ECOLOGY OF BIOLOGICAL SULFATE REMOVAL

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Thesis presented in partial fulfillment of the requirements for the degree of Master of Science at the University of Stellenbosch.

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature: Date:

SUMMARY

A laboratory-scale model was used to simulate biological sulfate removal. The focus of the research was microbial community response, such as the relative abundance of functional groups to changes in influent medium composition. Specific oligonucleotide probes were obtained that recognised sulfate reducing bacteria (SRB) within the biofilm community. Terminal restriction fragment length polymorphism (T-RFLP) and BIOLOG™ Ecoplate analyses were used to study the SRB community when provided with sodium lactate, sucrose or ethanol as carbon sources in complex Postgate C broth. These two analyses, as well as conventional methods, were applied to follow succession patterns in the laboratory scale reactors, and to determine the possible presence and relative abundance of microorganisms other than bacteria under sulfate reducing conditions. T-RFLP and BIOLOG™ Ecoplate analyses indicated a few dominant organisms in the community and a slight decline after a shift to another carbon source. Fluorescent hybridization showed higher numbers of SRB relative to the total microbial community than conventional culturing techniques. Furthermore, microscopic observations showed that not only SRB and other bacteria, but also yeast and filamentous fungi were integrated in a biofilm under sulfate reducing conditions. These microscopic observations were verified with fluorescent *in situ* hybridization (FISH) and yeast Live / Dead viability probes.

OPSOMMING

Biologiese sulfaat-verwydering is met behulp van 'n laboratoriumskaalmodel gesimuleer. Die doel van die navorsing was om die respons van 'n mikrobiese gemeenskap met byvoorbeeld die relatiewe hoeveelheid van funksionele groepe op veranderinge in invloeiende medium samestelling te bestudeer. Spesifieke oligonukleotiedpeilers wat sulfaatreduserende bakterieë (SRB) in 'n biofilmgemeenskap kan opspoor is gebruik. Die SRB gemeenskap is bestudeer met behulp van terminale-restriksiefragmentlengtepolimorfisme (T-RFLP) en BIOLOG™ Ecoplate analise waar natriumlaktaat, sukrose of etanol as koolstofbronne toegevoeg is. Hierdie twee tipes analise en konvensionele metodes is aangewend om suksessiepatrone in die laboratoriumskaalreaktor te volg en die moontlike teenwoordigheid en relatiewe hoeveelheid van organismes, uitsluitende bakterieë, onder sulfaatreduserende kondisies te bepaal. Analise van T-RFLP en BIOLOG™ Ecoplate het aangedui dat 'n paar dominante organismes in die gemeenskap teenwoordig was, wat effens afgeneem het na verskuiwing na 'n ander koolstofbron. Fluoresserende hibridisasie het hoër getalle van SRB relatief tot die totale mikrobiese gemeenskap aangedui as konvensionele kultuur tegnieke. Mikroskopiese analises het verder getoon dat benewens SRB en ander bakterieë ook giste en filamentagtige swamme onder sulfaatreduserende kondisies in 'n biofilm geïntegreer was. Hierdie mikroskopiese waarneminge is bevestig deur fluoresserende *in situ* hibridisasie (FISH) en gis Lewe / Dood lewensvatbaarheid peilers.

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1. INTRODUCTION

Since microbes have been present on earth longer than other organisms, they have evolved the ability to thrive in almost any environment. There are microorganisms that flourish inside of eukaryotic cells, some survive at temperatures greater than 100°C, others in the presence of toxic metals like copper or mercury. There are also microorganisms that grow at pH values of 2.0 and others that grow at pH 11.0, or 3.5 km below the earth's surface and even in saturated salt solutions or at 0°C. Microbes require an energy producing system (including an electron acceptor) to sustain life and nutrients and liquid water in order to grow and reproduce (Hurlbert, 1999). Energy can come from one of two sources: from light or from the oxidation of reduced organic and/or inorganic molecules.

Microbes have extended the environment they can live in by developing enzymes that allow them to make use of sunlight for energy as well as a diversity of electron donor/acceptors pairs so they can transact energy-yielding oxidative reactions on available energy sources. The range of electron acceptors includes gaseous oxygen, sulfate, nitrate, nitrite, carbon dioxide, carbon monoxide, iron and magnesium. Since these conditions cover the entire earth, even that portion under the oceans, these bacterial forms may make up the largest single mass of life on earth (Hurlbert, 1999).

All life-giving nutrients endlessly turnover in a cyclic way and each cycle involve one group or more of microorganisms that are accountable for carrying out this process. A given cycle is often viewed as starting with basic elements being converted into larger, complex organic polymers. Once the cells containing these

polymers die, degradation, or mineralization as the process is often called, occurs and the polymers are converted to the basic chemical precursors of new life (Hurlbert, 1999).

Microbial sulfur (S) transformations, as can be seen in the sulfur-cycle, are closely connected with the carbon–cycle. In this cycle, sulfur reduction is coupled with organic matter utilization, which is a major mineralization pathway in anaerobic habitats. Organisms that oxidise sulfur, can be autotrophic and/or phototrophic (Jørgensen 1988; Voordouw 1995), and can grow under aerobic and anaerobic conditions.

Microorganisms involved in the sulfur–cycle are extremely diverse. The anaerobic sulfate reducing bacteria (SRB), which are unique physically and genetically, are represented by several genera, most of which were discovered recently (Widdel and Bak, 1991; Devereux and Stahl, 1993). Sulfur can be found in a range of valence states from the highly reduced sulfide (-2) to the most oxidized form in sulfate SO_4^{2-} (+6). There are several intermediate valence forms of sulfur that can act as both electron donors and electron acceptors. Many sulfur compounds are highly reactive. Microorganisms must therefore often compete with abiotic reactions. These characteristics can be used in the treatment of industrial effluents containing high sulfate concentrations. Maree and co-workers developed a biological sulfate reduction process (Maree and Strydom, 1985; Maree *et al.*, 1986). This is a reactor system based on the principle of a single-stage completely-mixed reactor configuration. This reactor, introduced by Maree *et al.*, (1997), can remove sulfate and sulfide simultaneously, due to air introduction into the reactor system. Anaerobic digesters and reactors, where the methanogenic bacteria (MB), the SRB and the acetogenic bacteria (AB) form a consortium, represent an area where

metabolic cooperation between bacteria has been studied. Such consortial activities are interactions between two or more populations in a given community, which enable organisms to maximize their metabolic capabilities and to maintain community integrity and stability.

The production of sulfides (in the gaseous form H_2S and in the dissociated forms HS^- and S^{2-}) during the sulfate removal/reduction process is a major problem. The produced sulfides are toxic to most bacteria at relative low concentrations and is fatally toxic to humans at gaseous concentrations of 800 – 1000 ppm (Speece, 1996).

Two different biotechnological processes can be used for the removal of the produced hydrogen sulfides. Cork (1985) suggested that the photosynthetic green sulfur bacteria (*Chlorobium limicola*) use light energy to produce organic energy, and reduce H_2S to elemental sulfur (S^0). Buismann (1989) showed that sulfide can also be oxidized to elemental sulfur microbially by a group of colorless sulfur bacteria under oxygen limitation conditions.

Wolfaardt *et al.*, (1994) described that microbial activities, which result in macro-scale environmental changes and which can be measured in physical and chemical terms, occur at micro-scale.

Anaerobic digestion of wastewater like industrial and municipal effluents is most efficient when microbial aggregates in the form of biofilms, sludge granules and flocs are present (Wolfaardt *et al.*, 1994). The term microbial aggregate is chosen to indicate those associations of microorganisms that are largely microbial biomass plus varying amounts of extra cellular polymeric matter produced by microbes themselves. A better understanding of the mechanisms which microbial communities apply in nature to reproduce under opposed environments, such as

biofilm and floc formation can, through microbial manipulations, result in process optimization. However, the microbial processes relevant to industrial SO_4 removal still remains a *black box*.

The structure of a microbial community was mainly defined by two parameters: Identity and abundance of its members (Amann *et al.*, 1995). Since both cultivation and fingerprint methods were not sufficient to address these questions, hybridization techniques applying rRNA-targeted oligonucleotide probes had been developed.

Most bacteria in aquatic habitats are not free floating but sessile (McGlohorn *et al.*, 1999). In their natural environment bacteria form coagulated masses called biofilms. A biofilm develops when bacteria attach to a surface, grow and produce polysaccharides and other extracellular material. As more and more bacteria adhere, a biofilm is formed. As a working community the bacteria on the surface of the biofilm absorb organic as well as inorganic matter from the bulk liquid and make them available as nutrients to the cells within the biofilm. In a microbial biofilm, microbes co-operate to remove waste and toxins from the biofilm. Antimicrobial agents, phagocytic white blood cells and chemical biocides are less effective against the microbes within a biofilm, compared to planktonic cells. The protection that the biofilm provides the microbes is a problem in medicine and industry (McGlohorn *et al.*, 1999).

To contribute to the knowledge base required for improved sulfate and sulfide removal rates, utilization and the effect of different carbon sources, sucrose and ethanol, were investigated in this study.

A major challenge was to develop a laboratory-scale system that would provide a realistic simulation of industrial settings for sulfate removal. This study followed a study by Greben (Greben *et al.*, 2000). The latter focussed on system optimization.

This study concentrated on microbial behavior, and specifically new approaches that can be applied to study microbial behavior in a complex community setting. Therefore the goal was to design a small reactor which would function in the same way as the industrial and industrial research reactors, but on a micro scale to apply different techniques to elucidate the microbiology and ecology of sulfate removal. Furthermore previous studies focused primarily on cell numbers, while this study focused on shifts in the community to evaluate the relative abundance.

The specific objectives of this study were thus to:

1) obtain oligonucleotide probes to recognize SRB in a biofilm community
2) evaluate the application of two types of analyses, terminal-restriction fragment length polymorphism (T-RFLP) and BIOLOG™ EcoPlates for community profiling, and apply the techniques in 1 and 2 together with conventional techniques to:
    a. evaluate community stability of SRB under different nutrient regimens (carbon sources);
    b. follow succession patterns in a micro-scale reactor; and
    c. determine the possible presence and relative abundance of microorganisms other than bacteria under sulfate reducing conditions.

2. LITERATURE REVIEW

2.1 The sulfur cycle - a basic process in the environment

The sulfur cycle is, like the carbon and nitrogen cycle, an essential process in nature. However, due to human activities, the cycle can easily be disturbed on a local level as well as globally (Kuenen and Beudekker, 1982). Among the major environmental pollutants in the sulfur cycle is the formation of SO_2/SO_3 and other sulfur compounds that arise by burning fossil fuels (like diesel fuel), due to global industrialization. The sulfur cycle consists of several oxidative and reductive steps, which are in balance in natural ecosystems. Sulfate and sulfur serve as electron acceptors in the reductive metabolic pathways, and are used by a wide range of anaerobic bacteria. In the oxidative component of this cycle the reduced sulfur compounds are electron donors for anaerobic phototrophic bacteria. The phototrophic bacteria gain their energy from light energy and provide growth energy for the colorless sulfur bacteria.

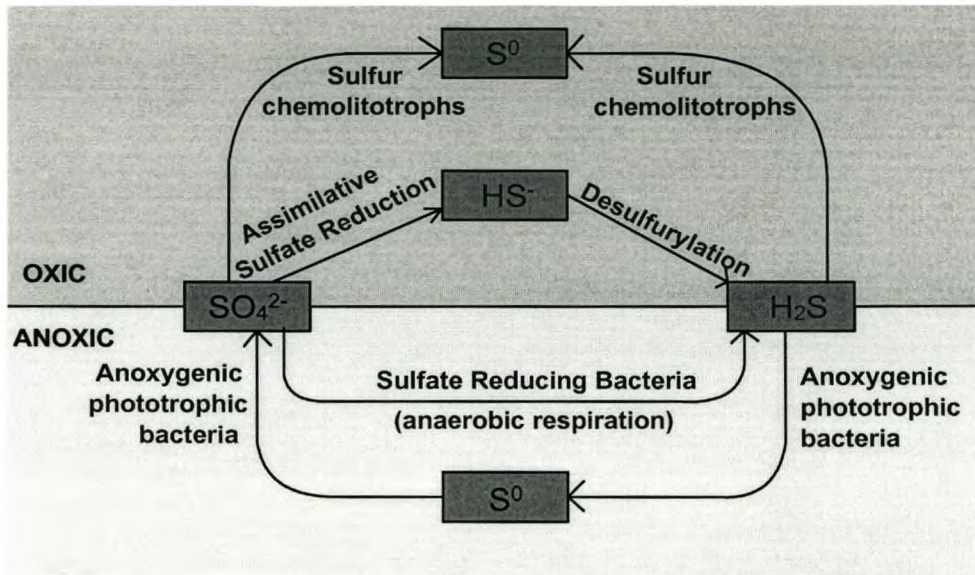

Figure 1. Diagrammatic representation of the Sulfur Cycle (adapted from Prescott *et al.*, 1996).

2.2 The sulfate reducing bacteria

The SRB are a specialized group of anaerobic bacteria that are responsible for the dissimilatory reduction of sulfate to sulfide. This reduction is coupled to organic matter oxidation (Lovly and Philips, 1994). SRB are often important in the anaerobic degradation of organic matter in aquatic habitats. Once thought to be restricted primarily to sulfate removal, SRB have been confirmed to be capable of utilizing iron, manganese, and even oxygen as electron acceptors. It is further generally accepted that SRB oxidize products of fermentative bacteria such as fatty acids, alcohols, some aromatic acids, amino acids, and hydrogen. Indeed, as illustrated in figure 2, the SRB are involved in various processes and/or associations in a variety of environments.

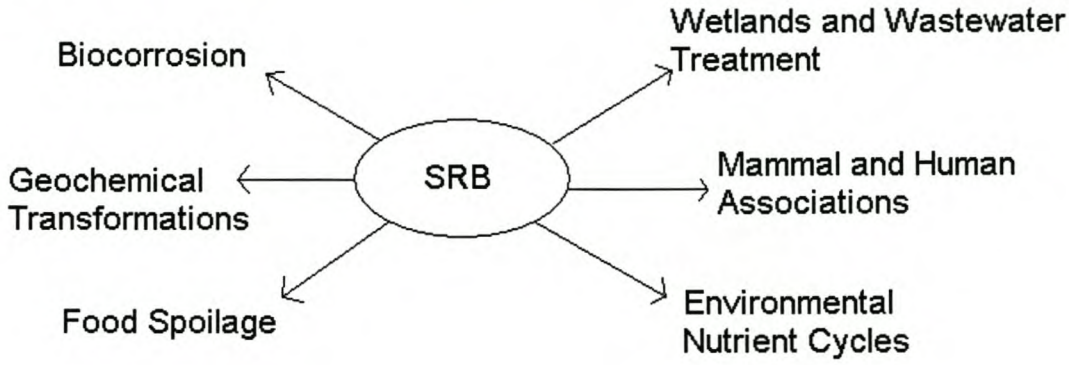

Figure 2. Interaction of Sulfate Reducing Bacteria with their environment (adapted from Barton, 1995).

Successful sulfate reduction/removal is associated with pH increase due to the production of sulfide and alkalinity (Greben, 2001). Therefore, the SRB and the sulfate reduction technology is beneficial to industries experiencing acid mine drainage problems, as it results in removal of sulfate (SO_4) and often in metal removal and an increase in the pH of the treated water.

The SRB, which are unique physically and genetically, are represented by several genera, most of which were discovered more recently (Devereux and Stahl, 1993; Widdel and Bak, 1991). The two most established genera of SRB are *Desulfovibrio* and *Desulfotomaculum*. More recently, several new genera have been identified. These include *Desulfobacter, Desulfonema, Desulfobulbus, Desulfoarculus, Desulfobacterium, Desulfococcus, Desulfohalobium, Desulfomicrobium, Desulfosarcina, Desulfobotulus, Desulfomonile, Thermodesulfobacterium* and *Archaeoglobus*. The *Desulfovibrio* are the best known group, which are relatively

easy to isolate and purify; they are mesophilic and can be halophilic and do not form spores. They are curved or sigmoid with some exceptions. The SRB are Gram-negative except *Desulfonema* spp. The principle end products of SRB metabolism are H_2S and CO_2 (Postgate, 1984).

SRB are present in most soil and water habitats, but are outnumbered by other microbes (Widdel and Bak, 1991). The enrichment of the population with respect to these bacteria is necessary prior to isolation. It is done mainly via batch culture. Such cultures can be prepared from soil, water or other samples using Postgate media (Postgate, 1984).

2.2.1 Bioenergetics of SRB in relation to their environmental impact

The cellular physiology of the SRB and of other sulfidogenic species is determined by the energetic requirements that result from their respiratory mode of metabolism with sulfate and other oxyanions of sulfur as terminal electron acceptors (Hamilton, 1998). As a further consequence of their relatively restricted catabolic activities and their requirement for anaerobic conditions, sulfidogenic bacteria are almost always found in nature as component organisms within microbial consortia. The capacity to generate significant quantities of sulfide influences the overall metabolic activity and species diversity of these consortia and is the root cause of the environmental impact of the sulfidogenic species, which include corrosion, pollution and the souring of hydrocarbon reservoirs.

2.3 Research towards a better understanding of the SRB and their involvement in the Sulfur-Cycle

The importance of the sulfur cycle in natural processes has been recognized. In addition, the key role of this cycle in industry became increasingly evident. In order to optimize this process for industrial application, various studies were performed on key factors that influence the sulfur-cycle.

2.3.1 Nutrient requirements for SRB

Several nutrient requirements were determined by White and Gadd (1996) to better the competence of a sulfate-reducing bacterial mixed culture for biotechnological removal of sulfate, acidity and toxic metals from waste waters. In batch culture, lactate results in the most biomass. Ethanol is more effective than lactate in stimulating sulfide production and acetate is less effective. Additional bicarbonate and hydrogen only stimulates sulfide production slightly. When ethanol is used as substrate, the sulfide output per unit of biomass is the greatest. In continuous culture, ethanol and lactate can be used directly as efficient substrates for sulfate reduction, while acetate yields reduced growth. Glucose may have a damaging effect on pH, as it is utilized following fermentation to organic acids. Ethanol is not only the most efficient substrate because of its efficient yield of sulfide, but is also the most cost effective substrate. The presence of additional carbon sources does not stimulate growth or sulfate reduction in batch culture. However, the presence of complex nitrogen sources (yeast extract or cornsteep) encourage both sulfate reduction and growth. Addition of cornsteep demonstrate the strongest effect on growth and sulfate reduction and is relatively inexpensive. In continuous culture, cornsteep considerably increases the yield of sulfate reduced per unit of ethanol consumed.

The most effective nutrient organization for bioremediation using SRB requires ethanol as carbon source and cornsteep as a complex nitrogen source.

A novel sulfate-reducing bacterium was isolated from the roots of the macrophyte *Zostera marina* (Nielsen *et al.,* 1999). The name *Desulfovibrio zosterae* sp. nov. was proposed for this SRB, which utilized lactate, pyruvate, malate, ethanol, L-alanine, fumarate, choline and fructose with sulfate as electron acceptor. This SRB can also degrade fumarate, pyruvate and fructose without an external electron acceptor and sulfate can be substituted with thiosulfate, sulfite and elemental sulfur.

Magot *et* al. (1997) characterized a strictly anaerobic thiosulfate-reducing bacterium that was isolated from a corroding offshore oil well in Congo. The name of this new bacterium was proposed as *Dethiosulfovibrio peptidovorans*. The thiosulfate-reducing bacterium utilizes peptides and amino acids, but not sugars or fatty acids. It is also able to ferment serine, histidine, and casamino acids. Arginine, glutamate, leucine, isoleucine, alanine, valine, methionine, and asparagine are only utilized when thiosulfate is present. *D. peptidovorans* ferments peptides to acetate, isobutyrate, isovalerate, 2-methylbutyrate, hydrogen, and carbon dioxide. When thiosulfate or sulfur is added, peptide utilization, growth rate and biomass increase. Addition of sulfate, however, had no effect on growth rate, peptide utilization and biomass. Hydrogen sulfide is produced during growth of *D. peptidovorans* and is associated with decrease in H_2. Addition of thiosulfate or sulfur can reverse hydrogen inhibition.

The growth kinetics of the sulfate-reducing bacterium *Desulfovibrio desulfuricans* was examined under a variety of conditions, including hydrogen sulfide inhibition, initial lactate concentration, sulfate concentration and pyruvate as carbon source (Cooney *et al.*, 1996). Hydrogen sulfide inhibits growth and decreases growth yields

and the sulfate-specific reduction rate. This inhibition is direct and reversible. A high initial lactate concentration delays bacterial growth, reduces the specific sulfate reduction rates and provides inconsistent biomass growth yields. This may result in a bottleneck in the lactate oxidation pathway that induces the production of butanol. Pyruvate as a carbon source is more efficient to use than lactate as it yields better growth rates and more biomass with only a minor decrease in the rate of specific sulfate reduction, but pyruvate required nearly 40% less sulfate than lactate.

Syntrophic propionate and ethanol conversion was probably achieved mainly by SRB, while hydrogen, formate, and acetate were consumed mainly by methanogens in a study of granules from an up-flow anaerobic sludge blanket system that contained primarily ethanol, propionate, and acetate as carbon sources, and sulfate (Wu *et al.*, 1991). Hydrogen and formate were formed during syntrophic ethanol conversion by the granules. The concentrations of hydrogen and formate were kept at a thermodynamic equilibrium, which indicates that both are intermediate metabolites in degradation. Formate is consumed during methanogenesis from H_2-CO_2 after an accumulation. The absence of sulfate led to higher concentrations of accumulated formate than cases where sulfate was present. Maximum substrate degradation rates of propionate and ethanol was better by the addition of sulfate (8 to 9 mM). At this sulfate concentration SRB do not play an active role in the metabolism of hydrogen, formate, and acetate, but SRB are responsible for ethanol and propionate conversion through sulfate reduction. The results from Wu *et al.* (1991) indicates that in this granular microbial consortium, methanogens and SRB did not compete for common substrates.

2.3.2 SRB in extreme temperature

Sulfate reducing bacteria were investigated in two Arctic sediments with *in situ* temperatures of 2.6 and -1.7 °C (Knoblauch *et al.,* 1999). Most-probable-number counts at an incubation temperature of 10 °C were higher than at 20 °C, which indicates that a high proportion of the community were psychrophilic. The mean specific sulfate reduction rates of 19 isolated psychrophiles were compared to corresponding rates of 9 marine, mesophilic SRB. The results indicated that, as a physiological adaptation to the permanently cold arctic environment, psychrophilic sulfate reducers have considerably higher specific metabolic rates than their mesophilic counterparts at similarly low temperatures.

2.3.3 SRB in correlation with oxygen

The study by Minz *et al.* (1999) revealed a special localization of SRB within the region defined by the oxygen chemocline. *Desulfonema*-like populations dominated between the different groups of SRB that were quantified and accounted for up to 30% of total rRNA extracted from certain depth intervals of the chemocline. Recognized genera of SRB are not restricted by high levels of oxygen in a mat community. There is a possibility of significant sulfur cycling within the chemocline.

Magot *et al.* (1997) demonstrated the first experimental proof of the participation of thiosulfate reduction by microbial corrosion of steel. Pure cultures of a strictly anaerobic thiosulfate-reducing bacterium *D. peptidovorans*, induced a vastly active pitting corrosion of mild steel. The penetration rates were up to 4 mm per year. Optimum growth occurred in the presence of 3% NaCl at pH 7.0 and 42 °C.

Molecular information about the bacterial composition of a co-culture capable of sulfate reduction after exposure to oxic and microoxic conditions was used by Teske *et al.* (1996b) to identify and subsequently isolate the components of the mixture in pure culture. Sequencing showed that *Desulfovibrio* strain and an *Acrobacter* strain was present.

Ramsing *et al.* (1993) used oligonucleotide probes, binding to 16S rRNA, to study the vertical distribution of SRB in photosynthetic biofilms. SRB were unequally dispersed in the biofilm and present in all states from single scattered cells to dense clusters of thousands of cells. SRB were counted along vertical sections through the biofilm to quantify their vertical distribution. There was a negative correlation between the vertical distribution of SRB cells and the measured oxygen profiles. Although scattering of SRB through the biofilm differed in light- and dark-incubated samples, likely because of the different extensions of the oxic surface layer, SRB were largely restricted to anoxic layers in both cases.

Okabe *et al.* (1999) studied the vertical distribution of SRB within a biofilm with the use of fluorescent *in situ* hybridization (FISH). A relatively high abundance of SRB-stained (with 16S rRNA-targeted oligonucleotide probes) cells were evenly distributed throughout the biofilm. SRB were also present in the oxic surface of the biofilm. Further microelectrode measurements demonstrated a high sulfate-reducing activity in the narrow anaerobic zone located about 150 to 300 μm below the biofilm surface and above which an intensive sulfide oxidation zone was found. It was found that elemental sulfur (S^0) is an important intermediate of the sulfide reoxidation in the thin wastewater biofilms (approximately 1,500 μm), which accounts for about 75% of the total sulfur pool in the biofilm. The involvement of an

inner Fe-sulfur-cycle to the overall sulfur-cycle in aerobic wastewater biofilms was not significant (less than 1%) due to the relatively high sulfate reduction rate.

2.3.4 Microbial community analyses

Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA and DNA encoding rRNA (rDNA), isolated from a stratified marine water column gave an indication of specific bacterial populations in several water column layers in a study by Teske *et al.* (1996a). A highly differentiated pattern of rRNA- and rDNA-derived PCR amplifies was revealed, which probably reflected active and resting bacterial populations.

In a study by Raskin *et al.* (1995), the microbial community structure of twenty-one single-phase and one two-phase full-scale anaerobic sewage sludge digesters were evaluated using oligonucleotide probes that were complementary to conserved regions of the 16S rRNAs of phylogenetically defined groups of methanogens and SRB. It was found that methanogens in mesophilic, single-phase sewage sludge digesters accounted for approximately 8-12% of the total community. Methanosarcinales and methanomicrobiales made the majority of the total methanogen population. Methanobacteriales and methanococcales were present at lower levels in the digesters. Phylogenetic groups of mesophilic, Gram-negative SRB were constantly present in the digesters. *Desulfovibrio* and *Desulfobulbus* species made the majority of the sulfate-reducing populations. *Desulfobacter* and *Desulfobacterium* spp. played a insignificant role in the digesters and *Desulfosarcina*, *Desulfococcus* and *Desulfobotulus* species were not detected in the digesters.

Sulfate reduction by one or more of these populations played a significant role in two-phase and single-phase digesters evaluated in a study by Raskin *et al.* (1995). SRB played a role in making conditions for methanogenesis favorable by providing the substrates needed. Significant levels of active methanogens were present in the first phase of two-phase digesters. Although true phase separation was not accomplished, the dominant populations in the second phase were different from those in the single-phase digesters.

PCR amplification of 16S ribosomal DNA fragments from a co-culture able to reduce sulfate were analyzed by DGGE. Two distinct 16S ribosomal DNA bands that were present indicated two different bacterial components present in the co-culture (Teske *et al.*, 1996a). DNA sequencing showed that the bands came from a *Desulfovibrio* strain and an *Acrobacter* strain. The phylogenetic locations of bacteria are frequently consistent with their physiological properties and culture requirements, therefore, molecular identification of the two components of this co-culture permitted the design of specific culture conditions to separate and isolate both strains in pure culture. Through this approach it was possible to combine molecular and physiological analysis of mixed cultures and microbial communities.

2.3.4.1 Fluorescent *in situ* hybridization (FISH)

By using i*n situ* hybridization, single-stranded DNA molecules are permitted to form hybrids with molecules that have sufficiently similar, complementary sequences. Oligonucleotide probes with a fluorescent dye can be hybridized to the cells in biofilms (Amann *et al.*, 1998).

Oligonucleotides (18 nucleotides) are preferred to polynucleotides (~ 50 nucleotides) as they allow for single mismatch discrimination of target nucleic acids. Ribosomal RNA is a suitable target for phylogenetic probes for a number of reasons:

1) Ribosomes are ubiquitous.

2) Ribosomal sequences are functional conserved molecules and are nontransferable between species.

3) The primary structures of 16 S and 23 S rRNA are composed of regions of higher and lower evolutionary conversation (Fox *et al., 1977* and Amann *et al.,* 1995). If microbial species and subspecies needed to be distinguished, probe specificity could be adjusted freely to target the most variable regions of the molecule in contrast to highly conserved regions, which may be targeted for universal probes (Amann *et al.,* 1995, Manz *et al.,* 1993, Muyzer and Ramsing, 1995 and Amann *et al.,* 1996).

4) Extensive rRNA sequence databases are available, especially for 16 S rRNA, allowing the computer-assisted design and testing of oligonucleotide probes.

5) Ribosomes usually occur in high copy numbers throughout the cytoplasm (e.g., more than 1000 copies) so that the entire cell content becomes flourescent on hybridization to a specific probe (Amann *et al.,* 1998).

In a study by Teske *et al.* (1996a) DGGE patterns were hybridized with rRNA probes. This revealed that there was an increased presence and activity of SRB within and below the chemocline of a stratified fjord. Most-probable-numbers (MPN), a non molecular method, showed a similar distribution of SRB (approximately 25 cells and 250 cells per ml) in the water column of a Mariager Fjord. *Desulfovibrio-* and *Desulfobulbus*-related strains were found to be present in the oxic zone. DGGE was used to show that the MPN isolates had phylogenetical

similarity with sulfate-reducing delta subdivision proteobacteria (members of the genera *Desulfovibrio*, *Desulfobulbus* and *Desulfobacter*). In contrast, the molecular isolates constituted an independent lineage of the delta subdivision proteobacteria.

The vertical distribution of SRB in aerobic wastewater biofilms grown on rotating disk reactors was investigated by Okabe *et al.* (1999) by means of FISH with 16 rRNA-targeted oligonucleotide probes. To find the vertical distribution of SRB populations with their activity, microprofiles of oxygen, hydrogen sulfide, nitrate, nitrite, ammonium and pH were measured with microelectrodes.

2.3.4.2 Terminal-restriction fragment length polymorphisms (T-RFLP)

Terminal restriction fragment length polymorphisms (T-RFLP), also known as terminal restriction fragment (TRF) patterns, is a quantitative molecular technique that was developed for rapid analysis of microbial diversities, structure and development in various environments (Liu *et al.*, 1997; Kitts, 2001). This technique makes use of PCR with one or both of the primers being fluorescently labeled. In most cases the primers are designed to amplify sections of bacterial genes encoding the 16S rRNA. The PCR products are digested and fluorescently labeled terminal restriction fragments can be measured with a DNA sequencer (Liu *et al.,* 1997). T-RFLP data has the advantage of being automatically converted to a digitized form that can be analyzed with statistical techniques (Kitts, 2001). In studies involving complex microbial communities, T-RFLP has a better resolution than other DNA-based methods for evaluating community structure, since the identification of specific elements in a TRF pattern is possible by comparison to entries in a good sequence database or by comparison to a clone library.

T-RFLP is a powerful tool for assessing the diversity of complex bacterial communities and for rapidly comparing the community structure and diversity of different ecosystems. Liu *et al.* (1997) used computer-simulated analysis of T-RFLP for 1002 eubacterial sequences. It was shown that 686 sequences could be amplified by PCR and classified into 233 unique terminal restriction fragment lengths. All bacterial strains in a model bacterial community were distinguished with T-RFLP, which revealed a consistent pattern with a predicted outcome. Their analysis of complex bacterial communities in activated sludge, bioreactor sludge, aquifier sand, and termite guts, with T-RFLP, revealed great species diversity.

In a study by Regan *et al.* (2002), T-RFLP was used to characterize the diversity of ammonia-oxidising bacteria and nitrite-oxidising bacteria in the distribution systems of a pilot-scale chloraminated drinking water treatment system. T-RFLP indicated the presence of ammonia oxidising *Nitrosomas* in each of the distribution systems in contrast to a significantly smaller peak attributable to *Nitrosospira*-like ammonia-oxidizing bacteria.

Osborn *et al.* (2000) evaluated the reproducibility and robustness of T-RFLP. Environmental DNA samples were isolated from polluted or pristine soil and 16S rDNA of the total community was amplified using the polymerase chain reaction. They were able to produce consistently almost identical community profiles from the same sample. Also, very little variation was detected between replicate restriction digestions, PCR amplifications and DNA isolations. A decrease in template DNA concentration produced a decline in the complexity and intensity of fragments present in the community profile. Higher template DNA concentrations

did not increase the complexity of the fragments. A variation between profiles were generated from the same DNA sample when different Taq polymerases were used. Different annealing temperatures caused lower levels of variability between PCR products. Incomplete digestion by restriction enzymes can lead to an overestimation of the overall diversity within a community. The authors concluded that standardized T-RFLP analysis is highly reproducible and robust and that the technique yields high-quality fingerprints that consist of fragments of precise sizes, which could be phylogenetically assigned once an appropriate database has been constructed.

T-RFLP was employed to study the structure of microbial communities as a function of community composition and the relative abundance of specific microbial groups to observe the consequences that plant community and land-use history have on microbial communities in soil (Buckley and Schmidt, 2001). It was shown that despite differences in plant community composition, plots with a long-term history of agricultural use have remarkably similar microbial community structures. However, microbial community structures vary considerably between fields that had never been cultivated and those having a long-term history of cultivation.

T-RFLP and randomly amplified polymorphic DNA (RAPD) were used to observe microbial succession in a fed-batch culture where the anaerobic degradation of phenol was studied (Guieysse *et al.*, 2001). T-RFLP and RAPD both generally provided similar results, which indicated a community shift after each phenol amendment.

2.3.5 Oil and Tar sands

The microbial treatment of sour waste streams resulting from the production or refining of natural gas and crude oil have been investigated by Sublette *et al.* (1998). The application of this technology for the treatment of sour wastes on a commercially feasible scale has been hindered by several technical barriers including substrate inhibition, product inhibition, the need for septic operation, biomass recycle and recovery, mixed waste issues, and the need for large-scale cultivation of the organism for process startup. SRB appear to be inhabitants of waters of oil-bearing shales and strata (Postgate, 1984). SRB have been injected into oil wells in an attempt to increase oil production (Barton, 1995). *D. desulfoforicans* secretes mucin, which is important in the release of oil from oil-containing sands (Postgate, 1984).

2.3.6 Negative implications of SRB to industry.

The case studies given above are all examples where the activity of SRB was beneficial to industry. However, their activity may also have a negative impact on industry. Below are two examples to demonstrate this.

2.3.6.1 Food spoilage

SRB have been implicated in deterioration of food in commercial processing. They are involved in the spoilage of olives in olive brines and in canned corn, possibly due to the introduction of sulfur dioxide during the preservation (Barton, 1995). SRB e.g. *Desulfomaculum* spp. are known for forming 'sulfur stinkers' of canned vegetables.

2.3.6.2 Biocorrosion

SRB play an important role in the biocorrosion of iron, various metal alloys, and the degradation of concrete (Gibson *et al.,* 1988). Although SRB are obligate anaerobes, they can be readily isolated from aerobic environments. They are usually component members of mixed microbial consortia, within which, aerobic and facultative organisms supply the nutrients for the SRB from their metabolic products of primary nutrients, and hence generate the necessary reducing conditions to allow growth of SRB. On iron, the corrosion brought about by SRB is restricted to anaerobic environments like waterlogged soil. When exposed, the soil neighboring the corroded metal smells of H_2S, and the metal is pitted rather than evenly corroded. It has been hypothesized that SRB like *Desulfovibrio* and *Desulfomaculum* corrode iron by their hydrogenase activity.

The corrosion of concrete pipes and buildings is a consequence of the process caused by SRB metabolism (Gibson *et al.*, 1988). There are two types of metabolism involved in the sulfur cycle in the environment. The anaerobic process is one where hydrogen sulfide (H_2S), is produced by anaerobic bacteria. The aerobic process occurs where the H_2S is converted to elemental S^0 or sulfuric acid (H_2SO_4). The sulfur oxidizing bacteria grow on and within the concrete. This oxidizes the H_2S present and produces H_2SO_4. The sulfuric acid dissolves any CaOH and $CaCO_3$ within the cement binder, thus causing degradation of concrete.

2.3.7 Mammalian Associations

Although SRB are the complex heterogeneous microflora typically associated with environmental and intestinal habitats, they have been found inhabiting the human

oral cavity (Willis *et al.*, 1995). The comensals of the human oral cavity give rise to numerous organic and short chain fatty acids from fermentative growth e.g. acetate, propionate, and lactate. These acids may serve as substrates that can be oxidized for further anaerobic respiration of which the SRB are capable.

SRB have been demonstrated in the rumen of cattle and many other animals including humans (Roediger *et al.,* 1997). In the human large gut, mucin, a sulfated polysaccharide serves as a source of sulfate for the bacteria. The release of this mucin is attributed to enzymes from *Bacteriodes*. The diet strongly influences the type of bacteria in the large intestine. When sulfate is copious, SRB are abundant, but when it is limited, methanogens are abundant.

In the gut, the SRB have a good supply of sulfurous compounds and are provided with adequate anaerobic conditions. Principally, the *Desulfovibrio* are the SRB that are able to outcompete methanogenic bacteria to dispose H_2 in the large bowel. The main metabolic product of colonic fermentation is hydrogen sulfide (H_2S) followed by carbon dioxide (CO_2) and methane (CH_4) gas. The H_2S is highly toxic. If it is not efficiently removed in the gut its accumulation may result in damage to colonic epithelial cells.

Ulcerative colitis (UC) is an acute and chronic inflammatory disease of the large bowel (Gibson *et al.*, 1988). Its cause is unknown, but it may be caused by an interaction between genetic factors, which may determine the immune response or the expression of enzymes that control intracellular metabolism, and environmental factors such as diet and the nature of bacterial flora (Pitcher *et al.,* 1996).

It has been hypothesized that UC may be caused by SRB (Roediger *et al.,* 1997). The combination of these factors results in the breakdown in the integrity of the colonic epithelial cell barrier and the perpetuation of inflammation. However, it is

unclear whether SRB have an initiating or perpetuating role in this disease. 50 % of healthy individuals harbor significant populations of SRB in feces compared to the 96 % of UC sufferers especially the *Desulfovibrio* genus (Vines *et al.,* 1998).

2.3.8 SRB in reactors for waste water treatment

The treatment of commercial effluent results in a system that encourages the growth of SRB. SRB have been used to treat a variety of industrial waste, including waste from distilleries (Postgate, 1984), brewery wastewater (Wu *et al.,* 1991), and bioprecipitation of toxic metals as sulfides (White and Gadd, 1996). This causes the resulting sludge to have a lower wastewater content.

The ability of *Thiobacillus denitrificans* to deodorize and detoxify water containing sulfides produced by an oil-field was evaluated under full-scale field conditions at Amoco Production Co. Salt Creek Field in Midwest, WY. More than 800 m^3/d of water containing 100 mg/l sulfide and total dissolved solids of 4800 mg/l, were successfully biotreated in an earthen pit (3000 m^3) over a six-month period (Sublette *et al.,* 1998). Complete removal of sulfides and elimination of associated odors were observed. The system could be upset by severe hydraulic disturbances; however, the system recovered rapidly when normal inflow rates were restored.

Granules from an up-flow anaerobic sludge blanket system treating brewery wastewater that contained mainly ethanol, propionate, and acetate as carbon sources and sulfate (0.6 to 1.0 mM) were characterized for their physical and chemical properties, metabolic performance on various substrates, and microbial composition (Wu *et al.,* 1991). Transmission electron microscopic examination showed that at least three types of microcolonies existed inside the granules. One type consisted of methanothrix-like rods with low levels of methanobacterium-like rods; two other

types appeared to be associations between syntrophic-like acetogens and methanobacterium-like organisms.

Selvaraj *et al.* (1997) investigated a column reactor with BIO-SEP polymeric beads, which were inoculated with a mixed SRB community. Using this approach they achieved high sulfite conversion rates, in the range of 16.5 mmol/liter/hour, with 100% conversion to 20 mmol/liter/hour, with 95% conversion. This resulted in an average specific activity for sulfite reduction in the column, in terms of dry weight of SRB biomass, of 9.5 mmol of sulfite/gram/hour. It was found that in addition to flue gas desulfurization, possible applications of the studied microbial process could be the treatment of sulfate/sulfite polluted wastewater from the pulp and paper, petroleum, mining, and chemical industries. Sulfur dioxide (SO_2) is one of the major pollutants in the atmosphere that cause acid rain. Microbial processes for reducing SO_2 to hydrogen sulfide (H_2S) have been demonstrated by utilizing mixed cultures of SRB with municipal sewage digest as the carbon and energy source. To maximize the productivity of the bioreactor for SO_2 reduction in this study, various immobilized cell bioreactors were investigated. A stirred tank with SRB flocs and columnar reactors with cells immobilized in either potassium-carrageenan gel matrix or polymeric porous BIO-SEP beads were used. The maximum volumetric productivity for SO_2 reduction in the continuous stirred-tank reactor (CSTR) with SRB flocs was 2.1 mmol of SO_2/litre/hour. The potassium-carrageenan gel matrix used for cell immobilization was not durable at feed sulfite concentrations greater than 2000 mg/l 1.7 mmol/liter/hour.

The biological sulfate removal process was also studied by Greben (2001). The results from this study showed that a single stage completely-mixed reactor system can be used successfully to reduce in a biological way the sulfate from acid mine

drainage (AMD). Sulfate and the produced sulfides were removed via sulfur (S^0) using ethanol as carbon source, treating acid mine water for a period of 95 days. Sulfate (SO_4) levels were lowered from 3000 mg/l to less than 200 mg/l, while the sulfide concentration was lower than 200 mg/l. The maximum sulfate (SO_4) reduction rate that was achieved was 8.4 g SO_4 /liter/day. They found that the experimental chemical oxygen demand (COD)/sulfate ratio was between 0.55 and 0.84, which was in accordance with their calculated theoretical value of 0.50 and 0.67. The study showed that the experimental sulfide/sulfate ratio was less than the calculated theoretical value of 0.33 due to the conversion of sulfate to sulfur and due to metal sulfide precipitation. It was also shown that it was possible to completely remove iron and copper, whereas the level of aluminum, manganese and zinc were lowered to less than 4 mg/l. Sulfate (SO_4) removal rates resulted in alkalinity production, causing the pH to increase from 2.9 to 7.9.

2.4 Bioreactors

A bioreactor is a system which supports and may contain an organism(s) during a desired process (Müller *et al.,* 1993). Bioreactors can be used to produce biomass, which in turn produce desired products. The bioreactors have defined and controllable environmental factors and are therefore an efficient way of exploiting capabilities of biological systems. They have proven to be useful in both research and industry since the internal environment is usually easily manipulated and monitored.

In order for a process to be cost effective and feasible certain criteria have to be met. The final goal needs to be reached in reasonable time frame and production cost should be as low as possible. Factors affecting the efficiency of a process and a

system therefore have to be defined and optimized. Factors may be temperature, nutrient availability, oxygen supply and demand, pH, mixing, agitation and mass transfer. In simple systems (like shake flasks) the control of dissolved oxygen (DO) has proven difficult, as the mass transfer is dependent on the surface to liquid ratio and the availability of gas in the flask. Attempts to increase the surface area of the media and increase the flow of air proved to increase the DO in flasks (Tunac, 1989). In more advanced bioreactors the DO is controlled by directly adding gases to the media. Supplementing the medium with gases, along with good mixing greatly increases the oxygen transfer rate and thereby also the DO in the medium. Factors affecting the biological system can be controlled by using cooling water jackets (temperature), acid/base addition (pH), feed lines (supplies nutrients), and baffles/agitators (mixing and shear stress). There are numerous bioreactors with varying configurations to meet different requirements. Individual reactors can be classified in four different ways: 1. Based on the combination of the mode of substrate addition and the reactor geometry (i.e. continuous tubular packed bed), 2. Based on the configuration of the biomass in the reactor (i.e. freely suspended or immobilized), 3. The mode providing the mixing within the reactor (i.e. mechanical agitation or gas agitation), and 4. Based on the type of biocatalyst or enzyme used in the reactor (i.e. aerobic microorganisms or anaerobic microorganisms), (Atkinson and Mavitina, 1991).

Alternative methods to the bioreactor being investigated to remove high sulfate concentrations can be physical or chemical. Both physical (reverse osmosis, electrodialysis and ion exchange) and chemical (precipitation with barium salts and lime and limestone precipitation) methods have been tested and applied (Greben, 2001).

Improved knowledge of microbial activity will assist in improved reactor design. The use of small (laboratory scale) reactors is preferred to evaluate parameters that will be difficult (financially not feasible) with full scale reactors.

2.5 Pitfalls in microbial community analysis

2.5.1 Cell lysis and extraction of DNA

A critical step in a PCR-mediated evaluation of environmental habitats is the lysis of microbial cells (Liesack *et al.*, 1991). Insufficient or preferential disruption of cells will cause some DNA or RNA, which is not released from the cells, to bias the view of the composition of microbial diversity. Unreleased DNA or RNA will not contribute to the final analysis of diversity. On the other hand rigorous conditions required for cell lysis of Gram-positive bacteria should be avoided as this treatment may lead to highly fragmented nucleic acids from Gram-negative cells. Fragmented nucleic acids are sources of artefacts in PCR amplification experiments and may contribute to the formation of chimeric PCR products. In addition, various biotic and abiotic components of environmental ecosystems, such as inorganic particles or organic matter, affect lysis efficiency and may interfere with subsequent DNA purification and enzyme steps.

2.5.1 PCR amplification

Full length 16S rDNA can be amplified directly with a set of primers binding conserved regions of the 16S rDNA (Lane *et al.*, 1985 and Weisburg *et al.*, 1991). Although PCR amplification has become the method of choice for obtaining rRNA sequence data from microbial communities or pure cultures, several problems arise

when the methods are applied to environmental communities: (i) inhibition of PCR amplification by co-extracted contaminants, (ii) differential amplification, (iii) formation of artefactual PCR products. One has also to consider that (iv) contaminating DNA would unavoidably lead to a biased reflection of the microbial community (Stackebrandt and Liesack, 1993).

2.5.2 Formation of chimeric molecules

In vitro recombination of homologous DNA leading to chimeric molecules composed of parts of two different sequences has been widely observed (Shuldiner *et al.*, 1991) and is not restricted to 16 S rDNA amplification from complex microbiota. Chimers between two different DNA molecules with high sequence similarity (i.e. homologous genes) can be generated during the PCR process as DNA strands compete with specific primers during the annealing step.

In addition to incomplete strand synthesis during the PCR process, DNA damage has been suggested to promote the formation of chimeric molecules in PCR co-amplification of templates with high sequence similarities. All types of DNA damage were shown to support production of recombinant PCR products. Since rigorous cell lysis conditions for DNA preparation from environmental samples are likely to cause damages (Pääbo, 1990). These findings could have significant impact on 16 S rDNA amplification from complex microbiota.

3. MATERIALS AND METHODS

3.1 Reactor design and operational parameters

3.1.1 Bioreactor

The bioreactors were 100 ml borasilicate glass bottles, which were modified to have a medium inlet and effluent outlet (Figure 3). These bioreactors were filled with glass dices and microscope slides to increase the surface to volume ratio and for later microscopic biofilm analysis. The bioreactors were single stage, packed upflow bioreactors, and could hold 30 ml of medium.

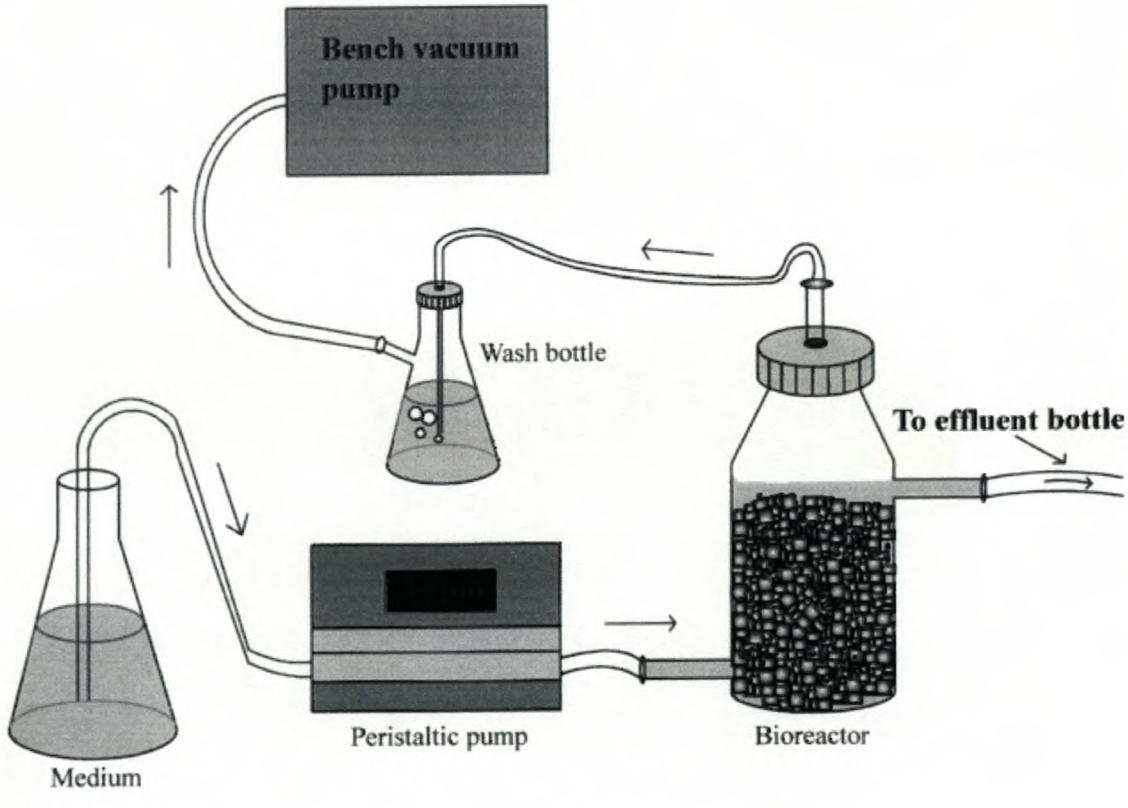

Figure 3. Diagrammatic presentation of bioreactor constellation.

Postgate Medium C (for Sulfate Reducing bacteria), (Atlas, 1995) was pumped continuously from the reservoir with a Watson Marlow peristaltic pump (model 205

S) at a constant rate of 5 ml per hour to the inlet of each bioreactor resulting in a hydraulic retention time of 6 hours. The lid from each reactor had an outlet for gas release. The evacuated gas was washed in a washing bottle filled with deionised water and a mixture of ironous sulfate and ferric chloride to reduce the hydrogen sulfide gas to elemental sulfur. The reduction process was evident from a yellow precipitate (sulfur) that was formed in the glass bottle. As a safety precaution, the effluent from each bioreactor was collected in a ten liter waste bottle, from which the gas was stripped of H_2S as described for the bioreactor gas. Samples were submitted to CSIR, Pretoria for SO_4/S^{2-} analysis.

The silicon tubes, as well as the medium reservoirs, were changed regularly to prevent back-growth and subsequent contamination of the growth medium.

3.1.2 Growth conditions

Each bioreactor was inoculated with 20 ml from an existing SRB reactor. Modified Postgate Medium C was used to cultivate a SRB community. The content of the medium was: sodium lactate (Lacolin) ($C_3H_5NaO_3$), 1.5 g/l as carbon source; sodium sulfate anhydrous (Na_2SO_4), 2 g/l; ammonium chloride granular ($NH_4C\lambda$), 0.075 g/l; yeast extract, 0.05 g/l; potassium dihydrogenorthophosphate (KH_2PO_4) 0.015 g/l; calciumchloride-2-hydrate crystalline ($CaCl \cdot 2\ H_2O$), 0.06 g/l; magnesium sulfate heptahydrate ($MgSO_4 \cdot 7\ H_2O$), 0.06 g/l; iron(-ous) sulfate ($FeSO_4$), 0.004 g/l, pH 7.5.

3.1.2.1 Effect of carbon source on community stability

The reactor feed was changed after 15 weeks from Postgate Medium C with sodium lactate as carbon source, to either ethanol or sucrose as carbon source. In each case, the alternative carbon source was added to provide the same amount of carbon: 3.11 g/l sodium lactate, 2.38 g/l sucrose and 1.92 g/l ethanol. The reactors were then fed with these carbon sources for 10 days, after which feeding of Postgate Medium C with sodium lactate as carbon source was reintroduced. These treatments were performed in duplicate. Samples from the bioreactors were collected on days 0, 1, 3, 5, 7, 10 and 20 (10 days after shift back to sodium lactate as carbon source).

The samples were then subjected to the following analysis: terminal restriction fragment length polymorphism (T-RFLP), fluorescent *in situ* hybridization (FISH), BIOLOG Ecoplates™ and plate counts (total anaerobic and total aerobic).

3.2 Community diversity analysis

3.2.1 Growth media to detect presence of functional groups

The sole carbon source was changed from sodium lactate to ethanol, and sucrose respectively.

C: control reactors maintained with sodium lactate over the total time period.

E: reactors maintained on sodium lactate for 15 weeks, then changed to ethanol for 10 days, then switched back to sodium lactate.

S: reactors maintained on sodium lactate for 15 weeks, then changed to sucrose for 10 days, then switched back to sodium lactate.

3.2.1.1 Confirmation of the presence of Sulfate Reducing Bacteria

One 1 ml sample was collected from the control bioreactor and a triplicate dilution series was made in physiological saline solution. The dilutions were plated out on Postgate medium C for sulfate reducing bacteria with 2 % agar and were incubated anaerobically for two months at room temperature in a anaerobic cabinet (Forma Scientific, Centrotec, Cape Town, South Africa) with the following gas mixture: 9.5% H_2, 9.6% CO_2, balance N_2.

3.2.1.2 Total aerobic and anaerobic microbial cell counts

From each bioreactor 1 ml was collected at each of the time points (day 0, 1, 3, 5, 7, 10 and 20) and used to make dilution series (10^{-3} to 10^{-8}) in triplicate under aerobic and anaerobic conditions (anaerobic cabinet). Dilutions were plated out on 10 % tryptone soy agar (TSA) using the spread plate technique. Plates were incubated for 3 days at room temperature. Colony forming units per ml (CFU's / ml) were determined from plates containing between 25 and 250 colonies.

3.2.1.3 Enumeration of fungi

Medium for the isolation of Lipomycetous yeast and fungi. 2% malt extract agar (MEA) plates containing Streptomycin (0.5 g/l) were used to select Lipomycetous and some Basidiomycetous fungi and yeast. Plates were incubated aerobically for 3 days at room temperature.

Glass dices were collected from the bioreactors with a sterile pair of forceps, rinsed with sterile distilled water and streaked over the surface of the plates.

3.2.1.4 Medium for the isolation of Lipomycetous and Basidomycetous yeasts

The composition of the medium was as follows: glucose (5 g/l), KH_2PO_4 (1 g/l), $MgSO_4$ (0.5 g/l), NaCl (0.1 g/l), $CaCl_2$ (0.1 g/l), thymine (nitrogen source) (0.1 g/l), chloramphenicol, (0.2 g/l), trace element solution (0.1 %), vitamin solution (0.5 %), cyclohexamide (200 µg/l), agar (10 g/l), pH set to 5.2.

Composition of the trace element solution: H_3BO_3 (0.5 g/l), $CuSO_4 \cdot 5\ H_2O$ (0.04 g/l), KI (0.1 g/l), $FeC\lambda_3 \cdot 6\ H_2O$ (0.2 g/l), $MnSO_4 \cdot H_2O$ (0.4 g/l), $Na_2MoO_4 \cdot H_2O$ (0.2 g/l), Na_2MoO_4 (0.16 g/l), $ZnSO_4 \cdot 7\ H_2O$ (0.4 g/l), NaCl (1g/l), $CoSO_4$ (0.1 g/l), $CoSO_4 \cdot 7H_2O$ (0.18 g/l); $CaCl_2$ (0.1 g/l), $AlK(SO_4)_2 \cdot 12\ H_2O$ (0.01 g/l).

Composition of the vitamin solution: biotine (0.2 mg/l), calcium pentothenate (40 mg/l), folic acid (0.2 mg/l), inositol (200 mg/l), *p*-aminobenzoic acid (20 mg/l), pyridoxine hydrochloride (40 mg/l), riboflavin (20 mg/l), thiamine (100 mg/l).

The same approach as described in 3.2.1.3 was followed to inoculate the plates.

3.2.2 Microscopy

Microscopy was performed with a Nikon Eclipse E 400 epifluorescent microscope, with filters for DAPI, rhodamine and FITC labeled probes, with a 60 x oil immersion objective and pictures were taken with a COHU high performance CCD camera (model no. 4912-5010/0000).

A viability test for fungi and yeasts was performed by using a Live/Dead *Bac*Light Viability Kit (Catalogue no. 7009 Molecular Probes Inc., Eugene, Oregon, USA). The kit provided two nucleic acid stains: Calcofluor ™ White M2R (fungal cell wall stain) and FUN-1(viable fungal cell stain) that distinguishes live cells from non-viable cells. Only metabolically active cells are marked with fluorescent

intravacuolar structures, whereas dead cells are bright, diffuse, green-yellow fluorescent.

Four µl of each of the two components of the *Bac*Light Fluorescent probe were mixed with 1 ml dH_2O to form a stock solution (40 µM). In a typical staining procedure, 50 µl from the stock solution was pipetted on the samples, and left for 10 minutes at room temperature, after which unbound probe was washed off with dH_20.

3.2.3 Fluorescent *in situ* hybridisation (FISH)

Microscope slides were carefully removed from bioreactors with a sterilized pair of forceps at each of the time points (day 0, 1, 3, 5, 7, 10 and 20) and gently rinsed with physiological salt solution. Slides were incubated in a solution of 5 g/l glucose and 5 g/l yeast extract for 2 hours at 30 °C to increase the ribosomal content in the cells. The biofilms present on microscope slides were fixed with 4 % (w/v) paraformaldehyde solution for 1 hour at room temperature. After fixation, slides were carefully washed with phosphate-buffered saline, (130 mM sodium chloride, 10 mM sodium phosphate, pH 7.2). The biofilms were then dehydrated through an ethanol series of 50%, 80%, 96% (v/v) for 3 minutes each. Lysozyme (1mg/ml in 100 mM Tris-HCl, pH 7.5, 5 mM EDTA) was added after fixation for permeabilizing of the outer membrane of the cell at room temperature for 15 minutes. In order to halt enzymatic activity, the slides were washed carefully with phosphate-buffered saline solution. Nine µl hybridization buffer (25 % (v/v) formamide, 0.9 M NaCl, 0.01 % (w/v) sodium dodecyl sulfate (SDS), 5 mM EDTA, 20 mM Tris-HCl, pH 7.2) was applied to each of the microscopic slides. Three oligonucleotide probes were used that were obtained from Invitrogen (Pty, Ltd). Probe Eub338 (5' Rhod-GCT GCC TCC CGT AGG AGT-3') designed to bind to

most known bacteria was conjugated to rhodamine. The next oligonucleotide probe, SRB385 (5' Fluoro (FITC)-CGG CGT CGC TGC GTC AGG-3') was designed to bind to SRB and was conjugated to FITC, while the third probe (5' Fluoro (FITC)-TCT GGA CCT GGT TGA GTT TCC-3') was also conjugated to FITC, designed for yeasts. One µl of each probe (50 ng for Eub 338, 1000 ng for SRB385 and 50 ng for yeasts) was added to the area where the hybridization buffer was applied before and incubated in a moist chamber for 3 h at 46 °C. Excess hybridization buffer and unhybridized probe was gently removed through a washing step for 30 minutes at 46°C with prewarmed washing buffer (112 mM NaCl, 20 mM Tris HCl pH 7.2, 0.01 % (w/v) SDS, 5 mM EDTA). Slides were rinsed with distilled water and dried at room temperature in the dark in order to minimize photobleaching. Microscopy was performed directly after drying the slides.

3.2.4 Terminal Restriction Fragment Length Polymorphism's (T-RFLP)

3.2.4.1 Genomic DNA extraction (Zhou *et al.*, 1996)

One ml samples were collected from the plantonic phase of each of the reactors on each of the sampling dates (days 0, 1, 3, 5, 7, 10 and 20) centrifuged at 4500 x g for 5 minutes, then resuspended and mixed with 1 ml DNA extraction buffer (100 mM Tris-HCl pH 8, 100 mM EDTA pH 8, 100 mM sodium phosphate pH 8, 1.5 M NaCl , 1% (w/v) CTAB). Samples were frozen at -70°C and thawed at 60°C for three successive times. After the freeze-thaw process, 5 µl proteinase K (20 mg/ml) was added and the samples were incubated at 37°C for 30 min. with shaking (225rpm). After this step, 150 µl 20% (w/v) SDS was added and incubated at 65°C in a water bath for 2 hours with gentle end over end inversions every 15 minutes.

Samples were then centrifuged at 6800 x g for 10 minutes. The supernatants were transferred to 2 ml tubes and mixed with equal volume of chloroform isoamylalcohol (24:1 vol/vol). Nucleic acids were precipitated from the aqueous phase by adding 0.6 volume isopropanol. After incubation for 1 hour at room temperature, a crude nucleic acid preparation was obtained by centrifugation at 9800 x g for 20 minutes at room temperature. The pellet was washed with 70% (v/v) ethanol, dried and dissolved in 200 µl dH_2O. Five µl DNA from each of the 38 samples was electrophoresed on 0.8% (w/v) agarose gels containing 40 µg ethidium bromide after addition of loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 40% (w/v) sucrose). DNA was electrophoresed for 1h at 75 V. The concentration of the DNA was estimated by comparison to standard markers.

3.2.4.2 T-RFLP PCR

Isolated colonies that appeared black (and therefore assumed to be SRB due to H_2S production and the characteristic coloration on the appropriate growth medium) were chosen from Postgate C Agar to perform colony PCR and subsequent restriction enzyme digestion and T-RFLP analysis. For each of the samples (those taken from the bioreactors at sampling dates (days 0, 1, 3, 5, 7, 10 and 20) and the samples from colonies that appeared black: SRB1, SRB2, SRB3) the following agents were added in the same order as mentioned (4°C):

Five µl 10x Amplification buffer, (500 mM KCl, 100 mM Tris-Cl pH 8.3, 15 mM $MgCl_2$), 0.5 µl 20 mM solution of dNTPs (Roche, Diagnostics, South Africa, PTY, LTD, 9 Will Scarlet Road, P.O. Box 1927 Randburg 2125), 2.5 µl of a 20 µM solution of primer 1389R (5'-ACG GGC GGT GTG TAC AAG-3', conjugated to HEX; (Invitrogen), 2.5 µl of a 20 µM solution of primer 63F (5'-CTG AAC GTA

CAC AAT CCG GAC-3', conjugated to FAM; (Invitrogen, Pty, Ltd), 33.75 µl dH_2O, 5 µl template DNA and 0.75 µl Taq polymerase (5 U/µl; Promega, 2800 Woods Hollow Road, Madison WI 53711-5399).

For PCR of the colonies that appeared black, a sterile toothpick was used to pick each colony up and to resuspend it in the PCR mixture. These cells served as the template DNA.

The concentration of the genomic DNA in the different samples were estimated from a 0.8% (w/v) agarose gel in comparison with a λ-marker with a known concentration. Samples that had a high DNA concentration, in comparison to each other, were diluted 10 times. These dilutions served as the template DNA. In samples where the DNA concentration was lower, undiluted DNA served as the template.

PCR was carried out in a Perkin-Elmer Gene Amp 2400 thermocycler. The machine was programmed to have an initial denaturation period of 4 minutes at 94°C, followed by 30 cycles of amplification (30 seconds at 94°C, 30 seconds at 55°C, 30 seconds at 72°C). Finally, an extension step of 4 minutes at 72°C was performed. Optimized reaction conditions that were used are given in Table 1.

Table 1. Summary of the reaction conditions for T-RFLP PCR.

Mg^{2+}	KCl	dNTPs	Primers	DNA polymerase	Template DNA
1.5 mM	50 mM	200 µM	1 µM	2.25 - 3.75 units	1 - 50 ng

5 µl from each PCR product was electrophoresed on 0.8 % (w/v) agarose gels.

Restriction Enzyme digestion

Sixteen µl DNA from each PCR product was digested in a 20 µl reaction with 2 µl *Msp*I restriction enzyme (10 U/µl; Promega, 2800 Woods Hollow Road Madison, WI 53711-5399) and 2 µl buffer M (100 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$, 10 mM Dithiothreitol, 500 mM NaCl) for 90 minutes in a 37°C water bath. Eight µl from each restriction enzyme digestion was loaded on 0.8 % (w/v) agarose gel. DNA was electrophoresed for 1h at 75 V. The concentration of the DNA was estimated by comparison to standard markers.

T-RFLP analysis

The fluorescent products as obtained from PCR (as described in section 3.2.4.2) were digested with the restriction enzyme, *Msp*I (Amersham Pharmacia Biotech Inc.), and analyzed by capillary electrophoresis via laser-induced fluorescence on an ABI PRISM 3100 Genetic Analyzer, which separated the restriction fragments solely by size. The abundance and length of the fluorescent terminal fragments labeled by the fluorescent dye, FAM, were determined. This yielded specific patterns or "community fingerprints" of the samples from the bioreactors and the isolates obtained from Postgate C medium.

3.2.5 BIOLOG Ecoplate™ analysis

Biolog EcoPlates (BIOLOG Inc., Hayward, CA, USA) were used in triplicate (one plate per sample) to obtain whole-community metabolic profiles of the reactors. These plates contain 31 carbon sources that have been demonstrated to be useful for community analysis (Table 3.2). Each series contains a control without carbon source. Each well contains colourless tetrazolium, which is reduced to purple formazan by NAD from bacterial oxidation of organic matter.

From each reactor, 1 ml planktonic sample was collected at each of the sampling dates (day 0, 1, 3, 5, 7, 10 and 20) and used for the determination of metabolic fingerprints under aerobic and anaerobic conditions. These planktonic samples were diluted one to ten with dH_2O and mixed carefully by pipetting. Biolog Ecoplates were inoculated with 150 µl in each well and incubated at room temperature for 24 hours, aerobically and anaerobically (anaerobic cabinet). Carbon source utilization caused a color reaction in the wells, which was from clear to purple. STATISTICA 5 (StatSoft Ltd, 21-23 Mill Street, Bedford, MK 40 3 EU) was used for cluster analysis of the metabolic fingerprints.

Table 2. Carbon sources of Biolog Ecoplates

WATER	β-METHYL-D-GLUCOSIDE	D-GALACTONIC ACID γ-LACTONE	L-ARGININE
PYRUVIC ACID METHYL ESTER	D-XYLOSE	D-GALACTURONIC ACID	L-ASPARAGINE
TWEEN 40	I-ERYTHRITOL	2-HYDROXY BENZOIC ACID	L-PHENYLALANINE
TWEEN 80	D-MANNITOL	4-HYDROXY BENZOIC ACID	L-SERINE
α-CYCLODEXTRIN	N-ACETYL-D-GLUCOSAMINE	γ-HYDROXYBUTYRIC ACID	L-THREONINE
GLYCOGEN	D-GLUCOSAMINIC ACID	ITACONIC ACID	GLYCYL-L-GLUTAMIC ACID
D-CELLOBIOSE	GLUCOSE-1-PHOSPHATE	α-KETOBUTYRIC ACID	PHENYLETHYLAMINE
α-D-LACTOSE	D,L-α-GLYCEROL PHOSPHATE	D-MALIC ACID	PUTRESCINE

4. RESULTS AND DISCUSSION

4.1 Reactor design and operational parameters

Sulfate-reducing conditions developed in the reactors after a relatively short period (12 days). This was evident by the black precipitation that formed (Figure 4). Although not an objective of the study, the SO_4^{2-} and S^{2-} levels were evaluated by CSIR in Pretoria. The results indicated that there was a reduction of $SO4^{2-}$ in the influent from 1652 to 696 mg/l and an increase in S^{2-} from 4 to 52 mg/l due to sulfate reduction that took place in the bioreactors.

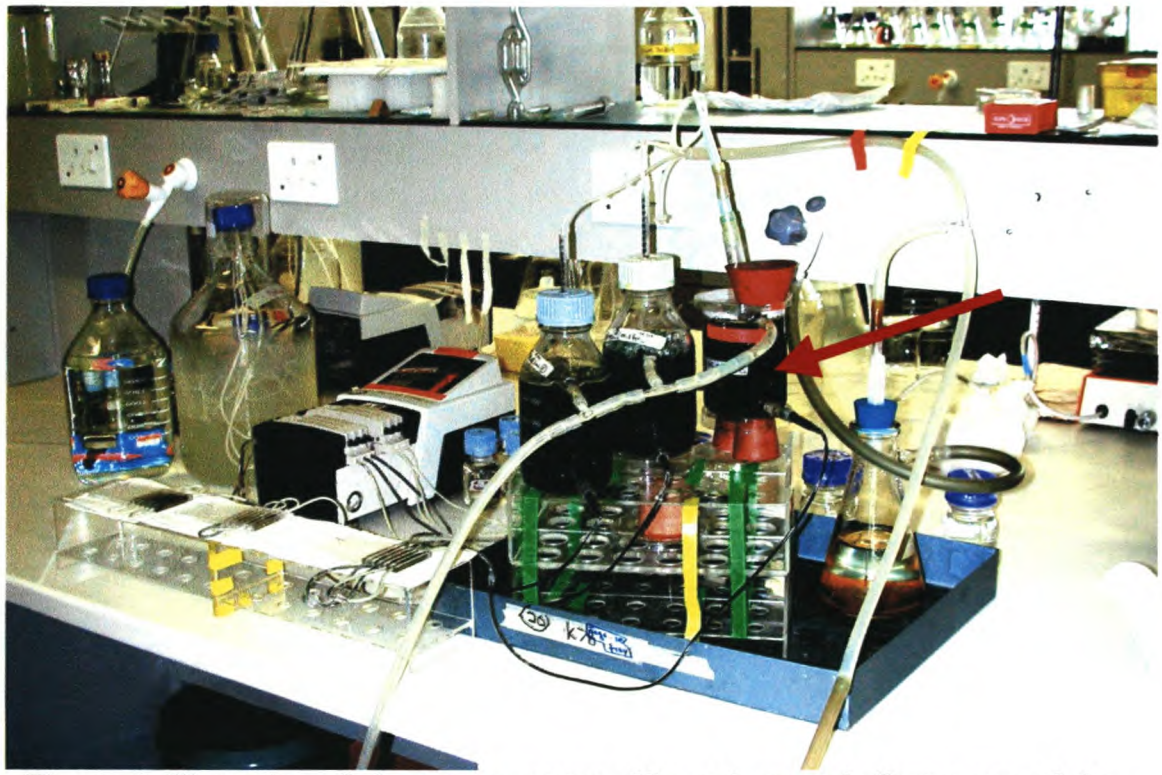

Figure 4. Illustration of the reactor system. The red arrow indicates one of the bioreactors with evident black precipitation that was formed.

4.2 Community diversity analysis

4.2.1 Growth media to detect presence of functional groups

The following discussion refers to cultivation with three nutrient conditions, namely sodium lactate, sucrose and ethanol, were evaluated. Sodium lactate served as the control (C). There were two bioreactors that received sucrose (S1 and S2) and two bioreactors that received ethanol (E1 and E2) as carbon source.

4.2.1.1 Isolation of Sulfate Reducing Bacteria

Micro-aerophilic microorganisms, not reducing SO_4 (colonies that were not black), were dominant on the plates. Colonies that appeared black were assumed to be SRB due to H_2S production and the characteristic coloration on the growth medium (Postgate C agar). Relatively low cell numbers were observed for the SRB counts. In average there were less than 2 % of SRB per plate of evident colonies.

4.2.1.2 Total aerobic and anaerobic heterotrophic counts

One day before the shift to ethanol or sucrose as carbon sources (day 0), aerobic cell numbers, as determined with plate counts on 10 % TSA, ranged between 4 $x10^8$ and 7 $x10^8$ CFU/ml (Figure 5 A). On day three after the change of carbon source, the cell numbers for E1, E2, S1 and C increased to about 1 $x10^9$ CFU/ml. On day ten the cell numbers from C, E1, E2 and S1 decreased to less than 5 $x10^8$ CFU/ml. The cell numbers for S2 remained at 7 $x10^8$ CFU/ml. On day twenty (ten days after the shift back to the original carbon source) cell numbers from all samples increased and varied between 5 and 10 $x10^8$ CFU/ml. Although the changes in cell numbers throughout the series were not significant, it might indicate that although the

communities in the bioreactors appeared to be at a steady state, they were indeed still constantly evolving.

The numbers of anaerobic cells, as determined through plate counts on 10 % TSA, remained relatively constant in all reactors for the duration of the experiment. They varied around 2 $x10^8$ CFU/ml (Figure 5 B). Exceptions were on day three, where the cell numbers for all reactors (except C) increased to about 2.2 $x10^8$ CFU/ml. The cell counts peaked on day three after the change in carbon source with their maximum at 2.8 $x10^8$ CFU/ml, decreased to about 2.5 $x10^8$ CFU/ml on day five and increased on day seven again slightly to about 2 $x10^8$ CFU/ml. Once again, the changes in cells numbers through the series were not significant.

Plate counts were frequently complicated by relative few organisms that spread across the agar surface, these plates were not used.

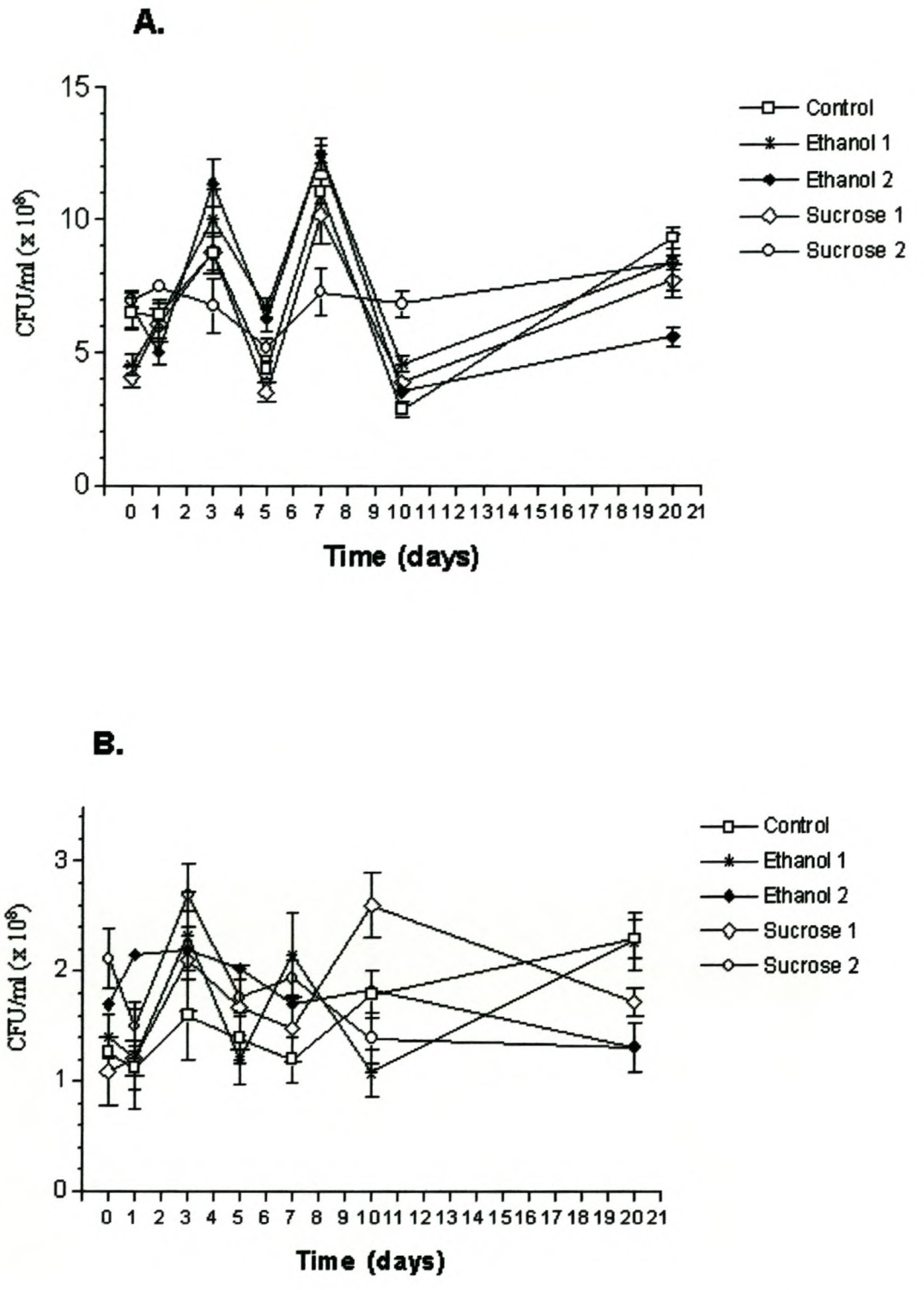

Figure 5. Total heterotrophic plate counts as determined through aerobically (A) and anaerobically (B) incubated 10 % TSA plates. Symbols refer to the average of triplicates.

4.2.1.3 2% MEA plates with Streptomycin for fungi and yeast

Biofilm from glass dices that were incubated in the bioreactors through the series of carbon shifts, were used to streak on 2% MEA plates with Streptomycin to determine the presence of fungi and yeast. Figure 6 shows representative plates that contained fungi and yeast. These yeast and fungi were identified by microscopy and morphological characteristics as *Rhodotorula* (pink yeast), black yeast, *Fusarium* (white/pink filamentous fungi), *Aspergillus* (yellow) and *Rhizopus* (blue-green).

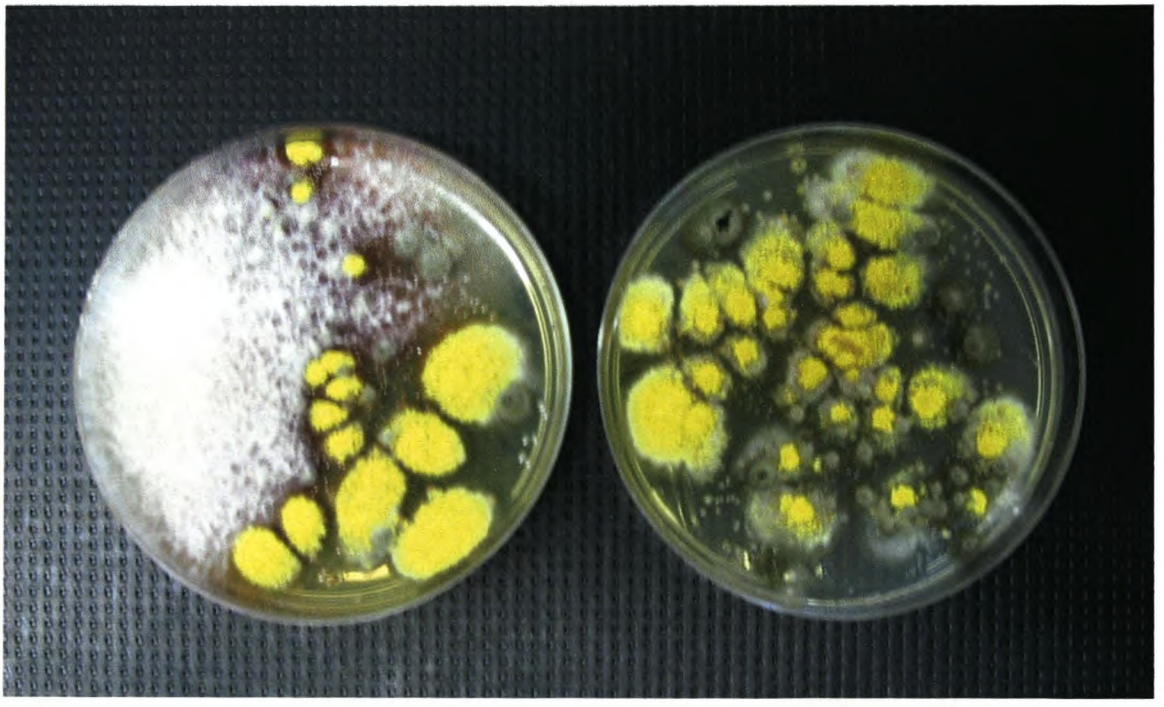

Figure 6. Plates containing fungal and yeast growth.

4.2.1.4 Lipomycetous and Basidomycetous yeast

Similar to Figure 6, glass dices from the bioreactors were streaked out on plates that specifically allowed growth of lipomycetous and basidomycetous yeasts. Plates contained mostly colonies of *Aspergillus* and black yeast as seen in Figure 7. This showed that yeast was present in the biofilm that was culturable.

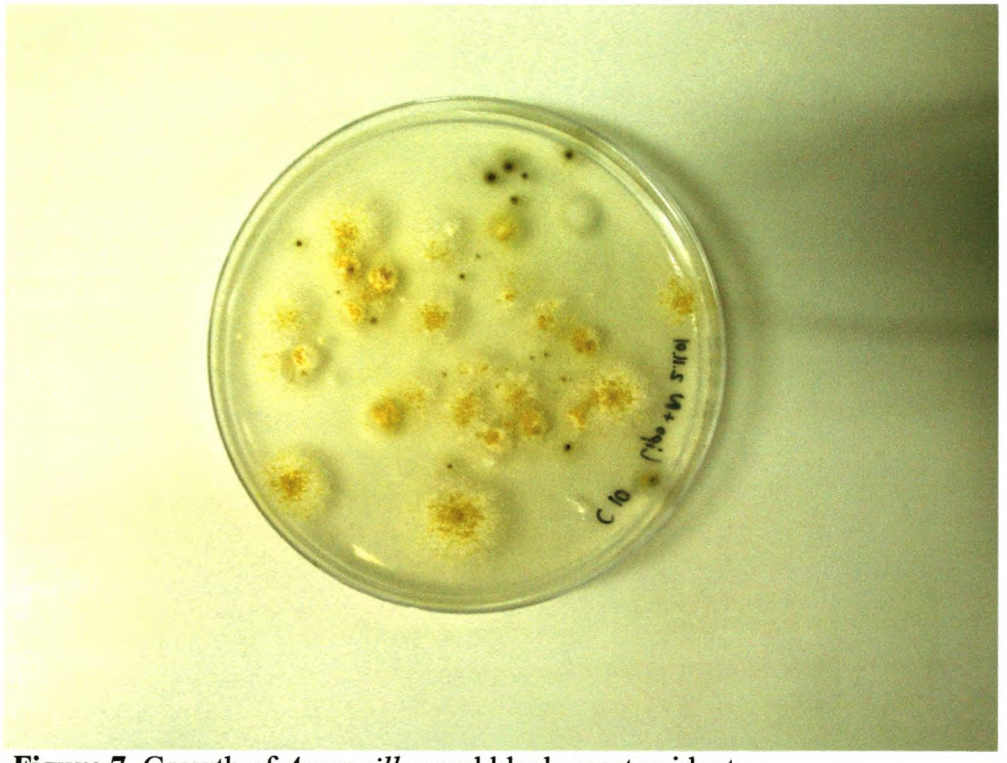

Figure 7. Growth of *Aspergillus* and black yeast evident.

4.2.2 Microscopy

*Bac*Light for filamentous and unicellular fungi

During routine microscopic analysis of the communities from the different reactors, cells that resembled a typical yeast morphology were observed. Therefore, further experiments were carried out to verify that these cells were indeed of yeast taxa. The *Bac*Light probe for filamentous and unicellular fungi was used. This probe discriminates between live and dead fungal cells. Only metabolically active cells are marked with fluorescent intravacuolar structures. Whereas dead cells are bright, diffuse, green-yellow fluorescent. Images from these fungi and yeast are shown in Figures 8 A to 8 E. These images aim to verify the presence of yeast and fungi present in the bioreactors where they formed part of a biofilm with bacteria grown under anaerobic conditions. These microorganisms represented different life stages. Yeast cells were growing actively in the biofilm as can be seen in Figures 8 A and 8 E where a typical basidomycetous and another unknown yeast respectively were captured in the process of budding. These results indicate that the conditions present in the bioreactors were favorable for the growth of fungi and the formation of aleurospores (Figure 8 D).

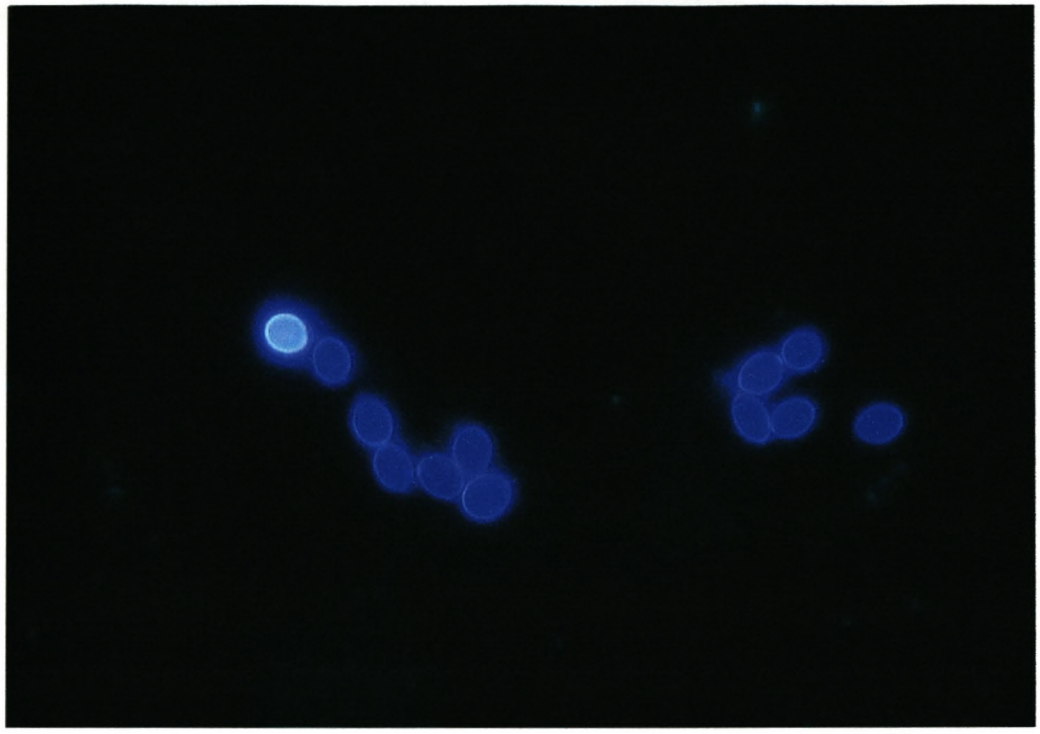

Figure 8 A. Bipolar budding typical of many basidomycetous yeast taxa.

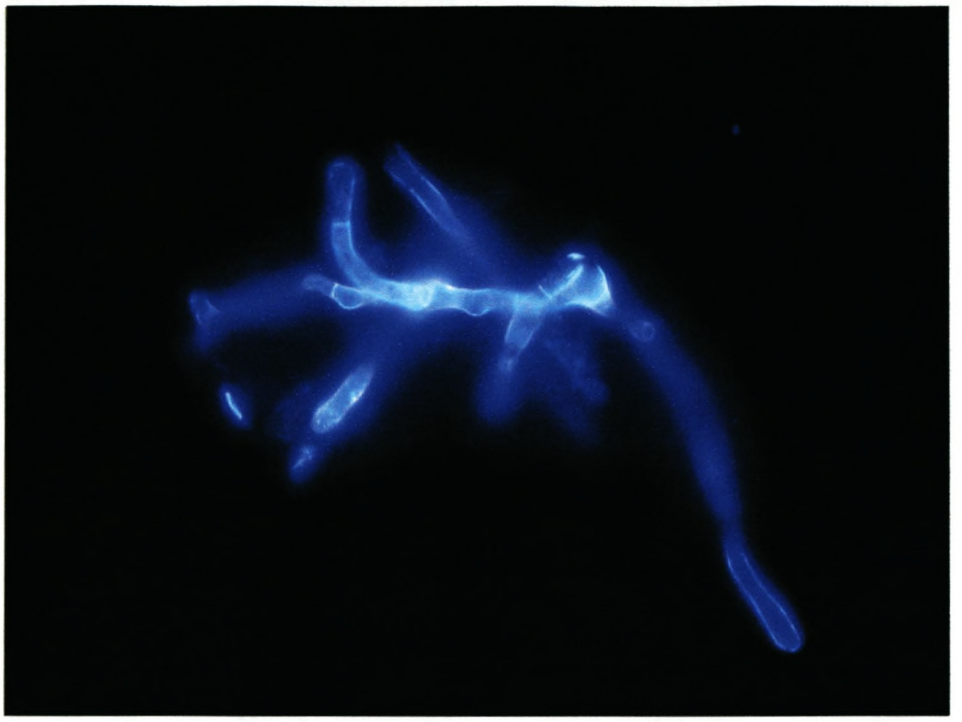

Figure 8 B. Fungal hyphae.

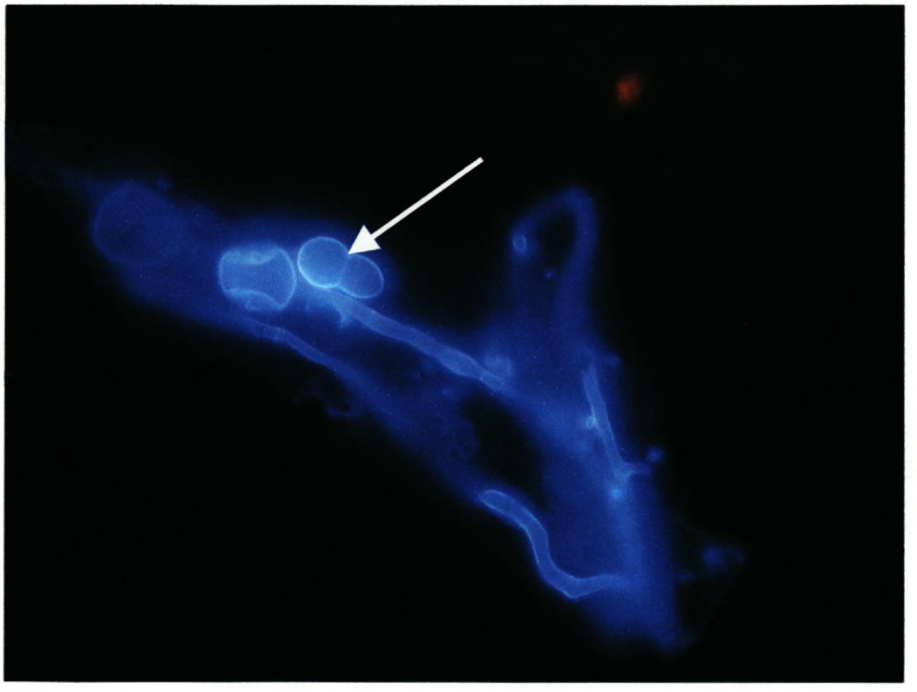

Figure 8 C. Fungal hyphae with aleurospores including a budding yeast on a broad base (arrow).

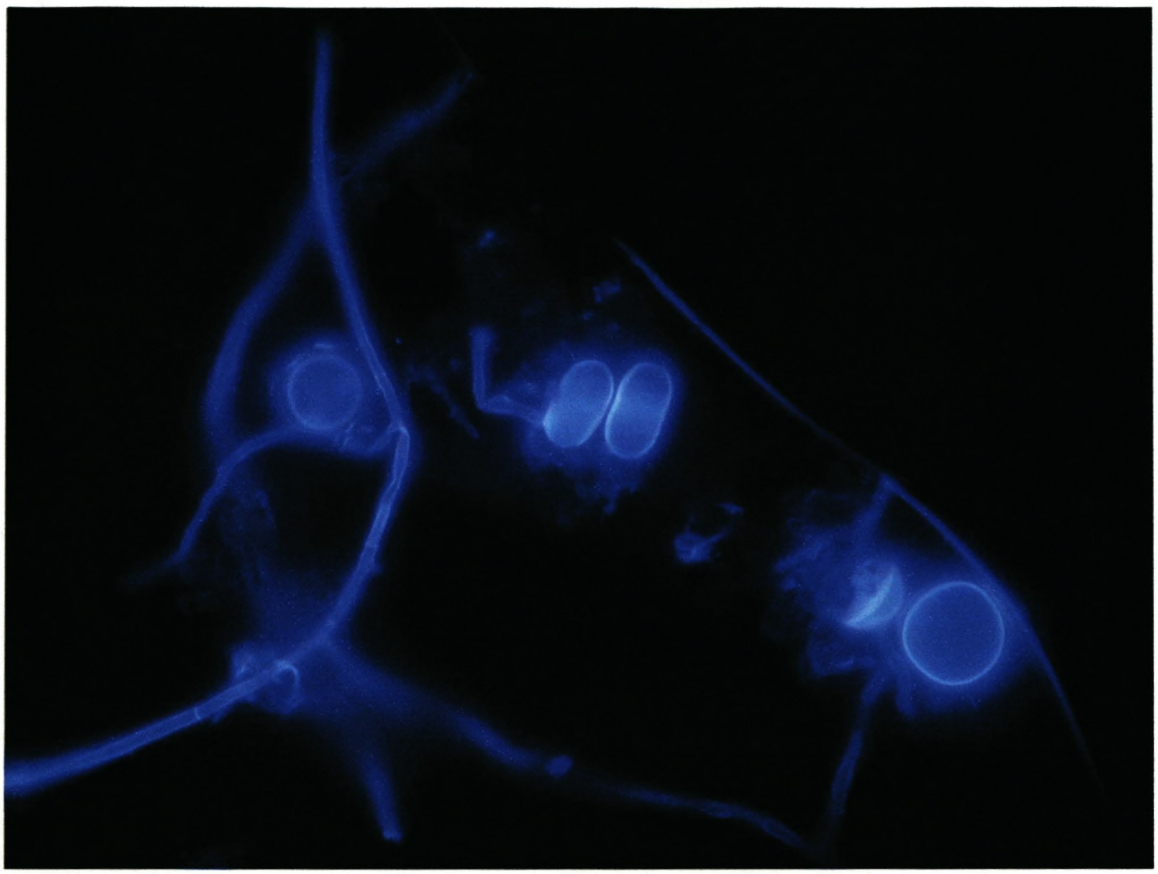

Figure 8 D. Aleurospores and other spore-like structures.

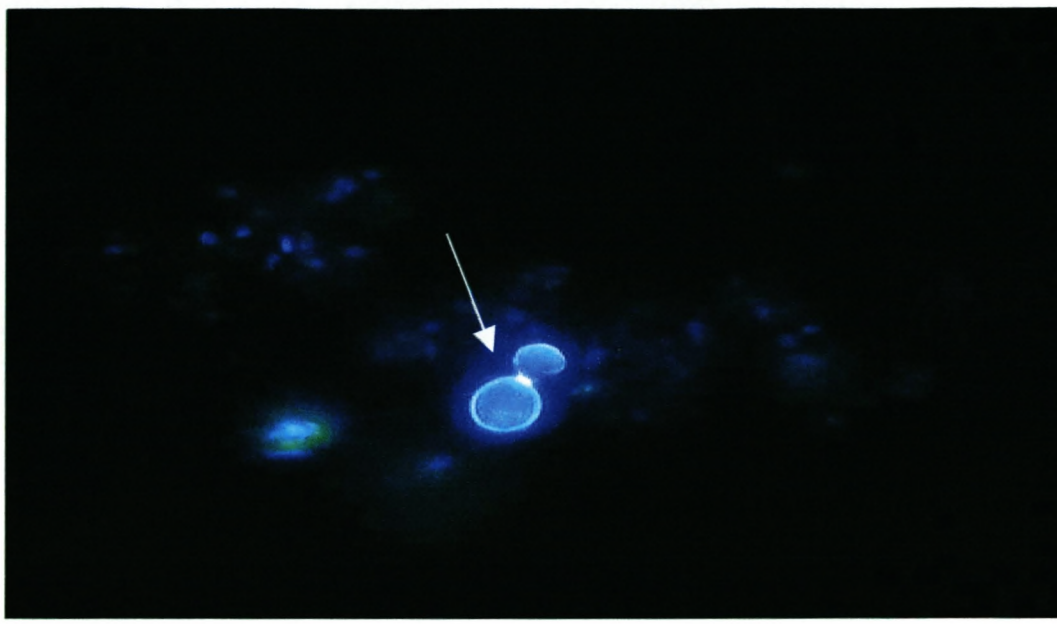

Figure 8 E. Budding yeast cell (arrow).

4.2.3 Flourescent *in situ* hybridization (FISH)

In situ hybridization was a technique in which single-stranded DNA molecules were permitted to form hybrids with molecules that had sufficiently similar, complementary sequences. Oligonucleotide probes with a fluorescent dye was hybridized to the cells in biofilms. Oligonucleotides (18 nucleotides) were preferred to polynucleotides (~ 50 nucleotides) as they allowed for single mismatch discrimination of target nucleic acids (Amann *et al.,* 1998).

16 S rRNA *in situ* hybridization detected actively growing microorganisms with sufficient content of target molecules (i.e. rRNA). In contrast to the analysis of the T-RFLP electropherogram, fluorescent *in situ* hybridization (FISH) provided a powerful tool for quantitative analysis as single cells could be specifically detected and counted under the microscope.

4.2.3.1 Detection of SRB in biofilm

Figures 9 (A and B) show typical results obtained with the SRB specific probe (green cells) and with the Eubacterial probe (red cells). Figure 10 also shows a typical result obtained with the fungi specific probe (green cells) and Eubacterial cells (yellow/red). In Figure 10 yeast cells (green) were attached to a bacterial colony (yellow/red). The conditions in the bioreactor biofilm were favourable so that these yeast cells were budding.

It is likely that unicellular and filamentous fungi and bacteria cooperated with each other in the biofilm to sustain life in anaerobic conditions where nutrient levels were relatively low.

Figure 9 A and B verified that SRB (green cells) were also part of the biofilm in the bioreactors. The relative amount of SRB as compared to other bacteria present in the biofilm were different in A and B. The relative amount of SRB was higher in B than in A. Some regions of the biofilm had a higher concentration of SRB. This may imply that all the microorganisms present in the biofilm were not spread evenly through the biofilm and that different micro niches may have been formed. In Figure 9 B, where the proportion of SRB was higher, the amount of other cells present was relatively low. Large areas were left where no bacterial growth was present. This may also indicate that SRB grew more abundantly when not surrounded by relative high numbers of bacteria.

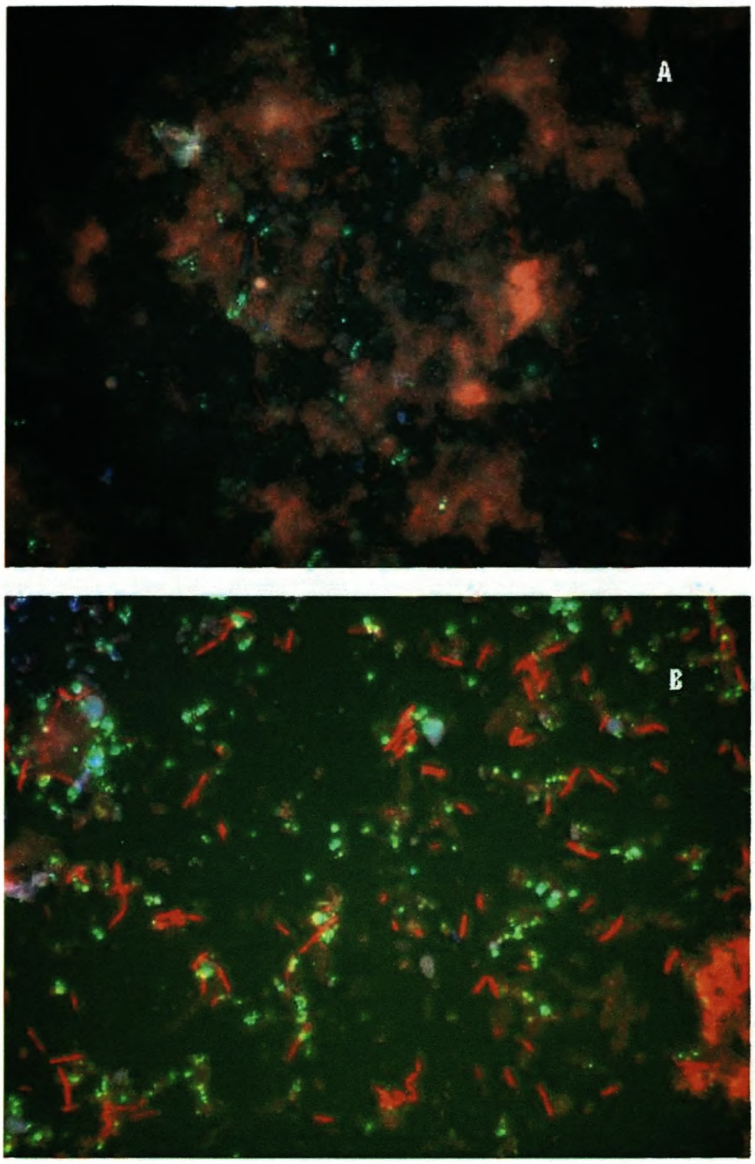

Figure 9 A and B. Sulfate reducing bacteria (green cells) incorporated in a heterogeneous biofilm community coexisting among bacterial populations (red cells), and yeast/fungi (blue cells).

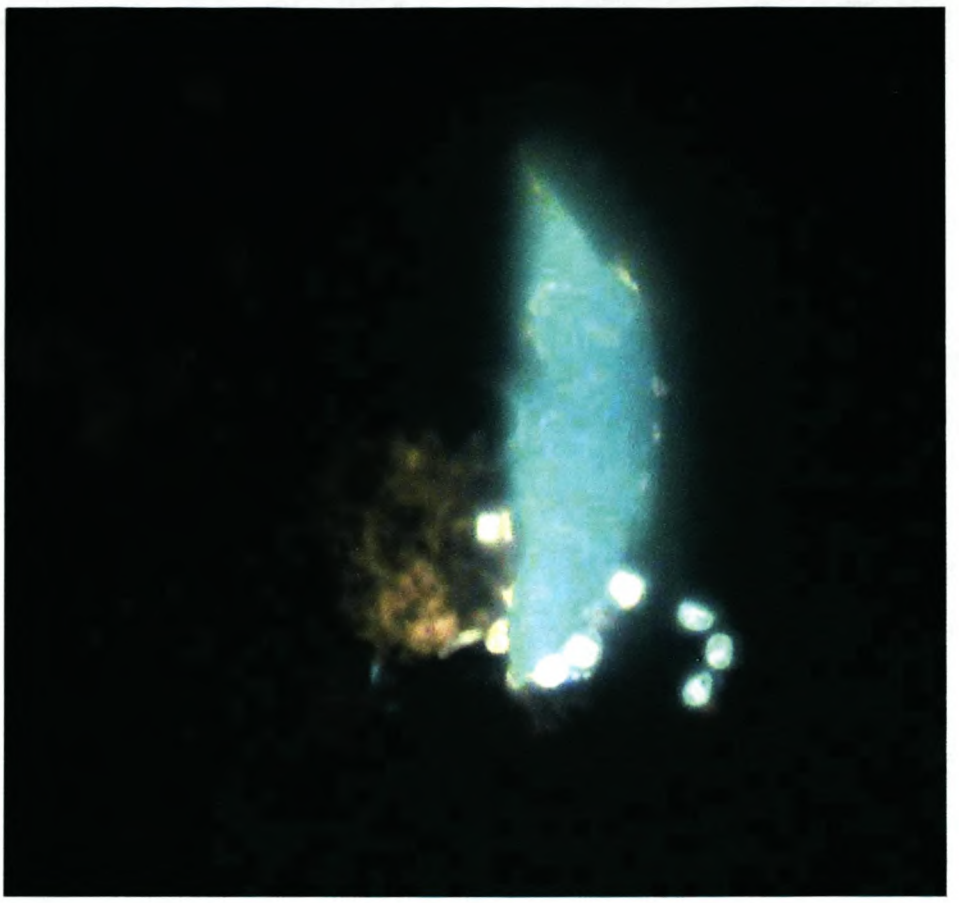

Figure 10. Yeast cells (green) showing bipolar budding in a chain, attached to a bacterial colony (yellow/red cells). Note the large blue area, which likely consists of microbially produced exopolymers.

4.2.3.2 Relative numbers of Sulfate Reducing Bacteria

Microscopy, combined wit FISH, as illustrated in Figure 11 was used to determine the relative numbers of SRB present in a selected area of a biofilm (Table 3). The percentage of SRB present in the biofilm was calculated as the fraction green cells present in the biofilm (total number of cells green, red, blue). The fraction of SRB was higher than that of other bacteria and of fungi. From Figure 9 A, it was seen that the proportion of

bacteria in the biofilm is not consistent. Depending on the specific area of the biofilm that was selected for determining relative proportion of SRB, the numbers would vary. Some regions of the biofilm had a higher concentration of SRB. Although there were sections of biofilm where the proportion of other microorganisms was much higher than the proportion of SRB, no section was observed where no SRB was present. This is in contrast with the observations made with culturing techniques (4.2.1.1) where SRB were hardly detectable. This demonstrates the need to apply various techniques when working with complex communities, especially when considering the relative abundance of populations with multifaceted growth requirements, such as SRB.

In biofilms labelled with the SRB-specific and Eubacterial probe, unicellular and filamentous fungi appeared blue as shown in Figure 11.

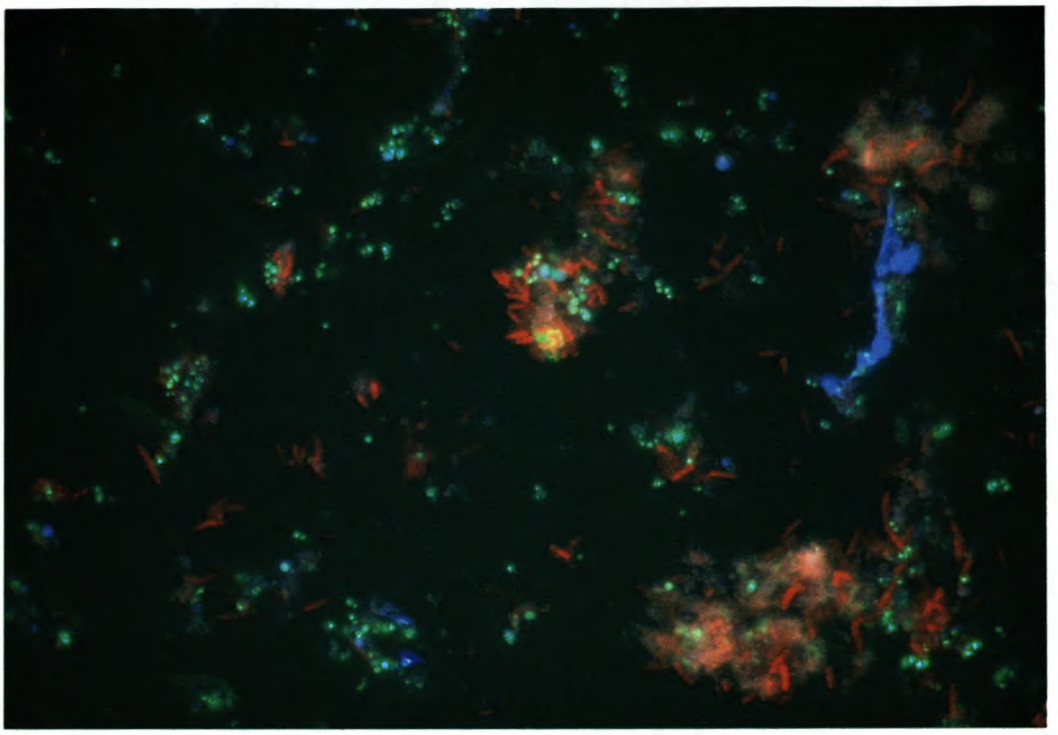

Figure 11. Biofilm on which FISH technique was performed showing yeast/fungi (blue), SRB (green) and other bacteria (red cells).

Table 3. Example of proportions of SRB (green cells), Eubacteria (red cells) and unicellular and filamentous fungi (blue cells) present in the biofilm.

Microorganism	Cell number	Fraction of biofilm (as percent)*
SRB	190	53 %
Eubacteria	135	37 %
Yeast/fungi	35	10 %

*calculated from Figure 11.

4.2.4 Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis.

T-RFLP analysis measured the size polymorphism of terminal restriction fragments (T-RF´s) from a PCR amplified DNA. The use of fluorescently tagged primer focussed the analysis to the T-RF´s of the digestion only. The T-RFLP pattern produced from

amplified 16S rDNA was a simplified representation of the bacterial community in natural environments. Data from the T-RFLP are presented in Table 4.

T-RFLP analysis indicated community diversity in the different reactors. Table 4 provides a summary of the data obtained. Because of the inherent difficulties in obtaining data from electropherograms, this discussion will focus on the results presented in Table 4. There were some deviations such as fragment 108 for Ethanol 1, day 3 and Ethanol 2, day 3; the Ethanol 2 had nearly the double peak height as Ethanol 1 (see Figure 12). Fragments of 108 base pairs (bp) disappeared after three days and did not re-appeared. This might also be, because the peak height were close to the threshold value of 20. This might also mean that the microorganisms from which these fragments came were not dominant in the community. Furthermore there were also changes in the control suggesting that a steady-state was not reached in the community, even after 15 weeks of adaptation.

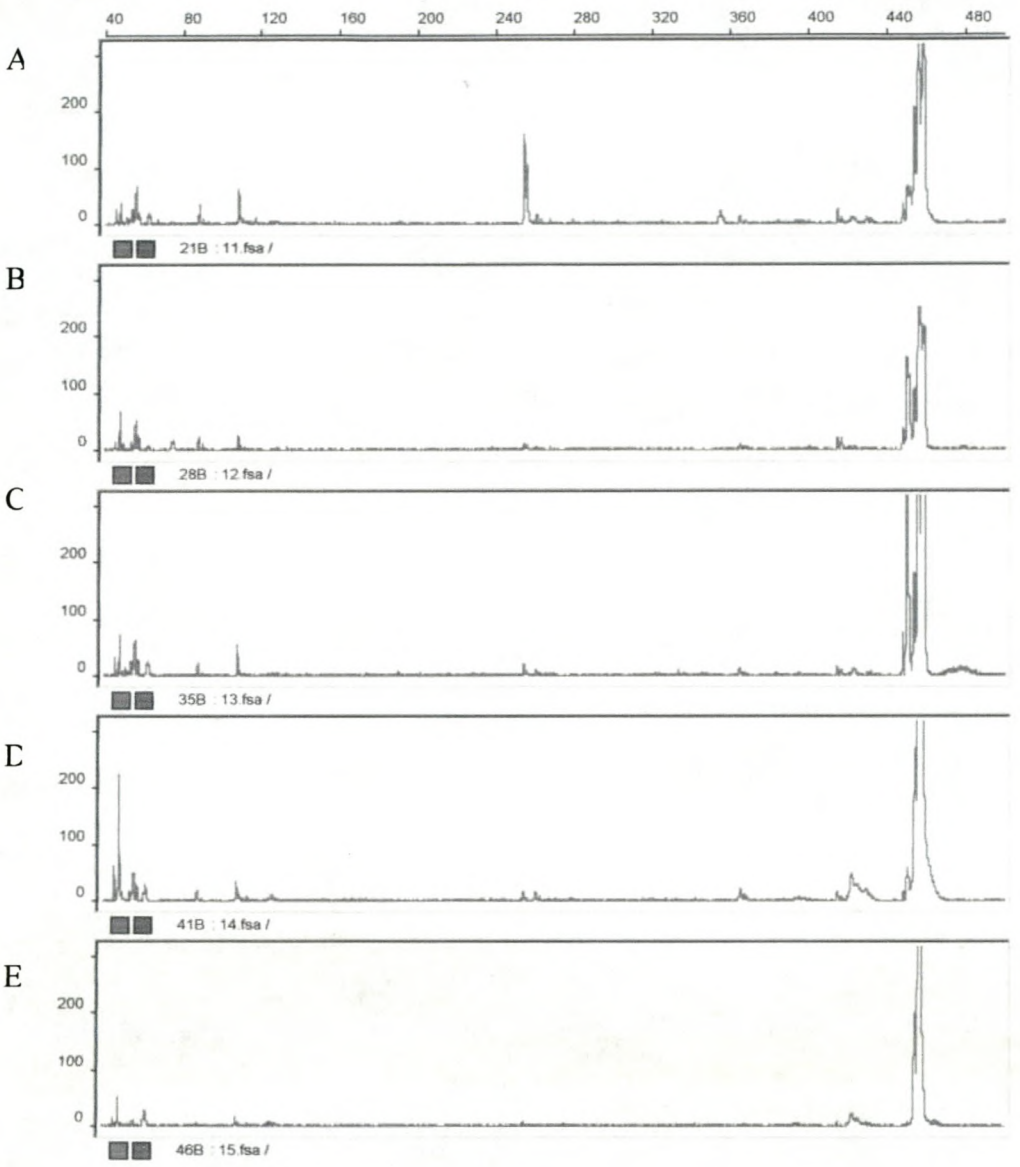

Figure 12. Electropherograms from T-RFLP analysis. Control day 3 (A), Ethanol 1 day 3 (B), Ethanol 2 day 3 (C), Sucrose 1 day 3 (D) and Sucrose 2 day 3 (E). The size (in bp) of terminal restriction fragments is illustrated in the x-axis. The intensity of fluorescence (arbitrary units), which gave rise to the third column of Table 2, peak height is illustrated on the y-axis.

Table 4. Terminal restriction fragments from samples taken from the bioreactors at the various time points (day 0, 1, 3, 5, 7, 10 and 20). The first column shows the size, in base pairs, of the terminal restriction fragments. The values given under day 0 to day 20 are the peak heights (relative units). Only significant peaks with a value above 20 are shown.

Size (in base pairs) of Terminal Restriction Fragment	Day 0	Day 1	Day 3	Day 5	Day 7	Day 10	Day 20
Ethanol							
108	36	52	44	0	0	0	0
255	28	35	23	0	0	0	0
256	22	33	22	0	0	0	0
448	61	0	59	0	28	0	0
450	239	0	305	99	102	78	0
456	368	0	421	142	100	132	36
458	429	0	797	0	267	114	44
459	0	0	222	371	236	113	68
Sucrose							
108	21	37	29	0	0	0	0
255	21	32	20	0	0	0	34
256	32	22	22	0	0	0	35
448	0	29	0	0	0	0	0
450	90	82	0	0	32	0	0
456	459	900	808	345	95	199	35
458	376	0	0	0	0	0	201
459	0	0	0	0	0	0	0
Control							
108	0	23	64	28	0	32	0
255	23	130	163	33	46	57	20
256	29	33	107	27	56	53	20
448	24	29	37	37	31	22	0
450	161	306	69	240	0	78	0
456	267	341	403	236	100	120	43
458	245	471	530	296	116	220	0
459	0	0	0	0	0	0	156

The peak height indicates the relative concentration of the specific restriction fragment present. The concentration of a restriction fragment gives an indication of the relative abundance of the organisms represented by the fragment. There were some fragments that were present only in one sample. These fragments always had small peak heights, indicating that they probably did not form part of any dominant community members. These fragments were: 261, 280, 329, 330, 331, 354, 355, 365, 419, 425, 427, 432, 433, 463, 464, 467 and 480.

Fragments 255 and 256 disappeared from the bioreactors fed with sucrose and ethanol samples after three days, but returned to the sucrose samples on day 20. This might mean that the organisms represented by fragments 255 and 256 did not grow in ethanol and in sucrose and 10 days after the shift back to sodium lactate as carbon source, only the microorganisms in the bioreactors that were fed with sucrose, occurred again in relatively high numbers (the concentration of fragments were high).

Fragment 448 disappeared after one day in the sucrose fed bioreactors and after 7 days in the ethanol fed bioreactors. The organisms represented by these fragments did not return after the 10 days shift back to sodium lactate. It seemed that the organisms represented by fragment 448 persisted longer on ethanol than on sucrose.

Some fragments were present only in ethanol and sucrose fed bioreactors. These fragments might represent microorganisms that preferred sucrose and ethanol as carbon sources. These fragments included 422, 423, 431, 451 and 457 (results not shown). It is also important to note that SRB 2 and SRB 3 (sulfate reducing colonies, which appeared black, obtained from plates) also shared some of these peaks. These black colonies were

collected and streaked out, but nevertheless white colonies appeared and just a few black colonies were present. This might be due to not enough H_2S production by these cells.

It must also be noted that a similar T-RFLP size is not a proof of identity, since different species might have DNA sequence homologies. Some fragments tend to be specific for sucrose bioreactors. Fragment 420 and 429 (results not shown) were only present in sucrose samples. These fragments were, however, not present throughout the whole time period and disappeared at the latest after 5 days. Some fragments appeared randomly in all reactors, without any distinct pattern (fragments 417, 455 and 414, results not shown). These fragments might represent microorganisms that were present throughout the entire time period. It is possible that there were peaks for these fragments that were under the threshold of 20. Values under 20 were considered by the computer software as background noise.

Fragment 450 were present in control samples, ethanol and sucrose samples, but disappeared after 10 days (see Table 4). This showed that there were changes in the communities that were not only caused by the shift to other carbon sources.

Fragment 453 (results not shown) were present only in samples from SRB 3, day 0 and in sucrose samples from day 20. These fragments were not present in any control samples and therefore it cannot be concluded that it was the shift in carbon source that let the organisms represented by these fragments to disappear.

Fragment 456 were present in nearly all samples. The peak heights from these fragments were high therefore it might be assumed that fragments 456 represented microorganisms that were well established in the community and were not visibly affected by the change in carbon source. It is important to note that SRB 2 and 3 (sulfate reducing colonies,

obtained from plates) also shared fragment 456. This could mean that the organisms represented by fragment 456 are sulfate reducing bacteria and that these bacteria were unaffected by ethanol and sucrose as carbon sources. It may, however, also be possible that fragment 456 represented more than one species. This would be possible if the region of base pair 456 of the PCR product is relatively conserved.

Fragment 458 was present only in day 0 bioreactors and in the control, ethanol and recovered sucrose (day 20) samples. The organisms represented by fragment 458 were not able to use sucrose as carbon source, but re-appeared in the community after the shift back to sodium lactate as carbon source. Fragment 458 was absent in all of the sulfate reducing colonies that were isolated. This might mean that the organisms represented by fragment 458 were not directly involved in the sulfate reduction process or that these bacteria were unculturable. The peak heights of these fragments on the electropherograms were high, meaning that these bacteria constituted a significant proportion of the community. Once again, high peak heights might mean that there was more than one organism which DNA sequence could be cut with *Msp*I at that specific site.

Fragment 459 was present only in SRB 2 and 3 and in ethanol samples and in the control sample of day 20. This fragment represented microorganisms that could not grow with sucrose as carbon source. Since the fragment was present in only one of the control samples, it can be said that these organisms preferred ethanol as carbon source.

T-RFLP analysis from the three colonies (SRB 1, 2, and 3, sulfate reducing colonies, obtained from plates) showed different profiles (results not shown). The T-RFLP profile

obtained for the colony grown on anaerobic plates had two peaks, one of which was similar to a peak from SRB 2 and to another peak from SRB 3. The second peak was not present in either of the other two colonies. This peak was also not visible in any of the samples from the carbon source shift experiments. This peak might represent an organism, which made a small contribution to the complex community and could not be picked up in an overall community analysis but had such a great effect that only such a small number of cells was present in the sample. This organism might have found more optimal growth conditions in the Postgate medium C agar.

Although sterile laboratory techniques were used, it can not be ruled out, that this peak was contamination by other bacteria.

Different species may have the same terminal restriction fragment lengths, hence leading to an underestimation of the actual bacterial diversity.

It is possible that some fragments were not included in the results, due to limitations of the software used. Some peaks that were high, were also wide. In some cases there may have been two peaks very near to each other on the electropherogram, but the software only detected the highest peak and not the lower peak because the peaks shared the same base.

4.2.5 Advantages and pitfalls of T-RFLP and FISH

Organisms that might have been abundant and culturable under certain conditions might have developed into dormant and possibly uncultered forms. However, due to the power of the PCR to amplify small amounts of DNA, organisms occurring in small numbers in an environment were detectable through T-RFLP.

It may be possible that not all cells were disrupted equally, which then could bias the view of the composition of microbial diversity as DNA or RNA, which is not released from the cells, will not contribute to the final analysis of diversity. On the other hand rigorous conditions required for cell lysis of Gram-positive bacteria were avoided as this treatment may lead to highly fragmented nucleic acids from Gram-negative cells, which could contribute to the unwanted formation of chimeric PCR products (Liesack *et al.*, 1991).

Application of FISH showed a difference in signal intensity between pure cultures and environmental samples (results not shown). There is a critical step to take to increase the signal intensity by environmental samples. The cells have to be treated with a lysozyme solution. However, with this solution there is a possibility of not only increasing cell wall permeability but also to induce cell lysis.

4.2.6 BIOLOG ECOPLATES

4.2.6.1 Biolog EcoPlate™

Microbial communities provide useful data for studying both applied and basic environmental events. Microorganisms are typically the first organisms to react to chemical and physical changes in the environment. Because they are at the bottom of the food chain, changes in microbial communities are often precursors to changes in the health and viability of the environment as a whole (Konopka *et al.,* 1998).

The evaluation of the results for the Biolog Ecoplates that were incubated in this study under aerobic conditions showed the following (there were seven time points where samples were collected). On day 0, all five bioreactors (control, sucrose1, sucrose 2, ethanol 1 and ethanol 2) had the same metabolic fingerprint. One day after the shift to another carbon source, the control and the sucrose fed bioreactors had similar metabolic profiles, but the bioreactors, which had ethanol as new carbon source were different. Between the cluster control/sucrose 1 and the cluster ethanol 1 and 2/sucrose 2 was a linkage distance of 0.03 percent disagreement. On day three the linkage distance increased to 0.06 percent disagreement between the cluster ethanol 1 and 2/control 1 and the cluster sucrose 1 and 2. On day five there was no change from day three. On day seven occurred a linkage distance between the control/ethanol 2 and cluster of sucrose 1 and 2. Their linkage distance was 0.03 percent disagreement. The linkage distance of the cluster ethanol 1 still increased to a linkage distance of 0.21 percent disagreement. The cluster ethanol 1 had a linkage distance of 0.18 percent disagreement from the cluster control/ethanol 2 and cluster sucrose 1 and 2. On day ten after the carbon source shift, the linkage distance of the cluster ethanol 1 and 2 stayed the same as on day seven, but the

group sucrose 1 and 2 disagreed more from the cluster control. The cluster sucrose 1 and 2 then had an increased linkage distance with 0.18 percent disagreement towards the group control (see Figure 13). On day ten the carbon source was changed to the initial carbon source sodium lactate, which was the carbon source of the control bioreactor. A time period of ten days was given for recovery. On day twenty, recovery to the initial state was evident. The cluster containing the control and the cluster containing the data from the bioreactor which was fed with sucrose were identical again and the linkage distance between the cluster control/sucrose towards the cluster ethanol 1 and 2 decreased to 0.18 percent disagreement. The bioreactors fed with ethanol as carbon source showed less recovery than the bioreactors fed with sucrose as carbon source.

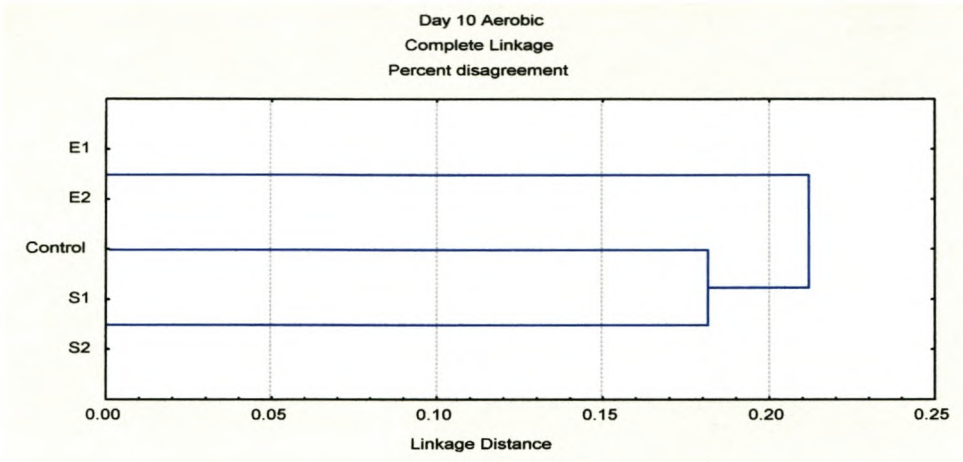

Figure 13. A typical diagram of the cluster analysis of the microbial community from the bioreactors. Shown here is the Biolog data of the different fed bioreactors 10 days after the carbon source was changed from sodium lactate to sucrose and ethanol respectively (S=sucrose; E=ethanol; C=sodium lactate as carbon source).

In Table 5 and Figure 14 the ANOVA analysis is shown as statistical verification of the finding that there was a significant difference in the community metabolic profiles between the bioreactors fed with ethanol as carbon source as opposed to the control. There was no significant difference between the bioreactors fed with sucrose as carbon source and the control that had sodium lactate as carbon source.

Table 5. Statistical analysis of BIOLOG Ecoplates™ (aerobic). Shown are the arcsin(p) of the bioreactors. Bioreactor C (1), bioreactor sucrose 1 (2), bioreactor sucrose 2 (3), bioreactor ethanol 1 (4) and bioractor ethanol 2 (5).

ANOVA

Source of Variation	*df*	*MS*	*F*	*P-value*
Rows	6	0.125	14.553	0.000
Columns	4	0.035	4.078	0.012
Error	24	0.009		
Total	34			

SEM 0.035018

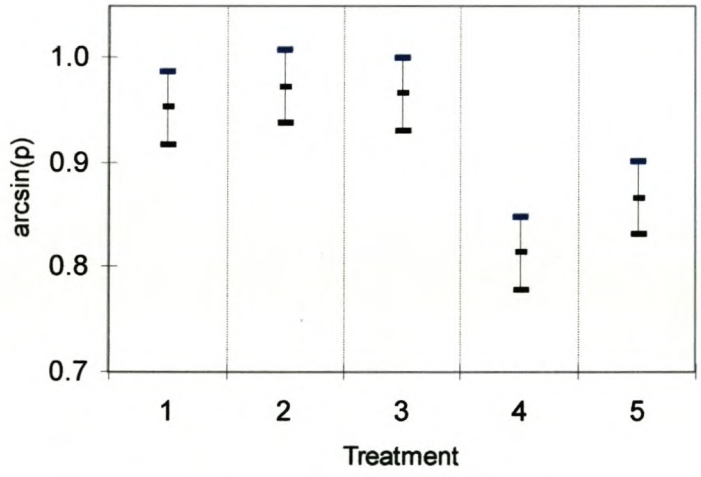

Figure 14. Statistical analysis of BIOLOG Ecoplates™ (aerobic). Shown are the arcsin(p) of the functional diversity, the substrate utilization profiles. Bioreactor C (1); bioreactor sucrose 1 (2); bioreactor sucrose 2 (3); bioreactor ethanol 1 (4) and bioractor ethanol 2 (5).

Anaerobically incubated Biolog Ecoplates.

There were four time points (day 0, 5, 10 and 20; results not shown) where samples were taken. On day 0 all five bioreactors had the same metabolic fingerprint. On day five (five days after the shift to another carbon source), a linkage distance of 0.03 percent disagreement between the cluster control/sucrose and the cluster ethanol appeared. In Figure 15 the cluster for day 10 is not shown, as there was no disagreement between sucrose and ethanol. The linkage distance did increase again to 0.03 percent disagreement between the cluster control/sucrose and ethanol on day 20.

In Table 6 and Figure 15 results as obtained from the ANOVA analysis is shown as statistical verification of the finding that anaerobically, there was no significant difference in the community profile between the bioreactors which had ethanol as carbon source. There was no significant difference between all anaerobically kept Biolog Ecoplates^{TM}. Because the samples were taken from an anaerobic habitat it may not have been suitable to analyze the substrate utilisation profile under aerobic conditions.

Table 6. Statistical analysis of BIOLOG Ecoplates™ (anaerobic). Shown are the arcsin(p)of the bioreactors. Bioreactor C (1), bioreactor sucrose 1 (2), bioreactor sucrose 2 (3), bioreactor ethanol 1 (4) and bioractor ethanol

Source of Variation	*Df*	*MS*	*F*	*P-value*
Rows	3	0.129547	17.51621	0.000111
Columns	4	0.011822	1.598441	0.237907
Error	12	0.007396		
Total	19			
	SEM	0.043		

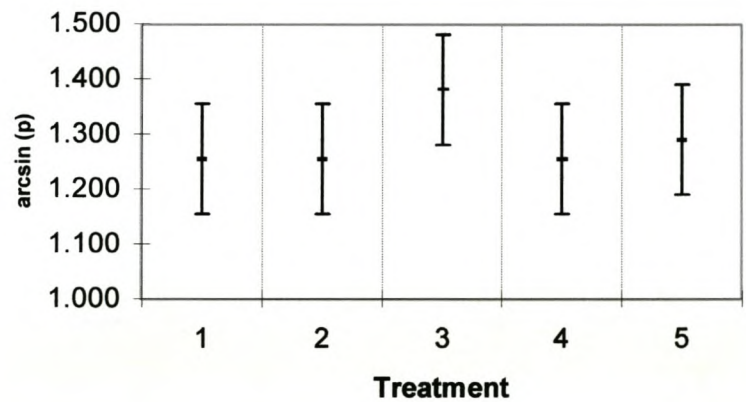

Figure 15. Statistical analysis of BIOLOG Ecoplates™ (anaerobic). Shown are the arcsin(p) of the functional diversity, the substrate utilization profiles. Bioreactor C (1); bioreactor sucrose 1 (2); bioreactor sucrose 2 (3); bioreactor ethanol 1 (4) and bioractor ethanol 2 (5).

4.3. CONCLUSIONS

The T-RFLP results showed that the microbial community consisted of a variety of microorganisms in all the samples. A few dominant microorganisms were present in the bioreactors, since a few T-RF's were present over the entire time period of the study. After the carbon source shift, the T-RFLP was able to show a slight decline in microbial diversity and the disappearance of members of the community which were present in the control and before the carbon source shift.

SRB formed a complex bacterial group, difficult to isolate and identify with conventional cultivation methods. With FISH, it was possible to show the presence of SRB in biofilm.

It was interesting to find that unicellular and filamentous fungi were integrated into the microbial community in the bioreactors. The obtained oligonucleotide probes that recognised SRB in a biofilm community showed the integration of SRB in a heterogeneous biofilm community coexisting among bacterial populations and fungal taxa.

The study implemented the use of BIOLOG Ecoplate^{TM} which were kept anaerobically during the analysis. Biolog Ecoplates have not been reported to be incubated anaerobically before. This analysis showed that the use of sucrose in comparison to ethanol as carbon source is more favourable. The shift in carbon source from sodium lactate to sucrose had a smaller impact on the community profile than ethanol. Ethanol as carbon source changed the metabolic fingerprint of the community more notably.

The advantages of molecular methods in microbial ecology were quite obvious. T-RFLP provided means to study community complexity and to monitor population shifts. Oligonucleotide probes provided means to rapidly indicate members of SRB, in both pure

cultures and environmental samples. Furthermore, FISH allowed visualisation and quantification of specific bacteria in their environment without cultivating them. The BIOLOG Ecoplate™ analysis as a different means of studying community complexity and profiles (metabolic fingerprint) confirmed the results of the T-RFLP analysis (genetic fingerprint). According to this study, the combination of T-RFLP and FISH provided insight into the *black box* of the microbial community structure and the dynamics in mixed community, the ecology, of a sulfate-reducing bioreactor. Further studies may concentrate more on changes in the T-RFLP profile according to different sulfate reduction rates.

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