Development of a Toolkit to Enable Quantitative Microbial Ecology Studies of Sulphate Reducing and Sulphide Oxidising Systems

Report to the
Water Research Commission

by

Robert P van Hille, Liabo Motleleng, Nathan van Wyk, Rob Huddy, Mariette Smart and Susan T L Harrison
Centre for Bioprocess Engineering Research
University of Cape Town

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Obtainable from
Water Research Commission
Private Bag X03
GEZINA, 0031

orders@wrc.org.za or download from www.wrc.org.za

EXECUTIVE SUMMARY

BACKGROUND

Acid rock drainage (ARD), particularly that arising from diffuse sources such as waste rock dumps, tailings impoundments and coal spoils, represents one of the most significant threats to the sustainability of water resources in the northern and eastern regions of South Africa. Large areas are likely to be impacted by the ARD, which can persist for decades or even centuries. There is a need for sustainable remediation systems to address the challenge.

Biological treatment systems, based on biological sulphate reduction, potentially offer a sustainable alternative to conventional physical and chemical processes. Stable performance of sulphate reducing systems depends on the maintenance of a stable, robust microbial community. Currently, there is a lack of techniques available for the quantitative evaluation of microbial communities in sulphate reducing systems. The development of tools, with a relatively rapid turnaround, to assess the structure of sulphate reducing communities would be valuable in the management of remediation systems based on sulphate reduction.

AIMS

The following were the aims of the project:

1. Literature review covering a critical evaluation of techniques used for qualitative and quantitative microbial ecology. Collation of a database of organisms, with 16S sequences, isolated from sulphate reducing and sulphide oxidising systems.
2. Construction of a 16S clone library from DNA extracted from laboratory sulphate reduction and sulphide oxidation reactors as well as samples from test site investigating concrete corrosion accelerated by sulphide oxidising bacteria.
3. Design of group and species specific primer sets and FISH probes for organisms isolated.
4. Testing primer sets for amplification efficiency and cross reactivity. Development of a “toolkit” consisting of acceptable primer sets.
5. Quantitative characterisation of microbial communities in the sulphate reduction and sulphide oxidation reactors. Assessing the impact of substrate (sulphate and sulphide) loading and hydraulic residence on the microbial community structure in a sulphate reduction and sulphide oxidation system respectively.

METHODOLOGY

The first part of the project involved generating comprehensive clone libraries of the sulphate reducing and sulphide oxidising communities. The total genomic DNA was extracted from community samples and the 16S rRNA gene from all component species was amplified using universal PCR primers. The amplified gene fragments were cloned into vectors and used to transform competent *Escherichia coli* cells. The transformed cells were plated out and over 150 cloned colonies were selected for amplified ribosomal DNA restriction analysis to identify unique sequences, which were then sent for DNA sequences. The resulting sequence data were compared to known sequences in the National Centre for Biotechnology Information database to identify closest known relatives.

The 16S sequence information was used to design qPCR primer sets and fluorescent *in situ* hybridisation probes for the sulphate reducing bacteria group and individual species. These were evaluated for specificity and cross-reactivity.

In parallel, a series of sulphate reducing and sulphide oxidising reactors were operated, under conditions likely to induce changes in the community structure. Samples were taken for DNA extraction at regular intervals and these were used as templates to test the molecular tools.

RESULTS AND DISCUSSION

The clone library constructed from the mixed sulphate reducing community consisted of 48 unique species, of which 17 grouped closely with known sulphate reducing species. Of these, members of the genus

Desulfomicrobium were most common, followed by *Desulfovibrio.* The non-sulphate reducing species were distributed among the *Bacteriodetes*, followed by the *Acholeplasma* and *Mesotoga*.

Three separate clone libraries were constructed for the sulphide oxidising community. The first, using samples taken from carbon deficient reactors, showed low diversity and was dominated by autotrophic sulphur oxidisers, such as *Chromatium* and *Chlorobium*. By contrast, under carbon replete conditions the diversity was much greater and the dominant sulphur oxidisers were *Thiobacilli* and *Halothiobacilli.* In addition, a number of heterotrophic bacteria were detected. The third library was generated using samples obtained from an experimental sewer site, investigating accelerated corrosion of concrete pipes due to sulphur cycling. The data generated in this study showed clear evidence of microbial succession and evolution of the microbial community in response to increasingly acidic conditions. This represents one of the first studies to characterise a complete community in that environment and coupled with the performance data being generated in that study could provide a significant breakthrough.

The development of species specific qPCR primer sets was less successful. A number of primer sets were designed, based on the 16S sequences of known species. These were evaluated using software packages to assess specificity and the likelihood of dimerisation. While the *in silico* evaluation suggested the primers would work well, in practice this was not the case. Primer sets and fluorescence *in situ* hybridisation (FISH) probes were developed for the sulphate reducing bacterial group and *Desulfomicrobium*, the most abundant of the species identified in the clone library. These were successfully used to assess the impact of reduced hydraulic retention time on the relative proportion of sulphate reducers to non-sulphate reducing bacteria.

GENERAL

The aims of the project were achieved to varying degrees of success. Comprehensive clone libraries were constructed to reflect the diversity in both the sulphate reducing and sulphide oxidising systems. The libraries represent a useful repository of data for ongoing development, while the data on the community associated with the concrete corrosion environment is groundbreaking.

The development of specific tools to quantify individual species within the mixed community was less successful, although success was achieved in quantifying total sulphate reducers and members of the *Desulfomicrobium*. Valuable lessons have been learned with regard to probe and primer design and progress toward achieving all the goals is continuing.

CONCLUSIONS

The most significant conclusions are:

- Comprehensive clone libraries have been constructed for the sulphate reducing and sulphide oxidising communities.
- The microbial community associate with accelerated concrete corrosion has been characterised and clear evidence of microbial succession provided.
- Fluorescence *in situ* hybridisation probes and qPCR primer sets to quantify total sulphate reducing bacteria and the *Desulfomicrobium* group have been designed and tested.
- The qPCR primer sets have been used to illustrate changes in the relative proportion of sulphate reducing to non-sulphate reducing bacteria in stirred tank reactors, as a function of reducing hydraulic retention time.

RECOMMENDATIONS

It is recommended that the information generated to date be used to continue designing and testing primers and probes to allow for quantification of a more complete set of species within the mixed community. The potential of utilising next generation sequencing facilities in South Africa should be investigated.

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Mr Niks Lesufi	*Chamber of Mines*
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Others	
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CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACRONYMS and ABBREVIATIONS

AMD	Acid mine drainage
ARD	Acid rock drainage
ARDRA	Amplified ribosomal DNA restriction analysis
ARISA	Amplified ribosomal intergenic spacer analysis
BLAST	Basic Local Alignment Search Tool
COD	Chemical oxygen demand
DGGE	Denaturing gradient gel electrophoresis
DPBR	Degrading packed bed reactor
FISH	Fluorescence *in situ* hybridisation
LFCR	Linear flow channel reactor
NCBI	National Centre for Biotechnology Information
PCR	Polymerase chain reaction
qPCR	Quantitative real-time polymerase chain reaction
SEM	Scanning electron microscopy
SOB	Sulphur oxidising bacteria
SRB	Sulphate reducing bacteria

GLOSSARY

ARDRA An extension of the restriction fragment length polymorphism technique to the 16S rRNA. The technique involves PCR of the conserved regions at the ends of the 16S gene, followed by digestion using tetracutter restriction enzymes. The pattern obtained is representative of the species analysed.

ARISA Technique for microbial community analysis that uses fluorescently-labelled PCR to amplify the highly variable intergentic spacer region between the 16S and 23S ribosomal RNA genes. The fluorescent primers can be translated into peaks in abundance of the different fragments lengths on an electropherogram.

BLAST Computer based tool for comparing gene sequence information to known sequences in the NCBI database. The analysis identified the most closely related sequences.

Ribotyping Fingerprinting of genomic DNA restriction fragments that contain all or part of the genes coding for the 16S and 23S rRNA. By digesting the genes with a specific restriction enzyme, fragments of different lengths are generated. By performing a gel electrophoresis with the digested samples, the fragments can be visualised as lines on the gel, where larger fragments are close to the start of the gel, and smaller fragments further down. Each unique set of fragments is referred to as a ribotype.

CHAPTER 1: BACKGROUND

1.1 INTRODUCTION

Biological treatment of mining-impacted waters has been under development for several years. Although it has been demonstrated at scale (ref. Grootvlei plant) and commercialised (ref. Pacques), the biological principles underpinning biological treatment of sulphate- and/or sulphide-rich mine water are still not fully understood. This project forms part of a broader programme aimed at developing a deeper understanding of sulphate reduction and sulphide oxidising communities, to facilitate the rational design and optimisation of systems for the remediation of acid mine drainage (AMD) and acid rock drainage (ARD). The performance of such systems is dependent on the development and maintenance of a robust microbial community. Poor performance and process failure are often associated with the loss of key organisms or a shift in microbial community composition. In the absence of information on the community structure and how it changes in response to process conditions it is difficult to predict when and why process failure will occur and more difficult to arrest the failure before it occurs.

Advances in molecular biology techniques over the past decade have made it possible to qualitatively characterise complex microbial communities relatively inexpensively, although techniques such as denaturing gradient gel electrophoresis (DGGE) and the construction of clone libraries are labour intensive and time consuming. Once the 16S rRNA gene sequence information is available it is possible to develop secondary tools, such as fluorescence *in situ* hybridisation (FISH) probes and quantitative real-time PCR (qPCR) primer sets that allow a more rapid and potentially quantitative picture of the microbial community to be developed. This approach has been used successfully to follow changes in the microbial community in commercial bioleaching tanks. However, the bioleaching communities are significantly less diverse than in sulphate reducing or sulphide oxidising systems, with less than five dominant members in most cases.

1.2 PROJECT AIMS

The following were the aims of the project:

1. Literature review covering a critical evaluation of techniques used for qualitative and quantitative microbial ecology. Collation of a database of organisms, with 16S sequences, isolated from sulphate reducing and sulphide oxidising systems.
2. Construction of a 16S clone library from DNA extracted from laboratory sulphate reduction and sulphide oxidation reactors as well as samples from test site investigating concrete corrosion accelerated by sulphide oxidising bacteria.
3. Design of group and species specific primer sets and FISH probes for organisms isolated.
4. Testing primer sets for amplification efficiency and cross reactivity. Development of a “toolkit” consisting of acceptable primer sets.
5. Quantitative characterisation of microbial communities in the sulphate reduction and sulphide oxidation reactors. Assessing the impact of substrate (sulphate and sulphide) loading and hydraulic residence on the microbial community structure in a sulphate reduction and sulphide oxidation system respectively.

1.3 SCOPE AND LIMITATIONS

The project was ambitious, as the microbial communities in both environments are complex. Furthermore, there have been very few attempts to use quantitative molecular tools to characterise sulphate reducing communities and the success achieved in those studies has been limited.

CHAPTER 2: LITERATURE REVIEW

2.1 INTRODUCTION

Acid rock drainage (ARD) is currently a significant problem within the mining and minerals processing sector of South Africa. The biological treatment of mine waters has received increased attention due to its potential as a sustainable and economically attractive alternative to chemical treatment. A considerable amount of research has been conducted on the development of biological systems, particularly those based on biological sulphate reduction (BSR) technologies. One of the major challenges associated with BSR is the provision of an economically viable carbon source and electron donor, and this has been the subject of numerous studies. A second challenge, that arises when efficient sulphate reduction is achieved, is how to manage the resulting sulphide. A number of chemical options, ranging from precipitation to sulphide stripping and controlled oxidation, exist although the cost and potential sustainability of these are often questionable.

Research in South Africa, conducted over the past ten years by the Environmental Biotechnology Research Unit (EBRU) at Rhodes University, Pulles Howard and de Lange, Golder Associates Africa (GAA) and the University of Cape Town, has led to the development of a semi-passive system based on BSR using low-cost, complex organic carbon sources coupled with microbial sulphide oxidation. The research has highlighted the necessity for the correct consortium of microorganisms for both the sulphate reduction and sulphide oxidation stages. The component species making up the relevant populations have been determined using qualitative molecular biology tools, but these analyses only provide a "snapshot" of the population at a particular time and are at best able to provide a semi-quantitative characterisation. The development of new molecular methods, such as quantitative real-time PCR (qPCR), provides the potential for rapid and affordable quantitative assessments of the microbial ecology of these complex systems. However, in order to make use of the technology the populations must be qualitatively assessed and the information used to develop group and species specific primer sets.

Therefore, the primary aim of this project is to develop and test the necessary primers to allow the rapid and quantitative characterisation of mixed microbial populations in both the sulphate reduction and sulphide oxidation components of ARD treatment systems.

2.2 ACID ROCK DRAINAGE

Acid rock drainage (ARD) and similar effluents continue to be a significant problem within the industrial sector, not only in South Africa but other parts of the world. As the global population and the demand for commodities continue to expand, the rapid increase in industrial activity is resulting in a greater generation of wastewaters. These wastewaters currently pose a threat to the surrounding ecosystems and habitats. These wastewaters are typically generated by the following industries: pulp, paper, chemical, metallurgical and mining (Oyekola, 2008).

The effluents from the above-mentioned industries are generally rich in sulphates, sulphides and dissolved metals. The mining and minerals processing industries are the largest contributor to ARD, posing the greatest risk to the environment and receiving water bodies. As a result, the mitigation and treatment of ARD warrants considerable attention and management (Naicker *et al*., 2003).

2.3 TREATMENT TECHNOLOGIES

A variety of technologies have been developed for the treatment of ARD. The established methods are based on oxidation, neutralisation, precipitation and sedimentation (INAP, 2009). The oxidation converts iron and aluminium to their less soluble oxidised form which makes subsequent precipitation more efficient. The most appropriate treatment is dependent upon the volume of the effluent, concentration, type of contaminants and pH of the water (Gazea *et al*., 1996). These treatment technologies can be divided into two broad categories, active and passive treatment systems.

Active systems are generally abiotic and typically involve neutralisation by addition of alkaline chemicals, inducing precipitation of the metals, or physical separation techniques, such as ion exchange or membrane processes. There are, however, a number of active systems that rely on bacterial sulphate reduction processes.

2.3.1 Biological sulphate reduction principles and practice

In the anaerobic biological sulphate reduction treatment process, sulphide and bicarbonate are produced by sulphate reducing bacteria (SRB) in the presence of a suitable electron donor and carbon source. In an AMD treatment context the bicarbonate alkalinity neutralises acidity while dissolved metals are precipitated as metal sulphides. These are less soluble than their hydroxide equivalents allowing lower residual metal concentration in solution (Hammack *et al*., 1994). These reactions are summarised in Equations 1 to 3.

$$\textit{Electron donor} + SO_4^{2-} \rightarrow HS^- + HCO_3^- \qquad \text{Equation 1}$$

$$Me^{2+} + HS^- \rightarrow MeS_{(s)} + H^+ \qquad \text{Equation 2}$$

$$HCO_3^- + H^+ \rightarrow H_2O + CO_2 \qquad \text{Equation 3}$$

2.3.2 Mechanisms of sulphate reduction

There are two primary pathways for sulphate metabolism. In assimilatory sulphate reduction, sulphate is reduced into sulphide for the synthesis of amino acids and subsequent metabolic processes. This process is achieved by most bacteria, fungi and plants (Widdel, 1988; Cooney *et al*., 1996). During dissimilatory sulphate reduction, sulphate acts as an electron acceptor for the degradation of organic substrates (electron donor) or reaction of H_2. At near ambient temperatures, sulphate reduction to sulphide does not occur significantly in the absence of microorganisms (Liamleam and Annachhatre, 2007).

Dissimilatory sulphate reduction is a form of anaerobic respiration (Singleton, 1993). The biochemical dissimilatory sulphate reduction pathway for sulphate-reducing prokaryotes is initiated with the active transport of exogenous sulphate across the bacterial cell membrane into the cell (step 1, Figure 1). The intracellular sulphate is then reduced, through a sequence of reactions, to sulphide (Figure 1) (Lengeler *et al*., 1999; Shen and Buick, 2004). The intermediate sulphite is either reduced directly through a single step, involving the transfer of six-electrons in the presence of sulphite reductase, or through a series of intermediates such as metabisulphite ($S_2O_5^{2-}$), dithionite $S_2O_4^{2-}$, trithionate $S_3O_6^{2-}$ and thiosulphate $S_2O_3^{2-}$ to form sulphide (step 4) (Postgate, 1984; Lengeler *et al.*, 1999). Finally, the generated sulphide is excreted into the environment (Shen and Buick, 2004; Menert *et al*., 2004). Enzymes involved in dissimilatory sulphate reduction include pyrophosphatase, ATP sulphurylase, APS reductase and sulphite reductase (Figure 1) (Gibson, 1990; Sass *et al*., 1992; Visscher *et al*., 1992; Mudryk *et al*., 2000).

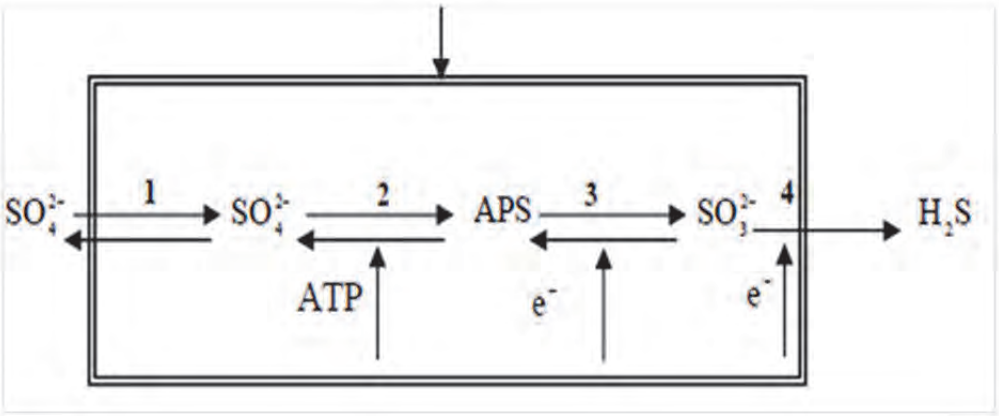

Figure 1: The pathway of dissimilatory sulphate reduction (e- = electron, ATP = adenosine triphosphate, APS = adenosine phosphosulphate). 1-4 represent the steps involved in the dissimilatory sulphate reduction process (Shen and Buick, 2004)

2.3.3 Electron donors for BSR

Various carbon sources and electron donors are available for BSR, including hydrogen, volatile fatty acids (lactate, acetate), short chain alcohols (ethanol, methanol) and sugars. The three main factors affecting the

choice of electron donor for BSR processes are: 1) the treatment efficiency or ability of electron donor to oxidise completely on reduction of sulphate, thus minimising the occurrence of other pollutants (residual organics) in the effluent; 2) the cost of electron donor per unit of sulphylkionate reduced to sulphide and 3) the availability of the carbon source (van Houten *et al.,* 1994). A preferred organic electron donor and carbon source should stimulate the SRB activity (Gibert *et al.,* 2003) and facilitate a robust BSR process.

Simple carbon sources such as lactate, acetate and methanol are readily available for SRB. Complex carbon sources, on the other hand, require the presence of fermentative microbial groups to facilitate degradation into easily assimilated substrates. Operation at higher hydraulic retention times will thus be required for reactors fed with complex carbon-sources (Christensen *et al*., 1996; Chang *et al*., 2000; Boshoff *et al*., 2004). Reactor design, feed sulphate concentration and choice of electron donor are critical factors that influence performance of biological sulphate reduction systems (Moosa *et al*., 2002; Boshoff *et al*., 2004; Dvorak *et al*., 1992). Other factors that affect BSR include temperature, pH, redox potential and hydraulic retention time.

2.3.4 Current applications for biological sulphate reduction

Commercial technologies such as Thiopaq® and BioSURE® make use of sulphate reduction to remove sulphate from wastewaters (Rose *et al*., 2000; Liamleam and Annachhatre, 2007). In the Thiopaq® technology, developed in Netherlands by Paques B.V., a gas-lift reactor is fed with hydrogen gas as the electron donor and CO_2 as the carbon source. Ethanol and methanol have also been used as carbon sources in this technology, although a change from autotrophic (CO_2) to heterotrophic (ethanol or methanol) operation requires a different microbial population. Sulphate is reduced into sulphide which can be partially oxidised into sulphur in two separate compartments of the Thiopaq® system. The sulphur produced can be used in the generation of sulphuric acid. This technology has been implemented on a full-scale at the Kennecott Bingham copper mine, USA, the Budelco zinc refinery, Netherlands and the synthetic fibre production plant of Akzo Nobel, Netherlands (Hulshoff Pol *et al*., 2001).

The BioSURE® system was developed in South Africa and makes use of a falling sludge bed reactor (FSBR) to facilitate the use of primary sewage sludge as the principal carbon source (Rose *et al*., 2000; Neba, 2006). The system was implemented at demonstration scale at the Ancor sewage treatment facility to reduce the sulphate load of partially treated (neutralised) AMD from the Grootvlei mine. The plant is no longer operational due to insufficient carbon source and problems with the stability of the final iron sulphide product.

Biological sulphate reduction plays an integral role in the integrated managed passive (IMPI) treatment process, another South African technology. Sulphate reduction takes place in a degrading packed bed reactor (DPBR), which is packed with a variety of complex and lignocellulosic carbon sources. The slow kinetics of lignocelluloses decomposition has resulted in insufficient organic carbon release to sustain effective sulphate reduction and the system has had to be supplemented with molasses (Coetser *et al*., 2004).

The effluent stream from the DPBR is rich in sulphide and requires additional treatment in order for the stream to meet discharge requirements. This effluent passes into a sulphide oxidation reactor, where the desire is to achieve partial oxidation of the sulphide to elemental sulphur, without further oxidation back to sulphate.

2.4 BIOLOGICAL SULPHIDE OXIDATION

In nature, sulphur metabolising microorganisms play a vital role in the conversion of the various forms of sulphur. Sulphide can be biologically oxidised by denitrifying organisms, colourless bacteria (in the presence of oxygen) or anaerobically by photosynthetic bacteria (Bowker, 2002). Sulphide, polysulphides, thiosulphate, elemental sulphur, polythionates, bisulphite and sulphate are all inorganic sulphur compounds that can be utilised within a microbial community as an electron acceptor. This may occur via dissimilatory or assimilatory pathways (Bruser *et al*., 2000). In terms of an ARD remediation process, the preferred product is elemental sulphur, with the removal of all dissolved sulphide species.

The natural sulphur cycle is controlled by heterotrophic bacteria as well as specialised bacteria such as colourless sulphur bacteria, which interconvert the various forms of the element.

Heterotrophic bacteria depend upon organic sources of carbon, whereas autotrophs are able to utilise carbon dioxide from the atmosphere (Bowker, 2002). Most sulphide that accumulates within the environment is formed due to sulphate reduction by sulphate reducing bacteria (SRB). Thereafter, sulphide oxidising bacteria (SOB) oxidise the sulphides to elemental sulphur and beyond.

Sulphide oxidising bacteria are chemoautotrophic and obtain their energy from the oxidation of sulphur compounds. Sulphide oxidising bacteria can be divided into two main groups, the photosynthetic sulphur bacteria and the colourless sulphur bacteria. Photosynthetic sulphur bacteria use sulphide as the electron donor, CO_2 as the carbon source and the energy is provided by light (Molwantwa, 2007). Colourless sulphur bacteria were among the first group of biogeochemically important bacteria to be studied, in part due to the fact that several species are large and produce macroscopically visible structures such as mats (Gray and Head, 1999). These organisms generally oxidise sulphide to sulphate, generating more metabolically useful energy as opposed to partial oxidation to S^0 (Lens and Kuenen, 2001). In order to obtain sulphur as a product, sulphide oxidation must occur under stringent conditions, such as high sulphide loads and within a narrow redox potential and pH window.

Sulphide oxidising microorganisms may be found in two distinctly different environments. The species which form an important component of the bioremediation system exist under neutral or mildly alkaline conditions. A second group of organisms exist under mild to strongly acidic conditions and play an important role in the generation of acid rock drainage, by oxidising the reduced sulphur in the mineral to sulphuric acid. These include mesophilic species of the genus *Acidithiobacillus*, moderately thermophilic species of the genus *Sulfobacillus* and a number of thermophilic archaea from the genera *Sulfolobus* and *Metallosphaera*, among others.

2.4.1 Contribution to concrete sewer corrosion

Apart from their role in ARD generation, mesophilic sulphide oxidising bacteria have been implicated in the accelerated corrosion of concrete sewers, where they are responsible for the oxidation of hydrogen sulphide gas, generated in stagnant launders and subsequently evolved when the liquid is subjected to turbulent flow in pipes. The gas penetrates the cement component in the non-submerged portion of the pipe, where it is oxidised to sulphuric acid, which reacts with the binder to reduce the structural integrity.

Research into sewer corrosion in South Africa has been conducted for over 60 years, spearheaded by the National Building Research Institute (NBRI) of the Council for Scientific and Industrial Research (CSIR). The Institute published findings of this work as early as 1959 (CSIR, 1959). Suggested interventions included careful consideration of hydrodynamics to minimise high velocity and turbulent flow, to reduce evolution of gaseous hydrogen sulphide and the use of dolomitic, rather than siliceous aggregate material as the higher inherent alkalinity would neutralise the acid generated. To date, limited information is available on the microbial community responsible for the sulphide oxidation, so targeted interventions have not been considered.

2.5 STRUCTURE-FUNCTION RELATIONSHIPS IN COMPLEX SYSTEMS

Many complex bioprocesses, such sulphate reduction, anaerobic digestion and bioleaching, depend on the action of a consortium of microorganisms, each performing specific roles within the process. The overall efficiency of the process and its ability to weather or recover from upset conditions depend on the correct organisms being present in the correct proportions. The relationship between process functionality and population structure is therefore critical.

Anaerobic digestion of complex organic material in sulphate-containing wastewaters has been demonstrated to occur through the synergistic interaction of hydrolytic, fermentative, acetogenic, sulphate-reducing and methanogenic reactions in a multistep process, with methanogenesis and sulphate reduction acting as the terminal processes (Menert *et al*., 2004; Patidar and Tare, 2005). The SRB rely on the activity of fermentative bacteria for the supply of carbon sources from complex organic materials (Ravenschlag *et al*., 2000). Some studies have reported the syntropic relationship between SRB and methanogens (Weijma, *et al*., 2002), while some have reported competition for substrate between these groups (Vossoughi *et al*., 2003). Studies have revealed that the microbial ecology is influenced by substrate amendment (Nagpal *et al*., 2000; Oyekola *et al*., 2007). Few strains of SRB are known to oxidise a broad range of electron donors

readily (Oude Elferink *et al*., 1995). Therefore, in mixed cultures, the dominant strains of SRB will be informed by substrate availability.

Studies have also demonstrated that sulphate loading influences the diversity of mixed cultures. When sulphate is in excess, SRB are the predominant group (Raskin *et al*., 1996). Methanogens outcompete SRB at low substrate concentrations (Koizumi *et al*., 2003). Chemical oxygen demand (COD) is a measure of the degree of reduction and is representative of the amount of potentially degradable organic compounds. The COD/sulphate ratio is a critical parameter in sulphate reducing systems. At a COD/sulphate ratio below 1.7, SRB dominate, while methanogens dominate at a COD/sulphate ratio above 2.7 (McCartney and Oleszkiewicz, 2011). Competition for sulphate also exists between members of SRB groups and is influenced by various factors including μ_{max}, KSO_4^{2-}, yield and maintenance requirement (Oude Elferink *et al*., 1999). A positive link between functional and structural dynamics of SRB has been reported (Santegoeds *et al*., 1998), and a direct relationship between *Desulfobulbus* predominance and sulphate reduction rate has also been reported (Li *et al*., 1999).

Oyekola (2008) demonstrated that the relative proportions of both SRB and fermentative bacteria varied with the COD/sulphate ratio, as did the members of the SRB community. Icgen and co-workers (2006, 2007) illustrated the shift in the relative dominance of members of the SRB community as a function of sulphate loading rate and sulphide concentration. The prevalence of bacterial groups typically corresponds to the levels of their activities and significance in a mixed environment (Santegoeds *et al*., 1999). Changes in operating conditions (pH, temperature, redox, substrate loading, inhibitors, etc.) of these systems can affect performance as a result of changes in population structure (loss of species or changes in relative proportions), changes in intrinsic performance (inhibition or activation of metabolic process) or both. However, without the correct analytical tools it is not possible to elucidate the relationship between population structure and intrinsic performance.

2.6 MOLECULAR TOOLS FOR MICROBIAL ECOLOGY STUDIES

Only a small fraction of known microorganisms have been isolated and characterised using traditional culturing techniques, owing to lack of definition of conditions under which most of the bacteria grow in their natural environment and the interdependency of species (Muyzer and Smalla, 1997). Approaches that complement the traditional culturing methods are needed, to better understand microbial ecology and its dynamics. Molecular techniques have been used to characterise microorganisms from various environments (Brikhoff and Muyzer., 1997; Smalla *et al*., 1998; Miletto *et al*., 2007). These molecular techniques are based on the identification of molecular markers, such as the 16S rRNA or its encoding gene, and genes related to important biogeochemical functions (Miletto *et al*., 2007). The 16S gene is most commonly used, due to the nature of the sequence. The gene is present in all bacteria and archaea (eukaryotes have a corresponding 18S gene) and contains regions that are highly conserved and regions that are hypervariable. The conserved regions are similar across all bacteria and archaea, allowing the design of universal primers, while the hypervariable regions allow discrimination to a species or strain level.

Recently, molecular methods such as fluorescence *in situ* hybridisation (FISH), quantitative real-time polymerase chain reaction (qRT-PCR), denaturing gradient gel electrophoresis (DGGE), and terminal restriction fragment length polymorphism (T-RFLP) have been used to study microbial ecology in various environments (Teske *et al*., 1998; Ito *et al*., 2002a; Stubner, 2004; Pérez-Jiménez and Kerkhof, 2005; Oyekola *et al*., 2008). Molecular techniques can be qualitative (T-RFLP and DGGE), semi-quantitative (FISH) or quantitative (qRT-PCR).

2.6.1 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a critical technique that has revolutionised molecular biology. The process was developed by Kary Mullis in the mid-1980s, for which he received a Nobel Prize in 1993. It is based on the mechanism that cells use to replicate their DNA. Central to the process is the use of a thermostable polymerase enzyme allows the denaturation (94°C), annealing (55-65°C) and elongation (72°C) cycles to be completed without the enzyme being inactivated. The enzyme was isolated from the thermophilic bacterium *Thermus aquaticus* (Chien *et al*., 1976). Each cycle results in a doubling of the target sequence, so after 25-30 cycles there are sufficient copies to allow for visualisation and manipulation. A schematic of the PCR reaction is shown in Figure 2.

Polymerase chain reaction has revolutionised the characterisation of environmental samples, as it facilitates the identification of species independent of the ability to culture them. The development of universal primers allowed the amplification of DNA from all component species in a sample, even if they make up a very small fraction of the total population. The amplified product can be subjected to a number of secondary techniques such as denaturing or temperature gradient gel electrophoresis (DGGE or TGGE) and restriction enzyme based techniques to separate the component species. Once isolated, the amplification product from a particular species can be purified, cloned into an expression vector and ultimately the specific DNA sequence can be determined. Classification based on molecular methods has superseded traditional techniques as the most widely used way of identifying and classifying new species.

Two qualitative PCR based techniques, DGGE and restriction endonuclease analysis, are described below.

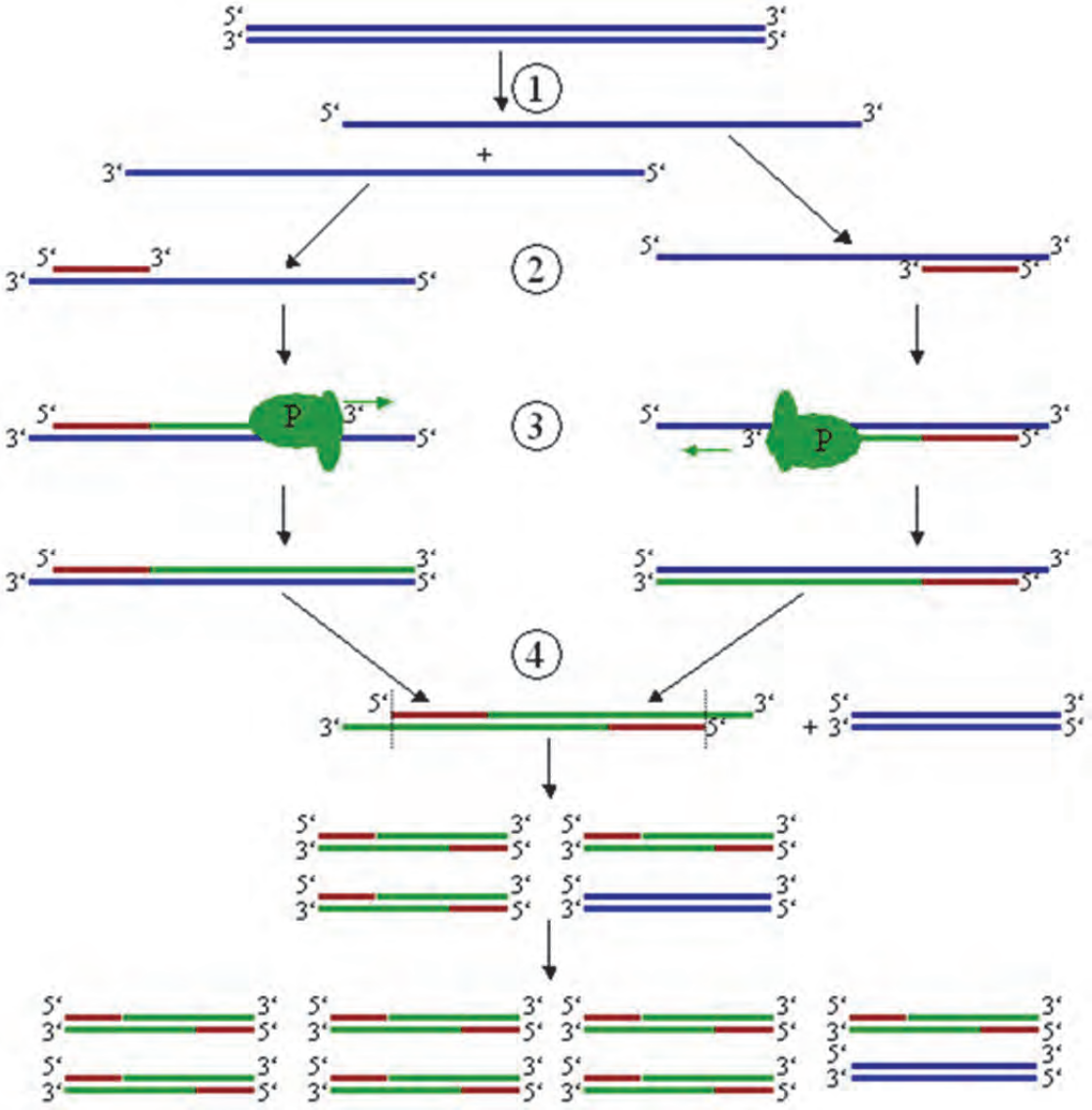

Figure 2: Schematic representation of the PCR reaction showing the three components of the cycle. The first phase (1) represents high temperature (94°C) denaturation, followed by (2) primer annealing and (3) elongation at 72°C. Upon completion of the first cycle (4) the resulting product strands make up the template for the subsequent cycle.

2.6.2 Denaturing gradient gel electrophoresis (DGGE)

Separation of DNA fragments in DGGE is based on the decreased electrophoretic mobility of partially denatured (unravelling of the double strand) double stranded DNA on polyacrylamide gel containing linear gradient of denaturing agent (urea and formamide) (Muyzer, 1999). Alternatively, the denaturing gradient may be achieved by increasing temperature (TGGE). DNA molecules with a high G-C content (characterised by three intermolecular hydrogen bonds) will denature more slowly than molecules with high A-T content (two hydrogen bonds). The separation of PCR-amplified DNA fragments by DGGE (PCR-DGGE)

is an extensively used screening method for fast assessment of microbial community diversity and dynamics. The individual bands can be cut from the gel, purified and sequenced. The sequence information can be used to identify the species from which it originated, using extensive online databases.

2.6.3 Restriction endonuclease analysis

This rapid quantitative technique was first described by Coram-Uliana and co-workers (2006) for the rapid and cost-effective characterisation of samples from bioleach environments and was subsequently adapted by Oyekola (2008) for application in a sulphate reducing system. The process is based on the amplification of the 16S rRNA gene using universal primers, followed by the digestion of the amplified product using a series of restriction endonuclease enzymes to generate products of various sizes. The products are visualised using standard agarose gel electrophoresis and the particular banding pattern can be compared to theoretical restriction patterns generated using published 16S sequences. The technique is rapid and relatively inexpensive, requiring only a basic PCR machine and standard electrophoresis equipment. However, it is restricted to confirming the presence or absence of target species, for which a complete 16S sequence is available. Notwithstanding this limitation, Coram-Uliana and co-workers were able to distinguish between eight groups of bioleaching organisms, using 10 restriction enzymes, while Oyekola (2008) was able to confirm the presence of six dominant SRB species in a bacterial sulphate reducing system utilising lactate as the carbon source.

2.6.4 Fluorescence *in situ* hybridisation (FISH)

Fluorescence *in situ* hybridisation is a semi-quantitative method and utilises fluorescence microscopy to visualise targeted cells. In this method a short piece (15-30 bases) of nucleic acid (probe) with a specific sequence, complementary to a specific DNA or RNA sequence in the target is used. The probe is tagged with a fluorescent molecule. Typically FISH probes target the 16S rRNA molecule in the ribosome. Once the probe has hybridised the slide (Figure 3) is visualised under incident UV light, causing the fluorochrome to emit light at a specific wavelength allowing positively tagged cells to be distinguished (Brown, 1995).

The probes rely on sequence data and are typically highly specific, targeting only one particular component of the community, although group (e.g. SRB) specific probes can be designed if a portion of the target molecule is conserved within that group. The probes typically target the 16S rRNA molecule which forms a component of the ribosome. The number of targets in a particular cell is therefore dependent on the level of metabolic activity and the technique is less effective for samples where the cells are not highly active (less protein synthesis so fewer ribosomes to act as targets). Amann and co-workers (1995) stated that a few thousand target rRNA molecules are required to obtain a detectable FISH signal. The problem of visualising cells with low metabolic activity has been addressed by utilising probes that are labelled with the enzyme horseradish peroxidise (HRP). The *in situ* hybridisation is followed by catalysis and accumulation of fluorescent tyramides at the HRP molecule. The modified process is referred to as catalysed reporter deposition (CARD) FISH. The process was further optimised by Pernthaler and co-workers (2002) who increased the permeability of the target cells, resulting in an almost 100% improvement in detection rates.

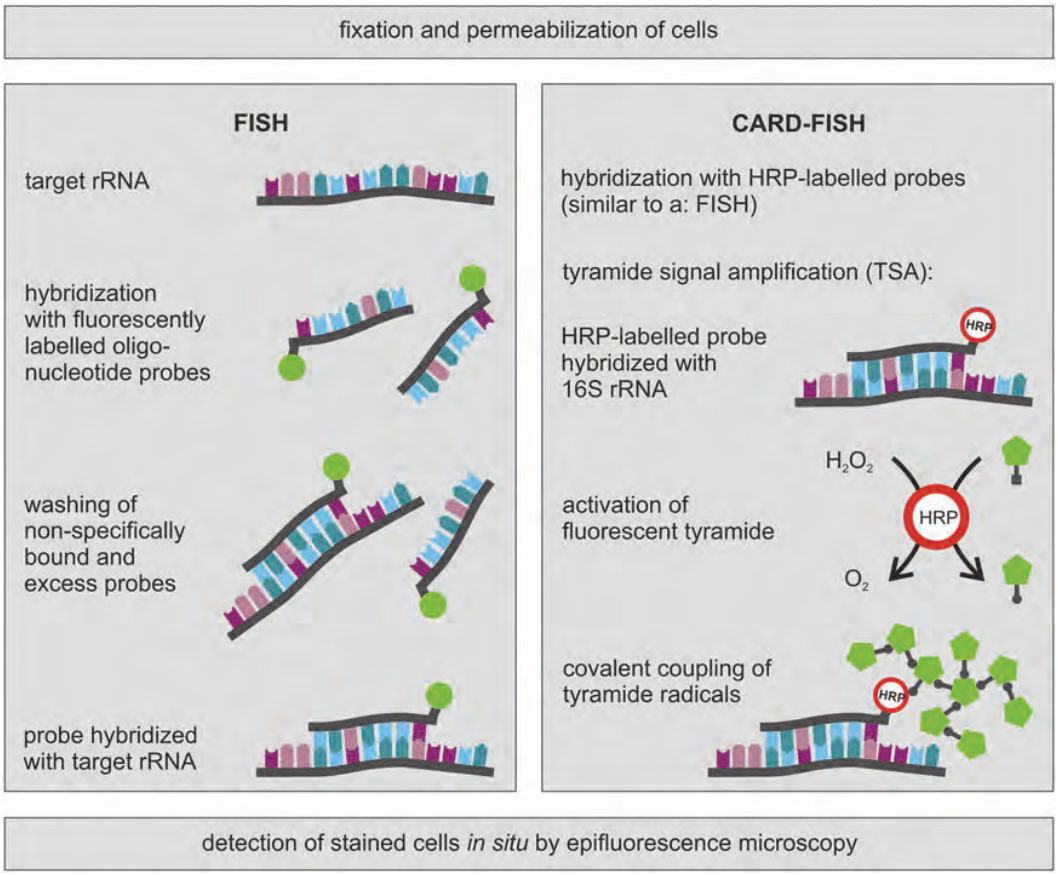

Figure 3: The principal steps in FISH and CARD-FISH (Eickhorst and Tippkotter, 2008)

Incgen and Harrison (2006) used FISH to assess the microbial population structure and function of a mixed culture of sulphate-reducing bacteria (SRB) maintained in anaerobic continuous bioreactors before and after a major perturbation. This involved the rapid increase of sulphate in the feed of a bioreactor operated at steady state (35°C, pH 7.8 and a 2.5 day hydraulic retention time (HRT)) from 10 to 15 g/ℓ. The community structure determined by FISH, by using 16S rRNA-targeted oligonucleotide probes, was linked to the functional performance of the SRB in the reactor. Hybridisation analysis using these 16S rRNA-targeted oligonucleotide probes revealed that a high concentration (15 g/ℓ) sulphate was toxic for *Desulfobacterium* and *Desulfobulbus*, while species belonging to the *Desulfococcus* group became dominant at the higher sulphate loading.

2.6.5 Quantitative real time PCR (qPCR)

This method combines the principles of PCR and FISH, by utilising a fluorescent dye that intercalates with the double stranded product. Following each cycle of denaturation, annealing and elongation the tube is exposed to radiation at the excitation frequency and the resulting emission signal, which is proportional to the amount of PCR product, is quantified. Based on the rate of increase in signal strength through successive cycles, the amount of template in sample can be determined. A series of standards of known concentration are used as the basis for the quantification. This method is accurate, sensitive and gives fast results.

While qPCR has the potential to allow the quantification of component species within a mixed population as cells/mℓ, there are a number of conditions that need hold for the output to be valid. These include:

- The DNA extraction efficiency needs to be 100% for all species present in the sample and there needs to be conservation of DNA throughout the extraction process.
- The efficiency of the qPCR reaction needs to be 100%, with no false positive signal due to non-specific binding or primer dimerisation.
- The number of copies of the target gene (typically the 16S rRNA gene) in the specific organism needs to be known.

A rigorous assessment of the process for samples from bioleach environments has been conducted at UCT and concluded that these assumptions cannot be met with rigorous certainty. However, results are typically accurate to at least within an order of magnitude. Results are often expressed as copy number/mℓ where the copy number of the gene is not known for certain and the characterisation of the population in terms of relative proportion is often preferred to presenting data as cells/mℓ.

The specificity of the amplification reaction can be gauged by performing a melt curve analysis (Figure 4) on the product following the amplification cycles (Stubner, 2002). This involves heating the tubes, typically from 72°C to 95°C in increments of 0.2°C. When the melting temperature of the product is reached the fluorescence signal is lost and this is integrated to produce a melt curve. If the melt curve for the sample matches that obtained with the standard it can be assumed that the amplification was specific. The presence of multiple peaks on the melt curve implies some non-specific amplification. The melt curve can also be used to gauge the diversity within a particular sample when universal primers are used. In this case, each amplification product is likely to have a different melting temperature, due do difference in the GC content and size of the amplicon. The number of peaks gives an indication of the diversity of the population.

However, to maximise its quantitative potential, fastidious attention is required to the efficiency of the DNA extraction step, particularly its consistency across different species and the quality of the DNA used. Further the design of efficient and definitive primers for qRT-PCR analysis (Derveaux *et al*., 2009) is required for all microbial species or groups contributing to the ecology of the community.

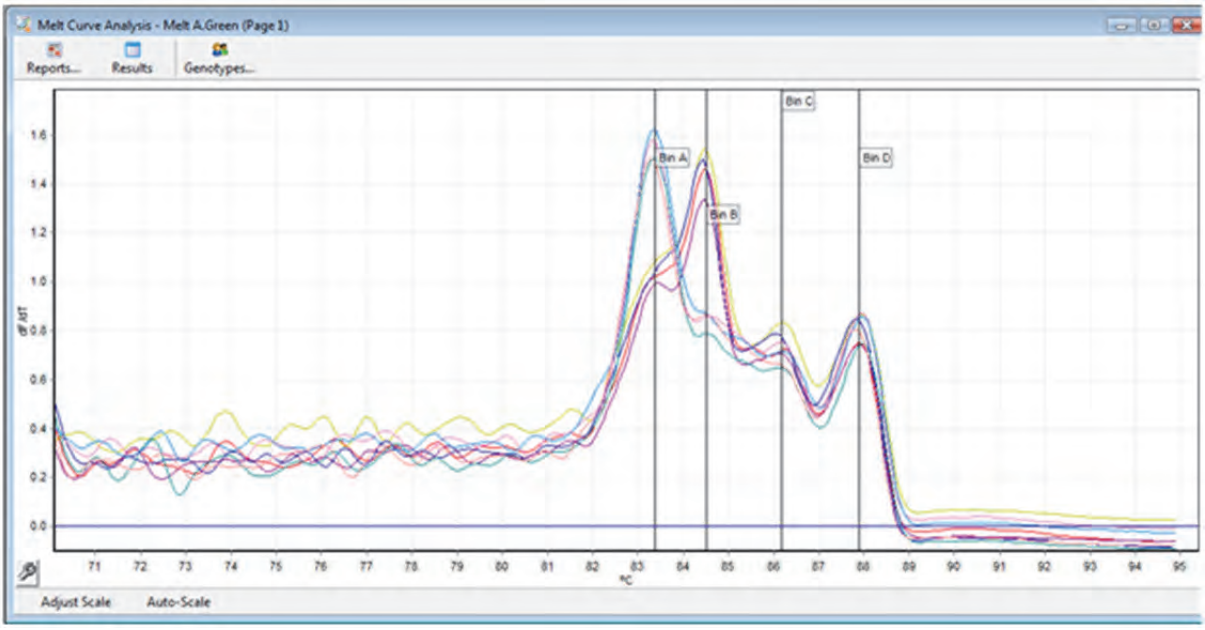

Figure 4: Example of a melt curve analysis illustrating the presence of four different amplification products

There are few reported instances where qPCR has been applied to sulphate reducing environments, although some pioneering work in the field has been performed Stephan Stubner from the Max Planck Institute for Terrestrial Microbiology. His work initially focussed on the quantification of *Desulfotomaculum* lineage 1 organisms in rice field soil. These Gram +ve sulphate reducing organisms were thought to play an important role in sulphate reduction in rice field soil, but constituted only a small percentage of the total bacterial population. Determination of 16S rDNA target numbers was not possible with dot blot hybridisation due to the insensitivity of the method. Other methods, like FISH, also lack the possibility for the accurate determination of the abundances of ‘‘minor’’ microbial groups (Amann *et al*., 1995). The study showed that amplification with a primer combination specific for *Desulfotomaculum* lineage 1 demonstrated an abundance of this group of approximately 2% and 0.5% of the eubacterial 16S rDNA targets in rice bulk soil and rice root samples, respectively.

Stubner extended the research associated with SRB populations in rice field soil to focus on the Gram –ve SRB (Stubner, 2004). He developed 11 real-time PCR primer sets targeting the 16S rRNA genes, combined with SybrGreen detection. Three of the primer sets were specific for the ‘‘main’’ groups, i.e. the *Desulfovibrionaceae*, the *Desulfobacteraceae* and *Desulfobulbus* species, while the remaining eight primer

sets were developed for subgroups within the first two main groups. The detection limits corresponded to less than 0.02% of the eubacterial 16S rDNA targets in bulk soil, rhizosphere soil and rice root DNA extracts, highlighting the sensitivity of the technique.

The 16S rRNA gene is not the only suitable target for qPCR primers. Kondo and associates (2008) demonstrated a technique for the rapid enumeration of sulphate reducing bacteria by qPCR using the dissimilatory sulphate reductase (DSR) gene as the target.

While there have been a number of studies using qPCR to assess sulphate reducing communities, success has been limited and this technique does not appear to have been applied to sulphide oxidising systems.

2.7 DEMONSTRATION OF THE VALUE OF QUANTITATIVE MICROBIAL ECOLOGY STUDIES

The qPCR based method proposed for this study has recently been used in a collaborative study between UCT and BHP Billiton to assess microbial succession in a simulated heap leach environment (Dew *et al*., 2011). Microbial succession refers to a microbial species, or group, succeeding another due to changes in the growth environment. In heap bioleaching microbial succession is normally a function of a change in the heap temperature or percolation solution chemistry, particularly the solution Eh and pH. A total of 17 species specific primer sets, as well as universal primer sets for bacteria and archaea were used to track changes in population structure as the simulated heap increased in temperature from ambient to extreme thermophile conditions (> 65°C). The results confirmed that microbial succession had taken place, but also provided a number of fascinating insights into the process. For example, there was a lag period of approximately a month between the temperature in the simulated heap changing and the noticeable shift in population structure, which suggested that species are able to tolerate temperatures outside of their optimal range for a relatively extended period before being lost from the population (Figure 5). In addition, such species persisted at temperatures well outside their operational range for the duration of the experiment, suggesting the presence of microenvironments where which could support their growth. In this case it was the persistence of the iron oxidising mesophile *Leptospirillum ferriphilum*. This persistence of this organism, which has a high affinity for ferrous iron, explained the higher than expected redox potentials measured in the column effluent.

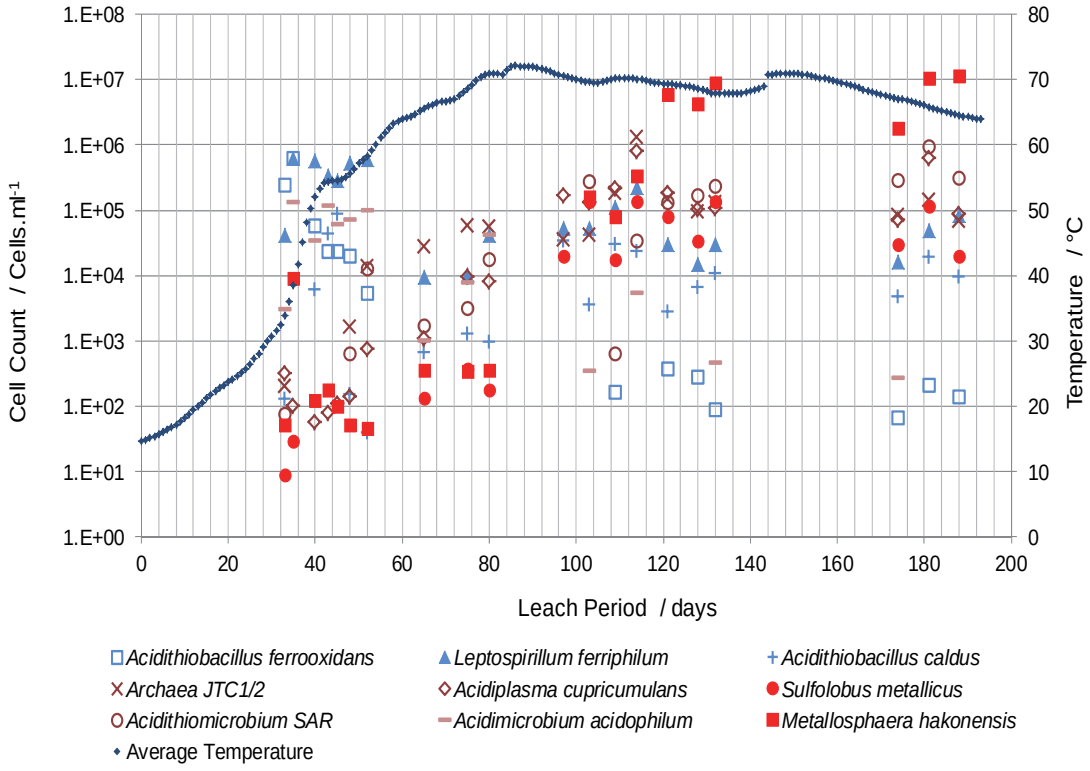

Figure 5: Succession of dominant microbial species as a function of time and ore temperature for a six metre simulation column (Dew et al., 2011).

The same primer sets have been used to determine the microbial population within a number of tank leaching (BIOX) reactors (Tupikina *et al.*, 2013). These studies have clearly shown that the microbial population associated with tank leaching of gold containing concentrates (pyrite/chalcopyrite) has evolved significantly over the past 10 years, with *Leptospirillum ferriphilum*, the species previously considered dominant (Coram and Rawlings, 2002) no longer playing a major role in any of the reactors samples from various operations around the globe. Their role as primary iron oxidisers appears to have been taken over by moderately thermophilic archaea belonging to the genera *Ferroplasma* or *Acidiplasma*.

The discussion above has highlighted the value of quantitative microbial studies in assessing population dynamics in both tank and heap bioleaching systems. Current work on optimising the performance of a passive system for the treatment of ARD has shown that organic carbon flux through the system is critically important and ultimately affects the composition of the microbial consortium. The presence of a number of autotrophic sulphide oxidisers has been confirmed under organic carbon limiting conditions. The performance of the reactor depends on the present of heterotrophic organisms, which provide structural integrity to the floating sulphur biofilm. The demonstrated potential of the qPCR technique to quantify target species, even when they make up only a small percentage of the total population suggests it could be an important tool in further describing the structure-function relationship in these complex environments.

CHAPTER 3: MATERIALS AND METHODS

3.1 INTRODUCTION

This chapter describes the microbial cultures, reactor systems and analytical tools used to generate the data presented in the report. The specific experimental approaches followed for each set of experiments are described in greater detail in the relevant chapters. In addition, a description of the Virginia experimental sewer site, from where the concrete pipe samples were obtained, is provided.

3.2 VIRGINIA EXPERIMENTAL SEWER SITE

3.2.1 Background

Ongoing interest in role of biological sulphur cycling in the corrosion of concrete prompted the establishment of a test site where the monitoring of actual performance of various materials used in the manufacture of concrete sewer pipes could be performed. Virginia, in the Free State, was selected as a suitable site, due to the high sulphate load in the sewage, the flat terrain, which necessitates pumping and the climatic conditions, with daily temperatures up to 35°C in summer and as high as 27°C in winter. Under these conditions, sulphide concentrations in the sewage stream can exceed 100 mg/ℓ (Alexander *et al*., 2008). The Virginia experimental sewer (VES) was set up alongside a bypass line (Figure 6).

Figure 6: Photograph of the Virginia experimental sewer site showing the location of the manholes

Three categories of sewer pipe materials were installed in 1988. These included unprotected cementitious, protected cementitious and high density polyethylene (HDPE) pipes. The unprotected concrete samples consisted of Portland cement (PC) with siliceous (SIL) aggregate (PC/SIL), Portland cement with dolomitic (DOL) aggregate (PC/DOL), calcium aluminate cement (CAC) with siliceous aggregate (CAC/SIL), and asbestos fibre reinforced cement (FC). The protected samples consisted of PC/SIL with a post-installed HDPE lining and FC coated with epoxy tar, polyurethane and epoxy (Goyns *et al.*, 2008).

In 2004, the original pipes that had suffered extensive corrosion were removed from the sewer line leaving 'gaps'. Thereafter, three manholes were built in the 'gaps'. A fourth manhole was added in 2007 (Figure 6**Error! Reference source not found.**). Cylindrical rings (870 mm internal diameter, 300 mm long and 80 mm in thick), representing 17 different concrete sewer pipe materials, were installed in each of manholes I, II and III. The cylindrical rings in Manhole IV have an internal diameter of 870 mm internal diameter, a length of 200 mm and a thickness 80 mm and represent 28 different concrete sewer pipe materials. These rings

have ‘lids’ sawn in the top 120° so that they can be removed for observations, measurements and sampling (Figure 7). Samples from manholes I, II and III were provided for analysis in the work presented in this report.

Figure 7: Photograph through a portion of a manhole showing five experimental rings

3.2.2 Test material composition

The composition of the 15 experimental rings sampled for this study are summarised in Table 1. CEM I and CAC are standard types, while SCM refers to supplementary cementing materials. Supplementary materials used were ground granulated blast furnace slag (GGBS), fly ash and silica fume.

Table 1: Composition of the experimental rings from which microbial samples were obtained. The numbers in parenthesis represent the sample numbers for the community analysis

Sample	Binder type and content in mix				Total binder in mix (%)	Aggregate		Sand[1]	
	Cement		SCM						
	Type	%	Type	%		Type	%	Type	%
PC/DOL 16 (1)	CEM I	16	-	-	16	DOL	48	DOL/SIL	36
PC/DOL 18 (2)	CEM I	18	-	-	18	DOL	47	DOL/SIL	35
PC/DOL 23 (3)	CEM I	23	-	-	23	DOL	44	DOL/SIL	33
PC/SL/DOL (4)	CEM I	12	GGBS	6	18	DOL	47	DOL/SIL	35
PC/FA/DOL (5)	CEM I	13.5	Fly ash	4.5	18	DOL	47	DOL/SIL	35
PC/SF/DOL (6)	CEM I	16.5	SF	1.5	18	DOL	47	DOL/SIL	35
CAC/SIL 23 (9)	CAC	23	-	-	23	SIL	44	SIL	33
CAC/DOL 16 (10)	CAC	16	-	-	16	DOL	48	DOL/SIL	36
CAC/DOL 18 (11)	CAC	18	-	-	18	DOL	47	DOL/SIL	35
CAC/DOL 23 (12)	CAC	23	-	-	23	DOL	44	DOL/SIL	33
CAC/FA/SF/DOL(14)	CAC	11	Fly ash SF	4.5 1.5	17	DOL	47	DOL/SIL	36
CAC/ALAG™ (15)	CAC	20	-	-	20	ALAG™	50	ALAG™	30

[1] dolomitic and siliceous sand in 50/50 proportion when mixed

ALAG™ is an artificial calcium aluminate aggregate

3.2.3 Sampling protocol

The test rings were sampled by lifting off the top part and scraping off approximately 20 g of loose binder material into sealable bags and transported to the laboratory at UCT. A portion of the sample (4 g) was transferred into 10 mℓ of sterile phosphate buffered saline in a sterile McCartney bottle and agitated on a vortex mixer. Samples were spun down at low speed (2000 × *g*) to remove the particulate material and the cells recovered from the supernatant by high speed centrifugation (10 000 × *g*). Genomic DNA was extracted from the recovered cells as described below and subjected to ARISA (Section 3.6.7) analysis to assess community diversity. Based on the indicated diversity, small clone libraries were constructed for each sample, sequenced and analysed as described in Sections 3.6.5 and 3.6.8.

3.3 MICROBIAL CULTURES

3.3.1 Sulphate reducing bacteria (SRB) culture

The SRB stock community was obtained from the Department of Microbiology, Biochemistry and Biotechnology at Rhodes University, originally from the anaerobic compartment of a facultative pond at the Grahamstown sewage treatment works, and has been maintained at UCT since 2001. The stock community has been maintained on modified Postgate B medium consisting of: (0.5 g/ℓ KH_2PO_4, 1 g/ℓ NH_4Cl, 2 g/ℓ $MgSO_4.7H_2O$, 1 g/ℓ Na_2SO_4, 1 g/ℓ yeast extract, 6 mℓ/ℓ 60% sodium lactate solution, 0.3 g/ℓ sodium citrate). Unless otherwise stated, all chemical reagents were analytical grade from Merck.

3.3.2 Sulphide oxidising culture

The sulphide oxidising culture was enriched from effluent from packed bed sulphate reducing reactors, provided by Golder Associates Africa. The effluent was used to inoculate a small (2.125 ℓ) linear flow channel reactor. Sulphide (100 mg/ℓ as Na_2S) and an organic carbon source (1 g/ℓ acetate) were added to the reactor. A floating sulphur biofilm began to develop after 48 hours. Samples of the biofilm and bulk liquid were maintained in 1 ℓ Schott bottles on a medium containing 0.5 g/ℓ KH_2PO_4, 1 g/ℓ NH_4Cl, 0.5 g/ℓ $MgSO_4.7H_2O$, 1 g/ℓ acetate and 100 mg/ℓ HS^-. The bottles were sub-cultured every two weeks.

3.4 REACTOR UNITS

3.4.1 Continuously stirred biological sulphate reduction reactor

Continuous experiments were performed in glass reactors with a working volume of 1 ℓ. These were refined from the reactor setup reported by Moosa *et al*. (2002) and Oyekola *et al*. (2009) for kinetic studies of biological sulphate reduction using suspended culture and are shown in Figure 8. The reactor height was 200 mm, with a liquid volume height of 118 mm, and diameter 104 mm. Agitation was provided by an overhead stirrer powering a four-bladed marine impeller (58 mm diameter) at 300 rpm. The reactor was fitted with four vertical baffles (10 mm width) to prevent vortex formation. The reactor lid was specially designed and constructed to reduce the possibility of air ingress. The impeller shaft passed through a column containing three rubber lip seals, which effectively sealed the unit. In addition, the lid was fitted with a feed port, a sampling port and a gas venting port. Off gas was passed through a series of sulphide stripping units (1 M NaOH) for health and safety reasons and to allow the quantification of any sulphide lost as H_2S gas. Temperature was controlled at 30°C by pumping heated water through the external jacket or placing the reactors in a temperature-controlled water bath. Feed solution was continuously pumped into the reactor using a variable speed peristaltic pump.

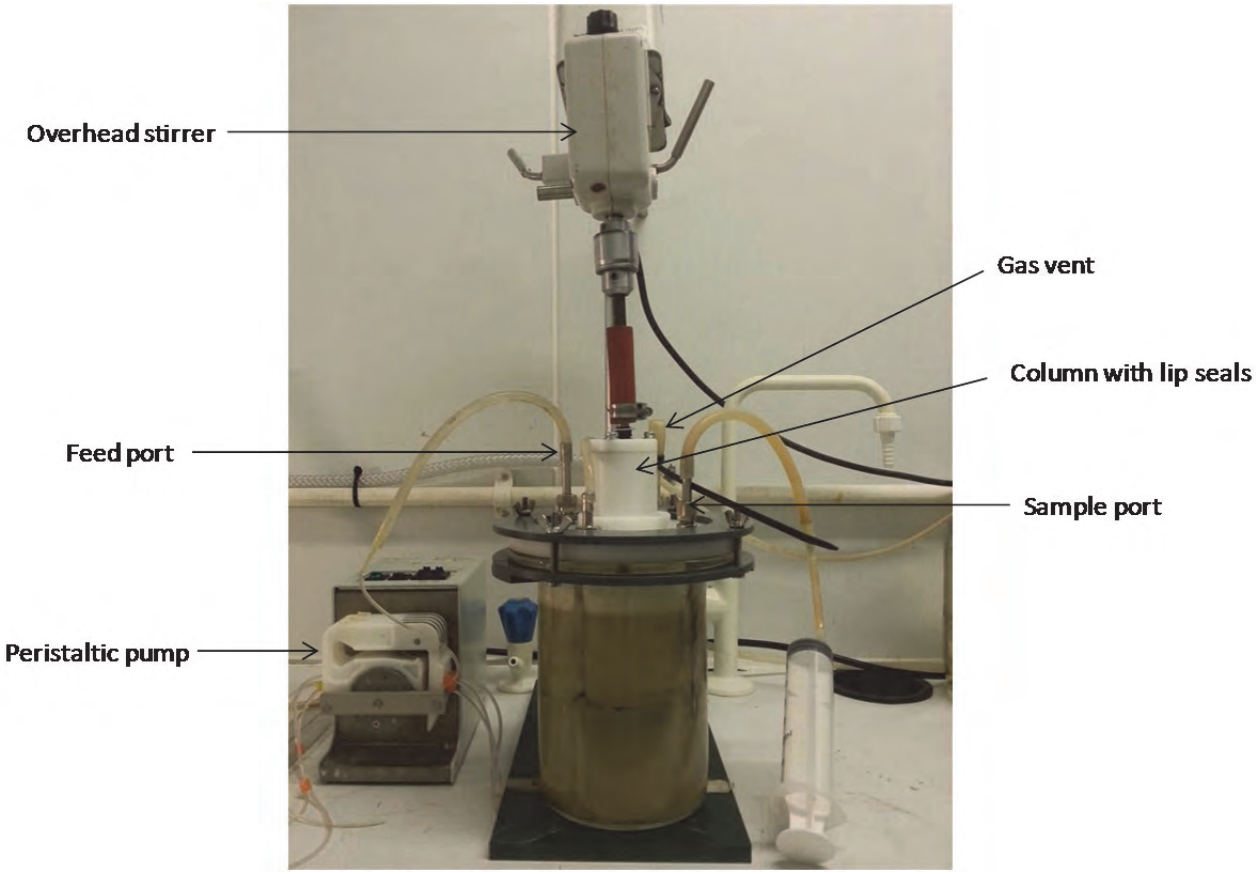

Figure 8: Photograph of the 1 ℓ CSTR used to generate baseline data for biological sulphate reduction, using suspended culture under reactor conditions designed for biokinetic studies

3.4.2 Linear flow channel reactor (LFCR)

Studies were carried out using three identical 37.5 ℓ purpose built Perspex reactors. The dimensions of the reactor are shown in Figure 9. The reactors were housed in a custom built support with a drip tray in case of leakage (Figure 10). Typically, these were operated with a liquid working volume of 25 ℓ and a headspace of 12.5 ℓ. Air was blown into the headspace at a rate of 48 ℓ/day and exited via the gas outlet port, which then passed through the sodium hydroxide scrubber. Each reactor was sealed with a silicone gasket, which ensured that any sulphide gas liberated was not lost and could be recovered in the sodium hydroxide (NaOH) scrubber. The LFCRs were operated at room temperature (23-25°C) and atmospheric pressure. Two additional, smaller, LFCRs were constructed with a volume 3.75 ℓ (liquid volume 2.125 ℓ). These reactors were used for culture enrichment. The reactor drawings are detailed in Appendix A.

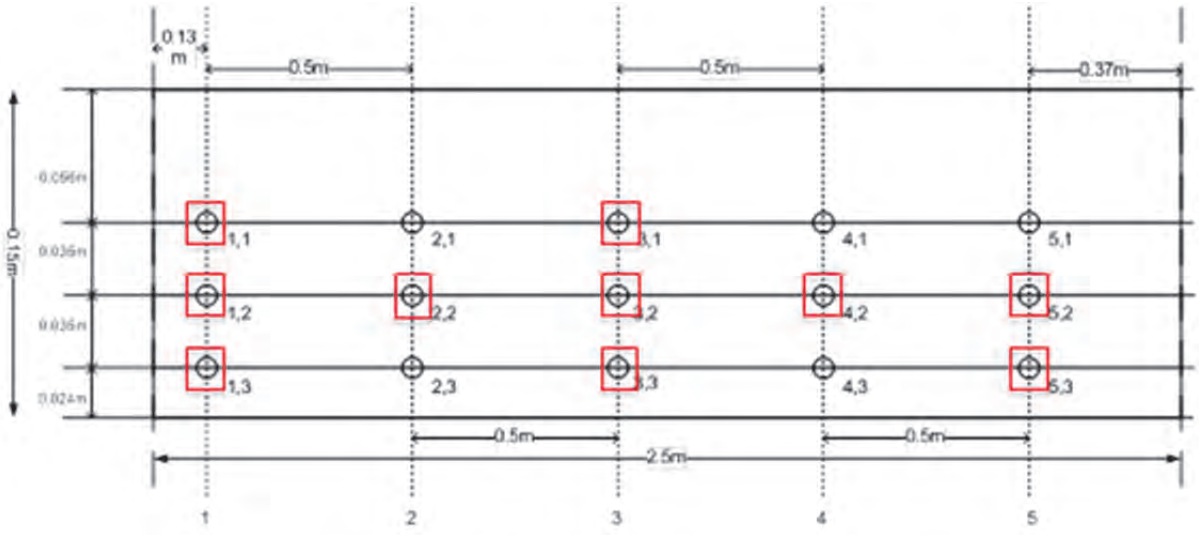

Figure 9: Schematic diagram showing dimensions of the LFCR. The nine sample ports highlighted are those which were routinely sampled

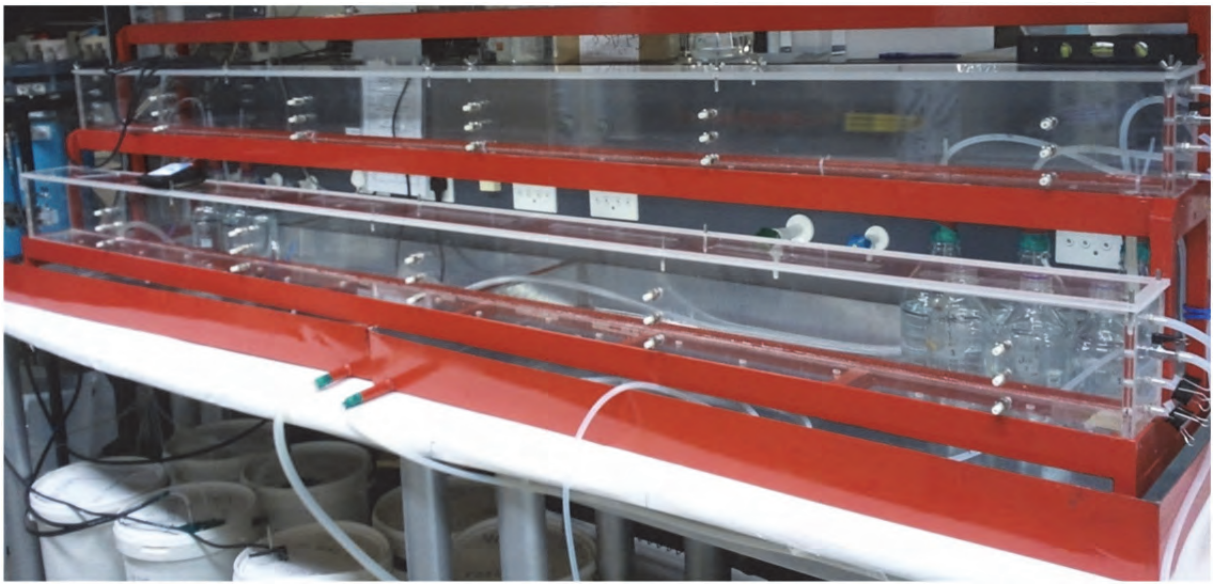

Figure 10: Photograph showing two of the LFCR reactors

3.5 ANALYTICAL METHODS

3.5.1 pH and redox potential

All pH testing was done on a Cyberscan 2500 micro pH meter. The meter was calibrated daily using standard (pH of 4.0 and 7.0) buffer solutions. Redox potential was measured using a Metrohm pH lab 827 redox meter.

3.5.2 Chemical oxygen demand (COD)

All COD measurements were carried out using the Merck reagent test protocols for high (1500-10000 mg/ℓ) and low (100-1500 mg/ℓ) concentrations. The method is based on the oxidation of the sample with a hot sulphuric acid solution containing potassium dichromate, with silver sulphate as the catalyst. The chloride is masked with mercury sulphate. The concentration of unconsumed yellow $Cr_2O_7^{2-}$ ions or green Cr_3^{+} ions is then determined photometrically and used to quantify oxygen demand. To quantify the COD concentrations, standard solutions (0, 250, 500, 750, 1000, 1250, 1500 (low range) and 2500, 5000, 7500 and 10000 mg/ℓ COD (high range)) were prepared using potassium hydrogen phthalate.

3.5.3 Sulphide

Aqueous sulphide was quantified using the colorimetric DMDP method. The principle of the method is reaction of aqueous sulphide with N,N-dimethyl-p-phenylene diamine, catalysed by ferric ions, to produce methylene blue. An appropriate volume of sample (10-4800 µℓ) is added to 200 µℓ of 1% zinc acetate. The volume is made up to 5 mℓ with deoxygenated water, after which 500 µℓ of 0.4% N,N-dimethyl-p-phenylene diamine (in 6 M HCl) and 500 µℓ of 1.6% ferric chloride (in 6 M HCl) are added. The sample is mixed well and left to react for a minimum of 5 minutes after which the absorbance is read at 670 nm and the concentration determined relative to a standard curve. The assay has a maximum detection limit of just over 1 mg/ℓ so significant dilution is required. This is typically achieved by using a small volume (20-50 µℓ) of sample. Extensive testing confirmed the precision and accuracy of the assay, using the small volume, with standard deviations < 2%.

3.5.4 Volatile fatty acids (VFAs)

A full volatile fatty acids (VFAs) analysis was conducted to quantify the concentration of lactic, acetic, propionic, iso-butyric, butyric, iso-valeric and valeric acids present in the reactors. The concentration of each VFA was determined using HPLC on a Waters Breeze 2 HPLC system equipped with a Bio-Rad Organics Acids ROA column and a UV (210 nm wavelength) detector. The system was run isocratically using a mobile phase of 0.01 M H_2SO_4 at a flow rate of 0.6 mℓ/min. The pressure in the column did not exceed 13 780 kPa Sample injection volumes of 100 µℓ were used. To quantify the VFA concentrations standard solutions (250, 500, 750 and 1000 mg/ℓ for each acid) were prepared using HPLC grade standards (Sigma).

3.5.5 Anions

Dissolved anions (sulphate, chloride, nitrate and phosphate) were measured by HPLC using a Waters Breeze 2.0 system equipped with a Waters IC-Pak A HR (Anion High resolution) column and a conductivity detector. The system was run isocratically using a sodium borate-gluconate mobile phase at a flow rate of 1 mℓ/min. The pressure in the column did not exceed 13 780 kPa. Sample injection volumes of 100 µℓ were used. To quantify the ion concentrations standard solutions (50, 100, 150 and 200 mg/ℓ) of each ion were prepared using their respective sodium salt.

3.5.6 Alkalinity titration

A measured volume (10-15 mℓ) of the reactor effluent was titrated against 0.01 N H_2SO_4, first to pH 8.3 and then pH 4.5 in order to quantify the alkalinity of the solution. The carbonate and bicarbonate concentrations at the end of the run were determined according to the method presented by Clesceri *et al.* (1989).

3.6 CHARACTERISATION OF THE SULPHATE REDUCTION AND SULPHIDE OXIDISING MICROBIAL POPULATIONS

Initially sulphate reducing populations from the lactate and acetate fed digesters were analysed, along with samples from the sulphide oxidising stock reactors. The sequence information from these samples has been received and verified. A second round of cloning and sequencing was performed on environmental SRB samples (Middelburg demonstration plant DPBR overflow) and positive enrichment cultures (microalgae and AD overflow fed).

A preliminary characterisation of the sulphide oxidising microbial community was performed as a component of the WRC project K5/1834 and is described in van Hille *et al*. (2012). A more comprehensive characterisation is presented in the current study. The pertinent information relating to the DNA extraction and sequencing of the samples from the sulphide oxidising reactors is summarised below.

3.6.1 DNA extraction

3.6.1.1 Phenol chloroform method

DNA was extracted from stock sulphide oxidising cultures and operating linear flow channel reactors (LFCRs), as well as from stock sulphate reducing cultures, enrichment cultures and environmental samples. The protocol was similar for all samples. Samples (15 mℓ) were spun down in sterile 2 mℓ microcentrifuge

tubes to obtain pellets. A 1 mℓ volume of Buffer S ((100 mM Tris-HCl (pH 8.5), 100 mM NaCl, 50 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0, 2% (W/V) sodium docecyl sulphate (SDS)) was added to each sample. The samples were vortexed thoroughly and proteinase K was added to a final concentration of 0.05 mg/mℓ. The microcentrifuge tubes were incubated at 65°C for 2 hours with occasional inversions to mix the contents. Phenol: chloroform: isoamylalcohol (24:24:1) solution (1 mℓ) was then added to each tube. Mixing was achieved by gentle inversion to form an emulsion. The samples were then centrifuged at 1500 × *g* for 20 min to separate the phases. The centrifugation was repeated if the aqueous phase was still cloudy. The aqueous phases were then transferred to clean 2 mℓ microcentrifuge tubes and mixed with 0.6 volumes of propan-2-ol. Mixing by inversion was followed by centrifugation at 10000 × *g* for 10 min. The resulting pellets were then washed with 200 µL of cold 70% ethanol and dried at room temperature. The DNA was the resuspended 50 µℓ of sterile H_2O. The DNA was then quantified using the Nanodrop 2000.

3.6.1.2 Roche High Pure Template Preparation kit

Samples for DNA extraction were obtained from 1 ℓ continuously stirred tank reactors (CSTRs). Samples (2 mℓ) were centrifuged at 10000 × *g* and the pellet used for DNA extraction. Genomic DNA was extracted using the High Pure PCR Template Preparation Kit (Roche, Germany) according to the manufacturer instructions.

3.6.2 Conventional PCR

Both PCR amplification methods utilised KAPA master mix™ (KAPA Biosystems Cape Town, SA). PCR reactions were carried out in a 50 µℓ reaction volumes containing 1 X KAPA master mix™ and 0.25 µM primers (Universal bacteria primer set- 16S or Universal algal/fungal primer set- 18S). PCR steps for amplification: DNA was denatured at 94°C for 5 min. This was followed by 40 cycles of 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 20 sec. This was followed by a final extension at 72°C for 2 min.

3.6.3 Denaturing gradient gel electrophoresis (DGGE)

A new DGGE system was installed and commissioned. The optimisation has been performed using an approach similar to that of Miletto and co-workers (2007).

Prior to loading, PCR products were mixed with 6X bromophenol blue loading dye (1 µℓ per µℓ of PCR product). A range of denaturing gradients from 45-65%, 30-40% and 20-30 (w/v) denaturant were tested. The stock solution (100%) of denaturant contained 7 M urea and 40% (v/v) formamide. The gradient gels were 1 mm thick, with 8% (w/v) polyacrylamide used as the basis. Gels were initially run at 180 V for 10 min to facilitate the access of PCR products into the denaturing gradient gel, and then at a constant voltage of 1100 Vh. Gels were incubated in a 1 × TAE buffer at a stable temperature of 60ºC. Subsequent to electrophoresis, gels were silver stained for 30 min and destained in 1 × TAE buffer for 15 min. Images of the gels were then captured using the ChemiDoc™ system (Bio-Rad, USA)

3.6.4 Quantitative real-time polymerase chain reaction (qPCR)

PCR reactions were carried out in a 20 µℓ reaction volumes containing 1X KAPA master mix™ and 0.25 µM primers (Table 1) (Universal algal/fungal primer set- 18S PCR or Universal Archaeal primer set 16S) steps for amplification: DNA was denatured at 95°C for 5 min. This was followed by 35 cycles of 95°C for 10 sec, annealing at 60°C for 10 sec and extension at 72°C for 20 sec. This was followed by a final extension at 72°C for 5 min.

3.6.5 Cloning and sequencing

Polymerase chain reaction amplified DNA bands were excised from the gels and purified using the QIAquick Gel Extraction Kit (QIAGEN™), according to the manufacturer's instructions. The recovered DNA amplicon (3 µℓ) was ligated into pGEM-T Easy™ (Promega). The ligation mixture contained the following: 2 × ligase buffer, 1 µℓ T4 ligase, 1 µℓ pGEM-T Easy™ and 3 µℓ DNA. The ligation mix was incubated at 4°C overnight. The ligation mixture was used to transform competent *E. coli* cells, which were plated (100 µℓ) onto LB plates containing 0.5 mM ampicillin, 0.5 mM IPTG (Isopropyl-Beta-D-thiogalactoside) and 80 µg/mℓ X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactoside). The plates were incubated at 37°C overnight and plasmid

DNA was extracted from positive transformants using the GenElute^{TM} hp Plasmid Miniprep Kit (SIGMA-ALDRICH). Purified samples were sent for sequencing. Microbial identification was performed by comparing the resulting sequences against known sequences using the National Centre for Biotechnology Information's (NCBI) basic local sequence alignment tool (BLAST).

Table 2: Specific information on primer sets used for conventional and qPCR

Type of PCR	Primer name	Primer sequence	Product size
Standard PCR	Universal bacterial 16S	(Reverse- 1492rG ') 5' gtacggItaccttgttacgactt 3' (Forward- 27fG) 5' gagagtttgatcItggctcag 3'	1400 bp
	Universal algal/fungal 18S	(Reverse- Uni 18S1686) 5' aaagggcagggacgtaatcaa 3' (Forward- Uni 18S603) 5' cgcggtaattccagctcca 3'	1000 bp
qPCR	Universal bacterial 16S	(Forward- UniBactF336) 5' gactcctacgggaggcagca 3' (Reverse- UniBactR937) 5' ttgtgcgggcccccgtcaat 3'	600 bp
	Universal archaeal (16S)	(Forward- UniArchF342) 5' acggggigcaicaggcg 3' (Reverse- UniArchR932) 5' tgctcccccgccaattcc 3'	600 bp

3.6.6 Amplified ribosomal DNA restriction analysis (ARDRA)

The ARDRA analysis was performed on successfully transformed clones to identify unique ribotypes in order to minimise unnecessary sequencing. During cloning, the distribution of 16S rRNA inserts in the transformed colonies will represent the distribution of species in the community. If clones were selected for sequencing on a random basis they too would represent the original distribution and multiple sequences of the dominant community members would be obtained. While this provides some information on community structure sequencing multiple replicates becomes prohibitively expensive. Using ARDRA it is possible determine clones with similar inserts, on the basis of restriction band patterns, so only unique ribotypes are sequenced. Approximately 500 ng of purified PCR product was digested using *Hae*III and *Alu*I, respectively (Fermentas, South Africa), at 37°C overnight. The digested DNA fragments were separated by electrophoresis on a 1.2% (w/v) TAE agarose gel at 55 V for approximately 60 min. The gels were stained with 0.1% (w/v) ethidium bromide and destained with distilled water, before visualisation on a G-Box (SYNGENE, Germany) UV transilluminator. Putative unique ribotypes were identified on the basis of unique *Hae*III and *Alu*I restriction patterns. PCR products (section 2.5) representing ribotypes that were presumed to be unique, were sent for sequencing in the Netherlands (http://dna.macrogen.com/eng/) using the M13 forward and reverse primers.

3.6.7 Amplified ribosomal intergenic spacer analysis (ARISA)

Microbial abundance in the concrete sewer samples was determined using ARISA. A PCR reaction was performed using 50 of template per reaction and FAM-labeled primers (FAM-ITSF [5'-GTCGTAACAAGGTAGCCGTA-3'] and ITSReub [5'-GCCAAGGCATCCACC-3']) at a final concentration of 0.2 µM each using the 2G Robust polymerase (KAPA Biosystems). The subsequent amplicons were analysed at the Central Analytical Facility (CAF) at the University of Stellenbosch. The PCR products were subjected to a post PCR clean-up prior to electrophoresis. Two µℓ of cleaned PCR product was mixed with the appropriate internal size standard (Applied Biosystems) and Hi-Di, prior to denaturing for 5 minutes at 95°C. Directly after heating the samples were placed on ice for 5 minutes. Electrophoresis was performed on either an ABI3130xl or an ABI3730xl unit, using a 50 cm capillary array and POP7 (all supplied by Applied Biosystems). The data were further analysed using GeneMapper (Version 4.0, Life Technologies).

3.6.8 Analysis of sequences

The 16S rRNA sequences obtained from the clone library were edited and assembled using Chromas version 2.01 (Technelysium Pty Ltd., Australia) and DNAMAN for windows version 4.13 (Lynnon Biosoft, Canada), respectively. Homology and similarity searches of DNA sequences were performed using the basic local alignment search tool (BLAST) (Altschul *et al.,* 1989; Altschul *et al.,* 1997), as provided by the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/).

For phylogenetic analysis the 16S rRNA sequences obtained from the 16S rRNA clone library and reference sequences obtained from the NCBI database were aligned using CLUSTAL_X, version 2.0 (Thompson *et al*., 1997) and manually edited to a common length of 1520 nucleotides. Phylogenetic analyses were conducted using MEGA version 5.1 (Kumar *et al*., 2004) and a neighbour-joining (Saitou and Nei, 1987) tree constructed. Bootstrap values were based upon 1,000 re-sampled data sets (Felsenstein, 1985) and only bootstrap values greater than 40% are indicated.

3.7 FLUORESCENCE *IN SITU* HYBRIDISATION (FISH)

This method combines the visual information given by the use of microscopy and the precision provided by molecular genetics. Therefore, individual microbial cells can be visualised and identified within their natural microhabitats (Moter and Gobel, 2000). In 1969, two research groups independently developed *in situ* hybridisation (Pardue and Gall; 1969; John *et al*., 1969). The researchers hybridised radioactively labelled DNA or 28S RNA to cytological preparations from *Xenopus* oocytes and these were detected by microautoradiography. This technique offered the advantage of the nucleic acid being examined inside a cell without altering the cell morphology or the integrity of it various compartments (Moter and Gobel, 2000). In 1988, Giovannoni *et al.,* introduced FISH into bacteriology by using radioactively labelled rRNA-directed oligonucleotide probes for the microscopic detection of bacteria (Giovannoni *et al.,* 1988). With time, radioactive labels were steadily superseded and replaced by non-isotopic dyes due to the development of fluorescent labels (Landegent *et al.,* 1984; Pinkel *et al*., 1988). Fluorescently labelled oligonucleotide probes were first used in 1989 by DeLong to detect single microbial cells (DeLong *et al*., 1989). These offered the advantage of being safer than radioactive probes, with better resolution, and no need for additional detection steps (Moter and Gobel, 2000). But perhaps the biggest advantage will be that fluorescent probes can be labelled with dyes of different emission wavelength thus enabling detection of several target sequences within a single hybridisation step; FISH has therefore become a powerful molecular tool in recent years (Icgen and Harrison, 2006a; Icgen and Harrison, 2006b).

The specific information for the probes used in this study is summarised in the table below. The design of the probes was informed by the sequence information obtained during the generation of the clone library, as well as published literature. The specificity of all probes and the potential for cross-reactivity was assessed *in silico*, using the Geneious software.

Table 3: Target species, formamide concentration used and specific sequence of the FISH probes used in this study

Probe	Target	Formamide (%)	Sequence (5'-3')
EUB338	Most bacteria	0-50%	GCTGCCTCCCGTAGGAGT
SRB129	*Desulfobacter*	10	CAGGCTTGAAGGCAGATT
DELTA495a	Most δ-proteobacteria and most Gemmatimonadetes	35	AGTTAGCCGGTGCTTCCT
DSM213	*Desulfomicrobium*	15	CATCCTCGGACGAATGCA

3.7.1 Methodology

Cell Fixation: Samples were collected from respective reactors and cells were harvested (10000 rpm, 10 min). These were washed (8000 rpm, 5 min) in 1 × PBS followed by resuspension in 375 µℓ of fresh 1 × PBS and three volumes (1.125 mℓ) of freshly prepared paraformaldehyde (4% [w/v] in 1 × PBS) was added.

This was followed by incubation at 4°C overnight. This was subsequently washed in fresh 1 × PBS and stored in 1 × PBS: ice cold absolute ethanol (1:1 [v/v], -20°C) until hybridisation (Amann, 1995).

Slide preparation: 5 µℓ of resuspended fixed cells were mounted onto glass slides and oven-dried at 46°C for 20 min. In order to increase permeability of the target cells for the oligonucleotide probes, 5 µℓ of lysosyme (10 mg/mℓ) was added on to the cell spot, followed by incubation on ice for 20 min. Lysosyme was then washed off using sterile Millipore water. The samples were then dehydrated using an increasing ethanol series (50, 80 and 98% ethanol) for 3 min each. The slides were then allowed to air dry and stored at -20°C prior to hybridisation (Li *et al*., 1997).

Hybridisation: 2 mℓ fresh hybridisation buffer was prepared by adding the following stock solutions consecutively: 36 µℓ NaCl (5 M); 40 µℓ Tris-HCl (1 M, pH 7.2); 2 µℓ SDS (10%); varying concentrations of formamide concentrations for different probes (Table 1). Sterilised ddH_2O was added to make up to 2 mℓ. Hybridisation buffer (9 µℓ) and 1 µℓ of each probe were added to the cell spots, resulting in a probe concentration of ~ 50 ng/µℓ. Cover slips were placed on top of the slides to ensure that the entire sample was exposed to the hybridisation mix. Slides were placed in 50 mℓ tubes containing the excess hybridisation buffer. These were sealed and laid horizontally in the oven for incubation (46°C, 2 h).

Washing: Slides were immersed in pre-warmed (48°C) washing buffer (varying NaCl concentrations; 1 mℓ Tris-HCl (1 M, pH 7.2); 50 µℓ SDS (10%), made up to 50 mℓ with sterilised ddH_2O) followed by incubation (48°C, 20 min). The slides were rinsed in a beaker containing ice-cold ddH_2O, and then air-dried in the dark. Samples were then counterstained by adding 10 µℓ DAPI (1 µg/mℓ in ddH_2O) on to the slides. Cover slips were placed on top of the slides. This was followed by incubation (ambient temperature, 5 min), rinsing with ddH_2O and air drying. Aliquots of antifade solution, AFI (Citifluor Ltd., London UK) were added to the wells, with the cover slips pressed down gently to remove excess antifade, prior to microscopy.

Microscopy: This was performed using an Olympus model BX40 epifluorescence microscope.

CHAPTER 4: BIOREACTOR STUDIES AND COMMUNITY ANALYSIS

4.1 INTRODUCTION

The primary aim of the research was to develop a set of molecular tools that could be used to perform a quantitative or semi-quantitative analysis of the microbial community, specifically to help understand how the microbial community changes in response to operational parameters. In order to test the probes and primer sets it was necessary to operate reactors under conditions that were likely to induce a change in community structure. For the sulphate reducing community hydraulic retention time was chosen, while for the sulphide oxidising community the presence or absence of organic carbon was selected.

The performance of the reactor systems are summarised here, but are presented in greater detail elsewhere (Harrison *et al.,* 2014; Mooruth, 2013).

4.2 EXPERIMENTAL PROGRAMME

4.2.1 Sulphate reducing reactor studies

4.2.1.1 Lactate-based growth medium

The baseline data were generated using a similar set of experimental reactors and operating conditions used by Moosa (2001) and Oyekola (2008). A series of standard CSTRs were operated at 30°C, using the modified Postgate B medium described in section 3.2.1 as the feed source. Reactors were operated at feed sulphate concentrations of 1, 2.5 and 5 g/ℓ. The lactate concentration in the feed was adjusted to maintain a COD/sulphate ratio of 0.7 in all the reactors. The reactors were started at a hydraulic retention time of 5 days and sampled at least once per HRT. Once steady state had been established and monitored for at least three HRTs, the feed rate was increased, reducing the HRT to 4 days. The process was repeated a number of times, collecting data at 3, 2, 1.5, 1 and 0.5 days. For the reactors with feed sulphate concentrations of 2.5 and 5 g/ℓ, the experiment was terminated at a HRT of 1 day.

The reactors were sampled by removing 10 mℓ of solution and immediately using 20 µℓ to perform a sulphide assay. The pH and redox potential were measured using the remaining sample. A subsample (2 mℓ) was transferred to a 2 mℓ Eppendorf tube, to which 40 µℓ of zinc chloride solution was added. The tubes were mixed on a vortex mixer, then centrifuged at 14000 × *g* for 7 minutes. The supernatant was used to prepare samples for analysis of anions and VFAs by HPLC.

4.2.1.2 Algal digestate-based growth medium

Two continuous stirred tank reactors were set up and fed effluent from the anaerobic digester as the carbon source and electron donor, in order assess the proof of concept. No additional nutrients were provided. Constraints on the number of reactors available and the amount of effluent that could be generated from the digester meant that a limited number of reactors could be maintained. The decision was taken to test the 2.5 and 5 g/ℓ sulphate concentrations, as the data generated at these conditions using lactate as the feed identified challenges. Effluent from the digester was collected until 2 ℓ was available. The effluent was filtered through a fine mesh (50 µm) cloth, then centrifuged at 5 000 × *g* for 10 minutes to remove particulate material that could block the feed pipes. The COD of the supernatant was determined. The centrifuged effluent and sulphate stock solution were placed in separate feed bottles and the pumping rates calibrated to achieve the desired overall HRT in the reactor and maintain a COD/SO_4^{2-} ratio of 0.7. The concentration of the sulphate stock solution was adjusted appropriately. The calculation and calibration was performed each time fresh digester effluent was added, although the COD of the effluent was relatively consistent so only small changes were required.

Both sets of reactors were initially operated at a HRT of 5 days (dilution rate 0.0083/h). The reactors were sampled at least twice per HRT and the pH, redox potential and sulphide concentration determined immediately. A fraction (2 mℓ) of the sample was treated with 40 µℓ of zinc chloride (100 g/ℓ) and centrifuged at 14000 × *g* for 7 minutes to remove sulphide as zinc sulphide. The supernatant was filtered through a 0.45 µm nylon membrane filter and retain for HPLC analysis (VFAs and sulphate). A COD analysis was performed on selected samples, with a minimum of one analysis per HRT.

The data were used to determine when steady state was achieved. Once steady state had been maintained for three consecutive HRTs the feed rate was increased to achieve the next HRT.

4.2.2 Sulphide oxidising reactors

The LFCRs were operated with a working liquid volume of 25 ℓ, unless otherwise stated. They were closed to the surroundings (gasket sealed lid) creating a headspace of 12.5 ℓ. The headspace was flushed with air at a flow rate of 48 ℓ/day, with the exit gas passing through an alkaline scrubber to recover any H_2S gas. Unless otherwise stated the reactors were fed, via the uppermost inlet port, at a set flow rate and the effluent exited via the uppermost exit port. For each experiment the reactor was filled with 25 ℓ of sulphide effluent from packed bed sulphate reducing reactors and was allowed to stand for 24 hours. This was to allow the microbial consortium to acclimatise to the new conditions prior to starting the experiment. A series of experiments were performed without addition of acetate to the channel feed, while for the second series the feed was supplemented with acetate at concentrations between 1 and 2.25 g/ℓ.day. The details of the individual runs have been reported previously (van Hille and Mooruth, 2013).

4.2.3 Characterisation of the microbial communities

A clone library approach was selected to classify the sulphate reducing and sulphide oxidising communities. Initial attempts to determine the diversity of the communities by DGGE were not successful, in part due to technical challenges and in part due to the diversity of the communities. A number of bands were visible on the degrading gel, but the resolution was not sufficient to excise separate bands.

Samples from the lactate-fed and digestate-fed reactors, as well as the packed column reactors were pooled for DNA extraction, to ensure coverage of the diversity of sulphate reducers. Approximately 150 clones were selected from the successful transformants and subjected to the ARDRA analysis. This reduced the diversity down to 48 unique ribotypes, which were subsequently sequenced.

The sulphide oxidising community from the stock reactors was characterised separately to that from the experimental LFCRs, as diversity was lower. The community in the LFCR was determined under two sets of conditions, to assess the impact of organic carbon on the community. The first set of samples was taken from LFCRs that were operated without acetate supplementation. Under these conditions the sulphur biofilm was thin and brittle. The second set of samples was taken from reactors supplemented with a minimum of 250 mg/ℓ acetate. Under these conditions a thick, continuous biofilm formed. The progression of biofilm formation suggested an organic carbon “scaffold” which supported the sulphide oxidisers and deposited sulphur.

4.3 RESULTS AND DISCUSSION

4.3.1 Sulphate reducing reactors

4.3.1.1 Lactate-based growth medium

The data presented in this section represent a summary of steady state data at the different hydraulic retention times in all three reactors. Steady state was assumed when the change in key parameters, particularly residual sulphate concentration, was less than 10% for three successive HRTs following a change in system conditions.

The steady state profiles for sulphate consumption and measured sulphide, at a feed sulphate concentration of 1.0 g/ℓ are shown in Figure 11. The graph also shows the theoretical sulphide concentration, based on the molar concentration of sulphate reduced. The sulphate reduction is relatively consistent across the range of

HRTs from 5 days down to 1 day, with between 870 and 920 mg/ℓ of the 1000 mg/ℓ feed being consumed. Based on molar stoichiometry, the measured sulphide concentration should have been just below 300 mg/ℓ. However, the actual measured sulphide concentration was significantly lower, suggesting either a loss of sulphide to the surroundings or further reaction of the sulphide. The reactor unit was sealed and any off gas passed through a sodium hydroxide sulphide scrubber, where gaseous hydrogen sulphide (H_2S) would be converted to aqueous bisulphide (HS^-). Analysis of the bisulphide concentration indicated no significant loss of H_2S from the reactor. This is consistent with the steady state pH (7.4 ± 0.15). Under these conditions the majority of the aqueous sulphide would exist as the HS^-. The result suggests partial oxidation or precipitation of a portion of the sulphide. No precipitates were observed, while a layer of elemental sulphur was observed at the air-liquid interface.

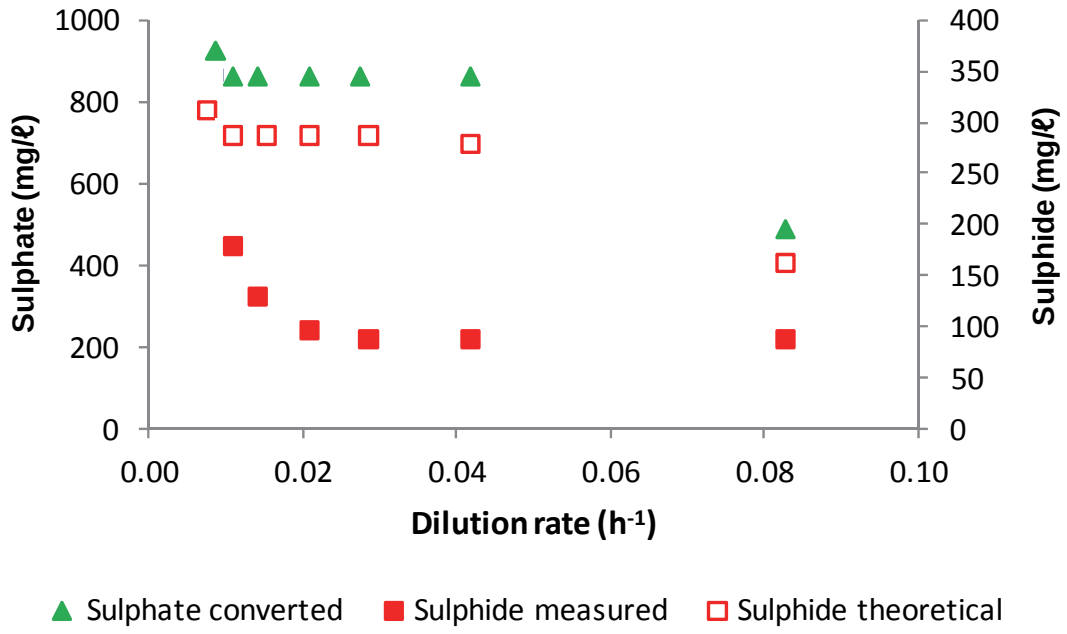

Figure 11: Sulphate converted and measured and theoretical sulphide concentrations for the CSTR receiving 1 g/ℓ SO_4^{2-}

There was a significant decrease in the amount of sulphate reduced at a HRT of 12 hours (dilution rate of 0.083/h), with the conversion efficiency falling to around 50%. Despite the reduction in the sulphate conversion, the conversion of lactate remained at 99% (Figure 12). Based on the amount of sulphate reduced, the expected residual lactate concentration at this HRT should have been over 1000 mg/ℓ. However, a residual concentration of <30 mg/ℓ was detected. This indicates that the sulphate conversion was not limited by lactate concentration. This observation also suggests that while sulphate reducers oxidised lactate at a rate near their μ_{max} another group of microorganisms characterised by higher μ_{max} and K_s values for lactate utilisation were able to proliferate due to increased lactate loading at the high volumetric loading rate. Consequently, there was no accumulation of lactate. The decline in sulphate conversion was most likely a consequence of wash out of a portion of the sulphate reducing community when the reactor was operated at a dilution rate greater than their μ_{max}. These data are similar to those obtained by Oyekola *et al.* (2009, 2010, 2012) and Baksaran and Nemati (2006). The study showed that the decrease in sulphate reduction efficiency coincided with a decrease in acetate formation and increase in propionate formation (Oyekola *et al*., 2009). Propionate production is an indication of lactate fermentation (Heimann *et al*., 2005). In addition Oyekola *et al.* (2012) performed a qualitative assessment of microbial community structure, which confirmed that the diversity of sulphate reducers decreased with increasing dilution rate.

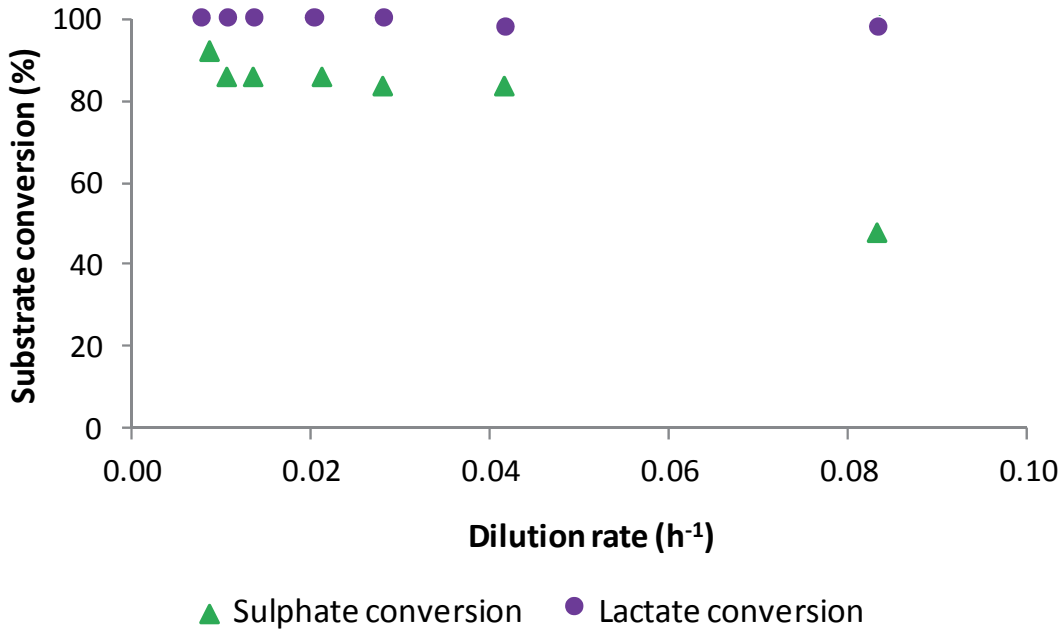

Figure 12: Proportion of sulphate and lactate converted as a function of dilution rate (HRT) for the CSTR receiving 1 g/ℓ SO_4^{2-}

4.3.1.2 Algal digestate-based growth medium

The dataset for the reactors fed on digestate from the anaerobic digester was limited to the five, four and three day HRTs due to a shortage of algae for digestion and limited capacity in the digester, so sufficient digestate could not be generated to sustain the reactors at low HRTs.

Due to the fact that data are only available for three steady states, the graphs for this section show time course data, rather than steady state data.

4.3.1.3 Feed sulphate concentration 2.5 g/ℓ

The data presented in Figure 13 show a period of unsteady state operation for the first 23 days, during which the residual sulphate concentration decreased from >400 mg/ℓ to <100 mg/ℓ, at which point steady state was achieved. The sulphate conversion efficiency under steady state conditions was between 96% and 97%, which was significantly higher than the corresponding reactor receiving lactate as the electron donor (54 55%).

The hydraulic retention time was decreased from five days to four days on day 34. This led to a short term increase in residual sulphate concentration, with the reactor appearing to approach the new steady state by day 53. At this point the feed supply to the reactor became partially blocked and the lower volumetric loading created the impression of very efficient performance. Once the problem was detected and rectified the residual sulphate concentration increased again and the new steady state was achieved from day 80, with the residual sulphate concentration remaining stable between 50 mg/ℓ and 60 mg/ℓ. Once again, the sulphate conversion was significantly higher than for the lactate-fed reactor.

There are two factors that contribute to the very efficient sulphate reduction. The first is the composition of the feed, which contains primarily acetate, propionate and iso-valerate. Acetate is a two-carbon organic acid and cannot be fermented further, while propionate and iso-valerate are not easily fermented. Therefore, the competition for the electron donor that occurred in the lactate-fed reactors did not take place. Secondly, a significant amount of the sulphide that would be produced from the efficient sulphate reduction is not retained in solution (Figure 14). If all the sulphide had been retained in solution the concentration would have exceeded the levels that have been reported as inhibitory to sulphate reducing species. The sulphide is probably partially oxidised to elemental sulphur, which was observed on the surface of the reactor and as a suspended colloid in the effluent, polysulphides or thiosulphate.

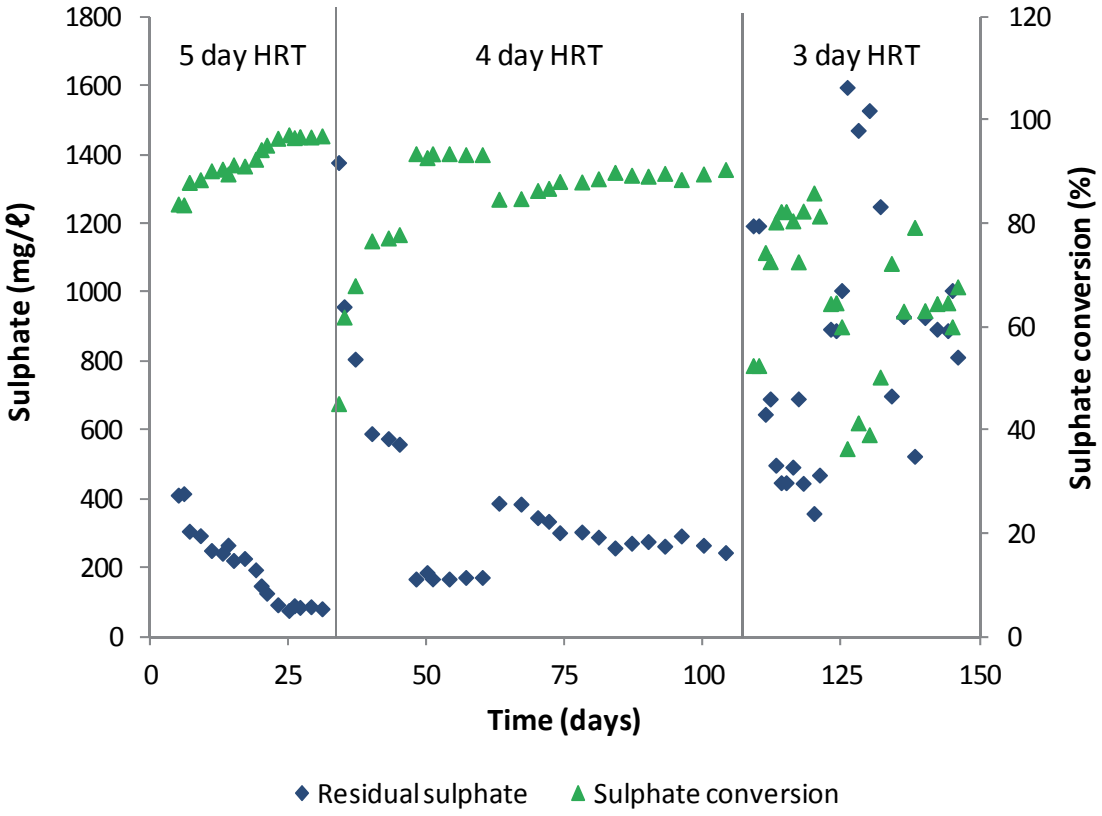

Figure 13: Residual sulphate and sulphate conversion data for the CSTR receiving feed with a sulphate concentration of 2.5 g/ℓ and digestate as the electron donor

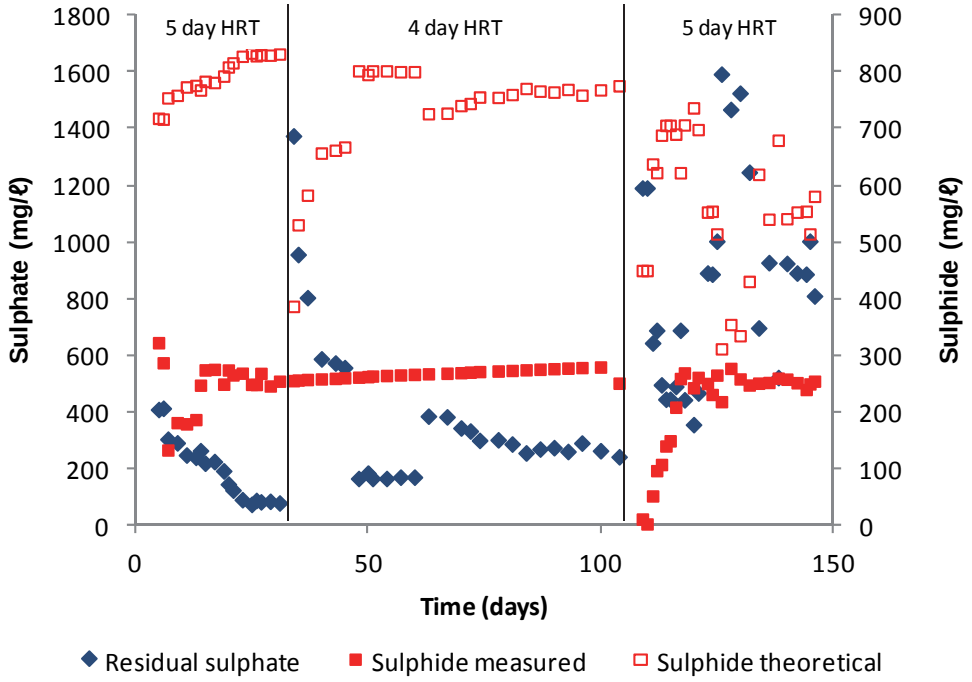

Figure 14: Residual sulphate, measured and theoretical sulphide values for the CSTR receiving feed with a sulphate concentration of 2.5 g/ℓ and digestate as the electron donor

During December 2013, for a two week period, the reactor was poorly maintained, with only intermittent feeding. As a consequence, the feed was exhausted and air was pumped into the reactor. This resulted in

the complete elimination of aqueous sulphide (Figure 14) and slight increase in redox potential. Upon resumption of correct maintenance the sulphide concentration recovered to the previous levels, but the sulphate reduction performance became inconsistent. The performance approached steady state again from about day 140.

Analysis of the VFA profile in the effluent from the reactor showed almost complete conversion of the acetate, propionate and iso-valerate in the feed. Acetate was the only VFA detected in the effluent, but the concentration did not exceed 10 mg/ℓ. The complete conversion of the VFAs is consistent with the extent of sulphate reduction achieved, given that the COD/sulphate ratio in the feed was 0.7.

4.3.1.4 Feed sulphate concentration 5 g/ℓ

The data for the reactor receiving feed with sulphate at a concentration of 5 g/ℓ show very similar trends to the 2.5 g/ℓ case. There was an initial period of unsteady state, with steady state being achieved after 23 days at a five day HRT (Figure 15). The residual sulphate concentration at steady state was around 150 mg/ℓ, which represented a very high sulphate reduction efficiency. The residual sulphate increased briefly to over 1400 mg/ℓ following the decrease in HRT to three days, but fell rapidly thereafter. Steady state at the four day HRT was achieved shortly after the tubing has been replaced (day 82). The residual sulphate concentration at steady state was significantly higher (550-600 mg/ℓ) than at the five day HRT, resulting in a decrease in sulphate reduction efficiency to around 85%. This is still significantly higher than the 55% achieved in the lactate-fed reactor.

The 5 g/ℓ reactor was subjected to the same period of neglect as the 2.5 g/ℓ reactor. While the consistency of the residual sulphate data was negatively affected, the overall sulphate reduction efficiency remained high. The higher concentrations of sulphate and electron donor in the feed would result in a higher biomass density, which may have resulted in the greater resilience observed.

The measured sulphide is again very low, relative to the theoretical value based on the amount of sulphate reduced, which is a clear indication that some oxygen entered the reactor (Figure 16). The efficient mixing achieved by the marine impeller ensured continuous transport of sulphide to the reaction zone, so a significant portion of the generated sulphide was consumed. However, the amount of oxygen entering the system was relatively small so the reaction stoichiometry favours partial oxidation.

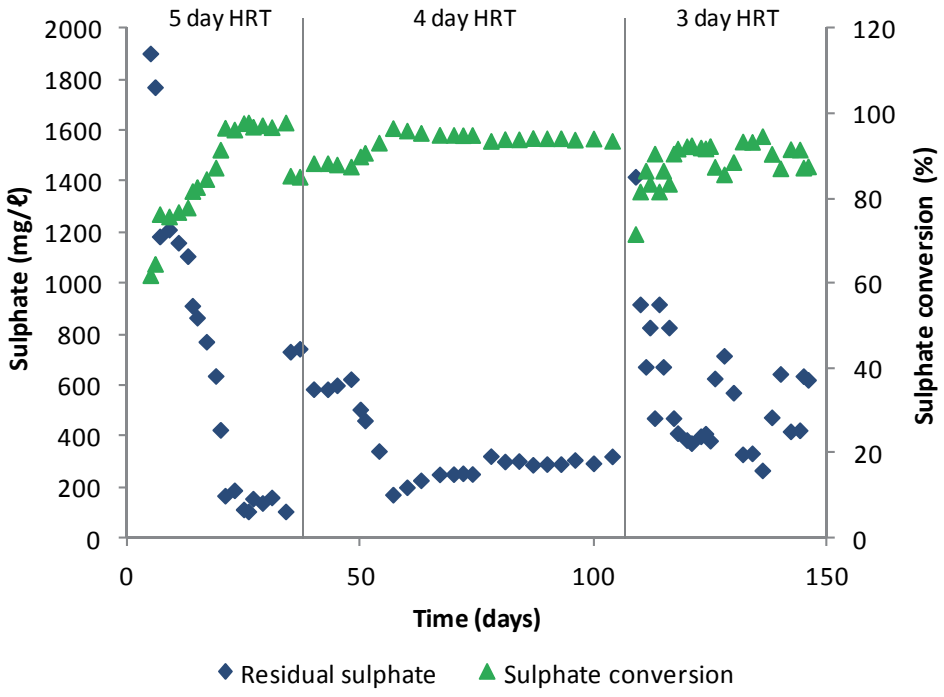

Figure 15: Residual sulphate and sulphate conversion data for the CSTR receiving feed with a sulphate concentration of 5 g/ℓ and digestate as the electron donor

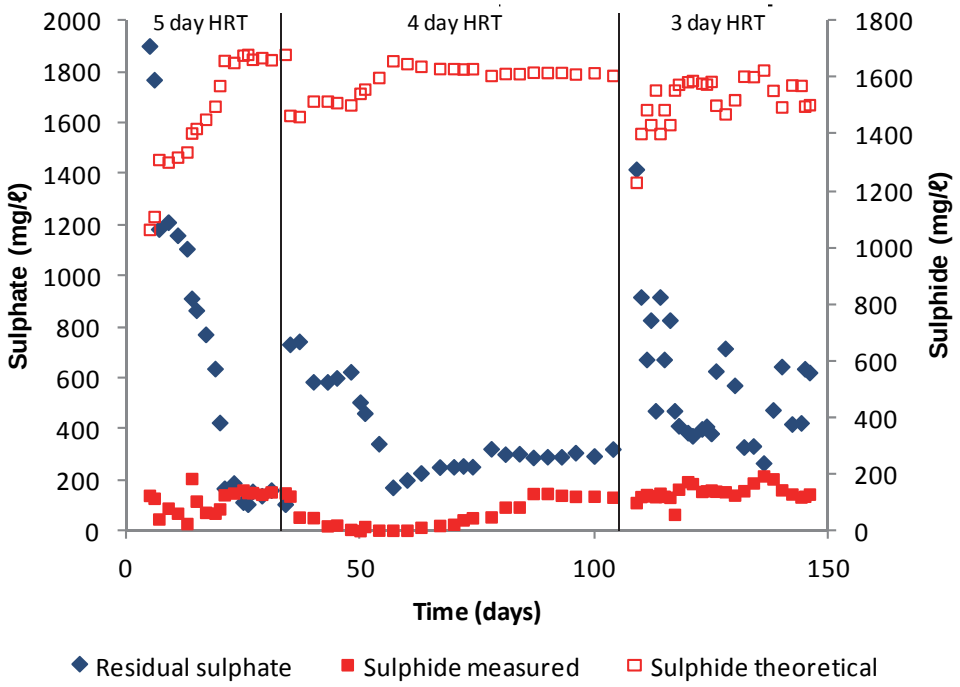

Figure 16: Residual sulphate, measured and theoretical sulphide values for the CSTR receiving feed with a sulphate concentration of 2.5 g/ℓ and digestate as the electron donor

4.3.1.5 Volumetric sulphate reduction rates (VSSRs)

The VSRR is the most relevant measure of reactor efficiency and the data for the digestate reactors are summarised in Figure 17. During steady state at the five day HRT the VSRR achieved in the 5 g/ℓ reactor (40.4 mg/ℓ.h) is double that achieved in the 2.5 g/ℓ reactor, reflecting the similar conversion efficiencies. By contrast, the VSRRs achieved in the corresponding lactate-fed reactors were 11.3 mg/ℓ.h and 24.3 mg/ℓ.h for the 2.5 g/ℓ and 5 g/ℓ reactors respectively. Following the reduction of the HRT to four days the VSRR for the 2.5 g/ℓ reactor increased to 25.5 mg/ℓ.h which is consistent with the linear increase observed in the 1 g/ℓ lactate-fed reactor. The VSRR for the 5 g/ℓ reactor also increased by a similar proportion, to 49.2 mg/ℓ.h. The data for the 2.5 g/ℓ reactor at the three day HRT is inconsistent, so a steady state value could not be derived. The performance of the 5 g/ℓ reactor was more consistent, with a mean VSRR of 62.4 mg/ℓ.h, which was substantially higher than the 27.7 mg/ℓ.h obtained in the corresponding lactate-fed reactor.

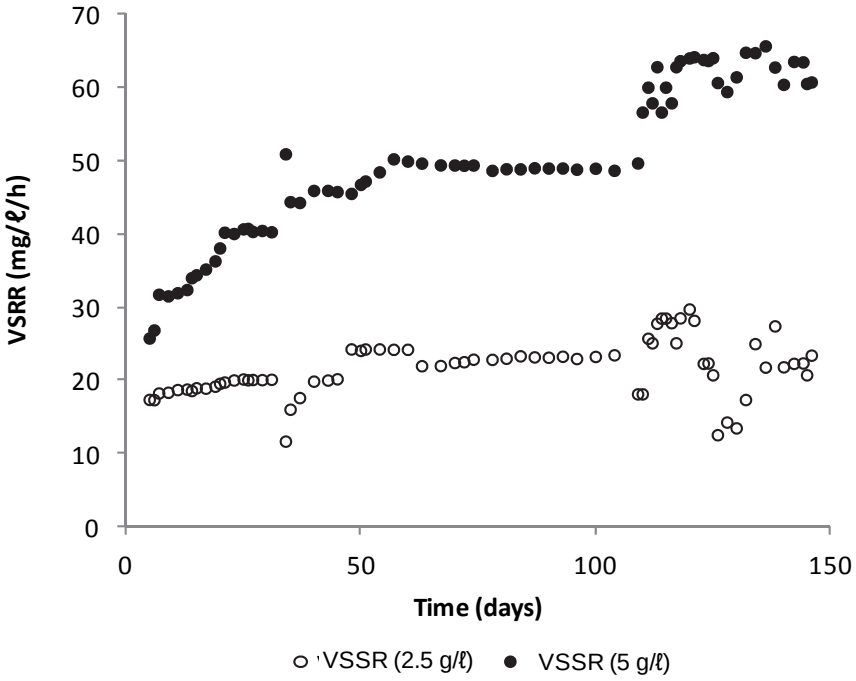

Figure 17: Volumetric sulphate reduction rates achieved in the two CSTRs at five, four and three day HRTs

4.3.1.6 Effect of hydraulic retention time on reactor performance

The data for both the lactate-fed reactor and digestate-fed reactors showed that performance was affected by a reduction in the HRT. The decrease in overall efficiency may have been due to kinetic limitations of the community, a change in community structure or a combination of both. The goal of the molecular toolkit is to provide the ability to unpack the reasons. At HRTs below 1 day there appeared to be a shift in the microbial community in the lactate-fed reactor, as the sulphate conversion decreased significantly, but complete conversion of the lactate still occurred. This phenomenon was more obvious in reactors operated at feed sulphate concentrations of 2.5 and 5 g/ℓ, where the discrepancy between lactate utilised and sulphate reduced was far more pronounced (Oyekola *et al*., 2009; 2012).

4.3.2 Sulphide oxidising systems

4.3.2.1 Linear flow channel reactor

The performance of the sulphide oxidising reactors under acetate supplemented and non-supplemented conditions was described in detail by van Hille and Mooruth (2013). In summary (Table 4), in the absence of acetate supplementation the biofilm took longer to form, was thinner and more brittle than in the reactors receiving the acetate supplement, and in some cases was incomplete. The thin, incomplete biofilm did not provide a sufficient barrier to oxygen mass transfer. As a result, sulphide conversion was high, but a significant amount of the sulphide was fully oxidised back to sulphate and sulphur recovery was low.

Acetate supplementation resulted in a very different biofilm structure. The biofilm formed more quickly, with complete coverage of the reactor surface within 24 hours. The biofilm developed through a “sticky” phase, when the organic content was high, to the brittle phase, following the deposition of more elemental sulphur. The presence of acetate in the medium led to the formation of a biofilm with greater structural integrity and substantially more sulphur could be deposited in the biofilm before it showed signs of collapse.

Table 4: Summary of operating conditions and performance of LFCRs from which samples were taken for community structure analysis. S recovery represents the proportion of the oxidised sulphide recovered as elemental sulphur in the biofilm. Adapted from van Hille and Mooruth (2013)

Run	Feed rate (ℓ/day)	HRT (days)	Acetate addition (g/ℓ.day)	HS^- loaded (mmoles)	HS^- oxidation (%)	S recovery (%)
1	6.25	4	0	580.7	96.1	59
2	6.25	4	0	498.3	93.3	52
3	12.5	2	0	572.7	82.2	92
4	12.5	2	0.99	754.2	91.0	66
5	12.5	2	2.26	617.4	84.7	78
6	25	1	1.17	1023.7	85.0	68
7	25	1	2.24	879.0	80.7	75
8	25	0.5	0.86	378.8	93.5	75
9 (1)	18.75	1.5	1.26	452.6	65.2	74
9 (2)	18.75	1.5	1.77	572.1	82.0	76

4.3.2.2 Experimental sewer system

The performance of the different types of concrete used in the Virginia experimental sewer site falls outside the scope of the current study, a brief summary is included to provide context for the microbial community analysis. Detailed historical data are available (Alexander *et al*., 2008), while current data will be reported in the PhD of Moses Kiliswa.

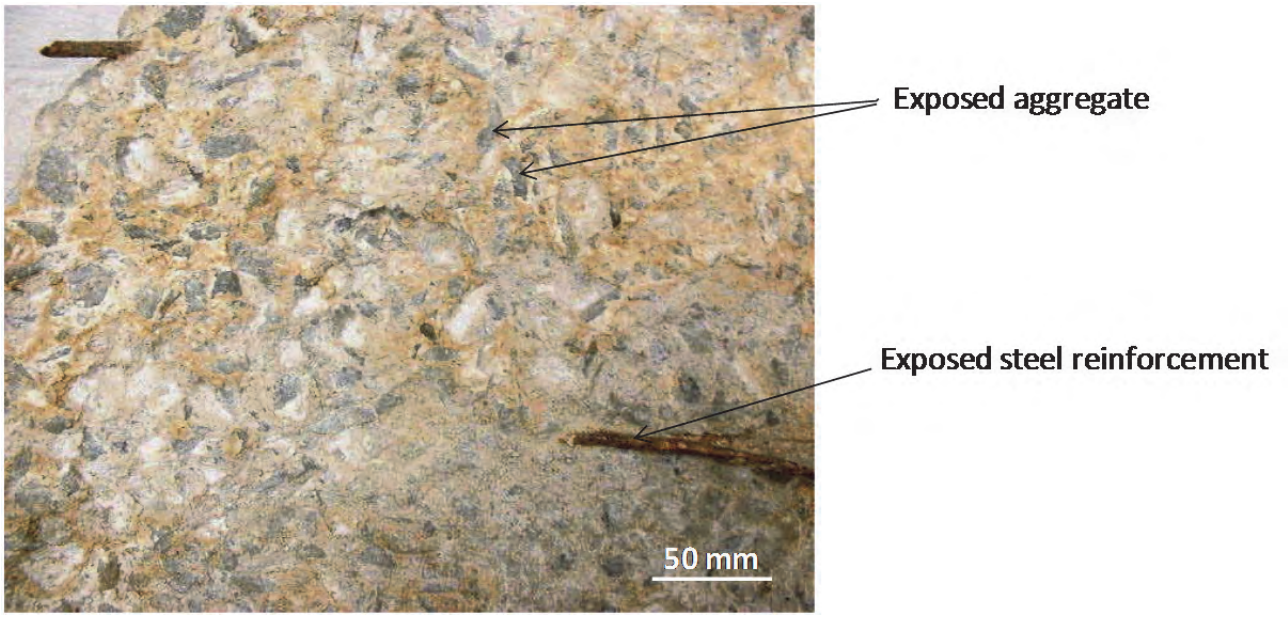

Figure 18: Photograph of severely corroded concrete pipe showing exposure of aggregate material and reinforcing rods

Data from the experimental sewer site revealed that samples containing the standard CEM I cement showed significant acid attack within five months of instillation, irrespective of the nature of aggregate used (dolomitic or siliceous). The pH within the cement matrix had decreased to between pH 1.2 and pH 2.4, indicating sufficient acid formation to overcome the inherent alkalinity of the cement matrix. After 17 months, mass loss was between 4 and 6.3%, with aggregate and the steel reinforcing rods exposed (Figure 18).

4.3.3 Sulphate reducing community

A total of 48 unique ribotypes were obtained during the analysis of the microbial community from the different sulphate reducing systems. A simplified phylogenetic tree showing the evolutionary relationship of the

different species is shown in Figure 19. Due to the large number of sequences, reference sequences are not included in the tree. Instead, the individual clusters of sequences have been annotated to reflect the closest known relatives. Of the 48 unique sequences 17 clustered with putative sulphate reducers, of which the majority were identified as members of the genus *Desulfomicrobium*. Of the remaining SRB, three members of the *Desulfovibrio,* two *Desulfocurvus* and a single representative of the *Desulfuromonas* were identified.

The greatest diversity was encountered among the *Bacteriodetes*, followed by the *Acholeplasma* and *Mesotoga*.

4.3.4 Sulphide oxidising communities

4.3.4.1 Stock reactors

The microbial species identified in the stock reactors consist of a mixture of sulphide oxidising organisms and anaerobic, sulphate reducers. The latter would have dominated the DPBR and been carried over with the LFCR feed. Previous work on the microbial ecology of floating sulphur biofilms, conducted by Bowker (2002) and Molwantwa (2007) found similar broad groups of organisms. The most significant difference between this and previous work is the predominance of autotrophic sulphide oxidisers in the current system. The three dominant sulphide oxidisers, *Chlorobium limicola, Thiomonas intermedia* and *Chromatium okenii* are all photosynthetic sulphur bacteria. While these are efficient sulphide oxidisers, *Chlorobium* has been recorded to oxidise sulphide at up to 286 mg/ℓ/h, the majority of the sulphur is retained intracellularly.

The dominant organisms from the three stock reactors and LFCRs were identified as follows (Table 5).

Table 5: Dominant microorganisms identified in the sulphide oxidising stock reactors

Species	Primer set	16S gene target	% identity
Stock reactor 1			
Thiomonas intermedia	Standard PCR 16S bact	yes	99
Chlorobium limicola	Standard PCR 16S bact	yes	99
Rhodotorula mucilaginosa	Standard PCR 18S	yes	99
Stock reactor 2			
Chromatium okenii	Standard PCR 16S bact	yes	97
Cupriavidus metallidurans	Standard PCR 16S bact	yes	99
Rhodobacter sp.	qPCR 16S bact	yes	97
Stock reactor 3			
Desulfovibrio sulfodismutans	Standard PCR 16S bact	yes	94
Desulfovibrio magneticus	Standard PCR 18S	No[1]	86
Spirochaeta sp.	Standard PCR 16S bact	yes	99
Rhodotorula mucilaginosa	Standard PCR 18S	yes	100

[1]sequence similar to elongation factor functional gene

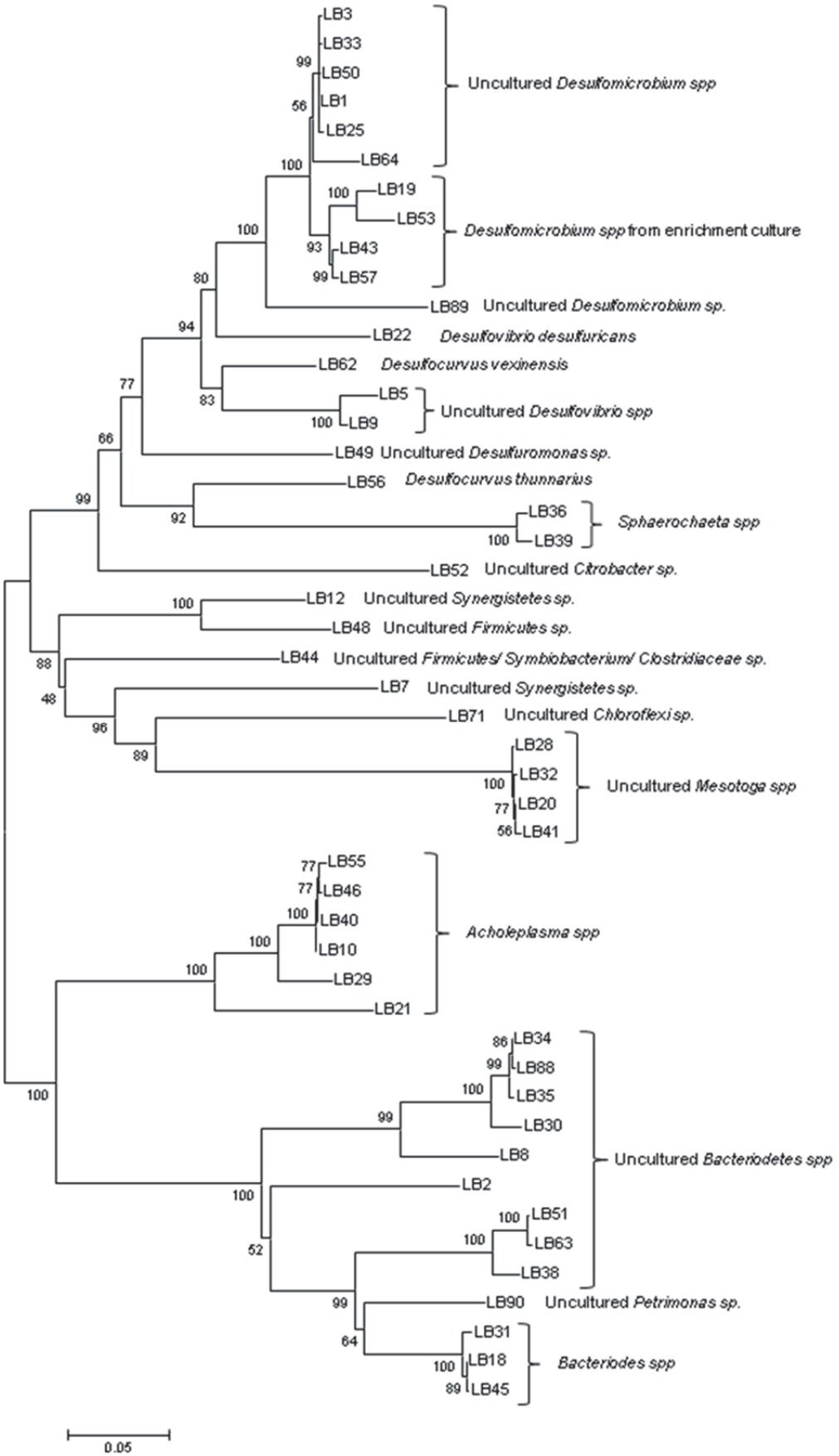

Figure 19: Unrooted 16S rRNA gene phylogenetic tree of SRB community 16S rRNA library clones, generated with universal bacterial primers. The tree was based on the sequence alignment of a common length portion (1420 nucleotides) and was obtained using the neighbour-joining method. Bootstrap values are based upon 1000 re-sampled data sets and only values of greater than 40% are indicated.

4.3.4.2 Organic carbon deficient reactors

The operation of the LFCR in the absence of supplementation with organic carbon resulted in an incomplete, thin and brittle biofilm (Figure 20). Under these circumstances sulphide oxidation was complete, but a significant amount of the sulphide was fully oxidised back to sulphate, rather than the desired sulphur product.

The microbial diversity in the system was relatively low, with only 9 unique ribotypes detected (Figure 21). Comparison of the sequences obtained to those in the NCBI database showed the majority of the species were autotrophic sulphur oxidisers, with a smaller number of sulphate reducers and heterotrophic bacteria. The *Chromatium* and *Chlorobium* species, as well as *Tiomonas intermedia*, that were detected in the stock reactors were found in the LFCR. The *Desulfovibrio* species and the rumen bacterium were not unexpected as the feed to the LFCR was derived for the packed column reactors and these species have previously been detected in DPBR reactors.

The heterotrophic species, *Pseudomonas* and *Cupriavidus*, are typically associated with anaerobic and wastewater treatment systems.

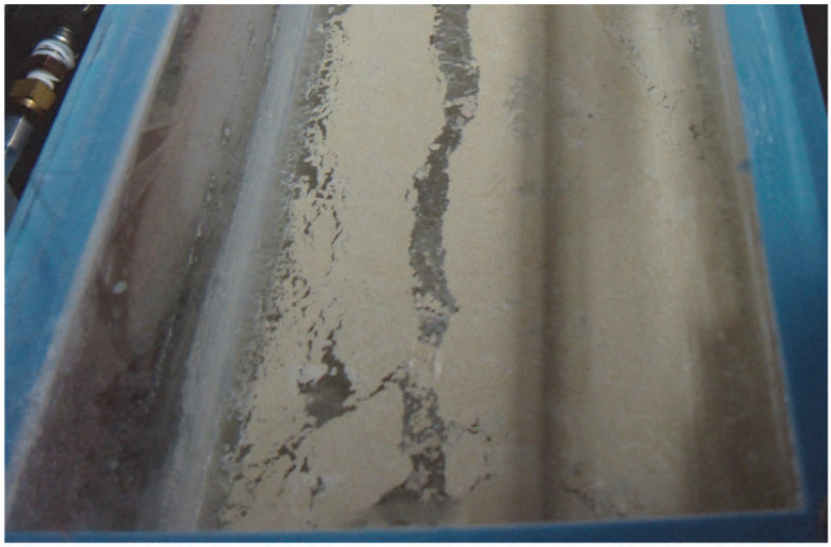

Figure 20: Photograph showing the thin, brittle structure of the biofilm formed in the absence of acetate supplementation

4.3.4.3 Acetate supplemented reactors

The clone library generated for the LFCR system after acetate supplementation showed a much greater microbial diversity, with several heterotrophic organisms identified (Figure 22). A total of 24 unique clones were identified. However, not all clones could be conclusively identified to the species level (>97% similarity to specific species in the NCBI database). *Geobacter lovelyi, Geobacter thiogenes* and *Desulfovibrio sp.* were identified again. Various sulphur-oxidising bacteria were identified, such as *Thiobacillus sp.* and/or *Halothiobacillus sp*. *Thiobacillus sp.* are colourless sulphur bacteria which are able to derive energy chemolithotrophically from reduced sulphur compounds. An electron donor (reduced sulphur compound e.g. H_2S) and electron acceptor (i.e. oxygen) is necessary for proliferation. Therefore, the FSB would be the ideal environment for such an organism. *Halothiobacillus sp.* has been identified in a hypersaline Siberian soda lake, as well as in a microbial mat within an anaerobic digester employing biological desulphurisation (Banciu *et al*., 2008; Kobayashi *et al.,* 2012).

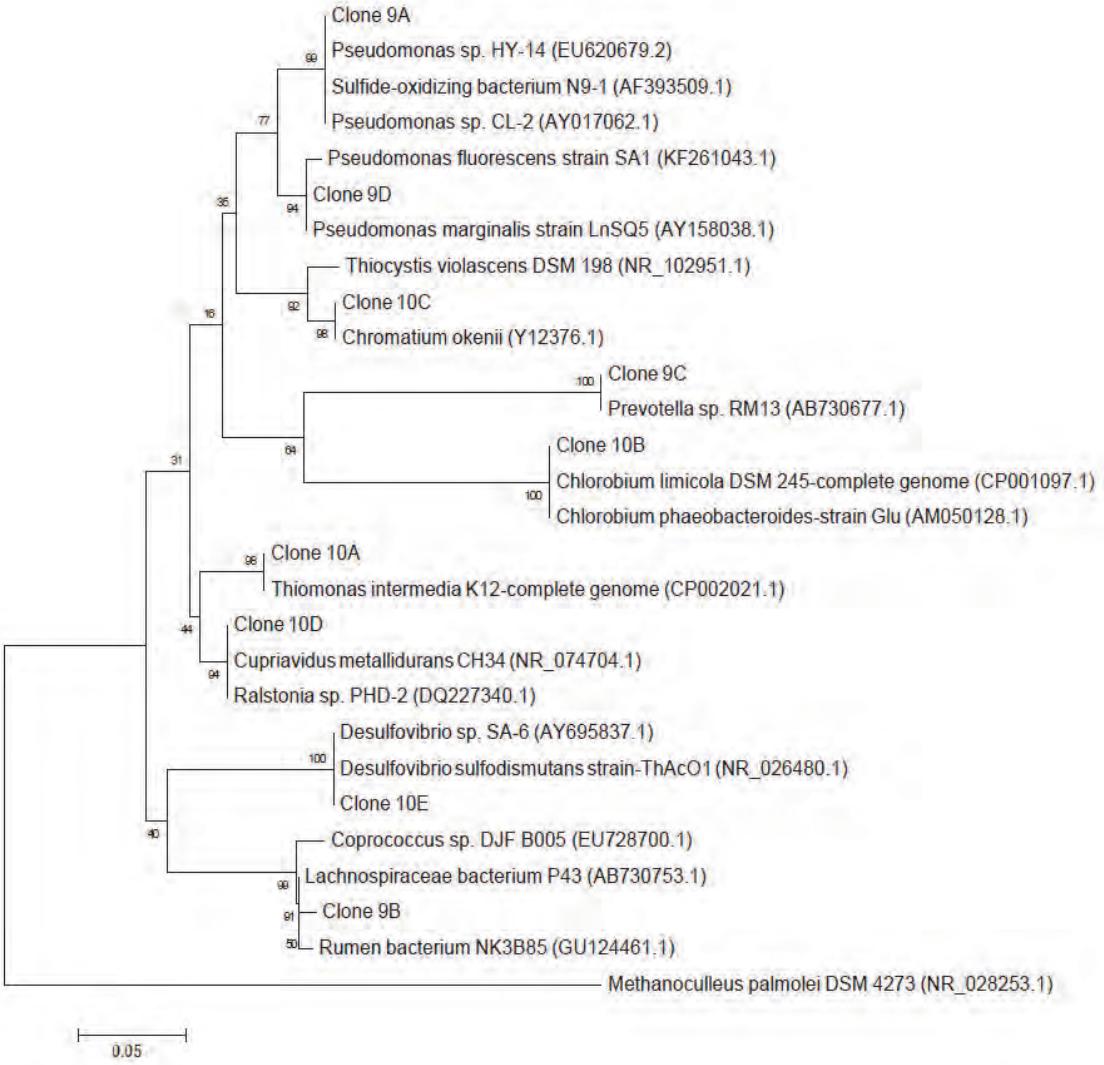

Figure 21: Unrooted 16S rRNA gene phylogenetic tree of non-supplemented LFCR community 16S rRNA library clones, generated with universal bacterial primers. The tree was based on the sequence alignment of a common length portion (1420 nucleotides) and was obtained using the neighbour-joining method. Bootstrap values are based upon 1000 re-sampled data sets and only values of greater than 40% are indicated

A larger number of the unique bacterial clones had a high similarity (>97%) to heterotrophic organisms that were identified, such as *Haematobacter sp.*, *Herbaspirillum huttiense subsp. putei*, *Spirochaete sp.*, *Paludibacter propionicigenes* (Ueki *et al*., 2006) and *Cloacibacillus sp. Herbaspirillum huttiense* is able to utilise acetic acid as a substrate (Dobritsa *et al*., 2010). The presence of a *spirochaete* species within the microbial population was also visually confirmed by SEM. Dubinina *et al.* (2011) identified a new novel spirocheata strain (*Spirochaeta perfilievii sp*. nov.) capable of oxidising sulphide to elemental sulphur under micro-oxic conditions. The strain was an aerotolerant anaerobe with fermentative metabolism. The photoautotrophic organism *Chlorobium limicola* was only identified in one of the LFCRs. Therefore, the microbial community structure was drastically altered by the supplementation of acetate to the LFCR, as no photoautotrophic organisms were detected in the LFCR under optimal conditions. Furthermore, there was a more and diverse microbial community structure, with a large number of heterotrophic and chemolithotrophic organisms.

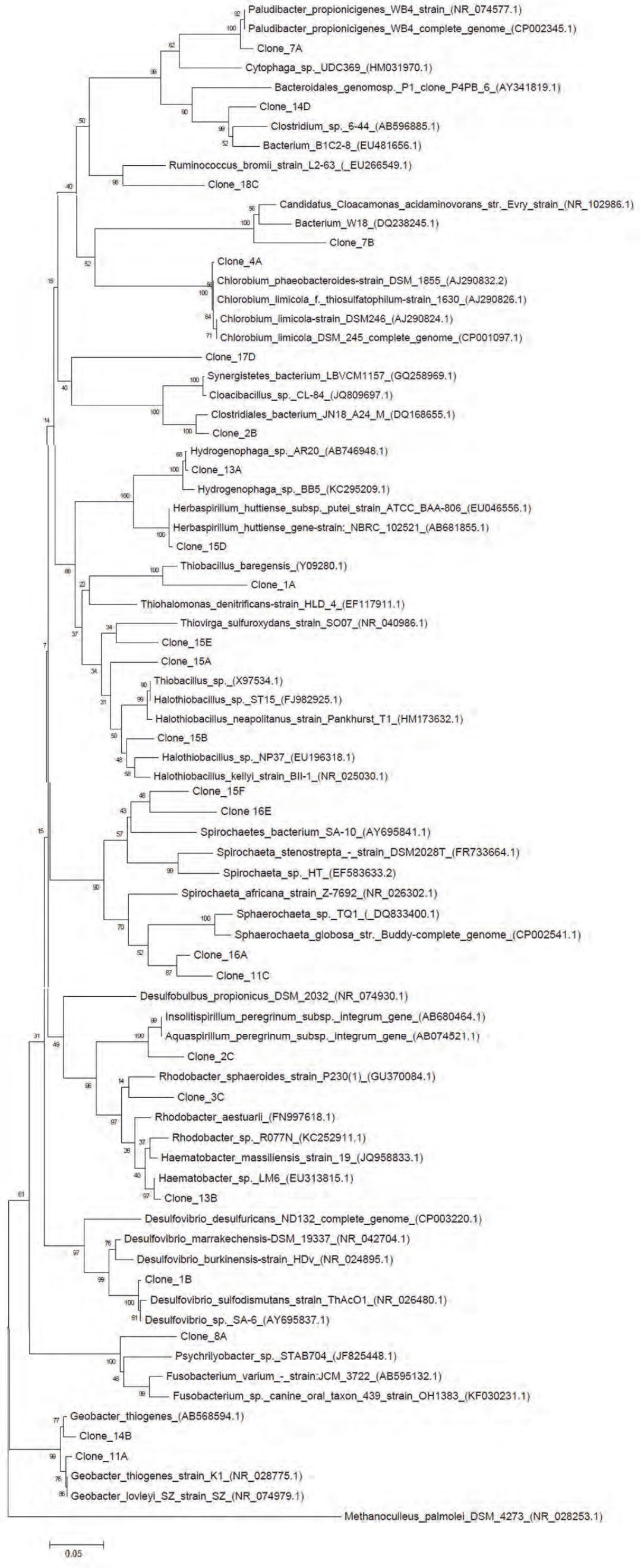

Figure 22: Unrooted 16S rRNA gene phylogenetic tree of non-supplemented LFCR community 16S rRNA library clones, generated with universal bacterial primers. The tree was based on the sequence alignment of a common length portion (1420 nucleotides) and was obtained using the neighbour-joining method. Bootstrap values are based upon 1,000 re-sampled data sets and only values of greater than 40% are indicated.

4.3.4.4 Experimental sewer concrete samples

The extraction of genomic DNA was successful for all 12 samples processed, with DNA concentrations ranging from 500 to 2,800 ng of DNA per g of sample processed (Figure 23). The quality of the extracted DNA was high.

The mini clone libraries were successfully constructed for all samples and between seven and ten unique clones, based on the ARISA analysis, were sequenced for each. The results of the sequencing are summarised in Table 6.

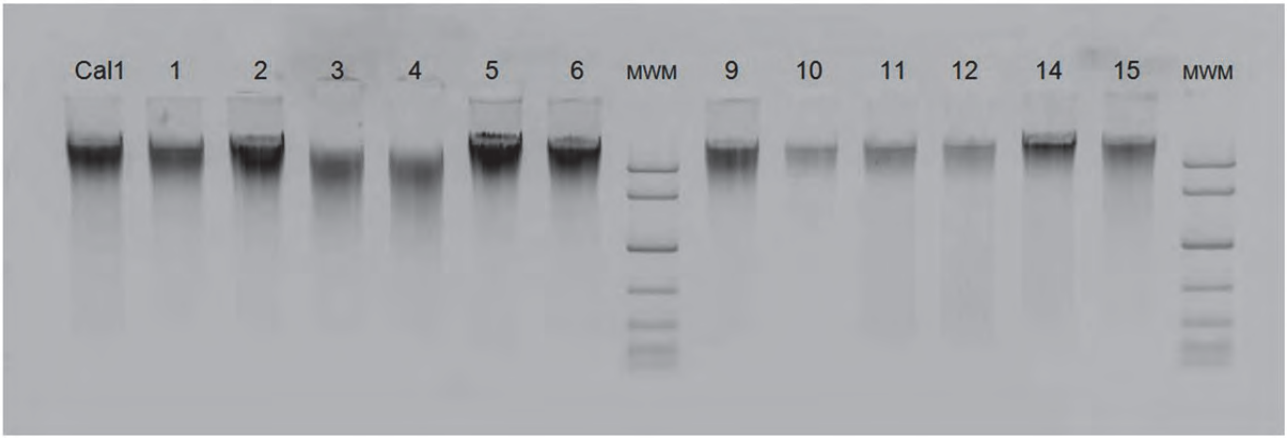

Figure 23: Image of agarose gel showing genomic DNA extracted from experimental sewer samples (1-15). Cal1 refers to a positive control (standard liquid culture of E. coli) and MWM refers to the molecular weight marker

Table 6: Summary of sequence data analysis for the experimental sewer concrete samples

Sample	Unique clones sequenced	Genera identified	Reduced sulphur oxidisers
PC/DOL 16 (1)	10	*Frateuria, Nevskia, Aminobacter, Dyella*	0
PC/DOL 18 (2)	10	*Frateuria, Nevskia, Citrobacter, Dyella*	0
PC/DOL 23 (3)	8	*Nevskia, Paenibacillus, Mesorhizobium*	0
PC/SL/DOL (4)	10	*Frateuria, Nevskia, Paenibacillus, Dyella, Halothiobacillus*	4
PC/FA/DOL (5)	9	*Sphingomonas, Citrobacter, Acidiphilium, Acidithiobacillus*	2
PC/SF/DOL (6)	9	*Mesorhizobium, Erythrobacter, Paenibacillus, Halothiobacillus, Thermithiobacillus*	4
CAC/SIL 23 (9)	8	*Novosphingobium, Burkholderia, Frateuria, Halothiobacillus*	1
CAC/DOL 16 (10)	9	*Dyella, Frateuria, Nevskia, Acidiphilium, Acidithiobacillus*	4
CAC/DOL 18 (11)	10	*Alicyclobacillus, Acidiphilium, Acidithiobacillus*	5
CAC/DOL 23 (12)	8	*Alicyclobacillus, Acidithiobacillus*	5
CAC/FA/SF/DOL(14)	7	*Alicyclobacillus, Sulfobacillus, Acidithiobacillus*	5
CAC/ALAG^{TM} (15)	10	*Acidocella, Acidiphilium, Thiomonas, Acidithiobacillus*	9

The results are interesting in that a clear progression, with respect to community structure was observed. Sample 1 (PC/DOL 16) was furthest upstream, while sample 15 was furthest downstream. No organisms capable of oxidising reduced sulphur species were detected in the first three samples and the most of those identified have been reported to have pH optima in the neutral to slightly acidic range, with optimum temperatures between 30°C and 40°C, which is consistent with the prevailing conditions. *Frateuria* sp. was originally classified as *Acetobacter aurantius*, but was reclassified in 1980 (Swings *et al*., 1980). It is capable of growing on glucose and a range of other carbon sources, generating acetate as a metabolic by-product. While optimum growth is observed at neutral pH it is able to grow under acidic conditions (pH 3.6). Members

of the genus *Dyella* have been isolated from various soil environments. They grow optimally between pH 4.5 and pH 8, across a wide range of temperatures (4-42°C), although optimum growth typically occurs between 25°C and 35°C (Jung *et al*., 2009). While most members of the genus are strictly heterotrophic a species (*Dyella thiooxydans*) capable of oxidising a range of reduced sulphur species (excluding sulphide and thiocyanate) has recently been classified (Amandham *et al.,* 2011). The third genus common to the first four samples was *Nevskia*, which form microcolonies, typically at an air-water interface or in damp soil (Stürmeyer *et al*., 1998). Members of the genus grow optimally at 25-28°C at pH 6-7, although growth was observed between pH 4 and pH 7 (Weon *et al*., 2008). Members of the genus *Paenibacillus* have been isolated from a range of environments, with most showing optimal growth at pH 7 and temperatures around 37°C (Osman *et al*., 2006), which are consistent with the conditions in the experimental sewer.

Organisms capable of reduced sulphur oxidation were identified from sample 4 onwards. Initially, the majority of positively identified clones belonged to the genus *Halothiobacillus*. Members of this genera are able to oxidise reduced sulphur compounds, but have an optimum pH range of pH 6.5 to pH 8 and temperature range of 30-40°C (Kelly and Wood, 2000). Therefore, while these organisms are capable of oxidising sulphur to produce acid, they are inhibited by the product of their metabolism. In samples where *Halothiobacillus* and *Thermithiobacillus* are present, the remaining species identified also had pH optima in the neutral range.

A second, significant shift in community structure is observed between sample 10 and sample 11, with the disappearance of all species which prefer neutral or mildly acidic habitats. These are replaced by acidophilic species, some of which can oxidise reduced sulphur species, such as *Acidithiobacillus, Thiomonas* and *Sulfobacillus*, while others, such as *Alicyclobacillus* are strictly heterotrophic. Members of the genus *Acidiphilium* are heterotrophic, although one species *Acidiphilium acidophilum* has been shown to be capable of sulphur oxidation (Rohwerder and Sand 2003). The species which were most common in the first four samples are effectively absent from the final four. This suggests that the establishment of a community capable of oxidising reduced sulphur species resulted in a significant change in environmental conditions, particularly pH, and was responsible for a complete shift in the microbial community to one dominated by acidophiles. While the importance of microbial activity in the acid-mediated corrosion of concrete is well recognised and genera such as *Halothiobacillus* and *Acidithiobacillus* have been identified as important (Monteny *et* al., 2000; O'Connell *et al*., 2010), this study represents an important step toward understanding the complex microbial community and the evolution of community structure in response to increasing acidity.

The experimental sewer study is still ongoing and at this stage it is not possible to conclude whether the differences in community structure observed were influenced by the position of the test sections, their composition, or a combination of both.

4.4 CONCLUSIONS

Sulphate reducing and sulphide oxidising systems were maintained in the laboratory to generate kinetic data and investigate the effect of operating conditions on the efficiency of sulphate reduction, sulphide oxidation and sulphur recovery. Samples from these reactor systems were used to generate clone libraries for the sulphate reducing community and the sulphide oxidising communities in the presence and absence of acetate supplementation. The libraries were generated to be as comprehensive as possible, so the available sequence data could be used to design FISH probes and qPCR primer sets.

A total of 48 unique ribotypes were isolated from the various sulphate reducing reactors, of which 17 corresponded to putative sulphate reducers. The majority of these were identified as members of the genus *Desulfomicrobium.* There was greater diversity among the non-sulphate reducing bacteria. However, the clone library approach does not address the relative abundance of the different species so it is not clear whether the reactors were numerically dominated by sulphate reducers or not.

In the sulphide oxidising systems the community structure was very different when soluble organic carbon was limiting, relative to acetate supplemented systems, both in terms of diversity and the type of sulphide oxidisers present. In non-supplemented reactors, autotrophic sulphide oxidisers, such as *Chlorobium* and *Chromatium* were dominant. Acetate supplemented reactors showed greater diversity and the major sulphide oxidisers were members of the *Thiobacilli* and *Halothiobacilli.*

The second sulphide oxidising environment characterised was an experimental sewer system, investigating the effect of concrete composition on rates of corrosion. The results clearly show evidence of microbial

succession and the evolution of the microbial community as the sulphide oxidation progresses. The study represents an important step forward in understanding the complexity of the microbial community and identifying microorganisms that have not traditionally been associated with concrete corrosion. A greater understanding of microbial community structure will allow for targeted interventions, both to optimise beneficial processes and inhibit destructive ones.

CHAPTER 5: DEVELOPMENT OF MOLECULAR TOOLS

5.1 INTRODUCTION

The data presented in the previous chapter clearly showed the complexity of the microbial communities in both the sulphate reducing and sulphide oxidising systems, with the exception of the LFCR in the absence of organic carbon, which selected for a predominantly autotrophic community. The diversity in the communities was significantly greater than that for bioleaching systems, for which tools for quantitative microbial ecology have previously been developed.

The second notable observation was that in both the sulphate reducing and sulphide oxidising systems there were a greater number of species not directly involved in sulphate reduction or sulphide oxidation. The contribution of these organisms to the overall system is not known. They may have a positive or negative impact on process performance.

Finally, while the microbial communities were described in terms of diversity, no information was provided on the relative abundance of species making up the community. This chapter details progress toward the development of tools for performing quantitative microbial ecology.

5.1.1 Principle behind qPCR primer design

The quantitative real-time polymerase chain reaction (qPCR) technique has the potential to provide a quantitative assessment of community structure, allowing it to be monitored as a function of time or in response to system perturbations. Polymerase chain reaction amplifies a fragment of DNA included between two short (approximately 20 bases long) oligonucleotides, theoretically doubling the quantity of product at every cycle. To detect, in real time, the quantity of products formed the most popular approach uses an intercalating fluorescent dye that binds to double stranded DNA. Therefore, the specificity of the assay is dependent on the primer pair and consequently its optimal design is essential for reliable qPCR experiments (Nonis *et al*., 2011). In theory, qPCR primers are similar to those for conventional PCR, however they need incorporate a number of special features, so primer design is the essential step (Nolan *et al*., 2006). There are a number of internet based tools available to assist with primer design although the majority use algorithms that have been conceived for conventional PCR. Nonis and co-workers (2011) developed a ranking tool specifically for qPCR primer sets that deducts points, from a zero base, if certain desirable features were absent. These scores are calculated according to the criteria listed below (Nonis *et al*., 2011):

- Optimal primer length between 15 and 25 bases: Shorter primers are not accepted, longer primers received a score of -1 for each nucleotide exceeding 25 bases.
- G/C content around 50%: A range between 40% and 60% was considered as acceptable (between 8 and 12 G/C for a 20 nucleotides primer). A score of -1 was applied for each 1% exceeding the acceptable range (e.g. 35% or 65% G/C were given a score of -5).
- Having one G or C at 3' terminal position preceded by one A/T was considered as positive for the control of mis-priming. When this feature was not found, a -1 score was assigned. No more than 3 G or C should be present in the last five bases of 3'-end to avoid ambiguous binding. If 4 or 5 G/C were found, a score of -7 or -10 was assigned respectively.
- Long runs of a single base should be avoided (especially G or C). If runs longer than 4 were present, a score of -1 was counted for each G or C, -0.75 for each A or T.
- Primers should have a $\Delta G > -10$ kcal/mol, since primer dimers have a negative DG value. Since ΔG is a mere index of primer-dimer potential formation, they preferred to focus on removing all the potential annealing sites. So, primer sequences were scanned for potential annealing sites made of 4 or more nucleotides. This search was conducted for each primer against itself, and between the two forming a primer pair. A negative score was given in an amount equal to half the length of the annealing site. If the annealing site was at 3' terminus of one primer, the score was double the length of the annealing site. While, if it was at 3' terminus for both primers, score was three times the length of the annealing site. Secondary structures of primers were scored in the same way.

To successfully utilise this technology for a specific system, the component organisms need to be identified and their 16S (prokaryotic) or 18S (eukaryotic) ribosomal rRNA genes sequenced. Primers are designed based on the sequence data. While tools such as PRaTo are able to assist in screening primers they still need to be extensively tested to demonstrate effective amplification and ensure cross reactivity (false positives) does not occur. False positives can occur if the if complimentary sequences are present outside the rRNA gene. These products will almost certainly be a different size to the desired product and can be identified by running the amplification product on a conventional agarose gel.

5.2 MATERIALS AND METHODS

5.2.1 Design of qPCR primers

The design of the first set of qPCR primers was based on data from the PhD thesis of Oyekola (2008). During this research a relatively basic technique was used to assess the microbial community. The DNA from the mixed community was extracted and amplified using domain specific PCR primers. The amplified product was then digested with a suite of 21 restriction endonucleases. The principle behind the approach was that the different microbial species in the reactors would have different 16S rRNA gene sequences and where the sequences differed in the region of a recognition site for a particular restriction enzyme the resulting product sizes would be different. These could be visualised by running the products on an agarose gel. By using 21 different restriction enzymes it was hoped that there would be sufficient discriminatory power to produce unique banding patterns for each of the component species. Individual species were identified by generating simulated banding patterns using the DNAMAN software package. Known 16S sequences, from the NCBI database, were entered into the software and simulated restriction digests performed. The predicted pattern could then be compared to actual patterns from the agarose gels. The technique was subjective, in aligning the banding patterns, and was limited to species whose full 16S sequences had been uploaded to the database. On the basis of this analysis Oyekola identified six SRB species from the lactate-fed reactors. The species and the qPCR primers are shown in Table 7.

Table 7: Details of qPCR primer design based on the species identified during the study of Oyekola (2008). For several of the species multiple primer sets were designed

Species name	Sequence 5'-3'	T_m (°C)	G/C content (%)	ΔG (kcal/mole) Homodimer	PRaTo score
Desulfobulbus propionicus	DPP131 F: CCT GTC TTC ATG TCT GG	55.0	52.9	-5.38	-14
	DPP135 F: CCTGTCTTCATGTCTGGCATAATACG	57.3	46.2	-5.38	-17
	DPP244 R: GCA GAC CCC TCT TCA TGC AG	58.2	60	-7.02	
Desulfobacter postgatei	DPG140 F: CTTCAAGCCTGGGATAAC	50.0	50.2	-3.54	-2
	DPG246 R: GGTACGCAAACTCATCTCCAAAC	56.2	47.8	-3.65	
	DPG1077 F: GAGCACAGAGACAGGTGCTGC	60.5	61.9	-8.03	-19
	DPG1334 R: CAA TCC GGA CTG AGA TAG GC	54.9	55	-12.9	
Desulfococcus multivorans	DM142 F: TTGGATTCGGGATAACCCTTCG	56.8	50%	-6.14	-8
	DM241 R: GCTCATCCCCAAACGGTAG	55.6	57.9	-3.61	
	DM1195 F: GAAGATACTGCCCCGGTTAAC	55.4	52.4	-9.75	-21
	DM1334 R: CTCCAATCCGAACTAAGGACTGC	57.4	52.2	-4.64	
Desulfosarcina variabilis	DS250 R: ATGGGCCCGCGTACCATTAGC	62.4	61.9	-15.42	-17
	DS459 R: GGTTCTTCCCACTTGACAGAGC	57.9	54.5	-3.17	
Desulfotomaculum ruminis	DR144 F: TAGACCGGGATAACAGTTGG			-9.75	-27
	DR246 R: CACCTCCAACTAGCTAATGGGAC	57.0	52.2	-8.28	

A second set of qPCR primers was designed, based on the information from the clone library generated during the current study. The primer sequences, melting temperature and GC content are detailed in **Error! Not a valid bookmark self-reference.**.

Table 8: Details of qPCR primers designed based on 16S rRNA clone library generated in this study

Name	Target	Sequence 5'-3'	Product size (bp)	T_m (°C)	G/C content	Reference
DSM442F	*Desulfomicrobium*	GGCATTGGTCTAATAGGCCTTTGTT	190	64.7	44	This study
DSM632R	*Desulfomicrobium*	TGGGATTTCACCCCTGACTTACAA	190	65.1	45.8	This study
SRB336F	All SRB	AGACTCCTACGGGAGGCAGCAG	159	68.2	63.6	This study
SRB495R	All SRB	GCACGGAGTTAGCCGGTGCTT	159	68.2	61.9	This study
TotalF	All bacteria	TCCTACGGGAGGCAGCAGT	466	59.4	63.2	1
TotalR	All bacteria	GGACTACCAGGGTATCTAATCCTGTT	466	58.1	42.2	1

1 – Nadkarni *et al*., 2002

5.2.2 Primer validation approach

The primers were designed based on sequence information and alignments against sequences for sulphate reducing bacteria available on the National Centre for Biotechnology Information (NCBI) website. A number of iron and sulphur oxidising organisms and non-SRB anaerobes were used as negative controls. Examples of the sequence alignments are presented in Appendix B.

Primer sets were synthesised by the DNA synthesis facility of the Department of Molecular and Cell Biology at the University of Cape Town. Primers were initially tested using conventional PCR to assess whether amplification was successful and the product bands corresponded to the desired size. Genomic DNA extracted from lactate or algae-fed SRB reactors was used as template, with genomic DNA from a mixed bioleaching population and a DNA free tube as the negative controls.

Following conventional PCR, selected primer sets were tested using qPCR, first with genomic DNA from SRB reactors and a bioleaching reactor as the template and then against 24 clones from the 16S rRNA library, representing at least one member of each clade based on the phylogeny (Figure 19). A melt step was included after the amplification cycles to confirm whether amplification was specific, or not.

5.2.3 Probes for fluorescence *in situ* hybridisation (FISH)

The FISH probes used in this study are summarised in Table 9. The probes were selected based on the information from the clone library from the sulphate reducing systems.

Table 9: Details of the FISH probes used in this study

Probe	Target	Target site	Formamide concentration (%)	Sequence (5'-3')
EUB338	Most bacteria	338-355	0-50	GCTGCCTCCCGTAGGAGT
SRB129	*Desulfobacter*	129-146	10	CAGGCTTGAAGGCAGATT
DELTA495a	Most Deltaproteobacteria and Gemmatimonadetes		35	AGTTAGCCGGTGCTTCCT
DSM213	*Desulfomicrobium*		15	CATCCTCGGACGAATGCA

5.3 RESULTS AND DISCUSSION

5.3.1 Sulphate reducing bacteria group specific primers

A set of primers (Uni 974 F and Uni 1241 R) were designed based on the aligned sequences of a large number of SRB available in the NCBI database. Despite excellent alignment of the forward primer and relatively good alignment of the reverse primer across the range of SRB, as well as poor homology to the non-SRB the primer set was not specific. Good amplification was achieved when genomic DNA from the SRB reactors was used, but positive amplification was achieved using the bioleach sample, where no SRB could survive (Figure 24). The amplification products, including that achieved using bioleaching culture DNA, were the correct size. This implies that the primers were binding in the correct position, but that the stringency was not sufficiently high.

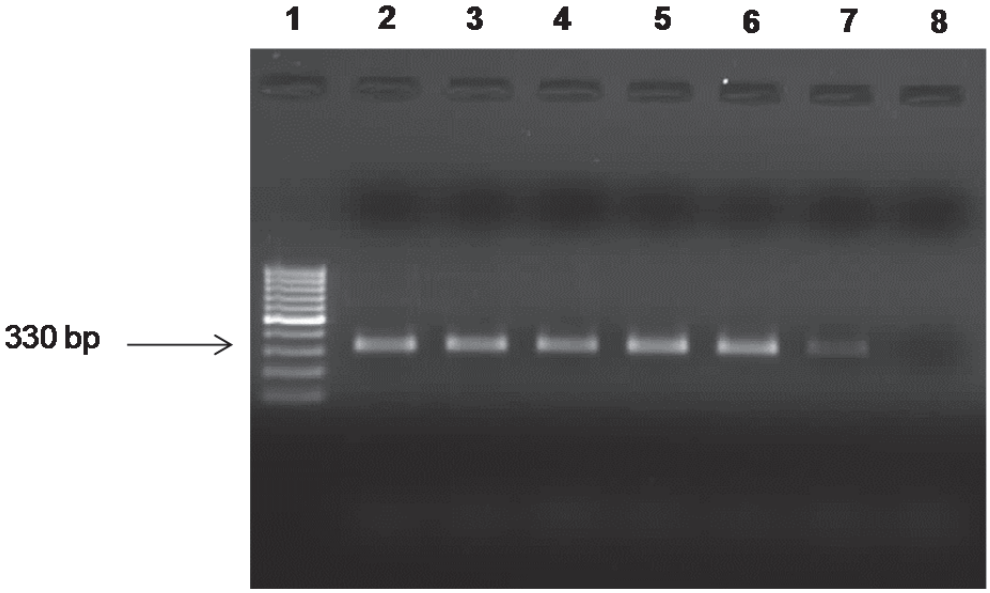

Figure 24: Amplification, by conventional PCR, of genomic DNA extracted from a lactate fed SRB reactor using the Uni974 F and Uni1241 qPCR primer set. Lane 1: Fermentas GeneRuler 100 bp; lanes 2-4: replicate samples from the lactate fed SRB reactor; lanes 5-6: duplicate samples from the algae fed SRB reactor; lane 7: non SRB control; lane 8: no DNA control.

The melt analysis following qPCR indicated that the amplification product obtained using the bioleach sample DNA had a distinctly different GC content, despite the amplicon being a similar size (Figure 25). The melt temperature was approximately 3.5°C higher, which relates to a significantly higher GC content.

The second group specific primer set (SRB336F and SRB495R), designed by aligning the sequences of the SRB identified in the clone library, yielded far better results. Amplification was specific to SRB and no non-specific amplification was observed, using both the bioleaching sample and a sample of methane producing archaea as negative controls. The primer set was used to quantify the proportions of sulphate reducing and non-sulphate reducing bacteria as a function of HRT in both the lactate and digestate-fed reactors.

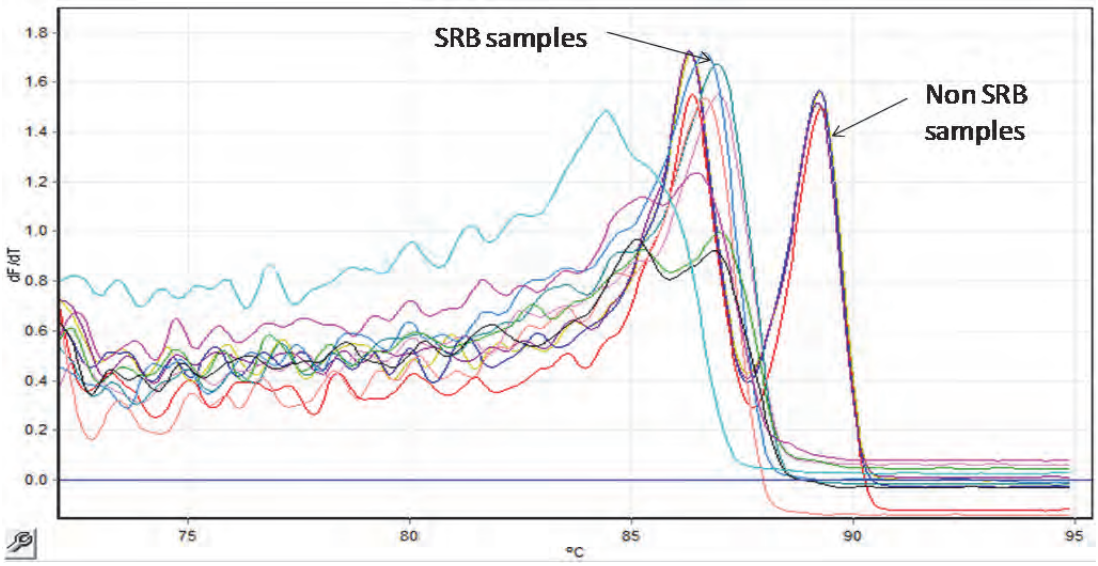

Figure 25: Melt curve analysis of SRB samples obtained from sulphate reducing environment and non SRB (bioleaching) samples after qPCR using the Uni974 F and Uni1241 R primer set. A total of 10 ng of genomic DNA was used as template for each reaction.

5.3.2 Sulphate reducing bacteria species specific primers

The species specific primer sets that were designed, based on the information from Oyekola's work, did not yield encouraging results. While amplification was achieved using the *Desulfobulbus propionicus* primer set when DNA from the SRB culture was used it was not specific; two bands were visible and neither were the correct size (Figure 26). Amplification of the bioleaching culture DNA was also visible, but again the product size was too large. This suggested that the primers annealed to regions outside the 16S rRNA gene.

The lack of amplification in the desired region could be accounted for by a three dimensional conformation in that region of DNA that makes primer annealing difficult or impossible, but it is more likely that the restriction digest technique used by Oyekola (2008) did not provide an accurate assessment of the microbial community. The clone library generated during the current project did not identify any species with a 16S sequence similar to *Desulfobulbus*, so the most obvious reason for the lack of amplification was the absence of the species from the mixed culture.

The *Desulfococcus multivorans* species specific primer set gave far more encouraging results. Positive amplification was achieved using template DNA from both the lactate fed and algae fed SRB reactors and the amplicon was the correct size (Figure 27). By contrast, the bioleaching sample DNA did not yield a discrete band.

The qPCR analysis showed the correct amplification profile, although amplification only started after about 20 cycles, which is indicative of a low concentration of the target DNA in the template mixture. *Desulfococcus multivorans* was not detected in the clone library generated during this study. However, species present in low numbers may be missed during generation of the library, despite over 150 clones having been selected for analysis.

The melt analysis confirmed the specificity of the primers, with only a single peak visible in the SRB culture samples and no significant peak visible in the non-SRB control samples (Figure 28).

Results obtained from qPCR analysis using the DSM442F and DSM632R primer set showed that they were able to selectively amplify the *Desulfomicrobium* 16S rRNA containing clones identified from the lactate reactor without non-specific amplification, with the exception of the *Citrobacter* sp clone, LB52 (Figure 29). The figure shows positive amplification for the *Desulfomicrobium* clones LB1 and LB64. The reactions containing the remainder of the clones started to increase in fluorescence at a similar cycle number as the no template control, indicating possible primer dimer formation. The amplification of LB52 is not unexpected as the primer binding sites are present within the 16S rRNA sequence of the clone. The identity of this clone

will again be confirmed by sequencing, to rule out mistaken annotation. LB52 is phylogenetically related to the SRB containing clade, although it appears on a separate branch (Figure 19).

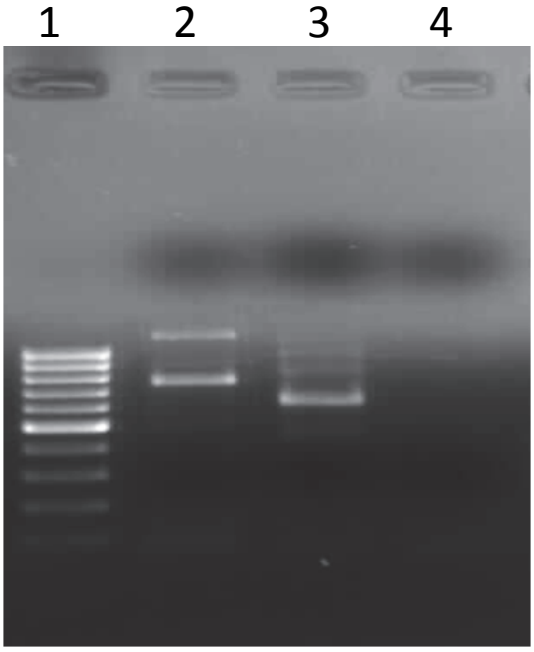

Figure 26: Genomic DNA amplification obtained using the Desulfobulbus propionicus species-specific forward primer (DPP478) and the Uni831 R reverse primer. Lane 1: Fermentas GeneRuler 100 bp; lane 2: DNA sample from the lactate fed SRB bioreactor; lane 3: non SRB control; lane 4: no DNA control.

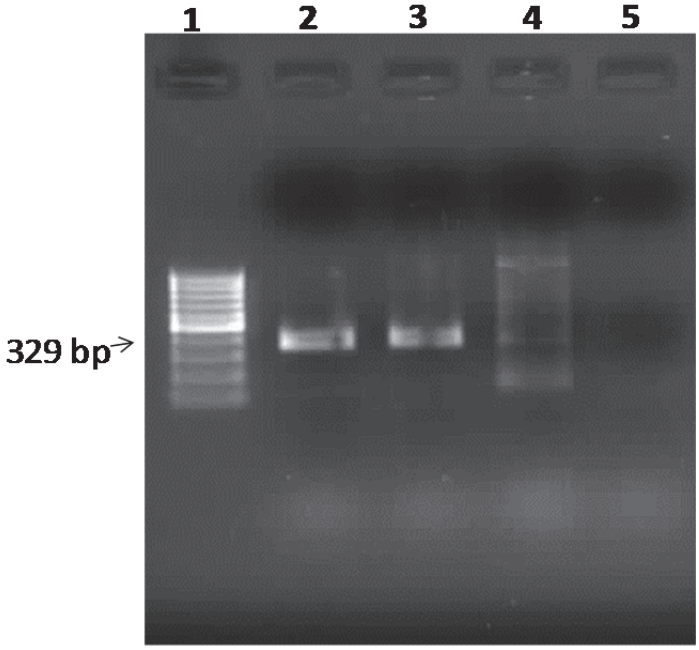

Figure 27: Amplification, by conventional PCR, of genomic DNA (10 ng) using the Desulfococcus multivorans species specific forward primer (DM502) and the Uni831 R reverse primer. Lane 1: Fermentas GeneRuler 100 bp; lane 2: DNA from the lactate fed SRB bioreactor; lane 3: DNA from the algae fed SRB bioreactor; lane 4: non SRB (bioleaching DNA) control; lane 5: no DNA control.

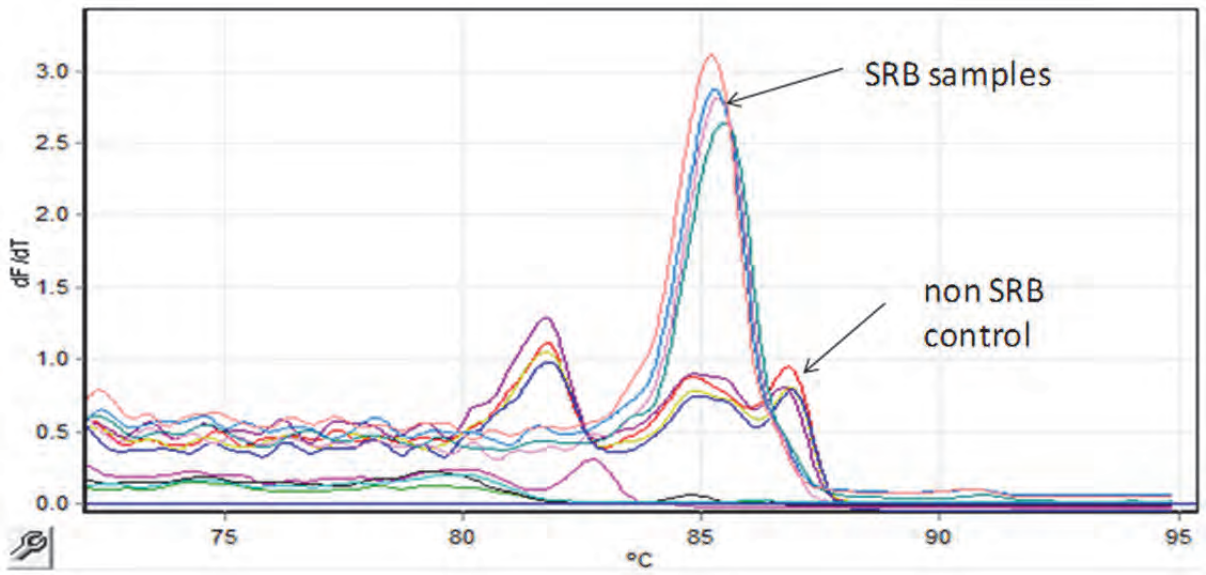

Figure 28: Melt curve analysis of SRB samples obtained from sulphate reducing environment and non SRB (bioleaching) samples after qPCR using the DM502 and Uni831 R primer set. A total of 10 ng of genomic DNA was used as template for each reaction.

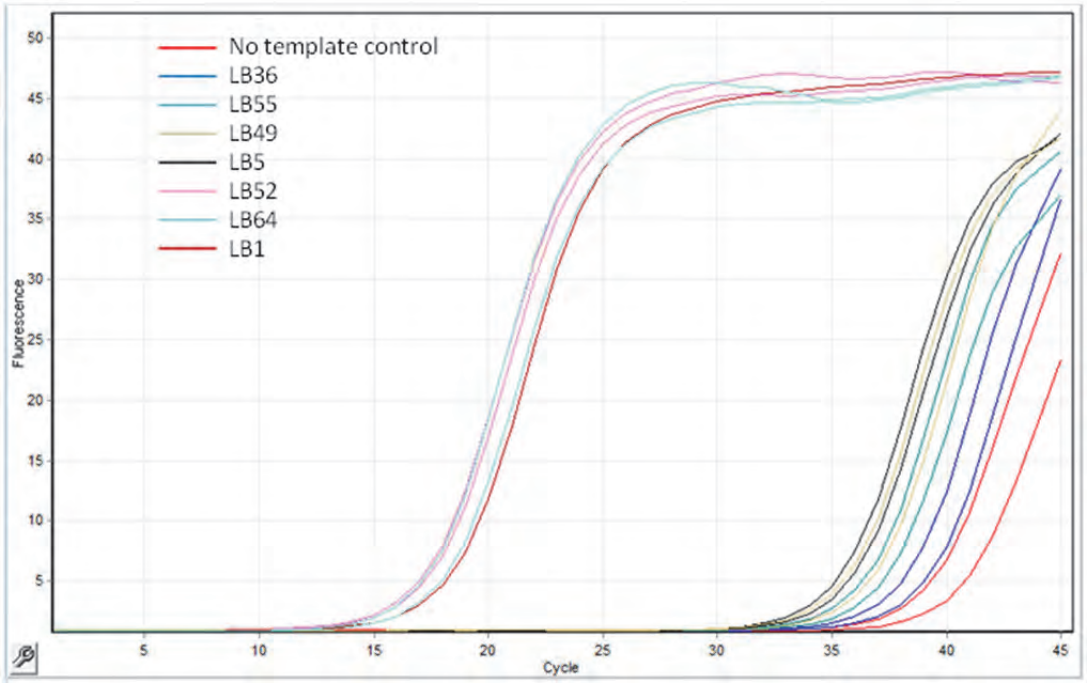

Figure 29: Graphical representation of the qRT-PCR results obtained using the DSM442F and DSM632R primer set

5.4 FISH DATA FROM REACTORS

The initial FISH studies were conducted using four oligonucleotide probes, labelled with fluorescein isothiocyanate (FITC), which results in green fluorescence. Samples were counterstained with DAPI as a positive, but non-discriminatory control. The images presented in Figure 30 show similar levels of fluorescence with both the DAPI and the EUB338 probe. This indicates that the community largely comprised bacteria, with insignificant archaeal populations. This is expected as the methanogenic archaea are more susceptible to inhibition by aqueous sulphide.

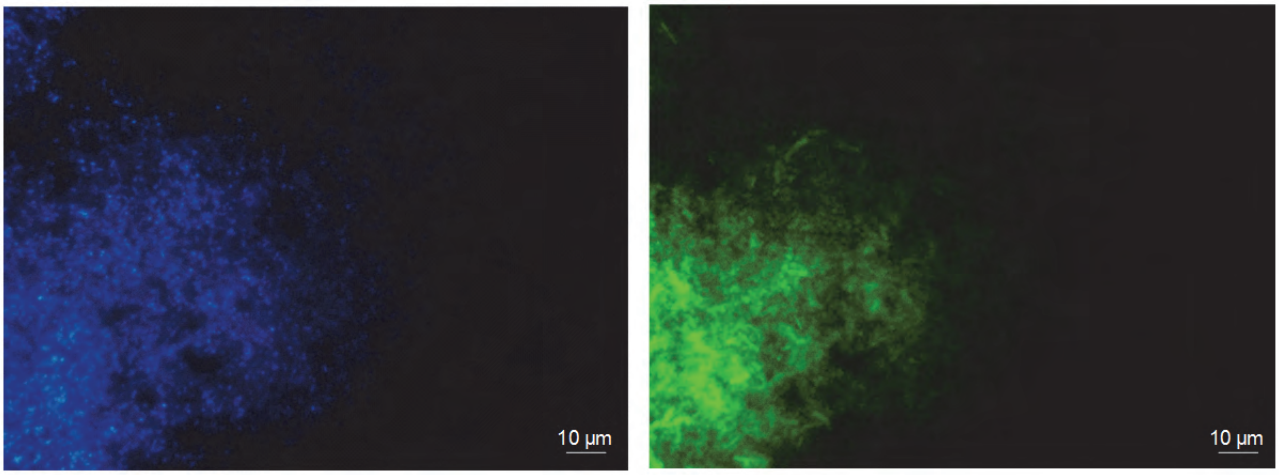

Figure 30: Epifluorescence images of sample from the reactor fed 2.5 g/ℓ sulphate, at a 3 day HRT. Left-hand image stained with DAPI, while the right-hand image is stained with the FITC-labelled EUB338 probe

The results obtained with the three group or species specific probes are shown in Figure 31, Figure 32 and Figure 33. The green signal is significantly lower, relative to the DAPI signal, than that obtained using the EUB338 probe. This indicates that a significant proportion of the community was made up of non-sulphate reducing bacteria. This is consistent with the results of the clone library and also a study performed on lactate-fed reactors.

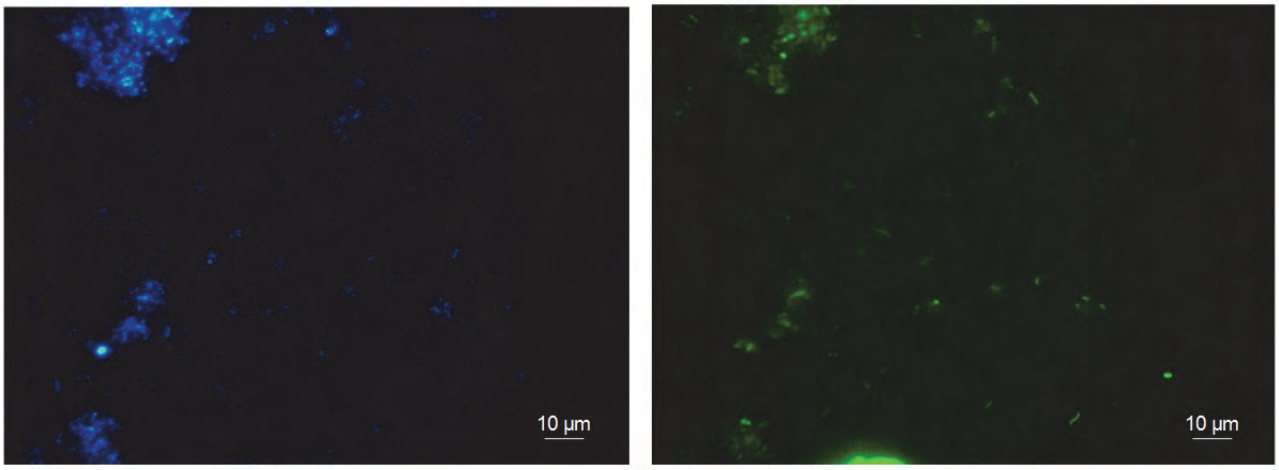

Figure 31: Epifluorescence images of sample from the reactor fed 5 g/ℓ sulphate, at a 3 day HRT. Left-hand image stained with DAPI, while the right-hand image is stained with the FITC-labelled SRB129 probe

The fluorescence signals from the FISH probes allow some discrimination of the cell morphology. These show that that majority of the SRB are rod-shaped, which is consistent with expectations, based on the information provided by the clone library. It is less easy to discern morphology from the DAPI signal, as DAPI only binds to double stranded DNA, which is relatively condensed, even in a bacterial cell. By contrast, the FISH probes bind to the 16S RNA in the ribosomes, which should be abundant and distributed throughout an active cell.

Of the three more specific probes tested, the SRB129 probe, which targets *Desulfobacter*, consistently resulted in the weakest signal, suggesting members of the *Desulfobacter* genus were not the dominant SRB. The strongest signal was obtained using the DSM312V probe, which is specific for *Desulfomicrobium*. This is consistent with the data from the clone library, which indicated that the greatest diversity among the SRB was among the genus *Desulfomicrobium*. However, it is difficult to justify the conclusion when only one group is targeted at a time. A more effective method is to use multiple fluorochromes and specific microscope filters, allowing simultaneous visualisation of multiple groups.

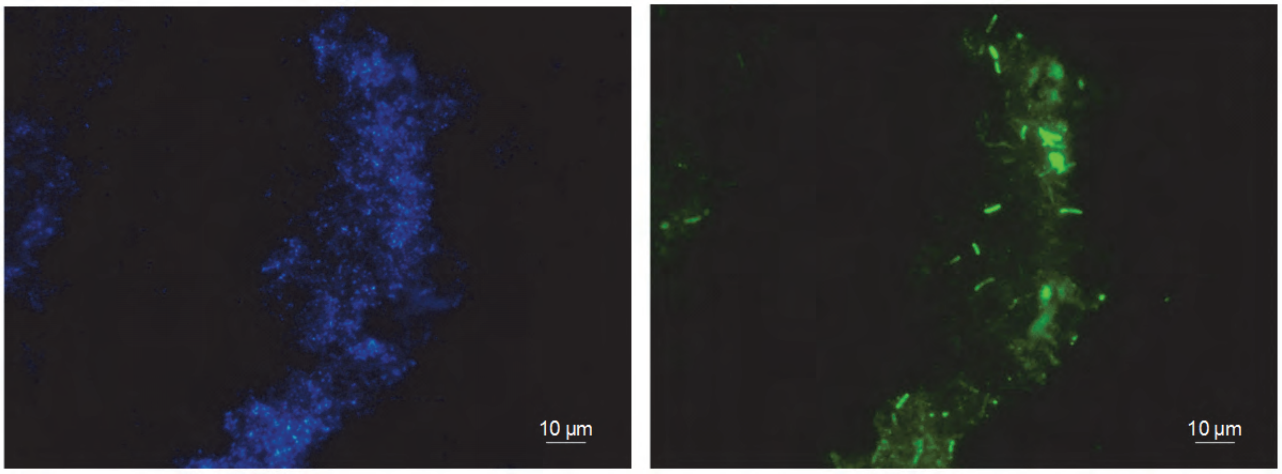

Figure 32: Epifluorescence images of sample from the reactor fed 5 g/ℓ sulphate, at a 3 day HRT. Left-hand image stained with DAPI, while the right-hand image is stained with the FITC-labelled Delta495a probe

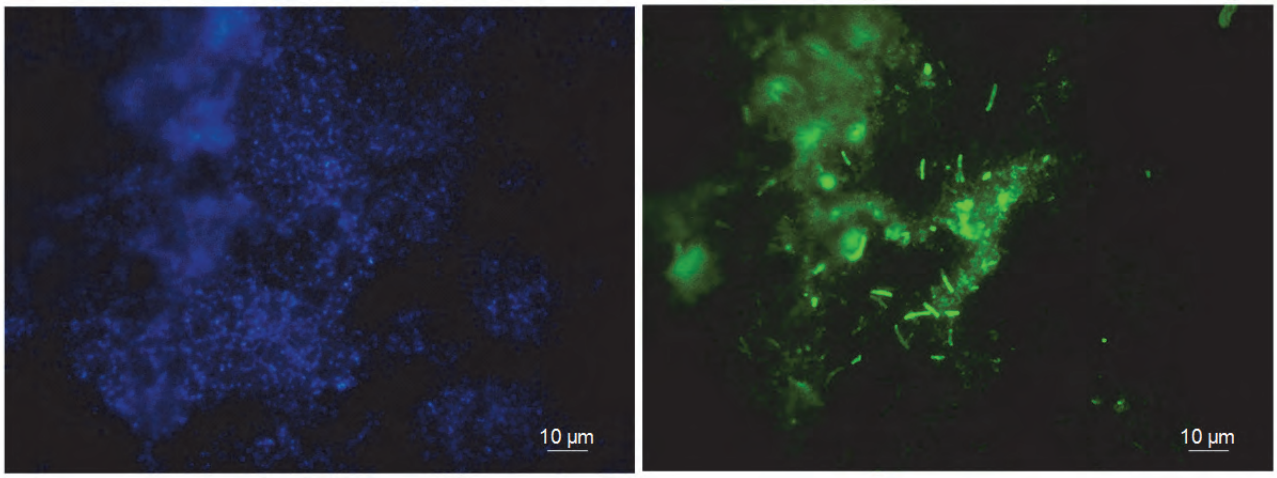

Figure 33: Epifluorescence images of sample from the reactor fed 5 g/ℓ sulphate, at a 3 day HRT. Left-hand image stained with DAPI, while the right-hand image is stained with the FITC-labelled DSM213V probe

5.5 QUANTIFYING THE EFFECT OF HYDRAULIC RETENTION TIME ON COMMUNITY STRUCTURE

The group specific qPCR primer set was used to quantify the relative proportion of sulphate reducers to non-sulphate reducing bacteria in the lactate-fed and digestate-fed reactors as a function of decreasing HRT.

The community in the lactate-fed reactor, operated at a feed sulphate concentration of 1 g/ℓ, was dominated by sulphate reducing bacteria at HRTs of between 1 and 4 days. A standard mass of template DNA (10 ng) was used in all reactions. Initially, the bacterial domain specific primer set, which should amplify DNA from most bacterial species, was used to quantify the total amplification value. After that, the same template DNA was used, but with the SRB group specific primer set. A comparison of the values allowed the calculation of the proportion of the total bacterial community made up of SRB. The value is a relative number, for several reasons. One of these relates to the number of copies of the 16S rRNA gene found in each of the component species. This number is not known, unless a full genome sequence is available.

The results showed that SRB made up between 74 and 82% of the total community at HRTs between 4 days and 1 day. The situation changes significantly at a HRT of 12 hours, where SRB made up just 47.5% of the total community. The results are consistent with performance data, which showed a significant decrease in sulphate reduction efficiency at a 12 hour HRT. The performance data suggested a loss of a portion of the SRB community and the qPCR data confirm this. The number of species specific primer sets will need to be expanded to determine if particular species were lost or if a portion of all species were lost. The former is more consistent with the concept of washout.

5.6 CONCLUSIONS

Some success has been achieved in the development of group and species specific qPCR primer sets and FISH probes, although not to the extent anticipated at the start of the project. The exercise has shown that while it is relatively easy to design primers and probes that are homologous to the target region and have the correct properties, in terms of melting temperature and lack of dimer formation, this does not guarantee success in practice.

Despite this, significant progress toward the goal of the project has been made. The sequences for all the clones described in Chapter 5 are now available and can be used to validate all future primer sets.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

The study has made some progress toward the realisation of the aims, but not to the extent anticipated at the start of the project.

The major successes have been the detailed characterisation of the sulphate reducing and sulphide oxidising communities that have been used to generate performance data in the UCT laboratories over the past four years as well as the community associated with the corrosion of concrete pipes. The latter represents a potentially ground-breaking study. The diversity of the sulphate reducing community was significantly greater than had been suggested by previous studies, which used FISH and a restriction enzyme based technique to characterise the community. A total of 48 unique ribotypes were identified, of which 17 aligned closely with known sulphate reducing species, compared to only six SRB species suggested by the restriction enzyme technique. The majority of the sulphate reducing species belonged to the genus *Desulfomicrobium.*

The sulphide oxidising community in the bioremediation system was strongly influenced by the presence or absence of soluble organic carbon. In carbon deficient systems the overall diversity was low and the community was dominated by autotrophic sulphur oxidisers, such as *Chromatium* and *Chlorobium* species. Few heterotrophs were detected. From a performance perspective, the biofilm took longer to form, was often incomplete and where it did form was thin and brittle. By comparison, in acetate supplemented reactors the microbial diversity was significantly higher and the primary sulphur oxidisers belonged to the genera *Thiobacillus* and *Halothiobacillus*. The biofilm formed more rapidly, was complete and was able to support the accumulation of a significant amount of elemental sulphur. These data suggested that the heterotrophic community members played a critical role in laying down the initial biofilm and ensuring the biofilm had sufficient structural integrity to support accumulated sulphur.

The analysis of the concrete sewer samples clearly illustrated microbial succession, which could be tied to the change in the micro-environment from neutral to acidic, as a consequence of sulphide oxidation by the pioneer species (*Thiobacillus* and *Halothiobacillus*). The major difference between the sewer and LFCR environments is the availability of oxygen. Complete oxidation of sulphide to sulphuric acid occurs in the sewer and the increasing acidity becomes inhibitory to the pioneer species. The community structure analysis showed the emergence of species from the genera *Acidithiobacillus*, *Acidiphilium* and *Alicyclobacillus*. The extent to which this is influenced by concrete composition and/or location along the length of the sewer is the subject of an ongoing investigation. This is one of the first studies to complete a more comprehensive characterisation of the microbial community. When coupled with the performance data being generated it could represent a watershed study.

The qPCR primer sets designed for the species suggested by the restriction digest method did not yield positive results, despite showing good alignment with the target sequences. However, the majority of the species indicated on the basis of the restriction digests were not identified in the clone library, suggesting that the restriction digestion method may have yielded inaccurate results.

Greater success was achieved with the SRB group specific primers and FISH probes, which were designed to target all SRB, based on the species represented in the clone library. The qPCR primers showed positive amplification when reactor samples were used as template, but not for a series of negative controls. Similarly, the FISH probe which was designed to target a similar portion of the gene yielded positive results. The qPCR primer set for the *Desulfomicrobium* genus has also shown promising results.

6.2 RECOMMENDATIONS

The project has generated positive results and much valuable information has been accumulated, specifically the clone libraries for the sulphate reducing and sulphide oxidising communities. The SRB group specific primers and FISH probes have yielded positive results and the lessons learned have opened the way for further success. It is recommended that this process continue.

During the course of the project the potential to use next generation sequencing techniques to perform metagenomic sequencing was explored. The cost of transporting extracted DNA on dry ice to facilities abroad and the continued devaluation of the South African Rand have made this option prohibitively expensive. However, the Centre for Proteomic and Genomic Research (CPGR), located in Cape Town, has just launched its Next Generation Sequencing (NGS) platform, which promises to provide a more cost effective alternative. It is recommended that a limited number of samples, from those that have been accumulated during the current project, be used for metagenomic sequencing to assess the capacity at the CPGR.

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APPENDIX A: Schematic diagrams of LFCR

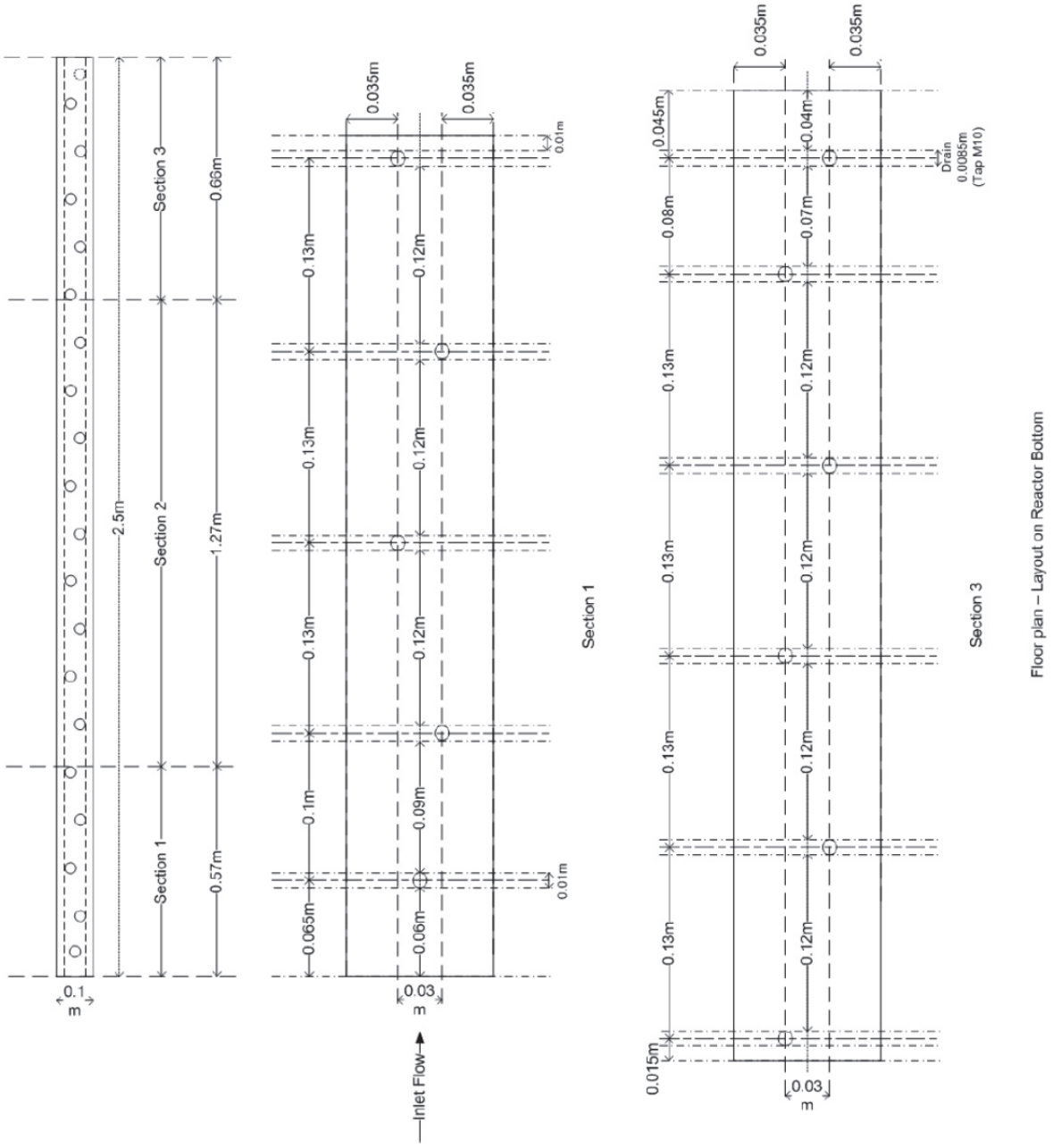


Figure A1: *Schematic of the top view of the entire LFCR, divided into three sections, showing the specific dimensions and spacing of the holes for placement of the cylindrical baffles*

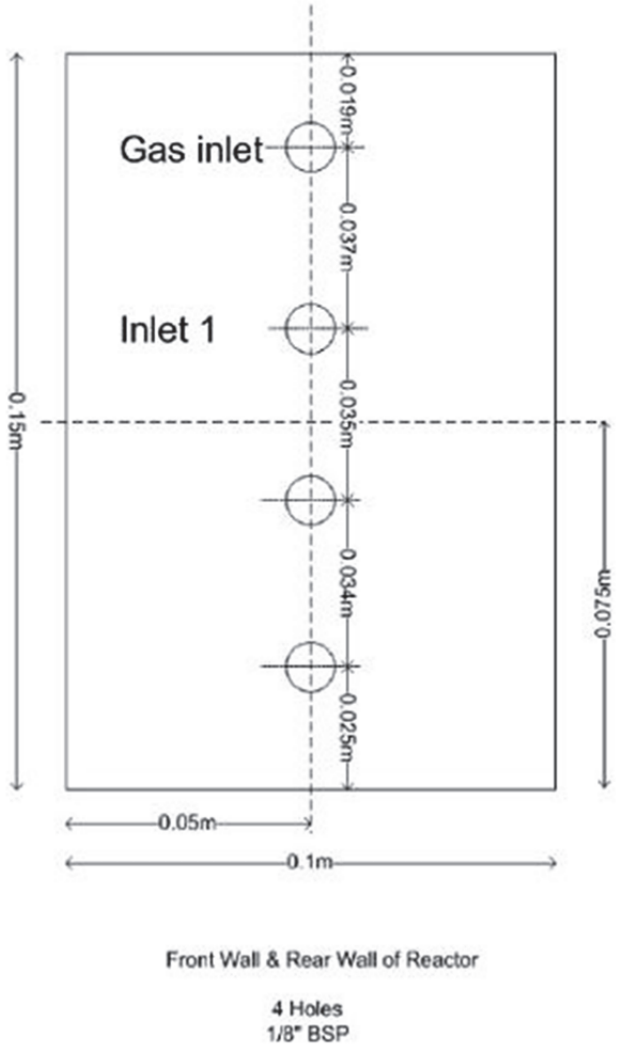


Figure A2: *Schematic drawing of the front and rear wall of the LFCR, showing the position of the three possible inlet/outlet ports for liquid and the gas inlet/outlet*

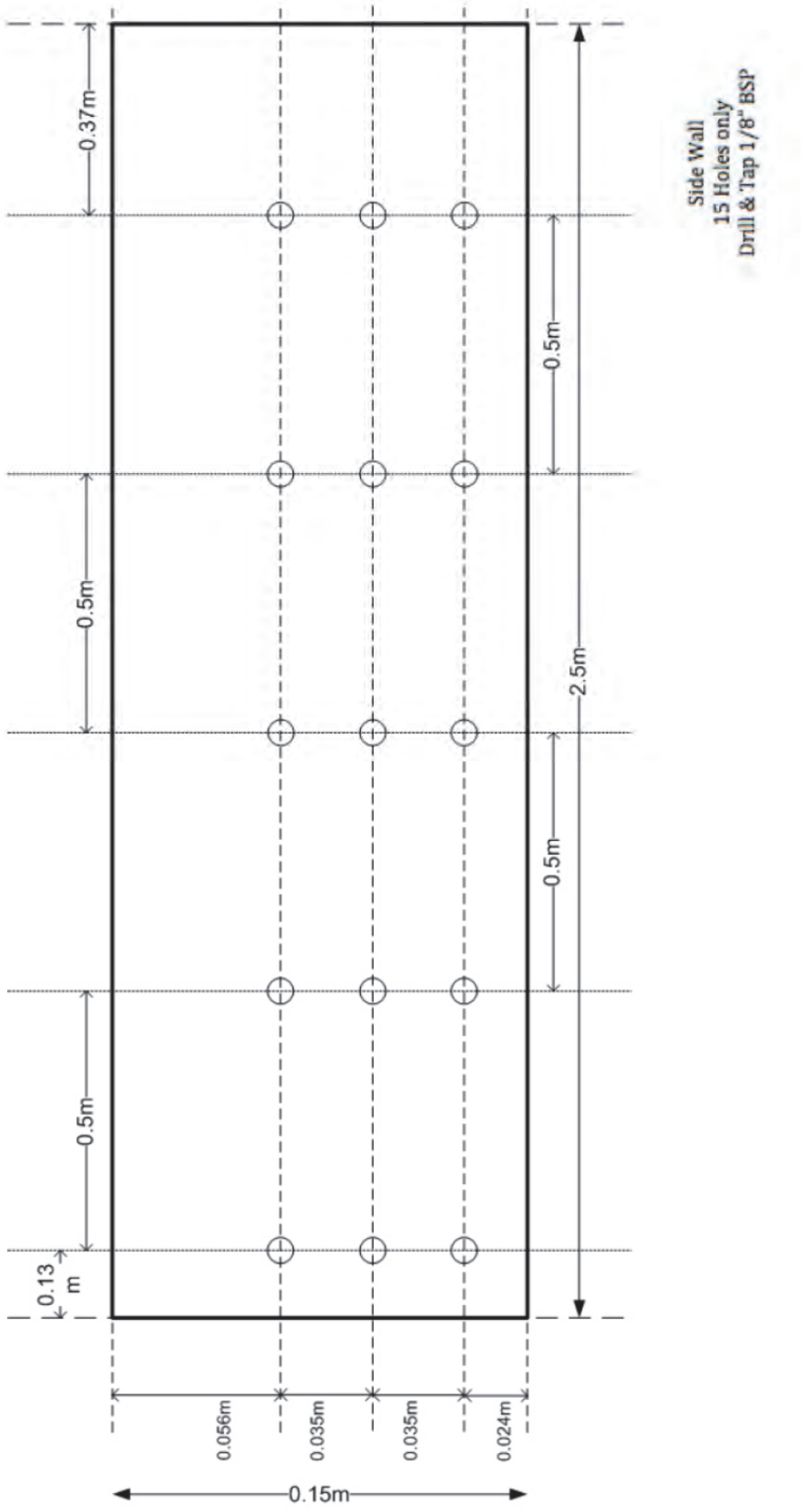
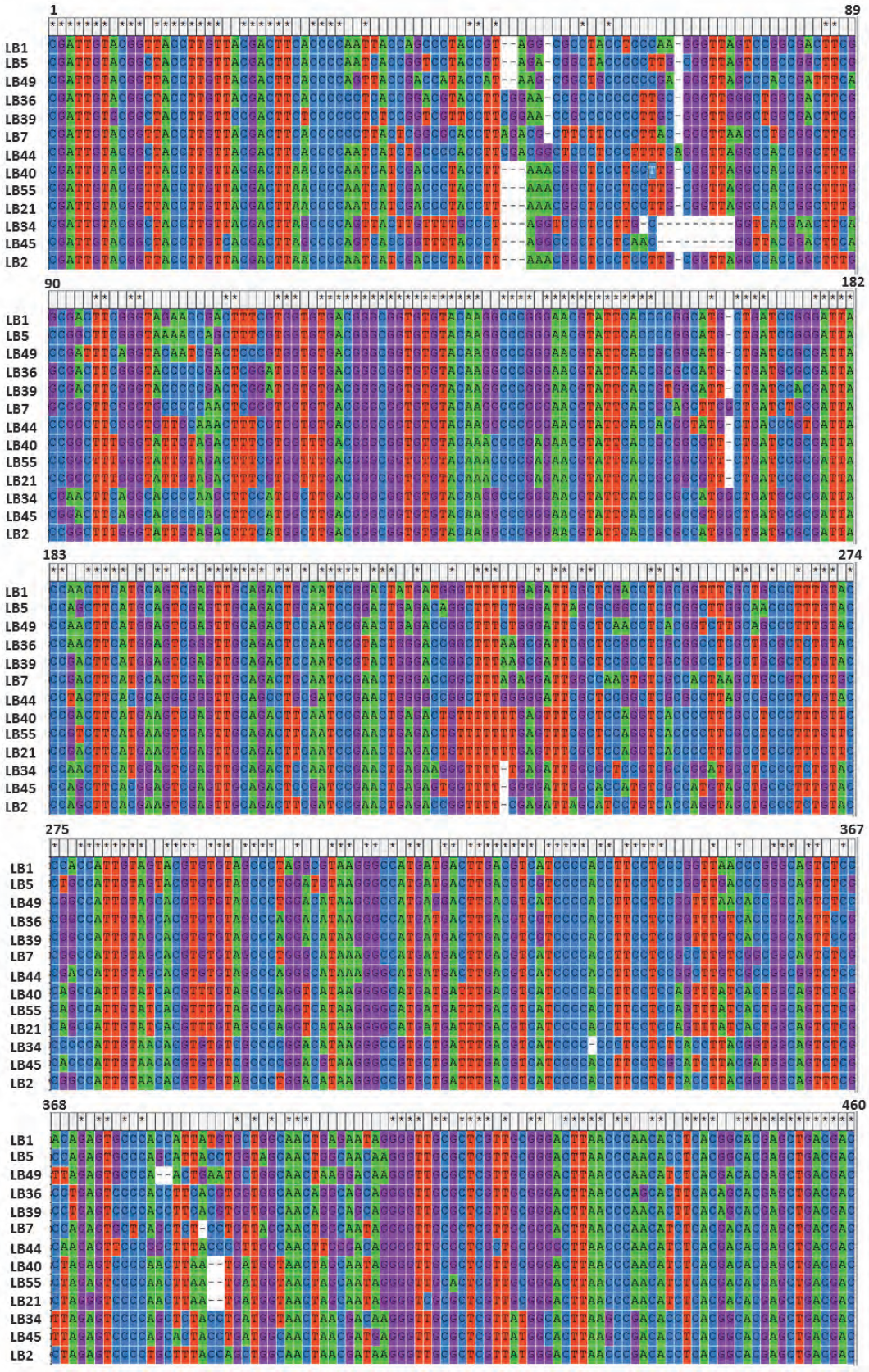
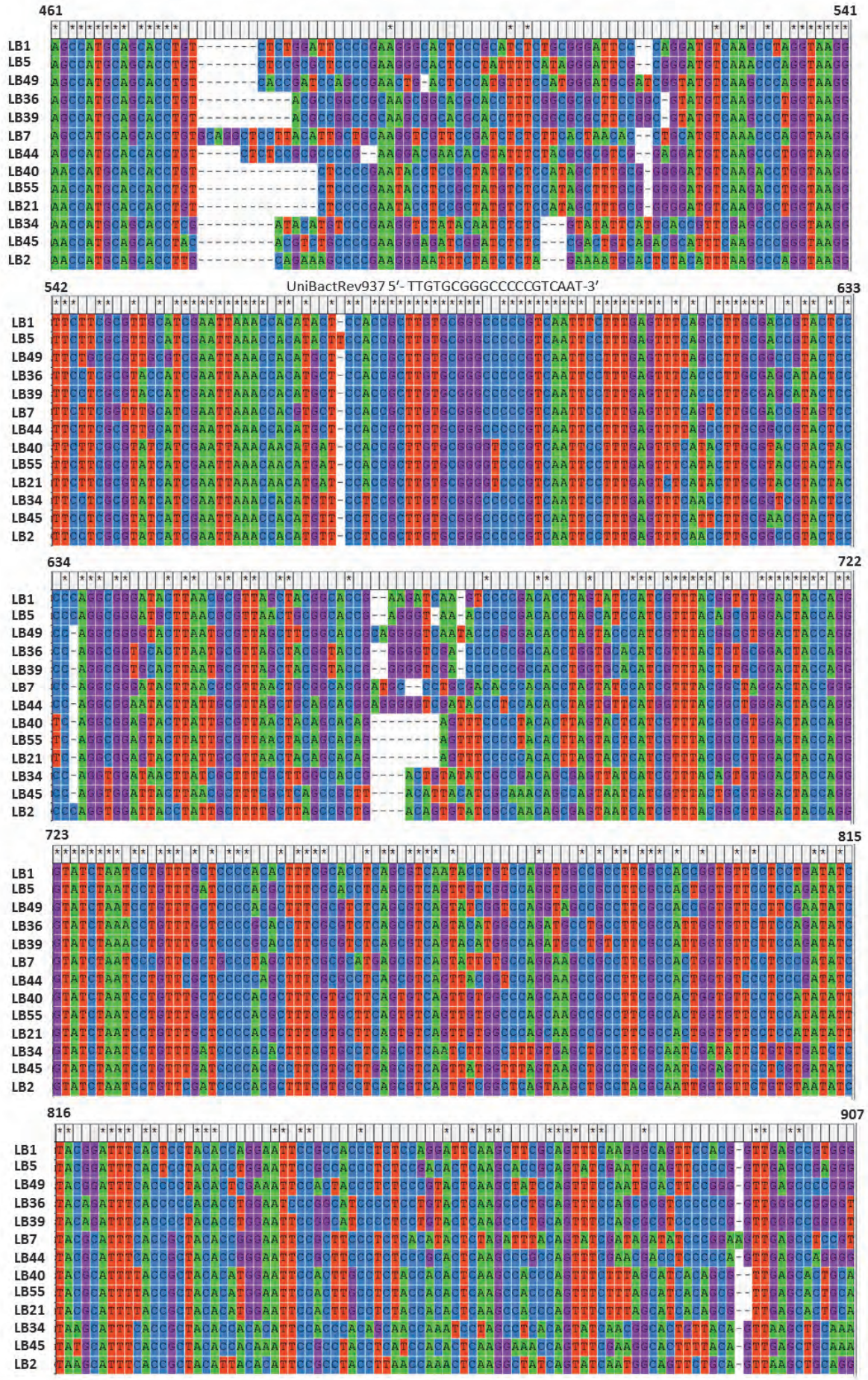


Figure A3: *Schematic diagram showing the location of the 15 sampling ports in the front wall*

APPENDIX B: Example of sequence alignment for qPCR primer design




461
541
LB1
LB5
LB49
LB36
LB39
LB7
LB44
LB40
LB55
LB21
LB34
LB45
LB2
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634
722
723
815
816
907

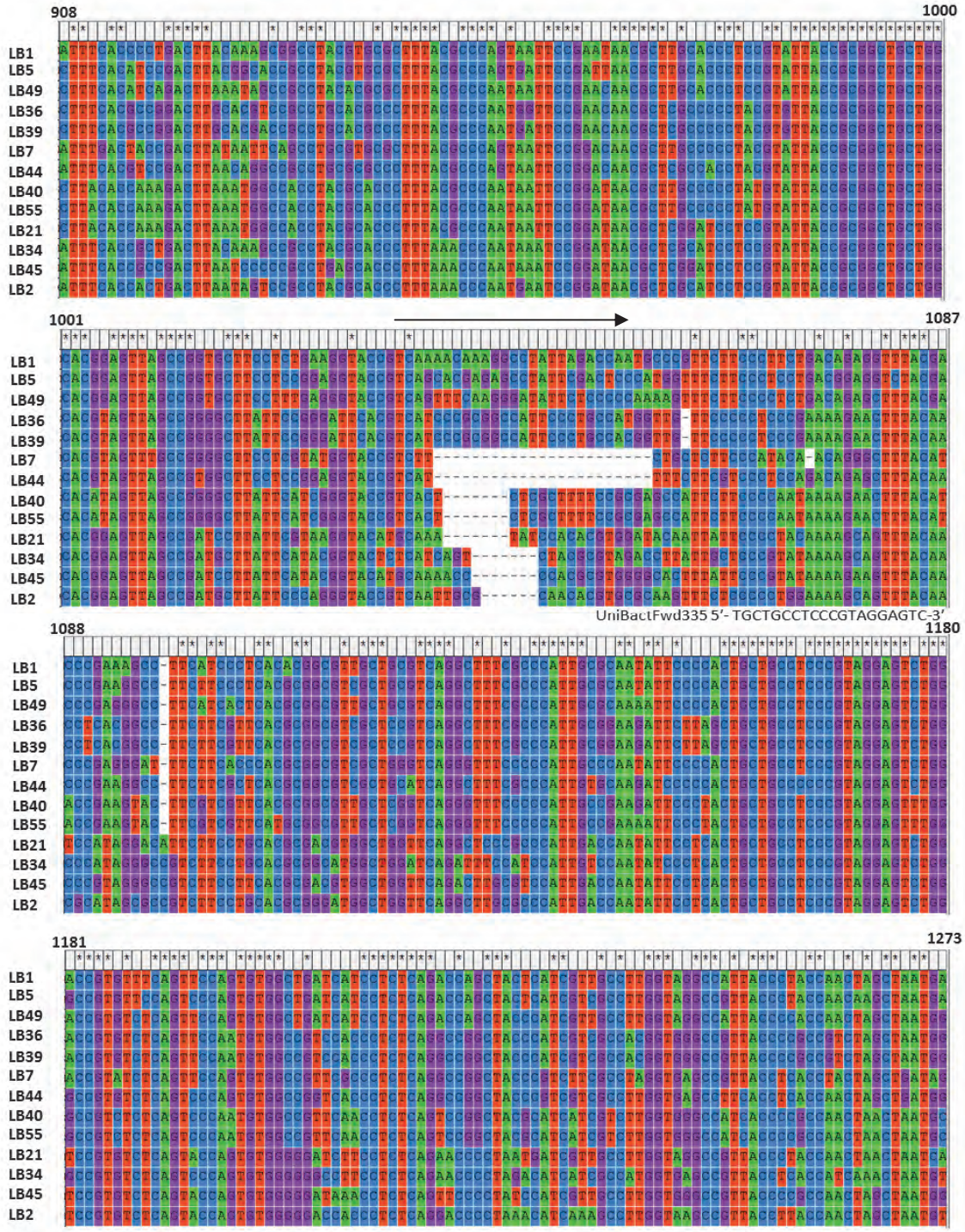

Figure B1: *Multiple sequence alignment of 16S rRNA genes of LB1, LB2, LB5, LB7, LB21, LB34, LB36, LB39, LB40, LB44, LB45, LB49 and LB55. The position of the Universal forward (UniBactFwd335) and reverse (UniBactRev937) primers are indicated. Potential regions suitable for designing qPCR primers to be used in conjunction with the universal forward and reverse primers will be designed based on regions of relatively low homology between the 16S rRNA sequences.*