
gi_174332_D.baculatus               -----ACTGGAGAGTTTGATTCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_1619915_Desulfomicrobium_ap      ----------AGAGTTTGATCCTGNCTCAGAATGA-ACGCTGNCGGCGTG
gi_9650687_Desulfomicrobium_or      ------------------------------AATGA-ACGCTGGCGGCGTG
gi_14422328_Desulfomonas_ovile      ------------------------------------------------
gi_6979515_Desulfomonas_pigra       ----------AGAGTTTGATCCTGGCTCAGATTGA-ACGCTGGCGGCGTG
gi_1816616_unidentified_sulfat      ------------------------------------------------
gi_18034276_Desulfococcus_mult      ----------AGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_11558175_Desulfococcus_biac      -------------------TCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_6707498_DesulfosarcinaCE105      ------------------------------------------------
gi_3820900deltaproteobacterium      ------------------------------AGAATGA-ACGCTGGCGGCGTG
gi_4581231_Desulfobacterium_ce      --------------------CCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_174347_D.variabilis              -NTTNATTGGAGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_11528067_deltaproteobacteri      ------------------------------------------GGCGGCGTG
gi_18034278_Desulfobacter_curv      ----------AGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_1816610_Desulfobacter_BG23       --------------TTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_1816607_Desulfobacter_BG8        ----------AGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_2388781_Desulfobacter_halot      ------------------------------------------------TG
gi_2501751_Desulfobacter_vibri      ----------------------TGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_1816614_Desulfobacter_BG72       ----------AGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_18034283_Desulfobacter_post      ----------AGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_1816609_DesulfobacteriumBG1      ----------AGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_1816612_Desulfobacterium_BG      ----------AGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_4826627_Desulfobacterium_BS      ---------TAGAGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_1293665_Desulfobacterium_ni      ------------------------------------------GGCGGCGTG
gi_18034281_Desulfobacterium_v      ------------AGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_18034280_Desulfobacterium_a      ------------AGTTTGATCCTGGCTCAGAATGA-ACGCTGGCGGCGTG
```

```
gi_4239810_sulfate-reducing_ba      ----------------------------AATGA-ACGCTGGCGGCGTG
gi_829103_D.amnigenus               ---------------TGATTATGGCTCAGAATGA-ACGCTGGCGGCGTG
gi_2808554_Desulfocapsa_sulfoe      ----------------------------AACGA-ACGTTGGCGGCGTG
gi_14269273_Uncultured_bacteri      ----------------------------------------------CAGG
gi_13625869_deltaproteobacteri      ---------------------------------AA-ACGCTGGCGGCGTG
gi_643670_Pyrococcus_furiosus       --------ATTCCGGTTGATCCTGCCGGAGGCCACTGCTATGGGGGTCCG
gi_1235787_T.hydrothermalis         ----------TCCGGTTGATCCTGCCGGAGGCCACTGCTATGGGGGTCCG
```

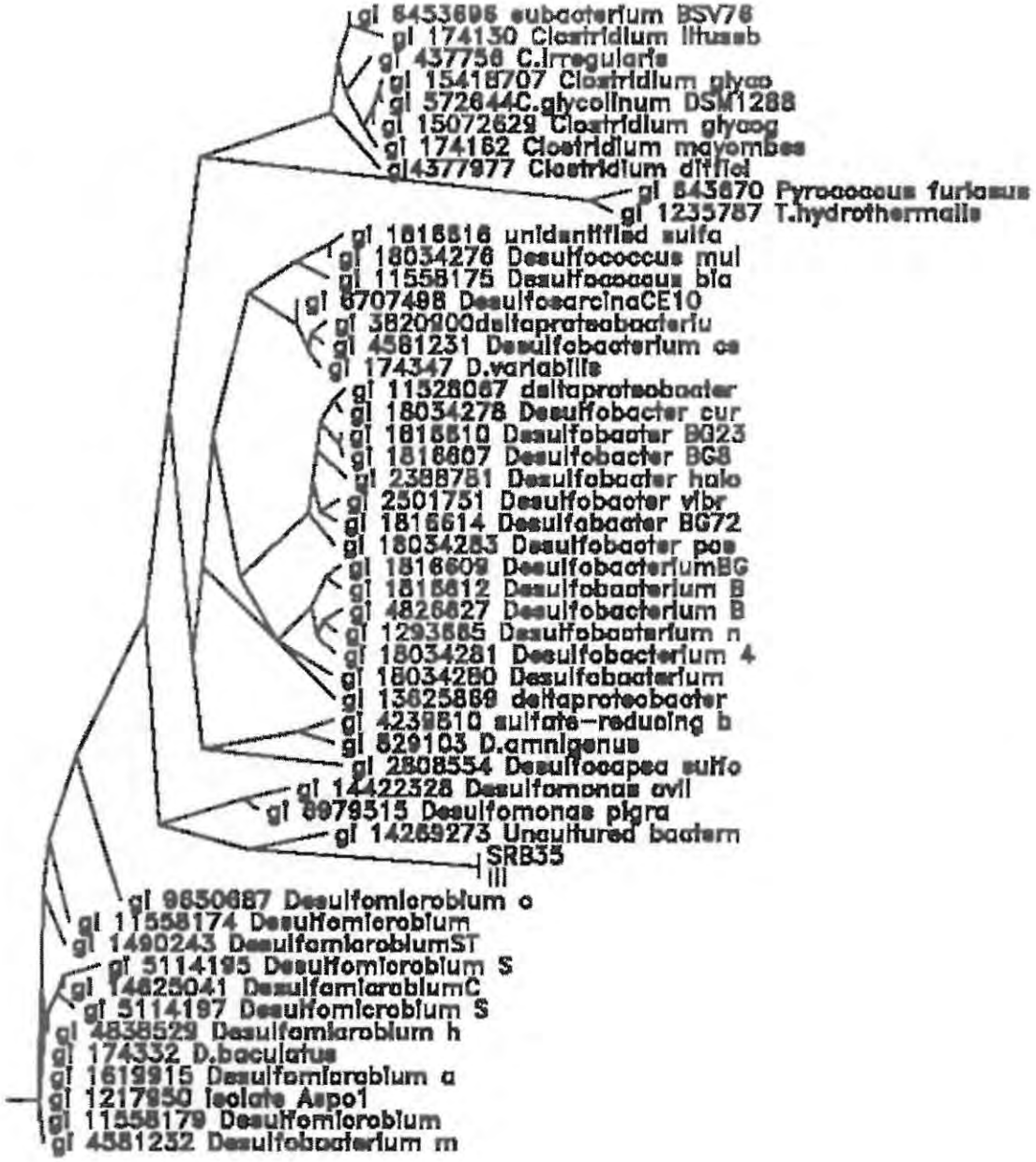
APPENDIX H: PHYLOGENETIC TREES

H-1: Group 1

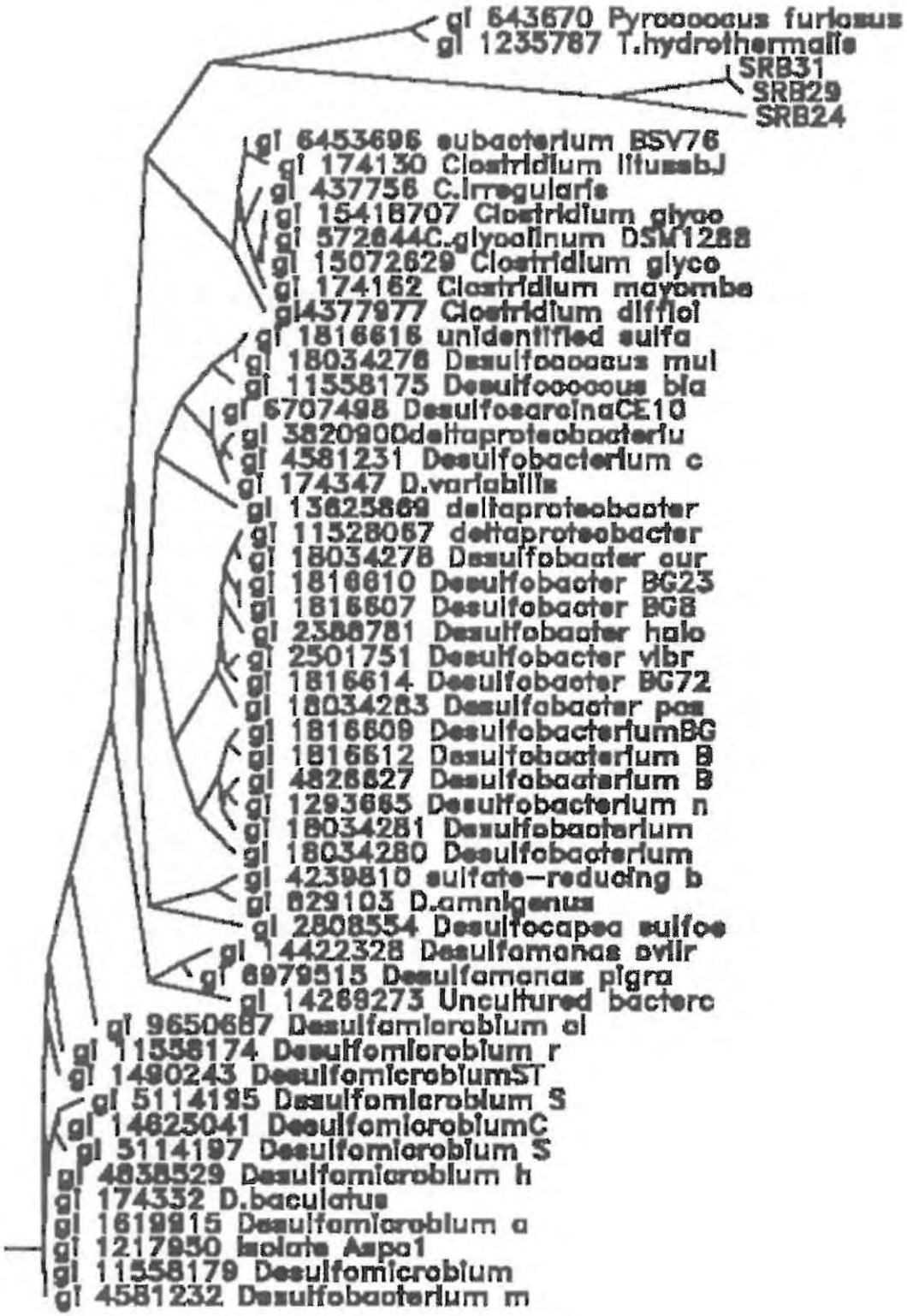
H-2: Group 2


gi 5453596 eubacterium BSV76
gi 174130 Clostridium ltiuseb
gi 437758 C.irregularis
gi 15418707 Clostridium glycoH
gi 572644C.glycolinum DSM1288
gi 15072629 Clostridium glyco
gi 174162 Clostridium mayombe
gi4377977 Clostridium difficl
gi 14269273 Unoultured bacter
ll
SRB30
gi 643670 Pyrococcus furiosus
gi 1235787 T.hydrothermalis
gi 1816616 unidentified sulfa
gi 18034278 Desulfococcus multG
gi 11558175 Desulfococcus biaO
gi 6707498 DesulfosarcinaCE10
gi 3820900deltaproteobacteriu
gi 4581231 Desulfobacterium a
gi 174347 D.variabilis
gi 11528067 deltaproteobacter
gi 18034276 Desulfobacter cur
gi 1816610 Desulfobacter BG23
gi 1816607 Desulfobacter BG8
gi 2388781 Desulfobacter halo
gi 2501751 Desulfobacter vibr
gi 1816614 Desulfobacter BG72
gi 18034283 Desulfobacter pos
gi 1816609 DesulfobacteriumBG
gi 1816612 Desulfobacterium B
gi 4826627 Desulfobacterium B
gi 1293865 Desulfobacterium nO
gi 18034281 Desulfobacterium
gi 18034280 Desulfobacterium
gi 4239810 sulfate-reducing bE
gi 829103 D.amnigenus
gi 2808554 Desulfocapsa sulfoM
gi 13625869 deltaproteobacter
gi 14422328 Desulfomonas avil
gi 6979515 Desulfomonas pigra
gi 9650687 Desulfomicrobium o
gi 11558174 Desulfomicrobium
gi 1490243 DesulfomicrobiumSTW
gi 4838528 Desulfomicrobium h
gi 174332 D.baculatus
gi 14825041 DesulfomicrobiumC
gi 5114195 Desulfomicrobium S
gi 5114197 Desulfomicrobium S
gi 1619915 Desulfomicrobium a
gi 1217950 Isolate Aspo1
gi 4581232 Desulfobacterium m
gi 11558179 Desulfomicrobium

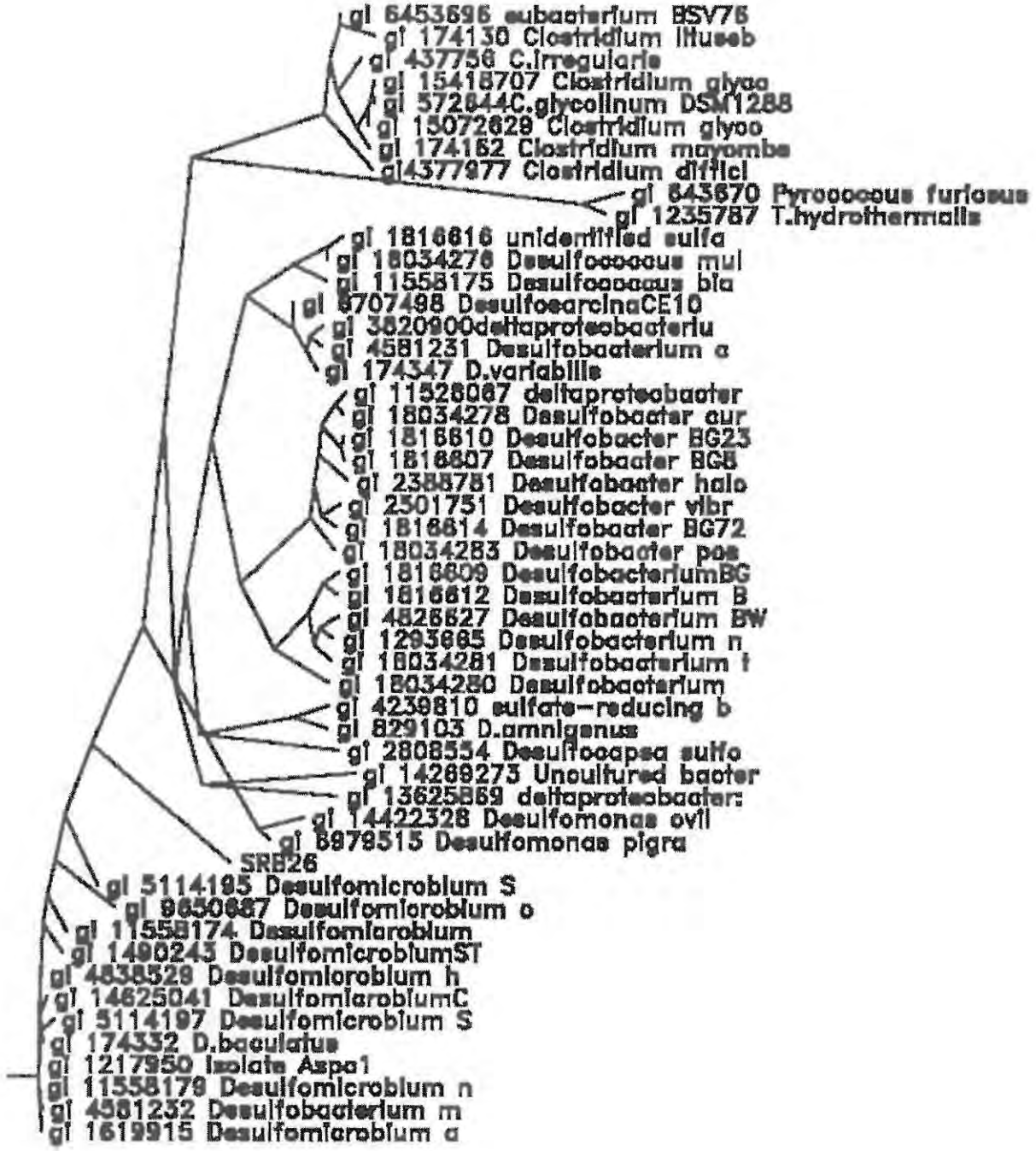
H-3: Group 3


gi 6453696 eubacterium BSV76
gi 174130 Clostridium lituseb
gi 437758 C.irregularis
gi 15418707 Clostridium glyco
gi 572644C.glycolinum DSM1288
gi 15072629 Clostridium glycog
gi 174162 Clostridium mayombee
gi4377977 Clostridium difficil
gi 843670 Pyrococcus furiosus
gi 1235787 T.hydrothermalis
gi 1816816 unidentified sulfa
gi 18034276 Desulfococcus mul
gi 11558175 Desulfococcus bia
gi 6707498 DesulfosarcinaCE10
gi 3820900deltaproteobacteriu
gi 4581231 Desulfobacterium ca
gi 174347 D.variabilis
gi 11528067 deltaproteobacter
gi 18034278 Desulfobacter cur
gi 1816810 Desulfobacter BG23
gi 1816807 Desulfobacter BG8
gi 2388781 Desulfobacter halo
gi 2501751 Desulfobacter vibr
gi 1816814 Desulfobacter BG72
gi 18034283 Desulfobacter pos
gi 1816809 DesulfobacteriumBG
gi 1816812 Desulfobacterium B
gi 4826827 Desulfobacterium B
gi 1293685 Desulfobacterium n
gi 18034281 Desulfobacterium 4
gi 18034280 Desulfobacterium
gi 13625869 deltaproteobacter
gi 4239810 sulfate-reducing b
gi 529103 D.aminigenus
gi 2808554 Desulfocapsa sulfo
gi 14422328 Desulfomonas avil
gi 6979515 Desulfomonas pigra
gi 14269273 Uncultured bacterm
SRB35
gi 9850687 Desulfomicrobium o
gi 11558174 Desulfomicrobium
gi 1490243 DesulfomicrobiumST
gi 5114195 Desulfomicrobium S
gi 14825041 DesulfomicrobiumC
gi 5114197 Desulfomicrobium S
gi 4838529 Desulfomicrobium h
gi 174332 D.baculatus
gi 1619915 Desulfomicrobium a
gi 1217950 Isolate Aspo1
gi 11558179 Desulfomicrobium
gi 4581232 Desulfobacterium m

H-4: Group 4


gi 643670 Pyrococcus furiosus
gi 1235787 T.hydrothermalis
SRB31
SRB29
SRB24
gi 6453696 eubacterium BSV76
gi 174130 Clostridium ljungdahlii
gi 437756 C.irregularis
gi 15418707 Clostridium glyco
gi 572644C.glycolinum DSM1288
gi 15072629 Clostridium glyco
gi 174162 Clostridium mayombe
gi4377977 Clostridium difficil
gi 1816616 unidentified sulfa
gi 18034276 Desulfococcus mul
gi 11558175 Desulfococcus bia
gi 6707498 DesulfosarcinaCE10
gi 3820900deltaproteobacteriu
gi 4581231 Desulfobacterium c
gi 174347 D.variabilis
gi 13625869 deltaproteobacter
gi 11528067 deltaproteobacter
gi 18034278 Desulfobacter cur
gi 1816610 Desulfobacter BG23
gi 1816607 Desulfobacter BG8
gi 2388781 Desulfobacter halo
gi 2501751 Desulfobacter vibr
gi 1816614 Desulfobacter BG72
gi 18034283 Desulfobacter pos
gi 1816609 DesulfobacteriumBG
gi 1816612 Desulfobacterium B
gi 4826827 Desulfobacterium B
gi 1293665 Desulfobacterium n
gi 18034281 Desulfobacterium
gi 18034280 Desulfobacterium
gi 4239810 sulfate-reducing b
gi 829103 D.amnigenus
gi 2808554 Desulfocapsa sulfo
gi 14422328 Desulfomonas pigr
gi 6979515 Desulfomonas pigra
gi 14269273 Uncultured bacter
gi 9650687 Desulfomicrobium ol
gi 11558174 Desulfomicrobium r
gi 1490243 DesulfomicrobiumST
gi 5114195 Desulfomicrobium S
gi 14625041 DesulfomicrobiumC
gi 5114197 Desulfomicrobium S
gi 4838529 Desulfomicrobium h
gi 174332 D.baculatus
gi 1619915 Desulfomicrobium a
gi 1217950 Isolate Aspa1
gi 11558179 Desulfomicrobium
gi 4581232 Desulfobacterium m

H-5: Group 5


gi 6453696 eubacterium BSV76
gi 174130 Clostridium lituseb
gi 437756 C.irregularie
gi 15418707 Clostridium glyco
gi 572644C.glycolinum DSM1288
gi 15072629 Clostridium glyco
gi 174152 Clostridium mayombe
gi4377977 Clostridium difficl
gi 643670 Pyrococcus furiosus
gi 1235787 T.hydrothermalis
gi 1816816 unidentified sulfa
gi 18034276 Desulfococcus mul
gi 11558175 Desulfococcus bia
gi 6707498 DesulfosarcinaCE10
gi 3820900deltaproteobacteriu
gi 4581231 Desulfobacterium a
gi 174347 D.variabilis
gi 11526067 deltaproteobacter
gi 18034278 Desulfobacter cur
gi 1816810 Desulfobacter BG23
gi 1816807 Desulfobacter BG8
gi 2388781 Desulfobacter halo
gi 2501751 Desulfobacter vibr
gi 1816814 Desulfobacter BG72
gi 18034283 Desulfobacter pos
gi 1816809 DesulfobacteriumBG
gi 1816812 Desulfobacterium B
gi 4826627 Desulfobacterium BW
gi 1293665 Desulfobacterium n
gi 18034281 Desulfobacterium t
gi 18034280 Desulfobacterium
gi 4239810 sulfate-reducing b
gi 829103 D.amnigenus
gi 2808554 Desulfocapsa sulfo
gi 14269273 Uncultured bacter
gi 13625869 deltaproteobacter
gi 14422328 Desulfomonas ovil
gi 8979515 Desulfomonas pigra
SRB26
gi 5114195 Desulfomicrobium S
gi 9650687 Desulfomicrobium o
gi 11558174 Desulfomicrobium
gi 1490243 DesulfomicrobiumST
gi 4838529 Desulfomicrobium h
gi 14625041 DesulfomicrobiumC
gi 5114197 Desulfomicrobium S
gi 174332 D.baculatus
gi 1217950 Isolate Aspa1
gi 11558179 Desulfomicrobium n
gi 4581232 Desulfobacterium m
gi 1619915 Desulfomicrobium a

H-6: Group 6