Evaluation of Hydrogen as Energy source for biological sulphate removal in industrial wastewaters.

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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.

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Abstract

Biological removal of sulphate from wastewater can be achieved by using a gas mixture consisting of 80% hydrogen and 20% carbon dioxide as energy and carbon sources. A novel reactor, including a venturi device for optimal hydrogen gas-liquid contact, and geotextile for immobilisation of the sulphate reducing bacterial community, was introduced. Efficient, relatively stable sulphate removal was obtained when the reactor was operated in continuous mode. The maximum sulphate removal rate obtained when the reactor was 8% packed with geotextile, was 1 g SO_4/(L.d) and 4 g SO_4/(L.d) when the reactor was 80% packed with geotextile. Kinetic batch studies showed that the highest sulphate removal rates were obtained at 29.5 °C; a pH of 7.5; initial sulphate concentration of 4000 mg/L; initial alkalinity of 1600 mg/L; cobalt concentration of 3 mg/L and when excess hydrogen gas was fed compared to what is stoichiometrically required (900 ml/min). Nickel addition showed inhibition at increased concentrations (>3 mg/L).

The biofilm structure was observed on the geotextile with electron microscopy, while the viability of the biofilm was indicated with fluorescence microscopy. These observations indicated the suitability of the geotextile as a support material for biofilm formation in the sulphate reducing system. The stability of the sulphate reducing community was analysed, using the T-RFLP protocol. It was shown that the composition of the community changed after a period of 3 months, when the reactor was subjected to environmental changes. The reactor was also observed to be more efficient in terms of sulphate removal after the environmental changes, of which the temperature change from an average of 39 to 29.5 °C was the most prominent. Subsequently, it was speculated that the population shift was in favour of a more efficient system for sulphate removal. A dynamic, viable, mesophilic sulphate reducing community was therefore observed on the geotextile support, responsible for successful sulphate removal in a novel venturi-reactor.

Defining optimal operating conditions, and a knowledge of biofilm structure and composition may contribute to the successful implementation of the biological sulphate removal component of the integrated chemical-biological process for the treatment of industrial wastewater, when hydrogen and carbon dioxide are supplied as the energy and carbon sources, respectively.

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Opsomming

Ongewenste industriële afval-water kan biologies behandel word deur 'n gasmengsel van 80% waterstof en 20% koolstofdioksied te gebruik vir sulfaat verwydering. 'n Reaktor wat 'n venturi apparaat bevat vir optimale waterstofgas-vloeistof kontak, asook geotekstiel vir die immobilisasie van die bakteriële sulfaatverwyderende gemeenskap, is bekend gestel. Effektiewe, relatief stabiele sulfaatverwydering is waargeneem sodra die reaktor op 'n kontinue basis gevoer is. Die optimale sulfaat verwyderingstempo wat bereik is as die reaktor 8% met geotekstiel gevul was, was 1 g SO_4/(L.d) en 4 g SO_4/(L.d) wanneer die reaktor 80% met geotekstiel gevul was. Kinetiese groepstudies het getoon dat die beste sulfaatverwydering bereik is by 'n gemiddelde temperatuur van 29.5 °C; pH van 7.5; aanvanklike sulfaatkonsentrasie van 4000 mg/L; aanvanklike sulfied konsentrasie van 268 mg/L; aanvanklike alkaliniteit van 1600 mg/L; kobalt konsentrasie van 3 mg/L, asook wanneer 'n oormaat waterstofgas gevoer is (900 ml/min), in vergelyking met wat stoichiometries benodig word. 'n Verhoogde byvoeging van nikkel by die voerwater (3 mg/L), het tekens van inhibisie getoon.

Die biofilm struktuur is waargeneem op die geotekstiel met behulp van 'n elektronmikroskoop, terwyl die lewensvatbaarheid van die biofilm aangedui is met behulp van fluoressensie mikroskopie. Hiermee is die bruikbaarheid van geotekstiel as 'n ondersteunings-matriks bevestig. Die stabiliteit van die sulfaatverwyderende gemeenskap is ondersoek deur die T-RFLP protokol te gebruik. Hiermee is aangedui dat die samestelling van die gemeenskap verander het na die 3 maande toets periode, toe die reaktor onderhewig was aan omgewings veranderinge. Die reaktor het ook 'n verbetering in sy sulfaatverwyderings vermoë getoon na hierdie tydperk van omgewingsveranderinge, waarvan 'n temperatuur verandering vanaf 'n gemiddeld van 39 na 29.5 °C die prominentste was. Dit is dus gespekuleer dat die populasie verskuiwing ten gunste was van 'n beter sisteem vir sulfaatverwydering. 'n Dinamiese, lewensvatbare, mesofiliese sulfaatreduserende gemeenskap, verantwoordelik vir die sulfaatverwydering in die venturi-reaktor, is dus waargeneem op die geotekstiel as 'n ondersteuningsmatriks.

Met hierdie evaluasie kan die insig wat verkry is in die reaktor samestelling en die optimale kondisies vir die reaktor werking, bydra tot die suksesvolle implementasie van die biologiese komponent, in die geïntegreerde chemies-biologiese proses vir die behandeling van

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industriële afval water, wanneer 80% waterstof en 20% koolstofdioksied gas as energie en koolstofbron respektiewelik, gebruik word.

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CHAPTER 1:LITERATURE REVIEW

1.1. GENERAL INTRODUCTION

Sulphate is present in the environment as a dissolved compound in the oceans, and it is an important stock of sulphur on earth, where sulphate is also present as an insoluble salt, eg. in gypsum-layers. Rainwater dissolves sulphates from their solid reservoirs and transports them into groundwater and deeper soil layers, which are mostly anaerobic. Sulphate reducing bacteria (SRB) reduce the sulphate under these anaerobic conditions, forming sulphide, which can precipitate with heavy metals when present (Lens *et al*., 1998). Sulphate reduction occurs in addition to methanogenesis in anaerobic environments, rich in oxidized sulphur compounds and in the presence of organic material. Re-oxidation of metal sulphides to sulphates sometimes takes place, which mostly occurs through the activities of *Thiobacillus* bacteria (Middleton and Lawrence, 1977). Water bodies can also be polluted by acid deposition of sulphur compounds from the atmosphere, which comes from sea salt aerosols or volcanic eruptions. H_2SO_4 forms indirectly, following atmospheric SO_2 and SO_3 emission, thus leading to acid, sulphate-contaminated rainwater (acid rain).

In addition to the natural occurrence of sulphate in the environment, it is also introduced into the environment through a variety of human activities. For instance, domestic sewage contains sulphate at concentrations that typically vary between 20 and 500 mg/L. In the pulp and paper industry, mills use sulphur in various forms and processes, and sulphate compounds exist in wood and in untreated water. In the fermentation industry, the use of sulphuric acid for pH control is the main source of sulphate contamination. In the photographic sector, thiosulphate is used as a fixer, and in the TNT (trinitrotoluene) manufacturing process, unsymmetrical TNTs are sulfonated by adding sulfite in the purification stage. Sulphate or sulphite is used as raw material in the production of sulphur-containing xenobiotics (environmental pollutants). The use of sulphur-containing fossil fuels results in atmospheric SO_2 and SO_3 emissions and ultimately in acid sulphate depositions (Lens *et al*., 1998).

Sulphate is a chemically inert, non-volatile, and non-toxic compound. Restrictions on the sulphate emission in environmental legislation mainly aim to reduce the salt content and/or to minimize acid in sewers. High sulphate concentrations can disrupt the natural sulphur cycle, while the biodegradation pathways and rates can be altered considerably as a result thereof.

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In South Africa, large areas of polluted underground or surface- acid mine drainage (AMD) exist (Figure 1.1). This is a major environmental concern especially where mining has been practiced on a large scale. AMD is acidic water, containing H_2SO_4, derived from the microbial oxidation of sulphide minerals. During this process, pyrite (FeS_2) is broken down in the presence of oxygen and water to form Fe^{2+} (Ferrous Iron) and H_2SO_4. The Fe^{2+} is oxidized at a low pH (pH2), to form Fe^{3+} (Ferric Iron) by *Thiobacillus ferrooxidans*. This causes the precipitation of insoluble ferric hydroxide and complex ferric salts, typically called 'yellow boy' in coalmines (Madigan *et al.*, 1997). The resulting AMD leaches into the surrounding streams, causing damage to natural vegetation, rivers, watersheds, natural habitat and aquatic life (Middleton and Lawrence, 1977).

Figure 1.1: AMD showing precipitation of Ferric salts at a coalmine in South Africa.

An integrated chemical-biological process, that uses ethanol and/or sugar as carbon and energy source, and that is capable of reducing sulphate to less than 200 mg/L, has been developed for the treatment of AMD (Figure 1.2).

Figure 1.2: Biological sulphate removal unit at Navigation, Witbank, South Africa, where ethanol/sugar is used as energy source for the SRB to remove sulphate from mine water.

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In addition to the environmental considerations of AMD, the price of the energy and carbon source for the SRB is an important consideration in the application of sulphate removal technology. In response to the relatively high operational costs associated with the use of ethanol and sugar as carbon and energy sources, the search is on for more cost effective options. One such option is hydrogen: when hydrogen is produced as an electrolytical by-product during the integrated treatment process, it can be supplied together with carbon dioxide as an alternative energy and carbon source, respectively, for the SRB (see Chapter 3, reaction 4). Therefore, the purpose of this study was to evaluate the use of hydrogen as the energy source for SRB in the reduction of sulphate during the treatment of industrial and mining wastewaters.

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1.2. LITERATURE REVIEW

1.2.1 Biological sulphate reduction

SRB are used in the biological sulphate reduction technology to achieve the reduction of sulphate (SO_4). This is a process in which SRB use sulphate as an electron acceptor during the oxidation of organic matter. Sulphate, in which sulphur is in the +6 oxidation state, is reduced to sulphide, the –2 oxidation state of sulphur. Organic compounds are oxidized simultaneously, and during this process, the SRB are provided with energy for growth and cell maintenance. As indicated previously, SRB can use several carbon and energy sources. A schematic representation of the anaerobic degradation of organic matter in the presence of sulphate is shown in Figure 1.3.

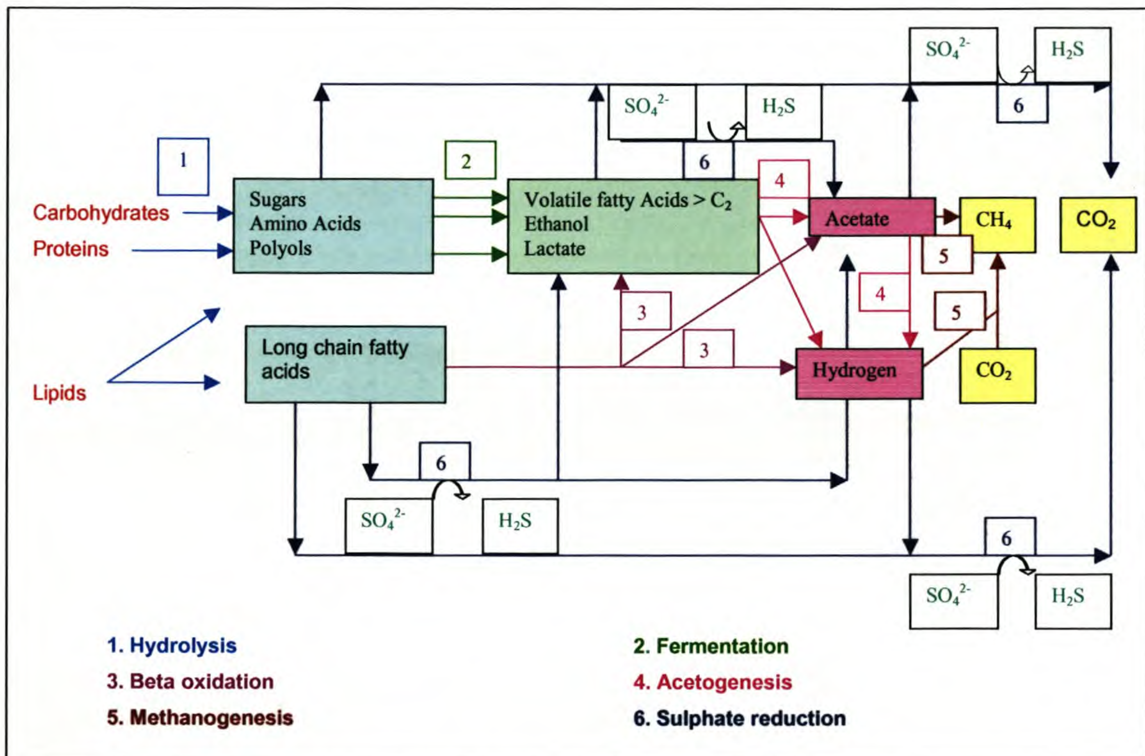

Figure 1.3: A schematic representation of the anaerobic degradation of organic matter in the presence of sulphate (adopted from Lens *et al*., 1998).

The two most commonly mentioned genera of sulphate-reducing bacteria are *Desulfovibrio* and *Desulfotomaculum*, with *Desulfovibrio* being the most abundant. These SRB are small, gram-negative, curved rods (vibrio) that perform best under mesophilic temperatures and strict anaerobic conditions. They differ from other SRB, because of their single polar flagellum.

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SRB are able to use several intermediates of the anaerobic mineralization process as a carbon and energy source in the presence of sulphate, sulfite or thiosulfate (Figure 1.3). For instance, it has been demonstrated that SRB can use decomposed saw dust (Tuttle *et al.*, 1969), hydrogen and CO_2 (80:20), gas mixture or producer gas (van Houten, 1996), formate (De Smul and Verstraete, 1999), acetate (Dries *et al.*, 1998, O'Flaherty *et al.*, 1998), methanol (Weijma *et al.*, 2000), pyruvate, propionate, butyrate (Lens *et al.*, 1997), higher and branched fatty acids (Lens *et al.*, 1998), lactate, ethanol, sucrose (De Smul *et al.*, 1997, Greben *et al.*, 2000), as well as higher alcohols, fumarate, succinate, malate, and aromatic compounds (Lens *et al.*, 1998) as energy sources in the sulphate removal process.

1.2.1.1 Biofilms and community interactions

The environment surrounding bacteria is in constant flux. According to Decho (1999), the successful adaptation of bacteria to changing natural conditions requires that the organisms are able to respond to their changing external environment by being able to modulate their gene expression accordingly.

Microorganisms use available energy sources and nutrients from their environment. In most cases, they have to adapt to conditions where their energy and nutrient sources are limited. Even when enough nutrients and energy sources are available, a high growth rate can lead to the release and build-up of toxic waste compounds, which will limit further growth of the microorganisms. In reaction to these changing conditions, competition often exists between different microorganisms, which require adaptation by the cells. A typical response of microbes to adapt to the conditions in their environment for better uptake of nutrients is to change their cell morphology, such as increasing the surface area for better absorption. The specific and non-specific attachment of these organisms can also increase to absorb the nutrients that are often found in higher localized concentrations on surfaces (Cloete, 1994; Wolfaardt *et al.*, 1994).

In the environment, microorganisms can be found in their specific habitats as populations of the related organisms, or as communities that consist of different types of organisms in interaction with each other (Cloete, 1994). Many microbial processes often found in the environment require consortial activities that are not possible with single-species populations (Wolfaardt *et al.*, 1994). Through co-operative interactions between two or more populations

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sharing a habitat, microorganisms can maximize their metabolic capabilities. In this way, the individual cells contribute to the overall maintenance, stability and integrity of populations or communities, and hence contribute to their own survival (Wolfaardt *et al*, 1999).

When microorganisms live and grow in aggregated forms (such as biofilms), the number of interacting species may vary from one environment to another. A common feature is usually that the microorganisms are embedded in a matrix of extracellular polymeric substances (EPS), which also play an important role in these interactions by facilitating communication between cells through participating in cell-cell recognition and by serving as an adhesin (Wolfaardt *et al*, 1999).

Biofilms can also be described as accumulations of microorganisms (prokaryotic and eukaryotic unicellular organisms), EPS, multivalent cations, biogenic and inorganic particles as well as colloidal and dissolved compounds (Wingender *et al.*, 1999). Microbial EPS are biosynthetic polymers (biopolymers). Proteins, nucleic acids and amphiphilic compounds such as phospholipids are commonly found in EPS. The EPS are mainly responsible for the structural and functional integrity of biofilms and are considered as the key components that determine the physicochemical and biological properties of biofilms. EPS form a three-dimensional, gel-like, highly hydrated and often charged biofilm matrix, in which the microorganisms are embedded and more or less immobilized. In general, the proportion of EPS in biofilms can vary between 50 and 90% of the total organic matter. The extracellular localization of EPS and their composition may be the result of different processes, such as active secretion, shedding of cell surface material, cell lysis and adsorption from the environment (Wingender *et al.*, 1999). The structure of the biofilm also develops in response to flow, where ridges are caused to develop parallel to flow direction. The highest bacterial biomass development takes place in a 'shell-type growth', located near the surface of the biofilm (Decho, 1999).

EPS production by environmental strains of anaerobes such as SRB has not been a subject of extensive study. However, it is known that the yield and composition of bacterial EPS depend on the species and growth conditions. When EPS formation by SRB (*Desulfovibrio desulfuricans spp.*) was studied, an amorphous viscous substance that was thought to be polysaccharide was observed (Beech *et al.*, 1999). Abundant slime formation was also

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observed in old cultures of *Desulfovibrio desulfuricans spp.* and in continuous cultures of *Desulfovibrio vulgaris spp.* (Beech *et al.*, 1999).

It is therefore evident that biofilms containing different types of microorganisms, adapted for maximum nutrient absorption and metabolic capabilities, often form in a specific environment. Biofilm formation does not only favour nutrient uptake, but attachment to a surface also stabilizes the organisms and enables maintenance of community integrity (Cloete, 1994; Wolfaardt *et al.*, 1994). Biofilm formation by cells involved in sulphate removal in a bioreactor may therefore contribute to a stable and efficient biological sulphate reduction process. Such a reactor can be packed with several types of support material, including geotextile, pumus particles or sponge (Van Houten, 1996).

1.2.1.2 Competition between organisms in biological sulphate reduction

Sulphidogenic bioreactors for the treatment of sulphate rich wastewater are complex ecosystems that contain many bacterial species. In these mixed cultures, competition exists between the different species for carbon and energy sources: methanogens use the carbon and energy source for CH_4 formation, SRB for sulphate reduction, and acetogens for acetate formation. The importance of this competition increases with the decrease in the COD/sulphate ratio of the wastewater (Lens *et al.*, 1998). The outcome of this competition will determine the formation of the end-products of the anaerobic mineralization processes.

When using ethanol as energy and carbon source in batch and continuous culture, competition exists for ethanol between the sulphate-reducing bacteria (such as *Desulfobulbus propionicus, Desulfotomaculum orientis, Desulfovibrio vulgaris Marburg*, *Desulfovibrio gigas*, *Desulfovibrio desulfuricans Essex*) and the fermenting bacteria (*Pelobacter propionicus* and *Acetobacterium carbinolicum*) (Szewzyk and Pfennig, 1990). The outcome of the competition in the latter study was found to depend on the maximum specific growth rate μ_{max} and the substrate concentration K_s that allow half maximum growth rate. It was also shown that an increasing dilution rate in continuous culture, (with the exception of *Acetobacterium carbinolicum*), caused an increase in the molar growth yields. This well-known phenomenon was thought to be caused by the increasing predominance of the energy consuming maintenance metabolism at low growth rates. Furthermore, competition experiments in continuous culture showed that under low dilution rates, SRB are able to successfully

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compete with fermenting bacteria, because of their higher affinity for ethanol (Szewzyk and Pfenning, 1990).

When using hydrogen as the energy source, the most important competition that should be noted is that between hydrogenotrophic sulphate reducing bacteria (HSRB) and hydrogenotrophic methanogenic bacteria (HMB). The HSRB out-compete the HMB, as HSRB gain more energy from the consumption of molecular hydrogen and therefore have a higher substrate affinity, growth rate and cell yield than HMB. HSRB also maintain the hydrogen concentration below the threshold value required by the HMB, thus preventing HMB to use the hydrogen. This theory explains the inhibition of HMB when sulphate enters an anaerobic bioreactor (Lens *et al*., 1998).

Yamamoto-Ikemoto et al. (1996) studied symbiosis and competition among SRB, filamentous sulphur bacteria (FSB), denitrification bacteria (DNB) and poly-P accumulation bacteria (PAB) in activated sludge of a municipal plant, operated under anaerobic-oxic conditions. Under anoxic conditions, the DNB were dominant because the DNB out-competed the PAB and the SRB for organic acids. Under anaerobic conditions, phosphate release and sulphate reduction occurred simultaneously. SRB produced 4 moles of acetate from 4 moles of propionate and/or unknown substances by reduction of 3 moles of sulphate. It was shown that, under these conditions, PAB and SRB competed for organic acids, such as propionate, however PAB utilized acetate produced by SRB. In addition, Lens *et al*. (1998), showed that SRB activity can stimulate propionate degradation, while the addition of propionate can enhance the sulphate reduction process.

According to Lens *et al*. (1998), acetotrophic SRB (ASRB) have an advantage over acetate utilizing MB (AMB) in their competition for acetate: ASRB gain more energy from the consumption of acetate than AMB and ASRB tend to have higher growth rates than AMB, especially at low acetate concentrations. Alphenaar *et al*. (1992) showed that acetate was oxidised by both SRB and methanogenic bacteria. It was also shown that the fraction of acetate used by sulphate reducers relative to methanogens increased with time, resulting in a predominance of SRB, especially at a relative long hydraulic retention time (40h). These authors also showed that granulation was favoured by the combination of high upward velocity and short hydraulic retention time, and no difference was found between the attachment capacity of SRB and methanogens.

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The composition of the feed water could also influence the competition between SRB and MB for acetate. It was found that the contribution of SRB to COD removal increased by increasing the glucose/acetate ratio of the feed. Although impractical, acetate-rich influent also promoted acetate removal by MB. In addition, at sufficiently high sulphate concentrations, ASRB could out-compete AMB. This is a very lengthy process and can take up to 1000 days or maybe even longer. SRB are also known to have a high demand for iron. However, it was found that dosing of extra iron to an anaerobic filter reactor, treating acetate, ethanol and formate did not exert a beneficial effect on the SRB (Lens *et al*., 1998).

The type of seed sludge and experimental runtime can also influence the outcome of the competition between acetate utilizing bacteria. A long period is needed for some types of bacteria to out-compete other species, where a steady state situation needs to be reached, and where the number of one of the competing species is very low compared with the other. Therefore, the choice of seed sludge is, in addition to the applied environmental conditions, of importance.

While the optimal pH range for ASRB (7.3 – 7.6) is comparable to that of AMB (6.5 – 7.8), the ASRB can tolerate higher pH values than the AMB. In addition to the direct influence of pH on the growth of the bacteria, an indirect influence is also possible, where pH determines sulphide and ammonia toxicity:

$$H_2S \underset{}{\overset{pH\ 6.5}{\rightleftarrows}} HS^- \qquad (1)$$

H_2S is the toxic form of sulphide that causes inhibition of both SRB and MB. H_2S (g) is formed at pH < 6.5, while HS^- is formed at pH >6.5.

Mesophilic SRB and MB have similar temperature ranges, and therefore respond similar to temperature conditions, however SRB appear to be less sensitive to temperature changes than MB. For example, in response to a decrease of reactor temperature in continuous reactors from 35 to 25 °C over a period of 30 days, the fraction of electron flow used by SRB increased from 43 to 80%. However, further studies are required in this regard for the development of practical guidelines (Lens *et al*., 1998).

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1.2.1.3 Factors that may lead to inhibition in biological sulphate reduction

Cation inhibition of sulphate reduction can take place, usually in the presence of high concentrations of Na^+ and Ca^{2+}. In the case of sodium, process failure is unlikely in practise. In contrast, calcium carbonate and phosphate precipitates can cause scaling of the reactor at calcium concentrations of 400 mg/L, and can therefore interfere with the reactor performance. Here, the precipitates are entrapped in the reactor biomass, where they gradually accumulate and can ultimately result in a loss of the sludge granule activity, due to a Ca^{2+}-layer, which can block substrate transport. Phosphate deficiency can also be the result of calcium-phosphate precipitation (Lens *et al.*, 1998).

It has been reported that sulphide toxicity is severe, and can cause process failure. The inhibitory effect of sulphide is reportedly caused by undissociated H_2S, because only neutral molecules can diffuse through the cell membrane. It is speculated that H_2S may interfere with the assimilatory metabolism of sulphur, while it may also affect the intracellular pH. Therefore, at low pH values and low temperatures, the sulphide toxicity increases, because the formation of undissociated sulphides is favoured (Hulshoff Pol *et al.*, 2001). Van Houten (1996) showed that a sulphide concentration of 350 – 400 mg/L could be toxic, however Greben *et al.* (2004) demonstrated that sulphide concentrations of as high as 1400 mg/L could be tolerated by SRB. Adaptation of SRB to the environment therefore plays a role in this regard. Furthermore, it was also shown that granular sludge was less inhibited by H_2S than suspended sludge at low and neutral pH, whereas both types of sludge are equally inhibited at high pH values (Hulshoff Pol *et al.*, 2001). Up to 50% inhibition of MB was found in suspended sludge reactors at H_2S concentrations ranging from 50 to 130 mg/L. Control of the pH is therefore important in sulphidogenic bioreactors, where the toxicity of H_2S is concerned (Lens *et al.*, 1998). In contrast to the inhibitory effects of sulphide, the addition of sulphide (20 mg/L) could be considered as an obligate nutrient requirement in some cases for anaerobic biotechnology, which could even ensure process stability (Speece, 1996).

Because oxygen has an inhibitory effect on SRB, and thus sulphate reduction, the sulphate reduction process occurs under anaerobic conditions. Marschall *et al.* (1993) showed that three strains (*Desulfovibrio desulfuricans CSN, Desulfovibrio vulgaris Marburg, Desulfobacter hydrogenophilus*) slowly formed sulphide at oxygen concentrations below 6% air saturation (15μM). In homogeneously aerated cultures (6 - 25μM maximum oxygen),

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most of the strains tested, even those isolated from oxic environments, did not grow. Upon prolonged incubation with oxygen, the cells typically lost their motility and the ability to grow in anoxic medium. When SRB were exposed to oxygen, they remained viable for hours or even days, offering a potential explanation for their survival in the environment where they periodically come in contact with oxygen. Despite the discovery of SRB that can respire to a limited extent with oxygen, bacteria carrying out dissimilatory sulphate reduction in the presence of O_2 have not yet been identified (Marschall *et al.*, 1993).

Cypionka (2000) confirmed that SRB could remain viable in the presence of oxygen, and indicated that certain SRB species (*Desulfovibrio*) could even reduce oxygen to water. Community structure analysis and microbiological studies in the oxic zones and near the oxic/anoxic boundaries of their specific environment, suggested that the SRB were in fact especially adapted to oxygen.

1.2.2 Carbon and Energy Sources in Biological Sulphate Removal

1.2.2.1 Hydrogen

When hydrogen is utilized by the sulphate-reducing bacteria, it is similar in principle to that of aerobic forms of hydrogen oxidizers, although the carriers in electron transport and the hydrogenase enzymes differ (Caldwell, 1995). It was indicated that sulphate reduction is coupled to ATP formation by a PMF (proton motive force) derived from electron transport. Sulphate is therefore reduced in a stepwise fashion to H_2S by intracellular enzymes, necessitating transport of sulphate from the external environment by active transport, involving ATP expenditure. Inside the cell, sulphate is reduced to sulphite and H_2S by a collection of enzymes (ATP sulfurylase and adenoxine phosphosulfate (APS) reductase). Sulphide is then formed by the action of sulphite reductase, during which action sufficient energy is released for the formation of 2 to 3 ATP moles per mole of sulphite reduced. Hydrogen serves as the electron donor for the reduction of sulphite by the action of an electron transport chain. This requires the presence of nickel containing hydrogenase enzymes (Caldwell, 1995). The necessity of the presence of nickel should therefore be kept in mind when developing an efficient system for the use of hydrogen as electron source for the biological reduction of sulphate.

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Studies by van Houten (1996) demonstrated the optimisation of biological sulphate reduction using a gas-lift reactor, fed with hydrogen and carbon dioxide as energy and carbon sources, respectively. Of particular interest was biofilm formation, sulphide toxicity, sulphate conversion rate and gas liquid mass transfer limitations. It was shown that:

- The SRB formed stable biofilms on pumice particles.
- High free H_2S concentrations caused reversible inhibition rather than acute toxicity.
- If the H_2S concentrations were kept below 450 mg/L at a pH of 7.0, a maximum sulphate conversion rate of 30g SO_4^{2-} /(L.d) could be achieved.
- The gas to liquid hydrogen mass transfer capacity of the reactor determined the maximum sulphate conversion rate.
- A gas mixture of hydrogen and carbon dioxide (80% : 20%) was used to cultivate the hydrogen-consuming SRB.

Several reactor criteria were consequently identified:

- The pH should be between 7.0 and 8.5;
- Temperature: 25 - 35ºC;
- The $[H_2S]$ < 450 mg/L;
- The reactor should be set up to function under anaerobic conditions;
- Active biomass should be retained;
- Viable biomass should be immobilized;
- The contents of the reactor should be well-mixed and
- To maximize H_2 mass transfer, a high gas hold-up & low bubble diameter should be aimed for in the design of the reactor.

Van Houten (1996) achieved a sulphate removal rate of up to 30 g/(L.d). The results of these experiments revealed that the HSRB were not autotrophic and needed acetate as carbon source. It was assumed that acetate was formed by homoacetogenic bacteria from the available CO_2 and H_2.

Carbon monoxide (CO) can be metabolised by a number of naturally occurring microorganisms, along with water to produce hydrogen (H_2) and carbon dioxide (CO_2). Wolfrum *et al.* (2001) demonstrated this 'water-gas shift' reaction at ambient temperatures by

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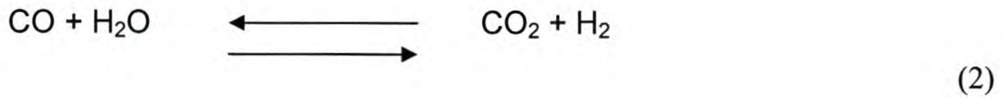
isolating *Rubrivivax gelatinosus* CBS2, a photosynthetic bacterial strain that can perform a reaction, which can be represented in the following stoichiometry:



$$CO + H_2O \rightleftharpoons CO_2 + H_2 \quad (2)$$

1.2.2.2. Bio-energy Sources

1.2.2.2.1 Biomass conversion

The production of bio-waste products by several industries can be used to the advantage of energy-consuming processes. This can be done in the form of renewable energy sources and bio-energy. The social benefits associated with the establishment of a biomass processing industry in South Africa will contribute to addressing important challenges such as the creation of rural employment. Lynd *et al.* (2002) suggested that this could be accomplished by building on South Africa's significant strengths, for example the country's large biomass production potential, as well as the expertise associated with the largest non-petroleum hydrocarbon processing industry in the world.

Plant material consists of primary components that can be described as lignocellulosic biomass. This biomass is composed of cellulose, hemicellulose and lignin. Cellulose is a primary component of most cell walls, and is made up of glucose (6 carbon sugar), arranged in bundles. Hemicellulose links these bundles, and is composed of Xylose (5 carbon sugar). Lignin is present in significant amounts and contributes largely to the structural strength of plants. The methods of extracting and dissolving the cellulose and hemicellulose have been improved over the last few years. This has resulted in the production of component sugars, which can be used as the energy source for organisms (Shleser, 1994). The primary use for plant biomass in Southern Africa, is for heating (fires) and commodity products (Lynd *et al.*, 2002).

Another source of energy for biological sulphate reduction could be lucerne, since it is produced at a large scale, mostly for feed for grass-eating animals – however this is likely to be an expensive option. Studies by Dill *et al.* (2001) showed that when using hay as the carbon and energy source, 99% SO_4 removal was obtained. A slightly lower percentage SO_4 removal (97.8%) was obtained when using Kikuyu grass.

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The Biosure process (Rose *et al.*, 2000) is an example of how sewage sludge disposal can be linked to AMD treatment. In this process, sewage sludge serves as the electron donor for the SRB, while it is simultaneously stabilized. The suspended solids settle and are recycled, while large particles are hydrolysed so that the small organic compounds become available for sulphate reduction. This process was implemented in a pilot-plant at Grootvlei Mine (South Africa), and proved to be a reliable method for treating mine drainage wastewater after 18 months of operation (Hulshoff Pol *et al.*, 2001).

Potato skin contains a large amount of nutrition and energy, mostly in the form of starch and cellulose, when compared to the amount of starch and cellulose found in the flesh of the potato. Therefore, this can be an excellent source of energy and sugars when hydrolysed. The big potato chips factories in South Africa typically produce 90 000 tons of potato peels per annum. However, the produced peels are not readily available as this kind of bio-waste product is used by pig-farmers.

In anaerobic bioreactors, the mineralisation or fermentation of organic material to methane (CH_4) and carbon dioxide (CO_2) is accomplished by a close cooperation of various metabolic groups of bacteria (Oude Elferink, 1998). Polymers are hydrolysed to mono and oligomers and then fermented to reduced organic compounds such as volatile fatty acids (VFAs), e.g. propionate and butyrate and to alcohols, lactate and succinate (Figure 1.3).

1.2.2.2.2 Volatile Fatty Acids

The use of a VFA mixture in a sulphidogenic granular sludge reactor was demonstrated by Lens *et al.* (1997). The VFA mixture consisted of a mixture of acetate:propionate:butyrate in the ratio 1:2:2 on COD basis at 30°C and pH 8, operating an UASB and an upflow staged sludge bed reactor. In this study, it was shown that the butyrate and propionate were utilized more rapidly than the acetate.

Omil *et al.* (1997) investigated the competition between acetate utilizing methane-producing bacteria and sulphate-reducing bacteria in mesophilic upflow anaerobic sludge bed reactors, operated at pH 8, and using volatile fatty acids as the carbon and energy sources. It was found that under a high reactor pH, a short solid retention time (<150 days) and the presence of a substantial SRB population in the inoculum, the time required for acetate-utilising SRB to out-compete MB was considerably reduced. It was also seen that more than 200 days of

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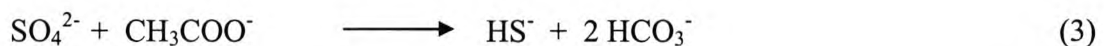
reactor operation was required for ASRB to out-compete AMB. It was further shown that the use of excess sulphate was also important to favour the sulphate reducers.

The SRB can be divided into two major groups, those that can completely oxidise organic matter to H_2S and CO_2, and those that carry out an incomplete oxidation, usually to acetate. Subsequently, the SRB compete with the methanogenic bacteria for both hydrogen/carbon dioxide, acetate and other substrates such as propionate, butyrate, and ethanol (O'Flaherty *et al.*, 1998).

De Smul and Verstraete (1999) showed that a maximum sulphate removal rate of 2.1 g/(L.d) could be achieved in an acetate-fed UASB reactor (see section 1.2.3 of this chapter). The reactor pH was kept between 8.0 and 8.5. It was found that if calcium-containing tap water was used, sulphide was produced, while demineralised tap water promoted methane production. The amount of COD added (in the form of acetate) was calculated per gram sulphate, which needed to be reduced, according to the stiochiometry for sulphate reduction by ASRB:



$$SO_4^{2-} + CH_3COO^- \longrightarrow HS^- + 2\,HCO_3^- \qquad (3)$$

The researchers concluded that low Ca^{2+} concentrations favoured dominance by acetate utilising MB (AMB) at the expense of acetate utilizing SRB (ASRB). It was speculated that the effect of calcium on the retention of ASRB in that type of reactor was probably calcium ions bridging between the cells (De Smul and Verstraete, 1999).

According to Hulshoff Pol *et al.* (1998), ASRB have a thermodynamic and kinetic advantage over AMB in their competition for acetate. However, these observations were less predictable in modern high-rate anaerobic reactors, with sludge retention based on sludge immobilisation. Therefore, besides the growth kinetics, many other factors influence the competition between ASRB and AMB.

Middleton and Lawrence (1997) used acetic acid as organic carbon source for SRB. In these experiments, it was concluded firstly that a monod-type kinetic growth model could be used to describe the growth of ASRB in continuous culture over the temperature range of 20 - 31°C. Secondly, the effect of temperature on the growth of SRB over the above mentioned

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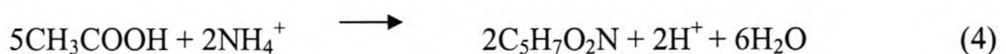
range (20 - 31°C), could be described by an Arrhenius type relationship i.e. any factor which increases the rate of collisions, e.g. increased concentration of the reactants or increased temperature, will increase the reaction rate. Thirdly, if sulphate concentrations were greater than 50 mg/L (as S at 20°C) or 10 mg/L (as S at 25°C and 31°C), the growth of acetate utilizing SRB is independent of sulphate concentration.

Middleton and Lawrence (1977) used the empirical formula $C_5H_7O_2N$, for bacterial cells, to compile the following equation to describe the synthesis of acetate utilizing bacterial cells from acetic acid:

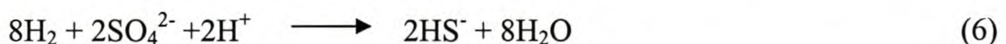


$$5CH_3COOH + 2NH_4^+ \longrightarrow 2C_5H_7O_2N + 2H^+ + 6H_2O \quad (4)$$

1.2.2.2.3 Sugar

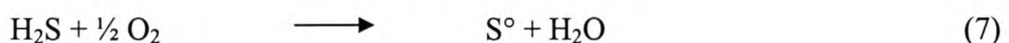
When sugar is the carbon and energy source, the SRB can utilize the sugar and produce hydrogen according to the following reactions (Greben, *et al.* 2000):

$$C_{12}H_{22}O_{11} + 5H_2O + 4SO_4^{2-} \longrightarrow 4CO_2 + 8H_2 + 4HS^- + 8HCO_3^- + 4H^+ \quad (5)$$



$$8H_2 + 2SO_4^{2-} + 2H^+ \longrightarrow 2HS^- + 8H_2O \quad (6)$$

In the presence of air, the produced sulphides can be oxidised by sulphide oxidising bacteria to form elemental sulphur, according to reaction (7) (Greben, *et al.* 2000):



$$H_2S + \frac{1}{2} O_2 \longrightarrow S^\circ + H_2O \quad (7)$$

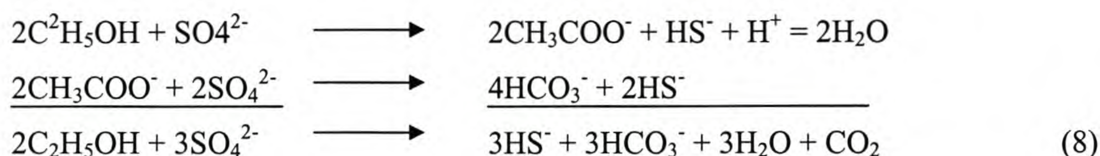
In the study by Greben *et al.* (2000), sugar utilization was compared to ethanol and methanol utilization, and it was found that sugar is a suitable carbon and energy source. The maximum volumetric and specific sulphate reduction rate for sugar was determined to be 10.4 g SO_4/(L.d) and 0.79 g SO_4/(g VSS.d), respectively. Optimum utilisation of the carbon and energy sources was also obtained at shorter residence times (less than 8 h). At longer residence times (longer than 8 h), sulphate-reducing bacteria were out-competed by methanogenic bacteria.

1.2.2.2.4 Ethanol

De Smul *et al.* (1997) demonstrated that ethanol could be used successfully as the carbon and energy source when applying biological sulphate reduction technology. They demonstrated ethanol usage by SRB in a mesophilic (30-35̊C), sulphidogenic expanded-granular-sludge-blanket reactor, removing 80% sulphate at loading rates of up to 10 – 12 g SO_4/(L.d). In these experiments, $CHCl_3$ was injected directly into the reactor to inhibit methanogenesis. The pH was between 7.7 and 8.3 and the ratio of ethanol to sulphur was near stoichiometry.

Additionally, it was indicated that the SRB growing on ethanol and acetate were retained in the reactor at a v_{up} of 3.5-5.0 m/h and were not selectively washed out as was expected. It was shown that, at alkaline pH, the activity of the acetotrophic sulphate-reducing bacteria (growing on acetate) was strongly enhanced, whereas at pH below 7.7, the acetotrophic sulphate-reducing bacteria were inhibited by aqueous H_2S.

Greben *et al.* (2000) also demonstrated the use of ethanol as carbon and energy source in a sulphidogenic, anaerobic single-stage bioreactor. They obtained a sulphate reduction rate of 4.8 g SO_4/(L.d) and 2.8 g SO_4/(g VSS.d) respectively. The reaction is as follows:



$$2C^2H_5OH + SO4^{2-} \longrightarrow 2CH_3COO^- + HS^- + H^+ = 2H_2O$$
$$\underline{2CH_3COO^- + 2SO_4^{2-}} \longrightarrow \underline{4HCO_3^- + 2HS^-}$$
$$2C_2H_5OH + 3SO_4^{2-} \longrightarrow 3HS^- + 3HCO_3^- + 3H_2O + CO_2 \qquad (8)$$

Research by Shleser *et al.* (1994) focused on the production of ethanol for the state of Hawaii, where there was an interest in establishing a new industry that would develop and use locally produced renewable fuels, and thereby reducing the dependence on imported petroleum. Ethanol production could help to transform organically based municipal solid waste materials to a higher value product, while at the same time reducing the negative impacts on the environment. In addition, such initiatives would attract the private sector and government investment in biomass energy projects in Hawaii. These principles could also be applied in South Africa. Ethanol can be used widely as a bio-energy. For example in biological sulphate reduction technologies, ethanol can be utilized as the carbon and energy source for the sulphate reducing bacteria. At the CSIR demonstration plant in Witbank, South Africa,

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the sulphate reduction technology is currently applied, where ethanol is the source of carbon and energy in a single stage reactor system (Greben *et al.*, 2000).

Traditionally, ethanol is produced from fermentation of sugars and the process usually consists of a pre-treatment step and a fermentation step. These sugars are typically obtained from soluble forms, such as sucrose, molasses from sugar cane, or fructose from corn. These soluble sugars are edible, coming also from the edible part of the plant, while the rest of the plant was considered waste materials. Over the last few years, however, research showed that the production of ethanol could also originate from sugars, produced from agricultural by-products such as corn stover, bagasse, yard and wood waste, etc. (Shleser *et al.*, 1994).

Ethanol production is a promising way to obtain bio-energy from bio-waste, and it could therefore be considered as an approach to fuelling biological sulphate reduction.

1.2.2.2.5 Other

De Smul and Verstraete (1999) showed that in a formate-fed, sulphate-reducing, expanded granular-sludge-blanket reactor operated at pH 8.25 to 8.65, a maximum sulphate removal rate of 9.5 g/(L.d) could be achieved at the slowest upflow velocity tested (3.0 m/h). However, this could only be achieved if methane-producing bacteria were repressed by two consecutive doses of 3.85 g/L 2-bromo-ethane sulfonate.

Several SRB are known to oxidize aromatic compounds including carboxylated, hydroxylated, methoxylated and aminated compounds. One pure culture, *Desulfomonile tiedjei* is also known to carry out reductive dehalogenation under sulphate reducing conditions. It was also shown by Drzyzga *et al.* (1994), that 4-fluorobenzoate could be mineralised incompletely in a benzoate-degrading, sulphate-reducing diculture, with the release of acetate. The produced acetate was subsequently used by two sulphate-reducing strains. A symbiotic relationship between these strains is therefore likely, in which the vibrio-shaped bacteria might profit from the acetate released.

Other electron donors that could be used as electron donors for growth include benzoate, 3- and 4-hydroxybenzoate, protocatechuate, catechol, phenol, 2,5-dimethoxyphenol and fatty acids up to C_8.

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1.2.3 Reactor systems for biological sulphate removal

Several anaerobic reactor systems can be used for biological sulphate removal from wastewater. A few basic reactor types described in literature are discussed below. It should be noted that variations on these designs are commonly found.

1.2.3.1 Passive treatment

Wastewater containing acid and metals can be treated using wetlands that are either constructed or natural. This treatment method is a passive, low-cost approach and has been applied worldwide. Anaerobic wetlands are based on sulphate reduction and the subsequent precipitation of metal sulphides. Examples of carbon sources often used to fuel this process include manure, compost, natural vegetation and sawdust (Johnson, 2000; Hulshoff Pol *et al.*, 2001).

A passive treatment (wetland) system was constructed at the former Wheal Jane tin mine in the UK, for the treatment of mine's drainage water (Johnson, 2000). The plant was based on a combined treatment in an aerobic constructed compartment and an anaerobic, buried compartment to exclude oxygen introduction through plant roots. The plant promoted aerobic iron and arsenic removal and anaerobic precipitation of copper, cadmium and zinc as metal sulphides, using hay, sawdust and manure as electron donors and sources of SRB for the reduction of sulphate to form sulphide. A limitation of this system is the large area required for treatment, which is not always available in practice.

Another example of a passive system for the treatment of acid mine water is at the Arnot Colliery in South Africa (Pulles *et al.*, 2001). A diagrammatic representation of the sulphate reducing units (SRUs), constructed as part of the pilot plant of this system, is shown in Figure 1.4. The diagram shows that the SRUs were designed to achieve extensive contact between the sulphate containing influent (mine water) and the carbon sources, which included sewage sludge, cow manure and hay. The SRUs were constructed in the form of an inverted truncated prism, containing a synthetic liner over the natural ground level and over the top of the horizontal flow units to exclude oxygen. The influent mine water was distributed across the top of the SRU and the water then migrated down into the carbon substrate. Stone

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packing was placed around the bottom collection manifold to prevent the clogging of the pipe work with carbon substrate (Pulles *et al.*, 2001).

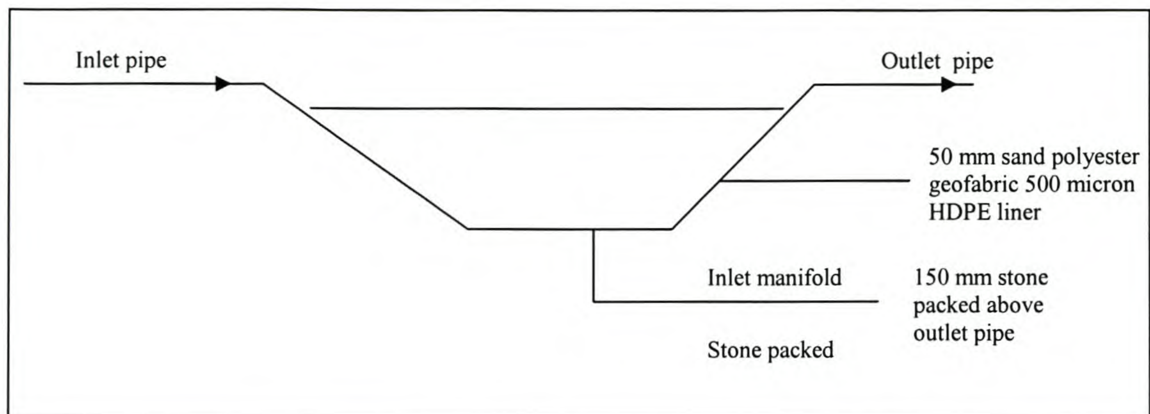

Figure 1.4: A cross section of the sulphate reducing units (adopted from Pulles *et al.*, 2001).

1.2.3.2 Upflow Anaerobic Sludge Bed (UASB) reactor

After the development of high-rate anaerobic treatment systems in the 1970s and dense granules in UASB reactors (Lettinga *et al.*, 1980), the cost of anaerobic treatment was reduced. Subsequently, anaerobic treatment of wastewater became more attractive. Since then, the UASB reactor has found wide application in anaerobic wastewater treatment and is, according to Visser (1995), the reactor type with the widest application.

In practice, UASB reactors can be applied for the treatment of wastewaters with low strength wastes, such as brewery waste (up to 1500 mg/L COD), to higher strength wastes (for example 10-15g/L COD) (O'Flaherty *et al.*, 1998) because of the ability to treat the latter at a high rate (Harada *et al.*, 1994). A typical UASB reactor consists of a cylindrical tube, which is topped by a spherical three-phase separator. Liquid recirculation is applied in order to achieve an up flow, which can create a continuous granular bed expansion (therefore, this system is also referred to as an expanded granular sludge bed reactor or EGSB) (Dries *et al*, 1998). An example of an EGSB reactor can be seen in Figure 1.5.

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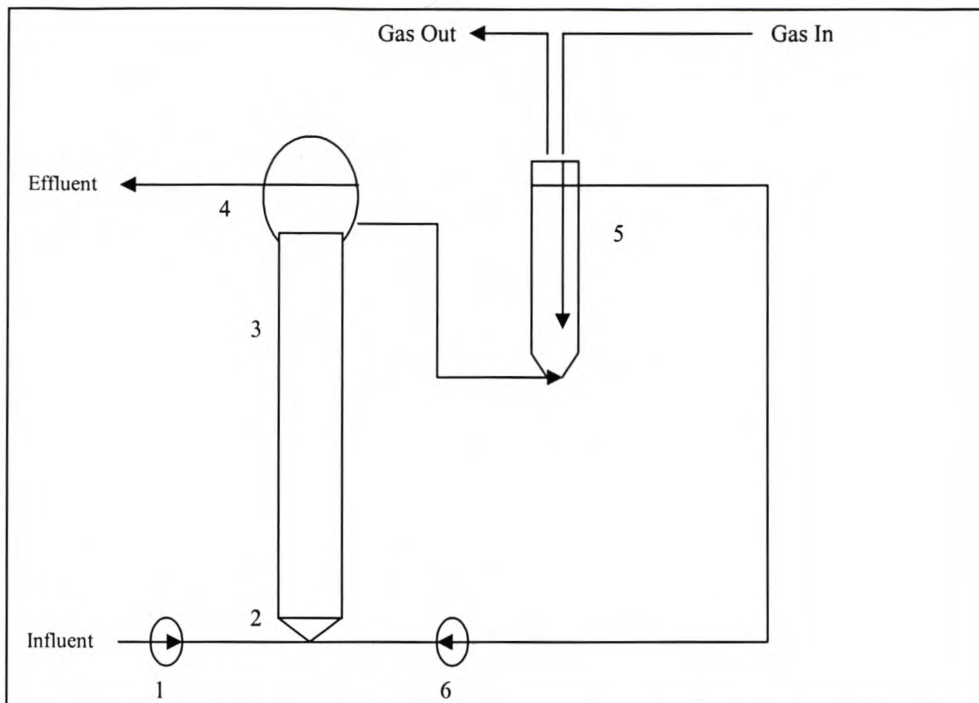

Figure 1.5: Configuration of an externally stripped sulphidogenic EGSB reactor: 1 Influent pump; 2 liquid distribution cone; 3 expanded sludge blanket; 4 sludge decantation sphere; 5 stripping unit; 6 recycle pump (adopted from De Smul *et al*., 1997).

The influent is pumped continuously in the recycle stream at the bottom of the reactor. Biomass retention can be enhanced by introducing a deflector in the recirculation stream, which essentially is an upward curving of the effluent tubing to avoid excessive biomass-washout. The head-space of the reactor can be connected to a column filled with an acidic solution, to allow monitoring of the rate of biogas formation and the composition thereof (Dries *et al*, 1998). In this reactor type, high sludge retention can also be achieved if the formation of a well settleable, highly active granular sludge is possible (Visser, 1995). Therefore, granular sludge is usually utilised to initiate sulphate reduction in this type of reactor. Alphenaar *et al*. (1992) found that the granulation process of the organisms could be favoured by a high upward velocity and a short hydraulic retention time.

It was shown in propionate metabolism that reactors containing aggregates or biofilms (for example UASB, fixed film or plug flow reactors) had an advantage over CSTR (continuously stirred tank reactors, or completely mixed) reactors. The reason for this was the consequential close proximity of the H_2-producers to the H_2 utilizers (Speece, 1996).

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1.2.3.3 Completely mixed reactor

A completely mixed reactor (Figure 1.6) usually consists of a cylinder or tank, containing sludge and influent, completely mixed with a stirrer (continuously stirred tank reactor or CSTR) and is often heated (Held *et al.*, 2001).

These reactors can also have fixed, floating or gas holding covers. Mixing can be achieved in several ways by using mechanical means, including recirculation pumps or gas (Dennis *et al.*, 2001).

An example of the use of a completely mixed reactor system is the biological treatment of acid mine effluent (Figure 1.2 and 1.6), where sulphate is converted via sulphide to sulphur in an anaerobic single-stage reactor (Greben *et al.*, 2000). In this system, ethanol was used as the carbon and energy source. A schematic diagram of the reactor that was used for this process is shown in Figure 1.6. A gravity clarifier, which is a method for biomass immobilization in anaerobic CSTR, can also be seen in Figure 1.6 (Speece, 1996).

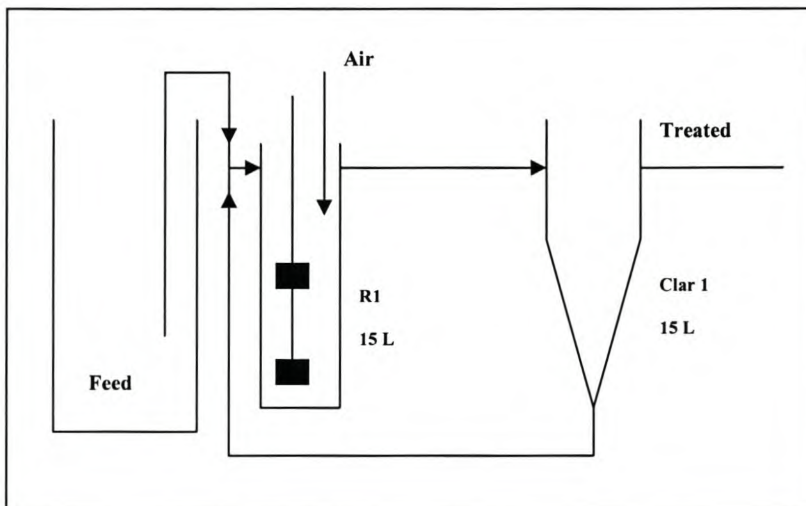

Figure 1.6: Completely mixed reactor system (Adopted from Greben *et al.*, 2000)

1.2.3.4 Anaerobic filter (AF) and Packed Bed Reactors (PBR)

Anaerobic filter reactors can be constructed to enable either upflow or downflow (UFAF and DFAF, respectively). These reactors contain anaerobic filters and can be filled with materials such as glass beads, plastic beads, sand or other support media that can serve as immobilisation materials for retention of the biomass (Held *et al.*, 2001). In some cases,

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anaerobic reactors can be called 'high rate digesters', since they operate at reduced hydraulic retention times from 20 days to a few hours (Dennis *et al.*, 2001). The upflow filter configuration usually allows for the majority of biomass to remain unattached under the non-turbulent conditions afforded by the packing (Speece, 1996). Two AF reactors can also be connected in series for improved reactor performance (Young *et al.*, 1989).

O'Flaherty *et al.* (1998) described an example of an anaerobic digester, treating citric acid production wastewater, randomly packed with polypropylene cascade rings (0.175 m x 0.05 m). The reactor was called a '*full-scale upflow fully-packed digester*'. Another example of a reactor containing a fixed-bed in the form of polyethylene cascade rings, treating sulphate-containing citric acid production wastewater, is mentioned by Colleran *et al.* (1998). A schematic representation of the reactor is shown in Figure 1.7. In the diagram, it can be seen that the reactor is a combination of an UASB and an AF reactor, containing a granular sludge bed in the lower section and an upper filter section, consisting of randomly-packed polyethylene cascade rings. Detailed analysis of the biomass after 5 years of operation indicated that hydrogen and propionate-utilising SRB had out-competed the hydrogenophilic methanogens and propionate syntrophs (Colleran *et al.*, 1998).

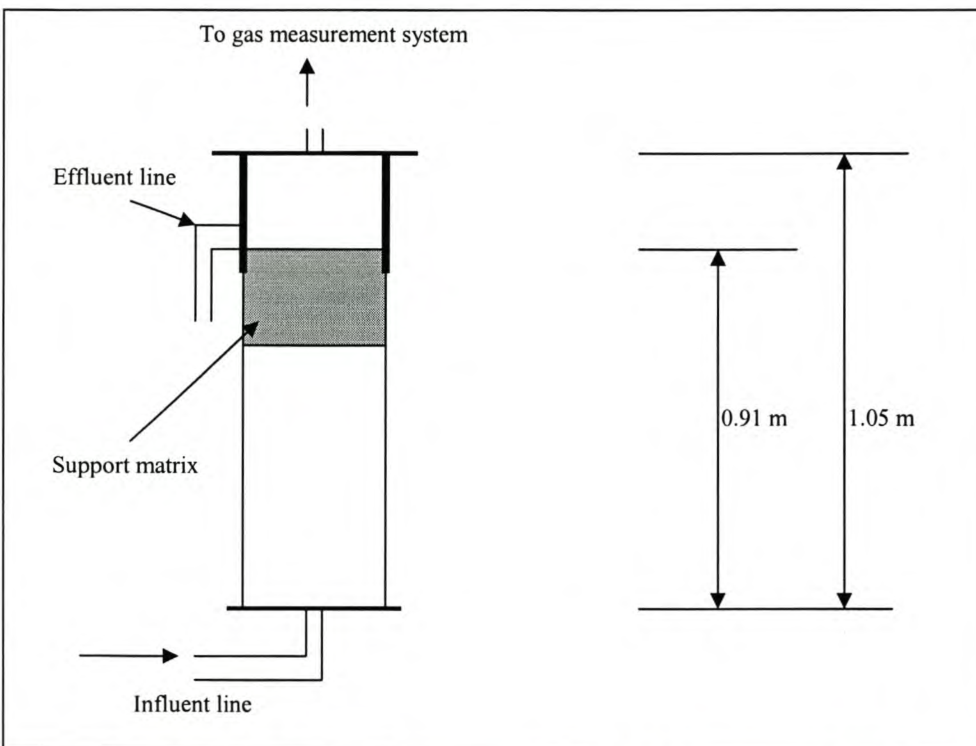

Figure 1.7: Schematic diagram of laboratory-scale hybrid reactor used for thermophilic anaerobic treatment of simulated citric acid production wastewaters (Adopted from Colleran *et al.*, 1998).

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When immobilization particles, such as sand, coal, granular activated carbon, reticulated polyurethane foam, fired clay or porous glass beads are added to a CSTR and fluidised (mixed or stirred), the reactor is often called a fluidised bed reactor. However, for this type of reactor, equipment for media/biomass separation must be located directly downstream from the reactor to avoid biomass loss (Speece, 1996).

1.2.3.5 Gas-lift Reactor

Van Houten (1996) used a gas-lift reactor to enable the use of hydrogen or coal gas as the energy source for biological sulphate removal. The reactor design is indicated in Figure 1.8. The reactor consisted of a plastic internal draft-tube, containing the biomass, immobilised on pumice particles, and an outer mantle, containing water at a temperature of 30 °C. The pH was controlled to be constant at 7.0 with a pH controller, while the gas and the feed were recycled.

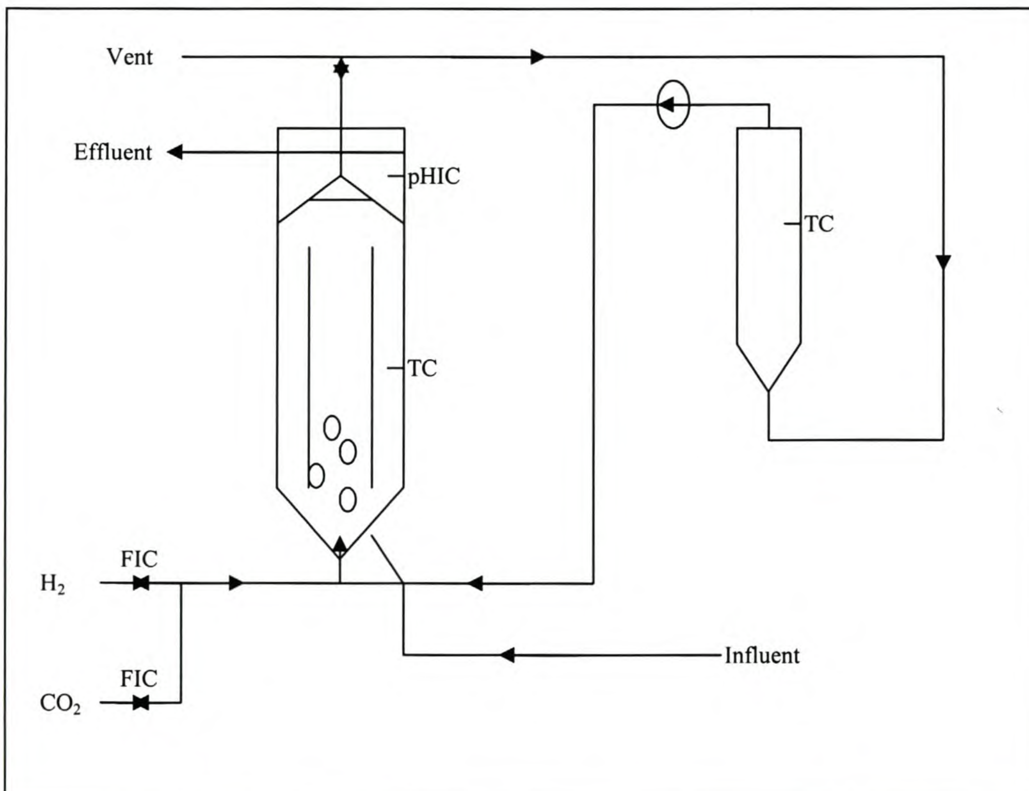

Figure 1.8: Diagrammatic representation of the gas-lift reactor: FIC: flow indication and control; pHIC: pH indication and control; TC: temperature control (Adopted from Van Houten, 1996).

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1.2.3.6 Sequential Batch Reactor (SBR)

Sequencing batch reactors are a type of a contact digester, which can utilize the same tank for digestion and separation (Dennis *et al*., 2001). The reactor generally consists of two or more columns, which can be operated with several cycles. Each cycle can consist of several stages, such as a feeding stage, where the column is filled; a reaction stage, where the biological reaction takes place; and draw stage, where the treated water is discharged so that the column can be filled again and the cycle repeated (Qin *et al*., 2004). The separation of the biomass is accomplished by gravity (Dennis *et al*., 2001).

According to Speece (1996), batch feeding results in kinetic and performance advantages as compared with CSTR operation. The time required for the reaction step depends on parameters, such as substrate characteristics and strength, required effluent quality, biomass concentration and waste temperature.

The venturi reactor, used in this study (Figure 2.1), was often operated in batch mode, where the reactor was filled with the feed water, mixed and recycled with the Hydrogen/CO_2 gas mixture for a period of 24 hours. Thereafter, the treated water was drained from the reactor and the reactor could be filled again with feed water. The advantage of using this type of reactor is that the feed water composition could be changed with each cycle, to determine the effect of the changes applied.

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1.2.4 The specific objectives of this study were:

1. The design of a stable, continuously operated biological sulphate removal reactor, using a gas mixture consisting of hydrogen and carbon dioxide as energy and carbon sources, respectively.

2. The maintenance of the biological sulphate removal reactor and the evaluation of the reactions involved, by determining the influence of hydrogen dosage, reactor temperature, pH, initial alkalinity, sulphate and sulphide concentration as well as the addition of trace elements on the sulphate removal ability of the system.

3. To observe microbial behaviour in the reactor with regards to biofilm formation and community stability.

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CHAPTER 2: MATERIALS AND METHODS

2.1. BIOREACTOR STUDIES

2.1.1 Reactor design

To apply the biological sulphate removal technology, using H_2 and CO_2 as energy and carbon source, the following key parameters were addressed in the design of a venturi reactor:

- The hydrogen gas solubilization and mass transfer were optimised by using a venturi device in a tall reactor, without the introduction of air.

- The reactor contained geotextile as a support medium for bacterial attachment, (*Geotextile Africa, 122 Charles street, Bridell, Kemptonpark*) and biofilm formation. Geotextile is a coarse, fibrous material, normally used in road construction. It was anticipated that microbial biofilms could form on the long geotextile strips.

During these studies, two identical reactors were operated (R1 and R2) (Figure 2.1, Table 2.1): The height of each reactor was 6 m; the total volume 190 L and the active volume 157 L. A gas mixture (80% H_2 : 20% CO_2) was introduced into the reactor through a venturi system. The delivery rate of the gas was controlled using a Watson-Marlow pump (*Watson-Marlow Bredel SA, Honeydew, Gauteng, SA*).

Sulphate rich feed water was fed into the reactor from the top, while the gas was pumped into the recycle line, thus providing efficient contact between the gas and liquid. Reactor R1 was packed with 8% geotextile strips and reactor R2 with 80% geotextile strips and sheets. R1 was initially used in continuous operational studies, while R2 was used for biofilm and kinetic batch studies.

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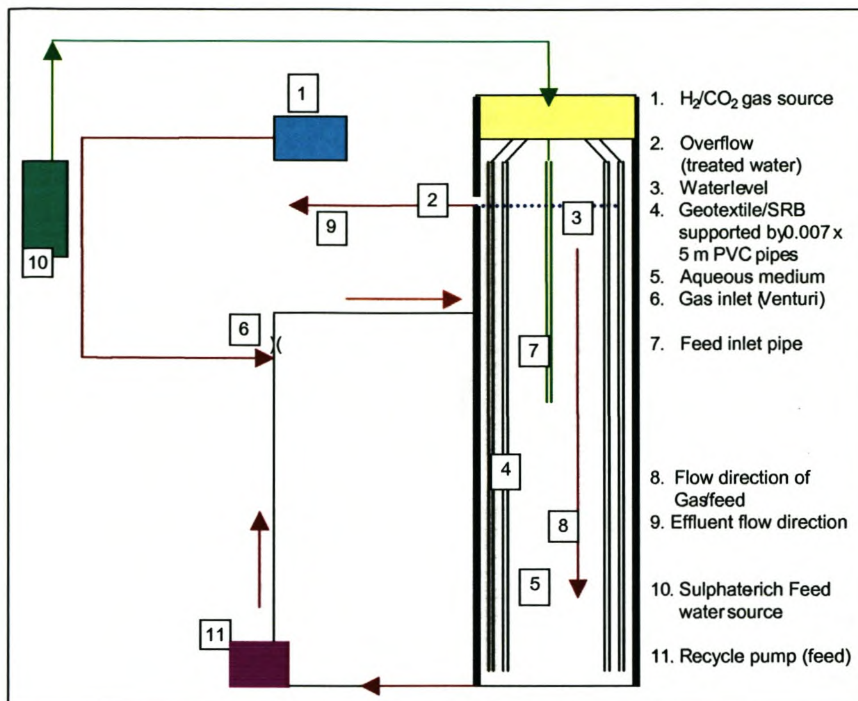

Figure 2.1: A schematic representation of the venturi reactor for sulphate removal by SRB. The total length of the reactors was 6m.

Table 2.1: The venturi reactor dimensions

Description	Unit	Measurement
Total volume:	L	190
Active volume:	L	157
Length thick pipe:	m	6
Length thin pipe:	m	5
Thick pipe radius:	cm	10
Thin pipe radius:	cm	2.5
Geotextile % of active volume R1:	%	8
Geotextile % of active volume R2 and R3	%	80

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2.1.2 Reactor Feed

The feed water contained 1700 mg/L sulphate and was prepared as described in Table 2.2.

Table 2.2: Composition of feed water

$MgSO_4$:	3.8 g/L
$(NH_4) SO_4$:	200 mg/L
$FeSO_4$:	2 mg/L
H_3PO_4:	50 mg/L
$NaHCO_3$	500 mg/L
Nutrients:	1 ml/L
Nutrient solution	
$MnCl_2.4H_2O$:	1 µg/L
$ZnCl_2$:	1 µg/L
Na_2MoO_{24}:	1 µg/L
H_3BO_3:	1 µg/L
$CuCl_2.2H_2O$:	1 µg/L
KCl:	1 mg/L
$FeCl_3.4H_2O$:	1 mg/L
$NiNO_3.6H_2O$:	1 mg/L
$CoCl_2.6H_2O$:	1 mg/L

During the batch studies, the pH was controlled by using NaOH or HCl. The alkalinity was raised by using $NaHCO_3$. The sulphide concentration was increased by adding Na_2S to the feed.

2.1.3 Biomass

The reactors were inoculated with the following sludge:

- Anaerobic digester sludge, obtained from the Daspoort Sewage Plant, Pretoria, South Africa as the inoculum for the reactor (50% of the total sludge added).
- Sulphate reducing bacteria mixture from the CSIRosure demo plant, Navigation Mine, Witbank, operating with sugar as the carbon and energy source (50% of the total sludge added).

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2.1.4 Routine analysis

The sulphate, sulphide, alkalinity, COD, and pH were manually determined according to the analytical procedures as described in Standard Methods (APHA, 1985). The analyses were all carried out on filtered samples, except for the feed COD and the sulphide samples. The alkalinity of the samples was determined by titrating with 0.1N HCl to a pH of 4.2. The COD samples were pre-treated with a few drops of H_2SO_4 and N_2 gas to correct for the COD value caused by the sulphide concentration. GC analyses were occasionally done to analyse the VFA content (see Appendix A1).

2.1.5 Reactor operation

2.1.5.1 Reactor 1 (R1)

Reactor R1 (8% geotextile) was operated continuously for 120 days. The hydraulic retention time (HRT) was kept constant at 24 h. The amount of hydrogen added was constantly an average 3 times the amount of hydrogen needed stoichiometrically to reduce the sulphate in the feed.

Due to variance in the ambient temperature, experiments were carried out at two temperatures, namely 21.2 and 35 °C. The duration of these experiments ranged between 9 and 19 days at each temperature.

2.1.5.2 Reactor 2 (R2)

R2 was operated continuously at a HRT of 9 hr for 35 days. The sulphate concentration, sulphide concentration, alkalinity and hydrogen introduction rate were kept constant. The temperature changed gradually with an average of 10 °C during the course of the experiment, because of pump heat build-up as well as an increase in the ambient temperature.

In a subsequent experiment, kinetic data were collected by operating the reactor in batch mode. The reactor was operated in continuous mode until stable (90%) sulphate removal was achieved, and then switched to batch mode. This involved draining the reactors, followed by filling with feed water (Table 2.2). Filtered and unfiltered samples were thereafter collected at regular intervals.

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The volume of gas mixture introduced into R2 was 700 ml/min (0.6 g/L H_2). Accordingly, the amount of gas introduced was 4.5 times the stoichiometric amount needed for total sulphate reduction per litre of feed water (0.13 g/L). This amount of gas oversupply was chosen (and not a higher amount), for both practical and safety reasons.

The batch experiments were carried out to determine the effect of the following parameters on the rate of sulphate reduction: temperature (29.5 °C; 39 °C); pH (7.2, 7.5, 8.0); sulphate concentration (1500; 4000 mg/L); calcium sulphate, alkalinity (230; 290; 1150; 1600 mg/L); sulphide concentration (0; 100; 268 mg/L); cobalt concentration (0.24µg/L; 1; 3 mg/L); nickel concentration (0.26µg/L; 3 mg/L); and hydrogen applied/ hydrogen stoichiometrically required (ratios of 1.5; 4.5; 6). When each different parameter was changed, all the other parameters listed were kept constant for comparison purposes. Each test was done in duplicate. The first set of results was obtained during the first temperature period, P1 (29.5 °C), while the second set of batch test results were either obtained during P1 or the second temperature period, P2 (39 °C).

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2.2 MICROBIAL BEHAVIOUR

2.2.1 Attachment to geotextile

A LEO 1430VP scanning electron microscope (SEM) was used to observe the microbial attachment and biofilm formation on the geotextile filaments. The high magnification SEM images were also used to visualise the geotextile structure and to speculate on the suitability of geotextile as an immobilization matrix for the SRB and biofilm development.

Wet samples of geotextile from R2 were mounted onto microscope stubs, and gold-coated under vacuum for visualization of the biofilm on the geotextile structures.

Despite the high resolution of SEM, and thus the ability to gain insight in the ultra-structure when applying this technique, SEM images do not provide information on the metabolic state of the microbes. Since only the live bacteria contribute to the substrate removal activity within the biofilm, it would be beneficial if an additional technique capable of indicating the relative amount of live and dead cells of the biofilm (on the geotextile) in the venturi reactor was used.

This was achieved by using an epifluorescent microscope in combination with a LIVE/DEAD *Bac*Light Bacterial Viability Kit (*Molecular probes, laboratory specialist services, Clareinch, SA*), which showed the live and dead bacterial cells as green and red cells, respectively. The kit consisted of two fluorescent nucleic acid stains: SYTO 9 green fluorescent nucleic acid stain and propidium iodide fluorescent nucleic acid stain. The propidium iodide penetrates only bacteria with damaged membranes, causing them to stain red, while the bacteria with intact cell membranes stain fluorescent green. The excitation/emission maxima for SYTO 9 stain are 480/500 nm, and 490/635 nm for propidium iodide. The staining suspension was prepared according to manufacturer's instructions, which included the combination of 3µl of component A and 3µl of component B and the addition of 994µl DI water for a total volume of 1ml.

Small geotextile samples were removed from the reactor, immersed in 1ml of the *Bac*Light staining solution and incubated overnight in the dark. Biofilm formation on the geotextile

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was then observed using a Nikon Eclipse E400 epifluorescent microscope equipped with excitation/barrier filter sets of 465-495/515-555 nm and 540-580/600-660nm.

2.2.2 Terminal restriction Fragment Length Polymorphisms (T-RFLP)

T-RFLP has been applied as an analytical technique for quantitative microbial community diversity analysis in various environments (Liu *et al.*, 1997). This approach was used in the present study to evaluate community stability in a venturi reactor. Four samples were analysed from R2 for this purpose: on day 370 (sample 1), day 375 (sample 2), day 377 (sample 3) and day 475 (sample 4) of reactor operation.

For the small-scale preparation of bacterial genomic DNA and subsequent t-RFLP analysis, modifications (personal communication; Dr MN Gardner) of previously described methods (Liu *et al.*, 1997; Blackwood *et al.*, 2003; Egert and Friedrich, 2003) were followed.

Two ml of each DNA sample was centrifuged for 10 minutes at 6 000 rpm, the supernatant removed and the pellet suspended in 565µl 10 mM Tris buffer pH8 and 5µl Lysozyme (50 mg/ml). This resuspended solution was incubated at 37 °C for 30 minutes. Thereafter, 5µl proteinase K (20 mg/ml) and 30 µl 10% SDS was added to the solution and incubated at 45 °C for an hour. After the incubation, 100µl of 5M NaCl was added, mixed and then 80µl NaCl/CTAB solution (4.1 g NaCl and 10 g CTAB in 100 ml distilled H_2O) was added, and the solution incubated again at 65° C for 25 minutes.

After incubation, an equal volume of chloroform/isoamyl alcohol (24:1[v/v]) was used to extract the CTAB-protein/polysaccharide complexes. The top aqueous supernatant was then transferred to a fresh tube, and the DNA was precipitated by adding a 0.6 volume of isopropanol, mixing and centrifuging the solution for 20 min. at 15 000 rpm. Following centrifugation the pellet was washed in 200 µl 70% ethanol. After the ethanol was extracted, the DNA was resuspended in 50 µl Tris buffer pH 8. The presence of genomic DNA and the estimation of the product concentration were confirmed by a 1% agarose gel.

One µl of the resuspended DNA was then added to the PCR master mix. The PCR reaction mix had a total reaction volume of 50 µl and contained (with final concentrations indicated): 5µl buffer, 4µl dNTP's (0.2 mM), 2 µl MgCl (1 mM), 0.5 µl forward primer 341F (0.5 µM),

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0.5 µl reverse primer 1387R (0.5 µM), 0.2 µl Taq polymerase, 33.8 µl nuclease free water and 4 µl template DNA. The PCR was performed with a *GeneAmp 2700* thermocycler *(PCR systems, Applied Biosystems (AB))*, which was set for an initial increase in temperature to 94 °C for 2 minutes in the first stage. The second stage consisted of 30 cycles of the following: 94 °C for 30 seconds, 56 degrees for 30 seconds and 72 °C for 1minute and 30 seconds. The third stage consisted of 4 minutes at 72 °C and during the final stage, the temperature decreased to 4 °C, where it was ready for further steps.

After the PCR reaction, the efficiency of the procedure was tested by running 5µl of the PCR product on a 1% agarose gel, stained with Ethidium bromide (10mg/ml). After the confirmation of the presence of amplified DNA, the DNA bands of interest were excised from the agarose gel, placed in an eppendorf tube, 400µl 6M NaI added and incubated at 55°C for 5-10 minutes. Subsequently, 7-10µl silica was added to the solution, incubated for 20 minutes on ice, centrifuged for 30 seconds and the supernatant was discarded. Then 500µl new wash solution (235ml EtOH, 25ml 1M NaCl, 2.5ml 2M Tris (pH 8.0), 2.5ml 0.5M EDTA, in 500ml ddH_2O) was added to the pellet and centrifuged at 15 000 rpm for 30 seconds. The supernatant was again discarded, and the last step was repeated four times. The pellet was resuspended in 7µl dH_2O, incubated at 55°C for 5-10 minutes and centrifuged for 30 seconds. Finally, the supernatant was transferred to a fresh eppendorf tube.

The purified DNA was digested with the *Rsa*I, *Msp*I and *Hha*I restriction enzyme in their buffers for 2 hours at 37 °C in 20-30µl total volume (Roche Molecular Biochemicals) (10 µl of the amplified, purified DNA was then added to 1µl of the enzyme, 2µl of the buffer and 7µl water and the solution was incubated at 37 °C for 3 hr). The restriction enzyme was thereafter heat inactivated at 70°C for 10 minutes.

After the restriction enzyme digestion, the DNA was purified again by using CentriSep spin columns from *Princeton Separations* (eluate was 20-30µl dH_2O containing the DNA fragments). For this purpose, sephadex gels were prepared so that the DNA could move through the gel beads, while the salts and proteins remained in the beads.

The purified DNA was sequenced by injecting 2-3µl DNA into a LI COR *genetic sequencer*.

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CHAPTER 3: RESULTS AND DISCUSSION

3.1. BIOREACTOR STUDIES

3.1.1 Reactor 1

3.1.1.1 Temperature

Table 3.1 shows an increase in the rate of sulphate reduction from 588 mg SO_4^{2-}/(L.d) at 21.2°C to 925 mg SO_4^{2-}/(L.d) at 35 °C. Figure 3.1 shows the difference in SO_4^{2-} concentration between the feed (influent) and the effluent.

Table 3.1: Effect of Temperature on SO_4 removal rate.

Determinant	Unit	Temperatures (°C)			
		21.2	std dev	35	std dev
Feed:					
Sulphate	mg/L	1481	454	1133	202
Alkalinity	mg/L	203	100	317	112
pH		7.1	0.1	7.1	0.1
Treated:					
Sulphate	mg/L	894	404	192	193
Alkalinity	mg/L	919	104	1281	160
pH		7.8	0.9	8.1	0.3
Sulphides	mg/L	198	50	196	123
Rates:					
Sulphate reduction	mg/(L.d)	588	250	925	52
Ratios:					
S^{2-}produced/SO_4^{2-} removed		0.3		0.2	
Alkalinity/SO_4^{2-} removed		1.2		1.0	

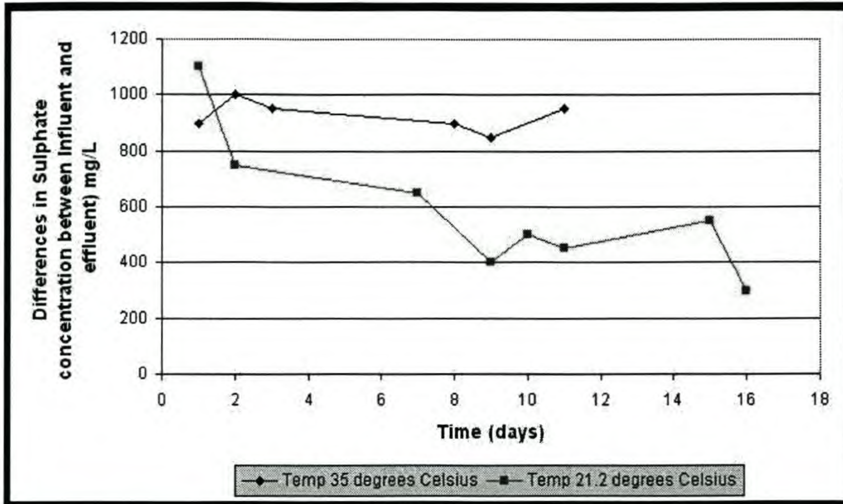

Figure 3.1: Effect of temperature on sulphate removal in the venturi reactor.

Studies by Lens *et al.* (1998) showed that mesophilic SRB and MB have similar temperature ranges, and therefore respond to similar temperature conditions. However, SRB appear to be less sensitive to temperature changes than MB (Lens *et al.*, 1998). Even though methanogenesis was not measured here, these results demonstrate the influence of temperature on sulphate removal: more efficient sulphate removal was observed at temperatures close to the mesophilic range (35 °C) than at lower temperatures (21.2 °C).

3.1.1.2 Sulphide production

When the temperature was 21.2 °C, it could be seen that the theoretical ratio of Sulphide $_{Produced}$/Sulphate $_{removed}$ (0.33), correlated well with the experimental ratios obtained (0.3) (Table 3.1). During the period where the temperature was 35 °C, however the S^{2-}/SO_4^{2-} ratio was 0.2 compared to the theoretical value of 0.3. A possible explanation for this could be that the system allowed the entrance of air at strategic joints, which were not properly sealed, to form sulphur (reaction 1). The period of 35°C was in the beginning of the experimental period, while the second period of 21.2°C was later in the experimental period, which possibly allowed the reactor to undergo structural improvements and therefore no air entered the system during the later stage.

$$2H_2S + O_2 \rightarrow 2S^\circ + 2H_2O \qquad (1)$$

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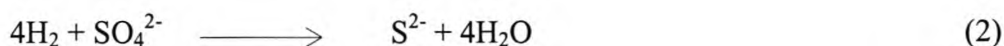
The average experimental alkalinity/sulphate ratio was 1.2 and 1.0 for temperature periods of 21.2 and 35 °C respectively (Table 3.1), which correlated well with the theoretical ratio of 1.04.

The standard deviation of the pH of the venturi reactor was 0.9 and 0.3 for the periods where the temperature was 21.2 and 35 °C respectively (Table 3.1). The pH of the reactor was therefore kept between 7.3 and 8.2 for optimal sulphate removal. When deciding on the optimal pH for operating the sulphidogenic bioreactor, there are several factors of pH that should be considered. The optimal pH for ASRB is 7.3 – 7.6, while the optimal pH for AMB is 6.5 – 7.8. ASRB can also tolerate higher pH values than the AMB (Van Houten, 1996; Lens *et al*., 1998).

3.1.1.3 Metabolic end products

Hydrogen can be utilized as an energy source by SRB for sulphate reduction or by homoacetogenic bacteria to produce volatile fatty acids (VFA) (such as acetic acid).

The stoichiometric amount of hydrogen required for sulphate reduction is 8 g H_2/96 g SO_4^{2-} (reaction 2).



$$4H_2 + SO_4^{2-} \longrightarrow S^{2-} + 4H_2O \qquad (2)$$

It was noted that acetate was the main VFA produced (16.8 mg/L average). Small amounts of propionic(1 mg/L), butyric (0.26mg/L) and valeric acid (< 0.2 mg/L) were detected.

The formation of volatile fatty acids during excess H_2 dosages could be ascribed to the activity of homoacetogenic bacteria, which use the CO_2 and H_2 to form acetate (reaction 3). Hydrogenotrophic SRB, a heterotrophic SRB, can use this acetate as its carbon source: the acetate consuming SRB cannot use the CO_2 directly, as only the autotrophic bacteria can utilise the CO_2.

$$4H_2 + 2CO_2 \longrightarrow CH_3COOH + 2H_2O \qquad (3)$$

This is consistent with the theory that sulphidogenic bioreactors treating sulphate rich wastewater are complex ecosystems, containing many bacterial species. When using

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hydrogen as energy source, competition exists between HSRB and HMB. The HSRB typically out-compete the HMB, as the HSRB gain more energy from the consumption of molecular hydrogen and therefore have a higher substrate affinity, growth rate and cell yield than the HMB. The HSRB could also maintain the hydrogen concentration below the threshold value required by the HMB, thus preventing the HMB from using the hydrogen. This theory explains the inhibition of the HMB when sulphate forms part of the chemical components in the wastewater (Lens *et al.*, 1998).

The type of seed sludge and experimental runtime may also influence the outcome of the competition between acetate utilizing bacteria. For some types of bacteria a long period of time is needed to out-compete other species (Lens *et al.*, 1998). It has been shown by Visser (1995) that the ASRB need 200 – 400 days to out-compete the acetogenic MB (AMB), when the COD/SO_4 ratio is <1. It was also noticed that when an oversupply of sulphate is present, the ASRB should out-compete the AMB in the competition for acetate (Visser, 1995).

3.1.2 Reactor 2

In Figure 3.2 it can be seen that during continuous operation of R2, the sulphate removal rate reached a maximum of 4g/(L.d) after 3 weeks of operation (day 20). This figure shows that the sulphate removal rate was not stable during the continuous operation of R2. The corresponding sulphide produced, indicated the possibility of the formation of toxic levels of H_2S concentrations. It was therefore necessary to obtain a better understanding of the factors that could potentially affect the sulphate removal efficiency. The effect of various parameters on the sulphate removal efficiency was subsequently investigated by means of batch studies in order to understand and obtain a stable sulphate removal system.

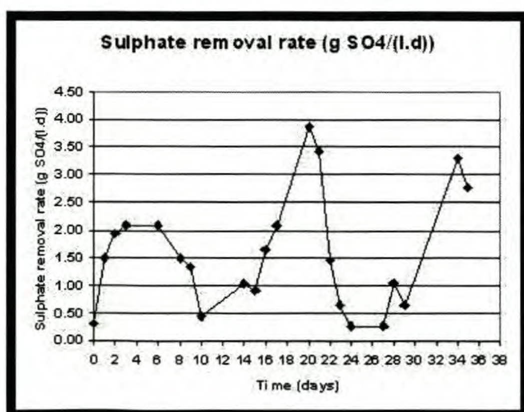

Figure 3.2a: The sulphate removal rate in the continuous reactor with 80% geotextile (R2).

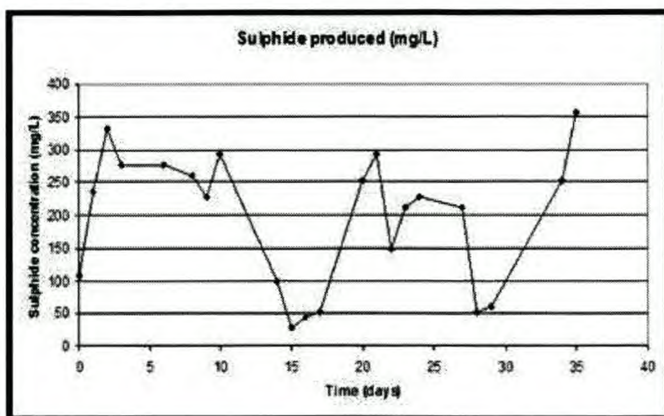

Figure 3.2b: The corresponding sulphide produced in the continuous reactor with 80% geotextile (R2).

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Figure 3.3 shows the average trends observed for various performance parameters during the batch experiments. The various graphs support the following chemical reactions:

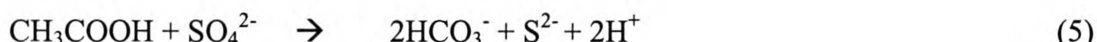
- Sulphate reduction and sulphur production:

Sulphate removal was associated with sulphide and alkalinity production (reaction 4). The average measured experimental $alkalinity_{produced}$ to $sulphate_{removed}$ ratio was 0.98, which compares well with the theoretical value of 1.04, while the experimental $sulphide_{produced}$ to $sulphate_{removed}$ ratio measured was 0.27, which also compares well with the theoretical value of 0.33. In this batch experiment, it was observed that the pH initially decreased. This decrease could be ascribed to oversupply of the mixture of H_2/CO_2 gas, which caused an initial oversupply of CO_2, and a subsequent decrease in pH.

$$CO_2 + 4H_2 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + 3H_2O \quad (4)$$

- Fatty acid formation:

Although no organic material was fed to the reactor, an average of 109 mg/L acetate were measured. This could be ascribed to reaction 3 (mentioned in 3.1.1.3) and reaction 5:



$$CH_3COOH + SO_4^{2-} \rightarrow 2HCO_3^- + S^{2-} + 2H^+ \quad (5)$$

Similar results were obtained in earlier experiments (3.1.1.3). Although no COD was added to the feed water, a low concentration of COD was measured in the effluent of the reactor. When analysing the volatile fatty acid (VFA) content, it was noticed that most of the COD present in the reactor could be ascribed to acetate. This acetate was presumably produced in the reactor by homoacetogenic bacteria that use CO_2 and H_2 to form acetate. The acetate is used as the carbon source by the hydrogenotrophic SRB (HSRB), which are heterotrophic SRB. The hydrogen consuming SRB cannot use CO_2 directly, in contrast to autotrophic bacteria (Hulshoff Pol *et al.*, 1998).

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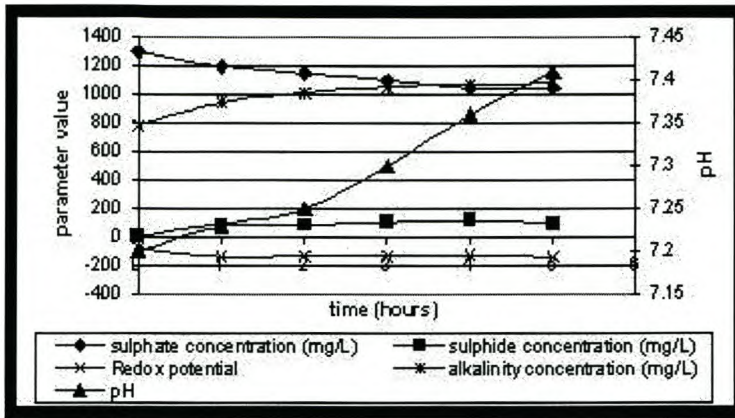

Figure 3.3 Average trends of various parameters during batch studies (values represent averages of 10 measurements).

3.1.2.1 The effect of some environmental parameters on sulphate removal.

3.1.2.1.1 Temperature

Figure 3.4 shows the effect of temperature on sulphate removal. The temperature at each hourly sample of the particular batch test was measured. Each sample showed a consistent increase in temperature with the progression of each batch test. The temperature increase and the maximum temperature reached during each batch test, was not only caused by the heat generated by the recycle pump, but was also influenced by the ambient temperature. Therefore, average temperatures were calculated and given for each batch test. In Figures 3.4 to 3.6, it can be seen that better sulphate reduction was obtained when the average temperature was 29.5 °C than when it was 39°C.

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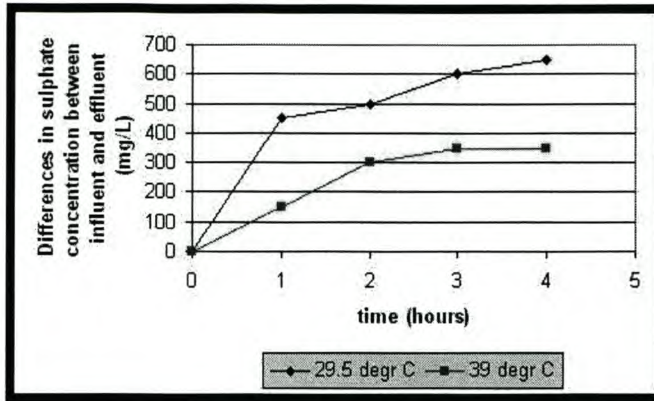

Fig.3.4 The effect of temperature on sulphate removal during a standard batch test at a pH of 7.5.

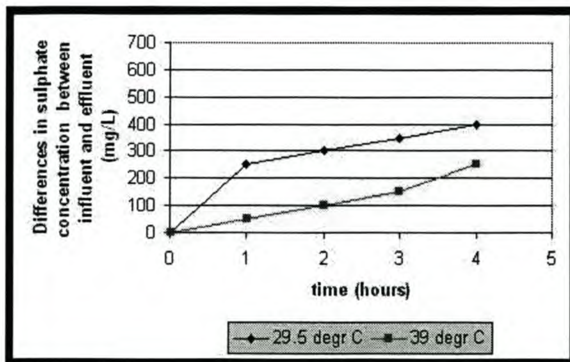

Figure 3.5: The effect of temperature on sulphate removal at an initial pH of 8.0.

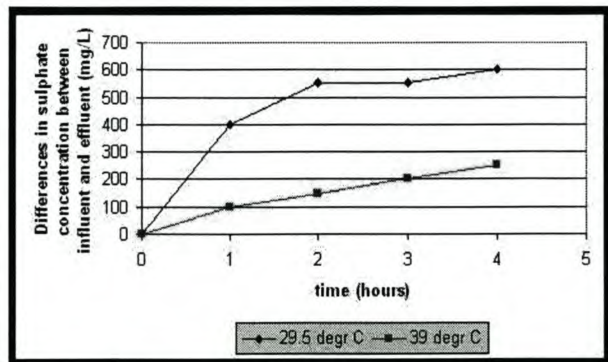

Figure 3.6: The effect of temperature on sulphate removal at an initial pH of 7.2.

The improved sulphate removal at an average temperature of 29.5°C suggests that continuous operation over the preceding months at an average temperature of 30 °C resulted in the selection of a mesophilic community in the venturi reactor. Previous literature shows that sulphidogenic reactors, using hydrogen and CO_2 or acetate as the energy source, were operated at temperatures of 30°C for optimal sulphate removal, when the experiments were designed under mesophilic conditions (Visser, 1995; Van Houten, 1996).

Corresponding with the higher sulphate removal rate at 29.5 °C were increased sulphide and alkalinity concentration production rates, respectively (results not shown).

3.1.2.1.2 pH

In the first set of batch tests in R2 (Figure 3.7), all the parameters were kept the same as in the standard batch test (for comparison purposes), while the pH was varied between 7.2, 7.5 and 8.0 at an average temperature of 29.5°C (P1). In the second set of batch tests (Figure 3.8),

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the parameters and conditions in R2 were the same as in the first set with the exception of temperature, which was at an average of 39°C (P2). Figure 3.7 shows the sulphate removal pattern during P1 as a function of pH. It was noted that optimal sulphate removal was achieved at pH 7.5, which agreed with the finding of van Houten (1996).

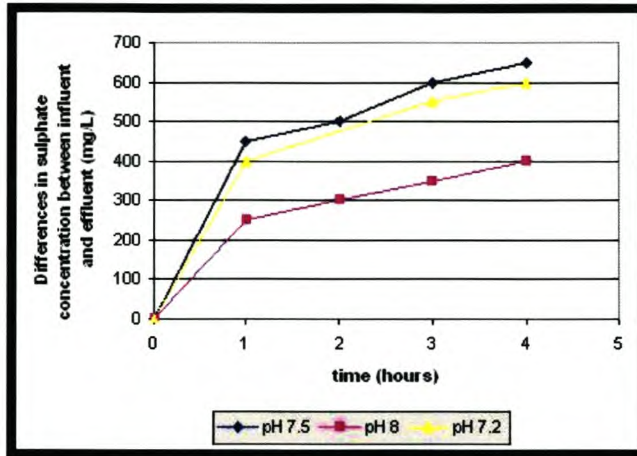

Figure 3.7: The average sulphate removal in R2 at different pH values at an average temperature of 29.5 °C).

The second set of batch tests (Figure 3.8) confirmed the findings shown in Figure 3.7: the optimum pH for sulphate removal was 7.5. However, in Figure 3.7 an overall better sulphate removal was seen than in Figure 3.8. In Figures 3.4 to 3.6, it was shown that the optimum average temperatures for sulphate removal was 29.5 °C (P1). Therefore, it can be assumed that the overall better sulphate removal seen in Figure 3.7 was the result of the more favourable temperature range at which these batch tests were done. When the average results obtained during the two series of tests were calculated, the same overall pattern of results was obtained, as shown in Figure 3.9.

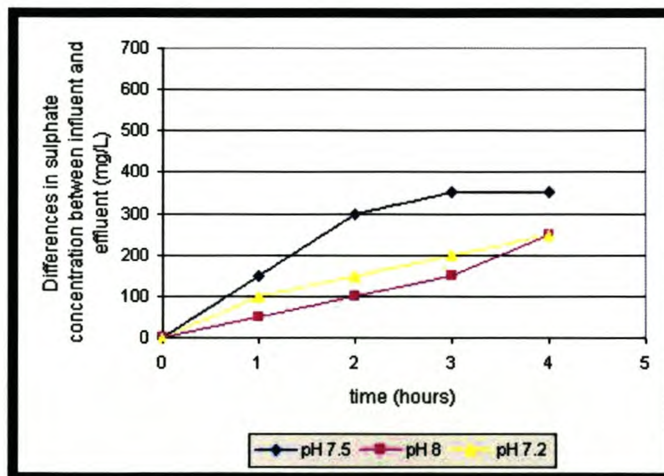

Fig. 3.8: The average sulphate removal in R2 at different pH during an average temperature of 39°C.

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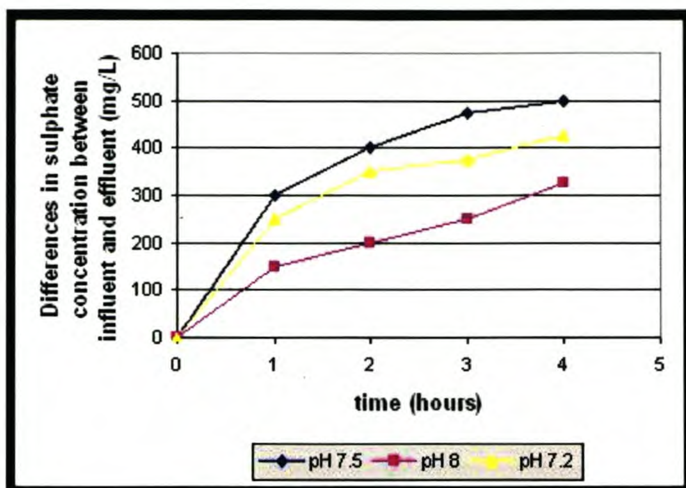

Fig.3.9: The average sulphate removal at different pH values.

3.1.2.1.3 Sulphate Concentration

Figures 3.10 to 3.12 show the effect of the sulphate concentration on the sulphate removal rate in R2. The highest sulphate removal rate was obtained at the batch experiment where the sulphate concentration was the highest (4000 mg/L). These results indicated that the rate of sulphate removal could be related to the sulphate concentration, if it is assumed that the rate of sulphate removal is controlled by diffusion of the sulphate-rich solution into the biofilm. Oude Elferink (1998) reported on the prediction of biological sulphate removal rates in biofilm systems. It was concluded that the limiting factor at high sulphate concentrations is the mass transfer of sulphate into the biofilm, while at low sulphate concentrations the growth rate of the SRB is limited.

In these results, as in the previous results, the second set of batch tests in Figure 3.11 confirmed the findings in the first set of results shown in Figure 3.10: an increased initial sulphate concentration (4000 mg/L) caused the best sulphate removal. Figure 3.10 (P1) showed an overall better sulphate removal than in Figure 3.11 (P2), presumably also because of the more favourable temperature range during P1. The average sulphate removal of the two periods can be seen in Fig. 3.12 and it is evident that the same sulphate removal pattern was followed for all the batch tests in P1 and P2.

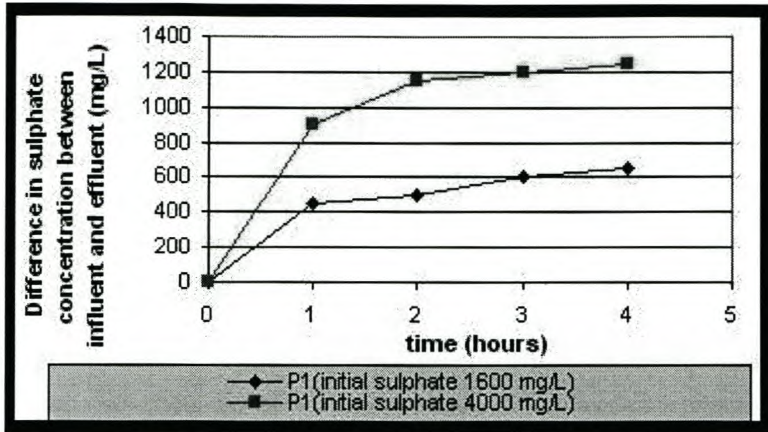

Figure 3.10: The sulphate removal pattern in R2 during P1 (average temperature of 29.5°C) at initial sulphate concentrations of 1600 and 4000 mg/L.

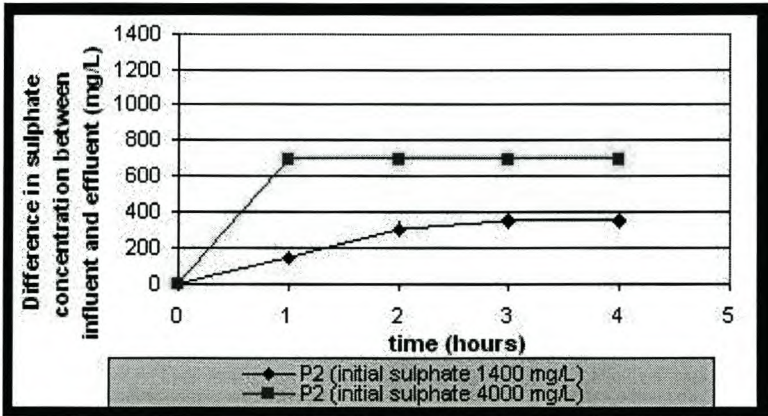

Figure 3.11: The sulphate removal pattern in R2 during P2 (average temperature of 39°C) at initial sulphate concentrations of 1400 and 4000 mg/L.

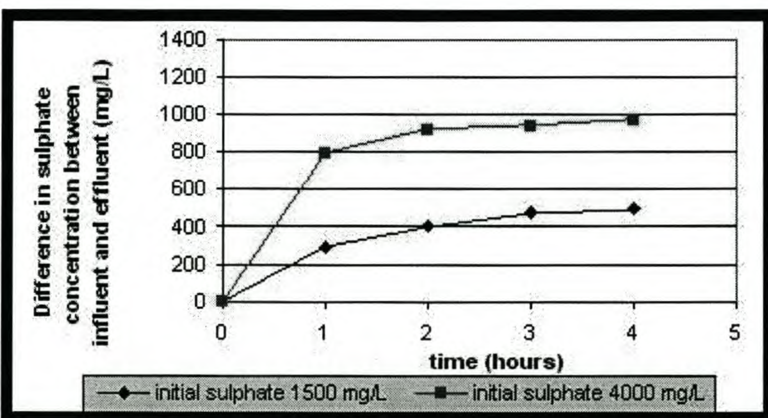

Figure 3.12: The average sulphate removal in R2 at average initial sulphate concentrations of 1500 and 4000 mg/L.

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3.1.2.1.4 Effect of calcium sulphate

Figures 3.13 and 3.14 show the hourly sulphate and corresponding sulphide concentrations, respectively, measured in batch tests. The increase in sulphide concentration shows that sulphate removal occurred (Figure 3.14), despite the initial increase in sulphate concentration. This increase in sulphate concentration (Figure 3.13 between hr 2 and hr 3) can be explained by the presence of $CaSO_4$ crystals, which could dissolve to increase the sulphate concentration after the initial decrease.

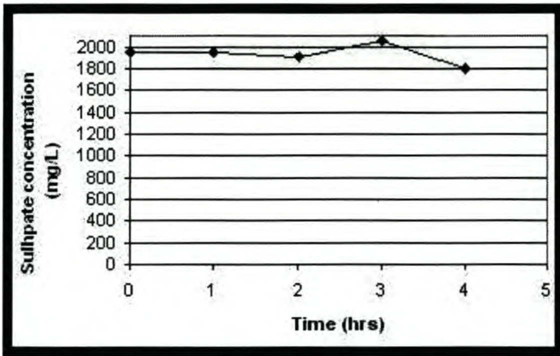

Figure 3.13: Hourly Sulphate concentration during batch test when the feed contained $CaSO_4$

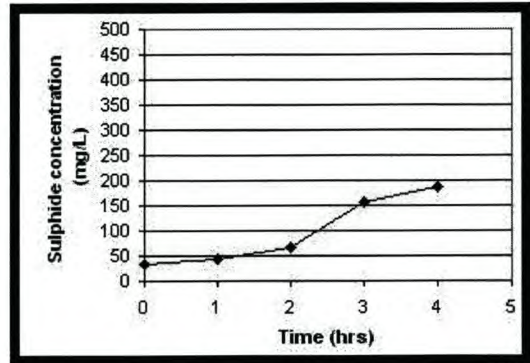

Figure 3.14: Change in sulphide concentration in batch test when feed contained $CaSO_4$.

When $CaSO_4$ was excluded from the feed-water in a subsequent batch experiment, improved corresponding hourly sulphate reduction results were observed (Figure 3.15a and 3.15b). In addition to the improved sulphate reduction efficiency, increases in sulphide and alkalinity concentration were observed.

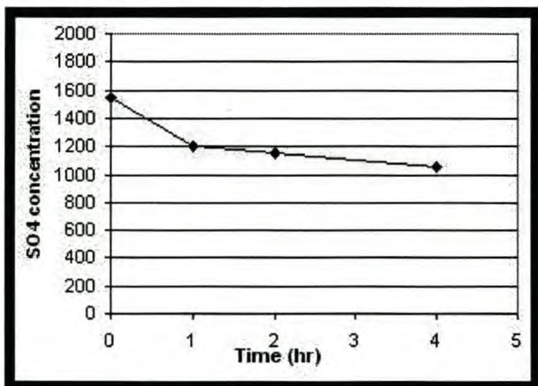

Figure 3.15a: Change in Sulphate Concentration during batch test when the feed did not contain $CaSO_4$.

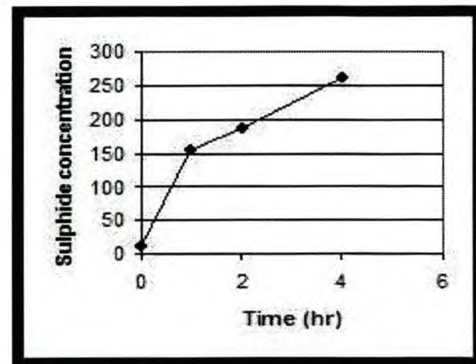

Figure 3.15b: Change in sulphide concentration during batch test when the feed did not contain $CaSO_4$.

From the second batch test conducted in R2, it could be concluded that if $CaSO_4$ was left out of the feed, the sulphate removal was more efficient. In accordance with the notable sulphate

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concentration decrease, notable increases in sulphide and alkalinity concentration were observed. Therefore, if the wastewater that has to be treated (in practice) contains $CaSO_4$, it could affect the efficiency of the sulphate removal in the reactor.

3.1.2.1.5 Sulphide Concentration

When the initial sulphide concentration was 100 mg/L as opposed to 0 mg/L, the sulphate removal rate increased under both temperature conditions (Figure 3.16-3.18). The improved sulphate removal rate could be explained by the lower reactor redox potential (Figure 3.20), caused by the addition of Na_2S. The average redox potential was -167 mV when 100 mg/L Na_2S was added, while the average potential was –72 mV when no Na_2S was added.

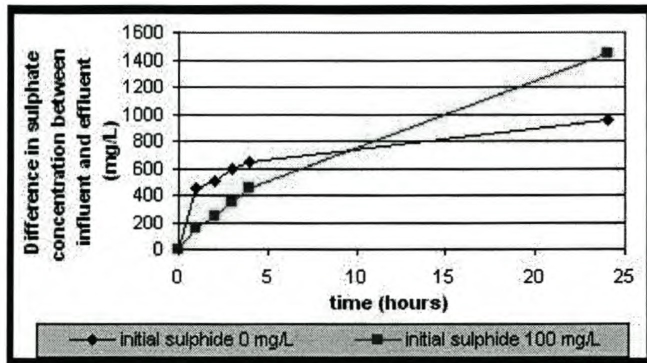

Figure 3.16: Sulphate removal efficiency at different initial sulphide concentrations; average temperature 29.5°C.

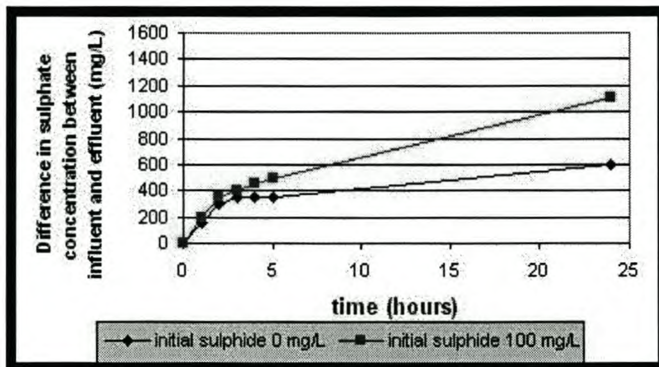

Figure 3.17: The sulphate removal operating R2 (average temperature 39°C) at different initial sulphide concentrations (0 and 100 mg/L)

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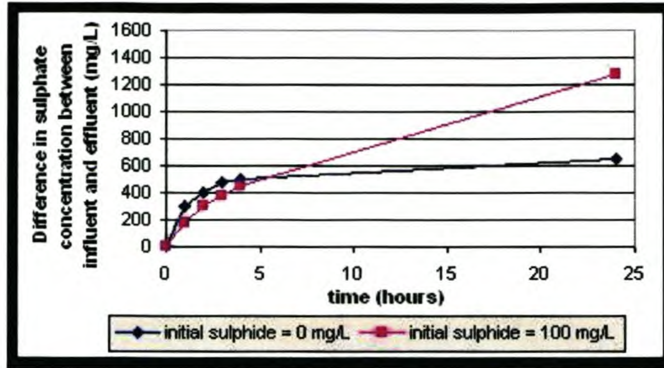

Figure 3.18: The average sulphate removal operating R2 at two different initial sulphide concentrations (0 and 100 mg/L); std dev 5%.

Figure 3.19 shows the average sulphate removal when the initial sulphide concentrations were 0, 100 and 268 mg/L (where 1475 mg/L was the average total sulphate removed of the batch tests when the initial sulphide concentration was 268 mg/L in R2; std dev of 14.8%). This relatively high standard deviation is likely the result of different pH values during the two batch tests, which varied between 7.01 and 7.8. As discussed earlier, the possible explanation for this occurrence is the equation that showed that H_2S (g) (the toxic form of sulphide that causes inhibition of SRB) is formed at pH < 6.5, while HS^- is formed at pH >6.5. (Lens *et al.*, 1998). Therefore, the possibility exists that more H_2S gas was formed initially at a lower pH of 7.02-7.5. This could then have caused the inhibition of SRB, resulting in lower sulphate removal efficiency than when the pH was higher (pH 7.7) and therefore less H_2S (g) could form theoretically.

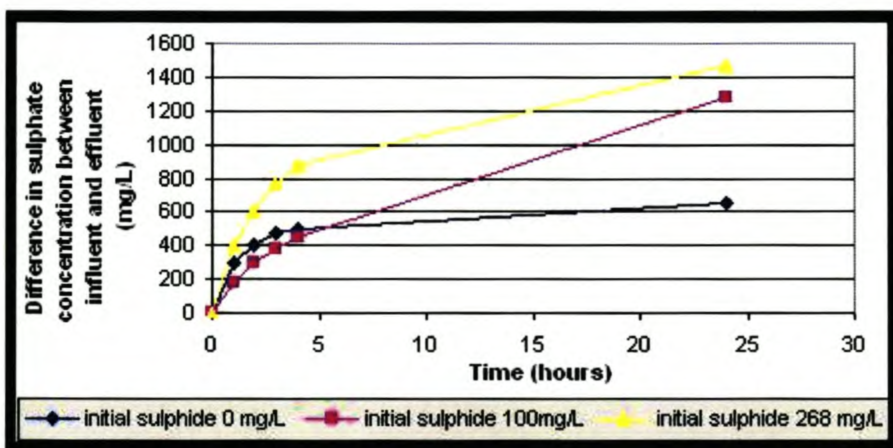

Figure 3.19: The average sulphate removal operating R2 at three different initial sulphide concentrations (0,100 and 268 mg/L)

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In Figure 3.20, it can be seen that the average redox potential was lower, and therefore more favourable for sulphate removal when Na_2S (100 and 268 mg/L, respectively) was added to the initial feed water.

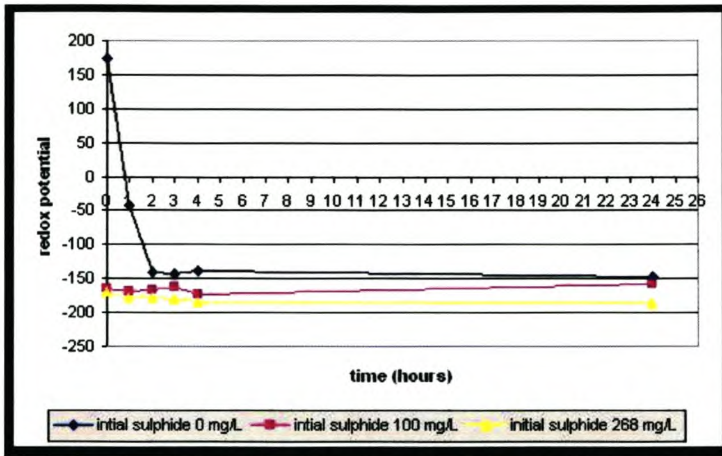

Figure 3.20: Effect of initial sulphide concentration on redox potential.

3.1.2.1.6 Alkalinity

Figures 3.21-3.22 show that a higher alkalinity promotes the efficiency of the sulphate removal process when all the other parameters were constant and kept the same as in the standard batch test. Figure 3.21 shows better overall sulphate removal at an averaged temperature of 29.5°C than 39°C, similar to the other parameters.

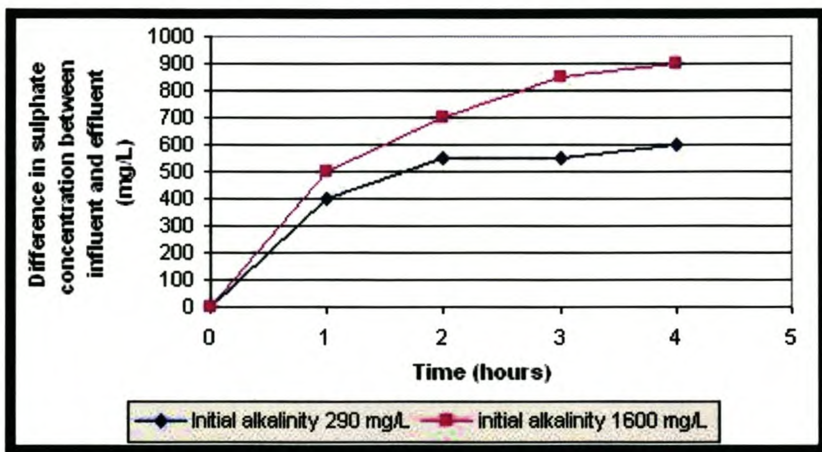

Figure 3.21: Sulphate removal efficiency at different initial alkalinity and average temperature of 29.5°C.

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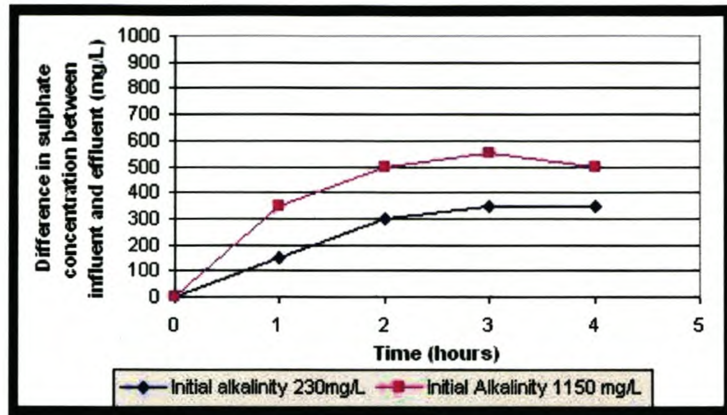

Figure 3.22: Sulphate removal efficiency at different initial alkalinity and average temperature of 39°C.

Figure 3.23 shows the hourly redox potential measured for the individual batch tests at each different initial alkalinity concentration. Here it can be seen that if the alkalinity is increased initially by using $NaHCO_3$, the initial redox potential is also more favourable (a more negative value). Therefore, the reason for the more efficient sulphate removal when the alkalinity was initially added could be because of the more favourable initial redox potential. Overall, these results show that, similar to the other parameters, alkalinity has an effect on the sulphate removal efficiency.

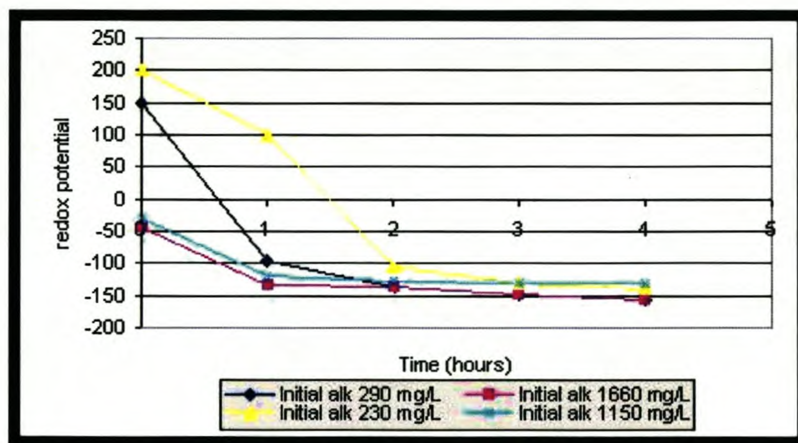

Figure 3.23: Redox potential measured during the batch experiments with different initial alkalinity concentrations (290, 230, 1660, 1150 mg/L).

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3.1.2.1.7 Trace Elements

The effect of two trace metals (cobalt and nickel) on the reactor performance was also investigated. This is an important consideration in anaerobic processes, because metals are involved in many of the enzymatic activities of sulphidogenesis and acidogenesis (van Hullebusch *et al.*, 2004). It was shown that cobalt, and to a lesser degree nickel, played a key role in methanogenic activity in UASB reactors where methanol and VFA's were treated (Zandvoort *et al.*, 2003). Therefore, these two trace metals were included in this study.

Cobalt

In Figure 3.24, it can be seen that when cobalt was added (1 and 3 mg/L, respectively) to the feed water (with a residual concentration of 0.25µg/L cobalt and 0.26 µg/L nickel), the average sulphate removal increased. Therefore, it can be deduced that the addition of cobalt is beneficial for the system and causes more efficient sulphate removal.

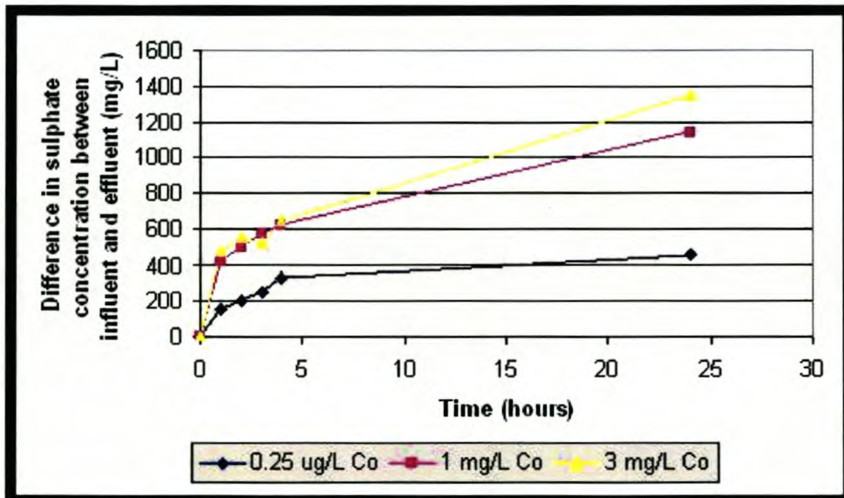

Figure 3.24: The average sulphate removal operating R2 at three different initial cobalt concentrations (0.25 µg/L, 1 and 3 mg/L)

Figure 3.24 shows the average sulphate removal measured in two batch sets conducted at the same temperature. There was little variation between replicate measurements (<6.3%). From these relatively constant values, it can be deduced that the results were reproducible, if the temperature range and all the other parameters were kept the same in the batch tests.

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Nickel

In Figure 3.25, it can be seen that addition of nickel (3 mg/L) in the feed water (with a residual concentration of 0.25μg/L cobalt and 0.26 μg/L nickel), resulted in a decrease in the average sulphate removal efficiency. Here was also little variation between replicate measurements (<2.5%), which again indicated the reproducibility of the results.

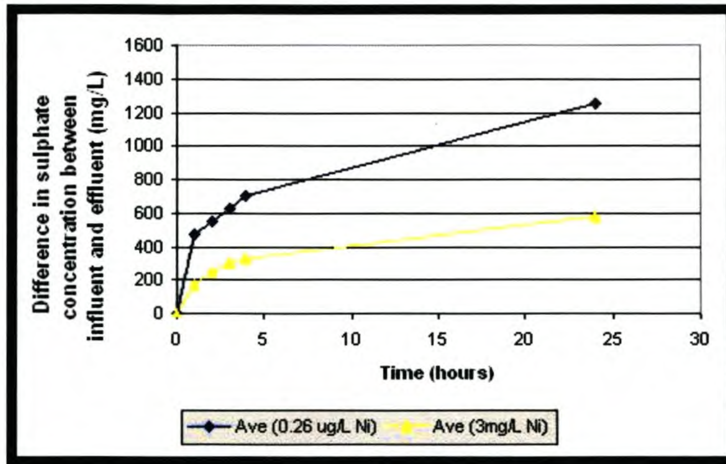

Figure 3.25: The average sulphate removal at two different initial nickel concentrations (0.26 μg/L and 3 mg/L)

It was previously mentioned that nickel is required in processes where hydrogen serves as the electron donor for the reduction of sulphite by the action of an electron transport chain, because this process requires the presence of nickel containing hydrogenase enzymes (Caldwell, 1995). Therefore, it is speculated that nickel is necessary in trace amounts for the above-mentioned process, although as indicated in Figure 3.25 it becomes inhibitory at higher concentrations.

3.1.2.1.8 Hydrogen requirement

During all the batch tests, the amount of hydrogen introduced, was 700 ml/min, which is 4.5 times more than the amount of hydrogen required for total sulphate removal:

Hydrogen applied = H_2 (ml/min)/22.4 x 2/qFeed (ml/min)

= g H_2/L applied

(a)

Hydrogen required = SO_4 to reduce (mg/L)/1000 x 8/96

= g H_2/L required to reduce the SO_4 in the reactor during batch

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(b)

When an average of 1600 mg/L sulphate in a total of 150 L feed water (qFeed) had to be reduced during a batch test, it was calculated that 0.13 g H_2 was required to reduce all the sulphate present in the feed water (b). Furthermore, it was calculated that 0.6 g H_2 was applied per litre feed water, if 700ml H_2 was introduced per minute (a). Therefore, the amount of hydrogen that was applied was 4.5 times more than the amount of hydrogen required for the total reduction of all the sulphates in the feed water. Similarly, if 900 and 250 ml/min H_2 is introduced into the reactor respectively, the hydrogen introduced is 6 and 1.5 times more (respectively) than the actual hydrogen requirement.

Figure 3.26, shows the effect of introduced hydrogen on sulphate removal: higher hydrogen input (900ml/min) increased the sulphate removal efficiency, while lower hydrogen input (250ml/min) decreased the sulphate removal efficiency, thereby demonstrating the correlation between hydrogen concentration and removal efficiency.

The variations between the replicate measurements were low, indicating the reproducibility of the results.

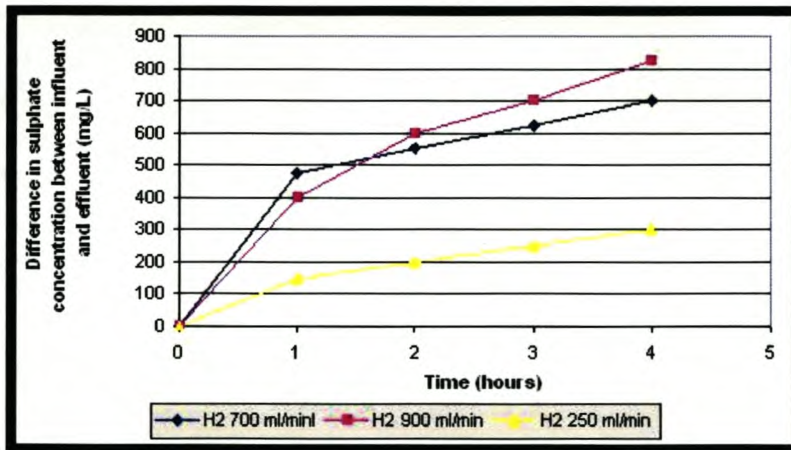

Figure 3.26: The effect of rate of hydrogen introduction on sulphate removal.

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3.2. MICROBIAL BEHAVIOUR

3.2.1 Attachment to geotextile

Despite major developments in the area of wastewater treatment technology, little knowledge has been acquired with regard to the structure and function of the microbial populations within the process (Collins *et al.*, 2002). Therefore, the viability, stability and structure of the biofilm in the venturi reactor were observed in this study.

The increased surface area associated with the geotextile that was installed in R2, and the subsequent biofilm formation on the geotextile, provided a probable explanation for the efficient sulphate reduction in R2. The immobilization of biomass associated with biofilm formation would prevent washout of cells from the reactor. A realistic assumption is that the SRB also formed part of the biofilm community. Figure 3.27 shows an example of biofilm formation on a geotextile strip that was removed from the venturi reactor.

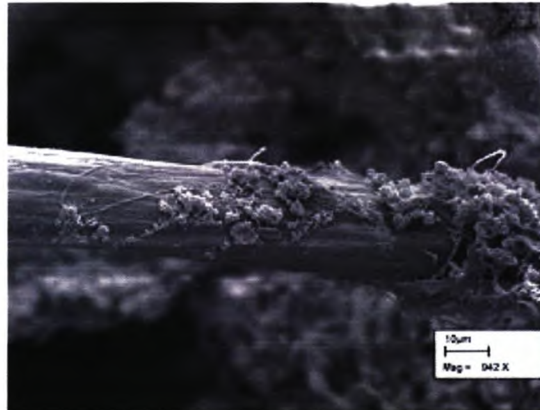
Figure 3.27: Geotextile used in the hydrogen contact reactor before biofilm formation (top) and after biofilm formation (bottom), showing the biofilm on the geotextile strip, as well as the black colour of the biofilm and the yellow colour of the plastic pipe, caused by the formation of sulphide compounds (as a result of S^{2-} production by the SRB).

Scanning Electron Microscopy (SEM)

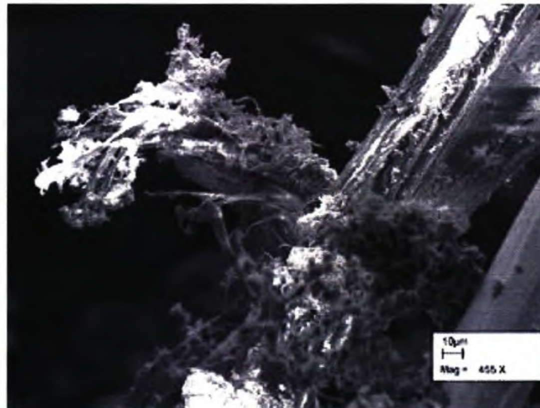
Figure 3.28 shows SEM images of biofilm formation on the geotextile filament. In these photomicrographs, the progression of biofilm development is evident, showing that the process could start at the endpoints of the finer geotextile structure, and through the production of extensive extracellular polymeric substance (EPS), could grow further to become established on the bulk filamentous structures of the geotextile. EPS typically form a three-dimensional, gel-like, highly hydrated and often charged biofilm matrix, in which the microorganisms are embedded and more or less immobilized. The yield and composition of

bacterial EPS depend on the species and growth conditions (Beech *et al.*, 1999). In general, the proportion of EPS in biofilms can vary between 50 and 90% of the total organic matter. The extracellular localization of EPS and their composition may be the result of different processes, such as active secretion, shedding of cell surface material, cell lysis and adsorption from the environment (Wingender *et al.*, 1999). Figure 3.28 suggests that the biofilms that form on the geotextile had similar properties, and that the increased attachment surface area afforded by the geotextile would thus enhance microbial activity in the reactors.

A



B



C

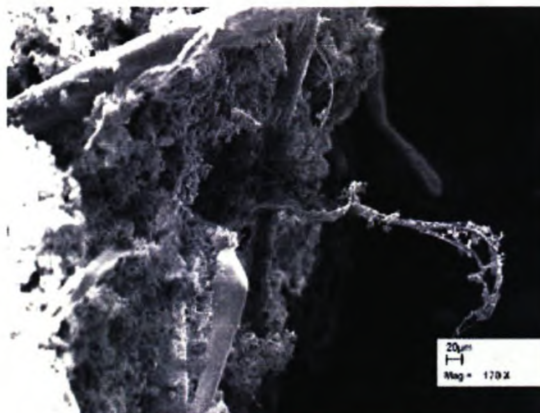


Figure 3.28: SEM photomicrographs, showing the progression of biofilm on the geotextile fibres.

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Previous studies showed that the biofilm density (of anaerobic wastewater treatment systems) and morphology are related, and that high density biofilms exhibit a non-filamentous structure, characterized by dense patches of microbial colonies, while low density biofilms exhibit a filamentous structure. It was also mentioned that since biofilm accumulation increases fluid frictional resistance, the frictional resistance should increase in proportion to the biofilm thickness once a critical biofilm thickness is reached (Speece, 1996). Based on the SEM photographs, it can be speculated that high-density colony formation occurred in the reactor on the geotextile.

It was mentioned that the diffusional resistance could influence the efficiency of the bulk reaction when the biofilm reaches a thickness of greater than 1mm (Henze *et al.*, 1982; Speece, 1996). According to McCarty and Smith (1986), the substrate concentration within a granule or biofilm can be significantly less than in the bulk liquid and the H_2 concentration in the bulk liquid may also not be in equilibrium with the H_2 in the gas phase (Speece, 1996; McCarty *et al.*, 1986). Therefore, effluent recycling was practiced to reduce mass transfer limitations due to biofilm thickness and diffusional resistance.

Epifluorescence Microscopy (BacLight Staining)

Epifluorescence microscopy combined with viability stains showed a mixture of viable and non-viable cells in the biofilm community. Such variety in viability is a general phenomenon in biofilms, where old cells are trapped in the EPS matrix. Cell lysis and release of their cellular content typically results in the breaking up of these cells, part of which can be utilized by living cells. Biofilm studies often report on mature biofilms containing >20% non-viable cells.

According to Tresse *et al.* (2003), who studied the dynamics of living and dead bacterial cells within a mixed-species biofilm in a biotrickling filter, the relative abundance of live cells reached a maximum concentration in the biofilm where after the biofilm was characterized by the accumulation of dead cells and organic matter. The live cells were dominant in the biofilm during the establishment phase of biofilm formation (>72%) and subsequently decreased in fraction of the total cells (51%) in the maturation phase.

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The photomicrographs in Figure 3.29 shows typical examples of the biofilms collected from R2 after 200 days of operation. As expected, biofilm micro-colonies consisted of dead (red fluorescent; on average >33%) and live (green fluorescent) cells, suggesting that the microbial community in the reactor formed active biofilms that were in steady-state with the physical-chemical environment of the reactor.

A B

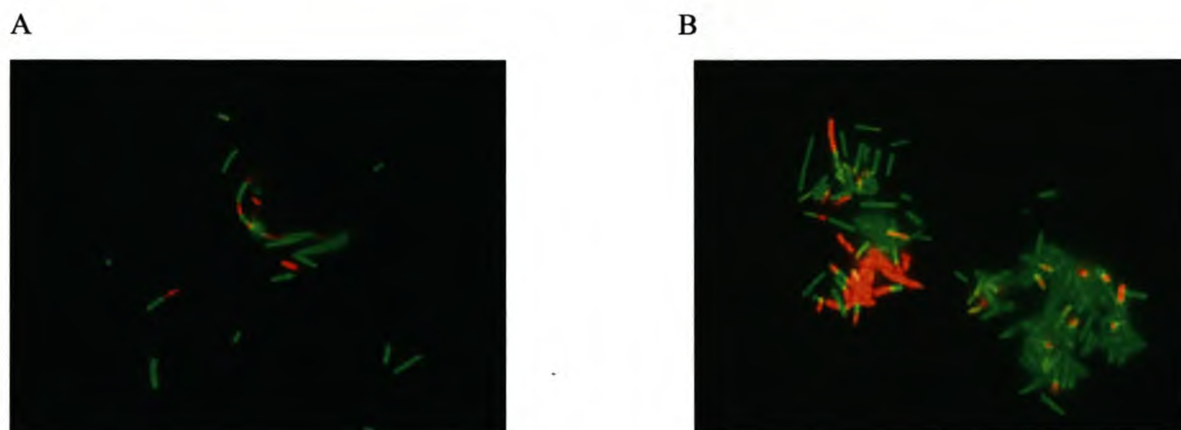

Figure 3.29: Red and green fluorescent cells representing dead and live cells respectively.

3.2.2 Terminal Restriction Fragment Length Polymorphisms (T-RFLP)

According to Collins *et al.* (2002), T-RFLP is very effective and useful as a tool for molecular biomonitoring of engineered ecosystems, such as wastewater treatment systems.

During the T-RFLP analysis in this study, the DNA from the whole community of R2 was extracted and the 16S rRNA genes were amplified, using fluorescently labelled primer pairs (only the forward primer was labelled). The 16S rRNA genes are usually chosen as target genes for T-RFLP, because they are highly conserved regions, of which the sequence and restriction enzyme sites are known. When a labelled primer is used, fluorescently labelled amplicons are synthesized, digested with restriction enzymes and size separated via gel electrophoresis automated sequencers. Thus, only the labelled terminal fragments are detected and quantified.

Four samples were analysed from R2: on day 370 (sample 1), day 375 (sample 2), day 377 (sample 3) and day 475 (sample 4) of reactor operation. Sample 1, 2 and 3 were collected when the reactor operation and environmental conditions were stable, while sample 4 was

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taken after several environmental and condition changes were made (batch tests, section 1). The PCR reaction result was visualised on an agarose gel. A molecular marker, a negative control (nuclease free water) and a positive control (*Salmonella* DNA) was included. Each sample was done at least in triplicate for both the PCR reaction and the gel electrophoresis. This process was repeated several times for optimisation purposes.

After the PCR products were digested with the restriction enzymes (*Rsa*I, *Msp*I, *Hha*I), the products were electrophoresed. The gel (Figure 3.30) also contained molecular markers as well as negative (nuclease free water) and positive (*Salmonella* DNA) controls. All the samples that were treated with a particular enzyme, were subsequently compared (Figure 3.31).

Theoretically, when an organism's 16S rDNA PCR product is digested with a restriction enzyme (for T-RFLP), a single band of a certain size (depending on the location of the restriction enzyme's target sequence), specific for that organism should be obtained on the sequencing gel (if the target sequence of that particular restriction enzyme is found on the 16S rDNA). Similarly, if there are for instance seven different organisms in a community, each one containing a specific restriction enzyme's target sequence, seven bands of different sizes should be obtained after treatment with a particular restriction enzyme. When three different enzymes were used to treat all the samples, each enzyme should generate the same amount of bands for each sample and thus by using three different enzymes, the different digest results of each sample should confirm the amount of bands obtained when compared.

When analysing the gel in Figure 3.30, the presence of pseudo-terminal fragments in the PCR products was suggested: each lane of the particular restriction digest in the *Salmonella* control, gave more than one band (the *Salmonella* control should only have one band in each lane, because the 16S rDNA PCR product from a pure culture should all have the same sequence). Pseudo-terminal fragments could form, where some of the produced amplicons are partly single stranded, during the PCR of the 16S rRNA genes. One reason for this formation of single stranded DNA, is thought to be the consequence of the ability of the 16S RNA molecule to form secondary structures, which could be responsible for the incomplete synthesis of a portion of the 16S rRNA gene amplicons during PCR. At greater PCR cycle numbers, the amplicon concentration increases, which increases the chances of interstrand and intrastrand annealing, hence the increased chance of the formation of local secondary structures during the last few cycles. The consequence to the polymerase enzyme of these

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secondary structures is the dissociation of the polymerase enzyme from the template containing the secondary structures and the subsequent incomplete synthesis of a fraction of the 16S rRNA gene amplicon during PCR. Furthermore, restriction enzymes need double stranded DNA (dsDNA) to digest a sequence, as a consequence, a DNA template which is partial double stranded will result in the restriction enzymes only cutting where the sequence is dsDNA, resulting in a product that is significantly larger than one expects (Egert *et al.*, 2003 and personal communication; Dr MN Gardner). This finding significantly complicated the results. A solution to this problem could be the future use of the Mung Bean nuclease enzyme (Egert *et al.*, 2003), which digests the single-stranded DNA parts from the partly single stranded 16S rRNA gene amplicons, thereby also removing the fluorescent signal from the defective amplicons and consequently render it undetectable.

However, if the overall result was compared to assess if the population changed during the three-month period (Figure 3.31), some changes were observed in the dominant bands obtained in the sequencing gel. Samples 1-3 were taken from R2 over a short period, when the environmental conditions were stable. By combining the T-RFLP pattern of each of the three samples, a master pattern can be created. In doing so, this takes into account problems with the DNA extraction (i.e. the DNA extraction of one sample could be more efficient than the other) as well as problems that could have occurred during the PCR reaction such as the formation of pseudo-transcription fragments (Egert *et al.*, 2003).

Thus, in samples 1-3, digested with *Hha*I (Figure 3.31A), the presence of four bands was showed (755-bp, 228-bp, 180-bp and 40-bp). When the conditions changed (Sample 4), the 180-bp band was lost and two new bands (117bp and 285 bp) appeared. For the *Msp*I digest (Figure 3.31B), the first set of environmental conditions (Sample 1-3) showed the presence of five bands: 202-bp, 172-bp, 165-bp, 157-bp, and 143-bp. Upon changing the conditions (Sample 4), the 165-bp is lost, and two new bands (132-bp and 98-bp) were observed. For the *Rsa*I digest (Figure 3.31C), the first set of environmental conditions (Sample 1-3) showed the presence of four bands: 612-bp, 540-bp, 390-bp, and 142-bp. Upon changing the conditions (Sample 4) the 390-bp band is lost, and two new bands (136-bp and 437-bp) appeared.

Taken together, these results suggested that there were at least 3 organisms that were unaffected by the environmental changes. Upon changing conditions, one organism was lost from the original community (Sample 1-3), and two new bands were noted in the T-RFLP's

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of Sample 4. The new bands were probably the consequence of conditions favouring the growth of these organisms whose numbers could initially have been too low to be detected by T-RFLP in the original samples. Therefore, these results suggest a probable shift in the population in the reactor over a three-month period during which the reactor conditions were changed.

Similar results were obtained by Collins *et al*. (2002) who showed that shifts in the population structure occurred in response to environmental conditions.

During this experimental three-month period, a change in the reactor performance was observed: the reactor performance efficiency increased in terms of sulphate removal. A major environmental change during the three months was a decrease in temperature (39°C to 29.5°C). The lower temperature proved to be the optimal temperature for sulphate removal in the kinetic studies; however it could also have played a role in causing the population shift, as shown by the T-RFLP. Therefore, the two organisms that dominated after the environmental conditions were changed and the one organism that was lost, could be a partial explanation for the better reactor performance observed.

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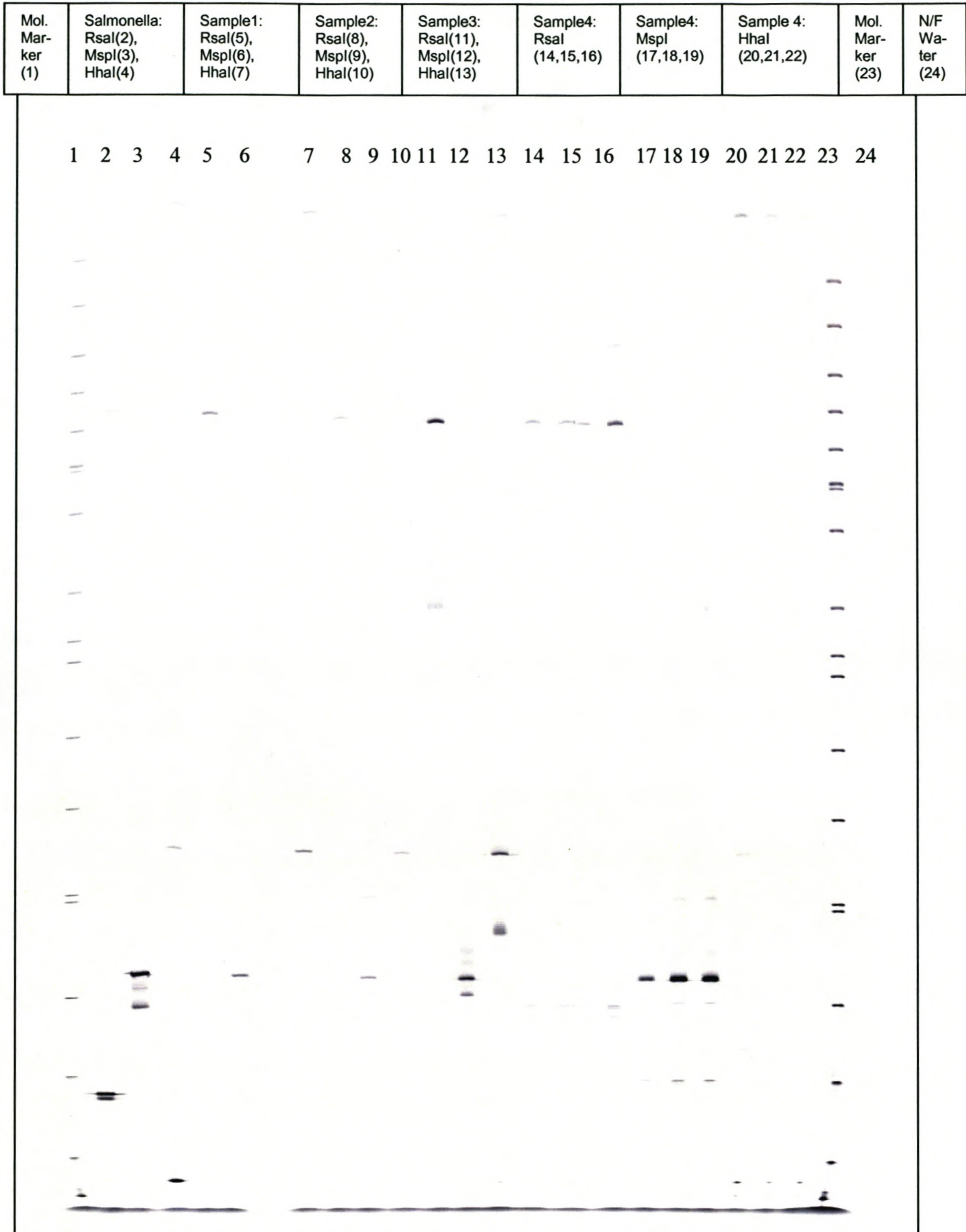

Figure 3.30: Sequenced restriction enzyme treated PCR product. Lanes 1 and 23 indicates the molecular markers and lane 24 the negative control, containing nuclease free water.

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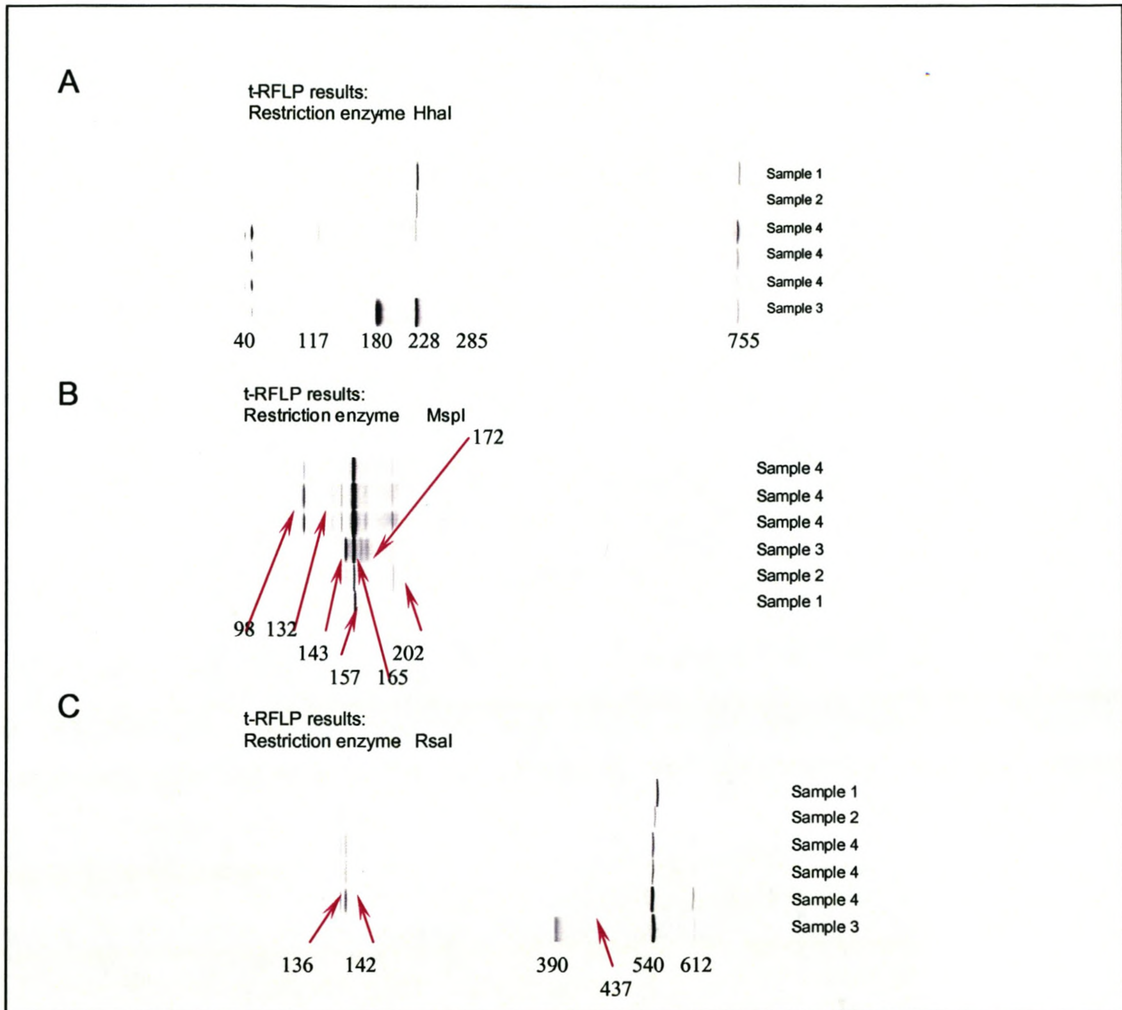

Figure 3.31: Comparison of different samples, treated with similar restriction enzymes.

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CHAPTER 4: GENERAL CONCLUSIONS

From the tests performed under continuous operation, it can be concluded that:

Efficient, relatively stable sulphate removal was achieved in the venturi reactor when supplied with a mixture of hydrogen and carbon dioxide as the energy/carbon source to the sulphate reducing bacteria. At a temperature of 35 °C in a reactor packed with geotextile (8% of the reactor volume), a volumetric SO_4^{2-} reduction rate of 1g SO_4^{2-}/(L.d) was achieved.

It was demonstrated that geotextile is a suitable support matrix to immobilize biomass, and to prevent washout from the reactor, as notable retention of microorganisms was observed.

An increase in the surface area for microbial attachment (reactor filled 80% with geotextile) improved sulphate removal to a maximum of 4g/L.d.

From the kinetic studies performed when the reactor was operated under batch conditions, it was concluded that:

The highest sulphate removal rates were obtained during the kinetic studies when the average temperature was maintained at 29.5 °C, when the reactor pH was 7.5, the $CaSO_4$ concentration in the feed was decreased and when the following initial concentrations was increased in the feed: sulphate (4000 mg/L); sulphide (268 mg/L, due to a more favourable redox potential), alkalinity (1600 mg/L), cobalt (3mg/L) and hydrogen gas (an input of 900 ml/min) (when compared to the amount of hydrogen gas stoichiometrically needed for the reduction of sulphate in the reactor).

Furthermore, it was observed that although the addition of nickel might be necessary in trace amounts for the above-mentioned process, it becomes inhibitory at higher concentrations.

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Biofilm studies:

Geotextile was used as an immobilization material for the sulphate reducing community. The immobilization of biomass was associated with biofilm formation in the venturi reactor. This would prevent the washout of cells from the reactor.

Macroscopically, biofilm formation was observed on the geotextile strips in the venturi reactor in the form of a black biomass. SEM images showed the progression of biofilm formation on the geotextile in addition to extensive EPS formation. SEM images also showed that the biofilms had a high density, with extensive micro-colony formation in the biofilms. Therefore, it was evident that the geotextile was a suitable material for immobilisation of biomass in a sulphidogenic bioreactor.

It is speculated that the biofilm could be too thick for optimal mass transfer between sulphate or hydrogen gas and the bacterial communities that form the biofilm. This possibility can be circumvented by practicing effluent and gas recycling to ensure optimal surface contact between the organisms and the substrate for optimal diffusion conditions in support of sulphate reduction.

Biofilm viability studies showed several dead microbial cells (>33%). Because of this, as well as the known fact that the biofilm sample was taken on day 200 of reactor operation, it was apparent that the biofilm was mature and established and contained mostly viable cells. This proved once more the suitability of geotextile as a support matrix for the formation of a stable and viable biofilm for sulphate reduction in the venturi reactor.

Community stability:

When the venturi reactor was subjected to environmental condition changes, the community stability was analysed over a period of 3 months by T-RFLP studies. The results suggested the presence of stable, dominant species, as well as the appearance of some, and the disappearance of other species during the three-month period. This further suggested the presence of species that were eliminated by the changes made in the reactor, as well as the probable shift in the population in the reactor over the test period.

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During the experimental period, a change in the reactor performance was observed: the reactor performance efficiency increased in terms of sulphate removal, after the three-month period. The main change during the three months was a decrease in temperature from 39 to 29.5 °C. This could have played a role in causing the population shift, as shown by T-RFLP. Such a population shift may be an explanation for the better reactor performance observed in addition to the more favourable reactor temperature (29.5 °C).

This aspect of the study could be investigated in more detail in the future, to establish the specific organisms responsible for the better sulphate removal observed in the reactor at the optimal temperature of 29.5°C. If these organisms are subsequently isolated and used as an inoculum for a new similar sulphidogenic reactor, it could aid in the establishment of a highly efficient biological sulphate removal system in wastewater treatment technology, especially when used in addition to the optimal conditions determined in this study, for optimal sulphate removal using hydrogen as the energy source.

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APPENDIX

1. GC Analysis

All VFA analyses were done using a gas chromatograph (Hewlett Packard. HP 5890 Series II) equipped with a flame ionisation detector (GC/FID), while the data analyses were done using the Chem Station, supplied by Hewlett Packard, software package. The column used was a HP-FFAP, 15 m x 0.530 nm, 1 micron. An outline of the GC/FID programme used is depicted in Table 3. The N_2 flow rate was set at 1 mℓ/min.

Table 3: The GC/FID programme for the detection of VFA

Parameter	Setting
Initial oven temperature (°C)	30
Initial time (Min)	2
Temperature programme: (°C)	80
Rate (°C/min)	25
Final temperature (°C)	200
Final time (min)	1
FID temperature (°C)	240