THE BIOLOGY AND MOLECULAR ECOLOGY OF FLOATING SULPHUR BIOFILMS

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Michelle Louise Bowker

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ABSTRACT

Floating sulphur biofilms have been observed to occur on sulphate-containing natural systems and waste stabilization ponds. It has been postulated that these biofilms form on the surface of the water because sulphate reducing bacteria present in the bottom layers of the water body reduce sulphate to sulphide which then diffuses upwards and is oxidized under the correct redox conditions to sulphur by sulphide oxidizing bacteria. Very little information exists on these complex floating systems and in order to study them further, model systems were designed.

The Baffle Reactor was successfully used to cultivate floating sulphur biofilms. Conditions within the reactor could be closely scrutinized in the laboratory and it was found that sulphate levels decreased, sulphide levels increased and that sulphur was produced over a period of 2 weeks. The success of this system led to it being scaled-up and currently a method to harvest sulphur from the biofilm is under development.

It is thought that biofilms are highly complex, heterogeneous structures with different bacteria distributed in different layers. Preliminary work suggested that bacteria were differentially distributed along nutrient and oxygen gradients within the biofilm. Biofilms are very thin structures and therefore difficult to study and Gradient systems were developed in an attempt to spatially separate the biofilm species into functional layers. Gradient Tubes were designed; these provided a gradient of high-sulphide, low oxygen conditions to high-oxygen, low-sulphide conditions. Bacteria were observed to grow in different layers of these systems. The Gradient Tubes could be sectioned and the chemical characteristics of each section as well as the species present could be determined. Silicon Tubular Bioreactors were also developed and these were very efficient at producing large amounts of sulphur under strictly controlled redox conditions.

Microscopy and molecular methods including the amplification of a section of Ribosomal Ribonucleic acid by Polymerase Chain Reaction were used in an attempt to characterize the populations present in these biofilm systems. Denaturing Gradient Gel Electrophoresis was used to create band profiles of the populations; individual bands were excised from the gels and sequenced. Identified species included *Ectothiorhodospira* sp., *Dethiosulfovibrio russensis, Pseudomonas geniculata, Thiobacillus baregensis* and *Halothiobacillus kellyi.*

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ABBREVIATIONS

A	-	Adenine
BLAST	-	Basic Local Alignment Search Tool
bp	-	Base Pairs
C	-	Cytosine
cm	-	Centimetre
CO_2	-	Carbon dioxide
$dddH_2O$	-	Triple Distilled Water
DGGE	-	Denaturing Gradient Gel Electrophoresis
dNTPs -	-	deoxynucleoside triphosphate
EDTA	-	Ethylene Diamine tetra-Acetic Acid
EPS	-	Extracellular Polysaccharide
FISH	-	Fluorescent in situ Hybridisation
G	-	Guanine
g/l	-	Grams per litre
HPLC	-	High Performance Liquid Chromatography
H_2S	-	Hydrogen sulphide
LB	-	Luria Broth
M	-	Molar
ì	-	Micron
ìl	-	Microlitre
mg	-	Milligram
mg/l	-	Milligram per litre
mg/l C	-	Milligram per litre Carbon
ml	-	Millilitre
mm	-	Millimetre
mM	-	Milli-Molar
nm	-	Nanometre
PCR	-	Polymerase Chain Reaction
%	-	Percentage
ppm	-	parts per million
rDNA	-	Ribosomal Deoxyribonucleic Acid
rRNA	-	Ribosomal Ribonucleic Acid

rpm	-	Revolutions per Minute
sec	-	Seconds
SEM	-	Scanning Electron Microscopy
SOB	-	Sulphide Oxidizing Bacteria
SRB	-	Sulphate Reducing Bacteria
STB	-	Silicon Tubular Bioreactor
T	-	Thyamine
TE	-	Tris EDTA Buffer
TEM	-	Transmission Electron Microscopy
TOC	-	Total Organic Carbon
TSP	-	Tannery Stabilization Pond
UASB	-	Upflow Anaerobic Sludge Bed
U/ìl	-	Units per microlitre
V	-	Volts

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CHAPTER 1:

SULPHIDE OXIDIZING BACTERIA IN FLOATING BIOFILM SYSTEMS

1.1 Sulphur and the Sulphide problem

Sulphur occurs abundantly throughout the earth's crust and is an essential nutrient for all forms of life on earth. The majority of sulphur on earth is found in sediments and rocks in the form of sulphate and sulphide minerals. But due to its presence in fossil fuels and in metal sulphide deposits, environmental contamination by sulphur compounds has increased in step with man's utilization of these raw materials (Brown, 1982).

Sulphur transformations are complex due to the wide variety of its oxidation states and also the fact that the transformations can occur chemically as well as biologically (Brock & Madigan, 1991).

Sulphur compounds in the atmosphere are subjected to a range of photochemical and free radical reactions, resulting in their oxidation to sulphate. Sulphates occur in all natural waters where the maximum concentration allowed is 400ppm. One of the most important causes of sulphate pollution is contamination by acid mine water (Brown, 1982). Discharging industrial effluents containing high sulphate concentrations into surface waters contributes directly to mineralisation and corrosion potential (Maree & Hill, 1989). The presence of sulphate in the water can result in the formation of highly toxic hydrogen sulphide (Brown, 1982).

Hydrogen sulphide has a high affinity for most metallo-proteins, this means that it acts as a respiratory poison and is toxic to most forms of life (Brown, 1982). This malodorous gas also has a high oxygen demand and thus causes significant depletion of oxygen in receiving waters (Henshaw *et al.*, 1998). Hydrogen sulphide must be removed from wastewaters because of its toxicity, oxygen demand, corrosivity and bad odour (Janssen *et al.*, 1997).

Sulphide-containing waste is generated by a number of industries such as petrochemical plants, tanneries, viscose rayon manufactures, the gasification of coal for electricity production or by the anaerobic treatment of sulphate wastewaters (Janssen *et al.*, 1997). The emission of sulphide is a major problem associated with the anaerobic treatment of sulphate and sulphite containing wastewaters, its release must therefore be stringently controlled (Buisman *et al.*, 1989).

Methods for sulphide removal include physicochemical processes that involve direct air stripping, oxidation and chemical precipitation (Buisman *et al.*, 1989). The high energy requirements or the high chemical and disposal costs are important drawbacks of these treatment processes.

Direct air stripping leads to a voluminous air stream contaminated with hydrogen sulphide which then has to be treated (Buisman *et al.*, 1989). Oxidation processes used for sulphide removal are aeration and chemical oxidation (chlorination, ozonation, potassium permanganate treatment and hydrogen peroxide treatment). In all these oxidation processes thiosulphate and sulphate will be the end products (Buisman *et al.*, 1989). Chemical precipitation also generates sludge that must be disposed.

Demineralization processes such as reverse osmosis may be applied for sulphate removal but they are expensive, hence the need for alternative treatment methods (Maree & Hill, 1989).

The removal of sulphur compounds from waste streams may be mediated by the application of bacteria involved in the natural sulphur cycle (Janssen *et al.*, 1997).

1.2 The Sulphur Cycle.

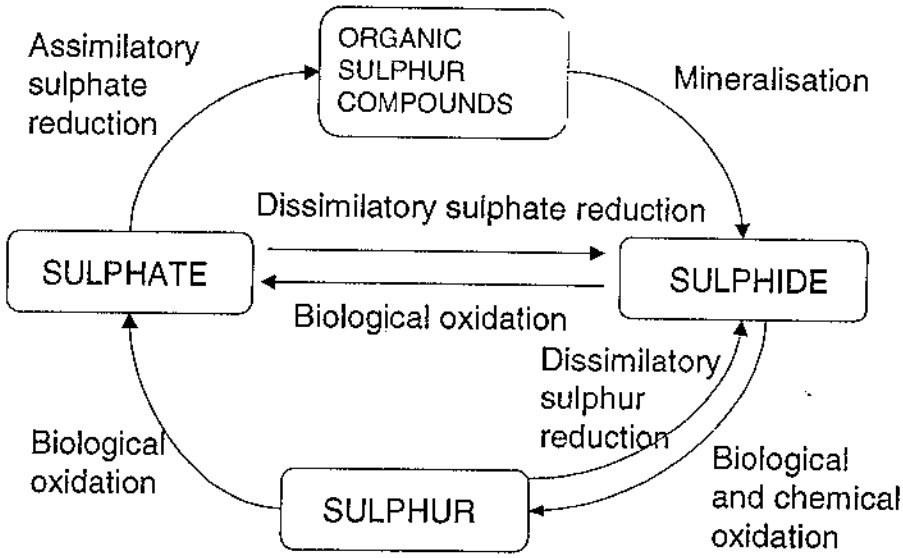

Figure 1.1: The Sulphur Cycle (Janssen *et al.*, 1999).

Sulphur microorganisms play a crucial role in the sulphur cycle, they are responsible for the interconversion of various inorganic forms of sulphur, the mineralisation of organic sulphur compounds and for the immobilization of sulphur in microbial protoplasm (Brown, 1982). In nature sulphide can be biologically oxidized anaerobically by photosynthetic bacteria, denitrifying organisms and in the presence of oxygen by colourless sulphur bacteria (Buisman *et al.*, 1989).

Under oxygen-limiting conditions, sulphur is the major end-product of bacterial sulphide oxidation, whereas sulphate is formed under high redox oxygen-enriched conditions (Janssen *et al.*, 1997). The formation of sulphur is preferred because elemental sulphur is nonsoluble, non-toxic, non-corrosive and can therefore be removed from water and reused as a valuable by-product, also sulphate formation requires more oxygen and therefore a higher energy consumption is needed for aeration (Janssen *et al.*, 1997; Henshaw *et al.*, 1998). The desired function of this biological process is the removal of sulphide and the production of sulphur. Conversions between different species of sulphur are accomplished by several types of naturally occurring bacteria (Henshaw *et al.*, 1998).

The most common sulphur compounds used as energy sources are hydrogen sulphide, elemental sulphur and thiosulphate, the final product of sulphur oxidation is in most cases sulphate. Less energy is available when one of the intermediate oxidation states is used instead of sulphate (Brock & Madigan, 1991). The oxidation of the most reduced sulphur compound (H_2S) occurs in stages, the first oxidation step results in the formation of elemental sulphur, Figure 1.1.

Inorganic sulphur compounds are found in nature in various oxidation states with sulphides as the most reduced and sulphates as the most oxidized forms (Suzuki, 1999). Many microorganisms derive their energy for growth from the oxidation of fully or partially reduced sulphur compounds (Suzuki, 1999). Sulphur is the immediate oxidation product of sulphide (Suzuki, 1999). In sulphur oxidation the production of protons (H^+) result in the acidification of the media (Brock & Madigan, 1991).

1.3 Bacterial utilization of sulphur compounds

On earth deposits of elemental sulphur are of either volcanic or microbial origin (Steudal, 1998). Sulphur bacteria are ubiquitous organisms and are important in the global recycling processes of inorganic and organic sulphur compounds.

When oxygen enters previously anaerobic environments, sulphide is chemically and microbiologically oxidized to elemental sulphur, this is then subject to attack by autotrophic and heterotrophic microorganisms, eventually being converted to sulphate (Brown, 1982). Autotrophs derive energy from the oxidation of inorganic compounds (chemoautotrophs) or from light (photoautotrophs). Heterotrophs depend on organic sources of carbon whereas autotrophs can utilize CO_2 for their entire carbon demand (Brown, 1982).

Microbial attack on elemental sulphur is determined by pH, temperature and moisture, and under favourable conditions, oxidation to sulphate is rapid (Brown, 1982). Plants form reduced compounds from sulphate whereas animals oxidize reduced compounds to sulphates. Microorganisms perform both reactions and some bacteria even use sulphate in place of oxygen as their terminal electron acceptor while others make use

of the oxidation of reduced inorganic sulphur compounds as a source of energy (Brown, 1982).

The natural sulphur cycle is controlled by heterotrophic bacteria which interconvert organic and inorganic forms of the element, and also by specialized sulphur bacteria which carry out oxidation-reduction reactions on inorganic forms of the element (Brown, 1982). Sulphate reducing bacteria (SRB) utilize organics such as lactate or pyruvate as their carbon source (Maree & Hill, 1989).

1.3.1 Sulphate Reduction

Most of the sulphide that accumulates in the environment is formed by the reduction of sulphate (Brown, 1982)(Smith & Klug, 1981). Sulphate reduction is performed by bacteria such as *Desulfuromonas, Desulfovibrio* and *Desulfotomaculum.* But there are also certain bacteria that reduce elemental sulphur to sulphide, for example, *Shewanella putrefaciens, Beggiatoa spp.* and *Thiorhodaceae* (Moser & Nealson, 1996). SRB are found in nearly all anaerobic organically rich environments. They occur in marine, estuarine and freshwater sediments. *Shewanella putrefaciens* can grow either aerobically or anaerobically utilizing many different electron acceptors and is often found in abundance at redox interfaces in nature (Nealson *et al.,* 1995).

SRB conduct dissimilatory sulphate reduction, which is the use of SO_4^{2-}, $S_2O_3^{2-}$, SO_3^{2-}, $S_2O_4^{2-}$ and S^0 as the terminal electron acceptor instead of oxygen (Brown, 1982). Energy is derived from the dissimilation of simple organic compounds like ethanol, lactic acid and carbohydrates.

SRB are important for the formation of some geological deposits. It was estimated that about 95% of the world's sulphur deposits occur due to the activities of SRB (Brown, 1982). Metal sulphide ores such as iron, zinc, copper and lead are also formed by SRB (Brown, 1982).

1.3.2 Sulphur Oxidation

Both chemical and microbiological processes are important for the oxidation of reduced inorganic compounds of sulphur (Brown, 1982). Most sulphide oxidizing bacteria (SOB) are chemoautotrophic. They obtain their energy from the oxidation of

inorganic sulphur compounds rather than organic carbon compounds (Brown, 1982). Their carbon requirements are satisfied through the fixation of atmospheric carbon dioxide (Brown, 1982). *Thiobacillus denitrificans* is a SOB and a facultative anaerobe that can use nitrate rather than oxygen as its terminal electron acceptor (Brown, 1982). Other common SOB such as *Arthrobacter, Bacillus and Flavobacterium* can oxidize S^0, SO_3^{2-}, $S_2O_3^{2-}$ and S^{2-} to SO_4^{2-} heterotrophically (Brown, 1982).

The aerobic sulphide oxidizers have to live where oxygen and sulphide coexist, under oxic conditions sulphide rapidly oxidizes spontaneously at neutral pH and therefore the bacteria must compete with the chemical oxidation of sulphide. Bacterial oxidation therefore occurs only in areas where gaseous sulphide rises from the anoxic zone and meets oxygen descending from the oxic areas (Brock & Madigan, 1991). Some sulphide oxidizing bacteria deposit the elemental sulphur formed inside the cell. These internal sulphur deposits serve as an energy reserve and when the supply of H_2S has been depleted, the sulphur is further oxidized to sulphate (Brock & Madigan, 1991). *Beggiatoa* species convert sulphide into extracellular sulphur which is not autooxidizable, in this way it withdraws its energy source from the chemical oxidation process. As an intermediate *Thiobacillus spp.* may form intracellular sulphur (Zehnder. 1988)

Elemental sulphur is chemically stable but is readily oxidized by SOB, for example *Thiobacillus spp.* which attaches to insoluble sulphur crystals.

Mixed-culture studies of sulphide oxidizing bacteria have shown that the formation of sulphur is dependent on the sulphide load and the availability of oxygen (Visser *et al.*, 1997). The capacity of bacteria to produce sulphur was found to be strain dependent, *Thiobacillus spp.* Strain W5 is capable of stoichiometrically converting sulphide to sulphur under acrobic conditions (Visser *et al.*, 1997). Studies showed that an increase in sulphide load led to increased sulphur production and decreased sulphate production but if sulphide accumulated in the reactor then the sulphide-oxidizing capacity of the biomass decreases (Visser *et al.*, 1997). *Thiobacillus spp.* initially converts sulphide to sulphate but if sulphate continues to be the sole product, then incoming sulphide will accumulate in the media. Due to the toxic nature of sulphide,

conditions become unfavourable for the bacteria so they redistribute the electrons by converting some of the sulphide to sulphur (Visser *et al.*, 1997).

Oxidizing species like *Thiobacilli spp.* and *Chromatium vinosum* turn reduced sulphur compounds such as H_2S or the terminal sulphur atom of the thiosulphate anion into elemental sulphur which is stored either inside or outside the bacterial cells (Steudal, 1998)(Prange *et al.*, 1999). The formation of these sulphur globules is reversible. If no reduced sulphur compounds are available then the elemental sulphur is oxidized to sulphate to produce energy and to reduce CO_2 for biomass production (Steudal, 1998). Sulphur bacteria metabolism is under intense study since it has been demonstrated that they may be used economically to remove poisonous or otherwise unwanted sulphur compounds from industrial waste gases or waters (Steudal, 1998). For example, sulphate may be reduced enzymatically to sulphide utilizing cheap organic substances such as sewage as reductants. Other bacteria can then be used to oxidize the sulphide with air to elemental sulphur which can be recovered. It is hoped that these developments will soon be available on a large enough scale as an alternative to the processes operated today which are not always perfect from an ecological perspective (Steudal, 1998).

1.3.3 Photosynthetic Sulphur Bacteria

Photosynthetic sulphur bacteria are abundant in anaerobic environments rich in H_2S and they are often found in association with SRB. Sulphide released by the SRB is converted by the photosynthetic sulphur bacteria to sulphur and sulphate. This is then available for utilization by SRB found in deeper layers (Brown, 1982). Organic matter is supplied to the system by carbon dioxide fixing chemo- and photoautotrophic bacteria, meaning that an ecosystem based on the sulphur cycle can be self-sustaining (Brown, 1982).

In oxygen deficient environments where light intensity is high, hydrogen sulphide can be oxidized to elemental sulphur by photosynthetic sulphur bacteria (Brown, 1982). Green and purple bacteria use sulphide as an electron donor for cell synthesis from carbon dioxide with light as sole energy source for anoxygenic photolithoautotrophic growth. The final product is sulphate.

As intermediate to sulphate, green sulphur bacteria and *Ectothiorhodospira* species form extracellular sulphur, whereas *Chromatium* species store intracellular sulphur globules. The sulphur oxidizers utilize sulphide from the underlying sulphate reduction zone. These photosynthetic bacteria use carbon dioxide to oxidize hydrogen sulphide, they depend on sunlight for their energy source (Brown, 1982).

1.4 Biological treatment processes

Sulphur-containing waste streams are generally treated by chemical methods which involve high chemical and disposal costs (Visser *et al.*, 1997). Under anaerobic conditions sulphate, sulphite and organic sulphur compounds present in wastewater are reduced by bacterial activity to sulphide (Buisman *et al.*, 1991). The odour, toxicity, oxygen demand and corrosion problems associated with sulphide in wastewater frequently necessitate wastewater treatment i.e. oxidation of sulphide to a less harmful form (Buisman *et al.*, 1991). Biological oxidation of sulphide with oxygen is significantly faster than the chemical noncatalyzed oxidation of sulphide with oxygen (Buisman *et al.*, 1991).

The ability of autotrophic bacteria to oxidize sulphide at high rates has led to the development of biotechnological methods to remove sulphide from effluent streams (Visser *et al.*, 1997). A major environmental advantage of a biotechnological process for sulphide removal is the recovery of sulphur thereby preventing eutrophication of streams through the discharge of sulphate containing waste-water (Visser *et al.*, 1997).

The principle of biotechnological sulphide removal is that sulphide is microbiologically converted to elemental sulphur using oxygen (Buisman *et al.*, 1991). The advantages of this process are:-

1. no catalyst or oxidants other than air are necessary;
2. no chemical sludge needs to be disposed of;
3. little biological sludge is produced;
4. low energy consumption;
5. possible reuse of sulphur;
6. little sulphate or thiosulphate discharge.

The bacteria present in sulphide removal reactors are probably obligate and facultative chemolithotrophic colourless sulphur bacteria. The end-products of the biological sulphide oxidation are elemental sulphur and sulphate (Buisman *et al.*, 1991).

Microbial processes operate around ambient temperature and at atmospheric pressure, thus eliminating the high costs for heat and pressure generation as required for many chemical treatment processes (Janssen *et al.*, 1997). The use of colourless sulphur bacteria provides one of the most reliable biotechnological approaches for hydrogen sulphide removal. These organisms have very simple nutritional requirements, i.e. they utilize reduced inorganic sulphur compounds as their electron donors and carbon dioxide as their carbon source (Janssen *et al.*, 1997).

Instead of using expensive chemical methods a natural biological solution for the problem of sulphur compound polluted wastewater was therefore sought. The basis for this technique was bacterial biofilms which had been observed to form naturally on sulphur contaminated wastewaters (Dunn, 1997).

1.5 Previous work on biofilms

Biofilms have previously been used to treat wastewater streams, such as in the Thiopaq process which was highly energetic, costly, unable to treat large volumes and unsuitable for passive treatment.

Floating sulphur biofilms were observed to form on the surface of Tannery effluent ponds (Dunn, 1997) and this served as the basis for biotechnological process innovation. Bioreactor development in order to create model systems for the growth of these biofilms was undertaken in the Rhodes Biosure Process by Corbett and Whittington-Jones (1998).

Very little was known about floating sulphur biofilms, the Baffled Reactor was used to simulate the conditions necessary for floating sulphur biofilm formation. Preliminary studies were performed on the floating sulphur biofilm system by Gilfillan (2000). It was shown that the biofilm was not a homogenous structure but consisted of distinctive layers of bacterial with different morphologies, Figure 1.2.

Shorter rod-like bacteria were visible in the top sections of the biofilm, while longer rods were visible in the bottom sections of the biofilm

The arrows in Figure 1.2 indicate voids and channels present within the biofilm. The Floating film was very thin and therefore difficult to study. In this project it was hypothesized that the floating biofilm was not amorphous but was both morphologically and physiologically differentiated. The floating film occurred at the air / liquid interface where there is a steep redox gradient from strongly positive to strongly negative, this provided the ideal conditions for sulphide oxidation to sulphur. A better understanding of the ongoing processes occurring in this system would hopefully result in the industrial application of floating sulphur biofilms to remove sulphide from waste ponds and recover sulphur as a valuable by-product.

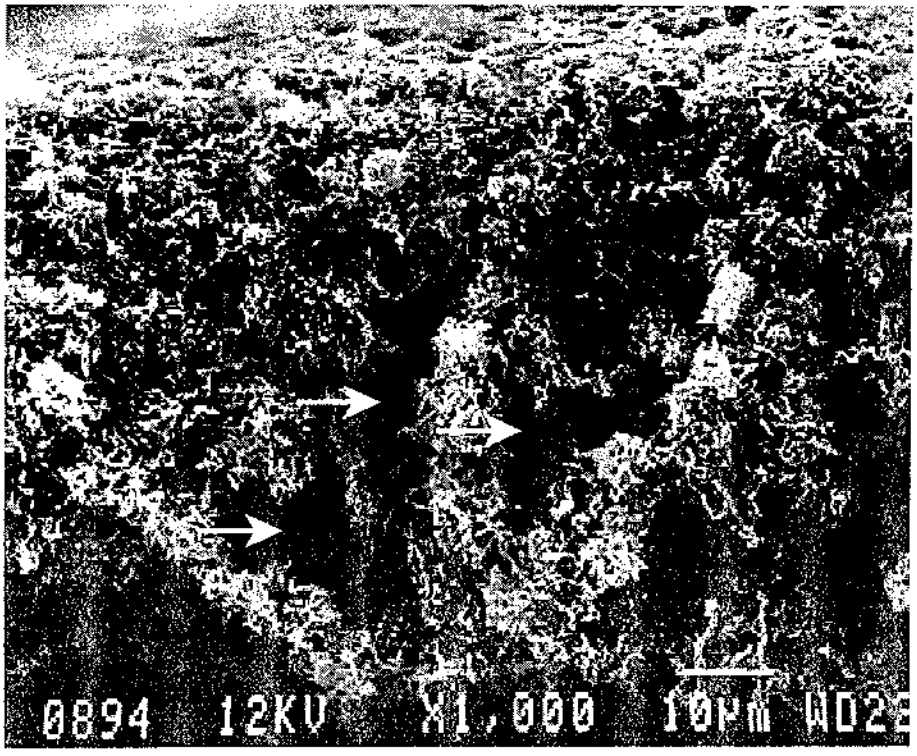

Figure 1.2: The Floating sulphur biofilm (Gilfillan, 2000).
The arrows indicate the water channels that anastamose throughout the biofilm.

1.6 Biofilms

Planktonic life as individual cells living in aqueous suspensions is just one of the possible survival strategies available to bacteria. Another strategy is the colonization of solid surfaces or other interfaces by the formation of biofilms (Amann *et al.*,

1995). Microorganisms flourish in most terrestrial and aquatic ecosystems as biofilms (Korber *et al.*, 1999). Biofilm bacteria predominate numerically and metabolically in virtually all nutrient-sufficient ecosystems (Costerton *et al.*, 1995).

Biofilms are defined as matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces (Costerton *et al.*, 1995). Bacteria initiate biofilm development in response to specific environmental cues like nutrient availability. (O'Toole *et al.*, 2000). In oligotrophic environments, organic nutrients tend to associate with available surfaces (Costerton *et al.*, 1995).

The life of attached bacteria as opposed to planktonic life forms is very different, biofilm communities develop internal heterogeneity, structure/function relationships exist in biofilms that are important to biological activity and individual cells and entire communities respond to environmental change (Christensen *et al.*, 1999). This lifestyle offers many advantages, including the higher availability of nutrients and the possibility of optimal long-term positioning in relation to other microorganisms or physicochemical gradients (Amann *et al.*, 1995).

One of the key features of environmental biotechnology are mixed cultures. These arise from the presence of multiple electron-donors and multiple electron-acceptors in the wastewaters treated by biological processes (Rittmann & Manem, 1992). Even where only one microbial reaction is desired to operate a process, several other reactions occur because of the substrates present in the influent (Rittmann & Manem, 1992). Wastewater biofilms are very complex multispecies structures displaying considerable heterogeneity with respect to both the microorganisms present and their physicochemical microenvironments (Okabe *et al.*, 1999).

Due to the presence of multiple electron donors and electron acceptors present in the wastewaters, successive vertical zonations of predominant respiratory processes have been found to occur in close proximity. Bacteria within the biofilm may be distributed in different layers as oxygen limitation occurs. Oxygen is a limiting factor in aerobic biofilms due to its rapid consumption (Xu *et al.*, 1998). It is also important to take the role of rate limiting nutrients into account as they establish physiological

gradients causing the pattern of population distribution to be very complicated in mixed populations. Both aerobes and anaerobes may be present (Xu *et al.*, 1998).

Aerobic wastewater biofilms typically display a thickness of only a few millimetres (Okabe *et al.*, 1999). These compact microbial communities exhibit high specific rates in their metabolic processes. The high activity in these biofilm systems is due to both dense populations of microorgansims and a high availability of organic substrates. Heterotrophic biofilms found in sewers and wastewater treatment plants receive most of their nutrients from water loaded with nutrients and organic matter (Kuhl & Jorgensen, 1992). Biodegradable organic materials are ubiquitously present in surface waters, groundwater and wastewaters, thus the substrates for the growth of heterotrophic bacteria are always present (Rittmann & Manem, 1992).

1.6.1 The Characteristics of Biofilms

Biofilms form interesting microbial ecosystems with a characteristic internal architecture (Sekiguchi *et al.*, 1999). Biofilms consist of microorganisms embedded in a matrix of extracellular substance (De Beer *et al.*, 1994). The biofilm matrix, which is typically composed of polysaccharides, may contain one or more anionic uronic acids and is densely concentrated around the microcolonies of cells that produce the polymeric substance (Costerton *et al.*, 1994). EPS production by biofilm bacteria facilitates the initial attachment of the bacteria to surfaces, the formation and maintenance of microcolony and biofilm structure, enhanced biofilm resistance to environmental stress and antimicrobial agents and biofilm nutrition (Costerton *et al.*, 1995).

Mass transfer within the biofilm is assumed to be diffusional and perpendicular to the surface on which it forms. However, microscopic observations indicate that biofilms are not flat and the distribution of microorganisms is not uniform (De Beer *et al.*, 1994). Instead, multispecies biofilms form highly complex structures containing voids, channels, cavities, pores and filaments with cells arranged in clusters and layers (De Beer *et al.*, 1994). The intercolonial spaces do contain the same polymer as found in the dense matrix but it is sparsely distributed, producing water channels that allow convective flow (Costerton *et al.*, 1994).

It was hypothesized that the biofilm structure is not a chance occurance but represents an optimal arrangement for the influx of nutrients (De Beer *et al.*, 1994). Recurrent patterns of various

structural elements in both pure and mixed-species biofilms support the premise that basic functional requisites underlie biofilm structure and that structural diversity actually reflects the adaptation of unicellular organisms to a diverse range of physical, chemical and communal circumstances on surfaces (Costerton *et al.*, 1995).

Voids may enhance substrate and product fluxes by decreasing diffusional resistance or by facilitating convection. Biofilms are therefore nonuniform structures consisting of discrete cell aggregates and interstitial voids that facilitate oxygen transport (De Beer *et al.*, 1994).

The biofilm should be treated as a continuum rather than as a complex multiphase system (Wood & Whitaker. 1999). The water channels that anastomose throughout microbial biofilms and provide direct access from the bulk fluid to the colonized surface (Costerton *et al.*, 1994). Biofilm bacteria therefore enjoy some of the advantages of multicellular life, as the channels deliver nutrients and remove metabolic products just like a primitive circulatory system (Costerton *et al.*, 1994).

Compact biofilm communities are stratified with morphologically distinctive layers in which many different processes may occur simultaneously in close proximity (Birger Ramsing *et al.*, 1993). Metabolic rates in these communities are extremely high and respond fast to changes in ambient light, oxygen, nutrient content etc. The predominant metabolic processes are stratified and these subdivide the biofilm into functional layers (Birger Ramsing *et al.*, 1993).

1.6.2 Interactions within the Biofilm

The biofilm microenvironment is typified by a wide range of biological, chemical and physical factors (Korber *et al.*, 1999). Biofilms can be considered as microecosystems in which different microbial strains and species efficiently cooperate in order to protect themselves against environmental stresses and to facilitate more efficient nutrient uptake (Gottenbos *et al.*, 1999). Such complex associations between microbial community members within the biofilms have allowed for the growth of attached organisms in a wide variety of environments. The interactions that occur between biofilms and their physical and chemical micro- and macro environments determine the manner and success whereby these biological systems cycle nutrients,

degrade toxins, survive in hostile macro environments and resist antimicrobial agents (Korber *et al.*, 1999).

Studies revealed that different populations are restricted to well-defined depth intervals. It is likely that this highly structured distribution is intimately linked to the flow of substrates through the community, this suggests the general hypothesis that each phylogenetic group fill a general community function, and that individual members within a group are then specialized within that general role (Risatti *et al.*, 1994). A general principle defining the structure of any microbial community is that the end products of metabolism by one microbial population are often utilized by another (Risatti *et al.*, 1994). Biofilms can therefore be considered as microecosystems in which different microbial strains and species efficiently cooperate in order to protect themselves against environmental stresses and to facilitate more efficient nutrient uptake (Gottenbos *et al.*, 1999).

Within the biofilm, mixed-species microcolonies may form. This allows metabolically cooperative species to benefit from interspecies substrate exchange and mutual end product removal (Costerton *et al.*, 1994). This level of structural organization and metabolic specialization within the biofilm make sustained metabolic cooperation possible, this may explain the existence of anaerobes within aerobic biofilms (Costerton *et al.*, 1994). Biofilm bacteria are therefore distributed where the availability of nutrients and the concentrations of end products are optimal, this may depend on the metabolic activity of neighbouring cells (Costerton *et al.*, 1994).

Biofilms can support a wide range of bacterial species, this is due to the development of different microhabitats within the biofilm (Santegoeds *et al.*, 1998). Due to resistance to mass transfer; microzonations develop within the biofilm, providing a broad range of niches for bacteria with different physiological characteristics (Santegoeds *et al.*, 1998). SRB may therefore also be important in aerobic biofilms, as they can proliferate in anaerobic areas of the biofilm (Santegoeds *et al.*, 1998).

Despite a typical thickness of only a few millimetres, a vertical zonation of respiratory processes can be found in biofilms (Kuhl & Jorgensen, 1992). Experiments done

showed that oxygen penetrated only 0.4mm into the biofilm due to high oxygen respiration in the upper layers where the organic substrate concentration was the highest (Kuhl & Jorgensen, 1992). High oxygen respiration also occurred at a narrow zone where sulphide was oxidized, about 0.2-0.475mm below the biofilm surface (Kuhl & Jorgensen, 1992). Sulphide was produced by sulphate reduction 1.05 to 2.15mm below the biofilm surface; sulphate only partially penetrated the biofilm and was depleted at the bottom of the sulphate reduction zone. Sulphate oxidation and sulphate reduction were found in two spatially separated zones (Kuhl & Jorgensen, 1992).

The development of anaerobic zones in the biofilm created an optimal environment for sulphate reducers. Reduced chemical species (sulphide) generated in the anoxic bottom layers of stratified water bodies are rapidly oxidized by populations at the oxic-anoxic interface (Minz *et al.*, 1999; Santegoeds *et al.*, 1998). Reduced substrates diffuse from the permanently anoxic region, providing electron donors to support an active respiratory population within the chemocline (Minz *et al.*, 1999).

1.6.3 The Mechanism of Biofilm Formation

The formation of a biofilm in an aqueous environment is thought to proceed as follows:

1. when organic matter is present a conditioning film of adsorbed components is formed on the surface prior to the arrival of the first organisms;
2. microorganisms are transported to the surface through diffusion, convection, sedimentation, or active movement;
3. initial microbial adhesion occurs;
4. attachment of adhering microorganisms is strengthened through exopolymer matrix production and unfolding of cell surface structures;
5. surface growth of attached microorganisms and continued secretion of exopolymer matrix occurs. (Gottenbos *et al.*, 1999).

Microbial adhesion is mediated by specific interactions between cell surface structures and specific molecular groups (Gottenbos *et al.*, 1999). The primary adhesion of bacteria to surfaces is governed by cell surface-to-surface and substrate-to-surfaces changes and hydrophobicities (Ahearn *et al.*, 1999).

1.6.4 Problems with Biofilm Study

The examination of native biofilm communities is complicated by the difficulties in identifying constituent biofilm members *in situ*, in quantifying physical, chemical and spatial aspects of biofilms and in linking processes and activity with specific biofilm bacteria (Korber *et al.*, 1999). Traditional studies of microbial communities are incomplete because of the inability to identify and quantify all contributing populations (Risatti *et al.*, 1994).

Phylogenetic relationship is a very important part of an ecological study. Phylogenetic dimension is taken for granted in the macroecological world where animals and plants are obviously different (Risatti *et al.*, 1994). Their diverse morphologies make distinguishing orders, families and genera quite straightforward. In contrast distinguishing differences in the microbial world are more complex despite their rigorous metabolic and phylogenetic diversity. Even when the environment contains bacteria less closely related to each other than animals are to plants, this is not apparent from direct microscopic examination (Risatti *et al.*, 1994).

The characterization of biofilm populations is difficult as not all natural populations are amenable to pure-culture isolation (Amann *et al.*, 1992). In addition, spatial heterogeneity and aggregation compromise culture enumeration. Even if some bacteria do grow on defined media the phenotype expressed in pure culture does not necessarily reflect the phenotype expressed under natural conditions (Risatti *et al.*, 1994).

1.7 The elucidation of the members of the biofilm population

In natural systems biofilms are often located in places that are difficult to access, this makes the direct analysis of the individual organisms very complicated (Christensen *et al.*, 1999). Why use molecular techniques to study microbial diversity and ecology? These techniques have allowed the development of tools to address a central problem in microbial ecology, the inability to cultivate more than a small percentage of the bacteria that can be visualized by direct count procedures (Head *et al.*, 1998).

Ecological studies of mixed-species biofilms require the localization of members of specific bacterial populations in relation to others, as well as the activity of individual cells (Korber *et al.*, 1999). In the past the only way of obtaining any information was by microscopy and this did not provided much information. One of the disadvantages of microscopic work is the subjectivity of morphological data, this is because images must be selected but an advantage is that it is direct and therefore unaffected by extrapolation (Costerton *et al.*, 1994). The morphology of microorganisms is however too simple to serve as a basis for accurate classification and allow reliable identification (Amann *et al.*, 1995).

A reliable method for determining the diversity of microbes in an environmental sample has long been sought by microbiologists (Ferris *et al.*, 1996). Microscopy has limited usefulness since diverse microorganisms can share simple morphologies and cultivation limits detection to organisms that grow under the defined conditions of culture media (Ferris *et al.*, 1996).

More detailed information on biofilm population structure was obtained using disruptive techniques where cells were sampled and plated out on selective media in order to isolate pure cultures. The pure cultures then underwent testing for multiple physiological and biochemical traits in order to identify them but this method was inaccurate considering that the majority of bacteria cannot be cultured on defined media. Conventional microbial techniques based on selective culturing are therefore of limited usefulness for quantification and characterization of environmental populations, as it is widely recognized that culture media poorly represent natural growth conditions or because different strains of microorganisms are interdependent (Santegoeds *et al.*, 1998).

Also the majority of microscopically visualized cells are viable but do not form visible colonies, these are either known species for which applied cultivation conditions are just not suitable or which have entered a nonculturable state and unknown species that have never been cultured before due to a lack of suitable methods (Amann *et al.*, 1995). No information on the spatial relationships of the different bacteria could be achieved using this disruptive technique.

Determining the physiological and biochemical properties of a single species in a laboratory pure culture may also bias the phenotype since gene expression is strongly influenced by environmental constraints and the growth mode of the cells. Since isolated strains are known to adapt genetically to the prevailing environmental conditions they may also not be genetically representative of their environmental counterparts (Lee *et al.*, 1999). With the advent of rRNA techniques in microbial ecology, cultivation-independent examination of the structure and dynamics of complex microbial communities became possible (Lee *et al.*, 1999). The application of molecular methods has revolutionized the routine identification of bacteria from environmental and industrial samples (Korber *et al.*, 1999).

Techniques based on the analysis of genetic material complement the conventional microbiological approach and are routinely used to determine the presence and distribution of individual bacterial species, including those in complex communities like bacterial biofilms (Santegoeds *et al.*, 1998). One of the problems associated with molecular work is the extraction of nucleic acids.

1.7.1 Limitations of nucleic acid extraction

The quantitative recovery of nucleic acids from environmental samples imposes major limitations. These problems are caused by spores being less readily lysed than vegetative cells and gram-positive cells being more resistant to cell lysis than gram-negative cells (Head *et al.*, 1998). It has been demonstrated that a combination of physical and chemical treatments such as freeze thawing, lysis with detergents and bead beating lysed approximately 96% of soil bacteria, smaller cells seem to be more resistant to lysis (0.3-1.2μm) (Head *et al.*, 1998). It was also found that even without bead beating, up to 99.8% lysis could be achieved by merely using extended lysis incubations and up to 6 freeze thaw cycles (Head *et al.*, 1998).

One of the major obstacles encountered in studying the ecology of these organisms is the difficulty involved in isolating, identifying and enumerating individual species and strains with similar metabolic requirements (Goebel & Stackebrandt, 1994). The application of 16S rRNA sequence analysis has revolutionized the study of both microbial ecology and phylogeny (Goebel & Stackebrandt, 1994). Amann and co-

workers, 1996, successfully used 16S rRNA technologies to monitor the development of a mixed population biofilm of SRB in a continuous anaerobic bioreactor. Several rRNA-based methods have been developed to identify and quantify microorganisms in complex environments. These methods can be used without isolation and cultivation.

The earliest attempts to analyze the diversity of naturally occurring microbial populations began about a decade ago and relied on the direct extraction, purification and sequencing of 5S rRNA from environmental samples. But the limited length of the 5S rRNA molecule (120bp) does not allow for high resolution analysis, these were only used to study low diversity communities (Head *et al.*, 1998). 16S rRNA molecules consist of ~ 1500-nucleotides and 23S rRNA molecules of ~ 3000-nucleotides, both of these molecules therefore contain sufficient information for a reliable phylogenetic analysis of more complex communities (Amann *et al.*, 1995).

Ribosomal RNA is a suitable target for primers as ribosomes are ubiquitous, ribosomal sequences are functionally conserved molecules and are non-transferable between species, the primary structures of 16S and 23S rRNA are composed of regions of high and low evolutionary conservation (Korber *et al.*, 1999). The V6 hypervariable region is the optimal primer target because it has the highest average phylogenetic variability and in contrast to other variable regions is flanked by conserved sites which are suitable for the annealing of eubacterial primers (Heuer *et al.*, 1999).

If microbial species are to be distinguished, primer sequence can be adjusted to target the most variable regions. Extensive rRNA sequence databases are available, especially for 16S rRNA, allowing easy identification of species using computer programs (Korber *et al.*, 1999). Ribosomes occur in high copy number in most species (Korber *et al.*, 1999).

The rRNA approach, together with other molecular techniques holds great potential for an analysis of microbial diversity which is unbiased by the limits of pure-culture techniques (Amann *et al.*, 1995).

The Polymerase Chain Reaction (PCR) is used to speed up the process, by using PCR 16S rRNA gene fragments can be selectively amplified from mixed DNA (Amann *et al.*, 1995). Gene libraries derived from mixed amplification products should contain only defined fragments which can be rapidly sequenced from known priming sites, this approach reduces the need for lengthly screening procedures which are necessary to identify the rRNA containing clones in shotgun libraries (Amann *et al.*, 1995).

1.7.2 Biases and limitations of PCR

But PCR amplification introduces a bias in that it is not quantitative, and preferential amplification of certain templates may occur so that the representative assessment of natural abundance of rRNA genes would be inaccurate (Santegoeds *et al.*, 1998). Relating community analysis obtained by molecular techniques to processes occurring within microbial consortia is of great practical relevance but the techniques currently available are insufficient to accurately predict the behaviour of such a complex community (Santegoeds *et al.*, 1998).

Selectivity in PCR amplification of rRNA genes is a source of bias that can affect the results of molecular biological measures of diversity (Head *et al.*, 1998). Small differences in the sequence of universally conserved regions may result in selective amplification of some sequences, particularly when primer annealing is at high stringency (Head *et al.*, 1998). The frequency of different sequence types in PCR products has been assumed to represent the relative abundance of different components of a microbial community, this is incorrect however as the copy number of rRNA genes present within the genomes of different bacteria can range from 1 to 14 (Head *et al.*, 1998). There is also concern that less abundant sequences are discriminated against, and also that high %G+C templates are discriminated against due to lower efficiency of strand separation during the denaturing step of the PCR (Head *et al.*, 1998).

1.7.3 Formation of chimeric rDNA sequences

A potential risk of gene amplification by PCR from a mixed culture is the formation of chimeric sequences assembled from sequences of different species (Amann *et al.*, 1995). Two main factors will increase the occurance of chimeras. The availability of partial length rDNA fragments present in low molecular weight genome DNA

preparations or generated by premature termination of elongation during PCR. Also the percentage of highly conserved stretches along the primary structure of rDNA where, after denaturation, single strands originating from different rDNA can anneal in highly complementary regions (Amann *et al.*, 1995).

Biases can also occur during the cloning step, different cloning efficiencies for rRNA fragments from different organisms can not be ruled out (Amann *et al.*, 1995).

Quantification of the relative abundance of certain populations from the relative abundance of certain rDNA clones will always be biased because the number of rRNA gene operons present in a bacterial chromosome vary between species (Amann *et al.*, 1995). In order to decrease the bias introduced by PCR and cloning alone another technique was sought.

1.7.4 Denaturing Gradient Gel Electrophoresis

Denaturing Gradient Gel Electrophoresis (DGGE) is frequently applied in microbial ecology to compare the structure of complex microbial communities and to study their dynamics (Heuer *et al.*, 1999). The steps in the procedure are extraction of genomic DNA from environmental samples, amplification of 16S rRNA genes (16S ribosomal DNA) in PCR and electrophoretic separation of PCR products with differing sequences in a linear gradient of denaturants (Heuer *et al.*, 1999). This allows for the analysis of many samples which is essential when studying spatial and temporal variations of microbial community structures in relation to environmental factors and shifts due to perturbation or experimental treatment (Heuer *et al.*, 1999).

The 16S rDNA sequence divergence of different bacterial species has been exploited as an indicator of diversity (Muyzer *et al.*, 1993). To assess this diversity, PCR amplification, cloning and sequence analysis have been used but these are expensive and time consuming and species present in low numbers are not likely to be detected (Muyzer *et al.*, 1993). A direct approach for directly determining the genetic diversity of complex microbial populations must be taken. In denaturing gradient gel electrophoresis (DGGE) DNA fragments of the same size are separated depending on their GC content (Muyzer *et al.*, 1993).

Separation in DGGE is based on the electrophoretic mobility of a partially melted DNA molecule in polyacrylamide (Muyzer *et al.*, 1993). GC-rich sequences can be incorporated into one of the primers (GC-clamp primers) in order to modify its melting behaviour and prevent the double stranded DNA from separating completely. Individual DNA molecules denature along their length adjacent to the GC clamp according to their melting characteristics, the melting characteristics depend on the sequence of the fragment as AT denature at a lower temperature than GC (Ferris *et al.*, 1996). Once the melting point of the fragment is reached the migration of the fragment in the gel stops. Sequence variants of particular fragments therefore stop migrating at different positions (Muyzer *et al.*, 1993). DGGE analysis of 16S rRNA has been used to profile the members of complex communities and to infer the phylogenetic affiliation of the community members (Ferris *et al.*, 1996).

DGGE analysis of different biofilm samples demonstrated the presence of many distinguishable bands, most likely derived from as many different bacterial species (Muyzer *et al.*, 1993). Information can also be inferred from the analysis of banding patterns (Ferris *et al.*, 1996). Band intensities cannot be used to infer the abundance of a particular bacterial population (Santegoeds *et al.*, 1998). Also, the number of bands appearing on the DGGE depends not only on the number of different bacterial species but also on the amount of DNA loaded (Santegoeds *et al.*, 1998). Individual bands from DGGE fingerprints can have their sequences determined and be phylogenetically analysed, this removes the biases introduced by shotgun cloning of PCR products (Heuer *et al.*, 1999). Fingerprinting of bacterial communities by separation of amplified 16S rDNA fragments with DGGE provides the opportunity to compare community structure of multiple environmental samples.

It is essential to initially identify a band by sequencing because some sequence types migrate to similar positions. The position of the band in the gel alone is therefore not a reliable means of identification. This can be solved by narrower gradients or longer gels (Ferris *et al.*, 1996). Because DGGE bands are sequenced directly this removes the bias introduced through cloning, it also eliminates lengthly screening of redundant clones this all serves to increase the number of samples that are able to be processed, all of which enables a more aggressive pursuit of the ecology of natural populations (Ferris *et al.*, 1996).

DGGE detects the occurrence of sets of phylogenetically related populations as a consistent pattern of community structure (Ferris *et al*, 1996).

The main reason that spatial and temporal heterogeneity of bacterial populations has not been thoroughly investigated is because of the labour-intensiveness of most traditional techniques, as a result information comes from a limited sample set. DGGE will provide a faster approach to screening large numbers of samples allowing for rapid assessment of the populations making up complex communities (Ferris *et al.*, 1996).

DGGE provides an immediate display of the members of a population in both a qualitative and semiqualitative way (Muyzer *et al.*, 1993). If further analysis is required DNA fragments can be excised from the gel, reamplified and sequenced directly, this makes DGGE analysis a rapid and efficient approach for the analysis of mixed microbial populations form natural environments (Muyzer *et al.*, 1993). By using very narrow gradients, high resolution results can be achieved. The DGGE profiling method can be useful for diagnosing the presence and relative abundance of microbes and it will aid us in understanding the genetic diversity of complex microbial populations (Muyzer *et al.*, 1993). Isolation has been facilitated by the use of the polymerase chain reaction (Amann *et al.*, 1992). The use of mechanical disruption, offers the most unbiased release of nucleic acid from a diverse community (Amann *et al.*, 1992).

1.8 Research Objectives

Previous work indicated that floating sulphur biofilms may provide a useful basis for biotechnological bioprocess development which would provide an alternative to high-cost sulphide oxidation processes like Thiopaq. Floating sulphur biofilms would be suitable for large volume flow wastewater treatment and also passive systems.

Very little information is available on floating sulphur biofilms, possibly due to the difficulties involved in their study. Previous studies suggested that they were complex differentiated structures with high levels of microbial activity. There was a great need to understand these systems in order to proceed with process development.

The objectives of this study were to develop laboratory methods to reproduce and study the floating sulphur biofilm systems. These research objectives included the development of a low cost reactor system with high biomass retention and oxidation capacity, identification of control parameters for the production of sulphur and the evaluation of environmental factors.

To analyse the conditions in these model systems, and determine whether structural and functional differentiation occurred. To elucidate the species compositions using molecular techniques and to compare the species found in the model systems to those found in natural populations. And finally to determine the processes underlying the formation of these biofilms.

CHAPTER 2:

FLOATING SULPHUR BIOFILMS

2.1 Introduction

Under oxic conditions sulphide rapidly oxidizes spontaneously at neutral pH. Sulphur-oxidizing bacteria are able to catalyse the oxidation of sulphide but due to the rapid spontaneous reaction, bacterial oxidation occurs only in areas where gaseous sulphide rises from the anoxic zone and meets oxygen descending from the oxic areas (Brock & Madigan, 1991).

SOB can convert sulphide into extracellular or intracellular sulphur which is not autooxidizable, by doing this they withdraw their energy source from the chemical oxidation process (Steudal, 1998). The formation of these sulphur globules by SOB is reversible; if no reduced S compounds are available then the elemental sulphur is oxidized to sulphate to produce energy and to reduce CO_2 for biomass production (Steudal, 1998).

Sulphur bacteria metabolism is under intense study since it has been demonstrated that they may be used economically to remove poisonous or otherwise unwanted sulphur compounds from industrial waste gases or waters (Steudal, 1998). For example, sulphate may be reduced enzymatically to sulphide utilizing cheap organic substances as reductants. Other bacteria can then be used to oxidize the sulphide with air to elemental sulphur that can be recovered. It is hoped that these developments will soon be available on a large enough scale to be used as an alternative to the processes operated today, which are not always perfect from an ecological perspective (Steudal, 1998).

In most natural and bio-engineered systems with a sufficient nutrient supply bacteria occur as spatially organized, matrix-enclosed, multispecies communities, biofilms rather than as single planktonic cells (Lee *et al.,* 1999). Bacterial biofilms have been observed to form on the surfaces of sulphur lakes and certain sulphidic effluent ponds; this phenomenon formed the basis for the development of model laboratory systems for sulphur recovery from sulphide containing wastewater.

These biofilms are thought to oxidize hydrogen sulphide at the liquid/air interface. When the hydrogen sulphide approaches the air/liquid interface it comes into contact with the correct redox conditions for SOB to oxidize the sulphide into sulphate and under the correct conditions, sulphur. Most of the sulphide that accumulates in the environment is formed by the reduction of sulphate by SRB but some originates from the decomposition of organic matter (Brown, 1982).

Biofilms were observed to form naturally on Tannery Stabilization Ponds (TSP) at Wellington (Dunn, 1997). These ponds were high in sulphate and the biofilms were postulated to contain SOB, which were producing sulphur (Dunn, 1997). Hardly any information is available on these biofilms in literature and because they may form the basis for bioprocess development there was a need for further study. The first objectives in this study were then to confirm the occurrence of these natural biofilms and then to create model systems, which mimicked the natural conditions encouraging the growth of these biofilms in order to facilitate further study in the laboratory.

The first model system used was the Baffle Reactor. The Baffle Reactor was designed to operate according to the same principles as the natural system (Gilfillan, 2000). In the natural system it was postulated that a SRB layer in the mud at the bottom of the TSP reduced sulphate into hydrogen sulphide which then diffused towards the surface of the TSB where it was oxidized to sulphur by SOB.

The Baffle Reactor was seeded with sludge from the sulphidogenic upflow anaerobic sludge bed (UASB) reactor. This sludge consisted of well-settled microbial aggregates that develop by mutual attachment of bacterial cells, these aggregates contain a variety of bacterial species involved in the anaerobic degradation of organic matter, including hydrolytic, fermentative, acidogenic, acetogenic, methanogenic bacteria and a well established population of SRB (Santegoeds *et al*, 1999).

The bacteria present in the sludge layer used a simple carbon source, lactate, which was present in the SRB lactate media (Appendix 1**)** to reduce sulphate into hydrogen sulphide. The hydrogen sulphide then diffused towards the air/liquid interface where it was oxidized to sulphate and sulphur by a SOB population, resulting in the formation of a floating sulphur biofilm.

In order to gain a better understanding of this complex system the chemical characteristics of this model system were studied in depth. Daily sulphate, sulphur, sulphide, pH and TOC samples were taken and analysed and were used to draw conclusions as to what was occurring in this complex system. Bacterial samples were also taken for later DNA analysis and identification.

2.2 Methods

2.2.1 The Tannery stabilization pond

Field observations were performed at the TSP at Wellington, these observations included a series of photographs that were taken as the biofilm formed on the surface of the TSP. Samples were collected from the liquid layer at the surface of the TSP at Wellington on the 12th March 2001. These triplicate samples included part of the biofilm and were taken by breaking a sterile petri dish in half and using it to scoop samples into sterile Eppendorf tubes. The samples were taken in triplicate from the edge of the TSP where the biofilm was well developed. These samples were used for sulphate, sulphur, sulphide, TOC and pH analysis, part of the sample was also stored for molecular analysis. Samples for molecular analysis were made to 30% glycerol and stored at -70^{o}C.

2.2.2 Setting up and sampling of the Baffle Reactor

The first model system used to grow floating sulphur biofilm was the Baffle Reactor developed by Gilfillan (2000) (Figure 2.1).

Approximately 9L of sludge was collected from the Upflow Anaerobic Sludge Bed (UASB) Reactor at the Grahamstown sewage works. This sludge was poured into the Baffle Reactor and allowed to settle overnight. The Baffle Reactor was then fed SRB-Lactate medium (Appendix 1) at a flow rate of 2l/day. The Baffle Reactor was covered with a lid to restrict oxygen flow and tin foil so that no photosynthetic bacteria could establish themselves.

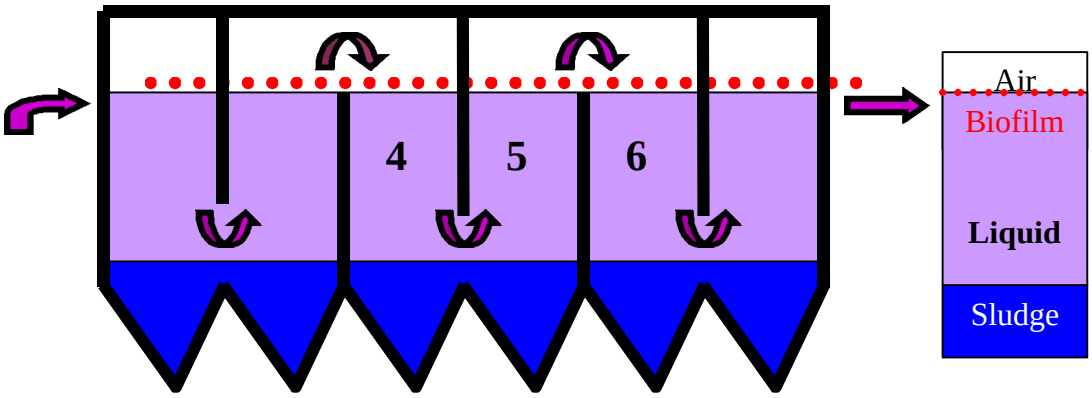

Figure 2.1: The laboratory-scale Baffle Reactor

The arrows (from left) indicate SRB lactate medium flowing into the reactor. In the first well, the medium was forced to flow downwards towards the sludge layer and then upwards, towards the air/liquid interface. The medium then flowed through the biofilm over the baffle and then down towards the sludge layer. This trend of flow occurred through out the rest of the reactor and then the excess medium flowed out the outflow pipe. Wells 4, 5 and 6 represent the positions where samples were taken for chemical analysis.

The Reactor was photographed periodically in order to monitor the development of the biofilm and liquid samples were taken in triplicate from the reactor every second day. These samples were taken from Wells 4, 5 and 6 at a depth of approximately 3 cm. The samples were not taken from the biofilm itself as the biofilm was not a homogenous structure and these samples would therefore have been inaccurate. Taking samples from this depth enabled one to establish exactly what conditions were prevalent in the liquid before it entered the biofilm and then after it exited the biofilm layer, this would allow useful insights as to what processes were occurring within the biofilm. These samples were used for sulphate, sulphide, sulphur, TOC and pH analysis, samples were also stored for molecular analysis.

2.2.3 Sulphate analysis

Sulphate analysis was performed using High Performance Liquid Chromatography (HPLC). The samples were diluted 1 in 10 for sulphate analysis and then 1ml of this sample was filtered through a 0,45µ nylon filter and then a Waters C18 solid-phase extraction cartridge.

The Waters C18 solid-phase extraction cartridge was activated by running 1ml of triple distilled water ($dddH_2O$) through it slowly, followed by 1ml of methanol and then the column was rinsed with 1ml of $dddH_2O$. The cartridge was then shaken dry and the samples were filtered twice through the cartridge. The cartridge was regenerated each time using the activation method described above. These filtration steps were done to remove excess organics that would interfere with the HPLC or damage the HPLC column.

The samples were run on a Waters model 510 pump**,** the HPLC column used was a Hamilton PRP-X100 10um 150x4.1mm column**.** The detector was a Waters 430 Conductivity Detector and the injection port was a Rheodyne injection port. The software used for data analysis was BORWIN version 1.5x. A standard curve was prepared using sodium sulphate. The 20µl samples were run at a flow-rate of 2ml/min, the sulphate peak had a retention time of approximately 10 minutes and the mobile phase is detailed in Appendix 1.

2.2.4 Sulphide analysis

The 100µl sample for sulphide analysis was added to 100µl of zinc acetate (Appendix 1), this was then made up to 5ml with $dddH_2O$. The samples were vortexed briefly and 500µl of Ferric chloride (Appendix 1) and 500µl of amide-sulphuric acid stock solution (Appendix 1) was added. The samples were allowed to stand at room temperature for 1 hour and then the absorbance was read on a Shimadzu UV-160A UV-visible recording spectrophotometer at 670nm.

The blanks for the spectrophotometer were prepared as above except 100µl of $dddH_2O$ instead of sample was added. A standard curve ranging from 0-10mg/l was prepared from a 100mg/l sulphide stock solution.

2.2.5 Sulphur analysis

500µl samples were used for sulphur analysis. Each sample was placed in an eppendorf tube and microfuged at 13 000rpm in a F45-24-11 rotor in an Eppendorf centrifuge 5415D for 10 minutes, the supernatent was discarded and then the pellet was resuspended in 1ml of HPLC grade acetone. The sample was allowed to stand

for 1 hour and then it was filtered through a 0.45µ nylon membrane to remove any particulate matter.

The samples were analysed using HPLC. The samples were run on a Waters model 510 pump**,** the HPLC column used was a Phenomenex LUNA 5µ C18 (2) size 150x4.6mm column**.** The detector was a Waters 484 Tunable Absorbance Detector and the injection port was a Rheodyne injection port. The software used for data analysis was BORWIN version 1.5x. A mobile phase was 95:5 Methanol: water (Appendix 1), this was run at a flow rate of 2ml/min.

2.2.6 Total Organic Carbon Analysis

Samples for TOC analysis were microfuged for 10 minutes at 13 000rpm in a F45-24-11 rotor in an Eppendorf centrifuge 5415D. The supernatant was transferred to a fresh tube and injected into a ROSEMOUNT Dohrmann Total Organic Carbon Analyzer DC-180**.** The TOC buffer and TOC standard are detailed in Appendix 1.

2.2.7 pH Analysis

1ml samples for pH analysis were placed in Eppendorf tubes and pH readings were taken using the CyberScan pH 2000 pH meter.

2.3 Results

2.3.1 Visual observations of floating sulphur biofilm on the surface of TSP.

The development of a mature floating sulphur biofilm was observed on the surface of a TSP at Wellington in the Western Cape, Figure 2.2.

Figure 2.2 shows the initial stages of biofilm formation, this phenomenon was viewed on a sunny windless day and the entire process occurred in under an hour. Initially there were dispersed clumps of white biofilm visible on the surface of the water, then a thin white scum layer started to develop over the entire surface of the pond (Figure 2.3), this layer became thicker and thicker until the biofilm shown in Figure 2.4 was properly developed.

Figure 2.2: The initial stages of biofilm formation on the TSP at Wellington**.**

Figure 2.3: The intermediate stages of biofilm formation on the TSP at Wellington.

Figure 2.4: The final stages of biofilm formation of the TSP at Wellington.

These observations led to the hypothesis that sulphur biofilms occur naturally on sulphidic waters and led to the development of model laboratory systems for sulphur recovery from sulphide containing waste water. The first of these model systems was a baffled reactor (Figure 2.1).

2.3.2 Chemical analysis of the samples from the TSP

Table 2.1: The prevailing chemical conditions within the TSP.

Sulphate analysis	Sulphide analysis	Sulphur analysis	TOC analysis	pH analysis
*	52mg/l	46mg/l #	5230ppmC	9.23

* Sulphate analysis could not be performed on samples taken from the TSP at Wellington, as the high salinity interfered with the HPLC analysis of the sample. Sample dilution was attempted but the sulphate peak was reduced below detection levels before the salt peak was eliminated. Sulphate levels measured by Dunn (1997) were however found to be 943mg/l.

The sulphur concentration of the TSP was found to be 46mg/l, this sample was not only supernatant, it did include part of the biofilm, during collection this was unavoidable.

2.3.3 Visual observations of floating sulphur biofilm formation on the Baffle Reactor.

The formation of this film was seen to occur within hours of the start up of the reactor. Figure 2.5 shows the formation of a thin white layer on the surface of the Baffle Reactor within 3 hours of start up, this is the initial stage of formation of the floating sulphur biofilm.

Figure 2.6 shows the Baffle Reactor 1 day after start up, floating sulphur biofilm was seen to be present in a thick white layer. The biofilm was observed to be thicker on the upflow wells in comparison to the downflow wells, this was possibly due to more sulphide being drawn up out of the sludge towards the surface of the upflow well with the flow of the feed, resulting in the biofilm forming faster in that position. This

supported the postulation that sulphide diffusing from the lower levels of the reactor is primarily responsible for the formation of the floating sulphur biofilms.

Figure 2.5: Biofilm formation on the surface of the Baffle Reactor within 3 hours of start up.

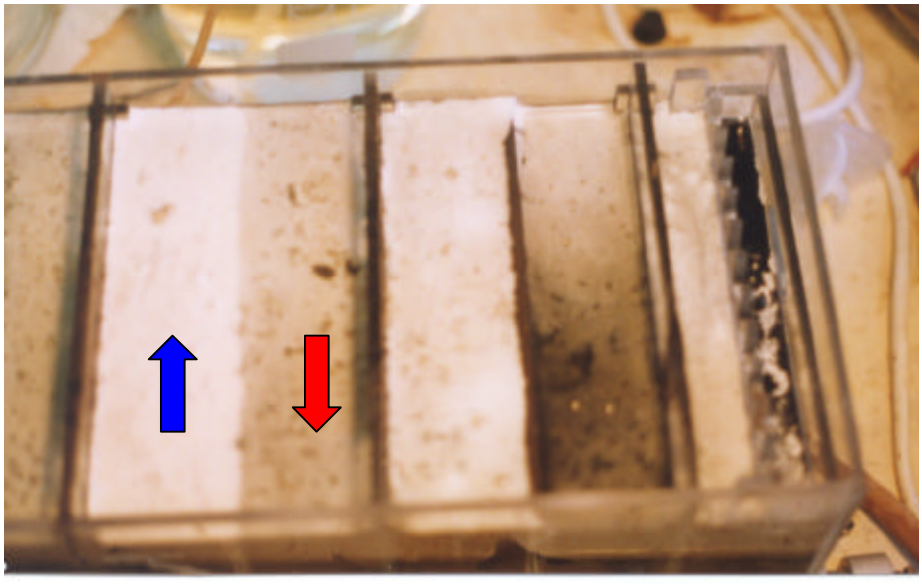

Figure 2.6: Floating sulphur biofilm forming on the surface of the Baffle Reactor 1 day after start up.

The blue arrow indicates and upflow well and the red arrow indicates a downflow well, the biofilm was observed to be thicker on the upflow wells.

Figure 2.7 shows the Baffle Reactor completely covered with floating sulphur biofilm, total coverage occurred within 2 days of start up of the Baffle Reactor.

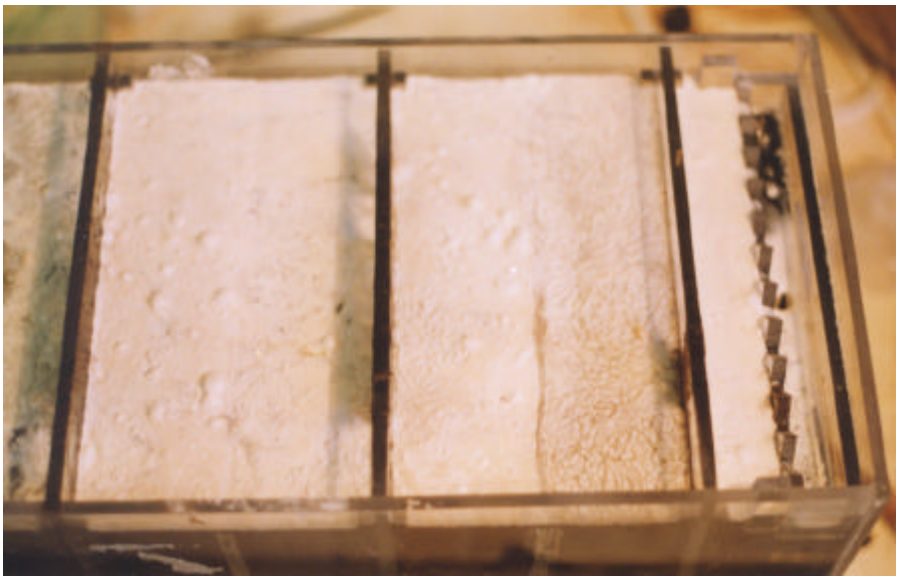

Figure 2.7: A Photograph of the floating sulphur biofilm on the surface of the Baffle Reactor 2 days after start up.

2.3.4 Sulphate Analysis

The sulphate concentration of the SRB lactate medium fed to the Baffle Reactor was approximately 1455mg/l. By the time that the feed had reached the first sampling point at well 4 the sulphate concentration had been reduced to 700mg/l on day 1 (Figure 2.8). For all three wells the sulphate concentration showed a general decreasing trend over time down to a final concentration of approximately 400mg/l on the day 14 (Figure 2.8). Well 4 consistently had the highest sulphate concentration, followed by well 5 and then well 6. This sulphate removal through the wells was expected as well 4 was the closest to the feed inlet.

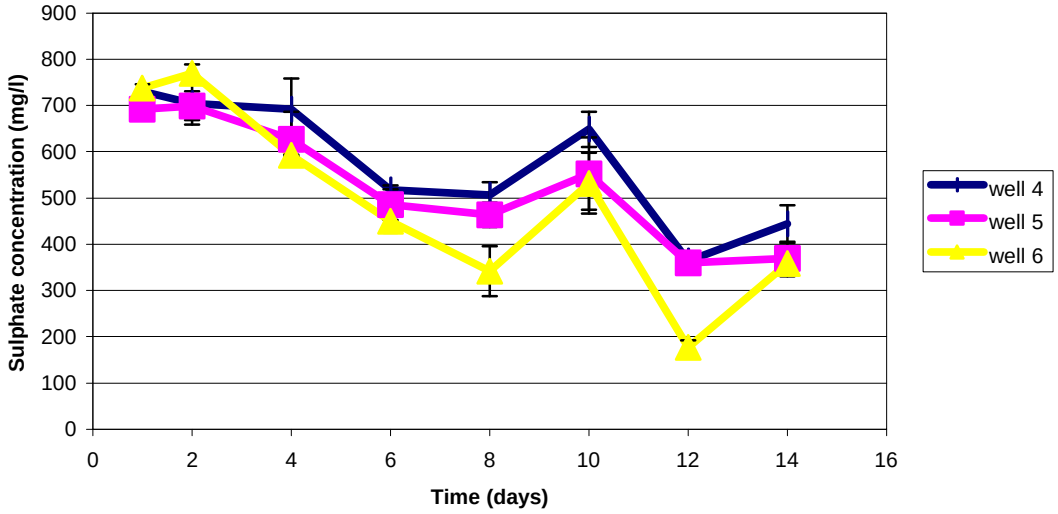

Figure 2.8: Sulphate concentration in Wells 4, 5 and 6 of the Baffle Reactor**.**

2.3.5 Sulphide Analysis

The sulphide concentration increased in the Baffle Reactor (Figure 2.9) from 17mg/l on day 1 to approximately 150mg/l on day 12. The sulphide concentration did however show a decrease on the final day, day 14, to approximately 100mg/l. The sulphide concentration was highest in Well 6 and lowest in Well 4, which could have been due to the accumulation of sulphide as the feed progressed through the reactor. On the day 14 the sulphide concentration in all of the wells showed a dramatic decrease.

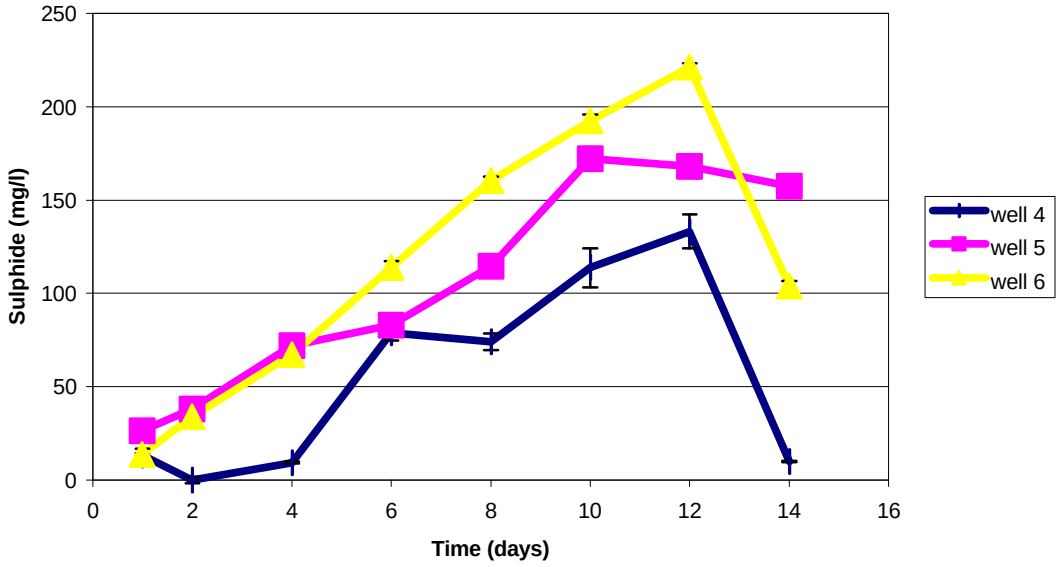

Figure 2.9: Sulphide Concentration of Wells 4, 5 and 6 of the Baffle Reactor

2.3.6 Sulphur Analysis

The sulphur analysis (Figure 2.10) showed an increase from approximately 20mg/l on day 1, to approximately 100mg/l on day 4 and then decreased to less than 20mg/l on the day 14. Well 6 had the highest sulphur concentration and Well 4 had the lowest sulphur concentration, possibly due to the sulphur accumulating in the media as it moved through the reactor.

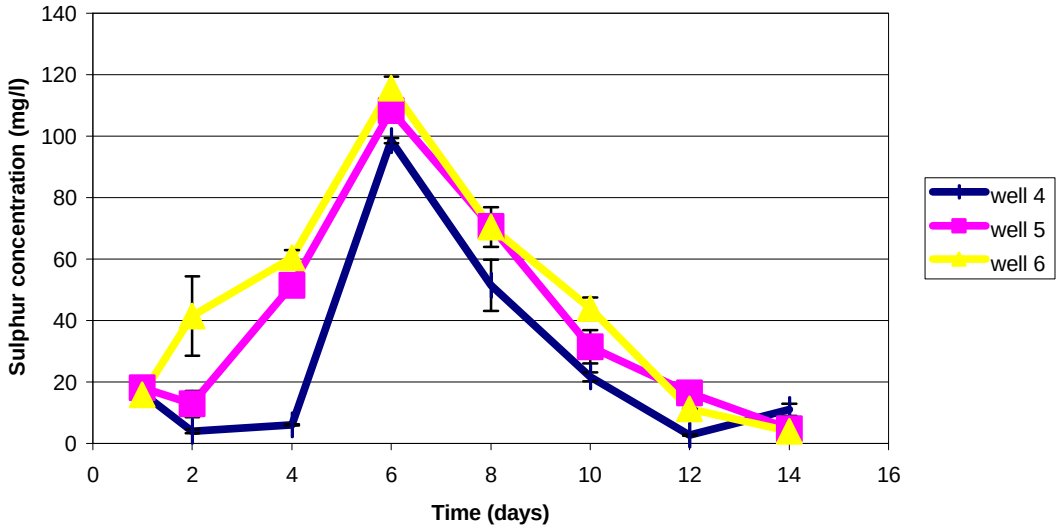

Figure 2.10: Sulphur concentration of Wells 4, 5 and 6 of the Baffle Reactor

2.3.7 TOC Analysis

Figure 2.11 showed that the TOC decreased from 1110 mg/l C to approximately 950mg/l C where it remained until day 12. The TOC decreased dramatically by day 14, this corresponded with the decline in sulphide levels.

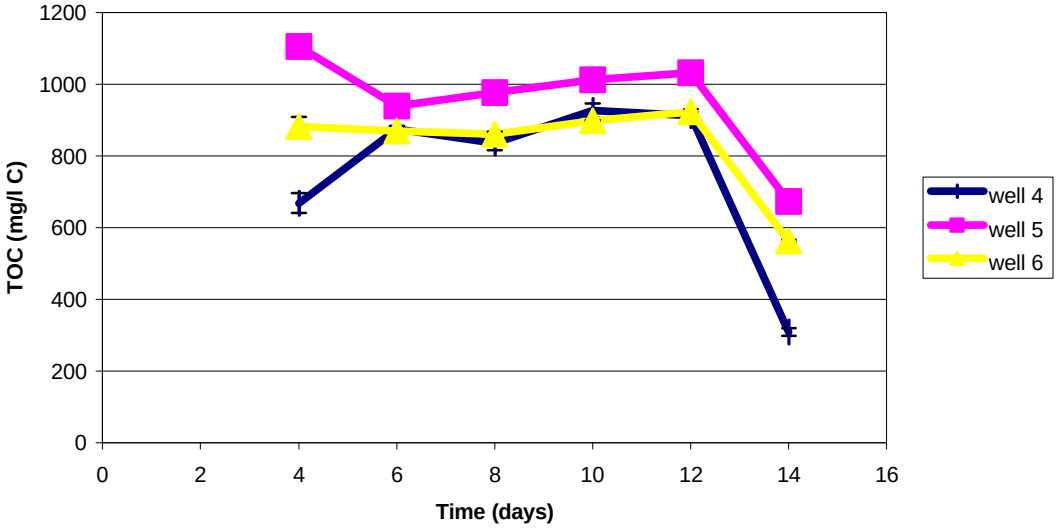

Figure 2.11: Total Organic Carbon of Wells 4, 5 and 6 of the Baffle Reactor**.**

2.3.8 pH Analysis

The pH profile in the Baffle Reactor showed a decline from a starting value of pH 7.3 to approximately pH 6.6 on day 8 (Figure 2.12). Thereafter the pH increased again to approximately 6.8. Well 6 had the highest values and well 5 had the lowest values.

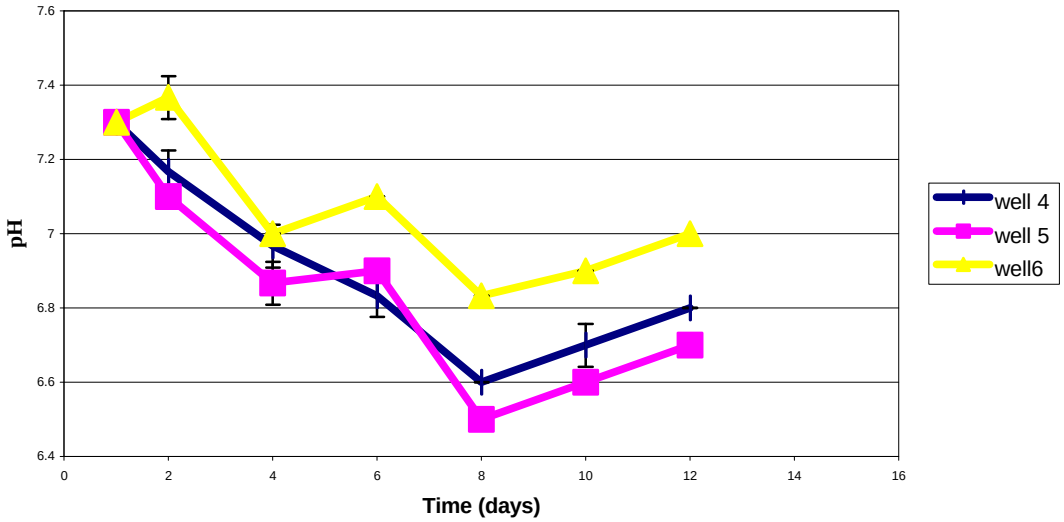

Figure 2.12: pH of Wells 4, 5 and 6 of the Baffle Reactor**.**

2.4 Discussion

The TSP was observed to develop a floating sulphur biofilm over the period of an hour, Figure 2.4. This floating film did not develop completely due to the effect of the prevailing weather conditions, wind interfered with the formation of the biofilm. The Baffle Reactor was observed to develop a complete floating sulphur biofilm within 3 days of start-up, refer to Figure 2.7**.** Analyses were performed on three wells of the Baffle Reactor every second day, these analyses included sulphate, sulphide, sulphur, TOC and pH. The same analyses were carried out on the single triplicate sample from the TSP.

The sulphate analysis of the Baffle Reactor, Figure 2.8, confirmed that an overall reduction in the sulphate concentration of the reactor was occurring. Figure 2.8 showed that by the time that the medium reached well 4 the sulphate concentration had decreased to approximately half of the total concentration of sulphate in the media.

Well 4 was always observed to have a higher sulphate concentration than well 5 and well 6. This may have been due to the fact that well 4 was closer to the inflow pipe through which the media containing a high concentration of sulphate was supplied to the reactor. The results suggested that as the medium moved through the reactor the sulphate was converted to other sulphur containing compounds by the bacterial species present within either the sludge or the biofilm.

The reactor also seemed to become more efficient over time at utilizing the sulphate in the medium. By day 14 the sulphate concentration in the wells was approximately 75% less than the sulphate concentration in the feed. These results suggest that the bacterial population either became more developed and efficient or that the bacteria just increased in number, resulting in them being able to utilize more of the sulphate present in the medium by day 14 when compared to day 1.

The sulphate concentration of the TSP was unable to be determined due to the high salt content of the pond but a previous study by Dunn (1997) proved that the pond had a high sulphate concentration of approximately 943mg/l. This sulphate concentration

was higher than the concentration found in the wells that were sampled from the Baffle Reactor but the SRB lactate medium did contain higher sulphate levels. The sulphate concentration within the TSP was therefore found to be in a similar range to the medium in the Baffle Reactor.

The sulphide analysis performed on the samples from the Baffle Reactor, refer to Figure 2.9, revealed that the sulphide concentration within the reactor increased over time. This suggested that the bacteria in the reactor were utilizing the sulphate present in the medium and other sulphur compounds present in the sludge and using it to produce sulphide. The bacteria responsible for this were most likely the SRB present in the sludge layer. Well 4 was observed to contain a lower concentration of sulphide when compared to well 5 and well 5 had a lower concentration of sulphide than well 6. This was thought to be due to the accumulation of sulphide in the medium as it travelled through the reactor. The sulphide concentration of all of the wells decreased towards the end (Figure 2.9), this coincided with the subsidence of the biofilm within the reactor and the degradation of the sludge layer which became thinner over time. It was postulated that with fewer SRB in the sludge layer producing sulphide, due to its degradation, that methanogenic bacteria started to outcompete the SRB's resulting in a change in the bacterial population within the reactor and ultimately the subsidence of the biofilm.

The sulphide concentration of the TSP was low (52mg/l) when compared to that of the Baffle Reactor (150mg/l), this could have been due to there not being a well-developed sludge layer present at the bottom of the pond, the SRB's were thought to be present in the thick black mud layer at the bottom of the pond. The low sulphide concentration would suggest that they were not present in significant numbers.

The sulphur analysis of the Baffle Reactor, refer to Figure 2.10**,** indicated that there was an initial increase in sulphur from the start-up of the reactor. This suggested that the bacteria present in the reactor were oxidizing the sulphide produced by the SRB in the sludge layer into sulphur. The analysis was not performed on the biofilm itself but on the underlying liquid layer, a lot more sulphur was probably produced and caught up in the biofilm either as intracellular sulphur or extracellular sulphur or sulphur crystals trapped in the matrix produced by the bacteria within the biofilm.

From day 6 to the shutdown on day 14, the sulphur concentration decreased to below 20mg/l. This suggested that with the increasing age of the biofilm, less free sulphur was present in the reactor system. The previous high levels of sulphur could have been reoxidized to sulphate but the sulphate analysis does not reflect this, alternatively most of the sulphur could have been held within the biofilm, either internally within the SOB or externally, trapped by the EPS matrix.

The sulphur analysis of the TSP sample revealed that the sulphur concentration of the sample was low, at approximately 46mg/l. The presence of both sulphur and sulphide in the samples that were analysed seem to suggest that similar processes were occurring in the TSP and the Baffle Reactor. Although the presence of sulphate could not be confirmed, it was assumed to be present (Dunn, 1997), this would then have been reduced to sulphide by the SRB present in the sediment layer at the bottom of the pond. The sulphide would then have risen towards the surface where it was oxidized to sulphur by SOB.

The TOC analysis of the Baffle Reactor, Figure 2.11, suggested that the TOC of the reactor system remained basically constant until just before the shutdown when the biofilm subsided. The sudden decline in utilizable carbon could have been responsible for the death of the biofilm and hence its subsidence. The TOC of well 5 was consistently higher than that of the other wells, this was due to the fact that it was an upflow well and therefore the flow of the medium up out of the sludge layer would have carried more carbon.

The TOC of the TSP samples was very high compared to that of the reactor, confirming that it contained far more organic carbon than the liquid layer of the Baffle Reactor. This result was expected as a large number of dissolved compounds were present in the wastewater while few were present in the reactor system

The pH analysis of the Baffle Reactor, refer to Figure 2.12, revealed that the pH of the reactor showed a general decreasing trend. The pH at start-up on the day 1 was 7.3 which was approximately the same pH as the SRB lactate medium, the pH decreased to a low of 6.4 on day 8 and then increased to approximately 6.8 on day 12 just before shut-down. This decrease in pH could be due to the production of sulphuric acid as a

result of the high levels of sulphide present in the reactor. The increase in pH just before shut-down may suggest the change-over from a sulphidogenic system to a methanogenic system. The pH of well 6 was consistently higher than the other wells, it being a downflow well may suggest that the biofilm is producing alkalinity, while the upflow well 5 generally had the lowest pH, suggesting that the sludge was contributing to overall acidity of the system. The increase in alkalinity nearing the end of the lifespan of the reactor coincides with the increasing degradation of the sludge layer and finally the subsidence of the biofilm.

The pH of the TSP was shown to be 9.23, which is quite high when compared to that within the Baffle Reactor but this was mainly due to all the alkaline compounds used during the tanning process.

The occurrence of floating sulphur biofilms in nature was confirmed by observations made at the TSP in Wellington. The process occurring in this system was postulated to be the reduction of sulphate to sulphide by SRB present in the anaerobic zone, the diffusion of this sulphide towards the surface where it was oxidized to sulphur by SOB which were found in the form of a biofilm.

The floating sulphur biofilms were only observed to occur on windless days, if too much mixing of the water layer occurred, it prevented the formation of a film. This indicated that the conditions at the surface of the water column had to be very specific in order to encourage the growth of the biofilm. This observation suggested that a stable chemocline had to occur at the surface, before the correct conditions for biofilm formation were provided.

The Baffle Reactor system was successfully used to mimic the processes occurring in the natural system and proved to be efficient at encouraging the growth of a floating sulphur biofilm. It was thought that whatever chemical gradient occurred on the surface of the TSP was mimicked by the conditions provided within the Baffle Reactor. This reactor system was so successful for the production of floating sulphur biofilms that it was scaled–up by other members of the research team and is now run at the Grahamstown sewage works as part of the Biosure process. A biofilm

harvesting mechanism is currently under development for the harvesting of sulphur from the biofilm, Figure 2.13.

Figure 2.13: The Scaled-up version of the Baffle Reactor**.**

2.5 Conclusions

The natural occurrence of floating sulphur biofilms on the surface of sulphate containing wastewaters was confirmed by observations made at a TSP at Wellington. It was postulated that SRB in the anaerobic layer of the TSP were reducing sulphate to sulphide which was then oxidized to sulphur at the surface of the pond by SOB which were present in a floating biofilm. The biofilm only formed in the presence of a stable chemocline. The Baffle Reactor was successfully used in the laboratory to mimic the processes occurring within the TSP in order to encourage the growth of a floating sulphur biofilm.

CHAPTER 3:

STRUCTURE OF THE BIOFILM

3.1 Introduction

Microbial biofilm communities are characterized by various cell-to-cell interactions and by pronounced architectural and chemical heterogeneity (Lee *et al.,* 1999). Wastewater biofilms are very complex multispecies structures displaying considerable heterogeneity with respect to both the microorganisms present and their physicochemical microenvironments (Okabe *et al.,* 1999). Due to the presence of multiple electron donors and acceptors present in the wastewaters, successive vertical zonations of predominant respiratory processes have been found to occur in close proximity to each other.

Chemical gradients occur at the surface of water bodies, bacteria within biofilms occurring in these areas may be distributed in different layers as oxygen and nutrient limitation occurs. Rate limiting nutrients establish physiological gradients causing complex patterns of population distribution (Xu *et al.,* 1998).

Compact biofilm communities are stratified with morphologically distinctive layers in which many different processes may occur simultaneously in close proximity, the biofilm is therefore effectively subdivided into functional layers (Birger-Ramsing *et al*., 1993). Each phylogenetic group fills a general community function and individual members within a group are then specialized within that general role (Risatti *et al.,* 1994).

Aerobic wastewater biofilms display a typical thickness of only a few millimetres (Okabe *et al.,* 1999) and this causes serious methodological problems in the study of structures.

The laboratory-scale Baffle Reactor described in Chapter 2 proved successful at simulating the conditions for floating sulphur biofilm formation but unfortunately did not allow for a better understanding of the ongoing processes within the biofilm or allow for closer study of the bacterial populations present. The biofilm that formed on

the surface of the Baffle Reactor was fragile and only a few millimetres thick which made its sampling and study a complex operation. Therefore new methods had to be devised in order to undertake the study of these complicated systems.

Preliminary studies by Gilfillan (2000) had suggested that floating sulphur biofilms were complex differentiated structures with bacterial populations distributed at different levels across the thin biofilm. In order to investigate the possible presence of a structural and functional differentiation in these systems and whether a chemocline was established, it would be necessary to expand the biofilm in such a way that would make meaningful sampling possible. A way had to be found to distribute the bacteria within the biofilm over a greater distance. As it was assumed that gradients form within the natural biofilms and that these microzonations influence the species distribution, the concept of gradient systems was developed. The Gradient Tube system (Figure 3.1) and the Silicon Tubular Bioreactor were developed for this purpose.

The first of these systems was the Gradient Tube system, Figure 3.1. This system was designed in an attempt to separate the bacterial species and bioprocesses spatially. The Gradient Tube consisted of a sulphide plug overlayed with agarose containing medium, which was inoculated with biofilm samples from the Baffle Reactor. In this way the sulphide plug would serve the same function as an SRB sludge layer, releasing sulphide towards the liquid/air interface. The sulphide in this case diffused out of the plug into the overlying media, creating a gradient of low oxygen and high sulphide at the bottom of the tube, and high-oxygen and low-sulphide at the top of the tube. If different bacterial species with specific chemical requirements were present these should have proliferated in the specific chemical niche that suited them, resulting in a separation of the different bacterial species along the gradient. This would have resulted in the 1-2mm thick biofilm being expanded over a distance of 12cm.

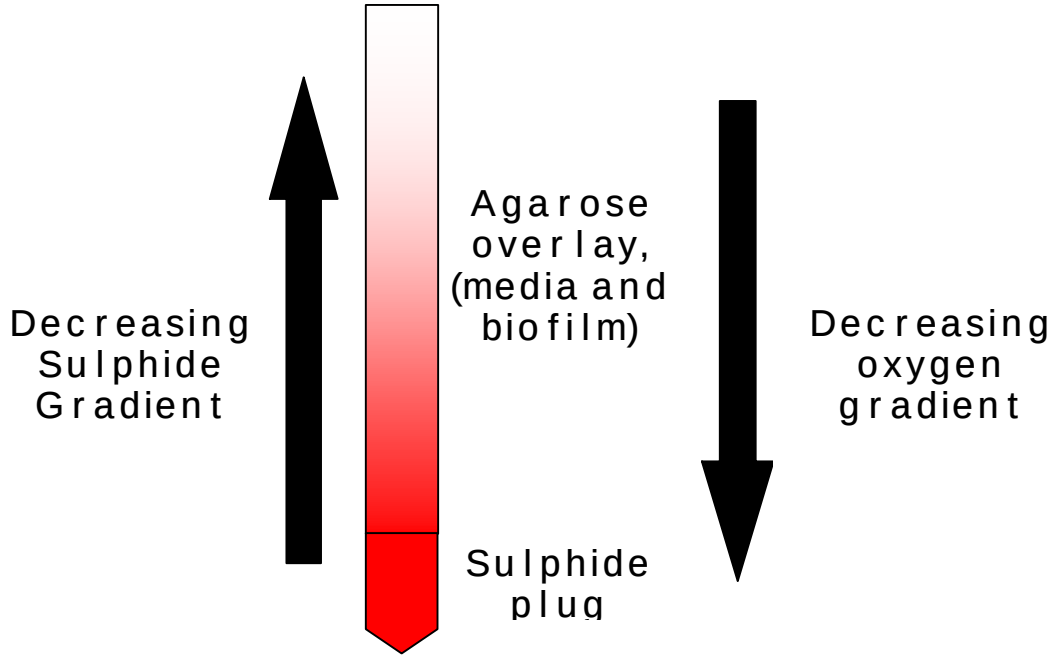

Figure 3.1: The layout of the Gradient Tube System**.**

The sulphide plug allows for the upward diffusion of sulphide into the overlay, oxygen diffuses down the length of the tube establishing sulphide and oxygen gradients.

Sectioning of the agarose overlay would provide information on the conditions and bacterial species present in each section of the tube, this would provide great insight into the processes going on within the biofilm.

Another Gradient system was the Silicon Tubular Bioreactor (STB) designed by Neil Rein (2001). This reactor system consisted of a length of silicon tubing spiralled around a central support structure. An organic sulphide-containing medium was fed through the tube. This configuration provided the bacteria near the top of the spiral with more nutrients than those further down while the species further down the spiral could however utilize the overlying bacteria's byproducts. Oxygen diffusion was controlled by the wall thickness of the silicon tube and also by the consumption of oxygen by heterotrophic bacteria associated with the biofilm established on the inner surface of the tubing. This system resulted in a stratification of different bacterial populations and therefore a separation of different species and bioprocesses along a downward gradient.

While the performance of the STB was described by Rein (2001) the investigation of the bacterial populations involved will be described here.

Spatial separation of the biofilm species would simplify the study of these communities, their interactions and the conditions under which they exist. This in turn would provide insight into the specific function that each species carries out within the community and allow for optimisation of a system that would provide the correct conditions for the growth of the most successful sulphur oxidizing species. Thus allowing for the development of industrial applications based on these biological processes.

The expansion of the biofilm would prove useful in determining whether structural and functional differentiation occurred and whether the main influencing factor was a chemical gradient influenced by the environment and the bacterial populations themselves.

3.2 Methods

3.2.1 Setting up the Gradient Tube system

A 5ml sulphide plug was poured into the bottom of a test tube (Appendix 2). This plug consisted of SRB lactate medium, 1% agarose and hydrogen sulphide. The lactate medium was included so that nutrients would not diffuse down into the plug from the medium/biofilm overlay. The plug was allowed to set in the fume hood and then an overlay was poured (Appendix 2).

The overlay (Appendix 2) consisted of 1% low melt agarose and SRB lactate medium and a biofilm inoculum taken from the Baffle Reactor. The biofilm inoculum consisted of a 500ìl biofilm sample that had been mixed with 500ìl of glycerol and frozen at –70°C. A bacteria-free control tube was also prepared. The overlay was poured as cool as possible, with minimal air bubbles and allowed to set an in the Forma Scientific anaerobic hood.

3.2.1.1 Gradient Tubes set 1

Two different nitrogen sources were used in the growth medium for the Gradient Tube system set 1. The SRB lactate medium was modified, one set of tubes contained ammonium chloride as the nitrogen source and the other contained potassium nitrate (Appendix 2).

Different sulphide concentration were used for the plugs, these were 0,125g/l, 0,25g/l and 0,5g/l. A pH control tube was poured, this tube identical to the other tubes but it contained Neutral Red as a pH indicator and no biofilm. The 0,125g/l sulphide plug tubes included the biofilm and the pH indicator. pH indicator was not included in all the tubes in case it influenced the growth of the bacteria. Tables 3.1 and 3.2 detail the contents of the Gradient Tubes set 1 and 2.

Table 3.1: Gradient Tubes, set 1.

Tube number	Sulphide concentration in the plug	Nitrogen source	Biofilm inoculum	pH indicator
1	0.5g/l	Potassium nitrate	yes	none
2	0.5g/l	Ammonium chloride	yes	none
3	0.25g/l	Potassium nitrate	yes	none
4	0.25g/l	Ammonium chloride	yes	none
5	0.125g/l	Potassium nitrate	yes	Neutral red
6	0.125g/l	Ammonium chloride	yes	Neutral red
7	0.125g/l	Potassium nitrate	none	Neutral red

3.2.1.2 Gradient Tubes set 2

Another set of Gradient tubes were set up, these tubes were variations of the 0.5g/l sulphide plug tubes from set 1, as this concentration of sulphide provided the best results.

Table 3.2: Gradient Tubes, set 2

Tube number	Sulphide concentration in the plug	Nitrogen source	Biofilm inoculum	pH indicator
A	0.5g/l	Ammonium chloride	none	none
B	0.5g/l	Potassium nitrate	none	none
C	0.5g/l	Ammonium chloride	yes	none
D	0.5g/l	Potassium nitrate	yes	none
E	0.5g/l	Ammonium chloride	yes	Neutral red
F	0.5g/l	Potassium nitrate	yes	Neutral red

The Gradient Tubes were incubated in the dark at room temperature for 7 days. After the 7 day incubation period, the 0,5g/l tubes (both the ammonium chloride and the potassium nitrate tubes) were broken open. The agarose overlay was sliced into 0,5g sections, these sections were placed into sterile eppendorf tubes and 1ml of sterile Tris-EDTA buffer (Appendix 2) was added. The samples were then heated to 50°C and vortexed. The samples were stored at 4°C overnight to facilitate diffusion, these samples were used for sulphate, sulphur, sulphide and TOC analysis, part of these samples was also stored for molecular analysis.

3.2.2 Sulphide Analysis

20µl samples were taken from each of the 0.5g sections and added to 100µl of zinc acetate (Appendix 1) and this was then made up to 5ml with dddH_2O and the assay was performed as previously described in Chapter 2.

3.2.3 Sulphur Analysis

200µl of each 0.5g sample was placed in an eppendorf tube and microfuged at 13000rpm for 10 minutes. The supernatant was discarded and then the pellet was resuspended in 100µl of HPLC grade acetone. The assay was performed as described previously in Chapter 2.

3.2.4 TOC Analysis

200µl samples were taken from each 0.5g section and these were microfuged for 10 minutes at 13 000rpm. The supernatent was transferred to a fresh tube and used for analysis as previously described in Chapter 2.

3.2.5 Sulphate Analysis

100µl of each 0.5g sample was diluted by adding 200µl of dddH_2O and then filtered through a 0,45µ nylon filter. The sulphate analysis was completed as previously described in Chapter 2.

The 0,5g/l Gradient Tube Experiments were used for analysis as these had given the best visual results, 2 sets of data was analysed: Tubes 1 and 2 from set 1 (see Table 3.1) and Tubes C and D from set 2 (see Table 3.2).

3.3 Results

3.3.1 Visual Observations of Gradient Tubes

Figure 3.2 showed that after 1 day's incubation, dark bands began to appear in the tubes that had been inoculated with biofilm. The absence of these dark bands in tubes that had not been inoculated with biofilm suggested that the bands were due to bacterial growth. Tubes 1 and 2 displayed the thickest bands suggesting that the higher sulphide concentration encouraged more growth or that more sulphide had been oxidized at this point. The bacteria also seemed to have grown better in tube 1 when compared to tube 2, this indicated that the bacterial grew better when potassium nitrate was used as a nitrogen source when compared to ammonium chloride.

The pH indicator in tube 7 (Figure 3.2), turned yellow most of the way up the tube, indicating the successful diffusion of sulphide through the medium, the presence of sulphide would cause an increase in pH. A different colour profile was observed in tubes 5 and 6, which contained pH indicator as well as the biofilm inoculum. The yellowing of the indicator seemed to stop at the dark bands of growth near the bottom of the tube. This could have indicated either that the bacteria were utilizing the sulphide at this point, thereby preventing its further diffusion or that the bacteria were maintaining the pH further up in the tube, thus preventing a colour change.

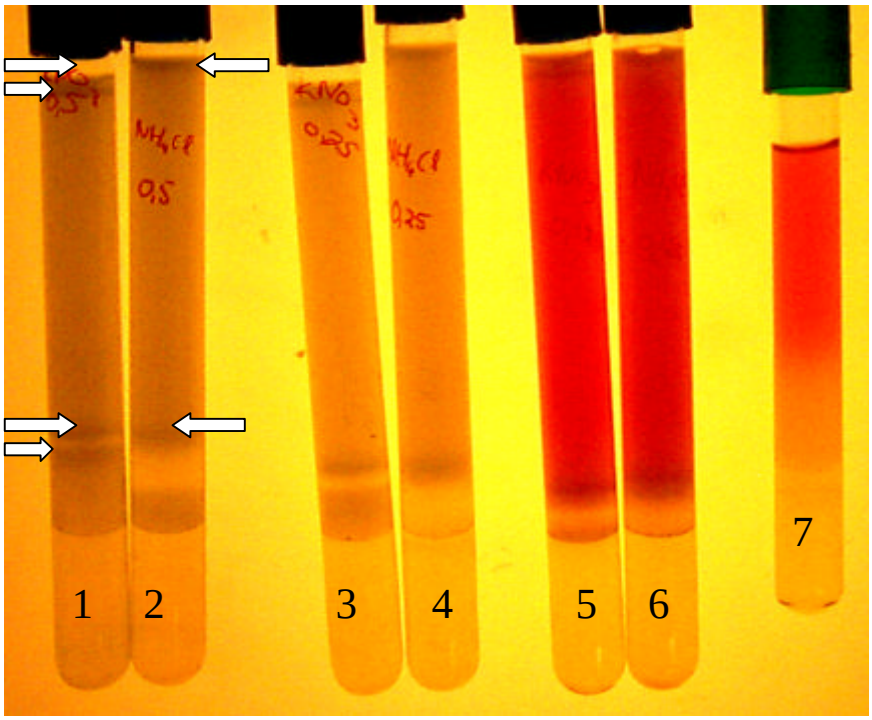

Figure 3.2: The Gradient Tubes (set 1), photographed after one day.

1 and 2 were the 0,5g/l sulphide plug tubes, 1 was the potassium nitrate tube and 2 was the ammonium chloride tube. 3 and 4 were the 0,25g/l sulphide plug tubes, 3 was the potassium nitrate tube and 4 was the ammonium chloride tube. 5 and 6 were the 0,125g/l sulphide plug tubes that contained biofilm and pH indicator, 5 was the potassium nitrate tube and 6 was the ammonium chloride tube. 7 was the pH indicator tube that contained no biofilm inoculum. The white arrows on tubes 1 and 2 indicate zones of growth.

Figure 3.3 shows the same tubes two days after inoculation. It can be seen that distinctive dark bands were present at different levels within the tubes and also that these seemed to influence the pH within the tubes. Tube 5 was pink all of the way up its length while tube 6 was mostly yellow, having only one pink section which coincided with a darker band of growth. This meant that the pH in Tube 5 did not change at all, possible reasons for this are that the sulphide was prevented from diffusing up the overlay because it was all being utilized by bacteria present in the overlay or that the bacteria present in the tube were maintaining the pH of the overlay in order to maintain a favourable microenvironment, thereby preventing a colour change form pink to yellow.

There were differences between tube 5 and tube 6, the difference in these tubes was the nitrogen source. This suggested that the nitrogen source influence the bacterial growth and therefore the utilization of sulphide.

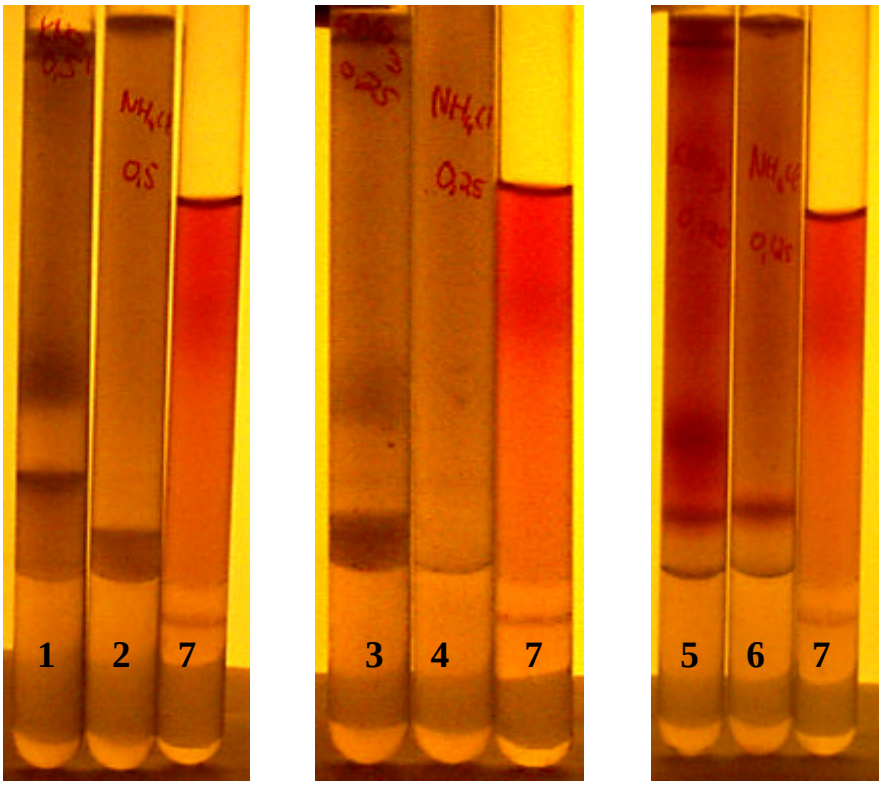

Figure 3.3: A close up view of the Gradient Tubes set 1, after 2 days.

1 and 2 were the 0,5g/l sulphide plug tubes, 1 was the potassium nitrate tube and 2 was the ammonium chloride tube. 3 and 4 were the 0,25g/l sulphide plug tubes, 3 was the potassium nitrate tube and 4 was the ammonium chloride tube. 5 and 6 were the 0,125g/l sulphide plug tubes that contained biofilm and pH indicator, 5 was the potassium nitrate tube and 6 was the ammonium chloride tube. 7 was the pH indicator tube that contained no biofilm inoculum.

The colour difference, indicative of a difference in pH, was quite clear in Figure 3.3, tube 5 was definitely pink most of the way up its length, while tube 6 only had bands of pink and was mostly yellow. The no bacteria control tube, 7, was yellow for about half of its length and then pink the rest of the way, no distinctive bands were visible. Tubes 1, 2, 3 and 4 all showed distinctive bands of growth at different levels in the overlay.

Figure 3.4 to 3.6 were diagrammatic representations of the Gradient Tube set 2. Figure 3.4 showed that the darker bands present in the Gradient Tubes were not just

the result of chemical reactions and preparation but actually occurred due to bacterial growth. This could be seen from the fact that no dark bands appeared in the bacteria-free control tubes A and B while dark bands did appear in tubes C and D which contained a biofilm inoculum. The potassium nitrate tube, C, differed from the ammonium chloride tube, D, more bands of growth were present in tube C. The evidence suggested that the bacteria grew better when provided with potassium nitrate as their nitrogen source rather that ammonium chloride, this could have been due to the fact that potassium nitrate is a more readily oxidizable nitrogen source than ammonium chloride and also because nitrate can be used as a terminal electron acceptor. Potassium nitrate was possibly used instead of oxygen as the terminal electron acceptor and this therefore allowed for the growth of bacteria under low oxygen, high sulphide conditions at the bottom of the tube.

The pH indicator tubes E and F, Figure 3.4, displayed bands of colour that corresponded closely to the bands of growth seen in tubes C and D, thereby indicating that the pH was influenced by the bacterial growth and not only the diffusion of sulphide. The pH profiles of tubes E and F differed, again suggesting that the nitrogen source was playing an important role in this system, influencing the growth of the bacteria and therefore the utilization of the sulphide and the maintenance of a favourable pH in the bacteria's microenvironment.

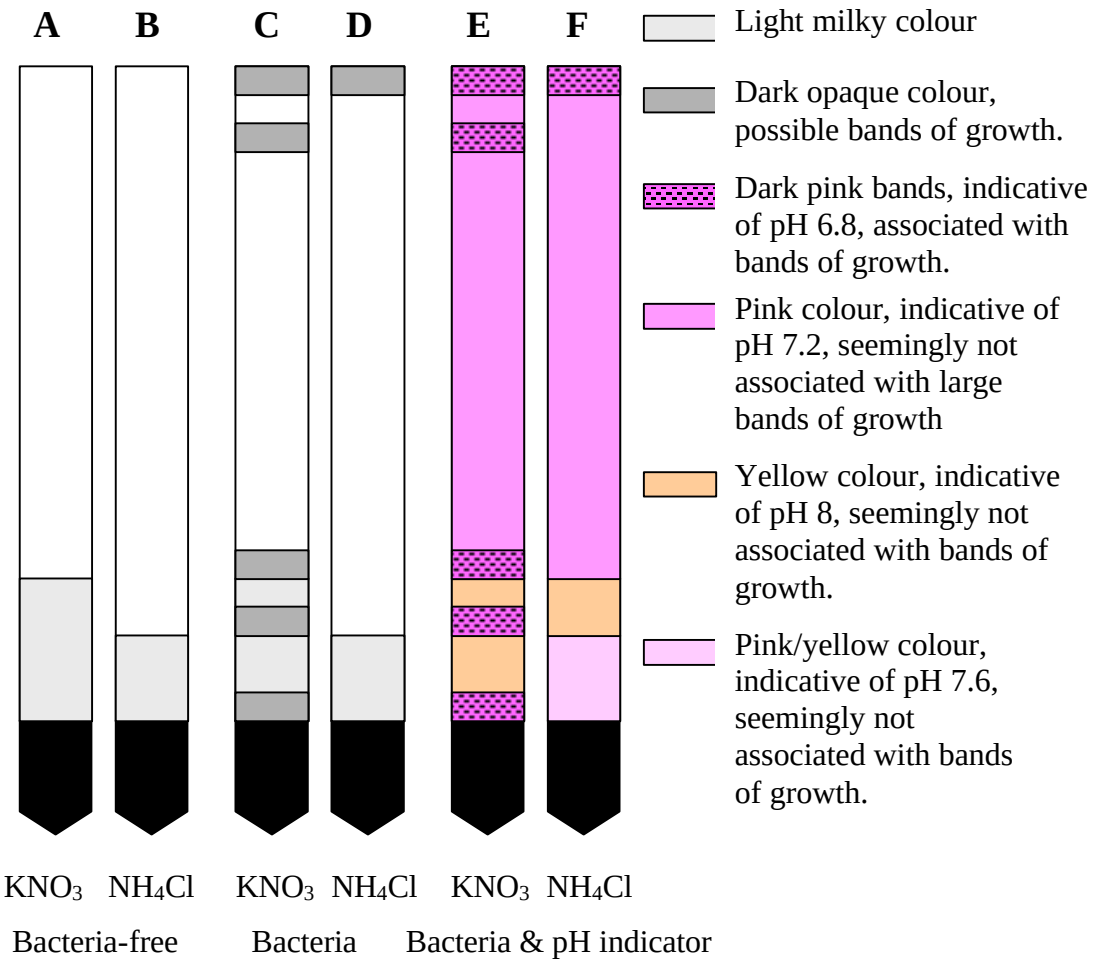

Figure 3.4: Diagrammatic Representation of the Gradient Tubes set 2, one day after start up.

A, B, C, D, E and F were all 0,5g/l sulphide plug tubes. A, C and E were the potassium nitrate tubes and B, D and F were the ammonium chloride tubes. A and B contained no biofilm inoculum. C and D contained a biofilm inoculum. E and F contained both biofilm inoculum and neutral red as a pH indicator.

Figure 3.5 should be looked at in comparison with Figure 3.4 and Figure 3.6 as these all represent changes occurring in the same system over a period of time. Figure 3.5 shows that more diffusion of sulphide has occurred, this could be seen in all the tubes as the darker bands had all moved further up the length of the overlay.

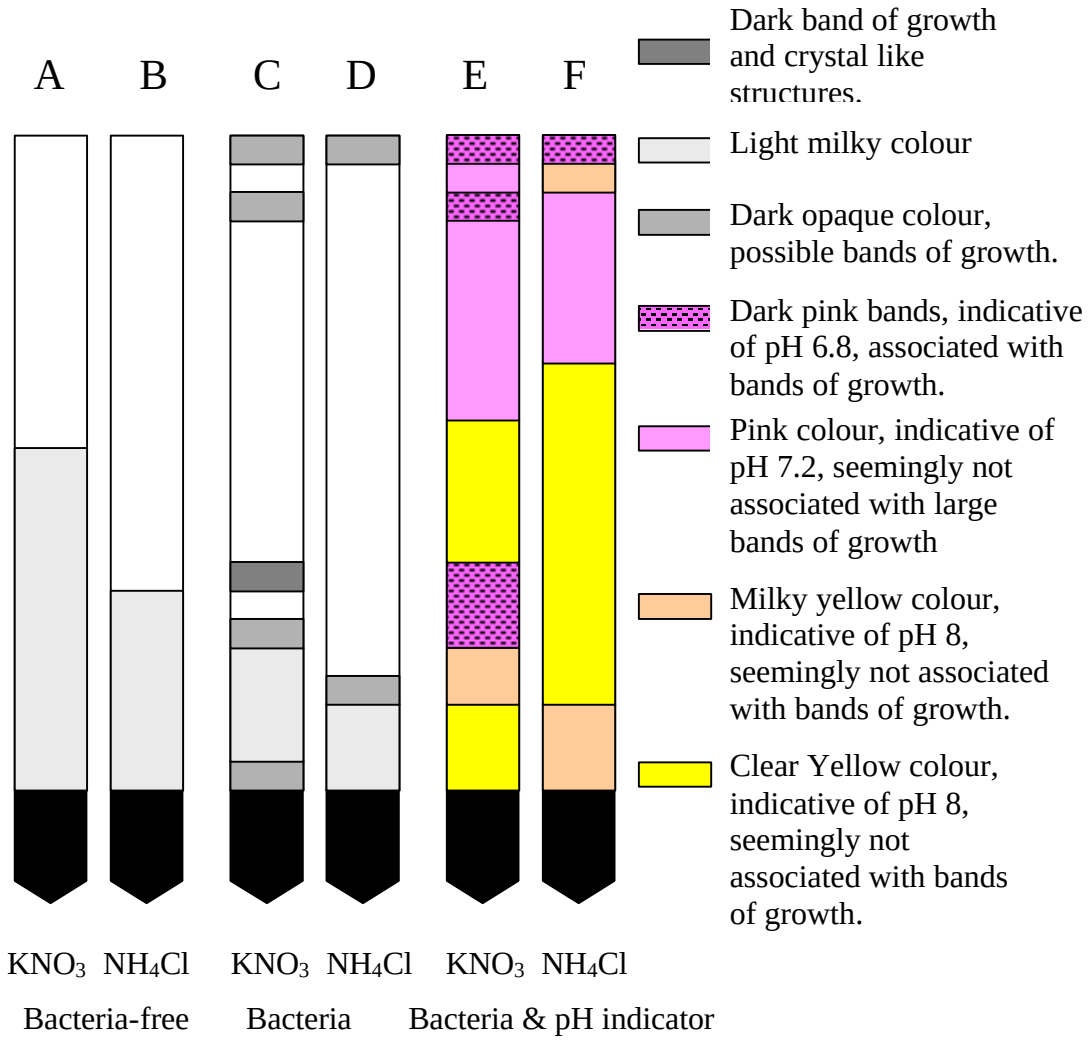

Figure 3.5: Diagrammatic Representation of the Gradient Tubes set 2, three days after start up.

A, B, C, D, E and F were all 0,5g/l sulphide plug tubes. A, C and E were the potassium nitrate tubes and B, D and F were the ammonium chloride tubes. A and B contained no biofilm inoculum. C and D contained a biofilm inoculum. E and F contained both biofilm inoculum and neutral red as a pH indicator.

Figure 3.5 showed the results of set 2 after 3 days. The pH indicator tubes, E and F, Figure 3.5, displayed different pH profiles, the yellowing phenomenon associated with a rise in pH was seen to move to a higher level in the tube. While the yellow level did rise in tubes E and F, the bands corresponding to the darker bands of growth seen in tubes C and D remained pink in colour suggesting that the bacteria were still maintaining their microenvironment at around pH 6.8. Other changes could be seen in tubes C and D, one of the bands in tube C seemed to have crystal-like structures present in it that were not previously visible.

When Figure 3.6 was looked at in comparison to Figure 3.5 and Figure 3.4 it was evident that further changes had occurred in the tubes.

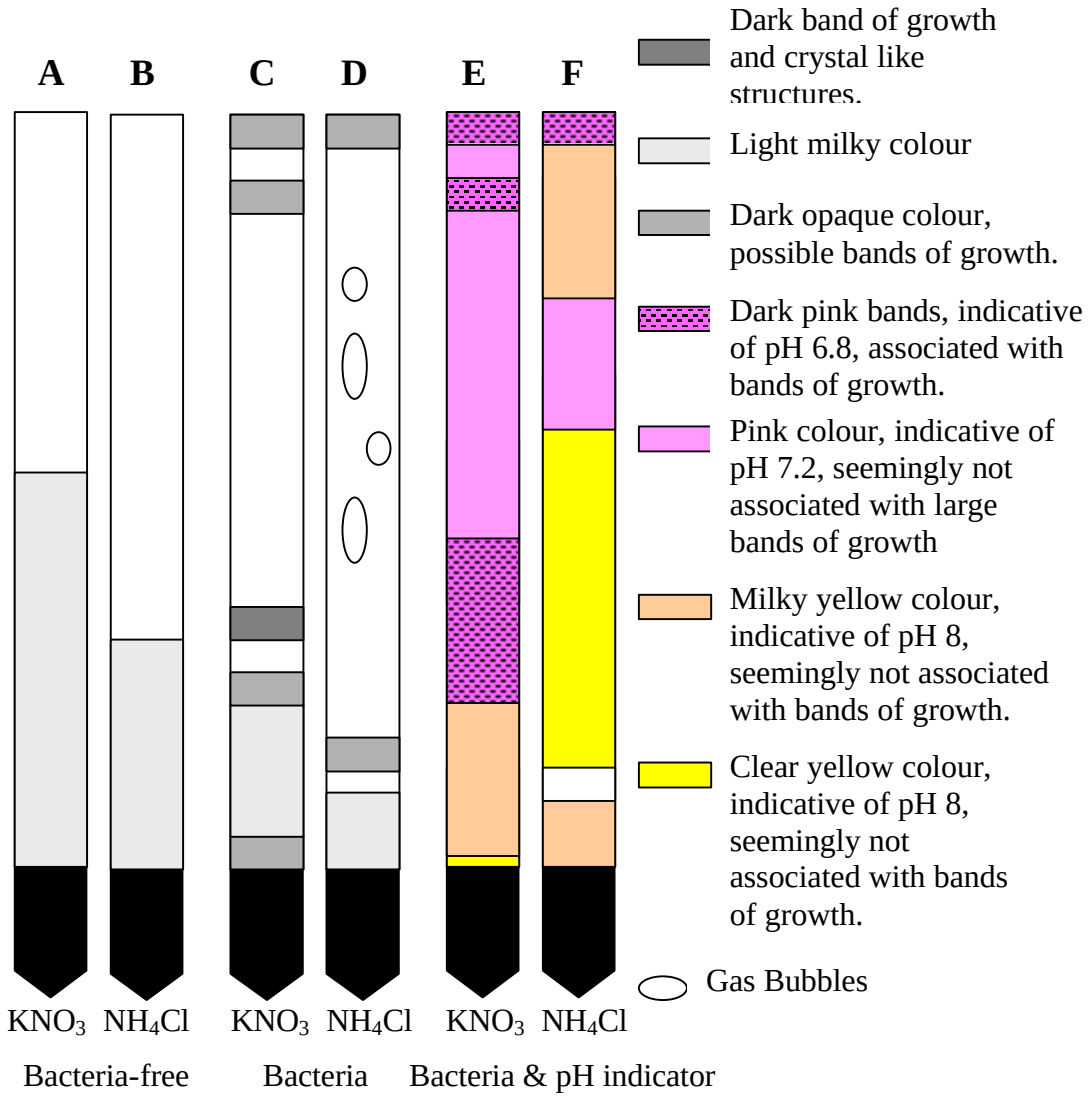

Figure 3.6: Diagrammatic Representation of the Gradient Tubes set 2, six days after start up.
A, B, C, D, E and F were all 0,5g/l sulphide plug tubes. A, C and E were the potassium nitrate tubes and B, D and F were the ammonium chloride tubes. A and B contained no biofilm inoculum. C and D contained a biofilm inoculum. E and F contained both biofilm inoculum and neutral red as a pH indicator.

Tube D, Figure 3.6, had developed gas bubbles quite high up in the overlay, these could have been due to bacterial respiration. The same gas bubbles were not present in tube C, this could only have been due to different bacteria having grown in the two different tubes due to the differing nitrogen source. Further changes had occurred in the pH indicator tubes, E and F, but the bands of growth had maintained their pH near 7 rather than let the diffusion of the sulphide bring the pH closer to 8.

3.3.2 Sulphide Analysis.

Figure 3.7 and 3.8 showed that the sulphide concentration was highest near the bottom of both tubes 1 and 2 and tubes C and D. This was expected as the sulphide plug was at the bottom of the tube. The sulphide concentration gradually decreased towards the top of the overlay, where it was present in very small quantities. Tubes 1, 2, C and D showed basically the same sulphide profile, suggesting that the nitrogen source had no bearing on the diffusion of sulphide through the medium. Both data sets were represented because even though they were similar each one provided valuable information about the system.

Figure 3.8 showed that the sulphide concentration in tubes C and D decreased from the plug towards the top of the overlay, this was expected as the plug was the source of the sulphide. The potassium nitrate tube, C, had a slightly higher sulphide concentration than the ammonium chloride tube, D, but this was probably due to experimental method as tube D samples were read first on the spectrophotometer and therefore tube C samples had more time to develop. The sulphide levels may also have been higher due to SRB converting sulphate in the lactate medium into sulphide.

The arrows in figure 3.8 represent the positions of the bands of growth in the Gradient Tubes. The bands of growth do not appear to have a dramatic effect on the diffusion of sulphide throughout the overlays.

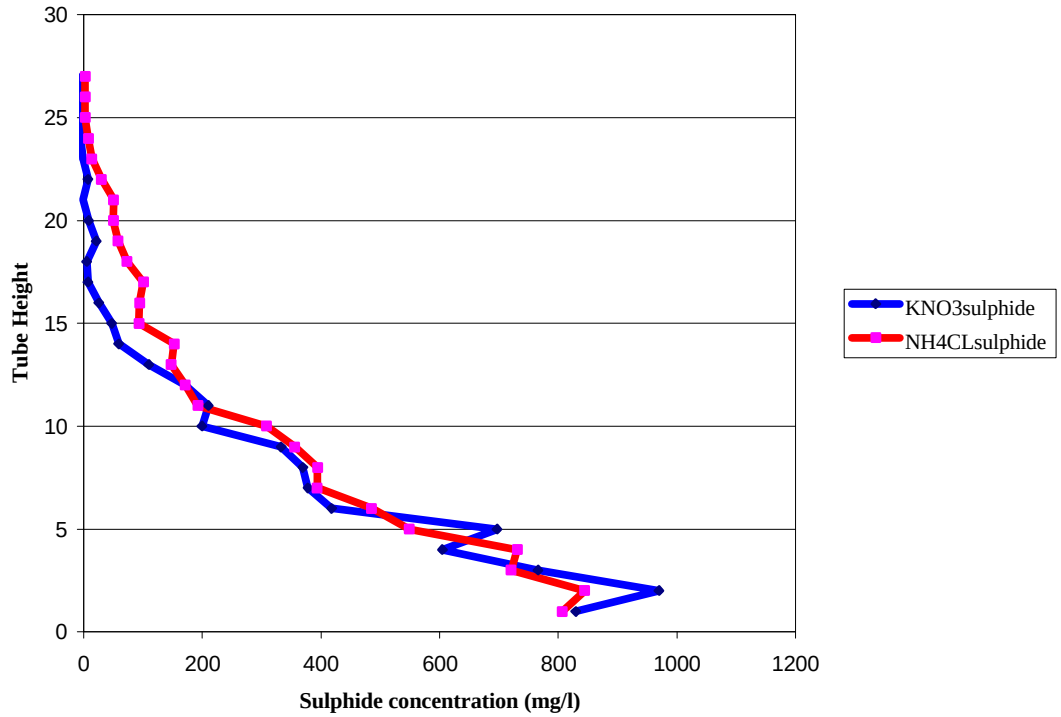

Figure 3.7: Graph of the Sulphide concentration in Tubes 1 and 2, set 1**.**

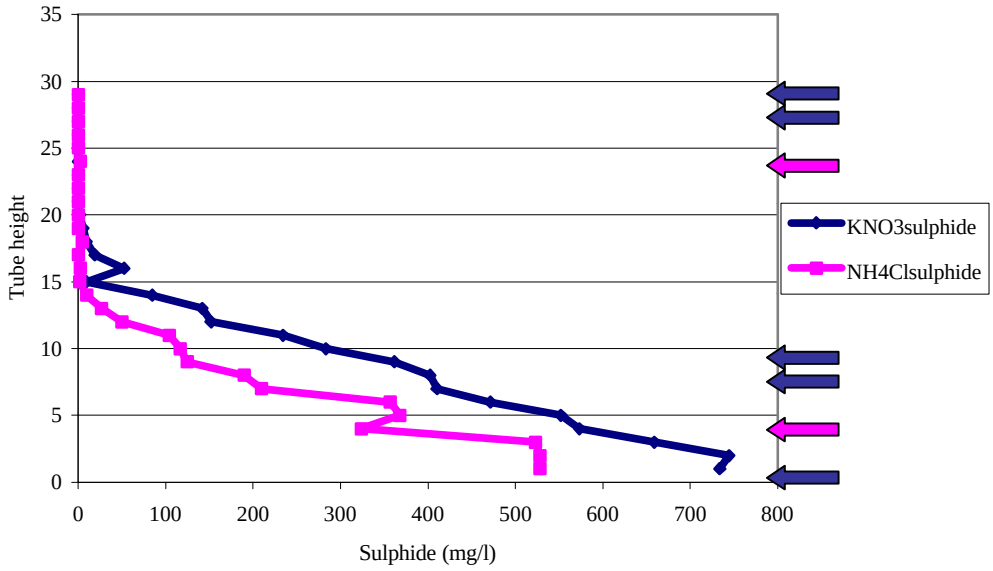

Figure 3.8: Graph of the Sulphide concentration in Tubes C and D, set 2.

The blue arrows represent the positions of the bands of growth within the potassium nitrate Gradient Tube and the pink arrows represent the positions of the bands of growth within the ammonium chloride Gradient Tube.

3.3.3 Sulphur Analysis

There were distinct zones within the Gradient Tubes where the sulphur concentration was high (Figure 3.9 and 3.10). There were also differences in the distribution of sulphur between the ammonium chloride containing tubes and the potassium nitrate containing tubes. The ammonium chloride tube only had high concentrations of sulphur at the top of the tube where the oxygen level was the highest. The potassium nitrate tube had four zones of high sulphur concentration; there was sulphur near the bottom, two high sulphur zones just above the halfway mark and then another sulphur zone at the top of the tube. Potassium nitrate is able to act as the terminal electron acceptor during bacterial respiration and sulphide oxidation, this therefore must have played a big role in the growth and distribution of bacteria within the tubes and therefore the production of sulphur.

Figure 3.10 when compared to Figure 3.9 proved that the two sets of experiments had similar sulphur production zones. The ammonium chloride tube had a very low sulphur concentration near the bottom but at about the halfway point, the sulphur concentration increased slightly, the highest level of sulphur was found at the air/overlay interface. The potassium nitrate tube again displayed a zone with a high sulphur concentration at the bottom of the tube, three high sulphur zones above the halfway point and then a sulphur zone at the air/overlay interface. This seemed to suggest that the bacteria that had ammonium chloride as their nitrogen source grew best in the presence of oxygen and could only oxidize sulphide to sulphur at the top of the tube. The bacteria that had potassium nitrate as their nitrogen source seemed to proliferate through-out the tube and were able to produce sulphur at all levels of the tube including just above the plug where the sulphide concentration was extremely high and the presence of oxygen was highly unlikely.

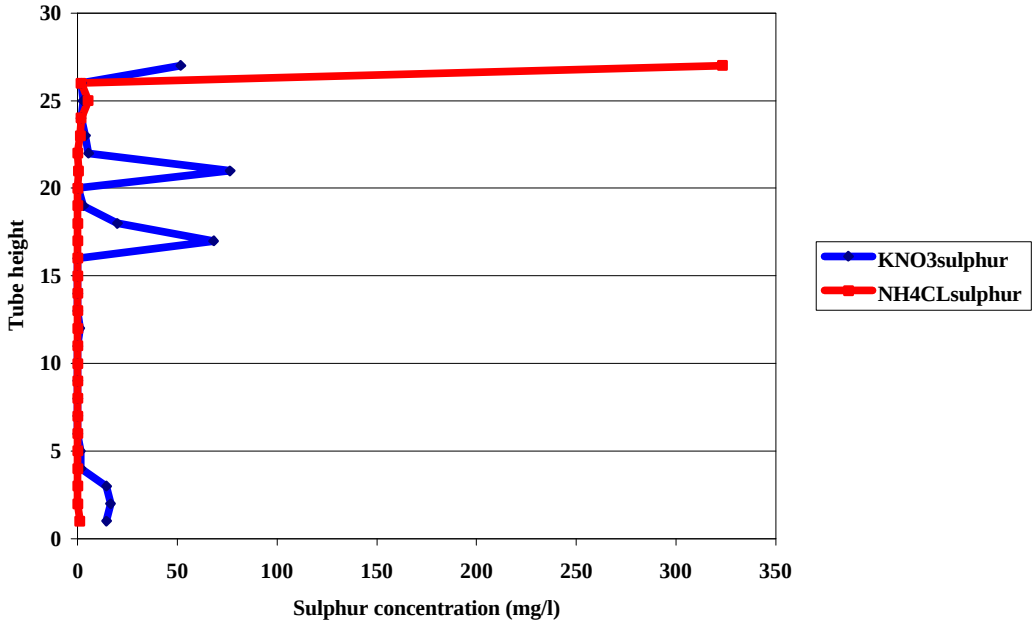

Figure 3.9: Sulphur concentration in Gradient Tubes 1 and 2, set 1.

Bacterial growth was possibly occurring throughout the Gradient Tube but specific bands of growth were documented for the Gradient Tubes set 2. The bands of growth in the Gradient Tubes, Figure 3.10, were represented by arrows. The band of growth at the bottom of the potassium nitrate Gradient Tube seemed to correspond with an area high in sulphur, suggesting that the bacteria present in that particular band were oxidizing sulphide to sulphur. The bands of growth did not always correspond with high levels of sulphur indicating that even though bacterial growth was occurring, it did not necessarily mean that sulphide oxidation was taking place. The bands of growth present in the top sections of both the potassium nitrate and ammonium chloride Gradient Tubes did however correspond to areas where the sulphur concentration was high.

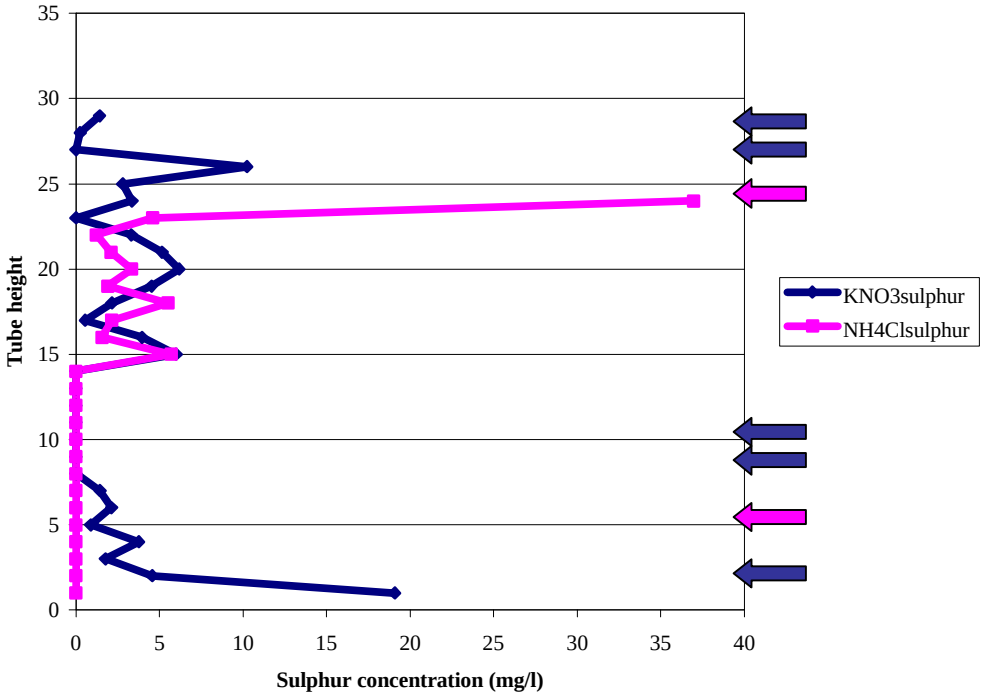

Figure 3.10: Sulphur concentration in Gradient Tubes C and D**,** set 2.

The blue arrows represent the positions of the bands of growth within the potassium nitrate Gradient Tube and the pink arrows represent the positions of the bands of growth within the ammonium chloride Gradient Tube.

3.3.4 TOC Analysis

Figures 3.11 and 3.12 showed that the TOC of both tubes 1 and 2 and C and D had a general decreasing trend towards the air/overlay interface. This suggested that the carbon was being utilized more efficiently near the top of the tube where the oxygen concentration was higher.

The areas where bands of growth occurred are indicated in Figure 3.12 by arrows. Areas of low TOC seem to correspond with areas where bands of growth occur. The highest levels of TOC occur in the centre of the Gradient Tubes where no bands of growth were visible.

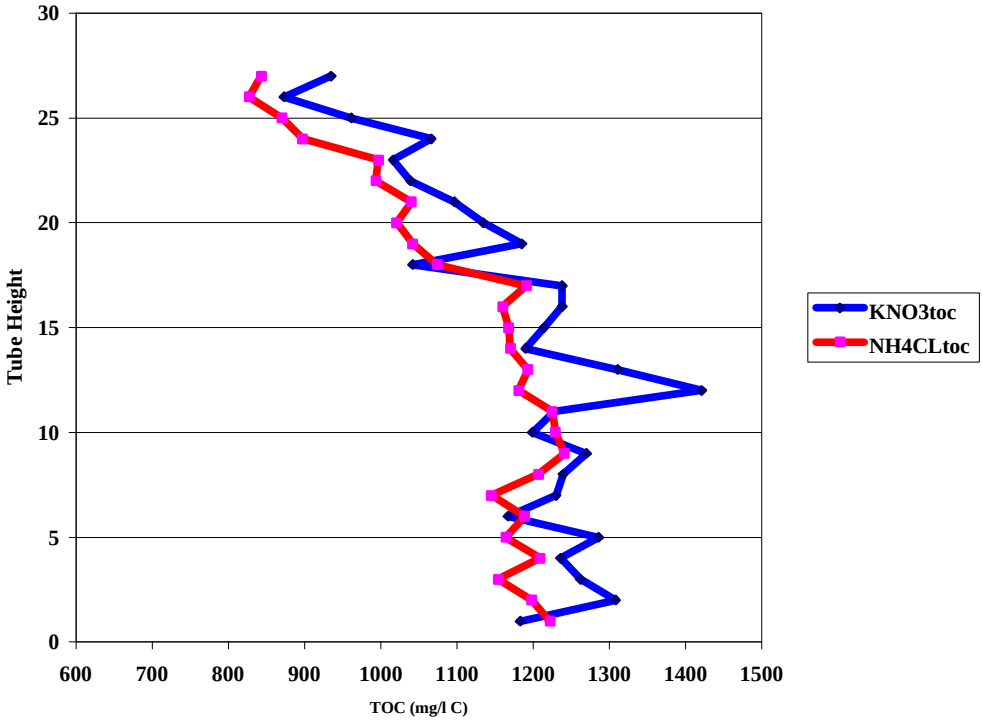

Figure 3.11: TOC of the Gradient Tubes 1 and 2, set 1.

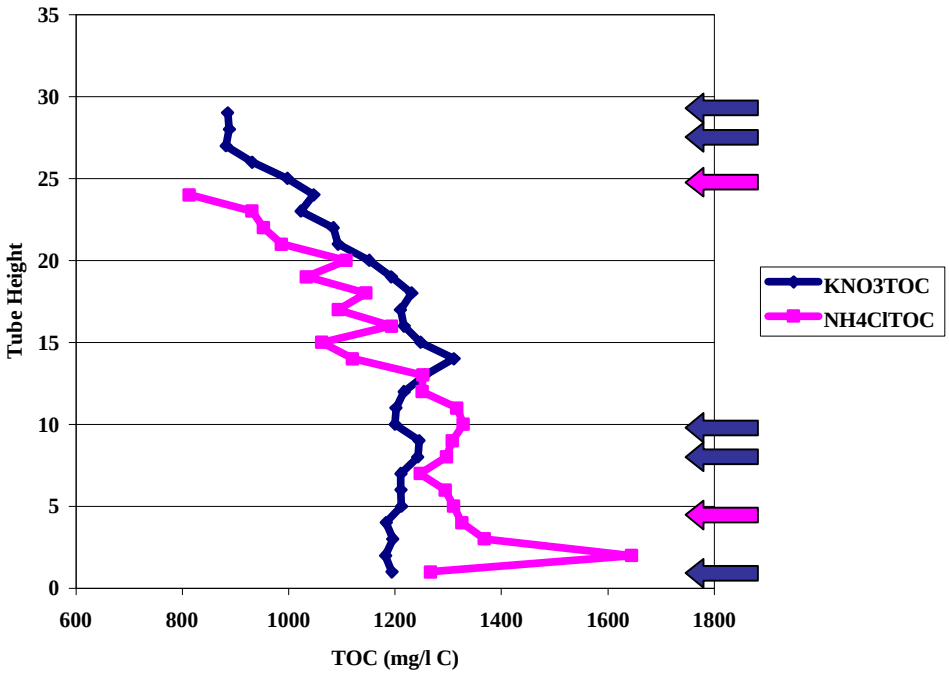

Figure 3.12: TOC of the Gradient Tubes C and D, set 2.

The blue arrows represent the positions of the bands of growth within the potassium nitrate Gradient Tube and the pink arrows represent the positions of the bands of growth within the ammonium chloride Gradient Tube.

3.3.5 Sulphate Analysis

Figures 3.13 and 3.14 showed that the sulphate concentration for both tubes 1 and 2 and tubes C and D was high at the bottom, decreased slightly and stabilized for most of the length of the tube and then finally increased towards the air/overlay interface. This suggested that sulphide was oxidized to sulphate more efficiently near the bottom of the tube and also near the top of the tube. The areas of higher sulphate at the bottom and top of the Gradient Tubes correspond to bands of growth, Figure 3.14. This trend could suggest the presence of two different populations of bacteria that oxidize sulphide under different conditions, one at the bottom of the tube under high-sulphide, low-oxygen conditions and the other at the top of the tube, under high-oxygen, low-sulphide conditions. However there were bands of growth that did not correspond to areas high in sulphate, suggesting that non-sulphide oxidizing bacteria were proliferating within the Gradient Tube system.

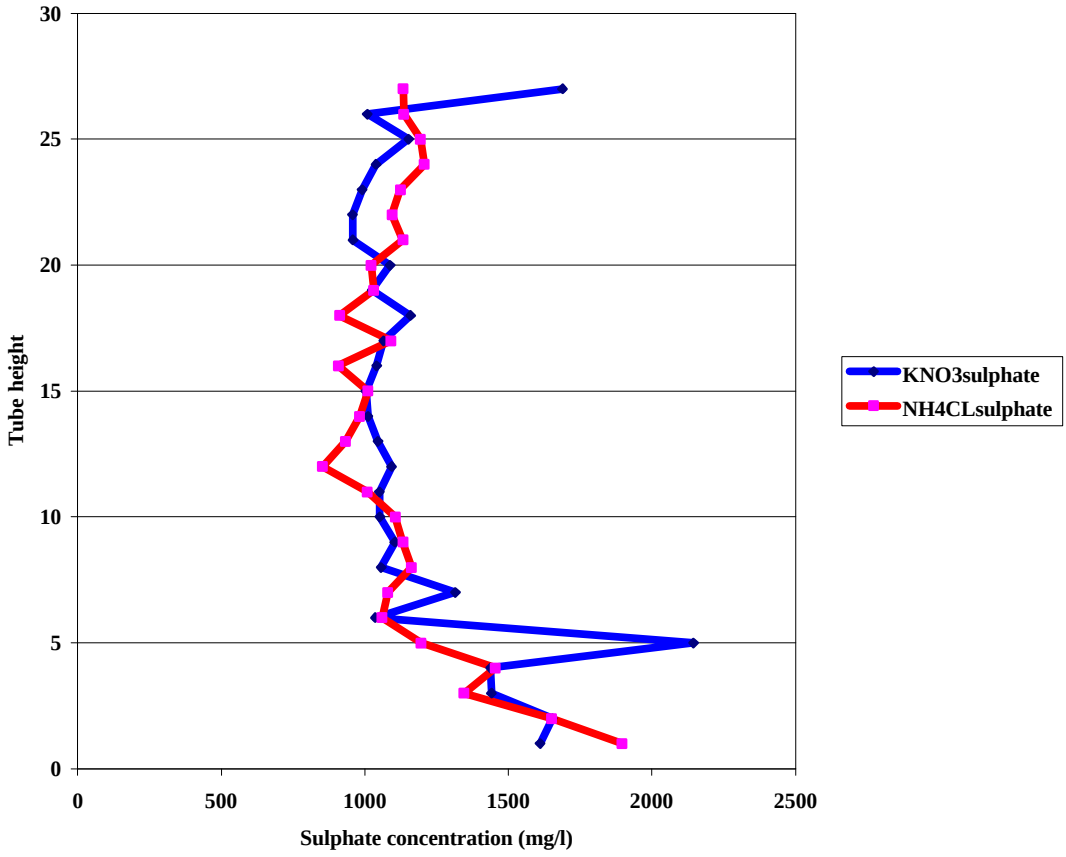

Figure 3.13: Sulphate concentration of the Gradient Tubes 1 and 2, set 1.

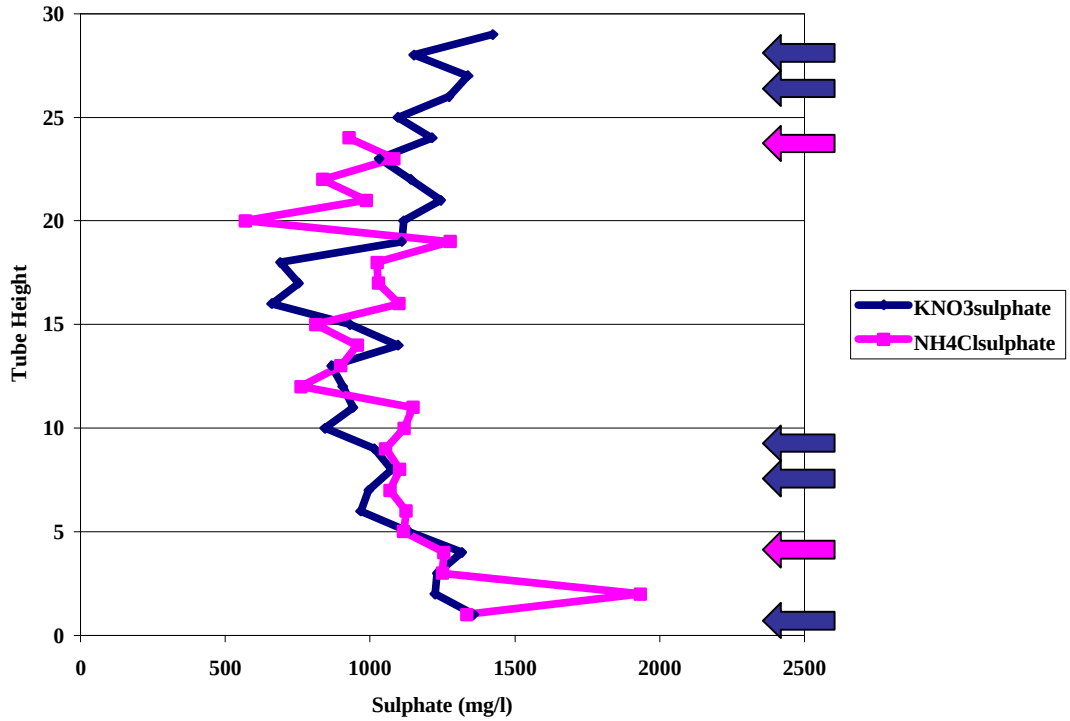

Figure 3.14: Sulphate concentration of the Gradient Tubes C and D, set 2.
The blue arrows represent the positions of the bands of growth within the potassium nitrate Gradient Tube and the pink arrows represent the positions of the bands of growth within the ammonium chloride Gradient Tube.

3.4 Discussion

The Gradient Tubes proved successful in sustaining bacterial growth. Figures 3.2, 3.3, 3.4, 3.5 and 3.6 all displayed tubes with dark coloured bands, these bands were considered to be indicative of bacterial growth. The bacterial-free control tubes A and B in Figures 3.4, 3.5 and 3.6, did not have any dark bands present, while tubes C and D, which did contain biofilm inoculums had distinctive dark bands. This evidence showed that the dark bands were not artefacts of setting up the tubes or present due to any abiotic phenomena, the most likely explanation for their appearance was bacterial growth.

The Gradient Tubes that were set up promoted bacterial growth at different positions in the tubes, clearly visible in Figures 3.2-3.6, these observations suggested that bacteria were proliferating only at certain positions where the different conditions established by the diffusion of the sulphide out of the plug and the diffusion of oxygen down into the medium suited them best. The gradient of conditions that occurred

throughout the tube encouraged the growth of bacteria in microenvironments that suited their optimum growth conditions. This hopefully resulted in a separation of the different species into functional layers over the gradient.

Figures 3.2-3.6 demonstrated that the nitrogen source included in the SRB lactate medium played a very important role in influencing where the bacteria could grow and possibly which species proliferated. The Gradient Tubes that had potassium nitrate instead of ammonium chloride as the nitrogen source displayed more bands of growth, especially under high sulphide, low oxygen conditions. This was due to potassium nitrate being able to function as a terminal electron acceptor and therefore allowing for bacterial growth. .

The pH control tubes that contained biofilm inoculums, 5, 6, E and F in Figures 3.2-3.6 were clearly different to the pH control tube that did not contain bacteria, tube 7**.** The bacteria-free control tube 7, Figure 3.2 and 3.3**,** had no bands forming, only a colour change was observed. This indicated that the bacteria present were influencing the pH of the system.

The sulphide diffused upwards out of the plug into the overlay, this raised the pH, causing the pH indicator to change from pink to yellow, this colour change eventually occurred along the entire length of tube 7. When comparisons were made between the bacteria-free control tube 7 and the pH indicator tubes that did contain bacteria, 5, 6, E and F, it could be seen that the colour change did not occur in all parts of these tubes. The darker bands of growth in tubes 5, 6, E and F remained pink in colour, this indicated that the pH was still approximately 6.8 while the pH of the surrounding medium was closer to pH 8. A possible reason for this was that the bacteria were maintaining the pH of their immediate microenvironment in order to establish favourable growth conditions. Another possible reason could be the production of acid by bacteria growing in these regions, this has been reported in Brock & Madigan, (1991), where *Thiobacillus* species were reported to oxidize sulphide minerals, in the process producing sulphuric acid.

Figures 3.4-3.6 clearly showed that the pink bands in tubes E and F closely corresponded to the dark bands of growth in the tubes C and D which did not contain

indicator. The presence of the bands of growth also seemed to influence pH changes higher up in the tubes. This could be seen especially clearly in tubes 5 and 6 in Figure 3.3, the whole length of tube 5 above the first band of growth remained pink while the whole length of tube 6 changed from pink to yellow with the exception of the one dark band of growth which remained pink. This suggested that the bacteria in tube 5 were altering the microenvironment within the tubes and that bacteria throughout the overlay were maintaining the pH of the medium at 6.8.

The analyses performed on all of the 0.5g sections that the tubes were cut into proved to be very useful, the chemical conditions in each section could be elucidated. The sulphide concentration in both the Gradient Tubes from the set 1 and the set 2 were similar for all sections of the tubes, Figure 3.7 and 3.8. The results showed that the sulphide concentration was highest at the bottom of the tube and very low near the top of the overlay, this was true for both the ammonium chloride tubes and the potassium nitrate tubes. These results were expected as the source of the sulphide (the sulphide plug) was at the bottom of the tube. The sulphide diffused out of the plug into the overlying medium causing a gradient increasing sulphide from the bottom of the tube to the top, also air diffused down the length of the overlay. This created high-sulphide, low-oxygen conditions at the bottom of the tube and low-sulphide, high-oxygen conditions at the top of the tube. This provided many different microenvironments, bacteria could then proliferate in the chemical niche that suited them the best, thereby separating species out along a gradient.

The results of the analysis of the sulphur concentrations within the different sections of the tubes gave very interesting trends, refer to Figures 3.9 and 3.10**.** The sulphur concentrations of the different sections of ammonium chloride tubes, 2 and D, from both the Gradient Tubes set 1 and set 2 displayed a similar trend, refer to Figures 3.9 and 3.10. This was also true for the potassium nitrate tubes, 1 and C. Tubes 2 and D both had very little sulphur, if any, in the lower sections of the tube, in fact in tube 2 the only section of the overlay that contained sulphur was the very top section where the oxygen concentration was highest. Tube D was similar but more of the sections near the top did contain sulphur, sulphur was only present above the halfway point. These results suggested that the bacteria growing in the Lactate medium with ammonium chloride as the nitrogen source could only produce sulphur under high-

oxygen, low-sulphide conditions. The presence of sulphur in the top sections seemed to correspond to a dark band of growth. Sulphur producing bacteria for example *Beggiatoa* and *Thiothrix* have been documented to grow under conditions of low-oxygen and high-sulphide (Basu *et al.*, 1995).

Tubes 1 and C both had sulphur present in the lowest fractions of the tube, just above the sulphide plug, these areas of elevated sulphur concentration also seemed to correspond to dark bands of growth. They also contained a few sections of high sulphur concentration above the halfway mark and sulphur in the very top section. These results showed that the bacteria growing in the lactate medium with potassium nitrate as the nitrogen source could produce sulphur under high sulphide, low oxygen conditions and also under low-sulphide, high-oxygen conditions. This suggested that the potassium nitrate in the medium was possibly acting as the terminal electron acceptor, allowing for bacterial growth and sulphide oxidation. The potassium nitrate may also have encouraged the proliferation of different bacteria than those in the ammonium chloride tube, bacteria that could oxidize sulphide to sulphur under basically anaerobic conditions with high sulphide levels.

These results were supported by the visual observations, which showed dark bands of growth at the bottom of the potassium nitrate tubes but not at the bottom of the ammonium chloride tubes.

The TOC analysis performed on the different sections of each of the tubes from both the set 1 and the set 2 displayed similar trends, refer to Figures 3.11 and 3.12**.** The trends for both the ammonium chloride and the potassium nitrate tubes are basically the same. The TOC was higher at the bottom of the tubes and decreased towards the top. These results suggest that the carbon in the lower levels of the tubes, where the sulphide levels were high and the oxygen levels were low, was not being utilized by bacteria. But at the top of the tube where the sulphide levels were low and the oxygen levels were high and where bands of growth were visible, the carbon was being used up. This suggested that the bacteria in both the potassium nitrate and the ammonium chloride tubes were growing better under high-oxygen, low-sulphide conditions.

The results of the sulphate analysis of the sections of the potassium nitrate and ammonium chloride tubes from both set 1 and set 2 showed similar trends, refer to Figures 3.13 and 3.14**.** The general trend was that the sulphate concentration was high at the bottom of the tube and then decreased slightly and remained stable for most of the length of the tube before increasing again at the top of the tubes. The sulphate concentration was expected to be higher near the top of the tubes because as the sulphide came into contact with the air it could begin to undergo chemical oxidation to sulphate, also there were bands of growth present at the top of both the ammonium chloride and the potassium nitrate tubes so the sulphide would be undergoing bacterial oxidation as well. The fact that the sulphate was high at the bottom of the tube also suggested that oxidation was occurring, this pointed towards the presence of a sulphide oxidizing bacterial population. The evidence of bacterial growth at the bottom of the tube was observed as dark bands in the potassium nitrate tubes, Figures 3.2-3.6. The sulphur concentration was also high at the bottom of the tube again suggesting the presence of a SOB population.

3.5 Conclusion

The Gradient Tubes proved to be a useful system that allowed for the separation of bacteria into their different optimal microniches by providing them with a gradient of conditions, the bacteria would only proliferate in the niche that suited them and therefore only occurred at certain points along the chemocline.

After observing where the bacteria were growing, the tubes were sectioned and the specific chemical conditions of the each section were established. These chemical results were duplicated in two sets of experiments, proving the reproducibility of the system.

The bacteria were seen to develop within the tubes as dark bands, these bands maintained their pH at around 6.8, possibly their growth optimum, rather than let it increase with the diffusion of sulphide. The two different media types used had a profound impact on the distribution of bacteria throughout the tubes, the medium that contained potassium nitrate, seemed to promote more bacterial growth, especially at the bottom of the tube where oxygen was low and sulphide high. This was due to the

fact that the potassium nitrate could take the place of oxygen as the terminal electron acceptor. No bands of bacterial growth were visualized at the bottom of the ammonium chloride tubes. Sulphide oxidation to sulphur also only occurred at the bottom of the potassium nitrate tubes and not the ammonium chloride tubes, although it was observed to occur at the top of both of the different types of tubes.

The different bacterial species in each section could be determined using molecular techniques and conclusions could be drawn about these bacteria and their function within the system by looking at the conditions under which they were growing.

CHAPTER 4:

THE MICROSTRUCTURE OF SULPHUR BIOFILMS.

4.1 Introduction

Multispecies biofilms form highly complex structures containing voids, channels, cavities, pores and filaments with cells arranged in clusters and layers (De Beer *et al.*, 1994). Direct light and electron microscopic observation have shown that biofilm bacteria are generally enveloped in large amounts of a fibrous, highly hydrated, exopolysaccharide matrix whose chemical composition is species specific (Costerton *et al.*, 1994).

The morphology of microorganisms is too simple to serve as a basis for reliable identification but it does allow a provisional estimate of the diversity of forms present (Amann *et al.*, 1995). A microscopy study was therefore undertaken to provide an initial indication of the structure of the biofilm. First gramstains were performed on biofilm samples in order to form an idea of whether the bacteria were gram-positive or gram negative. Then the basic structure of the biofilm had to be studied and the different forms of bacteria present within the biofilm were looked at. This was done using Scanning Electron Microscopy (SEM) because the basic biofilm structure could be preserved even with the rigorous preparation procedures. SEM provided a much more detailed picture of the bacteria within the biofilm even allowing for the observation of extracellular sulphur. Intracellular sulphur could only be visualize using Transmission Electron Microscopy (TEM) and confirmed using X-ray diffraction.

The Electron microscope was therefore used to get an initial picture of the heterogeneous structure of the biofilm, the basic morphology of its inhabitants and whether or not they produced extracellular or intracellular sulphur.

4.2 Methods

4.2.1 Light Microscopy

Biofilm samples were taken from the Baffle Reactor, a small amount of biofilm was placed onto a microscope slide along with a drop of water. This was then mixed around and the standard method for gramstaining was used (Sambrook *et al.*, 1989).

4.2.2 Collection of samples for Scanning Electron Microscopy

4.2.2.1 Collection of Biofilm samples.

In an attempt to preserve the integrity and structure of the biofilm, the samples were floated onto a 0.45µ nylon filter, another filter was placed on top of the first one, sealing the biofilm inside. The filters were then stapled together in order to prevent disturbance of the biofilm during the specimen preparation.

4.2.2.2 Collection of Gradient Tube samples

The Gradient tubes were cut into top, middle and bottom sections, these were placed in beakers and heated until they melted completely. The melted samples were then filtered onto 0.45µ nylon filters.

4.2.3 Preparation of samples for SEM (Cross *et al.*, 2001)

The 0.45µ nylon filters with the biofilm samples on them were dried and then fixed in a 2.5% solution of buffered glutaraldehyde (Appendix 3) at 4°C overnight. The buffered glutaraldehyde was poured off and the sample was washed twice with phosphate buffer (Appendix 3). Once the wash steps were completed the sample was put through an alcohol dehydration series. The alcohol concentrations used were 30% through to absolute ethanol in a 10% dilution series, each step was left for 10 minutes. The sample was then placed into a Critical Point Dryer apparatus basket where it was covered with Absolute Ethanol.

Critical point drying dried the sample without causing any distortion or collapse. The Critical point drying technique avoided these problems by preventing the passage of the liquid/vapour interface through the specimen during the drying process (Cross *et al.*, 2001). This was achieved by raising the temperature and pressure of the liquid filling and surrounding the specimen to above its critical point, at which stage there

was no longer an interface between the liquid and vapour phases. The temperature was then maintained at above its critical point and the pressure was slowly reduced, the liquid in the specimen was bled out as a vapour from the pressure vessel (Cross *et al.*, 2001).

After critical point drying the desired part of the filter was cut out and stuck onto a clean specimen stub with double-sided conductive tape. If a side view of the biofilm was desired, the biofilm, still between the filters was placed in a stub which had a clamp to hold the biofilm at 90° to the specimen stub.

The sample was then gold-coated in order to improve the secondary electron emission and to reduce charge build up. The sample was then placed in the Scanning Electron microscope JEOL JSM 840 for viewing.

4.2.4 Collection and Preparation of samples for Transmission Electron Microscopy (Cross *et al.*, 2001).

The TSP biofilm sample was used for TEM. First 100µl cells were spun down at 10000 rpm for 2 minutes in a sterile eppendorf, the supernatant was poured off and the small pellet was covered with buffered glutaraldehyde (Appendix 3) and left at 4°C overnight. The pellet was then spun down again, this was done in case the pellet had resuspended over night. The buffered glutaraldehyde was then poured off and the tube was refilled with cold phosphate buffer (Appendix 3) and allowed to wash for 10 minutes. This step was repeated to ensure that all the glutaraldehyde was washed off (Cross *et al.*, 2001).

Osmium tetroxide (Appendix 3) was then added to one of the samples, the other was used for X-ray diffraction so no osmium tetroxide was added as this would interfere with the accuracy of the results. The one sample was left in osmium tetroxide for 1 hour, the osmium tetroxide was then decanted and the tube refilled with phosphate buffer. This was left to stand for 10 minutes and then decanted. Another wash step with phosphate buffer was performed (Cross *et al.*, 2001).

The buffer was decanted and the tube was refilled with 30% Ethanol, this was allowed to stand for 5 minutes. The procedure was repeated for the 50%, 70%, 80%, 90% Ethanol concentrations and then two changes of absolute ethanol were done. The ethanol was poured off and replaced with propylene oxide, this was poured off after 15 minutes. Fresh propylene oxide was added and left for a further 15 minutes before it was decanted (Cross *et al.*, 2001).

The tube was refilled with 75:25 propylene oxide : resin which was allowed to infiltrate for 1 hour before it was decanted and replaced with 50:50 propylene oxide : resin. This was allowed to infiltrate for a further 1 hour and then it was replaced with a 25:75 propylene oxide : resin mixture. After 1 hour the mixture was poured off and replaced with pure resin which was allowed to infiltrate overnight (Cross *et al.*, 2001).

Pure resin was used to fill a BEEM capsule and the sample was transferred into the capsule which was placed in an oven at 60°C for 48hours in order to allow the resin to polymerise (Cross *et al.*, 2001).

In order to obtain satisfactory ultrathin serial sections from the sample, the resin block had to be trimmed to the correct shape, a trapezium with 0.2mm long sides. The sample was then sliced into thin sections (approximately 50nm) using an ultramicrotome. These samples were scooped out of the water bath onto copper grids, these were left on filter paper to dry before viewing on the Transmission Electron Microscope JEOL 1210.

4.3 Results

4.3.1 Gram-staining

Figures 4.1 showed that there were many different forms of bacteria present these included long gram-positive rods, shorter gram-negative rods and chains of gram-negative cocci.

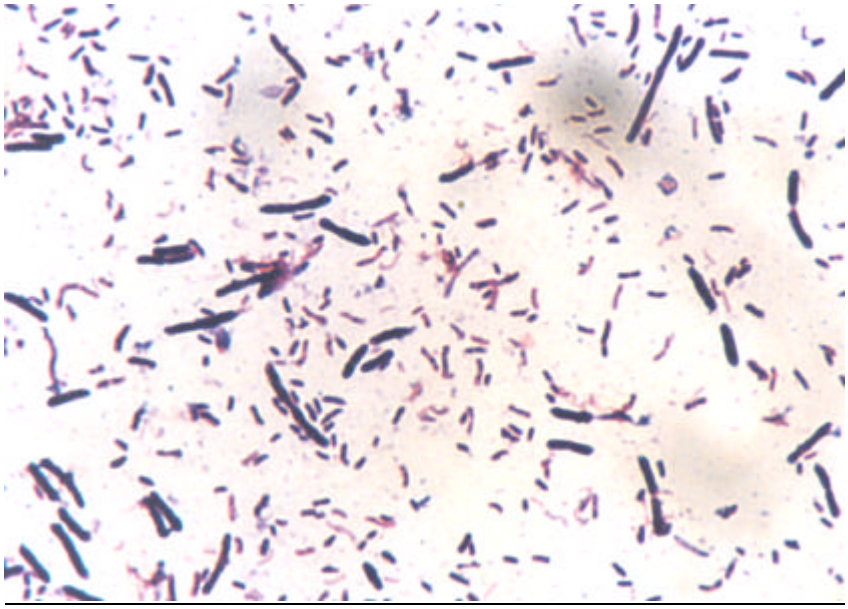

Figure 4.1: Gram-stain of bacteria present in a biofilm sample from the Baffle reactor. Magnification x 1000.

4.3.2 Scanning Electron Microscopy

4.3.2.1 SEM of the Baffle Reactor

Figure 4.2 shows a side-on perspective of the biofilm. This was done using a stub with a 90° support. The photograph shows bacteria with diverse morphologies embedded in an exopolysaccharide matrix. Also present in the biofilm were large crystals visible in the foreground of the photograph, these could possibly have been sulphur crystals. The crystals seemed to be embedded within the biofilm with bacteria surrounding them and some bacteria even seemed to colonize the crystal surface.

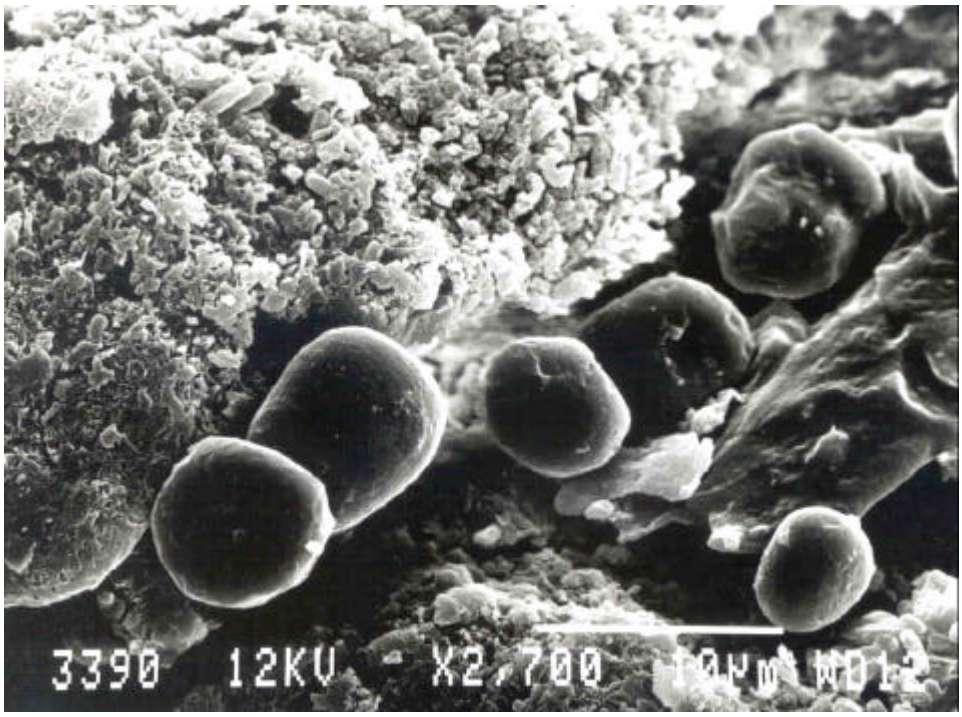

Figure 4.2: SEM photograph of the biofilm from the Baffle Reactor taken with the biofilm positioned at a 90° angle.

The sheer mass of bacteria clumped together in the biofilm is clearly visible in Figure 4.3**.**

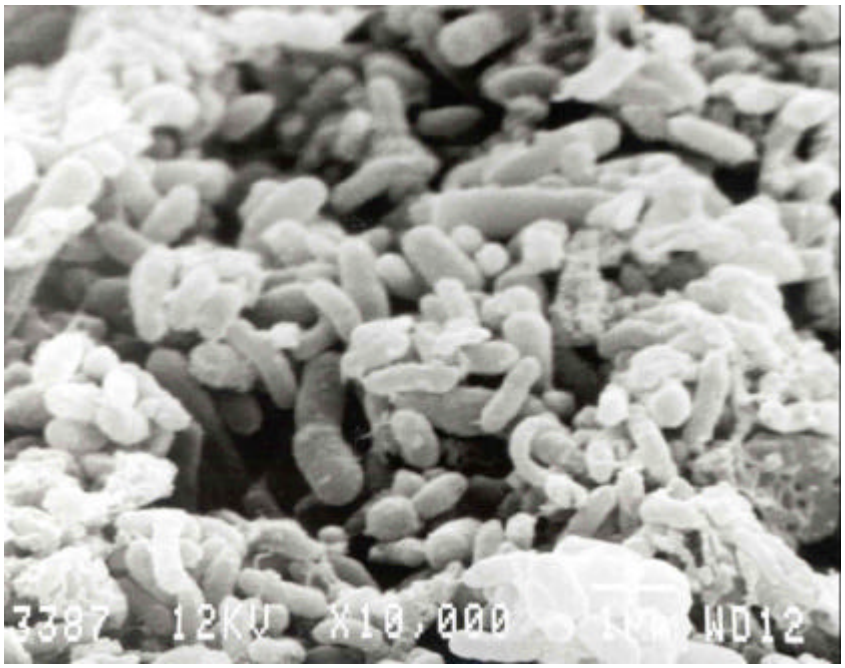

Figure 4.3: SEM photograph of the bacteria present in the biofilm from the Baffle Reactor.

The large orthorhombic crystal visible in Figure 4.4, was surrounded by bacteria, some of which appeared to be growing on the surface of the crystal. This crystal could possible be an orthorhombic sulphur crystal but its exact identification is unknown.

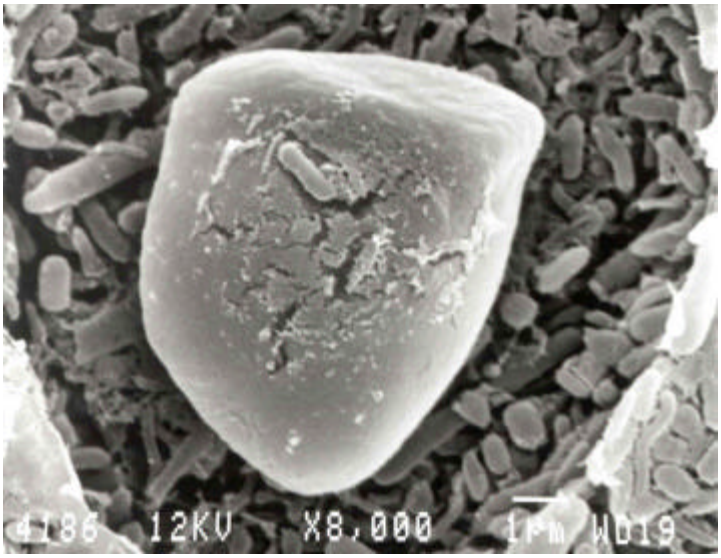

Figure 4.4: SEM of bacteria surrounding and colonizing a crystal in the floating biofilm from the Baffle Reactor.

Figure 4.5 shows the diversity of the inhabitants of the floating sulphur biofilm. Visible in the photograph are cocci, vibrio, long rods and short rods.

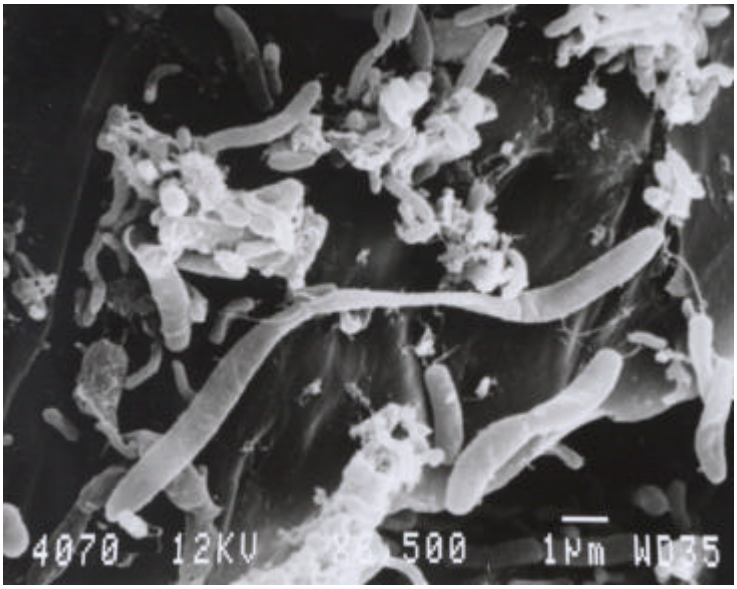

Figure 4.5: SEM photograph demonstrating the diverse morphologies that occurred within the biofilm present on the surface of the Baffle Reactor.

4.3.2.2 SEM of the Gradient Tubes

A comparison was made between the top, middle and bottom sections of the potassium nitrate and ammonium chloride Gradient Tubes, Figure 4.6.

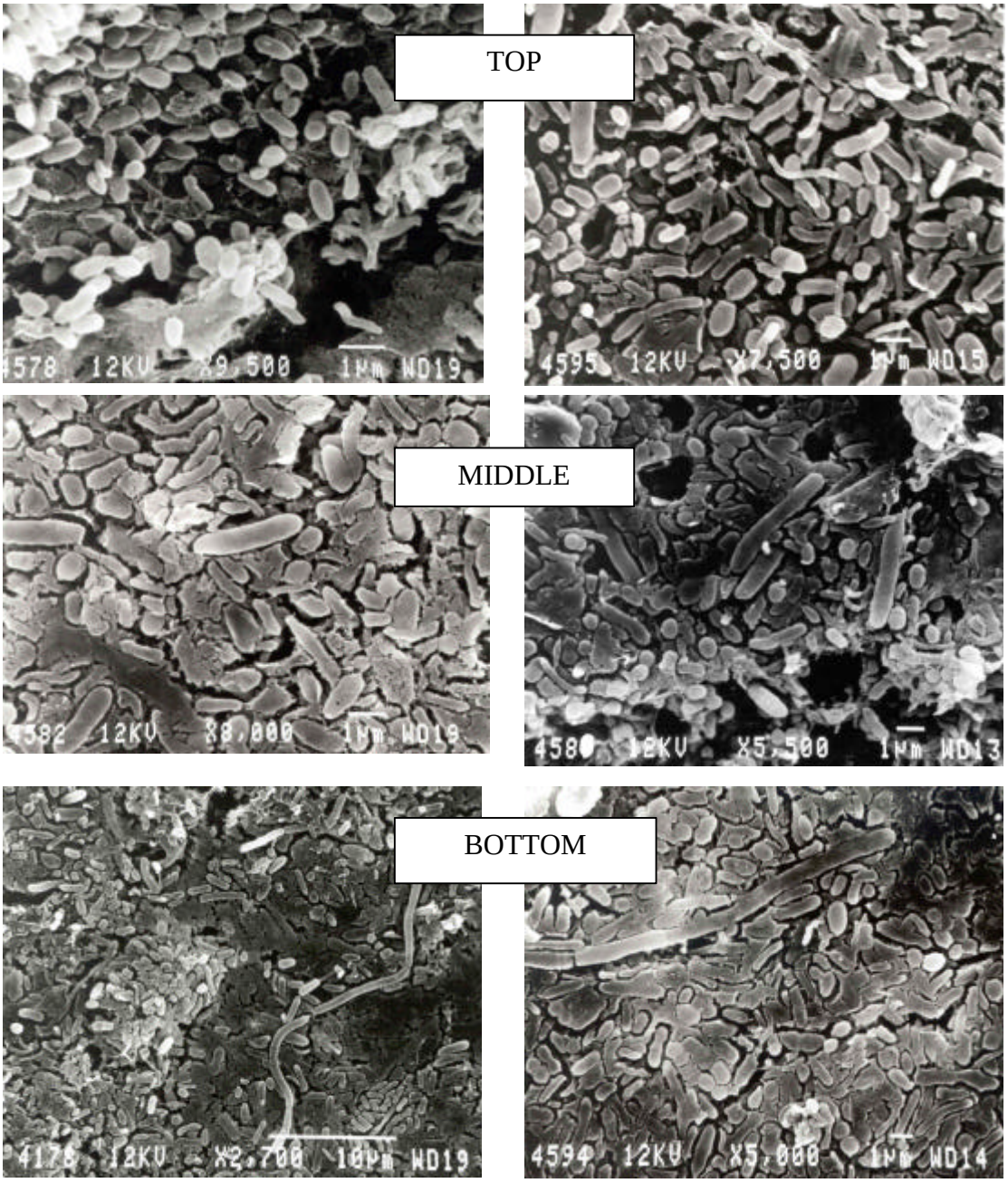

Figure 4.6: SEM photographs of bacteria from the top, middle and bottom sections of the potassium nitrate and ammonium chloride Gradient Tubes.

The SEM photographs on the left side of the page are those taken of the top, middle and bottom sections of the potassium nitrate Gradient Tubes. The SEM photographs on the right side of the page are those taken of the top, middle and bottom sections of the ammonium chloride Gradient Tubes.

Figure 4.6 shows that many different bacterial morphological types were also present in all the sections of the potassium nitrate and ammonium chloride Gradient Tubes, these included cocci, vibrio, short rods and long rods. The top sections of both of the ammonium chloride Gradient Tubes and the potassium nitrate tubes were very similar and seemed to contain mainly cocci, vibrio and short rod-like bacteria. The middle sections of both of the Gradient Tubes were also similar, these contained cocci, vibrio, short rod-like bacteria and long rod-like bacteria. The bottom sections of both of the different Gradient Tubes contained cocci, vibrio, short rod-like bacteria, long rod-like bacteria and also chains of very long rods.

Figure 4.6 showed that there were definitely bacteria present in the different sections of the Gradient Tubes. Similar bacteria were observed in corresponding sections of both the potassium nitrate Gradient Tubes and the ammonium chloride Gradient Tubes. There was however a distinct difference between the top, middle and bottom sections of the tubes. The top sections contained mainly cocci, vibrio and short rods while the middle sections contained all of the above morphologies and also long rods. The bottom sections of the Gradient Tubes contained all of the different morphologies listed above but also included chains of very long rods which were absent from the upper sections.

Another interesting bacterium was observed in the top section of the potassium nitrate Gradient Tube, this bacterium appeared to be coated in extracellular sulphur globules, Figure 4.7.

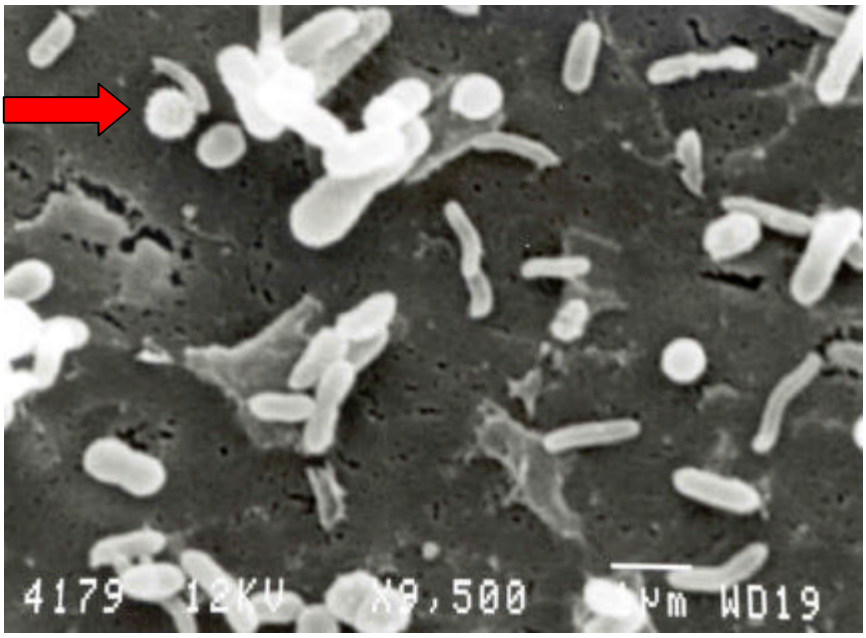

Figure 4.7: SEM photograph of bacteria from the top section of the potassium nitrate Gradient Tube.

The red arrow indicates a coccoid cell that appears to be coated with extracellular crystals, possibly sulphur.

4.3.2.3 SEM of the STB

The most interesting observation made when looking at the Phosphate Silicon Tube Bioreactor system were the bacteria pictured in Figure 4.8. Figure 4.8 is a SEM photograph of a chain of short rod-like bacteria with extracellular crystals, possibly sulphur present on the outside of their cell membranes. It was postulated that these silicon reactors were the most successful converters of sulphate into sulphur due to the abundance of these extracellular sulphur producers.

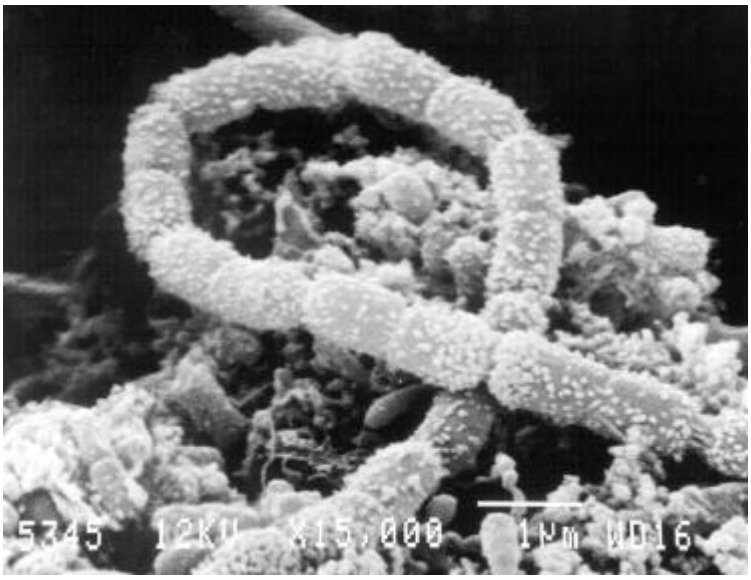

Figure 4.8: A SEM photograph of a chain of rod-like bacteria which are producing extracellular crystals, possibly sulphur in the Phosphate STB (Rein, 2001).

4.3.2.3 SEM of the Tannery Stabilization pond biofilm

Figure 4.9 depicted some bacteria within a fibre-like matrix, unfortunately better SEM photographs could not be taken as the high salinity of these samples interfered with the preparation process. In fact the salinity was so high that gram-stains could not be performed because due to the heat fixing process all that remained to be seen were salt crystals.

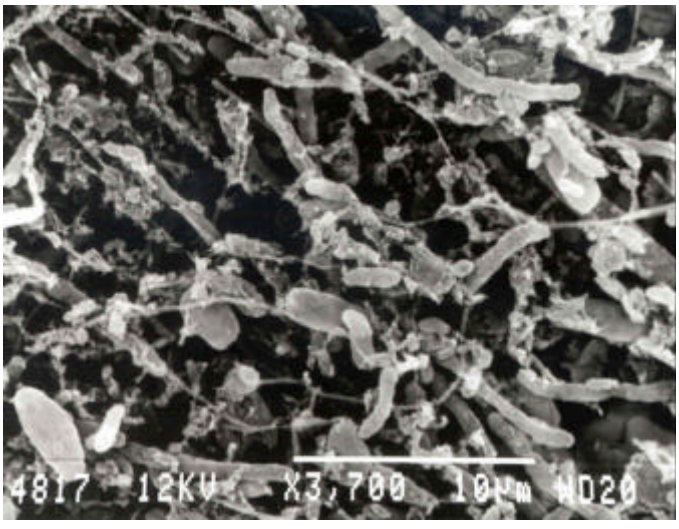

Figure 4.9: A SEM photograph of the biofilm found on the TSP.

4.3.3 Transmission Electron Microscopy

TEM photographs were taken of the bacteria found in the biofilm at the tannery, this was done to see whether they produced intracellular sulphur, as no extracellular sulphur was evident in the SEM photographs and very low sulphur concentrations were detected in the chemical analysis. Sections of the bacteria are visible in Figures 4.10 and 4.11, the electron dense regions on the inside of the cells were thought to be intracellular sulphur.

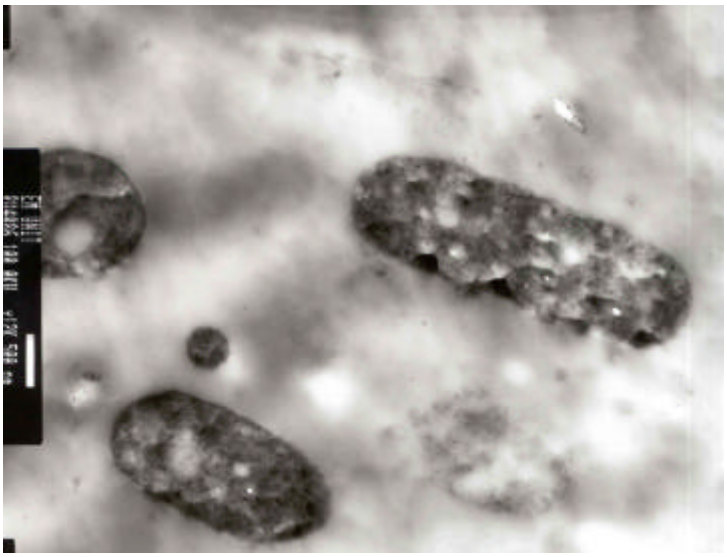

Figure 4.10: A TEM photograph of sections through the bacteria present in samples from the TSP

It could not be confirmed visually that the white globules visible in Figures 4.10 and 4.11 were intracellular sulphur, so the samples were prepared for X-ray diffraction. These samples were not stained with osmium tetroxide. X-ray diffraction measurements were undertaken at The University of Port Elizabeth, Department of Physics**.**

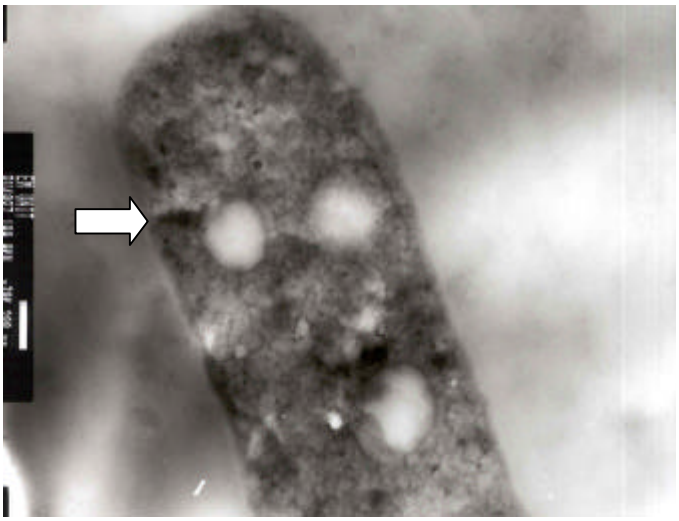

Figure 4.11: A SEM photograph of a section through a bacterial cell present in the biofilm from the TSP.

The white arrow indicates an electron dense region.

Sulphur was not detected in any large quantity, as can be seen from the X-ray diffraction results, Figure 4.12**.** This could possibly have been due to a loss of sulphur during the TEM preparation (sulphur is soluble in acetone), these results were therefore inconclusive. It could not be proved that the cells contained intracellular sulphur.

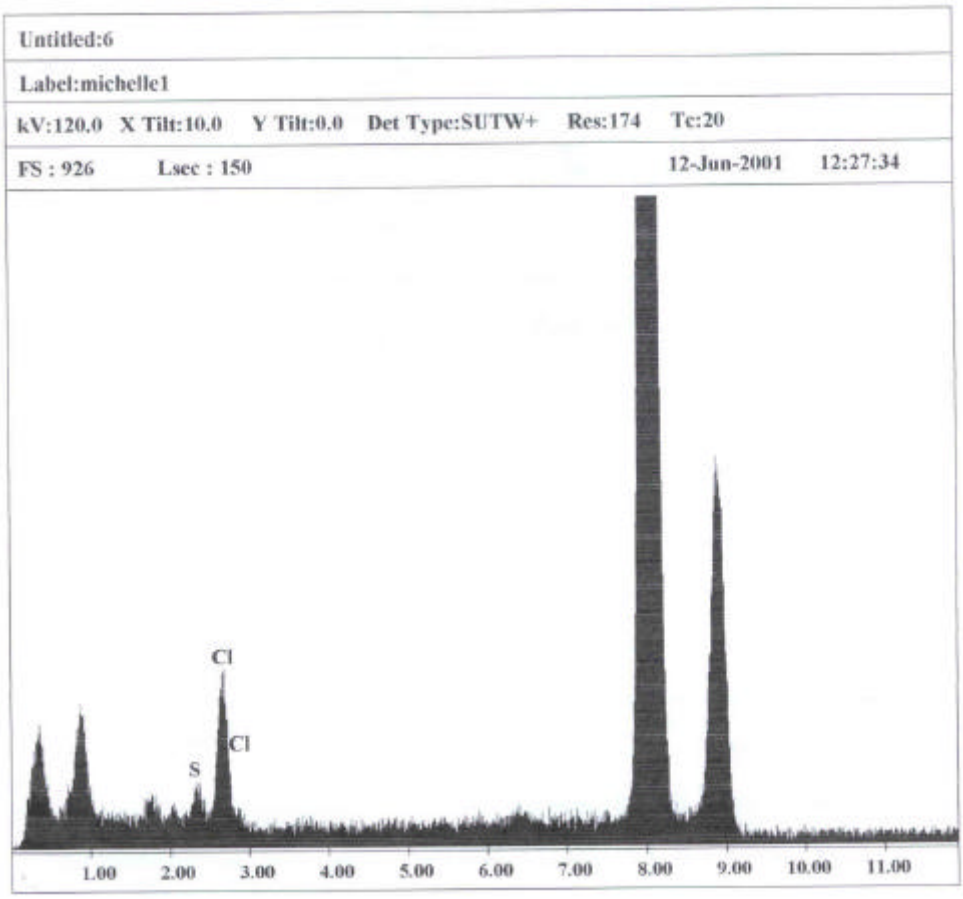

Figure 4.12: The Results of the X-ray Diffraction analysis of the Tannery sample.

A small peak labelled S in the diagram represented the amount of sulphur in the sample.

The X-ray Diffraction sample was prepared for TEM, leaving out any steps which involved the addition of a salt or metal. The samples were mounted on copper grids because copper is far away from sulphur on the periodic table and would not diffract in a zone of the same wavelength as sulphur. The peaks on the graph indicate the elements detected to be present, the two largest peaks were copper (from the grid that the sample was mounted on), sulphur and chloride were also detected but not in very high amounts.

4.4 Discussion

The microscopy studies all indicated that the floating sulphur biofilm community was both diverse and complex, and that many different bacteria with varied morphologies were present. Biofilm systems are generally not homogenous due to spatial and temporal chemical variation within the biofilm allowing for the growth of many different types of bacteria (Costerton *et al.*, 1995), this would account for the many different bacterial forms present.

While no definite identification of the inhabitants of the biofilm could be made the results still allowed for a comparison of the different reactor systems on the basis of the different bacterial morphologies occurring the different reactor systems.

Figure 4.1 demonstrated that there were both gram-positive and gram-negative bacteria present in the biofilm and that there were many different shapes and sizes of these bacteria. The floating sulphur biofilm community could therefore be considered as extremely diverse, the inner heterogeneity of the biofilm provided many different ecological niches which promoted the growth of many different bacterial forms.

The SEM work on the biofilm provided far more information that the gramstains. The gramstain procedure was very destructive so no information on the inner structure and distribution of the different populations could be elucidated. Also the magnification and resolution of the SEM is far superior to what could be achieved with a light microscope. The SEM preparation was less destructive than gramstaining, the biofilm was kept mostly intact during the preparation of the samples. Figure 4.2 was a side-on view of the biofilm, it depicted crystals surrounded on both sides by a diverse bacterial population on bacteria embedded in a matrix. These crystals have been documented previously in sulphur biofilms by Gilfillian (2000) Figure 4.3 and 4.5 demonstrated the high number and diversity of bacteria present in the biofilm. Figure 4.4 showed bacteria surrounding and colonizing the surface of an orthorhombic crystal, possibly sulphur. The visual observations supported this as the thick white biofilm did become brittle and definitely had crystalline characteristics, also chemical analysis confirmed the presence of sulphur in the system.

The Gradient Tubes were novel systems that were used in an attempt to separate the inhabitants of the biofilm along a gradient of sulphide and oxygen concentrations. A comparison was made between the populations that proliferated within the different systems and different areas within the tubes and also to the more natural floating sulphur biofilm that developed on the surface of the Baffle Reactor. SEM was performed on both the potassium nitrate set of experiments and the ammonium chloride set of experiments. These were also compared to each other with respect to the diversity of morphologies present at the different positions in the tubes.

In the top most section of the Gradient Tubes where the oxygen level was high and the sulphur level was low, many bacteria were seen to proliferate, refer to figures 4.6. Figure 4.6 showed the bacteria which had colonized the uppermost sections of the potassium nitrate and ammonium chloride Gradient Tubes, included vibrio, cocci and short rods, less common were the long rods that were visible in Figures 4.2 – 4.5 of the Baffle Reactor biofilm. Also present in Figure 4.7 was a single coccus that seemed to have extracellular sulphur present on the outside of its cell wall, this was observed in the potassium nitrate Gradient Tube. This microscopic evidence was supported by the chemical analysis of the tubes, both indicated the presence of sulphur in the top fractions of the tube. This sulphur was possibly produced by SOB for example *Beggiatoa* species which has been documented to produce sulphur under aerobic, high-sulphide conditions (Basu *et al.*, 1995).

The middle sections of the potassium nitrate and ammonium chloride Gradient Tubes, Figure 4.6**,** were seen to contain cocci, vibrio, short rods and long rods. Neither sample showed any evidence of extracellular sulphur producers.

The bottom sections of the potassium nitrate and ammonium chloride Gradient Tubes both contained cocci, vibrio, long and short rods and also chains of very long rods, no extracellular sulphur producers were seen. The tubes showed similar bands of growth at the bottom of their overlays so it was expected that the populations might have appeared similar.

The STB were the most successful systems for converting sulphate and sulphide into sulphur. Figure 4.8 showed some of the diverse forms of bacteria present in these

reactors, these morphologies were comparable to those found in the Baffle Reactor and the Gradient tubes but the main difference between these systems was the presence of extracellular sulphur producers**.** The extracellular sulphur producers were probably the reason that the reactors produced sulphur so efficiently while the other systems were quite inefficient in comparison. A well documented extracellular sulphur producer is *Beggiatoa* species (Basu *et al.*, 1995), also Ectothiorhodospira species have been observed to produce extracellular sulphur (Dunn, 1997).

The SEM of the natural floating film that formed on the TSP was also prepared for SEM but the high salinity of the samples interfered with some of the preparation steps hence good images were not achieved. Figure 4.9 depicted bacteria from the biofilm in a sort of fibrous matrix, many different bacterial forms were also seen to be present but not extracellular sulphur producers.

Due to the fact that the Baffle Reactor system, Gradient Tube systems and TSP biofilm did contain sulphur according to the chemical analysis but almost no extracellular sulphur producers were seen, TEM was performed in order to see if intracellular sulphur was produced. Figure 4.10 and 4.11 are TEM photographs of cells which seemed to contain electron dense globules inside their cell membranes. It could not be confirmed that these globules were sulphur merely by visual evaluation so X-ray Diffraction was attempted.

Figure 4.12 depicts the results of the X-ray Diffraction analysis, sulphur was found to be present but not in very high quantities, this and the fact that the Physics Department was having problems with the X-ray Diffraction system, led this study to be inconclusive as to whether the cells contained intracellular sulphur.

The floating sulphur biofilm was seen to contain sulphur but this sulphur was only thought to have been observed in the form of orthorhombic sulphur crystals and not as extracellular sulphur. These observations suggested that the biofilm was providing the correct conditions for the catalytic conversion of sulphide to polysulphide and then sulphur rather than its oxidation to thiosulphate and then sulphate. The fact that not many extracellular sulphur producers were observed does not mean that none

were present, they were possibly present in low numbers, in different sections of the biofilm where the redox conditions suited them best

4.5 Conclusion

The floating sulphur biofilm from the Baffle Reactor was found to contain a very diverse bacterial population. This was postulated to be due to the many different environmental niches created in the biofilm structure which in turn encouraged the growth of many different bacteria.

The Gradient Tube experimental system was found to encourage the growth of a diverse community of bacteria, similar looking to those found in the Baffle Reactor biofilm. The populations within the different sections of the Gradient Tubes were found to be similar but the potassium nitrate Gradient Tube was the only one that was seen to contain an extracellular sulphur producer.

There was a definite morphological differentiation throughout the different sections of the tube although no conclusive evidence could be found that species separation was occurring.

The success of the STB was concluded to be due to the presence of extracellular sulphur producers, the redox conditions in these systems were carefully controlled and seemed to be perfect to encourage the growth of these bacteria.

The presence of intracellular sulphur in the biofilms could not be confirmed by TEM and X-ray diffraction although it must have been present due to the results of the chemical analysis and the absence of extracellular sulphur producers.

Sulphur was present in the biofilm possibly in the form of orthorhombic sulphur crystals, suggesting that the biofilm was creating the perfect condition for the catalytic conversion of sulphide to sulphur.

CHAPTER 5:
MOLECULAR STUDY OF SULPHUR BIOFILM POPULATIONS

5.1 Introduction

One of the major obstacles encountered in studying the ecology of biofilms is the difficulty involved in isolating, identifying and enumerating individual species and strains with similar metabolic requirements (Goebel & Stackebrandt, 1994). Diverse natural communities like biofilms contain many different species of bacteria, culture techniques are inadequate to provide the correct growth conditions for all of these bacteria (identified and unidentified species) and microscopy, which has been a very useful technique in the past, is also not of much use as the simplicity of bacterial morphology makes distinguishing between all the different species impossible. Bacterial diversity can today be more directly assessed by the cultivation-independent comparative analysis of genetic material (Amann *et al.*, 1996).

The application of molecular methods has revolutionized the routine identification of bacteria from environmental and industrial samples (Korber *et al*., 1999). Techniques based on the analysis of genetic material complement the conventional microbiological approach and are routinely used to determine the presence and distribution of individual bacterial species, including those in complex communities such as bacterial biofilms (Santegoeds *et al.,* 1998).

The quantitative recovery of nucleic acids from environmental samples imposes major limitations on the molecular approach. The initial extraction of nucleic acids is a crucial step because not all microorganisms lyse equally well (Amann *et al.*, 1995). It has been demonstrated that a combination of physical and chemical treatments such as freeze thawing, lysis with detergents and bead beating lysed approximately 96% of soil bacteria, smaller cells seem to be more resistant to lysis (0.3-1.2μm) (Head *et al.*, 1998). It was also found that up to 99.8% lysis could be achieved by merely using extended lysis incubations and up to 6 freeze thaw cycles (Head *et al.*, 1998).

The application of 16S rRNA sequence analysis has revolutionized the study of both microbial ecology and phylogeny (Goebel & Stackebrandt, 1994). Several rRNA-based methods have been developed to identify and quantify microorganisms in complex environments. These methods can be used without isolation and cultivation.

The rRNA approach, together with other molecular techniques holds great potential for an analysis of microbial diversity which is unbiased by the limits of pure-culture techniques (Amann *et al.*, 1995).

rRNA has particular advantages, it is present in all organisms, it has conserved and variable regions (this enables the selection of general and specific target sequences), it contains enough sequence information to be used as a phylogenetic marker, the genes are not transferred horizontally between species and there are large data-bases of sequences available (Muyzer & Ramsing, 1995). With the aid of Polymerase Chain Reaction (PCR) a target rRNA sequence can be amplified.

To get a general impression of the heterogeneity of the community it is possible to separate the PCR products with DGGE. DGGE is a method by which fragments of DNA of the same length but different sequence can be resolved electrophoretically (Head *et al*, 1998). This method has recently been applied to the analysis of 16S rRNA genes from environmental samples and allows the separation of a heterogeneous mixture of PCR amplified genes on a polyacrylamide gel. Individual bands may be excised, reamplified and sequenced to give an indication of the composition and diversity of the microbial community (Head *et al.*, 1998).

Fingerprinting of bacterial communities by separation of amplified 16S rDNA fragments with DGGE provides the opportunity to compare community structure of multiple environmental samples. This method is now widely used to characterize microbial ecosystems. The number and position of bands on the gel allow a comparison of dominant rDNA types between communities. However the number and intensity of the bands does not accurately represent the number and abundance of species within the community because of the inherent bias of PCR amplification (Eichner *et al.*, 1999).

Selectivity and errors in PCR amplification of rRNA genes are however a source of bias that can affect the results of molecular biological measures of diversity (Head *et al.*, 1998). Small differences in the sequence of universally conserved regions may result in selective amplification of some sequences, particularly when primer annealing is at high stringency and errors may occur during amplification (Head *et al.*, 1998). PCR amplification can result in more than one band on the DGGE from a single organism with multiple heterogeneous rRNA operons (Eichner *et al.*, 1999). Also in a complex mixture of species, less abundant DNA templates are not sufficiently amplified in order for them to be visualized on a gel, the visible bands therefore only represent the most abundant species (Eichner *et al*, 1999).

In order to ensure that the PCR products were accurate copies of the target sequences the Expand™ High Fidelity PCR System from Roche was used to perform the PCR reaction. Expand™ High Fidelity PCR System is composed of a unique enzyme mix containing thermostable *Taq* DNA polymerase and Pwo DNA polymerase. This polymerase mixture is designed to give high yield and high specificity PCR products from episomal and genomic DNA. Also due to the inherent 3' – 5' exonuclease proofreading activity of the Pwo DNA polymerase, Expand™ High Fidelity PCR System results in a 3-fold increased fidelity of DNA synthesis when compared to Taq DNA polymerase. The Expand™ High Fidelity PCR System generates products that are a mixture of blunt ends and 3' single A overhangs, making insertion into plasmids easier.

The thermocycler temperatures used incorporated a touchdown cycle, this meant that the primer annealing started off at a high temperature, encouraging only exact primer binding, resulting in an increased specificity, the temperature then dropped over a number of cycles resulting in more primer binding but off the newly made exact copies.

To determine the optimal DGGE conditions for characterizing the microbial sample, the PCR amplified fragments can be run on a perpendicular DGGE gel from 10-100% denaturant. Denaturant concentration is specified as percent of an arbitrary standard: 100% denaturant is defined as 40% (v/v) formamide plus 7M urea (Abrams &

Stanton, 1992). By looking at the result of the perpendicular gel, it could be predicted at which denaturant concentration the DNA stopped migrating (Muyzer *et al*., 1993). To determine the amount of time and voltage of the electrophoresis many runs were performed and the optimal conditions were selected according to the best gel results.

PCR and DGGE are very useful techniques that can be used to quickly profile the community members from a wide variety of environmental samples.

Investigation of microbial community structure and diversity in environmental samples generally include cloning and sequence determination of 16S rRNA genes (Mau & Timmis, 1998). The detection of minor members in environmental samples generally requires the analysis of many cloned sequences (Mau & Timmis, 1998).

Cloning and sequencing are time-consuming; DGGE offered a faster and more comprehensive means to study varying bacterial communities. DGGE detects the occurrence of sets of phylogenetically related populations as a consistent pattern of community structure (Ferris *et al.*, 1996). The DGGE profiling method can be useful for immediately approximating the presence and relative abundance of microbes, but further cloning and sequencing are necessary before an accurate understanding of the genetic diversity of the complex microbial populations could be formed. DNA fragments were excised from the gel, these single DNA templates were reamplified and sequenced directly.

Because DGGE bands were sequenced directly this removed the bias introduced through shot-gun cloning, it also eliminated the lengthly screening of redundant clones which served to increase the number of samples that were able to be processed, all of which enabled a more aggressive pursuit of the ecology of the populations studied.

Although sequencing of bands for analysis provides insight into the community structure through phylogenetic affiliations of community members, the information about their physiological and ecological traits derived from the partial sequences is

rather limited. Molecular and chemical results were therefore combined to provide an overall picture of how the different systems functioned.

5.2 Methods

5.2.1 Sample collection

Biofilm samples were skimmed off the top of the Baffle Reactor, the Falling sludge bed reactor and the TSP. This was done by breaking a sterile petri dish in half and using it as a scoop, the samples (generally 1ml) were placed in sterile eppendorfs and frozen at -20°C. Samples from the test tube reactors were collected in 0,5g sections; these were melted in 1ml of TE buffer (10mM Tris, 1mM EDTA, pH 8.0) (Appendix 2**)** and used for further analysis. Samples from the STB were taken by cutting open the tubing using sterile blades and scraping the attached biofilm into sterile eppendorf tubes and then storing them at -20°C.

5.2.2 DNA extraction (manual method-Head *et al.*,1998)

Frozen biofilm samples were thawed at room temperature. These samples were then spun down at 13 000 rpm for 5 minutes in an Eppendorf 5415D desktop centrifuge. The supernatant was discarded and the pellets were resuspended in 500µl of TE buffer (Appendix 2) and 6µl of 50mg/ml of lysozyme stock solution was added. The tubes were then incubated for 3 hours at 37°C, these samples were vortexed periodically.

The samples were boiled for 1 minute and then five freeze-thaw cycles were performed using liquid nitrogen and an 80°C water bath. After the last freeze-thaw cycle, the sample was allowed to cool to room temperature and then 50µl of 10% Sodium Dodecyl Sulphate (SDS) (Appendix 4) and 2.5µl of 50µg/ml Proteinase K stock solution was added and the tube was mixed by inverting four times. The samples were incubated at 37°C overnight.

100µl of 10% CTAB (Appendix 4) and 200µl of 5M NaCl (Appendix 3) was added and the samples were incubated at 55°C for 1 hour. The samples were then aliquoted out in 500µl volumes into sterile eppendorfs, and an equal amount of Buffer-

Saturated Phenol pH 8 (Appendix 4) was added. The samples were vortexed for 30 seconds and then spun down for 2 minutes at 13 000rpm. The upper aqueous layer was then transferred to a fresh eppendorf tube and an equal amount of phenol:chloroform:isoamyl alcohol (24:24:1) was added. The samples were then vortexed and spun down as above. The upper aqueous layer was then transferred to a fresh eppendorf tube and an equal amount of chloroform:isoamyl alcohol (24:1) was added. The sample was vortexed and spun down as previously done, this step was repeated until the upper aqueous layer was clear.

The final clear upper aqueous layer was placed in a sterile eppendorf tube and 2.5 volumes of ice cold 96% rectified ethanol was added, the samples were then placed at -20°C for 12 hours. The samples were then microfuged at 13 000rpm for 20 minutes and the supernatant was discarded. The pellets were allowed to air dry and then resuspended in triple distilled water, the volume depending on the size of the pellet, but usually 500µl. The DNA concentration of the samples was generally calculated to be 0.5µg/µl. The DNA samples were placed at 4°C for short-term storage and for long-term storage they were placed at -20°C.

5.2.3 Agarose Gel Electrophoresis (Sambrook *et al*., 1989)

The results of the DNA extractions were viewed by using agarose gel electrophoresis. First a 1% agarose gel was prepared, this consisted of 0,5g of molecular grade agarose and 50ml 1 x TBE Buffer (Appendix 4), this mixture was boiled until the agarose was completely dissolved and then cooled to approximately 50C. 50 ìl of ethidium bromide stock solution (Appendix 4) was added and then the agarose gel was poured into a gel mould, a comb was placed into the gel and it was allowed to set. Once set the gel was placed in a gel tank containing 1 x TBE buffer.

The 10ì l DNA extraction samples had 2ì l DNA loading buffer (Appendix 4) added to them before they were loaded onto the gels. A *lPst1* molecular weight marker was loaded onto the gel in order to determine the molecular weight of the bands on the gel by drawing a standard curve (Appendix 4). The gels were generally run at 100V of 1 hour and then viewed on a UV lightbox and photographed using a Kodak Digital camera.

5.2.4 Polymerase Chain Reaction

PCR amplification was performed for each of the many different DNA samples, these included samples from the Baffle Reactor, TSP biofilm, each section of the Gradient Tubes and the three different sections from the STB. The PCR reactions were all performed using an increased magnesium concentration of 3mM, 1 in 100 dilutions of the DNA samples also had to be performed in certain cases.

Each 25µl PCR reaction contained: 1µl 10mM GM5F primer stock solution (Muyzer *et al.,* 1993) (Santegoeds *et al,* 1998) (Appendix 5), 1µl 10mM 907R primer stock solution (Muyzer *et al.,* 1993) (Santegoeds *et al,* 1998) (Appendix 5), 1µl 10mM dNTP stock solution (Appendix 5), 2.5µl Expand High Fidelity 10 x PCR buffer without $MgCl_2$, 3µl Expand High Fidelity 25mM $MgCl_2$ stock solution, 0.4µl Expand High Fidelity polymerase mixture (3.5U/µl), 1µl DNA (200-500ng/µl chromosomal DNA) and 10.1µl $dddH_2O$

The PCR reactions were placed in the PCR*Sprint* thermocycler from Hybaid and the Touchdown thermocycle program was run, Table 5.1.

Table 5.1: Thermocycler cycles used for PCR amplification

Initial Denaturation	95°C	2 minutes	1 cycle
Denaturation	94°C	30 seconds	4 cycles
Annealing	68°C	45 seconds	
Extension	72°C	2 minutes	
Denaturation	94°C	30 seconds	4 cycles
Annealing	66°C	45 seconds	
Extension	72°C	2 minutes	
Denaturation	94°C	30 seconds	4 cycles
Annealing	64°C	45 seconds	
Extension	72°C	2 minutes	
Denaturation	94°C	30 seconds	4 cycles
Annealing	62°C	45 seconds	
Extension	72°C	2 minutes	

Denaturation	94°C	30 seconds	12 cycles
Annealing	60°C	45 seconds	
Extension	72°C	2 minutes	
Final Extension	72°C	5 minutes	1 cycle

5.2.5 Denaturing Gradient Electrophoresis

The first step in DGGE was to optimize the denaturant concentration. This was done by using a perpendicular DGGE gel. Perpendicular gels differ from normal DGGE gels in the direction of the gradient, as outlined in Figure 5.1.

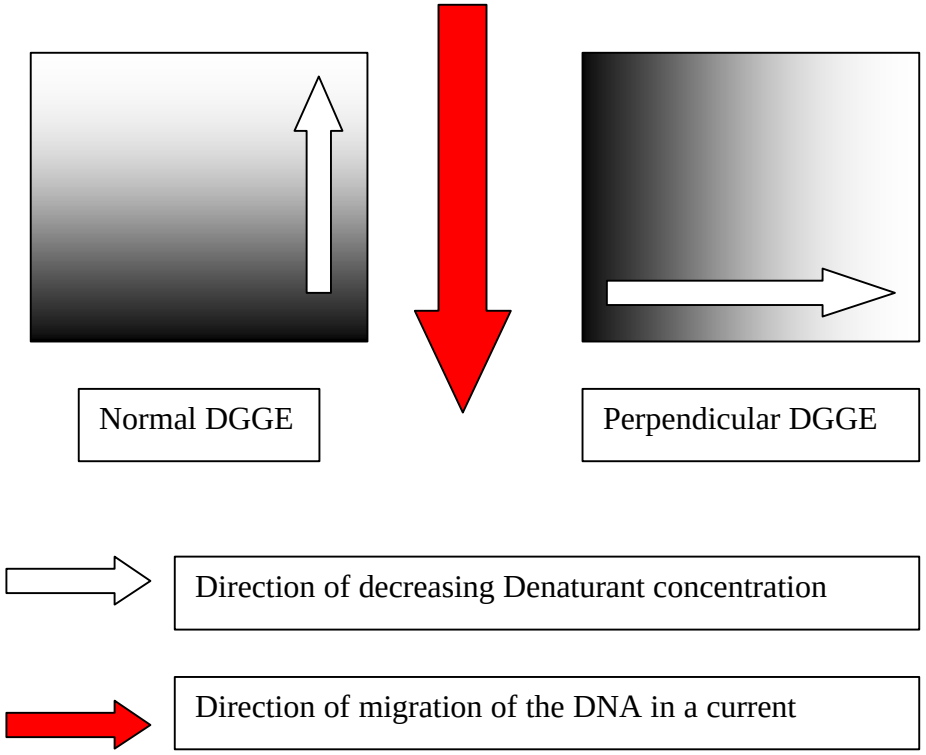

Figure 5.1: Diagram illustrating the difference between normal DGGE and Perpendicular DGGE.

Once the Perpendicular gel was run, it was easy to see at which denaturant concentration the DNA stopped migrating and therefore which concentration of denaturants provided optimum separation.

The Sigma-Aldrich Vertical Electrophoresis Unit model SV10-CDC was used for the DGGE. The Gel plates were cleaned first with acetone and then alcohol, these were assembled according to the manufacturer's manual and clamped into the SIGMA Gel Casting Unit. 1.5l of 1 x Tris-Acetic acid EDTA (TAE) Buffer (Appendix 5) was preheated to 65°C.

The solutions used for the Perpendicular gel were 0% denaturant (Appendix 5) and 100% denaturant (Appendix 5). These solutions were placed in the Bio-Rad model 385 Gradient Former, the 100% denaturant in the first well with a magnetic stirrer bar and the 0% denaturant in the second well, the channel between the wells was opened and an Eyela Microtube Pump MP-3 was used to pump the denaturants towards the gel plates. The Snijder Hotplate Magnetic Stirrer was used to mix the denaturants resulting in the formation of the gradient.

A MACMED Microlance®3 Sterile Needle was used to pour the gel between the gel plates, once the gel level was about 2cm from with the top of the glass plates, the pump was switched off and the gel was allowed to set in a 37°C incubator

The microtubing and gradient former were washed with dddH_2O in order to flush out all the acrylamide before it polymerized. Once the gel had set, the plates were separated and the gel turned onto its side, the spacers were repositioned and the plates replaced and reclamped into the casting stand. 0% Denaturant was then used to pour the well section of the gel, a 10 well comb was inserted and the gel was placed back at 37°C to set.

Once the gel had set, it was removed from the casting stand and placed in the Sigma-Eldrich Gel Tank, the gel tank was filled with the preheated 65°C 1 x TAE buffer. The comb was removed and the wells were flushed of excess acylamide using a syringe. A prerun was done to make sure that all the acylamide moved out of the wells, this was done at 200V for 5 minutes.

The perpendicular gel was loaded using Costar® Gel-Loading Tips, for the perpendicular gel a PCR product containing only one DNA template was used.

Approximately 5µl of PCR product and 3µl of DNA loading buffer (Appendix 4) were loaded into each well. The gel was run at 180V for 2hours. The buffer temperature was kept at 65°C by circulating 65°C water through the Base Core using a Watson-Marlow 504S pump.

Once the run was complete the gel was removed from the plates and the DNA bands were visualized using a silver stain technique (described in 5.2.6).

The optimized denaturant concentrations determined by perpendicular DGGE were 55% denaturant (Appendix 5) and 65% denaturant (Appendix 5). The normal DGGE gels were poured as above, the only difference being that the gel was poured up to the top of the plates and the comb inserted, the gel plates were never separated and the gel was never turned onto its side. Also PCR products containing many different DNA template were loaded onto the gel, duplicate gels were always poured, one of these gels had a 55% - 65% denaturant gradient and the other contained 0% denaturant (Appendix 5). This was done as a control to distinguish between non-specific bands and the 586bp PCR fragments.

5.2.6 Silver staining (Method adapted from the Qiagen TGGE Manual)

The gels were carefully removed from the plates and soaked in fixing solution (Appendix 5) for 6 minutes. The fixing solution was discarded and the gel was immersed in 0.1% silver nitrate for 10 minutes. The excess silver ions were removed by rinsing the gel twice in Milli-Q water. The gel was developed in a fresh solution of 1.5% sodium hydroxide, 0.01% sodium borohydride and 0.15% formaldehyde (Appendix 5). Once the gel had developed sufficiently the developing was stopped by washing the gel in fixing solution for 30sec. The gel was then left in water and photographed on a lightbox using a Kodak Digital Camera.

5.2.7 Extraction of DNA from DGGE bands (Adapted from Sambrook *et al.*, 1989).

Bands were excised from the gel, these were placed into sterile eppendorf tubes and 400µl of 1M sodium chloride in TE buffer pH 8 (Appendix 5) was added. The samples were placed at 65°C for 1 hour and then incubated at 37°C overnight. The

samples were microfuged at 13 000rpm for 10 minutes and the supernatant was transferred to a fresh tube. An equal amount of phenol : chloroform : isoamyl alcohol (24 : 24 : 1) was added and the sample was vortexed every 30 seconds for 5 minutes. The sample was then microfuged for 2 minutes at 13 000 rpm and the upper aqueous layer was transferred to a fresh tube. 3M sodium acetate (Appendix 5) was added to a final concentration of 10% and 2.5 volumes of 96% rectified ethanol was added. The samples were left at -20°C for 2 hours before being spun down at 13 000rpm for 10 minutes. The supernatant was discarded and the pellet was allowed to air dry. The dry pellet was resuspended in 12µ of dddH_2O. This sample was used for PCR, as above. The PCR samples were then ready for cloning.

5.2.8 Preparation of Competent *E.coli* DH5α (Adapted from Sambrook *et al.*, 1989).

Competent *E.coli* cells were prepared by inoculating a 5 ml test tube of Luria broth (LB) (Appendix 6) with DH5á cells, these were shaken overnight at 37ºC. Four 100ml LB flasks were inoculated with 1.5, 1, 0.7 and 0.3 ml of the pre-inoculum and incubated at 37ºC on the orbital shaker for approximately 2 hours. The cultures were allowed to grow until the absorbance of the 1.5ml inoculated flask reached an absorbance of 0.6–0.8 (OD_{600}). These flasks were then placed on ice for 5-10 minutes.

The cultures were centrifuged for 10 minutes at 5000rpm using the Beckman JA 14 rotor, all centrifugation steps were performed at 4°C. The supernatant was discarded and the pellet resuspended in 50 ml of RF1 (100 mM KCl, 50 mM $MnCl_2$, 30 mM CH_3COOK, 10 mM $CaCl_2$, 15 % m/v glycerol, pH 5.8) (Appendix 6), This was incubated on ice for 20 minutes. The cells were then pelleted by a 10 minute centrifugation step as above. The supernatant was decanted and the four pellets were resuspended in 4 ml RF2 (10 mM MOPS, 10 mM KCl, 75 mM $CaCl_2$, 15 % m/v glycerol, pH 6.8) (Appendix 6) and the flask contents were pooled. The cells were aliquoted in 150ì l volumes and were stored at – 80ºC, until use.

5.2.9 Setting up Ligations (pGEM-T Easy Vector System)

PCR products for cloning had to be ligated into the pGEM-T Easy Vector system (Appendix 6). Ligation reactions with a final volume of 10µl were set up as per Table 7.1 and then incubated at 4°C overnight.

Table 5.2: Ligation Reactions for pGEM-T Easy Vector.

	Standard Reaction	Positive Control	Background Control
2 x Rapid Ligation Buffer	5µl	5µl	5µl
pGEM-T Easy Vector	1µl	1µl	1µl
PCR product	1µl	-	-
Control Insert	-	2µl	-
T4 DNA Ligase 3	1µl	1µl	1µl
dddH_2O	2µl	1µl	3µl

5.2.10 Transformation (Sambrook *et al.*, 1989)

The ligations were incubated at 4°C overnight and then 2.5ul of each ligation reaction was added to 150ìl of thawed competent *E.coli* cells, the tubes were mixed and left on ice for 20 minutes. The cells were heat-shocked at 42ºC for 45 seconds and then cooled on ice for 5 minutes. 1 ml of SOC medium (Appendix 6) was added and the tubes were placed at 37ºC for 1 hour to allow the cells to recover.

200µl of the cells were plated onto LB plates containing 100µg/ml Ampicillin (Appendix 6) and spread with 4ìl IPTG stock solution (200mg/ml) (Appendix 6) and 40 ìl X-GAL stock solution (100mg/ml) (Appendix 6). The plates were incubated at 37ºC overnight.

Colonies containing an insert would have appeared white and those that just had the plasmid appeared blue. The white colonies were picked off the standard reaction plates into 5ml LB test tubes using sterile toothpicks and the cultures were grown overnight at 37ºC.

It had to be confirmed that the correct size insert had been inserted into the cells, this was done by performing a plasmid extraction on the overnight cultures and then a plasmid digest. The restriction endonuclease digestion products were resolved on a 1% agarose gel.

5.2.11 Plasmid extraction

The plasmids were extracted from the cells using the QIAprep Spin Miniprep Kit from QIAGEN®. The white colonies tooth-picked into 5ml LB test tubes were grown at 37ºC overnight. 1ml of each culture was placed into a sterile eppendorf, the cells were pelleted by centrifuging them at 13 000rpm for 1 minute. The supernatant was discarded and another 1ml volume of culture was added, the centrifugation step was repeated.

The pelleted cells were resuspended in 250µl of Buffer P1. 250µl of Buffer P2 was added and the tube was inverted 5 times in order to mix. 350µl of Buffer N3 was added and the tube was inverted immediately but gently 5 times. The sample was centrifuged at 13 000rpm for 10 minutes, the supernatant was then applied to a QIAprep spin column. The sample was centrifuged for 1 minute and the flow-through was discarded. The QIAprep spin column was washed using 750µl of Buffer PE and then centrifuged at 13 000rpm for 1 minute. The flow-through was discarded and the samples were centrifuged for an additional 1minute in order to remove any residual wash buffer.

The QIAprep column was placed into a sterile 1.5ml eppendorf tube and 50µl of Buffer EB was added to the centre of the column in order to elute the DNA. The samples were left to stand for 1 minute and then centrifuged at 13 000rpm for 1 minute. The DNA was placed at 4°C for short-term storage and at -20°C for long-term storage.

5.2.12 *Eco*RI Digest

The plasmid extraction had to be digested with *Eco*RI in order to check whether the insert was correct. The *Eco*RI enzyme cuts on either side of the inserted fragments

and in some cases in the centre of the fragment. The correct result would be a 586bp fragment or two smaller fragments whose combined size added up to 586bp.

10µl of each QIAprep plasmid extraction was used for the digest. The digest consisted of 0.2µl of *Eco*RI (10U/µl), 2µl Buffer H, 10µl plasmid and 7.8µl of dddH_2O. The plasmid digest was mixed and placed at 37°C for 3 hours. The restriction endonuclease digestion products were resolved on a 1% agarose gel.

5.2.13 Thermocycling using the BigDye™ Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems)

The Thermocycling reaction sample consisted of 200-500ng of plasmid DNA, 1µl 10ìM GM5F primer stock solution, 4µl BigDye, 2µl 5 x Dilution Buffer and distilled water (to volume of 20ì l). The reaction samples were placed in the GeneAmp® PCR system 9700 thermocycler and the cycle sequencing method in Table 7.2 was followed.

Table 5.3 Cycle Sequencing on the 9700 PCR System.

Step	**Action**
1	Place the tubes in the thermal cycler and set the volume to 20µl
2	Repeat the following for 25 cycles: • Rapid thermal ramp to 96°C. • 96°C for 10 seconds. • Rapid thermal ramp to 50°C • 50°C for 5 seconds. • Rapid thermal ramp to 60°C. • 60°C for 4 minutes.
3	Rapid thermal ramp to 4°C and hold.
4	Spin down the contents of the tube in a microcentrifuge
5	Purify the extension products

5.2.14. Purification of the Extension Products using the ZYMO Research DNA Clean & Concentrator™-5

100ìl of DNA binding buffer was added to each sample once cycle sequencing was complete. The samples were then loaded onto a Zymo-Spin column that was placed in a 2 ml collection tube. The samples were centrifuged at 10 000g for 10 seconds and the flow-through was discarded. 200ìl of wash buffer was added to the column and then the samples were centrifuged for 10 seconds at maximum speed. The flow-through was discarded and another 200ìl was applied to the column. The samples were spun for 30 seconds at 10 000g and the flow-through was discarded.

To elute the DNA 8ìl of dddH_2O was applied directly to the column matrix. The Zymo-Spin column was placed in a sterile eppendorf and centrifuged for 10 seconds at 10 000g.

The tubes containing the DNA were left open and placed at 37°C in order to dry the DNA. The dry DNA pellet was stored at –20°C for sequencing.

5.2.15 DNA sequencing on the ABI PRISM® 3100 Genetic Analyser (Applied Biosystems).

Each ZYMO purified DNA sample was resuspended in 10ìl of deionised formamide (Appendix 5), the samples were vortexed and spun down. The samples were then heated to 95°C for 2 minutes in order to denature them and then chilled on ice until ready to use.

Refer to the ABI PRISM 310 Genetic Analyser User's Manual (P/N 903565) for information on the sequencing process.

5.2.16 Analysis of the Sequencing Results

Chromatograms were generated by the ABI PRISM® Genetic Analyser Data Collection system 1.0.1 by Applied Biosystems. These chromatograms were converted into text format using Gene Tools and then put into the NCBI BLAST database. BLAST or Basic Local Alignment Search Tool is a set of similarity search programs designed to explore all of the available sequence databases (Altschul *et al.*,

1990). The percentage similarity to an identified species was recorded, also the length of the match and the E value, which is important for determining the accuracy of the result.

5.3 Results

5.3.1 DNA extraction

Figure 5.2 showed that DNA had been successfully extracted from the Baffle Reactor (duplicate samples) and from samples of the TSP biofilm. The DNA in all of the lanes was slightly degraded, indicated by the long smear in each lane, but there were very bright bands at the top of the gel, indicating a high concentration of large molecular weight fragments. This DNA was good enough to be used as template for PCR.

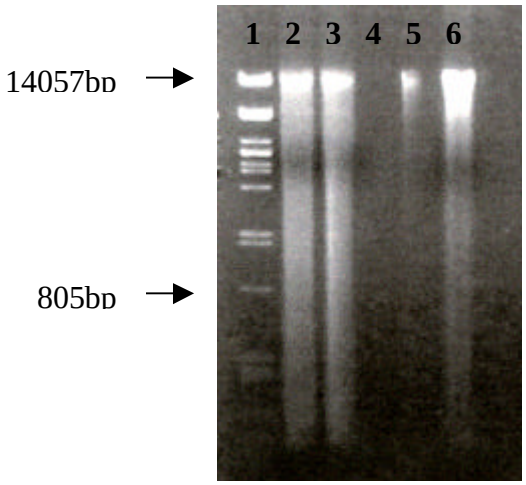

Figure 5.2: 1% Agarose gel showing the results of the DNA extractions.

Lanes 1-6 contain ë *Pst* DNA Molecular Weight Marker, DNA from the Baffle Reactor biofilm sample 1, DNA from the Baffle Reactor biofilm sample 2, blank, DNA from the TSP biofilm sample 1 and DNA from the TSP biofilm sample 2.

5.3.2 PCR of Biofilm DNA Extractions

PCR was performed on DNA extractions of the Baffle Reactor, the TSP biofilm, the Gradient Tubes and the STB. The PCR primers amplified up 586bp fragments, Figure 5.3, each band on the gel contained 586bp fragments with many different sequences.

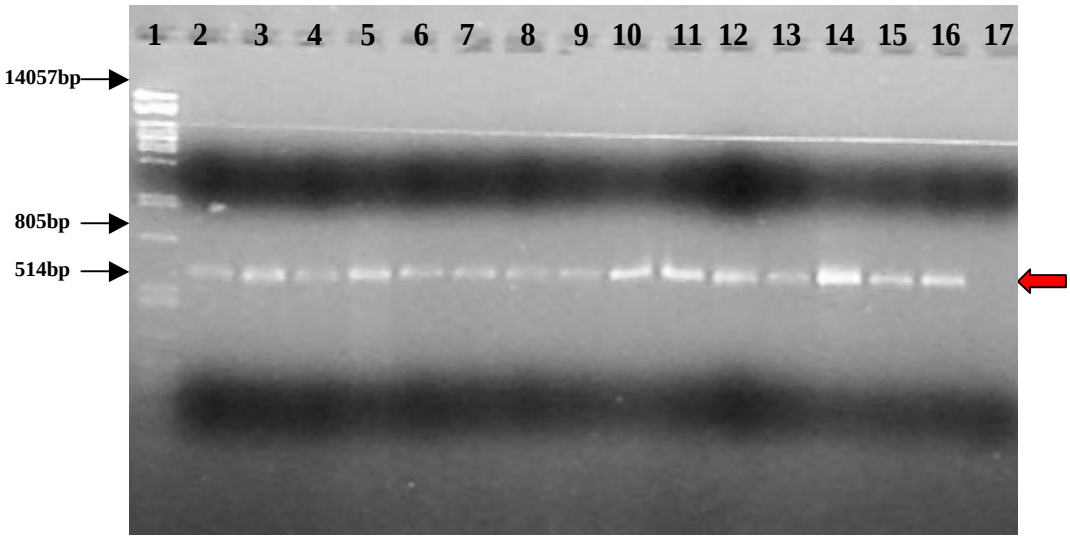

Figure 5.3: Agarose gel of PCR products amplified from different Biofilm DNA extractions.

Lanes 1-17 contain:λ *Pst* Molecular weigh marker, PCR product from the Baffle Reactor DNA extraction 1, PCR product from the Baffle Reactor DNA extraction 2, PCR product from the TSP DNA extraction 1, PCR product from the TSP DNA extraction 2, PCR product from the ammonium chloride Gradient Tube 1, PCR product from the ammonium chloride Gradient Tube 2, PCR product from the potassium nitrate Gradient Tube 1, PCR product from the potassium nitrate Gradient Tube 2, PCR product from the Phosphate STB 1, PCR product from the Phosphate STB 2, PCR product from the Phosphate STB 3, PCR product from the Chloride STB 1, PCR product from the Phosphate STB 2, PCR product from the Phosphate STB 3, PCR product from a Positive 586bp control and PCR product from a Negative water control.

5.3.3 Optimizing the Denaturing Gradient for DGGE

The gradient used to separate the 586bp PCR fragments had to be optimized, this was done using a perpendicular gradient of denaturants. Figure 5.4 showed that approximately halfway across the gel, the fragment had stopped migrating as its melting point at the optimum denaturant concentration had been reached. Towards the 0% denaturant concentration the fragment had migrated further in the gel indicating that it had not denatured. Towards the 100% denaturant concentration, no fragments were visible, this was due to the fragments separating completely in the high denaturant concentration and migrating off the gel.

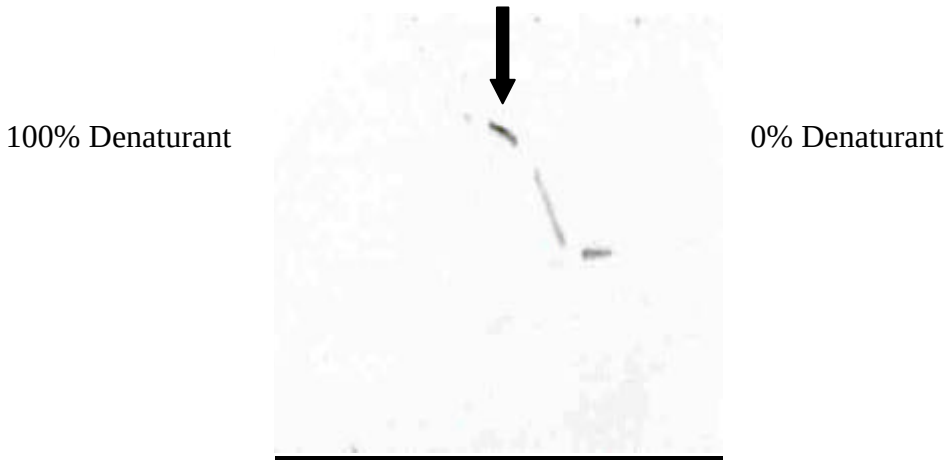

Figure 5.4: Optimization of DGGE using a 0% - 100% perpendicular gradient.

The black arrow indicates the position in the gel where the optimal concentration of denaturant occurred; this is indicated by the band at the top of the gel. The top band of DNA stopped migrating because strand separation occurred under the correct denaturing conditions.

5.3.4 DGGE analysis of the PCR products

DGGE was used to separate some of the different sequence 586bp PCR fragments, Figure 5.5. A 0% denaturant gel was used as a control gel where any bands appearing were considered to be non-specific bands from the DNA sample, the positions of these bands were compared to those on the 55% - 65% denaturant gel, any similar sized bands were ignored.

The 55% - 65% denaturant gel displayed many different bands (refer to the black arrow markers in Figure 5.5). Each sample had its own unique banding profile, suggesting that the species within each sample were different. Lane 1 contained a sample from the TSP, this lane showed unique and common bands when compared to the other samples, suggesting that the TSP had some species unique to it but also that it could have some of the other species in common with some of the other systems. Lanes 2 and 3 contained samples from the Baffle Reactor, the bands in these lanes were in the same regions of the gel but when compared to the other lanes (1, 5, 6, 7, 8, 9) they were different suggesting a different species composition to the TSP and Gradient Tube samples. The samples in lanes 4, 5, 6 and 7 all came from the potassium nitrate Gradient Tubes. The samples loaded in lanes 4, 5 and 6 were taken from the top of the tube, these lanes showed a banding pattern different to that in lane 7 which was a sample taken from the bottom of the Gradient Tube. This indicated

that different species occurred at different levels within the tube. The banding profiles were also distinctly different from those in Lanes 1-3, 8 and 9. Lanes 8 and 9 contained samples from the ammonium chloride Gradient Tubes, the banding profiles were distinctly different from those in the other lanes and also there were differences in the banding patterns between the top and the bottom of the Gradient Tubes. Suggesting possible differences in species composition in the different areas of the Gradient Tubes.

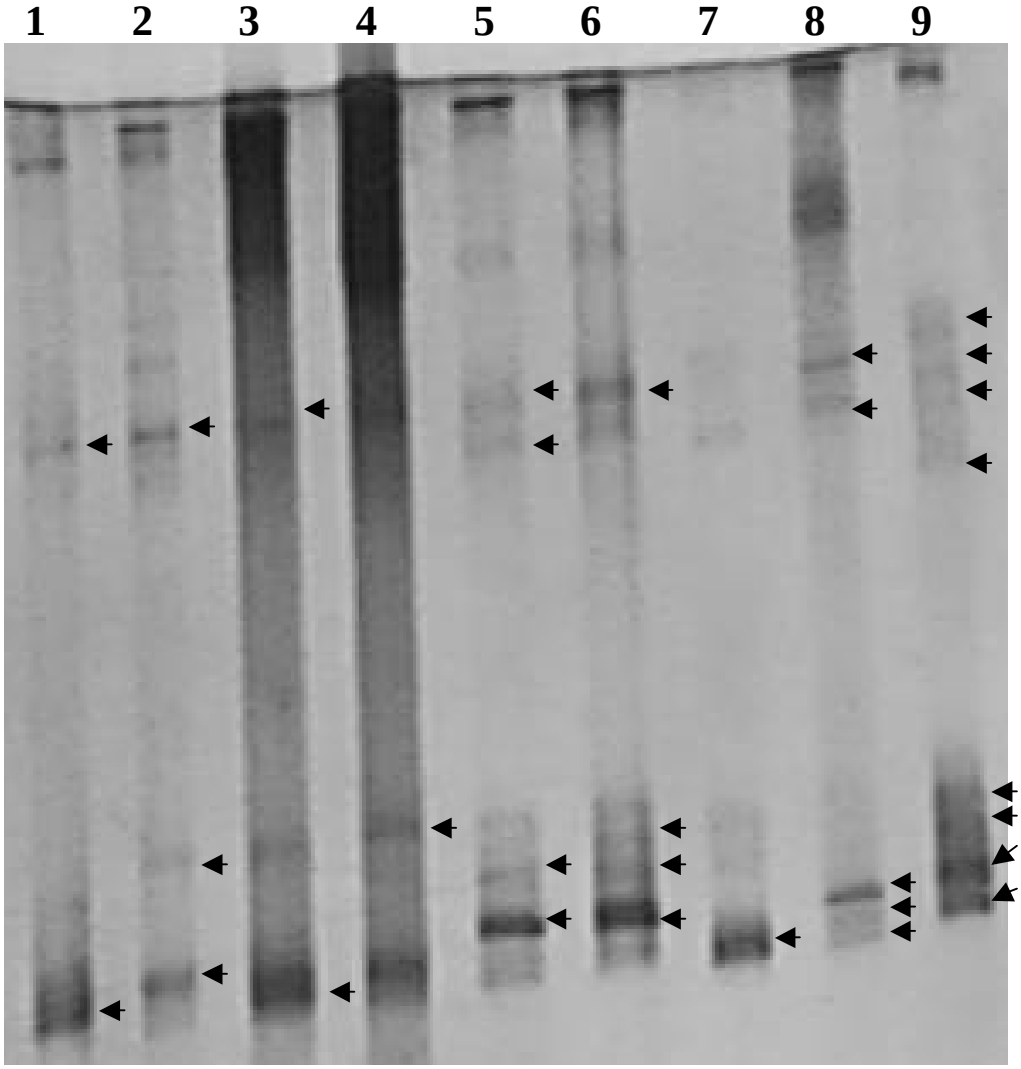

Figure 5.5: The 55%- 65% DGGE used to separate the 586bp PCR products.

Lanes 1-9 contained:- TSP Biofilm PCR product 1, Baffle Reactor biofilm PCR product 1, Baffle Reactor biofilm PCR product 2, Potassium nitrate Gradient Tube (top), Potassium nitrate Gradient Tube (top), Potassium nitrate Gradient Tube (top), Potassium nitrate Gradient Tube (bottom), Ammonium chloride Gradient Tube (top) and Ammonium chloride Gradient Tube (bottom). The black arrows in the figure point out some of the most prominent bands that appeared on the gel.

5.3.5 Reamplification from the DGGE

The bands on the DGGE in Figure 5.5 were cut out, extracted and PCR was performed again in order to get a single sequence PCR product for cloning and sequencing. Bands of 586bp were visible after reamplification. These PCR products were cloned into p-GEM T vector system and used for sequencing.

5.3.6 *Eco*RI digest results of the Plasmid extracts

*Eco*RI digests were performed on the plasmid extracts. *Eco*RI was expected to cut on either side of the 586bp fragment, the result on the gel would then be a 3018bp plasmid fragment and a 586bp insert. Some of the inserts may contain an internal *Eco*RI site and therefore the correct result would be a 3018bp plasmid band and two smaller bands whose size added up to 586bp. The results of the agarose gel electrophoresis were seen in Figure 5.6. All lanes contained the correct sized bands, lanes 3, 6, 7 and 10 all had a 586bp insert and a 3018bp plasmid. Lanes 4, 5, 8 and 9 contained inserts with an internal *Eco*RI site, the gel showed that there were 3 bands present, a large 3018bp plasmid band and 2 smaller bands whose combined size was approximately 586bp. Once it was established that the correct size inserts were present in the plasmids, the samples could be prepared for sequencing.

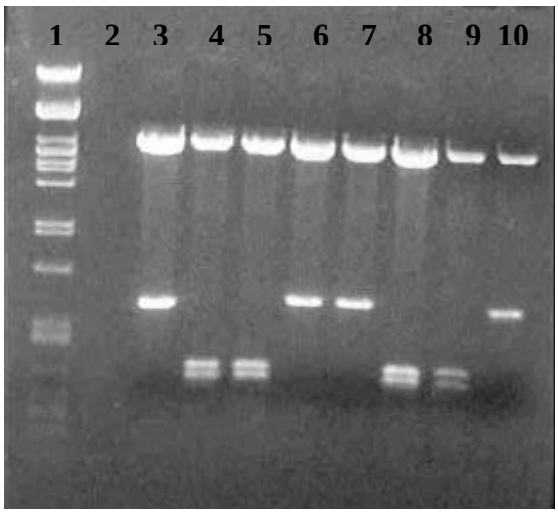

Figure 5.6: Plasmid constructs with unidentified DNA inserts digested with *Eco*RI.

Lanes 1-10 contained: λ *Pst* Molecular Weight Marker, Blank, Unidentified Clone 1 from the Baffle Reactor, Unidentified Clone 2 from the Baffle Reactor, Unidentified Clone 1 from the TSP, Unidentified Clone 2 from the TSP, Unidentified Clone 1 from the ammonium Gradient Tube, Unidentified Clone 1 from the potassium Gradient Tube, Unidentified Clone 1 from the chloride STB and Unidentified Clone 1 from the phosphate STB.

5.3.7 Sequencing results

A number of plasmid inserts from each of the different Biofilm system DNA extractions were sequenced and put through the BLAST database, the sequence matches are given below. The percentages given are the percentage homology between the sequence sent in for BLAST and the actual species listed, Table 7.3.

Table 5.4: The Sequence similarities from all the isolates from the Biofilm systems.

Tannery Stabilization pond			
Species	**Percentage similarity of isolate**	**E-value**	**Length (base pairs)**
Ectothiorhodospira sp.	98%	0.0	428
Ectothiorhodospira sp.	98%	0.0	469
Ectothiorhodospira shaposhnikovii	95%	0.0	437
Veillonella sp.	98%	0.0	445
Laboratory Scale Baffle Reactor			
Species	**Percentage similarity of isolate**	**E-value**	**Length (base pairs)**
Comamonas denitrificans	97%	0.0	432
Bacteroides uniformis	93%	0.0	468
Elbe River Snow isolate	98%	0.0	403
Veillonella sp.	98%	0.0	391
Clostridium celerecrescens	98%	0.0	455
Pseudomonas geniculata	98%	0.0	477
Pseudomonas testosteroni	79%	2e-74	338

Scaled-up Baffle Reactor at the sewage works			
Species	**Percentage similarity of isolate**	**E-value**	**Length (base pairs)**
Uncultured bacterium mlel-2	97%	0.0	459
Trichlorobacter thiogenes	98%	0.0	434
Arcobacter butzleri	96%	0.0	454
Uncultured epsilon proteobacterium	98%	0.0	450
Fusibacter paucivorans	95%	0.0	438
Bacteroides sp.	91%	e-129	384
Dethiosulfovibrio russensis	96%	0.0	453
Acinetobacter sp. Isolate LY1	98%	0.0	481

Ammonium chloride Gradient Tube				
Area in the Tube (number in brackets refers to the specific section of the tube)	**Species**	**Percentage similarity of isolate**	**E-value**	**Length (base pairs)**
Top (N21)	*Veillonella atypica*	95%	0.0	401
Top (N21)	*Veillonella atypica*	96%	0.0	483
Top (N21)	*Veillonella atypica*	96%	0.0	490
Top (N21)	*Anaerofilum pentosovorans*	99%	0.0	492
Middle (N12)	*Bacteroides acidofaciens*	94%	0.0	482
Middle (N12)	*Succinispira mobilis*	83%	7e-65	276
Middle (N12)	*Clostridium botulinum*	99%	0.0	490
Middle (N12)	Elbe River Snow isolate	96%	0.0	406
Bottom (N4)	*Clostridium akagii*	95%	0.0	408
Bottom (N4)	*Clostridium thiosulforeducens*	95%	e-172	398
Bottom (N4)	*Anaerobacter polyendosporus*	92%	0.0	421
Bottom (N4)	Unidentified eubacterium from anoxic bulk soil	96%	0.0	411

Potassium nitrate Gradient Tube				
Area in the Tube (number in brackets refers to the specific section of the tube)	**Species**	**Percentage similarity of isolate**	**E-value**	**Length (base pairs)**
Middle (K8)	*Clostridium akagii*	92%	0.0	407
Middle (K8)	*Anaerobacter polyendosporus*	92%	e-176	424

Chloride Silicon Tubular Bioreactor				
Area in the Reactor	**Species**	**Percentage similarity of isolate**	**E-value**	**Length (base pairs)**
Top	*Thiobacillus baregensis*	95%	0.0	400
Top	*Thiobacillus baregensis*	96%	0.0	403
Top	*Achromobacter sp.*	99%	0.0	424
Top	*Achromobacter sp.*	99%	0.0	411
Top	*Halothiobacillus kellyi*	87%	e-105	374
Middle	*Achromobacter sp.*	99%	0.0	431
Bottom	*Comamonas testosteroni*	97%	0.0	403
Bottom	*Brevundimonas vesicularis*	100%	0.0	566
Bottom	*Novosphingomonas sp.*	99%	0.0	482
Bottom	*Rhodopseudomonas palustris* strain KD1	100%	0.0	461

Phosphate Silicon Tubular Bioreactor				
Area in the Reactor	**Species**	**Percentage similarity of isolate**	**E-value**	**Length (base pairs)**
Middle	*Thiobacillus baregensis*	93%	e-146	346
Middle	*Thiobacillus baregensis*	96%	0.0	433
Middle	*Halothiobacillus kellyi*	93%	0.0	406
Middle	*Thiobacillus baregensis*	95%	0.0	400
Middle	*Thiobacillus baregensis*	96%	0.0	365
Middle	*Thiobacillus baregensis*	96%	0.0	421
Bottom	*Thiobacillus neapolitanus*	98%	0.0	449

The sequencing data for the naturally occurring biofilm on the TSP included species such as *Ectothiorhodospira sp.* and *Veillonella sp.* *Ectothiorhodospira sp.* is a known producer of extracellular sulphur (Brock & Madigan, 1991).

Ectothiorhodospira belongs in the purple sulphur oxidizing family and can use reduced sulphur compounds as electron donors for anoxygenic photosynthesis and carbon dioxide fixation (Prange *et al.*, 1999). During the oxidation of sulphide or thiosulphate to sulphate, globules of sulphur are accumulated as an intermediate, these globules can reach diameters of 1 μm and are deposited outside

Ectothiorhodospiraceae bacterial cells (Prange *et al.*, 1999). The TSP also contained *Veillonella sp.* which was found in the ammonium chloride Gradient Tube and the Baffle Reactor.

The laboratory-scale Baffle Reactor contained many different species such as *Comamonas denitrificans, Bacteroides uniformis, Veillonella sp., Clostridium celerecrescens, Pseudomonas geniculata* and *Pseudomonas testosteroni.* No specialist sulphur oxidizing species were found in this system, however some *Pseudomonads* are capable of sulphide oxidation, *Pseudomonads* can oxidize thiosulphate to tetrathionate (Brock & Madigan, 1991). The Baffle Reactor contained *Veillonella sp.* this was common to the TSP and the Gradient Tube system, the Elbe River Snow isolate was also common to both the Baffle Reactor and the Gradient Tube system.

The Baffle Reactor was scaled-up, the larger version contained a few specialist sulphide oxidizing species such as *Trichlorobacter thiogenes* which belongs to the *Desulfuromonas* group, *Fusibacter paucivorans* which is an anaerobic thiosulphate reducing bacterium and *Dethiosulfovibrio russensis (*Staley *et al.,* 1989*).*

The sequencing results for the Gradient Tube systems also showed similarities, for both the ammonium chloride and potassium nitrate Gradient Tube systems no known specialist sulphide oxidizing species were identified. The top section of the ammonium chloride Gradient Tube contained mainly *Veillonella atypica,* the middle section contained *Bacteroides acidofaciens, Clostridium botulinum* and *Succinispira mobilis* and the bottom section contained mainly *Chlostridium* species. Poor sequence data was generated for the potassium nitrate Gradient Tube but the middle section did contain *Clostridium akagii* and *Anaerobacter polyendosporus.*

The sequence data for the STB systems showed that both the chloride and the phosphate STB contained a number of known sulphide oxidizing species, for example *Thiobacillus baregensis* (Brown, 1982), which occurred in both reactor systems. There were differences in the two reactor systems, in the chloride system the known sulphide oxidizing species were found in the top sections of the tube only, the middle

and bottom sections contained general species which did not have any specialist sulphide oxidizing capabilities such as *Comamonas testosteroni* and *Rhodopseudomonas*.. These species are part of the Pseudomonad group and are related to the purple sulphur bacteria. Pseudomonads are able to utilize a wide variety of compounds as carbon sources and for energy generation, Pseudomonads have been documented as having some sulphide oxidizing ability (Brock & Madigan, 1991). The phosphate system differed because it had specialist sulphide oxidizing species such as *Thiobacillus baregensis* and *Halothiobacillus kellyi* in the middle and bottom sections, no assumptions could be made about the species composition of the top section as poor sequence data for that section was generated. The presence of specialist sulphide oxidizing species could account for the success of these reactors at producing large quantities of sulphur.

These results suggested that although most of the biofilm systems were proved to contain or produce sulphur, they did not necessarily contain sulphide oxidizers in large numbers. Only the STB systems which had their redox conditions closely controlled seemed to have an abundance of specialist sulphide oxidizers.

5.3.8 Sequencing results related back to DGGE

The sequencing data presented in Table 5.4 were sequences amplified from bands visualized on DGGE gels. These are therefore just some of the species that were present in these systems, not every single species within the systems could be sequenced. More than one species per band was not found during the course of this experiment but this phenomenon has been reported (Eichner *et al.*, 1999). The DGGE, figure 5.7, clearly shows that although the bands on the DGGE can represent single species, a single species can be represented by more than one band.

Figure 5.7 also showed that the 55% - 65% DGGE gel had many distinct bands at different positions in the different lanes. When comparing the different lanes it was seen that some of the bands were common, this suggests the presence of common bacterial species in the different samples. However there were some bands at different positions in lanes 4 and 5, suggesting that there were different species in the phosphate STB compared to the chloride STB.

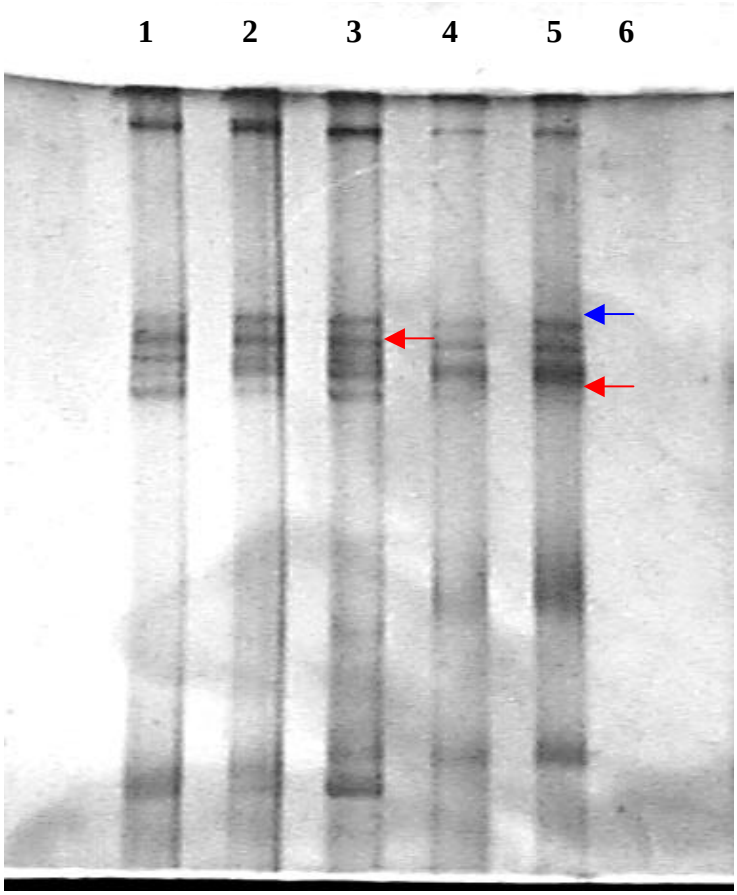

Figure 5.7: 55%-65% DGGE of the PCR products from the STB.

Lanes 1-6 contain: Chloride STB (top), Chloride STB (middle), Chloride STB (bottom), Phosphate STB (bottom), Phosphate STB (middle) and Phosphate STB (top). The red arrows indicate the bands that were identified as containing *Halothiobacillus kellyi*, the blue arrow indicates the position of the band that was identified as containing *Thiobacillus baregensis.*

5.4 Discussion

The stringent DNA extraction method that was used to prepare the biofilm samples involved extended lysozyme treatments and many freeze thaw cycles; as recommended by Head *et al.*, 1998. This lengthly procedure was used in the hope of disrupting all of the bacterial cells present in the biofilm, thus releasing their DNA and resulting in a sample containing a comprehensive collection of all of the different DNA from the different members of the biofilm community.

Other methods for DNA extraction were considered, these included the QIAamp DNA Mini Kit-Cat. No. 51399 method but these were not thought to be stringent enough to result in the breaking open of the majority of the bacterial cells, this

method was therefore not used as it would have resulted in inaccurate results. Some cells are more resistant to lysis than others and these may have withstood extraction methods, resulting in their DNA not being present in the final sample. These would then have contained an incomplete representation of all members of the bacterial population.

Figure 5.2 provided evidence that the DNA extraction procedures used were successful. All of the lanes showed the presence of DNA degradation products but large DNA fragments were also present. They were therefore considered to be of sufficiently high quality to be used for PCR.

At this stage it was not known how many different DNA templates were present in the samples but according to Head *et al.,* (1998) it should have been representative of the vast majority of the bacteria present in the biofilm community.

The PCR amplification of the different DNA extraction samples was successful, Figure 5.3. The primers flanked a 586bp fragment that was amplified off the different DNA templates present in each sample using PCR. Each of these different sequence 586bp fragments represented a different species but when the PCR products were resolved on a 1% agarose gel (Figure 5.3), only a single band was visible.

The fragments were separated according to their sequence differences, this was done by running them on a linear gradient of denaturants. This technique takes advantage of the fact that GC bonds break at a higher melting point than AT bonds. Sequences with a high GC content would therefore migrate further along the gradient of denaturants. Once the melting point was reached the fragment would partially denature. One of the primers included a GC clamp that prevented total denaturation, and stopped migrating or migrated very slowly. This resulted in a profile of bands of different melting points/sequences down the denaturant gradient. Each band was thought to represent a different bacterial species.

The DGGE, Figure 5.5, clearly showed that the 586bp PCR products contained many different sequences, as seen by the presence of the many bands on the gel. When the banding profiles of the different samples were compared it was found that they did

not match, indicating the presence of different bacterial species within the samples. Figure 5.5 clearly showed that the banding profile of the TSP was different to that from the Baffle Reactor and the Gradient Tubes indicating that although some species / bands were common, there were individual DNA bands that were only derived from certain samples. Not only was it clear that the different systems had different bacteria, Figure 5.5 suggested that within the same Gradient Tube there were species differences. This was seen particularly clearly when comparing the banding profiles of the top and bottom sections of the potassium Gradient Tubes (Lanes 6, 7, and 8). This differentiation of species at different levels within the Gradient Tubes may indicate functional separation and suggest a complex distribution of bacteria within the biofilm into many functional layers.

Figure 5.6 also indicated that differences in the banding profiles of the phosphate STB and chloride STB did occur, though there were also many common bands present suggesting that the species composition of the two systems was very similar. DGGE proved to be a fast technique at creating an overall profile of species differences between systems and allowed for separation of these species. The separation of the DNA into distinctly different bands made the isolation of single species easier.

Cutting out individual bands, presumably derived from single species, from the DGGE and reamplifying them by PCR was also successful. Because of the possibility that one band might contain more than one sequence, DGGE was used in conjunction with cloning (Felske *et al.*, 1998). The PCR products were cloned and then sequenced in order to establish exactly which species were present in the different systems.

Many of the reactor systems shared common species but there were also unique bands present in some of the samples. Samples from the same systems but at different positions within the system were also seen to have species differences. This suggested a separation of species along a gradient of environmental conditions established within the biofilm.

The TSP biofilm was found to contain mainly *Ectothiorhodospira sp.* this was expected as the colour of the water suggested the occurrence of a purple

photosynthetic species. The *Ectothiorhodospira sp.* possibly outcompeted most of the other bacterial species that could have existed under this systems high saline conditions but *Veillonella sp.* was also detected. *Ectothiorhodospira sp* is an extracellular sulphur producing species that had been documented to grow in high saline conditions (Dunn. 1997). The presence of this sulphur producer in the biofilm suggested that sulphide was being oxidized into sulphur and stored within the bacterial cells present. This evidence suggests that the sulphur cycle was occurring within the Tannery ponds at Wellington.

The laboratory-scale Baffle Reactor system while encouraging biofilm growth and clearly producing sulphur was not seen to contain many specialist sulphide oxidizing species according to the sequence data. However there were *Pseudomonad* species present which have been known to oxidize sulphur but these were probably not the only sulphide oxidizing species present, there were possibly other sulphide oxidizers in the system but these were possibly present in low numbers or were difficult to detect. The redox conditions in this reactor system were not closely controlled as it was first developed to model naturally occurring floating sulphur biofilms, if closer redox monitoring was done, it would probably result in a more specialist population of sulphide oxidizing bacteria. The bacterial species present in the Baffle Reactor did however reduce sulphate, oxidize sulphide and produce sulphur (reported in Chapter2), effectively setting up the complete sulphur cycle process within the confines of this model system

The scaled-up Baffle Reactor at the sewage works was found to contain a number of sulphide oxidizing species, such as *Trichlorobacter thiogenes* which belongs to the *Desulfuromonas* group, *Fusibacter paucivorans* which is an anaerobic thiosulphate reducing bacterium and *Dethiosulfovibrio russensis* (Staley *et al.,* 1989*).* This larger system also had minimal redox control over the prevailing conditions but it seemed to encourage the growth of a more specialist sulphide oxidizing population. The dimension of the larger reactor and the air space between the fluid level and the lid was different to the smaller Baffled Reactor, this possibly influenced the redox conditions at the liquid / air interface and encouraged the growth of a more specialist population of bacteria. The bacteria found within this reactor included SRB and SOB, these bacteria were therefore capable of supporting the complete sulphur cycle within

the biofilm. The SRB reduced sulphate to sulphide which then became available to the SOB to oxidize into sulphur. Gradients within the biofilm would have facilitated the growth of this broad range of bacteria, allowing for the development of anaerobic zones where the SRB could thrive and oxygen rich areas for the SOB.

Another model system used to separate the biofilm into its functional sections was the Gradient Tube system. These systems, while successfully encouraging the growth of a population of bacteria and allowing for the utilization of sulphide and sulphate to oxidize it to sulphur (see chemical analysis results chapter 3), possibly had few specialist sulphide oxidizers present but none were identified in the sequencing results. The fact that none were identified did not mean that none were present, just that they were not present in large numbers or were undetectable. This could possibly be due to the fact that the redox of this system was not under tight control, many different bacterial population could therefore grow under these conditions. The microenvironment may have favoured the growth of a more general bacterial population which may have out competed the slow growing sulphide oxidizing species.

There seemed to be differences in species composition of the different sections of the tubes, *Veillonella atypica* seemed to occur mainly in the top section of the tubes while other species like *Bacteroides* and *Clostridium* occurred in the middle and bottom sections of the tubes. This was expected as the conditions at the different levels of the tubes differed greatly. The exact chemical conditions that these species were found to be growing at could be found by looking at the data presented in Chapter 3. For example the *Veillonella atypica* grew at a sulphide concentration of 50mg/l, a sulphur concentration below 5mg/l, a TOC value of 1020mg/l C and a sulphate concentration of 1200mg/l.

These Gradient Tube systems were a very effective way of separating species into differentiated layers and allowing for the elucidation of the prevailing conditions that occurred in those sections. For future work the redox conditions in these Gradient Tube systems should be more tightly controlled in order to encourage the growth of more specialized populations of bacteria.

Sequence data for the STB systems showed that there were many specialist sulphide oxidizing species present in both the chloride and the phosphate reactor systems, these included *Thiobacillus baregensis* and *Halothiobacillus kellyi* (Brock & Madigan, 1991). When compared to the other biofilm systems studied, these reactors had the greatest abundance of specialist sulphide oxidizing species present, this was thought to be due to the fact that the redox conditions within these reactors were closely monitored and the perfect redox conditions were provided for sulphur formation. This controlled microenvironment seemed to select for or encourage the growth of specialist sulphide oxidizing bacteria and favour the production of sulphur while removing sulphide from the system

There were differences in species composition at different levels within these reactors, for the chloride STB the sulphide oxidizing species were detected only in the top section of the reactor, closest to where the feed entered the system. This suggested that the feed which was altered to help maintain the correct redox conditions was quickly utilized by these sulphide oxidizing bacteria, as the feed progressed further down the reactor, one or more of the essential components that encouraged sulphide oxidizer growth must have become depleted as no specialist sulphide oxidizing species were detected in the middle or bottom sections of the reactor.

The Phosphate STB system however had a population of mostly sulphide oxidizers, these were found in the middle and bottom sections of the reactor, this suggested that the phosphate containing feed made the systems redox conditions more stable or the conditions more favourable throughout its entire length and thereby encouraged the growth of mainly sulphide oxidizers.

The prevailing redox conditions within a system seemed to have a profound effect on whether specialist sulphide oxidizers occur in large numbers in the biofilm or not. Most of the systems that did not have a tightly regulated redox contained generalist species, some of which could oxidize sulphur but no really specialist species, specialist sulphide oxidizing species occurred in the greatest numbers in the STB that had the tightest redox control.

There were also found to be differences in species composition at different levels in the gradient systems which indicated a segregation of species into functional levels. This differentiation may or may not reflect what actually occurs in the biofilm but all research so far points to the fact that floating sulphur biofilms are not homogenous and are highly heterogeneous with different species occurring at different levels depending on the prevailing microenvironment.

5.5 Conclusion

The species identity of members of the different biofilm systems were analysed using DNA extraction, PCR, DGGE, cloning and sequencing. The results suggested that there were species similarities between some of the different biofilm communities but that most of the systems studied contained unique species.

Specialist sulphur-oxidizing species were not detected in all of the systems, but there were specialist species detected in the STB. This was postulated to be due to the fact that the STB provided the most stable redox conditions.

The STB and Gradient Tube systems promoted the growth or segregation of different species along the gradient of conditions provided within these systems. This separation of species into different layers was the objective of the Gradient systems.

CHAPTER 6:

GENERAL CONCLUSIONS

6.1 Floating Sulphur Biofilms

Floating sulphur biofilms have been observed to form on the surface of sulphide containing wastewater systems. It was postulated that these floating films contain large numbers of SOB which oxidize sulphide into sulphur. This sulphur occurs either as intracellular sulphur which are sulphur globules stored inside the bacterial cells or extracellular sulphur which are orthorhombic sulphur crystals caught up in the EPS matrix. It was proposed that these floating sulphur biofilm systems could be used to bioremediate sulphide containing wastewater and at the same time be used for sulphur recovery. Very little information is available on floating sulphur biofilms so this study was undertaken in order to establish a better understanding of the ongoing processes within these systems so that biotechnological process development could be implemented.

6.2 Model Systems

The first objective of this project was to develop model systems that would allow for the study of floating sulphur biofilms. The first model system, the Baffle Reactor, was based on observations of a TSP at Wellington where floating sulphur biofilm formation was observed. These ponds contained high levels of sulphate (Dunn, 1997), with a thick black sediment layer that contained SRB. These SRB were thought to reduce the sulphate in the wastewater to sulphide which then diffused towards the air / liquid interface. The redox gradient at this interface was very steep, ranging from highly positive to highly negative over the space of a few millimetres. These turned out to be ideal conditions for the oxidation of sulphide and the formation of floating sulphur biofilms were observed. It was proposed that SOB were responsible for the oxidation of sulphide into elemental sulphur.

The Baffle Reactor was developed in an attempt to model this system. The Baffle Reactor was seeded with a sludge layer containing SRB and was fed a SRB lactate medium. This medium contained sulphate, the SRB reduced the sulphate to sulphide which was then oxidized to sulphur at the air / liquid interface by SOB which were

present in the form of a thick white biofilm layer. This theory was supported by observations made during the formation of the biofilm. The biofilm formed faster on the upflow wells in the Baffle Reactor, this was proposed to be because a greater concentration of sulphide was being carried to the surface by the current and therefore more sulphide was available for oxidation. Sulphide production and its subsequent oxidation on the liquid surface of the reactor provided a basis for the reliable production of the biofilms.

The Baffle Reactor was so successful at encouraging floating sulphur biofilm growth that this reactor system was scaled up by other members of the research team. The scaled-up reactor is run at the Grahamstown sewage works and a system for harvesting the sulphur in the biofilm is currently under development as part of the Rhodes Biosure process.

The floating sulphur biofilm that developed on the surface of these reactors was only a few millimetres thick and very brittle, and this made studying the internal structure of the biofilm and the differential distribution of the population into functional layers quite difficult. In the natural floating films it is proposed that the biofilm develops over a wide gradient of redox conditions, which develop within a narrow area. The conditions of the biofilm also vary widely between the surface of the liquid, within the biofilm and on the underside of the floating film. In an attempt to get a clearer picture of the biofilm structure, gradient systems were developed. This provided for a greater spatial separation of the different bacterial populations within the biofilm and instead of the narrow gradient of only a few millimetres, a wider gradient could be set up.

This first system investigated was the Gradient Tube system. These systems were set up in test tubes. The gradient ran from high sulphide, low oxygen conditions at the bottom of the tube to high-oxygen, low-sulphide conditions at the top of the tube, over a distance of approximately 12cm. This gradient was established by sulphide diffusing upwards out of a sulphide plug at the bottom of the tube and oxygen diffusing down into the tube from the atmosphere. Over a 7 day period, dark bands of growth were observed to form at certain points in the overlays that contained biofilm inoculum and not in the control tubes that contained no inoculum.

Two different types of media were used to form the overlays, the one had ammonium chloride as the nitrogen source and the other had potassium nitrate. There were distinct differences in the number and positions of the bands of growth between these two different types of overlays. The overlay that had potassium nitrate seemed to promote more bacterial growth, especially at the bottom of the tube where oxygen concentration was low and sulphide concentration was high, this was due to the fact that nitrate can act as the terminal electron acceptor in the absence of oxygen. No bands of bacterial growth were visualized at the bottom of the ammonium chloride tubes, growth only occurred at the top of the tube where conditions of high-oxygen and low-sulphide existed.

pH control tubes were also set up, using neutral red as a pH indicator. The bands of growth were seen to sometimes prevent the diffusion of sulphide up the length of the tube, possibly by utilizing it before it could diffuse any further or maintaining the pH by acid production. This was seen as the sulphide caused the pH indicator to change from pink (pH 6.8) to yellow (pH 8) as it diffused. The bacteria-free control tubes were seen to turn yellow the entire way up its length while the tubes that contained biofilm inoculum only turned yellow up to a band of growth where sulphide oxidation was presumed to occur. The bands of growth maintained the pH of the surrounding media at 6.8. As seen by the fact that the indicator remained pink within the dark bands even if the surrounding medium had turned yellow.

The agar overlay in the tubes could be removed and cut into sections, and the prevailing conditions in each section could be established. When combined with molecular analysis, the species which developed in a band of growth that existed under a particular set of conditions could be established.

The other Gradient system studied was the STB system (Rein, 2001). This system consisted of spirals of silicon tube wrapped around a central core. Media was fed through the tubes. The bacteria at the top of the tube utilized part of the medium and excreted waste products that would then influence the population growing further down the silicon tube. This system was also sectioned and using molecular analysis the possible spatial separation of different populations of bacteria was investigated.

6.3 The Conditions within the Model systems

In order to gain a better understanding of the ongoing processes occurring in the model systems, chemical analyses were undertaken. These included studies of sulphate, sulphide, sulphur, TOC and pH.

Samples were taken from the liquid layer between the sludge and the biofilm in the Baffle Reactor. The sulphate analysis of the reactor showed that the overall sulphate in the reactor was decreasing, this corresponded to an increasing sulphide concentration. It was assumed that SRB in the sludge layer were reducing the sulphate to sulphide. The sulphur concentration also increased over this period and it was assumed that the SOB in the biofilm were oxidizing the sulphide into sulphur. The TOC analysis indicated that the TOC of the system remained relatively constant until the sludge layer ultimately degraded and the biofilm subsided. The pH of the reactor decreased from 7.3 to 6.8 over the time it was run but this was thought to be due to the formation of sulphuric acid from the sulphide.

The overall results of the analyses provided evidence that the reactor system was operating in accordance with what we first proposed, which was that the SRB reduce the sulphate in the feed to sulphide, the sulphide diffuses up out the sludge and comes into contact with the correct redox conditions where it is then oxidized to sulphur by SOB present in the biofilm. The Baffle Reactor was concluded to be a good model system for the reproducible formation of floating sulphur biofilms.

The Gradient Tube systems also had sulphate, sulphide, sulphur, TOC and pH analyses performed. These however were done for each separate section of the tube, approximately 0.5cm sections. Knowing the exact conditions in each section is extremely valuable as it would allow deductions to be made about the bacteria present in that section and provide insight on their function within the system.

The analyses confirmed that a gradient of decreasing sulphide concentrations occurred from the bottom to the top of the tubes, it was assumed that with the top of the tube being in contact with the atmosphere that a decreasing oxygen gradient from the top to the bottom of the tube was also established.

The sulphate analysis revealed that the sulphate concentration was high at the bottom of the tube and then decreased slightly and remained stable for most of the length of the tube before increasing again at the top of the tubes. The sulphate concentration was expected to be higher near the top of the tubes because as the sulphide came into contact with the air it could begin to undergo chemical oxidation to sulphate, also there were bands of growth present at the top of both the ammonium chloride and the potassium nitrate tubes so the sulphide would be undergoing bacterial oxidation as well. The fact that the sulphate was high at the bottom of the tube also suggested that oxidation was occurring, this pointed towards the presence of a sulphide oxidizing bacterial population. The evidence of bacterial growth at the bottom of the tube was observed as dark bands in the potassium nitrate tubes. The bacteria definitely seemed to favour potassium nitrate as a nitrogen source, as more growth bands were observed to form in the tubes containing it.

The sulphur analysis revealed that the bacteria growing in the lactate medium with potassium nitrate as the nitrogen source could produce sulphur under high sulphide, low oxygen conditions and also under low-sulphide, high-oxygen conditions. This suggested that the potassium nitrate in the medium allowed for the proliferation of different bacteria than those in the ammonium chloride tube, bacteria that could oxidize sulphide to sulphur under basically anaerobic conditions with high sulphide levels.

The TOC was higher at the bottom of the tubes and decreased towards the top. These results suggested that the carbon in the lower levels of the tubes, where the sulphide levels were high and the oxygen levels were low, was not being utilized by bacteria. But at the top of the tube where the sulphide levels were low and the oxygen levels were high, the carbon was being used up. This suggested that the bacteria in both the potassium nitrate and the ammonium chloride tubes were growing better under high-oxygen, low-sulphide conditions.

6.4 Microscopy of the Model systems

Previous microscopy work done on the floating sulphur biofilm showed that they were highly differentiated heterogeneous structures (Gilfillan, 2000). The biofilm contained a wide variety of bacterial forms which seemed to be present in two distinct layers, short rods near the surface of the biofilm and long rods on the underside of the biofilm.

Electron microscopy work done during this study on the floating sulphur biofilm from the Baffle Reactor and the Gradient Tubes agreed with the previous observations, many different bacterial morphologies were observed, these included very long rods, short rods, cocci and vibrio. Also observed were orthorhombic sulphur crystals with bacteria present on their surfaces. What was not observed in the floating sulphur biofilm from the Baffle Reactor and the Gradient Tubes were extracellular sulphur producers. These extracellular sulphur producers may have been present but not in significant enough numbers to be visible. It was assumed that the bacteria in the biofilm may have been storing sulphur as intracellular globules, TEM work did show globules on the inside of the cells but X-ray diffraction could not confirm whether or not these were sulphur globules. The sulphur in the floating sulphur biofilms was observed mainly as orthorhombic sulphur crystals, this suggested that the biofilm was setting up the correct conditions for the catalytic conversion of sulphide into sulphur.

In electron microscope work on the STB (Rein, 2001), extracellular sulphur was clearly seen on a large number of the bacteria present in the system. This system was the most successful reactor system for producing sulphur in the research group and it was also the only one that had an abundance of extracellular sulphur producers. The STB were operated under tightly controlled redox conditions, this seemed to encourage the growth of large numbers of extracellular sulphur producers and in turn make these reactors very successful sulphur producing systems.

What became apparent from the microscope studies was that there was structural and functional differentiation within the Gradient Tube systems. This suggested that the same differentiation would also be observed in the thin floating biofilm, as the same gradient of conditions existed there but over a shorter space.

6.5 Species composition of the floating sulphur biofilms

Molecular analysis was performed on the different reactor systems in order to elucidate the species composition of the biofilms and make comparisons between the gradient systems, model systems and natural biofilm populations. PCR of 16s rRNA was performed on total DNA extracts from each system. The PCR products were separated according to GC content on DGGE gels. The banding profiles from the gels were used to make comparisons between the systems, most systems had common bands suggesting common species but they also had unique bands present only in particular systems suggesting different species being present in the varied conditions in each system.

The DGGE bands were reamplified by PCR, ligated into plasmids and sequenced. The sequences were put through the BLAST database and species matches were made. The population present in the natural biofilm observed on the TSP was found to contain mainly purple photosynthetic sulphur oxidizing bacteria, *Ectothiorhodospira sp* but it also contained *Veillonella sp.* *Veillonella sp* are bacteria normally found in the oral cavity associated with plaque formation (Staley *et al*., 1989). *Ectothiorhodospira sp* are able to perform anoxygenic photosynthesis, growth occurs anaerobically with reduced sulphur compounds as electron donors (Staley *et* al., 1989). *Ectothiorhodospira sp* oxidize sulphide to sulphur which is deposited outside the cell as extracellular sulphur (Staley *et al*., 1989). The presence of *Ectothiorhodospira sp* had been previously suggested by Dunn, (1997) but this was the first sequencing evidence to prove that *Ectothiorhodospira sp* dominated this system.

The Baffle Reactor samples were found to contain many different species, these included *Comamonas denitrificans, Bacteroides uniformis, Veillonella sp., Clostridium celerecrescens, Pseudomonas geniculata* and *Pseudomonas testosteroni*. There were however no specialist sulphide oxidizing species detected in the biofilm, this could mean that they were only present in small numbers. Pseudomonads have however been documented to oxidize sulphide (Brock & Madigan, 1991).

The scaled-up version of the Baffle Reactor did however contain some known sulphide oxidizing species like *Trichlorobacter thiogenes* which belongs to the *Desulfuromonas* group, *Fusibacter paucivorans* which is an anaerobic thiosulphate reducing bacterium and *Dethiosulfovibrio russensis.* Other species present included *Bacteroides sp* and *Acinetobacter sp.*

The ammonium chloride Gradient Tube was found to contain mainly *Veillonella atypica* in the upper sections of the tube where the sulphide concentration was low and the oxygen concentration was high. The middle sections of the tube contained many different species, these included *Bacteroides acidofaciens, Succinispira mobilis* and *Clostridium botulinum.* The bottom section of the tube was found to contain *Clostridium akagii, Clostridium thiosulforeducens* and *Anaerobacter polyendosporus,* these bacteria were growing under conditions of high sulphide and low oxygen. These sequencing results suggested that there was a differentiated distribution of species along the gradient within the tube.

By far the most successful sulphur producing reactor system which was also observed to contain extracellular sulphur producers was the STB system. The species that were present in these systems were mostly all known sulphide oxidizing species. These species also seemed to be distributed in different sections of the reactor. In the chloride STB the species at the top of the tube were mainly *Thiobacillus baregensis,* which derive their energy from the oxidation of reduced sulphur compounds (Staley *et al*., 1989) and *Halothiobacillus kellyi.* Further down along the gradient were other generalist species, these included *Comamonas testosteroni, Brevundimonas vesicularis, Novosphingomonas sp* and *Rhodopseudomonas palustris.* The specialist sulphur oxidizers were only found in the top sections of this reactor near the feed inlet. The conditions of the media as it came into the reactor must have been ideal for encouraging the growth of these species, further down the tube, where some of the nutrients had been used up, there were only generalist species. This suggests that these specialist sulphur oxidizing species need to be provided with very stringent conditions in which to grow other wise they are outcompeted by other faster growing species. These results also suggested the presence of a population which displayed spatially differentiated separation of bacterial species performing different functions.

The phosphate STB system showed a different species distribution to that seen in the chloride system, specialist species were found in the bottom sections of the tubing. These species included *Thiobacillus baregensis, Halothiobacillus kellyi* and *Thiobacillus neapolitanus.* The fact that sulphide oxidizing species occurred even in the bottom section of the tubing suggested that the phosphate enriched medium was better suited to encouraging the growth of sulphide oxidizing species than the chloride medium. This could possibly also have to do with the redox potential, these systems had very strictly controlled redox conditions, the phosphate medium possibly maintained these conditions for the whole length of the tubing while the chloride medium could only provide the correct redox conditions in the upper most sections of the tubing.

The model reactor systems and biofilm studies proved that the floating sulphur biofilm was a very diverse heterogeneous system which supported many different species of bacteria. The model systems were able to simulate the correct conditions for natural sulphur biofilm formation and were successful at producing floating sulphur biofilms. In an attempt to spatially separate the constituent members of the biofilm population, gradient systems were designed, these reactors provided a gradient of conditions along their length and it was hoped that the bacteria would establish themselves in the microenvironment that best suited them.

The gradient systems were successful at encouraging the growth of different species in different parts of the gradient. This allowed for their physical separation and also the elucidation of the exact conditions that they were found in. One fact that became clear was that the redox potential of these systems was very important. The correct redox conditions for sulphide oxidation occur near the surface of the liquid / air interface and range from strongly positive to strongly negative over a very short distance. The reactor system that had the most success at producing sulphur from sulphide had a stringently controlled redox potential. This tight control seemed to select for or allow the growth of specialist sulphide oxidizers without them being out competed by other faster growing generalist species.

Very little is known about floating sulphur biofilms but they seem to have great potential for the removal of sulphide from sulphide containing wastewater and also

for the production of sulphur. More study needs to be done on these systems in order to optimise their conditions for maximum sulphur production and eventual industrial application.

6.6 Descriptive model of biofilm formation.

The extensive studies on the floating sulphur biofilm resulted in the proposal of a descriptive model to explain the mechanism of its formation. It was proposed that the surface of the wastewater was initially colonized by heterotrophic bacteria which consumed the oxygen at the surface, resulting in a steep redox gradient. These heterotrophic organisms also produced an EPS matrix, which appeared as a thin slimy layer on the surface of the water. *Pseudomonas aeruginosa* has been documented to respond to favourable nutrient conditions by aggregating at available surfaces and producing EPS in order to develop into mature biofilms (Costerton *et al.*, 1995).

The heterotrophic organisms were thought to pave the way for the SOB by setting up a conditioning film at the surface and providing the correct redox conditions. The SOB then colonized the biofilm at positions which provided the optimal conditions for their growth, thus preventing them from being outcompeted by other generalist species. The SOB polymerise sulphide to polysulphide (Rein, 2001) and at low redox, approximately -155mV, this crystallizes to orthorhombic sulphur.

The fact that predominately the crystalline form of sulphur was observed in the system (Gilfillan, 2000) seemed to indicated that the conditions within the biofilm encouraged the catalytic conversion of sulphide into crystalline sulphur. Extracellular sulphur producing bacteria were observed but not in high numbers unless the redox conditions were strictly controlled. It was postulated that these extracellular sulphur producers were present in the biofilm but only in a very narrow band around their optimal redox conditions.

A model for what was occurring within the floating sulphur biofilms was developed, refer to Figure 6.1.

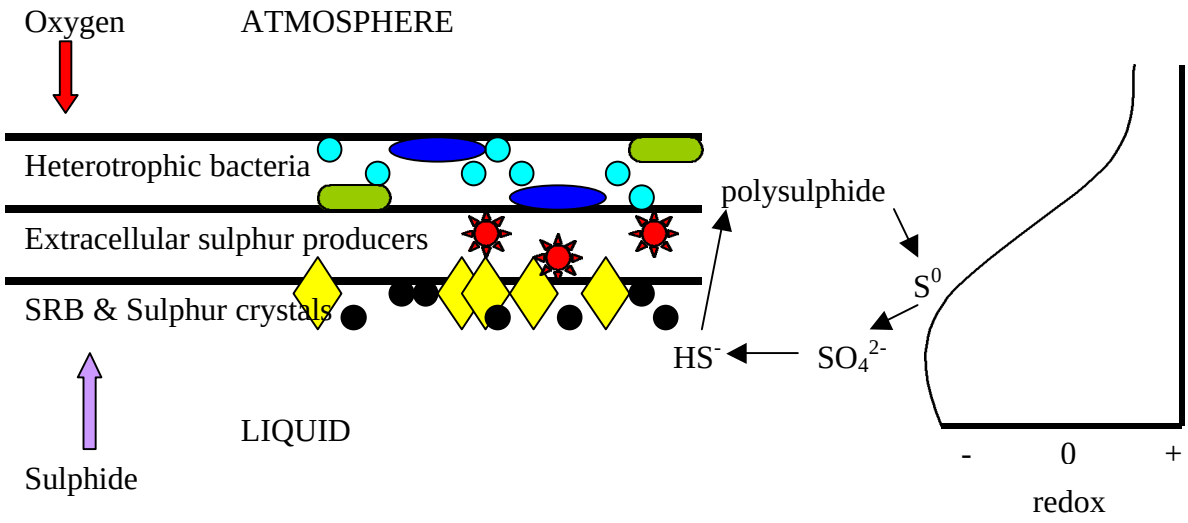

Figure 6.1: A Diagrammatic representation of the processes proposed to be occurring within the floating sulphur biofilm.

Figure 6.1 shows that the top most layer of the biofilm, contains mainly heterotrophs which are proposed to be the initial colonizers and the main producers of EPS, previously documented by Costerton *et al. (1995).* As the heterotrophs grow at the surface they use up the oxygen and set up a steep redox gradient with areas favourable for extracellular sulphur producer colonization. The extracellular sulphur producers only colonize the narrow band of redox conditions that provides them with the optimal conditions for their growth. The conditions at the base of the biofilm seem to encourage the formation of sulphur crystals, these were often observed in the lower layers of the biofilm (Gilfillan, 2000). These lower layers were also proposed to be anaerobic, creating a microenvironment for SRB and thereby setting up a complete sulphur cycle within the biofilm as well as the larger scale sulphur cycle involving the SRB in the bottom layers of the water body.

The middle stages of the film are flexible but towards the end of the films lifespan, it becomes brittle due to the build up of sulphur crystals. The sulphur crystals eventually result in the biofilm becoming so heavy that it subsides and then another film begins to form.

In order for the floating film to be used in a biotechnological process future work would include a study on the optimal harvesting time for the film, it would have to be at a stage when the biofilm contains a high amount of sulphur but is not so brittle that it sinks when it is disturbed. This work has begun on the scaled-up reactor at the Grahamstown sewage works and will hopefully result in a biotechnological process that will enable the large- scale passive treatment of sulphate and sulphide containing wastewater with the recovery of sulphur as a valuable by-product.

This model is only a proposal and future work to substantiate these claims could include Fluorescent in situ hybridisation (FISH), Confocal laser microscopy, and microprobes, in order to profile the chemocline and further work on the gradient systems which would help to expand the chemocline.

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APPENDIX 1:

Media and Reagents for Chemical Analysis.

MEDIA

SRB Lactate Medium (Modified from Atlas, 1993).

3.5ml	60% Sodium Lactate
2g	$MgSO_4.7H_2O$
1g	NH_4Cl
1g	Na_2SO_4
1g	Yeast Extract
0.5g	K_2HPO_4
0.15g	$CaCl_2.6H_2O$

Make up to 1l with dddH_2O

HPLC

Anion (Sulphate) HPLC Mobile Phase

5.52g	para-Hydroxybenzoic acid
250ml	Methanol

Make up to 2l with dddH_2O.

For Running buffer, use 300ml of the above concentrate, make up to 1l, filter through 0.45µ filters and degas.

Sulphur HPLC Mobile Phase

950ml	Methanol
50ml	dddH_2O

Filter through 0.45µ nylon filter and degas.

SULPHIDE ANALYSIS

Zinc Acetate

10.55g	$(CH_3.COO)_2Zn.2H_2O$

Make up to 1l with $dddH_2O$.

Ferric Chloride

8g	$FeCl_3$
500ml	6M HCl

Store in a dark bottle at room temperature.

Amide-sulphuric acid stock solution

2g	N-N dimethyl-p-phenylene diamine dihydrochloride
500ml	6M HCl

Store in a dark bottle at room temperature.

TOC

TOC Buffer

20g	Potassium peroxodisulphate
400ml	$dddH_2O$
2ml	Phosphoric acid

Make up to 1l with $dddH_2O$.

TOC Standard

425mg	Potassium pthalate
40ml	$dddH_2O$
0.2ml	Phosphoric acid

Make up to 100ml with $dddH_2O$, store in a dark bottle

APPENDIX 2:

Gradient Tubes

GRADIENT TUBES

Quadruple strength SRB lactate medium-ammonium chloride

3.5ml	60% Sodium Lactate
2g	$MgSO_4.7H_2O$
1g	NH_4Cl
1g	Na_2SO_4
1g	Yeast Extract
0.5g	K_2HPO_4
0.15g	$CaCl_2.6H_2O$

Make up to 250ml with $dddH_2O$, autoclave.

Quadruple strength SRB lactate medium-potassium nitrate

3.5ml	60% Sodium Lactate
2g	$MgSO_4.7H_2O$
1g	KNO_3
1g	Na_2SO_4
1g	Yeast Extract
0.5g	K_2HPO_4
0.15g	$CaCl_2.6H_2O$

Make up to 250ml with $dddH_2O$, autoclave.

Double strength SRB lactate medium-ammonium chloride

1.75ml	60% Sodium Lactate
1g	$MgSO_4.7H_2O$
0.5g	NH_4Cl
0.5g	Na_2SO_4
0.5g	Yeast Extract
0.25g	K_2HPO_4
0.05g	$CaCl_2.6H_2O$

Make up to 250ml with $dddH_2O$, autoclave.

Double strength SRB lactate medium-potassium nitrate

1.75ml	60% Sodium Lactate
1g	$MgSO_4.7H_2O$
0.5g	KNO_3
0.5g	Na_2SO_4
0.5g	Yeast Extract
0.25g	K_2HPO_4
0.05g	$CaCl_2.6H_2O$

Make up to 250ml with $dddH_2O$, autoclave.

Sulphide plug (5ml-0.5gHS^-)

0.05g	Agarose
1.25ml	$dddH_2O$
1.25ml	Quadruple strength media (1 tube for each different nitrogen source).

The medium, water and agarose were heated until the agarose dissolved completely. The solution was then allowed to cool to 55°C and 2.5ml 1g/l HS^- was added, the plug was poured into the bottom of a sterile test tube and allowed to set before the overlay was poured.

Agarose Overlay (50ml)

0.5g	Low Melt Agarose
25ml	$dddH_2O$
25ml	double strength media (1 tube for each different nitrogen source).

The medium, water and agarose were heated until the agaorse dissolved completely. The solution was then allowed to cool to 45°C and 1ml Biofilm inoculum was added, this was not added in the case of the bacteria-free control tubes.
For the pH control tubes 4 drops of Neutral red indicator (0.125g/l) was added.
Each overlay was approximately 15ml, these were poured on top of the plugs and the tubes were allowed to set in the anaerobic hood.

Tris-EDTA Buffer (0.01M Tris; 0.05M EDTA)

0.61g	Tris(hydroxymethyl)aminomethane
0.093g	EDTA

Add 400ml of $dddH_2O$ and pH to 8, then make up to 500ml volume with $dddH_2O$. Sterilize by autoclaving.

APPENDIX 3:

Electron Microscopy

Phosphate buffer

Solution A	35.8g/l	$Na_2HPO_4.2H_2O$
Solution B	13.6g/l	KH_2PO_4

To achieve a molarity of 0.1 and a pH 7.3 these solutions are mixed in the ratio 4 parts Solution A : 1 part Solution B.

Buffered glutaraldehyde

10ml	25% ultrastructure grade glutaraldehyde
90ml	Phosphate buffer

This solution is stored in a dark bottle at 4°C.

1% Buffered Osmium Tetroxide

Solution 1	2.55% $Na_2HPO_4.2H_2O$
Solution 2	2.52% NaOH
Solution 3	5.4% glucose
Solution 4	41.5ml Solution 1
	8.5ml Solution 2

Fixative consists of 45ml Solution 4, 5ml Solution 3 and 0.5g OsO_4.

Dissolve the osmium tetroxide crystals in Solution 4 by warming in the fume hood.

Cool and decant into a clean flask, add Solution 3 and pH to 7.3 with 0.1M HCl.

Store in a dark bottle at 4°C. (Cross *et al.*, 2001)

APPENDIX 4:

DNA Extractions and Agarose Gel Electrophoresis.

10 % SDS (100 ml)

10 g	SDS
100 ml	$dddH_2O$

Warm to 65°C to allow SDS to dissolve.

10% CTAB

10g	Cetyltrimethylammonium bromide
100ml	$dddH_2O$

Warm to 65°C to allow CTAB to dissolve.

5M NaCl

292g	NaCl
1l	$dddH_2O$

Autoclave to sterilize.

TRIS-BUFFERED PHENOL (pH 8)

Melt 100g of phenol at 68ºC and then add an equal volume of 0.5M Tris-HCl (pH 8) at room temperature. Stir on magnetic stirrer for 15 minutes. When the two phases have separated, aspirate the upper aqueous phase. Add an equal volume of 0.1M Tris-HCl (pH 8) to the phenol and stir. Extract the upper aqueous phase. Repeat the extraction procedure until the pH of the phenol is greater than 7.8. Add 0.1 x volume of 0.1M Tris-HCl (pH 8) containing 200ì l of â-mercaptoethanol and store the phenol at 4ºC in dark bottle. The phenol should be made in a fume hood and pH checked using pH paper.

CHLOROFORM: ISOAMYL ALCOHOL (24:1) (100 ml)

96ml	chloroform
4ml	isoamyl alcohol

10 x TBE BUFFER (1l)

107,8g	Tris base
55g	Boric Acid
7.44g	di-sodium EDTA

Make up to 800 ml with dddH_2O and pH to 8.3 with boric acid. Make up to 1l and autoclave. Dilute 1 in 10 for 1 x TBE.

ETHIDIUM BROMIDE (500 mg/ml)

Dissolve 0.5 g of ethidium bromide powder in 1ml of dddH_2O, store in a dark bottle at room temperature.

DNA LOADING BUFFER (6 x)

0.25%	Bromophenol blue
0.25%	Xylene cyanol
30%	Glycerol

***l Pst1* Molecular Weight Marker**

Digest 200ìl ëDNA (0.25 ug/ul) with 24ìl of 10 x buffer H and 10ìl of *Pst1* enzyme for 3 hours at 37º C. Add 550ìl TE buffer (pH 8) and 150ìl 6 x loading buffer. Aliquot 100 ìl into eppendorfs and store at -20°C.

Band sizes of *λPst1* Molecular Weight Marker:

- 14057bp
- 5077bp
- 4749bp
- 4507bp
- 2838bp
- 2459bp
- 2443bp
- 2140bp
- 1986bp
- 1700bp
- 1159bp
- 1093bp
- 805bp
- 514bp
- 468bp
- 448bp
- 339bp
- 264bp
- 247bp
- 216bp
- 211bp
- 200bp
- 164bp
- 150bp

APPENDIX 5:-

- **Polymerase Chain Reaction and Denaturing Gradient Gel Electrophoresis.**

Primer 907R

Oligonucleotide Information

Reference # :	1093548	Product :	200 nmole DNA Oligo
Purification :	PAGE purification	Unit Size :	200 nmole
Sequence Name :	907R GC	Bases :	60

Sequence : 5'- CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GCC GTC AAT TCC TTT GAG TTT -3'

Molecular Weight :	22,375.00
GC Content :	78.3 %
Tm (50nm NaCl) :	89.59 °C

Printed on: 6/14/00 M595063

Amount of Oligo

5.5	=	8.11	=	0.18
OD_{260}		nanomoles		mg

Modifications and Services	Qty
PAGE purification	1

Primer GM5F

Oligonucleotide Information

Reference # :	1093549	Product :	DNA Oligo
Purification :	Standard Purification	Unit Size :	200 nmole
Sequence Name :	GM5F	Bases :	17

Sequence : 5'- CCT ACG GGA GGC AGC AG -3'

Molecular Weight :	6,436.00
GC Content :	70.6 %
Tm (50mM NaCl) :	57.76 °C

Amount of Oligo

11.1	=	56.91	=	0.37
OD_{260}		nanomoles		mg

Both primers were manufactuered by Integrated DNA technology, Iowa, USA.

10mM dNTP`S

30ìl of each nucleotide (dATP, dTTP, dCTP & dGTP) is added into an eppendorf. 180ìl of dddH_2O is added, bringing the total volume to 300ìl. Aliquot into eppendorfs (60 ìl volumes) and store at –20º C.

DGGE (Adapted from Myers *et al., 1987).*

10 x TAE BUFFER (1L)

48.4g	Tris base
3.72g	EDTA
11.42ml	Glacial acetic acid

Make up to 1l with dddH_2O and autoclave.

40% Acrylamide Stock solution

100g	Acylamide
2.7g	bis-acylamide

Make up to 250ml with dddH_2O.

20% APS stock solution

0.2g	Ammonium persulphate
1ml	dddH_2O

Make fresh each time, keep away from light.

Deionized Formamide

Mix 250ml formamide with 12.5g Amberlite® IRN-150 resin, stir for 1 hour at room temperature and filter to remove the resin. Store in a dark bottle at 4°C.

0% Denaturant (50ml)

5ml	10 x TAE
7.5ml	40% Acrylamide stock solution

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

Use 7ml for 1 complete 0% Denaturant control gel or 3.8ml for a gradient gel.

Add 40µl 20% APS and 4µl TEMED to set the gel.

100% Denaturant (50ml)

5ml	10 x TAE
7.5ml	40% Acrylamide stock solution
21g	Urea
20ml	deionised Formamide

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

Use 3.8ml for a gradient gel.

Add 40µl 20% APS and 4µl TEMED to set the gel.

55% Denaturant (50ml)

5ml	10 x TAE
7.5ml	40% Acrylamide stock solution
11.55g	Urea
11ml	deionised Formamide

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

Use 3.8ml for a gradient gel.

Add 40µl 20% APS and 4µl TEMED to set the gel.

65% Denaturant (50ml)

5ml	10 x TAE
7.5ml	40% Acrylamide stock solution
13.65g	Urea
13ml	deionised Formamide

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

Use 3.8ml for a gradient gel.

Add 40µl 20% APS and 4µl TEMED to set the gel.

SILVER STAINING

Fixing Solution

100ml	Absolute Ethanol
1ml	Glacial Acetic acid

Make up to 1l with dddH_2O.

Developing Solution

4.5g	NaOH
300ml	dddH_2O
0.03g	Sodium borohydride
1.2ml	Formaldehyde

Make fresh every time, light sensitive.

DNA EXTRACTION FROM ACRYLAMIDE GEL

1M NaCl in TE Buffer pH 8

Make a 1 in 5 dilution of 5M NaCl in TE buffer, pH 8.

3 M SODIUM ACETATE

408.1g	Sodium acetate
800ml	dddH_2O

Adjust the pH to 7 with acetic acid. Make up to 1l and sterilise by autoclaving

APPENDIX 6:

Cloning

COMPETENT CELLS

RF1

100mM	KCl
50mM	$MnCl_2$
30mM	CH_3COOK
10mM	$CaCl_2$
15%	Glycerol

Make up to 1l with dddH_2O and adjust the pH to 5.8 with Acetic acid.

RF2

10mM	MOPS (4-Morpholine-propanesulfonic acid)
10mM	KCl
75mM	$CaCl_2$
15%	Glycerol

Make up to 200ml with dddH_2O and adjust the pH to 6.8 with KOH.

Luria Broth (LB)

5g	Tryptone
2.5g	Yeast Extract
2.5g	NaCl

Make up to 500ml with dddH_2O. Adjust the pH to 7 with NaOH and sterilize by autoclaving.

LB-Ampicillin Agar Plates

5g	Tryptone
2.5g	Yeast Extract
2.5g	NaCl
7.5g	Agar

Make up to 500ml with dddH_2O. Adjust the pH to 7 with NaOH and sterilize by autoclaving, allow to cool to 60° and add 500µl of 100mg/ml Ampicillin stock.

SOC medium

2g	Tryptone
0.5g	Yeast extract
0.05g	NaCl
10ml	250mM KCl

Dissolve in 85ml $dddH_2O$, adjust pH to 7. Make volume up to 95ml with $dddH_2O$, sterilize by autoclaving and add 1ml filter sterilized 2M $MgCl_2$ and 1ml filter sterilized 2M glucose stock solution. Aliquot in 1ml volumes and store at -20°C.

IPTG Stock solution (200mg/ml)

0.2g	IPTG
1ml	$dddH_2O$

Store at -20°C, use 4µl per agar plate.

X-Gal (100mg/ml)

0.1g	X-Gal
1ml	dimethylformamide

Store at -20°C, in a dark bottle, use 40µl per agar plate.

PGEM-T Easy Vector Plasmid Map.