The Microbial Ecology of Sulphidogenic Lignocellulose Degradation

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By

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ABSTRACT

Acid mine drainage is a well known environmental pollutant, not only in South Africa, but throughout the world, and the use of microbial processes in the treatment of these wastes has been the subject of investigation over past decades. Lignocellulose packed-bed reactors have been used in passive treatment systems, and, although effective initially, they show early decline in performance while the packing material remains largely un-utilized. Little is known about this phenomenon which remains a severe constraint in the development of efficient passive mine water treatment systems. It has been proposed that the degradation pathways of the complex lignocellulose substrate may be limited in some way in these systems during the manifestation of this effect.

This study has addressed the problem using a molecular microbial ecology methodology in an attempt to relate trophic functions of the microbial population to the physico-chemical data of the system.

A field–scale lignocellulose packed-bed reactor located at Vryheid Coronation Colliery (Northern Kwa-Zulu Natal province, South Africa) was monitored for six years and the results showed the classic profile of performance decline related to a slowdown in sulphate reduction and alkalinity production. The reactor was decommissioned, comprehensive samples were collected along the depth profile and the microbial populations investigated by means of 16S rRNA gene methodology.

The population was found to include cellulolytic *Clostridia* spp., *Cytophaga/Flavobacter/Bacteroidetes, Sphingomonadaceae* and as yet uncultured microorganisms related to microbiota identified in the rumen and termite gut. These are all known to be involved as primary fermenters of cellulose. *Desulphosporosinus* was present as sulphate reducer. A comparison of substrata sampling and population distribution suggested that spatial and temporal gradients within the system may become established over the course of its operation. Based on these findings, a laboratory-scale

reactor was constructed to simulate the performance of the packed-bed reactor under controlled experimental conditions.

The laboratory-scale reactor was operated for 273 days and showed comparable performance to that in the field in both biomolecular and physico-chemical data. Clearly defined trophic niches were observed. These results suggested that a sequence of events does occur in lignocellulose degradation over time. Based on the spatial and temporal column studies, a descriptive model was proposed to account for these events.

It was found that fermentative organisms predominate in the inlet zone of the system using easily extractable compounds from the wood, thus providing feedstock for sulphate reduction occurring in the succeeding compartments. Production of sulphide and alkalinity appears to be involved in the enhancement of lignin degradation and this, in turn, appears to enhance access to the cellulose fraction. However, once the readily extractables are exhausted, the decline in sulphide and alkalinity production leads inexorably to a decline in the overall performance of the system as a sulphate reducing unit operation.

These observations led to the proposal that with the addition of a limited amount of a readily available carbon source, such as molasses, in the initial zone of the the reactor, the ongoing generation of sulphide would be sustained and this in turn would sustain the microbial attack on the lignocellulose complex. This proposal was tested in scale-up studies and positive results indicate that the descriptive model may, to some extent, provide an account of events occurring in these systems. The work on sustaining lignocellulose degradation through the maintenance of sulphate reduction in the initial stages of the reactor flow path has led to the development of the Degrading Packed-bed Reactor concept and that, has subsequently been successfully evaluated in the field.

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A	-	Adenine
ALD	-	Anoxic Limestone Drains
AMD	-	Acid Mine Drainage
APS	-	Alkalinity Producing Systems
bp	-	Base pair
BLAST	-	Basic Local Alignment Search Tool
carbon/d	-	Carbon per day
C	-	Cytosine
cDNA	-	complementary DNA
CFB	-	*Cytophaga/Flavobacter/Bacteroides*
Cy3	-	Cynine dye
CLSM	-	Confocal Laser Scanning Microscopy
CTAB	-	Cetyltrimethylammonium bromide
$C_6H_{12}O_6$	-	Glucose
CO_2	-	Carbon dioxide
CH_4	-	Methane
Da	-	Dalton
DACST	-	Department of Arts, Culture, Science and Technology
$dddH_2O$	-	Triple Distlled Water
DGGE	-	Deanaturing Gradient Gel Electrophoresis
DNA	-	Deoxynucleic Acid
DNS	-	Dinitrosalicylic acid
dNTP	-	Deoxynucleoside triphosphate
DPBR	-	Degrading Packed Bed Reactor
EBG	-	Environmental Biotechnology Group
EDTA	-	Ethylene Diamine tetra-Acetic Acid
EPS	-	Extracellular Polysaccharide
EtOH	-	Ethanol
IC	-	Ion chromatography
IF	-	Innovation Fund
FA	-	Fluorescent Antibodies
FISH	-	Fluorescent *In Situ* Hybridization
FOB	-	Iron-Oxidizing Bacteria
G	-	Guanine
ID	-	Internal diameter
IDT	-	Integrated DNA Technology
HPLC	-	High Performance Liquid Chromatography
HSRU	-	Hydrogen Sulphide Removal Unit
LSRU	-	Large Sulphate Reducing Unit
LB	-	Luria broth
MME	-	Molecular Microbial Ecology
MPN	-	Most Probable Number
ng	-	nanogram
nm	-	nanomitre
NCBI	-	National Centre for Biotechnology Information

N-J	-	Neighbor Joining
OD	-	Optical Density
O_2	-	Oxygen
PCA	-	Prochatechuate
PCR	-	Polymerase Chain Reaction
PDA	-	Photo – diode array
PHD	-	Pulles, Howard and de Lange
RAPS	-	Reducing and Alkalinity Producing System
RISCs	-	Reduced Inorganic Sulfur Compounds
RP	-	Redox poising
RT	-	Reverse Transcriptase
RT-PCR	-	Reverse Transcriptase PCR
rDNA	-	Ribosomal Deoxynucleic Acid
rRNA	-	Ribosomal Ribonucleic Acid
SA	-	South Adit
Sec	-	Second
SAPS	-	Successive Alkalinity Producing System
SEM	-	Scanning Electron Microscopy
SSCP	-	Single Strand Conformation Polymorphism
SOB	-	Sulphur-Oxidizing Bacteria
SRB	-	Sulphate Reducing Bacteria
SRP	-	Sulphate Reducing Prokaryotes
SPARRO	-	Slurry Precipitation Recycle Reverse Osmosis
SRU	-	Sulphate Reducing Unit
T	-	Thymine
TE	-	Tris EDTA buffer
TEM	-	Transmission Electron Microscopy
T_m	-	Melting temperature
SSRU	-	Small Sulphate Reducing Units
TGGE	-	Temperature Gradient Gel Electrophoresis
T-RFLP	-	Terminal Restriction Fragment Length Polymorphism
UPVC	-	Unplastisized poly vinylidine chloride
VCC	-	Vryheid Coronation Colliery
VFA	-	Volatile Fatty Acids
WA	-	West Adit
ZnS	-	Sphalerite
ZnAc	-	Zinc Acetate

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CHAPTER ONE

Complex Organic Carbon Utilization in Mine Wastewater Treatment

1.1. Acid Mine Drainage Wastewaters

Both biological and physico-chemical processes are initiated by the geophysical trauma of mining operations and, among a range of effects, give rise to pyrite oxidation, acid formation and heavy metal solubilization (Johnson, 1995). These acid mine drainage (AMD) leachates, produced in mine drainage wastewaters and waste dump runoff, cause severe aquatic habitat degradation downstream of the mining operation and represent a major environmental hazard associated with mining activities, both in South Africa and throughout the world (Pulles *et al.*, 2001).

Metal sulphides are rapidly oxidized in a number of chemical and microbial processes (Davidson *et al.,* 1989; Evangelou, 1995; Gazea *et al.,* 1996). The resulting hydrogen ion production lowers the pH and thereby releases soluble metal and sulphate ions into the environment. Under the acidic conditions resulting from AMD, the oxidation of pyrite proceeds by the following reaction (Davidson *et al.,* 1989).

$$FeS_2 + 14\,Fe^{3+} + 8\,H_2O \rightarrow 15\,Fe^{2+} + 2\,SO_4^{2-} + 16\,H^+ \qquad (1)$$

According to this reaction, every mole of pyrite is converted to 16 moles of hydrogen thus causing a substantial lowering in pH, known as proton acidity. In addition to the above process, dissolved iron, and other metals, may also lower the pH of the effluent resulting in mineral acidity (Johnson *et al.,* 2002). Effluents from coal and metal mines often also contain reduced inorganic sulphur compounds (RISCs). According to Johnson *et al.* (2002), RISCs arise during the oxidative dissolution of sulphide minerals and the following reaction illustrates their contribution to acidity in mine waters.

$$6\,S_2O_3^{2-} + 2O_2 + 2H_2O \rightarrow 3SO_4^{2-} + 4H^+ \qquad (2)$$

The oxidative dissolution of sulphide minerals is a process that is greatly accelerated by chemolithotrophic bacteria (Johnson 1995; Schippers *et al.*, 1996), and Figure 1.1 illustrates the involvement of microorganisms in these systems.

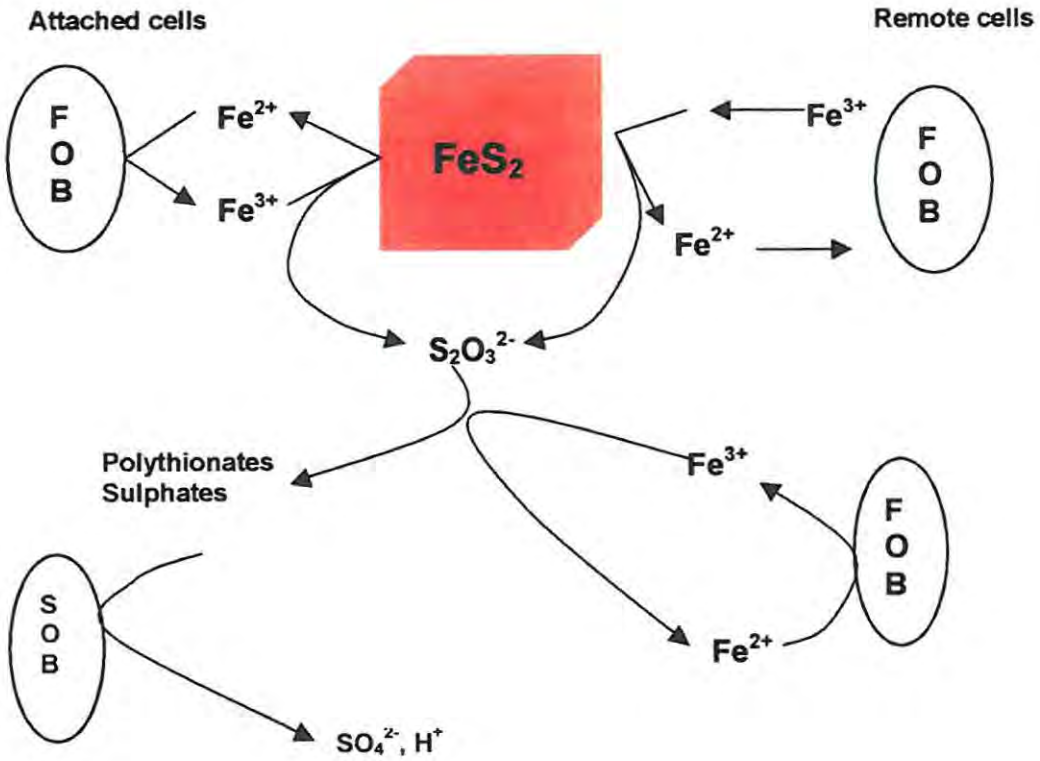


Fig. 1.1: The oxidative dissolution of pyrite by acidophilic bacteria. FOB denotes iron-oxidizing bacteria and SOB sulphur-oxidizing bacteria. (After Schippers *et al.*, 1996 and Johnson *et al.*, 2002).

The cycling of iron between its ferrous and ferric ionic states initiates the mineral oxidative dissolution of pyrite. Iron is reduced on reaction with pyrite and the reduced iron is subsequently oxidized by microorganisms that use the resultant energy for their metabolism. Therefore, knowledge of the organisms

involved in the process and their selection and control could be applied in the bioremediation of AMD.

The chemical reactions involved in generating AMD may have profound impacts on the receiving ecosystem. Firstly, the ferric precipitate that is associated with AMD destroys vegetation by covering the soil layer and clogging the clay particles. Secondly, AMD has also been responsible for a marked decline in aquatic species and ecosystem diversity. The toxicity of the high concentrations of heavy metals and low pH has been linked to the total elimination of certain fish species in some aquatic ecosystems (Barnes and Romberg, 1986; Banks *et al.,* 1997). In South Africa the majority of gold and coal mines are located in the most populated and industrialized areas of the country where the demands on fresh water supplies for domestic and industrial use are both very high. The discharge of AMD, whether from underground workings, or as leachate from slimes and waste dumps, passes into the natural drainage systems and profoundly affects the quality of water downstream. Consequently, it has long been argued that the AMD problem must be viewed in the context of the critical balance existing between the present and future demands for available water (Hanze and Pieterse, 1978).

Although mines consume only 4.2 per cent of total water usage in South Africa, the discharge of AMD into fresh water resources may lower the quality of this water to the point where it is no longer useable. It has been estimated that when mine pumping operations finally cease, filling of the voids would occur within a decade, and subsequent long term surface flows of AMD may last for decades or possibly even centuries (Scott, 1995).

1.1.1. Treating Acid Mine Drainage

A considerable body of research has been undertaken to deal with the AMD problem and both physico-chemical and biological systems have been investigated. The subject has been comprehensively reviewed by Gazea *et al.* (1996) and Pol *et al.* (2001).

1.1.1.1. Physico-chemical Treatment Process

Physico-chemical treatment processes are usually implemented during the operational phase of a mine when human and financial resources are available for operational control and maintenance of plant, or during the closure or rehabilitation phases when large volumes of water require treatment. Several physico-chemical processes have been applied, which include Slurry Precipitation Recycle Reverse Osmosis (SPARRO), lime neutralization (Thompson, 1980; Barnes and Romberg, 1986; Maree *et al.*, 1996; Elliot *et al.*, 1998) and limestone addition which is more cost effective than lime. Limestone (Maree *et al.*, 1992) dissolves when the feed water pH is below 7, however, it was noted by Sasowsky *et al.* (2000) that metal precipitates coat the limestone surface resulting in an armoring which inhibits further reactivity. The coating of limestone has been prevented either by the use of fluidized bed reactors (Cole *et al.*, 2001) or by anoxic limestone drains (Younger, 1998; Johnson, 2001). Other physico-chemical methods employed for remediation of AMD include pyrite microencapsulation to control pyrite oxidation (Evangelou, 2001) and a membrane filtration system (Schoeman and Steyn, 2001).

1.1.1.2. Biological Treatment Process

A biological treatment system has many advantages over the traditional physico-chemical approach. Biological systems have been used in both active and passive treatment configurations (Gazea *et al.*, 1996; Harris and Ragusa, 2000). These systems are distinguished mainly in terms of their operational and management requirements. In passive treatment systems the addition of chemicals and energy consuming treatment operations are practically eliminated. Furthermore, the operation and maintenance requirements are considerably lower than those of active treatment systems (Gazea *et al.*, 1996; Dill *et al.*, 2001; Van der Merwe, 2002). Pulles *et al.* (2001) have defined passive treatment as:

"A water treatment system that utilizes naturally available energy sources such as topographical gradient, microbial metabolic energy, photosynthesis and chemical energy and requires regular but infrequent maintenance to operate successfully over its design life."

Younger (2001) emphasized that the effective passive treatment of AMD should be based on anoxic geochemical procedures incorporating carbonate dissolution in the presence of bacterial sulphate reduction and in the absence of Fe^{3+}. This kind of approach would secure bicarbonate alkalinity, raised pH and, through sulphate reduction, affect the immobilization of toxic metals by precipitations of metallic sulphides (Younger *et al.*, 1997).

Present passive treatment technologies are typically modeled after wetlands and other naturally occurring processes, with modifications of methods directed towards meeting specific treatment goals (Younger *et al.*, 1997). Early research included investigation of natural *Sphagnum* sp. wetlands. It was noted by Tuttle *et al.*, (1969) and Wieder and Lang (1982) that the use of wetlands was able to improve the water quality of AMD without causing other damaging effects to the ecosystem. Although this process had its limitations, they were reportedly able to raise the pH and lower the iron concentration.

These studies had a pronounced impact on the development of other passive treatment approaches.

In recent years, a variety of passive treatment systems have been developed that do not require continuous chemical inputs but rather take advantage of naturally occurring chemical and biological processes to purify contaminated mine water (Johnson, 1995; Gazea *et al.*, 1996; Banks *et al.*, 1997; Younger *et al.*, 1997; Younger, 1998). The primary passive technologies include constructed wetlands (aerobic and anaerobic), alkalinity producing systems, anoxic limestone drains, limestone ponds and channels or a combination of the above. Constructed wetlands, both aerobic and anaerobic, mimic the filtration process, thereby removing contaminants from wastewater. Aerobic wetlands are artificially created shallow ponds with dense populations of reeds. By providing numerous points for precipitation and settlement, the main pollutants, such as Fe, Mn and Al, are effectively removed. They were found to be the most suitable for waters with the pH close to 7, and containing sufficient alkalinity to neutralize any acidity that might be produced within the wetland during the hydrolysis of ferric iron (Hedin *et al.*, 1994; Zipper and Jage, 2001). Anaerobic wetlands, on the other hand, consist of a thick layer of compost, which creates the anoxic environment supporting the establishment of sulphate reducing bacteria, known for their ability to remove sulphate and elevate alkalinity.

A particular advance in the design of anaerobic wetlands was the development of the so-called vertical-flow systems, which have now been successfully operating throughout the world for a number of years. (Zipper and Jage, 2001). These systems have been given a variety of names such as: Successive Alkalinity Producing System (SAPS), (Kepler and McCleary, 1994). Alkalinity Producing Systems (APS) (Scousen *et al.*, 2000), and Reducing and Alkalinity Producing System (RAPS), which is the presently accepted and used name (Zipper and Jage, 2001). RAPS combines the treatment mechanisms of anaerobic wetlands and anoxic limestone drains (ALD) in an attempt to compensate for the limitations of both (Kepler and

McCleary, 1994). In this system, the water is first passed through a bed of compost, then through the layer of limestone from which it is drained off, back to the bed of compost, thereby ensuring the continuous flooding of the organic matter. Younger (2001) reported that RAPS generates five times more alkalinity per unit and volume than the compost wetland alone. However, both the classical anaerobic wetland and RAPS require post-treatment of the aerobic wetland to ensure the removal of any dissolved iron.

Regardless of the design, the effectiveness of the passive treatment system depends largely on the rate at which the alkalinity can be produced by microorganisms and the efficiency can be improved by the addition of suitable organic substrates, which would promote the growth of alkalinity-producing microorganisms (Kalin *et al.,* 1991; Johnson, 1995; Younger *et al.,* 1997). Over the years a variety of lignocellulosic materials have been proven to encourage the growth of bacteria under sulphidogenic conditions. These include straw and hay, spent mushroom compost, sawdust, oak chips, paper and peat (Wakao *et al.*, 1979; Dvorak and McIntire, 1992; Bechard *et al.*, 1993; Pareek *et al.*, 1998; and Chang *et al.*, 2000).

1.2. Sulphate Reducing Microbial Consortia

Anaerobic microbial processes may play an important role in the efficient purification of waste streams contaminated with organic compounds. The low solubility of molecular oxygen in water is a limiting factor in the application of aerobic processes and this may result in an incomplete breakdown of the organic compounds present (Hanze and Harremones, 1983). Moreover, anaerobic processes do not incur the costs of aeration associated with aerobic reactors and there is no danger of aerosols containing cells being released from reactor vessels.

The sulphate reducing bacteria (SRB), also recently termed sulphate-reducing prokaryotes (SRP) (Wagner *et al.*, 1998), occur among the microorganisms involved in anaerobic degradation of organic substances. From the point of view of modern rRNA-based taxonomy, SRB form a heterogeneous group, however, their general geobiological importance justifies grouping them in a functional microbial association. Figure 1.2 illustrates the similarity the relationship between *Bacteria* and *Archaea*.

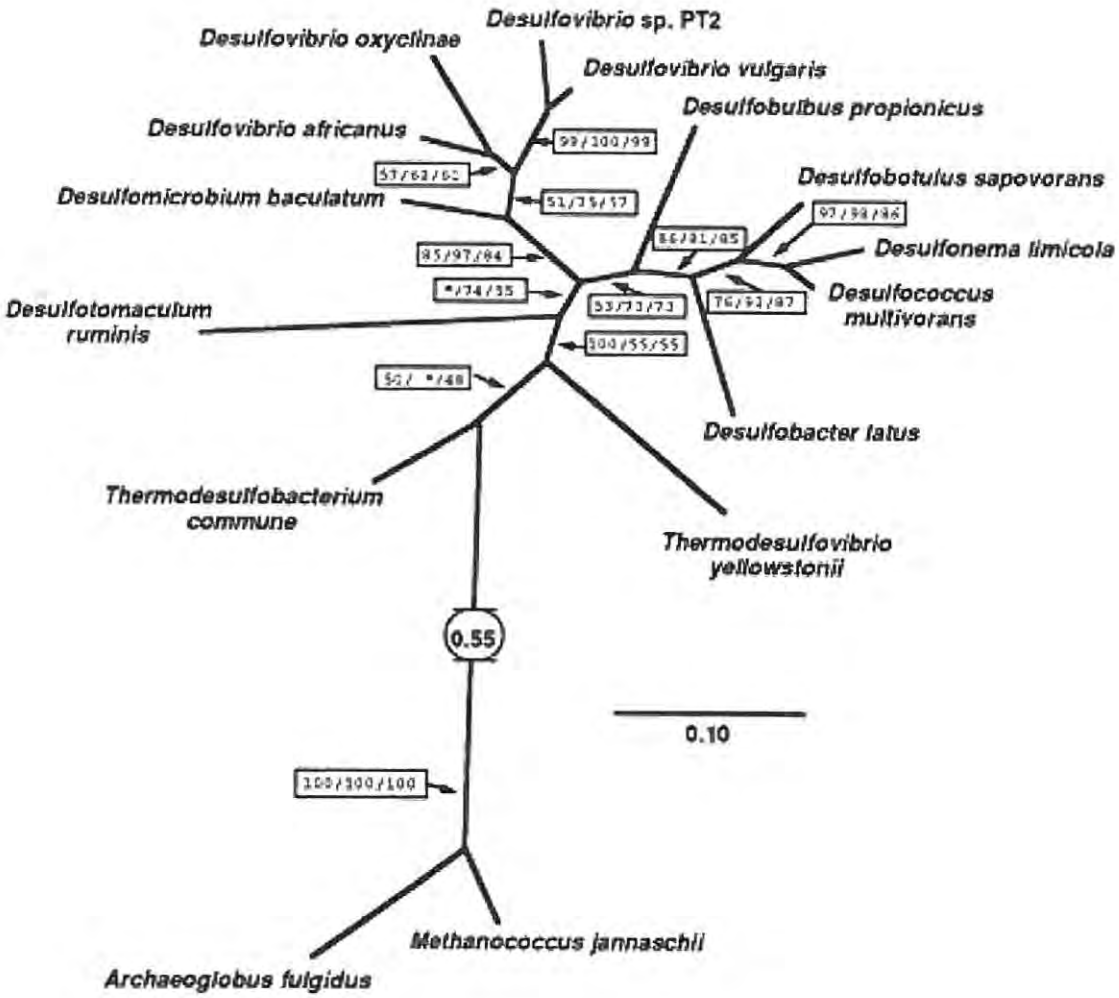


Figure 1.2: Phylogenetic relationship of *Archaea* and *Bacteria* inferred from comparisons of 16S rRNA genetic relationships. (After Wagner *et al.*, 1998).

The unifying physiological trait of these microorganisms is their ability to use sulphate as terminal electron acceptor for the dissimilation of organic compounds (Widdel and Pfenning, 1984; Wagner *et al.*, 1998). The most significant aspect of their metabolism is the production of H_2S, which inhibits the growth of many aerobic microorganisms because it is a very strong reducing agent (Postgate, 1984). According to Widdel and Pfenning, (1984) during dissimilatory sulphate reduction, the sulphate ion is utilized as an oxidant for the degradation of organic matter.

$$2CH_2O + SO_4^{2-} \rightarrow H_2S + 2HCO_3^- \qquad (3)$$

From the above equation it is evident that for each mole of reduced sulphate, an equivalent amount of sulphide is formed (Widdel and Pfenning, 1984).

1.2.1. Occurrence of Sulphate Reducing Bacteria

SRB are among the most ancient physiological groups of bacteria. The results of their activity have been detected as far back as 2900 to 3900 million years ago, when the oxygen concentration in the Earth's atmosphere was still very low (Vasconcelos and McKenzie, 2000, Shen *et al.*,2001).

Today they occupy ecological niches where access to oxygen is limited, e.g. waterlogged soils like rice fields and tidal zones, bottom sediments; marine and freshwater, subterranean water systems and oil-bearing formations. SRB predominate at depths of 1–5 cm from the surface in the bottom sediments of tidal zones, with the maximum population occurring at a depth of 3–5 cm, but they may be found as deep as 1100 m below the ocean surface (Radchenko and Tashirev, 1991). This occurrence is mainly related to the distribution of organic matter, which comes from the land or diffuses from the bottom sediments. Not all SRB are strict anaerobes. Labrenz *et al.* (2000)

succeeded in retrieving an aerotolerant community of SRB from a flooded mine with Pb-Zn ore deposits. It was demonstrated that the specific, aerotolerant assemblage of SRB was able to form a pure precipitate of sphalerite (ZnS) (Vasconcelos and McKenzie, 2000). There is no doubt that this reaction has important implications for biotechnological applications of these organisms. Knowledge of the ability of SRB to utilize various organic matters is an important criterion for the identification and assessment of the prospects for the use of these microorganisms in wastewater purification. SRB are known to catalyze the final stages of the anaerobic mineralization of organic compounds. They depend on other organisms in mixed consortia to convert complex organics into simpler compounds, which may serve as their substrates (Widdel and Pfenning, 1984; Raskin *et al.*, 1996; DeLong, 2000). This relationship may be symbiotic or syntrophic. Dubillier *et al.* (2001) described syntrophic cycling of oxidized and reduced sulphur compounds between the sulphate-reducing and sulphide-oxidizing bacteria living in a symbiotic relationship with a marine oligochaete worm. Sulphate-reducing bacteria in that relationship produce sulphide that serves as an electron donor for the autotrophic fixation of CO_2 by sulphide-oxidizing bacteria. The electron donors for sulphate-reducing bacteria are supplied here by the worm during anaerobic metabolism. External electron donors such as organic matter or H_2 are taken up from the sediment or hydrothermal vents. Another fascinating syntrophic interaction was recently described by DeLong (2000), in which methane-derived carbon may be efficiently transferred from methane-oxidizers to sulphate-reducing bacteria probably via acetate. Figure 1.3 shows the syntrophic association between the methane-consuming archaea and the sulphate reducing bacteria.

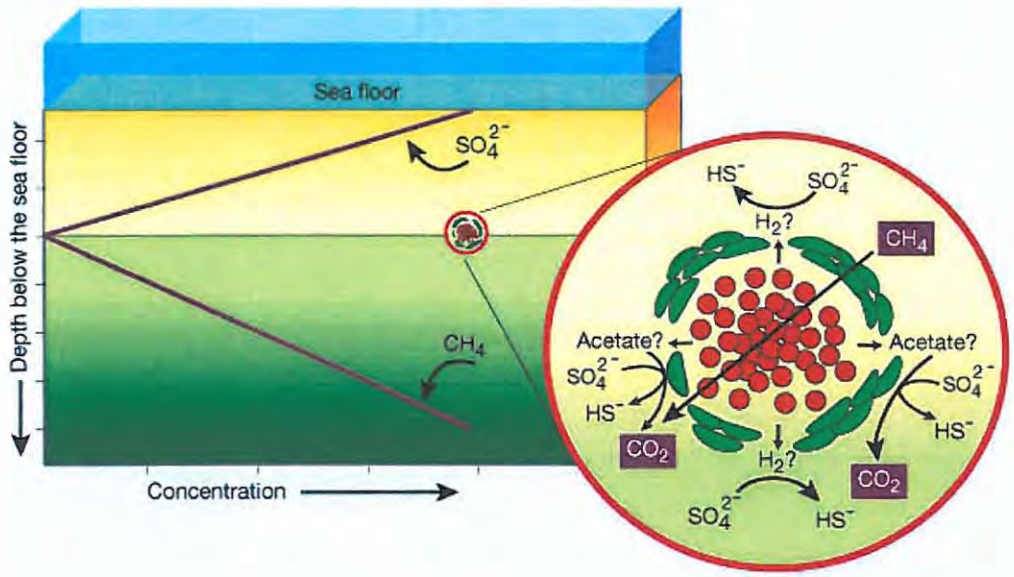


Figure 1.3: The sulphate-methane interface below the sea floor showing microorganisms spatially organized in tight clusters. (After DeLong, 2000).

1.2.2. The role of SRB in Degradation of Wood and Lignin

The Sulphate Reducing Bacteria are known to produce sulphide and alkalinity. Madikane (2002) demonstrated that the combination of sulphide and alkalinity at room temperature accelerates the release of extractives and wood aromatics, and that these compounds may be used as carbon and electron donors by SRB (Bak and Widdel, 1986; Harms *et al.*, 1999; Meckenstock *et al.*, 2000).

1.3. Lignocellulose

The term lignocellulose refers to lignin, cellulose and hemicellulose compounds, which are the basic structural components of plants.

1.3.1. Cellulose

Despite the great differences in composition and anatomical structure of cell walls across plant taxa, cellulose represents the highest percentage of plant biomass, ranging from 35 to 50% of plant dry weight. (Crawford, 1981). In a few cases, like cotton bolls, cellulose is present in a nearly pure state. In other cases the cellulose fibers are embedded in a matrix of other structural biopolymers, mainly hemicellulose and lignin. Although these matrix interactions vary with the plant cell type and with the maturity of the plant, they are a dominant structural feature that limits the rate and extent of utilization of whole, untreated biomass materials (Lynd *et al.*, 2002).

Chemically, cellulose occurs in a semicrystalline state with a molecular weight of approximately 10^6 Da. It appears as an unbranched polymer of several thousand D-glucose units joined via β-1, 4-glycosidic bonds. Individual chains are linked together by hydrogen bonds (Crawford, 1981). The repeated β-1-4 bonds in cellulose (Figure 1.4) require only a limited number of enzymes to degrade this material.

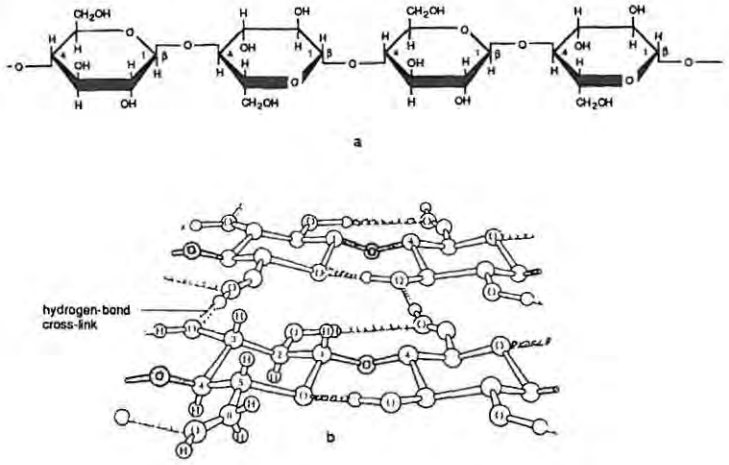


Figure 1.4: Cellulose structure showing β-1, 4 glycosidic bonds and hydrogen links between two parallel chains of glucose. (After Paul and Clark, 1989).

1.3.2. Hemicellulose

Hemicellulose, another group of polysaccharide compounds occurring in plants, represents 20 to 35% of plant dry weight (Crawford, 1981). The main component of hemicellulose is xylan. Chemically, it is a polymer of xylose linked by β-1, 4-bonds with branched structures including L-arabinose, uronic acids, and hexoses such as D-glucose, D-mannose, and D-galactose. The degree of branching depends on the source of the hemicellulose (Biely, 1985). An interesting characteristic of some xylans, mainly those of hardwoods, is the fact that they are acetylated. According to Biely (1985), birch xylan contains over 1 mol of acetic acid per mol of xylose. The presence of acetyl groups makes the xylan more soluble in water. Figure 1.5 illustrates the most important structural characteristics of xylan. Hemicelluloses bind bundles of cellulose fibrils to form microfibrils, which enhance the stability of the cell wall. They also cross-link with lignin, creating a complex web of bonds, which provide structural strength, and challenge microbial degradation (Biely, 1985).

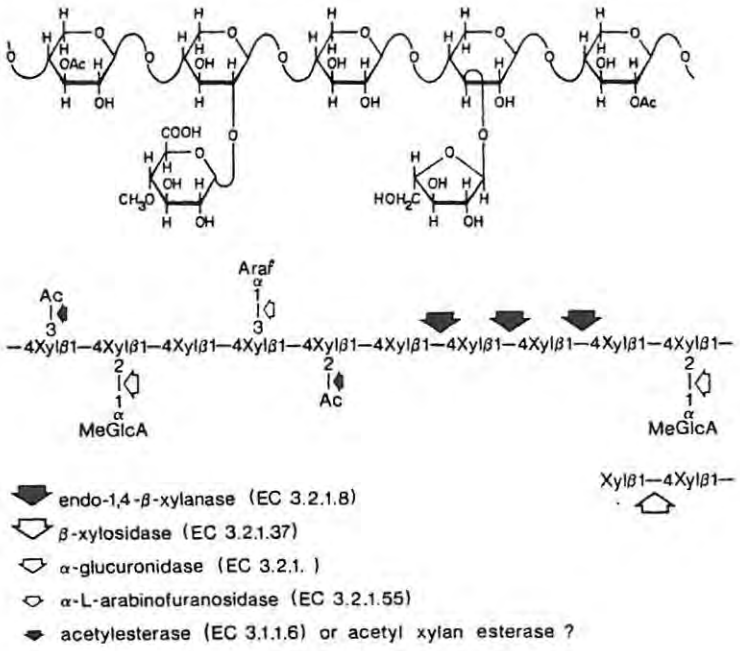


Figure 1.5: Plant xylan and the sites of its attack by microbial enzymes. (After Biely, 1985).

1.3.3. Lignin

Lignins represent an important proportion of plant biomass (up to 30% dry weight in some woody plants). Although lignin is important for the adaptation to the environment of the vascular plants they have a negative impact on the agroindustrial utilization of many crops. They reduce forage digestibility and are difficult to separate from the other components of lignocelluloses during the pulping process (Clayton, 1969).

Since the first characterization of "encrusting material" embedded in the cellulose in wood and the introduction of the term "lignin" by Schulze in 1865, to identify this material, much data have been accumulated on the chemistry, biosynthesis, distribution and properties of lignins. These phenolic polymers are the second most abundant plant polymer on earth (Deobald and Crawford, 1997).

1.3.3.1. Lignin Structure

Lignin is basically composed of three monomeric units (Figure 1.6). The monolignols are biosynthesized in the plant via the shikimic acid pathway, and include p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. The relative proportions of these aromatic alcohols may vary dramatically among species. Lignins are typically considered to be deposited only in the secondary walls of specialized tissues involved in mechanical support (e.g. sclerenchyma), conduction (e.g. vessels and tracheoids) and for protection and defense (e.g. periderm). The deposition of this polymer into the cell wall provides reinforcement and confers new properties such as relative invulnerability and resistance to biodegradation (Pareek *et al.*, 1998).

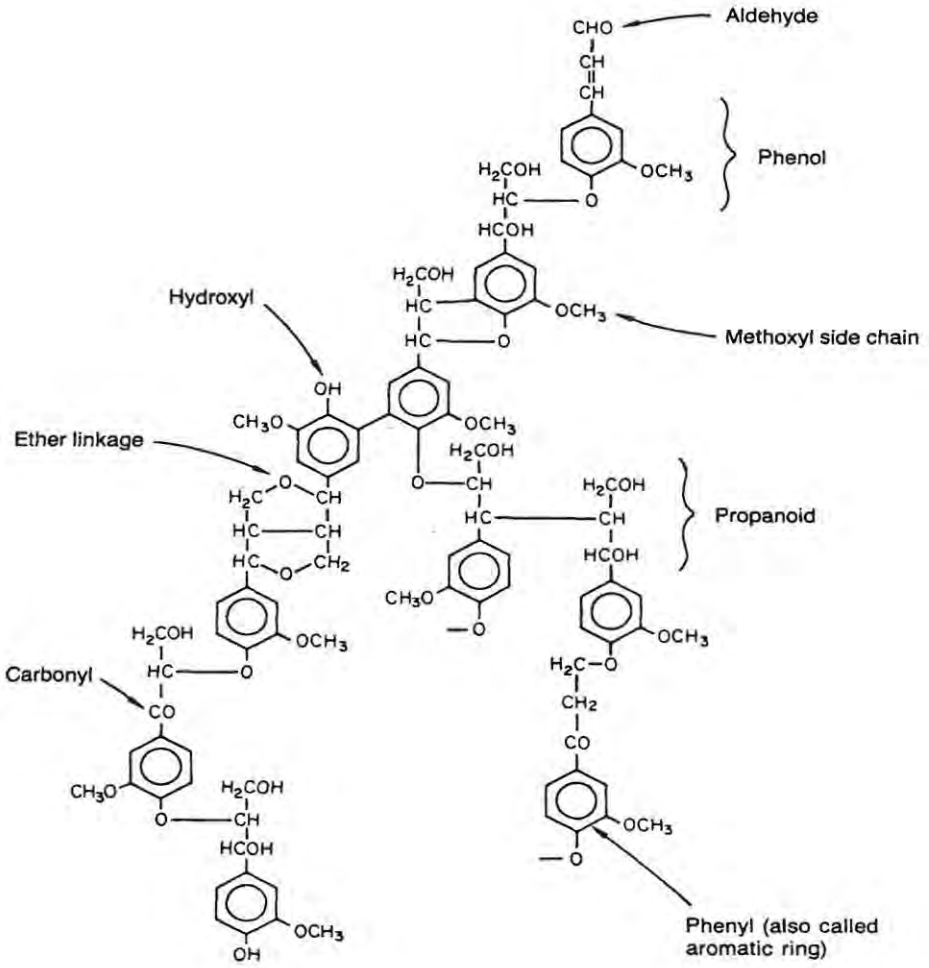


Figure 1.6: Generalized structure of lignin. (After Paul and Clark, 1989). Lignin is formed by free radical reactions and therefore has a random structure of which the above provides an indication of what the polymer might look like.

From an environmental point of view, lignin is a major source of reduced carbon in the biosphere, and its recalcitrance to biodegradation is considered to restrict the cycling of other carbons, both in the natural environment and in biomass conversion systems (Crawford, 1981).

1.4. Diversity of Lignocellulose Degrading Organisms

Microorganisms exclusively carry out the hydrolysis and utilization of lignocellulose in sufficient amounts to provide usable energy. Many of the higher animal species are also able to utilize the lignocellulose material, this, however, is due to the microbial consortia present in their gut. (Wang *et al.*, 1995). Fungi are known to decompose lignocellulosic material and their ability to utilize cellulose is distributed across the entire kingdom, from the primitive, protist-like Chytridiomycetes to the advanced Basidiomycetes (Agrios, 1978). The latter have been studied in detail with regard to their capacity to degrade lignocellulose and the following are of special interest:

1. Brown-rot fungi as the preferential degraders of the polysaccharide components of wood, namely cellulose and hemicellulose, leaving the lignin unaffected.
2. White-rot fungi as the effective degraders of cellulose, hemicellulose and lignin. They are able to attack hardwoods, normally resistant to brown-rot fungi.
3. Soft-rot fungi, which are able to utilize both the polysaccharide and lignin components of wood and usually affect the surface layer of wood, which has been exposed to high moisture content for a long time.

The involvement of bacteria in wood degradation had been noted by several investigators, as early as the middle of the last century, when Ellwood and Ecklund (1959) reported bacteria attached to pine logs from log storage ponds. Later, Boutelje and Kiessling (1964) attributed the decay of oak wood from two submerged ships in the Baltic Sea to bacteria. Unfortunately, for many decades no follow up research was done to explain that incident. More recent investigations point to the ability of bacteria from several taxa to degrade lignin model compounds, chemically depolymerized lignins, and to some extent, natural and synthetic lignin (Crawford and Crawford, 1984; Kern,

1984; Kirk and Farrell, 1987; Haider *et al.*, 1999). However, their ability to mineralize high molecular weight lignin has not been established. It appears that bacteria participate in lignin degradation by mineralizing low molecular weight lignin fragments present in lignocellulose samples. These fragments may be released by abiotic means or by fungal degradation. The role of bacteria as primary degraders is not clear. One of the most active bacteria in degrading synthetic lignin appears to be *Xanthomonas* sp. strain 99 isolated from soil (Kern, 1984).

Kern and Kirk (1987) suggested that bacterial cells are able to take up lignin molecules of 1000 or less and metabolize them to CO_2. The same action may apply to findings by Haider *et al.* (1999) with *Nocardia* spp. and by Crawford with various species of Actinomycetes (Crawford and Crawford, 1984). Jokela *et al.* (1985), showed that mixed bacterial cultures are able to metabolize a synthetic tetrameric lignin model (MW=650). Recent findings by Burnes *et al.* (2000) show that bacteria are able to degrade wood extractives, including a wide range of resin and fatty acids.

1.5. Anaerobic Degradation of Lignin-related Aromatic Compounds

Until recently, little has been known of the fate of aromatic compounds derived from lignin breakdown under strictly anaerobic conditions. Several studies under aerobic conditions have shown that a considerable portion of the lignin polymer is incompletely mineralized, resulting in the release of water-soluble compounds (Phelan *et al.*, 1979; Crawford, 1981). These soluble compounds of reduced molecular size may eventually enter the anaerobic environment and become potential carbon sources for the anaerobic microflora. In 1979, Healy and Young demonstrated degradation of eleven aromatic compounds to methane and carbon dioxide under strictly anaerobic conditions. These findings were supported by numerous reports from subsequent researchers

(Healy *et al.,* 1980; Grbic-Galic, 1983; Li *et al.,* 1996; Peng *et al.*, 2003). Recently Hara *et al.* (2003) characterized a prochatechuate (PCA) 4,5-cleavage pathway genes of *Sphingomonas paucimobilis* SYK-6. These genes consist of four transcriptional units: a cluster *ligK-orf1-ligI-lsdA,* a cluster *ligJAB* and the monocistronic genes *ligR* and *ligC.* These are involved in degradation of low molecular weight aromatic compounds derived from lignin. Figure 1.7 illustrates the catabolic pathway for vanillate and syringate by *S. paucimobilis* SYK-6 and the organization of the PCA 4,5-cleavage pathway genes.

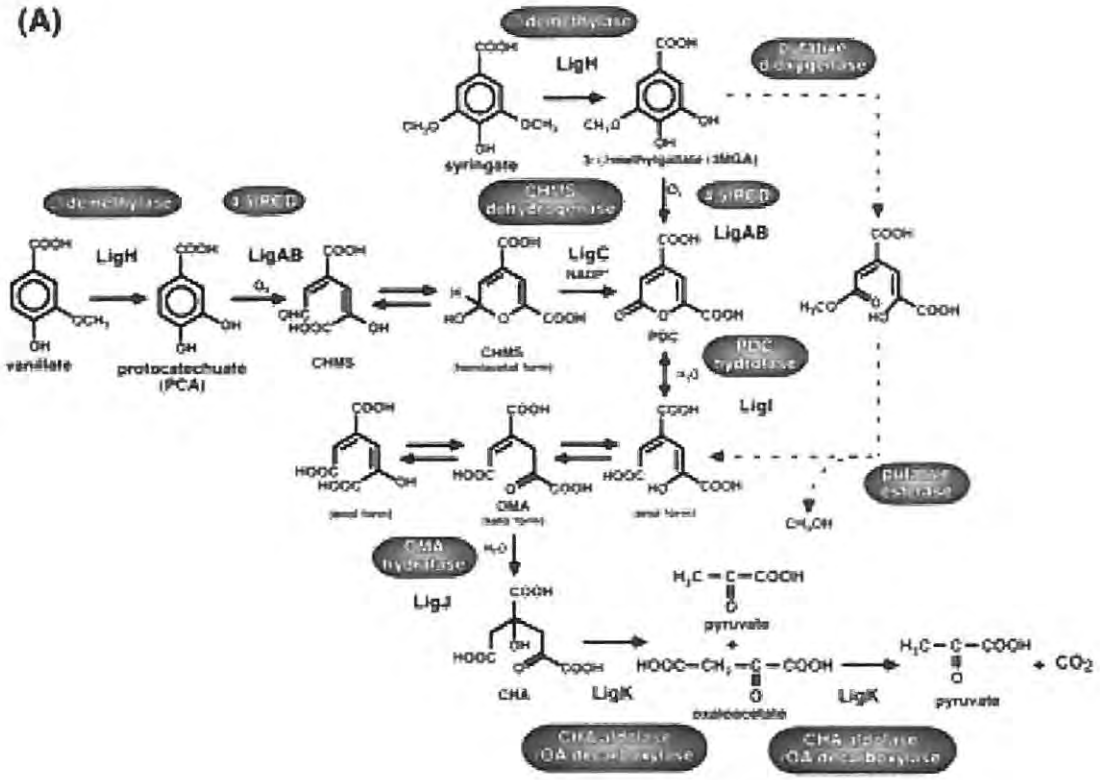


Figure 1.7: Catabolic pathway of vanillate and syringate of *Sphingomonas paucimobilis* SYK-6 (A) and organization of the PCA 4,5-cleavage pathway genes (B). (After Hara *et al.*, 2003)

1.6. Taxonomic Diversity of Cellulose Degrading Microorganisms

In examining the distribution of cellulolytic species across taxonomic groups, it is very useful to consider microbial taxonomy based on phylogeny, rather than on set random morphological or biochemical characteristics as used in classical taxonomy. The evolutionary relatedness of organisms is currently based largely on phylogenetic trees constructed from measurements of sequence divergence of the 16S rRNA gene of prokaryotes and 18S rRNA gene of eukaryotes (Olsen *et al.*, 1994). These trees reveal that the ability to digest cellulose is widely distributed among many genera in the domain *Bacteria* and in the fungal groups within the domain *Eucarya*, but no cellulolytic members of the domain *Archaea* have yet been identified. Within the eubacteria, there is considerable concentration of cellulolytic capabilities among the aerobic order *Actinomycetales (*phylum *Actinobacteria*) and the anaerobic order of *Clostridiales (*phylum *Firmicutes*).

Physiologically the cellulolytic bacteria can be found in several groups (Lynd *et al.,* 2002):

1. Fermentative anaerobes, typically Gram-positive organisms (*Clostridium, Ruminoccocus and Caldicellulosiruptor*) but also including a few Gram-negative species, most of which are phylogenetically related to the *Clostridium* assemblage (*Butyrivibrio* and *Acetovibrio*).
2. Aerobic Gram-positive bacteria (*Cellulomonas*, *Thermobifida* and *Streptomyces.*
3. Aerobic gliding bacteria (*Cytophaga* and *Sporocytophaga*)

The above classification is oversimplified because the noncellulolytic *Clostridium acetobulyticum* contains a complete cellulosomal gene cluster that is not expressed due to disabled promoter sequences (Schwarz, 2001).

Examination of the rapidly expanding genomic database may reveal similar surprises in future.

Among the bacteria, there is a well-defined difference in cellulolytic strategy between the aerobic and anaerobic groups (Rainey *et al.*, 1994). Aerobic cellulose degraders are known to produce substantial amounts of extracellular cellulase enzymes that can be recovered from culture supernatant (Schwarz, 2001). The summary of cellulose degradation under aerobic conditions is carried out according to the following reaction (Ljungdahl and Ericksson, 1985):

$$nC_6H_{12}O_6 + n6\ O_2 \rightarrow n6\ CO_2 + n6\ H_2O \qquad (4)$$

The bacteria sometimes adhere to the cellulose substrate in the medium, but physical contact between cells and cellulose does not seem to be essential for cellulase production (Kauri and Kushner, 1985). Anaerobic organisms degrade cellulose mainly via a complex cellulase system known as the cellulosome (Mechaly *et al.*, 2001; Fierobe *et al.*, 2001; Schwarz, 2001). Most anaerobic cellulolytic species grow best on cellulose when attached to the substrate itself, and this adhesion is usually obligate. According to Ljungdahl and Ericksson (1985) the degradation of cellulose is carried out via fermentation and acetogenesis coupled to ferric iron reduction. The following reaction summarizes the degradation reaction:

$$C_6H_{12}O_6 \xrightarrow{\text{Fermentation, acetogenesis}} 3CH_4 + 3CO_2 \qquad (5)$$

A more detailed pathway of this reaction is depicted in Figure 1.8.
In cellulolytic anaerobes the volume of substrate converted to various fermentation end products like ethanol, organic acids, CO_2, and H_2, is similar to other fermentative anaerobes (Lynd *et al.*, 2002). These microorganisms

seldom exist in isolation. In the anaerobic environment cellulose degradation is a combined effort between the primary cellulolytic species and the secondary and ancillary microorganisms. A primary cellulose degrader converts cellulose to cellobiose and glucose, which in turn are fermented to lactate, acetone, ethanol, CO_2 and H_2. Excess sugars are used by secondary organisms and, depending on the species, are also converted to lactate, acetate, ethanol, CO_2 and H_2. The ancillary bacteria, e.g. *Desulfotomatuculum* can convert the excess glucose, lactate or ethanol arising from the above to acetone, CO_2 and H_2. Hydrogen-utilizing bacteria assimilate the hydrogen arising from the above and use it for the reduction of CO_2 to acetone or methane; sulphate to H_2S; nitrate to ammonia. Figure 1.8 illustrates simplified metabolic steps in anaerobic degradation of complex organic compounds.

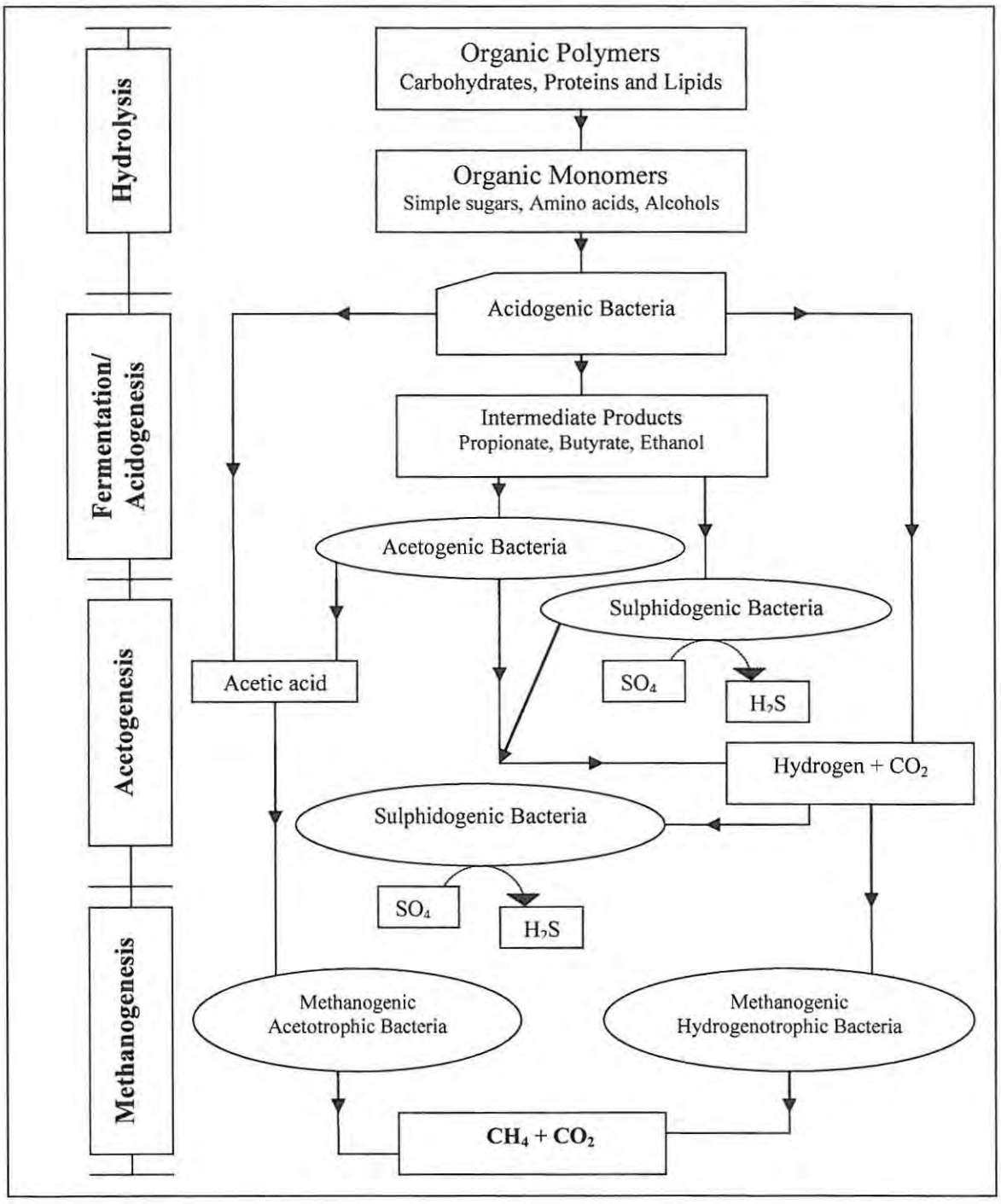


Figure 1.8. Multi-step nature of anaerobic degradation of complex organic matter. The energy yield is comparably low, since organic compounds are used both as electron donors and acceptors. Modified from Giraldo and Eugenio (1993).

Many examples of primary/secondary associations within the anaerobic environment have been quoted in the literature including *Bacteroides* spp., *Treponema bryantii* (Stanton and Canale-Parola, 1980); *Treponema bryantii/Bacteroides succinogenes/Ruminococcus albus* (Kudo *et al.,* 1987); *Acetovibrio cellulolyticus/Desulfovibrio* spp. *Methanosarcina barkeri* (Laube and Martin, 1981); *Cellulomonas* spp. */Bacillus mascerans/Azospirillum brasiliense* (Halsall and Gibson, 1985). *Clostridium thermocellum/Clostridium thermosaccharolyticum* (Avgerginos and Wang, 1980).

1.7. Techniques Applied in the Identification of Microorganisms in Environmental Samples

A full understanding of the processes occurring in any natural or engineered system must also account for the microorganisms that contribute to the reaction. Wastewater treatment is one of the most important biotechnological processes used worldwide to treat polluted water and to alleviate anthropogenically-induced damage to the environment. Prokaryotic microorganisms dominate in all types of wastewater treatment plants. These organisms represent both the "causative agent" responsible for the observed conversions and they also contribute to the most frequently encountered problems (Kampfer *et al.,* 1996). Therefore the efficiency and robustness of wastewater treatment system depends mainly on the composition and activity of the microbial communities present at different stages of the treatment. Although biological treatment of wastewater has been used for more than a century, the advent of molecular biology has greatly increased our understanding of the process. Consequently, these microbial communities were considered as a "black box", and progress in the design and control of wastewater treatment plants was derived mainly from empirical research in civil engineering. It has been claimed that around 99 % of all microorganisms in nature cannot be isolated in pure culture, since their culture requirements

are not known (Amman *et al.*, 1995), and therefore little is known about the identity of the microbes in those communities. Microbial identity has important ramifications in environmental engineering, like wastewater treatment and bioremediation, in which the key process may be governed by uncharacterized microbiota. In order to obtain a better understanding of microbial diversity and its role in the ecosystem other techniques, which complement the traditional microbiological approach, are necessary.

Recent advances in molecular biology and molecular systematics provide methods for the direct characterization of environmental populations, without the need for prior growth in the laboratory. This section of the chapter aims to review these new insights and compare them with traditional techniques.

1.7.1. Light Microscopy and Cultivation

Classically, two approaches have been used to investigate the microorganisms in environmental samples. Firstly, samples were analyzed by standard light microscopy and a provisional identification was made using Gram-staining and morphological characteristics (Eikebloom and Van Buijsen, 1975). However, these techniques, are not able to distinguish between the different bacterial domains. Furthermore, polymorphism of certain filaments is known (Wagner *et al.*, 1994), which further complicates morphology-based identification.

The second approach was based on cultivation and isolation of bacteria (Eikebloom *et al.* 1998). The number of active bacteria in this method is estimated by the most probable number (MPN) technique and/or total plate counts. After isolation, bacteria are identified using either physiological parameters or chemotaxonomic markers.

Numerous bacteria have been isolated and identified from environmental samples using cultivation-based techniques, leading to the perception that for example pseudomonads, enterobacteriaceae, and acinetobacter are key components of the microbial flora of these systems. After the introduction of molecular techniques for community analyses, however, it became obvious that only a limited number of the microorganisms present can be isolated from the environment by standard cultivation techniques and that those bacteria forming colonies on the respective media are generally of minor numerical importance *in situ* (Wagner *et al.*, 1993; Manz *et al.*, 1994; Wagner *et al.*, 1994; Kampfer *et al.*, 1996;). Therefore, cultivation offers only a biased and incomplete view of the bacterial diversity in natural systems. The lack of knowledge is most severe for complex, multispecies microbial communities. Here, populations are frequently arranged in a very specific way, such as in biofilms, and such communities have activities that cannot be achieved by individual microorganisms (Lawrence *et al.*, 1994; Santegoeds *et al.*, 1998; Stickler, 1999; Davey and O'Toole, 2000; Watnick and Kolter, 2000). On the other hand, direct cultivation of *in situ* important microorganisms is still a prerequisite to allow complete physiological and genetic analysis.

1.7.2. Immunofluorescence

A more direct identification and quantification of bacteria is offered by the use of fluorescent antibodies (FA). However, the production of these antibodies usually requires the prior isolation of the target organism and therefore restricts the method to culturable bacteria. Once available, the FA-technique can be used to identify and quantify defined organisms in an environmental sample. However, the high serological diversity of many bacterial genera or even species (Wagner *et al.*, 1995) would require the simultaneous use of a large number of FA for their detection thereby rendering the method awkward and expensive. Furthermore, FA are relatively large molecules and for that reason they do not easily penetrate dense extracellular polymeric substances

of flocs and biofilms. However, this problem could be overcome if they were applied to cryosectioned samples. Another difficulty with using the FA-technique is that it often suffers from a high background fluorescence caused by unspecific binding of the FA to debris and filament sheaths (Wagner *et al.*, 1995). Despite these limitations, the FA-technique has the unique advantage in that detection does not require the killing of the cells under investigation.

1.7.3. The 16S rRNA Gene Approach: The Revolution in Wastewater Treatment Microbiology

The comparative sequence analysis of environmentally retrieved 16S rRNA sequences has become the gold standard for cultivation-independent assessment of bacterial diversity in natural and engineered systems (Amann *et al.*, 1995). Current 16S rRNA gene databases contain more than 20 000 entries and thereby provide a high resolution framework for the assignment of those sequences obtained in 16S rRNA gene libraries from environmental diversity surveys (Hugenholtz *et al.*, 1998). The approach consists of DNA extractions, subsequent amplification of the 16S rRNA gene using primers targeting regions conserved in the bacterial genome, cloning and sequencing. The obtained sequences are analyzed together with adequate reference sequences to deduce their phylogenetic affiliation.

It is however important to realize that even if the complete diversity of an environmental 16S rRNA gene library is harvested, the obtained species inventory might not represent the naturally occurring diversity. In other words, not all numerically important bacterial populations of an environmental sample will be found in a retrospective 16S rRNA gene library. This failure might be caused by:

1. Inefficient DNA extraction (Juretschko *et al.*, 1998).
2. Inadequate coverage by the selected PCR primers.

3. Kinetic and stochastic biases introduced by the PCR amplification (Suzuki and Giovannoni, 1996; Suzuki *et al.*, 1998).
4. Cloning bias.
5. Different numbers of copies of rRNA genes.

1.7.3.1. Polymerase Chain Reaction (PCR)

PCR employs oligonucleotide primers and thermostable DNA polymerase to amplify target DNA sequences by temperature-controlled cycles which enable strand separation, primer annealing and primer extension. For a general diversity survey, the best-suited PCR conditions should avoid extremely stringent annealing conditions to allow binding of the primers to organisms with nucleotide changes at the target position (Soondrum and Neumaier, 2000). Failing that, the procedure might often lead to the amplification of undesired PCR products and the necessity to purify the expected PCR amplificate by agarose gel electrophoresis and band excision before subsequent investigation.

Furthermore, the PCR cycle number has a profound influence on the composition of the PCR amplificate if a complex template mixture is used (Suzuki and Giovannoni, 1996). Highly abundant and less abundant organisms are more likely to be represented in comparable numbers in the PCR product if high cycle numbers are used, an effect caused by template reannealing at high concentrations. In contrast, the 16S rRNA composition of PCR products generated with low cycle numbers should more accurately represent the composition of the 16S rRNA sequences in the isolated nucleic acid, however this is not always the case (Suzuki and Giovannoni, 1996).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Modification of traditional PCR, RT-PCR is a very useful technique employed both in diagnostic genetics and in the molecular microbial ecology nvestigation. The principle of this technique is based on an RNA strand being reverse transcribed into its DNA complement (cDNA), which subsequently is followed by amplification of the resulting DNA using PCR. Therefore the first step in RT-PCR is reverse transcription (RT) during which an RNA strand is transcribed into its DNA with the use of an RNA-dependent DNA polymerase. This reaction is followed by synthesis of the second strand of DNA with the use of deoxyoligonucleotide primer and DNA-dependent DNA polymerase. The cDNA and its matching part are then exponentially amplified through PCR. This technique is extremely sensitive, where a very low number of RNAs can be detected (Gilbert, *et al.,*1996)

Real-Time PCR

Real-Time PCR was designed for quantitave purposes, where a quantitative thermocycler monitors the progress of polymerase chain reactions as they proceed (Gibson, *et al.,* 1996).

1.7.3.2. 16S rRNA Gene-based Fingerprinting Techniques

16S rRNA-based fingerprinting techniques supplement the 16S rRNA approach. The common principle of these techniques is to separate PCR products of the same length but different sequence to visualize the diversity within the amplificate by banding pattern. Many of these techniques were developed to analyze mutations in medical research and were later adapted in environmental microbiology (Fisher and Lerman, 1979; Myers *et al.*, 1985;). The most frequently applied fingerprinting techniques are denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE)

(Muyzer *et al.*, 1993; Wawer and Muyzer, 1995; Nielsen *et al.*, 1999; Liu *et al.*, 2001), terminal restriction fragment length polymorphism (T-RFLP) (Liu *et al.*, 1997), and single strand conformation polymorphism (SSCP) (Lee *et al.*, 1996; Dabert *et al.*, 2001). The main advantage of these fingerprinting techniques is that a high sample number can be processed in a relatively short time in order to gain an overview on the diversity of the complex microbial community in wastewater treatment plants. Bands of interest can be excised, cloned and sequenced for subsequent identification.

1.7.3.2.1. Denaturing Gradient Gel Electrophoresis (DGGE) and Temperature Gradient Gel Electrophoresis (TGGE)

DGGE/TGGE procedure is based on electrophoresis of PCR-amplified 16S rDNA fragments in polyacrylamide gels containing a linearly increasing gradient of denaturant (DGGE) or temperature (TGGE). Using these techniques DNA fragments of the same length, but of different base-pair sequences, can be separated. Separation 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 gel, a transition of helical to partially melted molecules occurs, and migration of the molecule will stop. Sequence variation within such domains causes their melting temperatures to differ thereby allowing separation during electrophoresis (Myers *et al.*, 1985; Muyzer *et al.*, 1993, Heuer *et al.* 1999). However, in order to obtain optimal separation and high resolution banding patterns, incorporation of a GC-rich clamp at the 5' prime end of the fragment is necessary (Muyzer *et al.*, 1993).

1.7.3.2.2. Hybridization Techniques Using rRNA-targeted Probes

Dot blot and *in situ* hybridization techniques using rRNA-targeted oligonucleotide probes allow, in contrast to all other techniques, the quantitative determination of the composition of complex microbial communities. These probes can be adjusted to different phylogenetic levels. Presently, a considerable number of ready-to-use domain-, division-, genus-, and species-specific probes are available for identification of the respective target organisms within their natural habitat (Amann *et al.*,1995; Hugenholtz *et al.*, 1998). However, for high-resolution analysis of a certain environmental sample, additional probes should be designed from cloned sequences obtained with the 16S rRNA approach. Application of these probes in the dot blot or *in situ* techniques allows one to detect and quantify the corresponding microorganisms present in the habitat for which the 16S rRNA gene library was established. The complete procedure including the 16S rRNA gene library analysis, clone-specific probe design, and quantitative hybridization experiments are referred to as the full-cycle rRNA approach (Amann *et al.*, 1995).

1.7.3.2.3. Dot-blot Hybridization of rRNA

A molecular approach that does not depend on amplified genes is the dot-blot hybridization assay. In this approach, ribosomal RNA isolated from environmental samples is spotted onto membranes and hybridized with radioactive 16S rRNA-targeted oligonucleotide DNA probes. The relative abundance of specific taxons can be estimated by comparing the hybridization signal obtained with the taxon specific probe to the signal obtained with a universal probe (Raskin *et al.*, 1994; Lin and Stahl, 1995; Stahl, *et al.*, 1995). Quantitative dot blot-hybridization measures the abundance of the target population via its rRNA content. Since the rRNA of a microbial cell varies

depending on its activity and physiological history and on prevailing environmental conditions, the obtained data cannot be translated to cell numbers. Furthermore, the method can be biased by varying efficiency of RNA extraction from different microbial populations and degradation of the rRNA (which might affect different probe target sites in a different way) during the procedure. However, quantitative dot-blot analyses have been successfully used in wastewater microbiology (Rossello-Mora *et al.*, 1995; Mobarry *et al.*, 1996; de los Reyes *et al.*, 1997) but they are more widely applied in environments like soil microbial mats, which due to their high background fluorescence are not well-suited for fluorescent *in situ* hybridization (FISH) (Minz, *et al.*, 1999).

*1.7.3.2.4. **Fluorescent** In Situ **Hybridization***

In situ hybridization with fluorescently-labeled, rRNA targeted oligonucleotide DNA probes, is well suited to identify the target organisms within activated sludge flocs or biofilms by specific staining. In contrast to dot-blot hybridization, simultaneous binding of multiple probes to the same target cells can be proven by FISH through microscopic observation of the hybridized cells and the use of different probe labels. Therefore, sets of probes with hierarchical or identical specificity can be used to increase reliability of identification (Wagner *et al.*, 1993; Daims *et al.*, 1999). The combination of FISH and confocal laser scanning microscopy (CLSM) (Wagner *et al.*, 1994) dramatically improved the image quality by exclusion of out of focus fluorescence and allowing the development of semiautomatic quantification protocols by use of digital image analysis (Bouchez *et al.*, 2000). These methods measure the specifically stained biovolume of a target population and refer to it as the volume of those microorganisms stained by the bacterial probe set or a DNA-binding dye. In the manual technique only a few hundred or thousand cells are counted whereas the semi-automated protocols detect

more than 100 000 cells per measurement and are therefore much more reliably reproduced (Daims *et al.*, 2001).

Confocal laser scanning microscopy of FISH results may also be used to investigate the spatial distribution of microorganisms in activated sludge flocs and biofilms and to study co-localization of microbial populations (Wagner *et al.*, 1994; Juretschko *et al.*, 1998; Schramm *et al.*, 1999). However, such analysis can only be performed successfully if the architecture of the flocs or biofilms is preserved by embedding or cryosectionning and is not destroyed during fixation and hybridization (Moter *et al.*, 1998; Schramm *et al.*, 1999;). Recently, Lee *et al.* (1999) have developed an elegant method for studying simultaneously the architecture of biofilms and the function of relevant prokaryotes. This method involved a direct combination of fluorescent *in situ* hybridization and microautoradiography thereby allowing *in situ* identification and determination of substrate uptake patterns of individual cells within complex microbial consortia.

In summary, several molecular techniques are available for investigating microbial diversity in wastewater treatment plants. In particular, the full-cycle rRNA approach allows one to precisely measure the composition and dynamics of microbial communities in these systems.

Some of the methods used in molecular microbial ecology studies are illustrated in figure 1.9.

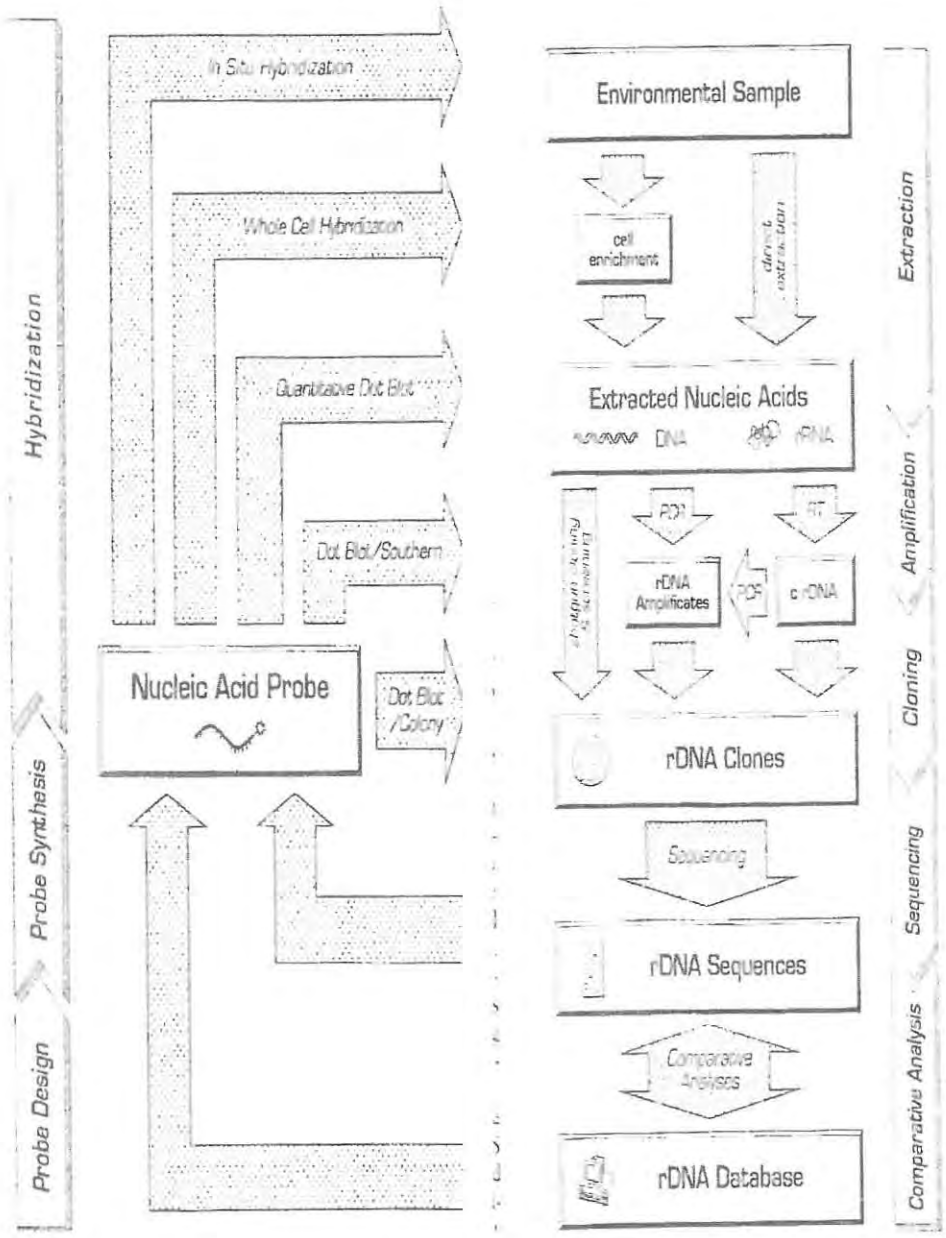


Figure 1.9: Methods used in molecular microbial ecology study. (After Amann, 1995).

1.8. Biases and Limitations of Molecular Techniques

Although application of molecular techniques in microbial ecology has revolutionized the analysis of environmental samples and revealed remarkable results, there are still many problems to be overcome. The most essential step in molecular techniques, the extraction of DNA, must be reliable and reproducible in order to obtain a real view of the genetic diversity of microorganisms in environmental samples. However, inefficient extraction of DNA from natural samples remains a problem (Juretschko *et al.*, 1998). PCR amplification of specific genes, which is straightforward for genomic DNA of a single species, might become unpredictable when working with a complex mixture of bacteria from a polluted environmental sample. The main reasons for this are the presence of PCR inhibitors and preferential amplification of target DNAs from some organisms rather than others (Reysenbach *et al.*, 1992).

A specific limitation of the DGGE approach is that separation of PCR products obtained from a very complex mixture of microorganisms, such as those found in soils, can be difficult (Torsvik *et al.*, 1990). Furthermore, only limited sequence information is obtained with the DGGE/TGGE approach, since the separation is limited for fragments shorter than 500 bp (Muyzer and Ramsig, 1995).

1.9. Microarray Technology

From the microbial ecology point of view, it is essential to gain knowledge not only of the composition and architecture of microbial populations in any given environment, but also to understand microbial functions and behaviour in such an environment. Conventional molecular methods, such as the PCR, gene cloning, terminal-restriction fragment length polymorphism, denaturating gradient gel electrophoresis and *in situ* hybridization are effective for these

kinds of study but very labour intensive and are able to address only one gene at a time. A rapid development in the field of molecular biology and especially genome sequencing projects, has dictated a drastic change in experimental methods (Dennis *et al.*, 2003) with an aim to focus on the expression of many genes at one time instead of concentrating on one gene at a time. One of these new technologies, which developed also from rapid advances in both robotics and miniaturization, is DNA microarray technology (Schena *et al.*, 1995).

DNA microarrays were originally developed for rapid sequence analysis of genomic DNA (Khrapko *et al.*, 1991; Southern *et al.*, 1992; Guo *et al.*, 1994; Chee *et al.*, 1996; Drmanac and Drmanac, 1999; Anthony *et al.*, 2000). This technology was also successfully used to analyze total gene expression in pure cultures (deRisi *et al.*, 1997; Richmond *et al.*, 1999; Ye *et al.*, 2000; Thompson *et al.*, 2002). However according to Zhou (2003) and Sharkey *et al.* (2004) applying microarrays for use in environmental studies are challenging mainly in terms of specifity, sensitivity and quantification. That probably explains why this technique, although it has attracted attention among environmental microbiologists, has only in recent years seen wider implementation in the study of microbial populations in the environment (Gushin *et al.*, 1997; Call *et al.*, 2001; Liu,*et al.* 2001; Small *et al.*, 2001; Wu *et al.*, 2001; Valinsky *et al.*, 2002; Cho and Tiedje, 2002; Loy *et al.*, 2002; Taroncher-Oldenburg *et al.*, 2003; Rhee, *et al.*, 2004, Franke-Whittle *et al.* 2005).

The microarrays employed in environmental investigations can be divided into three groups (Zhou and Thompson, 2002; Zhou, 2003):

1. Functional gene arrays (FGA)
2. Community genome arrays (CGA)
3. Phylogenetic oligonucleotide arrays

FGA contain probes that correspond to genes encoding enzymes involved in different environmental processes that are very useful in studying the physiological activities of microbial assemblages in the environment (Wu *et al.*, 2001; Zhou and Thompson, 2002; Rhee *et al.,* 2004). CGA are made using whole genomic DNA isolated from pure culture and can be used for characterizing a microbial population from the cultivability point of view (Zhou, 2003). Phylogenetic oligonucleotide arrays are usually produced using short primers from rRNA genes. These arrays can be used for phylogenetic analyses of a microbial population and their architecture in environmental samples (Rhee *et al.,* 2004). Figure 1.10 outlines two different approaches for the production of microarrays.

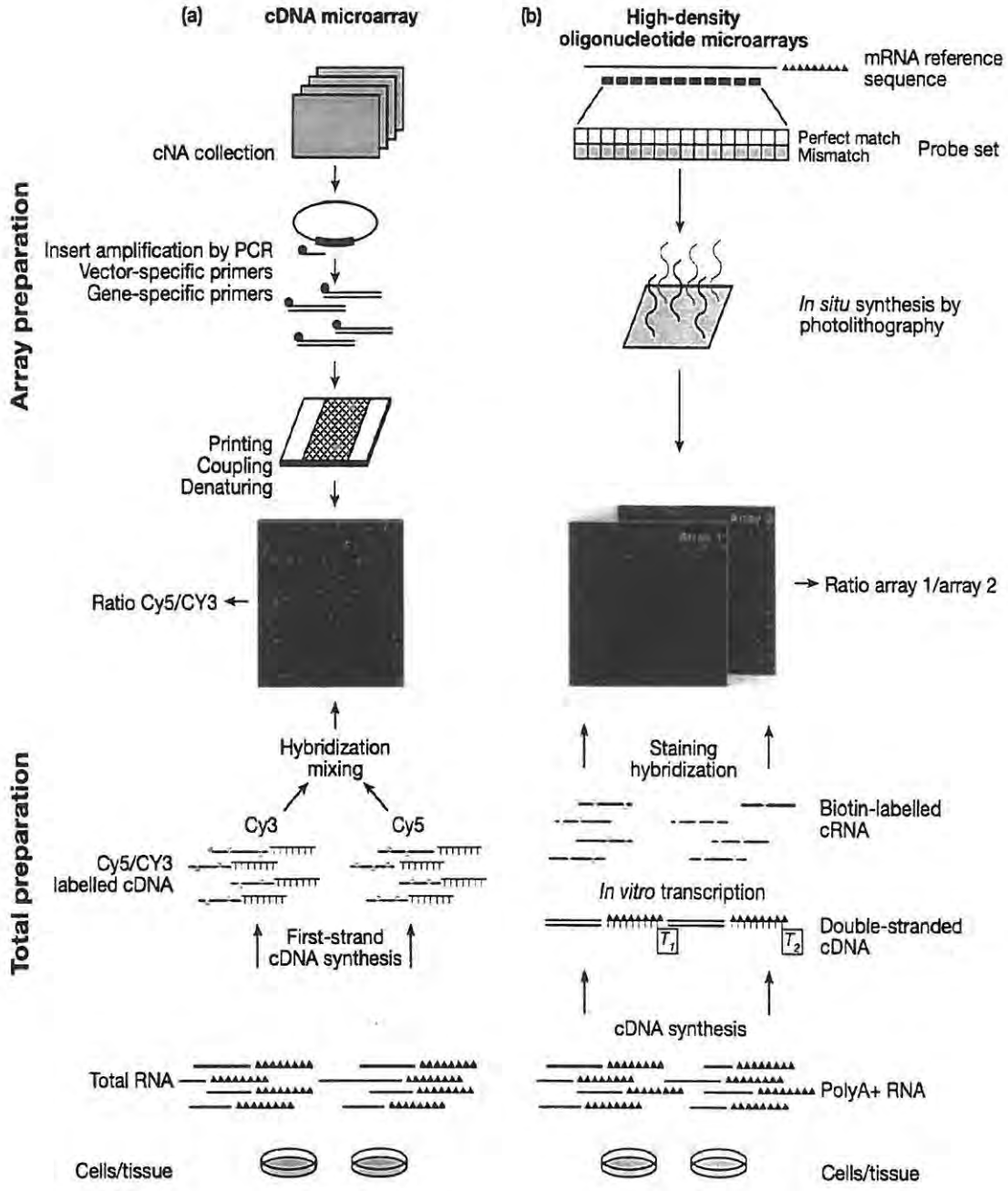


Figure 1.10: Schematic representation of microarray development for analyzing gene expression. (a) cDNA array development; (b) oligonucleotide arrays. (After Sharkey *et al.*, 2004).

1.10. The Microbial Ecology of Lignocellulose Biodegradation in AMD Passive Treatment

Biological systems for passive treatment of AMD are dependent on the availability of low-cost carbon and electron donor sources and, among these, lignocellulosic materials have received considerable attention (Dvorak and McIntire, 1992; Bechard *et al.*, 1993; Pareek *et al.*, 1998; and Chang *et al.*, 2000). To address this problem a R9 million inter-disciplinary research programme was initiated by the Department of Arts, Culture, Science and Technology (DACST) Innovation Fund (IF) and managed by Pulles, Howard and de Lange (PHD), and the Environmental Biotechnology Group (EBG) at Rhodes University. This group was contracted to undertake a number of fundamental studies related to the development of passive treatment systems, and to include findings relating to prior work in the following areas:

- Sulphidogenic hydrolysis of complex organic carbon substrates (Whittington-Jones, 2000);
- The Rhodes BioSURE Process in AMD treatment (Corbett, 2001);
- Enhanced hydrolysis of sewage sludge (Molwantwa, 2002);
- The manipulation of immobilized sulphate reducing systems (Sanyahumbi, 2003);
- Degradation of aromatic compounds in sulphate reducing environments (Ehlers, 2003);

The IF programme in the EBG laboratories was based on these prior studies and included the following investigations:

- The Microbial Ecology of Sulphate Reduction in the Rhodes BioSure Process ® (Chauke, 2002);

- The Microbial Ecology of Floating Sulphur Biofilms (Bowker, 2002);
- Biological Sulphide Oxidation in heterotrophic environments (Rein, 2002);
- Biosulphidogenic Hydrolysis of Lignin and Lignin Model Compounds (Madikane, 2002);
- The Degradation of Lignocellulose in a Biologically-Generated Sulphidic Environment (Roman, 2005)
- The Microbial Ecology of Sulphidogenic Lignocellulose Degradation (Author of this thesis)

1.11. Research Hypothesis

Characterization of the microorganisms active within mixed microbial consortia, the determination of trophic activity, and spatial and temporal gradients within biosulphidogenic lignocellulose degrading systems, provide a basis for establishing a structural and functional explanatory model describing the performance of these systems. If successful, such an explanatory model may be used to improve the operational performance of lignocellulose-based AMD passive treatment systems.

1.12. Research Objectives:

In using a molecular microbial ecology approach to understand the operation and performance of sulphidogenic lignocellulose packed-bed reactor systems, the following research objectives were identified:

- To investigate and describe the operation and performance of an existing field-scale lignocellulose packed-bed reactor treating AMD;

- To use laboratory-scale reactors to simulate sulphidogenic lignocellulose packed-bed systems in order to investigate the establishment of microbial consortia both in linear and temporal distribution in these systems;

- To use phylogenetic analysis of the obtained microbial populations, to determine the distribution of trophic activity within the lignocellulose packed-bed reactor, and using this as a means of assigning functional activity to its components;

- To use the data derived, to design an explanatory model accounting for the mobilization of lignocellulose as a carbon and electron donor source for sulphate reduction;

- To evaluate the possible application of these findings in the improved performance of sulphidogenic lignocellulose packed-bed AMD passive treatment systems.

CHAPTER TWO

Materials and Methods

2.1. Introduction

The research effort to develop integrated passive minewater treatment systems for the sustainable removal of sulphates, metals and acidity commenced in South Africa in 1995 (Pulles *et al.*, 1995). In its first phase the programme targeted the development of preliminary management guidelines which could be used by the gold and coal mining industries to select, design, operate and maintain the most appropriate integrated passive treatment systems during the operation and closure of mining projects. At that stage, two mines, Western Areas Gold Mine and Arnot Colliery were identified as suitable pilot plant sites, and plants were constructed. The results of those investigations, however, were not satisfactory in terms of the envisaged goals. The sulphate removal of the units was highly variable, and the average sulphate removal rate of 11.7g/m^3/day was well below the economic target of 60 g of sulphate removed per m^3 of organic matter per day that had been established at the outset. This led to the construction of a pilot plant at the Vryheid Coronation Colliery (VCC) near the town of Vryheid in northern Kwa-Zulu Natal.

The VCC pilot plant was used in the investigation as reported here as the source for the baseline study of minewater passive treatment systems operated at field-scale. The author was directly involved in the sampling phase of the investigations reported here. Based on the findings of this study a laboratory–scale lignocellulose packed-bed reactor was designed to investigate the system under controlled conditions.

This chapter reports the materials and methods used in the studies undertaken.

2.2. The VCC Pilot Plant

2.2.1. Overview

VCC is a closed mine situated 30 km from Vryheid in northern Kwa-Zulu Natal. At the site there are two adits actively decanting AMD from the old mine, namely the South Adit (SA) and West Adit (WA). Under normal operational mode, the influent into the sulphate reducing units is a mixture of the WA and SA water. The physical location of the VCC pilot plant is shown in Figure 2.1.

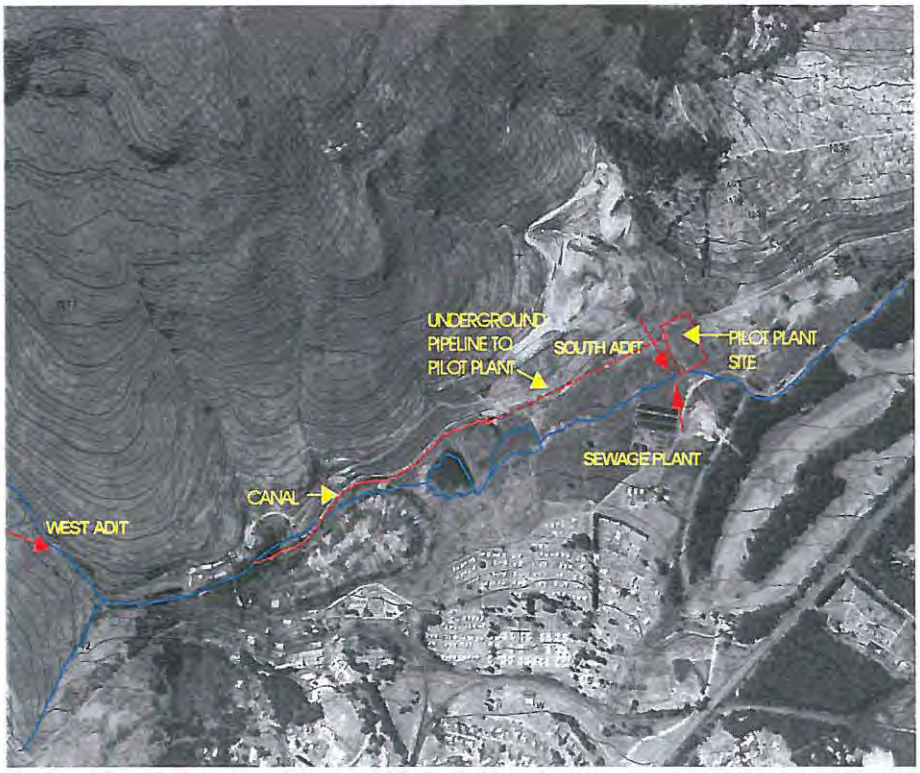


Figure 2.1: Location of pilot plant site at Vryheid Coronation Colliery. (After Pulles, 1999).

The pilot plant consisted of four Small Sulphate Reducing Units (SSRU), two Large Sulphate Reducing Units (LSRU) and a post-treatment facility comprising an aerobic wetland and oxidation cascade. All units received their

feed water from the splitter boxes where the SA and WA water were mixed in a 1:1 ratio. Treated water from the SSRU was "polished" in a post-treatment facility, consisting of a wetland and an oxidation cascade to remove residual nutrients added to the system (Figure 2.2). Effluent from the oxidation cascade was then discharged into a natural stream flowing outside the pilot plant.

2.2.1.1. Small Sulphate Reducing Units (SSRU)

The SSRU were constructed as inverted truncated pyramids, filled with layers of the various organic substrates (Table 2.1). In order to increase the permeability of the organic matter in SSRU 3 and SSRU 4, 50 % inert dolerite layers were incorporated between the carbon matters. Although originally designed as downflow units, these units were subsequently changed to upflow units due to the headloss problems experienced. SSRU 3 was operated in series i.e. the effluent from SSRU 2 was fed anaerobically to SSRU 3.

An iron augmentation unit was investigated as a component of the process design but was later decommissioned due to its limited effect on the system.

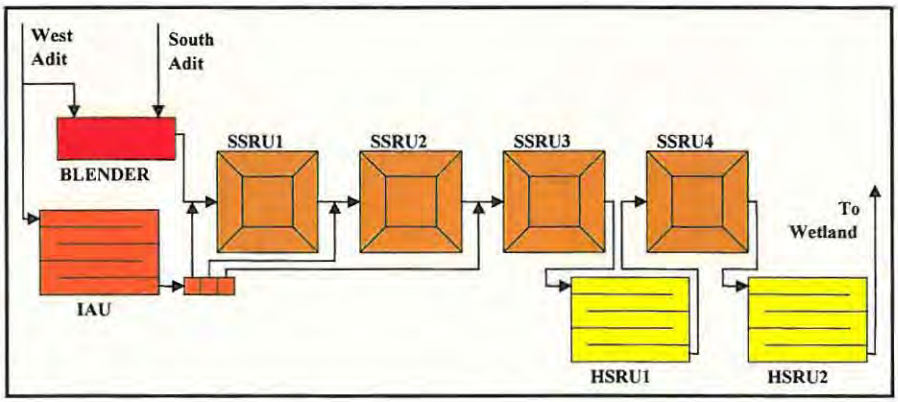


Figure 2.2: Small sulphate reducing units process stream as originally constructed (phase 2). (After Pulles, 1999).

Table 2.1: SSRU organic matter layers.

Carbon substrate	SSRU1	SSRU2	SSRU3	SSRU4
Hay	2 x 2 m^3		2 x 6 m^3	2 x 4 m^3
Silage		3 x 7 m^3		
Woodchips	3 x 6 m^3	3 x 6 m^3		
Sawdust	2 x 2 m^3	2 x 2 m^3	2 x 2 m^3	2 x 6 m^3
Sewage sludge	2 x 2 m^3	2 x 2 m^3	2 x 2 m^3	2 x 4 m^3
Cow Manure	3 x 4 m^3	1 x 1 m^3	2 x 4 m^3	2 x 2m^3
Chicken litter	2 x 2 m^3		2 x 4 m^3	2 x 4 m^3
Bulking agent–dolerite*			3 x 8 m^3	3 x 8 m^3
Total volume	46 m^3	48 m^3	60 m^3	64 m^3

*25 mm according to engineer's specification

2.2.1.2. Large Sulphate Reducing Units (LSRU)

The LSRU were constructed in a similar manner to the SSRU, i.e. as inverted truncated pyramids, filled with layers of the various carbon substrates (Table 2.2). The two LSRUs running in parallel were designed to remove sulphates in a single stage reactor (Figure 2.3). LSRU 1 was operated as a down flow unit, while LSRU 2 was operated as an up flow unit. Effluent from the LSRUs was then gravity fed to the post-treatment facility.

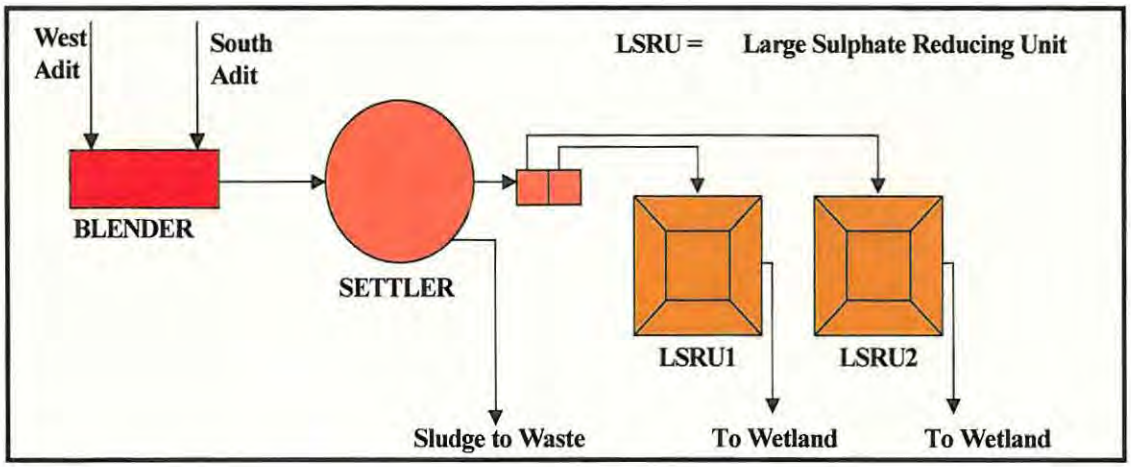


Figure 2.3: Large sulphate reducing units process stream (as constructed, after Pulles, 1999).

Table 2.2: LSRU organic matter layers

Carbon Substrate (m^3)	LSRU1	LSRU2
Hay	46 m^3 x 6	50 m^3x 6
Wood chips	12 m^3x 2	20 m^3x 3
Sawdust	30 m^3x 2	20 m^3x 3
Sewage Sludge	12 m^3x 2	12 m^3x 2
Cow Manure	30 m^3x 2	22 x 3
Chicken litter	5 m^3x 1	10 m^3x 2
Bulking agent–dolerite*	121 m^3 x 8	None
Total Carbon	135 m^3	134 m^3

*25 mm according to engineer's specification

2.2.1.3. Hydrogen Sulphide Removal Units (HSRU)

The HSRU were constructed with a horizontal flow along a tortuous path. A 100 mm thick layer of inert dolerite rock (25 mm according to engineer's specification) was placed on the bottom of the units to act as a growth medium for the anaerobic photosynthetic sulphur oxidizing bacteria which oxidize sulphide and deposit sulphur (Pulles, 1999).

2.2.1.4. Acid Mine Drainage Feed

WA and SA seepage from the former VCC mine were mixed in equal volumes before sending it through the SSRU for treatment. Acid mine drainage (AMD) from the WA was fed to a constant header box via a pipeline while seepage from the SA was pumped to a storage tank from where it was gravity fed to a constant header box (Figure 2.4 and Figure 2.5).

The SA and WA water were gravity fed into two constant header boxes from where the AMD flowed into separate compartments of the splitter boxes (Figure 2.4 and Figure 2.5). Each SSRU is equipped with a splitter box to enable passive control of flow rates. Each splitter box consists of two water

collection compartments (SA and WA equipped with a V-notch and an overflow plate). Excess water flows over the overflow plate and yields a constant flow by means of a V-notch. SA and WA water are then mixed in the mixing chamber after which it is gravity fed through pipelines to the sulphate reducing units.

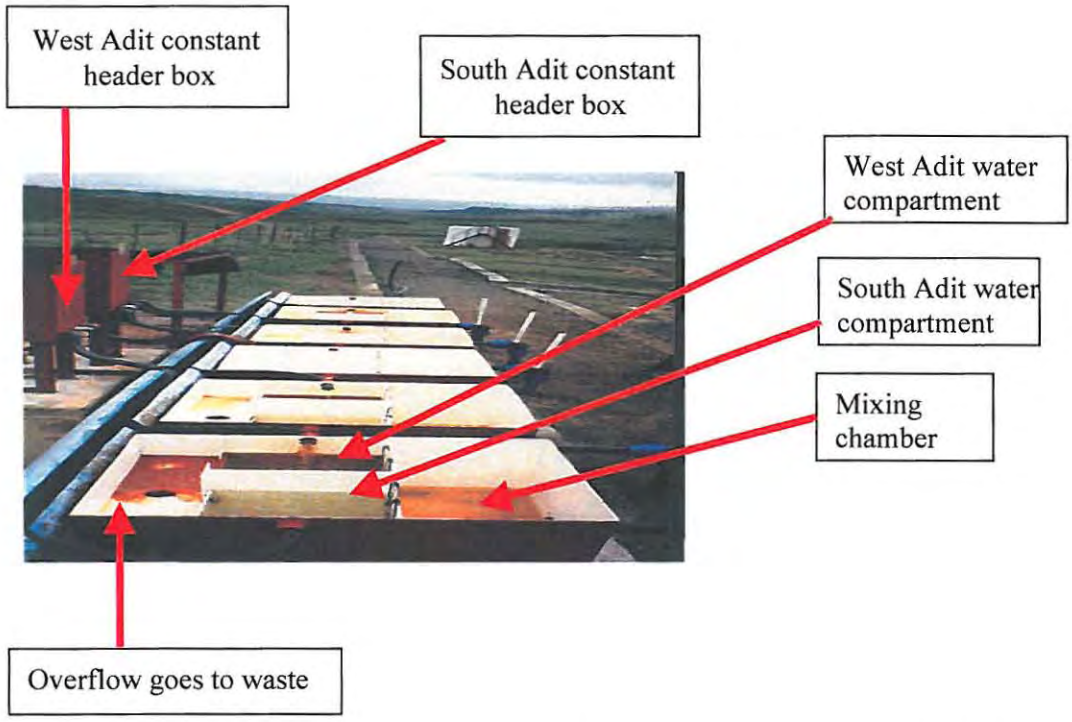


Figure 2.4: Splitter boxes at the VCC pilot plant showing the different feed compartments.

Figure 2.5: Close-up view of splitter boxes and mixing chambers.

2.2.1.5. Post-treatment Units

The SRU are responsible for an addition of nutrients and organic loading to the treated water, making it unsuitable for discharge without any form of post-treatment. The post-treatment facility consisted of an aerobic wetland and an oxidation cascade (Figure 2.6). The aerobic wetland was constructed as a shallow reedbed in a zigzag flow configuration with the intention of removing organic nutrient loads. The oxidation cascade is responsible for the oxygenation of the treated water and the possible removal of manganese not removed in the upstream SRU.

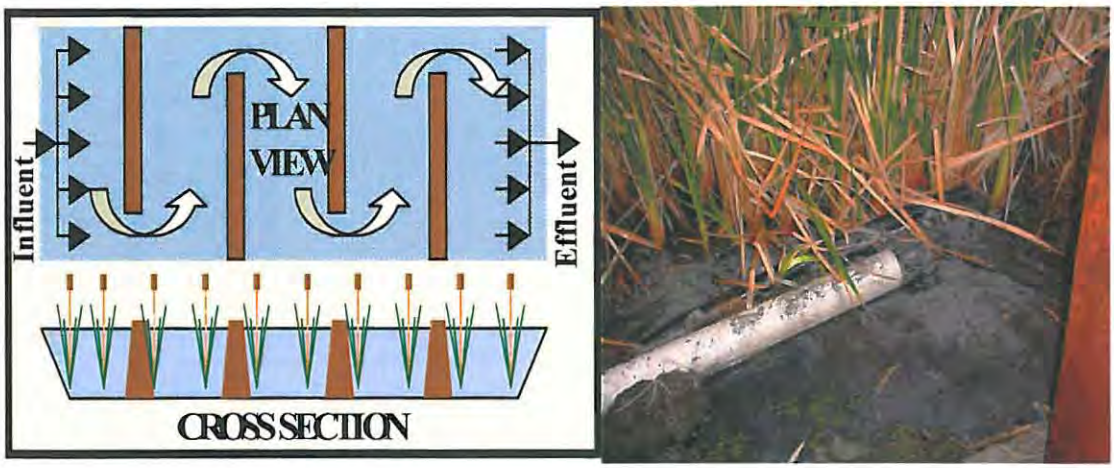


Figure 2.6: Aerobic wetland at VCC pilot plant: A: schematic drawing (Pulles, 1999); B: photograph taken at the site.

2.2.1.6. Sampling Regime and Data Acquisition

Daily Collection of Data

The pilot plant at VCC is maintained and operated by an operator appointed by PHD. Flow rate, temperature, conductivity and pH are measured on weekdays. Evaporation and rainfall are also recorded.

Monthly and Fortnightly Collection of Data

The sampling frequency and analysis done during the different phases of operation at the pilot plant are outlined in Table 2.3.

Table 2.3: Summary of sampling frequency and analyses done at VCC pilot plant

Period	Phase	Frequency	Acidity	Alkalinity	Aluminium	Ammonia as N	Biological oxygen	Calcium	Chemical oxygen	Chloride	Electrical	*Escherichia coli*	Iron	Kjeldahl nitrogen	Magnesium	Manganese	Nitrate	Nitrite	Ortho-phosphate	pH	Redox potential	Sodium	Sulphate	Sulphide	Total dissolved	Total Phosphate	Volatile fatty acids
December 1996-November 1997	1	Fortnightly			√	√		√	√			√	√		√	√							√		√	√	
December 1997-December 1998	1	Fortnightly	√	√	√	√	√	√	√	√	√		√	√	√	√	√	√	√	√		√	√		√	√	√
January 1999-November 2001	2	Monthly		√	√	√			√		√		√			√			√	√			√	√	√		
December 2001-April 2002	2	Monthly		√	√	√			√		√		√			√			√	√			√	√			
May 2002-March 2004	2	Monthly		√	√				√		√		√			√				√	√		√	√			

The sampling procedure for the influent and effluent samples of the sulphate reducing units and post-treatment facility was as follows: sulphate concentration: a 20 ml sample was preserved with 10 ml (1N) HCl; sulphide concentration: a 50 ml sample was preserved with 4 drops zinc acetate (APHA, 1989). A 1 litre sample bottle was filled to the brim and the unpreserved sample was analyzed for redox potential using an ORP probe (Hanna), pH, and alkalinity and metal concentrations.

One litre samples were collected from each SSRU. Five sub-samples were taken at different points in the SSRU to make up the one litre composite sample, whereas in the LSRU sub-samples were collected at nine different sampling points to make up the one litre composite sample. In the up flow

reactors (SSRU 3 and LSRU 2), the samples were collected just below the organic matter layer in order to measure loss due to re-oxidation of sulphide to sulphate. In the down flow reactors (SSRU 1, SSRU 2 and LSRU 1), inside samples were collected just above the organic matter layer to measure the effect of evaporation and rainfall on sulphate and sulphide concentrations (Pulles, 1999).

2.2.1.7. Data Reporting – Sulphate Load

An economic feasibility study performed by PHD indicated that in order for a passive treatment system to be viable, 60g of sulphate must be removed per cubic metre of organic matter per day (g/m^3 organic matter/d) (Pulles, 1999). Sulphate load removed was calculated using the monthly influent and effluent sulphate concentrations and measured influent flow taking the effect of rainfall and evaporation into account according to the following equations:

Sulphate load in (g/m^3 organic matter/d) =

$$\frac{[SO_4^{2-}\text{ concentration IN (mg/ℓ) x influent flow (m}^3\text{/d)}] + [SO_4^{2-}\text{ concentration in rain (mg/ℓ) x rainfall (m}^3\text{/d)}]}{\text{volume of organic matter (m}^3\text{)}} \quad \textbf{(6)}$$

Sulphate load out (g/m^3 organic matter/d) =

$$\frac{SO_4^{2-}\text{ concentration OUT (mg/ℓ) x [influent flow (m}^3\text{/d) + rainfall (m}^3\text{/d) – evaporation (m}^3\text{/d)]}}{\textbf{volume of organic matter (m}^3\textbf{)}} \quad \textbf{(7)}$$

Sulphate load removed (g/m^3 organic matter/d) =

Sulphate load in (g/m^3 organic matter/d) - Sulphate load out (g/m^3 organic matter/d)

(8)

2.2.1.8. Evaluation of Sulphate Reducing Units

The main functions of sulphate reducing units at VCC are to rapidly condition the influent by removing dissolved oxygen, establishing the desired redox conditions and producing elevated levels of sulphides and alkalinity. This process optimizes hydrolysis of the organic matter and the production of volatile fatty acids (VFA). Therefore, the effluent from such a reactor should contain reduced levels of metals and sulphate and elevated levels of sulphides, alkalinity and VFAs (van der Merwe, 2002). It was noted, however, that after a certain period of time the performance of those units had declined. Figures 2.7-2.14 illustrate the performance of the SSRU at VCC plant over the period of six years (Molwantwa *et al.*, 2003).

Sulphate Removal in the SSRU

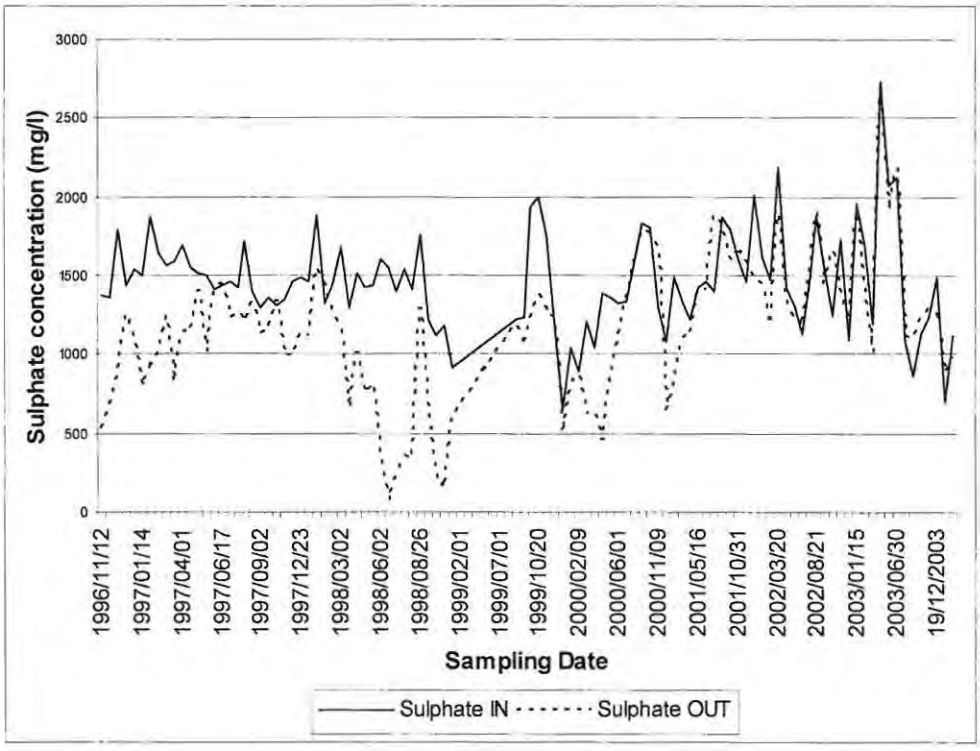


Figure 2.7: Monthly sampling dates for inlet and outlet sulphate concentration for SSRU 1 (After Molwantwa *et al.*, 2003).

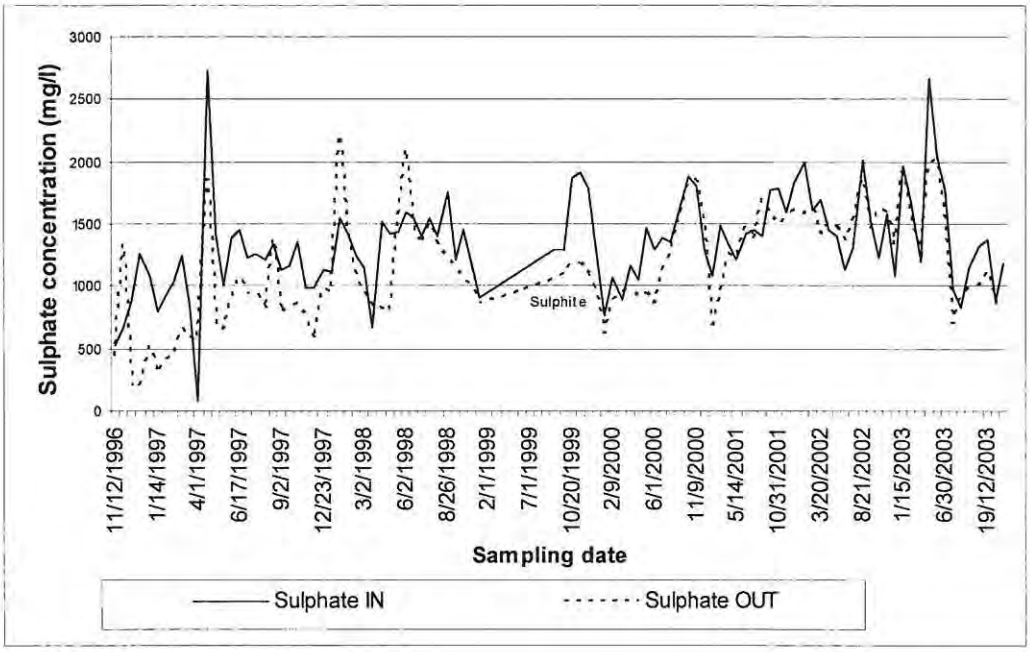


Figure 2.8: Monthly sampling dates for inlet and outlet sulphate concentration for SSRU 2 (After Molwantwa *et al.*, 2003)

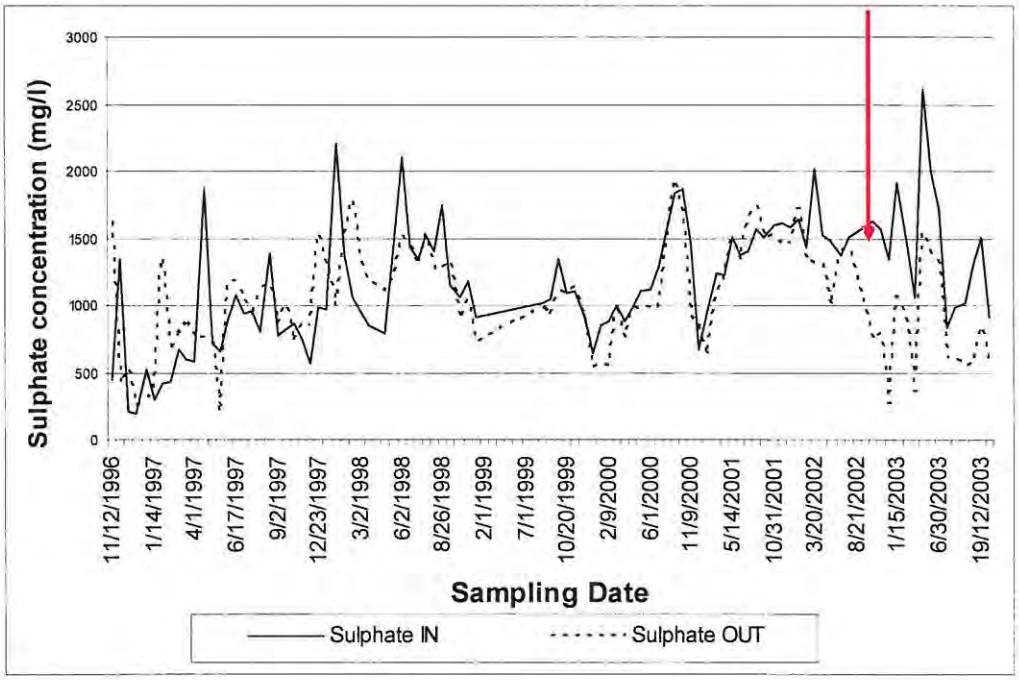


Figure 2.9: Monthly sampling dates for inlet and outlet sulphate concentration for SSRU 3. (After Molwantwa *et al.*, 2003). (Red arrow indicates time of decommissioning of the rector)

The red arrow indicates the time of decommissioning of SSRU 3 due to poor performance. The data after the arrow illustrate the performance of the reactor after recommissioning as a Degrading Packed-bed Reactor (DPBR) and the implementing of improvements suggested by the laboratory scale study reported below.

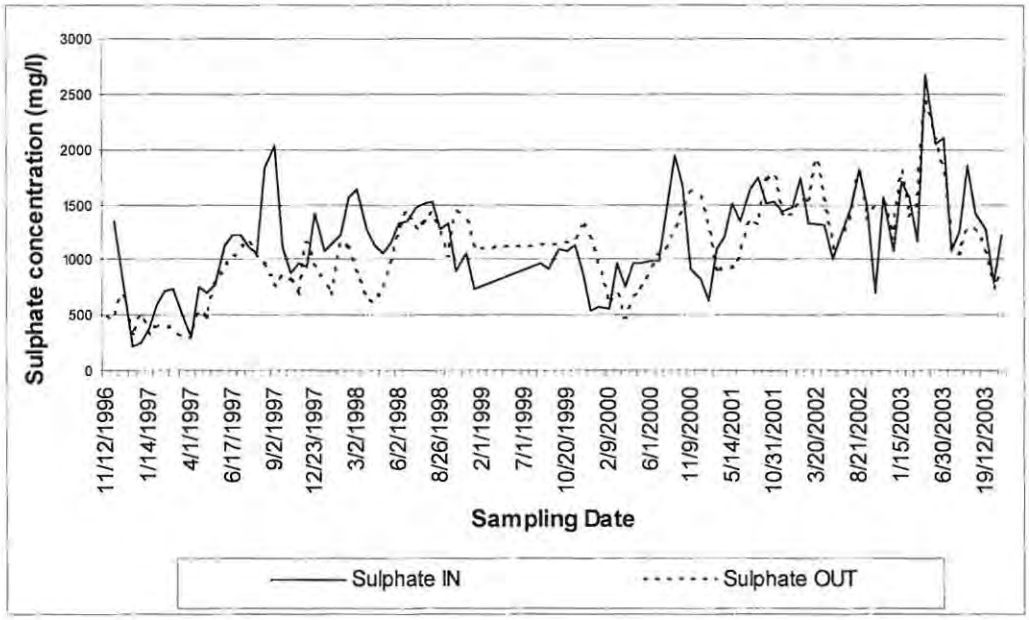


Figure 2.10: Monthly sampling dates for inlet and outlet sulphate concentration for SSRU 4. (After Molwantwa *et al.*, 2003).

It was mentioned before that in order to maintain the economic viability of this plant a removal efficiency of 60g SO_4/m^3 organic matter/day would need to be achieved. However, data for SSRU in terms of sulphate removal clearly indicate the poor performance of these units. There was evidently a decrease in removal of sulphate over a period of time (Figure 2.7-2.10). It was noted by Molwantwa *et al.* (2003), that the average sulphate removal for all the units was 27,9 g SO_4/m^3 organic matter/day, which is less that half of the target value.

2.2.1.9. Alkalinity Production in the SSRUs

$CaCO_3$ concentration was used as a measure of the alkalinity in the influent and effluent. During the process of sulphate reduction, sulphate is reduced to sulphide and bicarbonate ion is produced. Figures 2.11-2.14 illustrate the alkalinity in the SSRU at the VCC pilot plant (Molwantwa *et al.*, 2003).

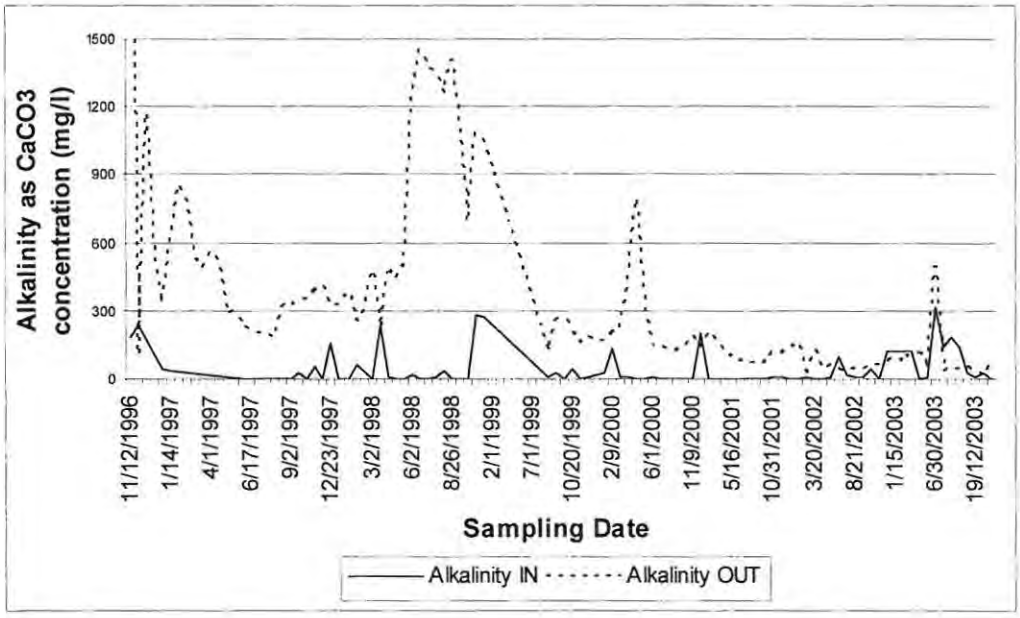


Figure 2.11: Monthly sampling dates for inlet and outlet alkalinity concentration for SSRU 1. (After Molwantwa *et al.*, 2003).

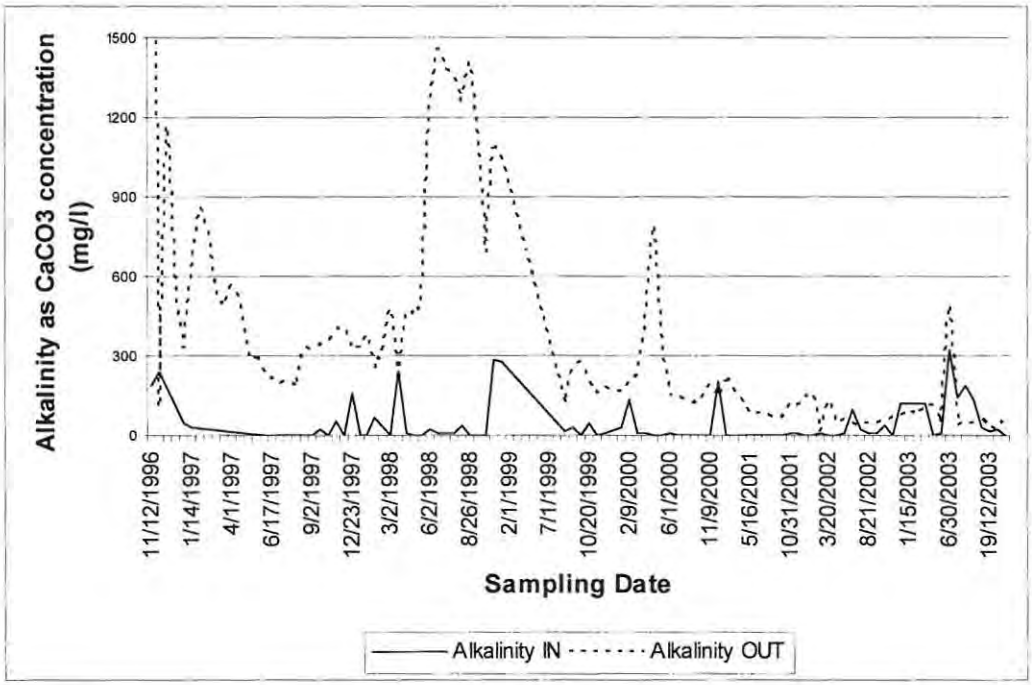


Figure 2.12: Monthly sampling dates for inlet and outlet alkalinity concentration for SSRU 2. (After Molwantwa *et al.*, 2003).

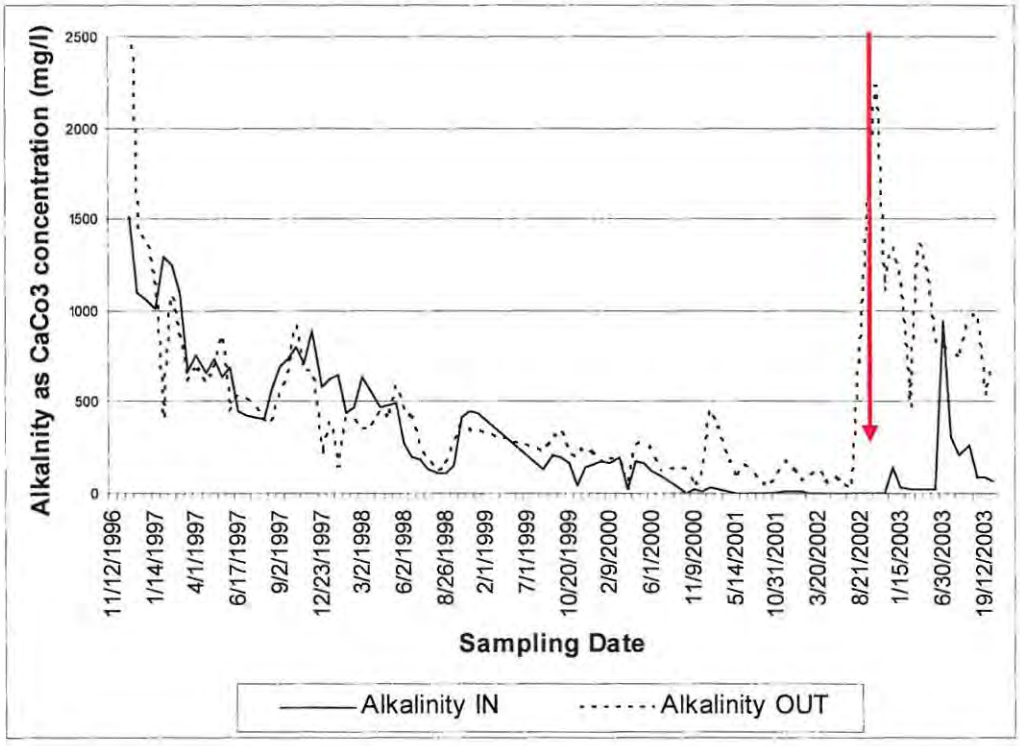


Figure 2.13: Monthly sampling dates for inlet and outlet alkalinity concentration for SSRU 3. (After Molwantwa *et al.*, 2003) (Red arrow indicates time of decommissioning of the reactor).

The red arrow indicates the time of decommissioning of SSRU 3 due to poor performance with regards to alkalinity. The data after the arrow illustrate the performance of the reactor after recommissioning as a Degrading Packed-bed Reactor (DPBR) and implementing improvements suggested by the laboratory scale study.

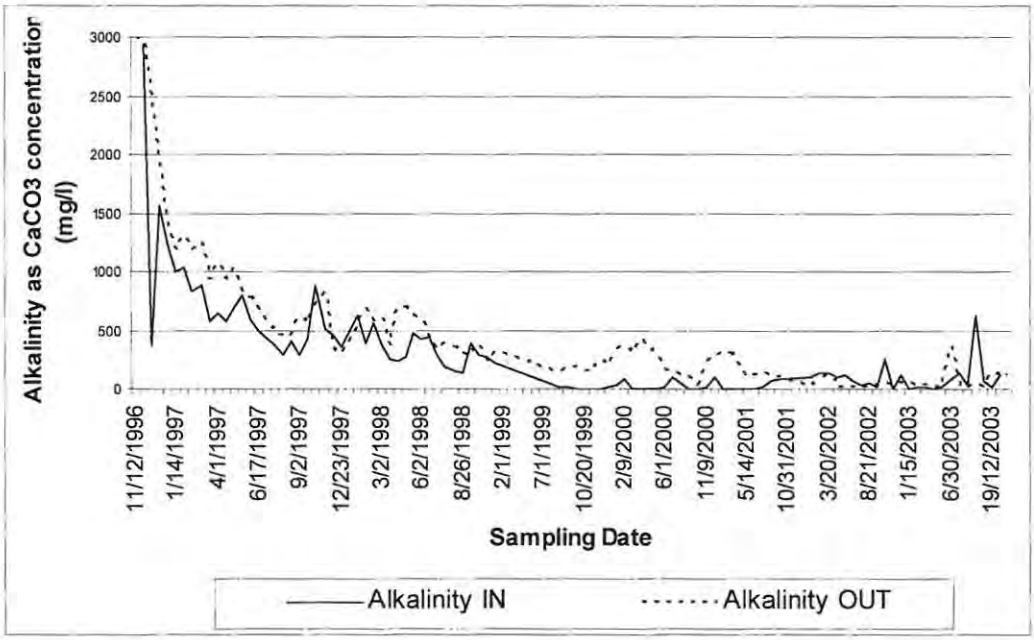


Figure 2.14: Monthly sampling dates for inlet and outlet alkalinity concentration for SSRU 4. (After Molwantwa *et al.*, 2003).

The data presented above reveal the poor performance of the SSRU. Such a high concentration of sulphates and a low alkalinity in the effluent could not warrant the continued use of the reactor. Furthermore, the performance of the units drastically declined as the time progressed. This resulted in one of the units being decommissioned and converted into a degrading packed-bed reactor.

2.2.2. Decommissioning of SSRU 3

Because of the poor performance of the plant, it was decided to decommission one SSRU in order to re-evaluate its design and implement new findings in the packing of the new reactor.

SSRU 3 was selected to be decommissioned based on the lack of long-term performance of the unit and because it was the only SSRU which was operated in series with another. Decommissioning of SSRU 3 was done between the 19th and 21st August 2002.

2.3. Sampling of Field-scale Reactor

Various samples were taken during the decommissioning of SSRU 3 in an attempt to explain the low sulphate removal efficiencies obtained. Prior to draining SSRU 3, liquid samples were taken from the water column above the organic matter surface. SSRU 3 was then emptied by means of a pump. Solid samples were taken at each of the visible organic matter layers before removing them to reach the next visible layer using manual labour. Sampling and removal of the different carbon layers were repeated until the bottom of the unit was reached.

Although 10 different layers of waste carbon were packed into the reactor at commissioning in 1996, compaction of the carbon sources occurred, making clear identification of all of the initial carbon layers impossible. Therefore a limited number of samples were taken. Figure 2.15 and Figure 2.16 illustrate SSRU No. 3 before decommissioning. It was noted that the surface of the reactor was covered with algae commonly known as duckweed (*Lemna* spp.)

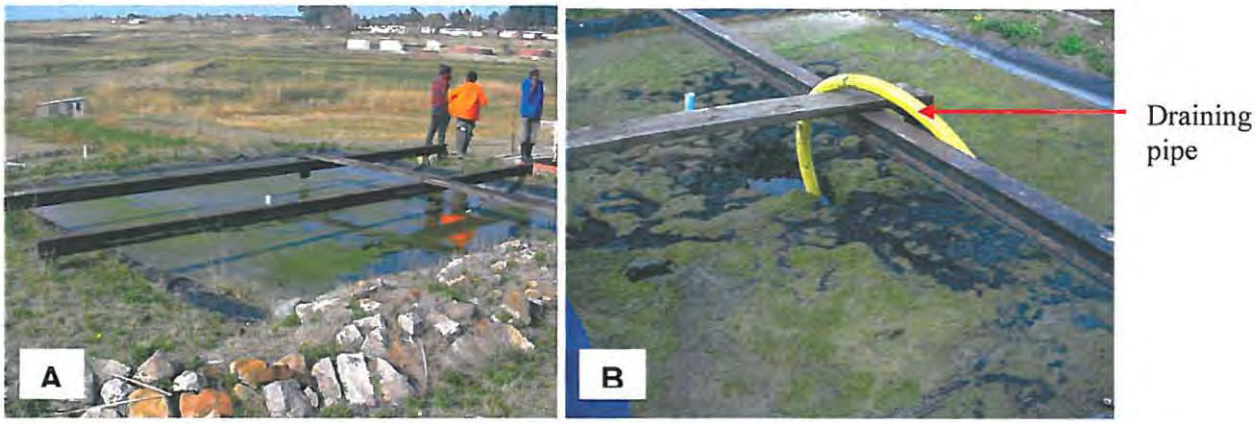


Figure 2.15: SSRU 3 before decommissioning. (A) surface of the reactor covered with actively growing duckweed, (B) close view of the same reactor.

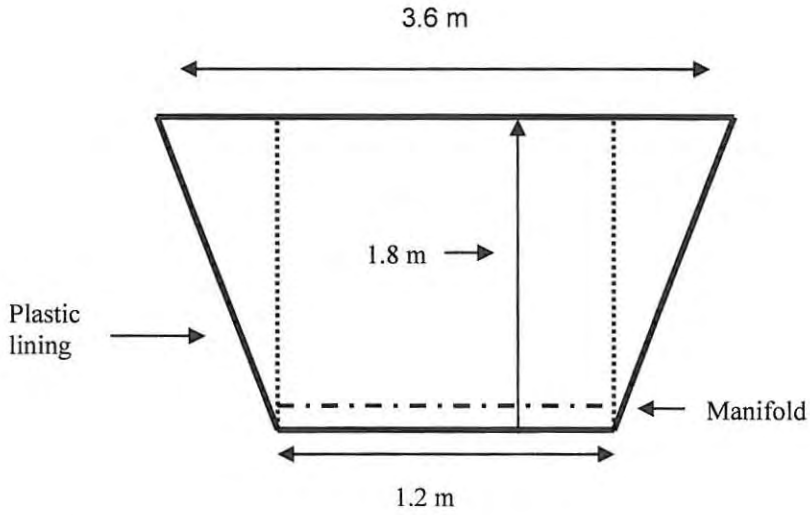


Figure 2.16: The inverted pyramid configuration of SSRU 3 before decommissioning (not to scale).

Upon the commissioning of the reactor in 1996, 10 different layers of waste material were packed into the unit. However, Figure 2.17 shows how over a period of six years of operation the compacting of the material was evident, making the identification of the various fractions difficult.

Figure 2.17: Depth profile of the waste material packed in SSRU 3 showing the evidence of heavy compaction.

Solid samples were taken from the sections where the carbon layers could still be identified. Table 2.4 lists all the samples collected for analyses.

Table 2.4: Samples collected for analysis during decommissioning of SSRU 3

Area	Sample type	Analyses
Organic sawdust	Solid	EM[1] MME[2]
Organic-wood chip	Solid	EM, MME
Organic-hay	Solid	EM, MME

1. EM = scanning electron microscopy
2. MME = molecular microbial ecology

During the decommissioning process, the first visible carbon layer was sawdust (approximately 15-20 cm thick) followed by the layer of dolerite. The thickness of the sawdust varied and appeared to be deepest at the centre (Figure 2.17). Among the sawdust samples big blocks of wood were found, which did not complied with the packing protocol. Figure 2.18 represents one of the big block of wood found in the packing material of the reactor.

Figure 2.18: A block of wood recovered during decommissioning of the SSRU 3. It was noted that the wood serving as a carbon source in the reactor had not degraded after 6 years, however, there was distinct development of bacterial biofilm.

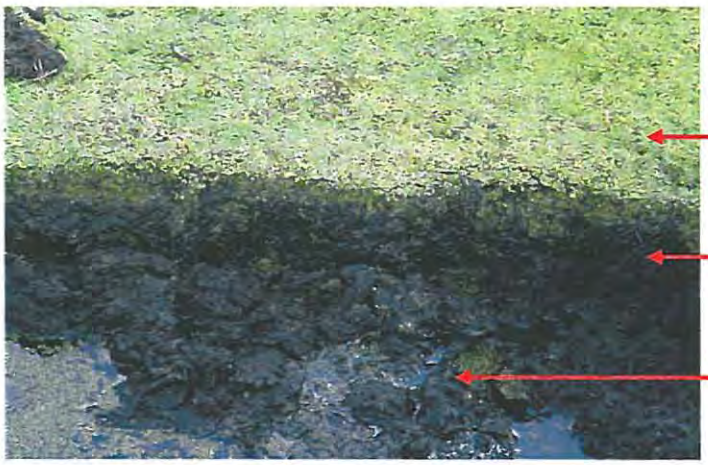


Figure 2.19: The first layer of sawdust under the algal biomass followed by the thick layer of dolerite.

It was noted during the unpacking of the reactor that the sawdust had undergone considerable visible degradation while hay remained almost intact (Figure 2.20).

Figure 2.20: A layer of hay between the dolerite showing clearly that the material was not degraded over a period of six years of operation.

2.4. A Laboratory-scale Packed-bed Reactor

The investigation of the microbial ecology in the VCC reactor identified apparent diversity in the microbial populations present. However the lack of clarity on how these organisms might interact in the system led to the design of a linear reactor system on laboratory-scale, which would facilitate the comparison of both the physico-chemical analysis and the profiling of microbial populations within the system.

2.4.1. Designing the Reactor

A laboratory-scale lignocellulose column reactor (Figures 2.21 and 2.22) was constructed of unplastisized polyvinylidine chloride (uPVC) tubing, ID 200 mm and uPVC fittings and set up in the Robertsville Laboratories of PHD in Gauteng. In order to enable easy sampling of the bioreactor, the uPVC taps were fitted at various points along the length of the reactor. Inlet and outlet

ports were fitted with distribution units (Figure 2.21 and 2.22) to ensure even distribution of the inlet and outlet water.

Some of the sampling ports were modified in order to allow the insertion of wooden rods providing a lignocellulose substrate which could be withdrawn for easy monitoring of biofilm formation without interfering with the rest of the packed column. Figure 2.23 B represents a schematic drawing of the modified port and figure 2.22 shows the bioreactor operating in the laboratory.

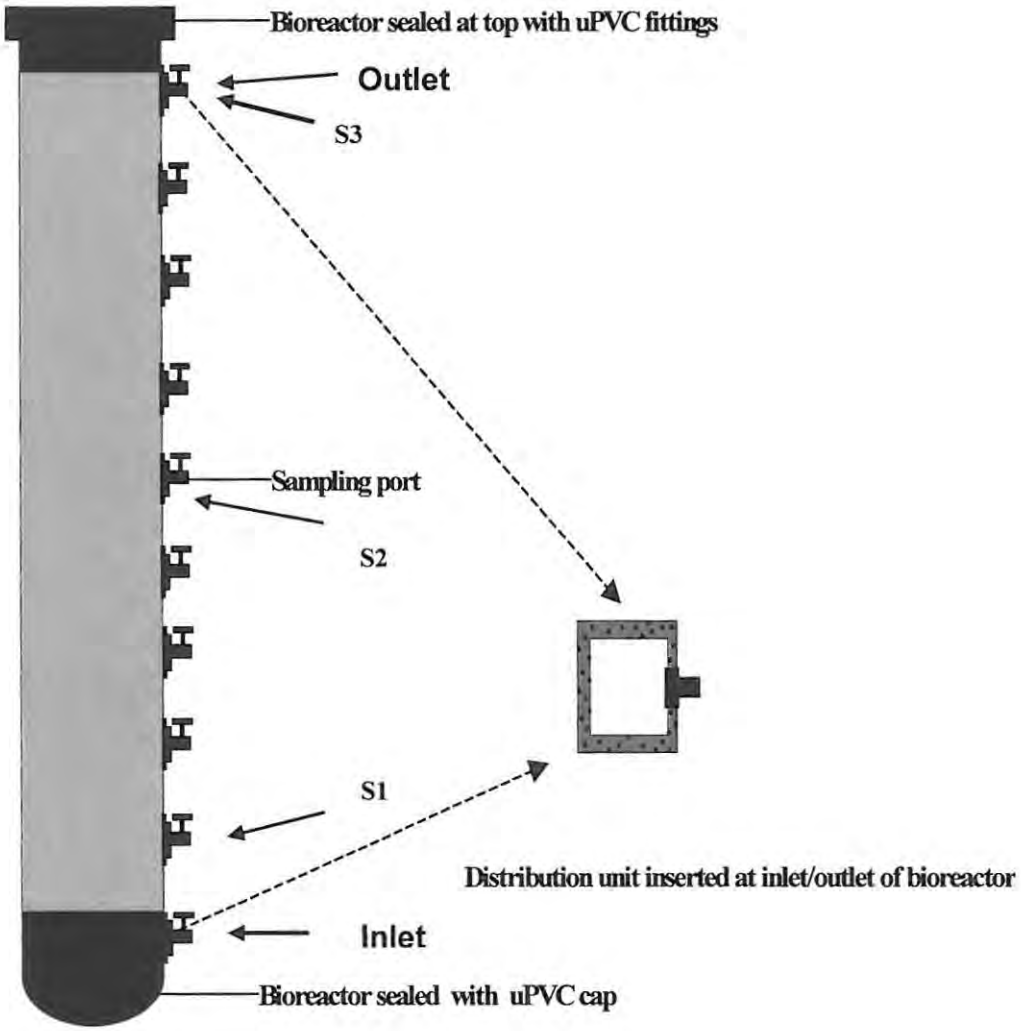


Figure 2.21: Schematic diagram of the lignocellulose laboratory-scale column reactor. S1, S2, and S3 show where modified sampling ports were inserted.

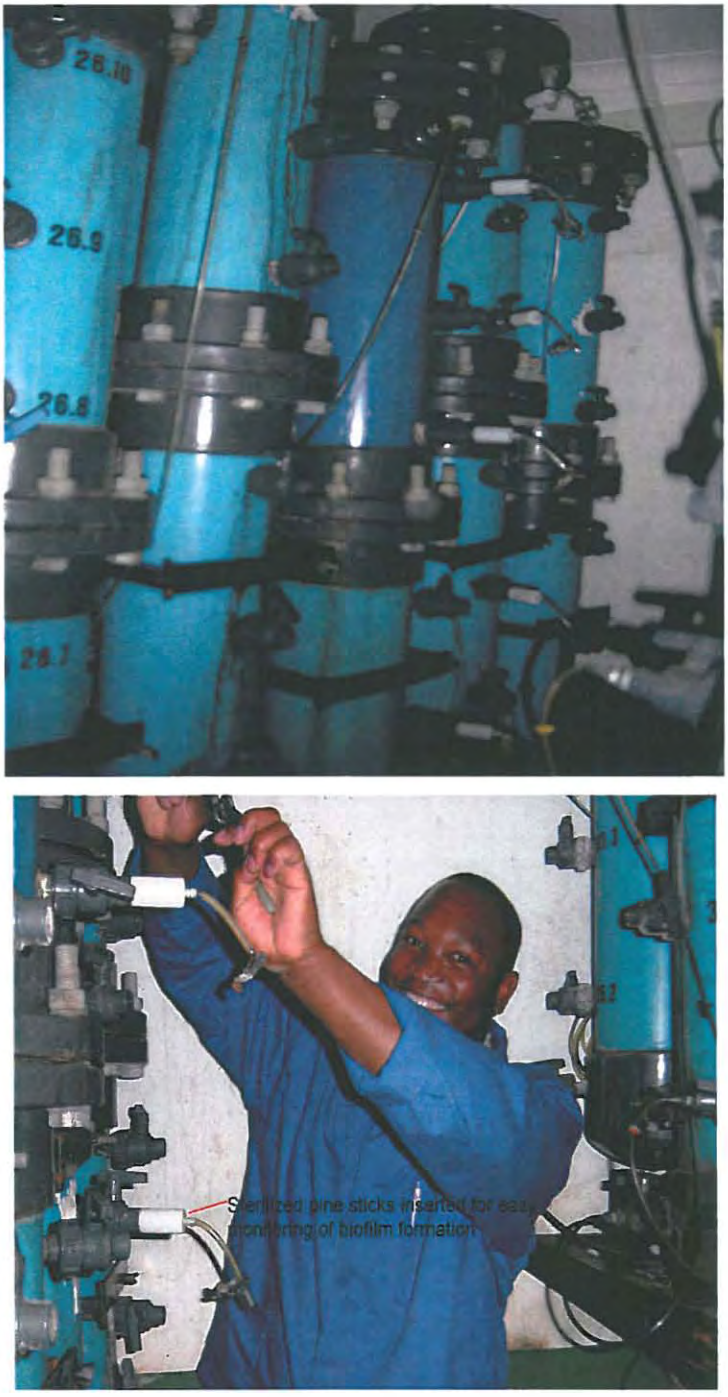
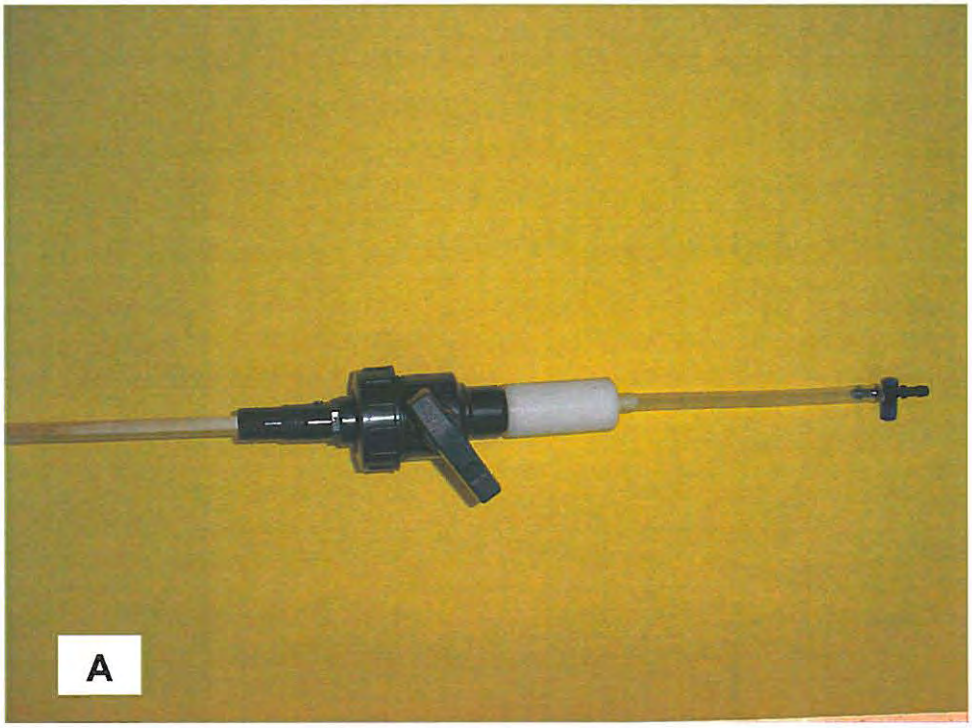
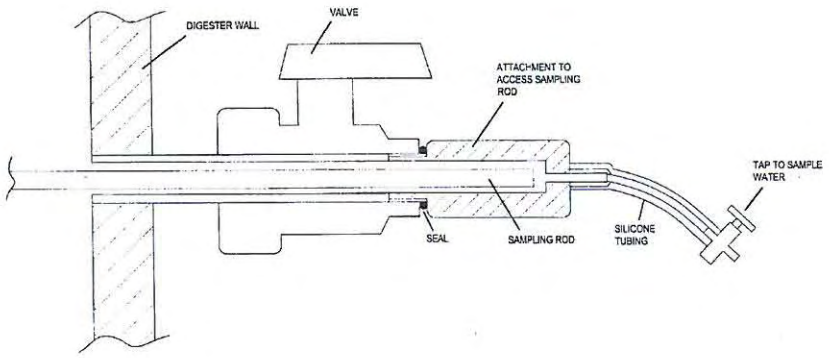


Figure 2.22: Lignocellulose laboratory-scale packed-bed reactor illustrating modified sampling ports.





B

Figure 2.23 Modified sampling port (A) photograph, (B) schematic drawing (not to scale).

The reactor was packed with three layers of the following materials:

Blue Buffels grass (*Buchloe dactuloides)*	500 grams x 3
Wattle chips of random size	500 grams x 3
Bagasse	250 grams x 3
Beads	180 grams
Inoculum (Sludge)	1000 ml x 3

The reactor was operated in an upflow configuration with a retention time calculated to be 96 hours and monitored for 273 days. After packing, seven days was allowed for bacterial colonization of the carbon source. After 81 days of operation the reactor was fed 350 ml of 1 % (v/v) molasses solution in order to commence feed supplementation.

2.4.2. Feed Water: Acid Mine Drainage (AMD)

AMD was collected from South Witbank Mine and pre-treated to neutralize the pH using Anoxic Lime Stone Drainage (ALD). Figure 2.24 below illustrates the set-up for AMD neutralization. The AMD from the feed tank was passed via a redox poising reactor (RP) to the ALD and then to the lignocellulose laboratory-scale column reactor.

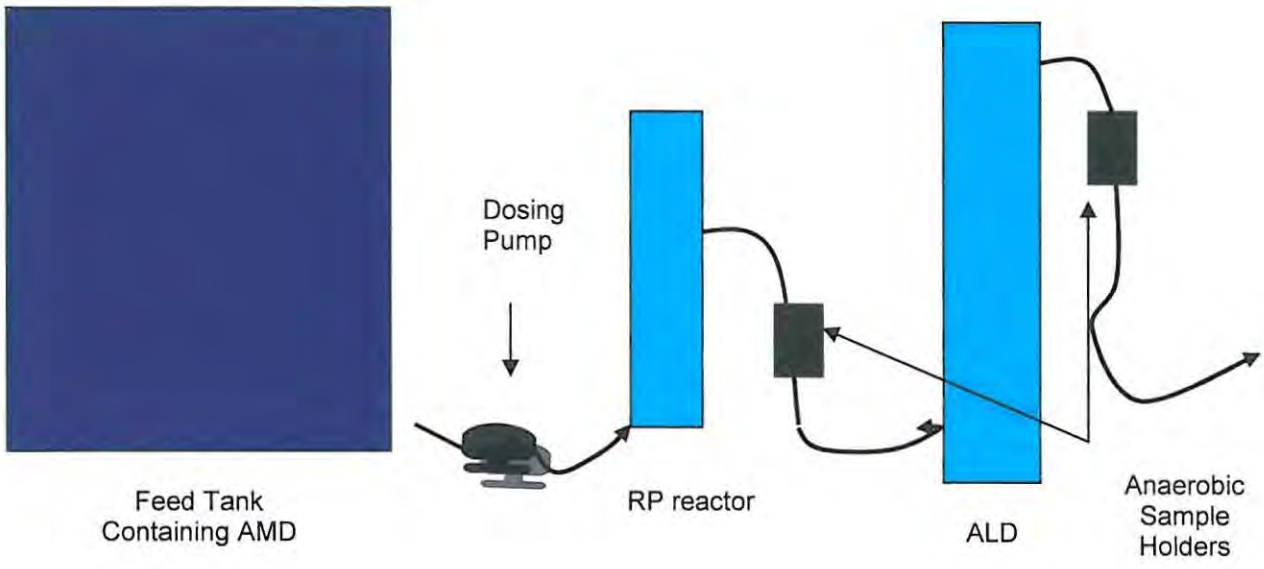


Figure 2.24: Schematic illustration of AMD pre-treatment using ALD.

2.4.3. Sampling of the Packed-bed Reactor.

The Lignocellulose Laboratory-scale Column Reactor was sampled on a daily basis and its performance monitored for sulphate and sulphide concentrations, pH, alkalinity and redox potential. Additionally, every two months over a one year period, specimens for DNA extraction and electron microscopy (EM) were collected. Sample wooden rods with the biofilm attached were removed and a section was cut using a sterile scalpel blade, placed into sterile 50 ml Falcon tubes (Laboratory & Scientific Equipment Co.) and kept on ice or at 4° C until processed (usually within 4 days) (Figure 2.25 and 2.26). For the purpose of the EM study a section of the wood with biofilm attached was placed immediately in an Eppendorf tube containing 2.5 % glutaraldehyde in phosphate buffer. At the same time, liquid samples were collected for chemical depth profile analyses in which the following were monitored:

- Sulphate removal
- pH
- Reducing sugars
- Volatalile Fatty Acids (VFA)
- Aromatic compounds

Since the objective of the project was to analyze the temporal distribution of microbiota in the reactor and comparing it to the chemical status of the reactor on the particular day of sampling. Thus only one sampling could be undertaken.

Figure 2.25: Author drawing samples from the reactor at the Robertsville Laboratory.

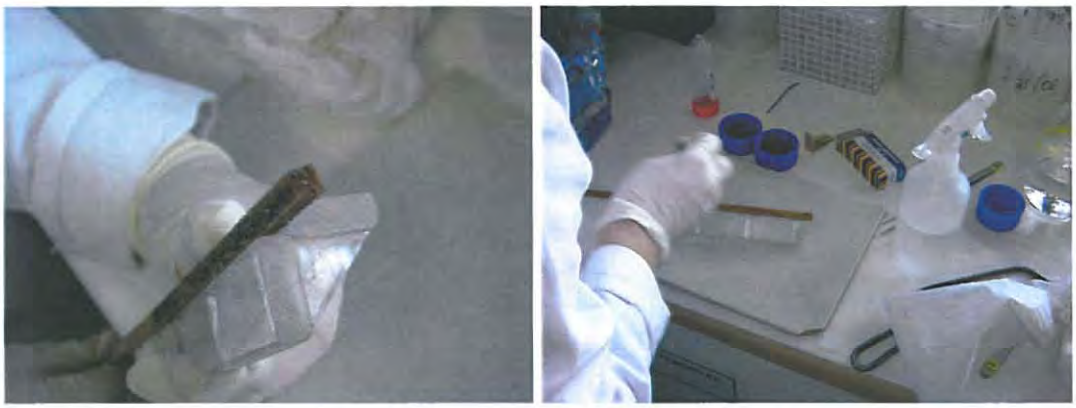


Figure 2.26: Sampling of biofilm growth which had developed on the wooden rods drawn from the column at regular sampling intervals.

2.4.4. Analytical Methods

2.4.4.1. Reducing Sugars

Reducing sugars were measured using the method of Wood and Bhat (1988). A 100 µl sample was added to 150 µl of dinitrosalicylic acid (DNS) reagent (Appendix G). The reaction mixture was heated at 100° C for 10 minutes. Following heating, 1 ml of distilled water was added to the reaction vessel and thereafter the absorbance was read at 540 nm. Absorbance values after subtraction of the reagent blank were then translated into glucose equivalents using a glucose standard curve (Figure 2.27).

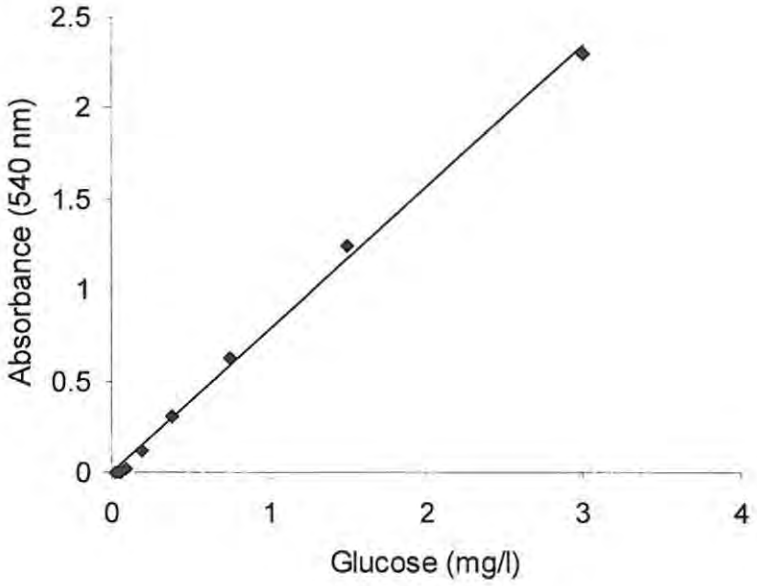


Figure 2.27: Standard curve for the determination of reducing sugars using the method of Wood and Bhat, 1988. Glucose was used as the standard (r^2 = 0.997).

2.4.4.2. Sulphate Analyses

Sulphate concentrations were determined using high performance liquid chromatography (HPLC) with a Hamilton PRP-X100 10 µm column (15mm x 4.1mm). The mobile phase was 4mM p-hydroxybenzoic acid, 2.5% methanol, pH 8.5. The flow rate was 2 ml/min. maintained with a Waters 510 pump and a Waters 430 conductivity detector was used. The software used for data analysis was BORWIN version 1.5x. Prior to chromatography, the samples were cleaned by mixing in a 1:1 ratio sample: ZnAc (10.44 %) to allow for removal of sulphide, followed by filtration through 0.45 µm nylon filter then passing it through a 25 mg C_{18} Isolute solid phase extraction column to bind contaminating organics (Rein, 2002). Sulphate concentrations were determined using a sulphate standard illustrated in Figure 2.28.

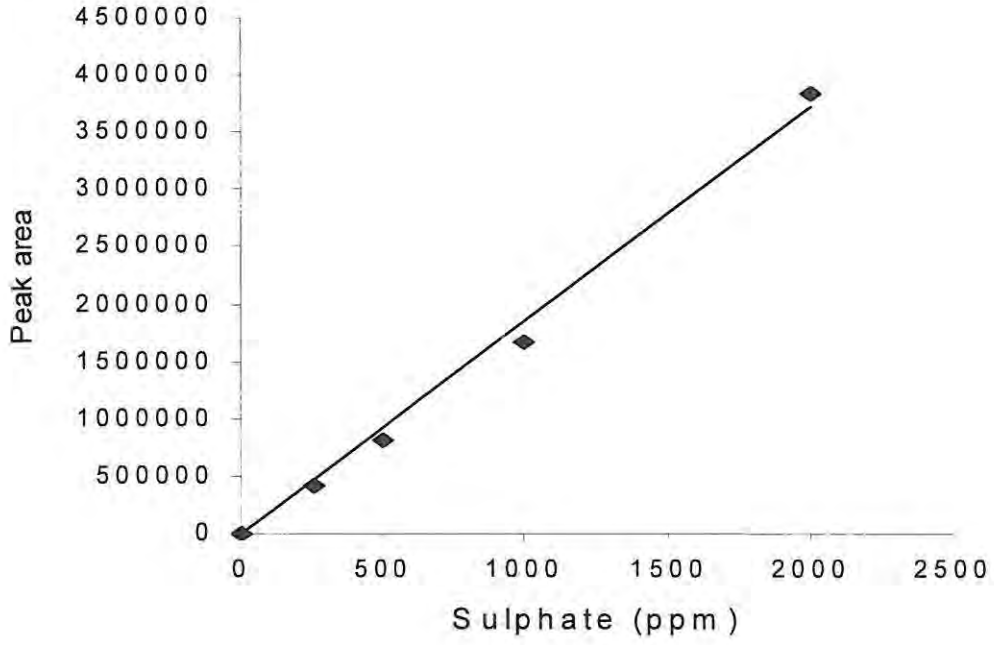


Figure 2.28: Standard curve for the determination of sulphate (r^2 = 0.9933).

2.4.4.3. Aromatic Compounds

Reversed–phase HPLC was used to analyse aromatic compounds released from lignin in the laboratory-scale column reactor. Analysis was done using a Beckman System Gold HPLC, module 126, with detection by Photo–diode array (PDA), module 168. A Waters Symmetry C_{18} column (4.6 x 250 mm) with 5-µm particle size was used for analysis with readings made at 310 nm. The mobile phase was composed of acetonitrile: water in the ratio 60:40 with the water containing 0.1 % acetic acid. Naphthalene was used as the internal standard (Figure 2.29). Flow rate was 1 ml/min.

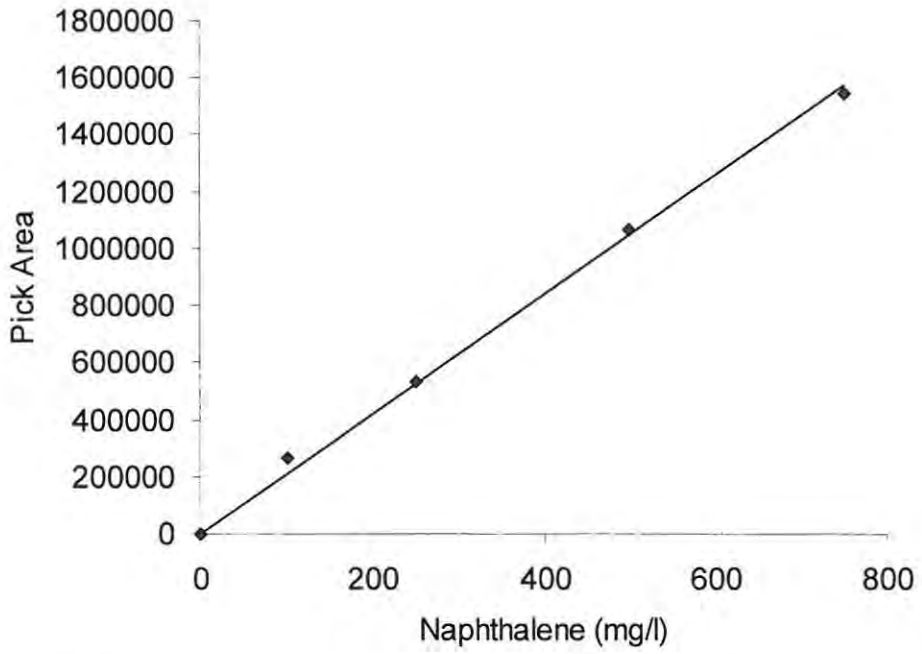


Figure 2.29: Standard curve of naphthalene, used as internal standard for the determination of aromatic compounds (r^2 = 0.9971).

2.4.4.4. Volatile Fatty Acids

Gas chromatography (GC) was used to determine the concentration of volatile fatty acids in the samples. Analyses were performed using Hewlett Packard 5890 Gas Chromatograph. To 1 ml of sample 0.5 ml of DCM was added. The mixture was centrifuged at 3000 rpm for 3 minutes. The supernatant was filtered through 0.45 µm membrane filter (Whatman) and samples were injected into GC at initial temperature of 40° C, initial time 1 minute, rate 4° C per minute and final temperature of 200° C. The ethyl butyrate was used as an internal standard. The ratio of the area under the curve of results was compared to the initial standard (Figure 2.30).

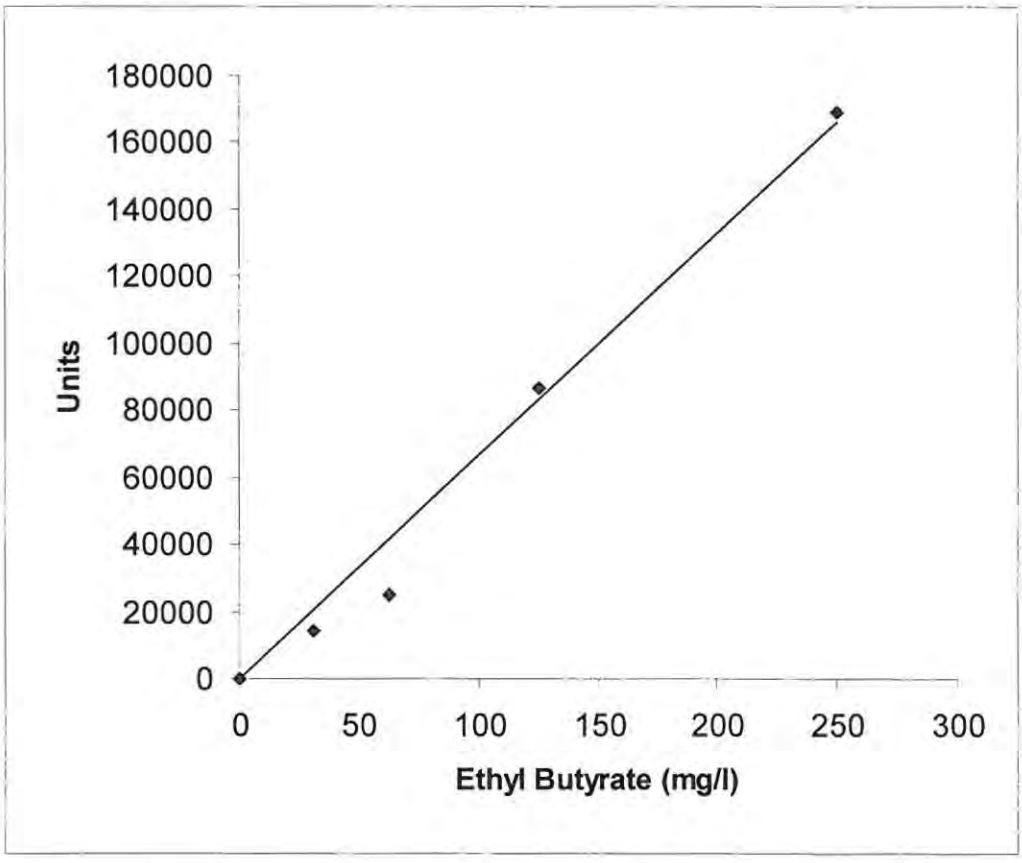


Figure 2.30: Standard curve of ethyl butyrate, used as internal standard for the determination of volatile fatty acid (r^2 = 0.9828).

2.4.5. Microscopy

2.4.5.1. Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM)

The samples of hay, sawdust and wood were prepared for scanning electron microscopy (SEM) and transmission electron microscopy by fixing in 2.5 % glutaraldehyde and stored on ice or at 4° C until examination. They were processed as described in Appendix F1 and F2 respectively.

2.4.5.2. Fluorescent In Situ Hybridization

Environmental samples were pre-treated by the addition of chemical fixatives in order to kill the cells, to preserve their morphology, and to make them accessible for oligonucleotide probes. For Gram-negative bacteria a paraformaldehyde based fixative (Appendix F 3.2) fulfils these requirements and allows the samples to be stored at –20° C for several years (Amann *et al.*, 1995). Fixation of Gram-positive cells creates a problem (Wagner *et al.*, 1994) and the methods still have to be developed. However, most Gram-positive organisms can be successfully permeabilized for FISH by the addition of 50 % ethanol (Roller *et al.*, 1994). Others, for example microorganisms containing mycolic acid in their cell wall, require additional pretreatment including the use of cell wall lytic enzymes (Davenport *et al.* 2000). Fixed samples were then hybridized with probes labeled with fluorescent dye (Appendix F) and examined using a confocal laser scanning microscope (Zeiss LSM 510) based at the Munich Technical University in Freising.

In this study the specimen from the sampling rods was prepared on Teflon coated slides and dried for about 10 min at 46° C. Following drying, the specimen was dehydrated on the slide using an increasing ethanol series (3 min each in 50, 80, and 100 % ethanol). Ten microlitres of hybridization buffer

(Appendix F 3.3) was placed into the wells on top of the dehydrated specimen. One microlitre of probe, SRB 385 specific for most of sulphate reducing bacteria (Amann, *et al.,* 1990) (Appendix F 3.5) labelled with Cy3 was added to the dehydrated specimen and the slide was transferred into the hybridization chamber and incubated at 46° C for 1.5 hours. Following the hybridization the slide was rinsed with the washing buffer (Appendix F 3.3) and incubated in the washing buffer at 48° C for 10 min. The slide was rinsed with distilled water and then quickly dried. The dried specimen on the slide was embedded with embedding liquid and covered with a cover slip. The preparation was analyzed using confocal laser scanning microscopy.

2.5. Molecular Microbial Ecology

2.5.1. Total Nucleic Acid Extraction.

The first step in successful genomic DNA extraction from environmental samples involves optimization of the extraction protocol. Due to the many inhibitors present in environmental samples and the unpredictable behaviour of microbial consortia, the most stringent conditions of extraction are employed in order to target the entire population. Many microorganisms in nature, in order to survive harsh environmental conditions, have developed a tough cell wall, which generally results in low permeability of chemicals used for DNA extraction. Therefore, the first step towards successful bacterial DNA extraction involves lysis of the cell.

Conventional methods for the lysis of the bacterial cells make use of an enzyme such as lysozyme, and detergents such as sodium dodecyl sulphate (SDS), Tween 20, Nonidet P-40 and Triton X-100 (Hermans *et al.,*1990; Buck *et al.,* 1992; Hass *et al.,* 1993; Plikaytis *et al.,* 1993; van Scoolingen *et al.,* 1994).

Mechanical methods have also been used to lyse bacterial cells. A number of protocols utilize glass beads in conjunction either with vortexing (Plikaytis *et al.,* 1993) or sonication (Folgueira *et al.,* 1993). Extremes of temperatures have also been used, with repeated freeze-thaw cycles being followed by boiling. It was found that up to 99.8 % lysis could be achieved by extended lysis incubations and up to 6 freeze-thaw cycles (Buck *et al.,* 1992).

Once cells have been lysed, conventional protocols usually include a number of steps to remove cellular debris, in particular bacterial protein. This can be done by using a nonspecific protease like proteinase K, plus phenol, chloroform and isoamyl alcohol (Hermans *et al.,* 1990). A number of commercial DNA extraction kits have also been developed (for example, Roche, Qiagen, Promega). They usually capture any PCR inhibitors released during the extraction process, thereby providing a PCR ready product in much shorter time as compared to the conventional protocols. However, the yield of recovered DNA by that method was not considered satisfactory for the purpose of this study.

Phenol-chloroform Protocol for Purified DNA

Prior to extraction, samples from wood, hay and sawdust were vortexed in the presence of 2 mm sterilized glass beads, in order to dislodge the biofilm from the carbon source. Following that, 2 ml samples were aseptically aliquoted into sterilized microcentrifudge tubes (Eppendorf, Merck) and pelleted by centrifugation at 13 000 rpm for 5 minutes in an Eppendorf 5415D desktop centrifuge. The supernatant was discarded and the pellets were washed once with 2 x buffer A (Appendix A 2.7), and one part 50 % glycerol (Bond *et al.,* 2000), and then resuspended in 0.5 ml of 2 x buffer A. It was necessary to include this washing step as the low pH of the specimen may cause the hydrolysis of DNA, and a high concentration of metals could contaminate the extracted DNA and have an inhibitory effect on subsequent PCR analyses.

Polyadenilic acid (Appendix A 2.9) and lysozyme (Appendix A 2.8) were added to the suspension and the sample was incubated with shaking for 3 hours at 37° C. Samples were subjected to four cycles of freezing in liquid nitrogen followed by heating for one minute at 80° C. SDS and Proteinase K (Appendix A 2.1 and 2.3) were added to the mixture, and this mixture was incubated overnight at 37° C. One hundred µl of 10 % CTAB (Appendix A 2.2) and 200 µl of 5M NaCl (Appendix A 2.4) were then added and the samples were incubated at 55° C for 1 hour. The samples were then aliquoted into 500 µl volumes in sterile Eppendorf tubes and the cell lysate was extracted with phenol-chloroform-isoamyl alcohol (24:24:1) (Appendix A 2.5). Nucleic acids were precipitated with 2.5 volumes of ice cold 96% rectified ethanol overnight at -20° C, pelleted by centrifugation and resuspended in TE buffer (Appendix A 2.6). The DNA samples were placed at 4° C for short-term storage. Duplicate DNA samples were stored at -20° C.

2.5.2. DNA Quantification

Quantification of the DNA is essential as a starting point for the subsequent optimization of the DNA template in the PCR reaction. DNA quantification may be done in a number of ways. The most common method is a spectrophotometric analysis at an optical density of 260 nm (Plikaytis *et al.* 1993; Harn *et al.,* 1997). Many molecular researchers prefer quantification of DNA by means of electrophoresis in the presence of ethidium bromide (Appendix C 2.2) (Sambrook *et al.,* 1989). Ethidium bromide contains a planar group that intercalates between the stacked bases of DNA, and the amount of ultra-violet-induced fluorescence subsequently emitted is proportional to the total mass of DNA present in the sample (Sambrook *et al.,* 1989). In this method a sample of DNA is electrophophoresed in the presence of a series of standards of known concentration, or of a molecular weight marker, which produces a ladder of bands of known concentration. The quantity of DNA in the sample can then be estimated by comparing the fluorescent yield of the sample with that of the molecular weight marker bands

or by comparison to the fluorescence of the standards (Ausubel *et al.*, 1987; Sambrook *et al.*, 1989).

The method of choice in this project was by spectrophotometric determination. The readings were taken at wavelengths of 260 and 280 nm. The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample. An OD of 1 corresponds to approximately 50 μg/ml for double-stranded DNA and 40 μg/ml for single-stranded DNA and RNA. The 280 nm reading determines the amount of protein in the sample, therefore the ratio between the readings at 260 and 280 nm (OD_{260}/OD_{280}) provides an estimate of the purity of nucleic acid. A pure preparation of DNA should have an OD_{260}/OD_{280} value of between 1.8 to 2.0. A ratio of less than 1.8 indicates contamination with protein or phenol, and then an accurate quantification of the amount of nucleic acid using spectrophotometric method is not possible. Based on spectrophotometric reading, the amount of DNA in a sample is calculated according to the following formula:

DNA concentration = OD_{260} x 50 μg/ml x Dilution factor (9)

2.5.3.DNA Amplification: Polymerase Chain Reaction

Optimization of PCR Reaction

The development of the polymerase chain reaction has enabled rapid and efficient analysis of specific DNA sequences (Mulllis and Faloona, 1987). Optimization of PCR is extremely important for the production of large quantities of a single, specific amplification product and for obtaining reproducible and reliable data (Welsh and McClelland 1990). Variation in the concentration of the components in the PCR reaction may result in the production of different products from any one sample of DNA.

Welsh and McClelland (1990) found that magnesium chloride concentration, primer annealing temperature, template DNA purity, primer length and primer sequence all affected the number, reproducibility and intensity of any bands produced. Variation may also arise from using different thermocyclers and different PCR cycle conditions (Mullis *et al.*, 1994). Many investigators have found that different DNA polymerases may also influence the amount of product produced. Therefore, such sensitivity to reaction conditions makes it imperative to optimize PCR reactions thoroughly and ensure that all parameters are standardized for all the samples. It is also important to establish the reproducibility of the results obtained under the optimized conditions (Mullis *et al.*, 1994).

Primers; design, concentration, annealing temperature and primer extension

The key to successful PCR lies in the design of appropriate primers. Typical primers are of 18 to 28 nucleotides in length and have 50 to 60 % G + C composition. This length of primer is usually selective enough to specify a single site in the genome. However, longer primers and annealing at higher temperature can make the PCR reaction more selective. The melting temperature (T_m) of 40mer primer is estimated to be 15° C higher than that of 20mer oligonucleotide of similar base composition (Sambrook *et al.*, 1989). An applicable annealing temperature is usually 5° C below true T_m of the amplification primers (Innis *et al.*, 1988). Increasing the annealing temperature enhances discrimination against incorrectly annealed primers and reduces misextension of incorrect nucleotides at 3'end of primers. Therefore, stringent annealing temperatures, especially during the first cycles of PCR, will favour an increase in specificity. Many investigators, for maximum specificity in the initial cycle, add *Taq* DNA polymerase after the first denaturation step during primer annealing (so called hot-start PCR). However, recent advances in designing and formulating DNA polymerases do

not necessitate interruption of the PCR reaction (Hot Start ™*Taq* Polymerase).

Primer Extension

Extension time depends mainly upon the length and concentration of the target sequence and upon temperature. Primer extensions are usually performed at 72° C. Estimates for the rate of nucleotide incorporation at 72° C vary from 35 to 100 nucleotides per second depending upon the buffer, pH, salt concentration and the nature of the DNA template (Innis *et al.,* 1988; Saiki and Gelfand, 1989). An extension time of one minute at 72° C is considered sufficient for products up to 2 kb in length. However, longer extension times may be helpful during early cycles, when product concentration exceeds enzyme concentration (Innis, *et al.*,1988).

Primer Concentration

The optimal primer concentration is generally between 0.1 and 0.5 µM. Higher concentrations have been found to promote mispriming and accumulation of nonspecific products as well as increasing the probability of generating a template-independent artifact like primer-dimers. Nonspecific products and primer-dimer artifacts are themselves substrates for PCR and compete with the desired product for enzyme, dNTPs and primers resulting in a lower yield of the desired product.

Fidelity of Amplification

According to Eckert and Kunkel (1990) DNA polymerase makes errors at a low but finite rate that varies depending on the enzyme, condition of the reaction, and the sequence. The errors produced by *Taq* DNA polymerase are mainly single-base substitutions. The rate can be higher than 10^{-3} per

nucleotide at high Mg^{2+} and high nucleotide concentrations, and less than 10^{-6} per nucleotide under other conditions (Eckert and Kunkel, 1990). Other enzymes with proof reading activity are believed to produce errors at a much lower frequency than the *Taq* DNA polymerase (Eckert and Kunkel, 1990).

Enzyme Concentration

The recommended concentration range for *Taq* polymerase is between 1 and 2.5 units per 100 µl reaction when other parameters are optimal (Chamberlain *et al.*, 1988). However, enzyme requirements may vary with respect to the manufacturer of the enzyme as well as individual target templates or primers. Innis and Gelfand (1990) recommend testing enzyme concentrations ranging from 0.5 to 5 units per 100 µl reactions and assaying the results by gel electrophoresis. If the enzyme concentration is too high, non-specific background products may accumulate, and if too low, an insufficient amount of desired product is obtained.

Deoxynucleotide Triphosphates (dNTP)

Deoxynucleotide concentrations between 20 and 200 µM result in an optimal balance between yield, specificity and fidelity. The four dNTPs should be used at equivalent concentrations to minimize misincorporation errors. Both the specifity and fidelity of PCR are increased by using lower dNTP concentrations in order to minimize mispriming at nontarget sites and to reduce any extension of misincorporated nucleotides (Innis *et al.*, 1988).

Magnesium Concentration

It is essential to optimize the magnesium ion concentration. According to Welsh and McClelland (1990) the magnesium concentration may affect all of the following: primer annealing, strand dissociation temperatures of template

and PCR product, product specificity, formation of primer-dimer artifacts and enzyme activity and fidelity. *Taq* DNA polymerase requires free magnesium in excess of that bound by template DNA, primers and dNTPs. Accordingly, PCRs should contain 0.5 to 2.5 mM magnesium over the total dNTP concentration (Welsh and McClelland, 1990; Innis and Gelfand, 1990).

Denaturation Time and Temperature

The most likely cause for failure of a PCR reaction is incomplete denaturation of the target DNA. Typical denaturation conditions are 95° C for 30 seconds or 97° C for 15 seconds, however, higher temperatures may be appropriate, especially for G + C rich targets.

In addition to the problem of reproducibility, great care must be taken to eliminate the effect of contamination. However, it has been shown that contaminating DNA, because it is usually present in much smaller quantities than the sample DNA, may not be able to compete against the latter for primer and will therefore not always amplify to sufficient levels to be visible as a band on the gel. However, dealing with mixed populations and using general primers, which anneal to all bacterial species (16S rRNA genes), creates a larger risk of contamination especially in the environmental microbiology laboratory, where exposure to undesirable microorganisms is very common Thus, it is essential that a "blank" PCR reaction, i.e. one containing sterilized water in place of DNA template, be included with each PCR experiment. The appearance of bands in the blank invalidates the results of such an experiment.

Polymerase Chain Reaction Amplification of the Bacterial 16S rRNA gene

In this study, in order to ensure that the PCR products were accurate copies of the target sequences, the Expand™ High Fidelity PCR System from Roche

was used to perform PCR reaction. Expand™ High Fidelity PCR System is composed of a unique enzyme mix containing thermostable *Taq* DNA polymerase and *Pwo* DNA polymerase. This polymerase mixture is designed to give high yield and high specificity products from genomic DNA. In addition, due to inherent 3'- 5' exonuclease proofreading activity of *Pwo* DNA polymerase, Expand™ High Fidelity PCR System results in a 3-fold increased fidelity of DNA synthesis when compared to *Taq* DNA polymerase alone. Bacterial 16S rRNA genes were amplified by PCR in 25 µl reaction containing approximately 30 ng of purified DNA per ml, 1 x PCR buffer, 200 µM concentration of each of the four deoxynucleoside triphosphates, 2.5 mM $MgCl_2$, 0.4 U of Expand™ High Fidelity PCR System (Roche Biochemicals), 350 mM reverse and forward primers per microlitre. Both primers GM5 F (Appendix B 2.1) and 907 R, incorporating GC-clamp at the 5'-end of the primer (Appendix B 2.2) was obtained from Integrated DNA Technology (IDT), USA. The primers, GM5 forward and 907 reverse (Muyzer *et al.*,1995) were used to amplify a 586 bp rDNA fragment, corresponding to position 341-927 in the 16S rDNA of *Escherichia coli*. The choice of primers was dictated by the aim of the experiment, where the goal was to target the total microbial population in the system, rather than functional organisms. Amplification was performed in a Hybaid PCR Sprint thermocycler using a touch-down PCR procedure (Table2.5).

Table 2.5: Detailed touch-down program used for PCR amplification.

Reaction	Temperature	Duration	No of cycles
Initial Denaturation	95°C	2 minutes	1 cycle
Denaturation	94°C	30 seconds	
Annealing	68°C	45 seconds	
Extension	72°C	2 minutes	4 cycles
Denaturation	94°C	30 seconds	
Annealing	66°C	45 seconds	
Extension	72°C	2 minutes	4 cycles
Denaturation	94°C	30 seconds	
Annealing	64°C	45 seconds	
Extension	72°C	2 minutes	4 cycles
Denaturation	94°C	30 seconds	
Annealing	62°C	45 seconds	
Extension	72°C	2 minutes	4 cycles
Denaturation	94°C	30 seconds	
Annealing	60°C	45 seconds	
Extension	72°C	2 minutes	12 cycles
Final extension	72°C	5 minutes	1 cycle

The PCR product was analyzed on 1 % agarose gels (Appedix C 1) containing ethidium bromide (Appendix C 2.2), visualized using a UV transiluminator and photographed using a Kodak Digital Camera system. PCR product was purified using QIAquick PCR purification columns (Qiagen) for further analysis.

2.5.4. Denaturing Gradient Gel Electrophoresis of the PCR Product

Denaturing Gradient Gel Electrophoresis (DGGE) is frequently applied in microbial ecology to compare the structure of complex microbial communities and to study their dynamics. The technique is based on the reduction in DNA fragment mobility in a dense medium when part of the double helix unravels. Strand separation is induced by an increase in the concentration of chemical

denaturants such as formamide and urea. The method offers a high probability of detecting any difference between two sequences. The fragments can be recovered from the gel and used in further analysis such as sequencing (Myers *et al.*, 1985; Myers *et al.*, 1987).

A typical DGGE gel is a polyacrylamide gel with a gradient of urea and formamide increasing from the top to the bottom of the gel, parallel to the direction of electrophoresis. During electrophoresis the chemical denaturants induce melting and strand separation of the DNA fragments. The melting temperature (T_m) is dependent on the base composition of the fragment. In general, AT-rich fragments have a lower T_m than GC- rich fragments. In order to modify the DNA melting behaviour and prevent the double helix from separating completely it was recommended by Myers *et al.* (1985) that one of the primers should incorporate a GC-rich sequence (GC clamp). Individual DNA molecules denature along their length adjacent to the GC clamp according to their melting characteristics. Once the melting point of the fragment is reached, the migration of that fragment in the gel stops. Therefore sequence variants of particular fragments stop migrating at different positions.

Optimization of DGGE

In order to determine the optimal conditions for the separation of a 568 bp PCR fragment amplified from a 16S rRNA gene, a perpendicular gel was set up with 0-100 % urea/formamide denaturant (Appendix C 3.2.1 and C 3.2.4). It was established that approximately halfway across the gel, the fragment had stopped migrating as its melting point that is the optimum denaturant concentration had been reached.

DGGE Analyses of 568 bp Fragments

PCR products were analyzed in DGGE gels consisting of 55-65 % urea/formamide gradient in 6 % acrylamide gels according to previously described protocols (Muyzer *et al.* 1993). The gradient was prepared using a BIORAD Model 385 gradient former. A 0 % denaturant gel (Appendix C 3.2.4) was used as a control where any appearing bands were considered to be non-specific carry-over band from the DNA sample. The position of the bands was compared to those on the 55–65 % denaturant gel and any similar sized bands were ignored. The electrophoresis was performed in an electrophoresis cell (Sigma–Aldrich 10x10 Vertical Electrophoresis Unit (Z33, 956-3) with 1 x TAE buffer (Appendix C 3.2.7) at 60° C and 120 V for 2 – 3 hours. DNA was stained with ethidium bromide, (Appendix C2.2) visualized on the transiluminator and photographed with a Kodak digital camera. Bands of interest were excised with a sterilized scalpel blade and transferred to 200 µl TE buffer (Appendix A 2.5). The DNA was extracted according to the protocol described by Sambrook *et al.*, (1989)(Appendix D), reamplified and confirmed by subsequent DGGE that the product consisted of single band. The reamplified PCR product was cloned into the pGEM-T easy vector system (Promega, USA), transformed into *Escherichia coli,* strain JM 109 from which extracted plasmid was prepared for sequencing.

2.5.5. Cloning and EcoR1 Digest of Extracted Plasmids

Cloning was performed using the pGEM-T easy vector system obtained from Promega, USA. Ligation reactions (Appendix E 2) were set up in a 0.5 ml tubes and incubated overnight at 4° C for the maximum number of transformants. Transformation of ligation reactions was done using high efficiency competent cells of *Escherichia coli* strain JM 109. Transformants were screened on LB/IPTG/X-Gal plates (Appendix E 1) after 16–24 hours incubation at 37° C. Transformants lacking β–galactosidase activity appear

white on X-Gal plates as opposed to blue colonies, which lack insert. Colonies containing insert were picked with a sterile toothpick and transferred to LB broth (Appendix E 1.1). Plasmid was extracted using Qiagen plasmid extraction kit according to the manufacturer's instruction.

In order to confirm the presence of inserts, *Eco*R1 digestion was performed. *Eco*R1 was expected to cut on either side of the 568 bp fragment, therefore the results on the gel would be a 3018 bp plasmid fragment and 568 bp insert. Some of the inserts may contain an internal *Eco*R1 site and therefore the correct results would show on the gel 3018 bp plasmid band and two smaller bands whose size should add to 568 bp. Plasmids with the correct insert were prepared for sequencing.

2.5.6. Sequencing

2.5.6.1. Template Preparation and Cycle Sequencing

Plasmids containing inserts were sequenced using Big Dye Terminator 3 sequencing kit (Applied Biosystems) with 100 to 200 ng of template DNA according to the manufacturer's instructions. For initial analysis, a partial sequence was obtained using primer GM5 F (Appendix B 2.1). Extended sequences were obtained by using universal sequencing primers T7 (Appendix H 2.1) and SP6 (Appendix H 2.2). All primers were obtained from Integrated DNA Technology (IDT), USA. The reaction mixture for cycle sequencing was prepared as outlined in Table 2.6.

Table 2.6: Reaction mixture used for template preparation prior to cycle sequencing.

Reagent	Concentration	Volume
Terminator v.3 ready reaction premix	2.5X	4 µl
Big Dye sequencing buffer	5X	2 µl
Primer	3.2 pool	
Template	100 – 200 ng	
Water		to 20 µl
Total volume		20 µl

Cycle sequencing was performed in Perkins Elmer 9700 thermocycler starting with initial denaturation at 96°C for 1 min with rapid thermal ramp of 1°C/sec. This was followed by 25 cycles of rapid thermal ramp to 96°C for 10 sec, rapid thermal ramp to 50°C for 5 sec, rapid thermal ramp to 60°C for 4 min and finally rapid thermal ramp to 4°C.

The extension products were purified using DNA Clean & Concentrator ™ – 5 columns (Zima Research, USA). Each sample received 100 µL of DNA binding buffer. The samples were then loaded into spin columns (Zima Research, USA) that were placed in a 2 ml collection tube. The samples were centrifuged at 10 000 g for 10 seconds and the flow-through was discarded. 200 µl of wash buffer was added to the column and the samples were again centrifuged at 10 000 g for 10 seconds. The wash step was repeated twice. The flow-through was discarded. The DNA was eluted with 8 µl of sterile $dddH_2O$. The eluted DNA was dried at 37°C and then stored at -20°C until sequenced.

2.5.6.2. DNA Sequencing

DNA sequence was determined on an automated ABI 3100 Prism® Genetic Analyzer at Rhodes University, Grahamstown, South Africa. Each sample was resuspended in 10µl deionised formamide, vortexed and spun down.

The samples were then transferred to a 96 well microtitre plate, rapidly heated to 95° C in order to denature the DNA and then immediately chilled on ice. The microtitre plate was placed in the 3100 ABI PRISM Genetic Analyser and the sequencing process was performed using 50 cm capillary array filled with POP-6 ™ polymer (Applied Biosystems).

2.5.7. Analyses of the Sequencing Results

Data generated by the ABI 3100 Prism® Genetic Analyzer were converted to text format using Chromas software and then analyzed using the National Centre for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) database (Altschul *et al.,* 1997). To obtain the phylogenetic relationship between the clones, the data were analyzed using the Neighbor Joining (N-J) algorithm.

2.6. Molecular Microbial Ecology of the Laboratory–scale Packed- bed Reactor

The slow rate of degradation of the lignocellulose substrate in the packed- bed reactor has been related to the highly complex structure of this biopolymer. Both this study of the VCC reactor and other investigations (Johnson *et al.,* 2002; Loy *et al.,* 2002; Johnson and Hallberg, 2003; Clarke *et al.,* 2004) have shown that complex microbial communities are involved in these systems and that cellulolytic, fermentative, acetogenic, and aromatic compound degrading bacteria, and probably other syntrophic bacterial populations, are needed to cleave lignocellulose in order to support bacterial sulphate reduction activity. However, while the SSRU 3 reactor at VCC had shown the presence of this diverse community of microbiota, with multiple, and probably interactive, electron donors and acceptors, the survey of plant operation over the six year period of observation had revealed poor overall performance. Setting up the laboratory-scale column reactor had created an opportunity to investigate the population under controlled conditions where their spatial and temporal

development could be directly monitored in a manner which had not been possible in the VCC plant.

2.6.1. Sampling Methods

Specimens for DNA extraction were collected from the reactor every three months over a period of one year. Wood sampling rods were installed as shown in Figure 2.22 A. At sampling times they were removed from the reactor and a small amount of biofilm was aseptically detached. This was placed in a sterile 50 ml Falcon tube (Laboratory & Scientific Equipment Co), and kept on ice or at 4° C until processed (within 4 days). In the event where immediate processing was not possible, the samples were "snap" frozen in liquid nitrogen and stored at -80° C until processed. The development of biofilm on the rods progressively increased with time over the sampling period as shown in Figure 2.31.

Figure 2.31: Discoloration and biofilm formation on wood sampling rods at different stages: (1) After one month, (2) after four months, (3) after six months. Red arrows point to the bacterial biofilm developed on the sampling rods.

2.6.2. Molecular Techniques

The analyses of microbial population in the laboratory packed-bed reactor were performed as described earlier in this chapter (section 2.5).

CHAPTER THREE

Performance of the Lignocellulose Packed-bed Reactor in Field and Laboratory-scale Studies.

3.1. Introduction

The study undertaken on the VCC reactor, and the evidence of its poor performance, was related to the extensive operating conditions of the system, which were inherently difficult to control under rigorous experimental conditions. In order to understand the processes at work in the system, and to possibly suggest ways in which problems, such as early failure, may be overcome, a laboratory-scale bioreactor study was initiated. Among the questions to be answered were how the microbial population develops during the bioremediation process and what involvement it has in the chain of chemical reactions occurring during the bioremediation process.

Previous investigations have shown that consecutive vertical zonations of predominant microbial species occur simultaneously in the wastewater environment, depending on the organic matter provided (Ramsing *et al.,* 1993; Lettinga, 1995; deBeer *et al.,* 1997; Santegoeds *et al.*, 1998; Okabe *et al.*, 1999; Santegoeds *et al.*, 1999;). Given the mix of organisms in the microbial consortia identified in the VCC reactor, and the uncertainty with respect to how these might interact in the system, it was decided to design a linear reactor system on laboratory-scale, which would enable the correlation of both physico-chemical data and the profiling of microbial populations within the system.

3.2. Results and Discussion

3.2.1. Field-scale Lignocellulose Packed-bed Reactor

The performance of the Field–scale reactor was monitored over six years by the Environmental Consultancy firm Pulles, Howard and de Lange and results of their study are reported in chapter two, section 2.2.1 (Overview).

3.2.2. Laboratory-scale Lignocellulose Packed-bed Reactor

The Laboratory-scale reactor column in this study was configured and operated as discussed in chapter 2, section 2.3.1.

Daily monitoring of the performance of the column reactor revealed that the bioreactor achieved operational sulphate removal from day 39 (43.2 mg/l/day) and sulphate removal continued to increase until day 97 (212.6 mg/l/d). Thereafter the rate of the reaction decreased, an effect that was also observed from the field-scale study noted previously. Shock sulphate loading of the system on day 200 and 207 with 13 484 mg/l and 9 184 mg/l respectively, resulted in the increase of sulphate reduction to 419 mg/l/day between days 200 and 273 and the sulphide production for the duration of study was between 157.2 mg/l and 271.4 mg/l (Coetser, 2004).

3.2.2.1. Sulphate Depth Profile

Sulphate analyses were undertaken on samples drawn as previously described. Figure 3.1 shows sulphate concentration at different sampling depths over a 273 day period.

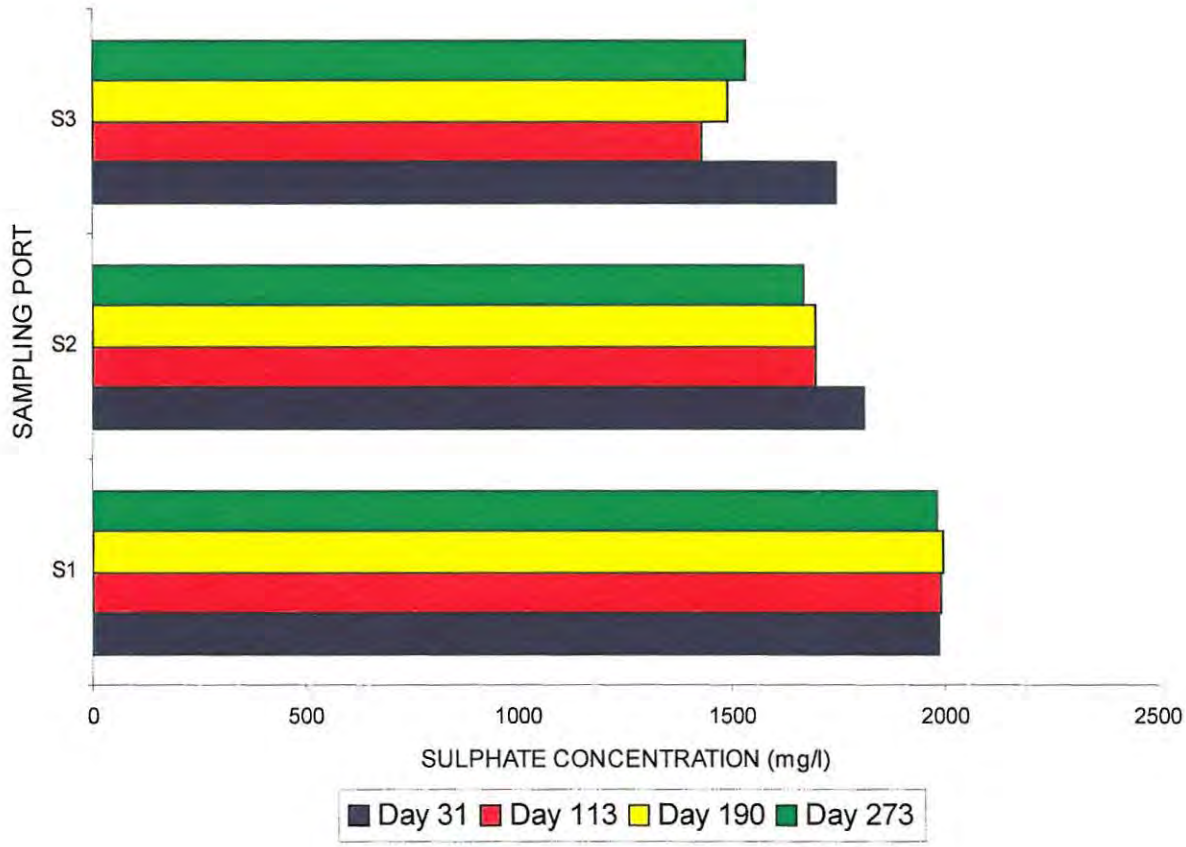


Figure 3.1: Sulphate concentration at different sampling depths in the lignocellulose column reactor monitored over a 273 day period (see figure 2.21 for sampling port configuration).

Sulphate concentration in the first zone of the reactor (inlet zone) remained the same during the operation of the reactor, however a noticeable decrease

occurred in the middle and the top of the column, evidently the result of an increase in efficiency with time. Day 113 of operation of the reactor shows the highest sulphate removal at the top of the column followed by a slight decrease by day 190 and day 273. Inlet zone (S1) (Figure 2.21) did not show significant change in sulphate reduction over the period of study, however, at sampling ports S2 and S3, (Figure 2.21) sulphate reduction showed continuous improvement over the full period of the study.

3.2.2.2. pH Depth Profile

The pH depth profile over the operational time of the laboratory–scale reactor is reflected in the Figure 3.2.

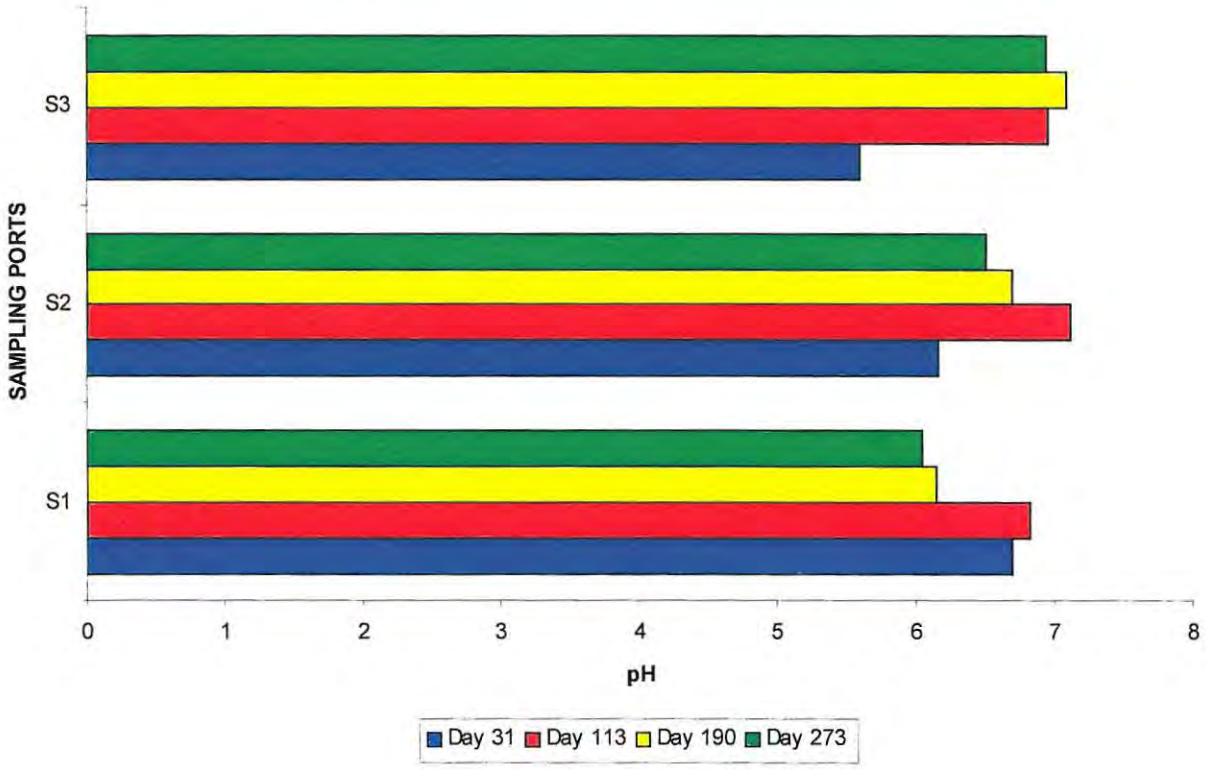


Figure 3.2: Change in pH of the effluent from different depths of the reactor over a period of 273 days.

Figure 3.2 shows the pH profile in the lignocellulose column reactor on the sampling days reported. In the initial period of operation of the reactor (sampling day 31) pH decreased from sampling ports S1 to S3 (inlet to outlet) with values falling from 6.7 at the inlet to 5.6 at the outlet. Thereafter, pH increased at day 113 with the highest value reaching 7.12 in the middle zone of the column. At day 190 and 273, pH appeared to rise with passage up the length of the column. These observations are consistent with acid production, possibly due to fermentation activity, firstly throughout the reactor and then, with stable operating conditions established, confined more to the inlet zone of the reactor. The elevation of the pH up the length of the reactor is consistent with sulphate reduction activity and accords well with the sulphate profile in Figure 3.1.

3.2.2.3. Depth Profile of Aromatic Compounds

The release of aromatic compounds in the lignocellulose column reactor was monitored as an indicator of lignin breakdown within the lignocellulose complex (Figure 3.3)

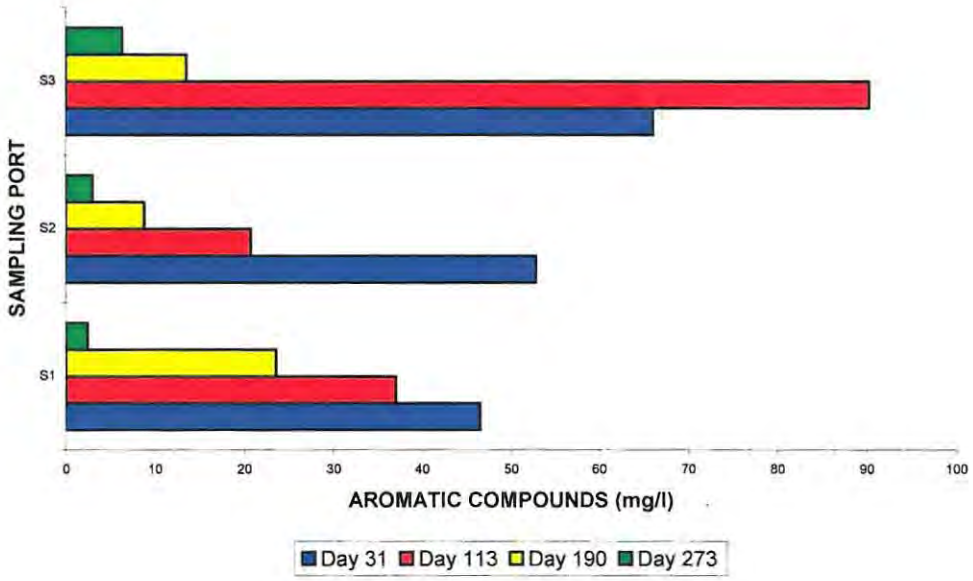


Figure 3.3: Change in the content of wood aromatics from different depths of the reactor over a period of 273 days.

Results of Figure 3.3 show high levels of lignin breakdown in the initial phase of operation and subsequent decrease both over time and along spatial arrangement in the reactor. The highest level of aromatic compound release from lignocellulose compounds was observed on day 113 at the outlet zone of the column. From day 190 there was a noticeable decrease in release of aromatic compounds throughout the column. These results may be compared with the concentration of the reducing sugars which show an inverse response. The result accords well with an understanding of lignocellulose degradation where access to cellulose by microbial systems is limited by prior cleavage of the lignin structure (Crawford and Crawford, 1984; Crawford and Crawford, 1988).

3.2.2.4. Depth profile of Reducing Sugars

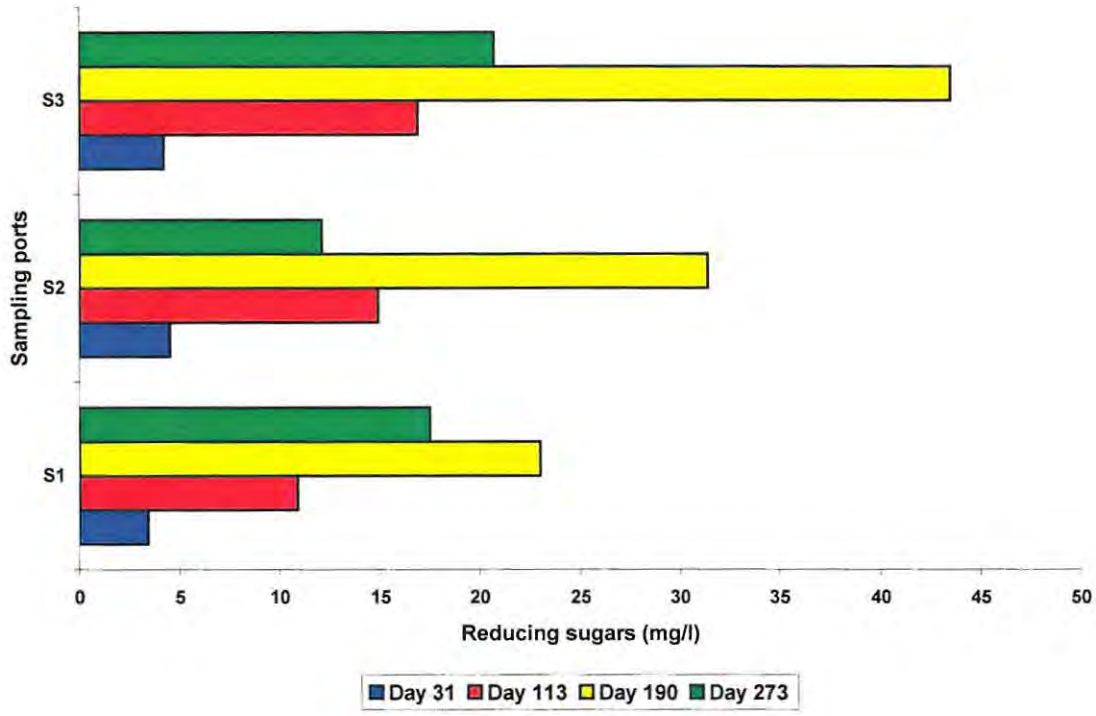


Figure 3.4: The concentration of reducing sugars at different depths of the reactor over a period of time.

The production of reducing sugars in the different zones of the reactor shows low initial levels during the early period of operation (Figure 3.4). However, the concentration then increased at each sampling port reaching its highest value on day 190 at the top zone of the reactor. Thereafter the content of the reducing sugars decreased indicating either improved utilization or reduced mobilization of the substrate.

3.2.2.5. Depth Profile of Volatile Fatty Acids

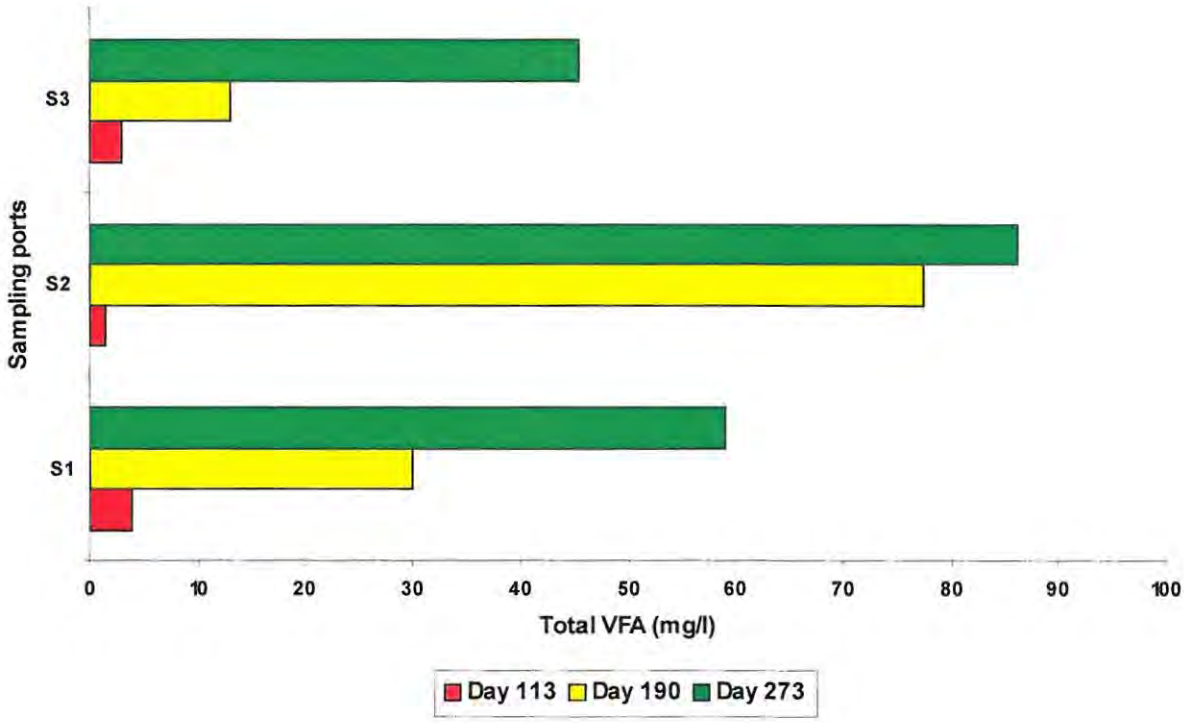


Figure 3.5: Change in the total volatile fatty acids (VFA) from different depths of the reactor over a period of time.

Unfortunately the decision to monitor the production of volatile fatty acids was taken after the analysis of the day 31 samples were completed. The release of VFAs was minimal in the first stages of the process and over a period of time showed a marked increase with the highest concentration being reached on day 273 (Figure 3.5). Throughout the operation of the reactor the highest concentration of VFAs occurred in the middle zone of the reactor and decreased along its length. This observation relates closely to data demonstrating the release of wood aromatics in the system. Since VFAs are the metabolic products of the degradation of wood aromatics, as the amount of wood aromatics released into the reactor increase and are degraded, the concentration of VFAs would also be expected to increase. The consumption

of the aromatic compounds in this way would manifest as a reduced level of these compounds.

3.3. Decommissioning of Lignocellulose Laboratory-scale Packed-bed Reactor

After almost a year of continuous operation the lignocellulose laboratory-scale reactor was decommissioned and unpacked. The condition of the packing material was observed and final samples drawn for chemical and microbiological analysis. At the same time a second reactor was decommissioned, which had not been part of the monitoring study reported here. This reactor had performed poorly over the period of its observation and this provided an opportunity to compare this with the test reactor which had shown the typical symptoms of an early good performance followed by late failure. The reactor was set up on the same principle as the one used in this study, however it differed only in the organic matter content, which in this case was wood chips only. It was noted that the organic matter in the badly performing reactor remained intact for a period of over a year. No biofilm formation was visible upon unpacking of the column (Figure 3.6 B and 3.7 B).

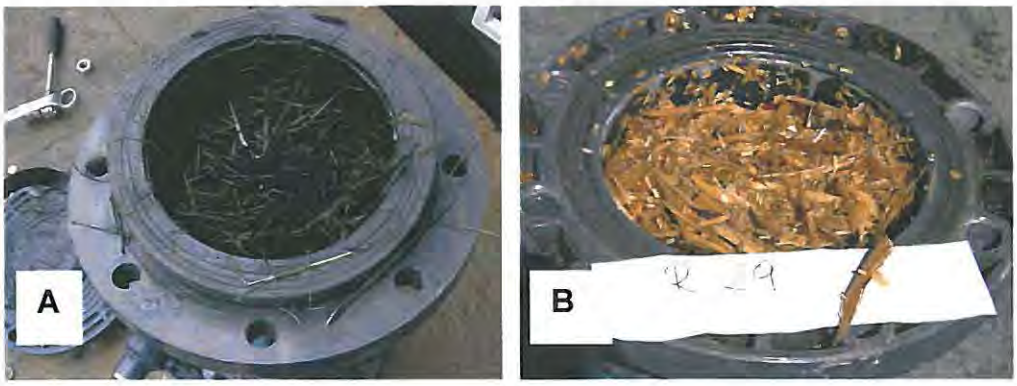


Figure 3.6: Organic matter at the top of reactors, examined upon unpacking. (A): reactor under investigation in this study, packed as described previously, (B): poorly performing reactor packed with wood chips only.

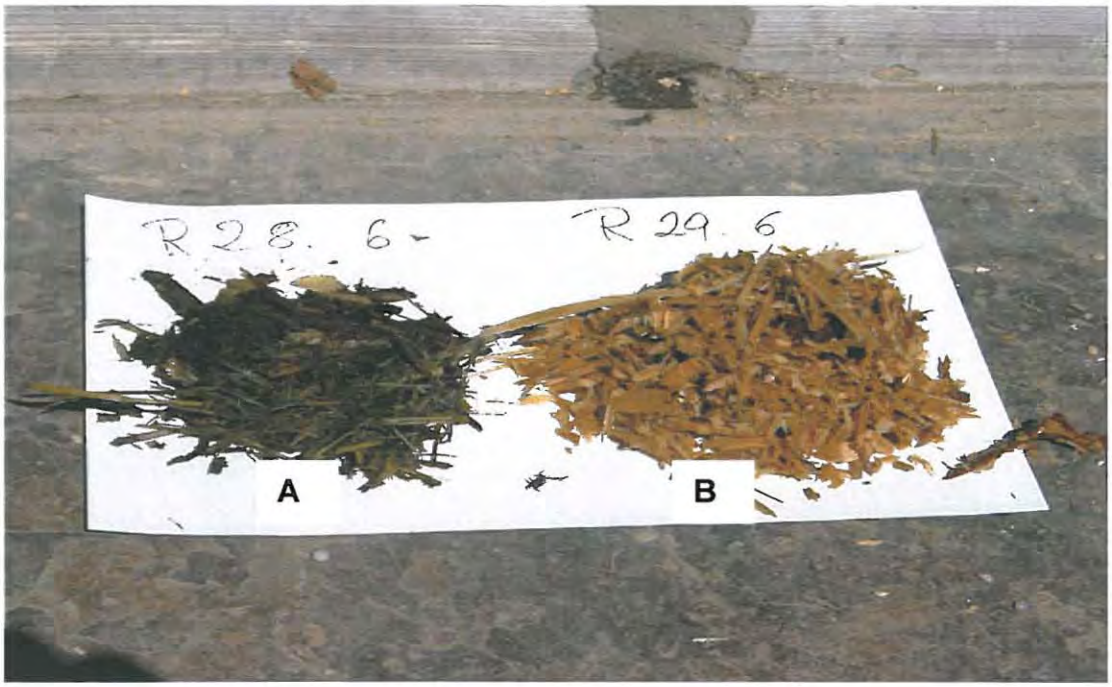


Figure 3.7: Organic matter from the middle sections of two reactors. (A): reactor under investigation, (B): poorly performing reactor.

Monitoring of the performance of reactor B revealed no sulphate reduction (Coetser, personal communication) in contrast to the reactor under study in which biofilm formation was observed and sulphate reduction took place.

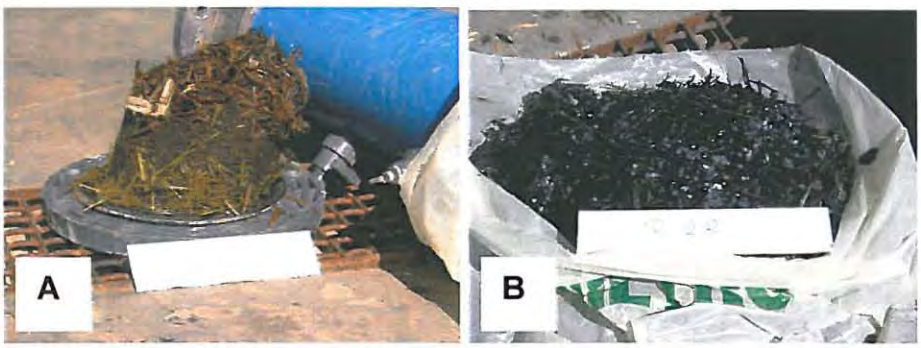


Figure 3.8: Organic matter from the bottom (A) and top of reactor (B) under study.

The above photographs show the organic matter content of the column reactor under study. Figure 3.8 A illustrates the organic matter recovered

from the inlet zone of the column. The organic matter material had undergone partial decomposition, however judging by the colour and smell of the material no evidence of major sulphate reduction could be detected. Figure 3.8 B illustrates the organic matter content from the outlet zone of the reactor. The organic matter material had undergone visible decomposition, and the colour and smell of the sample revealed that sulphate reduction had taken place.

The chemical reactions occurring in the reactor during the process of bioremediation apparently indicate a series of sequential steps involved in degradation of the complex organic lignocellulitic material. The significant release of wood aromatics in the beginning of the remediation process evidently decreases over the period of time (Figure 3.3). This reaction is directly coupled to the concentration of volatile fatty acids and reducing sugars in the reactor (Figure 3.5 and 3.4). With the decrease of wood aromatics in the system, the concentration of volatile fatty acids and reducing sugars increases. This may be observed as both temporally and spatially arranged effects. Comparing the data generated from analyses of the pH and sulphate concentration, it is apparent that highest sulphate removal occurs with increase of pH in the reactor, which again may be observed as both temporal and spatial effects (Figure 3.1 and 3.2).

3.4. Microscopy

3.4.1. Electron Microscopy Study

Scanning and Transmission Electron Microscopy study were performed as described in Material and Methods, chapter 2, section 2.4.5.1. The reason for undertaking these studies was to investigate the penetration of a microbial population into the tissues of the lignocellulose material. The SEM study showed the rich microbial population on the surface of the substrate, but no conclusions could be made as to the kind of organisms present. The TEM did

not show any microorganisms present inside the tissue of the substrate. The decision was made to omit these results as insignificant.

3.4.2. Fluorescent In Situ Hybridization (FISH)

Attempts had been made to investigate the microbial population in the reactor applying fluorescent *in situ* hybridization (FISH). This study however was done only on a preliminary basis due to the time constraints, project finances and the lack of availability of a suitable microscope at that time. In the preliminary study sulphate reducing bacteria were targeted with fluorescent labeled probe SRB 385 labelled with Cy3 (emission 570 nm) (Appendix F 6). Probe EUB was used to detect total population of eubacteria in a sample. Specimen was counterstain with DAPI. Figure 3.9 shows the presence of sulphate reducing bacteria in the biofilm developed on sampling rods.

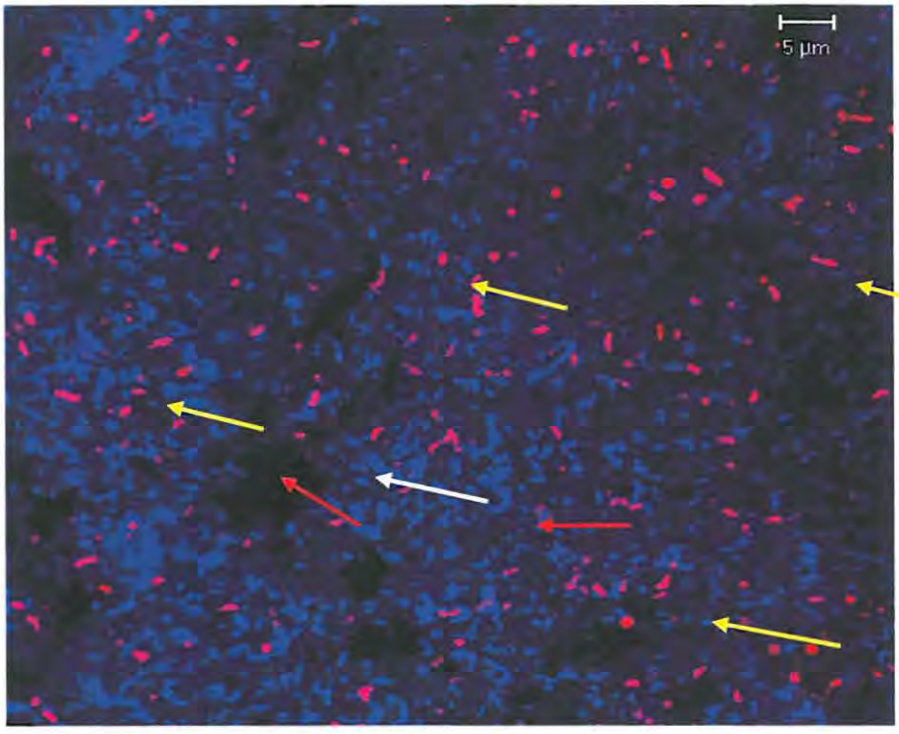


Figure 3.9: Photomicrograph of fluorescence *in situ* hybridization showing stained biofilm collected from lignocellulose packed-bed reactor. Yellow arrows indicate different Sulphate Reducing Bacteria targeted by probe SRB 385, red arrows indicate background DAPI stain of microbial population, white arrow points to black background that could be EPS. Preparation was analyzed using Confocal Laser Scanning Microscope (CSLM). Courtesy of Munich Technical University.

Probe SRB in Figure 3.9 is targeting only some sulfate-reducing bacteria of the Deltaproteobacteria, other Deltaproteobacteria and Gram-positive bacteria. Probe EUB failed to bind to bacteria in the sample, which could be explained by the fact that probably was not specific for all bacteria. The paper by Daimes, *et al.*, 1999, supports above statement,

It is worth mentioning that the specimen above was obtained from sample earlier subjected to DGGE and sequencing. No sulphate reducing bacteria were detected by sequencing following DGGE. In 1993, Muyzer *et al.*, working with mixtures of pure cultures found that DGGE could not detect populations of bacteria whose abundance was less than approximately 1 % of the total cell count. No further investigation using FISH was possible, however, it crucial that any fingerprinting technique should be supplemented by more advanced investigation.

According to the literature, the lignocellulose packed-bed laboratory-scale column reactor study described here is one of the longest spatial investigations undertaken on such systems. It was reported that Colberg and Young (1985) performed a 43 day study on a soluble fraction of cellulose, Kim *et al.* (1997) studied the degradation of filter paper, and newspaper for 30 days, Benner *et al.* (1985) did a 25 day study on the rate of lignocellulose biodegradation in two separate wetland samples. Clarkson and Xiao (2000) performed a 300 day study on the production of biogas from the anaerobic conversion of paper. This lignocellulose column reactor study operating under sulphidogenic conditions was undertaken over a period of 273 days.

3.5. Conclusions

The concept underpinning integrated passive water treatment systems is to provide prolonged removal of sulphate, while sustaining the elimination of metals and acidity from AMD. The Vryheid Coronation Colliery (VCC) pilot plant was constructed with this hypothesis in mind. However, the data obtained over six years of operation of the pilot plant clearly indicate that the

plant was not performing satisfactorily (Molwantwa *et al.*, 2003). The following conclusions were reached after six years of operation:

- The SSRU at the VCC pilot plant has shown that the degradation of complex organic matter sources takes place in stages over time. In the start-up period, when there is an abundance of readily degradable organic matter driving sulphate reduction, there is a higher rate of sulphate reduction. This is characterized by AMD neutralization (pH increase and alkalinity generation), sulphate removal and sulphide production. Over time when the readily degradable organic matter is depleted, and only the recalcitrant organic matter remains, the rate of sulphate reduction decreases.

- The target sulphate load removal of 60g/m^3 organic matter/d was not achieved by these units.

- Since the redox potential for the sulphate/sulphide couple depends on pH reduction, the redox potential values for SSRU were not conducive for active sulphate reduction.

- Metal removal in the SSRU was higher in the start-up period and decreased with time. This is probably due to the high sulphide concentration and change in pH, which are involved in metal precipitation.

- During the operation of the process nutrients present in the reactor packing material are solubilized in influent water and this elevation of nutrients status requires a post-treatment stage in order to meet water quality standards.

- Effective removal of nutrients and metals in the aerobic wetland was observed.

- Re-oxidation of sulphide to sulphate took place in the oxidation cascade. The oxidation cascade was responsible for the removal of residual manganese.

Based on the data reported in this chapter, for a laboratory-scale lignocellulose packed- bed reactor the findings may be summarized as follows:

- Increased sulphate removal, and possibly associated pH elevation, in the reactor system, over time, appears to be related to the release of aromatic compounds from the wood tissue;

- Release of aromatic compounds from the lignin structure appears to precede the appearance of reducing sugars;

- Release of reducing sugars in turn appears to precede the appearance of VFAs, with the major part of the production (or accumulation thereof) occurring in the middle of the column, and the major part of consumption in the bottom and top zones;

- It is possible that the sequence of events which dictate the chain of chemical reactions, resulting in degradation of lignocelluloses material within the bioreactor, is governed by the spatial arrangement of the diverse microbial community occurring within it. The previous study of the microbial ecology in the VCC reactor had suggested that this might be so, and that in a linear flow path regime, as apposed to a mixed reactor system, the continuity of the interaction between the consortia may become dislocated by the occurrence of limiting conditions at a lower level.

In order to further investigate this hypothesis, the molecular microbial ecology study of the samples drawn from the laboratory-scale lignocellulose-system was undertaken.

CHAPTER FOUR

Molecular Microbial Ecology of a Field-scale Lignocellulose Packed- bed Reactor

4.1. Introduction

Although a physical and chemical analysis of the laboratory–scale decommissioned lignocellulose packed-bed reactor was undertaken, as outlined in the previous chapter, these parameters provided little indication of the trophic environments affecting microbial growth within the system. It had been proposed that an understanding of the distribution of microbial consortia within the reactor, and relating this information to the known physiological behavior of the identified forms, would provide a basis for erecting a descriptive model accounting for biological activity within the system.

The structure of microbial communities in environmental systems is usually highly complex and diverse, yet the study of their architecture and functions is crucial to an understanding of the dynamics and stability of natural ecosystems (Davey and O'Toole, 2000; Watnick and Kolter, 2000). The intricacy of these communities is both fascinating from a research point of view and also presents challenges for the investigator. Many approaches presently used to investigate the diversity and functions of the microbial population are biased because of the limitations of cultivation methods and current estimates indicate that less than 1 % of the microorganisms present in nature can be cultured in the laboratory (Amann *et al.*, 1990; Amann *et al.*, 1995; Amann and Kuhl, 1998). For these reasons a molecular approach using total genome DNA extracts was followed in identifying the main forms of bacteria present in the system.

4.2. Results and Discussion

The procedures followed in a microbial ecology study of the field-scale lignocellulose packed-bed reactor were outlined in Materials and Methods in chapter two, section 2.5.

4.2.1. Total Genomic DNA Extraction

Samples of hay, sawdust and wood collected from SSRU 3 were used for extraction of total genomic DNA using phenol–chloroform extraction as described in section 2.5.2.1. Extracted DNA was visualized on 0.8 % agarose gel (Appendix C 1.1) and quantified using a Shimoda spectrophotometer as described previously.

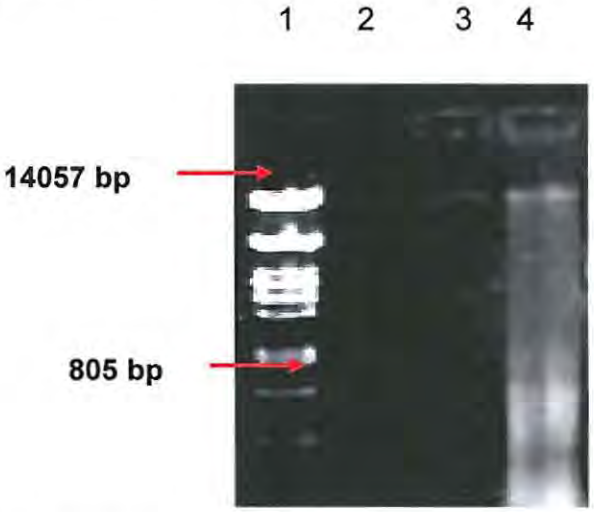


Figure 4.1: Agarose gel electrophoresis of total genomic DNA extracted from hay (lane 2), sawdust (lane 3) and block of wood (lane 4) visualized on 0.8 % agarose gel stained with ethidium bromide. From the gel it is apparent that the amount of genomic DNA recovered from hay (lane 2) and sawdust (lane 3) is minimal, while the DNA recovered from block of wood (lane 4) is degraded. However it was sufficient to proceed with the analysis. Lane 1 represents molecular marker λ*pst*.

4.2.2. Polymerase Chain Reaction

Bacterial community 16S rRNA genes (and therefore 16S rDNA) from organic matter samples recovered from the SSRU 3 reactor at VCC field plant were PCR amplified using primers GM5F and 907R, the latter, incorporating GC clamp (Appendix B 2.2). Figure 4.2 shows the photograph of the 1 % agarose gel on which the 568 bp product was visualized.

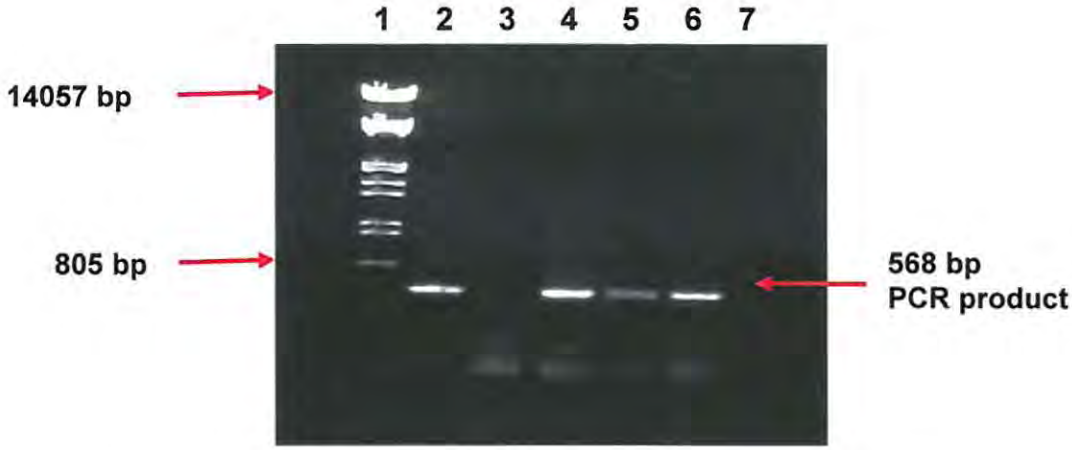


Figure 4.2: Agarose gel electrophoresis of 568 bp product. Lane 1 represents molecular marker λ *pst*, lanes 2 - 6 show PCR products, lanes 2 and 3 represent product obtained from sawdust samples (lane 3 - lack of product), lanes 4 and 5 show product obtained from hay samples, lanes 6 and 7 show product from wood sample (where lane 7 is lacking a product)

4.2.3. Denaturing Gradient Gel Electrophoresis

In order to determine the community structure of the amplicon, the PCR products were fingerprinted using the DGGE technique as described in section 2.5.2.4. Figure 4.3 illustrates the different banding pattern of the various samples. The bands of interest were excised, DNA was extracted (Appendix D), reamplified and cloned into pGEM-T Easy Vector as described previously and sequenced.

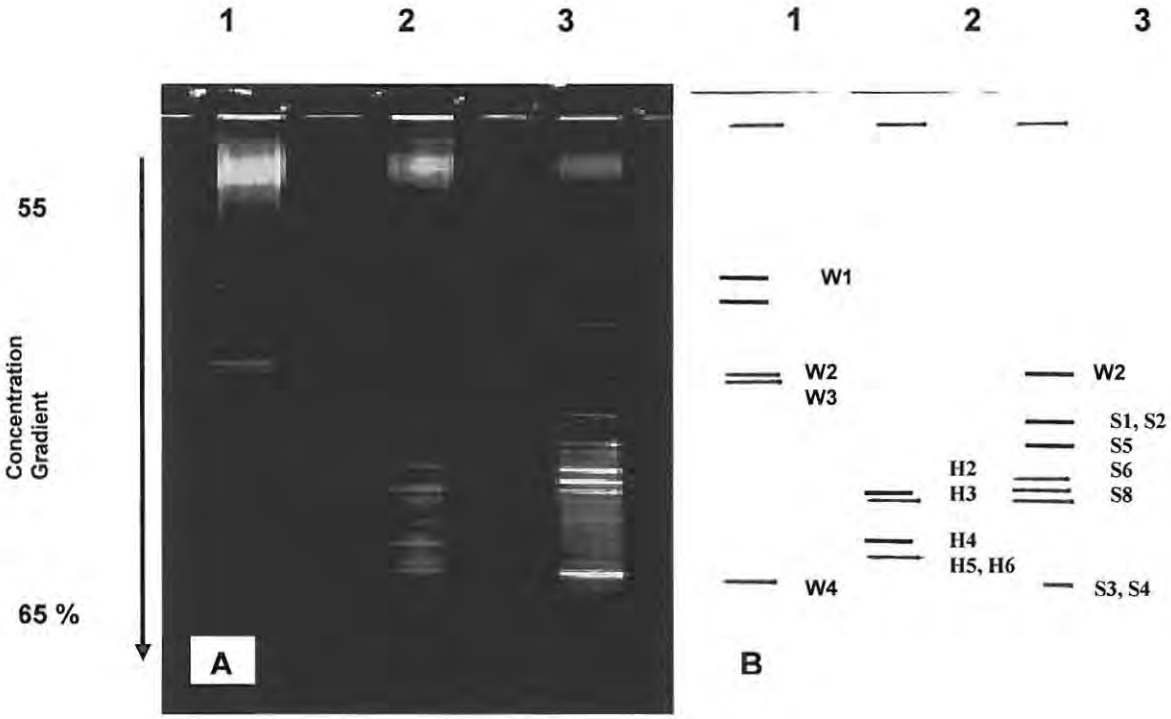


Figure 4.3: Comparison of bacterial community in SSRU 3 at VCC pilot plant. A: DGGE gel stained with ethydium bromide and photographed with Kodak digital gel documentation system. B: Copy of the bands excised from original gel. Lane 1: wood sample, Lane 2: hay, Lane 3: sawdust.

DGGE gel electrophoresis in this study reveals the distinct pattern, which is illustrated by the similarity of band migration from different samples. It is evident that band H3 and S8 representing hay and sawdust samples respectively stopped migrating in the same position of the gel. According to BLAST results followed by phylogenetic analysis (Table 4.1 and Figure 4.4) these bands exhibit the presence of closely related species of *Sphingomonas* and *Novosphignobium.*

The same appears to be apparent for bands W4, H5 and S4 representing wood, hay and sawdust samples respectively. According to BLAST results and phylogenetic analyses (Table 4.1 and Figure 4.4) these represent the consortium of closely related *Clostridium* sp. namely *C. thermosuccinogenes,*

C. termiditis and C.cellobioparum, all being known to posses cellulolytic activity.

4.2.4. DNA Sequencing and Phylogenetic Analysis

A total of 20 clones obtained from the DGGE bands were sequenced and analyzed using NCBI BLAST database. Table 4.1 shows the BLAST results of the various 16S sequences obtained with the closest relative from Genbank database and the percent divergence of the strain from this relative while Figure 4.4 illustrates phylogenetic analysis of the clones.

Table 4.1: The BLAST results of the 16S rRNA sequences for SSRU 3 obtained with the closest relative from the Genbank database.

Sample	Band	Clone	Closest relatives to the clones as determined by BLAST	Accession Number	% Gene identity
Wood	1	W1	Bacteria from anoxic soil	AJ229237	93
	2	**W2**	**Uncultured bacterium**	**AB063829**	**98**
	3	**W3**	**Uncultured *Cytophaga/Flavobacter/Bacteroides* group**	**AF351234**	**99**
	4	**W4**	***Clostridium thermosuccinogenes***	**Y18180**	**98**
	4	**W5**	***Clostridium* from anoxic soil**	**AJ229251**	**95**
	4	W6	*Bacteroides stercoris*	X83953	92
Hay	1	W7	Uncultured bacterium	AF414580	92
	2	**H2**	**Uncultured rumen bacterium**	**AB009228**	**96**
	3	**H3**	***Sphingomonas* spp.**	**AJ001052**	**99**
	4	H4	Uncultured bacterium	AY159185	93
	5	**H5**	***Clostridium cellobioparum***	**X71856**	**99**
	5	**H6**	***Clostridium termitidis***	**X71854**	**98**
Sawdust	1	S1	*Clostridium cellobioparum*	X71856	93
	2	S1	*Clostridium thermosuccinoges*	Y18180	90
	3	**S3**	***Clostridium termitidis***	**X71854**	**98**
	3	**S4**	***Clostridium* spp.**	**AJ229249**	**97**
	4	**S5**	***Desulfosporosinus***	**AJ493052**	**99**
	5	**S6**	**Unidentified eubacteria**	**AJ229190**	**99**
	6	S7	Uncultured rumen bacterium	AB009228	89
	7	**S8**	***Novosphingobium***	**AY151394**	**98**

On the basis of percent similarity obtained from the BLAST results (more than 95 %) only 13 out of 20 clones were selected for phylogenetic analysis by Neighbor Joining algorithm. These are shown in Table 4.1 and are depicted in bold.

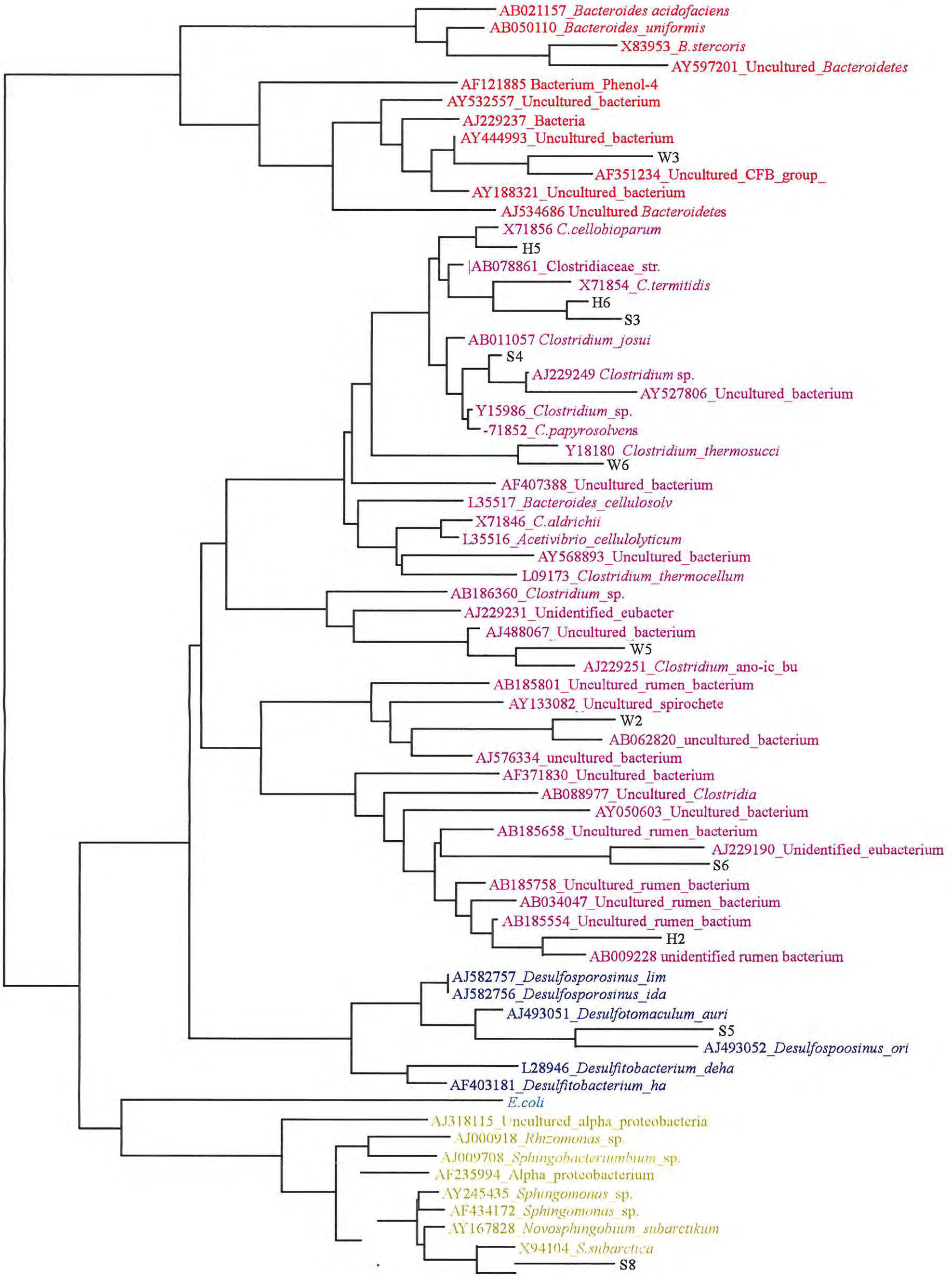


Figure 4.4: Phyologenetic analysis obtained from the sequences of the clones from VCC reactor. Taxonomic groups are depicted in different colours and the clones in black. Space bar indicates 1% sequence divergence

4.3. Description of Clones

4.3.1. Wood Samples

Clone W2

Bacterial population colonizing the wood sample was represented mainly by the division of Firmicutes and Bacteroidetes. Clone W2 is affiliated with uncultured bacterium (AB062820) recovered from a termite gut *Captotermes formosanus* (Tokuda *et al.*, 2000). The closest known relation of this bacterium are bacteria belonging to phylum *Spirochaetes.* In the same cluster appears the yet uncultured bacterium (AJ576334), recovered from the gut of humus-feeding larva of *Pachnoda ephippiata* (Egert *et al.,* 2003). The presence of these organisms is quite noteworthy from the physiological and nutritional point of view. It is known that the diverse microbiota of the termite gut enable the host to feed on lignocellulosic material. Therefore, any resemblance between the termite gut population and that of the reactor population implies that the microbiota which have developed in the lignocellulose field reactor have the ability to utilize the complex lignocellulose material or intermediates of its degradation (Ohkuma *et al.,* 1999; Tokuda *et al.*, 2000).

Clone W3

Clone W3 belonging to division Bacteroidetes is affiliated with yet uncultured CFB bacterium (AF351234) recovered from coal-tar-waste-contaminated waters (Bakermans and Madsen, 2002). In the same cluster appear uncultured bacterium (AY188321), bacterium recovered from biofilms growing on membranes transferring oxygen (AY444993) (Cole *et al.,* 2004), bacterium

XB45 (AJ229237) belonging to the division of Bacteridetes and recovered from anoxic bulk soils of rice paddy microcosms (Chin *et al.,* 1999). In more distant relation with clone W3, but in the same cluster, appears bacterium Phenol-4 (AF121885) known to be involved in degradation of aromatic compounds (Knight *et al.,* 1999).

Clone W4

Clone W6 affiliated with *Clostridium thermosuccinogenes* (Y18180) (Stackebrandt *et al.* 1999) bunches together with the cluster of numerous other *Clostridium* spp. such as *C. papyrosolvens* (-71852), *C. josui* (AB011057), *C. termiditis* (X71854) and *C. cellobioparum* (X71856). In the same cluster appear clones isolated from hay and sawdust samples of the VCC reactor.

Clone W5

The Division Firmicutes is represented here by two clones: W5 and W6. Clone W5 is affiliated with *Clostridium* spp. FCB90-3 (AJ229251) recovered from anoxic bulk soil of rice paddy microcosms (Chin *et al.,* 1999). Evident in the same cluster is the presence of other *Clostridium* spp. namely AJ488067 involved in partial degradation of chlorobenzenes (Adrian *et al,.* 2002), AJ229231, clone BSV87 recovered from anoxic rice paddy soils (Hengstmann *et al.,* 1999) and *Clostridium* spp. EBR-02E-0046 isolated from microbial consortia developing in a methanogenic fermentor (Shiratori *et al.,* 2004).

4.3.2. Hay Samples

Clone H2

This clone is affiliated (96 % similarity) with yet unidentified rumen bacterium RFN80 (AB009228) recovered previously from environmental samples (Tajima, 1997). In the same cluster (Figure 3.4) appear other uncultured rumen bacteria namely AB185554 (Ozutsumi *et al.,* 2004) and AB 034047 recovered from bacterial community in the rumen during adaptation to high grain diet (Tajima *et al.,* 2000). The closest known relationship to this clone is a cellulolitic *Clostridium.*

Clone H3

The above clone according to phylogenetic analysis (Figure 4.4) is in relation (99 % similarity) with *Sphingomonas* sp. (AJ001052) identified earlier in the samples of microbial communities recovered from paper printing machines (Vaisanen *et al.,* 1998). Different clones of *Sphingomonas* sp. are evident in the same cluster, namely clone FI012 (AY349411) obtained from environmental samples (Paster and Dewhirst, 2003), IW3 (AB076396) known to produce exoplysaccharides (Matsuyuma *et al.,* 2001), AV6C (AF434172) is capable of degrading monocyclic aromatic compounds (Di Gioia *et al.,* 2002). Closely related to *Sphingomonas* sp. are *Novosphingobium* sp. which branch together with clone H4 (Figure 4.4). In the same cluster the uncultured eubacterium WD249 (AJ292599) appears, which is known to be involved in degradation of polychlorinated biphenyl (Nogales *et al.,* 2001).

Clone H5 and H6

Both of these are clustering with a wide diversity of *Clostridium* spp. Clone H5 (Figure 4.4) is closely affiliated (99 % similarity) with *Clostridium cellobioparum*-X71856, while clone H6 is in the close relation (98 % homology with *Clostridium termiditis*-X71854 (Rainey and Stackebrandt 1993). These clostridia specifically are known to be involved in degradation of cellulose and hemicellulose. *C. cellobioparum* should be of main focus in this population as, according to Collins *et al.* (1994), it belongs to the clostridial cluster III. The species belonging to this cluster are known to produce the multiprotein complex known as the cellulosome (Van Dyk and McCarthy, 2002; Doi *et al.*, 2003).

4.3.3. Sawdust Samples

Clones isolated from sawdust samples were affiliated with a wide diversity of microbial populations that may be found across the whole phylogenetic tree drawn for the samples recovered from the field reactor at VCC (Figure 4.4).

Clone S3 and S4

Clone S3 is very closely affiliated (Figure 4.4) with clone H6, both of them clustering together with *C.termiditis*, X71854 (Rainey and Stackebrandt, 1993). Clone S4 is affiliated with yet uncultured *Clostridium* strain FCB90-1, AJ229249 isolated from anoxic rice paddy soils (Chin *et al.*, 1999). This cluster brunches together on one side with uncultured bacterium (AY527806), isolated from environmental samples contaminated with uranium and nitrate

(North *et al.,* 2004), and on the other side with *Clostridium josui* (AB011057) known to be involved in cellulose degradation via the cellulosome (Kakiuchi *et al.,* 1998). According to Kakiuchi *et al.* (1998), *Clostridium josui* genes *cipA* and *celD* encode for scaffolding protein CipA, however smaller in size, CipA protein of *Clostridium thermocellum,* is similar to CbpA of *Clostridium cellulovorans* and CipC of *Clostridium cellulolyticum.* CelD of *Clostridium josui* consists of a N-terminal signal peptide and a dockerin domain. Sequence analysis of amino acids from *C. josui* proteins revealed that both CipA and CelD are major component of the cellulose enzymes.

Clone S5

Clone S5 emerge in the cluster of sulphate reducing bacteria. The closest affiliation (99 % similarity) seem to be with *Desulfosporosinus orientis*, AJ493052 (Stackebrandt *et al.* 2003). In the same cluster appear *Desulfospororinus idahoense*, AJ582756 and *Desulfospororinus limeticum*, AJ582756, both isolated from fresh water lake sediments (Ramamoorthy *et al.,* 2003) as well as *Desulfitobacterium hafniense*, AF403181 (Davis and Tjedie, 2001) and *Desulfitobacterium dehalogenans*, L28946, known to reductively dechlorinate chlorophenolic compounds (Utkin *et al.,* 1994).

Clone S6

S6 clusters together (99 % similarity) with yet unidentified eubacterium clone BSV28, AJ229190 isolated from anoxic rice paddy soils (Hengstmann *et al.,* 1999). This clone emerges in the cluster of unidentified rumen bacteria, namely AB185658, AB185554 and AB185758 (Ozutsumi *et al.,* 2004) as well as rumen bacterium 5C0d-14, AB034047 isolated from rumen microbiota

during adaptation of cattle to high-grain diet The closest known relationship to this clone is a cellulolitic *Clostridium* (Tajima *et al.,* 2000).

Clone S8

S8 is affiliated with *Novosphingobium subarcticum*, AY151394 with 98 % similarity, previously isolated from environmental samples and characterized as a PAH-degrading bacterium (Bastiaens *et al.,* 2000). This clone appears in the cluster of various *Sphingomonas* sp. and *Novosphignobium* sp. as discussed with clone H3.

4.4. Conclusions

This chapter involved the investigation and characterization of microbial population colonizing lignocellulosic organic matter material in a field reactor in the passive treatment configuration. Methods used involved molecular techniques namely DNA extraction, partial or complete amplification of 16S rRNA genes, DGGE, cloning, sequencing and phylogenetic analysis of the obtained data. The molecular analyses were performed on the samples retrieved from the bioreactor during decommissioning and these included hay, wood and sawdust. According to phylogenetic analysis performed on the sequences of clones obtained from different DGGE bands, the microorganisms colonizing the organic matter material included cellulolitic *Clostridium spp..,* CFB species, as yet uncultured bacteria closely related to microbiota inhibiting rumen and termite gut, and *Sphingomonadaceae,* all being known to be primary fermenters of cellulose. Sulphate reducing bacteria were represented only by one clone closely related to *Desulfosporosinus* sp.

According to chemical analysis carried out over six years, the reactor demonstrated clear decline in its performance (chapter two), however the microbial population which was identified in the reactor appeared to be appropriate for utilization of organic matter and consequently, through its degradation, should provide the microenvironment for remediation of AMD. These observations posed the question as to why the system does not work effectively. Previous work done by numerous researchers (Radchenko and Tashirev, 1991; Davey and O'Toole, 2000; Watnick and Kolter, 2000) clearly indicate that both in the natural and engineered systems the successful performance depends on interspecies sequential nutrient mobilization. The examination of the organic matter content of the reactor during decommissioning (Figure 2.17 - 2.19) and the results obtained from DNA analysis (Table 4.1 and Figure 4.4) suggest that spatial and temporal gradients were apparently disturbed with time. This disturbance consequently resulted in a limitation of critical nutrients, which in turn restricted the consortia assortment. To test these hypotheses, a laboratory-scale column reactor was designed which incorporated the experimental methods to investigate spatial and temporal gradients within the column. The following chapters will exemplify in more detail the approach taken to test the hypothesis.

CHAPTER FIVE

Molecular Microbial Ecology of a Laboratory-scale Lignocellulose Packed-bed Column Reactor

5.1. Introduction

The slow rate of degradation of the lignocellulose substrate in the packed-bed reactor has been related to the highly complex structure of this biopolymer. Both this study of the VCC reactor and other investigations (Johnson *et al.*, 2002; Loy *et al.*, 2002; Johnson and Hallberg, 2003; Clarke *et al.*, 2004) have shown that complex microbial communities are involved in these systems and that cellulolytic, fermentative, acetogenic, and aromatic-compound degrading bacteria, and probably other syntrophic bacterial populations, are needed to cleave lignocellulose in order to support bacterial sulphate reduction activity. However, while the SSRU 3 reactor at VCC has shown the presence of this diverse community of microbiota, with multiple, and probably interactive, electron donors and acceptors, the survey of plant operation over the six year period of observation revealed poor overall performance and it was not possible to examine the microbial communities over both spatial and temporal distribution. Setting up the laboratory-scale column reactor created an opportunity to investigate the population under controlled conditions where their spatial and temporal development could be directly monitored in a manner which was not possible in the VCC plant.

5.2. Results and Discussion

Sampling of the reactor was described in chapter two, section 2.4.3. Molecular microbial investigations were performed according to the protocols described in chapter two, section 2.5.

5.2.1. Total Genomic DNA Extraction

Extracted DNA was visualized on 0.8 % agarose gel and quantified spectrophotometrically.

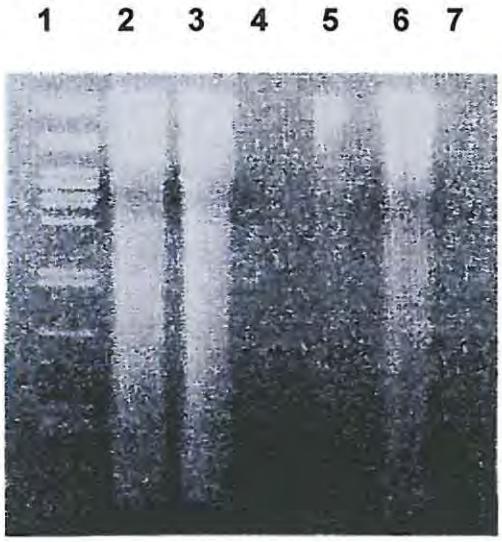


Figure 5.1: Total genomic DNA extracted from the reactor biofilm. Lane 1: molecular marker λ pst. Lanes 2–6 show total genomic DNA and lane 7 negative control as examined on the 0.8 % agarose gel stained with ethidium bromide and photographed with Kodak digital system.

DNA was successfully extracted from the biofilm formed on the wood sampling rods during bioremediation of AMD. Figure 5.1 shows that DNA was degraded. However, there was sufficient concentration of DNA to perform the PCR.

5.2.2. Polymerase Chain Reaction

Community 16S rRNA genes from the column reactor were PCR amplified with GM5F and 907R primers (Appendix B 2) using the touch down program. The 568 bp amplified product was electrophoresed on 1 % agarose at 90 V for about 45 minutes and visualized on the UV transilluminator. The gel was photographed with Kodak digital gel documentation system (Figure 5.2)

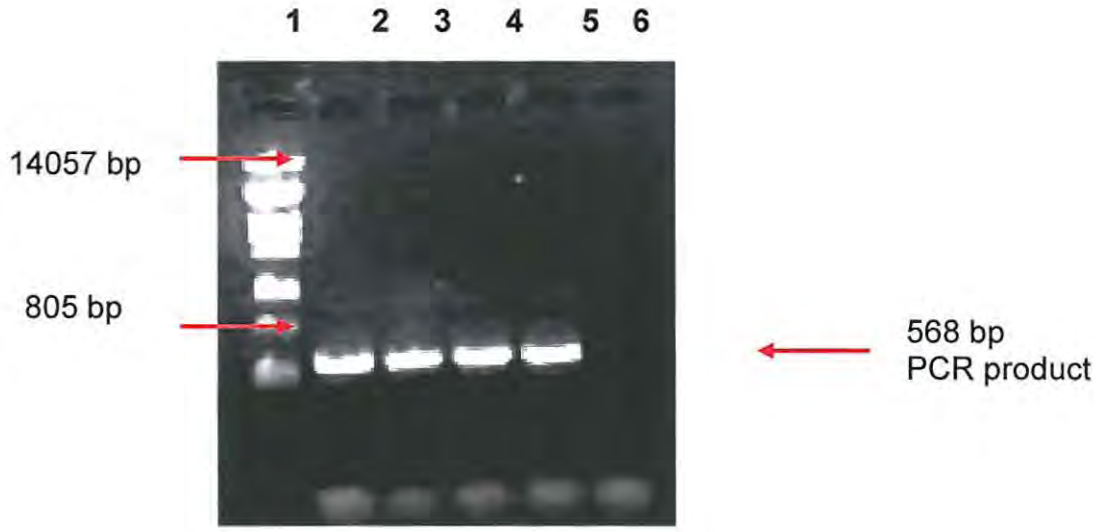


Figure 5.2 Agarose gel electrophoresis of 568 bp product. Lane 1: molecular marker λ *pst*; lane 2–4: 568 bp PCR product amplified from total genomic DNA isolated from laboratory-scale column packed-bed reactor (lane2: inlet zone, lane 3: middle zone, lane 4: outlet zone); lane 5: positive control (568 bp construct); lane 6: negative control.

5.2.3. Denaturing Gradient Gel Electrophoresis.

In order to determine the community structure of the amplificate, the PCR products were separated using the DGGE as described previously. Figure 5.5 illustrates the different banding pattern of the various samples.

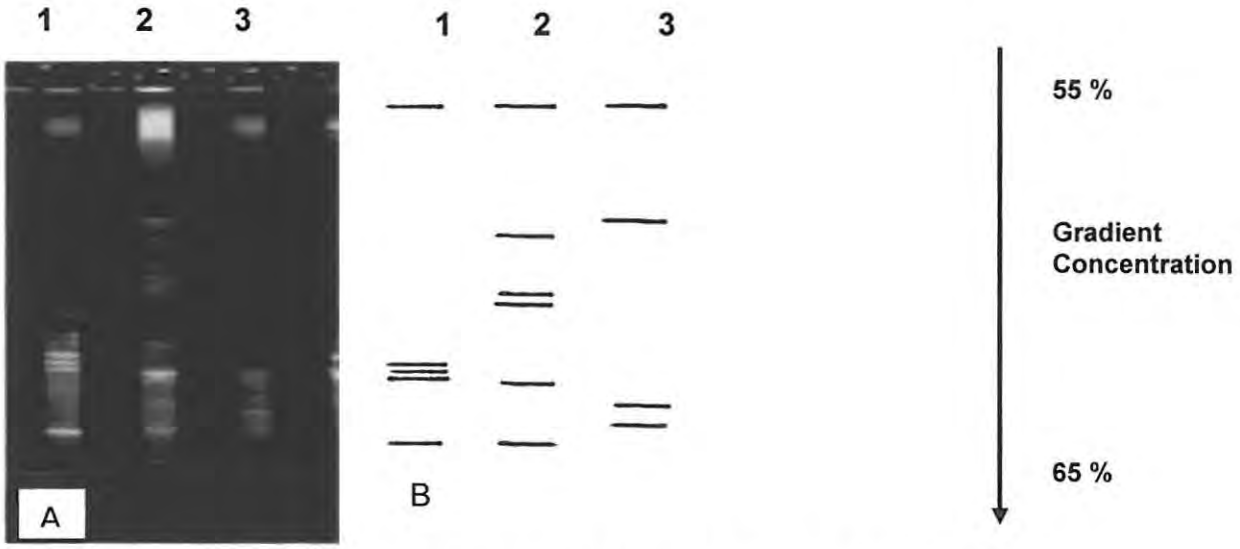


Figure 5.3: Comparison of the bacterial community in the Column Packed-bed Reactor after first sampling. (A) DGGE gel stained with ethidium bromide and photographed with Kodak digital system. Each lane represents DGGE pattern of CR product. Lane 1: Bottom of the reactor; lane 2: middle; lane 3: top of the reactor. B: Copy of the bands of interest traced from the gel.

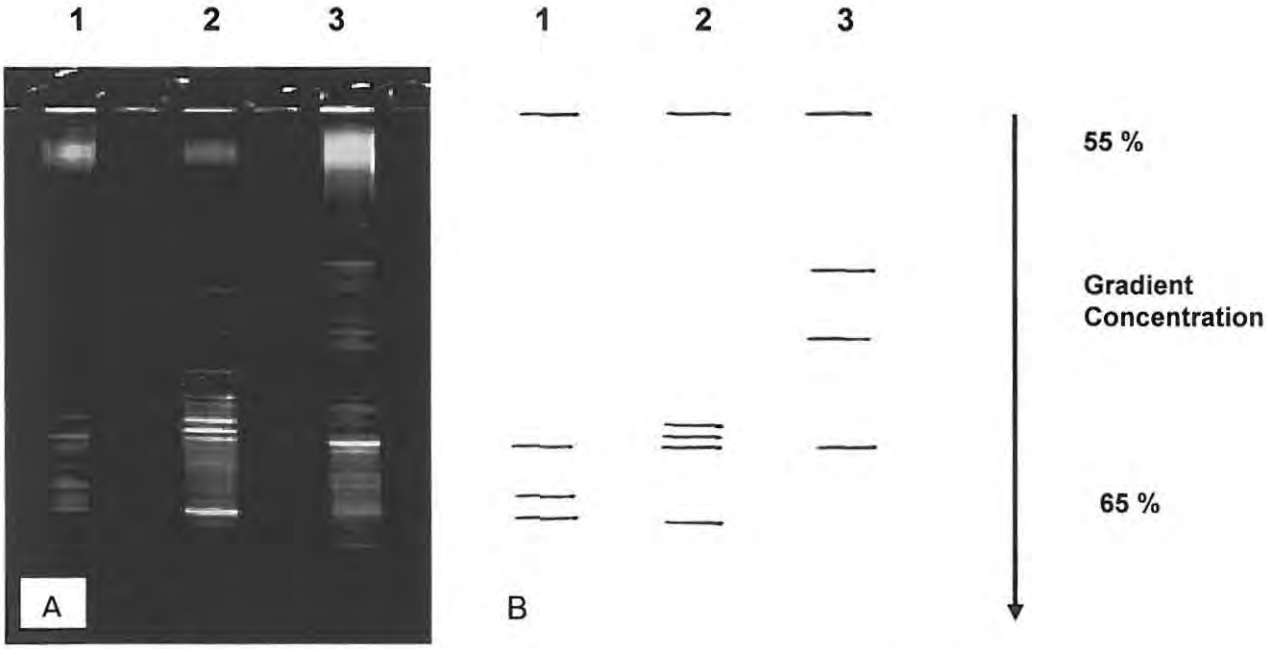


Figure 5.4: Comparison of the bacterial community in the Packed-bed Reactor after second sampling. (A) DGGE gel stained with ethidium bromide and photographed with Kodak digital system. Each lane represents DGGE pattern of PCR product. Lane 1: Bottom of the reactor; lane 2: middle; lane 3: top of the reactor. B: Copy of the bands of interest traced from the gel.

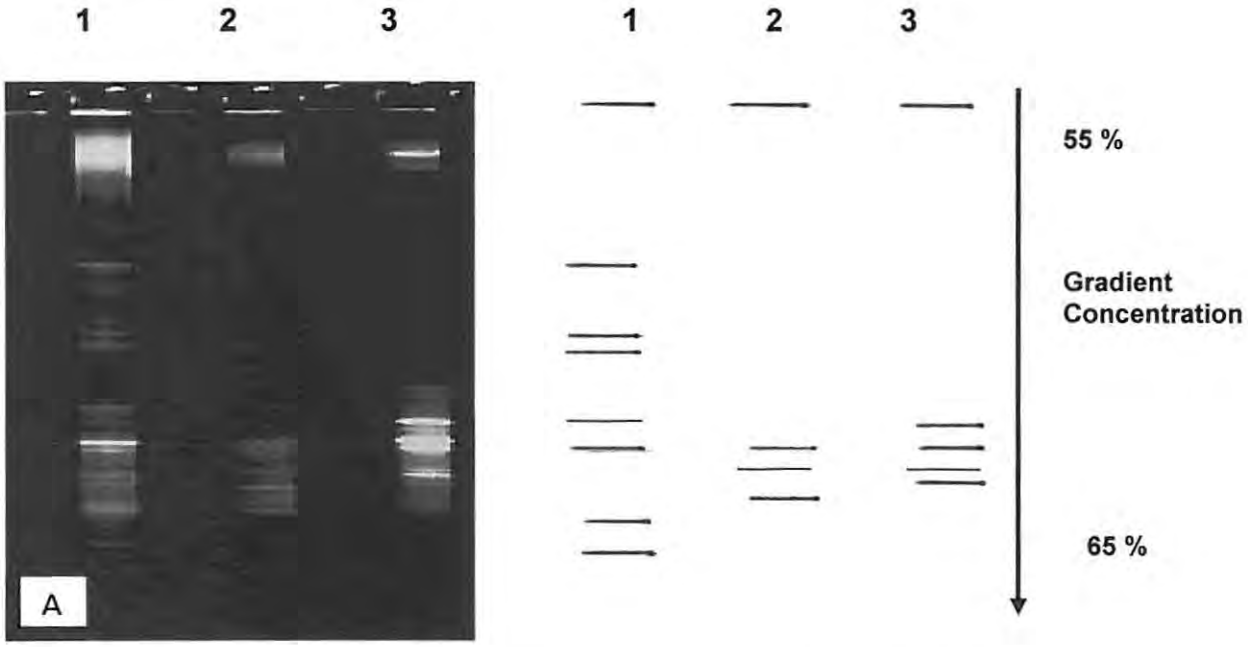


Figure 5.5: Comparison of the bacterial community in the Column Packed-bed Reactor after third sampling. (A) DGGE gel stained with ethidium bromide and photographed with Kodak digital system. Each lane represents DGGE pattern of PCR product. Lane 1: Bottom of the reactor; lane 2: middle; lane 3: top of the reactor. B: Copy of the bands of interest traced from the gel.

The bands of interest were excised, the DNA was extracted (Appendix D) and PCR amplified again. Bands of 568 bp were visible after reamplification. In order to confirm that the new amplificates were indeed single sequence PCR products an additional DGGE was performed.

5.2.4. Cloning of the 568 bp PCR Product

PCR products from DGGE bands were cloned into pGEM-T Easy Vector (Appendix E) and transformed into *E. coli* JM109 as described previously. Extracted plasmids containing inserts (Figure 5.6) were prepared for sequencing.

5.2.5. EcoR1 Digest of the Plasmid Extracts

*Eco*R1 digests were performed on the plasmid extracts. *Eco*R1 was expected to cut on either side of the 586 bp fragment (Appendix H), bands on the gel would then be a 3018 bp plasmid fragment and 586 bp insert. Some of the inserts may contain an internal *Eco*R1 site and therefore the correct result would be a 3018 bp plasmid band and two smaller bands whose sizes would add up to 568 bp. The result of the agarose electrophoresis of the digest is illustrated in Figure 5.6. All lanes contained the correct sized bands, lane 3, 6, 7 and 10 all had 568 bp insert and 3018 bp plasmid. Lanes 4, 5, 8 and 9 contained inserts with an internal *Eco*R1 site, the gel showed the bands present, a large 3018 bp plasmid band and two smaller bands whose combined size was approximately 568 bp. Once it was established that the correct size inserts were present in the plasmids, the samples were prepared for sequencing.

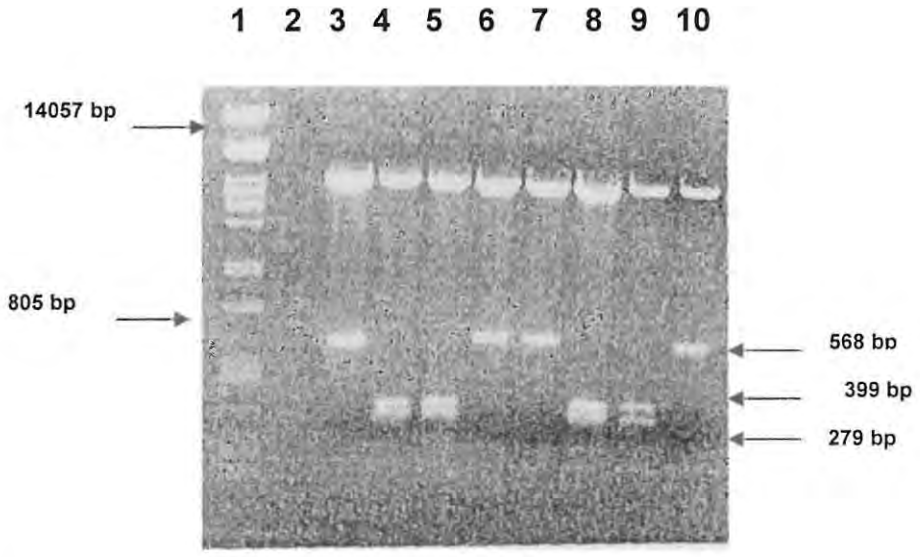


Figure 5.6: Agarose gel of plasmids digested with *Eco*R1. Lane 1 represents molecular marker *λpst*, lanes 3–4 represent digested plasmids. The plasmids were extracted from transformants. Arrows indicate inserts.

5.2.6. DNA Sequencing

A total of 85 clones were obtained from the DGGE bands. Prior to sequencing each of these were screened for inserts using *Eco*R1 restriction digest. The plasmids were extracted as described previously and inserts were sequenced using an automated ABI 3100 Prism® Genetic Analyser as described previously. The data were analyzed firstly by using NCBI BLAST database and then phylogenetically using Neighbor Joining algorithm as described previously. Tables 5.1 and 5.2 show the BLAST (Altschul *et al.*, 1997) results of the various 16S sequences obtained with the closest relative from Genbank database and the % divergence of the strain from this relative.

Table 5.1: The BLAST results of the 16S rRNA sequences for column packed-bed reactor obtained with the closest relative from the Genbank database. Clone numbering refers as follows: A=Inlet; B=mid column; C=outlet. Roman numerals refer to first, second and third sampling.

Clones	Group	Closest Relative to the Clone	Access Number	Taxonomic Affiliation of the Closeset Relative to the clone according to Bergey's Manual Phylum	Class	Genus	% Similarity
Ci1	1A	Cluster III termite bacterium	AB055736	Unassigned	Unassigned	Unassigned	96
Cii1	1A	Bacterium Phenol-4	AF121885	Unassigned	Unassigned	Unassigned	96
Bi2	1A	Bacterium Phenol-4	AF121885	Unassigned	Unassigned	Unassigned	97
Ci3	1A	Bacterium Phenol-4	AF121885	Unassigned	Unassigned	Unassigned	98
Ai5	1A	Bacterium Phenol-4	AF121885	Unassigned	Unassigned	Unassigned	98
Ci4	1A	Bacterium Phenol-4	AF121885	Unassigned	Unassigned	Unassigned	99
Bi6	1A	Bacterium Phenol-4	AF121885	Unassigned	Unassigned	Unassigned	100
Bi4	1A	Bacterium Phenol-4	AF121885	Unassigned	Unassigned	Unassigned	100
Bi7	1A	Benzene mineralizing assemblage	AF029039	Unassigned	Unassigned	Unassigned	97
Ai4	1A	Benzene mineralizing assemblage	AF029039	Unassigned	Unassigned	Unassigned	100
Bi14	1A	Benzene mineralizing assemblage	AF029039	Unassigned	Unassigned	Unassigned	100
Cii2	1B	*Cytophaga fermentans*	M58766	*Bacteroidetes*	*Sphingobacteria*	*Cytophaga*	97
Bi13	1C	Uncultured CFB group	AF351234	Unassigned	Unassigned	Unassigned	
Bi1	1C	Uncultured CFB group	AF351234	Unassigned	Unassigned	Unassigned	96
Bi5	1C	Uncultured CFB group	AF351234	Unassigned	Unassigned	Unassigned	97
Bii1	1D	Uncultured CFB group	AF351234	Unassigned	Unassigned	Unassigned	97
Bii2	1D	Bacteria from anoxic soil	AJ229237	Unassigned	Unassigned	Unassigned	98
Bi1	1C	Uncultured CFB group	AF351234	Unassigned	Unassigned	Unassigned	96
Bi5	1C	Uncultured CFB group	AF351234	Unassigned	Unassigned	Unassigned	97

Bii1	1D	Uncultured CFB group	AF351234	Unassigned	Unassigned	Unassigned	97
Bii2	1D	Bacteria from anoxic soil	AJ229237	Unassigned	Unassigned	Unassigned	98
Bii3	1D	Bacteria from anoxic soil	AJ229237	Unassigned	Unassigned	Unassigned	96
Bii9	1E	*Bacteroides* spp.	AF157056	*Bacteroidetes*	*Bacteroides*	*Bacteroides*	97
Cii3	1E	*Bacteroides* spp.	AF157056	*Bacteroidetes*	*Bacteroides*	*Bacteroides*	97
Cii4	1E	*Bacteroides* spp.	AF157056	*Bacteroidetes*	*Bacteroides*	*Bacteroides*	97
Cii5	1E	*Bacteroides* spp.	AF157056	*Bacteroidetes*	*Bacteroides*	*Bacteroides*	96
Cii6	1E	*Bacteroides* spp.	AF157056	*Bacteroidetes*	*Bacteroides*	*Bacteroides*	96
Biii4	2A	Uncultured bacterium	AB062829	Unassigned	Unassigned	Unassigned	97
Bii5	2A	*Spirochaeta zuelzerae*	M88725	*Spirochaetes*	*Spirochaetales*	*Spirochaeta*	99
Bii4	2A	*Spirochaeta zuelzerae*	M88725	*Spirochaetes*	*Spirochaetales*	*Spirochaeta*	100
Bii6	2B	*Treponema brennaborense*	Y16568	*Spirochaetes*	*Spirochaetales*	*Treponema*	98
Bii7	2C	Uncultured bacterium	AJ009481	Unassigned	Unassigned	Unassigned	97
Biii1	3A	Manganese oxidizing bacterium	U53824	Unassigned	Unassigned	Unassigned	97
Biii2	3A	Manganese oxidizing bacterium	U53824	Unassigned	Unassigned	Unassigned	97
Biii3	3B	Uncultured bacterium	AY159185	Unassigned	Unassigned	Unassigned	97
Bi8	3C	*Hyphomicrobium* spp.	Y14306	Proteobacteria	Alpha subclass	*Hyphomicrobiaceae*	97
Bi3	3D	*Sphingomonas*	AJ001052	Proteobacteria	Alpha subclass	*Shingomonas*	99
Bi9	4	*Azospirillum* spp.	AF411852	Proteobacteria	Alpha subclass	*Azospirillum*	98
Cii7	4	*Azospirillum* spp.	AF411852, AY118225	Proteobacteria	Alpha subclass	*Azospirillum*	97
Cii8	4	*Azospirillum* spp.	AF411852, AY118225	Proteobacteria	Alpha subclass	*Azospirillum*	97
Cii9	4	*Azospirillum* spp.	AF411852, AY118225	Proteobacteria	Alpha subclass	*Azospirillum*	97
Cii10	4	*Azospirillum* spp.	AF411852, AY118225	Proteobacteria	Alpha subclass	*Azospirillum*	97
Bii10	5	*Thiobacillus aquaesulis*	U58019	Proteobacteria	Beta subclass	*Thiobacillus*	96
Bii15	5	*Thiobacillus aquaesulis*	U58019	Proteobacteria	Beta subclass	*Thiobacillus*	96
Biii3	5	*Thiobacillus aquaesulis*	U58019	Proteobacteria	Beta subclass	*Thiobacillus*	98
Bii6	5	Uncultured bacterium	AF414580	Unassigned	Unassigned	Unassigned	98

Cii11	5	Uncultured bacterium	AF414580	Unassigned	Unassigned	Unassigned	99
Ciii4	6A	Uncultured bacterium	AB074934	Unassigned	Unassigned	Unassigned	97
Biii6	6A	Uncultured bacterium	AB074934	Unassigned	Unassigned	Unassigned	95
Ciii6	6A	Uncultured bacterium	AB074934	Unassigned	Unassigned	Unassigned	95
Biii7	6A	Uncultured bacterium	AB074934	Unassigned	Unassigned	Unassigned	95
Aiii1	6A	Uncultured bacterium	AJ229251	Unassigned	Unassigned	Unassigned	96
Ci2	6B	*Desulfosporosinus*	AJ493052	*Fermicutes*	*Clostridia*	*Desulfosporosinus* spp.	98
Ci6	6B	*Desulfosporosinus*	AJ493052	*Fermicutes*	*Clostridia*	*Desulfosporosinus* spp.	97
Ciii9	6B	*Desulfosporosinus*	AJ493052	*Fermicutes*	*Clostridia*	*Desulfosporosinus* spp.	97
Bi10	6B	*Desulfosporosinus*	AJ493052	*Fermicutes*	*Clostridia*	*Desulfosporosinus* spp.	98
Bi11	6B	*Desulfosporosinus*	AJ493052	*Firmicutes*	*Clostridia*	*Desulfosporosinus* spp.	97
Ciii10	6B	*Desulfosporosinus*	AJ493052	*Firmicutes*	*Clostridia*	*Desulfosporosinus* spp.	99
Biii5	6C	Unidentified eubacterium	AJ229190	Unassigned	Unassigned	Unassigned	96
Ciii2	6C	Unidentified eubacterium	AJ229190	Unassigned	Unassigned	Unassigned	97
Ciii5	6C	Unidentified eubacterium	AJ229190	Unassigned	Unassigned	Unassigned	96
Aii1	6D	Uncultured bacterium	AY050603	Unassigned	Unassigned	Unassigned	99
Ciii3	6D	Uncultured bacterium	AY050603	Unassigned	Unassigned	Unassigned	99
Aii2	6E	Uncultured rumen bacterium	AB009228	Unassigned	Unassigned	Unassigned	96
Bii8	6E	Uncultured rumen bacterium	AB009228	Unassigned	Unassigned	Unassigned	97
Aii4	7A	Uncultured bacterium	AJ488074	Unassigned	Unassigned	Unassigned	98
Biii8	7A	Uncultured bacterium	AJ488074	Unassigned	Unassigned	Unassigned	97
Aii5	7A	Uncultured bacterium	AJ488074	Unassigned	Unassigned	Unassigned	98
Aii3	7A	Uncultured bacterium	AJ488074	Unassigned	Unassigned	Unassigned	100
Aiii2	7B	Uncultured bacterium	AF129865	Unassigned	Unassigned	Unassigned	97
Aiii3	7B	Uncultured bacterium	AJ009493	Unassigned	Unassigned	Unassigned	98
Aiii4	7B	Uncultured bacterium	AJ009493	Unassigned	Unassigned	Unassigned	98
Ai2	7C	*Clostridium peptidovorans*	AF156796	*Firmicutes*	*Clostridia*	*C.peptidovorans*	99

Ai1	7C	*Clostridium peptidovorans*	AF156796	*Firmicutes*	*Clostridia*	*C.peptidovorans*	99
Ciii1	7D	*Clostridium* from anoxic soil	AJ229251	*Firmicutes*	*Clostridia*	*Clostridium* sp.	
Ai10	7D	*Clostridium thermosuccinogenes*	Y18180	*Firmicutes*	*Clostridia*	*C.thermosuccinogenes*	97
Ai3	7D	*Clostridium thermosuccinogenes*	Y18180	*Firmicutes*	*Clostridia*	*C.thermosuccinogenes*	98
Ai9	7D	*Clostridium thermosuccinogenes*	Y18180	*Firmicutes*	*Clostridia*	*C.thermosuccinogenes*	
Ai6	7D	*Clostridium termitidis*	X71854	*Firmicutes*	*Clostridia*	*C.termiditis*	98
Ai7	7D	*Clostridium termitidis*	X71854	*Firmicutes*	*Clostridia*	*C.termiditis*	99
Ai8	7D	*Clostridium cellobioparum*	X71856	*Firmicutes*	*Clostridia*	*C.cellobioparum*	100
Ai11	7D	*Clostridium cellobioparum*	X71856	*Firmicutes*	*Clostridia*	*C.peptidovorans*	98
Aiii5	7D	*Clostridium cellobioparum*	X71856	*Firmicutes*	*Clostridia*	*C.peptidovorans*	97
Aiii6	7D	*Clostridium cellobioparum*	X71856	*Firmicutes*	*Clostridia*	*C.peptidovorans*	97
Ciii8	7D	*Clostridium cellobioparum*	X71856	*Firmicutes*	*Clostridia*	*C.peptidovorans*	96
Ciii7	7D	*Clostridium cellobioparum*	X71856	*Firmicutes*	*Clostridia*	*C.peptidovorans*	95
Aiii7	8	*Selenomonas ruminantium*	AF221600	*Firmicutes*	*Clostridia*	*Selenomas* spp.	
Ci5	8	*Streptococcus*	AF479580	Unassigned	Unassigned	Unassigned	95
Biii6	8	*Streptococcus*	AF479580	Unassigned	Unassigned	Unassigned	

Table 5.2: Summary of the results: total count of clones designated to different taxonomic groups. Number in bold indicates total number of clones in the group. Clone numbering refers as follows: A=Inlet; B=mid column; C=outlet. Roman numerals refer to first, second and third sampling.

GROUP/SUBGROUP	CLONE	TOTAL No
1A	Cii1, Bl2, Ci3, Ai5, Ci4, Bi6, Bi4, Bi7, Ai4, Bi4,Bi14	11
1B	Cii2	1
1C	Bi13, Bi1, Bi5	3
1D	Bii1, Bii2, Bii3,	3
1E	Bii9, Cii3, Cii4, Cii5, Cii6,	5
TOTAL 1		**23**
2A	Bii5, Bii4, Biii4	3
2B	Bii6	1
2C	Bii7	1
TOTAL 2		**5**
3A	Biii1, Biii2,	2
3B	Biii3	1
3C	Bi8	1
3D	Bi3, Bi9	2
TOTAL 3		**5**
4	Cii7, Cii8, Cii9, Cii10	**5**
5	Bii10, Cii15, Bii6, Cii11, Biii3	**4**
6A	Ciii4, Biii6, Ciii6, Biii7	4
6B	Aiii1	1
6C	Ci2, Ci6, Cii9, Bi10, Bi11, Ciii10,	6
6D	Biii5, Ciii2, Ciii5, Aii1, Aiii3, Aii2, Bii8	7
TOTAL 6		**19**
7A	Aii4, Biii8, Aii5, Aii3	4
7B	Aiii2, Aiii3, Aiii4	3
7C	Ai2, Ai1	2
7D	Ciii1, Ai10, Ai13, Ai9, Ai6, Ai7, Ai8	7
7E	Ai11, Aiii5, Aiii6, Ciii8, Ciii7	5
TOTAL 7		**21**
8	Aiii7, Ci5, Biii6	3
TOTAL 8		**3**

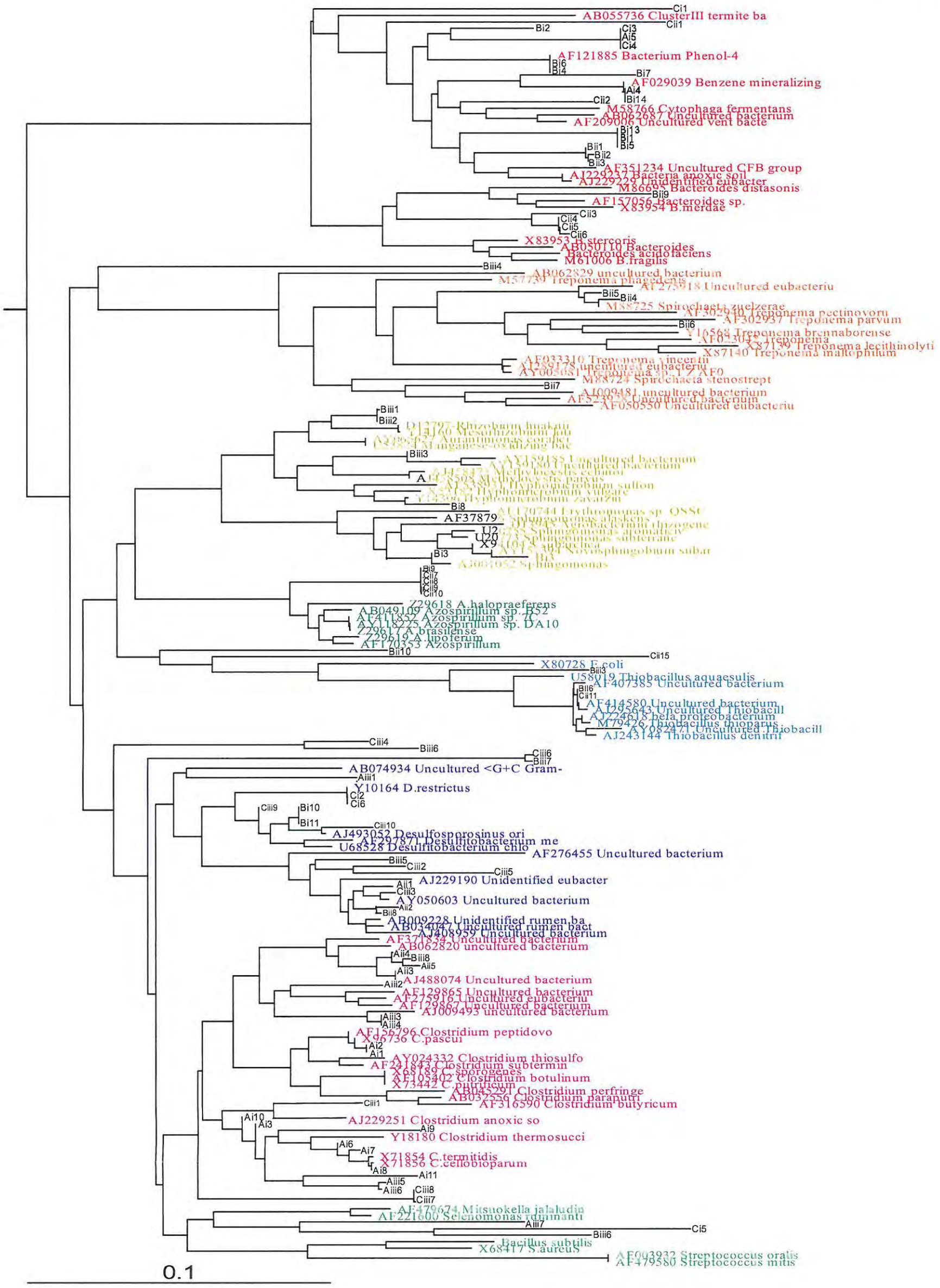


Figure 5.7: Phylogenetic relationship of the total clone population in the lignocellulose packed column reactor. Taxonomic groups are depicted by different colours and the clones in black. Space bar indicates 1% sequence divergence.

5.2.7. Description of Clones

Figure 5.7 shows the large diversity of microorganisms, which established themselves within the lignocellulose column reactor during the bioremediation process. The most abundant appears to be "group one".

Group One

This group is subdivided into 5 subgroups (A, B, C, D, and E) and consists of bacteria belonging to the Division *Bacteroidetes*, mainly represented by genera *Bacteroides* and *Cytophaga* (Table 5.2). This group, although quite diverse, cluster together on a single branch of the dendrogram (Figure 5.7). One group of clones Cii1; Bi2; Ci3; Ai5; Ci4; Bi6 and Bi4 are related to bacterium phenol-4 AF121885 (Knight *et al.*, 1999). In very close relation with this species appear to be the benzene-mineralizing bacterium AF029039 (Phelps *et al,*. 1998) represented here by clones Bi7, Ai4 and Bi14. Both species based upon their 16S rRNA sequences are placed within the ε-subdivision of the Proteobacteria and are the closest to the genus *Cytophaga* (Knight *et al.*, 1999). They are both related to a strain from a site involved in intrinsic bioremediation of a hydrocarbon- and chlorinated solvent-contaminated water (Dojka *et al.*, 1998). This suggests that this group may be involved in aromatic compound degradation.

Phenolic compounds enter the environment as an intermediate during the biodegradation of natural polymers containing aromatic rings, such as lignins and tannins, and from aromatic amino-acid precursors. In addition, they may also enter the environment as intermediates during the biodegradation of xenobiotic compounds. Phenolic compounds are also associated with pulp mills, coal mines, refineries, wood preservation plants, and various chemical industries, as well as their waste waters. Therefore the detection of the organisms involved in the degradation of the phenolic compounds in this

bioremediation system is of utmost importance. The first evidence of sulphidogenic phenol degradation was reported by Bak and Widdel in 1986 who have described a pure culture, *Desulfobacterium phenolicum* that oxidizes phenol to CO_2 using SO^{2-}_4 as terminal electron acceptor. Oremland and Capone (1988) noted that phenol degradation was dependent on the presence of sulphate and inhibited by the addition of molybdate, a specific inhibitor of sulphate reduction. This inhibition indicates that degradation of phenol is coupled to sulphate reduction, however, it is noteworthy that the Phenol-4 clone does not fall within previously described sulphate reducers (Dojka, *et al.,* 1998). Assuming that the bacteria represented by these clones are able to reduce sulphate, the ability to reduce sulphate may be a function of a more diverse group of microorganisms than previously believed. Several sulphate-reducing bacteria have been reported to degrade aromatic compounds including phenol, benzoate, catechol and aniline and utilize their end product as carbon and energy sources (Haggblom and Young, 1995).

Aromatic hydrocarbons were considered to be recalcitrant in the environment under anaerobic conditions until the first evidence of anaerobic degradation of BTEX (benzene, toluene, ethylbenzene and xylene) compounds was reported by Kuhn *et al.* (1985). It was found that the most easily degradable aromatic hydrocarbon was toluene. Detailed studies on the degradation of toluene by the denitrifying bacteria had shown that the first degradation step is the addition of fumarate to the toluene methyl group (Fraser *et al.,* 1993; Beller and Sportman, 1997). They demonstrated that this reaction is catalyzed by benzylsuccinate synthase which is responsible for degradation of such environmental pollutants as *xylene and cresol (*Beller and Sportman, 1997).

Anaerobic degradation of polycyclic aromatic hydrocarbons has been demonstrated by Langenhof *et al.* (1996) and Coates *et al.* (1997 and 2002). Naphthalene-degrading denitrifying and sulphate reducing cultures have been

reported by several researchers (Moller *et al.,* 1998; Meckenstock *et al.,* 2000; and Rockoe *et al.*, 2000.)

Annweiler *et al.* (2000) reported the anaerobic degradation of 2-methylnaphthalene by a sulphate reducing enrichment culture and they identified the first enzyme in the pathway as naphthyl-2-methyl-succinate synthase. They identified two groups of metabolites, namely naphthyl-2-methyl-succinic acid and naphthyl-2-methylene-succinic acid. Both of these compounds appeared to be structural analogs of the first metabolites in anaerobic toluene degradation, namely benzylsuccinic acid and phenylitaconic acid (Haider *et al.,* 1999).

A number of clones in this group are related to *Cytophaga fermentens* M58766 (Gherna and Woese, 1992) and two other uncultured bacteria AB 062687, a member of an ethylbenzene degrading, sulphate reducing bacteria and AF209006, isolated from a shallow submarine hydrothermal vent. Both are closely related to *Cytophaga* spp. Other clones in this group Bi3, Bi1, Bi5, Bii1, Bii2 and Bii3 group together with the uncultured CFB group isolated from anoxic environmental samples (Ruimy *et al.,* 1996; Chin *et al.,* 1999; Plumb *et al.,* 2001; Bakermans and Madsen, 2002). Clone Bii9 branches together with *Bacteroides* spp. AF157056 from marine gut biota as closest match (Dewhirst *et al.,* 1999).

Group Two

Group 2 in these analyses may be subdivided into three subgroups, A, B and C (Table 5.2, Figure 5.9), and is represented by the clones clustering together with a variety of *Treponema* and *Spirochaeta* spp. This group is very diverse and members have previously been assigned mainly as human pathogens. However, recent findings point to its close symbiosis with various termites and therefore, it may be involved in lignocellulose degradation. Von Wintzigerode

et al. (1999) demonstrated that some *Treponema* spp. are members of the trichlorobenzene-transforming microbial consortium.

Leadbetter *et al.* (1999) isolated from termites' gut two strains of *Treponema* sp. (strain ZAS-1 and ZAS-2), which proved to be CO_2–reducing acetogens. This finding was further confirmed by Noda *et al.* (2003) during their study on ectosymbiotic spirochetes in termite gut. Kasperowicz and Michalowski (2002) noted that *Treponema saccharophilum*, an inhabitant of the rumen, is responsible for fructan degradation in Timothy grass.

The protein of *Treponema pallidum* originally reported to be an outer membrane protein of the syphilis spirochete has been shown to be actually a component of the ABC (ATP-binding cassette) system which shuffles Zn(II) and possibly other metals like manganese across the cytoplasmic membrane (Lee *et al.*, 2002). This finding together with the claim of Langendijk-Genevaux *et al.* (2001), that the presence of SRB is positively correlated with the occurrence of *T. denticola* explains the occurrence of this phylum in the bioremediation process.

Group Three

This group belongs to phylum Proteobacteria, class Alphaproteobacteria. According to Bergey's Manual (1974) it can be further subdivided on the family level with leading families such as *Rhizobiacea, Hyphomicrobiaceae* and *Sphingomonadaceae* (Table 5.2; Figure 5.9). Family *Hyphomicrobiaceae* is represented here mainly by clone Bi8 closely related to *Hyphomicrobium vulgare* and *Hyphomicrobium zavarzinea.* This clone branches together with clone Biii2 and Biii1, which are clustered with a manganese oxidizing bacterium. Family *Sphingomonadaceae* is represented by clones Bi3 and Bi9, both very closely related to *Sphingomonas* spp. and this phylum has been a focus area of many environmental researchers. Nohynek *et al.* (1996) isolated strains KF1^T, KF3 and NKF1 of previously *Pseudomonas,* later

reclassified as *Sphingomonas,* which became known to degrade chlorophenol. This finding was later confirmed by Crawford and Ederer (1999). At the same time research by Kim and Zylstra (1999) demonstrated that *Sphingomonas* spp. are able to utilize toluene, *m*-xylene, *p*-xylene, biphenyl, naphthalene, phenanthrene and anthracene as sole sources of carbon and energy for growth. Masai *et al.* (1999) isolated the strain of *Sphingomonas paucimobillis* SYK-6 that was able to grow on a variety of dimeric lignin compounds. These compounds were degraded via vanillate and syringate by a unique enzymatic system composed of etherases, *O*-demethylases, ring cleavage oxygenases and side chain cleaving enzymes. Further studies of the same research group revealed that SYK-6 has developed two *O*-demethylation systems in order to get energy from C_1 compounds. Sonoki *et al.* (2000) proposed two different *O*-demethylation systems in SYK-6 (Figure 5.8).

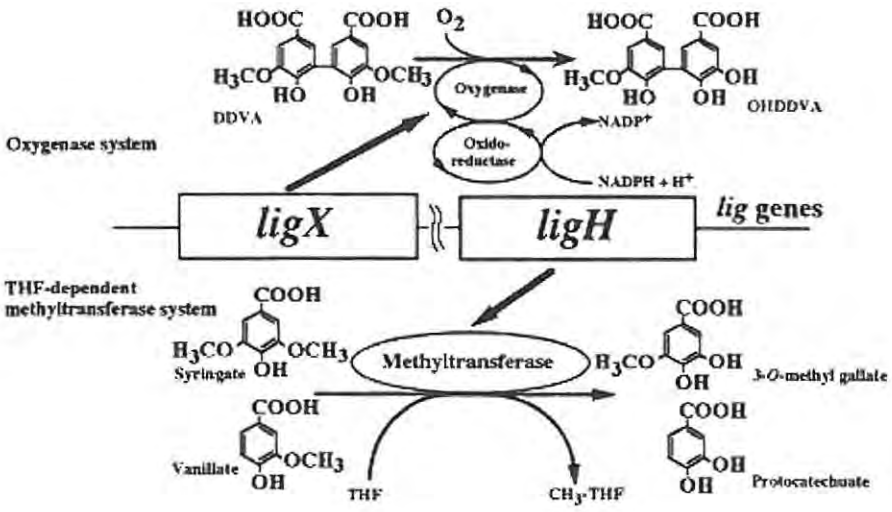


Figure 5.8: Proposed different O-demethylation systems for cleavage of the methyl ether linkage in *S. paucimobilis* SYK-6 (After Sonoki *et al.*, 2000).

They also found that ligH was similar to FTHS (formyltetrahydrase syntheses) of *Clostridium thermoaceticum* (Sanoki *et al.*, 2000). *Sphingomonas*

paucimobillis SYK-6 was also found to degrade ferulic acid to vanillin, which is further metabolized through 4,5-cleavage pathway to pyruvate and oxaloacetate (Figure 5.9) (Masai *et al.*, 2002).

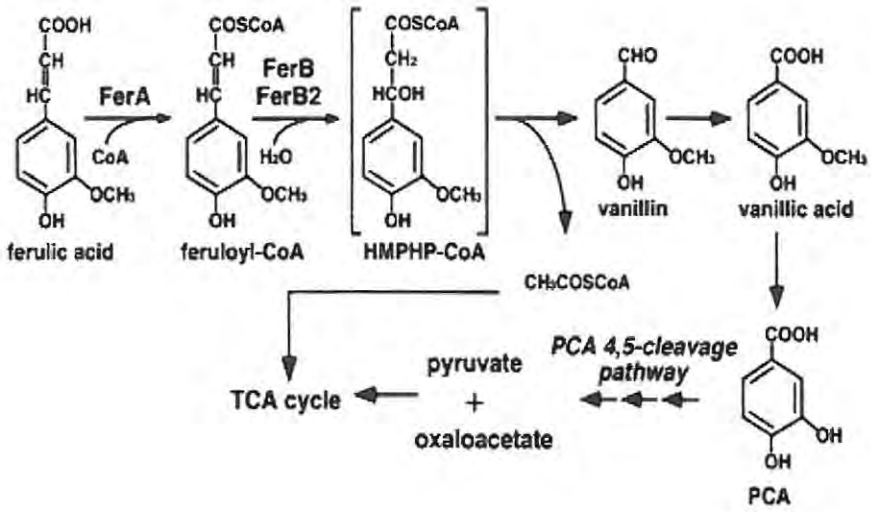


Figure 5.9: Ferulic acid catabolic pathway of *S. paucimobilis* SYK-6. (After Masai *et al.*, 2002).

Group Four

Group 4 is represented by strains related to *Azospirillum* which are mostly similar to three *Azospirilium brasilense*., including one nitrogen fixer from fuel contaminated Arctic soil, AF411852 (Eckford *et al.*, 2002). This strain branches together with clones Cii7, Cii8, Cii9 and Cii10. It is noteworthy that according to Lopez-de-Victoria and Lovell (1993), many *Azospirillum* species possess strong chemotaxis to aromatic substrates such as benzoate, protocatechuate, 4-hydroxybenzoate and catechol.

Group Five

This group is represented by two clones, namely Bii6 and Cii11 which cluster together with *Thiobacillus* spp, These species branch within *Thiobacillus*

aquaesulis and *Thiobacillus denitrificans*, but closest to *Thiobacillus thioparus*, a thiosulphate-oxidizing bacteria, AF414580 from a uranium reducing community (Chang *et al.,* 2000) and AJ224608 from a thiosulphate oxidizing community.

Group Six

Group 6 consisting of the following clones: Ciii4, Biii6, Ciii6, Biii7, Ciii1, Aiii1, CI2, Ci6, Cii9, Bi10, Bi11, Ciii10, Biii5, Ciii2, Ciii5, Aii1, Aiii3, Aii and Bii8 branch together with a number of sulphate reducing bacteria such as *Desulfosporosinus* and *Desulfitobacterium.* According to phylogenetic analysis the closest species in this cluster are *Desulfitobacterium metallireducens* (AF297871) known to be involved in the reduction of metals and humic acids as well as chlorinated compounds. The attention in this group is drawn by strain 853-15A(T) isolated from a uranium-contaminated aquifer, clustering together with clones Ciii; Bi10; Bi11; Ciii10. It was noted by Finneran *et al.* (2002) that the end products of the metabolism of this strain appear to be acetate and carbon dioxide. Additional electron donors included formate, ethanol, butanol, butyrate, malate, and pyruvate. *Desulfosporosinus orientis* appearing in the same cluster has the ability to use malate and glycerol as electron donors and fumarate and arsenate as electron acceptors (Stackebrandt *et al.,* 2003).

On the same cluster appear yet unidentified and uncultured rumen bacteria AY050603/AB009228/AB034047/AJ408959, represented here by clones Ciii2, Ciii5, Ciii3, and Ciii4.

Group Seven

This group is subdivided into four groups, A, B, D, and C. The clones from group A and B: Aii4, Biii8, Aii5, Aii3, Aiii2, Aiii3, Aiii4, cluster together with a range of as yet uncultured species obtained from environmental samples. The latter include AJ488074, part of a consortium involved in dechlorination of chlorobenzene (direct submission, 2002), AJ229251, a polysaccharide degrading bacterium from anoxic soil, AF129865/Ay275916, both from lignocellulose anaerobic digesters, AF371834, a pig gut bacterium, and AB062820, a termite gut bacterium. The presence of the latter is quite noteworthy from a physiological and nutritional point of view. It is known that the diverse microbiota of the termites gut enable the host to feed on lignocellulosic material. Therefore, any resemblance between the termites gut population and that of the reactor population implies that the microbiota which have developed in the lignocellulose degrading packed-bed reactor have the ability to utilize the complex lignocellulose material or intermediates of its degradation (Ohkuma *et al.,* 1999 and Tokuda *et al.,* 2000).

The clones from groups C and D: AI2, AI1, Ai10, Ai13, Ai9, Ai6, Ai7, Ai8, Ai11, Aiii5, Aiii6, Ciii8, Ciii7 which are predominantly of *Clostridium* species with the closest species *Clostridium thermosuccinogenes*-Y18180 (Stackebrandt *et al.,* 1999), *C. termiditis*-X71854 and *C. cellobioparum*-X71856 (Rainey and Stackebrandt, 1993). These clostridia specifically are known to be involved in degradation of cellulose and hemicellulose. Especially *C. cellobioparum* should be of main focus in this population as, according to Van Dyke and McCarthy (2002), it belongs to the clostridial cluster III. The species belonging to this cluster are known to produce a multiprotein complex, the cellulosome (Van Dyke and McCarthy, 2002; Doi *et al.,* 2003).

Group Eight

This group of clones is clustered with tannin-tolerant organisms, both belonging to order *Clostridiales*, family *Acidaminococcaceae*, namely *Selenomonas ruminantum* and *Mitsuokella jalalundii*. Since these organisms occur in the rumen they are likely to be associated with lignocellulose degradation.

5.3. Conclusions

As was observed in the VCC reactor study (section 4.2.4) a rich diversity of microorganisms, representing a range of different microbial physiological properties, was recorded in the laboratory-scale column reactor. Again the consortia included fermentative, cellulolytic, acetogenic and aromatic compound-cleaving forms, which may be trophically related to the components of the lignocellulose compound being degraded. This raises the question as to why the packed-bed system failed, after a short period of operation, even though the appropriate microbial populations appear to have been in place.

Previous investigators have observed that consecutive vertical zonations of predominant species occur simultaneously in the wastewater environment, and depend on the organic matter provided by other members of their consortium. This is particularly evident in biofilm architectures (Ramsing *et al.*, 1993; Lettinga, 1995; deBeer *et al.*, 1997; Santegoeds *et al.*, 1998; Okabe *et al.*, 1999; Santegoeds *et al.*, 1999). It is thus apparent that, although the appropriate biocatalysts may be present in a system, in tackling the degradation of complex organic structures, such as lignocellulose, the spatial and temporal arrangements of the system might critically influence events, particularly given a linear flow regime.

The laboratory-scale lignocellulose packed-bed study provided the opportunity to draw samples both over time and along the length of the column flow path, and thereby to interrogate the system for the possible presence of spatial and temporal distribution patterns indicating trophic gradients within the system.

CHAPTER SIX

Spatial and Temporal Distribution of the Microbial Population Colonizing Lignocellulosic Material in the Laboratory-scale Packed-bed Reactor

6.1. Introduction

It has been observed that natural assemblages of bacteria within a biofilm matrix can function as a cooperative consortium, in a relatively complex and coordinated manner (Costerton *et al.,* 1995; Davey and O'Toole, 2000). Instead of being seen as simple slime layers containing microorganisms randomly adhering to surfaces, biofilms are now thought to represent biological systems with a high level of organization, and where bacteria form structured, coordinated and functional communities (O'Toole *et al.,* 2000). In this regard, in natural environments, biofilms are composed of a multispecies microbial community of anchored bacteria that share genetic material and fill distinct trophic niches (Watnick and Kolter, 2000). They distribute themselves according to their ability to survive in that particular environment and also according to any symbiotic relationships between the groups of bacteria. Therefore, the bacteria in a multispecies biofilm are not randomly distributed but rather organized to meet the needs of each other (Moller *et al.,* 1996; and 1998; Okabe *et al.,* 1999).

In nature, bacterial communities play a key role in the production and degradation of organic matter, the degradation of many environmental pollutants and the cycling of nitrogen, sulphur, carbon and many metals. This process has been demonstrated for both surface-attached biofilms and the sludge granules that are readily formed in anaerobic reactors (MacLead *et al.,* 1990). Furthermore, the development of these biofilms results in more efficient processing of contaminants in wastewater treatment (Wydham and Kennedy, 1995).

In view of the above, there is a critical need to understand microbial diversity both in natural and in engineered environments. Over the past few decades,

the outlook on microbial diversity has changed substantially, mainly due to molecular phylogenetic studies that try to establish objective relationships between organisms in complex natural consortia (Hugenholtz *et al.,*1998).

In the studies reported on earlier at least some of the organisms, with the biocatalytic capacity necessary for lignocellulose degradation, were present in both the VCC and the laboratory-scale packed-bed reactors. The manner in which the sampling had been undertaken in the laboratory-scale reactor made it possible to also investigate possible spatial and temporal distribution patterns within the system, and thus to determine whether the presence of a complex consortial architecture could be detected. Moreover, whether these patterns could be related in any way to the observed performance of these systems in sulphate reduction activity is discussed in chapter 3.

The use of modified sampling ports in the laboratory- scale reactor (described in Material and Methods, chapter 2) enabled samples to be drawn along both the length of the reactor and at intervals of a one year period of operation of the system. The data acquired in this procedure enabled the investigation of the spatial distribution patterns of microbial communities in the reactor system.

Analyses of the microbial communities identified have been summarized in Tables 6.1–6.3 and Figures 6.1–6.3.

6.2. Inlet Zone

The laboratory-scale reactor was operated as an upflow system with the inlet zone (first third of the reactor) as shown in Figures 2.21 and Figure 2.22. The sampling port 2 was used to sample the inlet zone (Figure 2.21)

Table 6.1: Distribution of microbiota in the inlet zone (first third) of the column reactor.

Zone	Sampling time	Clone	Group	Inferred trophic activity
IN	**1**	Ai11	7	Cellulose degraders
		Ai5	1	Aromatic degraders
		Ai2	7	Cellulose degraders
		Ai1	7	Cellulose degraders
		Ai3	7	Cellulose degraders
		Ai6	7	Cellulose degraders
		Ai7	7	Cellulose degraders
		Ai9	7	Cellulose degraders
		Ai10	7	Cellulose degraders
		Ai8	7	Cellulose degraders
	2	Aii1	6	Sulphate reducers
		Aii2	6	Sulphate reducers
		Aii5	7	Cellulose degraders
		Aii4	7	Cellulose degraders
		Aii3	7	Cellulose degraders
	3	Aiii7	8	Rumen bacteria
		Aiii2	7	Cellulose degraders
		Aiii3	7	Cellulose degraders
		Aiii4	7	Cellulose degraders
		Aiii5	7	Cellulose degraders
		Aiii6	8	Cellulose degraders

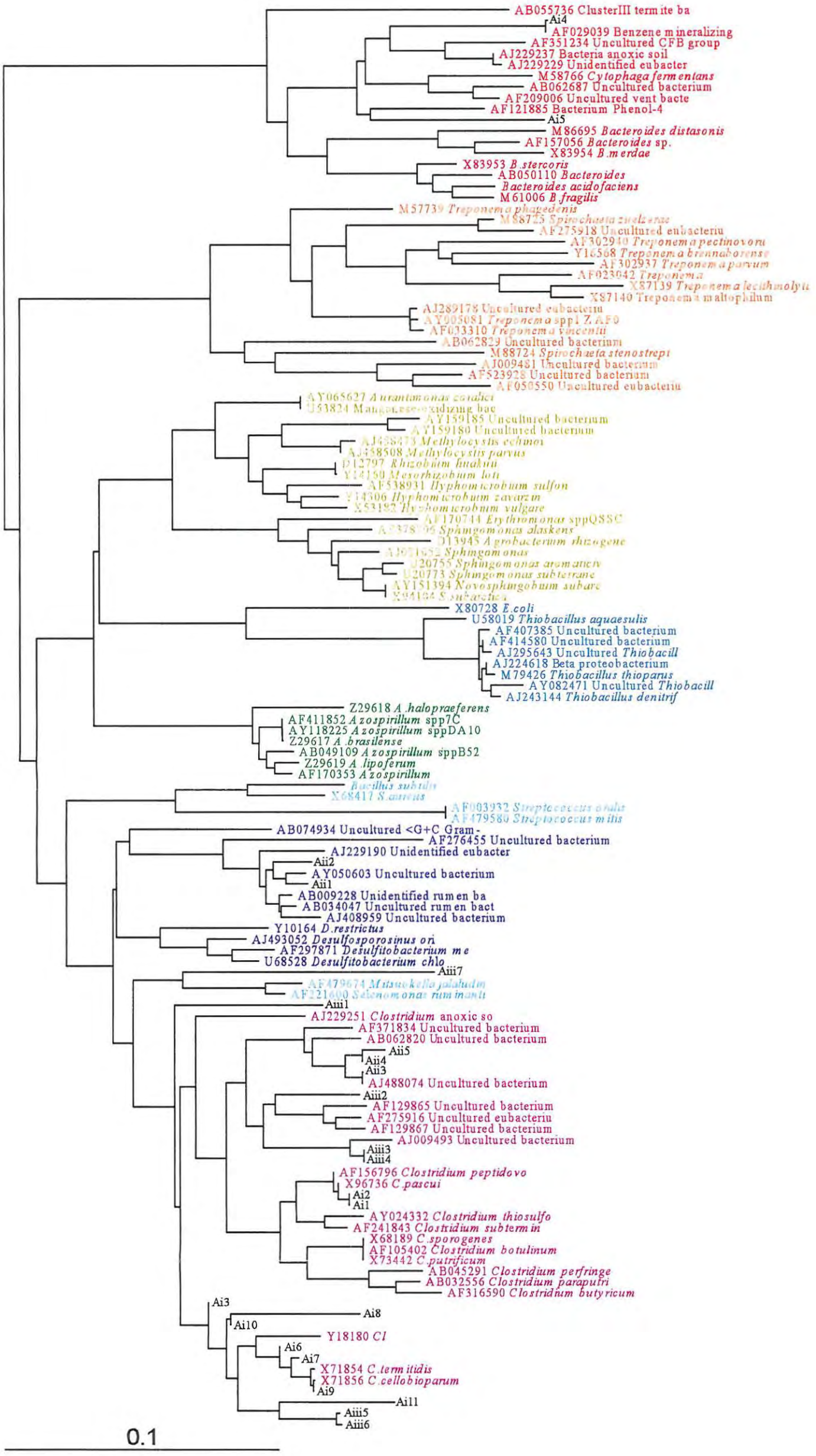


Figure 6.1: Phylogenetic analysis of the population in the inlet zone of the column packed reactor. Taxonomic groups are depicted by different colours and clones in black. Space bar indicates 1% sequence divergence.

Table 6.1 and Figure 6.1 show that the first third of the column was dominated by the Gram–positive eubacteria with low G+C values, and they are related to the genus *Clostridium* according to the phylogenetic analysis. These bacteria predominated in this zone of the column during the whole of the study period. However, during the second and third sampling periods the consortium developed to include a range of uncultivated species that have been identified from other environmental samples. The latter include AJ488074, part of a consortium involved in dechlorination of chlorobenzene, AJ229251, a polysaccharide degrading bacterium from anoxic soil, AF129865/Ay275916, both from lignocellulose anaerobic digesters, AF371834, a pig gut bacterium, and AB062820, a termite gut bacterium. Although the *Clostridium* species predominate in this section of the column, the presence of two other clones can be detected, namely Ai4, closely related to a bacteria from benzene mineralizing consortium AF029039 and Ai5, closely related to bacterium phenol-4, AF121885.

6.3. Middle Zone

The middle zone of the reactor S2 (second third of the column) was sampled from port 6 (Figure 2.21)

Table 6.2: Distribution of microbiota in the middle zone (second third) of the column reactor.

ZONE	SAMPLING TIME	CLONE	Group	Inferred trophic activity
MIDDLE	**1**	Bi2	1	Aromatic degraders
		Bi6	1	Aromatic degraders
		Bi4	1	Aromatic degraders
		Bi7	1	Aromatic degraders
		Bi1	1	Aromatic degraders
		Bi14	1	Aromatic degraders
		Bi13	1	Aromatic degraders
		Bi5	1	Aromatic degraders
		Bi8	3	Aromatic degraders, involved in lignin degradation
		Bi3	3	Aromatic degraders, involved in lignin degradation
		Bi9	3	Aromatic degraders, involved in lignin degradation
		Bi10	6	Sulphate reducers
		Bi11	6	Sulphate reducers
	2	Bii1	1	Aromatic degraders
		Bii2	1	Aromatic degraders
		Bii3	1	Aromatic degraders
		Bii4	2	CO_2 – reducing acetogens,
		Bii5	2	CO_2 – reducing acetogens,
		Bii6	5	CO_2 – reducing acetogens,
		Bii7	2	CO_2 – reducing acetogens,
		Bii8	6	Sulphate reducers
		Bii9	1	Aromatic degraders
		Bii10	4	*Azospirillum*
	3	Biii1	3	Manganese oxidizers
		Biii2	3	Manganese oxidizers
		Biii3	3	Manganese oxidizers
		Biii5	6	Sulphate reducers
		Biii6	8	Rumen bacteria
		Biii7	6	Sulphate reducers
		Biii8	7	Cellulose degraders

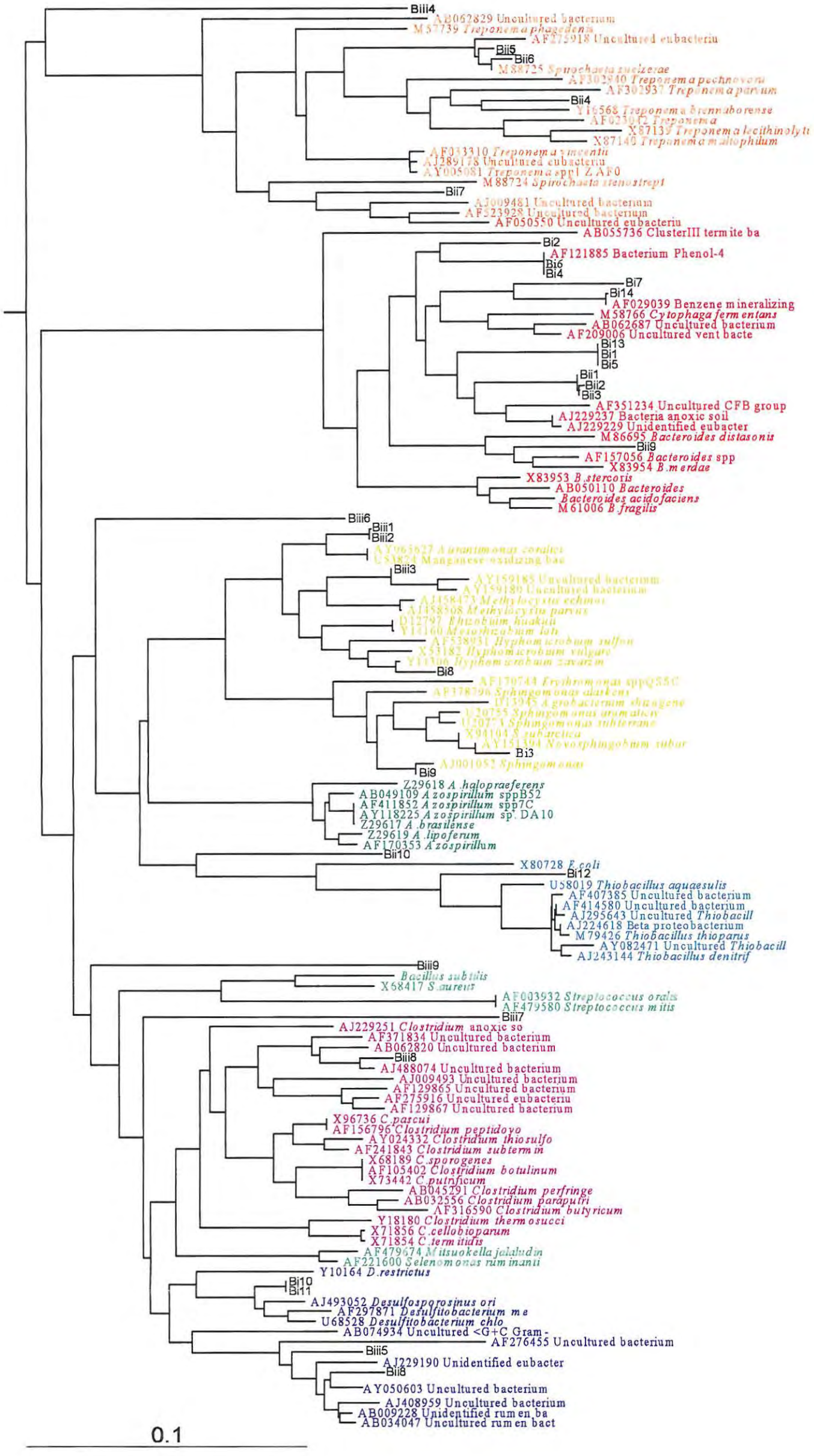


Figure 6.2. Phylogenetic analysis of the microbial population in the middle zone of the column packed-bed reactor. Taxonomic groups are depicted by different colours and the clones in black. Space bar indicates 1% sequence divergence.

The second third of the column, at sampling time 1, was dominated by the clones belonging to the genus *Bacteroides* and *Cytophaga*. Among them the most abundant are those related to the Bacterium phenol-4, which could be detected, but with fewer types in the first zone of the column. At this time, clones related to the genera *Sphingomonas, Novosphingobium, Desulfosporosinus* and *Desulfitobacterium* also appear.

At the later stages (second and third sampling times) new species were detected such as *Spirochaeta, Treponema,* and an uncultured *Clostridium* spp. that branches together with an unidentified sulphate reducing bacteria.

6.4. Upper Zone

Sampling port 10 corresponding to S3 (Figure 4.1) was used to draw samples from the upper zone of the reactor.

Table 6.3: Distribution of microbiota in the upper zone (last third) of the column reactor.

Zone	Sampling time	Clone	Group	Inferred trophic activity
OUT	1	Ci1	1	Bacteroides spp.
		Ci3	1	Bacterium Phenol-4
		Ci4	1	Bacterium Phenol-4
		Ci2	6	*Desulfosporosinus*
		Ci6	6	*Desulfosporosinus*
	2	Cii1	1	Aromatic degraders
		Cii2	1	Aromatic degraders
		Cii3	1	Aromatic degraders
		Cii4	1	Aromatic degraders
		Cii5	1	Aromatic degraders
		Cii6	1	Aromatic degraders
		Cii7	4	Nitrogen fixers, possessing strong chemotaxis to aromatic substrates
		Cii8	4	Nitrogen fixers, possessing strong chemotaxis to aromatic substrates
		Cii9	4	Nitrogen fixers, possessing strong chemotaxis to aromatic substrates
		Cii10	4	Nitrogen fixers, possessing strong chemotaxis to aromatic substrates
		Cii15	5	Thiosulphate oxidizers

		Cii11	5	Thiosulphate oxidizers
3		Ciii8	6	Sulphate reducers, involved in reduction of metal and humic colloids
		Ciii7	6	Sulphate reducers, involved in reduction of metal and humic colloides
		Ciii6	6	Sulphate reducers, involved in reduction of metal and humic colloides
		Ciii2	6	Sulphate reducers, involved in reduction of metal and humic colloides
		Ciii5	6	Sulphate reducers, involved in reduction of metal and humic colloides
		Ciii3	6	Sulphate reducers, involved in reduction of metal and humic colloides
		Ciii4	6	Sulphate reducers, involved in reduction of metal and humic colloides

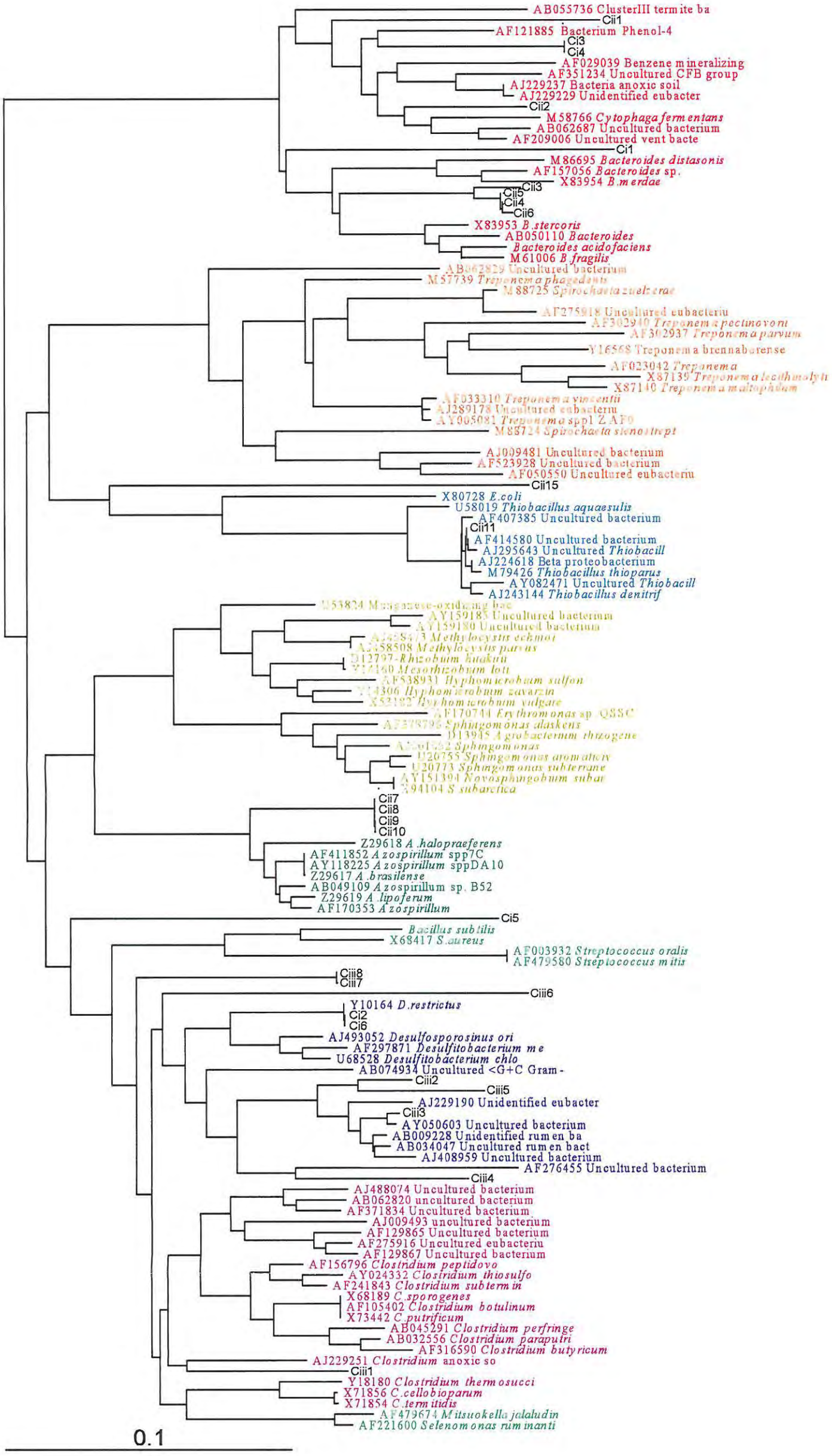


Figure 6.3: Phylogenetic analysis of microbial population in the upper zone of the column packed reactor. Taxonomic groups are depicted by different colours and the clones in black. Space bar indicates 1% sequence divergence.

In the upper zone of the bioremediation column (Figure 6.3 and Table 6.3) the microbial population was similar in some respects to that in the first two zones of the column. However, there was clear evidence of a change of pattern. The predominant microorganisms in this section were those belonging to *Cytophaga* phylum with a distinct majority of Phenol-4 bacterium (clones: Cii1, Ci3, Ci4) which branch together with the benzene mineralizing consortium AF029039, CFB group AF351234 and yet unidentified eubacterium isolated from anoxic soil environment AJ229237/AJ229229. Clone Cii2 from the same cluster branch together with *Cytophaga fermentans,* M58766 and uncultured bacterium AB062687/AF209006. Clones Cii3, Cii5, Cii4, cluster together with *Bacteroides stercosis* 83953, *B. acidofaciens* and *B. fragilis* M61006.

6.5. Correlation of Population and Biophysical Environment in the Reactor

At the same time as samples were drawn for DNA extraction and phylogenetic analysis, the chemical environment occurring within the reactor at the various zones was monitored. Figures 6.4 to 6.10 summarize the spatial and temporal gradients in the reactor and show changes across the system over time for sulphate concentration, pH, reducing sugars, aromatic compounds and volatile fatty acids.

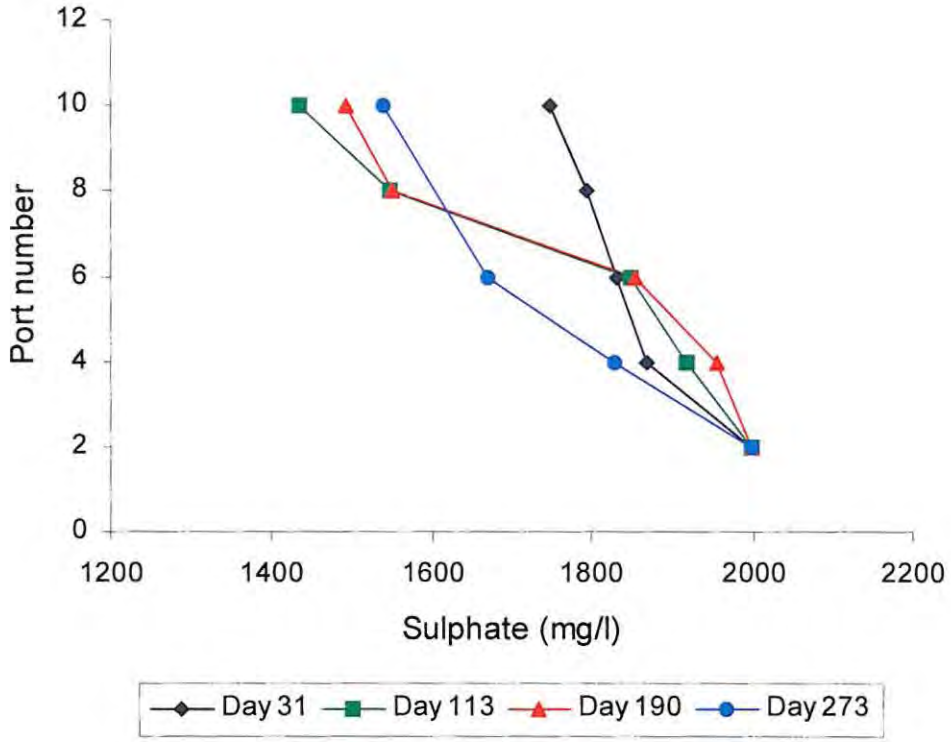


Figure 6.4: Depth profile of sulphate reduction measured across the packed-bed column reactor from the inlet to the outlet.

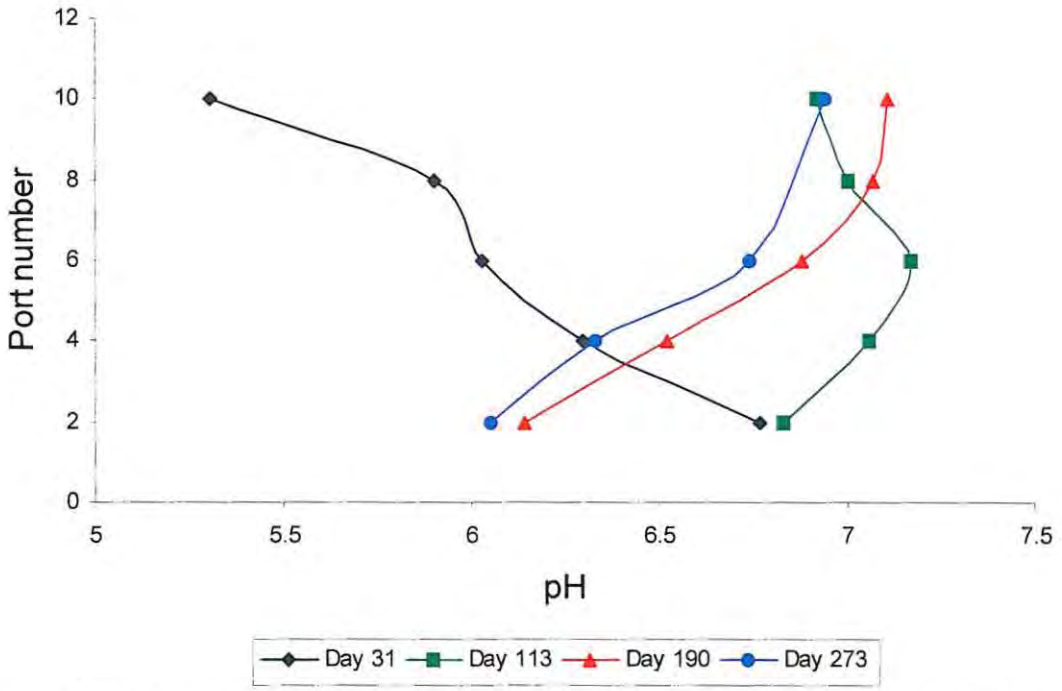


Figure 6.5: Depth profile of pH measured across the packed-bed column reactor from the inlet to the outlet.

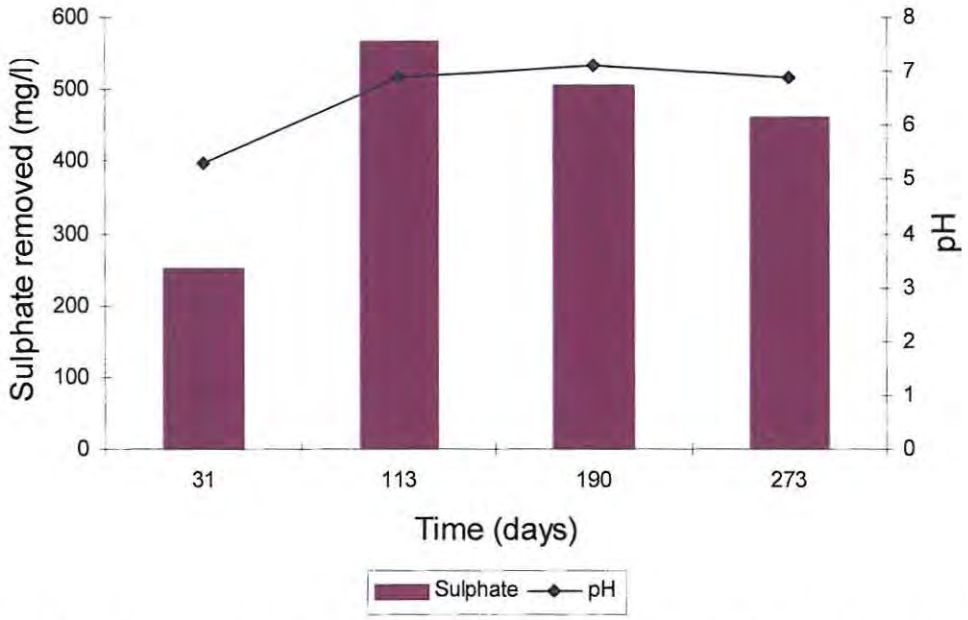


Figure 6.6: Comparison of sulphate removed and pH on the days on which sampling of the column reactor was undertaken and measured at port 10.

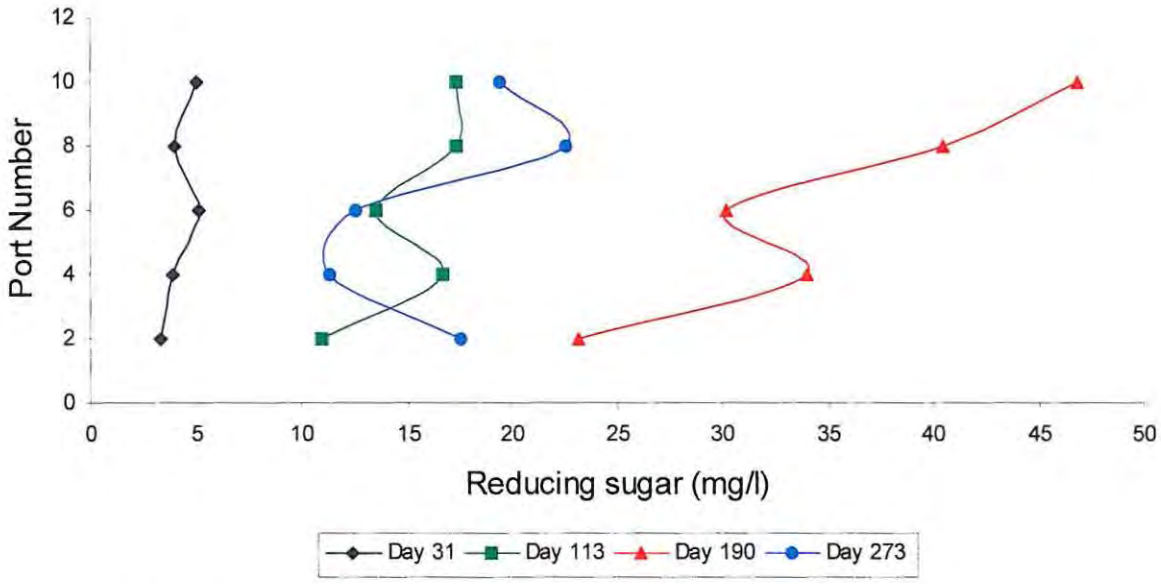


Figure 6.7: Depth profile of reducing sugar measured across the packed-bed column reactor from the sampling port 2 to the outlet port 10.

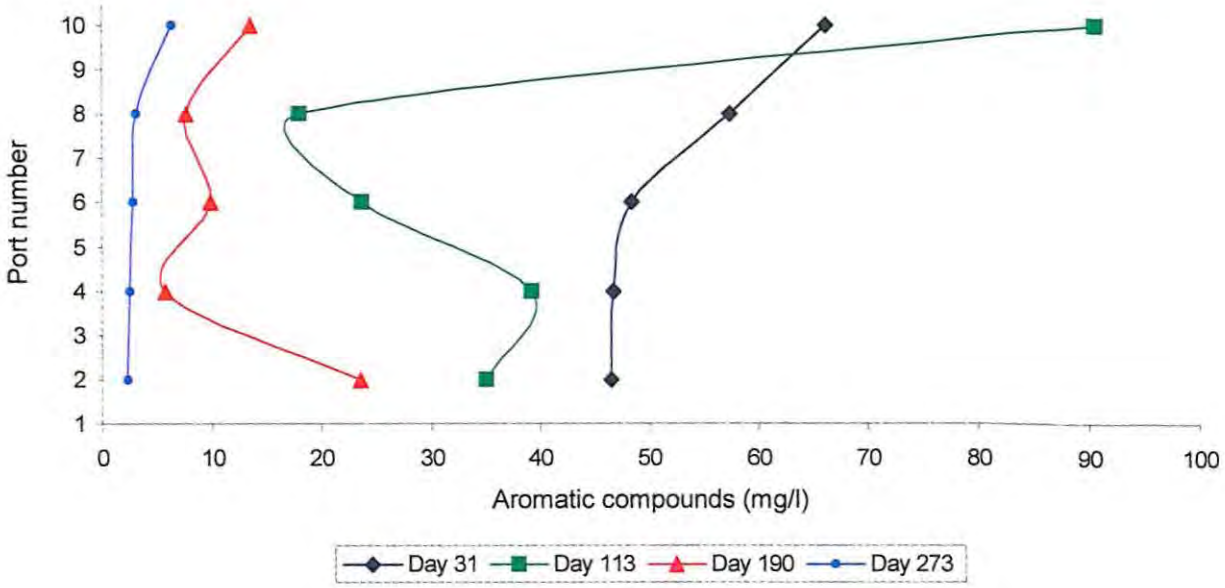


Figure 6.8: Depth profile of aromatic compounds measured across the packed-bed column reactor from the sampling port 2 to the outlet port 10.

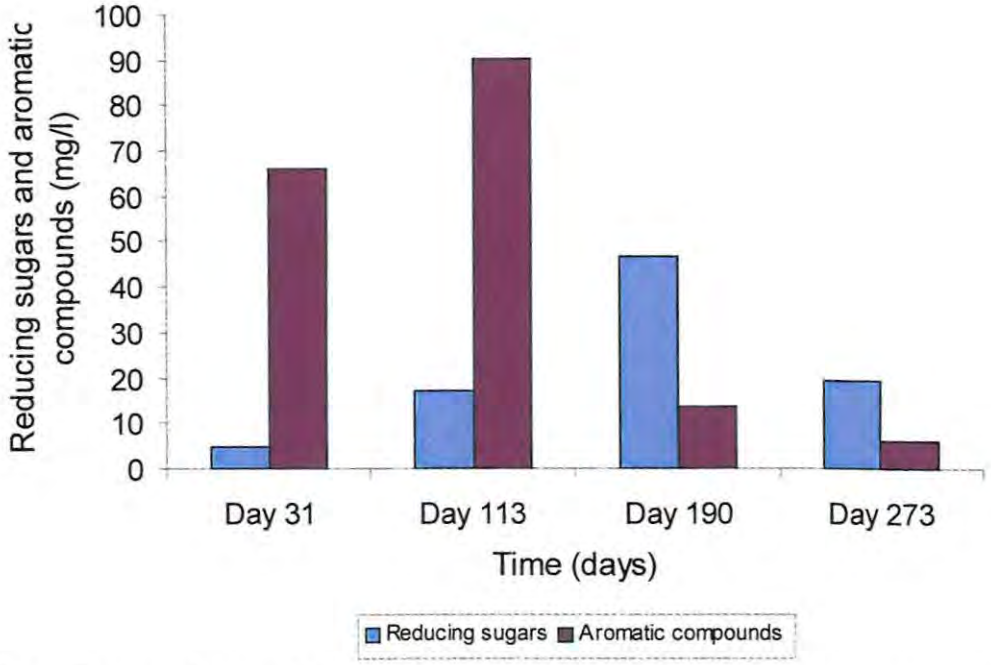


Figure 6.9: Comparison of reducing sugar and aromatic compound release for the packed-bed column reactor measured at port 10.

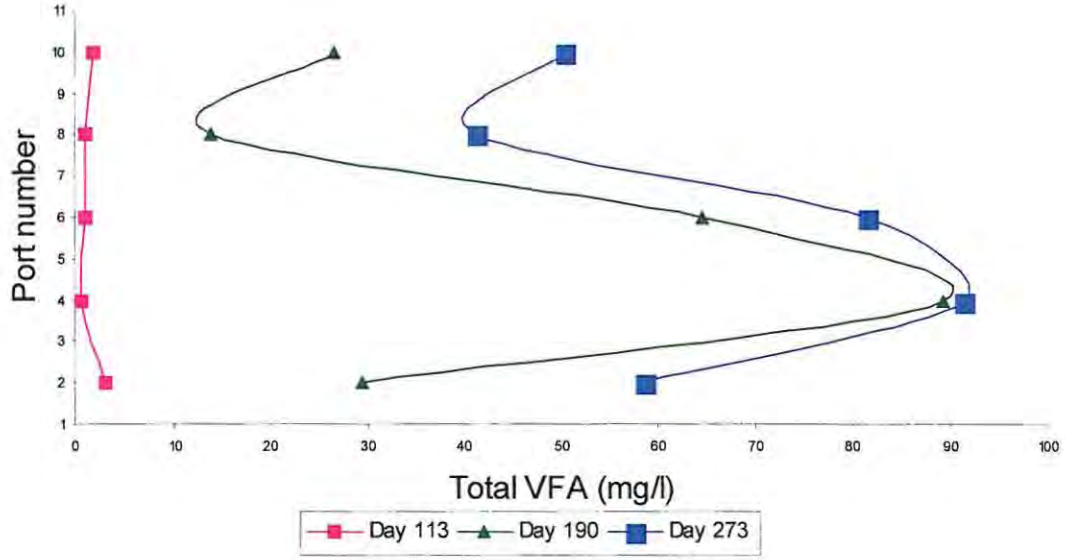


Figure 6.10: Depth profile of volatile fatty acids measured across the packed-bed column reactor from sampling port 2 to the outlet port 10.

Figure 6.5 shows how the column reactor changed with respect to pH over time. In the early stages acidification was observed, probably due to fermentation reactions within the column. Inspection of figure 6.9 reveals that there was a small amount of reducing sugars present and these were probably being fermented to volatile fatty acids (VFAs), also explaining the low pH observed during the early stages.. Day 113 shows an alkalizing effect up to port 6 (Figure 6.5) after which acidification occurs once again. Figure 6.10 shows that from port 8 there is a marked increase in aromatic content, also in the middle of the reactor which may also be linked to the decrease in pH observed at this point in Figure 6.5. Days 190-273 show a pronounced alkalizing effect through the depth of the reactor (Figure 6.5). Reducing sugar content except for day 190 was low and aromatic content for this period was also low.

Comparing sulphate reduction and pH through the column reactor, it was observed that the greater the sulphate reduction the higher the pH of the effluent (Figure 6.6)

Figure 6.11 summarizes the occurrence of the clones identified across the spatial and temporal gradients described above. Although it would have been most useful to also have an indication of the population size of each group, this information is not available. Thus only the presence of each group type can be shown, with diversity possibly providing a loose indication of the diversity of the particular trophic niche being exploited. It is also noted that, with hindsight, group-specific primers could have been used to determine the full extent of the sulphate reducing populations more thoroughly. As only bacterial 16S rRNA primers (Appendix B) were used it may be assumed that the sulphate reducers were substantially under-reported.

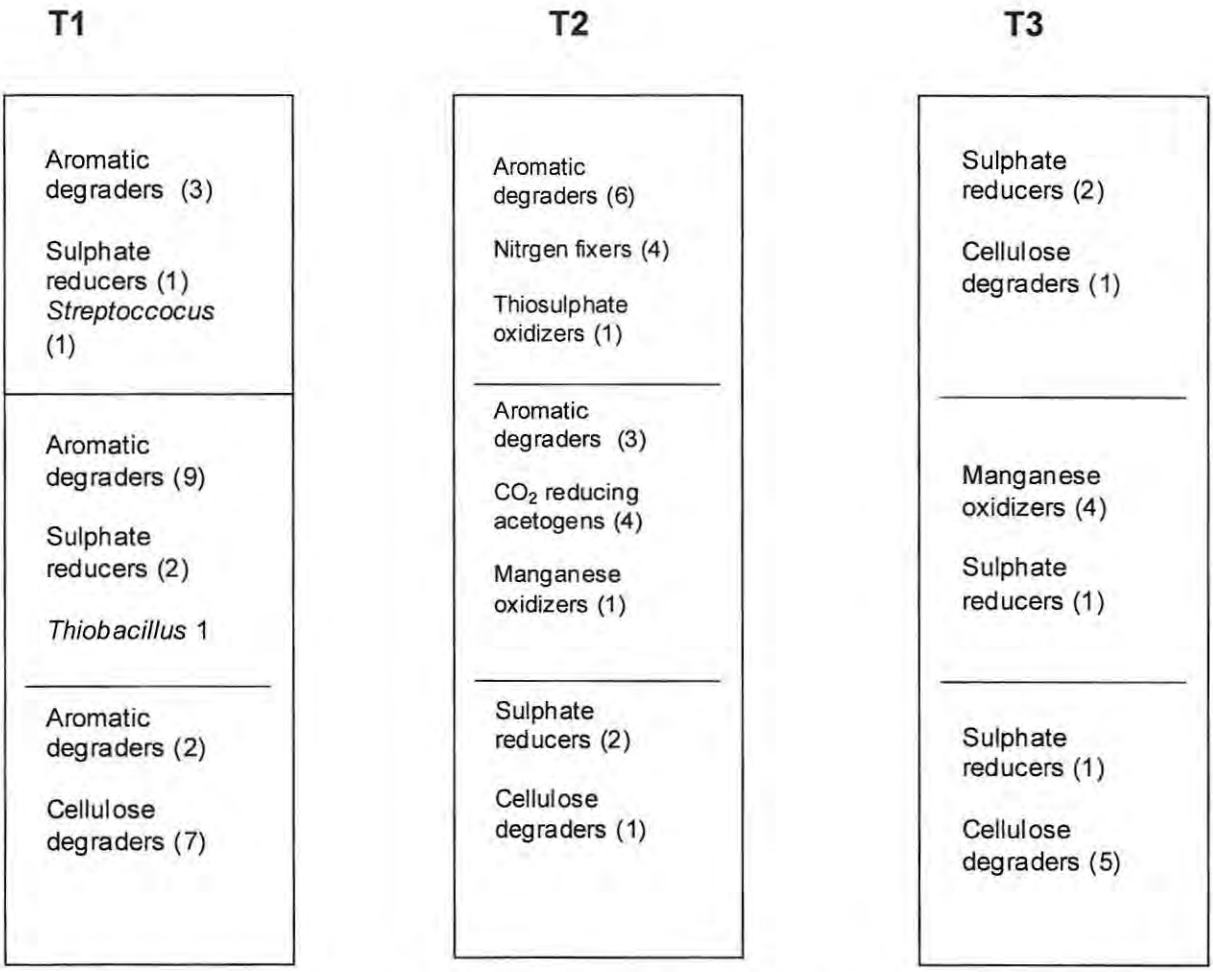


Figure 6.11: Occurrence of bacterial clones in the laboratory packed-bed reactor showing their appearance distributed spatially across the column, and at thee separate sampling times (T1 – 113 days; T2 – 190 days; T3 – 273 days) as based on inferred trophic activity.

6.6. Conclusions

The research undertaken for this dissertation involved the comparison of one of the longest running lignocellulose packed reactors in the country, operating under a sulphidogenic passive treatment configuration at VCC and a laboratory-scale reactor operated for one year, and set-up at a distance of 450 km from VCC. Although widely separated in both space and time, the outcome of the investigation of the two systems showed pronounced similarities of the clones isolated. These include the CFB group, various

Clostridium spp., microflora similar to that of the termite gut and rumen, *Sphingomonas* spp. and various sulphate reducers.

These results provide evidence that common microbial processes seem to be at work in both field and laboratory units. The observations made in the laboratory reactor may therefore be taken as an indication of what happens in the field-scale systems, which were not monitored as closely, as this was not possible.

From the spatial and temporal analysis of the laboratory packed-bed column it is evident that organisms generating aromatic compounds predominated throughout the column during the first sampling time (T1 Figure 6.11). This may be correlated with a high level of aromatic compounds at day 31 followed by a steady decline through to day 273 (Figure 6.8). This also correlates with lower pH and lower sulphate reduction. At the middle and the outlet zone, in addition to aromatic degraders, only sulphate reducers can be found, which correlates with enhanced sulphate removal and increasing pH recorded in these zones (Figures 6.4 to 6.6). The presence of one clone related to *Thiobacillus* sp. in the middle zone of the column is uncertain, however, it may be due to a synthrophic relationship, which is known to occur in biofilm assemblages (Radchenko and Tashirev, 1991).

The inlet zone of the reactor was dominated by cellulose degraders throughout the bioremediation process, but in middle and upper zones they were found only during the second and third sampling periods (T1 and T2, Figure 6.11). Their presence may be related to the rise of both reducing sugars and VFAs at these times (Figure 6.7 and 6.10) and, together with the decline in numbers of aromatic degrading clones, may indicate the sequence in which the substance was attacked. Sulphate reducing bacteria appear throughout the system with the exception of middle and upper zones at time 2 (T2) (Figure 6.11).

The results of this study indicate the presence of defined gradients in both space and time with respect to the appearance of different trophic groups of bacteria and the chemical changes in the system. On the basis of these findings it may be possible to construct an explanatory model which would elucidate the relationship between the microbial population within the system and bio-physical changes occurring across the reactor. This could enable the establishment of a possible structural/functional relationship to offer insight into the question of why these systems fail over time. This will be dealt with in the following chapter.

CHAPTER SEVEN

Conclusions on the Biodegradation of Lignocellulose in the Sulphidogenic Environment

The generally accepted view of lignin biodegradation has been that the ether linkages in the lignin compound can only be cleaved biologically under aerobic conditions (Burnes *et al.*, 2000). This apparent dependence on oxidizing enzymatic systems infers that anaerobic degradation of this substrate does not occur, and that aeration may inhibit the ability of sulphate reducing systems to mobilize these substrates. Roman (2005), has however, shown that wood tissue is degraded under biosulphidogenic conditions and that specifically lignin, among the various components of the lignocellulose complex, is degraded in purified form provided a readily available organic matter source is also available to the sulphate reducing consortia present. Madikane (2002) showed that linked model compounds synthesized in the group's laboratory, were successfully cleaved by sulphate reducing microbial consortia.

Roman (2005) used various extracts of pine wood from which firstly the water-soluble extractables had been removed, secondly the pectin fraction and finally the cellulose and hemicellulose fractions, thereby ultimately leaving the lignin component. Together with other members of the research group, including this author, the potential of each of these components to release aromatic compounds under biosulphidogenic conditions was evaluated, and these findings related to the observations of whole wood degradation, which have been reported in chapter 6 for the VCC and the laboratory-scale packed-bed reactors. Roman, together with Madikane (2002), found that the preliminary increase in sulphate reduction that occurs in whole wood packed-bed reactors is associated with the initial release of the readily extractable portion of the substrate. Consumption of the readily extractable fraction was linked to the generation of sulphide and alkalinity in the biological system, and the environment established in this way played some role in mobilizing the lignin and cellulose fractions. It was shown that comparable effects could be

demonstrated where the sulphide/alkaline environment was established using inorganic reagents, and Madikane (2002) suggested that the reaction mechanisms involved may be analogous in some way to the sulphidic "Kraft process" in which the lignocellulose complex is degraded in paper manufacture. The problem associated with rigorous confirmation of these proposals, within the complex environment of a holistic lignocellulose packed-bed system, are not trivial, and therefore it was decided to use the phylogenetic study to provide additional evidence from a separate, but related system to attempt to further understand the events observed.

The study reported here, on the microbial ecology of the sulphidogenic lignocellulose-degrading system, was undertaken concurrently with Roman and Madikane's work, and it was a basic proposition of the research hypothesis that an understanding of the activity of the organisms involved, and what information this offers about the trophic environments established within the system, would, together with the chemical and biochemical changes monitored, provide a basis on which a descriptive model might be constructed that could account for the events observed.

The laboratory packed-bed reactor provided a linear flow experimental system in which it was proposed that aspects of the different mechanisms involved in lignocellulose degradation might be observed as spatially separated sequential events. Both the physico-chemical and the phylogenetic studies showed well defined stratification of environments in the column reactor, and these findings indicated that the sequence of events occurring in the lignocellulose degradation might indeed be reflected as spatially separated events in this experimental system. Fermentation reactions appear to predominate at the inlet zone of the column, but whether this is due to the release of easily available compounds from exposed cellulose substrate, is uncertain. In either event the redox of the system will be appropriately poised in this part of the reactor, and the VFA produced provides for the sulphate reducing organisms occurring in the succeeding functional compartments

(Figure 6.11). The SRB were found to predominate mainly in the upper compartments. At the first sampling for profiling of the microbial population, high levels of aromatic compounds were generated but relatively lower amounts of reducing sugars and VFA, accompanied by lower rates of sulphate reduction. Second and third samplings showed a pronounced increase of reducing sugars and VFA from the inlet to upper zone of the column, which also was coupled with a decrease in the content of aromatic compounds (Figure 6.11). This observation suggests that lignin has to be removed in some way in order to expose the underlying cellulose and hemi-cellulose to microbial attack.

Indications were provided that the diversity of aromatic degraders in the microbial population in these zones had decreased by the end of the run (Figure 6.11), which also correlated with the decline in the concentration of aromatic compounds measured. In contrast, cellulose degraders and SRB populations were observed to dominate at these times. Although the laboratory-scale reactor system was not run to the well known "collapse" phase, as observed at the VCC reactors after a period of time (Figure 7.1), evidence of slow down in performance of the laboratory reactor was noted by day 273 (Figure 6.4).

It may thus be proposed that the loss of aromatic degrading organisms from first to third sampling periods, associated with the decline in levels of aromatic compounds observed over the same period, is an indication that the residual underlying lignin becomes increasingly unavailable in some way, possibly due to masking by the degraded cellulose and biofilm debris. Once the available cellulose is consumed, the production of reducing sugars and VFA would decline, and together with that declining sulphide and alkalinity production will occur. Based on Roman and Madikane's findings, this would in turn affect the further degradation of lignin and also the release of further reducing sugars and VFA. Consequently, in this way, the once accelerating cycle of lignin cleavage, initiated by sulphide generation fueled by the early release of the

easily extractable fraction, followed in turn by cellulose breakdown and reducing sugar release and VFA production, and again leading to further sulphate reduction and lignin breakdown, becomes interrupted. Thereafter, the reduction in sulphide production leads to an unwinding of the apparently interrelated events, with reductions in lignin and cellulose breakdown, and reducing sugar and VFA release leading to less sulphide generated. The system then settles down to relatively low levels of performance, which were observed in the reported decommissioning study reported, to coincide with large amounts of residual lignocellulose packing remaining unutilized in the reactor.

Based on these findings, and the interpretation of events observed, a descriptive model has been outlined in Figure 7.1.

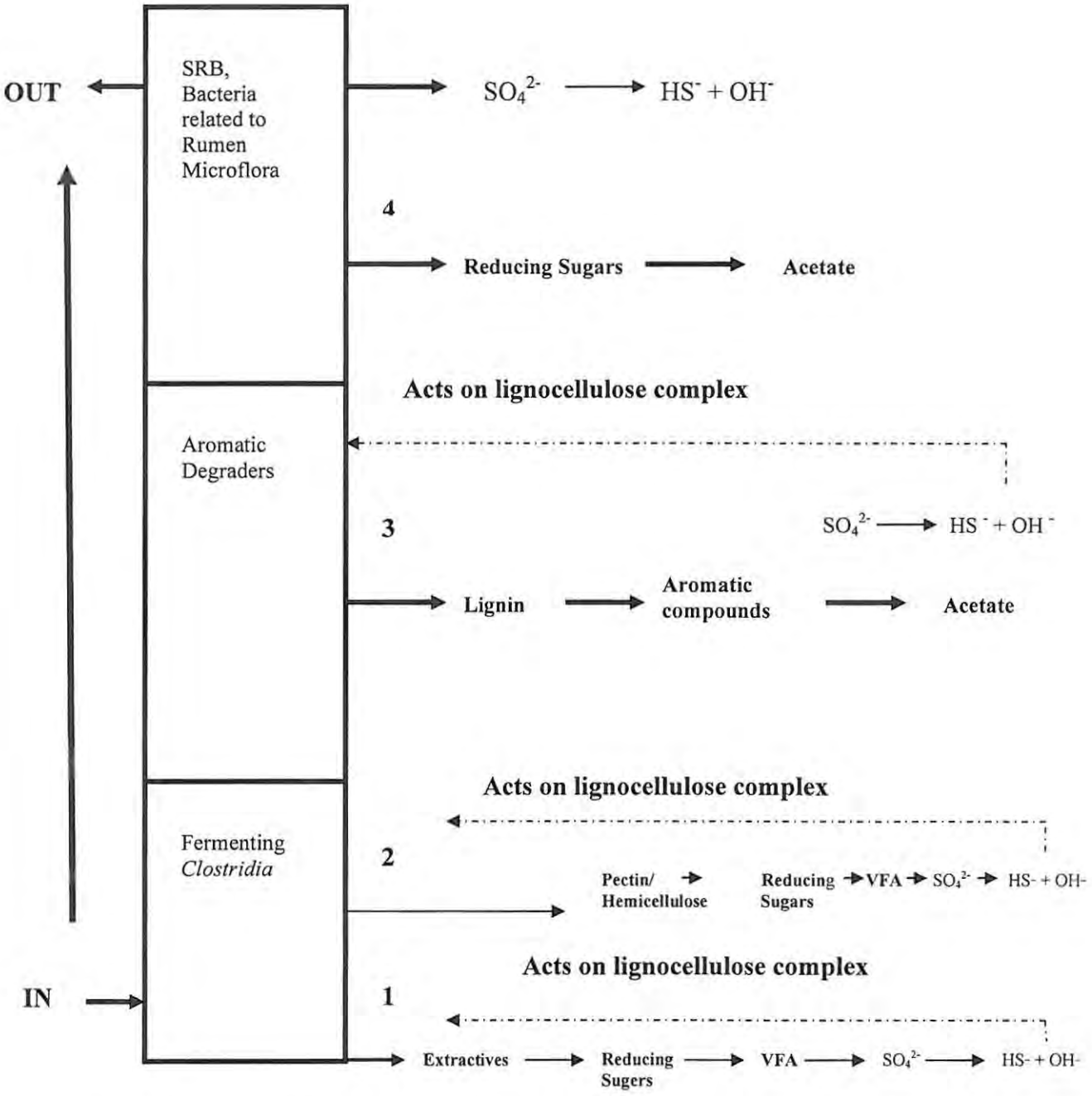


Figure 7.1: A schematic representation of the steps involved in the degradation of lignocellulose in biosulfidogenic systems as reflected in the results reported in this study. The steps involved may include the following: 1) Soluble extractives are metabolized to produce VFAs that are utilized as electron donors for sulphate reduction. 2) The sulphide and alkalinity produced contributes together with enzyme systems present to the breakdown of the lignocellulose complex with the release of aromatic compounds in the second compartment. 3) These further contribute to VFA production and further sulphate reduction. 4) Exposure of the cellulose structure by cellulolytic bacteria leads to increasing reducing sugars levels, VFA and sulphate reduction.

7.1. Development of the Degrading Packed-bed Reactor

Apart from offering potentially useful insights into the operation and functioning of naturally occurring sulphidogenic lignocellulose degrading sediment environments, the results of this study could also have useful outcomes for practical applications in mine water treatment which have been noted in chapter 1. Given the descriptive model outlined above, it was proposed that the eventual decline observed in these lignocellulose packed-bed systems is characterized by a slowdown in sulphide and alkalinity generation in the system. If a decline in a feed availability is the cause, then stimulation of this part of the sequence of events by feed augmentation could, possibly, ensure an ongoing supply of sulphide, and concomitant mobilization of the lignocellulose complex.

Based on this reasoning, an experimental system was set up for laboratory and VCC packed-bed reactors, in which (0.1 %) molasses was added to the feed (Coetser, 2004). Results obtained (Coetser, 2004) indicated that the addition of 0.1 (v/v) molasses to SSRU 3 lead to a 47-fold increase in sulphide levels through the depth of the reactor, compared to a 9.7 fold increase during phase 1.

These findings led to the development of the Degrading Packed-bed Reactor concept and, in scale-up studies, this has been shown to provide the desired performance profile without the decline previously observed. The positive outcome in this study served to strengthen the hypothesis on which the model system has been based.

7.2. Future Work

The analysis of the system, and especially the opportunity to compare results obtained from the laboratory-scale packed-packed reactor with those of the VCC reactor raised a number of important issues. Both reactors were set up at widely different points in time and at considerable distance from each other. Both were used for remediation of AMD using lignocellulose as a carbon source. Both operated in a sulphidogenic, passive treatment configuration. It is noteworthy that 16S rRNA derived analysis revealed striking similarities in the microbial community. This raises the question whether the system could be used in the future as a standard approach to bioremediation of AMD and possibly other environmental pollutants. However, in order to answer this question, further work should be done on confirming biosulphidogenic lignin breakdown, possibly using radiotracer studies, and on the architecture of biofilms established to try to improve an understanding of the three-dimensional interaction of the biological and physico-chemical environments. In this regard the following would be useful:

- Fluorescent *in situ* hybridization (FISH) studies, using probes based on the population profiled in this study could provide additional information on the syntrophic associations proposed between the populations.

- The application of microarray technology by targeting functional genes of interest would provide a real time mechanism for following the microbial role in the processes of lignocellulose breakdown.

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APPENDIX A

DNA EXTRACTION PROTOCOL, REAGENTS AND BUFFERS

1. Phenol-chlorofom DNA Extraction Protocol.

Prior to extraction samples were pelleted by centrifugation, washed once with 2 x buffer A, and then resuspended in 500 µl TE buffer in 2 ml microcentrifuge tube. Polyadenilic acid, and lysozyme were added to the suspension and the mixture was incubated for 1 hour at 37°C. Proteinase K, SDS, CTAB and NaCl were added to the mixture, and this was incubated overnight at 37°C. Cell lysates were extracted with phenol-chloroform-isoamyl alcohol (24:24:1). Nucleic acids were precipitated with 2.5 volumes of 96% rectified ethanol overnight at -20°C, pelleted by centrifugation and resuspended in TE buffer.

2. Reagents and Buffers:

2.1. 10 % SDS (Sodium dodecyl sulphate)

SDS 10 g
dddH_2O 100 ml
Warm to 65° C to allow SDS to dissolve.

2.2. 10 % CTAB (Cetyltrimethylammonium bromide)

CTAB 10 g
dddH_2O 100 ml
Warm to 65° C to allow CTAB to dissolve.

2.3. Proteinase K

2mg/ml stock solution

2.4. 5 M NaCl

NaCl 292 g
dddH_2O 1 L
Autoclave to sterilize.

2.5. Chloroform/Isoamyl Alcohol (24:1)

Chloroform 96 ml
Isoamyl alcohol 4 ml

2.6. TE (Tris/EDTA) buffer pH 8.0

Tris/HCl pH 8.0	10 mM
EDTA pH 8.0	1 mM

2.7. Buffer A

200 mM Tris [pH 8.0]
50 mM EDTA
200 mM NaCl
2 mM sodium citrate
10 mM $CaCl_2$)
one part 50% glycerol

2.8. Lysozyme Solution

Lysozyme	10 mg
dddH_2O	1 ml

2.9. Polyadenilic Acid

Polyadenilic Acid	200 µl
ddd H_2O	1 ml

APPENDIX B

PCR REAGENTS AND BUFFERS

1. PCR Master Mix:

Master mix 1:

	Volume	Final concentration
H_2O	4.0 µl	
dNTP's mix	2.5 µl	0.2 mM
Primer 907 R	2.5 µl	0.5 µM
Primer GM5 F	2.5 µl	0.5 µM
Template DNA	1.0 µl	

Master mix 2:

H_2O	9.5 µl	
PCR Buffer 10x conc.	2.5 µl	1 x
Enzyme Taq DNA Polymerase	0.5 µl	0.5 U/25 µl

NB. The preparation of two separate master mixes helps to avoid enzyme/primer interaction and thus prevent degradation of primer and template.

Combine two master mixes, briefly vortex and spin.

2. PCR Primers

2.1 GM5 F (5' - cct acg gga gcagc ag - 3')

2.2 907 R (5' - cgc ccg ccg cgc ccc gcg ccc gtc ccg ccg ccc ccg ccc gcc gtc aat tcc ttt gag ttt - 3')

APPENDIX C

GEL ELECTROPHORESIS PROTOCOLS, REAGENTS AND BUFFERS

1. Agarose Gel Electrophoresis

An agarose solution was prepared in 1 x TBE buffer (pH 8.0) by melting in the microwave. Depending on the size of the DNA 0.8 % (for total genomic DNA) and 1.0 % (for 586 bp PCR product) of agarose was used. After melting of agarose 0.5 µg/ml of ethidium bromide was added before pouring mixture into horizontal gel casting tray. The combs were inserted and once gel slab has set, the combs were removed and the casting tray was placed in the horizontal buffer chamber filled with 1 x TBE. DNA sample was mixed with loading buffer and electrophoresed at 90 volts for approximately 45 minutes. The product of electrophoresis was visualized on the UV transiluminator and photographed with Kodak digital camera.

2. Reagents and Buffers

2.1. 10 x TBE (Tris-borate/EDTA) buffer pH 8.0

Tris base	107.8 g
Boric acid	55.0 g
EDTA 0.5 M pH 8.0	40.0 ml

Make up to 1 L with $dddH_2O$.

2.2. Ethidium Bromide

0.5 g of ethidium bromide
1 ml of $dddH_2O$
Store in the dark bottle at 4° C.

2.3. DNA Loading Buffer

Bromophenol blue	0.25 %
Xylene cyanol	0.25 %
Glycerol	30.00 %

2.4. λ *Pst*1 Molecular Weight Marker

200 µl λDNA (0.25 µl/µl)
24 µl of 10 x buffer H
10 µl of *Pst* 1 enzyme
Digest for 3 hours at 37° C.
Add 550 µl of TE buffer (10 mM pH 8.0) and 150 µl of 6 x loading buffer.

3. DGGE (Denaturing gradient gel electrophoresis)

3.1. DGGE protocol

The PCR products were separated on denaturing gradient from 40 % to 50 %. Prior to forming the gradient 20 µl of 20 % APS and 4 µl of TEMED were added to denaturing solution. The gradient was formed using gradient maker (BIORAD Model 385 Gradient Former).

The electrophoresis was performed in an electrophoresis cell (Sigma-Aldrich) with 1 x TAE buffer at 60°C and 150 V for 4.5 hours using BIORAD Power Pac 3000, 165-5057 with magnetic stirrer going continuously. After electrophoresis the gel was stained with ethidium bromide for 25-20 minutes and then examined on UV transilluminator.

3.2. Reagents and Buffers

3.2.1. 100 % Denaturant (50ml)

10 x TAE buffer	5.0 ml
40% Acrylamide	7.5 ml
Urea	21.0 g
Formamide	20.0 ml

Make up to 50 ml with dddH_2O and store at 4° C

3.2.2. 55 % Denaturant (50 ml)

10 x TAE buffer	5.0 ml
40% Acrylamide	7.5 ml
Urea	11.55 g
Formamide	11.00 ml

Make up to 50 ml with dddH_2O and store at 4° C

3.2.3. 65 % Denaturant (50 ml)

10 x TAE buffer	5.0 ml
40% Acrylamide	7.5 ml
Urea	13.65 g
Formamide	13.00 ml

Make up to 50 ml with dddH_2O and store at 4° C

3.2.4. 0 % Denaturant (50 ml)

10 x TAE buffer	5.0 ml
40% Acrylamide	7.5 ml

Make up to 50 ml with dddH_2O and store at 4° C

3.2.5. 40 % Acrylamide Stock Solution

Acrylamide	100.0 g
Bis-acrylamide	2.7 g

3.2.6. 20 % APS Stock Solution

Ammonium persulphate	0.2 g
dddH_2O	1.0 ml

3.2.7. 10 x TAE

Tris base	48.40 g
EDTA	3.72 g
Glacial acetic acid	11.42 ml
dddH_2O	1.0 L

APPENDIX D

EXTRACTION OF DNA FROM DGGE BANDS (Adapted from Sambrook *et al.*, 1989).

Bands of interest were excised with sterilized scalpel blade from DGGE gel and transferred to 200 µl Tris-EDTA buffer and incubated overnight at 37° C. Following the incubation, samples were centrifuged at 13 000 rpm for 10 minutes. The supernatant was transferred to a fresh tube and DNA was extracted using phenol : chloroform : isoamyl alcohol (24 : 24 : 1). Extracted DNA was precipitated with 3M sodium acetate pH 8.0 and 2.5 volumes of ice cold rectified 96% ethanol. Pellet was resuspended sterile ddd H_2O.

APPENDIX E

CLONING PROTOCOL

1. Media and Reagents:

1.1. **Luria Berthrani Broth (LBB)**

Bacto tryptone	10.0 g
Bacto yeast extract	5.0 g
NaCl	10.0 g
H_2O	1.0 L

1.2. **Luria Berthrani Agar (LBA)**

Bacto tryptone	10.0 g
Bacto yeas extract	5.0 g
NaCl	10.0 g
Bacto Agar	18.0 g
H_2O	1.0 L

1.3. **LBA/Ampicilin**
Add 100 µg /ml of ampicillin to preccoled agar medium

1.4. **IPTG** (Isopropylthio-β-o-galactiside). M.W. = 228.3
Make up 1 M solution of IPTG in H_2O. Sterilize by filtrationthrough 0.22 micron disposable membrane filter. Store in on one ml aliquots at -20° C.

1.5. **X-gal** (5-Bromo-4-chloro-3-indolyl-β-o-galactoside.
Stock solution of X-gal is made by dissolving 20mg in I ml of dumethylformamide. Protect from sun damage by covering the bottle with tin foil or alternatively use glass or polypropylene bottle. Store at -20° C.

1.6. **LBA/AMP/IPTG/X-Gal** plates.
Add 100 µg /ml of ampicillin to preccoled LBA medium. Allow the plates to solidify. Use 100 µl of 100mM IPTG and 20 µl of 50 mg/ml X-Gal. Spread over LBA/AMP plates and allow to absorb for 30 min at 37° C.

1.7. SOC Medium

Bacto tryptone	2.0. g
Bacto yeast extract	0.5 g
1 M NaCl	1.0 ml
0.25 M KCl	1.0 ml
2 M Mg^{2+} stock, filter sterilized	1.0 ml
2 M glucose filter sterilized	1.0 ml
H_2O	97.0 ml

1.8 2 M Mg^{2+} Stock

$MgCl_2$	20.33 g
$MgSO_4$	24.65 g
H_2O up to 100 ml and filter sterilize.	

2. Ligation Reaction

	Standard reaction	+ve control	Back-grown control
2 x rapid ligation buffer, T4 DNA ligase	5 µl	5 µl	5 µi
PGEM-T easy vector	1 µl	1 µl	1 µl
PCR product	X µl	—	—
Consort insert DNA	—	2 µ	—
T4 DNA ligase	1 µl	1 µl	1 µl
H_2O up the final concentration	10 µl	10 µl	10 µl

Mix the reaction by pippeting and incubate overnight at 4° C for most number of transformants.

3. Transformation using ligation reaction

Transformation was performed according to Promega protocol.

Blue-white screening was performed using Luria Agar plates supplemented with X-Gal and IPTG.

APPENDIX F

MICROSCOPY

1. SCANNINING ELECTRON MICROECOPY:

Prior to examination the samples were washed twice in 0.1 M phosphate buffer for 10 min then passed through an alcohol dehydration series: 30, 50, 60, 80, 90 and 100 % ethanol for 10 min, followed by two 100 % ethanol washes for 10 min. 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 SEM.

2. TRANSNISSION ELECTRON MICROSCOPY:

Wood tissues were flooded with cold buffered gluteraldehyde (2.5 % in 0.1 M phosphate buffer consisting of 80 parts Solution 1: 35.8 $g.L^{-1}$ $Na_2HPO_4.12H_2O$ and 20 parts Solution 2: 13.6 $g.L^{-1}$ KH_2PO_4) and dissected into small cubes. The tissue pieces were transferred to a petri dish containing cold buffered gluteraldehyde and cut into smaller cubes (no larger than 1 mm^3). Thereafter, the pieces were placed in specimen tubes containing cold buffered gluteraldehyde and allowed to fix for a minimum period of 12 h at 4 °C. During the following secondary fixation, dehydration and embedding procedure, the gluteraldehyde was decanted and tubes washed twice with phosphate buffer at 10 min each wash. Thereafter, the buffer was decanted and 1 % buffered osmium tetroxide solution (0.5 g osmium tetroxide, 45 ml Solution D and 5 ml Solution C; Solution D = 41.5 ml Solution A: 2.26 % $NaH_2PO_4.H_2O$, 2.25 %, $NaHPO_4.2H_2O$ and 8.5 ml Solution B: 2.52 % NaOH; Solution C: 5.4 % glucose) was added to cover tissue samples. After 90 minutes the osmium tetroxide was decanted off and washed twice with buffer (10 min each wash).

The tubes were refilled with 30 % ethanol and left for 5 min whereafter the procedure was repeated for 50, 70, 80, 90 % and absolute ethanol. The dehydrating procedure for all ethanol concentrations used two changes of absolute ethanol. Absolute ethanol was decanted and refilled twice with propylene oxide and left 15 min in each case. Propylene oxide : embedding mixture (75:25) was added to tubes and allowed to infiltrate for 90 minutes. The procedure was repeated for 50:50, 25:75 and pure embedding media and allowed to infiltrate overnight. The embedding mixture consisted of: Mixture A (Epon 812, 6.8 g; DDSA, 8.3g) and Mixture B (Epon 812, 10.3 g; NMA, 9.1g). Final mixture comprised of Mixture A (12.1 g), Mixture B (17.0 g) and DMP 30 (0.8 g). The mixture was stirred for 20 min and poured in McCartney bottles and allowed to mix at 1 rpm on a shaker until use. Flat specimen molds were filled to about ⅔ full with pure embedding media and samples from the tubes were transferred singly to the capsules. The molds were placed in an oven (set at 60 °C) to allow embedding media to polymerize for 48 h. Thereafter, the molds were cooled and polymerized blocks removed and stored in labelled specimen tubes. Blocks were trimmed and ultra thin slices cut using glass knives and a RMC MT7 ultramicrotome. Tissue slices were seized onto grids and heavy metal staining using uranyl acetate and lead citrate concluded the procedure. The grids were examined in a JEOL 1210 TEM.

3. FLUORECENCE *in situ* HIBRIDIZATION (FISH)

3.1 Standard Protocol for Hybridization on Slides

The specimens for FISH analyses were set up in the wells on Teflon coated slides obtained from Zeiss and dried for 10 minutes at 46° C. After drying, the slides were subjected to dehydration in an increasing ethanol series (3min each in 50, 80, and 100 % ethanol). Following the dehydration, 10 µl of hybridization buffer and l.0 µl of probe was dropped onto the wells. The slides were placed in hybridization chamber and incubated in the hybridization oven at 46° C for 1.5 hours. After incubation the slides were rinsed with washing

buffer and incubated at 48° C for 10 minutes. Following the incubation, slides were rinsed with distilled water, dried, embedded and analyzed using CSLM.

3.2. **Fixation Protocol**

Gram –ve cells were fixed with 4 % paraformaldehyde solution, while Gram +ve cells were fixed with ice cold absolute ethanol.

3.3. **Buffers**

Hybridization Buffer

M NaCl	360 µl
1 M Tris/HCl pH 8.0	40 µl
Formamide (0 – 70 % v/v)	1600 µl
10 % (w/v) SDS	4 µl

Washing Buffer

M Tris/HCl pH 8.0 1000 µl
5 M NaCl and 0.5 M EDTA pH 8.0 according to the following table:

% formamide in hybridization buffer	NaCl in mol/l	NaCl (µl)
0	0,900	9.000
5	0,636	6.300
10	0,450	4.500
15	0,318	3.180
20	0,225	2.150
25	0,159	1.490
30	0,112	1.020
35	0,080	700
40	0,056	460
45	0,040	300
50	0,028	180
55	0,020	100
60	0,008	40
70	0,000	No NaCl, only 350 µl EDTA

- 10 % (w/v) SDS 50 µl

3.4. Coating of Slides

Gelatin Coating

The slides cleaned in a solution of ethanolic KOH (10 % KOH in 85 % EtOH) for 1 hour and air dried were dipped in a gelatin solution (0.075 % gelatin, 0.01 % chromium potassium sulfate dodecahydrate) at 70° C.

Poly-L-Lysine Coating

The slides cleaned in a solution of 1 % HCl in 70 % EtOH and air dried were immersed in 0.01 % poly – L – Lysine for 5 minutes, drained and dried for 1 hour at 60° C or overnight at room temperature.

3.5. Probes

3.5.1. SRB 385

Position: 385-402
Sequence: 5'-cgg cgt cgc tgc gtc agg -3'
G + C content: 77.8
Tm: 59
MW: 5526
Formamide: 35%

3.5.2. EUB

Position: 338-355
Sequence: 5'-gct gcc tcc cgt agg agt-3'
G + C content: 66.7
Tm: 55

APPENDIX G

DNS reagent

DNS reagent was made up as follows (Wood and Bhat, 1988):

Dinitrosalicyclic acid	10.0 g
Phenol	2.0 g
Sodium sulphite	0.5 g
Rochelle salt	200.0 g

Dissolve these components in 500 ml of a 2 % (w/v) NaOH solution and thereafter dilute to 1 000 ml with d.H_2O.

APPENDIX H

Sequencing Primers:

1. SP6 Promoter Primer: tac gat tta ggt gac act ata g
2. T7 Promoter Primer: taa tac gac tca cta tag gg

APPENDIX I

Molecular weight marker: λ Pst 1:

- 14057 bp
- 5077 bp
- 4749 bp
- 2838 bp
- 2459 bp
- 2443 bp
- 2143 bp
- 2140 bp
- 1986 bp
- 1700 bp
- 1159 bp
- 1093 bp
- 805 bp
- 514 bp
- 468 bp
- 339 bp
- 264 bp
- 247 bp
- 216 bp
- 211 bp
- 200 bp
- 164 bp